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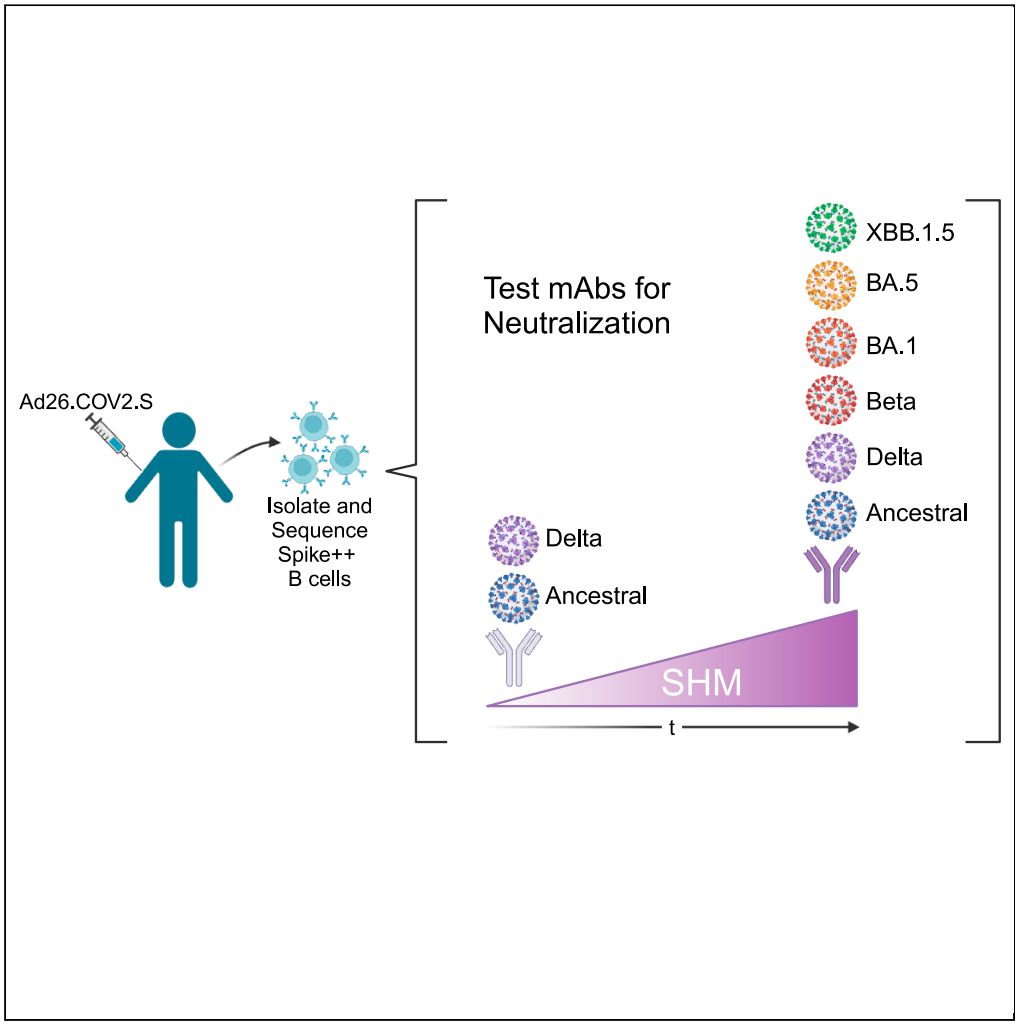


Catherine Jacob-Dolan, Michelle Lifton, Olivia C. Powers, ..., Jerald Sadoff, Aaron G. Schmidt, Dan H. Barouch

dbarouch@bidmc.harvard.edu

## Highlights

Ad26.COV2.S induced neutralizing antibodies increase in breadth over 8 months

Somatic hypermutation in spike specific B cells also increases over 8 months

Highly mutated monoclonal antibodies neutralize more variants than less mutated

Ad26.COV2.S induces long term affinity maturation

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# B cell somatic hypermutation following COVID-19 vaccination with Ad26.COV2.S

Catherine Jacob-Dolan,[1,2,3,4] Michelle Lifton,[1] Olivia C. Powers,[1] Jessica Miller,[1] Nicole P. Hachmann,[1] Mya Vu,[2] Nehalee Surve,[1] Camille R. Mazurek,[1] Jana L. Fisher,[1] Stefanie Rodrigues,[1] Robert C. Patio,[1] Trisha Anand,[1] Mathieu Le Gars,[5] Jerald Sadoff,[5] Aaron G. Schmidt,[2,3] and Dan H. Barouch[1,2,4,6,*]

## SUMMARY

**The viral vector-based COVID-19 vaccine Ad26.COV2.S has been recommended by the WHO since 2021 and has been administered to over 200 million people. Prior studies have shown that Ad26.COV2.S induces durable neutralizing antibodies (NAbs) that increase in coverage of variants over time, even in the absence of boosting or infection. Here, we studied humoral responses following Ad26.COV2.S vaccination in individuals enrolled in the initial Phase 1/2a trial of Ad26.COV2.S in 2020. Through 8 months post vaccination, serum NAb responses increased to variants, including B.1.351 (Beta) and B.1.617.2 (Delta), without additional boosting or infection. The level of somatic hypermutation, measured by nucleotide changes in the VDJ region of the heavy and light antibody chains, increased in Spike-specific B cells. Highly mutated mAbs from these sequences neutralized more SARS-CoV-2 variants than less mutated comparators. These findings suggest that the increase in NAb breadth over time following Ad26.COV2.S vaccination is mediated by affinity maturation.**

## INTRODUCTION

Due to the continued mutation of SARS-CoV-2, understanding and designing vaccines which can induce broad immune responses that cover emerging and future variants is a research priority. SARS-CoV-2 variants arise due to mutations that often confer advantages in viral replication, infectivity, fitness, and/or allow for escape of immune pressure.[1–9] However, the humoral immune response is not static, and B cells can affinity mature for weeks to months in germinal center (GC) reactions via somatic hypermutation (SHM) and select for higher affinity antibodies then produced in the differentiated plasma cells.[10]

Through this process of mutation and selection, the B cells affinity mature to the antigen. Resulting B cells and plasma cells exiting the GC express BCRs or secrete antibodies, respectively, with higher antigen affinity to the specific antigen.[10–13] Here we present data showing B cell affinity maturation measured by SHM for longer than anticipated, at least 8 months after Ad26.COV2.S vaccination, and correlated with an increase in serum and monoclonal antibody neutralization breadth. These data align with the observed increase in serum neutralizing breadth previously reported for Ad26.COV2.S vaccinated individuals and the similar expansion of memory breadth reported for Ad26.COV2.S and mRNA vaccinated individuals.[14–16]

Almost all COVID-19 vaccines represent novel vaccine platforms (e.g., mRNA vaccines, vector vaccines, or adjuvants used in protein vaccines).[17–24] Here we study a closely followed early pandemic cohort from 2020 which allows us to study vaccine responses in the absence of previous vaccination or infection, which is no longer possible given widespread boosting and infection.[1,25–29] In a previous study, we analyzed the durability and breadth of NAb responses from SARS-CoV-2 naive individuals vaccinated with Ad26.COV2.S and reported an expansion in the breadth of viral variant neutralization from month 1 to month 8 following vaccination.[14] In our current study, we evaluated the mechanism for this observation.

Here, we describe the cross-neutralization potential of serum NAbs and levels of SHM in SARS-CoV-2 Spike-specific B cells over 8 months following Ad26.COV2.S vaccination in individuals who did not receive any booster immunizations and who remained uninfected, as tested by longitudinal nucleocapsid serology, during this time period. We find that Ad26.COV2.S vaccination induced serum NAb responses that increased in breadth and B cell responses with increased SHM over 8 months. Additional boosts and SARS-CoV-2 infection then further increased serum NAb responses at later timepoints. Recombinant monoclonal antibodies (mAbs) produced based on the BCR (B cell receptor) sequencing also increased in neutralizing breadth over time and with increased SHM.

[1]Center for Virology and Vaccine Research, Beth Israel Deaconess Medical Center, Boston, MA, USA
[2]Ragon Institute of Mass General, MIT, and Harvard, Cambridge, MA, USA
[3]Harvard Medical School, Department of Microbiology, Boston, MA, USA
[4]Harvard Medical School, Department of Immunology, Boston, MA, USA
[5]Janssen Vaccines and Prevention B.V., Leiden, the Netherlands
[6]Lead contact
*Correspondence: dbarouch@bidmc.harvard.edu
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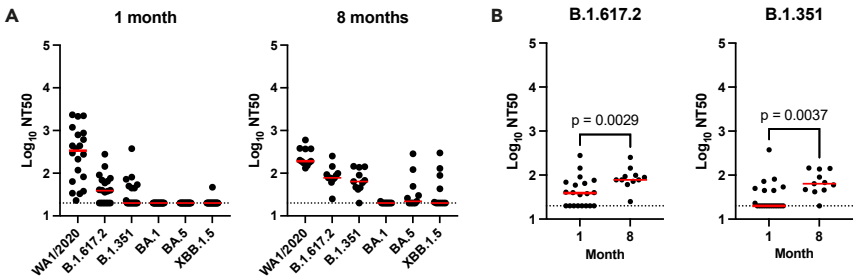


**Figure 1. Serum neutralization of SARS-CoV-2 Variants over 8 months**

(A) Neutralizing antibody (NAb) titers measured by luciferase based pseudovirus neutralization assay for ancestral WA1/2020, B.1.617.2 (Delta), B.1.351 (Beta), BA.1 (Omicron), BA.5, and XBB.1.5 matched pseudoviruses for 1 month, 2 months, 3 months, and 8 months post first vaccination.

(B) Comparison of neutralization of B.1.617.2 and B.1.351 at 1 and 8 months timepoints ($p$ = 0.0029 and $p$ = 0.0037 respectively). The limit of quantitation (NT50 = 20) is represented by a dotted line. Medians are shown with red bars.

## RESULTS

### Serum binding and neutralization breadth over time

To assess serum neutralization, we performed pseudovirus neutralization assays using the ancestral (WA1/2020) or variant B.1.351, B.1.617.2, Omicron BA.1, BA.5, or XBB.1.5 in a cohort of 20 individuals that comprised the non-placebo participants in the Ad26.COV2.S phase 1/2a study run at BIDMC in 2020.[14,30] Serum was tested from early (1 month post immunization, $n$ = 20) and late (8 months post immunization, $n$ = 11 available samples) timepoints against these pseudoviruses. All participants received 1 or 2 Ad26.COV2.S immunizations and were followed clinically and longitudinally tested for nucleocapsid serology at each visit to confirm that these individuals remained uninfected during this time period in 2020. Samples from three participants were excluded from the 8-month timepoint, as these participants had been either boosted or infected before the 8-month timepoint and displayed markedly higher NAb profiles (Figure S1).[14] As previously reported, we detected increases in serum NAb responses to the B.1.351 and B.1.617.2 variants over 8 months, although the Omicron variants (e.g., BA.1, BA.5 and XBB.1.5) remained relatively resistant to neutralization after 1 or 2 Ad26.COV2.S immunizations (Figure 1A). The individual paired data, when available as not all samples were available at month 8, shows a mixture of increases and decreases in responses to WA1/2020 over time but clear trends of increased B.1.617.2 and B.1.351 at month 8 compared to month 1 (Figure S2A). The increases in B.1.617.2 and B.1.351 serum neutralization from 1 month to 8 months were significant ($p$ = 0.0029 with a 2-fold change and $p$ = 0.0037 with a 3.2-fold change respectively) (Figure 1B). The individual paired data also details significant increases in B.1.617.2 and B.1.351 serum neutralization from 1 month to 8 months ($p$ = 0.0056 and $p$ = 0.0281 respectively) (Figure S2B). Binding antibody data by electrochemiluminescence assay (ECLA), capturing both neutralizing and non-neutralizing binding antibodies, similarly showed initially high responses which waned over time but remained durably detectable in most participants. (Figure S3).

Serum neutralization of these Omicron variants was observed in individuals after they received additional vaccines or were infected, which occurred in most individuals by month 14 during the B.1.617.2 surge in 2021. These additional antigen exposures increased serum neutralizing titers against the ancestral strain as well as Omicron variants (Figure S4); increased breadth was also observed in binding antibody titers at these late time points (Figure S5). These data show that serum neutralization breadth increased over eight months following initial Ad26.COV2.S vaccination and further increased following further antigenic exposure by boosting or infection. In order to ascertain the specific responses to Ad26.COV2.S, only samples with no confirmed breakthrough infection or known additional vaccination were included in our further analysis, the 8-month time point was therefore the latest timepoint included in further analysis.

### Somatic hypermutation levels in BCRs over time

To assess the molecular basis for the expansion of serum NAb breadth from month 1 to month 8, we sorted Spike-specific single B cells from multiple different donors and sequenced the BCR variable regions (Figure S6). In sorting we also determined that the memory B cell compartment contained significant percentage Spike-specific B cells though the compartment remained roughly the same percentage of the total B cell pool at both 1 and 8 months (Figure S7). By comparing the sequences from the sorted B cells to germline human BCRs using IMGT HighV-QUEST, we determined the number of nucleotide changes (SHM) per heavy ($IgV_H$) or light ($IgV_L$) chain variable region from successful variable chain sequencing reads. We observed significantly higher levels of SHM in both the $IgV_H$ and $IgV_L$ chains by month 3 ($p$ = 0.0366 and $p$ = 0.0060 for $IgV_H$ and $IgV_L$ respectively) and particularly by month 8 ($p < 0.0001$ for both $IgV_H$ and $IgV_L$) (Figure 2). The sequenced $IgV_H$, $IgV_K$, and $IgV_L$ were distributed over a diverse set of germlines, including emphasis on $IgV_H$ 3–30, $IgV_H$ 1–69, though mostly at the 8 months timepoint, and $IgV_H$ 3–9, with no significant changes in levels of clonality across the timepoints, similar to previous reports (Figures S8 and S9).[15]

We next assessed the potential correlation between the serum neutralization breadth and SHM levels from one to 8 months. The neutralization potency NT50 for the variants B.1.617.2 and B.1.351 correlated with the median SHM level of the $IgV_H$ genes for each individual tested (B.1.617.2 r = 0.3827, $p$ = 0.0488; B.1.351 r = 0.5952, $p$ = 0.0011) (Figures 3A and 3B). These data show a significant increase in SHM levels in

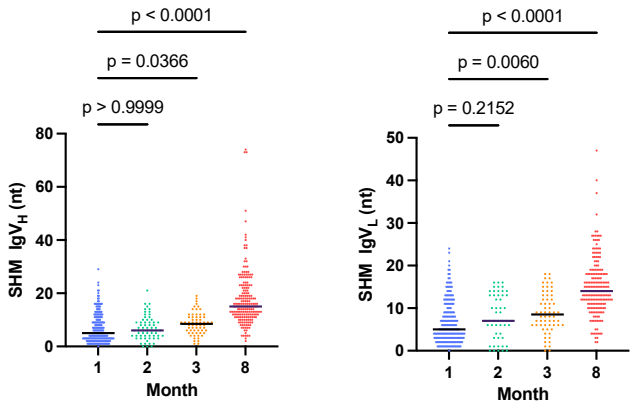


**Figure 2. Somatic hypermutation of spike specific B cells over 8 months**

Nucleotide changes in the $V_H$ and $V_L$ chain sequences of Spike-specific B cells at 1 month (blue dots), 2 months (green dots), 3 months (orange dots), and 8 months (red dots) post vaccination. 2m, 3m, and 8m time points were compared to 1m by two sided Mann-Whitney tests ($p > 0.9999$, $p = 0.0366$, and $p < 0.0001$ respectively for $V_H$ and $p = 0.2152$, $p = 0.0060$, and $p$, 0.0001 respectively for $V_L$. Median lines are shown with black bars.

Spike specific B cells over 8 months following Ad26.COV2.S vaccination, which correlates with the increase in serum neutralization breadth to include greater B.1.351 and B.1.617.2 neutralization. Previous reports have shown increases in SHM levels in Spike specific B cells over 6 months following Ad26.COV2.S vaccination, we have expanded upon this to show the continuation of SHM increases through 8 months here.[15]

### Monoclonal antibody analysis

To determine whether the increases in serum neutralization breadth were linked to increased SHM in Spike-specific B cells, we recombinantly produced 99 mAbs from the 1-month timepoint and 103 mAbs from the 8-month timepoint and tested them for Spike binding and neutralization of variants. The mAbs tested from the 8-month timepoint samples included more robust neutralizers and significantly more antibodies that were able to neutralize variants such B.1.617.2 and B.1.351 with also some increase in the number able to neutralize Omicron lineage variants such as BA.1, BA.5, and XBB.1.5, though these were not highly potent (Figure 4A). Though there was no significant increase in mAbs neutralizing potency against the ancestral strain from 1 to 8 months, the increases in neutralization of variants were significant both for early variants B.1.617.2 and B.1.351 with $p = 0.0097$ and $p < 0.0001$ respectively, and for later variants i.e., BA.1, BA.5, and XBB.1.5 with $p = 0.0002$, $p = 0.0470$, and $p = 0.0007$ though the number of mAbs neutralizing these later variants at either timepoint were low (Figure 4B). Variant Spike binding as measured by ECLA also shows higher binding and greater numbers of cross-reactive antibodies at the 8-month time point compared to the 1-month time point (Figure S10).

We next tested whether total (IgV + IgH) SHM of the neutralizing mAbs tested correlated with the breadth of the neutralization capacity of the mAbs, defined as number of variants neutralized. We found a positive correlation ($r = 0.64$, $p < 0.0001$) between the SHM level and detectable neutralization breadth of the mAbs tested (Figure 5). These data show increased binding and neutralization of variants at the mAb level over 8 months post Ad26.COV2.S immunization and a correlation between the level of SHM and the breadth of the mAb neutralization capacity.

## DISCUSSION

Our data demonstrate that Ad26.COV2.S vaccination leads to serum NAb responses that increase in breadth over 8 months, in the absence of further boosting or infection, as demonstrated by the increased neutralization of the B.1.617.2 (Delta) and B.1.351 (Beta) variants. The WA1/2020 serum NAb responses waned slightly in some participants and increased in others, this may be due to the waning of the initial blast response as is often seen shortly after vaccination.[31] However, serum NAb responses against Omicron variants required boosting or infection. We observed affinity maturation with increased SHM in Spike-specific B cells that correlated with the increased serum NAb breadth. Taken together, our findings suggest that continued affinity maturation may be responsible for the increased serum NAb breadth over time.

These data align with prior observations from our group and others. Previous studies have described 8-month durability and increase in serum neutralization breadth in Ad26.COV2.S vaccinated individuals as well as durable B cell memory responses and increasing SHM in Spike specific B cells up through 6 months post Ad26.COV2.S vaccination.[14,15] SHM is necessary to produce high affinity humoral immune responses specific to invading pathogens. After SHM, higher affinity BCRs recognizing the antigen are selected via $T_{FH}$ (T Follicular helper) cell help. These selected BCRs are then produced as antibodies by plasma cells after differentiation.[10–13] Broadly neutralizing antibodies (NAbs) against pathogens are often highly mutated, having gone through many rounds of SHM and selection.[32–37] However, continued SHM relies on the presence of the antigen in the GCs.[10,13,38–40] Here, we show that SHM occurs for at least 8 months post vaccination with Ad26.COV2.S,

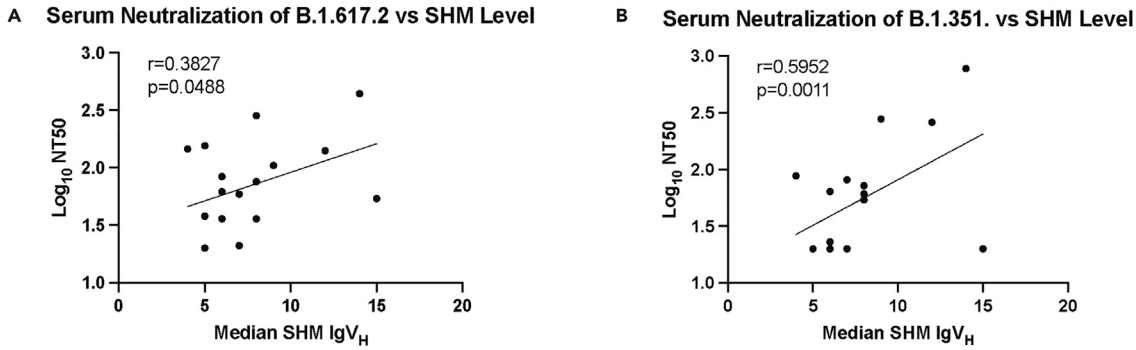


**Figure 3. Correlation of SHM and serum neutralization for B.1.351 and B.1.617.2**

Median nucleotide changes in the variable regions of the heavy ($IgV_H$) chain sequences of Spike specific B cells at 1m, 2m, 3m, and 8m post vaccination plotted against the corresponding pseudovirus serum neutralization NT50 values for (A) both B.1.617.2 (Delta) r = 0.382, $p$ = 0.048 and (B) B1.351 (Beta) r = 0.0.595, $p$ = 0.001.

which is longer than expected for a protein and inactivated virus vaccine but has been reported for virus infection and live virus vaccines.[13,40–43]

Our data confirm and extend prior observations that mRNA and Ad-based vaccines lead to increasing SHM levels through at least six months.[15,39,44,45] We show that increased SHM correlates increased breadth of serum neutralization, which was not reported for protein and inactivated virus based COVID-19 vaccines. This may be due to the differences in the timing of antigen availability from nucleic acid vaccines and protein or inactivated virus vaccines. Gene-based vaccines allow for more sustained antigen expression than protein or inactivated virus vaccines,[13,46,47] which may then allow persistence of antigen in the GC which enhances antibody potency.[13,40]

Increased SHM reported here correlated with increased SARS-CoV-2 variant neutralization breadth. SHM and subsequent selection elicits increased antibody affinity to antigen.[10,48] This increased affinity often results from improved interactions at the antibody-antigen binding interface (e.g., salt bridge, polar, hydrophobic interactions, rigidification or reorientation of the CDR loops, or any combination of these).[49–52] An increase in affinity may be coupled with enhanced specificity and either increased or decreased sensitivity to mutations[53–55]; antibodies elicited by infection with SARS-CoV-2 have previously been shown to be less susceptible to viral escape and have increased binding and neutralization breadth.[54,56] Here, we see that Ad26.COV2.S vaccination in humans induced continued affinity maturation and increased affinity and neutralization breadth contributing to a broader serum neutralization profile. At the monoclonal antibody level, we also observed that an increase in SHM correlated with an increase in neutralization breadth. The monoclonal antibodies that neutralized the greatest numbers of variants were among the most highly mutated antibodies, pointing toward long term affinity maturation-based increases in SHM leading to increases in neutralization breadth. The role of non-NAbs is also important to consider, it is known that these antibodies can be important in shaping immune responses to infection.[57] Further research will be required to probe the non-neutralizing Fc-domain based functionalities of responses to Ad26.COV2.S at the monoclonal level including Fc-region sequencing.[30]

Over 200 million doses of Ad26.COV2.S have been administered across Africa, USA, Europe, and Latin America, and an Ad26 based vaccine for Ebola has also been approved.[58–61] The work presented here provides data on the development of B cell responses over time following Ad26.COV2.S vaccination and emphasizes that B cell affinity maturation kinetics should be a future focus of research with gene-based vaccines. Ad26.COV2.S continues to be used as a booster for vaccinated or SARS-CoV-2 exposed individuals, and thus future work should ascertain whether this long-term expansion of breadth and SHM is also observed in pre-immune recipients who receive Ad26.COV2.S.

In summary, we show that Ad26.COV2.S induces durable immune responses in an early pandemic uninfected clinical trial cohort. We found that NAb responses increased in breadth over at least 8 months following vaccination with Ad26.COV2.S. We showed that this increase in NAb breadth correlated with increases in levels of SHM in peripheral Spike-specific B cells. Further investigation into mAb responses showed that the more highly mutated antibodies also correlated with greater NAb. These data suggest that low levels of antigen may persist following Ad26.COV2.S vaccination and may drive continued GC reactions, resulting in broader NAbs. Further research on the kinetics of antigen production and retention in GCs after vaccination would help increase our understanding of COVID-19 and other vaccines.

## Limitations of the study

This study was unable to follow the response to Ad26.COV2.S vaccinees significantly longer than 8 months due to the high level of circulating virus at the time and the approval of booster doses. Most individuals in the Phase 1/2b study were either infected or boosted soon after the 8-month time point, thus changing the trajectory of the immune response. It remains to be seen just how long affinity maturation induced by an Ad26 based vaccine can last in the absence of additional boosts or infection. Additionally, this study is unable to address questions about IgG subtypes which may be of import due to the use of variable region only sequencing. Future studies may be able to shed light on the IgG

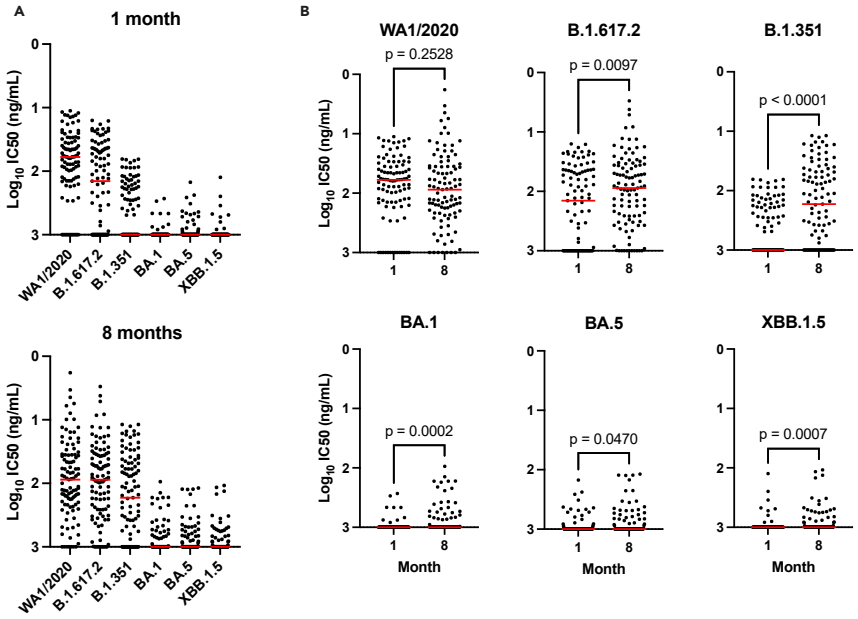


**Figure 4. Monoclonal antibody (mAb) neutralization of variants at 1 and 8 months**

(A) Inhibitory concentration 50 (IC50) in ng/mL of mAbs to the WA1/2020 (ancestral), B.1.617.2 (Delta), B.1.351 (Beta), BA.1 (Omicron), BA.5, and XBB.1.5 variants at 1- and 8-month post vaccination. (A) Comparison of IC50 at 1- and 8-month post vaccination for WA1/2020 ($p$ = 0.2528), B.1.671.2 ($p$ = 0.0097), B.1.351 ($p$ < 0.0001), BA.1 ($p$ = 0.0002), BA.5 ($p$ = 0.0470), and XBB.1.5 ($p$ = 0.0007). The limit of quantitation (IC50 = 1000 ng/mL) is represented by the x axis. Medians are shown with red bars.

subtype profile of responses induced by Ad26.COV2.S or other Ad26 based vaccines through systems serology approaches or broader sequencing approaches.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE

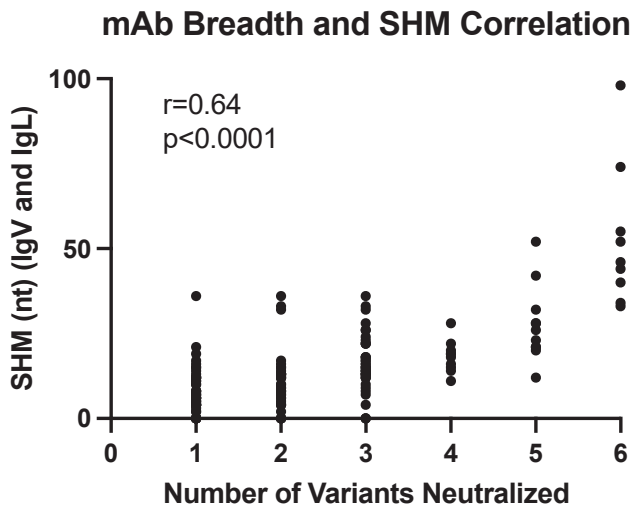


**Figure 5. Correlation of monoclonal antibody (mAb) neutralization breadth and SHM level**

The number of variants neutralized is plotted against the corresponding total SHM (IgV and IgL) level in nucleotide changes (nt) for each tested mAb that detectably neutralized (<1 μg/mL) at least one variant, r = 0.64, $p$ < 0.0001.

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.109716.


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## AUTHOR CONTRIBUTIONS

C.J.D., A.G.S., and D.H.B. designed the study. C.J.D. and M.L. performed the cell enrichment, staining, and sorting. C.J.D., O.P., J.M., N.P.H., N.S., C.R.M., J.L.F., S.R., R.C.P., and T.A. performed the immunologic and virologic assays. C.J.D. and M.V. produced the recombinant proteins. C.J.D. produced the probes and performed the BCR sequencing. C.J.D. analyzed the data and performed any statistical analyses. M.L.G. and J.S. led the clinical trial which generated the samples used in this study. C.J.D. and D.H.B. wrote the paper with the involvement of all the co-authors.

## DECLARATION OF INTERESTS

D.H.B. is a co-inventor on provisional vaccine patents licensed to Janssen (63/121,482; 63/133,969; 63/135,182). M.L.G. and J.S. are employees of Janssen. The authors report no other conflict of interest.


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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| anti-human CD3-APCR700 | BD Pharmigen | UCHT1; RRID: AB_2744385 |
| anti-human CD20-BV650 | BD Pharmigen | 2H7; RRID: AB_2744327 |
| anti-human CD19-PECy7 | BD Pharmigen | SJ25C1; RRID: AB_396893 |
| anti-human CD27-BV711 | BioLegend | O323; RRID: AB_11219201 |
| anti-human CD71-BV786 | BD Pharmigen | L10.1; RRID: AB_2743078 |
| anti-human CD14-BV421 | BD Pharmigen | M5E2; RRID: AB_2739154 |
| anti-human CD16-BV421 | BD Pharmigen | 3G8; RRID: AB_2716865 |
| anti-human CD38-PECy5 | BioLegend | HIT2; RRID: AB_313459 |
| anti-human CD8-BV421 | BioLegend | SK1; RRID: AB_2629583 |
| anti-human IGD-BV510 | BD Pharmigen | IA6-2; RRID: AB_396111 |
| anti-human IgG-BB700 | BD Pharmigen | G18-145; RRID: AB_287143 |
| anti-human IgM-BV605 | BD Pharmigen | G20-127; RRID: AB_2737928 |
| Biological samples | | |
| EDTA, SST, Paxgene collection tubes with whole blood, from humans | BIDMC Clinical Research Center | N/A |
| Chemicals, peptides, and recombinant proteins | | |
| SARS-CoV-2 Spike S.PP | A.G.Schmidt Laboratory | N/A |
| NIR Live/Dead | Invitrogen | L34994 |
| Strep-Tactic APC | IBA Life Sciences | 6-5010-001 |
| Strep-Tactic PE | IBA Life Sciences | 6-5000-001 |
| TALON resin | Takara | 635503 |
| Superscript IV | Thermo Fisher | 18090010 |
| RNAseOUT | Invitrogen | 10777019 |
| Critical commercial assays | | |
| EasySep™ Human Pan-B Cell Enrichment Kit | StemCell Technologies | 19554 |
| V-PLEX COVID-19 Platform (Multiplex ELISA) | MesoScale Discovery | K15567U |
| Deposited data | | |
| GenBank: PP563986-PP564389 | This paper | https://www.ncbi.nlm.nih.gov/genbank/ |
| Experimental models: Cell lines | | |
| HEK293T-hACE2 | This paper | N/A |
| HEK293T | ATCC | CRL_3216 |
| Expi293F | Thermo Fisher Scientific | A14527 |
| Oligonucleotides | | |
| Tiller human BCR sequencing primers | Tiller et al. J Immunol Methods | https://doi.org/10.1016/j.jim.2007.09.017 |
| Recombinant DNA | | |
| S2P Plasmid | J.S.McLellan Laboratory | N/A |
| Human IgG Plasmids | A.G.Schmidt Laboratory | N/A |
| psPAX2 | AIDS Resource and Reagent Program | 11348 |

(*Continued on next page*)

*Continued*

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| pLenti-CMV Puro-Luc | Addgene | 17477 |
| pcDNA3.1-SARS CoV-2 SdCT | This paper | N/A |
| Software and algorithms | | |
| FlowJo 10.8.1 | BD Bioscience | http://www.flowjo.com/ |
| GraphPad Prism 10.1 | GraphPad Software | http://www.graphpad.com/scientific-software/prism/ |
| Diva SoftMax Pro 6.5.1 | SoftMax Pro Software | http://www.moleculardevices.com/products/microplate-readers/acquisition-and-analysis-software/softmax-pro-software |
| BioRendr | BioRendr | https://biorender.com/ |
| Other | | |
| A Study of Ad26.COV2.S in Adults (COVID-19) | Janssen Vaccines & Prevention B.V. | ClinicalTrials.gov Identifier: NCT04436276 |

## RESOURCE AVAILABILITY

### Lead contact

Dan H. Barouch: dbarouch@bidmc.harvard.edu.

### Materials availability

Materials are available via contact with the corresponding author/lead contact.

### Data and code availability

Data reported in this paper will be shared by the lead contact upon request. Antibody sequences have been deposited at GenBank: PP563986 - PP564389. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

## EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

The aim of this study was to perform an in-depth longitudinal analysis of the development of the humoral immune response to Ad26.COV2.S vaccination in humans. This original study was conducted at Beth Israel Deaconess Medical Center with IRB approval and has been previously published.[30] See Figure S1 for demographic information.

We isolated serum and SARS-CoV-2 Spike specific B cells from the peripheral blood of non-placebo Ad26.COV2.S Phase 1b trial participants at early and late timepoints, sequenced the BCRs of these B cells, and recombinantly expressed a diverse panel of IgGs from the donors for further characterization.

Samples were selected from frozen PBMCs of non-placebo participants originally collected in the CoV1001 Phase 1b trial of Ad26.COV2.S immunogenicity in humans run at Beth Israel Deaconess Medical Center in Boston, MA.[30]

## METHOD DETAILS

### Expression and purification of recombinant SARS-CoV-2 antigens

Plasmids encoding the SARS-CoV-2 RBDs were designed based on GenBank sequences MN975262.1 (SARS-CoV-2); constructs were codon-optimized and synthesized by Integrated DNA Technologies (IDT). SARS-CoV-2 RBD contained HRV 3C–cleavable 8xHis and an SBP tag. The plasmid encoding the SARS-CoV-2 Spike S.PP was generously provided by the McLellan Lab. SARS-CoV-2 Spike contained a C-terminal fold on trimerization domain and HRV 3C–cleavable 6xHis and 2xStrep II tags. Proteins were transiently expressed in Expi293F cells (Thermo Fisher Scientific). Five to 7 days after transfection, supernatants were harvested by centrifugation and purified using cobalt TALON resin (Takara) followed by size exclusion chromatography on a Superdex 200 Increase 10/300 GL column (GE Healthcare).

### Probe generation

SARS-CoV-2 Spike was expressed and purified as described above. The Strep II tagged Spike trimers were mixed with fluorescently labeled Strep-Tactin (APC and PE) to form Spike-APC and Spike-PE. Both labeling steps were performed for 30 min at 4°C before sorting.

### Single B cell sorting

B cells were purified from PBMCs using EasySep Human Pan-B Cell Enrichment Kit (Stemcell Technologies) and incubated with 25 nM of each SARS-CoV-2 Spike probe for 30 min at 4°C. Cells were stained with anti-human CD3-APCR700 (UCHT1 BD Pharmigen), CD20-BV650 (2H7, BD Pharmigen), CD19-PECy7 (SJ25C1, BD Pharmigen), CD27-BV711 (O323, BioLegend), CD71-BV786 (L1.01, BD Pharmigen), CD14-BV421 (M5E2, BD Pharmigen), CD16-BV421 (3G8, BD Pharmigen), CD38-PECy5 (HIT2, BioLegend), CD8-BV421 (SK1, Biolegend), IgD-BV510 (IA6-1, BD Pharmigen), IgG-BB700 (G18-145, BD Pharmigen), IgM-BV605 (G20-127, BD Pharmigen), and NIR Live/Dead (Invitrogen) for an additional 30 min. SARS-CoV-2 Spike specific single B cells, defined as L/D-$CD3^{-}CD8^{-}CD14^{-}CD16^{-}$CD20+IgD-Spike++, were single cell index sorted using BD FACSAria II (BD Biosciences) into 96-well plates containing lysis buffer supplemented with RNAseOUT. Plates were stored at −80°C for subsequent analysis. Flow cytometry data were analyzed using FlowJo software version 10.8.1.

### BCR sequencing

BCR sequencing was carried out as described previously.[42] In brief, mRNA from lysed B cells, 100–200 sorted B cells per donor used, was reverse transcribed with SuperScript IV (ThermoFisher) with poly dT and random hexamers. Two rounds of PCR were performed to amplify heavy and light chain transcripts. Amplified products from the second round PCR were detected by 2% agarose gel and sequenced by Sanger sequencing (Genewiz), success rate of sequencing was in the range of 50%. Sequences of all sorted B cells were analyzed using IMGT HighV-QUEST. Sequences were uploaded in bulk to IGMT HighV-QUEST which returned summary files from which the SHM level in amino acid change and the germline identity of the mAbs were sourced for graphical representation. SHM data were analyzed and graphed using GraphPad Prism 10.1 (GraphPad Software).

### Electrochemiluminescence assay (ECLA)

ECLA plates (MesoScale Discovery SARS-CoV-2 IgG, Panels 22, 23) were designed and produced for multiplex binding assays with up to 10 antigen spots in each well, including either Spike or RBD proteins from multiple SARS-CoV-2 variants.[62] The plates were blocked with 150 μL of Blocker A (1% BSA in distilled water) solution for at least 30 min at room temperature shaking at 700 rpm with a digital microplate shaker. During blocking the serum was diluted to 1:5,000 or 1:50,000 in Diluent 100. The calibrator curve was prepared by diluting the calibrator mixture from MSD 1:10 in Diluent 100 and then preparing a 7-step 4-fold dilution series plus a blank containing only Diluent 100. The plates were then washed 3 times with 150 μL of Wash Buffer (0.5% Tween in 1x PBS), blotted dry, and 50 μL of the diluted samples and calibration curve were added in duplicate to the plates and set to shake at 700 rpm at room temperature for at least 2 h. The plates were again washed 3 times and 50 μL of SULFO-Tagged anti-Human IgG detection antibody diluted to 1x in Diluent 100 was added to each well and incubated shaking at 700 rpm at room temperature for at least 1 h. Plates were then washed 3 times and 150 μL of MSD GOLD Read Buffer B was added to each well and the plates were read immediately after on a MESO QuickPlex SQ 120 machine. MSD titers for each sample was reported as Relative Light Units (RLU) which were calculated as Sample RLU minus Blank RLU and then fit using a logarithmic fit to the standard curve. The upper limit of detection was defined as 2x106 RLU for each assay and the signal for samples which exceeded this value at 1:5,000 serum dilution was run again at 1:50,000 and the fitted RLU was multiplied by 10 before reporting. The lower limit of detection was defined as 1 RLU and an RLU value of 100 was defined to be positive for each assay. Data were analyzed using GraphPad Prism 9.5.1 (GraphPad Software).

### Pseudovirus neutralization assay

The SARS-CoV-2 pseudoviruses expressing a luciferase reporter gene were used to measure pseudovirus neutralizing antibodies in the sera as described previously.[63] In brief, the packaging construct psPAX2 (AIDS Resource and Reagent Program), luciferase reporter plasmid pLenti-CMV PuroLuc (Addgene) and Spike protein expressing pcDNA3.1-SARS-CoV-2 SΔCT were co-transfected into HEK293T cells (ATCC CRL_3216) with lipofectamine 2000 (ThermoFisher Scientific). Pseudoviruses of SARS-CoV-2 variants were generated by using WA1/2020 strain (Wuhan/WIV04/2019, GISAID accession ID: EPI_ISL_402124), B.1.351 (Beta, GISAID accession ID: EPI_ISL_712096), B.1.617.2 (Delta, GISAID accession ID: EPI_ISL_2020950), BA.1 (Omicron, GISAID ID: EPI_ISL_7358094.2), BA.5 (GSAID ID: EPI_ISL_12268495.2), BQ.1.1 (GISAID ID: EPI_ISL_14752457), XBB.1 (GISAID ID: EPI_ISL_15232105), or XBB.1.5 variants (GISAID ID: EPI_ISL_16418320). The supernatants containing the pseudotype viruses were collected 48h after transfection; pseudotype viruses were purified by filtration with 0.45-μm filter. To determine the neutralization activity of human serum, HEK293ThACE2 cells were seeded in 96-well tissue culture plates at a density of 1.75 × 104 cells per well overnight. 3-fold serial dilutions of heat-inactivated serum samples were prepared and mixed with 50 μL of pseudovirus. The mixture was incubated at 37°C for 1 h before adding to HEK293ThACE2 cells. After 48 h, cells were lysed in Steady-Glo Luciferase Assay (Promega) according to the manufacturer's instructions. SARS-CoV-2 neutralization titers were defined as the sample dilution at which a 50% reduction (NT50) in relative light units was observed relative to the average of the virus control wells. Data were analyzed using GraphPad Prism 9.5.1 (GraphPad Software).

## QUANTIFICATION AND STATISTICAL ANALYSIS

Descriptive statistics were calculated using GraphPad Prism 9.5.1 (GraphPad Software). Non-parametric Mann-Whitney tests were used to test for significance between groups and Spearman's rank correlation tests were used to test for correlations between datasets. P-values less than or 0.05 were considered significant.

## ADDITIONAL RESOURCES

Samples used in this analysis derived from the clinical trial: A Study of Ad26.COV2.S in Adults (COVID-19) (ClinicalTrials.gov Identifier: NCT04436276) sponsored by Janssen Vaccines & Prevention B.V.