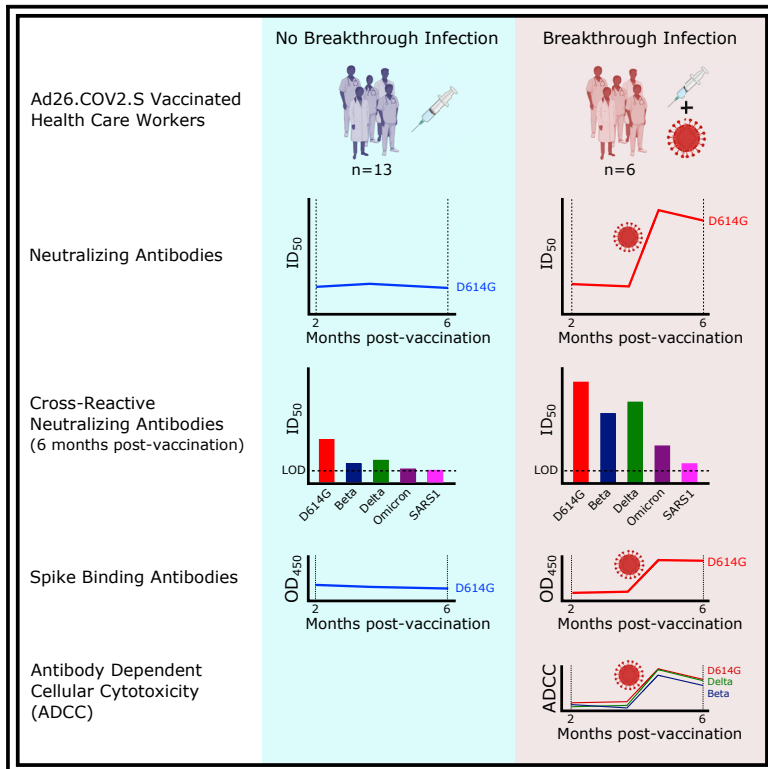


Ad26.COVID.S breakthrough infections induce high titers of neutralizing antibodies against Omicron and other SARS-CoV-2 variants of concern

Graphical Abstract



Authors

Dale Kitchin, Simone I. Richardson, Mieke A. van der Mescht, ..., Theresa M. Rossouw, Veronica Ueckermann, Penny L. Moore

Correspondence

pennym@nicd.ac.za

In brief

Kitchin, Richardson et al. show that responses to Ad26.COVID.S persist for 6 months after vaccination. Furthermore, mild breakthrough infection after Ad26.COVID.S vaccination results in dramatic boosts in binding and neutralizing antibody titers and Fc effector function. This includes high titers of neutralizing antibodies against the highly resistant Omicron variant.

Highlights

- Ad26.COVID.S neutralizing antibodies persist 6 months post-vaccination
- Breakthrough infection boosts binding antibodies, ADCC, and neutralization
- Boosted neutralizing antibodies cross-react with SARS-CoV-2 variants including Omicron
- Vaccination and infection synergistically contribute to high levels of immunity



Report

Ad26.COVS breakthrough infections induce high titers of neutralizing antibodies against Omicron and other SARS-CoV-2 variants of concern

Dale Kitchin,^{1,2,13} Simone I. Richardson,^{1,2,13} Mieke A. van der Mescht,³ Thopisang Motlou,^{1,2} Nonkululeko Mzindle,^{1,2} Thandeka Moyo-Gwete,^{1,2} Zanele Makhado,^{1,2} Frances Ayres,^{1,2} Nelia P. Manamela,^{1,2} Holly Spencer,^{1,2} Bronwen Lambson,^{1,2} Brent Oosthuysen,^{1,2} Haajira Kaldine,^{1,2} Marizane du Pisanie,⁴ Mathilda Mennen,⁵ Sango Skelem,⁵ Noleen Williams,⁵ Ntobeko A.B. Ntusi,^{5,6,7} Wendy A. Burgers,^{7,8,9} Glenda G. Gray,¹⁰ Linda-Gail Bekker,^{7,11} Michael T. Boswell,⁴ Theresa M. Rossouw,³ Veronica Ueckermann,⁴ and Penny L. Moore^{1,2,7,12,14,*}

¹National Institute for Communicable Diseases (NICD) of the National Health Laboratory Service (NHLS), Johannesburg, South Africa

²SAMRC Antibody Immunity Research Unit, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

³Department of Immunology, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa

⁴Division for Infectious Diseases, Department of Internal Medicine, Steve Biko Academic Hospital and University of Pretoria, Pretoria, South Africa

⁵Cape Heart Institute, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa

⁶Department of Medicine, University of Cape Town and Groote Schuur Hospital, Cape Town, South Africa

⁷Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Cape Town, South Africa

⁸Division of Medical Virology, Department of Pathology, University of Cape Town, Cape Town, South Africa

⁹Wellcome Centre for Infectious Diseases Research in Africa, University of Cape Town, Cape Town, South Africa

¹⁰The South African Medical Research Council, Tygerberg, South Africa

¹¹The Desmond Tutu HIV Centre, University of Cape Town, Cape Town, South Africa

¹²Centre for the AIDS Programme of Research in South Africa, Durban, South Africa

¹³These authors contributed equally

¹⁴Lead contact

*Correspondence: pennym@nicd.ac.za

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SUMMARY

The Janssen (Johnson & Johnson) Ad26.COVS non-replicating viral vector vaccine has been widely deployed for COVID-19 vaccination programs in resource-limited settings. Here we confirm that neutralizing and binding antibody responses to Ad26.COVS vaccination are stable for 6 months post-vaccination, when tested against multiple SARS-CoV-2 variants. Secondly, using longitudinal samples from individuals who experienced clinically mild breakthrough infections 4 to 5 months after vaccination, we show dramatically boosted binding antibodies, Fc effector function, and neutralization. These high titer responses are of similar magnitude to humoral immune responses measured in convalescent donors who had been hospitalized with severe illness, and are cross-reactive against diverse SARS-CoV-2 variants, including the neutralization-resistant Omicron (B.1.1.529) variant that currently dominates global infections, as well as SARS-CoV-1. These data have implications for population immunity in areas where the Ad26.COVS vaccine has been widely deployed, but where ongoing infections continue to occur at high levels.

INTRODUCTION

A phase 3 clinical trial of Ad26.COVS in eight countries demonstrated 85% protection against severe disease,¹ including in South Africa, where the trial coincided with the emergence of the Beta (B.1.351) variant, which was shown to have increased resistance to neutralizing antibodies.^{2,3} As a result, Ad26.COVS was made available to South African health care workers (HCWs) in early 2021 through the Sisonke open-label, phase 3b clinical trial. Globally, this vaccine has also been used widely in several countries, including the United States

and European Union member states, with 5.38, 15.68, and 16.16 million doses administered in these regions, respectively, by the beginning of November 2021.

Subsequently, South Africa has experienced a third and fourth wave of infection, driven by the Delta (B.1.617.2) and Omicron (B.1.1.529) variants, respectively, with reports of Ad26.COVS breakthrough infections (BTIs) occurring. Infections following mRNA vaccination result in boosted neutralizing antibody titers,⁴⁻⁶ but less is known about the immunological consequences of BTI after Ad26.COVS vaccination. With the emergence in late November 2021⁷ of the Omicron variant, which



now dominates global infections and has more spike mutations in key neutralizing epitopes than any variant to date, a key question is whether Ad26.COV2.S-vaccinated individuals who experienced breakthrough infections in the previous Delta-driven wave would have substantial neutralizing responses against this variant.

Here, we evaluated the durability and breadth of vaccine-elicited humoral responses in 19 HCWs vaccinated with Ad26.COV2S in February–March 2021 (Figure 1A). Second, we characterized the humoral response to BTI in a subset of six individuals with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) PCR-confirmed infections 4 to 5 months (median number of months: 4.4; interquartile range [IQR]: 4.1–4.8) following vaccination. Five of these participants were followed longitudinally 2 to 6 months post-vaccination, whereas for the sixth BTI participant only 2- and 6-month post-vaccination samples were available (Table S1). These BTIs occurred between June and August 2021, during the third wave of SARS-CoV-2 infections in South Africa. This wave was driven by the more transmissible Delta variant, which accounted for between 40% and 95% of genomes sequenced in South Africa over this period.⁸ BTIs were thus most likely caused by the Delta variant, though sequencing data for these participants were not available. Participants, of whom 16 of 19 were female, had a median age of 34 (IQR: 30–40 years) and all presented with mild disease (Table S1). All 19 participants were SARS-CoV-2 naive prior to vaccination, as confirmed by nucleocapsid ELISA (Figure 1B).

RESULTS

We first assessed the durability of vaccine-elicited antibody responses in individuals who were confirmed to have remained uninfected up to 6 months post-vaccination, by nucleocapsid ELISA (Figure 1B). Spike binding responses against the original D614G variant were measured at 2, 4, and 6 months post-vaccination. No significant reduction in binding was observed over this period (Figure 1C), as has been previously reported.^{9–11} We also used a spike-pseudotyped lentivirus neutralization assay to measure longitudinal neutralization titers against the ancestral D614G variant (which differs from the vaccine spike protein by a single D614G mutation), six SARS-CoV-2 variants with increased transmissibility and/or immune escape mutations, and SARS-CoV-1. The SARS-CoV-2 variants included Beta, Delta, Gamma (P.1), and Omicron, as well as C.1.2, and A.VOI.V2, isolated in South Africa and Angola, respectively, which share common mutations with variants of concern (VOCs) and are of local relevance to the Southern African region.^{12,13}

For the ancestral D614G variant, geometric mean titers (GMTs) were stable up to 6 months post-vaccination (GMTs of 104, 117, and 96 at 2, 4, and 6 months post-vaccination), consistent with previous studies^{10,11} (Figures 1D and 2A). Where detectable, titers against the six variants were similarly stable over 6 months, showing no significant differences over time (Figure 2A), as observed in two previous studies.^{10,14} However, for all variants tested, titers were 1.9- to 4.2-fold lower at 2 months post-vaccination compared with the D614G variant, as reported elsewhere^{10,14–17} (Figure 2A). For the Beta and Delta variants, in

particular, half of non-BTI vaccinees showed no detectable neutralization at 6 months post-vaccination (Figure 2C). As expected, titers against SARS-CoV-1 were low, with GMTs of 28 and 21 at 2 and 4 months, respectively, and undetectable at 6 months post-vaccination (Figure 2A).

We next assessed the breadth and magnitude of humoral immune responses following BTI. In all participants, BTI occurred between 3 and 5 months post-vaccination. Prior to BTI, the nucleocapsid binding responses in both the BTI and non-BTI participants were negative, and only detected following BTI (Figure 1B). There were also no significant differences in D614G spike binding responses between the BTI and non-BTI participants prior to 3 to 4 months post-vaccination (Figure 1C). However, following infection, there was a 3.3-fold increase in spike responses, which peaked at approximately 2 weeks post-infection (5 months post-vaccination) and remained constant until 1 month post-infection (6 months post-vaccination) (Figure 1C).

We also assessed the impact of BTI on Fc effector functions, which have been implicated in protection from severe coronavirus disease 2019 (COVID-19) disease, and which generally retain activity against VOCs.^{18,19} We examined whether levels of antibody-dependent cellular cytotoxicity (ADCC), measured by ability to cross-link Fc γ R1IIa were boosted following BTI. Similar to binding, ADCC against D614G remained stable up to 3 to 4 months post-vaccination, with a rapid 3.1-fold increase in activity after BTI (Figure 1E). These responses peaked (geomean RLU: 712) at approximately 2 weeks post-infection (5 months post-vaccination), but declined slightly (geomean RLU: 558) by 1 month post-infection (6 months post-vaccination) (Figure 1E). Both before and after infection, ADCC was cross-reactive against D614G, Beta, and Delta variants, showing only slight decreases against VOCs relative to D614G across all time points (geomean RLUs of 712, 626, and 702 against D614G, Beta, and Delta, respectively, at 2 weeks post-infection). This illustrates the resilience of Fc effector function against VOCs in Ad26.COV2.S BTI participants.

Neutralization titers against D614G closely mirrored the spike binding and ADCC response, with no significant differences in titers between the BTI and non-BTI participants prior to 3 to 4 months post-vaccination, but with a dramatic increase in titers for all participants (407-fold increase from 102 to 41,528 GMT) following infection (Figures 1D and 2B). This increase in neutralization titers is similar in magnitude to what was previously reported for a single individual with Ad26.COV2.S BTI.¹⁰ We observed extremely high neutralization titers at approximately 2 weeks post-infection (5 months post-vaccination), which declined by approximately 4.7-fold 1 month thereafter (Figure 1D). Neutralization titers after BTI were also significantly higher against six SARS-CoV-2 variants relative to non-BTI participants (40- to 154-fold difference in GMT), and SARS-CoV-1 (9-fold difference in GMT) (Figures 2B and 3). This includes the highly neutralization-resistant Omicron variant, against which titers ranged from 161 to 1,858 (GMT of 843) for the BTI participants (Figure 3). Thus, in contrast to vaccine-elicited responses, BTI after a single dose of Ad26.COV2.S resulted in neutralization of all SARS-CoV-2 variants, with a GMT >800 against Omicron, and >3,000 against all other SARS-CoV-2 variants (Figures 2B, 2C, and 3).

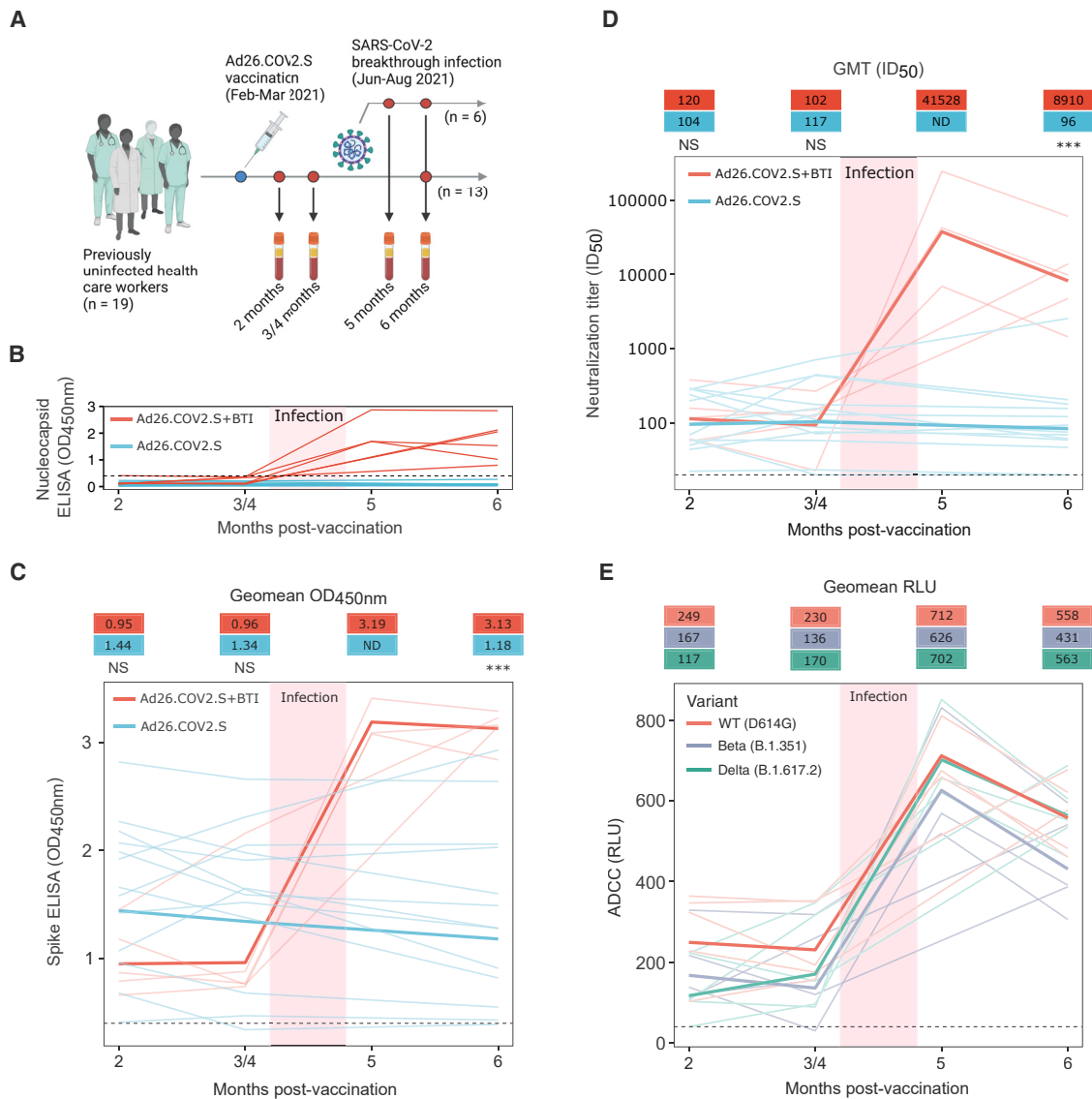


Figure 1. Ad26.COVID.S BTI boosted plasma binding and neutralization antibody titers against the ancestral variant (D614G) and increased ADCC activity against the D614G, Beta, and Delta variants

(A) Nineteen HCWs, vaccinated with a single dose of Ad26.COVID.S, were recruited, with six having BTIs. Longitudinal blood draws occurred between 2 and 6 months post-vaccination. However, for one BTI participant, only 2- and 6-month post-vaccination plasma samples were available.

(B) Nucleocapsid ELISA binding (OD_{450nm}), from 2 to 6 months post-vaccination, is shown for each BTI and non-BTI participant by red and blue lines, respectively.

(C) Spike binding responses to the D614G spike protein (OD_{450nm}) by ELISA for BTI and non-BTI participants are shown in red and blue, respectively, with each line representing individual responses over time. Lines in bold show the geomean OD_{450nm} for each group.

(D) Neutralization titers (ID₅₀) against the D614G variant, from 2 to 6 months post-vaccination are shown for each BTI and non-BTI participant with longitudinal data by red and blue lines, respectively. Lines in bold indicate the GMTs for the BTI and non-BTI groups.

(E) Cross-reactive ADCC activity for each BTI participant with longitudinal data up to 6 months post-vaccination, shown as relative light units (RLUs). ADCC activity for each participant against the D614G, Beta, and Delta variants is shown by the red, blue, and green lines, respectively. Lines in bold show the geomean RLU for each variant. The threshold for positivity for each assay is indicated by a dashed line in each figure. All results are the mean of two independent experiments. Statistical analyses were performed using the Mann-Whitney test between groups, with *** denoting $p < 0.001$, NS for non-significant, and ND for no data.

DISCUSSION

Overall our data confirm durable vaccine-elicited humoral immune responses 6 months after a single dose of Ad26.COVID.S, consistent with other studies.^{10,14–17} Moreover, despite relatively

modest titers after vaccination, we observed significantly boosted binding antibodies, ADCC, and neutralization activity following BTI. This boost resulted in neutralization titers in BTI participants at 1 month post-infection (GMT 8,249) that were higher than those elicited by a two-dose Pfizer-BioNTech

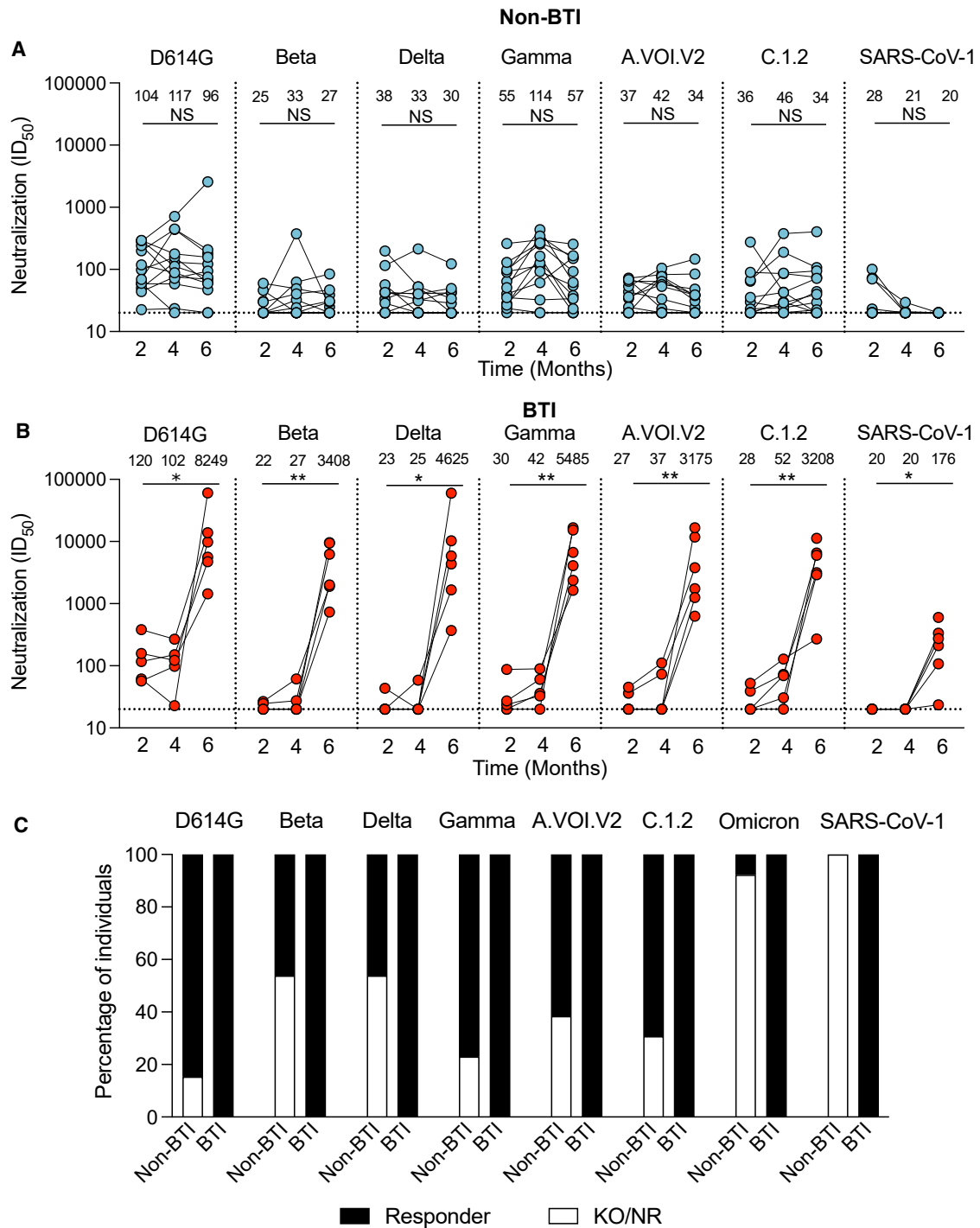


Figure 2. Longitudinal neutralization responses over 6 months for BTI and non-BTI participants against SARS-CoV-2 variants and SARS-CoV-1

(A and B) Neutralization ID₅₀ titers are shown for (A) Ad26.COVS2.S vaccinees who did not have breakthrough infection (non-BTI) and (B) BTI Ad26.COVS2.S vaccinees at 2, 3, 4, and 6 months post-vaccination, against the D614G, Beta, Delta, Gamma, C.1.2., and A.VOI.2 variants, and SARS-CoV-1. All results are the mean of two independent experiments. Significance is shown as per Friedman test, across all time points where NS denotes non-significant, * denotes $p < 0.05$, and ** denotes $p < 0.01$.

(C) Percentage of individuals who are neutralization responders (Black; ID₅₀ > 20), or are either non-responders or show knockout relative to D614G (KO/NR, ID₅₀ < 20; white) at 6 months post-vaccination.

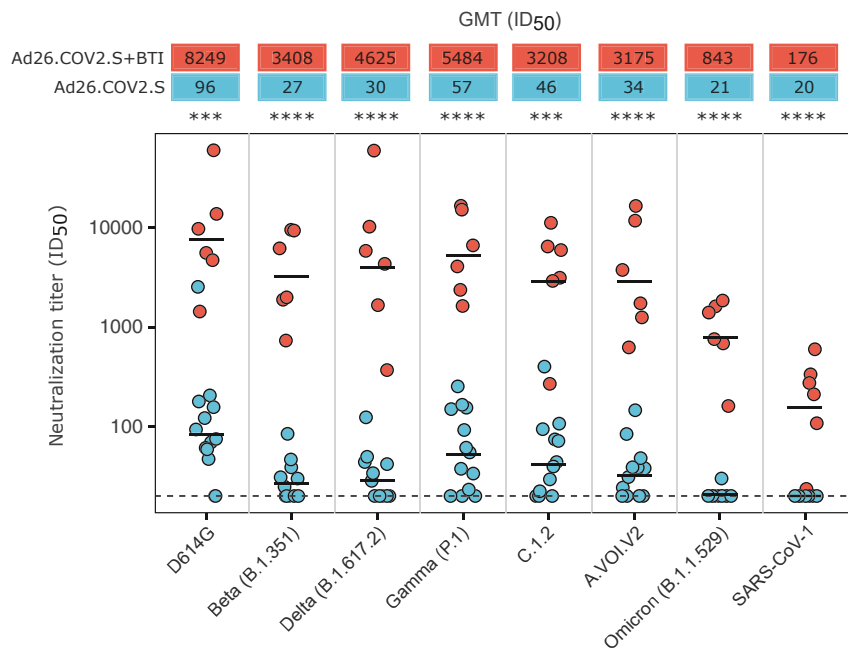


Figure 3. BTI results in increased plasma neutralization titers against all SARS-CoV-2 variants, and SARS-CoV-1, 6 months post-vaccination

The neutralization titers against the ancestral (D614G), Beta (B.1.351), Delta (B.1.617.2), Gamma (P.1), C.1.2, A.VO1.V2, and Omicron (B.1.1.529) SARS-CoV-2 variants, and SARS-CoV-1, for six BTI participants relative to 13 non-BTI participants at the 6-month post-vaccination visit (approximately 1 month post-BTI). Each dot represents the neutralization titer of a single participant, with the BTI participants and non-BTI participants shown in red and blue, respectively. The GMT for each group against each variant is shown by a black horizontal bar in the plot, with the values given in the red and blue boxes above the plot. Neutralization titers in the BTI group were significantly higher than those of the non-BTI group (40- to 154-fold higher GMT against the SARS-CoV-2 variants and 9-fold higher against SARS-CoV-1). All results are the mean of two independent experiments. Statistical analyses were performed using the Mann-Whitney test between groups, with *** denoting $p < 0.001$ and **** denoting $p < 0.0001$.

(BNT162b2) vaccine regimen (GMT: 1,128) 2 month post-vaccination, or those observed in convalescent donors who had previously been hospitalized with moderate (GMT: 993) or severe disease (GMT: 3,747) (Figure S1). Though we note that these comparisons differ by number of antigen exposures and timing, among other variables, this illustrates the extremely high level of boosting observed in BTIs. Similar to BTI individuals, we have previously confirmed that ADCC, binding, and neutralization are also significantly boosted following vaccination in individuals who were previously infected,²⁰ but not to the same levels we report here for BTI (GMT of 1,372 versus 8,249 for neutralization, respectively) (Figure S1). Whether this is a result of increased affinity maturation broadening the response against variants, or rather the boosting of vaccine-elicited cross-reactive memory B cells to higher titers, remains to be determined and will be the focus of future work.

These data add to previous reports of BTI following mRNA vaccination, which results in >30-fold increased neutralization potency, suggesting broad relevance across multiple vaccine modalities.^{4,5,21} Affinity maturation following SARS-CoV-2 infection can lead to the development of broader and more potent neutralizing antibodies.^{22,23} We have previously shown that the sequence of the spike in a prior infection influenced the breadth and potency of neutralizing antibodies following vaccination with Ad26.COV2.S.²⁰ Whether spike identity in BTIs (here, likely a heterologous exposure to the Delta variant after priming with ancestral sequence) similarly contributes to the selection of cross-reactive B cells is not yet known.²⁴ Taken together, these findings suggest a strongly synergistic effect of vaccination and infection, which will contribute to higher levels of protective community immunity in areas with high burden of infection, and may have contributed to the low hospitalization rates seen during the Omicron-driven fourth wave in South Africa.²⁵ As homologous and heterologous boosters are deployed, this effect may be

further enhanced.²⁶ Overall, this study provides insight into the magnitude and quality of humoral immune responses elicited by BTIs after an adenovirus-based vaccine, with implications for public health interventions in regions that have experienced high levels of SARS-CoV-2 transmission.

Limitations of study

This study is limited by the relatively small number of BTIs that were characterized. We also note that the median age of the BTI participants was higher than that of the non-BTI participants (BTI participants median age: 39 years, IQR: 32–59 years; non-BTI participants median age: 33 years, IQR: 29–36 years), which may impact comparisons between the two groups.^{11,27} We do not have sequencing data to determine the variant responsible for BTI, and it is possible that the infecting viral sequence may impact the quality of the response, as we have previously shown.^{20,28} We note that the pseudotyped virus neutralization assay used here can only assess the effectiveness of neutralizing antibodies against the spike protein in preventing viral entry into host cells, and cannot detect any effects antibodies may have on viral replication or cell-to-cell spread.^{29,30} Inherent differences between the pseudotyped viruses and authentic, replication-competent SARS-CoV-2 viruses, such as spike density and geometry on the virion surface, may also result in differences in sensitivity.^{29,30} However, neutralization titers from pseudovirus and replication-competent SARS-CoV-2 assays generally correlate well.^{31,32} We also focused only on humoral responses, and T cell responses following BTI will need to be defined in future studies.

STAR★METHODS

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

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
 - Lead contact
 - Materials availability
 - Data and code availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
 - Human subjects
 - Cell lines
- **METHOD DETAILS**
 - SARS-CoV-2 antigens
 - SARS-CoV-2 spike enzyme-linked immunosorbent assay (ELISA)
 - Spike plasmid and lentiviral pseudovirus production
 - Pseudovirus neutralization assay
 - Antibody-dependent cellular cytotoxicity (ADCC) assay
- **QUANTIFICATION AND STATISTICAL ANALYSIS**

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xcrm.2022.100535>.

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AUTHOR CONTRIBUTIONS

D.K., S.I.R., and P.L.M. conceived the study, designed experiments, analyzed data, and wrote the paper. Z.M., F.A., B.O., and B.E.L. made molecular constructs and expressed antibodies. Z.M. and T.M.G. expressed and purified recombinant antigens. Z.M. and F.A. performed spike and nucleocapsid ELISAs. T.M., N.M., and H.K. made pseudoviruses and performed neutralization experiments. S.I.R., N.P.M., and H.S. performed ADCC assays. L.G.B. and G.G.G. conceptualized and led the Sisonke Ad26.COVS2 trial. S.I.R. established the NICD HCW cohort of vaccinees. M.A.v.d.M., M.d.P., M.T.B., T.M.R., and V.U. established the Steve Biko HCW cohort and provided samples. M.M., S.S., N.W., N.A.B.N., and W.A.B. established the Groote Schuur Hospital cohort and provided samples. All authors critically reviewed and approved the final manuscript.

DECLARATION OF INTERESTS

P.L.M. is a member of the advisory board for *Cell Reports Medicine*. All other authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CR3022	Genscript (https://www.genscript.com)	N/A
BD23	Dr Nicole Doria-Rose, VRC, USA	N/A
P2B-2F6	Dr Nicole Doria-Rose, VRC, USA	N/A
anti-IgG APC (clone QA19A42)	Biologend	Cat#366905 RRID:AB_2888847
Palivizumab	Medimmune	Synagis; RRID: AB_2459638
Bacterial and virus strains		
SARS-CoV-2 pseudoviruses for ancestral (D614G), Beta, Delta, Gamma, Omicron, C.1.2, A.VOI.V2	Wibmer et al., 2021; Richardson et al., 2021 This paper	N/A
Biological samples		
Convalescent hospitalized blood samples	Groote Schuur Hospital	https://www.gsh.co.za
Convalescent hospitalized blood samples	Steve Biko Academic Hospital	https://www.sbah.org.za
AD26.COVID.S vaccinee blood samples	Groote Schuur Hospital	https://www.gsh.co.za
AD26.COVID.S vaccinee blood samples	Steve Biko Academic Hospital	https://www.sbah.org.za
AD26.COVID.S vaccinee blood samples	National institute for Communicable Diseases	https://www.nicd.ac.za
Chemicals, peptides, and recombinant proteins		
SARS-CoV-2 original (D614G) spike protein	Dr Jason McKellan	N/A
Critical commercial assays		
PEI-MAX 40,000	Polysciences	Cat # 24765-1
QUANTI-Luc luciferase	Invivogen	Cat# rep-qlc2
Luciferase	Promega	Cat# PRE263B-C
Experimental models: Cell lines		
Human Embryonic Kidney (HEK) 293F	Dr Nicole Doria-Rose, VRC, USA	N/A
HEK293T/ACE2.MF	Dr Michael Farzan, Scripps, USA	N/A
Jurkat-Lucia™ NFAT-CD16 cells	Invivogen	Cat # jkti-nfat-cd16
Human Embryonic Kidney (HEK) 293T cells	Dr George Shaw, UPenn,USA	N/A
Recombinant DNA		
Spike Hexapro plasmid	Dr Jason McKellan	N/A
SARS-CoV-2 ancestral variant spike (D614G) plasmid	Wibmer et al., 2021	N/A
Beta spike (L18F, D80A, D215G, K417N, E484K, N501Y, D614G, A701V, 242-244 del) plasmid	Wibmer et al., 2021	N/A
Delta spike (T19R, R158G L452R, T478K, D614G, P681R, D950N, 156-157 del) plasmid	Keeton et al., 2021	N/A
Gamma spike (L18F, T20N, P26S, D138Y, R190S, K417T, E484K, N501Y, D614G, H655Y, T1027I, V1176F)	Richardson et al., 2021	N/A
Omicron spike ((A67V, Δ69-70, T95I, G142D, Δ143-145, Δ211, L212I, 214EPE, G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493K, G496S, Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, L981F) plasmid	This paper	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
C.1.2. spike (P9L, C136F, Δ144, R190S, D215G, Δ242-243, Y449H, E484K, N501Y, D614G, H655Y, N679K, T716I, T859N) plasmid	This paper	N/A
A.VOI.V2 spike (D80Y, Δ144, I210N, Δ211, D215G, R246M, Δ247-249, W258L, R346K, T478R, E484K, H655Y, P681H, Q957H) plasmid	This paper	N/A
SARS-CoV-1 spike plasmid	Dr Elise Landais, Scripps	N/A
Firefly luciferase encoding lentivirus backbone plasmid	Dr Michael Farzan, Scripps	N/A
Software and algorithms		
Geneious software	Biomatters Ltd	https://www.geneious.com
FACSDiva 9	BD Biosciences	https://www.bdbiosciences.com
FlowJo 10	FlowJo, LLC	https://www.flowjo.com
R version 4.1.0	The R Foundation for Statistical Computing	https://www.r-project.org
ggplot2 package	Tidyverse	https://ggplot2.tidyverse.org
Graphpad Prism 9	Graphpad	https://www.graphpad.com
Biorender	Biorender	https://www.biorender.com

RESOURCE AVAILABILITY

Lead contact

Further information and reasonable requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Penny Moore (pennym@nicd.ac.za).

Materials availability

Materials will be made available by request to Penny Moore (pennym@nicd.ac.za).

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- 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 SUBJECT DETAILS

Human subjects

HCWs vaccinated with one dose of Ad26.CoV2.S (5×10^{10} viral particles) as part of the Sisonke implementation trial were followed longitudinally and plasma sampled at 2-, 3- (in some cases 4-) and 6-months post-vaccination. An additional plasma sample was collected from BTI participants at 5-months post-vaccination, which was approximately 2-weeks post-infection. Non-BTI participants were recruited from HCWs at the National Institute for Communicable Diseases (NICD) (Johannesburg), while BTI participants were recruited from HCWs at the NICD, Steve Biko Academic Hospital (Tshwane, South Africa) and Groote Schuur Hospital (Cape Town, South Africa). Lack of prior infection in these individuals was confirmed by Nucleocapsid ELISA as described.²⁰ Ad26.CoV2.S vaccinees with prior SARS-CoV-2 infection were recruited from a longitudinal study of healthcare workers enrolled from Groote Schuur Hospital, with plasma samples collected 2-months post-vaccination. Plasma was also collected from thirteen participants that had received two doses of the Pfizer BioNTech vaccine (BNT162b2) 2-months after they had received their last dose (Johannesburg, South Africa). Convalescent participants were recruited as part of a hospitalised cohort at the Steve Biko Academic Hospital between May and August 2020, with plasma samples collected 10-days after the initial positive PCR test. Ethics approval was obtained from the Human Research Ethics Committees of the University of the Witwatersrand (ethics reference number: M210465),

University of Pretoria (ethics reference number: 247/2020) and University of Cape Town (ethics reference numbers: 190/2020 and 209/2020). Written informed consent was obtained from all participants.

Cell lines

Human embryo kidney HEK293T cells were cultured at 37°C, 5% CO₂, in DMEM containing 10% heat-inactivated fetal bovine serum (Gibco BRL Life Technologies) and supplemented with 50 µg/mL gentamicin (Sigma). Cells were disrupted at confluence with 0.25% trypsin in 1 mM EDTA (Sigma) every 48–72 hours. HEK293T/ACE2.MF cells were maintained in the same way as HEK293T cells but were supplemented with 3 µg/mL puromycin for selection of stably transduced cells. HEK293F suspension cells were cultured in 293 Freestyle media (Gibco BRL Life Technologies) and cultured in a shaking incubator at 37°C, 5% CO₂, 70% humidity at 125 rpm maintained between 0.2 and 0.5 million cells/mL. Jurkat-LuciaTM NFAT-CD16 cells were maintained in IMDM media with 10% heat-inactivated fetal bovine serum (Gibco, Gaithersburg, MD), 1% Penicillin Streptomycin (Gibco, Gaithersburg, MD) and 10 µg/mL of Blasticidin and 100 µg/mL of Zeocin was added to the growth medium every other passage.

METHOD DETAILS

SARS-CoV-2 antigens

For ELISA, SARS-CoV-2 full ancestral spike (L18F, D80A, D215G, K417N, E484K, N501Y, D614G, A701V, 242-244 del) proteins were expressed in Human Embryonic Kidney (HEK) 293F suspension cells by transfecting the cells with the respective expression plasmid. After incubating for six days at 37°C, 70% humidity and 10% CO₂, proteins were first purified using a nickel resin followed by size-exclusion chromatography. Relevant fractions were collected and frozen at –80°C until use.

SARS-CoV-2 spike enzyme-linked immunosorbent assay (ELISA)

Two µg/ml of spike protein (ancestral D614G) was used to coat 96-well, high-binding plates and incubated overnight at 4°C. The plates were incubated in a blocking buffer consisting of 5% skimmed milk powder, 0.05% Tween 20, 1× PBS. Plasma samples were diluted to 1:100 starting dilution in a blocking buffer and added to the plates. IgG or IgA secondary antibody was diluted to 1:3000 or 1:1000 respectively in blocking buffer and added to the plates followed by TMB substrate (ThermoFisher Scientific). Upon stopping the reaction with 1 M H₂SO₄, absorbance was measured at a 450 nm wavelength. In all instances, mAbs CR3022 and BD23 were used as positive controls and Palivizumab was used as a negative control.

Spike plasmid and lentiviral pseudovirus production

The SARS-CoV-2 Wuhan-1 spike, cloned into pCDNA3.1 was mutated using the QuikChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies) and NEBuilder HiFi DNA Assembly Master Mix (NEB) to include D614G (ancestral) or lineage defining mutations for Beta (L18F, D80A, D215G, Δ242-244, K417N, E484K, N501Y, D614G and A701V), Delta (T19R, Δ156-157, R158G, L452R, T478K, D614G, P681R, D950N), Gamma (L18F, T20N, P26S, D138Y, R190S, K417T, E484K, N501Y, D614G, H655Y, T1027I, V1176F), C1.2. (P9L, C136F, Δ144, R190S, D215G, Δ242-243, Y449H, E484K, N501Y, D614G, H655Y, N679K, T716I, T859N), A.VOI.V2 (D80Y, Δ144, I210N, Δ211, D215G, R246M, Δ247-249, W258L, R346K, T478R, E484K, H655Y, P681H, Q957H) and Omicron (A67V, Δ69-70, T95I, G142D, Δ143-145, Δ211, L212I, 214EPE, G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493K, G496S, Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, L981F). SARS-CoV-1 spike was also cloned into pcDNA.

Pseudotyped lentiviruses were prepared as previously described.³ Briefly, pseudoviruses were produced by co-transfecting HEK293T cell line with one of the SARS-CoV-2 variant spike plasmids or the SARS-CoV-1 spike plasmid, in conjunction with a firefly luciferase encoding lentivirus backbone plasmid (HIV-1 pNL4.luc encoding the firefly luciferase gene) with PEIMAX (Polysciences). Culture supernatants were clarified of cells by a 0.45-µm filter and stored at –70°C.

Pseudovirus neutralization assay

For the neutralization assay, plasma samples were heat-inactivated and clarified by centrifugation. Heat-inactivated plasma samples from vaccine recipients were incubated with the SARS-CoV-2 or SARS-CoV-1 pseudotyped virus for 1 hour at 37°C, 5% CO₂. Subsequently, 1 × 10⁴ HEK293T cells engineered to over-express ACE-2 (293T/ACE2.MF, kindly provided by M. Farzan at Scripps Research) were added and incubated at 37°C, 5% CO₂ for 72 hours upon which the luminescence of the luciferase gene was measured. Titers were calculated as the reciprocal plasma dilution (ID₅₀) causing 50% reduction of relative light units. CB6 and CA1 were used as positive controls, while Palivizumab was used as a negative control.

Antibody-dependent cellular cytotoxicity (ADCC) assay

The ability of plasma antibodies to cross-link and signal through FcγRIIIa (CD16) and spike expressing cells was measured as a proxy for ADCC. HEK293T cells were transfected with 5 µg of either SARS-CoV-2 original variant (D614G), Beta or Delta spike plasmids using PEI-MAX 40,000 (Polysciences) and incubated for 2 days at 37°C. Expression of spike was confirmed by differential binding of CR3022 and P2B-2F6 and their detection by anti-IgG APC staining measured by flow cytometry. Subsequently, 1 × 10⁵ spike transfected cells per well were incubated with heat inactivated plasma (1:100 final dilution) or monoclonal antibodies (final

concentration of 100 $\mu\text{g}/\text{mL}$) in RPMI 1640 media supplemented with 10% FBS 1% Pen/Strep (Gibco, Gaithersburg, MD) for 1 hour at 37°C. Jurkat-LuciaTM NFAT-CD16 cells (Invivogen) (2×10^5 cells/well) were added and incubated for 24 hours at 37°C, 5% CO₂. A volume of 20 μL of supernatant was then transferred to a white 96-well plate with 50 μL of reconstituted QUANTI-Luc (Invivogen) secreted luciferase and read immediately on a Victor 3 luminometer with 1s integration time. Relative light units (RLU) of a no antibody control was subtracted as background. Palivizumab was used as a negative control, while CR3022 was used as a positive control, and P2B-2F6 to differentiate the Beta from the D614G variant. To induce the transgene 1 \times cell stimulation cocktail (ThermoFisher Scientific, Oslo, Norway) and 2 $\mu\text{g}/\text{mL}$ ionomycin in R10 was added as a positive control to confirm sufficient expression of the Fc receptor. CR3022 (for spike and RBD) or 4A8 (NTD) were used as positive controls and Palivizumab were used as negative controls. RLUs for original and Beta spikes were normalised to each other and between runs using CR3022. A cut off of 40 was determined by screening of 40 SARS-CoV-2 naive and unvaccinated individuals. All samples were run head-to-head in the same experiment as were all variants tested.

QUANTIFICATION AND STATISTICAL ANALYSIS

Analyses were performed in Prism (v9; GraphPad Software Inc, San Diego, CA, USA) and graphs generated using the ggplot2 package in R version 4.1.0. Where neutralization titers were below the limit of detection, these were assigned a nominal value of 20 in geometric mean titer (GMT) calculations, which is the lowest plasma dilution factor used in the neutralization assay. Non-parametric tests were used for all comparisons and all t tests were 2-sided. The Mann-Whitney test was used for unpaired comparisons between two groups, while the Kruskal-Wallis ANOVA with Dunns correction was used for multiple comparisons for unpaired groups. The Friedman test was used for multiple comparisons between paired groups. *P* values less than 0.05 were considered to be statistically significant.

Supplemental information

**Ad26.COVS breakthrough infections induce high
titers of neutralizing antibodies against
Omicron and other SARS-CoV-2 variants of concern**

Dale Kitchin, Simone I. Richardson, Mieke A. van der Mescht, Thopisang Motlou, Nonkululeko Mzindle, Thandeka Moyo-Gwete, Zanele Makhado, Frances Ayres, Nelia P. Manamela, Holly Spencer, Bronwen Lambson, Brent Oosthuysen, Haajira Kaldine, Marizane du Pisanie, Mathilda Mennen, Sango Skelem, Noleen Williams, Ntobeko A.B. Ntusi, Wendy A. Burgers, Glenda G. Gray, Linda-Gail Bekker, Michael T. Boswell, Theresa M. Rossouw, Veronica Ueckermann, and Penny L. Moore

Supplementary Information

Table S1: Study participant details relating to Figure 1, 2 and 3

PTID^a	Month of Vaccination	Age range at Vaccination	Gender	Month of BTI^b	Severity of infection
BTI01	2021/02	56-60	F	2021/06	Mild
BTI02	2021/02	36-40	F	2021/06	Mild
BTI03	2021/02	36-40	F	2021/07	Mild
BTI04	2021/02	56-60	F	2021/07	Mild
BTI05	2021/03	31-35	F	2021/08	Mild
BTI06*	2021/02	31-35	M	2021/07	Mild
V01	2021/03	56-60	F	N/A	N/A
V02	2021/03	31-35	F	N/A	N/A
V03	2021/03	31-35	M	N/A	N/A
V04	2021/03	26-30	F	N/A	N/A
V05	2021/03	31-35	M	N/A	N/A
V06	2021/03	31-35	F	N/A	N/A
V07	2021/03	51-55	F	N/A	N/A
V08	2021/03	26-30	F	N/A	N/A
V09	2021/03	31-35	F	N/A	N/A
V10	2021/03	26-30	F	N/A	N/A
V11	2021/03	26-30	F	N/A	N/A
V12	2021/03	36-40	F	N/A	N/A
V13	2021/03	26-30	F	N/A	N/A

^a Participant identifier (PTID)

^b Breakthrough infection (BTI)

* No longitudinal samples available, only 2- and 6-month post-vaccination samples

N/A: Not applicable

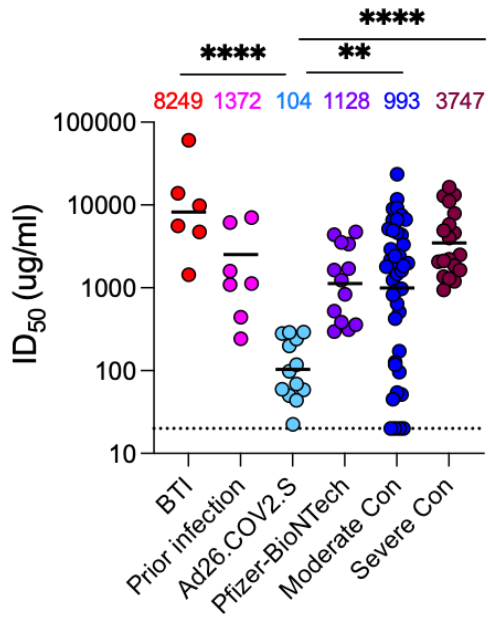


Figure. S1: BTI neutralization responses to the D614G variant compared to vaccinees and convalescent plasma related to Figure 3. Neutralization ID₅₀ titers of two-dose Pfizer-BioNTech (2-months post-vaccination) and one-dose Ad26.COVS vaccinees (2-months post-vaccination) and hospitalised convalescent individuals with moderate and severe infection 10-days after a positive PCR test are shown compared to BTI individuals (1-month post-infection). Individuals who were previously infected by SARS-CoV-2 prior to Ad26.CoV2.S vaccination are shown 2-months post-vaccination. Limit of detection is indicated by a dotted line. GMT is indicated by the black bars and above the plot, while significant differences between groups, as calculated by Kruskal-Wallis ANOVA with Dunns correction, are shown above the graph with with ** denoting p<0.01 and **** denoting p<0.0001.