Ad26.COV2.S breakthrough infections induce high titers of antibodies capable of neutralizing variants of concern

Dale Kitchin1,2,* , Simone I. Richardson1,2,* , Mieke A. van der Mescht3, Thopisang Motlou1,2, Nonkululeko Mzindle1,2, Thandeka Moyo-Gwete1,2, Zanele Makhado1,2, Frances Ayres1,2, Nelia P. Manamela1,2, Holly Spencer1,2, Bronwen Lamson1,2, Brent Oosthuysen1,2, Mathilda Mennen4, Sango Skelem4, Noleen Williams4, Ntobeko A.B. Ntusi2,5,6, Wendy A. Burgers6,7,8, Glenda G. Gray9, Linda-Gail Bekker6,10, Michael T. Boswell11, Theresa M. Rossouw3, Veronica Ueckermann11 and Penny L. Moore1,2,6,12#

Affiliations
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
4Cape Heart Institute, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa
5Department of Medicine, University of Cape Town and Groote Schuur Hospital, Cape Town, South Africa
6Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Cape Town, South Africa
7Division of Medical Virology, Department of Pathology, University of Cape Town, Cape Town, South Africa
8Wellcome Centre for Infectious Diseases Research in Africa, University of Cape Town, Cape Town, South Africa
9The South African Medical Research Council, Tygerberg, South Africa
10The Desmond Tutu HIV Centre, University of Cape Town, Cape Town, South Africa
11Division for Infectious Diseases, Department of Internal Medicine, Steve Biko Academic Hospital and University of Pretoria, Pretoria, South Africa
12Centre for the AIDS Programme of Research in South Africa, Durban, South Africa

*Equal contribution
# Corresponding author: email - pennym@nicd.ac.za

Abstract

The Janssen (Johnson & Johnson) Ad26.COV2.S non-replicating viral vector vaccine, which requires only a single dose and conventional cold chain storage, is a valuable tool for COVID-19 vaccination programs in resource-limited settings. Here we show that neutralizing and binding responses to Ad26.COV2.S vaccination are stable for 6-months post-vaccination, with responses highest against the ancestral vaccine-similar D614G variant. Secondly, using longitudinal samples from individuals who experienced clinically mild breakthrough infections 3-4 months after vaccination, we show dramatically boosted binding antibodies, Fc effector function and neutralization. These responses, which are cross-reactive against diverse SARS-CoV-2 variants and SARS-CoV-1, are of similar magnitude to humoral immune responses measured in severely ill, hospitalized donors. These data highlight the significant priming capacity of Ad26.COV2.S, and have implications for population immunity in areas where the single dose Ad26.COV2.S vaccine has been deployed.

(142 words)

NOTE: This preprint reports new research that has not been certified by peer review and should not be used to guide clinical practice.
A phase 3 clinical trial of Ad26.COV2.S in eight countries demonstrated 85% protection against severe disease, including in South Africa, where the trial coincided with the emergence of the neutralization-resistant Beta (B.1.351) variant. As a result, Ad26.COV2.S was made available to South African health care workers (HCWs) in early 2021 through the Sisonke open-label, phase 3b clinical trial. Globally the Ad26.COV2.S vaccine has also been adopted as part of COVID-19 vaccination programs in a number of countries, including South Africa, USA, and EU member states, with 5.38, 15.68 and 16.16 million doses administered in these regions, respectively, by the beginning of November 2021.

Here, we evaluated the durability and breadth of vaccine-elicited humoral responses in nineteen HCWs vaccinated with Ad26.COV2.S in February-March 2021 (Fig. 1a). Secondly, we characterised the humoral response to breakthrough infection (BTI) in a subset of six individuals with SARS-CoV-2 PCR-confirmed infections 4-5 months (median number of months: 4.4; interquartile range: 4.1-4.8) following vaccination. Five of these participants were followed longitudinally 2-6 months post-vaccination, while for the sixth BTI participant only a single sample from 1 month post-infection was available (Table S1). These BTIs occurred during the third wave of SARS-CoV-2 infections in South Africa, which was driven by the more transmissible Delta (B.1.617.2) variant.

Participants, of whom 16/19 were female, had a median age of 34 (interquartile range: 30-40 years) and all presented with mild disease (Table S1). All nineteen participants were SARS-CoV-2 naive prior to vaccination, as confirmed by nucleocapsid ELISA (Fig. 1b).

We first assessed the durability of vaccine-elicited antibody responses in individuals who remained uninfected. 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 (Fig. S1a). We also measured longitudinal neutralization titers against the ancestral D614G variant (which differs from the vaccine insert by a single D614G mutation), five SARS-CoV-2 variants with increased transmissibility and/or immune escape mutations (Beta, Delta, Gamma [P.1], C.1.2 and A.VOI.V2) and SARS-CoV-1. For the ancestral D614G variant, geometric mean titers (GMT) were stable up to 6 months post-vaccination (GMTs of 104, 117 and 96 at 2-, 4- and 6-months post-vaccination) (Fig. 1c, S2a). Where detectable, titers against the six variants were similarly stable over 6 months, showing no significant differences over time (Fig. S2a). However, for all variants tested, titers were 1.9-4.2-fold lower at 2-months post-vaccination compared to the D614G variant, as reported elsewhere (Fig. S2a). For the neutralization resistant Delta and Beta variants, in particular, approximately half of non-BTI vaccinees showed no detectable neutralization at 6-months post-vaccination (Fig. S2c). 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 (Fig. S2a).

We next assessed the 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 as expected (Fig 1b). There were also no significant differences in D614G spike binding responses between the BTI and non-BTI participants prior to
3/4-months post-vaccination (Fig. S1a). 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) (Fig. S1a).

Similar to binding, antibody-dependent cellular cytotoxicity (ADCC) against D614G remained stable up to 3/4-months post-vaccination, with a rapid 3.1-fold increase in activity after BTI (Fig. S1b). 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) (Fig. S1b). Both before and after infection, ADCC was cross-reactive against D614G, Beta and Delta variants, showing only slight decreases against variants of concern (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 3/4-months post-vaccination, but with a dramatic increase in titers for all participants (407-fold increase from 102 to 41528 GMT) following infection (Fig. 1c). This increase in neutralization titers is similar in magnitude to what was previously reported for a single individual with Ad26.COV2.S BTI. Neutralization titers peaked at approximately 2 weeks post-infection (5-months post-vaccination) and declined by approximately 4.7-fold one month thereafter (Fig. 1c). Neutralization titers after BTI were also significantly higher against five SARS-CoV-2 variants relative to non-BTI participants (70-154-fold difference in GMT), and SARS-CoV-1 (9-fold difference in GMT) (Fig. 2, S2b). Thus, in contrast to vaccine-elicited responses, BTI after a single dose of Ad26.COV2.S resulted in complete coverage of SARS-CoV-2 variants at titers >1:3,000 (Fig. 2, S2a, S2b).

Overall, our data shows durable vaccine-elicited humoral immune responses 6 months after a single dose of Ad26.COV2.S, consistent with other studies. Furthermore, despite relatively modest titers after vaccination, we observe 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 2 dose Pfizer-BioNTech (BNT162b2) vaccine regimen (GMT: 1,128), or those observed in acutely infected hospitalised individuals with moderate (GMT: 993) or severe disease (GMT: 3,747) (Fig. S3). 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, but not to the same levels we report here for BTI (1,372 vs 8,249 for neutralization, respectively) (Fig. S3). This confirms previous data indicating that Ad26.COV2.S is a potent priming immunogen11.

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. 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 disease burden. Furthermore, the potent priming of humoral immune responses by Ad26.COV2.S may be beneficial for homologous and
heterologous boosting strategies. Overall, this study provides insight into the magnitude and quality of humoral immune responses elicited by breakthrough infections after an adenovirus-based vaccine, with implications for public health interventions in regions with high SARS-CoV-2 transmission.

(1114 words)

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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. and N.M. 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.COV2.S trial. S.I.R. established the NICD HCW cohort of vaccinees. M.A.v.d.M, M.T.B, T.M.R., 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.

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Figure Legends

Fig. 1: Ad26.COV2.S breakthrough infection (BTI) results in boosted plasma neutralization titers against the ancestral variant (D614G). (a) Nineteen South African HCWs, vaccinated between February and March 2021 with a single dose of Ad26.COV2.S, were recruited into a vaccine durability study, with longitudinal blood draws occurring at 2-, 3- (or 4), 5- and 6-months post-vaccination. Six of the HCWs had BTIs (PCR-confirmed) between June and August 2021. (b) Nucleocapsid ELISA binding (OD450nm), from 2-6-months post-vaccination, is shown for each BTI and non-BTI participant by red and blue lines, respectively. The threshold of positivity is indicated by the dashed horizontal line. (c) Neutralization titers (ID50) against the D614G variant, from 2-6-months post-vaccination, are shown for each BTI and non-BTI participant by red and blue lines, respectively. Lines in bold indicate the GMTs for the BTI and non-BTI groups, with the values indicated above the figure. The horizontal dashed line indicates the lower limit of quantitation. 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.

Fig. 2: Breakthrough infection (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 and A.VOI.V2 SARS-CoV-2 variants, and SARS-CoV-1, for six BTI participants relative to thirteen non-BTI participants at the 6-months 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. Neutralization titers in the BTI group were significantly higher than those of the non-BTI group (70 to 154-fold higher GMT against the SARS-CoV-2 variants and 9-fold higher against SARS-CoV-1). Statistical analyses were performed using the Mann-Whitney test between groups, with *** denoting p<0.001 and **** denoting p<0.0001.

Materials and methods

Human samples
HCWs vaccinated with one dose of Ad26.CoV2.S (5x1010 viral particles) as part of the Sisonke implementation trial were followed longitudinally and plasma sampled at 2-, 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). 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.

SARS-CoV-2 antigens
For ELISA, SARS-CoV-2 original variant (D614G) full spike 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. A commercial, recombinant nucleocapsid protein (BioTech Africa, Cat. no.: BA25-C) was used as the antigen in the nucleocapsid ELISAs.

SARS-CoV-2 Spike and nucleocapsid enzyme-linked immunosorbent assay (ELISA)
Spike or nucleocapsid protein (2 μg/mL) 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, 1x PBS. Plasma samples were diluted to 1:100 starting dilution in a blocking buffer and added to the plates. IgG secondary antibody was diluted to 1:3000 or 1:1000 respectively in blocking buffer and added to the plates followed by TMB substrate (Thermo Fisher Scientific). Upon stopping the reaction with 1M 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 gene sequence, cloned into pcDNA3.1, was mutated using the QuikChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies) and NEBuilder HiFi DNA Assembly Master Mix (New England Biosciences) to include D614G (ancestral) or lineage defining mutations for Beta (L18F, D80A, D215G, Δ242-244, K417N, E484K, N501Y, D614G and A701V), Gamma (L18F, T20N, P26S, D138Y, R190S, K417T, E484K, N501Y, D614G, H655Y, T1027I, V1176F), Delta (T19R, Δ156-157, R158G, L452R, T478K, D614G, P681R, D950N), C1.2. (P9L, C136F, Δ144, R190S, D215G, Δ242-243, Y449H, E484K, N501Y, D614G, H655Y, N679K, T716I, T859N) and A.VOI.V2 (D80Y, Δ144, I210N, Δ211, D215G, R246M, Δ247-249, W258L, R346K, T478R, E484K, H655Y, P681H, Q957H). SARS-CoV-1 spike was also cloned into pcDNA3.1. Pseudotyped lentiviruses were prepared as previously described. Briefly, 293T/17 cells (HEK293T cell line) were co-transfected with a SARS-CoV-2 spike plasmid in conjunction with a firefly luciferase encoding lentivirus backbone plasmid (pNL4-3.Luc.R-E-) with PEI MAX (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-1/2 pseudotyped virus for 1 hour at 37°C, 5% CO₂. Subsequently, 1x10⁴ HEK293T cells engineered to over-express ACE-2 (293T/ACE2.MF), kindly provided by M. Farzan (The Scripps Research Institute) were added and incubated at 37°C, 5% CO₂ for 72 hours upon which the luminescence of luciferase was measured. Titers were calculated as the reciprocal plasma dilution (ID₅₀) causing 50% reduction of relative light units (RLU). Monoclonal antibodies CB6 and CA1 were used as positive controls.

**Antibody-dependent cellular cytotoxicity (ADCC) assay**

The ability of plasma antibodies to cross-link spike expressing cells and signal through FcγRIIIa (CD16) was measured as a proxy for ADCC. For spike assays, HEK293T cells were transfected with 5μg of SARS-CoV-2 original variant spike (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. Spike transfected cells (1x10⁵ per well) were incubated with heat inactivated plasma (1:100 final dilution) or monoclonal antibodies (final concentration of 100 μg/mL) in RPMI 1640 media supplemented with 10% FBS 1% Pen/Strep (Gibco, Gaithersburg, MD) for 1 hour at 37°C. Jurkat-Lucia™ NFAT-CD16 cells (Invivogen) (2x10⁵ cells/well) were added and incubated for 24 hours at 37°C, 5% CO₂. Twenty microliters of supernatant was then transferred to a white 96-well plate with 50 μl of reconstituted QUANTI-Luc secreted luciferase and read immediately on a Victor 3 luminometer with 1s integration time. The 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, 1X cell stimulation cocktail (Thermofisher Scientific, Oslo, Norway) and 2 μg/mL ionomycin in R10 was added to confirm sufficient expression of the Fc receptor.

**Statistical analysis**

Analyses were performed in Prism (v9; GraphPad Software Inc, San Diego, CA, USA). Non-parametric tests were used for all comparisons. 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.

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Fig. 1
Fig. 2