Prior infection with SARS-CoV-2 boosts and broadens Ad26.COV2.S immunogenicity in a variant dependent manner

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Summary

The Johnson and Johnson Ad26.COV2.S single dose vaccine, designed as an emergency response to the pandemic, represents an attractive option for the scale-up of COVID-19 vaccination in resource-limited countries. We examined the effect of prior infection with ancestral (D614G) or Beta variants on Ad26.COV2.S immunogenicity approximately 28 days post-vaccination. We compared healthcare workers who were SARS-CoV-2 naive (n=20), to those infected during the first wave prior to the emergence of Beta (n=20), and those infected in the second wave (n=20), when Beta was the dominant variant. We demonstrate that a priming exposure from infection significantly increased the magnitude of spike binding antibodies, neutralizing antibodies and antibody-dependent cellular cytotoxicity activity (ADCC) against D614G, Beta and Delta variants. The magnitude of antibody boosting was similar in both waves, despite the longer time interval between wave 1 infection and vaccination (7 months), compared to wave 2 (2 months). ADCC and binding cross-reactivity was similar in both waves. However, neutralization cross-reactivity varied by wave, showing that the antibody repertoire was shaped by the spike sequence of the infecting variant. Robust CD4 and CD8 T cell responses to spike of similar or higher magnitude as those elicited by infection were induced after vaccination. In contrast to antibody responses, prior infection was not required for the generation of high magnitude T cell responses, and T cell recognition of the Beta variant was fully preserved. Therefore, Ad26.COV2.S vaccination following prior infection, even >6 months previously, may result in substantially enhanced protection against COVID-19, of particular relevance in settings of high SARS-CoV-2 seroprevalence. Furthermore, the dominant impact of the infecting variant on neutralization breadth after vaccination has important implications for the design of second-generation vaccines based on variants of concern.

Introduction

The Johnson and Johnson Ad26.COV2.S vaccine is a single dose adenovirus 26 vectored vaccine expressing the SARS-CoV-2 Wuhan-1 stabilized spike. A phase 3 clinical trial of Ad26.COV2.S on three continents demonstrated 66% efficacy against moderate disease and 85% protection against severe disease 28 days after vaccination (Sadoff et al., 2021). Moreover, the South African component of the trial showed similar levels of efficacy despite the emergence of the neutralization resistant SARS-CoV-2 Beta (B.1.351/501Y.V2) variant. Vaccination with Ad26.COV2.S triggers neutralizing responses that gradually increase in magnitude and breadth, and potent Fc effector functions and T cell activity, both of which

retain activity against variants of concern (Moore et al., 2021; Barouch et al., 2021; Stephenson et al., 2021; Alter et al., 2021).

Prior infection boosts titers of binding and neutralizing antibodies elicited by mRNA vaccines (Manisty et al., 2021; Saadat et al., 2021; Stamatatos et al., 2021; Vanshylla et al., 2021; Wang et al., 2021b). These increased titers conferred the ability to neutralize SARS-CoV-2 variants, illustrating that only one dose of these vaccines may be sufficient to protect previously infected individuals from multiple variants. Similarly, a single dose of the BNT162b2 vaccine boosted antibody-dependent cellular cytotoxicity (ADCC) in previously infected individuals, and T cell cross-reactivity was largely retained (Geers et al., 2021; Reynolds et al., 2021; Tauzin et al., 2021). The impact of prior infection on immune responses elicited by vectored vaccines is less well defined (Havervall et al., 2021). Furthermore, whether the duration between infection and vaccination, or the genotype of the infecting virus impacts the subsequent immune response, is unknown. As vectored vaccines are cost-effective, and widely used in low- and middle-income countries, this has implications for vaccine efficacy in regions with high levels of infection, like South Africa (Hsiao et al., 2020; Mutevedzi et al., 2021; Sykes et al., 2021).

South Africa experienced a first wave of infections in mid-2020, dominated by the ancestral SARS-CoV-2 D614G variant. From November 2020 to February 2021, a second wave of infections was dominated by the Beta variant (Tegally et al., 2021; Wibmer et al., 2021; Cele et al., 2021; Wang et al., 2021a). The two virologically distinct waves provided the unique opportunity to assess whether the time between infection and vaccination impacted the magnitude and breadth of boosted antibody and T cell responses, and to compare immunogenicity in the context of vaccine-matched and mismatched infection.

Results

We established a longitudinal observational study of 400 healthcare workers (HCWs) with serial sampling since the first wave of COVID-19 in South Africa. We studied 60 of these HCWs who were vaccinated as part of a Phase 3b implementation trial of single dose Ad26.COV2.S vaccine (Takuva et al., 2021). HCWs were recruited into three groups, namely those without prior SARS-CoV-2 infection (n=20), and those with PCR-confirmed infection either during the first wave (n=20) or second wave (n=20) in South Africa (**Figure 1A** and **Supplementary Table S1**). The Beta variant accounted for >90% of infections in the Western Cape at the time (**Figure 1A**), making it likely that this variant was responsible for

infections in the latter group. Indeed, whole genome sequencing performed for 8/20 of the second wave participants confirmed that all 8 were infected with the Beta variant. Serological profiles were generated for each participant by measuring nucleocapsid and spike antibodies at all time points since July 2020 (3-8 monthly study visits) (**Supplementary Figure S1A-C**). These data confirmed the absence of infection (or re-infection), or the timing of first or second wave SARS-CoV-2 infections. As expected, infection-naive participants developed antibodies to spike after vaccination but remained negative for nucleocapsid antibodies. For those infected in the first wave, a median of 232 days pre-vaccination (IQR 200-261), waning spike antibodies were boosted after vaccination, and nucleocapsid antibodies continued their decline. Second wave participants demonstrated more diverse profiles, due to the upward trajectory of antibodies from recent infection, which occurred a median of 73 days (IQR 54-82) prior to vaccination. We identified one potential vaccine breakthrough infection and one suspected re-infection in the infection-naive and second wave groups, respectively (**Supplementary Figure S1A and C**). These two participants were excluded from subsequent analyses.

Peripheral blood mononuclear cells (PBMC) and plasma were collected at a study visit prior to vaccination (median 22 days pre-vaccination, IQR 14-29) and approximately one month after vaccination (median 29 days, IQR 28-34). We tested pre- and post-vaccination plasma for IgG binding antibodies to the ancestral D614G spike. Binding antibodies elicited by vaccination in the absence of infection (geometric mean titer [GMT]: 0.22) were comparable with those in both infected groups prior to vaccination (GMT: 0.28 and 0.32 for first and second wave, respectively). However, vaccination in HCWs with prior infection in both waves resulted in universally boosted binding responses (**Figure 1B**). In those infected in the first wave, binding antibodies were boosted three-fold to a GMT of 0.87. Similarly, for wave 2 infections we observed a three-fold boost in GMT to 0.9. In all HCWs, regardless of prior infection, spike-specific binding antibodies were highly cross-reactive, with no significant difference in binding between the D614G and Beta spike (**Figure 1C**).

Using a SARS-CoV-2 pseudovirus assay with the D614G spike, we tested neutralizing antibodies elicited by vaccination alone. Consistent with previous studies (Moore et al., 2021), we saw relatively low titers post-vaccination in the infection naïve group, with a GMT of 74. In both groups with prior infection, we observed a significant boost in neutralizing responses after vaccination. For first wave HCWs, titers were boosted 13-fold from a GMT of 210 to 2,798 (**Figure 2A**). Similarly, second wave HCWs were boosted 12-fold from a GMT of 99 to 1,157. To determine cross-reactivity of neutralizing antibodies, we compared neutralization of D614G with Beta and Delta. For antibodies induced by vaccination alone, all

participants showed significantly lower titers against Beta (85% showing no neutralization, GMT of 28) and Delta (78% showing no neutralization, GMT of 29). In both groups of previously infected HCWs we saw cross-neutralization of Beta and Delta, but the degree of cross-reactivity varied by wave of infection (**Figure 2B and C**). For HCWs infected in the first wave, while neutralization of Beta and Delta was maintained, titers were significantly lower for both variants of concern (VOCs) (a reduction in GMT from 2,798 to 606 and 443, respectively, compared to D614G). In contrast, plasma from those infected in the second wave with Beta showed no significant difference in neutralization of D614G (GMT: 1157) but 6-fold lower neutralization of Delta (GMT: 200, $p<0.001$) (**Figure 2B and C**). Overall, prior infection followed by vaccination triggered high titer neutralizing antibodies able to neutralize multiple variants. However, the pattern of neutralization varied by wave, suggesting that the neutralizing antibody repertoire was shaped by the genotype of the infecting variant.

The impact of prior infection on Fc effector responses to vectored vaccines is not known. We measured the ability of plasma antibodies from vaccinees to crosslink FcγRIIIa (CD16) expressing cells and cell surface D614G, Beta or Delta spikes on target cells, as a surrogate for ADCC. Responses to D614G elicited by vaccination alone (GMT: 39) were similar to those elicited by natural infection (GMT: 86 for wave 1 and 54 for wave 2) (**Figure 3A)**. However, in previously infected individuals, post-vaccination responses following both waves were significantly higher (wave 1: GMT of 218; wave 2: 197) (**Figure 3A)**. ADCC assays performed using the Beta and Delta variants showed only slight loss in activity compared to D614G in the vaccine-only group (**Figure 3B**). However, in individuals with prior infection, there was no significant difference between ADCC responses to D614G, Beta and Delta (**Figure 3B and C)**, demonstrating cross-reactive ADCC responses to variants of concern.

We examined the effect of prior SARS-CoV-2 infection on post-vaccination T cell responses. We measured total cytokine production (IFN-γ, TNF-a and IL-2) in response to peptides covering the Wuhan-1 spike protein by intracellular cytokine staining (**Supplementary Figure 2A**). Vaccination induced spike-specific CD4 and CD8 T cell responses in all groups (**Figure 4A and B**). Infection-naive participants or those infected in the first wave had significantly higher CD4 T cell responses after vaccination (median: 0.051 and 0.064, $p=0.0004$ and 0.016, respectively). The group infected during the second wave, reflecting more recent infection, had pre-existing responses that were significantly higher than the first wave baseline infection responses ($p=0.006$). This group had a more modest response to vaccination, with similar medians (0.132% and 0.147%, p=ns).

The induction of CD8 responses to Ad26.COV2.S vaccination differed markedly from CD4 responses (**Figure 4B**). CD8 responses were present in few individuals prior to vaccination (15 and 32%, compared to 85 and 100% for CD4 responses in the first and second wave groups, respectively). In contrast to CD4 responses, vaccine-induced CD8 T cell response magnitudes did not differ significantly between the groups (**Supplementary Figure S2B**). **Figure 4C** summarises the trajectories of vaccine T cell responses in responders. The fold-change in median CD4 T cell frequencies from pre- to post-vaccination decreased with higher magnitude of pre-existing responses, with a 5.7-fold change in the infection-naïve group, 1.5-fold in the first wave group and 1.1-fold in the recently infected second wave group. For CD8 responses, the fold-increase was similar for the three groups (**Figure 4C**). In addition to differential response trajectories, the proportion of individuals who mounted a T cell response in each group differed for CD4 and CD8 responses. CD4 T cell responses were more frequent, with 14/19 (74%), 17/20 (85%) and 18/19 (95%) responders in the uninfected, first wave and second wave groups, respectively. CD8 responses occurred in 8/19 (42%), 9/20 (45%) and 12/19 (60%) participants in each of the three groups, respectively (**Figure 4C**). Finally, we analysed whether T cells induced by vaccination recognized the Beta variant. We tested peptide pools based on S1 and S2, corresponding to D614G ancestral strain or Beta variant sequences. Mutations in Beta are located predominantly in S1 (8/9 mutations), with S2 having the single A701V change. We found no difference in CD4 recognition of Beta S1 or S2 compared to D614G in any of the three groups, demonstrating that recognition of Beta is fully preserved (**Figure 4D**). Cross-reactivity was also demonstrated for CD8 responses targeted at the Beta variant (**Supplementary Figure S2C**). These results demonstrate that cross-reactive CD4 and CD8 T cell responses are generated after vaccination, regardless of prior infection.

Discussion

A range of studies of mRNA vaccines have demonstrated a boosting effect of prior infection (Manisty et al., 2021; Reynolds et al., 2021; Saadat et al., 2021; Stamatatos et al., 2021; Wang et al., 2021b). However, whether this is true of viral vectors, including the single dose Johnson and Johnson vaccine is less clear. We show that infection prior to vaccination with Ad26.COV2.S significantly boosts the magnitude and cross-reactivity of binding antibodies, neutralizing antibodies and Fc effector function. T cell responses were robustly generated even in the absence of prior infection and were preserved against Beta. These data have particular significance in countries like South Africa, where SARS-CoV-2 seropositivity is 20-

40% (Mutevedzi et al., 2021; Hsiao et al., 2020; Sykes et al., 2021). Indeed, in the cohort of HCW in which this sub-study is embedded, we detected a seropositivity rate of 56%. Thus, prior infection may enhance the protective efficacy of this vaccine, which is frequently used in resource-limited settings.

Neutralization breadth was shaped by the variant responsible for infection. Prior exposure to D614G resulted in reduced titers against both Beta and Delta, consistent with previous studies (Liu et al., 2021). However, while Beta infection resulted in the preserved neutralization of D614G, we, like others, observed significant loss of activity against Delta, now dominant in South Africa (Liu et al., 2021). Therefore, while all participants were exposed to the same vaccine, the genotype of the infecting virus determined the specificity of the responses, prior to vaccine boosting. These findings have important implications for vaccine design: heterologous prime-boost regimens may not be sufficient to drive breadth, and the sequence of VOC spikes incorporated into second generation vaccines may impact the repertoire of vaccine-induced antibodies.

Fc effector functions are important in vaccine-elicited protection against many infectious diseases (Richardson and Moore, 2021). Reduced SARS-CoV-2 severity/mortality has been correlated with Fc effector activity (Zohar et al., 2020) and antibodies isolated from convalescent donors require Fc function for optimal protection (Winkler et al., 2021; Schafer et al., 2020). We show that Ad26.COV.2 vaccination in SARS-CoV-2 naïve HCWs elicits significant ADCC responses, consistent with previous data (Stephenson et al., 2021). In addition, prior infection significantly enhanced vaccine-elicited ADCC responses, independent of time post-infection, as for the BNT162b2 vaccine (Geers et al., 2021; Tauzin et al., 2021). Finally, we show that unlike neutralizing antibodies, ADCC activity through vaccination alone, or boosted by prior infection, was cross-reactive for Beta and Delta. This is consistent with previous findings, and suggests that ADCC-mediating antibodies target regions of the spike beyond the major neutralization epitopes (Alter et al., 2021).

SARS-CoV-2-specific T cells play a key role in modulating COVID-19 disease severity (Rydyznski Moderbacher et al., 2020) and provide protective immunity in the context of low antibody titers (McMahan et al., 2020). We show that robust spike-specific CD4 and CD8 memory T cell responses were induced by AD26.COV2.S vaccination, consistent with Alter et al (2021). The magnitude of vaccine-induced T cell responses was similar to convalescent responses in the first and second wave participants prior to vaccination. The effect of prior infection and the kinetics of T cell boosting was distinct from the antibody response. CD4 responses in the infection naïve group were induced to a similar magnitude as the first wave

group, with existing CD4 T cells only moderately boosted. This effect was more marked in the second wave group and may reflect some degree of immune regulation. During infection, SARS-CoV-2-specific CD4 T cells exhibit high expression of the activation marker PD-1 (Sattler et al., 2020; Riou et al 2021a), which may limit proliferative capacity in those with recent infection. There was a marked increase in the magnitude and proportion of CD8 responses to spike induced *de novo* after vaccination, compared to infection, with no difference between the three study groups. Vaccination induced T cell responses that cross-recognized pooled peptides based on Beta spike, suggesting that most vaccinees target conserved epitopes in spike. These data are in accordance with several recent studies (Reynolds et al., 2021; Gallagher et al., 2021; Geers et al, 2021; Tarke et al., 2021), including our own (Riou et al., 2021b), describing a minimal impact of mutations in SARS-CoV-2 variants on T cell responses in the context of infection and vaccination.

Overall, we show a dramatic effect of recent or distant infection on the magnitude and breadth of neutralizing responses and ADCC. Ad26.COV2.S vaccination alone drives continued maturation of B cell responses, conferring enhanced neutralization of variants, and durability (Barouch et al., 2021). Whether prior infection will also enhance maturation of neutralizing antibodies and extend durability still further is unknown. T cell responses, though more modestly impacted by prior infection, were robust and cross-reactive. This suggests that an infection ‘prime’ boosts Ad26.COV2.S immunogenicity, and in areas of high seroprevalence, may have a positive impact on effectiveness of this single dose vaccine. Most significantly, we show for the first time that breadth of neutralization after vaccination is dictated by the infecting variant, which has important implications for adapted vaccines based on variants of concern.

Figure Legends

Figure 1. Spike-specific antibody responses in Ad26.COV2.S-vaccinated healthcare workers with and without previous SARS-CoV-2 infection. A. Study design showing three groups of 20 participants each (left panel), either with no prior infection, infection in the first wave (May to August 2020) and infection in the second wave (November 2020 to January 2021). Samples were taken pre-vaccination (baseline), and approximately 28 days after vaccination. SARS-CoV-2 epidemiological dynamics in the Western Cape (South Africa) are shown (top right panel). Prevalence of SARS-CoV-2 lineages on the left y-axis (n=1178 sequences). The ancestral strain (D614G) is depicted in blue and Beta in red. The number of COVID-19 cases is represented on the right y-axis. The bars on top of the graph indicate the periods when participants were infected in the first and second waves (confirmed by PCR and serology). Vertical dotted lines denote the period when vaccination occurred. Demographic and clinical characteristics of participants in the three groups (bottom right panel). Sex, age (median and interquartile range) and days since PCR-confirmed SARS-CoV-2 infection. **B.** Plasma samples from participants with no prior infection (green, n=19), first wave infection (blue, n=20) or second wave infection (red, n=19) were tested for binding to the full D614G spike protein pre- and post-vaccination (OD_{450nm}) **C.** Cross-reactivity of vaccine-induced antibody responses to the spike of the D614G and Beta variants are shown. Threshold for positivity is indicated by a dotted line. Horizontal black or red bars indicate geometric mean titers, with values shown below. Statistical analyses were performed using the Mann-Whitney test between groups, and the Wilcoxon test for pre- and post-vaccine time points or D614G compared to Beta responses. *** denotes $p<0.001$, ns = non-significant. Experiments were performed in duplicate with the average value shown.

Figure 2. Neutralizing antibody responses to Ad26.COV2.S vaccination after prior infection. A. Neutralization of the SARS-CoV-2 D614G pseudovirus by plasma pre- and post-vaccination from participants with no prior infection (green, n=19) and those infected in the first (blue, n=20) and second waves (red, n=19). Neutralization is reflected as an ID_{50} titer. The threshold for positivity is indicated by a dotted line **B.** Cross-reactive neutralization post-vaccination against D614G, Beta and Delta variants. Pie charts show the proportion of vaccine non-responders (NR; grey), knock-out of neutralization of Beta or Delta (KO; black), titer of 20-400 (orange), or >400 (red). **C.** Fold change of post-vaccination D614G

neutralization titers relative to Beta or Delta. The dotted line indicates a fold change of 1 (no change). The horizontal black and red bars indicate geometric mean titers, with values indicated on the graphs. Statistical analyses were performed using the Mann-Whitney test between groups, and the Wilcoxon test for paired analyses. * denotes p<0.05, *** p<0.001, ns, non significant. Experiments were performed in duplicate with the average value shown.

Figure 3. Antibody-dependent cellular cytotoxicity (ADCC) responses to Ad26.COV2.S vaccination after prior infection. A. ADCC activity represented as relative light units (RLU). **B.** Cross-reactive ADCC activity 28 days post-vaccination against D614G, Beta and Delta. Pie charts show the proportion of vaccine non-responders (NR; grey), knock-out of Beta/Delta neutralization (KO; black), or detectable ADCC activity (41-150, orange and >150, red). **C.** Fold-change of post-vaccination D614G ADCC levels relative to the Beta/Delta variants. The dotted line indicates a fold change of 1 (no change). For all before-after plots, the threshold for positivity is indicated by a dotted line. The horizontal black and red bars indicate geometric mean titers, with these values indicated on the graphs. Statistical analyses were performed using the Mann-Whitney test between groups, and the Wilcoxon test for pre- and post-vaccine time points or D614G compared to Beta/Delta responses. * denotes p<0.05, ** p<0.01, *** p<0.001, ns, non significant. Experiments were performed in duplicate with the average value shown.

Figure 4. T cell responses to Ad26.COV2.S vaccination in the context of prior infection. A. Frequency of total cytokine-producing spike-specific CD4 T cells, and **B.** CD8 T cells, in those with no prior infection (green, n=19), infection in the first wave (blue, n=20), and infection in the second wave (red, n=19), in PBMC stimulated with a peptide pool based on Wuhan spike. **C.** Median fold-change of CD4 and CD8 T cell frequencies after vaccination in responders. Error bars indicate interquartile range. Pie charts indicate the proportion of responders (black) and non-responders (grey), indicated in the centre. **D.** Cross-reactivity of T cell responses post-vaccination. The frequency of cytokine-producing CD4 T cells post-vaccination in response to peptide stimulation with the S1 or S2 regions of spike from D614G or Beta is shown. Horizontal black or red bars indicate medians. The dotted line indicates the threshold for positivity and values are background-subtracted. Statistical analyses were performed using the Wilcoxon test. * denotes p<0.05, ** p<0.01, ns = non-significant.

Methods

Study design and study participants

Participants were recruited from a longitudinal study of healthcare workers (HCW; n=400) enrolled from Groote Schuur Hospital (Cape Town, Western Cape, South Africa). HCW in this cohort were recruited between July 2020 and January 2021, and vaccination with single dose Johnson and Johnson Ad26.COV2.S in the Sisonke Phase 3b trial took place between 17 February and 26 March 2021. Sixty participants were selected for inclusion in this study, based on the availability of PBMC and plasma prior to vaccination and approximately one month after vaccination, and who fell into one of three groups: (1) No evidence of previous SARS-CoV-2 infection by diagnostic PCR test or serial serology; (2) infection during the 'first wave' of the pandemic in South Africa, prior to 1 September 2020, with known date of laboratory (PCR)-confirmed SARS-CoV-2 infection; and (3) infection during the 'second wave', with known date of laboratory (PCR)-confirmed SARS-CoV-2 infection between 1 November 2020 and 31 January 2021. Full demographic and clinical characteristics of participants are summarized in **Supplementary Table 1**. The study was approved by the University of Cape Town Human Research Ethics Committee (HREC 190/2020 and 209/2020) and the University of the Witwatersrand Human Research Ethics Committee (Medical) (no M210429). Written informed consent was obtained from all participants.

SARS-CoV-2 spike whole genome sequencing and phylogenetic analysis

Whole genome sequencing of SARS-CoV-2 was performed using nasopharyngeal swabs obtained from 19 of the hospitalized patients recruited during the second COVID-19 wave. Sequencing was performed as previously published (Moyo-Gwete et al., 2021). Briefly, cDNA was synthesized from RNA extracted from the nasopharyngeal swabs using the Superscript IV First Strand synthesis system (Life Technologies, Carlsbad, CA) and random hexamer primers. Whole genome amplification was then performed by multiplex PCR using the ARTIC V3 protocol (https://www.protocols.io/view/ncov-2019-sequencing-protocol-v3-locost-bh42j8ye). PCR products were purified with AMPure XP magnetic beads (Beckman Coulter, CA) and quantified using the Qubit dsDNA High Sensitivity assay on the Qubit 3.0 instrument (Life Technologies Carlsbad, CA). The Illumina® DNA Prep kit was used to prepare indexed paired end libraries of genomic DNA. Sequencing libraries were normalized to 4 nM, pooled, and denatured with 0.2 N sodium hydroxide. Libraries were sequenced on

the Illumina MiSeq instrument (Illumina, San Diego, CA, USA). The quality control checks on raw sequence data and the genome assembly were performed using Genome Detective 1.132 (https://www.genomedetective.com) and the Coronavirus Typing Tool (Cleemput et al., 2020). The initial assembly obtained from Genome Detective was polished by aligning mapped reads to the references and filtering out low-quality mutations using bcftools 1.7-2 mpileup method. Mutations were confirmed visually with bam files using Geneious software (Biomatters Ltd, New Zealand). Phylogenetic clade classification of the genomes in this study consisted of analyzing them against a global reference dataset using a custom pipeline based on a local version of NextStrain (https://github.com/nextstrain/ncov) (Hadfield et al., 2018).

Roche serology

Serial serum samples were analysed from longitudinal study visits from enrolment to post-vaccination (3-8 time points per participant) at Public Health England, Porton Down. The Elecsys anti-SARS-CoV-2 Spike and the Elecys anti-SARS-CoV-2 electrochemiluminescent immunoassays were performed (Roche Diagnostics, GmbH), which enable detection of total antibodies against the SARS-CoV-2 spike (S) receptor binding domain (RBD) and nucleocapsid (N) proteins, respectively. Samples were analysed on a Cobas e801 instrument and a result ≥0.8 U/mL was considered positive in the S assay, and ≥1.0 U/mL positive in the N assay, according to the manufacturer's instructions.

Isolation of peripheral blood mononuclear cells (PBMC)

Blood was collected in heparin tubes and processed within 3 hours of collection. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient sedimentation using Ficoll-Paque (Amersham Biosciences, Little Chalfont, UK) as per the manufacturer's instructions and cryopreserved in freezing media consisting of heat-inactivated fetal bovine serum (FBS, Thermofisher Scientific) containing 10% DMSO and stored in liquid nitrogen until use.

SARS-CoV-2 antigens

For serology assays, SARS-CoV-2 original and beta variant spike proteins were expressed in Human Embryonic Kidney (HEK) 293F suspension cells by transfecting the cells with the spike plasmid. After incubating for six days at 37°C, 70% humidity and 10% CO_2, proteins were first purified using a nickel resin followed by size-exclusion chromatography. Relevant fractions were collected and frozen at -80 °C until use.

For T cell assays, we used peptides covering the full length SARS-CoV-2 spike protein, by combining two commercially available peptide pools of 15mer sequences with 11

amino acids (aa) overlap (PepTivator®, Miltenyi Biotech, Bergisch Gladbach, Germany). These peptides are based on the Wuhan-1 strain and cover the N-terminal S1 domain of SARS-CoV-2 from aa 1 to 692, as well as the majority of the C-terminal S2 domain. Pools were resuspended in distilled water at a concentration of 50 µg/mL and used at a final concentration of 1 µg/mL. To determine T cell responses to SARS-CoV-2 variants, we constructed pools based on S1 and S2 for D614G and Beta viruses, respectively. Individual peptides (15mers with 10 aa overlap) spanning D614G or Beta spike mutation sites (L18F, D80A, D215G, del 242-244, R246I, K417N, E484K, N501Y and A701V) were synthesized (GenScript Biotech, Piscataway, NJ, USA) and individually resuspended in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, Missouri, United States) at a concentration of 20 µg/mL. We obtained a peptide array of individual S peptides spanning the entire S protein (17mers with 10 aa overlap, 181 peptides) from BEI Resources (NIAID, NIH), which were individually resuspended in DMSO at a concentration of 20 µg/mL. D614G or Beta full-length S1 and S2 pools were created by pooling aliquots of the GenScript peptides spanning the mutated regions with aliquots of the BEI peptides making up the remainder of the S1 or S2 region, at a final concentration of 80 µg/mL. S1 and S2 ancestral or Beta pools were used at a final concentration of 1 µg/mL. Since these peptides are 17mer in length, they were non-optimal for detecting CD8 T cell responses to the D614G and Beta variants.

SARS-CoV-2 Spike Enzyme-linked immunosorbent assay (ELISA)

Two µg/ml of spike protein were 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. Secondary antibody was diluted to 1:3000 in blocking buffer and added to the plates followed by TMB substrate (Thermofisher Scientific). Upon stopping the reaction with 1 M H_2SO_4, absorbance was measured at a 450nm wavelength. In all instances, mAbs CR3022 and BD23 were used as positive controls and palivizumab was used as a negative control. All values were normalized with the CR3022 mAb.

Pseudovirus neutralization assay

SARS-CoV-2 pseudotyped lentiviruses were prepared by co-transfecting the HEK 293T cell line with either the SARS-CoV-2 ancestral variant spike (D614G), the Beta spike (L18F, D80A, D215G, K417N, E484K, N501Y, D614G, A701V, 242-244 del) or the Delta spike (T19R, R158G L452R, T478K, D614G, P681R, D950N, 156-157 del) plasmids in conjunction with a firefly luciferase encoding lentivirus backbone plasmid. For the neutralization assay, heat-inactivated plasma samples from vaccine recipients were incubated with the SARS-

CoV-2 pseudotyped virus for 1 hour at 37°C, 5% CO_2. Subsequently, 1x104 HEK 293T cells engineered to over-express ACE-2 were added and incubated at 37°C, 5% CO_2 for 72 hours upon which the luminescence of the luciferase gene was measured. CB6 was used as a positive control.

Antibody-dependent cellular cytotoxicity (ADCC) assay

The ability of plasma antibodies to cross-link FcγRIIIa (CD16) and spike expressing cells was measured as a proxy for ADCC. 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 binding of CR3022 and P2B-2F6 and their detection by anti-IgG APC staining measured by flow cytometry. Subsequently, $1x10^5$ spike transfected cells per well were incubated with heat inactivated plasma (1:100 final dilution) or control mAbs (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) ($2x10^5$ cells/well) were added and incubated for 24 hours at 37°C, 5% CO_2. Twenty µl 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. Relative light units (RLU) of a no antibody control were 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 as a positive control.

Cell stimulation and flow cytometry staining

Cryopreserved PBMC were thawed, washed and rested in RPMI 1640 containing 10% heat-inactivated FCS for 4 hours prior to stimulation. PBMC were seeded in a 96-well V-bottom plate at ~2 x 10^6 PBMC per well and stimulated with SARS-CoV-2 spike peptide pools: full spike pool (Miltenyi), and ancestral and beta mutated S1 and S2 pools (1 µg/mL). All stimulations were performed in the presence of Brefeldin A (10 µg/mL, Sigma-Aldrich, St Louis, MO, USA) and co-stimulatory antibodies against CD28 (clone 28.2) and CD49d (clone L25) (1 µg/mL each; BD Biosciences, San Jose, CA, USA). As a negative control, PBMC were incubated with co-stimulatory antibodies, Brefeldin A and an equimolar amount of DMSO.

After 16 hours of stimulation, cells were washed, stained with LIVE/DEAD™ Fixable VIVID Stain (Invitrogen, Carlsbad, CA, USA) and subsequently surface stained with the

following antibodies: CD14 Pac Blue (TuK4, Invitrogen Thermofisher Scientific), CD19 Pac Blue (SJ25-C1, Invitrogen Thermofisher Scientific), CD4 PERCP-Cy5.5 (L200, BD Biosciences, San Jose, CA, USA), CD8 BV510 (RPA-8, Biolegend, San Diego, CA, USA), PD-1 BV711 (EH12.2H7, Biolegend, San Diego, CA, USA), CD27 PE-Cy5 (1A4, Beckman Coulter), CD45RA BV570 (HI100, Biolegend, San Diego, CA, USA). Cells were then fixed and permeabilized using a Cytofix/Cyto perm buffer (BD Biosciences) and stained with CD3 BV650 (OKT3) IFN-g Alexo 700 (B27), TNF BV786 (Mab11) and IL-2 APC (MQ1-17H12) from Biolegend. Finally, cells were washed and fixed in CellFIX (BD Biosciences). Samples were acquired on a BD LSR-II flow cytometer and analyzed using FlowJo (v10, FlowJo LLC, Ashland, OR, USA). A median of 282 848 CD4 events (IQR:216 796 - 355 414) and 153 192 CD8 events (IQR 109 697 - 202 204) were acquired. Cells were gated on singlets, CD14-CD19-, live lymphocytes and memory cells (excluding naive CD27+ CD45RA+ population). Results are expressed as the frequency of CD4+ or CD8+ T cells expressing IFN-g, TNF-a or IL-2. Due to high TNF-a backgrounds, cells producing TNF-a alone were excluded from the analysis. Cytokine responses presented are background subtracted values (from the frequency of cytokine produced in unstimulated cells), and the threshold for a positive cytokine response was defined as >0.02%.

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 and Wilcoxon tests were used for unmatched and paired samples, respectively. All correlations reported are non-parametric Spearman's correlations. *P* values less than 0.05 were considered statistically significant.

Supplementary data

Supplementary Table 1: Clinical and demographic details of study participants

Supplementary Figure 1: Serological profiles of study participants. Spike and Nucleocapsid antibody profiles in A. No prior infection group; B. First wave infection; C. Second wave infection group. Serial serum samples were analysed from all available study visits prior to vaccination (3-8 samples per participant). Anti-spike (S; closed circles) and nucleocapsid (N; open circles) antibodies were measured by the Elecsys ECLIA system (Roche Diagnostics). The horizontal lines indicate the cut-off for a positive response (≥0.8

U/mL in the S assay, and ≥1.0 U/mL in the N assay). The vertical line with "v" indicates when vaccination took place. The asterisk indicates a potential breakthrough infection in A (both S and N antibodies increasing after vaccination); a serological non-responder despite a confirmed PCR test for SARS-CoV-2 in B; and a re-infection in C (a positive PCR in the second wave but serological evidence of infection in the first wave). The potential breakthrough and re-infection participants were excluded from further study.

Supplementary Figure 2: Analysis of T cell responses after Ad26.COV2.S vaccination. **A.** Representative flow cytometry plots of CD4 and CD8 T cell cytokine responses (IFN-g, TNF-a and IL-2) in response to a pool of spike peptides, with the unstimulated control shown. The pre- and post-vaccination plots are shown, from one second wave participant. T cell responses were calculated from boolean gates of all cytokines and the background (unstimulated sample) was subtracted. The single TNF-a-producing subset was excluded due to high background responses. **B.** Summary of median frequencies of cytokine-producing spike-specific CD4 and CD8 T cells, in those with no prior infection (green, n=19), infection in the first wave (blue, n=20), and infection in the second wave (red, n=19). Symbols represent medians and error bars interquartile range. Statistical comparisons between groups were performed with the Kruskal Wallis test with Dunn's multiple comparisons test. **C**. Cross-reactivity of spike-specific CD8 T cell responses post-vaccination. The frequency of total cytokine-producing CD8 T cells post-vaccination in response to peptide stimulation with the S1 or S2 regions of spike from D614G or Beta is shown. Horizontal black or red bars indicate medians. The dotted line indicates the threshold for positivity and values are background-subtracted. Statistical analyses were performed using the Wilcoxon test for paired samples. * denotes $p<0.05$, ns = non-significant.

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Author contributions

W.A.B, P.L.M. and N.A.B.N. designed the study. W.A.B and P.L.M. analyzed the data and wrote the manuscript. R.K. S.I.R. and T.M.G. generated and analyzed the data and wrote the manuscript. S.I.R., T.M.G., T.H., N.P.M., Z.M. and T.M. performed ELISAs, neutralization assays and ADCC assays. R.K., M.B.T., N.B., R.B. and A.N. performed flow cytometry assays. M.M., S.S. and L.R.C. managed the HCW cohort and contributed clinical samples and data. A.O. and T.B. characterized the serological profiles of the cohort. N.Y.H. contributed samples and D.D., A.I. and C.W. performed viral sequencing and data analysis. H.M. and J.B. contributed to cohort characterization and sample selection. C.R. contributed to data analysis and study design. A.G., N.G., L.G.B. and G.G. established and led the Sisonke vaccine study. N.A.B.N., J.M. and M.N. established and led the HCW cohort and contributed samples. All authors reviewed and edited the manuscript.

Competing interests

The authors declare that they have no competing interests.

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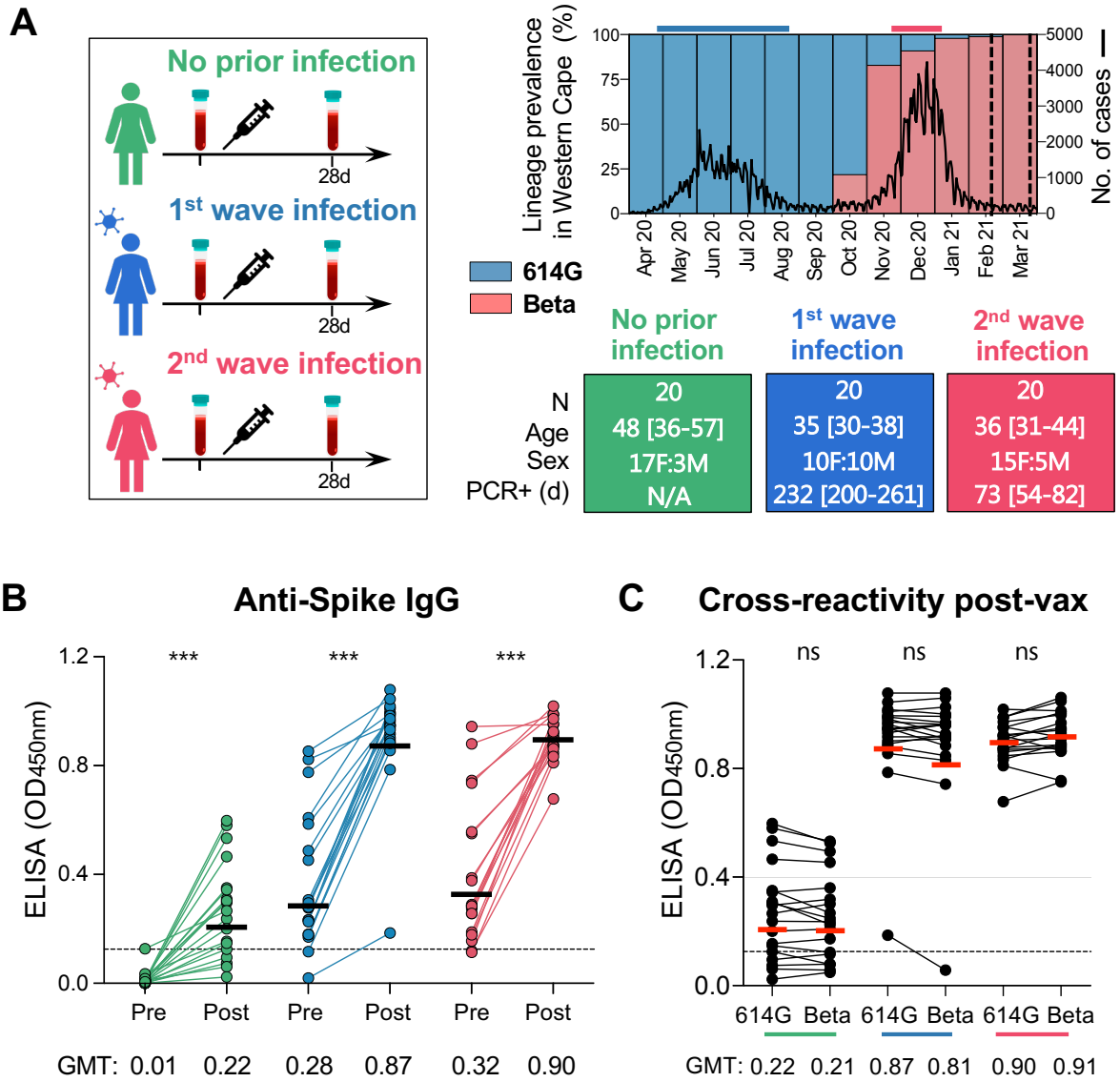

Figure 1. Spike-specific antibody responses in Ad26.COV2.S-vaccinated healthcare workers with and without previous SARS-CoV-2 infection.

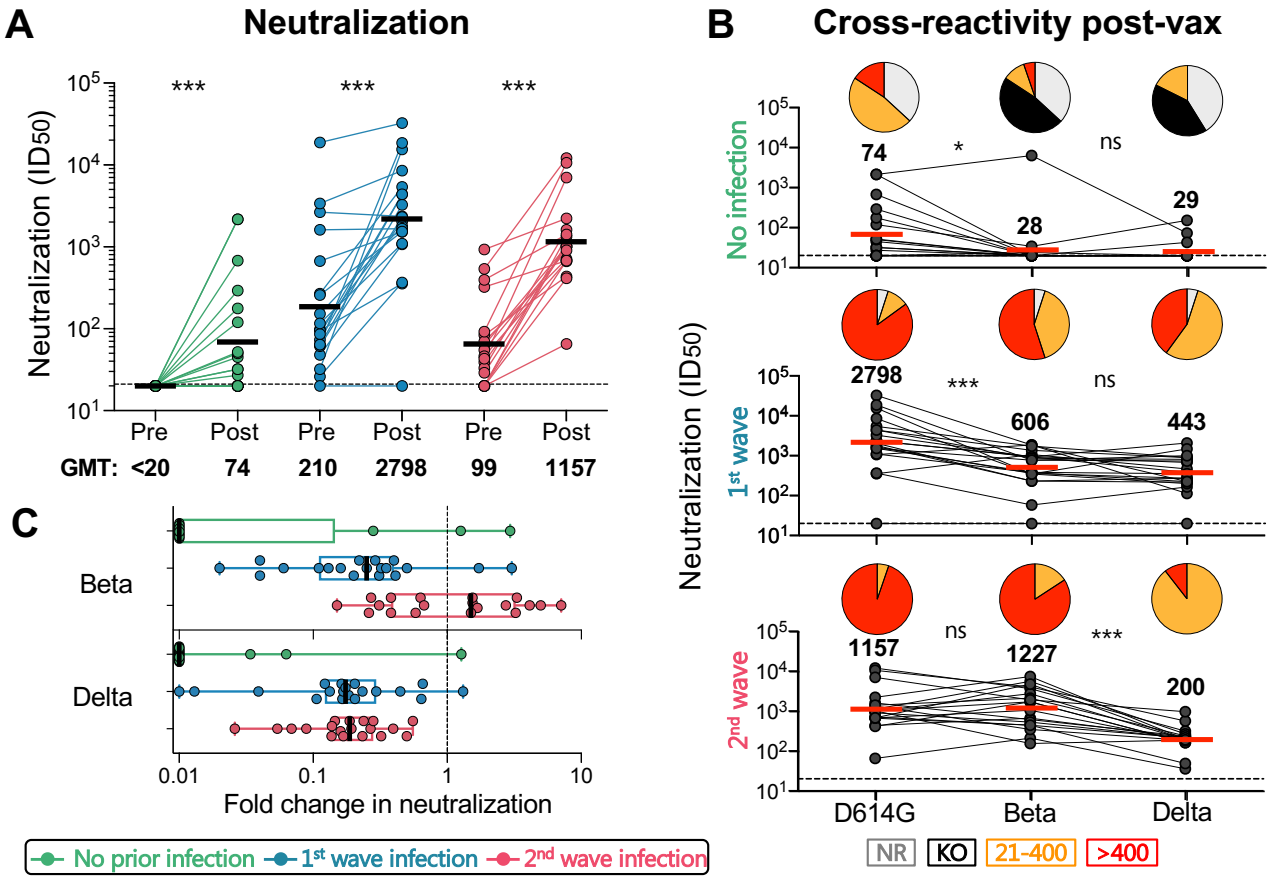

Figure 2. Neutralizing antibody responses to Ad26.COV2.S vaccination after prior infection.

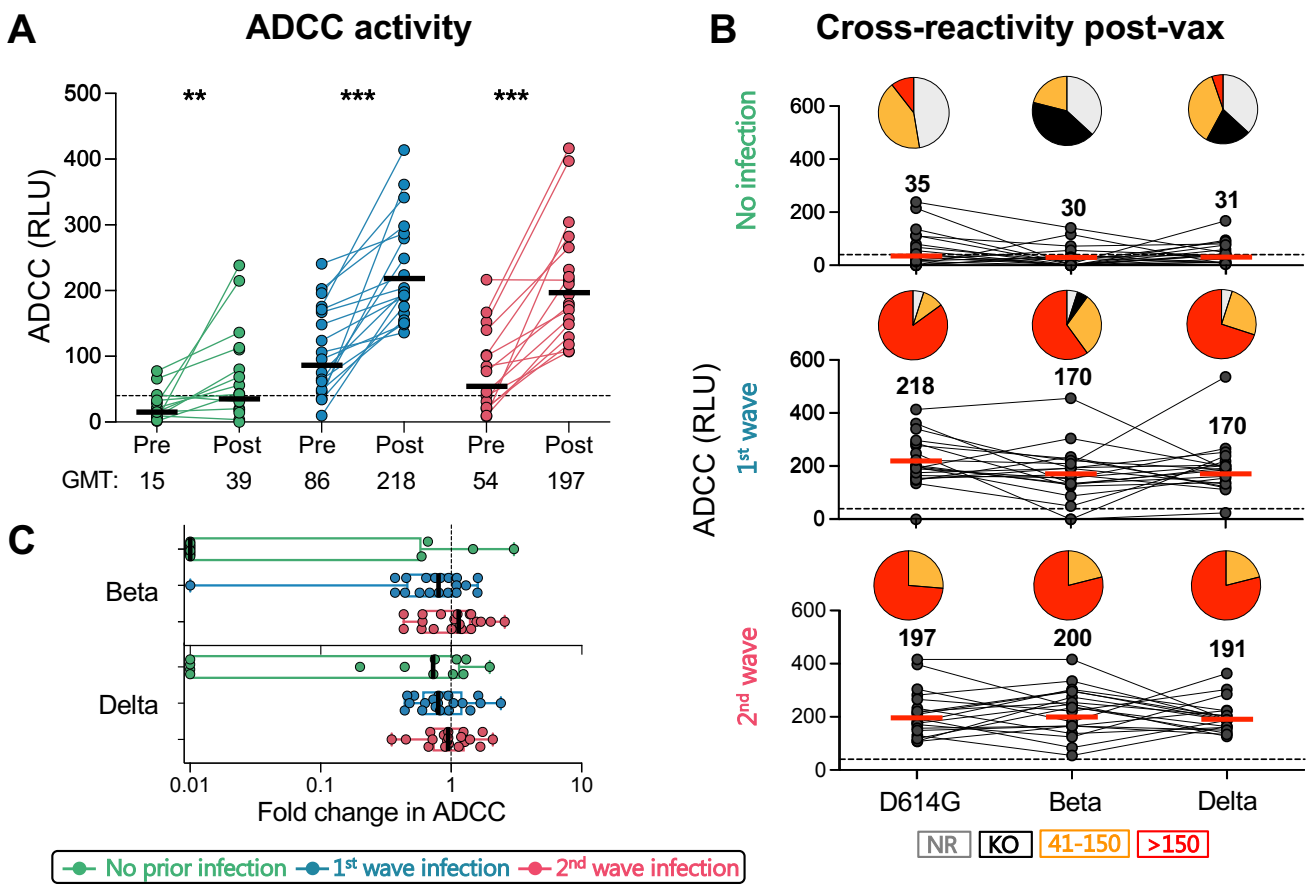

Figure 3. ADCC responses to Ad26.COV2.S vaccination after prior infection.

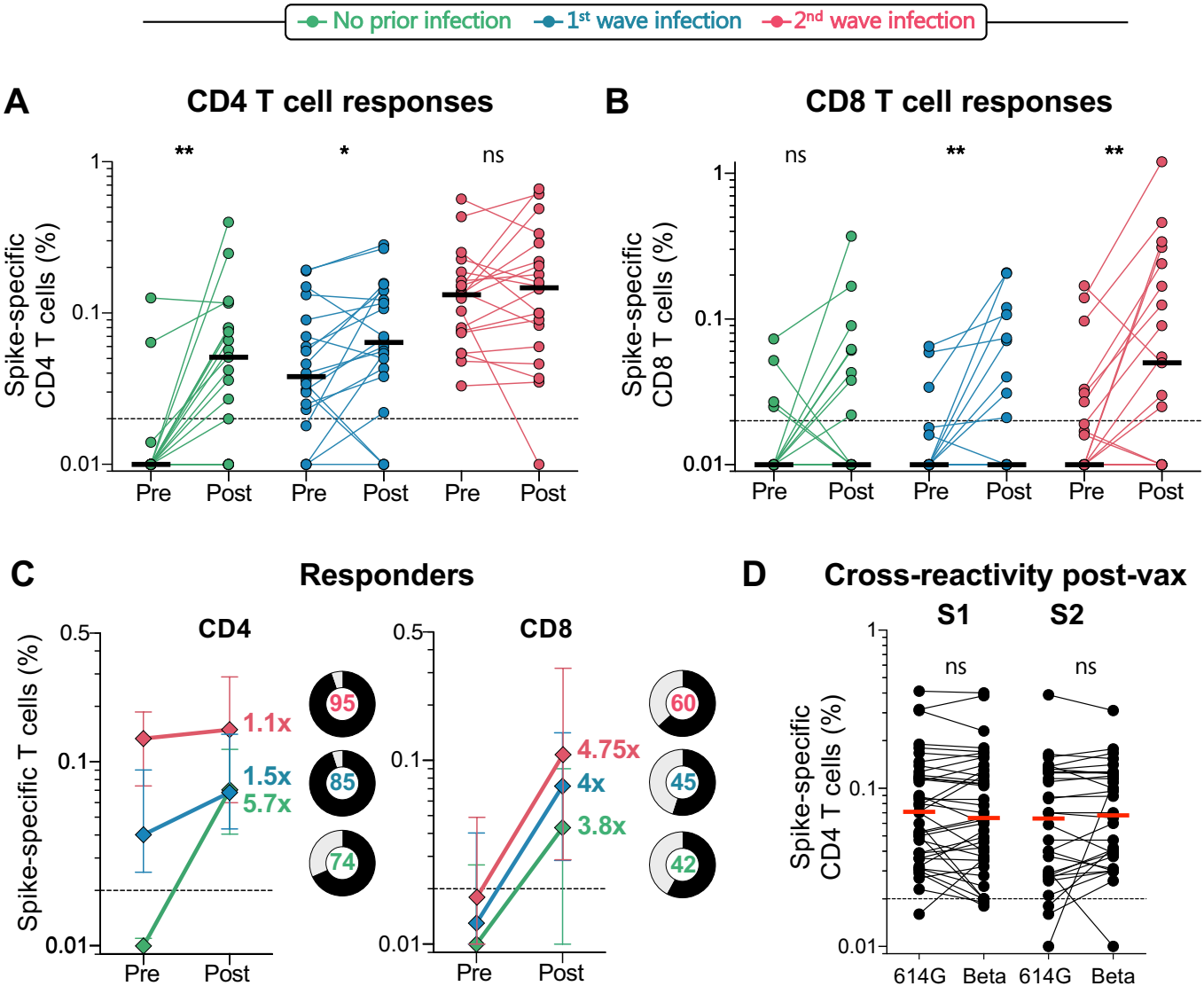

Figure 4. T cell responses to Ad26.COV2.S vaccination in the context of prior infection.