



Delayed-interval BNT162b2 mRNA COVID-19 vaccination enhances humoral immunity and induces robust T cell responses

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Delayed dosing intervals are a strategy to immunize a greater proportion of the population. In an observational study, we compared humoral and cellular responses in health care workers receiving two doses of BNT162b2 (Pfizer-BioNTech) vaccine at standard (3- to 6-week) and delayed (8- to 16-week) intervals. In the delayed-interval group, anti-receptor-binding domain antibody titers were significantly enhanced compared to the standard-interval group. The 50% plaque reduction neutralization test (PRNT50) and PRNT90 titers against wild-type (ancestral) severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and Alpha, Beta and Delta variants were higher in the delayed-interval group. Spike-specific polyfunctional CD4⁺ and CD8⁺ T cells expressing interferon- γ and interleukin-2 were comparable between the two groups. Here, we show that the strategy of delaying second doses of mRNA vaccination may lead to enhanced humoral immune responses, including improved virus neutralization against wild-type and variant SARS-CoV-2 viruses. This finding has potentially important implications as vaccine implementation continues across a greater proportion of the global population.

Currently, a substantial portion of the world's population remains unvaccinated against coronavirus disease 2019 (COVID-19). Multiple vaccine types have been investigated and implemented worldwide to impede the spread of COVID-19, proving extremely successful in the prevention of infection and symptomatic disease¹. The BNT162b2 mRNA vaccine (Pfizer-BioNTech) has been authorized for administration with a 3-week interval between the two doses¹. In Canada, a 4-month delay between vaccine doses was announced in March 2021 as part of a strategy to accelerate the proportion of the population that receives one dose and achieve higher overall protection in the context of limited vaccine supply. Shortages of COVID-19 vaccines continue in many jurisdictions, even as oversupply exists in others. Delayed-interval vaccine dosing is an unproven and controversial public health strategy whose effect on vaccine immunogenicity is not well studied, especially in relation to predominant circulating variants of concern (VOCs). VOCs have generally supplanted wild-type virus as the major strains causing infection in the population. A meta-analysis of two doses of the ChAdOx1 nCoV-19 vaccine has supported a delayed interval of 3 months between vaccine doses for improved vaccine efficacy and binding-antibody response². More recently, it was found that delaying the administration of a second dose of BNT162b2 vaccine

up to 6–14 weeks continues to provide strong cellular and humoral responses when compared to standard-interval vaccination³.

In our current study, a total of 93 health care workers (HCWs) were enrolled and received vaccination with two doses of the BNT162b2 vaccine from January to June of 2021 (Fig. 1a). Of these, 39 of 93 (41.9%) received both doses of the vaccine at the standard interval and 54 of 93 (58.1%) received both doses of the vaccine at the delayed interval (Fig. 1a). Median time to the second dose was 35 days in the standard-interval group (interquartile range (IQR), 21–35; full range, 21–38 days) and 88 days (IQR, 83–90; full range, 53–123 days) in the delayed-interval group. The median age of the cohort was 40.9 years (IQR, 34.5–52.5), and participants were predominantly female (74/93 (79.6%)). Median age was similar between the two groups (40.3 years in the standard-interval group and 41.4 years in the delayed-interval group); female sex was also similarly distributed (31/39 (79.5%) versus 43/54 (79.6%) participants in the two groups, respectively). Blood work after the second dose of vaccine was taken at a median of 35 days (IQR, 29–40) and 32 days (IQR, 28–39) in the standard- and delayed-vaccination groups, respectively. Two participants had detectable anti-receptor-binding domain (anti-RBD) antibody prior to vaccination, although they did not have a history of documented COVID-19. No participant developed symptomatic COVID-19 infection after vaccination over the study period.

There were robust humoral immune responses after two doses of the BNT162b2 vaccine in all participants. However, anti-RBD antibody titer was significantly greater (Fig. 1b) in the delayed-interval group compared to the standard-interval group (median: 10,816 U ml⁻¹ (IQR 6,642–16,695) versus 3,420 U ml⁻¹ (IQR, 1,792–7,765), respectively, $P < 0.0001$). This increase in titer had a moderately positive correlation to time interval (Fig. 1c) between the two doses (Spearman $r = 0.501$, $P < 0.0001$).

Neutralization titer was determined against wild-type and Alpha, Beta and Delta variants using PRNT assays. PRNT50 and PRNT90 represented the maximum dilution titers at which 50% and 90% viral inhibition occurred, respectively. Prior to vaccination, all HCWs were negative for neutralization except for one HCW in the delayed-interval group who had a low positive neutralization titer of 1:20. After two doses of vaccine, all HCWs had positive neutralization with PRNT50 for wild-type, Alpha and Delta and 97.8% for Beta. Geometric mean PRNT50 titers were significantly higher in the delayed-interval group against wild-type, Alpha, Beta and Delta

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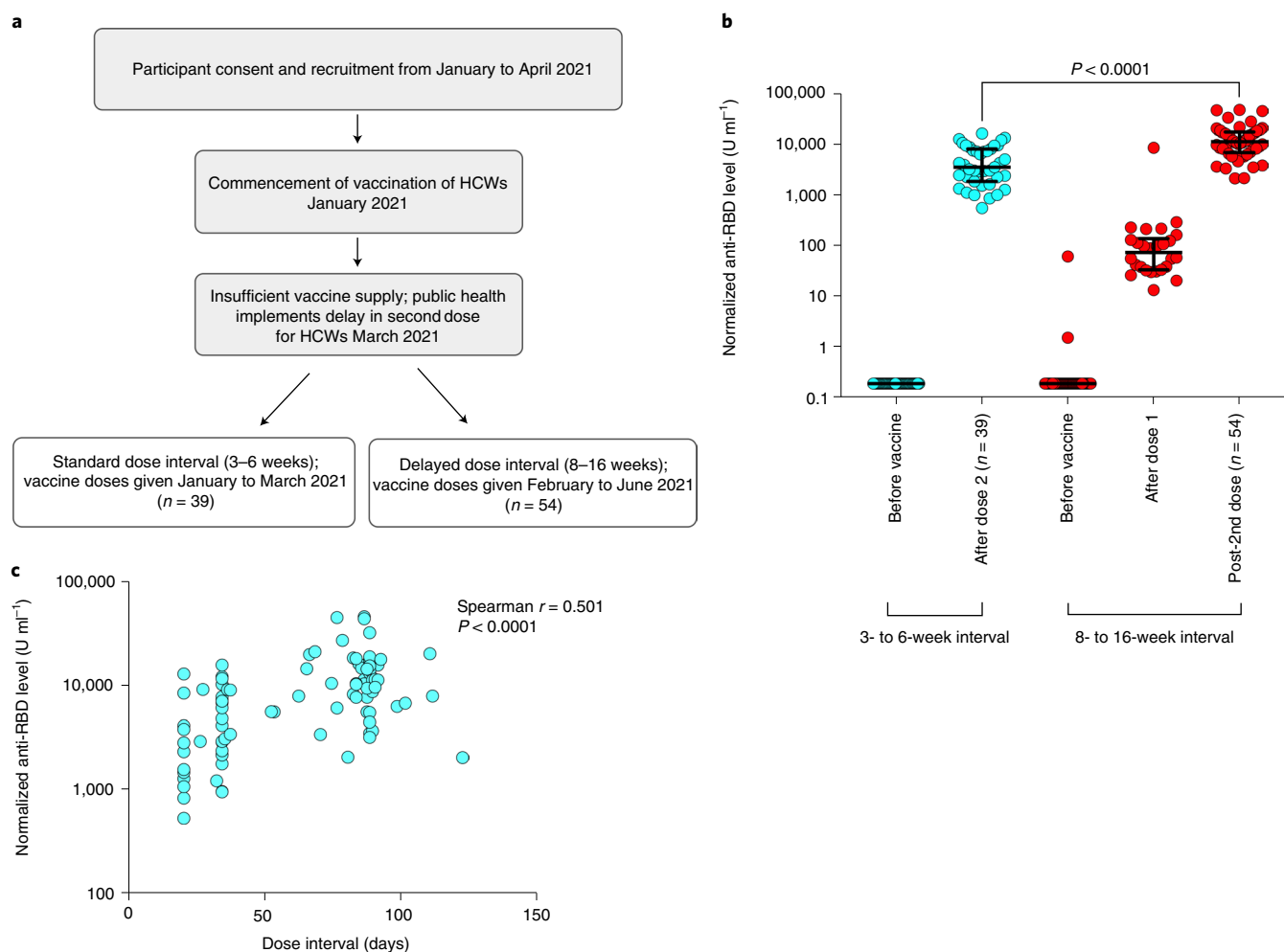


Fig. 1 | Study flow chart and anti-RBD titers between a standard (3–6 weeks) and delayed (8–16 weeks) second-dose interval in HCWs receiving two doses of BNT162b2 vaccine. a, Experimental design detailing time intervals of participant recruitment and dose intervals over the study period.

b, Anti-RBD titer in standard ($n = 39$) and delayed ($n = 54$) groups (U ml^{-1}). Each dot represents an individual participant. Horizontal lines represent median, and vertical lines indicate the IQR. Between-group comparisons were performed using a two-sided Mann–Whitney U test. **c**, Correlation of anti-RBD titer with dose interval (in days) between doses 1 and 2 of the BNT162b2 vaccine. Spearman's correlation test was performed; Spearman r and corresponding two-tailed P values are shown in the top right corner.

variants of SARS-CoV-2 (Fig. 2a–e). PRNT90 titers were generally lower than PRNT50 titers (Fig. 3a–e), although the majority of HCWs had positive neutralization by both measures. For PRNT90, 98.9% of HCWs had positive neutralization against wild-type, 94.6% for Alpha, 76.3% for Beta and 91.4% for Delta. Neutralization titers were significantly reduced against the Beta variant for both groups. Reverse cumulative distribution curves for each variant are shown and represent the proportion of participants reaching a threshold titer for PRNT50 and PRNT90 (Fig. 2b–e and Fig. 3b–e). These visually demonstrate improved responses in the delayed-interval group. Within the delayed-interval group, a comparison of post-first dose and post-second dose PRNT50 and PRNT90 is shown in Extended Data Fig. 1.

Cell-mediated immune responses were determined following stimulation with overlapping spike protein peptides. Spike-specific polyfunctional CD4^+ T cells, defined by double positivity for interferon- γ (IFN- γ) and interleukin-2 (IL-2) (Fig. 4a), were not significantly different in the delayed-interval group compared with the standard-interval group (median polyfunctional CD4^+ T cell frequency: 208 (IQR, 128–407) versus 429 (IQR, 191–792) cells per 10^6 CD4^+ T cells, respectively, Holm–Šídák adjusted $P = 0.17$).

The proportion of patients above the predefined cutoff of 0.01% (or 100 cells per million) was similar between the two groups (97.4% standard interval versus 84.6% delayed interval, $P = 0.156$), suggesting the majority of participants mounted spike-specific CD4^+ T cell responses, regardless of vaccine interval. Polyfunctional CD8^+ T cell responses tended to be lower than CD4^+ responses in both cohorts (Fig. 4b), and overall, only 6 of 51 participants (11.8%) elicited a vaccine-specific polyfunctional CD8^+ T cell response if a threshold value of $\geq 0.01\%$ was applied. When compared to PRNT50 and PRNT90 titers, polyfunctional CD4^+ T cell responses were similar regardless of neutralizing antibody titer (Fig. 4c,d). Monofunctional CD4^+ T cells expressing IL-2 in response to spike were lower in the delayed-vaccine group (Fig. 4a) (Holm–Šídák adjusted $P = 0.0052$). Monofunctional CD8^+ T cells were readily detected in both groups (Fig. 4c) at similar levels.

Adverse events (AEs) for dose 2 were comparable between the standard- and delayed-interval groups (Fig. 5a,b). Specifically, systemic inflammatory events such as fever and chills occurred with comparable frequency in both groups. However, myalgia was reported more commonly in the delayed-interval group (32.0% versus 15.8%, respectively). Among those who provided

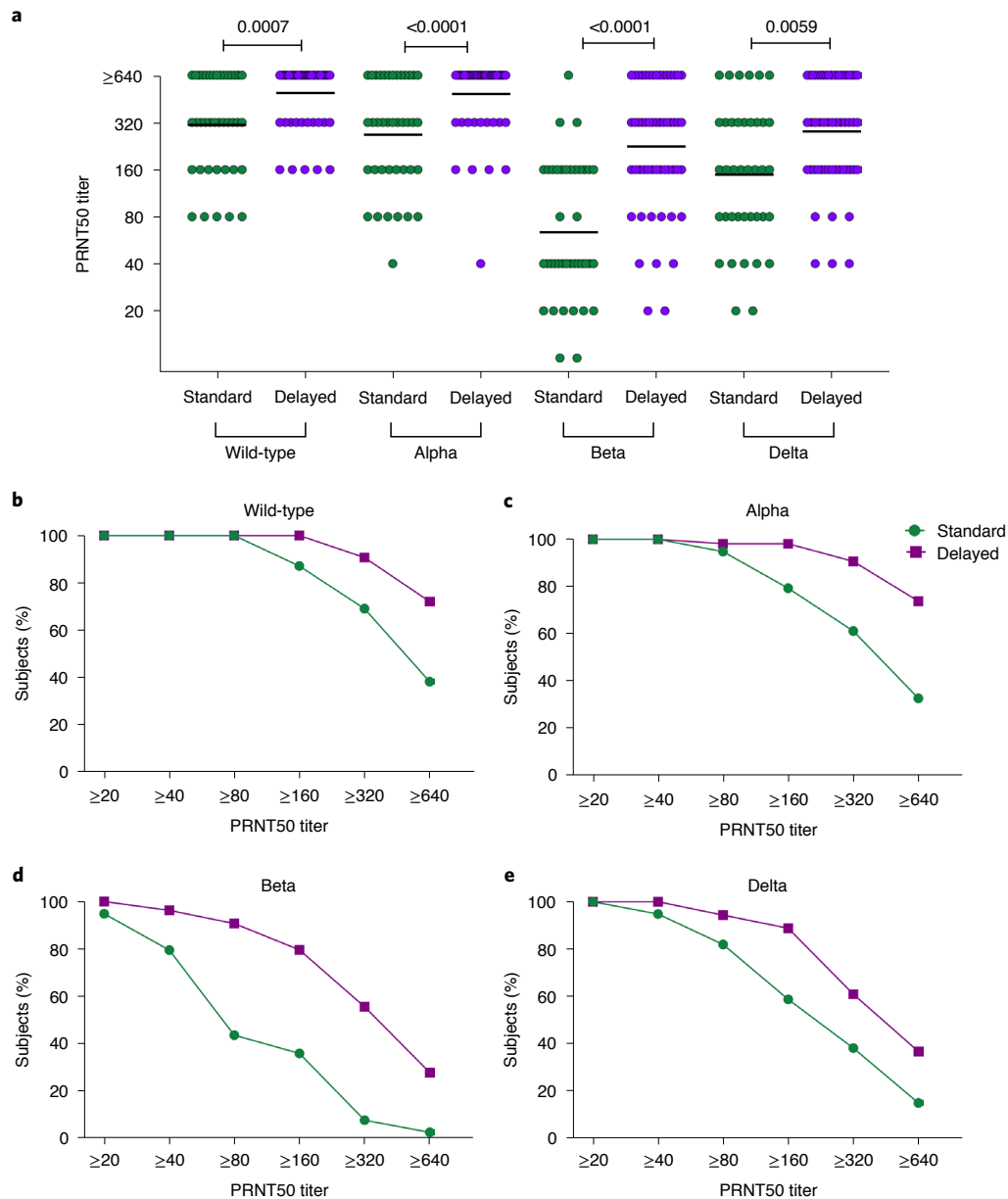


Fig. 2 | PRNT50 titers in participants receiving standard versus delayed vaccination. **a**, Comparative analysis of PRNT50 titers in standard ($n=39$) and delayed ($n=54$) groups for wild-type and predominant circulating VOCs of SARS-CoV-2 (Alpha, Beta and Delta). Horizontal lines represent the geometric mean. Between-group comparisons were performed using a Mann-Whitney U test; two-sided P values are reported above each comparison. Participants with undetectable neutralizing antibodies were arbitrarily set to 10 (half of the lowest positive titer). **b–e**, PRNT50 reverse cumulative distribution curves, commencing at titers ≥ 20 , for the standard versus delayed group against wild-type (**b**), Alpha (**c**), Beta (**d**) and Delta (**e**) variants of SARS-CoV-2. Reverse cumulative distribution curves denote the percentage of participants that reach each titer threshold.

AE data ($n=77$), the severity of AEs correlated with the degree of anti-RBD response. In those participants who experienced grade 2 events (14/77 (18.2%)) with the second dose of vaccine (Fig. 5c), median anti-RBD antibody titer after the second dose was 14,617 U ml⁻¹ (IQR, 7,692–21,702) compared with 7,679 U/ml (IQR, 3,211–11,363) in those who experienced only grade 1 or no AEs ($P=0.0025$).

Delayed dosing of mRNA COVID-19 vaccines has been a controversial issue, based primarily on pragmatic reasoning aimed at mitigation of vaccine shortages rather than scientific justification. The main findings of this comparative assessment of humoral and cellular immune response in HCWs receiving two doses of the BNT162b2 vaccine were as follows: (1) all HCWs, regardless

of regular or delayed dosing intervals, produced robust immune responses after two doses of the vaccine; (2) anti-RBD titers were significantly higher in the delayed-interval group; (3) neutralizing antibody titers based on PRNT50 and PRNT90 were significantly higher in the delayed-interval group (this was observed for wild-type as well as Alpha, Beta and Delta SARS-CoV-2 variants); and (4) cellular immune responses represented by spike-specific monofunctional and polyfunctional CD4⁺ T cells, and CD8⁺ T cells were detectable in both groups and generally not significantly different, with the exception of monofunctional IL-2-producing CD4⁺ T cells. The data presented provide scientific rationale to support delayed-dosing policies for mRNA vaccines, especially because there are limited peer-reviewed published data reporting on this.

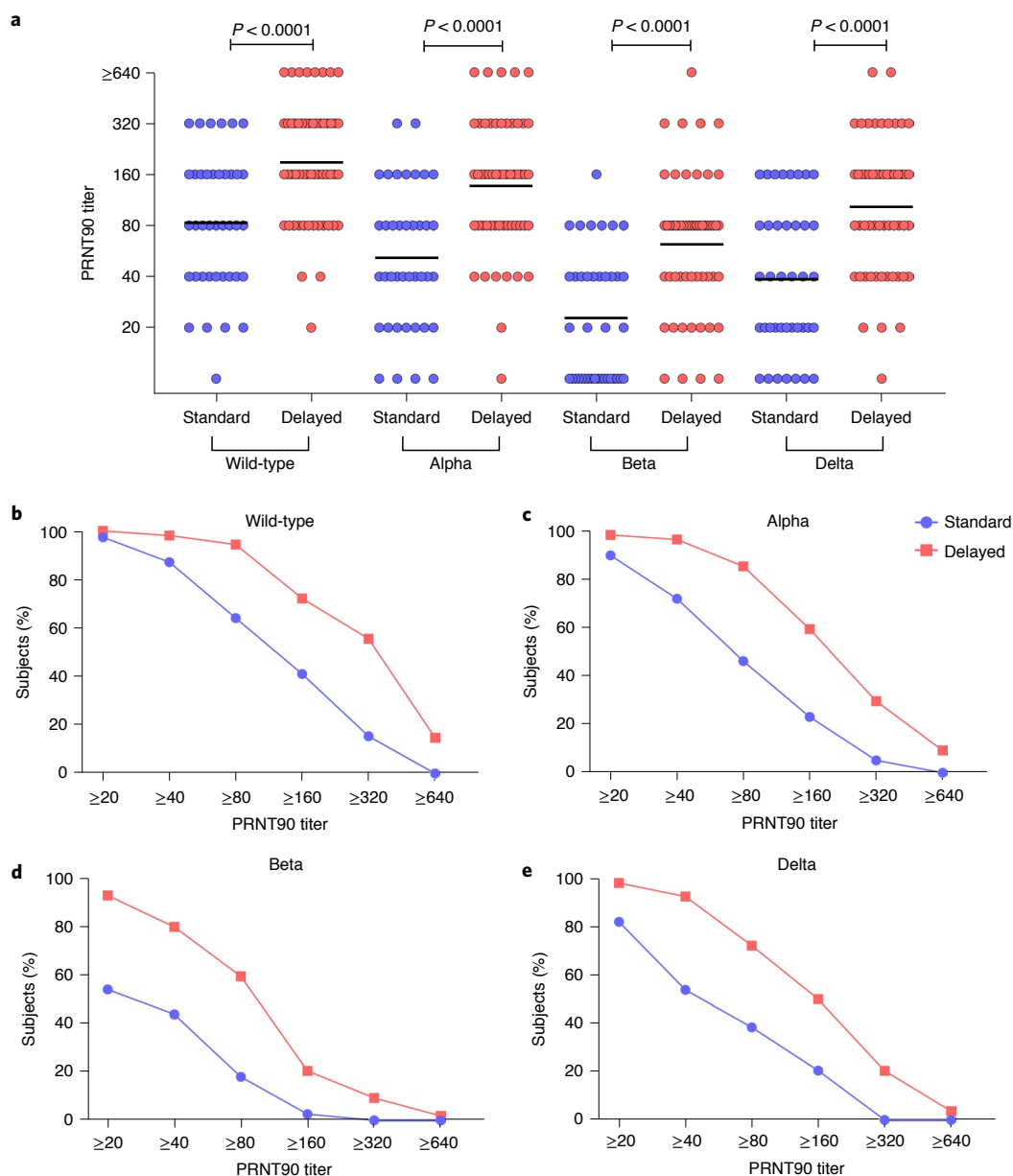


Fig. 3 | PRNT90 titers in participants receiving standard versus delayed vaccination. a, Comparative analysis of PRNT90 titers in the standard ($n=39$) and delayed ($n=54$) groups for wild-type and predominant circulating VOCs of SARS-CoV-2 (Alpha, Beta and Delta). Horizontal lines represent the geometric mean. Between-group comparisons were performed using the Mann-Whitney U test; two-sided P values are reported above each comparison. Participants with undetectable neutralizing antibodies were arbitrarily set to 10 (half of the lowest positive titer). **b–e**, PRNT90 reverse cumulative distribution curves, commencing at titers ≥ 20 , for the standard versus delayed group against wild-type (**b**), Alpha (**c**), Beta (**d**), and Delta (**e**) variants of SARS-CoV-2. Reverse cumulative distribution curves denote the percentage of participants that reach each titer threshold.

Concern has been raised that delaying the interval of the two doses of vaccine may encourage the development of new SARS-CoV-2 variants in the interim while individuals await the second dose⁴. This concern may be mitigated, however, by improved neutralization titers against circulating variants achieved in the delayed-vaccination group, possibly conferring a more durable response and longer-term protection, as well as potentially increasing the likelihood of sufficient antibody levels to confer efficacy against heterologous and partially vaccine-resistant variants. The greater humoral response achieved by delaying mRNA vaccination is consistent with studies of delaying the second dose of vector-based (ChAdOx1) vaccine² and BNT162b2 (ref. ³). Our study additionally provides PRNT90 data in addition to PRNT50 data. The former

data may have better discriminatory ability in healthy cohorts. Also, we used flow cytometry (versus an enzyme-linked immunosorbent spot assay), which may allow for more detailed discrimination of cell populations and account for differences in our findings related to IL-2 monofunctional CD4⁺ T cells.

The mechanism resulting in enhanced antibody responses in the delayed vaccine group are unknown. It has been described previously⁵ that memory B cells against SARS-CoV-2 spike increase between 1 month and 8 months after infection, whereas memory CD4⁺ T cells decline. Similarly, it has been observed that by 6 months after infection, the number of RBD-specific memory B cells remains largely unchanged but displays greater resistance to RBD mutations and has increased potency⁶. This effect is likely a result of

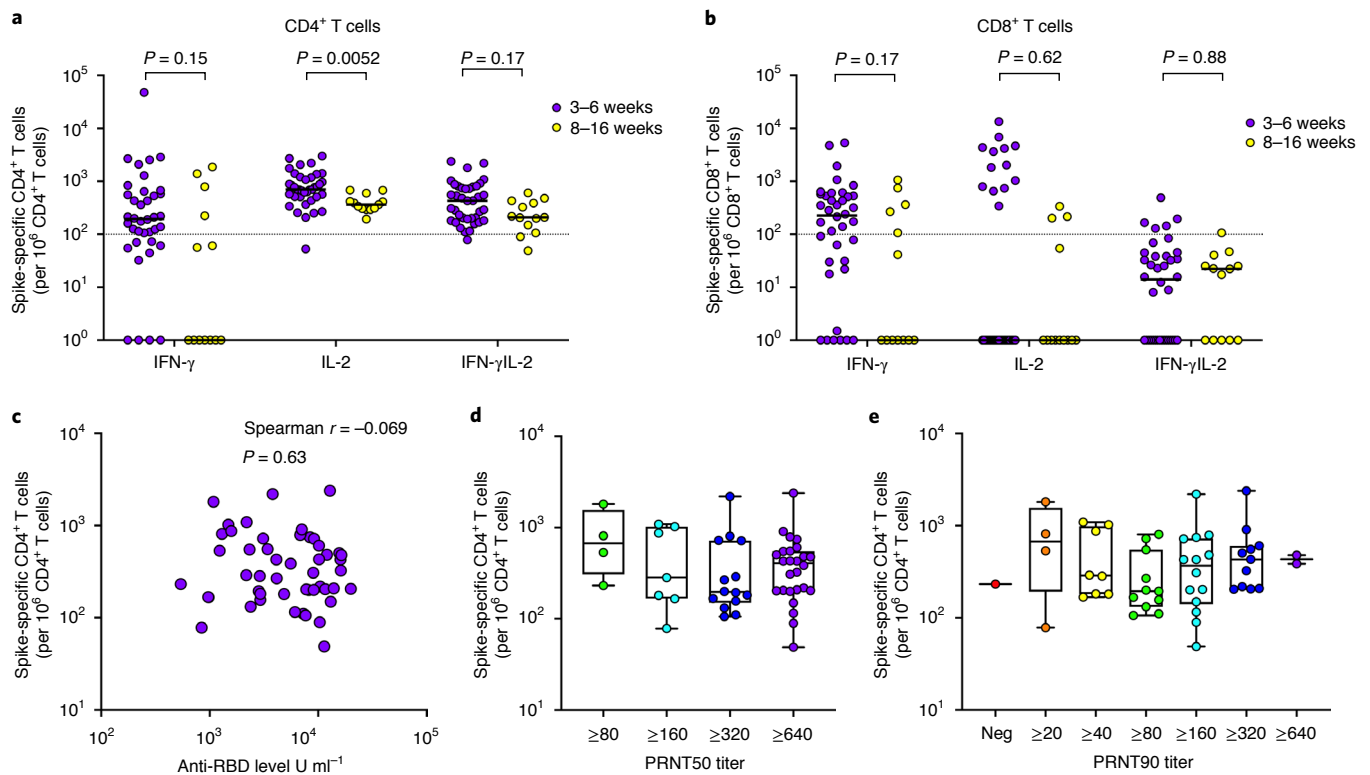


Fig. 4 | Spike-specific CD4⁺ and CD8⁺ T cell responses, and correlation of cellular with humoral responses. **a,b**, Proportions of IFN- γ ⁺ monofunctional, IL-2⁺ monofunctional and IFN- γ ⁺IL-2⁺ polyfunctional CD4⁺ T cells (**a**) and CD8⁺ T cells (**b**) in HCWs with standard or delayed vaccination. Horizontal bars denote the median for each group. A two-sided Mann–Whitney *U* test with Holm–Šidák correction for multiple comparisons was used. Adjusted *P* values are shown above each respective comparison. Comparisons were performed between $n = 38$ HCWs with standard vaccination and $n = 13$ HCWs with delayed vaccination. **c**, Correlation of IFN- γ ⁺IL-2⁺ polyfunctional CD4⁺ T cells versus anti-spike RBD levels (U ml⁻¹). Spearman’s correlation test was performed; Spearman r and corresponding two-tailed *P* values are shown in the top right corner. **d,e**, Box-whisker plots showing frequencies of IFN- γ ⁺IL-2⁺ polyfunctional CD4⁺ T cells in $n = 51$ HCWs according to corresponding PRNT50 (**d**) and PRNT90 (**e**) titers. Comparison across PRNT titers done using Kruskal–Wallis test, $P = \text{NS}$ (not significant). Plots show median (horizontal line), with the upper and lower bounds of the vertical lines indicating the maximum and minimum values, respectively. The upper and lower bounds of boxes represent the upper and lower limits of the IQR, respectively. In all plots, each participant is represented by a dot. Neg, negative PRNT90 result.

germinal center reactions in peripheral lymphoid tissues, where follicular helper T cells help guide processes like somatic hypermutation and affinity maturation that evolve the humoral response over time. Both mRNA vaccines have demonstrated potent SARS-CoV-2-specific germinal center responses, even with just a single dose of vaccination⁷. As such, we propose that the memory B cell repertoire at the time of delayed vaccination may simply be of a greater caliber, leading to a higher quantity and/or quality of antibody response. Analogous maturation processes are not known to occur for T cells, which are thought to contract at a faster rate than B cells⁸. Although we did not measure memory B cells or follicular helper T cells in our study, we did identify lower frequencies of IL-2-monofunctional CD4⁺ T cells in the delayed-immunization protocol. However, no statistical difference was measured between groups with respect to the polyfunctional CD4⁺ and CD8⁺ T cells, suggesting that delayed second-dose immunization is still capable of inducing robust vaccine-specific T cell responses. Importantly, no cellular correlate of immunity has been identified.

Our study has some limitations. Our cohort was younger and female predominant, and both factors can positively influence the immune response⁹. However, given similar age and sex distribution in the standard and delayed groups, this factor would not influence the group comparisons. Although we did not collect blood from the standard-interval group after the first dose of vaccine, there is no rationale to expect this to be different between the two groups

(after the first dose). An analysis of circulating T follicular helper and spike-specific B cells was not performed owing to limited numbers of cells available for analysis. Our study was not powered to determine real-world vaccine effectiveness; however, in keeping with our results, recent Canadian epidemiologic data (in preprint at the time of writing) support improved vaccine effectiveness with the delayed second dose¹⁰. A strength was the duration of the study and follow-up after vaccination, with all participants observed for several months and at least 6 weeks after their second dose of vaccine.

In summary, our study demonstrates that a delayed-vaccination schedule does not appear to be detrimental to overall immunogenicity and actually results in improved neutralizing antibody responses to wild-type, Alpha, Beta and Delta variants. This is of public health importance in global jurisdictions, where significant portions of the population have yet to be vaccinated because of either vaccine unavailability or hesitancy. Our study also provides immunogenicity data to support recent epidemiologic observations¹⁰. Further studies of the significance of these findings, including the durability of the immune response over time, correlates of protective efficacy, and immunogenicity as it relates to heterologous variant viruses, would be helpful to define the optimal dosing interval and the necessity or timing of a booster dose of vaccine. Our cohort will be followed for 12 months after enrolment, with testing at 6 and 12 months after the second dose of vaccine, to provide longer-term assessment of the

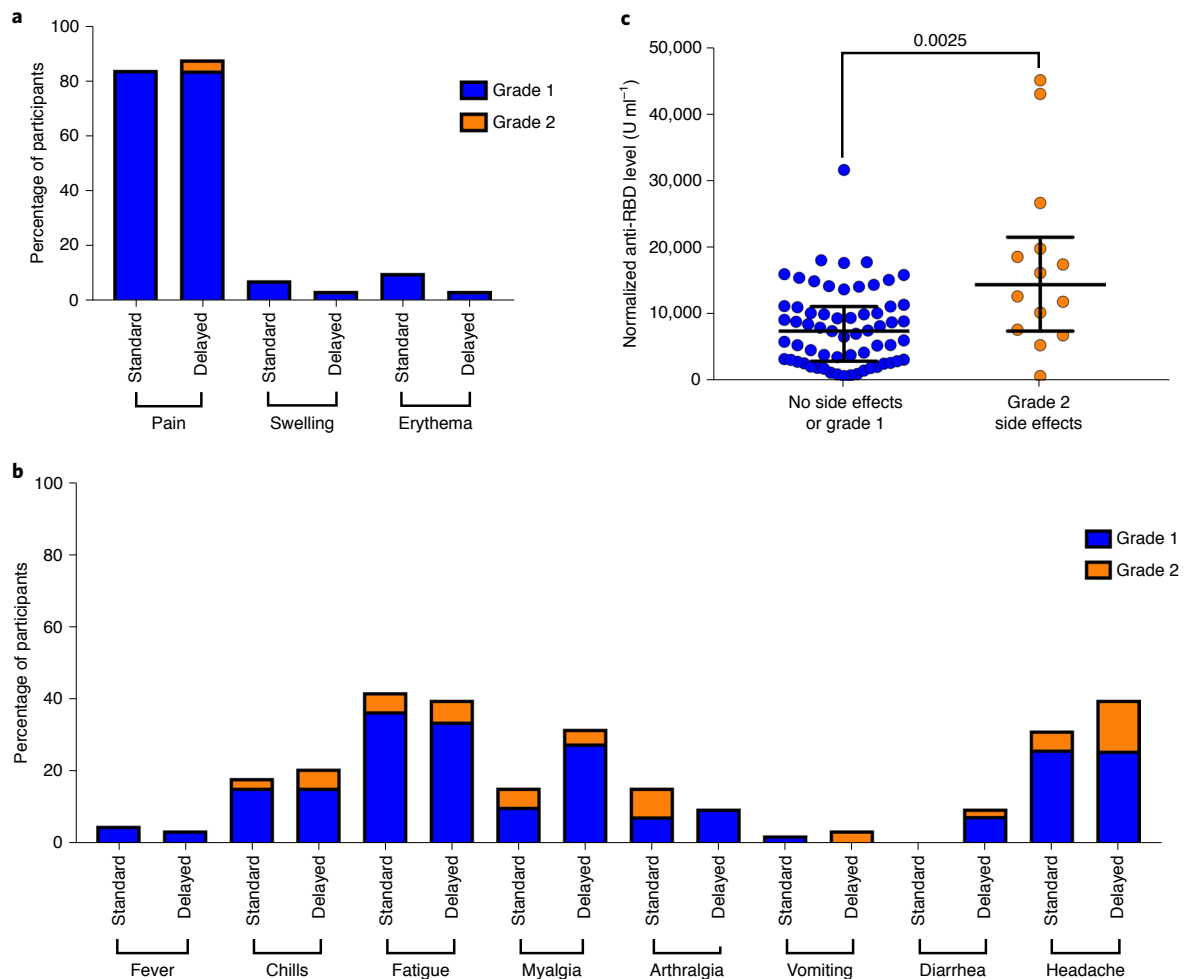


Fig. 5 | AEs from the second dose of BNT162b2 in the standard- (3–6 weeks) and delayed-interval (8–16 weeks) groups. a, Local AEs (grade 1 and 2) according to percentage of participants. **b**, Systemic AEs (grade 1 and 2) according to percentage of participants. **c**, Anti-RBD titer in participants with no or grade 1 AEs ($n=63$) versus grade 2 AEs ($n=14$). Horizontal lines represent median, and vertical lines indicate the IQR. Each participant is represented by a dot. Anti-RBD titer was analyzed using a Mann–Whitney U test, and a two-sided P value is shown for comparison.

effect of a standard versus delayed vaccination schedule on humoral and cell-mediated vaccine immunogenicity.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41590-021-01126-6>.

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Methods

Patient population and study design. This was a prospective observational cohort study of HCWs recruited from a tertiary, university-affiliated hospital network in Toronto, Canada, who were eligible to receive the two scheduled doses of the BNT162b2 (Pfizer/BioNTech) mRNA vaccine (Fig. 1a). Potential participants were recruited via mass emails to HCWs working at several hospital sites. Those who completed the two-dose vaccine series between 4 January 2021 and 13 June 2021 and consented were included and followed for at least 6 weeks after the second dose. Participants were excluded if they had known previous COVID-19 infection or had an immunosuppressive condition. Delayed dosing for HCWs came into effect in March 2021. This allowed for two study cohorts: those who received two doses at the standard interval (3–6 weeks) and those who received two doses at a delayed interval of >8 weeks. All participants provided blood samples prior to vaccination and 4–6 weeks after the second dose of vaccine. In the delayed-interval group, an extra blood sample was obtained 4 weeks after dose 1. The local institutional research ethics board approved the study (University Health Network Research Ethics Board (CAPCR 20-6069)), and all participants provided signed informed consent.

Anti-RBD antibody and neutralization responses. Antibody against the spike RBD (anti-RBD) was measured using the commercial Roche Elecsys anti-SARS-CoV-2 S immunoassay. Briefly, the assay is a double-antigen sandwich electrochemiluminescence immunoassay that quantitatively detects antibodies to the RBD of the SARS-CoV-2 spike protein. Sera were tested as per the manufacturer's instructions in a certified biochemistry laboratory. The assay's lower limit of detection was 0.4 U ml⁻¹, and a positive test result was defined as ≥0.8 U ml⁻¹.

Neutralizing antibodies were assessed by PRNT50 and PRNT90 assays using ancestral (wild-type) SARS-CoV-2 (Canada/ON_ON-VIDO-01-2/2020, EPI_ISL_42517), titrated as previously reported^{11,12}, as well as the SARS-CoV-2 variants B.1.1.7 (Alpha), B.1.351 (Beta) and B.1.167.2 (Delta). In a 96-well plate, antibodies were serially diluted and mixed with diluted SARS-CoV-2 (100 plaque-forming units (p.f.u.) per 100 µl), yielding final antibody dilutions of 1:20 to 1:640 and a final virus concentration of 50 p.f.u. per 100 µl. No neutralization, 50% neutralization and 90% neutralization controls were prepared by diluting SARS-CoV-2 at 50, 25, and 5 p.f.u. per 100 µl, respectively. We used 2% FBS and 1× penicillin supplemented DMEM as a no-virus control. After 1 h of incubation at 37°C and 5% CO₂, 100 µl of each antibody–virus mixture was added in duplicate to 12-well plates containing Vero E6 cells (ATCC; cell lines used were evaluated for *Mycoplasma* contamination via polymerase chain reaction and tested negative) at 95–100% confluence, and 100 µl of each control was added in triplicate to two sets of 12-well plates containing Vero E6 cells. All plates were incubated at 37°C and 5% CO₂ for 1 h of adsorption, followed by adding liquid overlay containing equal volumes of 3% carboxymethylcellulose and 2× modified Eagle's medium (Temin's modification). After a 3-day incubation for the ancestral virus, Alpha and Beta and a 4-day incubation for Delta, the liquid overlay was removed and cells were fixed with 10% neutral-buffered formalin. The monolayer in each well was stained with 0.5% crystal violet (w/v), and the average number of plaques was calculated at each dilution and compared with the average number of plaques in the 50% and 90% neutralization controls. The reciprocal of the highest dilution resulting in 50% and 90% reduction in plaques compared with controls was defined as the PRNT50 and PRNT90 endpoint titers, respectively. PRNT50 titers and PRNT90 titers ≥20 were considered positive for SARS-CoV-2 neutralizing antibodies, whereas titers <20 were considered negative.

Cell-mediated immunity assessment. In a subset of participants who provided additional blood, spike-specific CD4⁺ and CD8⁺ T cell responses were assessed as previously described¹³. Briefly, peripheral blood mononuclear cells (PBMCs) underwent Ficoll-based isolation and were cryopreserved in liquid nitrogen until use. A total of 10⁶ PBMCs were rested for 2 h and subsequently incubated with 15-mer overlapping peptides encompassing the full SARS-CoV-2 spike protein (PepTivator, Miltenyi Biotec) at a final concentration of 5 µg ml⁻¹ per peptide, along with a CD28/CD49d costimulatory antibody cocktail (BD Biosciences) and a protein transport inhibitor to prevent cytokine release (Thermo Fisher Scientific). Cells were fixed, permeabilized and incubated with an antibody cocktail to detect intracellular markers (IFN-γ and IL-2). PMA/ionomycin was used as a positive control, and cells with media alone were used as a negative control. Frequencies of CD4⁺ and CD8⁺ T cells that expressed both cytokines (polyfunctional cells) or only one cytokine (monofunctional) were measured. Flow cytometry was performed on an LSR II BGRV (BD Biosciences) at the SickKids-UHN Flow Cytometry Facility. The frequency of vaccine-antigen-specific T cells was determined by subtracting the frequencies of cytokine-positive T cells in unstimulated comparators from peptide-stimulated samples. A representative gating strategy is shown in Extended Data Fig. 2. A positive T cell response was defined at a frequency of 0.01% or greater (limit of quantitation). A minimum of 100,000 live CD3⁺ T cells were required for samples to be included in the flow analysis. Flow data were collected using BD FACS Diva version 6.1.3, and flow data were analyzed using FlowJo v10.7.1.

Antibodies used in this study were obtained from commercial vendors and included anti-human CD3-BV786 (clone SK7, 563799, BD Biosciences; dilution 1:80), anti-human CD4-Pacific Blue (clone RPA-T4, 558116BD, Biosciences; dilution 1:40), anti-human CD8-APC-Cy7 (clone RPA-T8, 557760, BD Biosciences; dilution 1:40), anti-human IFN-γ-FITC (clone B27, 554700, BD Biosciences; dilution 1:40), and anti-human IL-2-APC (clone MQ1-17H12, 500310, BioLegend; dilution 1:40). Validation of antibodies for specificity and application was directly performed by vendors, and corresponding data are available from the suppliers. Antibodies were titrated in-house to evaluate the best concentrations for discrimination of negative and positive cells using PBMCs collected from healthy human volunteers.

Safety and AEs. Safety assessments included monitoring through a participant-directed vaccine diary for local and systemic AEs for 7 days after each injection. AEs were categorized by the U.S. Food and Drug Administration toxicity grading scale for volunteers in vaccine trials as follows: grade 1, no interference in daily activities; grade 2, some interference in daily activities; grade 3, participants unable to perform daily activities; and grade 4, potentially life threatening¹⁴.

Statistical analysis. The primary analysis included post-second dose anti-RBD titers, neutralizing antibody and spike-specific polyfunctional and monofunctional CD4⁺ and CD8⁺ T cells between the standard- and delayed-interval groups. These were compared between groups using Mann–Whitney *U* test, and a significant *P* value was set at 0.05. To adjust for multiple comparisons in the T cell analysis, the Holm–Šidák method was used to calculate adjusted *P* values for monofunctional and polyfunctional T cells. Secondary analyses were as follows: (1) correlation between anti-RBD titer and time between doses using Spearman's test, (2) correlation between spike-specific polyfunctional CD4⁺ T cell response and anti-RBD level using Spearman's test, (3) a comparison polyfunctional CD4⁺ T cell response across PRNT50/PRNT90 titers using Kruskal–Wallis and (4) the median anti-RBD titer in those with grade 2 events compared to those no or grade 1 events using Mann–Whitney *U* test. All measurements were taken from distinct samples, and all tests were two sided. Statistical analysis was performed using SPSS v29.0 and Prism GraphPad v9.1.1.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All numerical data found in the figures and extended data that support the findings of this study are fully available as source data files. Source data are provided with this paper.

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Author contributions

D.K., A.H., V.G.H. and V.H.F. designed the study; V.G.H., V.H.F., D.K. and A.H. analyzed data; V.G.H., V.H.F., M.I., B.M.-K., V.K., K.M., H.W. and A.R. performed the study; and D.K., A.H., V.G.H., V.H.F. and H.W. wrote the manuscript.

Competing interests

D.K. has received clinical trials grants from Roche and GSK and advisory fees from Roche, GSK, Sanofi, Merck and Exevir. A.H. has received a clinical trial grant from Roche and advisory fees from Merck. V.K. has received consultancy fees from Abbott

Diagnostics. All competing interests are unrelated to the current study. The remaining authors declare no competing interests.

Additional information

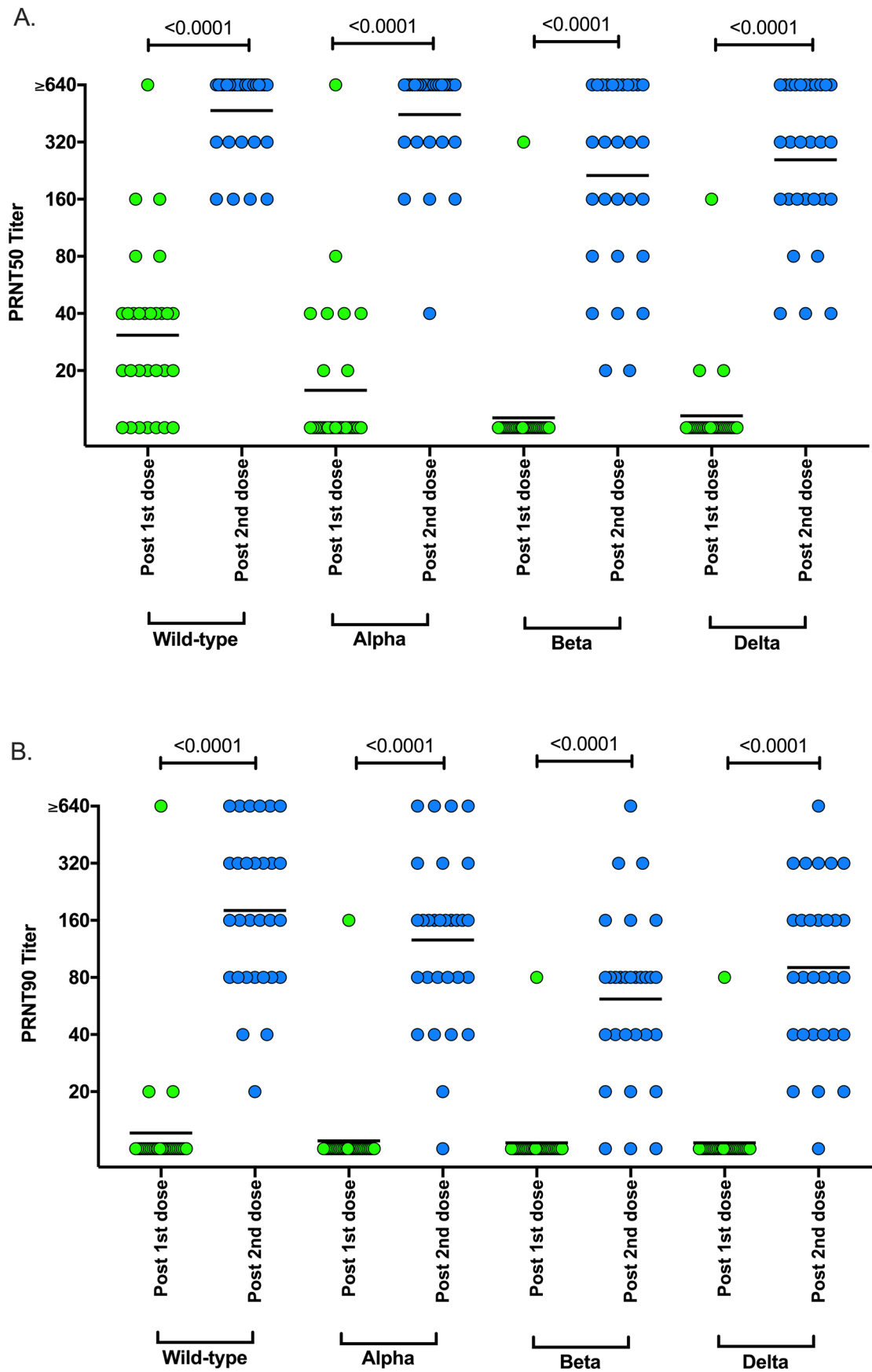
Extended data is available for this paper at <https://doi.org/10.1038/s41590-021-01126-6>.

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41590-021-01126-6>.

Correspondence and requests for materials should be addressed to Victoria G. Hall or Deepali Kumar.

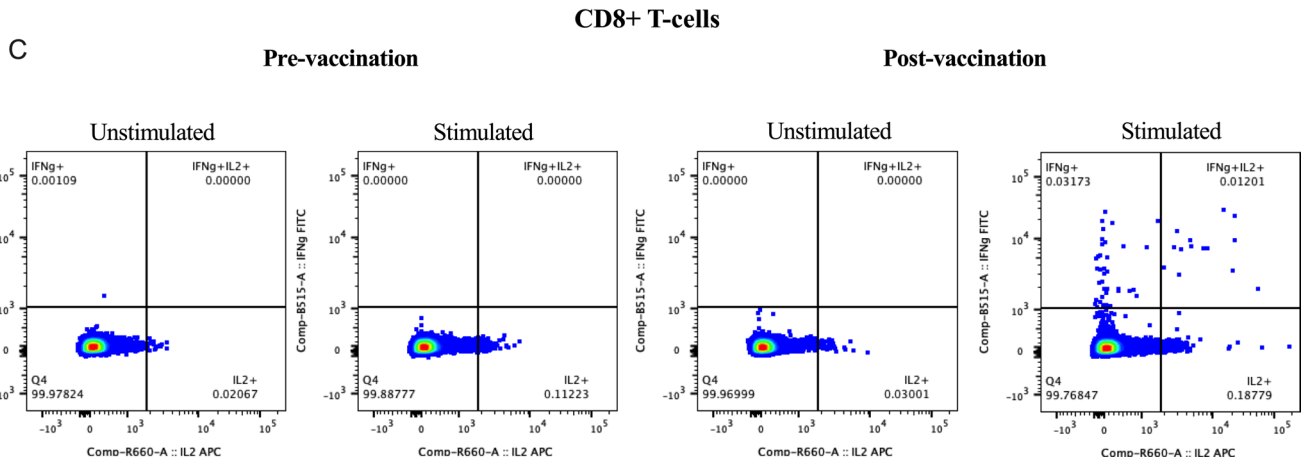
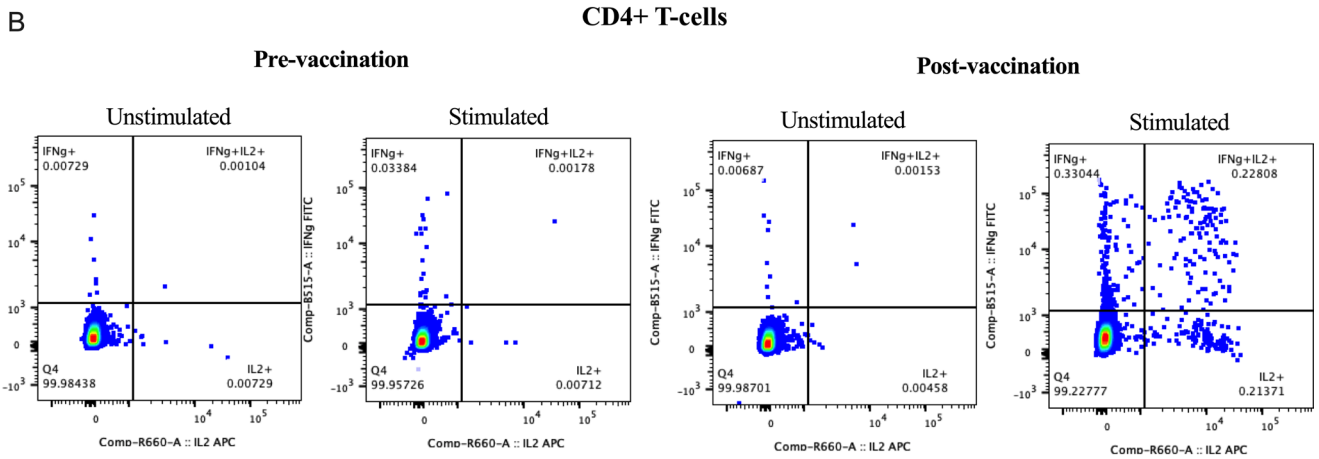
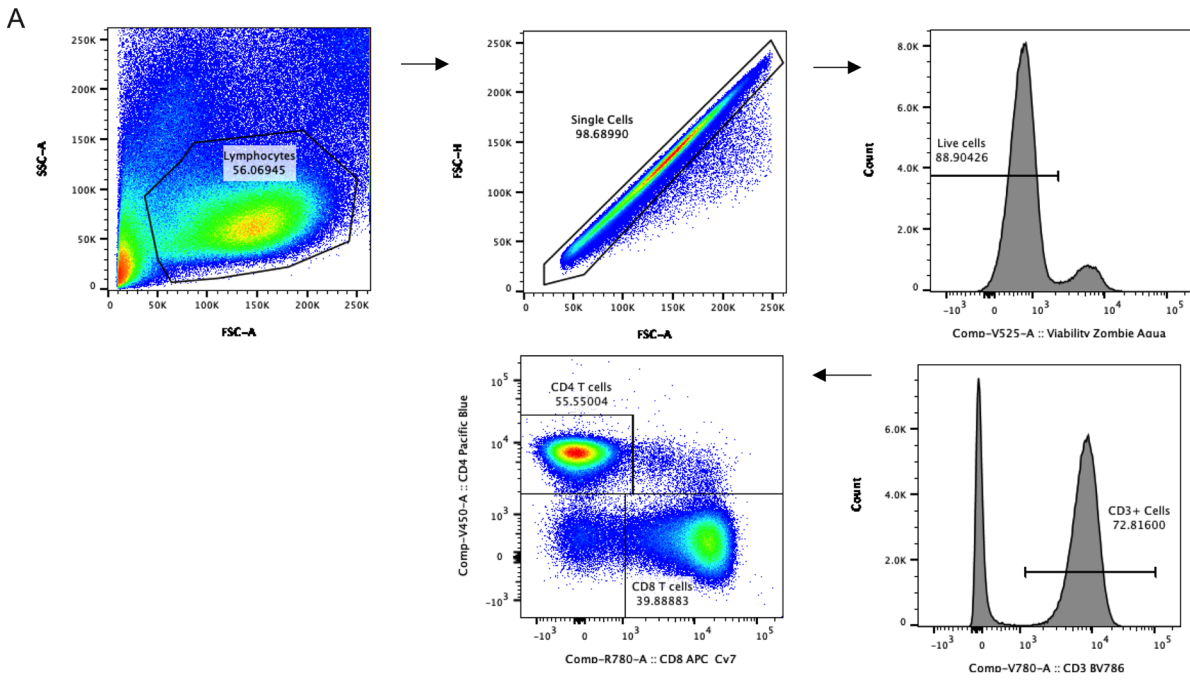
Peer review information *Nature Immunology* thanks Stephen Kent and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Zoltan Fehervari was the primary editor on this article and managed its editorial process and peer review in collaboration with the rest of the editorial team.

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Extended Data Fig. 1 | See next page for caption.

Extended Data Fig. 1 | PRNT titers after first and second dose in participants with delayed dosing. PRNT50 (A) and PRNT90 (B) titers in a subgroup of participants with delayed dosing who gave samples after both first-dose and second dose of vaccine. Comparisons against ancestral SARS-CoV-2, and variants of concern (alpha, beta and delta) are shown. Horizontal lines denote the median. Between group comparisons were performed using the Wilcoxon signed-rank test; two-sided p-values are shown above each corresponding comparison (n = 29).



Extended Data Fig. 2 | See next page for caption.

Extended Data Fig. 2 | Gating strategy and representative cytokine dot plots. (A) The sequential gating strategy for identifying CD4⁺ and CD8⁺ T cells. Arrows indicate the sequence of hierarchical gates. Number in each plot refers to the frequency of the gated subset in the parental population. (B, C) Plots showing IFN- γ (y-axis) vs. IL-2 (x-axis) production, gated on CD4⁺ T cells (B) or CD8⁺ T cells (C). Plots are taken from the same participant. Plots show T cells stimulated with SARS-CoV-2 spike peptides, or media alone for the unstimulated control. Left two panels show response with 3-6-week interval, and right two panels show T cell responses following administration of delayed (8-16-week interval).

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Life sciences study design

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Sample size	The healthcare worker was recruited as part of a larger study looking at COVID-19 vaccine immunogenicity in transplant patients. In the larger study, the sample size for healthy controls was 120 for comparison to transplant patients. However, because vaccine in Canada was delayed for healthcare workers, this control group could no longer be compared to the transplant group. For the analysis presented here, no formal sample size calculation was performed since the recommendations about the interval between first and second dose of COVID-19 vaccine for healthcare workers were rapidly changing in Canada due to vaccine shortages, and it was unclear how many healthcare workers would be vaccinated at the standard vs. delayed intervals (real world recruitment with convenience sampling).
Data exclusions	Healthcare workers with prior documented COVID-19 infection were excluded.
Replication	Binding Antibody - For antibody testing, this was done using commercially validated assays in a CLIA-certified biochemistry lab. Live virus neutralization - The neutralization assay was done at the National Microbiology Laboratory/Public Health Agency of Canada using a validated plaque reduction neutralization test. This is a central reference lab performing live-virus PRNT testing for Canada. T cell assays - Steps were taken during the performance of the T-cell assays to ensure robustness including having an adequate number of live cells. All the assays used in this study including T-cell assays have been previously published by our group in peer-reviewed journals (most recently Hall et al., NEJM, Aug 11, 2021). No replicates were performed due to limited sample material.
Randomization	Not applicable. This was a cohort study and therefore was not randomized. It was not possible to randomize due to uncertainties in vaccine distribution.
Blinding	Not applicable. Laboratory personnel conducting the experiments were blinded to dosing intervals and other clinical details.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
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<input type="checkbox"/>	<input checked="" type="checkbox"/> Eukaryotic cell lines
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<input checked="" type="checkbox"/>	<input type="checkbox"/> Animals and other organisms
<input type="checkbox"/>	<input checked="" type="checkbox"/> Human research participants
<input checked="" type="checkbox"/>	<input type="checkbox"/> Clinical data
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Methods

n/a	Involved in the study
<input checked="" type="checkbox"/>	<input type="checkbox"/> ChIP-seq
<input type="checkbox"/>	<input checked="" type="checkbox"/> Flow cytometry
<input checked="" type="checkbox"/>	<input type="checkbox"/> MRI-based neuroimaging

Antibodies

Antibodies used	Flow cytometry: anti-human CD3-BV786 (BD Biosciences) clone SK7, cat no: 563799, dilution 1:80 anti-human CD4-Pacific Blue (BD Biosciences) clone RPA-T4, cat no: 558116, dilution: 1:40 anti-human CD8-APC-Cy7 (BD Biosciences) clone RPA-T8, cat no: 557760, dilution: 1:40 anti-human IFN- γ -FITC (BD Biosciences) clone B27, cat no: 554700, dilution: 1:40 anti-human IL-2-APC (BioLegend) clone MQ1-17H12, cat no: 500310, dilution: 1:40
Validation	All antibodies were obtained from commercial vendors and corresponding Data Sheets are available and provided by the manufacturers describing validation of specificity. All antibodies were separately titrated in-house to evaluate best concentrations for discrimination of negative and positive cells.

Eukaryotic cell lines

Policy information about [cell lines](#)

Cell line source(s)	Vero E6 (purchased through ATCC)
Authentication	Cell lines were authenticated by the provider. Additional information about the cell line can be found at the following link: https://www.atcc.org/products/crl-1586 .
Mycoplasma contamination	Cell lines were tested for Mycoplasma (and were negative by PCR).
Commonly misidentified lines (See ICLAC register)	No commonly misidentified cell lines were used.

Human research participants

Policy information about [studies involving human research participants](#)

Population characteristics	We prospectively recruited immunocompetent healthcare workers at the University Health Network, Toronto, who intended to receive two doses of COVID-19 vaccine. The 93 participants who enrolled in the study were predominantly female (79.6%) and median age was 40.9 years. Initially, vaccine was provided at the standard interval as stated in the product monograph; however, due to limited supply, second doses for healthcare workers were delayed for up to 16 weeks. Therefore, 39/93 participants received vaccine at standard intervals and 54/93 participants had a delay in their second dose.
Recruitment	Participants were recruited by sending a mass email to healthcare workers. This was a prospective observational study and all participants provided written informed consent. The study cohort represents the demographics of healthcare workers at our institution and there is no apparent self-selection bias.
Ethics oversight	University Health Network Research Ethics Board

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	PBMCs were isolated using conventional Ficoll-based separation protocols. After isolating the PBMCs from the peripheral blood, they were treated for 15 min in red cell lysis buffer according to the manufacturer's instruction (BD Pharmalysse). Cells were cryopreserved in 10% DMSO + 90% FBS (ThermoFisher Scientific). Cells were stored in a Mr. Frosty Freezing Container (ThermoFisher Scientific) for 24 hours at -80oC prior to storage in the vapor phase of liquid nitrogen. Samples were assessed in batches. Cells were gently thawed in a 37oC water bath and washed in complete RPMI (10% FBS, L-glu, pen/strep, HEPES) to remove residual DMSO. Cells were counted using a Vi-Cell instrument (Beckman Coulter) and seeded in 96-well plates at 1 million cells per well in complete RPMI. Cells were rested for 2 hours at 37oC prior to undergoing stimulation with/without peptides. Peptides were added at 5ug/mL, as determined by prior experiments and co-stimulatory antibodies were added (BD FastImmune anti-CD28/CD49d). An hour after stimulation of cells, a protein transport inhibitor was added to prevent cytokine release (ThermoFisher Scientific). Cells were incubated overnight (16 hours) and subsequently stained for viability using the Zombie Aqua viability dye (BioLegend) at 1:1000 dilution. Cells were Fc blocked (BD Biosciences), and stained with a surface antibody cocktail consisting of human anti-CD3, -CD4 and -CD8 antibodies for 30 min on ice. Cells were then fixed in 4% paraformaldehyde (Fixation Buffer, BioLegend, Cat no: 420801) at room temperature for 30 min. Cells were incubated with an intracellular antibody cocktail targeting anti-human IFN- γ and IL-2 prepared in Intracellular Staining Permeabilization Wash Buffer (BioLegend) for 20 min at room temp. After staining, cells were washed thoroughly and resuspended in wash buffer. Tubes were wrapped in foil to protect from light, and kept at 4oC until acquisition of data. Data was acquired within 24 hours of staining.
Instrument	Data was analyzed on an LSR II VBGR instrument (Becton Dickinson) at the SickKids-UHN Flow Cytometry Core Facility.
Software	Acquisition was performed using BD FACSDiva. Analysis was performed using FlowJo, v10.7.1.
Cell population abundance	N/A. No sorting was performed.
Gating strategy	Gating Strategy: SSC-A vs FSC-A (lymphocytes) -> FSC-H vs FSC-A (single cells) -> count vs viability-Zombie Aqua (live cells;

Gating strategy

gated on Zombie Aqua-negative cells -> count vs CD3-BV786 (CD3+ T-cells; gated on CD3+ cells) -> CD4-pacific blue vs CD8-APC-Cy7 (CD4+ T-cells are CD8-negative, and CD8+ T-cells were CD4-negative). Live CD4+ and CD8+ T-cells were then gated in terms of IFN- γ -FITC vs IL-2-APC to display cytokine production. To gate live/dead cells properly, control specimens were prepared where PBMCs were heat-killed by incubation at 65oC for 10 min and then mixed in a 1:1 ratio with live (non-heat killed cells). These cells were then stained for flow analysis. To determine the proper placement of cytokine gates for determining cut-offs, a combination of negative controls (unstimulated PBMCs) and positive controls (PBMCs stimulated with PMA/ionomycin for 16 hours, a known potent inducer of IFN γ and IL2 production in T-cells) were used.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.