# Poor antigen-specific responses to the second BNT162b2 mRNA vaccine dose in SARS-CoV-2-experienced individuals

One sentence summary: Immune responses to the booster dose of mRNA vaccine BNT162b2 are poor in subjects with a prior history of SARS-CoV-2 infection.

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#### Abstract:

The advent of COVID-19 vaccines will play a major role in helping to end the pandemic that has killed millions worldwide. Vaccine candidates have demonstrated robust humoral responses and have protected against infection. However, efficacy trials were focused on individuals with no prior exposure to SARS-CoV-2, and, as a result, little is known about immune responses induced by these mRNA vaccines in individuals who recovered from COVID-19. Here, we evaluated immune responses in 32 subjects who received two-dose BNT162b2 mRNA vaccination. In individuals naive to SARS-CoV-2, we observed robust increases in humoral and antigen-specific antibody-secreting cell (ASC) responses following each dose of vaccine, whereas individuals with prior exposure to SARS-CoV-2 demonstrated strong humoral and antigen-specific ASC responses to the first dose but muted responses to the second dose of the vaccine for the time points studied. These data highlight an important gap in our knowledge and may have major implications for how these vaccines should be used to prevent COVID-19.

#### Main text

SARS-CoV-2 has caused hundreds of millions of infections and millions of deaths. Vaccines are critical to ending the pandemic. Humoral responses are thought to be an important, although unproven, correlate of protection following COVID-19 vaccines, as novel mRNA vaccines against SARS-CoV-2 have induced robust humoral responses against SARS-CoV-2<sup>1-6</sup> and were efficacious in large-scale clinical trials<sup>7,8</sup>. However, large-scale clinical trials excluded individuals with a prior diagnosis of COVID-19. As a result, little is known about the immune responses in SARS-CoV-2-experienced individuals, a setting which is relevant to hundreds of millions of people worldwide. Furthermore, it is unknown whether durability of antibody responses following vaccination, which was evaluated in SARS-CoV-2-naive subjects who received mRNA-1273<sup>9</sup>, is adversely affected by prior response to SARS-CoV-2 infection. Better understanding of the effects of vaccination in the context of immunological history is urgently needed.

## Results

Prior immune history can affect subsequent responses to antigen. To test the effects of immunological history in the setting of COVID-19, we recruited 13 subjects who had documented infection with SARS-CoV-2 and 19 subjects who were SARS-CoV-2-naive. Age ranged from 24 to 62. The median age was 39 years for naïve adults and 41 for SARS-CoV-2-experienced individuals. All SARS-CoV-2-experienced subjects had mild or asymptomatic COVID-19. Two individuals were infected with SARS-CoV-2 within 30 days prior to vaccination, whereas the remaining 11 were at least six months beyond diagnosis of COVID-19. All subjects received two doses of the BNT162b2 mRNA vaccine in accordance with FDA Emergency Use Authorization, and immune responses assessed at approximate intervals before and after each dose of vaccine (**Figure 1A**).

Given the likely importance of humoral responses as a correlate of protection, we first assessed antibody responses to the S protein. IgG antibodies were detectable in individuals who had recovered from SARS-CoV-2 infection and not detectable in those who were SARS-CoV-2-naive (median titers 25 and 7224, respectively; P=0.00012; Wilcoxon test) (**Figure 1B**). Following first dose immunization,

SARS-CoV-2-experienced subjects had a median fold-change of 47 whereas SARS-CoV-2-naive subjects had a median fold-change of 2.6 (P=0.02; Wilcoxon test). However, after second dose immunization, SARS-CoV-2-experienced subjects had a median fold-change of 1.4 whereas the SARS-CoV-2-naive subjects had a fold-change of 13 (P=0.0025; Wilcoxon test). Indeed, the two cohorts had similar anti-S1 IgG titers one week after the second dose (P=0.24; t-test). These data demonstrate rapid and robust humoral responses after vaccination in both cohorts, but there was minimal further increase in SARS-CoV-2-experienced subjects after the second vaccine dose.

In a subset of subjects, we next asked if neutralizing antibody titers changed following immunization. SARS-CoV-2-experienced subjects had antibodies with low neutralization activity at baseline in two of three subjects, and sera from SARS-CoV-2-naive subjects had no neutralization activity in four of four subjects (P=0.12; Wilcoxon test) (**Figure 1C**). Following first dose immunization, however, we found a rapid increase in neutralizing titers in all three subjects to a median titer of 9860 in SARS-CoV-2-experienced subjects compared to a very low level increase in only one of four subjects with a median titer of 10 in SARS-CoV-2-naive subjects (P=0.04; Wilcoxon test). In contrast to the first dose of vaccine, there was no increase after the second dose of the vaccine in SARS-CoV-2-experienced subjects whereas the SARS-CoV-2-naive subjects continued to increase in titer. Thus, similar to the S1-specific IgG responses, the second dose of vaccine did not stimulate further neutralizing antibody responses in individuals who had had prior COVID-19.

Humoral responses are associated with antigen-specific B cell responses, so we next asked whether antigen-specific antibody-secreting cell (ASC) responses increased with vaccination by performing ELISpot analysis of freshly-acquired samples one week after vaccination. After the first dose of vaccine, SARS-CoV-2-naive subjects had few or no antigen-specific ASCs detected, whereas SARS-CoV-2-experienced subjects had robust IgG- and IgA-secreting ASC responses to RBD, S1, and S2 proteins (**Figure 1D-I**). After the second dose of vaccine, SARS-CoV-2-naive subjects demonstrated increased antigen-specific ASC responses with a fold-change of 7 for S1-specific ASC, whereas the SARS-CoV-2-experienced subjects had a fold-change of 0.13 for S1-specific ASC (**Figure 1J**). Similar reductions in circulating antigen-specific ASC were noted for S2 and RBD (**Figure 1K-L**). Together, these data demonstrate that while antigen-specific ASC responses continued to increase with each of two vaccine doses in SARS-CoV-2-naive subjects, fewer antigen-specific ASC were in circulation one week after the second vaccine dose than the first dose in SARS-CoV-2-experienced subjects.

## **Discussion:**

Humoral responses are likely to be critical mediators of protection against SARS-CoV-2 exposure, thus understanding factors that affect humoral responses will be critical to optimal use of these vaccines. Indeed, more studies are needed to fully understand the breadth and quality of the immune response to these vaccines. Here, we observed robust antibody responses in individuals who were SARS-CoV-2-naive, with increases in total binding antibody titers, neutralizing antibody titers, and antigen-specific ASC following each dose of the vaccine. In contrast, SARS-CoV-2-experienced subjects responded strongly to the first dose of vaccine with increased antibodies and antigen-specific ASC responses, but did not respond as strongly following a second vaccine dose.

Several possibilities exist for why those who have prior immune responses to SARS-CoV-2 would respond strongly to a first, but not second, vaccine dose. For example, the reduction in antigen-specific ASC may indicate that the peak ASC response is earlier, or perhaps later, than one week after second immunization, and that the humoral response may not peak soon after the second dose as was observed in immunogenicity studies<sup>2</sup>. Alternatively, the high titer of antibodies after one dose in those with prior SARS-CoV-2 infection could be high enough to bind and prevent presentation of S epitopes on MHC molecules by the antigen-presenting cells, thereby limiting further stimulation of the immune response. Another possibility is that the prior infection by SARS-CoV-2-induced antibodies reactive to the lipid nanoparticle carrier of the BNT162b2 product which were then re-induced with the first dose of the vaccine. Indeed, poly(ethylene glycol), which may be used in the formulation of lipid nanoparticles<sup>10</sup>, may be the target of antibody responses<sup>11,12</sup>. Furthermore, COVID-19 may lead to a predisposition to anti-phospholipid antibodies in a substantial proportion of adults<sup>13</sup>. One or more of these mechanisms may account for the discordant responses to the second dose of vaccine, and this information will be helpful to determine optimal strategies for vaccination.

Together, these results highlight the need for further investigation into the role of prior immunological experience on the subsequent response to COVID-19 vaccines, and future studies may be needed to determine whether more optimal dosing regimens are needed for durable, protective immunity to infection by the SARS-CoV-2 virus.

## Methods

## **Recruitment and enrollment**

Thirty-two individuals (19 SARS-CoV-2-naïve and 13 SARS-CoV-2-experienced) provided written consent for enrollment with approval from the NYU Institutional Review Board (IRB 20-00595 and IRB 18-02037)

## Blood samples processing and storage

Venous blood was collected by standard phlebotomy. Blood collection occurred at baseline, day 6-13 (Post 1st Dose), 0-4 days prior to second vaccination (Pre 2nd Dose) and day 6-12 Post 2nd Dose. Peripheral blood mononuclear cells (PBMC) were isolated from heparin vacutainers (BD Biosciences) that were stored overnight at room temperature (RT) prior to processing. Serum was collected in SST tubes (BD Biosciences) and frozen immediately at -80°C.

# ELISA

Direct ELISA was used to quantify antibody titers in participant serum. Ninety-six well plates were coated with 1 µg/ml S1 protein (100 µl/well) or 0.1 µg/ml N protein diluted in PBS and were then incubated overnight at 4°C (Sino Biological Inc.). Plates were washed four times with 250 µl of PBS containing 0.05% Tween 20 (PBS-T) and blocked with 200 µl PBS-T containing 4% non-fat milk and 5% whey, as blocking buffer at RT for 1 hour. Sera were heated at 56°C for 1 hour prior to use. Samples were diluted to a starting concentration of 1:50 (S1), or 1:100 (N) were first added to the plates and then serially diluted 1:3 in blocking solution. The final volume in all wells after dilution was 100 µl. After a 1-hour incubation period at RT, plates were washed four times with PBS-T. Horseradish-peroxidase conjugated goat-anti human IgG, IgM, and IgA (Southern BioTech, 2040-05, 2020-05, 2050-05) were diluted in blocking buffer (1:2000, 1:1000, 1:1000) and 100 µl was added to each well. Plates were incubated for 1 hour at RT and washed four times with PBS-T. After developing for 5 min with TMB Peroxidase Substrate 3,3',5,5'-Tetramethylbenzidine (Thermo Scientific), the reaction was stopped with 1M sulfuric acid. The optical density was determined by measuring the absorbance at 450 nm on a Synergy 4 (BioTek) plate reader.

In order to summarize data collected on individuals, the area under the response curve was calculated for each subject and end point titers were normalized.

## ELISpot

A direct enzyme-linked immunospot (ELISpot) assay was used to determine the number of SARS-CoV-2 spike protein subunit S1-, S2-, and receptor-binding domains (RBD)-specific IgG, IgA, and IgM ASCs in fresh PBMCs. Ninety-six well ELISpot filter plates (Millipore, MSHAN4B50) were coated overnight with 1 mg/mL recombinant S1, S2, or RBD (Sino Biological Inc.), or 10 mg/mL of goat anti-human IgG/A/M capture antibody (Jackson ImmunoResearch Laboratory Inc., 109-005-064). Plates were washed 4 times with 200 µl PBS-T and blocked for 2 hours at 37°C with 200 µl RPMI 1640 containing 10% fetal calf serum (FCS), 100 units/ml of penicillin G, and 100 ug/ml of streptomycin (Gibco), referred to as complete medium. Fifty µl of cells in complete medium at  $10 \times 10^6$  cells/ml were added to the top row of wells containing 150 µl complete medium and 3-fold serial diluted 3 times. Plates were incubated overnight at 37°C with 5% CO<sub>2</sub>. Plates were washed 1 time with 200 µl PBS followed by 4 times with 200 µl PBS-T. Biotinylated anti-human IgG, IgM, or IgA antibody (Jackson ImmunoResearch Laboratory Inc., 709-065-098, 109-065-129, 109-065-011) were diluted 1:1000 in PBS-T with 2% FCS (Ab diluent) and 100 µl was added to wells for 2 hours at RT or overnight at 4°C. Plates were washed 4 times with 200 µl PBS-T and incubated with 100 µl of Avidin-D-HRP conjugate (Vector Laboratories, A-2004) diluted 1:1000 in Ab diluent for 1 hour at RT. Plates were washed 4 times with 200 µl PBS-T and 100ml of AEC substrate (3 amino-9 ethyl-carbazole; Sigma Aldrich, A-5754) was added. Plates were incubated at RT for 5 minutes and rinsed with water to stop the reaction. Developed plates were scanned and analyzed using an ImmunoSpot automated ELISpot counter (Cellular Technology Limited).

# SARS-CoV-2 microneutralization assay

Viral neutralization activity of serum was measured in an immunofluorescence-based microneutralization assay by detecting the neutralization of infectious virus in cultured

Vero E6 cells (African Green Monkey Kidney; ATCC #CRL-1586). These cells are known to be highly susceptible to infection by SARS-CoV-2. Cells were maintained according to standard ATCC protocols. Briefly, Vero E6 cells were grown in Minimal Essential Medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2mM L-glutamine, and 1% of MEM Nonessential Amino Acid (NEAA) Solution (Fisher #MT25025CI). Cell cultures were grown in 75 or 150 cm<sup>2</sup> flasks at 37°C with 5% CO<sub>2</sub> and passaged 2-3 times per week using trypsin-EDTA. Cell cultures used for virus testing were prepared as subconfluent monolayers. All incubations containing cells were performed at 37°C with 5% CO<sub>2</sub>. All SARS-CoV-2 infection assays were performed in the CDC/USDA-approved BSL-3 facility in compliance with NYU Grossman School of Medicine guidelines for biosafety level 3. SARS-CoV-2 isolate USA-WA1/2020, deposited by the Centers for Disease Control and Prevention, was obtained through BEI Resources, NIAID, NIH (NR-52281, GenBank accession no. MT233526). Serial dilutions of heat-inactivated serum (56°C for 1 hour) were incubated with USA-WA1/2020 stock (at fixed 1x10<sup>6</sup> PFU/ml) for 1 hour 37°C. One hundred microliters of the serum-virus mix was then added to the cells and incubated at 37°C with 5% CO<sub>2</sub>. Twenty-four hours post-infection, cells were fixed with 10% formalin solution (4% active formaldehyde) for 1 hour, stained with an α-SARS-CoV-2 nucleocapsid antibody (ProSci #10-605), and a goat  $\alpha$ -mouse IgG AF647 secondary antibody along with DAPI and visualized by microscopy with the CellInsight CX7 High-Content Screening (HCS) Platform (Thermo Fisher) and high-content software (HCS) <sup>14</sup>.

# **Statistical analyses**

Statistical analyses were carried out using the R environment using the "rstatix" library (version 0.6.0). Use of parametric or nonparametric tests was guided by Shapiro-Wilk normality testing. A log(x+1) transformation was performed prior to significance testing for 2-sample t-tests where sample variances were unequal as identified by Levene's test. All tests were two-tailed tests with  $\alpha$ =0.05. Study schematic was made with BioRender.

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## Contributions

M.I.S., A.C., R.S.H., and M.J.M. conceived the study. M.I.S., A.C., J.P.W., T.K., S.L.G-G., and J.R.A. carried out experiments. S.W.H., G.M., and M.A. were involved in clinical recruitment. M.J.M. and R.S.H. supervised the study. M.I.S., A.C., S.L.G-G.,

S.B.K., and R.S.H. were involved in data analysis and interpretation. M.I.S. and R.S.H. wrote the manuscript, and all authors provided critical feedback.

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#### Disclosures

MJM reported potential competing interests: laboratory research and clinical trials contracts with Lilly, Pfizer (exclusive of the current work), and Sanofi for vaccines or MAB vs SARS-CoV-2; contract funding from USG/HHS/BARDA for research specimen characterization and repository; research grant funding from USG/HHS/NIH for SARS-CoV-2 vaccine and MAB clinical trials; personal fees from Meissa Vaccines, Inc. for Scientific Advisory Board service.

# References

- 1. Mulligan, M. J. *et al.* Phase I/II study of COVID-19 RNA vaccine BNT162b1 in adults. *Nature* **586**, 589–593 (2020).
- 2. Vogel, A. B. *et al.* BNT162b vaccines are immunogenic and protect non-human primates against SARS-CoV-2. *Cold Spring Harbor Laboratory* 2020.12.11.421008 (2020) doi:10.1101/2020.12.11.421008.
- 3. Sahin, U. *et al.* COVID-19 vaccine BNT162b1 elicits human antibody and TH1 T cell responses. *Nature* **586**, 594–599 (2020).
- 4. Walsh, E. E. *et al.* Safety and Immunogenicity of Two RNA-Based Covid-19 Vaccine Candidates. *N. Engl. J. Med.* **383**, 2439–2450 (2020).
- 5. Jackson, L. A. *et al.* An mRNA Vaccine against SARS-CoV-2 Preliminary Report. *N. Engl. J. Med.* (2020) doi:10.1056/NEJMoa2022483.
- 6. Anderson, E. J. *et al.* Safety and Immunogenicity of SARS-CoV-2 mRNA-1273 Vaccine in Older Adults. *N. Engl. J. Med.* **383**, 2427–2438 (2020).
- 7. Baden, L. R. *et al.* Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine. *N. Engl. J. Med.* **384**, 403–416 (2021).
- 8. Polack, F. P. *et al.* Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine. *N. Engl. J. Med.* **383**, 2603–2615 (2020).
- 9. Widge, A. T. *et al.* Durability of Responses after SARS-CoV-2 mRNA-1273 Vaccination. *N. Engl. J. Med.* **384**, 80–82 (2021).
- Ilinskaya, A. N. & Dobrovolskaia, M. A. Understanding the immunogenicity and antigenicity of nanomaterials: Past, present and future. *Toxicol. Appl. Pharmacol.* 299, 70–77 (2016).
- 11. Armstrong, J. K. *et al.* Antibody against poly(ethylene glycol) adversely affects PEG-asparaginase therapy in acute lymphoblastic leukemia patients. *Cancer* **110**, 103–111 (2007).
- Hashimoto, Y. *et al.* Generation, characterization and in vivo biological activity of two distinct monoclonal anti-PEG IgMs. *Toxicol. Appl. Pharmacol.* 277, 30–38 (2014).
- 13. Zuo, Y. *et al.* Prothrombotic autoantibodies in serum from patients hospitalized with COVID-19. *Sci. Transl. Med.* **12**, (2020).
- 14. Seifert, L. L. *et al.* The ETS transcription factor ELF1 regulates a broadly antiviral program distinct from the type I interferon response. *PLoS Pathog.* **15**, e1007634 (2019).

# Figures:



Figure 1. Antigen-specific B cells are reduced in circulation one week after the second vaccine dose in SARS-CoV-2-experienced individuals.

Figure 1. Antigen-specific B cells are reduced in circulation one week after the second vaccine dose in SARS-CoV-2-experienced individuals. A. Study schematic. B. S1 antibody titers were assessed for SARS-CoV-2 experienced (purple) and SARS-CoV-2-naive (yellow) subjects. Connected lines indicate repeated measurements of the same subjects. C. Neutralizing antibody titers were assessed using *in vitro* neutralization assay. D-E. ELISpot assays shown for a representative SARS-CoV-2-naive (D) or SARS-CoV-2-experienced (E) subject one week after each dose of vaccine. F-H. Summary statistics shown for ELISpot assays. For each panel, S1 (left), S2 (middle), or RBD (right) antigens are shown for IgG one week after first dose (F) or second dose (G), or for IgA one week after first dose (H) or second dose (I).
J-L. ELISpot data shown for SARS-CoV-2-naive (left) or SARS-CoV-2-experienced (right) subjects for S1 (J), S2 (K), or RBD (L). Connected lines indicate repeated measurements from the same subjects.