Evidence of premature lymphocyte aging in people with low anti-spike antibody levels after BNT162b2 vaccination

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Highlights
Some relatively healthy individuals mounted weak responses to BNT162b2 vaccination

“Low responders” had impaired spike-specific antibody, B and T cell responses

Transcriptomic analysis revealed evidence of premature lymphocyte aging

Premature lymphocyte aging could reduce vaccination efficacy
Evidence of premature lymphocyte aging in people with low anti-spike antibody levels after BNT162b2 vaccination

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SUMMARY
SARS-CoV-2 vaccines have unquestionably blunted the overall impact of the COVID-19 pandemic, but host factors such as age, sex, obesity, and other co-morbidities can affect vaccine efficacy. We identified individuals in a relatively healthy population of healthcare workers (CORALE study cohort) who had unexpectedly low peak anti-spike receptor binding domain (S-RBD) antibody levels after receiving the BNT162b2 vaccine. Compared to matched controls, “low responders” had fewer spike-specific antibody-producing B cells after the second and third/booster doses. Moreover, their spike-specific T cell receptor (TCR) repertoire had less depth and their CD4+ and CD8+T cell responses to spike peptide stimulation were less robust. Single cell transcriptomic evaluation of peripheral blood mononuclear cells revealed activation of aging pathways in low responder B and CD4+T cells that could underlie their attenuated anti-S-RBD antibody production. Premature lymphocyte aging may therefore contribute to a less effective humoral response and could reduce vaccine efficacy.

INTRODUCTION
The devastating impact of the COVID-19 pandemic led to over 5 million deaths by the end of 2021 and has resulted in one of the largest drops in life expectancy in recent history (Andrasfay and Goldman, 2021). The cumulative deaths would have been worse if not for modern technology that provided for the rapid development of safe and effective vaccines toward the SARS-CoV-2 virus. In particular, the mRNA vaccines were demonstrated to have over 90% efficacy in preventing COVID-19 in clinical trials (Baden et al., 2021; Polack et al., 2020). Real-world studies also demonstrated a similar effectiveness of the mRNA vaccines (Dagan et al., 2021). Unfortunately, breakthrough infections still occur among vaccinated populations, especially with the emergence of new viral variants, and can be significant events with associated morbidity and mortality (Cohn et al., 2021).

Various arms of the innate and adaptive immune systems must coordinate to resolve infections in COVID-19 patients (Ferraccioli et al., 2022; Sette and Crotty, 2021). However, prevention of SARS-CoV-2 infections after vaccination is largely mediated by neutralizing antibody (Corbett et al., 2021; Israelow et al., 2021; Pegu et al., 2021; Rydzynski Moderbacher et al., 2020). Solid organ transplant recipients are particularly vulnerable to breakthrough infections as a large proportion of this population fail to seroconvert after vaccination (Boyarsky et al., 2021; Sattler et al., 2021; Werbel et al., 2021). In contrast, patients with autoimmune diseases, who are generally less immunosuppressed, largely develop neutralizing antibodies after 2 doses of mRNA vaccination (Deepak et al., 2021; Melmed et al., 2021). However, immunosuppressed patients are still at elevated risk of breakthrough infection despite a high rate of seroconversion because this group generally has a less robust vaccine response with lower neutralizing antibody titers (Farroni et al., 2022; Levin et al., 2021; Picchianti-Diamanti et al., 2021). Similarly, cancer patients undergoing treatment may have impaired neutralizing antibody production after vaccination and are a population at risk for breakthrough infections with decreased effectiveness of the mRNA vaccines (Figueiredo et al., 2021; Wu et al., 2021). Age is another...
within the B cell compartment in terms of naive and memory cell numbers or B cell activation machinery. Cells from the controls and low responders and found that levels of both were comparable between the naive and memory B cells and CD40 (a vital costimulatory signal necessary for optimal B cell activation) by naive B cells from both groups (Figure 2C). Thus, low responders have no immediately apparent defects and controls (Figures 2B, S2B and S3). Moreover, we evaluated surface expression of MHCII (HLA-DR; preparations of naive (IgD+ CD27–) and memory (CD27+) B cell subsets were also similar between low responders and controls (Tables 1 and S1). None of the participants in the control and low responder groups had any evidence of prior SARS-CoV-2 infection based on a negative anti-N IgG level (Ebinger et al., 2021). Although we did not directly interrogate the plasmablast response, prior work established a strong interdependence between plasmablast levels and anti-spike antibody titers, suggesting that the low anti-S-RBD antibody levels are a consequence of inadequate development of spike-specific plasmablasts after BNT162b2 vaccination (Rincon-Arevalo et al., 2021).

The average age of controls and low responders was 48.2 and 48.6, respectively (Tables 1 and S1). Notably, most individuals in our cohort were relatively healthy and not of advanced age or receiving powerful immunosuppressants or immune modulators, providing an opportunity to understand factors that lead to poor effectiveness of the mRNA vaccine in the general population. Anti-S-RBD IgG levels after a third vaccine dose/booster (timepoint T7; Figure 1D) were also lower in the low responders compared to controls. We also calculated the half-life of the decay of antibody levels in each individual and found no difference between groups (Figure 1E). The decay constant of antibodies after BNT162b2 vaccination is constant after vaccine boosters (Goel et al., 2021a), which allowed us to estimate the time for the anti-S-RBD antibody level to drop below 4160 AU/mL after the booster dose (Figure 1F). We found that the control would reach this threshold 133.0 ± 16.8 days after the booster shot of BNT162b2 whereas low responders would reach this level after 67.8 ± 10.1 days.

Low responders have fewer spike-specific memory B cells

We next performed cytometric profiling of PBMCs and whole blood from low responders and controls, which revealed no substantial differences in the proportions of major immune cell types (B cells, T cells, NK cells, monocytes) that might be indicative of immune deficiencies (Figures 2A, S2 and S3). The proportions of naive (IgD+ CD27–) and memory (CD27+) B cell subsets were also similar between low responders and controls (Figures 2B, S2B and S3). Moreover, we evaluated surface expression of MHCII (HLA-DR; presents antigen to T follicular helper cells to promote germinal center B cell differentiation into plasma cells and memory B cells) and CD40 (a vital co-stimulatory signal necessary for optimal B cell activation) by naive B cells from the controls and low responders and found that levels of both were comparable between the groups (Figures 2C and 2D). Furthermore, LPS stimulation induced HLA-DR upregulation to a similar degree on naive B cells from both groups (Figure 2C). Thus, low responders have no immediately apparent defects within the B cell compartment in terms of naive and memory cell numbers or B cell activation machinery.

factor that increases the risk of breakthrough infection, with those ≥80 years old having lower neutralizing antibodies compared to those <80 years old (Collier et al., 2021; Levin et al., 2021). Thus, seroconversion after COVID-19 vaccination may not provide a complete picture of the protection from SARS-CoV-2 infection, which requires sufficient antibody production for optimal protection. Indeed, the level of neutralizing antibody correlates with the protective effects from SARS-CoV-2 infection, and the rate of SARS-CoV-2 breakthrough infections in vaccinated individuals is inversely related to neutralizing antibody levels (Bergwerk et al., 2021; Corbett et al., 2021; Khoury et al., 2021; Pegu et al., 2021; Tartof et al., 2021).

To investigate potential mechanisms that could explain lower antibody responses after vaccination, we identified BNT2162b2 vaccine recipients within a longitudinal study of COVID-19 vaccinated healthcare workers (CORALE) who had attenuated spike-specific antibody levels after vaccination (henceforth called low responders). Compared to age- and sex-matched controls, low responders typically had fewer spike-specific antibody-producing B cells, but an intact although qualitatively reduced T lymphocyte response toward spike peptide stimulation. The diminished lymphocytic response in low responders persisted after booster doses, indicating inherent defects in the humoral response to mRNA vaccination. Transcriptomic evaluation of PBMCs by scRNAseq identified alterations to signaling pathways in CD4+T cells and B cells of low responders that were consistent with a premature aging phenotype.

RESULTS

Persistently reduced levels of anti-S-RBD antibodies in low responders to BNT162b2 vaccination

We collected blood longitudinally from individuals in the CORALE cohort at regular intervals over 10 months and measured anti-S-RBD IgG levels at 7 different timepoints (Figures 1A–1C and S1). We identified low responders (n = 16) in the CORALE cohort whose anti-S-RBD IgG levels after the second vaccine dose were <4160 AU/mL, the cutoff that represents a 95% probability of a plaque reduction neutralization test (PRNT) ID50 at a 1:250 dilution (Ebinger et al., 2021), and matched them with controls of the same sex and with similar age, BMI, race, ethnicity, and co-morbidities (n = 17; 1 control was replaced part way through the study) (Tables 1 and S1). We did not directly interrogate the plasmablast response, as prior work established that the decay of antibodies after BNT162b2 vaccination is constant after vaccine boosters (Goel et al., 2021a), which allowed us to estimate the time for the anti-S-RBD antibody level to drop below 4160 AU/mL after the booster dose (Figure 1F). We found that the control would reach this threshold 133.0 ± 16.8 days after the booster shot of BNT162b2 whereas low responders would reach this level after 67.8 ± 10.1 days.

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We therefore next evaluated spike-specific IgG-producing B cells by performing B cell ELISpot assays on PBMCs from the T4 timepoint (13–17 weeks after the second dose of the BNT162b2 vaccine). Total IgG-producing B cell numbers were similar in low responders compared to the control group (Figure 2E), but low responders had fewer spike-specific IgG-producing B cells than controls at this timepoint (Figure 2F). Of interest, there were more spike-specific IgG-producing B cells in both groups 1–4 weeks after the booster dose (T7 timepoint), but the low responders continued to have fewer spike-specific IgG-producing B cells compared to controls (Figures 2G and 2H), which mirrored the trend of the anti-S-RBD antibody levels (Figures 1B and 1F). Collectively, these results indicate that low responders have decreased production of spike-specific memory B cells after the BNT162b2 vaccine, which may result in an attenuated recall response after antigenic rechallenge.

Low responders and controls generate a diverse T cell repertoire

The BNT162b2 mRNA vaccine also stimulates the cellular immunity arm to train both CD8+ and CD4+ T lymphocytes in their respective duties to kill infected cells and facilitate the development of humoral immunity (Guerrera et al., 2021; Israelow et al., 2021). Mass cytometry and flow cytometry of T4 and T6 samples, respectively, did not reveal reductions in CD4+ or CD8+ T cell numbers or clear differences in subset...
composition in low responders that could explain the reduced production of anti-S-RBD antibodies, although CD27+ TEM (both CD4+ and CD8+) were lower at T6 and trended lower at T4 (Figures 3A–3C, S2 and S3).

We also performed T cell receptor (TCR) sequencing as an initial step in evaluating the T cell response to vaccination in the control and low responder groups. We did not have access to samples prior to vaccination but our group and others have found that spike-specific T cell clonal expansion occurs specifically after BNT162b2 vaccination (Sahin et al., 2021; Xu et al., 2022), so we performed TCR sequencing using T4 samples (Figures 3 and S4). The Shannon information entropy of the TCR describes the diversity of T cell clones present in each patient. After normalization to read counts, T cell diversity was correlated with age.

Table 1. Demographics and clinical characteristics of control and low responders

|                        | Total (33) | Control (17)* | Low Responder (16) |
|------------------------|------------|---------------|-------------------|
| **Age, n**             |            |               |                   |
| Mean (SD)              | 48.4 (12.4) | 48.2 (12.4)   | 48.6 (12.9)       |
| Median (range)         | 47 (29, 73)| 47 (30, 73)   | 46.5 (29, 73)     |
| **Sex, n (%)**         |            |               |                   |
| Male                   | 18 (54.5)  | 9 (52.9)      | 9 (56.3)          |
| Female                 | 15 (45.5)  | 8 (47.1)      | 7 (43.8)          |
| **BMI**                |            |               |                   |
| Mean (SD)              | 26.0 (5.2) | 25.4 (4.3)    | 26.6 (6.1)        |
| Median                 | 24.8       | 25.8          | 24.4              |
| **Race/Ethnicity, n (%)|            |               |                   |
| White                  | 16 (48.5)  | 8 (47.1)      | 8 (50)            |
| Black or African American| 0 (0)     | 0 (0)        | 0 (0)             |
| Asian                  | 12 (36.4)  | 6 (35.3)      | 6 (37.5)          |
| Native Hawaiian/Pacific Islander | 1 (3.03) | 1 (5.9) | 0 (0) |
| American Indian/Alaska Native | 0 (0) | 0 (0) | 0 (0) |
| Multiple/Other         | 4 (12.1)   | 2 (11.8)      | 2 (12.5)          |
| Hispanic/Latinx        | 4 (12.1)   | 3 (17.6)      | 1 (6.3)           |
| Non-Hispanic/Latinx    | 29 (87.9)  | 14 (82.4)     | 15 (93.8)         |
| **Co-morbidities, n (%)|            |               |                   |
| Asthma                 | 5 (15.2)   | 2 (11.8)      | 3 (18.8)          |
| Cancer                 | 2 (6.1)    | 2 (11.8)      | 0 (0)             |
| Cardiac                | 3 (9.1)    | 2 (11.8)      | 1 (6.3)           |
| COPD                   | 2 (6.1)    | 1 (5.9)       | 1 (6.3)           |
| Diabetes               | 2 (6.1)    | 1 (5.9)       | 1 (6.3)           |
| Hypertension           | 11 (33.3)  | 5 (29.4)      | 6 (37.5)          |
| Autoimmune             | 11 (33.3)  | 6 (35.3)      | 5 (31.3)          |
| **Bloodtype, n (%)**   |            |               |                   |
| O                      | 15 (45.5)  | 9 (52.9)      | 6 (37.5)          |
| A                      | 7 (21.2)   | 3 (17.6)      | 4 (25)            |
| B                      | 5 (15.2)   | 2 (11.8)      | 3 (18.8)          |
| AB                     | 3 (9.1)    | 1 (5.9)       | 2 (12.5)          |
| Unknown                | 3 (9.1)    | 2 (11.8)      | 1 (6.3)           |
| Breakthrough infection*| 6 (18.2)   | 3 (17.6)      | 3 (18.8)          |

*aOne control patient was lost to follow-up and replaced with a newly matched control for subsequent experiments.

*bBreakthrough infection was defined as a newly elevated anti-N and anti-S-RBD above baseline or documentation of a positive test for SARS-CoV-2 as of March 3, 2022.
Because of the age-associated effect on entropy, we performed paired t-tests between the control and low responder samples that were matched by sex and age (Table S1) and found no difference in the Shannon information entropy between low responders and controls (Figure 3E), suggesting that low responders have a diverse T cell repertoire that is similar to controls. To assess T cell specificity against the spike protein, we compared TCR repertoires to the MIRA dataset, a published set of 154,000 putative SARS-CoV-2-responsive TCR sequences (Alter et al., 2021). We evaluated the breadth, which assesses the number of unique clones, and depth, which reflects expansion of specific clones (Figure 3F). When comparing sequenced TCRs to MIRA to find SARS-CoV-2-specific sequences, we observed no difference in breadth or depth between controls and low responders (Figure S4). However, when we allowed single
amino acid substitutions, insertions, or deletions to also include additional sequences that have not previously been demonstrated to be spike-specific but have a high probability to bind SARS-CoV-2 epitopes, we found no change in breadth with age and no difference in breadth between the two groups (Figures 3G and 3H), but that depth declined with age (Figure 3I) and was attenuated in low responders compared to controls (Figure 3J).

TCR sequencing demonstrated that low responders develop a diverse T cell repertoire recognizing the spike peptide. However, although the TCR diversity serves as a global metric of immune robustness, we wanted to understand the vaccine-specific recall response of T lymphocytes. We therefore measured the induction of IFN-γ, TNF-α, and IL-2 by CD4+ and CD8+ T cells after stimulation of PBMCs from the T4 timepoint with spike peptide (Table S2 and Figure S5). We quantified the monofunctional (IFN-γ+, TNF-α+, IL-2+) and polyfunctional (IFN-γ+ and TNF-α+, IFN-γ+ and IL-2+, TNF-α+ and IL-2+) activation of CD4+ T cells after spike peptide stimulation and observed a trend toward lower responses in the low responders compared to the controls after the second and third shot of the BNT162b2 vaccine. Collectively, these data indicate that the low responders have defects in spike-specific T cells after BNT162b2 vaccination.

Figure 3. Evaluation of T cell subsets and TCR diversity in low responders and controls

(A–C) Flow cytometry evaluation of total CD3+ T cells and CD4+ and CD8+ T cells (A), CD4+ T cell subsets (B), and CD8+ T cell subsets (C) among PBMCs collected at the T6 timepoint. Naive T cells were CD45RA+CCR7+CD27+, central memory T cells (TCM) were CD45RA–CCR7+CD27+, and effector memory T cells (TEM) were CD45RA–CCR7–CD27++ (see gating strategy in Figure S2B). Statistical significance was evaluated by Student’s t test. Data are represented as mean ±SEM.

(D–J) TCR sequencing was performed using PBMCs collected at the T4 timepoint.

(D) Shannon entropy of normalized read counts of TCR sequencing data (% maximum) plotted against the age of each individual demonstrates T cell diversity (all clones) correlated with age. Statistical significance was calculated by combining controls and low responders; trend lines are shown for each group ($R^2 = 0.39$ and 0.52 for controls and low responders, respectively).

(E) Pairwise comparison of the normalized Shannon entropy between matched individuals.

(F) Diagram illustrating the concept of depth and breadth of TCR clones. Breadth represents the number of distinct clones as a proportion of the total clonal repertoire without considering clone size, whereas depth reflects the frequency of specific T cells.

(G–J) Evaluation of TCR sequences similar to or exactly matching SARS-CoV-2-specific TCR sequences defined in the MIRA database.

(G and H) Evaluation of the breadth of TCR clones to assess the relationship between breadth and age (G, $R^2 = 0.036$ and 0.0017 for controls and low responders, respectively), and breadth and vaccination response (H, with matched pairs indicated).

(I and J) Evaluation of the depth of TCR clones to assess the relationship between depth and age (I, $R^2 = 0.23$ and 0.17 for controls and low responders, respectively), and depth and vaccination response (J, with matched pairs indicated). A generalized linear model was used to determine correlation to age. Statistical significance between low responder and control was evaluated by paired Wilcoxon Signed-Rank Test.
Lymphocytes from low responders have aging-like transcriptomic signatures

To further explore the qualitative differences in the lymphocyte compartment between groups, we performed scRNAseq on PBMCs from 4 matched control and low responder pairs, none of whom were receiving any immunosuppressants or immune modulators, at the T4 timepoint (Figures 4A and S6). We determined the differentially expressed genes between controls and low responders in unstimulated CD4+ T cells based on an adjusted p value cutoff of 0.05 (Figure 4B and Table S3) and used Ingenuity Pathway Analysis (IPA) to identify the canonical pathways that are affected by the pattern of differentially expressed genes (Figure 4C). The transcriptomic analysis of CD4+ T cells in the low responders compared to controls indicated suppression of EIF2 and sirtuinsignaling, both of which are pathways that diminish with aging (Kurupati et al., 2019; Lee et al., 2021; Ottens et al., 2021; Sabath et al., 2020). Conversely, the coronavirus pathogenesis pathway, leukocyte extravasation signaling, and oxidative phosphorylation were higher in low responders compared to controls. Next, we used the Upstream Regulator Analysis
module in IPA to predict the factors that would lead to these patterns of transcriptomic changes (Figure 4D). Similar to the canonical pathway analysis, decreased sirtuin signaling (SIRT1, SIRT3) was also identified as potentially contributing to the transcriptomic differences in CD4+T cells of low responders compared to controls. Moreover, low responders also had downregulation of c-Myc signaling components (MYC, MYCN), which are important mediators in T cell development and regulation of cellular senescence (Douglass et al., 2001; Guney et al., 2006). Of interest, genes induced by several cytokines were elevated in the low responders, which may indicate that chronic low level basal cytokine signaling in low responder CD4+T cells underlies their defective cytokine responsiveness to spike stimulation.

Finally, we interrogated the transcriptomic differences between controls and low responders in the B cell compartment (Figure 5A). Like the CD4+T cell analysis, canonical pathway analysis of the B cell compartment revealed attenuated EIF2 and sirtuin signaling. Moreover, upstream regulator analysis once again predicted that downregulation of MYC family members (MYC, MYCN) and SIRT3 could account for the transcriptomic changes in low responders compared to controls (Figure 5B). Altogether, the evaluation of baseline transcriptome differences suggests that B and CD4+T cells from low responders exhibit suppression of signaling pathways consistent with a premature aging phenotype.

DISCUSSION

Here, we identify factors in a relatively healthy population of healthcare workers that may cause a diminished vaccine response. We found that low responders have numerically normal T and B lymphocyte compartments and similar activation of naive B cells after LPS stimulation. However, functional deficiencies were apparent in low responders that could reduce the robustness of the humoral response to vaccination. Indeed, when compared to age- and sex-matched controls, the low responders had deficient T and B lymphocyte recall responses to spike peptide stimulation. Both groups responded to a booster dose of the mRNA vaccine, but the low responders had a persistent attenuation of the CD4+T cell and B cell responses to spike peptide stimulation and lower levels of anti-S-RBD antibodies. Finally, transcriptional changes in the peripheral blood lymphocytes of low responders suggested suppression of signaling pathways in CD4+T cells and B cells consistent with a premature aging phenotype.

The effectiveness of COVID-19 vaccines in preventing infection is directly proportional to neutralizing antibody levels (Corbett et al., 2021; Khoury et al., 2021; Pegu et al., 2021). We found that low responders have quantitatively lower amounts of antibodies even after a vaccine booster vaccine. The antibodies appear to
be qualitatively similar between groups with no difference in the decay constant. However, our data suggested that antibody levels would drop below 4160 AU/mL (95% probability of a plaque reduction neutralization test (PRNT) ID50 at a 1:250 dilution) approximately 2 months earlier in low responders than controls. Although our sample size was too small to detect any differences in the rates of breakthrough infections, the reduced level of antibodies in the low responder group suggests an increased susceptibility to breakthrough infections (Corbett et al., 2021; Pegu et al., 2021).

The recent emergence of Omicron as a SARS-CoV-2 variant of concern has generated attention for its increased infectability and resistance to vaccines (Dejnirattisai et al., 2021; Garcia-Beltran et al., 2021). Unlike cellular immunity, which has stability across different variants of concern, the humoral immune arm has a blunted response toward Omicron (Petrone et al., 2022a, 2022b; Tarke et al., 2022). Booster doses of BNT162b2 have demonstrated increased breadth of the humoral response due to re-activation of memory B cells, which undergo further affinity maturation to increase diversity (Gruell et al., 2022; Muecksch et al., 2021), but the emerging data have also demonstrated that a higher post-immunization titer is required for viral neutralization (Nemet et al., 2021). As such, generating a higher neutralizing antibody titer after vaccination may become paramount in effectively controlling the continued spread of the SARS-CoV-2 virus and emergence of new variants. Moreover, our data show that low responders also have a reduced population of spike-specific memory B cells compared to controls. This difference was evident after the second dose of BNT162b2 and persisted after the third dose suggesting an inherent defect in the response to the mRNA vaccine. Notably, the reduced number of memory B cells in low responders reflects a smaller available pool of cells to generate an appropriate and increasingly diverse humoral response against emerging variants of concern (Sokal et al., 2021).

A large gap remains in our understanding of the mechanisms that drive the heterogeneity of the vaccine response that leads to breakthrough infections. Aging is one of the largest risk factors that is common to both severe illness and vaccine failure. Decreased vaccine efficacy and an accelerated waning of immunity are observed in the elderly (Ciabattini et al., 2018; Frasca et al., 2020). Furthermore, neutralizing antibody levels after BNT162b2 vaccination are lower and breakthrough infections are higher among older compared to younger people (Collier et al., 2021; Levin et al., 2021). Of interest, we found alterations in signaling pathways consistent with aging-related defects in both B and CD4+ T cells from low responders, a cohort largely comprised of young/middle-aged people. We identified decreased sirtuin signaling in B and CD4+ T cells from low responders when compared to age-matched controls. Aged immune cells exhibit downregulation of sirtuin proteins, which are important senolytic regulators (Lee et al., 2021). Moreover, low responders to the influenza vaccine have lower SIRT1 expression by antibody-secreting cells (ASCs) compared to high responders (Kurupati et al., 2019). Sirtuin signaling also supports proteostasis, which becomes disrupted with increased age and decreases in senescent cells (Ottens et al., 2021; Sabath et al., 2020).

In addition, the EIF2 pathway is an important arm of the unfolded protein response (Costa-Mattioli and Walter, 2020), and our transcriptomic analyses revealed suppressed EIF2 signaling in B and CD4+ T cells from low responders compared to controls, suggesting alterations in proteostasis. We also found that low responders have reduced c-Myc signaling, which can trigger cellular senescence (Guney et al., 2006). Aging also causes dysfunctional hematopoietic cell differentiation (Frasca et al., 2020), and the Myc family members have an important role in B and T cell differentiation (Douglas et al., 2001; Habib et al., 2007) signifying additional deficiencies in the development of the immune response after vaccination.

Low responders had fewer spike-specific antibody-producing B cells compared to controls, even after booster, indicating an attenuated anamnestic response (Goel et al., 2021b). Generation of antigen-specific memory B cells after mRNA vaccination is inversely correlated with age (Goel et al., 2021a), which is congruous with the premature lymphocyte aging phenotype in low responders. Although B cells are the central effectors of humoral immunity, the process of generating an adequate immunogenic response after vaccination is not possible without CD4+ T cells (Crotty, 2015). The spike-specific recall response in CD4+ T cells was also blunted in low responders compared to controls. Strikingly, monofunctional and polyfunctional IL-2, TNF-α, and IFN-γ CD4+ T cell responses to spike peptide stimulation were reduced in low responders, and this persisted even after the booster dose. Thus, the CD4+ T cells have intrinsic deficits that may impair their ability to provide sufficient “help” in the generation of neutralizing antibodies after vaccination.
We primarily focused on the humoral response in these studies, although our analysis also demonstrated a blunted spike-specific CD8\(^+\) T cell recall response in low responders. Altogether, these results are consistent in that the BNT162b2 vaccine induces B cells and CD4\(^+\) and CD8\(^+\) T cells to recognize the spike protein of the SARS-CoV-2 virus (Goel et al., 2021a; Israelow et al., 2021; Sahin et al., 2020). However, low responders have broad impairments in both humoral and cell-mediated immunity that could in combination result in less protection from infection and more severe disease if infected (Israelow et al., 2021; Rydyznki Moderbacher et al., 2020; Sette and Crotty, 2021).

Our findings demonstrate that some people have inherent defects consistent with an aging-related phenomenon that could contribute to an ineffective vaccine response. The elderly population develops a chronic, low-grade inflammatory state called inflammaging that contributes to the development of several age-related diseases (Akbar et al., 2016). Of interest, CD4\(^+\) T cells in low responders have augmented inflammatory pathways (e.g., TNF, IFNG, etc.) compared to controls in the scRNA-seq analysis, and this pattern is consistent with a senescent-associated secretory phenotype that is the hallmark of inflammaging. People with multiple co-morbidities have decreased effectiveness of BNT162b2 vaccination (Brosh-Nissimov et al., 2021; Dagan et al., 2021; Sattler et al., 2021); thus, the general adult population, which includes the elderly and those who are unable to work due to illness and associated co-morbid conditions, likely has a higher rate of people with a reduced humoral response to vaccination than we observed in this study of a relatively healthy population of healthcare workers. Indeed, chronic renal disease is a risk factor for decreased neutralizing antibody levels after BNT162b2 vaccination and for breakthrough infection (Agrawal et al., 2021; Grupper et al., 2021; Sattler et al., 2021). Similarly, obesity and diabetes have also been associated with increased breakthrough SARS-CoV-2 infection after vaccination (Agrawal et al., 2021; Antonelli et al., 2021; Juthani et al., 2021). Notably, obesity, diabetes, and chronic kidney disease have disruptions in metabolic pathways (e.g., sirtuin signaling) that cause immunological dysfunction through premature aging and maladapted proteostasis (Ottens et al., 2021; Stefan et al., 2021). However, some of the low responders in our study had no apparent co-morbidities suggesting a senescence-like phenotype may also be induced in otherwise healthy individuals that reduce their overall responsiveness to the BNT162b2 vaccine. Future directions could include testing adjunct therapies that correct metabolic imbalances, such as methods to augment the sirtuin signaling, to improve the vaccine response in at-risk populations.

In conclusion, our longitudinal study identified low responders who have a diminished ability to generate anti-S-RBD antibodies to the BNT162b2 vaccine, even after three doses of the BNT162b2 vaccine. Low responders have basal changes in their B and CD4\(^+\) T cells that suggest a premature senescence phenotype, which could contribute to the reduced humoral response after vaccination. Because anti-S-RBD antibody levels correlate with viral neutralization and the protective effects from vaccination, emergence of new variants of concern such as Omicron may require a refined strategy to ensure adequate protection in those with an attenuated response after vaccination against SARS-CoV-2.

Limitations of the study

One of the main limitations of our study is the sample size. We identified 16 low responders within the 2022 individuals in the CORALE study, and although this is a relatively small number, it is not easily repeated considering the small proportion (0.79%) of low responders in a generally healthy population. Nevertheless, considering the hundreds of millions of people in the US alone getting COVID-19 vaccinations, the number of people in the population who are likely affected is quite substantial.

STAR METHODS

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AUTHOR CONTRIBUTIONS
Patient recruitment, clinical information, and sample collection were performed by S.J., M.W., M.D., and S.C. All patient samples were processed and stored by Y.H. and J.E.S. Y.H., J.E.S., C.Y., A.X., H.S.G., and P.C. were involved in data analysis and interpretation. Y.H., J.E.S., A.X., C.Y., J.F., R.Z., M.E.M., J.G.B., C.A.J., K.S., A.M., S.A.G., S.C.J., S.C., H.S.G., and P.C. helped with methodological development. The manuscript was written by H.S.G. and P.C. with input by all authors. S.C., H.S.G., and P.C. were responsible for overall supervision and funding. Y.H. and J.E.S. were collectively considered before A.M.X. and C.Y. because they performed all of the biobanking. The order of YH and JES was determined by seniority.

DECLARATION OF INTERESTS
Authors declare that they have no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Flow cytometry antibodies | See Table S4.1 | See Table S4.1 |
| CyTOF antibodies    | See Table S4.2 | See Table S4.2 |
| Cell hashing antibodies | See Table S4.3 | See Table S4.3 |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Human TruStain FcX™ (Fc Receptor Blocking Solution) | BioLegend | Cat #: 422302 |
| Zombie Red™ Fixable Viability Kit | BioLegend | Cat #: 423109 |
| Cell-ID™ Cisplatin | Fluidigm | Cat #: 201064 |
| EQ Four Element Calibration Beads | Fluidigm | Cat #: 201078 |
| Spike peptide: PepMix SARS-CoV-2 | JPT | Cat #: PM-WCPV-S-1 |
| **Critical commercial assays** |        |            |
| IgG (S-RBD) - SARS-CoV-2 IgG II assay | Abbott | N/A |
| IgG (N) - SARS-CoV-2 IgG assay | Abbott | N/A |
| Human IgG ELISpot BASIC kit (HRP) | Mabtech | Cat #: 3850-2H |
| Human IgG (SARS-CoV-2, RBD) ALP | Mabtech | Cat #: 3850-4APW-R1-1 |
| QIAseq Immune Repertoire RNA Library Kit | Qiagen | Cat #: 333705 |
| Chromium Next GEM Single Cell 5’ Kit v2 | 10x Genomics | Cat #: 1000265 |
| Chromium Next GEM Chip K Single Cell Kit | 10x Genomics | Cat #: 1000286 |
| Dual Index Kit TT Set A | 10x Genomics | Cat #: 1000215 |
| Library Construction Kit | 10x Genomics | Cat #: 1000190 |
| Collibri™ Library Quantification Kit | ThermoFisher | Cat #: A38524500 |
| **Deposited data** |        |            |
| Antibody titers from vaccinated healthcare workers | Ebinger et al., 2021 [https://doi.org/10.1038/s41591-021-01325-6](https://doi.org/10.1038/s41591-021-01325-6) | N/A |
| SARS-CoV-2 spike-specific TCR sequences (MIRA) | Adaptive Biotech | N/A |
| TCR sequencing data | SRA: PRJNA869780 | N/A |
| scRNAseq data | GEO: GSE199123 | N/A |
| **Software and algorithms** |        |            |
| FlowJo | BD Biosciences | N/A |
| CyCombine | Pedersen et al., 2022 [https://doi.org/10.1038/s41467-022-29383-5](https://doi.org/10.1038/s41467-022-29383-5) | N/A |
| GigaSOM | Kratochvil et al., 2020 [https://doi.org/10.1093/gigascience/giaa127](https://doi.org/10.1093/gigascience/giaa127) | N/A |
| PhenoGraph | Levine et al., 2015 [https://doi.org/10.1016/j.cell.2015.05.047](https://doi.org/10.1016/j.cell.2015.05.047) | N/A |
| Seurat v4.0 | Stuart et al., 2019 [https://doi.org/10.1016/j.cell.2019.05.031](https://doi.org/10.1016/j.cell.2019.05.031) | N/A |
| Ingenuity Pathway Analysis | Qiagen | N/A |
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Peter Chen (peter.chen@cshs.org).

Materials availability
This study did not generate new unique reagents.

Data and code availability
- TCR sequencing data and single-cell RNA-seq data have been deposited at SRA and GEO, respectively, and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.
- Additional Supplemental Items are available from Mendeley Data: https://doi.org/10.17632/c2vfpvbrbz.1
- This article does not report original code.
- Any additional information required to reanalyze the data reported in this article is available from the lead contact on request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human subjects: Low responder and control selection criteria
From a source cohort of N = 2022 healthcare workers in a longitudinal serology study (Ebinger et al., 2021), we identified 16 individuals without evidence of SARS-CoV-2 infection prior to vaccination who had IgG (S-RBD) antibody values < 4,160 AU/mL (conversion factor 7 AU/mL = 1 BAU/mL) after 2 doses of BNT162b2 (identified at 8 weeks after the second dose of BNT162b2) and for whom we could identify a matched control (i.e., T3 timepoint). The 4,160 AU/mL threshold for IgG (S-RBD) has been shown to correspond to a 0.95 probability of obtaining a PRNT ID50 at a 1:250 dilution as a representative high titer (SARS-CoV-2 IgG II Quant Assay User Manual (Abbott Laboratories, Diagnostics Division, 2000). Individuals were considered to have a prior history of SARS-CoV-2 infection if they had an elevated IgG (N), self-reported having prior COVID-19, and/or have a positive PCR test for SARS-CoV-2 PCR in the medical chart. The 16 “low responder” individuals were matched to 16 control individuals with medium to high spike-specific antibody values (also negative for prior COVID-19) by age, sex, and co-morbidities (determined by chart review). The control for Pair 2 was lost to follow-up early in the study and was replaced by another matched control for all studies from the T5 collection timepoint onward. Table S1 provides detailed information about each individual. Our PBMC collections began at the T4 timepoint. The PBMCs that we obtained were limited in quantity, which required us to be judicious when selecting the timepoints at which experiments were conducted. The majority of experiments were performed at the T4 timepoint (B cell ELISpot, scRNA-seq, TCR sequencing, T cell activation with spike peptide stimulation, mass cytometry). We also did the B cell ELISpot and spike peptide stimulation experiments at the T7 timepoint to evaluate spike-specific lymphocytes after the third BNT162b2 dose. We had insufficient samples from the T4 timepoint for the flow cytometry. Regardless, BNT162b2 vaccination is not expected to affect steady-state immune cell numbers so using T6 samples to characterize basal immune cell populations is appropriate as it would not be expected to differ between timepoints.

METHOD DETAILS

Sample collection and storage
The CORALE study was approved by the Cedars-Sinai Institutional Review Board, and all enrolled participants provided written informed consent for blood and clinical data collection.

Blood was drawn serially to monitor antibody levels and provide samples to study the immune response after vaccination. PBMCs were isolated from whole blood using Ficoll-Paque™ PREMIUM (Fisher Scientific, Waltham, MA). Ficoll was added to the bottom of a Sepmate 50 tube (STEMCELL; Cambridge, MA) and then blood was added, the tubes were centrifuged at 1000 g for 15 min, and the PBMC layers were collected. Cells were then washed 3 times with PBS, counted and stored at 1 × 10^6 cells per vial in liquid nitrogen.
Antibody measurement and ABO typing

Serological assays for antibodies to the receptor binding domain (RBD) of the S1 subunit of the viral spike protein [IgG (S-RBD)] and the nucleoprotein [IgG (N)] were performed at all blood collection timepoints using the Abbott SARS-CoV-2 IgG II assay and SARS-CoV-2 IgG assay, respectively (Abbott Labs, Abbott Park, IL). An IgG (N) S/C of $\geq 1.4$ was denoted as definitive seropositive status because of prior SARS-CoV-2 exposure based on a previously established threshold (Chew et al., 2020). IgG (S-RBD) less than 50 AU/mL (7.1 BAU/mL) is considered negative. The limit of quantitation for IgG (S-RBD) is 22 AU/mL (3.1 BAU/mL). Antibody data for both controls in pair 2 was included when available. The initial antibody response to the BNT162b2 vaccine in the CORALE cohort was previously described, and the anti-S-RBD antibody levels at timepoints T1 through T3 presented within the current study for several of the individuals overlap with those previously published (Ebinger et al., 2021). Half-life of the antibody levels was calculated for each individual using anti-S-RBD levels at T3 and T5 (https://www.calculator.net/half-life-calculator.html). ABO typing was performed using the Ortho Vision Platform from Ortho Clinical Diagnostics.

SARS-CoV-2 spike-specific and total IgG ELISpot

SARS-CoV-2 and total IgG ELISpot assays were performed using Mabtech IgG ELISpot kits (Mabtech; Cincinnati, OH). Cryopreserved PBMCs (T4 and T7 collection timepoints) were thawed, counted, and resuspended in complete medium RPMI 1640 (Corning; Corning, NY) supplemented with 10% fetal bovine serum (Fisher Scientific; Waltham, MA). After resting the cells for 30 min at 37°C, 3 x 10⁶ cells were stimulated with 1 μg/mL R848 and 10 ng/mL rhIL-2 to induce IgG secretion by memory B cells and then incubated at 37°C, 5% CO2 for 3 days. PVDF plates were coated with mAbs91/145 one day before performing ELISpot. 1 x 10⁵ and 1 x 10⁴ stimulated cells were seeded in coated plates for SARS-CoV-2 spike-specific IgG and total IgG detection, respectively, and the cells were incubated at 37°C, 5% CO2 for 24 h. RBD-WASP and mAbs MT78/145-biotin were used to detect spike-specific IgG and total IgG, respectively. Detection solution containing anti-WASP-ALP and streptavidin-HRP was then added to the cells and incubated at room temperature for 1 h. BCIP/NBT (Mabtech, Cincinnati, OH) and AEC substrates (BD Biosciences; San Diego, CA) were then used to reveal the presence of spots. Spots formed by IgG-secreting cells were imaged by dissection microscopy and counted using ImageJ.

Mass cytometry (CyTOF)

Whole blood was incubated with 1 KU/mL heparin solution for 20 min at room temperature prior to antibody cocktail and cisplatin viability staining for 30 min at room temperature. Red cells were then lysed by incubation with Cal-Lyse for 10 min at room temperature in the dark, followed by washing with dH2O and PBS. Leukocytes were then fixed with paraformaldehyde and washed with PBS prior to freezing at ~80°C. Thawed cells were subsequently permeabilized using Cytoperm permeabilization buffer and stained for intracellular markers by incubation in Cytoperm buffer for 1 h at room temperature. EQ Four Element Calibration Beads were then added to the samples prior to analysis using the Helios system (Fluidigm). Following acquisition, files were pre-processed to normalize samples to EQ Four Element Calibration Beads using Premessa (https://github.com/ParkerICI/premessa) to adjust for variations in machine performance. Live, intact cells were then gated using FlowJo. CyCombine was used for batch normalization to correct for variance between batches stained separately for intracellular markers (Pedersen et al., 2022). Normalization parameters were subsample size for batch effect detection: $5 \times 10^4$ cells, normalization method: scale, and ties method: average. Primary clustering was performed using GigaSOM (Kratzchvil et al., 2020). The primary clustering for this dataset was carried out with a 64-by-64 node map, trained on the staining panel’s “lineage” subset of markers (CD11c, CD19, CD3, CD4, CD8α, CD56, TCRVa24Ja18, CD14, gdTCR) and on the total cell yield of all viable samples (27 samples total). The resulting map assigns a total of 1.4 x 10⁶ cells to 4096 nodes (primary clusters), which were meta-clustered to obtain cell phenotypes used for analysis. PhenoGraph (Levine et al., 2015) was then used to meta-cluster the nodes into immune cell subsets. A first meta-clustering of all nodes was done with the nearest-neighbor parameter set to 50 on the following markers: CD11c, CD19, CD3, CD8α, CD56, TCRVa24Ja18, CD14, gdTCR. Subsequent clustering was performed on the cells falling into the CD19⁺, CD3⁺ CD4⁺, and CD3⁺ CD8α⁺ clusters, respectively. This was run with the nearest-neighbor parameter set to 30, on the following marker sets: B cells (CD19⁺) – CD27, CCR7; CD4⁺ T cells (CD3⁺ CD4⁺) – CD27, CCR7, CD45R0, CD25, FoxP3; CD8⁺ T cells (CD3⁺ CD8α⁺) – CCR7, CD27, CD45R0. The Leiden community detection algorithm option was used for all of these runs (Traag et al., 2019).
B and T Cell flow cytometry

Frozen PBMC vials from the T6 collection timepoint were thawed in a warm water bath and cells were re-suspended in warm RPMI supplemented with 10% FBS and rested at 37°C for 30 min. Cells were then divided into two groups that were either immediately processed for flow cytometry or were stimulated with 100 ng/mL LPS as a non-specific B cell activator (Xu et al., 2008) for 48 h prior to flow cytometry. Flow cytometry was performed to evaluate T and B cell subsets and surface expression of MHCII and CD40 by B cells. PBMCs were incubated in PBS containing 0.2% viability dye for 20 min at 4°C and then stained with antibodies for 30 min at 4°C. Cells were then fixed and stored at 4°C protected from light prior to acquisition using a BD LSRFortessa™ cell analyzer. Data analysis was performed using FlowJo™ v10.8.1.

TCR sequencing

RNA was isolated from PBMCs (T4 collection) using a Qiagen Total RNeasy Mini kit. Immunosequencing of the CDR3 regions of human TCRβ chains was performed using the QiAseq Immune repertoire RNA library kit (Qiagen) followed by sequencing on the NovaSeq 6000 system (Illumina). To assess patient T cell specificity, we compared patient TCR repertoires to the MIRA dataset, a published set of 154,000 putative SARS-CoV-2-responsive TCR sequences (Alter et al., 2021). After sorting for spike protein-responsive TCRs, we matched spike-specific TCRs to patient repertoires to determine vaccine responsiveness. An antigen-specific response is usually highly specific to each target, making exact matches between patient TCRs and TCR repositories like MIRA rare, but a slightly mismatched TCR is likely to bind the same antigen target. We therefore also searched for similar TCR sequences that were slightly mismatched to MIRA.

T Cell activation assays

Functional activation of T cells was measured using an assay developed for whole blood stimulation with spike peptides (Jordan et al., 2021). Whole blood samples collected from participants at the T4 and T7 collection timepoints were stimulated with a pool of 315 overlapping peptides (15-mer) that spanned the SARS-CoV-2 spike protein (PepMix SARS-CoV-2; JPT) plus Brefeldin A and anti-CD28/CD49d (BD Bioscience, San Jose, CA). After 9 h, cells were immunostained with CD3, CD4, and CD8-specific antibodies followed by fixation and permeabilization for intracellular staining for IL-2, IFN-γ, and TNF-α, and analysis by flow cytometry.

Single Cell RNA sequencing

Frozen PBMCs (T4 collection timepoint) were thawed and recovered in RPMI with 10% FBS at 37°C for 2 hours. PBMCs from each patient were further labeled with Total-seq C hashing antibodies (BioLegend) and equal numbers were mixed. The cells were then stained with DAPI (3μM) for 5 min to assess viability. Live cells were sorted using a BD Influx (BD Biosciences, San Jose, CA, USA) in the Cedars-Sinai Flow Cytometry Core. Single cells were captured using a 10X Genomics Chromium Controller and libraries were prepared according to the Single Cell 5’ Next GEM V3.1 Reagent Kits User Guide. The barcoded sequencing libraries were quantified by quantitative PCR using the KAPA Library Quantification Kit (KAPA Biosystems, Wilmington, MA). Libraries were sequenced using a Novaseq 6000 (Illumina) with custom sequencing settings of 28bp and 91bp for read 1 and 2, respectively, to obtain a sequencing depth of ~2.5 × 10^4 reads per cell.

CellRanger v6.0.0 software was used with the default settings for demultiplexing, aligning reads with STAR software to a human GRCh38 transcriptome reference provided by 10X Genomics https://support.10xgenomics.com/single-cell-gene-expression/software/downloads/latest. Single cell analysis R package Seurat v4.0 was used to demultiplex hashtag oligos (HTO) and for further data analysis (Stuart et al., 2019). For quality control and filtering out low quality cells, only cells expressing more than 200 genes (defined as genes detected in at least 3 cells) and fewer than 10% mitochondrial genes were selected. We also analyzed the expression of mitochondrial and ribosomal genes across all samples to ensure any differences in these genes were biologically relevant and not due to technical variation (Figure S7). To minimize doublet contamination for each dataset, we kept only singlets identified during the hashtag oligo demultiplex step. We used default normalization and data scaling from the Seurat package, which is a log normalization and linear model for data scaling. Batch correction package Harmony with was used for data integration (Korsunsky et al., 2019). The batch correction was processed with Principal Component Analysis (PCA) using the 3000 most variable genes,
and the first 20 independent components were used for downstream unbiased clustering with a resolution of 0.5. The Uniform Manifold Approximation and Projection (UMAP) method was used for visualization of unsupervised clustering. Cell cluster identities were determined using known gene markers of major immune cell types (Yao et al., 2021). Differentially expressed genes between different clusters and groups were calculated using Mode-based Analysis of Single-cell transcriptomics (MAST) (Finak et al., 2015).

Differentially expressed genes between control and low responders within respective cellular compartments were analyzed with Ingenuity Pathway Analysis (Qiagen). For the canonical and upstream pathway analysis results, unbiased pathways with a Z score ≥ 2.0 were considered significant (Table S2).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Statistics**

All statistics were determined with Prism version 9.3.0 (GraphPad). Unless otherwise indicated, Student’s t-test was used to test statistical significance between two groups. Two-way ANOVA was used for time course evaluation of antibody levels. Statistical significance between low responder and control TCR clones was evaluated by paired Wilcoxon Signed-Rank Test. A generalized linear model was used to determine correlation to age. All graphs present means +/- SEM unless otherwise indicated. All significances are denoted in the respective figure panels; the absence of a p value indicates non-significance. Additional details of statistical analyses are included in the figure legends.