Durable T cell responses contrast with faster antibody waning in BNT162b2-vaccinated elderly at 6 month

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Abstract

Efficient COVID-19 vaccines have been developed in record time. Here, we present findings from a comprehensive and integrated analysis of multiple compartments of the memory immune response in 312 individuals vaccinated with the BNT162b2 mRNA vaccine. Two vaccine doses induced high antibody and T cell responses in most individuals. However, antibody recognition of the Spike protein of delta variant was less efficient than that of the Wuhan strain. Age stratified analyses identified a group of low antibody responders where individuals ≥ 60 years were overrepresented. Waning of the antibody and cellular responses was observed in 30% of the vaccinees after six months. However, age did not influence the waning of these responses. Taken together, while individuals ≥ 60 years old took longer to acquire vaccine-induced immunity, they develop more sustained acquired immunity at six months post-vaccination. However, the higher proportion of older individuals in the group of antibody low responders and the lower antibody reactivity the Delta variant call for a booster immunization to increase immune responses and protection.

Introduction

By the beginning of September 2021, over 250 million SARS-CoV-2 confirmed cases and five million associated deaths were reported worldwide (https://covid19.who.int/). Several vaccines developed in record time have shown high efficacy against symptomatic infection and severe COVID-19. The Pfizer/Biontech BNT162b2 mRNA vaccine and one of the most deployed worldwide, is a two-dose regimen, administered 21 days apart. Initial phase 3 data showed an efficacy of ~50% after the first dose and >90% after the second dose against severe disease caused by the original SARS-CoV-2 strain in naïve individuals [1]. This was further supported by real-world data [2–6]. Recent reports have shown that it still provides significant protection against the emerging SARS-CoV-2 variants [7–9]. The BNT162b2 vaccine induces anti-Spike antibody, memory B cells and T cell responses in humans [8–15], which are both required for protection against infection and disease [16–22]; the former being considered as the main correlate of protection [23–25]. Different factors such as age, gender, microbiome, comorbidities or environment influence the development of effective immune responses [26]. Since the elderly are at major risk of COVID-19 severe disease [27], it is necessary to assess their immune responsiveness to COVID-19 vaccination. Initial studies reported that antibody responses were similar to the younger groups [28], while others described lower responses in older groups [29–34]. Despite lower immune responsiveness, the two-dose BNT162b2 vaccine has demonstrated similar vaccine efficacy in the elderly [10]. In the early days of vaccination implementation, there was discussion on delaying the second dose in order to offer more individuals with a first dose [35]. In addition, it was recently shown that delaying the second dose provided higher immunogenicity and maintained vaccine efficacy [36]. However, a suboptimal immune response would favor breakthrough infections due to the ancestral or variant viruses [37, 38]. Thus, the kinetics of induction and maintenance of the adaptive immune responses in the elderly, which tend to respond less efficiently to vaccination [26], remain to be fully understood.

Here, we compared the kinetics of specific antibodies, B and T cell memory responses in a cohort of BNT162b2-vaccinated healthcare workers and elderly individuals in Singapore up to six months post-immunization. We specifically investigated longitudinal and integrated data from the same individuals with a variety of quantitative laboratory antibody, B and T cell assays, allowing a comprehensive analysis of the establishment and persistence of the vaccine-induced responses.

Results

A cohort of 312 individuals was vaccinated with the Pfizer-BioNTech BNT162b2 vaccine from the beginning of January 2021 to May 2021 in Singapore (Supplementary table 1). The median age was 50.9 years (range, 22 to 82) and volunteers were predominantly female (58.3%) and Chinese (72.4%). Participants' characteristics differed across the different vaccination groups, which reflected vaccine prioritization for healthcare workers and elderly individuals. None of the participants had known or reported history of SARS-CoV-2 infection and were all negative for antibodies against the N protein using the commercial Roche serology assay. At the time of vaccination, Singapore had a low case count which corroborated with low sero-prevalence. Participants were also all negative for antibodies against the N protein using the commercial Roche serology assay. To monitor immune responses, longitudinal blood samples were acquired at baseline corresponding to the day of the first dose, 21 days later at the time of the second dose and up to 180 days post first dose (Fig. 1A).

Antibody response to SARS-CoV-2 mRNA vaccine

All volunteers (n=312) were analyzed for vaccine-induced anti-Spike (S) protein-specific antibody levels and neutralizing efficacy using various assays. The flow cytometry-based assay (SFB) is based on the recognition of SARS-CoV-2 Spike protein stably expressed on the surface of HEK293T cells, allowing the detection of antibodies binding to different epitopes present on the full Spike protein [39, 40]. The majority of volunteers seroconverted after the first dose (95% had higher antibodies than the cohort baseline, and above their individual baseline) (Fig. 1B and Supplementary table 2). After the second dose, all participants but one had developed anti-Spike protein antibodies by day 90. Immunoglobulin isotyping revealed that the proportion of vaccinees with detectable IgM (above both cohort and individual baseline) was >85% at day 21 but dropped to 12% by day 90 (12%) and was negligible by day 180 (Supplementary fig. 1), indicating rapid maturation of the antibody after vaccination. Interestingly, IgG1 dominated the antibody response, followed by IgG3 and IgG2, while IgG4 was barely detected (Fig. S1). However, by day 180, anti-Spike antibody levels had declined in 95% of the vaccinees (Supplementary table 3) on average by 39% (median binding percentage from 40.5% at day 90% to 24.1 at day 180). We also observed a sizeable proportion of low responders (individuals with responses below median cohort response at consecutive time points (37.2% at day 90 and 22.2% at day 180) (Supplementary table 4).

We next profiled antibodies specific to the receptor binding domain (RBD) of the S protein, which is the immunodominant target of anti-SARS-CoV-2 neutralizing antibodies [41] using a commercial assay (Roche S). The first vaccine dose induced antibodies in all but two vaccinees (Fig. 1C and Supplementary

table 2). After the second dose, all individuals seroconverted by day 90. However, 36.5% of individuals mounted a poor secondary anti-RBD IgG response (Supplementary table 5). A significant decline in anti-RBD antibody levels was also observed at day 180 in 77.8% of the vaccinees (Supplementary table 3), on average by 30% (median value from 1140 U/ml at day 90 to 799.8 U/ml at day 180).

We next measured the level of neutralizing antibodies in these vaccinees using a surrogate virus neutralization test (sVNT) for the Wuhan strain, that has a good concordance with the live-virus neutralization test [42]. It was observed that >79.1% of the plasma had neutralizing antibodies above individual baseline after the first dose, 99% after the second dose and ~93% at day 180. However, between day 90 and day 180, serum neutralization efficacy declined in 77.5% of the participants and on average by 25% (from a median inhibition of 89.9% to 67.4%) (Fig. 1D and supplementary table 3). One third of the vaccinees mounted a poor secondary neutralization response (Supplementary table 6) and 19 out of 312 (6%) had no neutralizing antibodies (below baseline inhibition) at day 180 (Fig. 1D).

Notably, when the data from different serological assays were analyzed according to age, we observed a significant negative correlation of the age of the individuals with the antibody response at day 21 (after the first dose) and also with the antibody response at day 90 (after the second dose) (Supplementary fig. S2). Sample distribution revealed two clusters of individuals, low responders (1) <60 and (2) ≥ 60 years. This was confirmed by principal component analysis (PCA) of the three combined serological assays at days 21, 90 and 180 (Fig. 2A). Thus, the data age-stratified and analyzed (<60 versus ≥60). For all assays, vaccinees ≥ 60 years had a lower response compared with younger vaccinees after the first dose (Fig. 2B). After the second dose, the antibody responses were boosted in all groups. However, the increase was less pronounced in the older age group who displayed lower antibody levels against the whole spike protein and RBD and had serum neutralizing capacity at day 90 (Fig. 2B). At day 180, while the elderly had more antibodies against the whole S-protein compared with the younger population (Fig. 2B, left panel), they still had lower levels of anti-RBD antibodies and lower neutralizing antibody capacity (Fig. 2B, middle and right panel respectively). Similarly, there were more older individuals (participants with responses below median cohort response at consecutive time points) among the low responders (Supplementary tables S4, S5 and S5).

We next assessed the waning of antibodies between age groups by measuring the difference in antibody levels between days 180 and 90 in paired samples (Fig. 2C). Although antibody levels were lower at the cohort level, decline in antibody levels was significantly more pronounced in the older population than in the younger one (Fig, 2C left and middle panels, and supplementary table 3). The waning of neutralization capacity did not differ between both age groups (Fig. 2C right panel and supplementary table 3).

We also examined the binding efficiency of the vaccinated plasma to the spike protein of the Delta variant using the SFB assay. In a previous study, we reported that the levels of IgG against the Wild type or its variants strongly was strongly correlated with their capacity to inhibit pseudovirus and live virus neutralization expressing the respective various Spike proteins [43, 44]. Here, we show that any time

points the antibody response was lower against the Delta variant than the wild-type original strain (Fig. 3A). However, the difference in recognition was only different between age groups after the first dose at day 21 (Fig. 3A, left panels). This was not observed at later time points similar in both age groups (Fig. 3B, middle and right panels).

Memory B cell response to SARS-CoV-2 mRNA vaccine

To measure the induction of RBD-specific circulating memory B cells by the vaccine, a B cell ELISPOT assay was performed on a subset of randomly selected age-matched individuals (n=78, from which we had 36 paired samples for the four time points). After the first dose, there was no significant increase at day 21, even though 47% of the individuals of the paired groups had higher memory B cells than their individual baseline (Supplementary table 2). After the second dose, a significant increase in RBD-specific memory B cell percentage was observed at day 90 (Fig. 4A). Analysis of paired samples confirmed these observations (Fig. 4B), where 76.5% had positive responses above their baseline levels. By day 180, the numbers of RBD-specific B cells continued to increase (Fig. 4A), with 85.3% of individuals having responses above their baseline levels at day 180 (Supplementary table 2). Generally, all individuals had produced RBD-specific circulating B cells in the six-month period.

When the data were age-stratified, we observed that the memory B cell response was lower in vaccinees ≥ 60 years at day 21, compared with the younger vaccinees after the first dose, (Fig. 4C). However, after the second dose, the memory B cell response increased in vaccinees ≥ 60 years at day 90 and 180 (Fig. 4C), corresponding to an overall increase in the number of total memory B cells (Fig. 4D). After two doses, the memory B cell response continued to increase for both age groups over time (Fig. 4C). At day 180, the difference between the two age groups disappeared, with both age groups having similar levels of memory B cell response (Fig. 4C-D). By comparing differences in the memory B cell response between time points (Fig. 4E), we found that younger individuals responded faster, with a greater increase right after the first dose at day 21. Therefore, the second dose is critical for the older age group as observed by the significant increase at day 90 (Fig 4E). .

T cell responses to SARS-CoV-2 mRNA vaccine

T cell stimulation was determined by quantifying cytokines (IL-2 and IFN-γ) directly secreted by Spike–specific CD4 and CD8 T cells in whole blood after overnight incubation with peptide pools covering 75-80% of Spike protein [45]. This was done in a subset of volunteers (n=155) randomly selected from the cohort but age matched (n=81 < 60 and 82 ≥ 60). At baseline, majority (~95%) of the individuals had very low production of IL-2 or IFN-γ (less than 10 pg/ml) after Spike peptide pool stimulation (Fig. 5A and 5B). After one dose, most of the vaccinees had a T cell response that increased further after the second dose. 100% of the vaccinees had a peptide-mediated IL-2 response above individual baseline after the first dose at day 21, days 90 and 180 (Fig. 5A and supplementary table 2). A robust IFN-γ response above baseline was also observed (~93 to 95% after the first and second doses) and sustained up to day 180 (Fig. 5B and supplementary table 2).

We next performed a detailed analysis of the T cell subsets by Elispot in a smaller subset of the volunteers due to cell availability. We used peptides covering potential CD8 or CD4 T epitopes (see materials and methods). For the CD8 assay using Spike protein peptide pools covering potential 9mers CD8 epitopes [46], we showed that, at baseline, some vaccinees already showed a high level of spots (Fig. 5C), suggesting a cross-reactive CD8 T cell response from exposure to other circulating coronaviruses. After the first dose, 54% had an increase in spots above their individual baseline values at day 21. After the second dose, 75% of the vaccinees had a response above their individual baseline at day 90 (Fig. 5C and supplementary table 2). By day 180, only 40.3% still had a CD8 T cell response (above their own baseline values, Supplementary table 2). Overall, 88.9% (64/72) mounted a CD8 T cell response during the 6-month follow-up. However, a comparison between responses at day 180 and 90 revealed that the response waned in 48% of the vaccinees (Supplementary table 3).

We next stimulated PBMC with a 15mer peptide pool corresponding to potential CD4 epitopes and measured the response by Elispot. CD4 Th1 (IL-2 and/or IFN-γ) responses were low at baseline, except for a few individuals (Fig. 6D). After one dose, 69% of the vaccinees and 84.6% after the second dose had a response higher than their baseline by day 90 and 83.33% by day 180 (Supplementary table 2). Overall, the CD4 Th1 cell response was significantly different after the first dose and further significantly boosted after two doses. Over 96.2% (75/78) mounted a response during the six-month follow-up. Comparison between responses at day 180 and 90 revealed that the CD4 Th1 response waned in 46.6% of the vaccinees (Supplementary table 3). A CD4 Th2 cell response was observed but was not as strong as the Th1 response (Supplementary fig. 3). At day 21, 59% of the vaccinees had values above their own baseline, a percentage which remained constant at day 90 but started to wane by day 180 (Supplemental tables S2 and S3).

Age-stratified analysis showed that post-vaccination Spike peptide pool-mediated IL-2 response was similar in both age groups at all time points (Fig. 6A). The IFN-γ response was lower at baseline in the <60 group. However, after the first dose (day 21), it reached similar level to that of ³60 group. At days 90 and 180, the older age group had T cells producing significantly more IFN-γ than the younger individuals (Fig. 6B). We did not observe any age effect on the CD8 ELISpot response (Fig.6C). CD4 Th1 was significantly lower at baseline for the older age group but the responses were similar at days 21, 90 and 180 (Fig. 5D). Post-immunization Th2 cell responses were also similar at the different times (Supplementary fig. 3 and supplementary table 3).

We next assessed the waning of T cell responses between age groups by measuring difference in response levels between days 180 and 90 in paired samples (Fig. 6E and supplementary table 3). Although T cell responses were lower at the cohort level, the decline was not significantly different between age groups. On the contrary, IFN-γ T cell response was even higher in the older age group (Fig 6E, middle left panel).

Discussion

In this study, we show that the two-dose regimen BioNTech/Pfizer BNT162b2 COVID-19 vaccine is highly immunogenic and generates robust antibody, B and T cell responses in most vaccinees. Despite the strong immunogenicity, a sizeable proportion of vaccinated mounted a low antibody response. Further analysis showed that individuals ≥ 60 years developed antibody responses at a slower pace, with a lower peak, and were more represented in the low responders' fraction. (Fig. 2 and supplementary tables 3, 4 and 5). However, antibody responses decreased less rapidly in the older age group as seen 6 months post-immunization (Fig. 2B, middle and right panel and Fig. 2C). These data are in line with the memory B cell data, which show lower levels of memory B cell in the older age group after the first dose at day 21, but eventually were at similar levels at day 90 and continued to increase at day 180. This indicates the building of equivalently strong B cell memory in responding individuals. Spike-specific T cell responses were induced in most vaccinees and remained high until day 180 (Fig. 4). Unlike the antibody response, both age groups were equally represented in the low T cell responder group (Supplementary tables 7, 8 and 9). Although around 30–50% of the whole cohort had a decrease in response between days 90 and 180 (except for the IFN-γ T cell responses), this waning was observed in both age groups. T cell help is also essential for the development of SARS-CoV-2 memory CD8 T cells. A recent report supports an important role for these cells in protection against the virus [21].

The overrepresentation of older individuals in the low responder groups is likely a consequence of immunosenescence, which is characterized by the reduced adaptive immune responses [47–49]. This has been well described for influenza vaccines in Caucasian populations [50–51]. However, studies on the Chinese population in Singapore showed no impact of age on immunogenicity after influenza vaccination [52]. Here, we showed that, following COVID-19 vaccination, antibody responses were partially affected by age. Additional genetic, behavioral, nutritional or environmental factors might account for this phenomenon and deserve further studies.

The low neutralizing antibody response in a larger subset of the older age group (20-30% more than in the younger groups) has important clinical implications as it suggests reduced vaccine efficacy in the older individuals, especially against new emerging variants. High neutralizing antibody levels have been proposed as one of the essential protective against reinfection with the original Wuhan strain of SARS-CoV-2 or new emerging variants that can escape antibody neutralization [42, 43, 53–57]. In this study, we showed that the antibody reactivity against the Delta variant by the vaccine-induced response was lower, compared with the WT. However, this lower response against the Delta variant was similar across the different age groups, and not limited to the older individuals. In addition, the older population that we have shown here were less prone to develop an efficient primary response, increasing the dose of vaccine may overcome initial low responsiveness, as shown for Influenza vaccination [58]. Second generation vaccines with better immunogens or adjuvants [59] that induce a more rapid and efficient helper T cell response may be designed for this population [60].

The other main finding of our study is that the waning of the vaccine-induced T cell response is not affected by age. This is particularly important as T cells are thought to protect against severe disease [20, 22]. They recognize peptide epitopes distributed throughout the SARS-CoV-2 Spike protein [45, 61–63]

and are less susceptible to antibody escape mutations in variant strains [62, 64]. Our finding, that the levels and activities of the T cell response were maintained in all age groups up to 180 days, suggesting protection in all age groups. This agrees with recent studies which have reported similar efficacy of the BNT162b2 vaccine against severe disease after infection with the Wild type or the Delta variant, as compared with the WT strain [7–9, 65].

Lastly, we show here that the antibody responses induced by mRNA COVID-19 vaccines is not long–lasting, with responses starting to wane after 3-6 months in a proportion of vaccinated individuals, which has been recently observed in other studies [66, 67]. Antibody levels waning was more pronounced in the elderly population. There are emerging reports of waning protection after 6-month vaccination [68]. This calls for the identification of the low responders, individuals who are not able to develop an efficient induction of immunity following the recommended two-doses 21 days apart regime. These individuals may require additional booster vaccine doses with the same mRNA construct [69] and/or with constructs coding for the variant viruses. They may also benefit from booster vaccine doses of the same platforms or other vaccine platforms. These approaches are already under investigation [70–73] and could help these individuals to elicit an efficient immunity and protection against infection and disease in the current COVID-19 pandemic.

Material And Methods

Cohorts and Ethics

A cohort of 312 individuals was recruited (Table S1) comprising healthcare workers and older individuals. The study design and protocol for the COVID-19 PROTECT study group were assessed by National Healthcare Group (NHG) Domain Specific Review Board (DSRB) and approved under study number 2012/00917. Collection of healthy donor samples was approved by SingHealth Centralized Institutional Review Board (CIRB) under study number 2017/2806 and NUS IRB 04-140. Written informed consent was obtained from all study participants in accordance with the Declaration of Helsinki for Human Research. The experiments adhered to the principles set out in the Department of Health and Human Services Belmont Report.

Sample collection

Blood was collected in VACUETTE EDTA tubes (Greiner Bio, #455036) or in Cell Preparation Tubes (CPT) (BD, #362761) for volunteers at various timepoints (day 0, 21, 90 and 180 post first-dose) [74].

Serological assays for the detection of anti-SARS-CoV-2 antibodies

Serum specimens were stored at -25°C and equilibrated at room temperature before time of analysis. Samples were analyzed using two commercial assays, in accordance with the manufacturer's protocol. The Elecsys® Anti-SARS-CoV-2 S (Roche S) and Elecsys® Anti-SARS-CoV-2 (Roche N) immunoassays using the Roche Cobas e411 Analyzer (Roche) allow the quantitative detection of total antibodies against the SARS-CoV-2 spike (S) protein receptor binding domain (RBD) and the qualitative detection of total

antibodies against the SARS-CoV-2 nucleocapsid (N) antigen respectively. Plasma were incubated with either a mix of biotinylated and ruthenylated SARS-CoV-2 S-RBD antigens or N antigens to form immune complexes. Complexes were attached to streptavidin-coated microparticles upon incubation and then transferred to a measuring cell. For the Roche S assay, the electro-chemiluminescent signal representing the level of antibodies was measured and samples within the linear range of quantitation (0.4 – 250 U/mL) were assigned a value. Samples with antibody levels ≥ 0.8 U/mL were considered positive. For the Roche N assay, the cut-off index (COI) was derived from the measured signal, where samples with COI ≥ 1.0 were considered reactive.

Spike protein flow cytometry-based assay (SFB assay) for antibody detection

The SFB assay was performed as previously described (Goh et al, 2021a; 2021b). S protein-expressing cells were seeded at 1.5 x 10^5 cells per well in 96 well V-bottom plates. Cells were incubated with human serum (diluted 1:100 in 10% FBS) followed by a secondary incubation with a double stain, comprising Alexa Fluor 647-conjugated anti-human IgG (1:500 dilution) and propidium iodide (PI; 1:2500 dilution). Cells were acquired using a BD Biosciences LSR4 laser and analyzed using FlowJo (Tree Star). The assay was performed as two independent experiments within technical duplicates each time.

Determination of SARS-CoV-2 neutralizing antibody level using sVNT

Neutralizing antibodies against SARS-CoV-2 was measured using the surrogate virus neutralization (sVNT) platform [42] and conducted according to manufacturer's protocol (cPass^{TM}, GenScript). HRP-conjugated RBD (RBD-HRP) provided was diluted with HRP Dilution Buffer to 1:1000. Test plasma was diluted with the Sample Dilution Buffer to 1:10. The diluted plasma were then mixed with the diluted RBD-HRP in 1:1 ratio (e.g., 60 µL diluted plasma with 60 µL diluted RBD-HRP). The mixtures were incubated at 37°C for 30 minutes. After first incubation, 100 µL of the mixtures was added into each well of the ACE2-coated plate provided. The plate was covered with a plate sealant and incubated at 37°C for 15 minutes. After second incubation, the plate was washed four times with 260 µL 1× wash buffer to remove the unbound RBD-HRP. For measurement of RBD-HRP bound onto the plate, 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB) was added into each well. The chromogenic signal was allowed to develop for 15 minutes in the dark before 50 µL of the TMB stop solution was added into the well. Absorbance at 450 nm was acquired using Cytation 5 microplate reader (BioTek). cPass^{TM} percentage inhibition was calculated according to the manual, and a 30% cut-off was used to determine a positive result.

Memory B cell ELISpot

SARS-CoV-2 RBD-specific memory B cell numbers were counted using the ELISpot Path: Human IgG (SARS-CoV-2, RBD) ALP kit (Mabtech), following manufacturer's instructions. Fresh PBMCs (1,000,000) were resuspended in 1ml RPMI + 10% FBS + 1 µg/ml R848 + 10 ng/ml IL-2, and incubated at 37°C, 5% CO_2 for 4-5 days to differentiate memory B cells into antibody-secreting cells. After incubation, cells were counted, and 100,000 or 400,000 live cells were taken for ELISpot plating to determine RBD-specific memory B cell numbers. Total IgG secreting cells were detected by plating 1,500 or 3,000 live cells to

normalize the results. Plates were then read on an IRIS ELISpot reader (Mabtech). Spots were calculated based on the average of two wells.

Whole-blood culture with SARS-CoV-2 peptide pools

This was performed as described previously (Tan, JCI, 2021). Whole blood (320 µl) drawn on the same day was mixed with 80 µl RPMI and stimulated with pools of spike protein peptides (2 µg/ml) or a DMSO control. After 15 h of culture, the culture supernatant (plasma) was collected and stored at −80°C until quantification of cytokines. Cytokine concentrations in the plasma were quantified using an Ella machine with microfluidic multiplex cartridges measuring IFN-γ and IL-2 following the manufacturer's instructions (ProteinSimple). The positivity threshold was set at 10 x times the lower limit of quantification of each cytokine (IFN-γ = 1.7 pg/ml; IL-2 = 5.4 pg/ml) after DMSO background subtraction.

IFN-γ/IL-2 FluoroSpot assays. Donor PBMCs were first thawed in RPMI-1640 with 10% Fetal Bovine Serum (R10 medium) and incubated overnight for recovery in high density (10 million PBMCs per 2mL) in AB medium (RPMI-1640 + 10% Human AB Serum + 1% Penicillin Streptomycin + 1% 200g/mL D-glucose). PBMCs were then used for FluoroSpot assays to measure CD8, CD4 Th1 and Th2 responses. CD8 and CD4 Th1 responses were measured using Human IFN-γ/IL-2 FluoroSpot PLUS kits as per manufacturer's protocol (Mabtech, Sweden). In brief, PVDF plates pre-coated with IFN-γ mAb (1-D1K) and IL-2 mAb (MT2A91/2C95) were washed with sterile phosphate buffered saline (PBS) and blocked with R10 medium for at least 30 minutes at room temperature (RT). After overnight rest, PBMCs were harvested and suspended in AB medium. PBMCs were seeded at 250,000 cells per well and stimulated in duplicates with SARS-CoV-2 spike glycoprotein peptide pool (JPT Peptide Technologies, Germany) with 0.1µg/mL co-stimulator anti-CD28 (mAb CD28A as per MabTech protocol). Medium containing 1% DMSO was used as negative control, while 0.02µg/mL anti-CD3 mAb (CD3-2) was used as positive control. Cells were incubated overnight at 37˚C and 5% CO2. Following overnight incubation, plates were washed with PBS and incubated with detection antibodies anti-IFN-γ mAb (7-B6-1-BAM) and anti-IL2 mAb (MT8G10, biotinylated) diluted in PBS with 0.1% BSA for two hours at RT. Plates were then washed with PBS and incubated with fluorophore conjugates for IFN-γ (anti-BAM-490) and IL-2 (SA-550) in PBS with 0.1% BSA for one hour at RT. Plates were washed and incubated with ready-to-use fluorescent enhancer II for 15 minutes at RT. All incubations were performed in the dark. Plates were emptied and dried overnight at RT and analysed the next day with MabTech IRIS FluoroSpot and ELISpot reader using FITC filter (excitation 490 nm/emission 510 nm) for IFN-γ and Cy3 filter (excitation 550 nm/ emission 570 nm) for IL-2.

IL-4/IL-5/IL-13 FluoroSpot assays. CD4 Th2 responses were measured using custom Human IL-4/IL-5/IL-13 FluoroSpot FLEX kits as per manufacturer's protocol (MabTech). In brief, PVDF plates were activated with 15µL 35% EtOH per well for a maximum of one minute. Plates were washed with cell culture water and incubated with IL-4 mAb (IL4-I), IL-5 mAb (TRFK5) and IL-13 mAb (MT1318) in PBS at 4°C overnight, protected from light. After overnight incubation, plates were washed with sterile PBS and blocked with R10 medium for at least 30 minutes at RT. After overnight rest, PBMCs were harvested and suspended in

AB medium. Stimuli were prepared in AB media with 0.1µg/mL co-stimulator anti-CD28 (mAb CD28A). PBMCs were seeded at 250,000 cells per well and stimulated with peptide pool from the Spike protein (JPT Peptide Technologies). 1% DMSO only medium was used as negative control. Cells and stimuli were incubated overnight at 37˚C and 5% CO_2. Following overnight incubation, plates were washed with PBS and incubated with detection antibodies anti-IL4 mAb (IL4-II), anti-IL5 mAb (5A10) and anti-IL13 mAb (25K2) diluted in PBS with 0.1% BSA for 2 hours at RT. Plates were then washed with PBS and incubated with fluorophore conjugates for IL-4 (SA-550), IL-5 (anti-WASP-640) and IL-13 (anti-BAM-490) in PBS with 0.1% BSA for 1 hour at RT. Plates were washed and incubated with ready-to-use fluorescent enhancer II for 15 minutes at RT. All incubations were performed in the dark. Plates were emptied and dried overnight at RT and analysed the next day with Mabtech IRIS FluoroSpot and ELISpot reader using Cy3 filter (excitation 550 nm/ emission 570 nm) for IL-4, Cy5 filter (excitation 640 nm/ emission 660 nm) for IL-5 and FITC filter (excitation 490 nm/emission 510 nm) for IL-13.

Low responder population definition

Low responders were defined as fully vaccinated individuals with antibody response below cohort's median response at consecutive timepoints (%). As an example, the low responders, at day 90, had responses below cohort's median response at both day 21, 90 and 180.

Statistical analysis

To assess immune positivity after the vaccine doses, we used two methods to define baseline values. In method 1, we define a cohort baseline using the upper range of the data set (for data which did not follow a normal distribution). For data which follow a normal distribution, a cohort mean + 3 SD was used to define a cut-off. In method 2, positivity was defined when values were above individual baseline values.

Statistical analysis was performed using GraphPad Prism 7 software. Paired comparisons for samples taken at different times or unmatched pairwise comparisons (when comparing between age group) were performed using the Mann Whitney U test, while matched pairwise comparisons were performed using the Wilcoxon matched pairs signed rank test. To compare between multiple groups, Kruskal-Wallis tests and post hoc tests using Dunn's multiple comparison tests were used to identify significant differences. Spearman's correlation analyses were performed to calculate correlation coefficient rho and P value. P values less than 0.05 were considered significant. Statistical analysis was performed using GraphPad Prism 9.1.2. FluoroSpot results were analyzed with Welch's t test for parametric unpaired comparisons. All tests were two-tailed and $p < 0.05$ was considered statistically significant. Principal Component Analysis (PCA) was performed on the data from SFB, Elecsys RBD assay and commercial C-pass sVNT assay, using Singular Value Decomposition (SVD) method in ClustVis [75].

Declarations

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Author contributions

Conceptualization: LR, DCB, YSL, RTPL, LFW, EER, AB, BEY and LFPN. Sample collection: YD, SP, LJS, JS, ESL, DLSO, DCB, BEY. Formal analysis: YSG, AR, NLB, WC, JMC, SWF, LR, DCB, YSL, RTPL, LFW, EER, AB, BEY and LFPN. Investigation: YSG, AR, NLB, WC, JMC, SWF, ZWC, NZZ, MZT, YHC, NKMY, SNA, YH, PXH, CYL, GC, SD, AJL. Writing—original draft preparation: LR, YSG, DCB, ABC, REC. Writing: review and editing: all authors, and H. S. Supervision: LR, LFPN, DBL, BEY, RTPL, LFW, ECR, AB.

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Data availability

The source data are provided as a Source Data file. Other data can be obtained upon reasonable request to the corresponding author. Source data are provided with this paper.

Conflict of interests

A patent application for the SFB assay has been filed (Singapore patent #10202009679P: A Method Of Detecting Antibodies And Related Products). The authors declare no other competing interests.

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Figures

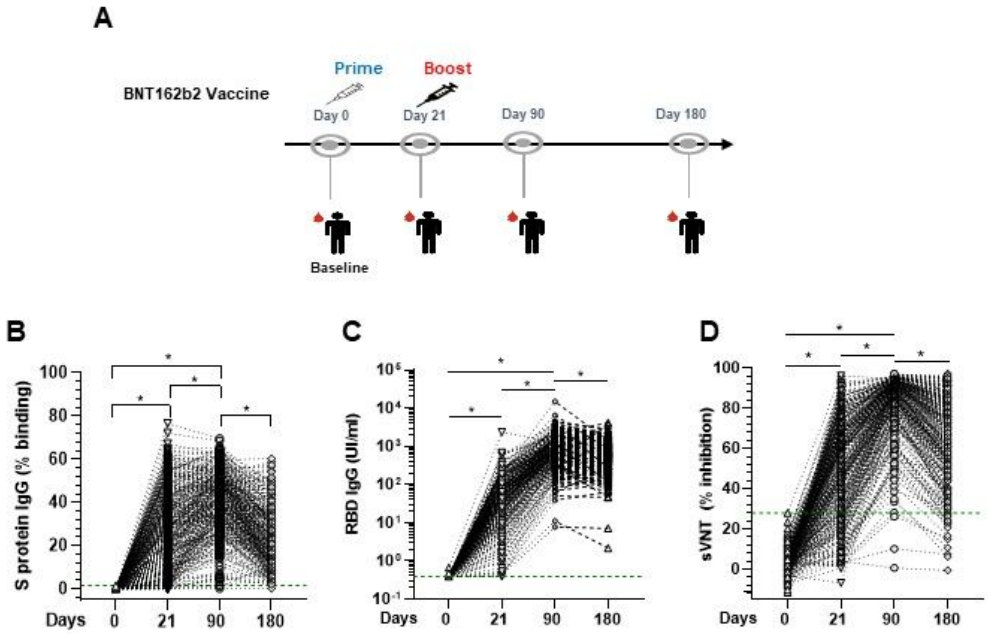

Figure 1

Figure 1

Anti-SARS-CoV-2 spike protein antibody response after vaccination. (A) Schematic description of the longitudinal vaccination and blood sampling strategy in a cohort of Singaporean individuals (n=312). Kinetics of IgG response were analyzed using 3 serological assays on paired samples. Overall cohort baseline value is defined as the value greater than maximum range of all samples in the cohort. The green dotted line represents the maximum range of the samples in the different assays. (B) A flow

cytometry-based assay using the full Spike protein (SFB) assay. Median (range) of values at day 0 was 0.06% (0.002, 1.7). Antibody levels below the maximum range (1.7%) were considered baseline values. *, p < 0.001, Friedman test; (C) The Roche S assay using the RBD protein fragment. Median (range) of values at day 0 was 0.39 U/ml (0.39, 0.67). Antibody levels below the maximum range (1.38 U/ml) were considered baseline values; *, p < 0.001, Friedman test; (D) a surrogate virus neutralization test (sVNT). Median (range) of values at day 0 was 0.39% (-11.54, 27.94). Inhibition below the maximum range (27.94%) were considered baseline values. *, p < 0.0001, Friedman test.

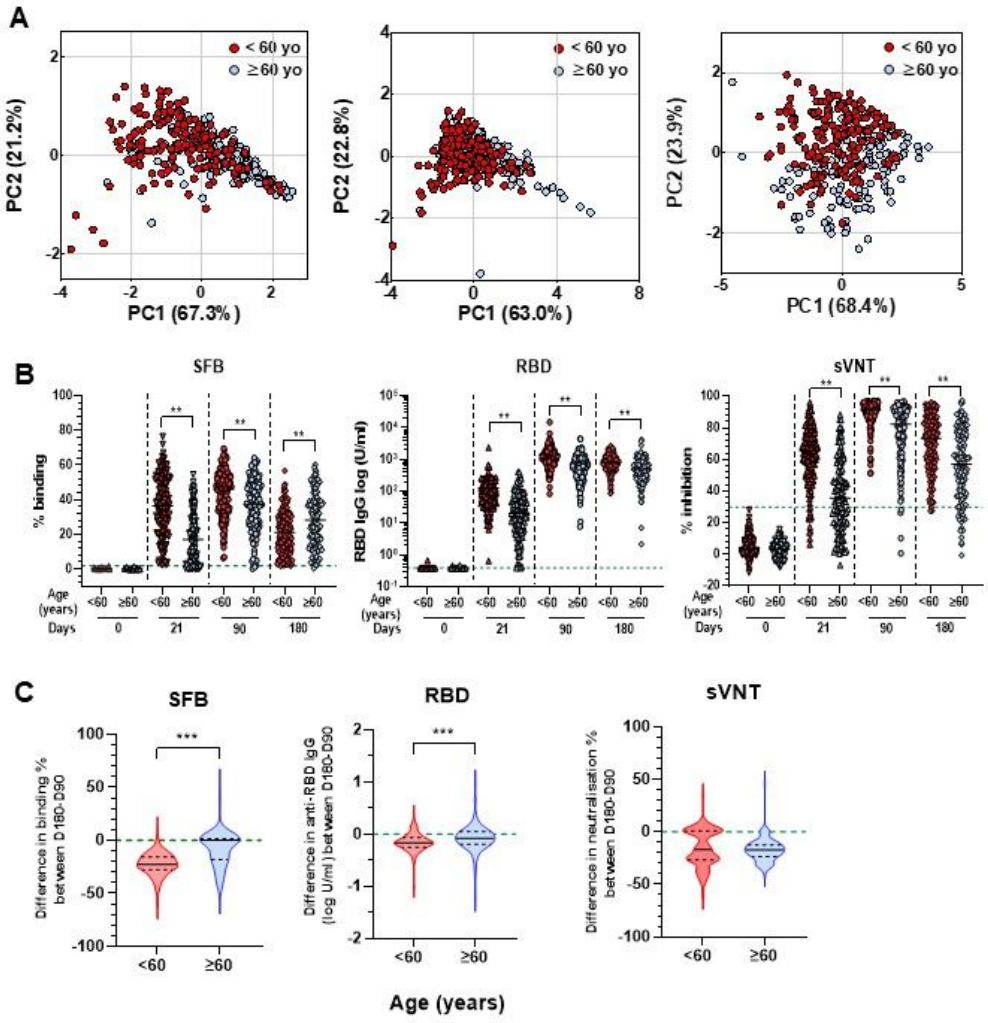

Figure 2

Figure 2

Age stratification of antibody responses. (A) Principal component analysis of antibody responses determined by the 3 different serology assays at different times. (B) Comparison between age groups: <60 (n=178) and ≥60 (n=134), at different time points (day 0, 21, 90 and 180) using SFB (left panel); RBD Roche S assay (middle panel); and sVNT assay (right panel). The median value of each age group is represented by the black line. The green dotted lines represent the maximum range of the samples for the whole cohort baseline. *, $p < 0.01$, Mann Whitney test. (C) Violin plots (with median and quartiles) showing difference in antibody response between days 180 and 90 for paired samples for both age groups. The green dotted lines represent no difference. *, $p < 0.01$, Mann Whitney test. (D) Comparison between age groups: <60 (n=17) and ≥60 (n=18), at different time points (days 21, 90 and 180) using SFB with cells expressing either the wild-type Wuhan original virus (WT) or the delta variant of the Spike protein. Median (range) values at day 0 against the WT and the Delta strains for all samples were respectively 0.07% (0.012-0.3) and 0.18 %(0.03-.0.825).

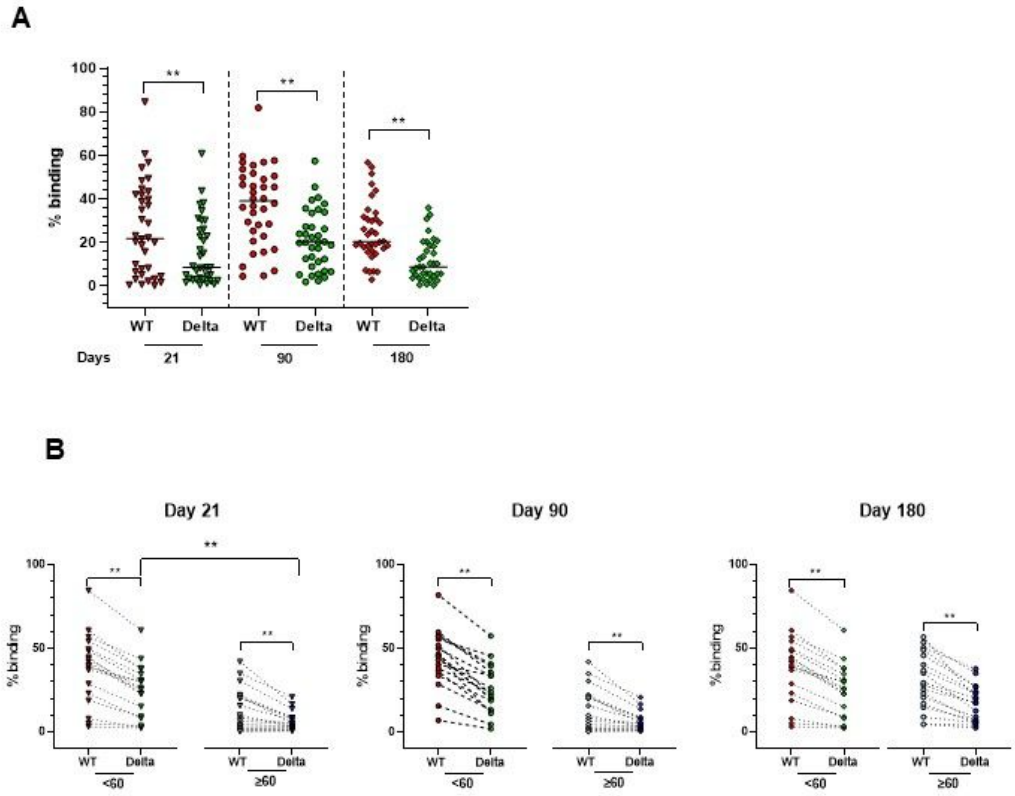

Figure 3

Figure 3

Antibody recognition of wild type and Delta strains. (A) Comparison of antibody response of the vaccinees (n=36) at days 21, 90 and 180 using the SFB assays with cells expressing either the wild-type Wuhan original virus (WT) or the delta variant of the Spike protein. Median (range) values at day 0 against the WT and the Delta strains for all samples were respectively 0.07% (0.012-0.3) and 0.18 % (0.03-.0.825). Median (range) values at day 0 against the WT and the Delta strains for all samples were

respectively 0.07% (0.012-0.3) and 0.18 %(0.03-.0.825). **, p < 0.001, Mann Whitney test. (B) Comparison between age groups: <60 (n=18) and ≥60 (n=18), at different time points (days 21, 90 and 180) using SFB with cells expressing either the wild-type Wuhan original virus (WT) or the delta variant of the Spike protein.

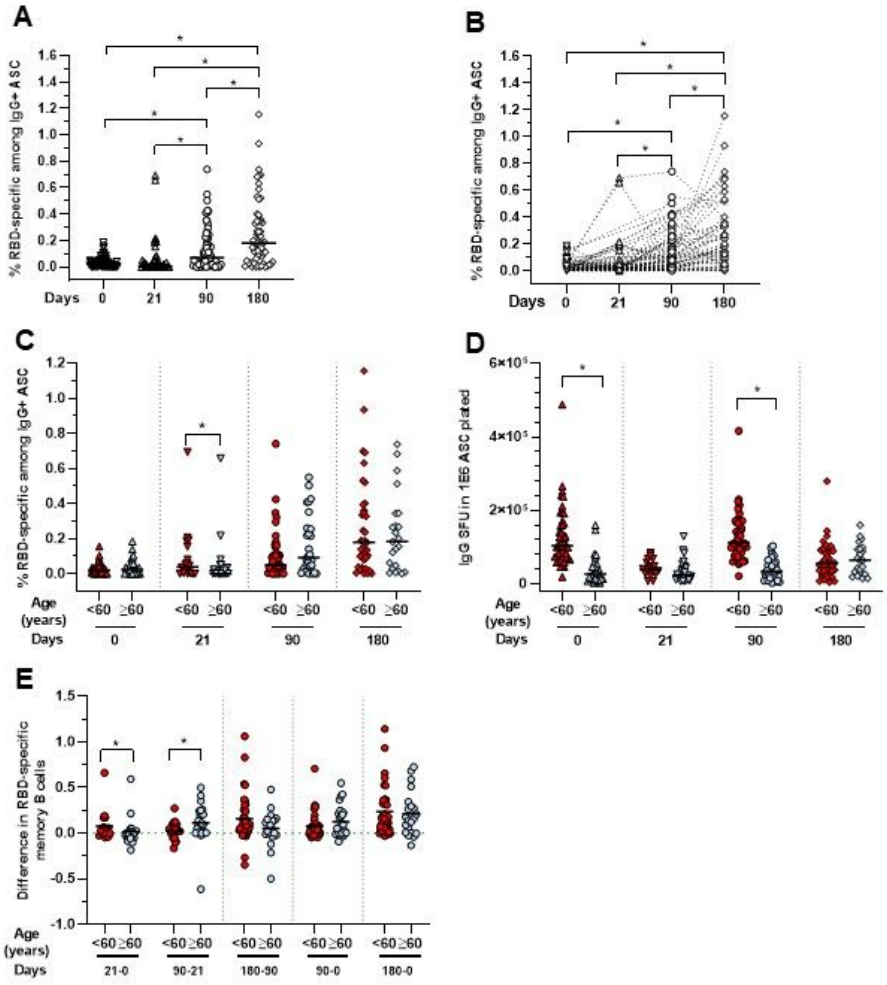

Figure 4

Figure 4

Circulating RBD-specific memory B cells after vaccination. (A) RBD-specific memory B cells were determined by ELISPOT using a RBD protein. Measurement was done on PBMC from vaccinated participants at baseline or day 0 (n= 73), at day 21 (n=43), at day 90 (n=76) and at day 180 (n=60). *, $p < 0.01$, Dunn's test after Kruskal Wallis ($p < 0.001$). (B) Paired wise comparison of total RBD-specific memory B cells for the analyzed aged group at different days post doses is shown (n=35). *, $p < 0.01$, Friedman test. (C) RBD-specific memory B cells comparison between the analyzed age groups. Samples were from individuals: aged <60: at day 0 (n=46), day 21 (n=18), day 90 (n=46), and day 180 (n=37); and aged ≥60, at day 0 (n=27), at day 21 (n=25), at day 90 (n=30), and day 180 (n=23). (D). Total IgG producing memory B cells comparison between the same analyzed age groups as above in (C). *, $p < 0.01$, Mann Whitney test. (E) Difference in RBD-specific memory B cells between paired samples and different time points (n=35). *, $p < 0.01$, Mann Whitney test.

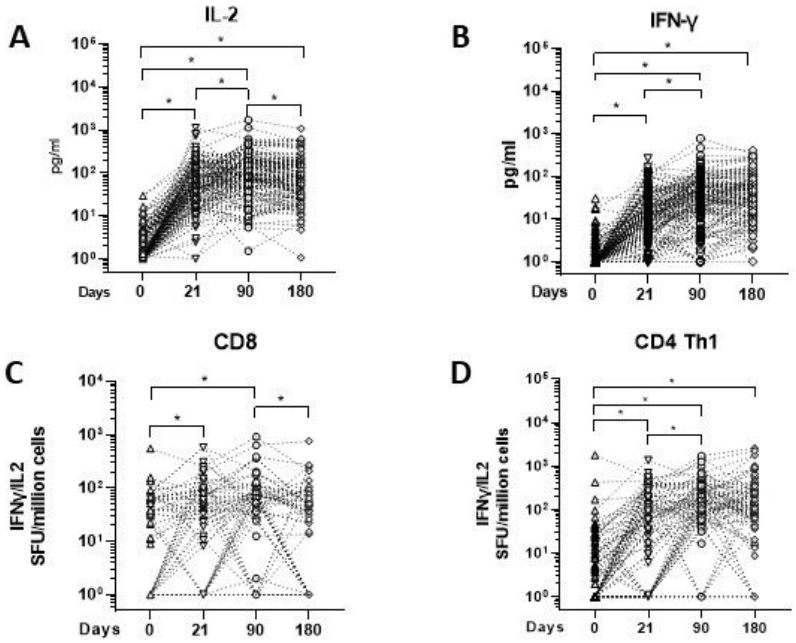

Figure 5

Figure 5

Anti-SARS-CoV-2 spike protein T cell responses. (A) IL-2 and (B) IFN-γ secretion profile of whole-blood cultures stimulated with S protein peptide pool compared at different time points of paired samples from vaccinated individuals (n=155). The limit of detection for each cytokine (IL-2 = 5.4 pg/ml; IFN-γ = 1.7 pg/ml). Values below limit of detection levels were plotted as 1. *, p <.001, ANOVA on log-transformed data, which follow a normal distribution. Kinetics of Spike-protein-specific CD8 (C) or (D) CD4 Th1 cells

overtime in vaccinees. T cells were assayed on a subset of vaccinees (n=80) by IL-2/ IFN-γ ELISPOT using 9 mer or 15 mer pool peptides respectively. Data are presented are spot forming units (SFU) per million of PBMC from paired samples from vaccinated individuals at 4 time points. Each data point represents the normalized mean spot count from duplicate wells for one study participant, after subtraction of the medium-only control *, $p < 0.01$, Dunn's test after Kruskal Wallis ($p < 0.001$).

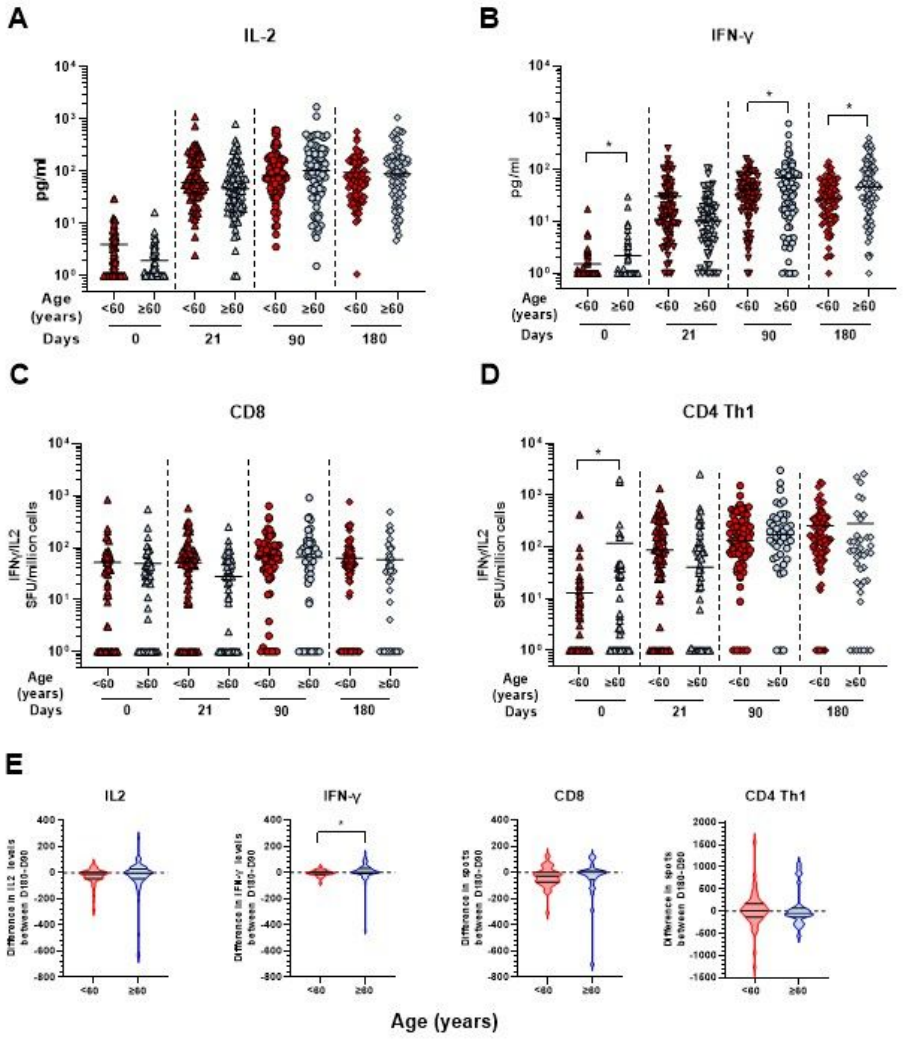

Figure 6

Figure 6

Age stratification of T cell responses. Comparison of the T cell response between age groups of samples from vaccinated individuals at different time points post immunization. (A) IL-2 and (B) IFN-γ production induced by Spike-peptide pool stimulation from individuals aged <60: (n=82), and ≥60 (n=75). Mean values are indicated by a dark line. *, p < 0.01, Student t test on normalized log values. (C) CD8 T cells comparison between the analyzed age groups. < 60 group: day 0 (n=66), day 21 (n=66), day 90 (n= 66) and day 180 (n=85), and ≥60: day 0 (n=43), day 21 (n=44) and day 90 (n=43 and day 180 (n=41). Median values are indicated by a dark line. *, p < 0.01, Mann Whitney test on log values. (D) CD4 Th1 cells comparison between the analyzed age groups. <60 group: day 0 (n=72), day 21 (n=72), day 90 (n= 72) and day 180 (n=83), and ≥60: day 0 (n=43), day 21 (n=44) and day 90 (n=43 and day 180 (n=41). Median values are indicated by a dark line. *, p < 0.01Mann Whitney test. *, p < 0.01, Mann Whitney test on log values. (E) Violin plots (with median and quartiles) showing difference in T cell responses measure in the different assays between days 180 and 90 for paired samples for both age groups (IL2 and IFN-γ: <60: (n= 82), and ≥60 (n=75); CD8 T cells: <60: (n= 45), and ≥60 (n=27)) and CD4 T cells, <60: (n= 51), and ≥60 (n=28). p = 0.003, Mann Whitney test.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- MS2021Reniasupplementarysubmitted.pdf