Cellular and humoral functional responses after BNT162b2 mRNA vaccination differ longitudinally between naive and subjects recovered from COVID-19

Graphical abstract

Highlights

- History of SARS-CoV-2 infection affects longitudinal responses to BNT162b2 vaccine
- Lower humoral but enhanced cellular responses early after vaccine in naive subjects
- Comparable humoral and cellular responses almost 8 months after vaccination
- Similar S-specific B cells late after vaccine in those naive and recovered from COVID-19

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In brief

Lozano-Rodríguez et al. show that naive subjects have enhanced SARS-CoV-2 spike-specific T reactions but reduced humoral-specific responses compared with individuals recovered from COVID-19. However, almost 8 months after vaccination, comparable specific responses are observed with equivalent levels of SARS-CoV-2-specific B cells and neutralizing antibodies.
Report

Cellular and humoral functional responses after BNT162b2 mRNA vaccination differ longitudinally between naive and subjects recovered from COVID-19

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SUMMARY

We have analyzed BNT162b2 vaccine-induced immune responses in naive subjects and individuals recovered from coronavirus disease 2019 (COVID-19), both soon after (14 days) and later after (almost 8 months) vaccination. Plasma spike (S)-specific immunoglobulins peak after one vaccine shot in individuals recovered from COVID-19, while a second dose is needed in naive subjects, although the latter group shows reduced levels all along the analyzed period. Despite how the neutralization capacity against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) mirrors this behavior early after vaccination, both groups show comparable neutralizing antibodies and S-specific B cell levels late post-vaccination. When studying cellular responses, naive individuals exhibit higher SARS-CoV-2-specific cytokine production, CD4+ T cell activation, and proliferation than do individuals recovered from COVID-19, with patent inverse correlations between humoral and cellular variables early post-vaccination. However, almost 8 months post-vaccination, SARS-CoV-2-specific responses are comparable between both groups. Our data indicate that a previous history of COVID-19 differentially determines the functional T and B cell-mediated responses to BNT162b2 vaccination over time.

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection and its associated pathology, coronavirus disease 2019 (COVID-19), have had an enormous impact on healthcare systems worldwide and still constitute a challenge. Several vaccines have been authorized for emergency use by both the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA). Among them, the BNT162b2 messenger RNA (mRNA) vaccine has been widely used following an accelerated two-dose vaccination schedule, which has exhibited specific humoral and cellular responses in 95% of individuals (Polack et al., 2020).

A number of studies have suggested a strong spike-specific antibodies generation by individuals recovered from COVID-19 after a first vaccine shot and that the second dose appears to be redundant (Ebinge et al., 2021; Gobbi et al., 2021; Levi et al., 2021; Prendecki et al., 2021a). In contrast, a second dose seems to be needed for a strong immunization in naive subjects (Ebinge et al., 2021; Levi et al., 2021; Mulligan et al., 2020; Walsh et al., 2020). Besides, the effect of this mRNA vaccine on spike-specific T cell responses has gained much attention (Ni et al.,...
In this regard, to understand the cellular responses generated after vaccination, considering previous SARS-CoV-2 exposure is crucial for future adjustments in vaccination regimes. Some reports are warning about the waning of the BNT162b2-vaccine-induced protection a few months after vaccination despite still showing a robust efficacy against suffering from COVID-19 (Chemaitelly et al., 2021; Goldberg et al., 2021). This wane has been linked to a decay in the levels of SARS-CoV-2-specific neutralizing antibodies (Bayart et al., 2021; Doria-Rose et al., 2021), although neither the role of SARS-CoV-2 spike-specific T (Guerrera et al., 2021) and B (Turner et al., 2021) cells is fully understood nor the relationship between both humoral and cellular responses triggered by COVID-19 vaccines against (re)infection.

Herein, we aimed to evaluate the immune responses triggered by immunization with the BNT162b2 vaccine in a cohort of naive subjects and individuals recovered from COVID-19. Both humoral and cellular responses were thoroughly analyzed using blood samples taken before vaccination, after the first dose, 14 days, and almost 8 months after the vaccination regime was completed. Our data indicated that previous SARS-CoV-2 exposure conditioned early responses post-vaccination, as naive subjects showed enhanced SARS-CoV-2 spike-specific CD4+ T cells but reduced humoral spike-specific responses compared with individuals recovered from COVID-19. However, almost 8 months after vaccination, comparable humoral and cellular responses were observed in both groups, importantly, with equivalent levels of SARS-CoV-2-specific memory B cells and neutralizing antibodies. Therefore, our findings suggest that previous exposure to the virus determines early functional T and B cell-mediated responses to BNT162b2 vaccination. However, both naive subjects and individuals recovered from COVID-19 show comparable memory SARS-CoV-2-specific immunity almost 8 months after vaccination.

RESULTS

Humoral responses triggered after vaccination show specific kinetics in naive subjects and individuals recovered from COVID-19

Following the BNT162b2 vaccination strategy recommended by both the FDA and the EMA, a total of 27 individuals were vaccinated with a two-dose regime administrated 21 days apart. Of them, 16 had not been previously exposed to SARS-CoV-2 coronavirus (naive), while 11 were reported as having recovered from COVID-19 (Table S1). For all participants, four blood samples were taken: 5 days before the first dose (sample 0), 14 days after the first dose (sample 1), 14 days after the second dose (sample 2), and a final long-term sample collected 230 days (almost 8 months) after the second dose (sample 3) (Figure 1A).

We first analyzed the levels of SARS-CoV-2-specific plasma immunoglobulins. One vaccination dose induced the presence of both anti-spike S1 immunoglobulin A (IgA) and anti-receptor binding domain (RBD) IgAs, whose levels were further boosted by the second immunization dose in naive individuals (Figure 1B). Although subjects recovered from COVID-19 showed higher levels of IgA than naive participants after the first dose, the concentrations after the second vaccination shot were comparable between both groups and, again, slightly higher in subjects recovered from COVID-19 after almost 8 months post-vaccination (sample 3) (Figure 1B). The analysis of IgGs (anti-spike S1, anti-RBD, and anti-full spike) in sample 0 confirmed that the subjects recovered from COVID-19 had been previously exposed to SARS-CoV-2, and these participants showed higher levels of specific IgGs than naive individuals throughout the observation period (Figure 1C). It is noteworthy that the titers of all the analyzed antibodies dropped in sample 3, but individuals recovered from COVID-19 maintained slightly higher levels (Figures 1B and 1C).

Beyond the Ig concentrations, we evaluated the neutralization capacity of plasma against the spike antigen. The neutralization capacity was measured using a competitive immunoassay. In naive individuals, two doses were required to induce neutralizing antibodies, whereas in recovered individuals, one dose induced high neutralization titers. Of note, after a second dose, subjects recovered from COVID-19 further increased their neutralization activity, which was higher than in naive individuals 14 days after full vaccination (Figure 1D). Note that, although the neutralization capacity was still measurable, the neutralizing antibodies dropped dramatically in sample 3. Importantly, this neutralization ability was similar between naive subjects and individuals recovered from COVID-19 at this long-term post-vaccination time (Figure 1D). To further characterize the differential neutralization capacity conditioned by previous exposure to SARS-CoV-2, a functional assay based on the neutralization of a pseudovirus expressing the spike protein of SARS-CoV-2 was done. We accomplished this analysis in sample 2, the first one where both naive subjects and individuals recovered from COVID-19 showed neutralizing activity. This analysis confirmed that individuals recovered from COVID-19 exhibited a better neutralizing capacity (Figure S1A). Altogether, these data indicated a differential expression pattern of humoral responses between naive individuals and subjects recovered from COVID-19 over time post-vaccination (Figure 1E).

Next, we focused on circulating B cell-derived populations because of their role in humoral responses. Based on a fluorescence-activated cell sorting (FACS) panel of 39 extracellular markers and an unsupervised uniform manifold approximation and projection (UMAP) dimensional reduction followed by manual gating, we identified canonical cell subsets in peripheral blood mononuclear cells (PBMCs) (Figure 1F). Again, we performed this analysis in sample 2, where naive subjects and individuals recovered from COVID-19 showed neutralizing activity. This analysis confirmed that individuals recovered from COVID-19 exhibited a better neutralizing capacity (Figure S1A). Altogether, these data indicated a differential expression pattern of humoral responses between naive individuals and subjects recovered from COVID-19 over time post-vaccination (Figure 1E).
Figure 1. SARS-CoV-2 spike-specific humoral response following BNT162b2 mRNA vaccination in naive subjects and individuals recovered from COVID-19

(A) Experimental design. Blood samples were collected 5 days before BNT162b2 mRNA vaccination (sample 0), 14 days after the first dose (sample 1), and 14 days (sample 2) and 230 days (sample 3) after the second dose.

(B) Concentrations of plasma anti-spike S1 IgA (left panel) and anti-receptor binding domain (RBD) IgA (right panel) antibodies.

(C) Concentrations of plasma anti-spike S1 IgG (left panel), anti-RBD IgG (central panel), and anti-full spike IgG (right panel) antibodies.

(D) Concentration of neutralizing antibodies in plasma by means of a competitive assay; $10^8$/free anti-spike signal is depicted.

(E) Heatmap of $Z$ score of IgA, IgG, and anti-spike neutralizing antibodies.

(F) Uniform manifold approximation and projection (UMAP) of peripheral blood mononuclear cells (PBMCs) followed by manual gating to identify the indicated populations.

(G) UMAP of B cells followed by manual gating to identify the indicated populations in sample 2.

(H) UMAP clustering expressions of HLA-DR, IgD, IgM, and IgG on B cells.

(I) Frequency of SARS-CoV-2 spike-specific B cells in gated CD19+ cells in sample 3.

(B, C, D, and I) Data shown as mean ± SEM (ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001). Unpaired Student’s t-test in samples 0, 1, 2, or 3.

(B, C, and D) Two-way ANOVA analyzing the time course (denoted by vertical bar, †), n = 16 naive, n = 11 recovered from COVID-19.

See also Figures S1, S2, and S3A.
Considering the lack of differences in the levels of neutralizing antibodies between naive subjects and individuals recovered from COVID-19 almost 8 months after complete vaccination (sample 3; Figure 1D), we decided to analyze the levels of SARS-CoV-2 spike-specific B cells in this long-term time point (Figure S3A). Remarkably, all of the participants showed SARS-CoV-2 spike-specific B cells, with comparable levels between groups (Figure 1I).

**Naive individuals show enhanced SARS-CoV-2-specific T cell lymphoproliferative responses early after vaccination but are similar to those recovered from COVID-19 at later time points**

To explore whether previous exposure to SARS-CoV-2 could modulate specific cellular responses against this coronavirus in fully vaccinated individuals, PBMCs from both naive and recovered subjects were ex vivo-exposed to a peptide pool covering the SARS-CoV-2 spike protein, henceforth called the S-peptide (Figure 2A). First, we analyzed the presence of several chemokines and cytokines in culture supernatants after this ex vivo stimulation for 5 days. The production of both CCL-2 and CXCL10 was induced by the SARS-CoV-2 spike peptide pool except for CCL-2 in individuals recovered from COVID-19 almost 8 months post-vaccination (Figure 2B). However, only naive individuals showed a robust induction of most of the cytokines analyzed (interleukin [IL]-2, IL-4, IL-6, IL-10, and tumor necrosis factor alpha [TNF-α]), although this S-peptide-specific response in naive subjects was exclusive to sample 2 (Figure 2C). Interestingly, interferon (IFN)-γ expression showed a specific pattern, mirroring CCL2 production (Figures 2B and 2C).

To further examine this differential outcome, cytokine production was analyzed by intracellular FACS staining. The expression of IL-2, TNF-α, IFN-γ, and granzyme B were consistently induced in CD4+ T cells after ex vivo stimulation with the S-peptide pool in both naive subjects and individuals recovered from COVID-19 early after vaccination (sample 2) (Figures 2D and S4), with less robust responses in CD8+ T cells. However, more than 7 months after vaccination (sample 3), this SARS-CoV-2-specific response was negligible in both groups (Figures 2D and S4).

Interestingly, the analysis of the intracellular cytokine production increment induced by SARS-CoV-2 spike antigen ex vivo stimulation showed a much more intense induction of IL-2 in both CD4+ and CD8+ T cells from naive subjects than from individuals recovered from COVID-19 early after vaccination (sample 2) (Figure 2E). Considering the crucial role of IL-2 in the lymphoproliferative capacity of CD4+ T cells, we decided to analyze this function. Proliferation ability was explored based on carboxyfluorescein succinimidyl ester (CFSE) dilution of total PBMCs after ex vivo stimulation with the S-peptide pool. Both CD4+ and CD8+ T cells proliferated in response to the spike antigen in naive subjects and individuals recovered from COVID-19, although with an apparent stronger effect on CD4+ T cells from naive subjects (Figure 2F). The increment of proliferation between SARS-CoV-2 spike-antigen-stimulated and non-stimulated PBMCs confirmed a more powerful CD4+ lymphoproliferative activity in naive subjects than in individuals recovered from COVID-19, specifically, early after vaccination (sample 2) (Figure 2G).

These data suggest a strong SARS-CoV-2 spike-specific T cell response early after vaccination in naive subjects (but not in COVID-19-recovered individuals) that declines over time.

**SARS-CoV-2-specific effector memory T cell enhanced responses in naive individuals decline along the timeline**

Next, we dissected the SARS-CoV-2 spike-specific T cell responses. First, we analyzed the phenotype of both proliferative (CFSEdim) and non-proliferative (CFSEbright) CD4+ T cells after antigen-specific stimulation (Figure S3B). As expected, this challenge induced the transition from a naive to effector memory (EM) phenotype in proliferated CD4+ T cells (Figures 3A and 3B). Of note, early after vaccination (sample 2), an increase in the frequency of EM re-expressing CD45RA (EMRA) cells was observed, while almost 8 months after vaccination (sample 3), a significant central memory (CM) response was induced (Figures 3A and 3B). Interestingly, the study of these populations in terms of proliferative capacity showed that previous exposure to SARS-CoV-2 had no impact on these transitions along the timeline (Figure 3C). Although in a less robust way, similar behaviors were observed for CD8+ T cells (Figures S5A–S5C).

We next analyzed intracellular cytokine production in CD4+ T cell subpopulations induced by ex vivo SARS-CoV-2 spike peptide pool stimulation. A consistent IL-2 production was observed in naive individuals early after vaccination (sample 2) that was maintained in effector populations (EMRA and EM) in the long term (sample 3) (Figure 3D). However, CD4+ T cells from subjects recovered from COVID-19 did not respond to the SARS-CoV-2 spike peptide pool stimulation at any time (Figure 3D). In line with previous observations, naive individuals showed a stronger increment of IL-2 production in the EMRA and EM population than did subjects recovered from COVID-19 early after vaccination that declined almost 8 months post-vaccination (Figure 3E). Again, despite a less robust response after antigen-specific stimulation, a similar IL-2 expression pattern was observed in CD8+ T cells (Figures S5D and S5E).

These data indicated that previous infections of SARS-CoV-2 dampened T EM cellular responses early after a complete BNT162b2 vaccination. However, almost 8 months post-vaccination, the SARS-CoV-2 spike-specific T responses were comparable between naive subjects and individuals recovered from COVID-19.

**Humoral and cellular activation features are inversely correlated early after vaccination**

Based on the differential behavior of SARS-CoV-2 spike-specific humoral and cellular responses between naive subjects and individuals recovered from COVID-19, we explored whether these features could identify subjects belonging to these two groups, in an unsupervised manner, in samples 2 and 3. To address this question, we performed a clustering analysis based on the different immunological variables studied in this work (Figure 4A). This algorithm generated a clearer discrimination between naive subjects and individuals recovered from COVID-19 in sample 2 than in sample 3 (Figure 4A).

Next, we depicted the correlation between the analyzed variables once they were classified based on their functionality. Along
Figure 2. SARS-CoV-2 spike-specific cellular ex vivo response following BNT162b2 mRNA vaccination in naive subjects and individuals recovered from COVID-19.

(A) Experimental design of the T cell cellular response ex vivo in PBMCs in samples 2 and 3 after stimulation with SARS-CoV-2 spike peptide pool.

(B) CCL-2 and CXCL10 chemokines production.

(C) IL-2, IL-4, IL-6, IL-10, TNFα, and IFNγ production.

(D) Percentage of IL-2+ cells in CD4+ (left panel) and CD8+ (right panel) T cells.

(E) Increment of IL-2+ cells comparing SARS-CoV-2 spike peptide pool-stimulated and non-stimulated CD4+ and CD8+ T cells.

(F) Frequency of proliferative (CFSE dim) CD4+ and CD8+ T cells.

(G) Increment of proliferation comparing SARS-CoV-2 spike peptide pool-stimulated and non-stimulated CD4+ and CD8+ T cells.

(B–G) Each dot represents an individual. (B–D and F) Paired Student’s t test (ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001). (E and G) Mann Whitney test (ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001). n = 16 naive, n = 11 recovered from COVID-19.

See also Figure S4.
these lines, humoral parameters quantified in plasma were confronted to the cellular response after *ex vivo* cellular stimulation with SARS-CoV-2 spike peptide pool (Figure 4B). This representation suggested inverse correlations early after vaccination (sample 2) between humoral and cellular responses, particularly IgG production and neutralization capacity of SARS-CoV-2 spike-specific pro-inflammatory cytokine (CCL2, CXCL10, IFNγ, and IL-2) production and CD4+ T cell proliferation (Figure 4B). However, these correlations were attenuated more than 7 months after vaccination (sample 3) (Figure 4B). The analysis of these correlations confirmed the statistically significant inverse association between IgG-based and cellular responses such as CD4+ T cell proliferation or IL-2 production in sample 2 (Figure 4C). However, no significant correlations were found between these variables in sample 3 (Figure 4C). Altogether, these analyses revealed that early after a complete vaccination regimen with BNT162b2, differential humoral
and cellular responses were triggered between naive subjects and individuals recovered from COVID-19 almost eight months after vaccination, both responses were comparable between both groups.

**DISCUSSION**

The generation of mRNA COVID-19 vaccines such as mRNA-1273 (Baden et al., 2021) and BNT162b2 (Polack et al., 2020) represents a revolution in vaccinology and is one of the key pillars of humanity’s eventual success against the pandemic caused by the SARS-CoV-2 infection. These vaccines are based on a lipid-nanoparticle-encapsulated mRNA encoding the full-length spike protein of the SARS-CoV-2 virus (Corbett et al., 2020; Walsh et al., 2020). The BNT162b2 vaccine was the first COVID-19 vaccine approved for emergency use by both the FDA and the EMA. This approval was based on the results of a clinical trial declaring an efficacy of 95% in preventing COVID-19 after a two-dose regime 21 days apart (Polack et al., 2020). Since then, a global vaccination campaign began aiming to face off the pandemic.

It is worth noting that a medical history of COVID-19 was an exclusion criterion to be enrolled in the above-mentioned clinical trial (Polack et al., 2020). Therefore, the potential effect of a previous infection with SARS-CoV-2 was not anticipated. Here, we have performed a broad analysis of both humoral and cellular responses triggered by BNT162b2 vaccination by comparing naive subjects and individuals recovered from COVID-19 along a timeline after receiving the complete vaccine regime. We performed a massive phenotypic study of PBMCs after vaccination, but more importantly, it was accompanied by the analysis of functional immunological capabilities such as antibody neutralization and
T cell activation and proliferation in response to specific SARS-CoV-2 spike antigens. Notably, our analysis covers responses after only one vaccine dose but also early (14 days) and late (more than 7 months) responses after the complete two-dose vaccination regime.

Previous studies addressing differential responses between naïve subjects and individuals recovered from COVID-19 have focused on the analysis of specific antibodies as a subrogate of vaccine efficacy. In phase 1/2 studies, mRNA immunization with BNT162b2 showed that two doses were requested to elicit high titers of neutralizing antibodies in naive individuals; in contrast, in recovered patients, the first immunization acted as a booster, thus inducing neutralization titers higher than those observed after the full immunization of naïve patients (Mulligan et al., 2020; Walsh et al., 2020). Along the same lines, a pioneer study indicated that individuals with a previous SARS-CoV-2 infection generated stronger humoral responses than infection-naïve subjects after just a single dose of the BNT162b2 vaccine (Prendecchi et al., 2021a). These findings were confirmed in a cohort of volunteers that received either the BNT162b2 or the mRNA-1273 vaccine (Krammer et al., 2021) but also in individuals receiving only one shot of the vaccine based on the SARS-CoV-2 spike protein-expressing adenovirus (Sasikala et al., 2021; Voysey et al., 2021). Nevertheless, follow-up studies showed that anti-spike SARS-CoV-2 IgGs titers were comparable between naïve subjects and individuals recovered from COVID-19 after 11 to 21 days of the complete two-dose regime (Ebing et al., 2021; Levi et al., 2021). Of note, our data on early humoral responses support these findings. We also observed that antibody levels and the neutralizing capability of plasma from individuals recovered from COVID-19 were higher than that of naïve subjects early after vaccination (samples 1 and 2), an effect suggested but not fully analyzed in a previous study (Gobbi et al., 2021). However, more than 7 months after vaccination, individuals recovered from COVID-19 still showed higher antibody titers but comparable neutralizing antibodies to naïve subjects. These data highlight the need to discriminate between antibody titers and neutralizing capacity. In addition, the analysis of this neutralizing capacity against specific SARS-CoV-2 variants of concern (Carreno et al., 2021; Noori et al., 2021) would improve our understanding about the breadth of the vaccine-conferred protection.

The relevance of cellular responses after SARS-CoV-2 virus infection have been studied (Grifoni et al., 2020; Ni et al., 2020), but data regarding how previous exposure to SARS-CoV-2 impacts these immunogenic responses along the timeline after vaccination are still scarce. Memory B cell responses one week after vaccination were boosted in individuals recovered from COVID-19 after just one shot of the mRNA vaccine, while naïve subjects required two doses to reach comparable memory B cell levels (Goel et al., 2021). This response mirrors the production of SARS-CoV-2 spike-specific antibodies early after vaccination, as previously discussed.

The analysis of the vaccine-induced humoral responses 7 months after the complete vaccination regime showed a drop in antibody titers in the long term, in accordance with other studies (Doria-Rose et al., 2021; Naaber et al., 2021), along with a marked decrease in the neutralizing capacity, reaching comparable low levels in both naïve subjects and individuals recovered from COVID-19. Of note, these data do not necessarily indicate the lack of specific protection against the SARS-CoV-2 virus because, in line with other studies (Ciabattini et al., 2021; Turner et al., 2021), we detected circulating SARS-CoV-2 spike protein-specific B cells even more than 7 months after full vaccination. Importantly, the levels of these B cells were comparable between naïve subjects and participants recovered from COVID-19. Considering that SARS-CoV-2 spike-specific memory B cells showed a switch to an anti-RBD neutralizing phenotype (Sokal et al., 2021), it is tempting to speculate that long-term protection already documented for the BNT162b2 vaccine (Thomas et al., 2021) is warranted, based at least in part on the restimulation of these cells during a SARS-CoV-2 reinfection. Future studies will shed light on the efficacy of this specific protective mechanism.

In our study, we analyzed SARS-CoV-2 spike-specific responses in T cells after restimulation with a peptide pool covering this antigen. This assay showed a differential response between naïve subjects and individuals recovered from COVID-19 early after vaccination, with a more pronounced activation of CD4+ T cells in naïve subjects. This was revealed by a higher induction of cytokine production and proliferation after restimulation, particularly in EM cells. The mechanistic implication of regulatory T cells (Tregs) in this effect (Campbell and Koch, 2011) deserves further studies, as we observed higher levels of this immunomodulatory population in individuals recovered from COVID-19 at this early time point after vaccination. Of note, high levels of SARS-CoV-2 spike-specific CD4+ T cells correlate with a lower COVID-19 predisposition (Sattler et al., 2020), stressing the relevance of a robust cellular response. Of note, previously published data indicated a reduction of the SARS-CoV-2-specific T cell-mediated responses along the timeline after vaccination (Guerrera et al., 2021). Along these lines, we observed that T cell responses dropped to comparable levels in both naïve subjects and individuals recovered from COVID-19.

Our data point toward boosted T cell responses in naïve individuals early after complete BNT162b2 vaccination in a scenario of reduced humoral reactions such as lower SARS-CoV-2 spike-specific IgGs titers and neutralizing capabilities. This concerted response allowed an unsupervised clustering of naïve subjects and individuals recovered from COVID-19 that anticipated inverse correlations between cellular and humoral immune responses. This is relevant, as it is known that cellular immunity may contribute to protection against SARS-CoV-2 infection if antibody responses are suboptimal (McMahan et al., 2021). Therefore, our data suggest that this differential mechanism could take place early after vaccination in naïve individuals compared with in subjects recovered from COVID-19. However, more than 7 months after vaccination, humoral and cellular responses dropped similarly in both naïve subjects and individuals recovered from COVID-19, showing no correlations. Still, memory SARS-CoV-2 spike-specific B cells were present at comparable levels in both groups, suggestive of an equivalent long-term protection mechanism (Ciabattini et al., 2021; Turner et al., 2021).
In summary, our data indicate that concerted humoral and cellular responses over time after vaccination should be considered to define vaccination regimes against COVID-19. This notion could apply to proposals such as the delay of the second vaccination dose (Kadire et al., 2021), the administration of just one shot to a population previously infected with SARS-CoV-2 (Goel et al., 2021), or of a third boosting dose (Mahase, 2021).

Limitations of the study
Sample size is a limitation of this study. Considering the high number of immune variables analyzed and their complexity, we decided to perform our study with a not-so-large but well-controlled cohort of participants. We believe that this approach allowed us to reach clear conclusions, but a multicentre cohort with a larger number of patients would be desirable. Furthermore, all stimulations and detections of SARS-CoV-2-specific responses have been performed against the original S-protein. The analysis of such responses against SARS-CoV-2 variants of concern would expand the relevance of our study. Finally, mechanistic studies would help to explain the divergent responses observed between naive subjects and individuals recovered from COVID-19.

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AUTHOR CONTRIBUTIONS
E.L.-C., C.d.F., R.L.-R., J.A.-O., and L.A.A. designed the study. E.L.-C. and C.d.F. wrote the manuscript. A.M.-Q., M.A.G.-G., A.d.d.-C., M.P., L.G., I.L.-F., G.M.-M., and C.H.-B. recruited the participants and collected the samples. R.L.-R., J.A.-O., J.V.-Q., K.M.-H., J.C.C.-D., M.B.-G., and V.T. performed the analysis and the immunological biomarkers quantification. J.M.B. and N.R. provided the know-how and critical reagents for the intracellular FACS staining. J.V.-Q. and C.d.F. performed the UMAP analysis. J.A., J.G.-P., A.C., C.V.-O., and L.L.-M. performed design and neutralization testing. A.P.-I. performed the clustering heatmap analysis. E.L.-C., C.d.F., R.L.-R., J.A.-O., J.V.-Q., C.C.-Z., and L.A.A. discussed the results. R.L.-R., J.A.-O., J.V.-Q., and L.A.A. performed a critical review of the manuscript. All authors read and agreed to submit the manuscript for publication.

DECLARATION OF INTERESTS
The authors declare no competing interests.

INCLUSION AND DIVERSITY
We worked to ensure gender balance in the recruitment of human subjects. We worked to ensure ethnic or other types of diversity in the recruitment of human subjects. We worked to ensure that the study questionnaires were prepared in an inclusive way. One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in science. One or more of the authors of this paper self-identifies as a member of the LGBTQ+ community. One or more of the authors of this paper received support from a program designed to increase minority representation in science. The author list of this paper includes contributors from the location where the research was conducted who participated in the data collection, design, analysis, and/or interpretation of the work.

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-human CD45RA BUV395 (clone 5H9) | BD | Cat# 740315; RRID:AB_2740052 |
| Anti-human CD16 BU496 (clone 3G8) | BD | Cat# 612944; RRID:AB_2870224 |
| Anti-human CCR5 BUV563 (clone 3A9) | BD | Cat# 741401; RRID:AB_2870893 |
| Anti-human CD62L BUV615 (clone SK11) | BD | Cat# 751364; RRID:AB_2875371 |
| Anti-CD11c BU661 (clone B-Ly6) | BD | Cat# 612967; RRID:AB_2870241 |
| Anti-human CCR7 BUB737 (clone 2-L1-A) | BD | Cat# 749676; RRID:AB_2873937 |
| Anti-human CD56 BUB737 (clone NCAM 16.2) | BD | Cat# 612766; RRID:AB_2813880 |
| Anti-human CD8 BU805 (clone SK1) | BD | Cat# 612889; RRID:AB_2833078 |
| Anti-human IgD BV480 (clone IA6-2) | BD | Cat# 566138; RRID:AB_2739536 |
| Anti-human IgG BV605 (clone G18-145) | BD | Cat# 563246; RRID:AB_2738932 |
| Anti-human CXCR5 BV750 (clone RF8B2) | BD | Cat# 747111; RRID:AB_2871862 |
| Anti-human CD141 BB515 (clone 1A4) | BD | Cat# 566017; RRID:AB_2739462 |
| Anti-human CD127 APC/R700 (clone HIL-7R-M21) | BD | Cat# 565185; RRID:AB_2739099 |
| Anti-human IL-2 APC/R700 (clone MQ1-17H12) | BD | Cat# 565136; RRID:AB_2739079 |
| Anti-human CD163 BB790 (clone GHI/61) | BD | Cat# 624296; RRID:AB_2214940 |
| Anti-human NKG2C BB700 (clone 134519) | BD | Cat# 748162; RRID:AB_2872623 |
| Anti-human CD123 SuperBright436 (clone 6H6) | ThermoFisher Scientific | Cat# 62-1239-42; RRID:AB_2662727 |
| Anti-human CD161 eFluor 450 (clone HP-3G10) | ThermoFisher Scientific | Cat# 48-1619-42; RRID:AB_10854273 |
| Anti-human CD8 Pacific Orange (clone 3B5) | ThermoFisher Scientific | Cat# MHCD0380; RRID:AB_10372066 |
| Anti-human CD20 Pacific Orange (clone 2H7) | ThermoFisher Scientific | Cat# MHCD2030; RRID:AB_10375578 |
| Anti-human TCRγδ PerCP-eFluor 710 (clone B1.1) | ThermoFisher Scientific | Cat# 46-9959-42; RRID:AB_2573926 |
| Anti-human CD25 PE-AlexaFluor 700 (clone CD25-3G10) | ThermoFisher Scientific | Cat# MHCD2524; RRID:AB_2539740 |
| Anti-human CCR7 BV421 (clone G043H7) | Biolegend | Cat# 353208; RRID:AB_11203894 |
| Anti-human CD3 BV510 (clone OKT3) | Biolegend | Cat# 317332; RRID:AB_2561943 |
| Anti-human CD3 BV570 (clone UCHT1) | Biolegend | Cat# 300436; RRID:AB_2562124 |
| Anti-human CD4 BV570 (clone RPA-T4) | Biolegend | Cat# 300534; RRID:AB_2563791 |
| Anti-human CD4 cFluor-YG584 (clone K3) | Cytek Biosciences | Cat# R7-20042; RRID:AB_2885083 |
| Anti-human IgM BV570 (clone MHM-88) | Biolegend | Cat# 314517; RRID:AB_10913816 |
| Anti-human CD28 BV650 (clone CD28.2) | Biolegend | Cat# 302946; RRID:AB_2616855 |
| Anti-human TNFα BV660 (clone MAb11) | BD | Cat# 563418; RRID:AB_2738194 |
| Anti-human CCR6 BV711 (clone G034E3) | Biolegend | Cat# 353436; RRID:AB_2629608 |
| Anti-human IFNγ BV711 (clone 4S.B3) | Biolegend | Cat# 502540; RRID:AB_2563506 |
| Anti-human PD-1 BV785 (clone E12.2H7) | Biolegend | Cat# 329929; RRID:AB_11218984 |
| Anti-human CD57 FITC (clone HNK-1) | Biolegend | Cat# 359604; RRID:AB_2562387 |
| Anti-human CD3 SparkBlue550 (clone SK7) | Biolegend | Cat# 344852; RRID:AB_2819985 |

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Anti-human CD14 SparkBlue550 (clone 63D3) | Biolegend | Cat# 367148; RRID:AB_2832724 |
| Anti-human CD45 PerCP (clone 2D1) | Biolegend | Cat# 368506; RRID:AB_2566358 |
| Anti-human CD4 PerCP/Cy5.5 (clone SK3) | Biolegend | Cat# 344608; RRID:AB_1953236 |
| Anti-human CD11b PerCP/Cy5.5 (clone ICRF44) | Biolegend | Cat# 301328; RRID:AB_10933428 |
| Anti-human PD-L1 PE (clone 10F.9G2) | Biolegend | Cat# 124308; RRID:AB_2073556 |
| Anti-human CD24 PE/Dazzle594 (clone ML5) | Biolegend | Cat# 311134; RRID:AB_2566349 |
| Anti-human CD95 PE/Cy5 (clone DX2) | Biolegend | Cat# 305610; RRID:AB_314548 |
| Anti-human CXCR3 PE/Cy7 (clone G025H7) | Biolegend | Cat# 353720; RRID:AB_11219383 |
| Anti-human Granzyme B PE/Cy7 (clone QA16A02) | Biolegend | Cat# 372214; RRID:AB_2728381 |
| Anti-human CD1c AlexaFluor647 (clone L161) | Biolegend | Cat# 331510; RRID:AB_1186032 |
| Anti-human CD27 APC (clone M-T271) | Biolegend | Cat# 356410; RRID:AB_2561957 |
| Anti-human CD19 SparkNIR685 (clone HIB19) | Biolegend | Cat# 302270; RRID:AB_2832581 |
| Anti-human HLA-DR APC/Fire750 (clone L243) | Biolegend | Cat# 307658; RRID:AB_2572101 |
| Anti-human CD38 APC/Fire810 (clone HIT2) | Biolegend | Cat# 303550; RRID:AB_2860784 |

### Biological samples

| Blood samples of Healthy Health Personnel | This paper | N/A |

### Chemicals, peptides, and recombinant proteins

| Ficoll-Plus | GE Healthcare | Cat# 17-1440-03 |
| RPMI 1640 Medium | Thermo Fisher Scientific | Cat# 11594506 |
| DMEM Medium | Thermo Fisher Scientific | Cat# 11965092 |
| Phosphate buffer saline (PBS) | Sigma | Cat# P4417-100TAB |
| Bovine serum albumin (BSA) | Sigma | Cat# A9647-1KG |
| Foetal Bovine Serum (FBS) | Thermo Fisher Scientific | Cat# 11560636 |
| Carboxyfluorescein succinimidyl ester (CFSE) | Thermo Fisher Scientific | Cat# C34554 |
| PepTivator SARS-CoV-2 Prot_S | Miltenyi Biotec | Cat# 130-126-701 |
| Protein Transport Inhibitor (Containing Brefeldin A) | BD | Cat# 555029 |
| Protein Transport Inhibitor (Containing Monensin) | BD | Cat# 554724 |
| LIVE/DEAD Fixable Blue Dead Cell Stain Kit | Thermo Fisher Scientific | Cat# L34962 |
| True –Stain Monocyte Blocker | Biolegend | Cat# 426103 |
| Brilliant Stain Buffer | BD | Cat# 566349 |
| Dimethyl sulfoxide (DMSO) | Sigma | Cat# 67-68-5 |

### Critical commercial assays

| Cytofix/Cytoperm Fixation/Permeabilization Kit | BD | Cat# 554714 |
| COVID-19 (SARS-CoV-2) quantitative IgG ELISA | Demeditec | Cat# DECOV1901Q |
| LEGENDplex SARS-CoV-2 Serological IgA Panel (2-plex) | Biolegend | Cat# 741139 |
| LEGENDplex SARS-CoV-2 Serological IgG Panel (3-plex) | Biolegend | Cat# 741131 |

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RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Eduardo López-Collazo (elopezc@salud.madrid.es).

Material availability
This study did not generate new unique reagents.

Data and code availability
- Data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Healthy health personnel volunteers and longitudinal samples
A total of 27 healthy health personnel volunteers of the Research Institution of La Paz University Hospital of Madrid (Spain) were enrolled for this study before vaccination against Spike protein of SARS-CoV-2 (BNT162b2 SARS-CoV-2 mRNA vaccine of Pfizer & BioNTech). Blood samples were taken at four times: 5 days before the vaccination (sample 0), 14 days after the first dose of vaccine (sample 1), 14 days after the second dose of vaccine (sample 2) and 230 days after the second dose (sample 3) (Figure 1A). Informed consent was obtained from all volunteers in accordance with the ethical standards and following the ethical guidelines of the 1975 Declaration of Helsinki. All healthy health personnel data were anonymized before study inclusion and their details are summarized in Table S1.

Culture conditions of primary and Vero E6 cells
Fresh and thawed Peripheral Blood Mononuclear Cells (PBMCs) were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 25 mM HEPES, 2 mM L-glutamine and 1% Penicillin and Streptomycin Mix (Gibco) before some stimulation to their activation or proliferation. PBMCs were cultured at 37 °C at 5% CO2 in a humidified incubator. Vero E6 were cultured in DMEM containing 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1% Penicillin and Streptomycin Mix (Gibco).
**METHOD DETAILS**

**PBMCs isolation, storage and thawing procedure**
Peripheral blood mononuclear cells (PBMCs) from healthy health personnel vaccinated with BNT162b2 SARS-CoV-2 mRNA vaccine were isolated from EDTA anticoagulant venous blood using Ficoll-Plus (GE Healthcare Bio-Sciences) solution according to the manufacturer’s instructions. PBMCs were washed twice with phosphate buffer saline (PBS) and counted using Trypan blue staining. A part of cells was resuspended in two aliquots of 6 x 10^8 cells in fetal bovine serum (FBS) containing 10% DMSO (Sigma-Aldrich). Then, aliquoted PBMCs were slowly frozen (−1° C/minute) using a controlled-grade freezing device (Mr. Frosty, ThermoFisher Scientific) and stored for 24 hours at −80° C before storage in liquid nitrogen. For use PBMCs, they were rapidly thawed in a water bath at 37° C and washed twice with RPMI 1640 medium containing 10% fetal bovine serum (FBS), 25 mM HEPES, 2 mM L-glutamine and 1% Penicillin and Streptomycin Mix (GenScript, Gibco).

**Plasma collection**
Plasma samples from healthy health personnel vaccinated with the Pfizer vaccine against SARS-CoV-2 were obtained from EDTA anticoagulant venous blood using Ficoll-Plus (GE Healthcare Bio-Sciences) solution according to standard density gradient centrifugation method. Then, they were aliquoted and stored at −80° C until use.

**Algorithm for dimensionality reduction**
Uniform Manifold Approximation and Projection (UMAP) analysis was carried out using all markers listed in Table S2. Data were manually gated to remove aggregates, dead cells, debris, and CD45 negative events, and then they were sub-sampled to include 60% of CD45+ live singlets from each sample. Subsequently, the UMAP analysis was performed to visualize the different subpopulations in groups. CD3+ and CD19+ subpopulations were defined as CD3+/TCRε4/CD56−/CD14−/CD4+ or CD8+, and CD3+/CD56−/CD14−/CD19−/CD20+, respectively, prior to the UMAP analysis. UMAP settings for CD3+ subpopulation used CD45RA, CD28, CCR7, PD-1, CD27, CD57, CD127, CD25, CD95, CD38 and HLA-DR fluorescent parameters. UMAP settings for CD19+ subpopulation used CD38, CD27, CD19, CD24, IgD, IgM, IgG and CD20 fluorescent parameters. All fluorescent parameters were used besides lived and CD45+ cells. UMAP was run using 15 nearest neighbors, a minimal distance of 0.5, in 2-dimensions and spectral initialization mode (McInnes et al., 2020). Data were analyzed using FlowJo (TreeStar) v10.6.2 software.

**Detection of anti-spike IgA and IgG SARS-CoV-2 antibodies**
For detection of specific antibodies IgA and IgG against the Spike protein of SARS-CoV-2, reserved plasma samples from healthy health personnel vaccinated with the Pfizer vaccine against SARS-CoV-2 stored at −80° C were thawed and centrifuged at 1000 relative centrifugal force for 30 minutes to remove particulates prior to use. The title of IgA antibodies in plasma samples were performed by the bead-based multiplex assay, LEGENDplex SARS-CoV-2 Serological IgA Panel (2-plex, Spike (S1) and receptor binding domain (RBD) of Spike protein) (Biolegend) according to the manufacturer’s instructions. The title of IgG antibodies in plasma samples were performed by the bead-based multiplex assay, LEGENDplex SARS-CoV-2 Serological IgG Panel (3-plex, Spike (S1), receptor binding domain (RBD) of Spike protein and nucleocapsid (N)) (Biolegend) according to the manufacturer’s instructions. Samples were acquired on a FACSCalibur flow cytometer (BD Biosciences) and data were analyzed using LEGENDplex (Biolegend) v.8 software. For quantification of IgG antibodies against full Spike protein of SARS-CoV-2, COVID-19 quantitative IgG ELISA kit from Demeditec Diagnostics GmbH (Ref.: DECOV1901Q) was used. Data obtained were corroborated by Eurofins-Ingenasa kits: INGE-ZIM®-NP-COVID 19 DR and INGEZIM®-RBD-COVID 19 DR.

**Detection of neutralization capacity of plasma against SARS-CoV-2 Spike antigen**
The neutralizing antibodies in plasma samples were performed by a competitive immunoassay of ACE2-conjugated beads, LEGENDplex SARS-CoV-2 Neut. Ab Assay (1-plex) according to the manufacturer’s instructions.

To measure neutralising antibodies titres by means of viral pseudoparticles, diluted plasma samples were preincubated with pseudoviruses generated by co-transfection of the plasmid pNL4–3ΔenvRen and an expression vector for the viral SARS-CoV-2 Spike (pcDNA3.1-ScOvu2Δ19-D614) and added at the concentration of 10 ng p24Gag per well to Vero E6 cells in 96-well plates. At 48 h post infection, viral infectivity was assessed by measuring luciferase activity (Renilla Luciferase Assay (Promega, Madison, WI, USA) using a 96-well plate luminometer LB 960 Centro XS (Berthold Technologies, Oak Ridge, TN, USA). The titre of neutralising antibodies was calculated as 50% inhibitory dose (neutralising titre 50, NT50), expressed as reciprocal of four-fold serial dilution of heat-inactivated sera (range 1:32–1:8192), resulting in a 50% reduction of pseudovirus infection compared with control without serum. Samples below the detection threshold (1:32 serum dilution) were given 1:16 value. Positive and negative controls were included in the assay and non-specific neutralisation was assessed using a nonrelated pseudovirus expressing the vesicular stomatitis virus envelope.

**Antibodies and immunophenotyping by flow cytometry**
Stored PBMCs were thawed as we have described above and they were rested in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 25 mM HEPES, 2 mM L-glutamine and 1% Penicillin and Streptomycin Mix (Gibco) for 1 hour previous the staining
protocol. Then, PBMCs were stained with fluorochrome-conjugated antibodies to a multi-colour panel of surface markers listed in Table S2. Dead cells were excluded using LIVE/DEAD Blue fluorescent reactive dye purchased from Invitrogen and True-Stain Monocyte Blocker (BioLegend) reagent was added prior to the label protocol to block the nonspecific binding of some fluorochromes on monocytes. Labeled cells were acquired on a Cytek Aurora Spectral Cytometer (Cytek Biosciences). Data were analyzed using FlowJo (TreeStar) v10.6.2 software.

**SARS-CoV-2 Spike-specific B cells detection**

SARS-CoV-2 Spike-specific B cells were detected in sample 3 by means of the SARS-CoV-2 Spike B cell analysis kit provided by Miltenyi Biotec, following the manufacturer’s instructions. Labeled cells were acquired on a Cytek Aurora Spectral Cytometer (Cytek Biosciences). Data were analyzed using FlowJo (TreeStar) v10.6.2 software.

**SARS-CoV-2 Spike-specific T cell proliferation assays and supernatant collection**

Fresh PBMCs from healthy health personnel 14 (sample 2) and 230 (sample 3) days after the second dose of BNT162b2 SARS-CoV-2 mRNA vaccine, stimulated with Peptivator SARS-CoV-2 Prot_S (Miltenyi Biotec) for 5 days, were thawed. The concentration measurements of cytokines in supernatant samples was performed by the bead-based multiplex assay, LEGENDplex Human Essential Immune Response Panel (13-plex: IL-1β, IL-2, IL-4, IFN-γ, TNF-α, MCP-1 (CCL2), CXCL10, IL-6, IL-8 (CXCL8), IL-10, IL-12p70, IL-17A and Free Active TGF-β1), according to the manufacturer’s instructions. Samples were acquired on a FACSCalibur flow cytometer (BD Biosciences) and data were analyzed using LEGENDplex (BioLegend) v.8 software.

**PBMCs stimulation and intracellular cytokine staining**

Thawed PBMCs were stimulated with Peptivator SARS-CoV-2 Prot_S (Miltenyi Biotec) consisting in a pool of 15-mer sequences with 11 amino acids overlap covering the immunodominant sequence domains of the Spike glycoprotein of SARS-CoV-2. Incubation was performed for 6 hours at 37°C 5% CO2 in presence of Golgi-Plug containing Brefeldin A (BD) and Golgi-Stop containing Monensin (BD) added after 1 hour of the stimulation according to the manufacturer’s instructions. After that, PBMCs were washed and stained with the surface markers (listed in Table S3) for 30 minutes at room temperature, twice washed, fixed and permeabilized using the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD) and stained with fluorochrome-conjugated antibodies to multi-colour panel of surface markers listed in Table S3. Dead cells were excluded using LIVE/DEAD Blue fluorescent reactive dye purchased from Invitrogen and True-Stain Monocyte Blocker (BioLegend) reagent was added prior to the label protocol to block the nonspecific binding of some fluorochromes on monocytes. Labeled cells were acquired on a Cytek Aurora Spectral Cytometer (Cytek Biosciences). Data were analyzed using FlowJo (TreeStar) v10.6.2 software.

**SUPPLEMENTARY MATERIALS**

**SUPPLEMENTARY TABLES**

Table S2. Dead cells were excluded using LIVE/DEAD Blue fluorescent reactive dye purchased from Invitrogen and True-Stain Monocyte Blocker (BioLegend) reagent was added prior to the label protocol to block the nonspecific binding of some fluorochromes on monocytes. Labeled cells were acquired on a Cytek Aurora Spectral Cytometer (Cytek Biosciences). Data were analyzed using FlowJo (TreeStar) v10.6.2 software.

**SUPPLEMENTARY FIGURES**

Figure S1. SARS-CoV-2 Spike-specific T cell proliferation assays and supernatant collection.

**SUPPLEMENTARY METHODS**

**SUPPLEMENTARY REFERENCES**

Statistical analysis of biological data

Data are expressed as mean ± SEM, and single dots representing an individual subject each. Student’s t-test for two groups comparison of quantitative variables, either unpaired (t-test or Mann-Whitney) or paired (t-test or Wilcoxon), and ANOVA or Kruskal-Wallis for multiple groups comparisons of quantitative variables were performed. Correlation between quantitative variables were evaluated by Spearman’s analysis. All along figures, p-values (P) are denoted as ns: non-significant, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

In order to perform a visual correlation analysis between the expression of different immune factors, and COVID-19 history of the subjects, the raw data of each one was normalized using the Z-Score strategy [(value-μ)/σ]. The hierarchical clustering analysis was developed by heatmap, geom tile and ggplot2 packages (version 1.16.0) in R language (version 4.0.2). This package is available at https://www.r-graph-gallery.com/heatmap. The clustering was analyzed and distributed by average linkage method, in which the distance between two clusters is defined as the mean of distances between all pairs of objects, where each pair is made up of one object from each group. Measurement method between rows and columns was performed by Manhattan method.