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The SARS-CoV-2 spike residues 616/644 and 1138/1169 delineate two antibody epitopes in COVID-19 mRNA COMINARTY vaccine (Pfizer/BioNTech)

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Key-words: coronavirus, COVID-19, SARS-CoV-2, mRNA vaccine, spike protein, immunity, antibody, B cell epitope, synthetic peptide, protein-peptide conjugate, ELISA, immunization, mouse model.

Running Title: Linear antibody epitopes in COMINARTY vaccine

ABSTRACT

The newly identified coronavirus SARS-CoV-2 is responsible for the worldwide pandemic COVID-19. Considerable efforts have been made for the development of effective vaccine strategies against COVID-19. The SARS-CoV-2 spike protein has been assigned as major antigen candidate for the development of COVID-19 vaccines. The COVID-19 mRNA BNT162b2 vaccine (COMIRNATY, Pfizer/BioNTech) is a lipid nanoparticle-encapsulated mRNA encoding a full-length and prefusion-stabilized SARS-CoV-2 spike protein. In the present study, synthetic peptide-based ELISA assays were performed to identify linear B cell epitopes that contribute to elicitation of antibody response in vaccinated individuals with COMIRNATY. The synthetic S2P6 peptide containing the spike residues 1138/1169 and to a lesser extent, the synthetic S1P4 peptide containing the spike residues 616/644 were recognized by the immune sera from COMIRNATY recipients but not COVID-19 recovered patients. The S2P6 peptide has been identified as immunogenic peptide in adult BALB/c mice that received protein-peptide conjugates in a prime-boost schedule. Based on our data, we propose that the synthetic S2P6 peptide and to a lesser extent the synthetic S1P4 peptide, would be of interest to measure the dynamic of antibody response to COMIRNATY vaccine. The synthetic S2P6 peptide is a SARS-CoV-2 spike peptide candidate for the development of peptide-based vaccines against COVID-19.

INTRODUCTION

SARS-CoV-2 virus is an enveloped RNA virus belonging to betacoronavirus genus of Coronoviridae family (1). The COVID-19 disease associated with the emerging SARS-CoV-2 infection is a pandemic public health threat since early 2020 with millions of deaths to date (1). The main route for SARS-CoV-2 transmission is through respiratory droplets necessitating the implementation of effective control measures (2-6). Convalescent COVID-19 patients develop neutralizing anti-SARS-CoV-2 antibodies produced by the antiviral immune response (7-9). The structural spike protein playing a crucial role in eliciting the immune response during COVID-19 disease has been considered as the predominant viral antigen target for vaccine development (10-13). The transmembrane S-protein spike homo-trimer at the virus surface mediates receptor binding through interaction with cell entry receptor ACE2 (14-18). The trimeric S protein contains the S1 and S2 subunits (10, 11, 14). The N-terminal S1 subunit which comprises the Receptor Binding Domain (RBD) is able to bind ACE2 and anti-RBD antibodies exert potent neutralizing activity against SARS-CoV-2 (10, 11, 13, 14). The C-terminal S2 subunit containing the fusion elements is responsible for viral membrane fusion with host-cell membranes (10, 11, 14). A furin-like cleavage site has been identified at the S1/S2 junction playing a major role in the pathogenesis of SARS-CoV-2 infection (18-20). The virus binding to ACE2 receptor via the RBD elicits the proteolytic cleavage of pre-protein S into S1 and S2 subunits by cellular proteases (16, 18). Such processing results in large conformational changes causing S1 shedding and exposure of the fusion elements in S2 which trigger fusion process between viral and host-cell membranes (10, 11, 21, 22).

Accelerated vaccine programs for COVID-19 prevention have led to the development of commercially available vaccines targeting the SARS-CoV-2 spike protein (23-25). To date, the most performing current vaccines are based on nucleoside modified mRNA strategy (26-28). The commercialized COVID-19 mRNA BNT162b2 known as COMIRNATY vaccine (Pfizer/BioNTech) is a lipid nanoparticle-encapsulated mRNA encoding a full-length and prefusion-stabilized S protein (29, 30). BNT162b2 (COMIRNATY) vaccine is administered in two doses, 21 days apart. The approval of this candidate vaccine has been based on a large group of individuals who had been immunized with BNT162b2 in an international and placebo-controlled phase III trial (29, 30). Vaccinated individuals developed high neutralizing SARS-CoV-2 antibody titers which are indicators of COVID-19 protection

(29, 30). The efficacy study demonstrated that the COVID-19 mRNA vaccine candidate induced 95% protection against COVID-19 in a 2-dose regimen (29, 30).

Serological tests on COVID-19 patients identified immunodominant antibody epitopes in SARS-CoV-2 spike protein (31-34). Analysis of SARS-CoV-2 S protein by IEDB resource (for The Immune Epitope Database and Analysis Resource) has led to predict immunodominant antibody epitopes exposed to the spike protein surface (35). To our knowledge, there has been little information on specificities of antibodies induced by COMIRNATY vaccine. With the aim to design peptide-based vaccine candidates for COVID-19 prevention, we were interested in identifying continuous B cell epitopes which are potentially targets of anti-spike antibodies in COMIRNATY-vaccinated individuals. In the present study, we identified a limited number potential linear antibody epitopes spanning the two S1 and S2 subunits of SARS-CoV-2 spike protein using a B cell epitope prediction tool available on the IEDB server. A set of synthetic peptides representing potential linear B cell epitopes in the spike protein was synthesized. Synthetic peptide-based ELISA tests were performed with COMIRNATY recipient sera to identify candidate linear antibody epitopes in the SARS-CoV-2 spike protein.

RESULTS

Synthetic peptides representing potential linear B-cell epitopes in the spike protein

To search for possible B cell epitopes in SARS-CoV-2 spike protein, we used a predictor tool available in IEDB website. The predictor tool BepiPred-2.0 can generate a score for potential B cell epitopes and we have submitted the spike protein sequence. Thirty-four sequences were identified as potential linear B cell epitopes using 0.50 as cutoff parameter (Table S1). Although B cell epitope for antibody recognition usually spans 3 to 8 continuous amino acids, we decided to prioritize candidate epitopes which sequence lengths at least 19 amino-acid residues. Such long, continuous linear epitopes should be more appropriate to retain conformational information related to the efficiency of antibody binding. Thus, sequences n°1, 11, 12, 18, 19 and 31 (Table S1) were selected to generate a set of synthetic peptides representing potential SARS-CoV-2 spike epitopes (Table 1). As sequence n°18 ($_{166}$NCTEVPVAIHADQLTPT$_{232}$) is directly adjacent to sequence n°19 ($_{234}$RVYSTGSNVFQ$_{244}$), we decided to generate a synthetic 28-mer peptide containing the spike residues 166/244. The six synthetic

peptides spanning 19 to 32 continuous amino-acid residues are located into the S1 and S2 subunits of SARS-CoV-2 spike protein (Fig. 1). The synthetic S1P1 to S1P5 peptides map into the N-terminal domain (NTD), receptor binding domain (RBD), and C-terminal domain 2 (CTD2) sequences of the S1 subunit whereas the synthetic S2P6 peptide spans the connector domain (CD) and heptad repeat-2 (HR2) sequences into the C-terminal region of the S2 subunit.

The Alpha, Beta, Gamma and now Delta variants of SARS-CoV-2 have been recently classified as VOC (Variants of Concern) by World Health Organization. Given that VOC display an increased transmissibility and/or change on viral pathogenicity, we decided to generate S1P1$^{[S]}$, S1P3$^{[N]}$, and S1P5$^{[H]}$ peptide variants bearing amino-acid substitutions that have been recently identified in Gamma, Beta, or Alpha variant of SARS-CoV-2, respectively (Table 1). The Delta variant with amino-acid substitutions L452R, T478K, D614G, P681R, and D950N is becoming a dominant SARS-CoV-2 strain worldwide. We noted that amino-acid changes in Delta variant have no effect on the S1P1, S1P2, S1P3, S1P4 and S2P6 peptide sequences. S1P5 peptide which spans the S1/S2 cleavage site was the only peptide sequence affected by Delta variant. However, S1P5 peptide was not mutated with the amino-acid change P681R from the Delta variant. Thus, only the S1P5$^{[H]}$ peptide variant was used for further analysis.

Antigenic reactivity of synthetic peptides with COMIRNATY recipient sera

To evaluate the antibody response against SARS-CoV-2 spike protein in COMIRNATY-vaccinated individuals, we developed an indirect ELISA assay based on soluble recombinant RBD protein (rRBD) produced from transfected CHO cells. A recombinant soluble SARS-CoV-2 nucleoprotein N protein (rN) was also produced as control antigen for natural infection. Both both rN and rRBD were preceded by a heterologous signal peptide and ended by the IgG2A high chain region as C-terminal protein tag. The secreted rN and rRBD proteins from transient transfected CHO cells were detected by immunoblot assay (Fig. 2A). Soluble rN and rRBD proteins were quantified by direct ELISA assay using goat anti-mouse IgG heavy chain HRP antibody. Serum samples from COVID-19 immune subjects (Table S2) and infection-naive individuals (Table S3) were used to validate the antigenic reactivity of rN and rRBD proteins by indirect ELISA (Fig. 2B).

The antigenic reactivity of synthetic peptides was evaluated with the serum sample from an infection-naive individual who has been fully vaccinated with COMIRNATY in April 2021. The immune reactivity of COMIRNATY recipient serum was first evaluated on SARS-CoV-2 spike protein by indirect ELISA using rRBD protein for spike protein-based antibody capture (Fig. 3A). A pool of serum samples from ten infection-naive subjects (Table S3) served as negative serum control. Administration of COMIRNATY vaccine resulted in significant production of anti-RBD antibodies in vaccinated subject (Fig. 3A). A high anti-RBD antibody titer was maintained for 2 months after the injection of a second vaccine dose (Fig. 3A). As a measure of immunity to natural infection, a lack of antibody response against rN confirmed that individual remained negative for SARS-CoV-2 infection at the time of the vaccination (Fig. 3A).

The immune serum from COMIRNATY-vaccinated individual was assayed for reactivity of antibodies against the synthetic peptides through a peptide-based ELISA (Fig. 3B). Among the six synthetic peptides and their variants, only S1P4 and S2P6 were significantly recognized by the immune serum from the vaccinated subject at dilution serum 1:50. The immune reactivity of COMIRNATY recipient serum against the synthetic S1P4 and S2P6 peptides was observed up to two months after the injection of a second vaccine dose (Fig. 3B). We wondered whether administration of COMINARTY vaccine to an individual who had experienced SARS-CoV-2 infection induced production of antibodies capable of binding to the synthetic peptides. It has been reported that a single dose of a such COVID-19 mRNA vaccine is sufficient to maximize immune protection in post-infection individuals (34). In line with this finding, antibody capture assays were performed on an immune serum from a COVID-19 immune subject who had received a single-dose of COMIRNATY three months after recovery from mild disease. The diagnosis of SARS-CoV-2 infection had been performed by PCR during the acute phase of COVID-19. Administration a single dose of vaccine significantly increased the level of anti-RBD antibodies but not anti-N antibodies in COVID-19 immune subject (Fig. 4A). Thus, COMIRNATY vaccine elicits antibody response against the SARS-CoV-2 spike protein in a COVID-19 immune subject. The reactivity of COVID-19 patient serum was assayed few weeks after recovery through synthetic peptide-based ELISA (Fig. 4B). There was a lack of antigenic reactivity for all the synthetic peptides tested in relation to COVID-19 donor serum. Thus, the stretches of spike residues 616/644 and 1138/1169 were

not or weakly immunogenic in an individual who experienced SARS-CoV-2 infection. Administration of COMIRNATY vaccine in COVID-19 recovered individual resulted in production of antibody capable of binding to the synthetic S1P4 and S2P6 peptides 0.5 month after the receipt of a single dose (Fig. 4B). Thus, COMIRNATY vaccine was efficient to elicit antibody response against the stretches of spike residues 616/644 and 1138/1169 in a COVID-19 patient who previously mounted a humoral immunity against SAR-CoV-2 spike protein in the course of a natural infection.

We next evaluated the antigenic reactivity of the synthetic S1P4 and S2P6 peptides using a group of infection-naive individuals who had been immunized with COMIRNATY in January-February 2021(n = 9, median age: 50, female to male ratio: 0.9) (Table S4). The serum samples were collected few weeks after injection of a second dose of COMIRNATY vaccine. The pre-immune serum of each vaccinated subject was used as negative serum control. The COMIRNATY recipient sera (serum dilution 1:100) were first assessed for anti-RBD antibodies by indirect ELISA (Fig. 5A). All vaccinated individuals developed anti-RBD antibodies confirming that administration of COMIRNATY was effective in eliciting an antibody response against SARS-CoV-2 spike protein. The absence of anti-rN antibodies in vaccine recipient sera indicated that all nine subjects were not affected by SARS-CoV-2 infection at the time of the vaccination (Fig. 5A). Synthetic peptide-based ELISA was performed with the serum samples (dilution 1:50) from the group of COMIRNATY recipients (Fig. 5B). The peptide S1P5 served as negative peptide control. The synthetic S2P6 peptide but not the synthetic S1P4 peptide showed a significant antigenic reactivity with the COMIRNATY recipient sera as compared to pre-immune sera. The ability of vaccine recipients to produce antibodies capable of binding to synthetic S2P6 peptide highlights a role for the stretch of residues 1138/1169 as linear B cell epitope in the recombinant spike protein expressed by COMIRNATY vaccine. The fact that immune reactivity to the synthetic S1P4 peptide was not significantly different between the nine COMIRNATY recipient sera compared to their pre-immune sera highlights individual differences in the development of an effective antibody response targeting the stretch of spike residues 616/644.

Immune reactivity of COVID-19 patient sera with the synthetic S1P4 and S2P6 peptides

We evaluated antigenic reactivity of the synthetic S1P4 and S2P6 peptides with a group of COVID-19 immune subjects. We selected a panel of thirty COVID-19 donor sera (median age: 52.5 years, female to male ratio: 4) collected in November and December 2020 in France (Table S2). The COVID-19 serum donors developed mild COVID-19 symptoms without requirement for hospital admission. Symptomatic COVID-19 patients with progression to severe disease or admitted in intensive care units in hospital were not included in this study. Serological tests based on semi-quantitative SARS-CoV-2 specific antibody assays indicated that COVID-19 immune subjects developed anti-SARS-CoV-2 IgG and most of them also had specific IgM titers (Table S2). There were no significant differences in humoral immune reactivity among COVID-19 immune subjects related to women/men ratio or different age categories. Although durability of IgM and IgG can vary among COVID-19 patients, the IgM titers are consistent with serum samples collected within the first weeks following acute infection.

The immune reactivity of COVID-19 donor sera (Table S2) was first verified for the presence of anti-spike antibodies by indirect ELISA using rRBD for antigen-based antibody capture (Fig. 6A). The rN protein was used as control viral antigen for natural SARS-CoV-2 infection. The serum samples collected before the emergence of COVID-19 in France were used as negative control sera (Table S3). There was a significant immune reactivity of COVID-19 donor sera against both rN and rRBD proteins. The serum samples (n = 23) among the COVID-19 recovered patients who developed higher anti-RBD antibody titers (serum dilution 1:100) were assayed through synthetic peptide-based ELISA (Fig. 6B). Serum samples from COVID-19 immune subjects showed no significant reactivity with the S2P6 peptide although a great variability in antibody response of relevant specificity. A lack of significant antigenic reactivity for S1P4 peptide with COVID-19 donor sera was also observed (Fig. 6B). Such results suggest that individuals who had experienced mild SARS-CoV-2 infection did not develop a significant antibody response against the stretches of spike residues 615/644 and 1138/1169 in the course of natural infection.

Immunization of BALB/c mice with protein-peptide conjugates

To evaluate whether the stretches of SARS-CoV-2 spike residues 616/644 and 1138/1169 were immunogenic, the synthetic S1P4 and S2P6 peptides were N-terminally coupled to Keyhole Limpet Hemocyanin (KLH) protein carrier. The protein-peptide conjugates were assessed with serum sample from a COVID-19 immune subject who received COMIRNATY vaccine. The KLH-S1P5 served as negative protein-peptide conjugate control. The antigenic reactivity of KLH-S1P4 and KLH-S2P6 conjugates was verified by indirect ELISA using immune serum from vaccinated COVID-19 patient with COMIRNATY (Fig. 7A). The antigenic reactivity of protein-peptide conjugates was comparable to that observed with synthetic S1P4 and S2P6 peptides, confirming that both KLH-S1P4 and KLH-S2P6 are suitable for further experiments.

To assess the immunogenicity of KLH-peptide conjugates in inbred laboratory mice, three groups of adult BALB/c mice were subcutaneously (s.c.) inoculated with 20-30 µg of KLH-S1P4, KLH-S1P5 or KLH-S2P6 conjugates in a prime-boost schedule. Immune sera were collected two weeks after the third immunization. The ability of the protein-peptide conjugates to elicit antibody response in mice was assessed by indirect ELISA using protein-peptide conjugates for antigen-mediated antibody capture. Immunization with KLH-S1P4 and KLH-S2P6 conjugates but not KLH-S1P5 conjugate elicited a strong antibody response against protein-peptide conjugates (Fig. S1). To evaluate the ability of the KLH-peptide conjugates to elicit antibody production of relevant specificity, individual mouse immune sera were tested on the synthetic S1P4, S1P5, and S2P6 peptides through a peptide-based ELISA (Fig. 7B). Mouse pre-immune serum collected prior to antibody production against protein-peptide conjugates served as control serum. Most of BALB/c mice (n= 5) that received KLH-S2P6 conjugates developed S2P6 peptide-reactive antibodies with a median $O.D._{450\ nm}$ value about 2.0 at serum dilution 1:50 (Fig. 7B). We noted that KLH-S1P4 conjugate was mostly inefficient to elicit a significant production of S1P4 peptide-reactive antibodies (Fig. 7B). Also, there was a lack of antibody response against KLH-S1P5 conjugate as a negative control. The immunogenicity of the synthetic S2P6 peptide reinforces the notion that the SARS-CoV-2 spike protein expressed by COVID-19 mRNA COMIRNATY vaccine comprises a B cell epitope which is delimited by the continuous residues 1138/1169.

DISCUSSION

The COVID-19 mRNA COMIRNATY vaccine is highly effective to prevents SARS-CoV-2 infection among individuals of diverse ages. With the aim to identify specific antibody epitopes in COMIRNATY vaccine, a set of potential linear B cell epitopes in the SARS-CoV-2 spike protein was assayed for their antigenic reactivity in relation with serum samples from COMIRNATY vaccine recipients and COVID-19 recovered patients.

In the present study, we showed that administration of COMIRNATY vaccine to infection-naive individuals elicits production of antibodies capable of binding to the synthetic peptides S2P6 and to a lesser extent the synthetic S1P4 peptide, representing the SARS-CoV-2 spike residues 1138/1169 and 616/644, respectively. The continuous spike residues 616/644 are located at the C-terminal domain 2 (CTD2) of S1 subunit upstream of the spike furin-cleavage site RRAR at the junction of S1 an S2 subunits whereas the spike residues 1138/1169 are positioned at the junction of the CD and the HR2 in the C-terminal region of S2 subunit (Fig. 1). In contrast to what it has been observed with COMIRNATY recipient sera, no significant immune reactivity of COVID-19 immune subjects was observed with both synthetic S1P4 and S2P6 peptides. However, administration of COMIRNATY vaccine to a COVID-19 recovered patient was efficient to elicit a production of antibodies capable of binding to synthetic S1P4 and S2P6 peptides. Thus, the COMIRNATY vaccine is effective to elicit an antibody response targeting the SARS-CoV-2 spike residues 1138/1169 and to a lesser extent, the residues 616/644. The recombinant SARS-CoV-2 spike S protein expressed by a such COVID-19 mRNA vaccine is stabilized in a prefusion state due to amino-acid substitutions S-K986P and S-V987P (25-27, 29). One privileged hypothesis is that the immune recognition of spike residues 616/644 and 1138/1169 as linear B cell epitopes would be an antigenic characteristic of the prefusion-stabilized spike protein. Whether the two antibody epitopes containing the spike residues 616/644 and 1138/1169 are a feature of COMIRNATY vaccine is an important issue that remains to be validated by analyzing a larger cohort of serum samples from vaccinated individuals. It would be also of priority to evaluate whether the other commercially available COVID-19 mRNA vaccine mRNA-1273 licensed as SPIKEVAX by Moderna/NIAID also elicits an antibody response targeting the spike residues 616/644 and 1138/1169. Taken into consideration with the singularity of the synthetic S1P4 and S2P6 peptides, it could be interesting to

design of peptide-based diagnosis assays to monitor antibody production in COMIRNATY-vaccinated, infection-naive individuals as well as COVID-19 immune subjects who received a single vaccine dose. It is of note that, to date, the stretches of spike residues 616/644 and 1138/1169 are highly conserved among the identified SARS-CoV-2 variants.

To our knowledge, it is not yet known whether the SARS-CoV-2 spike residues 616/644 and 1138/1169 contribute to the protective efficacy of COVID-19 mRNA COMIRNATY vaccine (31, 32-34, 35, 36). There is some evidence that potent neutralization of SARS-CoV-2 requires both spike conformational changes and receptor blockade elicited by antibody binding (10, 13, 15, 17, 18, 37). The antibody recognition of the spike residues 616/644 into the C-terminal domain 2 of S1 subunit might have an impact on the efficiency of S1/S2 processing at the spike residue 685 by the cell surface protease TMPRSS2 (15-20). The antibody recognition of spike residues 1138/1169 might have an effect on fusion process between viral and host-cell membranes by trapping the pre-fusion state of trimer spike (21, 22, 37). Immunization of adult BALB/c mice with the synthetic S2P6 peptide conjugated to a protein carrier identified the SARS-CoV-2 spike residues 1138/1169 as immunogenic peptide. The production of mouse S2P6-peptide reactive antibodies highlights the ability of such a synthetic 32-mer peptide to elicit antibody response. A synthetic peptide representing a neutralizing antibody epitope in spike protein could be capable of inducing specific antibodies that can confer protection against SARS-CoV-2 virus infection. It is therefore of priority to determine whether mouse S2P6-peptide reactive antibodies display immune activity against SARS-COV-2 from which the peptide sequence was derived of. Such a study would broaden our understanding on the role of spike residues 1138/1169 in the protective efficacy of COVID-19 mRNA COMIRNATY vaccine by providing information helping the future development of peptide-based effective vaccines against SARS-CoV-2 (25).

METHODS

Antibody epitope mapping

The IEDB (for Immune Epitope Database, https://www.ied.org) as B cell epitope-prediction tool was applied to the B cell epitope candidates in the SARS-CoV-2 spike protein (UniprotKB-P0DTC2

(SPIKE_SRAS2)). The BepiPred-2.0 algorithm was used to predict antibody epitopes using 0.50 as the cut-off parameter.

Expression of recombinant N and RBD proteins in mammalian cells

Mammalian codon-optimized genes coding either for the SARS-CoV-2 N protein or the RBD domain of spike protein (residues 328/583) (UniProtKB- P0DTC9 (NCAP_SARS2)) were established using Cricetulus griseus codon usage as reference (38). The sequence encoding the mouse Ig gamma-2A (IgG2A) chain C region sequence (UniprotKB-P01863 (GCAA_MOUSE) was fused in frame to the carboxy-terminus of N and RBD proteins with a spacer serine-glycine spacer. The protein sequence is preceded by the Secrecon signal peptide (39). The synthesis of gene sequences and their cloning into Kpn-I and Xho-I restriction sites of the pcDNA3.1 vector plasmid to generate pcDNA3/N-IgG2A and pcDNA3/RBD-IgG2A were performed by Genecust (Boynes, France). The plasmid sequences were verified by Sanger method (Genecust). Chinese Hamster Ovary CHO cells were transiently transfected with pcDNA3/N-IgG2A and pcDNA3/RBD-IgG2A using Lipofectamine 3,000 (Thermo Fisher Scientific, les Ulis, France). After 72 h of transfection, cell supernatants were harvested and clarified cell culture supernatant (C3S) fractions were obtained by centrifugation at 1,000 x g for 10 min at room temperature. Secreted N-IgG2a (rN) et RBD-IgG2A proteins in C3S fractions were detected by immunoblot assay using goat anti-mouse IgG heavy chain HRP antibody (Abcam, Cambridge, UK). The amounts of soluble rN and rRBD proteins in C3S fractions were estimated at 1.5 and 0.8 $\mu g.mL^{-1}$, respectively, by direct ELISA test using goat anti-mouse IgG heavy chain HRP antibody. Standard for ELISA calibration was done using a serial dilution of mouse IgG at 1 $mg.mL^{-1}$ as coating antigen (Vectors, Clinisciences, France).

Synthetic SARS-CoV-2 S peptides

The synthetic peptides S1P1 to S2P5 and S2P6 representing potential linear B cell epitopes into the S1 and S2 subunits of SARS-CoV-2 S protein, respectively, and their variants $S1P1^{[S]}$, $S1P3^{[N]}$, and $S1P5^{[H]}$ were chemically synthesized by Genecust (Boynes, France). Peptides sequences and their position on SARS-CoV-2 spike protein are indicated in the Table 1. Peptides were dissolved in DMSO at concentration of 10 $mg.mL^{-1}$ and then diluted in sterile H_2O at the final concentration 1 $mg.mL^{-}1$. The

stock peptide solutions were stored at -80°C. Working peptide solutions at the final concentration 0.2 $mg.mL^{-1}$ in sterile H_2O were stored at -80°C.

ELISA methods

For indirect ELISA assay using recombinant soluble rN and rRBD proteins as antibody capture antigens, a 96-well plate was coated with 0.1 ml of CS3 fractions at final concentration of 150 $ng.mL^{-1}$ of rN or 80 $ng.mL^{-1}$ of rRBD in PBS at 4°C overnight. For peptide-based ELISA, a 96-well plate was coated with 0.1 ml of peptide at final concentration of 200 $ng.mL^{-1}$ in PBS at 4°C overnight. For indirect ELISA, a 96-well plate was coated with 0.1 ml of KLH-peptide at final concentration of 1 $\mu g.mL^{-1}$ in PBS at 4°C overnight. At the end of incubation period, the solution was discarded, the wells were washed with PBS supplemented with 0.01% Tween-20 (PBST) and then incubated with a commercial ELISA blocking agent (EBA) at room temperature (RT) for 1 h. After washing with PBST, the wells were incubated with human serum sample at final dilution 1:50 in EBA or mouse serum sample at final dilution 1:100 at 37°C for 2 h. After washing with PBST, the wells were incubated with goat anti-human IgG-HRP or goat anti-mouse IgG-HRP at final dilution 1:2,000 in EBA at room temperature for 1 h. After washing with PBST and PBS, the wells were incubated with TMB substrate solution at RT for 3 min and the reaction was stopped with acidic stopping solution. Absorbance was measured using microplate reader at 450 nm.

Immunoblot assay

Aliquots of clarified cell culture supernatant (CS3) fractions from transfected CHO cells expressing soluble rN or rRBD proteins were loaded on a 4-12% SDS-PAGE and transferred to nitrocellulose membrane. After blocking of the membrane with PBST supplemented with non-fat milk, the membrane was incubated 1 h with goat anti-mouse IgG heavy chain HRP antibody. After several washes with PBST, the membrane was developed with Pierce ECL Western blotting substrate (ThermoFisher Scientific, Illkirsh-Graffenstaden, France) and then exposed on an Amersham imager 600 (GE Healthcare).

Commercial serum samples from COVID-19 immune subjects

Serum samples were collected from de-identified COVID-19 immune subjects (n = 30 total) in 2020 (Table S2) and infection-naive individuals (donation in 2019; n = 10 total) (Table S3) in France. All serum samples were purchased from Cerba Xpert (Saint-Ouen l'Aumône, France).

Serum samples from COMIRNATY vaccine recipients

Blood samples were collected from de-identified healthy COMINARTY® vaccine recipient adults (n = 9) by professional medical caregivers in the Centre Hospitalier Universitaire de La Reunion (CHU de La Reunion, France) (Table S4). Ethical approval for this study was obtained from Research Ethics Committee "Comité de Protection des Personnes, Nord Ouest IV de Lille, France (approval number EudraCT/ID-RCB 2020-A01253-36)". Written informed consent was obtained from all volunteers before. Age, gender, origin, date of vaccination and date of specimen collection were collected for all volunteers. Study individuals were fasting before venous puncture. Blood was sampled in EDTA tubes and plasma was stored at − 80 °C. All methods were carried out in accordance with relevant guidelines and regulations. Blood samples were collected in infection-naive individual prior vaccination and few weeks after the receipt of the second dose of COMINARTY vaccine.

In this study, two anonymized COMINARTY vaccine recipients who have given their consent for the use of their serum samples were also assessed for the reactivity of their antibodies. An infection-naive individual (Male, 59 years, France) who had been fully vaccinated with COMIRNATY in a two-dose regimen in April 2021. Serum samples were collected 0.5 and 2 months after the receipt of the second dose. A COVID-19 immune subject (Female, 62 years, France) who received the COMIRNATY vaccine three months after recovery. Serum samples were collected few weeks after COVID-19 recovery in March 2021 and then 0.5 month after the receipt of a single dose of COMIRNATY in June 2021.

Mouse experiment and ethical statement

The Animal Ethics Committee of CYROI n°114 approved all the animal experiments with reference APAFIS#32599-202106010905895 8v2 (July 2021). All animal procedures were performed in accordance with the European Union legislation for the protection of animals used for scientific

purposes (Directive 2010/63/EU). The study was conducted following the guidelines of the Office Laboratory of Animal Care (agreement n° 974 001 A) at the Cyclotron and Biomedical Research CYROI platform, Sainte-Clotilde, La Reunion, France and in accordance with the ARRIVE guidelines (https://arriveguidelines.org). Three groups of five 6-week-old female BALB/cJRj mice (Janvier Labs, France) were hosted in individually ventilated plastic cages (5 animals per cage) under 50-60% humidity and 22-25°C temperature in a 12/12-hour light dark cycle. Food and drinking were provided *ad libitum*. The adult BALB/cJRj mice (Janvier Labs, France) were s.c. inoculated with KLH-peptide conjugates in complete Freund's adjuvant (Sigma, France). The protein-peptide conjugate samples in a final volume of 0.1 ml were distributed over two injection sites. Immunized mice were boosted with the same protein-peptide conjugate in incomplete Freund's adjuvant at Days 7 and 21 after primary immunization (Day 0). Two weeks after the last immunization, retro-orbital blood sampling was performed in anesthetized mice. All animals were daily observed to detect any stress or suffering

Statistical analysis

Unpaired and paired t tests were used to compare quantitative data. GraphPad Prism 9 was used for all statistical analysis.

Author Contributions:

J.A. contributed to formal analysis, investigation, methodology and validation.; W.V. contributed to formal analysis, investigation, methodology, supervision, and writing review & editing; C.C. contributed to investigation, methodology, and resources; C.P. contributed to investigation and resources; B.R. contributed to methodology, resources, and writing review & editing; M. L-X. contributed to methodology and resources; O.M. contributed to methodology, resources and writing review & editing.; P.M. contributed to methodology, funding acquisition, project administration and writing review & editing.; D.C. contributed to methodology, resources and writing review & editing; G.G. contributed to formal analysis, investigation, methodology, and writing review & editing.; P.D. contributed to conceptualization, formal analysis, investigation, methodology, resources, visualization, validation, writing-original draft and writing review & editing.

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Competing interests:

All authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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FIGURE LEGENDS

Figure 1. **Description of synthetic peptides representing potential linear SARS-CoV-2 spike antibody epitopes.** The organization of SARS-CoV-2 spike protein with the S1 and S2 subunits and their different domains and motifs is shown. The synthetic S1P1 to S1P5 and S2P6 peptides and their variants S1P1$^{[S]}$ (Gamma variant of SARS-CoV-2), S1P3$^{[N]}$ (Beta variant of SARS-CoV-2), and S1P5$^{[H]}$ (Alpha variant of SARS-CoV-2) are positioned on the spike protein.

Figure 2. **Antigenic reactivity of recombinant N and RBD proteins with COVID-19 donor sera.** In (A), CHO cells were transfected 72 h with plasmids expressing RBD-IgG2A (rN) and N-IgG2A (rRBD) proteins or empty vector plasmid (control). Aliquots of clarified cell culture supernatants (C3S) were analyzed by immunoblot assay using goat anti-mouse IgG heavy chain HRP antibody. In (B), pools of four COVID-19 donor serum samples (Table S2, serum donors n°16, 18, 19, and 20) (COVID-19 donor serum) and four negative donor serum samples (Table S3) (negative donor serum) at dilution 1:100 were tested for anti-N and anti-rRBD antibodies by indirect ELISA using rN and rRBD for protein-based antibody capture. The intensity values of serum samples were measured at O.D. 450 nm. The results are the mean (± SEM) of three replicates. Unpaired *t* tests between COVID-19 immune subject and infection-naive individual were performed and statistically significant comparisons are shown as * $p < 10^{-4}$.

Figure 3. **Antigenic reactivity of synthetic peptides with COMIRNATY recipient sera.** Serum samples from an infection-naive individual who received COMIRNATY vaccine in a 2-dose regimen were collected at 0.5- or 2-month after the injection of a second dose. A pool of ten infection-naive individuals collected in 2019 (Table S3) served as negative control serum. In (A), serum samples at dilution 1:100 were assessed for the detection of antibodies against SARS-CoV-2 N and S proteins by indirect ELISA using soluble rN and rRBD proteins for antigen-based antibody capture. The intensity values of serum samples were measured at O.D. 450 nm. The results are the mean (± SEM) of three replicates. Statistical comparisons were performed between serum samples. Statistically significant comparisons are shown as * $p < 10^{-4}$ (ns: non-statistically significant, $p > 0.05$). In (B), the serum samples were assayed at dilution 1:50 on synthetic peptides and their variants (200 ng.ml^{-1}) through synthetic peptide-based

ELISA. The intensity values of vaccine serum sample were measured at O.D. 450 nm. The results are the mean (± SEM) of three replicates. Pairwise comparisons between peptides showed that the experimental points for S1P4 and S2P6 peptides are significatively different from the other peptides (** $p < 10^{-4}$, * $p < 10^{-3}$). The differences between 0.5 and 2-month for the synthetic S1P4 and S2P6 peptides are non-statistically significant (ns, $p > 0.05$).

Figure 4. **Antigenic reactivity of synthetic peptides with immune sera from a COVID-19 Immune subject who received COMIRNATY vaccine**. Serum samples from a COVID-19 patient were collected few weeks after recovery of SARS-CoV-2 infection (post-infection) and then two weeks after the injection of a single dose of COMIRNATY vaccine (vaccination). Vaccine administration was performed three months after COVID-19 recovery. In (A), serum samples at dilution 1:100 were assayed for the detection of antibodies against SARS-CoV-2 N and spike proteins by indirect ELISA using recombinant rN and rRBD proteins for antigen-based antibody capture. A pool of serum samples from infection-naive individuals (Table S3) served as negative control serum. The intensity values of serum samples were measured at O.D. 450 nm and their immune reactivity was estimated as a fold increase of intensity values obtained with negative control serum. The results are the mean (± SEM) of three replicates. Statistically significant comparisons are shown as * $p < 10^{-4}$ (ns: non-statistically significant, $p > 0.05$). In (B), serum samples at dilution 1:50 were assayed for the detection of specific antibodies through peptide-based ELISA. The intensity values of serum samples were measured at O.D. 450 nm. The results are the mean (± SEM) of three replicates. Paired *t* tests on experimental points between post-infection and vaccination immune were performed and statistically significant comparisons are shown as * $p < 10^{-4}$. Differences between experimental points considered as non-statistically significant are not shown.

Figure 5. **Immune reactivity of COMIRNATY recipient sera against the synthetic S1P4 and S2P6 peptides.** Serum samples from COMIRNATY vaccine recipients (n= 9) (Table S3) were collected few weeks after the receipt of the second vaccine dose. The pre-immune serum of each individual was collected prior vaccination. In (A), serum samples at dilution 1:100 have been tested for SARS-CoV-2 N and S antibodies by indirect ELISA using soluble rN and rRBD proteins for antigen-based antibody capture. The intensity values of serum samples were measured at O.D. 450 nm. Paired *t* tests were

performed between pre-immune serum and vaccine recipient serum (ns: non-statistically significant, $p > 0.05$). In (B), the serum samples were assayed on the synthetic S2P4 and S2P6 peptides (200 $ng.ml^{-1}$) through peptide-based ELISA at serum dilution 1:50. The synthetic S1P5 peptide served as negative control serum. The intensity values of vaccine serum samples were measured at O.D. 450 nm. Paired *t* tests were performed between pre-immune serum and vaccine recipient serum.

Figure 6. **Antigenic reactivity of the synthetic peptides with COVID-19 immune subjects.** Serum samples from COVID-19 recovered patients (n = 30) (Table S2) and ten infection-naive individuals (n = 10) (Table S2) were tested for the antibody reactivity against the N and RBD proteins through indirect ELISA (A), or the synthetic S1P4, S1P5, and S2P6 peptides through peptide-based ELISA (B). The intensity values of serum samples were measured at O.D. 450 nm. In (A), the serum samples at dilution 1:100 were assayed for the detection of antibodies against SARS-CoV-2 N and spike proteins by indirect ELISA using recombinant rN and rRBD proteins (1 $\mu g.ml^{-1}$) for antigen-based antibody capture. The intensity values of serum samples were measured at O.D. 450 nm. Unpaired *t* tests between infection-naive individuals and COVID-19 immune subjects were performed and statistically significant comparisons are shown as * $p < 10^{-4}$. In (B), The serum samples (n = 23) among the COVID-19 recovered patients who developed higher anti-RBD antibody titers (were assayed for the detection of antibodies against the synthetic S1P4, S1P5, and S2P6 peptides (200 $ng.ml^{-1}$) through peptide-based ELISA at serum dilution 1:50. The results are the mean (± SEM) of three replicates. The intensity values of serum samples were measured at O.D. 450 nm. Unpaired *t* tests between infection-naive individual and COVID-19 immune subject were performed and differences between experimental points considered as non-statistically significant ($p > 0.05$) are not shown.

Figure 7. **Antibody reactivity in immunized mice with protein-peptide conjugates**. In (A), serum sample from a COVID-19 recovered patient who had been immunized with a single dose of COMIRNATY was collected 0.5 month after vaccine administration and serum dilutions were assayed on synthetic peptides through peptide-based ELISA (top) or protein-peptide conjugates through indirect ELISA (bottom). The synthetic S1P5 peptide and KLH-S1P5 conjugate served as negative controls. The intensity values of serum samples were measured at O.D. 450 nm. In (B), serum samples from mice (n

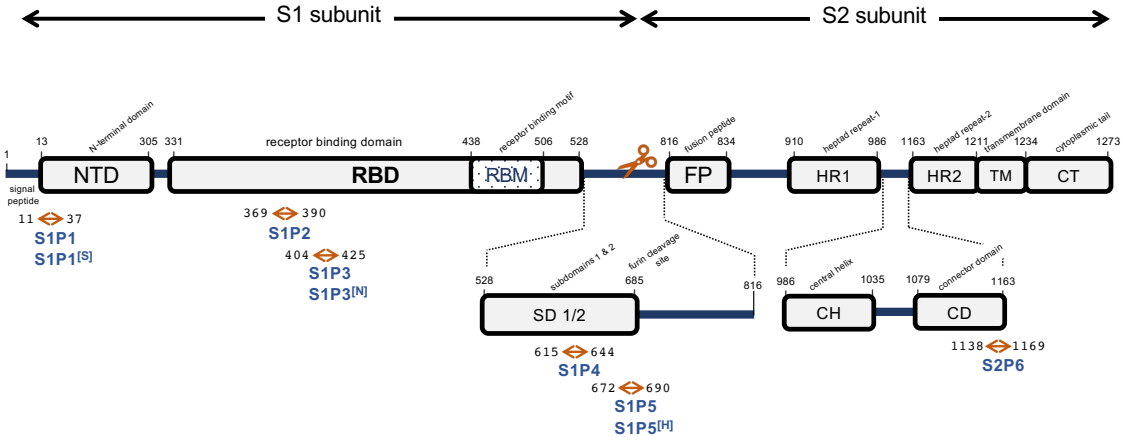
=5) that received the KLH-peptide conjugates were assessed for peptide-reactive antibodies through peptide-based ELISA using the synthetic S1P4, S1P5, and S2P6 peptides (0.2 $\mu g.mL^{-1}$) for antigen-based antibody capture. The pre-immune serum of each individual that received the KLH-peptide conjugates was tested. The intensity values of serum samples at dilution 1:50 were measured at O.D. 450 nm. Paired *t* tests between pre-immune serum and KLH-peptide serum were performed (ns: non-statistically significant, $p > 0.05$). The results are representative of two independent experiments.

Table 1. Sequences of synthetic peptides and their variants.

Peptide	Amino acid sequence	Spike residues	Length
S1P1	SQCVNLTTRTQLPPAYTNSFTRGVY	11-37	25-mer
S1P1[S]*	SQCVNFTNRTQLPSAYTNSFTRGVY	11-37	25-mer
S1P2	YNSASFSTFKCYGVSPTKLNDLCF	369-392	24-mer
S1P3	GDEVRQIAPGQTGKIADYNYKL	404-425	22-mer
S1P3[N]**	GDEVRQIAPGQTGNIADYNYKL	404-425	22-mer
S1P4	NCTEVPVAIHADQLTPTWRVYSTGSNVFQ	615-644	29-mer
S1P5	ASYQTQTNSPRRARSVASQ	672-690	19-mer
S1P5[H]***	ASYQTQTNSHRRARSVASQ	672-690	19-mer
S2P6	YDPLQPELDSFKEELDKYFKNHTSPDVDLGDI	1138-1169	32-mer

* gamma, ** beta, and *** alpha variants of SARS-CoV-2 according to WHO label. The amino-acid substitutions are underlined.

Figure 1


S1 subunit
S2 subunit
1
13
N-terminal domain
305
331
receptor binding domain
438
receptor binding motif
506
528
816
fusion peptide
834
910
heptad repeat-1
986
1163
heptad repeat-2
1211
transmembrane domain
1234
cytoplasmic tail
1273
NTD
RBD
RBM
FP
HR1
HR2
TM
CT
signal peptide
11 37
S1P1
S1P1[S]
369 390
S1P2
404 425
S1P3
S1P3[N]
528
subdomains 1 & 2
685
furin cleavage site
816
986
central helix
1035
1079
connector domain
1163
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S1P5
S1P5[H]
1138 1169
S2P6

Figure 2.

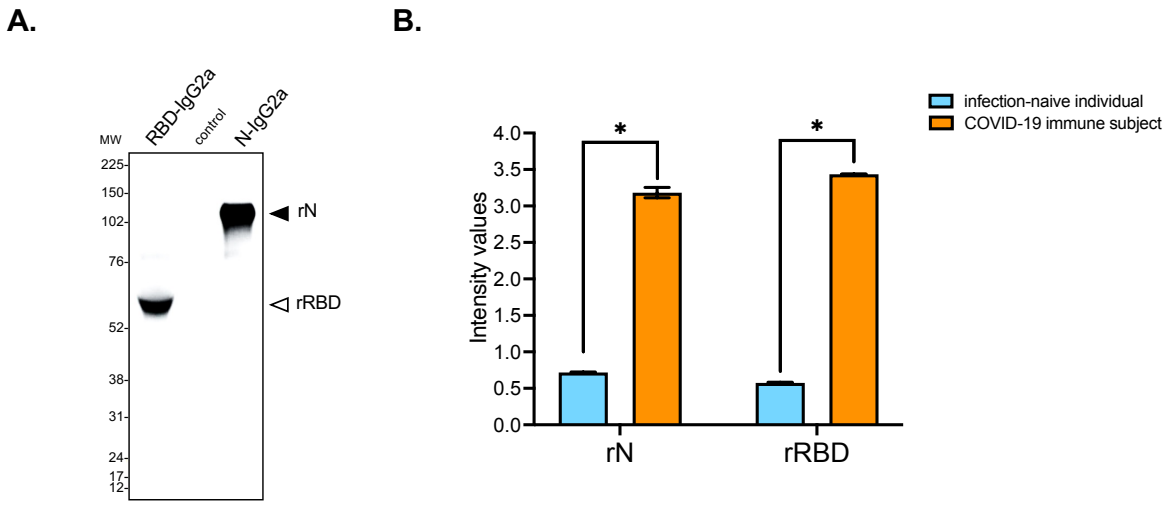

A.
B.
MW
RBD-IgG2a
control
N-IgG2a
225
150
102
76
52
38
31
24
17
12
rN
rRBD
Intensity values
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
rN
rRBD
infection-naive individual
COVID-19 immune subject

Figure 3.

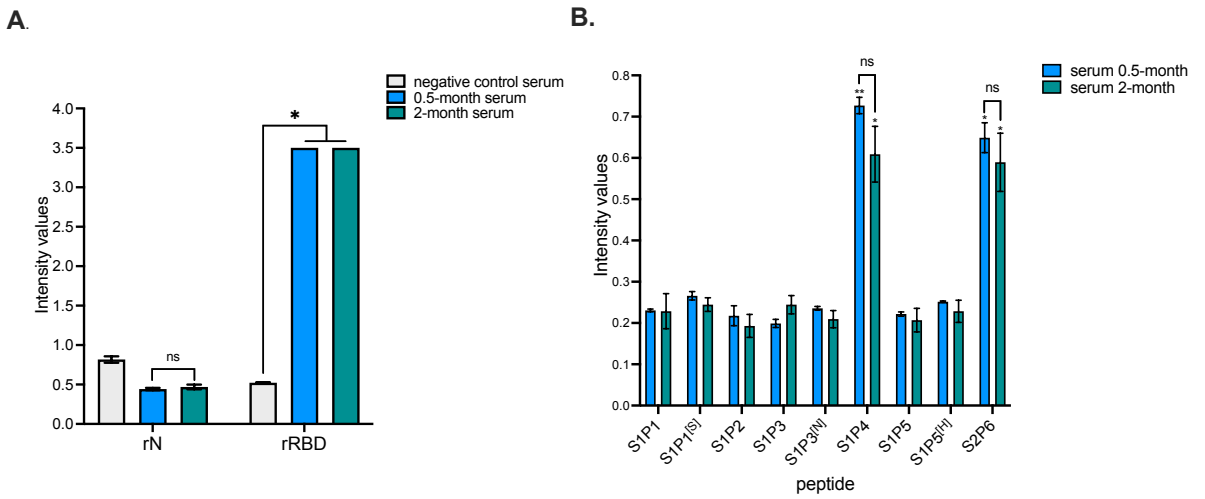

A.
B.
negative control serum
0.5-month serum
2-month serum
Intensity values
ns
*
rN
rRBD
serum 0.5-month
serum 2-month
peptide
S1P1
S1P1[S]
S1P2
S1P3
S1P3[N]
S1P4
S1P5
S1P5[H]
S2P6

Figure 4.

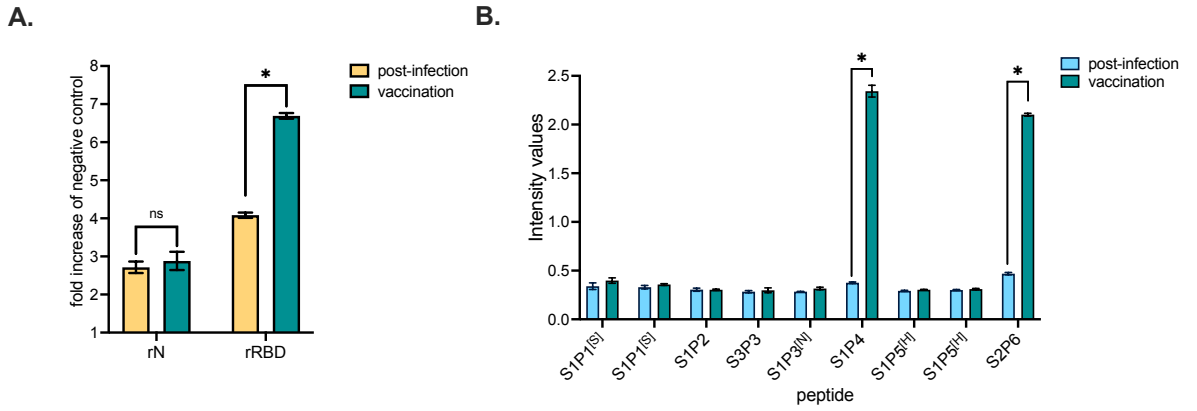

A.
B.
fold increase of negative control
post-infection
vaccination
ns
*
rN
rRBD
Intensity values
peptide
S1P1[S]
S1P1[S]
S1P2
S3P3
S1P3[N]
S1P4
S1P5[H]
S1P5[H]
S2P6

Figure 5.

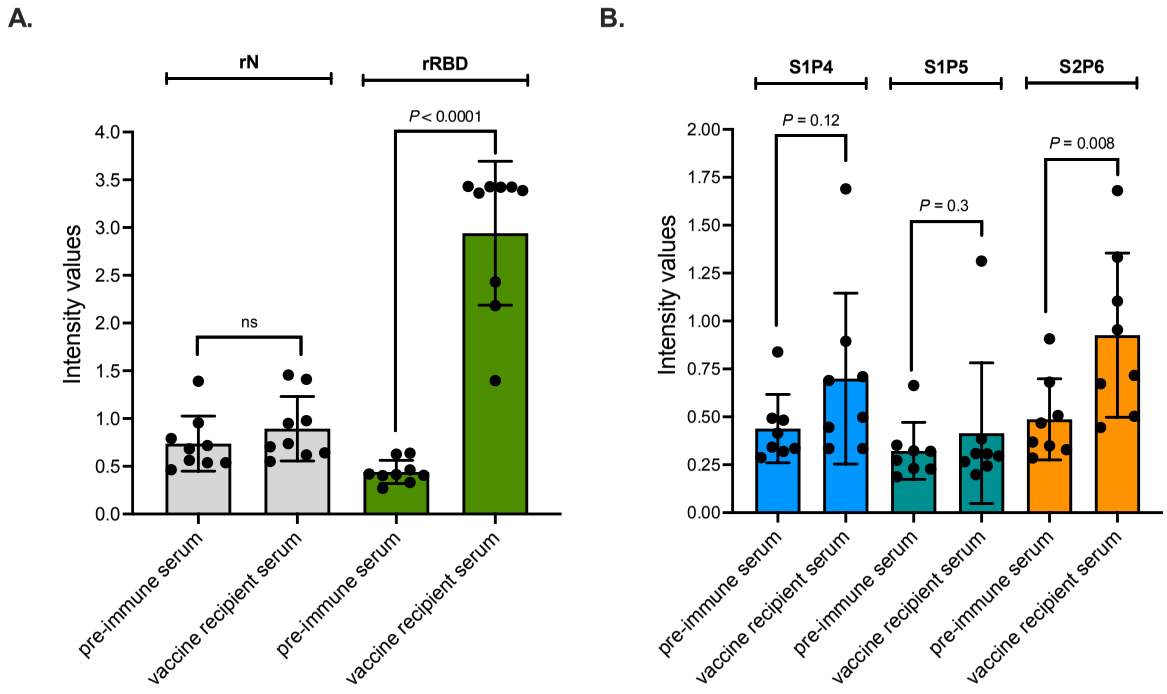

A.
rN
rRBD
P < 0.0001
ns
Intensity values
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
pre-immune serum
vaccine recipient serum
pre-immune serum
vaccine recipient serum
B.
S1P4
S1P5
S2P6
P = 0.12
P = 0.3
P = 0.008
Intensity values
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
pre-immune serum
vaccine recipient serum
pre-immune serum
vaccine recipient serum
pre-immune serum
vaccine recipient serum

Figure 6.

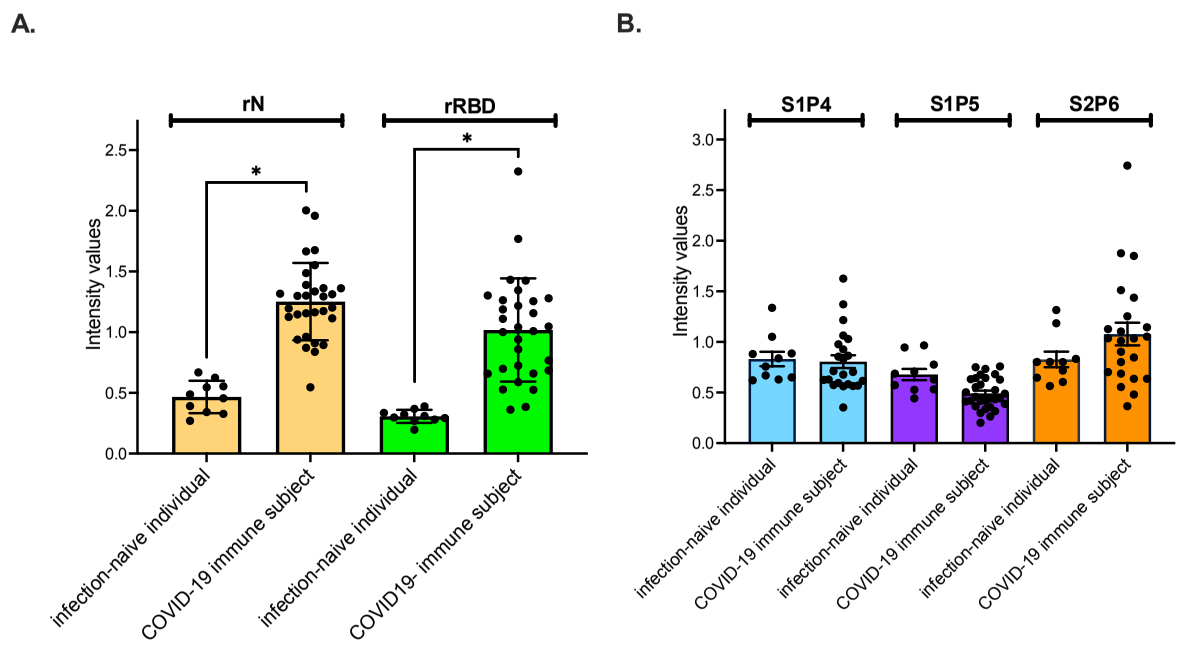
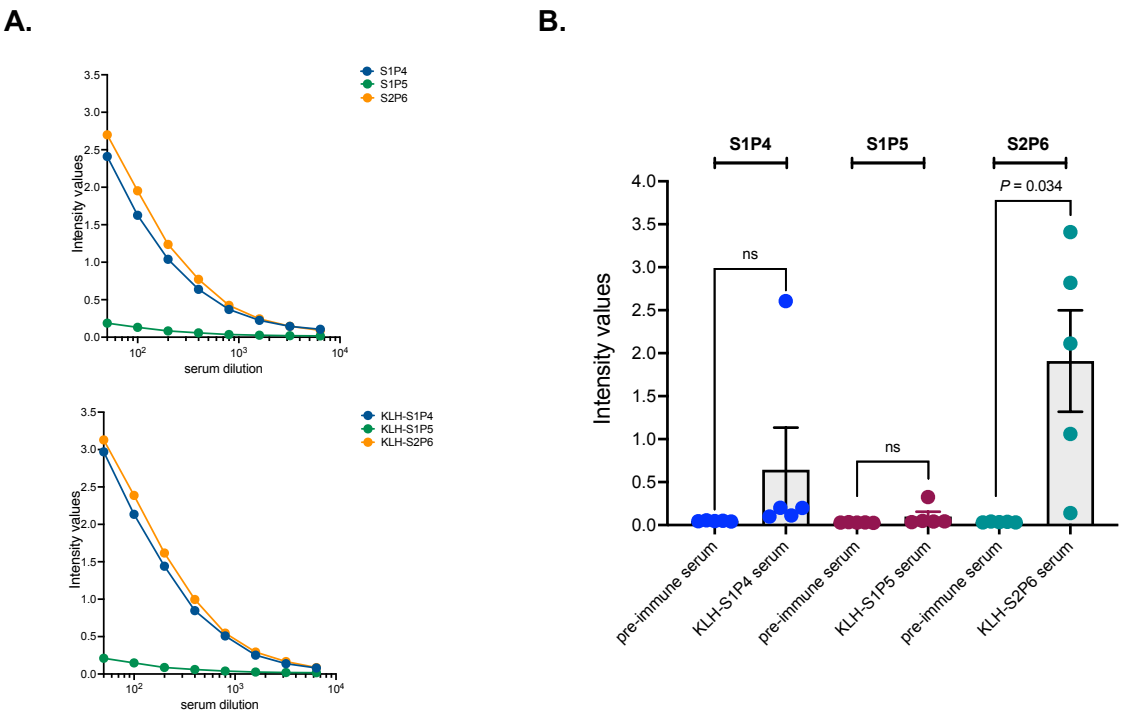

A.
rN
rRBD
*
*
Intensity values
2.5
2.0
1.5
1.0
0.5
0.0
infection-naive individual
COVID-19 immune subject
infection-naive individual
COVID19- immune subject
B.
S1P4
S1P5
S2P6
Intensity values
3.0
2.5
2.0
1.5
1.0
0.5
0.0
infection-naive individual
COVID-19 immune subject
infection-naive individual
COVID-19 immune subject
infection-naive individual
COVID-19 immune subject

Figure 7.


A.
S1P4
S1P5
S2P6
Intensity values
serum dilution
KLH-S1P4
KLH-S1P5
KLH-S2P6
Intensity values
serum dilution
B.
S1P4
S1P5
S2P6
P = 0.034
ns
ns
Intensity values
pre-immune serum
KLH-S1P4 serum
pre-immune serum
KLH-S1P5 serum
pre-immune serum
KLH-S2P6 serum

Supplementary Files

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