Article

Not peer-reviewed version

Assessment of the Humoral Immune Response to the SARS-CoV-2 Spike Protein Receptor Binding Motif

Maria E.S. Monteiro , Guilherme C Lechuga , Larisa R Gomes , João PRS Carvalho , Paloma Napoleão-Pêgo , Carlos M Morel , David W Provance-Jr , Salvatore G De-Simone *

Posted Date: 7 November 2023

doi: 10.20944/preprints202311.0296.v1

Keywords: SARS-CoV-2; Variants; Spike Glycoprotein; Receptor Binding Motif, Receptor Binding Domain; Neutralizing Antibodies; Ig subclasses; IgG; IgA

Article

Assessment of the Humoral Immune Response to the SARS-CoV-2 Spike Protein Receptor Binding Motif

Maria E.S. Monteiro [1,4], Guilherme C. Lechuga [1,2], Larissa R. Gomes [1], João P.R.S. Carvalho [1,3], Paloma Napoleão-Pêgo [1,2], Carlos M. Morel [1], David W. Provance [1,2] and Salvatore G. De-Simone [1,2,3,4,*]

[1] Center for Technological Development in Health (CDTS)/National Institute of Science and Technology for Innovation in Neglected Population Diseases (INCT-IDPN), Oswaldo Cruz Foundation (FIOCRUZ) Rio de Janeiro, 21040-900, Brazil; pegopn@gmail.com (P.N-P.); gclechuga@gmail. com (G.C.L.); carlos.morel@fiocruz.br (CMM); bill.provance@fiocruz.br.

[2] Epidemiology and Molecular Systematics Laboratory (LEMS), Oswaldo Cruz Institute, FIOCRUZ, Rio de Janeiro, 21040-900, Brazil.

[3] Program of Post-Graduation on Science and Biotechnology, Department of Molecular and Cellular Biology, Biology Institute, Federal Fluminense University, Niterói, 22040-036, Brazil. joaopedrorsc@gmail.com (J.P.R.S).

[4] Program of Post-Graduation on Parasitic Biology, Oswaldo Cruz Institute, FIOCRUZ, Rio de Janeiro, RJ, Brazil, 21040-900. monteiro.meduarda@gmail.com (M.E.S.M.)

* Correspondence: salvatore.simone@fiocruz.br; Tel.: +55-213-865-8180

Abstract: Global economic and social burden was caused by the SARS-CoV-2 spread worldwide. Despite the end of the pandemic, there is a concern about virulent evolving variants of the virus which can bypass the humoral immune response induced by vaccination or infection. Crucial to the viral entrance, amino acid residues in the RBM region, which interacts with cellular receptor ACE2, can elicit neutralizing antibody response. Herein we determine the immunogenicity of one-dose or heterologous dose vaccinated serum against wild-type and mutated RBM region. Despite low antibody response to wild-type SARS-CoV-2 RBM, omicron variants possess four mutations in RBM (S477N, T478K, E484A, F486V) that induce even less antibody titers. The most predominant responses were against IgA and IgG. While neutralizing antibodies (nAbs) predominantly target the RBD, our investigation revealed a diminished seroreactivity within the RBD's crucial region, the receptor-binding motif (RBM), potentially impacting the production of protective nAbs. S1WT and S2WT RBM peptides binding to nAbs were evaluated through microscale thermophoresis, and higher affinity (35 nM) was obtained for sequence S2WT (GSTPCNGVEGFNCYF), containing the FNCY patch. Our data indicates that SARS-CoV-2 RBM is not an immunodominant region in vaccinated individuals. Understanding the intricate dynamics of the humoral response and its interplay with viral evolution and host genetics is essential for the formulation of effective vaccination strategies, not only against SARS-CoV-2 but also for future emerging coronaviruses.

Keywords: SARS-CoV-2; variants; spike glycoprotein; receptor binding motif; receptor binding domain; neutralizing antibodies; Ig subclasses; IgG; IgA

1. Introduction

COVID-19 is attributed to the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which initially emerged in Wuhan, China, and quickly disseminated across the globe, leading to substantial economic and societal challenges. [1]. Despite the recent announcement by the World Health Organization declaring the end of the COVID-19 pandemic [2], the virus continues to evolve through mutations and recombination. The emergence of new SARS-CoV-2 variants raises concerns regarding their potential to evade host immunity. For instance, the Omicron variants have been reported to evade neutralizing antibodies (nAbs) induced by vaccination or prior infection [3]. Mutations, especially in the Spike (S) protein, pose a threat to the efficacy of current vaccines due to their capacity to evade antibody recognition, a critical component of the immune response [4]. The

 doi:10.20944/preprints202311.0296.v1

study of the humoral response to SARS-CoV-2 is crucial for understanding population-wide and individual responses to viral infection and vaccination. The variation in the antibody response to linear B-cell epitopes among individuals is of particular importance for serodiagnosis and vaccine development.

Neutralizing antibodies (nAbs) can target different regions of the Spike protein, with the majority binding to the N-terminal domain (NTD) and the receptor-binding domain (RBD) [5]. The binding modes of RBD-specific nAbs can be classified into four primary categories, depending on the epitopes they specifically target. Neutralization can occur through competition with the ACE-2 receptor, direct binding to RBD, and by inducing steric hindrance and restraining conformational changes in the Spike protein [6]. The RBD, located in the S1 domain of the S protein, has been extensively studied due to its high variability and its critical role in interacting with the virus entry receptor in mammalian cells, angiotensin-converting enzyme 2 (ACE2). Previous research has shown that the S protein is highly immunogenic, and protective nAb responses are predominantly directed against the RBD domain [7–9].

It is estimated that neutralizing antibodies (nAbs) binding to the RBD constitute approximately 90% of the neutralizing activity in convalescent sera [7]. Within the RBD, there is a receptor-binding motif (RBM) that plays a crucial role in interacting with the ACE2 receptor. The RBM can be divided into three flexible coil regions: knob (aa 444-449 and 496-505), base (aa 490-494 and 450-456), and tip (aa 473-489) [8].

Neutralization is primarily mediated by IgG, IgA, and IgM, with IgG being the most abundant neutralizing antibody [9]. IgM and IgG are produced simultaneously or sequentially in response to SARS-CoV-2 infection, reaching peak concentrations within the first two weeks and remaining in the bloodstream for at least six months [10]. IgG can be further subdivided into four subclasses with diverse effector functions based on the constant region: IgG1, IgG2, IgG3, and IgG4 [11]. Viral proteins predominantly elicit IgG1 and IgG3 responses, while IgG2 and IgG4 have been associated with polysaccharide antibacterial responses [11,12]. In terms of binding capacity, convalescent sera IgG3 has been shown to have the most pronounced ability to bind to the SARS-CoV-2 RBD [13]. High levels of anti-RBD IgG4 subclass have been correlated with increased mortality and severe disease [14].

While some SARS-CoV-2 mutations may be neutral or harmful, a subset can enhance viral fitness and enable immune evasion [4]. The interaction between the Spike protein and ACE2 is predominantly facilitated by amino acid residues within the RBM, which are the focal points of neutralizing antibodies (nAbs) [15–17]. Contact points of potent nAbs like B38 and CC12.1 overlap with the Spike-ACE2 interaction residues [15]. Mutations in the RBM, including E484K and N501Y, whether alone or in combination, have been associated with decreased binding of nAbs [8,17]. Notably, the N501Y mutation not only compromises the affinity of neutralizing antibodies but also increases the binding affinity to ACE2 [18].

While nAbs directed towards the RBM have been isolated by various research groups, little is known about their contribution to the pool of antibodies in the serum of SARS-CoV-2-infected or vaccinated individuals. However, evidence suggests that they are produced in lower frequencies [15,19]. Given the critical role of the RBM in viral entry and ACE2 interaction, it is reasonable to assume that it is highly immunogenic and can provide a structure-based framework for rational vaccine design and the selection of immunotherapeutic agents. Consequently, the RBM sequences were selected for synthesis and subsequent evaluation of the humoral response in a panel of vaccinated individuals. To understand the impact of SARS-CoV-2 mutations on host antibody responses, sequences from Omicron BA.4 and BA.5 were analyzed. Additionally, IgG class and subclasses specific to the RBM region were characterized in vaccinated individuals, and the interaction of antibodies with the RBM region was assessed using Microscale thermophoresis. The comprehensive peptide analysis of the RBM tip revealed that this region is not immunodominant.

 doi:10.20944/preprints202311.0296.v1

2. Materials and Methods

2.1. Serum samples

A panel comprising 31 sera samples was obtained from one dose vaccinated individuals (Oxford/AstraZeneca) and four doses (15 sera). Serum from healthy donors, also collected before the pandemic, was provided by HEMORIO, a centralized network of blood donor facilities in Rio de Janeiro, Brazil. To ensure patient privacy, all samples were provided without identifying information.

2.2. Peptides Synthesis

SARS-CoV-2 RBM 15 mer peptides [(S1WT) FERDISTEIYQAGST); S2WT (GSTPCN GVEGFNCYF) and S3WT (YFPLQSYGFQPTNGV)were chosen to be synthesized by the F-moc strategy in a synthesizer machine (MultiPep-1 CEM Corp, Charlotte, NC, USA) [20]. Once sequence assembly was completed, the F-moc groups were removed, and the peptide-resin (Wang resin) was cleaved and fully deprotected with TFA/H2O/EDT/ TIS (94/2.5/2.5/1.0 v/v, 90 min). The peptide was precipitated by adding chilled diethyl ether, centrifuged for 3 × 10 min at 4 ◦C, and the pellet was taken up in aqueous AcOH (10% v/v), dried, and stored as a lyophilized powder. When necessary, the peptides were dissolved in water, centrifuged (10,000 g, 60 min, 15 ◦C) and the supernatant filtered on a centricon 10 filter. The single peptides were used without previous purification, but their identity was checked by MS (MALDI-TOF or electrospray).

2.3. Enzyme-Linked Immunosorbent Assay (ELISA)

In-house ELISA was performed using Immunolon 4HB plates (Immunochemistry Technologies, Bloomington, MN, USA) coated overnight at 4 °C with 500 ng of peptides per well in coating buffer (50 mM Carbonate-Bicarbonate buffer, pH 9.6). Plate wells were washed with PBS-T (phosphate-buffered saline plus 0.05% Tween® 20) between each step. Plates were incubated for one hour at 37 °C with 1% BSA (200 ul) in PBS-T to block free binding sites. Next, the patient's sera were diluted (1:25) in coating buffer, and 100 µl were applied onto immunosorbent plates and incubated for 1 h at 37°C. Following several washes with PBS-T, the plates were incubated with 100 µl goat anti-human IgG-HRP (1:10,000, Sigma-Aldrich, St Louis, MO, USA) for 1 h at 37°C. Finally, 3,3′,5,5′-tetramethylbenzidine (1-Step™ Ultra TMB-ELISA, Scienco Biotech Ltda, Lages, SC, Brazil) was added for 15 min, and the reaction was stopped by adding 0.5 M sulfuric acid.

The plate was read within 2 h of adding the Stop Solution. Values of blank wells, which contained only peptides, were subtracted from the sample's optic density.

2.4. Purification of RBM antibodies

Antibodies anti-RBM were purified from a pool (n=10) of serum from one dose vaccinated individuals, through an affinity column (3 cm × 1 cm i.d.) prepared using a RBD SARS-CoV2 recombinant protein [21] coupled on Sepharose-4B beads according to the procedures previously described [22]. Anti-RBM antibodies were eluted with glycine buffer pH 2.8 and collected in Eppendorf tubes containing Tris-HCl pH 10 buffer and subsequently concentrated on centricon-P30 filters (Sigma Chemical Co, Saint Louis, MO, U.S.A.) (Figure S1). Protein concentration was estimated by measuring absorbance at 280 nm. The purification of antibodies was analyzed by in-house ELISA. Briefly, Immunolon 4HB plates (Immunochemistry Technologies, Bloomington, MN, USA) were coated overnight at 4 °C with 200 ng of multi-epitope (Dx-SARS-RBD) recombinant protein containing RBM peptides or non-SARS-CoV-2 sequences (Control). In-house ELISA followed the methods described above, but incubation was performed using purified antibodies (100 µg/mL) diluted at 1:1.000.

2.5. Microscale thermophoresis (MST)

Purified neutralizing antibodies to the RBM regions (Figure S1) were labeled with the fluorescent dye NT-647 using Monolith NT™ Protein Labeling Kits and mixed at a 3:1 molar ratio with unlabeled

 doi:10.20944/preprints202311.0296.v1

protein. For the interaction experiments, the fluorescent anti-RBM antibodies were kept at a constant concentration (12.5 µg/mL), while the concentration of the unlabeled S1WT and S2WT peptides varied from 0.5 µg/mL–0.12 ng/mL. The assay was performed in PBS containing 0.05% Tween 20 and after a short incubation time, the samples were loaded into standard MST NT.115 glass capillaries. Kd data were calculated using the NanoTemper software package [23].

2.6. Statistical Analysis

Data were analyzed by Prism software (GraphPad version 6, San Diego, CA, USA). The Kruskal–Wallis test was applied to identify statistical differences, followed by Dunn's multiple comparisons tests. A $p < 0.05$ was a significant difference. The reactivity index (RI) reflected the absorbance divided by the cutoff determined by the ROC analysis of each protein. All results >1.1 were considered positive and <0.90 were deemed to be negative. Samples with an RI value of 1.0 ±10% were defined as being in a gray zone and deemed inconclusive [21].

3. Results

The Spike sequences from VoCs were aligned and further subjected to phylogenetic analysis. The Omicron variants presented twelve mutations in RBM, including, mutations that were reported in the previous VOCs such as L452R, T478K, E484 and N501Y, also N440K, G446S, L452Q/R, S477N, T478K, E484A, F486V, Q493R, G496S, and Y505H (Figure 1a) most of these mutations alter viral fitness and immune evasion. The interaction of several described human nAbs with the binding residues on RBM were evaluated (Figure 1b), most of the binding occur on RBM tip (aa 473-489), where 6 amino acids residues (37,5%) are mutated in Omicron variants (S477N, T478K, E484A, F486V, Q493R, G496S). Interactions of Spike with ACE-2 were highlighted, including residues between 438–506 of the RBM, that makes the maximum contact with the N-terminal PD domain of ACE2. Described human nAbs evaluated displayed contact residues in RBM which overlaps amino acids interacting with ACE2. Since most of Omicron mutations were concentrated in RBM tip we synthetized peptides with different lengths to analyze antibody production and binding affinity. First RBM including tip region were fragmented in three 15-mer peptides S1WT (^{464}FERDISTEIYQAGST^{478}) S2WT (^{476}GSTPCNGVEGFNCYF^{490}) and S3WT (^{489}YFPLQSYGFQPTNGV^{503}) (Figure 1c).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 7 November 2023 doi:10.20944/preprints202311.0296.v1

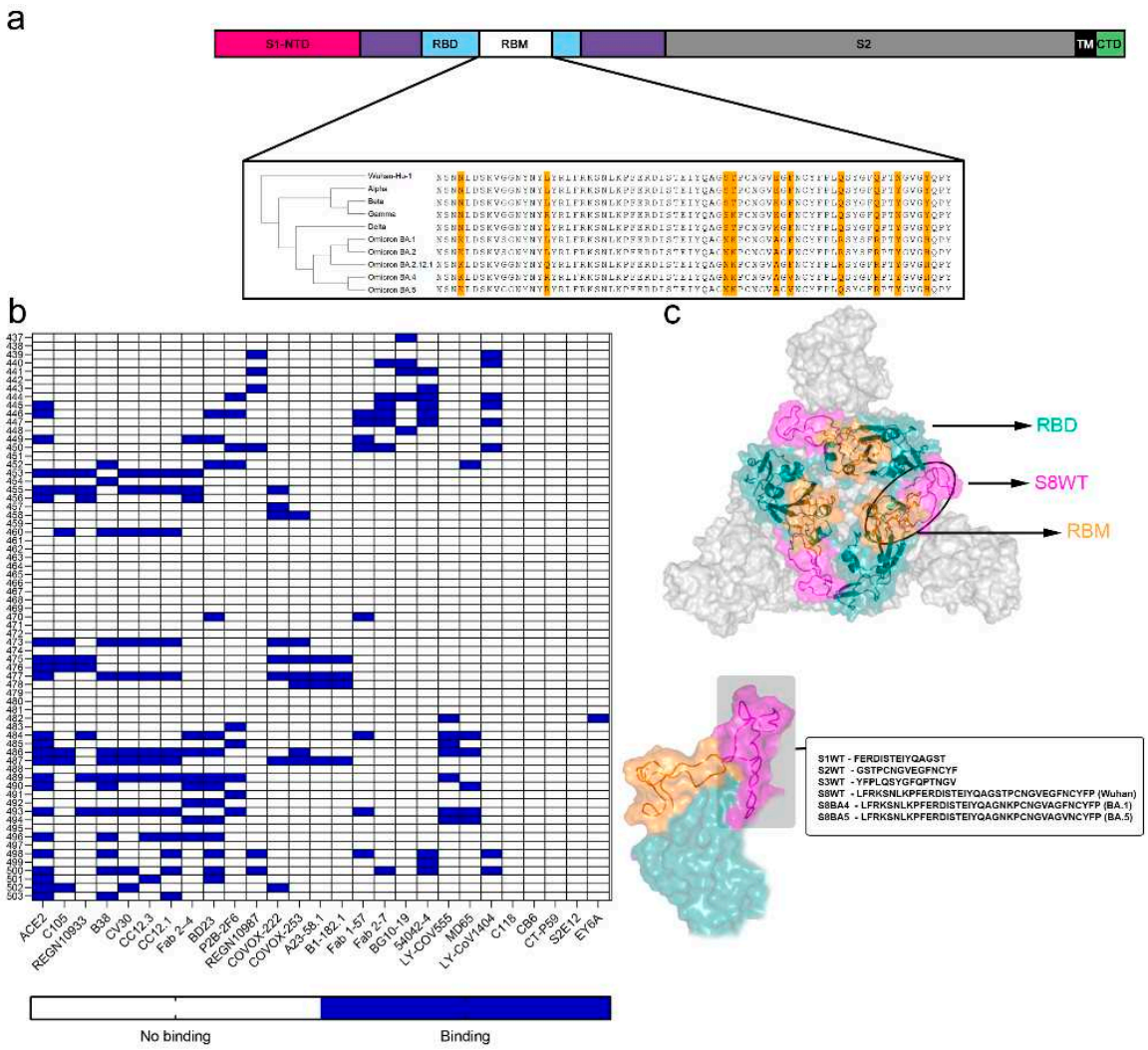

Figure 1. Structural organization of Spike protein and receptor binding motif (RBM), phylogenetic analysis of Variants of concern (VoCs) and interactions of nAbs to residues in RBM. (a) Organization of Spike protein domains and phylogenetic analysis of VoCs, highliting mutations (yellow) in RBM. (b) Interactions of RBM residues (437-503) with ACE-2 and human nAbs. (c) Tridimensional model of Spike protein trimer showing RBD (green), RBM (yellow) and the synthetic peptide S8WT (magenta) comprising the tip portion of RBM.

Seroreactivity of these three regions were tested by peptide ELISAS, using a serum panel of vaccinated individuals with one dose of Oxford/AstraZeneca (ChAdOx1-S) (Table S1) and four doses of heterologous boosting (Oxford/AstraZeneca - ChAdOx1-S; Pfizer-BioNTech - BNT162b2 or Janssen - Ad26.COV2.S) (Figure 2 and Table S2). A panel of pre-pandemic sera was used as control for the cut-off calculation. Few individuals responded for IgG to the RBM tip peptides, with reactivity index >1.1, seroreactivity for peptides S1WT and S2WT were similar for prime immunization 4/30 (13.3%) and for booster heterologous doses 1/7 (14.3%). Peptide S3WT had a lower performance with only two positive samples for first-dose vaccination and one for booster heterologous doses (Figure 2a). Although low antibody concentration, one open question is the affinity of produced Spike nAbs to these regions. Microscale thermophoresis (MST) was performed to measure the Kd of the peptide-antibody interactions. Specific purified antibodies to the RBM were separated using a recombinant protein affinity column (Figure S1), and then antibodies were tagged with a fluorescent probe, interactions of antibodies with different concentrations of peptides (S1WT and S2WT) were measured in glass capillaries and MST traces recorded (Figure S2). The dissociation constant (Kd) calculated from the dose-response curve was strikingly different from peptides S1WT (Kd= 640,73 nM) and

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 7 November 2023 doi:10.20944/preprints202311.0296.v1

S2WT (Kd=35 nM) (Figure 2b). Results demonstrated that the binding affinity of RBM antibodies to peptide S2WT was stronger. Aiming to increase the sensitivity of the analysis a 37-mer peptide was synthesized (S8WT, ^{452}LFRKSNLKPFERDISTEIYQAGSTPCNGVEGFNCYFP^{488}), also two other peptides from Omicron BA.1 (S8BA1, ^{452}LFRKSNLKPFERDISTEIYQAGNKPCNGVAGFNCYFP^{488}) and Omicron BA.5 (S8BA5, ^{452}LFRKSNLKPFERDISTEIYQAGNKPCNGVAGVNCYFP^{488}) included these VoCs mutations (Figures 1c). There was a slightly increase in seroreactivity for IgG of the first dose vaccination 6/30 (20%) for peptide S8WT in comparison with fragmented residues (13-14%) (Figures 2a and c). Mutation at the RBM region of Omicron BA.1 (S8BA1) and BA.5 (S8BA5) reduced both the reactivity index mean and the number of positive samples 4/30 and 2/30, respectively. IgM levels were low and significant different from IgG and IgA in one dose or heterologous dose vaccinated individuals (Figures 2c and d). Additionally, some individuals with one dose of vaccine, produced IgA response to the RBM tip of Spike wild type (5/30) and reduced response to Omicron BA.1 (2/30) and BA.5 (2/30) (Figure 2c). Analyzing the group of heterologous vaccination booster doses, only one sample had seroreactivity for IgG and IgA against wild-type sequence S8WT (Figure 2d). Mutations in Omicron variants reduced almost three times the reactivity index of this sample. Subclasses of IgG positive samples was also analyzed for the group with the first dose vaccination, a predominancy of IgG1 was found (5/12), but reactivity significantly decreased for Omicron variants (Figure S3).

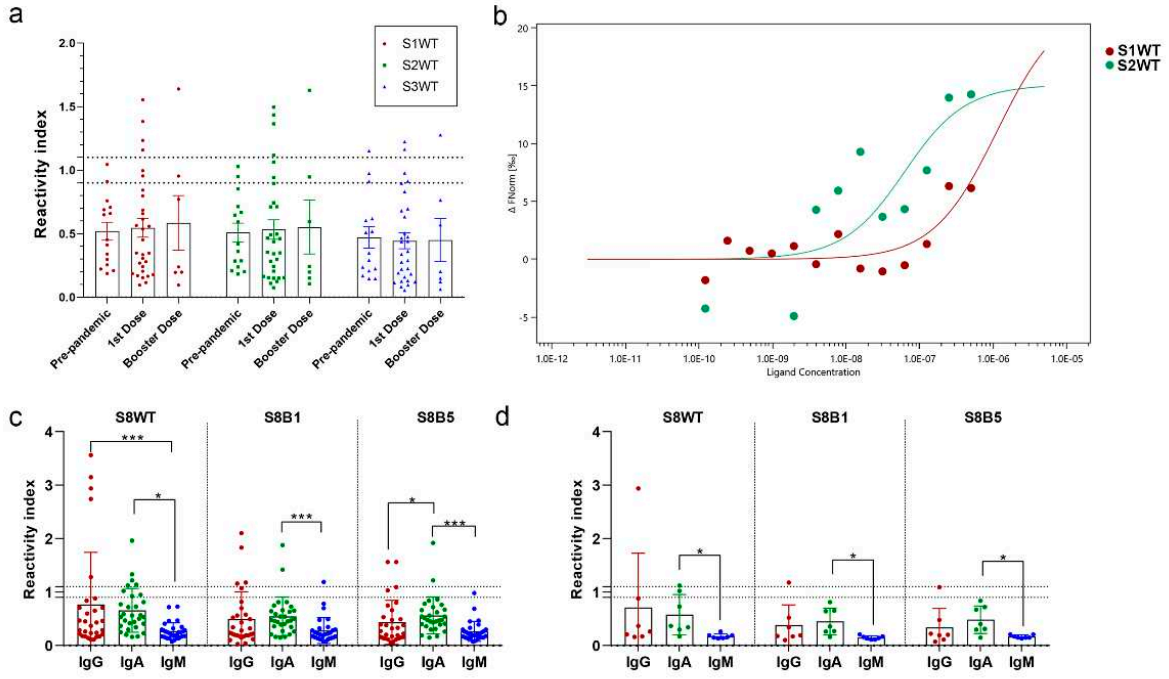

Figure 2. RBM tip humoral response and affinity in vaccinated individuals. (a) In-house peptide-ELISAS performed with peptides S1WT, S2WT and S3WT and a coort of pre-pandemic sera (n=15), individual vaccinated with the first dose of Oxford-AstraZeneca after 15 days (n=30) and booster heterologous doses (n=7). (b) Representative dose–response curve showing the binding of antibodies from vaccinated serum with peptides S1WT and S2WT, the Kd of 640.7 nM and 35 nM, respectivelly. A serial dilution of the peptide from 0.5 μg/mL–0.12 ng/mL was used. (c) Imunoglobulin classes reactivity of RBM peptides S8WT, S8B1 and S8B5 using the coort of vaccinated individuals with the first dose of Oxford-AstraZeneca (n=30) and (d) booster doses (n=7). A Kruskal–Wallis test was applied to identify statistical differences, followed by Dunn's multiple comparisons tests. A $p < 0.05$ was considered to be a significant difference. *p<0.05; **p<0.01 and ***p<0.001.

 doi:10.20944/preprints202311.0296.v1

4. Discussion

The humoral response against the Spike protein can be elicited through natural infection or immunization, and immune protection against symptomatic SARS-CoV-2 hinges on the levels of neutralizing antibodies [24]. It is suggested that nAbs against the Spike protein may be both low in quantity and of short duration due to the structural properties of coronaviruses [25]. Notably, the N-terminal domain and the receptor-binding domains are considered immunodominant and are the primary targets of nAbs. However, nAbs can also target regions such as the S2 stem helix (SH) and the S2 fusion peptide (FP) [17,26]. Antibodies that specifically bind to the RBD constitute over 90% of the neutralizing activity in convalescent sera [7].

nAbs exhibit four main classes based on the location of their epitopes within the Spike protein [3]. Classes 1 and 2 nAbs primarily target the RBD, including the receptor-binding motif, and can compete with ACE2 binding. Class 3 RBD nAbs bind to regions flanking the ACE2-binding region, while class 4 nAbs are highly conserved in the RBD but do not directly block ACE2-RBD binding [3]. Various monoclonal antibodies have been identified to bind to the RBM region [15]. However, there remains limited knowledge regarding the diversity of antibodies produced. The RBM region is pivotal for ACE2 receptor binding and comprises the knob (aa 444-449 and aa 496-505), base (aa 490-494 and aa 450-456), and tip (aa 473-489) [8]. Many nAbs against SARS-CoV-2 are found to bind to the tip of the RBM, particularly in or around the FNCY patch [15]. The MST analysis conduce in this study corroborates the significance of these residues in antibody binding affinity.

Immune evasion by variants of concern is primarily driven by mutations in the Spike protein that pose a threat to both natural and vaccine-induced immunity. The Omicron variant accumulates mutations in the RBM, including 10 mutations in this region. Some of these mutations directly interfere with ACE2 binding, affecting important residues in the RBM, such as those at positions Q493, Q498, N501, and Y505 [27,28]. A comparison of the immunogenicity of RBM, RBD, and the entire S protein reveals that the RBM region displays reduced seroreactivity [15,19]. However, immunization in mice with RBD or RBM vaccines has been shown to induce nAbs [29,30]. According to our data, adenovirus vaccines containing Spike mRNA, such as AstraZeneca-Oxford, or combined heterologous mRNA vaccines, seem to induce low titers of anti-RBM antibodies. The diminished sera reactivity observed in our study may be attributed to MHC-II restriction [31]. A study examining common MHC-II alleles predicted poor binding for the RBM, suggesting a lack of MHC-II support in T-B cooperation, which impacts the production of nAbs in the general population [31]. Effective antibody responses necessitate cooperation between a B cell and a CD4 T cell (helper cell) activated by an epitope on the same antigen recognized by the B cell (T-B cooperation). Nonetheless, T-B cooperation is limited by the presence of Major Histocompatibility Complex class II (MHC-II) molecules [32]. Thus, the study of B and T cell epitopes and the human leukocyte antigen (HLA) polymorphism in different populations is relevant for vaccines design [33]. Recent study demonstrated that the variability of both humoral and cellular responses to the mRNA vaccine is influenced by the HLA profile. An association was found between HLA haplotype and high antibody concentration and or low humoral responses to Spike antigens [34]. Another study revealed that variation in humoral responses against SARS-CoV-2 spike and the RBD at 28 days after first vaccination (ChAdOx1-S) shows genome-wide significant association with major histocompatibility complex (MHC) class II alleles [35].

5. Conclusions

The humoral response to SARS-CoV-2 infection and vaccination primarily targets the receptor-binding domain (RBD) of the Spike protein. Despite the production of neutralizing antibodies (nAbs) directed to Spike, the immunodominance of the RBD region does not guarantee long-term protective immunity. The emergence of SARS-CoV-2 variants, such as the Omicron variant, with mutations in the RBD, presents a challenge to the efficacy of current vaccines. Our findings suggest that the RBM region, crucial for viral entry and ACE2 interaction, displays reduced seroreactivity, potentially impacting the production of nAbs in vaccinated individuals. The interplay between B and T cell epitopes, the MHC-II restriction, and HLA polymorphism underscores the need for comprehensive

 doi:10.20944/preprints202311.0296.v1

population-based studies for effective vaccine design. Understanding the complexities of the humoral response and the impact of genetic variability on vaccine efficacy is essential for developing robust and inclusive vaccination strategies against SARS-CoV-2 and other evolving coronaviruses.

6. Patents

The antigenic peptides described in this study are protected under Brazilian and US provisional patent applications BR 10.2019.017792.6 and PCT/BR2020/ 050341, respectively, filed by FIOCRUZ. They may serve as a future source of funding.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. **Figure S1.** Purification of human antibodies against the RBM region of the SARS-CoV-2 spike protein using a recombinant RBM Sepharose 4B affinity column (3 x 1 cm, inner diameter) (a) Evaluation of absorbance at 280 nm of the eluate different fractions, highlighted in green the eluate fractions used to concentrate polyclonal antibodies (b) In-house ELISA showing the specificity of purified antibodies to the multiepitope Dx-SARS-RBD containing RBM fragments. **Figure S2**: Microscale thermophoresis (MST) traces of anti-RBM antibodies binding to different concentrations of S1WT (red) and S2WT (green) peptides by MST. Relative fluorescence (RF) between the bound and unbound state was determined over a time of 21s with 20s MST-on time for evaluation. The blue bar indicates the ΔRF before the temperature gradient was applied, whereas the red bar shows the ΔRF during the thermophoresis. For interaction experiments, the amount of NT.647-labeled antibodies was kept constant, while the concentration of unlabeled peptides varied from 0.5 μg/mL–0.12 ng/mL. The assay was performed in PBS containing 0.05% Tween 20 and after a short incubation period, the samples were analyzed in standard glass MST NT.115 capillaries. **Table S1:** One-dose regime AstraZeneca-Oxford vaccinated serum information. **Table S2**: Heterologous booster dose vaccinated serum information. **Figure S3**: IgG subclass neutralizing response against RBM peptides in vaccinated sera. Subclass Immunoglobulin subclasses reactivity of RBM peptides S1WT, S2WT, S8WT, S8BA1, and S8BA5 using a cohort of vaccinated individuals positives for IgG and with the first dose of Oxford-AstraZeneca (n=12) and booster doses. For analysis purposes were done multiple comparisons using Tukey's multiple comparisons test where a $p < 0.05$ was considered a significant difference (* = $p < 0.05$).

Author Contributions: Conceptualization, S.G.D-S.; G.C.L.; C.M.M.; methodology, M.E.S.M.; L.R.G.; J.P.R. S.C.; P.N-P. software, M.E.S.M.; G.C.L.; D.W.P.; formal analysis and data curation, G.C.L.; writing—original draft preparation, M.E.S.M; G.C.L.; writing—review and editing, G.C.L. and S.G.D.-S.; project administration, S.G.D.-S.; funding acquisition, C.M.M; S..G.D.-S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Carlos Chagas Filho Foundation for Research Support of the State of Rio de Janeiro/FAPERJ (#110.198-13; #SEI21078002021; #SEI26000306611722021) and the Brazilian Council for Scientific Research (CNPq, #467.488/2014-2 and #301744/2019-0). Funding was also provided by FAPERJ (#210.003/2018) through the National Institutes of Science and Technology Program (INCT) to C.M.M. (INCT-IDPN).

Institutional Review Board Statement: The study was approved by the Human Research Ethical Committee of the Oswaldo Cruz Institute/FIOCRUZ (CAAE:49971421.8.0000.5248), the University of Estacio de Sá (CAAE: 3309 0820.8. 0000.5284) and UNIGRANRIO (CAAE: 21362220.1.0000.5283) study center ethics committee and conducted under good clinical practice and applicable regulatory requirements, including the Declaration of Helsinki.

References

1. Pal, M.; Berhanu, G.; Desalegn, C.; Kandi, V. Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2): An Update. *Cureus* **2020,** *e7423*. doi:10.7759/cureus.7423.
2. Lenharo, M. WHO declares end to COVID-19's emergency phase. *Nature* 2023, *d41586-023-01559-z*. doi:10.1038/d41586-023-01559-z.
3. Harvey, W.T.; Carabelli, A.M.; Jackson, B.; Gupta, R.K.; Thomson, E.C.; Harrison, E.M.; Ludden, C.; Reeve, R.; Rambaut, A.; COVID-19 genomics UK (COG-UK) Consortium; et al. SARS-CoV-2 variants, spike mutations and immune escape. *Nat Rev Microbiol* **2021**, *19*, 409–424, doi:10.1038/s41579-021-00573-0.

4. Liu, L.; Iketani, S.; Guo, Y.; Chan, J.F.-W.; Wang, M.; Liu, L.; Luo, Y.; Chu, H.; Huang, Y.; Nair, M.S.; et al. Striking antibody evasion manifested by the omicron variant of SARS-CoV-2. *Nature* **2022,** *602*, 676–681, doi:10.1038/s41586-021-04388-0.
5. Chi, X.; Yan, R.; Zhang, J.; Zhang, G.; Zhang, Y.; Hao, M.; Zhang, Z.; Fan, P.; Dong, Y.; Yang, Y.; et al. A Neutralizing human antibody binds to the n-terminal domain of the spike protein of SARS-CoV-2. *Science* **2020**, *369*, 650–655, doi:10.1126/science. abc6952.
6. Huang, Q.; Han, X.; Yan, J. Structure-based neutralizing mechanisms for SARS-CoV-2 antibodies. *Emerg Microbes Infect* **2022**, *11*, 2412–2422. doi:10.1080/22221751.2022.2125348.
7. Piccoli, L.; Park, Y.-J.; Tortorici, M.A.; Czudnochowski, N.; Walls, A.C.; Beltramello, M.; Silacci-Fregni, C.; Pinto, D.; Rosen, L.E.; Bowen, J.E.; et al. Mapping neutralizing and immunodominant sites on the SARS-CoV-2 Spike receptor-binding domain by structure-guided high-resolution serology. *Cell* **2020**, *183*, 1024-1042.e21, doi:10.1016/j.cell.2020.09.037.
8. Deshpande, A.; Harris, B.D.; Martinez-Sobrido, L.; Kobie, J.J.; Walter, M.R. Epitope classification and RBD binding properties of neutralizing antibodies against SARS-CoV-2 variants of concern. *Front Immuno* **2021**, *12*, 691715, doi:10.3389/ fimmu.202 1.691715.
9. Gasser, R.; Cloutier, M.; Prévost, J.; Fink, C.; Ducas, É.; Ding, S.; Dussault, N.; Landry, P.; Tremblay, T.; Laforce-Lavoie, A.; et al. Major Role of IgM in the Neutralizing Activity of Convalescent Plasma against SARS-CoV-2. Cell Rep 2021, 34, 108790, doi:10.1016/j.celrep.2021.108790.
10. Long, Q.-X.; Liu, B.-Z.; Deng, H.-J.; Wu, G.-C.; Deng, K.; Chen, Y.-K.; Liao, P.; Qiu, J.-F.; Lin, Y.; Cai, X.-F.; et al. Antibody responses to SARS-CoV-2 in patients with COVID-19. *Nat Med* 2020, *26*, 845–848, doi:10.1038/s41591-020-0897-1.
11. Schroeder, H.W.; Cavacini, L. Structure and function of immunoglobulins. *J Allergy Clin Immunol* **2010,** *125*, S41–S52, doi:10.1016/ j.jaci.2009.09.046.
12. Ferrante, A.; Beard, L.J.; Feldman, R.G. IgG subclass distribution of antibodies to bacterial and viral antigens. *Ped Infect Dis J* **1990,** *9*, 516.
13. Korobova, Z.R.; Zueva, E.V.; Arsentieva, N.A.; Batsunov, O.K.; Liubimova, N.E.; Khamitova, I.V.; Kuznetsova, R.N.; Rubinstein, A.A.; Savin, T.V.; Stanevich, O.V.; et al. Changes in anti-SARS-CoV-2 IgG subclasses over time and in association with disease severity. *Viruses* **2022**, *14*, 941, doi:10.3390/v14050941.
14. Rubio-Casillas, A.; Redwan, E.M.; Uversky, V.N. Does SARS-CoV-2 induce IgG4 synthesis to evade the immune system? *Biomolecules* **2023,** *13*, 1338. https://doi.org/10.3390/biom13091338
15. Almanza, G.; Clark, A.E.; Kouznetsova, V.; Olmedillas, E.; Castro, A.; Tsigelny, I.F.; Wu, Y.; Gao, G.F.; Leibel, S.L.; Bray, W.; et al. Structure-selected RBM immunogens prime polyclonal memory responses that neutralize SARS-CoV-2 variants of concern. *PLoS Pathog* **2022**, *18*, e1010686, doi:10.1371/journal.ppat.1010686.
16. Pratesi, F.; Errante, F.; Pacini, L.; Peña-Moreno, I.C.; Quiceno, S.; Carotenuto, A.; Balam, S.; Konaté, D.; Diakité, M.M.; Aréva-lo-Herrera, M.; et al. A SARS–CoV-2 spike receptor binding motif peptide induces anti-spike antibodies in mice and is recognized by COVID-19 patients. *Front. Immunol* **2022**, *13*, 879946, doi:10.3389/fimmu.2022.879946.
17. Chen, Y.; Zhao, X.; Zhou, H.; Zhu, H.; Jiang, S.; Wang, P. Broadly neutralizing antibodies to SARS-CoV-2 and other human coronaviruses. *Nat Rev Immunol* **2023,** *23*, 189–199, doi:10.1038/s41577-022-00784-3.
18. Liu, Y.; Liu, J.; Plante, K.S.; Plante, J.A.; Xie, X.; Zhang, X.; Ku, Z.; An, Z.; Scharton, D.; Schindewolf, C.; et al. The N501Y spike substitution enhances SARS-CoV-2 transmission. *Nature* **2022,** *602*, 294-299. doi: 10.1038/s41586-021-04245-0.
19. Bachmann, M.F.; Mohsen, M.O.; Zha, L.; Vogel, M.; Speiser, D.E. SARS-CoV-2 Structural features may explain limited neutralizing-antibody responses. *npj Vaccines* **2021**, *6*, 1–5, doi:10.1038/s41541-020-00264-6.
20. De-Simone, S.G.; Gomes, L.R.; Napoleão-Pêgo, P.; Lechuga, G.C.; de Pina, J.S.; da Silva, F.R. Epitope mapping of the diphtheria toxin and development of an ELISA-specific diagnostic assay. *Vaccines* (Basel) **2021**, *9*, 313. doi: 10.3390/vaccines9040313.
21. Gomes, L.R.; Durans, A.M.; Napoleão-Pêgo, P.; Waterman, J.A.; Freitas, M.S.; De Sá, N.B.R.; Pereira, L.V.; Furtado, J.S.; Aquino, R.G.; Machado, M.C.R.; et al. Multiepitope proteins for the differential detection of IgG antibodies against RBD of the spike protein and non-RBD regions of SARS-CoV-2. *Vaccines* (Basel) **2021**, *9*, 986, doi:10.3390/vaccines9090986.

22. Lechuga, G.C.; Napoleão-Pêgo, P.; Bottino, C.C.G.; Pinho, R.T.; Provance-Jr, D.W.; De-Simone, S.G. Trypanosoma cruzi prese-nilin-like transmembrane aspartyl protease: characterization and cellular localization. *Biomolecules* **2020**, *10,* 1564, doi:10.3390/ biom10111564.
23. De-Simone, S.G.; Nascimento, H.J.; Prado, I.C.; Aguiar, A.S.; Melgarejo, A.R.; Pina, J.L.S.; Ferreira, P.F.; Provance, D.W. Puri-fication of equine IgG3 by lectin affinity and an interaction analysis via microscale thermophoresis. *Anal Biochem* **2018,** *561–562,* 27–31, doi:10.1016/j.ab.2018.09.010.
24. Khoury, D.S.; Cromer, D.; Reynaldi, A.; Schlub, T.E.; Wheatley, A.K.; Juno, J.A.; Subbarao, K.; Kent, S.J.; Triccas, J.A.; Davenport, M.P. Neutralizing antibody levels are highly predictive of immune protection from symptomatic SARS-CoV-2 infection. *Nat Med* **2021,** *27,* 1205–1211, doi:10.1038/s41591-021-01377-8.
25. Bachmann, M.F.; Mohsen, M.O.; Zha, L.; Vogel, M.; Speiser, D.E. SARS-CoV-2 Structural features may explain limited neutralizing-antibody responses. *npj Vaccines* **2021**, *6,* 1–5, doi:10.1038/s41541-020-00264-6.
26. Li, Y.; Lai, D.; Zhang, H.; Jiang, H.; Tian, X.; Ma, M.; Qi, H.; Meng, Q.; Guo, S.; Wu, Y.; et al. Linear epitopes of SARS-CoV-2 spike protein elicit neutralizing antibodies in COVID-19 patients. *Cell Mol Immunol* **2020**, *17*, 1095–1097, doi:10.1038/s41423-020-00523-5.
27. Sun, C.; Xie, C.; Bu, G.-L.; Zhong, L.-Y.; Zeng, M.-S. Molecular characteristics, immune evasion, and impact of SARS-CoV-2 variants. *Sig Transduct Target Ther* **2022**, *7*, 202, doi:10.1038/s41392-022-01039-2.
28. Lupala, C.S.; Ye, Y.; Chen, H.; Su, X.-D.; Liu, H. Mutations on RBD of SARS-CoV-2 Omicron variant result in stronger binding to human ACE2 receptor. *Biochem Biophys Res Communi* **2022**, *590*, 34–41, doi:10.1016/j.bbrc.2021.12.079.
29. Chen, W.-H.; Hotez, P.J.; Bottazzi, M.E. Potential for developing a SARS-CoV receptor-binding domain (RBD) recombinant protein as a heterologous human vaccine against coronavirus infectious disease (COVID)-19. *Hum Vaccine Immunother* **2020**, *16,* 1239–1242, doi:10.1080/21645515.2020.1740560.
30. Liu, X.; Chang, X.; Rothen, D.; Derveni, M.; Krenger, P.; Roongta, S.; Wright, E.; Vogel, M.; Tars, K.; Mohsen, M.O.; et al. AP205 VLPs based on dimerized capsid proteins accommodate RBM domain of SARS-CoV-2 and serve as an attractive vaccine candidate. *Vaccines* 2021, *9,* 403, doi:10.3390/vaccines9040403.
31. Castro, A.; Ozturk, K.; Zanetti, M.; Carter, H. In silico analysis suggests less effective MHC-II Presentation of SARS-CoV-2 RBM Peptides: Implication for neutralizing antibody responses. *PLoS ONE* ***2021****, 16,* e0246731, doi:10.1371/journa.pone. 0246731.
32. Vainio, O.; Toivanen, P.; Toivanen, A. Major histocompatibility complex and cell cooperation. *Poul Sci* **1987**, *66,* 795–801, doi:10. 3382/ps.0660795.
33. Lani, R.; Senin, N.A.; AbuBakar, S.; Hassandarvish, P. Knowledge of SARS-CoV-2 epitopes and population HLA types is important in the design of COVID-19 vaccines. *Vaccines* **2022**, *10,* 1606. https://doi.org/10.3390/vaccines10101606
34. Bertinetto, F. E.; Magistroni, P.; Mazzola; G. A., Costa, C.; Elena, G.; Alizzi, S.; Scozzari, G.; Migliore, E.; Galassi, C.; Ciccone, G.; et al. The humoral and cellular response to mRNA SARS-CoV-2 vaccine is influenced by HLA polymorphisms. *HLA* **2023**, *102,* 301–315. https://doi.org/10.1111/tan.15049
35. Mentzer, A. J.; Connor, D., Bibi, S., Chelysheva, I., Clutterbuck, E. A., Demissie, T., Dinesh, T., Edwards, N. J., Felle, S., Feng, S., et al. Human leukocyte antigen alleles associate with COVID-19 vaccine immunogenicity and risk of breakthrough infection. *Nat Med* **2023**, *29*, 147–157. https://doi.org/10.1038/s41591-022-02078-6