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Determination of SARS-CoV-2 Spike Protein Epitopes Recognized by Pooled IgGs from COVID-19 Plasma

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Abstract

COVID-19 has brought an unprecedented health crisis around the world. This study aimed to reveal peptide epitopes on the SARS-CoV-2 spike antigen targeted by IgGs in COVID-19 convalescent and vaccine plasma to better understand immune responses to COVID-19 infection and vaccination. Peptide epitopes were isolated at picogram level by passing a synthetic peptide library mixture of SARS-CoV-2 spike protein through columns with immobilized IgGs isolated from blood donated by COVID-19 convalescent and vaccinated individuals in a rural community. Multiple spike antigen peptide epitopes were identified by mass spectrometry based proteomic analysis and about two thirds of the epitopes had specific binding with both the convalescent IgGs and the vaccine IgGs. The 3D mapping of the epitopes demonstrated polyclonal nature of COVID-19 IgGs which recognized broad regions of the SARS-CoV-2 spike antigen.

Keywords: COVID-19 convalescent plasma**,** COVID-19 vaccine plasma**,** SARS-CoV-2spike protein**,** Epitope**,** Mass spectrometry

1. Introduction

Coronavirus disease 2019 (COVID-19) was first identified in Wuhan China in December 2019 and quickly evolved into a worldwide pandemic within a few months. Individual COVID-19 patients have shown unpredictable wide range of clinical manifestation from no symptom to severe illness such as dyspnea, hypoxia and multi-organ dysfunction that needs hospitalization or ICU admission.Coronaviruses are a family of linear positive single-stranded RNA viruses packed as coiled helical nucleocapsid inside spherical envelope covered with crown-like spikes. The causative agent for COVID-19 is a novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Zhou et al., 2020). Genomic analysis indicates SARS-CoV-2 is zoonotic and probably transmitted to human through some intermediate hosts from bats being the original animal reservoir(Andersen et al., 2020). The threat from future coronavirus epidemic is unpredictable as there are hundreds of known coronaviruses circulating among animals and some may adapt to new hosts and eventually jump to human, like SARS-CoV-2. Currently there is still no specific antiviral treatment available for COVID-19, but fortunately two mRNA-based vaccines, BNT162b2 from Pfizer-BioNTech and mRNA-1273 from Moderna, as well as an adenovirus-based DNA vaccine, Ad26.COV2.S from Johnson & Johnson, have been approved by FDA with emergency use authorization (EUA) in USA. Efficacy for preventing COVID-19 illness from phase 3 clinical trials was 95%, 94 % and 66% for BNT162b2,mRNA-1273 and Ad26.COV2.S, respectively(Baden et al., 2020; Polack et al., 2020; Sadoff et al., 2021).

The genomic RNA (gRNA) of SARS-CoV-2 encodes 14 open reading frames for 29 proteins (Gordon et al., 2020). The spike (S) protein forms the spikes protruding from the virion surface that gives the virion a crown-like appearance. The receptor binding domain (RBD) of the S protein (AA330-530) specifically binds the angiotensin-converting enzyme 2 (ACE2) receptor on host cells, which initiates virus fusion with the host cell and virus uptake(Yan et al., 2020). Therefore, the S antigen is the target immunogen for Moderna, Pfizer-BioNTech, and J&J vaccines, which deliver mRNA or DNA of the S antigen. When human is infected with SARS-CoV-2, a variety of antibodies are produced by plasma B cells against multiple epitopes on virus proteins as adaptive immune response. In general, only a subset of induced antibodies can neutralize virus and block infection, therefore, classified as neutralizing antibodies (NAbs). Occasionally, no NAbs can be induced to effectively prevent virus infection as seen in vaccine development for Hepatitis C virus (HCV) and human immunodeficiency virus (HIV) over many years of investigation (Burton et al., 2012; Klein et al., 2013).

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Some antibodies might even facilitate viral entry and enhance host infection(Wan et al., 2020). Therefore, mapping antibody epitopes in immune response to SARS-CoV-2 infection is important to understand the immune response to infection at molecular level and provide advice for effective vaccination to prevent possible repeated waves of infection with SARS-CoV-2 new variants.

Since the outbreak of COVID-19, multiple reports have been published on study of SARS-CoV-2 epitopes. Those reported epitopes were either predicted using bioinformatics tools, or experimentally identified through ligand binding immunoassays(Ahmed et al., 2020; Baruah & Bose, 2020; Grifoni et al., 2020; Tilocca et al., 2020; Wang et al., 2020). We experimentally identified SARS-CoV-2 S antigen peptide epitopes though a different strategy. We pulled down epitopes from a synthetic peptide library mixture spanning the entire SARS-CoV-2 S protein through affinity columns packed with immobilized IgG antibodies isolated from blood donated by COVID-19 convalescent and vaccinated individuals, and then identified peptide epitopes through high performance liquid chromatography-mass spectrometry (HPLC-MS) shotgun proteomic analysis. We also mapped the identified S antigen epitopes on the 3D structure of SARS-CoV-2 S protein trimer.

2. Material and methods

2.1. Preparation of COVID-19 convalescent plasma and vaccine plasma

A rapid point-of-care (POC) serological IgG/IgM cassette (Biohit) targeted at SARS-CoV-2 nucleocapsid antigen (anti-N) was used for COVID-19 convalescent antibody screening following the instruction of the manufacturer. Another IgG/IgM cassette (CoronaCHEK) targeted at SARS-CoV-2 S antigen (anti-S) was used for screening of COVID-19 vaccinated individuals. Both were FDA EUA granted colloidal gold based lateral flow immunochromatographic assay. EDTA blood samples were collected from ten eligible donors in each group. Criteria for convalescent blood donor selection include: 1) SARS-CoV-2 anti-N IgG positive with or without documented positive COVID-19 diagnostic test; 2) complete resolution of COVID-19 symptoms at least 14 days before the donation. Criteria for vaccinated blood donor include: 1) complete COVID-19 vaccination and tested positive for anti-S IgG; 2) No prior known COVID-19 infection or hospitalization. All donors were adult (>18 years old) males or females who are not pregnant. A consent form was signed by each donor before blood draw. Protocol and consent for blood collection were approved by Cape Fear Valley Health System IRB committee.

Blood was centrifuged at 2000 × g for 10 minutes at room temperature. Tri(n-butyl) phosphate (Sigma) and Triton X-100 (Fisher Scientific) were added to the harvest plasma to a final concentration of 1% and incubated at room temperature for one hour to inactivate possible enveloped viruses. Aliquoted plasma was stored at -20 ^{o}C prior to use. Blood samples were processed in a biosafety level 2 laboratory with appropriate PPE protection.Anti-N IgGs in each convalescent plasma were confirmed and determined with a commercial quantitative ELISA kit (MP Biomedicals). SARS-CoV-2 NAbs were determined with ELISA based cPass surrogate virus neutralization test (sVNT) (GenScript).

2.2. COVID-19 IgGs purification

Total IgGs were isolated from convalescent and vaccine plasma samples using an AKTA FPLC system (Amersham Biosciences). Thawed plasma was centrifuged at 10, 000 × g for 5 minutes at room temperature and 3 mL clean supernatant was diluted with 12 mL loading/wash buffer, 0.02 M sodium phosphate, pH 7.0. The mixture was filtered through a 0.45 μm cellulose acetate syringe filter before loading onto a 5 mL HiTrap Protein G affinity column (Cytiva) pre-equilibrated with the loading/wash buffer. Column was then washed with 5 column volume (CV) loading buffer, eluted with 5 CV elution buffer, 0.1 M glycine-HCl, pH 2.7, and finally re-equilibrated with 10 CV loading buffer. Elution peak was collected by a fraction collector into tubes containing 100 μL neutralization buffer, 1 M sodium phosphate, pH 9.0 for each 1 mL fraction. Fractions 1-9 were combined and concentrated to approximately 0.5 Ml with Amicon Ultra centrifugal filter, Ultra-15, MWCO 30 kDa (Millipore Sigma). Protein was quantified by directly measuring 280nm absorbance using Synergy H1 microplate reader (BioTek). The concentrated total IgGs from ten convalescent plasma were combined as one pool. Total IgGs isolated from ten vaccine plasma were combined as another pool. Negative control IgGs were also isolated from a human plasma collected prior to the COVID-19 outbreak that was confirmed being anti-N and anti-S negative.

2.3. IgG column preparation

A Convalescent-IgG column, a Vaccine-IgG column, and a Negativecontrol-IgG column were prepared by immobilizing aliquots of pooled IgGs isolated from ten convalescent plasma, ten vaccine plasma, and a negative human plasma collected prior to the COVID-19 outbreak. Prior to column immobilization, IgGs obtained in step *2.2* were buffer exchanged by ×25 diluting with loadingbuffer and then concentrating back to the initial volume. This was repeated two more times to reduce glycine concentration to below 1 μM as glycine competes with IgGsfor protein coupling reaction.

Aliquots of buffer exchanged IgGs was immobilized onto a 1 mL Hitrap HP NHS-activated agarose column (Cytiva) following the protocol recommended by the vendor. In brief, aliquot containing 60 mg pooled IgGs was diluted with equal volume of coupling buffer, 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.0. Hitrap NHS column was opened and washed with 6 mL ice-cold 1 mM HCl immediately. Then IgG aliquot was applied onto the column. The column was sealed and incubated for 30 minutes at room temperature, and then washed with 3 × 2 ml of deactivation buffer A, 0.5 M Tris, 0.5 M NaCl, pH 8.3, followed by 3 × 2 ml of deactivation buffer B, 0.1 M sodium acetate, 0.5 M NaCl, pH 4. The column was then incubated in deactivation buffer A for 30 min to quench remaining active sites before the washing was repeated two more times. The column was finally washed with 5 mL of loading buffer and used directly for affinity purification of peptide epitopes or equilibrated with PBS buffer containing 0.05% sodium azide and stored at 4°C. Columns prepared were tested to purify COVID-19 nucleocapsid (N) antigen from a mixture of 20 μg recombinant SARS-CoV-2 N protein (Thermo Scientific) and 200 μg bovine serum albumin (BSA)(Sigma) in 1 mL loading buffer following the affinity purification procedure described in section *2.4*. Flowthrough, wash, and elution fractions were concentrated to 35-45μL with 10KD MWCO Amicon Ultra-0.5 and were analyzed with SDS-PAGE.

2.4. Affinity isolation of SARS-CoV-2 S peptide epitopes

Three IgG columns prepared were pre-equilibrated with loading/wash buffer, 0.02 M sodium phosphate, pH 7.0, immediately prior to use. A total 100 μg of a peptide library (GenScript) containing 316 synthetic peptides (15mers with 11 amino acid overlap) covering the entire SARS-CoV-2 S protein in 1 mL loading buffer was loaded to each IgG column. The columns were sealed and incubated at room temperature for 30 min, washed with 20 CV wash buffer, eluted with 5 CV elution buffer, and thenre-equilibrated with 10 CV loading buffer. Flowthrough, wash, and elutioncollected in 1.25-mL fractions were concentrated in a SpeedVac to dryness. Each fraction was reconstituted with 30 μL 2% acetonitrile in water, 0.1% formic acid. Samples were stored at -20 °C prior to HPLC-MS analysis.

2.5. Peptide HPLC-MS analysis

An FPLC (Shimadzu) with a ZORBAX 300Extend-C18 3.5 μm, 0.3×100 mm column (Agilent) coupled to a LTQ ion trap MS (Thermo Fisher Scientific) was used to generate low resolution MS data. A splitter was used to deliver mobile phase at a flow rate of approximately 8 μL/min. Mobile phase A was water, 0.1% formic acid. Mobile phase B was acetonitrile, 0.1% formic acid. HPLC was set at 5% B for injection and held on for 15 min to desalt, followed by gradient elution from 5%-25% B for 40 min, and then 95% B for 5 min before set back to 5% B for 9 min to re-equilibrate the column. Injection volume was 5 uL. The LTQ MS was equipped with an ESI probe set at positive mode with the following parameters: sheath gas 8; aux gas 2; sweep gas 1; spray voltage 3.90 kV; capillary temperature 275 °C; full MS in 300-2000 m/z followed by three collision induced dissociation (CID) data-dependent scan acquisition (DDA). Dynamic exclusion was set for 30 s after a precursor was scanned for MS/MS. High resolution MS data were generated using an Orbitrap Exploris 480 Mass Spectrometer equipped with a C18 nanoLC(Thermo Fisher Scientific) at the Molecular Education, Technology, andResearch Innovation Center (METRIC) of North Carolina State University. Samples (2uL) were injectedand separatedby a 50 min linear gradient elution at 300 nl/min from 2% - 50% mobilephase B with a 10 min online desalt prior to the elution. The Orbitrap was operated as follows: positive ion mode,acquisitionfull scan (m/z 300–1,600) with 120,000 resolving power, DDA MS/MS acquisition at 15,000 resolving power implementing higherenergy collisional dissociation (HCD) with a normalized collision energy setting of 25% with cycle time of 1.5 sec. Dynamicexclusion was set for 20 s after a precursor was scanned for MS/MS. The HPLC-MS raw data files were searched for peptide identification using MSFragger 3.3, an open-source proteomics database search tool (Kong et al., 2017). A FASTA database was generated by combining the SARS-CoV-2 S protein sequence in the virus reference genome downloaded from NCBI (Accession number: NC_045512.2) with common contaminants and decoyed sequences. The default non-specific search was performed with non-enzymatic digestion option and peptide length of 15 plus variable modification for methionine oxidation. Mass tolerance was set at ±1Da and ± 50ppm for LTQ and Orbitrap mass spectral data, respectively. Peptides and proteins were assembled from the MSFraggeroutput .pepXML files using IDPicker 3.1 with 2% protein FDR threshold(Ma et al., 2009).

2.6. SARS-CoV-2 S epitope 3D visualization

SARS-CoV-2 S antigen peptide epitopes identified from HPLC-MS were displayed as highlights on SARS-CoV-2 S protein trimer 3D structure using Cn3D software, an open-source macromolecular structure viewer(Wang et al., 2000). Structural data of SARS-CoV-2 S protein trimer was retrieved from protein databank (PDB ID: 6VXX) (Walls et al., 2020).

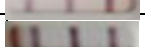
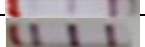
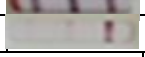
3. Results and Discussion

3.1. COVID-19 plasma samples

3.1.1. Convalescent donor and plasma

We conducted serologic survey of COVID-19 prevalence in rural Harnett County, North Carolina since the start of the pandemic. Among 420 participants tested between September 2020 to March 2021, 14% of the cohort were anti-N IgG/IgM positive and almost 30% of individuals that tested positive had no prior known exposure or infection(Subramanian et al., 2021). Convalescent plasma samples were prepared from blood collected from ten donors in the cohort who were screened positive for COVID-19 anti-N IgG antibodies. Information about the donors and the corresponding test results were shown in Table 1. Anti-N IgGs were confirmed for all plasma samples collected using a commercial quantitative ELISA assay and the measured concentrations were also listed in the table. Some donors had stronger COVID-19 antibody signals than others, but no obvious correlation was found between the antibody level and age, gender, COVID-19 symptom severity, or the time past the confirmed infection. In addition, it was unknown whether any donors had repeat exposure or infection before the serological screening. Nine of the ten convalescent plasma samples showed >30% inhibition of interaction between SARS-CoV-2 S-RBD and human ACE2 in the sVNT assay, thus positive in COVID-19 NAbs, and contained anti-S IgGs. Plasma #8 in Table 1,like the negative control plasma, was negative in COVID-19 NAbasas its neutralization inhibition was far below 30% - the assay cutoff for positive NAb. Meanwhile, plasma #8 also had lowest amount of anti-N IgGs among ten convalescent plasmas, so it might contain NAbs at a much lower concentration which was not detectable for the sVNT assay.

Table 1 Attributes of ten COVID-19 anti-N IgG positive blood donors.

No.	Collection Date	Gender	Age	COVID-19 symptoms in last 3 months	[a]Test image	Anti-N IgG μg/mL	[b]Neutralizing Inhibition, %	[c]SARS-CoV-2 NAb
1	9/14/2020	F	33	none		8	64.9±0.6	Positive
2	10/30/2020	M	46	none		10	81.3±0.2	Positive
3	11/10/2020	F	68	none		11	98.15±0.06	Positive
4	11/18/2020	F	28	none		6	98.07±0.06	Positive
5	11/19/2020	F	46	none		9	96.2±0.2	Positive
6	11/23/2020	F	52	cough		6	70±1	Positive
7	11/27/2020	M	69	cough, dyspnea, fever		15	97.77±0.03	Positive
8	11/27/2020	F	25	cough		0.5	2.5±0.9	Negative
9	11/27/2020	F	54	dyspnea		11	94.53±0.06	Positive
10	12/1/2020	M	65	none		1.5	50.6±0.1	Positive
Negative control						not detected	2±2	Negative

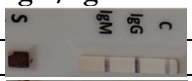
[a] Line C-control, Line G-IgG, Line M-IgM;
[b]cPass assay, mean and SD, n=2;
[c]NAb positive cutoff: neutralizing inhibition > 30%.

3.1.2.Vaccinated donor and plasma

Table 2 showed donor information and anti-S IgG/IgM test results for 22 participants who had completed COVID-19 vaccination for 2-22 weeks from December 28, 2020, to May 5, 2021. Though it was not our intention to compare immune responses among different vaccines, we observed that three who had Pfizer vaccine, and other three who had J&J vaccine did not have visible IgG/IgM signals, indicating their anti-S IgG/IgM were below the detection limit of the serological kit. All five participants who had Moderna vaccine showed positive results. Blood samples were collected from the first 10 individuals who had visible IgG responses and consent to donate blood. Three of them had Moderna vaccine and seven had Pfizer vaccine.

Plasma from all ten donors were confirmed positive in COVID-19 NAbs, which further indicated these donors all contained SARS-CoV-2 anti-S antibodies. However, quantity of their anti-S IgGs was not determined due to lack of a quantitative ELISA available for the measurement.

Table 2 Screening results of 22 vaccinated individuals.

[a]No.	Age	Sex	COVID-19 Vaccine	Vaccine Completion	[b]IgG/IgM Test	[c]NAb Inhibition, %
*1	50	F	Moderna	4 weeks		65.26±0.09
2	52	M	Pfizer	18 weeks		
3	44	F	J&J	9 weeks		
4	52	M	Pfizer	18 weeks		
*5	29	M	Pfizer	20 weeks		93.54±0.05
*6	65	F	Pfizer	15 weeks		94.9±0.2
7	44	F	Pfizer	15 weeks		
*8	26	F	Moderna	15 weeks		98.68±0.02
*9	44	F	Pfizer	17 weeks		97.96±0.01
10	65	M	Pfizer	20 weeks		
*11	40	F	Moderna	13 weeks		97.53±0.05
*12	29	M	Pfizer	22 weeks		98.2±0.1
13	47	F	J&J	12 weeks		
14	31	M	Pfizer	21 weeks		
15	36	F	J&J	12 weeks		
*16	62	F	Pfizer	21 weeks		97.66±0.05
*17	38	F	Pfizer	22 weeks		83.4±0.3
*18	25	M	Pfizer	22 weeks		98.69±0.01
19	71	F	Moderna	8 weeks		
20	51	M	Pfizer	2 weeks		
21	17	F	Pfizer	2 weeks		
22	65	M	Moderna	15 weeks		

[a] *Blood samples were collected from individuals with * symbol*

[b] *S-blood sampling well; IgG/IgM-anti-S Abs; C-control; No visible IgG/IgM: sample # 3,4,7,10,13,15*

[c] *Mean and SD, n=2; NAb positive cutoff: inhibition > 30%*

3.2. COVID-19 IgGs purification

Protein G affinity chromatography (AC) is useful to isolate class specificIgGs from human plasma. Table 3 listed total IgGs with protein yield% obtained from 3 mL convalescent plasma using protein G AC purification. Similarly, IgGs from vaccine plasma and the negative control plasma were also obtained with a yield about 2-5% total protein of each plasma. Pooled convalescent IgGs were confirmed both anti-S and anti-N positive, while the pooled vaccine IgGs were anti-S positive but anti-N negative.

IgGs antibodies are generated by human body to defense numerous pathogens, and the concentration of specific antibodies for a particular pathogen varies tremendously. The amount of COVID-19 anti-N IgGs measured (Table 1) were found below 0.1% of the total IgGs in the convalescent plasma samples as shown in Table 3. To avoid potential sample loss and deactivation of COVID-19 specific IgGs in further isolations, total IgGs were directly immobilized to construct COVID-19 affinity column without additional isolation of COVID-19 specific anti-S or anti-N IgGs.

Table 3 The amount of IgGs isolated and total plasma proteins in 3 mL convalescent plasma samples.

Plasma No.	Total plasma protein, mg	IgGs purified, mg	IgGs / total plasma protein, %
p1	846	18	2
p2	1042	30	3
p3	892	29	3
p4	911	31	3
p5	772	16	2
p6	820	14	2
p7	661	13	2
p8	672	19	3
p9	702	19	3
p10	690	34	5

3.3. IgG column preparation

Aliquots of 60 mg pooled IgGs isolated from ten COVID-19 convalescent plasma, ten COVID-19 vaccine plasma, and a COVID-19 negative control plasma were each applied to a 1 mL NHS-activated agarose columns. Approximately 40±10 mg IgGs were immobilized onto each column while the rest were recovered from the flow-through and washes collected in the immobilization procedure. The IgG columns prepared were examined with affinity purification of antigen mixed with excess BSA. SARS-CoV-2 S protein was the preferred testing antigen; however, recombinant SARS-CoV-2 N protein was used in the column testing due to its better stability and affordability. When 200μg BSA and 20μg SARS-CoV-2 N protein mixture was loaded onto the columns, proteins were mainly recovered in the flowthrough and first few wash fractions. N protein was detected in the elution from the Convalescent-IgG column (Figure 1 Lane 8), indicating selective binding of the N protein, but was not observed in elution of the other two columns (Figure 1 Lane 7 and Lane 9). This was expected as pooled vaccine IgGs and negative control IgGs both were anti-N negative. The N protein was exclusively detected in the second elution fraction from the Convalescent-IgG column (Lane 8) without BSA contamination, though the amount of BSA loaded was ten times that of the N protein. Columns were extensively washed with 20 CV of wash buffer for complete removal of non-specific binding. Small BSA overflow smear was observed in lane 3, 6 and 8 due to the high concentration of BSA loaded in adjacent lanes. However, no BSA smears were observed when reduced samples sizes were loaded. The result demonstrated immobilized IgGs on the columns were still active for antigen binding. TheConvalescent-IgG column had a binding capacity of approximately 2 μg N protein estimated from gel band intensity.

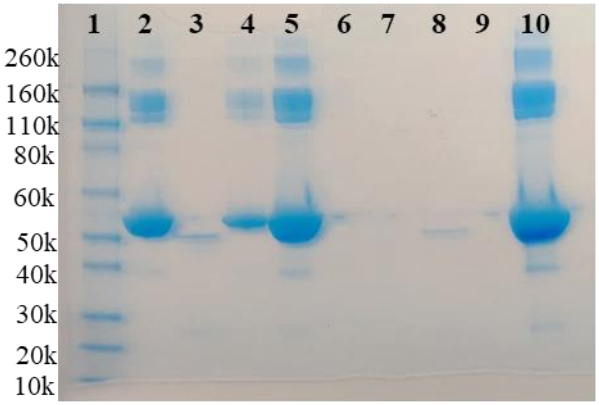


Figure 1. Protein gel analysis of affinity purification of 20μg N-protein and 200μg BSA. Convalescent-IgG column: Lane: **1**-marker; **2**-10μg BSA; **3**-1μg N protein; **4**-5μL flowthrough; **5**-5μL1st wash; **6**-10μL last wash; **8**-10μL 2nd elution. Vaccine-IgG column: **7**-10μL 2nd elution. Negative control-IgG column: **9**-10μL 2nd elution; **10**-5μL1st wash.

3.4. Epitope purification and identification by HPLC-MS

Aliquot of synthetic SARS-CoV-2 S peptide library was loaded onto each of the IgG columns made. Columns were extensively washed using 20 CV wash buffer, which was significantly larger than a volume used in typically AC purification. The intention was to completely remove non-specific binding.Wash and elution fractions were first examined using in-house HPLC low resolution LTQ MS. Spectral count results from MSFragger search of the RAW files against protein database showed that majority of SARS-CoV-2 peptides loaded were in early washes, especially the first wash fraction from each column (Table 4). No peptides were identified in the last wash fraction indicates the wash completely cleaned non-specifically bound non-epitope peptides off the columns. Peptides were identified in the second elution fraction of the Vaccine-IgG column and the Convalescent-IgG column, but not the Negative-Control column. The spectral counts corresponding to the second fraction elution were much smaller than that of the first wash fraction, indicating the IgG columns were heavily overloaded and only a very small portion of total peptides were retained by the COVID-19 IgG columns from specific binding.

Table 4. Protein database search of low resolution HPLC-MS data for epitope affinity purification.

Column	Injection	Spectral count	Distinct peptide matches	Protein sequence coverage
Vaccine-IgG	1st wash fraction	264	173	87%
	last wash fraction	--	None	--
	2nd elution fraction	5	5	5%
Convalescent-IgG	1st wash fraction	522	186	87%
	last wash fraction	--	None	--
	2nd elution fraction	3	3	4%
Negative control-IgG	2nd elution fraction	--	None	--

The S antigen epitope-containing elution fraction from the Convalescent-IgG column and Vaccine-IgG column was reanalyzed using the high resolution nanoLC-MS, which is more sensitive than the low resolution HPLC-MS system. Unique peptide epitopes identified were listed in Table 5. About 2/3 of them were observed in elution of both the Vaccine-IgG and the Convalescent-IgG columns. Six of them are in the RBD region of SARS-CoV-2 S antigen, which explained the NAb activity of the pooled convalescent and vaccineIgGs. The list included three of the four B cell epitopes identified by(Amrun et al., 2020) through peptide microarray immunological assays, and our data uncovered multipleadditional epitopes on the S antigen.However, the yields were so low that the extracted ion chromatogram (EIC) peak for identified epitopes in .RAW data files was not intensive enough for peak integration and quantification analysis. By comparing peak heightwith the values of method standards, the amount was estimated to be < 300 pg epitope eluted from the Vaccine-IgG column, and even lower from the Convalescent-IgG column. This epitope binding capacity was much lower than the N antigen binding capacity observed for the Convalescent-IgG column, which wasat microgram level. This indicates majority of the anti-S IgGs in the pooled antibody isolated from COVID-19 convalescent and vaccine donors either did not target peptide epitopes, or the affinity toward peptide epitopes were weak and did not sustain the extensive wash step of the AC purification. We recognized a pitfall in our work by using a linear peptide library of the SARS-CoV-2 S antigen for this study. IgG antibodies generally recognize conformational epitopes of an antigen with variable posttranslational modifications (PTM). However, the library used for epitope pull-downwas designed as potential T-cell epitopes of COVID-19 S antigen, which are short synthetic 15mer linear peptides without PTMs.Since SARS-CoV-2 was heavily glycosylated(Watanabe et al., 2020), peptides in the library may not be a good representative of potential epitopes of SARS-CoV-2 S antigen, resulting in poor affinity towards antibodies of the antigen. In addition,whether epitopes we identified are truly linear determinants or part of conformational determinants of COVID-19 S antigen was uncertain. Thus, identifications listed in Table 5 should not be considered as pure linear epitopes of the S antigen targeted by the anti-S IgGs in recruited donors responded to COVID-19 infection and vaccine in the community.

Table 5 SARS-CoV-2 S peptide epitopes identified by high resolution HPLC-MS.

Sequence (charge state)	Retention time, min	Precursor, m/z
DEDDSEPVLKGVKLH(+2)(+3)^{V,C}	23.61	841.4260/561.2880
SYQTQTNSPRRARSV(+3)^{C}	17.14	584.9466
HVSGTNGTKRFDNPV(+3)^{V,C}	17.96	543.6094
RALTGIAVEQDKNTQ(+3)^{C}	18.79	548.6280
KEELDKYFKNHTSPD(+4)^{C}	20.28	463.4786
VIRGDEVRQIAPGQT(+3)^{V,C,R}	20.45	546.9695
FHAIHVSGTNGTKRF(+4)^{V,C}	20.58	418.7230
DEVRQIAPGQTGKIA(+3)^{V,R}	21.42	528.6177
EQDKNTQEVFAQVKQ(+3)^{V}	21.67	598.2952
DKYFKNHTSPDVDLG(+3)^{C}	21.88	579.2810
RKSNLKPFERDISTE(+4)^{V,C,R}	22.02	455.7467
NSNNLDSKVGGNYNY(+2)^{V,R}	22.13	829.8734
GKAHFPREGVFVSNG(+3)^{V,C}	22.44	534.6110
EKGIYQTSNFRVQPT(+3)^{V}	22.57	589.9714
NFSQILPDPSKPSKR(+3)^{V,C}	22.63	571.9807

KIQDSLSSTASALGK(+3)[V,C]	22.79	502.6105
RTQLPPAYTNSFTRG(+3)[V,C]	23.45	570.2972
FGRDIADTTDAVRDP(+3)[C]	23.46	550.2692
NLTTRTQLPPAYTNS(+2)[V,C]	23.89	838.9341
HRSYLTPGDSSSGWT(+3)[V,C]	23.94	550.9217
IADTTDAVRDPQTLE(+2)[C]	24.49	822.9075
GGVSVITPGTNTSNQ(+2)[C]	24.83	716.3571
QIAPGQTGKIADYNY(+2)[C,R]	25.15	819.9084
VTYVPAQEKNFTTAP(+2)[C]	25.35	833.4281
PRRARSVASQSIIAY(+3)(+4)[V,C]	25.39	558.9847/419.4899
LLALHRSYLTPGDSS(+3)[V,C]	25.55	543.9577
TESNKKFLPFQQFGR(+3)[C]	25.57	609.6568
GIAVEQDKNTQEVFA(+2)[V,C]	25.59	824.9124
KLIANQFNSAIGKIQ(+3)[V]	26.13	548.9860
VIAWNSNNLDSKVGG(+2)[C,R]	27.06	787.4024
TNGTKRFDNPVLPFN(+2)(+3)[V,C]	27.27	860.9447/573.9658
TLDSKTQSLLIVNNA(+2)[V]	29.07	808.9466
KTQSLLIVNNATNVV(+2)[V]	30.15	807.9653
VITPGTNTSNQVAVL(+2)[V,C]	31.27	757.4157
GTNTSNQVAVLYQDV(+2)[V]	33.30	804.8967
VNNTVYDPLQPELDS(+2)[C]	35.42	852.4101

[V] Peptides in the elution from the COVID-19 Vaccine-IgG column;
[C] Peptides in the elution from the COVID-19 Convalescent-IgG column;
[R] Peptides within RBD of SARS-CoV-2 spike protein.

3.5. 3D mapping of S protein antigen epitopes

SARS-CoV-2 S protein is a class I transmembrane protein protruding on SARS-CoV-2 virus surface. The functional S protein is homotrimer stabilized by multiple inter-chain and intra-chain disulfide linkage. SARS-Cov-2 S antigen epitopes identified were displayed on the protein 3D structure of the S trimer available in PDB protein databank (PDB ID: 6VXX) (Figure 2). The 3D mapping of the S antigen epitopes demonstrated polyclonal nature of COVID-19 IgGs which recognized broad regions including RBD of the S protein as well as close similarity between epitopes targeted by the pooled convalescent IgGs and those targeted by the pooled vaccinate IgGs.

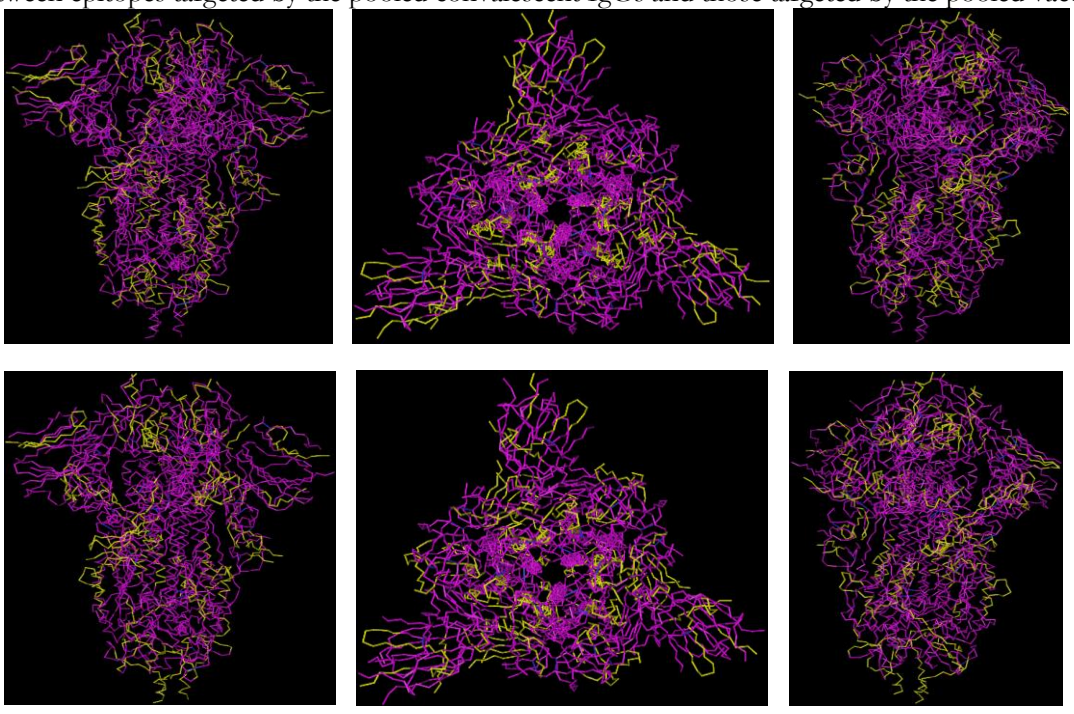


Figure 2. S antigen peptide epitopes identified for COVID-19 vaccine IgGs (top) and convalescent IgGs (bottom) by HPLC-MS were labeled in tube 3D structures of SARS-CoV-2 S protein trimer as front, top and side views. Identified epitopes were in yellow highlight, protein backbone was in purple, and blue connections in tube structures were cysteine-cysteine crosslinks.

In conclusion, our study identified multiple peptide epitopes of SARS-CoV-2 S antigen that were recognized by IgGs in community donors responding to COVID-19 vaccine and SARS-CoV-2 infection. Though only a very small portion of the total anti-S IgGs were specific to those epitopes, the broad polyclonal nature of IgG responses revealed at molecular level sheds light on protection from COVID-19 vaccine and infection against SARS-CoV-2 and its new variants unless mutations occur extensively across broad regions of the S antigen.

4. Declarations of competing interest

The authors declare no conflict of interests.

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