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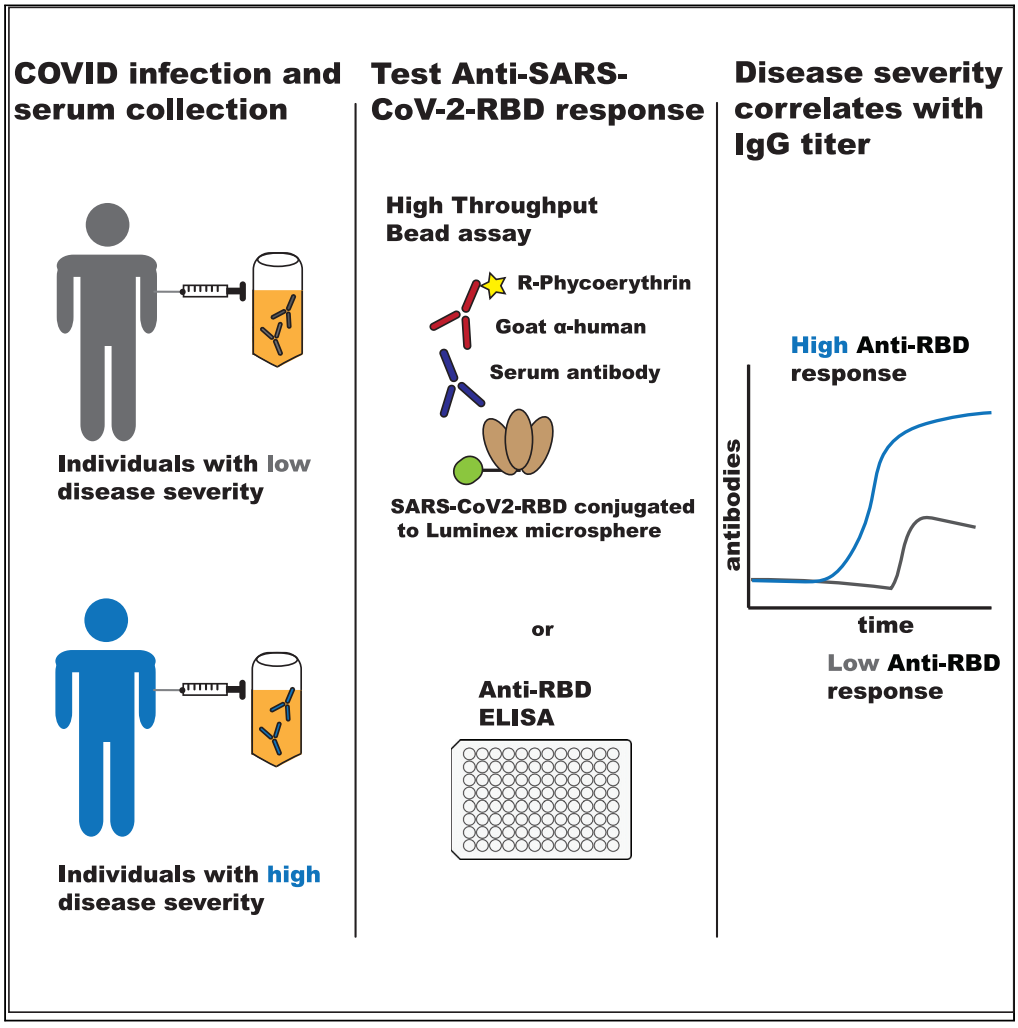
A high-throughput liquid bead array assay confirms strong correlation between SARS-CoV-2 antibody level and COVID-19 severity



Monique Bennett, Sandra Yoder, Eric Brady, ..., C. Buddy Creech, Allison P. Wheeler, Isaac Thomsen

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HIGHLIGHTS

We developed a sensitive, high-throughput assay for quantification of SARS-CoV-2 IgG

The assay uses liquid bead array technology for efficient and reproducible results

COVID-19 symptom severity was strongly correlated with SARS-CoV-2 S_{RBD} IgG levels

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A high-throughput liquid bead array assay confirms strong correlation between SARS-CoV-2 antibody level and COVID-19 severity

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SUMMARY

A detailed understanding of the adaptive host response to SARS-CoV-2 infection in humans is urgently needed. We developed a sensitive, high-throughput, and efficient assay using liquid bead array technology. We observed advantages over traditional ELISA for the detection and quantification of binding IgG against the receptor binding domain (RBD) of SARS-CoV-2. To determine whether COVID-19 symptom severity correlates with SARS-CoV-2 IgG, we measured anti-RBD IgG levels from 67 subjects recovered from PCR-confirmed COVID-19. We found that COVID-19 symptom severity strongly correlated with RBD IgG level ($p < 0.001$). These findings have substantial implications for public policy surrounding assessments of antibody responses and possible immunity, as not all cases of COVID-19 can be assumed to generate a protective antibody response, and mild disease in particular is capable of generating very low-level anti-RBD IgG levels. These findings also have important implications for the selection of donors for convalescent plasma to be used therapeutically.

INTRODUCTION

Much is unknown regarding the adaptive immune response to the coronavirus SARS-CoV-2 (Coronaviridae Study Group of the International Committee on Taxonomy of, 2020). Due to the novel nature of this virus, the population has little or no pre-existing immunity to SARS-CoV-2, which has major implications for optimal management and prevention of this globally important pathogen. As with other coronaviruses, the spike protein (S) of SARS-CoV-2 plays a crucial role in pathogenesis and serves as the immunodominant antigen in the host response to infection. The spike protein is a large, membrane-bound glycoprotein that is divided into the S1 and S2 domains: the S1 domain, or receptor binding domain (RBD), binds to the human angiotensin-converting enzyme 2 (ACE2) receptor and facilitates viral attachment to human cells (Jiang et al., 2020; Wrapp et al., 2020; Zhou et al., 2020; Letko et al., 2020; Yan et al., 2020).

During the SARS outbreak in 2003, it was reported that anti-SARS-CoV antibody levels correlated with disease severity, with clinically sicker patients mounting a higher-titer response (Lee et al., 2006). A number of neutralizing monoclonal antibodies (mAbs) were also reported to bind specifically to the RBD of SARS-CoV (Pak et al., 2009; Hwang et al., 2006). Emerging data strongly suggest that RBD is a highly specific, immunodominant target of the host response following COVID-19 (Premkumar et al., 2020). Antibody titers to RBD correlate with reduction of plaque formation by SARS-CoV-2 in ACE2-producing Vero cells (Okba et al., 2020), and sera with high-titer anti-RBD antibodies neutralize SARS-CoV-2 more potently (Hansen et al., 2020; Yuan et al., 2020). It remains unclear, however, whether disease severity is clearly correlated with antibody production. This correlation has major implications as a correlate of immunity is sought, as mild disease may not generate optimal protection against future SARS-CoV-2 exposures.

To investigate this, we developed SARS-CoV-2 S_{RBD} ELISA and liquid bead array assays to quantify binding antibody levels in human sera. The bead-based assay is intended to offer a superior degree of discrimination and accuracy, particularly at low-level antibody concentrations, along with a much wider dynamic range, compared with traditional indirect ELISA methodology. We then obtained human sera from subjects with proven, symptomatic SARS-CoV-2 infection at disease convalescence (~6 weeks post

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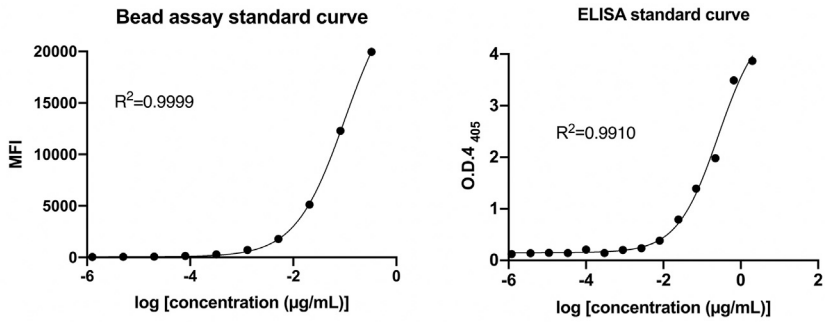

	Accuracy (%)	LOD (ng/ml)
Bead assay	95 – 108	$<3.07x10^{-07}$
ELISA	83 – 143	$1.86x10^{-06}$

Figure 1. Liquid bead array assay performs favorably in comparison with standard indirect ELISA

Standard curves were generated by 4-fold serial dilutions of a cross-reactive SARS-CoV-1 monoclonal antibody known to bind SARS-CoV-2 RBD. Both the bead array and standard ELISA generate valid binding curves, although the bead array exhibits higher accuracy (defined as percent recovery against known antibody concentrations) and greater sensitivity with a lower limit of detection.

symptom onset). We used these samples to determine whether clinical symptom severity is correlated with SARS-CoV-2 RBD antibody titer following COVID-19.

RESULTS

Assay performance characteristics

The liquid bead array assay was designed to detect the presence of anti-SARS-CoV-2 S_{RBD} -specific antibodies from human sera. Recombinant RBD was conjugated to Luminex MagPlex microspheres and incubated with serially diluted serum samples and a cross-reactive SARS-CoV monoclonal standard. Over 20 replicate runs were conducted to define the accuracy, precision, and range of the bead array assay. Accuracy, defined as percent recovery of the known value of the mAb reference, ranged between 94.9% and 107.5%. The lower limit of detection (LOD), defined as the mean fluorescent intensity (MFI) of IgG-depleted serum plus 3 times the standard deviation of the mean, was $<3.07 \times 10^{-7}$ ng/mL. Precision was assessed by repeated measurements of a high control, negative control, and three samples known to span the range of the assay. An inter- and intra-assay coefficient of variation (CV) of under 25% for all samples was obtained. Last, the assay was found to be highly efficient, requiring only 0.8 µL of human sera per run.

Bead array assay versus standard indirect ELISA

To evaluate the performance of the liquid bead array assay, a subset of sera were tested by traditional indirect ELISA. The subset was chosen to include high-, medium-, and low-titer samples based on the bead array data. Standard curves were determined for both assays using the mAb CR0322 (Figure 1); although the linear portion of the curves largely overlap for both assays, the bead array assay shows a higher degree of sensitivity for all samples tested. For equivalent curves, standard ELISA required a higher concentration of 2 µg/mL compared with the bead assay initial concentration of 0.33 µg/mL (see Table S1 for specific sample data from dilutions across the linear portion of each sample curve). The performance of the bead assay at the lower end of the curve was also more sensitive than ELISA, with the bead assay achieving a LOD of less than 3.07×10^{-7} ng/mL when compared with the LOD by ELISA of 1.86×10^{-6} ng/mL. Thus, indirect ELISA confirmed the results of the bead array assay, while highlighting the potential advantages of a bead array platform.

Correlation of disease severity with SARS-CoV-2 IgG

Convalescent sera were obtained from 67 subjects following PCR-confirmed COVID-19. The median age in the study population was 36.0 years (interquartile range [IQR] 24.0–52.5), and 33% of the subjects were male

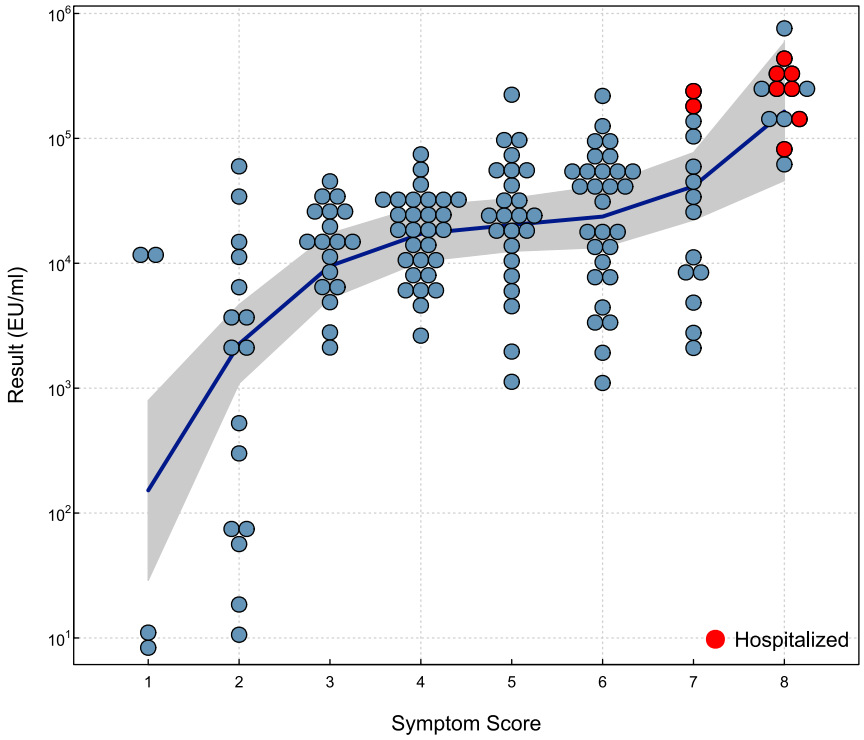

Figure 2. SARS-CoV-2 receptor binding domain IgG levels are strongly correlated with COVID-19 symptom severity

Partial effect plot of log antibody measurement (EU/mL, calculated via liquid bead array normalized to a monoclonal standard) of binding IgG against SARS-CoV-2 RBD in samples obtained ~6 weeks post-COVID-19. Increasing symptom severity is strongly associated with increased anti-SARS-CoV-2 RBD IgG ($p < 0.001$, see Table 1). Measurements were performed in duplicate, with a triplicate value obtained if the duplicate values differed by >15%, and the repeated measures were accounted for in the model by compound symmetric correlation. Gray region indicates point wise 95% confidence interval limits. Red points indicate IgG values from subjects hospitalized for COVID-19.

(see Table S2 for full demographic information and RBD IgG levels per subject). Symptom scores ranged from very mild (scores of 1 or 2; n = 2 and 7, respectively) to severe symptoms that warranted hospitalization (scores of 7 or 8; n = 8 and 4, respectively). The most frequent symptom scores were 4, 5, and 6 (n = 14, 10, and 13, respectively).

Raw MFI from the bead array assay was converted to EU/mL by standardization against a monoclonal antibody. Samples from subjects who had recovered from COVID-19 had a markedly wide range of IgG levels against SARS-CoV-2 RBD, ranging from 9.6 to 731,768 EU/mL. Increasing COVID-19 symptom severity was strongly and significantly correlated with quantity of SARS-CoV-2 RBD binding IgG in both unadjusted and covariate-adjusted analyses ($p < 0.001$, Figure 2; Table 1). For example, the mean increase in IgG levels for a patient who reports a 2 versus a 7 on the COVID-19 severity scale was 40,906 EU/mL (95% confidence interval: 16,785, 69,951). Furthermore, this correlation was independent of time to sample collection. All samples were collected approximately 6 weeks post symptom onset (median days from symptom onset: 45; IQR 41–55]. Four subjects were hospitalized for COVID-19; these four subjects exhibited four of the five highest SARS-CoV-2 IgG levels in the cohort.

DISCUSSION

As physicians and public health officials across the globe work to rapidly identify best practices for COVID-19 pandemic response and containment, a detailed understanding of the natural adaptive host response to SARS-CoV-2 infection is crucial. We identified a highly significant correlation between clinical severity of COVID-19 disease and the amount of SARS-CoV-2 binding antibody against the receptor binding domain. This finding has significant implications for defining immunity following infection. Although RBD titer has been shown to correlate with viral neutralization *in vitro* (Premkumar et al.,

Table 1. Multivariate model of association with SARS-CoV-2 RBD IgG level with *a priori* selected variables

Predictor	Chi-square statistic	p value
Severity of COVID-19 symptoms	50.730	<0.001
Age[a]	0.509	0.775
Sex[b]	0.735	0.391
Time from symptom onset to sample collection[c]	1.127	0.288
Total	51.584	<0.001

[a]Median age in the study population was 36.0 years [IQR 24.0–52.5].
[b]67% of subjects were female and 33% male.
[c]Median time from symptom onset: 45 days [IQR 41–55 days].

2020; Okba et al., 2020), a true "correlate of protection" remains unknown. It appears increasingly likely, however, that total antibody amount (particularly anti-RBD IgG) will serve as a surrogate for functional protection following infection. Our data suggest that more severe COVID-19 symptoms may be associated with increased protection from subsequent SARS-CoV-2 infection, although these responses are known to wane over time, and true correlates of durable protection are urgently needed.

The liquid bead array assay described in the report offers advantages over traditional ELISA, including improved discrimination at low-level antibody titers. We found the reported assay methodology to be highly accurate and reproducible, and the assay allowed for discrimination of anti-RBD IgG at very low levels. Furthermore, the wide dynamic range of the assay (with read-outs in this study ranging from 10 to 410,700 EU/mL) allows for a highly granular visualization and analysis of data, which can be advantageous for research questions such as the one reported in this article. While the bead array is unlikely to be deployable in clinical settings due to a relatively higher cost and required expertise compared with standard techniques, this assay is ideally suited to a research environment for situations in which higher dynamic range and immunologic granularity are desirable.

Clinical aspects of COVID-19 are highly variable between individuals (Huang et al., 2020; Holshue et al., 2020), and asymptomatic disease is also common. Recent reports have suggested that asymptomatic disease generates a fundamentally different host response compared with critical illness, but it was previously unclear if this distinction persisted across milder, but symptomatic, patients with COVID-19 (Long et al., 2020). Our study benefits from access to nearly 70 samples from patients with confirmed COVID-19 and associated symptom severity scoring. Our clinical correlation data are somewhat limited because the majority of patients were not hospitalized, symptom scores were subjective, and judgments of severity may vary across study subjects. However, it is notable that of the 67 patients, 4 required hospitalization (a more objective marker of increased severity), and each of the hospitalized subjects mounted a very high-titer IgG response to SARS-CoV-2 RBD.

In conclusion, we report an efficient and reproducible platform for the measurement of SARS-CoV-2 antibodies in human serum and have found, consistent with data from SARS-CoV-1, that the severity of COVID-19 correlates with the amount of antibody produced against the viral receptor binding domain. As pandemic responses begin to investigate the use of serology and consideration of "immunity passports," it is important to note that not all cases of COVID-19 can be assumed to generate a protective antibody response, and mild disease in particular may generate virtually no detectable anti-RBD IgG. This has widespread implications, including the use of COVID-19 symptom severity as a simple screen for which patients might represent ideal donors for convalescent plasma, as hospitals move toward use of plasma as a potential therapeutic. Further work to define a true correlate of protection and predictors of protective immunity is urgently needed.

Limitations of the study

This study uses convalescent plasma from infected patients to quantify anti-SARS-CoV-2 antibody titer. Presence of antibody alone does not confer an assumption of protection (i.e., virus neutralization), and ongoing work will investigate correlation between RBD-IgG-mediated neutralization and protection

from SARS-CoV-2 and to determine whether the reported liquid bead assay can be bridged to a correlate of protection.

Resource availability

Lead contact

Further information and request for resources can be directed to Isaac Thomsen, MD, MSCI, Vanderbilt University Medical Center (isaac.thomsen@vumc.org).

Materials availability

The study did not generate new or unique reagents or materials.

Data and code availability

This study did not involve the use of any custom code, algorithms, or software.

METHODS

All methods can be found in the accompanying Transparent methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102052.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.Y., J.M.P., C.B.C., A.P.W., and I.T.; Methodology, M.B., S.Y., T.G.S., and I.T.; Formal Analysis, T.G.S.; Investigation, M.B., S.Y., E.B., A.P.W., and I.P.T., Resources, J.M.P., J.P.P., G.R.B., C.B.C., and I.T.; Writing – Original Draft, M.B., S.Y., and I.T.; Writing – Review & Editing, all authors; Supervision, J.M.P., G.R.B., and I.P.T.; Project Administration, J.M.P. and J.P.P.; Funding Acquisition, J.M.P., J.P.P., G.R.B., C.B.C., and I.T.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

A high-throughput liquid bead array assay confirms strong correlation between SARS-CoV-2 antibody level and COVID-19 severity

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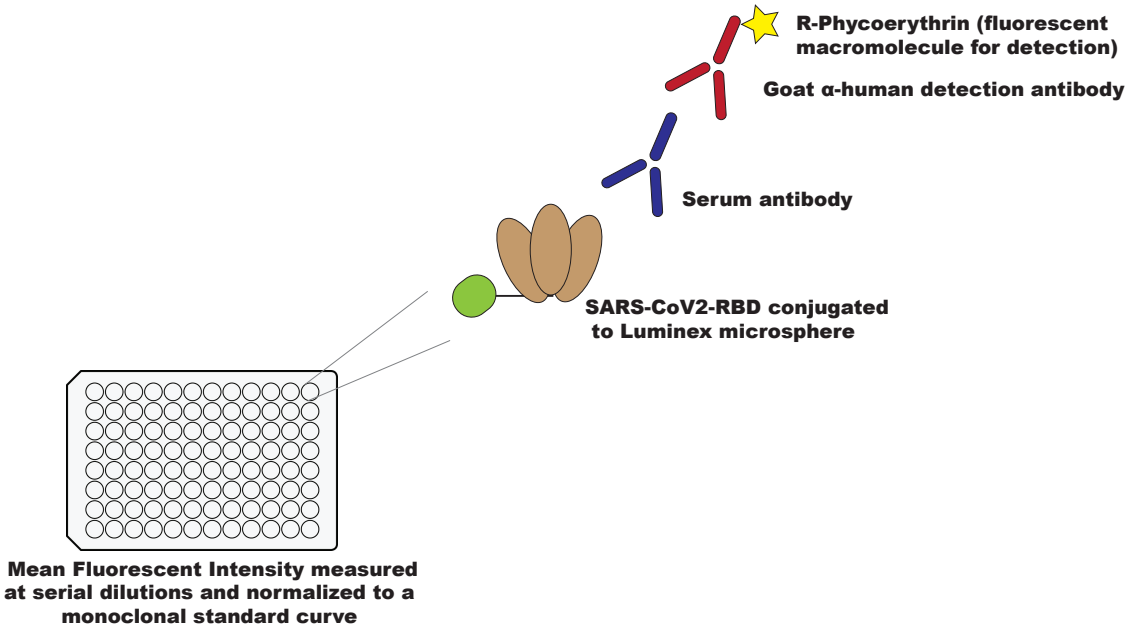

Supplemental Figure 1. *Liquid bead-based assay used to detect the presence of anti-SARS-CoV-2 S_{RBD} -specific antibodies, related to Figure 1*

Recombinant RBD was conjugated to Luminex MagPlex microspheres and incubated in 96- well plates with serially diluted serum samples and a cross-reactive SARS-CoV monoclonal antibody as a standard. Serum antibodies bound to SARS-CoV-2 S_{RBD} were detected by R-Phycoerythrin conjugated F(ab')2 fragment goat anti-human IgG Fc gamma conjugate. Plates were acquired using a Luminex MagPix Instrument at 100 beads per well, with Xponent software version 4.3.

Supplemental Table 1. ELISA and Liquid bead assay sample data from the linear portion of each sample curve, related to Figure 1

Sample	ELISA EU/mL	Dilution	O.D.	Bead EU/mL	Bead Dilution	MFI
1006	855	900	0.951	145818	1200	9398.00
		2700	0.489		4800	3522.50
		8100	0.224		19200	1197.50
1058	1983	2700	0.735	184707	1200	11228.00
		8100	0.360		4800	4952.00
		24300	0.184		19200	1636.00
1060	2592	2700	0.785	235467	1200	497.00
		8100	0.458		4800	118.00
		24300	0.242		19200	46.00
					76800	27.00
1043	3210	2700	1.189	731769	4800	7745.50
		8100	0.767		19200	2942.00
		24300	0.352		76800	1057.50
					307200	345.50
1054	2539	2700	0.941	235236	1200	9740.00
		8100	0.484		4800	5079.00
		24300	0.258		19200	1775.00
1055	283	300.0	0.945	3223	300	6598.00
		900	0.461		1200	2180.00
		2700	0.225		4800	552.00
					19200	151.00
1104	172	300.0	0.573	6987	300	2456.00
		900	0.281		1200	928.50
		2700	0.152		4800	270.00
					19200	80.00
1101	164	300.0	0.545	4605	300	1532.00
		900	0.254		4800	222.50
		2700	0.144		19200	75.00
1124	122	300.0	0.408	4417	300	1551.00
		900	0.211		1200	680.00
		2700	0.129		4800	191.00
					19200	64.00
1045	80	300.0	0.267	6402	300	1880.00
		900	0.161		1200	775.50

		2700	0.110		4800	153.00
1052	69	300.0	0.230	8	300	119.50
		900	0.137		1200	64.00
		2700	0.103			
1068	51	300.0	0.171	19	300	69.00
		900	0.109		1200	51.00
		2700	0.097			
1151	47	300.0	0.156	LOD	300	
		900	0.118		1200	
		2700	0.101		4800	
1150	40	300.0	0.134	26	300	140.00
		900	0.105		1200	
		2700	0.093		4800	
3	37	300.0	0.122	442	300	583.00
		900	0.103		1200	
		2700	0.092		4800	
1025	38	300.0	0.128	10	300	35.00
		900	0.105		1200	
		2700	0.093		4800	

Supplemental Table 2. Individual values and demographics, related to Figure 2 and Table 1

Mean RBD IgG (EU/mL)	Symptom Score	Days from symptom onset to sample	Age	Gender
12016	5	43	22	Male
19228	4	53	24	Female
3360	7	46	24	Female
41319	3	37	37	Female
145818	8	72	75	Male
50463	6	56	37	Male
6190	6	44	22	Female
22234	5	46	38	Female
22586	4	45	30	Female
48970	4	33	36	Male
10130	3	56	22	Female
14881	4	48	53	Female
52422	6	36	45	Female
16714	4	43	38	Female
50314	5	36	56	Female
7691	5	65	26	Female
30855	3	39	21	Female
10	1	40	67	Female
250483	8	38	58	Male
2720	3	39	54	Male
10998	3	37	22	Female
79975	6	44	68	Female
24101	6	50	40	Female
21609	3	42	20	Male
26007	5	53	56	Female
1494	2	33	66	Male
52173	6	45	51	Female
15880	6	30	20	Female
17965	4	41	58	Female
374211	8	42	70	Female
1906	2	48	59	Male
56004	5	42	43	Male
12832	4	59	20	Female

13107	2	43	20	Male
17195	4	40	21	Female
42607	2	38	22	Female
45	2	44	40	Female
13739	6	46	21	Female
201545	7	49	27	Male
4750	2	48	22	Female
10488	1	53	29	Female
18447	7	44	25	Female
184707	6	41	47	Female
410717	8	55	59	Male
122567	5	44	62	Female
59081	6	41	28	Female
27367	4	46	22	Female
48054	4	34	25	Female
6606	6	57	52	Female
185848	8	44	46	Female
52	2	33	34	Male
42633	7	56	26	Female
39376	5	63	26	Female
38377	6	58	44	Male
20527	5	44	23	Female
13177	3	53	22	Female
4493	4	54	28	Female
107531	7	45	52	Male
2617	5	59	40	Female
5647	3	65	36	Male
5232	4	52	36	Male
25270	3	67	36	Male
5215	7	70	30	Female
2467	6	65	61	Female
18020	4	63	68	Male
26508	7	68	33	Male
32730	4	70	61	Male

Transparent Methods

Development of liquid bead assay

Uncoated Luminex MagPlex-C superparamagnetic carboxylated xMAP-microspheres were coupled to 100 µg SARS-CoV2 region binding domain (RBD) (courtesy of the laboratory of Dr. James E Crowe, Jr), per manufacturer recommendations. Bridging studies were later done using commercially available protein (Leinco Technologies, Fenton, MO) and found to be comparable. Briefly, coupling uses carboxyl groups activated by 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride in the presence of N-hydroxysulfosuccinimide to form a reactive intermediate that is then replaced by the primary amine of the RBD protein. The protein coupled bead was then washed and blocked with buffer containing bovine serum albumin before use.

For the magnetic microsphere antibody binding assay, a 96-well round bottom dilution plate was used. A monoclonal standard (CR0322, a known cross-reactive SARS-CoV-1 monoclonal antibody kindly gifted from the laboratory of Dr. James E Crowe, Jr.) was diluted using 4-fold dilutions starting at 1:3000. The high control was diluted with four, 4-fold dilutions starting at 1:1200, and the low control was diluted with four, 4-fold dilutions starting at 1:20. Unknown samples were diluted with four, 4-fold dilutions starting at 1:300. The assay plate consisted of 25 µl of prepared dilutions plus 25 µl of coated microspheres (1000 beads per well) in a film bottom black walled plate (Greiner). After a 40-minute incubation at room temperature on a plate shaker, the plate was washed twice using a microplate washer with magnetic adapter (BioTek 405LS) with wash buffer consisting of 0.1% BSA and 0.02% sodium azide in PBS. The detection reagent, R-Phycoerythrin conjugated F(ab')2 fragment goat anti-human IgG Fc gamma specific conjugate (Jackson ImmunoResearch), was diluted 1:200 and added to each well. The plate was incubated for 30 minutes at room temperature on a plate shaker. A final wash was performed before resuspending in 130µl of wash buffer. The plate was acquired on a

Luminex MagPix Instrument acquiring 100 beads per well, with Xponent software version 4.3 (**Supplemental Figure 1**).

ELISA methods for comparison

96-well microtiter plates were coated with recombinant SARS-CoV-2 S_{RBD} protein overnight at a concentration of 1 µg/ml. Plates were blocked with 1% BSA and TBST for 1 hour. Plasma samples were diluted at a starting concentration of 1:300 in 1% BSA and TBST and microtiter plates were incubated for 90 minutes at room temperature. Goat anti-human secondary conjugated to alkaline phosphatase was added for 1 hour and used to detect IgG antibodies bound to RBD. The absorbance was measured at 405 nm using a Biotek spectrophotometer.

Samples for correlation with severity

All human samples used during development of the SARS-CoV-2 receptor binding domain assay were obtained with informed consent and subject to Vanderbilt University Medical Center (VUMC) Human Subject Protection Program oversight. Samples from subjects participating in the VUMC-led Passive Immunity Trial of Nashville (PassItOn; NCT 04362176) who provided informed consent for use of residual samples for secondary research were used to correlate antibody titers with symptom severity. Samples were processed and analyzed in a coded manner, linked only to age, sex, and symptom score. Symptom severity score was a patient-reported variable on a 1-10 scale, where 1 represented very mild symptoms and 10 represented critical illness, with a breakdown of ranges as follows:

1 – 3: Very mild to mild disease (upper respiratory symptoms, minimal fever)

4 – 6: Moderate disease (multiple days of fever, significant respiratory symptoms)

7 – 9: Severe disease (Respiratory difficulty with decreased oxygenation; hospitalization was considered or required; or prolonged high fever and severe systemic symptoms)

10: Critical illness (intensive care unit admission; “was or should have been in the ICU”)

Statistical analysis of correlation

A linear model of log IgG measurements was constructed with generalized least squares with the following covariates: symptom severity, age, sex, and days between symptom onset and sample draw. Both symptom severity and age were included in the model as polynomials in order to capture potential non-linear associations with the outcome. To account for repeated measures, a compound symmetric correlation structure within subjects was incorporated into the model. The overall impact of each predictor was evaluated with a likelihood ratio test on the model estimated with maximum likelihood. Statistical analyses were performed in R-4.0.0 (R Core Team, 2020).

References

R Core Team (2020). A Language and Environment for Statistical Computing (R Foundation for Statistical Computing).