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Research paper

SARS-CoV-2 diagnostics: Towards a more comprehensive approach to routine patient testing

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ABSTRACT

The SARS-CoV-2 pandemic has provided the stimulus for the rapid development of a variety of diagnostic testing methods. Initially these were deployed as screening tools to evidence spread of the virus within populations. The recent availability of vaccines against the virus and the need to better understand the parameters of post-infection protective immunity requires development of methods, suitable for use in the routine diagnostic laboratory, capable of characterising the viral immune response in greater detail. Such methods need to consider both cellular and humoral immunity. Toward this aim we have investigated use of a commercial multiplex assay (COVID Plus Assay, One Lambda), providing assessment of the SARS-CoV-2 response at structural level, and developed an in-house cell stimulation assay using commercially available viral peptides (Miltenyi). This paper reports our experience in use of these methods in extended investigation of a cohort of healthcare workers with prior screening results indicative of viral infection. The antibody response generated is shown to be both qualitatively and quantitatively different in different individuals. Similarly a recall response to SARS-CoV-2 antigen involving the T cell compartment can be readily demonstrated in recovered individuals but is of variable magnitude.

1. Introduction

The SARS-CoV-2 pandemic has provided the stimulus for the rapid development of a variety of diagnostic testing methods. These test for presence of virus as a means of determining current infection or screen for evidence of antibody production against virus as a means of establishing past exposure (Smithgall et al., 2020). This two pronged approach, with focus on detection of infected or recovered individuals has formed the basis for clinical management of SARS-CoV-2 infection in the routine setting since mid-2020.

There is a major gap in our understanding of SARS-CoV-2 that is not addressed by and represents a limitation of our currently utilised test systems. This relates to the generation of protective immunity. Although it is tempting to speculate on the basis of the presence of SARS-CoV-2 antibody, demonstrated in systems above, this has been obtained the assumption would entirely overlook the role of the cellular arm of the immune response in viral immunity (Hellerstein, 2020). It would also neglect the need to understand the precise target of detected antibody reactivity and the relationship of this to protection from infection, most particularly since some antibodies having been found to enhance infectivity (Huang et al., 2020). Lastly, the measurement of circulating antibody provides no insight into persistence of post-infection immunity which is based in the generation of cellular immune memory (Woodland et al., 2002).

An urgent need for development of this capability in the setting of the routine diagnostic laboratory exists in assessment of herd immunity and evaluation of response to vaccination.

Against this background we have performed investigations of SARS-CoV-2 immunity in a small cohort of individuals utilising an adaptation of a well-established methodological approach for the determination of cellular immunity together with a novel multiplex assay for semi-quantitative determination of antibody target of reactivity at structural level. Both methods are entirely suited to performance in the diagnostic laboratory setting and together provide a means of examining and longitudinally monitoring the immune status of patients with potential for prediction of clinical outcome.

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2. Methods

2.1. Identification of SARS-CoV-2 exposed individuals

Staff members ($n = 12$) from Leeds Teaching Hospitals that had reported symptoms consistent with COVID-19, with the diagnosis confirmed clinically or by PCR, and now at various times post recovery were identified and asked to provide a serum sample. These were tested for SARS-CoV-2 IgG antibodies using commercial ELISA systems (Euroimmun/IDK) in accordance with manufacturer's instruction. Results of this testing are shown in Table 4.

Individuals that had generated an antiviral response as assessed by reactivity were then tested for functional T cell immunity against SARS-CoV-2 and a more detailed analysis of their SARS-CoV-2 antibody response performed.

2.2. Lymphocyte phenotyping

A standard panel providing values for percentage and absolute number of $CD3^+$, $CD4^+$, and $CD8^+$ T cells as well as $CD19^+$ B cells and $CD3^-CD56^+$ NK cells was employed in initial evaluations. In brief, whole blood was added to a commercially available mix of flourochrome labelled antibodies (Becton Dickinson, BD) that recognised cell surface determinants on T, B and NK cells (CD45/CD3/CD4/CD8/CD19/CD16–56). Following erythrocyte lysis the samples were acquired on FACSCanto II flow cytometer using BD Clinical Software. This indicated the percentage and absolute value of the subsets measured.

B cells were additionally analysed to assess naïve ($CD27^-IgD^+$) and memory ($CD27^+IgD^{+/-}$) phenotype in addition to $CD24^{hi}CD38^{hi}$ transitional B cell and $CD38^{hi}CD27^{hi}$ plasmablast populations. Naïve and memory $CD3^+CD4^+$ T cells were determined by staining with antibodies against CD3/CD4/$CD45^{RA}$ and CD27. CD4 T-helper phenotype analysis by flow cytometry, using antibodies against CXCR3 and CCR6, gated from $CD3^+CD4^+$ T-helper cells was additionally performed. For all additional phenotyping, washed whole blood was added to antibody mixes for 20 min in the dark at room temperature. The samples were washed, after red cell lysis, by centrifugation in PBS + 0.1% FBS. After washing steps were complete, the cell pellet was resuspended in approximately 300 μl PBS + 0.5% formaldehyde. Results were acquired using FACSCanto II flow cytometers and DIVA software.

2.3. $CD4^+$ T Lymphocyte re-stimulation with SARS-CoV-2 antigen preparations

SARS-CoV-2 peptides (Miltenyi) were used to stimulate an *in-vitro* recall response using peripheral blood mononuclear cells (PBMCs) obtained from individuals that had recovered from infection. Briefly, PBMC were isolated using *Lympholyte H (*Cedarlane). Following washing in tissue culture media, isolated cells were adjusted to a density to 5×10^6/ml using TC199 + 5% AB serum. 1 ml aliquots of cell suspension were incubated with SARS-CoV-2 peptides (10 μg/peptide using mix of pepTivator SARS-CoV-2 M, N and S peptides - Miltenyi Biotech). Following overnight incubation, BD Golgistop was added to the cells and they were incubated for a further 6 h at 37 °C and 5% CO_2. At the end of the incubation, the cells were harvested, washed, fixed and permeabilised prior to the addition of antibodies against CD4, IFNɣ, IL-4 and IL-17, following manufacturer's (BD) instructions. Data was acquired using a FACSCanto II flow cytometer, collecting data from 100,000 $CD4^+$ T cells.

For initial work in verification of this assay system, PHA and PMA/ionomycin were used to stimulate isolated PBMC from healthy volunteers to confirm technical performance. IFNɣ, IL-4 and IL-17 production was observed following 6 h stimulation. No cytokines were detected in unstimulated cells.

2.4. Antibody measurement

The LABScreen™ COVID Plus Assay (OneLambda, Canoga Park, California), was used to detect and monitor the SARS-CoV-2 antibody response. Data was acquired using a LABScan™ 200 platform. Testing was performed according to the manufacturer's instruction locally adapted for performance at half-volume.

The LABScreen™ COVID Plus Assay panel detects antibodies to SARS-CoV-2 Spike (extracellular domain), S1, S2 and receptor binding domain (RBD) as well as nucleocapsid protein (NP). The panel also detects antibodies to Spike S1 for other common coronavirus (HCoV-229E, HCoV-HKU1, HCoV-NL63 and HCoV-OC43), as well as markers for SARS-CoV-1 and MERS, reducing potential false positive results caused by other common coronaviruses.

3. Results

3.1. Lymphocyte phenotyping

In order to examine the basic composition of lymphocyte populations from individuals that had recovered from SARS-CoV-2 infections, we initially tested samples from those individuals ($n = 12$) using our standard cell marker panel, which measures the percentage and absolute numbers of T, B and NK cells in whole blood. The test was also performed in 6 individuals that were assumed to have not encountered SARS-COV-2 based on their personal history. The values obtained from samples taken from individuals that had tested positive for SARS-CoV-2 antibodies were all within the normal range for all subsets and were similar to the results from healthy adults. This was true both for the percentage of lymphocyte subpopulations as well as for the absolute numbers of each population assessed.

B cells were further analysed to assess naïve ($CD27^-IgD^+$) and memory ($CD27^+IgD^{+/-}$) phenotype in addition to $CD24^{hi}CD38^{hi}$ transitional B cell and $CD38^{hi}CD27^{hi}$ plasmablast populations. All populations were readily detected in individuals post COVID-19 and at levels that were similar to the levels obtained from the healthy control (HC) cohort. Transitional B cells were found at an average of 6.64% in the COVID-19 group and 7.25% in the HC cohort. Plasmablasts were barely detectable in both groups (0.3 vs 0.37). Memory B cells were similar between the two groups with 17.45% of B cells being $CD27^+IgD^-$ switched in the COVID-19 groups compared to 17.68% in HCs. Non-switched memory B cells ($CD19^+CD27^+IgD^+$) were found to represent 11.69% of B cells in the COVID-19 group and 11.82% of B cells in HCs.

A similar approach was taken to assess the levels of naïve and memory $CD3^+CD4^+$ T cells. To that end whole blood was stained with antibodies against CD3/CD4/CD45RA and CD27. The levels of naïve and memory $CD4^+$ T cells were similar in individuals who had suffered from COVID-19 infections compared to healthy COVID-19 naive individuals for all cell types assessed (44% vs 46% for $CD45RA^+CD27^+$ naïve T; 45% vs 45% for the $CD45RA^-CD27^+$ T central memory (TCM); 7% vs 6.75% for the $CD45^{RA-}CD27^-$ T effector memory (TEM) cells; and 3.95% vs 2.15% for $CD45^{RA+}CD27^-$ T effector memory RA (TEMRA) cells.

We also used a cell surface phenotypic approach to identify Th-1, −2 and − 17 $CD4^+$ T cells in peripheral blood (Fig. 1a shows a representative dot plot). Examination of Th-1 cells ($CXCR3^+CCR6^-$), Th-2 cells ($CXCR3^-CCR6^-$) and Th-17 cells ($CXCR3^-CCR6^+$) revealed little difference between individuals who had experienced SARS-CoV-2 infection compared to healthy SARS-CoV-2 naive individuals (Fig. 1b). For Th-2 cells ($CXCR3^-CCR6^-$) mean values of 40.25% vs 44.97%, Th-1 cells ($CXCR3^+$ $CCR6^-$) 27.7% vs 24.5% and Th-17 ($CXCR3^-CCR6^+$) 12.62% vs 13.78%.

Fresh whole EDTA blood was stained with flurochrome labelled antibodies against CD3, CD4, CXCR3 and CCR6. Following lysis and washing, the cells were acquired on a FACSCanto II flow cytometer. Th-1 cells were defined as $CXCR3^+CCR6^-$, Th-2 cells, $CXCR3^-CCR6^-$ and Th-17 as $CXCR3^-CCR6^+$, analysing $CD3^+CD4^+$ T cells. Fig. 1a shows a

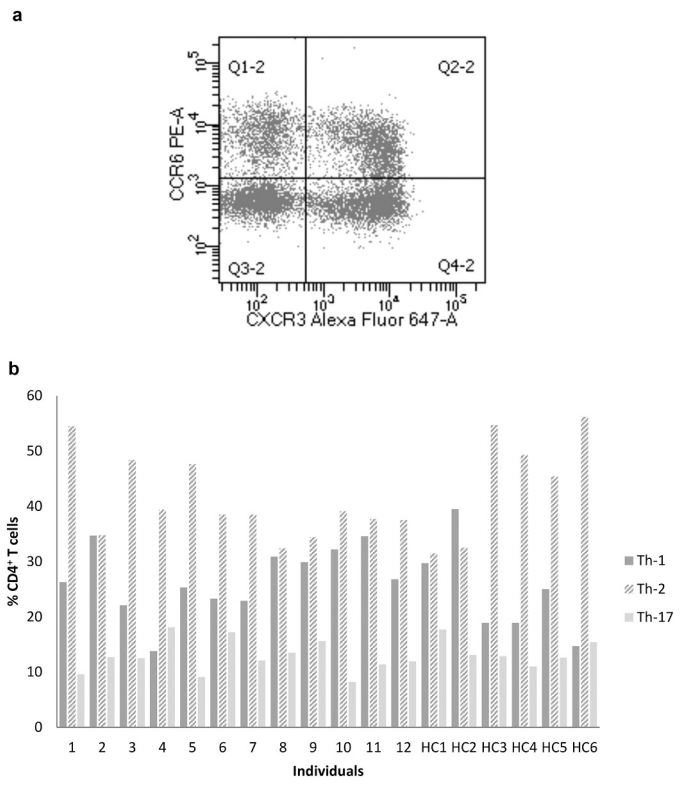

Fig. 1. T-helper frequency established using cell surface staining.

representative dot plot. Fig. 1b shows the values for each subtype in individuals post COVID-19 and HCs.

3.2. $CD4^+$ T Lymphocyte re-stimulation with SARS-CoV-2 antigen preparations

SARS-CoV-2 T cell recall response was determined as described in the section above.

Samples taken from the same individuals used as HC for phenotyping with no clinical history or evidence of SARS-CoV-2 infection were used as negative controls in the cytokine secretion assay. We observed a detectable $CD4^+$ T cell response, with IFNɣ and IL-17 generated in $CD4^+$ T cells in all cases although at lower level than in the confirmed post COVID-19 group (Tables 1 and 2).

Results for individuals with evidence of prior virus infection by means of a PCR test and/or SARS-CoV-2 IgG antibodies as detected by ELISA (Table 4) showed that for the majority of patients tested where there was evidence of an immune response against SARS-CoV-2 (detectable anti-IgG SARS-CoV-2 antibodies), there was also evidence of a CD4 T cell response measured by IFNɣ release from activated $CD4^+$ T cells. No cytokine release was detected from the unstimulated cells.

Table 1
Isolated PBMC from healthy control individuals with no history of COVID-19 exposure were re-stimulated with COVID-19 antigen preparations and cytokines measured using intracellular flow cytometry. Values are presented as % of CD4+ T cells expressing each cytokine and as number of events.

	IFNɣ		IL-4		IL-17	
	%	No. of events	%	No. of events	%	No. of events
HC 1	0.2	35	0.0	20	0.1	46
HC 2	0.0	24	0.0	8	0.1	4
HC 3	0.0	78	0.0	4	0.1	26
HC 4	0.1	40	0.0	16	0.1	24
HC 5	0.0	22	0.0	7	0.1	32
HC 6	0.0	26	0.0	15	0.1	51

Table 2
Mean positive events following stimulation of post COVID-19 individuals and healthy controls for the three cytokines measured.

	IFNɣ	IL-4	IL-17
Post COVID Individuals	130	8	92
Healthy Controls	38	12	31

Fig. 2a shows representative IFNɣ/IL-4 dot plots from unstimulated and antigen stimulated T cells.

The level of the SARS-CoV-2 CD4 T cells IFNɣ response was low (around 0.1% - 0.2% of $CD4^+$ T cells), although this is in line with expectation.

To attempt to further clarify these findings we also recorded the number of events in the cytokine gates in response to the antigenic re-stimulation. These results are shown in Fig. 2b/c and Table 3 and indicate that in the majority of antibody positive individuals an in-vitro T cell response could be generated following re-stimulation with peptide. The level of IL-4 was low in all cases in contrast to interferon levels. Interestingly IL-17 was also observed in a number of individuals following stimulation with antigen preparations.

Isolated PBMC were stimulated with antigen preparations as described in methods and cytokine generation measured using intracellular flow cytometry, using $CD4^+$ T cell generation of IFNɣ IL-4 and IL-17 as readout. Fig. 2a shows a representative example of staining from unstimulated and antigen stimulated CD4+ T cells. Fig. 2b shows percentage of CD4 T cell expressing cytokines, Fig. 2c expresses the results as number of positive events.

3.3. Antibody measurement

Table 4 presents results of testing by LABScreen COVID Plus assay and Euroimmun/IDK ELISA methods. The Euroimmun ELISA detects IgG antibody vs S protein whilst the IDK method detects IgG antibody vs NP protein. Thresholds for positivity in each assay were those defined by the manufacturer.

Comparison of results generated in both systems showed good correlation for S and NP reactivity. The LABScreen COVID Plus assay additionally identified that S reactivity was limited in some cases to certain structural components of SARS-CoV-2 S protein.

Comparative contributions of component antibody levels to overall individual level are shown in Fig. 3. Intra-individual differences in reactivity to viral structural components is demonstrable as well as marked inter-individual differences in total antibody level.

Contributory and total antibody levels measured in the LABScreen™ COVID Plus assay.

4. Discussion

We have performed immunological testing of a small cohort of individuals with history of SARS-CoV-2 infection utilising tests of both cell and antibody mediated immunity that are suited to performance in routine diagnostic laboratories. These provide an approach toward initial and ongoing assessment of SARS-CoV-2 immunity (and protection from reinfection) that is needed as part of laboratory activity to improve patient clinical management.

Although commercial ELISPOT methods are becoming available in this study we have taken a flow cytometry approach, measuring intracellular cytokines as a measure of COVID-19 T cell immunity. This approach was largely determined by available technology within our laboratory. Furthermore, given the general use of similar platforms in major clinical immunology laboratories in the UK we feel that the approach is one that could be readily adopted by other centres. Lymphocyte populations in post COVID-19 individuals were within the normal range and similar to healthy controls. This is in contrast to the significant perturbations reported for acute infection (Deng et al., 2020)

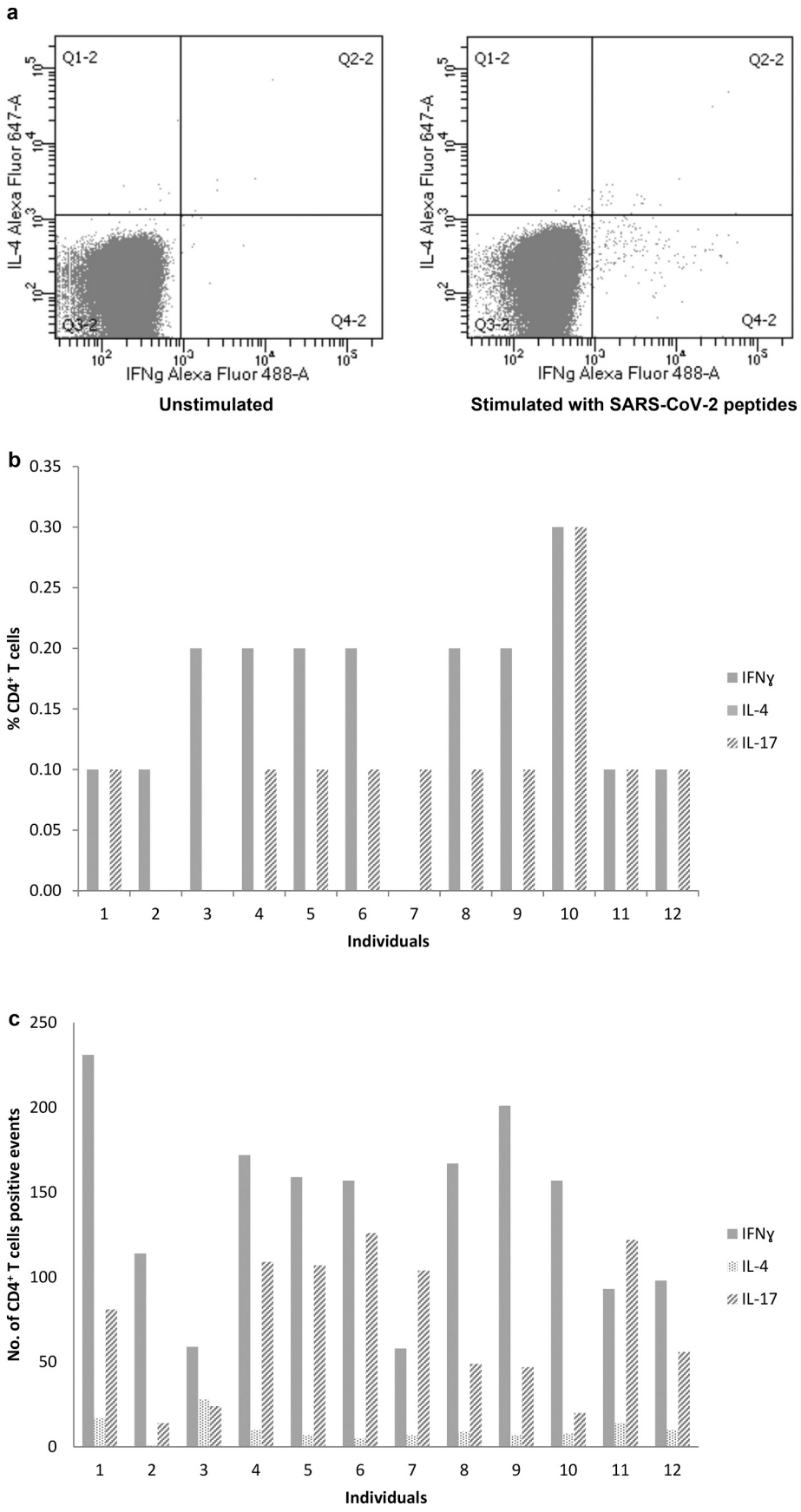

Fig. 2. PBMCs stimulation.

Table 3
T cell production of cytokines following in-vitro stimulation of PBMC. Isolated PBMC were stimulated with antigen preparations and cytokines detected by flow cytometry. Results presented describe the percentage and event number of CD4+ T cells positive for each cytokine.

Individuals	IFNɣ		IL-4		IL-17	
	% Positive	No. of events	% Positive	No. of events	% Positive	No. of events
1	0.1	231	0.0	17	0.1	81
2	0.1	114	0.0	1	0.0	14
3	0.2	59	0.0	28	0.0	24
4	0.2	172	0.0	10	0.1	109
5	0.2	159	0.0	7	0.1	107
6	0.2	157	0.0	5	0.1	126
7	0.0	58	0.0	7	0.1	104
8	0.2	167	0.0	9	0.1	49
9	0.2	201	0.0	7	0.1	47
10	0.3	157	0.0	8	0.3	20
11	0.1	93	0.0	14	0.1	122
12	0.1	98	0.0	10	0.1	56

and identifies that in individuals that have recovered from infection there are no on-going abnormalities in respect of quantitative markers of system integrity.

A recall response to SARS-CoV-2 antigen involving the T cell compartment can be readily demonstrated in recovered individuals. This is a feature of viral infections in general and important towards assessing immune memory and, potentially, on-going immunity to reinfection. The relatively low number of SARS-CoV-2 reactive cells demonstrated in the recall assay is consistent with expectation in that typically fewer than 0.001% of an individuals' T cells respond to virus (Alberts et al., 2002). The demonstration of lower-level reactivity in controls is intriguing and although this may represent assay system background it has previously been reported that T cell reactivity exists in up to 50% of individuals with no history of infection or of exposure to the virus (Grifoni et al., 2020). Evidence that this reflects pre-existing immunity to the virus resulting from exposure to other coronaviruses has been recently been provided (Ng et al., 2020).

We utilised a novel multiplex, bead based, methodology for detection of SARS-CoV-2 antibodies. Using this approach the response is shown to comprise sub-structural reactivities showing differential intra and inter-individual responsiveness with likely implications for protective immunity. An immunogenetic link with risk for infection, likely based in capacity for generation of a protective immune response has previously been demonstrated (Poulton et al., 2020). It is also of note in this regard that antibodies targeting the S2 protein provide protective immunity against SARS-CoV-2 (Zhong et al., 2005). In contrast anti S1 monoclonal antibodies have been demonstrated to facilitate virus entry into cells (Wang et al., 2014).

It is noteworthy that the test cohort includes individuals with positive antibody test results against common cold varieties of coronavirus, SARS-CoV and MERS. Whilst it would not be unexpected to detect IgG seroreactivity to antigens from common human coronaviruses with known seasonal circulation, including 229E, HKU1, NL63 and OC43, the low prevalence and geographic spread of SARS-CoV and MERS means there would be less expectation for exposure to these viruses. The possibility cannot be entirely discounted however since the cohort included members of staff from the Infectious Diseases unit where there has been one confirmed case of MERS and who therefore may have experienced workplace exposure. A more likely scenario is that such reactivity reflects conservation of sequences between different human coronaviruses as has been reported (Lei and Zhang, 2020). Bystander reactivation of humoral memory may also play a part (Horns et al., 2020). Lastly, manufacturer defined thresholds for antibody positivity were applied and may be set too low, resulting in the generation of false positive results. Further work would be required to establish which of these possibilities accounts for the findings.

Table 4
Results of LABScreen COVID Plus/Euroimmun IgG and IDK IgG ELISA assays interpreted in accordance with manufacturer's instructions.

Individual	LABScreen COVID Plus									ELISA	
	SARS-CoV-2 Spike	SARS-CoV-2 Spike S1	SARS-CoV-2 Spike RBD	SARS-CoV-2 Spike S2	SARS-CoV-2 NP	HCoV-229E Spike S1	HCoV-HKU1 Spike S1	HCoV-NL63 Spike S1	HCoV-OC43 Spike S1	MERS-CoV	SARS-CoV Spike S1	IgG Euro	IgG IDK
1	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE
2	POSITIVE	POSITIVE	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE
3	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE
4	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	NEGATIVE	NEGATIVE	POSITIVE	NEGATIVE
5	POSITIVE	POSITIVE	POSITIVE	POSITIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE
6	POSITIVE	POSITIVE	POSITIVE	POSITIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE
7	POSITIVE	POSITIVE	POSITIVE	POSITIVE	NEGATIVE	NEGATIVE	POSITIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE
8	POSITIVE	POSITIVE	POSITIVE	POSITIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
9	POSITIVE	POSITIVE	POSITIVE	POSITIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	NEGATIVE
10	POSITIVE	NEGATIVE	POSITIVE	POSITIVE	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE
11	POSITIVE	POSITIVE	POSITIVE	POSITIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	NT
12	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE	NEGATIVE	NEGATIVE	POSITIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE	POSITIVE

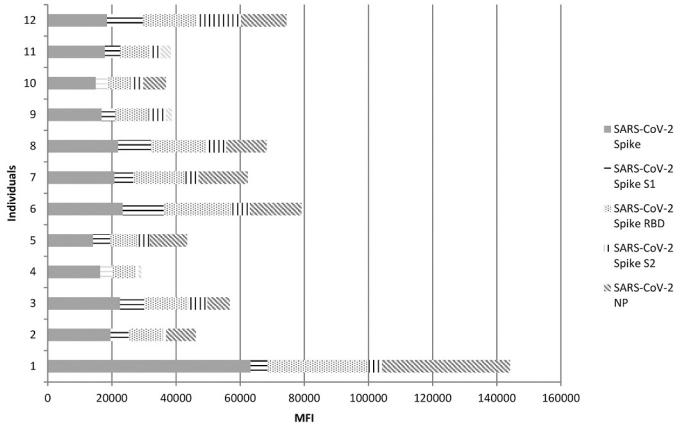

Fig. 3. SARS-CoV-2 Antibody levels.

Although T cell and neutralising antibody responses have been described to play a role in virus clearance (Braun et al., 2020) correlates of protective immunity are not yet firmly established. In particular, it is not clear how strong a response is needed to prevent reinfection and the duration of immunity is not defined. Addressing such questions requires diagnostic laboratories to begin to establish test systems for ongoing quantitative evaluation of immune status of previously infected or vaccinated individuals.

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