Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
SARS-CoV-2 diagnostics: Towards a more comprehensive approach to routine patient testing

Clive Carter\textsuperscript{a}, Pamela Hughes\textsuperscript{a}, Anna McHugh\textsuperscript{a}, Fatima Nadat\textsuperscript{a}, Penny Lewthwaite\textsuperscript{b}, Sinisa Savic\textsuperscript{c}, Brendan Clark\textsuperscript{a,*}

\textsuperscript{a} Departments of Immunology, Leeds Teaching Hospitals, UK
\textsuperscript{b} Departments of Infectious Diseases, Leeds Teaching Hospitals, UK
\textsuperscript{c} Departments of Clinical Immunology, Leeds Teaching Hospitals, UK

ARTICLE INFO

Keywords: SARS-CoV-2 COVID-19 Virus Immunity Humoral Cellular

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.

* Corresponding author.
E-mail address: brendanclarke@nhs.net (B. Clark).

https://doi.org/10.1016/j.jim.2021.113044
Received 4 December 2020; Received in revised form 17 March 2021; Accepted 20 March 2021
Available online 27 March 2021
0022-1759/Crown Copyright © 2021 Published by Elsevier B.V. All rights reserved.
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 results of this testing are shown in Table 4.

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 fluorochrome labelled antibodies (Becton Dickinson, BD) that recognised cell surface determinants on T, B and NK cells (CD45/CD3/CD4/CD8/CD19/CD16–). Following erythrocyte lysis the samples were acquired on FACSCan 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^hiCD38^- transitional B cell and CD38^hiCD27^- plasmablast populations. Naïve and memory CD3^+ CD4^+ T cells were determined by staining with antibodies against CD3/CD4/CD45RA 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% BSA. After washing steps were complete, the cell pellet was resuspended in approximately 300 µL PBS + 0.5% formaldehyde. Results were acquired using FACSCan 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 Lymphocyte H (Cedarlane). Following washing in tissue culture media, isolated cells were adjusted to a density to 5 × 10^5/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% CO2. At the end of the incubation, the cells were harvested, washed, fixed and permeabilised prior to the addition of antibodies against CD4, IFNy, IL-4 and IL-17, following manufacturer’s (BD) instructions. Data was acquired using a FACSCan 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. IFNy, 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^hiCD38^- transitional B cell and CD38^hiCD27^- 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 CD45RA^-CD27^- T effector memory (TEM) cells; and 3.95% vs 2.15% for CD45RA^-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 fluorochrome labelled antibodies against CD3, CD4, CXCR3 and CCR6. Following lysis and washing, the cells were acquired on a FACSCan 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
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 IFNγ 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 IFNγ release from activated CD4+ T cells. No cytokine release was detected from the unstimulated cells.

#### Table 1

|                | IFNγ | IL-4 | IL-17 |
|----------------|------|------|-------|
|                | %    | No. of events | %    | No. of events | %    | No. of events |
| Healthy Controls | 0.2 | 35 | 0.0 | 20 | 0.1 | 46 |
| Post COVID Individuals | 0.0 | 24 | 0.0 | 8 | 0.1 | 4 |

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

The level of the SARS-CoV-2 CD4+ T cells IFNγ 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 antigen 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 IFNγ-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.
...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 reinfestation. 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 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 T cell production of cytokines following in-vitro stimulation of PBMC. Isolated T cells positive for each cytokine.

Table 3

| Individuals | IFNγ | 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   | 17   | 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   | 3    | 0.3    | 20              |
| 11          | 0.1   | 93    | 0.0   | 14   | 0.1    | 122             |
| 12          | 0.1   | 98    | 0.0   | 10   | 0.1    | 56              |

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.
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.

References

Alberts, B., Johnson, A., Lewis, J., et al., 2002. Molecular Biology of the Cell, 4th ed. Garland Science, New York. T Cells and MHC Proteins. Available from: https://www.ncbi.nlm.nih.gov/books/NBK26926/.

Braun, J., Loyal, L., Frentsch, M., Wendisch, D., Georg, P., Kurth, F., et al., 2020. Presence of SARS-CoV-2-reactive T cells in COVID-19 patients and healthy donors. medRxiv. https://doi.org/10.1101/2020.04.17.20061440.

C. Carter et al.