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Short Communication

A flow cytometry-based proliferation assay for clinical evaluation of T-cell memory against SARS-CoV-2

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ABSTRACT

In general, the method of choice for evaluating immunity against Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is detection of antibodies against the virus in patient sera. However, this is not feasible in patients who do not produce antibodies, either due to a primary immunodeficiency or secondary to treatment with immunosuppressive drugs. Assessment of the antiviral T cell response is an alternative to serological tests, but most T cell assays are labor-intensive and unsuitable for a clinical routine laboratory.

We developed a flow cytometry-based assay for T cell proliferative responses against SARS-CoV-2, based on the detection of blast transformation of activated cells. The assay was validated on previously SARS-CoV-2 infected individuals and healthy seronegative blood donors, displaying 74% sensitivity and 96% specificity for previous infection with SARS-CoV-2. The usefulness of the assay was demonstrated in a patient with common variable immunodeficiency with a history of COVID-19. The described T-cell assay is a clinically relevant complement to serology in the evaluation of cellular immunity against SARS-CoV-2, which can be emulated by any routine lab with flow cytometric competence.

1. Introduction

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has spread rapidly across the globe and coronavirus disease 2019 (COVID-19) was declared a pandemic by the World Health Organization (WHO) on the 11th of March 2020. This evoked an intense effort from the scientific community to characterize the host response against the virus and to develop vaccines. Much interest has been directed towards the antibody response to SARS-CoV-2, whereas less attention has been given to concomitant T cell responses.

The role of T cells in antiviral responses and formation of immunological memory in general is well-recognized. Indeed, several studies have demonstrated T cell memory and effector responses against a broad selection of epitopes from SARS-CoV-2, as well as cross-reactive responses in unexposed individuals (Sekine et al., 2020; Braun et al., 2020; Tarke et al., 2021). However, most available methods for assessment T cell reactivity are labor-intensive and unsuitable for a clinical routine laboratory.

The Flow-cytometric Assay for Specific Cell-mediated Immune response in Activated whole blood (FASCIA) is a well-established, versatile method for assessment of T lymphocyte reactivity against a wide variety of stimuli, including mitogens, superantigens and microbial antigens (Marits et al., 2014). Since the assay is based on whole blood, it requires no laborious preparation of PBMCs prior to set-up. The read-out is blast transformation of activated cells, detected by flow cytometry as an increase in forward scatter, with addition of fluorophore-conjugated antibodies against lymphocyte lineage antigens.

Herein, we describe the development of a FASCIA protocol for detection of T cell responses against SARS-CoV-2, where the method is validated on samples from COVID-19 convalescent plasma (CCP) donors and compared to healthy control samples from seronegative healthy blood donors with no history of COVID-19. The clinical applicability of the test is illustrated by analysis of cells from a patient with common variable immunodeficiency (CVID) and a history of natural COVID-19

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infection.

2. Methods

2.1. Study subjects

Peripheral blood samples were collected from 65 COVID-19 convalescent plasma (CCP) donors and 55 seronegative healthy blood donors with no history of COVID-19 (Table 1). CCP donors were characterized as positive for SARS-CoV-2 IgG, fully recovered from infection and afebrile for >14 days.

The study was approved by the Swedish Ethical Review Authority (Approval No: 2020-02522). Informed consent was obtained from the CCP donors and the CVID patient.

2.2. Enumeration of CD3+ T-lymphocytes

Analysis of CD3+ T-lymphocytes was performed by flow cytometry on a Aquios CL (Beckman coulter) which utilizes a direct volumetric single-platform method with incorporated sample preparation with a monoclonal antibody mixture (anti-CD45-FITC [clone B3821F4A], anti-CD56-RDI [clones N901 + NKH-1], anti-CD16-RDI [clone 3G8], anti CD19-ECD [clone J3–119], anti-CD3-PC5 [clone UCHT1]) Beckman Coulter.

2.3. FASCIA

The FASCIA-method has been described in detail elsewhere (Marits et al., 2014). Briefly, heparinized whole blood (diluted 1:10) was stimulated in GlutaMAX RPMI 1640 (supplemented with 2 mM L-glutamine, 100 IU/mL penicillin, 100 IU/mL streptomycin) in a final volume of 250 uL in U-bottom 96-well plates (Falcon) for 7 days at 37 °C, 5% CO_2 and 95% humidity. At day 7, the samples were stained with CD3-FITC/CD4-PE Simultest (BD Biosciences) and erythrocytes were lysed using IOTest lysing solution (Beckman Coulter) before 20% of the sample was acquired on a Cytoflex S flow cytometer (Beckman Coulter). Blasts were identified as cells with high FSC and divided into CD4 (CD3 + CD4+) and CD8 (CD3 + CD4-) in KaluzaC software (Beckman Coulter) (Fig. 1a). Results are expressed as blasts/μL whole blood which is calculated as follows: Acquired blasts x 5 (in order to get total blasts) / 25 μL (volume of whole blood in the FASCIA-assay).

2.3.1. Stimuli

Pokeweed mitogen (PWM) at 5 μg/mL was used as positive control of T cell blast formation ability. The SARS-CoV-2 proteins and peptide pools tested in the FASCIA-assay were derived from nucleocapsid (N) protein and spike (S) protein and used together with 0.1 μg/mL anti-CD28 (Mabtech AB). SARS-CoV-2 N-protein and S-protein RBD-domain (kind gifts from M. Sällberg and J. Nordin, respectively) were used at 1–2 μg/mL. Scanning peptide pools (15-mer sequences with 11 amino acids overlap) were used at 0.25, 0.5, 1 and 2 μg/mL. The peptide pools were from Miltenyi Biotech (PepTivator SARS-CoV-2 prot N and PepTivator SARS-CoV-2 prot S) and from JPT (N- and C-terminal PepMix SARS-CoV-2 Spike B.1.429).

SARS-CoV-2 (isolate SARS-CoV-2/human/SWE/01/2020; accession number MT093571) was grown on Vero E6 cells for 3 days. Supernatants were collected and spun at 600 *g* for 6 min to remove cell debris. Supernatants were then UV-inactivated using a VL-215.G UV-lamp (Vilber Lourmat). Supernatants containing inactivated SARS-CoV-2 were then used at a 10-fold dilution.

Table 1
Characteristics of study subjects.

	Healthy controls (n = 55)	CCP-donors (n = 65)
Age, y, median (range)	50 (24–68)	48 (21–63)
Sex, F / M, n (%)	10 (18) / 45 (82)	29 (45) / 36 (55)
T-cells, cells/uL, median (range)	1121 (685–2009)	1132 (454–2238)
SARS-CoV-2 IgG, ratio, median (range)	0.2 (<0.1–0.6)	4.5 (2.2 - >12)

2.4. SARS-CoV-2 IgG ELISA

Titres of anti-SARS-CoV-2 antibodies in serum were measured with SARS-CoV-2 IgG ELISA (Euroimmun), which uses a recombinant structural spike 1 (S1) protein as target, and the assay was performed according to the manufacturer's instructions. Results are expressed semi-quantitatively as a ratio and values <0.8 are considered negative, 0.8–1.1 borderline and ≥ 1.1 are classified as positive.

2.5. Statistics

Statistical analyses were performed using GraphPad Prism software 9.0 (GraphPad Software). Differences between HC and CCP-donors were determined by Mann-Whitney *U* test and Fisher's exact test. The ability of the FASCIA-assay to differentiate between positive and negative samples was determined by ROC-analysis. Correlation analyses were performed using Spearman correlation. Repeated samples were compared by Two-way ANOVA or Wilcoxon signed-rank test.

3. Results and discussion

FASCIA is a well-established clinically available analysis at Karolinska University Laboratory for detection of memory T-cell immune responses against infectious agents. Hence, it was our first option for assessment of cellular immunity against SARS-CoV-2. FASCIA enables evaluation of both CD4+ and CD8+ T-cell responses with the gating strategy outlined in Fig. 1a. To validate the assay, we used heparinized blood samples from COVID-19 convalescent plasma (CCP) donors and seronegative, healthy blood donors with no history of COVID-19 (Table 1). Pokeweed mitogen was used to confirm general T cell responsiveness, normal blast formation was observed in all tested subjects.

No significant differences were observed in age or T-cell counts between healthy coontrols and CCP-donors, but the frequency of females was significantly lower in the healthy control-group.

3.1. T-cell responses against spike peptide pools

First, commercially available SARS-CoV-2 antigens were evaluated as stimuli in CCP donors, including peptide pools and whole protein from nucleocapsid (N) and spike (S) proteins. In most cases, the blast responses against these antigens were very low (data not shown), except for peptide pools from the spike protein, where CD4+ T-cell responses above background were consistently detectable. However, when comparing the responses in CCP donors with those of healthy controls, there was no significant difference (Fig. 1b).

The low responses are presumably due to inefficient antigen presentation and/or absence of innate adjuvant signals in the peptide pools. Theoretically, such issues may be overcome by increasing the antigen concentration, but the appearance of unspecific background fluorescence with higher concentrations of the peptides precluded such measures.

Cross-reactivity may contribute to the lack of discriminative ability, but the assay performance was not improved by selective use of peptides derived from the N-domain of the spike protein, which is less homologous to other coronaviruses (data not shown).

3.2. T-cell responses against inactivated whole virus

To find a more potent stimulus in the FASCIA, supernatants containing SARS-CoV2 whole viral particles, which are processed through

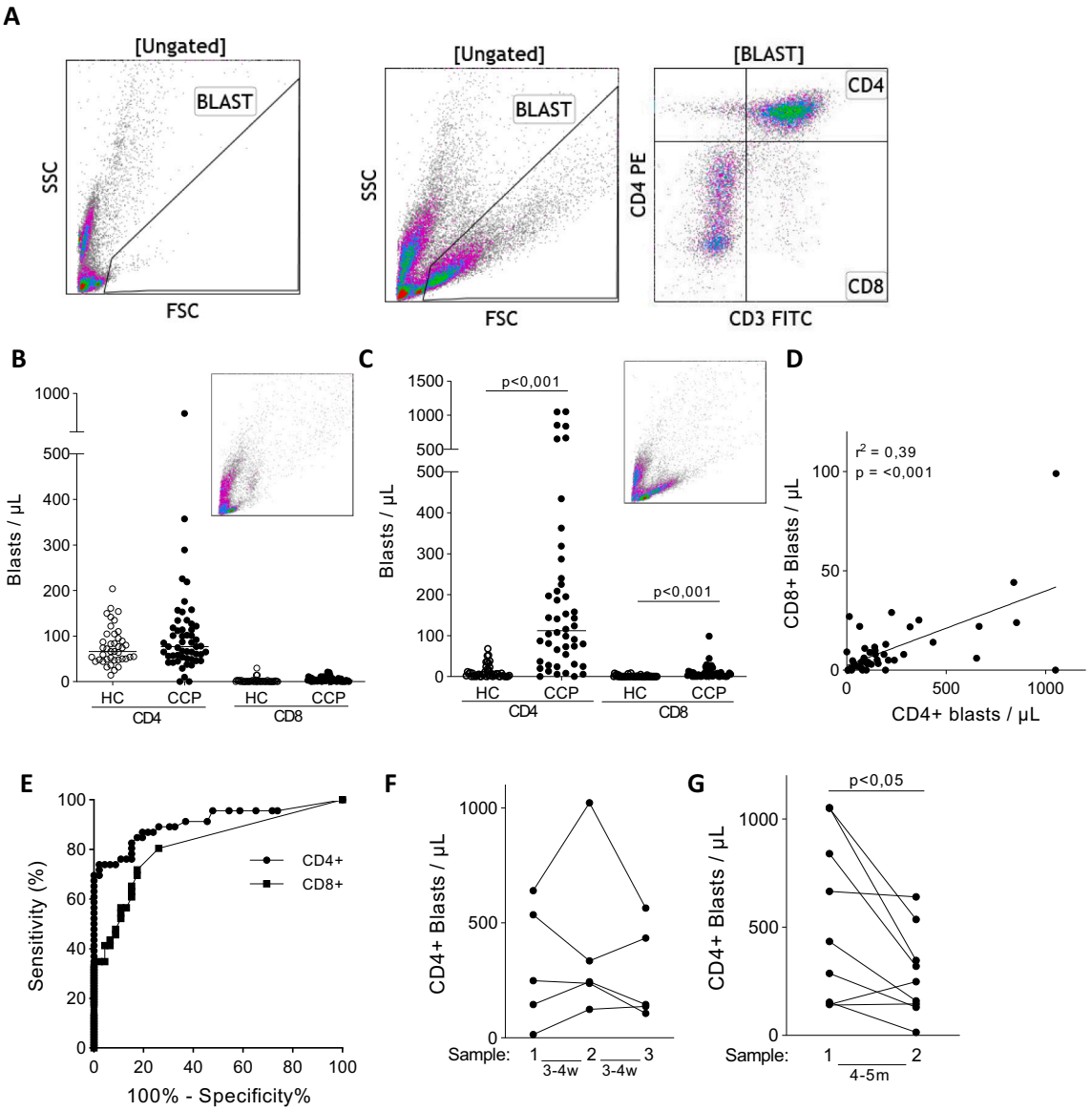

Fig. 1. Memory T-cell response against SARS-CoV-2 measured by FASCIA.
(A) Gating strategy for blasts (high FSC) in unstimulated (left) and PWM-stimulated (right). CD4+ blasts were defined as CD3 + CD4+ and CD8+ blasts were defined as CD3 + CD4-. (B) T-cell responses to spike peptides in HC ($n = 41$) and CCP-donors ($n = 56$). Insert shows typical FSC/SSC plot in CCP-donor. (C) T-cell responses to whole viral particles in HC ($n = 46$) and CCP-donors (n = 46). Insert shows typical FSC/SSC plot in CCP-donor. (D) Correlation between CD4 + and CD8+ blasts in CCP-donors stimulated with whole viral particles (n = 46). (E) ROC-curve of CD4+ and CD8+ blasts in HC and CCP-donors stimulated with whole viral particles (n = 46). (F-G) CD4+ blasts in CCP-donors over time. Samples were taken 3–4 weeks (F) and 4–5 months (G) apart. *P*-values calculated using Mann-Whitney *U* test (C) or Wilcoxon signed-rank test (G). Correlation calculated with Spearman's correlation (D).

other routes of antigen presentation as compared to peptides, were evaluated. This resulted in much more pronounced T cell responses in CCP donors and displayed less cross-reactivity in the seronegative control group, as compared to peptide pools from the spike protein. The difference was observed in both CD4+ and CD8+ T-cells but was most pronounced in the CD4+ T cell population (Fig. 1c). The CD8+ T cell responses were of clearly lower magnitude, which presumably reflects presentation of extracellular antigens on MHC class II and poor conditions for cross-presentation in the FASCIA. However, the correlation between CD4 and CD8 responses (Fig. 1d) indicates that the CD4+ T cell response in the FASCIA is a valid surrogate marker for total T cell reactivity against SARS-CoV-2. There was no difference in the background T-cell reactivity against

SARS-CoV-2 when comparing male and female healthy controls. A significantly higher number of CD4+ T cell blasts was observed in female (median 197; range 20-1052) as compared to male CCP donors (median 80; range 0-853), which is in agreement with observations made by others (Takahashi et al., 2020). There was no significant correlation between age and the FASCIA-results (data not shown).

3.3. Assay performance

The FASCIA against whole viral particles displayed a sensitivity of 74% and a specificity of 96% for previous infection with SARS-CoV-2, using a cut-off of 50 CD4+ T cell blasts/µl (Fig. 1e). The area under the ROC curve for the CD4+ T-cell response was 0.91. A cut-off above 7 blasts/µl was necessary to achieve a specificity >95% for CD8+ responses, however this resulted in a sensitivity of 41% which was considered too low to be clinically useful (Fig. 1e).

In a subset of CCP donors, samples were obtained at multiple time

points, allowing for evaluation of short- and long-term reproducibility. No significant differences were observed with respect to the magnitude of CD4+ blast responses in 3 repeated samples obtained within a time frame of 2 months (Fig. 1f). Over an extended observation period of up to 5 months, a small, but significant, decline in the CD4+ blast response was observed in a majority of CCP donors (Fig. 1g). Importantly, the exact time of infection in the CCP donors in our study is unknown but was approximately 2–4 months before the first sample, thus the second sample was obtained 6–9 months post infection. In concordance, Feng et al. recently described retained T cell reactivity up to 1 year after SARS-CoV2 infection (Feng et al., 2021).

3.4. Correlation with antibody titers

There was no apparent correlation between the CD4+ T cell response in the FASCIA and antibody titers. Notably, a subset of CCP-donors displayed high numbers of CD4+ T cell blasts (>500) and intermediate antibody levels, whereas another group had lower CD4+ T cell responses and, conversely, higher antibody titers (Fig. 2). This is in concordance with other studies where discordant antibody and T cell responses have been reported (Sekine et al., 2020), (Rydyznski Moderbacher et al., 2020), (Schwarzkopf et al., 2021). If this observation reflects an underlying difference in the biology of the immune response remains to be investigated. However, it implies that the FASCIA has an inherent diagnostic value, independent of the antibody response.

3.5. Patient case report

The patient was a 42-year-old male with common variable immunodeficiency (CVID), who fulfilled all diagnostic criteria for CVID, including low IgG (around 2 g/L prior to IgG replacement therapy), absent IgM and IgA as well as a weak response to vaccination (DiTee-Booster and Pneumovax). The patient was on immunoglobulin replacement therapy since the onset of the disease 20 years ago. The disease course has been dominated by bacterial respiratory tract infections and more recently with the development of granulomatous lymphocytic interstitial lung disease (GLILD). Treatment has been given in the form of high dose corticosteroids and ibrutinib to inactivate B-cells. Approximately 3 months after initiation of Ibrutinib-treatment, the patient contracted COVID-19, which was verified by PCR. The symptoms were mild and consisted of a sore throat, back pain, fatigue, and a low-grade fever. The patient did not have to seek medical attention and tested himself via a COVID-19 test-center located outside an outpatient clinic in his hometown. The patient is seen regularly at the primary immunodeficiency clinic. Upon one of his regular visits, 3 weeks after the infection, blood was drawn for analysis of COVID-19 antibodies and FASCIA. The patient had a clear positive response in the FASCIA-analysis (131 CD4+ blasts/µl), whereas he was negative for COVID-19 IgG antibodies. A follow-up sample for COVID-19 serology 5 months later revealed a low positive IgG response against the spike-protein. This low IgG response is probably endogenous, although it cannot be ruled out that it is a trace from the commercial immunoglobulin preparation. However, the most likely explanation is that the patient has a very low endogenous capacity to produce antibodies after COVID-19 and that this capacity was further reduced by the ibrutinib treatment.

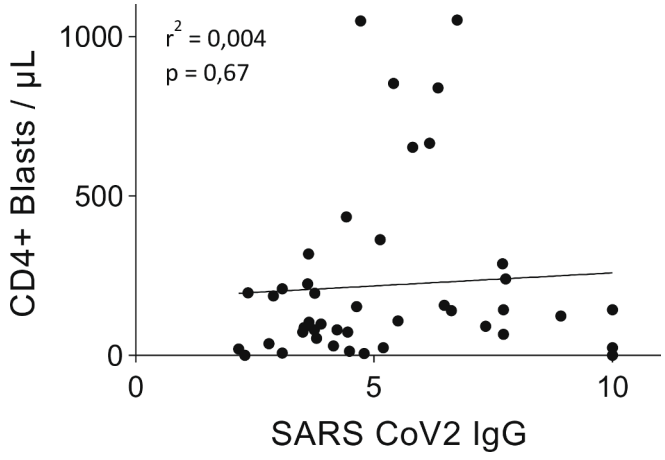

Fig. 2. Correlation analysis between the CD4+ T-cell response and antibody titer in CCP-donors (n = 44). Correlation calculated with Spearman's correlation.

The disease course of COVID-19 in patients with CVID has been reported previously (Ameratunga et al., 2021; Gupta et al., 2021). Interestingly, the clinical picture seems to be very variable, where a majority have a mild/moderate disease, and a few suffer from very severe/life-threatening disease. A worse outcome seems to be connected to dysfunctional T-cells, either caused by concomitant malignancy, T-cell suppressive therapy or for idiopathic reasons. Thus, T-cells seems to be central for a protective immune-response against COVID-19. The importance of T-cell immunity is further illustrated by patients with X-linked agammaglobulinemia, who do not have any endogenous antibody production, and yet are fully able to recover from COVID-19 infection (Soresina et al., 2020; Almontasheri et al., 2021).

This case-presentation serves to illustrate that FASCIA, a clinically available T-cell assay, can be useful to evaluate immune responses against SARS-CoV-2 in patients with low or non-existent antibody production. Importantly, the lack of a proper antibody response extends beyond patients with CVID and XLA and comprise solid organ transplant patients, stem cell transplant patients and patients undergoing treatment with B-cell depleting drugs, including rituximab. Thus, the SARS-CoV-2 FASCIA is an excellent clinical tool that could be used to assess immune responses in a wide range of immunocompromised patients.

4. Conclusion

In the present study, we developed a flow cytometry-based assay for detection of T cell proliferative responses against SARS-CoV-2. With this assay, we demonstrated that CD4+ T cell responses against whole, inactivated SARS-CoV-2 are readily detectable in most CCP donors. Multiple studies (Braun et al., 2020; Sekine et al., 2020; Tarke et al., 2021) have demonstrated cross-reactive T cell responses against SARS-CoV-2 in unexposed individuals, presumed to be directed against epitopes shared with common cold coronaviruses. These responses were usually of lower magnitude than those seen post-COVID-19, which is in accordance with our results, since the seronegative control group rarely displayed responses above 50 CD4+ T cell blasts/µl.

The limitations of the assay are to a large extent embedded in its design: The use of whole blood instead of PBMCs or selected cells is efficient but does not allow for correction of the initial number of cells, which naturally will vary depending on the donor. Furthermore, the blast response recorded only reflects the number reacting cells but does not provide any information about cytokine profile or type of T cell response beyond the CD4/CD8 distinction. The use of whole viral particles instead of defined peptides precludes epitope mapping. Finally, whole viral particles are not commercially available and thus requires a P4 laboratory for manufacturing. There are other viable options for assessing T cell reactivity against SARS-CoV-2, including ELISpot, intracellular cytokine staining (ICS) and AIM assay (Sekine et al., 2020). ELISpot has the advantage of a lower limit of detection but requires specialized instrumentation. A diagnostic approach for assessment of SARS-CoV-2 T cell reactivity involving ICS was recently described by Carter et al. (2021). Certainly, ICS provide information about the cytokine profile of responding CD4+ T-cells that is not obtained in the FASCIA. On the other hand, ICS is significantly more labor intensive and less suitable for large scale analyses. AIM assays are not dependent on detection of a specific cytokine, but their sensitivity may vary dependent on the choice of activation markers (Reiss et al., 2017). The FASCIA

relies on detection of proliferation, a universal response of activated memory T-cells. In addition, it is an uncomplicated and cost-effective assay format that can be performed with minimal equipment. Since it is based on whole blood, it does not require PBMC preparation and it can easily be adopted to a 96-well format, further decreasing hands-on time.

In summary, we have developed an assay for T cell reactivity against SARS-CoV-2 suitable for clinical use. Assessment of SARS-CoV-2 T cell reactivity is of obvious importance in antibody deficient and otherwise immunocompromised patients, as exemplified by the patient described herein. It can also be useful in evaluation of vaccine responses and assessment of the durability of SARS-CoV-2 immunity on the population level. Metrics that correlate to long-term protection from SARS-CoV-2 are still poorly defined. To this end, large-scale, prospective studies of antibody levels and cellular immunity are needed, and the FASCIA may be a valuable tool in such studies.

Declaration of Competing Interest

None.

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