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Development of an in-house SARS-CoV-2 interferon-gamma ELISpot and plate reader-free spot detection method

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ABSTRACT

Coronavirus disease 2019 (COVID-19) vaccination programs rolled out in an attempt to stop the COVID-19 pandemic. Besides neutralising antibodies, effective T cell responses are also crucial for protection against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection and COVID-19 disease severity.

To assess SARS-CoV-2-specific T cell immunity, we developed an interferon-gamma (IFN-γ) enzyme-linked immunospot (ELISpot) that can be deployed in research and diagnostic settings. We optimised our ELISpot by testing multiple antigen concentrations to stimulate peripheral blood mononuclear cells of SARS-CoV-2-unexposed, COVID-19 convalescent and COVID-19 vaccinated volunteers. Also, we developed an ELISpot plate reader-free method to detect and quantify spots, which we compared to manual spot counting and automated analysis by an ELISpot plate reader.

We observed strong SARS-CoV-2-reactive T cell responses in COVID-19 convalescent, and COVID-19 vaccinated volunteers but absent or only weak responses in unexposed volunteers. Overall, antigens with concentrations from 0.1 to 5.0 μg/mL per peptide elicited similar T cell responses. Also, our plate reader-free detection method reliably detected and quantified SARS-CoV-2-specific T cells, demonstrated by an excellent reliability when compared to manual analysis and automated analysis by an ELISpot plate reader.

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused a pandemic and a worldwide healthcare crisis. The situation's urgency demanded the rapid development of diagnostic tests to determine current and past SARS-CoV-2 infection and to assess immune protection following prior infection or coronavirus disease 2019 (COVID-19) vaccination (Ong et al., 2021). COVID-19 vaccination campaigns are massively rolled out in an attempt to end the pandemic, while the persistence of immune protection after COVID-19 disease or vaccination remains to be elucidated (Poland et al., 2020).

Protective immunity against COVID-19 is considered to be associated with the presence of neutralising antibodies that target the SARS-CoV-2 spike's receptor-binding domain (RBD) (Sette and Crotty, 2021). Therefore, serological analyses of neutralising antibodies are routinely performed to evaluate the immune status after COVID-19 disease and vaccination. However, there is emerging evidence that an effective $CD4^+$ and $CD8^+$ T cell response is also crucial for protection against SARS-CoV-2 infection and COVID-19 resolution (Bertoletti et al., 2021). For example, T cell cytopenia is a well-characterised observation in severe COVID-19 patients (Li et al., 2020; Liao et al., 2020), and the presence of robust SARS-CoV-2-specific $CD4^+$ and $CD8^+$ T cell responses is associated with successful recovery from COVID-19 (Grifoni et al., 2020). Hence, the assessment of SARS-CoV-2-specific T cell responses may also be essential to evaluate the SARS-CoV-2 immune status after natural infection or COVID-19 vaccination.

Various assays can assess functional T cell responses, with each having its specific strengths and limitations. One of these assays is the enzyme-linked immunospot (ELISpot), a modified enzyme-linked immunosorbent assay (ELISA) technique (Calarota and Baldanti, 2013; Ji and Forsthuber, 2014). In the ELISpot, peripheral blood mononuclear cells (PBMCs) are stimulated with antigens in a microtiter plate, either in the form of whole antigens or a pool of peptides. Antigen-specific reactive T cells secrete the cytokine interferon-gamma (IFN-γ), which

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is captured and stained. Subsequently, captured IFN-γ becomes visible as precipitated 'spots' on the membrane, representing the footprints of antigen-specific cytokine-secreting cells. The number of spots is a direct measurement of antigen-specific IFN-γ-producing T cells (Smith et al., 2001).

Spots are usually detected and quantified with automated reader systems (Calarota and Baldanti, 2013). However, such systems are often not part of medical microbiology and immunology laboratories and require substantial investment. Therefore, there is a need for a more economical and alternative standardised method to analyse ELISpot results.

In this technical note, we aimed to describe the performances of our in-house SARS-CoV-2 ELISpot assay and how different peptide concentrations influence SARS-CoV-2-specific T cell responses in SARS-CoV-2 unexposed, COVID-19 convalescent, and COVID-19 vaccinated individuals. Furthermore, we compared the performances of our partially automated reader-free spot-detection method to manual spot counting and spot counting by an automated ELISpot plate reader.

2. Materials and methods

2.1. Voluntary participants

Whole blood was obtained from 12 hospital staff members of our department by venepuncture using lithium-heparin blood collection tubes. SARS-CoV-2 unexposed (n = 3), COVID-19 convalescent (n = 3; 99, 153, and 349 days after SARS-CoV-2 positive reverse transcriptase quantitative polymerase chain reaction), and vaccinated (n = 6, range 10–61 days after administration of second COVID-19 vaccine dose) volunteers were included. Vaccinated volunteers were vaccinated with BioNTech/Pfizer's BNT162b2 (n = 5) or Moderna's mRNA-1273 (n = 1) mRNA vaccine. All staff members agreed to volunteer in the study.

2.2. PBMC isolation

Within eight hours after blood collection, PBMCs were isolated from blood samples using the Ficoll® paque density gradient separation. Cells were washed twice by the addition of pre-heated (37 °C) RPMI cell culture medium (RPMI 1640 medium; Gibco) and centrifugation. The pellet was resuspended in pre-heated (37 °C) AIM-V medium (AIM-V® + AlbuMAX® (BSA); Gibco), which is a cell culture medium containing lipid-rich bovine serum albumin, L-glutamine, streptomycin sulfate at 50 μg/mL, and gentamicin sulfate at 10 μg/mL. The PBMC concentration was determined in an automated cell counter (WBC System; HemoCue®), whereafter PBMCs were diluted in pre-heated (37 °C) AIM-V medium to a final concentration of 5×10^6 cells per mL.

2.3. SARS-CoV-2 ELISpot assay

The ELISpot is a simple but extremely sensitive assay that detects antigen-specific cytokine-secreting T cells at a single-cell level by stimulating T cells with an antigen. The ELISpot distinguishes from other T cell assays by its simpler method and the redundancy of additional expensive laboratory equipment. Also, results are obtained relatively fast, enabling high sample throughput (Negri et al., 2020). These assets make the ELISpot cost-effective and particularly suitable for routine screening purposes in diagnostic medical microbiology and immunology laboratories to determine the presence of SARS-CoV-2-specific T cells.

On day 1, microtiter strip plates precoated with a monoclonal anti-IFN-γ antibody (mAb 1-D1K; Mabtech) were washed three times with phosphate-buffered saline (PBS; bioMérieux) and conditioned for 30 min with AIM-V medium at room temperature. The following stimulators were added in a volume of 50 μL per well: AIM-V as negative control, anti-CD3 (1:1000; mAb CD3-2; Mabtech) as positive control, and SARS-CoV-2 peptide pools consisting of 15-mer sequences with 11 amino acids overlap that cover the predicted immunodominant regions of spike (S_{id}) (PepTivator® SARS-CoV-2 Prot_S; Miltenyi Biotec) or overlap the entire spike S1 subunit (S1) (PepTivator® SARS-CoV-2 Prot_S1; Miltenyi Biotec), nucleocapsid protein (N) (PepTivator® SARS-CoV-2 Prot_N; Miltenyi Biotec), or membrane protein (M) (PepTivator® SARS-CoV-2 Prot_M; Miltenyi Biotec). Peptide pools were diluted in AIM-V medium to a final concentration of 0.1, 0.5, 1.0, 2.0, and 5.0 μg/mL peptide. The pre-diluted PBMCs were added at 50 μL per well in a final cell number of 2.0×10^5 or 2.5×10^5 PBMCs per well. The microtiter plate was incubated for 16−20 h at 37 °C with 5% CO_2 in a humidified atmosphere.

On day 2, the polyvinylidene fluoride membranes were washed three times with PBS, and the alkaline phosphatase conjugated antibody (1:200; Mouse-7-B6-1-ALP; Mabtech), specific for IFN-γ, was added to the wells and was incubated for two hours at room temperature. The plate was washed three times with PBS, and 100 μL of the substrate (BCIP-NBT-plus; Mabtech) was added to the wells and was incubated at room temperature for 10 min, whereafter the reaction was stopped with demineralised water.

2.4. ELISpot spot quantification

Spots were visualised with a digital microscope (DX1; Veho®) in a standardised illuminated environment. Images were analysed using the open-source FIJI software (Schindelin et al., 2012) and were converted into 32-bit black-and-white images (see Fig. 1A and B). An intensity threshold of 75 and particle size threshold of 5 $pixel^2$ were set to automatically select all distinct dark-coloured spots using the Particle Analysis tool. The sample was excluded if <100 spots were present in the positive control well. The number of spots in the negative control well was subtracted from the antigen-stimulated wells, and the results were expressed as spot-forming cells per 10^6 PBMCs (SFCs /10^6 PBMCs), unless stated otherwise.

In the validation process of our spot detection and quantification method, spots were independently manually counted by two laboratory technicians without prior knowledge of prior infection, vaccination status and the results derived by the other quantification methods, whereafter the average of the two counts was calculated. Secondly, spots were automatically counted by an automated ELISpot reader system (ELR03; AID-GmbH), using minimal threshold settings for intensity (20), size (8), and gradient (1).

2.5. Statistical analysis

Kruskal-Wallis test with Dunn's post hoc analysis was performed to compare multiple groups at a two-tailed level of $\alpha = 0.05$ using GraphPad Prism v9.0.2 for MacOS. Intraclass correlation coefficient (ICC) was calculated with 95 % confidence interval (CI) to determine the level of reliability of our spot detection and quantification method using IBM® SPSS® statistics v26.00.00 for MacOS.

3. Results

3.1. Antigen concentrations

To determine how different peptide concentrations affect our ELISpot results, we first stimulated PBMCs of two SARS-CoV-2 unexposed, two COVID-19 convalescents, and two fully COVID-19 vaccinated volunteers with our SARS-CoV-2 peptide pools at a final concentration of 0.1, 0.5, 1.0, 2.0, and 5.0 μg/mL per peptide (Fig. 2). Overall, we observed a similar number of SFCs across different concentrations in all volunteers. However, in one unexposed and convalescent case, peptides in concentrations from 0.1 to 2.0 μg/mL evoked zero response, whereas 5.0 μg/mL did evoke a T cell response (i.e., Unexposed #1 S_{id} and Convalescent #2 S_{id}). In contrast, we also observed stronger responses at low concentrations than at high concentrations in two cases (i.e., Unexposed #2 N and M).

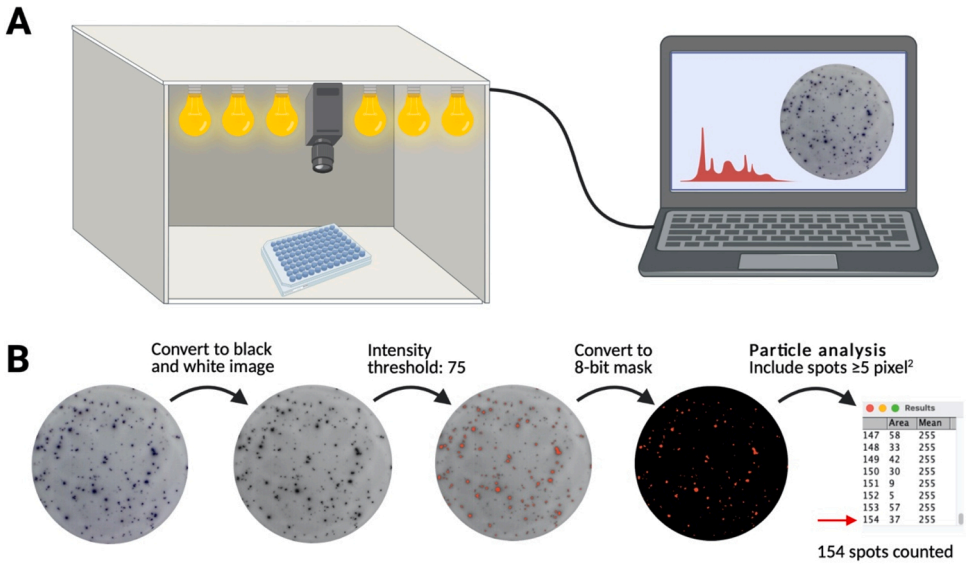

Fig. 1. ELISpot imaging, detection, and quantification process (**A**) Spots were visualised by imaging membranes with a digital microscope in a standardised illuminated environment. (**B**) Steps of spot detection and quantification using the Particle Analysis tool of FIJI. Created with BioRender.com.

3.2. Validation of in-house ELISpot assay and spot detection and quantification method

We stimulated PBMCs of three SARS-CoV-2 unexposed, three COVID-19 convalescents, and six fully COVID-19 vaccinated volunteers with our SARS-CoV-2 peptide pools (Fig. 3A). In the unexposed, we observed low numbers of reactive T cells. These volunteers presented no S1-reactive T cells and up to 10 S_{id}, N, and M-reactive T cells. In contrast, all COVID-19 convalescent volunteers presented reactive T cells to all antigens, except for one S_{id} unreactive volunteer. Stimulation by all the tested antigens resulted in similar response magnitudes up to 100 SFCs, respectively. Vaccinated volunteers presented robust responses after stimulation of spike peptide pools ranging from 12 to 480 SFCs. Although the number of vaccinated volunteers is limited, S1 elicited significant higher responses than N and M, and S_{id} elicited significant higher responses than N but not M (all $p < 0.05$). Furthermore, we validated our spot detection and quantification method by comparing the number of spots detected by this method to the number of spots detected by manual spot counting and spot counting by an automated ELISpot plate reader (Fig. 3B). We observed excellent reliability of spot counting by our FIJI Particle Analysis method when compared to manual spot counting or spot counting by an ELISpot plate reader (ICC > 0.99).

4. Discussion

Our data suggest that PBMC stimulation with different antigen concentrations ranging from 0.1 to 5.0 μg/mL per peptide does not lead to significantly different T cell responses in our in-house SARS-CoV-2 ELISpot assay. In addition, we obtained similar results in the quantification of the number of T spots with our plate reader-free spot-detection method compared to manual spot counting and spot counting by an automated ELISpot plate reader.

The IFN-γ ELISpot is a relatively simple technique to assess antigen-specific IFN-γ-secreting $CD4^+$ Th1 and $CD8^+$ cytotoxic T cells that are considered to be essential for an effective host defence against intracellular pathogens. Diagnostic laboratories most commonly apply the technique to identify latent tuberculosis infections, often in immunocompromised patients (Wagstaff and Zellweger, 2006). Also, researchers apply the technique to study T cell immunity against pathogens, for instance, against SARS-CoV-2. There is considerable variation in ELISpot assay design depending on the targeted cell types and antigen characteristics. For example, studies that performed SARS-CoV-2 ELISpots used antigen concentrations ranging from 0.5 to 10.0 μg/mL per peptide (Demaret et al., 2020; Woldemeskel et al., 2020). Our study suggests similar results may be achieved when PBMCs are stimulated with peptides ranging from 0.1 to 5.0 μg/mL.

Notably, our ELISpot showed weak T cell responses against N and M peptides in unexposed volunteers and COVID-19 vaccinated volunteers who received a SARS-CoV-2 spike mRNA vaccine. Previous studies also reported SARS-CoV-2-specific T cell responses against spike, N, and M in SARS-CoV-2 unexposed individuals, indicating the presence of pre-existing cross-reactive T cells populations that developed after prior common cold coronavirus infections (Braun et al., 2020). However, the protective role of these cross-reactive T cells is not yet proven. We observed no pre-existing S1-reactive T cells, possibly because the spike S1 of SARS-CoV-2 has low resemblance to the spike S1 of endemic coronaviruses (Braun et al., 2020). Accordingly, the assessment of S1-specific T cells might be the most specific approach to detect SARS-CoV-2-specific T cells.

Most laboratories use an automated ELISpot plate reader to detect and quantify spot-forming cells. Since these analysers are large investments and manual counting of spots likely leads to higher variability of results when compared to objective software-dependent spot detection (Ansari et al., 2013), we developed an ELISpot plate reader-free but software-dependent method to detect and quantify spots. Similar to the study of Hayashi et al. that assessed response to cancer vaccine immunotherapy (Hayashi et al., 2020), we used the Particle Analysis tool of the open platform software FIJI in which we set a particle intensity and size threshold to distinguish distinct spots from background noise. Unlike the previous study, we did not punch out the membranes from the microtiter plates and scan the membranes. Instead, we left the membranes in the microtiter plate while imaging them in an illuminated environment, thereby standardising the imaging conditions. The excellent reliability of our reader-free spot detection and quantification method suggests that our reader-free method could be used to detect and quantify spot-forming cells in both research and diagnostic settings in the absence of a plate reader.

Our ELISpot may provide information on whether an individual has protection against SARS-CoV-2 infection since an effective T cell response inversely correlates with COVID-19 severity (Rydyznski Moderbacher et al., 2020). However, there are also several limitations of our study and the interpretation of our findings. The protective role of SARS-CoV-2 specific T cells is not yet fully crystallised (Bertoletti et al., 2021). Also, it is unknown whether the number of SARS-CoV-2-reactive

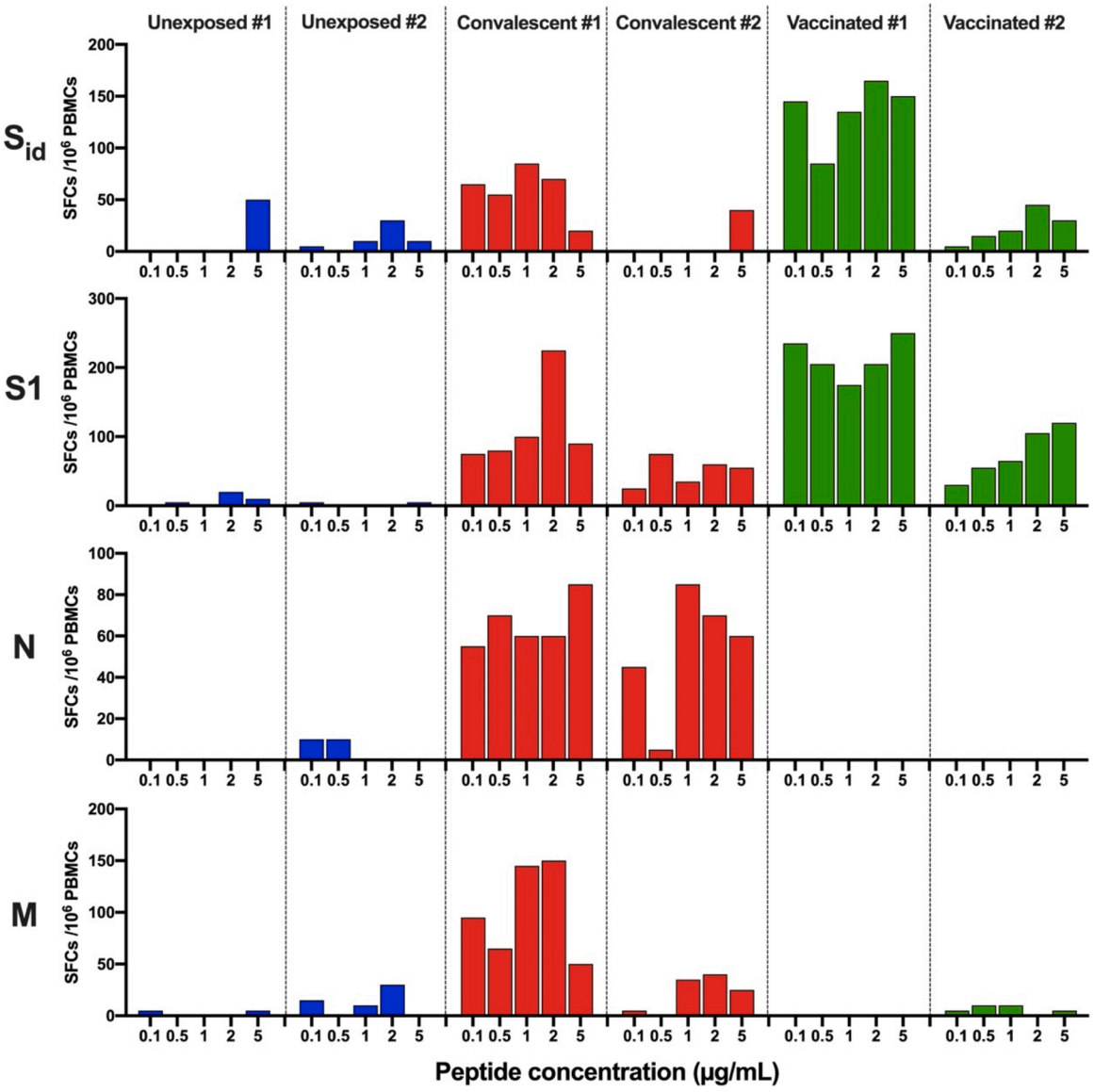

Fig. 2. SARS-CoV-2-specific T cell responses after stimulation with different peptide concentrations. Magnitude of background-subtracted IFN-γ responses to tested SARS-CoV-2 peptide pools at concentrations from 0.1 to 5.0 µg/mL. PBMCs of two unexposed, two COVID-19 convalescents, and two fully vaccinated volunteers were stimulated for 16-20 h with SARS-CoV-2 peptides in the ELISpot assay and were responsive to anti-CD3. Spots were detected and quantified using the protocol described in Fig. 1.

T cells correlates with immune protection. Furthermore, this IFN-γ ELISpot assay does not provide a complete overview of SARS-CoV-2 cellular immunity since the ELISpot only determines immediate IFN-γ responses of $CD4^+$ and $CD8^+$ effector T cells and effector memory T cells. The second type of memory T cells, i.e., the central memory T cells, are not immediately responsive and have to differentiate into effector T cells before producing cytokines. Therefore, the assessment of these central memory T cells requires a 'cultured ELISpot', in which PBMCs are cultured for multiple days (Calarota and Baldanti, 2013). Finally, we included 12 volunteers, of which six volunteers for the peptide concentrations comparison. Therefore, our findings should ideally be confirmed in a larger cohort.

In conclusion, we demonstrated that our IFN-γ ELISpot assay can adequately detect SARS-CoV-2-specific T cells in COVID-19 convalescent and COVID-19 vaccinated individuals and that antigens concentrations varying from 0.1 to 5.0 µg/mL per peptide may result in similar T cell responses. Also, we provide a software-dependent ELISpot plate reader-free spot detection method for the analysis of ELISpot results that demonstrated excellent reliability when compared to analysis by an ELISpot plate reader. Further research has to elucidate the potential protective role for SARS-CoV-2-specific reactive T cells and cross-reactive T cells.

Data availability

No data was used for the research described in the article.
Data will be made available on request.
The data that has been used is confidential.

Author contributions

Conceptualisation: JGMK, DSYO; Data curation: WAM; Formal analysis: WAM; Funding acquisition: JGMK, DSYO; Investigation: WAM; Methodology: WAM, JGMK, DSYO; Project administration: WAM, JGMK, DSYO; Resources: WAM, JGMK, DSYO; Software: WAM; Supervision: JGMK, DSYO; Validation: WAM, JGMK, DSYO; Visualisation: WAM; Writing – original draft: WAM; Writing – review & editing: JGMK, DSYO

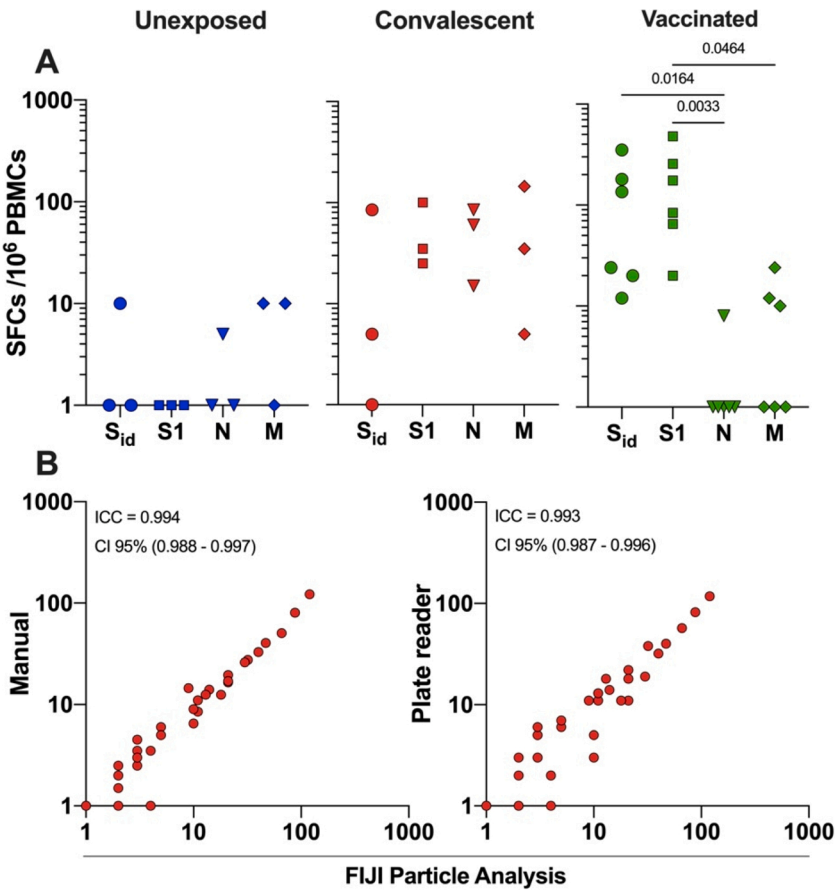

Fig. 3. Magnitude of SARS-CoV-2-specific T cell responses and comparison between ELISpot analysis methods.
(**A**) Magnitude of background-subtracted IFN-γ responses after stimulation with SARS-CoV-2-derived peptides. PBMCs of three SARS-CoV-2 unexposed, three COVID-19 convalescents, and six fully COVID-19 vaccinated volunteers were stimulated for 16–20 h with SARS-CoV-2 peptide pools (1 μg/mL) in the ELISpot assay and were responsive to anti-CD3. P values were calculated with the Kruskal-Wallis test followed by Dunn's post-hoc multiple comparison.
(**B**) Comparison of spot counts by FIJI's Particle Analysis and spots counts by manual counting and counting by an automated ELISpot plate reader. Data comprises direct spot counts after stimulation by S_{id}, S1, N and M antigen of 12 volunteers as shown in Fig. 2 (n samples = 48). ICCs were calculated to compare ELISpot analysis methods.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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