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# Laboratory Evaluation of SARS-CoV-2 Specific T-Cell Immunity by ELISpot Interferon-γ Based Immunoassay

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**Abstract**

Cellular and humoral immune responses are fundamental for SARS-CoV-2 elimination, infection resolution, and protection against reinfection. Data on long-term SARS-CoV-2-specific T-cell responses are limited. We conducted this study to evaluate the presence of SARS-CoV-2 specific T lymphocytes using the ELISpot interferon-γ-based kit for outpatients with or without a history of SARS-CoV-2 infection. SARS-CoV-2 activated T-cell response was defined from peripheral blood mononuclear cells (PBMCs) of nine (n=9) outpatients- 6 women and 3 men, aged 32 to 73 years old, all from Varna city, Bulgaria. They were tested between November and December 2021. Eight of them – 88.9% were in contact with the COVID-19 virus from 1 to 20 months ago. The number of spots obtained provides a measure of the abundance of SARS-CoV-2 – sensitized effector T cells in the peripheral blood. A reactive T-cellular immune response was observed 12 months after SARS-CoV-2 viral infection. In addition, IgG humoral immunity was analyzed and compared with the specific T-cell response in some participants. Compared with humoral immunity-related studies, those focusing on SARS-CoV-2-specific cellular immunity using the ElISpot immunoassay are relatively falling behind standardization. The SARS-CoV-2 specific ELISpot Interferon-γ immunoassay, described in our study, conducted with a small group of patients, can serve as an effective immunoassay to measure host T-cell responses and help further detailed understanding of pathogenetic mechanisms and persistence of cellular and humoral immunity against SARS-CoV-2.

**Keywords:** SARS-CoV-2, T-cell immunity, ElISpot, Interferon-γ, humoral immunity

**Резюме**

Клетъчният и хуморален имунен отговор са в основата на елиминирането на SARS-CoV-2 вируса, възстановяване на макроорганизма, както и защита срещу повторно заразяване. Данните, изнесени в литературата за дългосрочния специфичен Т-клетъчен имунитет при SARS-CoV-2-инфекцията са ограничени. Проведохме настоящото проучване, за да оценим наличието на активирани, интерферон-γ продуциращи и специфични за SARS-CoV-2 Т-лимфоцити, като използвахме ELISpot при амбулаторни пациенти със или без анамнеза за инфекция със SARS-CoV-2. Т-клетъчният отговор беше дефиниран чрез изследване на мононуклеарни клетки от периферна кръв на девет (n=9) амбулаторни пациенти – 6 жени и 3 мъже, на възраст от 32 до 73 години в град Варна, България. Пациентите са взели участие в проучването между месец ноември и декември 2021 г. Осем от тях – 88.9% са били в контакт с вируса на SARS-CoV-2 в интервала от 1 до 20 месеца преди тестването. Броят на получените петна (spots) се базира на наличието на SARS-CoV-2-сенсибилизирани ефекторни Т-лимфоцити в периферната кръв на участниците в проучването. Проследихме наличие на Т-клетъчен имунен отговор 12 месеца след SARS-CoV-2 вирусната инфекция. При част от участниците, изследвахме и хуморалния имунен отговор (SARS-CoV-2 IgG) антитела, като го сравнихме със специфичния Т-клетъчен имунен отговор. За разлика от проучванията отчитащи хуморалния имунитет, тези, фокусирани върху специфичния SARS-CoV-2 клетъчен имунитет, дефиниран с ElISpot имуноанализа, относително изостават от стандартизацията. ELISpot анализа, може да служи като ефективен метод за дефиниране на интерферон-γ продуциращите Т-клетки на макроорганизма, както и разбирането на подробните патогенетични механизми на клетъчния и хуморален имунен отговор срещу вируса на SARS-CoV-2.

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## Introduction

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) belongs to the family *Coronaviridae*, genus Betacoronavirus. The virus was first discovered in Wuhan (China) and has caused the COVID-19 pandemic worldwide. Since its appearance in December 2019, SARS-CoV-2 has infected more than 768 million individuals, resulting in the death of 6.95 million (WHO, 2023). SARS-CoV-2 infection can result in a range of clinical manifestations, from asymptomatic or mild to severe COVID-19, which requires hospitalization. A harmonized innate and adaptive immune response is crucial for the control and clearance of most viral infections (Moga *et al*., 2022). Cellular and humoral responses are fundamental for SARS-CoV-2 elimination, infection resolution, and long-term protection against reinfection. (Nielsen *et al*., 2021; Sette and Crotty, 2021; Safont *et al*., 2022). The role of T-cells in the resolution or exacerbation of COVID-19 and their potential to provide long-term protection from SARS-CoV-2 reinfection is crucial. Similar to other respiratory viral infections, adaptive immune responses, particularly T cells, play a prominent role in SARS-CoV-2 infection (Chen and Wherry, 2022 ). Studies on the strength and duration of adaptive immune responses in COVID-19 patients and convalescents may help to better understand how immune protection develops (Brodin, 2021). According to the literature, SARS-CoV-2 antibodies show up to decrease quicker than the SARS-CoV-2-specific memory T-cells, and a few years after infection individuals were considered SARS-Covid antibody negative, while memory T-cells were recognized a long time after viral infection had passed (Wu *et al*., 2007; Le Bert *et al*., 2020). T cells can identify past SARS-CoV-2 infections at a time when PCR tests would be negative and antibody levels may be fading away (Dan *et al*., 2021; Kruse *et al*. 2021).

In general, it takes up to 10 days on average for viral infections to elicit adaptive T-cell immune responses (St. John and Rathore, 2019). Natural killer (NK) and natural killer T cells (NKT), as well as antigen-specific CD4+ and CD8+ T cells, are the main producers of interferon-gamma (IFN- γ), which is crucial for protection against intracellular viruses like SARS-CoV-2 (Wykes and Renia, 2017). There are a few available protocols to measure the level of IFN- γ because of the important part this cytokine plays in immune responses. The ELISpot test, one of the most popular ELISA-based variants, measures cytokine levels in serum samples and cell cultures by binding the cytokine with labeled antibodies (Schreiber, 2001). The ELISpot method was initially developed to detect antigen-specific antibodies and has been modified to detect cytokines produced by antigen-specific cells (Czerkinsky *et al*., 1983). This assay allows the quantification of specific T cell types that produce IFN-γ in response to a determined antigen. The main principle of SARS-CoV-2 immunoassay is based on T-cell stimulation in cell culture with peptides from the target protein (S), and the supernatants afterward are tested in ELISA for IFN-γ levels. Compared with humoral immunity-related studies, those focusing on SARS-CoV-2-specific cellular immunity using the ELISpot technique are relatively falling behind standardization, and data on long-term SARS-CoV-2 specific T-cell responses are still restricted (Hao *et al.*, 2022).

## Materials and Methods

SARS-CoV-2 activated T-cell response was defined from peripheral blood mononuclear cells (PBMCs) of nine (n=9) outpatients from Varna city, Bulgaria, tested at Medical Diagnostic Laboratory "Status". T-cell immunity testing is highly specialized and requires specific equipment and well-trained personnel.

Eight of the patients were in contact with the COVID-19 virus from 1 to 20 months ago, and one (n=1) was not infected and was not vaccinated. Four patients (n=4) were tested for cellular and humoral SARS-CoV-2 immune responses. All subjects were tested in the laboratory between November 2021 and December 2022. The results were interpreted by a clinical virologist.

*Limitations of the study*

As respondent inclusion criteria were considered: outpatients who have recovered from COVID-19; who have been vaccinated against COVID-19, with date of application of the last dose of vaccine at least 7-10 days before the survey; those suspected of asymptomatic COVID-19 infection; with mild and moderate clinical manifestation of COVID-19, without definitive PCR result; with a low antibody titer against COVID-19 after illness or vaccination; who have not been ill and have not been vaccinated; outpatients examined on another occasion.

As respondent exclusion criteria were considered outpatients: outpatients with severe clinical manifestation of COVID-19 and positive PCR result at the time of conducting the study; people who have been vaccinated against COVID-19, with a date of application of the last dose of vaccine less

than 7 days ago before the test; outpatients without complete data necessary to carry out the survey; outpatients who refused to participate into the trial.

*Ethics statement*

The current study and the corresponding results reported in submitted papers and involving human participants were based on the 1964 Helsinki Declaration and its later amendments. Ethical approval was obtained by the Research Ethics Committee at the Medical University, Varna (Approval number: 026-75; Protocol number: 130/20.04.2023).

All the participants completed a quick start-up questionnaire (Table 1) and provided written informed consent for the use of their blood and blood components (such as sera and plasma) and acknowledged that they cannot be identified via the paper as they have been fully anonymized.

*IFN-γ ELISpot assay procedure*

PBMCs were isolated from anticoagulated whole blood using CTP vacutainers with buffered sodium citrate or sodium heparin anticoagulant, liquid density medium, an inert gel barrier, and vacutainers with lithium heparin. For the patients included in the study, we used one 8 ml (two from 4 ml) CPT vacutainer or one 6 ml vacutainer with lithium heparin. Blood samples were stored at room temperature and processed within 8 h of blood collection. No potential stimulatory factors were observed during PBMC isolation or storage. PBMCs were isolated from whole blood within 6 h after collection. Heparinized whole blood was diluted 1-3 fold with D-PBS (phosphate physiological solution), depending on the hematocrit level of the blood sample. A Pancoll lymphocyte separating medium was used (Pan Biotech Germany). The Pancoll solution was covered with a layer of diluted blood in a centrifuge vial without mixing the phases. After a short centrifugation step at room temperature, lymphocytes, together with monocytes and platelets, were harvested from the white blood cell layer between the plasma sample layer and the Pancoll. The separated cells were then washed twice in D-PBS to purify the lymphocytes by removing the platelets. Mononuclear cells were counted using a standardized cell count. This ensures that even in

**Table. 1**. Patients' start-up questionnaire

| QUESTIONNAIRE<br>Cellular immune response against SARS-CoV-2 virus |
|---|
| Dear patients,<br>The short questionnaire aimed to define the immune status of the SARS-CoV-2 virus.<br>The provided information will support a more accurate interpretation of the results obtained from cellular and/or humoral immune response testing for COVID-19.<br>Your personal data will not be associated with the answers you have provided, but the results obtained can be used for scientific purposes.<br>Your personal data will not be used for purposes other than those for which you provided them.<br><br>Thank you! |
| 1. Gender:<br>❑ Male<br>❑ Female |
| 2. Age: |
| 3. My health status regarding COVID-19 is *(Please mark your answer. More than one answer is possible)*:<br>❑ I have already had clinical and/or laboratory symptoms of COVID-19 and have recovered from it.<br>❑ I am with post- COVID-19 infection<br>❑ I am vaccinated against COVID-19<br>❑ I have had COVID-19 vaccine booster dose(s)<br>❑ I assume I have had an asymptomatic COVID-19 infection<br>❑ I have never been infected and never had symptoms of the infection<br>❑ I am not vaccinated |
| 4. Please mark the type of vaccine (if applicable), the schedule and the date of your last vaccine dose, for more detailed interpretation of your cellular immunity test:<br>……<br>…… |

patients with low T cell counts due to a weakened immune system (immunocompromised and immunosuppressed), the sample will have a sufficient number of cells added to the ElIspot test wells. To measure T-cell viability, we used the trypan blue staining assay in a hemocytometer. Trypan blue is an azo dye that is cell membrane-impermeable and, therefore, only enters cells with compromised membranes. Upon entry into the cell, trypan blue binds to intracellular proteins, thereby rendering the cell blue. The trypan blue staining assay allows for the direct identification and enumeration of live (unstained) and dead (blue) cells in the T-cell population. For stimulation and proliferation of T-cells, the initial concentration should be approx. 1-3×$10^6$/ml/well in the culture medium. Cell isolation and the addition of cell suspensions and mitogen/antigen solutions were performed under strict aseptic conditions in a safety cabinet. The serum-free medium used for the culture and expansion of T-cells from PBMCs was Pancerin 413, according to the manufacturer's instructions (Pan Biotech, Germany). The peptide pools with synthetic origin contain sequences of immunodominant epitopes of specific regions of SARS-CoV-2 peptides. A pool of 15-20 mer peptides overlapping with 11 amino acids, covering the whole spike protein of SARS-CoV-2 (S-SARS-CoV-2), was adjusted for ELISpot T-cell stimulation.

The principle of the IFN-γ ELISpot procedure was based on the incubation of a Multitestplate (MTP) with 96 wells fitted with membranes and coated with 5 µg/mL anti-human IFN-γ antibodies. It was incubated with 100 µL PBMCs (2×$10^6$/ml PBMCs/well)/Pokeweed (PC)/SARS-CoV-2 peptide mix (Ag solution) in a pair of wells, for the Ag, per patient, and then 100 µl serum-free media (Pancerin 413-5 to 7%), according to the instructions of the manufacturer (GenID GmbH, Germany). The plate was placed in a $CO_2$ incubator at 37°C, 5-7% $CO_2$ for 20 to 24 h without vibration (S-Bt Smart Biotherm, Biosan). After overnight incubation, the secondary antibodies, substrates, and distilled water were used. The plate was dried thoroughly (preferably overnight) before evaluation of the results. During incubation, cytokines are secreted by the cells, which are then captured by the primary antibody. After the cells were removed, the plates were washed and incubated for 2 h with alkaline phosphatase (AP)-labeled secondary antibody. After washing, the plates were developed with TMB substrate-100 µl/well. The plates were thoroughly dried before evaluation. Spots were analyzed.

The minimum detectable unit of response of the ELISpot assay was one spot. The number of cytokine-producing cells was determined by counting spots. The size of the spots can provide additional information regarding the number of cytokine(s) produced by the cells. The limit of detection is typically up to 1 in 200000 cells. However, a negative result does not exclude the possibility of COVID-19 exposure. A negative control (NC media with cells) and positive control (PC-Pokeweed mitogen with cells) were run in duplicate (S1 and S2) per patient. A negative control with more than 10 spots and a positive control with fewer than 50 spots were considered as suspected. The positive control establishes that the ELISpot assay provides an expected positive result and is used to document an adequate number of inducible cells. The negative control was used to establish the non-specific baseline response of the assay; however, routine experience has shown that some patients tend to have higher spot counts depending on their general individual immunological constitution, medication, infections, and nutritional habits. The basic steps of the ELISpoT assay are shown in Fig. 1. Detection of reactive T-cells indicates exposure to SARS-CoV-2. ELISpot results for the used SARS-CoV-2 peptide mix showed 100% analytical sensitivity when compared with serological- and/or RT-PCR-based findings, resulting in 100% specificity and 100% sensitivity.

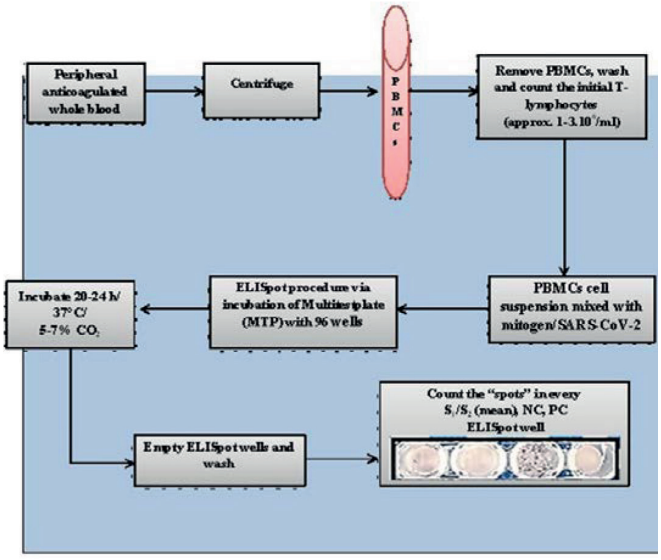


**Fig. 1.** General phases of SARS-CoV-2 ELiSpot Interferon-γ immunoassay

*Interpretation of ELISpot assay results*

ElISpot plates were evaluated manually under a dissecting microscope and the frequency of secreting cells was calculated as follows: one cell = one spot; number of spots = number of cells secreting cytokines; intensity and size of spot = relative

cytokine-secreting ability of T cells. To interpret the test results, a stimulation index (SI) was used. The mean number of spots from the patient's PBMC sample (stimulated cultured in two wells, S1 and S2) was divided by the mean number of spots from the negative control (unstimulated wells-NC). In our study, the number of spot-forming cells per $2\times10^6$/ml PBMCs plated was used to calculate the SI. Results were defined as reactive when SI>3, borderline between 2 and 3, or negative when SI <2.

*Anti-SARS –COV-2 QuantiVac ELISA (IgG)*

The ELISA test procedure conducted in parallel for some of the patients was done, as per, the instructions of the manufacturer (Euroimmun, Germany), via the automated system Euroimmun analyzer I-2P. The reagent wells were coated with the S1 domain of the spike protein of SARS-CoV-2, isolated Wuhan-Hu-1, and expressed recombinantly in the human cell line HEK 293. The results for the neutralizing antibodies are expressed in BAU/ml (binding antibody units/ml), according to the "First WHO International Standard". The result interpretation is <25.6 BAU/ml-negative; ≥25.6<35.2 BAU/ml-borderline; ≥35.2 BAU/ml-positive result.

*Statistical analysis*

Qualitative variables were reported as numbers, relative proportions (%), and confidence intervals (95% CI). Figures and tables were created using Microsoft Office Pack, 2010.

**Results**

The research case series was conducted with nine outpatients- 66.7 % (95% CI: 29.9% - 92.5%, n = 6) of them women and 33.3 % men (95% CI: 7.5% - 70.0%, n = 3), from 32 to 73 years old. Eight of them-88,9% (95% CI: 51.7% - 99.7%, n =8) were in contact with the COVID-19 virus from 1 to 20 months ago. One of the subjects was naïve and unexposed to the virus-11.1 % (95% CI: 0.3% - 48.2%, n =1). The summarized data for all 9 patients are shown in Table 2.

The stimulation index (SI) was calculated using the mean number of spots from the patient's PBMCs sample (cultured in two wells, S1 and S2) divided by the mean number of spots from the negative control varied from <2 SI up to >50 SI. A detailed look at the results of the ELISpot interferon-γ assay for each of the nine patients in our study is shown in Fig. 2.

Four of the patients- 44.4% (95% CI: 13.7% - 78.8%, n =4) were tested for both cellular and humoral immune responses. The S-SARS-CoV-2 IFN-γ T cell response was correlated with S1-SARS-CoV-2-specific serum antibody concentrations. The quantitative ELISA results from the determination of anti-SARS-CoV-2 antibodies were above the threshold (≥35,2 BAU/ml) and considered seroconversion for all four tested patients. Three of the participants in the study were vaccinated -33.3% (95% CI: 7.4% - 70.0%, n =3) at least one week before

**Table. 2.** Summarized data of the included individuals (n=9)

| Patient | COVID-19 immune status | Stimulation index (SI) in ELiSpot T-cell response | COVID-19 Ab in ELISA (BAU/ml) B-cell response | COVID-19 immunization status | Clinical presentation |
|---|---|---|---|---|---|
| 1 | Infected 7 months ago | SI=3 | 151.7 BAU/ml | Not vaccinated | Moderate |
| 2 | Infected 12 months ago | SI=2,5 | Not tested | Not vaccinated | Mild |
| 3 | Infected 12 months ago | SI=3 | Not tested | Not vaccinated | Mild |
| 4 | Infected 20 months ago | SI=2,5 | 2544,86 BAU/ml | Vaccinated (Pfizer/ BioNtech) 4 months ago 2 doses | Mild |
| 5 | Not infected | SI<2 | Not tested | Not vaccinated | Not infected |
| 6 | Infected 1 month ago (reconvalescent) | SI>50 | Not tested | Not vaccinated | Mild |
| 7 | Infected 6 months ago | SI=13 | Not tested | Vaccinated (Pfizer/ BioNtech) 2 doses and (a booster dose 1 week ago) | Mild |
| 8 | Infected 8 months ago | SI=4 | 241,22 BAU/ml | Vaccinated (Janssen) 2 months ago 1 dose | Mild |
| 9 | Infected 10 months ago | SI=3 | 48,63 BAU/ml | Not vaccinated | Mild |

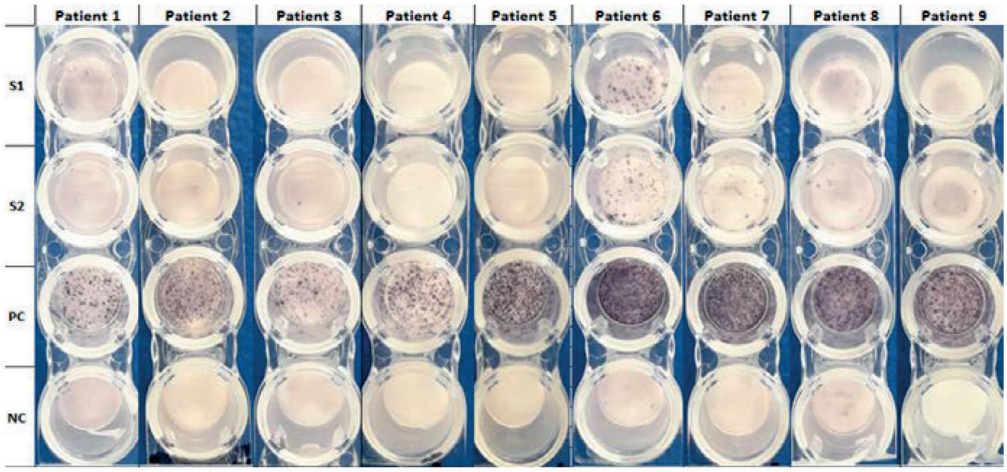


**Fig. 2.** ELISpot Interferon-γ immunoassay performance results for evaluation of SARS-CoV-2 specific T-cell responses (n=9). $S_1$ and $S_2$- Patient's PBMCs in a duplicate well; PC-positive control (Pokeweed mitogen); NC-negative control (Panserin 413 medium)

the test for cellular immunity and at least 6 months after COVID-19 infection. Six of the patients had not been vaccinated-66.7% (95% CI: 29.9% - 92.5%, n =6).

As per WHO severity definitions based on clinical indicators, adapted from WHO COVID-19 disease severity categorization there is: non-severe COVID-19 (asymptomatic, mild, and moderate); severe COVID-19 (oxygen saturation < 90% on room air and signs of severe respiratory distress) and critical COVID-19 (acute respiratory distress syndrome (ARDS), sepsis, septic shock, etc). Asymptomatic are people who test positive for SARS-CoV-2 without clinical symptoms. Individuals with mild clinical presentation are considered those who have any of the various signs and symptoms of COVID-19 from the upper respiratory tract but not shortness of breath or dyspnea. Patients with moderate illness show evidence of lower respiratory disease during clinical assessment or imaging and who have an oxygen saturation measured by pulse oximetry ($SpO_2$) ≥94% on room air. Patients with mild and moderate illnesses usually do not require emergency interventions or hospitalization (WHO, 2023).

Eight of the presented cases -88.9% (95% CI: 51.7% - 99.7%, n =8) had been infected with SARS-COV-2 and were re-convalescent. Regarding the WHO criteria, seven of the participants in the trial- 87.5% (95% CI: 47.3% - 99.7%, n =7) had a mild clinical presentation of COVID-19 and symptoms of fever, cough, sore throat, malaise, headache, muscle pain, nausea, vomiting, diarrhea, loss of taste and smell. One patient-12.5 % (95% CI: 0.3% - 52.6%, n =1) had a moderate clinical presentation and evidence of lower respiratory disease and oxygen saturation measured by pulse oximetry ($SpO_2$) ≥94%. Asymptomatic COVID-19 patients were not registered in the survey. All of the patients fully recovered from the viral infection, except for one patient who is with permanent anosmia-12.5 % (95% CI: 0.3% - 52.6%, n =1).

## Discussion

This study evaluated the presence of SARS-CoV-2-specific T lymphocytes as an essential component of protective immunity. An ELISpot interferon-γ-based kit was used for 9 outpatients with or without a history of SARS-CoV-2 infection. A coordinated and adequate protective immune response is the key to the control and recovery of most viral infections. Protective immunity is acquired via vaccination or infection and is conducted by humoral immunity (B-cells), as well as cellular immunity (T-cells) (Collier *et al*., 2021). In order to restrict viral spread T-cells are able to respond quicker once infection has advanced, although they cannot prevent permissive cells from infection. Despite the fact that clearance of SARS-CoV-2 involves both cellular and humoral immune responses T cell immunity plays a basal role against severe infections and further complications (Wherry and Barouch, 2022).

The ELISpot procedure is considered to be one of the most sensitive and highly effective cell-immunity evaluation assays and has been widely used to diagnose and interpret the results of tuberculosis infection (T-spot test) (Pai *et al*., 2007). To establish the presence of T-cell reactivity in an individual, ELISpot results were evaluated in terms of spot counts in antigen-containing versus medium control wells (Karulin *et al*., 2015). González et al. evaluated a commercially available SARS-CoV-2 IFN-γ

secreting kit with 81.1% and 90.9% sensitivity and specificity in 3- and 12-month COVID-19 patients, respectively (Fernández-González *et al.*, 2021). Our case series study evaluated an IFN-γ-releasing ELISpot assay for SARS-CoV-2-specific T-cell detection in a small cohort of patients. The high sensitivity of the assay makes it particularly useful for examining a small population of cells found in specific T-cell immune responses, with IFN-γ captured directly on the ELISpot 96 wells Multitestplate.

Calculation of the concentration of viable T-cells present in the patient's cell suspension is of ultimate importance for the interpretation of the ELISpot result, and the use of an insufficient number or an excess of T-lymphocytes may lead to misinterpretation, as T-cell activation is dependent on the interaction of T-lymphocytes with antigen-presenting cells (APCs), highlighting that APCs are vital for an effective adaptive immune response. In ELISpot assays, such interactions are critically dependent on the density of PBMC in ELISpot assay wells. A direct linear relationship, in the range of $2.5 \times 10^4$ and $10^6$ PBMC per well, was defined between the quantity of plated PBMC and the number of visualized spots (Karulin *et al.*, 2015). In case of being plated at less than $5 \times 10^4$/ml PBMC per well, the cells won't be able to form a monolayer, and T-cell responses may be unclear. However, when plated at more than $10^6$/ml PBMC per well, the cells become overcrowded which results in indefinite ELISpot result interpretation (Zhang *et al*., 2009; Hanson *et al*., 2015). For our study, a concentration of $2\times10^6$ T-cells per well was used to visualize spot-forming cells and define SI. Each spot represents the footprint of an individual cytokine-secreting T-cell. Spots were clear, focused, and easy to distinguish, although at higher numbers (Patient 6, SI>50), and the confluence of spots could interfere with the countability of the data. The number of spots obtained provides a measure of the abundance of SARS-CoV-2–sensitized effector T cells in the peripheral blood. The size of the spots provided additional information regarding the amount of cytokines produced by T cells. Based on our results, patients who had reported a recent encounter with SARS-CoV-2 and had a clinical presentation of the infection showed higher SI results.

After SARS-CoV infection in 2003, T-cell responses have been shown to persist for up to 17 years (Kaaijk *et al*., 2022). Virus-specific T-cells were present in almost all individuals who had a prior episode of COVID-19 and have been reported to have a half-life of approximately 3–5 months (Grifoni *et al*., 2020; Mateus *et al*., 2020; Rydyznski *et al*., 2020 Weiskopf *et al*., 2020; Rodda *et al*., 2021). The relationship between T-cell response and disease severity should be assessed at different time points post-infection (Fujii *et al*., 2022). Some studies have investigated T cell immunity induced after SARS-CoV-2 infection in mildly symptomatic adult cases (Sekine *et al*., 2020), showing weaker T cell responses in mild than in moderate or severe COVID-19 cases. In this study, we observed a weak reactive cellular immune response 12 months after the COVID-19 infection. Previous studies have suggested that SARS-CoV-2-specific T cells are present in peripheral blood 10 months after infection (Lu *et al*., 2021). According to other studies, functional memory B and T cells in response to SARS-CoV-2 can be maintained for 6 months to 12 months after natural infection (Zuo *et al*., 2021; Moga *et al*., 2022). However, compared with antibody-related research, studies focusing on SARS-CoV-2-specific cellular immunity using ELISpot assays are relatively lagging behind standardization, and data on long-term responses are limited (de Candia *et al*. 2021; Goletti *et al*., 2021).

The humoral immune response is essential for viral clearance in the host. Antibodies from SARS-CoV-2 convalescent patients have been found to persist for more than 14 months (Rosati *et al.,* 2021). The numbers and quality of SARS-CoV-2-specific antibody responses differ between asymptomatic people and those with mild, moderate, or severe disease (García-Abellán *et al*., 2021). Eight patients in our case series had mild-to-moderate illness. Anti-SARS-CoV-2 IgG antibodies were found in all four participants with humoral immunity. Previous research found that the humoral response to SARS-CoV-2 infection was widespread among infected people, and the magnitude of anti-SARS-CoV-2 IgG titters was closely linked with the breadth of circulating virus-specific T-cell responses (de Candia *et al*., 2021). Several studies have found that the level and quality of SARS-CoV-2-specific antibody responses differ between asymptomatic and symptomatic individuals (Govender *et al*., 2022). Some investigations have found larger amounts and longer periods of COVID-19 in more severe cases, whereas others have found no discernible difference (Robbiani *et al*., 2020). According to the literature, SARS-CoV-2 exposure induces interferon (IFN)-producing T cells without seroconversion, implying that cellular immunological responses, rather than humoral responses, maybe more reliable indications of SARS-CoV-2 infection

(Gallais, 2020). Virus-specific T-cells are present in nearly all patients who have had a previous COVID-19 episode (Rodda *et al*., 2021).

SARS-CoV-2-specific T lymphocytes are related to diminished infection severity in infected subjects (Rydyznski *et al.*, 2020). There are reports of healthy people with little to no neutralizing antibodies detectable after infection successfully controlling SARS-CoV-2, while having considerable SARS-CoV-2-specific T-cell memory (Nelde *et al*., 2021; Schulien *et al*., 2021). In general, the synthesis of specific T cells is interconnected with milder clinical presentation, and both T cells and antibodies are associated with convalescence in the majority of the SARS-CoV-2 infected individuals (Tan *et al*., 2021; Govender *et al*., 2022). In our study, seven of the clinically presented cases of SARS-CoV-2 viral infection were mild and one patient had symptoms of a moderate infection. One of the participants with mild COVID-19 infection, one month after the infection, showed a very strong T-cell immune response (SI>50). Approximately 80% of SARS-CoV-2 infections are mild-to-moderate, with patients fully recovering from infection, while the remaining cases show severe (15%, requiring oxygen) and critical (5%, requiring ventilation) pneumonia (Chen, 2020; Guan, 2020; Huang, 2020; de Candia *et al*., 2021).

## Conclusion

The SARS-CoV-2 specific ELISpot Interferon-γ immunoassay described in our study, although conducted with a small group of patients, can serve as an effective immunoassay to measure host T-cell responses and help further detailed understanding of pathogenetic mechanisms and persistence of cellular and humoral immunity against SARS-CoV-2.


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## Ethical approval

This study was performed after written informed consent was obtained from all the participants and approved by the Research Ethics Committee at the Medical University, Varna (Approval number: 026-75; Protocol number: 130/20.04.2023) in Bulgaria.

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