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Differences in Characteristics of T-Cell Immunity to SARS-CoV-2 in Clinically Healthy Subjects

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We studied the differences in the characteristics of T-cell immunity in clinically healthy volunteers of three groups: "no previous COVID-19, not vaccinated", "recovered", and "vaccinated" as well as the relationship between the presence of IFNγ-releasing T cells in response to stimulation with peptide pools overlapping the main S, N, M, ORF3, and ORF7 protein sequences and the presence of IgG to the SARS-CoV-2 S protein. In the "no previous COVID-19, non-vaccinated" group, T cells specific to both S protein and other virus proteins were absent in 95% subjects. In the "recovered from COVID-19" group, T cells specific to the spike protein were present in samples from 39% subjects. In the same group, T-cell immunity to other viral proteins was present in 58% subjects. In vaccinated subjects, specific T cells responding to stimulation with S protein peptides were found in 47% cases and T cells specific to N, M, ORF3, ORF7 proteins were detected in only 22% subjects.

Key Words: *T-cell immunity; humoral immunity; COVID-19; IgG SARS-CoV-2*

Antiviral immunity is a multifaceted phenomenon. The nonspecific innate immune response can to a certain extent suppress viral replication and activate antigen-presenting cells. At the same time, inflammatory cytokines and chemokines attract effector cells to infected tissues. T-helper cells produce cytokines and co-stimulatory signals necessary for maturation, replication, and switch of the B-cell isotype. T helpers also contribute to cytotoxic T-lymphocyte activation, clonal expansion, and effector function. B cells produce antibodies that agglutinate, opsonize, and neutralize viral particles. Activated $CD8^+$ T cells lyse infected cells via the perforin, granzyme, and FasL system. IFNγ and TNF secreted by T cells also contribute to virus inactivation. The coordinated interaction of the humoral and cellular responses stops viral replication and leads to destruction of infected cells and virus removal from the body. Then, the number of virus-specific lymphocytes gradually decreased to a small population of long-lived memory cells capable of responding quickly to reinfection.

The success in combating viral infections depends primarily on the presence of high-affinity antibodies in the body capable of effectively neutralizing the pathogen even in low concentrations. Insufficient formation or rapid loss of neutralizing antibodies can limit the effectiveness and duration of immunity. To prevent this, revaccination aimed at induction of memory B cells and long-living plasma cells is performed.

T cells are regulators of cellular and humoral immunity. There is increasing evidence that they play an important role in resolving COVID-19 [6]. It has been suggested that SARS-CoV-2 infection can induce long-term T-cell immunity. This follows from the fact that SARS-CoV-specific T cells can be detected in some patients even 17 years after infection [11]. Specific $CD4^+$ and $CD8^+$ T cells are present in the blood of COVID-19 patients for 6-9 months after infection, irrespective of the disease severity [15]. Memory cells predominantly belong to the $CD4^+$ subpopulation, although during the acute phase, equivalent presence of $CD4^+$ and $C84^+$ T cells is registered [8]. According

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to published data, most COVID-19 patients show a robust multivalent T-cell immune response [14]. Moreover, most T cells demonstrate specificity to a wide range of viral proteins (peptide pools overlapping the main epitopes of adhesion, membrane and nucleocapsid proteins, nonstructural components, and orphan reading frames). This multispecific T cell response is optimal for reducing the probability of virus escape via mechanisms of mutations or variable antigen presentation. It is important to note that T cells recognize infected cells or antigen-presenting cells, rather than the virus itself. This means that T cells do not prevent infection, but contribute to reducing the viral load in the host after infection [9].

In the human population, the course of COVID-19 varies: from asymptomatic and/or mild infection to the development of severe complications and even death. Even at the beginning of the pandemic, it became obvious that old age was a significant risk factor of severe course of the disease. Some experts believe that this can be due to the fact that the pool of naive T cells decreases with age and the response to new pathogens, including SARS-CoV-2, becomes weaker as the protective primary T-cell response against viruses requires mobilization of naive $CD4^+$ and $CD8^+$ T cells. So far, there is no evidence that the formation of T-cell immunity and T-cell memory can be impaired in the elderly as a result of natural infection or vaccination [10], although the production of IFNγ and cytokines by $CD4^+$ T cells is markedly reduced in people over 80 years [7].

Understanding whether SARS-CoV-2-specific T cells are associated with protection or pathogenesis is critical to determining future therapeutic and prophylactic strategies in managing the current pandemic [3]. Recently, indirect evidence for a protective role of SARS-CoV-2-specific T lymphocytes has emerged. Patients with COVID-19 against the background of hematological diseases with reduced humoral responses and B cell deficiency show better survival when a sufficient pool of specific $CD8^+$ T cells is available [2].

It has already been shown that rapid induction of specific T- and B-cell responses was associated with a milder course of the disease [1,4,17]. The role of T cells during severe COVID-19 remains poorly studied, because most studies have been performed with recovering volunteers.

The importance of rapid mobilization of the T-cell response was demonstrated in the study of immunological parameters in 12 patients with acute SARS-CoV-2 infection from onset to convalescence. In this work, the authors quantified SARS-CoV-2 viral RNA in the upper respiratory tract as well as antibodies and circulating T cells specific for various structural (NP, M, ORF3a, and S) and nonstructural proteins (ORF7/8, NSP7, and NSP13). Early induction of IFNγ-secreting T lymphocytes was observed only in patients with the mildest form of the disease and high viral clearance [19]. Other studies have shown activation of T cells, especially $CD8^+$, the in peripheral blood of patients with severe COVID-19 [16,18,21]. In these patients, pronounced lymphopenia was observed [5,12], the number of $CD8^+$ T cells decreased most drastically [20], probably due to their active recruitment to the lungs.

Our aim was to study T-cell immunity in clinically healthy subjects of the following groups: “no previous COVID-19, not vaccinated”, “recovered”, and “vaccinated” and to compare these indicators with the parameters of humoral immunity.

MATERIAL AND METHODS

The study used material from 118 volunteers (54.5 (45.25; 62.75) years; 48 women, 70 men). All patients self-referred to a medical facility for information on their immune status. All participants signed informed consent for participation in the Biomedical research project “Biomedical Research of Human Tissue and Fluid Samples”, approved by the Expert Ethics Council of the St. Petersburg City Hospital No. 40 (February 12, 2019).

Detection of IgG to the spike (S) protein of SARS-CoV-2. IgG to SARS-CoV-2 S protein was assayed in blood serum on an Alisei (Radim) automatic analyzer using SARS-CoV-2-IgG-IFA-BEST reagents (Vector-Best) according to the manufacturer’s instructions.

Detection of specific T cells. Detection of T cells secreting IFNγ in response to stimulation with peptide pools overlapping the main sequences of S, N, M, ORF3 ,and ORF7 proteins was performed by ELISpot assay. Peripheral blood mononuclear cells (PBMC) were isolated in a Ficoll gradient from whole blood stabilized with 3.2% sodium citrate. PBMC were counted on an XN-1000 hematology analyzer (Sysmex). Detection of specific cells was performed using TigraTest SARS-CoV-2 reagent kit (Generium,) including two inducing peptide pools. Pool 1 overlaps the sequence of spike (S) protein, pool 2 overlaps the sequences of other structural (NP, M, and ORF3) and nonstructural (ORF7) proteins.

Statistical processing of data. Quantitative variables were converted into categorical variables according to the reference value of the corresponding study (Table 1). For statistical processing we used R programming language version 3.6.1 and Python programming language version 3.9. Fisher’s test and Mann—Whitney test were applied and Spearman’s correlation was calculated. The differences between the groups “no previous COVID-19, not vaccinated”,

"recovered", and "vaccinated", Kruskal–Wallis test with Mann–Whitney tests as post-hoc analysis was used. The differences between the samples were considered significant at $p<0.05$. Two machine learning models (gradient binning over decision trees) were constructed to recover missing data. The overall accuracy of the model was used as the metric for successful model training. The models were optimized using genetic algorithms, using tournament selection as the selection operator [13].

RESULTS

In the group "no previous COVID-19, not vaccinated", T cells specific to both the SARS-CoV-2 spike protein sequence and other proteins of the virus were absent in 95% the subjects. Also, 100% subjects in this group lacked humoral immunity to the new coronavirus infection. In the "recovered" group, S protein-specific T cells were present in 39% subjects. In the same group, T-cell immunity to other viral proteins was present in 58% subjects. Antibodies to SARS-CoV-2 were detected in all (100%) individuals recovered from COVID-19. Specific T cells responding to stimulation with S protein peptides were found in 47% patients vaccinated with Gam-COVID-Vac (Sputnik V) for prevention of new coronavirus infection, and only 22% had cells specific to peptides of N, M, ORF3, and ORF7 proteins. Humoral immunity to SARS-CoV-2 was found in 72% vaccinated patients.

Figure 1 demonstrates the data on specific T-cell immunity to S protein in different groups. Specific T cells are found with approximately equal frequency among vaccinated and recovered from COVID-19 patients. This can indicate high efficiency of vaccination. The presence of specific T cells in a small percentage of unvaccinated patients in whom SARS-CoV-2 has never been detected before suggests that coronavirus infection was probably asymptomatic in these individuals. There is also the possibility that T-cell immunity has been developed through repeated exposure to low doses of the virus that do not result in infection, but are sufficient for antigenic presentation.

Figure 2 shows a diagram of the distribution of volunteers with T-cell immunity specific to structural and nonstructural SARS-CoV-2 proteins (excluding S protein). The diagram shows that the majority of volunteers having this type of T cells were COVID-19 recovered patients. This is fully consistent with the theory, because vaccines contain exclusively the RBD-domain of the SARS-CoV-2 spike protein and the immune response to other viral proteins does not develop. In the group of vaccinated volunteers, T cell to a wide range of viral proteins were found in a high percentage of patients. This can be explained

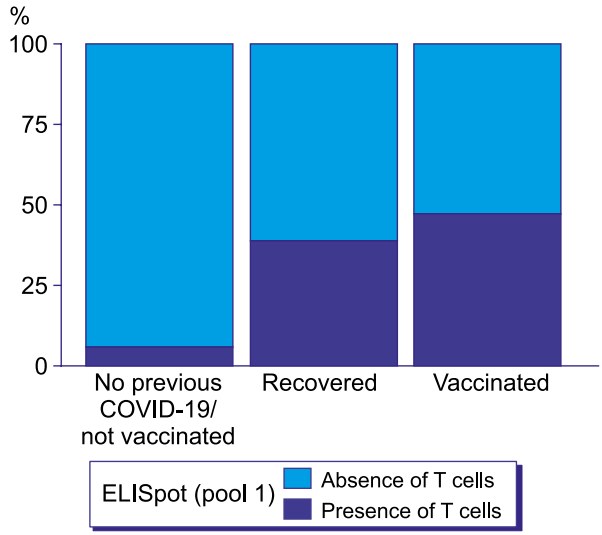

Fig. 1. Presence/absence of T-cell immunity according to ELISpot values (pool 1) in different groups. Peptide pool 1 overlaps the S protein sequence SARS-CoV-2.

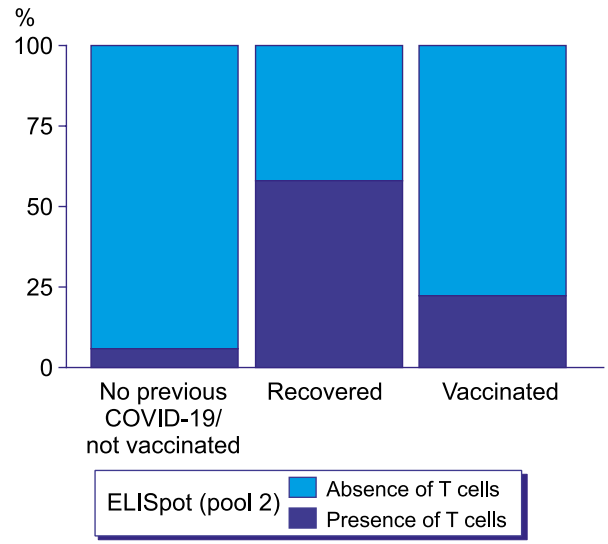

Fig. 2. Presence/absence of T-cell immunity according to ELISpot values (pool 2) in different groups. Peptide pool 2 overlaps the N, M, ORF3, and ORF7 protein sequences of SARS-CoV-2.

by the fact that these subjects contacted with the infectious agent either before vaccination (and were asymptomatic) or after it. The presence of specific T cells in non-vaccinated individuals without previous COVID-19 as in the case of T cells responding to stimulation by S protein peptides, suggests that they were probably asymptomatic or developed immunity through repeated contact with low doses of virus that did not lead to infection, but were sufficient for antigenic presentation.

Significant differences were revealed by comparing samples of categorical variables reflecting the presence/absence of humoral immunity and the

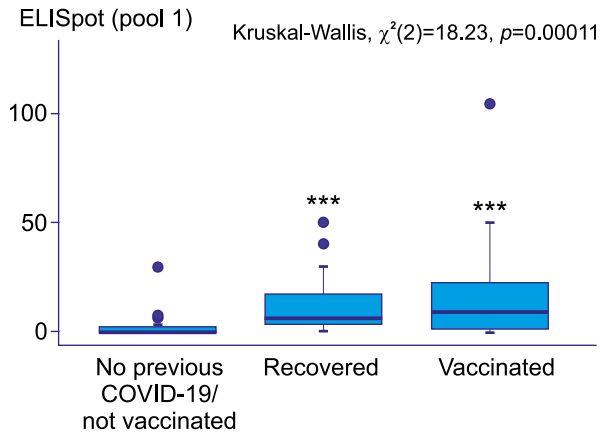

Fig. 3. Box plot with intergroup differences in ELISpot values (Pool 1: S protein). ***p<0.0001 in comparison with the group "no previous COVID-19/not vaccinated".

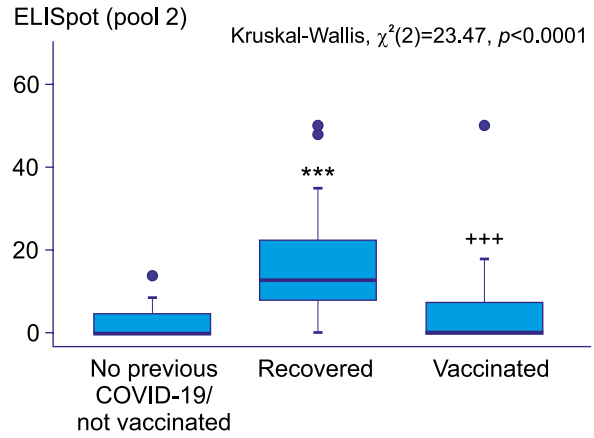

Fig. 4. Box plot with intergroup differences in ELISpot values (pool 2: proteins N, M, ORF3, and ORF7). ***p<0.0001 in comparison with the group "no previous COVID-19/not vaccinated".

presence/absence of specific T cells induced by peptide pool 2 using Fisher's test (p=0.01), suggesting that these values may be correlated. At the same time, a similar comparison involving a sample of variables reflecting the presence/absence of specific T cells induced by peptide pool 1 shows no significant difference (p=0.138). In contrast to Fisher's test, a comparison of the same samples using the Mann–Whitney test shows significant differences for both peptide pool 1-induced T cells (p=0.009) and peptide pool 2-induced T cells (p=0.01).

The Spearman's correlation coefficient was calculated from the absolute values of samples (Table 1). The results of the correlation coefficient calculation are consistent with the results obtained using the Mann–Whitney test: "positivity coefficient" and "number of spots in ELISpot (pool 1)" – r=0.51 (p<0.0001); "positivity coefficient" and "number of spots in ELISpot (pool 2)" – r=0.42 (p=0.0015) and variables "number of spots in ELISpot (pool 1)" and "number of spots in ELISpot (pool 2)" – r=0.52 (p<0.0001). It should be noted that the obtained correlation coefficients reflect medium strength of the connection between the variables. The findings indicate that disease and/or vaccination led to the formation of a complex balanced humoral and cellular immune.

A Kruskal–Wallis test with the Mann–Whitney test as post-hoc analysis was used to detect the differences between the groups ("no previous COVID-19, not vaccinated", "recovered", and "vaccinated"). The absolute numbers of T cells specific to S protein were significantly lower in the "no previous COVID-19, not vaccinated" group in comparison with the "recovered" (p=0.0002) and "vaccinated" groups (p=0.0004). Analysis of these data and the chart in Figure 1 clearly showed that this immunity variant has not only equal frequency among vaccinated and recovered subjects, but also approximately the same intensity.

The absolute numbers of T-cells specific to M, N, ORF3, and ORF7 proteins in the group "no previous COVID-19, not vaccinated" were lower than in the "recovered" group (p=0.00002). At the same time, this value was higher in the "recovered" group than in the "vaccinated" group (p=0.0001). It is worth noting that the groups "no previous COVID-19, not vaccinated" and "vaccinated" did not differ significantly by this parameter (p=0.227) (Fig. 4). The fact that the number of this type of T cells in vaccinated subjects did not significantly differ from that in non-vaccinated individuals without previous COVID-19, but they occur much more frequently in vaccinated individuals (Fig. 2) can indicate that vaccinated subjects can contact SARS-CoV-2 quite frequently without developing the disease.

The positivity coefficient indirectly reflecting the presence of IgG antibodies to SARS-CoV-2 was significantly lower in the group "no previous COVID-19, not

TABLE 1. Instrumental Measured Parameters of Cellular and Humoral Immunity to SARS-CoV-2

Sample	Study	Unit of measurement	Value for a positive result
No previous COVID-19/ not vaccinated	IgG SARS-CoV-2	Positivity coefficient, non-dimensional value	>1.1
Recovered	ELISpot (Pool 1)	Number of spots, units	>10
Vaccinated	ELISpot (Pool 2)	Number of spots, units	>10

vaccinated" compared to both "recovered" (p=0.0002) and "vaccinated" (p=0.0003) groups.

Two machine learning models were built to recover missing data. Two nested models with binary variable prediction were used to solve the multiple classification problem (over-infected/vaccinated/unvaccinated, and not vaccinated). We selected quantitative test values for the positivity ratio reflecting the level of antibodies to SARS-CoV-2 (IgG), for specific T cells (ELISpot pool 1), and for specific ELISpot T cells (ELISpot pool 2) as traits (Table 1).

The first model constructed solved the binary classification problem: "subject recovered from COVID-19 or was vaccinated against COVID-19"/"subject had no previous COVID-19 and was not vaccinated against COVID-19" (accuracy on the training set 100%, accuracy on the test set 91%). The second model solved the binary classification problem: "subject recovered from COVID-19"/"subject vaccinated against COVID-19" (accuracy on the training set 100%, accuracy on the test set 71%). The models were optimized using genetic algorithms, with tournament selection as the selection operator. Missing data (13 subjects) were recovered from model predictions. The reconstructed data were used in the comparison of groups using the Kruskal–Wallis test.

Our data clearly demonstrate that a complex humoral and cellular immune response develops as a result of disease and vaccination. At the same time in vaccinated patients predominantly T cells specific to SARS-CoV-2 protein were detected. T cells specific to M, N, ORF3, and ORF7 proteins were also detected in 22% subjects in this group. This may suggest that vaccinated subjects may experience SARS-CoV-2 quite frequently without developing the disease.

A limitation of this study is the fact that virus-specific T cells were analyzed in the bloodstream using the IFNγ ELISpot. This assay can only detect peripheral Th1 T cells secreting IFNγ. Consequently, it is not possible to assess the full diversity of the T-cell response as well as its intensity in the lung tissues where the main interaction with the virus occurs. The positivity coefficient measured to assess humoral immunity does not directly reflect the number of antibodies and their neutralizing ability, so one should be very cautious in drawing any conclusions from the absolute value of this parameter.

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