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HEALTH SCIENCES

Memory elicitation, T-cell response and antibody production: an independent study of an inactivated entire virus vaccine (Coronavac)

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Abstract: Health professionals working to mitigate the COVID-19 pandemic are one of the main risk groups for the disease, being prioritized for vaccination. Considering this, the aim of this study was to analyze the immune response of these professionals immunized with CoronaVac in the first and second doses. Blood samples were collected after the first and second doses of the vaccine (CoronaVac) and used to investigate hematological and biochemical parameters, analysis of immunoglobulin production, cytokines, and gene expression profile, as well as the identification of subsets of immune cells. Post-first dose immunological phenotypic memory ($CD27^+$) profiles (T $CD4^+$, $TCD8^+$ and $CD19^+$) showed a significant increase, as did Monocyte APCs ($CD80^+HLA\text{-}DR^+$) in relation to the second dose. The cytokines IL-2, IL-6 and IFN-° showed increased values in relation to the other analyzed cytokines. The Th2/Th17 profile in the second dose was characterized by gene expression analysis. The production of IgM and IgG after vaccination showed statistically significant values in the comparison between doses. CoronaVac showed activation of APCs monocytes, memory response of T and B lymphocytes, with immunoglobulins production. This set of responses is characterized by the Th2/Th17 immunological profile.

Key words: CoronaVac, health professionals, immunological memory, immunoglobulin production, COVID-19, SARS-CoV-2.

INTRODUCTION

Coronavirus 2019 disease (COVID-19), which emerged in China, is currently considered the most serious outbreak of pneumonia in the last 100 years, affecting more than 200 countries around the world (Li et al. 2020a, Lu et al. 2020, Melo et al. 2020). According to the Johns Hopkins University Center for Systems Science and Engineering (CSSE) COVID-19 Panel, the disease had generated an estimated 195,767,462 cases and 4,183,074 deaths worldwide (on July 28, 2021), and 19,749,073 cases and 551,835 deaths from Brazil (JHU, 2021).

The transmissibility of COVID-19 is due to the high viral load in the upper respiratory tract, even in asymptomatic patients, which differentiates it from other respiratory diseases (Arons et al. 2020, Li et al. 2020b). Virus propagation occurs through direct contact with contaminated surfaces,

aerosols and respiratory droplets (Chan et al. 2020). In the course of the infection, the patient may not present symptoms or develop different clinical manifestations, such as fever, cough, mild pneumonia, dyspnea, hypoxia, pulmonary impairment on imaging tests, respiratory failure, systemic shock or multiple organ failure and death (Buitrago-Garcia et al. 2020, De Souza Silva et al. 2020, Furukawa et al. 2020, Merad & Martin 2020).

Although the susceptibility to contagion is for everyone, healthcare workers are at high risk of contracting COVID-19 due to their direct or indirect contact with biological fluids from patients, visitors and/or other healthcare workers exposed to the disease. In China, about 29% of healthcare workers were infected with COVID-19 during the first months of the pandemic (Wang et al. 2020). At the same time, Spain and Italy had 22% and 20% of health workers infected, respectively (Arons et al. 2020, Suárez-García et al. 2020). According to the Pan American Health Organization, of the infected healthcare workers in all the Americas, Brazil had 54% of those infected, which corresponds to 307,000 professionals. The most affected were nursing professionals, especially technicians and assistants (OPAS 2020).

In this context, with the still insufficient supply of COVID-19 vaccines around the world, governments prioritized high-risk groups to receive the immunizer at the initial stage of availability. At first, these high-risk groups included healthcare workers, the elderly (especially those with chronic comorbid conditions), and those working in essential services. In Brazil, the first vaccines were approved for emergency use in January/2020 by the Brazilian Health Regulatory Agency, being an inactivated whole virus vaccine (CoronaVac) and a DNA vaccine carried by adenoviral vector (AstraZeneca) (ANVISA 2021). Thus, the aim of this study is to analyze the immune response of healthcare workers immunized with the inactivated whole virus vaccine (CoronaVac) in the first and second doses.

MATERIALS AND METHODS

Blood Sample Collection

The study volunteers are 50 healthcare workers at Hospital das Clínicas of Federal University of Pernambuco (HC-UFPE) who worked throughout the COVID-19 pandemic and were vaccinated exclusively with the CoronaVac in two doses (Sinovac Research and Development Co®). Blood collection was performed in two stages: 15 days after the first dose and 15 days after the second dose. Laboratory and immunological analyzes were performed with blood samples collected in different tubes (BD Vacutainer®) for hematological, coagulogram, serological, biochemical and immunological exams. All volunteer health professionals who participated in this study signed a '*Free and Informed Consent Form*' agreeing with the research approved by the Ethics Committee for Research with Human Beings of Federal University of Pernambuco (nº 4.206.047/2020, CAAE 30332120.8.3001.5191).

Laboratory analysis of blood samples

After blood collection from healthcare workers, the blood and serum samples were sent to the Laboratory of Analysis HC-UFPE for laboratory analysis. The blood count was performed on a Yumizen H2500® hematology analyzer, followed by review of the blood smear under an optical microscope. The coagulogram was analyzed in a STA Compact Max® automatic analyzer. Sample serum was used to evaluate serological and biochemical parameters using Architect i2000SR® fully automated immunopanels and CMD 800i automated biochemical analyzer (Wiener Lab®), respectively.

Immunological tests

Analysis of serum cytokines and immunoglobulins production

The serological samples collected, according to Blood Sample Collection section were used for cytokine measurements and for quantitative detection of anti-SARS-CoV-2 immunoglobulins produced by the vaccine. Th1/Th2/Th17 human cytokines were measured with a Cytometric Bead Array (CBA) kit (BD Bioscience®) with all data acquired by the Accuri cytometry platform and processed with the BD Accuri® software. Quantitative detection of IgG and IgM was performed using IchromaTM COVID-19 Ab kit (Boditech Med Incorporated®) by the fluorescent immunoassay technique. Qualitative detection of IgG and IgM was performed using SARS-CoV-2 antibody test kit by colloidal gold immunochromatography technique (Lepu Technology®).

Immunophenotyping Assay

The isolation of peripheral blood mononuclear cells (PBMC), previously collected in Blood Sample Collection section was performed by PBMC centrifugation (2,000 RPM, 30 min) under a Ficoll separation gradient (1.077 g/mL; GE Healthcare Life Sciences®), as cells were washed twice with 1X PBS (1600 RPM, 10 min) and stained with specific antibodies. Labeling was done using anti-CD3-FITC, anti-CD4-PercPCy5, anti-CD8-PE, anti-CD14-FITC, anti-CD19-FITC, anti-CD27-PECY7, anti-CD80-PE and Anti-HLA-DR-PercPCy5 (all from BD Biosciences®). Cell acquisition was performed in 10,000 events in a FACSVerse flow cytometer (BD Biosciences®) and fluorescence data analysis was performed using BD FACSuite® software.

Gene expression analysis

RNA extraction and cDNA synthesis

Total RNA extraction was performed using isolated PBMC, at a cell concentration of 5x106 and homogenized using 1 mL of liquid Trizol (Invitrogen®). Purification of isolated total RNA was performed using the RNeasy® Mini Kit (QIAGEN®) following the manufacturer's instructions. RNA quality was assured by a NanoDrop 2000 spectrophotometer (Thermo Scientific® Wilmington, USA) and 1% agarose gel electrophoresis. Subsequently, 1µg of purified RNA of suitable quality (an OD260/280 of 1.8 to 2.1 and intact rRNA subunits - 28S and 18S) was used to synthesize cDNA using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase (Thermo Scientific® Wilmington, USA). Water was used as a negative control RT reaction for each sample.

Primer design and efficiency estimation for qPCR

The primers used to detect the STAT4, JAK2, STAT6, STAT3 and FOXO4 genes were designed based on previous studies (Park et al. 2019a, b, Su et al. 2014, Usui et al. 2003, Zhao et al. 2018). The reference genes, ACTB and GAPDH, were used for relative quantification. These genes were previously validated in PBMC samples (Eyerich et al. 2009, Lewkowicz et al. 2011, Mousset et al. 2019). A cDNA from a positive PBMC sample was used to exemplify a real test condition.

Expression evaluation by qPCR

The RT-qPCR reaction was performed in a final volume of 10 µL using the Fast SYBR® Green Master Mix kit (Applied Biosystems®). For reading, the AriaMx Real-Time PCR System (Agilent Technologies®) was used according to the following parameters: 95 ºC for 20s for polymerase activation, 40 cycles of 95 ºC for 3s

for denaturation and 60 ºC for 30s for annealing and extension. Thereafter, the geometric means of reference genes (ACTB and GAPDH) was used to calculate the relative expression of all targets (Livak & Schmittgen 2001).

Statistical analysis

The D'Agostino test was applied to test the normality of the hypothesis and the statistical differences between groups were analyzed by one-way analysis of variance (ANOVA) with the student's t-test confirmation. As for the results of genes expression, it was used the Mann-Whitey test. All results are expressed as the means of the values of the groups ± standard deviation and analyzed considering the value of p <0.05 as statistically significant. The graphs were processed by GraphPad Prism 9 Software®.

RESULTS

This study aimed to investigate, independently, the immunological profile promoted by the CoronaVac vaccine (Sinovac Research and Development Co®) in healthcare workers continuously exposed to patients contaminated by SARS-CoV-2, and symptomatic, in the scope of the Hospital's Wards and ICU of the Clinics of the Federal University of Pernambuco. Although some articles that are being published demonstrate the effectiveness of the vaccine, including for some variants that have started to circulate around the world, our main focus was to observe the effects in a group at risk for continuous infection by the virus in the inter-dose and post-second dose period.

Blood parameters of vaccinated healthcare workers (red blood cells, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (HCM), mean corpuscular hemoglobin concentration (MHCM), red cell distribution width (RDW), leukocytes totals, neutrophils, eosinophils, basophils, lymphocytes, atypical lymphocytes, monocytes, and platelets) were evaluated comparing the results of individuals in the first and second doses of the vaccine. Most of the hematological parameters did not show changes in relation to the reference values nor in the comparison of the two doses. However, it was observed that healthcare workers vaccinated with the second dose had increased hematocrit, monocyte and lymphocyte parameters (Figure 1).

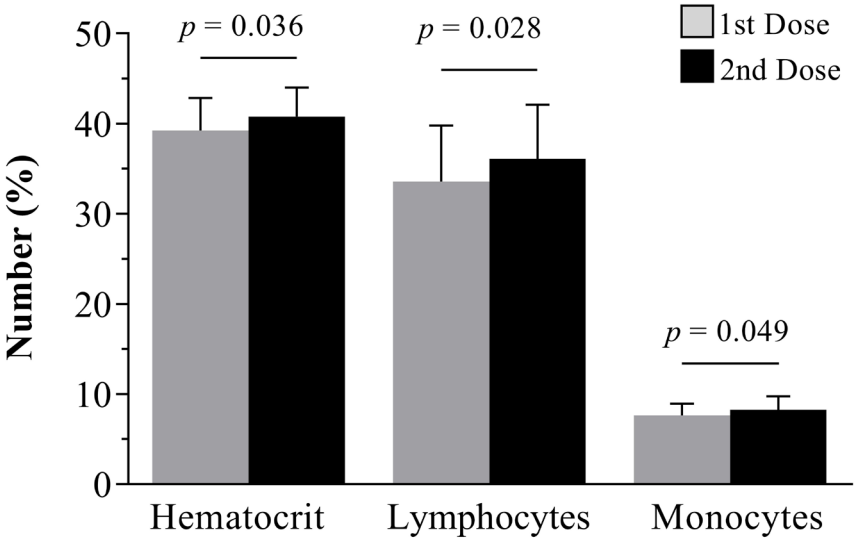

Figure 1. Hematological results of 50 healthcare workers vaccinated with CoronaVac. Gray vertical bars are the values observed 15 days after the first dose and black vertical bars are the values observed 15 days after the second dose.

The biochemical parameters investigated were creatinine, creatine phosphokinase (CPK), lactate dehydrogenase (LDH), ferritin, gamma glutamyl transferase (GGT), oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), urea, C-reactive protein (CRP), bilirubin, sodium, potassium, chloride and fibrinogen. Regarding these parameters, both groups did not present statistical differences between the first and the second dose.

The immunological study of vaccines followed two lines of reasoning, the elicitation of the cellular immune response (which investigates the cellular subtypes and activation pathways of the immune response directed by the T helper lymphocyte) and the humoral immune response (investigation of cytokines and immunoglobulins).

Cellular immunological analysis investigated the cellular memory (using the CD27 antibody) and monocytic effector action ($CD80^+HLA\text{-}DR^+$) profiles. In this sense, the results of immunophenotyping showed that there was a significant increase in monocytes and lymphocytes, with a decreasing prevalence of monocytes, B lymphocytes, $CD8^+$ T lymphocytes and $CD4^+$ T lymphocytes (Figure 2). When comparing doses, $CD4^+$ and $CD19^+$ lymphocytes (B lymphocytes) were increased after the first dose of the vaccine, but with no change to $CD8^+$ T lymphocytes. Furthermore, this post-first dose increase was associated with phenotypic profiles of immunological memory ($CD27^+$), where all sublines investigated (T $CD4^+$, $TCD8^+$ and $CD19^+$) showed a significant increase in relation to the second dose. Monocyte lineage profiles corroborated the results of increased monocyte counts (Figure 1) for the second dose ($CD14^+$). It is worth noting that about 60% of these monocytes had an activated profile, that is, an effector function for antigen presentation ($CD80^+HLA\text{-}DR^+$) in both the first and second doses of the vaccine, although the highest values were in the first dose (Figure 2).

Regarding the results of the production of Th1, Th2 and Th17 cytokines, we could observe a certain balance in the values of the production of cytokines IL-2, IL-4, IL-6, IL-10 and TNF-α between the first and second dose (Figure 3). However, the cytokines IL-2, IL-6 and IFN-° showed increased values in relation to the other analyzed cytokines, with a predominant

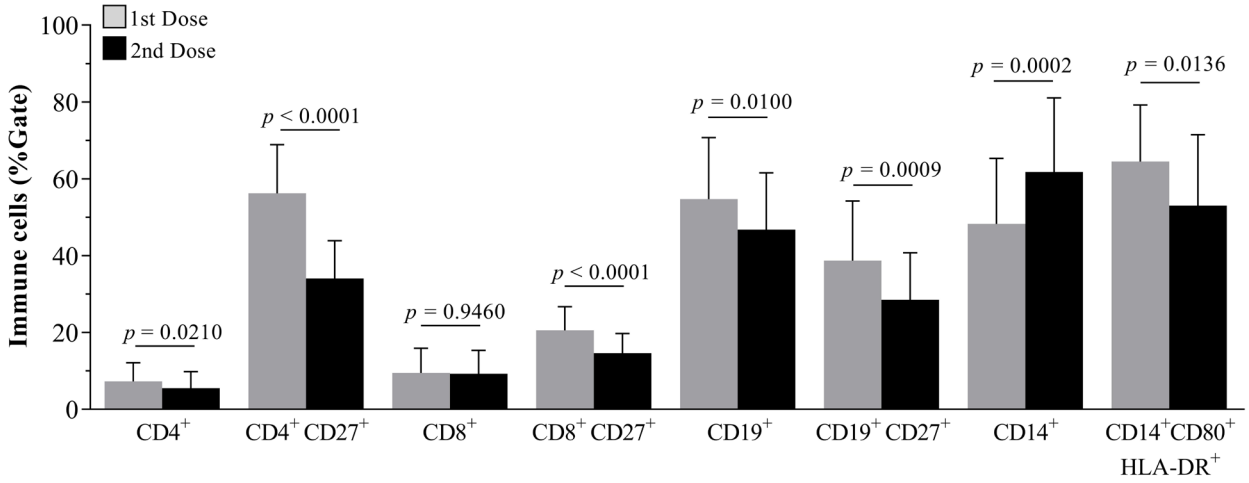

Figure 2. Immunophenotyping of immune cells of 50 healthcare workers vaccinated with CoronaVac. Gray vertical bars are the values observed 15 days after the first dose and black vertical bars are the values observed 15 days after the second dose.

emphasis for IFN-. In addition, IL-17 cytokine values were statistically increased in the second dose (p = 0.0068) and TNF-α also showed a slight increase in the second dose (176.5±1.65; 179.3±7.5 for first and second doses, respectively) but with no statistical difference between them.

In the analysis of gene expression for the genes STAT4 (Th1), JAK2 (pleiotropic gene for Th1 and Th17 responses), STAT3 (Th17), STAT6 (Th2) and FOXO4 (Th22) there was an activation of all these genes in the second dose (Figure 4). However, the JAK2, STAT3 and STAT6 genes were predominant in relation to the others, characterizing a major T helper response of the Th2/Th17 type after the second dose.

For the analysis of immunoglobulins, three screening conditions for anti-SARS- CoV-2 antibodies were performed, the first condition occurred in the pre-vaccination period, the second in 15 days after the first dose and the third also 15 days after the second dose of the vaccine. The objective of the first screening was to elucidate whether professionals had contact with the virus in the pre-vaccination stage. With these results, we could elucidate whether the increase in immunoglobulins was due to the vaccine or whether the professionals already had an increased profile because they had previously been contaminated with the virus. In this case, two variables would need to be clarified: 1) the detection of pre-vaccination anti-SARS-CoV-2 immunoglobulins would increase or not the production of post-vaccination antibodies and 2) the detection of pre- vaccination anti-SARS-CoV-2 immunoglobulins-vaccination is a condition of better immune response for the post-vaccination phase. The results obtained were interpreted as follows: 1) individuals not detected by the kit and individuals negative for both immunoglobulins belong to the same line of reasoning, that is, they did not develop the disease. The results showed that these individuals presented production of both IgM and IgG after vaccination with statistically significant values in the comparison between doses. 2) The groups that showed detection of immunoglobulins in the pre-vaccination phase belong to another line of reasoning, that is, they had the disease, even if asymptomatically, as reported by all in their own questionnaire. In this case, despite the increased values in the second dose of the vaccine, only professionals reactive for IgG showed significant values in the production of IgG in the second dose.

The results of pre-vaccination screening detected 28 (56%) health professionals

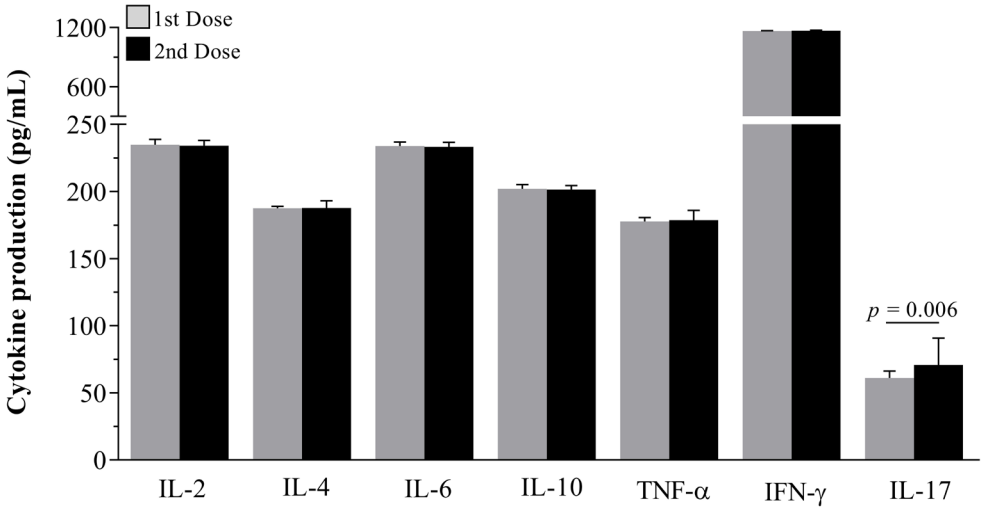

Figure 3. Levels of Th1, Th2 and Th17 cytokines found in the serum of 50 healthcare workers in the first and second doses with CoronaVac. Gray vertical bars are the values observed 15 days after the first dose and black vertical bars are the values observed 15 days after the second dose.

considered non-reactive or negative and 22 (44%) reagents to immunoglobulins (revealing previous contact with the virus). Figure 5 shows the comparison of immunoglobulin production in first and second doses between these two groups. The results demonstrate that there was no statistical difference in the production of immunoglobulins between negative individuals and those who presented an anti-SARS-CoV-2 antibody profile in any analyzed dose.

The results that consider only the 50 vaccinated individuals, with no relevance to the history of laboratory detection for previous infection (or not) with the SARS-CoV-2 virus, can be seen in Figure 6 and demonstrated a significant increase in IgM and IgG for the vaccinated in the second dose.

DISCUSSION

CoronaVac immunologically reproduces the same steps observed by wild virus, maintaining a cellular antigenic memory profile. In addition, we also needed to understand whether the individuals used as research subjects, healthcare workers exposed daily to patients with COVID-19, was a limiting factor in the action of the immunobiological, since we did not intend to study/question the efficacy and safety of vaccine that have already been proven in several multicenter studies (Faria et al. 2021, Hitchings et al. 2021, Tanriover et al. 2021, Wu et al. 2021a, b).

The health professionals vaccinated in this study showed an increase in the number of immune cells, both for lymphocytes and monocytes after the second dose of the vaccine (Figure 1), these data were corroborated by the characterization of sublines with increased monocytes, $CD19^+$ lymphocytes, $CD8^+$ T lymphocytes and T $CD4^+$ (Figure 4). The increase in monocytes and especially their M1 activated effector phenotype ($CD14^+/CD80^+/HLA\text{-}DR^+$), observed in this study, reveals an important finding for mucosal protection (site of action of SARS-CoV-2), antiviral action and for the antigen presentation mechanism in COVID-19 (Burdo et al. 2015).

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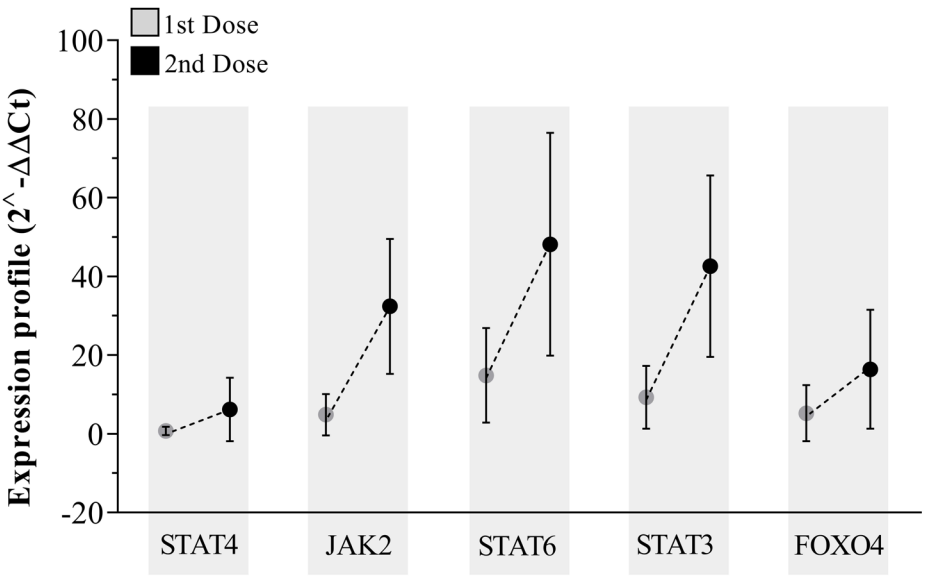

Figure 4. Results of the immune response pathways in healthcare workers vaccinated in the first and second doses with CoronaVac. All genes showed a statistical increase between the first and second dose, value of p<0.001. Genes associated with the Th2/Th17 response (JAK2, STAT3 and STAT6) were seen at high values for healthcare workers who received the second dose of the vaccine.

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compared to the first. These cytokines are directly involved with the immune response against SARS-CoV-2, often so exacerbated that it can promote the cytokine storm in severe disease. An important point to be highlighted is the majority of IFN-° production that was observed, which, as previously mentioned, is suppressed by the virus during the infectious phase (Singh et al. 2020). What is known so far is that, in COVID-19, IFN-° is activated and helps epithelial cells and macrophages in the intracellular antiviral defense and prevents the advance to severe COVID-19 (Singh et al 2020). Furthermore, IL-6 and IL-17 are cytokines that activate the JAK2/STAT3 pathway (Wu & Yang 2020), and IL-6 promotes the recruitment of neutrophils and cytotoxic T cells (Singh et. 2020, Wu & Yang 2020) and IL-17, a molecule associated with protection against a variety of pathogens on the mucosal surfaces of the respiratory tract, including influenza viruses and SARS-CoV-2 itself (Norton et al. 2012, Gallorini et al. 2014, Wu et al. 2020).

The results of the analyzed immunological pathways corroborate all the studies mentioned above, with specific activation JAK2/STAT3/STAT6 corresponding to a Th2/Th17 response, especially overexpressed in the second dose of the vaccine (Figure 5). Since the Th2 response observed in this study (which was not confirmed by the prevalent production of IL-4) is not a response directed towards antiviral action, we suggest that it is being confirmed by only one gene (STAT6), with others being prevalent two canonical genes confirming the Th17 pathway (JAK2 and STAT3). In this case, it is possible that STAT6 elicitation is associated with the

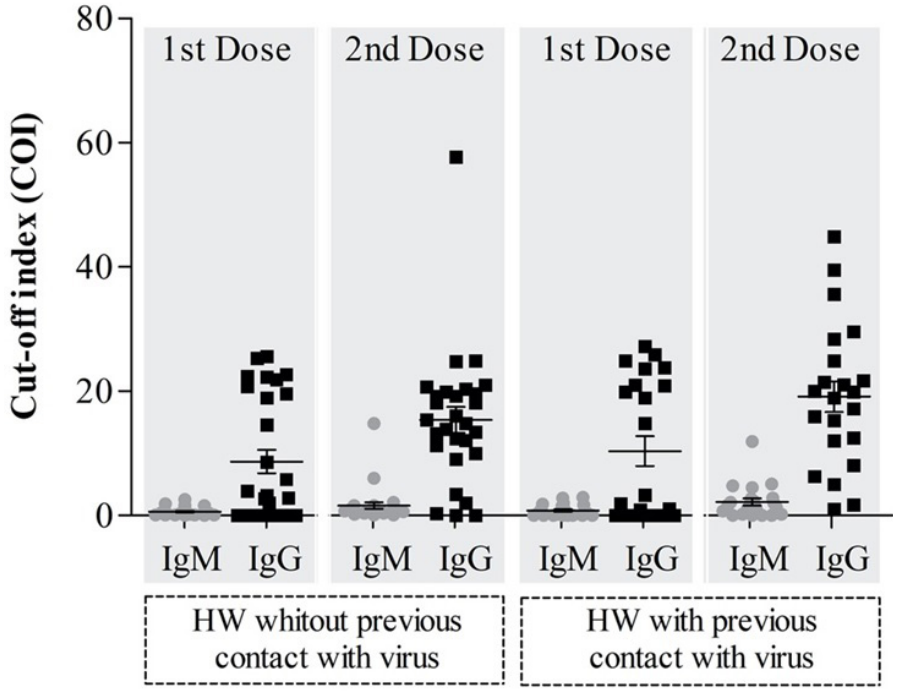

Figure 5. Production of anti-SARS-CoV-2 IgM and IgG immunoglobulins in non-reactive or negative healthcare workers 28 (56%) for immunoglobulins and positive 22 (44%) in the pre-vaccination screening. HW = Healthcare workers.

CoronaVac vaccine adjuvant and not with the inactivated virus components. Similar to the findings of this study with CoronaVac, Miskulin et al. (2021) analyzing the immune response of an mRNA vaccine (Pfizer/BNT162b2) observed that there is activation of $CD4^+$ and $CD8^+$ T lymphocytes, inductors of robust production of IFN-° and, consequently, with a Th1 profile (Vogel et al. 2020, Cascella et al. 2021, Wu et al. 2021a, b).

This same response linked to a Th1 profile can also be observed for other vaccines against SARS-CoV-2, such as the single- dose adenoviral vector-based vaccine (Janssen and Beth Israel Deaconess Medical Center/JNJ-78436735/Ad26.COV2.S) (Mercado et al. 2020) and the adenoviral vector- based booster dose vaccine (AstraZeneca/Oxford University/AZD1222) (Graham et al. 2020, Silva-Cayetano et al. 2021, Watanabe et al. 2021).

Studies show that the response of anti-SARS-CoV-2 T cells during COVID-19 may not depend on circulating antibodies (Sewell et al. 2020) and have pointed to the need for the formulation of immunobiological that strongly activate T lymphocytes and B, memory, since these cells are critical for the protection and maintenance of the individual's defense system against reinfections and the establishment of COVID-19 (Grifoni et al. 2020, Sekine et al. 2020) By specifically analyzing patients recovered from COVID-19, it is possible to identify a robust immune response of neutralizing antibodies (IgG and IgA) Spike-specific, which can decay around two months after the absence of symptoms, memory B cells and cells TFH (follicular T lymphocytes), which indicates elicitation of plasma cells (effector B lymphocytes) and maturation of the humoral immune response (Cox & Brokstad 2020, Huang et al. 2020). All these parameters were observed and elicited by CoronaVac in health professionals immunized in this study.

As previously reported, there was a large elicitation of B lymphocyte response, especially memory, and in the first dose of the vaccine. However, the proof of effector activity of these cells (activated plasma cells) occurred through the increasing immunoglobulin release profile between prime and boost (Figure 6). Other independent studies like this one corroborate our findings. Özdemir et al. (2021) demonstrated

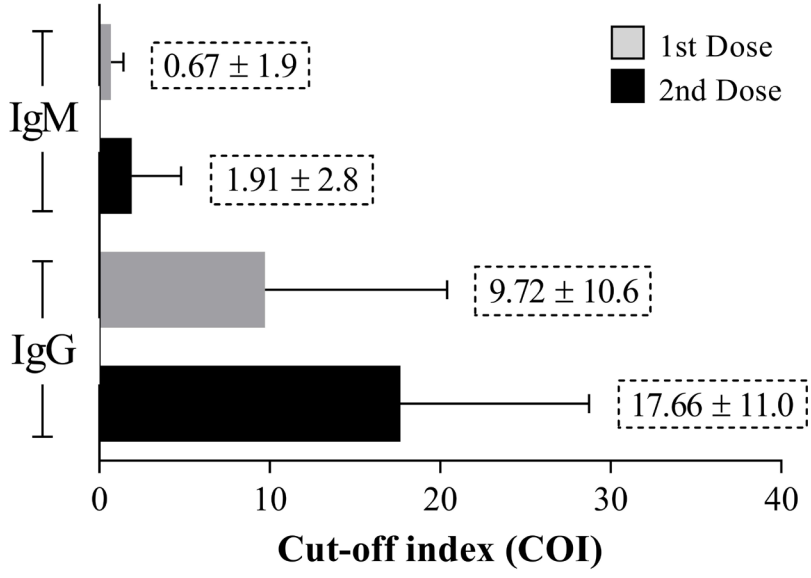

Figure 6. Index of serum immunoglobulin analysis of 50 healthcare workers vaccinated in the first and second doses with CoronaVac. Horizontal bars in gray represent the first dose and horizontal bars in black represent the second dose of the vaccine.

that 91.6% of the 264 healthcare workers vaccinated with CoronaVac had anti-SARS-CoV-2 IgG titres after the second-dose, Calil et al. (2021) investigating breast milk samples from 16 vaccinated mothers showed a significant increase in anti-SARS-CoV-2 IgA and no decay for one month after the second vaccine dose and Bochnia-Bueno et al. (2021), investigating the seroconversion of antibodies from 133 healthcare workers vaccinated with CoronaVac showed that after 20 days of the second dose, 97% of the sera had anti- Spike IgG and 52% had anti-N SARS-CoV-2 IgG.

CONCLUSIONS

The healthcare workers in this study had two doses of the CoronaVac vaccine and did not show side effects. The immunological response balance was interesting, showing memory activation in the first dose (increasing of $CD4^+CD27^+$, $CD8^+CD27^+$, and $CD19^+CD27^+$ subsets) and effector activation (increasing of $CD4^+$ and $CD19^+$ lymphocytes, $CD80^+$HLA-DR^+ monocytes, and IgM and IgG immunoglobulins) in the second dose. Thus, the results point to the fact that the second dose of CoronaVac enhanced the immunological response through the increase of the Th2/Th17 immunological profile, linked to a response of T lymphocytes, B lymphocytes, monocytes, memory formation, and immunoglobulin production.

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