SARS-CoV-2 cellular immune response in uninfected health care workers with prolonged and close exposure to COVID-19 patients

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Abstract

Health care workers (HCW) are at an increased risk since they are directly exposed to SARS-CoV-2 infected patients, nevertheless, some remained without the development of anti-SARS-CoV-2 antibodies, suggesting lesser susceptibility to infection\(^1-5\). This study aimed to ascertain a potential specific cellular immune response to SARS-CoV-2 in these largely exposed HCWs.

In this cross-sectional, case-control study, we analyzed 39 exposed uninfected HCWs and 17 convalescent HCWs. Cellular immune response was evaluated after SARS-CoV-2 stimulation with peptide pools (proteins S, M, and N), using bead-based multiplex assay (12 cytokines).

Overall, 94.8% of uninfected HCWs had some degree of specific cellular response to SARS-CoV-2 structural proteins that could be classified, according to the number of cytokine production, as strong (61.5%), partial (33.3%), and weak/no response (5.1%). Strong responders showed a higher anti-inflammatory cytokine production (IL5 and IL10, \(p<0.001\) and \(0.002\), respectively), and similar (IFN-\(\gamma\) and TNF-\(\alpha\), \(p=0.435\) and 0.532, respectively) or higher (IL12, \(p=0.021\)) pro-inflammatory production compared to convalescents, resulted in a predominantly Th2 response.

This study demonstrated a consistent and polyfunctional immune cellular response after stimulation with SARS-CoV-2 peptides in extensively exposed individuals that should be considered to establish the infection susceptibility, the impact in herd immunity, and the risk of relapses.

Introduction

SARS-CoV-2 epidemic started in December 2019 in Wuhan (China) and has spread rapidly worldwide becoming pandemic threatening public health\(^1-6\). Health care workers (HCW) are at increased risk of SARS-CoV-2 infection since they are continuous and directly exposed to infected individuals\(^7,8\). Data about the seroprevalence of SARS-CoV-2 infection among health care workers are still scarce. It is estimated to be up to 38.9% (23.7% in an intramural survey including 4968 health professionals of our hospital, data not published)\(^9-11\), while it is up to 5.7% in the general population\(^12\). This higher prevalence among HCW is closely related to risk factors such as exposure to aerosol-generating procedures, suboptimal handwashing after patient contact, longer work hours, and suboptimal protective personal equipment use\(^13-14\).

Although many HCWs referred poor access to protective equipment while having very close contact with SARS-CoV-2 infected patients, they have not developed positive serology, suggesting less susceptibility to the infection. We hypothesized that these HCWs could have, at least in part, a cellular immune response that could prevent infection or antibody development. Hence, this study aimed to investigate the potential cellular immune reactivity to SARS-CoV-2 among uninfected HCWs despite long-term direct exposure to infected patients.
Materials And Methods

This was a cross-sectional, case-control study (2:1 ratio), performed in a tertiary University Hospital. The exposure level among HCWs included the continuous care to COVID-19 patients, shortage of complete personal protective equipment, the exposure to aerosol-generating procedures\textsuperscript{7,8}, and additional close contact with infected households. Cases and controls were identified through informal interviews with hospital staff and by self-identification, and were included from May 6 to June 1, 2020.

Uninfected HCWs with proved direct and continued COVID-19 patient care for more than two weeks with no diagnosis of current or past SARS-CoV-2 infection, ascertain by a negative serology for anti-SARS-CoV-2 antibodies in an intramural survey (IgM/IgA and IgG antibodies, Novatec Immunodiagnostica, Germany), were included as cases. Convalescent HCWs, with similar exposure to COVID-19 patients, who had been diagnosed by RT-PCR or/and specific serology, were included as controls.

This study was approved by our Institutional Review Board (EC162/20) and performed according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all the participants. The global study was registered at the clinicaltrials.gov (NCT04402827).

Variables and laboratories measurements

Age, sex, COVID-19 symptoms, exposure to SARS-CoV-2-infected patients, handling of aerosol-generating procedures, and additional close contacts with an infected household were collected at the inclusion visit. At the inclusion, anti-SARS-CoV-2 IgG and IgM antibodies were again determined in all participants, both anti-SARS-CoV-2 IgG/IgM antibodies (COVID-19 IgG/IgM Rapid Test Kit, UNscience Biotechnology, Wuhan, China), and anti-SARS-CoV-2 IgA antibodies (COVID-19 -SARS-CoV-2 IgA ELISA, Demeditech, Germany) for a correct inclusion.

Multiple plasma cytokines were quantified using a human Th cytokine panel (Human MACSplex Cytokine 12 kit, Myltenyi). Briefly, 50 µL of plasma was mixed with beads coated with capture antibodies specific for IFN-α, IFN-γ, TNF-α, IL12, GM-CSF, IL9, IL10, IL4, IL5, IL17A, IL2, IL12p70, and IL6, following the manufacturer’s instructions. Peripheral blood mononuclear cells (PBMC) were isolated from EDTA blood samples and stored in liquid nitrogen until use (Supplementary material). SARS-CoV-2 peptide pools of proteins S, M, and N were used for stimulation of PBMCs. Each peptide pool is composed by a pool of peptides consisting mainly of 15-mer sequences with 11 amino acids overlap, covering the immunodominant sequence domains of the surface glycoprotein S, the complete sequence of the membrane glycoprotein M, and the complete nucleocapsid phosphoprotein N of SARS-CoV-2 (PepTivator SARS-CoV-2 Prot S, M, and N, Miltenyi-Biotec, Cologne, Germany). Cytokine production was quantified by the above mentioned bead-based multiplex assay in these five conditions: stimulation with the three different SARS-CoV-2 peptides (proteins S, M, and N), a negative medium control, and a positive T-cell activator (CytoStim, Miltenyi-Biotec, Germany) control (A sample nonresponsive to T-cell activator would be excluded from the analysis). PBMCs were plated in 96-wells flat-bottom plates at 7x10^5 cells/well.
complete medium and after 18 hours of stimulation, supernatants were harvested for cytokine multiplex quantification in duplicate using human Th cytokine panel (Human MACSPlex Cytokine 12 kit, Miltenyi-Biotec, Germany).

**Statistical analysis**

Estimation of sample size was not done since no data about immune response to SARS-CoV-2 was available in this population, hence, HCWs were specifically selected and not randomly assigned. To categorize the immune response as the outcome for this study, since no other categorization has been described so far, we defined strong responders to those individuals with a total of the cytokines produced to the three viral proteins (a maximum of 30 positive cytokine production per sample) within the three highest quartile range, while partial responders were defined as those within the lowest interquartile range. Characteristics of both groups were compared using two-tailed statistic tests, chi-square or Fisher's exact tests for categorical variables and Student's t-test or Mann-Whitney U-tests for continuous variables. Categorical variables are shown as frequencies and proportions where continuous variables are shown as mean and standard deviation or median and interquartile ranges (IQR). A p-value below 0.05 was considered to be statistically significant.

**Results**

A total of 60 patients, 40 cases, and 20 controls, were included in the study. Four individuals were finally excluded, one case with positive serology, and three controls with negative serology. Hence, a total of 39 cases and 17 controls were finally included. The baseline characteristics of the participants are shown in table 1. The mean age was 38 years, and 55.4% were female. The handling of aerosol-generating procedures and/or additional risk contact with an infected household were similar in both groups (p=0.224 and p=0.440, respectively).

The level of the soluble plasma cytokines was similar in both groups of participants, as shown in supplementary figure 1, with a slightly lower level of IL2 found in uninfected HCWs (p=0.063).

**High cellular response in uninfected HCWs**

Although almost all the uninfected HCWs showed specific response by the production of at least one cytokine after SARS-CoV-2 protein S, M, and N stimulation of the PBMCs, they had lower total number of cytokines compared to convalescents (p<0.001, figure 1A, left). Nonetheless and strikingly, uninfected HCWs had a similar total number of cytokine production to viral peptide S (p=0.289) compared to that found in convalescents (figure 1A, right). None of the samples tested negative for the positive control.

The median number of positive cytokine production in all the participants was 12 (IQR 8-17), and therefore the production of at least eight cytokines was considered as a strong response. Convalescent
HCWs had a median number of cytokine production of 18 (minimum 10 and maximum 25), fulfilling our definition of strong responders in all cases. Thus, a total of 24 uninfected HCWs (61.5%) were classified as strong immune responders, 13 HCWs as partial responders with less than eight cytokine production, (33.3%), whereas 5.1% were classified as weak/no responders (two participants with two or one cytokine production, only IL6). No differences in age, sex, time of exposure, exposure to aerosol-generating procedures, or additional contact with an infected household (p=0.460, p=0.420, p=0.404, and p=0.509 respectively) were found between strong and partial responders. Uninfected HCWs with strong response had lower global cytokine production compared to convalescents (figure 1B, left), but they produced higher number of cytokines in response to viral protein S (p=0.010), that was lower to proteins M and N (p<0.001 in both cases) (figure 1B, right). As expected, the number of total cytokines in those classified as partial responders was lower compared to convalescents for the response to the three structural viral peptides (p<0.001 in all cases).

The level of each cytokine among uninfected and convalescent HCWs are shown in supplementary figure 2. Strikingly, uninfected strong responders had higher levels of Th2 cytokines IL5 and IL10 (p<0.001 and 0.002, respectively), and similar levels of IL4 (p=0.342) to viral protein S compared to convalescents (figure 2). To protein M, they had similar levels of IL5 and IL10 (p=0.284 and p=0.115, respectively), and lower levels of IL4 (p=0.001). To protein N, only the levels of IL5 were similar to that found in convalescents, while they were lower for IL10 and IL4 (p=0.020 and p<0.001, respectively). Also, in comparison with convalescents, the level of Th1 cytokine IL12 to protein S was higher (p=0.021), while IFN-γ and TNF-α were similar (p=0.435 and 0.532, respectively), and they were lower to proteins M and N (figure 2). Finally, the level of IL17A in strong responders was similar to that found in convalescents to protein S and M (p=0.278 and p=0.080), but lower to protein N (p=0.018). In contrast, HCWs with partial response showed lower levels of cytokine production in almost all cytokines, except for IL5, IL10, and IL12 with similar levels to protein S compared to convalescents, and mostly lower to proteins M and N, as shown in figure 2. To graphically show the changes observed in the 10 cytokines according to strong or partial response to the different structural proteins, and in comparison with convalescent, statistically significant differences are detailed in figure 3. As it could be observed, there was different cytokine response to protein S among uninfected HCWs with a strong or partial response (p=0.004), especially in Th2 cytokines. We also observed a higher Th1/Th2 ratio higher in convalescents in response to protein S compared to that found in uninfected strong and partial responders (supplementary figure 3). The IFN-γ/IL10 ratio in convalescents was 6.65 [1.39-24.36], while in strong responders was 0.81 [0.46-1.23] and in partial responders was 0.35 [0.15-1.00]. Again, TNF-α/IL10 ratio in convalescents was 1 [0.74-4.47], while as 0.58 [0.46-0.91] in strong responders and 0.31 [0.11-0.92] in partial responders.

**Discussion**

This study showed that the presence of SARS-CoV-2 cellular immune response in the absence of specific antibodies could be more important than previously considered for immune protection. In 39 uninfected HCWs largely exposed to COVID-19 patients, we demonstrated a consistent and polyfunctional
production of cytokines in response to SARS-CoV-2 peptides, covering viral structural proteins S, M and N, that was similar to that observed in 17 convalescent HCWs, adjusted by age and sex.

These findings are in agreement with other works focused on SARS-CoV-2-specific T cells that started to be characterized in COVID-19 patients and uninfected individuals\(^{15-20}\). Le Bert et al found specific cellular responses in recovered patients and uninfected individuals mainly to structural protein (NP) and non-structural proteins (NSP), although NSP response was rarely detected in SARS-COV-2 recovered individuals, measured by the production of IFN-\(\gamma\) and TNF-\(\alpha\) by CD4 and CD8 T cells\(^{17}\). Grifoni et al found that 40-60\% of unexposed individuals had SARS-CoV-2-reactive CD4 T cells. However, our data are of the greatest value at reflecting the possible protective factor of cellular response in a population with a high risk for SARS-CoV-2 infection\(^{15}\).

According to the total number of cytokine production after protein S, M, and N stimulation of the PBMCs, we described different profiles. Notably, 61.5\% of the uninfected HCWs were classified as having a strong response, and 33.3\% a partial response. This high proportion of cellular response have to be taken in the context of subjects having been exposed to more than eight weeks, when most of the infection-susceptible individuals have already got infected. Furthermore, even a partial response could have been enough to prevent infection since a response to SARS-CoV-2 protein S was observed in most of them. Unfortunately, a predictive clinical or soluble factor to identify individuals with specific immune response was not found.

The pre-existing cellular responses could have been induced by cross-reaction to seasonal endemic coronaviruses, such as OC43, HKU1, NL63, and 229E that present different degree of amino acid homology\(^{21-24}\). Immunity to these coronaviruses appears to be short-lived as antibody titers decay at 4-12 months after infection\(^{25}\). Nevertheless, re-infection with these coronaviruses can occur repeatedly within a single year, eventually activating the cross-reactive immunity. The role of preexisting SARS-CoV-2 reactive cells as a correlate of protection is somehow unclear and needs to be addressed in prospective and larger studies including individuals not exposed to SARS-CoV-2 infection. On the other hand, our data could suggest the development of a cellular response after contact with SARS-CoV-2 in this so high exposed population.

In the recently published ENE survey, among those who reported COVID-19 symptoms before the study only 16.9\% had specific antibodies\(^{26}\). The presence of cellular response to structural and/or accessory proteins suggesting a past SARS-CoV-2 infection was described in six out of eight household contacts, in the absence of antibodies production\(^{27}\). The lack of association of strong response with the time of exposure or handling aerosol-generating procedures does not invalidate this hypothesis, since the probability of infection is multifactorial. Moreover, the different immune response observed in uninfected HCWs, with predominance of Th2 response, could suggest a response to an aborted infection.

Interestingly, among uninfected strong responders, a higher anti-inflammatory cytokine production (IL5 and IL10) to protein S, and a similar pro-inflammatory response (IFN-\(\gamma\) and TNF-\(\alpha\)) or even higher (IL12)
was observed, compared to convalescents.

Of great interest, we demonstrate that the immune response is mostly driven against protein S, both in convalescent and uninfected HCW, especially in those with a strong response. These data support the role of this structural protein as the basis for vaccine development, as even subjects with partial response in our study, having a low response to protein M and N, are probably protected because of the response to this protein S.

Our study has limitations, including the cross-sectional design that limits the association between the exposure and the immune response. Also, there was a selection bias towards the inclusion of subjects, by including highly and prolonged exposed HCWs with negative specific serology as the population of the study. Finally, specific CD4 and CD8 T cell responses was not determined yet in this study. Instead, a wide spectrum of cytokines produced by many different immune cells was quantified to better understand the cytokine changes found in many SARS-CoV-2 infected patients, and how they may play a role in the immune system.

In summary, this study confirms the presence of SARS-CoV-2-specific cellular immunity in seronegative highly-exposed individuals, and this can be efficiently protective. This is an important issue in terms of recommendations of the general population since herd immunity is crucial for estimating the risk of reinfections. Nonetheless, it is of great importance to investigate whether this cellular immunity is long-lasting.

**Declarations**

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**Declaration of interest**

The authors declare no competing interests.

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**Author contributions**
AV and JLC designed the study. JLC, PV, AM, CQ recruited the participants for this study. JLC, PV and AV collected data from participants to generate a database. AV did all the laboratory work. AV and JLC analysed and interpreted data. AV and JLC prepared the manuscript on the basis of comments from all authors. All authors provided data, reviewed and contributed to the final version of the manuscript and agreed to be accountable for the work.

Data sharing

Requests for materials or data should be addressed to corresponding authors upon request.

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Tables

Table 1: Characteristics of the health care workers included in this study.
|                                        | Uninfected HCWs (N=39) | Convalescent HCWs (N=17) | p   |
|----------------------------------------|------------------------|--------------------------|-----|
| **Age (years)**                        | 39 [31-51]             | 38 [30-50]               | 0.894 |
| **Gender (Female)**                    | 20 (51.2%)             | 9 (52.9%)                | 0.519 |
| **Health profession**                  |                        |                          | 0.232 |
| Physicians                             | 26 (66.6%)             | 14 (83.3%)               |     |
| Nurses                                 | 13 (33.3%)             | 3 (17.6%)                |     |
| **Time of exposure (weeks attending COVID-19 patients)** | 8 [6-10]               | 6 [5-8]                  | 0.084 |
| **Additional exposure**                |                        |                          |     |
| Aerosol generating procedures          | 33 (84.6%)             | 12 (70.6%)               |     |
| SARS-CoV-2-infected household          | 5 (12.8%)              | 1 (5.9%)                 |     |
| **Time from COVID-19 diagnosis to serological survey (weeks)** | -                     | 4 [2-6]                  |     |
| **Time from serological survey to study inclusion (weeks)** | 3 [2-4]               | 5 [3.5-5]                | <0.001 |
| **Serology at serological survey**     |                        |                          |     |
| IgM/IgA positive                       | 0                      | 9                        |     |
| IgG positive                           | 0                      | 17                       |     |
| **Serology at study inclusion**        |                        |                          |     |
| IgA positive                           | 0                      | 3                        |     |
| IgM positive                           | 0                      | 3                        |     |
| IgG positive                           | 0                      | 17                       |     |
| **SARS-CoV-2 RT-PCR**                  |                        |                          |     |
| Positive                               | -                      | 11                       |     |
| Negative                               | 6                      | -                        |     |
| Not tested                             | 33                     | 6                        |     |

Data are expressed as median and interquartile range, and percentage. Mann-Whitney U test for statistical differences between variables. HCW, health care workers; ¹, chi-square test; ², aerosol-generating procedures included airway suction, application of a high-flow O₂ instrument, bronchoscopy,
endotracheal intubation, tracheostomy, nebulizer treatment, sputum induction, positive pressure ventilation, manual ventilation, and cardiopulmonary resuscitation; \(^3\) by ELISA; \(^4\) IgA by ELISA and IgG and IgM by rapid chromatographic immunoassay.

**Figures**

A. **Total cytokine production**

![Graph A](image)

B. **Total cytokine production according to response profiles**

![Graph B](image)

Figure 1
A. Total number of cytokine production in uninfected (UI) and convalescent (CO) HCWs after in vitro PBMCs specific stimulation: to the combination of protein S, M and N, left and to each of the proteins separately, right. B. Total cytokine production according to different profiles: Strong responders (SR, with a total production of more than 8 cytokines, partial cellular responders (PR, with equal or less than 8 cytokine production, and weak/no responders (NR, with 2 or less total cytokine production). Left, total cytokine production to the combinations of protein S, M and N; and right, total cytokine production to each of the proteins separately. Significant when p<0.05. Not significant results are not shown.
Figure 2

Level of cytokines produced by strong (SR) and partial (PR) cellular responders compared to convalescent (CO) HCWs according to SARS-CoV-2 specific peptides S, M and N used for PBMC stimulation. Significant when p<0.05. Not significant results are not shown.

Cytokine level increments compared to convalescents

![Diagram showing cytokine levels for SR and PR responders to proteins S, M, and N.](image)

- Higher
- Similar
- Lower
- Undetectable

Figure 3

Cytokine level increments compared to convalescents according to strong (SR), and partial (PR) cellular responders to specific SAR-CoV-2 peptides S, M and N. Each cytokine level in each of the groups has been labeled as higher, similar lower or undetectable compared to the levels found in convalescents. Cytokine levels to protein S (main responses), M and N are shown. Significant when p<0.05. Not significant results are not shown.

Supplementary Files

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- Supplementaryfigures.pptx