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## Pre-existing T-cell immunity to SARS-CoV-2 in unexposed healthy controls in Ecuador, as detected with a COVID-19 Interferon-Gamma Release Assay

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### ABSTRACT

**Background:** Studies of T-cell immune responses against SARS-CoV-2 are important in understanding the immune status of individuals or populations. Here, we use a simple, cheap, and rapid whole blood stimulation assay - an Interferon-Gamma Release Assay (IGRA) - to study T-cell immunity to SARS-CoV-2 in convalescent COVID-19 patients and in unexposed healthy contacts from Quito, Ecuador.

**Methods:** Interferon-gamma (INF- $\gamma$ ) production was measured in the heparinized blood of convalescent and unexposed subjects after stimulation for 24 h with the SARS-CoV-2 Spike S1 protein, the Receptor Binding Domain (RBD) protein or the Nucleocapsid (NP) protein, respectively. The presence of IgG-RBD protein antibodies in both study groups was determined with an “in-house” ELISA.

**Results:** As measured with INF- $\gamma$  production, 80% of the convalescent COVID-19 patients, all IgG-RBD seropositive, had a strong T-cell response. However, unexpectedly, 44% of unexposed healthy controls, all IgG-RBD seronegative, had a strong virus-specific T-cell response with the COVID-19 IGRA, probably because of prior exposure to common cold-causing coronaviruses or other viral or microbial antigens.

**Conclusion and Discussion:** The high percentage of unexposed healthy subjects with a pre-existing immunity suggests that a part of the Ecuadorian population is likely to have SARS-CoV-2 reactive T-cells. Given that the IGRA technique is simple and can be easily scaled up for investigations where high numbers of patients are needed, this COVID-19 IGRA may serve to determine if the T-cell only response represents protective immunity to SARS-CoV-2 infection in a population-based study.

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### Introduction

The immune response to SARS-CoV-2 infection involves not only antibody production, but the infection also triggers a T-cell response in patients (Lipsitch et al., 2020, Ni et al., 2020). In general, the determination of the presence and magnitude of a specific memory T-cell response to this coronavirus infection is

performed with an ELISPOT assay by establishing the number of lymphocytes that produce IFN- $\gamma$  after stimulation with disease-specific antigens or peptides (Ni et al., 2020, Pai et al., 2014, Abate et al., 2013, Braun et al., 2020, Grifono et al., 2020, LeBert et al., 2020, Sekine et al., 2020). For this technique, peripheral blood mononuclear cells (PBMCs) are isolated from fresh heparinized blood by density-gradient centrifugation; the secretion of cytokines by individual T-cells is quantified by microscope or with a special apparatus—the ELISPOT plate reader. The ELISPOT assay is a “for research laboratories only” technique that is highly laborious, technically demanding, relatively expensive, and cannot easily be used in a clinical laboratory or be applied in population-based studies. Due to the cost, infrastructure, and the laboratory

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requirements needed to perform the assay, its use in resource-limited settings such as Ecuador may be minimal.

Cellular immune responses can also be determined with a more straightforward assay: an interferon-gamma release assay (IGRA). This assay, an *in vitro* blood test, quantifies T-cell secreted cytokines (i.e., gamma interferon (IFN- $\gamma$ ); however, other cytokines like TNF $\alpha$  or IL-2 can also be quantified) upon stimulation of the whole blood (not just the isolated lymphocytes) with specific antigens (Pai et al., 2014, Abate et al., 2013). The secreted cytokine IFN- $\gamma$  is measured with an ELISA assay, a readily available technique in most laboratories, and the results are read out with an ELISA plate reader. IGRAs have found an application in the clinical laboratory and are used to determine a latent infection with *Mycobacterium tuberculosis* (MTB) as well as to measure cytomegalovirus (CMV)-specific cell-mediated immunity and predict CMV infection in transplant recipients (Pai et al., 2014, Abate et al., 2013). These IGRA tests have been extensively evaluated and, compared with the ELISPOT assay, have been shown to display similar capabilities for predicting MTB or CMV infection. Moreover, several commercial and FDA or CE marked assays to detect immunity against TB or CMV are available and are widely performed.

In the present study, we explore the use of a COVID-19 IGRA and assess the magnitude of IFN- $\gamma$  release in convalescent COVID-19 patients and healthy COVID-19 naïve controls in response to specific antigens of the SARS-CoV-2 virus: the RBD protein; the S1 part of the Spike protein; or the Nucleocapsid (N) protein. Additionally, we determine the presence of SARS-CoV-2 specific IgG antibodies in those participants with an “in-house” ELISA (Amanat et al., 2020, Guevara et al., 2021). We show that a significant percentage of unexposed donors possess SARS-CoV-2 reactive T-cells, and we discuss these results.

## Material and methods

### Convalescent COVID-19 patients, healthy controls, and the setting

This study was conducted in Quito, Ecuador, in September 2020. No statistical methods were used to predetermine the sample size. Table 1 shows the characteristics of the 32 patients in this study: thirteen convalescent COVID-19 patients, all with non-severe COVID-19 symptoms/signs and a positive RT-PCR diagnosis in June 2020; and 17 unexposed healthy controls. These healthy controls had been extremely cautious during the pandemic, avoiding contact with other people and self-reported none of the typical signs and symptoms of COVID-19 for the duration of the pandemic, such as fever, a persistent cough, or a loss of smell or taste. Also, two asymptomatic SARS-CoV-2 carriers were included in this study. These two subjects never showed any symptoms or signs of COVID-19 disease; however, the RT-PCR technique was carried out on them as a requirement for a work permit in June 2020 and resulted in positive diagnoses.

**Table 1**

Demographic and diagnostic characteristics of the convalescent COVID-19 patients and healthy controls.

Subjects n = 33	Male / Female Ratio	Mean age (SD)	RT-PCR positive	COVID-19 related symptoms and signs	RBD serodiagnosis	T-cell response positive
13 convalescent patients	10/3	34 (8.0)	13/13	13	13/13 Positive	10/13 (77%)
2 asymptomatic patients	1/1	32 (2.1)	2/2	None	Negative	2/2 (100%)
17 healthy controls	8/9	40 (12.8)	None	None	Negative	8/17 (47%)

Thirty-two subjects were included in this study: thirteen RT-PCR positive patients with clinical signs and symptoms of COVID-19, two asymptomatic RT-PCR positive patients, and 17 healthy COVID-19-naïve patients. All COVID-19 patients had at least three of the five most common signs or symptoms of COVID-19: fever, respiratory problems, loss of smell/taste, and fatigue. They were positive for serology with an “in-house” SARS-CoV-2 Receptor Binding Domain (RBD) IgG ELISA (5, 6). No severe clinical cases were admitted in this study, and patients were all treated at home. The 17 healthy controls and the two asymptomatic RT-PCR positive subjects reported no signs or symptoms of COVID-19 or other health-related problems since the COVID-19 outbreak in Ecuador in March 2020, and all 19 were negative for RBD-IgG serology. All convalescent patients were tested three months after their positive RT-PCR.

## Serology

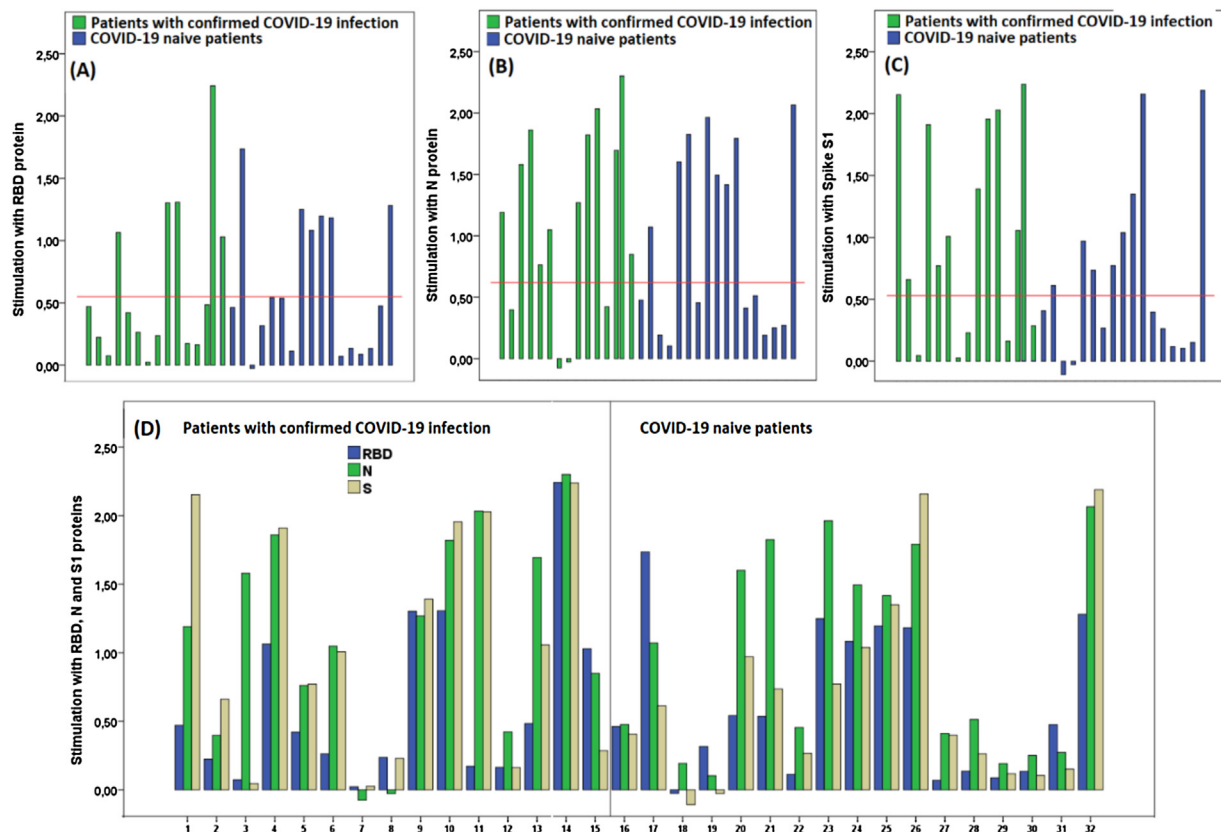
Serology and IGRAs for all patients and controls were performed during the last two weeks of September 2020, three months after the RT-PCR diagnosis of the convalescent patients. To determine the presence of coronavirus-specific IgG antibodies in our study group, an “in-house” SARS-CoV-2 Receptor Binding Domain (RBD) IgG ELISA (9, 10) was used.

### Interferon Gamma Release Assay (IGRA)

For the IGRA, a heparinized blood sample was taken (4 mL) and divided into aliquots of 250  $\mu$ L and stimulated with respectively 2  $\mu$ g/mL of the RBD protein (isolation procedure from reference 9) or 2  $\mu$ g/mL of the commercially available Nucleocapsid (N) protein or Spike (S1) protein (ViroGen, [www.virogen.com](http://www.virogen.com), Catalog Numbers: 00,221-V and 00,226-V respectively). A dose concentration-response curve with blood from IGRA positive and IGRA negative patients was used to determine the antigens' optimum concentration for stimulation in the IGRA. At 2  $\mu$ g/mL, a maximum IFN- $\gamma$  response in blood from COVID-19 subjects was found without stimulation in the uninfected controls. For each participant, an unstimulated control (NIL) was included, and we assayed a 250  $\mu$ L blood sample with the mitogen phytohaemagglutinin (PHA), a positive control to assess the cytokine response rate of the blood sample. Stimulation was realized in a 96-well flat-bottom ELISA plate at 37 °C for 24 h. Plasma was harvested, and IFN- $\gamma$  production was determined with a Human IFN- $\gamma$  ELISA (MaxTM Standard Set Biologend cat. No. 430,101).

## Results

The RBD IgG ELISA showed that all COVID-19 symptomatic patients (RT-PCR positive) were positive for IgG antibodies against the RBD protein three months after the RT-PCR diagnosis. The two asymptomatic RT-PCR positive patients and all healthy unexposed control patients were seronegative with this ELISA (See Table 1). The results of whole blood stimulation in the COVID-19 IGRA assay are shown in Figure 1. Strong induction of IFN- $\gamma$  production was found in 80% of the COVID-19 symptomatic patients with all three antigens, but the N protein and S protein were the most potent stimulators of an IFN- $\gamma$  response (Figure 1 A, B, C). The two asymptomatic but RT-PCR positive patients (patient 14 and 15), both without specific SAR-CoV-2 antibodies, also showed a strong inducible T-cell immunity response. Of the COVID-19 naïve patients, 45% showed inducible T-cell immunity. The response in these healthy unexposed controls with a T-cell response was statistically not significantly different in magnitude from the convalescent COVID-19 patients for any of the stimulating antigens (p-value = 0.271, 0.437, and 0.719 for RBD, N, and S1 proteins, respectively).



**Figure 1.** IFN- $\gamma$  production after stimulating whole blood of convalescent COVID-19 patients and healthy unexposed controls with RBD, N, or S1 protein.

The results of stimulation of convalescent patients and controls with, respectively, the RBD, N, or S1 protein are shown in Fig. 1A, B, and C. In Fig. 1D, the IFN- $\gamma$  responses of each patient and to the three antigens are grouped together. Patients with confirmed COVID-19 infection are numbered 1–15, of which patients 14 and 15 were asymptomatic. Subjects 16–33 are COVID-19 naïve patients. After 24 h of stimulation, IFN- $\gamma$  concentrations in plasma were determined using a commercially available human IFN- $\gamma$  enzyme-linked immunosorbent assay-based kit (Biolegend cat. No. 430,101). The magnitude of the stimulation was expressed as the optical density at 450 nm ( $OD_{450}$ ) for blood stimulated with antigen minus the  $OD_{450}$  for blood without antigen stimulation. An OD of 1.0 corresponded with approximately 400 pg/mL IFN- $\gamma$ . Phytohemagglutinin (PHA) was used as the mitogen at a final concentration of 5  $\mu$ g/mL in the IGRA to assess the quality and response capacity of the blood. All patients and controls showed a maximum production of the cytokine with this mitogen with a mean optical density ( $OD_{450}$ ) of 2.54 (SD = 0.13), representing approximately 1000 pg/mL of IFN- $\gamma$ . There was no difference in the median IFN- $\gamma$  level in the unstimulated blood of convalescent patients and healthy controls ( $OD_{450}$  = 0.38, SD = 0.14 versus  $OD_{450}$  = 0.32, SD = 0.16). These results are not shown. A horizontal line has been drawn in each graph to represent the cut-off value for stimulation. This value was determined by calculating the mean plus two standard deviations of the response of nine subjects (patients 16, 18, 19, 22, 27–31) of the control group with the lowest IFN- $\gamma$  production after stimulation.

## Discussion and conclusions

Using an easy-to-perform whole blood assay that assesses T-cell responses, we found that most convalescent patients and a significant proportion of healthy unexposed individuals had a specific and strong T-cell response determined with the production of IFN- $\gamma$  after stimulation with SARS-CoV-2 specific antigens (Figure 1). Several studies, all using the ELISPOT assay, have reported the presence of a T-cell response in most or all convalescent COVID-19 patients (Braun et al., 2020, Grifoni et al., 2020, LeBert et al., 2020, Sekine et al., 2020, Moderbacher et al., 2020, Mateus et al., 2020, Peng et al., 2020). However, concerning a T-cell response in healthy unexposed controls, response rates between 28 and 50% have been reported (Doshi, 2020, de Vries, 2020, Lipsitch et al., 2020, Sagar et al., 2020), and several studies report no T-cell response in COVID-19 naïve control patients (de Vries, 2020, Moderbacher et al., 2020, Schwarzkopf et al., 2021). Most of these studies were performed with the ELISPOT assay, but in Italy and the USA, an IGRA has also been used to detect the SARS-CoV-2 T-cell response (Petroni et al., 2020, Murugesan et al., 2020). In both studies, a specific T-cell response was detected in convalescent patients; however, the IGRA did not detect an IFN- $\gamma$  response in healthy unexposed subjects.

Pre-existing T-cell immunity to SARS-CoV-2 in COVID-19 naïve subjects has been reviewed in several publications (Altmann and

Boyton, 2020, Dosho et al., 2020, de Vries, 2020, Sette and Crotty, 2020). The reason why a specific COVID-19 T-cell response might be detected in a healthy unexposed subject remains unclear and is the subject of speculation. Most ELISPOT studies with COVID-19 naïve subjects used human blood samples derived before the SARS-CoV-2 virus was discovered in 2019, thus before any chance of exposure. For this reason, these ELISPOT studies concluded that the T-cell response in unexposed healthy subjects probably comes from prior contact with circulating “common cold” coronaviruses (Grifoni et al., 2020, Matheus et al., 2020) or previous exposure to flu and/or CMV viruses (Mahajan et al., 2020) or other viral or microbial antigens (Tan et al., 2020). Our study used blood taken during the pandemic from volunteers who self-reported zero contact, were seronegative for the COVID-19 antigen RBD, and had never shown any signs or symptoms of COVID-19. Therefore we cannot exclude that the T-cell response in some of our COVID-19 “naïve” healthy controls comes from asymptomatic SARS-CoV-2 virus infections. Patients 14 and 15 of our study are an example of this. These individuals were classified as COVID-19 patients because of a positive RT-PCR; however, they were asymptomatic and did not develop an antibody response against the RBD protein, but both showed a strong T-cell response. It is possible that, after SARS-CoV-2 colonization of the nasopharynx, the immune system of these two asymptomatic patients left them with a memory T-cells-only response, and no SARS-CoV-2 specific antibodies were



induced. Another explanation is that the T-cell response comes from a previous corona-like infection, and the pre-existing T-cell immune response protected these two patients against the development of a more invasive disease pattern. Pre-existing cross-reactive T-cells could be significant in explaining some of the differences in infection rates or pathology (Le Bert et al., 2020, Sagar et al., 2020). Concerning the cohort of naïve controls, we excluded that this group contains a considerable number of SARS-CoV-2 infected individuals with undetected asymptomatic infection, basically because all the integrands of the control group are IgG seronegative for the RBD antigen. Another reason is that the infection prevalence in Ecuador is relatively low. At the time of this study, September 2020, Ecuador had reported about 200,000 confirmed COVID-19 cases (about 1.1% of the population). Of course, the actual infection prevalence could have been several times higher, but even so, it is improbable that 45% of this control group got infected, and thus that we are looking at a T-cell response specific for a SARS-CoV-2 infection.

In conclusion, the finding of a high percentage of unexposed healthy subjects with a pre-existing immunity in Quito, Ecuador, raises the expectation that a significant part of our population is likely to have SARS-CoV-2 reactive T-cells because of prior exposure to flu, CMV viruses, or common cold-causing coronaviruses. Future investigations should aim to determine whether the pre-existing immune response is protective and/or can overcome a more severe COVID-19 disease or contributes to a faster recovery.

### Importance and limitation of this study

Our study showed that the COVID-19 IGRA is a useful and sensitive new tool that can assess exposure to coronaviruses or cross-reactivity to similar viruses by determining the release of IFN- $\gamma$  after stimulation with virus-specific antigens. The technique is simple, relatively cheap, and can be used in a clinical laboratory. This is certainly not the case with the ELISPOT assay. Moreover, the IGRA assay can be easily scaled up for population-based studies and investigations where high numbers of patients are required. Together with serology, the IGRA can be used to screen both antibody and T-cell responses of individual patients to better understand population-level immunity and determine pre-existing immunity in seronegative individuals.

An important limitation of our study is that the study sample is not representative of the population. No statistical methods were used to predetermine the sample size. Furthermore, our assay didn't differentiate between IFN- $\gamma$  production of CD4+ or CD8+ cells, but stimulation with defined CD8(+) and CD4(+) peptides can be considered (Grifoni et al., 2020, Peng et al., 2020) and has been used in the IGRAs described in Petrone et al., 2020 and Murugesan et al., 2020). Also, the induction of other cytokines can be measured with this IGRA (Petrone et al., 2020). Likewise, the IGRA can be employed for follow-up on vaccination to determine the development of the T-cell response in vaccinated individuals or can be used to determine if the presence of cross-reactive memory T-cells is associated with protective immunity and can diminish the disease manifestations from SARS-CoV-2 infection (Sagar et al., 2020).

### Ethics approval and participation consent

Blood was collected following informed written consent. The Ethics Committee of the Health Ministry of Ecuador approved the study protocol 004–2020.

### Conflict of interests

The authors declare that they have no competing interests.

### Author contributions

Study design; JHW and GE; Data collection and laboratory experiments: GE, AG, JC, AMR, and MMV; Data analysis; JHW, GE, and ET; Writing: JHW, GE, and ET.

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