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Immunity in the SARS-CoV-2 infection
Helping in patients' clinical management

Guillem Safont Gonzalez

Ph.D. Thesis

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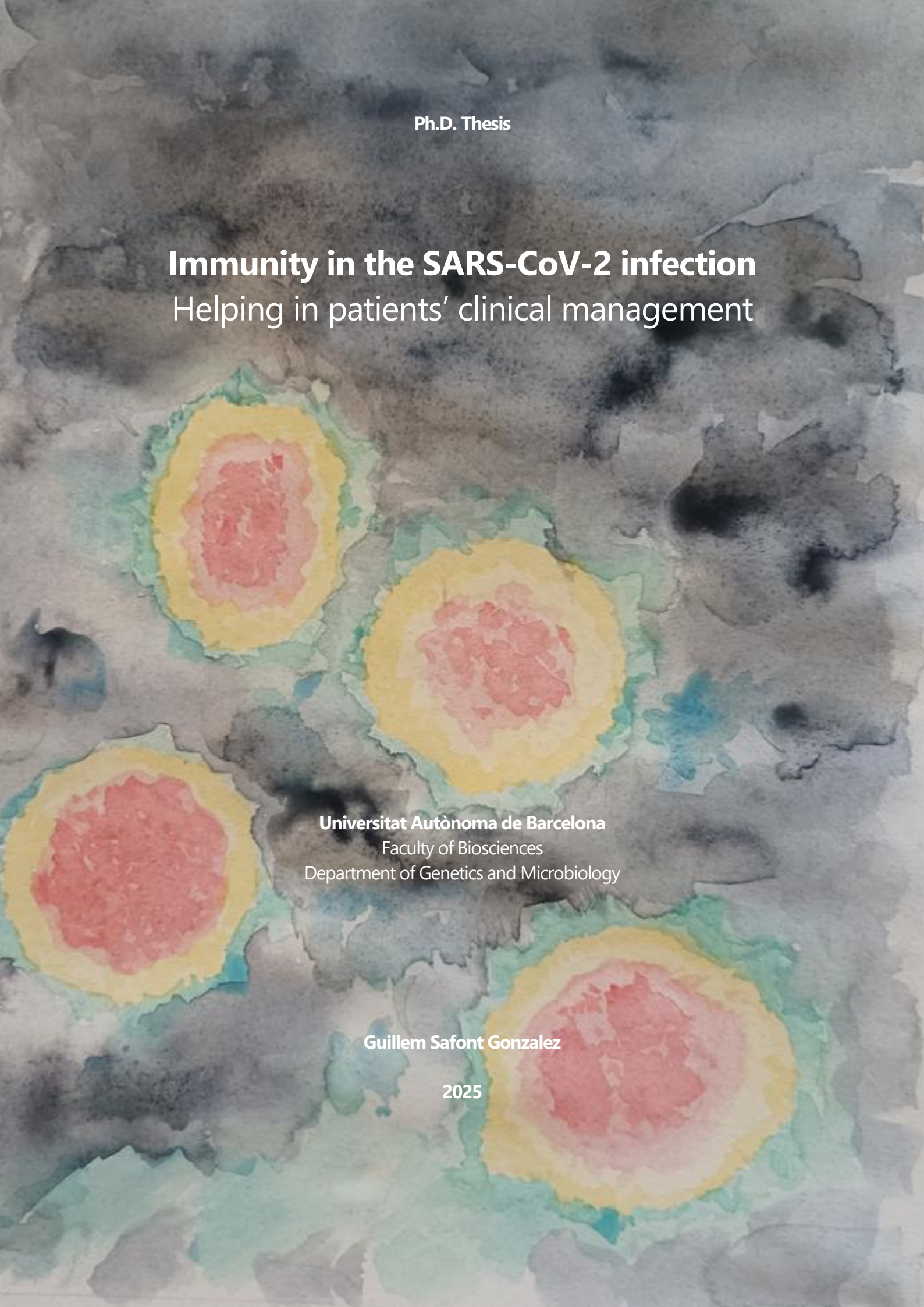

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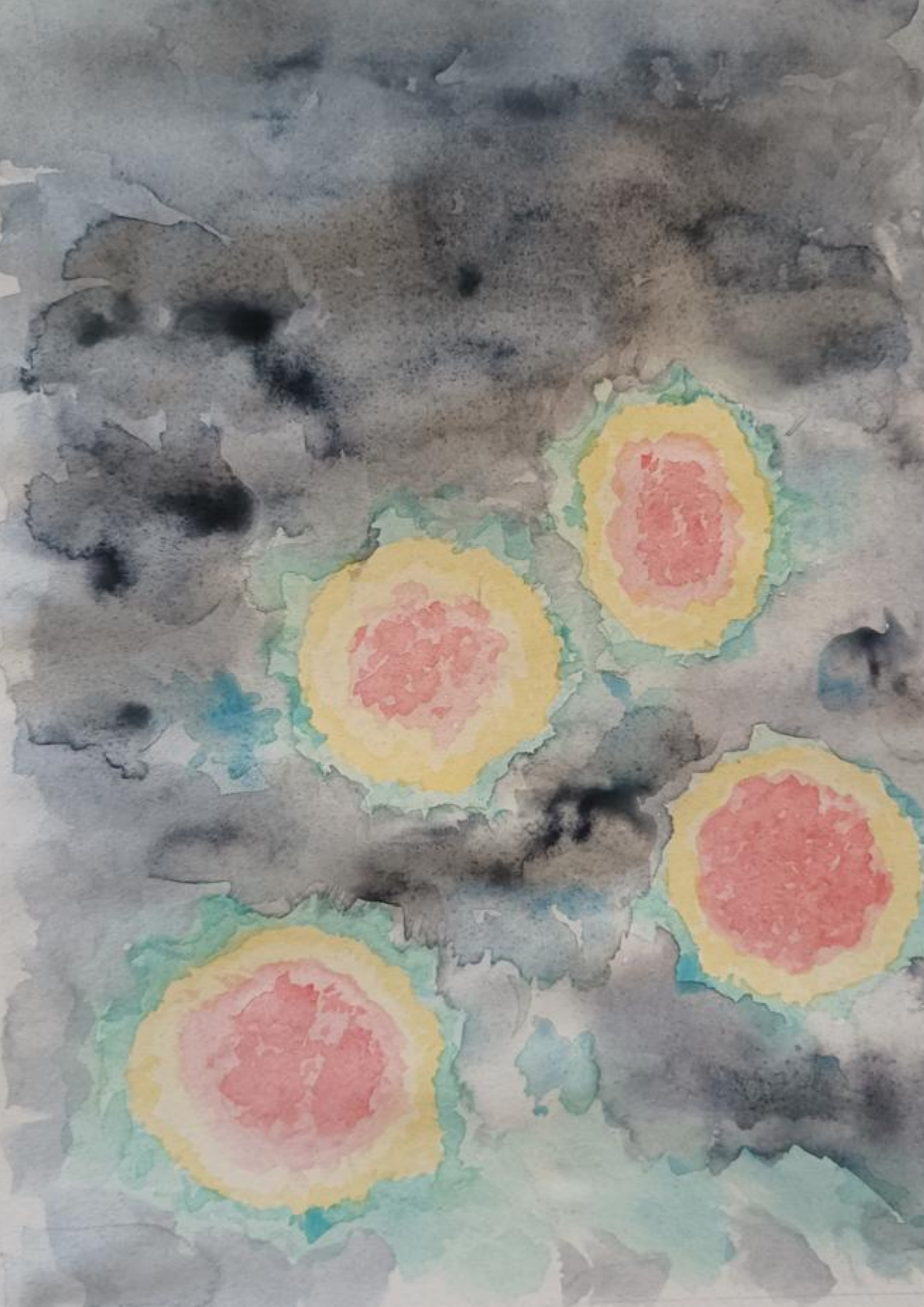

Ph.D. Thesis

Immunity in the SARS-CoV-2 infection

Helping in patients' clinical management

Thesis directors

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Wachupec 𝄞,

Antonia Gonzalez Navarro (i presumptament, Take That), des de 1995

El Guillem ya no es el que era,

Josefa Navarro Ruiz, després de portar-li la contrària, 2016

Acknowledgments

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Contents

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SUMMARY

Summary

The COVID-19 pandemic exposed important gaps in clinical and scientific preparedness for pandemics. Among them, the need to improve routine immunity assessment, based mainly on serology alone, which wanes over time and may not represent the whole picture of effectively fighting an infection, especially in those individuals with impaired humoral immunity. The thesis aims to assess the measurement of cellular immune responses against SARS-CoV-2 in different contexts to provide new tools and improve the evaluation of adaptive immunity for clinical decision-making in the post-pandemic situation.

Article 1 demonstrates the value of using the IFN-γ measurement against different SARS-CoV-2 antigens during acute disease, in convalescence, and post-vaccination. IFN-γ cellular responses were low during acute disease, consistent with early sampling and with lymphopenia and anergy found in severe manifestations. In contrast, convalescent individuals showed robust IFN-γ responses, particularly in those patients with previous severe disease, which could be attributed to greater antigenic exposure. Vaccinated individuals showed a lesser amount of response compared to those with a previous recorded infection. Spike triggered the highest immunogenicity among the three antigens tested. However, both Nucleocapsid and Membrane elicited significant IFN-γ cellular responses, making them important options to improve future vaccine designs. Finally, moderate correlations were obtained when the cellular assessment was compared with humoral responses, with discordances highlighting the importance of measuring cellular responses.

Article 2 extends the approach to a dual-cytokine evaluation (IFN-γ and IL-2), increasing the responder detection in acute, convalescent, and vaccinated groups, providing a more sensitive and functionally informative interpretation. IFN-γ predominated during acute infection, whereas IL-2 was increased after immunization or convalescence (memory response maintenance). The dual measurement captured individuals missed by the single-marker assays. In line with the previous study, severity impacted responses, being increased in survivors after severe infection, but blunted in fatal cases. When integrated with humoral data, moderate correlations were detected, with discordances between both evaluations.

Article 3 focuses on immunocompromised candidates for monoclonal antibody prophylaxis, for whom treatment is determined by their serology status. Cellular responses assessed by cytokine release (IFN-γ, IL-2, IL-21, and IL-5) against an antigen pool from the virus were studied in participants with both negative and positive serology against SARS-CoV-2. Serology underestimated the presence of adaptive responses to SARS-CoV-2 in these patients, with nearly 40% of those with negative serology

displaying cellular immune responses to at least one cytokine, demonstrating that the absence of humoral response does not imply the absence of cellular immunity. Despite that, cellular responses in patients with negative serology were less intense and less polyfunctional than in those with positive serology. Previous COVID-19 (hybrid immunity) was associated with stronger IFN-γ and IL-2 responses, whereas corticosteroid treatment promoted diminished Th1 responses, particularly in negative serology individuals. IL-5 was comparatively maintained in negative serology patients, suggesting a relative Th2 shift that may compromise effective antiviral control. IL-21, which reflects T and B cell cooperation, was also diminished in patients with negative serology. These findings provide insights into the prioritization of prophylaxis in immunocompromised patients, both assessing their functionality and the clinical factors affecting them.

Altogether, the studies reveal a consistent picture of SARS-CoV-2 immune response and its implications on infection management. Cellular responses are diminished during SARS-CoV-2 infection but consolidate afterwards during convalescence, where the disease severity and outcome play a crucial role. IFN-γ and IL-2 can capture cellular adaptive responses against the pathogen, with increased performance during the disease and after immunization, respectively. Responses to vaccination are detected but are lower when compared to natural immunization or hybrid immunity. Serology is informative on its own, but lacks a deeper assessment provided by cellular responses measurement, with functional analysis relying on expanded cytokine panels, and longer windows for detecting previous exposure and memory response assessment against reinfections. Clinical conditions such as previous exposure, underlying diseases, and immunosuppressive treatments significantly influence these responses, informing patient management and prophylaxis strategies. Despite some limitations, our studies provide a framework with the viability to be standardized, with rapid and accessible methods that, combined with serology, could provide full assessments of adaptive responses for clinical management of new SARS-CoV-2 variants or future emerging pathogens.

Resum

La COVID-19 va posar de manifest importants mancances en la preparació clínica i científica per afrontar noves pandèmies. Entre elles, destaca la necessitat de millorar l'avaluació rutinària de la immunitat, que actualment es basa principalment en la serologia, la qual disminueix amb el temps i pot no reflectir completament la capacitat de combatre eficaçment una infecció, especialment en individus amb immunitat humoral compromesa. Aquesta tesi té com a objectiu avaluar les respostes immunitàries cel·lulars davant del SARS-CoV-2 en diferents contexts, amb la fi de proporcionar noves eines i millorar l'avaluació de la immunitat adaptativa per a la presa de decisions clíniques en la situació post-pandèmica.

L'**Article 1** demostra el valor de l'ús de la mesura de l'IFN-γ davant diferents antígens del SARS-CoV-2 durant la fase aguda de la malaltia, en la convalescència i després de la vacunació. Les respostes cel·lulars d'IFN-γ van ser baixes durant la malaltia aguda, cosa que és coherent amb la recollida precoç de mostres i amb la limfopènia i anergia observades en les manifestacions greus. En canvi, els individus convalescents van mostrar respostes robustes d'IFN-γ, especialment aquells que havien sofert una malaltia greu, la qual cosa podria atribuir-se a una major exposició antigènica. Les persones vacunades van mostrar una resposta cel·lular menor en comparació amb aquelles amb infecció prèvia documentada. La proteïna Spike va desencadenar la major immunogenicitat entre els tres antígens analitzats (Spike, Nucleocàpsida i Membrana). No obstant, tant els antígens de la Nucleocàpsida com la Membrana van desencadenar respostes cel·lulars significatives d'IFN-γ, cosa que les converteix en opcions rellevants per millorar futurs dissenys vacunals. Finalment, es van obtenir correlacions moderades en comparar l'avaluació cel·lular amb les respostes humorals, destacant les discordances i la importància de mesurar també les respostes cel·lulars.

L'**Article 2** amplia l'enfocament cap a una avaluació dual de citoquines (IFN-γ i IL-2), cosa que augmenta la detecció de individus que responen davant d'antígens del SARS-CoV-2 en els grups amb patologia aguda, convalescents i vacunats, proporcionant una interpretació més sensible i funcionalment informativa. L'IFN-γ va predominar durant la infecció aguda, mentre que la IL-2 va augmentar després de la immunització o la convalescència (manteniment de la resposta de memòria). La mesura dual va permetre identificar individus que no van ser detectats amb els assaigs d'un sol marcador. En línia amb l'estudi anterior, la gravetat va afectar les respostes cel·lulars, observant-se un augment d'aquesta resposta en els casos amb bon pronòstic després d'infecció greu, però una resposta atenuada en els casos fatals. Al integrar-se amb les dades humorals, es van detectar correlacions moderades, amb discordances entre les dues avaluacions.

L'**Article 3** se centra en candidats immunocompromesos a profilaxi amb anticossos monoclonals, per a qui el tractament es determina segons el seu estat serològic. Es van estudiar respostes cel·lulars mitjançant la producció de citoquines (IFN-γ, IL-2, IL-21 i IL-5) davant d'un conjunt d'antígens del virus, en participants amb serologia tant negativa com positiva davant del SARS-CoV-2. La serologia va subestimar la presència de respostes adaptatives enfront al SARS-CoV-2 en aquests pacients, amb aproximadament el 40% dels individus amb serologia negativa mostrant respostes immunitàries cel·lulars a almenys una citoquina i demostrant que l'absència de resposta humoral no implica absència d'immunitat cel·lular. Malgrat això, les respostes cel·lulars en pacients amb serologia negativa van ser menys intenses i menys polifuncionals que en aquells amb serologia positiva. La infecció prèvia per COVID-19 (immunitat híbrida) s'associa amb respostes més intenses d'IFN-γ i IL-2, mentre que el tractament amb corticoides va promoure una disminució de les respostes Th1, especialment en individus amb serologia negativa. La IL-5 es va mantenir comparativament en els pacients amb serologia negativa, cosa que suggereix un viratge relatiu cap a una resposta Th2 que podria comprometre un control antiviral efectiu. La IL-21, que reflecteix la cooperació entre cèl·lules T i B, també es va reduir en els pacients amb serologia negativa. Aquestes troballes ofereixen pistes per a la priorització de la profilaxi en pacients immunocompromesos, avaluant tant la seva funcionalitat com els factors clínics que les afecten.

En conjunt, els estudis revelen una imatge coherent: les respostes cel·lulars es veuen disminuïdes durant la infecció per SARS-CoV-2, però es consoliden posteriorment durant la convalescència, sent la gravetat i el desenllaç de la malaltia factors crucials. L'IFN-γ i la IL-2 permeten detectar respostes immunitàries adaptatives cel·lulars davant del patogen, amb un rendiment incrementat durant la malaltia i després de la immunització, respectivament. Les respostes a la vacunació són detectables, però inferiors si es comparen amb la immunització natural o la immunitat híbrida. La serologia resulta informativa per si sola, però manca de l'avaluació més profunda que proporciona la mesura de les respostes cel·lulars, ja que l'anàlisi funcional dependent de panells ampliats de citoquines permet que hi hagi finestres temporals més llargues per detectar exposicions prèvies i també avaluar la memòria davant de reinfeccions. Les condicions clíniques com exposicions prèvies, malalties subjacents i tractaments immunosupressors influeixen significativament en aquestes respostes, orientant el maneig del pacient i les estratègies de profilaxi. Malgrat algunes limitacions, els nostres estudis ofereixen un marc amb viabilitat per ser estandarditzat, mitjançant mètodes ràpids i accessibles que, combinats amb la serologia, podrien proporcionar una avaluació completa de les respostes adaptatives per al maneig clínic de noves variants del SARS-CoV-2 o de futurs patògens emergents.

Resumen

El COVID-19 ha puesto de manifiesto importantes carencias en la preparación clínica y científica ante pandemias. Entre ellas, destaca la necesidad de mejorar la evaluación rutinaria de la inmunidad, que actualmente se basa principalmente en la serología, la cual disminuye con el tiempo y puede no reflejar completamente la capacidad de combatir eficazmente una infección, especialmente en individuos con inmunidad humoral comprometida. Esta tesis tiene como objetivo evaluar la medición de las respuestas inmunitarias celulares frente al SARS-CoV-2 en distintos contextos, con el fin de proporcionar nuevas herramientas y mejorar la evaluación de la inmunidad adaptativa para la toma de decisiones clínicas en la situación postpandémica.

El **Artículo 1** demuestra el valor del uso de la medición de IFN-γ frente a diferentes antígenos del SARS-CoV-2 durante la fase aguda de la enfermedad, en la convalecencia y tras la vacunación. Las respuestas celulares de IFN-γ fueron bajas durante la enfermedad aguda, lo cual es coherente con la recogida temprana de muestras y con la linfopenia y anergia observadas en las manifestaciones graves. En cambio, los individuos convalecientes mostraron respuestas robustas de IFN-γ, especialmente aquellos que habían sufrido una enfermedad grave, lo que podría atribuirse a una mayor exposición antigénica. Las personas vacunadas mostraron una respuesta celular menor en comparación con aquellas con infección previa documentada. El antígeno Spike desencadenó la mayor inmunogenicidad entre los tres antígenos analizados (Spike, Nucleocápside y Membrana). No obstante, los antígenos Nucleocápside y Membrana provocaron respuestas celulares significativas de IFN-γ, lo que los convierte en opciones relevantes para mejorar futuros diseños de vacunas. Por último, se obtuvieron correlaciones moderadas al comparar la respuesta celular con la humoral, destacando las discordancias y la importancia de medir también las respuestas celulares.

El **Artículo 2** amplía el enfoque hacia una evaluación dual de citoquinas (IFN-γ e IL-2), lo que incrementa la detección de respuesta contra el virus en los grupos de enfermedad aguda, convalecientes y vacunados, proporcionando una interpretación más sensible y funcionalmente informativa. IFN-γ predominó durante la infección aguda, mientras que IL-2 aumentó tras la inmunización o la convalecencia (mantenimiento de la respuesta de memoria). La medición dual permitió identificar individuos que no fueron detectados con los ensayos de un solo marcador. En línea con el estudio anterior, la gravedad afectó las respuestas celulares, observándose un aumento de esa respuesta en los pacientes con buen pronóstico tras infección grave, pero una respuesta atenuada en los casos fatales. Al integrarse con los datos humorales, se detectaron correlaciones moderadas, con discordancias entre ambas evaluaciones.

El **Artículo 3** se centra en inmunodeprimidos candidatos a profilaxis con anticuerpos monoclonales, para quienes el tratamiento se determina según su estado serológico. Se estudiaron sus respuestas celulares mediante la liberación de citoquinas (IFN-γ, IL-2, IL-21 e IL-5) frente a un *pool* de antígenos del virus, en participantes con serología tanto negativa como positiva frente al SARS-CoV-2. La serología subestimó la presencia de respuestas adaptativas al SARS-CoV-2 en estos pacientes, con cerca del 40% de los individuos con serología negativa mostrando respuestas inmunitarias celulares a al menos una citoquina y demostrando que la ausencia de respuesta humoral no implica ausencia de inmunidad celular. A pesar de ello, las respuestas celulares en pacientes con serología negativa fueron menos intensas y menos polifuncionales que en aquellos con serología positiva. La infección previa por COVID-19 (inmunidad híbrida) se asoció con respuestas más intensas de IFN-γ e IL-2, mientras que el tratamiento con corticoides promovió una disminución de las respuestas Th1, especialmente en individuos con serología negativa. IL-5 se mantuvo comparativamente al resto de citoquinas en los pacientes con serología negativa, lo que sugiere un cambio relativo hacia una respuesta Th2 que podría comprometer el efectivo control antiviral. IL-21, que refleja la cooperación entre células T y B, también se redujo en los pacientes con serología negativa. Estos hallazgos ofrecen claves para la priorización de la profilaxis en pacientes inmunodeprimidos, evaluando tanto su funcionalidad como los factores clínicos que la afectan.

En conjunto, los estudios revelan una imagen coherente: las respuestas celulares se ven disminuidas durante la infección por SARS-CoV-2, pero se consolidan posteriormente durante la convalecencia, siendo la gravedad y el desenlace de la enfermedad factores cruciales. IFN-γ e IL-2 permiten detectar respuestas inmunitarias adaptativas celulares frente al patógeno, con un rendimiento incrementado durante la enfermedad y tras la inmunización, respectivamente. Las respuestas a la vacunación son detectables, pero inferiores si se comparan con la inmunización natural o la inmunidad híbrida. La serología resulta informativa por sí sola, pero carece de la evaluación más profunda que proporciona la medición de las respuestas celulares, siendo el análisis funcional dependiente de paneles ampliados de citoquinas, y con mayores ventanas temporales para detectar exposiciones previas y evaluar la memoria frente a reinfecciones. Las condiciones clínicas como exposiciones previas, enfermedades subyacentes y tratamientos inmunosupresores influyen significativamente en estas respuestas, orientando el manejo del paciente y las estrategias de profilaxis. A pesar de algunas limitaciones, nuestros estudios ofrecen un marco con viabilidad para ser estandarizado, mediante métodos rápidos y accesibles que, combinados con la serología, podrían proporcionar una evaluación más completa de las respuestas adaptativas para el manejo clínico de nuevas variantes del SARS-CoV-2 o de futuros patógenos emergentes.

2

GENERAL INTRODUCTION

1. The COVID-19 pandemic: epidemiology, etiology, and clinical characteristics.

1.1. COVID-19 general emergency: global impact on public health and economy.

At the end of 2019, a cluster of atypical pneumonia cases were identified in Wuhan, Hubei Province, China[1]. These unexplained cases were rapidly linked to the city's Huanan Wet Seafood Wholesale Market, indicating a possible zoonotic transmission of pneumonia, presumed to originate from bats and pangolins acting as intermediate hosts[2]. Subsequent investigation confirmed the novel severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) as the causative agent, and the name was chosen for its genetic and phylogenetic similarities with SARS-CoV, responsible for the SARS epidemic in 2003. The disease associated with SARS-CoV-2 was designated as coronavirus disease-2019 (COVID-19)[3]. At the beginning of 2020, the majority of infected individuals had visited the Huanan Market, so direct contact with wild animals and their consumption was considered the initial source of the infection. The Chinese government closed animal wet markets due to their poor hygiene and to stop the spread of the pathogen. However, the high transmissibility of SARS-CoV-2, in combination with its ability to produce severe pneumonia as well as mild or asymptomatic infection, led to the rapid worldwide spread of the infection[4]. On March 11th 2020, the WHO officially declared COVID-19 a pandemic, highlighting the great impact of the disease on the global healthcare systems[5].

The pandemic has had an unprecedented impact on public health, with approximately 780 million cases reported, with more than 7 million people dying from COVID-19, as of May 2025[6]. At the beginning of the health emergency, Spain was among the most affected European countries. Until June 2023, the total number of cases was close to 14 million, with more than 120,000 deaths associated with the infection[7]. The severity of the disease varied widely, with clinical presentations spanning from asymptomatic infections to severe pneumonia and acute respiratory distress syndrome (ARDS)[8]. Moderate to critical cases required hospitalization, gravely saturating hospitals and intensive care units, leading to a shortage of beds, personnel, medical supplies, personal protective equipment, and other essential resources[9,10]. In response to the crisis, most countries implemented restrictive measures to palliate the consequences of the disease, including restrictions on mobility, compulsory confinement, and increased epidemiological surveillance. These measures shaped the economy for some years, severely debilitating global trade and mobility, and gravely affecting the regions relying most on it[11,12].

1.2. SARS-CoV-2 characteristics: etiology, transmission, and variants.

SARS-CoV-2 is a single-stranded, positive-sense RNA virus that belongs to the *Coronaviridae* family and the *Orthocoronavirinae* subfamily. The family is divided into four genres: Alpha, Beta, Gamma, and Delta. The first two have clinical relevance in humans, as they cause disease in mammals. Seven types of coronaviruses have been described to infect humans, four of them only causing mild upper respiratory tract disease. The remaining three have been responsible for two epidemics (MERS-CoV and SARS-CoV) and the COVID-19 pandemic (SARS-CoV-2)[4,13]. SARS-CoV-2 is an enveloped virus with a thirty-kilobase genome, making it one of the largest RNA viral genomes. It encodes sixteen non-structural proteins

involved in the virus's cycle, and four structural proteins: Spike (S), Nucleocapsid (N), Membrane (M), and Envelope (E). Spike is a trimeric glycoprotein disposed on the envelope, forming the peculiar crown-like ("corona") shape that gives the name to the family (Figure 1)[4,14]. The glycoprotein plays a pivotal role in the pathogenesis of the virus, binding specifically to the human angiotensin-converting enzyme 2 (ACE2) receptor, thus facilitating the entrance of the virus into human cells. It has two domains called S1 and S2. The S1 domain is responsible for binding to the target cells, while the S2 domain facilitates membrane fusion with the target cell. The receptor-binding domain (RBD) found in the S1 subunit is the most variable part of the virus, interacting directly with ACE2 to enter the host cells (Figure 1). This receptor plays a fundamental role in blood pressure control and is abundantly found in the intestine, lungs, kidneys, and heart, but is distributed throughout almost the whole body[15–17].

Respiratory transmission is the predominant route of SARS-CoV-2 infection. The virus is carried by either large droplets after sneezing or coughing, or aerosols generated by speaking, or even breathing[18]. While larger droplets tend to settle on surfaces within a short distance, aerosols remain sustained in the air for extended periods. Therefore, close contact with infected individuals combined with enclosed environments with poor ventilation heightens the risk of getting infected with SARS-CoV-2[19,20]. The virus can enter the body through contact with mucous tissue that can be in contact with droplets and aerosols, with the mouth, the nose, and the eyes mucosae being the main entry routes of the virus[18]. Other transmission pathways have also been described but are less relevant in the overall spread of the virus (fomites and faecal-oral)[4,21]. The efficiency of the transmission can be influenced by host, environmental, and viral factors. Viral load and variant, absence or presence of symptomatology, immune status, and comorbidities of the infected individual, combined with behavioural factors and environmental conditions, heavily impact the risk of transmitting the virus[21–23]. Mitigation strategies during the pandemic included wearing surgical masks, social distancing, hand hygiene, and adequate ventilation[24,25].

Since its emergence, SARS-CoV-2 has undergone a continuous accumulation of genome mutations due to its error-prone RNA polymerase and its high mutation rate, leading to the appearance of new variants[26]. The majority of the mutations, although also affecting nucleocapsid, are located in the spike glycoprotein, precisely in the RBD. Although some mutations do not confer any advantage to the virus, others enhance its fitness in human hosts. These mutations have led to changes regarding transmissibility, immune evasion, and the severity of the infection[26,27]. Through close epidemiologic surveillance, the variants were phylogenetically connected and organized under lineages, and then classified according to their potential public health implications by the WHO as variants of concern (VOCs), variants of interest (VOIs), and variants under monitoring (VUMs)[28,29]. The key VOCs during the pandemic have been, in chronological order, Alpha, Beta, Gamma, Delta, and Omicron. Alpha had increased transmissibility, Beta and Gamma had mutations contributing to immune evasion, and Delta had both characteristics enhanced[30,31]. The most significant accumulation of mutations has been seen in the Omicron variant, highly increasing immune escape (even after vaccination and natural infection), and enhancing ACE2 binding, making it highly transmissible. There is still a need for continued genomic surveillance as a great number of sublineages are still appearing[26,32].

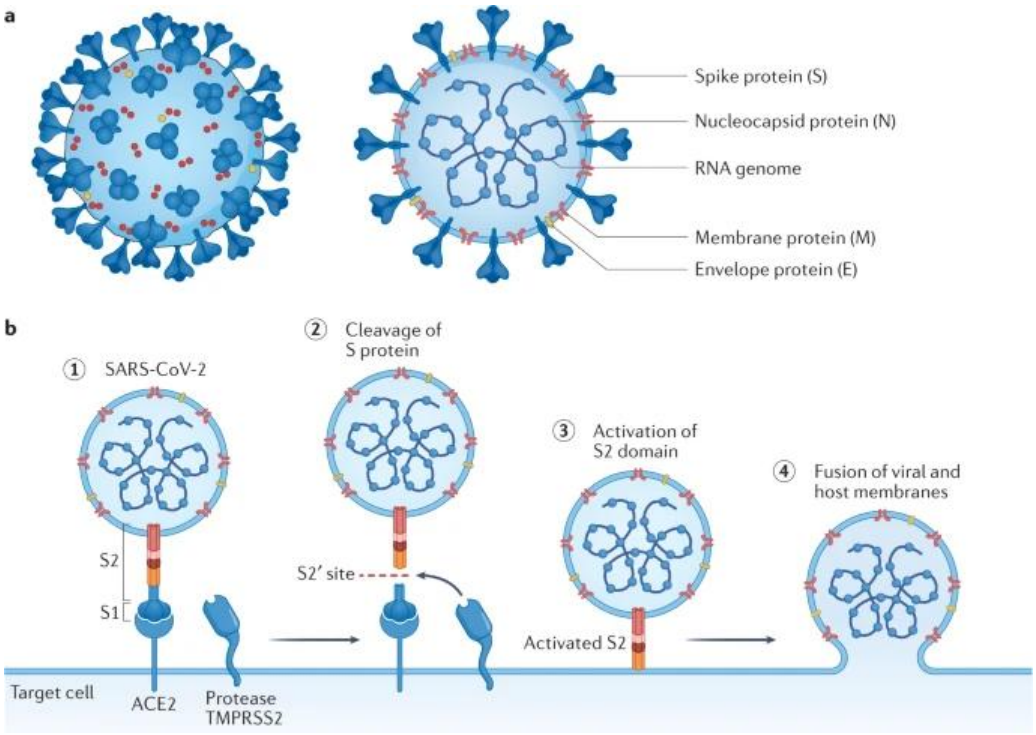

Figure 1. Molecular architecture and cellular pathogenesis of SARS-CoV-2. a) SARS-CoV-2 consists of the following structural proteins: spike protein (S), nucleocapsid protein (N), membrane protein (M), and envelope protein (E). b) The S protein attaches to the receptor ACE2 on the host cell using the S1 domain. This allows the cleavage of the S protein, leading to activation of the S2 domain for fusion. Activated S2 fuses viral and host lipid bilayers, leading to deposition of the viral positive-sense, single-stranded RNA genome into the host cell. Reprinted from Lamers, M.M., Haagmans, B.L. SARS-CoV-2 pathogenesis. Nat Rev Microbiol 20, 270–284 (2022), Copyright © 2022, reproduced with permission from Springer Nature.

1.3. Clinical characteristics and symptoms of SARS-CoV-2 infection.

SARS-CoV-2 infection is the causative agent of COVID-19. The spectrum of the disease manifestations ranges from asymptomatic infection to severe pneumonia with ARDS, the latter being particularly prevalent in vulnerable populations. The severity of the disease is influenced by several factors, including age, sex, underlying comorbidities, viral load, and immune response[33].

The incubation period of the virus takes place between 2 and 14 days after exposure, after which infected individuals may or may not exhibit symptomatology, but are already capable of transmitting the infection[34,35]. Forty percent of symptomatic individuals show mild respiratory disease, displaying a variety of symptoms including dry cough, fever, and cold-like symptoms such as congestion, runny nose, or sore throat[36,37]. These individuals may also experience head and muscle aches, myalgia, fatigue, loss of smell and taste, and diarrhea[38]. The majority of these individuals recover within 10 to 14 days without complications. In contrast, moderate infection (40% of the symptomatic individuals) additionally manifests with shortness of breath, requiring hospitalization with or without oxygen therapy[36,39].

Severe COVID-19 is characterized by significant hypoxia and respiratory distress, requiring at least high-flow ventilation. Apart from lung damage, this form of the disease can be associated with systemic inflammation, multiorgan involvement such as kidney or myocardial damage, and even neurological complications. The severe manifestations of the disease involve ARDS, septic shock, and multiple organ

dysfunction, requiring the patient's non-invasive ventilation (NIV) or mechanical ventilation[36,39]. Case fatality rates varied from 0.7% to more than 4%, depending on the variant and the geographical location, and have decreased thanks to the global vaccination strategy[40].

1.4. SARS-CoV-2 diagnostic methods.

Given that SARS-CoV-2 transmission occurs from both asymptomatic and symptomatic individuals, primarily through respiratory droplets, tracing infected individuals during the pandemic was crucial to control its spread[41,42]. Different diagnostic techniques were used for early detection and containment, accurate diagnosis, and monitoring of close contacts to prevent the spread of the pathogen[43].

At the onset of the pandemic, the confirmation of SARS-CoV-2 infection was crucial for patient management and for performing epidemiological studies to understand the extent of the transmission. Nucleic acid amplification tests (NAATs) have been employed as the reference diagnosis of COVID-19 for their high sensitivity and specificity. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) has been the most used method for detecting the virus and is regarded as the gold standard by the WHO[44,45]. While the RT-qPCR method itself can optimally detect SARS-CoV-2 and inform the patient within 4 to 6 hours, the overall turnaround time for results is typically 12 to 24 hours, and in remote areas, it can be delayed up to 3 days due to sample transportation and processing constraints, as specialized laboratory equipment and trained personnel are required[43,46]. Several samples are adequate for RT-qPCR, including saliva, sputum, oropharyngeal swab, and nasopharyngeal swab, which is considered the gold standard[47,48]. The limit of detection of the technique is 10^2-10^3 copies/mL, much lower than the threshold of copies considered infectious (10^5 to 10^6), and it detects the infection even before the onset of symptoms (Figure 2)[43].

To address the need for faster, more accessible, and cost-effective diagnostic tests, rapid antigen tests (RATs) were developed as an alternative to RT-qPCR. The test is based on the use of specific capture antibodies in a lateral flow format to detect SARS-CoV-2 antigens. The potential for self-testing with single-use tests, eliminating the need for expensive equipment, along with its lower costs and the rapid results (15-20 minutes), made RATs a key alternative for case detection, therefore avoiding a delay in implementing measures to interrupt disease transmission[4,43]. The limit of detection of RATs is 10^5 to 10^6 viral copies per milliliter, significantly higher than RT-qPCR (Figure 2). Although RATs have a higher limit of detection compared to RT-qPCR, their ability to identify individuals with viral loads exceeding 10^6 copies per milliliter makes them particularly useful for detecting those most likely to transmit the virus. However, some false negative results can be obtained in incubation periods when viral loads are lower to the limit of detection[43,49].

In addition to RT-qPCR and RATs, other techniques such as ELISA for viral antigen detection and alternative NAATs were used at different stages of the pandemic. Loop-mediated isothermal amplification (LAMP) tests were also produced during the pandemic. However, the technique was disregarded in favor of RT-qPCR and TARs due to the difficulty in designing sets of robust primers to detect the virus, combined with the good performance and implementation of RT-qPCR[50].

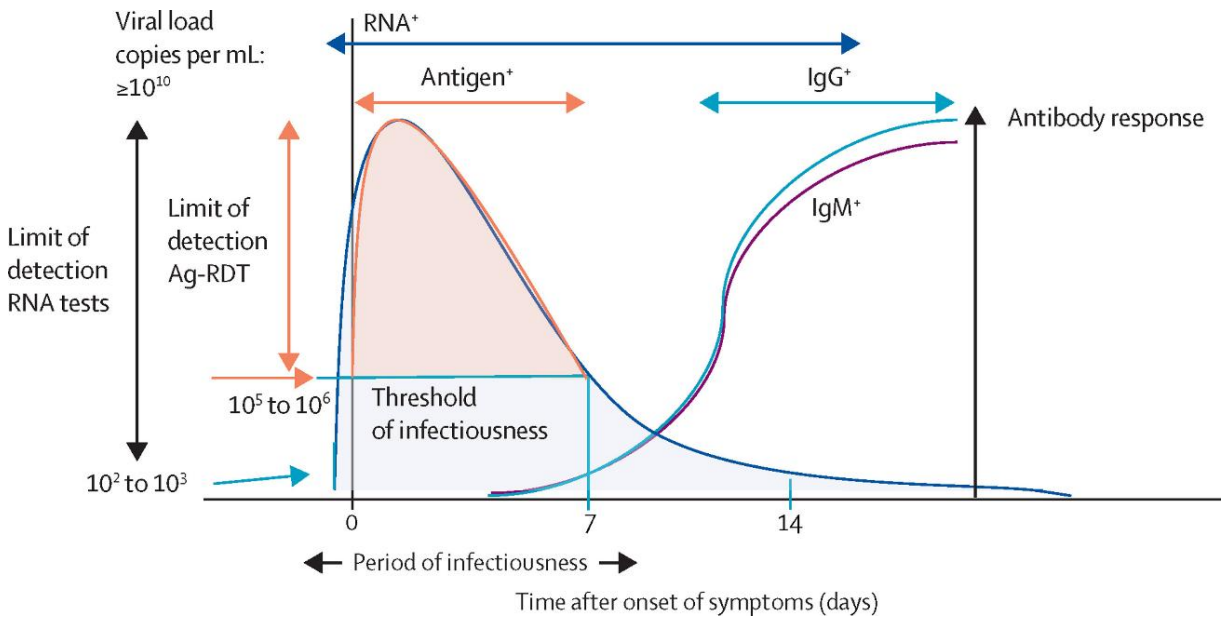

Figure 2. Optimal timelines for the different diagnostic techniques for SARS-CoV-2 detection. Schematic of the viral dynamics of, and antibody response to, SARS-CoV-2 infection in a symptomatic patient, and the optimal timeframe for deployment of different types of tests. Reprinted from Peeling, RW. et al., Diagnostics for COVID-19: moving from pandemic response to control, Lancet, Vol. 399, 757-768, Copyright © 2021, with permission from Elsevier.

Complementary techniques like genomic sequencing have been crucial in monitoring SARS-CoV-2 evolution and variant surveillance[29]. Finally, antibody serology testing was used at the beginning of the pandemic as an infection diagnostic, but has been repurposed to support clinical patient management. This test can provide information about previous exposure and humoral response triggered by vaccination, which can help guide vaccination and pre-exposure prophylaxis, but also has provided epidemiological information about serology levels at a population level[4,51].

1.5. Treatment approaches against COVID-19.

Since the emergence of the pandemic, there has been a significant evolution in the treatment landscape against SARS-CoV-2. Initial treatment approaches were empirical, with primary focus on supporting care measures such as ventilation therapies[52,53]. The absence of therapies targeting the virus resulted in an urgent need for clinical research to find or repurpose new medications to face the infection. According to the National Institutes of Health (NIH), the pathogenesis of COVID-19 is driven by the viral replication at the beginning of the infection and the immune response dysregulation against SARS-CoV-2[33,54]. Antiviral agents and immunomodulators have played a pivotal role in treating COVID-19, in the early and late phases of the disease, respectively. Most strategies were derived from previous treatments against other coronaviruses (MERS and SARS)[4].

Antiviral agents directly target the SARS-CoV-2 virus to inhibit its replication. Their efficacy is often heightened at the beginning of the infection, suppressing the viral replication before reaching uncontrollable viral loads. This prevents hyperinflammatory responses associated with severe manifestations of COVID-19, thus leading to improved clinical outcomes[55,56]. Remdesivir is an adenosine

analogue that inhibits the viral RNA polymerase, thereby preventing the virus from replicating. It is a repurposed medication originally developed for the Ebola virus, administered intravenously with full FDA approval for hospitalized COVID-19 patients. Moreover, Molnupiravir (ribonucleoside analog) and Paxlovid (SARS-CoV-2 protease inhibitors) offered an effective outpatient option for treating mild and moderate COVID-19 high-risk individuals[57,58].

Immunomodulators have played a crucial role in managing severe COVID-19, helping to modulate the immune response to prevent hyperinflammation. During 2020, corticoids such as dexamethasone were employed to treat severe manifestations of the disease, as it was demonstrated that they could reduce mortality up to one third in patients receiving supplemental oxygen or mechanical ventilation[59]. Furthermore, the administration of Interleukin 6 inhibitors (Tocilizumab) and Janus Kinase (JAK) inhibitors (Baricitinib) has been used as directed therapies, aiming to inhibit exacerbated immune reactions to SARS-CoV-2 infection. Tocilizumab binds to the IL-6 receptor, blocking the interaction with IL-6 and preventing its proinflammatory effects, leading to a reduction in inflammatory reactions[60]. Baricitinib inhibits the kinase activity of JAK proteins, inhibiting the JAK-STAT signaling pathway, thereby disrupting pathogenic immune responses[61]. Several other therapies related to immunity, such as type I IFNs, IL-1 and TNF inhibitors, monoclonal antibodies, and convalescent plasma, were used in different contexts and had different effectiveness[62,63].

1.6. Global vaccination strategy: pursuing herd immunity and protecting at-risk populations.

In response to the rapid propagation of the disease, research groups and pharmaceutical companies started the development of vaccines using established technologies, such as inactivated and attenuated pathogen vaccines. However, the development of mRNA vaccines signified a key milestone in the fight against the infection, to prevent further transmission and protect vulnerable populations[64,65]. These types of vaccines were developed remarkably in less than a year following the onset of the pandemic. The rapid approval of various mRNA vaccines by regulatory authorities such as the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) was facilitated by great research efforts to enhance mRNA quality and delivery methods, and to reduce immunogenicity. All research resulted in great safety, efficacy, and scalability of production of these vaccines[64,66]. Pfizer-BioNTech (BNT162B2) and Moderna (Spikevax) are both based on the use of mRNA from the virus's Spike protein, and have been the most widely used with this technology. However, other vaccine types have been used, such as viral vector vaccines (Oxford-AstraZeneca, Janssen), inactivated virus vaccines (CoronaVac), and protein subunit vaccines (Novavax), which also had great importance in protection against infection around the world[67].

As of August 2024, 70.7% of the global population has received at least one dose of the COVID-19 vaccine, with 92% of those complying with the initial vaccination protocol[68]. The initial vaccination protocol in Spain consisted of four authorized vaccines: three required two separate doses (Pfizer-BioNTech (3 weeks), Moderna, and AstraZeneca (4 weeks)), while Janssen only required one. Initially, priority was given to high-risk groups, but as dose availability increased, the protocol was expanded to cover the entire population[69]. The global vaccination campaign has achieved significant milestones,

administering a total of almost 14 billion vaccine doses. However, dramatic disparities between high-income and low-income countries have been produced (Figure 3). While the first achieved close to 80% of coverage, in low-income countries, only around 33% of people received at least one dose of the prophylaxis, relying on vaccine donations from developed countries[70]. The inequity has resulted in preventable deaths and illnesses, and has made countries vulnerable to future infection waves and the appearance of new virus variants[71].

In almost all studies, vaccines have between 80 and 90% efficacy against symptomatic and asymptomatic infections. mRNA vaccines are highly effective against infection and provide a high level of protection against severe disease, hospitalization, and death (around 95%). This percentage is reduced for the rest of the vaccine designs[72]. Although some adverse events have been reported, most of them are mild and of short duration, while severe events are very improbable, and most of them are related to comorbidities[73]. Overall, the impact of the vaccine has proven to be beneficial in several aspects. These include the reduction of severe cases and hospitalization (thus avoiding millions of deaths), the protection of vulnerable individuals, and the alleviation of the healthcare system's burden. All these benefits contributed to the lifting of restrictions[74–76].

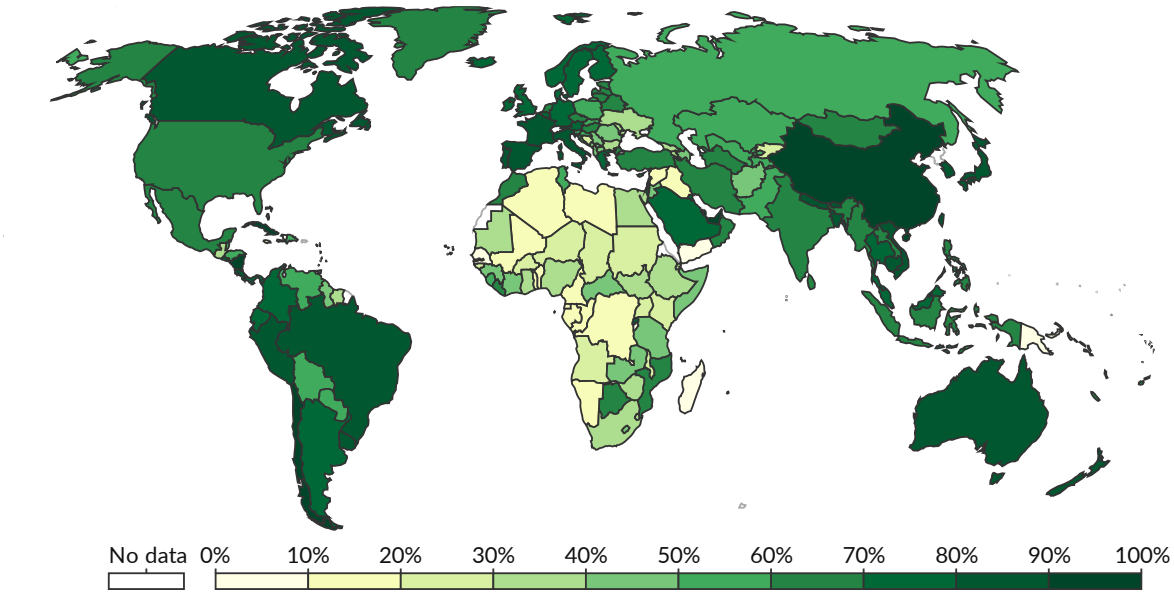

Figure 3. Share of people who completed the initial COVID-19 vaccination protocol, Aug 12, 2024. Total number of people who received all doses prescribed by the initial vaccination protocol, divided by the total population of the country. Published online at Our World in Data – Last updated 12 August 2024. Retrieved from ourworldindata.org/covid-vaccinations | CC-BY.

2. Immune response against SARS-CoV-2.

2.1. Innate immune response: the first barrier.

SARS-CoV-2 initially targets the respiratory epithelium, initiating the infection in the upper airways, but potentially spreading to the lower respiratory tract. The virus enters the cells through the ACE2 receptor, which is abundantly expressed in type II alveolar epithelial cells, but is also found in other cell types[77]. Viral replication induces the death of cells located in the airways and tissue damage. As a result of that, danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) are released, which are recognized by innate immune sensors[78].

The innate immune response is the first line of defense against SARS-CoV-2, acting rapidly to prevent an exacerbated viral replication and to initiate downstream immune responses. Dendritic cells, macrophages, neutrophils, epithelial cells, and fibroblasts express different pattern recognition receptors (PRRs) that detect viral components when the virus enters the respiratory tract. This interaction triggers intracellular signaling cascades with adaptor proteins, leading to the activation of transcription factors. These factors are responsible for the production of type I and III interferons (IFNs), locally inducing an antiviral state in infected epithelial cells. This would ideally limit viral replication without provoking excessive inflammation that may damage lungs[79,80].

However, SARS-CoV-2 has developed different strategies to evade innate immunity. Viral proteins such as NSP1, NSP3, ORF6, and ORF9b can inhibit production and signaling through interferons, allowing the virus to replicate inside respiratory tract cells[81]. Different pro-inflammatory cytokines such as IL-1β, IL-6, IL-18, and TNF are also produced to fight the infection, and different cell types such as neutrophils, monocytes, natural killer (NK) cells, and dendritic cells are recruited to the site through chemokine signaling[82]. These cells allow the clearance of infected cells through apoptosis induction, neutrophil extracellular traps (NETs), and phagocytosis, and the amplification of the inflammatory response. Additionally, the complement system is activated[80,83]. Both early dysregulated type I interferon responses and late IFN hyper-responsiveness are correlated with higher rates of severe disease and mortality, coinciding with exacerbated inflammation in severe COVID-19 patients, and in the disruption of the production of balanced adaptive immune responses.

2.2. Adaptive immune response: fighting the infection.

The innate immune response initiates the antiviral response through three mechanisms: restricting viral replication, creating an antiviral state, and priming the adaptive immune response. The first two tasks focus on limiting the span of the infection, while the third is crucial to trigger the development of the adaptive response, with antigen-presenting cells (APCs), particularly dendritic cells, processing and presenting SARS-CoV-2 peptides to lymphocytes in the lymph nodes[84,85]. Adaptive immune responses require between 6 and 10 days to achieve an extensive proliferation and differentiation of naïve cells into SARS-CoV-2-specific effector populations. Although some days are required, coordinated responses

are generated involving virus-specific $CD4^+$ and $CD8^+$ T-cells, and antibodies, which will control viral replication and will shape long-term responses[84,86].

$CD4^+$ T-cells play a pivotal role in coordinating the adaptive immune response, and are usually detected between 2 and 4 days after the infection onset. Their frequency is generally higher than that of $CD8^+$ T-cells, particularly in the early stage of the infection. These cells differentiate predominantly into Th1 and circulating T follicular helper (cTfh) cells[84,87]. Th1 cells secrete antiviral cytokines such as IFN-γ, IL-2, and TNF to mediate antiviral activity, and chemokines such as CCL3, CCL4, and CXCL1 for immune cell recruitment and inflammation. Instead, cTfh cells are involved in supporting B cell maturation and the production of neutralizing antibodies, but also help in the development of memory B-cells and long-term humoral immunity through the secretion of cytokines like IL-21. Effective $CD4^+$ T-cells are related to mild and moderate manifestations of the disease, and are correlated to viral clearance and local tissue repair[88]. However, severe COVID-19 is characterized by dysregulated $CD4^+$ T-cells with atypical differentiation to Th2 and Th17 phenotypes, an activation phenotype including the expression of $CD38^+$, $CD95^+$, $HLA\text{-}DR^+$, $Ki67^+$, and PD-1, and compromised effector functionalities central to pathogenesis[89,90]. This dysregulation is accompanied by elevated expression of exhaustion markers and increased apoptosis, leading to an increase in IL-6 and IL-10 levels, all contributing to lymphopenia and impairment of the cellular immune response[89,91]. Regulatory T-cells (Tregs) can mitigate lung and systemic damage by limiting hyperinflammatory responses, and their dysfunction could lead to more severe clinical manifestations. Therefore, Tregs are vital in a balanced adaptive immune response[92].

$CD8^+$ T cells are critical to eliminate SARS-CoV-2-infected cells through the production of granzyme B and perforin, while also being great IFN-γ producers. Effective $CD8^+$ T-cell responses, characterized by having a great cytotoxic activity and polyfunctionality, are correlated with milder symptoms and more favorable outcomes. However, in severe cases, even though they may exhibit an activation phenotype (CD38, HLA-DR, CD69, and CD25), they are in decreased numbers, and exhibit inhibitory receptors and reduced cytotoxic activity, indicating a dysfunctional state. This dysfunction can lead to worse outcomes due to inefficient viral clearance[84,88].

B-cells play a central role in the immune response to SARS-CoV-2 by producing antibodies targeting the virus and resulting in its clearance. Following antigen recognition and $CD4^+$ T helper cell support, B-cells become activated and start proliferating, differentiating into short-lived plasmablasts and long-lived plasma cells, producing specific antibodies against SARS-CoV-2[93]. B-cells begin the production of different antibody isotypes, mainly IgA, IgM, and IgG. This production starts almost simultaneously, different from other infections where IgM is primarily produced[94]. Antibodies mainly target the spike protein (especially the RBD), although other epitopes are targeted, and prevent the virus from entering cells through neutralization. However, some B-cells undergo an extrafollicular pathway and differentiate into antibody-secreting cells with low affinity to the virus, providing rapid protection against the pathogen[95]. Although high antibody titers can help reduce infection, they are not correlated with mild infection, as during severe illness, patients develop stronger antibody responses[96]. Some patients with mild infection may eliminate the virus before developing high antibody titers. B-cells not only produce

neutralizing antibodies but also secrete pro- and anti-inflammatory cytokines, and help in the antigen presentation, having also a role in T-cell activation and in the shaping of the adaptive immune response[97].

2.3. Immunopathogenesis.

SARS-CoV-2 induces a significant dysregulation of the immune system, particularly in severe and critical COVID-19 cases. In those cases, the balance between a protective immune system and damaging inflammatory responses is altered. One of the most important alterations is lymphopenia, marked by a significant reduction in NK cells and $CD8^+$ T-cells, but also in $CD4^+$ T-cells and sometimes in B-cells[98,99]. This can serve as an early indicator of disease severity, dropping lymphocyte numbers below 20%, impairing patients' antiviral defenses, and leaving them susceptible to secondary infections[100]. The different hypotheses for lymphocyte depletion are the following: the virus enters lymphocytes through ACE2 and kills them, increased proinflammatory cytokine levels promote T-cell exhaustion and depletion, lymphatic organs are targeted by the virus and are destroyed, and increased lactic acid production may inhibit lymphocyte proliferation[98,100] (Figure 4).

Although the absolute number of T-cells drops dramatically, the remaining cells are usually activated, showing upregulation of markers such as CD69, CD38, and CD44, and high expression of OX40 and 4-1BB, promoting clonal expansion and priming immune responses[101]. The expression of these markers is associated with a vigorous response to the viral infection[102]. However, T-cells also show exhaustion markers, as they become rapidly overstimulated, losing functional capacity or "burning out", contributing to disease progression[99,103]. Apart from lymphocytes, granulocyte and monocyte cell populations are also altered. Neutrophil numbers increase significantly, being recruited to the infection site and increasing NET release, promoted by the dysfunctional control of the infection[104]. On the other side, eosinophil, basophil, and monocyte numbers decrease, gravely impacting the immune homeostasis[105–107] (Figure 4).

Another factor inducing pathogenesis is the cytokine storm, which is characterized by extremely high proinflammatory cytokine levels leading to lung injury, shock, and multiorgan damage, including heart, liver, and kidney[108]. Th1 and Th17 cells are usually the responsible to recruit other immune cells, promoting the production of proinflammatory cytokines that participate in the cytokine storm: IL-1β, IL-2, IL-6, IL-7, IL-8, IL-10, G-CSF, GM-CSF, IP10, MCP1, MIP-1α, IFN-γ, and TNF; but IL-1β, IL-6, and IL-10 have been seen to be the most elevated ones[98,108–110]. In non-severe patients, altered levels of these cytokines have been seen but not so abruptly (Figure 4).

The hyperactivation seen in B-cells leads to increased antibody production, increasing IgGs and total antibody levels. In some individuals, this exacerbated antibody response is correlated with severity. These antibodies can form immune complexes that bind to Fc receptors on myeloid cells, triggering the production of proinflammatory cytokines and therefore increasing tissue injury[98,111]. In critical patients, IgGs against spike usually show aberrant patterns of glycosylation, increasing affinity to Fc and worsening the pathogenesis through cytokine storms, and complement and platelet activation[98,103]. In

addition, autoantibodies against type I IFN have been detected in some severe patients. These antibodies severely compromise the initial antiviral defenses, which allow increased viral replication and inflammatory damage[103,112] (Figure 4).

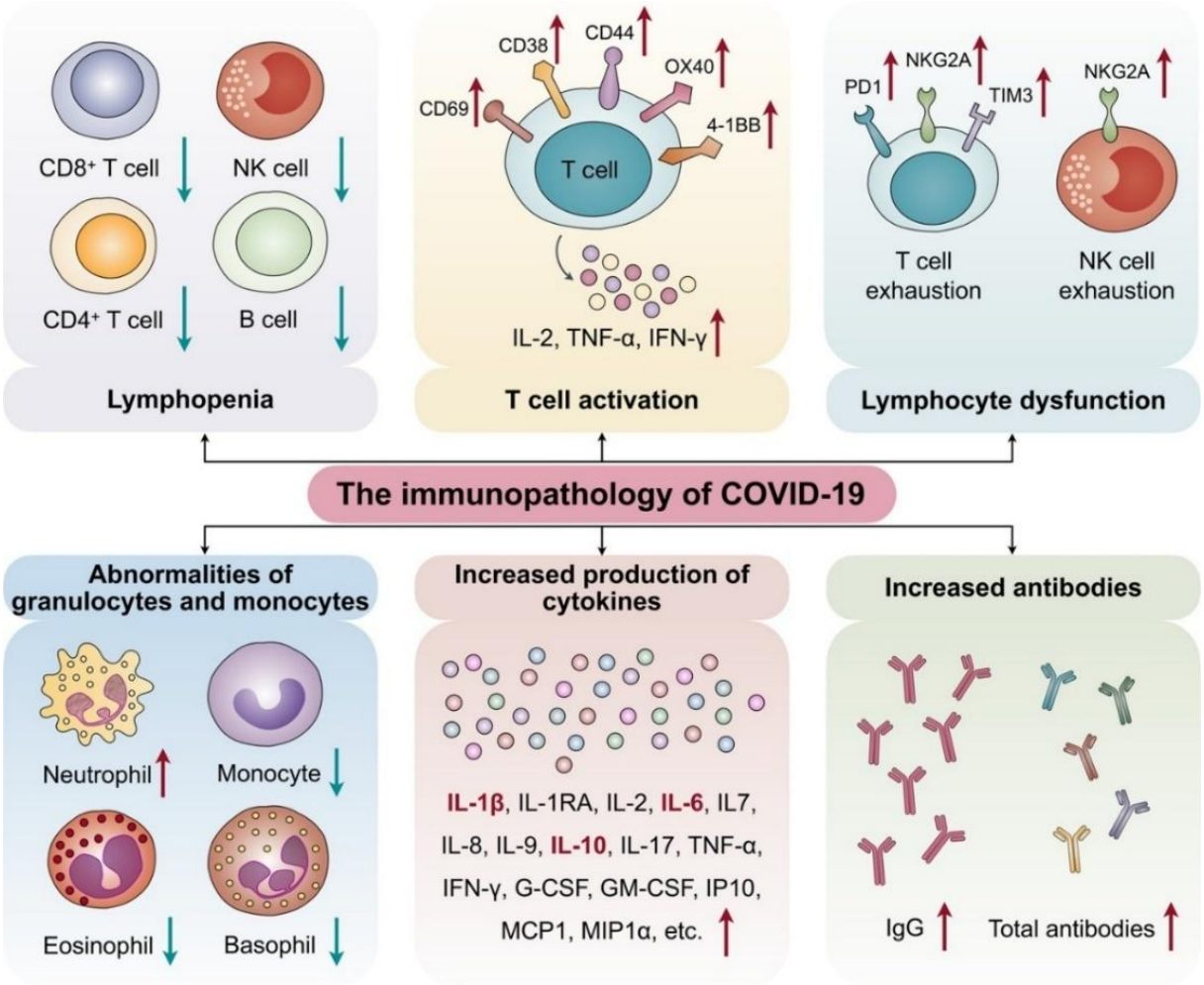

Figure 4. COVID-19 immunopathology. The immunity manifestations of COVID-19 contain different facets: lymphopenia, the activation and dysfunction of lymphocytes, abnormalities of granulocytes and monocytes, an increased production of cytokines, and increased antibodies. Reprinted from Yang, L. et al. The signal pathways and treatment of cytokine storm in COVID-19. Sig Transduct Target Ther 5, (2020). Copyright © 2020, Springer Nature | CC-BY

3. Factors affecting immunity against COVID-19.

3.1. Protection against reinfection: the impact of previous infections and vaccination.

After fighting SARS-CoV-2 infection and/or after vaccination, the human immune system is capable of mounting a memory adaptive immune response with memory $CD4^+$ and $CD8^+$ T-cells, memory B-cells, and through antibody production. The combination of the different immune components is fundamental for protection and long-term memory against the pathogen[113]. $CD4^+$ T-cells play a fundamental role in the direct control of viral infections and in supporting humoral responses. After the infection, 90% of the individuals show stable $CD4^+$ T-cell memory subsets for at least 6 to 8 months[114]. These cells differentiate predominantly into Th1, Tfh, and cytotoxic $CD4^+$ T-cells, the last secreting IFN-γ, TNF, CD40L, and granzyme B[115,116]. Memory $CD4^+$ T-cells exhibit effector and central memory phenotypes, and are detected in the blood, bone marrow, spleen, lymph nodes, and lungs[117]. Asymptomatic individuals show reduced memory T-cell numbers, but the relation of the quality of the memory T-cell responses with severity is still under discussion[87,113]. Vaccination induces a robust $CD4^+$ T-cell memory in nearly all individuals after two mRNA vaccine doses, with $CD4^+$ memory T-cells at equivalent frequencies to naturally infected individuals, showing a similar duration of response after infection of more than one year[118,119]. They mainly show polyfunctional Th1 memory phenotypes and Tfh cells maintaining germinal centers and promoting the production of neutralizing antibodies[120]. cTfh abundance has been seen to be correlated with neutralizing antibodies[121]. While only modest differences have been detected regarding circulating $CD4^+$ memory T-cells in vaccinated individuals compared with previously infected individuals (asymptomatic or symptomatic), increased response in hybrid immunity has been detected[113,122].

Early and strong $CD8^+$ T-cell responses during infection are correlated with milder outcomes during acute infection, showing their protective role. $CD8^+$ T-cell memory is observed in 70% of individuals one month after the infection, and in 50% after 8 months[114,115]. Phenotypically, these cells exhibit effector and terminally differentiated effector markers, which retain functional capacities and produce IFN-γ and granzyme B[123]. In addition, tissue-resident memory $CD8^+$ T-cells are also detected in the lung, and are critical for local protection[117,124]. Vaccination with mRNA vaccine design induces spike-specific $CD8^+$ T-cells in more than 70% of individuals after immunization, but it varies among the different vaccine platforms[120]. Memory cells are found in around 40 to 65% of individuals for 6 months, and have a functional effector memory phenotype, persistently secreting IFN-γ[125]. The magnitude of $CD8^+$ responses elicited by vaccination is lower compared to $CD4^+$ responses[116]. Similar to $CD4^+$ T-cells, only modest differences have been detected in circulating $CD8^+$ memory T-cells with hybrid immunity[118].

B-cell memory is an essential component of long-term immunity, capable of providing a rapid anamnestic antibody response upon reinfection with the virus. After SARS-CoV-2 infection, memory B-cells against spike and RBD develop within 2 weeks and increase in frequency for several months until 4 to 6 months, reaching a plateau[115,126]. These cells show a predominantly IgG-producing phenotype, with some producing IgA, but with a rapidly waning IgM-producing subset. Apart from blood, they are found

in lymph nodes, bone marrow, and the lungs, rapidly responding upon reinfection[117]. Severe COVID-19 has been associated with high memory B-cell frequencies. Vaccination induces similar memory B-cell frequencies to natural infection, with specific B-cells increasing between 3 and 6 months after infection. However, affinity maturation has been seen to be lower as the short interval between doses could reduce the memory B-cell quality[120,127]. In the case of memory B-cells, hybrid immunity has been seen to enhance substantially its frequency and quality[118].

Finally, neutralizing antibodies are fundamental for preventing infection and are generated by plasmablasts and plasma cells. After infection, most individuals seroconvert, and neutralizing antibody titers are detectable in 80 to 90% of infected individuals one year post-infection, and although they decay, they are maintained for a longer time[113,115]. Antibody titers have been seen to be lower in asymptomatic individuals. However, durable antibody responses have been detected in the majority of infected individuals[128]. Two mRNA dose vaccination induces high neutralizing antibody levels, but a rapid decay occurs after 6 to 8 months. This decay is steeper than that seen after infection. However, a third dose has been seen to improve the strength and the durability of neutralizing antibodies[129,130]. On the other hand, hybrid immunity confers the most robust and broad humoral protection, with one single vaccine dose combined with previous infection inducing substantially elevated antibody titers. This is mainly due to memory B-cells encoding antibodies with great potency and breadth after infection being recalled after vaccination[131,132]. The opposite order (first vaccination and after infection) also produces the same effect[131]. Mucosal immunity mirrors antibody kinetics, also being enhanced in hybrid immunity[133].

3.2. Individual variability: risk of being infected and worse outcomes.

The heterogeneity in the population makes some individuals more susceptible to infection and to have severe manifestations of the disease than others. Several risk factors and comorbidities are closely related to SARS-CoV-2 infection and worse COVID-19 outcomes.

Regarding demographic risk factors, elderly individuals have increased vulnerability to SARS-CoV-2 infection, mainly due to decreased immune system effectiveness and higher prevalence of comorbidities. People aged 70 and older have a 65% higher risk for COVID-19 compared to younger individuals, and 5.1 times higher probabilities of dying from COVID-19 in patients over 59 years old[134,135]. Aging reduces the ability to combat infections through immunosenescence, with baseline chronic proinflammatory status and low innate immune responses contributing to the disease, combined with different comorbidities.

Sex has also been seen to influence the predisposition and worse outcomes of COVID-19. According to a meta-analysis, men had an 8% higher risk of being infected compared to women, and an 18% higher risk of suffering a severe disease. In addition, an increased risk of death was detected[134,135]. Other meta-analyses found increased risk for ICU admission and death, but no association with increased risk of infection in men[136]. Several factors could be contributing to the sex disparity. Hormone production can influence immune responses, as estrogen in women enhances both innate and adaptive responses,

whilst low testosterone levels when aging may contribute to increased severity of the infection. Increased levels of ACE2 have also been detected in men compared to women, which also enhances the probability of the virus entry into cells and their infection[137]. Additionally, lifestyle factors and comorbidities more common among men are associated with increased severity and mortality in COVID-19, such as smoking[135].

Comorbidities play a fundamental role in both the probability of infection and the severity of the disease. Cardiovascular diseases and hypertension constitute one of the major risk factors. Viral binding to ACE2 disrupts the vasoconstriction and vasodilation balance, promoting endothelial dysfunction, vasoconstriction, and inflammation. This leads to the development of myocarditis, arrhythmias, and increased coagulation dysfunction[139,140]. The elevation of cardiac biomarkers combined with coagulation abnormalities is correlated with worse outcomes and increased mortality, particularly in patients with previous cardiovascular conditions[141].

Diabetes mellitus is another important risk factor for COVID-19. Type 1 and 2 diabetes contribute to an immunocompromised state, impairing both rapid response and adaptive response against SARS-CoV-2, promoting viral replication, and complicating outcomes. In addition, the disease courses with different complications, which exponentially increase unfavorable outcomes[141,142]. Cancer also induces a state of immunosuppression from the malignancy itself or as a result of the treatment received. Treatments also predispose cancer patients to cardiotoxicity and other pathologies, worsening the severity of the disease[140,143]. In that line, patients with immunodeficiency (such as primary immunosuppression or HIV), transplant recipients, or patients with autoimmune disorders receiving immunosuppressive treatments, are at higher risk of being infected with SARS-CoV-2[144,145]. However, the effect on the risk of infection and the disease severity depends on the specific immunosuppressive treatments and the type of underlying immune disease the patients have[144].

Respiratory diseases such as chronic obstructive pulmonary disease (COPD), chronic respiratory disease (CRD), and asthma also have an impact on worse complications during acute disease and worse outcomes. Viral replication on the damaged respiratory airways can further increase symptomatology and tissue and vascular injury, as previously discussed[146,147]. Other diseases, such as chronic liver and chronic kidney diseases, are also responsible for increasing both severe outcomes and mortality[148,149].

Additionally, other factors can have a great influence on COVID-19 outcomes. Obesity is associated with a proinflammatory state and an overall dysregulated immune response, with the adipose tissue possibly serving as a virus reservoir. These factors provoke more severe manifestations of the disease and prolong viral shedding[139,150]. Smoking and heavy alcohol consumption can also interfere with the correct functioning of the immune system and the cardiovascular system, although there is controversy about their effect on COVID-19[139,151,152]. Physical activity has also been correlated to a lower risk of getting infected, developing severe disease, or dying[153].

3.3. Variants and immune evasion.

SARS-CoV-2 is still evolving genetically. Since October 2020, it has been evolving into mutated variants, especially in the spike protein (Figure 5). They are mainly distinguished by a high number of non-synonymous mutations, and with different transmissibility and pathogenicity[154]. Initial variants appearing had enhanced mutations to facilitate cell entry, particularly in the furin binding site[154,155], whereas Omicron and its sublineages have benefited from an altered entry system and enhanced immune evasion[156]. The immune escape is a major driver of the success of the new Omicron sublineages, increasing their fitness and transmissibility, although manifesting lower severity degrees in immunocompetent individuals[157]. The mutations accumulated in key regions of the spike protein, particularly the amino-terminal region and the RBD, have led to poor neutralization capacity of antibodies produced from earlier infections and the first-generation vaccines. A markedly reduced neutralization activity has been detected, particularly in mRNA vaccines, as they elicit the production of spike-directed antibodies (33-fold for the Pfizer vaccine, and 78-fold for Moderna)[154,156]. A booster dose of mRNA vaccine partially restores neutralization capacity, but does not overcome Omicron's immune evasion[158]. These factors are directly associated with an increased probability of SARS-CoV-2 infection in comparison with older variants of the virus, such as Delta[156].

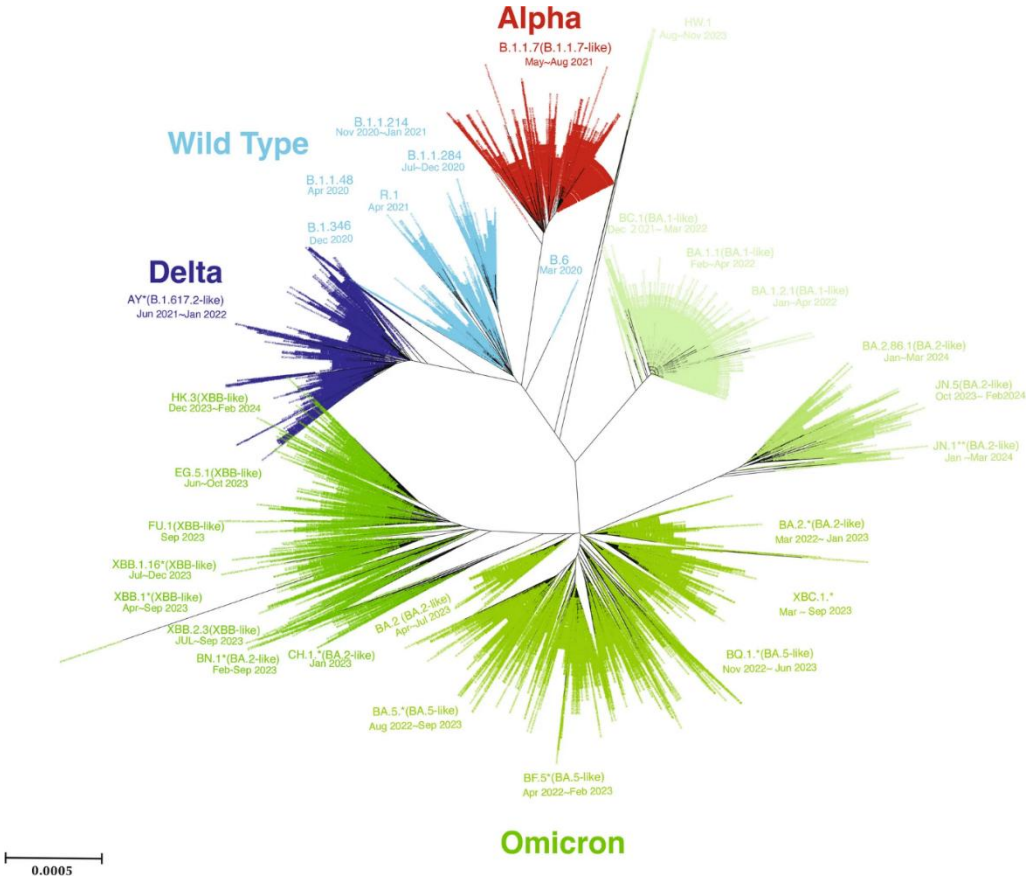

Figure 5. Dynamics of SARS-CoV-2 variant evolution between March 2020 and July 2024. Comprehensive phylogenetic tree analysis illustrating a total of 7383 SARS-CoV-2 full genomes submitted from Hiroshima. From Chhoung, C. et al. Sustained applicability of SARS-CoV-2 variants identification by Sanger Sequencing Strategy on emerging various SARS-CoV-2 Omicron variants in Hiroshima, Japan. BMC Genomics 25, 1063 (2024). Copyright © 2024, Springer Nature | CC-BY https://bmcgenomics.biomedcentral.com/articles/10.1186/s12864-024-10973-0/figures/4

In contrast to antibody neutralizing capacity, T-cell responses are directed against a range of different viral peptides, predominantly spike, nucleocapsid, and membrane proteins, but also other structural and non-structural proteins[159]. As T-cell recognizes epitopes distributed across different proteins, the escape mutations are more widely distributed in comparison to escape mutations seen with antibody responses, which are concentrated within the most variable zone of the spike protein. Each individual typically presents between 30 and 40 epitopes following infection[154,159]. For this reason, T-cell immune escape of new VOCs after old infections and first-generation vaccines is reduced, and response is maintained. Therefore, mutations on the spike RBD have a smaller impact on the overall T-cell response compared to the effect on humoral response, despite some epitope-specific responses being lost[102,160]. Individuals with only a spike-specific T-cell repertoire, but limited against the rest of the virus antigens, might be more susceptible to being reinfected with the new VOCs. Despite this, the main variant evolutionary drivers appear to be increased transmissibility and antibody evasion, which is enhanced in the last SARS-CoV-2 lineages KP.3.1.1 and XEC[154,156,161].

4. From pandemic to endemic.

4.1. Post-pandemic situation: prophylaxis and protection against new variants.

The COVID-19 emergency committee declared the end of the public emergency in May 2023 due to a considerable decline in the disease's morbidity and mortality[162]. The reduced severity of infections with new variants, combined with the protective immunity from vaccination and previous infection, has played an important role in this decline. Nevertheless, due to its ability to evade immune responses and the emergence of new variants, SARS-CoV-2 continues to pose a significant threat to susceptible individuals[154,163].

In response to the emergence of new variants and the waning immunity of the overall population, countries started to administer booster vaccine doses to individuals at high risk of being infected or worse outcomes[164]. A single booster dose was seen to significantly reduce infection with SARS-CoV-2

and severe disease in patients older than 60, being both safe and effective. A robust immune response was observed after homologous or heterologous vaccination, with increased protection against symptomatic infection. This led to a recommendation for its administration six months after the second dose[165,166]. Annual boosters have also been recommended for groups at high risk of COVID-19 after the first boosters, as studies have shown that this can reduce the risk of severe disease[167,168]. Nowadays, these annual boosters have been adapted into variant-specific vaccines, thereby enhancing the protection and preventing the immune escape of new variants[169]. In addition to specific vaccines against the virus, the design of new pancoronavirus vaccines has been proposed, as they could provide substantial value in the case of the appearance of a new virus. It has been discussed that, despite probably having low efficacy against a novel coronavirus, it would offer an initial degree of protection, enhancing pandemic preparedness.

However, vaccination has been seen to be less effective in immunocompromised individuals, particularly those unable to produce neutralizing antibodies. Monoclonal antibodies (mAbs) are an effective tool for preventing and treating COVID-19 in these groups, as they mimic neutralizing SARS-CoV-2 antibodies targeting the spike protein of the virus, thereby blocking SARS-CoV-2 entry into the cells[170]. Several mAb therapies have been evaluated, showing great performance before the appearance of the Omicron variant. While some maintained some activity, such as tixagevimab and cilgavimab (EVUSHELD), many lost efficacy against novel Omicron sublineages[170,171]. It is of utmost importance to continue working on the development of both adapted vaccination and mAbs to new variants to protect those most susceptible to COVID-19.

4.2. Long COVID and pulmonary sequelae.

Long COVID, also known as post-COVID condition, is defined as the persistence or emergence of new symptoms 3 months after the initial SARS-CoV-2 infection, with these symptoms lasting for at least 2 months with no other explanation[172]. This condition has affected approximately 65 million individuals worldwide, representing 10% of the total cases, although among hospitalized patients, the prevalence ranges from 50% to 70%. While vaccination reduces the risk of developing long COVID, breakthrough infections can still lead to the condition[173,174]. Individuals from all age groups can experience long-term effects, especially those with a more severe disease, although most long-COVID cases are found in mild-disease patients, as they account for the majority of cases reported[175].

The clinical manifestations of long-COVID are diverse, affecting a wide range of organ systems. Cardiovascular, thrombotic, and cerebrovascular complications are among the most common conditions of the disease. Furthermore, type 2 diabetes, myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS), and dysautonomia have been observed in some patients, with these symptoms persisting for extended periods of time[175–177]. Further symptoms may include lung sequelae, articular pain, “brain fog”, depression and anxiety, loss of smell, taste, or appetite, gastrointestinal symptoms, and other complications that can significantly impact day-to-day life[178]. The timing and progression of the symptoms vary, with neurological symptoms having a delayed onset and even worsening over time, while gastrointestinal and mild respiratory symptoms are likely to resolve[175,179].

The underlying mechanisms of long-COVID appear to be complex and multifactorial. Viral persistence in a wide range of tissues has been detected, with the spike antigen being detected in long-COVID individuals 12 months after diagnosis[180]. On the other hand, there is evidence of sustained immune activation being reported, with decreased numbers of effector memory cells, elevated markers of exhaustion, and persistent cytokine production for months after the infection. This is accompanied by elevated levels of both type I and III IFNs[181]. In addition, other alterations include the increase of autoantibodies, reactivation of latent viruses, alterations in microbiota, thrombosis, and endothelial dysfunction[175,182].

Most diagnostic tools for long COVID are still under development, with several techniques for detecting different abnormalities and biomarkers still under investigation. The limited testing accessibility, combined with the challenges in diagnosis, resulted in elevated false-negative rates[175,183]. Establishing a long COVID diagnosis and defining treatment is of utmost importance for these patients. Consequently, the identification of sensitive biomarkers is crucial. Regarding treatments, the current options are based on small-scale studies or treatments effective against other diseases, with several trials in progress[184]. Finally, vaccination reduces the risk of developing long COVID, with the risk depending on the viral variant. Nevertheless, reinfections heighten the risk of long-COVID even following vaccination[185,186].

4.3. Preparedness for future pandemics: scientific strategies and European calls

The COVID-19 pandemic revealed significant vulnerabilities in countries' preparedness and healthcare systems to combat emerging infectious diseases. Insufficient coordination and data sharing of research, delays in diagnostics and outbreak detection, and gaps in vaccine and treatment development impeded a better response to the pandemic[187,188].

In response to these gaps, the European Health Emergency Preparedness and Response Authority (HERA) was created as a directorate-general of the European Commission to prepare the European Union (EU) for future pandemics. Its key priorities are identifying pathogen families with the potential to start a pandemic and funding research against these pathogens, with a major focus on the advancement of flexible vaccine platforms[189]. In parallel, partnership programs and Horizon calls like the European Partnership for Pandemic Preparedness have been developed to improve the EU's capacity in prevention, preparedness, surveillance, risk assessment, early warning, and response against future pandemics[188].

Apart from flexible vaccine platforms, the EU is investing in longitudinal immune profiling platforms through standardized immunological assays to better understand early immune activation, adaptive memory formation, and correlates of protection against infections. Investments have been made in clinical trial networks and infrastructure to improve, harmonize, and scale up the knowledge obtained from research. In addition, secure data-sharing is facilitated through platforms such as the European COVID-19 Data Platform and the Versatile Emerging Infectious Disease Observatory[190,191].

Finally, it is important to integrate the One Health approach in the preparation for new infectious disease emergencies. Given that over 60% of emerging infectious diseases originate in animals, zoonosis is the principal route of pandemic emergence. Therefore, the integration of human medicine, veterinary science, and environmental health would be key in preventing the onset of future pandemics, with a defined surveillance and prevention strategy. This perspective is already found in EU programs such as the One Health European Joint Programme[192–194].

3

JUSTIFICATION

The COVID-19 pandemic, caused by the coronavirus SARS-CoV-2, has represented one of the greatest global health emergencies in modern history. Since its emergence in 2019 in Wuhan, China, it has infected around 780 million individuals and has been responsible for more than 7 million deaths worldwide. The pandemic has had a devastating impact on healthcare systems, exposing their vulnerabilities, but also had detrimental economic and social consequences of unprecedented magnitude. Despite transitioning to an endemic state, SARS-CoV-2 is still circulating and evolving, challenging acquired immunity through previous infection and vaccination, highlighting the need for scientific research on the topic.

One of the challenges in COVID-19 management has been the evaluation of protective immunity against the infection, usually determined by serological testing. Antibody-based diagnostics dominated the immunological assessment of SARS-CoV-2 exposure and vaccine efficacy during the pandemic, but they are unable to represent the complexity of the adaptive response. This is accentuated in individuals with waning antibody levels or impaired humoral immunity.

The cellular immune response is key in the antiviral response, participating in viral clearance, limiting disease severity, and determining memory responses. It involves a complex network of cell subsets capable of recognizing pathogens, producing effector cytokines, and specifically responding against them. Cellular immune responses not only contribute to the clearance of infected cells during the acute phase of infection but also establish long-term immune surveillance mechanisms that can prevent reinfection and limit the severity of future exposures to the pathogen. These specific responses have been observed to persist after COVID-19, even when circulating antibodies wane or become undetectable, and are fundamental against new variants, as they act against a broader set of viral epitopes.

Although several platforms exist to assess cellular immunity, their integration into clinical practice has been limited, as they are used to a lesser degree in routine diagnostics and public health strategies. Accessible tools could help evaluate the functionality and quantity of these cellular immune responses, acting as key components in personalized clinical decision-making. They could be essential in identifying those individuals who could benefit from prophylactic therapies, assessing protection against reinfection, and estimating the risk of severe disease. This is fundamental for patients with compromised humoral immunity, who, despite lacking a humoral response, may still be able to exhibit a functional cellular memory. On the other hand, patients with established humoral responses might also benefit from this measurement, as positive serology does not always indicate a complete and functional response against the pathogen, since they could lack a specific cellular response.

For this reason, this thesis aims to address, from a practical perspective, the need to test and explore the measurement of cellular immune responses to SARS-CoV-2 infection and vaccination. It contains complementary studies that contribute to giving evidence and provide pragmatic solutions to implement in clinical practice in different situations regarding COVID-19.

The first study (Safont G et al. 2022, **Article 1**) investigates T-cell responses during acute SARS-CoV-2 infection, through convalescence, and following mRNA vaccination using an IFN-γ ELISPOT. It aims to characterize IFN-γ-producing cells in response to immunogenic peptides from the virus in participants with different immunological backgrounds, establishing the IFN-γ ELISPOT assay as a viable tool to monitor immune responses, complementing humoral response evaluation.

The second study (Safont G et al., 2024; **Article 2**) builds on these findings by evaluating the added value of measuring both cellular IFN-γ and IL-2 responses to SARS-CoV-2 using a dual FluoroSPOT assay. The dual measurement improves the assessment of the patients' immunological state, both during the acute phase with an IFN-γ-centered response, and after infection or vaccination with a prominent IL-2 response. This test can help detect participants with cellular immune response even in the absence of detectable antibodies, emphasizing the importance of incorporating cellular immune response measurement for immune surveillance during the infection and after immunization.

The third study (Safont G et al., 2025; **Article 3**) focuses on immunocompromised patients who are candidates for prophylaxis with monoclonal antibodies against SARS-CoV-2. The lack of humoral response in some of these patients challenges the determination of the levels of protection against the pathogen. Prophylaxis management would substantially benefit from cellular assessment assays, uncovering protected individuals considered unprotected, and further understanding their adaptive cellular response against the virus.

Taken together, the thesis addresses the need to evaluate SARS-CoV-2 immunity beyond the serological measurement by incorporating cellular immune measurements across different clinical contexts. It demonstrates the ability of ELISPOT to accurately detect antigen-specific cellular immune responses during and after acute infection, after vaccination, and in immunocompromised states. By providing different studies that evaluate this type of assay, the thesis contributes to a more comprehensive understanding of the adaptive immune response, promoting the integration of cellular immunity assessment into the management of COVID-19.

4

OBJECTIVES

The aims of this study are as follows:

1. To study the cellular immune response against SARS-CoV-2 by measuring IFN-γ secretion in individuals with acute COVID-19, during convalescence, and after vaccination (**Article 1**).
   a. To evaluate the specific cellular immune response against different SARS-CoV-2 antigens, including spike, nucleocapsid, and membrane proteins.
   b. To quantify IFN-γ responses against the pathogen during the acute phase of COVID-19, across different severity degrees (mild, moderate, and severe disease).
   c. To compare the quantity of IFN-γ T-cell responses during and after COVID-19 according to severity, as well as after vaccination, to better understand the kinetics of cellular immune responses regarding COVID-19.
   d. To study correlations between IFN-γ cellular immune responses and IgG and IgM antibody levels and neutralizing activity against the virus to explore discordances between both branches of the adaptive immunity.

2. To characterize SARS-CoV-2-specific cellular immune responses by the dual detection of IFN-γ and IL-2 secretion, and to evaluate their potential to help in COVID-19 patient management (**Article 2**).
   a. To investigate if the combined detection of IFN-γ and IL-2 increases the sensitivity to identify SARS-CoV-2-specific T-cell responses compared to measuring each cytokine alone.
   b. To compare the quantity and pattern of release of IFN-γ and IL-2 in different states regarding SARS-CoV-2 infection (acute COVID-19, convalescence, and vaccination), and correlate them with disease severity and time since infection or vaccination.
   c. To study the correlation between both IFN-γ and IL-2 cellular immune responses and humoral immunity, including IgG, IgM, and neutralizing antibodies, to identify discordances between both branches of the adaptive immunity against the virus.
   d. To validate the use of fluorescence ELISPOT technology for the detection of IFN-γ and IL-2 specific responses to SARS-CoV-2, as a rapid and accessible tool to improve patient management.

3. To evaluate SARS-CoV-2-specific cellular immune responses in immunocompromised patients by assessing the secretion of IFN-γ, IL-2, TNF, IL-21, and IL-5, and to explore its value in the prioritization of prophylaxis administration (**Article 3**).

    a. To determine the presence and functional quality of SARS-CoV-2-specific T-cell responses in participants with negative versus positive serology using FluoroSpot assays.

    b. To compare the intensity and polyfunctionality of cytokine responses between negative and positive serology immunosuppressed patients.

    c. To explore the production of IFN-γ, IL-2, TNF, IL-21, and IL-5 and the imbalances between them in immunocompromised patients.

    d. To determine clinical factors influencing cellular immune responses in negative serology patients, such as previous COVID-19 and immunosuppressive treatment.

    e. To validate the measurement of IFN-γ, IL-2, IL-21, and IL-5 as a tool assisting in the management of immunocompromised individuals against COVID-19.

5

RESULTS

Article 1

Measuring T-Cell Responses against SARS-CoV-2 Is of Utility for Disease and Vaccination Management

Safont, Guillem; Latorre, Irene; Villar-Hernández, Raquel; Stojanovic, Zoran; Marín, Alicia; Pérez-Cano, Cristina; Lacoma Alicia; Molina-Moya, Bárbara; Solis, Alan Jhúnior; Arméstar, Fernando; Matllo Joan; Díaz-Fernández Sergio; Cendón, Arnau; Sokalchuk, Liliya; Tolosa, Guillermo; Casas, Irma; Rosell, Antoni; Domínguez, Jose

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Article

Measuring T-Cell Responses against SARS-CoV-2 Is of Utility for Disease and Vaccination Management

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Abstract: The measurement of specific T-cell responses can be a useful tool for COVID-19 diagnostics and clinical management. In this study, we evaluated the IFN-γ T-cell response against the main SARS-CoV-2 antigens (spike, nucleocapsid and membrane) in acute and convalescent individuals classified according to severity, and in vaccinated and unvaccinated controls. IgG against spike and nucleocapsid were also measured. Spike antigen triggered the highest number of T-cell responses. Acute patients showed a low percentage of positive responses when compared to convalescent (71.6% vs. 91.7%, respectively), but increased during hospitalization and with severity. Some convalescent patients showed an IFN-γ T-cell response more than 200 days after diagnosis. Only half of the vaccinated individuals displayed an IFN-γ T-cell response after the second dose. IgG response was found in a higher percentage of individuals compared to IFN-γ T-cell responses, and moderate correlations between both responses were seen. However, in some acute COVID-19 patients specific T-cell response was detected, but not IgG production. We found that the chances of an IFN-γ T-cell response against SARS-CoV-2 is low during acute phase, but may increase over time, and that only half of the vaccinated individuals had an IFN-γ T-cell response after the second dose.

Keywords: SARS-CoV-2; T-cell response; humoral response; IFN-γ; vaccination; ELISPOT

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1. Introduction

The Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has infected a total of 552.5 million individuals causing the death of 6.35 million since its appearance in December 2019 [1]. Considered a pandemic since March 2020 after a large global outbreak, it is a major public health concern, filling ICUs and neglecting the control of other diseases [2–4]. Whilst most people experience an asymptomatic or a mild SARS-CoV-2 infection, others can present a severe condition usually associated with some specific comorbidities. The severe condition is associated with pneumonia, involving chest pain and hemoptysis, but can also cause organ failure, which can lead to severe sequelae, and even death [5].

Currently, the main available vaccines against SARS-CoV-2 are based on the spike protein of the virus. This glycoprotein has a high antigenicity and is significantly immunogenic when activating the adaptive immune response [6]. At present, the principal vaccines administered against the pathogen are the following: mRNA-1273 (Spikevax, ModernaTX, Inc., Cambridge, MA, USA) and BNT162b2 (COMIRNATY, Pfizer, Inc., and BioNtech, New York, NY, USA), both based on mRNA vaccine design; and ChAdOx1-S (Oxford/AstraZeneca, UK) based on carrier vaccine design. Two doses of these vaccines are recommended, and a third dose is also administered as a booster. Recently, a second booster has been recommended for immunocompromised individuals with a suboptimal immune response to an earlier vaccination [7]. Currently, 67.4% of the world population has received at least one dose of a COVID-19 vaccine, and 12.46 billion doses have been administered globally, contributing to a decrease in the severity and the mortality associated with the pathogen [8].

Humoral, and cellular adaptive responses are fundamental for the virus elimination, the infection resolution, and the protection against reinfection [9,10]. Looking closer at the adaptive response, specific antibodies seem to decline faster than the cellular response. In fact, individuals infected with SARS-CoV, did not show any specific antibody response within two to three years after infection, however, SARS-CoV-specific memory T-cells were detected even after 11 years [11]. Nevertheless, the cellular response against the virus and its mechanisms are far from being completely characterized, so studying it is substantial for a better understanding of the immune response against the virus, the pathogenicity associated, and the long-term immunity that SARS-CoV-2-specific T-cells could be conferring. In addition, the role that vaccination plays in triggering specific T-cell responses against the virus must not be overlooked, as analyzing it can contribute to determine the robustness and the durability of protection [12,13].

In this study, we assessed the cellular immune response by detecting the IFN-γ secreting T-cells after specific stimulation with different SARS-CoV-2 antigens during acute disease and convalescence, and after vaccination. In addition, the IgG and IgM humoral immunity were analyzed and compared with the specific T-cell response.

2. Materials and Methods

2.1. Study Groups and Clinical Definitions

Blood sample collection took place in the Hospital Universitari Germans Trias i Pujol from July 2020 to November 2021. A signed written consent form was obtained from all subjects included in the study after being informed about the project. The study was approved by the Ethics Committee of the Hospital Germans Trias i Pujol (PI-20-117).

In this study, patients were classified according to disease severity following the advice of the Pulmonology Department COVID-19 Commission of the Hospital Universitari Germans Trias i Pujol. Severity degrees were defined as asymptomatic, mild, moderate, and severe. In our study, patients classified as asymptomatic were health care workers who had a positive SARS-CoV-2 PCR in the context of work setting routine screenings and had no symptoms during the course of infection. Patients were defined as having mild infection if they did not require oxygen support or were only in need of nasal prongs, independently if they were hospitalized or not. Moderate infection was reported in patients admitted to respiratory semi-critical care unit requiring non-invasive ventilation, and severe infection in those hospitalized in intensive care unit (ICU) requiring invasive mechanical ventilation.

A total of 259 samples from 230 individuals were obtained and classified as follows:

1. One hundred twenty-eight samples from controls who were health care workers. selected based on having no prior/present positive SARS-CoV-2 PCR and/or rapid antigen test, and/or having no detectable IgG or IgM plasma antibodies at the moment of inclusion. They were grouped according to SARS-CoV-2 vaccination status as follows: (a) unvaccinated individuals (n = 80), and (b) vaccinated individuals with Pfizer (n = 47) or Moderna (n = 1). Days between sampling and the first/second dose administration were recorded.

2. Seventy-one samples from patients with asymptomatic (n = 6), mild (n = 3), moderate (n = 33), and severe (n = 29) COVID-19 disease enrolled during the acute phase of the disease. All patients had a reported positive SARS-CoV-2 PCR and/or rapid antigen test. Inside this group, 19 patients with moderate or severe COVID-19 were followed-up (having two or more consecutive samples, being the overall number of samples 48) during days 0, 2, 7, 28 or discharge after admission into semi-critical or ICU.
3. Sixty samples from individuals recruited during the convalescence phase after mild (n = 22), moderate (n = 18), or severe disease (n = 20). All of them had a record of the number of days between sampling and COVID-19 diagnosis with a positive test.

Overall, a total of 322 samples were collected for the study, although 63 were excluded for not having enough cell counts for T-cell studies. Descriptive demographic and clinical data from individuals included in the analysis have been summarized in Table 1. Data concerning intrinsic information of the samples such as time since diagnosis and vaccination, or lymphopenia at sampling have been included in Supplementary Table S1.

2.2. Peripheral Blood Mononuclear Cells (PBMCs) Isolation and Cryopreservation

A total of 16 mL of blood was collected from each patient in CPT tubes (Becton Dickinson Diagnostics, Franklin Lakes, NJ, USA). Next, PBMCs were isolated from blood by density gradient centrifugation. Afterwards, they were washed twice with RPMI (Biowest, Nuaillé, France) supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaillé, France) and finally counted using trypan blue in an inverted microscope. For cryopreservation, cells were suspended in 10% DMSO FBS (Sigma-Aldrich, Saint Louis, MO, USA), transferred into cryovials, and then stored at −80 °C in a cold Nalgene Mr. Frosty Cryo 1 °C Freezing Container (ThermoFisher, Waltham, MA, USA). Cells were then transferred to liquid nitrogen within a week.

2.3. ELISPOT Assay for IFN-γ T-Cell Response Detection

The T-cell response from each sample was evaluated by means of an ELISPOT assay (T-SPOT Discovery SARS-CoV-2, Oxford Immunotec, Abingdon, UK). The cells were thawed, and their concentration was adjusted to 2.5×10^6 cells/mL. Next, a total of 250,000 cells per well were stimulated overnight (16–20 h) at 37 °C, 5% CO_2 with: specific SARS-CoV-2 (a) spike antigen, (b) nucleocapsid (NCP) antigen, (c) membrane antigen, (d) antigens with homology regions of endemic human coronaviruses, (e) AIM-V medium (negative control), and (f) phytohemagglutinin (mitogen; positive control). Peptides from the assay covered the most immunogenic regions of the virus genome, spanning the full length of those proteins, which allowed the study of the extent of T-cell immunity and guaranteed the effect of point mutations was minimized [14]. As detailed by the manufacturer, following the incubation, the IFN-γ released was revealed with a detection antibody and a substrate that showed the IFN-γ secreting cells as spot-forming cells (SFCs). The SFCs were counted for each of the antigens using an automated plate reader (Autoimmun Diagnostika GmbH, Straßberg, Germany) and checked by the naked eye.

The test scored as positive when the final SFCs count was more than 7, even when the sample was unresponsive for the positive control. A borderline result was considered between 5–7 SFCs (both included). SFCs counted in the negative control were always subtracted from the SFCs counted for every specific antigen (antigen SFCs—negative control SFCs). Samples with less than 20 SFCs in the positive control and/or more than 10 SFCs in the negative control were considered indeterminate. The number of reactive T-cells (SFCs) for each of the patient groups enrolled in the study was also analyzed to investigate the quantity of the IFN-γ response.

Table 1. Descriptive table from the patients included in the study.

Participants Variables	Controls (n = 128)		Acute (n = 42)				Convalescent (n = 60)			
							Mild (n = 22)			
	Unvaccinated (n = 80)	Vaccinated (n = 48)	Asymptomatic (n = 6)	Mild (n = 3)	Moderate (n = 16)	Severe (n = 17)	Non-Hospitalized (n = 12)	Hospitalized (n = 10)	Moderate (n = 18)	Severe (n = 20)
Age (years ± SD)	39 ± 13.3	42.9 ± 13.9	37.8 ± 18.1	25.3 ± 3.5	56.4 ± 16.1	65.8 ± 14.1	42.9 ± 12.7	63.8 ± 9.7	58.1 ± 14.6	56.9 ± 11.3
Male N (%)	24 (30)	9 (18.8)	4 (66.7)	0 (0)	13 (81.3)	13 (76.5)	3 (25)	6 (60)	10 (54.5)	15 (75)
Pneumonia N (%) *	0 (0)	0 (0)	0 (0)	0 (0)	16 (100)	17 (100)	0 (0)	9 (90)	18 (100)	20 (100)
Unilobar	0 (0)	0 (0)	0 (0)	0 (0)	1 (6.3)	0 (0)	0 (0)	2 (20)	2 (11.1)	0 (0)
Multilobar	0 (0)	0 (0)	0 (0)	0 (0)	32 (93.7)	17 (100)	0 (0)	7 (70)	16 (88.9)	20 (100)
ICU admission N (%) *	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	17 (100)	0 (0)	0 (0)	0 (0)	20 (100)
Oxygen support N (%) *	0 (0)	0 (0)	0 (0)	0 (0)	16 (100)	17 (100)	0 (0)	5 (50)	18 (100)	20 (100)
Nasal prongs	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	5 (50)	0 (0)	0 (0)
Non-invasive mechanical vent.	0 (0)	0 (0)	0 (0)	0 (0)	16 (100)	0 (0)	0 (0)	0 (0)	18 (100)	0 (0)
Invasive mechanical vent.	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	17 (100)	0 (0)	0 (0)	0 (0)	20 (100)
Vaccinated with 1st dose N (%)	0 (0)	48 (100)	2 (33.3)	0 (0)	1 (6.3)	3 (17.6)	6 (50)	0 (0)	0 (0)	0 (0)
Vaccinated with 2nd dose N (%)	0 (0)	40 (83.3)	0 (0)	0 (0)	0 (0)	0 (0)	4 (33.3)	0 (0)	0 (0)	0 (0)
Comorbidities N (%)	2 (2.6)	9 (18.8)	0 (0)	0 (0)	14 (87.5)	12 (70.6)	3 (25)	6 (60)	12 (66.7)	12 (60)
Respiratory disorders (asthma, OSAS, COPD)	0 (0)	2 (4.2)	0 (0)	0 (0)	9 (56.3)	1 (5.9)	0 (0)	4 (40)	1 (5.9)	0 (0)
Cardiovascular diseases (AHT, ictus, atrial fibrillation)	1 (1.3)	2 (4.2)	0 (0)	0 (0)	7 (43.8)	10 (58.8)	1 (8.3)	3 (30)	8 (44.4)	9 (45)
Autoimmune disorders (DM2, psoriasis, Jorgen, other)	1 (1.3)	4 (8.3)	0 (0)	0 (0)	4 (25)	6 (35.3)	1 (8.3)	1 (10)	4 (22.2)	5 (25)
Central nervous system disorders (dementia, epilepsy, Parkinson)	0 (0)	1 (2.1)	0 (0)	0 (0)	2 (12.5)	4 (23.5)	0 (0)	0 (0)	1 (5.5)	1 (5)
Malignant neoplasies	0 (0)	0 (0)	0 (0)	0 (0)	1 (6.3)	3 (17.6)	0 (0)	1 (10)	1 (5.5)	2 (10)
Obesity	n/a	n/a	0 (0)	0 (0)	3 (18.8)	4 (23.5)	1 (8.3)	1 (10)	6 (33.3)	6 (30)
Immunosuppressive treatment N (%)	2 (2.5)	4 (6.3)	0 (0)	1 (16.7)	4 (25)	1 (5.9)	1 (9)	1 (10)	3 (16.7)	2 (10)
Oral (betamethasone, prednisone, NSAIDS)	1 (1.3)	1 (2.1)	0 (0)	1 (16.7)	1 (6.3)	1 (5.9)	0 (0)	0 (0)	0 (0)	1 (5)
Inhaled	1 (1.3)	2 (4.2)	0 (0)	0 (0)	3 (18.8)	0 (0)	0 (0)	1 (10)	3 (16.7)	1 (5)
Topic	0 (0)	2 (4.2)	0 (0)	0 (0)	0 (0)	0 (0)	1 (9)	0 (0)	0 (0)	0 (0)
Deaths N (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	5 (29.4)	0 (0)	0 (0)	0 (0)	0 (0)

* In the convalescent group, these variables are referring to the characteristics of their acute COVID-19 episode. n/a = not available.

2.4. ELISA for Humoral Response Detection

The IgG concentration against spike was quantified with the QuantiVac ELISA kit according to the manufacturer's instructions (Euroimmun, Lübek, Germany). Six calibrators at different known concentrations were used to perform a calibration curve. Binding IgG units against the antigen [BAU/mL] were calculated through extrapolation to the curve.

The IgG and IgM against NCP were evaluated with the semi-quantitative Anti-SARS-CoV-2 (IgG) and Anti-SARS-CoV-2 (IgM) ELISA kits (Euroimmun, Lübek, Germany) according to the insert instructions. A ratio was performed between the sample's and calibrator's absorbance. Cut-off values for positive, negative, and borderline values were provided by the manufacturer for each test.

2.5. Statistical Analysis

The results comparing the IFN-γ response between groups were performed using the two-tailed Mann-Whitney U-test for unpaired comparisons. The differences were considered statistically significant when a p-value was <0.05. The correlations were calculated using the two-tailed non-parametric Spearman test. The statistical analyses together with graphical representations were carried out using GraphPad Prism version 8 (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1. Positivity Rate between Patient Groups

Looking at the overall T-cell response results for specific antigens, the one that triggered more positive responses was spike (46.8% (118/252)). NCP and membrane antigens elicited a positive response in 30.9% (77/249) and 29.3% (73/249) of the samples, respectively (Table 2). In addition, the antigens with homology regions of endemic (human) coronaviruses also induced a response in 25.9% (65/251) of the samples (Supplementary Table S2). In 7 samples the results of the 3 antigens were considered indeterminate due to inadequate responses in the negative or positive controls. These indeterminate samples corresponded to 3 unvaccinated controls and 4 acute patients (1 moderate and 3 severe cases). In addition, 2 samples presented less than 20 SFCs in the positive control; however, as they had a response against the spike and/or the membrane antigens, they were considered positive only for that particular antigen and indeterminate for the others.

Table 2. Overall positivity results for the T-cell IFN-γ secretion against each antigen, including borderline results (%).

Groups	Spike (n = 252)	Nucleocapsid (n = 249)	Membrane (n = 249)	Any SARS-CoV-2 Antigen (n = 252)
Overall (n = 259)	118/252 (46.8)	77/249 (30.9)	73/249 (29.3)	130/252 (51.6)
Controls (n = 128)	27/125 (21.6)	2/125 (1.6)	3/125 (2.4)	27/125 (21.6)
Vaccinated (n = 48)	24/48 (50)	1/48 (2.1)	3/48 (6.3)	24/48 (50)
Unvaccinated (n = 80)	3/77 (3.9)	1/77 (1.3)	0/77 (0)	3/77 (3.9)
Acute disease (n = 71)	38/67 (56.7)	31/64 (48.4)	21/64 (32.8)	48/67 (71.6)
Asymptomatic (n = 6)	2/6 (33.3)	2/6 (33.3)	1/6 (16.7)	3/6 (50)
Mild (n = 3)	1/3 (33.3)	1/3 (33.3)	1/3 (33.3)	1/3 (33.3)
Moderate (n = 33)	22/32 (68.8)	14/32 (43.8)	8/32 (25)	24/32 (75)
Severe (n = 29)	13/26 (50)	13/23 (56.5)	10/23 (43.5)	20/26 (76.9)
Convalescent (n = 60)	53/60 (88.3)	44/60 (73.3)	49/60 (81.7)	55/60 (91.7)
Mild * (n = 22)	16/22 (72.7)	13/22 (59.1)	15/22 (69.6)	17/22 (78.3)
Non-hospitalized (n = 12)	6/12 (50)	6/12 (50)	5/12 (41.7)	7/12 (58.3)
Hospitalized (n = 10)	10/10 (100)	7/10 (70)	10/10 (100)	10/10 (100)
Moderate * (n = 18)	17/18 (94.5)	13/18 (72.2)	16/18 (88.9)	18/18 (100)
Severe * (n = 20)	20/20 (100)	18/20 (90)	18/20 (90)	20/20 (100)

Positivity percentages were calculated excluding the indeterminate results from the total number of samples tested for each group. Denominator of each ratio indicates the total n for that antigen and that group of individuals that had a valid result. * Severity considered during their acute COVID-19 episode.

As shown in Table 2, the specific response against spike antigen was present in 50% (24/48) of the vaccinated controls. The positivity rate in unvaccinated controls was low (3.9% (3/77) for spike, 1.3% (1/77) for NCP and 0% for membrane). The positive results against NCP and membrane in vaccinated individuals, and the positive results against the three antigens in control unvaccinated individuals, could be due to asymptomatic and/or non-reported SARS-CoV-2 infections. In the acute disease patient's group, the percentage of positive results against spike, NCP, and membrane was 56.7% (38/67), 48.4% (31/64), and 32.8% (21/64), respectively. In addition, acute severe patients showed a lower percentage of positive results against spike (50% (13/26)) than moderate ones (68.8% (22/32)). In convalescent patients, a total of 91.7% (55/60) of the samples were responsive against at least one SARS-CoV-2 antigen, being the spike and membrane the ones with the highest positivity percentage (88.3% (53/60) and 81.7% (49/60), respectively). Moreover, positivity percentages against any of the antigens among convalescent patients increased according to the severity during the acute phase of the disease.

Regarding the 19 patients with follow-up, positivity percentage against spike was the highest (54.2% (26/48)), increasing according to days of hospitalization (Supplementary Table S3). When analyzing SFCs in these monitored patients, high inter-individual variability was observed although most responses tended to increase. No association was found between the disease outcome (mortality vs. non-mortality) and the T-cell response detected during the days admitted into semi-criticals or ICU (Supplementary Figure S1).

3.2. Quantitative IFN-γ Response against SARS-CoV-2 Antigens

In addition to positivity rates described in the previous section, the number of responding T-cells after specific stimulation was also investigated. When the quantity of this response (measured as SFCs) against spike was analyzed in acute COVID-19 patients, a reduced IFN-γ response was observed in asymptomatic and mild subgroups. Moderate acute COVID-19 patients displayed a greater response than severe acute COVID-19 patients, however, there was no statistical significance when comparing the four subgroups (Figure 1). Moreover, the number of lymphocytes/µL in acute COVID-19 patients moderately correlated with the IFN-γ T-cell and IgG response against spike (SR = 0.318; $p = 0.019$ for T-cells; SR = 0.553; $p = 0.009$ for IgG; Supplementary Figure S2a,b). Regarding convalescent patients, the number of SFCs against spike increased according to disease severity in the acute phase, and it was significantly higher in patients who passed severe COVID-19 compared to mild COVID-19 patients who were not hospitalized ($p < 0.05$). Moreover, mild, moderate, and severe COVID-19 convalescent subgroups had a significantly higher response than vaccinated controls ($p < 0.01$ when comparing mild or moderate; $p < 0.0001$ in severe) (Figure 1). No more significant differences were found between groups.

Regarding NCP and membrane, none of the patients with acute COVID-19 showed a median response above the positivity threshold. Although not significant, convalescent subgroups had a higher average response than acute and control groups for both antigens. In convalescent individuals, the response against the membrane antigen was significantly lower in mild non-hospitalized individuals than in the rest of the groups ($p < 0.0001$) (Supplementary Figure S3a,b).

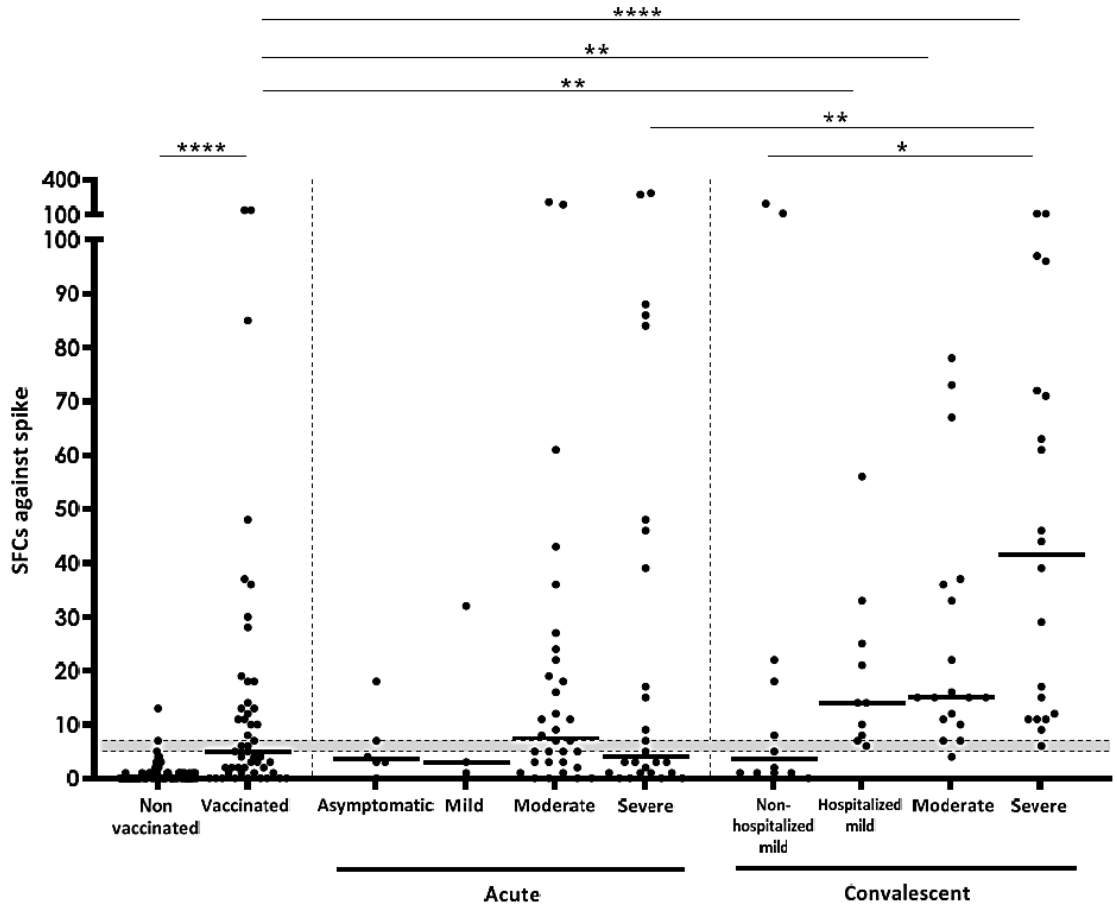


Figure 1. Number of SFCs after stimulation with spike in the different study groups. Horizontal lines represent medians. Grey area shows borderline results. Differences between conditions were calculated using the two-tailed Mann-Whitney U test. *p* is considered significant when <0.05 (* <0.05, ** <0.01, and **** <0.0001).

3.3. IFN-γ Response According to Days after Vaccination and after COVID-19 Diagnosis

The days between sampling and the first/second vaccine dose administration were documented. As shown in Figure 2a, there was not a significant correlation (SR = 0.133; $p = 0.369$) between the IFN-γ response and time after the first vaccine dose. A total of 46.5% (20/43) of individuals did not show response against spike after receiving the two vaccine doses (between 21–77 days after the first dose administration) (Figure 2a).

The T-cell response against any of the three SARS-CoV-2 specific antigens tested was analyzed in convalescent patients since diagnosis. Although responses tended to increase along time, no correlation was found between the antigens evaluated and time (SR = 0.197; $p = 0.132$ for spike, SR = 0.087; $p = 0.51$ for NCP; and SR = 0.212; $p = 0.103$ for membrane) (Figure 2b–d).

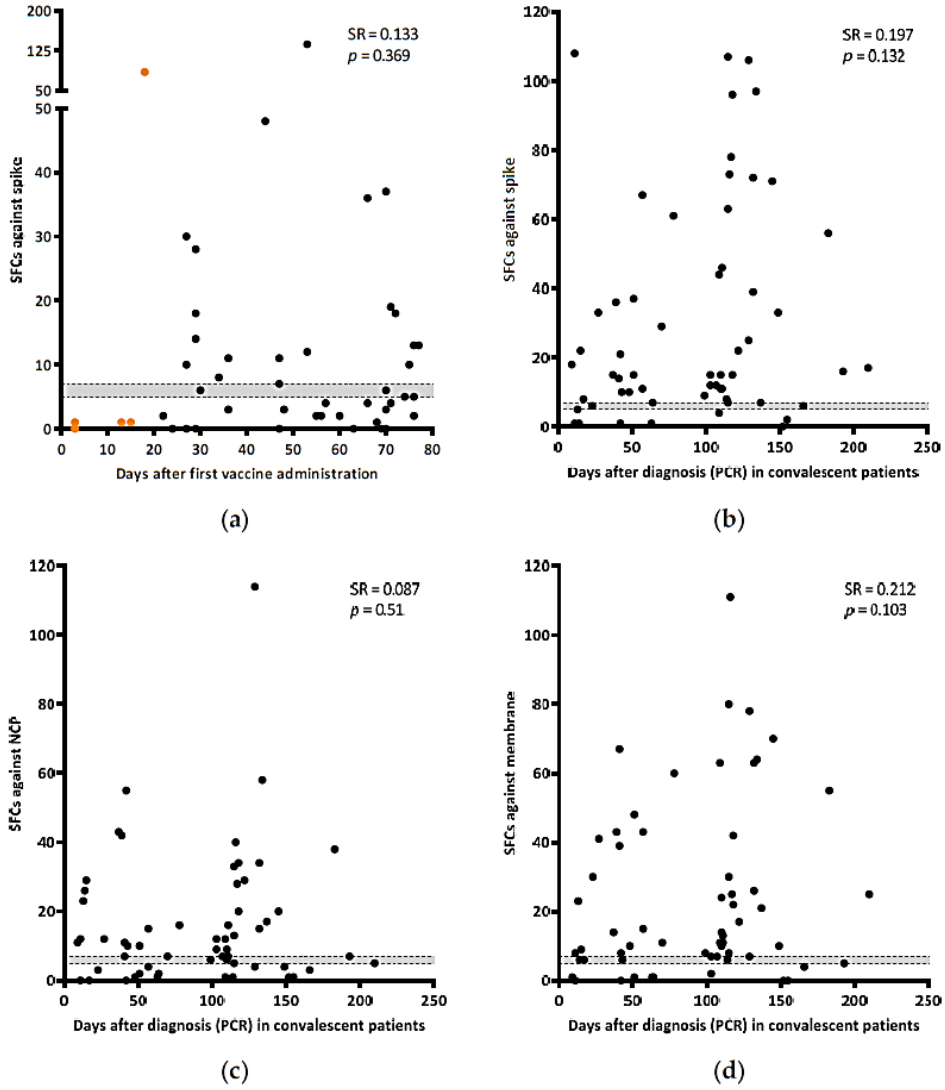


Figure 2. Correlation of the IFN-γ response against spike (SFCs) with days after the first dose of the vaccine administration (a). Orange dots are samples from individuals with only one dose. Correlation of the IFN-γ response (SFCs) in spike (b), NCP (c), and membrane (d) with days after diagnosis (PCR) in convalescent patients. Grey area shows borderline results. Correlations were calculated using the two-tailed non-parametric Spearman test.

3.4. T-Cell IFN-γ Production and Humoral Responses

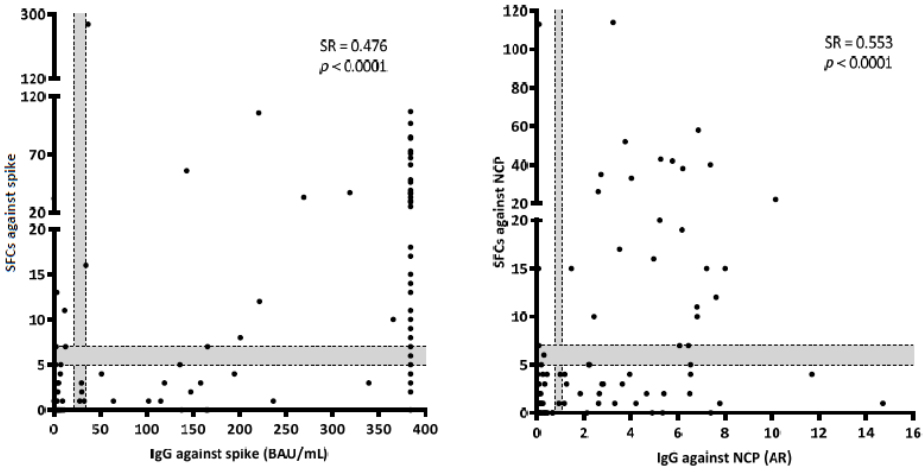
In a subgroup of 159 samples, it was possible to evaluate IgGs against spike and NCP, as well as IgMs against NCP. Four of them had indeterminate results for the T-cell test. Overall, the number of positive results obtained by detecting IgG was higher than that obtained when detecting the IFN-γ producing T-cells (55.5% (86/155) IgG vs. 40.6% (63/155) T-cell against spike; 35.7% (55/154) IgG vs. 20.8% (32/154) T-cell against NCP) (Table 3). In addition, levels of IgG specific for spike and NCP antigens significantly correlated with SFCs. For both cases, there was a moderate correlation between antibody and cellular responses (for IgG spike SR = 0.476, p = <0.0001; for IgG NCP SR = 0.553, p = <0.0001) (Figure 3a,b). Unvaccinated controls showed low percentages of positive responses against both spike (9.7% (6/62) and 4.8% (3/62) for IgG and T-cell responses, respectively) and NCP (6.5% (4/62) and 1.6% (1/62) for IgG and T-cell responses, respectively). In the vaccinated group, the number of positive results when evaluating spike was higher when

detecting the humoral response than when detecting T-cell responses (90% (27/30) and 46.7% (14/30), respectively). Considering patients with acute COVID-19, IgGs against spike were present in 67.6% (23/34) of the samples compared to 52.9% (18/34) when detecting a T-cell response against the same antigen. Similarly, 69.7% (23/33) and 38% (12/33) of the samples were positive for IgG and T-cell responses against NCP, respectively. The number of positive results were comparable between IgG and cellular responses in convalescent patients for spike (100% (29/29) and 95.6% (28/29), respectively), but not for NCP (95.6% (28/29) and 69% (20/29), respectively) (Table 3). Regarding IgM against NCP, patients with acute disease showed an amount of positive results, similar to that obtained by studying T-cell responses (27.3% (9/33) for IgM and 38% (12/33) for T-cells). A lower rate of positive IgM results was observed in controls and convalescent participants (no response in controls (0/92), and 3.6% (1/29) in convalescents; data not shown).

Table 3. Overall positivity results obtained using antibody detection and T-cell IFN-γ production detection after spike and NCP stimulation including borderline results (%).

Groups	Antibody Response		T-Cell Response	
	IgG Spike (*n* = 155)	IgG NCP (*n* = 154)	Spike (*n* = 155)	NCP (*n* = 154)
Overall (*n* = 159)	86/155 (55.5)	55/154 (35.7)	63/155 (40.6)	32/154 (20.8)
Controls (*n* = 94)	33/92 (35.9)	4/92 (4.3)	17/92 (18.5)	1/92 (1.1)
Vaccinated (*n* = 30)	27/30 (90)	0/30 (0)	14/30 (46.7)	0/30 (0)
Unvaccinated (*n* = 64)	6/62 (9.7)	4/62 (6.5)	3/62 (4.8)	1/62 (1.6)
Acute (*n* = 36)	23/34 (67.6)	23/33 (69.7)	18/34 (52.9)	12/33 (38)
Asymptomatic (*n* = 6)	4/6 (66.7)	3/6 (50)	2/6 (33.3)	2/6 (33.3)
Mild (*n* = 3)	1/3 (33.3)	1/3 (33.3)	1/3 (33.3)	1/3 (33.3)
Moderate (*n* = 12)	8/12 (66.7)	9/12 (75)	9/12 (75)	4/12 (33.3)
Severe (*n* = 15)	10/13 (76.9)	10/12 (83.3)	6/13 (46.2)	5/12 (41.7)
Convalescent (*n* = 29)	29/29 (100)	28/29 (95.6)	28/29 (95.6)	20/29 (69)
Mild (*n* = 8)	8/8 (100)	7/8 (87.5)	8/8 (100)	6/8 (75)
Moderate (*n* = 10)	10/10 (100)	10/10 (100)	9/10 (90)	5/10 (50)
Severe (*n* = 11)	11/11 (100)	11/11 (100)	11/11 (100)	9/11 (81.8)

Positivity percentages were calculated excluding the indeterminate results from the total number of samples tested for each group. Denominator of each ratio indicates the total *n* for that antigen and that group of individuals that had a valid result.



(a) (b)

Figure 3. Correlations between the IFN-γ T-cell (SFCs) and IgG responses against spike (a) and NCP (b). AR refers to Absorbance Ratio (sample Abs/calibrator Abs). BAU refers to Binding Antibody Units. Grey areas show borderline results. Correlations were calculated using the two-tailed non-parametric Spearman test. In Figure 3a, results out of the calibration curve (equal or over 384 BAU/mL) were excluded from the Spearman test.

Thirty-three samples (21.3%) showed discrepancies when comparing IgG response against spike and/or NCP and T-cell response against any SARS-CoV-2 antigen. In this case, 27 of the 33 (81.8%) discrepancies were as a result of having IgGs but no T-cell response, and the opposite happened with the other 6 samples. Most of the samples with IgGs but no T-cell response were from vaccinated controls (51.9% (14/27)). In this case, 11 discrepancies were found in acute patients, being 4 of them (36.4%) positive for T-cell but not for IgG. Convalescent individuals did not show any discrepancies (Supplementary Table S4).

4. Discussion

The study and monitoring of the specific cellular and humoral responses against SARS-CoV-2 are of foremost importance for a better understanding of immunity during the acute phase of the disease and the long-lasting immunity after infection and/or vaccination, and probably in the long-COVID-19 syndrome. In this study we assessed the IFN-γ T-cell immune response against specific SARS-CoV-2 antigens and compared it with the humoral response in patients undergoing acute disease, during convalescence, and in unvaccinated and vaccinated individuals. Our results indicate that the IFN-γ T-cell response against SARS-CoV-2 is low during the acute phase of the disease but can increase over time as seen in this study in the case of convalescent individuals. Moreover, the overall cellular immune response triggered by vaccination was low, as around half of the vaccinated individuals did not show a response after the second dose administration, but humoral response is detected in the majority of cases. Finally, IgG levels correlated with the number of IFN-γ releasing T-cells.

In our study, a low IFN-γ T-cell response was observed in individuals undergoing the acute phase of the disease, particularly in severe patients. One plausible reason that can explain this is the inclusion of patients who have not yet developed an adaptive response in the initial phases of the disease. The IFN-γ release is essential for fighting viral infections, however, it is still not yet clear the strength of T-cell immune response during severe illness, as controversial data indicate that severe COVID-19 patients can have an insufficient but also an excessive response. Some manuscripts have reported that lymphopenia and anergy in severe acute patients may be a reason for that lack of cellular response [15–18]. Our results are consistent with these previous studies, indicating that lymphopenia and lower IFN-γ T-cell responses could correlate with disease severity [15,17–19]. In fact, the majority of samples initially excluded from the study with not enough cell counts were samples from moderate or severe COVID-19 patients (68.3%). Although, humoral response was seen in a higher number of acute COVID-19 patients than the T-cell response, in some samples no humoral response was seen while an IFN-γ T-cell response was detected. That strengthens the fact that the study of the T-cell responses could be important in COVID-19 management.

In addition, our numbers also indicate that convalescent individuals have a robust response against SARS-CoV-2, as has been also stated by other studies [20–22]. So, presumably, this response may play a fundamental role in the immune response against reinfection, as has been reported in other studies [23–26]. Moreover, convalescent individuals with a previous severe acute disease showed a higher cell response than those with milder forms. That increase in severe cases may be a result of either an exacerbated immune response during the disease, in combination with a greater initial viral inoculum (causing a severe disease and a larger adaptive response to the virus) [27,28]. In our study, convalescent individuals remained positive for both cellular and humoral responses, some of them even seven months after resolving the disease. It can therefore be assumed that after an acute SARS-CoV-2 episode, an individual is less susceptible to being infected due to the adaptive response generated. Despite this, reinfections have been reported to occur in convalescent individuals and are associated with lower risk of severe disease [13,29]. In fact, the risk of reinfection with older variants has been seen to be low, but increases with the emergence of new variants [30,31]. Information on variants affecting patients from our study can be of utility for understanding immune protection against reinfection. Despite not being this specific information recorded, the main circulating variants during the study period

can be traceable according to our national Ministry of Health registries. Briefly, Alpha started to account for more than half of the circulating variants in January 2021. Next, Delta prevalence reached 5% in April 2021 and 95% in August 2021. Omicron represented more than 5% of cases for the first time in December 2021 [32].

When comparing the response between infection and vaccination, our study shows that an IFN-γ T-cell response is stronger in those patients who have undergone the disease than in those individuals who have been vaccinated. This should be further investigated, as the response has been described to be variable in vaccinated individuals and vaccines may not be equally effective against new SARS-CoV-2 variants [8]. The study published by Gazit S et al. showed that unvaccinated individuals had around a 13-fold increased risk to be infected with Delta variant than people with a past infection [33]. On the contrary, other studies have shown that vaccinated individuals had similar responses to convalescent ones, producing a cellular response at the same level as the humoral, even at an earlier stage. Moreover, in some vaccinated individuals a reduced IFN-γ T-cell response but an increased IL-2 T-cell response has been detected and may play a role in the long-term protection against infection [34,35]. Hence, for a better understanding of the T-cell response generated after vaccination and infection, other inflammatory cytokines should be studied. Spike has been seen to be the most immunogenic antigen of the three studied, although responses against NCP and membrane antigens must not be overlooked when comparing the immune response between natural infection and vaccination, as they also play a role in the protection against reinfection, generating a dominant response by cytotoxic T-cells, as reported in previous studies [20,36,37]. Finally, attending to the humoral response, overall results in our study indicate a moderate correlation between IgG and T-cell response levels for spike and NCP, as reported earlier [38]. However, we also observe that vaccination elicits a higher IgG response when compared to the IFN-γ T-cell one. Altogether, understanding which factors are responsible for these different responses is of great importance.

In addition to IFN-γ T-cell response, other inflammatory cytokines may be interesting to understand the immunopathology and the protection against reinfection. Looking into our results on IFN-γ, a high individual inter-variability was observed when monitoring COVID-19 patients and their outcomes, so it would be interesting to study other different cytokines and cell populations as possible prognosis markers. In line with this, sustained IL-6 and TNF-α production has been reported to be related to a low maturation of monocytes, also influencing the depletion of different cellular subsets including CD4+ T-cells [39]. These and other cytokines involved in the immunopathology of acute patients such as IL-1β and IL-10 could be fundamental for the discrimination of the outcome of the infection as they have been seen to be significantly higher in severe than in mild forms of the disease [15,40]. On the other hand, a strong and specific IL-2 response has been detected in COVID-19 recovered individuals [13,18,23], hence this cytokine's role in immune protection should be considered. Further studies should aim to assess the secretion of other cytokines in the context of SARS-CoV-2 infection and vaccination as well as the involvement of different cell populations.

This study has several drawbacks that should be addressed. First, some groups such as acute COVID-19 or vaccinated individuals have a relatively small sample size; consequently, statistical strength can be reduced. However, even though this limitation, patients from these study groups are clinically and microbiologically well-characterized, being possible to investigate the immune response according to each clinical situation. Second, SARS-CoV-2 variants were not documented for the individuals that have been or were diagnosed with the virus, limiting the interpretation of the results depending on this factor. Despite not being able to classify each sample according to the virus variant causing the infection, this information could be traceable as periods of prevalence of the different variants are updated in epidemiological documents from our national Ministry of Health [32]. Third, vaccinated individuals were not followed-up, impeding the assessment of the response of each individual through time and the comparison of the response after first and second doses. In future studies the impact of third and coming fourth doses of the vaccine in T-cell

response should be studied. Finally, classification according to severity of the COVID-19 disease followed in this study, as happens in other studies, did not completely fulfill with the classification recommended by WHO Working Group on the clinical characterization and management of COVID-19 [41], however it is rigorous and scientifically based to provide reliable results and conclusions.

Taking these facts into consideration, our findings show that measuring the T-cell responses is valuable to understand the picture of the immunity against SARS-CoV-2. We have provided data sustaining that spike, NCP, and membrane antigens from the virus can elicit the release of IFN-γ by specific T-cells, indicating that the last two antigens should not be overlooked in potential vaccine design and identification of the immune status. In addition, the IFN-γ T-cell response was low in the active phase of the disease, particularly in severe individuals. This response increased during convalescence, indicating that the adaptive T-cell response against the pathogen needs some time to be generated. According to the findings obtained for vaccinated individuals, our data suggest that the T-cell response is not always triggered after vaccination with an mRNA vaccine, however, it is compensated by the humoral immune response. Altogether, both types of responses are important against infection and towards protection and offer valuable information to understand the overall picture of the adaptive immunity against SARS-CoV-2.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jcm11175103/s1, Table S1. Descriptive table with information of the samples included in the study (time since diagnostic and vaccination, and lymphopenia at the moment of sampling); Table S2: Overall positive results for the T-cell IFN-γ secretion against high homology regions of endemic (human) coronaviruses included in the assay; Table S3: Overall positive results for SARS-CoV-2 antigens in samples from patients undergoing acute disease according to days after semi-intensive care admission including borderline results (%); Table S4: Comparison of the IFN-γ T-cell response with IgG response results. Overall comparison means positive for each of the antigens studied by both techniques (spike, NCP, and membrane for the IFN-γ T-cell studies, and spike and NCP for IgG detection); Figure S1: T-cell response (SFCs) follow-up in acute patients. Red lines indicate patients who died during the SARS-CoV-2 acute phase. Grey area shows borderline results; Figure S2: Correlation of the (a) IFN-γ T-cell response (SFCs; $n = 54$) and (b) IgG ($n = 21$) against spike in patients with acute disease and their lymphocyte concentration (lymphocyte/μL). The borderline area is shown in grey. On the left side of the discontinuous vertical line (<1200 lymphos/μL) lymphopenia is considered. Correlation was calculated using the two-tailed non-parametric Spearman test; Figure S3. Number of SFCs after stimulation with NCP (a), and membrane (b) in the different study groups. Horizontal lines represent medians. Grey area shows borderline results. Differences between conditions were calculated using the two-tailed Mann-Whitney U test. p is considered significant when <0.05 (** <0.01, *** <0.001, and **** <0.0001).

Author Contributions: I.L., G.S., R.V.-H., I.C., A.R. and J.D. designed the study. R.V.-H., I.L. and G.S. designed the experiments. B.M.-M., S.D.-F., A.C., L.S., R.V.-H., G.T., I.L. and G.S. performed the experiments. Z.S., A.M., C.P.-C., A.L., A.J.S., F.A., J.M. and I.C. contributed with resources. A.R. and J.D. supervised the study. J.D., I.L. and G.S. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy reasons.

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Article 2

Measurement of IFN-γ and IL-2 for the assessment of the cellular immunity against SARS-CoV-2

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Measurement of IFN-γ and IL-2 for the assessment of the cellular immunity against SARS-CoV-2

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The study of specific T-cell responses against SARS-CoV-2 is important for understanding long-term immunity and infection management. The aim of this study was to assess the dual IFN-γ and IL-2 detection, using a SARS-CoV-2 specific fluorescence ELISPOT, in patients undergoing acute disease, during convalescence, and after vaccination. We also evaluated humoral response and compared with T-cells with the aim of correlating both types of responses, and increase the number of specific response detection. Blood samples were drawn from acute COVID-19 patients and convalescent individuals classified according to disease severity; and from unvaccinated and vaccinated uninfected individuals. IgGs against Spike and nucleocapsid, IgMs against nucleocapsid, and neutralizing antibodies were also analyzed. Our results show that IFN-γ in combination with IL-2 increases response detection in acute and convalescent individuals ($p = 0.023$). In addition, IFN-γ detection can be a useful biomarker for monitoring severe acute patients, as our results indicate that those individuals with a poor outcome have lower levels of this cytokine. In some cases, the lack of cellular immunity is compensated by antibodies, confirming the role of both types of immune responses in infection, and confirming that their dual detection can increase the number of specific response detections. In summary, IFN-γ/IL-2 dual detection is promising for characterizing and assessing the immunization status, and helping in the patient management.

The coronavirus disease 2019 (COVID-19) pandemic caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has been the source of a great number of infections, hospitalizations, and deaths worldwide[1]. Although a lot of efforts to control the pandemic, such as developing vaccines and achieving herd immunity, have been accomplished, the post-acute phase of the pandemic is still a concern, as host immunity differs among individuals leading to different disease outcomes[2,3]. A well-stablished specific adaptive response is necessary to eliminate the virus and avoid an aggravation of the disease; however, an imbalanced immune response can lead to a worse outcome due to lymphopenia or inflammation's exacerbation[4,5]. In addition, this response is elemental in the protection against severe outcomes and reinfections[6,7]. Therefore, the study and measurement of T-cell and humoral responses against SARS-CoV-2 are relevant for understanding the correlates of protection to reinfection and for the proper clinical management of COVID-19.

The study of IFN-γ release by T-cells has been widely used and measured by IFN-γ release assays (IGRAs) to evaluate adaptive cellular responses, as is the case for *Mycobacterium tuberculosis* infection diagnosis[8,9]. IFN-γ secretion is involved in multiple functions, including increasing antigen presentation, inducing antiviral status (prevention of viral replication and induction of apoptosis) and stimulating the expression of numerous genes

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related to the inflammatory process[10]. Recently, the utility of measuring the specific IFN-γ released by T-cells in the context of SARS-CoV-2 has also been assessed, showing that IFN-γ is increased during convalescence compared to the acute disease phase. In addition, a large proportion of fully vaccinated individuals (with two doses of a mRNA SARS-CoV-2 vaccine) do not show IFN-γ release[11,12]. Although a good correlation between humoral and IFN-γ T-cell responses has been previously reported[13,14] a higher presence of humoral response is usually found in vaccinated and convalescents[11]. These findings show that IFN-γ T-cell responses can give a great overview of the specific SARS-CoV-2 cellular immunity, and that the assessment of other cytokines can provide more information on individuals' immunological status and outcome, especially in cases with a lack of IFN-γ response[15]. IL-2-secreting T-cells have been proven to be essential in the modulation of the development, homeostasis, and regulation of T-cells, having a very important role in the adaptive immune response. IL-2 secretion is crucial for memory T-cell development, and their proliferation and maintenance when facing a specific antigen[16–18]. Therefore, analyzing the production of this cytokine against specific SARS-CoV-2 antigens can contribute to having a better picture of memory T-cell responses and protection after vaccination and throughout convalescence[15,19,20].

Despite the many efforts to understand T-cell responses against SARS-CoV-2, more studies are required to shell the complexity of these responses during infection and to characterize the long-term immunity conferred by infection and vaccination. As IFN-γ and IL-2 are cytokines with a key role in the adaptive immune response during acute infection and T-cell memory, we hypothesize that the combined study of both cytokines will help in the management of SARS-CoV-2 infection, providing information on the immunological status in different clinical situations. Therefore, the main objective of this study was to assess the dual IFN-γ and IL-2 detection, using a SARS-CoV-2 specific fluorescence ELISPOT, in patients undergoing acute disease, during convalescence, and after vaccination. Additionally, as previously seen in other studies, humoral and T-cell response is impaired in some situations, thus, in this study, IgG, IgM, and neutralizing antibodies were also evaluated and compared with T-cells with the aim of correlating both types of responses, and increase the number of specific response detection.

Results

IFN-γ and IL-2 T-cell responses increased the number of positive responses

A total of 263 samples from 232 individuals were included in the study from July 2020 to May 2022. Ninety-three (35.4%) were from uninfected participants, 66 (25.1%) were from 35 acute patients, and 104 (39.5%) were from individuals during the convalescent phase. Overall, 19 of the 263 samples (7.2%) were indeterminate for CoV-iSpot and therefore excluded from the analysis. From the indeterminate samples, 11 were from acute COVID-19 patients, 7 from convalescent, and 1 from an uninfected unvaccinated participant. Ten samples (3.8%) were indeterminate only for IFN-γ and 15 (5.7%) for IL-2; 6 samples were indeterminate for both tests.

The number of individuals detected with immune response against SARS-CoV-2 increased when IFN-γ and also IL-2 T-cell response was studied [76.6% (187/244)]. The pancoronavirus panel triggered an IFN-γ or IL-2 T-cell positive responses in 35.2% and 28.7% of the samples, respectively (Supplementary Table 1). The pancoronavirus panel is a pool of spike, nucleocapsid, membrane, envelope and orf1 protein (conservative region) of the coronavirus family. According with the manufacturer description, the pool excludes homologies with the SARS-CoV-2 panel. Therefore, having a positive response for this particular panel means a response or previous exposition to seasonal coronaviruses. Positive responses for one of the cytokines and negative for the other were also analyzed for the SARS-CoV-2 specific panel (Supplementary Table 2).

IFN-γ and IL-2 T-cell responses differ according to the clinical situation

T-cell responses in unvaccinated uninfected individuals were only found in 4 out of 20 individuals (20%), 3 (15%), and 1 (5%) of them being positive for IFN-γ and IL-2 respectively. These results evidence a previous SARS-CoV-2 infection not detected before the inclusion. Vaccinated uninfected individuals showed a lower rate of positive IFN-γ T-cell response than IL-2 [68% (49/72) for IFN-γ response vs 81% (58/72) for IL-2]. Sixty of the 72 vaccinated individuals (83%) showed a positive response for either IFN-γ or IL-2 responses (Table 1).

Acute COVID-19 patients had a slightly higher rate of positive IFN-γ responses than IL-2 [61.9% (34/55) IFN-γ vs 56.4% (31/55) IL-2; and 65.4% (36/55) had at least a positive result for one of the two cytokines ($p = 0.66$)]. Positive responses in the acute group tended to increase with severity for both cytokines, but severe patients under IMV had a lower number of IL-2 positive results than those under NIV [71% (22/31) for NIV vs 62.5% (5/8) for IMV]. When positivity was analyzed according to the days of hospitalization in the acute COVID-19 patients, positive responses tended to increase after hospitalization except for samples taken on day 28 (Supplementary Table 3). Quantity of IFN-γ or IL-2 response was analyzed in the 17 monitored patients, and high inter-individual variability was observed over time. No differences were observed when comparing the evolution of the response through the follow-up according to disease outcome (Supplementary Fig. 1).

Convalescent individuals had higher rates of positivity when compared to acute patients for both cytokines [82.5% (80/97) in convalescent vs 61.8% (34/55) in acute patients for IFN-γ; 86.6% (84/97) in convalescent vs 56.4% (31/55) in acute for IL-2]. High amount of positive responses for at least one of the cytokines was detected in 87/97 (89.7%) of the convalescent individuals in comparison with detected IFN-γ or IL-2 alone ($p = 0.023$). Positive responses for IL-2 increased with the severity of the disease and for IFN-γ they followed a similar pattern except for severe NIV patients (Table 1).

As shown in Fig. 1a and b, when analyzing the quantity of specific IFN-γ and IL-2 T-cell responses (SI values) in the SARS-CoV-2 panel, as expected, significantly higher response was triggered in the uninfected vaccinated individuals compared to the unvaccinated for both cytokines ($p < 0.0001$). On the other hand, when analyzing the quantity of specific IFN-γ and IL-2 T-cell responses (SI values), they were significantly higher in convalescent

	IFN-γ$^+$	IL-2$^+$	IFN-γ$^+$ and/or IL-2$^+$
Uninfected (n = 92)	52/92 (56.5)	59/92 (64.1)	64/92 (70)
Unvaccinated (n = 20)	3/20 (15)	1/20 (5)	4/20 (20)
Vaccinated (n = 72)	49/72 (68)	58/72 (81)	60/72 (83)
Acute disease (n = 55)	34/55 (61.9)	31/55 (56.4)	36/55 (65.4)
Mild (n = 5)	2/5 (40)	1/5 (20)	2/5 (40)
Moderate (n = 4)	2/4 (50)	2/4 (50)	3/4 (75)
Severe NIV (n = 31)	22/31 (71)	22/31 (71)	23/31 (74.2)
Severe IMV (n = 8)	6/8 (75)	5/8 (62.5)	6/8 (75)
Dead (n = 7)	2/7 (28.6)	1/7 (14.3)	2/7 (28.6)
Convalescent (n = 97)	80/97 (82.5)	84/97 (86.6)	87/97 (89.7)
Mild (n = 23)[a]	15/23 (65.2)	16/23 (69.6)	17/23 (73.9)
Moderate (n = 19)[a]	17/19 (89.5)	16/19 (84.2)	18/19 (94.7)
Severe NIV (n = 26)[a]	21/26 (80.8)	23/26 (88.5)	23/26 (88.5)
Severe IMV (n = 29)[a]	27/29 (93.1)	29/29 (100)	29/29 (100)

Table 1. Percentage of samples with positive T-cell responses against SARS-CoV-2 peptides (%). Positivity percentages were calculated excluding the indeterminate results from the total number of samples tested for each group. *NIV* non-invasive ventilation, *IMV* invasive mechanical ventilation. [a]Severity considered during their acute COVID-19 episode.

than in acute cases ($p = 0.023$ and $p < 0.0001$, respectively). Although not significant, IFN-γ and IL-2 responses in acute COVID-19 patients showed an increase with severity. Severe acute patients with IMV and NIV displayed significantly higher responses than those who did not survive the disease (for IFN-γ: $p < 0.05$ for both comparisons; for IL-2: $p < 0.05$, only significant when comparing IMV with death). In addition, the SI increased with severity in convalescent individuals. Moreover, moderate convalescent individuals showed an increased IFN-γ or IL-2 response when compared to those with moderate acute disease ($p < 0.05$ for both cytokines). For IL-2, differences were also significantly higher in severe patients with NIV between the convalescence and the acute phase ($p < 0.01$). Interestingly, IFN-γ and IL-2 responses were higher in severe convalescent cases than in vaccinated individuals (for both cytokines: $p = 0.0001$ in convalescent individuals with IMV vs uninfected vaccinated; and $p = 0.001$ in convalescent individuals with NIV vs uninfected vaccineted).

Considering the ratio between both responses (SI IL-2/SI IFN-γ) (Fig. 2), acute patients showed a median ratio below 1, indicating a higher quantity of IFN-γ specific responses than IL-2. In contrast, median ratios higher than 1 were obtained for vaccinated individuals and severe convalescent individuals. Although no significant differences were observed on these comparisons, we found these results relevant as there is a trend that IFN-γ is highly secreted in acute patients (ratios below 1) and IL-2 in convalescent ones (ratios for NIV and IMV above 1). To compare both responses, a correlation between the SI of both IFN-γ and IL-2 from each of the samples was performed. Globally, a high correlation was observed between both responses ($SR = 0.743$, $p < 0.0001$). This correlation was also performed in uninfected vaccinated, acute, and convalescent groups, showing a moderate correlation in vaccinated individuals ($SR = 0.58$, $p < 0.0001$), and a high correlation in acute and convalescent patients ($SR = 0.805$, $p < 0.0001$ for acute patients; $SR = 0.7$, $p < 0.0001$ for convalescent, Supplementary Fig. 2a–c, respectively).

T-cell response with time differs according to immunization through vaccination or disease

Correlations between IFN-γ/IL-2 T-cell responses and time after vaccination were performed. No significant correlation was found between T-cell responses and time after vaccination for uninfected vaccinated individuals ($SR = 0.164$, $p = 0.169$ for IFN-γ; $SR = 0.04$, $p = 0.732$ for IL-2, Fig. 3a). In addition, after the second dose of the vaccine, 47 of the 66 samples tested (71.2%) showed an IFN-γ T-cell response, while 56 of the 66 (84.8%) showed an IL-2 T-cell one. Fifty-eight of the 66 samples with at least two vaccine doses (87.9%) had any response to IFN-γ and/or IL-2 response.

Responses for both cytokines tended to increase over time in acute patients, being significant in the case of IL-2 response ($SR = 0.226$, $p = 0.097$ for IFN-γ; $SR = 0.332$, $p = 0.012$ for IL-2, Fig. 3b). The same correlation was performed for convalescent patients and no significant increased response through time was seen for either IFN-γ or IL-2 ($SR = 0.049$, $p = 0.64$ for IFN-γ, $SR = 0.004$, $p = 0.97$ for IL-2, Fig. 3c). Regarding time since symptomatology appearance, a significant increase through time was seen for both IFN-γ and IL-2 responses ($SR = 0.341$, $p = 0.0165$ for IFN-γ; $SR = 0.376$, $p = 0.008$ for IL-2, Fig. 3d).

T-cell and humoral immune response had moderate correlations but were impaired in some clinical situations

When analyzing humoral response excluding vaccinated uninfected individuals, IgG against nucleocapsid (NCP) was found in 133 of the 172 samples (77.3%, Table 2). Eighteen of the 55 acute patients showed IgM against NCP response (32.7%). Neutralizing antibodies were detected in 200 samples (82%), 197 of them also positive for IgGs against Spike (98.5%). IgGs against Spike had the highest rates of positive responses in the uninfected vaccinated

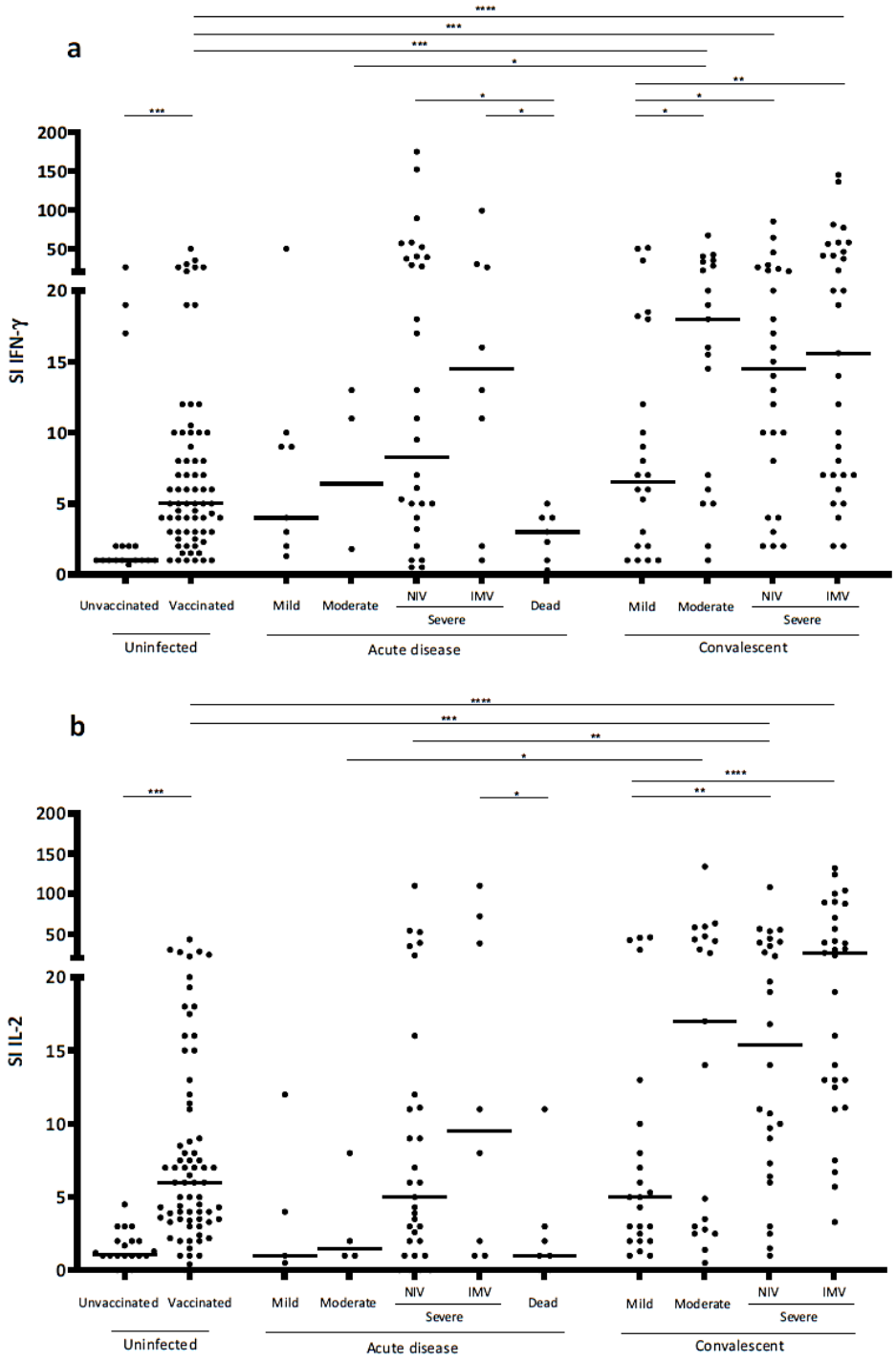

Figure 1. IFN-γ (a) and IL-2 (b) T-cell responses against the SARS-CoV-2 antigens. The specific response for each cytokine is represented using the SI (stimulation Index). Differences between two group conditions were calculated using the two-tailed Mann–Whitney U test. P is considered significant when < 0.05 (* < 0.05, ** < 0.01, *** < 0.001, and *** < 0.0001). SI: Stimulation index; Non-Invasive Ventilation (NIV); Invasive Mechanical Ventilation (IMV).

and convalescent groups [70/72 (97.2%) in uninfected vaccinated and 96/97 (99%) in convalescent]. IgG against NCP was the humoral response most found in acute patients [45/55 (81.8%)]. Neutralizing antibodies specific for mutations in the spike antigen from other variants different to the ancestral one, as omicron variants, are

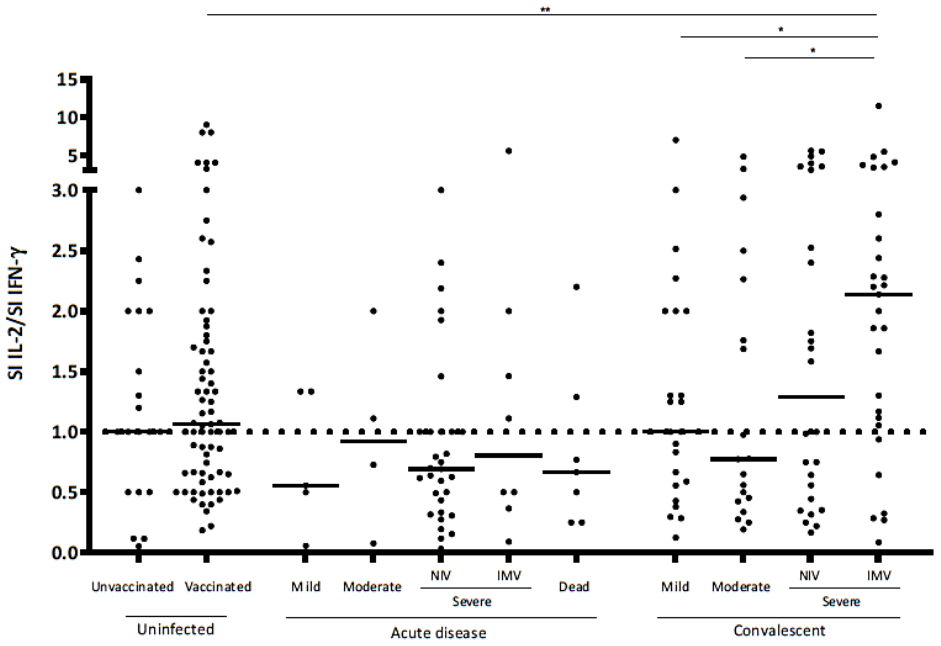

Figure 2. SI ratios between both cytokine responses (SI IL-2/SI IFN-γ) for the different study groups. Values over 1 meant higher IL-2 response whilst values below 1 meant higher IFN-γ response. Samples with a 0 value for IFN-γ SI and IL-2 higher than 0 were excluded for avoiding indeterminate results in the ratio calculation. Differences between conditions were calculated using the two-tailed Mann–Whitney U test. P is considered significant when < 0.05 (* < 0.05, ** < 0.01, *** < 0.001, and *** < 0.0001). SI: Stimulation index; Non-Invasive Ventilation (NIV); Invasive Mechanical Ventilation (IMV).

detected with this assay with a low sensitivity, therefore, the percentage of neutralization can be underestimated for some participants[21].

Both T-cell and humoral immune response were analyzed and correlated to detect those impaired cases and thus increase the number of specific response detections. IgG response showed significant moderate correlations with both IFN-γ (SR = 0.451, p < 0.0001 for IgGs against Spike excluding > 384 BAU/mL results, and SR = 0.397, p < 0.0001 for IgG against NCP) and IL-2 responses (SR = 0.583, p < 0.0001 for IgG against Spike excluding > 384 BAU/mL results, and SR = 0.464, p < 0.0001 for IgG against NCP; Fig. 4a and b). A moderate significant correlation was seen in acute individuals between IgM response against NCP and IFN-γ response (SR = 0.456, p = 0.0005), and a weak correlation with IL-2 response (SR = 0.304, p = 0.02, Fig. 4c). Weak correlations were found between the percentage of neutralization and IFN-γ/IL-2 T-cell responses (SR = 0.282, p < 0.0001 for IFN-γ and SR = 0.382, p < 0.0001, Fig. 4d).

Discrepancies between T-cells (positive for IFN-γ and/or IL-2) and the different humoral responses (IgG against Spike, IgG against NCP, and neutralizing antibodies) were analyzed (Supplementary Tables 4, 5 and 6). Most of the discrepancies were due to having a humoral but not a T-cell response. When comparing T-cell with Spike IgG response, 38 discrepancies were found [38/244 (15.6%)], most of them (28/38, 73.7%) due to having positive IgG response against Spike but no cellular one (neither IFN- γ nor IL-2) [in particular, 11/72 (15.3%) of these discrepancies were found in uninfected vaccinated individuals, 7/55 (12.7%) in acute patients, and 9/97 (9.3%) in convalescents; supplementary Table 6]. Similarly, positive humoral and negative T-cells responses discrepancies were found when comparing nucleocapsid IgGs [13/55 (23.6%) in acute patients, and 7/97 (7.2%) in convalescents; Supplementary Table 6] or neutralizing antibodies with cytokine responses [11/72 (15.3%) in uninfected vaccinated individuals; 15/55 (27.3%) in acute patients, and 9/97 (9.3%) in convalescents; Supplementary Table 6]. On the contrary, more positive cellular immune responses were detected when compared with IgM against nucleocapsid in acute patients. Interestingly, a total of 21 from 55 patients (38.2%) with acute disease had a positive T-cell response (IFN-γ and/or IL-2), but a negative IgM against nucleocapsid response (Supplementary Table 6). Therefore, the T-cell response still contributed by detecting some specific responses against the pathogen when the humoral response was not found, particularly in acute patients (Supplementary Tables 4, 5 and 6).

Discussion

Understanding the T-cell response to SARS-CoV-2 is crucial to determine cellular immunity generated after infection or vaccination as well as to characterize long-term immunity and protection against reinfection. While IFN-γ has been shown as crucial, other cytokines such as IL-2 may also play an important role in memory and protection against SARS-CoV-2. In this study, we assessed IFN-γ and IL-2 released by T-cells from COVID-19

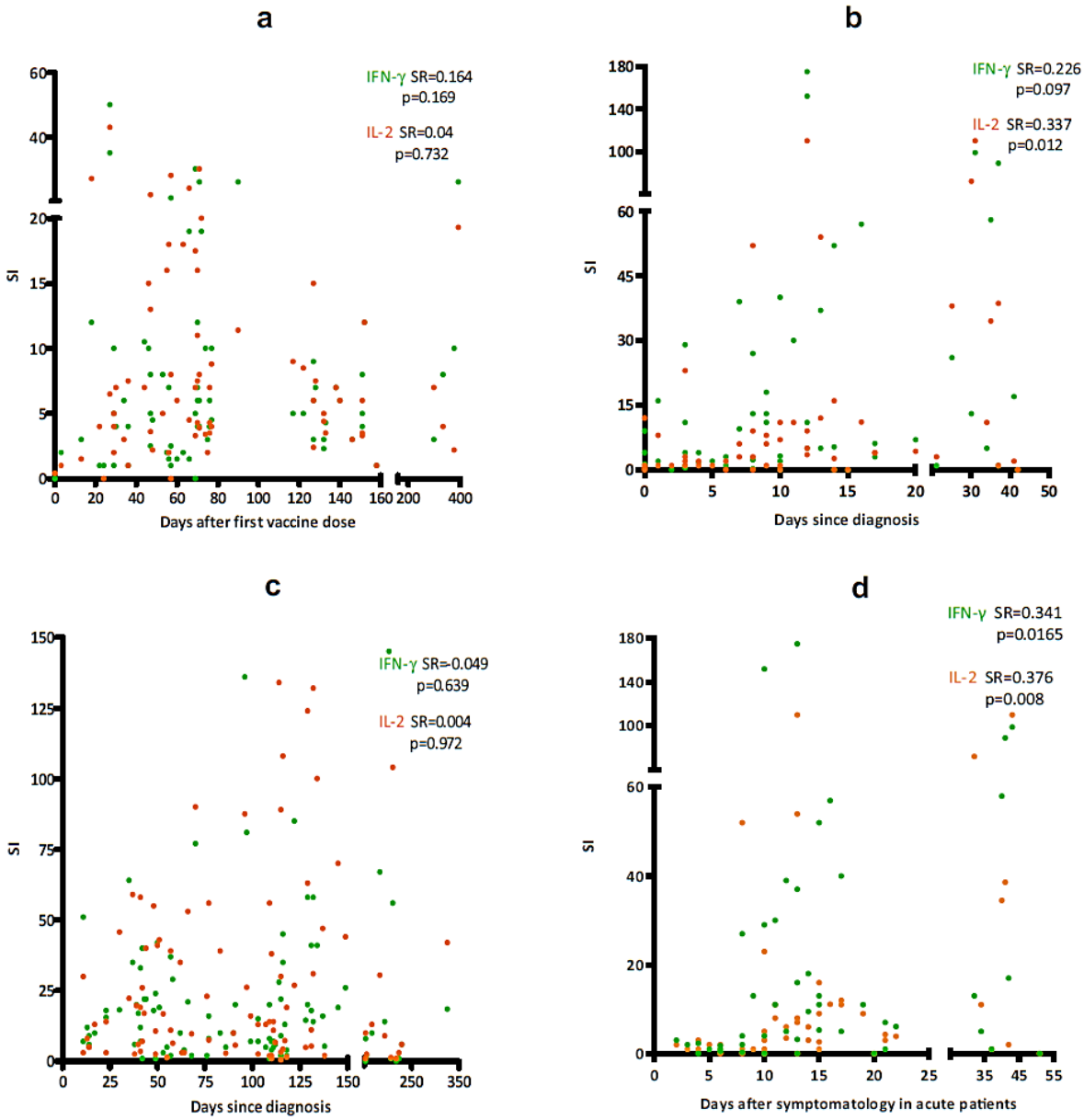

Figure 3. Correlations between IFN-γ (green) and IL-2 (orange) T-cell response with time since the first dose of the vaccine administration (**a**); time since diagnosis in acute patients (**b**) and convalescent individuals (**c**); and time since symptomatology appearance in acute patients (**d**). For figure (**b**), some samples were collected 35 days post-diagnosis during patient hospitalization in semi-critical or in intensive care units with severe symptomatology. For (**c**), some samples were collected 10 days after diagnosis but are considered convalescent as they correspond to healthcare workers with mild symptoms or without symptoms during the infection and have either a posterior negative PCR during their routine examinations or a finalization of the symptomatology. The specific response for each cytokine is represented using the SI. Correlations were calculated using the two-tailed non-parametric Spearman test. SI: Stimulation index.

patients and from vaccinated as well as unvaccinated uninfected individuals. This response was also compared to the humoral one.

Our main findings suggest that T-cell response is lower during the acute phase of the disease in comparison to convalescence. Moreover, IL-2 cytokine was higher than IFN-γ in vaccinated and convalescent individuals. Our results also indicate that although some discrepancies in antibody-positive and T-cell-negative responses were detected, a good correlation between humoral-cellular responses was observed. In our study, an increased response of both IFN-γ and IL-2 cytokines was seen in convalescent individuals, in comparison to acute COVID-19 patients. This finding has been previously described for IFN-γ, being attributed to a presumably not well-established adaptive immune response and, as a consequence, a very low specific cytokine secretion in acute patients[11,22,23]. In addition, when comparing results between both cytokines, IFN-γ release was higher than that

	IgG spike	IgG NCP	IgM NCP	Neutralizing	Any Ab
Uninfected (n = 92)	72/92 (78.3)	4/92 (4.4)	2/92 (2.2)	70/92 (76.1)	73/92 (79.3)
Unvaccinated (n = 20)	2/20 (10)	1/20 (5)	1/20 (5)	3/20 (15)	3/20 (15)
Vaccinated (n = 72)	70/72 (97.2)	3/72 (4.2)	1/72 (1.4)	67/72 (93.1)	70/72 (97.2)
Acute disease (n = 55)	38/55 (69.1)	45/55 (81.8)	18/55 (32.7)	36/55 (65.5)	48/55 (87.3)
Mild (n = 5)	3/5 (60)	3/5 (60)	1/5 (20)	3/5 (60)	3/5 (60)
Moderate (n = 4)	1/4 (25)	3/4 (75)	1/4 (25)	2/4 (50)	3/4 (75)
Severe NIV (n = 31)	25/31 (80.6)	29/31 (93.5)	11/31 (35.5)	22/31 (71)	30/31 (96.8)
Severe IMV (n = 8)	7/8 (87.5)	7/8 (87.5)	5/8 (62.5)	6/8 (75)	7/8 (87.5)
Dead (n = 7)	2/7 (28.6)	3/7 (42.9)	0/7 (0)	3/7 (42.9)	5/7 (71.4)
Convalescent (n = 97)	96/97 (99)	87/97 (89.7)	9/97 (9.3)	94/97 (96.9)	96/97 (99)
Mild (n = 23)	22/23 (95.7)	14/23 (60.9)	1/23 (4.3)	21/23 (91.4)	22/23 (95.7)
Moderate (n = 19)	19/19 (100)	18/19 (94.7)	1/19 (5.3)	18/19 (94.7)	19/19 (100)
Severe NIV (n = 26)	26/26 (100)	26/26 (100)	4/26 (15.4)	26/26 (100)	26/26 (100)
Severe IMV (n = 29)	29/29 (100)	29/29 (100)	3/29 (10.3)	29/29 (100)	29/29 (100)

Table 2. Percentage of positive responses against SARS-CoV-2 peptides.

of IL-2 during acute disease, contrary to the convalescent phase where higher IL-2 levels were observed instead. Moreover, when combining IL-2 with IFN-γ the percentage of positive responders increased, pointing out the overlooked information when only measuring IFN-γ[15,24]. As reported in other studies, effector T-cells probably secrete IFN-γ, since it is the cellular subset actively fighting the infection, whereas IL-2 would be secreted by memory T-cells[25]. Furthermore, IL-2 can represent a good protection biomarker as it has a central role in the maintenance of memory T-cell populations and their effector functions, being secreted mainly by memory CD4+ T-cells and enhancing the activity of both NK and CD8+ T-cells[26]. In this sense, knowing the roles that both cytokines play in the adaptive response against SARS-CoV-2, would allow an understanding of immune protection against the virus and reinfection, as reported for other viruses[26]. It should also not be overlooked that innate immune response is also participating in the release of IFN-γ in a smaller level by means of NK cells found inside the PBMCs population, and that this cellular subset is fundamental in the beginning of the infection. Further studies in this way should also be performed[27].

It is important to mention that COVID-19 severe patients with a fatal outcome (death) showed particularly diminished T-cell responses when compared to other groups. Lymphopenia and anergy due to apoptosis in some immune cellular subsets, particularly cytotoxic T-cells, have been reported in critical COVID-19 patients[23,28–30]. These patients are described as having a weakened immunity to SARS-CoV-2 due to an inability to mount a functional specific adaptive response to preclude the infection and stop viral replication, and as a result, have an unrestrained dissemination of the infection leading to death[28–31]. Moreover, it is observed here that T-cell responses increased according to the severity (excluding death) in those acute and convalescent patients, resulting in high levels of IFN-γ and even higher of IL-2 during severe disease compared with mild forms. This can be explained as a result of high viral loads in those severe patients during the acute phase[32,33]. On the contrary, it has been reported that disease severity is not related to specific CD4+ T-cells and CD8+ T-cell subsets secreting IFN-γ and IL-2, but to other inflammatory cytokines being secreted aberrantly, and being related to a worse infection and tissue damage[34]. Although our results indicate that specific IFN-γ and IL-2 cytokines are increased during severity, we hypothesize that their release is a consequence of a severe inflammatory scenario to avoid a fatal outcome, and could be used as a prognosis biomarker for clinical management of severe COVID-19 patients. Therefore, the lack of response would indicate that the patient is not producing a good inflammatory response and could die due to the inability to fight SARS-CoV-2 infection. These findings collide with our previous study[11], as we described that severe COVID-19 patients had lower IFN-γ responses. A possible explanation for this contradiction is that in this prior study, severe patients were not divided into two groups depending on the ventilatory requirements, including also patients with fatal outcomes in the same group, and as a consequence, affecting directly the decrease of T-cell responses in severe individuals. Moreover, another immunoassay was used for the previous study and therefore different approaches in the design between the tests may be affecting to the final results.

When comparing the specific response after infection with that generated after vaccination, our data indicates that specific T-cell responses were significantly higher in convalescents than in non-infected vaccinated individuals. Controversial data in this matter has been described, as some studies report a similar response after vaccination than that found during convalescence; nevertheless, others are in line with those reported here[11,35]. In this sense, some studies have reported an increased risk of reinfection in vaccinated individuals when compared to convalescent ones or individuals with hybrid immunity[24,35,36]. In line with that, assessing the overall T-cell response with a pool of SARS-CoV-2 antigens can provide information about this increased protection against reinfection, as response against all the antigens is found when having had previous infection (convalescent individuals and individuals with hybrid immunity). Differences observed between the immune response generated after vaccination and infection reside on the type of immunity triggered. Vaccination is focused both in humoral and cellular induction. The role of humoral response is to prevent infection, but it has been shown to be waning few years after immunization; instead, memory T-cell response has an essential role in long-term immunity, as

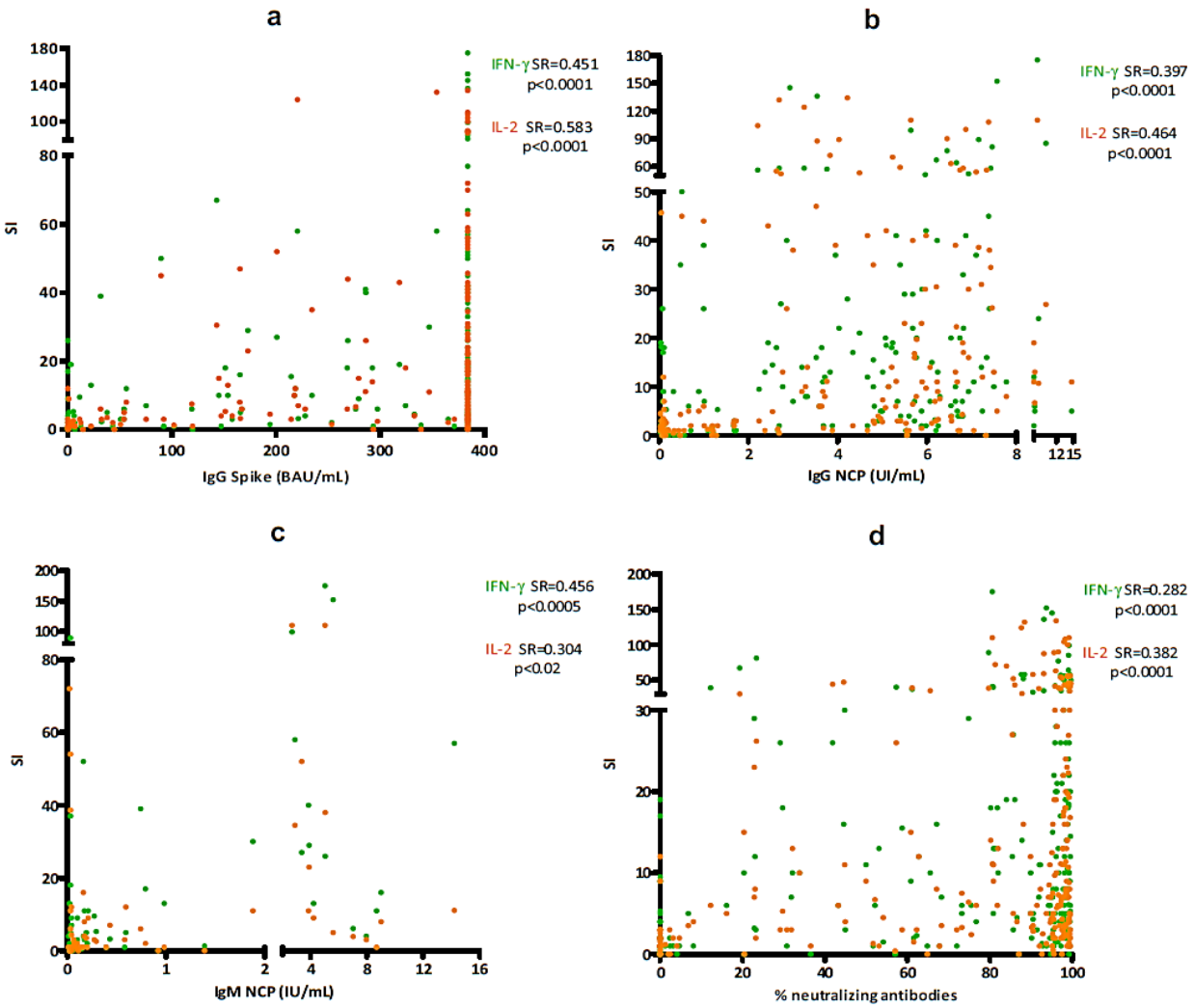

Figure 4. Correlations between IFN-γ (green) and IL-2 (orange) T-cell response with IgGs against Spike (a), IgGs against NCP (excluding the uninfected vaccinated individuals) (b), IgMs in acute patients (c), and percentage of neutralization (d). The specific response for each cytokine is represented using the SI. Correlations were calculated using the two-tailed non-parametric Spearman test. In (a), results equal to or over 384 BAU/mL were excluded for statistical analysis (the results including the ones over > 384 BAU/ml maintained significance and moderate correlations: SR IFN-γ = 0.317; SR IL-2 = 0.429). SI: Stimulation index. BAU: Binding antibody units.

seen during convalescence, and has been seen to be more durable[37–39]. In addition, vaccine design has only been centered on triggering a limited response against Spike antigen, leaving behind nucleocapsid and membrane antigens which would give a broader protection against new infections. Moreover, T-cell response has been seen to be cross-protective to fight variants of concern (VOCs) when the humoral response is compromised[28]. As discussed before, it has been shown that IL-2 is increased during convalescence, suggesting the importance of IL-2 in memory T-cell response. Therefore, the measurement of IL-2, in combination with IFN-γ, could be considered in diagnostics and the immune status evaluation, as they may determine the response conferred after vaccination or disease, providing an accurate definition of the immune cellular status of the individual against SARS-CoV-2[26]. Thus, these measurements could be a useful tool for clinicians to better manage their patients, especially to know whether a T-cell response is present or not in vaccinated immunocompromised patients with no detectable antibody responses. Interestingly, in our study, vaccinated mild convalescent individuals showed higher percentage of positive responses for both cytokines than the unvaccinated ones [9/12 (75%) vs 6/11 (55.6%) for IFN-γ, 10/12 (83.3%) vs 6/11 (55.6%) for IL-2, respectively] and is in concordance with the bibliography[24].

When comparing T-cell and humoral responses, correlations were found between both IFN-γ and IL-2 with IgGs against Spike and NCP, and neutralizing antibodies. Nevertheless, higher amount of positive IgGs and neutralizing antibodies were obtained when compared with T-cells. Concerning humoral responses, IgGs against Spike together with neutralizing antibodies were the ones detected the most, particularly during convalescence and after vaccination. The roles of humoral-cellular immunity on SARS-CoV-2 infection still remain controversial

as some authors report T-cell responses appearing previously than neutralizing antibodies after vaccination, whereas others report the opposite[40–43]. Although humoral responses are seen in a larger number of individuals after the disease and vaccination, memory T-cell responses related to the release of IL-2, are fundamental when the humoral response wanes after some time, and when the virus escapes humoral response due to the appearance of new VOCs[44,45]. Therefore, both memory T-cell immune status and humoral response evaluation are indispensable for comprehending protection against new variants and possible reinfections. Detecting both responses might indicate increased protection[44,45].

The study has some limitations that should be addressed. In the first place, statistical strength could be affected as mild and moderate subgroups of acute COVID-19 patients have small numbers. Notwithstanding, patient groups from our study were clinically well-characterized, making it possible to assess the immune response in each clinical status. In the second place, the most prevalent SARS-CoV-2 VOCs were not reported in our study. Although this can affect the immune response interpretation, data concerning VOCs has been registered by Spanish authorities, and therefore, variants can be traced: Wuhan (until February 2021), Alpha (until June 2021), Delta (until December 2021), and Omicron (from December 2021)[46]. Another limitation of the study is the impossibility of assessing long-term immunity and protection, as we do not have a follow-up cohort through time after infection to assess possible reinfections according to its IL-2 status. However, it is well known that IL-2 secretion is associated with the presence of a memory T-cell response, which is important in the protection against possible reinfection, and it is highly produced in convalescents and after the second dose of the vaccine. Finally, vaccinated individuals could not be followed-up, therefore, the response through time and administrated doses could not be assessed. Despite that, time since the administration of the different doses was registered for all vaccinated individuals and response through time could be evaluated.

Altogether, the measurement of IFN-γ and IL-2 cytokines can have a value for SARS-CoV-2 infection management. Our results show that IFN-γ in combination with IL-2 increases response detection in acute and convalescent individuals, having IFN-γ response a role during the acute phase of the disease, and IL-2 on long-term immunity against natural immunization or vaccination. In addition, IFN-γ detection can be a useful biomarker for monitoring severe acute patients, as our results indicate that those individuals with a poor outcome have lower levels of this cytokine. Moreover, fluorescence ELISPOT technology allows the detection of these specific immune responses against SARS-CoV-2 easily, being able to be adapted in the majority of laboratories. Finally, according to the findings observed here, T-cell responses generated in acute COVID-19 patients are lower compared to convalescence, indicating that T-cell responses need time to be generated. In some cases, the lack of cellular immunity is compensated by antibodies, confirming the role of both types of immune responses in infection and vaccination, and confirming that their dual detection can increase the number of specific response detections. All these data suggest the possible role of IFN-γ and IL-2 as effector and memory cytokines, respectively. Such dual detection is promising for assessing the post-immunization status and managing the infection, but more studies are needed in this direction evaluating other cytokines or cell markers related to diagnosis and disease outcome.

Methods

Study samples

Two-hundred sixty-three blood samples were drawn from 232 participants at Hospital Universitari Germans Trias i Pujol (Badalona, Spain) from July 2020 to May 2022. All the participants of the study filled out and signed a written informed consent form. The study was approved by the Ethics Committee of the Hospital Universitari Germans Trias i Pujol (PI-20-117), and the experiments were performed according to current regulations and guidelines. Clinical and demographic data from the individuals included in the study are summarized in Table 3, and data from the samples concerning time since diagnosis, time since vaccine doses and lymphopenia are included in Supplementary Table 7. Individuals were classified following WHO 2020 guidelines as follows[47]:

(i) Ninety-three uninfected healthcare workers with no previous nor present positive SARS-CoV-2 test (PCR or rapid antigen test (RAT)), and/or detectable IgM or IgG plasma antibodies against the virus. They were classified into two groups: (a) unvaccinated individuals (n = 21), and (b) vaccinated individuals (n = 72), in which 91.7% (66/72) had received two doses of the vaccine.

(ii) Sixty-six samples from 35 COVID-19 patients during the acute phase of the disease and with present positive SARS-CoV-2 test. Participants were classified according to disease severity by hospitalization and required ventilation criteria into: (a) healthcare workers with mild infection (n = 8) who had a positive SARS-CoV-2 PCR in work routine screenings and were neither hospitalized nor required ventilation support during infection; (b) moderate (n = 2) when the patient required hospitalization and ventilation with nasal prongs or Ventimask (VMK); (c) severe (n = 22) when the patient required high-flow ventilation or non-invasive ventilation (NIV) (n = 17), or invasive mechanical ventilation (IMV) (n = 5); and (d) dead (n = 3) when the patient died during infection. From acute COVID-19 group, 17 patients were followed-up and two or more samples were collected during days 0, 2, 7, 28, and/or at discharge after admission in semi-critical or intensive care units (48 samples in total).

(iii) One-hundred and four individuals after overcoming the acute phase of SARS-CoV-2 infection with a previous COVID-19 positive diagnostic. These individuals were also classified following WHO 2020 guidelines according to the severity of the previous disease as: (a) mild (n = 25); (b) moderate (n = 22); and (c) severe (n = 57), with high-flow ventilation or NIV (n = 26), or IMV (n = 31).

Patients variables (n = 232)	Controls (n = 93)		Acute (n = 35)					Convalescent (n = 104)			
	Unvaccinated (n = 21)	Vaccinated (n = 72)	Mild (n = 8)	Moderate (n = 2)	Severe (n = 22)		Dead (n = 3)	Mild (n = 25)	Moderate (n = 22)	Severe (n = 57)	
					NIV (n = 17)	IMV (n = 5)				NIV (n = 26)	IMV (n = 31)
Age (years ± SD)	34.6 ± 10.4	41.4 ± 13.9	34.5 ± 16.6	55.1 ± 26.7	59.6 ± 13.1	63.2 ± 11.3	79.6 ± 2.3	39.9 ± 12.9	61 ± 14.2	60.6 ± 14.1	58.6 ± 10.2
Male N (%)	3 (14.3)	18 (25)	4 (50)	2 (100)	15 (88.2)	4 (80)	2 (66.7)	5 (20)	11 (50)	14 (53.8)	22 (74.2)
Pneumonia N (%)[a]	0 (0)	0 (0)	0 (0)	2 (100)	17 (100)	5 (100)	3 (100)	0 (0)	19 (86.4)	26 (100)	31 (100)
Unilobar	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	5 (22.7)	0 (0)	1 (3.2)
Multilobar	0 (0)	0 (0)	0 (0)	2 (100)	17 (100)	5 (100)	3 (100)	0 (0)	14 (63.6)	26 (100)	30 (96.8)
ICU admission N (%)[a]	0 (0)	0 (0)	0 (0)	0 (0)	5 (29.4)	5 (100)	2 (66.7)	0 (0)	0 (0)	2 (7.7)	30 (96.8)
Oxygen support N (%)[a]	0 (0)	0 (0)	0 (0)	2 (100)	17 (100)	5 (100)	3 (100)	0 (0)	16 (72.7)	26 (100)	31 (100)
Nasal prongs or VMK	0 (0)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)	16 (72.7)	1 (3.8)	0 (0)
Non-invasive mechanical vent	0 (0)	0 (0)	0 (0)	0 (0)	17 (100)	0 (0)	3 (100)	0 (0)	0 (0)	26 (96.2)	0 (0)
Invasive mechanical vent	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	5 (100)	0 (0)	0 (0)	0 (0)	0 (0)	31 (100)
Vaccinated with 1st dose N (%)[b]	0 (0)	72 (100)	2 (25)	0 (0)	1 (5.9)	0 (0)	0 (0)	13 (52)	1 (4.5)	0 (0)	0 (0)
Vaccinated with 2nd dose N (%)[b]	0 (0)	66 (91.7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	10 (40)	0 (0)	0 (0)	0 (0)
Vaccinated with 3rd dose N (%)	0 (0)	3 (4.2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (8)	0 (0)	0 (0)	0 (0)
Comorbidities N (%)	1 (4.7)	8 (11.1)	0 (0)	1 (50)	15 (88.2)	3 (60)	3 (100)	3 (12)	11 (50)	21 (80.8)	21 (67.7)
Respiratory disorders (asthma, OSAS, COPD)	0 (0)	2 (2.8)	0 (0)	1 (50)	6 (35.3)	0 (0)	0 (0)	0 (0)	8 (36.4)	3 (11.5)	3 (9.7)
Cardiovascular diseases (AHT, ictus, atrial fibrillation)	1 (4.7)	2 (2.8)	0 (0)	1 (50)	7 (41.2)	3 (60)	3 (100)	1 (4)	6 (27.3)	14 (53.8)	15 (48.4)
Autoimmune disorders (DM2, psoriasis, Jorgen, other)	0 (0)	3 (4.2)	0 (0)	0 (0)	6 (35.3)	1 (20)	1 (33.3)	2 (8)	1 (4.5)	6 (23.1)	6 (19.4)
Central nervous system disorders (dementia, epilepsy, Parkinson)	0 (0)	1 (1.4)	0 (0)	0 (0)	2 (11.8)	0 (0)	1 (33.3)	0 (0)	1 (4.5)	0 (0)	1 (3.2)
Malignant neoplasies	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (20)	1 (33.3)	0 (0)	1 (4.5)	3 (11.5)	2 (6.4)
Obesity	0 (0)	0 (0)	0 (0)	0 (0)	7 (41.2)	1 (20)	0 (0)	0 (0)	3 (13.6)	4 (15.3)	7 (22.6)
Immunosuppressive treatment N (%)	0 (0)	4 (5.6)	1 (12.5)	0 (0)	4 (23.5)	0 (0)	1 (33.3)	1 (4)	4 (18.2)	3 (11.5)	2 (6.4)
Oral (prednisone, NSAIDS, etc.)	0 (0)	1 (1.4)	1 (12.5)	0 (0)	2 (11.8)	0 (0)	1 (33.3)	0 (0)	4 (18.2)	0 (0)	1 (3.2)
Inhaled	0 (0)	2 (2.8)	0 (0)	0 (0)	2 (11.8)	0 (0)	0 (0)	0 (0)	0 (0)	2 (7,7)	1 (3.2)
Topic	0 (0)	2 (2.8)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (4)	0 (0)	1 (3.8)	0 (0)
Deaths N (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)	0 (0)	0 (0)

Table 3. Descriptive table from patients included in the study. *NIV* non-invasive ventilation, *IMV* invasive mechanical ventilation, *n/a* not available. [a]In the convalescent group, these variables refer to the characteristics of their acute COVID-19 episode. [b]Sixty-six of 72 uninfected vaccinated individuals (91.7%) with two Pfizer doses.

Isolation of peripheral blood mononuclear cells (PBMCs)

Sixteen milliliters of blood were collected in cell preparation tubes (CPT; Becton Dickinson Diagnostics, Franklin Lakes, NJ) for density gradient PBMCs isolation. After centrifugation, PBMCs were collected, washed with 10% FBS RPMI (Biowest, Nuaillé, France), and counted using trypan blue. Cells were cryopreserved in FBS 10% DMSO (Sigma-Aldrich, Saint Louis, United States of America), first at − 80 °C in a Nalgene Mr. Frosty Cryo 1 °C Freezing Container (ThermoFisher, Waltham, United States of America) and were then transferred to liquid nitrogen within a week.

Detection of IFN-γ and IL-2 T-cell responses using a fluorescence ELISPOT assay

Fluorescent ELISPOT is a technique that allows the multiple detection of cytokines release against a pathogen with a very accessible protocol and without demanding a complicated analysis when compared to alternative ways to assess cellular immunity. It can also provide a result within 1 or 2 days and it can be easily implemented (not demanding expensive infrastructure and highly trained personnel). In this study, IFN-γ and IL-2 T-cell responses were measured with a fluorescence ELISPOT assay (CoV-iSpot, Autoimmun Diagnostika GmbH, Straßberg. Germany). Two-hundred thousand cells were dispensed in each well/condition in a 96-well plate coated with antibodies specific for IFN-γ and IL-2. Cells were stimulated overnight at 37 °C with two antigen pools: (a) pancoronavirus peptide mix, based on homology regions of the coronavirus viral family, and (b) SARS-CoV-2 peptide mix, based on peptides from Spike, NCP, membrane and envelope proteins unique for the Wuhan strain of the virus which can cross-react with new Spike mutations from the different variants. Negative (AIM-V) and positive controls (pokeweed-mitogen) were included for each sample. Samples were tested in duplicates for all conditions, strictly following manufacturer's instructions. Fluorescent spots [spot forming cells (SFCs)] were counted with an automated plate reader (Autoimmun Diagnostika GmbH, Straßberg. Germany). Specific response against pancoronavirus and SARS-CoV-2 antigens was analyzed by performing a ratio between the average of SFCs for the specific panel and the average of SFCs in the negative control (stimulation index, SI). Results were interpreted as follows: when the negative control had less than 2 SFCs, a SI < 5 was considered a negative response, between 5 and 7 borderline, and ≥ 7 positive. On the contrary, if the negative control had 2 or more SFCs, a SI < 2 was considered negative, between 2 and 3 borderline, and ≥ 3 positive. Samples with less than 50 IFN-γ or IL-2 spots in the positive control or with more than 10 IFN-γ or 20 IL-2 spots in the negative control were considered indeterminate, unless positive control was invalid and response against one of the antigen pools was found. Ratios between IL-2 and IFN-γ T-cell responses were also performed (SI IL-2/SI IFN-γ).

Detection of humoral responses with ELISA

Levels of IgGs against Spike and NCP proteins, IgM against NCP, and neutralizing antibodies were analysed by ELISA (Euroimmun, Lübek, Germany). IgGs against Spike were quantified with the QuantiVac kit; IgGs and IgMs against NCP were semi-quantified with the Anti-SARS-CoV-2 (IgG or IgM) kits; and neutralizing antibodies were measured with the NeutraLISA kit.

Plasma was incubated in a 96-well plate with specific fixed SARS-CoV-2 antigens for the measurement of IgGs against Spike, and IgG and IgM against NCP. For measuring neutralizing antibodies, plasma was incubated with the ACE2 human receptor which acts as a competitor to bind the S1/RBD domain from the SARS-CoV-2 Wuhan strain, fixed in the well.

Samples were tested in batches, having each batch a positive and a negative control to validate the test. For the semi-quantification of IgGs and IgMs against NCP, a calibrator was included to perform an absorbance ratio (AR = absorbance of the sample/absorbance of the calibrator). To quantify levels of IgG against Spike, a six-point calibration curve was used to quantify anti-Spike IgG levels [concentration given in Binding Antibody Units (BAU)/mL]. Finally, for the neutralizing antibodies ELISA, two replicas of a blank were included to calculate the percentage of neutralization [% of neutralization = 100 − ((absorbance of the sample*100)/absorbance of the blank)]. Positive, borderline, and negative cut-off values for each test were provided by the manufacturer's instructions.

Statistical analysis

The statistical analysis used to compare T-cell responses and ratios among groups was the two-tailed Mann–Whitney U-test for unpaired comparisons. Comparisons among the number of positives obtained in acute and convalescent cases in detecting IFN-γ, IL-2 response or response for at least one of the cytokines was calculated by a Cochran test. Statistical significance was considered when a p-value < 0.05 was obtained. Correlations were assessed by two-tailed non-parametric Spearman test. Both statistical analysis and graphical representations were done with GraphPad v8 (GraphPad Software, Inc, San Diego, CA).

Data availability

Without any reservation, raw data supporting the findings of this study will be made available by the corresponding author.

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Author contributions
R.V.-H., I.L., J.D. and G.S. contributed to conception and design of the study. D.S., I.L. and G.S. designed the experiments. D.S., S.D.-F., I.R., B.M.-M. and G.S. performed the experiments. Z.S., A.M., A.L., C.P.-C., A.L.-M., A.J.S., F.A., J.M., I.C., K.S., R.P. and A.R. contributed with resources. A.R., I.L. and J.D. supervised the study. I.L., J.D. and G.S. wrote the paper. All authors contributed to manuscript revision, read, and approved the submitted version.

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Competing interests
RVH, IL, and JD are registered as inventors on a patent (WO 2019/234296 A1) not related with the topic of this study that has been licensed by Genome Identification Diagnostics (GenID) GmbH. The remaining authors do not have any competing interests to declare.

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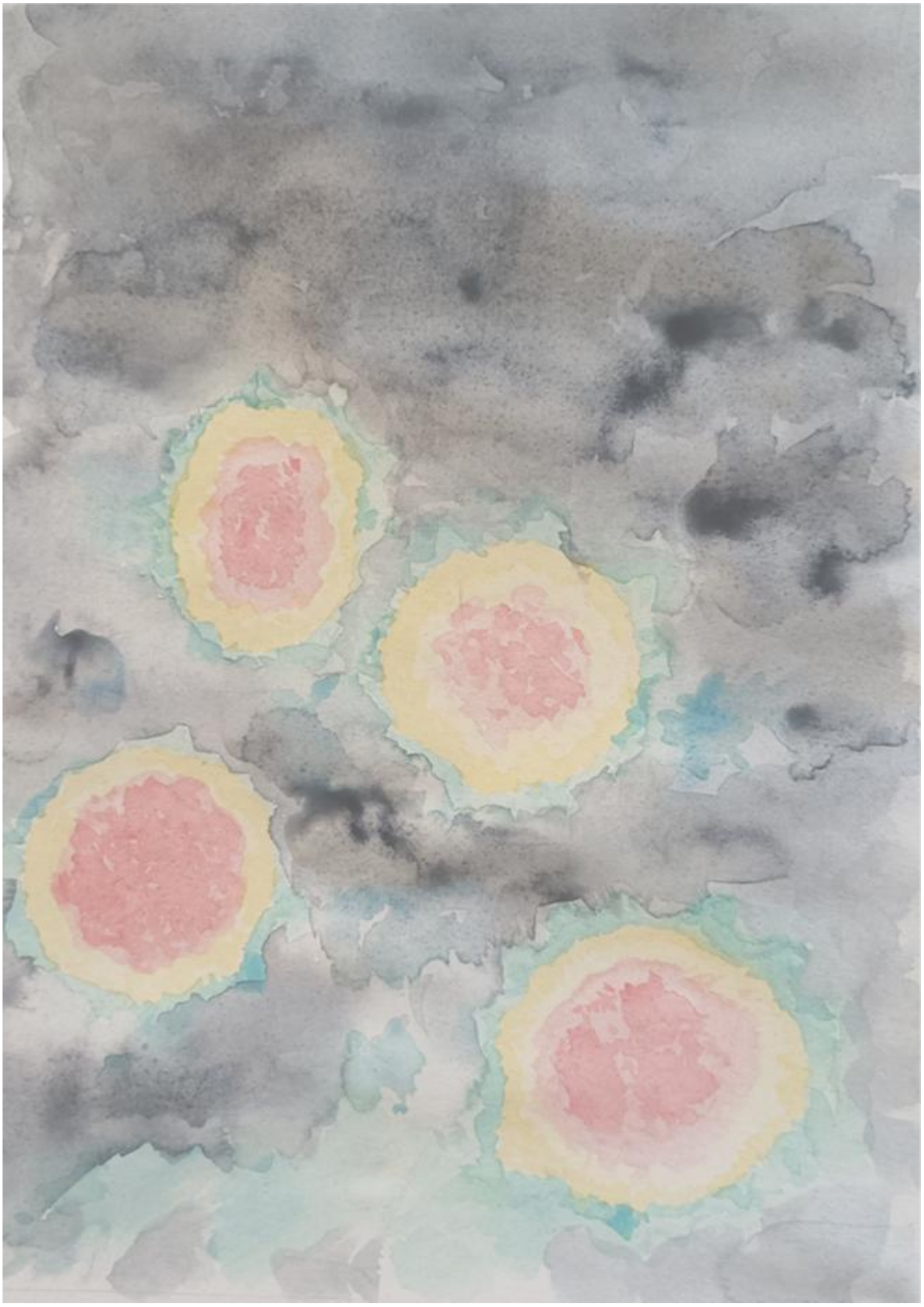

Article 3

Cellular response evaluation in immunosuppressed individuals to help guiding monoclonal antibody COVID-19 prophylactic treatment administration

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Cellular response evaluation in immunosuppressed individuals to help guide monoclonal antibody COVID-19 prophylactic treatment administration

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Immunocompromised patients are highly susceptible to viral infections and often have suboptimal humoral responses to vaccination. SARS-CoV-2-specific cellular responses were assessed in candidates for tixagevimab/cilgavimab (Evusheld) prophylaxis to support clinical decision-making in addition to serology. Between June and September 2022, 146 immunocompromised individuals were classified according to their serology (negative <260 vs. positive ≥260 BAU/mL). Peripheral blood mononuclear cells (PBMCs) were stimulated with a Spike peptide pool, and IFN-γ, IL-2, IL-21, and IL-5 responses were measured using a Fluorospot assay. Only 38.3% of negative serology patients showed a cellular response compared to 55.6% of those with positive serology. Negative serology was linked to lower polyfunctionality (38.8% vs 72.2%) and decreased IFN-γ and IL-2 responses. Previous COVID-19 increased the probability of IFN-γ response (OR 2.33) and IL-2 (OR 3.15), while corticosteroid intake reduced the probability of IFN-γ response (OR 0.33). Multivariate analysis estimated that less than 15% of negative serology patients, with no previous COVID, and on corticosteroids, would mount an IFN-γ response, compared to 68-90% in positive serology individuals, with previous infection, and not receiving corticosteroids. Evaluating cellular responses can provide additional information to serology and can help identify patients most at risk of insufficient immune protection.

The global outbreak of coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), had a considerable impact on healthcare systems worldwide. Following its emergence, from 2020 to 2023, it was the deadliest infectious disease in the world, accounting for almost 800 million reported infected individuals and more than 7 million deaths[1]. In May 2023, the COVID-19 Emergency Committee declared the end of the public health emergency. This assertion is further corroborated by the most recent reports from the WHO, which document a decline in both morbidity and mortality compared to previous years[2]. Global efforts to achieve herd immunity through vaccination, immunity acquired through previous infections, and the reduced severity of the disease caused by new variants have all played a role in the decline of transmission and severity. Nevertheless, SARS-CoV-2 continues to adapt, with the still prevalent Omicron variant mutating and infecting new hosts every day, leaving us in a novel epidemiological context[3,4].

Immunocompromised individuals and patients undergoing immunosuppressive treatment are highly susceptible to viral infections, including SARS-CoV-2. Although most of them have been vaccinated at least three times against SARS-CoV-2, a large proportion still have a suboptimal humoral response, and COVID-19 remains a potentially life-threatening disease for this group[5,6]. Then, COVID-19 monoclonal antibody prophylaxis has been administered to patients with defective humoral responses to neutralize the pathogen and prevent the spread of the infection and severe disease. In this sense, tixagevimab/cilgavimab (Evusheld, AstraZeneca) was a combination of two monoclonal antibodies that acted passive immunization in those patients with an inadequate humoral response after vaccination (<260 Binding Antibody Units [BAU)/ml])[7]. The lack of humoral response has been shown to be closely related to the lack of cellular response, leaving the individual with no defense against the pathogen[8,9]. However, in previous studies, immunocompromised patients such as solid organ transplant recipients and patients with autoimmune diseases who lack antibodies to SARS-CoV-2 appear to have a cellular response to the virus[10,11]. In such cases, information on the patient's cellular immune status would provide a better insight into the overall immune situation and may be of interest in deciding whether or not they should receive the prophylactic treatment.

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The adaptive immune response against SARS-CoV-2 is one of the crucial variables determining the course of the infection. Subsequently, a late and compromised response can lead to critical outcomes. IFN-γ and IL-2 have been seen to be valuable in COVID-19 management and the immunization status assessment after vaccination and after infection. This combination gives a broad idea of the cellular response against SARS-CoV-2, as both cytokines take an important part in an antiviral Th1 response[12,13]. Some studies analyzed the substantial role of baseline production of IFN-γ against SARS-CoV-2 in preventing infection and hospitalization, while others focused on the fundamental role of IL-2 in lymphocyte recovery in COVID-19 patients. Consequently, deficient production of the two cytokines would imply having an inadequate cellular response against the virus. This deficiency has been closely associated with acute respiratory distress, multiorgan failure, and death[14–16]. In contrast to a Th1 phenotype, a Th2 polarized response has been associated with symptomatology in SARS-CoV-2 infection and poor prognosis, as it is a mediator of pneumonia and tissue damage[17–19]. IL-5 is one of the main cytokines produced by Th2 cells, recruiting and helping in the maturation of eosinophils and basophils. Apart from Th1 and Th2 responses, other viral-specific cellular subsets are important in building an effective immune response against COVID-19. For example, T follicular helper (Tfh) cells are fundamental for the maintenance of antibody production over time. This cell type, usually localized in germinal centres, produces IL-21, a fundamental cytokine for the differentiation of B-cells into plasma cells[20]. Reciprocally, plasma cells are responsible for the differentiation of CD4 T-cells into circulating Tfh (cTfh)[21]. This cellular subset has been identified in whole blood after SARS-CoV-2 infection and has been directly correlated with neutralizing antibody titers[22,23]. Therefore, an impairment of any of these cytokines would have negative effects on building an efficient immune response against the virus.

Altogether, monoclonal antibody prophylaxis candidates are patients who are challenging to manage due to their diversity and complexity. A wide range of treatments and conditions affect their humoral and cellular responses. Serological levels against the virus offer a limited view of the patient's condition, as they do not provide information about the overall immune status. Assessing both Th1 and Th2 cellular responses and other cytokine profiles in peripheral blood mononuclear cells (PBMCs) would provide a good overview of the patient's cellular immune status and help in the clinical management of these patients in the context of COVID-19. In this sense, the main objective of this study was to evaluate the SARS-CoV-2 cellular response in immunocompromised patients who were candidates for monoclonal COVID-19 antibodies by studying IFN-γ, IL-2, IL-5, and IL-21 cytokines after SARS-CoV-2-specific stimulation.

Methods

Study samples

One hundred forty-six participants were recruited in the Hospital Universitari Germans Trias i Pujol (Badalona, Spain) for serology testing to determine their eligibility for tixagevimab/cilgavimab prophylaxis from June 2022 to September 2022. The recruitment followed the recommendations published by the Spanish Ministry of Health, where the criteria to select the candidates were detailed[24]. The distribution in the different candidate groups, indicating the specific immunosuppression, is shown in Table 1. Participants were given a written informed consent form for participating in the study, and blood was extracted for later immune analysis. The study was approved by the Ethics Committee of the Hospital Germans Trias i Pujol (PI-23-036). Clinical and demographic data from the participants are outlined in Table 1.

Serology evaluation

Blood samples were extracted from patients on the day of recruitment to measure the levels of neutralizing antibodies against SARS-CoV-2 by a chemiluminescent immunoassay (SARS-CoV-2 Trimeric IgG LIAISON XL test [DiaSorin, Vercelli, Italy]). The test was performed routinely in Hospital Universitari Germans Trias i Pujol. Individuals were classified according to their serological status against SARS-CoV-2 following the recommendations of tixagevimab/cilgavimab prophylaxis by the Spanish Health Ministry[24]: (i) forty-seven participants with less than 260 BAU/ml were considered to have negative serology against SARS-CoV-2, and (ii) ninety-nine participants with more than 260 BAU/ml were considered to have positive serology against the pathogen.

Peripheral blood mononuclear cells (PBMCs) isolation

Blood samples were collected in two 8ml cell preparation tubes (CPT; Becton Dickinson Diagnostics, Franklin Lakes, USA) for PBMCs isolation by density gradient. After separation, PBMCs were washed two times with 10% FBS RPMI (Biowest, Nuaillé, France) and counted with trypan blue. Cells were cryopreserved with a 10% DMSO FBS dilution (Sigma-Aldrich, Saint Louis, USA) in liquid nitrogen.

Evaluation of PBMCs cytokine release

Cytokine release by PBMCs was evaluated by means of FluoroSPOT assays (CoV-iSpot, Genome Identification Diagnostics GmbH, Straßberg, Germany). Cells were thawed and adjusted to a concentration of $2{\cdot}10^6$ cells/mL with AIM-V (ThermoFisher, Waltham, United States of America). Two hundred thousand cells were dispensed in each well in two 96-well plates. The first plate was coated with antibodies specific for IFN-γ and IL-2, and the second was coated with antibodies specific for IL-21 and IL-5. Each sample was tested in both plates with the SARS-CoV-2 Spike protein pool (S-pool), based on the whole Spike antigen from the Wuhan strain. The first plate was incubated for 20 hours, while the second one was incubated for 40 hours. Negative control (AIM-V; NC) and positive controls (pokeweed-mitogen, PC) were performed for each of the samples in both plates. Plates were developed following the manufacturer's instructions.

Participant's variables	Overall (n=146)	Negative serology (n=47)	Positive serology (n=99)	p-value[a]
Age (years ± SD)	58.5 ± 13.3	55.1 ± 15.8	60.1 ± 11.7	0.063
Male N (%)	78/146 (53.4)	18/47 (38.3)	60/99 (60.6)	**0.013**
Previous COVID-19 N (%)				
Overall	58/146 (39.7)	20/47 (42.6)	38/99 (38.4)	0.718
Mild	41/58 (70.7)	17/20 (85)	24/38 (63.1)	0.13
Moderate	11/58 (19)	3/20 (15)	8/38 (21)	0.73
Severe	6/58 (10.3)	0/20 (0)	6/38 (15.9)	0.165
Vaccinated with at least 2 doses N (%)	146/146 (100)	47/47 (100)	99/99 (100)	>0.99
Vaccinated with at least 3 doses N (%)	142/146 (97.3)	46/47 (97.9)	96/99 (97)	>0.99
Immunosuppressive treatment N (%)				
Overall	137/146 (93.8)	45/47 (95.7)	92/99 (92.9)	0.719
Biologic	56/137 (40.9)	27/45 (60)	29/92 (31.5)	**0.002**
B-cell depleters (B-cell depl.)	41/56 (73.2)	23/27 (85.2)	18/29 (62.1)	0.072
DMARD**	107/137 (78.1)	30/45 (66.7)	77/92 (83.7)	**0.029**
Prednisone	96/137 (70.1)	24/45 (53.3)	72/92 (78.3)	**0.005**
Treatment combination N (%)				
Only biologic	22/137 (16.1)	11/45 (24.4)	11/92 (12)	0.082
B-cell depl.	19/22 (86.4)	10/11 (100)	9/11 (81.8)	>0.99
Only DMARD[b]	7/137 (5.1)	2/45 (4.4)	5/92 (5.4)	>0.99
DMARD + prednisone	73/137 (53.3)	16/45 (35.6)	57/92 (62)	**0.006**
Biologic + prednisone	8/137 (5.8)	4/45 (8.9)	4/92 (4.3)	0.438
B-cell depl. + prednisone	3/8 (37.5)	2/4 (50)	1/4 (25)	>0.99
Biologic + DMARD	10/137 (7.3)	8/45 (17.8)	2/92 (2.2)	**0.002**
B-cell depl. + DMARD	9/10 (90)	8/8 (100)	1/2 (50)	0.2
Biologic + DMARD + prednisone	15/137 (10.9)	4/45 (8.9)	11/92 (12)	0.773
Anti B-cell + DMARD + prednisone	9/15 (60)	3/4 (75)	6/11 (54.5)	0.604
Tixagevimab/cilgavimab recipients N (%)	73/146 (50)	44/47 (93.6)	29/99 (29.3)	**<0.001**
Tixagevimab/cilgavimab candidate group N (%)				
HPT or CART-T receptors	1/146 (0.7)	0/47 (0)	1/99 (1)	>0.99
Solid organ transplant recipients[c]	75/146 (51.4)	16/47 (34)	59/99 (59.6)	**0.005**
Primary immunodeficiency	4/146 (2.7)	1/47 (2.1)	3/99 (3)	>0.99
Biologic immunosuppressive treatment	56/146 (38.4)	27/47 (57.4)	29/99 (29.3)	**0.002**
Cancer under chemotherapy	1/146 (0.7)	0/47 (0)	1/99 (1)	>0.99
High risk of infection[d]	9/146 (6.2)	3/47 (6.4)	6/99 (6.1)	>0.99

Table 1. Descriptive table of the participants included in the study. [a]p-value when comparing negative versus positive serology groups. [b]DMARD treatment only refers to synthetic disease-modifying antirheumatic drugs. [c]70 kidney transplant, 5 kidney and pancreas transplant recipients. [d]Citation from the Spanish Health Ministry recommendations for tixagevimab/cilgavimab administration24: Individuals at very high risk of severe disease following SARS-CoV-2 infection and who are contraindicated for vaccination against COVID-19 due to severe allergy (including anaphylaxis) to any of the vaccine components or who have developed severe adverse reactions associated with the administration of a dose of vaccine against COVID-19 and who are medically unable to complete the vaccination regimen.

Plates were read with an automated plate reader (Autoimmun Diagnostika GmbH, Straßberg. Germany) with software associated with fluorescent spot counting. For IFN-γ and IL-2, the ratio between the response against a particular antigen and basal response (spots in the NC) was assessed (Stimulation index [SI]). When the NC had less than 2 spots, an SI <5 was considered negative and ≥5 positive. Instead, if the NC had 2 or more spots, negative was considered when SI <2 and positive if ≥2. For IL-21 and IL-5, an SI over 2 was considered a positive response against the antigen.

Moreover, for IFN-γ and IL-2, results were considered indeterminate according to the manufacturer's instructions. Briefly, for IFN-γ, samples with more than 10 spots in the NC or less than 50 spots in the PC were considered indeterminate; whereas for IL-2, was considered when having more than 20 spots in the NC or less than 50 spots in the PC. Samples with an absent response in the PC but present against the antigens were not considered indeterminate. When a sample had an indeterminate result for both IFN-γ and IL-2, the results for the remaining cytokines were also considered indeterminate due to the lack of cut-offs to validate the results.

Statistical analysis

Statistical comparisons of the percentage of cellular responses between patients with positive and negative serology were performed using Fisher's exact test. Cellular responses were quantitatively compared according to serology status and different variables (previous COVID-19 and treatment regimens), using the two-tailed Mann-Whitney U-test for unpaired comparisons. Significance was considered when $p<0.05$ was obtained. Logistic regression models were used to assess the association of the result of the serology with IFN-γ, IL-2, IL-21, and IL-5 response. Raw models and models adjusted by age, sex, previous COVID-19, and previous treatments were constructed. OR and 95% CI were reported. With the best model for each cytokine, estimated probabilities of positive IFN-γ, positive IL-2, positive IL-21, and positive IL-5 were calculated and plotted graphically. The conditions of use of the models were validated. All analyses were performed with the program R version 4.4.1 (2024-06-14) for Windows. The levels of significance were represented as follows: <0.05 (*), <0.01 (**), <0.001 (***), and <0.0001 (****). Graphical representations were performed with GraphPad Prism v10 (GraphPad Software, Inc, San Diego, CA).

Results

Participants with negative serology display a cellular response against SARS-CoV-2

Forty-seven out of the 146 participants included in the study (32.2%) had negative serology (<260 BAU/ml) against SARS-CoV-2. Among them, eighteen (18/47, 38.3%) showed a response against the S-pool for at least one of the cytokines studied for cellular response evaluation (IFN-γ, IL-2, IL-21, and IL-5). Within the 99 participants with positive serology (67.8% of the total), fifty-five (55/99, 55.6%) also displayed cellular response against the S-pool for at least one of the cytokines studied. There was no significant statistical difference between the percentage of participants with cellular response having negative or positive serology against SARS-CoV-2 ($p=0.125$). Cellular responses for each of the cytokines studied are shown in Table 2. Of the 146 participants, 16 (10.9%) had an indeterminate result for both IFN-γ and IL-2. Seven of the participants with an indeterminate response for both cytokines had negative serology against SARS-CoV-2, while 9 had positive serology. Three of them were indeterminate for having an invalid NC (1 negative and 2 positive serology), and the rest were due to having fewer spots in the PC than the required (6 negative and 7 positive serology). One sample was indeterminate only for IFN-γ (negative serology), and 15 only for IL-2 (5 with negative serology and 10 with positive serology).

Cytokine response	**Number of patients with a T cell response**	
	Negative serology 18/47 (38.3%)	**Positive serology 55/90 (55.6%)**
To at least one cytokine N (%)		
IFN-γ+	8/18 (44.4)	35/55 (63.6)
IL-2+	9/18 (50)	40/55 (72.7)
IL-21+	3/18 (16.7)	24/55 (43.6)
IL-5+	10/18 (55.5)	33/55 (60)
To only one cytokine N (%)		
IFN-γ+ IL-2- IL-21- IL-5-	3/18 (16.7)	4/15 (26.7)
IFN-γ- **IL-2+** IL-21- IL-5-	2/18 (11.1)	4/15 (26.7)
IFN-γ- IL-2- **IL-21+** IL-5-	1/18 (5.6)	1/15 (6.7)
IFN-γ- IL-2- IL-21- **IL-5+**	5/18 (27.8)	6/15 (40)
Overall	11/18 (61.1)	15/55 (27.3)
To more than one cytokine N (%)		
IFN-γ+ IL-2+ IL-21- IL-5-	1/7 (5.6)	7/40 (17.5)
IFN-γ- **IL-2+ IL-21+** IL-5-	0/7	2/40 (5)
IFN-γ- IL-2- **IL-21+ IL-5+**	0/7	2/40 (5)
IFN-γ+ IL-2- **IL-21+** IL-5-	0/7	2/40 (5)
IFN-γ- **IL-2+** IL-21- **IL-5+**	2/7 (28.6)	3/40 (7.5)
IFN-γ+ IL-2- IL-21- **IL-5+**	0/7	1/40 (2.5)
IFN-γ+ IL-2+ IL-21+ IL-5-	0/7	2/40 (5)
IFN-γ+ IL-2+ IL-21- **IL-5+**	2/7 (28.6)	7/40 (17.5)
IFN-γ+ IL-2- **IL-21+ IL-5+**	0/7	0/40
IFN-γ- **IL-2+ IL-21+ IL-5+**	0/7	3/40 (7.5)
IFN-γ+ IL-2+ IL-21+ IL-5+	2/7 (28.6)	12/40 (30)
Overall	7/18 (38.9)	40/55 (72.7)

Table 2. Percentages of T cell response positivity according to the serology result.

Cellular response in participants with negative serology is less intense and less polyfunctional

Regarding the 18 participants with negative serology showing cellular response, IFN-γ response was detected in 8 participants (44.4%), IL-2 in 9 (50%), IL-21 in 3 (16.7%), and IL-5 in 10 (55.6%). In comparison, from the 55 participants with positive serology and cellular response, 35 of them (63.4%) showed IFN-γ response, 40 (72.7%) showed IL-2 response, 24 (43.6%) showed IL-21 response, and 33 (60%) showed IL-5 response (Table 2). There was no statistical significance when comparing the results from each cytokine between negative and positive serology for IFN-γ, IL-2, and IL-5 (p=0.1761, p=0.09, and p=0.787, respectively), nor IL-21, although it was close to achieving it (p=0.051).

To better understand the overall cellular response depending on the serology, the combination of cytokines induced in each patient was also assessed. Eleven of the participants with negative serology and with cellular response (61.1%) showed a response for only one cytokine, whilst 7 (38.9%) showed a polyfunctional response. In positive serology responders, this pattern was different, as 15 out of the 55 (27.3%) showed response for only one cytokine, while the rest (72.7%) showed response for at least two of them. As a result, participants with a negative serology showed significantly less polyfunctionality than those with a positive serology (p=0.0124). The pattern of cytokine production for participants with both negative and positive serology can be found in Table 2. No significance was found in the quantity of response for any of the cytokines regarding serology in those participants with cellular response for at least one cytokine (IFN-γ p=0.191, IL-2 p=0.103, IL-21 p=0.287, and for IL-5 p=0.156; Supplementary Figure 1).

Previous COVID-19 infection and corticosteroid intake are factors that influence the cellular immune response

Risk factors associated with low levels of cellular response according to serology were assessed for each cytokine. First, when analysing patients with no previous COVID-19 infection, the cellular response was significantly higher in those with positive than negative serology for IFN-γ and IL-2 (p=0.039 and p=0.0006, respectively), and close to significance for IL-21 (p=0.058). No significance was obtained for IL-5 (p=0.074, Figure 1a). Second, focusing on the treatment received, patients with positive serology had a significantly higher response than the ones with negative serology for both IFN-γ and IL-2 when they were treated with corticosteroids (p=0.0016 and p=0.0002, respectively), or DMARDs (p=0.0111 and p=0.0038, respectively), but neither for IL-21 nor for IL-5 for any of the treatments (Figure 1b and 1c).

Previously to performing a multivariate model, a univariate analysis was performed to analyze if the presence of cellular response was associated with positive serology. Statistical significance was detected for IFN-γ, IL-2, and IL-21 (p=0.042, p=0.015, and p=0.011, respectively), whereas no significance was found for IL-5 (p=0.165). Afterwards, the multivariate model was built incorporating age, sex, and previous COVID-19 infection variables for the three cytokines (IFN-γ, IL-2, and IL-21) that exhibited significance in the univariate analysis to assess their additional influence on positive cellular response (Supplementary Table 1-3). Multiparametric analysis in IL-5 was not considered because no initial significances between positive and negative serology were observed. Previous COVID-19 significantly influenced IFN-γ and IL-2 responses, with OR [CI95%] values of 2.33 [1.06-5.25] (p=0.037) and 3.15 [1.38-7.50] (p=0.007), respectively. However, no significant effect was observed for IL-21 (p=0.4). No effects from sex or age on the cellular response were observed, except for sex in IL-2 (p=0.048). Finally, the impact of different treatments on cellular response was also incorporated in the model, with findings indicating a significant effect on IFN-γ production in the adjusted model. Specifically, corticosteroid treatment was associated with reduced probability of IFN-γ responses (0.33 [0.13-0.80], p=0.017; Supplementary Table 4). However, no significant effects were observed for IL-2 and IL-21 responses (Supplementary Tables 5 and 6).

Logistic regression models were plotted with the estimated probability of a cellular response by integrating multiple variables. Participants with positive serology and prior COVID-19 exhibited a higher estimated probability of generating a cellular response for all three cytokines, particularly when compared to individuals with negative serology and no prior infection (Figures 2 and 3). When assessing IFN-γ response in the context of treatment, the models revealed that participants with negative serology, no previous COVID-19, and undergoing corticosteroid treatment had a lower estimated probability of mounting an IFN-γ response (ranging from 3% to 13%). In contrast, participants with positive serology and previous COVID-19 showed a significantly higher estimated probability of IFN-γ response, ranging from 65% to 88% in those not receiving corticosteroid treatment (Figure 2).

Discussion

Despite the conclusion of the COVID-19 pandemic, immunosuppressed patients are still at high risk of infection and hospitalization. Vaccine boosters have been crucial in protecting this group of patients from severe infection, but not as effectively as in the general population[25,26]. To improve this situation, prophylactic monoclonal antibody therapies have been developed to reduce the risk and severity of infection in immunosuppressed patients[27,28]. A meta-analysis including 25,435 immunocompromised patients reports that tixagevimab/cilgavimab administration protected from reinfection around 40% of the participants, and prevented hospitalization, ITU admission, and COVID-19 specific mortality in 66.19%, 82.13%, and 92.39% of patients, respectively[29]. Only serological levels have been used to decide who should receive prophylactic treatment, disregarding the cellular component of the immune response. Information on the patient's cellular immune status would help to guide decisions on prophylaxis and to better understand the patient's immune status against SARS-CoV-2. To this end, the cellular response of monoclonal antibody prophylaxis candidates was assessed in this study.

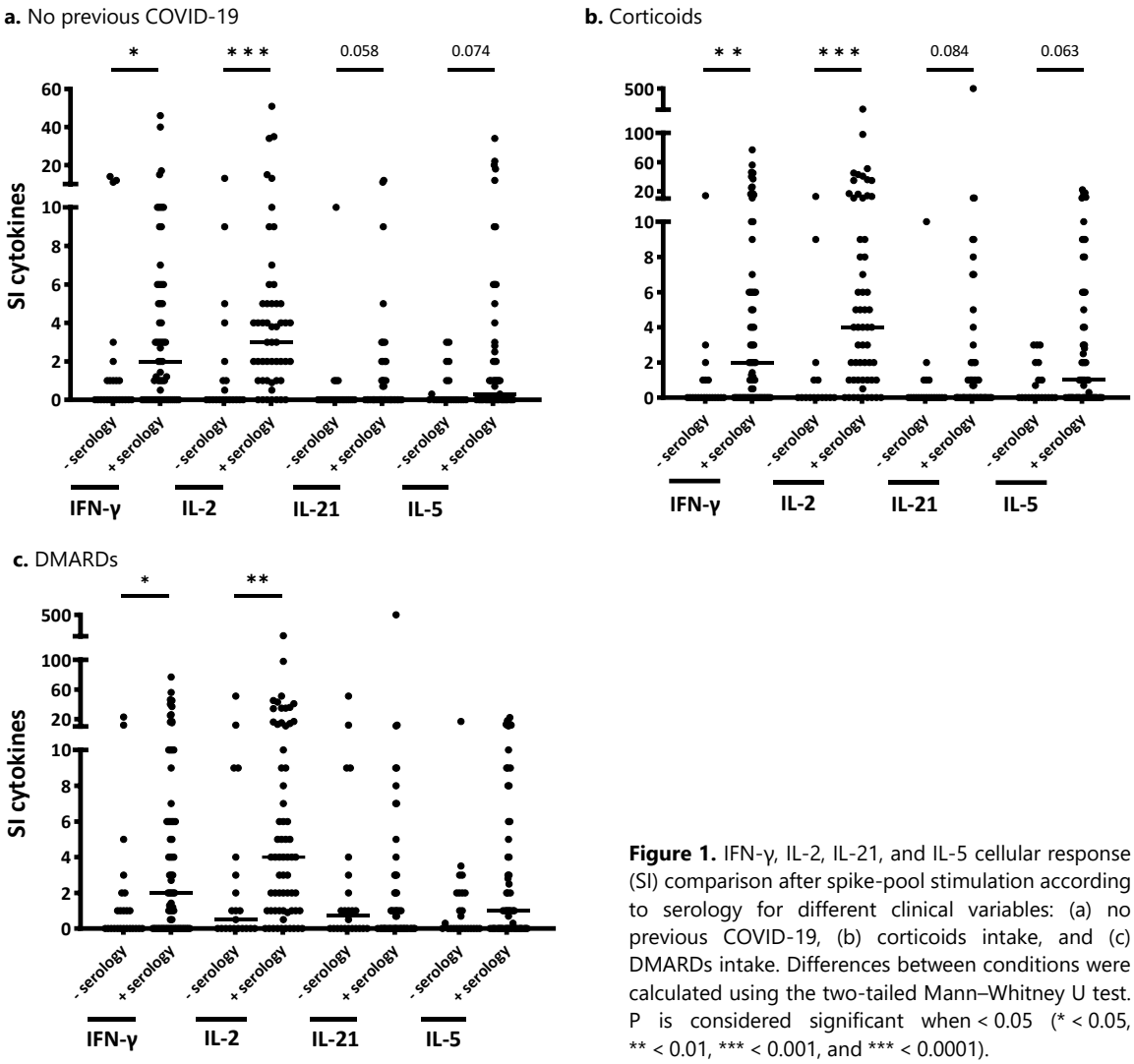

Figure 1. IFN-γ, IL-2, IL-21, and IL-5 cellular response (SI) comparison after spike-pool stimulation according to serology for different clinical variables: (a) no previous COVID-19, (b) corticoids intake, and (c) DMARDs intake. Differences between conditions were calculated using the two-tailed Mann–Whitney U test. P is considered significant when < 0.05 (* < 0.05, ** < 0.01, *** < 0.001, and *** < 0.0001).

Our objective was to assess whether participants with negative serology exhibited a cellular response to SARS-CoV-2. While both humoral and cellular responses are correlated[30,31], previous studies have described discrepancies between the two, even in the immunosuppressed population[12,32]. Therefore, the absence of a humoral response to SARS-CoV-2 does not necessarily indicate the lack of a cellular response in immunosuppressed patients[33,34]. In our study, we have observed that 38.3% of immunosuppressed patients without a humoral response still display a cellular immune response. However, the quantity of the cellular response observed in participants with negative serology was found to be lower than that observed in participants with positive serology. In this regard, it has been described that inefficient CD8 and CD4 T-cell responses are observed in immunocompromised patients, contributing to disease severity, combined with the delayed clearance of the virus due to insufficient antibody production[35]. Furthermore, imbalances between CD4 and CD8 adaptive responses have been observed to increase the risk of mortality, indicating the crucial influence of a balanced adaptive response in efficiently overcoming the disease[35]. Regarding the functionality of the detected response, we have observed that immunosuppressed patients with a negative serology exhibit less polyfunctional responses than those with a positive serology. In this sense, the different immunosuppressive treatment regimens may play a role in these results, particularly among participants with negative serology.

The combination of corticosteroids and DMARDs has been demonstrated to inhibit lymphocyte proliferation and the maturation of CD4 T-cells into helper cells[32], which also affects humoral responses indirectly. Additionally, patients receiving this treatment combination exhibit low seroconversion rates following the standard two-dose regimen of vaccination[36]. Some reports indicate preserved effector CD4 Th1 and CD8 type 1 responses, while others have suggested an overcompensation for the lack of neutralizing antibodies[37–39]. In our study, the use of corticosteroids was associated with a significantly lower response for both IFN-γ and IL-2 in patients with negative serology compared to those with positive serology, leaving these patients with both cellular and humoral depletion and designating them as clear candidates for prophylactic treatment[40,41]. Although DMARDs alone also exert detrimental effects on cellular immune responses[43], we hypothesize that these effects are mainly produced due to their coadministration with corticosteroids,

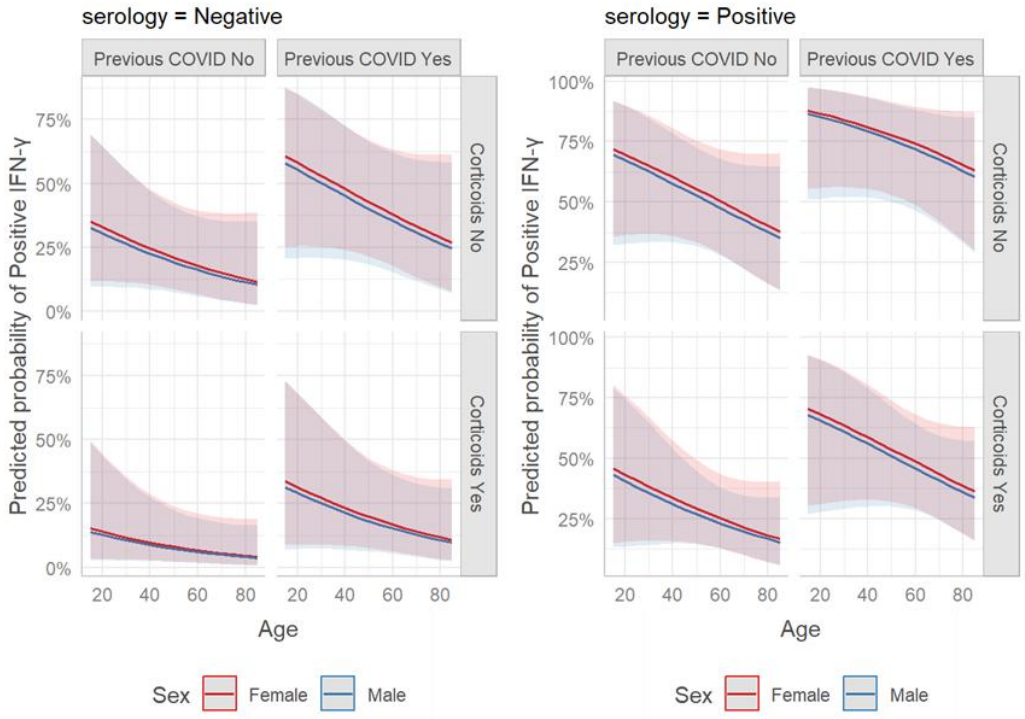

Figure 2. Plotted estimated probabilities of positive IFN-γ cellular response according to logistic regression models adjusted by serology, age, sex, previous COVID-19, and corticosteroid treatment. According to the estimated model, given an individual with negative serology, 50 years old, with no previous COVID, and under corticoid treatment, the estimated probability of having an IFN-γ cellular response would be around 7%, independent of sex. Instead, an individual with positive serology, 50 years old, with previous COVID, and not taking corticoids, would have an estimated probability of around 75% of having an IFN-γ cellular response independent of sex.

which have broader adverse effects on Th1-mediated immunity. Additionally, participants with negative serology receiving B-cell–depleting biologics (Ocrelizumab and Rituximab) exhibited higher IFN-γ and IL-2 responses. Unlike corticosteroids, these anti-CD20 therapies specifically target B cells without broadly suppressing cellular immunity. Previous studies have reported robust T-cell responses in patients treated with anti-CD20 therapies following vaccination, suggesting a compensatory T-cell activation that may reduce disease severity despite humoral depletion[43,44].

Regarding Th2 functionality, IL-5 release was quantitatively lower in participants with negative serology than in those with positive serology; however, the percentage of responders was nearly equivalent (55.6% vs. 60%). In this sense, corticosteroids promote a Th2-biased response by inhibiting IL-12 production, fundamental for T-cell differentiation into Th1 cells[45]. This bias has been observed to produce vaccine-associated enhanced respiratory disease upon infection in animal models and leads to worse prognoses in some diseases[46–48]. Finally, the production of IL-21 by Tfh cell subsets is pivotal in the development of humoral responses and is closely associated with B-cell activation in germinal centers, leading to rapid proliferation and differentiation into memory and plasma cells, which are essential for controlling future infections[49]. In line with our results, corticosteroids in combination with DMARDs are associated with a reduction in IL-21 production following vaccination, significantly impacting antibody production against SARS-CoV-2[50]. The same reduction has been observed following B-cell–depleting therapy[51]. Therefore, measuring IL-21 could provide additional information concerning the communication between T helper responses and B-cell maturation in these patients.

Participants with no previous COVID-19 and negative serology had a significantly lower cellular response for IFN-γ and IL-2 compared to those with a positive serology. Considering that all the patients were vaccinated, this finding highlights the fact that vaccination has a much bigger impact on the development of cellular responses in patients able to mount a good humoral response than in those with negative serology values. This goes in line with previous studies, where immunosuppressive treatment poses a risk for impaired cellular and humoral responses triggered by vaccination, particularly in patients naïve to the infection[52–54]. Interestingly, the response in vaccinated patients previously infected became comparable, independently of the patient's serology. In this sense, hybrid immunity (natural infection and vaccination) has been seen to be superior to vaccination alone in the generation of immune responses in the overall population and immunosuppressed patients, explaining our findings[52,55]. Our results also indicate that individuals with negative serology, no previous COVID-19, and receiving corticosteroids had the lowest probability of mounting a protective immune response (especially for IFN-γ). While negative serology was the primary criterion for determining prophylactic treatment administration, this study has described previous infection and treatment regimens as determinant factors for patient classification. In scenarios of limited prophylactic drug availability, patients with the lowest probability of generating an immune response should be prioritized for treatment. Our findings provide valuable

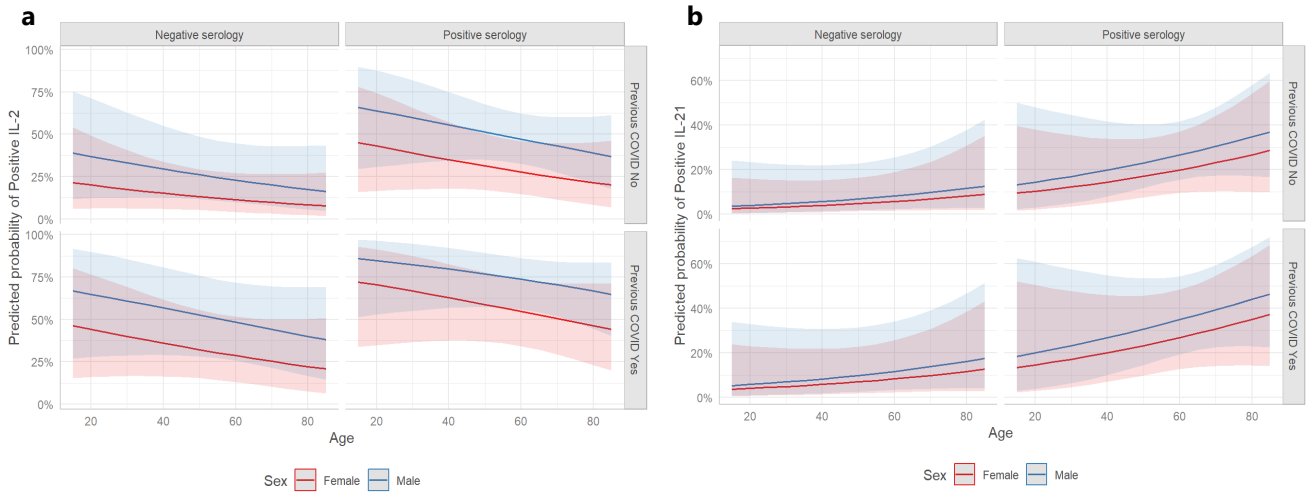

Figure 3. Estimated probabilities of positive IL-2 (a) and positive IL-21 (b) according to logistic regression models adjusted by serology, age, sex, and previous COVID-19. According to the estimated model, given an individual with negative serology, 50 years old, and with no previous COVID, the estimated probability of having an IL-2 cellular response would be of 26.0% (95%CI 11.6% to 48.6%) for a man, and of 13.1% (95%CI 5.2% to 29.3%) for a woman. Instead, an individual with positive serology, 50 years old, and with previous COVID, would have an estimated probability of having an IL-2 cellular response of 76.9% (95%CI 57.4% to 89.2%) for a man, and of 58.8% (95%CI 36.9% to 77.7%) for a woman.

insights into the importance of cellular response assessment for the future management of immunosuppressed patients who are highly susceptible to infection and undergo prophylactic treatment.

In our study, no severe COVID-19 infections were detected in the patients included after receiving tixagevimab/cilgavimab. Nevertheless, three patients were hospitalized, one of them requiring non-invasive ventilation. This one had a positive serology together with a positive cellular immunity (only for IL-21), whereas the other two patients had negative serology results, with one showing a negative and the other positive cellular response (for all cytokines). However, protection conferred by tixagevimab/cilgavimab has been reported to be less effective against new variants, and is no longer recommended by the FDA[56,57].

It is important to note that the study has some limitations. First, the participants exhibited a diverse range of treatment regimens, which presented a challenge in classification. In consequence, the assessment of the impact of different medications was inherently influenced by their co-administration with other medications. Second, the low number of patients with cancer receiving chemotherapy, under CART-T therapy, and with primary immunodeficiencies, complicated the analysis of the response and therefore could not be compared to other groups of tixagevimab/cilgavimab prophylaxis candidates. For instance, most haematological patients were not included in our study because they were managed for this specific purpose by another institute. Finally, information regarding the variant causing the infection in participants with previous COVID-19 was not registered; therefore, it was not possible to assess with certainty the impact of the different variants on the cellular immune response observed. However, given that the data concerning the most prevalent SARS-CoV-2 variants of concern (VOCs) during the pandemic were registered by the Spanish Ministry of Health58, we could correlate with our cohort to deduce the variant that caused the previous infection. As a consequence, we have seen that there was no significant impact on the variants in the capacity of generating cell immune response (9 responders from 13 potential Wuhan infections [69.2%], 2 from 3 potential Alpha infections [66.7%], 3 from 3 potential Delta infections [100], and 17 from 27 potential Omicron infections [63%]). In addition, we tested the SARS-CoV-2 Spike Omicron pool from the peptides differing from the Wuhan strain, in the 10 patients with a previous COVID-19 by a potential Omicron infection with any response against the Spike Wuhan peptide pools. Only in 2 cases a cellular response was observed, evidencing that adding specific variant peptides could help to identify extra responder cases, but does not significantly increase the detection of cases with cellular immune response.

The findings of this study suggest that the absence of a humoral response to SARS-CoV-2 does not necessarily indicate a lack of cellular response to the pathogen in immunocompromised patients. However, the cellular response is lower and less polyfunctional, highlighting potential imbalances between Th1 and Th2 responses. Patients with negative serology, no previous infection, and under corticosteroid treatment are at a higher risk of not being sufficiently protected after vaccination. Consequently, they should be considered as candidates for prophylaxis. We conclude that measuring IFN-γ, IL-2, IL-21, and IL-5 in monoclonal antibody prophylaxis candidates is an effective method for assessing the immune status of these patients, thereby assisting in the discrimination of patients eligible for prophylactic therapies and their prioritisation. Altogether, this study provides important information for clinically testing and managing immunosuppressed candidates for infectious diseases prophylaxis by measuring both humoral and cellular immune responses, and identifies specific non-responder profiles at most at risk of infection.

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Author contributions
I.L., J.D., and G.S. contributed to the conception and design of the study. D.S., I.L., and G.S. designed the experiments. D.S., N.T., and G.S. performed the experiments. D.G., R.U., I.C., L.M., J.V., M.M., C.C., I.N., R.P., and A.L. contributed with resources. I.L. and J.D. supervised the study. I.L., J.D., and G.S. wrote the paper. All authors contributed to manuscript revision, read, and approved the submitted version.

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Competing interests
IL and JD are registered as inventors on a patent (WO 2019/234296 A1) not related to the topic of this study that has been licensed by Genome Identification Diagnostics (GenID) GmbH. The remaining authors do not have any competing interests to declare.

6

GENERAL DISCUSSION

The COVID-19 pandemic has posed a very significant challenge to global health systems, exposing critical gaps in clinical and scientific preparedness for epidemiological crises. One of the main difficulties regarding the management of SARS-CoV-2 has been understanding immune responses to the pathogen, which has evolved in parallel with the progression of the pandemic stages. At the beginning of the pandemic, the need to comprehend immune correlates of protection against infection and disease progression was crucial, particularly to shed light on reinfections and vaccine efficacy. Since the beginning of the SARS-CoV-2 outbreak, the evaluation of neutralizing antibodies against the pathogen has provided a practical tool to assess previous exposure and the later response elicited after vaccination. However, it should be noted that antibodies alone are insufficient to characterize the protective immune status. Not all individuals mount humoral responses, especially in certain populations, and these responses are more labile than specific cellular responses.

In light of the limitations of solely evaluating humoral responses, the main objective addressed in the thesis is the evaluation of SARS-CoV-2-specific cellular responses. In this context, the purpose of the three studies included in this thesis shifts from initially measuring cellular responses in different contexts regarding SARS-CoV-2 infection, to evaluating the functionality of these responses, and finally to applying this assessment in a relevant clinical setting within the post-pandemic paradigm. The first study established the basis of the thesis by evaluating SARS-CoV-2–specific IFN-γ responses to Spike, Nucleocapsid, and Membrane proteins during acute infection, convalescence, and after vaccination, comparing the cellular findings with humoral responses against the virus. Building on these results, the second study incorporated IL-2, combining it with IFN-γ in a dual detection format to enhance detection. It also aimed to functionally assess responses by measuring effector and memory-related cytokines, thereby identifying responders that the one-cytokine approach could miss. Lastly, the third study adapted this approach for immunocompromised candidates receiving monoclonal antibody prophylaxis, expanding the panel with IL-5 and IL-21 to assess Th1/Th2 balance and T-B-cell communication, respectively. Overall, this sequence of studies connects the development of cellular assessments with their integration in clinical practice.

Following the order of the thesis studies, **Article 1** aimed to characterize the detectability of cellular responses against different SARS-CoV-2 antigens during the acute phase of the infection, in convalescence, and following vaccination. The evaluation was performed using IFN-γ as a representative cytokine of this response, as has been used in other pathogens such as *Mycobacterium tuberculosis*, or Cytomegalovirus (CMV). Spike has been described as the most immunogenic antigen from the virus, over both Nucleocapsid and Membrane antigens. We corroborated that finding as Spike was the one triggering response in most individuals during and after COVID-19 infection. Despite that, Nucleocapsid and Membrane triggered non-negligible immunogenicity. Vaccinated individuals showed a significant response against Spike but lower compared to those with previous infection. Vaccine design has focused on Spike for its immunogenicity, but Nucleocapsid and membrane could additionally strengthen vaccine designs[195–197]. IFN-γ responses against the pathogen were low during acute COVID-19, most likely due to the early collection of samples before the full development of the adaptive cellular response, and could also be related to lymphopenia and anergy previously reported in severe COVID-19 patients[198–

[200]. Active immunization through vaccination also achieved a certain degree of IFN-γ response against SARS-CoV-2. However, it was not comparable to that triggered after infection, which was more potent and found in a higher number of individuals[201]. Building directly on these findings based on IFN-γ detection, IL-2 was incorporated in **Article 2** as a complementary readout to assess memory response maintenance and to evaluate responses more comprehensively in the same clinical contexts. This combined assessment increased the detection of responders against a SARS-CoV-2 peptide pool across all the groups tested[202,203]. Through this approach, a more refined functional assessment could be performed, finding differences in the pattern of cytokine release depending on the clinical status of the individual. IFN-γ was predominant during acute infection, as was in **Article 1**, while elevated IL-2 responses were seen after immunization from previous infection or vaccination, linked to memory cellular responses[204,205]. The identification of individuals with isolated cellular responses, only releasing one of the cytokines, indicates that single-marker approaches could underestimate cellular immunity and reinforces the utility of the dual detection method. In this way, the second study helped contextualize the findings of the first with a distinguished functionality between IFN-γ and IL-2.

Disease severity markedly impacted the amount and pattern of responses, particularly during the infection. Robust cellular responses were detected in those patients with severe COVID-19 who had a good outcome compared to those with milder forms of the disease, explained by a severe inflammatory scenario[206,207]. This could be attributed to increased exposure to the virus, resulting from uncontrolled infection and consequent higher viral load[208,209]. The idea of memory responses persisting months beyond infection, in consonance with previous coronaviruses such as SARS-CoV, is reinforced with these findings, which are in line with bibliography[210–212]. The results were consistent in both **Article 1** and **Article 2**, where severity amplified post-infection responses. Instead, individuals with severe COVID-19 that led to a fatal outcome had diminished IFN-γ and IL-2 responses, reflecting insufficient adaptive activation, possibly due to persistent anergy and lymphopenia, unable to preclude viral replication[102,213,214]. The findings suggest that measuring specific cytokine release against the virus could be associated with different clinical outcomes. In both studies, cellular responses were higher after infection (particularly in severe forms) than after vaccination, which is aligned with different studies reporting lower reinfection risk in previously infected individuals compared with vaccinated individuals naïve to the infection[201]. Although other studies report quantitatively similar specific responses[215], our results could be related to the broader cellular response conferred by infection, exposing the immune system for longer periods and to multiple viral antigens not targeted by vaccination[102]. The integrated observations from both papers enabled the comprehensive evaluation of time after immunization (acute and post-immunization phases), function (IFN-γ and IL-2, effector and memory responses), and clinical outcomes (recovery and fatal outcomes) in a single assessment.

The relationship between cellular and humoral immunity was also examined in both studies. IgGs and neutralizing antibodies were detected in more individuals after vaccination and during the infection than IFN-γ and/or IL-2 responses, and correlations were moderate[216,217]. Discrepancies between both results (cellular and humoral) were found in nearly one-sixth of the patients in both studies[218]. From these discrepancies, individuals presenting cellular response but no humoral response accounted for around

20%. Considering that humoral immunity declines over time, while cellular memory, particularly reflected by IL-2, can persist, incorporating cellular immune assessment can be essential in estimating protection, especially taking into consideration new circulating variants and the greater immune escape against antibodies[219,220]. This reinforces the potential diagnostic and clinical value of assessing both branches of the adaptive immune response against SARS-CoV-2 simultaneously. These findings from **Article 1** and **Article 2** contributed to reporting insights into the dynamics of cellular responses to SARS-CoV-2 immunization and their utility in combination with serology evaluation.

At the latter stage of the pandemic, after vaccination was broadly implemented and disease severity had decreased in the overall population, concerns about protection against the virus in immunocompromised individuals emerged. For this group, serological evaluation has been used to determine their eligibility for monoclonal antibody prophylaxis against COVID-19, disregarding the role of cellular immune responses against SARS-CoV-2 infection. Information about these responses could provide a better understanding of their immune status against the virus and help guide decisions on prophylaxis. In this regard, the main objective of **Article 3** of this thesis was to evaluate the SARS-CoV-2 cellular response in immunocompromised candidates for monoclonal antibody prophylaxis by studying specific IFN-γ, IL-2, IL-5, and IL-21 cytokine production.

The findings revealed that nearly 40% of immunosuppressed patients with negative serology had SARS-CoV-2-specific cellular responses, demonstrating that negative serology does not necessarily indicate a lack of cellular response, although the magnitude and polyfunctionality were generally lower than in those patients with positive serology[221,222]. Regarding functionality, Th1 responses, marked by IFN-γ and IL-2 release, were particularly reduced in these patients, suggesting their direct compromise to clear the infection and form long-lasting immune memory[176,223,224]. The proportion of individuals responding with IL-5 against the pathogen was maintained in patients with negative serology, compared to those with positive serology. This could reflect a Th2-biased profile that disrupts Th1-Th2 balance, potentially impairing the effective resolution of the infection and worsening disease outcomes in negative serology individuals[222,225,226]. In addition, IL-21, key to the development of humoral responses and B-cell activation, was diminished both in quantity and frequency among patients with negative serology, indicating impaired communication between T and B cell populations and reinforcing the measurement of IL-21 as a marker that could improve the understanding of the interplay between cellular and humoral responses[227].

Factors influencing cellular immune responses were also studied in our cohort of immunocompromised patients. Previous COVID-19 was associated with stronger IFN-γ and IL-2 responses than those observed in infection-naïve individuals, reinforcing the advantage of hybrid immunity as seen in the other studies[228]. These results indicate both a clear link between the ability to mount a humoral response and the production of cellular responses after vaccination, and that previous COVID-19, in combination with vaccination, is related with the generation of a cellular response in patients with negative serology[229,230]. Immunosuppressive treatments, particularly corticosteroids, were associated with diminished Th1 responses, with the greatest effect in patients with negative serology. As has been previously discussed,

this impairment is directly involved in the Th1-Th2 imbalance and the absence of seroconversion[231]. These findings from **Article 3** evidence that relying on the serology assessment alone may overlook patients with severely weakened immune responses, and support considering previous COVID-19 and immunosuppressive treatments to identify candidates for monoclonal antibody prophylaxis.

Some limitations across the three studies should be considered. First, we did not specify which cellular subsets are responsible for the release of cytokines, which thus precludes phenotypic analysis of cellular responses. However, our main objective was not to fully characterize responses but to assess functionality and help in patient management through the measurement of detectable cytokines related to the adaptive response to SARS-CoV-2. Second, some cohorts had a relatively small number of individuals, which could be reducing the statistical strength of comparisons and impeding the performance of particular analyses. This happened in the three studies (acute COVID-19 mild and moderate cohorts across **Article 1** and **Article 2**, acute severe individuals dying from the disease, and specific immunosuppressive conditions in **Article 3**). Besides this, all cohorts were clinically deeply characterized, preventing inaccurate analyses and imprecise results due to low numbers across cohorts, and all the comparisons crucial for the studies could be performed. Third, SARS-CoV-2 variants were not documented at an individual level, which could hamper the interpretation of results. However, that could be traced to well-defined predominance periods of each variant, which were reported by the national health ministry. Additionally, in **Article 3**, no association was found between variants and the presence of a cellular response against the virus. Fourth, follow-up of responses could not be performed in different cohorts, limiting long-term response assessments. However, for some acute COVID-19 patients, follow-up from days 0 to 27 after hospitalization could be studied in both **Article 2** and **Article 3** manuscripts. Additionally, IL-2 was included to evaluate possible specific memory responses, avoiding the limitation, and giving information on those individuals with functionality associated with memory cellular responses. Finally, some treatment combinations challenged the analysis and classification of patients in **Article 3**, complicating the attribution of the effects seen on cellular immune responses.

Taken together, the findings from the three studies in this thesis demonstrate the value of measuring SARS-CoV-2-specific cellular immune responses across diverse clinical scenarios. **Article 1** showed IFN-γ detection both after infection and vaccination, providing insights into the presence of cellular responses in the absence of humoral immunity. **Article 2** expanded the approach by adding the IL-2 measurement, revealing distinct cytokine patterns in various clinical contexts regarding COVID-19, and emphasizing the need for assessing cellular responses rather than relying only on serology. Finally, **Article 3** focused on immunocompromised individuals, finding specific cellular responses (measuring IFN-γ, IL-2, IL-21, and IL-5) even in participants lacking humoral responses but with lower functionality. It also demonstrated that previous COVID-19 and corticosteroids act as the main factors influencing these responses. These findings highlight the importance of evaluating cellular immune responses in different clinical settings, especially in vulnerable populations. In summary, the three studies provide a comprehensive analysis beyond serology in favor of including this assessment in clinical practice. This approach improves the ability to identify patients with functional cellular responses and to guide

personalized management of COVID-19, which would additionally aid in the preparedness for future pandemics.

In the current post-pandemic context, the primary objectives are to continue expanding knowledge about COVID-19 diagnostics and treatments. Although the overall burden has decreased, high-risk groups, together with Long COVID and pulmonary sequelae, still require significant interventions from healthcare systems. In this new paradigm, it is fundamental to continue studying SARS-CoV-2-specific cellular responses to complement serology and its implementation in clinical practice, which could help refine risk stratification and guide booster vaccination, prophylaxis, and follow-up, especially in individuals most at risk of infection. Future work should focus on easy-to-use and standardized assays to allow rapid translation into clinical practice. All this knowledge and technology can be easily adapted to new variants and serve as a basis for novel pathogens, strengthening preparedness and improving the management of infections by emerging pathogens through individualized patient care.

7

CONCLUSIONS

Article 1

1. The evaluation of IFN-γ against SARS-CoV-2 antigens Spike, Nucleocapsid, and Membrane provides insight into the adaptive cellular immune response during acute infection, convalescence, and post-vaccination. Spike is the most immunogenic antigen, however, Nucleocapid and Membrane also induce significant IFN-γ secretion, supporting their consideration in future vaccine designs.
2. IFN-γ T-cell responses are low in the acute phase of the disease, which is consistent with early sampling and lymphopenia or anergy in severe disease.
3. Convalescent individuals display durable and robust IFN-γ responses, particularly after severe disease, related to greater antigenic exposure. Instead, vaccination elicits lower and more variable IFN-γ cellular responses.
4. Correlations between humoral and cellular are moderate, and discordances are not infrequent, pointing out that neither serology nor cellular immune response alone captures the whole adaptive immunity complexity.

Article 2

1. The dual-cytokine evaluation (IFN-γ and IL-2) increases the responder detection in comparison to a single-marker approach in acute, convalescent, and vaccinated cohorts.
2. Cytokine patterns align with the individual's COVID-19 situation. During acute disease, IFN-γ predominates, whereas IL-2 is enhanced after immunization or infection, reflecting its memory-related functionality. The severity of COVID-19 has a significant influence on the responses detected. It has a significant impact on cellular responses during convalescence, with increases detected according to severity, but also during critical acute disease, with fatal cases showing blunted IFN-γ measurements compared to the high levels found in their counterparts.
3. Integrating both serology and the dual-cytokine measurement increases the number of specific response detections, with discordances highlighting the importance of their assessment in combination.
4. Overall, the combined IFN-γ and IL-2 measurement provides a rapid and implementable technique for assessing cellular responses during acute COVID-19 and for monitoring post-infection and post-vaccination immunity.

Article 3

1. Evaluating serology alone in immunocompromised candidates for monoclonal antibody prophylaxis underestimates adaptive immune responses against SARS-CoV-2: 40% of patients with negative serology display SARS-CoV-2-specific cellular responses to at least one cytokine, confirming that the absence of humoral response does not imply the absence of cellular immunity.

2. Patients with negative serology exhibit less intense and less polyfunctional cellular responses, underscoring potential imbalances between cellular subsets and functionalities.

3. Previous COVID-19 (hybrid immunity) is associated with stronger IFN-γ and IL-2 responses in patients with negative serology, whereas corticosteroid treatment is correlated with diminished Th1 responses in these patients, identifying those patients with increased risk of cellular response absence. IL-21, a marker of T and B-cell communication, is detected in a lesser number of patients with negative serology and at a lower intensity, while IL-5 is maintained, suggesting a Th2 shift that may compromise an effective antiviral response.

4. Negative serology patients, without prior SARS-CoV-2 infection, and receiving corticosteroids, are the principal candidates for prophylaxis.

5. Measuring IFN-γ, IL-2, IL-21, and IL-5 can help in the discrimination of eligible patients among candidates for monoclonal antibody prophylaxis who are at high risk of COVID-19 in combination with serology.

Taken together, the thesis shows that measuring SARS-CoV-2-specific cellular immunity is feasible and can help in the clinical management of patients in combination with serology. IFN-γ and IL-2 provide insight into the cellular adaptive immune response against the virus, comprehensively assessing its antiviral functionality and understanding its dynamics across acute disease, convalescence, and after vaccination in both immunocompetent and immunocompromised individuals. Extended panels (with IL-5 and IL-21) uncover functional imbalances and alterations that cannot be assessed by serology alone. Moderate correlations between humoral and cellular responses, with not infrequent discordances, justify the addition of this assessment in the clinical management of patients, such as monoclonal antibody prophylaxis. Integrating standardized platforms for cellular response (such as ELISPOT) assessments together with serology can be the key to personalized clinical decisions with the ability to rapidly adapt to new variants and future pandemics.

8

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