Immunity & Ageing

RESEARCH

Open Access

Characterisation of specific responses to three models of viral antigens in immunocompetent older adults

Beatriz Rioseras[1,2,3], Eva Bueno-García[1,2,3], Alejandra García-Torre[1,2,3], Rocío López-Martínez[1,2], Marco Antonio Moro-García[2,3,4], Sara Alonso-Álvarez[2,3,5], Victoria Menéndez-García[1,2], Alba Lluna-González[2,6], Alejandra Sousa-Fernández[2,6], Marta Fernández-Gudin[2,6], Laura Campos-Riopedre[2,6], Corina Castro-del Cueto[2,6], Ana Belén Pérez-Fernández[2,6], Ana Alonso-Rodríguez[2,6], Carla Menéndez-Peña[2,6], Lara Menéndez-Peña[2,6], Noelia García-Arnaldo[2,6], Estefanía Feito-Díaz[2,6], Adriana Fernández-Lorences[2,6], Agustín Fraile-Manzano[2,6], Carolina Fernández-Iglesias[2,6], Jose Arturo Rivera[2,6], Carmen Pérez-Fonseca[2,6], Estíbaliz Urdiales-Ruano[2,6], María Debán-Fernández[2,6], Hugo Mendes-Moreira[2,6], Pablo Herrero-Puente[2,6] and Rebeca Alonso-Arias[1,2,3*]

Abstract

Background Memory responses to the antigens that an individual encounters throughout life may vary with the intensity and duration of antigen contacts or even with changes in immune status over time. This work aims to characterise specific responses to latent CMV, seasonal influenza and novel SARS-CoV-2 infections in immunocompetent individuals over 60 years of age. Specific cellular and humoral responses were identified by IFN-γ and granzyme B release by ELISpot and antibody level measurement. T lymphocyte subpopulation phenotypes were characterised by flow cytometry.

Results Cellular and humoral responses to these viruses were detected in almost all patients. Influenza and SARS-CoV-2 cellular responses were positively correlated. There was no significant correlation between CMV and influenza or SARS-CoV-2 responses although both were consistently lower in CMV-seropositive patients. CMV responses were negatively correlated with the levels of the least differentiated subsets of T lymphocytes, and positively correlated with the most differentiated ones, contrary to what happened with the influenza responses. Nevertheless, SARS-CoV-2 cellular responses were negatively correlated with the most differentiated $CD8^+$ T lymphocytes, while humoral responses were negatively correlated with the least differentiated T lymphocytes. Responses to the three viruses were correlated with a Th1/Th2/Th17 balance in favour of Th1.

Conclusions The results indicate that memory responses differ depending on the durability of the antigen stimulus. Cellular responses to novel pathogens resemble those generated by seasonal but not CMV infection. Subpopulation distribution and the level of specific T lymphocytes against previous pathogens could be used as immunocompetent status biomarkers in older adults reflecting their ability to generate memory responses to new pathogens.

*Correspondence:
Rebeca Alonso-Arias
ralonsoarias@hotmail.es

Full list of author information is available at the end of the article

Keywords Immunosenescence, Anti-viral immune memory, Cytomegalovirus, Influenza, SARS-CoV-2

Background

Contacts with antigens throughout an individual's life lead to the generation of a specific memory cellular and humoral immune response. Two consequences of this process are a reduction in the number of naïve T lymphocytes and an increase in the abundance of memory cells. In the case of viruses, the type of infection they cause, and the stage of life when it occurs, may determine the type and intensity of the memory generated. The effect will be more pronounced in older adults, whose memory T repertoire is more conditioned by successive reactivations of the viruses that cause chronic or latent infections, or by repeated immunizations, through infection or vaccination, against seasonal viruses.

Older adults may be more susceptible to infections by viruses and other pathogens as a consequence of the changes in the immune system that accompany aging, known as immunosenescence [1]. During ageing, a chronic low-grade inflammation known as inflammaging also appears [2]. Some of the changes that occur in the T lymphocyte compartment during immunosenescence have been associated with clinical consequences such as the generation of weaker vaccine responses, a reduction in the ability to secrete antibodies and a defective immune response to viruses, mainly those to which there has been no previous exposure [3].

The consequences of viral infection vary depending on the virus characteristics, the viral lifecycle and the ability of the host's immune system to eliminate the infection, where age is an important factor. Thus, some viral infections cause acute disease after a short incubation period, while others can remain in a latent state or cause chronic infections or diseases. Latent cytomegalovirus (CMV), seasonal influenza and the novel SARS-CoV-2 infection are examples of three distinct models of viral infection, all of which have important implications for older adults and can induce different immune memory mechanisms.

CMV is a DNA herpesvirus that is ubiquitous in human populations worldwide. As is typical of all herpesviruses, CMV has biological properties of latency and reactivation, whereby once an individual has been infected, the virus remains latent for the rest of their life [4]. The important contribution of CMV infection to immunosenescence in older adults is well known [3, 5–7], but it is also of significance in immunosuppressed situations such as in kidney transplant, in onco-haematological patients [8, 9], and in some chronic diseases such as chronic heart failure and renal disease [10–12].

Conversely, influenza is a highly contagious, annual respiratory illness caused by several RNA viruses belonging to the Orthomyxoviridae family. Most people come into contact with these viruses several times during their life and are able to eliminate them without any complication. However, these viruses can increase morbidity and mortality in some groups of individuals, particularly the immunocompromised, as older adults tend to be. It is well documented that older adults are at higher risk of developing severe influenza disease and serious complications than younger individuals [13, 14]. Vaccination is the main preventive measure against influenza infection, and it is recommended that chronically ill individuals and adults over the age of 65 years be vaccinated annually [15, 16].

SARS-CoV-2 is a very recently emerged coronavirus that infects humans. It was first detected in 2019 as the causative agent of the current coronavirus disease 2019 (COVID-19) pandemic. It differs significantly from previously identified coronaviruses and offers an opportunity to study the immune response generated by individuals to a new viral antigen. COVID-19 disease follows a course with very diverse clinical presentations and symptoms. The severity of this infection is also highly variable, ranging from completely asymptomatic, through very mild, to extremely serious symptoms [17]. The main risk factors include age, obesity, hypertension, diabetes mellitus, heart disease and lung disease. In the case of age, patients over 60 years were found to be around five times more likely to die after developing symptoms than patients aged between 30 and 59 years [18]. Our group previously reported that specific immunity to SARS-CoV-2 is preserved in older surviving adults [19].

The aim of the current study was to characterise and compare the specific cellular and humoral responses to these three different examples of viral infection models (latent with reactivations, repeated and new) in immunocompetent older adults. The ultimate goal is to find new biomarkers of the immunocompetence status of older adults, based on the characterisation of responses against known previous infections that could reflect their ability to generate specific memory responses to new pathogens.

Methods

Donors

Fifty-nine volunteers with a positive PCR for SARS-CoV-2 were recruited by the Emergency Service of the Hospital Universitario Central de Asturias (Oviedo, Spain). Inclusion criteria for this study were an age of over 60 years old and their SARS-CoV-2 infection had been asymptomatic or very mildly symptomatic not requiring hospital admission. Peripheral blood samples were drawn for analysis from all participants an average of 5 months after being infected with SARS-CoV-2

for the first time between March and May 2020, ranging from 3 to 7 months after the SARS-CoV-2 PCR test was negative. It is important to remark that these volunteers had not received any SARS-CoV-2 vaccine at the time of the study and that SARS-CoV-2 infection suffered a few months before was the first time that the individuals had come into contact with this virus. Informed consent was obtained from all volunteers before they participated.

Haemogram and biochemical characteristics

Counts of leukocytes, overall and separately for lymphocytes, monocytes and neutrophils, were obtained from donors' whole blood, anticoagulated with EDTA, by fluorescent flow cytometry and hydrodynamic focusing in a Sysmex XT-2000*i* analyser (Sysmex, Kobe, Japan) following the manufacturer's specifications.

The levels of D-dimer and NT-proBNP were measured in donor serum by turbidimetric immunoassay using an ACL TOP 750 analyser (Werfen, Barcelona, Spain), and by electrochemiluminescence using a COBAS e801 analyser (Roche, Basel, Switzerland), both following the manufacturer's specifications.

Immunophenotyping

For flow cytometry analysis of lymphocyte subpopulations, peripheral blood cells were surface-stained with a combination of antibodies appropriate for the cell population being analysed. T/B/NK populations were analysed with anti-CD45 (FITC), anti-CD56+16 (RD1), anti-CD19 (ECD) and anti-CD3 (PC5) using the AQUIOS Tetra-2+Monoclonal Antibody Reagents Panel (Beckman-Coulter, Brea, CA, USA). The naïve cells and the different maturation stages of memory $CD4^+$ and $CD8^+$ T lymphocytes were analysed using anti-CD3 (FITC), anti-CD8a (PE), anti-CD45 (PerCP), anti-CD27 (PECy7), anti-CCR7 (APC), anti-CD45RA (APCFire), anti-CD28 (BV) (BioLegend, San Diego, CA, USA), and anti-CD4 (ECD) (Beckman-Coulter). This staining made it possible to discriminate the different subpopulations of $CD4^+$ and $CD8^+$ T lymphocytes: naïve (N) ($CD45RA^+CCR7^+$), central memory (CM) ($CD45RA^-CCR7^+$), effector memory (EM) ($CD45RA^-CCR7^-$) and terminally differentiated effector T cells re-expressing CD45RA (EMRA) ($CD45RA^+CCR7^-$). The absolute frequency of cells per millilitre and the percentage of $CD4^+$ or $CD8^+$ T lymphocytes of these subsets of cells were measured. In the EM subpopulation it was possible to detect several maturation stages: less differentiated effector memory cells that are memory-like, i.e., EM1 ($CD27^+CD28^+$) and EM4 ($CD27^-CD28^+$); and the more differentiated effector memory cells that are effector-like, i.e., EM3 ($CD27^-CD28^-$) and EM2 ($CD27^+CD28^-$). The EM2 subtype is only present in $CD8^+$ T lymphoyctes but is absent from $CD4^+$ T lymphocytes. Functional differentiation of memory $CD4^+$ T lymphocytes was studied with anti-CXCR3 (AF488), anti-CD4 (ECD), anti-CCR6 (PC7) (Beckman-Coulter), anti-CCR7 (PerCP), anti-CD45RA (APCFire) and anti-CD28 (BV) (BioLegend), which were able to detect various Th subpopulations: Th1 ($CCR6^+CXCR3^+$), Th2 ($CCR6^-CXCR3^-$), Th17 ($CCR6^+CXCR3^-$) and Th1.17 ($CCR6^-CXCR3^+$). All information regarding the monoclonal antibodies used can be found in Supplementary Table 1.

These different stains were performed with 100 µL of whole blood anticoagulated with EDTA from the donors. Samples were stained with the corresponding combination of labelled monoclonal antibodies for 20 min at room temperature. Red blood samples were lysed for 10 min at room temperature with FACS Lysing Solution (BD Biosciences), washed in PBS and analysed using Kaluza software in a Navios cytometer (Beckman-Coulter). Appropriate isotype control mAbs were used for marker settings.

Specific T cellular response measurement

ELISpot assays were performed to quantify IFN-γ and granzyme B-producing specific T cells against CMV, influenza and SARS-CoV-2 viruses. Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood anticoagulated with EDTA by centrifugation on Ficoll–Hypaque gradients (Lymphoprep, Nycomed, Oslo, Norway). PBMCs (2.5×10^5/well) were then cultured for 18 h on a filter plate (Millipore, Billerica, MA, USA) previously coated with anti-IFN-γ or anti-granzyme B antibodies (15 µg/mL) (Mabtech, Nacka Strand, Sweden) and cultured in medium alone for negative control, in the presence of anti-CD3 (1 ng/mL) for positive control, and with peptides from the different viruses. The peptide pool contains 42 peptides from pp50, pp65, IE1, IE2 and envelope glycoprotein B antigens from CMV (28 of the peptides are MHC class I restricted and 14 are MHC class II restricted) (2 µg/mL) (Mabtech), the influenza virus quadrivalent vaccine used in the 2019–2020 campaign (1/100 dilution) or S1, S2 and N SARS-CoV-2 peptide pools ranging from 8 to 18 amino acids in length (2 µg/mL) (Mabtech). IFN-γ and granzyme B produced by T lymphocytes under specific stimulation were captured and detected by biotinylated anti-IFN-γ and anti-granzyme B antibody (1 µg/mL) (Mabtech), respectively, followed by streptavidin–horseradish peroxidase (Mabtech). Spots were developed using tetramethylbenzidine (TMB) substrate (Mabtech) and counted with ImageJ software. Results were considered to be negative when there were ten or fewer dots. The results were expressed as the frequency of IFN-γ or granzyme B producer T lymphocytes per 10^6 T cells.

Humoral response measurement

Levels of anti-CMV antigen antibodies (CMV-IgG) were detected in serum from the donors by chemiluminescence analysis using the LIAISON® CMV IgG II assay (DiaSorin, Milan, Italy). CMV seropositivity was defined as CMV-IgG > 14 U/mL.

Anti-influenza virus antibodies in serum obtained from individuals were measured semiquantitatively by ELISA as previously described [20], with some modifications [21]. The OD values of individual samples were compared against a calibration curve made by serial dilutions of the same internal control serum for all the experiments. The detection limit was 0.5.

Anti-SARS-CoV-2-specific IgG antibodies were quantified with a Human anti-SARS-CoV-2 (S) IgG ELISA kit and a Human anti-SARS-CoV-2 (N) IgG ELISA kit (Fine Test, Wuhan, China), following the manufacturer's specifications.

Statistical analysis and graphical presentation

Pearson's chi-squared test and Fisher's exact test were used to determine whether there were any significant associations between pairs of qualitative variables. The Kolmogorov–Smirnov test was used to determine whether quantitative variables were normally distributed. Haemogram and biochemical parameters data that were measured before and after the SARS-CoV-2 infection were analysed using Student's t test for paired samples or Wilcoxon's signed-rank test when the data were normally or non-normally distributed, respectively. The cellular responses to the three viral antigens were compared with a general linear model for repeated measures using Bonferroni-corrected post hoc pairwise comparisons. Group differences between quantitative variables were assessed with Student's t test or the nonparametric Mann–Whitney U test when the data were normally or non-normally distributed, respectively. Correlations between variables were assessed using the nonparametric Spearman test (ρ). Statistical analyses were carried out with SPSS 17.0 (SPSS Inc., Chicago, IL) and values of $p < 0.05$ were considered significant.

All graphs were created with GraphPad Prism (version 8.0.2).

Table 1 Participant characteristics

Variables	Patients ($n = 59$)
Age ± SD (years)	**72.2 ± 12**
Females	**39 (66)**
Underlying disease	**38/59 (64.4)**
Hypertension	25 (65.8)
Diabetes mellitus	13 (34.2)
Asthma	7 (18.4)
Heart disease	14 (36.8)
Renal disease	4 (10.5)
Smoker	3 (7.9)
Drinker	4 (10.5)
Altered D-dimer (> 500 ng/mL)	**18/49 (36.7)**
Altered NT-proBNP (> 300 pg/mL)	**9/58 (15.5)**
Influenza-Vaccinated_19/20	**33 (55.9)**
CMV-Seropositive	**49 (83)**
Symptomatic of COVID-19 disease	**36/56 (61)**
Fever	24 (66.7)
Cough	24 (66.7)
Dyspnoea	9 (25)
Chest pain	5 (13.9)
Ageusia	9 (25)
Anosmia	11 (30.6)
Mean days with symptoms ± SD	3.3 ± 2.7

Numbers in brackets represent percentages. Frequencies of patients with the different diseases are normalised to the total number of individuals with underlying diseases. Frequencies of the different COVID-19 symptoms are normalised to the total symptomatic individuals of COVID-19 disease

Results

Features of studied group

All the 59 donors recruited were more than 60 years old, with a mean age of 72.15 years (SD: 12 years), of whom 39 were female (66%) and 20 were male (34%). Details of underlying diseases (64.4% of the patients) and symptoms of patients that had mild symptomatic SARS-CoV-2 infection (61%) are summarised in Table 1. Laboratory characteristics, such as the level of the leukocyte subsets, D-dimer and NT-proBNP, were also measured in most patients. Regarding influenza immunology status, 33 patients had received the influenza vaccine some months before the samples were collected, while 26 had not been vaccinated in the most recent vaccination campaign (2019–2020). Forty-nine patients were CMV-seropositive and 10 were CMV-seronegative (Table 1).

Haemogram and biochemical characteristic recovery after SARS-CoV-2 infection

Since recruited patients had recently been infected with SARS-CoV-2, we wanted to determine whether some of the characteristics that are known to be altered during this infection had already returned to normal. As is well known, SARS-CoV-2 infection produces lymphopenia, as was seen in five of the patients for whom these data were available during the infection process (Supplementary Fig. 1). We wanted to establish whether patients showed normal levels of leukocytes when the study began. It was possible to obtain the basal haemogram information from 2 months to 4 years before the SARS-CoV-2 infection in 52 of the 59 patients. When the levels of the different leukocyte populations were compared before and after the infection, no statistically significant differences, or statistically significant but not biologically significant differences, were seen in the total leukocytes (Wilcoxon test, $p < 0.01$), lymphocytes (paired-samples t-test, $p < 0.01$),

monocytes (Wilcoxon test, $p<0.01$) or neutrophils (Wilcoxon test, $p>0.05$) (Fig. 1A and Supplementary Fig. 2). The differences observed were not biologically significant since the total leukocytes, lymphocytes and monocytes are all within normal limits for the age range of the patients (4–10, 1–5 and 0.2-1×10^6 cells/mL respectively) [22]. So, SARS-CoV-2 infection appeared not to affect leukocyte populations substantially once patients had completely recovered.

We now consider the biochemical characteristics that are known to be altered in SARS-CoV-2-infected patients and are considered indicators of a more severe COVID-19 disease. We measured D-dimer and NT-proBNP levels in serum samples of 49 and 58 of the 59 patients, respectively. Eighteen and nine individuals had an elevated level of D-dimer (>500 ng/mL) and NT-proBNP (>300 pg/mL), respectively, when the study began (Table 1). This finding was not associated with the patients' gender or COVID-19 symptomatology. Elevated levels of both factors were more likely to occur in older patients, since individuals with altered characteristics were significantly older than those whose parameters were within the normal range (Student's t test, $p<0.05$, for both) (Fig. 1B). With respect to individuals' existing underlying pathologies a higher prevalence of altered NT-proBNP was observed in patients who were suffering from an underlying cardiopathy (Pearson's chi-squared test, $p<0.05$) (Fig. 1C).

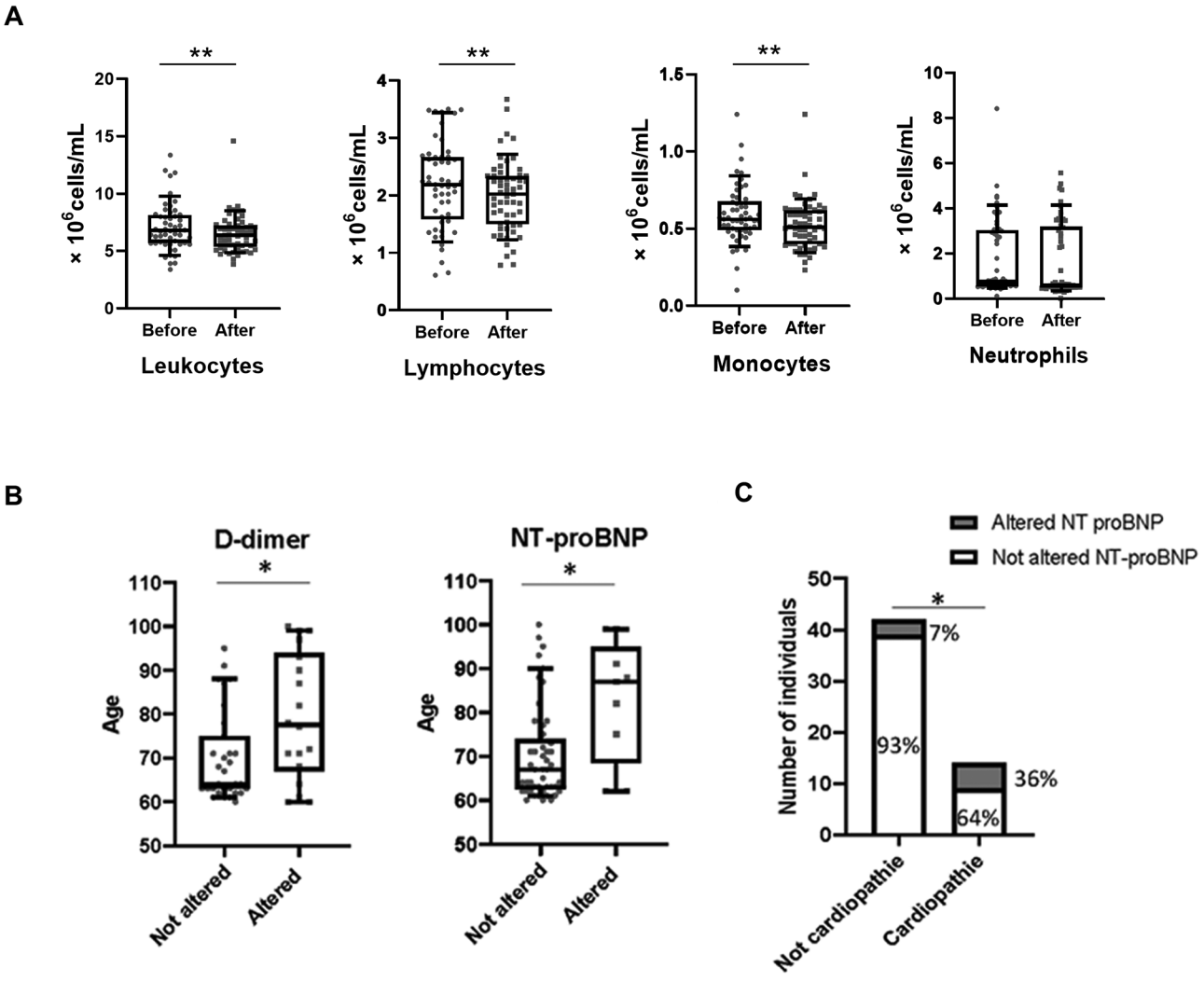

Fig. 1 Biochemical factors affected by SARS-CoV-2 infection. (**A**) Comparison between the level of total leukocytes (Wilcoxon test), lymphocytes (paired-samples t-test), monocytes (Wilcoxon test) and neutrophils (Wilcoxon test) populations before and after the SARS-CoV-2 infection. (**B**) Comparison of patients' age between individuals with altered and unaltered D-dimer and NT-proBNP biochemical indicators (Student's t-tests) (**C**) Representation of number of individuals without and with cardiopathie underlying disease (Pearson's chi-squared test). It is indicated the percentage of individuals with altered NT-proBNP in each case. ** statistically significant differences ($p<0.01$). * statistically significant differences ($p<0.05$)

Cellular and humoral responses to the three virus models

Cellular and humoral memory responses to the three viral antigens were determined. Cellular responses were measured by IFN-γ ELISpot and granzyme B ELISpot. Humoral responses were measured as the level of specific antibodies against the different viruses in sera. It was not possible to obtain all results for all patients, especially in the case of granzyme B ELISpot since not enough cells could be isolated from some patients. Complete results of the three measurements were achieved in 74.6% of patients for CMV, 84.7% for influenza and 83% for SARS-CoV-2 of the 59 patients enrolled in the study.

No responses were obtained for some measurements, especially cellular memory measured by granzyme B ELIspot (Fig. 2A). No cellular or humoral response was detected in 17% of CMV (corresponding to seronegative CMV patients) and 3% of SARS-CoV-2 cases. However, most patients showed both cellular (with at least one of the two measurements obtained) and humoral responses to CMV, influenza and SARS-CoV-2 viruses (83%, 83% and 85%, respectively) (Fig. 2B).

Isolated cellular responses with no accompanying humoral response were seen for influenza in 17% of the patients (Fig. 2B). Conversely, an isolated humoral response with no detectable cellular response was observed for SARS-CoV-2 in 12% of the patients. Of these, 14% showed anti-N antibodies, and the remaining 86% had both anti-N and anti-S antibodies. Likewise, 96% of the patients with humoral and cellular responses had both anti-N and anti-S antibodies, while the other 4% had isolated anti-N (2%) or anti-S (2%) antibodies (Fig. 2B).

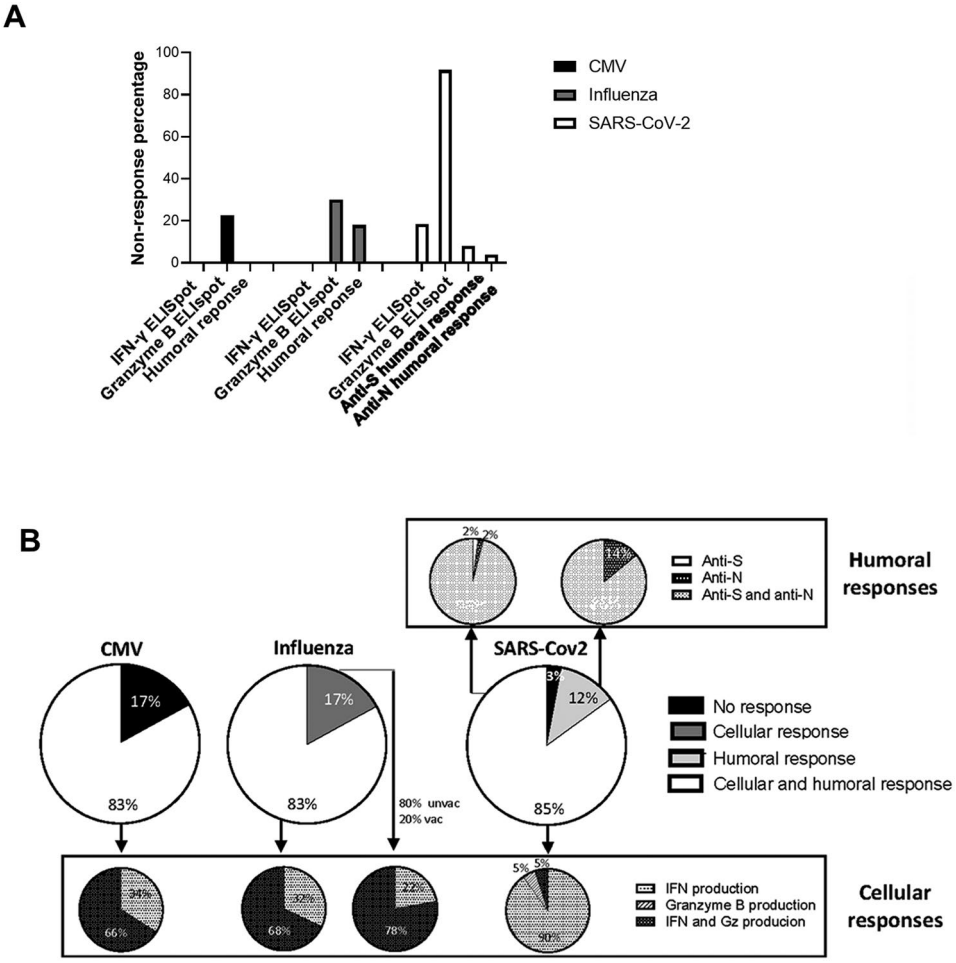

Fig. 2 Cellular and humoral responses detected. (**A**) Representation of the percentage of cases in which responses to the viruses (CMV among CMV-positive individuals in black, influenza in grey and SARS-CoV-2 in white) were not detected with each of the different measurements performed. (**B**) Schematic representation of the type of memory responses detected for the three viral infections (CMV, influenza and SARS-CoV-2). The percentage of patients in the different situations found for each virus is indicated (no detectable response in black, cellular response with no humoral response detected in dark grey, humoral response with no cellular response detected in light grey, and cellular and humoral responses detected in white). In the upper part is represented the percentage of patients that showed anti-S, anti-N or both humoral responses against SARS-CoV-2. In the bottom it is represented the percentage of patients that showed IFN-γ production, granzyme B production or both types of cellular responses against the three viruses. Vac: vaccinated_19/20. Unvac: unvaccinated_19/20

In all cases, positive cellular responses were detected mainly with IFN-γ-ELISpot measurement or both IFN-γ and granzyme B ELISpot. Just in the case of cellular responses to SARS-CoV-2, 5% of the patients showed granzyme B-ELISpot-positive results with negative IFN-γ-ELISpot. (Fig. 2B).

Anti-CMV responses

83% of the patients were CMV-seropositive. Distributions of the different response measurements against CMV are represented in Fig. 3A. As mentioned above, humoral and cellular responses to CMV matched perfectly, whereby all seronegative patients were also negative for cellular responses, while the seropositive group of patients showed cellular responses. There was a positive correlation in the seropositive CMV patients between the two cellular responses measured as IFN-γ and granzyme B-producer-specific T lymphocytes (Spearman test; $r=0.6$, $p<0.01$). However, no significant correlation was observed between the anti-CMV cellular and humoral responses (Supplementary Fig. 3A).

Anti-influenza responses

The influenza-vaccinated status of all patients was known: 55.9% of them had been vaccinated in the most recent vaccination campaign. Nevertheless, it can be assumed that all the patients, vaccinated and unvaccinated, had been exposed to this seasonal virus during

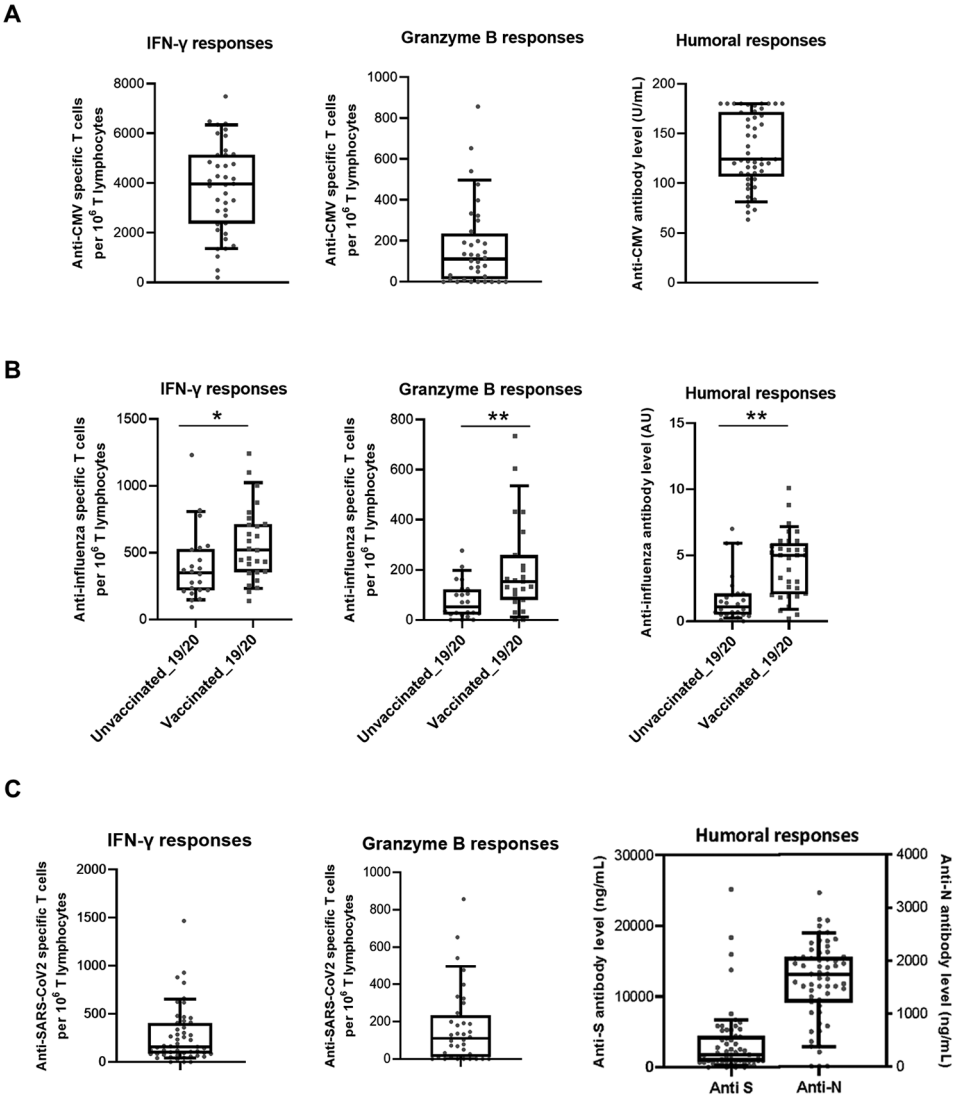

Fig. 3 Characterisation of anti-CMV, anti-influenza and anti SARS-CoV-2 memory responses (**A**) Anti-CMV cellular (left and middle graphs) and humoral (right graph) responses among the CMV-seropositive patients. (**B**) Comparison of anti-influenza cellular (left and middle graphs) and humoral (right graph) responses between vaccinated and unvaccinated patients from the most recent vaccination campaign (2019–2020) (Mann–Whitney test). (**C**) Anti-SARS-CoV-2 cellular (left and middle graphs) and humoral (right graph) responses. * statistically significant differences ($p<0.05$) Mann-Whitney U test. ** statistically significant differences ($p<0.01$) Mann-Whitney U test

their lives. In fact, the cellular memory response to influenza (IFN-γ and/or granzyme B-producing T lymphocytes) was detected in all the patients. Regarding the humoral response, 94% of vaccinated and 69% of unvaccinated patients had antibodies against influenza at a level above our detection threshold. The great majority (80%) of the 17% patients who showed cellular responses without antibodies to the influenza vaccine were unvaccinated in the last vaccination campaign (2019–2020) (Fig. 2B). As expected, the group of individuals vaccinated in the last vaccination campaign had a significantly higher specific cellular memory response (Mann–Whitney test, $p<0.05$ for IFN-γ and $p<0.01$ for granzyme B producer T lymphocytes per 10^6 T lymphocytes) and anti-influenza antibody titre (Mann–Whitney test, $p<0.01$) compared with the unvaccinated group (Fig. 3B).

The two different measurements of cellular response (IFN-γ and granzyme B production) and the values for IFN-γ-producer T lymphocytes and humoral response against influenza virus were both positively correlated (Spearman test; $r=0.6$, $p<0.01$ and $r=0.3$, $p<0.05$, respectively) (Supplementary Fig. 3B).

Anti-SARS-CoV-2 responses

All the patients had been infected with SARS-CoV-2 for the first time and an adequate response to the virus was expected as they were asymptomatic or suffered only very mild symptoms. As described above, cellular or humoral responses to SARS-CoV-2 virus were observed in 97% of the cases (85% showed both types of response, while 12% presented only a humoral response). No response was detected in two patients studied by any of the methods used (Fig. 2B). Distributions of the different response measurements against SARS-CoV-2 are represented in Fig. 3C. As described above, some biochemical characteristics related to the severity of the SARS-CoV-2 infection, D-dimer and NT-proBNP levels were measured. No significant differences were found in the responses to SARS-CoV-2 between patients with and without altered parameters (Supplementary Fig. 4).

Positive correlations were noted between cellular responses, measured as specific IFN-γ-producer T lymphocytes, and humoral responses to SARS-CoV-2. The correlation was higher for anti-N antibodies (Spearman test, $r=0.5$, $p<0.01$) than for anti-S antibodies (Spearman test; $r=0.3$, $p<0.05$). As expected, the two humoral response measurements were significantly positively correlated (Spearman test, $r=0.5$, $p<0.01$). However, no significant correlation was observed between the two cellular response measurements (Supplementary Fig. 3C).

Relation between viral antigen model responses

Comparing the cellular responses to the three viruses showed that the specific anti-CMV cellular response was stronger, in the CMV-seropositive group of patients, than the cellular response to the influenza virus, and both were stronger than the response to SARS-CoV-2, particularly when the cellular response was measured with IFN-γ ELISpot, since the differences between all three viral antigens' responses were statistically significant (repeated measures ANOVA followed by Bonferroni adjustment, $p<0.01$). When measured by granzyme B ELISpot, there were significant differences between CMV and SARS-CoV-2 (repeated measures ANOVA followed by Bonferroni adjustment, $p<0.05$) and between influenza and SARS-CoV-2 (repeated measures ANOVA followed by Bonferroni adjustment, $p<0.01$) but not between CMV and influenza in either of the unvaccinated_19/20 and vaccinated_19/20 patient groups (Fig. 4). Humoral responses could not be compared because they were measured in different ways.

We found a significant positive correlation between cellular responses to influenza and SARS-CoV-2 for IFN-γ-producer T lymphocytes (Spearman test; $r=0.4$, $p<0.01$) and granzyme B-producer T lymphocytes (Spearman test; $r=0.4$, $p<0.05$) although these were not apparent at the humoral level (Fig. 5A). On the other hand, there were no significant correlations of any of the measurements of the responses between CMV and SARS-CoV-2 or between CMV and influenza. However, comparing influenza and SARS-CoV-2 specific responses in CMV-seropositive and CMV-seronegative patients showed them to be consistently lower in CMV-seropositive than in CMV- seronegative patients, in both cases (Fig. 5B and C). This comparison was not significant probably because there were too few CMV-seronegative individuals in the sample for a difference of that magnitude to be significant.

Characterisation of the responses to the three antigen models in relation to the T lymphocyte phenotype

Immunosenescence may be related to the intensity of responses to the different virus infections. The immunophenotype of T lymphocytes by their degree of maturation (naïve/memory) and functional differentiation of $CD4^+$ T lymphocytes (Th1/Th2/Th17) and their correlations with the viral responses were analysed.

CMV

Agreeing with what is already well known and established, differences in the distribution of the T lymphocyte subpopulations were observed between CMV-seropositive and CMV-seronegative individuals (Supplementary Fig. 5A). CMV-seronegative patients had a significantly lower proportion of $CD4^+$EM3 than did CMV-seropositive individuals (median: 0.03% vs. 6.8%; Mann–Whitney test, $p<0.01$). However, the median proportions of $CD4^+$CM and $CD8^+$N were significantly higher in

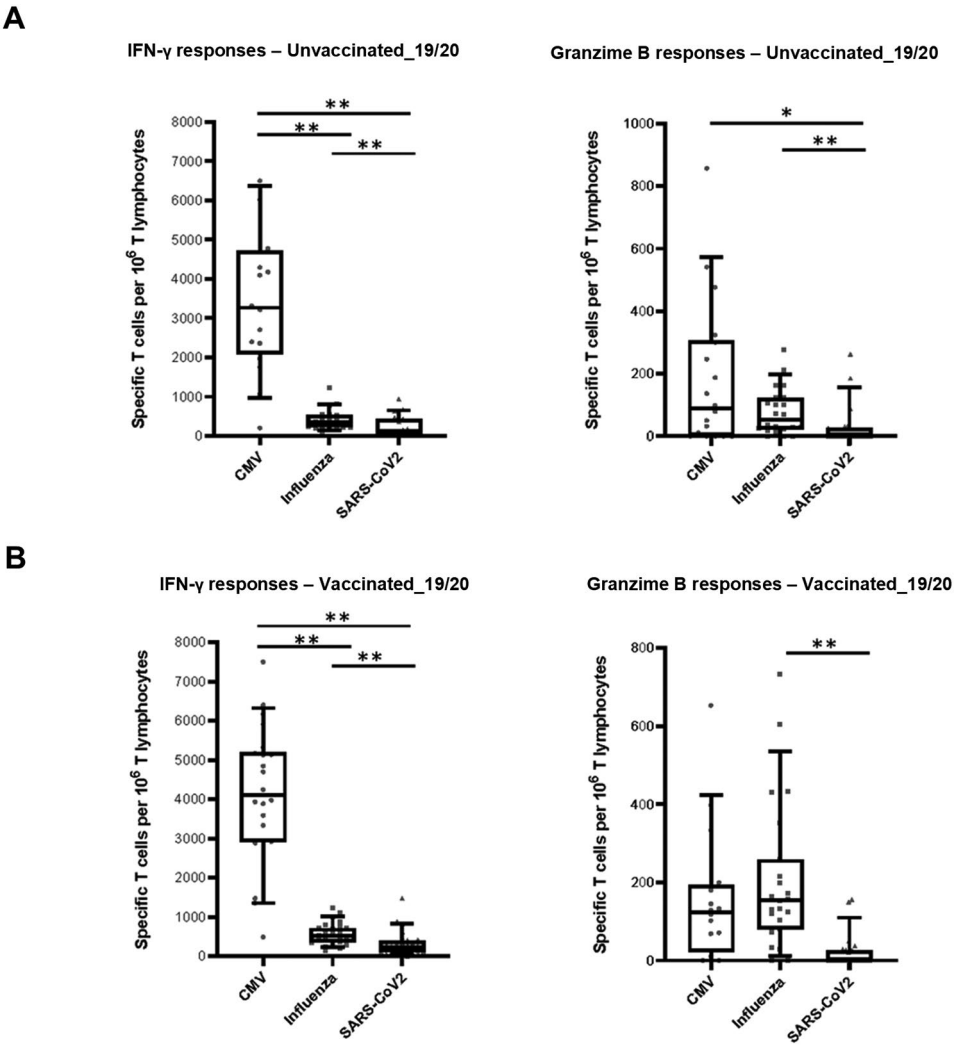

Fig. 4 Comparison of cellular responses generated against the three viruses. (**A**) Influenza unvaccinated patients. (**B**) Influenza vaccinated patients. Statistical tests used: repeated measures ANOVA followed by post-hoc Bonferroni. * statistically significant differences ($p < 0.05$). ** statistically significant differences ($p < 0.01$)

CMV-seronegative than in CMV-seropositive patients (30.4% vs. 22.6%; Mann–Whitney test, $p < 0.05$, and 16.2% vs. 7.1%; Mann–Whitney test, $p < 0.01$, respectively) (Supplementary Fig. 5A). With respect to the distribution of the functional subpopulations of $CD4^+$ memory T lymphocytes, there was a significantly lower mean percentage of Th1 in CMV-seronegative than in CMV-seropositive patients (29.2% vs. 39.2%; Student's t test, $p < 0.01$) (Supplementary Fig. 5B).

Considering the CMV-seropositive group of patients, the cellular response (IFN-γ and/or granzyme B-producing T lymphocytes) was negatively correlated with $CD4^+$N, $CD4^+$EM1 and $CD8^+$N while there was a significant positive correlation with $CD4^+$EM4. Regarding the humoral responses to CMV, there were negative correlations with $CD4^+$CM, $CD4^+$EM1, $CD8^+$N, $CD8^+$CM and $CD8^+$EM1. However, a significant positive correlation was seen with the $CD4^+$EM4, $CD4^+$EM3, $CD4^+$EMRA, $CD8^+$EM3 and $CD8^+$EMRA subsets (Fig. 6A). In the case of the functional differentiation of memory $CD4^+$ T lymphocytes, the humoral response to CMV was positively correlated with Th1 type and negatively correlated with Th2 and Th17 (Fig. 6B).

Influenza

It is expected that recently vaccinated patients are a more homogeneous group with respect to the immune responses generated against influenza seasonal pathogen. In patients vaccinated in the last vaccination campaign (2019–2020), the cellular memory responses to the influenza virus (IFN-γ and/or granzyme B-producing T lymphocytes) were negatively correlated with $CD4^+$EM3, $CD8^+$EM3 and $CD8^+$EMRA cells, and positively correlated with $CD4^+$EM4 and $CD4^+$EM1 cells. No

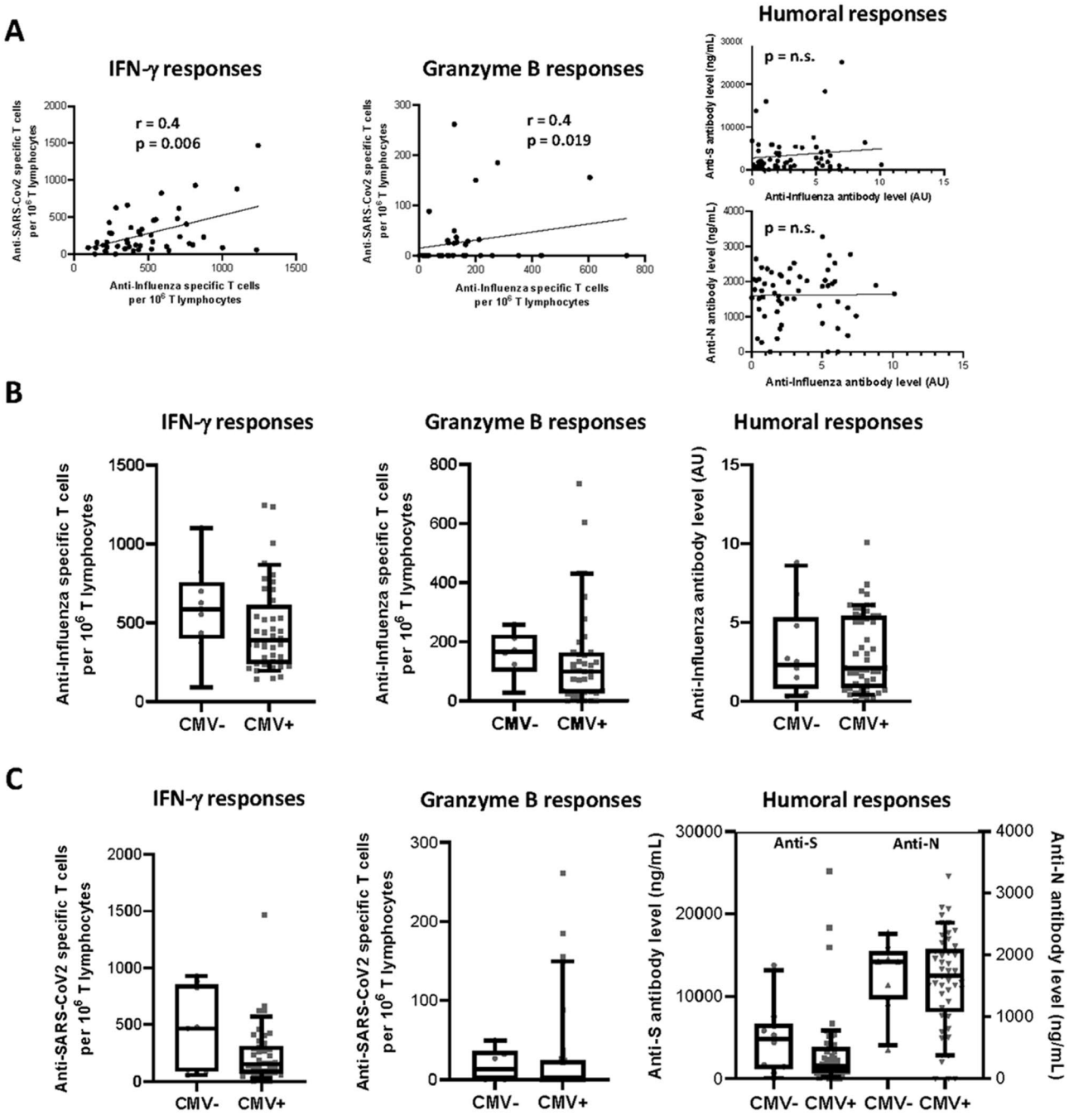

Fig. 5 Relation between viral antigen model responses. (**A**) The relationship between anti-influenza and anti-SARS-CoV-2 cellular (left and middle) and humoral responses (right). Spearman correlation coefficients and p-values are shown. n.s. = no significant. (**B**) Anti-influenza specific responses comparison between seropositive and seronegative CMV patients (Mann–Whitney test). (**C**) Anti-SARS-CoV-2 specific responses comparison between seropositive and seronegative CMV patients (Mann–Whitney test)

significant correlations were noted between the level of antibodies and the distribution of any of the subpopulations (Fig. 6A). Regarding the functional differentiation of memory $CD4^+$ T lymphocytes, the cellular response measured as IFN-γ-producer-specific T lymphocytes was negatively correlated with the Th2 subpopulation but positively correlated with the Th1.17 subpopulation (Fig. 6B).

SARS-CoV-2

T lymphocyte cellular responses to SARS-CoV-2 (IFN-γ and/or granzyme B-producing T lymphocytes) were correlated negatively with $CD4^+$EM4 and positively

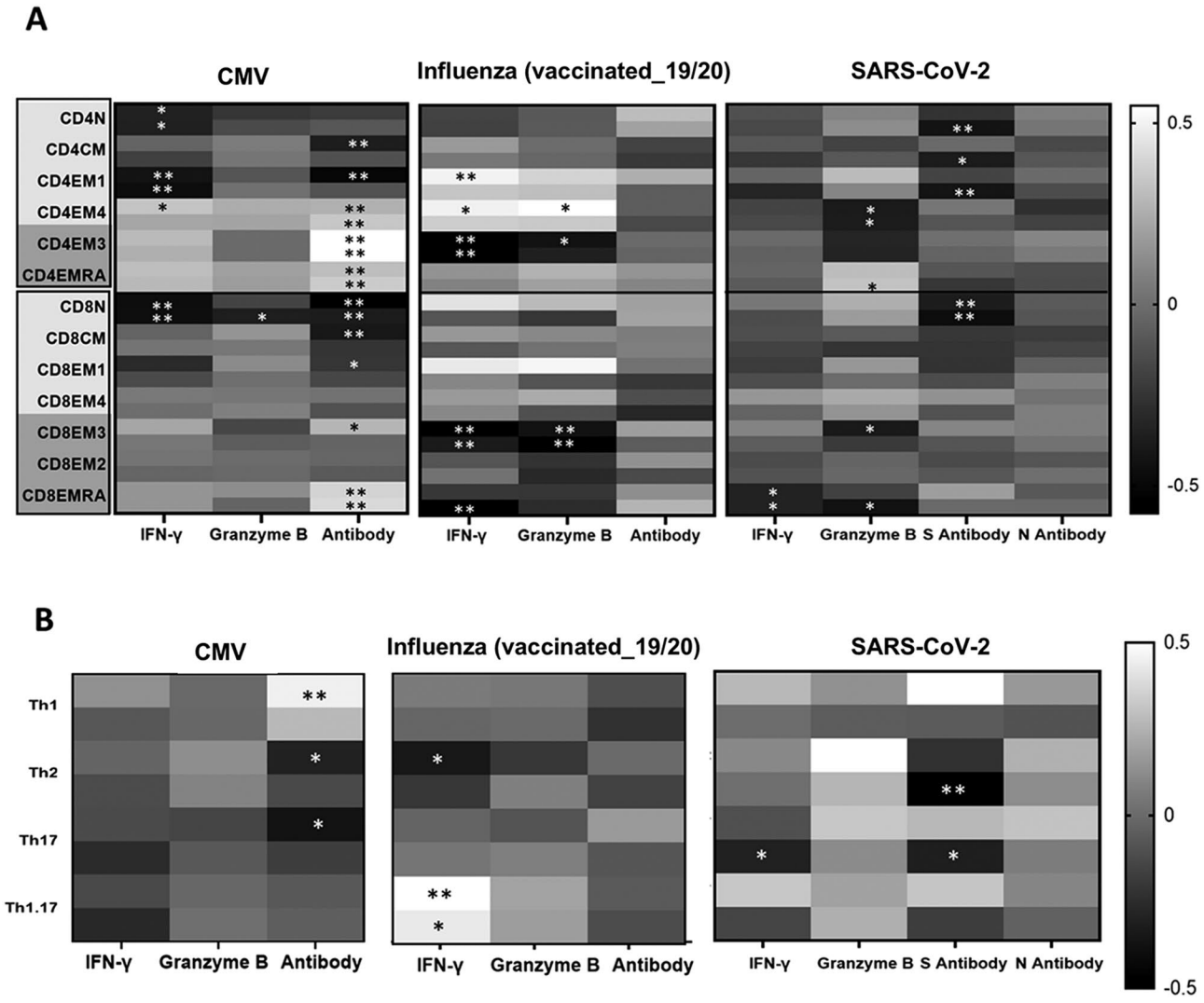

Fig. 6 Heat maps showing the correlations between the viral responses and the different T lymphocytes subpopulations distribution. Vertical columns represent the different types of cellular or humoral responses (labelled at the bottom). Horizontally are represented T lymphocyte subpopulations (labeled on the left); for each subpopulation the upper line is expressed as a percentage while the bottom line is expressed as absolute values in cells/μL. The correlation scale (Spearman correlation coefficients) is represented in a greyscale from darker (negative correlation) to lighter (positive correlation). An asterisk indicates a statistically significant correlation (Spearman correlation, $p < 0.05$) while two asterisks indicate a highly statistically significant correlation (Spearman correlation, $p < 0.01$). (**A**) Correlations of responses with the different T lymphocyte subpopulations according to their differentiation stage (naïve-memory). The subpopulations indicated on the left on a light background correspond to the least differentiated subpopulations while on a dark background to the most differentiated for both $CD4^+$ and $CD8^+$ T lymphocytes. Percentages are referred to the total $CD4^+$ or $CD8^+$ T lymphocytes but in the case of subtypes of EM they are referred to $CD4^+$EM or $CD8^+$EM. (**B**) Correlations of responses with the different functional differentiation subpopulations of memory $CD4^+$ T lymphocytes. Percentages are referred to the memory $CD4^+$ T lymphocytes

with $CD4^+$EMRA. Regarding $CD8^+$ T lymphocyte responses, there was a negative correlation between cellular responses and the $CD8^+$EM3 and $CD8^+$EMRA subsets. Analysis of the humoral responses revealed that the level of anti-S antibodies was negatively correlated with the $CD4^+$N, $CD4^+$CM, $CD4^+$EM1 and $CD8^+$N subsets, whereas anti-N antibodies were not significantly correlated with the distribution of any of the subpopulations (Fig. 6A). In terms of the functional differentiation of memory $CD4^+$ T lymphocytes, the humoral response, measured as anti-S antibodies, was negatively correlated with the Th2 and Th17 subpopulations. The negative correlation with Th17 lymphocytes was also seen at the cellular level (Fig. 6B).

Discussion

In this work we have characterised the cellular and humoral immune responses to three viral infection models in immunocompetent older adults. It is important to bear in mind that although it is feasible to classify the studied infections as examples of three different models of viral infection (latent, repeated and novel), it is not

possible to extrapolate our results to other viral infections. In fact, a limitation of the study is that we cannot confirm whether the observed differences are due to the type of infection or the biology of the virus (e.g. tropism, immune evasion mechanisms, viral escape, etc.). In the case of CMV infection, although it cannot be considered a model of chronic infection like other herpesviruses or the Epstein-Barr virus, its inclusion in the study is very important due to the characteristics of its interaction with the immune system. It is well known that gradual changes that trigger the deterioration of the immune system occur as an individual ages. These immunosenescence processes are also accompanied by a chronic low-grade inflammation, known as inflammaging, which is exacerbated by some factors, CMV infection being one of the most widely studied [1, 5–7].

All the patients studied in this work were older adults that had recently been infected with SARS-CoV-2 for the first time, but who had been asymptomatic or had experienced only very mild symptoms. This group of patients is hypothetically more immunocompetent, and potentially less affected by immunosenescence, than individuals in the same age range since their immune system were able to response adequately to a completely new antigen which it is uncommon in older adults [23]. In addition to not exhibiting clinical outcomes related to SARS-CoV-2 infection, the typical laboratory abnormalities associated with this infection, such as leukopenia or elevated levels of D-dimer and NT-proBNP serum biomarkers, were within normal ranges for the majority of the patients by the time the study began [24]. The elevated levels of the biochemical factors, D-dimer and NT-proBNP, detected in some of the patients studied are related to age and the presence of underlying cardiac pathology, as reported elsewhere [25, 26], rather than to the severity of the symptoms or the strength of the response to SARS-CoV-2. For these reasons, there was no bias regarding the clinical status of the patients in this study.

We know which individuals had been vaccinated in the previous influenza vaccination campaign (2019–2020), but information about the history of influenza infections or previous vaccinations was not available, which is a limitation of the study. The individuals studied therefore form a heterogeneous group with respect to influenza virus contact during their lives. Individuals vaccinated in the last vaccination campaign (2019–2020) constitute a more homogeneous group with respect to the immune memory that they would have developed to this seasonal pathogen. However, it is important to bear in mind that the immunoresponse generated in this group of patients is almost certainly not only due to the most recent vaccination received since it is very likely that they had been in contact with the virus at other times during their lives.

CMV-seronegative patients, as expected, did not show cellular responses either as they never have been infected by CMV. There were also two individuals in whom no cellular or humoral responses were detected against SARS-CoV-2. The lack of response of these two patients was surprising since all 59 individuals had been diagnosed with SARS-CoV-2 infection some months before. One possible explanation is that the RT-PCR used to diagnose SARS-CoV-2 infection has a specificity of greater than 95% [27], meaning that up to 5% of results could be false-positives. However, all the individuals studied were positive at least twice for the RT-PCR diagnostic test, making it unlikely that these two people were false-positives cases. On the other hand, it is important to bear in mind that all the individuals studied had had just one encounter with the SARS-CoV-2 virus, so it is possible that, in some cases, this stimulus was not strong enough to elicit a detectable memory response. This lack of response has been described before, especially in patients whose infection caused mild or no symptoms [19, 28], as is the case of the patients studied in this work. With the exception of these two cases, all the patients exposed to these antigens showed cellular and/or humoral responses. The responses generated are known to depend largely on the conditions of antigen exposure and persistence [29, 30], which could explain some of the differences found between the responses to the three viruses. CMV is a latent virus whose exposure persists over time because the immune system is exposed to the antigen at each reactivation [30, 31]. This explains why responses to this virus in CMV-infected individuals were the strongest and the most consistent. Conversely, the influenza virus is a seasonal, rather than a latent virus, exposure to which is frequent but not persistent over a lifetime, and the antigens to which people are exposed are continuously changing [15]. In this case, as expected, most of the patients recently vaccinated against influenza developed detectable humoral and cellular responses. Many of the people unvaccinated in the last vaccination campaign showed a cellular response but no detectable humoral response, possibly because most of them had not been in contact with the viral antigen for a longer time than the individuals vaccinated in the most recent campaign; it is well known that antibodies have a shorter lifespan than cellular memory [32, 33]. In the case of SARS-CoV-2 responses, some individuals had anti-SARS-CoV-2 antibodies but no detectable specific T cellular response. As mentioned above, asymptomatic or mild disease triggers a less intense memory response. It is likely that in this small group of patients the single encounter with the SARS-CoV-2 antigen enabled memory T cells to develop, although not to detectable levels. This is consistent with what happens in other coronavirus infections, such as SARS and MERS, in which higher levels of specific T

cells against the virus were found in patients with more severe symptoms [34]. In any case, the differences are also evident in the intensity of the cellular and humoral responses induced by each virus. While a positive correlation is observed between both types of response against influenza and SARS-CoV-2, the response to CMV does not exhibit this behaviour. The reason could be the type of memory response that successive encounters with viral antigens generate and, in fact, there are quite striking differences in the correlations between the subpopulation levels of T lymphocytes and the responses against the viruses. The persistence of exposure to the three viruses differs, with latent CMV being the most and SARS-CoV-2 the least persistent. This concurs with the results of the comparison of the strengths of cellular responses made in this study. However, it is also important to bear in mind that the antigenic stimuli used to measure the cellular responses could be immunogenically differently since peptide pools were used in the case of CMV and SARS-CoV-2 and whole vaccine in the case of influenza.

Besides this, the correlations between the different virus responses reflect the greater similarity of the responses to the novel SARS-CoV-2 virus to that of influenza than to the response to latent CMV. To investigate this in greater depth, the different responses were characterised in more detail taking into account the immunophenotyping of the lymphocyte subpopulations. CMV is a latent virus with successive reactivations whose infection is characterised by the generation of a more differentiated T lymphocyte phenotype, due to the persistent exposure of the immune system to this virus over time once the individual has been infected [11, 35]. This effect was also seen in our patients whereby CMV-seropositive individuals tended to have a higher frequency of highly differentiated T lymphocytes and a lower frequency of naïve T lymphocytes than CMV-seronegative patients. Consistent with this, it was observed that the stronger the responses were to CMV the more abundant were T lymphocytes with a differentiated phenotype. Many studies support this finding [11, 36]. CMV infection triggers the activation of B, T $CD4^+$ and T $CD8^+$ lymphocytes, and the persistent production of CMV virus results in a continuous and periodic stimulation of these cells, especially T $CD8^+$ [5, 37]. Each viral reactivation cycle generates a subset of CMV-specific T lymphocytes, which thereby reduces the T lymphocyte repertoire. The majority of these CMV-specific T lymphocytes are known to be terminally differentiated, corresponding to an exhausted and immunosenescent T cell phenotype [5, 38]. This situation of continuous immune stimulation also favours the pro-inflammatory chronic state that typically accompanies ageing [6, 39]. On the other hand, our results suggest that a better cellular response to the influenza virus is associated with a less T lymphocyte-differentiated phenotype. This is consistent with findings reported in young mice infected with the influenza virus, whose $CD4^+$ T lymphocyte compartment is comprised mainly of naïve cells that rapidly proliferate and differentiate into influenza-specific effector subsets that allow the clearance of the virus [40]. These correlations were seen at the cellular response level in recently vaccinated individuals, but there was no correlation with the humoral response in these patients. This might be because recently vaccinated patients, as expected, given the purpose of vaccination [15], had elevated levels of antibodies against influenza at the time of the study, perhaps independently of their T lymphocyte phenotype. Consistent with the correlations of the responses seen, SARS-CoV-2 showed mostly similar results to those of influenza with respect to cellular responses, especially $CD8^+$ T lymphocytes ones, provoking stronger cellular responses when less-differentiated $CD8^+$ T lymphocyte phenotypes were predominant. However, humoral responses to the SARS-CoV-2 virus (especially levels of anti-S antibodies) were negatively correlated with the abundance of naïve and less-differentiated memory T lymphocytes, similar to what happens in CMV. This could be because individuals with a more senescent immune system are less capable of generating an adequate cellular response —the main type of response for controlling viral infections— to this new viral antigen. Therefore, in these cases, the SARS-CoV-2 infection could persist for longer and could allow a stronger humoral response to be generated before the infection is resolved. This explanation is consistent with that previously proposed by Wu et al., who reported that severe SARS-CoV-2 patients had higher IgG-S and IgG-N titres [41], and with our earlier work, which determined that although there were no significant differences in cellular responses between hospital-admitted and non-admitted patients, anti-S and anti-N antibody titres were significantly higher in patients who required admission [19].

Regarding T $CD4^+$ differentiation into Th1/Th2/Th17 phenotypes, the Th1 response is the most prevalent in viral infections. Antiviral IFN-γ, mainly produced by this Th1 subset, enhances the stimulation of the adaptive antiviral response to clear the infection and generate a memory to protect against future infections. Accordingly, our results also implied that the immune response to the three viruses was associated with a Th1/Th2/Th17 balance in favour of Th1. These responses were positively correlated with the Th1 cells, as occurs in the case of CMV, coinciding with what was previously reported [42], or negatively correlated with the Th2 cells, as occurs in CMV, influenza and SARS-CoV-2. In the case of the response to influenza, it may seem to contradict our results that most of the licensed vaccines have been developed for the purpose of ensuring a Th2

response and the consequent production of antibodies [43]. However, these vaccines also collaterally induce cellular responses that favour the Th1 phenotype. This finding, and the greater persistence of the memory cellular response, are evidence supporting the proposal to modify the current criteria for developing influenza vaccines to ensure T cell-mediated protection, as other studies have already suggested [43, 44]. In the case of SARS-CoV-2 it is the humoral response that is negatively correlated with Th2 cells. Higher levels of Th2 cytokines, especially IL-4 and IL-5, can inhibit protective Th1 antiviral responses in COVID-19 patients. This was seen mainly in patients with severe COVID-19 disease, so it was hypothesized that Th2 inhibition might offer protection against severe COVID-19 symptoms, as seems to have happened in patients who were asymptomatic or who had mild COVID-19 disease [45]. Generally, there seem to be no differences in the three viruses' responses with respect to the distribution of the various functional differentiation subpopulations of memory $CD4^+$ T lymphocytes.

Conclusions

In conclusion, this work allowed us to accurately characterise the cellular and humoral responses generated against three viral infection models —latent with reactivations, seasonal and novel infection— in immunocompetent older adults. Average older adults, whose immunosenescence would be more pronounced, partly due to a higher prevalence and persistence of CMV latent infection over their lifetime, may be be less effective for fighting an infection generated by a novel antigen. The immune system was shown to react in a different way and with different intensity, depending on the durability and type of viral stimulus with which it is in contact. The results suggest that specific responses, especially cellular responses, to novel pathogens resemble the memory response enhanced by repeated, but not chronic, viral encounters. Both responses to novel and repeated pathogens may be favoured by a more naïve $CD8^+$ T lymphocyte phenotype compared with what happens when the immunosenescence is induced by latent CMV infection. The subpopulation distribution and the level of antigen-specific T lymphocytes acting against known previous pathogens could be good biomarkers of the immunocompetence status of older adults, reflecting their ability to generate specific memory responses to new pathogens.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12979-024-00488-3.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

Acknowledgements
The authors are grateful to the Instituto de Salud Carlos III for supporting the study and to the donors for their selfless contribution to help us better understand immunity to SARS-CoV-2 infection.

Author contributions
The authors' responsibilities were as follows: R Alonso-Arias, MA Moro-García and S Alonso-Alvarez designed the study; A García-Torre, E Bueno-García, B Rioseras, R López-Martínez, V Menéndez-García prepared protocols, collected and processed all the samples, performed or oversaw the experimental protocols, and analysed data; R Alonso-Arias, B Rioseras wrote the manuscript; A Lluna-González, A Sousa-Fernández, M Fernández-Goudin, L Campos-Riopedre, C Castro-Cueto, AB Pérez-Fernández, A Alonso-Rodríguez, C Menéndez-Peña, L Menéndez-Peña, N García-Arnaldo, E Feito-Díaz, A Fernández-Lorences, A Fraile-Manzano, C Fernández-Iglesias, JA Rivera, C Pérez-Fonseca, E Urdiales-Ruano, M Debán-Fernández, H Mendes-Moreira and P Herrero-Puente selected and recruited volunteers and organized their blood extractions and collected their clinical information; MA Moro-García and S Alonso-Alvarez reviewed the manuscript.

Funding
This research was partially supported by the Instituto de Salud Carlos III (COV20/0968) and by grant PI17/00714 from the Spanish I+D+i 2013–2016 State Program, which was cofounded by the Instituto de Salud Carlos III and the European Regional Development Fund (ERDF). E Bueno-García is sponsored by the Principado de Asturias (Programa de Ayudas "Severo Ochoa"; BP20-030).

Data availability
Data will be deposited in the Instituto de Salud Carlos III (ISCIII)-COVID19 repository, constituted to deposit data generated in the studies funded by the call for "Proyectos de investigación sobre el SARS-COV-2 Y LA ENFERMEDAD COVID-19".Also raw and derived data supporting the findings of this study are available from the corresponding author [R. Alonso-Arias] on request.

Declarations

Ethics approval and consent to participate
Informed consent was obtained from all the volunteers before they participated in the study. The study was approved in accordance with the Declaration of Helsinki by the ethics committee of the Hospital Central de Asturias (Oviedo, Spain) (nº 2020.269).

Consent for publication
Complete written informed consent was obtained from all the volunteers for the publication of this study.

Human Ethics and Consent to participate declarations
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]Immunology Department, Medicine Laboratory, Hospital Universitario Central de Asturias, Oviedo 33011, Spain
[2]Health Research Institute of the Principality of Asturias (ISPA), Oviedo 33011, Spain
[3]University Institute of Oncology of the Principality of Asturias (IUOPA), Oviedo 33011, Spain
[4]Medicine Laboratory Department, Hospital Universitario Central de Asturias, Oviedo 33011, Spain

[5]Haematology and Haemotherapy Department, Hospital Universitario Central de Asturias, Oviedo 33011, Spain
[6]Emergency Department, Hospital Universitario Central de Asturias, Oviedo 33011, Spain

Received: 13 July 2024 / Accepted: 13 November 2024
Published online: 05 December 2024

References

1. Pinti M, Appay V, Campisi J, Frasca D, Fülöp T, Sauce D, Larbi A, Weinberger B, Cossarizza A. Aging of the immune system: focus on inflammation and vaccination. Eur J Immunol. 2016;46(10):2286–301.
2. Wik JA, Skalhegg BS. T cell metabolism in infection. Front Immunol. 2022;13:840610.
3. Cunha LL, Valsecchi V, Ward LS. Investigating population-level immunosenescence: from bench to bedside. Front Immunol. 2022;13:949928.
4. Alford CA, Stagno S, Pass RF, Britt WJ. Congenital and perinatal cytomegalovirus infections. Rev Infect Dis. 1990;12(Suppl 7):S745–753.
5. Nikolich-Zugich J, van Lier RAW. Cytomegalovirus (cmv) research in immune senescence comes of age: Overview of the 6th international workshop on cmv and immunosenescence. *Geroscience* 2017;39:245–249.
6. Moro-Garcia MA, Alonso-Arias R, Lopez-Vazquez A, Suarez-Garcia FM, Solano-Jaurrieta JJ, Baltar J, Lopez-Larrea C. Relationship between functional ability in older people, immune system status, and intensity of response to cmv. Age (Dordr). 2012;34:479–95.
7. Solana R, Tarazona R, Aiello AE, Akbar AN, Appay V, Beswick M, Bosch JA, Campos C, Cantisán S, Cicin-Sain L, Derhovanessian E, Ferrando-Martínez S, Frasca D, Fulöp T, Govind S, Grubeck-Loebenstein B, Hill A, Hurme M, Kern F, Larbi A, López-Botet M, Maier AB, McElhaney JE, Moss P, Naumova E, Nikolich-Zugich J, Pera A, Rector JL, Riddell N, Sanchez-Correa B, Sansoni P, Sauce D, van Lier R, Wang GC, Wills MR, Zieliński M, Pawelec G. CMV and immunosenescence: from basics to clinics. Immun Ageing. 2012;9(1):23.
8. Alonso-Alvarez S, Colado E, Moro-Garcia MA, Alonso-Arias R. Cytomegalovirus in haematological tumours. Front Immunol. 2021;12:703256.
9. Iglesias-Escudero M, Moro-Garcia MA, Marcos-Fernandez R, Garcia-Torre A, Alvarez-Arguelles ME, Suarez-Fernandez ML, Martinez-Camblor P, Rodriguez M, Alonso-Arias R. Levels of anti-cmv antibodies are modulated by the frequency and intensity of virus reactivations in kidney transplant patients. PLoS ONE. 2018;13:e0194789.
10. Garcia-Torre A, Bueno-Garcia E, Lopez-Martinez R, Rioseras B, Diaz-Molina B, Lambert JL, Quiros C, Alonso-Alvarez S, Alonso-Arias R, Moro-Garcia MA. Cmv infection is directly related to the inflammatory status in chronic heart failure patients. Front Immunol. 2021;12:687582.
11. Moro-Garcia MA, Lopez-Iglesias F, Marcos-Fernandez R, Bueno-Garcia E, Diaz-Molina B, Lambert JL, Suarez-Garcia FM, de la Moris C, Alonso-Arias R. More intensive cmv-infection in chronic heart failure patients contributes to higher t-lymphocyte differentiation degree. Clin Immunol. 2018;192:20–9.
12. Yang TO, Chuang YF, Chiu YL. T-cell aging in end-stage renal disease: an evolving story with cmv. Med Microbiol Immunol. 2019;208:281–7.
13. Maruyama T, Fujisawa T, Suga S, Nakamura H, Nagao M, Taniguchi K, Tsutsui K, Ihara T, Niederman MS. Outcomes and prognostic features of patients with influenza requiring hospitalization and receiving early antiviral therapy: a prospective multicenter cohort study. Chest. 2016;149:526–34.
14. Garg S, Jain S, Dawood FS, Jhung M, Perez A, D'Mello T, Reingold A, Gershman K, Meek J, Arnold KE, et al. Pneumonia among adults hospitalized with laboratory-confirmed seasonal influenza virus infection-united states, 2005–2008. BMC Infect Dis. 2015;15:369.
15. Nypaver C, Dehlinger C, Carter C. Influenza and influenza vaccine: a review. J Midwifery Womens Health. 2021;66:45–53.
16. Keilman LJ. Seasonal influenza (flu). Nurs Clin North Am. 2019;54:227–43.
17. Forchette L, Sebastian W, Liu T. A comprehensive review of covid-19 virology, vaccines, variants, and therapeutics. Curr Med Sci. 2021;41:1037–51.
18. Gao YD, Ding M, Dong X, Zhang JJ, Kursat Azkur A, Azkur D, Gan H, Sun YL, Fu W, Li W, et al. Risk factors for severe and critically ill covid-19 patients: a review. Allergy. 2021;76:428–55.
19. Garcia-Torre A, Bueno-Garcia E, Lopez-Martinez R, Rioseras B, Moro-Garcia MA, Alonso-Alvarez S, Lluna-Gonzalez A, Sousa-Fernandez A, Fernandez-Gudin M, Campos-Riopedre L, et al. Surviving older patients show preserved cellular and humoral immunological memory several months after sars-cov-2 infection. J Gerontol Biol Sci Med Sci. 2022;77:33–40.
20. Ohishi K, Ninomiya A, Kida H, Park CH, Maruyama T, Arai T, Katsumata E, Tobayama T, Boltunov AN, Khuraskin LS, et al. Serological evidence of transmission of human influenza a and b viruses to caspian seals (phoca caspica). Microbiol Immunol. 2002;46:639–44.
21. Kang I, Hong MS, Nolasco H, Park SH, Dan JM, Choi JY, Craft J. Age-associated change in the frequency of memory cd4 + t cells impairs long term cd4 + t cell responses to influenza vaccine. J Immunol. 2004;173:673–81.
22. Bain BJ, Bates I, Laffan MA. Dacie and Lewis Practical Haematology. 12th ed. London: Elsevier; 2017.
23. Dadras O, SeyedAlinaghi S, Karimi A, Shamsabadi A, Qaderi K, Ramezani M, Mirghaderi SP, Mahdiabadi S, Vahedi F, Saeidi S, et al. Covid-19 mortality and its predictors in the elderly: a systematic review. Health Sci Rep. 2022;5:e657.
24. Ferrari D, Seveso A, Sabetta E, Ceriotti D, Carobene A, Banfi G, Locatelli M, Cabitza F. Role of time-normalized laboratory findings in predicting covid-19 outcome. Diagnosis (Berl). 2020;7:387–94.
25. Draper J, Webb J, Jackson T, Jones H, Rinaldi CA, Schiff R, McDonagh T, Razavi R. G SC-W. comparison of the diagnostic accuracy of plasma n-terminal pro-brain natriuretic peptide in patients < 80 to those > 80 years of age with heart failure. Am J Cardiol. 2018;122:2075–9.
26. Johnson ED, Schell JC, Rodgers GM. The d-dimer assay. Am J Hematol. 2019;94:833–9.
27. Healy B, Khan A, Metezai H, Blyth I, Asad H. The impact of false positive covid-19 results in an area of low prevalence. Clin Med (Lond). 2021;21:e54–6.
28. Boechat JL, Chora I, Morais A, Delgado L. The immune response to sars-cov-2 and covid-19 immunopathology - current perspectives. Pulmonology. 2021;27:423–37.
29. Harari A, Vallelian F, Meylan PR, Pantaleo G. Functional heterogeneity of memory cd4 t cell responses in different conditions of antigen exposure and persistence. J Immunol. 2005;174:1037–45.
30. Kalia V, Sarkar S, Ahmed R. Cd8 t-cell memory differentiation during acute and chronic viral infections. Adv Exp Med Biol. 2010;684:79–95.
31. Kim J, Kim AR, Shin EC. Cytomegalovirus infection and memory t cell inflation. Immune Netw. 2015;15:186–90.
32. Janeway C, Travers P, Walport M, Shlomchik M. Immunobiology: The immune system in health and disease, 5th edition ed. United Kingdom; Garland Science; 2001.
33. Le Bert N, Tan AT, Kunasegaran K, Tham CYL, Hafezi M, Chia A, Chng MHY, Lin M, Tan N, Linster M, et al. Sars-cov-2-specific t cell immunity in cases of covid-19 and sars, and uninfected controls. Nature. 2020;584:457–62.
34. Sariol A, Perlman S. Lessons for covid-19 immunity from other coronavirus infections. Immunity. 2020;53:248–63.
35. Khairallah C, Dechanet-Merville J, Capone M. Gammadelta t cell-mediated immunity to cytomegalovirus infection. Front Immunol. 2017;8:105.
36. Van den Berg SPH, Pardieck IN, Lanfermeijer J, Sauce D, Klenerman P, van Baarle D, Arens R. The hallmarks of CMV-specific CD8 T-cell differentiation. Med Microbiol Immunol. 2019;208(3–4):365–73.
37. Wertheimer AM, Bennett MS, Park B, Uhrlaub JL, Martinez C, Pulko V, Currier NL, Nikolich-Zugich D, Kaye J, Nikolich-Zugich J. Aging and cytomegalovirus infection differentially and jointly affect distinct circulating t cell subsets in humans. J Immunol. 2014;192:2143–55.
38. Karrer U, Sierro S, Wagner M, Oxenius A, Hengel H, Koszinowski UH, Phillips RE, Klenerman P. Memory inflation: continuous accumulation of antiviral cd8 + t cells over time. J Immunol. 2003;170:2022–9.
39. Pawelec G, Gouttefangeas C. T-cell dysregulation caused by chronic antigenic stress: the role of cmv in immunosenescence? Aging Clin Exp Res. 2006;18:171–3.
40. Lanzer KG, Johnson LL, Woodland DL, Blackman MA. Impact of ageing on the response and repertoire of influenza virus-specific cd4 t cells. Immun Ageing. 2014;11:9.
41. Wu J, Liang B, Chen C, Wang H, Fang Y, Shen S, Yang X, Wang B, Chen L, Chen Q, et al. Sars-cov-2 infection induces sustained humoral immune responses in convalescent patients following symptomatic covid-19. Nat Commun. 2021;12:1813.
42. Rentenaar RJ, Gamadia LE, van DerHoek N, van Diepen FN, Boom R, Weel JF, Wertheim-van Dillen PM, van Lier RA, ten Berge IJ. Development of virus-specific cd4(+) t cells during primary cytomegalovirus infection. J Clin Invest. 2000;105:541–8.
43. Kim YH, Hong KJ, Kim H, Nam JH. Influenza vaccines: past, present, and future. Rev Med Virol. 2022;32:e2243.
44. Pleguezuelos O, Robinson S, Fernandez A, Stoloff GA, Mann A, Gilbert A, Balaratnam G, Wilkinson T, Lambkin-Williams R, Oxford J, et al. A synthetic influenza virus vaccine induces a cellular immune response that correlates

with reduction in symptomatology and virus shedding in a randomized phase Ib live-virus challenge in humans. Clin Vaccine Immunol. 2015;22:828–35.
45. Pavel AB, Glickman JW, Michels JR, Kim-Schulze S, Miller RL, Guttman-Yassky E. Th2/th1 cytokine imbalance is associated with higher covid-19 risk mortality. Front Genet. 2021;12:706902.

Publisher's note