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Deregulated cellular circuits driving immunoglobulins and complement consumption associate with the severity of COVID-19 patients

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Abbreviationts list:

COVID-19: Coronavirus disease 2019

SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2

ARDS: Acute respiratory distress syndrome

ADCC: Antibody-dependent cell cytotoxicity

Tfh: Follicular helper T cell

NK: Natural killer

CRP: C-reactive protein

Ig: Immunoglobulin

mAb: Monoclonal antibody

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Abstract

SARS-CoV-2 infection causes an abrupt response by the host immune system, which is largely responsible for the outcome of COVID-19. We investigated whether the specific immune responses in the peripheral blood of 276 patients associated to severity and progression of COVID-19.

At admission, dramatic lymphopenia of T, B and NK cells associated to severity. Conversely, the proportion of B cells, plasmablasts, circulating follicular helper T cells (cTfh) and CD56-CD16+ NK-cells increased. Regarding humoral immunity, levels of IgM, IgA and IgG were unaffected, but when degrees of severity were considered, IgG was lower in severe patients. Compared to healthy donors, complement C3 and C4 protein levels were higher in mild and moderate, but not in severe patients, while the activation peptide of C5 (C5a) increased from the admission in every patient, regardless their severity. Moreover, total IgG, the IgG1 and IgG3 isotypes and C4 decreased from day 0 to day 10 in patients who were hospitalized for more than two weeks, but not in patients who were discharged earlier.

Our study provides important clues to understand the immune response observed in COVID-19 patients, associating severity with an imbalanced humoral response and identifying new targets for therapeutic intervention.

Introduction

Novel coronavirus disease (COVID-19), due to severe acute respiratory coronavirus 2 (SARS-CoV-2), is either asymptomatic or presents with mild symptoms in a majority of individuals. However, up to

20% patients develop a severe form of the disease with pneumonia, which in some cases results in acute respiratory distress syndrome (ARDS) and requires invasive mechanical ventilation. ARDS, together with myocardial damage, are main causes of mortality in COVID-19 [1].

A pathogenic hallmark of ARDS is the disruption of the alveolar-capillary barrier and a subsequent increase in permeability, which has been partially attributed to a maladaptive immune response. Thus, lung alveolar macrophages may be infected by the virus and become activated, leading to a cytokine release syndrome, which contributes to endothelial injury with the recruitment and activation of innate and adaptive immune cells and the extravasation of plasma components of the humoral immunity [2].

Humoral immunity plays a key role in the initial control of viral infections and cell-to-cell spread. It is mediated by the complement system and the immunoglobulins (Ig) [3, 4]. Different SARS-CoV-2 components such as pathogen-associated molecular patterns (PAMPs) and N protein, together with C reactive protein (CRP) from plasma, may activate either the alternative or the lectin pathways of the complement cascade early during infection [5]. Furthermore, early IgM isotype antibodies efficiently trigger the classical complement cascade. Secreted IgA antibodies neutralize viruses within the mucosa of the respiratory and gastrointestinal tracts. Finally, in a more advanced stage of the disease, virus-specific IgG antibodies opsonize viral particles. This may lead to the formation of immune complexes that contribute to the activation of the classical complement pathway. Furthermore, opsonized viral particles can attach to Fc receptors on phagocytes and NK cells. The latter constitute major innate immunity mediators during anti-viral responses, since they kill infected cells through different mechanisms, including antibody-dependent cell cytotoxicity (ADCC), which is mediated by the FcγRIIIA (CD16) binding to clustered IgG displayed on cell surface of virally infected cells [6]. Isotype switching and affinity antibody maturation, as well as the generation of memory B cells and long-lived plasma cells, are B cell responses driven by helper T cells, in particular follicular helper T cells (Tfh).

However, deregulated humoral immune response can damage host tissues. Complement-mediated tissue injury is elicited by an intense inflammatory loop secondary to C3a- and C5a-mediated recruitment and activation of neutrophils, monocytes, macrophages, lymphocytes, and platelets. Phagocytes in turn generate reactive oxygen species (ROS) and proteases, and neutrophils release neutrophil extracellular traps (NETs), which exacerbate tissue damage [7-10].

Several studies on SARS-CoV2 infection have attempted to elucidate phenotypic features of immune cell subsets either associated with severity or predictive of disease outcome. However, these studies have been conducted, in most cases, with a limited number of patients, and clinical parameters and disease severity are not homogeneously recorded in all of them [11, 12]. Likewise,

although specific antibodies and complement activation have been proposed to mediate some of the most severe complications of coronavirus infections, including that of SARS-CoV-2 [2, 5, 13, 14], solid evidence on the role of humoral immunity effector mechanisms in the pathogenesis of SARS-CoV-2-associated ARDS is clearly needed.

Results

COVID-19 patients have increased B cells and plasmablasts in peripheral blood

Our cohort of COVID-19 patients included 276 SARS-CoV-2 infected individuals who were further classified according to the severity of their clinical signs and symptoms in mild, moderate and severe, following recently described criteria [15]. The mean duration of symptoms before admission was 7.36 ± 5.2 days. Table 1 shows their main demographic and laboratory characteristics. The median (percentile 25 and 75) age was 63 (53.25-75) and 163 (59.05%) were men.

We conducted an initial analysis following the COVID-19 admission protocol, which comprised the quantification of the main peripheral blood lymphocyte subsets, including T, B and NK lymphocytes as well as plasmablasts, by multi-parametric flow cytometry (Fig.1A, Suppl. Fig 1). The proportion of T lymphocytes (either CD4+ or CD8+) and NK cells was similar in all the patients and healthy volunteers, with the exception of CD8+ T cells, which decreased in severe patients (Fig.1B). Conversely, the percentage of B cells was higher in COVID-19 patients and increased with disease severity, raising from a mean of 9.15% in healthy donors to 20.49% in severely ill patients. In accordance, plasmablasts were remarkably higher in patients, both in relative and absolute values (mean 1.51% vs 17.98%; and 2.83 cells/μl vs 27.37 cells/μl, respectively), and regardless severity degree (Figure 1B). This increase in absolute plasmablasts number is of special relevance, given the lymphopenia present in COVID-19 patients (Table 1), and the diminished absolute number of other lymphocyte subsets (Fig.1B). The elevated number of plasmablasts suggested a redistribution of the main maturation stages of the B lineage, including naïve, transitional, unswitched memory, IgM-only memory and class-switched memory B-cells, which were identified in a subgroup of 84 patients from our initial cohort, with the gating strategy shown in Suppl. Fig 2. In COVID-19 patients, the proportion of IgM-only memory B-cells increased, while unswitched memory cells decreased (Fig.1C). This redistribution was more marked in mild patients and progressively diminished in moderate and severe patients. Nevertheless, differences among patients with different severity degree were not statistically significant (Suppl. Fig 3).

Increased follicular helper T cells in the peripheral blood of COVID-19 patients

Given the role of Tfh in maturation and activation of B cells, we assessed whether circulating Tfh (cTfh) were increased in the peripheral blood of COVID-19 patients, in accordance with the increased number of plasmablasts. We observed that the cTfh proportion significantly increased with the severity of COVID-19 individuals, shifting from a median of 0.51% in healthy donors to 1.7 % in severely ill patients. Importantly, the absolute number remained steady despite the profound decrease of total CD4+ T cells (Fig.2A, Fig.1B). To further characterize this population, we assessed surface expression of CCR7 chemokine receptor, which has been related to lower B-cell activation capacity by Tfh [16, 17]. We found a significant increase of CCR7 expression in cTfh cells of patients with moderate to severe disease (Fig 2A). Finally, we found a direct correlation between cTfh proportion and total B-cells (r 0.33; p= 0.0090), class-switched B-cells (r 0.26, p= 0.0433), and plasmablasts (r 0.33, p= 0.0091) in peripheral blood (Fig.2B).

COVID-19 severity associates to serum levels of immunoglobulins and complement

The high number of plasmablasts prompted us to investigate possible alterations in Ig concentrations. At the time of admission, COVID-19 patients had serum concentrations of either IgG, IgA or IgM isotypes comparable to those in healthy volunteers (Fig.3A). On the other hand, when patients with different degree of severity were analysed, we observed that, in severe cases, the levels of IgA and IgM were similar, but the IgG concentration was decreased, compared to healthy volunteers (Fig.3B). In addition, a direct correlation was found between IgG (0.37; p=0.0007) and IgA (0.23; p=0.0444) serum concentrations and the absolute number of plasmablasts in peripheral blood, and between IgM levels and total number of peripheral blood IgM- only memory B-cells (0.36; p=0.0040) in COVID-19 patients. On the contrary, IgG (-0.31; p=0.0147) and IgA (-0.38; p=0.0021) were inversely related to the proportion of unswitched memory B cells (Fig.2B).

The increase in plasma concentrations of IL-6 and acute phase reactants that characterizes COVID-19 (Table 1) suggested that the concentration of C3 and C4 complement proteins, considered as acute phase reactants, could also be elevated. Therefore, we measured C3 and C4 levels in the sera of these patients, and could detect increased levels of both complement proteins (Fig.3A). Accordingly, to investigate whether complement activation occurs in COVID-19 patients, we quantified C5a, the activation peptide of complement component C5, which showed increased plasma levels in most patients (Figure 3A).

However, when considering different groups of severity, we observed that C3 and C4 levels increased in patients with mild to moderate disease, while returned to levels similar to healthy donors in those with severe disease (Fig.3B). Therefore, in contrast to other inflammatory parameters such as LDH, ferritin or CRP (Table 1), C3 and C4 values decreased as the severity

increased. Of note, unlike C3 and C4, C5a levels remained elevated in plasma regardless degree of severity (Fig.3B).

Next, we tested whether the decrease in these components of humoral immunity was actually related to the severity of the disease in critical patients, or rather mirrored an increased consumption along time. We then considered a hospitalization period longer than 15 days as a readout of the severity of the disease and collected the blood of a subgroup of 37 COVID-19 patients who were still hospitalized 10 days after admission and compared it to the initial blood test. With this approach, we observed that antibodies of the IgG, IgA and IgM isotypes, the IgG subclasses IgG1, IgG3, and IgG4 as well as C3, C4 and C5a complement proteins were similar in all patients at the time of admission (Fig.4A and B). However, after 10 days, serum concentration of IgG was significantly lower in patients hospitalized longer than 15 days, whereas levels of IgA and IgM did not change over this time. Interestingly, whereas IgG1 and IgG3 levels showed a significant fold decrease in long vs. short stay patients after 10 days from admission, IgG4 concentration diminished similarly in both groups of patients. In order to determine whether these changes corresponded to a specific anti-SARS-CoV-2 response, we quantified IgG against SARS-CoV-2 RBD and N proteins, which could be detected at a high titer at day 10 in all cases (Suppl. Fig.4). On the contrary, anti-CMV IgG levels decreased after 10 days of hospitalization in both groups (Suppl. Fig 4).

Regarding complement, C3 serum levels were stable over time in both groups of patients. On the other hand, a significant decrease of C4 concentration was observed after 10 days specifically in those patients whose severity eventually required a longer stay (Fig.4A). These patients however showed sustained C5a levels at this time point (Fig.4A). Conversely, activation product C5a decreased in patients who stayed less than 15 days, which did not show a parallel C4 significant reduction (Fig.4A).

The decrease in C4 and IgG serum concentrations in severe COVID-19 cases led us to hypothesize that antigen-antibody complexes were forming in excessive amounts and activating the classical pathway of complement system. Therefore, we quantified immune complexes by enzyme immunoassay in the serum of the SARS-Cov-2 infected individuals with different levels of severity, but we did not find detectable levels (data not shown).

Phenotypic features of NK cells from severe COVID-19 patients

The decrease of IgG that characterized severe patients also suggested a possible role of ADCC, which has been described to be mediated by specific NK cells subsets [18]. We therefore quantified the main functional subsets of NK cells ($CD56^{bright}CD16-$, $CD56^{dim}CD16+$, CD56-CD16+). A two-fold increase in the proportion of CD56-CD16+ NK cells was found in severe COVID-19 patients compared

to healthy donors and patients with mild disease (Fig. 5A). Furthermore, the absolute values of this population remained similar to those of healthy donors in all COVID-19 patients, in contrast to the decrease seen for other lymphocyte populations (Fig. 5A). Further analysis of this population showed a significant downregulation of CD16 expression irrespective of the degree of severity (Fig.5B). Interestingly, CD16 level in CD56-CD16+ cells slightly correlated to the serum concentration of IgG in COVID-19 patients (r-0.24; p=0.0496) (Fig.2B).

Discussion

It has been proposed that the severity of COVID-19 is related to a dysregulation of the immune response to SARS-CoV-2. However, there is no precise knowledge about the immune profile of COVID-19 patients with different clinical courses. To address this issue, we have performed a comprehensive characterization of the immune cell populations and soluble mediators of the humoral immunity in the peripheral blood of 276 patients, who presented from mild to critical illness in a single centre during the peak of the pandemic in Madrid, Spain.

Plasmablasts were significantly elevated in peripheral blood of most COVID-19 patients at the time of admission. In contrast, Ig levels were similar to those of healthy donors, or even decreased in the case of IgG in critically ill patients. Since patients were studied at admission, it was feasible that Ig production had not peaked yet. However, IgG levels further decreased 10 days after admission in patients with longer hospital stays, which suggests that IgG decrease was rather related to severe disease progression. Three possible explanations may account for these apparently contradictory observations. The first explanation is a primary antibody immunodeficiency that debuts with COVID-19. This is supported by the fact that several patients had very low levels of IgG, IgA and IgM in serum both at admission and after 10 days. We have not addressed this possibility yet, however we plan to carry out studies aimed at verifying this hypothesis by assessing Ig levels three months after discharge. Second, it is possible that certain individuals show impaired specific Ig production (as might appear from the decrease of the titer of CMV-specific antibodies shown in supplementary figure 4) after infection secondary to generalized lymphopenia, altered B cell maturation, or patient age. Regarding impaired specific Ig production, it is known that sepsis triggers immune cell hyperactivity followed, days later, by immune paralysis, which associates with a poor patient outcome [19]. Different mechanisms may underlie this process, including dysregulation of T cell subsets [20]. Isotype switching, which results in the production of IgA and IgG antibodies, is a feature of germinal centres in lymphoid follicles following the activation and interaction of specific B cells and Tfh cells. Despite the increase in cTfh cells in COVID-19 patients, cTfh from individuals with moderate to severe disease express higher levels of CCR7, which inversely correlates with Tfh

capacity for activating B cells [21] as CCR7 leads to their retention in the T zone of the lymph node, preventing their interaction with B cells in the germinal center [17].

Recent reports have described specific phenotypic features of cTfh involved in humoral response against SARS-CoV-2 proteins in convalescent COVID-19 patients [22]. Thus, although cTfh specific for spike protein were mainly cTfh17 (CXCR3-CCR6+), neutralizing antibody activities were directly related with the presence of cTfh1 (CXCR3+CCR6-) and cTfh2 (CXCR3-CCR6-) functional subsets. We have not studied in detail functional profile of cTfh in our cohort. Hence, further experiments are needed to establish a possible relationship between functional phenotype of cTfh and severity degree in these patients, and to confirm a Tfh–dependent defective B cell activation in COVID-19 that contributes to a possible immune paralysis at severe stages of the disease.

Likewise, plasmablasts number in peripheral blood decreases with age in healthy individuals [23] which could result in a reduced production of specific neutralizing antibodies and contribute to the severe course in the elderly. Finally, the decrease in circulating IgG could be secondary to its deposit in tissues, where they might activate different Fc-mediated effector mechanisms, including complement-dependent cytotoxicity. The absence of significant levels of immune-complexes in sera of these patients does not rule out their local formation and deposit in the tissues. Interestingly, evidence from autopsies and/or biopsies of COVID-19 patients suggests that a widespread complement activation could mediate microvascular injury and leukocyte infiltration, since deposits of different components of the complement system like C5b-9, C4d and MASP are observed in the microvasculature of the lung [24] or the skin [5, 13].

Furthermore, the role of complement in the pathogenesis and severity of other coronavirus infections had already been proposed [12, 25-28]. Thus, Gralinski et al demonstrated with C3-/-, C4-/- or Factor B-/- mice that complement activation significantly contributes to the pathogenesis of SARS caused by SASR-CoV. In addition, the N protein from either SARS-CoV, MERS and SARS-CoV-2 efficiently activates MASP-2, the primary enzymatic initiator of the lectin pathway [5]. Moreover, genomic/proteomic studies indicate a relevant role of different complement proteins in the pathogenesis and severity of COVID-19 [29].

The nature of specific antibodies which could activate complement should, therefore, require further research. For example, a recent study in a SARS-CoV macaque model that appeared before the emergence of first cases of COVID-19, demonstrated that a faster development of neutralizing IgG against SARS-CoV spike characterized animals who developed severe lung injury [14]. Moreover, S glycoprotein-specific Ab responses were higher and peaked earlier in deceased patients during the first 15 days after the appearance of symptoms, but dramatically dropped 5 days later, coinciding with clinical deterioration [30].

In our cohort, we have found that IgG1 and IgG3 isotypes, but not IgG4, decreased after 10 days from admission specifically in more severe patients, a finding that could be related to the greater capacity of those isotypes to mediate the activation of the classical pathway of complement. Of note, specific IgG3 antibodies are the first to appear in viral infections [31]. Likewise, C3 and C4 levels were increased in both mild and moderate patients but not in individuals with severe COVID-19, where C3 and C4 remained within the normal values or were even lower. The reasons for these findings include individual variability in C3 and C4 synthesis due to genetic deficiencies or individual genetic polymorphisms [32-34]. Alternatively, a higher C3 and C4 consumption may occur in severe patients by an excessive activation of either the lectins pathway or the classical pathway through specific anti-viral antibodies or immune-complexes. The selective C4 depletion observed is probably due to the limited amount of C4 in plasma compared to C3, which is one of the most abundant plasma proteins. In addition, a clear correlation between C3 and C4 was found in those patients (data not shown), suggesting a parallel consumption of both proteins which are characteristic of some autoimmune diseases and severe infections [35-38].

Accordingly, we found increased levels of C5a (the activation product of C5) also in patients with severe disease. Similarly, recent reports have demonstrated that C5a and other complement activation products like C5b-C9 are increased in COVID-19 patients [24, 39]. Hence, sustained high C5a concentration in the presence of normal or reduced levels of C3 and C4 would support complement activation in these patients. Quantification of C1q, mannose-binding lectin and factor B, among others, could further clarify which pathways are responsible for complement activation in COVID-19.

C5a is one of the strongest proinflammatory molecules, since it has chemoattractant activity and activates most leukocytes. In particular, C5a promotes the lung sequestration of myeloid cells [40] and pulmonary dysfunction and exerts procoagulant activity through several mechanisms, including the induction of tissue factor by endothelial cells and neutrophils, as well as the upregulation of plasminogen activator inhibitor-1 in mast cells. In addition, the cytolytically inactive terminal complement complex C5b-9 induces procoagulant activity through platelet prothrombinase and activates endothelial cells to express adhesion molecules and tissue factor [41-44]. In summary, our findings support the idea of complement being involved in the hyperinflammatory syndrome and thrombotic microangiopathies observed in COVID-19 patients.

NK cells are involved in the early immune defence against viral respiratory infections [45]. Actually, several studies from SARS-CoV and MERS infections, either in patients or animal models, had already revealed that these cells play an important role in fighting coronavirus [46-48]. The behaviour of NK cells in response to SARS-CoV-2 infection is not unique but similar to that described in other viral

infections [49] and in the previous outbreaks of coronavirus-associated SARS pneumonia. NK cell absolute numbers were reduced in patients compared to non-infected donors, with significantly lower counts in severe cases than in moderate or mild patients [50-52]. These authors have found no significant differences either in the proportion of whole NK population or in their main subsets CD56bright and CD56dim. However, to our knowledge, the CD56-CD16+ NK cell subset has not been analysed yet in the context of the COVID-19. We have observed that this subpopulation is clearly expanded in severe patients, and that CD16 expression is significantly lower in this subset when compared with healthy donors. This particular CD56-CD16+ population is expanded in some viral infections and demonstrates cytokine activation and ADCC activity in some settings. However, CD56-CD16+ NK cells have been shown to have less functionality and lower expression of natural cytotoxicity receptors in other situations [18, 53, 54]. Compared to peripheral blood, normal human lung is enriched in NK cells, which present a differentiated phenotype and have the capacity to respond to viral infections [55, 56]. But the persistence of highly active NK cells in the lung may exacerbate inflammation and result in an intense and irreversible damage of the airway epithelial cells [57, 58].

Many questions arise from the experimental data provided in this work. Whether the normal levels of C3 and C4 of our severe patients are reflecting a primary or secondary complement immunodeficiency or, on the contrary, an excessive activation of complement, is not known. Is the NK CD56-CD16+ subset promoting host defence or mediating tissue injury? Are they contributing to the antiviral response or reflecting a severely compromised innate immune response? At any rate, the increase of cTfh cells and the NK CD56-CD16+ subset, the low levels of total IgG or the decrease of IgG1 and IgG3 over time that characterize severe patients open new exploratory avenues of research.

Thus, while immune system is required for protection from SARS-CoV-2 infection, its response, if excessive or sustained over time, like that occurring in COVID-19, may perpetuate both ongoing inflammation and the hypercoagulable state. It is therefore necessary to provide more insights related to individual characteristics and the particular kinetics of the host response to SARS-CoV-2, to further understand the pathogenesis of COVID-19 and to decide the appropriate therapy and the treatment timing. This knowledge is of particular therapeutic relevance since it is the basis for clinical trials with the different immune modulators that are currently available including intravenous immunoglobulins, hyperimmune plasma or complement inhibitors, among others [59-61].

Material and methods

Study design and population

This is a retrospective observational study including 276 consecutive patients with confirmed detection of SARS-CoV-2 RNA, and admitted to the Accident and Emergency Department of the Hospital Universitario La Princesa because of mild to critical COVID-19 symptoms, from February 27th to April 29th.

Data collection

Demographic and laboratory data described in Table 1 were collected from electronic clinical records and included in an anonymized database. Baseline evaluation of immunity was performed around the 3rd day of admission (median=3 days; percentile 25-75 [p25-p75] 2 to 6). A second evaluation was obtained around the 14th day of admission (median=14 days; p25-p75 12 to 16.5) in a selected group of 37 patients.

SARS-CoV-2 RNA detection

Samples from nasopharyngeal and throat exudates were obtained with specific swabs as previously described [62]. As first line screening, we performed real-time RT-PCR assay targeting the E gene of SARS-CoV-2, with Real Time ready RNA Virus Master on Applied Biosystems TM Quant Studio-5 Real-Time PCR System. This assay was followed by confirmatory testing with the assay TaqPath™ COVID-19 CE-IVD Kit RT-PCR Applied Biosystems™ (ThermoFisher Scientific, Waltham, MA USA), which contains a set of TaqMan RT PCR assays for *in vitro* diagnostic use. This kit includes three assays that target SARS-CoV-2 genes (Orf1ab, S gene, N gene) and one positive control assay that targets the human RNase P RPPH1 gene [63]. Determinations were carried out in an Applied Biosystems^{TM} QuantStudio-5 Real-Time PCR System (CA, USA).

Sample collection and flow cytometry analysis

Peripheral blood samples from 276 COVID-19 patients were obtained in EDTA tubes for flow cytometry assessment around the 3rd day of admission. The distribution of the different leukocyte subsets was characterized by multiparametric flow cytometry within 24 h after extraction following the Guidelines for the use of flow cytometry and cell sorting in immunological studies [64]. To this end, 200 µL of whole fresh blood were stained in a BD OneFlow™ LST from BD Biosciences (San José, CA, USA) designed to identify and quantify the main lymphocyte populations of T (CD3+, CD4+,

CD8+, γδ+), B, and NK lineages. Absolute cell numbers were calculated from white blood cell counts obtained with an XN-10 Hematology System (Sysmex, Kobe, Japan- Roche, Basel, Switzerland).

A subgroup of 84 patients were randomly selected during the period of study for an extensive characterization of their lymphocyte subsets. These patients, as well as 19 healthy donors, were studied with an extended panel of multicolor monoclonal antibodies (mAbs): anti-CD4 FITC, anti-IgD FITC, anti-PD-1 PE, anti-CD56 PE, anti-CD27 PE, anti-CD38 PerCP/Cy5.5, anti-CXCR5 AF647, anti-IgM APC, anti-CD19 PE-Cy7, anti-CD8 APC/H7, anti-CD10 APC/H7, anti-CCR7 BV421, anti-CD16 V450, anti-CD3 V500 from BD Biosciences (USA); and anti-CD20 V450 from Immunostep (Spain).

Samples were incubated for 30 min at room temperature with the respective mAbs. Then, erythrocytes were lysed with FACS lysis solution (BD Biosciences) for 10 minutes and after washing with phosphate buffered saline, samples were analyzed in a FACS Canto II device (BD). At least 100 cells of the less represented subsets were collected and data were analyzed with the FACSDiva and FlowJo Softwares from BD Biosciences. The different leukocyte subsets were assigned according to the strategy showed in supplementary Figure 1.

Data are expressed as percentage or absolute number in cells/μl of a given lymphocyte subpopulation. A dual-platform method was used to calculate absolute cell counts. Mean fluorescence intensity (MFI) was employed to compare the level of expression of certain molecules.

Serum concentrations of immunoglobulins and complement proteins

Serum samples were tested by immunonephelometry for total IgG, IgA, IgM, C3 and C4 concentrations (Immage800, Beckman Coulter, California, USA) and for IgG1, IgG3 and IgG4 (Optilite, The Binding Site, Birmingham, UK). C5a quantification was performed with the MicroVue C5a ELISA kit (Quidel, Athens, USA) following manufacturer´s instructions. In addition, 19 serum samples from healthy donors obtained during the same period time were used to determine the variability of Ig, C3 and C4 and C5a levels in normal conditions. The absence of SARS-Cov-2 infection in healthy donors at the time of extraction was confirmed by retrospective determination of specific antibodies. The presence of circulating immunocomplexes was assessed with C1q CIC ELISA Kit (INOVA, San Diego, USA) following manufacturer´s instructions.

Detection of specific antibodies against SARS-CoV-2 and Cytomegalovirus

Nucleoprotein (NP) and the Receptor Binding Domain (RBD) of SARS-CoV-2 were produced and ELISA was performed as described by Martinez-Fleta et al.[65]. 96-well Maxisorp Nunc-Immuno plate were coated with 100 μL/well of recombinant proteins diluted in 0.1 M borate buffered saline (BBS) pH 8.8; NP at 0.5 μg/ml, RBD at 1μg/ml and incubated overnight at 4^{o}C. Coating solutions were then

aspirated, the ELISA plates were washed three times with 200 µl of PBS 0.05% Tween 20 (PBS-T) and blocked with 200 µl PBS-casein (Biorad,1x PBS blocker) for 1 hour at room temperature. The plates were washed again with PBS-T and 100 µl of patient serum sample diluted in PBS-casein, 0.02% Tween-20, was added and incubated for 2 hours at room temperature. The plates were washed again and 100 µL/well of AffiniPure Rabbit Anti-Human IgG (Fcγ fragment specific) from Jackson Labs was added and incubated for 1 hour at room temperature. The plates were washed with PBS-T four times and incubated at room temperature in the dark with 100 µL/well of Substrate Solution (OPD, Sigma prepared according to the manusfacturer's instructions) (typically for 3 minutes). 50 µL of stop solution (3M H2SO4) were then added to each well and the optical density (at 492nm) of each well was determined using a microplate reader.

Negative controls included wells coated just with blocking buffer and serum samples collected from healthy donors before 2019. The cut-off was established by comparison with the result of a positive serum sample.

Serum samples were also assessed with an anti-CMV/IgG detection kit (Enzygnos, Siemens), following manufacturer's instructions.

Statistics

Descriptive results were expressed as mean ± standard deviation (SD) or median and percentile 25-percentile 75 (p25-p75), as appropriate, while qualitative variables are presented as frequency (n) and relative percentages of patients (%). The unpaired, two-tailed, Student t-test was used to compare two independent groups and the paired Student t-test, to analyse two related samples. One-way ANOVA was employed to compare more than two groups and post-hoc multiple comparisons were made with Tukey's test. Spearman bivariate correlations were performed between serological quantitative markers and cell populations and corrplot R package (available from: https://github.com/taiyun/corrplot) was used for correlation map graphics. Variables in correlation map were reordered using hierarchical cluster method. The p-values were two-sided and statistical significance was considered when $p < 0.05$. To analyse distribution of lymphocyte in COVID-19 patients and healthy donors, an automated clustering and dimensionality reduction was performed using viSNE and FlowSOM tools (Cytobank). Population cells were normalized using log transformation for analysis. Differences in normalized cells between healthy donors and severity groups (adjusted by sex and age) were assessed with a moderated t-test using limma R package[66]. Cell populations that showed significant P-value (FDR = 5%) were considered as differentially expressed between groups. Stata v. 12.0 for Windows and R version 3.5.1 were used for analyses

and graphics. GraphPad Prism 4 software was also used for graphics. Data is presented making specification for $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****).

Study approval

This study was approved by the local Research Ethics Committee (register number 4070) and it was carried out following the ethical principles established in the Declaration of Helsinki. All included patients were informed about the study and gave an oral informed consent because of COVID-19 emergency as proposed by AEMPS (Agencia Española del Medicamento y Productos Sanitarios).

Author contributions

A.A, F.S.M and C.M.C developed the research idea and study concept, designed the study and wrote the manuscript; A.M.J, S.S.A and A.A.S designed and conducted most experiments, analysed the data and prepared the figures; M.S.N performed statistical analysis; M.L.T, E.M.G, M.J.C, S.C., H. F. and I.G.A. provided clinical data and peripheral blood samples from the study patient cohorts and critically revised the manuscript. J.M.R.F, J.M.C, H.T.R and M.V.G provided the reagents for the detection of specific antibodies against SARS-CoV-2. All other authors participated in patient samples processing and clinical data collection.

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The authors declare no commercial or financial conflict of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Tables.

Characteristics (missing data: n, %)	Study population n= 276	Mild n= 146	Moderate n= 89	Severe n= 41	Normal Values at H.U. La Princesa
Age	63 (53.25-75)	61 (52-75.25)	64 (53-75)	68 (58.5-72)	
Male sex	163 (59.05 %)	79 (54.10%)	53 (59.55%)	31 (75.61%)	
Leukocytes Count (cells/µl) (3, 1.08%)	6810 (4935-9640)	5885 (4675-7885)	7950 (5713-10313)	10580 (7470-13820)	4000-10000
Lymphocytes Count (cells/µl) (3, 1.08%)	1050 (675-1470)	1195 (860-1665)	870 (585-1308)	680 (480-1230)	1000-4000
Creatinine (mg/ dl) (3, 1.08%)	0.83 (0.67-1.03)	0.79 (0.66-1)	0.84 (0.69-0.99)	1 (0.63-1.55)	0.5-0.9
Bilirubin (mg/ dl) (5, 1.81%)	0.47 (0.36-0.72)	0.46 (0.35-0.67)	0.47 (0.36-0.72)	0.62 (0.41-1.16)	0-1.2
AST (U/L) (3, 1.08%)	34 (23-53.5)	31 (22-47.5)	38 (24-58)	45.5 (28.25-74)	4-32
ALT (U/L) (4, 1.44%)	34 (21-62.5)	32 (20-57.5)	35 (23-70.75)	40 (24-79)	5-33
GGT (U/L) (6, 2.17%)	54.5 (31-123)	50 (30-101)	55 (28-149)	82.5 (42-218)	6-42
LDH (U/L) (7, 2.53%)	288 (221-388.5)	244.5 (206-321.3)	321.5 (261.5-431.5)	369 (299-559)	135-214
CRP (mg/dl) (9, 3.26%)	5.77 (2.28-12.26)	3.97 (1.29-9.23)	9.04 (3.49-15.18)	8.83 (4.04-26.61)	0-0.5
Ferritin (ng/ml) (64, 23.18%)	288 (221-388.5)	619 (309-1441)	1037 (496.5-1740)	1587 (1030-2691)	30-400
D-dimer (µg/ml) (17, 6.15%)	0.81 (0.46-2)	0.72 (0.46-1.19)	0.91 (0.44-2.40)	3.46 (0.86-13.79)	0.15-0.5

IL-6 (pg/ ml) (12, 4.34%)	11.81 (0.8-42.35)	7.11 (0-23.42)	12.37 (0.95-41.33)	102 (32.83-272.7)	0-10

Table I. Demographic and laboratory characteristics of the study population classified by severity degree. All variables are expressed as median (p25-p75). AST: aspartate amino-transferase; ALT: alanine amino-transferase; GGT: gamma-glutamyl transferase; LDH: lactate dehydrogenase; CRP: C-reactive protein; IL-6: interleukin-6.

Figure legends

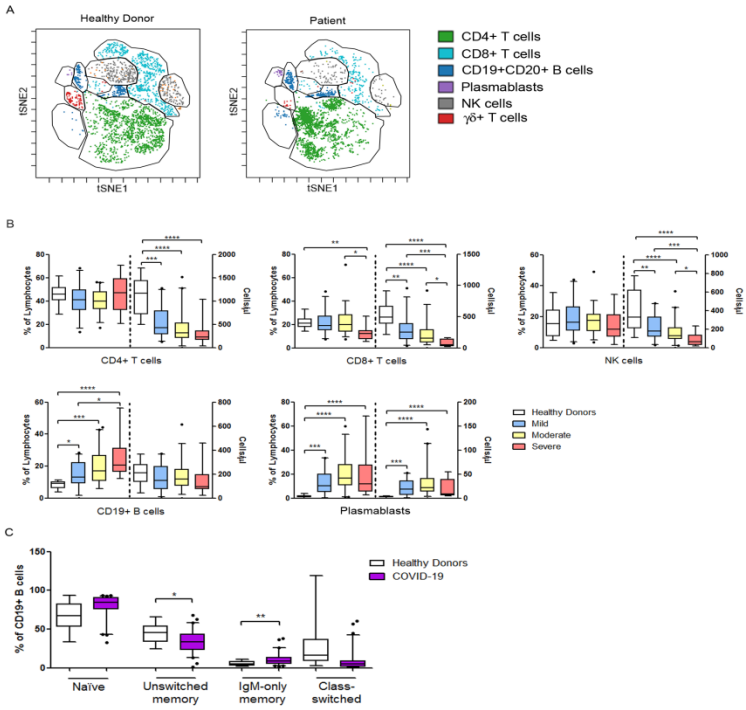

Figure 1. Distribution of lymphocyte subpopulations in the peripheral blood of COVID-19 patients characterized by multiparametric flow cytometry. (A) Analysis of distribution of lymphocyte subpopulations by automated clustering and dimensionality reduction FlowSOM tool (Cytobank) in a representative healthy donor and COVID-19 patient. **(B)** Boxplots show percentage and absolute number (cells/µl) of distinct lymphocyte subpopulations in healthy donors (white; n=19) and COVID-19 patients with different degrees of severity: mild (blue; n= 146), moderate (yellow; n= 89) and severe (red; n= 41), measured by flow cytometry. **(C)** Boxplots represent quantification of relevant B

cell subpopulations in healthy donors (n= 19) and selected COVID-19 patients (n= 84) assessed by flow cytometry. Data are from 19 experiments, with a median of 6 patients analyzed per experiment and day. Boxplots display percentiles 25 and 75 and median, and the whiskers correspond to percentiles 5 and 95. Asterisks indicate significant differences (p-values for ANOVA Tukey's contrast test: *p < 0.05, **p < 0.01, ***p < 0.001, **** p < 0.0001).

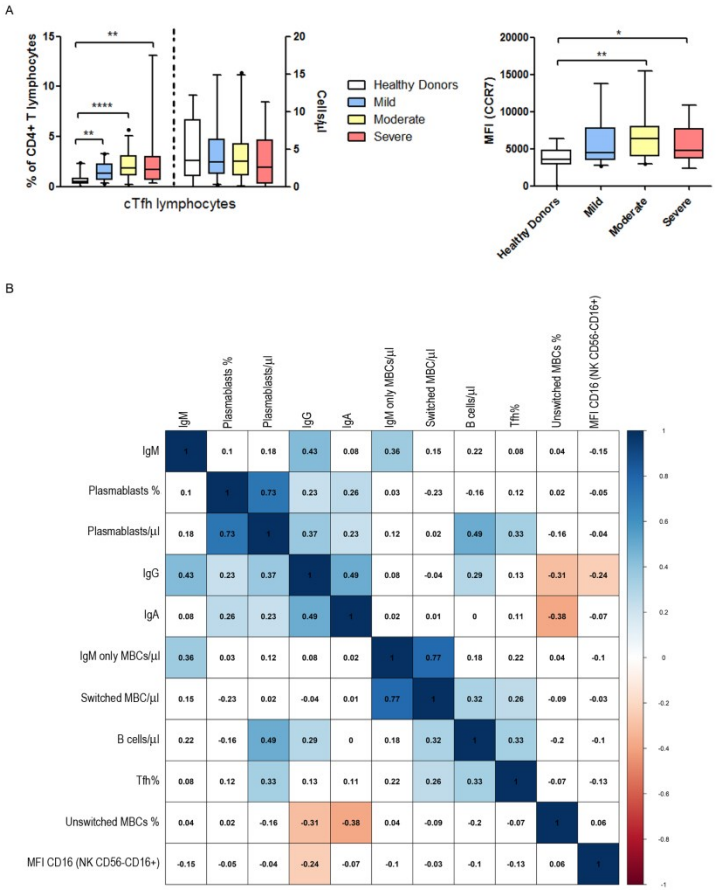

Figure 2. Circulating Tfh lymphocytes are increased in COVID-19 patients. (A) Left: Boxplots show quantification by multiparametric flow cytometry of cTfh lymphocytes as percentage or absolute number (cells/µl) in healthy donors (n= 19) and selected COVID-19 patients with different severity degree (mild (n= 29), moderate (n= 40) and severe (n= 15)). Right: Boxplots show CCR7 MFI of cTfh lymphocytes in healthy donors and COVID-19 patients with different severity degree (mild, moderate and severe). Asterisks indicate significant differences (p-values for ANOVA Tukey's contrast test: *p < 0.05, **p < 0.01, ***p < 0.001, **** p < 0.0001) **(B)** Annotated heatmap of a

correlation matrix for different variables in those same COVID-19 patients (n= 84). White squares include non-significant correlations ($p > 0.05$), red and blue squares include significant indirect and direct correlations ($p < 0.05$), respectively. Numbers inside squares and intensity of color correspond to Spearman's rank correlation coefficient. Variables in the correlation map were reordered using hierarchical cluster method. Data are from 19 experiments, with a median of 6 patients per experiment and day. Boxplots display percentiles 25 and 75 and median, and the whiskers correspond to percentiles 5 and 95.

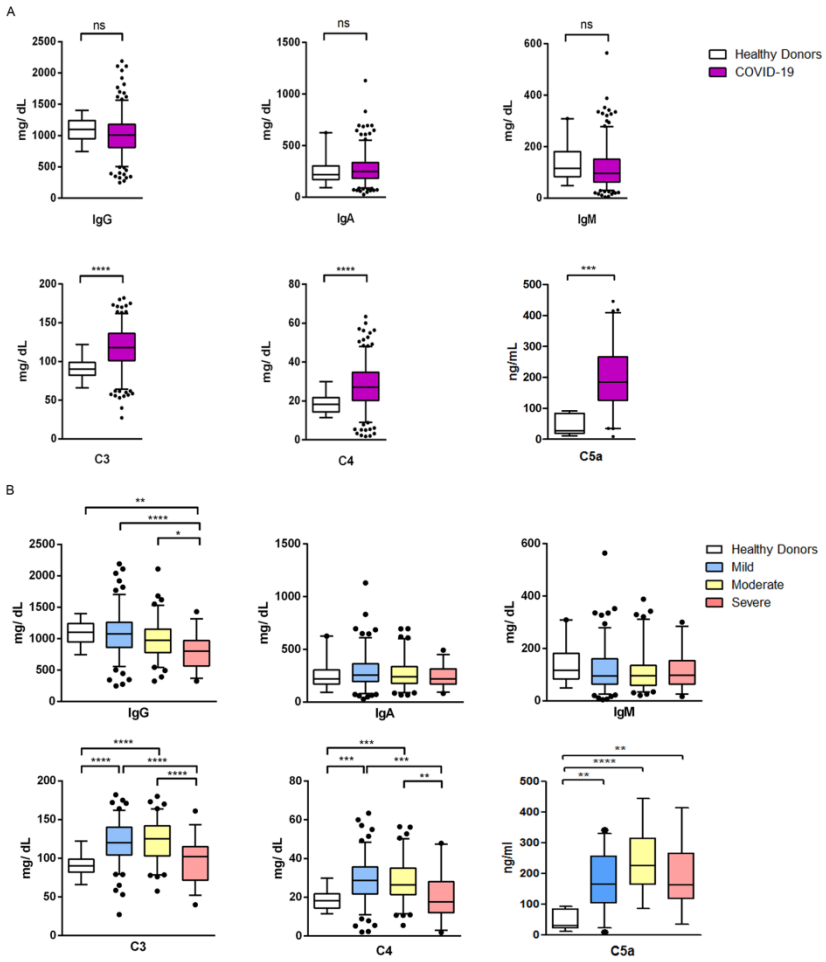

Figure 3. Immunoglobulins and complement levels are altered in COVID-19 patients. (A) Quantification of serum concentration (mg/dL) by nephelometry of IgG, IgA, IgM, C3 and C4 in healthy donors (n= 19) and COVID-19 patients (n= 255), and C5a by ELISA in healthy donors (n=10)

and COVID-19 patients (n=63). **(B)** Serum concentration (mg/dL) of IgG, IgA, IgM, C3 and C4 in healthy donors (n=19) and COVID-19 patients according to severity degree (mild (n= 138), moderate (n= 82) and severe (n= 35). Serum concentration (ng/mL) of C5a in healthy donors (n=10) and COVID-19 patients according to severity degree (mild (n= 29), moderate (n= 19) and severe (n= 15). Values represent quantification for each serum marker depicted as boxplots. Asterisks indicate significant differences (p-values for Mann-Whitney t-test or ANOVA Kruskal-Wallis test, as appropriate: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$). Data are from samples collected over a 6 weeks period and analyzed within 24 hours before freezing the serum. For C5a measurements, samples were thawed and assessed in 2 independent experiments. Boxplots depict percentiles 25 and 75 and the median, and the whiskers show percentiles 5 and 95.

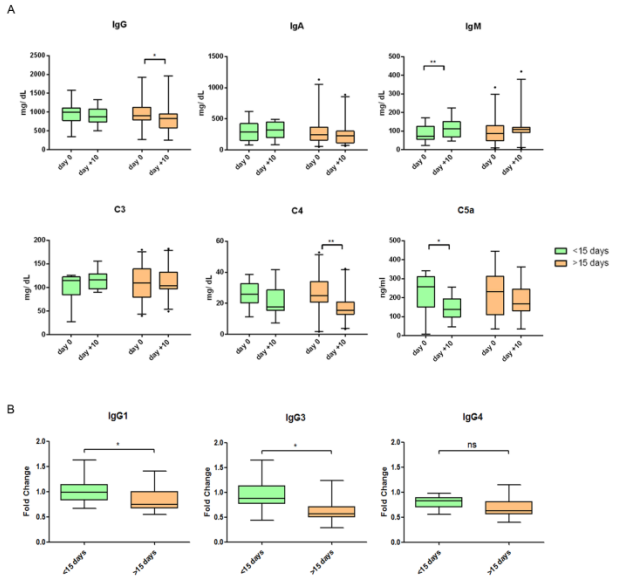

Figure 4. Humoral response is related to severity in COVID-19 patients. (A) Serum concentration (mg/dL) of IgG, IgA, IgM, C3, C4 and C5a in COVID-19 patients according to their hospitalization period was quantified by nephelometry or ELISA. **(B)** Fold change of IgG1, IgG3 and IgG4 levels

between day 0 and day +10 after admission. Green boxplots correspond to patients with a hospitalization period of less than 15 days (n= 13) and orange boxplots to those hospitalized more than 15 days (n= 24). C5a quantification in orange boxplots corresponds to n=15. Asterisks indicate significant differences (p-values for Mann-Whitney t-test or Wilcoxon t-test, as appropriate: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$). Data are from samples collected over a 6 weeks period and analyzed within 24 hours before freezing the serum. For IgG1, IgG3 and IgG4 levels measurements, samples were thawed and assessed within 2 independent experiments.. Boxplots display percentiles 25 and 75 and the median, and the whiskers correspond to percentiles 5 and 95.

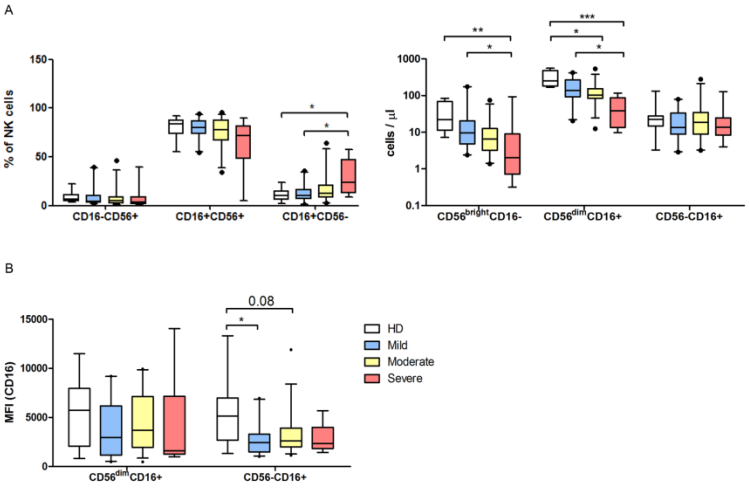

Figure 5. Phenotypic features of NK cells from severe COVID-19 patients. (A) Boxplots show quantification by flow cytometry of the proportion (left) and absolute values (right) of the three main functional subsets of NK cells in healthy donors (n= 19) and a selected group of COVID-19 patients with different severity degree (mild (n= 29), moderate (n= 40) and severe (n= 15). **(B)** Boxplot depict CD16 MFI of $CD56^{dim}$CD16+ and CD56-CD16+ NK subpopulations in those same individuals. Asterisks indicate significant differences (p-values for ANOVA Tukey's contrast test: *p < 0.05, **p < 0.01, ***p < 0.001, **** p < 0.0001). Data are from 19 experiments, with a median of 6 patients per experiment and day. Boxplots depict percentiles 25 and 75 and the median, and the whiskers correspond to percentiles 5 and 95.

Graphical abstract text for "Deregulated cellular circuits driving immunoglobulins and complement consumption associate with the severity of COVID-19 patients"

The pathogenesis of SARS-CoV-2 involves both cellular and humoral immunity. The proportion of B cells, plasmablasts, circulating follicular helper T cells (cTfh) and CD56-CD16+ NK-cells is increased in COVID-19 patients. However, levels of IgM, IgG and complement proteins are diminished in severe patients, this suggesting immunoglobulins and complement consumption.