Severity of SARS-CoV-2 infection is linked to double-negative (CD27− IgD−) B cell subset numbers

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Abstract

Objectives The role of B cells in COVID-19, beyond the production of specific antibodies against SARS-CoV-2, is still not well understood. Here, we describe the novel landscape of circulating double-negative (DN) CD27− IgD− B cells in COVID-19 patients, representing a group of atypical and neglected subpopulations of this cell lineage.

Methods Using multiparametric flow cytometry, we determined DN B cell subset amounts from 91 COVID-19 patients, correlated those with cytokines, clinical and laboratory parameters, and segregated them by principal components analysis.

Results We detected significant increments in the DN2 and DN3 B cell subsets, while we found a relevant decrease in the DN1 B cell subpopulation, according to disease severity and patient outcomes. These DN cell numbers also appeared to correlate with pro- or anti-inflammatory signatures, respectively, and contributed to the segregation of the patients into disease severity groups.

Conclusion This study provides insights into DN B cell subsets’ potential role in immune responses against SARS-CoV-2, particularly linked to the severity of COVID-19.

Keywords B cell · DN B cell · COVID-19 · Inflammation

Abbreviations

ALT Alanine aminotransferase
Alb Albumin
ASC Antibody-secreting cells
AST Aspartate aminotransferase
BCR B cell receptor
Breg Regulatory B cells

COVID-19 Coronavirus disease 2019
CRP C-reactive protein
DaSO Days after symptom onset
Dd D-dimer
DN Double-negative B cells
Fib Fibrinogen
FMO Fluorescence minus one
HIV Human immunodeficiency viruses
IP-10/CXCL10 Interferon gamma-induced protein
LDH Lactate dehydrogenase
Lk Leukocyte

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**Introduction**

B cells represent one of the main elements of the adaptive humoral immune system since they are responsible for mediating the production of antibodies directed against potential pathogens. The canonical classification strategies segregate human circulating B cells into different populations: transitional B cells (CD24<sup>hi</sup> CD38<sup>hi</sup> CD27<sup>−</sup>) that constitutes recent bone marrow emigrants, naïve B cells (CD38<sup>lo</sup>− CD27<sup>−</sup> IgD+) representing mature B cells that have never been stimulated by their cognate antigens, memory B cells (CD38<sup>lo</sup>− CD27<sup>−</sup> CD21<sup>+</sup>) that are developed following a primary infection and remain in a quiescent state until they encounter the same antigen to become activated and induce a robust secondary response that includes their differentiation into plasmablasts/plasma cells (CD38<sup>hi</sup> CD27<sup>hi</sup>) which are responsible for antibody secretion [1].

Recently, the development of single-cell genomic approaches and high-dimensional flow cytometry has allowed identifying emerging B cell populations with distinctive phenotypes and divergent functional characteristics [2, 3]. In 2007, it was described the expansion of an unknown B cell subset characterized by the absence of both IgD and CD27 (double-negative, DN) in systemic lupus erythematosus (SLE) patients, thus being postulated that they could represent a novel memory population [4]. Additional heterogeneity within the DN population has been recently established, where these cells comprise four major subsets: DN1 to DN4 B cells, based on their relative expression of CD21 and CD11c [3, 5, 6]. Although not clear yet, it has been suggested that DN1 may represent early activated memory cells, whereas DN2 cells would embody primed antibody-secreting cells (ASC) precursors derived from newly activated naïve cells [7]. The absence of CD21 and CD11c defines DN3 B cells, while both markers are expressed by DN4 B cells [3], but the function and significance of these subsets, particularly in contexts different from autoimmune diseases, remain to be elucidated.

Over the last couple of years, the worldwide Coronavirus disease 2019 (COVID-19) pandemic situation has been rushed the characterization of different anti-viral immune-mediated mechanisms aimed to resolve this emergent illness. COVID-19 is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that may evolve asymptomatically or with mild symptoms in most patients. In contrast, others suffer from acute respiratory distress syndrome (ARDS) with a poor prognosis [8]. The severity of COVID-19 depends on the balance of host immune responses against viral stimuli. In severe cases, this response is deregulated and characterized by a hyperinflammatory status originated by high levels of cytokines and pro-inflammatory molecules, known as cytokine storm [9].

Interestingly, COVID-19 patients display alterations in different myeloid and lymphoid cells that are associated with several clinical features [10, 11]; among these leucocyte subsets, B cells remain as one of the less-studied cell types in this disease with few reports that go beyond the analysis of a total CD19<sup>+</sup> B cell population in small or limited cohorts of patients [6, 12]. In this context, the contribution to disease of rare B cell subsets such as DN subpopulations remains poorly understood.

According to their phenotype, DN B cells could be contained in the subset commonly referred to as atypical B cells, observed at high frequencies during autoimmune disease including arthritis or SLE, and chronic infections with hepatitis C virus, human immunodeficiency virus (HIV), or malaria [4, 13–16]. Some authors consider these B cells as anergic or exhausted due to chronic antigenic stimulation since some express high levels of inhibitory receptors, such as Fc-receptor-like (FCRL) molecules [15]. However, this population’s “exhausted” description is conflicting with the previously mentioned SLE patients exhibiting high numbers of these atypical B cells, which have been proposed as activated lymphocytes in the process of differentiation to ASC [7]. Accordingly, it has been shown that their specific B cell receptors (BCRs) to *Plasmodium falciparum* could also contribute to the anti-parasitic antibodies’ generation [17]. As similar B cell phenotypes arise after influenza or yellow fever vaccination, vaccinia immunization, and primary HIV infection [18, 19], it could be suggested that these cells are part of protective immune responses.
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To elucidate if DN B cells could be friend or foe during SARS-CoV-2 acute infection, we analyzed the numbers of these cell compartments in peripheral blood of COVID-19 patients with different disease severity and found several differences that seem to contribute to segregate the disease severity status.

Methods

COVID-19 patients and healthy donors

91 COVID-19 patients and 15 healthy donors were recruited at Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán in Mexico City, Mexico. All patients confirmed by a positive PCR test for SARS-CoV-2 were invited to be included in the study. Upon admission, vital signs including
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**Fig. 1** DN B cell subsets in COVID-19 patients. A Gating strategy for the identification of the indicated B cell subsets in PBMCs (depicting representative results from a healthy control) previously selected from singlets gate (SSC-A vs. SSC-H), live Zombie Green− cells gate, and total CD19+ B cells gate. B Comparative analysis of total frequencies of CD19+ B cells on the left panel and absolute numbers on the right panel. C Comparative analysis of total DN cells; frequencies relative to CD19+ B cells on the left panel and absolute numbers on the right panel. D Comparative analysis of DN1 subset; frequencies relative to CD19+ B cells on the left panel and absolute numbers on the right panel. E Comparative analysis of DN2 subset. F Comparative analysis of DN3 subset. G Comparative analysis of DN4 subset. All frequency or absolute number values are displayed as mean (dashed line) plus lower and upper quartiles (dotted lines). Patients infected with SARS-CoV-2 (n=91), subdivided in mild/moderate (n=24), severe (n=35) or critical (n=32), were compared to healthy controls (n=35) or critical (n=24), severe (n=35) or healthy (n=35) controls. The included cytokines and chemokines were measured using the MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel kit (EMD Millipore) on a 2-laser Bio-Plex 200 suspension array system combined with a Bio-Plex Pro Wash Station (both from Bio-Rad), according to the manufacturer's instructions. Bead-fluorescence intensity readings for all the samples and standards were converted into the corresponding analyte concentrations employing the Bio-Plex Manager software v6.2 (Bio-Rad). The included cytokines and chemokines were selected among our available multiplex assays, according to the previous reports regarding their potential utility as blood-associated prognostic biomarkers in patients with COVID-19 [8, 21].

### Multiparametric flow cytometry analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors with Ficoll-Paque (GE Healthcare Life Sciences). Recovered cells were resuspended in RPMI-1640 (Gibco) and counted before staining procedures with the following conjugated monoclonal antibodies: BUV496 anti-human CD19 (BD Horizon), Brilliant Violet 650 anti-human CD38, APC/Cy7 anti-human CD27, Brilliant Violet 421 anti-human CD24, PerCP/Cyanine5.5 anti-human IgD, Alexa Fluor 700 anti-human CD21, PE/Dazzle 594 anti-human CD11c and Zombie Green dye (all from BioLegend). For staining, 2 × 10⁶ cells were treated with human a FcX blocker (BioLegend) for 10 min, then incubated for 30 min at 4 °C with the antibody cocktail, washed, and fixed with fixation buffer (BioLegend) for 1 h. Lastly, cells were washed once with cell staining buffer (BioLegend) and then resuspended in 300 μL of the same buffer for immediate flow cytometric analysis on a BD LSRFortessa using FACSDiva software (BD Biosciences), acquiring at least 1 × 10⁶ cells. Files were analyzed using FlowJo v10 software (BD Biosciences) with the strategy shown in Fig. 1A, using Fluorescence Minus One (FM0) controls to define gates plus CompBeads (BD Biosciences) and single stained fluorescent samples to achieve compensation.

### Cytokine and chemokine determinations

The concentrations of six cytokines and chemokines: IL-1RA, IL-6, IL-10, IL-18, monocyte chemoattractant protein 1 (MCP-1)/CCL2, and interferon gamma-induced protein (IP-10)/CXCL10, in serum of the patients, were measured using the MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel kit (EMD Millipore) on a 2-laser Bio-Plex 200 suspension array system combined with a Bio-Plex Pro Wash Station (both from Bio-Rad), according to the manufacturer’s instructions. Bead-fluorescence intensity readings for all the samples and standards were converted into the corresponding analyte concentrations employing the Bio-Plex Manager software v6.2 (Bio-Rad). The included cytokines and chemokines were selected among our available multiplex assays, according to the previous reports regarding their potential utility as blood-associated prognostic biomarkers in patients with COVID-19 [8, 21].

### Statistics and bioinformatics analysis

Principal component analysis (PCA) method was performed on RStudio (1.3), running on R software (4.0). All
numerical variables were scaled to have unit variance before the analysis. PCA was processed with FactoMineR library and graphically produced with Factoextra package. We used fviz_pca_biplot() function to represent DN variables contribution and their relation to patients disease severity. We presented a bar plot of variables using fviz_pca_biplot() to visualize the three dimensions global contribution. Moreover, we employed Matlab on 20b version for plotting three-dimensional PCA representation.

Results

DN B cell subsets are altered in COVID-19 patients

To identify DN subsets in peripheral blood, we segregate CD27− cells from total CD19+ B cells. We then exclude CD24+ cells to select a “mature” population composed by IgD naïve B cells and the remaining DN IgD− cells, further subdivided into four subsets: DN1, DN2, DN3, and DN4, based on their CD11c and CD21 expression. This gating strategy is depicted in Fig. 1A.

When total frequency or absolute CD19+ cell numbers were analyzed, we did not find significant differences in the B cell compartment between healthy subjects and COVID-19 patients of any category (Fig. 1B), as reported previously by our research group and also by other authors [12, 22]. We also found that conventional circulating B cell subsets, including transitional (T1/T2) CD24hi CD38hi CD27−, naïve CD38lo CD27− IgD+, unswitched-memory (USwM) CD27+ IgD+, switched-memory (SwM) CD27+ IgD+, and plasmablasts (ASC) CD38hi CD27hi exhibited similar trends to what has been informed in recent literature [6, 12] with the expansion of immature B cells in mild/moderate COVID-19, increase of plasmablasts in severe and critical patients, and a loss of memory subsets in almost all patients (Supplemental Fig. 1). Interestingly, some differences emerged when total DN cell frequencies were compared, particularly among mold/moderate and severe or critical patients (Fig. 1C). Outstandingly, several significant changes could be detected when each DN subset was analyzed: DN1 cell frequencies and absolute numbers from COVID-19 patients appeared as decreased when compared with healthy donors, a feature that seems to be more pronounced according to the disease severity (Fig. 1D). In contrast, DN2 cells exhibited a slight increase, most noticeable when critical patients were analyzed (Fig. 1E). Similarly, significant increments in the DN3 cell frequencies and absolute numbers in the total COVID-19 patients (n=91) in our cohort. The underlying color scale indicates Spearman’s coefficient values. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001
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patients were also detected (Fig. 1F) but appearing much more robust than the DN2 counterparts. Finally, the DN4 fraction was seen almost absent from circulation in all the groups analyzed, finding no significant differences in their numbers (Fig. 1G).

**DN subpopulations are associated with different clinical “signatures” of COVID-19**

To elucidate the potential roles of these rare B cell subsets, we tried to associate their amounts in peripheral blood to the patients’ available clinical and laboratory features, presented in Table 1 and compared among COVID-19 groups in Supplemental Fig. 2. When correlation analyses including all variables were performed, it becomes evident that each DN subset displayed a specific matrix pattern (Fig. 2). Interestingly, the DN1 frequencies and absolute numbers patterns stand out since they revealed mainly negative (several significant) correlations with many of the analyzed parameters, mainly related to a pro-inflammatory status in COVID-19 patients [23, 24]. Correspondingly, DN1 numbers positively correlated with oxygen parameters (SO2 and PaFi) and show a negative correlation with a surrogate of inflammation, such as albumin. Contrastingly, the remaining DN subsets exhibited opposite patterns, a feature that is particularly marked when the DN3 cell frequencies or absolute numbers are seen.

Additionally, when the outcomes of severe and critical (hospitalized) patients were analyzed, we observed exciting differences: DN1 cells were detected as significantly reduced in their frequency and absolute numbers in deceased individuals when compared to recovered ones (Fig. 3A); contrariwise, the DN3 subset appeared slightly increased in the deceased patients, with a significant difference only when frequencies were analyzed (Fig. 3C). Finally, no outcome differences were observed when DN2 or DN4 cell numbers were examined (Fig. 3B, D).

**DN B cell subset segregate critical and mild/moderate COVID-19 patients**

We segregate COVID-19 patients by analyzing all available clinical and laboratory variables plus DN1, DN2, and DN3 subsets frequencies and absolute numbers (DN4 values were...
excluded since these cells were almost absent in patients and did not contribute to groups segregation), using PCA in a 3D scatter-plot visualization (Fig. 4A). Axes include the three components with a higher proportion of the variation, 28.6%, 8.7%, and 6.4%, respectively. The plotted data represent 43.7% of the complete data variation, making it more informative and accurate for interpretation. Also, we have included 95% confidence ellipses to show intersection areas among severity.

We also deliver a 2D scatter-biplot to visualize our DN B cells’ influential degree independently between principal components 1 and 2 (Fig. 4B). We observed that the group of variables: DN2 frequency (where frequency is denoted as %), DN2 absolute counts (where absolute counts are symbolized as #), and DN3#, are positively correlated. A negative correlation among DN1% and the latter group of variables can be observed, and in the same way between DN1# and DN3%. The most explainable variables are determined by vectors magnitude (arrows size) being DN1%, DN2%, and DN2#. Finally, the fact that the presence of DN1 subset is correlated with mild/moderate patients is validated as well as for DN2/3 subsets with critical patients.

**Discussion**

Although B cells’ contribution to infection resolution is usually set at advanced or chronic stages, there are some reports suggesting that some subsets of this lineage could participate during acute phases of infectious disease, in other ways different from antibody secretion. Such “atypical” B lymphocytes, which certainly possess innate-like properties due to their high Toll-like receptors (TLRs) responsiveness, include the DN cells that are notably expanded in viral or parasitic infections, as well as autoimmune disorders [3, 25, 26].

Apart from the DN2 subset, that has been previously reported as expanded in SLE, Hepatitis C or HIV infection [3, 27], more recently in COVID-19 [12], and described as primed plasma cell precursors differentiated through an extra-follicular pathway [7]; almost nothing is known about the remaining DN subpopulations. Interestingly, we found significant alterations in DN1, DN2, and DN3 subsets during COVID-19 acute development in this work.

As expected, the DN2 subset was found expanded in COVID-19, increasing according to the disease severity but predominantly on the critical patients. That change could be explained by the prominent proinflammatory environment raised by these individuals; in our cohort, this could be evidenced by the positive correlation of these DN2 cells with IL-18 amounts, which could probably be involved in their expansion since it is associated with type 1 immune responses [28] that seem to be necessary for their

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**Fig. 4** DN B cells contribute to segregate COVID-19 patients by severity of the disease. 3-D (A) and 2-D (B) views of PCA considering all measured variables in COVID-19 patients, including the frequencies (%) and absolute numbers (#) of DN1, DN2, and DN3 subsets, clinical/laboratory values (respiratory rate, oxygen saturation, arterial partial pressure of oxygen/fraction of inspired oxygen ratio, days after symptom onset, leukocyte, lymphocyte, and neutrophil counts, alanine aminotransferase, aspartate aminotransferase, albumin, C-reactive protein, lactate dehydrogenase, fibrinogen, D-dimer, troponin, and National Early Warning Score) and cytokines (IL-1RA, IL-6, IL-10, IL-18, MCP-1, and IP-10). Each point represents a single patient projected to the first principal components, and colors are associated with the COVID-19 severity, as indicated. The 2-D view includes arrows representing the relative contributions of each of DN cell subsets, according to the indicated scale. The 3-D view only includes patients’ projection and severity but including three principal components. Both views contain 95% confidence ellipses.
differentiation [29]. As DN2 also demonstrated positive correlations with disease severity indicators (such as SO2 or NEWS), the notion of a contributing proinflammatory milieu is again supported. However, we cannot discard these cells’ direct influence on disease aggravation since their proinflammatory profile has been described previously [30, 31].

Like DN2, the DN3 subset displayed a similar but ever more robust expansion, enhanced in more severe patients; interestingly, this feature is more strongly associated with an inflammatory-associated pattern, where inflammation markers positively correlate with DN3 amounts whereas ventilatory parameters show the opposite. Additionally, DN3 augmented proportion appears to be linked to worse outcomes in more severe patients, making us suspect a proinflammatory function of these cells. Despite having a complete unknown function or origin, COVID-19 seems to be the only reported condition where DN3 cells are overrepresented, positioning them as exciting candidates for further studies.

On the other hand, DN1 cells exhibited a reduction in their amounts in COVID-19, being more evident in critical cases. When correlation analyses with clinical/laboratory features and cytokines were done, DN1 cell numbers displayed an opposite pattern than DN2 or DN3 cells, where several negative correlations with proinflammatory elements plus positive values for ventilatory parameters were observed. Hence, the high number of DN1 cells seems to be associated with a less severe disease course and even a better outcome in hospitalized patients. Again, since nothing is reported about this subset’s functional roles, we can only argue about their anti-inflammatory potential since it has been reported that cells with a similar phenotype to DNs have been documented to enhance CD4+CD25hi regulatory T cells (Treg) proliferation in vitro [32]. In this way, it is possible that the DN1 subgroup could contain a non-previously described regulatory B cells (Breg) population, helping to maintain homeostasis in mild/moderate COVID-19 but being lost in severe or critical cases.

Finally, as we employ the DN subset amounts together with some clinical/laboratory descriptors and cytokines (previously described as relevant as prognostic biomarkers in COVID-19 [8, 21]) for multivariate approaches, we conclude that the measurement of these cells can definitively support the segregation of patients according to disease severity, where mild/moderate and severe/critical patients exhibit major segregating-contributions of DN1 and DN2/ DN3, respectively, with similar or even better robustness than most of the variables studied here (Supplemental Fig. 3). The usefulness of these DN subset measurements as potential biomarkers for prognostic approaches is a feature that is not possible to determine in our cohort and needs to be addressed by a longitudinal study that possibly will shed light regarding the pathogenic or protective functional roles of these traditionally neglected B cells.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s00011-021-01525-3.

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Author contributions RC-D and VAS-H contributed equally to the design and performance of experiments, analysis, and interpretation of data. RC-D, VAS-H, JJT-R, and SR-R performed experiments and analyzed data. MC-H, JCP-F and DEM-S assisted in processing and preservation of control and patient samples. AP-F collected patient data, generated, and organized our clinical database. RC-D, VAS-H, and DG-M participate in writing and editing the manuscript. MP-R and VAS-H performed bioinformatics and statistical analyses. JLM-M designed and performed experiments, supervised general work, wrote, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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

Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical approval All recruited individuals signed an informed consent prior to the inclusion. The Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (Mexico City, Mexico) ethics and research committees approved the study (Ref. 3341) in compliance with the Helsinki declaration.

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