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RESEARCH ARTICLE

Immune cell kinetics and antibody response in COVID-19 patients with low-count monoclonal B-cell lymphocytosis

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Abstract

Low-count monoclonal B-cell lymphocytosis (MBL^{lo}) has been associated with an underlying immunodeficiency and has recently emerged as a new risk factor for severe COVID-19. Here, we investigated the kinetics of immune cell and antibody responses in blood during COVID-19 of MBL^{lo} versus non-MBL patients. For this study, we analyzed the kinetics of immune cells in blood of 336 COVID-19 patients (74 MBL^{lo} and 262 non-MBL), who had not been vaccinated against SARS-CoV-2, over a period of 43 weeks since the onset of infection, using high-sensitivity flow cytometry. Plasma levels of anti-SARS-CoV-2 antibodies were measured in parallel by ELISA. Overall, early after the onset of symptoms, MBL^{lo} COVID-19 patients showed increased neutrophil, monocyte, and particularly, plasma cell (PC) counts, whereas eosinophil, dendritic cell, basophil, and lymphocyte counts were markedly decreased in blood of a variable percentage of samples, and with a tendency toward normal levels from week +5 of infection onward. Compared with non-MBL patients, MBL^{lo} COVID-19 patients presented higher neutrophil counts, together with decreased pre-GC B-cell, dendritic cell, and innate-like T-cell counts. Higher PC levels, together with a delayed PC peak and greater plasma levels of anti-SARS-CoV-2-specific antibodies (at week +2 to week +4) were also observed in MBL^{lo} patients. In summary, MBL^{lo} COVID-19 patients share immune profiles previously described for patients with severe SARS-CoV-2 infection, associated with a delayed but more pronounced PC and antibody humoral response once compared with non-MBL patients.

1 | INTRODUCTION

Low-count monoclonal B-cell lymphocytosis (MBL^{lo}) is defined by the presence in blood of <500 clonal B cells/μL which usually display an immunophenotypic profile of chronic lymphocytic leukemia (CLL) cells, in the absence of other features of B-cell chronic lymphoproliferative disorder (B-CLPD).[1–4] Though MBL^{lo} is a highly prevalent condition in the general population (from 4% to 16% of otherwise healthy donors aged ≥40 years),[4–7] only a small fraction (≈1.8%) of MBL^{lo} cases develop high-count MBL (MBL^{hi}, ≥500 and < 5000 CLL-type B cells/μL)[2] in the medium term,[8] while no MBL^{lo} case has been described so far to evolve to CLL. In turn, MBL^{hi} shows a rate of progression to CLL-requiring treatment of 1.1% per year.[3] Despite its low rate of progression to CLL, MBL^{lo} has been associated with an increased risk of severe infections,[9] second lymphoid malignances,[7] and a shorter overall survival (OS) compared to non-MBL subjects.[8] The poorer clinical outcome of MBL^{lo} individuals points toward an underlying immune dysregulation, mostly involving the B-cell compartment and antibody-mediated immune responses,[10–13] that make MBL^{lo} subjects particularly susceptible to (more) severe infections.

Since December 2019, COVID-19 arose as a new infectious disease with a heterogenous clinical course in humans,[14] ranging from asymptomatic to severe disease requiring admission to the hospital, and even to intensive care units (ICU), and ultimately death.[15] Since then, several risk factors have been identified which contribute to explain the clinical heterogeneity of COVID-19. Among others, these include advanced age, male sex, and the presence of (certain) comorbidities.[15,16] More recently, MBL^{lo} has also emerged as a risk factor for the development of more severe COVID-19, independently of other comorbidities and prognostic factors.[17] Although many reports have extensively described the immune profiles associated with SARS-CoV-2 infection and demonstrated the clinical relevance of an adequate immune response for controlling SARS-CoV-2 infection and for clearance of the virus,[18–21] the precise immune profiles that might contribute to explain the poorer outcome of COVID-19 in MBL^{lo} versus non-MBL patients still remain to be identified. In this regard, previous cross-sectional studies have reported on the immune cell and humoral responses in the blood of MBL^{lo} COVID-19 patients at the acute phase of infection and after recovery from COVID-19,[17] but more detailed longitudinal analyses of the kinetics of immune cells and antibody responses in blood are still missing.

Here, we report on the longitudinal kinetics of the different populations of immune cells and of SARS-CoV-2-specific antibody levels in blood of a large cohort of MBL^{lo} subjects who had COVID-19 during the first and second waves of the pandemic, both during and after the infection, compared with age- and sex-matched non-MBL COVID-19 patients and healthy donors.

2 | METHODS

2.1 | Patients, controls, and samples

A total of 336 COVID-19 adult patients (149 men and 187 women; median age of 56 years [range: 20–99 years]) who were referred to the University Hospital of Salamanca between March 2020 and July 2021 (prior to vaccination against SARS-CoV-2), were studied. COVID-19 diagnosis was confirmed by the presence of SARS-CoV-2 by RT-PCR (real-time polymerase chain reaction) in a nasopharyngeal swab and/or a positive serology for anti-nucleocapsid (N) protein of SARS-CoV-2 IgM and/or IgG antibodies when a PCR-assay was not available. A small subgroup of COVID-19 patients (23/336, 7%) had received anti-viral therapy (either lopinavir/ritonavir or remdesivir) prior to sample collection at recruitment. All COVID-19 patients were screened for the presence of MBL and classified as follows:[1,2] (i) COVID-19 MBL patients (74/336 [22%], 41 men and 33 women, median age of 69 years [range: 38–95 years]), and (ii) COVID-19 non-MBL patients (262/336 [78%], 108 men and 154 women; median age of 52 years [range: 20–99 years]). Two out of 74 (3%) MBL^{lo} COVID-19 patients had been previously diagnosed with MBL^{lo} before the pandemic, in the context of a population-based screening. A total of 656 non-MBL healthy donors (HD) (301 men and 355 women; median age of 58 years [range: 19–97 years]) from the same geographical area who had been recruited and evaluated for the presence of MBL before the SARS-CoV-2 pandemic were studied as controls. The study was approved by the Ethics Committee of the University Hospital of Salamanca/IBSAL (approval codes: CEIC PI4705/2017 and PI 2020 03468).

2.2 | Immunophenotypic studies

A total of 676 peripheral blood (PB) samples were collected from the 336 patients at the time of COVID-19 diagnosis (179 samples from MBL^{lo} and 497 from non-MBL COVID-19 patients) and subsequently over a period of 43 weeks since the onset of infection (defined as the appearance of the first symptoms of COVID-19). All the samples were stained with the EuroFlow Lymphocyte Screening Tube (LST), the Immunemonitoring (IMM) BIgH tube, and the EuroFlow Innate Myeloid Cell (IMC) tube, using the EuroFlow bulk-lyse-stain-and-then-fix standard operating procedure[17,22] (Supplementary Methods; Supplementary Table 2). For each antibody combination, $\geq 10^7$ cells/tube were measured per sample. Automated data analysis was performed using the INFINICYT software (Cytognos SL, Salamanca, Spain) and the LST and IMM-BIgH data bases[23] (Supplementary Methods). The identification of the different white blood cell subsets (with the EuroFlow LST), B-cell and plasma cell subsets (using the IMM-BIgH tube), and dendritic cell subsets (with the EuroFlow IMC tube) was performed following standardized gating strategies, as described and illustrated elsewhere for each tube[23–25]; a summary of the phenotypic profiles used for the subsetting of these cell populations is shown in Supplementary Table 2 (panels B, D, and F for the EuroFlow LST, IMM-BIgH, and EuroFlow IMC tubes, respectively). For each cell population, absolute cell counts/μL of blood were calculated using a dual-platform approach,[26] based on their relative values (% from leukocytes) obtained with the flow cytometer, and then transformed into absolute cell counts after obtaining the absolute number of total leukocytes using the Sysmex XN-1000™ hematology cell analyzer (Sysmex, Kobe, Japan). For the major populations of leukocytes, correlations of the absolute number of cells/μL obtained with the hematology cell analyzer versus the flow cytometer using EuroFlow protocols were performed in a subgroup of samples ($n = 284$), with an overall high degree of (direct) correlation between both platforms for each subset analyzed (Supplementary Figure 1).

2.3 | Measurement of anti-SARS-CoV-2 IgM, IgG, and IgA antibody levels in plasma

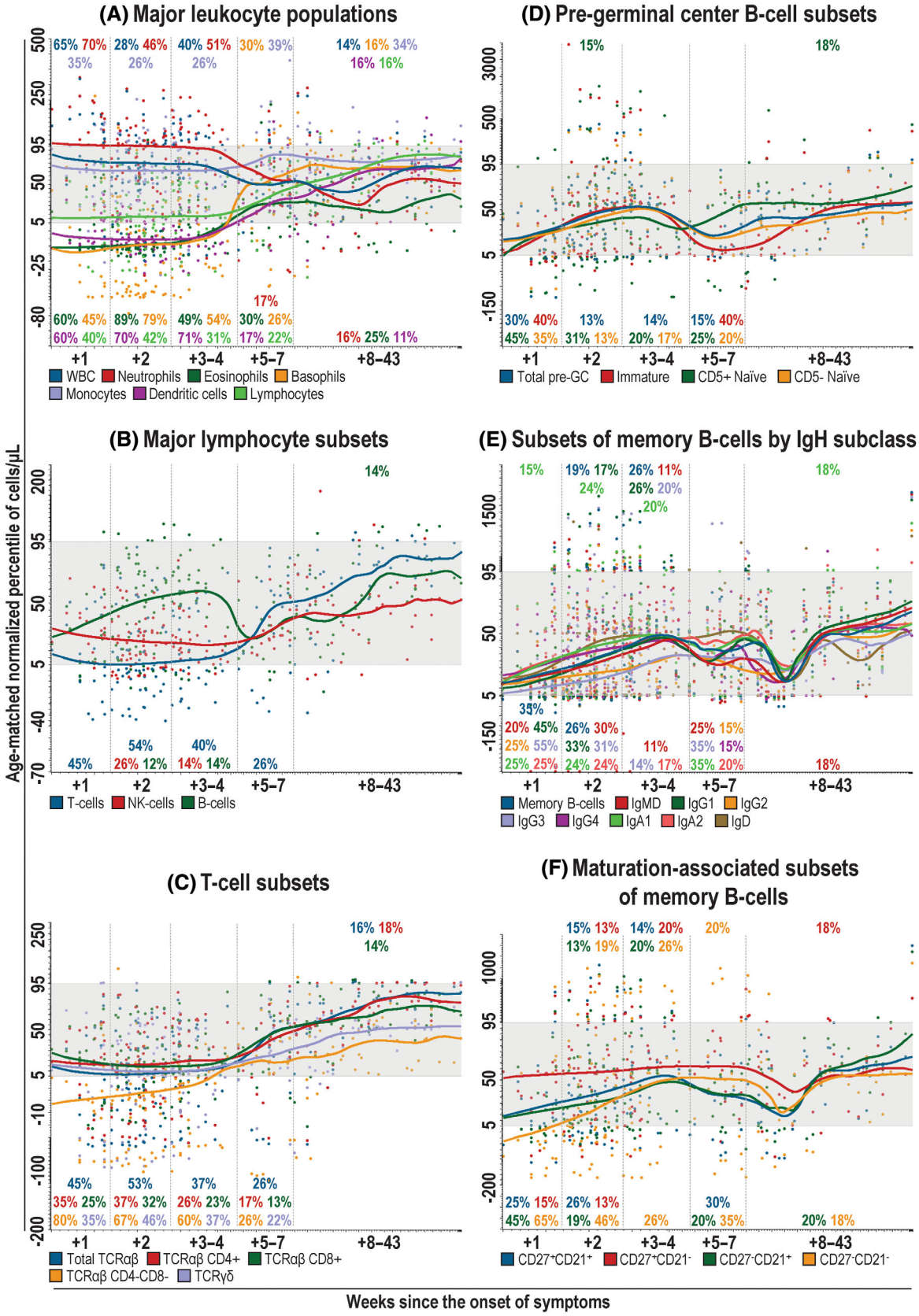
(Semi)quantitative determination of immunoglobulin (Ig) M, IgG, and IgA plasma levels against the spike (S) and the nucleocapsid (N) proteins of SARS-CoV-2 was performed in 668 and 454 samples, respectively. Commercially available ELISA kits for anti-S protein IgM antibodies (ThermoFisher, Waltham, MA), anti-S protein IgG and IgA antibodies (ImmunoStep S.L., Salamanca, Spain), anti-N protein IgM antibodies (AnshLab, Webster, TX), and anti-N protein IgG and IgA antibodies (Mikrogen Diagnostik, Neuried, Germany) were used. A high correlation of the results obtained by the anti-N IgM (AnshLab) and IgG (Mikrogen) kits employed in this study compared with reference methods used for the detection of specific antibodies against SARS-CoV-2 (e.g., Roche assays) has been previously reported and validated.[27–29]

2.4 | Measurement of viral load in plasma

Quantitation of viral load in plasma of COVID-19 patients was performed in 279 samples. Total RNA was extracted from plasma using the Viral RNA isolation kit (Macherey-Nagel GmbH, Düren, Germany) according to the manufacturer's specifications. Detection and quantitation of RNA viral copies was performed using the Bio-Rad SARS-CoV-2 ddPCR kit (Bio-Rad, Hercules, CA, USA) following the instructions of the manufacturer. The assay results were considered to be valid when the positive control had values ≥0.2 copies/uL; samples were classified as positive for SARS-CoV-2 when ≥0.1 copies/μL of viral RNA were detected, according to the kit instructions.

2.5 | Statistical methods

The Mann–Whitney *U* test (for non-paired continuous variables) or the Chi-square and Fisher exact tests (for categorical variables) were used to determine the statistical significance of differences observed between groups, using the MIDAS (v2.0.5.d) (Cytognos S.L.) and GraphPad Prism V8 (GraphPad Software, San Diego, CA) software



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programs. For comparisons between two groups, p-values ≤.05 (for categorical variables) and corrected (Benjamini-Hochberg procedure for continuous variables) p-values ≤.05 were used to define statistical significance. For multiple comparisons across all time-points analyzed, expressed as weeks since the onset of symptoms, a false discovery rate (FDR) of <5% was used as cutoff for statistical significance. For calculating correlation coefficients between continuous variables, the Spearman's rho test was used for non-Gaussian data distributions. Normalization of leukocyte values by age and their graphical representation were performed using MIDAS (v2.0.5.d), according to previously described algorithms[11] (Supplementary Methods). For box plot graphics, the GraphPad Prism V8 (GraphPad Software) was employed.

3 | RESULTS

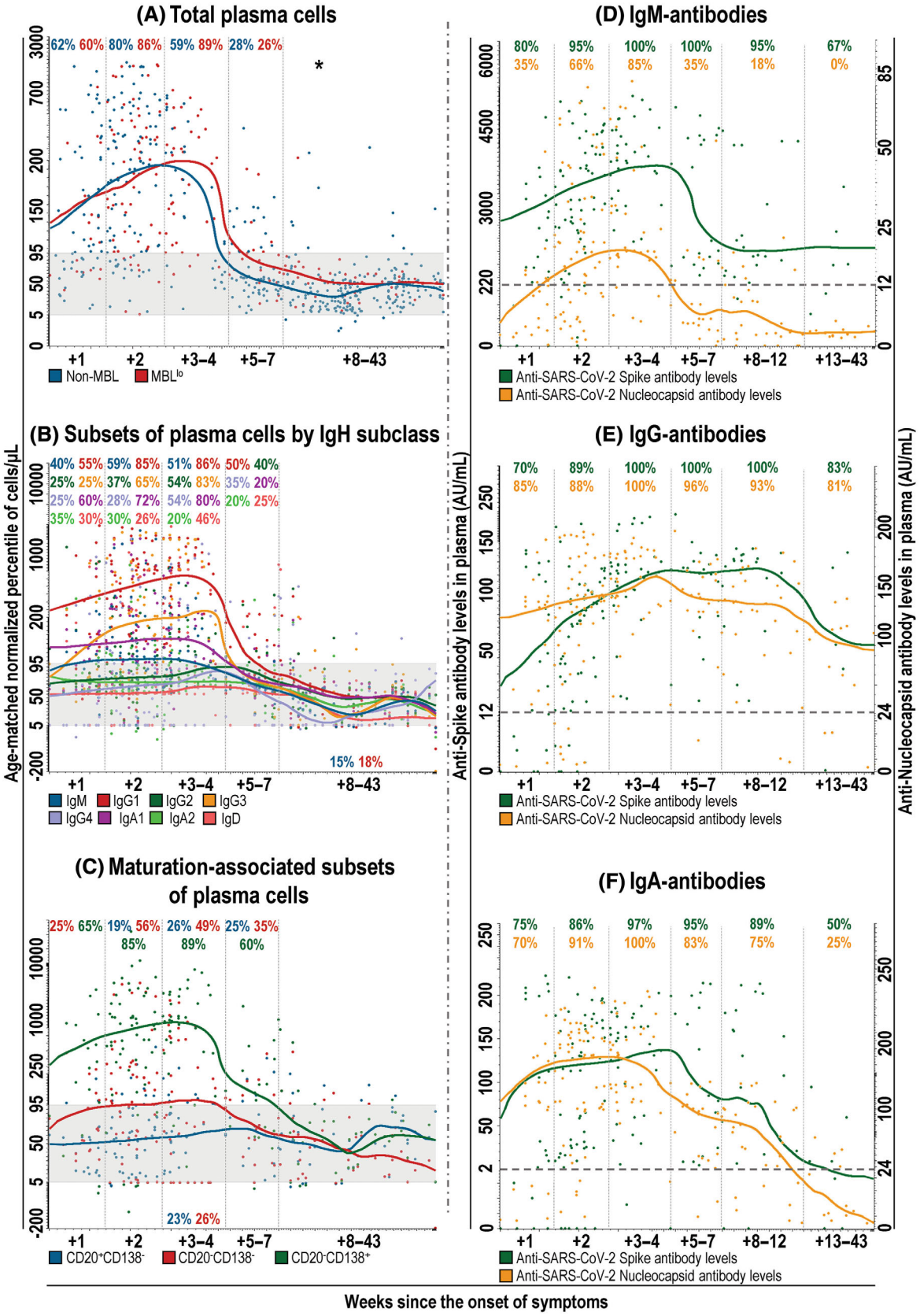
3.1 | Kinetics of the major populations of leukocytes and anti-SARS-CoV-2 antibodies in blood of MBL^{lo} patients during and after COVID-19

Absolute white blood cell (WBC) counts (after normalization by age against HD) were increased above the 95th percentile (95thp) of HD in 65% of blood samples ($p \leq .0001$) from MBL^{lo} patients, early (week +1) after the onset of COVID-19-related symptoms. This was due to increased neutrophil (70% of samples; $p \leq .0001$) and, to a lesser extent also, monocyte counts (35%, $p \leq .0001$) (Figure 1; Supplementary Tables 3 and 7). In contrast, eosinophil, basophil, and lymphocyte counts were markedly decreased below the 5th percentile (5thp) of HD in between 40% and 60% of samples ($p \leq .0001$) during the first week since the onset of COVID-19 (Figure 1A; Supplementary Tables 3 and 7). These altered profiles persisted through the first 4 weeks since the onset of symptoms, most samples (>60%) reaching normal values for these cell populations from week +5 onward. Despite this, monocyte counts remained increased in blood throughout the whole study period in between 26% and 39% of samples ($p \leq .0001$) (Figure 1A; Supplementary Tables 3 and 7). Among the innate leukocyte populations, eosinophil and basophil counts were the first that tended to recover toward normal levels (89% and 79% of samples with eosinophil and basophils counts below the 5thp of HD at week +2 vs. 49% and 54% at weeks +3–4 [$p \leq .0001$ and $p = .02$], respectively) (Figure 1A; Supplementary Tables 3 and 7). The WBC count almost completely recovered at week +5–7 (40% above the 95thp of HD at week +3–4 vs. 13% at week +5–7, $p = .04$), consistent with normalized neutrophil counts (51% above the 95thp of HD at week +3–4 vs. 13% at week +5–7, $p = .005$). Despite the overall tendency toward normal leukocyte levels described above, from week +8 to week +43 most major leukocyte populations still showed altered cell counts in up to one-fifth of samples, consisting of slightly increased WBC, basophil, monocyte, and lymphocyte counts ($p < .02$), together with decreased neutrophil, and eosinophil cell numbers ($p < .007$) (Figure 1A; Supplementary Tables 3 and 7). In addition, dendritic cell counts were also decreased in >60% of samples ($p \leq .0001$) during the first four weeks since the onset of symptoms, with partially normalized dendritic cell numbers at week +5–7 (71% below the 5thp of HD at week +3–4 vs. 17% at week +5–7, $p \leq .0001$). Afterward, dendritic cells still displayed altered cell counts in the longer term (Figure 1A; Supplementary Table 3).

More detailed analysis of the lymphocyte compartment in blood of MBL^{lo} patients showed that T cells had similar kinetics to those described above for the total lymphocyte counts, throughout the whole follow-up period (Figure 1B; Supplementary Table 3). Of note, this profile was similar for all major T-cell subsets, except for a more pronounced (early) decrease of TCR$\alpha\beta^{+}$CD4^{-}CD8^{-} T cells (80% below the 5thp of HD, $p \leq .0001$) at week +1 (Figure 1C; Supplementary Table 3). In contrast, NK-cell and B-cell counts barely changed along the study, except for a minor decrease of both cell populations between week +2 and week +4 ($p \leq .0001$ and $p = .03$, respectively) and the presence of increased B-cell counts at the latest follow-up time-points in 14% of cases ($p = .02$) (Figure 1B; Supplementary Table 3).

Despite this general B-cell behavior, more in-depth analysis of the B-cell compartment revealed that pre-germinal center (GC) B-cell counts were already decreased at week +1 in 30% of samples ($p = .001$), their levels remaining significantly low until week +8 in 13–15% of samples, when they finally reached normal values (Figure 1D; Supplementary Table 4). This profile was similar for all subsets of pre-GC B cells, except for immature B cells, which transiently recovered normal levels at week +2, to drop again at week

FIGURE 1 Kinetics of different populations of leukocytes in blood of MBL^{lo} patients during and after COVID-19. Kinetics of the major populations of leukocytes (A), lymphocytes (B), Tcells (C), pre-germinal center B-cell subsets (D), memory B cells grouped according to the IgH isotype and subclass expressed (E), and their maturation stage (F), present in blood of MBL^{lo} patients during COVID-19 infection, normalized by age. Data normalization by age is represented based on the distribution of absolute cell counts/μL for each cell population in pre-pandemic non-MBL age-matched HD, as reference. Colored dots depict the normalized value of the corresponding cell population for each individual sample studied, while colored horizontal curves represent the median value of each population along the study period (during and after COVID-19). Gray horizontal bands represent the 5th–95th percentile values (normality range) of pre-pandemic non-MBL HD. Vertical dotted lines delineate weeks +1, +2, +3–4, +5–7, and +8–43 showing a similar tendency (within each time interval). Percent values at the top and at the bottom of each panel indicate the percentage of MBL^{lo} samples in which the normalized absolute counts of the individual cell subsets (identified by the corresponding color-codes) were found to be significantly different ($p \leq .05$) from the age-matched HD control group (i.e., over the 95th percentile or below the 5th percentile of pre-pandemic non-MBL age-matched HD for each time interval, respectively). GC, germinal center; HD, healthy donor; IgH, immunoglobulin heavy chain; MBL, monoclonal B-cell lymphocytosis; MBL^{lo}, low-count monoclonal B-cell lymphocytosis; NK, natural killer; TCR, T-cell receptor; WBC, white blood cells.



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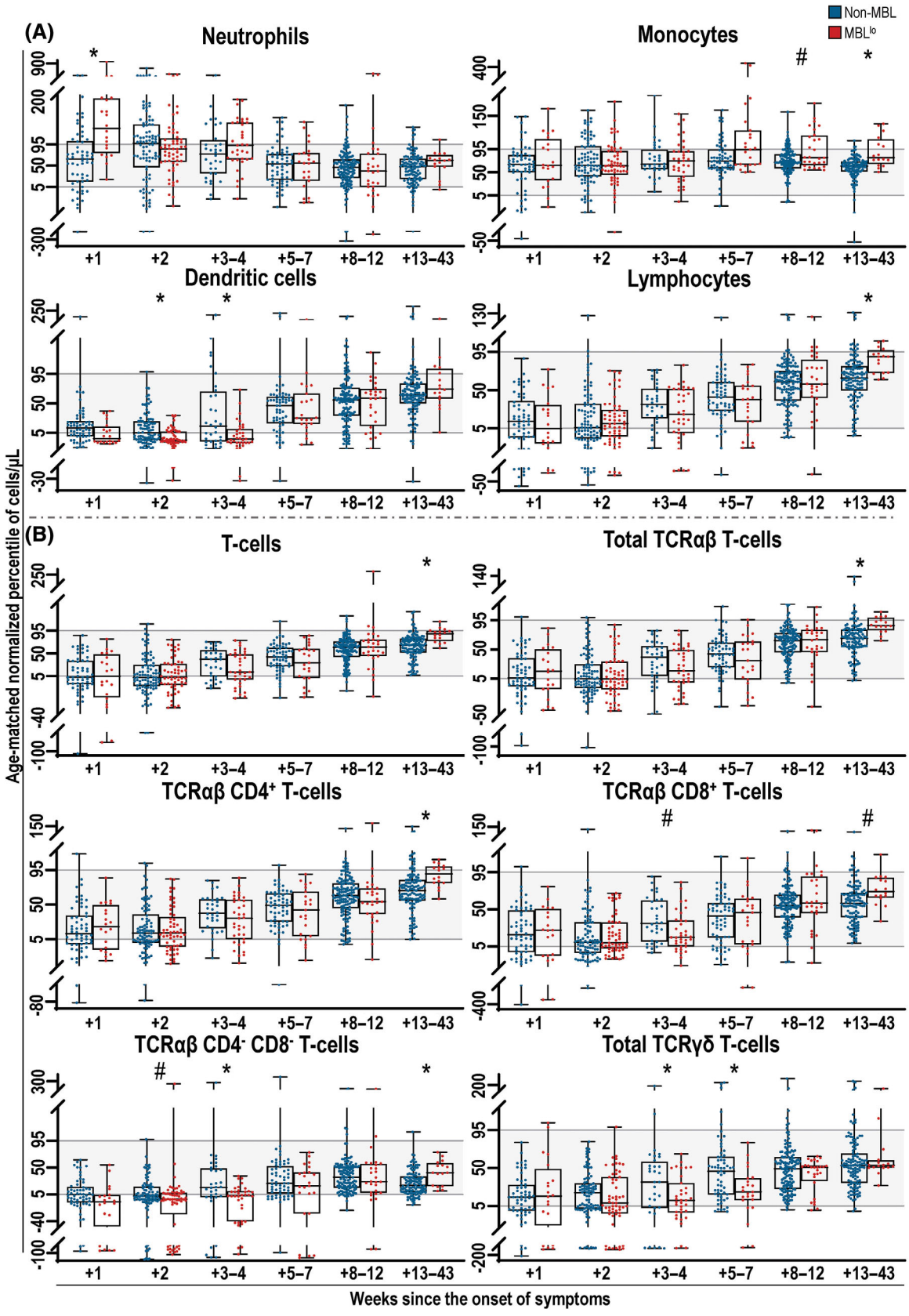
+5–7 (40% below the 5thp of HD at week +1 vs. 9% at week +2 vs. 40% at week +5–7; $p < .01$) (Figure 1D; Supplementary Table 4). Similarly to pre-GC B cells, memory B-cell (MBC) counts were also decreased in blood at week +1 in 35% of samples ($p = .0003$), but with an earlier recovery toward normal levels (26% below the 5thp of HD at week +2 vs. 3% at week +3–4, $p = .004$), independently of the IgH isotype and subclass expressed (Figure 1E; Supplementary Table 4). Similar kinetics were observed for the $CD27^+$ versus $CD27^-$ and $CD21^+$ versus $CD21^-$ subsets of MBC, except for $CD27^-CD21^-$ MBC, whose counts were markedly decreased in 65% of samples at week +1 ($p \leq .0001$) (Figure 1F; Supplementary Table 4).

In contrast to other B- and T-cell populations, plasma cells (PC) were significantly increased in blood already at the onset of COVID-19 symptoms (60% above the 95thp of HD at week +1, $p \leq .0001$), peaking at weeks +3–4 (89% above the 95thp of HD, $p \leq .0001$) and decreasing thereafter, until they returned to normal levels from week +8 onward (2% above the 95thp of HD, $p > .05$; Figure 2A; Supplementary Table 3). In contrast to MBC, PC subsets showed markedly different profiles depending on the pattern of expression of the different IgH isotypes and subclasses. Thus, the highest PC counts in blood at weeks+3–4 mostly corresponded to $IgG1^+$, $IgG3^+$, and $IgA1^+$ PC, which were increased in ≥80% of samples ($p \leq .0001$), while <60% of samples had increased counts of $IgG2^+$, $IgG4^+$, $IgA2^+$, and IgD^+ PC at weeks +3–4 (Figure 2B; Supplementary Table 5). IgM PC counts showed intermediate kinetics, as they peaked at week +2 (59% above the 95thp of HD, $p \leq .0001$), with an earlier recovery toward normal levels (51% above the 95thp of HD at weeks +3–4 vs. 5% at weeks +8–43, $p = .0003$; Figure 2B; Supplementary Table 5). In the medium-to-long-term (week +8–43), all PC subsets returned to normal levels (Figure 2B; Supplementary Table 5). Interestingly, once PC counts were elevated, this was mainly due to an increase in the more mature $CD20^-CD138^+$ PC, augmented in >60% of samples during the first 7 weeks ($p \leq .0001$) after the onset of symptoms. In turn, the number of $CD20^-CD138^-$ PC, and particularly, the less mature $CD20^+CD138^-$ PC, showed a lower increase in blood with a peak at week+2 (56% and 19% above the 95thp of HD, $p \leq .0001$ and $p = .03$; respectively) (Figure 2; Supplementary Table 5).

As regards the humoral response, anti-SARS-CoV-2 protein S and protein N specific antibodies were detectable in ≥50% of samples from MBL^{lo} patients early after the onset of symptoms (week +1) (Figure 2D–F), the only exception being anti-N IgM antibodies, which were positive at this time-point in only 35% of cases (Figure 2D; Supplementary Table 6). Subsequently, all antibody levels gradually increased, peaking at weeks +3–4 (Figure 2D–F). Thereafter, anti-N IgM median levels rapidly decreased below the cutoff level (median [IQR]: 20 [18 – 34] AU/mL at week +3–4 vs. 5 [4 – 16] AU/mL at week +5–7, $p = .001$). Although anti-S IgM median levels also decreased in parallel, they remained positive in >50% of cases along the whole study period (3986 [1150–4466] AU/mL at week +5–7 vs. 1350 [754–3289] AU/mL at week +8–12 vs. 993 [171–2627] AU/mL at week +13–43; $p > .05$) (Figure 2D). Likewise, IgA median levels started to progressively decrease at week +5–7 (78 [20–192] AU/mL at week +5–7 vs. 5 [0–22] AU/mL at week +13–43, $p = .03$ for anti-S IgA; and 195 [144–211] AU/mL at week +3–4 vs. 100 [32–117] AU/mL at week +5–7, $p = .002$ for anti-N IgA), until they were cleared from plasma in the majority of MBL^{lo} patients by the end of the study (5 [0–22] AU/mL for anti-S IgA; 6 [4 – 24] AU/mL for anti-N IgA) (Figure 2F). In contrast, anti-S and anti-N IgG levels remained elevated throughout the whole follow-up period, showing only a slight decrease at the latest time-points, such decrease being statistically significant for anti-N IgG (134 [102–156] AU/mL at weeks +8–12 vs. 99 [32–109] AU/mL at weeks +13–42, $p = .03$), but not for anti-S IgG levels (Figure 2E).

FIGURE 2 Kinetics of different populations of blood plasma cells and the anti-SARS-CoV-2-specific plasma antibody levels in MBL^{lo} patients during and after COVID-19. Kinetics of total plasma cells in blood of MBL^{lo} COVID-19 patients (A: vs. non-MBL COVID-19 patients) and their subsets as defined according to the IgH isotype and subclass expressed (B), and the specific PC-associated maturation stage (C, from less mature $CD20^+CD138^-$ to intermediate $CD20^-CD138^-$ and more mature $CD20^-CD138^+$ plasma cells) during and after COVID-19 infection, normalized by age. Plasma levels (AU/mL) of specific IgM (D), IgG (E), and IgA (F) antibodies against the nucleocapsid (N) and spike (S) proteins of SARS-CoV-2 in MBL^{lo} subjects, measured during and after COVID-19. Data normalization by age represented in panels A–C are based on the distribution of absolute cell counts/μL for each cell population in pre-pandemic age-matched non-MBL HD as reference. Colored dots depict the normalized value of the corresponding cell population for each individual sample, while colored horizontal curves represent the median value of each population along the study. Gray horizontal bands represent the 5th–95th percentile values (normality range) of age-matched pre-pandemic non-MBL HD. Vertical dotted lines delineate weeks +1, +2, +3–4, +5–7, and +8–43 intervals showing a similar tendency (within each time interval). Percentages at the top and at the bottom of each panel indicate the percentage of samples in which the normalized absolute counts of the individual cell subsets (identified by the corresponding color codes) are significantly different ($p \leq .05$) from age-matched HD (i.e., over the 95th percentile or below 5th percentile of pre-pandemic age-matched non-MBL HD for each time interval, respectively). *$p \leq .05$ for comparisons between median cell counts in MBL^{lo} vs. non-MBL (A). In panels D–F, colored dots correspond to the levels (AU/mL) of anti-spike (green dots) and anti-nucleocapsid (orange dots) antibodies for each sample, while horizontal curves represent the median value for each antibody along the study period (during and after COVID-19). Percent values at the top of the graphs indicate the percentage of positive samples (i.e., number of samples for which antibodies against the viral proteins were detected from all samples analyzed) for each time interval. Vertical dotted lines delineate week +1, +2, +3–4, +5–7, +8–12, and +13–43 showing a similar tendency (within each time interval). Ticked horizontal lines represent the preestablished cutoff value for positivity for each antibody isotype according to the corresponding manufacturer (220 AU/mL for IgM, 12 AU/mL for IgG and 2 AU/mL for IgA anti-spike antibodies and 12 AU/mL for IgM and 24 AU/mL for both IgG and IgA anti-nucleocapsid antibodies). Ab, antibody; AU, arbitrary units; HD, healthy donors; IgH, immunoglobulin heavy chain; MBL, monoclonal B-cell lymphocytosis; MBL^{lo}, low-count monoclonal B-cell lymphocytosis.



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Clonal B cells kinetics were investigated in a subset of 62/74 (84%) MBL^{lo} COVID-19 patients who were studied at least at two different time-points. The persistence of clonal B cells with a similar clonal size was confirmed for all patients at both time-points (recruitment and last follow-up). In addition, we reassessed a subgroup of MBL^{lo} COVID-19 patients 1 year after the onset of the disease, from which all of them (38/38, 100%) showed detectable clonal B cells in blood at similar levels to those observed in the first analysis (median [IQR] of 0.25 [0.07–3.83] at diagnosis vs. 0.21 [0.1–1.6] after 1 year, $p = .94$).

3.2 | Kinetics of the major populations of leukocytes and anti-SARS-CoV-2 antibodies in blood of MBL^{lo} versus non-MBL patients during and after COVID-19

Significant differences were found in the immune cell kinetic profiles of MBL^{lo} versus non-MBL patients during and after COVID-19. Thus, early after the onset of symptoms, MBL^{lo} patients showed (vs. non-MBL) significantly higher median WBC counts (+99thp vs. +52thp of HD, respectively; $p \leq .0001$), due to greater neutrophil levels (+129thp of HD in MBL^{lo} vs. +64thp of HD in non-MBL patients, $p \leq .0001$) (Figure 3A and Supplementary Figure 2). Conversely, the nadir of dendritic cells at week +2 was also significantly more pronounced in MBL^{lo} versus non-MBL patients (−6thp vs. +5thp of HD, $p = .01$) such profile extending until week +3–4 (−4thp vs. +15thp of HD, $p = .01$) (Figure 3A). Interestingly, similar kinetics were observed for all major subsets of DCs (plasmacytoid DCs, as well as both CD1c + and CD141+ myeloid DCs), though differences between the two patient groups were less pronounced for each dendritic cell subset (vs. the whole DC population), with only a trend toward lower levels in MBL^{lo} COVID-19 patients was found (Supplementary Figure 3). Of note, pre-GC B-cell counts were also significantly lower in MBL^{lo} versus non-MBL patients (+20thp vs. +58thp of HD, $p \leq .0001$) (Figure 4A), including all pre-GC B-cell subsets analyzed (Supplementary Figure 4). In addition, a tendency toward a lower decrease in MBC levels was observed in blood of MBL^{lo} versus non-MBL patients at week +2 (+29thp vs. +19thp of HD, $p = .08$; Figure 4A), due to lower CD27^{+}CD21^{+} B-cell numbers (+29thp vs. +15thp of HD, $p = .05$; Supplementary Figure 5), with a similar distribution of the IgH-isotype and subclass of MBC subsets (Supplementary Figure 6).

Once the blood leukocyte populations started to return toward normal counts at week +3–4, significantly lower levels of TCRαβ^{+}CD4^{-}CD8^{-} T cells (+2thp of HD vs. +16thp of HD, $p = .002$), TCRγδ T cells (+12thp vs. +33thp of HD, $p = .02$), and a trend toward decreased median TCRαβ^{+}CD8^{+} T-cell counts (+16thp vs. +33thp of HD, $p = .07$) (Figure 3B) were found in MBL^{lo} versus non-MBL patients. Conversely, at this time-point, total PC were significantly increased over normal levels in blood in 95% of MBL^{lo} versus only 59% of non-MBL cases ($p = .006$) (Figure 2A and Figure 4B), mainly due to more mature CD20^{-}CD138^{+} PC, which also remained more elevated in MBL^{lo} versus non-MBL patients from week +3 to week +8 (+808thp vs. +302thp of HD at weeks +3–4, $p = .02$; and + 139thp vs. +52thp of HD at weeks +5–7, $p = .02$) (Figure 4B and Supplementary Figure 7). These different PC kinetics observed at week +3–4 between both groups of COVID-19 patient, specifically involved the IgG3, IgA1, and IgA2 PC subsets (Supplementary Figure 8), which also accounted for the delayed recovery of normal PC values in MBL^{lo} versus no-MBL patients at week +5–7 (total PC counts: +56thp vs. +31thp of HD, $p = .03$; IgA1 PC: +49thp vs. +22thp of HD, $p = .03$; and IgA2 PC: +49thp vs. +19thp of HD, $p = .03$) (Figure 4B; Supplementary Figure 8).

In the longer-term (week +8–43), differences were still found between MBL^{lo} and non-MBL COVID-19 patients. These consisted of a trend toward higher WBC counts in MBL^{lo} (+73thp vs. +54thp of HD, $p = .07$) due to increased monocyte (+78thp vs. +62thp of HD, $p = .0006$) and lymphocyte numbers (+89thp vs. +64thp of HD, $p = .02$) (Figure 3A), particularly due to increased counts (all subsets) of TCRαβ^{+} T cells (+87thp vs. +67thp of HD, $p = .02$) (Figure 3B).

Overall, higher median antibody levels were also found in plasma of MBL^{lo} versus non-MBL COVID-19 patients prior to the PC peak in blood, at week +2 (3651 [2405–4190] AU/mL vs. 2414 [1048–3887] AU/mL, $p = .03$ for anti-S IgM; 135 [94–169] AU/mL vs. 94 [38–156] AU/mL, $p = .04$ for anti-N IgG; and 197 [112–209] AU/mL vs. 132 [36–196] AU/mL, $p = .02$ for anti-N IgA) (Figure 4C); such higher levels were still detectable at week +3–4 for anti-N IgA (195 [144–211] AU/mL vs. 149 [34–207] AU/mL, $p = .02$) and anti-S IgM (4063 [3816–4576] AU/mL vs. 3746 [2174–4252] AU/mL, $p = .07$). This was also associated with a trend toward higher median levels of anti-S IgG antibodies in plasma of MBL^{lo} vs. non-MBL patients in the medium term (107 [94–115] AU/mL vs. 99 [39–112] AU/mL at week +3–4, $p = .10$; and 115 [92–120] AU/mL vs. 80 [46–112] AU/mL at week +8–12, $p = .06$) and significantly

FIGURE 3 Kinetics of the major populations of leukocytes and T-cell subsets with a significantly different distribution in blood of MBL^{lo} vs. non-MBL patients during and after COVID-19. (A) Kinetics of the major leukocyte subsets; (B) Kinetics of different T-cell subsets. Data expressed as absolute cell counts/μL for each individual cell population analyzed, normalized by age. Data normalization by age is represented based on the distribution of absolute cell counts/μL for each individual population in pre-pandemic age-matched non-MBL HD as reference. Subjects without MBL (blue dots) and with MBL^{lo} (red dots) are grouped according to the following time intervals (in weeks) since the onset of symptoms of COVID-19: +1, +2, +3–4, +5–7, +8–12, and +13–43. Notched boxes represent 25th and 75th percentile values (IQR), whereas the line in the middle corresponds to median values. Whiskers represent the maximum and minimum values observed for each group. Gray horizontal bands represent the 5th-95th percentile values (normal range) in blood of pre-pandemic non-MBL HD. *Statistically significant differences ($p \leq .05$) between MBL^{lo} vs. non-MBL subjects; # tendency for statistically significant differences ($p > .05$ and $\leq .10$). HD, healthy donor; IQR, interquartile range; MBL^{lo}, low-count monoclonal B-cell lymphocytosis; TCR, T-cell receptor.

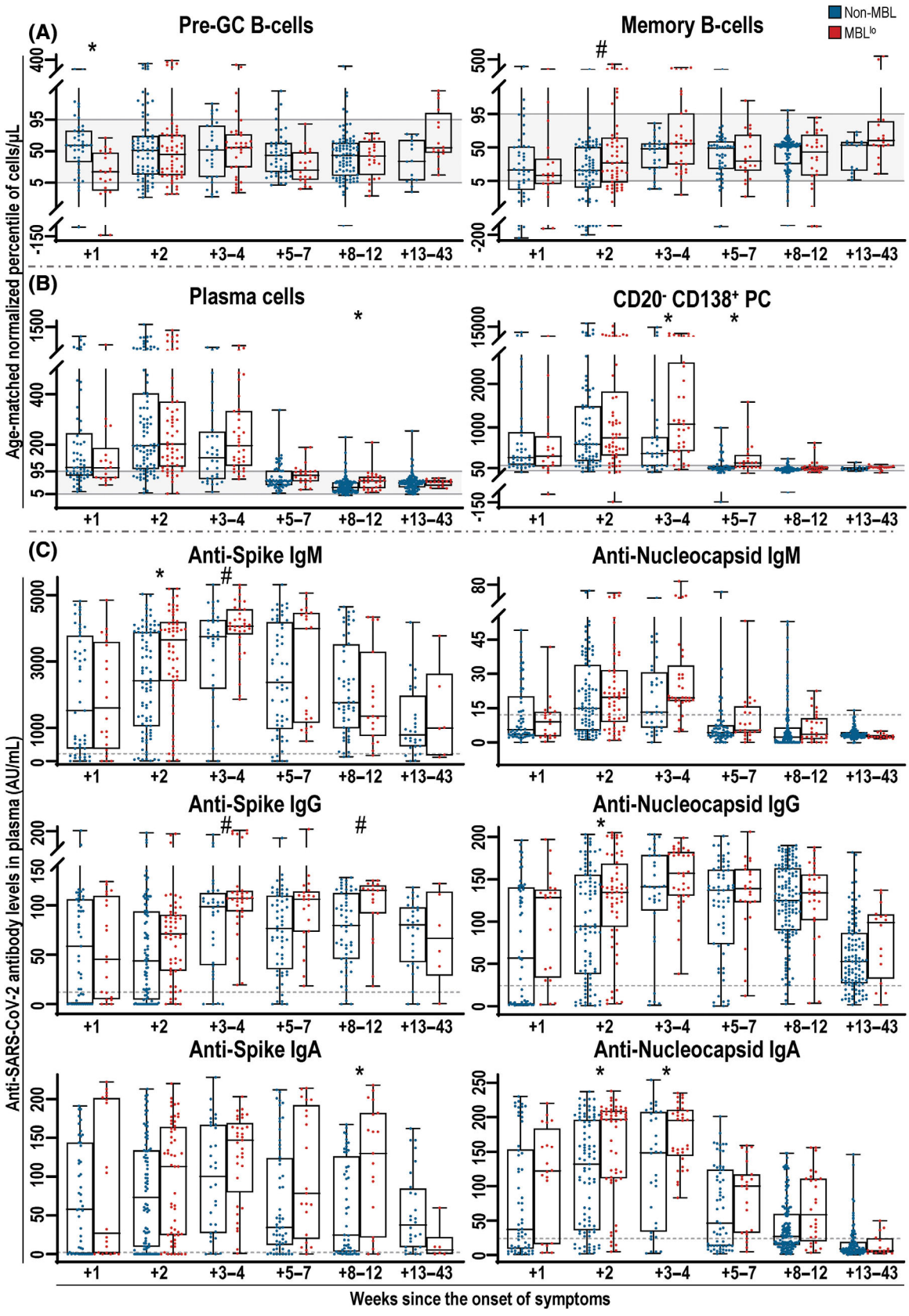

FIGURE 4 Legend on next page.

greater anti-S IgA median levels at week +8–12 (130 [21–182] AU/mL vs. 24 [3–127] AU/mL, $p = .03$) (Figure 4C).

3.3 | Detectable viral load in blood of MBL^{lo} versus non-MBL COVID-19 patients

A significantly higher percentage of MBL^{lo} samples were positive for SARS-CoV-2 RNA in plasma at week +1 after the onset of COVID-19-related symptoms (35% vs. 13% of non-MBL patients; $p = .05$) (Supplementary Table 8). Subsequently, no further differences were observed between both groups of patients at any time-point analyzed (Supplementary Table 8).

4 | DISCUSSION

MBL^{lo} has been recently identified as an independent risk factor for severe COVID-19,[17] but the underlying immune dysregulation still remains unknown. Here we investigated the long-term (longitudinal) immune cell and anti-SARS-CoV-2 antibody profile in blood of MBL^{lo} individuals infected by SARS-CoV-2 prior to the introduction of vaccination, aiming at better understanding the greater severity of COVID-19 in MBL^{lo} versus non-MBL patients. For more accurate identification of immune alterations associated with MBL^{lo} individuals during COVID-19, immune cell counts normalized according to age (which affects to blood leukocyte counts) and the onset of first COVID-19-related symptoms were used.

Previous reports have extensively described alterations in the distribution of the immune cells during the acute COVID-19 infection. These mainly consisted of increased neutrophil and monocyte counts in blood, associated with significantly decreased eosinophil, basophil, dendritic cell and lymphocyte counts, particularly among cases with more severe disease;[18–21] in addition, a substantial expansion of PC has been also described, such increase being more pronounced in patients with severe versus mild COVID-19.[18–21] Overall, our data show similarly altered immune profiles in blood of MBL^{lo} COVID-19 patients early after the onset of COVID-19-related symptoms, which persisted during the first 4 weeks of the disease. Afterward, blood cell counts started to recover, and in the majority of cases, they reached normal levels by week +5 after the onset of symptoms, in line with what has been described for patients hospitalized for severe COVID-19.[18,30] During the convalescence phase, several blood cell populations persisted altered in number in MBL^{lo} subjects, reflecting an incomplete recovery of the immune system, almost 1 year after the onset of the disease.[19] In line with the PC kinetics in blood, plasma levels of specific IgM, IgG, and IgA antibodies against different SARS-CoV-2 proteins were also detected in a substantial fraction of MBL^{lo} patients, early after the onset of symptoms (i.e., week +1), reaching a maximum at week +3–4 in parallel to the PC peak in blood. Once the viral infection was controlled, IgM and IgA levels decreased, whereas IgG antibody levels remained elevated until week +13, when they also started to decrease, in line with previous data. Of note, similar kinetics were observed for anti-S and anti-N antibody levels in plasma throughout the whole follow-up period. However, anti-S (i.e., IgM) antibodies persisted at low but still detectable levels in the longer-term compared with anti-N antibodies, in line with previous observations.[31] Altogether, the immune cell and antibody kinetics here reported for MBL^{lo} COVID-19 patients fully resemble those described for (more) severe COVID-19 patients,[21,32,33] and would support the previously reported association between MBL^{lo} and an increased risk for more severe COVID-19.[17]

Fully in line with this hypothesis, further specific comparison between MBL^{lo} and non-MBL COVID-19 patients revealed higher neutrophil counts at week +1 in MBL^{lo} patients, a well-established surrogate marker for more severe COVID-19.[18,21] In addition, MBL^{lo} COVID-19 patients also showed significantly lower pre-GC B-cell counts (vs. non-MBL COVID-19 patients) at this early time point. Of note, lower numbers of pre-GC B-cells have been previously reported in blood of otherwise healthy MBL^{lo} subjects,[12] which could contribute to an impaired and/or delayed B-cell response against new antigen challenges, due to a narrower naïve B-cell repertoire.[10] At week +2, a more pronounced decrease of dendritic cell counts was also observed in blood of MBL^{lo} versus non-MBL COVID-19 patients, such decrease extending until week +4. In parallel, a more marked decrease of TCR$\alpha\beta^{+}$CD4^{-}CD8^{-} and TCR$\gamma\delta^{+}$ T cells was observed in blood of MBL^{lo} patients, which extended from week +2 to week +7, while in non-MBL patients, these cell populations returned to normal levels

FIGURE 4 Kinetics of B-cell and plasma cell subsets, and SARS-CoV-2 specific antibody levels in plasma of MBL^{lo} vs. non-MBL subjects during and after COVID-19. Panels A and B show the kinetics of B-cell and plasma cell subsets, respectively, with data expressed as absolute cell counts/μL for each individual cell population analyzed, normalized by age. Data normalization by age is represented based on the distribution of absolute of cell counts/μL for each individual population in pre-pandemic age-matched non-MBL HD as reference. Gray horizontal bands represent the 5th–95th percentile values (normality range) in blood of pre-pandemic age-matched non-MBL HD. (C) Plasma levels (AU/mL) of anti-spike and anti-nucleocapsid SARS-CoV-2 proteins specific IgM, IgG, and IgA antibodies in MBL^{lo} subjects. Ticked horizontal lines represent the cutoff for positivity for each antibody isotype (220 AU/mL for IgM, 12 AU/mL for IgG and 2 AU/mL for IgA anti-spike; 12 AU/mL for IgM and 24 AU/mL for both IgG and IgA anti-nucleocapsid protein antibodies). For the three panels (A, B and C), subjects without MBL (blue dots) and with MBL^{lo} (red dots) are grouped according to the following time intervals (in weeks) since the onset of COVID-19 symptoms: +1, +2, +3–4, +5–7, +8–12, and +13–43. Notched boxes represent 25th and 75th percentile values (IQR), whereas the line in the middle corresponds to median values. Whiskers represent the maximum and minimum value for each group. *Statistically significant differences ($p \leq .05$) between MBL^{lo} vs. non-MBL subjects; # tendency for statistical differences ($p > .05$ and $\leq .10$). AU, arbitrary units; d, days; GC, germinal center; HD, healthy donors; Ig, immunoglobulin; IQR, interquartile range; MBL^{lo}, low-count monoclonal B-cell lymphocytosis.

already at week +4. Decreased dendritic cell and T-cell subset counts in blood of COVID-19 patients has been associated in the acute phase of infection with cell migration from blood to specific tissues, including secondary lymphoid organs for antigen-presentation to inexperienced B cells and infected tissues where a higher viral load exists, respectively.[34] Conversely, a more pronounced increase in PC counts, particularly due to more mature $CD20^{-}CD138^{+}$ PC, was observed in blood of MBL^{lo} patients early after the onset of COVID-19 symptoms, followed by a peak at week +4, when PC counts were already decreasing toward normal levels in non-MBL subjects. In line with these findings, higher levels of SARS-CoV-2-specific antibodies were also detected during the acute phase of infection, between weeks +2 and +4. In contrast to this data, recent studies have reported a defective humoral response (i.e., lower plasma levels of specific antibodies) to vaccination against SARS-CoV-2 in high-count MBL (MBL^{hi}) and CLL patients, resulting in failure to seroconversion after complete vaccination schedules in a significant proportion of cases.[35,36] Although data from vaccination might not be comparable with a complex disease such as COVID-19, higher antibody levels in MBL^{lo} COVID-19 patients might reflect the different biology of MBL^{lo} versus MBL^{hi} and CLL. Altogether, these findings reveal a delayed humoral immune response in MBL^{lo} versus non-MBL COVID-19 patients, which might be related to the more restricted BCR repertoire of pre-GC B cells in MBL^{lo},[10] and the higher probability for a delayed ability to recognize a new virus like SARS-CoV-2, with the subsequent need for higher antibody levels for an effective clearance of the meanwhile higher viral load. In this regard, previous studies have also shown that anti-SARS-CoV-2 antibody levels typically peak later in severe versus mild COVID-19 patients, while reaching higher plasma levels in the former cases.[33,37,38] Fully in line with our data, recent studies confirmed that COVID-19 patients aged >60 years (who are presumably enriched in MBL^{lo} cases) had significantly higher antibody responses and more severe COVID-19 compared with younger subjects, particularly children, who have a significantly broader pre-GC B-cell repertoire. Such higher antibody titres observed in older patients undergoing more severe COVID-19 are most probably required for complete clearance of the greater viral load achieved in the acute phase of infection prior to an effective humoral response.[32,33,38,39] Consistent with these data, here we found a higher frequency of MBL^{lo} versus non-MBL COVID-19 patients to have detectable SARS-CoV-2 RNA in plasma during the first week after the onset of symptoms.

After COVID-19, still some differences were observed in our cohort between MBL^{lo} and non-MBL patients, further supporting the persistence of immune alterations for relatively long period of time among MBL^{lo} subjects. These included higher monocyte and T-cell counts in blood of MBL^{lo} versus non-MBL COVID-19 patients. However, increased T-cell counts have been previously described in blood of MBL^{lo} versus non-MBL HD aged 60–70 years, suggesting these longer-term alterations might be related to MBL^{lo} itself rather than COVID-19.[11]

In summary, our data reveal altered immune cell and SARS-CoV-2 specific antibody profiles in blood of MBL^{lo} patients which mimic those previously described for severe COVID-19 disease in adults and that are consistent with a delayed and significantly more pronounced innate (i.e., dendritic cell and T cell) and PC immune response in MBL^{lo} versus non-MBL patients.

AUTHOR CONTRIBUTIONS

Julia Almeida, Miguel Marcos, and Alberto Orfao contributed to the conceptualization, design, and supervision of the study, as well as to recruitment of funding. Julia Almeida, Jacques J.M. van Dongen, and Alberto Orfao contributed to the design and development of antibody panels. Guillermo Oliva-Ariza, Blanca Fuentes-Herrero, Alba Pérez-Pons, Alba Torres-Valle, Julio Pozo, Óscar González-López, Marta Bernal-Ribes, Oihane Pérez-Escurza, Francisco Javier García Palomo, and Miryam Santos Sánchez performed the experiments. Guillermo Oliva-Ariza, Blanca Fuentes-Herrero, Quentin Lecrevisse, Alba Pérez-Pons, Alba Torres-Valle, Marta Bernal-Ribes, Martín Pérez-Andrés, F. Javier Morán-Plata, Daniela Damasceno, Vitor Botafogo, Noemí Muñoz-García, Rafael Fluxa, Julia Almeida, and Alberto Orfao analyzed and interpreted data. Cristina Carbonell, José Ángel Martín-Oterino, Amparo López-Bernús, Moncef Belhassen-García, Lourdes Vazquez, Guillermo Hernández-Pérez, Pilar Leoz, Pilar Costa-Alba, Elena Pérez-Losada, Ana Yeguas, Marta García-Blázquez and Miguel Marcos selected COVID-19 patients for the study and collected their relevant clinical information. Guillermo Oliva-Ariza, Blanca Fuentes-Herrero, Cristina Carbonell, Julia Almeida, Miguel Marcos, and Alberto Orfao coordinated the planning and execution of the experiments. Guillermo Oliva-Ariza, Blanca Fuentes-Herrero, Julia Almeida, and Alberto Orfao had full access to and verified the underlying data. Guillermo Oliva-Ariza, Julia Almeida, and Alberto Orfao wrote the paper. All the authors critically reviewed the manuscript and approved the final version of the document.

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CONFLICT OF INTEREST STATEMENT

Alberto Orfao, Julia Almeida, and Jacques J.M. van Dongen report being one of the inventors on the EuroFlow-owned European patent 119646NL00 registered on November 2019 ("Means and methods for multiparameter flow cytometry-based leukocyte subsetting"), and they are also authors of the PCT patent WO 2010/ 140885A1 ("Methods, reagents and kits for flow cytometric immunophenotyping"). The Infinicyt software is based on intellectual property of the University of Salamanca (Salamanca, Spain). All above mentioned intellectual property and related patents are licensed to Becton/Dickinson Biosciences (San José, California), which pays royalties to the University of Salamanca and the EuroFlow Consortium. These royalties are exclusively used for continuation of the EuroFlow collaboration and sustainability of the EuroFlow Consortium. The other authors declare no competing financial interests or any other conflicts of interest.

DATA AVAILABILITY STATEMENT

De-identified data collected during the study on individual patients will be available via the Spanish National DNA Bank Carlos III, immediately after publication for researchers who provide a proposal that is approved by the external scientific committee and ethics committee of the Spanish National DNA Bank.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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