

Effect of Ocrelizumab in Blood Leukocytes of Patients With Primary Progressive MS

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Abstract

Objective

To analyze the changes induced by ocrelizumab in blood immune cells of patients with primary progressive MS (PPMS).

Methods

In this multicenter prospective study including 53 patients with PPMS who initiated ocrelizumab treatment, we determined effector, memory, and regulatory cells by flow cytometry at baseline and after 6 months of therapy. Wilcoxon matched paired tests were used to assess differences between baseline and 6 months' results. *p* Values were corrected using the Bonferroni test.

Results

Ocrelizumab reduced the numbers of naive and memory B cells ($p < 0.0001$) and those of B cells producing interleukin (IL)-6, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor- α (TNF α) ($p < 0.0001$ in all cases). By contrast, the proportions of plasmablasts and B cells producing GM-CSF and TNF α increased significantly, suggesting the need for treatment continuation. We also observed a decrease in CD20⁺ T-cell numbers ($p < 0.0001$) and percentages ($p < 0.0001$), and a clear remodeling of the T-cell compartment characterized by relative increases of the naive/effector ratios in CD4⁺ ($p = 0.002$) and CD8⁺ ($p = 0.002$) T cells and relative decreases of CD4⁺ ($p = 0.03$) and CD8⁺ ($p = 0.004$) T cells producing interferon- γ . Total monocyte numbers increased ($p = 0.002$), but no changes were observed in those producing inflammatory cytokines. The immunologic variations were associated with a reduction of serum neurofilament light chain (sNfL) levels ($p = 0.008$). The reduction was observed in patients with Gd-enhanced lesions at baseline and in Gd- patients with baseline sNfL >10 pg/mL.

Conclusions

In PPMS, effector B-cell depletion changed T-cell response toward a low inflammatory profile, resulting in decreased sNfL levels.

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Glossary

EM = effector memory; **GM-CSF** = granulocyte-macrophage colony-stimulating factor; **IFN- γ** = interferon-gamma; **Ig** = immunoglobulin; **IL** = interleukin; **NK** = natural killer; **PBMC** = peripheral blood mononuclear cell; **PD-L1** = programmed death-ligand 1; **PPMS** = primary progressive MS; **RRMS** = relapsing-remitting MS; **sNfL** = serum neurofilament light chain; **TD** = terminally differentiated; **TNF α** = tumor necrosis factor-alpha.

MS is the most prevalent demyelinating disease of the CNS. Most patients initially show with a relapsing-remitting (RR) course. However, in about 10% of the cases, the disease starts with a progressive disability worsening without remission periods.¹ This form of the disease is known as primary progressive MS (PPMS) and is associated with a poorer prognosis.² Classically, patients with PPMS do not benefit of disease-modifying treatments approved for the relapsing form of the disease.³ This changed recently with the approval of ocrelizumab (Ocrevus; Roche, Grenzach-Wyhlen, Germany) as a disease-modifying treatment for PPMS. Its efficacy and safety were demonstrated in the ORATORIO phase III clinical trial.^{4,5}

At the molecular level, these humanized antibodies selectively target cells that express CD20 on their surface. The CD20 molecule is expressed in most B-cell subsets as pre-B, naive, and memory B cells, whereas it is absent in stem cells, pro-B cells, and plasma cells. Accordingly, ocrelizumab treatment results in B depletion mediated by complement, cellular cytotoxicity, or apoptosis.⁶ However, its effect on other immune cell subsets has not been fully addressed. The effects of B-cell depletion by rituximab, another CD20 monoclonal antibody, were studied in patients with RRMS. Flow cytometry demonstrated reduced CSF B cells and T cells in most patients 6 months after treatment.⁷ CD4⁺ and CD8⁺ T-cell reduction remained stable with subsequent rituximab cycles. This was also observed in other immune cell types.⁸ Future studies will address whether additional changes are observed in patients with RRMS treated with ocrelizumab. In this line, a nearly complete depletion of B cells was observed in patients with PPMS 2 weeks after the administration of a single dose of this drug.^{9,10} However, CD20 is also expressed on a small subset of CD3⁺ T cells, a highly activated subset of T cells displaying increased expression of activation markers and production of proinflammatory cytokines.^{11,12} These cells are found in blood, CSF, and chronic brain lesions of patients with MS^{12,13} and have shown to be effectively depleted by rituximab in patients with RRMS¹⁴ and ocrelizumab in a small cohort of 21 patients with MS (only 4 of them classified as patients with PPMS).^{9,10} Despite these data, less is known about the effect of ocrelizumab in different T- and B-cell subsets as well as on natural killer (NK) cells and monocytes.

We describe the changes induced by ocrelizumab in blood immune cells of patients with PPMS to further understand the effect of the drug in the abnormal inflammatory response taking place in these patients.

Methods

This multicenter prospective longitudinal study included 53 patients diagnosed with PPMS according to the McDonald criteria¹⁵ who consecutively initiated ocrelizumab treatment in 10 university hospitals. Basal patient data are depicted in table 1.

MRI examination was performed within 1 month before treatment initiation following clinical protocols established in each of the centers.

Sample Collection

Patient heparinized blood specimens were obtained just before initiating ocrelizumab treatment and 6 months thereafter, before the second dosing. Samples were sent to the Immunology Department of Hospital Ramón y Cajal (Madrid) where peripheral blood mononuclear cells (PBMCs) were separated 24 hours after blood collection, including those collected at the same hospital, and cryopreserved until studied. Basal and 6-month samples were studied simultaneously to avoid interassay variability. Serum samples were stored at -80°C until processed. A second aliquot of fresh blood collected in an EDTA tube was used to explore total lymphocyte and monocyte counts in a Coulter counter.

Monoclonal Antibodies

CD8-FITC, CD20-FITC, CD24-FITC, interferon-gamma (IFN γ)-FITC, interleukin (IL)-1 β -FITC, CD27-PE, IL-10-PE, CD197 (CCR7)-PE, GM-CSF-PE, CD3-PerCP, tumor necrosis factor-alpha (TNF α)-PerCP-Cy5.5, CD19-PE-Cy7, CD25-PE-Cy7, programmed death-ligand 1 (PD-L1)-PE-Cy7, CD45RO-APC, CD56-APC, IL-12-APC, IL-6-APC, CD4-APC-H7, CD8-APC-H7, CD14-APC-H7, CD38-APC-H7, CD3-BV421, CD127-BV421, IL-6-BV421, CD45-V500 (BD Biosciences, San Jose, CA), and IL-17-APC (R&D Systems, Minneapolis, MN).

Labeling of Surface Molecules

We prepared aliquots of 10^6 PBMCs in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA), labeled them with adequate amounts of fluorescence-labeled monoclonal antibodies during 30 minutes at 4°C in the dark. Cells were washed twice with PBS and analyzed by flow cytometry as detailed below.

In Vitro Stimulation and Intracellular Cytokine Staining

We studied intracellular production of pro- and anti-inflammatory cytokines by B and T lymphocytes as previously described.¹⁶ In

Table 1 Baseline Data and Patient Characteristics (n = 53)

Age, y, median (range)	52.0 (33.0–67.0)
Sex, F/M	23/30
Disease duration, y, median (range)	8.8 (1.4–15.4)
EDSS score, median (range)	6.0 (2.0–8.0)
Patients showing contrast-enhancing lesions (n = 48), n (%)	12 (25.0)
T2 lesions on MRI (n = 48), n (%)	
<10 lesions	11 (22.9)
10–50 lesions	29 (60.5)
50–100 lesions	4 (8.3)
>100 lesions	4 (8.3)

Abbreviation: EDSS = Expanded Disability Status Scale.

In addition, we explored intracellular cytokine production by monocytes by stimulating aliquots of 10^6 PBMCs with 1 mg/mL lipopolysaccharide (from *Escherichia coli* O111: B4; Merck, Kenilworth, NJ) during 4 hours at 37°C in 5% CO₂.

Flow Cytometry

PBMCs were analyzed within 1 hour after antigen labeling. Isotype controls were used for setting mean auto-fluorescence values. Results obtained were analyzed using FACSDiva software V.8.0 (BD Biosciences) as previously described.¹⁶ A minimum amount of 5×10^4 events were analyzed. The gating strategy is shown in figure e-1 (links.lww.com/NXI/A368). For intracellular cytokine staining, nonstimulated PBMCs were used as control of basal production (figure e-2, links.lww.com/NXI/A369). We explored intracellular production of IL-1 β , IL-6, IL-10, IL-12, and TNF α by monocytes; IFN γ , granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF α , IL-17, and IL-10 by CD4 and CD8 T cells; and IL-6, IL-10, TNF α , and GM-CSF by B cells.

Flow Cytometry Analyses

To avoid bias due to B-cell depletion, we analyzed total cell counts per microliter for every leukocyte subset. This was calculated by exploring percentages over total mononuclear cells (CD45⁺) and total lymphocyte and monocyte numbers as described above. In addition, we recorded the values of every T, B, NK, and monocyte subset relative to total T, B, NK, and monocyte cells, respectively.

Immunoglobulin and sNfL Quantification

Immunoglobulin (Ig) G, IgA, and IgM levels were measured by nephelometry on a BN ProSpec analyzer (Siemens Healthcare Diagnostics). Serum neurofilament light chain (sNfL) levels were quantified in a SR-X instrument (Quanterix, Lexington, MA) using the single molecule array NF-light Advantage Kit technique (Quanterix, Billerica, MA).¹⁷

Statistical Analysis

Statistical analyses were performed with GraphPad Prism 6.0 software (GraphPad Prism Inc., San Diego, CA). Differences between basal and 6 months samples were assessed by Wilcoxon matched paired tests. *p* Values were adjusted using the Bonferroni method. *p* Values below 0.05 were considered significant.

Standard Protocol Approvals, Registrations, and Patient Consents

This study was approved by the Ramón y Cajal University Hospital Clinical Research Ethics Committee. Written informed consent was obtained from every patient before entry.

Data Availability

Anonymized data supporting the findings of this study will be shared by any qualified investigator during 3 years after the publication of the study on reasonable request.

Results

Fifty-three patients with PPMS (43% females) treated with ocrelizumab for at least 6 months were included in this study. Median (range) age and disease duration were respectively 52.0 (33.0–67.0) and 8.8 (1.4–15.4) years, respectively, and the median Expanded Disability Status Scale score was 6 (2–8) at baseline. MRI data from 48 patients were available. A low baseline activity (defined as less than 10 lesions) was observed in 22.9% of patients, with a moderate activity (10–50 lesions) in 60.5% and a high (50–100 lesions) or very high activity (>100 lesions) in 16.6%. Twelve patients (25%) of our cohort showed at least 1 contrast-enhancing lesion (table 1).

We studied the changes induced by ocrelizumab in the peripheral blood mononuclear cell counts after 6 months of treatment. Patients experienced a discrete decrease in the absolute lymphocyte counts, not reaching statistical significance (ns) after Bonferroni correction and a clear increase in absolute CD14⁺ monocyte counts (*p* = 0.002, table e-1, links.lww.com/NXI/A372). We further addressed the impact of this drug on the absolute numbers and population percentages of different leukocyte subsets.

B Cells

As expected, total CD19⁺ B-cell counts were strongly reduced after ocrelizumab treatment (*p* < 0.0001, figure 1A and table e-1, links.lww.com/NXI/A372). We first explored effector and memory B-cell subsets. Ocrelizumab induced a decrease in naive and memory B-cell numbers (both *p* < 0.0001, figure 1A and table e-1) and of plasmablasts although the last one did not reach statistical significance (*p* = 0.06, figure 1A and table e-1). On the other hand, it caused a clear increase in percentages of plasmablasts and transitional B cells (both *p* < 0.0001, figure 1B and table e-1) relative to total CD19⁺ B cells.

When we evaluated intracellular cytokine production by B cells, we observed a drastic reduction in IL-6, IL-10, GM-

CSF, and TNF α B-cell numbers (all $p < 0.0001$, figure 1C and table e-2, links.lww.com/NXI/A373). Ocrelizumab also induced a decrease in the percentage of B cells producing TNF α ($p < 0.0001$) relative to total CD19 $^+$ B cells and a clear increase in the proportion of regulatory B cells producing IL-10 ($p < 0.0001$). By contrast, we also observed relative increases in the percentages of B cells producing IL-6 ($p = 0.004$) and GM-CSF ($p < 0.0001$) (figure 1D and table e-2).

T Cells

We next studied the effects of ocrelizumab in CD4 $^+$ T-cell subsets. No significant differences were observed in the total cell numbers except for a trend toward an increase in Treg CD4 $^+$ cells ($p = 0.03$, ns after Bonferroni correction, table e-1, links.lww.com/NXI/A372). However, we found a clear decrease in the percentage of terminally differentiated (TD) ($p < 0.0001$) CD4 $^+$ T subset relative to total CD4 $^+$ T cells and a trend toward relative increases in the percentages of naive CD4 $^+$ T cells ($p = 0.005$, ns after Bonferroni correction) as shown in figure 2A and table e-1. When we explored CD8 $^+$ T cells, we found a decrease in effector memory (EM) CD8 $^+$ T-cell percentages relative to total T CD8 $^+$ cells ($p = 0.001$, figure 2B and table e-1). We also observed an increase in the percentage of naive CD8 $^+$ T cells ($p = 0.01$, figure 2B and table e-1). In addition, we found clear increases in the ratios between naive/TD CD4 $^+$ T cells ($p = 0.002$, figure 2A and table e-1) and naive/EM CD8 $^+$ T cells ($p = 0.002$, figure 2B and table e-1). These increases were maintained for the ratios between naive and effector subsets (naive/EM + TD ratio) in both CD4 $^+$ ($p = 0.009$, figure 2A and table e-1) and CD8 $^+$ ($p = 0.002$, figure 2B and table e-1) T cells.

CD20 $^+$ T cells were analyzed in 39 patients with PPMS of our cohort. We observed a marked decrease in this subset both in absolute numbers ($p < 0.0001$) and in the percentage of CD20 $^+$ T cells relative to CD3 $^+$ T cells ($p < 0.0001$) (table e-1, links.lww.com/NXI/A372). In addition, we explored changes after 6 months of ocrelizumab treatment in CD4 $^+$ and CD8 $^+$ T-cell subsets expressing or not CD20 (figures e-3, links.lww.com/NXI/A370 and e-4, links.lww.com/NXI/A371). We found significant decreases in the percentages of all CD4 $^+$ CD20 $^+$ and CD8 $^+$ CD20 $^+$ subsets related to total CD4 $^+$ and CD8 $^+$ T cells, respectively. However, when we explored CD20 $^-$ T-cell subsets, we observed only a decrease of TD CD4 $^+$ ($p = 0.002$) and EM CD8 $^+$ ($p = 0.0008$) T-cell subsets and an increase of naive CD8 $^+$ T cells ($p = 0.007$), similar to that detected in total CD4 $^+$ and CD8 $^+$ T-cell subsets.

On studying intracellular cytokine production by CD4 $^+$ and CD8 $^+$ T cells, no changes were found in absolute cell counts except for a tendency to increase in IL-10-producing CD4 $^+$ cells ($p = 0.04$, ns after Bonferroni correction, table e-2, links.lww.com/NXI/A373). However, we observed a clear decrease in the percentages of CD4 $^+$ ($p = 0.03$) and CD8 $^+$ ($p = 0.004$) T cells producing IFN γ , respectively, to total CD4 $^+$ and CD8 $^+$ T cells (table e-2).

Innate Immune Cells

When we explored innate immune cells, we observed only a discrete decrease in the total numbers of CD56 bright NK cells ($p = 0.005$, table e-1, links.lww.com/NXI/A372), an increase in total monocyte numbers ($p = 0.002$, table e-1), and a trend toward an increase in the numbers of PD-L1-expressing monocytes ($p = 0.007$, ns after Bonferroni correction, table e-1). No changes were found in numbers or proportions of monocytes producing pro- or anti-inflammatory cytokines (table e-2, links.lww.com/NXI/A373).

Serum Igs and NfL Levels

IgG and IgA levels remained stable after ocrelizumab treatment. Only serum IgM levels decreased ($p < 0.0001$), but no patient reached levels below the normal range (data not shown). sNfL levels decreased after ocrelizumab treatment ($p = 0.008$, figure 3A).

Influence of Inflammatory Status in Ocrelizumab-Induced Changes

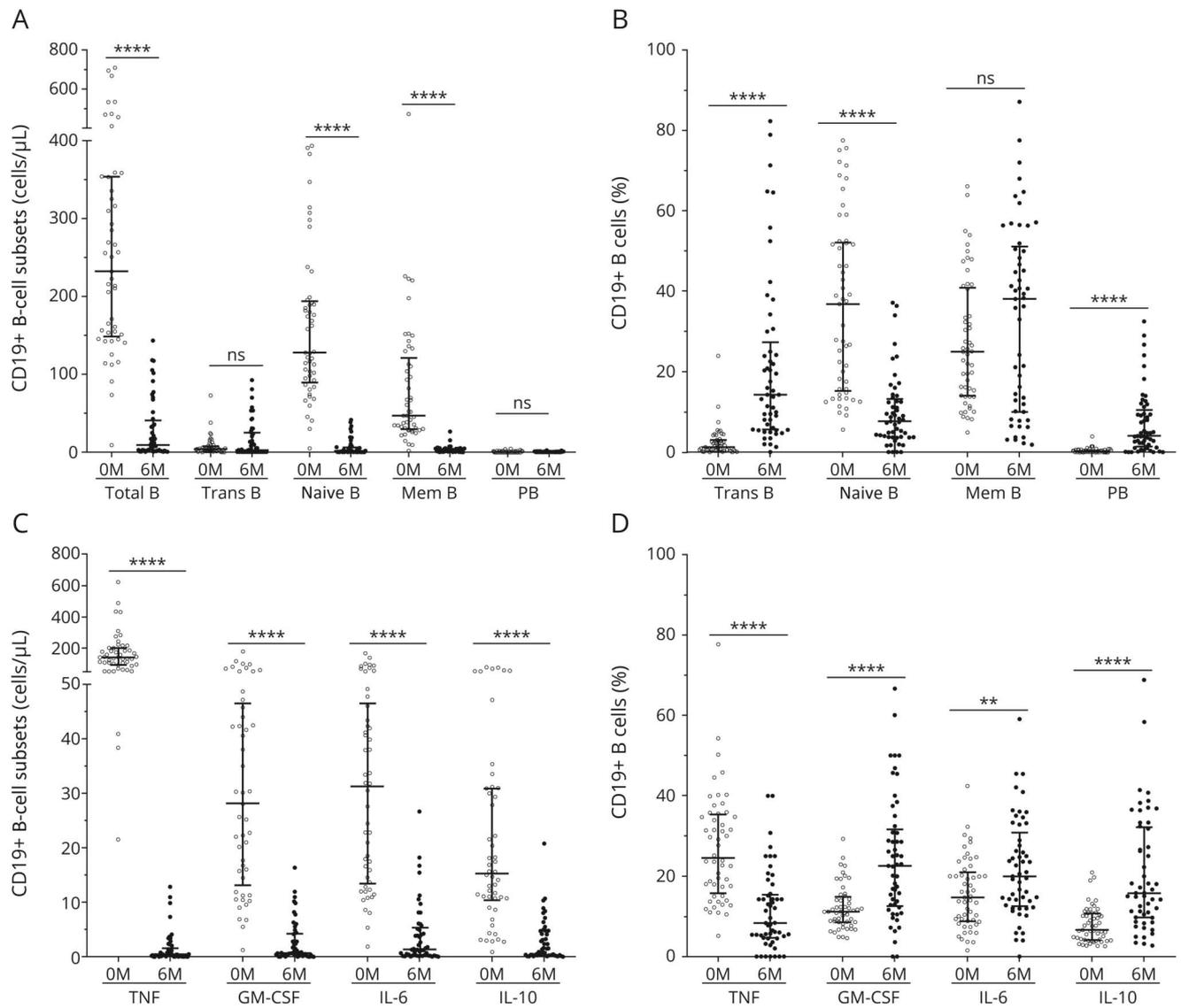
We finally evaluated changes in blood leukocyte subsets and in serum Igs and NfL values in patients showing ($n = 12$, Gd+) or lacking ($n = 41$, Gd-) gadolinium-enhanced lesion at baseline to elucidate whether the inflammatory status could condition ocrelizumab effects described above. No significant differences were observed in the leukocyte subsets or serum Igs between both groups. However, when studying sNfL, we found a significant decrease in patients showing gadolinium-enhancing lesions ($p = 0.03$, figure 3B) at baseline and only a trend ($p = 0.06$) in those lacking them. Of note, when we divided Gd- patients according to their baseline sNfL values, we found that those with values higher than 10 pg/mL ($n = 22$) experienced a clear decrease on ocrelizumab treatment ($p = 0.006$, figure 3C), whereas those with baseline sNfL below 10 pg/mL ($n = 19$) did not experience significant changes (figure 3D).

Discussion

Ocrelizumab is a humanized monoclonal antibody that selectively depletes CD20-expressing B cells, preserving the capacity for B-cell reconstitution and preexisting humoral immunity.¹⁸ The changes in the different peripheral blood immune cell subsets induced by this treatment have not been totally identified yet. We explored changes of a wide variety of leukocytes including different T, B, NK, and monocyte subsets in a multicenter prospective cohort of 53 patients with PPMS treated with this drug, by exploring these cells in baseline and 6 months' samples before treatment with the second dose of ocrelizumab.

Ocrelizumab induced a drastic depletion of CD19 $^+$ B-cell counts mainly because of a reduction in naive and memory subsets. In addition, we observed a trend toward a decrease in the number of plasmablasts. If confirmed in larger series, this

Figure 1 Changes in Blood B-Cell Subsets on Ocrelizumab Treatment



B-cell subsets were obtained before (0M) and at 6 months (6M) of ocrelizumab treatment ($n = 53$). (A) Absolute numbers (cells/ μ L) of the different CD19⁺ B-cell subsets. (B) Percentages of the CD19⁺ B-cell subsets related to total CD19⁺ cells. (C) Absolute numbers (cells/ μ L) of CD19⁺ cytokine-producing cells. (D) Percentages of CD19⁺ cytokine-producing cells related to total CD19⁺ cells. Median and 25%–75% interquartile range values are shown. ** $p < 0.01$, **** $p < 0.0001$. GM-CSF = granulocyte-macrophage colony-stimulating factor; IL = interleukin; MemB = memory B cell; PB = plasmablasts; TNF = tumor necrosis factor; TransB = transitional B cell.

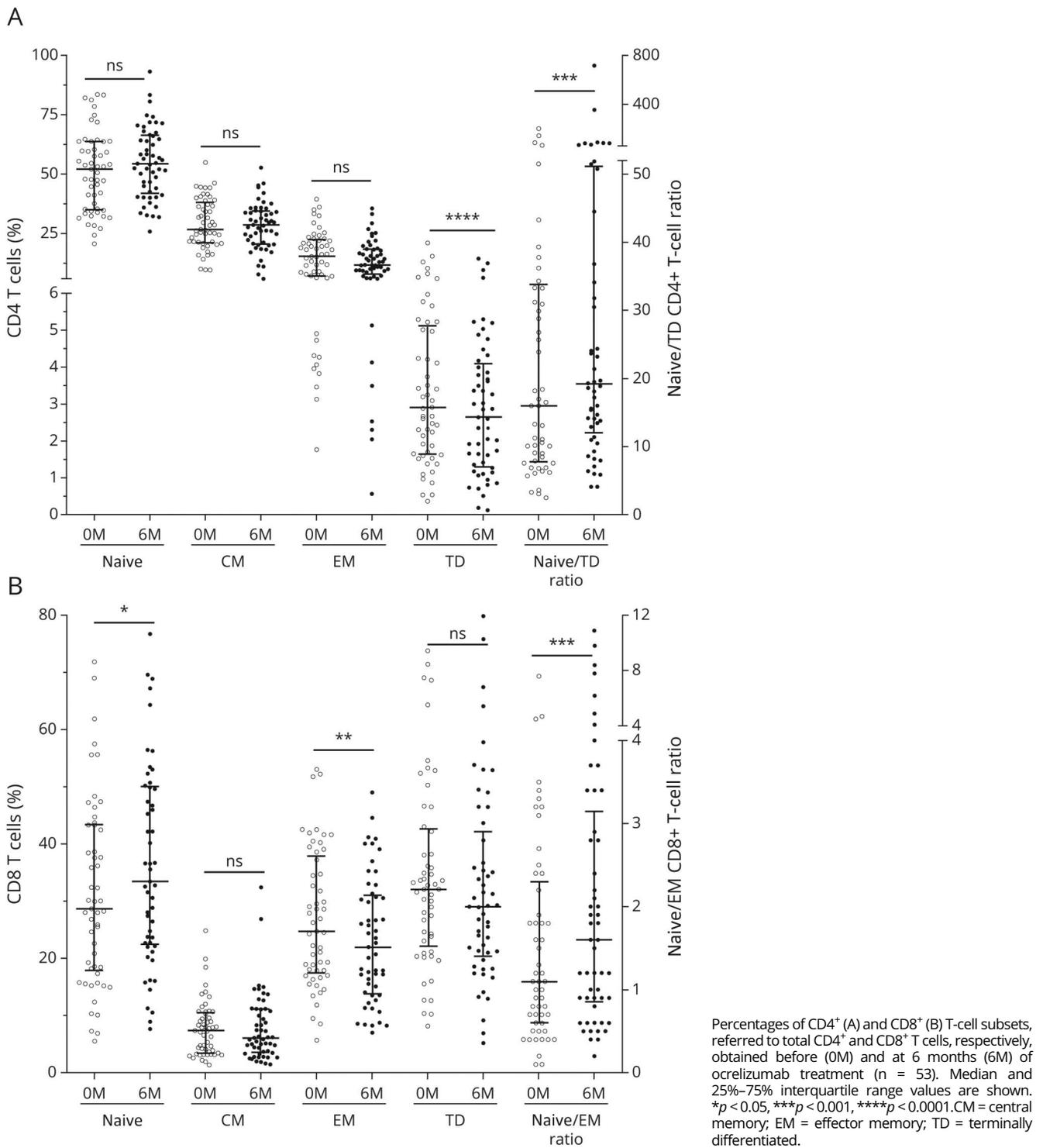
will be relevant because they are an important effector subset in MS¹⁹ being the effect of anti-CD20 antibodies on this B-cell subset questioned because of their low CD20 expression.²⁰ The only B-cell subset not experiencing a decrease at 6 months was transitional B cells. In fact, the proportion of these cells increased within the B-cell compartment, confirming that the B-cell repopulation is not affected by ocrelizumab¹⁸ because it was also observed on rituximab²¹ and fingolimod treatments.²² The proportion of plasmablasts also increased 6 months after ocrelizumab administration, suggesting a rapid B-cell differentiation to effector subsets.

We also observed a dramatic decrease in the numbers of B cells secreting TNF α , IL-6, IL-10, and GM-CSF. Moreover, there

was a relative decrease in the proportion of TNF α -producing cells and a relative increase of IL-10–producing cells in the B-cell compartment as reported for patients with RRMS.²³ However, there were also relative increases in GM-CSF- and IL-6-producing B cells, showing that some effector B cells can promptly arise after ocrelizumab treatment and strongly suggesting that anti-CD20 treatment does not reconstitute a fully healthy immune system or re-establish immune tolerance in all patients,²⁴ supporting the need for retreatment.

Anti-CD20 treatment also alters T-cell activation and cytokine production.⁸ We observed no significant changes in T-cell numbers after ocrelizumab treatment, with the exception of CD20⁺ T cells, which clearly decreased both in number

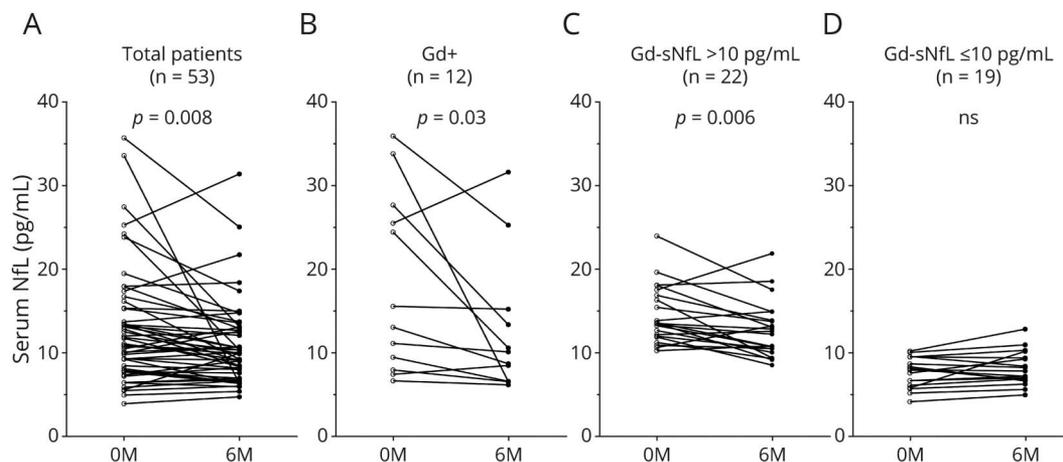
Figure 2 Changes in Blood T Cells Induced by Ocrelizumab Treatment



and percentages. This represents a unique cell population with a highly activated phenotype, proinflammatory and migratory properties, which has been proposed to play an important role in MS pathology.¹² Its downregulation may also be part of the beneficial effect of ocrelizumab in PPMS. Decreases in total CD20⁺ T-cell counts were also described for alemtuzumab, fingolimod, and dimethyl fumarate, but no reductions of the

proportions within the T-cell compartment were observed for these drugs.¹² Apart from this, ocrelizumab caused in the CD20⁻ T cells a decline of the proportion of effector T cells, an increase of CD8⁺ naive T cells, and of the ratio of naive/effector T cells. These data confirm that B-cell depletion induces a redistribution of the T-cell compartment, which favors naive vs effector cells.

Figure 3 Ocrelizumab Treatment Induces Changes in sNfL Levels



sNfL levels (pg/mL) obtained before (0M) and at 6 months (6M) of ocrelizumab treatment (n = 53). (A) All patients. (B) Patients showing gadolinium-enhanced lesions (Gd+) at baseline. (C) Patients not showing gadolinium-enhanced lesions (Gd-) with sNfL levels >10 pg/mL at baseline. (D) Patients not showing gadolinium-enhanced lesions (Gd-) with sNfL levels ≤10 pg/mL at baseline. sNfL = serum neurofilament light chain.

Ocrelizumab also shows an effect in cytokine-producing T cells. It induced decreases of CD4⁺ and CD8⁺ T cells producing IFN γ in our cohort. This decrease could be observed 6 months after ocrelizumab administration. The durable effect on IFN- γ -producing T cells can contribute to the clinical benefit of ocrelizumab in PPMS.

Moreover, we observed an increase in total numbers of monocytes expressing PD-L1, the ligand of the cell surface receptor PD-1, which promotes self-tolerance by suppressing T-cell inflammatory activity.²⁵ This could be important to modulate the abnormal response in MS.

By contrast, our data showed a decrease in the numbers, but not in percentages, of CD56 bright NK cells, thus suggesting that, opposite to that observed in response to other treatments in patients with RRMS,^{16,26,27} these cells do not play a role in the response to ocrelizumab treatment in PPMS.

Regarding serum Igs, ocrelizumab induced a decrease in serum IgM levels after treatment as previously described with rituximab with no changes in IgG and IgA values.

We finally explored changes in sNfL levels. Increasing data support that sNfL levels associate with disease activity and treatment response in patients with RRMS.²⁹ In this line, we observed a clear decrease of sNfL in patients showing Gd-enhanced lesions at baseline, but remarkably, it also significantly reduced the sNfL values in more than 50% of Gd- patients, who showed basal sNfL higher than 10 pg/mL, which suggests that these patients with PPMS still could have some inflammatory activity that can be modulated on ocrelizumab treatment.

Our data contribute to show the changes induced by ocrelizumab in blood leukocytes of patients with PPMS, indicating

that in addition to its impact on B cells, it can reshape the T-cell response toward a low inflammatory profile and induce a clear decrease in sNfL levels. These data should be confirmed in larger cohorts.

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Name	Location	Contribution
José I. Fernández-Velasco, BSci	Ramón y Cajal University Hospital, Madrid, Spain	Wrote the manuscript draft, stored blood samples, and performed the experiments
Jens Kuhle, MD, PhD	Basel University Hospital, Switzerland	Contributed to sNfL measurement and made a critical review of the manuscript
Enric Monreal, MD	Ramón y Cajal University Hospital, Madrid, Spain	Visited patients with MS, collected samples and clinical data, and made a critical review of the manuscript
Virginia Meca-Lallana, MD	La Princesa University Hospital, Madrid, Spain	Visited patients with MS, collected samples and clinical data, and made a critical review of the manuscript

Appendix (continued)

Name	Location	Contribution
José Meca-Lallana, MD, PhD	Virgen de la Arrixaca University Hospital, Murcia, Spain	Visited patients with MS, collected samples and clinical data, and made a critical review of the manuscript
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Paulette E. Walo-Delgado, MD	Ramón y Cajal University Hospital, Madrid, Spain	Stored blood samples, performed the experiments, and made a critical review of the manuscript
Aleksandra Maceski, PhD	Basel University Hospital, Switzerland	Contributed to sNfL measurement and made a critical review of the manuscript
Eulalia Rodríguez-Martín, PhD	Ramón y Cajal University Hospital, Madrid, Spain	Supervised flow cytometry studies and made a critical review of the manuscript
Ernesto Roldán, PhD	Ramón y Cajal University Hospital, Madrid, Spain	Supervised flow cytometry studies and made a critical review of the manuscript
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Appendix (continued)

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Luisa M. Villar, PhD	Ramón y Cajal University Hospital, Madrid, Spain	Designed and supervised the study and corrected the manuscript

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