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Making Minds Matter

Ocrelizumab associates with reduced cerebrospinal fluid B and CD20^{dim} CD4^{+} T cells in primary progressive multiple sclerosis

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Short title:

OCR reduces CSF B and CD20^{dim} T cells in PPMS

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Abstract

The anti-CD20 monoclonal antibody ocrelizumab reduces disability progression in primary progressive multiple sclerosis. CD20 is a prototypical B cell marker, however subpopulations of $CD4^+$ and $CD8^+$ T cells in peripheral blood and cerebrospinal fluid (CSF) also express low levels of CD20 ($CD20^{dim}$). Therefore, direct targeting and depletion of these $CD20^{dim}$ T-cell-subpopulations may contribute to the therapeutic effect of ocrelizumab. The aim of this observational cohort study was to compare $CD20^+$ B cell and $CD20^{dim}$ T cell distributions between peripheral blood and CSF of ocrelizumab-treated or untreated people with primary progressive multiple sclerosis. Ocrelizumab treatment was associated with depletion of circulating B cells and $CD20^{dim}$ $CD4^+$ and $CD20^{dim}$ $CD8^+$ T cells ($P<0.0001$, $P=0.0016$, and $P=0.0008$, respectively), but in CSF only with lower proportions of B cells and $CD20^{dim}$ memory $CD4^+$ T cells ($P<0.0001$ and $P=0.0043$, respectively). The proportional prevalence of CSF $CD20^{dim}$ memory $CD8^+$ T cells was not significantly reduced ($P=0.1333$). Only in CSF, the proportions of $CD20^{dim}$ cells within $CD4^+$ and not $CD8^+$ T cells positive for CCR5, CCR6 and CXCR3 were reduced in ocrelizumab-treated participants. The proportion of $CD20^{dim}$ $CD4^+$ T cells and abundance of $CD4^+$ relative to $CD8^+$ T cells in CSF correlated positively with age ($R=0.6799$, $P=0.0150$) and Age Related Multiple Sclerosis Severity Score ($R=0.8087$, $P=0.0014$), respectively. We conclude that, in contrast to CSF $CD20^{dim}$ $CD8^+$ T cells, B cells and $CD20^{dim}$ $CD4^+$ T cells are reduced in CSF of people with primary progressive multiple sclerosis with an ocrelizumab-associated depletion of circulating B cells and $CD20^{dim}$ T cells. Therefore, these cells are likely to contribute to the therapeutic effects of ocrelizumab in people with primary progressive multiple sclerosis.

Key words: primary progressive multiple sclerosis (PPMS), ocrelizumab, $CD20^{dim}$ T cell, B cell, cerebrospinal fluid (CSF)

Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS), in which T cells have a long-established pathogenic role. However, more recent studies on the effectiveness of CD20-targeting therapies in the reduction of disease activity in people with MS (pwMS) highlighted the role of B cells in MS pathogenesis.[1] Nowadays, the anti-CD20 monoclonal antibody ocrelizumab (OCR) is widely used as a highly effective disease modifying treatment (DMT) for relapsing remitting multiple sclerosis (RRMS) and is currently the only DMT that has shown an attenuation of disability progression in primary progressive multiple sclerosis (PPMS).[2-6]

CD20 is a membrane-spanning phosphoprotein strongly expressed on B cells and is widely regarded as a prototypical B cell-restricted marker.[7] However, small populations of $CD4^+$ and $CD8^+$ T cells have been shown to express CD20 at approximately 15 fold lower levels as well.[8] These $CD20^{dim}$ T cells represent a highly activated population with an increased capacity to produce pro-inflammatory cytokines.[8-11] Circulating $CD20^{dim}$ T cells are found to be expanded in several chronic inflammatory diseases.[12] In addition to this, CD20 is enriched on T cells isolated from non-diseased post-mortem human brain tissue, serving as a marker for a subset of CNS-homing T cells.[13,14] Accordingly, the relative numbers of $CD20^{dim}$ T cells are increased in the circulation and even more so in the cerebrospinal fluid (CSF) and white matter lesions of people with MS.[11,13-17] There is also evidence that $CD20^{dim}$ T cells are pathogenic in experimental autoimmune encephalomyelitis (EAE) mice and associated with disease activity in people with RRMS (pwRRMS) and disease severity in people with PPMS (pwPPMS).[15,18,19] Moreover, the depletion of circulating $CD20^{dim}$ T cells has been argued to contribute to OCR treatment efficacy.[19-24]

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Compared to rituximab,[25-27] the effects of OCR on lymphocytes in CSF of pwMS are less known. Interestingly, a recent study showed that the frequencies of circulating or CSF CD20^{dim} T cells were not affected in pwPPMS who received dimethylfumarate, a disease-modifying treatment commonly used for pwRRMS.[15] Since OCR did and dimethyl fumarate did not show significant effects in clinical trials for pwPPMS, divergent effects on the intrathecal lymphocyte composition could reveal important mechanisms in the modulation of PPMS.[5,28] Studying lymphocyte fractions in the CSF is especially relevant, since the pathology of PPMS has been argued to be more dependent on compartmentalized inflammatory and degenerative processes, and not on CNS recruitment of circulating lymphocytes.[29] Additionally, phenotypically distinct populations have been reported to patrol the intrathecal compartment in people with advanced progressive MS, and monoclonal antibodies as OCR do not cross the blood brain barrier (BBB).[30] The aim of our current study was to investigate the association of OCR therapy with both B cells and T cells and particularly CD20^{dim} subsets in the blood and CSF of pwPPMS.

Methods

Study design

We performed this research as a part of our longitudinal, prospective and observational cohort study "Study to Predict Inflammation and Neurodegeneration in PPMS" (SPIN-P). In this study, we included adults fulfilling the 2017 McDonald criteria for PPMS.[31] The only exclusion criterion was a life expectancy of 6 months or less. No other in- or exclusion criteria such as age, treatment, Expanded Disability Status Scale (EDSS) score or disease duration were used. PwPPMS were asked to voluntarily participate in this study by donating a blood and CSF sample at inclusion and at 1 year follow-up.[32] The study was approved by the medical ethics committee of the Erasmus Medical Center. All participants provided informed consent. The first participant was included on June 12th, 2020. For this research, pwPPMS who underwent a lumbar puncture (LP) when treated with OCR were matched with pwPPMS who did not receive OCR, based on age and sex. All included pwPPMS did not receive any other immune modifying therapy (IMT) in the at least three months prior to the LP.

Cell isolation and flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood according to the manufacturer's instructions with the use of vacutainer CPT® tubes containing sodium heparin (BD Biosciences, Erembodegem, Belgium). CSF of pwPPMS was obtained through LP. Cells from CSF were isolated by spinning down at 500g for 10 minutes. PBMC and CSF samples were taken on the same day and immediately used for phenotyping using conventional or spectral flow cytometry. Due to advancement in laboratory procedures during the course of our study, phenotyping data were acquired using both a BD LSRFortessa™ flow cytometer and a Cytek® Aurora™ spectral analyzer (5-laser; 355nm, 405nm, 488nm, 561nm and 640nm). Repeated samples were analyzed using the same machine and the comparability of both approaches was validated. We used a 13 and 37 color-based panel, respectively, with fluorochrome-labeled monoclonal anti-human antibodies as described in Supplementary Table 1 and Supplementary Table 2). The thirteen colour-based flow cytometry was performed using a variety of fluorochrome-labelled monoclonal anti-human antibodies (Supplementary Table 1) as described previously.[33] All cells were incubated with Human TruStain FcX Fc Receptor Blocking Solution (Biolegend) for 10 minutes at room temperature (RT) in the dark. All pre-titrated antibodies targeting chemokine receptors were added sequentially to the cells re-suspended in 1/5 BSB plus staining buffer (BD Biosciences) with a total volume of 50 µl. These were incubated ranging from 5-15 minutes at RT in the dark. After washing, all antibodies with fluorochromes peaking in the UV or violet chanel were added sequentially to the cells, which were incubated for 15 minutes at RT in the dark after the last addition. Lastly, after two washing cycles, a master mix of all remaining antibodies was added and incubated for 20 minutes at RT in the dark. Cells were then washed and re-suspended in 150 µl PBS+0,2% BSA for measurement with the Cytek® Aurora™ using

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SpectroFlo Software (V3.1.0). The cells were unmixed using single stained PBMCs from a healthy donor as reference controls and a fraction of unstained cells per tissue compartment of each MS donor. Analysis was performed using OMIQ software version 9.5.1 (from Dotmatics; https://www.omiq.ai/). Due to the switch in machines and fluorescent-labeled antibodies, we only compared percentages of positive cells and were not able to use mean fluorescence intensities. To ensure high quality of data, samples with <50 cell events within the analyzed gate were censored. Representative plots and the used gating strategy can be found in Supplementary Figure 1. Cells measured with spectral flow cytometry are represented by open dots in the graphs.

Clinical outcomes

The percentages of B cells and accompanying T-cell fractions in blood and CSF were correlated to age and Age Related Multiple Sclerosis Severity (ARMSS) score as parameters relevant for disease progression.[34]

Statistical analysis

Comparative analyses of differences in baseline characteristics between both groups were performed using the appropriate relevant statistical methods: Fisher's exact test or Mann-Whiney U test. Statistical analyses were performed using GraphPad Prism (version 9.0.0, San Diego, CA, USA) or SPSS (version 28.0.1.0, IBM SPSS Statistics); specific details are given in each figure legend. P-values of < 0.05 were considered significant.

Ethical statement

The studies involving human participants were reviewed and approved by the Medical Ethics Committee Erasmus MC (MEC-2014-033). The participants provided their written informed consent to participate in this study.

Results

Participants and CSF sampled

A total of 13 pwPPMS treated with OCR were matched to 13 untreated pwPPMS. One person in the OCR-treated group was excluded, since the LP was done 454 days after the last dose of OCR. Therefore, we were not able to consider this sample as an OCR-treated or untreated sample. Relevant clinical characteristics were comparable between the OCR-treated and untreated group. Particularly, these groups were similar regarding number of males, age at time of sampling, age at diagnosis, disease duration and EDSS at time of sampling (Table 1). Within one year prior to sampling, none of the OCR-treated pwPPMS and 2 untreated pwPPMS displayed inflammatory disease activity, defined as the presence of relapses, gadolinium enhancing lesions or the presence of a new lesion on follow-up MRI (0% versus 15.4%, respectively; P=0.260). Of the OCR-treated group, 6 pwPPMS (50%) had disease activity identifiable on MRI in the year before start of OCR, with 1 of these people also experiencing clinical relapses. People in the OCR-treated group received a median of 4.5 doses of OCR at time of sampling (range 2-5). Median number of days since the last dose of OCR at time sampling was 117 (range 14-189). Lastly, besides OCR, there were no substantial differences regarding IMT-use prior to the sample collection. In the OCR-treated group 4 pwPPMS (33.3%) had previously had pulse corticosteroids, with a median amount of 35.3 months since stop of this treatment before the sample collection for the current study (range 5.3-99.3 months) and 1 person (8.3%) had previously had teriflunomide, until 13.5 months before sample collection. In the untreated group, only 1 person (7.7%) had had prior IMT, namely pulse corticosteroids, until 2.9 months

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before the current sample collection. The clinical characteristics of included pwPPMS are summarized in Table 1.

CD4$^+$ and CD8$^+$ memory T cells expressing CD20, CCR5 and CXCR3 are enriched in the CSF of untreated pwPPMS

First, we analyzed the relative numbers of B cells and both CD4$^+$ and CD8$^+$ memory (CD45RA$^-$) T cells in paired PBMC and CSF samples from pwPPMS without OCR treatment. In particular, we zoomed in on CD45RA$^-$ memory T cells for the expression of CSF- and/or brain residency-associated T cell-markers CD20,[13] CCR5,[35] CXCR3 and CCR6,[35-38] as well as CCR4, a more skin-homing and T helper cell-defining marker.[39,40] We showed low prevalence of B cells and an enrichment of CD4$^+$ and CD8$^+$ CD45RA$^-$ memory T cells in PPMS CSF versus PBMCs (Figure 1A-B). Phenotypically, compared to PBMC fractions, these CSF memory T cells were characterized by a higher proportion of CD20$^+$ (Figure 1C), CCR5$^+$ (Figure 1D), and CXCR3$^+$ T cells (Figure 1E), yet with a similar abundance of CCR6$^+$ T cells (Figure 1F). CCR4 expression was more abundant on CSF CD8$^+$ CD45RA$^-$ memory T cells, yet lower on the CD4$^+$ fraction, compared to PBMCs (Figure 1G).

OCR treatment is associated with reduced frequencies of B cells and CD20^{dim} T cells in the PBMC fraction of pwPPMS

Next, we compared the distribution of B cells and CD4$^+$ and CD8$^+$ CD45RA$^-$ memory T cells in PBMCs of pwPPMS with versus without OCR treatment. As expected, PBMCs of OCR-treated individuals hardly contained B cells compared to untreated participants (Figure 2A-B). The frequencies of both total and CD45RA$^-$ memory CD4$^+$ and CD8$^+$ T cells, as well as their ratios were not different between the treated and untreated group (Figure 2 C-E). OCR-treated individuals did show lower proportions of CD20^{dim} cells within both the CD4$^+$ and CD8$^+$ CD45RA$^-$ memory T cell fractions (Figure 2F). No decrease was seen in CD20-negative T cells within the CD4$^+$ and CD8$^+$ CD45RA$^-$ memory PBMC compartments (Supplementary Figure 2), nor a significant correlation of CD20^{dim} T cell proportions with time since infusion (Supplementary Figure 3). This lower CD20^{dim} proportion coincided with lower proportions of CD8$^+$ and not so much CD4$^+$ CD45RA$^-$ memory T cells being CCR5$^+$ or CXCR3$^+$ (Figure 2G, H). Distribution of CCR6$^+$ and CCR4$^+$ fractions within CD4$^+$ and CD8$^+$ CD45RA$^-$ memory T cells was similar between both treatment groups (Figure 2I-J). In contrast to CCR6 and CCR4, CD20^{dim} T cells showed higher levels of CCR5 and CXCR3 (Figure 3A-B) compared to CD20-negative counterparts, which was in line with previous work.[15] Accordingly, we found a prominent loss of CD20^{dim} T cells within specifically the CXCR3$^+$ and CCR5$^+$ T-cell fractions in the OCR-treated group (Supplementary Figure 3). This shows that indeed the lowering of CXCR3$^+$ and CCR5$^+$ subsets within CD45RA$^-$ memory CD8$^+$ T cells correlates with a loss of CD20^{dim} cells in the treated group. These data indicate that in PBMCs of pwPPMS, OCR treatment profoundly depletes B cells as well as CD20^{dim} CD4$^+$ and CD8$^+$ T cells, and that this depletion contributes to an overall loss of CXCR3$^+$ and CCR5$^+$ T cells especially in the CD8$^+$ memory pool.

B-cell and CD20^{dim} CD4$^+$ memory T-cell fractions are significantly reduced in the CSF from OCR-treated pwPPMS

In the CSF of pwPPMS, OCR treatment was associated with a lower proportion of B cells (Figure 4A, B), but a similar distribution of total and memory T cells for both the CD4$^+$ and CD8$^+$ population (Figure 4C-E). OCR-treated pwPPMS had a lower proportion of CD20^{dim} cells within the CSF CD45RA$^-$ memory CD4$^+$ T-cell pool, which was not significantly lower within the CSF CD8$^+$ CD45RA$^-$ memory T cell pool (Figure

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4F). In contrast to PBMC (Figure 3), there was no association of OCR treatment with both total $CD4^+$ and $CD8^+$ memory T-cell proportions positive for CCR5 (Figure 4G) or CXCR3 (Figure 4H) in CSF. Also, the presence of $CCR6^+$ and $CCR4^+$ $CD45RA^-$ memory T cells in the CSF did not differ in the OCR treatment group (Figure 4I, J). However, $CCR5^+$, $CXCR3^+$, $CCR6^+$ and $CCR4^+$ cells within the $CD4^+$ $CD45RA^-$ memory T-cell pool of the CSF of OCR-treated pwPPMS were all depleted for $CD20^{dim}$ T cells, which was not the case for the $CD8^+$ memory T-cell pool (Supplementary Figure 4). These results suggest that for $CD4^+$ $CD45RA^-$ memory T cells, the presence of $CD20^{dim}$ fractions in the CSF of pwPPMS (Figure 1) is more closely associated with the proportions of its PBMC-counterparts and the depletion thereof, than for $CD8^+$ $CD45RA^-$ memory T cells.

The presence of $CD20^{dim}$ $CD4^+$ memory T cells in the CSF is associated with higher age and ARMSS score in treatment-naive pwPPMS

To explore clinical relevance of our findings, we correlated the proportions of PBMC and CSF B cells as well as $CD20^{dim}$ $CD4^+$ and $CD20^{dim}$ $CD8^+$ $CD45RA^-$ memory T cells to age and ARMSS score in untreated pwPPMS. Both a high age and disability score are important predictors of the development of progressive MS.[29] In accordance with earlier findings in healthy donors,[41] the percentage of CSF B cells negatively correlated with age, while the frequency of CSF $CD20^{dim}$ $CD4^+$ but not $CD8^+$ memory T cells correlated positively with age (Figure 5). In addition, both in PBMCs and CSF of untreated pwPPMS, a relative over-abundance of $CD4^+$ compared to $CD8^+$ $CD45RA^-$ memory T cells associated with a higher ARMSS score in. No significant correlations were found in OCR-treated participants (Supplementary Figure 5).

Discussion

Here, we showed that treatment of pwPPMS with OCR not only depletes both B cells and $CD20^{dim}$ T cells in the PBMC fraction, but is also associated with a significant reduction of total B cells and especially $CD4^+$ $CD20^{dim}$ $CD45RA^-$ memory T cells in the CSF. In sharp contrast to PBMC fraction, the proportional prevalence of $CD20^{dim}$ memory $CD8^+$ T cells and their brain-residency-associated chemokine receptor profile in the CSF was only marginally lower in OCR-treated pwPPMS. Since specifically the kinetics of CSF recruitment of B cells and $CD20^{dim}$ $CD4^+$ T cells are affected by OCR, we conclude that these cell types are more likely to contribute to the therapeutic effects of OCR in pwPPMS compared to $CD8^+$ T cells. The positive correlation of $CD20^{dim}$ $CD4^+$ and not $CD8^+$ memory T-cell presence with both a higher age and ARMSS score, two core hallmarks of progressive MS, supports the latter hypothesis.

The origin and function of $CD20^{dim}$ T cells have been a topic of debate. Although trogocytosis was recently shown as a mechanism for T cells to acquire CD20 in the context of EAE,[18] the expression of CD20 mRNA by human brain $CD4^+$ and $CD8^+$ T cells suggests this molecule to be part of the CNS-residency transcriptional program.[14] This program is also characterized by expression of CCR5 and CXCR3.[35,37,42,43] CXCR3 and its ligands CXCL9, CXCL10 and CXCL11 have previously been described in the context of MS and even as potential therapeutic targets.[44] Increased CXCR3 expression on $CD4^+$ lymphocytes in peripheral blood has been correlated with MS relapses.[45] Within MS lesions, lymphocytic cells express CXCR3 in nearly all perivascular inflammatory infiltrates.[35,46,47] It's ligand, CXCL9 was shown to act as a homing chemokine in microvascular endothelial cells and astrocytes from the human brain, while CXCL10 and CXCL11 are induced in response to inflammatory stimuli.[48] Moreover, CXCL10 has shown a significant correlation with the expression of CXCR3 on CSF $CD4^+$ T cells.[37,49] In pwRRMS a higher concentration of CXCL10 in the CSF has been reported.[50] Lastly, $CD8^+$ $CD20^{dim}$ T cells in the CSF also express higher levels of CCR6. This might suggest that these T cells travel between CSF and brain parenchyma rather than from the peripheral blood compartment to CSF, since CCR6 expression has previously been associated with the transmigration across the choroid plexus.[38] The precise role of these

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chemokines and their receptors on the exact kinetics and timing of recruitment of T cells into the CNS of pwPPMS remains to be elucidated.

The disease process of progressive MS is not fully understood, but is characterized by compartmentalized inflammation and loss of axons behind a closed BBB.[29] Despite a plethora of therapies for RRMS, no efficacious treatment for PPMS was available until the seminal phase 3 trial with OCR.[3] This trial drew a focus on the B cell as a contributor to progressive MS. Indeed, in advanced MS, we and others showed B cell infiltrates and intrathecal antibody production to remain a hallmark of MS pathobiology.[51] A reduction of PBMC B cells and CD20^{dim} T cells has been demonstrated in OCR-treated people with RRMS and PPMS,[21-23,52] yet effects of this therapy on intrathecal cell populations is most relevant in the context of compartmentalized progressive MS. Interaction of lymphocyte populations in the perivascular space and meninges has been suggested to rather drive lesion expansion and associated disability progression.[53] Similar to the CSF T cells in the current study, perivascular CD4$^+$ and CD8$^+$ T cells express tissue residency-associated programs and phenotypic markers, including CXCR3, and CCR5.[14,54,55] Interestingly, for CD4$^+$ T cells, these programs share a substantial overlap with phenotypic markers of peripheral helper T cells prone to interact with B cell populations locally at sites of inflammation, including expression of CCR2, CCR5 and PD-1.[56] These specific T cell populations could be instrumental in the association of local antibody secreting cell formation with a higher local CD4$^+$ / CD8$^+$ T cell ratio in the context of MS white matter lesions.[51] Although CD8$^+$ T cells are most prevalent in MS white matter lesions and even infiltrate the parenchyma, their effector profile in advanced MS remains uncertain.[14,54] As recently suggested by Ostkamp et al.[57], trafficking of CD8$^+$ T cells with tissue resident memory T cell characteristics between perivascular space and CSF could be a dynamic process within the borders of the CSF. Alternatively, swiftness of CSF-repopulation after CD20-treatment by peripheral CD20^{dim} cells might differ between CD4$^+$ and CD8$^+$ T cells. Nevertheless, our findings show that, in contrast to CD4$^+$ cells, a profound depletion of circulating CD20^{dim} CD8$^+$ T cells does not affect the phenotypic composition of CD8$^+$ T cells within the CSF for the markers investigated. This does not exclude a role of CNS resident CD8$^+$ T cells in PPMS,[58] yet does not support a clear association of this subset with the therapeutic effects of ocrelizumab in PPMS.

A higher age and higher disability score are two extensively consolidated predictors of progressive disease.[29] Therefore, the effect of age and disability on underlying immunological mechanisms is relevant to understand the immunological nature of progressive disease. The negative correlation between the percentage of B cells in the CSF and age that we found in pwPPMS is likely a consequence of the significant decrease of the number and percentages of B cells with age, which has been extensively described in previous studies.[59-63] Our study also supports previous observations that the percentage of CD20^{dim} T cells increases with age,[64] and reports an age-associated expansion of specifically the CD20^{dim} CD4$^+$ and not CD8$^+$ CD20^{dim} memory T cells. In this line, the association of a relative abundance of CSF CD4$^+$ compared to CD8$^+$ memory T cells with a higher ARMSS score – a powerful method for measuring relative severity of disability in MS – [34] provides further support to our hypothesis that this expansion could be a contributor to progressive disease.

In the context of our study, it is important to acknowledge certain limitations. First, we were able to analyze a relatively limited number of participants with PPMS. Second, the presence of OCR in the circulation of treated pwPPMS could possibly mask the CD20 epitope recognized by the 2H7 clone that we used for cytometric detection in this study.[19] However, Shinoda et al.[19] showed a similar reduction of cells comparing stainings with the anti-CD20 2H7-clone and an intracellular stained CD20 (clone: 1412) after treatment with OCR, indicating that these cells were genuinely depleted rather than merely masked in detection. Additionally, therapeutic antibodies such as OCR induce antibody-dependent cellular and complement-dependent cytotoxicity rapidly, and have a terminal half-life of 28 days.[65,66] Combined with the long time between OCR-infusion and blood sampling, a reduction of CD20-positive B and T cells is most likely. Moreover, there is evidence suggesting OCR penetrates the CSF poorly, as has been shown for

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the anti-CD20 monoclonal antibody rituximab,[67] which makes covering of the CD20 epitope intrathecally unlikely. Third, an indirect effect of OCR on circulating $CD20^{dim}$ T cells via B cell depletion cannot be excluded. B cell depletion was found to change the immune cell profile in MS.[68] Therefore, the depletion of B cells might have a significant effect on the T-cell compartment. Although we cannot exclude this mechanism, no reductions of CD20-negative T cells were observed.

In conclusion, the use of OCR therapy associates with reduction of B cells but also a decreased presence of particularly $CD4^+$ $CD20^{dim}$ T cells in the CSF of pwPPMS. Given that $CD20^{dim}$ T cells display characteristics linked to CNS infiltration, their depletion from the PBMC fraction could potentially play a role as mediators of the effectiveness of OCR treatment for people with PPMS. Lastly, a higher $CD4^+/CD8^+$ memory T cell-ratio associated with a higher ARMSS score in blood and CSF but not in OCR-treated individuals, further supporting the putative benefit of preventing the accumulation of $CD20^{dim}$ $CD4^+$ memory T cells into the CSF through life even of people with the progressive form of MS by treatments such as OCR.

Data Availability Statement

The data presented in this study are available upon reasonable request.

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Competing interests

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Figure 1: Relative frequencies and brain-homing phenotypes of CD4$^+$ and CD8$^+$ T cells in paired PBMC and CSF of untreated pwPPMS. This figure shows untreated people with primary progressive multiple sclerosis (pwPPMS) (n=13). (A) Percentages of B cells within total lymphocyte population in PBMC and CSF. (B) Percentages of memory (CD45RA$^-$) cells within the total CD4$^+$ (left) or CD8$^+$ (right) T-cell pool in PBMC and CSF. (C-G) Representative dotplots and percentages of CD20, CCR5, CXCR3, CCR6 and CCR4 within total CD4$^+$/CD8$^+$ memory T cells of a pwPPMS. Statistical significance was tested using Wilcoxon tests. P-values of < 0.05 were considered significant. Data acquired through traditional flow cytometry is denoted by solid dots (UNTX; n= 10, TX; n=8), while data obtained via spectral flow cytometry is indicated by open dots (UNTX; n= 3, TX; n=4). Due to changes in measured markers overtime the amount of dots may differ per graph.
Abbreviations: PBMC = Peripheral Blood Mononuclear Cell; CSF = cerebrospinal fluid; SSC-A = sideward scatter area

Figure 2: Frequencies of B cells and T cell subsets within the PBMC-fraction of untreated and OCR-treated pwPPMS. (A) Representative dotplots showing CD20 vs. CD3 in PBMCs of an untreated (top) and OCR-treated (bottom) pwPPMS. (B) Violin plot showing the percentage of B cells of total lymphocytes in age- and gender-matched untreated (UNTX; n=13) and OCR treated (TX; n=12) people with primary progressive multiple sclerosis (pwPPMS). (C) Violin plot showing the percentage of CD4$^+$ (top) and CD8$^+$ (bottom) of total T cells (D) and showing the CD4$^+$/CD8$^+$ ratio. (E) Violin plot showing the percentage of CD4$^+$ and CD8$^+$ memory (CD45RA$^-$) T cells within total lymphocytes. (F-I) Violin plot showing the percentage of CD20^{dim}, CCR5$^+$, CXCR3$^+$, CCR6$^+$ and CCR4$^+$ subsets within CD4$^+$ (left) and CD8$^+$ (right) memory T cells. Statistical significance was tested using Mann-Whitney U tests. P-values of < 0.05 were considered significant. Each violin plot shows median and quartiles through dotted lines. Data acquired through traditional flow cytometry is denoted by solid dots (UNTX; n= 10, TX; n=8), while data obtained via spectral flow cytometry is indicated by open dots (UNTX; n= 3, TX; n=4). Due to changes in measured markers overtime the amount of dots may differ per graph.
Abbreviations: PBMC = Peripheral Blood Mononuclear Cell; OCR = ocrelizumab; UNTX = untreated, TX = treated

Figure 3: Expression of brain-homing markers on paired CD20^{dim} versus CD20^{neg} subsets within memory T cells of untreated pwPPMS. Samples with frequencies of CD20^{neg} and CD20^{dim} CD4$^+$ (left panel) or CD8$^+$ (right panel) memory (CD45RA$^-$) T cells expressing CCR5 (A), CXCR3 (B), CCR4 (C) and CCR6 (D) in PBMC and CSF from untreated pwPPMS (n=13). Statistical significance was tested using Wilcoxon tests. P-values of < 0.05 were considered significant. Data acquired through traditional flow cytometry is denoted by solid dots dots (UNTX; n= 10), while data obtained via spectral flow cytometry is indicated by open dots (UNTX; n= 3). Due to changes in measured markers overtime the amount of dots may differ per graph.
Abbreviations: PBMC = Peripheral Blood Mononuclear Cell; CSF = cerebrospinal fluid; neg = CD20^{neg}; dim = CD20^{dim}

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Figure 4: Frequencies of B cells and T-cell subsets with a brain-homing phenotype in the CSF of pwPPMS with and without OCR treatment. (A) Representative dotplots showing CD20 vs. CD3 in CSF of an untreated (top) and OCR-treated (bottom) pwPPMS. (B) Violin plot showing the percentage of B cells of total lymphocytes in age- and gender-matched untreated (UNTX; n=13) and OCR-treated (TX; n=12) pwPPMS. (C) Violin plot showing the percentage of $CD4^+$ (top) and $CD8^+$ (bottom) of total T cells. (D) and showing the $CD4^+/CD8^+$ ratio. (E) Violin plot showing the percentage of $CD4^+$ and $CD8^+$ memory ($CD45RA^-$) T cells within total lymphocytes. (F-I) Violin plot showing the percentage of $CD20^{dim}$, $CCR5^+$, $CXCR3^+$, $CCR6^+$ and $CCR4^+$ subsets within $CD4^+$ (left) and $CD8^+$ (right) memory T cells. Statistical significance was tested using Mann-Whitney tests. P-values of < 0.05 were considered significant. Each violin plot shows median and quartiles through dotted lines. Data acquired through traditional flow cytometry is denoted by solid dots (UNTX; n= 10, TX; n=8), while data obtained via spectral flow cytometry is indicated by open dots (UNTX; n= 3, TX; n=4). Due to changes in measured markers overtime the amount of dots may differ per graph.
Abbreviations: CSF = cerebrospinal fluid; OCR = ocrelizumab; UNTX = untreated, TX = treated

Figure 5: Correlations between the presence of B cells and $CD20^{dim}$ T-cell subsets with age and ARMSS score in untreated pwPPMS. The percentages of B cells within total lymphocytes, $CD4^+/CD8^+$ memory ($CD45RA^-$) T-cell ratios as well as $CD20^{dim}$ cells within the $CD4^+$ and $CD8^+$ memory ($CD45RA^-$) T cell pool in both PBMC (left) and CSF (right) were associated with age (years) and Age Related Multiple Sclerosis Severity (ARMSS) scores of untreated people with primary progressive multiple sclerosis (n=13). Statistical significance was tested using Pearson r tests. P-values of < 0.05 were considered significant and are indicated using a bold font. Data acquired through traditional flow cytometry is denoted by solid dots (UNTX; n= 10), while data obtained via spectral flow cytometry is indicated by open dots (UNTX; n= 3). Due to changes in measured markers overtime the amount of dots may differ per graph.
Abbreviations: PBMC = Peripheral Blood Mononuclear Cell; CSF = cerebrospinal fluid; ARMSS score = Age Related Multiple Sclerosis Severity score

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	Untreated (n=13)	OCR-treated (n=12)	P
Male; n (%)	4 (30.8)	5 (41.7)	P=0.440[A]
Age at sample (years); median (range)	50.0 (43.2-61.4)	57.6 (37.2-62.0)	P=0.320[B]
Age at diagnosis (years); median (range)	47.6 (39.2-56.2)	50.0 (36.1-59.5)	P=0.852[B]
Disease duration (years, since symptom onset); median (range)	7.1 (2.0-13.2)	8.1 (2.7-14.1)	P=0.295[B]
EDSS at sample; median (range)	4.0 (2.0-6.5)	4.25 (3.5-7.5)	P=0.406[B]
Prior IMT; n (%)			
- None	12 (92.3%)	7 (57.8%)	P=0.073[A]
- Pulse corticosteroids	1 (7.7%)	4 (33.3%)	
- Months since stop of prior IMT; median (range)	2.9	35.3 (5.3-99.3)	
- Teriflunomide	0	1 (8.3%)	
- Months since stop of prior IMT; median (range)	N.A.	13.5	
Disease activity in year before sample*; n (%)	2 (15.4%)	0 (0%)	P=0.260[A]
- MRI; n (%)	2 (15.4%)	0 (0%)	
- Relapses; n (%)	0 (0%)	0 (0%)	
Disease activity in year before start of OCR*; n (%)	N.A	6 (50%)	
- MRI; n (%)		6 (50%)	
- Relapses; n (%)		1 (8.3%)	
Doses of OCR at sample; median (range)	N.A.	4.5 (2-5)	
Days since last dose of OCR at sample; median (range)	N.A.	117 (14-189)	

[A] Fisher's exact; [B] Mann-Whitney U. *Disease activity defined as the presence of relapses, gadolinium enhancing lesions or the presence of a new lesion on follow-up MRI within one year. In cases where no MRI within the year before sample or before start of ocrelizumab was performed, we scored the disease activity to be absent. Abbreviations: OCR = ocrelizumab; IMT = immunomodulating treatment; EDSS = Expanded Disability Status Scale; MRI = Magnetic Resonance Imaging; N.A. = not applicable

Table 1. Baseline characteristics

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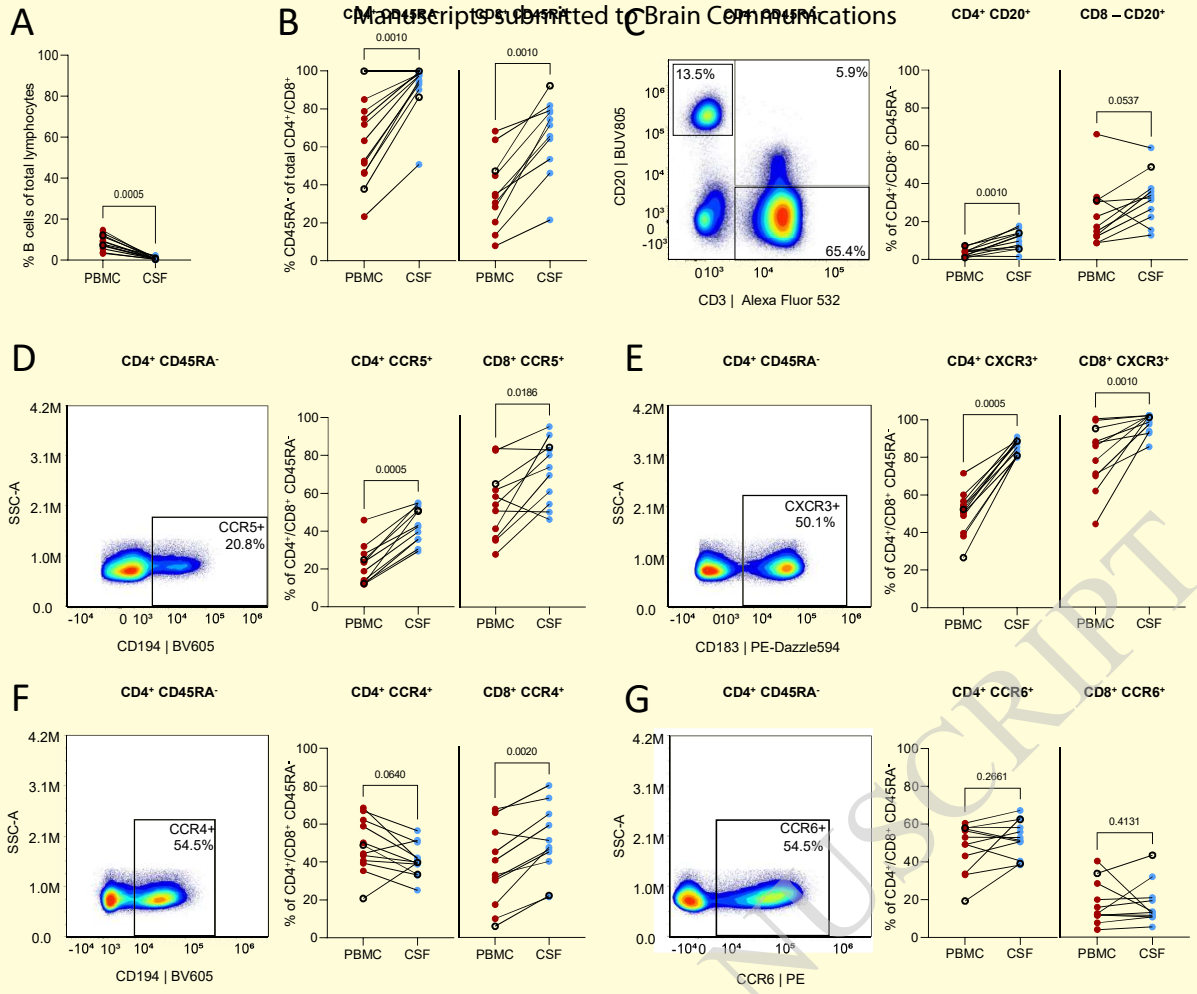

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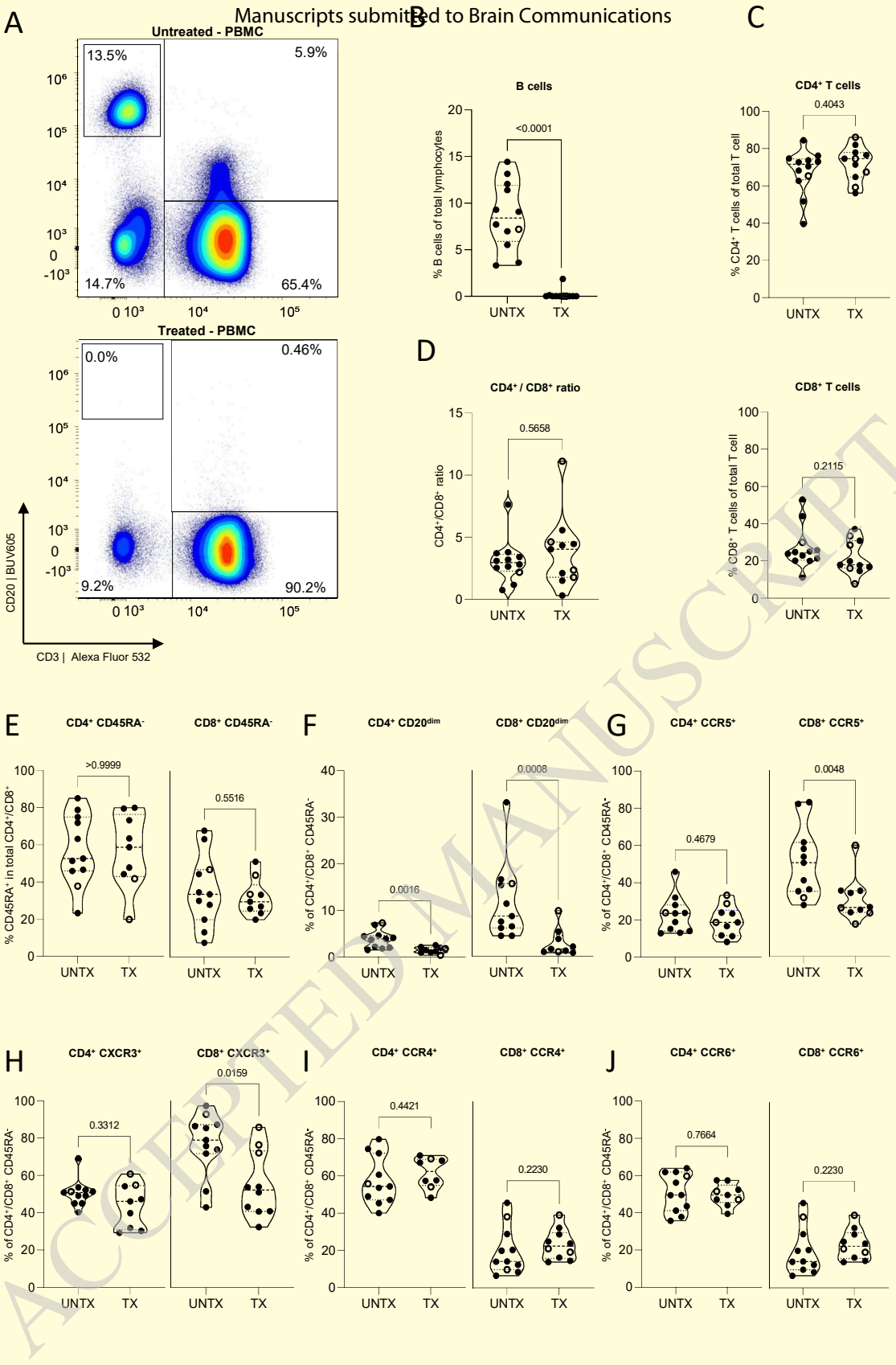

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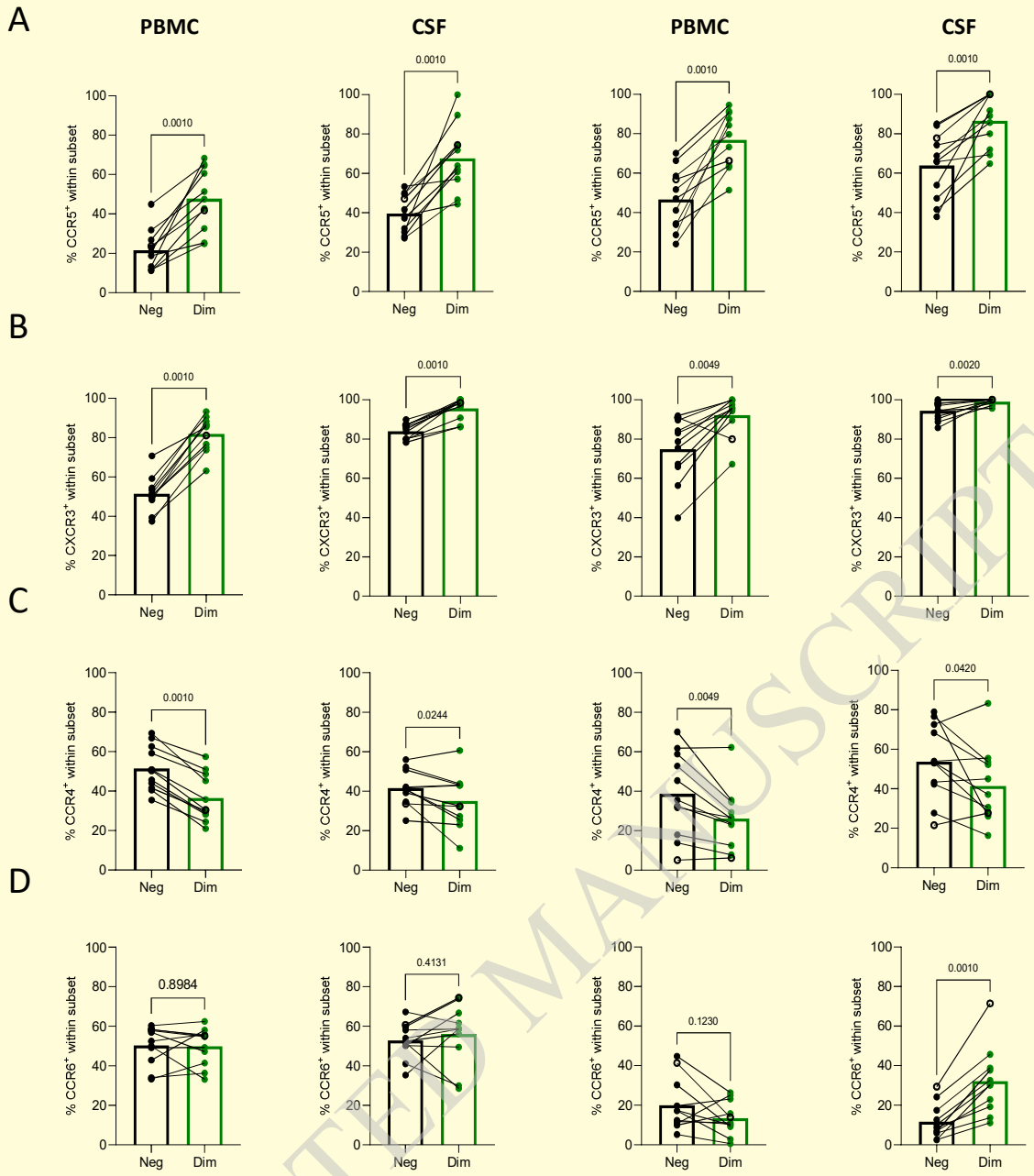

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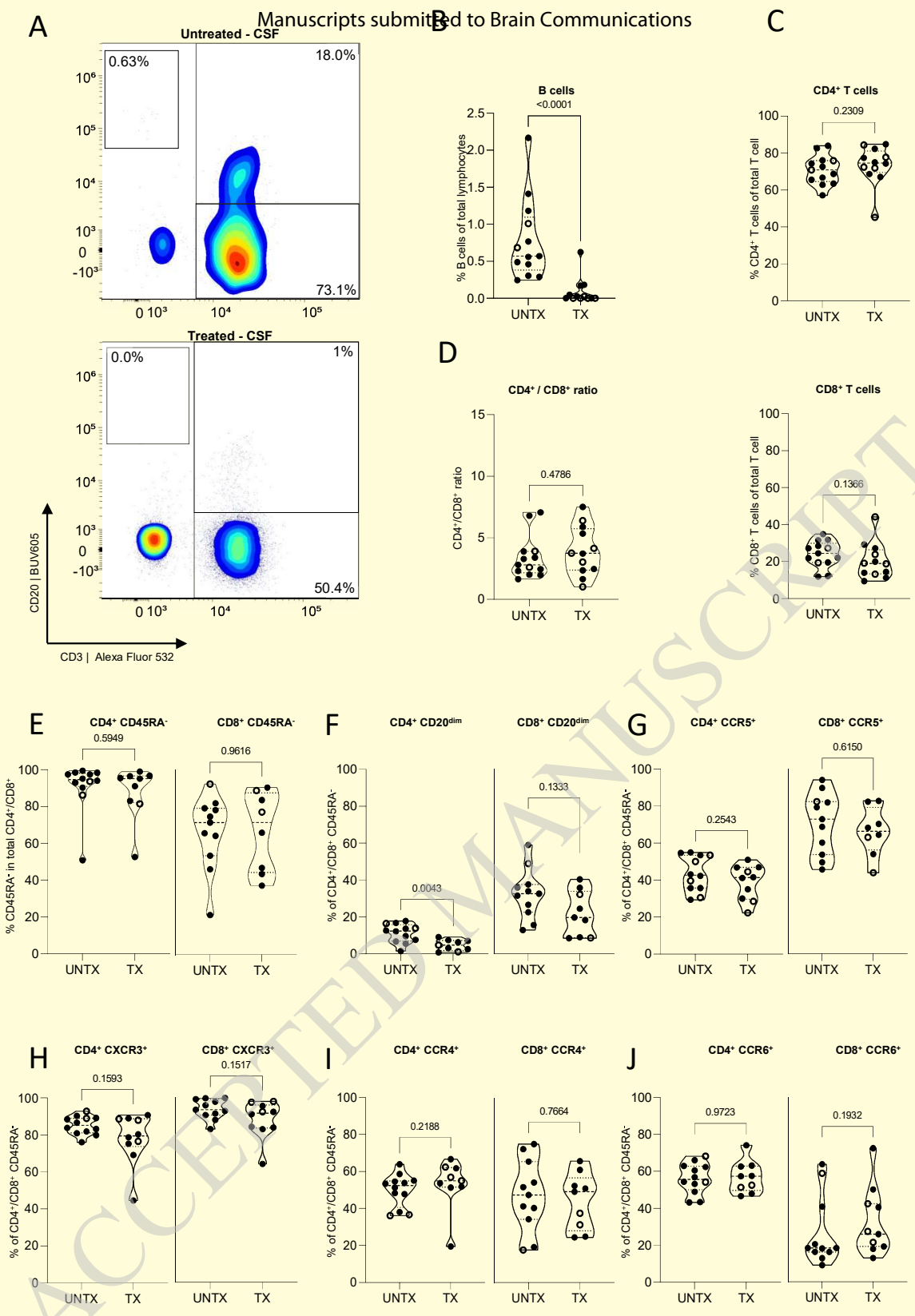

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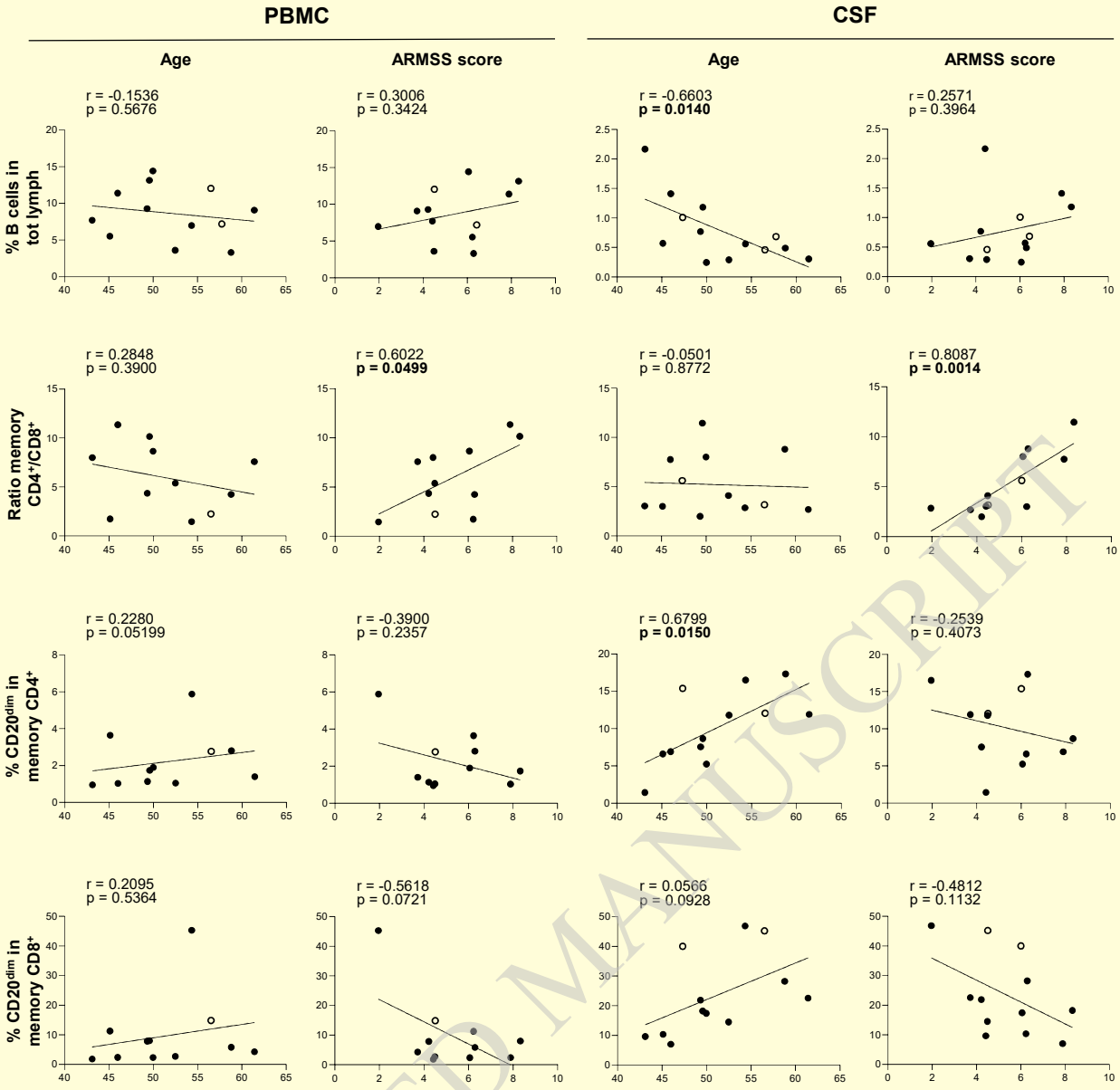

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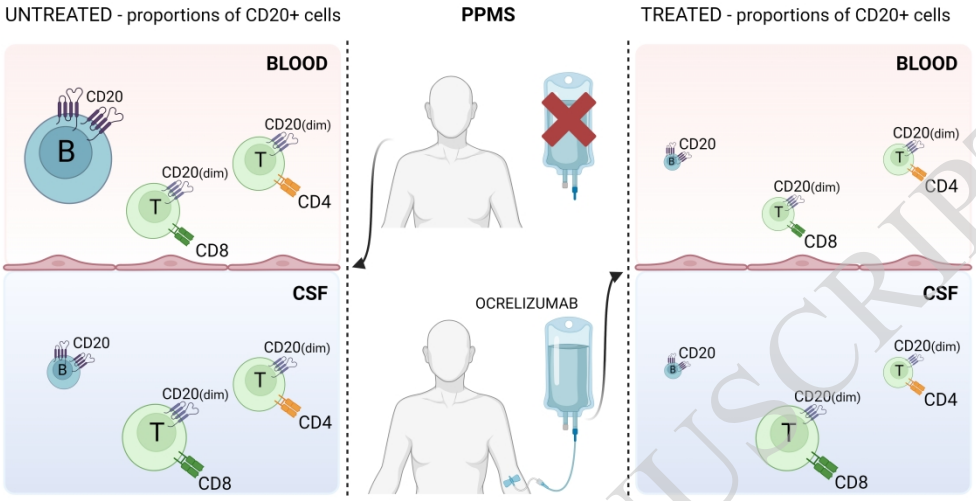

Graphical abstract

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