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BRIEF RESEARCH REPORT
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Mass Cytometry Identifies Expansion of T-bet$^+$ B Cells and CD206$^+$ Monocytes in Early Multiple Sclerosis

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Multiple sclerosis (MS) is an immune-driven demyelinating disease of the central nervous system. Immune cell features are particularly promising as predictive biomarkers due to their central role in the pathogenesis but also as drug targets, even if nowadays, they have no impact in clinical practice. Recently, high-resolution approaches, such as mass cytometry (CyTOF), helped to better understand the diversity and functions of the immune system. In this study, we performed an exploratory analysis of blood immune response profiles in healthy controls and MS patients sampled at their first neurological relapse, using two large CyTOF panels including 62 markers exploring myeloid and lymphoid cells. An increased abundance of both a T-bet-expressing B cell subset and a CD206$^+$ classical monocyte subset was detected in the blood of early MS patients. Moreover, T-bet-expressing B cells tended to be enriched in aggressive MS patients. This study provides new insights into understanding the pathophysiology of MS and the identification of immunological biomarkers. Further studies will be required to validate these results and to determine the exact role of the identified clusters in neuroinflammation.

Keywords: biomarker, multiple sclerosis, mass cytometry, immunology, B cells, monocytes

INTRODUCTION

Multiple sclerosis (MS) is a frequent chronic inflammatory demyelinating disease of the central nervous system (CNS) and is a leading cause of nontraumatic disability in young adults. Although its precise etiology remains to be identified, immune cells have been proposed as one of the main players in MS, especially during the early phase of the disease (1). Indeed, immunotherapies targeting lymphocytes and monocytes have a beneficial effect on MS disease activity. Besides T cells,

that are well-known as major players in MS, B cells have been recently pointed out for their diverse pathogenic effects: as pro inflammatory cytokine- and antibody-secreting cells, but also as antigen-presenting cells (2). These cells have also probably an important role in the progressive form of MS by contributing to the formation of tertiary ectopic lymphoid follicles inside the CNS (3, 4). Besides the adaptive immune system, the innate arm of the immune system (including monocytes, macrophages, dendritic cells, and microglia) has been investigated in mouse models of MS and was shown to be involved in MS pathogenesis. In particular, activated microglia and macrophages are the main cells described in MS active lesions (5) and myeloid cells act as antigen-presenting cells and effector cells in the context of neuroinflammation (6).

Immune cell features are thus particularly promising from a biomarker perspective due to their central role in MS pathogenesis but also as drug targets. Although the phenotypic and functional analysis of immune cells is an appealing strategy for understanding immune-mediated disease processes, immune cell profiling has currently no impact in clinical practice. Recently, high-resolution approaches, such as mass cytometry (CyTOF), helped to better understand diversity and function of the immune system and to highlight potential targets for novel therapies. CyTOF, combined with high-dimensional analysis, is a robust method to identify numerous and poorly-described cell subsets from heterogeneous populations, including in the autoimmune context (7, 8).

In this study, we deeply analyzed peripheral blood mononuclear cell (PBMCs) samples from drug-naïve early MS patients compared to age and sex-paired controls. We herein describe an increased abundance of a T bet-expressing B cell subset and a $CD206^{+}$ classical monocyte subset in the blood of MS patients opening new immunological pathways to investigate.

MATERIAL AND METHODS

Cohort

This study was registered and approved by the Ethics Committee of Rennes Hospital (notice n° 20.05) and was registered in clinicaltrials.gov (NCT04510350). All participants provided written informed consent. Blood samples were obtained from 11 early MS patients and 8 age and sex-paired healthy controls (HC).

MS patients included in this work participated to the OFSEP (Observatoire Français de la Sclérose en Plaques) cohort. Participating centers were Rennes, Lille, Nancy, Nimes and Bordeaux.

Inclusion criteria were: i) age > 18 years old, ii) MS diagnosis according to McDonald 2017 criteria at the last visit (9), iii) sampled during their first neurological episode, and iv) with at least one visit/year during the follow-up. Progressive MS patients were excluded. At time of PBMC sampling, all MS patients included were drug-naïve and so had never been treated by a disease modifying therapy (DMT).

Patients were classified into two groups according to the severity of the disease. "Aggressive MS" patients were defined as displaying a disease activity under disease modifying therapy (DMT) (minimum 6 months of treatment), or as having experienced two or more relapses within one year with residual disability associated with radiological activity (defined by the occurrence of at least one new T2 lesion or at least one gado-enhanced lesion on MRI). "Non-aggressive MS" patients were defined as having no disease activity during the follow-up, or as having disease activity without fulfilling the criteria for "aggressive MS". Disease activity was defined by the occurrence of at least one relapse and/or the occurrence of radiological activity.

PBMCs were collected and frozen in each center during the first neurological episode before transfer to Rennes Hospital for analysis. Frozen PBMCs from healthy controls were obtained from a biobanking from Rennes Hospital (NCT03744351).

Mass Cytometry

Antibodies (**Supplementary Table 1** for Lymphoid panel and **Supplementary Table 2** for Myeloid panel) were purchased either already metal-tagged (Fluidigm) or in purified form. For the last ones, they were labeled using the Maxpar Antibody Labeling Kit (Fluidigm), titrated, and stored at 4°C in Ab-stabilization buffer (Candor Bioscience). Cell labeling was performed as previously described (10). Briefly, cells were stained 5 minutes in RPMI supplemented with 0,5 µM Cisplatin Cell-ID™ (Fluidigm, San Francisco, CA) in RPMI 1640 before washing with 10% FBS in RPMI 1640. Cell were resuspended in 80µl of 0,5% BSA in PBS. Then, 60µl of each surface staining cocktail (lymphoid or myeloid) were added to 40µl of resuspended cells. After staining, cells were washed in 0,5% BSA in PBS before fixation and permeabilization with the transcription factor staining buffer set (Miltenyi, Bergisch-Gladbach, Germany). Then 60µl of each intracellular staining cocktail (lymphoid or myeloid), were added to 40µl of resuspended cells in Perm Buffer. After intracellular staining, cells were washed twice before staining in DNA intercalator solution (2.5% Paraformaldehyde, 1:3200 Cell-ID™ Intercalator-Ir (Fluidigm, San Francisco, CA) in PBS). Samples were cryopreserved at -80°C until acquisition on Helios™ System (Fluidigm, San Francisco, CA).

Data Analysis

The experimental design of the study is summarized in **Figure 1**.

After acquisition, intrafile signal drift was normalized and.fcs files were obtained using CyTOF software. To diminish batch effects, all files were normalized on EQ Beads (Fluidigm Sciences) using the premessa R package (https://github.com/ParkerICI/premessa). Files were then uploaded to the Cytobank cloud-based platform (Cytobank, Inc.). For all files, live single cells were selected by applying a gate on DNA1 vs. DNA2 followed by a gate on DNA1 vs. Cisplatin, then beads were removed by applying a gate on the beads channel (Ce140Di) vs. DNA1.

Each file was first analyzed using viSNE, based upon the Barnes–Hut implementation of t-SNE. The analysis was performed based on the event count of the file or on the maximum total events allowed by Cytobank. The following parameters were used: perplexity = 30; iterations = 5000;

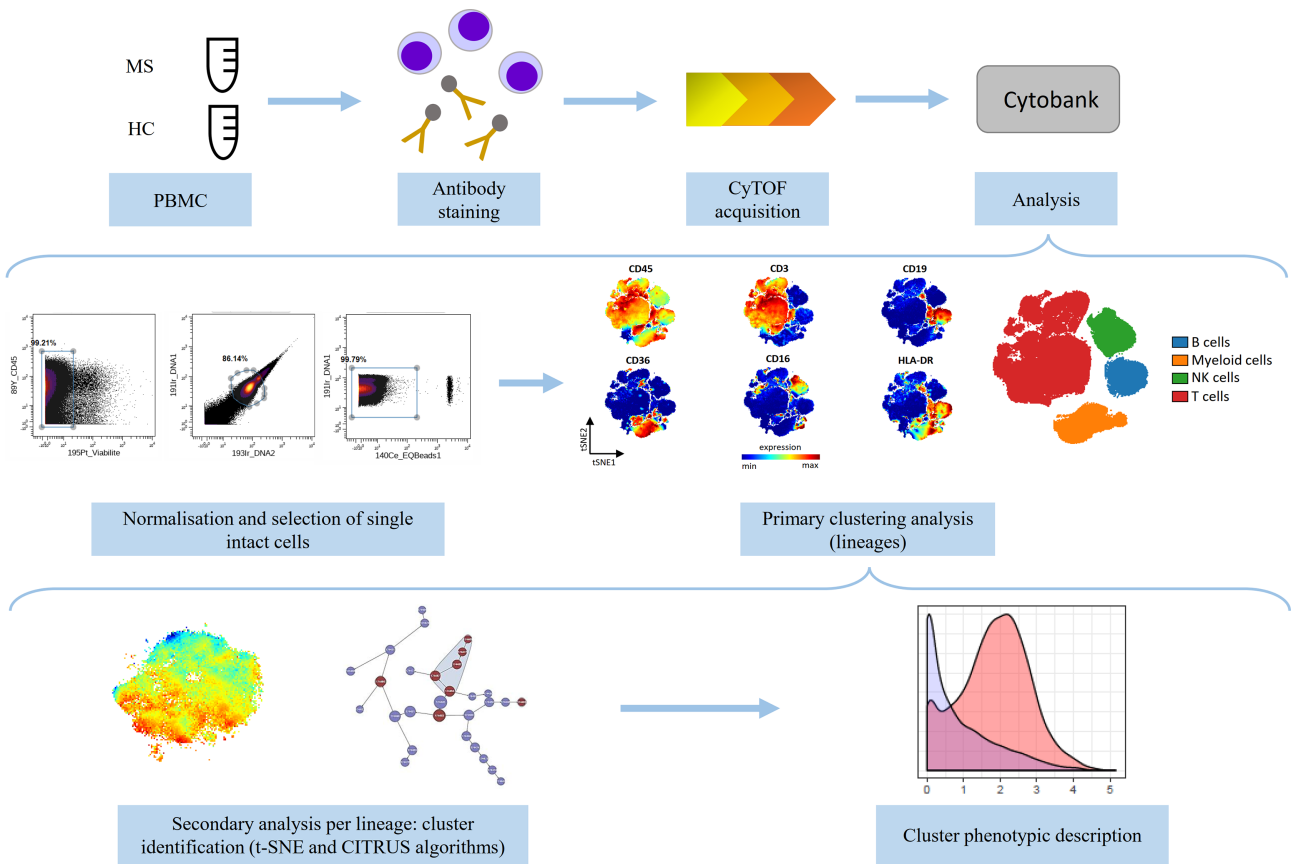


FIGURE 1 | Schematic representation of the experimental design of the study. Peripheral blood mononuclear cells (PBMCs) from multiple sclerosis (MS, n=11) patients and healthy controls (HC, n=8) were divided into two equal parts and stained with two antibody panels (Lymphoid panel and Myeloid panel, **Supplementary Tables 1**, **2**) and acquired on the CyTOF instrument. The data were normalized and single intact cells were manually selected. Primary analysis was performed to identify lineage cell subsets and secondary analysis was performed to describe phenotypic alterations per lineage between MS and HC (using t-SNE and CITRUS algorithms). Phenotypic description of the identified clusters was performed.

theta = 0.45. For the lymphoid panel, all 36 channels were selected and for the myeloid panel, all 33 channels were selected. The two panels included CD45, CD3, CD19, CD16, CD36, and HLA-DR, allowing selection of B-, T-, NK-, and myeloid cells. Then, we performed a t-SNE algorithm per lineage, to compare immune profile of MS patients and HC. Equal downsampling was performed, based on the lowest event count in all files. The following parameters were used: perplexity = 30; iterations = 5000; theta = 0.45.

We then applied a clustering method using the CITRUS algorithm. CITRUS proceeds through an unsupervised hierarchical clustering to identify clusters of cellular populations within the overall dataset, and then calculate biologically relevant features of these clustered populations. We performed the CITRUS algorithm on the previous viSNE results for each lineage, using all panel channels, and the following parameters: association model = SAM (Significance Analysis of Microarrays); mode of cluster characterization = abundance; event sampling = equal; minimum cluster size = 5%; FDR (False Discovery Rate) = 1%.

Finally, phenotypic description of identified clusters was performed.

Data generated during this study can be shared upon reasonable request.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism 8.4.2 Software. For comparisons, normality tests were first performed, and then Student's t test or Mann Whitney test were used when appropriate. A p value < 0.05 was considered statistically significant.

RESULTS

Patients

Analyses were performed on PBMC samples isolated from 11 MS patients and 8 HC. The demographic, clinical, and radiological data of MS patients and HC are detailed in **Table 1**. Gender and age did not differ between patients and controls [sex (% women): MS = 72.7, HC = 62.5, p > 0.99; age (years ± SD): MS = 31.4 ± 9.2,

TABLE 1 | Characteristics of MS patients and healthy controls at baseline.

	Controls	MS (Total)	Aggressive MS	Non-aggressive MS
n	8	11	5	6
Demographic characteristics				
Age in years (mean ± SD)	33.4 ± 9.5	31.4 ± 9.2	31.6 ± 8.3	31.2 ± 10.6
Women (%)	5 (62.5)	8 (72.7)	3 (60)	5 (83.3)
Clinical characteristics				
Delay between the first relapse and the sample collection in days (mean ± SD)	–	53 ± 43	52.4 ± 25.9	53.5 ± 56.3
Mains symptoms at first relapse				
Optic neuritis (%)	–	4 (36.4)	2 (40)	2 (33.3)
Motor symptoms (%)	–	3 (27.3)	1 (20)	2 (33.3)
Sensory symptoms (%)	–	3 (27.3)	1(20)	2 (33.3)
Vestibular/Cochlear symptoms (%)	-	1 (9.1)	1 (20)	0 (0)
Oculomotor symptoms (%)	-	1 (9.1)	1 (20)	0 (0)
EDSS Score at baseline (mean ± SD)	–	2.1 ± 1.5	2.2 ± 1.7	2 ± 1.5
Radiological characteristics				
Presence of at least one gadolinium-enhancing lesion on the MRI at baseline (%)	–	3 (27.3)	0 (0)	3 (50)

MS, multiple sclerosis; SD, standard deviation; EDSS, expanded disability status scale.

HC = 33.4 ± 9.5, p = 0.65]. Mean duration of follow-up was 30.8 ± 12.1 months. At last follow up, six patients were classified as « non-aggressive MS » and five as « aggressive MS ».

Identification of Lineage Cell Subsets

We characterized PBMCs from MS patients and HC using two separate mass cytometry panels exploring lymphoid and myeloid subsets; respectively (**Supplementary Table 1** for Lymphoid panel and **Supplementary Table 2** for Myeloid panel). We first compared the immune cell composition of PBMCs between MS patients and HC. We found that T cell abundance was lower in MS patients than in HC (MS=54.1%, HC=64.4%, p=0.002), whereas the proportion of the other cell lineages (B cells, NK cells, and myeloid cells) was not significantly modulated between the two groups (data not shown).

Phenotypic Alterations in the Lymphoid Compartment in Patients With Early MS

Using CITRUS algorithm, we first analyzed B cell subsets and highlighted 8 clusters with a significantly different abundance between MS patients and HC, including the parent cluster 174494 (**Figure 2A**). This memory B cell cluster ($CD19^+CD27^+CD38^-$) had a higher expression of the T-bet transcription factor. These $T\text{-}bet^+$ B cells also expressed migration and proliferation markers (CXCR3, CCR4, and Ki67) in a more pronounced way than other B cell clusters (**Figure 2B**). This cluster showed a trend towards a higher abundance in "aggressive MS" patients than in "non-aggressive MS" patients, even if the low number of patients precluded any definitive conclusion (**Figure 2C**). Cluster 174403 that identified activated naïve B cells ($CD19^+CD27^-CD38^+CXCR5^+HLA\text{-}DR^{++}CCR4^+$) had also a significantly higher abundance in MS patients than in HC (data not shown) whereas two clusters of naïve B cells ($CD19^+CD27^-CD38^+$) were reduced in MS patients compared to HC, including the cluster 174486 expressing low levels of CXCR3 and CCR4, and the cluster 174493 identifying naïve B cells with a low activation level (lower expression of CD44 and CD45RA than other clusters of B cells) (data not shown).

Concerning T cell subsets, we identified the "activated naïve" T cell cluster 1022457, expressing CD3, CD4, CD45RA, CD38, CCR7, and CD127 as significantly reduced in MS patients compared with HC (**Figures 2D, E**). Interestingly, this cluster showed a trend towards a lower abundance in "aggressive MS" patients than in "non-aggressive MS" patients (**Figure 2F**).

Finally, one cluster of NK cells, cluster 325121, had a significantly higher abundance in MS patients than in HC (**Figure 2G**). These cells were $CD56^{dim}$ $CD16^{hi}$ cytotoxic NK cells, and were positive for CD25, CD38, CXCR3, T-bet, and CD161, corresponding to "pro-inflammatory" NK cells. This cluster also expressed FoxP3, GATA3, and RORgt transcription factors in a more pronounced way than NK cells from other clusters (**Figure 2H**). Again, it showed a trend towards a higher abundance in "aggressive MS" patients than in "non-aggressive MS" patients (**Figure 2I**).

As a whole, we identified specific B cell, T cell, and NK cell clusters deregulated in MS patients with a tendency towards an amplification of these alterations in aggressive MS.

Phenotypic Alterations of the Myeloid Compartment in Patients With Early MS

We then performed viSNE analysis of the myeloid lineage. We detected, by manual gating, a peculiar myeloid subpopulation in 5 out of 11 MS patients and in none of 8 HC (chi square test = 0.026). This population expressed CD14, CD36, CD11b, CD11c, CCR2, CCR5, CD206, CD209, SIRPa, and S100A9 (**Figure 3A**). This subset was also identified (cluster 155313) using CITRUS as enriched in MS patients compared to HC, and corresponded to a subset of classical monocytes with an increased expression of pro-inflammatory (CD86, CD64, CD32) and regulatory (CD206, CD209, PD-L1) markers (**Figures 3B, C**). Using viSNE and CITRUS approaches, this subset was found in the same 3 "aggressive MS" patients, and 2 "non-aggressive MS" patients whereas it was not detected in HC **(Figure 3D).**

Contrary to the cluster 155313, cluster 155317 had a significantly lower abundance in MS patients than in HC (**Figure 3B**). Compared to cluster 155313, it expressed low level of CD206, CD209, CCR5, and S100A9 (**Figure 3C**), corresponding to classical monocytes with pro-inflammatory but not regulatory markers.

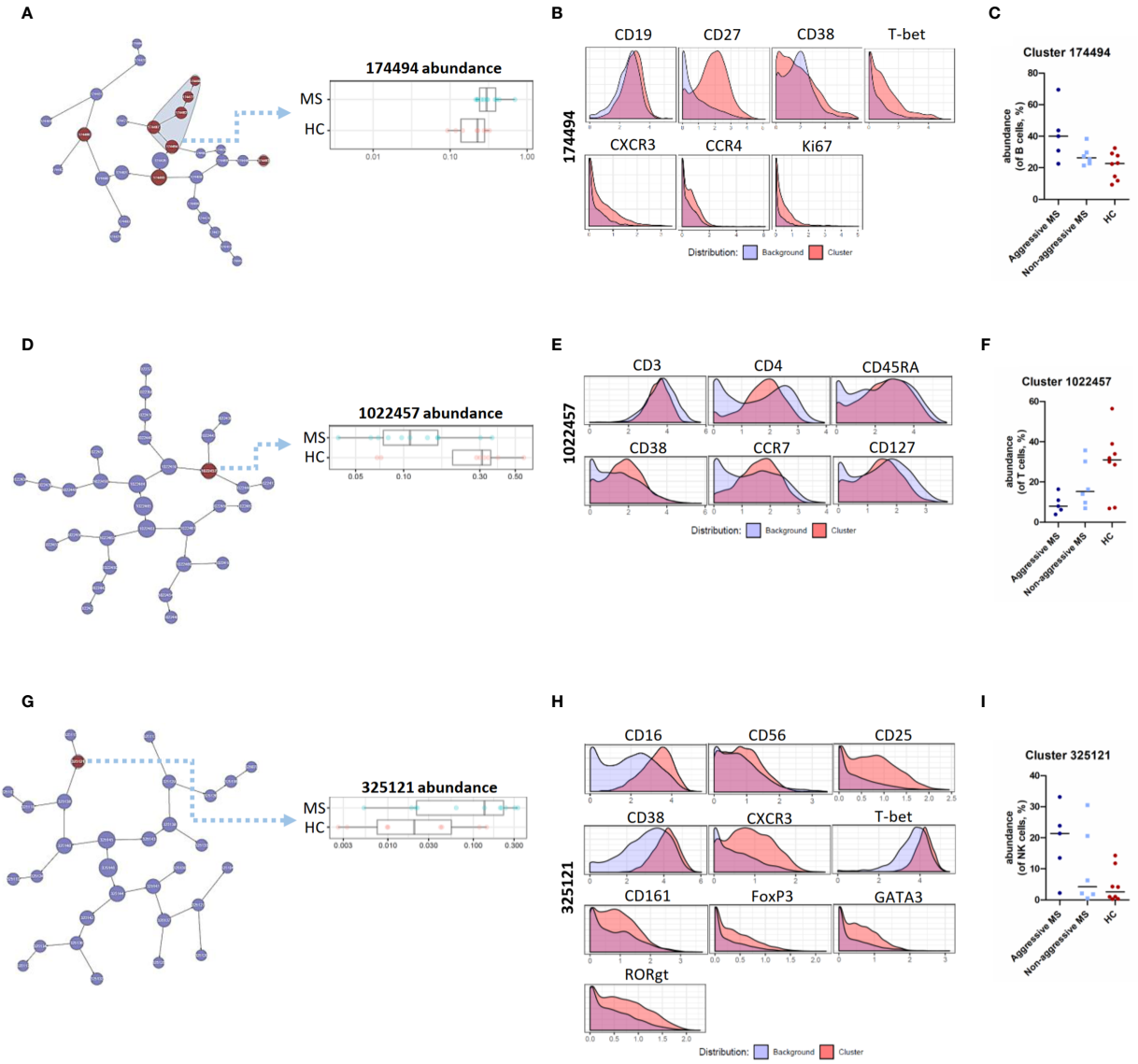


FIGURE 2 | CITRUS analysis of mass cytometry data from PBMC of MS patients and HC – B, T, NK cell lineages. **(A)** Visual representation of unsupervised hierarchical clustering of B cells and visualization of the clusters that are part of the significant results (in red). Abundance of the cluster 174494 (of B cells) in MS patients and HC. **(B)** Expression of CD19, CD27, CD38, T-bet, CXCR3, CCR4, and Ki67 on cells from cluster 174494 as compared to all other (background) B cells. **(C)** Abundance of cluster 174494 (of B cells, %) in « aggressive MS » patients, « non-aggressive MS » patients and HC. **(D)** Visual representation of unsupervised hierarchical clustering of T cells and visualization of the cluster that are part of the significant results (in red). Abundance of the cluster 1022457 (of T cells) in MS patients and HC. **(E)** Expression of CD3, CD4, CD45RA, CD38, CCR7, CD127 measured on cells from cluster 1022457 as compared to all other (background) T cells. **(F)** Abundance of cluster 1022457 (of T cells, %) in « aggressive MS » patients, « non-aggressive MS » patients and HC. **(G)** Visual representation of unsupervised hierarchical clustering of NK cells and visualization of the cluster that are part of the significant results (in red). Abundance of the cluster 325121 (of NK cells) in MS patients and HC. **(H)** Expression of CD16, CD56, CD25, CD38, CXCR3, T-bet, CD161, FoxP3, GATA3, and RORgt measured on cells from cluster 325121 as compared to all other (background) NK cells. **(I)** Abundance of cluster 325121 (of NK cells, %) in « aggressive MS » patients, « non-aggressive MS » patients and HC.

DISCUSSION

MS is a complex and heterogeneous disease with a highly variable disease course, reflecting, among other things, the interplay between genetic background and environmental factors. Even if many progresses have been made concerning the implication of the different subsets of immune cells in the pathological mechanisms, the crosstalk between the different immune cell subsets remains unclear.

Here, using extensive immune cell profiling exploring both adaptive and innate immune system, we describe a subset of T-bet$^+$ B cells particularly abundant in early MS patients, especially

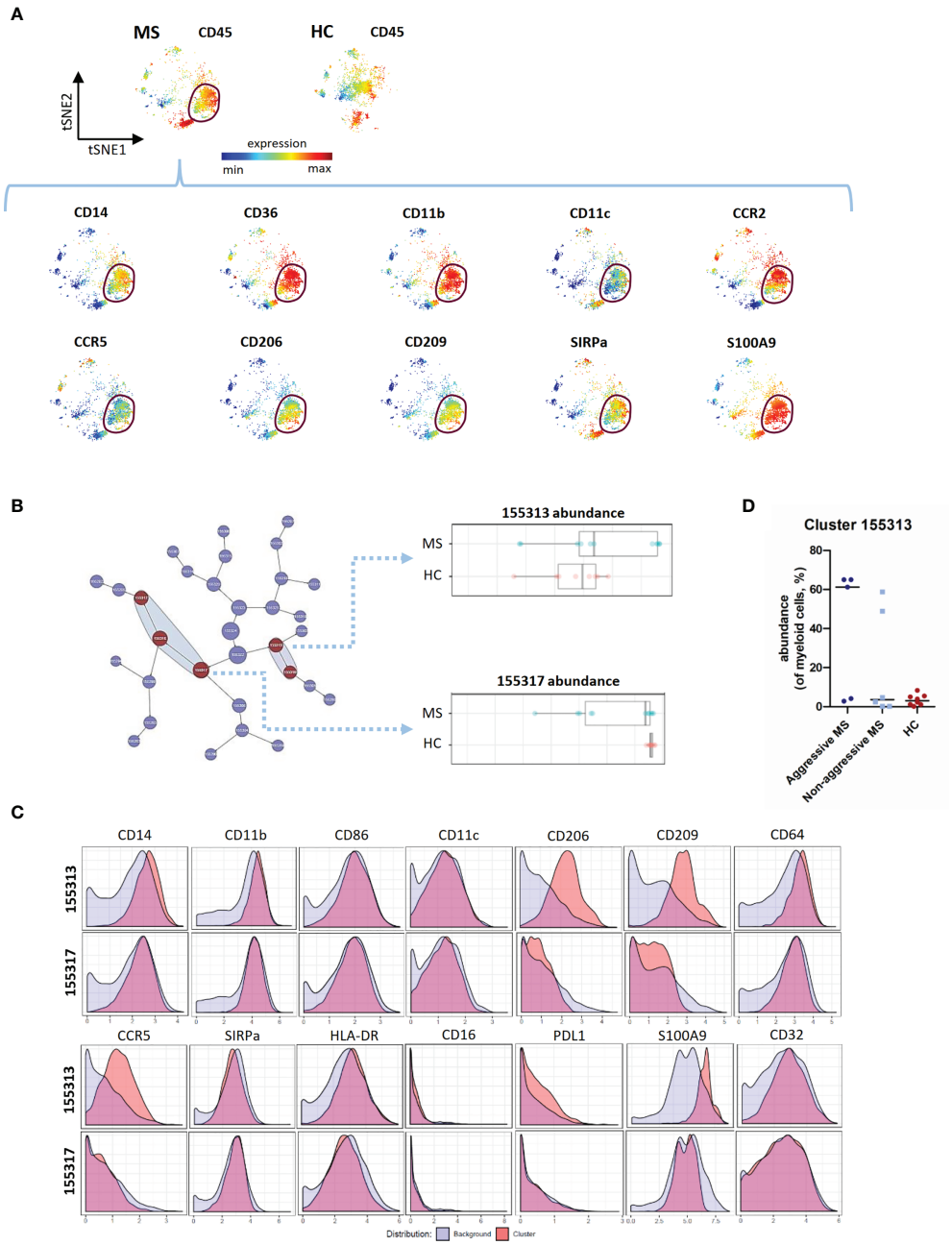


FIGURE 3 | CITRUS analysis of mass cytometry data from PBMC of MS patients and HC - Myeloid cell lineage. **(A)** Representative t-SNE maps, of a MS patient and a HC, regarding myeloid cells lineage. The t-SNE maps were generated based on expression levels of all markers of the myeloid panel (**Supplementary Table 2**). Description of a myeloid subset in a MS patient (circled). Each dot represents one cell and the color spectrum represents individual marker-expression levels (red : high expression; blue : low expression). **(B)** Visual representation of unsupervised hierarchical clustering of myeloid cells and visualization of the clusters that are part of the significant results (in red). Abundance of the cluster 155313 and 155317 (of myeloid cells) in MS patients and HC. **(C)** Expression of CD14, CD11b, CD86, CD11c, CD206, CD209, CD64, CCR5, SIRPa, HLA-DR, CD16, PD-L1, S100A9 and CD32 measured on cells from cluster 155313 and 155317 as compared to all other (background) myeloid cells. **(D)** Abundance of cluster 155313 (of myeloid cells, %), in « aggressive MS » patients, « non aggressive MS » patients and HC.

in the aggressive form of MS. We also found a decrease of a naïve T cell subset with activation markers, and an increase of a pro-inflammatory NK cell subset in the blood of MS patients. Finally, we found the presence of a CD206^{+} classical monocyte subset in the blood of 5/11 untreated early MS patients.

Multiple lines of evidence have recently pinpointed the key contribution of B lymphocytes to MS pathogenesis. In the relapsing remitting form of MS, the administration of B Cell Depleting Therapy (BCDT), using anti-CD20 monoclonal antibodies (mAb), was demonstrated to clinically and

radiologically improve MS patients (11, 12). The therapeutic benefit of the BCDT is thought to result from the elimination of B cells with pathogenic properties (13–15). In the peripheral blood of MS patients, pathogenic subsets of B cells were recently identified and characterized as pro-inflammatory GM-CSF- (16) and TNFα-secreting B cells (17, 18). In our study, we show that $CD27^+$ $CD38^-$ T-bet^+ B cells, that express CXCR3, CCR4 and Ki67, are significantly increased in early MS patients, with a trend towards a higher abundance in "aggressive MS" patients. In Human, T-bet expression can be induced in B cells by IL-27, IFNγ, or IL-21 (19). *In vivo*, T-bet is critical for maintaining antigen specific memory of IgG2a B cells (20). In autoimmunity, B-cell specific loss of T-bet in murine models of systemic lupus erythematosus (SLE) results in reduced disease manifestation (21), whereas $CD11c^{hi}$T-bet^+ B cells are overrepresented in SLE patients and correlated with clinical manifestations (22). These cells are poised to generate plasmablasts producing autoreactive antibodies (22). In MS, a recent study suggests that T-bet-expressing B cells are recruited in patient CNS (23). In our study, these T-bet^+ B cells seemed to be more represented in aggressive MS patients. The exact mechanism by which T-bet-expressing B cells are involved in neuroinflammation remains to be explored, and further studies are needed to link them to disease severity.

In this study, we also show that $CD4^+$ $CD38^+$ $CD45RA^+$ $CD127^+$ $CCR7^+$ T cells are decreased in early MS patients. Even if CD38 is considered as an activation marker, its expression on "naïve" T cells has already been reported (24). Recently, $CD4^+$ T cells with a similar phenotype were studied in HC. These cells were low proliferative, had a bias towards IL-13 secretion, and showed responsiveness to IL-7 (25). Among NK cells, we found an increase of an activated pro-inflammatory $CD56^{dim}CD16^{hi}$ $CD161^+$ subset in MS patients. In fact, a recent study suggests that CD161 defines a subset of pro-inflammatory NK cells that may contribute to inflammatory disease pathogenesis (26). The exact implication of these two last lymphocyte subsets in neuroinflammation remains to be precisely defined.

In MS, the different populations of myeloid cells, including monocytes, macrophages, microglia, and dendritic cells, have prominent roles in the pathogenesis as antigen-presenting cells (in periphery and inside the CNS) but also as effector cells (inside the CNS) (6). There are only few studies concerning circulating myeloid cells from MS patients, and data are not consistent (27, 28). Here we report an increased abundance of a myeloid cell subset with a phenotype corresponding to classical monocytes with pro-inflammatory markers (markers associated with M1 macrophage polarization, CD86, CD64, CD32), regulatory markers (markers associated with M2 macrophage polarization, CD206, CD209, PD-L1) and a high expression of S100A9. Very interestingly, the majority of activated macrophages in active MS lesions were shown to display a mixed pro-inflammatory and regulatory status (29). These results are consistent with those of a more recent study, in which iNOS/CD206 double positive macrophages were detected in all chronic active MS lesions examined with a higher frequency in the MS lesion center (30). It is tempting to speculate that the classical monocyte cluster highlighted in our study corresponds to circulating precursors of these CNS macrophages with intermediate activation status and their interest as biomarker related to MS severity deserves further evaluation.

By now, only few studies have used mass cytometry to deeply analyze in an unbiased manner PBMCs from MS patients. In particular, Böttcher et al. identified an increased abundance of $CCR7^+$ and $IL6^+$ T cells in early MS patients, whereas $CD141^{hi}$ $IRF8^{hi}$ $CXCR3^+$ $CD68^-$ dendritic cells were decreased (31). Galli et al. identified an expanded T helper cell subset characterized by the expression of GM-CSF and CXCR4 (32). Finally, Marsh-Wakefield et al. used a B-cell focused mass cytometry panel to compare peripheral $IgG3^+$ B cells of MS patients with inactive or active stages of disease (33). Overall, considering mass cytometry analyses of PBMCs in MS patients, the literature gathers only few studies using different panels and variable inclusion criteria, resulting in data that are difficult to compare and explaining the non-redundant results obtained.

Our study also has some limitations. The major one is the low number of MS patients and HC, sometimes resulting in data with high variability within the cohort. Moreover, the small number of patients did not allow us to make robust conclusions concerning the analysis between the different MS patient subgroups. Of note, enrolled patients were highly selected in order to constitute the most homogeneous cohort, including drug-naïve MS patients selected at the stage of the first neurological relapse. Nevertheless, these preliminary data will have to be confirmed using larger studies.

So, even if the number of patients was small, they were highly selected (first relapse, no DMT) and well characterized. Moreover, one of the strengths of our study is the use of two large antibody panels exploring both lymphoid and myeloid compartments. This pilot study opens the way to a bigger study with a high number of well-characterized patients to robustly compare immune profile of different MS patient subgroups according to disease severity, and so validate these potential biomarkers.

Using comprehensive immune profiling of PBMCs at the single cell level and unsupervised approach, we have been able to detect subsets of immune cells (T-bet^+ B cells, $CD206^+$ classical monocytes, pro-inflammatory NK cells) significantly increased in our cohort of early untreated MS patients compared to controls. These results open a path of biomarkers discovery using the power of high dimensional single cell techniques.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Rennes Hospital. The patients/participants provided their written informed consent to participate in this study.

OBSERVATOIRE FRANÇAIS DE LA SCLÉROSE EN PLAQUES

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SUPPLEMENTARY MATERIAL

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