

High frequency of circulating non-classical monocytes is associated with stable remission in relapsing-remitting multiple sclerosis

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ABSTRACT

'No evidence of disease activity (NEDA)', judged by clinical and radiological findings, is a therapeutic goal in patients with multiple sclerosis (MS). It is, however, unclear if distinct biological mechanisms contribute to the maintenance of NEDA. To clarify the immunological background of long-term disease stability defined by NEDA, circulating immune cell subsets in patients with relapsing-remitting MS (RRMS) were analyzed using flow cytometry. Patients showing long-term NEDA ($n=31$) had significantly higher frequencies of non-classical monocytes (NCMs) (6.1% vs 1.4%) and activated regulatory T cells (Tregs; 2.1% vs 1.6%) than those with evidence of disease activity ($n=8$). The NCM frequency and NCMs to classical monocytes ratio (NCM/CM) positively correlated with activated Treg frequency and duration of NEDA. Co-culture assays demonstrated that NCMs could increase the frequency of activated Tregs and the expression of PD-L1, contributing to development of Tregs, was particularly high in NCMs from patients with NEDA. Collectively, NCMs contribute to stable remission in patients with RRMS, possibly by increasing activated Treg frequency. In addition, the NCM frequency and NCM/CM ratio had high predictive values for disease stability (AUC = 0.97 and 0.94, respectively), suggesting these markers are potential predictors of a long-term NEDA status in RRMS.

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1. Introduction

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the CNS, of which pathogenesis would involve a wide variety of innate and acquired immune cells, including self-reactive T cells and B cells [1]. During early stages of MS, most patients are affected with relapsing-remitting multiple sclerosis (RRMS), characterized by occasional exacerbations of neurological symptoms (relapses) followed by various periods of recovery (remission). Fortunately, under current disease modifying therapy (DMT), more patients with RRMS are relatively well-controlled, compared with the pre-DMT era. To define the degree of disease stability, the concept of 'no evidence of disease activity (NEDA)' [2,3] is now widely used as an objective goal for treatment of

patients with RRMS in daily practice. NEDA-3, often used for this measure, is defined by 3 components: absence of clinical relapse, absence of new or active lesions on MRI images and absence of disease progression assessed by Expanded Disability Status Scale (EDSS) [3].

Even with a high efficacy DMT, it is not always possible to achieve NEDA-3 status, although some patients can maintain a long-term disease stability with a lower efficacy therapy or without DMT. Such varied responses to DMT may be explained by the differences in disease activity before treatment. Notably, even if NEDA-3 is achieved, it is not always continuous and we cannot predict how long it will be maintained. For example, approximately 30% of patients treated with interferon-beta (IFN- β) or glatiramer acetate (GA) could maintain NEDA-3 for

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two years and only less than 10% of IFN- β - or GA-treated patients could maintain a NEDA-3 status for seven years [4]. In another study, approximately 30% of IFN- β -treated patients who had a NEDA-3 status for over 2 years continue to maintain disease stability for more than 15 years with the same treatment [5]. Meanwhile, fingolimod (FTY) or natalizumab (NTZ) have been reported to increase the rate of NEDA-3 for two years to approximately 50% at the most [6]. Here, we hypothesized that common immunological mechanisms may be operative among patients who successfully maintain long-term disease stability. We made efforts to verify the hypothesis, hoping that it may lead to in-depth understanding of MS remission and identification of useful biomarkers for predicting long-term disease stability.

Monocytes are engaged in both the innate and adaptive immunity as phagocytes and antigen-presenting cells [7]. As they are observed in acute MS brain lesions [8,9], their involvement in MS pathogenesis is speculated [10]. Recently, studies in mice confirmed that monocyte-derived macrophages were responsible for the initiation of demyelination in the acute inflammatory phase [11,12]. On the other hand, GA or dimethyl fumarate (DMF) treatment induced anti-inflammatory monocytes in experimental autoimmune encephalomyelitis (EAE), an animal model of MS [13,14]. These findings indicate that monocytes can play both pathogenic and protective roles in MS.

In humans, monocytes are classified into three subpopulations based on the expression patterns of CD14 and CD16: classical monocytes (CMs; CD14^{high} CD16⁻), intermediate monocytes (IMs; CD14^{high} CD16^{high}) and non-classical monocytes (NCMs; CD14^{int} CD16^{high}) [7,15,16]. CMs are originated from hematopoietic cells in the bone marrow and differentiate into IMs and further into NCMs [17,18]. Accounting for 80–90% of monocytes, CMs egress into the peripheral blood and further into the inflammatory site, where they are mainly engaged in phagocytosis and secretion of inflammatory cytokines during bacterial infection [7,15]. In contrast, NCMs are a minor population that exhibit patrolling behavior with reduced phagocytic capacity [17]. Although altered frequencies of monocyte subpopulations have been reported in MS [19–22], it remains unclear whether anti-inflammatory monocyte subsets may play a role in MS pathogenesis.

In this study, we demonstrate that patients with RRMS maintaining a long-term NEDA-3 status are characterized by altered monocyte composition: a higher NCM frequency in the peripheral blood compared with patients with evidence of disease activity (EDA). Both the frequency of NCMs and NCM/CM

ratio were positively correlated with the frequency of activated regulatory T cells (Tregs) and the duration of the NEDA-3 status. These two markers had high predictive values for the relapse-free state in patients with RRMS over the following two years. Notably, NCMs increased the frequency of activated Tregs when co-cultured with CD4⁺ T cells, which is likely to be mediated by the high expression of PD-L1 in NCMs. Thus, NCMs play a protective role possibly by increasing the frequency of activated Tregs and the frequency of NCMs, as well as NCM/CM ratio, is associated with duration of disease stability (NEDA-3) in patients with RRMS.

2. Materials and methods

2.1. Participants

The demographic data of patients with RRMS and healthy controls (HCs) participated in the immune cell subset analysis are described in Table 1. All patients with RRMS were diagnosed using the McDonald criteria [23]. Patients were divided into two groups: NEDA group, which included patients with a NEDA-3 status over two years, and EDA group included patients with two or more relapses in the past two years under the same DMT. NEDA-3 was defined as the absence of (1) clinical relapse, (2) new or enlargement lesion of T2-weighted image or gadolinium-enhanced lesion on MRI and (3) disease progression [3]. We defined disease progression as an annual increase of 1.0 point or more on the EDSS. The duration of NEDA-3 status at the blood collection (mean [IQR] = 4.4 [2.0–7.0] in Cohort 1 and 3.0 [2.9–5.0] in cohort 2) was determined by checking the medical records retrospectively. The clinical course was followed up until April 2023 in all patients (eTable 1). The follow-up duration was two years for all patients in the ROC analysis showing predictive values and it was the entire study period for analysis of the survival curves.

The demographic information of the participants for co-culture assay is listed in eTable 2. Demographics of the participants in the PD-L1 expression analysis are shown in eTable 3. The patients in this analysis were also divided into the NEDA or EDA state group based on disease activity before blood collection, but using a different definition. To prioritize recruiting a larger number of participants, we assigned patients with NEDA-3 status over one year to the ‘NEDA state’ group and those with one or more relapse in the last year to the ‘EDA state’ group.

All patients who participated in this study were treated at the National Center Hospital of Neurology

Table 1. Demographic data of participants included in the immune cell subset analysis.

	Cohort 1		Cohort 2		
	NEDA (n=31)	EDA (n=8)	NEDA (n=17)	EDA (n=5)	HC (n=15)
Age (mean [SD])	46.7 [10.3]	41.0 [7.9]	44.7 [9.8]	38.2 [11.4]	47.1 [12.5]
Male:female	10:21	3:5	6:11	2:3	6:9
Disease duration (years)	12.0	15.0	12.0	4.0	
(median [IQR])	[6.5–19.0]	[11.5–18.5]	[6.5–19.0]	[4.0–4.0]	
EDSS (median [range])	1.5 [0.0–7.5]	4.5 [1.5–6.5]	1.5	3.0	
			[0.0–7.0]	[2.0–6.0]	
Duration of NEDA-3 (years)	4.4 [2.0–7.0]		3.0		
(median [IQR])			[2.9–5.0]		
OCB	6:13:12	5:0:3	6:4:7	3:2:0	
(positive:negative:unknown)					
Maintenance treatment		(daily dose of prednisolone; mean $\pm$ SD mg/day)			
Without DMT	5	1	2	0	
PSL	8(3.6 $\pm$ 1.8 mg)	0	0	1	
				(6.0 mg)	
PSL + IS	0	0	0	2	
				(8.5 $\pm$ 4.9 mg)	
IFN- β	8	3	2	0	
IFN- β + PSL	1 (2.0 mg)	1 (15 mg)	2 (2.3 $\pm$ 1.1 mg)	1 (1.25 mg)	
GA	5	1	0	0	
GA + PSL	0	0	4	0	
			(3.3 $\pm$ 1.5 mg)		
GA + IS	0	1	0	0	
DMF	0	1	0	0	
DMF + PSL	0	0	0	1 (4.0 mg)	
FTY	0	0	7	0	
NTZ	4	0	0	0	

Notes: Five patients in the NEDA group and three in the EDA group were also included in the PD-L1 expression analysis (eTable 2). The dose of prednisolone was listed in parentheses following the number of patients. NEDA: no evidence of disease activity; EDA: evidence of disease activity; HC: healthy control; IQR: interquartile range; EDSS: Expanded Disability Status Scale; OCB: oligoclonal band; DMT: disease modifying therapy; PSL: prednisolone; IS: immunosuppressant; IFN- β : interferon-beta; GA: glatiramer acetate; DMF: dimethyl fumarate; FTY: fingolimod; NTZ: natalizumab.

and Psychiatry, Japan, between 2018 and 2022. The Ethics Committee of the National Center of Neurology and Psychiatry, Japan approved this study and written informed consent was obtained from all participants. This study was performed in accordance with the Declaration of Helsinki. Patients who received steroid pulse therapy within one month before blood collection were excluded.

2.2. Cell staining and flow cytometry

Peripheral blood mononuclear cells (PBMCs) from donated blood were obtained *via* density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare Bioscience, Mississauga, ON, Canada). Fresh PBMCs were stained with fluorescent-labeled antibodies for cell surface markers. To assess the production of cytokines, including IFN γ and IL-17A, PBMCs were stimulated with 50 ng/mL phorbol myristate acetate (Sigma-Aldrich, MO, USA), 500 ng/mL ionomycin (Sigma-Aldrich) and 2 μ M monensin (Sigma-Aldrich) for 1 h. Stimulated cells were stained with cell surface antibodies followed by intracellular staining of Foxp3, IL-17A and IFN- γ using a Foxp3/transcription factor staining buffer set (eBiosciences, CA, USA), according to the manufacturer's instructions.

The following fluorescent-labeled antibodies were used for FACS analysis: FITC anti-Foxp3 (PCH101) (eBiosciences), Alexa Fluor 488 anti-CD56 (B159), PE anti-CD180 (G28-8), PE-Cy7 anti-CD45RA (L48),

APC-Cy7 anti-CD8a (RPA-T8), APC-Cy7 anti-CD19 (SJ25C1), APC-H7 anti-CD45RA (HI100), V500 anti-IgD (IA6-2), V500 mIgG1k (X40), V500 anti-CD4 (RPA-T4) and V500 anti-CD3 (UCHT1) (BD Biosciences, NJ, USA), PE anti-CD3 (UCHT1), PE anti-IFN- γ (B27), PE anti-PD-L1 (29E2A3), PerCP-Cy5.5 anti-CD38 (HIT2), PerCP-Cy5.5 anti-CD16 (3G8), PerCP-Cy5.5 anti-CD3 (UCHT1), PE-Cy7 anti-CD24 (ML5), PE-Cy7 anti-HLA-DR (L243), APC anti-CD14 (M5E2), APC anti-IL-17A (BL168), PB anti-CD27 (O323) and PB anti-HLA-DR (L243) (BioLegend, CA, USA), PE anti-CD56 (NKH1) (Beckman coulter, CA, USA) and FITC anti-CXCR3 (49801) and APC anti-CXCR5 (51505) (R&D Systems, MN, USA). Zombie Aqua Fixable Viability Kit (BioLegend) was used for evaluation of dead cells. Flowcytometry data were obtained using a FACS Canto II with FACSDiva software (BD Biosciences) and analyzed using a FlowJo software (BD Biosciences).

2.3. Cell culture

To evaluate whether monocyte subpopulations influence the frequency of activated Tregs, a T cell-monocyte co-culture experiment was designed. NCMs (CD16^{high}CD14^{int} monocytes) or CMs (CD14^{high}CD16^{low} monocytes) were sorted from fresh PBMCs of the participant using a FACS Aria II cell sorter (BD Biosciences). CD4⁺ T cells from the same individuals

were collected using a CD4⁺ T Cell Isolation Kit, human (Miltenyi Biotec, Germany) and an autoMACS Pro Separator (Miltenyi Biotec), according to the manufacturer's instructions. Ninety-six well plates were coated with 2 mg/mL anti-CD3 monoclonal antibody (OKT3) for 1 h at 37°C. Sorted CD4⁺ T cells were cultured with or without each sorted monocyte subset at a ratio of 2:1 in the presence of 50 ng/mL M-CSF (Recombinant human M-CSF, PeproTech, NJ, USA) in RPMI 1640 with 10% fetal calf serum in coated plates for four days. Incubated cells were stained with cell surface antibodies and the Zombie Aqua Fixable Viability Kit (BioLegend), followed by intracellular staining of Foxp3 as described above. The frequency of total Tregs (Foxp3⁺) or activated Tregs (Foxp3^{high} CD45RA⁻) in live CD4⁺ T cells was evaluated using a FACS Canto II (BD Biosciences).

2.4. Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 10; GraphPad Software, CA, USA). The Mann-Whitney *U* test or paired *t*-test was used to compare data between two groups, as appropriate. To compare data between three groups or more, the Kruskal Wallis test followed by Dunn's comparison test or one-way ANOVA followed by Tukey's multiple comparison test was used, as appropriate. Correlations was evaluated using Spearman's correlation analysis. The area under the receiver operating characteristic (ROC) curve (AUC) was used to evaluate the values of NCM frequency and the NCM/CM ratio for the biomarkers. The log-rank test was used to compare two survival curves. Data were considered statistically significant when the *p* value was under .05.

3. Results

3.1. High frequencies of NCMs and activated Tregs were observed in the peripheral blood of patients with RRMS in the NEDA group

To elucidate the immunological features of patients with RRMS showing long-term disease stability, the frequencies of B, T and non-T non-B immune cells in the peripheral blood of patients were analyzed using flow cytometry (Figure 1, eFigures 1 and 2). The patients were assigned to the NEDA and EDA groups as described in the Methods section. The most distinctive difference in the immunological feature between the NEDA and EDA groups was observed in the monocyte subpopulation: patients in the NEDA group had a higher frequency of NCMs among total monocytes than those in the EDA group

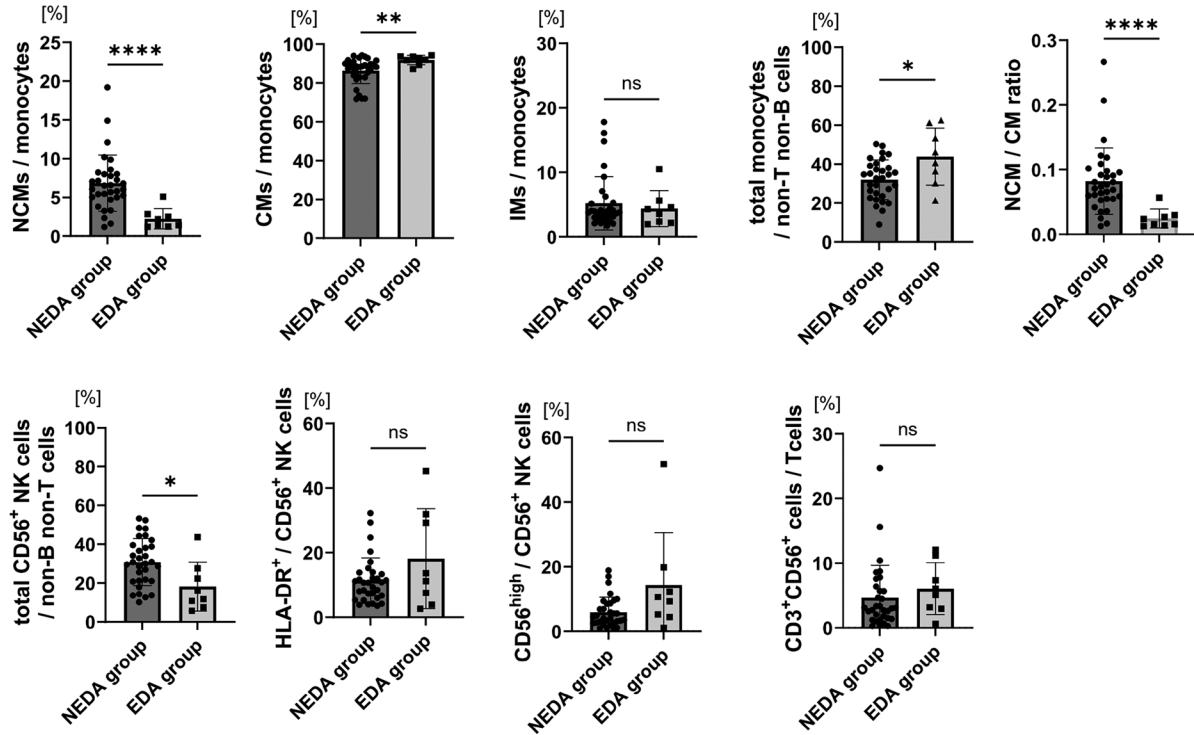
(Figure 1(A)). The frequency of total monocytes was lower in the NEDA group than in the EDA group, presumably reflecting a slight reduction in CMs, which account for the majority of the monocyte population. No significant difference in the IM frequency was observed between the two groups. Considering the difference in functions between NCM and CM [15,17], we considered that a balance of these two subsets might be important. As expected, the NCM/CM ratio was markedly higher in the NEDA group than in the EDA group (Figure 1(A)). While the frequency of total CD56⁺ NK cells was higher in the NEDA group, the frequencies of the HLA-DR⁺ CD56⁺ NK, CD56^{high} NK, and CD3⁺ CD56⁺ cells showed no significant difference between the two groups (Figure 1(A)).

Another notable finding in the NEDA group was the higher frequency of activated Tregs (Figure 1(B)). According to the previous studies, CD4⁺ Tregs consist of three functionally distinct fractions: CD45RA⁺ FoxP3^{low} (resting Tregs), CD45RA⁻ FoxP3^{hi} (activated Tregs) and CD45RA⁻ FoxP3^{low} (cytokine-secreting nonsuppressive T cells) [24]. Among these three subsets, activated Tregs exhibit the highest suppressive capacity [24,25]. There was no difference in the frequency of total Treg cells between the two groups (Figure 1(B)), and the percentages of memory CD4⁺T, Th1, Th17 and follicular T cells were similar between the two groups (Figure 1(B)). No significant differences were observed in the frequencies of B cell subsets, including total B cells, naïve, memory, transitional B cells and plasmablasts (eFigure 2).

Taken together, these results suggest that the altered frequency of non-T non-B cell subsets, including monocyte subsets, total monocytes and total NK cells may be specific immunological features associated with the NEDA-3 status in patients with RRMS.

To confirm the immunological features found in Figure 1(A), the experiment was performed with another cohort (Cohort 2; Table 1). Consistent with the data from Cohort 1, the frequency of NCMs was higher in the NEDA group than in the EDA group in Cohort 2 (Figure 2). The NCM frequency in the NEDA group was at a similar level to that in HCs, suggesting that the decrease in NCMs may reflect high disease activity. Moreover, the NCM/CM ratio in Cohort 2 was lower in the EDA group than in the HC or NEDA groups (Figure 2(B)). This ratio was not significantly different between the HC and NEDA groups (Figure 2(B)), suggesting that the decrease in this ratio was associated with high disease activity. When the patients were divided according to their medications, the patients with IFN- β tended to have a higher NCM frequency and NCM/CM ratio compared to those with PSL or GA $\pm$ PSL; although,

A



B

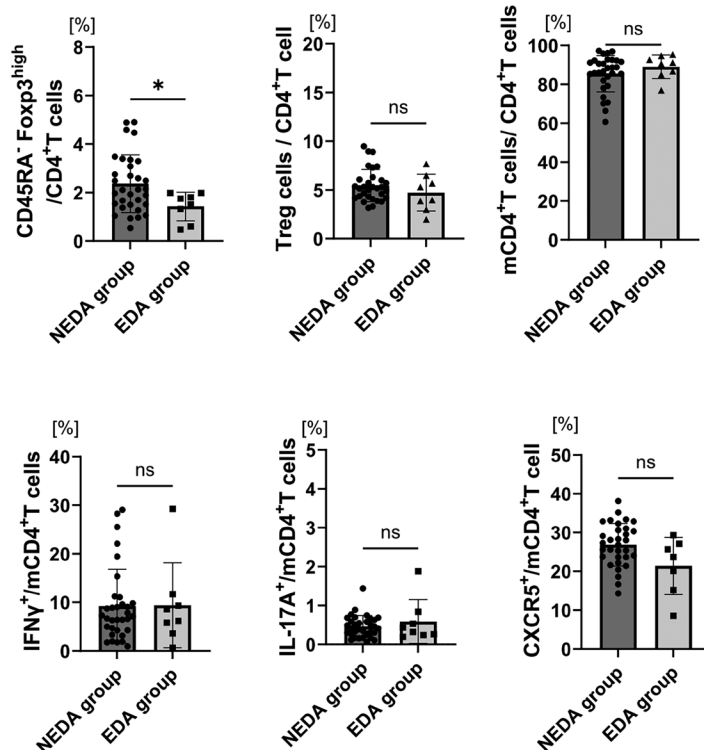


Figure 1. The monocyte composition and frequency of activated Tregs in the peripheral blood of patients are different between the NEDA and EDA groups. (A) Frequencies of the following in the peripheral blood of patients in the NEDA and EDA groups: monocyte subpopulations (NCMs, CMs and IMs) among monocytes, total monocytes among non-T non-B cells, the ratio of NCMs to CMs (NCM/CM ratio); total CD56⁺ NK cells among non-T non-B cells, CD56⁺ NK subsets (HLA-DR⁺ CD56⁺ NK cells, CD56^{high} NK cells) among total CD56⁺ NK cells and CD3⁺CD56⁺ cells among T cells. (B) Frequencies of the following in the peripheral blood of patients in the NEDA and EDA groups: activated Tregs (Foxp3^{high} CD45RA⁻ CD4⁺ T cells), Tregs (Foxp3⁺ CD4⁺ T cells) and memory CD4⁺ T cells and among CD4⁺ T cells; Th1 cells (IFN γ ⁺ memory CD4⁺ T cells), Th17 cells (IL-17A⁺ memory CD4⁺ T cells) and follicular helper T cells (CXCR5⁺ memory CD4⁺ T cells) among memory CD4⁺ T cells. The Mann-Whitney *U* test was used for statistical analysis. Error bars represent the mean $\pm$ SD. ns = not significant, * $.01 \leq p < .05$, ** $.001 \leq p < .01$, **** $p < .0001$. NEDA: no evidence of disease activity; EDA: evidence of disease activity; NCM: non-classical monocyte; CM: classical monocyte; IM: intermediate monocyte; Tregs: regulatory T cells; mCD4⁺ T cell: memory CD4⁺ T cell; Tfh cell: follicular helper T cell.

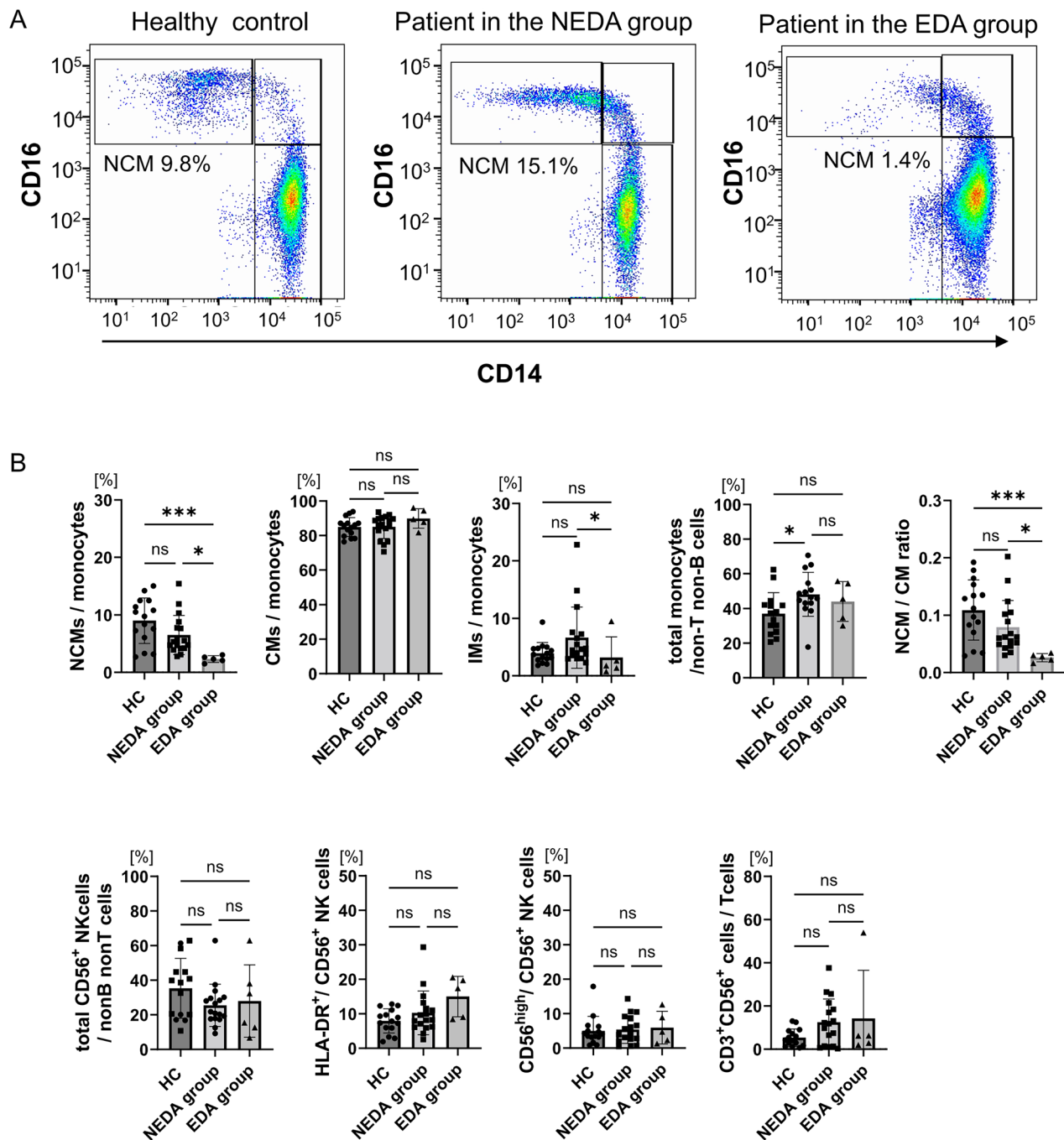


Figure 2. Patients in NEDA group are characterized by a high frequency of NCMs in the peripheral blood. (A) Typical flow cytometry plots showing three monocyte subpopulations in the peripheral blood of HC and patients in the NEDA and EDA groups. (B) Frequencies of non-T non-B cell subsets in Cohort 2 (Table 1). The frequency of each subset was analyzed using the same procedures as in Figure 1(A). The IM frequency was slightly different between the NEDA and EDA group, but this difference was not observed in Cohort 1. The Kruskal Wallis test with Dun's multiple comparison test was used to compare the three groups. Error bars represent the mean ± SD. ns = not significant, *0.01 ≤ p < 0.05, ***0.0001 ≤ p < 0.001. HC: healthy control; NEDA: no evidence of disease activity; EDA: evidence of disease activity; NCM: non-classical monocyte; CM: classical monocyte; IM: intermediate monocyte.

Tukey's multiple comparison tests showed no significant difference between any specific DMTs (eFigure 3(A,B)). Additional analysis showed that the NCM frequency and NCM/CM ratio were higher in the NEDA group than in the EDA group, when only the patients treated with PSL ± other DMTs are compared (eFigure 3(C)), indicating that the higher NCM frequency and NCM/CM ratio in the NEDA group cannot just be explained by the effects of DMTs.

These data confirmed that the NCM frequency and the NCM/CM ratio are higher in the NEDA group than in the EDA group. On the other hand, other differences between the NEDA group and EDA group observed in Cohort 1 were not reproduced in Cohort 2 (Figures 1(A) and 2(B)). Taken together, these results indicate that the altered composition of monocytes are likely to be associated with the NEDA-3 status.

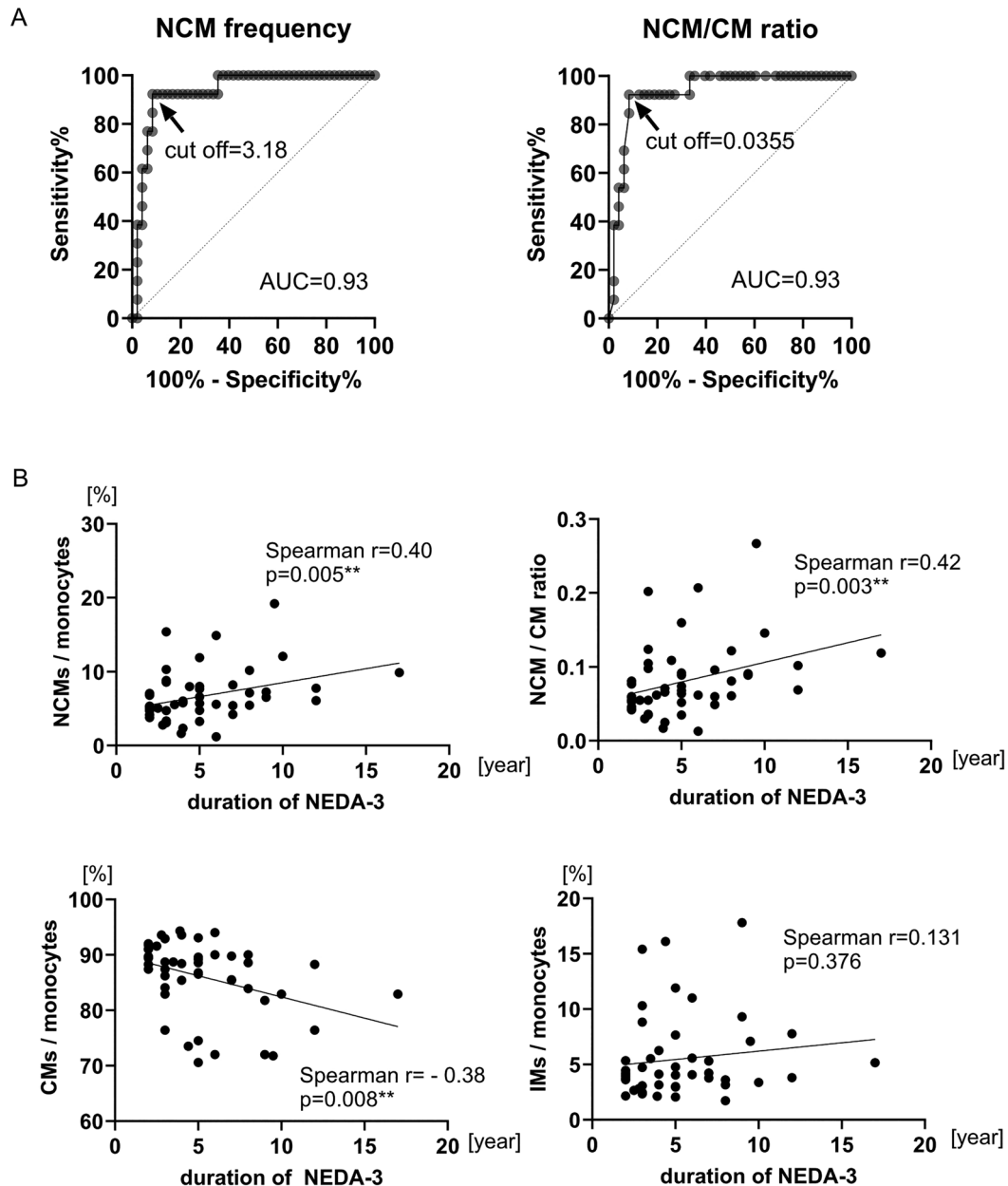


Figure 3. NCM frequency in peripheral blood is positively correlated with the duration of NEDA-3. (A) Receiver operating characteristic (ROC) curves showing the performance of the NCM frequency or the NCM/CM ratio to discriminate patients in the NEDA group from those in the EDA group. All patients listed in Table 1 ($n = 61$) were included in this analysis and the data from Figures 1(A) and 2(B) were used. The black arrow in each graph shows the optimal cutoff value of each marker (NCM, 3.18%; and NCM/CM ratio, 0.0355). (B) Correlation analysis between the duration of NEDA-3 at the blood collection and the frequency of monocyte subpopulations, or the NCM/CM ratio in the peripheral blood of patients in Cohort 1 and 2 from Figures 1(A) and 2(B). Only patients in the NEDA group were included in this analysis ($n = 48$), because the patients in the EDA group had a NEDA-3 status duration of 0. The Spearman correlation analysis was used for statistical analysis. $^{**}0.001 \leq p < 0.01$. AUC: area under the curve; NCM: non-classical monocyte; CM: classical monocyte; IM: intermediate monocyte; NEDA: no evidence of disease activity.

3.2. NCM frequency in peripheral blood was associated with the duration of NEDA-3 status

Next, we determined how clearly the NEDA and EDA groups could be differentiated based on the frequency of NCMs and the NCM/CM ratio. The NCM frequency and NCM/CM ratio had high sensitivity and specificity for two-year NEDA-3 status before sample collection, with an AUC of 0.93 (Figure 3(A)).

We then evaluated the correlation between some clinical features and the frequency of monocyte subpopulations in the NEDA and EDA groups. The duration of NEDA-3 status at the blood collection was positively correlated with the NCM frequency and NCM/CM ratio, and it was negatively correlated with the CM frequency in the NEDA group (Figure 3(B)). Although the frequency of NCMs in peripheral blood was previously reported to associate with aging in healthy

individuals [26], the NCM frequency and NCM/CM ratio did not associate with age in patients with MS in our cohort (eFigure 4(B)). The EDSS score was negatively correlated with the NCM frequency and NCM/CM ratio, and it was positively correlated with the CM frequency, but with a low correlation coefficient (Spearman $r=0.29$; eFigure 4(A)). No association was observed between the IM frequency and the duration of NEDA-3 status and EDSS score at the blood collection (Figure 3(B), eFigures 4(A)). The EDSS score was negatively correlated with the duration of NEDA-3 status at the blood collection in our cohort (eFigure 4(C)).

These data indicate that the altered monocyte composition is a possible immunological feature that is involved in disease stability in patients with RRMS.

3.3. NCM frequency and the NCM/CM ratio predict the disease course

As the frequency of NCMs and the NCM/CM ratio reflected disease stability before the collection of blood samples, we next examined whether these markers can predict the disease course for the two years after blood collection. One patient in the EDA group could not be followed up because the patient had moved to another hospital. During the follow-up period, 44 of the 48 patients (91%) in the NEDA group remained relapse-free. In contrast, one out of 12 patients (8.3%) in the EDA group remained relapse-free with a switch of medication during the follow-up period (eTable 1). Four patients in the NEDA group and 10 in the EDA group were excluded from this analysis because their medication was changed during the two years after sample collection. Among the remaining 46 patients, the NCM frequency and the NCM/CM ratio predicted the relapse-free status during the two years after sample collection with an AUC of 0.95 and 0.90, respectively (eFigure 5(A)).

When patients without a switch of medication until a relapse during the two-year follow-up period ($n=6$) were included in this analysis, the predictive values were even higher, with an AUC of 0.97 (NCM frequency) and 0.94 (NCM/CM ratio) (Figure 4(A)). When we analyzed only the patients in NEDA group ($n=48$), these parameters retained a high predictive value, with an AUC of 0.93 (NCM frequency) and 0.88 (NCM/CM ratio) (Figure 4(B)). Moreover, survival analysis using cutoff values from Figure 4(A) showed that these markers reliably predicted the disease course with or without a relapse for up to 52 months after the sample collection (log-rank test; $p<.0001$ (NCM frequency) and $p<.0001$ (NCM/CM ratio); Figure 4(C)).

Similar results were observed when using the cutoff values obtained from the ROC analysis after excluding patients who switched their medication during the two-year follow-up period (eFigure 5(B)).

These results suggest that the proportion of NCMs among monocytes and the NCM/CM ratio in the peripheral blood not only reflect disease stability before sample collection, but also serve as reliable predictors of disease stability in the following years.

3.4. NCMs increases frequency of activated Tregs

To gain insights into underlying mechanisms, further investigations focusing on the functions of NCMs were conducted. As we observed a higher frequency of activated Tregs in the NEDA group compared with the EDA group (Figure 1(B)), we examined if there were any correlations between the composition of monocytes and activated Tregs. Interestingly, the frequency of NCMs as well as the NCM/CM ratio positively correlated with the frequency of activated Tregs (Figure 5(A)). The frequency of IMs also had a slightly positive correlation with the frequency of activated Tregs (eFigure 6(A)), but the IM frequency data were inconsistent among the patient groups (Figures 1(A) and 2(B)). The frequencies of total monocytes, CMs and CD56⁺ NK cells were not associated with those of activated Tregs (eFigure 6).

Considering the positive correlation between the frequency of activated Tregs and NCMs (Figure 5(A)) and the antigen-presenting function of monocytes that interact with T cells, we hypothesized that NCMs affect the frequency of activated Tregs, which are known to regulate the pathology of MS [27,28]. To confirm our hypothesis, we cultured CD4⁺ T cells in the presence of NCMs or CMs from the same individual. Expectedly, the frequency of activated Tregs was significantly higher in co-cultures with NCMs compared to that with CMs or the control without monocytes, in both HCs and patients with RRMS (Figure 5(B)). The frequency of activated Tregs in the presence of CMs was similar to that in the control without monocytes (Figure 5(B)). The frequency of total Tregs showed a similar pattern to that of activated Tregs (Figure 5(C)).

Recently, circulating Ly6C⁺ monocytes, which correspond to human NCMs [17], have been shown to have a higher expression of PD-L1 than other monocyte subpopulations [29]. PD-L1 plays a pivotal role in the development of induced Treg cells (iTregs) and maintenance of their suppressive capacity [30]. Based on these findings, we examined the PD-L1 expression in NCMs or CMs in the peripheral blood of HCs and patients with NEDA and EDA states. Disease activity

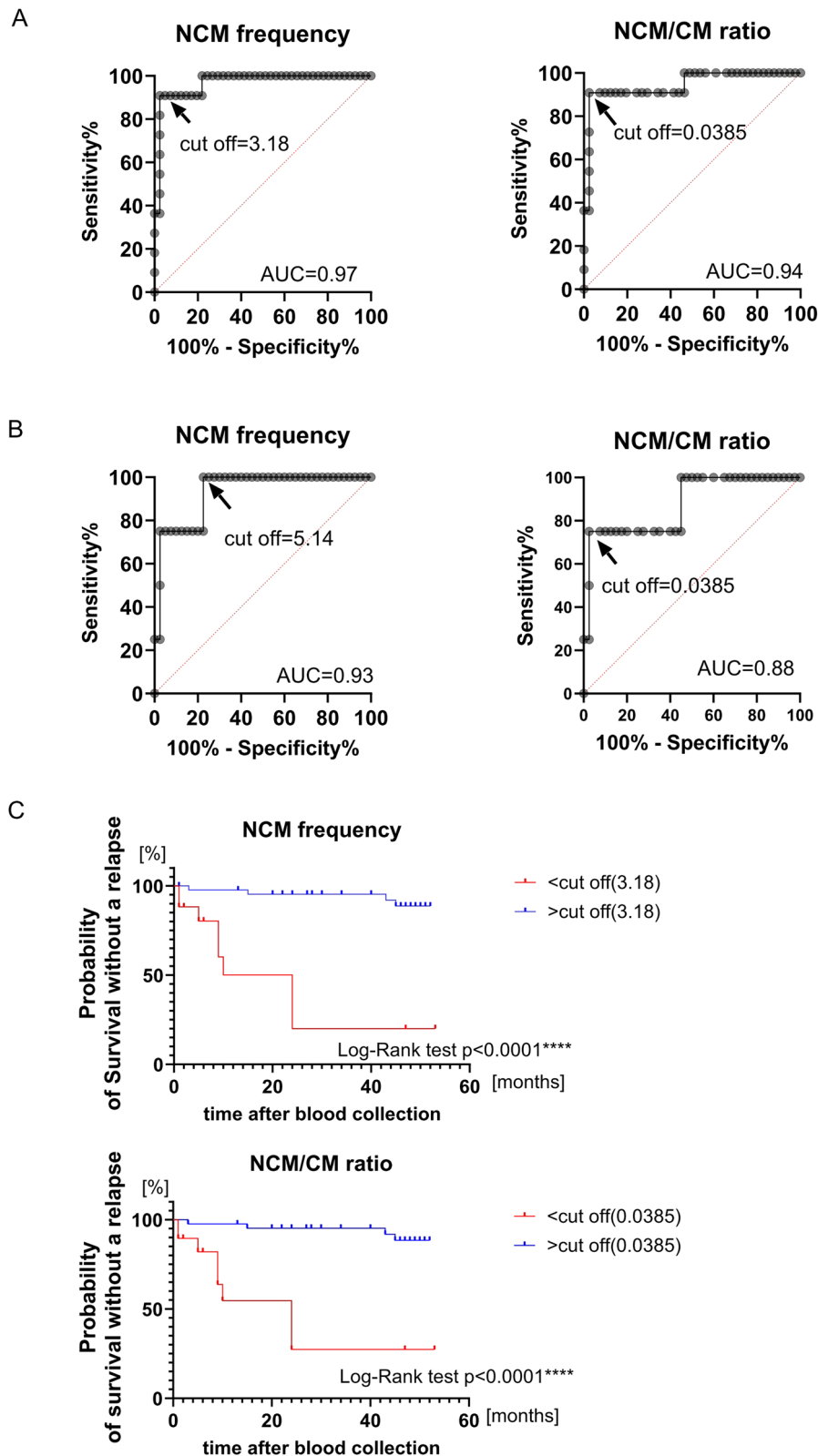


Figure 4. NCM frequency and the NCM/CM are possible predictive biomarkers for disease stability of RRMS. (A, B) ROC curves showing the predictive value of the NCM frequency or the NCM/CM ratio for absence of a relapse during the following two years after blood collection. Only the patients with a switch medication before a relapse for the following two years were excluded in (A) ($n = 52$). Patients in NEDA group under the same medication for the following two years ($n = 44$) were included in (B). (C) Kaplan–Meier curves showing the probability of survival without a relapse in all patients ($n = 61$) for the whole observation periods (ranging from 12 to 51 months). The patients were divided into two groups according to the cutoff value obtained from the ROC curve in (A). The cutoff value was set to 3.18% (NCM frequency) or 0.0385 (NCM/CM ratio). The patients were censored when they switched medication ($n = 13$) or the attending neurologist ($n = 1$). ‘Event’ was defined as a single clinical or radiological relapse. A Log-rank test was used to compare two survival curves. $****p < .00001$. NCM: non-classical monocyte; CM: classical monocyte; ROC: receiver operating characteristic; AUC: area under the curve.

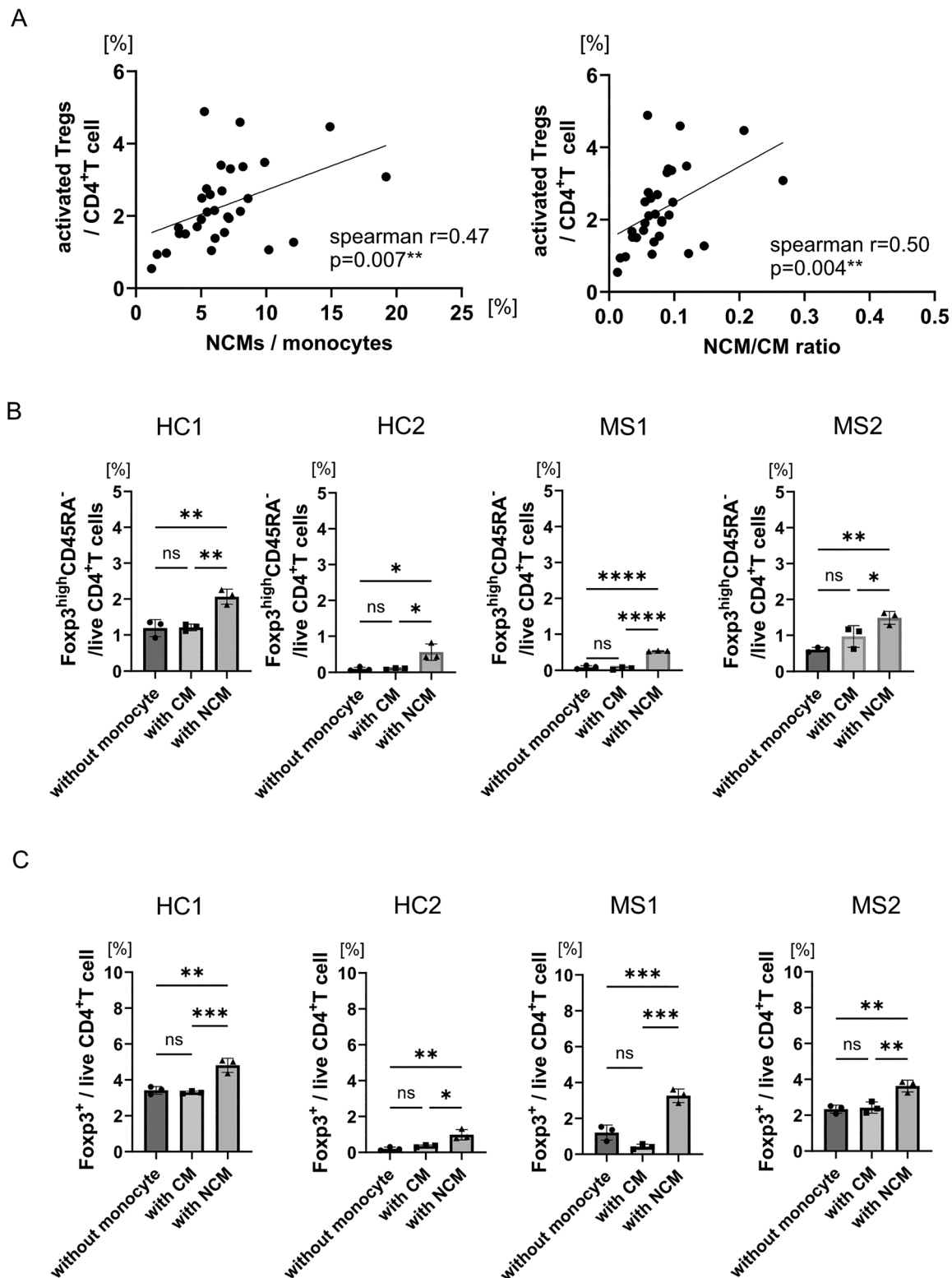


Figure 5. NCMs increase the proportion of activated Tregs. (A) Correlation analysis between the frequency of activated Tregs and the frequency of NCMs, or the NCM/CM ratio in the peripheral blood of patients in the NEDA group ($n = 31$; Cohort 1). The Spearman correlation analysis was used for statistical analysis. $^{**}0.001 \leq p < 0.01$. NCM: non-classical monocyte; CM: classical monocyte. (B, C) $CD4^{+}$ T cells and monocyte subpopulations (NCMs or CMs) were sorted from the peripheral blood of patients with RRMS ($n = 2$) and HCs ($n = 2$). The demographics of the participants are listed in eTable 2. Sorted $CD4^{+}$ T cells were co-cultured with sorted NCMs, CMs, or without monocytes (controls) in the presence of anti-CD3 and M-CSF for four days. After the exclusion of dead cells (Zombie Aqua-positive cells), the frequency of $CD45RA^{-}$ Foxp3^{high} activated Tregs (C) or Foxp3⁺ Tregs (D) among live $CD4^{+}$ T cells was analyzed using flow cytometry in each participant. Data were obtained in triplicates for each culture condition. p Values were calculated using one-way analysis of variance (ANOVA) with a Tukey's multiple comparison test. ns=not significant, $^{*}0.01 \leq p < 0.05$, $^{**}0.001 \leq p < 0.01$, $^{***}0.0001 \leq p < 0.001$, $^{****}p < 0.0001$. NCM: non classical monocyte; CM: classical monocyte; HC: healthy control; MS: multiple sclerosis.

was determined based on the disease course before sample collection. First, we found that PD-L1 expression was significantly higher in NCMs than in CMs in all three groups (Figure 6(A)). Notably, PD-L1 expression in NCMs was significantly higher in patients with NEDA status compared to those in the other two groups (Figure 6(B,C)). A similar trend was observed in the expression of PD-L1 in CMs, but the difference was not significant (Figure 6(B,C)). Collectively, these data suggest that NCMs increase the frequency of activated Tregs, possibly in part, *via* PD-L1 signaling.

4. Discussion

There is an unmet medical need for biomarkers that predict disease activity or stability of RRMS to manage patients' medications tailored to their disease activity. To date, several candidate predictors of disease activity have been proposed [31], including neurofilament light chain (NfL) in both the serum and cerebrospinal fluid (CSF) [32] and CXCL13 [33], glial fibrillary acidic protein (GFAP) [34] and 25-OH vitamin D [35] in the CSF. Although the survival analysis in a previous study showed that the

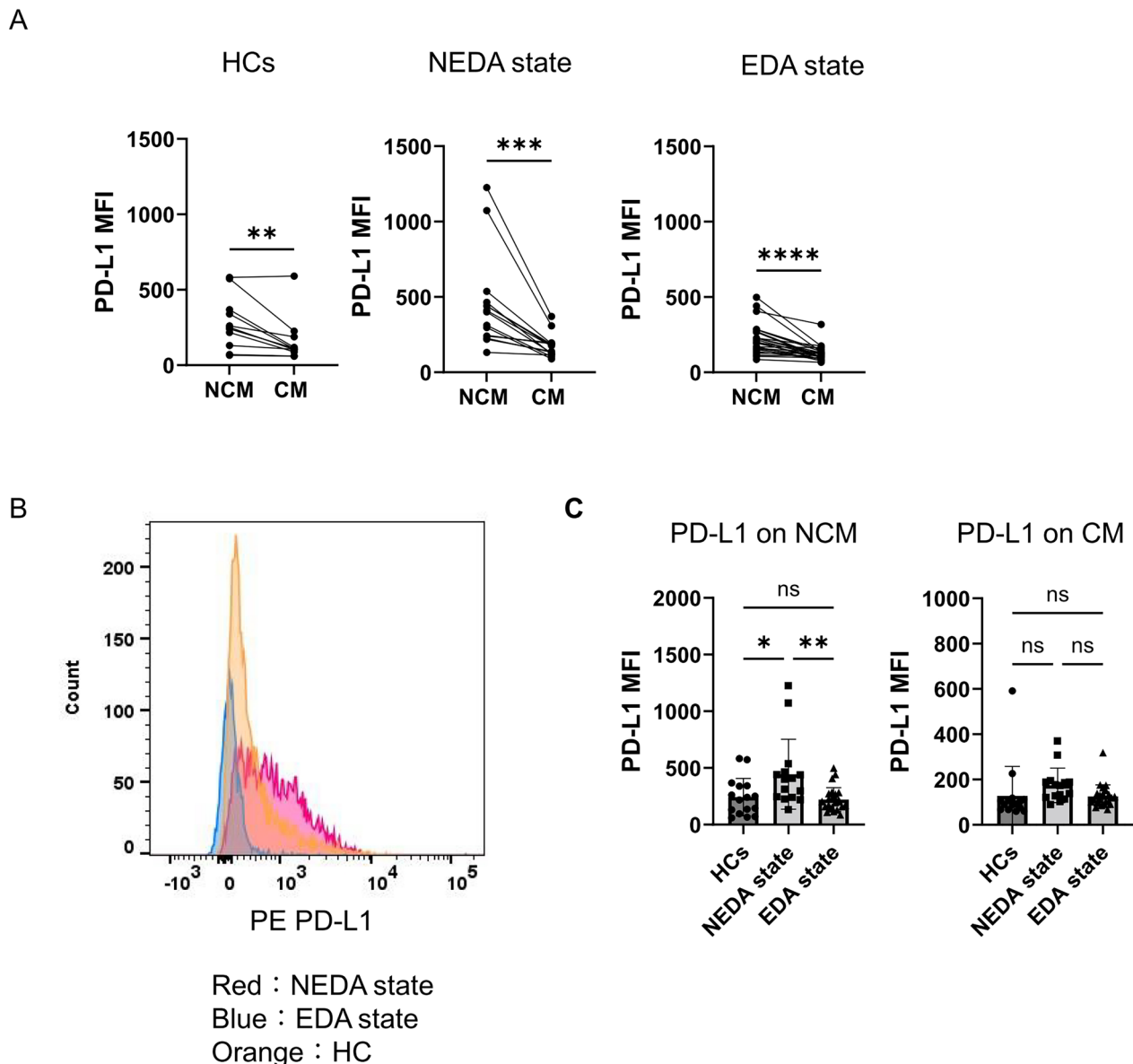


Figure 6. NCMs have a higher expression of PD-L1 than CMs and the PD-L1 expression in NCMs is higher in patients with the NEDA status than those with the EDA status. (A) The expression of PD-L1 on NCMs and CMs in the peripheral blood of HCs and patients with NEDA or EDA state. PD-L1 expression was compared based on the MFI, and the paired *t*-test was used for statistical analysis. (B) Representative histogram showing the MFI of PD-L1 on NCMs in the peripheral blood of HC (orange) and patients with NEDA (red) and EDA (blue) status. (C) Comparison of MFIs of PD-L1 on NCMs or CMs in the peripheral blood of HCs and patients with NEDA and EDA status. The Kruskal Wallis test followed by a Dunn's multiple comparison test was used for statistical analysis. ns=not significant, $^*0.01 \leq p < 0.05$, $^{**}0.001 \leq p < 0.01$, $^{***}0.0001 \leq p < 0.001$, $^{****}p < 0.0001$. MFI: mean fluorescence intensity; HCs: healthy controls; NEDA: no evidence of disease activity; EDA: evidence of disease activity; NCM: non-classical monocyte; CM: classical monocyte.

serum NfL level could distinguish the presence of a relapse over the next year [36], its predictive value for the two-year NEDA-3 status was not sufficient, with an AUC of 0.65 [32]. The AUC of NfL, CXCL13 and GFAP measured in the CSF for the two-year NEDA-3 status ranged from 0.75 to 0.85, with the highest AUC observed for CSF-NfL [32]. In this study, we found that the NCM frequency and the NCM/CM ratio predicted relapse-free status for two years with an AUC of 0.97 and 0.94, respectively (Figure 4(A)), suggesting that both the NCM frequency and the NCM/CM ratio could be reliable predictors of a long-term NEDA status.

Several studies have shown the altered proportion in B cell subsets (naïve, memory, or transitional B cells), T cell subsets (IFN γ ⁺CD4⁺T or Th1-like Th17 cells), or CD56^{high} NK cells in patients with MS who achieved NEDA-3 [37,38]. However, in these studies, the frequencies of immune cell subpopulations were compared before and after starting medication in each patient. By comparing patients with long-term NEDA-3 status to those with EDA status, we propose an altered monocyte composition, a high frequency of NCMs, as a distinctive immunological feature in the peripheral blood of patients with long-term disease stability of RRMS. Our results are supported by previous reports, which showed that the proportion of NCMs was higher in the peripheral blood of patients with inactive MS [21], but lower in treatment-naïve patients with RRMS than in HCs [20]. However, partially conflicting data are reported regarding the altered proportion of monocyte subsets in patients with RRMS: either a higher frequency of CD16⁺ monocytes [19] or NCMs [19,39], or a lower frequency of CD16⁺ monocytes [20,40]. These conflicting data might be due to the variety of disease activity at blood collection and monocyte lifecycles. Since monocytes differentiate into other monocyte phenotypes, or even macrophages and dendritic cells, and migrate out of circulation under host conditions [7,15], their composition in the peripheral blood is susceptible to host inflammatory conditions, including MS disease activity. Contamination of the intermediate phenotype to 'CD16⁺ monocyte' or 'CD14⁺CD16⁺ monocyte' may also explain the discrepancy. IMs are now recognized as a distinct subpopulation from NCMs and have been described as an inflammatory phenotype in rheumatoid arthritis [41,42]. We detected slight differences in the frequencies of CD56⁺ NK cells, total monocytes and CMs only in Cohort 1 (Figure 1(A)) and IMs only in Cohort 2 (Figure 2(B)). This inconsistency might be because the differences are small or highly variable and/or because of the small number of participants.

Considering the previous data that several disease modifying drugs induced anti-inflammatory monocytes in EAE mice [13,14] and recombinant IFN β could affect monocyte chemokine receptor and cytokine production [43], the higher frequency of NCMs seen in patients of the NEDA group might be partially affected by DMTs. Moreover, exogenous glucocorticoid was reported to induce expansion of CMs in bone marrow in rats [44], suggesting glucocorticoid therapy itself could influence monocyte composition in peripheral blood. However, IFN β -treated patients in the EDA group had lower frequency of NCMs and the NCM/CM ratio than the cutoff value obtained from ROC analysis for prediction of NEDA status (eFigures 3 and 5), while these markers were also above the cutoff values in IFN β -treated patients in the NEDA group. Moreover, all of treatment-naïve patients included in the NEDA group had both higher NCM frequency and NCM/CM ratio than the cutoff values. Furthermore, the NCM frequency and NCM/CM ratio were higher in the NEDA group than in the EDA group, when only the patients treated with prednisolone were analyzed (eFigure 3(C)). These data suggest that the NCM frequency and NCM/CM ratio reflect disease stability rather than the influence of their treatment.

Correlation analysis between clinical features and frequency of monocyte subsets revealed that the NCM frequency, as well as NCM/CM ratio, was positively correlated with the duration of NEDA-3 status (Figure 3(B)). Although NCMs in peripheral blood were previously reported as an age-dependent subset [26], age did not associate with the NCM frequency and NCM/CM ratio in this study (eFigure 4(B)). These data supported our hypothesis that the influence of aging might be quite small in a particularly high frequency of NCMs in patients with a long-term NEDA state. The discrepancy between the results from the previous study [26] and our study may be explained by the fact that the former analyzed healthy individuals and we have analyzed patients with MS. Our results further support the role of altered monocyte composition in MS. We also demonstrated that both the NCM frequency and NCM/CM ratio were negatively correlated with EDSS score (eFigure 4(A)). Patients who maintain a long-term NEDA-3 status generally have lower EDSS scores than those with disease activity owing to a lack of relapse or progression. Consistently, EDSS was negatively correlated with the duration of NEDA-3 status in our cohort (eFigure 4(C)), suggesting that higher frequency of NCMs, as well as higher NCM/CM ratio, were closely associated with long-term disease stability and not just the low severity, indicated by the lower EDSS score.

Tregs play a pivotal role in MS because of their ability to suppress excessive inflammation [24,27,45]. In this study, we demonstrated that the frequency of NCMs is positively associated with the frequency of activated Tregs, which are a highly suppressive subpopulation [24], and that NCMs can directly increase the frequency of activated Tregs *in vitro*. Previous reports demonstrated that iTregs development is promoted by PD-L1 signaling, which is involved in reducing T cell activation, proliferation and cytokine production [46] and as well as sustaining the suppressive function [30].

PD-L1 is a ligand of PD-1 and a previous study showed that PD-L1 was expressed in human monocytes [47]. In our study, a higher expression of PD-L1 was observed in NCMs than in CMs (Figure 6(A)), which is supported by a previous mouse study [29]. In addition, PD-L1 expression in NCMs was higher in the NEDA group than in the EDA group, but there was no significant difference in CMs between the two groups (Figure 6(C)). These findings suggested that NCMs were functionally different from CMs and that circulating NCMs can increase the frequency of activated Tregs, possibly via PD-L1 signaling.

A previous report showed that IFN- β treatment induced PD-L1 expression on human monocytes *in vitro*, and that mRNA expression of PD-L1 was increased in PBMC of patients with RRMS after induction of IFN- β [47]. Although we could not compare the PD-L1 expression in NCMs between IFN- β -treated patients with NEDA and EDA status due to lack of samples in the latter group, its expression tended to be higher in treatment-naïve patients with NEDA status compared to those with EDA status (data not shown). These data suggest that PD-L1 expression in NCMs cannot be explained only by the influence of DMTs but rather more related to disease stability.

The protective role of NCMs proposed in this study is supported by a previous mouse study, in which NR4A1^{-/-} mice lacking in Ly6C^{low} monocytes [48] exhibited exacerbated severity of EAE [49]. Ly6C^{low} monocytes have the CX3CR1^{high}CCR2^{low} phenotype and patrol peripheral tissues using CX3CR1 to maintain homeostasis rather than phagocytic function [7,15]. These data suggest that NCMs play an important role in disease stability of RRMS.

The major limitation of this study was the number of participants. Our study included a small number of participants from a single hospital. Due to the lack of DMT-matched data in the NEDA and EDA groups, it is not possible to accurately assess the influence of DMTs. We also have no CSF data that would provide information on whether the altered

monocyte composition in the peripheral blood is a consequence of the migration of specific monocyte subsets into the CNS. Further investigations with a larger number of participants and a DMT-matched cohort should strengthen our findings.

In summary, our findings suggest that NCMs play a protective role in RRMS by increasing the frequency of activated Tregs probably, in part, *via* PD-L1 signaling. In addition to its protective role, its frequency as well as altered monocyte composition (NCM/CM ratio) may be useful biomarkers for predicting current and foreseeable future disease stability.

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Author's contributions

MM, WS, KK, AK, LY, TO, RT and TY are drafting/revising of the article for content, including medical writing for content. MM, WS, LY, TO and TY have a major role in the acquisition of data. MM, WS, KK and TY have a major role in study concept or design; and analysis or interpretation of data.

Disclosure statement

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Data availability statement

All data relevant to this study are included in the article and [supplementary data](#).

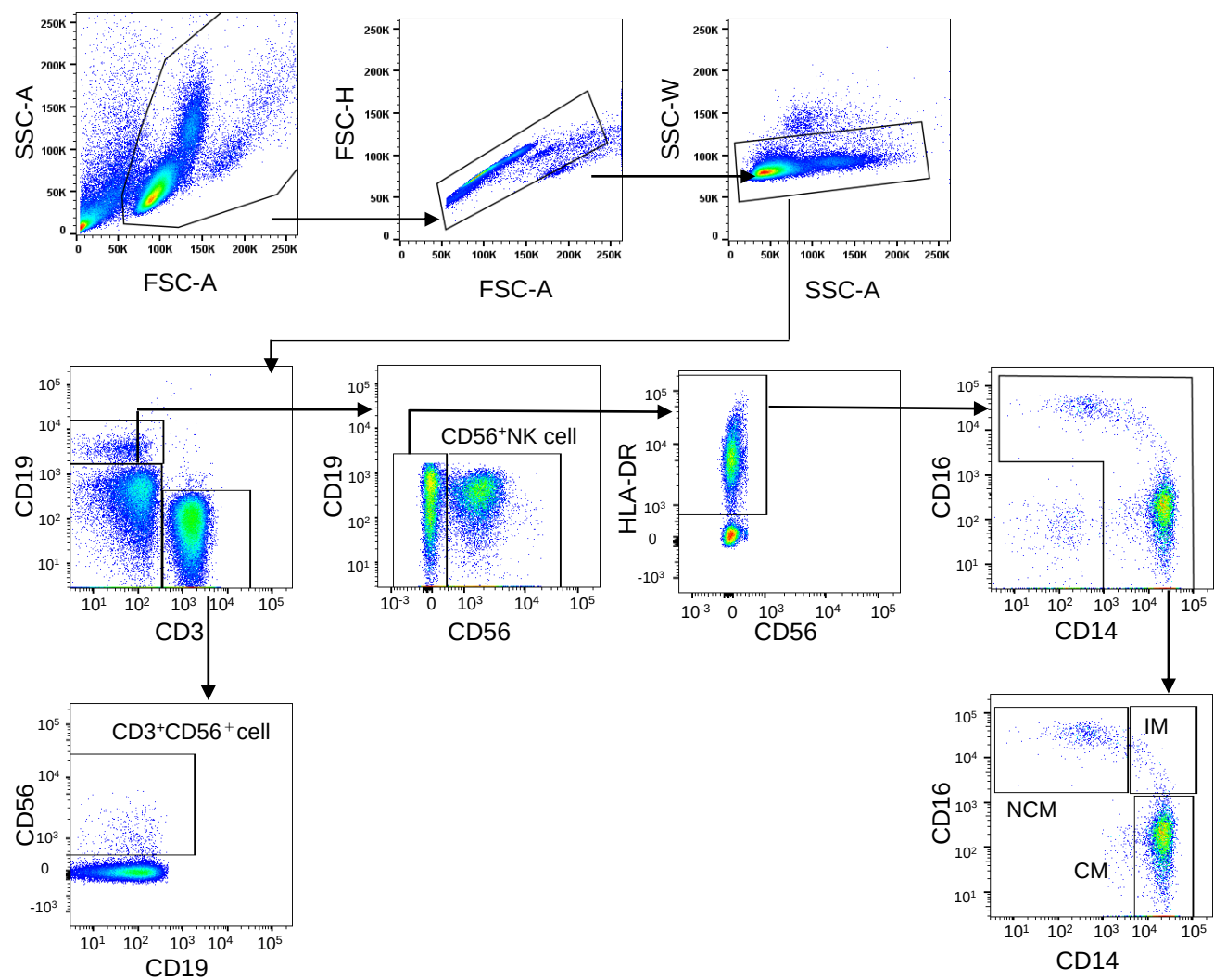
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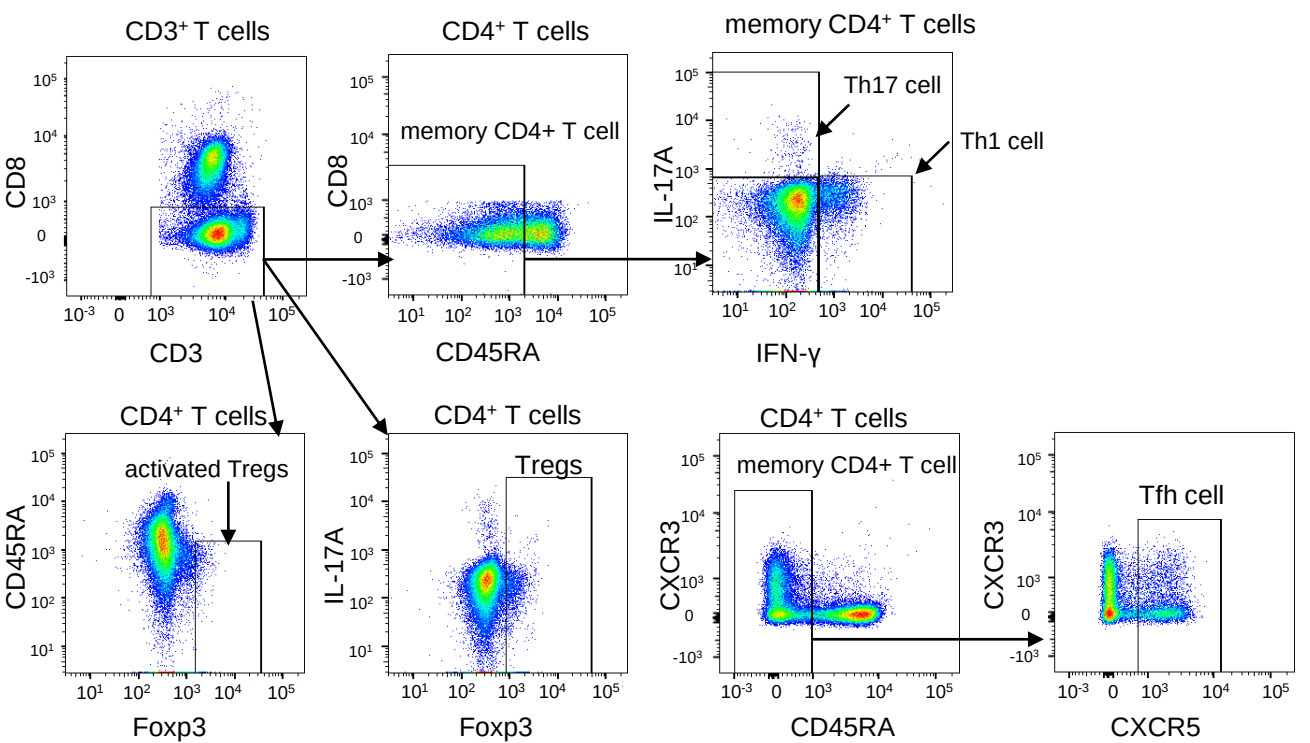
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eFigure 1

A

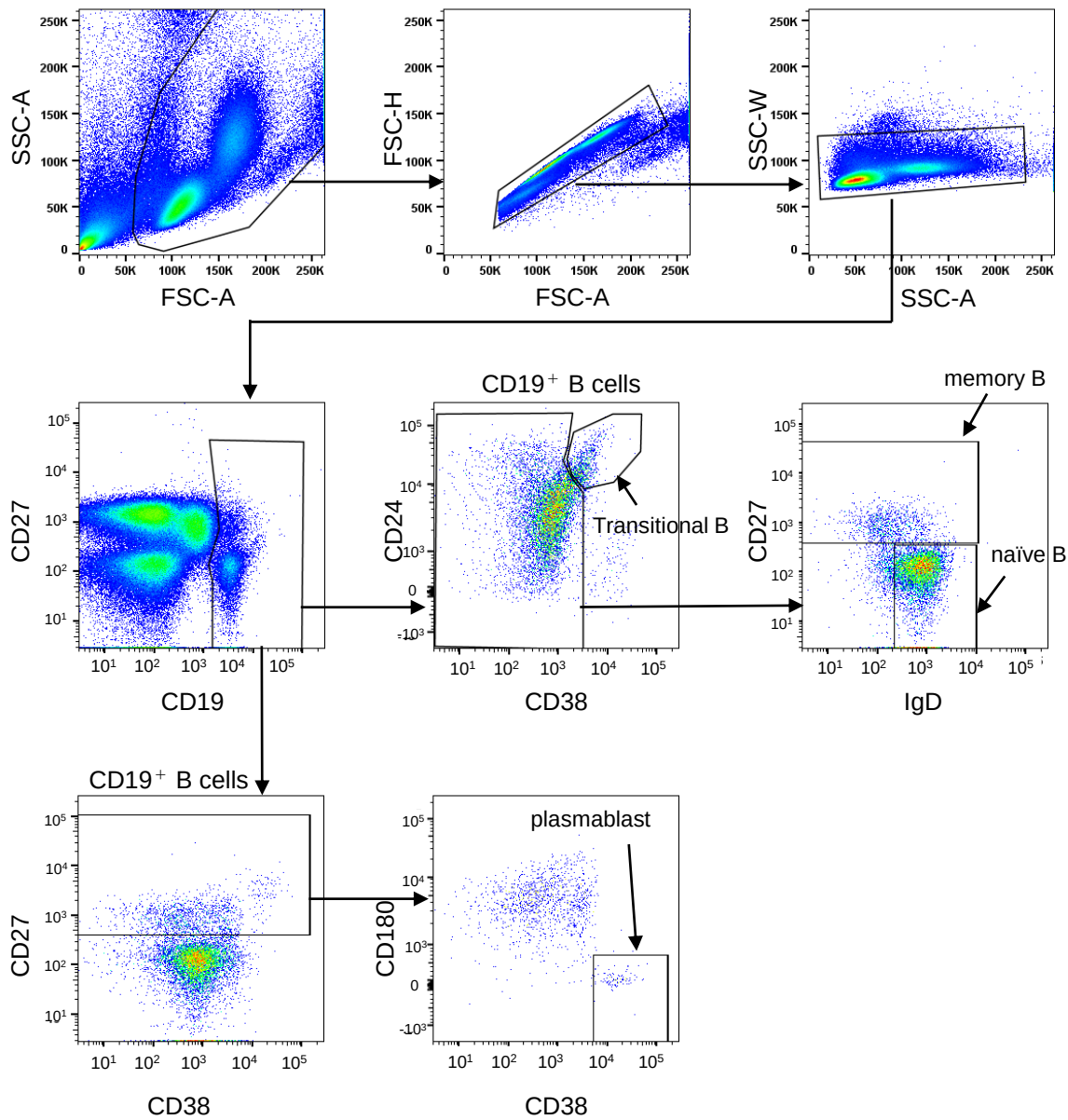


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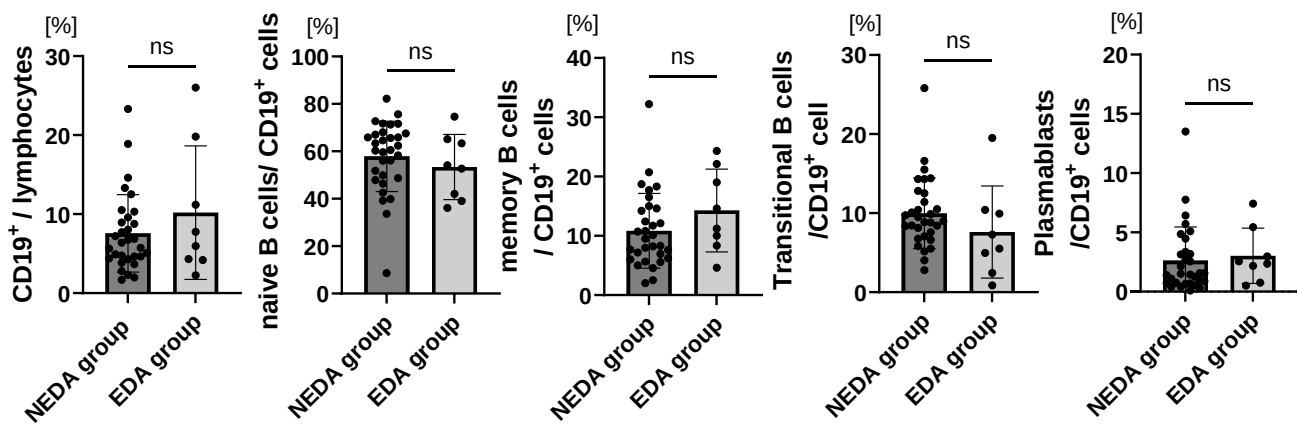


eFigure 2

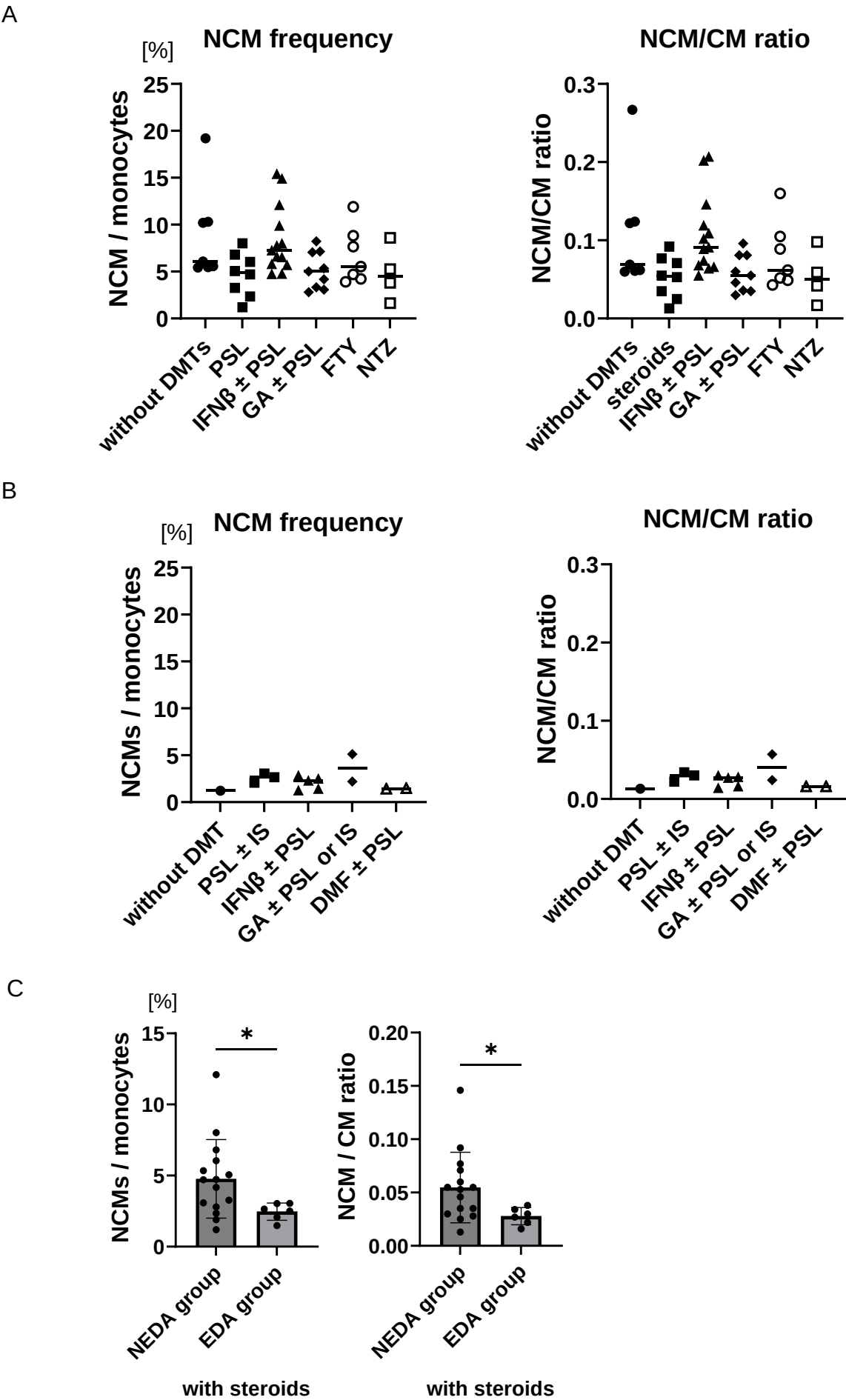
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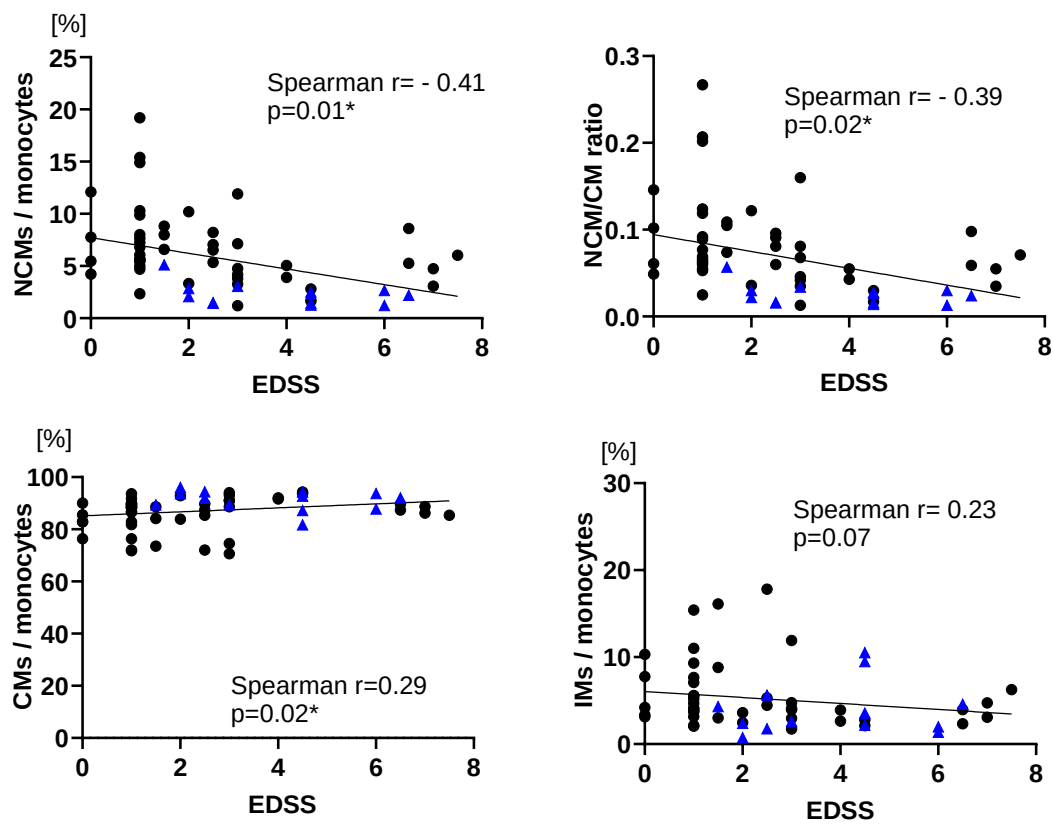


eFigure 3

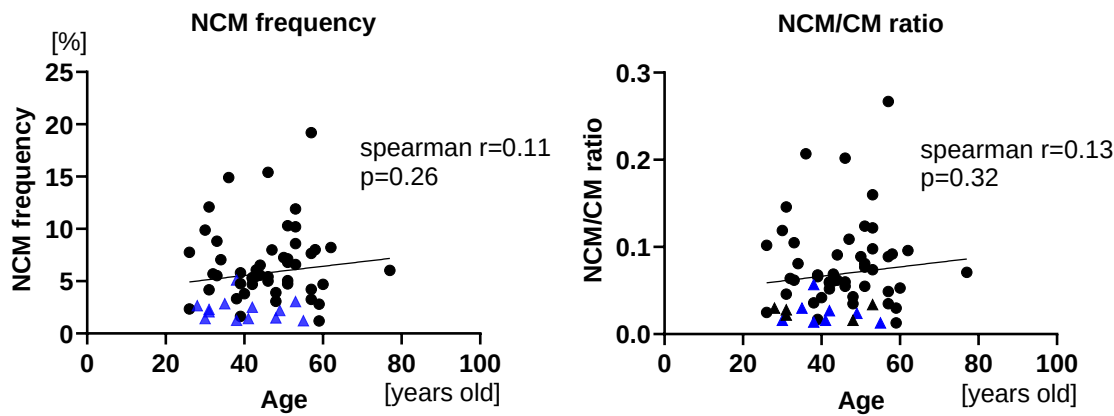


eFigure 4

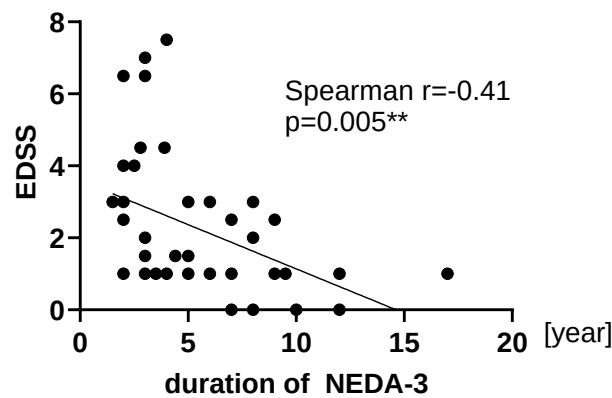
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B

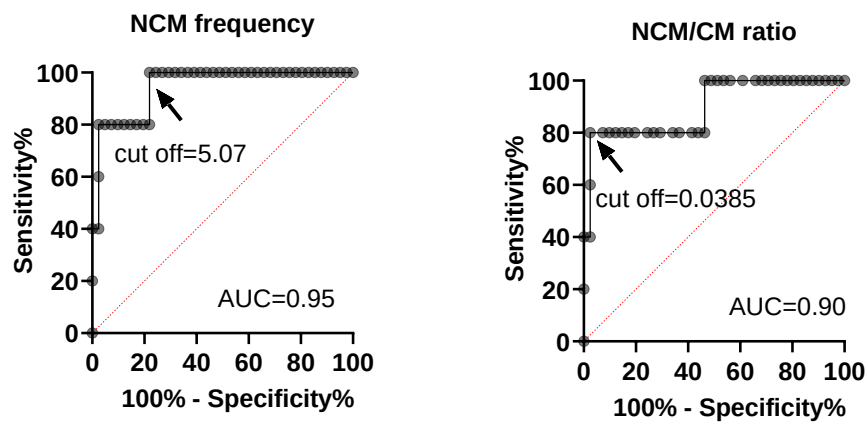


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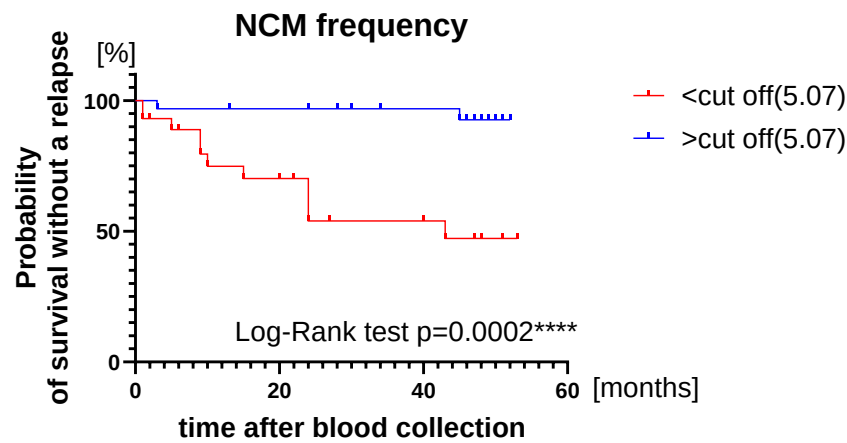


eFigure 5

A

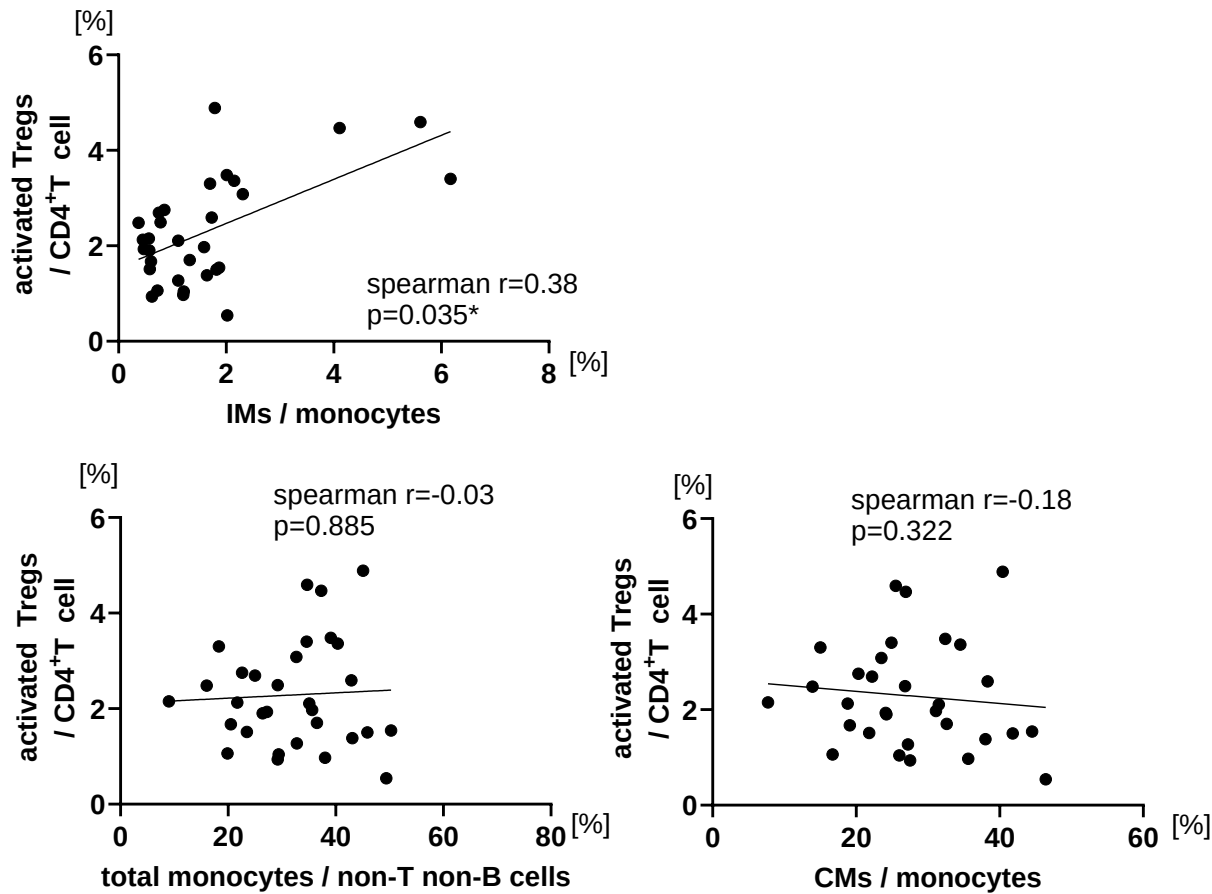


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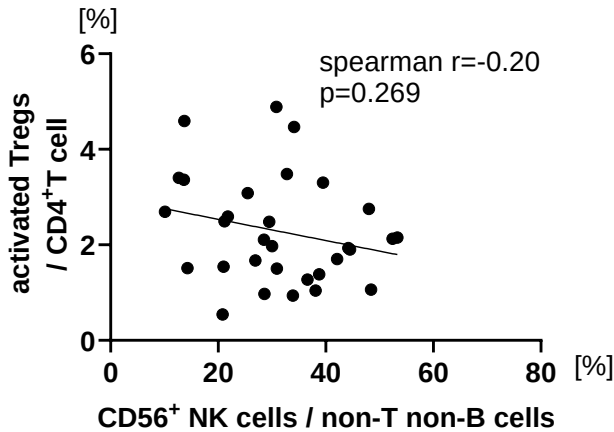


eFigure 6

A



B



Supplementary (eFigures1-6 and eTables1-3)

eFigure captions

eFigure 1 Gating strategy for non-T non-B cell and T cell subsets.

(A) Gating strategy and representative flow cytometry plots showing CD56⁺ NK cells, CD3⁺CD56⁺ cells, and monocyte subsets (classical [CMs], intermediate [IMs], and non-classical monocytes [NCMs]). (B) Gating strategy and representative flow cytometry plots showing the T cell subsets.

eFigure 2 Frequencies of B cell subsets in the peripheral blood are similar between the NEDA group and the EDA group.

(A) Gating strategy and representative flow cytometry plots showing B cell subsets. (B) PBMCs of patients in the NEDA and EDA groups were analyzed for frequencies of the following B cell subsets: CD19⁺ B cells among mononuclear cells and naïve B cells (CD27⁻ IgD⁺CD38^{low} B cells), memory B cells (CD27⁺CD38^{low} B cells), transitional B cells (CD24^{high} CD38^{high} B cells) and plasmablasts (CD27⁺CD38^{high}CD180⁻ B cells) among CD19⁺ B cells. The Mann-Whitney *U* test was used for statistical analysis. Error bars represent the mean $\pm$ SD. ns = not significant. NEDA: no evidence of disease activity; EDA: evidence of disease activity.

eFigure 3. NCM frequency and the NCM/CM ratio did not differ between the two specific DMT.

(A, B) The frequency of NCMs or the NCM/CM ratio in the peripheral blood of patients taking

medications as shown. Patients in the NEDA group (n = 48) (A) or those in the EDA group (n = 13) (B) of Cohorts 1 and 2 are included in this analysis. Although a slight difference was observed by one-way analysis of variance for patients in the NEDA group (A) (NCM frequency, $p = 0.04$; and NCM/CM ratio, $p = 0.03$), Tukey's multiple comparison tests showed no significant difference between any specific DMTs in both analyses. (C) The frequency of NCMs (left) and the NCM/CM ratio (right) in peripheral blood of patients in the NEDA and EDA group. Only the patients treated with prednisolone $\pm$ other DMTs (NEDA=15, EDA=6) were analyzed. Even when the effects of steroid treatment on NCM frequency are considered, the difference between the NEDA group and the EDA group in the frequency of NCMs or the NCM/CM ratio is apparent. NCM: non classical monocyte, CM: classical monocyte, DMT: disease modifying therapy; IS: immunosuppressant; IFN β : interferon-beta; GA: glatiramer acetate; DMF: dimethyl fumarate; FTY: fingolimod; NTZ: natalizumab.

eFigure 4 Correlation analysis between some clinical features and the frequency of monocyte subpopulations

(A) Correlation analysis between EDSS and the NCM/CM ratio or the frequency of three monocyte subsets (B) Correlation analysis between age and the NCM frequency or NCM/CM ratio in peripheral blood. All participants with MS in Cohort 1 and Cohort 2 (n=61) were included in (A) and (B). The data of EDA group are shown as blue triangles. (C) Correlation analysis between EDSS and duration of NEDA-3 at the blood collection in the NEDA group

of Cohort 1 and 2. Spearman's correlation analysis was used to evaluate correlations. $*0.01 \leq p < 0.05$, $**0.001 \leq p < 0.01$. EDSS: Expanded Disability Status Scale; NCM: non classical monocyte; IM: intermediate monocyte; CM: classical monocyte.

eFigure 5 NCM frequency and the NCM/CM ratio could be useful predictors for disease stability

(A) ROC curve analysis showing the predictive value of the frequency of NCMs and the NCM/CM ratio for absence of a relapse for the following 2 years. Patients without a switch of medication at any time point for the following two years ($n = 46$) were included in this analysis. The black arrow in each graph shows the optimal cutoff value. (B) Survival curve showing probability of survival without relapse in all patients ($n = 61$) same as that in Figure 4C. Patients were divided according to the cutoff value obtained from ROC curve of NCM frequency in (A), which was set to 5.07. The cutoff value of the NCM/CM in (A) was same as that in Figure 4A (0.0385); therefore, its survival curve was same as that in Figure 4C. Log-rank test was used to compare two survival curves. $***0.0001 \leq p < 0.001$. NCM: non-classical monocyte; CM: classical monocyte.

eFigure 6 Correlation analysis between the frequency of activated Tregs and the frequency of monocyte subsets or CD56⁺ NK cells

(A, B) Correlation analysis between the frequency of activated Tregs and the frequencies of

1 IMs, total monocytes and CMs (A), or CD56⁺ NK cells (B) in the NEDA group of Cohort 1.
2 Data from Figure 1 are used for this analysis. Spearman's correlation analysis showed that the
3 IM frequency was slightly correlated with the frequency of activated Tregs (p=0.035, Spearman
4 r=0.38). No association was observed between the frequency of activated Tregs and total
5 monocytes, CMs or CD56⁺ NK cells. *0.01 $\leq p < 0.05$. NEDA: no evidence of disease activity;
6 NCM: non-classical monocyte; IM: intermediate monocyte; CM: classical monocyte.

7

8

eTable 1. Clinical data of all patients with RRMS included in Table 1 during the follow-up periods, ranging from 12 to 51 months.

Clinical data of all patients with RRMS included in Table 1 during the follow-up periods are collected by checking medical records. Clinical data during the two-year observation period and overall observation period are listed. NEDA: no evidence of disease activity; EDA: evidence of disease activity; DMT: disease modifying therapy; IQR: Interquartile range.

	NEDA group (n = 48)	EDA group (n = 13)
For the following 2 years		
Patients who switched the attending hospital	0	1
Patients with a switch medication	4	10
Patients having a relapse	4	11
Having a relapse with a switching of DMT	0	9
Having a relapse under the same DMT	4	2
Patients with relapse-free state	44	1
Relapse-free state with a switch of DMT	4	1
Relapse-free under the same DMT	40	0
For the whole observation periods		
Observation periods after blood collection (median [IQR]; months)	49.0[47.0-51.0]	46.0[33.5-48.5]
Patients who switched the attending hospital	0	1
Patients with a switch medication	19	11
Patients having a relapse	10	11
Having a relapse with a switching of DMT	4	4
Having a relapse under the same DMT	6	7
Patients with relapse-free state	38	1
Relapse-free state with a switch of DMT	25	1
Relapse-free under the same DMT	13	0

eTable 2 Demographics of participants in the T cell-monocyte coculture assays. (Figure 5B, C).

HC: healthy control; EDSS: Expanded Disability Status Scale; OCB: oligoclonal bands; DMT: disease modifying therapy; IFN β : interferon-beta.

	HC (n=2)	Patients with RRMS (n=2)
Age (mean [SD])	33.5[2.1]	40.0[9.9]
Male : Female	1:1	0:2
Disease duration (years) (mean[SD])		7.0 [5.7]
EDSS (median [range])		1.0 [0.0]
OCB(positive: negative: unknown)		0:1:1
Maintenance treatment		
Without DMTs		1
IFN- β		1

1

2

eTable 3 Demographics of patients with RRMS and HCs in the PD-L1 expression analysis

(Figure 6)

NEDA: no evidence of disease activity; EDA: evidence of disease activity; HC: healthy control; EDSS: Expanded Disability Status Scale; ARR: annual relapse rate; OCB: oligoclonal bands; DMT: disease modifying therapy; PSL: prednisolone; IFN β : interferon-beta; DMF: dimethyl fumarate; FTY: fingolimod; NTZ: natalizumab.

	NEDA state (n = 15)	EDA state (n = 23)	HCs (n = 17)
Age (mean [SD])	43.0 [13.5]	43.6 [10.9]	47.4 [11.5]
Male: Female	2:13	9:13	5:12
Disease duration (years) (median [IQR])	11.0 [7.0-16.0]	8.0 [4.5-12.5]	
EDSS (median [range])	2.5 [1.0-8.0]	3.5 [1.0-6.5]	
Duration of NEDA-3 (years) (median [IQR])	3.0 [2.0-5.0]		
ARR (mean[SD])	0.0 [0.0]	2.0 [1.0-3.0]	
OCB (positive: negative: unknown)	7:1:7	12:4:7	
Maintenance treatment	(daily dose of prednisolone; mean $\pm$ SD mg/day)		
Without DMT	2	4	
PSL	1 (10mg)	4 (6.2 $\pm$ 4.3mg)	
PSL + IS	0	3 (4.7 $\pm$ 0.6mg)	
IFN- β	1	0	
IFN- β + PSL	1 (1.25mg)	0	
GA	2	0	
GA + PSL	3 (3.2 $\pm$ 2.0mg)	4 (3.9 $\pm$ 2.8mg)	
DMF	2	3	
DMF + PSL	0	4 (5.0 $\pm$ 3.7mg)	
FTY	2	0	
NTZ	1	1	