Research article

The ratio of circulating CD56$^{\text{dim}}$ NK cells to follicular T helper cells as a promising predictor for disease activity of relapsing-remitting multiple sclerosis☆

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A B S T R A C T

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system primarily mediated by CD4$^+$ T helper cells. This study investigated the dynamic changes of natural killer (NK) cells and follicular T helper (Tfh) cells and their associations in relapsing-remitting MS patients. The findings revealed inverse relationships between NK cells and CD4$^+$ T cells or Tfh cells. Specifically, CD56$^{\text{dim}}$ NK cells, not CD56$^{\text{bright}}$ NK cells, were negatively correlated with CD4$^+$ T cells and Tfh cells. However, no significant correlations were found between NK cells and sNfL levels or EDSS scores. The ratio of CD56$^{\text{dim}}$ NK cells to circulating Tfh (cTfh) cells demonstrated superior discriminatory ability in distinguishing relapsing MS patients from healthy controls (HCs) and remitting patients, as determined by receiver operating characteristic (ROC) analysis. Following treatment with immunosuppressants or disease-modifying therapies (DMTs), a significant increase in the CD56$^{\text{dim}}$ NK/cTfh ratio was observed. These findings suggest that the CD56$^{\text{dim}}$ NK/cTfh ratio holds promise as a prognostic indicator for clinical relapse and treatment response in MS.

1. Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) that is recognized as the primary cause of non-traumatic neurological disability among young adults [1]. The pathogenesis of MS is intricate, and it is believed that T cell- and B cell-dominated adaptive immunity plays a fundamental role in MS [2]. Oligodendrocytes and neurons are adversely affected by the direct cytotoxicity of CD8$^+$ T cells, as well as the inflammatory processes instigated by IFN-γ-secreting T helper (Th) 1 cells and IL-17-producing Th17 cells [3]. Furthermore, the significance of B cells in the pathogenesis of multiple sclerosis is substantiated by clinical trials evaluating B-cell depletion therapies [4], underlining the crucial role of follicular T helper (Tfh) cells in facilitating B cell development and immunoglobulin synthesis [5,6].

In recent years, there has been a growing body of evidence demonstrating the significant involvement of trained innate immunity in MS [7]. Of particular interest are natural killer (NK) cells, which exhibit a notable expansion in response to disease-modifying therapies (DMTs) [8–10]. NK cells are classified as group 1 innate lymphoid cells (ILC1) and possess the ability to rapidly identify and destroy "altered self" cells, such as tumor cells and virus-infected cells. The activation of NK cells is dependent on the dysregulation of signaling

mediated by activating and inhibitory receptors, as well as the presence of inflammation-related cytokines [11]. Apart from being the key defense in innate immunity, NK cells can promote or impair T cell immunity directly via IFN-γ or indirectly through dendritic cell modulation during the early phase of viral infections [12]. They can also contribute to chronic infection by diminishing Tfh cells and humoral immunity [13]. In the context of autoimmune diseases, NK cells, mainly the CD56^{bright} subset can eliminate autoreactive T cells by perforin [14]. Whereas in MS studies, the elimination of autologous autoreactive CD4^{+} T cells by CD56^{bright} NK cells was impaired [15,16]. CD56^{dim} NK cell was also shown to have the ability to kill activated T cells [17], despite its pro-inflammatory properties and potential antibody-dependent cellular cytotoxicity towards the CNS [18,19]. These findings imply the necessity of distinguishing between various subsets of NK cells in autoimmunity. Recently, a high ratio of NK cells to CD4^{+} T cells has been demonstrated to be beneficial for MS remission after interferon β or autologous hematopoietic stem cell transplantation therapies [20, 21], indicating a regulatory role of NK cells on CD4^{+} T cells. However, it is still undetermined regarding the dynamics of NK cell subsets and their correlations with CD4^{+} T cell subsets during the disease course of relapsing-remitting MS (RRMS). In this study, our goal is to investigate the relationship between NK cell subsets and CD4^{+} T cells, specifically Tfh cells, in RRMS to identify the potential predictors of clinical relapses and treatment effectiveness.

2. Materials and methods

2.1. Study population

Patients with RRMS admitted to our center from August 2021 to June 2023 fulfilling the 2017 McDonald's diagnostic criteria [22] were enrolled in this study. A clinical relapse was defined as the appearance of new neurological dysfunctions lasting over 24 h (hrs) with new lesions on MRI scanning. Remission status was considered as a stable clinical condition for more than one month since the last relapse. The Kurtzke's Expanded Disability Status Scale (EDSS) scores were evaluated by two attending physicians separately. Meanwhile, sex- and age-matched healthy controls (HCs) were included in this study. To assess the treatment efficacy for acute attacks, 8 patients with RRMS were further enrolled and sampled before and after treatment.

2.2. Peripheral blood mononuclear cells isolation

Peripheral blood samples were drawn from the cubital vein of all the patients and HCs and collected in anticoagulant tubes. Peripheral blood mononuclear cells (PBMCs) were isolated with Ficoll-Paque medium (Dakewe, China) by gradient-density centrifugation at 2000 rpm for 30 min (min) at room temperature without brake. Subsequently, the top layer, which consisted of plasma, was removed and the second layer, composed of lymphocytes and monocytes, was transferred into a fresh tube. PBMCs were then washed with phosphate-buffered saline (PBS) supplemented with 2 % fetal calf serum (Invitrogen, USA) followed by downstream staining procedures.

2.3. Flow cytometry

For surface antigen staining, cell suspensions were incubated with fluorescent antibodies and IgG isotypes at 4 °C for 30 min. After washing the cells with PBS containing 2 % fetal calf serum twice, cell frequencies and counts were detected by a Navios flow cytometer (Beckman coulter, USA) and analyzed with Kaluza software. The antibodies applied in this study were as follows: anti-human CD3-percp (UCHT1; Biolegend), anti-human CD4-FITC (OKT4; Biolegend), anti-human CXCR5-PE (J252D4; Biolegend), anti-human PD-1-allophycocyanin (EH12.2H7; Biolegend), anti-human CD56-FITC (5.1H11; Biolegend) and anti-human CD16-PE (3G8; Biolegend). Precision count beads™ (Biolegend) were used to determine the absolute count of cells.

2.4. Serum neurofilament light chain measurement

Serum neurofilament light chain (sNfL) levels were measured with ultrasensitive single molecule array technology using an HD-1 analyzer (Quanterix, Billerica, MA) by board-certified laboratory technicians blinded to clinical data, as previously described [23]. Briefly, target antibody-coated paramagnetic beads were combined with the serum sample and biotinylated detector antibody in the same incubation. Following a wash, a conjugate of streptavidin-β-galactosidase was mixed with the beads. After a final wash, the beads were resuspended in a resorufin β-D-galactopyranoside substrate solution and sealed within microwells in the array for signal measurement by the Simoa optical system.

2.5. Statistical analysis

Statistical analyses were performed with GraphPad Prism 8.0 (GraphPad Software, USA). Kruskal-Wallis H test was used to compare the age among relapsing MS patients, remitting MS patients and HCs. Mann-Whitney U test was used to assess the differences of onset age, disease duration, EDSS score and sNfL level among MS groups. Fisher's exact test was applied in the comparison of the ratio of females. For statistical analysis of multiple groups, one-way ANOVA was used to compare the differences. Paired t-test or Wilcoxon test was used to compare the differences between pre- and post-treatment according to the data normality. Nonparametric Spearman rank analysis or Pearson correlation analysis was used to assess the correlation between two variables of interest based on the characteristics of data distribution. A p value < 0.05 was considered statistically significant in all analyses.

3. Results

3.1. Demographic and clinical characteristics of RRMS patients and HCs

A total of 51 RRMS patients (22 relapsing, 29 remitting) and 20 HCs were enrolled in this study, with the demographic and clinical characteristics shown in Table 1. There were no statistical differences in the age and female ratio among the three groups ($P = 0.4350$; $P = 0.1612$). There were also no significant differences in the onset age and disease duration between relapsing and remitting MS patients ($P = 0.0886$; $P = 0.1323$). There was no difference in the EDSS score between the relapsing and remitting MS patients ($P > 0.9999$), and unexpectedly no difference in the serum NfL levels ($P = 0.1987$). Four of the 22 relapsing patients were receiving immunotherapies including steroids, intravenous immunoglobulin and DMTs alone or in combination at sampling. Moreover, 14 of the 29 remitting patients were on medication at sampling and 10 of them were treated with DMTs.

3.2. NK cells negatively correlate with CD4$^+$ T cells and cTfh cells in MS

To figure out the relationships between NK cells and cTfh cells and the disease activity of MS, the frequencies and counts of total NK cells (CD3$^-$CD56$^+$) and cTfh cells (CD3$^+$CD4$^+$CXCR5$^+$PD-1$^+$) in RRMS patients and healthy controls were evaluated by flow cytometry (Fig. 1A). Total NK cells decreased significantly during the relapsing phase, and recovered during the remission phase (Fig. 1B and C). Meanwhile, the frequency of cTfh cells increased significantly during the relapsing phase, but decreased profoundly during the remission (Fig. 1D). However, cTfh cell counts in MS patients only showed a slightly increase compared with HCs, and no significant difference was observed between the relapsing and remitting patients (Fig. 1E). There were pronounced negative correlations between total NK cells and CD4$^+$ T cells for all MS patients ($r = -0.6311$, $p < 0.0001$), the relapsing patients ($r = -0.7714$, $p < 0.0001$), and the remitting patients ($r = -0.4871$, $p = 0.0074$) (Fig. 1F–H; Supplementary Fig. s1A, left column). It was interesting to note that there was a significant inverse correlation between total NK cells and cTfh cells in all MS patients ($r = -0.3454$, $p = 0.0130$) but not in relapsing patients ($r = -0.3323$, $p = 0.1308$) or the remitting patients ($r = -0.1653$, $p = 0.3915$) (Fig. 1G–I; Supplementary Fig. s1B, left column). These results suggested sensitive changes of NK cells following disease activity and a potential role of NK cells in regulating cTfh cells in MS.

3.3. CD56^{dim} NK cells negatively correlate with CD4$^+$ T cells and cTfh cells in MS

We further evaluated circulating CD56^{dim} NK cells and CD56^{bright} NK cells to reveal their dynamic changes with disease activity (Fig. 2A). In accordance with total NK cells in the peripheral blood, both NK cell subsets were decreased markedly at relapsing compared with HCs (Fig. 2B–E). The frequencies and absolute counts of both CD56^{dim} NK cells and CD56^{bright} NK cells recovered in the remitting patients (Fig. 2B–E). Upon correlation analysis, only CD56^{dim} NK cell frequency displayed a significant inverse correlation with CD4$^+$ T cells ($r = -0.6052$, $p < 0.0001$) and cTfh cells ($r = -0.2987$, $p = 0.0463$) for all MS patients (Fig. 2F–H). There was also a remarkable negative correlation between CD56^{dim} NK cells and CD4$^+$ T cells ($r = -0.7313$, $p = 0.0013$) but not cTfh cells ($r = -0.2588$, $p = 0.3319$) in the relapsing patients (Fig. 2J–L). And similar inverse correlation was found between CD56^{dim} NK cells and CD4$^+$ T cells ($r = -0.4902$, $p = 0.0069$) in the remitting patients (Supplementary Fig. s1A, middle column). However, no significant correlations were found between CD56^{bright} NK cells and CD4$^+$ T cells or cTfh cells in all MS patients (Fig. 2G–I), the relapsing patients (Fig. 2K–M), and the remitting patients (Supplementary Fig. s1, right column). The results suggested the prominent role of CD56^{dim} NK cells in the crosstalk with CD4$^+$ T cells and cTfh cells in MS. Moreover, dysregulation of cTfh cells by CD56^{dim} NK cells may exist when the relapses occur.

Table 1
Demographic and clinical characteristics of the relapsing-remitting MS patients and HCs.

Variable	Relapsing MS (n = 22)	Remitting MS (n = 29)	HCs (n = 20)	P value
Age (y)	35.5 [21–55]	35 [18–53]	31.5 [12–51]	0.4350
Female, no. (%)	17 (77.3)	20 (69.0)	10 (50)	0.1612
Onset age (y)	29 [20–42]	28 [15–38]	NA	0.0886
Disease duration (m)	24 [0.5–192]	32 [3–192]	NA	0.1323
EDSS score	2.0 [1.0–9.0]	2.5 [0–4]	NA	>0.9999
sNfL (pg/mL)	7.255 (5.095–13.91)	4.94 (4.22–9.675)	NA	0.1987
Treatment at sampling				
Steroids, no. (%)	1 (4.5)	0		
Immunosuppressants, no. (%)	0	2 (6.9)		
Steroids plus immunosuppressants, no. (%)	2 (9)	2 (6.9)		
DMTs, no. (%)	1 (4.5)	10 (34.5)		
Steroids + DMTs, no. (%)	0	0		

Abbreviations: DMT, disease-modifying therapy; EDSS, Kurtzke's Expanded Disability Status Scale; HCs, healthy controls; m, month; MS, multiple sclerosis; NA, not applicable; no., number; sNfL, serum neurofilament light; y, year.
Data are presented as number (percentage) or median [range] or median (interquartile range). Differences among relapsing MS, remitting MS and HCs were analyzed by Kruskal-Wallis test (age), Fisher's exact test (female ratio), Mann-Whitney *U* test (onset age, disease duration, EDSS and sNfL). *P* value < 0.05 was considered statistically significant.
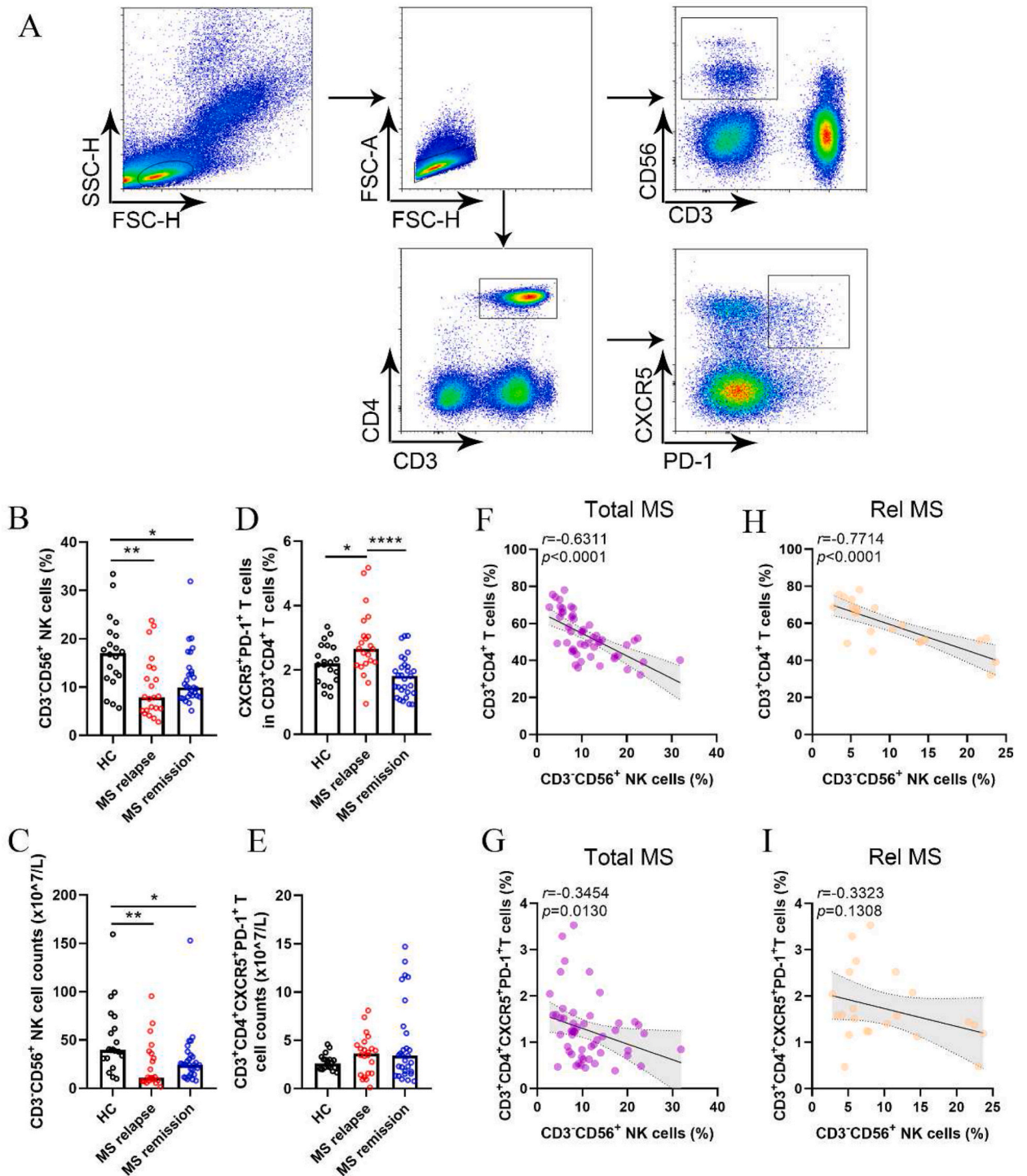
Fig. 1. Decreased circulating natural killer (NK) cells negatively correlate with CD4$^+$ T cells and T follicular helper (Tfh) cells in MS patients. (A) Gating strategy for NK cells (CD3$^-$CD56$^+$) and Tfh cells (CD3$^+$CD4$^+$CXCR5$^+$PD-1$^+$) from peripheral blood mononuclear cells of healthy controls (HCs) and MS patients. Representative plots are shown. Frequencies and cell counts of NK cells (B, C) and Tfh cells (D, E) from HCs (n = 20), relapsing MS patients (n = 22) and remitting MS patients (n = 29). Correlation analysis of NK cells with CD4$^+$ T cells in total MS patients (n = 51) (F) and relapsing MS patients (n = 22) (H). Correlation analysis of NK cells with Tfh cells in total MS patients (n = 51) (G) and relapsing MS patients (n = 22) (I). Data are represented as median. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$. Rel: relapsing.
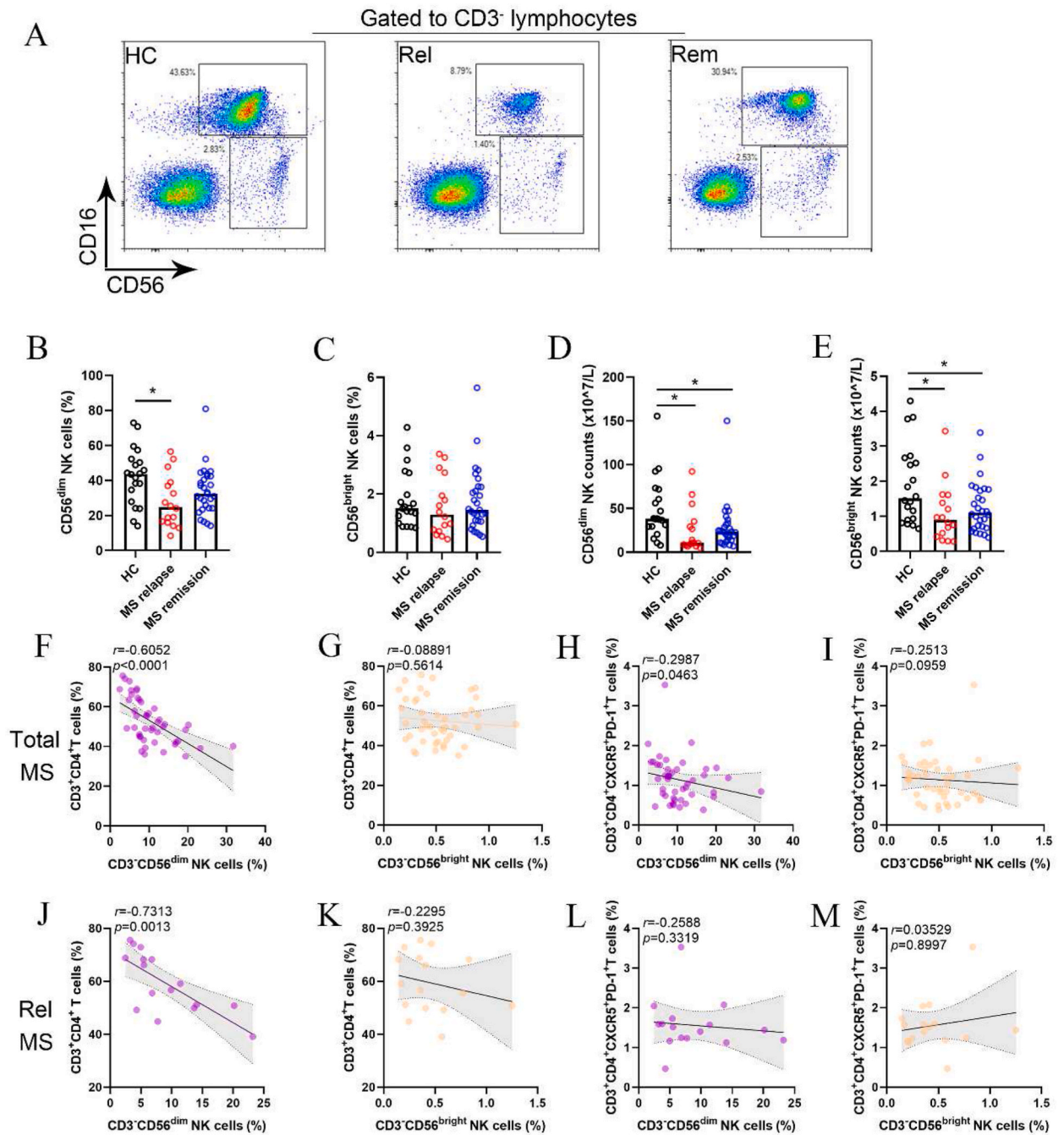
Fig. 2. CD56^{dim} NK cells, not CD56^{bright} NK cells, negatively correlate with CD4$^+$ T cells and T follicular helper (Tfh) cells in MS patients. (A) Representative plots of CD56^{dim} NK cells and CD56^{bright} NK cells from HCs and MS patients (CD56^{dim} NK cells: rectangle in the upper right; CD56^{bright} NK cells: rectangle in the bottom right). Frequencies (B, C) and cell counts (D, E) of CD56^{dim} NK cells and CD56^{bright} NK cells in CD3$^-$ lymphocytes from HCs (n = 20), relapsing MS patients (n = 16) and remitting MS patients (n = 29). Correlation analysis of CD3$^-$CD56^{dim} NK and CD3$^-$CD56^{bright} NK subset frequencies with CD4$^+$ T cells (F, G) and Tfh cells (H, I) in total MS patients (n = 45). Correlation analysis of CD3$^-$CD56^{dim} NK and CD3$^-$CD56^{bright} NK subset frequencies with CD4$^+$ T cells (J, K) and Tfh cells (L, M) in relapsing MS patients (n = 16). Data are represented as median. *P < 0.05. HCs: healthy controls; Rel: relapsing; Rem: remitting.

3.4. The ratio of CD56^{dim} NK cells to cTfh cells can discriminate the disease activity of MS more effectively

Receiver operating characteristic (ROC) curves were plotted to assess the value of NK cell subset ratios based on the above correlations with CD4$^+$ T cells and cTfh cells. Among the following ratios of CD56^{bright} NK cells to cTfh cells, CD56^{bright} NK cells to CD4$^+$ T

cells, CD56^{dim} NK cells to cTfh cells, CD56^{dim} NK cells to CD4$^+$ T cells, NK cells to cTfh cells, and NK cells to CD4$^+$ T cells, the ratio of CD56^{dim} NK cells to CD4$^+$ T cells was revealed to be best for the discrimination of MS patients from HCs with an area under the curve (AUC) of 0.7156 (70 % and 80 %, respectively) (Fig. 3). Although the ratio of NK cells to CD4$^+$ T cells showed a specificity of 100 % in distinguishing the relapsing patients and 90 % for MS patients compared to HCs, its AUC and sensitivity were not significant enough to serve as a reliable indicator.

Meanwhile, the ROC curve showed that the ratio of CD56^{dim} NK cells to cTfh cells could best discriminate the relapsing patients from HCs with the highest AUC of 0.8344 (65 % and 93.75 %, respectively) (Fig. 4A) or from the remitting patients with the highest AUC of 0.7974 (81.25 % and 68.97, respectively) (Fig. 4B), suggesting the most appropriate value of CD56^{dim} NK/cTfh cell ratio in the disease activity identification. Considering an important role of sNfL and EDSS score in MS activity and disease severity respectively, we also analyzed the correlations between the ratios above and sNfL levels or EDSS scores. No significant difference was observed in either sNfL or EDSS score with these ratios among NK cell subsets and CD4$^+$ T cells or cTfh cells (Supplementary Fig. s2, s3).

3.5. The ratio of CD56^{dim} NK cells to cTfh cells may be valuable for treatment efficacy assessment

To assess treatment efficacy, 8 patients with RRMS were sampled before and after treatment for acute attacks, with the clinical characteristics of each patient illustrated in Table 2. Ratios of CD56^{bright} NK cells to cTfh cells, CD56^{bright} NK cells to CD4$^+$ T cells, CD56^{dim} NK cells to cTfh cells, CD56^{dim} NK cells to CD4$^+$ T cells, NK cells to cTfh cells, and NK cells to CD4$^+$ T cells were compared between pre- and post-treatment in these patients. The ratios of NK cell subsets to CD4$^+$ T cells (Fig. 5A–C, E), as well as NK/cTfh cells and CD56^{dim} NK/cTfh cells (Fig. 5B–D), other than CD56^{bright} NK/cTfh cells (Fig. 5F), were significantly increased in patients after treatment. It was worth noting that the alterations in the ratios of NK/cTfh cells or CD56^{dim} NK/cTfh cells were particularly pronounced following immunotherapies, indicating the potential predictive value of ratios of NK/cTfh cells and CD56^{dim} NK/cTfh cells for treatment efficacy assessment.

4. Discussion

An increasing number of studies have pointed out diverse functions of NK cells in MS and experimental autoimmune encephalomyelitis (EAE) animal models [24–26]. Our study investigated the changes in NK cell subsets during different phases of RRMS and revealed an original negative correlation between CD56^{dim} NK cells and circulating Tfh cells. The findings also suggested a predictive value of the ratio of CD56^{dim} NK/cTfh cells for disease recurrence and the efficacy assessment of immunotherapies.

First, we detected the frequencies and absolute counts of circulating NK cells and the subsets in RRMS patients compared with healthy controls. Similar to previous reports, total NK cells, CD56^{dim} NK cells, and CD56^{bright} NK cells were significantly decreased when the disease recurred [21]. CD56^{dim} NK cells showed a more prominent reduction than CD56^{bright} NK cells. As the main subset of NK cells in the blood, CD56^{dim} NK cells possess stronger cytotoxicity than CD56^{bright} NK cells [27]. They were reported to be pathogenic in RRMS and primary progressive MS possibly due to the perforin and antibody-dependent cytotoxicity to the CNS [19,28,29]. However, CD56^{dim} NK cells could also lysis auto-active T cells as CD56^{bright} NK cells despite the core immunoregulation of the latter [17]. In addition, environmental risk factors like viral infections have been considered to play a role in MS pathogenicity [30]. Previous studies suggested the disease activity in MS could be concerned with the reactivation of Epstein-Barr virus (EBV) [31]. While, CD56^{dim} NK cells are the chief eradicator for those infected cells. The significant decrement of CD56^{dim} NK cells in relapsing MS patients may result in loss of control of viruses exactly as the expansion of EBV and the reaction of auto-immunity possibly due to molecular mimicry [30].

Second, the negative correlation between NK cells and CD4$^+$ T cells or Tfh cells in total MS patients were observed. Similar with those results achieved by Mimpen et al. [20], our data showed a high inverse relation between NK cells and CD4$^+$ T cells, especially in relapsing patients, suggesting an immunologic balance between innate immunity and adaptive immunity. In addition, NK cells might
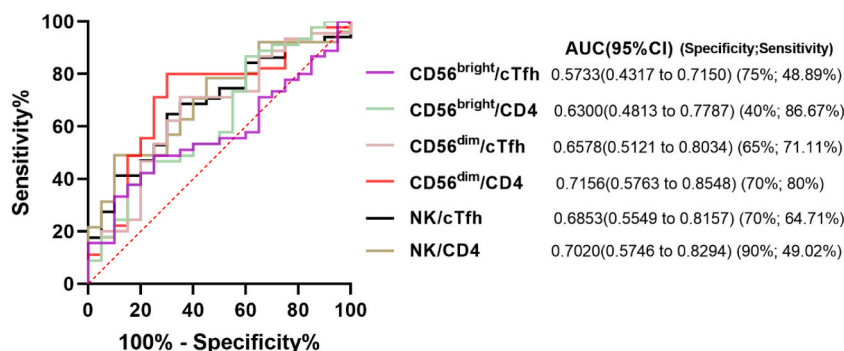
Fig. 3. Receiver operating characteristic (ROC) analysis shows CD56^{dim} NK/CD4$^+$ T cell ratio is the most discriminant value to distinguish MS patients from HCs. ROC curves of the ratios of CD56^{bright} NK/cTfh cells, CD56^{bright} NK/CD4$^+$ T cells, CD56^{dim} NK/cTfh cells, CD56^{dim} NK/CD4$^+$ T cells, NK/cTfh cells and NK/CD4$^+$ T cells. cTfh, circulating follicular T helper cells; AUC: area under the curve; CI: confidence interval.
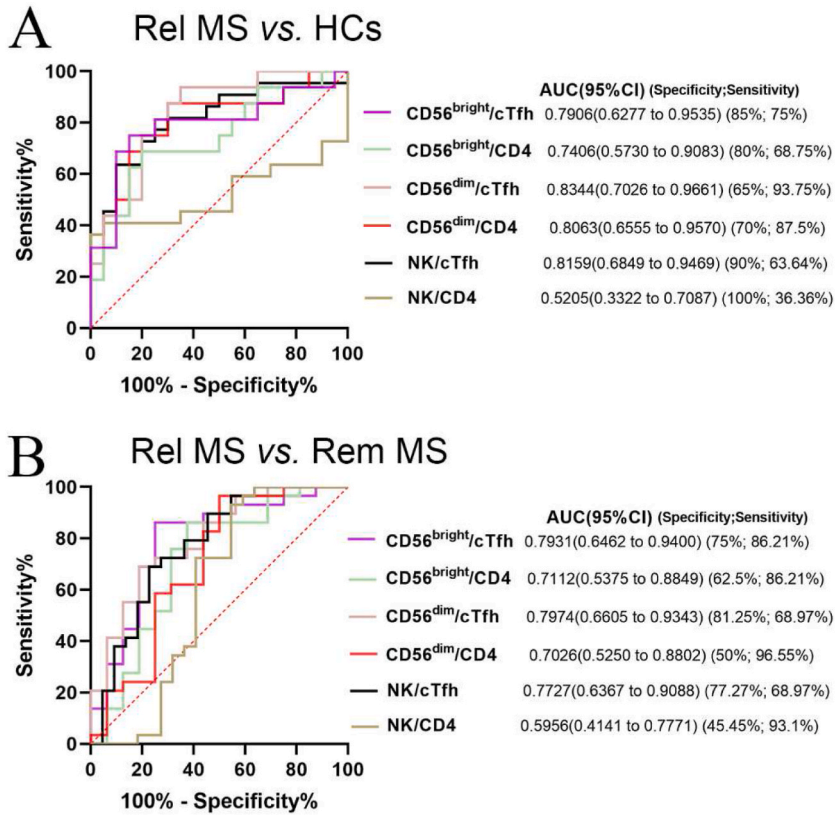
Fig. 4. Receiver operating characteristic (ROC) analysis shows CD56^{dim} NK/cTfh cell ratio is the most discriminant value to distinguish relapsing MS patients from HCs or remitting patients. ROC curves of the ratios of CD56^{bright} NK/cTfh cells, CD56^{bright} NK/CD4$^+$ T cells, CD56^{dim} NK/cTfh cells, CD56^{dim} NK/CD4$^+$ T cells, NK/cTfh cells and NK/CD4$^+$ T cells in relapsing patients with HCs (A) or remitting patients (B). cTfh, circulating follicular T helper cells; AUC: area under the curve; CI: confidence interval.

Table 2

Clinical characteristics of relapsing-remitting MS patients enrolled for treatment efficacy assessment.

Patient no.	Age (y)	Gender	Disease type	Treatment	Duration of treatment, (m)
1	37	F	RRMS	Steroids	1
2	35	F	RRMS	Steroids	1
3	53	F	RRMS	Steroids	1
4	21	F	RRMS	Ofatumumab	1[a]
5	39	M	RRMS	Ofatumumab	1[a]
6	38	M	RRMS	Teriflunomide	15
7	23	M	RRMS	Teriflunomide	12
8	23	F	RRMS	Rituximab	6[a]

Abbreviations: F, female; M, male; m, month; MS, multiple sclerosis; no., number; RRMS, relapsing-remitting multiple sclerosis; y, year.

[a] Intravenous injection was performed once.

control Tfh cells in MS unexpectedly. As an essential CD4$^+$ T cell subpopulation in humoral immunity, Tfh cells support B cell maturation and immunoglobulin production [32]. Studies focused on B cells in MS pointed out that the increased Tfh cells in the circulation and ectopic lymphoid structures in the CNS could contribute to disease progression [6,33]. NK cells were proven to control Tfh cells and humoral immunity in acute infections [34]. Depletion of NK cells early could increase Tfh cells and enhance virus-specific antibodies, thereby being beneficial for virus control [13]. Such suppression of Tfh cells may appropriately adjust T cell response and prevent T cell-driven immunopathology. Therefore, the missing negative correlation between NK cells and Tfh cells in the relapsing MS patients may suggest a mechanism of dysregulation of Tfh cells by NK cells underlying disease activity. The precise molecular target accounting for this needs to be studied further.

CD56^{bright} NK cells were prominent NK cell subsets in lymphoid organs to suppress autologous CD4$^+$ T cell proliferation during inflammation. However, the higher inhibitory ligand or lower activating ligand expressed on CD4$^+$ T cells render these cells not susceptible to NK cell-mediated killing in MS [15]. Our study showed CD56^{dim} NK cells, not CD56^{bright} NK cells, were the main NK cells
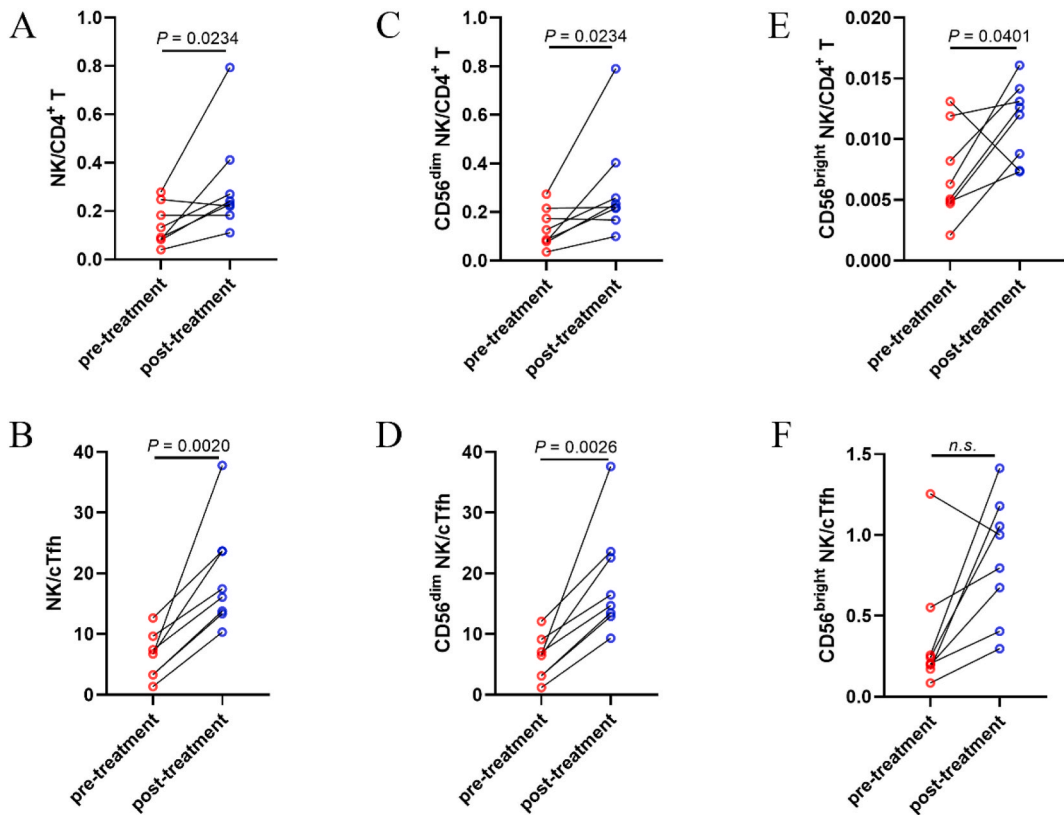
Fig. 5. The predictive value of CD56^dim NK/cTfh cell ratio for treatment efficacy. (A–E) The ratios of NK/CD4$^+$ T cells ($P = 0.0234$), NK/cTfh cells ($P = 0.0020$), CD56^{dim} NK/CD4$^+$ T cells ($P = 0.0234$), CD56^{dim} NK/cTfh cells ($P = 0.0026$) and CD56^{bright} NK/CD4$^+$ T cells ($P = 0.0401$) increase remarkably in the patients followed up after treatments with immunosuppressants or disease-modifying drugs. (F) No significant changes are shown in the ratio of CD56^{bright} NK/cTfh cells. n = 8. cTfh, circulating T follicular helper cells.

for regulating CD4$^+$ T cells and Tfh cells. It was in accordance with the preceding study that emphasized the negative correlation of IL-17$^+$CD4$^+$ T cells with CD56^{dim} NK cells [20]. We also found that the ratio of CD56^{dim} NK cells to CD4$^+$ T cells could help discriminate MS patients from the healthy. Meanwhile, the ratio of CD56^{dim} NK cells to Tfh cells was the best value among NK cell subsets to evaluate disease activity for MS patients. These results seem contradictory with previous reports on the main role of CD56^{bright} NK cells [35,36]. The difference may be related to the location of NK cells and our data focused on the NK cell subsets in the circulation. Despite the traditional consensus about the regulatory role of CD56^{bright} NK cells and the cytotoxicity of CD56^{dim} NK cells [29], our data stress a new side of CD56^{dim} NK cells in MS.

Third, we assessed the correlations between the ratios of NK subsets to CD4$^+$ T cells or cTfh cells and indicators for disease severity or disease activity. Analysis showed a negative trend for CD56^{dim} NK/cTfh cell ratio with serum NfL levels in MS. Studies have revealed a role of serum NfL level in the prediction of disease activity or progression of neuronal damage [37]. Therefore, a low ratio of CD56^{dim} NK cells to Tfh cells might be involved in massive neuronal destruction. Whereas, similar levels of NfL in the relapsing and remitting patients might be accountable for the minor trend. To increase the number of patients enrolled may be more befitting. Besides, there were no close relations between the ratios and EDSS scores of the patients, which indicates the predictive value of the ratio of CD56^{dim} NK cells to Tfh cells for disease activity than the cumulative disease damage.

Treatments including steroids, immunosuppressants or disease-modifying drugs can significantly attenuate disease activity [38]. In our report, the ratios of NK cell subsets to CD4$^+$ T cells or cTfh cells assessed pre- or post-treatments showed remarkable changes, especially for the ratio of NK cells or CD56^{dim} NK cells to cTfh cells in the peripheral blood. From clinical trials about the effect of disease-modifying drugs, CD56^{bright} NK cells were found to be immunoregulatory and protective with notable expansion [39]. The mechanisms were considered to relate to the IL-2 receptor, enough cytokines to access without the presence of CD4$^+$ T cells, or increased cytokines produced by monocytes [40–42]. We also found that the ratio of CD56^{bright} NK cells to CD4$^+$ T cells increased after treatments, but the elevating trends among the patients were not consistent. While, the significant and consistent increase in the ratios of NK cells or CD56^{dim} NK cells to cTfh cells suggests a possible role for them in the treatment efficacy assessment. Moreover, CD56^{dim} NK cells share part regulatory features with CD56^{bright} NK cells. Our study highlights that the ratio of CD56^{dim} NK cells to cTfh cells in circulation may reflect an underlying mechanism for immunotherapies.

There are several limitations in our study. Firstly, the lasting period of each relapsing MS patient enrolled in this study after recurrence was not specific and some patients received steroids or immunosuppressants at sampling. Secondly, the concrete molecular

mechanisms underlying NK cells and Tfh cells-crosstalk need further exploration. Finally, it will be better to increase the number of enrolled patients and refine them with different disease-modifying treatments for verifying the value of the ratio in the treatment efficacy.

In conclusion, our study reveals a negative inverse relation between NK cells and cTfh cells in RRMS. The immunologic balance is mainly regulated by CD56^{dim} NK cells. Moreover, the ratio of CD56^{dim} NK cells, not CD56^{bright} NK cells to cTfh cells can serve as a promising predictor for disease activity and additionally for the assessment of treatments. Future studies with larger patient samples are required to confirm the application value of this parameter in MS.

5. Conclusions

Our data explores a possible regulation of humoral immunity by CD56^{dim} NK cells in MS and may provide a novel promising predictor for disease activity and treatment efficacy assessment.

Ethics statement

All patients provided written informed consent in accordance with the Declaration of Helsinki. Study protocol was approved by the Ethics Committee of Tangdu Hospital of Air Force Medical University with the approval number KY20224208.

Data availability

The datasets used and/or analyzed in the current study are available from the first author or the corresponding author upon reasonable request.

Additional information

No additional information is available for this paper.

CRediT authorship contribution statement

Jiaqi Ding: Writing – original draft. **Xu Yan:** Data curation. **Cong Zhao:** Data curation. **Daidi Zhao:** Methodology. **Yan Jia:** Methodology. **Kaixi Ren:** Methodology. **Yao Wang:** Methodology. **Jiarui Lu:** Methodology. **Tangna Sun:** Methodology. **Sijia Zhao:** Methodology. **Hongzeng Li:** Conceptualization. **Jun Guo:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31533.

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