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RESEARCH ARTICLE

# CD8$^+$ T cell subpopulations and pro-inflammatory cytokines in neuromyelitis optica spectrum disorder

Ziyan Shi[1], Yuhan Qiu[1], Zhengyang Zhao[1], Dingke Wen[2], Hongxi Chen[1], Qin Du[1], Ying Zhang[1], Jiancheng Wang[1], Chao Yan[3], Mu Yang[4] & Hongyu Zhou[1]

[1]Department of Neurology, West China Hospital, Sichuan University, Chengdu, 610041, China
[2]Department of Neurosurgery, West China Hospital, Sichuan University, Chengdu, 610041, China
[3]Department of Neurology, Zigong fourth people's hospital, Zigong, 643000, China
[4]Translational Centre for Oncoimmunology, Sichuan Cancer Hospital and research Institute, Sichuan Cancer Center, Chengdu, 610000, China

**Correspondence**
Mu Yang, Translational Centre for Oncoimmunology, Sichuan Cancer Hospital and research Institute, Sichuan Cancer Center, No.55 South Renmin Road, Chengdu 610000, China. Tel: +86 028 85420569; Fax: +86 028 85420569; E-mail: mu.yang@uestc.edu.cn
Hongyu Zhou, Department of Neurology, West China Hospital, Sichuan University, No.28 Dianxin Nan Street, Chengdu 610041, China. Tel: +86 028 85423550; Fax: +86 028 85423550; E-mail: zhouhy@scu.edu.cn

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## Abstract

**Objective**: Our study aimed to investigate circulating CD8$^+$ T cell subpopulations and pro-inflammatory cytokines in the neuromyelitis optica spectrum disorder (NMOSD). **Methods**: A total of 121 peripheral blood samples were obtained from 57 patients with NMOSD, 34 patients with multiple sclerosis (MS), and 30 sex- and age-matched healthy controls (HCs) for detection of CD8$^+$ T cell subpopulations, including phenotypes of naïve ($T_N$, CD62L$^{hi}$CD45RO$^-$), effector/memory ($T_{E/M}$, CD62L$^{lo}$CD45RO$^+$), memory precursor ($T_{MP}$, CD127$^{hi}$KLRG1$^{lo}$), and short lived effector ($T_{SLEC}$, CD127$^{lo}$KLRG1$^{hi}$). In addition, 36 samples from 18 NMOSD, 12 MS, and 6 sex- and age-matched HCs for detecting pro-inflammatory cytokines (IFNγ and TNFα) using flow cytometry. **Results**: Compared with HCs, we found significantly reduced CD8$^+$ $T_N$ and increased CD8$^+$ $T_{E/M}$ in both NMOSD and MS, while decreased CD8$^+$ $T_{MP}$ was only observed in NMOSD. Patients treated with immunotherapy were associated with increased CD8$^+$ $T_N$ and decreased CD8$^+$ $T_{E/M}$ in NMOSD. Moreover NMOSD cohort showed significant higher proportions of IFNγ$^+$CD8$^+$ T cells and proportions of TNFα$^+$CD8$^+$ T cells than HC and MS cohorts. On the contrary, obviously decreased IFNγ and TNFα were found in NMOSD patients treated with immunotherapy. Furthermore, Multivariate linear regression analyses revealed that age was negatively correlated with CD8$^+$ $T_N$ and $T_{MP}$, and positively associated with $T_{SLEC}$; however, sex, EDSS scores and disease phase were not significantly associated with CD8$^+$ T subpopulations. **Interpretation**: This current study provides an evidence that circulating CD8$^+$ T cell with abnormal subpopulations and increased pro-inflammatory were associated with pathogenesis of autoimmune demyelinating disease of CNS, especially in NMOSD.

## Introduction

Neuromyelitis optica spectrum disorder (NMOSD) is a relapsing autoimmune demyelinating disease of central nerve system (CNS) that preferentially affects optic nerve, spinal cord and brainstem.[1,2] Other than severely visual impairment, patients with NMOSD also suffer from motor disabilities or even death at nadir.[3,4] Latest epidemiological studies also indicated that Asian and other non-Caucasians populations exhibited higher susceptibility of NMOSD onset than average level, which is ranging from 0.72 to 10 over 100,000 populations.[5-7] Unfortunately, even though immunosuppressive therapies concomitant with glucocorticoids are considered as the most efficient treatments for controlling NMOSD, still 30%-53% of patients were observed to have relapse and disease development under current medical managements.[8,9] Therefore, it is imperative to discover novel and appropriate treatments via revealing the immune cascades leading to onset and development of NMOSD.

NMOSD is characterized by a pathogenic autoantibody against aquaporin 4 (AQP4), which mainly expressed on astrocytes.[10,11] This humoral immune response also provokes NMOSD specific CD4$^+$ T cells in both circulating system and lesion sites.[12] Although CD4$^+$ T cells have been the main focus of NMOSD research for decades,[13,14] as one of the key players involved in neuroinflammation, CD8$^+$ T cells were found to participate the immune cascades in development of NMOSD.[15] Accumulating data from peripheral neuropathic diseases indicated that CD8$^+$ T cells played pivotal roles in triggering Guillain–Barre Syndrome (GBS), which is the prototypical autoimmune peripheral neuropathy.[16] Furthermore, as one of the most well studied autoimmune demyelinating diseases of CNS, MS was found to be correlated with abnormally increased frequencies and exaggerated pro-inflammatory responses of CD8$^+$ T cell subsets as well.[17] But, up to date, the detail information of CD8$^+$ T cells regarding to subpopulations and functions in NMOSD are not very extensively studied. Overall, the identification of decisive factors in connecting CD8$^+$ T cells and the occurrences of NMOSD is critical to investigate its underlying pathogenic roles and will provide an opportunity to develop new therapeutic targets.

Current pathogenic concepts implicate high frequency of CD8$^+$ $T_{E/M}$ cells as the consequence of viral induced "memory inflation," which are associated with autoimmune response in nervous system.[16,18] This distinct phenomenon is confirmed by eliminated expression of CD62L and acquired high expression level of CD45RO in CD8$^+$ T cells from animal models and human patients, respectively.[16,18,19] After acute infection, effector CD8$^+$ T cells generated into $T_{SLEC}$ cells (CD127$^{lo}$KLRG1$^{hi}$) and $T_{MP}$ cells (CD127$^{hi}$KLRG1$^{lo}$) capable of generating long-lived memory CD8$^+$ T cells to against infection.[20] Functionally, those CD8$^+$ T cells are strong secretors of pro-inflammatory cytokines of IFNγ and TNFα.[21] We previously reported a transgenic murine model with predisposed immune background, where CD8$^+$ T cells have been primed and possess typical effector/memory phenotypes.[16] This model exhibits large amount of circulating CD44$^{hi}$CD62L$^{lo}$ and CD127$^{lo}$KLRG1$^{hi}$ CD8$^+$ T cells, which initiate spontaneous autoimmune peripheral neuropathy via secreting high levels of IFNγ and TNFα. Here, in order to better dissect the relevant of CD8$^+$ T cell phenotypes and functions with clinical characteristics of autoimmune demyelinating CNS disease, we recruited patients with NMOSD under different treatment managements. Our investigation provides a potential framework to understand how corticosteroids and/or immunosuppressant prevent over activation of CD8$^+$ T cells thus prevent attacks in NMOSD.

## Participants and Methods

### Participants

All participants were recruited from West China Hospital, Sichuan University between March 2018 and May 2020. Overall, 57 patients with NMOSD with AQP4-IgG, 34 patients with MS, and 30 age-matched HCs were enrolled for detection of CD8$^+$ T cell subpopulations. Additionally, 36 participants including 18 patients with NMOSD, 12 patients with MS, and 6 HCs were also enrolled for detection of pro-inflammatory cytokines of IFNγ and TNFα secreted by CD8$^+$ T cells. Patients with NMOSD were diagnosed based on 2015 revised diagnostic criteria for NMOSD[2] and MS were based on 2017 revisions of the McDonald criteria for MS.[22] Sex, age, disease duration, Expanded Disability Status Scale (EDSS) scores, and treatments were included. This study was approved by the Medical Ethics Committee of the West China Hospital, Sichuan University (2018-29) and all participants given informed consent prior to their inclusion in this study.

### Sample collection and flow cytometry

Peripheral blood samples were collected from participants and prepared to be single cell suspensions as mentioned previously.[16] In brief, after washing with Alsevier's solution (Gibco™), samples were lysed in ACK buffer (Thermo Fisher Scientific) at room temperature for 5 min. Then the single cells were incubated with Fc Receptor Blocking Solution (422302, Biolegend™) at 4 °C for 30 min, followed by staining with surface Abs at the same condition, including CD8a-Percp (301030), CD62L-PE/Cy7 (304822), CD45RO-FITC (304204), CD127-APC/Cy7 (351348), and KLRG1-PE (367712) (Biolegend™). Subsequently, the staining samples were washed with 0.25% bovine serum albumin (Sigma™) and re-suspended with Phosphate Buffer solution (Gibco™) for detection. For intracellular staining was similar except for following steps: cells were suspended in RPMI-1640 medium (Gibco™) with 10% fetal bovine serum and 1% penicillin–streptomycin, then cultured at 37°C with 5% $CO_2$ for 4 h at the presence of 100 ng/ml Phorbol 12-myristate 13-acetate (PMA) (1652981), 1.5 μg/mL Ionomycin (5608212), and 1 μL/mL Brefeidin A solution (420601) (Biolegend™). After staining with surface antibodies CD3-FITC (317306) and CD8a-Percp (301030), the stimulated lymphocytes were then fixed and permeabilized using Foxp3/Transcription factor staining buffer (Invitrogen™) according to the manufacturer's protocol. Finally, intercellular staining with IFNγ-APC (506510) and TNFα-APC/Cy7 (506344) antibodies (Biolegend™) was performed. Stained cells were detected using LSRFortessa (BD) or FACS

 

Canto II (BD), and data analysis were conducted by Flowjo v10 (BD).

### Statistical analysis

Statistical analyses were conducted using GraphPad Prism v6.0 (GraphPad Software, San Diego, California, USA) and SPSS v25.0 software (IBM Corp., Armonk, NY, USA). Continuous variables were presented as mean ± standard deviation (SD) and categorical variables were shown as numbers and percentages. Student t test (normality) or Mann–Whitney U test (non-normality) was used to compare difference of CD8$^+$ T subpopulations between two groups. One-way ANOVA (normality) with Turkey's test or Kruskal–Wallis (non-normality) with Dunn's test was used for comparisons among three or more groups. Comparisons of CD8$^+$ T cell phenotypes between groups were corrected for age and sex using covariance analysis. In addition, the associations of sex, age, EDSS scores, disease phase (relapsing/progressive/remission), and treatments with CD8$^+$ T cell phenotypes were assessed by multivariate linear regression models. *P* values at two-tailed less than 0.05 were defined as statistically significant.

## Results

### Demographic and clinical characteristics of participants

In order to better understand the frequencies of CD8$^+$ T cell subpopulations in circulating system between NMOSD and MS, peripheral blood samples were collected from NMOSD, MS, and HCs. In detail, there were 57 (93% females) patients with NMOSD with the average age of 49 years old. During the time of blood collecting, 17 patients were admitted without any medication, whereas 22 and 18 patients were treated by glucocorticoids (GC) and immunosuppressive treatments (IST), respectively. In the IST group, there were four patients with azathioprine and 14 patients with mycophenolate mofetil. We also included blood samples from 34 (54% females) patients with MS with the average age of 35 years. 10 out of 26 patients with MS donated blood sample before any treatments, and other 16 patients were admitted by GC ($n = 11$) or disease modification treatments (DMT) ($n = 13$), respectively. In the DMT group, there were six patients with β-interferon, 3 patients with teriflunomide, and four patients with mitoxantrone. We also involved blood samples from 15 healthy controls 1 (HC1) (73% females) with average ages of 48 for comparing with NMOSD and 15 healthy controls 2 (HC2) (53% females) with average ages of 36 for comparing with MS (Table 1).

**Table 1.** Demographic and clinical characteristics of participants.

| | NMOSD $n = 57$ | MS $n = 34$ | HC1 $n = 15$ | HC2 $n = 15$ |
|---|---|---|---|---|
| Gender, Females: males | 54:3 | 21:13 | 11:4 | 8:7 |
| Age, mean(SD), years | 49(11.6) | 35(6.5) | 48(3.6) | 36(2.4) |
| Disease duration, mean (SD),years | 4.8(5.2) | 5.8(4.6) | NA | NA |
| EDSS scores, mean(SD) | 4.7(2.6) | 4.1(2.8) | NA | NA |
| Treatments, *n* | | | | |
| NT | 17 | 10 | NA | NA |
| GC | 22 | 11 | NA | NA |
| IST | 18 | 0 | NA | NA |
| DMT | 0 | 13 | NA | NA |
| Phase | | | | |
| Remission/relapse/ progressive, n | 28/29/0 | 5/18/11 | | |

NMOSD, neuromyelitis optica spectrum disorders; MS, multiple sclerosis; HC, healthy controls, HC1 compared with NMOSD and HC2 compared with MS respectively; SD, standard deviation; n, number; EDSS, Expanded Disability Status Scale; NT, non-treatment; GC, glucocorticoids; IST, immunosuppressive treatments (including 4 azathioprine and 14 mycophenolate mofetil); DMT, disease modification treatments (including 6 β-interferon, 3 teriflunomide, 4 mitoxantrone). NA, not applicable.

### Abnormality of circulating CD8$^+$ T cell subpopulations in NMOSD and MS

The flow cytometric gating strategy of CD8$^+$ T cell subpopulations was shown in Fig. S1. After correcting for age and sex between groups, significant reducing frequencies of CD8$^+$ $T_N$ cells were observed in untreated NMOSD and MS patients compared with HCs (23% vs. 45%, $P < 0.001$ and 34% vs. 51%, $P < 0.05$, respectively) (Fig. 1A). CD8$^+$ $T_{E/M}$ cells were significant higher in both NMOSD (31% vs. 17%, $P < 0.001$) and MS patients (36% vs. 15%, $P < 0.01$) than HCs (Fig. 1B). Moreover untreated patients with NMOSD solely exhibited up-regulation of circulating CD8$^+$ $T_{MP}$ cells (26% vs. 43%, $P < 0.001$), whereas only a similar trend was found in patients with MS (36% vs. 47%, $P > 0.05$) (Fig. 1C). Nevertheless, as the sign of short-lived effector phenotype, the population of CD8$^+$ $T_{SLEC}$ cells was relatively stable among all groups (Fig. 1D). No significant difference of CD8$^+$ T cell subpopulations were observed between NMOSD and MS.

### CD8$^+$ T cell subpopulations were influenced by the immunotherapy

As the well documented first-line drugs to against NMOSD, immunotherapies such as GC and IST are currently used for controlling severity of acute attacks and

 

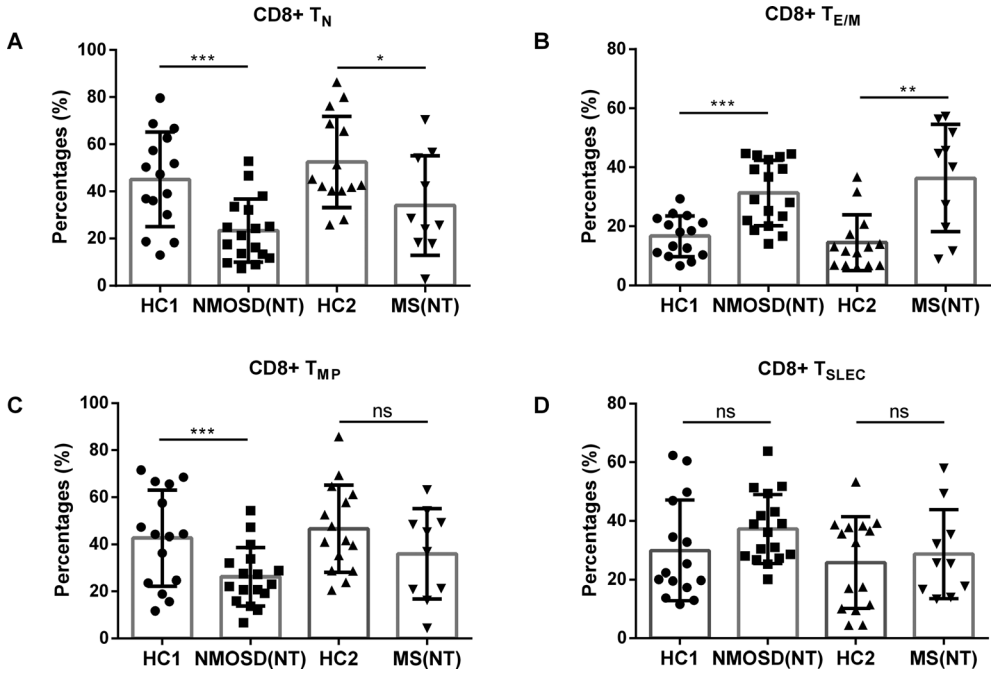


**Figure 1.** Proportions of circulating CD8+ T cell subpopulations. The proportions of CD8+ T subpopulations from NMOSD, MS, and HCs were detected using flow cytometry, including (A) $T_N$ ($CD62L^{hi}CD45RO^-$) cells, (B) $T_{E/M}$ ($CD62L^{lo}CD45RO^+$) cells, (C) $T_{MP}$ ($CD127^{hi}KLRG1^{lo}$) cells, and (D) $T_{SLEC}$ ($CD127^{lo}KLRG1^{hi}$.) cells. Differences in proportions of each subpopulation were compared between untreated NMOSD ($n$ = 17) and age-matched HC1 ($n$ = 15), and between untreated MS ($n$ = 10) and age-matched HC2 ($n$ = 15), respectively. NT, non-treatment; $T_N$, naïve T cells; $T_{E/M}$, effector/memory T cells; $T_{MP}$, memory precursor T cells; $T_{SLEC}$, short lived effector T cells. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, and ns (not significant) by Student t test (A and C) and Mann–Whitney U test (B and D), respectively. Each P value was corrected for age and sex.

preventing relapses of the disease.[8] Therefore, further investigation was employed to dissect whether these immunotherapies could influence subpopulations of $CD8^+$ T cells. According to the results from NMOSD cohort, patients treated with GC reported increased $CD8^+$ $T_N$ cell population (23% vs. 37%, $P < 0.01$) but decreased $CD8^+$ $T_{E/M}$ cell population (31% vs. 22%, $P < 0.01$) compared with untreated patients. Although $CD8^+$ $T_N$ cell population was comparable between NT group and IST group, decreased $CD8^+$ $T_{E/M}$ cell population was observed in patients treated with IST (31% vs 19%, $P < 0.01$) (Fig. 2A-B). Furthermore, the $T_{MP}$ and $T_{SLEC}$ subpopulations were not significantly differentiated between treated or untreated patients. Additionally, we performed a small longitudinal study analysis, five untreated patients with NMOSD showed moderately reduced $CD8^+$ $T_{E/M}$ cells after IST treatment ($P = 0.024$) (Fig. S2).

Furthermore, we also conducted the frequency comparison of each $CD8^+$ T cell subpopulation between treated and untreated patients in MS cohort. $CD8^+$ $T_{E/M}$ subset was decreased in DMT group compared with NT group while other subpopulations of CD8+ T cells were comparable between the two groups (Fig. 3). However, we did not find any significant difference in $CD8^+$ T cell subpopulations before and after DMT treatment from the longitudinal study analysis (Fig. S3).

Altogether, our observations suggested that immunotherapies would influence $CD8^+$ T cell subpopulations in patients with NMOSD and similar alterations could be seen in MS.

## Associations between $CD8^+$ T cell subpopulations and clinical characteristics in NMOSD and MS

According to the multivariate linear regression models from NMOSD cohort, $CD8^+$ $T_N$ and $CD8^+$ $T_{MP}$ populations were negatively associated with age ($\beta = -0.64$, $P < 0.001$ and $\beta = -0.82$, $P < 0.001$, respectively) (Table 2). In contrary, $CD8^+$ $T_{SLEC}$ was positively correlated with age ($\beta = 0.52$, $P < 0.01$). Moreover we found that immunotherapy was positively associated with CD8+ $T_N$ population ($\beta = 11.60$, $P < 0.01$) and was negatively associated with CD8+ $T_{E/M}$ population ($\beta = -10.77$, $P < 0.001$), which was line with the above results from groups' comparisons. No significant association between $CD8^+$ T cell subpopulations and sex, EDSS scores, or disease phase (Table 2).

 

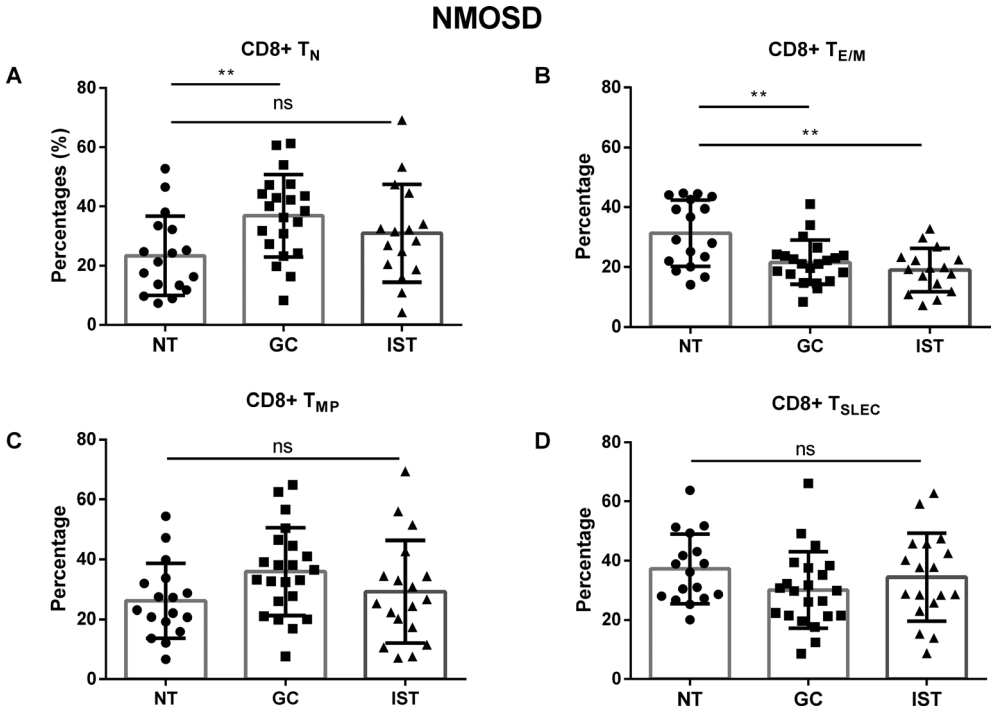


**Figure 2.** Comparison of circulating CD8$^+$ T cell subpopulations between different treatments in patients with NMOSD. The proportions of CD8$^+$ T subpopulations from NMOSD patients under different treatments, including 17 non-treatment (NT), 22 glucocorticoids (GC), and 18 immunosuppressive treatments (IST). $T_{N,}$ naïve T cells; $T_{E/M}$, effector/memory T cells; $T_{MP}$, memory precursor T cells; $T_{SLEC}$, short lived effector T cells. $^{**}P < 0.01$, and ns (not significant) by Kruskal–Wallis test with Dunn's correction for multiple comparison test. Each *P* value was corrected for age and sex.

Similarly, multivariate regression analyses also showed a negative association between DMT and CD8$^+$ $T_{E/M}$ ($\beta$=−13.59, $P < 0.001$) in MS patients, while no significant associations with other phenotypes was found (Table 3). In addition, age, sex, EDSS scores, and disease phase did not exhibit significant correlation with CD8$^+$ T cell subpopulations.

## Increased pro-inflammatory cytokines of CD8$^+$ T cells in NMOSD

Other than phenotypes, pro-inflammatory cytokine secreting was considered to deteriorate the severity of NMOSD lesions and play pivotal role in disease development.[23] Thus, we subsequently detect IFN$\gamma$ and TNF$\alpha$ secreting levels of CD8$^+$ T cell from 6 healthy controls, 12 patients with MS (6 NT and 6 DMT), and 18 patients with NMOSD (6 NT, 6 GC, and 6 IST) (Table S1). The flow cytometric gating strategy of IFN$\gamma$ and TNF$\alpha$ in CD8$^+$ T cell was shown in Fig. S2. NMOSD patients without treatments showed significant higher frequency of IFN$\gamma^+$CD8$^+$ T cells (56%) than healthy controls (22%) ($P < 0.01$) and MS patients (33%) ($P < 0.05$) (Fig. 4A). Similarly, TNF$\alpha^+$ CD8$^+$ T cells were increased in untreated patients with NMOSD (45%) compared with healthy controls (19%) ($P < 0.01$) and patients with MS (23%) ($P < 0.05$) (Fig. 4B). When compared with NT group, IFN$\gamma$ secreted by circulating CD8$^+$ T cells from patients with NMOSD were remarkably decreased in IST group (56% vs. 28%, $P < 0.01$) but not in GC group (Fig. 5A). Furthermore, both GC and IST treatments were found to be associated with decreased TNF$\alpha^+$CD8$^+$ T cells (45% vs. 19%, $P < 0.01$; and 45% vs. 16%, $P < 0.01$, respectively) (Fig. 5B). In contrast, there were no significant differences in IFN$\gamma$ or TNF$\alpha$ levels between DMT and NT groups in MS cohort (Fig. 5C and D).

Overall, our data indicated that circulating CD8$^+$ T cells biased toward activated phenotypes and pro-inflammatory functions in NMOSD, while immunotherapy seemed to be associated with reduced inflammatory pattern.

# Discussion

Circulating autoreactive T cells were considered as documented players in participating the progress of inflammatory demyelinating disease by triggering local inflammatory responses.[24] But few studies mentioned

 

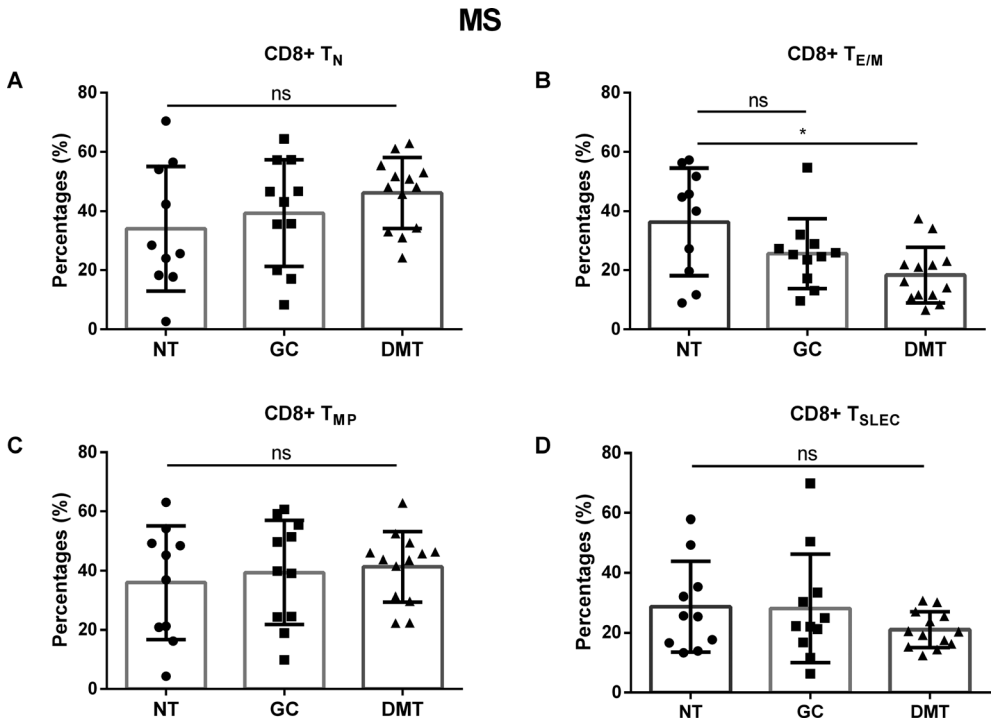


**Figure 3.** Comparison of circulating $CD8^+$ T cell subpopulations between different treatments in patients with MS. The proportions of $CD8^+$ T subpopulations were investigated in MS, including 10 non-treatment (NT), 10 glucocorticoids (GC), and 6 DMT, disease modification treatments (DMT). $T_{N,}$ naïve T cells; $T_{E/M}$, effector/memory T cells; $T_{MP}$, memory precursor T cells; $T_{SLEC}$, short lived effector T cells. $^*P < 0.01$, and ns (not significant) by Kruskal–Wallis test with Dunn's correction for multiple comparison test. Each *P* value was corrected for age and sex

about the dynamic changes of $CD8^+$ T cell subpopulations after treatment, as well as the development of autoimmune diseases. Recently, Sabatino et al. reported that anti-CD20 therapy depletes activated myelin-specific $CD8^+$ T cells in MS patients.[25] In this current study, we focus on circulating $CD8^+$ T cell phenotypes and functions in NMOSD and MS under different treatment managements. We found significantly reduced frequency of naïve $CD8^+$ T cells and increased frequency of effector/memory $CD8^+$ T cells in circulation of both NMOSD and MS, whereas patients with immunotherapy showed an another pattern where circulating increased naïve $CD8^+$ T cells with decreased effector/memory $CD8^+$ T cells. Additionally, a significantly reduced number of memory precursor $CD8^+$ T cells was observed solely in untreated NMOSD group, although it was comparable between treated and untreated groups. Furthermore, we further revealed that circulating $CD8^+$ T cells from patients with NMOSD presented significantly elevated secretions of pro-inflammatory cytokines including IFNγ and TNFα, but decreased in immunotherapy cohort. Taken together, our findings suggested that $CD8^+$ T cell subpopulations in the circulating system were associated with the pathogenesis of NMOSD and MS, while $CD8^+$ T cell pro-inflammatory cytokines were mainly associated with NMOSD.

Despite the previously reported pivotal role of $CD4^+$ T cells in the pathogenesis of NMOSD and MS, accumulating data based on animals' models and humans have initially explored the roles of $CD8^+$ T cells involved in autoimmune disease.[26-28] A study from the GBS suggested that peripheral myelin Ag specific $CD8^+$ T cells exhibited an effector/memory phenotype and produced many pro-inflammatory cytokines such as IFNγ and TNFα, which were required for the disease initiation in human and mice.[16] Meanwhile, research on experimental autoimmune encephalomyelitis (EAE) models further revealed that both $CD4^+$ and $CD8^+$ T cells recognized myelin oligodendrocyte glycoprotein (MOG) and contributed to trigger disease *in vivo*, even the disease was primarily driven by $CD4^+$ T cells.[26] Interestingly, recent study from human patients indicated that $CD8^+$ T cells instead of $CD4^+$ T cells presented a dominance in MS lesions and exhibited an activated cytotoxic phenotype, which; however, contradicted with the observations in animal models.[27,28] Lucchinetti et al. previously reported that numerous $CD3^+$ and $CD8^+$ T cells lymphocytes infiltrated in perivascular lesions of patients with NMO.[15]

 

**Table 2.** Associations between CD8$^+$ T phenotypes levels and clinical characteristics in patients with NMOSD.

| | $T_N$ | | $T_{E/M}$ | | $T_{MP}$ | | $T_{SLEC}$ | |
|---|---|---|---|---|---|---|---|---|
| | β | 95% CI | β | 95% CI | β | 95% CI | β | 95% CI |
| Age | −0.64 | (−0.97, −0.31)*** | −0.07 | (−0.16, 0.29) | −0.82 | (−1.10, −0.54)*** | 0.51 | (0.21, 0.80)*** |
| Gender | | | | | | | | |
| Females | −3.01 | (−19.05, 13.03) | 9.07 | (−1.99, 20.13) | −7.30 | (−21.94, 7.33) | 2.49 | (−12.72, 17.70) |
| Males | | | | | | | | |
| EDSS scores | −0.96 | (--2.92, 1.01) | 0.06 | (−1.29, 1.42) | 0.01 | (−1.69, 1.70) | 0.43 | (−1.33, 2.19) |
| Phase | | | | | | | | |
| Relapsing | −4.73 | (−14.55, 5.10) | 0.39 | (−6.38, 7.16) | −4.81 | (−13.14, 3.52) | 4.74 | (−3.92, 13.40) |
| Remission | | | | | | | | |
| Treatments | | | | | | | | |
| GC + DMT | 11.60 | (4.08, 19.12)** | −10.77 | (−15.95,-5.59)*** | 6.35 | (0.49, 13.19) | −5.23 | (−12.34, 1.87) |
| NT | | | | | | | | |

MS, multiple sclerosis; EDSS, Expanded Disability Status Scale; NT, non-treatment; GC, glucocorticoids; DMT, Disease modification treatment. TN, naïve T cells; TE/M, effector/memory T cells; TMP, memory precursor T cells; TSLEC, short lived effector T cells. β value, regression coefficients.
**$P < 0.01$.
***$P < 0.001$ by multivariate regression models.

**Table 3.** Associations between CD8$^+$ T phenotypes levels and clinical characteristics in patients with MS.

| | $T_N$ | | $T_{E/M}$ | | $T_{MP}$ | | $T_{SLEC}$ | |
|---|---|---|---|---|---|---|---|---|
| | β | 95% CI | β | 95% CI | β | 95% CI | β | 95% CI |
| Age | 0.34 | (−0.73, 1.42) | −0.09 | (−0.95, 0.77) | 0.40 | (−0.63, 1.42) | −0.36 | (−1.24, 0.52) |
| Gender | | | | | | | | |
| Females | 1.85 | (−10.71, 14.41) | −5.44 | (−15.46, 4.58) | 5.16 | (−6.82, 17.14) | −6.58 | (−16.84, 3.68) |
| Males | — | — | — | — | — | — | — | — |
| EDSS scores | 0.78 | (−2.91, 4.47) | 0.12 | (−2.83, 3.06) | 1.45 | (−2.08, 4.97) | −0.95 | (−3.97, 2.06) |
| Phase, *n* | | | | | | | | |
| Relapsing/Progressive | −16.26 | (−36.33, 3.81) | 7.11 | (−8.91,23.12) | −12.75 | (−31.89, 6.39) | 9.02 | (−7.37, 25.41) |
| Remission | — | — | — | — | — | — | — | — |
| Treatments | | | | | | | | |
| NT | −7.58 | (−21.36, 6.20) | 13.59 | (2.60, 24.58)* | −2.16 | (−15.30, 10.98) | 2.43 | (−8.82, 13.69) |
| GC/DMT | — | — | — | — | — | — | — | — |

MS, multiple sclerosis; EDSS, Expanded Disability Status Scale; NT, non-treatment; GC, glucocorticoids; DMT, Disease modification treatment. TN, naïve T cells; TE/M, effector/memory T cells; TMP, memory precursor T cells; TSLEC, short lived effector T cells. β value, regression coefficients.
*$P < 0.05$ by multivariate regression models.

Collectively, CD8$^+$ T cells are believed to be involved in the pathogenesis of autoimmune demyelinating disease of CNS, however, the underlying mechanism still needs to be further clarified.[29]

In this study, we reported significantly decreased proportions of both CD8$^+$ $T_N$ and CD8$^+$ $T_{MP}$ cells in patients with NMOSD, while only decreased CD8$^+$ $T_N$ population was found in patients with MS. However, it was worth noticing that both the two phenotypes of CD8$^+$ T cells were significantly reduced linearly with ages in healthy controls and patients with NMOSD. Previous literatures have demonstrated that the number of lymphoid-biased HSCs declines with increasing ages, that in turn leading to a diminished number of naive cells that migrate to secondary lymphoid tissues and peripheral circulations.[30] As a consequence, the down-regulation of CD8$^+$ $T_N$ cells results in reduction of diverse immune repertoire and perhaps contribute to explain the predisposition of NMOSD in middle-aged populations compared with young people. CD8$^+$ $T_{MP}$ cells are differentiated from effector CD8$^+$ T cells as acute infections resolve and then generate long-lived memory CD8$^+$ T cells, which protect humans from reinfection.[20] In contrast with MS, we further found significantly decreased number of CD8$^+$ $T_{MP}$ cells in NMOSD, which suggested a lower capacity of preventing from reinfection.

It has been well documented that corticosteroids and immunosuppressive agents including Azathioprine,

 

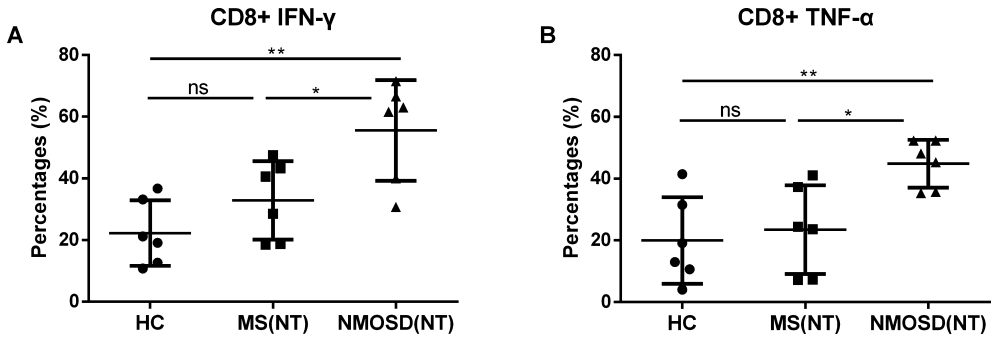


**Figure 4.** Analysis of pro-inflammatory cytokine (IFNγ and TNFα) secretion of $CD8^+$ T cells in peripheral blood. (A) IFNγ and (B) TNFα secreted by $CD8^+$ T cells were evaluated in six untreated MS patients, six untreated NMOSD patients, and six age- and sex-matched HCs. $T_{N,}$ naïve T cells; $T_{E/M}$, effector/memory T cells; $T_{MP}$, memory precursor T cells; $T_{SLEC}$, short lived effector T cells. NT, non-treatment; IMT, immunotherapies. $^{**}P < 0.01$, $^{*}P < 0.05$ and ns (not significant) by one-way ANOVA with Tukey's correction for multiple comparison tests. Each *P* value was corrected for age and sex.

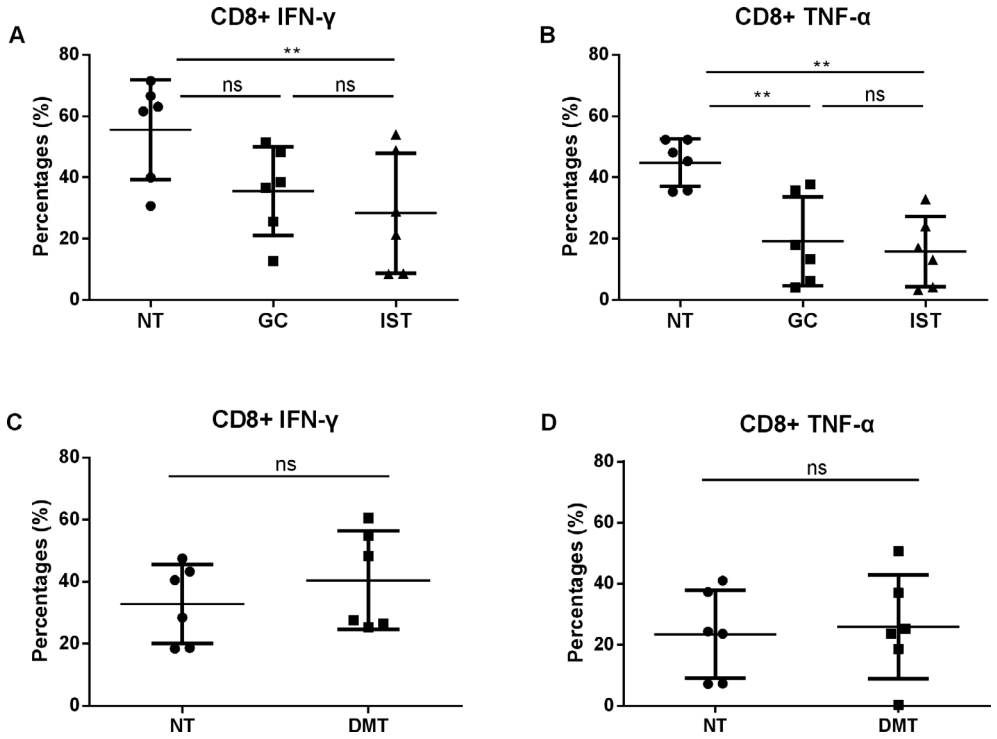


**Figure 5.** Comparison of pro-inflammatory cytokine (IFNγ and TNFα) secretion of $CD8^+$ T cells between different treatments in NMOSD and MS. IFNγ and TNFα secreted by $CD8^+$ T cells were evaluated in NMOSD patients (A and B) and MS patients (C and D). $^{**}P < 0.01$ and ns (not significant) by one-way ANOVA with Tukey's correction for multiple comparison tests. Each *P* value was corrected for age and sex.

Mycophenolate Mofetil, and Rituximab are recommended as first-line treatments for NMOSD to control the severity of acute attacks and to prevent relapse of disease, respectively.[8] Glucocorticoids are widely used to manage autoimmune diseases decades by inhibiting the expression of multiple inflammatory genes (cytokines, enzymes, receptors, and adhesion molecules).[31] As two of the most common immunosuppressive agents, Azathioprine and Mycophenolate Mofetil have been generally used in many autoimmune disease, through suppressing proliferation of activated B and T cells.[32,33] In this study, we discovered that immunotherapies were associated with increased naïve $CD8^+$ T cells and decreased effector/memory $CD8^+$ T cells in NMOSD. Similarly, MS patients treated with DMT also showed lower effector/memory $CD8^+$ T cells than untreated patients. Our study suggested that

 

immunotherapy might play an anti-inflammatory role via reducing the effector/memory CD8$^+$ T cells, which provide a potential therapeutic pathway for CNS demyelinating disease.

In addition to investigate the phenotypes, we also evaluated the functions alternation of CD8$^+$ T cells by detecting pro-inflammatory cytokines secretions (including IFN$\gamma$ and TNF$\alpha$), which were thought to be associated with the severity of both NMOSD and MS.[23,34] Surprisingly, when comparing with healthy controls and untreated MS patients, we found not only a higher proportion of IFN$\gamma^+$CD8$^+$ T cells but also a significantly greater number of TNF$\alpha^+$CD8$^+$ T cells in patients with NMOSD. More importantly, both the two pro-inflammatory cytokines were found decreased in patients with NMOSD that treated with immunotherapy. These results indicated that CD8$^+$ T cells' pro-inflammatory cytokines including IFN$\gamma$ and TNF$\alpha$ played a pivotal role in the pathogenesis of NMOSD, and also contributed to explain why patients with NMOSD often suffered from more serious attacks than patients with MS. However, whether these pro-inflammatory cytokines produced by CD8$^+$ T cells can be used as potential biomarkers to monitor the development of NMOSD and how about their specificity and stability require to be further studied.

There were some potential limitations in this study. Our results mainly from the cross-sectional study, although we performed a small longitudinal observation pre- and post-treatment. Therefore, a lager and well-designed longitudinal study is still required. Furthermore, study with a larger sample size should be performed to confirm the up-regulation of pro-inflammatory cytokines in NMOSD.

## Conclusions

This study provides an evidence from humans that excessively activated CD8$^+$ T cells were associated in pathogenesis of NMOSD and MS, while immunotherapy seemed to be associated with reduced inflammatory pattern. Therefore, activated CD8$^+$ T cells may be a potential biomarker of efficacy determination and therapeutic target for NMOSD, while more thoroughly further studies are necessary.

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## Conflict of interest

The authors declare that they have no conflict of interest.

## Authors' contributions

ZYS, MY, and HYZ contributed to study conception and design. All authors contributed to collection samples. ZYS, YHQ, ZYZ, DKW, HXC, and QD contributed to performing flow cytometry and analyzing data. ZYS and YHQ contributed to writing the manuscript. MY, HYZ, and DKW contributed to critical revision of the manuscript. YZ, JCW, and CY contributed to statistical analysis. MY and HYZ obtained funding. All authors substantially contributed to this manuscript and approving the final version.

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## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1**. Flow cytometric gating strategy of $CD8^+$ T cell subpopulations. (A) $CD8^+$ T cell subpopulations were detected in a healthy control (HC): (a) lymphocytes, (b) $CD8^+$ T cells, (c) naïve T ($T_{N,}$ $CD62L^{hi}CD45RO^-$) cells and effector/memory T ($T_{E/M}$, $CD62L^{lo}CD45RO^+$) cells, and memory precursor ($T_{MP}$, $CD127^{hi}KLRG1^{lo}$), and short lived effector ($T_{SLEC}$, $CD127^{lo}KLRG1^{hi}$). Comparisons of $CD8^+$ $T_N$ and $CD8^+$ $T_{E/M}$ (B), $CD8^+$ $T_{MP}$ and $CD8^+$ $T_{SLEC}$ (C) among a HC, a NMOSD patient, and a MS patient.

**Figure S2**. Pre- and post-treatment of $CD8^+$ T cell subpopulations in patients with NMOSD. The proportions of $CD8^+$ T subpopulations were detected in 5 NMOSD patients' pre-treatment and post-treatment with IST more than 6 months. IST, including mycophenolate mofetil ($n = 4$) and azathioprine ($n = 1$). $T_{N,}$ naïve T cells; $T_{E/M}$, effector/memory T cells; $T_{MP}$, memory precursor T cells; $T_{SLEC}$, short lived effector T cells. $^*P < 0.05$ by paired t test.

**Figure S3**. Pre- and post-treatment of $CD8^+$ T cell subpopulations in patients with MS. The proportions of $CD8^+$ T subpopulations were detected in 5 MS patients' pre-treatment and post-treatment with DMT more than

 

6 months. DMT, including β-interferon ($n$ = 3) and rituximab ($n$ = 2). $T_{N,}$ naïve T cells; $T_{E/M}$, effector/memory T cells; $T_{MP}$, memory precursor T cells; $T_{SLEC}$, short lived effector T cells. P values were calculated by paired t test.

**Figure S4**. Flow cytometric gating strategy of IFNγ and TNFα in CD8$^+$ T cells. Unstimulated (A) and stimulated samples(B) (100ng/ml PMA + 1.5ug/ml Ionomycin for 4 hours) from a healthy control (HC): (a) lymphocytes, (b) CD3$^+$CD8$^+$ T cells, (c) CD3$^+$CD8$^+$IFNg$^+$ T cells, and (d) CD3$^+$CD8$^+$TNF$^+$ T cells. Comparisons of CD8$^+$ IFNg$^+$ (C) and CD8$^+$ TNFα$^+$ T cells (D) among a HC, a NMOSD patient, and a MS patient.

**Table S1.** Demographic and clinical characteristics of participants for detecting pro-inflammatory cytokines secreted by CD8$^+$ T cells

 