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RESEARCH

Phenotypic and functional dysregulations of CD8 + T Cells in myasthenia gravis

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Abstract
Myasthenia Gravis (MG) is a heterogeneous autoimmune disorder characterized by fluctuating muscle weakness caused by autoantibodies targeting neuromuscular junction components. While the role of CD4 + T cells in MG is well established, the contribution of CD8 + T cells remains poorly understood. In this study, we analyze CD8 + T cells in 36 MG patients and 38 age- and gender-matched controls using flow cytometry to evaluate subset distribution, granzyme expression, and cytokine production. MG patients exhibit an altered CD4 + /CD8 + T cell ratio and significant changes in CD8 + T cell subsets, including increased central memory CD8 + T cell (Tcm) proportions and decreased effector memory CD8 + T cell (Tem) proportions. Granzyme B expression in Tcm cells is significantly elevated in MG patients, whereas no significant changes are observed in other subsets or GZMK expression. Cytokine analysis reveals increased IL-21, GM-CSF, and IL-17A production by CD8 + T cells in MG patients. These phenotypic and functional alterations of CD8 + T cells persist during the acute phase of the disease, regardless of immunotherapy usage, and vary between ocular and generalized MG. Subgroup and correlation analyses further identify age-dependent and age-independent dysregulations of CD8 + T cells, indicating complex and subtype-specific roles of CD8 + T cells in the immunopathological processes underlying MG. Our findings provide novel insights into the involvement of CD8 + T cells in MG pathogenesis, laying a foundation for future research and potential therapeutic strategies targeting CD8 + T cells.

Keywords Myasthenia gravis · CD8 + T cells · Pathogenesis · Granzyme B · IL-21 · GM-CSF

Introduction

Myasthenia Gravis (MG) is a chronic autoimmune neuromuscular disorder characterized by muscle weakness and fatigue, particularly affecting the skeletal muscles responsible for voluntary movements [1]. The hallmark of MG pathogenesis is the production of autoantibodies directed against key components of the neuromuscular junction (NMJ), most commonly the acetylcholine receptor (AChR), but also muscle-specific kinase (MuSK) and lipoprotein receptor-related protein 4 (LRP4), disrupting neuromuscular transmission and causing muscle weakness [2]. While B cells and autoantibodies are central to MG pathogenesis, it is increasingly recognized that specific T cell subsets are essential for the aberrant humoral responses that drive the disease.

The role of CD4 + T cells in MG has been extensively studied and well established, with a specific focus on T follicular helper (Tfh) cells. Tfh cells are a subset of CD4 + T cells crucial for regulating humoral immunity, particularly through their crosstalk with B cells in germinal centers. Tfh cells provide essential help for B cell activation, differentiation, class switching, and the production of high-affinity antibodies in both physiological and pathological

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conditions [3]. In the context of MG, higher frequencies and numbers of circulating Tfh cells are observed in patients, and their levels correlate with the disease severity to some extent [4, 5]. Furthermore, circulating Tfh cells from MG patients exhibit elevated expressions of ICOS and IL-21, which are essential for their helper functions in humoral responses [4, 5]. Tfh cells are also present in ectopic germinal centers within thymomas and hyperplastic thymus of MG patients, where they support dysregulated humoral responses [6, 7]. Moreover, dysfunctions in regulatory T (Treg) cells and expansions of pathogenic Th17 cells have been documented in MG, collectively contributing to compromised immune tolerance and disease progression [8–11].

While the role of CD4 + T cells in the development of MG has become increasingly clear, the precise contribution of CD8 + T cells remains incompletely characterized. CD8 + T cells are primarily known for their cytotoxic function, where they eliminate target cells through the release of cytotoxins such as perforin and granzymes, and the production of proinflammatory cytokines. This classical role is crucial for protecting the body from viral infections and tumorigenesis [12]. Nevertheless, the involvement of CD8 + T cells in autoimmune diseases is more complex. On one hand, CD8 + T cells can contribute to tissue damage and the impairment of specific target cells through direct cytotoxicity or the secretion of proinflammatory cytokines. These mechanisms are implicated in the immunopathological processes of several autoimmune disorders, such as rheumatoid arthritis, type 1 diabetes, ulcerative colitis and immune thrombocytopenia [13–16]. On the other hand, accumulating evidence indicates that some subsets of CD8 + T cells may exert a regulatory function, shaping the immune response and restricting autoimmune reactions under certain conditions [17, 18]. However, research on the role of CD8 + T cells in MG remains scarce. Existing literature predominantly addresses the overall frequency of CD8 + T cells in MG patients [19]. Systematic investigations into the distinct roles of CD8 + T cell subsets, as well as their functional alterations in MG, are still lacking, which hinders a comprehensive understanding of their contribution to MG pathogenesis.

Given the existing gaps in current knowledge, we aim to investigate the alteration in CD8 + T cells in MG patients, with a particular focus on molecules critical for their immunoregulatory and cytotoxic functions. Through subgroup analysis, we explore the potential roles of CD8 + T cells in different MG subtypes, providing novel insights into the immune mechanisms underlying the disease. Ultimately, our findings may contribute to a better understanding of CD8 + T cell involvement in MG and inform potential therapeutic strategies targeting these cells.

Materials and methods

Study population

This study included 36 MG patients who were admitted to Department of Neurology, the First Affiliated Hospital of Shandong First Medical University, between May 2023 and July 2024. MG is diagnosed based on typical clinical features, including fluctuating muscle weakness, and one of the following criteria: (1) pharmacological testing showing symptom improvement with acetylcholinesterase inhibitors, (2) abnormal electrophysiological findings (e.g., repetitive nerve stimulation or single fiber electromyography), or (3) detection of autoantibodies against AChR, MuSK, or LRP4. All patients were either newly diagnosed or complained of symptom exacerbation or relapse at the time of enrollment. The study included 38 age- and gender-matched control subjects, who had no history of autoimmune diseases or conditions affecting immune function and were not on medications known to significantly impact immune responses. Additionally, all participants, including both MG patients and controls, were free of acute or chronic infections and had no history of severe systemic conditions, such as acute cardiovascular events, malignancies, or renal disorders. The baseline characteristics of all participants, including demographic and clinical information, are summarized in Table 1.

The study was approved by The Ethics Committee of the First Affiliated Hospital of Shandong First Medical University. Written informed consent was obtained from all participants, and the study was conducted in accordance with the Declaration of Helsinki.

PBMC isolation and cryopreservation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Human Lymphocyte Separation Medium (Dakewe, DKW-KLSH-0100) according to the manufacturer's instructions. Blood samples were collected from both MG patients and controls into EDTA-coated tubes. PBMCs were separated by layering the blood over the separation medium and centrifuging at 700 × g for 30 min at room temperature. The mononuclear cell layer was carefully collected, washed with phosphate-buffered saline (PBS), and resuspended for further processing. Isolated PBMCs were cryopreserved in a freezing solution containing 90% fetal bovine serum (FBS; Gibco, 10099141C) and 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich, D2650), and stored in liquid nitrogen for future analysis.

Table 1 Demographic and clinical characteristics of the participants

Characteristic	CTR (n = 38)	MG (n = 36)	*P* value
Age, median (IQR), years	60 (52.25–66)	61 (52.5–69.75)	0.4589
Sex, No. (%)			
Male	20 (52.6)	19 (52.8)	> 0.9999
Female	18 (47.4)	17 (47.2)	
Autoantibody profile, No. (%)			
AChR (%)	-	27 (75.0)	-
MuSk (%)	-	3 (8.3)	
Negative (%)	-	6 (16.7)	
Thymic abnormality, No. (%)	-	12 (33.3)	-
LOMG, No. (%)	-	27 (75.0)	-
MGFA class, No. (%)			
MGFA I	-	13 (36.1)	-
MGFA II	-	19 (52.8)	
MGFA III	-	3 (8.3)	
MGFA IV	-	1 (2.8)	
MGFA V	-	0 (0)	
Immunotherapy, No. (%)			
None	-	19 (52.8)	
CS only	-	8 (22.2)	
CS and nonsteroidal IS	-	9 (25.0)	

IQR interquartile range, *AChR* acetylcholine receptor, *MuSK* muscle-specific tyrosine kinase, *LOMG* late-onset MG, *MGFA* Myasthenia Gravis Foundation of America classification, *CS* corticosteroid, *IS* immunosuppressants

Cell resuscitation

For analysis, cryopreserved PBMCs were thawed quickly in a 37 °C water bath and immediately diluted in complete RPMI 1640 medium (Gibco, C11875500BT) containing 10% FBS. Cells were then centrifuged at 300 × g for 5 min to remove the cryoprotectant, and resuspended in complete medium. Cell viability was assessed using the trypan blue exclusion method, with > 90% viability considered acceptable for subsequent experiments.

Flow cytometry

Flow cytometry was performed to characterize the surface molecules, intracellular granzyme profiles, and cytokine production of CD8 + T cells. For surface molecule detection, PBMCs were first stained with Zombie Aqua viability dye (Biolegend, 423,101) for 10 min at room temperature and then incubated with the following antibodies for 20 min in the dark: BV421 anti-human CD3 (UCHT1; BD Biosciences, 562,426), BV605 anti-human CD4 (RPA-T4; BD Biosciences, 562,658), BV650 anti-human CD8 (RPA-T8; BD Biosciences, 563,821), BV785 anti-human CCR7 (G043H7; Biolegend, 353,230), APC-eFluor780 anti-human CD45RA (HI100; Invitrogen, 47-0458-42), PE-Cy7 anti-human PD-1 (eBioJ105 (J105); Invitrogen, 25-2799-42), KIRAVIA Blue 520 (SA231A2; Biolegend, 367,726), and AF700 anti-human HLA-DR (LN3; Biolegend, 327014).

To assess granzyme expression, PBMCs were first stained with Zombie Aqua viability dye and specific surface antibodies (anti-human CD3, CD4, CD8, CCR7, and CD45RA) as described above. Following surface staining, cells were fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Biosciences, 554,714) according to the manufacturer's instructions. Intracellular staining was then performed with PE anti-human granzyme B (GB11; BD Biosciences, 561,142) and PerCP-Cy5.5 anti-human granzyme K (GM26E7; Biolegend, 370,514) for 30 min at 4 °C in the dark. After staining, cells were washed and prepared for detection.

For intracellular cytokine staining, PBMCs were restimulated in vitro with a cell stimulation cocktail containing protein transport inhibitors (Invitrogen, 00–4975-93) for 4 h at 37 °C. Following stimulation, cells were stained with Zombie Aqua viability dye and the following surface antibodies: FITC anti-human CD3 (UCHT1; Biolegend, 300,406), BV605 anti-human CD4, and BV650 anti-human CD8. After surface staining, fixation and permeabilization were performed using the BD Cytofix/Cytoperm kit, followed by intracellular staining for 30 min at 4 °C in the dark. The following antibodies were

used for cytokine detection: PerCP anti-human IFN-γ (4S.B3; Biolegend, 502,524), PE anti-human IL-21 (3A3-N2; Biolegend, 513,004), BV421 anti-human GM-CSF (BVD2-21C11; BD Biosciences, 562,930), and APC-eFluor780 anti-human IL-17A (eBio64DEC17; Invitrogen, 47–7179-42).

Stained cells were analyzed using a Beckman CytoFLEX flow cytometer (Beckman Coulter), and the data were processed and analyzed with FlowJo software.

Statistical analysis

All results are presented as means ± SEM. Normality was assessed using the Shapiro–Wilk test, and homogeneity of variance was evaluated using the Brown-Forsythe test. For data following a normal distribution, hypothesis testing was performed using Student's t-test or one-way ANOVA, with multiple comparisons conducted using the Šidák test for equal variances or Dunnett's T3 test for unequal variances. For data not following a normal distribution, non-parametric tests (Mann–Whitney test or Kruskal–Wallis test) were employed, followed by Dunn's multiple comparisons test. Correlations between variables were analyzed using Spearman's rank correlation. Statistical analyses were performed with Prism 9 software (GraphPad). A statistically significant difference is indicated as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$, $****P < 0.0001$.

Results

Alterations in CD8 + T cell subsets and functional markers in MG patients

We first examined the frequencies of CD4 + and CD8 + T cells among total T cells in MG patients. The frequency of CD8 + T cells was significantly decreased in MG patients compared to controls (Fig. 1B, middle panel), while the proportion of CD4 + T cells exhibited only a slight change (Fig. 1B, left panel). Consequently, the ratio of CD4 + T cells to CD8 + T cells showed an increasing trend in MG patients ($P = 0.0783$; Fig. 1B, right panel), reflecting an altered balance between these subsets.

Circulating CD8 + T cells are heterogeneous, consisting of subsets at different differentiation and functional stages [20]. Based on the expression of CCR7 and CD45RA, circulating CD8 + T cells can be classified into naïve T cells, central memory T (Tcm) cells, effector memory T (Tem) cells, and terminally differentiated effector memory T cells re-expressing CD45RA (Temra) (Fig. 1C). To further elucidate the role of CD8 + T cells in MG, we analyzed the distribution of these subsets. The proportions of naïve T cells and Temra cells were not significantly altered in MG patients compared to controls (Fig. 1D). Intriguingly, the proportion of Tcm cells was significantly increased, accompanied by a corresponding decrease in Tem cells in MG patients (Fig. 1D), suggesting changes in both the immunological memory and effector characteristics of CD8 + T cells.

We then examined the expression of HLA-DR, PD-1, and KLRG1 in CD8 + T cell subsets, which are associated with T cell activation, exhaustion, or senescence [21–23]. Evaluating the expression of these surface molecules provides valuable insights into the fundamental status of CD8 + T cells in MG. However, their expression levels were comparable between MG patients and controls across all three subsets (Fig. S1A–F), suggesting they may not contribute significantly to the altered distribution of CD8 + T cell subsets observed in MG.

Distinct patterns of granzyme expression in CD8 + T cell subsets from MG patients

To further investigate the functional status of CD8 + T cell subsets in MG, we examined the expression of granzyme B (GZMB), a key mediator of CD8 + T cell cytotoxicity under both physiological and pathological conditions [24]. First, we analyzed the overall expression of GZMB in CD8 + T cells and found no significant difference between MG patients and controls (Fig. 2A-B, left panel). Next, we assessed GZMB expression across individual CD8 + T cell subsets. The proportion of GZMB-positive cells was significantly increased in Tcm cells from MG patients compared to controls (Fig. 2A-B, middle-left panel), whereas no significant changes were observed in Tem and Temra subsets (Fig. 2A-B, middle-right and right panel). Consistently, a marked elevation in the proportion of GZMB + Tcm in total CD8 + T cells was observed in MG patients (Fig. 2C, left panel), while the proportions of GZMB + Tem and GZMB + Temra cells remained unchanged (Fig. 2C, middle and right panel).

CD8 + T cells expressing granzyme K (GZMK) are known to exhibit distinct phenotypes and functions compared to GZMB + CD8 + T cells. Recent studies have highlighted the critical role of GZMK + CD8 + T cells in immunosenescence and tissue inflammation in various autoimmune diseases [24, 25]. To explore their potential roles in MG, we analyzed GZMK expression in CD8 + T cells from MG patients and controls. The overall expression of GZMK in total CD8 + T cells and its subsets (Tcm, Tem, and Temra) showed no significant differences between the two groups (Fig. S2A-B). Notably, the proportion of GZMK + Tcm cells within total CD8 + T cells was significantly increased in MG patients compared to controls (Fig. S2C, left panel). This increase can be attributed to the higher proportion of Tcm cells within the CD8 + T cell population in MG patients, as described earlier.

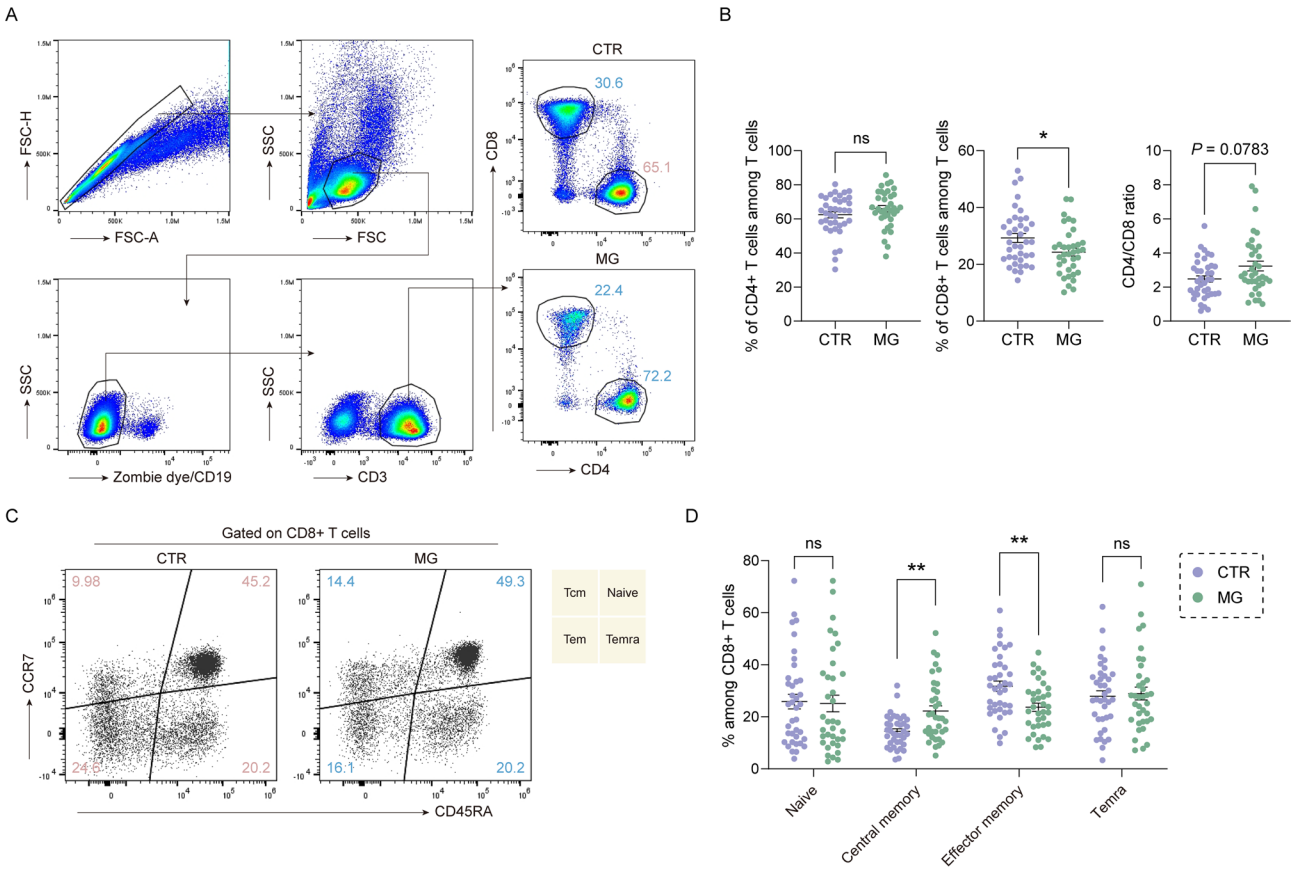

Fig. 1 Altered frequencies and subset distribution of CD8 + T cells in MG patients. (A) Representative flow cytometry plots showing the gating strategy for identifying CD4 + and CD8 + T cells among total T cells in MG and control group. (B) Quantification of CD4 + and CD8 + T cell frequencies among total T cells and the CD4/CD8 ratio in MG patients (n = 36) and controls (n = 38). (C) Representative flow cytometry plots demonstrating the classification of CD8 + T cell subsets based on CCR7 and CD45RA expression in MG and control group. (D) Quantification of naïve, central memory (Tcm), effector memory (Tem), and terminally differentiated effector memory (Temra) CD8 + T cell subsets in MG patients and controls. Data are presented as means ± SEM. Statistical significance was determined using Student's t-test for normally distributed data and Mann–Whitney test for not normally distributed data (ns, not significant; $*P < 0.05$, $**P < 0.01$)

These findings suggest that while the overall expression of GZMB and GZMK in CD8 + T cells remains unchanged in MG, the specific increase in GZMB + and GZMK + Tcm cells highlights the potential importance of this subset in the immunopathology of MG.

Altered cytokine production profiles in CD8 + T cells from MG patients

In addition to their cytotoxic activity, cytokine secretion represents another critical mechanism by which CD8 + T cells exert their effector functions. Beyond producing classical proinflammatory cytokines such as IFN-γ and TNF-α, CD8 + T cells are also capable of secreting IL-17A and GM-CSF [26], which are recognized as key effector molecules in Th17-mediated autoimmunity [27]. Furthermore, recent studies have identified a subset of CXCR5 + CD8 + T cells that support B cell responses through IL-21 secretion, underscoring their potential role in modulating humoral immunity [28]. Therefore, we analyzed the production of these cytokines by CD8 + T cells from MG patients and control subjects.

We found that the production of IFN-γ by CD8 + T cells did not show significant differences between MG patients and controls (Fig. 3B, left panel). In contrast, IL-21 production was significantly increased in CD8 + T cells from MG patients (Fig. 3B, middle panel), along with a corresponding increase in the subset of CD8 + T cells co-producing IFN-γ and IL-21 (Fig. 3B, right panel). Additionally, CD8 + T cells from MG patients exhibited a marked elevation in GM-CSF production (Fig. 3D, left panel). Furthermore, the proportion of CD8 + T cells producing IL-17A, as well as those co-producing IL-17A and GM-CSF, was also increased in MG patients (Fig. 3D, middle and right panel), albeit at a relatively low frequency in circulating CD8 + T cells.

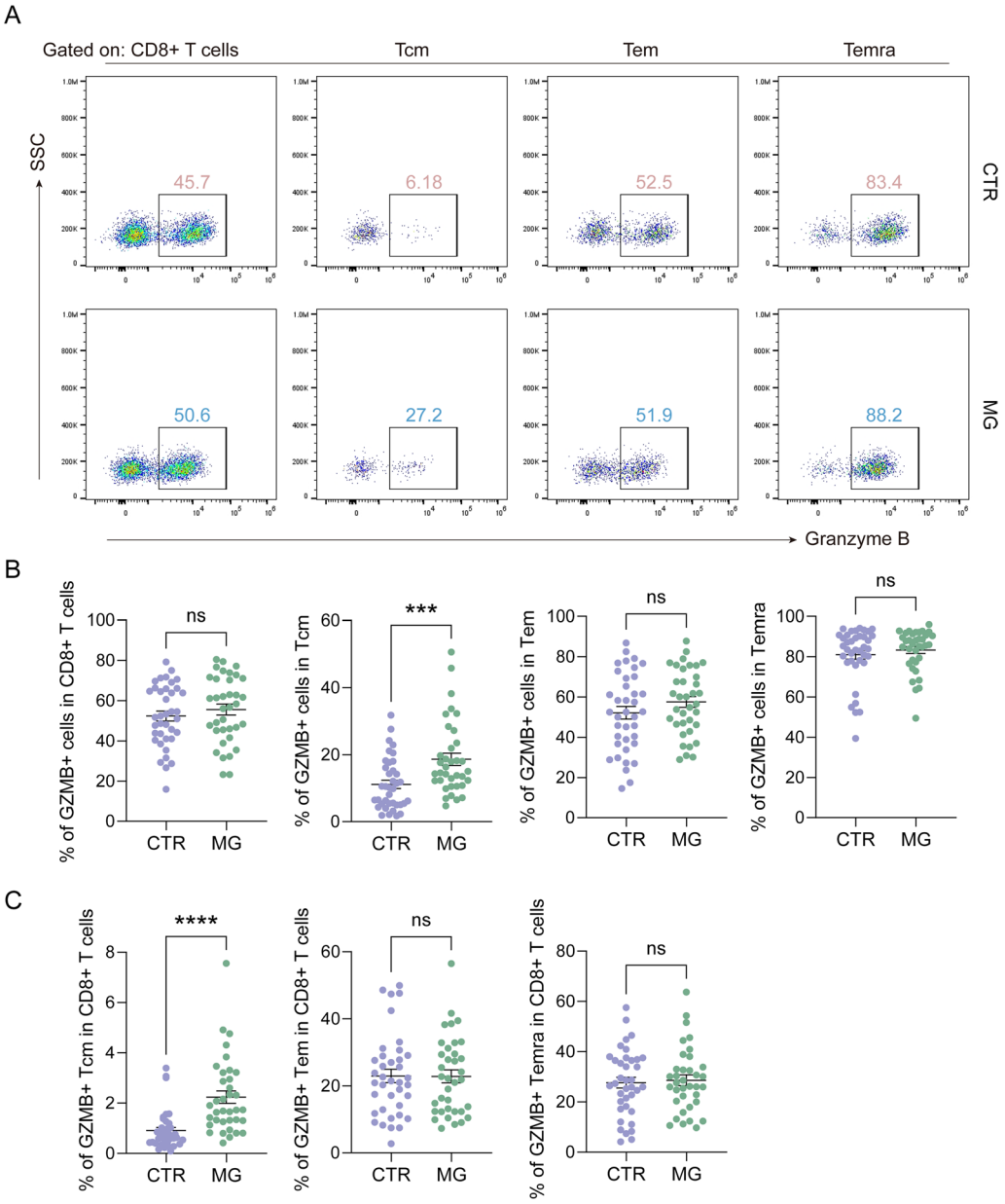

Fig. 2 Expression of GZMB in CD8 + T cells from MG patients and controls. (A) Representative flow cytometry plots showing GZMB expression in CD8 + T cells and their subsets from controls and MG patients. (B) Quantification of GZMB expression in total CD8 + T cells and across Tcm, Tem, and Temra subsets from MG patients (n = 36) and controls (n = 38). (C) Proportions of GZMB + Tcm, Tem, and Temra cells within total CD8 + T cells in MG patients and controls. Data are presented as means ± SEM. Statistical significance was determined using Student's t-test for normally distributed data and Mann–Whitney test for not normally distributed data (ns, not significant; ***$P < 0.001$, ****$P < 0.0001$)

These results highlight a shift in the cytokine production profile of CD8 + T cells in MG, characterized by increased IL-21 and GM-CSF production, as well as the emergence of IL-17A-producing subsets, which may reflect their involvement in the inflammatory processes underlying MG.

Persistent phenotypic and functional dysregulation of CD8 + T cells in MG development and symptom exacerbation

To better understand the roles of CD8 + T cells in MG, we performed a subgroup analysis based on immunotherapy

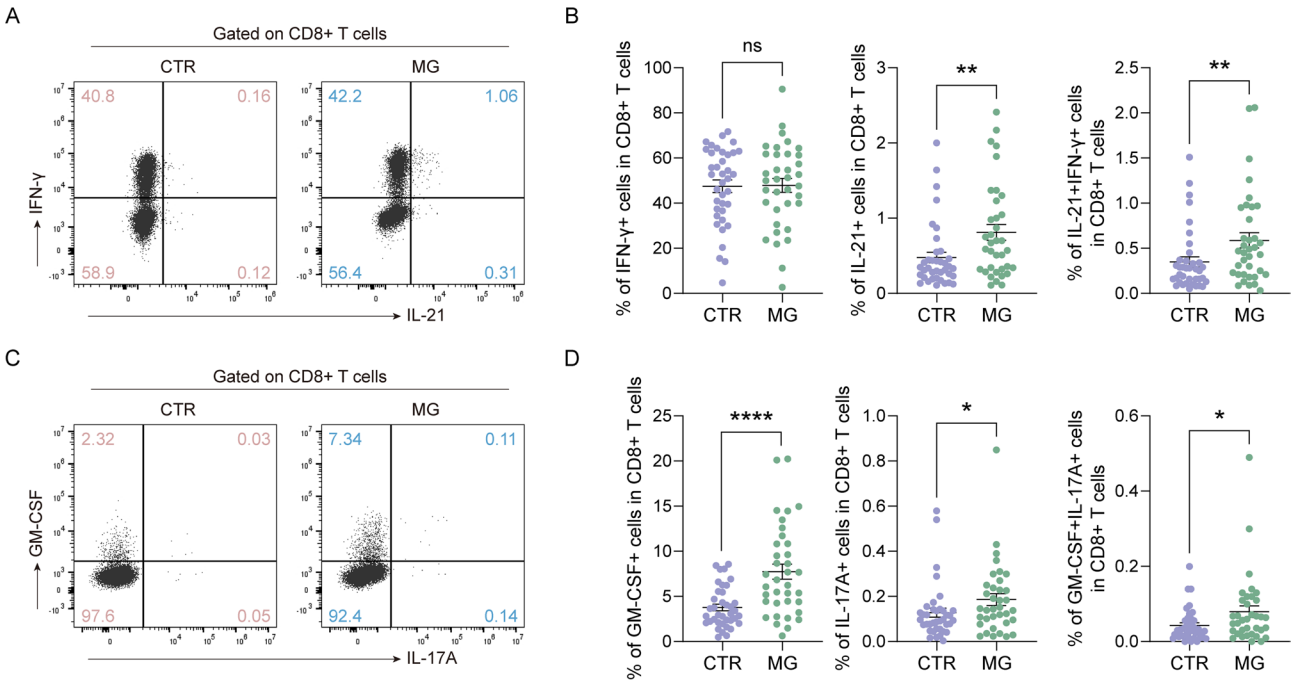

Fig. 3 Increased production of IL-21, GM-CSF, and IL-17A by CD8 + T cells from MG patients. (A) Representative flow cytometry plots showing the expression of IFN-γ and IL-21 in CD8 + T cells from controls and MG patients. (B) Quantification of IFN-γ, IL-21, and dual IFN-γ/IL-21-producing CD8 + T cells in MG patients (n = 36) and controls (n = 38). (C) Representative flow cytometry plots showing the expression of GM-CSF and IL-17A in CD8 + T cells from controls and MG patients. (D) Quantification of GM-CSF, IL-17A, and dual GM-CSF/IL-17A-producing CD8 + T cells in MG patients and controls. Data are presented as means ± SEM. Statistical significance was determined using Student's t-test for normally distributed data and Mann–Whitney test for not normally distributed data (ns, not significant; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$)

status. Patients were divided into two groups: an immunotherapy-naïve group (naïve, n = 19) and an immunotherapy-treated group (treated, n = 17). The treated group included patients receiving corticosteroids alone or in combination with immunosuppressants. Since all patients were either newly diagnosed or experiencing symptom exacerbation or relapse, this stratification provided an opportunity to assess the implications of CD8 + T cells in MG patients with uncontrolled autoimmune responses.

Our analysis revealed no significant differences in the proportion of CD8 + T cells, the CD4/CD8 ratio, or the distribution of CD8 + T cell subsets (naïve T, Tcm, Tem, and Temra) between the immunotherapy-naïve and immunotherapy-treated groups (Fig. 4A). Similarly, the expression levels of GZMB and GZMK within CD8 + T cell subsets were comparable between the two groups (Fig. 4B-C). Furthermore, cytokine production by CD8 + T cells, including IFN-γ, GM-CSF, IL-21, and IL-17A, also appeared unaffected by immunotherapy in patients experiencing symptom exacerbation (Fig. 4D-E).

These findings suggest that the phenotypic and functional changes of CD8 + T cells persist during the acute phase of the disease, reinforcing their potential involvement in the pathogenesis of MG.

Comparison of CD8 + T cells between OMG and GMG patients

We then compared CD8 + T cells between ocular MG (OMG, n = 13) and generalized MG (GMG, n = 23) patients. Our analysis revealed no significant differences in the proportion of CD8 + T cells, the CD4/CD8 ratio, or the distribution of CD8 + T cell subsets between the two groups (Fig. 5A). Similarly, the expression of GZMB and GZMK within CD8 + T cell subsets was also comparable (Fig. 5B-C). In terms of cytokine production, we found that the production of IFN-γ and IL-21 by CD8 + T cells did not differ significantly between OMG and GMG patients (Fig. 5D). However, the production of GM-CSF and IL-17A by CD8 + T cells was significantly higher in OMG patients (Fig. 5E).

These findings suggest that CD8 + T cell dysregulation may not be directly linked to disease severity but rather reflect the autoimmune processes underlying MG. Additionally, the differences in GM-CSF and IL-17A production highlight potential functional divergence of CD8 + T cells between OMG and GMG.

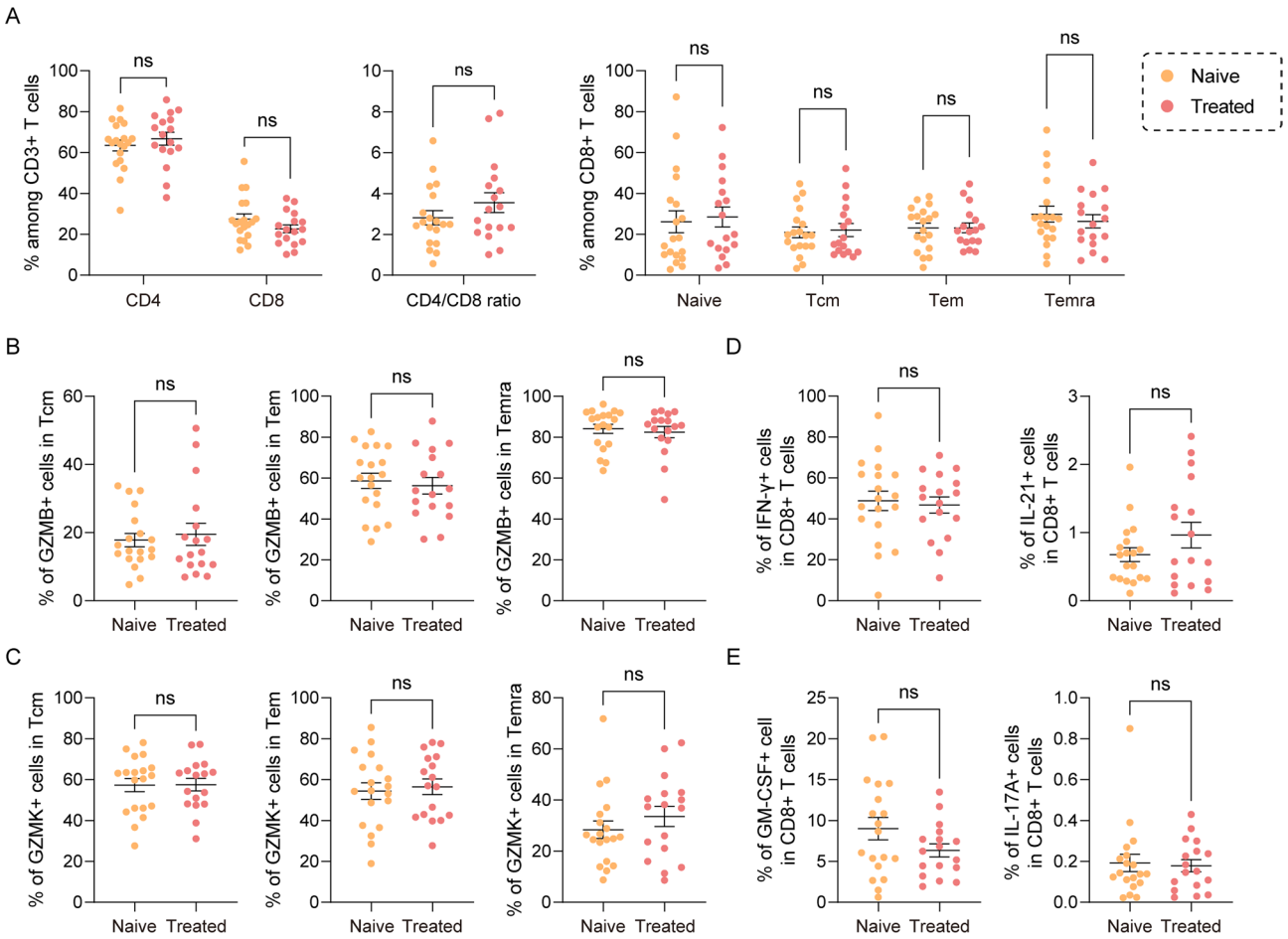

Fig. 4 Subgroup analysis of CD8 + T cells based on immunotherapy status in MG patients. (A) Quantification of the proportion of CD8 + T cells, the CD4/CD8 ratio, and the distribution of CD8 + T cell subsets (naïve T, Tcm, Tem, and Temra) in immunotherapy-naïve (n = 19) and immunotherapy-treated (n = 17) MG patients. (B) Comparison of GZMB expression within CD8 + T cell subsets between immunotherapy-naïve and immunotherapy-treated MG patients. (C) Comparison of GZMK expression within CD8 + T cell subsets between the two patient groups. (D) Quantification of IFN-γ and IL-21 production by CD8 + T cells in immunotherapy-naïve and immunotherapy-treated MG patients. (E) Quantification of GM-CSF and IL-17A production by CD8 + T cells in the two patient groups. Data are presented as means ± SEM. Statistical significance was determined using Student's t-test for normally distributed data and Mann–Whitney test for not normally distributed data (ns, not significant)

Distinct CD8 + T cell biology in EOMG and LOMG

MG is increasingly recognized as a heterogeneous disease, with early-onset MG (EOMG) and late-onset MG (LOMG) showing distinct clinical features and underlying mechanisms [29]. To determine whether CD8 + T cells play different roles in EOMG and LOMG, we stratified MG patients into two subgroups based on age of onset: EOMG (n = 9) and LOMG (n = 27). Given the significant impact of aging on phenotypes and functions of CD8 + T cells [25], we also divided the control group into two age-matched subgroups: individuals under 50 years of age (n = 9) and those over 50 years of age (n = 29). This stratification enabled the identification of disease-specific alterations in CD8 + T cells.

Subgroup analysis revealed that the proportion of naïve T cells within CD8 + T cells was lower in LOMG patients compared to EOMG patients (Fig. 6A, left panel). However, no significant differences in naïve T cell proportions were observed when comparing either EOMG or LOMG patients to their respective age-matched controls (Fig. 6A, left panel). Corresponding to the decrease in naïve T cells, other subset proportions exhibited varying degrees of increase in LOMG compared to EOMG, although these differences did not reach statistical significance (Fig. 6A, rightmost three panels). Notably, the increase in Tcm cell proportion was more pronounced in LOMG patients than in EOMG patients when compared to age-matched controls (Fig. 6A, middle-left panel). In contrast, Tem proportions were consistently decreased in both EOMG and LOMG patients,

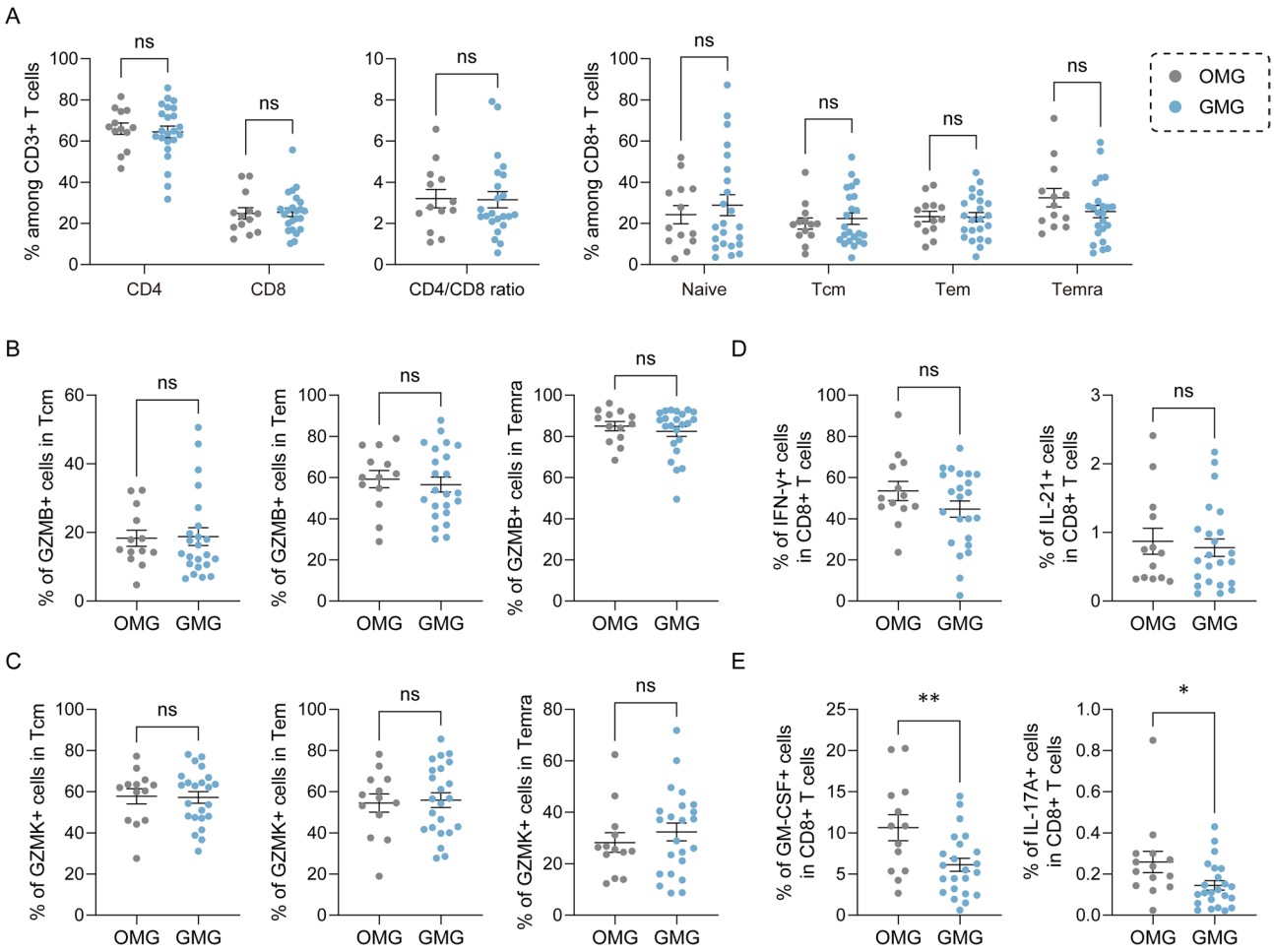

Fig. 5 Comparison of CD8 + T cells between OMG and GMG patients. (A) Quantification of the proportion of CD8 + T cells, the CD4/CD8 ratio, and the distribution of CD8 + T cell subsets (naïve T, Tcm, Tem, and Temra) in OMG (n = 13) and GMG (n = 23) patients. (B) Comparison of GZMB expression within CD8 + T cell subsets between OMG and GMG patients. (C) Comparison of GZMK expression within CD8 + T cell subsets in the two patient groups. (D) Quantification of IFN-γ and IL-21 production by CD8 + T cells in OMG and GMG patients. (E) Quantification of GM-CSF and IL-17A production by CD8 + T cells in OMG and GMG patients. Data are presented as means ± SEM. Statistical significance was determined using Student's t-test for normally distributed data and Mann–Whitney test for not normally distributed data (ns, not significant; $*P < 0.05$, $**P < 0.01$)

although the limited sample size may have precluded statistical significance in the EOMG group (Fig. 6A, middle-right panel). The proportion of Temra cells remained comparable between MG patients and their respective age-matched controls in both subgroups (Fig. 6A, right panel).

Regarding granzyme expression, an increased proportion of GZMB + cells within the Tcm subset was observed in MG patients compared to age-matched controls, with this elevation being more pronounced in LOMG patients (Fig. 6B, left panel). In contrast, no significant age-independent changes were detected for GZMB expression in other subsets (Fig. 6B, middle and right panels) or GZMK expression across any CD8 + T cell subsets (Fig. 6C).

For cytokine production, no significant differences in IFN-γ production by CD8 + T cells were found between MG patients and age-matched controls (Fig. 6D, left panel). However, IL-21 production was significantly higher in LOMG patients compared to both EOMG patients and age-matched controls (Fig. 6D, right panel). Similarly, GM-CSF production was increased in MG patients compared to controls, with a more pronounced elevation observed in LOMG patients (Fig. 6E, left panel). For IL-17A, an increasing trend was observed only in LOMG patients when compared to age-matched controls (Fig. 6E, right panel).

Finally, we analyzed the correlation between specific CD8 + T cell features and age. As expected, a significant negative correlation between age and the proportion of naïve T cells was observed in both MG patients and controls (Fig. 6F, left panel). Interestingly, a positive correlation between age and the proportion of Tcm cells was identified in MG patients, but not in controls (Fig. 6F, right panel). In controls, a weak positive correlation was

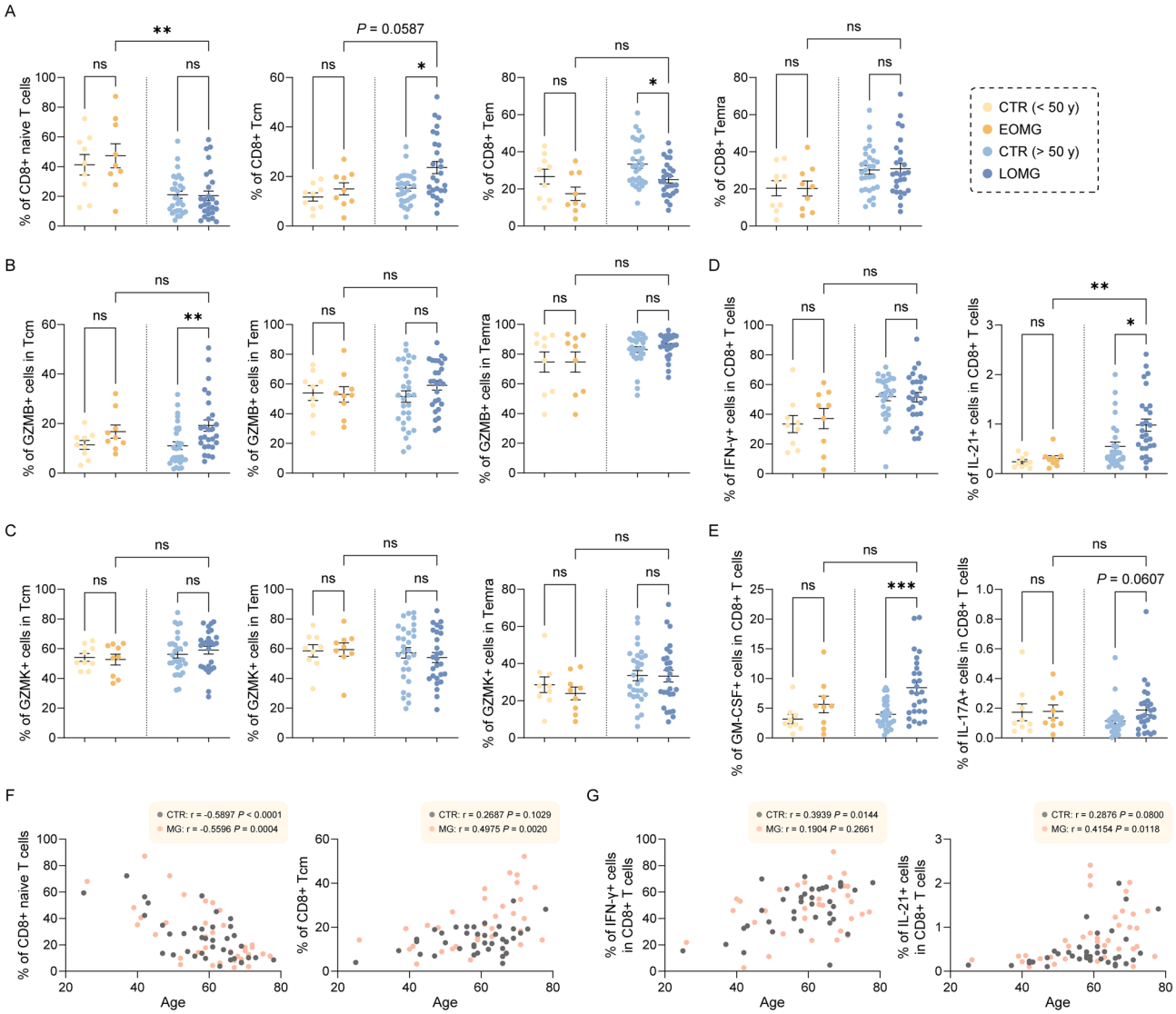

Fig. 6 Different phenotypic and functional characteristics of CD8 + T Cells in EOMG and LOMG. (A) Quantification of CD8 + T cell subsets (naïve T, Tcm, Tem, and Temra) in EOMG (n = 9) and LOMG (n = 27) patients compared to their respective age-matched controls (n = 9 for controls under 50 years and n = 29 for controls over 50 years). (B) Comparison of GZMB expression in Tcm, Tem, and Temra subsets between MG patients and age-matched controls. (C) Comparison of GZMK expression across CD8 + T cell subsets from MG patients and age-matched controls. (D) Quantification of IFN-γ and IL-21 production by CD8 + T cells from MG patients and age-matched controls. (E) Quantification of GM-CSF and IL-17A production by CD8 + T cells from MG patients and age-matched controls. (F) Correlation analysis between age and the proportion of naïve T cells as well as Tcm cells in MG patients and controls. (G) Correlation analysis between age and cytokine production (IFN-γ and IL-21) by CD8 + T cells in MG patients and controls. Data are presented as means ± SEM. Statistical significance in multiple comparsions (A-E) was determined by Šidák test for equal variances and Dunnett's T3 test for unequal variances, or by Dunn's test for not normally distributed data. (ns, not significant; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). Correlation analysis (F-G) was performed using Spearman's rank correlation coefficient

observed between age and IFN-γ production by CD8 + T cells, but this correlation was absent in MG patients (Fig. 6G, left panel). Instead, a weak positive correlation between age and IL-21 production by CD8 + T cells was identified in MG patients (Fig. 6G, right panel).

In summary, age-stratified analysis revealed multiple age-dependent and age-independent alterations in CD8 + T cells from MG patients, highlighting potentially distinct roles for CD8 + T cells in the pathogenesis of EOMG and LOMG.

Discussions

In this study, we investigated the phenotypic and functional dysregulations of CD8 + T cells in Myasthenia Gravis (MG) patients, focusing on their subset distribution, granzyme expression, and cytokine production. Our findings reveal significant alterations in CD8 + T cell profiles, highlighting the potential roles of CD8 + T cells in MG pathogenesis.

A major finding of this study is the altered distribution of CD8 + T cell subsets in MG patients, marked by increased Tcm proportions and decreased Tem proportions. Tcm and Tem represent two distinct subsets of memory T cells in circulation. Tcm cells exhibit greater longevity, stemness, and plasticity, characterized by high expression of the homing receptor CD62L and the chemokine receptor CCR7, which enable their recirculation between the blood and lymphoid tissues [30]. In contrast, Tem cells represent more differentiated memory subsets with enhanced effector functions, marked by the absence of CD62L and CCR7 expression, and are predominantly localized to peripheral blood and sites of inflammation [30]. The significance of altered CD8 + Tcm and Tem proportions in autoimmune diseases remains uncertain. Research in this area is still limited, with findings often inconsistent even in the same disease. For instance, Khanniche et al. and Maldonado et al. have reported increased Tcm proportions and decreased Tem proportions within peripheral blood CD8 + T cells in RA patients [31, 32]. Nevertheless, another study has reported a significant elevation of Tem proportions in CD8 + T cells in both peripheral blood and synovial fluid of RA patients, with no notable changes in Tcm proportions [33]. These discrepancies underscore the complexity of CD8 + T cell dynamics in autoimmune conditions. The shift in Tcm and Tem proportions may represent pathological immune memory in autoimmunity or could alternatively result from increased migration of Tem cells to sites of inflammation. Additionally, it remains unclear which memory T cell subset is enriched with autoreactive T cells in autoimmune conditions. Future investigations employing advanced techniques, including single-cell transcriptomics, single-cell TCR sequencing, and tetramer-based assays, are expected to address these questions and provide deeper insights into the role of CD8 + T cell memory in autoimmune responses.

We examined the expression of HLA-DR, PD-1, and KLRG1 on CD8 + T cells. HLA-DR is generally upregulated during T cell activation [21], whereas PD-1, an immune checkpoint molecule, is associated with both T cell activation and exhaustion [22]. KLRG1 is considered as a marker of T cell senescence and terminal differentiation [23]. Nevertheless, our analysis did not demonstrate significant differences in the expression of these markers among CD8 + T cell subsets in MG patients when compared to control subjects. This finding led us to further investigate the functional characteristics of CD8 + T cells. While the overall expression levels of GZMB and GZMK in CD8 + T cells did not differ significantly between MG patients and controls, a notable increase in GZMB expression was observed specifically within Tcm cells of MG patients. Moreover, the elevated proportion of Tcm cells in these patients resulted in increased frequencies of both GZMB + Tcm and GZMK + Tcm cells within the CD8 + T cell population. As previously discussed, Tcm cells exhibit greater stemness and differentiation potential, while Tem cells are more differentiated and possess stronger effector functions. Typically, GZMB expression is higher in Tem cells compared to Tcm cells. However, in MG patients, the elevated GZMB expression within the Tcm subset deviates from this conventional pattern. This observation may have several implications. Firstly, the increase in GZMB expression suggests that Tcm cells in MG patients may have acquired enhanced cytotoxic potential, indicating a shift towards pathological immune memory. In MG, Tcm cells might not merely serve as reservoirs of immune memory but also actively contribute to cytotoxic activity. Secondly, the elevated GZMB expression in Tcm cells may indicate that these cells are pre-activated or primed for cytotoxic responses, potentially as an adaptive response to chronic inflammation or ongoing immune activation in MG. Finally, the aberrant expression of GZMB in Tcm cells, normally circulating between peripheral blood and lymphoid tissues, might disrupt the intricate interactions between CD8 + T cells and other immune populations. In addition to altered granzyme expression, we found that CD8 + T cells from MG patients exhibited significantly higher secretion of IL-21, GM-CSF, and IL-17A, while IFN-γ production remained unchanged. To our knowledge, this is the first report documenting such changes in CD8 + T cells within the context of MG. Previous research has established an increased production of IL-21, GM-CSF, and IL-17A by CD4 + T cells in MG patients [4, 5, 34]. Our findings expand the understanding of T cell abnormalities in MG by highlighting the involvement of CD8 + T cells in shaping the immune microenvironment of the disease.

It is noteworthy that all the MG patients included in our study were in the acute phase of the disease, comprising both newly diagnosed, treatment-naïve individuals and those who had previously undergone immunotherapy but experienced symptom exacerbation or relapse. Importantly, the alterations in CD8 + T cells observed did not differ significantly between these two cohorts, implying that these changes are concomitant with uncontrolled autoimmune

responses in MG. These findings prompted us to consider the potential roles of CD8 + T cells in the immunopathological mechanisms underlying MG. CD8 + T cells are capable of inducing target cell damage and tissue destruction, a phenomenon observed in autoimmune disorders such as RA, type 1 diabetes, ulcerative colitis, and immune thrombocytopenia [13–16]. In MG, CD4 + T cell-driven humoral immunity and antibody-mediated compromised neuromuscular transmission are widely regarded as the core immunopathological mechanisms [2]. However, studies have also reported the presence of inflammatory cell infiltration in muscle tissues in a subset of MG patients [35, 36]. Based on these findings, we hypothesize that CD8 + T cells may contribute to the autoimmune process in MG through multiple mechanisms. They could interact with other immune cell subsets or shape an inflammatory microenvironment within the thymus or lymphoid tissues, thereby amplifying autoimmune responses. Additionally, in certain patients, CD8 + T cells might infiltrate muscle tissues, contributing directly to neuromuscular dysfunctions. On the other hand, the dual roles of CD8 + T cells in autoimmunity have garnered increasing recognition in recent studies. Specific subsets of CD8 + T cells, such as those expressing killer cell immunoglobulin-like receptors (KIRs), have been identified as having regulatory roles by eliminating autoreactive CD4 + T cells and restraining autoimmune responses in humans [18]. Besides, these KIR + CD8 + T cells are often associated with a Temra phenotype [37]. Whether KIR + CD8 + T cells are involved in MG pathogenesis remains an open question for future investigations.

Another intriguing finding of this study is the potential differences in the roles of CD8 + T cells in LOMG and EOMG. For instance, changes in Tcm proportions, elevated GZMB expression in Tcm, and augmented production of IL-21, GM-CSF, and IL-17 were all more prominent in LOMG. Additionally, in MG patients, we observed correlations between Tcm proportions, IL-21 production, and age, which were absent in controls. MG is increasingly recognized as a heterogeneous disease, with EOMG and LOMG potentially driven by distinct pathogenic mechanisms. EOMG is often associated with thymomas and thymic hyperplasia, while the precise mechanisms underlying LOMG await further elucidation [2, 29]. Our previous research identified type 2 diabetes as an independent risk factor for LOMG [38]. Based on these findings, we hypothesize that CD8 + T cells may play a more significant role in the pathogenesis of LOMG.

Indeed, our study has several limitations. First, the relatively small sample size reduced the statistical power of the analysis, particularly for subgroup comparisons. Second, we did not include patients in the stable phase or those with improved symptoms following treatment, making it unclear whether the observed changes in CD8 + T cells are dynamic or linked to symptom control. Third, the alterations detected in peripheral blood may not fully reflect the actual roles of CD8 + T cells in MG pathogenesis, as their activity in lymphoid tissues or affected tissues remains unexplored. Fourth, the use of conventional flow cytometry limited the breadth and depth of the analysis, preventing a comprehensive and unbiased characterization of CD8 + T cells. Finally, as a cross-sectional study, this work cannot establish causal relationships between CD8 + T cell changes and MG. Further longitudinal cohort studies, mechanistic investigations, and animal experiments are needed to elucidate the precise roles of CD8 + T cells in MG.

In summary, our study highlights significant phenotypic and functional alterations in CD8 + T cells in MG patients, including changes in memory subsets, granzyme expression, and cytokine production. These findings provide novel insights into the involvement of CD8 + T cells in the immunopathology of MG, suggesting distinct roles in different disease subtypes. While the results lay the groundwork for understanding the contribution of CD8 + T cells to MG, further studies are warranted to clarify their causal and mechanistic roles, potentially opening avenues for CD8 + T cell-targeted therapeutic strategies.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate All experiments were performed in accordance with the Declaration of Helsinki. The research received approval from the Research Ethics Committee of The First

Affiliated Hospital of Shandong First Medical University and all participants provided written informed consent.

Conflicts of interest The authors declare no competing interests.

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