**Role of perforin secretion from CD8+ T-cells in neuronal cytotoxicity in multiple sclerosis 31**

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**Abstract**

Multiple sclerosis is the most prevalent autoimmune disease of the central nervous system characterized by the presence of inflammation, myelin damage. While the immune system initiates the autoimmune response it remains unclear how it orchestrates neuronal damage. In previous studies, the presence of autoreactive CD4+ and CD8+ T lymphocytes, together with other inflammatory cells and cytokines in active MS lesions, suggest an autoimmune pathogenesis. In an extension of those studies, here we show that the role of CD8+ T-cell in MS and confirmed the perforin is an important factor. Furthermore, we determined that the CD4+ T-cell can enhance CD8+ T-cell cytotoxicity to neurons by induction intense inflammation.

***Keywords:*** *Multiple sclerosis, CD4+ T-cell, CD8+ T-cell,* *Perforin, neurodegeneration*

**Introduction**

Although multiple sclerosis (MS) was first diagnosed in 1894, the underlying pathomechanism of the disease remains unknown, and little evidence has been published regarding the efficacy of radical treatment options. Studies have demonstrated that focal demyelinating lesions are not restricted to the white matter in MS, but also affect the gray matter and normal-appearing white matter, suggesting that both axonal and neuronal loss are major causes for irreversible neurological disability[[1](#_ENREF_1)].

Inflammation is present at all stages of MS, leading to the predominant infiltration of macrophages, followed by CD8+ T-cells and lower numbers of CD4+ T-cells, B cells, and plasma cells[[2](#_ENREF_2)]. Progression of the disease results in diffuse inflammatory T-cell and B cell infiltration, microglia and astrocyte activation, and diffuse myelin reduction and axonal injury. These findings are associated with more pronounced atrophy of the gray and white matter.

Research has indicated that CD8+ T-cells exhibit oligoclonal expansion and are the most abundant T-cells in CNS lesions of patients with MS[[3-6](#_ENREF_3)]; however, the function of these cells in MS remains unclear and somewhat controversial. The pathogenic role of CD8+ T-cells in MS first became evident in a study in which an EAE model was developed using the self-protein myelin basic protein (MBP) [[7](#_ENREF_7)]. Various CNS-specific and therapeutically induced CD8+ T-cell responses have also been observed in patients with MS[[8-11](#_ENREF_8)].

Accumulating evidence has suggested that neurons indirectly interact with T-cells via microglia in the CNS[[12](#_ENREF_12)]. In a recent study, researchers recorded T-cell adhesion *in vivo* using PLP139-151-specific T-cells, observing a direct effect of T-cells on neurons in mouse brain slices[[13](#_ENREF_13)]. The authors observed that T-cells directly attacked neurons, resulting in glutamate receptor-mediated toxicity, which in turn resulted in calcium overload and neuronal death. However, as brain sections contain multiple rather than isolated neurons, it is difficult to determine the exact process by which damage to other brain cells occurs.

In our previous study, we observed that myelin basic protein (MBP)-induced lymphocytes can be applied directly to neurons of the brain *in vitro*[[14](#_ENREF_14)]. Most studies that have addressed the immunobiological aspects of multiple sclerosis (MS) in animal models of experimental autoimmune encephalomyelitis (EAE) have focused on CD4+ T-cells as the main orchestrators of pathogenesis and regulation. In 1984, Takenaka et al. transferred CD4+ T-cells in murine animals, observing that adoptive transfer of CD4+T-cells isolated from myelin antigen-primed animals is sufficient to induce disease in animal models of EAE[[15](#_ENREF_15)]. In 1989, Waldmann et al. reported that CD4+ T-cells from peripheral blood attack myelin in the CNS, leading to the development of MS. Therefore, studies have aimed to prevent disease recurrence and progression by blocking or inhibiting CD4+ T-cells in animal models of MS. Further studies have aimed to selectively remove CD4 + T-cells from the blood to treat MS in both the acute and recurrent stages[[16](#_ENREF_16)]. In 2013, [Zaguia et](http://www.ncbi.nlm.nih.gov/pubmed/?term=Zaguia%20F%5BAuthor%5D&cauthor=true&cauthor_uid=23396942) al. reported that NK-associated molecules expressed by human CD4+ T-cells confer oligodendrocyte-directed cytotoxicity. A significantly elevated proportion of *ex vivo* peripheral blood CD4+ T-cells from patients with MS express NKG2C when compared with controls, suggesting a novel mechanism via which infiltrating CD4+ T-cells may contribute to tissue injury in MS[[17](#_ENREF_17)].

Although studies have revealed that T-cells contribute to neuronal apoptosis and axonal degeneration in MS, the precise mechanism by which such damage occurs remains unclear, as some damage to the gray matter occurs in the very early stages of MS, despite the normal appearance of the white matter. Both CD4+ and CD8+ T-cells play important roles in MS, though their relationship to neuronal damage remains unclear. In the present study, we aimed to clarify the function of T-cell subgroups in the pathogenesis of MS in order to provide an experimental basis for preventing the loss of nerve function in patients with MS.

**Methods**

**EAE animal models**

Adult male Lewis rats were injected with myelin basic protein (MBP, Sigma) emulsified with Complete Freund's Adjuvant (CFA). Plantar injections of 100 μg MBP were administered on each side (200 μg per rat). The state of EAE was monitored each day.

**Cell cultures**

Purified CD4+ or CD8+ T-cells were isolated from heparinized peripheral blood (EAE animals and normal animals) via negative selection using RosetteSep (STEMCELL technologies, Grenoble, France), followed by a Ficoll density-gradient centrifugation. Purity of the isolated CD4+ or CD8+ T-cells within the T-cell population usually is between 97.8%–99.4%.

CD4+ cells were cultured with APC cells and stimulated with MBP87-99 [Val(87)-His-Phe-Phe-Lys-Asn-Ile-Val-Thr- Pro-Arg- Thr-Pro(99)], while CD8+ cells were directly cultured with MBP87-99. These cells were then co-cultured with neurons.

**ELISA**

Concentrations in cell culture supernatants were measured via enzyme-linked immunosorbent assay (ELISA) using the BD OptEIA™ Set Mouse kit (BD Biosciences, San Diego, USA), in accordance with manufacturer instructions.

**Western blot analysis**

Stimulated cells were lysed on ice in lysis buffer (15 mM PBS, 2% NP-40, 0.2% SDS, 10 mM EDTA, 1% protease inhibitor cocktail, 1% phosphatase inhibitor cocktail). The solution was then [centrifuge](D:/%E8%BD%AF%E4%BB%B6/Dict/6.3.69.4001/resultui/frame/javascript:void(0);)d at 14,000 g for 15 min at 4°C, following which the supernatant was collected and protein concentration was evaluated via BSA assay. Proteins were resolved on 10% acrylamide SDS-polyacrylamide gel electrophoresis and then electro-transferred to nitrocellulose membranes for Western blot (WB) analysis. The membranes were blocked in a 5% milk solution in TBS (0.1% Tween 20) and incubated overnight at 4°C with primary antibodies for Bax (1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Samples underwent incubation with HRP-conjugated secondary anti-body for 2 h at 25 °C, followed by chemiluminescence reaction performed via the use of electrochemiluminescence and film exposure. Expression of β-actin was used as a loading control.

**TUNEL**

The apoptosis of stimulated neurons with was evaluated using a TUNEL Apoptosis Assay Kit (Roche,TUN11684817), in accordance with manufacturer instructions.

**MTT**

Stimulated cells were cultured in 96-well plates. After 24 h, 20 μl MTT solution (5 mg/ml prepared with PBS, pH = 7.4) was added, and cells were incubated for an additional 4 h. The supernatant was carefully extracted, and 150 μl DMSO was added to stop the reaction. The OD value was tested at 490 nm.

**Statistical analysis**

Values are expressed as the mean ± SE. Statistics as indicated in the figure legends were calculated using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, USA). A *p* value of ＜0.05 was statistically significant.

**Results**

**Differential cytotoxic effects of CD4+ and CD8+ T-cells on neurons**

To address which type of T-cells play an important role in neuronal cell death in MS, we co-cultured T-cells stimulated by MBP peptide *in vitro* (MBP group) or T-cells from EAE modes (EAE group) with neurons. The degree of neuronal cell death was examined after 24 hours via TUNEL staining. Our results revealed that CD8+ T-cells were more toxic to neurons than CD4+ T-cells in both the MBP and EAE, although these effects were more pronounced in the MBP group (Figure 1). These data demonstrate that CD8+ T-cells, but not CD4+T-cells, play a major role in neuronal cell death in MS.

**The expression of Bax in neurons co-cultured with different T-cell types**

To further confirm that CD8+ T-cells play a greater role than CD4+ T-cells in neuronal cell death associated with MS, we examined the expression of Bax in neurons co-cultured with different T-cell types. We observed that, compared with controls, the level of Bax was slightly increased when neurons were co-cultured with CD4 in the MBP group, though a greater increase was observed in neurons co-cultured with CD8 (Figure 2 A and B). Similar results were observed in the EAE group (Figure 2 C and D). These data suggest that CD8+ T-cells provide a greater contribution to neuronal cell death than CD4+ T-cells in MS.

**Expression of various factors** [**secreted**](javascript:void(0);) **by different T-cell types co-cultured with neurons**

 In order to determine which factors secreted by each type of T-cell provided the greater contribution to neuronal cell death, we examined the secretion of various factors via ELISA. We observed significant increases in interleukin 17 (IL-17) secretion by CD4+ T-cells in both the MBP group and EAE group (Figure 3 A). Further, we observed increases in the secretion of perforin (Figure 3 B), granzyme B (Figure 3 C), and IFN-γ (Figure 3 D) by CD8+ T-cells. These results suggest that perforin, IL-17, and/or IFN-γ may play a primary role in neuronal cell death in MS.

**Direct effect of various factors on neuronal cell death**

To examine which of these factors was associated with damage to neurons, we examined their effects on neuronal cell death via MTT and Western blotting. Our findings indicated that perforin played a more important role than other factors: The MTT assay revealed that the viability of neurons treated with perforin was worse than that of other groups (Figure 4 A). Furthermore, Bax expression was significantly higher in neurons treated with perforin than under other conditions (Figure 4 B, \**P* < 0.05 ). These data suggest that perforin was more toxic to neurons.

**Discussion**

In the present study, neurons were co-cultured with CD4+ T-cells and CD8+ T-cells separated from EAE or MBP models to determine which T-cell type provides the greatest contribution to neuronal cell death in MS. We observed that CD8+ was associated with a significantly greater degree of apoptosis than CD4+ T-cells, and that the combination of CD8+ and CD4+ cells enhanced this apoptotic effect. Thus, these findings suggest that CD8+ T-cells may act to directly induce neuronal apoptosis via action on both neurons and axons in the CNS, potentially providing new insight into the pathogenesis of MS.

In the present study, we observed that simultaneous application of CD8+ and CD4+ T-cells resulted in increased neural apoptosis relative to application of CD8+ T-cells alone[[3](#_ENREF_3)]. Several previous studies have suggested that CD8+ and CD4+ T-cells exhibit functional interactions in the context of MS, although a growing body of evidence suggests that these interactions play a regulatory role in the pathogenesis of MS and MS-like disease. Jiang et al. reported that CD8+ T-cells modulated disease in an EAE model via actions on CD4+ T-cells. Previous researchers have also demonstrated that CD4+ T-cell vaccination protocol-mediated protection against EAE disease is dependent on the presence of Qa-1-restricted CD8+ T-cells[[18](#_ENREF_18)]. Other studies have suggested that these CD8+ T-cells may induce cell death in GA-loaded target T-cells and limit the proliferation of CD4+ T-cells[[19](#_ENREF_19)], thereby ameliorating the effects of EAE. We speculate that these effects may be result of inflammatory cytokine secretion by CD4+ T-cells. Effector cytokines from CD4+ T-cells may also act to enhance CD8 cytotoxic functions, thereby amplifying inflammatory cascades in the CNS. However, further studies are required to determine whether the role of CD8+ T-cells in MS is primarily pathological or regulatory.

In the present study, we evaluated levels of various cytokines (granzyme B, perforin, IL-17 and IFN-γ) in co-cultures of neurons and either CD8+ or CD4+ T-cells. We observed that increased levels of perforin resulted in increased neuronal cell death, suggesting that perforin secretion by CD8+ T-cells may play an important role in neuronal cell death. Previous studies have indicated that IFN-γreduces disease severity by mediating MBP-specific CD8+ T-cell-driven activity. Recent work has suggested that IL-10-producing CD8+ T-cells also act to diminish disease pathology[[20](#_ENREF_20), [21](#_ENREF_21)]. IFN-γ- and IL-17-producing CD8+ T-cells can be recruited into the CNS when responding to apoptotic T-cell associated self-epitopes[[22](#_ENREF_22)]. In the present study, we observed that increased levels of these cytokines were not associated with cytotoxic effects on neurons, further supporting the notion that they may act to neutralization cytotoxicity. Furthermore, previous studies have reported that CD8+ IL-17-secreting T-cell numbers are significantly elevated in acute CNS lesions in patients with MS[[23](#_ENREF_23)]. Giovanni et al. reported that patients with MS exhibit higher levels of circulating CD8+CD56-perforin+ T-cells than controls, suggesting that dysregulation of CD8+CD56-perforin+ T-cells may play a role in the development of MS[[24](#_ENREF_24)]. Consistent with these findings, perforin was associated with increased neuronal cell death in the present study, suggesting an important role for perforin secretion from CD8+ cells in MS.

In conclusion, our findings indicate that perforin-secreting CD8+ T-cells play a major role in MS, and that CD4 may enhance CD8 cytotoxicity to neurons by inducing inflammation. However, the precise roles of CD4 and CD8 in the pathophysiology of MS remain to be elucidated.

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**Figure Legends**

**Figure 1 Differences in the cytotoxicity of CD4+ and CD8+ T-cells to neurons.** CD4+, CD8+, or both CD4+ and CD8+ T-cells were co-cultured with neurons for 24 h. These T-cells were stimulated by MBP or harvested from EAE models. Neuronal viability was assessed via TNUEL staining (Bar = 100 μm). Data from three independent experiments were expressed as mean ± SEM and analyzed using a one-way ANOVA (\*P < 0.05).

**Figure 2 Expression of Bax in neurons co-cultured with different T-cell types.** (A) The expression of Bax in neurons co-cultured with different T-cell types for 24h stimulated with MBP. T-cells from control rats were stimulated with 50 μg/ml MBP on the first day, and another 100 μg/ml MBP was added on the fourth day. The different T-cells were then co-cultured with neurons. The control condition refers to T-cells stimulated with no related peptide. (B) Relative quantification of (A). Data were expressed as mean ± SEM from at least three independent experiments and analyzed using a one-way ANOVA (\*P < 0.05). (C) The expression of Bax in neurons co-cultured for 24 h with different T-cells from EAE models or stimulated with MBP. (D) Relative quantification of (C). Data were expressed as mean ± SEM from at least three independent experiments and analyzed using a one-way ANOVA (\*P < 0.05).

**Figure 3 The expression of various factors secreted by different T-cell types when co-cultured with neurons for 24 h**. Different T-cells were co-cultured with neurons for 24 h, and the expression of IL-17 (A), perforin (B), granzyme B (C), and IFN-γ (D) was examined via ELISA. Data were expressed as mean ± SEM from at least three independent experiments and analyzed using a one-way ANOVA (\*P < 0.05).

**Figure 4 Direct cytotoxic effects of various factors on neurons.** (A) Different factors (1.5 μg/ml) were added to neurons, and the neuronal viability was examined via MTT. Data were expressed as mean ± SEM from at least three independent experiments and analyzed using a one-way ANOVA (\*P < 0.05). (B) Different factors (1.5 μg/ml) were added to neurons, and the expression of Bax was examined via Western blotting.