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## Paucity of natural killer and cytotoxic T cells in human neuromyelitis optica lesions

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### Abstract

Neuromyelitis optica is a severe inflammatory demyelinating disease of the central nervous system. Most patients with neuromyelitis optica have circulating immunoglobulin G (IgG) antibodies against the astrocytic water channel protein aquaporin-4 (AQP4), which are pathogenic. Anti-AQP4 IgG-mediated complement-dependent astrocyte toxicity is a key mechanism of central nervous system damage in neuromyelitis optica, but the role of natural killer and cytotoxic T cells is unknown. Our objective was to determine whether natural killer and cytotoxic T cells play a role in human neuromyelitis optica lesions. We immunostained four actively demyelinating lesions, obtained from patients with anti-AQP4 IgG positive neuromyelitis optica, for Granzyme B and Perforin. The inflammatory cells were perivascular neutrophils, eosinophils and macrophages, with only occasional Granzyme B+ or Perforin+ cells. Greater than 95% of inflamed vessels in each lesion had no surrounding Granzyme B+ or Perforin+ cells. Granzyme B+ or Perforin+ cells were abundant in human spleen (positive control). Although natural killer cells produce central nervous system damage in mice injected with anti-AQP4 IgG, our findings here indicate that natural killer-mediated and T cell-mediated cytotoxicity are probably not involved in central nervous system damage in human neuromyelitis optica.

### Keywords

antibody-dependent cellular cytotoxicity; complement-dependent cytotoxicity; Devic's disease; mouse model; NMO-IgG

### Introduction

Neuromyelitis optica is an inflammatory demyelinating disease of the central nervous system (CNS) [1]. Most patients with neuromyelitis optica have circulating IgG<sub>1</sub> autoantibodies (called AQP4-IgG or NMO-IgG) that bind the water channel protein aquaporin-4 expressed in perivascular astrocyte foot processes [2]. In mice, AQP4-IgG damages the astrocytes by activating complement (complement-dependent cytotoxicity) [3] or by antibody-dependent natural killer cell-mediated cytotoxicity [4], depending on the experimental conditions.

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#### Conflicts of interest

There are no conflicts of interest.

There is strong evidence from mouse models and human studies that complement-dependent cytotoxicity plays a key role in CNS damage associated with neuromyelitis optica lesions. Intracerebral coinjection of AQP4-IgG and human complement in mice produces the characteristic features of human neuromyelitis optica lesions, including astrocyte damage with loss of AQP4 and glial fibrillary acidic protein (GFAP), inflammation, demyelination and perivascular deposition of activated complement components [3]. In this mouse model, complement inhibition prevented AQP4 IgG-mediated CNS damage. A key role for complement-dependent cytotoxicity in neuromyelitis optica is further supported by the marked perivascular deposition of IgG and activated complement components in human neuromyelitis optica lesions [5,6].

A possible role for antibody-dependent natural killer cell-mediated cytotoxicity in neuromyelitis optica has been suggested recently. Exposure of cultured cells [7] or ex-vivo spinal cord slices [8] to AQP4-IgG and natural killer cells damages the AQP4-expressing astrocytes. The coinjection of AQP4-IgG with natural killer cells in mouse brain in the absence of a complement produces some histological features of human neuromyelitis optica, including loss of AQP4 and GFAP expression, but no demyelination [4]. To date, although the presence of granulocytes and macrophages in human NMO lesions has been shown [5,6], there are no published data on the abundance of natural killer cells in human neuromyelitis optica lesions.

Here, we sought histological evidence from human neuromyelitis optica lesions supporting the involvement of antibody-dependent cell-mediated cytotoxicity (mediated by natural killer or cytotoxic T cells) in neuromyelitis optica pathology. Human neuromyelitis optica lesions were immunostained for Granzyme B and Perforin, which are selectively expressed in cytoplasmic granules of natural killer and cytotoxic T cells.

## Materials and methods

### Ethical approval

Ethical approval for the use of human tissue was obtained from the Wandsworth Local Research Ethics Committee.

### Human tissue

Tissue was obtained from the Thomas Willis Oxford Brain Collection and from a brain lesion biopsy of a neuromyelitis optica patient. Sections (7  $\mu$ m thickness) were cut from formalin-fixed, paraffin-embedded CNS lesions obtained from four AQP4-IgG-positive neuromyelitis optica patients and four multiple sclerosis patients. These lesions were extensively characterized (Table 1). Sections were stained with haematoxylin and eosin, Luxol Fast Blue and immunostained for AQP4 (Millipore, Livingston, UK), C5b-9 (Abcam, Cambridge, UK), Granzyme B (ANAspec, Fremont, California, USA) and Perforin (Abcam).

### Immunostaining

Tissues were treated with citric acid and then incubated with rabbit anti-AQP4 or anti-C5b-9 or anti-Granzyme B or anti-Perforin IgG (1: 100, 1 h, room temperature), followed by biotinylated anti-rabbit IgG and ABC (Vector Labs, Peterborough, UK). Immunoreactivity was visualized as brown with DAB/H<sub>2</sub>O<sub>2</sub> and counterstain was carried out with haematoxylin. Human spleen was used as a positive control. There was no immunostaining after excluding each primary antibody.

## Data analysis

The tissue sections were examined for myelin loss (pallor, intramyelinic oedema) and the presence of inflammatory cells as well as AQP4, C5b-9, Granzyme and Perforin immunoreactivity. After immunostaining for Granzyme B or Perforin, 20 inflamed vessels per lesion were examined, counting the number of Granzyme B+ or Perforin+ cells within 50  $\mu\text{m}$  of each vessel.

## Results

### General analysis

The neuromyelitis optica and multiple sclerosis lesions were characterized by degenerating myelin sheaths with myelin pallor after Luxol Fast Blue staining and intramyelinic oedema as well as multiple myelin-laden (foamy) macrophages, indicating active demyelination. Table 1 summarizes the features of these lesions. The samples had typical histological features of active neuromyelitis optica and multiple sclerosis (Fig. 1a and b). The neuromyelitis optica lesions showed loss of AQP4 and GFAP expression (indicating astrocyte damage) and perivascular inflammation, with infiltration of neutrophils, eosinophils and macrophages. The multiple sclerosis lesions showed preserved or increased AQP4 expression (indicating preserved astrocytes) and the perivascular inflammation was comprised of macrophages and lymphocytes (without neutrophils or eosinophils).

### Natural killer and cytotoxic T cells

Only occasional Granzyme B+ or Perforin+ cells were observed around inflamed vessels (Fig. 1a and b, middle and bottom rows). No Granzyme B+ or Perforin+ cells were observed distant from vessels. The Granzyme B+ and Perforin+ cells were mononuclear and immunostaining was evident as multiple cytoplasmic spots (Fig. 1c), consistent with Granzyme B and Perforin localization within cytoplasmic granules. Many Granzyme B+ and Perforin+ cells were present in human spleen (positive control; Fig. 1d) with morphology similar to the corresponding cells within the neuromyelitis optica and multiple sclerosis lesions. Table 2 shows that at least 95% of inflamed vessels within the neuromyelitis optica and multiple sclerosis lesions had no surrounding Granzyme B+ or Perforin+ cells. Five percent or fewer inflamed vessels had one or two Granzyme B+ or Perforin+ cells around them.

## Discussion

Granzyme B and Perforin, which are found in natural killer and cytotoxic T cells, are involved in the killing of target cells by apoptosis. After binding to target cells through Fc $\gamma$  receptors, natural killer and cytotoxic T cells insert Perforin into the target plasma cell membrane, which aggregates to form pores that facilitate the entry of Granzyme B. Granzyme B is a serine protease that activates the caspase-specific pathway, resulting in target cell apoptosis. The paucity of Granzyme B+ or Perforin+ cells in human neuromyelitis optica lesions indicates that antibody-dependent cell-mediated cytotoxicity caused by natural killer and cytotoxic T cells is unlikely to play a major role in human neuromyelitis optica.

The most abundant leucocytes in human [6,9,10] and mouse [3,9] neuromyelitis optica lesions are neutrophils, eosinophils and macrophages. T cells and macrophages predominate in human multiple sclerosis lesions with no neutrophils or eosinophils [6]. Several authors have reported a paucity of T cells in human [5,6] and mouse [11] neuromyelitis optica lesions. We have now also shown the paucity of natural killer cells in human neuromyelitis optica. Neutrophils play an important role in the early pathogenesis of neuromyelitis optica

lesions with evidence for the potential therapeutic efficacy of neutrophil elastase inhibitors [9]. Eosinophils, macrophages and microglia express Fc $\gamma$  receptors, and thus may cause antibody-dependent cell-mediated cytotoxicity in neuromyelitis optica, but their role has not yet been reported. The role of microglia in neuromyelitis optica also remains unknown.

Although natural killer and cytotoxic T cells are scarce in human neuromyelitis optica and multiple sclerosis lesions, these cell types may still play a role that is not related to antibody-dependent cell-mediated cytotoxicity. In multiple sclerosis, there is increasing evidence that circulating natural killer cells are protective by secreting immunosuppressive cytokines, such as interleukin-10, and by killing autoreactive T cells and antigen-presenting cells [12,13]. It is unclear whether circulating natural killer cells play a similar protective role in human neuromyelitis optica. In multiple sclerosis, the level of circulating natural killer cells decreases during acute attacks and increases during treatment, consistent with a protective role [12,13]. It may thus be interesting to monitor the level of circulating natural killer and cytotoxic T cells in acute neuromyelitis optica versus remission.

## Acknowledgments

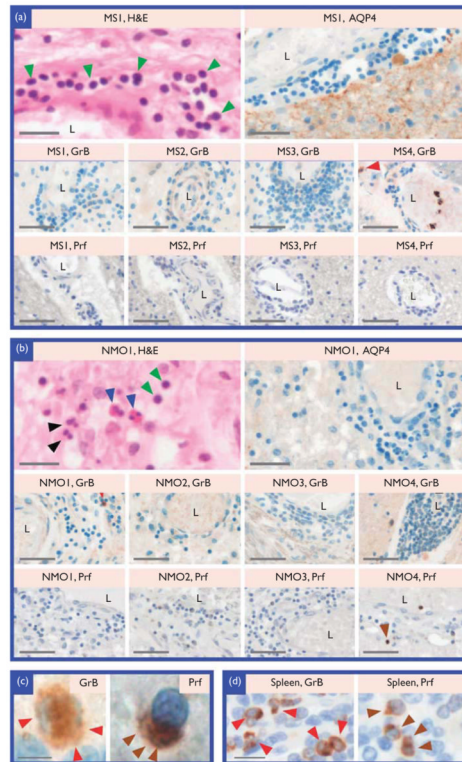
Human CNS tissue was obtained from the Thomas Willis Oxford Brain Collection and from a brain lesion biopsy of a neuromyelitis optica patient.

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**Fig. 1.** Granzyme B (GrB) and Perforin (Prf) immunostaining in human neuromyelitis optica and multiple sclerosis lesions. Inflamed CNS vessels in lesions from (a) four multiple sclerosis patients (MS1–MS4), and (b) four neuromyelitis optica patients (NMO1–NMO4) stained with H&E (top left), immunostained for AQP4 (top right), immunostained for Granzyme B (GrB, middle row) and immunostained for Perforin (Prf, bottom row). Perivascular neutrophils (black arrowheads), eosinophils (blue arrowheads), mononuclear (green arrowheads), Granzyme B+ cells (red arrowheads) and Perforin + cells (brown arrowheads). L, vessel lumen. (c) Magnified view of a Granzyme B+ and a Perforin+ cell. (d) Human spleen with Granzyme B+ (red arrowheads) and Perforin+ (brown arrowheads) cells. Scale bar=20  $\mu\text{m}$  (H&E: a, b), 40  $\mu\text{m}$  (AQP4: a, b), 50  $\mu\text{m}$  (GrB: a, b and Prf: a, b), 5  $\mu\text{m}$  (c), 12  $\mu\text{m}$  (d). CNS, central nervous system.

Lesion characteristics of the eight human central nervous system samples used in the study (four neuromyelitis optica and four multiple sclerosis)

**Table 1**

Diagnosis	Patient number	Site	AQP4	GFAP	Myelin	Inflam	Foamy mφ	Nφ/Eφ	Periv C5b-9
NMO	1	Cord	↓	↓↓	↓	↑↑	↑	↑	P
NMO	2	Brain	↓	↓↓	↓	↑↑	↑	↑↑	P
NMO	3	Brain	↓	↓	↓	↑v	↑	↑	P
NMO	4	Brain	↓	↓	↓	↑v	↑	↑↑	P
MS	1	Brain	→	→	↓	↑↑	↑	0	A
MS	2	Brain	↑	↑	↓	↑↑	↑	0	A
MS	3	Brain	→	→	↓	↑↑	↑	0	A
MS	4	Cord	↑	↑	↓	↑↑	↑	0	A

AQP4 immunostaining: ↓ extensive loss of staining, → mostly perivascular (normal), ↑ upregulated; Foamy mφ: 0 (absent), ↑ present, ↑↑ abundant; GFAP immunostaining: ↓↓, no staining, ↓ reduced staining, → normal, ↑ upregulated (reactive astrocytes); Inflam (inflammation): → nil (normal), ↑v mostly perivascular, ↑p mostly parenchymal, ↑↑ mostly parenchymal, ↑↑ mostly perivascular and parenchymal; MS, multiple sclerosis; Myelin (LFB) staining: → normal, ↓ partial axonal myelin loss, ↓↓ no myelin; Nφ/Eφ: 0(absent), ↑ present, ↑↑ abundant; NMO, neuromyelitis optica; Periv C5b-9 [Perivascular deposition of activated complement (C5b-9)]: P (present), A (absent).



**Table 2**

Granzyme B and Perforin immunostaining in neuromyelitis optica human lesions

Samples	% of inflamed vessels surrounded by					
	0 GrB+ cells	1 GrB + cell	2 GrB + cells	0 Prf + cells	1 Prf + cell	2 Prf + cells
NMO 1	95	5	0	100	0	0
NMO 2	100	0	0	100	0	0
NMO 3	100	0	0	100	0	0
NMO 4	90	10	0	95	1	0
NMO (mean±SE)	96.3±4.8	3.8±2.4	0.0±0.0	98.8±1.2	0.3±0.3	0.0±0.0
MS 1	95	0	5	95	0	1
MS 2	100	0	0	100	0	0
MS 3	100	0	0	100	0	0
MS 4	85	15	0	100	0	0
MS (mean±SE)	95.0±3.5	3.8±3.8	1.3±1.3	98.8±1.2	0.0±0.0	0.3±0.3

GrB, Granzyme B; MS, multiple sclerosis; NMO, neuromyelitis optica; Prf, Perforin.