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Neuroantigen-specific CD8+ regulatory T-cell function is deficient during acute exacerbation of multiple sclerosis

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ABSTRACT

Multiple sclerosis (MS) is an inflammatory, demyelinating disease of the central nervous system (CNS). MS is thought to be T-cell-mediated, with prior research predominantly focusing on CD4+ T-cells. There is a high prevalence of CNS-specific CD8+ T-cell responses in MS patients and healthy subjects. However, the role of neuroantigen-specific CD8+ T-cells in MS is poorly understood, with the prevalent notion that these may represent pathogenic T-cells. We show here that healthy subjects and MS patients demonstrate similar magnitudes of CD8+ and CD4+ T-cell responses to various antigenic stimuli. Interestingly, CD8+ T-cells specific for CNS autoantigens, but not those specific for control foreign antigens, exhibit immune regulatory ability, suppressing proliferation of CD4+CD25− T-cells when stimulated by their cognate antigen. While CD8+ T-cell-mediated immune suppression is similar between healthy subjects and clinically quiescent treatment-naïve MS patients, it is significantly deficient during acute exacerbation of MS. Of note, the recovery of neuroantigen-specific CD8+ T-cell suppression correlates with disease recovery post-relapse. These studies reveal a novel immune suppressor function for neuroantigen-specific CD8+ T-cells that is clinically relevant in the maintenance of peripheral tolerance and the intrinsic regulation of MS immune pathology.

1. Introduction

Multiple sclerosis (MS) is the most common disabling neurological disease of young people, typically presenting as a relapsing–remitting form (RRMS). MS is thought to be immune-mediated and is characterized by temporally and spatially separated central nervous system (CNS) lesions that may be accompanied by acute exacerbation of clinical symptoms, which remit over time with limited accumulating disability. The immune dysregulation that underlies the pathology of MS and its clinical exacerbations remain poorly understood. Much of our understanding of the immunology of MS derives from work in the murine model, experimental autoimmune encephalomyelitis (EAE). The vast majority of studies in MS and EAE have focused on the role of CD4+ T-cells as mediators and regulators of disease. The preponderant belief is that MS and EAE are mediated through CNS-specific CD4+ Th1/Th17 responses and regulated by CD4+ regulatory T-cells [1]. However, considerable evidence points to an important role for CD8+ T-cells in the pathogenesis and/or regulation of MS and EAE [2–18]. CNS lesions show a predominance of CD8+ T-cells with oligoclonal expansion [4], indicating an active role at the site of pathology. While it is thought that these cells represent a key pathogenic element of MS lesions, neither the antigenic specificity of these cells nor their role has been elucidated. MS patients show a high prevalence of CNS-specific CD8+ T-cell responses in their circulation [3]. These cells appear to have a mixed functional phenotype in that they express cytotoxic/inflammatory as well as regulatory effector molecules [3]. Again, the intuitive function attributed to these responses is that of pathogenesis. However, their role has not been adequately investigated. Moreover, healthy subjects also harbor such responses, raising the possibility that they may not be purely pathogenic.

Antigen-specific immune regulation has high therapeutic potential. Global defects in immune regulatory T-cell (T_{reg}) function have been demonstrated in a wide variety of human immune-mediated diseases [14,19–27]. In most cases, antigenic specificity of the regulatory population is poorly defined and this would be key in the ability to expand and utilize such populations. In the current

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study, we investigated the role of neuroantigen-specific CD8+ T-cells in MS and discovered an unexpected, novel and clinically relevant immune regulatory role for autoantigen-specific T-cells. This role has both biologic and therapeutic implications.

2. Methods and materials

2.1. Subject characteristics

MS patients were recruited and gave written informed consent at the UT Southwestern Clinical Center for Multiple Sclerosis. Table 1 summarizes patient characteristics. 11 treatment-naïve adult clinically definite RRMS patients (McDonald criteria) with quiescent disease were recruited. Exclusion criteria included pregnancy, HIV positivity, active cancer, other autoimmune, immunosuppressive, neurodegenerative conditions, clinical relapse or corticosteroid treatment within last 3 months and any history of disease-modifying immunomodulatory therapy. In addition, 9 treatment-naïve MS patients were recruited during an active acute clinical episode/relapse. 15 healthy subjects were recruited as controls (HC). All studies were approved by the UT Southwestern IRB according to Declaration of Helsinki principles.

2.2. Cell preparation and bead sorting

PBMC were isolated from whole blood using Ficoll Hypaque (GE Healthcare Biosciences, Pittsburgh, PA) density gradient. Purified CD8+ T-cells were isolated using CD8+ Microbeads positive selection kit (Miltenyi Biotec, Auburn, CA) and AutoMacs separation, according to the manufacturer's instructions. CD8+ enriched populations were >95% CD8+ and <0.1% CD4+ by flow cytometric analysis. "Untouched" CD4+ T-cells were isolated using CD4 negative selection kits (Miltenyi Biotech). CD25+ T-cells were depleted from the purified CD4+ using CD25 Microbeads (Miltenyi Biotec). CD4+CD25− enriched populations were >98% CD4+, <1% CD25+, and <0.1% CD8+ by flow cytometric analysis. CD4+CD25+ enriched populations were >98% CD4+ and <0.1% CD8+. CD25 expression ranged from 40.5 to 73.8%. The CD4+ and CD8+ T-cell-depleted PBMC population was irradiated with 3000 rad before being used as antigen-presenting cells (APC).

2.3. CFSE staining

To detect proliferative responses upon antigenic challenge, cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen Molecular Probes, Eugene, OR), as described previously [3,28]. Briefly, cells are suspended at 1×10^6 cells/mL and incubated for 7 min at 37 °C with 0.25 μM CFSE (Invitrogen), then washed twice with media containing 5% human serum.

Table 1
Summary of patient characteristics.

	Healthy Controls (HC)	RRMS: Quiescent (MS)	MS: Acute Exacerbation	MS: Exacerbation Follow-up
Number of Subjects	15	11	9	4
Average Age, y (Range)	44 (21–65)	40 (23–56)	45 (31–65)	44 (35–53)
Sex (M/F)	5/10	2/9	3/6	2/2
Days from Last Relapse [Mean (Range)]	N/A	599 (90–2920)	8 (2–50)	81 (31–118)

2.4. CMTPX staining

Cell Tracker Red CMTPX (Invitrogen Molecular Probes) was used to stain putative regulatory cells. CD8+ and CD4+CD25+ suppressor cells, or CD4+CD25− negative control cells were marked with CMTPX, as described previously [15]. Briefly, cells were suspended at 1×10^6 cell/mL and incubated for 15 min at 37 °C with 700 nM CMTPX, then washed twice with media containing 5% human serum. The longer-wavelength CMTPX exhibits bright red fluorescence that is easily distinguished from that of green fluorescent probes, such as CFSE.

2.5. Flow cytometry-based suppression assay cultures

1×10^6 CFSE-stained CD4+CD25− T-cells were used as responders in a 1 mL culture. 1×10^6 CD4- and CD8-depleted PBMC were irradiated with 3000 rad and used as APC. In replicate cultures, varying ratios of CMTPX-stained suppressors were added and cultured with various antigenic stimuli for 7 days in complete RPMI 1640 media containing 5% human serum, 100 U/mL Penicillin, 100 μg/mL Streptomycin, and 0.92 mg/mL L-glutamine. Cells were washed and stained for flow cytometry, as described below.

2.6. Antigenic stimulation

Pools of 15-mer peptides, overlapping by 10, spanning entire neuroantigenic proteins were used, as described previously [3]. These were used at 1 μg/mL final concentration for each peptide and covered myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), myelin associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (OMGP) and αβ-crystallin (CRAB). In addition, whole bovine MBP (wbMBP) was used at 20 μg/mL. For control foreign antigens, we utilized pools of known CD4 and CD8 epitopes of CMV (5 and 14 peptides, respectively) as well as whole cytomegalovirus (CMV) (Microbix Biosystems, Ontario, Canada) and tetanus toxoid (TT) (Accurate Chemical & Scientific Corp, Westbury, NY). 1 μg/mL anti-CD3 monoclonal antibody (OKT3) was used for mitogenic stimulation.

2.7. T-cell line generation

We generated neuroantigen- and control antigen-specific CD8+ and CD4+ T-cell lines by bead-sorting CD8+ (or CD4+) T-cells after 1 week of *in vitro* PBMC stimulation, followed by repeated antigen-specific expansion with autologous APC. CD8+ T-cell lines were maintained with 25 IU/mL IL-2 (Peprotech, Rocky Hill, NJ), 10 ng/mL IL-7 (Peprotech), 1 ng/mL IL-12 (Peprotech), and 1 ng/mL IL-15 (Peprotech), as previously described [29,30].

2.8. Flow cytometric antibody staining

On day 7 of *in vitro* stimulation, cells were washed with 0.1% (w/v) sodium azide/phosphate-buffered saline (Mediatech Cellgro). Cells were stained with anti-CD3-PE (BD Biosciences, San Jose, CA), anti-CD4-PECy5.5 (Invitrogen), anti-CD8-Pacific Blue (BD Biosciences), and anti-CD25-APC (BD Biosciences), then resuspended in 1% paraformaldehyde (Electron Microscopy Sciences, Hatfiled, PA). Flow cytometric data were acquired on a 4-Laser, 17-color LSRII using FACSDiva software (Becton Dickinson). CFSE was detected in the FITC channel and CMTPX in the PE-Texas red channel on the LSR.

2.9. Data analysis

Linear uncompensated data was transferred as FCS 3.0 files and analyzed after compensation and transformation using FlowJo