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A translatable molecular approach to determining CD8 T-cell epitopes in TMEV infection

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ABSTRACT

Defining the epitope specificity of CD8+ T cells is an important goal in autoimmune and immune-mediated disease research. We have developed a translational molecular approach to determine the epitope specificity of CD8+ T cells using the Theiler's murine encephalomyelitis virus (TMEV) model of multiple sclerosis (MS). TMEV-specific CD8+ T cells were isolated from brains and spleens of 7-day TMEV-infected C57BL/6J mice and stimulated by Cos-7 cells that were co-transfected with expression vectors encoding the D^b class I molecule along with overlapping segments of the TMEV genome. Both brain-infiltrating and spleen-derived CD8+ T cells expressed IFN-γ when Cos-7 cells were co-transfected with D^b class I molecule and the TMEV genomic segment that encoded the immunodominant TMEV epitope. This demonstrated that peripheral and brain-infiltrating CD8+ T-cell responses were focused on peptide epitope(s) encoded by the same region of the TMEV genome. We propose that a similar molecular approach could also be used to determine the antigen specificity of suppressor CD8 T cells by the measurement of transforming growth factor–β (TGF-β) production. In addition, with a randomly generated library and peripheral blood or isolated CSF CD8+ T cells, this would be an effective method of predicting the epitope specificity of CD8+ T cells in human inflammatory CNS diseases, in animal models of MS or other organ-specific inflammatory diseases with a protective or pathogenic role of CD8 T cells.

1. Introduction

The role of central nervous system (CNS)–infiltrating CD8+ T cells is an active area of CNS inflammatory disease research. Studies have demonstrated that CD8+ T cells are prevalent in multiple sclerosis (MS) lesions, outnumbering other lymphocytes at all stages of lesion development [1,2]. CD8+ T cells have also been shown to be clonally expanded in lesions, cerebrospinal fluid (CSF), and peripheral blood, suggestive of a common epitope, or a restricted number of epitopes being recognized by this cell type [2,3]. In addition, it has been demonstrated that T-cell receptor sequences from clonally expanded CD8+ T cells can persist for years indicating ongoing activation of memory CD8+ T-cell clones. Whether these epitope specific CD8+ T cells act as suppressors of inflammation or effectors of pathology remains to be determined.

A major hypothesis generated through human tissue and animal model studies have provided evidence for a suppressor role of CD8+ T cells. Work in human samples has relied on characterizing functional aspects of cultured CD8+ T cells derived from the peripheral blood of MS patients. Findings from this work have suggested that a deficiency of suppressor function by CD8+ T cells may be linked to MS pathologic conditions [4]. The majority of suppressor CD8+ T-cell work has been performed in the experimental allergic encephalomyelitis (EAE) animal model of MS. In EAE, the absence of CD8+ T cells results in the worsening of disease or susceptibility to secondary induction of EAE [5–7]. Mechanistic studies have provided evidence that suppressor CD8+ T cells act via T-cell receptor interaction with the Qa-1 molecule presented by activated CD4+ T cells to cull inflammatory responses. Qa-1–independent mechanisms involving cytokine mediated suppression by CD8+ T cells have also been proposed [8,9].

Although not mutually exclusive from the suppressor CD8+ T-cell hypothesis, independent studies also support an effector role for this cell type in the pathology in MS patients. Studies in human tissue have demonstrated that all CNS cell types within an MS lesion have the capacity to express major histocompatability complex (MHC) class I molecules and can potentially be targeted by CD8+ T cell–mediated cytotoxicity [10]. Also supporting a role for cytotoxicity by activated CD8+ T cells is their close proximity to oligodendrocytes and demyelinated axons in brain tissue from MS patients. In EAE models, CD8+ T cells have been shown to be sufficient to adoptively transfer disease [11,12]. In the Theiler mu-

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rine encephalomyelitis (TMEV) model, many aspects of clinical disease, including axonal dysfunction, demyelination, and motor dysfunction, have been linked to CD8+ T cells [13–16].

To determine the extent CD8+ T cells acquire suppressor and effector functions, the epitope specificity of CD8+ T cells in MS needs to be defined. Numerous approaches have been demonstrated to investigate the epitope specificity of CD8+ T cells in TMEV infection, including the use of overlapping peptide libraries, molecular expression of TMEV proteins, stable transfected cell lines, and recombinant vaccinia virus vectors [17–20]. These approaches have unequivocally demonstrated the utility in defining CD8 T-cell epitopes in TMEV infection. However, none of these previously developed systems are feasible approaches to define CD8 T-cell epitopes in human MS in which there are logarithmically higher numbers of potential antigenic targets and the genomic sequence encoding these antigenic determinants are unknown. We therefore have developed an unbiased translational molecular approach to identify CD8+ T-cell epitopes. In this proof-of-principle study, we successfully used this approach to identify the region of the TMEV genome that encodes the immunodominant epitope recognized by CNS-infiltrating CD8+ T cells in the C57BL/6 mouse strain. Although this mouse strain is resistant to chronic demyelination, as it effectively clears the virus in the acute meningoencephalitis stage, it has been demonstrated that demyelination may occur in mice with C57BL/6 background by an intravenous injection of the immunodominant peptide on day 7, or by the use of mice deficient in interferon-γ receptor [21,22]. Furthermore, there is no theoretical limitation for this method to detect epitopes in demyelinating strains or to detect polyclonal CD8 T-cell responses. In this study, CD8+ T cells from the spleen were stimulated by the identical TMEV library segment as CD8+ T cells isolated from the brain. This interesting observation strongly suggests a specific focus among CD8+ T cells to a conserved immunodominant epitope, regardless of being isolated from brain or a peripheral lymphoid compartment. This molecular approach with a large organ-specific cDNA library derived from MS lesions, similarly to what has already been accomplished in autoimmune diabetes studies [23], has the potential to be adapted for clinical use with peripheral human CD8+ T cells derived from the blood or the CSF as a noninvasive method to predict the epitope specificity of CNS-infiltrating CD8+ T cells in MS patients.

2. Subjects and methods

2.1. Animals

Five week old male C57BL/6 mice were obtained from Jackson laboratories (Bar Harbor, Maine). Mice were anesthetized with isoflurane and were intracranially infected with the Daniels strain of TMEV. Seven days postinfection the animals were euthanized, and tissue was harvested according to University of Cincinnati Institutional Animal Care and Use Committee standards.

2.2. Generation of TMEV library

Fifteen overlapping segments were amplified from the pDAFL plasmid containing the DA strain of TMEV using high-fidelity polymerase chain reaction (PCR), as presented in Table 1. Each segment was enzymatically digested then inserted into the pcDNA 3.1 His A vector (Invitrogen, Carlsbad, CA). The pcDNA 3.1 His A was digested with either EcoRI and XbaI to accommodate ligation of segments 1, 3, 5, 6, 7, 9, 10, 11, 12, 13, and 15 or BamHI and XbaI to accommodate ligation of segments 2, 4, and 8. In addition, the genomic sequence encoding the immunodominant TMEV peptide $VP2_{121-130}$ was inserted into the pcDNA 3.1 His B vector.

Table 1
Overlapping segments amplified from pDAFL plasmid containing Daniels strain of Theiler's murine encephalomyelitis virus (TMEV).

Segment	Location in TMEV genome	pcDNA3.1 His A cloning sites
TMEV 1	1038–1596	EcoRI 5′; XbaI 3′
TMEV 2	1536–2097	BamHI 5′; XbaI 3′
TMEV 3	2033–2596	EcoRI 5′; XbaI 3′
TMEV 4	2534–3099	BamHI 5′; XbaI 3′
TMEV 5	3031–3597	EcoRI 5′; XbaI 3′
TMEV 6	3532–4095	EcoRI 5′; XbaI 3′
TMEV 7	4030–4596	EcoRI 5′; XbaI 3′
TMEV 8	4531–5094	BamHI 5′; XbaI 3′
TMEV 9	5032–5596	EcoRI 5′; XbaI 3′
TMEV 10	5533–6096	EcoRI 5′; XbaI 3′
TMEV 11	6031–6597	EcoRI 5′; XbaI 3′
TMEV 12	6532–7095	EcoRI 5′; XbaI 3′
TMEV 13	7033–7597	EcoRI 5′; XbaI 3′
TMEV 14	7531–7971	EcoRI 5′; XbaI 3′
TMEV L*	1064–1547	EcoRI 5′; XbaI 3′

Fifteen overlapping segments were amplified from the pDAFL plasmid containing the TMEV genome using high-fidelity polymerase chain reaction. Segments were then digested with the specified restriction enzyme as defined by primer sequences and inserted into the pcDNA 3.1 His A vector to accommodate transfection into the Cos-7 cell line. The TMEV sequences overlap by 30 base pairs to ensure that all potential CD8+ T-cell epitopes are processed for antigen presentation. Note that the L* segment encodes a protein from an alternative reading frame important to TMEV infection.

2.3. Efficient co-transfection of Cos-7 cells with class I molecule and green fluorescent protein

Cos-7 cells (American Type Culture Collection number CRL-1651, African green monkey kidney cells) were plated into Costar 96-well culture plates (Corning Inc., Corning, NY) at approximately 70% confluency and incubated overnight. Cells were transfected using Fugene-6 (Roche Indianapolis, IN) according to the manufacturer's protocol. Cos-7 cells were co-transfected with green fluorescent protein (GFP) MigR1vector and a vector containing either the D^b or K^b MHC class I molecules (provided by Nilabh Shastri, University of California at Berkley, Berkeley, CA) which were expressed by the C57BL/6 strain of mouse. GFP MigR1 was co-transfected as a visual aid to distinguish wells that demonstrated efficient co-transfection. Peak GFP expression occurred in Cos-7 cells within 24–48 hours after transfection.

2.4. Isolation of brain mononuclear cells

Brains were pushed through 100-μm cell strainers into RPMI and 700 μg of collagenase type 4 (Worthington Lakewood, NJ) was added to each 5-ml quantity of slurry. Slurries were then incubated in a water bath at 42°C for 45 minutes. Each 5 ml of slurry was then added to Nalgene 50-ml ultra– high-speed centrifuge tubes (Nalge Nunc International, Rochester, NY) containing 1ml of 10X PBS, 9 ml of Percoll, and 35 ml of RPMI. Cell suspensions were then spun at 10,000 rpm (Sorvall SS-34 rotor) for 30 minutes. A lymphocyte layer was present in approximately the bottom 5 ml of media solution. Excess solution is aspirated off the lymphocyte layer. The lymphocyte layer was then resuspended into Falcon conical tubes, (Becton Dickinson, Franklin Lakes, NJ) and RPMI was added until 50 ml of total volume was reached. Cell suspensions were then spun at 1500 rpm for 10 minutes in a Sorvall Legend RT tabletop centrifuge (Thermo Scientific, Waltham, MA). Media was aspirated off and cell pellets were resuspended in RPMI media.

2.5. Isolation of whole spleen cells

Spleens were pushed though 100-μm cell strainers into RPMI 1640. Cell suspensions were then spun at 1500 rpm for 10 minutes in a Sorvall Legend RT tabletop centrifuge. Media was aspirated off and cell pellets were resuspended in 2ml RPMI and 3 ml ACK (0.15 mol/l NH4Cl, 1.0 mol/l KHCO3, 0.1 mmol/l Na2-ethylenediaminetetraacetate). Cell suspensions were then spun at