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# Increased Frequencies of Myelin Oligodendrocyte Glycoprotein/MHC Class II-Binding CD4 Cells in Patients with Multiple Sclerosis

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**Multiple sclerosis (MS) is an autoimmune disease characterized by infiltration of pathogenic immune cells in the CNS resulting in destruction of the myelin sheath and surrounding axons. We and others have previously measured the frequency of human myelin-reactive T cells in peripheral blood. Using T cell cloning techniques, a modest increase in the frequency of myelin-reactive T cells in patients as compared with control subjects was observed. In this study, we investigated whether myelin oligodendrocyte glycoprotein (MOG)-specific T cells could be detected and their frequency was measured using DRB1\*0401/MOG<sub>97-109</sub>(107E-S) tetramers in MS subjects and healthy controls expressing HLA class II DRB1\*0401. We defined the optimal culture conditions for expansion of MOG-reactive T cells upon MOG peptide stimulation of PMBCs. MOG<sub>97-109</sub>-reactive CD4<sup>+</sup> T cells, isolated with DRB1\*0401/MOG<sub>97-109</sub> tetramers, and after a short-term culture of PMBCs with MOG<sub>97-109</sub> peptides, were detected more frequently from patients with MS as compared with healthy controls. T cell clones from single cell cloning of DRB1\*0401/MOG<sub>97-109</sub>(107E-S) tetramer<sup>+</sup> cells confirmed that these T cell clones were responsive to both the native and the substituted MOG peptide. These data indicate that autoantigen-specific T cells can be detected and enumerated from the blood of subjects using class II tetramers, and the frequency of MOG<sub>97-109</sub>-reactive T cells is greater in patients with MS as compared with healthy controls. *The Journal of Immunology*, 2011, 187: 000–000.**

**M**ultiple sclerosis (MS) is a genetically mediated autoimmune disease of the CNS with loss of neurologic function (1–3). Specifically, focal T cell and macrophage infiltrates result in demyelination and loss of surrounding axons (4). It is postulated that T cell recognition of peptides derived from myelin proteins in the context of MHC presented by microglia is involved in the pathogenesis of the disease. Autoreactive T cells are found at approximately the same frequency in normal individuals and patients with MS (5–8), although in subjects with the disease, autoreactive T cells are in a more activated state as compared with T cells from normal individuals (9–11). Although the un-

derlying etiology for the dysregulated immune system in MS is not known, fully replicated genome-wide association scans have identified a number of allelic variants in immune-related genes, including *IL2RA*, *IL7R*, *CD58*, *CD226*, *CD6*, *IRF8*, and MHC class II, that are associated with MS susceptibility (12, 13).

We first identified T cell epitopes of myelin basic protein and proteolipid protein in both patients with MS and healthy controls during two decades ago and have demonstrated minor differences in the frequency of Ag-reactive cells in patients as compared with controls (6–8, 11, 14, 15); these autoreactive T cells can persist over long periods of time in patients (16, 17). Myelin oligodendrocyte glycoprotein (MOG) is another potentially important autoreactive T cell target in MS patients with epitopes identified that are restricted in the context of HLA DRB1\*0401 and HLA DRB1\*1501 (18–22).

Detecting myelin-reactive T cells and monitoring their frequency in the peripheral blood has been a difficult task for a number of reasons. First, there is nonspecific proliferation due to bystander activation of Ag-nonreactive T cells. Second, perhaps a more difficult issue is the rarity of circulating autoreactive T cells and the need to first stimulate with myelin Ags (23–25) to detect their presence followed by nonspecific readouts of function such as [<sup>3</sup>H]thymidine incorporation or cytokine secretion. This need for primary stimulation is not seen with HLA class I tetramers, which have been a useful tool to detect and characterize Ag-specific CD8 cells at the single cell level in a variety of studies, including infectious diseases, cancers, and type 1 diabetes (26–29). Finally, another hindrance to the use of MHC class II tetramers is the low TCR–MHC avidity, particularly for self-antigen. In this regard, hemagglutinin (HA) peptide-reactive, proinsulin-reactive, or glutamic acid decarboxylase (GAD)65 peptide-reactive T cells with specific peptide-loaded HLA DRB1\*0401 tetramers and other

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Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; GAD, glutamic acid decarboxylase; HA, hemagglutinin; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis.

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class II tetramers have been detected (30–42), although because of low autoreactive T cell frequency, expansion with autoantigen was necessary prior to T cell detection with tetramer.

In this study, we used HLA-DRB1\*0401 MHC class II tetramers loaded with an epitope of MOG and optimized culture conditions to detect these Ag-reactive CD4<sup>+</sup> T cells in patients with MS and control subjects. Similar to previous experience with peptides derived from the sequence of GAD in the investigation of Ag-reactive CD4 cells in patients with type 1 diabetes (36), we used a MOG peptide [MOG<sub>97–109(107E-S)</sub>] that has a greater binding affinity to DRB1\*0401 than does the native peptide to maximize detection of MOG-reactive CD4<sup>+</sup> T cells with the DRB1\*0401 tetramer. First, we demonstrated our ability to detect MOG<sub>97–109(107E-S)</sub> peptide-reactive T cells after a short-term culture of PMBCs obtained from patients with MS and healthy control subjects carrying the HLA DRB1\*0401 allele. We found that CD4<sup>+</sup> DRB1\*0401/MOG<sub>97–109(107E-S)</sub> peptide tetramer<sup>+</sup> cells were detected more frequently in cultures of PBMCs from subjects with MS than from cultures from healthy control subjects. We also replicated this finding in a subset of subjects using the native MOG<sub>97–109</sub> peptide loaded on DRB1\*0401 tetramers. Finally, reactivity and specificity of CD4<sup>+</sup>CD25<sup>+</sup> DRB1\*0401/MOG<sub>97–109(107E-S)</sub> peptide tetramer<sup>+</sup> cells were confirmed by single cell sorting cells of this phenotype followed by *in vitro* expansion and interrogation for responses to the MOG<sub>97–109(107E-S)</sub> peptide, the native MOG peptide, and control peptides in the context of DRB1\*0401. These data suggest that the DRB1\*0401/MOG<sub>97–109(107E-S)</sub> peptide tetramer can be used to differentiate MS and healthy control subjects by measuring the frequency of autoreactivity of MOG<sub>97–109</sub> peptide-reactive T cells and that this reagent is useful in isolating specific MOG<sub>97–109(107E-S)</sub> peptide and MOG<sub>97–109</sub> peptide-reactive T cells. Clinical investigations using these MHC class II tetramers in significantly larger cohorts of patients in relationship to MRI scanning are indicated.

## Materials and Methods

### Abs and reagents

HL-1 media was supplemented with 2 mM L-glutamine, 5 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.1 mM each nonessential amino acids, 1 mM sodium pyruvate (all from Lonza, Walkersville, MD), and 5% heat-inactivated human male AB serum (Immune Tolerance Network, San Francisco, CA). FBS was purchased from Atlanta Biologics. All Abs (anti-CD3, anti-CD4, and anti-CD25), 7-aminoactinomycin D (7-AAD), Golgi-Stop, and Vacutainer tubes were from BD Biosciences (San Jose, CA). PHA (PHA-P) was obtained from Remel (Lenexa, KS). PMA, ionomycin, trypan blue, DMSO, and neuraminidase were obtained from Sigma-Aldrich (St. Louis, MO). Human rIL-2 (Tecin) was obtained from the National Cancer Institute (Frederick, MD). Ficoll-Paque and [<sup>3</sup>H]thymidine were from Amersham Pharmacia Biotech (Piscataway, NJ). Viacount reagent was from Guava Technologies (Hayward, CA). The CD4-negative selection kit was from Miltenyi Biotec (Auburn, CA). Peptides were synthesized and purified by New England Peptide (Gardner, MA) or provided by the Immune Tolerance Network. Abs to human Vβ-chains were purchased from Beckman Coulter (Miami, FL).

### Isolation of PMBCs

Consenting volunteers, either healthy subjects or MS patients (all from the MS Clinic at Brigham and Women's Hospital, Boston, MA), expressing HLA class II DRB1\*0401 were selected and blood from these donors was collected by venipuncture in 10 ml lithium heparin Vacutainer tubes according to the guidelines and recommendations of the Brigham and Women's Hospital Institutional Review Board. The average age of the patients with MS was 54 ± 10 y (12 females, 2 males) and for controls 41 ± 13 y (10 females, 4 males), and the two groups were matched except for two subjects (MS patients that were 73 and 76 y old). All patients were in the relapsing-remitting phase of disease. For this phase I study, patients were on different treatments including IFN-β, glatiramer acetate, and daclizumab, although later analysis demonstrated no relationship between therapy and tetramer binding frequency. All tissue typing was performed at the Histocompatibility Laboratory at Brigham and Women's Hospital.

PMBCs were isolated by standard Ficoll-Hypaque methods and were used either fresh or after cryopreservation in 10% DMSO/90% human AB serum and storage in liquid nitrogen.

### Cell preparation

CD4<sup>+</sup> T cells were purified from PMBCs by negative selection using the Miltenyi Biotec CD4<sup>+</sup> T cell isolation kit. The non-CD4 cells were incubated in 24-well plates at a density of 2.5 × 10<sup>6</sup> cells/well for 2 h. Adherent cells were collected and used as APCs and were loaded with the peptides for 2 h before plating CD4<sup>+</sup> T cells (2.5 × 10<sup>6</sup>/well) in flat-bottom 12-well plates. The cells were cultured for 14 d in HL-1 medium containing 5% human serum. IL-2 (20 U/ml) was added on days 4, 7, and 10. The cultures were split in two wells and supplemented with fresh medium on day 7.

### Tetramer preparation

The generation of DRB1\*0401 soluble class II molecules has been described (43). The peptide MOG<sub>97–109</sub> was used to load the DRB1\*0401 molecule to generate the DRB1\*0401/MOG<sub>97–109</sub> tetramer. The MOG<sub>97–109</sub> peptide used in this study for tetramer construction (FFRDHSYQEEA, native sequence) has a mutation at position 107 (E-S) to stabilize binding to the DRB1\*0401/DRA\*0101 chains. The procedures for HA<sub>306–318</sub> (PRYVKQNTLKLAT) and GAD65<sub>555–567</sub> immunodominant epitope (NFFRMVISNPAAT, native sequence, the peptide used to load the DRB1\*0401 tetramer has a substitution at position 555, F-I, to stabilize binding) peptide loading were identical to those described earlier (43) and were used to generate DRB1\*0401/HA<sub>306–318</sub> and DRB1\*0401/GAD65<sub>555–567(557F-I)</sub> tetramers as control tetramers for staining. Streptavidin-PE (BioSource International, Camarillo, CA) was used for cross-linking. All of the tetramers used for direct staining were filtered through a Sephadex G-50 size exclusion column before use.

### Stimulation and tetramer staining

Cells were treated with or without 0.5 U/ml neuraminidase (type X from *Clostridium perfringens*) for 30 min at 37°C in HL-1 medium. The cells were washed with PBS, then stained with 10 µg/ml PE-labeled tetramer (DRB1\*0401/MOG<sub>97–109(107E-S)</sub> peptide or DRB1\*0401/GAD65<sub>555–567(557F-I)</sub>) at 37°C for 3 h in HL-1 medium with 2% human serum. Cells were stained for the last 30 min with allophycocyanin-labeled anti-CD25 and FITC-anti-CD4 mAbs; dead cells were discriminated using 7-AAD. After washing, the percentage of CD4<sup>+</sup>CD25<sup>intermediate</sup> tetramer<sup>+</sup> cells was determined within the live cell gate (7-AAD<sup>-</sup>) by flow cytometry. The data were acquired on an LSR II (BD Immunocytometry Systems, San Jose, CA) and analyzed with FlowJo software (Tree Star, Ashland, OR).

### Single cell cloning and specificity testing

CD4<sup>+</sup>CD25<sup>+</sup> DRB1\*0401/MOG<sub>97–109(107E-S)</sub> tetramer<sup>+</sup>7-AAD<sup>-</sup> cells were single cell sorted into 96-well plates using a FACSAria cell sorter (BD Immunocytometry Systems). Clones thus obtained were expanded for 28 d by stimulation with irradiated unmatched PMBCs in the presence of PHA-P (3 µg/ml) and IL-2 (20 U/ml) for two rounds. Clones were generated from two healthy control subjects and one MS subject, all bearing HLA DRB1\*0401.

### Proliferation assays

T cell clones were washed twice and added to U-bottom 96-well plates at 25,000 cells/well. The EBV-transformed B cell line Priess, which is homozygous for DRB1\*0401, was used as the APC. Irradiated (5000 rad) APCs were incubated with the peptides at 3 × 10<sup>5</sup> cells/ml for 2 h at 37°C. After washing and resuspending the cells in the original volume, 100 µl was added to each well in duplicates. After 72 h, plates were pulsed with 1 µCi/well [<sup>3</sup>H]thymidine to measure proliferation. Plates were harvested 16 h later to count the incorporated radioactivity (Wallac).

### Statistical analysis

Statistical differences were calculated using Prism 4.0 software (GraphPad Software, San Diego, CA) using an unpaired Student *t* test.

## Results

### Detection of MOG<sub>97–109(107E-S)</sub>-reactive T cells *ex vivo* and after *in vitro* culture

It has been previously shown that class II tetramers with modified GAD65 peptide and modified proinsulin peptide can be used to detect GAD and proinsulin-reactive T cells, respectively, in the peri-

pheral blood of human subjects (32, 35, 41, 43, 44). Using a similar strategy, we used a modified MOG peptide, MOG<sub>97-109(107E-S)</sub>. This peptide was designed with a 107E-S substitution at the P9 anchoring position such that it bound to DRB1\*0401/DRA1\*0101 MHC molecules with an affinity that was 50-fold higher compared with the wild type MOG<sub>97-109</sub> peptide (Supplemental Fig. 1). We cultured PBMCs from MS patients who expressed DRB1\*0401 with the MOG<sub>97-109(107E-S)</sub> peptide for 14 d and examined whether the DRB1\*0401/MOG<sub>97-109(107E-S)</sub> tetramer could detect CD4<sup>+</sup>CD25<sup>intermediate</sup> T cells (activated CD4<sup>+</sup> T cells) in the cultures (Fig. 1). Approximately 5% of the CD4<sup>+</sup>CD25<sup>intermediate</sup> T cells in culture bound the tetramer.

#### Defining the optimal conditions for MOG-specific cell detection in the peripheral blood

We performed a series of experiments to determine the optimal conditions for detecting MOG<sub>97-109(107E-S)</sub>-reactive T cells. We examined parameters including the length of culture after stimulation, dose response to the MOG<sub>97-109(107E-S)</sub> peptide, comparison of fresh versus cryopreserved samples, and finally reproducibility of the assay. We compared the culture of CD4<sup>+</sup> cells with MOG peptide and irradiated autologous adherent cells from the PBMCs for 7 d as compared with 14 d in the presence of IL-2. We observed that 14 d culture resulted in amplifying the number of MOG tetramer<sup>+</sup> CD4 cells while decreasing the background staining (Fig. 2A). This culture period resulted in a better signal-to-noise ratio of specific tetramer binding to irrelevant tetramer (DRB1\*0401/GAD65<sub>555-567(557F-I)</sub>) binding (9.7 as compared with 4.0, respectively). We then examined a range of concentrations of MOG<sub>97-109(107E-S)</sub> peptide to determine optimal dose responses of MOG-specific CD4 T cell expansion. As shown in Fig. 2B, 10 μg/ml induced maximal MOG tetramer binding as compared with binding to irrelevant GAD tetramer.

The use of fresh blood samples in clinical investigations is cumbersome in multicenter studies involving different participating centers; moreover, in most studies there is a need for immune monitoring over time. For these reasons, samples are collected and cryopreserved for a determined time before testing. We examined whether the detection of DRB1\*0401/MOG<sub>97-109(107E-S)</sub> tetramer<sup>+</sup> cells was affected in cryopreserved PBMC samples as compared with fresh samples. Using an optimal cryopreservation protocol for PMBCs developed by the Immune Tolerance Network (<http://www.immunetolerance.org/professionals/research/lab-protocols>) that allowed for 95% viability and >85% recovery, we did not find any significant decrease of the staining between fresh and cryopreserved PMBCs that were kept in liquid nitrogen for 3, 4, 5, and 20 wk prior to use (Fig. 2C).

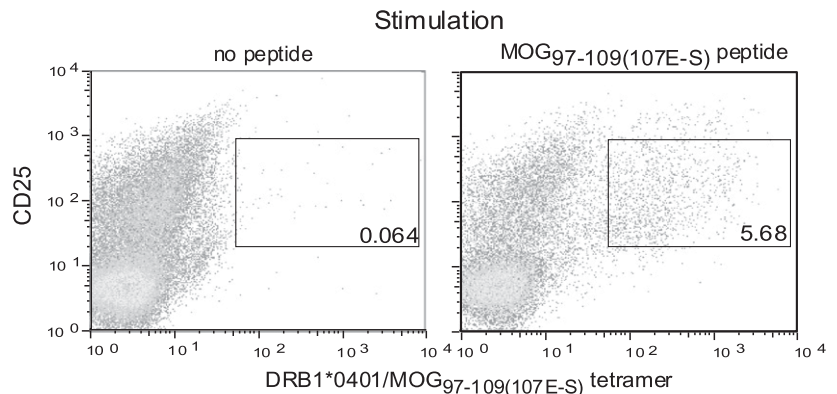
**FIGURE 1.** Detection of MOG<sub>97-109(107E-S)</sub>-reactive T cells using DRB1\*0401/MOG<sub>97-109(107E-S)</sub> tetramer in a MOG peptide-expanded culture. Cryopreserved PBMCs from a DRB1\*0401 MS subject were thawed. CD4<sup>+</sup> cells were isolated by negative selection and incubated with autologous adherent APCs from the same donor loaded with or without 10 μg/ml MOG<sub>97-109(107E-S)</sub>. The cocultures were carried for 14 d in the presence of recombinant human IL-2 (10 U/ml) on days 4, 7, and 10. The cells were stained with the DRB1\*0401/MOG<sub>97-109(107E-S)</sub> tetramer, anti-CD4, anti-CD25, and 7-AAD and analyzed using an LSR II flow cytometer. Live (7-AAD<sup>-</sup>) and CD4<sup>+</sup> cells were gated on and used to enumerate the percentage of CD25<sup>intermediate</sup> DRB1\*0401/MOG<sub>97-109(107E-S)</sub> tetramer<sup>+</sup> cells. This is a representative sample from four subjects examined.

#### Effect of adding cytokines during the culture and treating with neuraminidase before tetramer staining

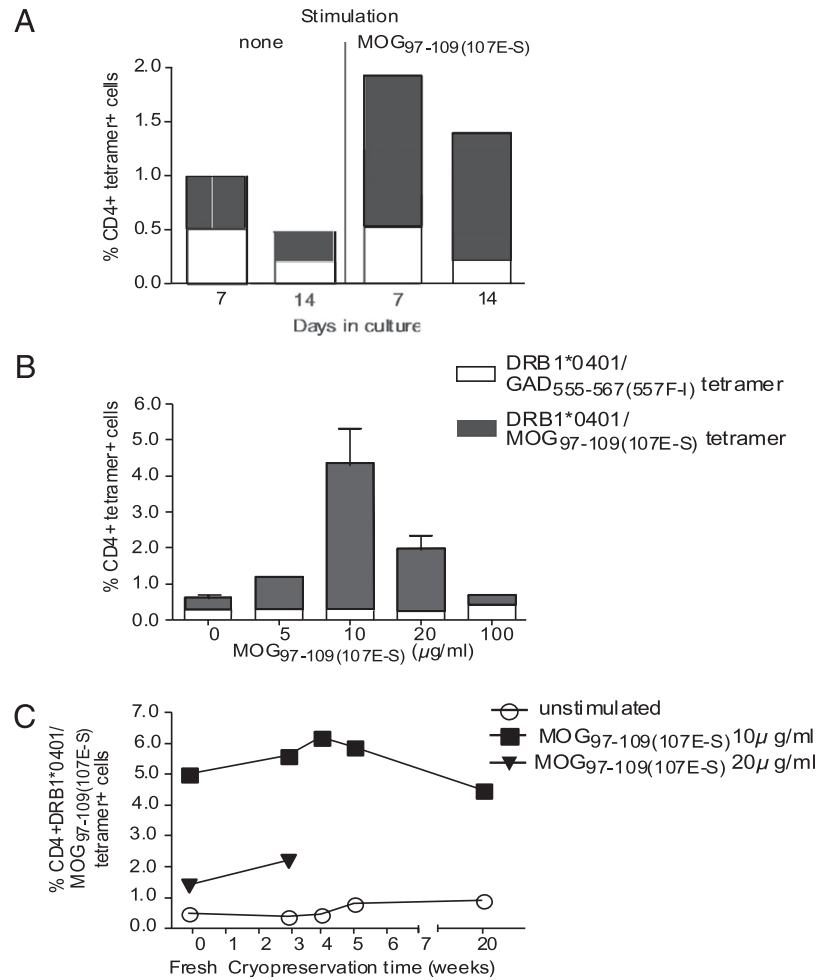
Because in vitro culture of PBMCs with Ag and IL-2 expands Ag-reactive T cells for detection, we examined whether IL-7, a T cell growth factor for memory T cells, improved the detection of CD4<sup>+</sup>DRB1\*0401/MOG<sub>97-109(107E-S)</sub> tetramer<sup>+</sup> cells; surprisingly, we found that adding IL-7 to the cultures resulted in a decrease of the intensity of staining and the percentage of MOG tetramer<sup>+</sup> cells (Fig. 3A). Neuraminidase treatment has been described to enhance CD4 tetramer staining of myelin-specific T cells in a mouse model of MS (45) and tetramer staining of CD8<sup>+</sup> T cells (46). We examined whether neuraminidase treatment increased DRB1\*0401/MOG<sub>97-109(107E-S)</sub> tetramer staining of human cells cultured with MOG<sub>97-109(107E-S)</sub>. We observed an increase in tetramer staining after treating the cells with neuraminidase, although T cell staining of both the DRB1\*0401/MOG<sub>97-109(107E-S)</sub> tetramer and the irrelevant DRB1\*0401/GAD65<sub>555-567(557F-I)</sub> tetramer was increased (signal-to-noise ratio, 3.9 and 4.4, respectively; Fig. 3B).

#### Cloning of MOG-reactive CD4 cells and specificity testing

To demonstrate that cells binding to the DRB1\*0401/MOG<sub>97-109(107E-S)</sub> tetramer were specific, we cultured CD4<sup>+</sup> cells from a MS patient with autologous APCs loaded with MOG<sub>97-109(107E-S)</sub> (Fig. 1). After 14 d stimulation we single cell cloned CD4<sup>+</sup>CD25<sup>intermediate</sup> DRB1\*0401/MOG<sub>97-109(107E-S)</sub> tetramer-binding cells and expanded them with PHA and IL-2. After expanding clones in vitro, they were rested and then examined for Ag specificity by using irradiated Pries B cells (DRB1\*0401<sup>+/+</sup>) pulsed with MOG<sub>97-109(107E-S)</sub> peptide. Twenty-three randomly chosen clones from 60 clones were examined for Ag reactivity as measured by tritiated thymidine incorporation. We found that 20 of 23 clones proliferated in response to the MOG<sub>97-109(107E-S)</sub> peptide-loaded APCs as defined by a stimulation index ≥5 and a Δcpm >10,000 (Fig. 4A). We then examined the dose responses of four representative clones to MOG<sub>97-109(107E-S)</sub> generated by single cell cloning. These clones responded to the MOG peptide with an approximate EC<sub>50</sub> dose of 1–10 μM (Fig. 4B). Because these clones were derived using the MOG<sub>97-109(107E-S)</sub> substituted peptide, it was important to show that these clones proliferated in response to the native MOG<sub>97-109</sub> peptide. The response of representative clones is shown in Table I. These clones responded to both the native and substituted MOG peptide with equivalent EC<sub>50</sub> doses, but not to an irrelevant GAD65<sub>555-567(557F-I)</sub> peptide. The magnitude of the response to the substituted MOG peptide was greater than the response to the native peptide for the clones that were tested (Supplemental Fig. 2).



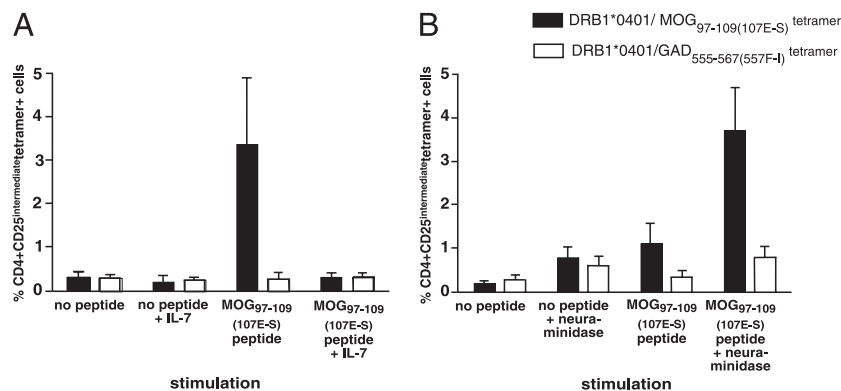
**FIGURE 2.** Optimum conditions for DRB1\*0401/MOG<sub>97-109(107E-S)</sub> tetramer staining. The conditions for optimal tetramer staining were determined using PMBCs from two DRB1\*0401 MS patients with respect to in vitro culture duration, concentration of MOG<sub>97-109(107E-S)</sub> in a 14-d in vitro assay, and evaluation of fresh versus frozen PMBCs for various periods of time. Triple-color FACS staining was conducted to include DRB1\*0401/MOG<sub>97-109(107E-S)</sub> tetramer-PE, CD4-allophycocyanin, and 7-AAD. The percentage of tetramer<sup>+</sup> cells was determined in the CD4 population by eliminating the 7-AAD-stained cells. The averages ( $\pm$ SD) from two samples are shown. *A*, The signal-to-noise ratio for cells stained with the specific tetramer (loaded with substituted MOG peptide) versus the irrelevant tetramer (loaded with the GAD peptide) was best at day 14 culture (ratio, 9.7) as compared with those at day 7 culture (ratio, 4.0). In 14-d cultures (*B*), the maximal number of DRB1\*0401/MOG<sub>97-109(107E-S)</sub> tetramer<sup>+</sup> cells was detected with an initial peptide stimulation concentration of 10  $\mu$ g/ml. *C*, The number of DRB1\*0401/MOG<sub>97-109(107E-S)</sub> tetramer<sup>+</sup> cells was similar from fresh as compared with PMBCs cryopreserved for various lengths of time in 14-d assays with the initial peptide stimulation of 10  $\mu$ g/ml MOG<sub>97-109(107E-S)</sub>.



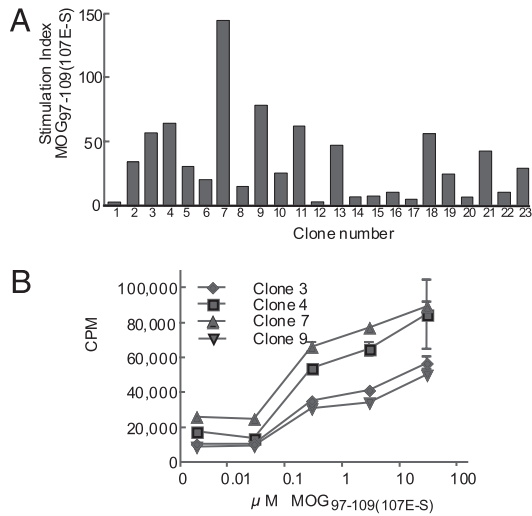
#### Detection of CD4<sup>+</sup> DRB1\*0401/MOG<sub>97-109(107E-S)</sub> tetramer<sup>+</sup> T cells after peptide culture with PMBCs from healthy controls and patients with MS

Finally, using the optimal conditions defined above, we examined whether there were differences in the frequency of autoreactive T cells after 14 d culture with the MOG<sub>97-109(107E-S)</sub> peptide between 12 healthy control subjects and 14 patients with MS, all of whom matched for expression of the DRB1\*0401 allele. For these experiments, CD4<sup>+</sup> cells were isolated by negative selection and

cultured with autologous APCs loaded with MOG<sub>97-109(107E-S)</sub> peptide in the presence of IL-2 added on days 4, 7, and 10. After 14 d stimulation, the percentage of CD4<sup>+</sup>CD25<sup>intermediate</sup> DRB1\*0401/MOG<sub>97-109(107E-S)</sub> tetramer<sup>+</sup> T cells were significantly higher in patients with multiple sclerosis as compared with healthy control subjects (Fig. 5A;  $p = 0.008$ ). We also examined the frequency of CD4<sup>+</sup>CD25<sup>intermediate</sup> DRB1\*0401/MOG<sub>97-109(107E-S)</sub> tetramer-binding CD4 cells in the absence of Ag stimulation. Surprisingly, we observed a significant difference between healthy



**FIGURE 3.** Addition of IL-7 or treatment with neuraminidase decreases specific binding of the DRB1\*0401/MOG<sub>97-109(107E-S)</sub> tetramer to MOG<sub>97-109(107E-S)</sub>-stimulated PMBCs. Cryopreserved PMBCs from DRB1\*0401 MS subjects were thawed. CD4<sup>+</sup> cells were isolated by negative selection and incubated with autologous adherent APCs from the same donor loaded with 10  $\mu$ g/ml MOG<sub>97-109(107E-S)</sub>. The cocultures were carried for 14 d in the presence of added IL-2 on days 4, 7, and 10. *A*, IL-7 (5 ng/ml) was added in the culture on the first day. *B*, Cells were incubated with or without 0.5 U/ml neuraminidase for 30 min at 37°C before staining with the tetramer and Abs on day 14. The cells were counterstained with anti-CD4, anti-CD25, and 7-AAD. Data shown are the average ( $\pm$ SE) of samples ( $n = 4$  for *A*, IL-7 addition;  $n = 5$  for *B*, neuraminidase treatment).



**FIGURE 4.** Reactivity of T cell clones generated with the DRB1\*0401/MOG<sub>97-109</sub> tetramer. T cell clones were generated by single cell cloning of CD4<sup>+</sup>CD25<sup>intermediate</sup> DRB1\*0401/MOG<sub>97-109(107E-S)</sub> tetramer<sup>+</sup> cells as described in *Materials and Methods*. Irradiated Priess (DRB1\*0401<sup>+/+</sup>) B cells were pulsed with MOG<sub>97-109(107E-S)</sub> peptide for 2 h, washed, and plated at 10,000 cells/well. T cell clones were added at 25,000 cells/well and incubated for 48 h. Wells were then pulsed with 1  $\mu$ Ci/well tritiated thymidine and harvested 28 h later. The stimulation index (Ag-pulsed culture cpm/no Ag-pulsed culture CPM) of representative 23 clones is shown in (A); concentration of MOG<sub>97-109(107E-S)</sub> peptide in this experiment was 10  $\mu$ M. A clone was considered to be positive to the peptide stimulation index >5 (horizontal line) and  $\Delta$ cpm >10,000. The dose response of four representative clones to the native MOG<sub>97-109(107E-S)</sub> peptide is shown in (B).

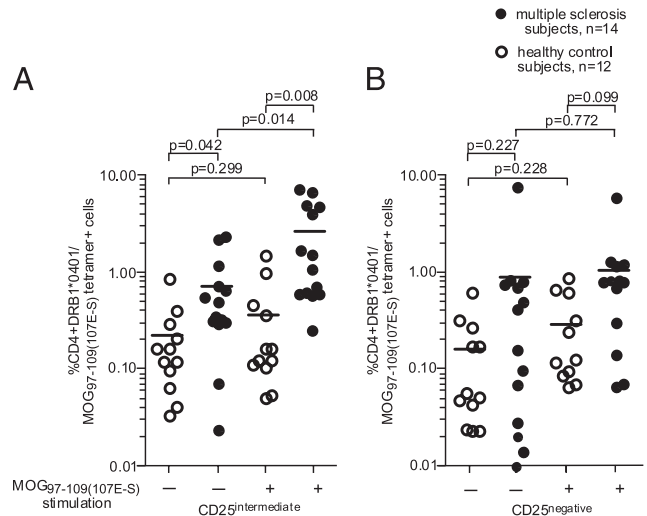
controls and patients with multiple sclerosis ( $p = 0.042$ ) after expansion for 14 d in the presence of IL-2 (Fig. 5A). The difference in the frequency of CD4<sup>+</sup>CD25<sup>-</sup> DRB1\*0401/MOG<sub>97-109(107E-S)</sub> tetramer-binding CD4 cells between healthy control subjects and MS patients did not reach significance (Fig. 5B). These experiments were also conducted with HA<sub>306-318</sub> peptide stimulation of PBMCs, culture, and subsequent reactive T cell detection with DRB1\*0401/HA<sub>306-318</sub> tetramer; there was no difference in the frequencies of HA-reactive T cells between MS and healthy control subjects (data not shown).

To demonstrate that CD4<sup>+</sup> T cells detected with the DRB1\*0401/MOG<sub>97-109(107E-S)</sub> tetramer reacted with the native peptide, we

Table I. CD4<sup>+</sup> T cell clones isolated with DRB1\*0401/MOG<sub>97-109(107E-S)</sub> tetramer responded to substituted and native MOG peptide

Clone No.	EC <sub>50</sub> ( $\mu$ M)		
	MOG <sub>97-109(native)</sub>	MOG <sub>97-109(107E-S)</sub>	GAD65 <sub>555-567(557F-1)</sub>
3	2	2	>100
4	10	10–30	>100
7	5	5	>100
6	10	10	>100
9	5	8	>100
11	5	5–10	>100
18	10	5	>100
21	5	5	>100
23	7	10	>100

T cell clones were assayed for reactivity to MOG<sub>97-108(native)</sub>, MOG<sub>97-109(107E-S)</sub> peptides, and GAD65<sub>555-567(557F-1)</sub> peptide as an irrelevant peptide with Priess cells (DRB1\*0401<sup>+/+</sup>) as APCs over a range of peptide concentrations (0.1–100  $\mu$ M). The EC<sub>50</sub> for each clone responding to peptides was determined. Each clone responded to each MOG peptide with approximately the same EC<sub>50</sub> whereas the magnitude of the response was greater to the substituted peptide and as compared with the response to the native peptide for eight of nine clones tested.



**FIGURE 5.** MOG<sub>97-109(107E-S)</sub>-reactive T cells are more frequent after culture with MOG<sub>97-109(107E-S)</sub> from MS subjects than from healthy subjects. Frequency of CD4<sup>+</sup>CD25<sup>intermediate</sup> DRB1\*0401/MOG<sub>97-109(107E-S)</sub>-reactive T cells (A) was determined by flow cytometry after 14 d culture with MOG<sub>97-109(107E-S)</sub> as described in *Materials and Methods*. CD4<sup>+</sup>CD25<sup>-</sup> DRB1\*0401/MOG<sub>97-109(107E-S)</sub> tetramer<sup>+</sup> T cells were enumerated as a comparison (B). CD4<sup>+</sup>CD25<sup>intermediate</sup> DRB1\*0401/MOG<sub>97-109(107E-S)</sub>-reactive T cells were more frequently detected from the MS patients after 14 d culture with or without MOG<sub>97-109(107E-S)</sub> stimulation. The  $p$  values comparing data between groups are shown in the figure.

examined PBMCs from DRB1\*0401<sup>+</sup> healthy controls and MS subjects cultured with no peptide, native MOG peptide, or substituted MOG peptide and then enumerated the frequency of CD4<sup>+</sup> T cells with the DRB1\*0401 tetramer loaded with either the native or substituted peptide (Supplemental Fig. 3). Culture of PBMCs from MS subjects with no peptide or culture of PBMCs from healthy controls in any of these conditions resulted in no detection of CD4<sup>+</sup> T cell expansion with either tetramer (loaded with either the native or substituted peptide). However, culture of PBMCs from MS subjects with either native or substituted peptide resulted in expansion of CD4<sup>+</sup> T cells binding the tetramer loaded with either the native or substituted peptide. This detection was greatest when PBMCs were cultured with substituted MOG peptide and detected with the DRB1\*0401/MOG<sub>97-109(107E-S)</sub> tetramer. To verify that the difference observed between MS and healthy subjects using the substituted MOG was relevant to MOG and not just due to an altered peptide ligand effect, we examined the frequency of CD4<sup>+</sup>CD25<sup>intermediate</sup> DRB1\*0401/MOG<sub>97-109</sub> tetramer<sup>+</sup> T cells in healthy subjects and patients with MS using the native MOG<sub>97-109</sub> to stimulate the cells and the DRB1\*0401/MOG<sub>97-109</sub>-loaded tetramer to detect reactive cells. We were able to confirm that the frequency of DRB1\*0401/MOG<sub>97-109</sub> was significantly higher in patients with multiple sclerosis as compared with healthy control subjects (Supplemental Fig. 4).

We analyzed the V $\beta$ -chain of a panel of CD4 clones by flow cytometry and found that each clone expressed a single V $\beta$ -chain type suggesting its clonality. Among the different clones, we found a normal distribution of expressed V $\beta$ -chains, indicating that there is no preference for one type of V $\beta$ -chain for reactivity to HA or MOG (data not shown).

## Discussion

MS is an inflammatory disease of the CNS. It fits into the group of autoimmune diseases that share allelic variations in common

gene regions, including the MHC locus, *IL2RA*, *IL7R*, and *CD226* (12, 13). However, it has been difficult to demonstrate differences in the frequency of myelin-specific T cells in the circulation of patients with the disease. An early study showed a higher frequency of anti-MOG<sup>Igd</sup> in the serum of MS patients, but no difference in the T cell proliferative response between healthy subjects and patients with MS (47). A more recent report by Bahbouhi et al. (48) has shown, using a sensitive detection method, increased frequencies of anti-myelin T cells in MS patients. Soluble MHC-peptide complexes allow the detection and isolation of Ag-specific T cells (26), and these recombinant MHC class II tetramers have been successfully used to detect Ag-specific CD4<sup>+</sup> T cells for viral, bacterial, and self-antigens (30–42). In this study, we successfully applied this approach to demonstrate increases in the frequency of myelin-specific T cells in patients with MS as compared with healthy controls using HLA-DRB1\*0401 class II tetramers loaded with MOG peptide.

Several staining conditions were also evaluated to provide optimal tetramer staining. One factor shown to influence tetramer detection of autoreactive T cells is the presence of moieties of sialic acid on cell surface proteins (45). It has been suggested that removal of sialic acid by neuraminidase treatment results in reduction of net cell charge in the interacting populations with increased membrane fluidity leading to enhanced cell-tetramer adhesiveness (45, 46). In contrast to previous reports, we observed that neuraminidase treatment increased the percentage of tetramer-binding cells; however, it also increased nonspecific binding of irrelevant tetramers to CD4<sup>+</sup> cells. This may indicate that the modifications to proteins on the cell surface differ between species or for this particular binding interaction.

Because IL-7 has been shown by our group (K. Raddassi, K. Bourcier, V. Seyfert-Margolis, and D. Hafler, unpublished observations) and others to increase the proliferation and cytokine production of Ag-reactive T cells (49–51), we examined whether this growth factor improved the expansion of MOG-reactive cells. Instead, we found that the addition of IL-7 to the culture decreased tetramer staining, which may be due to a decreased affinity of the TCR or a downregulation of the TCR. We also observed that tetramer staining was best observed in cells corresponding to the CD4<sup>+</sup>CD25<sup>intermediate</sup> subset that includes memory CD4 cells.

MOG<sub>97–109</sub> has been identified as a major target of T cells in subjects with the DRB1\*0401 allele (18–21). We used the higher affinity binding of the substituted MOG peptide for DRB1\*0401 to promote and stabilize binding of the peptide-MHC tetramer to the T cells, as quantitative enumeration of Ag-specific T cells by tetramer staining, particularly at low frequencies, critically depends on the quality of the tetramers and on the staining procedures. Specifically, suboptimal class II tetramer staining can be due to the low binding affinity of the MOG peptide to the class II molecule. As discussed above, we designed a modified MOG<sub>97–109(107E-S)</sub> peptide with an E-S substitution, which provided an optimal P9 anchoring position for DRB1\*0401/DRA1\*0101 binding. This peptide bound to DRB1\*0401 with a significantly higher affinity compared with the wild-type peptide.

To confirm that the CD4 cells binding to MOG tetramer produced with this modified peptide were indeed Ag-specific T cells, we directly examined the reactivity and specificity of the tetramer-binding cells by single cell sorting and cloning of tetramer<sup>+</sup> populations followed by in vitro expansion of clones and testing reactivity with peptides in the context of DRB1\*0401. The MOG<sub>97–109(107E-S)</sub>-reactive T cell clones derived in this manner had approximate EC<sub>50</sub> values of 1–10 μM of peptide. The MOG<sub>97–109</sub> peptide used here for tetramer construction (FFRDHSYQEEA, native sequence) has a mutation at position

107 (E-S) to stabilize binding to the DRB1\*0401/DRA\*0101 molecules. Thus, it was important to demonstrate that the T cell clones also had reactivity to the native peptide sequence (Table I). As can be seen, the EC<sub>50</sub> for each clone's reactivity to the native or substituted peptide is approximately equivalent, although the proliferative responses were less. The native peptide loaded in tetramer resulted in binding of CD4<sup>+</sup> cells to the tetramer after culture with either the native or the higher affinity peptide; however, in all of the MS samples examined, the frequency of tetramer-binding cells detected was increased when PBMCs were cultured with the substituted peptide and detected with the tetramer loaded with substituted peptide. Most importantly, we detected little tetramer staining in the PBMCs from the healthy controls and the MOG clones generated responded to both the substituted and the native MOG.

Limiting dilution frequency analysis has suggested that autoreactive T cells are found at approximately the same frequency in normal individuals as compared with patients with MS (5–8, 17). However, in subjects with MS, autoreactive T cells are in a more activated state as compared with T cells from normal individuals (9–11). Recently, the relative frequency and avidity of autoreactive CD4<sup>+</sup> T cells to GAD65 from PBMC samples from subjects at risk to type 1 diabetes and then after diagnosis for type 1 diabetes were followed using in vitro culture of PBMCs and detection of autoreactive T cells with DRB1\*0401/GAD65<sub>555–567(557F-I)</sub> tetramer (52). Additionally, the recurrence of T cell autoimmunity in type 1 diabetes patients who underwent pancreas-kidney transplants with immunosuppression was detected with DRB1\*0401/GAD65<sub>555–567(557F-I)</sub> tetramer reagents (53). These data indicate that self-peptide-loaded tetramer reagents are useful in following disease course and for generating information on the frequency and avidity of the T cell response to that Ag. We used the optimal tetramer conditions defined in this investigation and were able to detect MOG-reactive T cells in the peripheral blood after 14 d in vitro expansion in the presence of IL-2. As expected, MOG-reactive T cells were present in both healthy control and patients with MS; however, their frequency after culture with the peptide was higher in the MS population. We speculate that these culture conditions selectively allowed detection of MOG-reactive T cells that were either more activated or resistant to apoptosis. The ability to detect a difference in the frequency of MOG-reactive T cells in patients as compared with controls will allow future investigations to better elucidate the biologic characteristics of autoreactive T cells in the disease.

In summary, we have defined an MHC class II tetramer system using a mutated MOG peptide [MOG<sub>97–109(107E-S)</sub>] that allows us to detect MOG<sub>97–109(107E-S)</sub>-reactive T cells from subjects bearing the DRB1\*0401 allele. Even though the detection was better using the substituted MOG<sub>97–109(107E-S)</sub>, we were able to detect a higher frequency of tetramer-reactive cells from patients with MS as compared with healthy control using either the substituted or the native MOG. The parameters of culture are especially important when detecting rare Ag-specific T cells in the naive repertoire or in detecting low-avidity autoreactive T cells. MOG<sub>97–109</sub>-reactive T cells are present in the peripheral blood of both healthy controls and MS subjects, but the frequency in patients was higher after 14 d culture even without MOG peptide stimulation. The clones generated from MS subjects showed reactivity with the native and substituted MOG<sub>97–109</sub> peptide and showed specificity to that peptide. These strategies may be adapted for the detection of rare Ag-specific low-avidity autoreactive T cells in vivo for detecting the evolution of the autoreactive repertoire and following the phenotype of autoreactive T cells in the course of the autoimmune disease.

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## Disclosures

The authors have no financial conflicts of interest.

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