# Astrocytes Express Elements of the Class II Endocytic Pathway and Process Central Nervous System Autoantigen for Presentation to Encephalitogenic T Cells

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**Astrocytes are nonprofessional APCs that may participate in Ag presentation and activation of pathogenic CD4$^+$ T cells involved in central nervous system (CNS) inflammatory diseases. Using immortalized pure astrocytes as a complement to the study of primary astrocytes, we investigated whether these astrocytes express elements involved in the class II endocytic pathway and if they are capable of processing native myelin basic protein (MBP), a step that could be necessary for initiating or perpetuating T cell recognition of this self-Ag in vivo. Upon IFN-$\gamma$-stimulation, primary and immortalized astrocytes up-regulate class II transactivator (CIITA), invariant chain (Ii) (p31 and p41), H-2Ma, and H-2Mb. Analysis of CIITA cDNA sequences demonstrated that CIITA transcription in astrocytes is directed by a promoter (type IV) that mediates IFN-$\gamma$-inducible CIITA expression and encodes a CIITA protein that differs in its N-terminal sequence from CIITA reported in professional APC. Comparing live and fixed APC for Ag presentation, we show that Ag processing by APC is required for presentation of native MBP to autopathogenic T cells specific for the major MBP epitope, Ac1-11. We have observed that primary astrocytes and some, but not all, astrocyte lines in the absence of contaminating microglia are capable of processing and presenting native MBP, suggesting that there may be heterogeneity. Our study provides definitive evidence that astrocytes are capable of processing CNS autoantigen, indicating that astrocytes have potential for processing and presentation of CNS autoantigen to proinflammatory T cells in CNS autoimmune disease.** *The Journal of Immunology,* **1998, 161: 5959–5966.**

Astrocytes are the most abundant glial accessory cells in the central nervous system (CNS)[3] (1). Unlike professional APC, including dendritic cells, macrophages, and B cells, which express cell surface class II molecules constitutively (2), astrocytes and other nonprofessional APC require stimulation by IFN-$\gamma$ for class II expression (3, 4). Whether astrocytes participate in the activation and regulation of CD4$^+$ T cells within CNS inflammatory conditions in vivo is not clear. In vitro, astrocytes stimulated by IFN-$\gamma$ up-regulate class II as well as certain costimulatory molecules and can present Ag to CD4$^+$ T cells, indicating that they are also capable of processing native CNS autoantigens (3, 5–7). However, other data indicate that astrocytes are not efficient in Ag presentation and T cell activation, suggesting that they may be deficient in some element(s) necessary for Ag presentation (8–12). Processing of native CNS autoantigen may be required for initiating or perpetuating chronic activation of encephalitogenic T cells in vivo.

Processing of Ag by APC for presentation to CD4$^+$ T cells occurs through the endocytic pathway, which requires the coordinate regulation of molecules that participate in class II biosynthesis and maturation (13). The class II transactivator (CIITA) is the key intermediate responsible for constitutive and IFN-$\gamma$-inducible expression of class II (4). Recently, it was observed that CIITA expression is controlled in a tissue-specific manner through the differential activation of multiple nonhomologous promoters (14). One specific promoter, the type IV promoter, directed CIITA transcription in nonprofessional APC. Two other CIITA promoters were utilized in professional APC, type I in dendritic cells and type III in B cells. More recently, the type III promoter was shown to contain sequence(s) that also confers IFN-$\gamma$-inducible CIITA expression (15). CIITA also directs expression of invariant chain (Ii) and H-2M (HLA-DM), two molecules involved in class II maturation and Ag processing (16, 17). Deficiency in any of these elements can result in defects in Ag processing (18–21).

In this study, Ag processing was examined in primary and immortalized pure murine astrocyte lines. The latter offer several advantages, providing a useful complement to studies involving primary astrocytes (22–24). Immortalization by certain methods can capture astrocytes at different stages of differentiation, allowing one to examine whether there are developmental changes in their capability to serve as APC (22–26). Generation of separate lines from one population of primary astrocytes allows one to investigate whether there may be individual variation. Moreover, immortalized astrocytes are not contaminated with microglia, a more potent CNS APC, which can activate T cells in vitro and in vivo (27). In fact, potential contamination with microglia is regarded as an important concern when evaluating Ag presentation by primary

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[3] Abbreviations used in this paper: CIITA, class II transactivator; CNS, central nervous system; EAE, experimental allergic encephalomyelitis; Ii, invariant chain; MBP, myelin basic protein; MS, multiple sclerosis; TBS-T, TBS-Tween; nt, nucleotide.



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astrocytes (3, 5–7) and is also a concern when using sensitive techniques for examining gene expression in primary astrocyte cultures. By comparing live and fixed APC for their capability to present MBP, we demonstrate that processing is required for presentation of native MBP to encephalitogenic T cells that recognize the dominant MBP determinant, Ac1-11. Our results show that IFN-γ-stimulated astrocytes utilize the type IV CIITA promoter and define for the first time the protein sequence at the N terminus of the CIITA isoform transcribed from the murine type IV CIITA. In this study, we provide evidence that astrocytes express elements involved in the class II endocytic pathway and are capable of processing native CNS autoantigen.

## Materials and Methods

### *Development of primary astrocyte cultures*

Primary glial cultures were prepared from cerebral hemispheres of newborn B10.PL (H-$2^u$) mice as described (28). After removal of the meninges, dissociated tissue from the cerebral lobes was passed through a sterile 70-μm nylon cell strainer. This cell suspension was centrifuged, treated with trypsin-EDTA for 5 min at 37°C, washed, and plated in RPMI 1640 containing 10% heat-inactivated FCS, L-glutamine, and penicillin/streptomycin. Cells were initially plated at $5 \times 10^4$ cells/$cm^2$ in 75-$cm^2$ flasks that had been treated with poly-D-lysine (10 μg/ml) and washed 3 times. Nonadherent cells, including oligodendrocytes and microglia, were removed after 7 days of culture by agitation (200 rpm for 8 h). Supernatant was removed, media was replaced, and agitation was repeated (200 rpm for 18 h). Again, supernatant was removed and media was replaced. A total of 97% of the remaining cells were glial fibrillary acidic protein $(GFAP)^+$ and <1% were Mac-$1^+$.

### *Immortalization of primary astrocytes*

The SV40-406 cell line produces a replication-defective retrovirus encoding the SV40 large T Ag in the pZipNeoSV(X)1 construct (22, 24), which also contains neomycin phosphotransferase (Neo). Astrocytes cultured in vitro were exposed to SV40-406 culture supernatants for 2 h in the presence of polybrene (4 μg/ml). After one or more days of treatment, cells were exposed to G418 (400 μg/ml). Astrocytes were immortalized from two separate astrocyte cultures. Three colonies were recovered from cells immortalized after 7 days, providing lines 3.1, 3.2, and 3.3. Two colonies were obtained from cells immortalized after 45 days in culture, providing lines 1.1 and 2.1. Each colony was recovered from a separate plate. Thus, each line was the result of a separate immortalization event. All lines are $GFAP^+$, Mac-$1^-$ (a marker for microglia and macrophages), and negative for galactocerebroside (a marker for oligodendrocytes).

### *Antigens*

MBP peptide Ac1-11 (ASQKRPSQRHG) was synthesized by solid-phase F-moc chemistry by Quality Control Biochemicals (Hopkinton, MA) and purified by reversed-phase HPLC (C18 column, YMC, Raleigh-Durham, NC). The major peak contained 95% desired product, as determined by MALDI-TOF mass spectrometry and HPLC. Native murine, bovine, and human MBP were purified as described (29).

### *MBP-specific T cells*

PJR-25 is an encephalitogenic $CD4^+$ Th1 cell clone derived from PL/J mice (30) that is specific for MBP Ac1-11 in association with I-$A^u$. PJR-25 recognizes intact mouse, bovine, guinea pig, and human MBP.

### *Proliferation assays*

APC were treated with mitomycin C (60 μg/ml) for 1 h and washed three times with media. T cells ($10^4$/well) were cultured with either peptide or intact MBP and APC ($4 \times 10^4$/well) in a 96-well microtiter plate. In Ag fixation experiments, APC were treated with 0.5% paraformaldehyde for 30 min at room temperature and washed three times with media. APC were co-cultured with T cells in the presence of either peptide or intact Ag. All cultures were incubated for 48 h at 37°C. [$^3$H]Thymidine (1 μCi/ml) was added 18 h before harvesting and counting.

### *Flow cytometry*

After culture alone or with IFN-γ (100 U/ml) for 48 h, astrocytes were removed from flasks by treatment with PBS-EDTA, counted, dispensed at $10^6$ cells/tube, and washed with FACS buffer (PBS containing 0.5% BSA and 10 mM sodium azide). Cells were stained with either mAb 10-2.16 (anti-I-$A^{k,u}$) (31) or an isotype-matched control mouse Ab for 45 min at room temperature, washed, then incubated with a FITC-labeled rat anti-mouse secondary Ab for 45 min at room temperature. After three washes, samples were analyzed on a FACSort (Becton Dickinson, CA).

### *mRNA analysis*

mRNA was prepared from $1 \times 10^8$ cells using the Fast Track mRNA Isolation Kit (Invitrogen, San Diego, CA). Reverse transcription was performed using the GeneAmp RNA PCR Kit (Perkin-Elmer, Applied Biosystems, Foster City, CA) for 1 cycle: 42°C for 15 min, 99°C for 5 min, and 5°C for 5 min. The following PCR primers (designed from published sequences (14, 32–34)) were purchased from Genosys Biotechnologies (The Woodlands, TX): CIITA, 5′-CCCCTACATCTACCACCTCTAT-3′ and 5′-CTCCCTTTCCTGGCTCTTGTTGCT-3′; CIITA type I promoter, 5′-AAGAAGGAGAAGGCTACA-3′ and 5′-CTGGTCGCAGTTGATGGTAT-3′; CIITA type III promoter, 5′-GGCTGCCTGCTTCCCACC-3′ and 5′-TCGGGGAGACTGGGGATACT-3′; CIITA type IV promoter, 5′-GCAG GCAGCACTCAGAAGCA-3′ and 5′-CTGGTCGCAGTTGATGGTAT-3′; Ii exons 1–5, 5′-GGACCCCACAGGACTTCACATACT-3′ and 5′-AGAT GCTTCAGATTCTCTGG-3′; Ii exons 3–7, 5′-TGGCTACTCCCTTGCT-3′ and 5′-TCCTGCCTGGTCACTC-3′; Ii exon 6b, 5′-AGTGCCAGGAA GAAGT-3′ and 5′-TCTTGGTGTGAGGAAC-3′; H-2Ma, 5′-CTACGAG ATGTTGATGCGGGAAGT-3′ and 5′-GTGTAGCGGTCAATCTCGTGTG TC-3′; H-2Mb, 5′-GGACCCCACAGGACTTCACATACT-3′ and 5′-GCC GTCTTCTCCTTGTTGCTGTGG-3′; β-actin, 5′-CACCCTGTGCTGCTC ACCGAGGCC-3′ and 5′-CCACACAGATGACTTGCGCTCAGG-3′. For PCR detection of β-actin, CIITA, H-2Ma, H-2Mb, and Ii exons 3–7, cDNA was amplified 35 cycles: 95°C, 15 s; 58°C, 30 s; and 72°C, 30 s. For detection of Ii exon 6b, cDNA was amplified 35 cycles: 94°C, 15 s; 69°C, 30 s; and 72°C, 30 s.

### *DNA sequencing*

A 1.0 μl gel fragment containing the predominant 220-nucleotide (nt) cDNA band from electrophoresis of RT-PCR product was reamplified 10 cycles using the same parameters for initial PCR, then purified. DNA was sequenced using dideoxy-terminators on a Perkin-Elmer ABI 373 Stretch Sequencer.

### *Western blot analysis of Ii p31 and p41*

Cells were solubilized in PHEM lysis buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, and 1.0% Triton X-100 containing 1 mM PMSF, 1 mg/ml leupeptin, 20 mM benzamidine, and 5 mM iodoacetamide; pH 6.9), then centrifuged. Clarified supernatants were resolved by SDS-PAGE using 10% polyacrylamide gels under reducing conditions. Proteins were transferred to poly(vinylidene difluoride) membranes (Millipore, Bedford, MA), which were blocked for 90 min with 7.5% nonfat dry milk in TBS-Tween (TBS-T; 20 mM Tris, 137 mM NaCl, 0.1% Tween 20, pH 7.6). After three washes with TBS-T, membranes were incubated with primary rat mAb In-1 (35) diluted 1:10 in TBS-T for 90 min. Membranes were washed three times with TBS-T before addition of a horseradish peroxidase-conjugated goat anti-rat secondary Ab diluted 1:5000 in TBS-T. After 60 min incubation with the secondary Ab, membranes were washed five times with TBS-T. Ii proteins were then visualized by the enhanced chemiluminescent detection kit (ECL; Amersham, Arlington Heights, IL), followed by exposure to x-ray film.

## Results

### *CIITA expression by immortalized and primary astrocytes is directed by a promoter element that mediates IFN-γ-inducible CIITA expression*

Five individual astrocyte lines were obtained from immortalization of primary B10.PL (H-$2^u$) astrocyte cultures using a replication defective retrovirus containing SV40 large T Ag in the pZipNeoSV(X)1 construct (see *Materials and Methods*). Immortalization by this method does not confer tumorigenicity (22). Astrocytes were immortalized from multiple separate primary astrocyte cultures. Three colonies were recovered from one primary culture immortalized after 7 days, providing lines 3.1, 3.2, and 3.3. Two colonies were obtained from separate primary cultures immortalized after 45 days, providing lines 1.1 and 2.1. All astrocyte lines express glial fibrillary acidic protein (GFAP), an intermediate filament protein expressed primarily by astrocytes. They do not

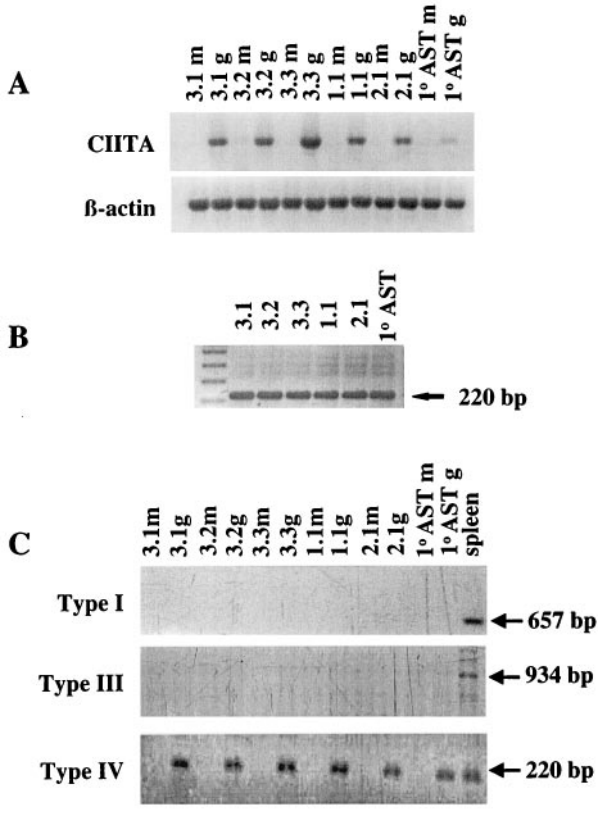


**FIGURE 1.** IFN-γ-inducible expression of CIITA and utilization of the type IV CIITA promoter by immortalized and primary astrocytes (1°AST). *A*, Expression of CIITA by astrocytes following treatment with IFN-γ. IFN-γ (100 U/ml for 24 h) treated (g) and media treated (m) astrocytes were examined for CIITA by RT-PCR. Analysis of *β*-actin by RT-PCR is shown as a positive control. *B*, The type IV CIITA promoter directs expression of CIITA in IFN-γ-treated immortalized astrocyte lines and primary astrocytes. *C*, CIITA expression directed by the type I and type III promoters can be detected in spleen but not astrocytes. RT-PCR was performed using a 5′ oligonucleotide primer corresponding to either the type I, III, or IV promoter and 3′ oligonucleotide primer corresponding to CIITA coding region.

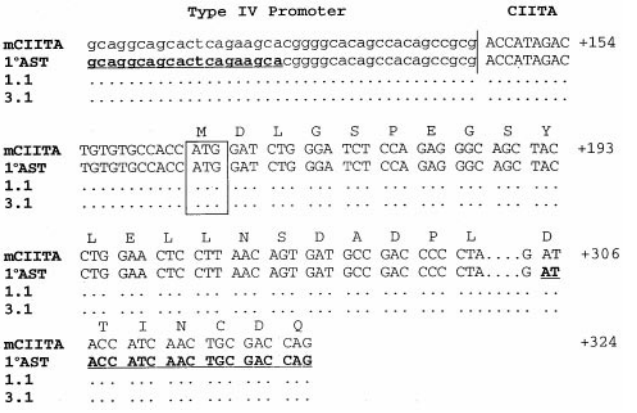


**FIGURE 2.** CIITA cDNA sequence corresponding to the 220-nt RT-PCR products from IFN-γ-stimulated immortalized and primary astrocytes (1°AST). The published mouse type IV CIITA promoter sequence is shown above for comparison. The junction between the 3′ end of the CIITA promoter in astrocytes and the published mouse CIITA occurs at CIITA nt 146. Dots indicate nt identity.

express Mac-1, an Ag expressed on microglia or macrophages (data not shown).

CIITA is considered essential for the IFN-γ-inducible expression of class II, Ii, and H-2M (16, 17). RT-PCR was used to evaluate CIITA expression in unstimulated and IFN-γ-stimulated immortalized astrocyte lines and primary astrocytes. As shown in Fig. 1*A*, CIITA transcripts were not detected by RT-PCR in unstimulated astrocyte lines or primary astrocytes, but were present in all astrocyte lines and primary astrocytes following stimulation with IFN-γ.

Of the distinct nonhomologous CIITA promoter elements described, the type IV promoter was initially identified as one that mediates IFN-γ-inducible CIITA expression (14). More recent studies indicate that utilization of the type III CIITA promoter can be involved in IFN-γ-inducible CIITA expression (15). The CIITA transcripts directed by the separate promoters differ at their 5′ ends (exon 1). When RT-PCR was performed with mRNA isolated from IFN-γ-stimulated astrocyte lines and primary astrocytes using 5′ primers from transcribed sequences of the type IV CIITA promoter and a 3′ primer located within the common CIITA coding sequence (32), CIITA expression was detected (Fig. 1*B*). In contrast, when RT-PCR was performed using a 5′ primer from either the type I or the type III promoter CIITA expression was not detected in astrocytes. Expression of CIITA was detected in splenic mRNA isolated from a mouse injected with CFA in the same experiment using primers from these promoter elements (Fig. 1*C*).

The cDNA products from the RT-PCR performed on two immortalized astrocyte lines and primary astrocytes (data presented in Fig. 1*B*) using a 5′ primer from the type IV CIITA promoter were sequenced. The sequences from the two immortalized astrocyte lines and primary astrocytes proved to be identical (Fig. 2). The 3′ end of the promoter sequence is contiguous with the CIITA coding sequence, starting at nt 146, which is 52 nt downstream of the AUG (start codon) used for initiating CIITA translation in B cells. The next AUG (identified as "ATG" in Fig. 2), which is located within a perfect Kozak sequence for initiation of translation (36), begins at nt 166. Initiation of translation at this AUG potentially encodes a CIITA protein that has 24 or 101 fewer N-terminal amino acids than the CIITA encoded by B-cells or dendritic cells, respectively (14). Thus, we define for the first time the N-terminal protein sequence of the CIITA isoform directed by the murine type IV CIITA promoter. Furthermore, our results indicate that there are no additional undiscovered exons containing N-terminal coding sequence transcribed from the type IV CIITA promoter.

### *Expression of Ii and H-2M by immortalized and primary astrocytes*

In Fig. 3*A*, it can be seen that all IFN-γ-stimulated astrocytes upregulated Ii mRNA. For primary astrocytes and certain lines, constitutive and inducible Ii mRNA was detected. APC can express two Ii isoforms, p31 and p41 (13, 33). Ii p41 differs by a 192-nt (64 amino acid) segment encoded by one additional exon, 6b (33). Ii p31 is the predominant form in most cells examined (13). Although Ii p31 and p41 both facilitate MHC class II assembly and prevent premature peptide binding to class II molecules, some data suggest that Ii p41 may also stabilize one or more proteases in the Ag processing compartment (37, 38). Using primers from sequences located within exon 6b, p41 mRNA was detected in IFN-γ-stimulated astrocytes (Fig. 3*A*, second panel from top). Utilizing primers that flanked exon 6b and distinguished p31 from p41 transcripts, both p31 and p41 transcripts were detected (Fig. 3*A*, third panel from top).

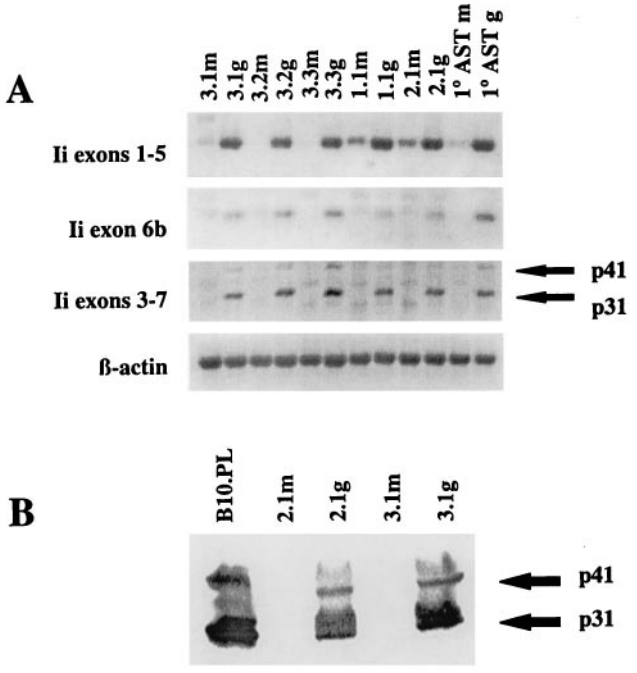


**FIGURE 3.** Ii expression by immortalized and primary astrocytes. *A*, RT-PCR analysis of Ii RNA expression from media (m) and IFN-γ (g) treated (100 U/ml) immortalized and primary astrocytes (1°AST). The *top panel* demonstrates transcripts containing exons 1–5. The *second panel from top* demonstrates expression of transcripts containing exon 6b (p41 specific). Both Ii p31 and p41 mRNA are distinguished (*third panel from top*). β-actin is shown as a positive control (*lowest panel*). *B*, Detection of individual Ii p31 and p41 expression by Western blot analysis. B10.PL splenocytes (B10.PL) are shown for comparison.

Western blot analysis was used to quantitate protein expression of Ii p31 and p41. It can be seen that the IFN-γ-stimulated astrocyte lines examined, as well as B10.PL splenocytes, expressed both Ii p31 and p41 proteins and that p31 is the predominant isoform (Fig. 3*B*). Despite some constitutive expression of Ii mRNA in untreated immortalized lines and primary astrocytes (Fig. 3*A*), Ii p31 and p41 protein were detected only when cells were treated with IFN-γ. All of the immortalized astrocyte lines, including 3.3, expressed significant levels of intracellular Ii protein by FACS analysis (data not shown).

Astrocytes were examined for expression of H-2Ma and H-2Mb mRNA by RT-PCR. In lines 3.3, 1.1, and 2.1, H-2Ma expression was primarily inducible by IFN-γ (Fig. 4). Constitutive H-2Ma expression was also observed in 3.1, 3.2, and primary astrocytes. In all astrocyte lines and primary astrocytes, stimulation with IFN-γ caused up-regulation of H-2Mb expression.

### *Immortalized and primary astrocytes process native MBP for presentation of the major encephalitogenic MBP determinant, Ac1-11*

Similar to primary astrocytes, the immortalized lines do not express cell surface class II without stimulation with IFN-γ (3, 7). As shown by FACS analysis in Fig. 5*A*, astrocyte lines and primary astrocytes can express I-$A^u$ class II molecules following stimulation with IFN-γ. In several separate experiments, it has been observed that line 3.2 expresses the lowest levels of I-$A^u$. Periodic re-examination over 1 yr has shown that each pattern of IFN-γ-inducible class II expression by individual lines has not changed.

After having demonstrated that IFN-γ-stimulated astrocyte lines can express elements involved in the class II endocytic pathway, the lines were tested for their capability to present the immunodominant MBP peptide Ac1-11 to encephalitogenic T cells that recognize this epitope (30). Splenocyte APC were tested in parallel. APC were pretreated with mitomycin C, which allowed APC to remain metabolically active but incapable of cell division. Four of five IFN-γ-stimulated astrocyte lines could present MBP Ac1-11. Representative data are shown in Fig. 5*B*. Line 3.2 was the only one of the five lines that was incapable of efficiently presenting Ac1-11 (data not shown), which is consistent with its low level of MHC class II expression.

Three of the four lines that could present MBP Ac1-11 to MBP-specific T cells could also present native MBP. Line 3.3, which could present Ac1-11, was not capable of presenting native MBP (Fig. 5*C*). This line could not present any form of native MBP, including guinea pig, mouse, bovine, or human MBP, although the other lines could present these heterologous MBP proteins. Thus, of five lines tested, three lines (1.1, 2.1, and 3.1) could present either native MBP or Ac1-11, one line (3.3) could present only Ac1-11, and one line (3.2) could not efficiently present Ac1-11 or native MBP. The inability to present MBP Ac1-11 or native MBP by line 3.2 does not reflect the lack of expression of costimulatory molecules. All lines express B7-2 (lines 1.1 and 2.1 can also express both B7-1 and B7-2 (data not shown), and activation of these encephalitogenic T cells requires B7-2 costimulation (J.M.S. and S.S.Z., unpublished data). As described by others (7), we have also observed that, in contrast to splenic APC (39), increasing the number of astrocyte APC does not enhance Ag-specific T cell proliferation.

Denatured Ag or partially degraded fragments of native Ag may not require processing by APC for presentation to T cells (40–42). Our observation that astrocyte line 3.3 presented MBP Ac1-11 but not native MBP suggests that processing by APC is required when native MBP is used as the Ag and that line 3.3 may possess a defect(s) in processing native MBP. To establish clearly whether processing of native MBP is necessary, we examined whether IFN-γ-activated astrocytes fixed by treatment with paraformaldehyde could present Ac1-11 and native MBP. Primary astrocytes and splenocytes were also examined. When fixed with paraformaldehyde, astrocyte lines could present MBP Ac1-11, but not native MBP (Table I). Similar to the astrocyte lines, primary astrocytes fixed with paraformaldehyde could only present Ac1-11, whereas

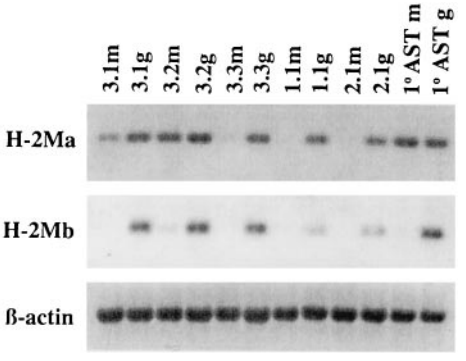


**FIGURE 4.** Immortalized and primary astrocytes express H-2Ma and H-2Mb. RT-PCR detection of H-2Ma in immortalized astrocyte lines and primary astrocytes (1°AST) is shown in the *upper panel*, H-2Mb in the *middle panel*, and β-actin in the *lower panel*. Astrocytes were treated with 100 U/ml IFN-γ (g) or media (m) alone.

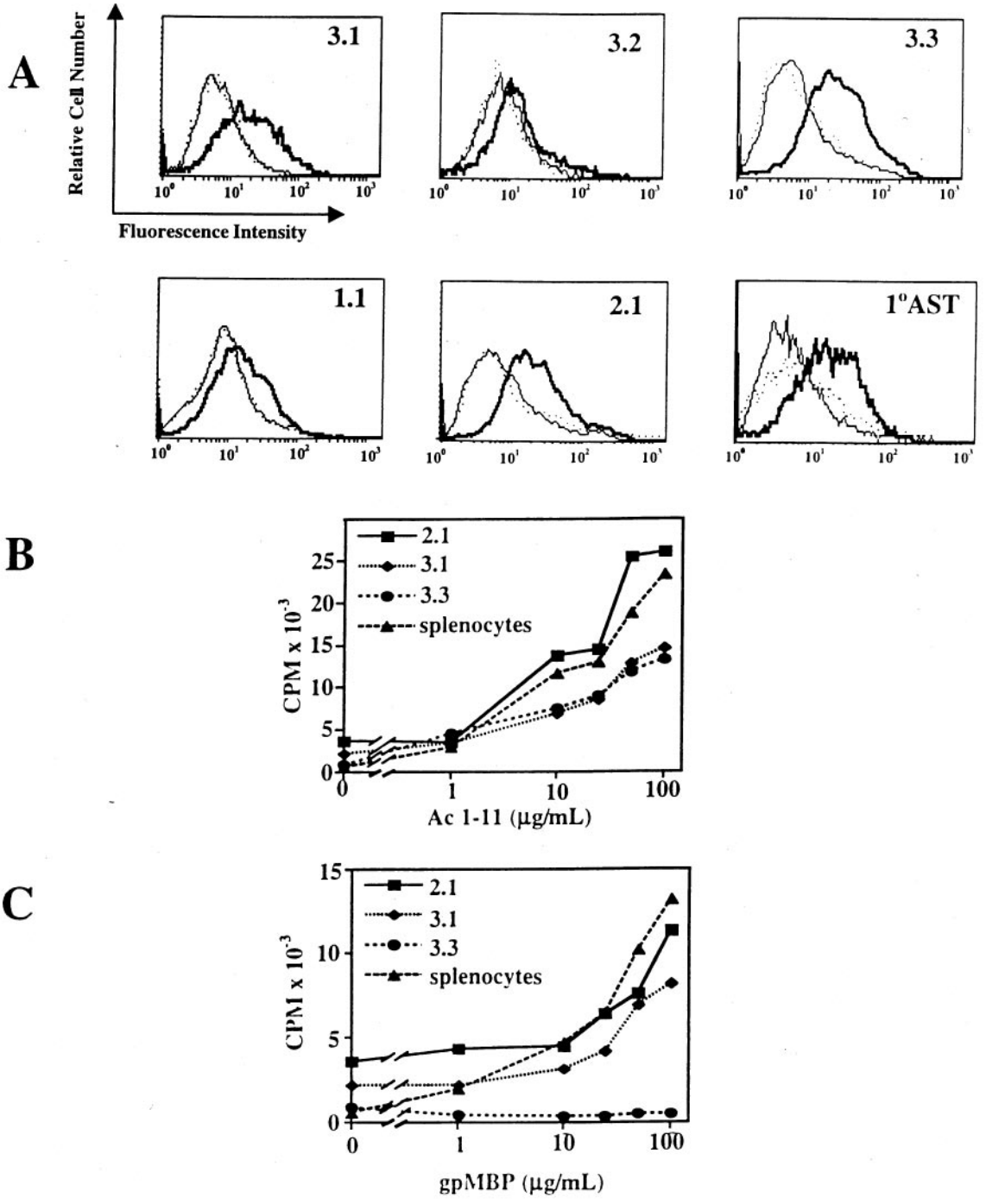


**FIGURE 5.** Expression of class II and presentation of MBP peptide Ac1-11 and native MBP by immortalized astrocyte lines. *A*, Expression of I-A class II molecules by unstimulated and IFN-γ-stimulated immortalized and primary astrocytes (1°AST). IFN-γ-stimulated astrocytes stained with anti-I-A (bold line), unstimulated astrocytes stained with anti-I-A (thin line), and IFN-γ-stimulated astrocytes stained with isotype-matched control Ab (dotted line). *B*, Presentation of MBP peptide Ac1-11. *C*, Presentation of native MBP. Immortalized astrocytes were stimulated with IFN-γ (100 U/ml) for 48 h, then treated with mitomycin C. In parallel, B10.PL splenocytes were treated with mitomycin C.

primary astrocytes treated with mitomycin C could present Ac1-11 or native MBP. These results demonstrate that processing is required for presentation of native MBP to T cells specific for MBP Ac1-11 and that some, but not all, of these astrocytes are capable of Ag processing.

## Discussion

In this report, we have established that Ag processing is required for presentation of native MBP to autopathogenic T cells specific for the dominant encephalitogenic MBP determinant, Ac1-11. If resident CNS APC initiate presentation of this autoantigen to pathogenic T cells in vivo, processing of MBP may be required. It has been shown that perivascular microglia can participate in Ag presentation and the activation of T cells in vivo (27). Whether astrocytes, the more abundant glial cell, also do so is more controversial (12). In inflammatory lesions of experimental allergic encephalomyelitis (EAE) and multiple sclerosis (MS), astrocytes express class II, suggesting that these accessory cells may participate in class II-restricted Ag presentation in vivo

Table I. *Astrocytes process native MBP for presentation of the major encephalitogenic determinant, Ac1-11*[a]

| APC[b] | Treatment[c] | Media (cpm) | Ac1-11 (cpm)[d] | MBP (cpm)[e] |
|---|---|---|---|---|
| 3.1 | PF | 1,438 | 43,125 | 2,079 |
| 3.3 | PF | 1,228 | 45,038 | 1,242 |
| 2.1 | PF | 1,816 | 52,380 | 1,847 |
| primary astrocytes | Mit C | 1,282 | 56,875 | 57,281 |
| primary astrocytes | PF | 2,093 | 47,992 | 2,011 |
| splenocytes | PF | 1,172 | 63,614 | 2,367 |

[a] Mean [$^3$H]thymidine incorporation was determined for replicate cultures. For cultures incubated with native MBP or Ac1-11, the SD from replicate cultures were within 10% of the mean. For cultures without (media), the SD were within 15% mean.
[b] $4 \times 10^4$ cells (splenocytes, primary and immortalized astrocytes) were provided as APC.
[c] For treatment of APC types, PF refers to paraformaldehyde fixation and Mit C refers to mitomycin C treatment.
[d] Ac1-11 was used at a final concentration of 50 $\mu$g/ml. Optimal concentration of Ac1-11 for stimulation by APC was 50-100 $\mu$g/ml.
[e] MBP was used at a final concentration of 100 $\mu$g/ml. Optimal concentration range for stimulation with this preparation of intact MBP was 100 $\mu$g/ml.

(43–45). Reports regarding Ag presentation by astrocytes have conflicted (3, 5–10). Some investigators have suggested that astrocytes may down-regulate T cell responses (12). A concern raised by other investigators studying Ag presentation by primary astrocytes is that microglia, considered a more professional APC (9), can contaminate primary astrocyte cultures and may contribute as APC (7). Potential contamination of primary astrocytes may also be a concern when evaluating gene expression by sensitive molecular techniques. We have examined both primary astrocytes and immortalized pure astrocyte lines, which virtually eliminates the possibility that a more professional APC, such as microglia, is responsible for Ag presentation observed in our studies. We have established that astrocytes are capable of expressing elements involved in the endocytic pathway and clearly demonstrated, by comparing live and fixed astrocytes, that murine astrocytes can process native MBP autoantigen for the activation of encephalitogenic MBP Ac1-11-specific T cells. Thus, although the role of astrocytes in CNS T cell activation may not be clear, our results suggest that some astrocytes have potential for Ag processing and presentation in vivo.

One astrocyte line (3.3) was capable of presenting MBP peptide but not native MBP, suggesting that it is deficient in some aspect(s) of processing MBP. However, this line expressed CIITA, Ii, H-2M, and class II after stimulation with IFN-$\gamma$, demonstrating that although expression of these elements may be necessary, it is not sufficient for processing and presentation of the major encephalitogenic MBP determinant. It was observed that, when melanoma cell lines deficient in CIITA, class II, Ii, and HLA-DM (H-2M) were transfected with CIITA, expression of all these elements was restored, although transfected cells could only present peptide Ag (17, 46). Processing of native Ag required exposure to IFN-$\gamma$, suggesting that IFN-$\gamma$ induces expression of another product(s) involved in Ag processing, independent of CIITA. In this regard, other transactivating factors related to this pathway may exist (47). Our observation that the IFN-$\gamma$-stimulated astrocyte line 3.3 is not capable of processing native MBP suggests that 3.3 cells require expression of a factor(s) that is not dependent upon IFN-$\gamma$ or that these cells are deficient in an aspect of IFN-$\gamma$ activation. Further analysis with these cells will permit examination of other elements, including specific proteases (48, 49), that may be required for MBP processing by astrocytes.

Astrocytes are not a uniform glial cell subpopulation (50, 51). Morphologic and biochemical characteristics have been used to distinguish astrocyte subtypes, suggesting that there may be functional heterogeneity (51–53). Some studies indicate that there may also be regional heterogeneity (52, 53). We have observed that three immortalized astrocyte lines (3.1, 3.2, and 3.3) isolated from the same primary culture differ from one another in their capability to present MBP, suggesting that there may be heterogeneity among individual astrocytes with regard to Ag presentation. We have also observed differences in expression of costimulatory molecules by murine astrocytes from one mouse strain (J.M.S. and S.S.Z., unpublished data). Thus, possible heterogeneity could, in part, account for the conflicting results reported regarding astrocyte function in T cell activation.

Immortalized astrocyte lines were used as a source of pure astrocytes, as primary astrocytes, in general, become senescent (23). Other investigators using this same approach to study CNS cells, including astrocytes, have also shown that immortalized cells retain important phenotypic characteristics of primary cells (22–24, 26). However, one concern in studying accessory cell function with immortalized astrocytes is that immortalization may confer properties not shared by primary astrocytes. For example, we cannot eliminate the possibility that deficiency in Ag processing by line 3.3 is related to its immortalization. However, we have shown that this astrocyte line, like primary astrocytes and astrocyte lines that do process and present MBP, is not deficient in expression of the elements addressed in this study, namely CIITA, Ii, H-2Ma, H-2Mb, or class II. If deficiency in a particular element involved in Ag processing is identified, it will be necessary to investigate whether immortalization, possibly by the integration within a particular gene locus, is related to deficient expression of that element. It is possible that interruption of gene expression by immortalization may facilitate the identification of additional genes involved in Ag processing. In this study, we have shown by examining immortalized astrocytes in parallel with primary astrocytes how immortalized astrocytes can serve as a valuable complement for the study of immune regulation by astroglia.

CIITA has been described as the "master control factor" for regulation of expression of MHC class II molecules (4). Although not a DNA-binding protein, CIITA is a transcriptional co-activator that associates with the RFX family of DNA-binding proteins to form a complex for activating class II gene transcription (54). Our results demonstrate that CIITA expression in astrocytes is IFN-$\gamma$-inducible and that its transcription is directed by the type IV CIITA promoter element. As it has recently been shown that distant promoter sequences confer IFN-$\gamma$ inducibility to the type III CIITA promoter (15), it is possible that this promoter element could be involved in regulation of CIITA expression in astrocytes under conditions not used in this study. Use of the type IV promoter produces a transcript encoding a CIITA protein that differs at its N terminus from the CIITA molecules expressed constitutively by professional APC (14). Analysis of the type IV CIITA transcripts in astrocytes also indicates that there are no additional exon-containing N-terminal coding sequences directed by the type IV CIITA promoter in these cells. The potential for distinct CIITA proteins that differ in N-terminal amino acid sequences raises the question of whether there is another level of complexity regarding CIITA expression in astrocytes and other nonprofessional APC. It is also possible that polymorphism exists within regions of this promoter element, which could contribute to class II dysregulation by nonprofessional APC that participate in Ag presentation to pathogenic T cells in organ-specific autoimmune disease, such as EAE and MS. It was observed that IFN-$\gamma$-stimulated astrocytes from certain mouse strains resistant to EAE expressed lower levels

of class II, suggesting a correlation between the level of expression of class II by astrocytes and susceptibility to EAE (55). In that study, the $F_1$ progeny of susceptible and resistant strains were susceptible to EAE and both parental class II alleles (haplotypes) were up-regulated, suggesting that a transactivating factor, possibly located outside the MHC, was deficient in the resistant parental strain. Thus, it will be of interest to determine whether there is polymorphism within this CIITA promoter and whether regulation of CIITA may contribute to EAE and MS susceptibility. Furthermore, learning how to manipulate expression of elements in the MHC class II endocytic pathway by CNS accessory cells in vivo could be beneficial in the therapy for T cell-mediated CNS autoimmune disease.

***Note added in revision.*** Since submission of this manuscript, two studies have been published that also address whether astrocytes are capable of processing native Ag (56, 57). The report by Tan et al. (56) indicates that astrocytes are capable of processing native Ag, whereas the study by Aloisi et al. (57) indicates that astrocytes are inefficient in processing and presentation of native Ag.

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## References

1. Shrikant, P., and E. N. Benveniste. 1996. The central nervous system as an immunocompetent organ: role of glial cells in antigen presentation. *J. Immunol. 157:1819.*
2. Steinman, R. M. 1991. The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol. 9:271.*
3. Fontana, A., W. Fierz, and H. Wekerle. 1984. Astrocytes present myelin basic protein to encephalitogenic T-cell lines. *Nature 307:273.*
4. Mach, B., V. Steimle, E. Martinez-Soria, and W. Reith. 1996. Regulation of MHC class II genes: lessons from a disease. *Annu. Rev. Immunol. 14:301.*
5. Takiguchi, M., and J. A. Frelinger. 1986. Induction of antigen presentation ability in purified cultures of astroglia by interferon-γ. *J. Mol. Cell. Immunol. 2:269.*
6. Frei, K., H. Lins, C. Schwerdel, and A. Fontana. 1994. Antigen presentation in the central nervous system: the inhibitory effect of IL-10 on MHC class II expression and production of cytokines depends on the inducing signals and the type of cell analyzed. *J. Immunol. 152:2720.*
7. Nikcevich, K. M., K. B. Gordon, L. Tan, S. D. Hurst, J. F. Kroepfl, M. Gardiner, T. A. Barrett, and S. D. Miller. 1997. IFN-γ activated primary murine astrocytes express B7 costimulatory molecules and prime naive antigen-specific T cells. *J. Immunol. 158:614.*
8. Sedgwick, J. D., R. Mössner, S. Schwender, and V. ter Meulen. 1991. Major histocompatibility complex-expressing nonhematopoietic astroglial cells prime only $CD8^+$ T lymphocytes: astroglial cells as perpetuators but not initiators of $CD4^+$ T cell responses in the central nervous system. *J. Exp. Med. 173:1235.*
9. Matsumoto, Y., K. Ohmori, and M. Fujiwara. 1992. Immune regulation by brain cells in the central nervous system: microglia but not astrocytes present myelin basic protein to encephalitogenic T cells under in vivo-mimicking conditions. *Immunology 76:209.*
10. Williams, J., K., E. Ulvestad, L. Cragg, M. Blain, and J. P. Antel. 1993. Induction of primary T cell responses by human glial cells. *J. Neurosci. Res. 36:382.*
11. Weber, F., E. Meinl, F. Aloisi, C. Nevinny-Stickel, E. Albert, H. Wekerle, and R. Holfeld. 1994. Human astrocytes are only partially competent antigen presenting cells: possible implications for lesion development in multiple sclerosis. *Brain 117:59.*
12. Meinl, E., F. Aloisi, B. Ertl, F. Weber, R. de Waal Malefyt, H. Wekerle, and R. Holfeld. 1994. Multiple sclerosis: immunomodulatory effects of human astrocytes on T cells. *Brain 117:1323.*
13. Wolf, P. R., and H. L. Ploegh. 1995. How MHC class II molecules acquire peptide cargo: biosynthesis and trafficking through the endocytic pathway. *Annu. Rev. Immunol. 11:267.*
14. Muhlethaler-Mottet, A., L. A. Otten, V. Steimle, and B. Mach. 1997. Expression of MHC class II molecules in different cellular and functional compartments is controlled by differential usage of multiple promoters of the transactivator CIITA. *EMBO J. 16:2851.*
15. Piskurich, J. F., Y. Wang, M. W. Linhoff, L. C. White, and J. P.-Y. Ting. 1998. Identification of distinct regions of 5′ flanking DNA that mediate constitutive, IFN-γ, STAT1, and TGF-β-regulated expression of the class II transactivator gene. *J. Immunol. 160:233.*
16. Chang, C.-H., and R. A. Flavell. 1995. Class II transactivator regulates the expression of multiple genes involved in antigen presentation. *J. Exp. Med. 181: 765.*
17. Siegrist, C.-A., E. Martinez-Soria, I. Kern, and B. Mach. 1995. A novel antigen-processing-defective phenotype in major histocompatibility complex class II-positive CIITA transfectants is corrected by interferon-γ. *J. Exp. Med. 182:1793.*
18. Viville, S., J. Neefjes, V. Lotteau, A. Dierich, M. Lemeur, H. Ploegh, C. Benoist, and D. Mathis. 1993. Mice lacking the MHC class II-associated invariant chain. *Cell 72:635.*
19. Bikoff, E. K., L.-Y. Huang, V. Episkopou, J. van Meerwijk, R. N. Germain, and E. J. Robertson. 1993. Defective major histocompatibity complex class II assembly, transport, peptide acquisition, and $CD4^+$ T cell selection in mice lacking invariant chain expression. *J. Exp. Med. 177:1699.*
20. Miyazaki, T., P. Wolf, S. Tourne, C. Waltzinger, A. Dierich, N. Barios, H. Ploegh, C. Benoist, and D. Mathis. 1996. Mice lacking H-2M complexes, enigmatic elements of the MHC class II peptide-loading pathway. *Cell 84: 531.*
21. Martin, W. D., G. G. Hicks, S. K. Mendiratta, H. I. Leva, H. E. Ruley, and L. Van Kaer. 1996. H-2M mutant mice are defective in the peptide loading of class II molecules, antigen presentation, and T cell repertoire selection. *Cell 84: 543.*
22. Jat, P. S., C. L. Cepko, R. C. Mulligan, and P. A. Sharp. 1986. Recombinant retroviruses encoding simian virus 40 large T antigen and polyomavirus large and middle T antigens. *Mol. Cell. Biol. 6:1204.*
23. Cepko, C. L. 1989. Immortalization of neural cells via retrovirus-mediated oncogene transduction. *Annu. Rev. Neurosci. 12:47.*
24. Frisa, P. S., M. N. Goodman, G. M. Smith, J. Silver, and J. W. Jaccobberger. 1994. Immortalization of immature and mature astrocytes with SV40 T antigen. *J. Neurosci. Res. 39:47.*
25. Noble, M., A. K. Groves, P. Ataliotis, Z. Ikram, and P. S. Jat. 1995. The H-2KbtsA58 transgenic mouse: a tool for the rapid generation of novel cell lines. *Transgenic Res. 4:215.*
26. McLaurin, J., G. Almazan, K. Williams, and J. P. Antel. 1995. Immortalization and characterization of rat microglia cells. *Neuropath. Appl. Neurobiol. 21:320.*
27. Hickey, W. F., and H. Kimura. 1988. Perivascular microglial cells of the CNS are bone marrow-derived and present antigen in vivo. *Science 239:290.*
28. McCarthy, K. D., and J. de Vellis. 1980. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J. Cell Bio. 85:890.*
29. Smith, M. E. 1969. An in vitro system for the study of myelin synthesis. *J. Neurochem. 16:83.*
30. Zamvil, S. S., D. J. Mitchell, A. C. Moore, K. Kitamura, L. Steinman, and J. B. Rothbard. 1986. T cell epitope of the autoantigen myelin basic protein that induces encephalomyelitis. *Nature 324:258.*
31. Oi, V. T., P. P. Jones, and L. A. Herzenberg. 1978. Properties of monoclonal antibodies to mouse Ig allotypes, H-2, and Ia antigens. *Clin. Top. Microbiol. Immunol. 81:115.*
32. Sims, T. N., J. F. Elliot, V. Ramassar, D. W. Denney, Jr., and P. F. Halloran. 1997. Mouse class II transactivator: cDNA sequence and amino acid comparison with the human transactivator. *Immunogenetics 45:220.*
33. Koch, N., W. Lauer, J. Habicht, and B. Dobberstein. 1987. Primary structure of the gene for the murine Ia antigen-associated invariant chains (Ii): an alternatively spliced exon encodes a cysteine-rich domain highly homologous to a repetitive sequence of thyroglobulin. *EMBO J. 6:1677.*
34. Cho, S., M. Attaya, M. G. Brown, and J. J. Monaco. 1991. A cluster of transcribed sequences between the Pb and Ob genes of the murine major histocompatiblity complex. *Proc. Natl. Acad. Sci. USA 88:5197.*
35. Koch, N., S. Koch, and G. J. Hammerling. 1982. Ia invariant chain detected on lymphocyte surfaces by monoclonal antibody. *Nature 299:644.*
36. Kozak, M. 1991. An analysis of vertebrate mRNA sequences: intimations of translational control. *J. Cell Biol. 115:887.*
37. Bevec, T., V. Stoka, G. Pungercic, I. Dolenc, and V. Turk. 1996. Major histocompatibility complex class II-associated p41 invariant chain fragment is a strong inhibitor of lysosomal cathepsin L. *J. Exp. Med. 183:1331.*
38. Fineschi, B., K. Sakaguchi, E. Appella, and J. Miller. 1996. The proteolytic environment involved in MHC class II-restricted antigen presentation can be modulated by the p41 form of invariant chain. *J. Immunol. 157:3211.*
39. Zamvil, S. S., P. A. Nelson, D. J. Mitchell, R. L. Knobler, R. B. Fritz, and L. Steinman. 1985. Encephalitogenic T cell clones specific for myelin basic protein: unusual bias in antigen presentation. *J. Exp. Med. 162:2107.*
40. Shimonkevitz, R., J. Kappler, P. Marrack, and H. Grey. 1983. Antigen recognition by H-2-restricted T cells. I. Cell-free antigen processing. *J. Exp. Med. 158: 303.*
41. Streicher, H. Z., I. J. Berkower, M. Busch, F. R. Gurd, and J. A. Berzofsky. 1984. Antigen conformation determines processing requirements for T-cell activation. *Proc. Natl. Acad. Sci. USA 81:6831.*
42. Lindner, R., and E. Unanue. 1996. Distinct antigen MHC class II complexes generated by separate processing pathways. *EMBO J. 15:6910.*
43. Traugott, U., L. C. Scheinberg, and C. S. Raine. 1985. On the presence of Ia-positive endothelial cells and astrocytes in multiple sclerosis lesions and its relevance to antigen presentation. *J. Neuroimmunol. 8:1.*
44. Sakai, K., T. Tabira, M. Endoh, and L. Steinman. 1986. Ia expression in chronic relapsing experimental allergic encephalomyelitis induced by long-term cultured T cell lines in mice. *Lab. Invest. 54:345.*

45. Traugott, U. 1989. Detailed analysis of early immunopathologic events during lesion formation in acute experimental autoimmune encephalomyelitis. *Cell. Immunol. 119:114.*
46. Martinez-Soria, E., C. Siegrist, and B. Mach. 1996. Highly efficient peptide binding and T cell activation by MHC class II molecules of CIITA-transfected cells. *Int. Immunol. 8:543.*
47. Douhan III, J., R. Lieberson, J. H. M. Knoll, H. Zhou, and L. H. Glimcher. 1997. An isotype-specific activator of major histocompatibility complex (MHC) class II genes that is independent of class II transactivator. *J. Exp. Med. 185:1885.*
48. Riese, R. J., P. R. Wolf, D. Brömme, L. R. Natkin, J. A. Villadangos, H. L. Ploegh, and H. A. Chapman. 1996. Essential role for cathepsin S in MHC class II-associated invariant chain processing and peptide loading. *Immunity 4:357.*
49. Ploegh, H. L. 1997. Destruction of proteins as a creative force. *Immunol. Today 180:269.*
50. Wekerle, H. 1988. Intercellular interactions in myelin-specific autoimmunity. *J. Neuroimmunol. 20:211.*
51. Wilkin, G. P., D. R. Marriot, and A. J. Cholewinski. 1990. Astrocyte heterogeneity. *T. Neurosci. 13:43.*
52. Hansson, E., L. Ronnback, and A. Sellstrom. 1984. Is there a "dopaminergic glial cell"? *Neurochem. Res. 9:679.*
53. Patel, A. J., A. Hunt, and F. Faraji-Shadan. 1986. Effect of removal of glutamine and addition of dexamethasone on the activities of glutamine synthetase, ornithine decarboxylase and lactate dehydrogenase in primary cultures of forebrain and cerebellar astrocytes. *Brain Res. 391:229.*
54. Scholl, T., S. K. Mahanta, and J. L. Strominger. 1997. Specific complex formation between the type II bare lymphocyte syndrome-associated transactivators CIITA and RFX5. *Proc. Natl. Acad. Sci 94:6330.*
55. Massa, P. T., V. Ter Meulen, and A. Fontana. 1987. Hyperinducibility of Ia antigen on astrocytes correlates with strain-specific susceptibility to experimental autoimmune encephalomyelitis. *Proc. Natl. Acad. Sci. USA 84: 4219.*
56. Tan, L., K. B. Gordon, J. P. Mueller, L. A. Matis, and S. D. Miller. 1998. Presentation of proteolipid protein epitopes and B7-dependent activation of encephalitogenic T cells by IFN-γ-activated SJL/J astrocytes. *J. Immunol. 160: 4271.*
57. Aloisi, F., F. Ria, G. Penna, and L. Adorini. 1998. Microglia are more efficient that astrocytes in antigen processing and in Th1 but not Th2 cell activation. *J. Immunol. 160:4671.*