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De Novo Central Nervous System Processing of Myelin Antigen Is Required for the Initiation of Experimental Autoimmune Encephalomyelitis

Stephen Mark Tompkins,* Josette Padilla,* Mauro C. Dal Canto, † Jenny P.-Y. Ting, ‡ Luc Van Kaer, § and Stephen D. Miller²*†

We demonstrate the absolute requirement for a functioning class II-restricted Ag processing pathway in the CNS for the initiation of experimental autoimmune encephalomyelitis (EAE). C57BL/6 (B6) mice deficient for the class II transactivator, which have defects in MHC class II, invariant chain (Ii), and H-2M (DM) expression, are resistant to initiation of myelin oligodendrocyte protein (MOG) peptide, MOG35–55-specific EAE by both priming and adoptive transfer of encephalitogenic T cells. However, class II transactivator-deficient mice can prime a suboptimal myelin-specific CD4⁺ Th1 response. Further, B6 mice individually deficient for Ii and DM are also resistant to initiation of both active and adoptive EAE. Although both II-deficient and DM-deficient APCs can present MOG peptide to CD4⁺ T cells, neither is capable of processing and presenting the encephalitogenic peptide of intact MOG protein. This phenotype is not Ag-specific, as DM- and Ii-deficient mice are also resistant to initiation of EAE by peptide-proteolipid protein peptide PLP178–191. Remarkably, DM-deficient mice can prime a potent peripheral myelin-specific CD4⁺ Th1 response. Further, B6 mice individually deficient for Ii and DM are also resistant to initiation of both active and adoptive EAE. Although both II-deficient and DM-deficient APCs can present MOG peptide to CD4⁺ T cells, neither is capable of processing and presenting the encephalitogenic peptide of intact MOG protein. This phenotype is not Ag-specific, as DM- and Ii-deficient mice are also resistant to initiation of EAE by peptide-proteolipid protein peptide PLP178–191. Remarkably, DM-deficient mice can prime a potent peripheral Th1 response to MOG35–55, comparable to the response seen in wild-type mice, yet maintain resistance to EAE initiation. Most striking is the demonstration that T cells from MOG35–55-primed DM knockout mice can adoptively transfer EAE to wild-type, but not DM-deficient, mice. Together, these data demonstrate that the inability to process antigenic peptide from intact myelin protein results in resistance to EAE and that de novo processing and presentation of myelin Ags in the CNS is absolutely required for the initiation of autoimmune demyelinating disease. The Journal of Immunology, 2002, 168: 4173–4183.

E xperimental autoimmune encephalomyelitis (EAE) is a CD4⁺ T cell-mediated disease of the CNS. In C57BL/6 (B6) mice, EAE can be induced by immunization with antigenic myelin components, or by the adoptive transfer of myelin-specific CD4⁺ T cells that have been reactivated in vitro (1–3). In both cases, T cells specific for CNS myelin infiltrate the CNS, causing the influx of macrophages and activation of CNS resident cells (4). The release of inflammatory mediators causes inflammation and subsequent tissue destruction and demyelination of axonal tracks. This results in acute paralysis with clinical and histopathological similarities to the human demyelinating disease, multiple sclerosis (MS) (5, 6).

Activation of CD4⁺ T cells requires that their TCR must recognize cognate Ags presented by class II MHC. The critical importance of class II MHC Ag presentation in EAE initiation is illustrated by the fact that treatment with anti-class II Abs inhibits or ameliorates the disease (reviewed in Ref. 7). A variety of proteins have important roles in the generation of the functional peptide/class II MHC protein complex. Invariant chain (Ii) and H-2M (DM) are critical components of the class II Ag processing pathway. Newly synthesized class II α- and β-chains associate with Ii in the endoplasmic reticulum (ER), forming a heterotrimeric complex, which then forms a homotrimer, or trimer of trimers (8). The Ii performs at least three critical functions in class II Ag processing and presentation: it aids in the assembly of the class II complex; it provides a signal sequence, targeting the class II/Ii complex through the Golgi apparatus to the endocytic pathway; and a portion of Ii, the class II-associated invariant chain peptide (CLIP) occupies the class II peptide-binding groove, preventing the binding of ER-resident peptides. Within endocytic and/or lysosomal compartments, the Ii is proteolytically degraded in a stepwise fashion by cathepsins (9), until only CLIP remains associated in the peptide-binding groove of the class II αβ heterodimer (10). DM colocalizes with the class II/CLIP complexes in the endocytic compartment, where it catalyzes the removal of the CLIP peptide from the class II MHC peptide-binding groove and enables the binding of antigenic peptides (11–13). DM also functions as a peptide editor, removing peptides with high off-rates and preferentially allowing the binding of high affinity peptides to class II complexes (14). Upon binding antigenic peptides, the class II/peptide complex traffics to the APC surface where CD4⁺ T cells can recognize the complex and be activated. Loss of expression of Ii or DM results in profound defects in Ag processing and presentation, class II expression, and CD4⁺ T cell development (12, 15–17).
The expression of all of these proteins; class II, Ii, and DM is regulated by the class II transcriptional activator (CIITA) (18–21). The CIITA is responsible for the constitutive expression of class II Ag processing proteins in “professional” APCs as well as the IFN-γ-inducible expression of class II, Ii, and DM in “nonprofessional” APCs. Mice deficient in CIITA have profound defects in class II, Ii, and DM expression (20, 22, 23) (although the effect on Ii expression is less severe) and substantial decreases in CD4+ T cells due to the absence of class II expression and defective thymic selection.

It is not clear which of a variety of candidate professional (e.g., macrophages, dendritic cells, and B cells) and nonprofessional (e.g., endothelial cells, microglia, and astrocytes) APCs present in the CNS during T cell-mediated demyelination are involved in presentation of myelin Ags to autoreactive CD4+ T cells active in disease initiation or relapses. In vivo studies using radiation bone marrow chimeras have demonstrated potential roles for both infiltrating macrophages, resident microglia, and CNS parenchymal cells in processing and presenting encephalitogenic Ags to CD4+ T cells (24–27). Each of these cell populations express different levels of CIITA, class II, Ii, and DM, and the expression of these proteins can change with the activation of the different cell populations (28, 29). Thus, there is significant interest in the expression requirements of the class II Ag processing and presentation proteins and their impact on activation of encephalitogenic CD4+ T cell populations. Thus, we investigated the role of these proteins in Ag presentation and myelin-specific CD4+ T cell activation in the context of CNS disease in vivo, using mice deficient in the expression of Ii, DM, and, via the CIITA knockout (KO) mouse, deficient in Ii, DM, and class II. Mice deficient in CIITA, Ii, or DM are resistant to initiation of EAE by both active priming and adoptive transfer of wild-type (wt) myelin oligodendrocyte protein, MOG35–55-specific encephalitogenic T cells. Although both Ii- and DM-deficient APCs can present MOG peptide to CD4+ T cells, neither is capable of processing and presenting the encephalitogenic peptide of intact MOG protein. Remarkably, DM-deficient mice can prime a potent, peripheral Th1 response to MOG35–55, comparable to the response seen in wt mice, yet maintain resistance to EAE initiation and can adoptively transfer EAE to wt, but not DM-deficient, mice. Together, these data demonstrate that the inability to process antigenic peptide from intact myelin protein results in resistance to EAE and de novo processing and presentation of myelin Ags in the CNS is absolutely required for the initiation of autoimmune demyelinating disease.

Materials and Methods

Mice

C57BL/6 female mice, 5–6 wk old, were purchased from The Jackson Laboratory (Bar Harbor, ME). CIITA-, DM-, and Ii-deficient mice are described elsewhere (12, 16, 20). CIITA-deficient mice have been backcrossed onto the C57BL/6 background six generations. DM- and Ii-deficient mice have been backcrossed onto the C57BL/6 background 10 and 13 generations, respectively. Mutant mice were bred by homozygous brother-sister matings and all mice were housed in barrier conditions with the Center for Comparative Medicine at Northwestern University (Chicago, IL). Mice were maintained on standard laboratory food and water ad libitum. Paralyzed animals were afforded easier access to food and water.

Abs, peptides, and protein

Hybridomas producing the anti-class I and anti-class II Abs (M1/42 and M5/114, respectively) were purchased from American Type Culture Collection (Manassas, VA) and cultured in DMEM 10% FCS. Supernatants were tested for Ab, filtered, and sterilized before storage. MOG35–55 (MEVGWYRSPFSRVHLYRNGK) was purchased from Genemed Synthesis (San Francisco, CA). PLP178 isotype Ab (NTWTTCQ5IAFSK) was purchased from Peptides International (Cleveland, OH). Amino acid composition was verified by mass spectrometry and purity (>98%) was assessed by HPLC. rMOG, consisting of the extracellular portion of MOG (aa 1–125) expressed in, and purified from Escherichia coli, was the generous gift of Dr. M. Gardinier (University of Iowa College of Medicine, Iowa City, IA).

Induction and clinical evaluation of peptide-induced EAE

For MOG35–55-induced EAE, 6- to 7-wk-old female mice were immunized s.c. with 200 μl of an emulsion containing 800 μg of Mycobacterium tuberculosis H37Ra (Difco, Detroit, MI) and 200 μg MOG35–55 distributed over three spots on the flank. Each mouse additionally received 200 ng pertussis toxin (PTx) (List Biological Laboratories, Campbell, CA) in 200 μl PBS i.p. on days 0 and 2 postimmunization. Animals that were immunized twice received the same immunization 7 days after the first immunization, without PTx. For PLP178/H9253-induced EAE, 6- to 7-wk-old female mice were immunized s.c. with 200 μl of an emulsion containing 800 μg of M. tuberculosis H37Ra and 50 μg PLP178/H9253 distributed over three spots on the flank. Each mouse additionally received 200 ng PTx in 200 μl PBS i.p. on days 0 and 2 postimmunization. Individual animals were observed daily and clinical scores assessed in a blinded fashion on a 0–5 scale as follows: 0 = no abnormality, 1 = limp tail, 2 = limp tail and hind limb weakness (legs slip through cage top), 3 = hind limb paralysis, 4 = hind limb paralysis and forelimb weakness, and 5 = moribund. The data are reported as the mean daily clinical score ± SEM for all animals in a particular group and/or as the mean peak clinical score ± SEM, i.e., the mean clinical score for all animals at the peak of disease. Unless otherwise mentioned, all mice were age and sex-matched for all experiments.

Initiation of EAE by adoptive transfer

Female donor mice (6- to 10-wk-old) were immunized i.p. with 200 μl of an emulsion containing 800 μg of M. tuberculosis H37Ra and 200 μg MOG35–55 distributed over three spots on the flank. Draining lymph nodes (LN) were harvested from donor mice after 7–11 days for in vitro stimulation. LN cells were cultured (10 × 106 cells/ml) in DMEM containing 10% FBS, 1 mM glutamine, 1 mM penicillin-streptomycin, 1 mM nonessential amino acids and 5 × 10–5 M 2-mercaptoethanol (D-ME; D-10; all products from Sigma-Aldrich, St. Louis, MO) with MOG35–55 peptide (30 μg/ml) and human IL-12 (20 ng/ml; R&D Systems, Minneapolis, MN). After 72 h incubation, cells were counted, washed, and resuspended (2.5 × 107 T cell blasts/ml; 10–15 × 106 LN cells/ml) in buffered salt solution. T cell blasts were differentiated from other LN cells by size under microscopic observation. On day 0, 6- to 7-wk-old female B6 or KO mice were injected i.p. with 5 × 107 T cell blasts/mouse (in 200 μl PBS i.p. on days 0 and 2. Individual animals were injected i.p. on the day of adoptive transfer with 200 μl PBS i.p. on days 0 and 2. Individual animals were injected i.p. 7 days after the adoptive transfer with 200 ng PTX in 200 μl PBS i.p. to control for background disease activity. clinical scores were as assessed above. For adoptive transfer of T cells from DM KO mice, donors and LN cells were prepared as described above, except that donors were primed with MOG35–55 on both days 0 and 7 and donors were sacrificed at day 14 postintrinsic priming.

Elicitation of delayed-type hypersensitivity (DTH) responses

DTH responses were measured using a 24-h ear-swelling assay. Prechallenge ear thickness was determined using a Mitutoyo model 7326 engineer’s micrometer (Schlesinger’s Tool, Brooklyn, NY). Immediately thereafter, mice were ear-challenged by injecting 10 μg of peptide (in 10 μl of saline) into the dorsal surface of the ear using a 100 μl syringe fitted with a 30-gauge needle. The increase in ear thickness was determined 24 h after ear challenge. Results are expressed in units of 10–4 inches ± SEM. Significance of ear swelling in experimental over naive mice was assessed by the Student’s t test.

Ag presentation assay

Spleens were collected from naive, C57BL/6 wt, or KO mice as indicated. RBCs were removed by hypotonic lysis and the remaining cells were used as APCs. The APCs were irradiated (3500 rad), washed, and cultured in 96-well microtiter plates at a density of 5 × 105 cells/well. Varying concentrations of MOG35–55, MOG141–191, or rMOG were added to the wells, and T cells were added as APCs. A MOG35–55-specific T cell line (103 cells/well) was cocultured with the APCs and Ags in a total volume of 200 μl D-10. Cocultures were incubated for 96 h, being pulsed with 1 μCi/well [3H]TdR for the final 24 h of the 96-h incubation period. [3H]TdT uptake was detected using a Topcount microplate scintillation counter (Packard Instrument, Meriden, CT) and results are expressed as the mean of triplicate cultures ± SEM. The long-term, MOG35–55-specific T cell line was derived from a C57BL/6 mouse primed with MOG35–55/CTA as previously described (28). In brief, draining LN cells were isolated and restimulated in D-10 for 4 days with
peptide, and rested in D10 plus human rIL-2 (2 U/ml; Roche, Indianapolis, IN) for a minimum of 2 wk. Peptide-specific restimulation and rest were repeated every 14–35 days.

**In vitro proliferation assays**

Draining LN were harvested from primed mice, counted, and cultured in 96-well microtiter plates at a density of 5 × 10^4 cells/well in a total volume of 200 μl HL-1 medium (BioWhittaker, Walkersville, MD; 1% penicillin/streptavidin, 1% glutamine). Cells were cultured with medium alone or different concentrations of peptide Ag for 72 h. Culture wells were pulsed with 1 μCi/well [3H]Tdr for the final 20 h of the 72-h incubation period. [3H]Tdr uptake was detected using a Topcount microplate scintillation counter and results are expressed as the mean of triplicate cultures ± SEM.

**ELISPOT assays**

Nitrocellulose-coated, 96-well flat-bottom microculture plates (Whatman, Clifton NJ) were precoated overnight at 4°C with 100 μl of anti-IFN-γ, anti-IL-4, or anti-IL-2 (R46A2 and 11B11 at 4°C, respectively) purchased from BD Pharmingen (San Diego, CA). Plates were washed four times with sterile PBS and wells were blocked with 200 μl sterile DMEM 1% BSA for 1 h at room temperature. LN cells (5 × 10^5) or 10^6 spleen cells were cocultured with Ag at varying concentrations in HL-1 medium (1% penicillin/streptavidin, 1% glutamine). Cultures were incubated at 37°C for 36 h. In mAb coculture experiments, Ab supernatants were diluted 1/5 for a final volume of 200 μl/well. Plates were subsequently washed three times with PBS and three times with PBS/0.05% Tween20 (PBS/Tween). Biotinylated anti-IFN-γ, anti-IL-2, or anti-IL-2 (XM1G1.2, BV6D-24G2, and JES6-5H4, respectively) at 2 μg/well diluted in PBS/Tween1/5% BSA, were added at 100 μl/well and incubated overnight at 4°C in a humidified chamber. Plates were washed four times with PBS/Tween and incubated for 2 h at room temp with 100 μl/well anti-biotin alkaline phosphatase (Vector Laboratories, Burlingame, CA) diluted 1/1000 in PBS/Tween1/5% BSA. Finally, plates were washed with PBS and developed in nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate substrate solution (Pierce, Rockford, IL). The developing reaction was quenched after 30–45 min using distilled water. ELISPOTs were counted using the ImmunoSpot series 1.0 analyzer (Resolution Technology, Cleveland, OH). Samples were set up in triplicate and anti-CD3 mAb were included as a positive control for each group.

**Immunohistochemistry**

Mice were anesthetized and perfused with 1× PBS on day 20 postimmunization or day 31 postadoptive transfer. Spinal cords were removed by dissection, and 2- to 3-mm spinal cord blocks were immediately frozen in OCT (Miles Laboratories; Elkhart, IN) in liquid nitrogen. The blocks were stored at −80°C in plastic bags to prevent dehydration. Six micrometer thick cross-sections from the lumbar region (approximately L2-L3) were cut on a Reichert-Jung Cyocut CM1850 cryotome (Leica, Deerfield, IL), mounted on Superfrost Plus electrostatically charged slides (Fisher, Pittsburgh, PA), air dried, and stored at −80°C. Slides were stained using a Tyramide Signal Amplification (TSA) Direct kit (NEL, Boston, MA) according to manufacturer’s instructions. Lumbar sections from each group were thawed, air-dried, fixed in 2% parafomaldehyde at room temperature, and rehydrated in 1× PBS. Nonspecific staining was blocked using anti-CD16/CD32, (FcγRIIIIR, 2.4G2; BD Pharmingen), and an avidin/biotin blocking kit (Vector Laboratories) in addition to the blocking reagent provided by the TSA kit. Tissues were stained with biotin-conjugated Abs anti-mouse CD4 (H129.19) and anti-mouse I-Ab (AF6-120.1) (BD Pharmingen). Sections were counterstained with 4’,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) and then coverslipped with Vectashield mounting medium (Vector Laboratories). Slides were examined and images were acquired via epifluorescence using the SPOT RT camera (Diagnostic Instruments, Sterling Heights, MI) and Metamorph imaging software (Universal Imaging, Downingtown, PA). Eight serial lumbar sections from each sample per group were analyzed at ×100 and ×400 magnification.

**Histologic evaluation**

Mice were anesthetized and sacrificed by total body perfusion through the left ventricle using chilled 3% glutaraldehyde in PBS (pH 7.3). Spinal cords were dissected out and cut into 1 mm thick segments and postfix in OsO_4_, dehydrated, and embedded in Epon. Toulidine blue stained sections from 10 segments/mouse were read and scored as follows: ± = mild inflammation without demyelination; 1+ = inflammation with focal demyelination; 2+ = inflammation with multiple foci of demyelination; 3+ = marked inflammation with bilateral, converging areas of demyelination; 4+ = extensive bilateral areas of demyelination and remyelination.

**Results**

CIITA-deficient mice can prime Th1 responses, but are resistant to initiation of EAE

Although the role of class II in the priming of myelin-specific T cells has been addressed using anti-MHC class II blocking Abs, susceptibility to EAE had not been studied with mice genetically deficient in class II expression. To investigate the possibility of nonclass II-restricted mechanisms for initiation of EAE, we tested CIITA KO mice for susceptibility to initiation of EAE by active priming and adoptive transfer of encephalitogenic T cells. Age- and sex-matched C57BL/6 wt and CIITA KO mice were primed with MOG<sub>35-55</sub>/CFA and observed for clinical symptoms as described in Materials and Methods. Although 100% of wt mice were susceptible to disease, none of the CIITA KO mice showed any clinical symptoms (Fig. 1a). In addition to defective class II expression, CIITA KO mice have a radically altered CD4<sup>+</sup> T cell repertoire (20, 22, 23) and might not have CD4<sup>+</sup> T cells capable of recognizing the MOG<sub>35-55</sub> epitope. Surprisingly, when MOG<sub>35-55</sub>-primed mice were tested for 24-h DTH responses, CIITA KO mice displayed measurable Ag-specific responses (Fig. 1c), albeit significantly reduced from those seen in wt mice. Analysis of the LN responses from MOG<sub>35-55</sub>-primed wt and CIITA KO mice 8 days postpriming by ELISPOT showed that the T cells produced the Th1 cytokines IL-2 and IFN-γ (Fig. 2, a and b, respectively), although the frequency of cytokine-producing LN cells from CIITA KO mice was lower and required higher Ag doses for activation than LN cells from wt mice. The IL-2- and IFN-γ-producing cells from CIITA KO mice were not MOG-specific CD8<sup>+</sup> T cell responses as coculture with anti-class I mAb

**FIGURE 1.** CIITA-deficient mice are resistant to initiation of EAE by active priming and adoptive transfer, but can prime/maintain peripheral Th1 responses in vivo. C57BL/6 wt (five mice per group) and CIITA KO mice (five mice per group) were primed with MOG<sub>35-55</sub> (a) or had MOG<sub>35-55</sub>-specific T cells adoptively transferred (b) i.p. to initiate disease. Mice were scored for clinical symptoms as described in Materials and Methods. On day 37, peripheral Th1 responses were measured by DTH in primed (c) and adoptively transferred (d) groups (*, p < 0.003 by Student’s t test).
FIGURE 2. CIITA-deficient mice can prime MOG35–55-specific Th1 responses. C57BL/6 wt or CIITA KO mice were primed with MOG35–55 in CFA without PTx. After 8 days, LN (a and b) or spleen (c and d) cells were isolated and assayed for production of MOG-specific cytokine production by ELISPOT. In a separate experiment, C57BL/6 wt or CIITA KO mice were primed with MOG35–55 in CFA and after 13 days, LN cells were isolated and cocultured with 10 μM MOG35–55 and anti-class I or anti-class II Ab supernatants. Cultures were assayed by ELISPOT for production of IFN-γ (e) or IL-2- (f) producing cells. The number of Th1 (g, IFN-γ) and Th2 (h, IL-4) cytokine-producing cells were also measured in LN and spleen cultures by ELISPOT analysis. Data is presented as the increase in number of spots above that seen in wells without Ag.
(M1/42) did not block cytokine production, while addition of anti-class II mAb (M5/114) completely abrogated Th1 cytokine production (Fig. 2, e and f). The lack of cytokine production with the addition of M5/114 was not due to nonspecific cytotoxicity, as cultures containing anti-CD3 mAb were unaffected by the addition of the anti-class II mAb (data not shown). Splenic Th1 responses were also reduced in CIITA KO mice compared with wt mice (Fig. 2, c and d), suggesting that residual class II MHC was present in both the spleen and LN of CIITA KO mice. The lack of disease in CIITA KO mice could potentially be due to a switch from IFN-γ production to IL-4 production by CD4+ T cells, resulting in an inhibition of the inflammatory response. To address this possibility, we compared IFN-γ and IL-4 responses from the LN and spleen of MOG35-55–primed wt and CIITA KO mice. There was little difference in the numbers of IFN-γ and IL-4–producing cells between wt and CIITA KO mice 13 days postpriming (Fig. 2, g and h).

To investigate the possibility that the failure to induce active disease in CIITA KO mice may be due to defective presentation of myelin peptides in the CNS, we determined the ability of MOG35-55–specific T cell blasts from wt B6 donors to adoptively transfer disease into wt vs CIITA KO mice. Similar to active disease, 100% of wt mice developed clinical EAE by adoptive transfer, while none of the CIITA mice were affected (Fig. 1b). Both wt and CIITA KO mice recipients showed significant DTH responses (Fig. 1d). This recall response in the CIITA KO mice eliminated the possibility that T cells primed by and restricted to wt IAβ were incapable of recognizing MOG peptide presented by the residual class II present in the CIITA KO mice.

**Priming of a peripheral Th1 response is insufficient for the initiation of EAE in Ii- and DM-deficient mice**

CITTA mutant mice have dramatically reduced levels of class II, Ii, and DM (20, 22, 23). Although the CIITA KO mice could present antigenic peptide to MOG–specific T cells, the possibility remained that due to the reduction of critical accessory molecules, the APCs could not process protein Ags. Thus, mice deficient in Ii or DM expression were tested for their susceptibility to initiation of EAE. Age and sex-matched wt C57BL/6, Ii KO, and DM KO mice were primed with MOG35-55/CFA and followed for disease as described in Materials and Methods. Neither Ii-deficient nor DM-deficient mice showed any clinical signs of disease (Fig. 3a), while 100% of wt mice got sick. wt mice had a mean peak clinical score of 2.9 ± 0.5 and histological analysis showed massive infiltration (Fig. 4a) and severe demyelination (Fig. 3c, data not shown). When tested for in vivo Th1 responses by DTH, Ii-deficient mice had no response, while, similar to CIITA-deficient mice, DM KO mice had reduced, but significant (p < 0.0002) MOG-specific ear swelling (Fig. 5a).

Both DM KO and Ii KO mice have significantly altered CD4+ T cell repertoires (12, 15–17, 30–33). Although DM KO mice could prime a MOG35-55–specific T cell response and elicit Th1 effector function (i.e., DTH), the Ii-deficient mice could not. Although DM- and Ii-deficient mice have diminished Ag processing and presentation in vivo, APCs from both can function in vitro to present peptide to CD4+ T cells (16, 34). To confirm our in vivo data, we tested LN cells from MOG35-55–primed wt, Ii-, and DM-deficient mice for Th1 recall responses by ELISPOT. LN cells from MOG35-55–primed DM and Ii KO mice produced IL-2 and IFN-γ (Fig. 6, a and c, respectively). In all cases, the frequency of Th1 cytokine-producing cells was less in the KO mice than in wt mice. Strikingly, even though the Ii-deficient mice did not have a MOG35-55–specific DTH response (Fig. 5a), their frequency of Th1 recall responses in vitro was greater than that seen in DM-deficient mice (Fig. 6, a and c).

As in the CIITA KO mice, while DM KO and Ii KO mice could prime attenuated MOG35-55–specific Th1 responses, the possibility remained that the response was below an encephalitogenic threshold. Alternatively, the class II expressed in the mutant APCs could be priming a repertoire of MOG-peptide–specific T cells, which could not recognize MOG peptide processed from the CNS (30). To rule out the possibility of a T cell defect, wt MOG35-55–specific T cells were adoptively transferred into C57BL/6 wt, Ii KO, and DM KO mice. Once again, 100% of wt mice developed EAE, while DM- and Ii-deficient mice remained healthy (Fig. 3, b and c). Upon measure of Th1 responses, mice from every adoptively transferred group, including Ii KO mice, elicited significant DTH responses (Fig. 5b), although the responses in the DM KO and Ii KO mice were slightly and dramatically reduced as compared with the wt response, respectively. Surprisingly, when analyzed for

**FIGURE 3.** Both Ii- and DM-deficient mice are resistant to initiation of MOG35-55–specific EAE by active priming and adoptive transfer. C57BL/6 wt, Ii KO, and DM KO mice were primed with MOG35-55 in CFA (a) or had MOG35-55–specific T cells adoptively transferred (b) i.p. to initiate disease as described in Materials and Methods. Mice were scored for clinical symptoms as described, disease parameters assessed, and in some experiments, the CNS analyzed histologically for inflammation and demyelination (c). In no experiment did Ii KO or DM KO mice display any disease symptoms. In some experiments, mice were observed out to 70 days postpriming/transfer without any sign of disease.
their ability to drive Th1 recall responses in vitro, LN cells from Ii-deficient animals showed no responsiveness (Fig. 6, b and d). LN cells from DM KO mice, in contrast, drove a high frequency of IL-2- and IFN-γ-producing T cells, although reduced from the responses seen in wt mice.

DM- and Ii-deficient mice have no CNS infiltration, inflammation, or demyelination after MOG₃₅₋₅₅-priming or adoptive transfer of MOG-specific T cell blasts

DM-deficient mice can both prime and recall significant Th1 responses (Figs. 5 and 6), yet fail to show any overt symptoms of EAE. Although adoptive transfer of wt encephalitogenic T cell blasts failed to initiate disease in DM KO mice, the possibility remained that there was a subclinical disease that exhibited no characteristic motor defects. To eliminate this possibility, MOG₃₅₋₅₅-primed or adoptively transferred wt, Ii KO, and DM KO mice were perfused and their spinal cords were analyzed histologically for CNS infiltration and inflammation as described in Materials and Methods. Although MOG-primed wt mice showed severe signs of inflammation and demyelination (Fig. 3c), Ii KO and DM KO mice showed no clinical pathology. Immunohistochemical analysis showed that spinal cords from wt mice either MOG₃₅₋₅₅-primed or adoptively transferred with MOG₃₅₋₅₅-specific T cell blasts showed extensive infiltration of CD4⁺ T cells mostly in the white matter with a lesser extent of staining in the gray matter (a, c, e, and g). In contrast, little CD4 or class II (green) positive cells were present in the spinal cords of DM-deficient mice (b, d, f, and h). No positive staining was observed in isotype-matched controls and in naive spinal cord tissue stained with CD4⁻ and MHC II-specific Abs (data not shown; ×100 magnification).

FIGURE 4. Immunohistochemical staining for T cells (CD4) and class II MHC (MHC II) in spinal cords of C57BL/6 wt and DM-deficient mice in MOG₃₅₋₅₅-specific EAE. Spinal cord tissues from day 20 postpriming (a–d) and from day 31 postadoptive transfer (e–h) were examined for the presence of CD4-positive T cells and class II (IAb) expression. Tissues were also counterstained with 4',6'-diamidino-2-phenylindole (blue). In both active and adoptively transferred EAE, C57BL/6 wt spinal cord tissues contained numerous CD4⁻ (red) and class II⁻ (green) positive cells mostly in the white matter with a lesser extent of staining in the gray matter (a, c, e, and g). In contrast, little CD4 or class II staining was present in the spinal cords of DM-deficient mice (b, d, f, and h). No positive staining was observed in isotype-matched controls and in naive spinal cord tissue stained with CD4⁻ and MHC II-specific Abs (data not shown; ×100 magnification).
DM- and Ii-deficient APCs cannot process and present the MOG35-55 epitope from intact MOG protein

The preponderance of data supported the hypothesis that DM KO mice, and to a lesser extent, Ii KO mice do not have defective MOG35-55-specific T cell responses. Both mutant mice could prime Th1 responses and present peptide for recall responses, yet failed to show any clinical signs of EAE. Indeed, the DM KO mice could prime and elicit a significant peripheral Th1 recall response to peptide in vivo. Thus, with the DM KO mice in particular, the defect appeared to be at the level of Ag presentation in the target organ. The Ii-deficient mice had a more profound defect in vivo; failing to elicit peripheral T cell responses, suggesting a broader defect than that seen in DM-deficient mice. Both Ii and DM deficient mice have defects in the processing of intact protein Ags, but the level of deficiency can be Ag-specific (35–37). To assess the ability of the mutant APCs to present intact MOG, splenocytes from wt, Ii KO, and DM KO mice were isolated and assessed for their ability to present peptide vs recombinant protein to a MOG35-55-specific T cell line. Although all of the APCs could present the MOG35-55 peptide and stimulate the line to proliferate (Fig. 7a), only the wt APCs could process and present the rMOG protein (Fig. 7b). Thus, resistance to EAE initiation in DM KO mice appeared to be at the level of protein Ag processing and presentation.

Resistance to EAE initiation in DM- and Ii-deficient mice is not Ag-specific

DM KO and Ii KO mice can differentially present peptide Ags, in an Ag-dependent manner (35–37). Although unlikely, it was possible that the defect in EAE initiation in the mutant mice could be specific to CNS processing and presentation of the MOG35-55 peptide. We thus tested the ability of a peptide of proteolipid protein, PLP178–191, to initiate disease in C57BL/6 mice. Similar to MOG35-55-induced disease, wt mice developed clinical disease with 100% incidence, while DM- and Ii-deficient mice failed to show any signs of disease (Fig. 8a). Immunohistochemical analysis of spinal cords from PLP178–191-primed mice showed significant CD4+ T cell infiltration and Iaα and F4/80 expression in wt tissues, while DM KO and Ii KO spinal cords showed little evidence of pathology (data not shown). Interestingly, when measuring Th1 recall responses in vivo, both DM- and Ii-deficient mice had significant PLP178–191 reactivities (Fig. 8b), although the response in Ii KO mice was not as significant as the response elicited in DM KO mice (p < 0.02 and p < 0.005, respectively). Ex vivo, LN cells from PLP178–191-primed Ii KO and DM KO mice proliferated to specific peptides in a dose-dependent fashion, albeit at reduced levels compared with wt LN cells (Fig. 8c). Thus, the phenotype of peripheral Th1 responsiveness upon myelin peptide immunization without initiation of clinical disease in DM- and Ii-deficient mice does not appear to be an Ag-specific phenomenon.

DM-deficient mice can prime potent encephalitogenic T cell responses

DM KO mice displayed the ability to prime significant peripheral MOG35-55-specific T cell responses without initiating EAE. This data, in conjunction with their inability to process rMOG protein

FIGURE 5. DM-deficient mice can prime and recall a peripheral, MOG35-55-specific Th1 response in vivo. C57BL/6 wt, Ii KO, and DM KO mice were primed with MOG35-55 in CFA (a) or had MOG35-55-specific T cells adoptively transferred (b) i.p. to initiate disease as described in Materials and Methods. On day 15 postpriming or day 24 postadoptive transfer, MOG35-55-specific peripheral Th1 responses from four to five mice per group were determined by DTH (*, p < 0.0002; **, p < 0.004).

FIGURE 6. Both Ii- and DM-deficient mice can prime MOG35-55-specific Th1 responses. C57BL/6 wt, Ii KO, and DM KO mice were primed with MOG35-55 in CFA (a and c) or had MOG35-55-specific T cells adoptively transferred i.p. (b and d) to initiate disease as described in Materials and Methods. On day 28 postpriming or day 30 postadoptive transfer, mice were sacrificed and LN cells were assayed for MOG35-55-specific Th1 responses by ELISPOT. (*, Cells from MOG35-55-primed wt mice responded vigorously to the highest Ag dose (100 μM peptide); the spots were confluent within each well and impossible to count.)
and the failure to initiate disease following adoptive transfer of wt encephalitogenic T cells, supported the hypothesis that the failure to initiate EAE in the DM mutant is the result of a failure to process CNS protein Ags. However, there remained a slight possibility that the wt T cells transferred into the KO mice were incapable of recognizing CNS Ags processed and presented by DM KO APCs and that the peptide-primed T cell population in DM KO mice was not potent enough to initiate clinical EAE. To address this concern, we primed C57BL/6 wt and DM-deficient mice with MOG35-55/CFA two times, 7 days apart, and compared their Th1 responses in vivo and in vitro to mice primed only once. Once again, DM KO mice were resistant to disease initiation, whether primed one or two times, while all of the wt mice developed EAE (Fig. 9b). Significantly, the DTH response in DM KO mice primed twice was equivalent to the response in wt mice primed once (Fig. 9b), suggesting that the magnitude of Th1 responses in the two groups was identical. ELISPOT analysis of LN cells from mice primed one or twice showed similar results, with DM KO mice primed twice displaying similar frequencies of IL-2- and IFN-γ-producing T cells as seen in wt mice primed once (data not shown). This suggested that unless there was a previously undescribed defect in T cells from DM-deficient mice, these T cells should have encephalitogenic potential. To test this hypothesis, we used DM KO mice primed twice as adoptive transfer donors. All of the wt recipients of MOG35-55-specific DM KO T cell blasts showed significant disease, while none of the DM KO recipients had any clinical signs (Fig. 9c), demonstrating that T cells from DM KO mice were encephalitogenic.

Discussion

The critical role of CD4+ T cells in the pathogenesis of MS and EAE has been clearly described. Indeed, the work of Ben Nun and colleagues (38, 39) demonstrating a lack of cross-reactivity of MOG-specific T cells from IAb and IAbm12 congenic mice demonstrates that the class II locus is absolutely critical for MOG-specific autoimmune T cell responses. Additionally, treatment with anti-IA Abs had been shown to inhibit EAE and a variety of other autoimmune diseases (7). Yet, the importance of different APC populations and the requirement for processing and presentation of CNS protein Ags remains controversial. Numerous studies have demonstrated differential capacities of CNS-resident APC populations to up-regulate the expression of the Ag-processing machinery and/or costimulatory molecules (24, 40–52) and activate myelin-specific CD4+ T cells. We have previously shown that following IFN-γ treatment, astrocytes from SJL/J mice up-regulate the expression of the CIITA, class II, Ii, and DM, as well as a variety of costimulatory molecules (28). Additionally, after IFN-γ exposure, the SJL/J astrocytes could process the immunodominant PLP epitope, PLP139–151, from intact protein and present it to encephalitogenic CD4+ T cells (28).

The CIITA has been described as the master switch for both constitutive and IFN-γ-induced class II expression. Findings from three distinct CIITA-deficient mice have demonstrated a profound, but not total, loss of class II (and accessory molecule) expression (20, 22, 23). Functional studies have shown that CD4+ T cells from CIITA-deficient mice cannot elicit a class II-restricted allogenic response or prime an anti-keyhole limpet hemocyanin T cell response (22). Mora et al. (53) demonstrated that while NOD mice deficient in CIITA expression did have pancreatic infiltration of CD8+ T cells, B cells, and macrophages, they did not develop diabetes, although an autoaggressive CD8+ T cell clone could initiate a delayed diabetes independent of CIITA function. To test for the requirement of class II in EAE, we attempted to initiate disease in CIITA-deficient mice. Not surprisingly, the CIITA KO mice

![FIGURE 7.](image7) Although both Ii- and DM-deficient APCs can present MOG35-55 peptide, neither can process and present MOG protein in vitro. Irradiated APCs from wt, Ii KO, and DM KO were assessed for their ability to process and present MOG peptide (a) or protein (b) Ag to a MOG35-55-specific T cell line. Cultures were pulsed with [3H]TdR after 72 h and harvested 24 h later as described in Materials and Methods. Data is representative of multiple experiments using distinct MOG-specific long-term T cell lines.

![FIGURE 8.](image8) Ii- and DM-deficient mice can prime PLP178-191-specific T cell responses, but are resistant to initiation of EAE by PLP178-191. C57BL/6 wt, Ii KO, and DM KO mice were primed with PLP178-191 in CFA to initiate disease (a) as described in Materials and Methods. On day 27 postpriming, mice were measured for PLP178-191-specific peripheral Th1 responses by DTH (*, p < 0.005; **, p < 0.02). Ag-specific T cell responses were measured on day 31 postpriming (c). Recall responses of LN cells from PLP178-191–primed wt, Ii KO, and DM KO were measured by in vitro proliferation assay as described in Materials and Methods.
were resistant to initiation of EAE. This defect was not solely due
to a failure in T cell activation, as adoptively transferred syngeneic
encephalitogenic T cells could not initiate disease. Additionally,
resistance to EAE was not the result of a failure in MOG peptide
presentation, as CIITA-deficient mice could present peptide
in vivo and in vitro. Because the CIITA-deficient mice express little
or no class II, there was a possibility that the DTH and IFN-γ
responses measured were class I-restricted. Other disease models
have provided evidence supporting the role of CD8+ T cells in
EAE, e.g., Huseby et al. (54) recently described a role for myelin
basic protein (MBP)-specific CD8+ cytotoxic T cells in the initia-
tion of EAE in C3H (H-2k) mice. In a more similar model, Sun et
al. (55) described a potential role for CD8+ T cells in the initiation
of MOG 35–55-specific EAE in B6 mice. Thus, there was a distinct
possibility that in the CIITA mutant mice, CD8+ T cells were
mediating the MOG-specific immune responses. To address this
possibility, we performed immunohistochemistry on DTH lesions
from MOG 35–55–primed mice. Immunohistochemical analysis of
ears following DTH showed significant CD4+, but not CD8+, T
cell infiltrates in MOG 35–55-challenged ears of both wt and CIITA
KO mice (data not shown), suggesting that the inflammatory
response was not mediated by CD8+ T cell responses. Additionally,
in ELISPOT analysis of MOG 35–55–primed wt and CIITA KO
mice, the inhibition of IL-2 and IFN-γ production by addition of
anti-class II mAb (Fig. 2, e and f) argues that trace levels of class
II were presenting MOG peptide to CD4+ T cells, and that CD8+
T cells were not mediating the inflammatory responses. Moreover,
in vitro MOG 35–55–specific IFN-γ and IL-2 responses were main-
tained using column purified CD4+ T cells (data not shown).
Lastly, both MOG 35–55- and PLP 178–191–specific proliferative
responses from C57BL/6 mice were inhibited by coculture with anti-
class II mAb, while the addition of anti-class I mAb had no effect
(data not shown). These data show that while there are almost
undetectable levels of class II proteins expressed in CIITA KO
mice, that level is sufficient for the priming of some peptide-spe-
cific, peripheral CD4+ T cell responses. This conclusion has
implications regarding the levels of class II required for the initiation
of peptide-induced immune responses and suggests that a broader
population of tissues may function as APCs as long as prerequisite
costimulatory molecules are present in cis or in trans. This in turn
may have important implications in peptide vaccine design. Our
data indicate that MHC class II levels are sufficient for priming a
Th1 peptide response in CIITA-deficient mice, but the mice are not
susceptible to EAE initiation perhaps due to the lack of Ag-pro-
cessing accessory molecules (e.g., Ii and DM) in the CNS or the
inability of nonprofessional APC to express functional class II.
Ii and DM KO mice have significant defects in class II expres-
sion and/or function. In the absence of Ii, class II α- and β-chains
fail to fold properly in the ER and the majority of the class II is
degraded. By associating with ER-resident peptides, a small but
significant population of class II αβ dimers does assemble, and is
shunted directly to the cell surface by the default pathway (16, 17).
Thus, Ii-deficient mice express low levels of class II that are oc-
cupied with peptides easily exchanged for high-affinity peptides at
the cell surface. Loss of DM function, in contrast, does not dra-
matically decrease class II expression. Class II is assembled and
transported to the endocytic compartment for peptide loading, but
without DM, CLIP is not removed from the class II peptide-bind-
groove. The class II αβ/CLIP complex is transported to the
APC surface where exogenous peptides could selectively ex-
change with CLIP and be presented (12, 15, 34, 56). Thus in both
cases, there was the possibility of priming a myelin-specific T cell
response and it was of considerable interest whether the specific
components of the class II Ag-processing machinery were required
for the activation of encephalitogenic T cells in vivo.

Professional APCs are incapable of processing and presenting
the MOG 35–55 determinant from a rMOG protein in the absence
of Ii or DM. Additionally, while the efficiency of peptide presenta-
tion by mutant APCs is decreased at lower Ag doses, both Ii- and
DM-deficient mice can present both MOG 35–55 and PLP 178–191.
Interestingly, the Ii-deficient mice had little response to MOG 35–55
challenge in vivo, yet responded more vigorously to in vitro Ag
restimulation than DM KO LN cells. This likely due to the
enhanced ability of mutant APCs to present peptide Ags in vitro,
which is often more efficient in Ii KO APCs than peptide presen-
tation by wt APCs (16). In vivo, we established that both Ii- and
DM-deficient mice are resistant to initiation of EAE by active
priming with either MOG 35–55 or PLP 178–191. In addition to de-
fective in vivo Ag presentation, DM- and Ii-deficient mice have
additional defects that could potentially contribute to disease re-
stance. Although both mutant mouse strains have altered CD4+ T
cell development (12, 16), the inability to transfer disease to KO
mice using wt MOG 35–55-specific encephalitogenic T cell blasts,
in conjunction with the demonstration of encephalitogenicity of
MOG 35–55-specific T cell blasts from DM-deficient mice when
transferred into wt mice, argues against a T cell defect preventing
disease. Indeed, the ability of DM KO mice to prime a T cell
response that is encephalitogenic in wt mice indicates that, in this
of transferred cells were CD8\(^+\), only CD4\(^+\) green fluorescent protein-positive cells were detected in the CNS of recipient mice (63). Although our results and those of others downplay the role of CD8\(^+\) T cells in our disease model, they do not conflict the work of Huseby et al. (54), as the authors specifically primed the class I Ag-processing pathway with a vaccinia virus expressing MBP to generate the encephalitogenic T cells from C3H.shi (MBP-mutant), H-2\(^b\) mice. In a disease model distinct from MOG-specific EAE in C57BL/6 mice, Huseby and colleagues demonstrate that CD8\(^+\) T cells can mediate encephalomyelitis. Their findings have important implications regarding factors contributing to autoimmune disease and MS in particular, but do not directly impact this study.

Note. After submission of this manuscript for publication, Slavin et al. (64) published a paper entitled “Requirement for endocytic Ag processing and influence of invariant chain and H-2 M deficiencies in CNS autoimmunity.” Similar to the results shown in this study, the authors use li- and DM-deficient mice to demonstrate the requirement of Ag processing in the CNS for the initiation of EAE in C57BL/6 mice by either active priming or adoptive transfer. We are very pleased by the similarities in results, as we strongly support each other’s conclusions.

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References


