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IFN-γ Determines Distinct Clinical Outcomes in Autoimmune Encephalomyelitis[1]

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Experimental autoimmune encephalomyelitis (EAE) is an inflammatory disease of the CNS initiated by autoreactive CD4$^+$ T cells. EAE classically presents with a progressive ascending paralysis and is a model of multiple sclerosis that recapitulates some aspects of the disease. In this report we describe a mouse strain that spontaneously develops a severe, nonclassical form of EAE with 100% incidence. The distinct clinical phenotype is marked initially by a slight head tilt, progressing to a severe head tilt, spinning, or a rotatory motion. Classical EAE spontaneously occurs in myelin basic protein (MBP)-specific TCR transgenic RAG-1$^{-/-}$ mice (referred to as T/R$^-$), whereas nonclassical EAE spontaneously occurs in T/R$^-$ IFN-$\gamma^{-/-}$ mice (T/R$^-\gamma^-$). Thus, the TCR recognizes the same Ag (MBP) and uses identical TCR in both cases. The cellular infiltrate in nonclassical EAE is predominantly found in the brainstem and cerebellum, with very little inflammation in the spinal cord, which is primarily affected in classical disease. Importantly, depending on the genetic makeup and priming conditions of the MBP-specific T cells, nonclassical disease can occur in the presence of an inflammatory infiltrate with eosinophilic, neutrophilic, or monocytic characteristics. Finally, we believe that nonclassical spontaneous EAE could be a useful model for the study of some characteristics of multiple sclerosis not observed in classical EAE, such as the inflammatory responses in the brainstem and cerebellum that can cause vertigo. ***The Journal of Immunology,*** **2005, 174: 1416–1423.**

Experimental autoimmune encephalomyelitis (EAE),[5] an animal model used to study multiple sclerosis (MS), is characterized by inflammation of the CNS mediated by autoreactive T cells specific for various myelin components that include myelin basic protein (MBP), myelin oligodendrocyte glycoprotein, and/or proteolipid protein (PLP) (1, 2). EAE usually manifests itself as an ascending progressive paralysis in a caudal to rostral direction starting with tail paralysis and eventually leading to forelimb paralysis and/or a moribund state in severe cases (classical EAE) (3, 4). The clinical signs of classical EAE correlate strongly with inflammation in the CNS, with a strong spinal cord involvement and limited brainstem and cerebellum inflammation. Interestingly, there have been reports of a nonclassical form of EAE with axial rotatory movement or front leg without hind leg involvement (5–10). Nonclassical EAE has been shown to primarily involve the brain, cerebellum, and/or brainstem, a feature also observed in MS (11).

EAE is primarily thought to be a Th1-mediated disease demonstrated by analysis of inflamed CNS tissue cell types (12–14) and the fact that polyclonal populations and select Th1 lines and clones specific for myelin components can transfer disease (12, 13, 15–19). However, EAE also develops when MBP-specific Th2 cells are adoptively transferred into immunodeficient recipients (18, 20).

The role of the IFN-γ, the prototypic Th1 cytokine, in the pathology of EAE and MS is complex. It is implicated both in the pathology of EAE, evidenced by the Th1 nature of CNS infiltrates and their adoptively transferred encephalitogenic potential, as well as in a protective role (21–25). In fact, IFN-γ disruption in susceptible and nonsusceptible strains can lead to a more severe form of EAE, characterized by differential chemokine expression patterns, increased encephalitogenic T cell proliferation, and the accumulation of polymorphonuclear infiltrate in the parenchyma of the CNS (22–28). This still does not eliminate IFN-γ as an essential factor for the initiation of disease in more physiological conditions where the initial triggering event is more subtle. That is, the overwhelming activation events in induction experiments with Ag, adjuvant, and/or pertussis toxin may bypass the necessity for IFN-γ to initiate disease, whereas IFN-γ may play a more important role in spontaneous EAE or the initiation of multiple sclerosis.

Using MBP-specific TCR transgenic mice, we have recently demonstrated that MBP-specific T cells that underwent an initial in vitro treatment with a neutralizing anti-IFN-γ Ab adoptively transferred a severe form of nonclassical EAE (20). Splenocytes from transgenic mice generated with IL-4 treatment alone (Th2) or IL-4 plus anti-IFN-γ (Th2Hi5) on day 0 of in vitro cell culture expressed prototypical Th2 cytokines, with IL-5 being reproducibly up-regulated 2- to 3-fold in Th2Hi5 cells vs Th2 cells. Both Th2-type cells induced disease, but the Th2Hi5 cells transferred a nonclassical or rotatory form of EAE in which the inflammatory site is

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[5] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; LFB, Luxol Fast Blue; MS, multiple sclerosis; MBP, myelin basic protein; MBP Ac_{1-17}, *N*-acetylated MBP peptide; PLP, proteolipid protein.

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skewed to a brainstem and cerebellum localization. In contrast, Th2 cells induced classical EAE with a spinal cord-centered infiltrate (20).

In this study we analyzed the role of IFN-γ in a spontaneous EAE model (29). We show that IFN-γ disruption in an MBP-specific TCR transgenic mouse system crossed into the RAG-1 deficient background results in a severe nonclassical form of spontaneous EAE characterized, most strikingly, by intense eosinophilia localized predominantly in the brainstem and cerebellum. This phenotype has nearly 100% penetrance and is indistinguishable from Th2Hi5 cell-transferred disease (20). Additionally, $T/R^-\gamma^-$ mice crossed to IL-5 gene-disrupted mice ($T/R^-\gamma^-5^-$) maintained the nonclassical clinical progression, with similar kinetics and severity, with the absence of eosinophilia observed in $T/R^-\gamma^-$ mice, indicating that IL-5 and the eosinophilia are not necessary for nonclassical disease development. Finally, transgenic T cells from IFN-$\gamma^{-/-}$ donors treated with Th1-polarizing IL-12 and anti-IL-4 Ab produce negligible levels of the Th2 cytokines IL-4, IL-5, IL-9, IL-10, and IL-13, yet still transfer nonclassical disease. Infiltrates in the cerebellum and brainstem from these afflicted mice have a much reduced granulocytic nature. Given the fact that, depending on the type of T cell injected, nonclassical EAE is associated with eosinophil, neutrophil, or mononuclear brainstem and cerebellum infiltrates, we conclude that the location of the infiltrate, not its cellular makeup, determines the clinical outcome.

Materials and Methods

Mice

The establishment of *N*-acetylated MBP peptide (MBP Ac_{1-11})-specific TCR transgenic mice has been described previously as well as the MBP-specific TCR transgenic mice with a disrupted RAG1 gene (29). In brief, the mice were made by injection of C57BL/6 zygotes with subsequent backcrossing with C57BL/10.PL (The Jackson Laboratory) to incorporate the $I.A^u$ restriction element. TCR$\beta^{-/-}$ (30), IFN-$\gamma^{-/-}$ (31), and IL-$5^{-/-}$ (32) backcrossed to C57BL/6 were purchased from The Jackson Laboratory, and subsequently H-2^u MHC was incorporated in homozygosity through crosses with C57BL/10.PL mice. Mice were kept under specific pathogen-free conditions in individually ventilated cages (Thoren) at the Skirball Institute Central Animal Facility, New York University Medical Center. All protocols involving mice handling were approved by New York University's Institutional and animal care use committee.

CNS tissue leukocyte isolation

This was performed as previously described (29). Briefly, mice were anesthetized with a mixture containing ketamine, xylazine, and acepromazine maleate (Fort Dodge) and perfused through the left ventricle of the heart with PBS and 5 mM EDTA. CNS tissue was harvested and treated with 10 mg of collagenase D (Roche) in PBS for 45 min at 37°C, followed by a 38% Percoll (Amersham Biosciences) gradient to isolate leukocytes. Isolated cells were subsequently trypan blue-stained and counted or stained with the anti-clonotypic MBP-specific TCR Ab 3H12 (33) conjugated to FITC and anti-CD4-PE (BD Pharmingen) and analyzed by FACS. The brainstem and cerebellum were dissected together, separated from the spinal cord.

Immunopathology

Mice were perfused as described above with the addition of 4% paraformaldehyde to the perfusion buffer. Tissue was incubated for at least 72 h in 10% buffered formalin phosphate (Fisher Scientific) to fix tissue, embedded, sectioned, and stained with H&E or combined Luxol Fast Blue (LFB)/H&E where indicated. LFB stains myelin in blue, and demyelinated white matter areas lose the blue color.

Generation of MBP-specific Th cells and adoptive transfer

MBP-specific cells were obtained from spleens of MBP-specific, TCR-transgenic, RAG-1^+ (referred to as T/R^+) or $T/R^+ \times$ IFN-$\gamma^{-/-}$ mice on the C57BL/10.PL genetic background. Spleen cells ($\sim 2 \times 10^6$/ml) were cultured in the presence of 5 μM MBP Ac_{1-17} and 100 U/ml IL-12 (BD Pharmingen) plus 10 μg/ml anti IL-4 (BD Pharmingen), IL-4 (100U/ml) only (BD Pharmingen), or IL-4 plus anti-IFN-γ (10 μg/ml) Ab (BD Pharmingen) to generate Th1, Th1γ^-, Th2, and Th2Hi5 cells. Cultures were restimulated 4 days after primary stimulation and subsequently 7 days thereafter with 50 μg/ml mitomycin C-treated syngeneic TCR $\beta^{-/-}$ or IFN-$\gamma^{-/-}$ splenocytes and MBP peptide (5 μM). The concentrations of IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13 (Quantikine IL-13 kit; R&D Systems), and IFN-γ in culture supernatants were determined by ELISA using Ab pairs and the protocol supplied by BD Pharmingen, unless otherwise noted. Cells were counted and injected i.v. at $\sim 5 \times 10^6$ cells/mouse 3 days after the third in vitro stimulation.

Results

MBP transgenic RAG$^{-/-}$ mice with a disrupted IFN-γ gene spontaneously develop a severe, nonclassical form of EAE

Spontaneous EAE arises in 100% of mice with a transgenic TCR specific for the MBP Ac_{1-11} + $I.A^u$ crossed onto the RAG-1-deficient background (T/R^-) as previously reported (29). These mice lack endogenously rearranged TCRs, and it has been shown that EAE can be prevented in these mice by injecting regulatory $CD4^+$ T cells from wild-type mice (33, 34). Spontaneous EAE in T/R^- mice exhibits the classical ascending progressive paralysis in a caudal-to-rostral fashion, beginning with tail paralysis, progressing to hind limb weakness, paralysis, and finally forelimb weakness and paralysis. We determined the effects of IFN-γ on this process, because it has been shown that the lack of IFN-γ in induced models of disease does not ameliorate, but actually results in an exacerbation of, disease (22–28). We therefore crossed T/R^- mice to IFN-γ gene-disrupted mice ($T/R^-\gamma^-$) and followed the kinetics of disease incidence and clinical signs. $T/R^-\gamma^-$ mice developed EAE with 100% incidence and with comparable kinetics to T/R^- mice (Fig. 1*a*). Extensive observation of spontaneous disease has indicated that there are no differences in the onset and

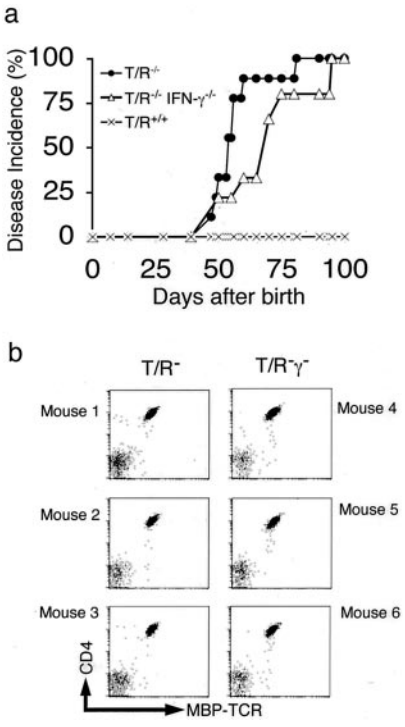

FIGURE 1. $T/R^- \times$ IFN-$\gamma^{-/-}$ mice ($T/R^-\gamma^-$) spontaneously succumb to nonclassical EAE. *a*, EAE incidence in untreated $T/R^-\gamma^-$ (△; $n = 9$), T/R^- (●; $n = 9$), and T/R^+ (X; $n = 7$). Data indicate the percentage of mice that developed EAE within each group. *b*, Blood analysis of three representative 3- to 4-wk-old T/R^- (mice 1–3) and $T/R^-\gamma^-$ (mice 4–6) mice. Vβ8.1.2 (Transgenic TCR) staining on the *x*-axis and CD4 staining on the *y*-axis are shown on forward/side scatter, lymphocyte-gated cells.

severity of spontaneous EAE between male and female mice, similar to what is observed in T/R$^-$ mice. However, the disease that develops in T/R$^-\gamma^-$ has a distinct clinical phenotype, marked initially by a slight head tilt progressing to a severe head tilt or the mouse lying on its side, which eventually leads to spinning or an axial rotatory motion (video of T/R$^-\gamma^-$ spontaneous nonclassical EAE at ⟨http://saturn.med.nyu.edu/~lafaille/nonclassical.mov⟩; video of T/R$^-$ spontaneous classical EAE at ⟨http://saturn.med.nyu.edu/~lafaille/classical.mov⟩; movies require Quicktime, which is available for PC and Macintosh at ⟨www.quicktime.com⟩). In addition, tail tonus is maintained in these mice, indicating that the affected sites within the CNS may be different in these mice compared with those in classical disease. Nonclassical disease is progressive, with no remission, and animals were euthanized when they reached the stage of spinning around their long axis. The progression of clinical signs in nonclassical spontaneous EAE closely follows the description by Muller et al. (9) of $PLP_{190-209}$-induced EAE in C3H mice.

By virtue of the cross of the same MHC class II-restricted MBP-specific TCR transgenic mice with RAG-1$^{-/-}$ mice, both IFN-γ$^+$ and IFN-γ$^{-/-}$ TCR transgenic strains harbor a population of T cells with identical TCR specificity and indistinguishable expression levels of the TCR and CD4 (Fig. 1*b*). Because it has been shown that after immunization the number of activated cells is significantly higher in IFN-γ$^{-/-}$ mice than in wild-type mice (23), we determined T cell compartment expansion in T/R$^-$ and T/R$^-\gamma^-$ mice. To this end, we analyzed mice at 4–6 wk of age and determined that there are no gross differences in cellularity of the T cell compartment in various organs and blood before clinical signs of EAE (Table I).

Infiltrate in nonclassical EAE is found predominantly in the brain, cerebellum, and brainstem

Given the clinical manifestation of nonclassical EAE in T/R$^-\gamma^-$ mice and the fact that previous reports of this form of EAE in various systems showed a predominantly upper motor neuron infiltrate (7–10), we performed cell counting and FACS analysis to analyze the cellular infiltrate of the brainstem and cerebellum (dissected together) and the spinal cord. T/R$^-$ with hindlimb weakness or paralysis and T/R$^-\gamma^-$ mice that had severe head tilt or were rolling were killed, and leukocytes were isolated as described in *Materials and Methods*. To quantify CNS infiltrates, whole organs from perfused mice were dissected (for instance, whole spinal cord), and all cells from that preparation were acquired and electronically counted by the FACS machine. We found that this method is more reliable in quantitative terms than histology, considering that cell losses are similar between different parts of the CNS. Fig. 2 shows that MBP-specific CD4$^+$ T cell numbers were markedly higher in the brainstem and cerebellum in T/R$^-\gamma^-$ compared with T/R$^-$ mice, and conversely, MBP-specific CD4$^+$ T cell numbers were dramatically lower in the spinal cord of T/R$^-\gamma^-$

Table I. *Comparison of the T cell compartment composition between T/R$^-$ and T/R$^-\gamma^-$ mice before signs of EAE*[a]

Organ	Cells	Mouse Type T/R$^-$	Mouse Type T/R$^-\gamma^-$
Spleen	Total cells	1.3×10^7	1.2×10^7
	CD4$^+$ cells	1.5×10^5	1.5×10^6
	Ratio T to CD11b	0.81	0.61
Inguinal lymph nodes	Total cells	2.7×10^6	2.4×10^6
	CD4$^+$ cells	2.0×10^6	1.8×10^6
	Ratio T to CD11b	9.3	10.5
Peripheral blood	% CD4$^+$ cells	44.8 ± 9.0	49.9 ± 7.4

[a] Average data from two 4- to 6-wk-old mice from each group with no clinical signs of EAE were analyzed. Less than 1% (<450 events/sample) of the total cells collected from the CNS (spinal cord and cerebellum plus brainstem) by Percoll separation were CD4$^+$3H12$^+$, analyzed by FACS. The average percentage of CD4$^+$ T cells from whole blood lysis staining (n = 10 mice/group) is given.

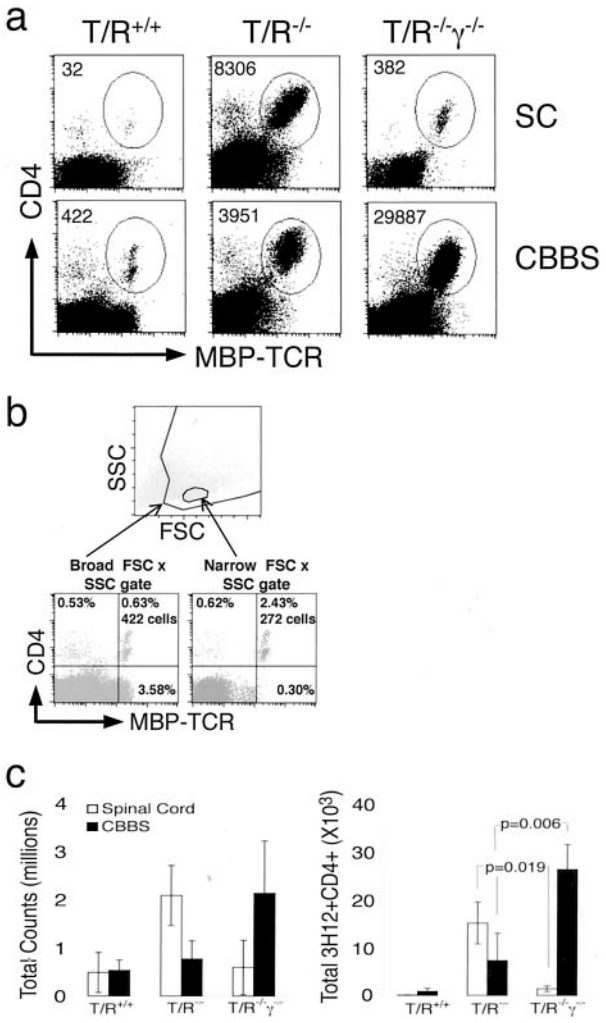

FIGURE 2. Differential localization of inflammatory infiltrates in T/R$^-$ vs T/R$^-\gamma^-$ mice. *a*, Representative FACS profile of MBP-specific (MBP-TCR) CD4$^+$ cells from a T/R$^+$ healthy mouse, a T/R$^-$ EAE L2.5-afflicted mouse, and a T/R$^-\gamma^-$ rolling mouse. Numbers in the plots are the number of events inside the indicated gate. SC, spinal cord; CBBS, cerebellum and brainstem, dissected together. Plots are representative of three mice from each group. Whole organs from perfused mice were dissected (whole spinal cord, whole brainstem, and cerebellum), and all cells from the preparations were acquired and electronically counted by a FACS machine. Numbers of MBP-specific T cells in the whole part of the CNS are indicated. The data shown are live (propidium iodide-negative) cells gated with a broad forward/side scatter gate that excludes subcellular debris, but allows all other populations to be analyzed. *b*, The broad forward/side scatter gate used in *a* as well as a tighter gate that enriches in lymphocytes are shown for the sample displayed in the *bottom left* of *a*. Note that the proportion of lymphocytes increases with use of the narrow gate, but the total number of T cells decreases. *c*, Analysis of the total cell counts (*left side*, hemocytometer count) and MBP-specific CD4$^+$ T cells (*right side*, FACS count) in the spinal cord (□) or the brainstem/cerebellum (■) from three mice from each group in *a*. The T/R$^-$ averaged level 2.5 EAE. The T/R$^-\gamma^-$ mice either were rolling or had severe head-tilt.

mice. We observed a higher cellularity overall in T/R$^-\gamma^-$ compared with T/R$^-$ mice, which is consistent with the antiproliferative properties of IFN-γ (23, 24, 31, 35).

Spontaneous nonclassical EAE inflammatory lesions are characterized by a polymorphonuclear infiltrate with marked eosinophilia

It is known that IFN-γ is a potent activator of macrophages in vitro and in vivo; indeed, characterization of EAE as a Th1 cell-mediated disease is supported by the predominance of monocytes and macrophages in the parenchyma and perivascular space in inflamed CNS tissues (10, 13, 14, 17–19). We therefore determined the characteristics of the lesions in nonclassical disease in the absence of the prototypic Th1 cytokine, IFN-γ. CNS tissue from T/R$^-\gamma^-$ mice with severe nonclassical disease was prepared, sectioned, and stained as described in *Materials and Methods*. Histological analysis of lesions from T/R$^-\gamma^-$ mice, both male and female, showed widespread granulocytic infiltrates with an elevated number of eosinophils (Fig. 3, *a* and *b*). Confirmatory of the large leukocyte cell counts and FACS data, we observed cellular infiltrates deep in the parenchyma, perivascular spaces, and meninges of the brainstem and cerebellum of sick mice. This is in marked contrast to the predominantly macrophage/monocytic infiltrate, which is mostly found in the perivascular areas and meninges of the spinal cord of T/R$^-$ mice (29).

Spontaneous EAE in mice lacking IFN-γ and IL-5 retains a nonclassical clinical progression, without eosinophilia

The preponderance of eosinophils in the lesions of T/R$^-\gamma^-$ mice along with the Th2Hi5 populations' high level of IL-5 production led us to speculate that IL-5 could be responsible for nonclassical disease. IL-5 is important for the differentiation, survival, and activation of eosinophils, and it has been show that IL-5 knockout mice do not develop eosinophilia (32). Eosinophils produce and store unique toxic molecules that can cause neurotoxicity directly or enhance inflammation (36–38) and could possibly play a major role in the pathogenesis of nonclassical EAE in T/R$^-\gamma^-$ mice. To assess the role of IL-5 and eosinophilia in nonclassical EAE, we crossed T/R$^-\gamma^-$ mice with IL-5$^{-/-}$ mice (T/R$^-\gamma^-5^-$) and determined the kinetics of disease incidence and clinical manifestations. Interestingly, T/R$^-\gamma^-5^-$ mice developed nonclassical EAE with approximately the same time course as T/R$^-$ and T/R$^-\gamma^-$ mice

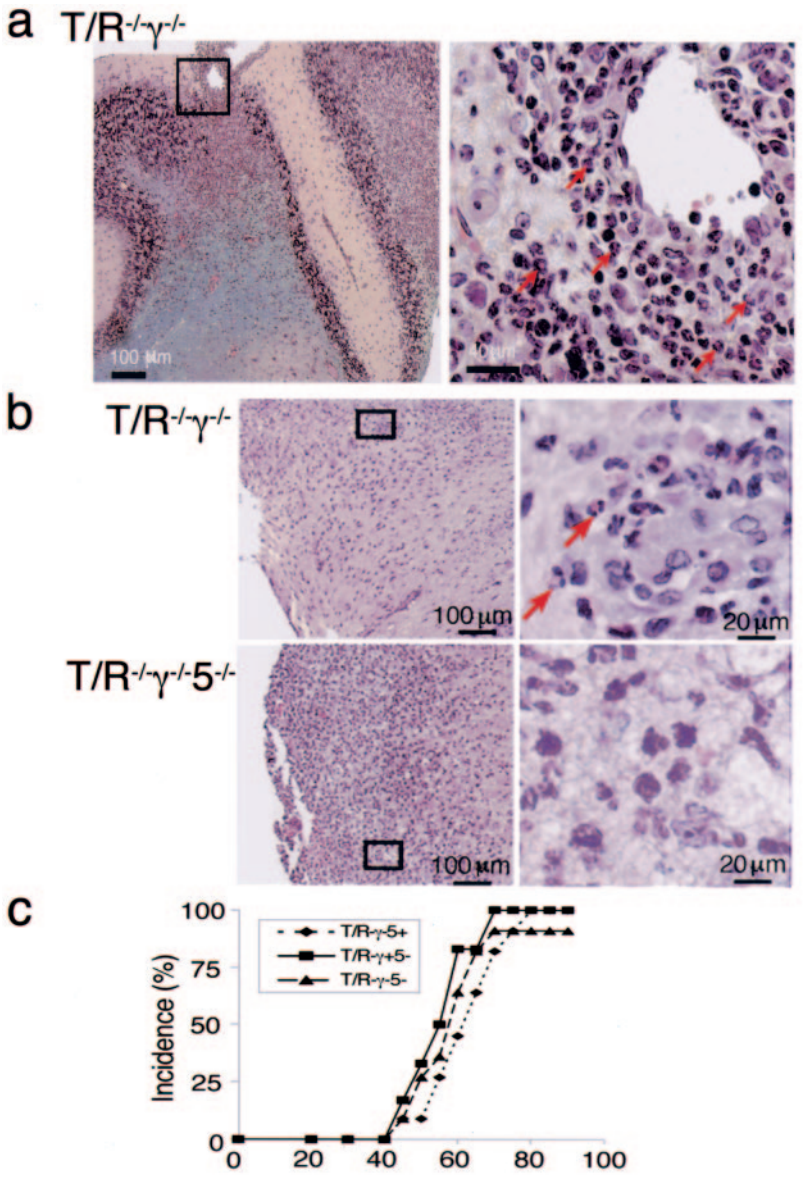

FIGURE 3. T/R$^-$ × IFN-$\gamma^{-/-}$ mice develop nonclassical EAE with intense eosinophilia, whereas T/R$^-$ × IFN-$\gamma^{-/-}$ × IL-5$^{-/-}$ mice (T/R$^-\gamma^-5^-$) develop nonclassical EAE without eosinophilia. *a*, Representative LFB/H&E staining of a cerebellum from a rolling T/R$^-\gamma^-$ mouse (representative of 12 mice). The *inset* box shows the area magnified in the *right panel*. Arrows point to some representative eosinophils. *b*, H&E-stained histological sections from infiltrated tissue in the brainstem of T/R$^-\gamma^-5^+$(representative of 12 mice) and T/R$^-\gamma^-5^-$ (representative of four mice) mice. The *inset* boxes show the area magnified in the *right panel*. Arrows point to some representative eosinophils in IL-5$^+$ mice. *c*, EAE incidence in untreated T/R$^-\gamma^+5^-$ (■; $n = 6$), T/R$^-\gamma^-5^+$ (●; $n = 9$), and T/R$^-\gamma^-5^-$ (▲; $n = 9$). Data indicates the percentage of mice that developed EAE within each group. The horizontal axis indicates the age of the mice in days.

and showed an incidence of nonclassical disease similar to that in T/R$^-\gamma^-$ mice (Fig. 3*c*). In addition, lesions from T/R$^-\gamma^-5^-$ mice, which exhibited severe nonclassical disease, displayed large numbers of polymorphonuclear leukocytes, but were devoid of the eosinophilia observed in T/R$^-\gamma^-$ mice (Fig. 3*b*). In summary, neither IL-5 nor eosinophilia is required for nonclassical EAE development.

Th2 cytokines and nonclassical EAE

We have previously shown that CD4$^+$ MBP-specific T cells initially primed in vitro in the absence of IFN-γ (Th2Hi5) can induce nonclassical EAE, whereas cells treated with IL-4 only (Th2) induce mostly classical EAE (20). As reported, both Th2 and Th2Hi5 cells produce high quantities of the Th2 cytokines IL-4, IL-9, IL-10, and IL-13, with the one major difference being levels of IL-5 that are 2- to 3-fold lower in Th2 populations compared with Th2Hi5 cells. However, we could not rule out the importance of Th2 cytokines in producing classical vs nonclassical disease, because small differences in the amounts produced between Th2 and Th2Hi5 cells (except in the case of IL-5) could potentially have major consequences on disease manifestation. In addition, we observed cases of nonclassical disease in Th2-transferred recipients (~17% incidence) (20), suggesting that subtle differences in Th2 cytokine levels could define which type of clinical EAE will be observed. To eliminate both IFN-γ and Th2 cytokines, we differentiated MBP-specific TCR transgenic T cells from IFN-$\gamma^{-/-}$ mice in the presence of IL-12 and neutralizing anti-IL-4 Ab (generating Th1γ^- cells). After three stimulations in vitro, we measured cytokine levels, injected the cells into RAG-deficient recipients, and evaluated disease progression. Th1γ^- cells produced much reduced levels of the Th2 cytokines IL-4, IL-5, IL-10, and IL-13 compared with Th2 and Th2Hi5 populations with, expectedly, no detectable IFN-γ production and an increase in IL-2 production over Th1, Th2, and Th2Hi5 populations (Fig. 4*a*). Th1γ^- cells display levels of T-bet mRNA comparable to those of Th1 cells and higher than those of Th2 and Th2Hi5 cells (data not shown). Interestingly, Th1γ^- cells injected into RAG-1$^{-/-}$ recipients produced severe, nonclassical EAE, indicating that the major Th2 cytokines play little or no role in determining the classical vs nonclassical EAE outcome. In addition, FACS and histological analysis of lesions from Th1γ^- cell-transferred mice indicated a predominantly monocytic type infiltrate similar to what is observed in T/R$^-$ mice and Th1-transferred mice (Fig. 4, *b* and *c*). The circled region in Fig. 4*b* shows the unique forward scatter/side scatter profile displayed by granulocytes. As clearly shown, whereas the infiltrate isolated from Th2Hi5 cell-injected mice has a strong granulocytic component, the CNS infiltrate of Th1 or, importantly, Th1γ^-cell-injected mice does not have a major granulocytic component. These observations were confirmed by histological analysis (Fig. 4*c*). These results demonstrate that neither the contribution of Th2 cytokines nor a granulocytic infiltrate (neutrophilic or eosinophilic) is necessary for the development of nonclassical EAE. The correlations (or lack thereof) among disease type, inflammatory infiltrate location, and cellular characteristics of the infiltrate are summarized in Table II.

Role of host-derived IFN-γ in nonclassical EAE

Up to this point, our adoptive transfer data into RAG-1$^{-/-}$ recipient mice (Fig. 4) demonstrated the key role of IFN-γ produced by the donor cells in determining the classical vs nonclassical disease outcome. However, RAG$^{-/-}$ recipient mice, which lack mature lymphocytes, have other sources of IFN-γ, such as NK cells. To establish whether host-derived IFN-γ could play a role in the adoptive transfer of classical vs nonclassical disease, we transferred

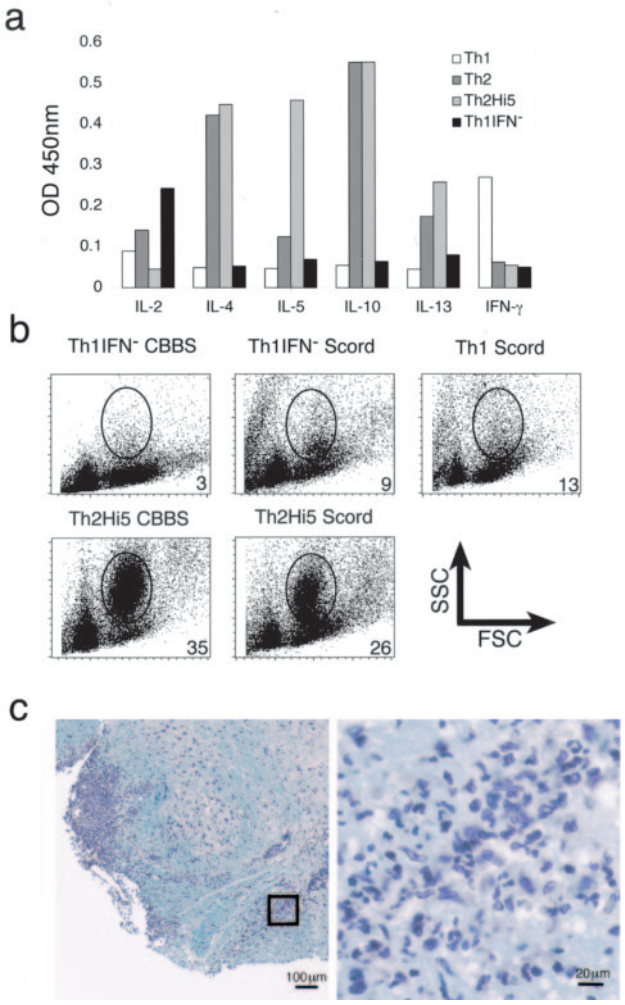

FIGURE 4. MBP-specific, Th1 IFN-$\gamma^{-/-}$ T cells (Th1γ^-) induce nonclassical EAE with a predominant mononuclear infiltrate. *a–c*, MBP TCR transgenic splenocytes from IFN-γ^+ and IFN-γ^- mice were stimulated three times in vitro under the indicated polarizing conditions. *a*, ELISA on supernatants of the indicated cell populations for IL-2, IL-4, IL-5, IL-10, IL-13, and IFN-γ. Data are representative of three independent cell supernatants. *b*, Forward/side scatter profiles from Th1γ^--transferred mice vs T/R$^-$ and T/R$^-\gamma^-$ mice. The circled regions indicate the Forward/side scatter profile of granulocytes. The numbers on the dot plots indicate the percentage of cells in the granulocyte gate. Data are representative of three independent experiments. *c*, LFB/H&E-stained histological analysis of brainstem of Th1γ^--transferred mice. Data are representative of two mice.

MBP-specific Th1, Th2, Th2Hi5, and Th1γ^- T cells into RAG$^{-/-}$ IFN-γ^+ and RAG$^{-/-}$ IFN-$\gamma^{-/-}$ recipient mice. As shown in Fig. 5, the lack of host-derived IFN-γ increased the tendency toward nonclassical disease. However, Th1 and Th2 cell administration resulted in a predominance of classical EAE even in RAG$^{-/-}$ IFN-$\gamma^{-/-}$ recipient mice. Thus, the capacity of the host to produce IFN-γ modifies the clinical outcome, but is not as determining a factor as the presence or the absence of IFN-γ during naive T cell priming.

Discussion

We described in this report a nonclassical form of EAE that spontaneously occurs in all T/R$^-$ IFN-$\gamma^{-/-}$ (T/R$^-\gamma^-$) mice that we observed. Clinically, the spontaneous disease appears to be similar to the disease reported by Greer et al. (8, 9) in $PLP_{190-209}$-immunized C3H mice (8, 9) and mice adoptively transferred mice with

Table II. *Infiltrate location, rather than infiltrating cell type, determines the type of EAE observed*

Mouse or Cell Type	Disease Induction	Clinical Manifestation	Predominant Infiltrate Location	Salient Feature of Infiltrate
T/R$^-$	Spontaneous	Classical	Spinal cord	Mononuclear
T/R$^-\gamma^-$	Spontaneous	Nonclassical	Cerebellum and brainstem	Eosinophilic
T/R$^-\gamma5^-$	Spontaneous	Nonclassical	Cerebellum and brainstem	Neutrophilic
Th1	Adoptive transfer	Classical	Spinal cord	Mononuclear
Th2	Adoptive transfer	Classical	Spinal cord	Neutrophilic
Th2Hi5	Adoptive transfer	Nonclassical	Cerebellum and brainstem	Eosinophilic
Th1γ^-	Adoptive transfer	Nonclassical	Cerebellum and brainstem	Mononuclear

$PLP_{190-209}$-specific cells (9). Systematic occurrence of a form of nonclassical EAE has also been reported in mice transferred with MBP_{79-87}-specific CD8$^+$ T cells (10) and upon reinduction of recovered Lewis rats with MBP and adjuvant (7). Our system has some distinct advantages, in that we have a completely reproducible spontaneous system that eliminates the need for immunization with adjuvants and pertussis toxin or in vitro culture of cells. In addition, using an MHC class II-restricted TCR transgenic crossed into the RAG-1$^{-/-}$ background, we determined that nonclassical EAE could occur in the absence of epitope spreading, CD8$^+$ T cells, or Abs. The latter point is important given a recent report showing the presence of lymphoid follicle-like structures containing B cells and follicular dendritic cells within the meninges of mice with EAE (39).

Muller et al. (9) showed that all C3H/HeJ (H-2^k) mice immunized with $PLP_{190-209}$ develop a severe nonclassical (axial rotatory) form of EAE. Disease induction with $PLP_{190-209}$-reactive cell lines led to mice displaying either classical or nonclassical forms of EAE, with nonclassical disease occurring in 10 of 14 animals (9). Another form of nonclassical EAE was reported by Huseby et al. (10) upon MBP_{79-87}-specific CD8$^+$ T cell-mediated induction of C3HeB/FeJ (H-2^k) mice. As pointed out by Huseby et al. (10), it was not determined whether the $PLP_{190-209}$-reactive cells that cause nonclassical EAE in C3H/HeJ mice were CD4 or CD8 cells, although the epitope was shown to bind to I.A^k (and I.A^d) (8). Given this uncertainty, Huseby et al. (10) speculated that the differences between classical vs nonclassical disease are due to CD4$^+$ cell-mediated disease vs CD8$^+$ CTL-mediated disease. However, in the monoclonal T/R$^-\gamma^-$ mice that we describe in this report, a nonclassical, rotatory EAE is clearly caused by CD4$^+$ T cells (Fig. 1*b*). Another significant difference between CD4$^+$ vs CD8$^+$ cell-mediated nonclassical disease lies in the role of IFN-γ; although anti-IFN-γ treatment reduced the severity of CD8$^+$ T cell-mediated adoptively transferred nonclassical EAE (10), the absence of IFN-γ was the determinant factor in our model of spontaneous nonclassical EAE. We have shown in this study that T/R$^-\gamma^-$ mice developed nonclassical EAE, whereas T/R$^-\gamma^+$ mice developed the classical form of disease. The absence of IFN-γ was also the determining factor in CD4$^+$ Th2Hi5 cell- and CD4$^+$ Th1γ^- cell-mediated, adoptively transferred, nonclassical EAE.

Muller et al. (9) performed a thorough histopathological analysis of nonclassical EAE induced by $PLP_{190-209}$ in C3H mice, observing, as we did in this study, relatively restricted distribution of the nonclassical lesions to the brainstem and cerebellum. Based upon the available data, they favored two alternatives to explain the development of nonclassical EAE: first, the target Ag could vary in distribution or accessibility within the CNS. Second, the concentration of APCs or MHC expression could be different between C3H mice and other mouse strains that develop classical EAE upon immunization with $PLP_{190-209}$. Neither alternative explains our observations. First, the target Ag in our studies of classical and nonclassical disease was the same. In both spontaneous models of EAE, the CD4$^+$ T cells recognize the same CNS Ag with identical affinity, ensured by the use of TCR-transgenic mice crossed with RAG$^{-/-}$ mice. Second, the same genetic background (B10.PL) could yield either classical or nonclassical disease. In our adoptive transfer studies, the same RAG$^{-/-}$ IFN-γ^+ recipients develop either classical EAE when injected with Th1 cells or nonclassical EAE when injected with Th2Hi5 cells (Figs. 4 and 5). The relevant factor determining the outcome of disease appears to be the differential priming of naive, MBP-specific T cells. T cell primed in the absence of IFN-γ, such as Th2Hi5 or Th1γ^- cells, induce nonclassical EAE, whereas T cells primed in the presence of (even small amounts of) IFN-γ, such as Th1 and Th2 cells, induce classical EAE.

We have also shown in this manuscript that the recipient's capacity to produce IFN-γ plays a role in classical vs nonclassical disease (Fig. 5); however, although the recipient's IFN-γ production accentuated the classical disease incidence, it did not revert in a major way the disease phenotype established by the different Th types. More than 200 genes, including genes involved in Ag processing/presentation and cell migration, are regulated by IFN-γ (40). It would be of great interest to establish which IFN-γ-dependent molecular mechanisms are responsible for the differential localization of the inflammatory infiltrate in classical vs nonclassical EAE. The study of adhesion molecules and chemokines/chemokine receptors whose expression is altered by IFN-γ appears to be particularly important.

Gordon et al. (7) described a nonclassical EAE phenotype in Lewis rats recovered from a first EAE episode and reinduced 28 days later with MBP and adjuvant. The secondary inflammation went from a lumbar/sacral location to a cervical spinal cord, cerebellum, brainstem, and cerebral localization. They speculated that target organ tolerance and other changes in the CNS prevented the reinitiation of disease in the spinal cord area and the increased inflammation in the brainstem and cerebellum. They showed that the encephalitogenic T cells were not responsible for this redistribution of inflammation, because adoptive transfer of reinduced splenocytes from nonclassical EAE rats produced classical disease. This is not the case in our system, where naive mice can develop classical or nonclassical disease depending on the type of T cell transferred into the mice. It would be interesting to examine the cytokine profiles in the cerebellum and brainstem of reinduced rats to determine whether there is a correlation with IFN-γ levels between our system and theirs.

Another important point raised by our studies is that clinically similar forms of nonclassical EAE occur when brainstem infiltrate is predominantly eosinophilic, neutrophilic, or monocytic. These data are summarized in Table II. Although the various types of leukocytes possess different mechanisms of toxicity, our results indicate that the location of the infiltrate, and not its cellular makeup, determines the clinical outcome.

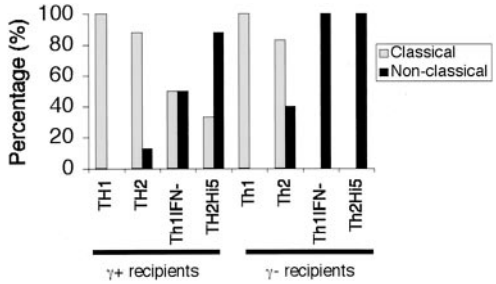

FIGURE 5. The role of recipient's IFN-γ. MBP-specific T cells, in vitro differentiated under the polarizing conditions shown in Fig. 4, were injected i.v. into RAG-$1^{-/-}$×IFN-γ^{+} (γ^{+} recipient) or RAG-$1^{-/-}$×IFN-$\gamma^{-/-}$ (γ^{-} recipient) mice. The incidences of classical (□) and nonclassical (■) EAE are indicated. Additions to >100% indicate rare mice displaying aspects of both phenotypes. γ^{+} recipients: Th1, $n = 9$; Th2, $n = 8$; Th1γ^{-}, $n = 8$; Th2Hi5, $n = 9$; γ^{-} recipients: Th1, $n = 5$; Th2, $n = 6$; Th1γ^{-}, $n = 5$; Th2Hi5, $n = 6$.

Furthermore, the data presented in this report may clarify some of the rules that govern Th cell migration to two different regions of the CNS and what type of inflammatory infiltrate different Th cells induce. As shown by the properties of Th1-IFN-$\gamma^{-/-}$ T cells, we can now design ways to stimulate Th cells that display predictable homing and inflammatory properties. For instance, if one wishes to create a neutrophilic infiltrate in the cerebellum, we would prime T cells in the absence of IFN-γ, (to minimize migration to the spinal cord), we would not have IL-5, (which would bring eosinophils), IL-4 would be present, etc. This information may, for instance, be relevant for the success of gene therapy/cellular therapy experiments in which specific gene products can be delivered by defined cell types migrating to particular locations within the CNS.

Given the data we have presented, there are two potential explanations for the different clinical manifestations we observed. 1) The adoptively transferred, activated Th populations or the in vivo-activated T cells in the spontaneous disease preferentially migrate to either the spinal cord (Th1, Th2, or in vivo-activated MBP transgenic cells from T/R^{-} IFN-γ^{+} mice) or the cerebellum and brainstem (Th2Hi5 cells or in vivo-activated MBP transgenic cells from T/R^{-} IFN-$\gamma^{-/-}$ mice). In support of this scenario, we have found that Th2Hi5 cells and Th1γ^{-} cells, which induce nonclassical EAE, express higher levels of CXCR4 mRNA than Th1 and Th2 cells, which induce classical EAE, whereas the ligand for CXCR4, stromal cell-derived factor-1/CXCL12, is expressed at higher levels in the brainstem and cerebellum than in the spinal cord of healthy mice (data not shown). The significance of this correlation, however, remains to be established. Post-transcriptional regulation of chemokine receptor expression is common, and mRNA data do not always correspond to the migratory capacity of cells. 2) The transferred or in vivo-activated T cells migrate throughout the CNS, but differential cytokine, chemokine, and chemokine receptor programs initiate site-specific damage that varies in different locations of the CNS. There is precedent for IFN-γ's site-specific role in CNS tissue damage that could account for differences between classical and nonclassical disease (41, 42). Additional evidence for this comes from the fact that adoptive transfer of fully activated, polarized cytokine and chemokine-producing T cells produce a disease that is essentially indistinguishable from in vivo-activated spontaneous disease ((Th1 and Th2 = T/R^{-}) (Th2Hi5 and Th1γ^{-} = T/$R^{-}\gamma^{-}$)) even though the site of activation and cell polarity are obviously different between adoptively transferred and spontaneous disease.

Additionally, there exists the possibility of MBP-specific T cells infiltrating the CNS, but surviving only in a particular section of the CNS (for instance, Th1 and Th2 cells would survive in the spinal cord, but Th2Hi5 and Th1γ- cells would not). However, this appears highly unlikely. Time-course studies have revealed that in both classical and nonclassical models, there is no gradual buildup of MBP-specific T lymphocytes in the CNS. Instead, lymphocytes are virtually absent from the CNS of healthy mice and invade the CNS in a wave. Once T lymphocytes infiltrate the CNS, clinical manifestations of EAE occur very quickly, literally within hours. The preferential localization of the infiltrates to the spinal cord (in classical EAE) and brainstem/cerebellum (in nonclassical EAE) is observed from the earliest time point at which any infiltrates were observed. Our observations are consistent with those of Flügel et al. (43) in adoptively transferred EAE in rats.

The relationship between TNF-induced apoptosis and IFN-γ has been highlighted by Furlan et al. (44). These authors showed that intrathecal delivery of IFN-γ protected mice from EAE by increasing apoptosis of CNS-infiltrating lymphocytes. This effect of IFN correlated with an increase in the mRNA level of TNF receptor type 1 in CNS-infiltrating cells. It is possible that in our experimental model, TNF levels are different in the brainstem/cerebellum vs spinal cord, and therefore, that cells primed in the absence of IFN-γ would be less sensitive to TNF-induced apoptosis. This mechanism would be inconsistent with the kinetics data discussed in the previous paragraph. However, our preliminary data indicate a 3-fold increase in TNF mRNA levels in the brainstem/cerebellum of mice undergoing nonclassical EAE compared with the spinal cord of mice undergoing classical EAE.

Finally, nonclassical EAE studies could generate useful information for some aspects of MS. In MS there is a high incidence of vestibular disturbances, and some human conditions, such as vertigo, can be caused by inflammatory responses in the brainstem and cerebellum (45–48), similar to what is observed in nonclassical EAE. The factors that determine the spatial localization of these lesions are not well understood, and the nonclassical model presented in this report holds promise in defining the mechanisms underlying these phenomena. For instance, it is possible that polymorphisms in the IFN-γ gene or downstream signaling may be associated with a high/low incidence of brainstem inflammation and vertigo. Although classical EAE has been and continues to be a useful model that recapitulates some characteristics of MS, we believe that nonclassical spontaneous EAE could be a useful model for the study of other aspects of MS not observed in classical EAE.

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