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CD1-Dependent Regulation of Chronic Central Nervous System Inflammation in Experimental Autoimmune Encephalomyelitis

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The existence of T cells restricted for the MHC I-like molecule CD1 is well established, but the function of these cells is still obscure; one implication is that CD1-dependent T cells regulate autoimmunity. In this study, we investigate their role in experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis, using CD1-deficient mice on a C57BL/6 background. We show that CD1−/− mice develop a clinically more severe and chronic EAE compared with CD1+/+ C57BL/6 mice, which was histopathologically confirmed with increased demyelination and CNS infiltration in CD1−/− mice. Autoantigen rechallenge in vitro revealed similar T cell proliferation in CD1+/+ and CD1−/− mice but an amplified cytokine response in CD1−/− mice as measured by both the Th1 cytokine IFN-γ and the Th2 cytokine IL-4. Investigation of cytokine production at the site of inflammation showed a CNS influx of TGF-β1-producing cells early in the disease in CD1+/+ mice, which was absent in the CD1−/− mice. Passive transfer of EAE using an autoreactive T cell line induced equivalent disease in both groups, which suggested additional requirements for activation of the CD1-dependent regulatory pathway(s). When immunized with CFA before T cell transfer, the CD1−/− mice again developed an augmented EAE compared with CD1+/+ mice. We suggest that CD1 exerts its function during CFA-mediated activation, regulating development of EAE both through enhancing TGF-β1 production and through limiting autoreactive T cell activation, but not necessarily via effects on the Th1/Th2 balance. The Journal of Immunology, 2004, 172: 186–194.

The CD1 molecules are cell surface glycoproteins with structural similarities to both MHC class I and II (1), but in contrast to classical MHC molecules, CD1 molecules are nonpolymorphic and TAP independent (2). The CD1 proteins are encoded in five different genes and are categorized into two distinct groups (3): CD1a, CD1b, and CD1c constitute group 1, and CD1d alone makes up group 2. CD1d is the only CD1 molecule expressed in mouse and is primarily seen on the surface of cells from the hemopoietic lineage (4), including B and T lymphocytes, macrophages, and dendritic cells. A relatively high expression of CD1 on APCs has been detected, and it has been shown that CD1 can present both peptidic and lipidic Ag to T cells (5–10).

CD1-restricted T cells have been described by many authors as a rather heterogeneous population, but the most characterized is a subset of T cells referred to as NKT cells, because they have been described to share properties with both NK and T cells. This population expresses the NK1.1 surface marker and an invariant TCR (11–17). Upon TCR ligation, NKT cells have been shown to very rapidly secrete high amounts of IFN-γ and IL-4, and it has been hypothesized that this can be of importance when shaping the adaptive immune response toward a Th1 or Th2 profile (18–20). Despite this, many studies using in vivo models with possible endogenous CD1-binding T cell ligands, have shown that neither mice depleted of NKT cells nor mice genetically deficient in CD1-restricted T cells have an altered Th1/Th2 balance, which indicates that this is not always their primary function (21–24). Furthermore, NKT cells have been shown to play a role in various pathogenic conditions such as infection (25) and tumors (26), but they have also been shown to be of importance in inducing tolerance (27–29) and controlling autoimmunity (20, 23, 30–36). Studies using animal models for organ-specific autoimmune diseases such as type 1 diabetes (nonobese diabetic (NOD)4 mice) and multiple sclerosis (experimental autoimmune encephalomyelitis (EAE)) have demonstrated that CD1-dependent cells have the ability to regulate the autoimmune attack on self. It has been shown that germline deletion of the CD1 gene exacerbates diabetes in the NOD mouse (23), and also that activation of NKT cells by the exogenous ligand α-galactosylerceramide (α-GalCer) protects from diabetes (37).

Mars et al. (33) and Fritz et al. (38) show in two independent studies that excessive numbers of NKT cells can protect mice from EAE development, and Jahng et al. (39), Singh et al. (20), Furlan et al. (40), and Miyamoto et al. (35) demonstrate that activation of NKT cells using α-GalCer or an analog of it, can prevent from EAE.

1 This work has been supported by grants from Swedish Strategic Research Foundation, Swedish Association of Neurologically Disabled, Swedish Natural Science Research Council, Swedish Medical Research Council, Alfred Osterlund Foundation, Tore Nilson Foundation, His Majesty Gustav V’s Foundation, Royal Swedish Academy of Science, Royal Physiographic Society in Lund, M. Bergvalls Foundation, Åke Wiberg Foundation, Boije Dahlin Foundation, and Crafoord Foundation.

2 A.T. and I.T. contributed equally to this work.

3 Address correspondence and reprint requests to Dr. Ingrid Teige, Section for Medical Immunology Research, Lund University, 111 Bismedicinskt Centrum, S-221 84 Lund, Sweden. E-mail address: Ingrid.Teige@inflam.lu.se

4 Abbreviations used in this paper: NOD, nonobese diabetic; EAE, experimental autoimmune encephalomyelitis; α-GalCer, α-galactosylerceramide; MOG, myelin oligodendrocyte glycoprotein; QTL, quantitative trait locus; p.i., postimmunization; PPD, purified protein derivate.
In our study, we were interested in elucidating whether normal numbers of CD1-dependent cells have an inherent ability to influence progression of EAE without stimulation with a nonnatural ligand such as α-GalCer. CD1 knockout (CD1−/−) mice in the EAE-susceptible C57BL/6 (B6) background were therefore immunized with myelin oligodendrocyte glycoprotein peptide 35–55 (MOG35–55) to induce encephalomyelitis (41). CD1−/− animals are deficient in all CD1-restricted cells, both the invariant TCR and NK1.1-bearing NKT cells, and other more poorly defined cells dependent on CD1 for development, homeostasis, or survival. In this study, we report that CD1−/− mice developed a more severe and chronic EAE compared with CD1+/+ mice, both in the sense of clinical symptoms as well as immune cell infiltration and demyelination in the CNS. We demonstrate that this was associated with a deficiency in TGF-β1 production at the site of the autoimmune attack. The lack of immunoregulation shown in CD1−/− mice was associated with a higher production of both the typical Th1 cytokine IFN-γ and the responding Th2 cytokine IL-4, showing that a Th1-skewed cytokine response was not the underlying mechanism for the augmented EAE. When using an adoptive T cell transfer model to induce EAE, we found that CD1−/− and CD1+/+ mice developed a similar EAE disease course, but when priming the immune system with CFA before adoptive transfer, the distinction in EAE development between the two groups was again observed. This demonstrates first, as has also been observed by others, that CD1-restricted T cells need activation to exert their regulatory function (20, 39, 40), and second, that this activation could be achieved by bacterial adjuvant stimulation only. This implies that CD1-restricted regulatory T cells need an inflammatory milieu to modulate an autoimmune response, either solely through bystander mechanisms or via an endogenous or bacterial ligand presented on an APC.

Materials and Methods

Mice
CD1−/− mice had been generated as described (22), backcrossed to C57BL/6 (B6) background for 10 generations and were kindly obtained from Prof. Dr. M. Grushy (Harvard School of Public Health, Boston, MA). Age-matched male and female B6.CD1−/− and B6.CD1+/+ at the age of 8–22 wk were used in all EAE experiments. All B6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were kept and bred at the conventional animal facility of Section for Medical Immunotechnology, Lund University, and all experiments were performed in accordance with the ethical committee in Malmö-Lund, Sweden.

EAE induction and clinical evaluation

EAE was induced as earlier described (41) in CD1+/+ and CD1−/− mice. Briefly, each animal was s.c. immunized in the flank with 200 μl of a 1:1 emulsion of 300 μg of MOG35–55 in PBS and CFA containing 500 μg of Mycobacterium tuberculosis H37Ra (Difco, Detroit, MI). An identical booster was given in the other flank 1 wk later. An i.p. injection of 500 ng of pertussis toxin (Bordetella pertussis; Sigma-Aldrich, Stockholm, Sweden) dissolved in 100 μl of PBS was given at the day of immunization (day 0) and at day 2. This dose of pertussis toxin was generally well tolerated in both CD1+/+ and CD1−/− mice. Hereafter, this will be referred to as the active-immunization EAE induction protocol. MOG35–55 was synthesized by Dr. Å. Engström (University of Uppsala, Uppsala, Sweden) using Fmoc/HBTU chemistry and purified by reversed-phase chromatography.

For adoptive T cell transfer experiments, each mouse was injected in the tail vein with a cell suspension containing 10 × 10^6 MOG35–55-specific T cells, treated as described below, in 300 μl of PBS. At the day of transfer (day 0) each animal was given an i.p. injection of 500 ng of pertussis toxin. Hereafter, this will be referred to as the passive-transfer EAE induction protocol.

For in vivo preactivation of the immune system before passive transfer of EAE, CD1+/+ and CD1−/− mice were immunized s.c. in the flank with 200 μl of a 1:1 emulsion of only PBS and CFA containing 500 μg of M. tuberculosis H37Ra. Two days later (day 0), the mice were subjected to the same treatment as in the passive-transfer EAE induction protocol. Hereafter, this will be referred to as the in vivo-preactivation and passive-transfer EAE induction protocol.

Mice were observed for clinical signs of EAE and weighed every second day. Clinical score was designated as follows: 0, no detectable signs of EAE; 1, affected tail tonus; 2, tail paralysis; 3, mild hindleg paresis; 4, severe hindleg paresis; 5, one hindleg paralysis; and 6, complete hindleg paralysis. Maximum score was defined as the highest clinical score for each animal during the experimental period.

For clinical EAE evaluation, the following parameters were used: day of onset was defined for each animal as the day of first appearance of EAE symptoms. EAE duration was calculated as the number of days each animal was scored sick divided by the total number of scored days and expressed as a percentage. Cumulative incidence was defined as the percentage of animals that developed EAE during the experimental period.

DNA from nine representative CD1−/− experimental mice was prepared from brain tissue by a proteinase K-based protocol (42). As references, DNA was prepared from a 129-D3 embryonic stem cell (E6.29), BALB/c, and B6, purchased from The Jackson Laboratory and bred in our animal facility. Forty-three fluorescence-labeled microsatellite markers (International, Ulm, Germany) were used for genotyping, covering chromosome 3 as well as some parts of chromosomes 1, 6, 7, 14, and 15. The latter were primarily located on known EAE quantitative trait loci (QTLs). PCRs were performed with 5 ng of DNA in a reaction volume of 10 μl containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl2, 0.3 pmol/μl respective forward and reverse primer, 0.4 mM dNTPs (Advanced Biotechnologies, Surrey, U.K.), and 0.25 μl of TaqDNA polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden). The PCR products were analyzed on a Megabace 1000 (Amersham Pharmacia Biotech), according to the manufacturer’s protocols.

The single nucleotide polymorphism marker analysis for frg1 on chromosome 3 was performed by Pyrosequencing (PSQ 96 System; Pyrosequencing, Uppsala, Sweden). Primer sequences for PCR were as follows: forward primer, 5′-CCG GAG GGT AGT CCG TGC ATC-3′; reverse primer, 5′-GGA CGT CCG TTA AGG ACA CTG-3′; and sequencing primer, fgr1-770, 5′-CAG CAT CTT TTC TTT CCG CCC TGG-3′. Biotinylated PCR products were cleaned up with streptavidin Sepharose beads on a Pyrosequencing Vacuum Prep workstation according to the manufacturer’s instructions (Pyrosequencing; http://www.pyrosequencing.com; June 2003).

The physical position of markers was determined using the Celera (Rockville, MD) database; if a marker name was unknown, the sequence was blasted against Celera to determine its position. Cenmorgon positions were retrieved from the Mouse Genome Informatics list (http://www.informatics.jax.org; June 2003).

Pathological examination and cell infiltration in CNS

Brain and spinal cord were dissected from three or four mice in each group at days 17, 35, and 56 postimmunization (p.i.), and tissues were divided and either formalin fixed or OCT embedded.

For demyelination studies, the lumbar part of spinal cords was collected and fixed in 4% formalin in PBS for a minimum of 24 h. Thereafter, they were dehydrated, embedded in paraffin, and transversally sectioned at 6 μm. Sections were incubated for 6 h at 60°C in 0.1% Luxol fast blue for visualizing myelin. Lithium carbonate (0.05%) was thereafter used to destain nonmyelinated areas. Hematoxylin-erythrosin was used for nuclear counter staining. Four transverse sections from each animal were analyzed using Easy Image Analysis 2000 (Bergström Instrument, Solna, Sweden).

First, the demyelination area in each section was measured, and then the total spinal cord area was measured. From these four sections, a mean value for each mouse was calculated. Demyelination was expressed as square millimeters of demyelination per 100-mm² total area of spinal cord section.

For immunohistochemical studies, brain tissue, including medulla oblongata, was sampled by dividing the organ midsagittally and sectioned parallel to the midline. Those sections in immediate vicinity of the midline were used for measurements. The thoracic part of the spinal cords was collected and sectioned longitudinally, and inner sections in close vicinity of the central canal were used for evaluation. Tissues were embedded in OCT compound (Sakura Finetek, Zoeterwoude, The Netherlands) and
CO2. Cells were stimulated in quadruplets with MOG35 through a sieve. Cell suspensions were treated with 0.84% NH4 Cl to lyse and 56 p.i., and single-cell suspensions were prepared in PBS by passing the last 15 h of culture. Cells were then harvested and analyzed with a flow beta counter (Matrix 96 Direct beta counter; Packard, Meriden, Connecticut) after a total of 20, 40, and 90 h in culture. Stimulation index was calculated by dividing the MOG35 CT) after a total of 20, 40, and 90 h in culture. Stimulation index was calculated by dividing the MOG35–55–specifc T cell line, this stimulation was repeated three to six times with intervals of 10–30 days. Between stimulations, the Ag-containing medium was removed, and the T cells were kept in a resting state in culture medium supplemented with IL-2; the medium was changed every fourth day. For passive-transfer experiments, the T cells were stimulated with MOG35–55 and APC for 96 h, and IL-2 was added to the cultures for the last 48 h to enhance the stimulation. After that, the cells were recovered, washed three times in PBS, set to the right concentration, and immediately injected into the mice. Before performing the comparing experiments, encephalitogenicity was tested on B6.CD1–/– mice using 3, 6, and 12 × 106 cells to transfer disease. All transfer experiments induced EAE but with a dose-dependent efficiency (data not shown).

**Statistical evaluations**

Statistical evaluation was performed using StatView software. For analyzing differences in clinical scores, Mann-Whitney tests were used. For analyzing the number of affected animals, χ² tests were used. When analyzing differences in cytokine production, T cell proliferation, and cell infiltration in CNS, Student’s unpaired t tests were used.

**Results**

CD1–/– mice have higher incidence of EAE and develop more severe and chronic disease

To investigate the role of CD1 and CD1-dependent cells in MOG-induced EAE, we immunized CD1–/– and CD1+/+ mice with MOG35–55, both on C57BL/6 background. As shown in Fig. IA and Table I, CD1–/– deficient mice developed a more severe EAE with higher clinical scores as compared with CD1+/+ mice. After initial paralysis, many CD1+/+ mice recovered from their neurological symptoms, whereas the CD1–/– mice remained severely affected for over 50 days. This is clearly seen both in Fig. IB and Table I when looking at the EAE duration and demonstrates a higher chronicity of disease in CD1–/– mice. The results also show that the day of onset is not affected by the expression of CD1. These results suggest that CD1 expression does not play a major role in the initial phase of the autoimmune attack, but rather in

**FIGURE 1.** CD1–/– mice develop augmented chronic EAE. Average clinical score (A) and percentage of EAE-affected animals (B) are significantly higher in CD1–/– (n = 14) compared with CD1+/+ mice (n = 12). Figures show results from two different experiments, with balanced groups, taken together. **, p ≤ 0.05, ***, p ≤ 0.01.
controlling the ongoing inflammation and preventing a chronic pathogenic reaction directed to self tissue.

**The CD1<sup>−/−</sup> flanking fragments do not influence the EAE outcome**

The CD1-deficient mouse used, as every knockout mouse created in an ES-129 cell line and backcrossed to the desired genetic background, generated a congenic line with linked 129 genes around the knocked-out gene. To determine the linked fragments of mice used in this study, CD1<sup>−/−</sup> mice have been genotyped with single nucleotide polymorphisms and microsatellite markers. The colony in our animal facility was established from five founder CD1<sup>−/−</sup> mice previously backcrossed to C57BL/6 for 10 generations. From genotyping the mice, it becomes obvious that, at an early stage, the mice have been crossed to BALB/c, making it necessary to distinguish three possible genotypes, 129-D3, B6, and BALB/c, respectively. Polymorphic microsatellite markers informative for all three parental made it possible to identify a linked homoyzogous 129-D3 fragment of 8 Mb (between D3Mit29 and the CD1<sup>−/−</sup> construct). B6 alleles can even be excluded for a 18-Mb fragment (D3Mit154 to D3Mit40). All the CD1<sup>−/−</sup> mice share this linked core fragment. Progressing telomeric, BALB/c alleles can be identified. D3Mit75 (positioned at 100 Mb) is the first informative marker for BALB/c, and D3Mit91 (positioned at 160 Mb) is the last of the used markers revealing some BALB/c alleles. The fragments from 85 to 160 Mb are heterozygous and of diverse genotypes demonstrating the heterogeneity of the five founder mice. For an overview, see Fig. 2, and for details including individual score comparisons, see supplementary data.<sup>5</sup>

### Table 1. Disease parameters in different EAE induction protocols<sup>a</sup>

<table>
<thead>
<tr>
<th>EAE Induction Protocol</th>
<th>Genotype</th>
<th>Day of Onset</th>
<th>EAE Duration (% of experimental period)</th>
<th>Cumulative Incidence (%)</th>
<th>Mean Score over Experimental Period</th>
<th>Maximum Score</th>
<th>EAE-Induced Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active immunization</td>
<td>CD1&lt;sup&gt;−/−&lt;/sup&gt; (n = 12)</td>
<td>9.7 ± 0.51</td>
<td>53 ± 6.4</td>
<td>100</td>
<td>1.4 ± 0.26</td>
<td>3.5 ± 0.34</td>
<td>0/12</td>
</tr>
<tr>
<td></td>
<td>CD1&lt;sup&gt;−/−&lt;/sup&gt; (n = 14)</td>
<td>9.1 ± 0.53</td>
<td>80 ± 2.5**</td>
<td>100</td>
<td>3.4 ± 0.33**</td>
<td>5.5 ± 0.39**</td>
<td>1/14</td>
</tr>
<tr>
<td>Passive transfer</td>
<td>CD1&lt;sup&gt;−/−&lt;/sup&gt; (n = 6)</td>
<td>6.8 ± 0.54</td>
<td>79 ± 1.3</td>
<td>100</td>
<td>2.1 ± 0.18</td>
<td>3.7 ± 0.21</td>
<td>0/6</td>
</tr>
<tr>
<td></td>
<td>CD1&lt;sup&gt;−/−&lt;/sup&gt; (n = 7)</td>
<td>7.9 ± 0.34</td>
<td>77 ± 0.8</td>
<td>100</td>
<td>1.9 ± 0.28</td>
<td>3.4 ± 0.37</td>
<td>0/7</td>
</tr>
<tr>
<td>In vivo preactivation and passive transfer</td>
<td>CD1&lt;sup&gt;−/−&lt;/sup&gt; (n = 11)</td>
<td>6.4 ± 0.57</td>
<td>31 ± 10.5</td>
<td>55</td>
<td>0.3 ± 0.12</td>
<td>0.6 ± 0.20</td>
<td>0/11</td>
</tr>
<tr>
<td></td>
<td>CD1&lt;sup&gt;−/−&lt;/sup&gt; (n = 13)</td>
<td>7.7 ± 1.14</td>
<td>62 ± 8.2**</td>
<td>100*</td>
<td>0.9 ± 0.14**</td>
<td>1.7 ± 0.17**</td>
<td>0/13</td>
</tr>
</tbody>
</table>

<sup>a</sup>EAE was induced using different protocols as described in Materials and Methods. Evaluations and analyses were done as described in Materials and Methods, comparing CD1<sup>−/−</sup> with CD1<sup>+/+</sup> within each experiment. Data represent the group mean value ± SEM.

*<sup>p</sup> < 0.05; **<sup>p</sup> < 0.01; and **<sup>p</sup> < 0.001.

<sup>5</sup>The on-line version of this article contains supplemental material.

**CD1<sup>−/−</sup> mice have elevated demyelination and CNS inflammation**

To investigate CNS histopathology, brains and spinal cords were dissected from mice with EAE development representative of the CD1<sup>−/−</sup> and the CD1<sup>+/+</sup> group, respectively, at day 17 (acute phase), day 35 (recovery phase), and day 56 (chronic phase) post-EAE immunization (p.i.) and stained for demyelination. As similar results were obtained at all time points, data are presented collectively. Results are shown in Fig. 3 and reveal that CD1<sup>−/−</sup> mice had a higher degree of demyelination when compared with CD1<sup>+/+</sup> mice. This finding correlates well with the clinical symptoms (Fig. 1) and shows that the sustained neurological dysfunction observed in CD1<sup>−/−</sup> are in part due to more severe demyelination in the CNS.

To evaluate infiltrating immune cells in CNS, identification of T and B cells, macrophages, and MHC class II-positive cells was performed by immunohistochemistry on CNS tissue from diseased CD1<sup>−/−</sup> and CD1<sup>+/+</sup> mice. Results from these stainings showed no difference in numbers of infiltrating immune cells between CD1<sup>−/−</sup> and CD1<sup>+/+</sup> mice at day 17 and CD1<sup>−/−</sup> on day 17 p.i., in the acute phase of disease. However, during progression of the disease, the number of infiltrating T cells and macrophages increased in CD1<sup>−/−</sup> mice, whereas this could not be seen in CD1<sup>+/+</sup> mice (see Fig. 4). This again indicates that CD1 expression is most important in preventing a chronic inflammation in the CNS rather than at earlier stages.

**Enhanced cytokine production but similar proliferation in CD1<sup>−/−</sup> mice**

When trying to elucidate the mechanism behind the difference in disease course between CD1<sup>−/−</sup> and CD1<sup>+/+</sup> mice, animals from both groups were sacrificed and spleens were dissected out on days 17, 35, and 56 p.i. Specific T cell in vitro proliferation to the disease-inducing peptide MOG<sub>35-55</sub> was assayed. Cells were also stimulated with other known CNS peptides (MOG<sub>79-90</sub> and myelin basic protein peptide 87–110) to investigate epitope spreading, but no proliferation to these Ags could be seen in any of the groups (data not shown). T cell proliferation to PPD as a positive control was specific in both CD1<sup>−/−</sup> and CD1<sup>+/+</sup> mice with no difference between the groups (data not shown).

When assaying the specific T cell proliferation after MOG<sub>35-55</sub> encountering in vitro, we could detect specific Ag response in both groups. The response increased in magnitude during the disease course, even in the CD1<sup>+/+</sup> animals that had no worsening of...
CD1-dependent regulation requires active immunization

Other authors (20, 39) have reported a requirement for activation of NKT cells with α-GalCer for the revelation of their positive effects on EAE. In this study, we were interested in further investigating whether the CD1-dependent regulation of EAE shown in Fig. 1 and Table I was dependent on active immunization. To study this, we established an encephalitogenic MOG_{35-55}-specific T cell line from CD1^{-/-} mice and induced EAE in both CD1^{+/+} and CD1^{-/-} mice by adoptive transfer of 10 × 10^6 of these T cells. Using this protocol, no difference in signs of clinical disease development could be detected between CD1^{+/+} and CD1^{-/-} mice (see Fig. 8 and Table I). All mice in both groups were affected chronically, and none of the mice recovered fully from neurological symptoms. This stands in sharp contrast to the disease course observed when EAE was induced using active immunization with MOG_{35-55} in CFA where CD1^{+/+} recover partially from CNS tissue, and stained cryosections for cells producing IFN-γ, IL-4, TNF-α, and TGF-β1. Sections from three to four representative animals per group and time point were counted for positively stained cells. When counting TGF-β1-producing cells, we found a striking difference between CD1^{-/-} and CD1^{+/+} mice at day 17 p.i., in the acute phase of the disease. In EAE-affected CD1^{-/-} mice, approximately eight times more TGF-β1-producing cells were found in the CNS compared with CD1^{+/+} mice (see Fig. 7). The number of TGF-β1-producing cells decreased dramatically in the CD1^{+/+} mice to be undetectable when the acute phase was over and the EAE entered a milder and more chronic phase (days 35 and 56 p.i.), and the TGF-β1 production in CD1^{-/-} mice remained very low or undetectable throughout the experiment (data not shown). These results indicate that CD1-dependent TGF-β1 production plays an important role in the breaking of a harmful autoantigen-driven inflammation in the CNS. No difference between the groups could be detected regarding IFN-γ, IL-4, or TNF-α (data not shown). The fact that IFN-γ and IL-4 production was similar in CD1^{+/+} and CD1^{-/-} mice again points toward a protective mechanism of CD1-restricted T cells other than a mere Th1/Th2 shifting, in this case rather a large burst of TGF-β1 production at the inflammatory site.

CD1-restricted regulation requires active immunization
mined the linked ES-129 fragment around the deleted backcrossed (10 generations). In addition, we have now deter-
experiments addressing the role of CD1 and has been extensively concerning the effects a gene, or rather its absence, exerts under EAE using CD1-decient mice. Gene knockout technology has
35 (H11005/nH11001/CNS compared with CD1– mice).

EAE induction protocols. [18–20]. Because EAE is a Th1-mediated autoimmune disease model, a skewing of the immune response toward a Th2 pro
(18–20). Because EAE is a Th1-mediated autoimmune disease model, a skewing of the immune response toward a Th2 pro

autoreactive T cell proliferation. MOG35–55-specific T cell proliferation (±SEM) in spleen cell cultures from EAE-immunized CD1+/+ and CD1−/− mice at days 17, 35, and 56 p.i. (n = 3–4/group and time point) does not differ among the groups.

and also excluded other contaminating gene fragments around this as well as QTLs mapped to be of importance for EAE (43, 44). Even if a contribution of polymorphic genes neighboring the CD1−/− cannot be completely excluded, it is more reasonable that the main effect comes indeed from the lack of the CD1. Thus, our study demonstrates that CD1 and CD1-dependent cells are of importance in the inherent regulation of actively induced EAE. We show that CD1−/− mice have a significantly higher clinical score after the EAE onset phase compared with their CD1+/+ counterparts. In contrast to CD1+/+ mice, they do not recover from the EAE, and they have a higher demyelination and infiltration of inflammatory cells in the CNS. This indicates that CD1 expression is crucial in the breaking of a pathogenic autoimmune inflammation in the CNS and for controlling a chronic attack with subsequent demyelination. Despite this finding, the capacity of the autoreactive T cells to proliferate in response to MOG35–55 was not enhanced in mice lacking CD1. However, when exploring the cytokine response of these autoreactive T cells, both the IL-4 and IFN-γ production was found to be elevated in the absence of CD1.

Discussion

CD1 and CD1-dependent cells have been implicated in tolerance and autoimmunity in earlier studies, but the mechanism behind this regulation is still to be elucidated (20, 23, 27–36). The focus of research has so far mainly been CD1-dependent NKT cells, which due to their prompt IL-4 production upon activation with following Th2 switching, have been proposed as possible regulatory cells (18–20). Because EAE is a Th1-mediated autoimmune disease model, a skewing of the immune response toward a Th2 profile has been regarded as protective (45–47).

In this study, we further investigate the importance of CD1 in EAE using CD1-deficient mice. Gene knockout technology has been a powerful tool over the last decades to address the question concerning the effects a gene, or rather its absence, exerts under physiological conditions. A drawback is that such strains often contain contaminations of genes derived from the founder 129 ES cell. The presently used mouse strain has been used in previous experiments addressing the role of CD1 and has been extensively backcrossed (10 generations). In addition, we have now determined the linked ES-129 fragment around the deleted CD1 gene neurological dysfunction. To test the hypothesis that the CD1-dependent regulatory pathway could be activated through adjuvant immunization, we immunized CD1+/+ and CD1−/− mice with CFA containing the same dose of mycobacteria as in the active immunization EAE induction protocol but without any MOG peptide; this was done 2 days before encephalitogenic T cell transfer. The results are shown in Fig. 9 and Table I and demonstrate that CD1−/− mice got a more severe EAE compared with CD1+/+ mice at day 56, and B cell infiltration is higher at day 35. No significant difference in invading MHC class II-positive cells could be seen, and no difference in invading cell number could be seen in the acute phase of EAE (day 17). *p ≤ 0.05, **p ≤ 0.01.

FIGURE 4. CD1−/− mice have more immune cell infiltration in the CNS compared with CD1+/+ mice. Numbers of infiltrating T cells, B cells, macrophages, and MHC class II-positive cells in cerebellum (±SEM) for EAE-immunized CD1−/− and CD1+/+ mice at day 17 (n = 3/group), day 35 (n = 3/group), and day 56 (n = 4/group). Number of infiltrating T cells in CD1−/− mice is significantly higher compared with that in CD1+/+ mice at days 35 and 56. Number of infiltrating macrophages is higher in CD1−/− mice at day 56, and B cell infiltration is higher at day 35. The results are shown in Fig. 9 and Table I and demonstrate that CD1−/− mice got a more severe EAE compared with CD1+/+ mice at day 56, and B cell infiltration is higher at day 35. No significant difference in invading MHC class II-positive cells could be seen, and no difference in invading cell number could be seen in the acute phase of EAE (day 17). *p ≤ 0.05, **p ≤ 0.01.

FIGURE 5. CD1+/+ and CD1−/− mice have similar autoreactive T cell proliferation. MOG35–55-specific T cell proliferation (±SEM) in spleen cell cultures from EAE-immunized CD1+/+ and CD1−/− mice at days 17, 35, and 56 p.i. (n = 3–4/group and time point) does not differ among the groups.

Autoreactive T cells produce higher amounts of cytokines in CD1−/− mice. MOG35–55-specific IFN-γ and IL-4 production in spleen cell cultures from individual CD1+/+ and CD1−/− mice at days 17, 35, and 56 p.i. Higher IFN-γ production could be measured in CD1−/− mice at day 35, but no difference could be detected at days 35 and 56. Higher IL-4 production could also be detected in CD1−/− mice at days 17 and 35, but not at day 56. *p ≤ 0.05, **p ≤ 0.01.
This difference was most prominent in the acute phase of the disease, which argues for an early influence of CD1 on controlling T cell cytokine production and hence the CNS inflammation and EAE outcome. Interestingly enough, this cytokine overproduction was true for the representative Th1 cytokine IFN-γ as well as the most prominent Th1 cytokine IFN-γ. This highlights the complex network controlling Th responses, showing that CD1-dependent regulation is not solely exerted through Th2 instruction, as has been proposed by other authors (20, 35, 39), but rather acts through an overall cytokine suppression.

Furthermore, TGF-β1-producing cells were present in the CNS of CD1+/+ mice in the acute phase of the disease, whereas in CD1−/− mice, the enhanced EAE was correlated with near absence of cells producing this cytokine in the inflamed CNS. TGF-β1 is a cytokine with demonstrated potent immunomodulatory effects in autoimmune diseases and the levels of TGF-β have been reported to increase in both EAE models and in multiple sclerosis patients during various disease-suppressing treatments (48–55). In agreement with this, we propose that TGF-β1 could be an important mediator for CD1-dependent regulation, preventing the CNS autoimmune inflammation from turning chronic. The induction of this regulatory pathway is impaired in the CD1−/− mice, and this could be a second explanation for the augmented and chronic EAE. This result is well in concordance with a study by Szalay et al. (56) showing that in vivo treatment with CD1-blocking Abs reduced TGF-β production.

CD1-dependent cells could be regarded as part of both innate and adaptive immune responses and might therefore provide an important communicatory link between these two interregulated systems. By using different passive-transfer systems, we were able to separate the adaptive disease-driving autoantigen-specific T cell response from the adjuvant-induced innate Ag-independent mechanisms. When encephalitogenic T cells were transferred alone to CD1+/+ and CD1−/− mice, we could clearly show that the CD1-dependent regulatory pathways were not active, because both groups developed equally severe EAE. As this result differed from the clinical pattern observed with the active immunization protocol, we concluded that the effect on EAE through CD1 is operating during the T cell priming or activation phase. This could result from the recently postulated effect on priming of dendritic cells through interactions with CD1-restricted T cells (57). Presumably, the dendritic cells could present lipid Ags from the oil or mycobacteria in the CFA, and the interaction with CD1-restricted T cells leads to regulation or modulation of APC function. Alternatively, as inflammation alone could cause a rapid influx of CD1-restricted T cells (58), the immunization might create a local inflammatory meeting point where CD1-restricted T cells could modify dendritic cells irrespective of the Ag involved. Another possibility is that CD1-restricted T cells are directly activated by mycobacterial cell wall components and CpG motifs from the CFA. These could bind...
and activate Toll like receptor 2- and 9-bearing cells, respectively (59–61). Both of these receptors have been found on NKT cells (62, 63), which are the most characterized regulatory cells missing in CD1−/− mice. To further strengthen this hypothesis, we repeated the experiment, but this time with preactivation of the immune system with CFA immunization. As predicted, CD1−/− mice developed a less severe EAE compared with CD1+/+ mice when the innate immune system was activated during the passive transfer of encephalitogenic T cells. Others (20, 35, 39, 40) have shown that activation of NKT cells through a CD1 binding synthetic ligand could confer protection from EAE, but no agent naturally seen by the immune system has so far been shown to stimulate CD1-dependent regulation. With this study, we demonstrate that this regulation can be achieved through activation with a bacterial-containing adjuvant. This more mimics a physiological occurring event and thus provides insight in the role for CD1-dependent cells and pathways in controlling autoreactive T cells in an immune system constantly facing different pathogens. Our results are well in concordance with a previous study in NOD mice where immunization with CFA protected against diabetes development. This protection could be transferred through T cells to naive mice, and the active part of the CFA was shown to be the bacterial component (64). Also, in a recently published study by Furlan et al. (40), α-GalCer-mediated protection from EAE in B6 mice could only be observed when the α-GalCer was administered s.c. in CFA, whereas no protection was conferred when α-GalCer was given i.p. in PBS. Three of the above-mentioned studies (20, 39, 40) have also looked at MOG-induced EAE in B6.CD1−/− mice but, in variance with our results, could not show significant differences in clinical scores of EAE between CD1−/− and CD1+/+ mice. This discrepancy could be explained by the fact that, in their hands, the actively induced EAE protocol results in very severe clinical symptoms. In such a situation, the enhanced EAE, seen as an effect of CD1 absence, might be masked. Alternatively, it could be stressed that ES-129 genes variably present in the different used strains are likely to differ even in mice with the same levels of CD1 deficiency of NK1.1 CD4+ T cells that resembles NK1.1 CD4+ T cells. Eur. J. Immunol. 28:3172. Brossay, L., S. Tangri, M. Bix, S. Cardell, R. Locksley, and M. Kronenberg. 1998. Mouse CD1-autoreactive T cells have diverse patterns of reactivity to CD1+ targets. J. Immunol. 160:3681. 17. Brossay, L., and W. E. Paul. 1997. A population of CD62L−/−NK1.1+ CD4+ T cells that resembles NK1.1+ CD4+ T cells. Eur. J. Immunol. 28:3172.

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Acknowledgments

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