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Caspase-1 Regulates the Inflammatory Process Leading to Autoimmune Demyelination

Roberto Furlan,* Gianvito Martino,‡ Francesca Galbiati,ι Pietro L. Poliani,* Simona Smiroldo,‡ Alessandra Bergami,* Gaetano Desina,*,§ Giancarlo Comi,† Richard Flavell,‖ Michael S. Su,‖ and Luciano Adorini‡

T cell-mediated inflammation is considered to play a key role in the pathogenic mechanisms sustaining multiple sclerosis (MS). Caspase-1, formerly designated IL-1β-converting enzyme, is crucially involved in immune-mediated inflammation because of its pivotal role in regulating the cellular export of IL-1β and IL-18. We studied the role of caspase-1 in experimental autoimmune encephalomyelitis (EAE), the animal model for MS. Caspase-1 is transcriptionally induced during EAE, and its levels correlate with the clinical course and transcription rate of proinflammatory cytokines such as TNF-α, IL-1β, IFN-γ, and IL-6. A reduction of EAE incidence and severity is observed in caspase-1-deficient mice, depending on the immunogenicity and on the amount of the encephalitogenic myelin oligodendrocyte glycoprotein (MOG) peptide used. In caspase-1-deficient mice, reduced EAE incidence correlates with defective development of anti-MOG IFN-γ-producing Th1 cells. Finally, pharmacological blockade of caspase-1 in Biozzi AB/H mice, immunized with spinal cord homogenate or MOG35–55 peptide, by the caspase-1-inhibitor Z-Val-Ala-Asp-fluoromethylketone, significantly reduces EAE incidence in a preventive but not in a therapeutic protocol. These results indicate that caspase-1 plays an important role in the early stage of the immune-mediated inflammatory process leading to EAE, thus representing a possible therapeutic target in the acute phase of relapsing remitting MS. The Journal of Immunology, 1999, 163: 2403–2409.

T he caspase family comprises thus far 13 different cysteine proteases that are mainly involved in the apoptotic pathway (1). Among them, caspase-1, formerly named IL-1β-converting enzyme, which is activated by caspase-11-mediated proteolytic cleavage (2), is less involved in the apoptotic cascade but is prominent in inflammation because of its pivotal role in regulating the cellular export of proinflammatory cytokines such as IL-1β. Caspase-1 is elevated in interstitial macrophages during inflammatory bowel disease (3) and in a variety of organs, including the brain, in response to bacterial LPS administration (4). Further evidence on the role played by caspase-1 in inflammation comes from studies on caspase-1-deficient (−/−) mice and caspase-1 pharmacological inhibitors. Caspase-1−/− mice display an alteration in the export of several proinflammatory cytokines, namely IL-1β, IL-1α, IL-6, and TNF-α, although neither IL-1α nor IL-6 nor TNF-α are substrates for caspase-1 (5). Furthermore, caspase-1 proteolytically activates IL-18, and caspase-1−/− mice have also reduced serum levels of IL-18 and IFN-γ in response to LPS administration (6). Caspase-1−/− mice are resistant to LPS-induced endotoxic shock (7) and to the induction of experimental pancreatitis (8). In vivo pharmacological inhibition of caspase-1 protects mice from TNF-α-induced liver failure (9) and collagen-induced arthritis (10).

MS3 is an immune-mediated demyelinating disease of the CNS of unknown etiology (11). The pathological hallmark of the disease is the presence within the CNS of inflammatory infiltrates containing few autoreactive T cells and many pathogenic nonspecific mononuclear cells (12). It is currently believed that Ag-specific T cells provide the organ specificity of the pathogenic process and regulate the recirculation within the CNS of activated mononuclear cells releasing inflammatory myelinotoxic substances. These latter cells can be activated in the periphery by polyclonal inflammatory stimuli, thus determining disease recurrence (12, 13). Proinflammatory cytokines participate either in Ag-specific T cell activation or in peripheral activation of nonspecific mononuclear cells. TNF-α, IFN-γ, and IL-6 levels increase before disease relapses (13, 14). An increased number of disease relapses was observed in MS patients treated with IFN-γ (15). Moreover, TNF-α, IFN-γ, and IL-1β are present in demyelinating plaques (16), and IL-1β has been shown to be a mediator of the inflammatory process sustaining EAE, the animal model for MS (17).

We evaluated the role of caspase-1 in EAE. We found that caspase-1 mRNA blood levels parallel those of proinflammatory cytokines, such as IL-1β, IL-6, TNF-α, and IFN-γ, during EAE and peak at the time of maximal EAE severity. A reduction of EAE incidence and severity was observed in caspase-1−/− mice depending on the immunogenicity and on the amount of the encephalitogenic MOG peptide used. Finally, pharmacological blockade of caspase-1 reduced the incidence of EAE, induced either with SCH or MOG35–55 peptide, in a preventive but not therapeutic

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3 Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; SCH, spinal cord homogenate; AU, arbitrary units; p.i., postimmunization.
protocol. These results indicate that caspase-1 plays an important role in the immune-mediated pathogenic events leading to EAE and might represent a suitable therapeutic target of the active phase of the immune-mediated inflammatory demyelination.

Materials and Methods

Mice and immunizations

Female Biozzi AB/H mice, 4–6 wk old, were purchased from Harlan U.K. (Blackthorn, U.K.). Female C57BL/6 mice, 4–6 wk old were obtained by Charles River (Calco, Italy). Female (SV129 × C57BL/6)F1, mice, hereafter designated (SV129 × B6)F1, 4–6 wk old, were obtained by The Jackson Laboratory (Bar Harbor, ME). Caspase-1−/− mice had been obtained as previously described (5). Briefly, chimeric mice were obtained by injection of embryonic stem cells, in which the caspase-1 gene was disrupted and replaced with a neomycin resistance gene cassette, into C57BL/6 blastocysts. The chimeric males were then mated with C57BL/6 mice. Homozygous mice with two copies of the disrupted caspase-1 gene were identified by Southern blot of genomic DNA, and the absence of caspase-1 mRNA in caspase-1−/− mice was confirmed by RT-PCR analysis. Homozygous mice were then interbred and used for the experiments. All animals were housed in specific pathogen-free conditions and treated according to the guidelines of the Animal Ethical Committee of our Institute. Mice were immunized with IFA (Difco, Detroit, MI) supplemented with 4 mg/ml Mycobacterium tuberculosis (strain H37Ra; Difco) and MOG 35–55 (Multiple Peptide Systems, San Diego, CA), MOG 40–55 (Roche Milano Ricerche, Milan, Italy), or SCH from Biozzi AB/H mice. Two immunization schedules were used for peptides: a single injection of 200 µg or two injections of 300 µg peptide 7 days apart. For SCH immunization, 1 mg of Ag was given twice, at days 0 and 7. All injections were followed by i.p. administration of 500 ng pertussis toxin (Sigma, St. Louis, MI) the same day and 48 h later. Body weight and clinical score (0 = healthy, 1 = flaccid tail, 2 = ataxia and/or paresis of hind limbs, 3 = paralysis of hind limbs and/or paresis of forelimbs, 4 = tetraparalysis, 5 = moribund or death) were recorded daily.

Miniosmotic pumps

Miniosmotic pumps (Alzet 2001, Alza, Palo Alto, CA) were implanted s.c. in the dorsal flank of mice. The mean fill volume of pumps was −220 µl, and the mean pumping rate was −1 µl/h, delivering continuously for 10 days. Pumps were filled with 50 µg/ml of the caspase-1 inhibitor Z-Val-Ala-d-phe-asp-fluoromethylketone (Bachem, Bubendorf, Switzerland) or with 50 µg/ml gentamicin (Sigma, St. Louis, MO) supplemented with 200 µM gentamicin, 50 µg/ml of MOG 35–55 peptide. Cultures were incubated for 3 days in 5% CO2 in air, and the mean pumping rate was 370 µl/h.

Ag-specific proliferation assays

For T cell proliferation assays, draining lymph nodes were removed, and 4 × 10^5 lymph node cells per well were cultured in 96-well culture plates (Costar, Cambridge, MA) in synthetic H-1 medium (Ventrex Laboratories, Portland, ME) supplemented with 2 mM l-glutamine and 50 µg/ml gentamicin (Sigma, St. Louis, MO) and serial concentrations (1, 3, 10 µM) of MOG 35–55 peptide. Cultures were incubated for 3 days in 5% CO2 in air, and the mean pumping rate was 710 µl/h before harvesting with 1 µl [3H]Thd (40 Ci/mmol, Pharmacia Biotech, Amersham, Colombo Monzese, Italy). Incorporation of [3H]Thd was measured by liquid scintillation spectrometry.

Intracytoplasmatic staining for cytokine production

Lymph node cells (6 × 10^5 cells/well) were cultured in 96-well culture plates in synthetic H-1 medium with 10 µM MOG 35–55, plus 10 µM LPS (Escherichia coli 0111:B4) for 24 h at 37°C with 5% CO2 and ionomycin (50 ng/ml) for 4 h at 37°C, with 5% CO2 and 10 µg/ml brefeldin A (Novartis, Basel, Switzerland) added for the last 2 h to prevent expression of newly synthesized cytokine. After fixation with 4% paraformaldehyde for 20 min at room temperature, cells were stained for IFN-γ and IL-4 using the method of Openshaw et al. (20) and Galiati et al. (21). Cells were washed, preincubated for 10 min with PBS/FCS/saponin, and then incubated with FITC rat anti-mouse IFN-γ (XMG1.2, Pharmingen, San Diego, CA) and PE rat anti-mouse IL-4 (11B11, Pharmingen) or with isotype controls FITC- and PE-labeled rat IgG1, x (B3-34, Pharmingen). After 30 min, cells were washed twice with PBS/FCS/saponin and then with PBS containing 5% FCS without saponin to allow membrane closure. Cell membranes were then stained with Cy-Chrome-labeled anti-CD4 (L3T4, Pharmingen) for 15 min at room temperature. Analysis was performed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) equipped with CellQuest software, and 50,000 events were acquired.

ELISA for IFN-γ secretion

IFN-γ was quantified by two-site sandwich ELISA using polyclonal microtiter plates (Falcon 3012) coated with AN-18,17 (24 µg/ml in carbonate buffer, 400 µg/ml FCS, previously described (21)). Samples (50 µl/well) were incubated overnight at 4°C, and the medium was aspirated. The plates were washed three times with PBS/FCS/saponin and blocked with 3% BSA (FCS) for 1 h at 37°C. The plates were washed and incubated overnight at room temperature with 10 µg/ml mouse IFN-γ (XMG1.2, Pharmingen) as described. Wells were washed and incubated with 1:1000 dilution of rabbit polyclonal anti-mouse IFN-γ (NovoaBiochem, Bensheim, Germany) for 1 h at room temperature, followed by a 1:1000 dilution of rabbit peroxidase-conjugated anti-rabbit IgG (Sigma, St. Louis, MO). The plates were washed three times with PBS/FCS. After washing, 10 µl of 5 mg/ml 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma, St. Louis, MO) and 10 µl of 100 µM H2O2 were added to each well. The plates were incubated for 30 min at room temperature, and the reaction was stopped with 10 µl of 4M glycine. The absorbance was read at 405 nm with an automated microplate ELISA reader (MR5000, Dynatech Laboratories, Chantilly, VA). IFN-γ level was expressed as pg/ml. Detection limit was 15 pg/ml.

Neuropathological features in EAE mice

At the time of sacrifice, mice were transcardially perfused with 4% paraformaldehyde. Brains and spinal cords were removed and postfixed in the same fixative for 2–4 h, washed in PBS, and then embedded in paraffin. Tissue sections were cut at 4 µm on a microtome and stained for histological examination. Hematoxylin and eosin staining was used to reveal tissue structure. For the detection of microglia/macrophages, Luminol Fast Blue staining was used to reveal demyelinated areas, and Bielschowsky staining was used to detect axonal loss. Macrophages were stained using peroxidase-labeled BS-1 isoclit B4 (Sigma), whereas T cells using a rat anti-CD3 Ab (pan-T cell marker; Serotec Ltd, Oxford, U.K.) revealed using a biotin-labeled secondary anti-rat Ab (Amersham). Neuropathological findings were quantified on a cross-section of the spinal cord per mouse. The number of perivascular inflammatory infiltrates was calculated and expressed as the numbers of inflammatory infiltrates per mm², whereas demyelinated areas and axonal loss were expressed as the percentage of...
Damaged area per mm² (22). T cells and macrophages were counted and expressed as the number of cells per mm².

Statistical analysis
Data are expressed as mean ± SE. Student’s t test for unpaired data was used to compare cytokine and caspase-1 mRNA levels and the cytokine protein levels. EAE cumulative score was calculated by summing up each individual score registered in any group of mice studied during the follow-up period levels. Comparison between cumulative scores was performed using Student’s t test. A χ² test was used to compare EAE incidence in the different groups of mice. p < 0.05 was considered significant.

Results
Caspase-1 mRNA is up-regulated in the course of EAE
C57BL/6 mice immunized with MOG₃₅–₅₅ were bled once a week for 5 weeks after immunization. RT-PCR was performed on blood samples to determine caspase-1, IL-1β, IL-6, TNF-α, and IFN-γ mRNA levels. Caspase-1 mRNA levels increased almost 8-fold (Fig. 1A) during EAE from basal levels recorded at day 7 p.i. This increase paralleled disease severity, peaking 4 wk p.i. (day 27 p.i., 32.9 ± 16.8 AU; p = 0.01 vs day 7 p.i.) (Fig. 1A) when EAE clinical score reached its maximum (Fig. 1C). Caspase-1 mRNA level increase paralleled that of the caspase-1 substrate IL-1β, which showed a 2-fold increase (day 27 p.i., 3.3 ± 0.9 AU; p = 0.03 vs day 7 p.i.) (Fig. 1B); IL-6 mRNA increased almost 8-fold (day 27 p.i., 15.4 ± 7.6 AU; p = 0.04) (Fig. 1B); TNF-α mRNA increased almost 3-fold (day 27 p.i., 12.2 ± 5.4; p = n.s. vs day 7 p.i.) (Fig. 1B), and IFN-γ mRNA 4-fold (day 27 p.i., 1.6 ± 0.5 AU; p = 0.02 vs day 7 p.i.) (Fig. 1B). These data indicate that caspase-1 is up-regulated at the transcriptional level during the course of EAE and that this up-regulation parallels that of proinflammatory cytokines such as IL-1β, IFN-γ, TNF-α, and IL-6.

Caspase-1−/− mice are less susceptible to EAE induction
To further explore the role of caspase-1 in EAE, we studied the susceptibility of caspase-1−/− mice to MOG peptide-induced EAE. We first analyzed EAE susceptibility of (SV129 × B6)F₁, C57BL/6, and caspase-1−/− mice. Caspase-1−/− mice are susceptible to EAE induction but develop a less severe disease than control mice. B. EAE immunization protocol with 200 or 600 μg MOG₃₅–₅₅ in C57BL/6 (n = 200 μg MOG₃₅–₅₅, ○ = 200 μg MOG₄₀–₅₅, ● = 600 μg MOG₄₀–₅₅). Immunization of caspase-1−/− mice with the low amount (200 μg) of the shorter peptide (MOG₄₀–₅₅) does not lead to EAE development. Irrespective of the dose of MOG₄₀–₅₅ used, C57BL/6 mice always show a more severe EAE than do caspase-1−/− mice.
Inhibition of the Th1 response in caspase-1-deficient mice

To elucidate the possible causes of reduced susceptibility of caspase-1−/− mice to EAE, we analyzed mice immunized with 200 μg of MOG35–55 peptide in which the most striking difference between caspase-1−/− and C57BL/6 was observed (Fig. 2B, Table I). Five caspase-1−/− mice and 5 C57BL/6 mice were sacrificed 10 days after immunization with MOG40–55, and cells from the draining lymph nodes were obtained. Proliferation assays to MOG40–55 showed no impairment in the responsiveness of T cells from caspase-1−/− compared with C57BL/6 mice (Fig. 3A). We detected, however, decreased levels of IFN-γ secreted in the supernatant of MOG40–55 restimulated cells (Fig. 3B) from caspase-1−/− compared with C57BL/6 mice (p = 0.01). Intracytoplasmic staining for IFN-γ and IL-4 of MOG40–55-restimulated cells showed reduced IFN-γ-producing cells and no induction of IL-4-producing cells (Fig. 3, C–E). These results suggest that cells from caspase-1−/− mice efficiently present the encephalitogenic peptide to T cells but are defective in Th1 development.

Caspase-1 inhibitor administration prevents but does not treat EAE

Pharmacological blockade of EAE with caspase-1 inhibitors was tested in Biozzi AB/H mice immunized with SCH or MOG35–55. In Biozzi mice, SCH induces a very aggressive relapsing-remitting EAE (25), whereas MOG35–55 induces a more chronic progressive disease. We administered vehicle or Z-Val-Ala-dl-Asp-fluoromethylketone, a highly specific, cell-permeable, and irreversible inhibitor of caspase-1-like proteases (26) to Biozzi mice before immunization with SCH or MOG35–55. Because this caspase-1 inhibitor is a peptide with a very short half-life in vivo, we implanted s.c. mini-osmotic pumps able to continuously release the inhibitor for 10 days. Continuous administration of the caspase-1 inhibitor induced a clearcut suppression of either SCH-induced or MOG35–55-induced EAE compared with vehicle-treated mice (Table II).

The cumulative EAE score, representing the disease burden, was significantly lower in caspase-1 inhibitor-treated mice than in vehicle-treated mice in both immunization protocols (Table II).

Table I. EAE in (SV129 × B6)F1, C57BL/6, and caspase-1−/− mice

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>MOG Peptide (amount)</th>
<th>EAE Incidence (%)</th>
<th>EAE Onset (range)</th>
<th>Maximum EAE Score (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(SV129 × B6)F1</td>
<td>MOG35–55 (200 μg)</td>
<td>4/4</td>
<td>20.0 ± 3.7</td>
<td>1.9 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>MOG35–55 (200 μg)</td>
<td>6/6</td>
<td>19.2 ± 3.0</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>MOG35–55 (200 μg)</td>
<td>11/12</td>
<td>17.3 ± 2.6</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>MOG40–55 (200 μg)</td>
<td>5/7</td>
<td>27.8 ± 2.4</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>MOG35–55 (600 μg)</td>
<td>7/9</td>
<td>23.2 ± 3.4</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>MOG40–55 (200 μg)</td>
<td>0/12</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Caspase-1−/−</td>
<td>MOG35–55 (200 μg)</td>
<td>0/12</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

* EAE onset is expressed as day post immunization ± SE.
* EAE score is expressed as mean maximum score ± SE.
* MOG40–55 was administered twice 1 wk apart (300 ± 300 μg).
* MOG35–55 has been administered twice 1 wk apart (600 ± 600 μg). MOG35–55 and MOG40–55 were administered twice 1 wk apart (100 ± 100 μg).
* NA, not applicable.
However, the mean day of onset and the mean clinical score of sick animals were comparable between groups (Table II). In the SCH-induced group, two animals for each treatment were sacrificed for neuropathology examination. Animals treated with the inhibitor had less infiltrates per mm² than did vehicle-treated controls (0.4 and 1.6 vs 6.6 and 3.1, respectively) (Fig. 4, A and B), less demy-
Caspase-1 is crucial in inflammatory process leading to EAE

Discussion

The central role of proinflammatory cytokines in orchestrating the pathogenic process leading to EAE has been extensively studied (27–29), but few data are thus far available on the posttranslational mechanisms of regulation of cytokine secretion during this experimental autoimmune CNS-confined disease. We focused our attention on the proteolytic enzyme caspase-1, because previous studies showed that its pharmacological inhibition or its absence (i.e., deletion mutant mice) mainly affects the secretion of proinflammatory cytokines such as IL-1β, IL-1α, IL-6, TNF-α, IL-18, and IFN-γ (5–7). We found that caspase-1 transcription is up-regulated during mouse EAE, reaching a peak at maximal disease severity. This induction is higher compared with other proinflammatory cytokines previously shown to be up-regulated during EAE (27) and is similar to that found in the Lewis rat EAE (30). These data suggest that proteolytic activation of proinflammatory cytokines is a crucial step in the immune-mediated process leading to EAE.

Caspase-1−/− mice were susceptible to MOG peptide-induced EAE. Disease susceptibility was associated with the number of immunizations and the dose and the MHC-binding affinity of the encephalitogenic MOG-peptide. In our hands, MOG40–55 showed a 3-fold weaker binding (IC50 = 360 pM) compared with MOG35–55 (IC50 = 1 nM) to the I-A8 molecule (data not shown). However, the disease induced in caspase-1−/− mice was always less severe than in C57BL/6 or SV129+/− mice. The most severely impaired Th1 cell development as indicated by the lower percentage of IFN-γ-producing cells from caspase-1−/− vs C57BL/6 mice. The most likely explanation of this finding possibly resides in the cleavage activity of caspase-1 on the precursor of IL-18 into its mature form (6). Although IL-18 in itself does not induce Th1 cell development (31, 32), which is mostly driven by IL-12 (33), Th1 cell development independent from IL-12 could be induced by the cooperative action of IL-18 and other factors (34). These factors may include IFN-γ itself (35) and IL-1αβ (33). Thus, the simultaneous caspase-1-dependent defect in IL-18 and IL-1αβ production could explain the impaired Th1 development and the reduced EAE in caspase-1−/− mice. A more aggressive immunization protocol, however, is able to overcome this limiting step in EAE induction, likely because other proteases may replace caspase-1 in the cleavage of pro-IL-1β and pro-IL-18.

Results obtained with caspase-1 inhibitor administration are consistent with the lower susceptibility to EAE of caspase-1−/− mice. The preventive administration of the caspase-1 inhibitor Z-Val-Ala-dL-Asp-fluoromethylketone dramatically reduced both relapsing-remitting or chronic-progressive EAE in two different mouse strains. However, the small proportion (35%) of mice not protected from EAE by caspase-1 inhibitor treatment developed a clinical course of EAE indistinguishable from that of controls. As in inflammatory fluids other proteases (i.e., trypsin, chymotrypsin, elastase, granzyme A) (36) can replace caspase-1 enzymatic activity, an immunization protocol able to induce a potent local inflammation may bypass its requirement. Nevertheless, recent data indicate that Z-Val-Ala-dL-Asp-fluoromethylketone is active also on other cysteine proteases involved in inflammatory reactions but not in the apoptotic cascade, such as caspasases-4 and -5 (37).

Administration of Z-Val-Ala-dL-Asp-fluoromethylketone during the effector phase of SCH-induced EAE (i.e., therapeutic protocol) had no effect on the disease course and severity. These data are suggestive for a crucial role played by caspase-1 in the peripheral activation of proinflammatory cytokines during the induction phase of EAE. The lack of efficacy of caspase-1 inhibitors once the inflammatory cascade has already determined CNS infiltration and damage might be also explained by the impaired blood-brain barrier crossing of caspase-1 inhibitors (26).

In conclusion, our results indicate that caspase-1 plays a crucial role in the development of the immune-mediated inflammatory process leading to CNS demyelination. EAE development is impaired in caspase-1−/− mice, although the requirement for caspase-1 in the inflammatory phase of EAE can be bypassed. The role of caspase-1 in autoimmune demyelination is further corroborated by the significant reduction of EAE incidence in mice preventively treated with the caspase-1-inhibitor Z-Val-Ala-dL-Asp-fluoromethylketone. However, the inefficacy of caspase-1 inhibitors in blocking ongoing EAE suggests that this enzyme is crucial in the early phase of the inflammatory process leading to immune-mediated demyelination. Thus, caspase-1 could represent both a marker of inflammation sustaining immune-mediated demyelination and a possible therapeutic target of the acute phase of relapsing-remitting MS.

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