IL-6-deficient mice are resistant to the induction of experimental autoimmune encephalomyelitis provoked by myelin oligodendrocyte glycoprotein

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Keywords: autoimmunity, cytokines, knockout mouse, neuroimmunology

Abstract

The role of IL-6 in experimental autoimmune encephalomyelitis (EAE) provoked by myelin oligodendrocyte glycoprotein (MOG) was investigated using IL-6-deficient mice. We show here that IL-6-deficient mice were resistant to the MOG-induced EAE as compared to wild-type mice (one out of 18 versus 17 out of 20). The delayed-type hypersensitivity response, lymphocyte proliferation response and antibody reactivity to MOG in IL-6-deficient mice were significantly lower than those in wild-type mice. Furthermore, the histological examination revealed that no infiltration of inflammatory cells was observed in the central nervous system of IL-6-deficient mice. These results indicate that IL-6 may play a crucial role in the induction phase of EAE. Given the potential relevance of this animal model for multiple sclerosis (MS), it is possible that anti-IL-6 therapy may be useful in the prevention of relapses of MS.

IL-6 is a multifunctional cytokine that regulates immune response. IL-6 provides multiple signals such as B cell differentiation, induction of acute phase proteins, neural cell differentiation, cytotoxic T cell differentiation and T cell growth, and it is considered to contribute to proliferative and autoimmune diseases (1,2). Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) and is believed to be an autoimmune disease associated with abnormalities in immunoregulation (3). Several studies have suggested that IL-6 plays a role in MS. Augmented levels of IL-6 in cerebrospinal fluid (CSF) and in serum of MS patients have been reported (4–6). IL-6 has been detected in MS lesions (7,8), and mononuclear cells in CSF and in blood of MS patients have expressed high levels of IL-6 mRNA (9). However, the role of IL-6 in MS has not clearly been understood, particularly since IL-6 is a pleiotropic cytokine involved in the regulation of immune responses (1,2).

To clarify the uncertainty, we investigated the role of IL-6 on experimental autoimmune encephalomyelitis (EAE), an inflammatory demyelinating disease that serves as a model for MS. The up-regulation of IL-6 gene expression in the CNS of animals with EAE correlated well with the severity (10–12) and the elevation of IL-6 gene expression in peripheral blood mononuclear cells was observed at the pre-stage of EAE (13). These results suggested that IL-6 might be a pathogenetic factor of EAE. However, there have been two conflicting results with regard to anti-IL-6 therapy in EAE. Willenborg et al. have shown that anti-IL-6 has no significant effect on EAE, but exogenous IL-6 inhibits EAE when delivered by the recombinant vaccinia virus system (14). By contrast, Gijbels et al. have reported that the administration of anti-IL-6 antibody prevented actively induced and adoptively transferred EAE in mice (15). In the latter study, however, it is uncertain whether neutralization of the IL-6 activity or enhancement of the IL-6 activity by anti-IL-6 antibody caused suppression of EAE.

To further define the role of IL-6 in autoimmune demyelinating disease, such as MS, we have investigated the influence...
IL-6-deficient mice are resistant to EAE

of IL-6 on the course of EAE using IL-6-deficient mice with the C57BL/6 background. Myelin oligodendrocyte glycoprotein (MOG) was chosen as an encephalitogen since EAE induced with MOG may closely resemble MS (16) and C57BL/6 mice (H-2b) are highly susceptible to MOG-induced EAE (17,18). Human recombinant MOG (rMOG) was prepared with the previously described method (19). The non-glycosylated glutathione sulfotransferase fusion protein representing the Ig extracellular domain (amino acids 1–125) of rMOG from Escherichia coli was solubilized with urea and then purified by CM-Sephadex 50 chromatography after being cleaved with thrombin. SDS–PAGE and Western blot analysis revealed that highly purified rMOG was obtained (19). Highly specific T cell and antibody responses to rMOG in C57BL/6 mice immunized with rMOG have also been confirmed (20). 129/Sv×C57BL/6 mice with a disrupted IL-6 gene have been previously described (21) and were bred under specific pathogen-free conditions. These mice were backcrossed onto the C57BL/6 background for seven generations. The presence of the defective IL-6 allele in mice was determined by PCR on genomic DNA as previously described (21). Female IL-6 knockout [IL-6(–/–)] mice, wild-type [IL-6(+/+) ] mice and heterozygous [IL-6(+/-)] mice of H-2b haplotype (C57BL/6) were used between the ages of 8 and 16 weeks for EAE experiments.

The course of the MOG-induced disease in IL-6(+/-), IL-6(+/-) and IL-6(+/-) mice is shown in Fig. 1(A and B) and summarized in Table 1. While IL-6(+/-) mice immunized with rMOG did not lose body weight, IL-6(+/-) and IL-6(+/-) mice showed weight loss after immunization (Fig. 1A). While only one out of 18 IL-6(+/-) mice developed mild EAE, IL-6(+/-) and IL-6(+/-) mice developed severe EAE at high frequency (Table 1 and Fig. 1B). No significant difference was observed between IL-6(+/-) mice and IL-6(+/-) mice. This result indicates that IL-6 is an important component in the pathogenesis of EAE. In an attempt to elucidate the mechanism for resistance of IL-6-deficient mice to EAE, we examined the immune response to MOG in the periphery and the histopathology in the CNS.

Delayed-type hypersensitivity (DTH) responses to rMOG were measured 15 days after the primary immunization with rMOG and CFA in IL-6(+/-) and IL-6(+/-) mice. As illustrated in Fig. 2, both IL-6(+/-) and IL-6(+/-) mice displayed a significant increase in footpad swelling in response to MOG; however, IL-6(+/-) mice had a significantly lower DTH response as compared to IL-6(+/-) mice. In addition, lymphocyte proliferation in response to rMOG was tested. As shown in Fig. 3, lymph node (LN) cells from IL-6(+/-) and IL-6(+/-) mice proliferated in response to rMOG, but a significantly lower response to rMOG was observed in IL-6(+/-) mice as compared to wild-type mice. These results indicate that T cell responses to MOG are lower in IL-6-deficient mice as compared to wild-type mice. Impairment in generation and/ or activation of encephalitogenic T cells could well explain the resistance to induction of EAE in IL-6-deficient mice, since EAE is mediated by CD4+ T cells of the Th1 phenotype in response to CNS myelin proteins (18,22).

To further elucidate the mechanisms for impairment of T cell responses to MOG in IL-6-deficient mice, phenotypic analysis of LN cells in IL-6(+/-) and IL-6(+/-) mice immunized with or without rMOG were performed. As shown in Table 2, a significantly lower population of T cells in IL-6(+/-) mice was observed, although the ratio of CD4/CD8 was not significantly
The difference in the footpad thickness before and after challenge for individual mice was recorded. Histograms represent the mean increase in footpad thickness (mm) of mice (n = 4).

Significant DTH responses were observed in both IL-6(+/+) and IL-6(–/–) mice (*P < 0.05 with Student’s t-test) when compared to OVA-treated controls, while a significantly lower DTH response was observed in IL-6(–/–) mice as compared to IL-6(+/+) mice (F<0.05 with Student’s t-test).

### Table 1. MOG induced EAE in IL-6(+/+), IL-6(–/–) and IL-6(–/–) mice

<table>
<thead>
<tr>
<th>Group</th>
<th>No. sick/Maximal clinical score</th>
<th>Day of onset</th>
</tr>
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<tbody>
<tr>
<td>IL-6(+/+)</td>
<td>17/20 3.0 ± 1.0</td>
<td>15.1 ± 3.1</td>
</tr>
<tr>
<td>IL-6(–/–)</td>
<td>14/20 2.9 ± 0.9</td>
<td>15.6 ± 3.9</td>
</tr>
<tr>
<td>IL-6(–/–)</td>
<td>1/18 1</td>
<td>28</td>
</tr>
</tbody>
</table>

*aThe method for EAE induction was described in Fig. 1.

*bNumber of mice with clinical signs per total number of mice from four experiments.

*cData were based on mice with clinical disease.

*dP<0.001 versus IL-6(+/+) mice with Fisher’s exact probability test.

The results in the present study suggesting that the immune response was measured as the mean c.p.m. ± SD based on three independent experiments.

A significantly lower response to rMOG was observed in IL-6(–/–) mice as compared with IL-6(+/+) mice (F<0.05 with Student’s t-test).

**Figure 2.** DTH reactions to rMOG in IL-6(+/+) and IL-6(–/–) mice. Twenty-five micrograms of rMOG or ovalbumin (OVA) in PBS were injected into each footpad 15 days after primary immunization with rMOG (100 µg/mouse) and CFA. Footpad swelling was measured at 0 and 24 h after the secondary antigenic challenge by using micrometer callipers. The difference in the footpad thickness before and after challenge for individual mice was recorded. Histograms represent the mean increase in footpad thickness (mm) for each group of mice (n = 4). Significant DTH responses were observed in both IL-6(+/+) and IL-6(–/–) mice (*P < 0.05 with Student’s t-test as compared to OVA-treated controls), while a significantly lower DTH response was observed in IL-6(–/–) mice as compared to IL-6(+/+) mice (F<0.05 with Student’s t-test).

**Figure 3.** Lymphocyte proliferation in response to rMOG in IL-6(+/+) and IL-6(–/–) mice. At 10–12 days after immunization, mice were killed and a single-cell suspension was prepared from the inguinal lymph nodes (LN). After two washes, the LN cells were cultured with or without 10 µg/ml or 25 µg/ml of rMOG or 25 µg/ml of myelin basic protein (MBP) (Sigma, St Louis, MO) at a concentration of 2×10^6/ml in RPMI 1640 medium supplemented with 5% FCS serum, HEPEIS (20 mM), 2-mercaptoethanol (3×10^-2 M), penicillin (100 U/ml) and streptomycin (100 µg/ml). Cultures were incubated for 72 h in 96-well, round-bottomed microtiter plates (0.2 ml/well) at 37°C in humidified 5% CO2. Wells were pulsed for the final 18 h of culture with 2 µCi [3H]thymidine. [3H]Thymidine uptake was measured by a Betaplate Counter (Wallac, Gaithersburg, MD). Results are expressed as the mean c.p.m. ± SD based on three independent experiments.

A significantly lower response to rMOG was observed in IL-6(–/–) mice as compared with IL-6(+/+) mice (F<0.05 with Student’s t-test).
response in IL-6-deficient mice might be generally suppressed are consistent with the previous reports concerning the function of IL-6 (1, 2, 21). However, as for DTH response, there have been conflicting results. Jayaraman et al. showed that IL-6 enhanced DTH (28), while Mihara et al. reported that IL-6 inhibited it (29). The mechanism for the inhibitory effect of IL-6 on DTH remains unclear but it may be mediated by indirect ways such as the induction of glucocorticoid or the inhibition of IL-1 and/or tumor necrosis factor (TNF)-α production (30, 31) as Mihara et al. suggested (29). Based

Table 2. Phenotype of LN cells from IL-6(+/+) and IL-6(−/−) mice immunized with or without rMOG

<table>
<thead>
<tr>
<th></th>
<th>CD3 (%)</th>
<th>CD4 (%)</th>
<th>CD8 (%)</th>
<th>B220 (%)</th>
</tr>
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<tbody>
<tr>
<td>LN from non-treated mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6(+/+)</td>
<td>66.3±2.1</td>
<td>37.3±2.1</td>
<td>27.3±1.2</td>
<td>28.0±2.7</td>
</tr>
<tr>
<td>IL-6(−/−)</td>
<td>61.0±1.7</td>
<td>32.0±1.7</td>
<td>30.0±1.4</td>
<td>33.5±2.7</td>
</tr>
<tr>
<td>LN from MOG-immunized mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6(+/+)</td>
<td>43.0±6.6</td>
<td>25.7±5.5</td>
<td>17.3±2.9</td>
<td>47.7±4.7</td>
</tr>
<tr>
<td>IL-6(−/−)</td>
<td>34.7±3.8</td>
<td>19.3±1.5</td>
<td>14.3±1.5</td>
<td>58.3±3.8</td>
</tr>
</tbody>
</table>

*aFreshly isolated inguinal LN cells (5×10^5 cells) from IL-6(+/+) and IL-6(−/−) mice 15 days after immunization with rMOG or from non-treated IL-6(+/+) and IL-6(−/−) mice were used to determine the phenotypes of these cells. After two washes, LN cells were incubated with rat anti-mouse mAb specific for FITC-conjugated CD3 (Serotec, Oxford, UK), phycoerythrin (PE)-conjugated CD4 (PharMingen, San Diego, CA), PE-conjugated CD8 (PharMingen) or FITC-conjugated B220 (Caltag, La Jolla, CA) for 45 min on ice and analyzed on FACScan flow cytometer with CellQuest software (Becton Dickinson, Mountain View, CA). Data for >1×10^4 cells were collected.*

The data are expressed as percentage (mean ± SD from three independent experiments) of total lymphocytes, characterized by their scatter properties.

bP<0.05 versus IL-6(+/+) mice with Student’s t-test.

tableCaptionTable 3. Analysis of spinal cords of IL-6(+/+) and IL-6(−/−) mice immunized with rMOG

<table>
<thead>
<tr>
<th></th>
<th>Mean clinical score</th>
<th>No. of histological abnormalities</th>
<th>Histological score (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6(+/+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 18</td>
<td>2.0</td>
<td>3/3</td>
<td>2.0 ± 1.3</td>
</tr>
<tr>
<td>day 35</td>
<td>1.3</td>
<td>3/3</td>
<td>1.7 ± 0.7</td>
</tr>
<tr>
<td>day 60</td>
<td>1.0</td>
<td>2/3</td>
<td>0.9 ± 0.7</td>
</tr>
<tr>
<td>IL-6(−/−)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 18</td>
<td>0</td>
<td>0/3</td>
<td>0</td>
</tr>
<tr>
<td>day 35</td>
<td>0</td>
<td>0/3</td>
<td>0</td>
</tr>
<tr>
<td>day 60</td>
<td>0</td>
<td>0/3</td>
<td>0</td>
</tr>
</tbody>
</table>

*aSpinal cords were obtained from three IL-6(+/+) mice and three IL-6(−/−) mice at day 18, 35 and 60 respectively. Each spinal cord was removed, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer and then embedded in paraffin. Sections (5 µm) were prepared and stained with H & E and KB for assessment of inflammation and demyelination respectively. The sections at cervical, thoracic and lumbar levels of the spinal cord from each mouse were examined.*

bMean clinical score of mice at each day used for histological examination.

cNumber of mice with histological abnormalities (inflammation and/or demyelination) per total number of mice (n = 3 at each day).

DatatableCaptionTable 3. Analysis of spinal cords of IL-6(+/+) and IL-6(−/−) mice immunized with rMOG

Response to rMOG was determined by ELISA as previously reported (16). Briefly, rMOG was diluted to a concentration of 1 µg/ml in 0.1 M carbonate buffer, pH 9.6, and coated to 96-well microtiter plates that had been preincubated with 0.2% glutaraldehyde. After coating overnight at 4°C, the plates were blocked with 3% BSA/PBS and were incubated with 1:10,000 dilution of mouse serum for 1 h at 37°C and the specific antibody binding was determined using an anti-mouse IgG biotin conjugate (Nittrei, Tokyo, Japan) and streptavidin–horseradish peroxidase. Tetramethylbenzidine was used as a substrate and the OD was measured at 450 nm. The results were expressed as OD values. Significantly lower anti-rMOG IgG levels in IL-6(−/−) mice were observed as compared with IL-6(+/+) mice at day 18 and 60 (*P<0.05 with Student’s t-test).
on the results of the present study, we believe that the relative reduction in the number of T cells and the impaired T cell proliferation and/or activation, which are possibly caused by the loss of IL-6, may explain the lower DTH response to MOG in IL-6-deficient mice. Therefore, it is likely that IL-6 itself plays an important role in DTH response.

Considering the report that the Tp1 response was impaired in IL-6-deficient mice (32) and the reduced immune response to MOG in IL-6-deficient mice as we observed in this study, either a shift from Tp1 to Tp2 and/or a diminished levels of their corresponding cytokines might well be responsible for the resistance of IL-6-deficient mice to EAE, since EAE is believed to be mediated by Tp1 cytokines (22). Taken together, we believe that IL-6 plays an important role in the induction phase of EAE, perhaps by enhancing the myelin antigen-specific T and B cell responses and/or by modulating production of cytokines in the periphery.

It is also possible that IL-6 may have an effect on the effector phase of EAE. The up-regulation of IL-6 has been detected within the CNS of animals with EAE and correlated with the severity and/or the course of EAE (10–12). In addition, overexpression of IL-6 in the CNS of mice has been reported to cause neurological symptoms (33), implying that IL-6 participated in CNS inflammation. However, since no inflammatory response was observed in the CNS of IL-6-deficient mice, we could not elucidate the role of IL-6 in the effector phase of EAE. To clarify the role of IL-6 in the induction and the effector phase of EAE, experiments on passively transferred EAE using IL-6(-/-) mice are necessary and currently being attempted.

Although inflammatory cytokines such as IL-1, IL-2, IL-6, IFN-γ, TNF-α and TNF-β have been considered to participate in the pathogenesis of EAE, recent studies using cytokine knockout mice have demonstrated that some of them are not essential for the induction of EAE: IFN-γ-deficient mice and IFN-γ-receptor-deficient mice have been shown to be susceptible to MBP- and MOG-induced EAE respectively (34,35). TNF-α-deficient mice have also been shown to be susceptible to MOG-induced EAE (20,36). TNF-β-deficient mice immunized with MOG have developed EAE (67%), but had significantly milder EAE (37). On the contrary, TNF-α and TNF-β double knockout mice immunized with the mouse spinal cord homogenate or with proteolipid protein developed a very severe form of EAE (38). Although there are some differences among those reports, which may be due to differences in materials such as encephalitogenic antigens and mouse strains, those results indicate that other cytokines can compensate for those three proinflammatory cytokines in induction of EAE. To the best of our knowledge, IL-6 is the only cytokine so far reported that may have a crucial role in induction of EAE.

In conclusion, the frequency of EAE was significantly low and the infiltration of inflammatory cells in the CNS was significantly reduced in IL-6 knockout mice immunized with MOG. Based on our findings, we believe that IL-6 may be an important factor for development of MS and anti-IL-6 therapy may be promising in MS, especially in the prevention of relapse.

Acknowledgements
We would like to thank Dr M. Kopf (Basel Institute for Immunology, Switzerland) for providing the IL-6 knockout mice and Dr T. Tanaka (Osaka University Medical School, Japan) for his advice for the antibody assay. This work was supported by Grant-in-Aid for Scientific Research (B) from the Ministry of Education, Science and Culture, Japan and by grants from the National Health and Medical Research Council and the National Multiple Sclerosis Society of Australia.

Abbreviations
CFA complete Freund’s adjuvant
CNS central nervous system
CSF cerebrospinal fluid
DTH delayed-type hypersensitivity
EAE experimental autoimmune encephalomyelitis
KB klüver–Barrera
LN lymph node
MBP myelin basic protein
MOG myelin oligodendrocyte glycoprotein
MS multiple sclerosis
OVA ovalbumin
rMOG recombinant MOG
TNF tumor necrosis factor

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IL-6-deficient mice are resistant to EAE

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