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Blockade of CD40 Ligand Suppresses Chronic Experimental Myasthenia Gravis by Down-Regulation of Th1 Differentiation and Up-Regulation of CTLA-4

Sin-Hyeog Im,* Dora Barchan,* Prasanta Kumar Maiti,* Sara Fuchs,2,* and Miriam C. Souroujon*†

Myasthenia gravis (MG) and experimental autoimmune MG (EAMG) are T cell-dependent Ab-mediated autoimmune disorders, in which the nicotinic acetylcholine receptor (AChR) is the major autoantigen. Th1-type cells and costimulatory factors such as CD40 ligand (CD40L) contribute to disease pathogenesis by producing proinflammatory cytokines and by activating autoreactive B cells. In this study we demonstrate the capacity of CD40L blockade to modulate EAMG, and analyze the mechanism underlying this disease suppression. Anti-CD40L Abs given to rats at the chronic stage of EAMG suppress the clinical progression of the autoimmune process and lead to a decrease in the AChR-specific humoral response and delayed-type hypersensitivity. The cytokine profile of treated rats suggests that the underlying mechanism involves down-regulation of AChR-specific Th1-regulated responses with no significant effect on Th2- and Th3-regulated AChR-specific responses. EAMG suppression is also accompanied by a significant up-regulation of CTLA-4, whereas a series of costimulatory factors remain unchanged. Adoptive transfer of splenocytes from anti-CD40L-treated rats does not protect recipient rats against subsequently induced EAMG. Thus it seems that the suppressed progression of chronic EAMG by anti-CD40L treatment does not induce a switch from Th1 to Th2/Th3 regulation of the AChR-specific immune response and does not induce generation of regulatory cells. The ability of anti-CD40L treatment to suppress ongoing chronic EAMG suggests that blockade of CD40L may serve as a potential approach for the immunotherapy of MG and other Ab-mediated autoimmune diseases. The Journal of Immunology, 2001, 166: 6893–6898.

Myasthenia gravis (MG) and its animal model, experimental autoimmune MG (EAMG), are caused by autoantibodies against the nicotinic acetylcholine receptor (AChR) at the neuromuscular junction of skeletal muscle. Even though these diseases are mediated by self-reactive Abs, T cells have been demonstrated to have a pivotal role in the autoimmune process. T cells produce proinflammatory cytokines (IL-12 and IFN-γ) and deliver costimulatory signals to autoreactive B cells, inducing them to produce pathogenic autoantibodies.

EAMG in rats mimics human MG in its clinical and immunopathologic manifestations and is a reliable model to investigate therapeutic strategies for myasthenia. In an attempt to develop an Ag-specific immunotherapy for MG, we have previously demonstrated that mucosal administration of recombinant fragments of human AChR prevents the induction of EAMG and suppresses ongoing EAMG in rats (1, 2). The mucosal tolerance was mediated by a shift from a Th1 to a Th2/Th3 AChR-specific response and down-regulation of costimulatory factors such as CD40 ligand (CD40L), B7-1, and B7-2 (3).

CD40L is expressed on activated CD4+ T cells, whereas CD40, the receptor for CD40L, is expressed on various APCs such as B cells, dendritic cells, and macrophages (4). CD40-CD40L interaction influences many T cell-mediated inflammatory responses as reviewed in detail elsewhere (5, 6). Binding of CD40L to CD40 triggers B cells to function as APC and up-regulates the expression of the costimulatory molecules B7-1 and B7-2 (7, 8). CD40-CD40L signaling also activates macrophages to produce inflammatory cytokines (9) and plays an important role in the in vivo activation and clonal expansion of Ag-specific T cells (10).

CD40-CD40L interactions were previously found to be essential for the development of several autoimmune diseases including collagen-induced arthritis (11), autoimmune oophoritis (12), experimental allergic encephalomyelitis (EAE) (13, 14), lupus nephritis (15, 16), experimental autoimmune thyroiditis (17), and spontaneous autoimmune diabetes (18).

The role of CD40-CD40L in EAMG was recently studied in CD40L knockout mice (CD40L−/−) (19). The mice were completely resistant to EAMG induction and had diminished Th1 and Th2 responses as well as severely impaired T cell-dependent AChR-reactive B cell responses (19). These observations suggested that signaling via CD40-CD40L plays an important role in the induction of EAMG. In this study we tested the effect of intervention in CD40-CD40L interactions on the progression of an already existing disease. We show that anti-CD40L treatment is an effective way of suppressing EAMG even when it is given during the chronic stage of disease. The underlying mechanism of this suppression seems to be mediated by down-regulation of B7-2 and up-regulation of CTLA-4 levels. Pathogenic Th1-type cytokines...
are decreased, but there are no significant changes in the levels of Th2- or Th3-type cytokines and no induction of regulatory cells.

Materials and Methods

Animals and Ag preparation

Female Lewis rats (6–7 wk of age) were purchased from the Animal Breeding Center of the Weizmann Institute of Science (Rehovot, Israel). Torpedo AChR was purified from *Torpedo californica* by affinity chromatography as previously described (20). Two recombinant human AChR fragments corresponding to the extracellular domain of the α-subunit were used to represent mammalian muscle AChR for determination of AChR-specific reactivity. The homology between rat and human muscle AChR in this region is 96%. Ho1-205, a fragment corresponding to residues 1–205 of human AChR α-subunit expressed without a fusion partner was used for determination of IgG isotypes of anti-AChR Abs. Trx-Ho1-210, which corresponds to residues 1–210 of the human AChR α-subunit and is expressed as a fusion protein with thioredoxin was used for determination of AChR-specific in vitro B cell proliferation. Trx-Ho1-210 was applied for B cell proliferation because it is similar in its conformation to native AChR, as assessed by its reactivity with α-bungarotoxin (α-BTX) and with conformation-dependent anti-AChR mAbs (3). All recombinant AChR fragments were expressed in *Escherichia coli* and purified as previously described (3, 21). The purified recombinant proteins exhibited a single fragment were expressed in medium supplemented with HEPES (25 mM), sodium pyruvate (1 mM), and diluted in PBS. For the alkaline phosphatase assay, 100-μl cell suspensions containing different cell concentrations were transferred to 96-well plates in which 100 μl of substrate solution (2-nil-nitrophenyl phosphate, disodium; 1 mg/ml) was added. The plates were incubated for 2–4 h at 37°C in 5% CO₂. Alkaline phosphatase activity was monitored by OD at 405 nm.

Determination of cytokines and costimulatory factors

PCR-ELISA was used to assess the levels of mRNA specific for cytokines (IL-2, IL-4, IL-10, IL-12, IFN-γ, and TGF-β) and costimulatory factors (CD40, CD40L, CD28, CTLA4, B7-1, and B7-2). Draining LNCs (1 × 10⁶/ml) were cultured with Torpedo AChR (0.25 μg/ml) for 40 h. Total RNA extraction was performed by using a total RNA extraction kit (Roche Molecular Biochemicals, Mannheim, Germany), and the extracted RNA was reverse transcribed into cDNA using MuLV reverse transcriptase (New England Biolabs, Beverly, MA) with oligo(dT) as a primer. PCR was performed in the presence of digoxigenin (DIG)-dNTP and specific primer pairs for specific cytokines and costimulatory factors as described (2) by using a PCR-ELISA kit (Roche Molecular Biochemicals). The sequences of primer pairs for PCR and internal primers specific for cytokines and costimulatory factors were the same as previously reported (3). The internal primers were all biotinylated by Biotin-Chem-Link (Roche Molecular Biochemicals) according to the manufacturer’s protocol. The amplified DIG-labeled PCR products were quantified by a peroxidase-conjugated anti-DIG Ab. PCR products were viewed with the peroxidase substrate ABTS, and signals were quantified by absorbance at 405 nm (2).

Statistical analysis

Student’s two-tailed t test was used to determine the significance of differences between group means.

Results

Anti-CD40L treatment suppresses clinical symptoms of chronic EAMG

The interaction of CD40L on T cells with its receptor, CD40, on B cells is critical for induction of T cell-dependent Ab production. Because MG and EAMG are T cell-dependent Ab-mediated autoimmune disorders, we reasoned that blocking of CD40-CD40L interaction could result in an immunosuppressive effect on the production of pathogenic autoantibodies. To test this hypothesis, anti-CD40L Ab was administered to rats at the chronic stage of EAMG, which usually appears ~4 wk after immunization with Torpedo AChR. Treatment was initiated either 4 (Fig. 1A) or 6 wk (Fig. 1B) after EAMG induction. Rats were injected four times a week with anti-CD40L Ab or anti-BSA as control Ab, and treatment was continued for 4–5 more weeks. At the beginning of treatment the mean clinical score of the rats was 1.5. Treatment with anti-CD40L Ab had a therapeutic effect on the severity of EAMG (Fig. 1, and Table I), whereas in the anti-BSA-treated group there was an aggravation in the severity of EAMG. Five weeks after initiation of treatment, two of eight rats died and two of eight rats recovered completely (Table I), whereas in the anti-BSA-treated group there was an aggravation in the severity of EAMG. Five weeks after initiation of treatment, two of eight rats died, and four were severely sick (mean clinical score: 2.5). A similar protective effect of anti-CD40L treatment was observed when treatment started 6 wk after disease induction (Fig. 1B). Three of seven rats recovered completely or partially, but none of the anti-BSA-treated rats showed any signs of recovery. It should be noted that the suppressive effect

Results are expressed as Δcpm after subtraction of background counts of unstimulated cultures from those of stimulated LNCs.

**B cell proliferation assay based on alkaline phosphatase activity**

B cell proliferation was assayed as described (3). Draining LNCs (1 × 10⁶/ml) were cultured in the medium used for lymphocyte proliferation supplemented by 10% FCS. Cells in 24-well plates were stimulated in vitro with Trx-Ho1-210 (50 μg/ml) alone, or in the presence of either anti-CD40L (200 μg/ml) or anti-BSA (200 μg/ml). LPS (5 μg/ml) was used as a positive control. After 4 days in culture, the cells were harvested, washed, and diluted in PBS. For the alkaline phosphatase assay, 100-μl cell suspensions containing different cell concentrations were transferred to 96-well plates in which 100 μl of substrate solution (2-nil-nitrophenyl phosphate, disodium; 1 mg/ml) was added. The plates were incubated for 2–4 h at 37°C in 5% CO₂. Alkaline phosphatase activity was monitored by OD at 405 nm.

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**Effect of anti-CD40L treatment on ongoing chronic EAMG.** 
Torpedo AChR was injected to induce EAMG, and rats were treated four times a week by anti-CD40L or anti-BSA Abs starting at the chronic phase, 4 wk (A), or 6 wk (B) after disease induction. Treatments and clinical score evaluations were performed as described in Materials and Methods and each group consisted of eight rats. Representative of three independent experiments. *, p < 0.005.

The effect of treatment with anti-CD40L on clinical symptoms of EAMG lasted only for a short period (2 wk) after the treatment has been discontinued. Also, adoptive transfer of splenocytes from anti-CD40L-treated rats into naive rats did not protect the recipients against subsequently induced EAMG (data not shown).

The expression of costimulatory factors was tested in the AChR-stimulated LNCs that were used for analysis of cytokine levels. As shown in Fig. 3, anti-CD40L treatment resulted in up-regulation of CTLA-4 and down-regulation of B7-2 levels compared with anti-BSA-treated rats. Three days after AChR injection, rats treated by anti-BSA or anti-CD40L were removed 4 or 5 wk after initiation of treatment and cultured for 40 h in the presence of Torpedo AChR. Total RNA was then prepared from the cells and subjected to PCR-ELISA with cytokine-specific or costimulatory factor-specific primers.

### Table I. Effect of anti-CD40L Ab treatment on EAMG

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Clinical Score (No./total)</th>
<th>Mean Clinical Score</th>
<th>Δ Weight 5–9 wk (g)</th>
<th>T Cell Proliferation (cpm)</th>
<th>Anti-Rat AChR Ab Titer (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-BSA</td>
<td>0/8 3/8 3/8 1/8 2/8</td>
<td>2.5</td>
<td>-8 ± 20</td>
<td>1834 ± 250</td>
<td>70</td>
</tr>
<tr>
<td>Anti-CD40L</td>
<td>2/8 2/8 3/8 1/8 0/8</td>
<td>1.4</td>
<td>-6 ± 14</td>
<td>1904 ± 190</td>
<td>22</td>
</tr>
</tbody>
</table>

* Evaluated 9 wk after the induction of EAMG and 5 wk after initiation of treatment. Representative experiment performed as in Fig. 1A, of three independent experiments.

* In response to Torpedo AChR (0.25 μg/ml), evaluated for three to four individual rats in each assay.

* Evaluated in pooled sera of rats, 5 wk after initiation of treatment. The anti-rat AChR titer of pooled sera before treatment was 40 nM.
IgG levels, as well as the levels of IgG1 and all IgG2 subtypes, were lower in anti-CD40L-treated rats as compared with control anti-BSA-treated rats. These data are in line with the observed cytokine profile and suggest that anti-CD40L treatment does not induce a shift from Th1 to Th2/Th3 regulation of the anti-AChR response. Such a shift would be expected to lead to an increase in IgG1, which in the rat is regulated by Th2-type cells. IgG2b and IgG2c, which have been previously suggested to be involved in rat EAMG (2, 23), are regulated by Th1 cells.

In vitro effect of anti-CD40L on B and T cell proliferation

To examine whether the observed effects of anti-CD40L treatment on EAMG and anti-self-AChR levels, are associated with changes in AChR-specific B cell proliferation, we compared the in vitro response to AChR of cells from myasthenic rats. LNCs were removed from myasthenic rats at the chronic phase of disease (mean clinical score: 2–3) and cultured for 4 days in the presence of Trx-Hα1-210 alone or together with either anti-CD40L or anti-BSA. Trx-Hα1-210 was applied for this assay because of the similarity of its conformation to native AChR, as assessed by its reactivity with α-BTX and with conformation-dependent anti-AChR mAbs (3). The level of B cell proliferation was determined by alkaline phosphatase activity, which is known to be specific for activated B cells (24, 25). As shown in Fig. 5 the presence of anti-CD40L strongly suppressed in vitro B cell proliferation, whereas anti-BSA Abs did not (p < 0.001). In contrast, in vitro anti-CD40L treatment did not induce suppression of T cell proliferation in response to AChR (data not shown). This is in agreement with the observation that anti-CD40L treatment in vivo down-regulates the anti-self AChR Ab levels but has no effect on AChR-specific T cell proliferation (Table I), and implies that anti-CD40L treatment suppresses proliferation of B cells but not of T cells. The lack of effect on the overall T cell proliferation and IL-2 levels, as well as the diminished Th1-dependent DTH response and reduced levels of Th1-type cytokines, suggests that anti-CD40L treatment affects Th1 differentiation rather than proliferation.

Discussion

In this study, we demonstrate that blockade of CD40L-CD40 signaling by anti-CD40L Abs is capable of suppressing chronic EAMG. Our results suggest that the underlying mechanism of this effect involves a suppression of Th1-type differentiation with no shift in the AChR-specific immune response from Th1 to Th2/Th3 regulation. These were reflected in suppressed levels of IFN-γ and IL-12 but no changes in IL-4, IL-10, and TGF-β as well as in a decreased DTH response to AChR and in reduced anti-self-AChR
Ab titers of all IgG isotypes. Anti-CD40L treatment affected the expression of specific costimulatory factors (B7-2 levels were reduced, whereas CTLA-4 levels were increased) but did not affect the levels of the other costimulatory factors (B7-1, CD28, CD40, and CD40L).

The production of autoreactive Abs in EAMG is regulated by AChR-specific CD44 and CD8+ helper T cells (26), and interaction of T and B cells is crucial for disease pathogenesis (27). B cells express both MHC II and costimulatory molecules on their cell surface and can work as APCs and provide costimulatory signals for Ag-specific T cell activation (28). In turn, the activated T cells express CD40L and secrete cytokines that provide costimulatory signals for B cell proliferation and differentiation. T cell activation, and B cell proliferation and differentiation, through this two-way T-B cell interaction can be a self-perpetuating process during disease progression.

The specificity of T cell help for B cell activation and differentiation is maintained by the expression of CD40L on the surface of T cells. Interaction of CD40L on T cells with CD40 on B cells induces B cell activation, cell surface expression of activation Ags, proliferation, and initiation of immunoglobulin isotype switch. This CD40L/CD40 costimulation is also an important regulator of T cell activation; therefore, up-regulation of CTLA-4 present on CD4+ T cells acts as a key negative immunomodulator of immune responses by blocking CD28-dependent T cell activation (45). CTLA-4 is also involved in the induction of peripheral T cell tolerance in vivo (46), and its blockade exacerbates clinical symptoms of EAE (47). The CTLA-4 gene is polymorphic, and certain alleles are linked to MG and other autoimmune diseases (48–54). CTLA-4 normally acts as a negative regulator of T cell activation; therefore, up-regulation of CTLA-4 levels by anti-CD40L treatment may attenuate Th1-type cell activation (55). Up-regulation of CTLA-4 was also found during remission periods in EAE (56), which is consistent with the role of CTLA-4 in the termination of immune responses. Therefore, the increased levels of CTLA-4 and down-regulated B7-2 levels induced by anti-CD40L observed in our study may lead to impaired T cell activation, which is necessary for the proliferation and differentiation of autoreactive B cells and may thus result in reduced Ab production. The unchanged levels of costimulatory factors other than B7-2 and CTLA-4 and the lack of effect on T cell proliferation by anti-CD40L treatment suggest that anergy is not induced and seems not to be involved in suppression of EAMG. Also, this may suggest that the up-regulation of CTLA-4 and down-regulation of B7-2 are not sufficient to affect T cell proliferation probably due to the activity of other cytokines such as IL-2 and costimulatory factors such as B7-1 and CD28. Nevertheless, they did affect T cell differentiation, resulting in specific down-regulation of IL-12 and IFN-γ levels.

It is noteworthy that the protective effect of anti-CD40L Abs disappeared within 2 wk after cessation of Ab treatment. This indicates that the immunosuppressive effect of anti-CD40L is reversible and does not induce any regulatory T cells. Indeed, adoptive transfer of splenocytes from anti-CD40L-treated rats did not provide protection against EAMG induction in the recipients, supporting the notion that no regulatory cells were induced by anti-CD40L treatment. This is in contrast to the long-lasting effect we have observed in rats in which mucosal tolerance was induced by recombinant AChR fragments (1, 2, 21). In addition, repetitive i.p. administration of rabbit anti-CD40L Abs to rats elicited an immune response to rabbit IgG. It is clear that when considering an analogous treatment of MG patients, one should avoid the repetitive administration of xenogeneic immunoglobulins, for instance, by the use of humanized anti-CD40L Abs.

In summary, treatment of chronic EAMG by anti-CD40L attenuates disease progression by suppressing Th1 differentiation and effector functions. The decreased level of anti-self-Ab was associated with reduced expression of B7-2, IL-12, and IFN-γ levels, and increased CTLA-4 levels, which may contribute to down-regulation of T cell effector functions. The effect of anti-CD40L treatment during the chronic phase of experimental myasthenia is of special relevance to human MG, which is a chronic disease. It is encouraging that an appropriate antagonist of CD40L-CD40 signaling could alleviate the symptoms of the chronic autoimmune process in EAMG. Therefore, targeting of CD40L-CD40-mediated signaling may be potentially suitable for immunotheraphy of myasthenia especially when combined with our previously described long-lasting Ag-specific effect of oral tolerance induced by AChR-derived fragments.

References


Igarashi, O., H. Yamanaka, S. Imajoh-Omji, and H. Naruichi. 1998. IL-12 receptor (IL-12R) expression and accumulation of IL-12R β1 and IL-12R β2 mRNAs in CD4+ T cells by costimulation with B7-2 molecules. J. Immunol. 160:1658.


