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B7-1 Costimulatory Molecule Is Critical for the Development of Experimental Autoimmune Myasthenia Gravis

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Following immunization with acetylcholine receptor (AChR), MHC class II-restricted, AChR-specific CD4 cell activation is critical for the development of experimental autoimmune myasthenia gravis (EAMG) in C57BL/6 mice. To study the contributions of B7-1 and B7-2 costimulatory molecules in EAMG, B7-1, B7-2, and B7-1/B7-2 gene knockout (KO) mice were immunized with Torpedo AChR in CFA. Compared with wild-type C57BL6 mice, B7-1 and B7-1/2 KO mice were resistant to EAMG development. B7-1 KO mice had reduced anti-AChR Ab compared with C57BL6 mice. However, neither B7-1 nor B7-2 gene disruption impaired AChR-induced or dominant α146–162 peptide-induced in vitro lymphoproliferative responses. Blocking of the B7-1 or B7-2 molecule by specific mAbs in vivo led to a reduction in the AChR-specific lymphocyte response, and the reduction was more pronounced in mice treated with anti-B7-2 Ab. The findings implicate B7-1 molecules as having a critical role in the induction of EAMG, and the resistance of B7-1 KO mice is associated with suppressed humoral, rather than suppressed AChR-specific, T cell responses. The data also point to B7-2 molecules as being the dominant costimulatory molecules required for AChR-induced lymphocyte proliferation.

Myasthenia gravis (MG) is a chronic autoimmune disease characterized by muscular fatigability resulting from a disturbance of the neuromuscular transmission. The main immunological feature appears to be, in most cases, the presence of serum autoantibodies directed against acetylcholine receptor (AChR). Abs to AChR and complement mediate the neuromuscular damage. EAMG is induced in susceptible strains of mice by s.c. immunizations with an emulsion of AChR and CFA. Mice subsequently produce serum anti-AChR Ab and develop muscle weakness and fatigability mimicking the human disease. This model has been extensively studied to determine the factors that could be involved in the development of this autoimmune disease (1). The production of autoantibodies to AChR is dependent on the help of T cells and results from complex, prior cellular activation events. MHC class II-restricted CD4 cells are crucial in EAMG pathogenesis (2, 3), which has also been shown to require complex cytokine regulation, with TNF, IL-6, and IL-18 playing a dominant role (4–15). Activation of T cells requires two signals. The first is provided by the cognate TCR/MHC peptide interaction. A second costimulatory signal is mediated through ligation of CD28 or CD152 (CTLA-4) by the costimulatory B7 molecules. Mouse B7-1 (CD80) and B7-2 (CD86) molecules have respective molecular masses of 55 and 80 kDa and are expressed mainly on APC. The mechanisms by which B7-1 and B7-2 molecules regulate the immune system at the cellular and molecular levels are becoming clearer. A study using CD28-knockout (CD28-KO) and CD40 ligand-KO mice has revealed the differential requirement of B7/CD28 and CD40/CD40 ligand interactions in EAMG development (16). Another study reported that the immune response to AChR was suppressed by blocking the B7-1 and B7-2 costimulatory molecules with CTLA4 lg (17). However, the respective involvement of B7-1 and B7-2 molecules in the pathogenesis of EAMG is still not understood.

In the present study we combined two complementary approaches to assess the respective roles of B7-1 and B7-2 in the immune response to AChR and EAMG. First, three genetically altered mouse strains (B7-1 KO, B7-2 KO, and B7-1/2 double KO) were used to examine and compare the contributions of the costimulatory molecules in the development and progression of EAMG. Second, B7-1 and B7-2 molecules were blocked in vitro or in vivo with Ab to transiently suppress the function of these molecules and further study their influence. Our findings implicate the B7-1 molecule as having a critical role in the development of the humoral immune response to AChR and clinical EAMG. Although B7-2 molecules are less critical for the development of clinical EAMG, they are important for the activation of AChR-specific T cells.

Materials and Methods

Mice

C57BL/6 and B6.C-H2b(12)/KhEg (bm12) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B10.TgEa(k) (B10.Aa(k)Eae(k)β) mice, expressing both I-A and I-E molecules, were a gift from Dr. C. David
B7-1 and B7-2 Involvement in EAMG

Mitogenic stimulation of splenocytes

Splenocytes from five naive B7-1 KO, B7-2 KO, or C57BL/6 mice were seeded into 96-well cell culture plates at a concentration of 4 × 10^4 cells/well and stimulated for 72 h in triplicate with 10 µl of PBS (control), 0.25–1.0 µg/ml LPS, or 1.25–5 µg/ml Con A. At 18–20 h before harvesting, cells were pulse-labeled with 0.1 µCi [3H]thymidine/well. [3H]Thymidine incorporation was measured using a beta scintillation counter (Beckman, Fullerton, CA).

In vitro B7 blockade assay

C57BL/6 mice were immunized at the base of tail with 20 µg of AChR/CFA. Seven days after immunization the mice were euthanized, and LNC were collected. LNC were cultured with 2.5 µg/ml AChR, and supernatants were added from hybridomas producing Abs to either B7-1 (clone 1G10) or B7-2 (clone GL-1) or to mouse IgD (clone 11-26C) as a control. The crude supernatants were diluted serially from 1/4 to 1/200 in culture medium. Proliferation was measured as described above using [3H]thymidine incorporation, and the inhibitory effect of the Abs was expressed as the percentage of specific inhibition (% inhibition with Ab to B7-1 – % inhibition with 11-26C).

In vivo B7 blockade of the response to AChR

C57BL/6 mice were immunized at the base of tail with 20 µg of AChR/CFA. Mice were injected i.p. with 100 µg of purified Ab to B7-1 (clone 1G10), B7-2 (clone GL-1), or mouse IgD (clone 11-26C) on days 0 and 3 after immunization. Seven days after immunization the mice were euthanized, and LNC were collected. LNC were cultured with no Ag, 2.5 µg/ml AChR, or 20 µg/ml peptide α146–162. Proliferation was measured by [3H]thymidine incorporation.

Flow cytometric analysis

Mice were immunized s.c. at the base of the tail with 20 µg of AChR/CFA in a final volume of 200 µl of emulsion. One week after immunization the mice were killed, and their draining para-aortic and inguinal LNC were collected and stained for the expression of CD4, CD25, CD28, CD80 (B7-1), or CD86 (B7-2) molecules (all Abs from BD Pharmingen) as described previously (8, 9).

Statistical analysis

To determine the significance of the observed results, two statistical tests were used. Incidences of EAMG were compared using Fisher’s exact test. Proliferative responses, cytokine production, and cell surface marker expressions were compared using Student’s t test.

Results

B7-1 gene deficiency protects H-2b mice from developing AChR-induced clinical EAMG

To determine the effects of B7-1 and B7-2 costimulations on the development of AChR-induced EAMG, we immunized C57BL/6, B7-1 KO, B7-2 KO, and B7-1/B7-2 KO mice with 20 µg of AChR in CFA on day 0 and again on day 28. As shown in Table I, the incidence of clinical EAMG was suppressed in B7-1 KO mice in both experiments. In the first experiment B7-1 KO mice were significantly more resistant than B7-2 KO mice (p = 0.0204), and their incidence of disease was not different from that of B7-1/2 double KO mice (p = 0.5294). In the second experiment B7-1 KO mice were more resistant than B7-2 KO mice (p = 0.00497) or C57BL/6 mice (p = 0.0196), and the susceptibility of B7-2 KO mice was not different from that of C57BL/6 mice (p = 0.4235). However, B7-2 KO mice had less severe disease compared with B6 mice. The data indicate that B7-1 costimulation has a critical role in the pathogenesis of EAMG.

B7-1 and B7-2 gene disruption impairs the AChR-specific Ab response

Anti-AChR Abs are a central factor in EAMG pathology. To assess whether the reduction of clinical signs of EAMG was associated with lower levels of anti-AChR Ab, sera from AChR-immunized mice were collected 14 days after the first and 14 days
KO mice had lower circulating anti-AChR Ab than B7-2 KO (p after the first immunization did not reach significance). However, the difference was not different from that of B7-1/2 double KO mice (p = 0.5294). In the second experiment B7-1 KO mice were more resistant than B7-2 KO mice (p = 0.00497) or C57BL/6 mice (p = 0.00196), and the susceptibility of B7-2 KO mice was not different from that of C57BL/6 mice (p = 0.4235).

**Table I. B7-1 gene disruption reduces the incidence and severity of T-AChR-induced clinical EAMG**

<table>
<thead>
<tr>
<th>Strains</th>
<th>No. of mice</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Disease Incidence no.%&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp. 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B7-1 KO</td>
<td>8</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0/8 (0)</td>
</tr>
<tr>
<td>B7-2 KO</td>
<td>9</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>5/9 (56)</td>
</tr>
<tr>
<td>B7-1/2 double KO</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1/9 (11)</td>
</tr>
<tr>
<td>Exp. 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B7-1 KO</td>
<td>8</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1/8 (12.5)</td>
</tr>
<tr>
<td>B7-2 KO</td>
<td>8</td>
<td>1</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>7/8 (87.5)</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>8</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>6/8 (75)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Disease incidences were compared using Fisher’s exact probability test. In the first experiment B7-1 KO mice were more resistant than B7-2 KO mice (p = 0.0204), and their susceptibility was not different from that of B7-1/2 double KO mice (p = 0.5294). In the second experiment B7-1 KO mice were more resistant than B7-2 KO mice (p = 0.00497) or C57BL/6 mice (p = 0.00196), and the susceptibility of B7-2 KO mice was not different from that of C57BL/6 mice (p = 0.4235).

**FIGURE 1. Anti-AChR Ab response in AChR-immunized B7-1 KO, B7-2 KO, and C57BL/6 (B6) mice.** Sera were collected 2 wk after the first immunization (day 14) and 2 wk after the second immunization (day 44). Results were compared using Student’s t test (*, p < 0.05). The data in the figure represent the cumulative results of two independent experiments.
B7-2 has a leading costimulatory role in regulation of the proliferative response to AChR in vitro

In KO mice a targeted deficiency can sometimes be compensated for by the use of a molecule with a similar activity. To evaluate the respective influences of the costimulatory molecules B7-1 and B7-2 in the lymphocyte response to AChR in mice with B7-1 and B7-2 molecules intact from birth, we first used Ab to B7-1 and B7-2 to block the in vitro lymphoproliferative response to AChR. Draining LNC were collected 1 wk after immunization of C57BL/6, bm12, or B10.TgE/H9251k mice with AChR/CFA and stimulated in vitro with AChR in the presence of B7-1 or B7-2 blocking Ab or isotype-matched control Ab. As shown in Table II, although B7-1 blocking had an efficient inhibitory effect on the lymphoproliferative response to AChR, blocking of B7-2 had a more pronounced inhibitory effect in EAMG-susceptible C57BL/6 mice. Even mouse strains (bm12 and B10.TgE/H9251k) that are relatively resistant to EAMG demonstrated a similar suppression of their AChR-specific lymphocyte response in the presence of B7-1 or B7-2 blocking Ab or isotype-matched control Ab. This also indicates that although B7-1 costimulatory molecules exhibit a central role in the pathogenesis of EAMG, B7-2 costimulation is mainly involved in the proliferative response of AChR-specific T cells.

The in vitro blocking of the B7 molecule only has an effect on the late response to AChR. It allows study of the effect of antigenic restimulation of AChR-immune cells. However, priming of lymphocytes to AChR occurs in vivo. To counteract the influence of
B7-1 and B7-2 in vivo, immediately after immunization with AChR/CFA. C57BL/6 mice were treated i.p. with 20 μg of Ab to B7-1 or B7-2, or with isotype-matched control Ab on the day of immunization (D0) and on day 3. The lymphoproliferative response was assessed on day 7. One representation of three independent experiments is shown.

Discussion
The critical role of CD28/CTLA4 molecules has been demonstrated in experimental autoimmune encephalomyelitis (EAE) (24–27) and in a variety of other autoimmune diseases, including collagen-induced arthritis (28), thyroiditis (29), and MG (17). There is an accumulated body of evidence that B7-1 and B7-2 molecules have differential roles in autoimmunity. For instance, anti-B7-1 Ab, but not anti-B7-2 Ab, decreases disease severity during the first phase of EAE (25–30). Also, treatment of relapsing-remitting EAE with anti-B7-1 Ab reduces disease severity, whereas treatment with anti-B7-2 Ab aggravates the disease (27, 30). A similar discrepancy exists in experimental murine diabetes and lupus, whereas anti-B7-2 Ab prevents disease expression, and treatment with anti-B7-1 Ab worsens the clinical course (31, 32).

Our finding indicates that B7-1 KO and B7-1/2 double KO mice are resistant to AChR-induced EAMG, while B7-2 deficiency has little effect on the development of EAMG (Table I). It is also evident from our results that the deficiency of B7-1 alone is sufficient to prevent disease initiation. This suggests that B7-1 costimulation must intervene earlier than B7-2 costimulation in the immune response to AChR. Therefore, like other autoimmune diseases, B7-1 and B7-2 are differentially required for EAMG induction. Although B7-1, but not B7-2, molecule deficiency suppressed the anti-AChR Ab response significantly following immunization with AChR (Fig. 1), it did not affect the in vitro lymphocyte responses to AChR (Fig. 2A). Therefore, the reduction of the susceptibility to AChR-induced EAMG in B7-1 KO mice cannot be explained by an absence of primary response to AChR. Thus, B7-1 and B7-2 molecules must be involved in the cellular communication between T and B cells, leading to anti-AChR Ab production, with a more important role for B7-1. T and B cells of B7-1 KO mice gave responses as powerful as the responses of C57BL/6 mice to nonspecific mitogen Con A or LPS. This indicates that there is no functional deficiency in B and T cells from B7-1 KO mice, as evaluated by the proliferative response. This finding further supports the assumption that the B7-1 molecule is critical for the costimulation events leading to the production of pathogenic anti-AChR Ab by B cells.

In contrast with congenital deficiency of the B7-1 receptor, both in vivo (Fig. 4) and in vitro (Table II) mAb blocking of B7-1 in C57BL/6 mice efficiently prevented lymphocyte proliferation. This inconsistency between acquired and congenital forms of B7-1 dysfunction may be explained by some compensatory mechanisms taking place in B7-1 KO mice. It is probable that in B7-1 KO mice the B7-2 molecule acts to replace part of the function of the B7-1 molecule. This is corroborated by the fact that B7-1/2 double KO mice exhibit a totally suppressed proliferative as well as a AChR-specific cytokine in vitro response.
Additionally, in vitro evaluation of the proliferative responses of lymphocytes might not genuinely reflecting the long term, in vivo status of an ongoing autoimmune disease. B7-1 and B7-2 molecules have been shown to have different expression kinetics (31, 33, 34). B7-2 seemed to be the dominant B7 molecule in the early stages of immunological response, whereas B7-1 had a more significant role in the continuation and preservation of the T cell response to immunogens (35). Accordingly, administration of anti-B7-2 Ab prevented experimental diabetes only when they were given in the earlier stages of autoimmune disease (32). Moreover B7-1 production was not restricted to immunological cells, and various different tissues, including endothelial cells, expressed this molecule (35). Other than lymphocyte interactions, B7-1 expression might also play a role in tissue response (from Ag presentation with MHC molecules to inflammatory events) (35, 36). Therefore, a reduction in AChR-specific B cell stimulation might not be the only disturbance in B7-1 KO mice. As indicated by their reduced IFN-γ response (Fig. 2B), a diminished inflammatory tissue response in B7-1 KO could also explain the reduction of EAMG susceptibility as well as why CD28 KO (16) and B7-2 KO mice (both strains lacking essential molecules in lymphocyte activation) are only partially resistant to EAMG, whereas B7-1 deficiency

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** Effect of in vivo treatment with anti-B7-1 or anti-B7-2 Ab on the expression of the cell surface markers CD28, CD4, CD25, B7-1, and B7-2. Treatment of C57BL/6 mice with anti-B7-1 or B7-2 Ab causes a reduction in CD4⁺ and CD4⁺CD25⁺ T cells without affecting B7-1, B7-2, or CD28 expression. Results were compared using Student’s t test. One representation of three independent experiments is shown.
renders mice almost totally resistant. Although it is highly possible that the suppressed IFN-γ production of B7-1 KO mice might have contributed to disease resistance, we do not believe that this has constituted a major effect on EAMG induction. In our experiments, B7-1 KO mice were almost completely resistant to disease. However, even complete deficiency of IFN-γ does not render mice entirely resistant to EAMG (4, 6, 7). Therefore, we do not suggest that a relatively small decrease in IFN-γ production could have made a major contribution to resistance against EAMG.

Another interesting finding was that in vitro blockade of B7-2 prevented the proliferation of AChR-immune LNC to AChR challenge more efficiently than B7-1 in a few mouse strains, with different MHC class II alleles. In contrast to B7-1, which in our system appears to play a more crucial role in the initiation of AChR-induced pathogenesis, B7-2 seems to be more important for in vitro lymphocyte proliferation of AChR-immune cells. This is possibly because this molecule is more important in memory T cell functions and the recall response to AChR.

One surprising observation was that CD4+CD25+ immunoregulatory T cells appear to be decreased substantially after anti-B7-1 Ab or B7-2 Ab treatment (Fig. 5). CD4+CD25+ T cells are a subset of CD4+ cells that are essential in the maintenance of tolerance to Ags. The absence of these cells results in several autoimmune diseases, such as thyroiditis, gastritis, and diabetes (37). CTLA-4/B7 interactions have also been demonstrated to be important in the regulation of peripheral tolerance (38), and CTLA-4 expression is dependent on the interaction of CD28 and B7 molecules (38). Since we did not test the in vivo effects of anti-B7-1 Ab treatment on the induction of EAMG, we are not certain whether this down-regulation of CD4+CD25+ T cells may adversely affect the clinical course of the autoimmune disease. However, to the best of our knowledge this is the first paper reporting a decrease in CD4+CD25+ cells following anti-B7-1 Ab treatment (although a similar decrease has been reported in B7 KO mice), which may indicate why anti-B7 treatment is detrimental in animal models of type 1 diabetes (31).

Although by the time patients present, MG is at an advanced stage, and B7-1 is expected to play an important role at the initiation of the immune response, it is also known that MG patients frequently present with exacerbations, and these exacerbations may continue for months. Therefore, it might be expected that AChR is presented to the immune cells via B7-1 molecule not only at the initiation, but also several times throughout the course of the disease. However, we only demonstrated that blockade of the B7 molecule may affect the induction of EAMG and did not examine the effects of anti-B7 Ab treatment on the course of established disease. Therefore, we strongly recommend that the role of B7 molecules in the clinical course and the exacerbations of MG be investigated in future studies.

In this study we have shown that the B7-1 molecule is required for Ab production after AChR immunization and in an AChR-reactive immune system response, B7-2 molecule plays an important role in reactivation of T cells against AChR. Whatever the reasons for disease resistance of B7-1 KO mice are, our results present B7-1 as a critical molecule in the initiation and development of AChR-induced EAMG. The significance of B7 molecules in both T cell (EAE) (26, 27) and immune complex (lupus) (32)-mediated autoimmune diseases has been previously reported. To our knowledge this report is the first study of the importance of B7 molecules in the induction of a classical Ab-mediated disease, MG.

Acknowledgments

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References

B7-1 AND B7-2 INVOLVEMENT IN EAMG


