The Journal of Immunology

# Anti-CTLA-4 Antibody Treatment Triggers Determinant Spreading and Enhances Murine Myasthenia Gravis

Hua-Bing Wang, Fu-Dong Shi, Hulun Li, Benedict J. Chambers, Hans Link and Hans-Gustaf Ljunggren



*J Immunol* 2001; 166:6430-6436; ;
doi: 10.4049/jimmunol.166.10.6430
http://www.jimmunol.org/content/166/10/6430



*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

# Anti-CTLA-4 Antibody Treatment Triggers Determinant Spreading and Enhances Murine Myasthenia Gravis[1]

**Hua-Bing Wang,[2]* Fu-Dong Shi,[2,3‡] Hulun Li,* Benedict J. Chambers,[‡] Hans Link,* and Hans-Gustaf Ljunggren[4†‡]**

**CTLA-4 appears to be a negative regulator of T cell activation and is implicated in T cell-mediated autoimmune diseases. Experimental autoimmune myasthenia gravis (EAMG), induced by immunization of C57BL/6 mice with acetylcholine receptor (AChR) in adjuvant, is an autoantibody-mediated disease model for human myasthenia gravis (MG). The production of anti-AChR Abs in MG and EAMG is T cell dependent. In the present study, we demonstrate that anti-CTLA-4 Ab treatment enhances T cell responses to AChR, increases anti-AChR Ab production, and provokes a rapid onset and severe EAMG. To address possible mechanisms underlying the enhanced autoreactive T cell responses after anti-CTLA-4 Ab treatment, mice were immunized with the immunodominant peptide $\alpha_{146-162}$ representing an extracellular sequence of the AChR. Anti-CTLA-4 Ab, but not control Ab, treatment subsequent to peptide immunization results in clinical EAMG with diversification of the autoantibody repertoire as well as enhanced T cell proliferation against not only the immunizing $\alpha_{146-162}$ peptide, but also against other subdominant epitopes. Thus, treatment with anti-CTLA-4 Ab appears to induce determinant spreading, diversify the autoantibody repertoire, and enhance B cell-mediated autoimmune disease in this murine model of MG.** ***The Journal of Immunology*****, 2001, 166: 6430–6436.**

The CTLA-4 (CD152) shares significant sequence homology with CD28, a costimulatory receptor that is constitutively expressed on resting human and murine T cells (1). Unlike CD28, CTLA-4 is not expressed by resting T cells, but is up-regulated after T cell activation (2) and appears to negatively regulate T cell activation (2–4). CTLA-4-deficient mice develop lethal lymphoproliferative disorders within a few weeks after birth (5, 6), supporting a role of CTLA-4 in down-regulating T cell activation and maintaining lymphocyte homeostasis. Furthermore, anti-CTLA-4 Ab treatment in vivo enhances host antitumor immunity (7), host antimicrobe immunity (8), and exacerbated T cell-mediated autoimmune diseases (9–11). However, the basis for the inhibitory effects of CTLA-4 remains largely unresolved.

Myasthenia gravis (MG)[5] and its animal model, experimental autoimmune myasthenia gravis (EAMG), are caused by autoantibodies against the nicotinic acetylcholine receptor (AChR) at neuromuscular junctions. The production of anti-AChR Abs in MG and EAMG is dependent on T cell help (12, 13). The AChR is a pentameric molecule consisting of four homologous subunits ($\alpha_2$, $\beta$, $\delta$, $\epsilon$ ($\gamma$)), whose sequences have been determined (12). In both rodent EAMG and human MG, the AChR-reactive $CD4^+$ cells preferentially recognize epitopes of the $\alpha$ subunit. In C57BL/6 (B6) mice, the sequence $\alpha_{146-162}$ is a dominant T cell determinant, whereas the sequences $\alpha_{111-126}$ and $\alpha_{182-198}$, respectively, are subdominant determinants (14, 15). The $\alpha$ subunit determinants recognized by AChR-reactive $CD4^+$ cells have been a subject of intensive investigation (16, 17). However, the costimulation requirements in the activation of AChR-specific T cells and the initiation of pathogenic Ab production remain largely unexplored.

We have recently shown that the costimulatory molecules CD28 and CD40 ligand are differentially required for the induction of EAMG (18). Consistent with this finding, Drachman and colleagues (19) have shown that treatment with CTLA-4Ig, a fusion protein of human Ig that binds to B7, prevented clinical EAMG in rats by reducing overall anti-AChR Ab production. In the present study, we have addressed the role of CTLA-4 in T cell-dependent B cell-mediated autoimmunity in murine MG. We demonstrate that anti-CTLA-4 Ab treatment enhances clinical EAMG with respect to both onset and severity. Our data further suggest that intramolecular determinant spreading, leading to an enhanced anti-AChR Ab response, is one mechanism responsible for the exacerbation of EAMG after anti-CTLA-4 Ab treatment.

## Materials and Methods

### *Mice*

B6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were bred and maintained under pathogen-free conditions in the animal facilities of the Microbiology and Tumor Biology Center, Karolinska Institute. Female mice, ages 8–10 wk at the initiation of the experiments, were used. Animal experimental procedures were performed in compliance with institutional guidelines.

### *Ags and synthetic peptide*

*Torpedo* AChR was purified from the electric organs of *Torpedo californica* (Pacific Biomarine, Venice, CA) by affinity chromatography on a

*Experimental Neurology Unit, Division of Neurology, and †Department of Medicine, Center for Infectious Medicine, Karolinska Institute, Huddinge University Hospital, Stockholm, Sweden; and ‡Microbiology and Tumor Biology Center, Karolinska Institute, Stockholm, Sweden

Received for publication October 31, 2000. Accepted for publication March 2, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants from the Swedish Medical Research Council, the Swedish Multiple Sclerosis Society, the Swedish Cancer Society, the Petrus and Augusta Hedlund Foundation, the Lars Hierta Foundation, the Magnus Bergwall Foundation, the Åke Wiberg Foundation, and the Karolinska Institute.

[2] H.-B.W. and F.-D.S. contributed equally and share first authorship.

[3] Current address: Department of Immunology, IMM-23, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037.

[4] Address correspondence and reprint requests to Dr. Hans-Gustaf Ljunggren, Microbiology and Tumor Biology Center, Karolinska Institute, S-171 77 Stockholm, Sweden. E-mail address: hanlju@ki.se

[5] Abbreviations used in this paper: MG, myasthenia gravis; AChR, acetylcholine receptor; EAMG, experimental autoimmune myasthenia gravis; LNC, lymph node cell; MBP, myelin basic protein; MNC, mononuclear cells; p.i., postprimary immunization; PILN, popliteal and inguinal lymph node; EAE, experimental autoimmune encephalomyelitis; IDDM, insulin-dependent diabetes mellitus.



0022-1767/01/$02.00

α-cobrotoxin-agarose resin (Sigma, St. Louis, MO) (20). The isolated oW-product was pure as judged by SDS-PAGE. Myelin basic protein (MBP) used as control Ag was purified from normal mouse brains (21). The AChR $\alpha_{111-126}$ (DKTGKIMWTPPAIFKS), $\alpha_{146-162}$ (LGIWTYDGTKVSISPES), $\alpha_{122-138}$ (AIFKSYCEIIVTHFPFD), and $\alpha_{182-198}$ (RGWKHWVYYTCCP DTPY) peptides (14) were synthesized at the Swedish Institute for Infectious Disease Control (Stockholm, Sweden). Unrelated peptide (KAIVELAFTYRSDSFEN) derived from Ku protein (22) was synthesized as a control.

### *Anti-CTLA-4 Ab treatment*

The hybridoma cell line (2), producing the anti-CTLA-4 Ab (UC10-4F10-11, hamster IgG) was a gift from J. A. Bluestone (University of California, San Francisco). Mice were treated with anti-CTLA-4 Ab (100 μg/mouse) or isotype control hamster IgG (100 μg/mouse; Sigma) i.p. for 5 consecutive days starting from the day of primary immunization (23). In some experiments, Ab treatment was started from day 40 postprimary immunization (p.i.) with the same dose regimen.

### *Induction and clinical evaluation of EAMG*

Mice were immunized s.c. with 20 μg of AChR in CFA in a total volume of 100 μl along the shoulders and back. Mice were boosted once after 1 mo with 20 μg of AChR in CFA s.c. at four sites on the shoulders and thighs. In some experiments, mice were immunized s.c. with 50 μg of $\alpha_{146-162}$ in CFA and boosted once as described above. The mice were observed every other day in a blinded fashion for signs of muscle weakness characteristic of EAMG. Clinical manifestation of EAMG was graded between 0 and 3 (24): 0, no definite muscle weakness; 1+, normal strength at rest but weak, with chin on the floor and inability to raise the head after exercise consisting of 20 consecutive paw grips; 2+, as grade 1+ and weakness at rest; and 3+, moribund, dehydrated, and paralyzed. Clinical EAMG was confirmed by injection of neostigmine bromide and atropine sulfate (24).

### *Mononuclear cell (MNC) suspensions*

MNC were obtained by grinding the popliteal and inguinal lymph nodes (PILN) through a wire mesh in medium. Cells were suspended in DMEM supplemented with 1% (v/v) MEM, 2 mM glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 10% (v/v) FCS (all from Life Technologies, Paisley, U.K.). The cells were washed three times and rediluted to a cell concentration of $2 \times 10^6$/ml for analysis of T cell responses and enumeration of IgG-secreting cells.

### *Lymphocyte proliferation responses*

Triplicate aliquots (200 μl) of MNC suspensions containing $4 \times 10^5$ cells were placed into 96-well round-bottom microtiter plates (Nunc, Copenhagen, Denmark). Ten-microliter aliquots of AChR, $\alpha_{111-126}$, $\alpha_{146-162}$, $\alpha_{122-138}$, $\alpha_{182-198}$, Ku peptide, MBP (all preparations 10 μg/ml), or Con A (5 μg/ml; Sigma) were added in triplicate into appropriate wells. After 4 days of incubation, the cells were pulsed for 18 h with 10-μl aliquots containing 1 μCi of [*methyl*-$^3$H]thymidine (sp. act., 42 Ci/mmol; Amersham, Arlington Heights, IL). Cells were harvested onto glass-fiber filters, and thymidine incorporation was measured. The results were expressed as counts per minute.

### *Cytokine ELISA*

Single-cell suspensions of AChR-primed draining lymph node cells (LNC) were cultured in the presence or the absence of AChR or $\alpha_{146-162}$ (10 μg/ml). Cell culture supernatants to be assayed for TGF-β1 content were generated in Aim V serum-free medium (Life Technologies). The supernatants were collected after 48 h in culture. IFN-γ and IL-4 production in culture supernatants was measured by optEIA kits (PharMingen, San Diego, CA). Biologically active TGF-β1 was measured with an ELISA kit (Promega, Madison, WI). The sensitivity of these ELISA was 31.3 pg/ml for IFN-γ, 7.8 pg/ml for IL-4, and 15.6 pg/ml for TGF-β1.

### *Assays of anti-AChR IgG Abs*

A solid-phase enzyme-linked immunospot assay was used with some modification (25). Briefly, wells of microtiter plates with nitrocellulose bottoms were coated with 100 μl of AChR or MBP (10 μg/ml in PBS). Aliquots (100 μl) of cell suspensions containing $2 \times 10^5$ MNC were added to individual wells in triplicate. After incubation for 24 h, the wells were emptied, followed by addition of rabbit anti-mouse IgG (Sigma), biotinylated swine anti-rabbit IgG (Dakopatts, Copenhagen, Denmark), and avidin-biotin peroxidase complex (Dakopatts). After peroxidase staining, the red-brown immunospots corresponding to cells that had secreted anti-AChR IgG were counted and standardized to numbers per $10^5$ MNC.

Anti-AChR IgG Abs were detected by ELISA as described previously (26). Microtiter plates (Corning Glass Works, Corning, NY) were coated with 100 μl/well of AChR (2 μg/ml) or 50 μl/well of $\alpha_{111-126}$, $\alpha_{146-162}$, $\alpha_{122-138}$, and $\alpha_{182-198}$ (5 μg/ml) at 4°C overnight. Uncoated sites were blocked with 10% FCS (Life Technologies). Serum samples (diluted 1/1000) were added and incubated for 2 h at room temperature. Then, plates were incubated for 2 h with biotinylated rabbit anti-mouse IgG (1/2000; Dakopatts), followed by alkaline phosphatase-conjugated avidin-biotin peroxidase complex (Dakopatts). The color was developed with *p*-nitrophenyl phosphate. Results were expressed as OD at 405 nm.

### *RIA for muscle AChR content*

The concentration of AChR/mg protein of mouse muscle carcass was determined according to a method described previously (20, 27). Briefly, triplicate 2 pM aliquots of $^{125}$I-α-bungrotoxin-labeled (Amersham), Triton X-100-solubilized mouse muscle extract were mixed with 5 μl of a standard pooled mouse anti-AChR serum. After overnight incubation, rabbit anti-mouse IgG (Dakopatts) was added. The resulting precipitate was pelleted by centrifugation, washed in 1 ml of Triton X-100 buffer, and pelleted again. The radioactivity of the pellet was counted in a gamma counter (Packard, Meriden, CT).

### *Statistical analysis*

Differences between groups were analyzed by Student's *t* test. Clinical scores were analyzed using the nonparametric Mann-Whitney *U* test. Differences between the groups with respect to disease incidence were analyzed by Fisher's exact test. The level of significance was set at $p = 0.05$.

## Results

### *Anti-CTLA-4 Ab treatment results in accelerated and exacerbated clinical EAMG*

To address the role of CTLA-4 in the development of AChR-induced EAMG, B6 mice were immunized twice with AChR in CFA and treated with anti-CTLA-4 Ab or control Ab as described in *Materials and Methods*. Anti-CTLA-4 Ab treatment at the time of primary immunization accelerated the onset (day 32 p.i. vs day 42 p.i. in control mice, $p < 0.01$) and enhanced the severity of muscle weakness (Fig. 1). Thirteen of 27 mice (48%) receiving control Ab showed mild or moderate muscle weakness after AChR boosting, among which only one mouse had died at the termination of the experiment. In contrast, 30 of 32 mice (94%; $p < 0.0001$) receiving anti-CTLA-4 Ab exhibited moderate to severe muscle weakness, among which 6 mice deteriorated progressively and died (Fig. 1). Of importance, B6 mice treated with anti-CTLA-4 Ab alone did not develop any signs of MG. Thus, we conclude that anti-CTLA-4 Ab treatment affects the initiation and progression of clinical EAMG.

### *Anti-CTLA-4 Ab treatment enhances anti-AChR Ab responses*

The pathogenic anti-AChR Abs in MG and EAMG are predominantly IgG Abs (12). These Abs are entirely responsible for the functional loss of AChR and impaired neuromuscular transmission (12, 13, 27). To address how anti-AChR Ab responses were influenced by anti-CTLA-4 treatment, we measured numbers of MNC from PILN-secreting IgG and IgG concentrations in serum. Mice that had received anti-CTLA-4 Ab had 2-fold higher numbers of anti-AChR IgG-secreting cells compared with mice treated with control Ab (Fig. 2*A*). The elevated numbers of anti-AChR-secreting cells are most likely due to a relative increment in anti-AChR-secreting cells rather than only reflecting a higher number of B cells in the cultures, as total numbers of B cells did not increase after CTLA-4 treatment (data not shown). Serum levels of anti-AChR IgG from anti-CTLA-4 Ab-treated mice were consistently higher than those from mice treated with control Ab (Fig. 2*B*). This was especially the case for Abs of the IgG2b isotype, even though all isotypes measured were increased (data not shown).

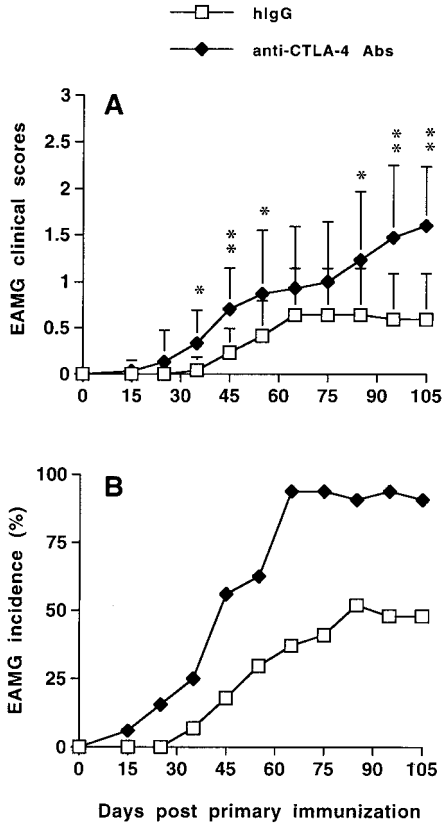


**FIGURE 1.** Anti-CTLA-4 Ab treatment exacerbates clinical EAMG in B6 mice. Mice were immunized with 20 $\mu$g of AChR in CFA, boosted 1 mo later, and monitored for signs of muscle weakness characteristic of EAMG. Mice were treated with either anti-CTLA-4 Abs ($n = 32$) or isotype control Abs ($n = 27$) for 5 consecutive days starting from the day of primary immunization. Symbols refer to mean values, and bars to SDs. *, $p < 0.05$; **, $p < 0.01$.

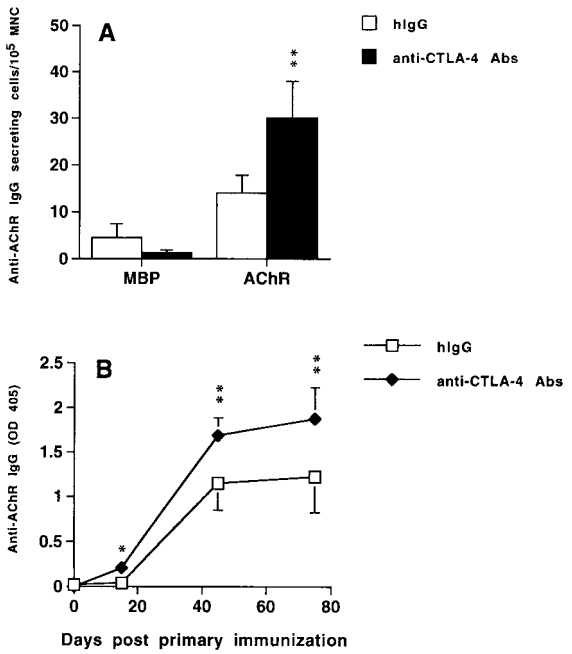


**FIGURE 2.** Anti-CTLA-4 Ab treatment up-regulates anti-AChR Ab production. *A*, Mice receiving control Abs ($n = 4$) or anti-CTLA-4 Abs ($n = 4$) were sacrificed on day 7 p.i. MNC isolated from PILN of individual animals were analyzed for anti-AChR IgG-secreting cells. The results are expressed as numbers of IgG-secreting cells per $10^5$ cells. Data are representative of three independent experiments. *B*, Sera were taken from the mice indicated in Fig. 1 by tail bleeding. Anti-AChR IgG from mice receiving control Abs ($n = 6$) and anti-CTLA-4 Abs ($n = 6$) were measured by ELISA. Symbols refer to mean values, and bars to SDs. *, $p < 0.05$; **, $p < 0.01$.

### *Anti-CTLA-4 Ab treatment enhances T cell responses to AChR*

In MG and EAMG the production of anti-AChR Abs is T cell dependent. Thus, the enhancement of anti-AChR Ab could be secondary to altered Th functions after anti-CTLA-4 Ab treatment. T cell proliferation responses to AChR may reflect expansion of autoaggressive T cells and may relate to disease severity (17). In initial experiments we examined T cell responses in AChR-immunized mice treated with anti-CTLA-4 or control Ab. Compared with mice treated with control Ab, MNC from anti-CTLA-4 Ab-treated mice showed enhanced proliferative responses to AChR and the determinants $\alpha_{111-126}$, $\alpha_{146-162}$, and $\alpha_{182-198}$ on the $\alpha$ subunit (Fig. 3*A*). Treatment with anti-CTLA-4 Ab also enhanced MNC proliferation upon Con A stimulation (control, 23,289 $\pm$ 6,812 cpm; anti-CTLA-4-treated, 46,322 $\pm$ 9,228 cpm). Up-regulation of T cell proliferation was also observed on day 105 (data not shown).

It has been shown that anti-CTLA-4 Ab treatment in vivo enhances both Th1 and Th2 cytokine production by autoreactive T cells (10, 28). It is not entirely clear whether CTLA-4 signaling can affect other types of cytokine responses, such as TGF-$\beta$ production by Th3 cells in vivo. To address how CTLA-4 regulates AChR-induced cytokine responses, we measured IFN-$\gamma$, IL-4, and TGF-$\beta$1 production in culture supernatants. Anti-CTLA-4 Ab treatment enhanced both AChR-specific IFN-$\gamma$ and IL-4 production compared with that in mice treated with control Ab. Interestingly, levels of TGF-$\beta$1 were significantly reduced in anti-CTLA-4 Ab-treated mice (Fig. 3*B*).

### *Anti-CTLA-4 Ab treatment triggers T cell determinant spreading within the $\alpha$ subunit of AChR, which leads to diversification of the anti-AChR Ab repertoire*

To elucidate mechanisms underlying the enhancement of T lymphocyte responses after anti-CTLA-4 Ab treatment, we immunized B6 mice with 50 $\mu$g of the $\alpha_{146-162}$ peptide in CFA. These mice were then treated with control Ab or anti-CTLA-4 Ab as described in *Materials and Methods*. We examined the immune responses to different epitopes on the AChR by exposing draining LNC to the $\alpha_{111-126}$, $\alpha_{122-138}$, $\alpha_{146-162}$, and $\alpha_{182-198}$ peptides and control Ku peptide as well as AChR on days 10, 40, and 70 p.i. In mice receiving anti-CTLA-4 Ab, proliferative responses were observed not only to the $\alpha_{146-162}$ peptide but also to the $\alpha_{111-126}$, $\alpha_{122-138}$, and $\alpha_{182-198}$ peptides on days 40 and 70 p.i. (Fig. 4, *A–E*). These results imply reactivity to determinants other than the one used for primary immunization, indicative of epitope (determinant) spreading (29, 30). It appeared that responses to these epitopes were not cross-reactive, since reactivity to the subdominant epitopes $\alpha_{111-126}$ and $\alpha_{182-198}$ and to the epitope $\alpha_{122-138}$ appeared not before day 10 p.i., at which time T cell proliferation had already been evoked to the $\alpha_{146-162}$ peptide in mice receiving anti-CTLA-4 Ab. Importantly, anti-CTLA-4 Ab treatment up-regulated T cell reactivity to the $\alpha_{111-126}$ peptide, but down-regulated that to $\alpha_{146-162}$ on day 75 p.i. (Fig. 4, compare *A* with *C*). Again, the amount of $\alpha_{146-162}$-specific IFN-$\gamma$ and IL-4 was higher in cell culture supernatants from anti-CTLA-4 Ab-treated mice compared with control Ab-treated mice (data not shown).

The AChR-$\alpha_{146-162}$ determinant is an immunodominant T cell epitope that can elicit anti-AChR Ab responses by triggering T cell

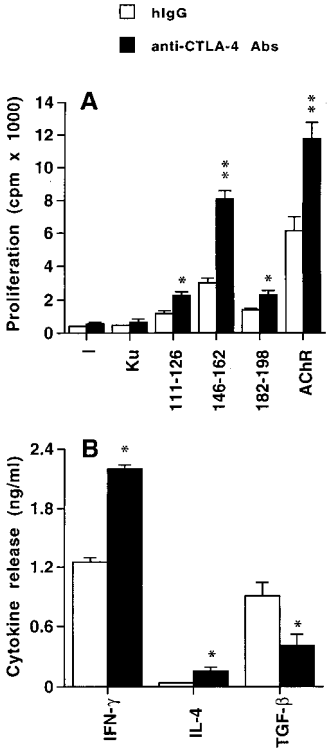


**FIGURE 3.** Anti-CTLA-4 Ab treatment enhances AChR-induced T cell responses. Samples taken from individual animals on day 7 p.i. were analyzed for proliferation and cytokine production. *A*, Proliferation of MNC derived from mice receiving control Abs ($n = 4$) or anti-CTLA-4 Abs ($n = 4$) after stimulation with Ags indicated. *B*, Productions of IFN-$\gamma$, IL-4, and TGF-$\beta$ were determined 48 h after in vitro stimulation with 10 $\mu$g/ml AChR. In the absence of AChR, levels of cytokines were very low or undetectable (430 $\pm$ 86 pg/ml for IFN-$\gamma$, 36 $\pm$ 5 pg/ml for IL-4, and 300 $\pm$ 25 pg/ml for TGF-$\beta$1). Data are representative of three independent experiments. *, $p < 0.05$; **, $p < 0.01$.

activation in B6 mice (14, 15). To determine whether T cell determinant spreading induced by anti-CTLA-4 Ab treatment can diversify the autoantibody repertoire, we measured serum levels of anti-peptide and anti-AChR Abs in the $\alpha_{146-162}$ peptide-immunized mice. As shown in Fig. 4, *F–J*, sera taken on days 15, 45, and 75 p.i. from mice receiving anti-CTLA-4 Ab revealed a significant reactivity to AChR as well as the $\alpha_{111-126}$, $\alpha_{146-162}$, and $\alpha_{182-198}$ peptides, whereas no significant Ab responses against these peptides could be detected from mice treated with control Ab. Importantly, our results demonstrated that the major enhancement in the production of anti-AChR Abs after anti-CTLA-4 Ab treatment can be attributed to anti-$\alpha_{122-138}$ Abs, a well-defined B cell epitope conserved in *Torpedo* and mouse AChR that is under the control of $\alpha_{146-162}$-specific T cells (31, 32).

### *Anti-CTLA-4 Ab treatment induces MG in mice sensitized with the $\alpha_{146-162}$ peptide*

Previous studies have demonstrated that immunization with the $\alpha_{146-162}$ peptide in CFA was not sufficient to induce clinical EAMG because of a failure to raise pathogenic and sustained levels of an autoantibody response (33). In the present experiment we showed that anti-CTLA-4 Ab treatment induced T cell determinant spreading to several other epitopes in addition to the immunizing peptide $\alpha_{146-162}$. Importantly, Ab responses against these determinants could only be detected in anti-CTLA-4 Ab-treated mice, not in control mice. These results imply a possible enhancement of the myasthenogenicity of the $\alpha_{146-162}$ peptide by breaking tolerance to an autoantibody response. To test this hypothesis, we monitored mice immunized with AChR-$\alpha_{146-162}$ in CFA for the development of MG. Surprisingly, but unequivocally, mice receiving anti-CTLA-4 Ab did develop mild muscle weakness in contrast to control mice (EAMG incidence, 30 vs 0%). Furthermore, a significant loss of AChRs was observed in muscle tissue in mice receiving anti-CTLA-4 Ab compared with control mice (AChR loss $\pm$ SD, 70 $\pm$ 19 vs 37 $\pm$ 20%; $p < 0.05$). Taken together, inhibition of CTLA-4 signaling in vivo enhanced the myasthenogenicity of the $\alpha_{146-162}$ peptide, most likely via processes that involve 1) T cell determinant spreading that lead to the diversification of autoantibody repertoire to self peptides, and 2) enhanced Th1 and Th2 responses. The combined effects may facilitate generation of autoantibodies cross-reacting with self-AChR and of MG susceptibility in $\alpha_{146-162}$ peptide-immunized mice.

### *Anti-CTLA-4 Ab treatment results in enhanced disease in established EAMG*

To define the time point when anti-CTLA-4 Ab exerts its effect on the development of EAMG, mice were divided into two groups after the second immunization. Ab treatment with either control Ab (10 mice) or anti-CTLA-4 Ab (10 mice) was initiated 10 days after the second immunization (day 40 p.i.), at which time a significant number of mice already exhibited clinical EAMG. Anti-CTLA-4 Ab treatment at this time point led to a more severe disease compared with control mice (Fig. 5*A*). At the termination of the experiment, two mice in the anti-CTLA-4 Ab-treated group had died, while none of the mice in the control group had died. The exacerbation of clinical symptoms was associated with enhanced AChR-induced Ab production (Fig. 5*B*) and T cell proliferation (Fig. 5*C*). Similarly, anti-CTLA-4 Ab treatment triggered determinant spreading and diversification of the autoantibody repertoire (data not shown). These results suggest that the role of CTLA-4 is not limited to the initial phase of immune response in this setting.

## Discussion

In this study, we show that both initial and ongoing autoimmunities to AChR in EAMG are enhanced when CTLA-4-dependent signaling is inhibited in vivo. Anti-CTLA-4 Ab treatment is known to enhance several T cell-mediated experimental autoimmune diseases, including murine models of encephalomyelitis (9, 10) and diabetes (11). The present study extends the negative regulatory role of CTLA-4 to a B cell-mediated autoimmune disease. Apart from these observations, the current study also suggests that anti-CTLA-4 Ab treatment may induce intramolecular determinant spreading within the AChR. Determinant spreading induced by inhibition of CTLA-4 signaling is thus one mechanism that may account for the enhanced development of MG in the present model.

An important phenomenon during autoimmune disease progression is the occurrence of recruited T cells that are sensitized to recognize epitopes distinct from and non-cross-reactive with inducing epitopes (29, 30). In rodent experimental autoimmune encephalomyelitis (EAE) models determinant spreading within the same myelin protein as well as between myelin proteins may be responsible for disease relapses (34). The driving factor(s) for the progression of the Ab-mediated MG is not clear. EAMG can be induced in animals immunized with sequences restricted to the extracellular AChR $\alpha$ subunit (35, 36), which probably involves intramolecular determinant spreading, resulting in pathogenic Abs that cross-react with self-AChR in situ and thus leading to AChR loss (37). Vincent and colleagues have provided the most convincing evidence for determinant spreading by immunization of rabbits with a pool of three overlapping peptides encompassing human

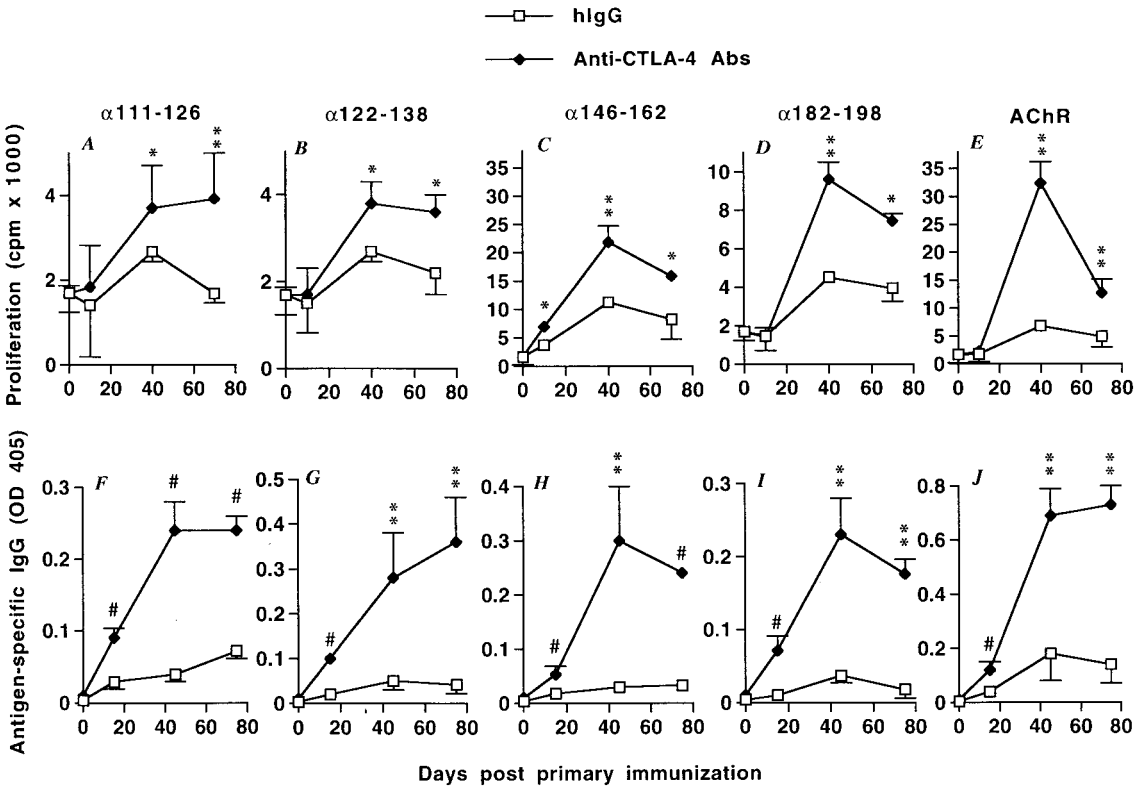


**FIGURE 4.** Anti-CTLA-4 Ab treatment triggers autoreactive T cell determinant spreading within the AChR $\alpha$ subunit and diversifies the anti-AChR Ab repertoire. B6 mice were immunized with 50 $\mu$g of $\alpha_{146-162}$ in CFA and treated with either control Abs or anti-CTLA-4 Abs. *A–E*, On days 10, 40, and 70 p.i. with $\alpha_{146-162}$ peptide, MNC ($n = 4-6$ in each group) were exposed to AChR, its dominant epitope $\alpha_{146-162}$, the subdominant epitopes $\alpha_{111-126}$ and $\alpha_{182-198}$, and the epitope $\alpha_{122-138}$. Proliferative responses to individual Ag/peptide stimulation were analyzed by [*methyl*-$^3$H]thymidine incorporation. Data are representative of two experiments with similar results. *F–J*, Sera from 10 mice in each group were taken on days 15, 45, and 75 p.i. IgG concentrations against AChR and individual peptides were measured by ELISA. Symbols refer to mean values, and bars to SDs. *, $p < 0.05$; **, $p < 0.01$; #, $p < 0.001$.

$\alpha_{138-199}$. This led to higher levels of Abs to rabbit AChR than to human AChR (35). T cell reactivity is skewed to the AChR $\alpha$ subunit in both MG and EAMG (12). In B6 mice $\alpha_{146-162}$-reactive CD4$^+$ T cells interact with AChR-specific B cells to produce pathogenic anti-AChR Abs in vitro and in vivo (32, 37), whereas direct immunization with this peptide fails to break self tolerance (33). Our study demonstrated that anti-CTLA-4 Ab treatment in mice immunized with $\alpha_{146-162}$ induced vigorous T cell responses not only to the $\alpha_{146-162}$ peptide, but also to the $\alpha_{111-126}$, $\alpha_{122-138}$, and $\alpha_{182-198}$ peptides. In addition, T cell reactivity to the whole autoantigen AChR in these mice was markedly enhanced. In support of this idea, three groups have independently shown that induction of tolerance to $\alpha_{146-162}$ is sufficient to prevent murine MG primed by whole AChR (38–40). Similar observations have recently been published for the EAE model (41).

The profound effects of inhibiting the CTLA-4-B7 interaction on disease development suggest that the CTLA-4 signaling pathway is critically involved in the evolution of autoantibody response in this model. In the present study significant levels of diversified Abs against extracellular regions on the $\alpha$ subunit could be detected only in anti-CTLA-4 Ab-treated mice. T cell recognition of $\alpha_{182-198}$, the cholinergic binding site on AChR, for instance, may lead to production of pathogenic Abs, which directly impair AChR function (42). Previous reports have demonstrated that two $\alpha_{146-162}$ specific T cell lines caused *Torpedo* AChR-primed B cells to differentiate and secrete fully cross-reactive Abs against the murine epitope $\alpha_{122-138}$ that dominate the Ab response in vitro (32). Our results confirm and extend this to an in vivo Ab response. Furthermore, these mice developed weakness accompanied by a significant loss of muscle AChR. Clinical EAMG appeared to result from broadened T cell responses and subsequent amplification of pathogenic Abs that bound to self peptide $\alpha_{122-138}$ and native AChR in situ, which led to AChR loss. These observations suggest that neo-autoreactive T cells play a pathogenic role by activating and driving AChR-specific B cells to diversify the autoantibody repertoire and may also, in turn, allow heightened presentation of certain subdominant or cryptic peptides at levels sufficient to stimulate CD4$^+$ T cells. Thus, determinant spreading induced by treatment with anti-CTLA-4 Ab appears to break tolerance to the T cell epitope $\alpha_{146-162}$ in B6 mice by broadening the cross-reaction of anti-peptide Abs with native AChR, which leads to full-blown clinical EAMG.

In addition to the observations on determinant spreading, it was observed that treatment with anti-CTLA-4 Ab was associated with augmented levels of IFN-$\gamma$ and IL-4 production. These cytokines can subsequently boost autoantibody production and exacerbate EAMG. MG has been associated with both Th1 and Th2 types of cytokines (43). Mice deficient in IFN-$\gamma$ or IFN-$\gamma$ receptor failed to mount sufficient anti-AChR Abs and were resistant to EAMG induction (44, 45). We have also demonstrated that exogenous IFN-$\gamma$ exacerbates clinical EAMG in the Lewis rat (46). Interestingly, TGF-$\beta$ production was suppressed after anti-CTLA-4 Ab treatment. TGF-$\beta$ is a potent inhibitor of T cell-mediated responses (47). Ag-specific triggering of Th3 cells to produce TGF-$\beta$ confers protection against EAMG (48). We have recently shown that NK cells may determine B cell-mediated autoimmunity via control of TGF-$\beta$ production by T cells (49). Chen et al. recently found that cross-linking of CTLA-4 induced TGF-$\beta$ production by murine

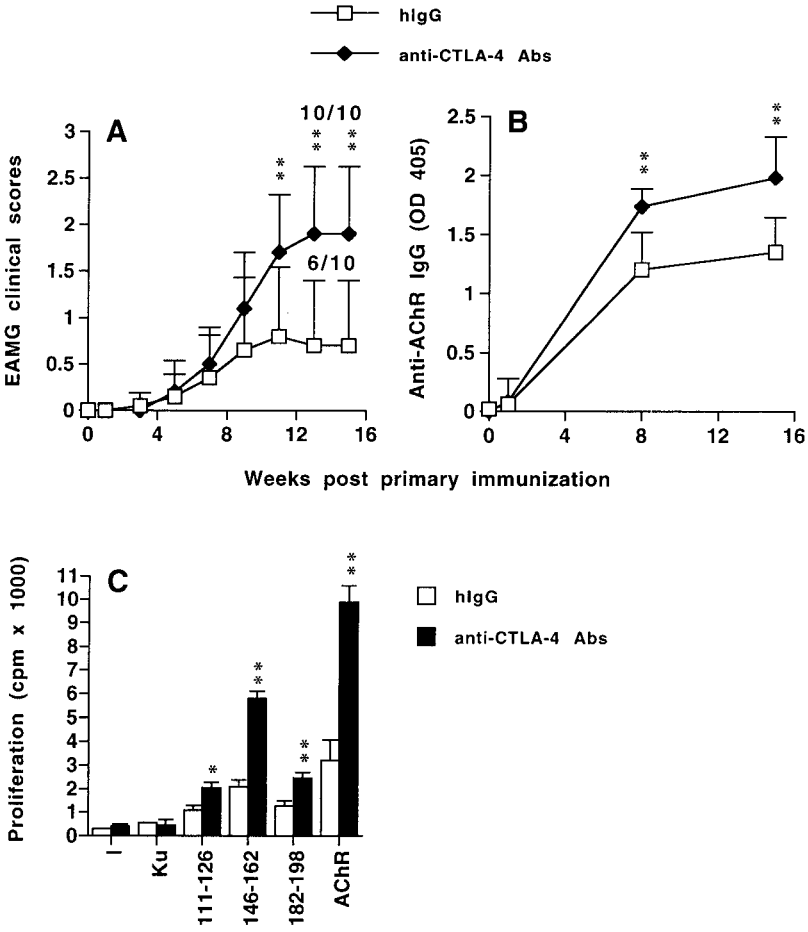


**FIGURE 5.** Effects of anti-CTLA-4 Ab treatment in established clinical EAMG. B6 mice were immunized with 20 $\mu$g of AChR in CFA without Ab treatment, then boosted on day 30 p.i. Ten days after AChR boost, i.e., day 40 p.i., mice were randomly separated into two groups and treated with either control Abs or anti-CTLA-4 Abs, respectively. *A*, Mice were monitored for signs of muscle weakness characteristic of EAMG. The mean clinical scores of mice receiving control Abs ($n$ = 10) and anti-CTLA-4 Abs ($n$ = 10) are indicated. *B*, Sera from these mice were taken at 1, 8, and 15 wk p.i., and anti-AChR IgG were evaluated by ELISA. *C*, Mice receiving control Abs ($n$ = 4) and anti-CTLA-4 Abs ($n$ = 4) were sacrificed on day 105 p.i. MNC were assayed for AChR-induced proliferation. Symbols refer to mean values, and bars to SDs. *, $p < 0.05$; **, $p < 0.01$.

$CD4^+$ T cells in vitro. In this study it was speculated that blockade of CTLA-4 signaling in vivo may lead to a loss of TGF-$\beta$ production by T cells, which in part contributes to the up-regulation of T cell activation (50). Furthermore, it has recently demonstrated that $CD25^+CD4^+$ regulatory T cells constitutively expressing CTLA-4 regulate autoimmune responses by TGF-$\beta$ production (51, 52). Therefore, anti-CTLA-4 Ab treatment may enhance EAMG via the aforementioned mechanisms.

In this model, treatment with anti-CTLA-4 Ab after the establishment of disease similarly enhanced the autoimmune responses to AChR and clinical EAMG. These results suggest that both primary and ongoing autoimmune responses can be down-regulated by CTLA-4 in the murine model of MG. The down-regulating effects of CTLA-4 on initial and ongoing autoreactive T cell responses have been shown in EAE, induced by myelin protein or peptide (10). In contrast, in the nonobese diabetes model of human insulin-dependent diabetes mellitus (IDDM), the effects of anti-CTLA-4 Ab were only observed during a narrow time window when potentially diabetogenic T cells are first activated (11). Currently, it is not clear why the role of CTLA-4 in the ongoing T cell responses may differ in induced autoimmune disease models (such as EAE and EAMG) from that in spontaneous autoimmune disease models (such as IDDM). In the initial prediabetic stage of IDDM, the immune response is targeted to a few determinants of glutamic acid decarboxylase, but later to other determinants in the glutamic acid decarboxylase molecules and, still later, to other $\beta$ cell Ags (53). It is likely that once the initial T cells are activated, diabetogenic determinants were exposed relatively more promptly and thoroughly when $\beta$ cell elimination began. Thus, the effect of anti-CTLA-4 Ab treatment on ongoing autoimmunity in IDDM is not as pronounced as in EAE and EAMG.

The current EAMG model allows us to uncover a possible mechanism underlying the regulation of autoimmune diseases by CTLA-4. It is most likely that the anti-CTLA-4 treatment enhances the ability of autoreactive T cells to provide help to B cells. The enhanced B cell function as Ab-producing cells or as APCs might contribute to disease development and enhance T cell determinant spreading. Furthermore, it cannot be excluded that the activities of other APCs may be enhanced as the result of Ab-induced opsonization leading to increased Ag presentation. Another not mutually exclusive possibility is that anti-CTLA-4 treatment may affect regulatory T cells such as the $CD4^+CD25^+$ cells that recently has achieved significant attention (54). Taken together, the present study suggests that CTLA-4 is a critical inhibitor of B cell-mediated autoimmune disease, thus providing a candidate target for immunointervention.

## Acknowledgments

We thank Dr. J. A. Bluestone for providing the 4F10 hybridoma cell line, Drs M. Levi and B. Wahren for help with peptide synthesis, and Drs. R. Wallin, M. T. Bejarano, and members of Drs. Ljunggren's and Link's groups for fruitful discussions and advice.

## References

1. Harper, K., C. Balzano, E. Rouvier, M. G. Mattei, M. F. Luciani, and P. Golstein. 1991. CTLA-4 and CD28 activated lymphocyte molecules are closely related in both mouse and human as to sequence, message expression, gene structure, and chromosomal location. *J. Immunol. 147:1037.*

2. Walunas, T. L., D. J. Lenschow, C. Y. Bakker, P. S. Linsley, G. J. Freeman, J. M. Green, C. B. Thompson, and J. A. Bluestone. 1994. CTLA-4 can function as a negative regulator of T cell activation. *Immunity 1:405.*
3. Waterhouse, P., L. E. Marengere, H. W. Mittrucker, and T. W. Mak. 1996. CTLA-4, a negative regulator of T-lymphocyte activation. *Immunol. Rev. 153: 183.*
4. Saito, T. 1998. Negative regulation of T cell activation. *Curr. Opin. Immunol. 10:313.*
5. Waterhouse, P., J. M. Penninger, E. Timms, A. Wakeham, A. Shahinian, K. P. Lee, C. B. Thompson, H. Griesser, and T. W. Mak. 1995. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science 270:985.*
6. Tivol, E. A., F. Borriello, A. N. Schweitzer, W. P. Lynch, J. A. Bluestone, and A. H. Sharpe. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity 3:541.*
7. Leach, D. R., M. F. Krummel, and J. P. Allison. 1996. Enhancement of antitumor immunity by CTLA-4 blockade. *Science 271:1734.*
8. Kathy, M., M. Camberis, and G. L. Gros. 1997. Protective immunity to nematode infection is induced by CTLA-4 blockade. *J. Exp. Med. 186:183.*
9. Karandikar, N. J., C. L. Vanderlugt, T. L. Walunas, S. D. Miller, and J. A. Bluestone. 1996. CTLA-4: a negative regulator of autoimmune disease. *J. Exp. Med. 184:783.*
10. Perrin, P. J., J. H. Maldonado, T. A. Davis, C. H. June, and M. K. Racke. 1996. CTLA-4 blockade enhances clinical disease and cytokine production during experimental allergic encephalomyelitis. *J. Immunol. 157:1333.*
11. Luhder, F., P. Höglund, J. P. Allison, C. Benoist, and D. Mathis. 1998. Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) regulate the unfolding of autoimmune diabetes. *J. Exp. Med. 187:427.*
12. Drachman, D. 1994. Myasthenia gravis. *N. Engl. J. Med. 330:1797.*
13. Lewis, R. A., J. F. Sela, and R. P. Lisak. 1995. Myasthenia gravis: immunological mechanisms and immunotherapy. *Ann. Neurol. 37:S51.*
14. Yokoi, T., B. Mulac-Jericevic, J. Kurisaki, and M. Z. Atassi. 1987. T lymphocyte recognition of acetylcholine receptor: localization of the full T cell recognition profile on the extracellular part of the $\alpha$ chain of *Torpedo californica* acetylcholine receptor. *Eur. J. Immunol. 17:1697.*
15. Oshima, M., A. R. Pachner, and M. Z. Atassi. 1994. Profile of the regions of acetylcholine receptor $\alpha$ chain recognized by T-lymphocytes and by antibodies in EAMG-susceptible and non-susceptible mouse strains after different periods of immunization with the receptor. *Mol. Immunol. 31:833.*
16. Hawke, S., H. Matsuo, M. Nicolle, G. Malcherek, A. Melms, and N. Willcox. 1996. Autoimmune T cells in myasthenia gravis: heterogeneity and potential for specific immunotargeting. *Immunol. Today 17:307.*
17. Conti-Fine, B. M., D. Navaneetham, P. I. Karachunski, R. Raju, B. Diethelm-kita, D. Okita, J. Howard, Jr., and Z. Y. Wang. 1998. T cell recognition of the acetylcholine receptor in myasthenia gravis. *Ann. NY Acad. Sci. 841:283.*
18. Shi, F. D., B. He, H. Li, D. Matusevicius, H. Link, and H. G. Ljunggren. 1998. Differential requirements for CD28 and CD40 ligand in the induction of experimental autoimmune myasthenia gravis. *Eur. J. Immunol. 28:3587.*
19. McIntosh, K. R., P. S. Linsley, P. A. Bacha, and D. B. Drachman. 1998. Immunotherapy of experimental autoimmune myasthenia gravis: selective effects of CTLA4Ig and synergistic combination with an IL2-diphtheria toxin fusion protein. *J. Neuroimmunol. 87:136.*
20. Lindstrom, J., B. Einarson, and S. Tzartos. 1981. Production and assay of antibodies to acetylcholine receptors. *Methods Enzymol. 74:432.*
21. Deibler, G. E., R. E. Martenson, and M. V. Kies. 1972. Large scale preparation of myelin basic protein from central nervous tissue of several mammalian species. *Prep. Biochem. 2:139.*
22. Prabhakar, B. S., G. P. Allaway, J. Srinivasappa, and A. L. Notkins. 1990. Cell surface expression of the 70-KD component of Ku, a DNA-binding nuclear autoantigen. *J. Clin. Invest. 86:1301.*
23. Kearney, E. R., T. L. Walunas, R. W. Karr, P. A. Morton, D. Y. Loh, J. A. Bluestone, and M. K. Jenkins. 1995. Antigen-dependent clonal expansion of a trace population of antigen-specific $CD4^+$ T cells in vivo is dependent on CD28 costimulation and inhibited by CTLA-4. *J. Immunol. 155:1032.*
24. Berman, P. W., and J. Patrick. 1980. Experimental myasthenia gravis: a murine system. *J. Exp. Med. 151:204.*
25. Shi, F. D., X. Bai, H. Li, Y. Huang, P. H. van der Meide, and H. Link. 1998. Nasal tolerance in experimental autoimmune myasthenia gravis (EAMG): induction of protective tolerance in primed animals. *Clin. Exp. Immunol. 111:506.*
26. Li, H., F. D. Shi, B. He, M. Bakheit, B. Wahren, A. Berglof, K. Sandstedt, and H. Link. 1998. Experimental autoimmune myasthenia gravis induction in B cell-deficient mice. *Int. Immunol. 10:1359.*
27. Christadoss, P., J. Lindstrom, S. Munro, and N. J. Talal. 1985. Muscle acetylcholine receptor loss in murine experimental autoimmune myasthenia gravis: correlated with cellular, humoral and clinical responses. *J. Neuroimmunol. 8:29.*
28. Walunas, T. L., and J. A. Bluestone. 1998. CTLA-4 regulates tolerance induction and T cell differentiation in vivo. *J. Immunol. 160:3855.*
29. Lehmann, P. V., E. E. Sercarz, T. Forsthuber, C. M. Dayan, and G. Gammon. 1993. Determinant spreading and the dynamics of the autoimmune T-cell repertoire. *Immunol. Today 14:203.*
30. Lehmann, P. V., T. Forsthuber, A. Miller, and E. E. Sercarz. 1992. Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. *Nature 358:155.*
31. Lennon, V. A., D. J. McCormick, E. H. Lambert, G. E. Griesmann, and M. Z. Atassi. 1985. Region of peptide 125–147 of acetylcholine receptor $\alpha$ subunit is exposed at neuromuscular junction and induces experimental autoimmune myasthenia gravis, T-cell immunity, and modulating autoantibodies. *Proc. Natl. Acad. Sci. USA 82:8805.*
32. Rosenberg, J. S., M. Oshima, and M. Z. Atassi. 1996. B-cell activation in vitro by helper T cells specific to region $\alpha$146–162 of *Torpedo californica* nicotinic acetylcholine receptor. *J. Immunol. 157:3192.*
33. Shenoy, M., M. Oshima, M. Zouhair, and P. Christadoss. 1993. Suppression of experimental autoimmune myasthenia gravis by epitope-specific neonatal tolerance to synthetic region $\alpha$146–162 of acetylcholine receptor. *Clin. Immunol. Immunopathol. 66:230.*
34. McRae, B. L., C. L. Vanderlugt, M. C. Dal Canto, and S. D. Miller. 1995. Functional evidence for epitope spreading in the relapsing pathology of experimental autoimmune encephalomyelitis. *J. Exp. Med. 182:75.*
35. Vincent, A., L. Jacobson, and P. Shillito. 1994. Response to human acetylcholine receptor $\alpha$138–199:determinant spreading initiates autoimmunity to self-antigen in rabbits. *Immunol. Lett. 39:269.*
36. Lennon, V. A., E. H. Lambert, K. R. Leiby, T. B. Okarma, and S. Talib. 1991. Recombinant human acetylcholine receptor $\alpha$-subunit induces chronic experimental autoimmune myasthenia gravis. *J. Immunol. 146:2245.*
37. Vincent, A., N. Willcox, M. Hill, J. Curnow, C. MacLennan, and D. Beeson. 1998. Determinant spreading and immune responses to acetylcholine receptors in myasthenia gravis. *Immunol. Rev. 164:157.*
38. Wu, B., C. Deng, E. Goluszko, and P. Christadoss. 1997. Tolerance to a dominant T cell epitope in the acetylcholine receptor molecule induces epitope spread and suppresses murine myasthenia gravis. *J. Immunol. 159:3016.*
39. Karachunski, P. I., N. S. Ostlie, D. K. Okita, and B. M. Conti-Fine. 1997. Prevention of experimental myasthenia gravis by nasal administration of synthetic acetylcholine receptor T epitope sequences. *J. Clin. Invest. 100:3027.*
40. Baggi, F., F. Andreetta, E. Caspani, M. Milani, R. Longhi, R. Mantegazza, F. Cornelio, and C. Antozzi. 1999. Oral administration of an immunodominant T-cell epitope downregulates Th1/Th2 cytokines and prevents experimental myasthenia gravis. *J. Clin. Invest. 104:1287.*
41. Karandikar, N. J., T. N. Eagar, C. L. Vanderlugt, J. A. Bluestone, and S. D. Miller. 2000. CTLA-4 downregulates epitope spreading and mediates remission in relapsing experimental autoimmune encephalomyelitis. *J. Neuroimmunol. 109:173.*
42. Counti-Tronconi, B. M., F. Tang, B. M. Diethelm, S. R. Spencer, S. Reinhardt-Maelicke, and A. Maelicke. 1991. Mapping of a cholinergic binding site by means of synthetic peptides, monoclonal antibodies, and $\alpha$-bungarotoxin. *Biochemistry 29:6221.*
43. Link, J., M. Soderstrom, A. Ljungdahl, B. Hojeberg, T. Olsson, Z. Xu, S. Fredrikson, Z. Y. Wang, and H. Link. 1994. Organ-specific autoantigens induce interferon-gamma and interleukin-4 mRNA expression in mononuclear cells in multiple sclerosis and myasthenia gravis. *Neurology 44:728.*
44. Balasa, B., C. Deng, J. Lee, L. M. Bradley, D. K. Dalton, P. Christadoss, and N. Sarvetnick. 1997. Interferon $\gamma$ (IFN-$\gamma$) is necessary for the genesis of acetylcholine receptor-induced clinical experimental autoimmune myasthenia gravis in mice. *J. Exp. Med. 186:385.*
45. Zhang, G. X., B. G. Xiao, X. F. Bai, P. H. van der Meide, A. Orn, and H. Link. 1999. Mice with IFN-$\gamma$ receptor deficiency are less susceptible to experimental autoimmune myasthenia gravis. *J. Immunol. 162:3775.*
46. Wang, H. B., F. D. Shi, H. Li., P. H. van der Meide, H. G. Ljunggren and H. Link. 2000. Role for IFN-$\gamma$ in rat strains with different susceptibility to experimental autoimmune myasthenia gravis. *Clin. Immunol. 95:156.*
47. Mason, D., and F. Powrie. 1998. Control of immune pathology by regulatory T cells. *Curr. Opin. Immunol. 10:649.*
48. Shi, F. D., H. Li., H. B. Wang, X. Bai, P. H. van der Meide, H. Link, and H. G. Ljunggren. 1999. Mechanism of nasal tolerance induction in experimental autoimmune myasthenia gravis: identification of regulatory cells. *J. Immunol. 162:5757.*
49. Shi, F. D., H. B. Wang, H. Li, S. Hong, M. Taniguchi, H. Link, L. Van Kaer, and H. G. Ljunggren. 2000. Natural killer cells determine the outcome of B cell-mediated autoimmunity. *Nat. Immunol. 1:245.*
50. Chen, W. J., W. W. Jin, and S. M. Wahl. 1998. Engagement of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) induces transforming growth factor $\beta$ (TGF-$\beta$) production by murine $CD4^+$ T cells. *J. Exp. Med. 188:1894.*
51. Takahashi, T., T. Tagami, S. Yamazaki, T. Uede, J. Shimizu, N. Sakaguchi, T. W. Mak, and S. Sakaguchi. 2000. Immunologic self-tolerance maintained by $CD25^+CD4^+$ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J. Exp. Med. 192:303.*
52. Read, S., V. Malmstrom, and F. Powrie. 2000. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of $CD25^+CD4^+$ regulatory cells that control intestinal inflammation. *J. Exp. Med. 192:295.*
53. Singh, R. R., and B. H. Hahn. 1998. Reciprocal T-B determinant spreading develops spontaneously in murine lupus: implications for pathogenesis. *Immunol. Rev. 164:201.*
54. Salomon, B., D. J. Lenschow, L. Rhee, N. Ashourian, B. Singh, A. Sharpe, and J. A. Bluestone. 2000. B7/CD28 Costimulation is essential for the homeostasis of the $CD4^+CD25^+$ immunoregulatory T cells that control autoimmune diabetes. *Immunity 12:431.*