A Critical Role for B7/CD28 Costimulation in Experimental Autoimmune Encephalomyelitis: A Comparative Study Using Costimulatory Molecule-Deficient Mice and Monoclonal Antibody Blockade[1]

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The B7/CD28 pathway provides critical costimulatory signals required for complete T cell activation and has served as a potential target for immunotherapeutic strategies designed to regulate autoimmune diseases. This study was designed to examine the roles of CD28 and its individual ligands, B7-1 and B7-2, in experimental autoimmune encephalomyelitis (EAE), a Th1-mediated inflammatory disease of the CNS. EAE induction in CD28- or B7-deficient nonobese diabetic (NOD) mice was compared with the effects of B7/CD28 blockade using Abs in wild-type NOD mice. Disease severity was significantly reduced in CD28-deficient as well as anti-B7-1/B7-2-treated NOD mice. B7-2 appeared to play the more dominant role as there was a moderate decrease in disease incidence and severity in B7-2-deficient animals. EAE resistance was not due to the lack of effective priming of the myelin peptide-specific T cells in vivo. T cells isolated from CD28-deficient animals produced equivalent amounts of IFN-γ and TNF-α in response to the immunogen, proteolipid protein 56–70. In fact, IFN-γ and TNF-α production by Ag-specific T cells was enhanced in both the B7-1 and B7-2-deficient NOD mice. In contrast, peptide-specific delayed-type hypersensitivity responses in these animals were significantly decreased, suggesting a critical role for CD28 costimulation in in vivo trafficking and systemic immunity. Collectively, these results support a critical role for CD28 costimulation in EAE induction. *The Journal of Immunology,* **2000, 164: 136–143.**

For complete activation, T cells require the delivery of at least two signals by APC. One signal is Ag specific and is delivered by TCR engagement of the peptide-MHC complex on the APC. The second "costimulatory" signal can be provided by a number of soluble and membrane-associated molecules. The CD28 molecule on T cells is a major costimulatory molecule through its ligation with the B7 family of molecules, B7-1 or B7-2, expressed on the APC (reviewed in Refs. 1 and 2). Following activation, T cells up-regulate surface expression of CTLA-4, a homologue of CD28, that binds the same ligands with higher affinity (3) and serves as a negative regulator of T cell activation (4–6). As the B7/CD28:CTLA-4 costimulatory system plays a critical role in determining the fate of immune responses (activation vs down-regulation), it is a highly promising therapeutic target for regulating immunopathological immune responses. In fact, inhibition of B7/CD28 costimulation has been shown to have significant immunosuppressive effects: reducing specific Ab production (7), prolonging the survival of organ transplants, inhibiting autoimmune diabetes, and lupus (8–12).

For this reason, we and others have explored the role of this costimulatory system in the induction and progression of murine experimental autoimmune encephalomyelitis (EAE),[4] a $CD4^+$ Th1-mediated inflammatory demyelinating disease of the CNS and a well-established animal model for the human disease, multiple sclerosis (13, 14). Studies employing CTLA-4Ig, a soluble CD28 antagonist that binds to both B7-1 and B7-2, in a proteolipid protein ($PLP_{139-151}$)-induced relapsing-remitting EAE model in SJL mice have shown a predominant role for CD28-mediated costimulation in EAE induction (15). Additionally, blockade of CD28 signaling with CD28 F(ab) ameliorates myelin basic protein-induced EAE (16), demonstrating the critical role for CD28-mediated costimulation in EAE induction and progression. However, the individual effects of anti-B7-1 and anti-B7-2 mAb therapies on disease induction were less clear. Kuchroo et al. reported that anti-B7-1 mAbs inhibited the disease while anti-B7-2 led to disease exacerbation. However, the effectiveness of the mAb required continuous therapy for 30 days postimmunization overlapping the first acute and relapse phases (17). In contrast, studies from our laboratory showed that treatment of SJL mice with anti-B7-2 during disease remission had no effect on further disease progression, while blockade of B7-1 with anti-B7-1 F(ab) suppressed clinical relapses (18, 19). These results demonstrate potential separate

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[4] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; DTH, delayed-type hypersensitivity; PLP, proteolipid protein; NOD, nonobese diabetic.

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functional roles for B7-1 and B7-2 during EAE induction and progression. However, the interpretation of these data are complicated by the fact that the efficacy of mAb therapy will depend on the effectiveness of the blockade based on the type or the dose of mAb used but, more importantly, may be influenced by the potential signaling capacity of the mAbs. This is perhaps best exemplified by the observation that treatment of SJL mice with intact anti-B7-1 mAbs during disease remission EAE results in accelerated relapses pathology and epitope spreading (19).

To more directly discern the potential roles of B7-1, B7-2, and CD28 in EAE induction, we compared disease induced in mice deficient for expression of CD28 ($CD28^{-/-}$) or B7 costimulatory molecules ($B7\text{-}1^{-/-}$ and $B7\text{-}2^{-/-}$) to the effects of mAb therapy for 10 days following the primary induction of EAE in wild-type mice. We describe the development of a new model of acute EAE in nonobese diabetic (NOD) mice using a proteolipid peptide, PLP_{56-70}, previously shown to be encephalitogenic in Biozzi AB/H ($I\text{-}A^{g7}$) mice (20). Disease severity was most significantly reduced in $B7\text{-}2^{-/-}$ NOD mice and absent in $CD28^{-/-}$ NOD mice as compared with wild-type NOD animals, despite the ability of these animals to produce normal or enhanced levels of IFN-γ and TNF-α to the immunizing Ag. Furthermore, disease induction was significantly delayed in mice treated for 10 days postimmunization with either CTLA-4Ig or with a combination anti-B7-1 plus anti-B7-2 mAbs, indicating a critical role for B7/CD28 signaling in EAE induction in the NOD mouse.

Materials and Methods

Mice

NOD female mice, 4–5 wk old, and $CD28^{-/-}$ mice on the C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). $B7\text{-}1^{-/-}$ and $B7\text{-}2^{-/-}$ mice were generated on the 129/S4AvJae background by gene targeting (21, 22). These knockout mice were backcrossed onto the NOD strain for five or more generations. The backcrossed animals were intercrossed, and female mice were used in all experiments. All mice were housed in barrier conditions with the Center for Experimental Animal Research at Northwestern University and were maintained on standard laboratory food and water ad libitum. Paralyzed animals were afforded easier access to food and water.

Peptides

PLP_{56-70} (DYEYLINVIHAFQYV) and $OVA_{323-339}$ (ISQAVHAAHAE INEAGR) were purchased from Peptides International (Louisville, Kentucky). Amino acid composition was verified by mass spectrometry, and purity (>98%) was assessed by HPLC.

Induction and clinical evaluation of PLP_{56-70}-induced EAE

Five- to 7-wk-old female NOD mice were immunized s.c. with 200 μl of an emulsion containing 800 μg of *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI) and 200 μg PLP_{56-70} distributed over two spots on the flank. Each mouse additionally received 200 ng pertussis toxin (List Biological Laboratories, Campbell, CA) in 200 μl PBS i.v. on days 0 and 2 postimmunization. Individual animals were observed daily, and clinical scores were assessed on a 0–5 scale as follows: 0, no abnormality; 1, limp tail; 2, limp tail and hind limb weakness (legs slip through cage top); 3, partial hind limb paralysis; 4, complete hind limb paralysis; and 5, moribund. The data are reported as the mean daily clinical score for all animals in a particular group and/or as the mean peak clinical score, i.e., the mean clinical score for all animals at the peak of disease. Animals were also monitored for the development of diabetes by determining urinary glucose levels. No animals were found to develop overt diabetes, probably due to the fact that CFA administration prevents clinical disease in NOD mice (23).

Ab treatments

The following mAbs were employed: hamster control Ig (Parsi12), anti-CD80 (B7-1) mAb 16-10A1, and anti-CD86 (B7-2) mAb GL-1 (24). Murine CTLA4-Ig was obtained from the Genetics Institute (Boston, MA). Abs were produced in an Acusyst Jr. Bioreactor and purified as previously described (10). Mice were treated i.p. with 50 μg of Ab in 500 μl saline every other day from day -2 until day 10 postimmunization (7 doses total).

Elicitation of delayed-type hypersensitivity (DTH) responses

DTH responses were quantitated using a 24-h ear swelling assay. Prechallenge ear thickness was determined using a Mitutoyo model 7326 engineer's micrometer (Schlesinger's Tool, Brooklyn, NY). Immediately thereafter, DTH responses were elicited by injecting 10 μg of peptide (in 10 μl of saline) into the dorsal surface of the ear using a 100-μl syringe fitted with a 30-gauge needle. The increase in ear thickness was determined 24 h after ear challenge. Results are expressed in units of 10^{-4} inches $\pm$ SEM. Background swelling ranged between 3–10 $\times$ 10^{-4} inches.

In vitro proliferation and cytokine assays

On day 10 postimmunization, draining lymph node and splenic cells were harvested and cultured in 96-well microtiter plates at a density of 5×10^5 cells/well in a total volume of 200 μl DMEM containing 10% FBS, 1 mM glutamine, 1% penicillin-streptavidin, 1 mM nonessential amino acids, and 5×10^{-5} M 2-ME (complete DMEM 10%; all products from Sigma, St. Louis, MO). Cells were cultured with media alone or different concentrations of peptide Ag for 96 h. Culture wells were pulsed with 1 μCi/well [^{3}H]TdR for the final 24 h of the 96 h incubation period. [^{3}H]TdR uptake was detected using a topcount microplate scintillation counter (Packard Instruments, Meriden, CT), and results are expressed as the mean of triplicate cultures $\pm$ SEM (background counts subtracted). Supernatants collected at 24 and 48 h from replicate cultures were assayed for IFN-γ, TNF-α, IL-4, and IL-5 levels using ELISA Minikits (Endogen, Cambridge, MA).

Histological evaluation

Mice were anaesthetized and sacrificed by total body perfusion through the left ventricle using chilled 3% glutaraldehyde in PBS, pH 7.3. Spinal cords were dissected out and cut into 1-mm thick segments and postfixed in OsO_4, dehydrated, and embedded in Epon (Electron Microscopy Sciences, Ft. Washington, PA). Toluidine blue-stained sections from 10 segments per mouse were read and scored blinded by Dr. Mauro Dal Canto at Northwestern University (Chicago, IL).

Statistical analyses

Comparison of the percentage of animals showing clinical disease between any two groups of mice was done by χ^2 using Fisher's exact probability. Comparisons of the mean day of onset of relapse and mean peak disease severity between any two groups of mice were analyzed by the Student's *t* test.

Results

Clinical signs of EAE are impaired in NOD mice deficient in CD28/B7 costimulation

To gain a clearer understanding of the role of the CD28/B7 costimulatory pathway in the initiation of EAE, we attempted to induce EAE in NOD mice deficient in CD28/B7 costimulation. Wild-type, $CD28^{-/-}$, $B7\text{-}1^{-/-}$, and $B7\text{-}2^{-/-}$ NOD mice were immunized with 200 μg PLP_{56-70} in CFA and monitored for clinical signs of disease for 35–50 days postimmunization. Clinical and histologic signs of EAE were not inducible in CD28-deficient NOD mice (Fig. 1*A* and Table I). B7-1-deficient mice developed a slightly milder disease course when compared with the wild-type NOD controls (Fig. 1*B*) displaying a similar disease incidence, but a slightly delayed onset and reduced clinical severity (Table I). Compared with wild-type NOD controls, clinical signs of EAE in B7-2-deficient NOD females were significantly impaired (Fig. 1*C*). $B7\text{-}2^{-/-}$ mice had a similar disease incidence, but a significantly delayed disease onset and reduced clinical and histologic disease severity compared to controls (Table I). The mild course of EAE seen in $B7\text{-}2^{-/-}$ mice was a consistent finding in four separate experiments. Thus, CD28 costimulation was required for the clinical and histological manifestations of EAE in the NOD mouse. As the clinical severity in $B7\text{-}2^{-/-}$ mice was significantly reduced ($p = 0.03$) as compared with $B7\text{-}1^{-/-}$ mice, the data also suggests that B7-2 plays a more predominant role than B7-1 in induction and progression of EAE in the NOD mouse. It was not possible to assess EAE induction in NOD mice deficient for both B7-1 and B7-2 as these mice develop overt diabetes in a very accelerated

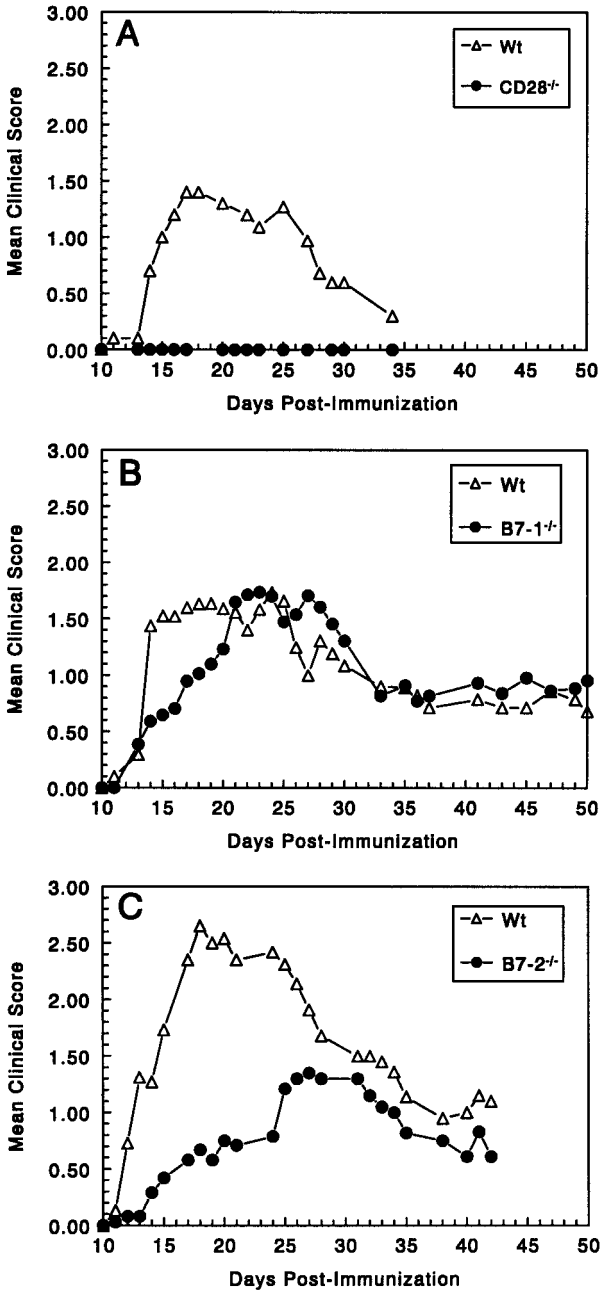

FIGURE 1. Comparison of PLP_{56-70}-induced EAE clinical disease courses between wild-type and costimulatory molecule-deficient NOD mice. Wild-type NOD mice and $CD28^{-/-}$ NOD mice (*A*), $B7\text{-}1^{-/-}$ (*B*), and $B7\text{-}2^{-/-}$ (*C*) were immunized with 200 μg of PLP_{56-70} in CFA on day 0 and scored for clinical signs of EAE as described in *Materials and Methods*. Results are plotted as the mean clinical score for all of the animals in each treatment group vs day postimmunization. Results are representative of four separate experiments.

fashion as compared with wild-type NOD mice (B. Salomon and J. A. Bluestone, manuscript in preparation) and were too sick to use in the EAE studies.

Myelin peptide-specific T cell responses in NOD mice deficient in CD28/B7 costimulation

We next determined the magnitude and phenotype of PLP_{56-70}-specific T cell responses in NOD mice deficient in CD28/B7 costimulation. PLP_{56-70}-specific proliferation and Th1-derived (IFN-γ and TNF-α) and Th2-derived (IL-4 and IL-5) cytokine responses in the lymph nodes draining the site of primary immunization and splenocytes of wild-type, $CD28^{-/-}$, $B7\text{-}1^{-/-}$, and $B7\text{-}2^{-/-}$ NOD mice were determined upon in vitro stimulation with PLP_{56-70} peptide 10 days following immunization. Despite their failure to develop clinical EAE, T cells from PLP-immunized CD28-deficient NOD mice responded similarly both in terms of proliferation (Fig. 2) and proinflammatory cytokine production (IFN-γ and TNF-α) (Fig. 3) when compared with wild-type NOD mice. By comparison, T cell proliferative responses were significantly enhanced in both lymphoid compartments in B7-1-deficient NOD mice and reduced in B7-2-deficient animals (Fig. 2). IFN-γ and TNF-α production in B7-1-deficient mice was significantly enhanced (21-fold and 3-fold, respectively) in lymph node T cells (Fig. 3). Despite the fact that clinical disease and T cell proliferation were significantly impaired in B7-2-deficient NOD mice, T cells from these animals made more IFN-γ (>20-fold) and TNF-α (2.4-fold) than wild-type NOD T cells (Fig. 3). IL-4 and IL-5 were not detectable in any of the culture supernatants (data not shown). These results suggest that PLP_{56-70}-specific Th1 cells are being primed in both B7-2- and CD28-deficient mice and that their failure to develop clinical EAE is not due to an inability to produce Th1-type cytokines.

NOD mice deficient in CD28/B7 costimulation exhibit reduced DTH responses to the immunizing myelin epitope

We next analyzed DTH reactivity as an in vivo measure of the relative abilities of wild-type, $CD28^{-/-}$, $B7\text{-}1^{-/-}$, and $B7\text{-}2^{-/-}$ mice to mount a Th1-dependent Ag recall response. At day 30 postimmunization, representative mice were ear challenged with 10 μg of the immunizing PLP_{56-70} peptide. As seen in Fig. 4, DTH responses were reduced in all of the knockout mice with the most significant decrease in CD28-deficient mice. These results suggest that disruption of the B7/CD28 costimulatory pathway abrogated the ability of CD28 knockout mice to mount a normal DTH response to the disease-initiating epitope. The results also suggest that severe defects in DTH responses, as seen in the CD28-deficient mice, can be more predictive of clinical outcome than are in vitro T cell proliferative responses or cytokine assays.

Treatments targeting both B7-1 and B7-2 at disease initiation effectively delay the onset of EAE

To attempt to compare the results obtained using the costimulation-deficient mice to previous reports that used in vivo therapy with intact mAbs, we immunized wild-type NOD mice with 200 μg PLP_{56-70} in CFA and treated them with CD28/B7 antagonists every other day from day -2 until day 10 postimmunization. Treatment with either CTLA-4Ig or with a combination of anti-B7-1 and anti-B7-2 mAbs resulted in a significant delay in disease onset in wild-type NOD mice (mean day of onset = 23 and 24 days, respectively) compared with that of control Ig-treated animals (mean day of onset = 18 days) (Fig. 5 and Table II). Disease severity was also reduced in three of the four animals treated with CTLA-4Ig (Fig. 5 and Table II). The individual contributions of B7-1 and B7-2 were examined by treating at disease initiation with either mAb. Clinical disease was not significantly affected by treatment with either anti-B7-1 or anti-B7-2 alone when compared with NOD mice treated with a control hamster Ig (Fig. 5 and Table II). Costimulatory blockade concomitant with myelin peptide priming did not result in long-term peripheral tolerance as indicated by the fact that clinical symptoms (Fig. 5), DTH, and T cell proliferative responses (data not shown) developed after the clearance of the Abs. DTH responses correlated with clinical severity, while T cell proliferative responses did not.

Table I. *EAE disease parameters in wild-type, CD28$^{-/-}$, B7-1$^{-/-}$, and B7-2$^{-/-}$ NOD mice*

Disease Parameter[a]	Genotype			
	Wild-Type NOD	CD28$^{-/-}$	B7-1$^{-/-}$	B7-2$^{-/-}$
Incidence	30/30 (100%)	0/13 (0%)*	(17/17) 100%	10/12 (83%)
Mean day of onset	13.3 ± 0.6	—	17.5 ± 1.0**	20.8 ± 1.9***
Mean peak clinical score	3.0 ± 0.1	—	2.5 ± 0.2**	1.7 ± 0.3***
Histology	Inflammation; demyelination	None	ND	Mild inflammation

[a] Mean day of onset and mean peak clinical score values were calculated as described in *Materials and Methods.*

*, Disease incidence significantly less than that of the wild-type NOD controls, $p < 0.00001$.

**, Mean day of onset was delayed and mean peak clinical score was reduced significantly compared to that of the wild-type NOD controls, $p < 0.01$.

***, Mean day of onset was delayed and mean peak clinical score was reduced significantly compared to that of the wild-type NOD controls, $p < 0.0001$.

When hamster Ig-treated control animals reached their peak of acute disease (day 25 postimmunization), representative mice from each mAb treatment group were sacrificed for histological examination of their spinal cords. This examination revealed significant inflammation and demyelination in the hamster Ig-treated controls (Fig. 6*A*). The groups treated with anti B7-1 or anti B7-2 alone had significant inflammation accompanied by a milder demyelination (Fig. 6, *B* and *C*). Histological signs of disease were absent in the group treated with both anti-B7-1 and anti-B7-2 (Fig. 6*D*), and only mild inflammation was observed in the CTLA-4Ig-treated group (Fig. 6*E*). Overall, the histological evaluation of the target organ corresponded closely with the clinical scores of each group at day 25 postimmunization (see Fig. 5). Collectively, these results imply that T cell activation sufficient to initiate EAE requires either B7-1 or B7-2 costimulatory signaling via CD28. However, in light of the significant reduction in disease noted in the B7-2$^{-/-}$ mice (Fig. 1), functional conclusions regarding the individual roles of B7-1 and B7-2 in EAE induction may be more complicated than is easily determined by mAb therapy.

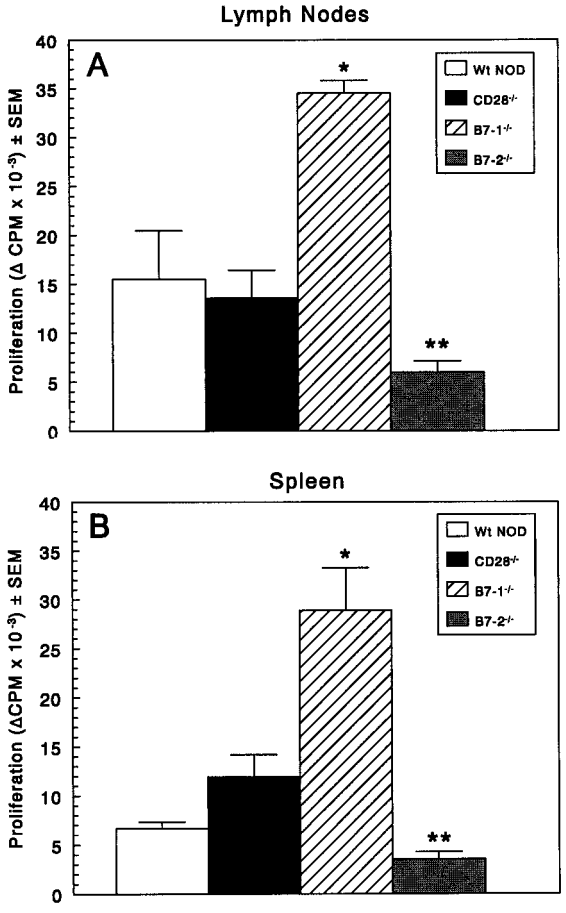

FIGURE 2. Myelin peptide-specific T cell proliferative responses in lymph node and spleen of wild-type and costimulatory molecule-deficient NOD mice. Draining lymph node and splenic cells isolated from PLP_{56-70}/CFA-primed mice 10 days postimmunization were cultured with PLP_{56-70}. Data represents the mean thymidine uptake of triplicate cultures stimulated for a total of 96 h with 50 μM peptide and is plotted as Δ cpm ± SEM (background counts subtracted). T cells from all groups failed to respond to $OVA_{323-339}$ as an irrelevant peptide control (data not shown). Results are representative of three separate experiments. *, Proliferative responses in B7-1$^{-/-}$ mice were significantly greater than wild-type controls. **, Proliferative responses of B7-2$^{-/-}$ mice were significantly less than controls, $p < 0.01$.

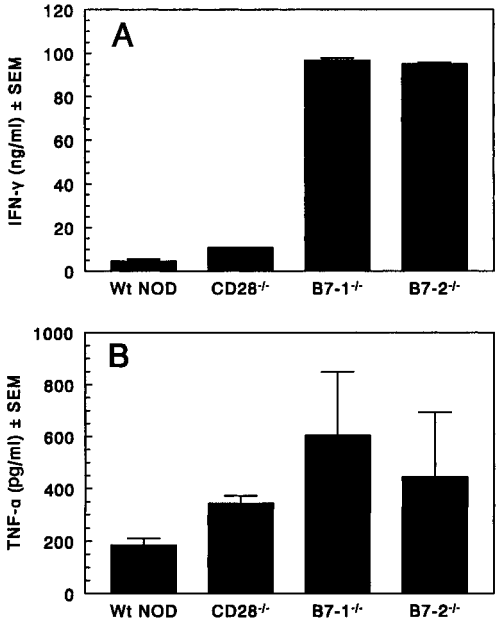

FIGURE 3. Myelin peptide-specific IFN-γ and TNF-α production from lymph node T cells of wild-type and costimulatory molecule-deficient NOD mice. Draining lymph node cells isolated from PLP_{56-70}/CFA-primed mice 10 days postimmunization were cultured with 50 μM PLP_{56-70}. Culture supernatants were collected after 48 h and assayed for IFN-γ and TNF-α levels by ELISA. Data is plotted as mean IFN-γ and TNF-α levels in triplicate cultures in ng/ml and pg/ml, respectively, ±SEM and is representative of two separate experiments. No Ag controls were below the range of our standard curves.

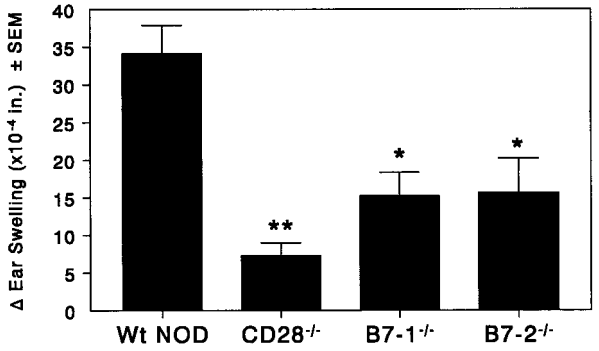

FIGURE 4. Myelin peptide-specific DTH responses are significantly reduced in costimulatory molecule-deficient NOD mice. DTH responses to PLP_{56-70} were assessed in four to five mice per group on day 30 postimmunization. The results are expressed as Δ24 h ear swelling (background swelling subtracted) in units of 10^{-4} inches ± SEM in response to ear challenge with 10 μg of PLP_{56-70}. Background ear swelling responses in unimmunized mice ($n = 4$) were 3.9 ± 1.0 U. These results are representative of two separate experiments. DTH responses in $CD28^{-/-}$, $B7\text{-}1^{-/-}$, and $B7\text{-}2^{-/-}$ NOD mice were significantly less than that in wild-type controls: *, $p < 0.05$; **, $p < 0.01$.

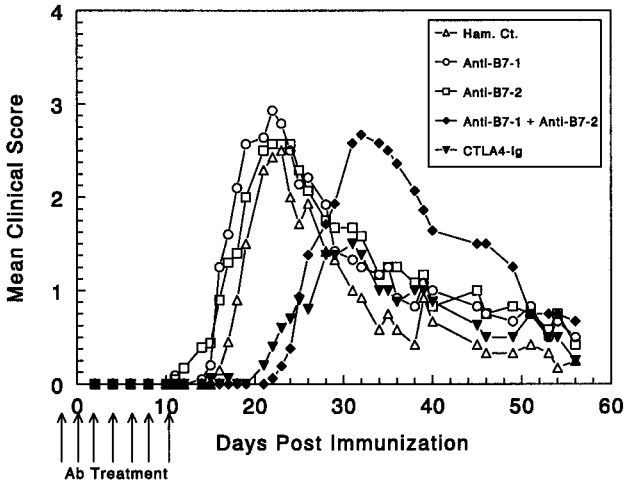

FIGURE 5. Comparison of costimulatory molecule antagonists on initiation of PLP_{56-70}-induced EAE in wild-type NOD mice. Wild-type NOD mice were immunized with 200 μg of PLP_{56-70} in CFA on day 0 and scored for clinical signs of EAE as described in *Materials and Methods*. Mice were treated i.p. with 50 μg of Ab every other day from day −2 until day 10 postimmunization (7 doses totaling 350 μg). Results are plotted as the mean clinical score for all of the animals in each treatment group vs day postimmunization.

Discussion

The B7/CD28 pathway provides critical costimulatory signals required for complete T cell activation, and members of this pathway have served as useful targets for immunotherapeutic strategies designed to regulate autoimmune diseases (10, 11, 17–19, 25). However, studies addressing the individual role of members of the B7/CD28 costimulatory pathway in induction and progression of autoimmune diseases using mAb therapy studies have yielded conflicting and confusing results. Although these studies clearly indicate a critical role for the B7/CD28 costimulatory pathway in either the initiation and/or effector phases of Th1-mediated autoimmune diseases, the individual functional roles of B7-1 and B7-2 in autoimmune disease pathogenesis have remained elusive. In the NOD mouse diabetes model, early treatment with anti-B7-2 or CTLA-4 Ig prevented clinical disease (10). However, later treatment with the same reagents had no effect on the incidence of overt diabetes. In contrast, both anti-B7-1 and combination anti-B7-1/anti-B7-2 therapy resulted in accelerated and exacerbated disease. The present study was designed to examine the roles of CD28 and its individual ligands, B7-1 and B7-2, in EAE, a Th1-mediated inflammatory autoimmune disease of the CNS. We examined disease induced in NOD mice deficient for expression of CD28, B7-1, or B7-2 costimulatory molecules in comparison to the effects of costimulatory molecule therapy using mAbs on PLP_{56-70}-induced EAE induction in wild-type NOD mice. CD28-deficient NOD mice were totally resistant to EAE, indicating a crucial role for CD28-mediated signals. Disease induction in B7-2-deficient NOD mice was more significantly impaired than that in B7-1-deficient NOD mice, indicating a more important role for B7-2-mediated signaling in disease induction. Also, we demonstrate that resistance to disease initiation is not due to the inability of T cells from B7-2- or CD28-deficient mice to produce Th1 cytokines as PLP_{56-70}-induced IFN-γ and TNF-α production was equivalent to or greater than wild-type levels. In contrast, peptide-specific DTH responses in these animals were significantly decreased. Lastly, development of EAE was significantly delayed in NOD mice in which CD28 signaling was inhibited by in vivo administration of a combination of anti-B7-1 plus anti-B7-2 or CTLA4-Ig. Interestingly, unlike disease in mice deficient for either B7-1 or B7-2, treatment with anti-B7-1 or anti-B7-2 alone was not effective in inhibiting EAE. However, it is clear that the anti-B7-1 and anti-B7-2 mAbs were functional because together they efficiently delayed disease onset in wild-type NOD mice (Fig. 5 and Table II), and anti-B7-1 mAb therapy of B7-2-deficient animals further reduced disease severity (A. M. Girvin and S. D. Miller, unpublished observation). In our studies, blocking B7 molecules with mAbs had moderate effects compared with NOD mice deficient in B7 molecules. Differential efficiency of mAb blockade in different tissues, particularly peripheral lymphoid organs vs the CNS, may

Table II. *EAE disease parameters in NOD mice treated at disease initiation with anti-B7-1, anti-B7-2, anti-B7-1 + anti-B7-2, or CTLA4-Ig*

	Monoclonal Ab Treatment				
Disease Parameter[a]	Hamster Ig Control	Anti-B7-1	Anti-B7-2	Anti-B7-1 + Anti-B7-2	CTLA-4 Ig
Incidence	7/7 (100%)	10/10 (100%)	9/10 (90%)	6/7 (86%)	3/4 (75%)
Mean day of onset	18.3 ± 0.7	16.6 ± 0.4	16.0 ± 0.7	23.3 ± 1.1*	24.0 ± 1.5*
Mean peak clinical score	2.8 ± 0.3	2.9 ± 0.3	2.8 ± 0.2	3.0 ± 0.5	2.2 ± 0.7
Histology	Inflammation; demyelination	Inflammation; mild demyelination	Inflammation; mild demyelination	None	Inflammation; no demyelination

[a] Mean day of onset and mean peak clinical score values were calculated as described in *Materials and Methods.*

*, Mean day of onset significantly greater than that of the hamster Ig-treated controls, $p \leq 0.005$.

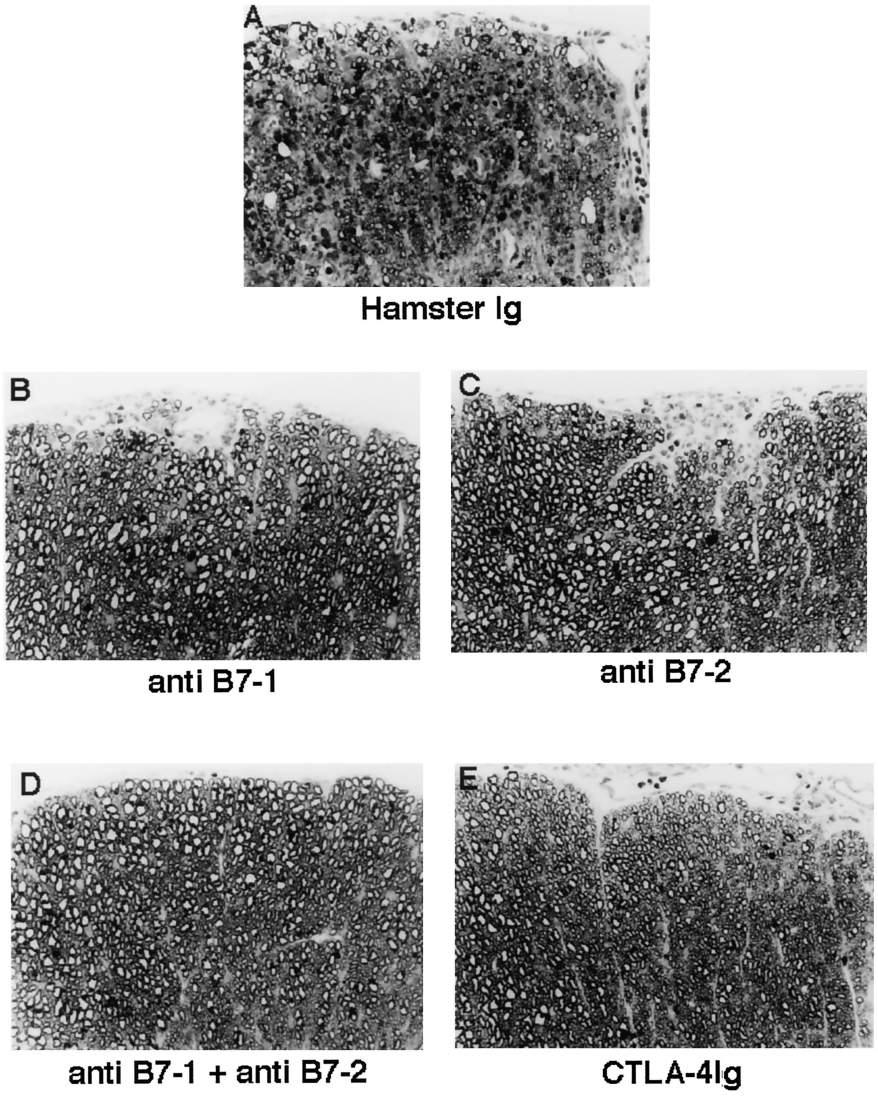

FIGURE 6. Histological analysis of spinal cord sections from wild-type NOD mice treated with costimulatory molecule antagonists. Spinal cords from two representative mice per group were examined for histological signs of mononuclear cell infiltration and demyelination at 25 days postimmunization. *A*, Spinal cord from hamster Ig control-treated NOD mouse (clinical score = 2) showing parenchymal inflammation and demyelination. *B*, Spinal cord from an NOD mouse treated with anti-B7-1 mAb (clinical score = 1) showing meningeal and parenchymal inflammation with some demyelination. *C*, Spinal cord from an NOD mouse treated with anti-B7-2 mAb (clinical score = 2) showing meningeal and parenchymal inflammation with some demyelination. *D*, Spinal cord from an NOD mouse treated with a combination of anti-B7-1 and anti-B7-2 mAbs (clinical score = 0) showing no inflammation or demyelination. *E*, Spinal cord from an NOD mouse treated with CTLA4-Ig (clinical score = 0) showing minimal inflammation, but no demyelination. All sections are 1 μm thick, Epon-embedded sections stained with toluidine blue. Original magnification, $\times$220.

explain this dichotomy. Also, mAb treatment only provides short-term blockade, while costimulatory molecule-deficient animals provide permanent blockade of costimulatory signaling. Each strategy has its own advantages and drawbacks. Thus, it is critical to analyze both before making definitive conclusions. Collectively, our results indicate that the initiation of EAE in the NOD mouse is regulated by B7/CD28 costimulation.

The failure to induce EAE in CD28-deficient NOD mice definitively demonstrates the necessity for B7/CD28 interaction in EAE initiation and progression. Despite their resistance to development of clinical EAE, T cells from PLP_{56-70}-primed CD28-deficient NOD mice proliferate and secrete IFN-γ and TNF-α at wild-type control levels, indicating the protected phenotype is not due to a blockade of T cell activation. The maintenance of T cell autoreactivity in the lymphoid system suggests that alternative costimulatory pathways, e.g., CD40/CD154 (26), can be sufficient for T cell expansion and cytokine production. Based on the lack of CNS inflammation and demyelination in these mice, it appears that CD28-deficient T cells are not present in the CNS. C-C chemokines play an important role in the recruitment of T cells to inflammatory sites, and macrophage inflammatory protein-1α (MIP-1α) has been shown to be critical for the acute phase of EAE (27, 28). Herold et al. have demonstrated that CD28-deficient T cells produce significantly less MIP-1α than wild-type T cells (29). Thus, decreased MIP-1α production by PLP_{56-70}-specific T cells from CD28-deficient NOD mice may in part account for the lack of clinical disease due to inefficient migration to the CNS. Ongoing studies are examining chemokine production by and the trafficking patterns of CD28-deficient T cells to determine whether these animals are protected from EAE because encephalitogenic T cells either cannot enter the CNS target organ and/or undergo rapid apoptosis in the CNS due to the absence of CD28 costimulation. Interestingly, CD28-deficient NOD mice undergo accelerated and exacerbated insulitis and diabetes (30). The reasons for these apparently contradictory findings are unclear, but they may be organ or disease specific, because the diabetes model is spontaneous while EAE is an induced autoimmune response. CD28 costimulation has been found to be critical for the spontaneous development of EAE (31). However, the requirement for CD28 signaling was overcome by immunization with very high doses of Ag.

Anti B7-1 mAb treatment of NOD mice results in a more severe and rapid onset of insulitis and diabetes (10). Interestingly, B7-1-deficient NOD mice had an EAE clinical course similar to wild-type controls. However, they developed enhanced PLP_{56-70}-specific proliferative responses, and proinflammatory cytokine responses of lymph node cells were significantly greater than wild-type controls. These observations suggest that lack of B7-1 results in increased T cell activation in the NOD mouse. Autoimmune

target tissue-specific up-regulation of B7-1 may function by binding to CTLA-4 with greater affinity than B7-2 and down-regulating T cell responses (32–34). However, B7-1-deficient animals exhibited significantly reduced DTH responses to the disease initiating peptide, suggesting that B7-1/CD28 interactions may also regulate the migration of T cells to nonlymphoid inflammatory tissues. Collectively, these results suggest that B7-1 plays dual roles in EAE by interacting with either CD28 or CTLA-4 to regulate disease.

It is interesting to speculate that the differences between the effects of anti-B7 mAbs on EAE induction in various reports may reflect strain differences in levels of expression of the individual B7 costimulatory molecules. A recent report has identified chromosomal regions encoding CD28/CTLA-4 and B7-1/B7-2 as susceptibility loci in the induction of EAE (35). It is possible that polymorphisms within these loci regulate differential expression of B7 costimulatory molecules in individual mouse strains. For instance, although B7-2 is predominantly expressed in naive SJL mice, we have reported that B7-1 cell-surface expression is highly up-regulated on APCs and T cells in the spleen and in the CNS of SJL mice undergoing relapsing EAE (36).

Based on our studies in costimulatory molecule-deficient NOD mice, it is clear that B7-2 plays an important role in initiation of EAE. B7-2-deficient mice developed a less severe clinical course of EAE with slower kinetics than wild-type animals and displayed reduced proliferative and DTH responses, suggesting that B7-2/CD28 interactions provide a critical costimulatory signal for expansion/survival of encephalitogenic T cells. The finding that B7-2 appears to play a dominant role in initiation of EAE is compatible with earlier reports examining the differential role of B7-1 and B7-2 in initiation of spontaneous diabetes in the NOD strain (10) and in graft rejection (37). We have reported that early treatment of NOD mice with anti-B7-2 mAb or human CTLA4-Ig resulted in a profound inhibition of diabetes onset. Interestingly, unlike anti-B7-1 therapy of diabetes, we failed to observe an exacerbated EAE disease course in either mice treated with anti-B7-1 mAb or in B7-1-deficient NOD mice, which may indicate fundamental differences in costimulatory molecule expression in naive NOD mice vs those injected with myelin peptides in CFA. The B7-2 predominance in initiation of EAE in the current study differs from EAE in the SJL mouse where blockade of B7-1-mediated costimulation in vivo with intact anti-B7-1 mAbs has been shown to inhibit disease initiation under suboptimal priming conditions (17, 38). However, these studies used intact Abs. Thus, it is unclear whether the effects on disease induction were due to specific "blockade" of B7-1-mediated signaling through CD28 and/or to up-regulation of B7-2 or other costimulatory molecules on relevant APCs, which in turn affected T cell activation. In this regard, we have previously reported that epitope spreading and subsequent disease relapses in SJL mice could be inhibited by treatment of mice during disease remission with anti-B7-1 F(ab) (18), but that administration of intact anti-B7-1 mAb resulted in accelerated clinical relapses, enhanced CNS pathology, and promoted epitope spreading (19). This dichotomy suggests that the anti-B7-1 F(ab) were blocking B7-1-mediated signaling, while the intact, bivalent mAb led to exacerbated disease via its ability to cross-link surface B7-1 molecules and signal APCs and/or activated T cells.

It is noteworthy that PLP_{56-70}-specific T cells from both B7-2- and CD28-deficient NOD mice produced normal or significantly enhanced levels of IFN-γ and TNF-α despite the fact that clinical disease was reduced or absent in these animals. Similar in vitro proliferation and cytokine production has been observed in T cells isolated from $CD28^{-/-}$/$RAG\text{-}1^{-/-}$ mice transgenic for a myelin basic protein-specific TCR despite their lack of spontaneous development of EAE (31). Proinflammatory cytokines, e.g., IFN-γ and TNF-α, are thought to play an important role in disease pathogenesis based on their coordinated expression in the CNS during acute and relapsing phases of EAE (39–43). However, our findings are consistent with a previous report showing that systemic administration of IFN-γ inhibits induction of EAE (44) and more recent studies showing that IFN-γ-, IFN-γ receptor-, and TNF-α-deficient mice develop EAE in some cases more severe than littermate controls (45–47). Whether the enhanced levels of IFN-γ and TNF-α contributed to disease protection in B7-2-deficient mice is not clear at this time.

In summary, our data show an absolute requirement for CD28 signaling and a predominant role for B7-2-mediated costimulation in the development of clinical EAE in NOD mice. Although continued investigation will be required to determine the separate functional roles of B7-1 and B7-2 in the initiation, progression and regulation of EAE, costimulatory molecules remain promising targets for immunotherapy of autoimmune diseases.

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