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Suppression of Experimental Autoimmune Encephalomyelitis Using Peptide Mimics of CD28[1]

Mythily Srinivasan,* Ingrid E. Gienapp,[§] Scott S. Stuckman,[§] Connie J. Rogers,[§] Scott D. Jewell,[†] Pravin T. P. Kaumaya,*[‡] and Caroline C. Whitacre[2§]

The B7:CD28/CTLA-4 costimulatory pathway plays a critical role in regulating the immune response and thus provides an ideal target for therapeutic manipulation of autoimmune disease. Previous studies have shown that blockade of CD28 signaling by mAbs can both prevent and exacerbate experimental autoimmune encephalomyelitis (EAE). In this study, we have designed two CD28 peptide mimics that selectively block B7:CD28 interactions. By surface plasmon resonance, both the end group-blocked CD28 peptide (EL-CD28) and its retro-inverso isomer (RI-CD28) compete effectively with the extracellular domain of CD28 for binding to B7-1. Both the CD28 peptide mimics inhibited expansion of encephalitogenic T cells in vitro. A single administration of EL-CD28 or RI-CD28 peptide significantly reduced disease severity in EAE. Importantly, we show that either CD28 peptide mimic administered during acute disease dramatically improved clinical signs of EAE, suppressing ongoing disease. The ratio of CD80:CD86 expression was significantly lower on $CD4^+$ and $F4/80^+$ spleen cells in CD28 peptide-treated mice. Peripheral deletion of Ag-specific $CD4^+$ T cells occurs following in vivo blockade of CD28 with synthetic CD28 peptides. ***The Journal of Immunology*****, 2002, 169: 2180–2188.**

Multiple sclerosis (MS)[3] is a demyelinating disease of the CNS characterized by myelin damage accompanied by inflammation and axonal severing. Experimental autoimmune encephalomyelitis (EAE) shares many of the clinical and histopathological features of MS and thus serves as a useful animal model. Considerable evidence suggests a central role for T cell-mediated immune responses in the pathogenesis of MS and EAE (1).

Activation of T cells requires a primary signal delivered via the TCR-CD3 complex interacting with a MHC-peptide complex on an APC and a second costimulatory signal provided primarily by CD28 interacting with the B7 molecules on the APC (2). Signaling via CD28 leads to transcriptional activation of several cytokine genes, principally IL-2, and up-regulation of anti-apoptotic molecules such as Bcl-x_L. High levels of IL-2 are required during the expansion phases of both primary and secondary T cell responses. Bcl-x_L regulates proliferation and survival of naive T cells (3). CTLA-4, expressed on activated T cells, also binds the B7 ligands on the APC, transmitting negative signals to terminate the immune response (4).

B7/CD28:CTLA-4 interactions play a critical role in the pathogenesis and/or regulation of EAE and MS (5–7). Mice genetically deficient in B7-1, B7-2, or CD28 are highly resistant to EAE (8). Therapeutic intervention in the B7:CD28/CTLA-4 pathway has led to varied results. Although administration of CTLA-4 Ig or anti-B7-1 mAb prevented the induction of EAE, CTLA-4 Ig was not effective in the treatment of established disease. Anti-B7-2 or anti-CTLA-4 mAb treatment exacerbated the clinical course of EAE (9–13). Interference with the delivery of negative signals via CTLA-4 may account for the differences in clinical outcome in these studies. Recently, Perrin et al. (14) have shown that treatment with Fab of CD28 mAb that specifically blocks B7:CD28 interactions ameliorates the clinical course of EAE.

Knowledge of the molecular topology of interacting surfaces can be used to develop antagonists of protein-protein interactions. For example, a peptide analog derived from the complementarity-determining region (CDR)-3-like region of CD4 inhibits T cell responses (15, 16). The premise is that the side-chain functional groups of the key residues of the binding epitope can be transferred to a much smaller fragment without loss of binding efficiency (17). A surface binding pocket consisting of CC′ or CDR-3 loops has been shown to mediate the binding of CD4 to MHC class II and of CD2 to LFA-3 (18). In the present study, we evaluated the therapeutic efficacy of a peptide antagonist for interfering with B7:CD28 interactions. Based on the differences in the kinetics of interaction of CD28 and CTLA-4 with B7 ligands, we hypothesized that a peptide derived from the ligand binding region of CD28 will selectively block B7:CD28 interactions without affecting the higher affinity B7:CTLA-4 interactions.

CD28 is a member of the Ig supergene family with a single IgV-like domain (19, 20). The residues implicated in ligand binding include the conserved hydrophobic motif "MYPPPY" and the adjacent charged residues, localized in the solvent-exposed CDR3-like loop region of CD28 (19–21). Similar proline-rich sequences in accessible regions of other globular proteins have been implicated in protein-protein interactions (22). Recently, we identified two biologically active CD28 peptide mimics derived from the

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[3] Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; CDR, complementarity-determining region; MBP, myelin basic protein; LNC, lymph node cell; PLP, proteolipid protein.

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CD28 CDR-3-like region. An end group-blocked CD28 peptide (EL-CD28) and its retro-inverso isomer (RI-CD28) were synthesized and characterized for their binding properties and biological activity (23). The synthetic CD28 peptides exhibited similar kinetics of interaction as CD28 for binding the CD80 ligand. Moreover, the CD28 peptides were observed to interfere with the activation of encephalitogenic T cells in vitro (23).

In the present study, we investigated the potential of the peptide mimics to suppress EAE in vivo. Our results indicate that treatment with the CD28 peptides prevented EAE induction and ameliorated established disease. Suppression of EAE was greater with the retro-inverso CD28 mimic than the L peptide analog of the parent sequence. The observed protection was accompanied by a decrease in the CD80:86 ratio on T cells and macrophages as well as apoptosis of Ag-specific $CD4^+$ T cells.

Materials and Methods

Peptide design and synthesis

All L- and D- peptides corresponding to the CD28 CDR3-like region were assembled by solid-phase peptide synthesis using F-moc/dicyclohexylcarbodiimide/hydroxybenzotriazole methodology on a fully automated peptide synthesizer as described (23). The free L-CD28 peptide was assembled on 4-methylbenzhydrylamine resin. The end-group blocked L-CD28 (EL-CD28) peptide, the retro-inverso CD28 (RI-CD28) peptide and the control peptides were assembled as peptide amides on Rink amide resin (Advanced ChemTech, Louisville, KY). The free NH_2 group of the terminal amino acid residue was acetylated with acetylimidazole and confirmed by a negative Ninhydrin test. The retro-inverso peptide was assembled in reverse order with respect to the parent peptide using F-moc-D-amino acid derivatives. The control peptides included an all D peptide, consisting of D amino acids in the forward sequence, and a reverse L-peptide, consisting of L amino acids in the reverse sequence as that of the parent peptide (Table I). The peptides were purified by semipreparative reverse-phase HPLC (Vydac, Hesperia, CA) and the identity of the purified peptide was confirmed by matrix-assisted laser desorption/ionization time of flight mass spectrometry.

Binding experiments

Competitive kinetic analyses between CD28-Ig or CTLA-4 Ig and the synthetic CD28 peptides for binding the B7-1 ligand were conducted using the BIAcore biosensor (Pharmacia Biosensor, Uppsala, Sweden) that employs surface plasmon resonance for directly measuring intermolecular interactions. CD80-Ig (282–368 resonance units (RU)) was immobilized indirectly via anti-mouse Fc (1210–1390 RU) to the sensor chip as described (24). Initially, the affinity constant of the interaction between CD28-Ig (the extracellular domain of CD28 fused to mouse IgG2a, a gift from Dr. Y. Liu, Ohio State University, Columbus, OH) or CTLA-4 Ig and CD80-Ig was determined by direct kinetic analysis. Competition was assessed by injecting a series of solutions containing a constant concentration of CD28-Ig (3.2 μM) or CTLA-4 Ig (0.8 μM) and increasing concentrations of EL-CD28 or RI-CD28 peptides. The CD80-Ig surface was regenerated between injections by washing with 5 mM NaOH. As control, the solutions were also injected onto an empty flow cell with no ligand immobilized.

Data was analyzed using BIAevaluation software 2.1 and BIAsimulation software version 2.1 (Pharmacia Biosensor). Before kinetic analysis, a zero baseline level was obtained by subtracting the background responses from injection of the analytes through a control flow cell with no ligand immobilized. In competitive kinetic experiments, when two analytes (CD28 peptides and CD28-Ig) were injected at the same time and react with the same site on the immobilized ligand (CD80-Ig), the observed response is the sum of the contributions from both analytes. The binding data was analyzed as previously described (24).

Table I. *Amino acid sequences of synthetic CD28 and control peptides*

Abbreviation	Sequence[a]
L-CD28	NH_2KIEFMYPPPYLDNERSNGTICOOH
EL-CD28	CH_3CO-*L*[KIEFMYPPPYLDNERSNGTI]-$CONH_2$
RI-CD28	CH_3CO-*D*[ITGNSRENDLYPPPYMFEIK]-$CONH_2$
D-CD28	CH_3CO-*D*[KIEFMYPPPYLDNERSNGTI]-$CONH_2$
RL-CD28	CH_3CO-*L*[ITGNSRENDLYPPPYMFEIK]-$CONH_2$

[a] Italicized *L* and *D* refer to L and D amino acid residues, respectively.

Antigens

Myelin basic protein (MBP) was extracted from guinea pig spinal cords (Harlan Sprague Dawley, Indianapolis, IN) as previously described (25). For EAE induction, MBP was further purified on a Sephadex G-50 column eluted with 0.01N HCl. The purified protein was dialyzed against water and lyophilized.

Induction of EAE and CD28 peptide treatment

Female B10.PL mice, 6–8 wk old, obtained from The Jackson Laboratory (Bar Harbor, ME) were injected s.c. over four sites on the flank with 200 μg of MBP in CFA containing 200 μg killed *Mycobacterium tuberculosis*, Jamaica strain. Pertussis toxin (List Biological Laboratories, Campbell, CA), 150 ng in 0.2 ml of PBS, was given i.p. at the time of immunization and 48 h later. Animals were observed daily for clinical signs and scored as follows: 0, no clinical signs; 1, limp tail or waddling gait with tail tonicity; 2, waddling gait with limp tail (ataxia); 2.5, ataxia with partial paralysis of one limb; 3, partial hind-limb paralysis; 3.5, full paralysis of one limb with partial paralysis of the second limb; 4, full paralysis of two limbs; 4.5 moribund; and 5, death. For treatment of EAE, mice were injected i.v. with 500 μg CD28 peptide (reconstituted in sterile PBS) either on the day of MBP immunization or 14 days later.

Proliferation analysis

Vehicle-, synthetic CD28 peptide-, and control peptide-treated mice were sacrificed either 10 or 26 days after immunization and in vitro proliferation of lymphocytes was assessed as described (26). Briefly, single cell suspensions prepared from spleens, mesenteric lymph nodes, and peripheral lymph nodes (inguinal, axillary, brachial, cervical, popliteal) were cultured in RPMI 1640 medium containing 10% FCS, 25 mM HEPES, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 5×10^{-5} M 2-ME in round-bottom 96-well plates (4×10^5 cells/well) and stimulated with MBP (40 μg/ml) or medium for 72 h, including a final 18-h pulse with [^{3}H]thymidine. Cultures were harvested onto glass-fiber mats using a Skatron harvester (Skatron, Sterling, VA) and counted by liquid scintillation using a Wallac betaplate (Wallac, Rockville, MD). The results are expressed as Δ cpm (mean cpm of cultures with Ag $-$ mean cpm of cultures with medium alone) $\pm$ SE.

ELISPOT analysis for cytokine-producing cells

ELISPOT analysis was performed as described (26). Briefly, 96-well unifilter plates (Polyfiltronics, Rockland, MD) were coated with anti-mouse IL-2 (clone JES6-1A12) or anti-mouse IFN-γ (clone R46A2) (BD PharMingen, San Diego, CA) at 4 μg/ml overnight at 4°C. After blocking with 1% BSA in DMEM for 2 h at room temperature, isolated lymph node cells (LNC) (5×10^5 cells/well) were resuspended in HL-1 medium (BioWhittaker, Walkersville, MD) and added to the plates in triplicate in the presence or absence of 40 μg/ml MBP. Following incubation for 24 h at 37°C, the plates were washed with PBS containing Tween 20 (1:2000) and cytokine-specific secondary Abs, biotinylated anti mouse-IL-2 (clone JES6-5H4) or biotinylated anti-mouse IFN-γ (clone XMG1.2) (BD PharMingen), 2 μg/ml, were added. After overnight incubation at 4°C, the plates were incubated with goat anti-biotin Ab conjugated to alkaline phosphatase (Vector Laboratories, Burlingame, CA) for 2 h at room temperature. The spots were visualized by adding 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium phosphatase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Image analysis of ELISPOT plates was performed using the KS ELISPOT system (Zeiss, Oberkochen, Germany). Data are expressed as the frequency of MBP-responsive cytokine producing cells per million $\pm$ SEM.

Detection of apoptosis

The terminal deoxynucleotidyl transferase assay was performed as described (27). LNC (10^6) from peptide- and control-treated mice were stained with PE-labeled anti-mouse CD4 (L3T4, clone RM 4-4; BD PharMingen) for 30 min at 4°C. After washing, the cells were fixed in 4% paraformaldehyde for 30 min at room temperature, followed by permeabilization with 0.1% sodium citrate containing 0.1% Triton X-100 for 2 min at 4°C. The cells were then incubated with FITC-labeled TdT reaction mixture for 60 min at 37°C using an in situ cell death detection kit (Boehringer Mannheim, Mannheim, Germany) according to manufacturer's instructions. As a positive control, cells were incubated in DNase (1 μg/ml) for 10 min at room temperature before incubation with TdT. A negative

control consisted of cells incubated without TdT. Cells were then washed, resuspended in PBS, and analyzed by flow cytometry.

mAb Staining and flow cytometric analysis

Single cell suspensions of LNC and spleen cells (0.5×10^6) were incubated with 1 μg of PE-conjugated anti-CD80 (clone 16-10A1), anti-CD86 (clone GL-1), anti-CD28 (clone 37.51), FITC-conjugated anti-CD11c (clone HL3), anti-CD19 (clone 1D3), or anti-CD4 (clone RM4-5) for 30 min at 4°C. For macrophages, a three-step staining procedure was used. LNC and splenocytes were incubated with 1 μg of anti-F4/80 (clone C1: A3-1) (R&D Systems, Minneapolis, MN) followed by washing and incubation in FITC-conjugated anti-rat IgG2b (clone A95-1) and PE-conjugated anti-CD80 or anti-CD86. All staining reactions were accompanied by appropriately matched isotype control reactions. All mAbs (unless specified) were purchased from BD PharMingen. Labeled cells were washed, resuspended in 1% paraformaldehyde, and analyzed by flow cytometry on an Epics XL flow cytometer (Beckman Coulter, Fullerton, CA).

Statistical analysis

For the mean clinical score, proliferation, and TUNEL assays, a one-way ANOVA with Tukey's posthoc was performed to determine the differences between the groups. Results were considered statistically significant at $p < 0.05$.

Results

Peptide design and synthesis

Mutagenesis and molecular modeling of the extracellular domain of CD28 were used to design a 20 residue CD28 peptide mimic encompassing the "MYPPPY" motif and the delineated flanking sequence (19–21). The charges at the end groups of the synthetic CD28 peptide were blocked (EL-CD28) to resemble the termini of the ligand binding epitope of the parent CD28 molecule (Table I). In addition, this modification stabilizes the secondary structure, which may be important for its functional interaction. A retro-inverso isomer of the CD28 peptide mimic (RI-CD28) was also synthesized. The use of D amino acids results in inverted chirality and the reversed order of amide bonds (-NHCO- instead of -CONH-) creates an analog that regenerates both the planarity of peptide bonds and spatial orientation of side chains closely related to that of the original peptide (28).

CD28 peptides compete with CD28 extracellular domain to bind CD80-Ig

Kinetic analysis of the sensograms for the binding of CD28-Ig or CTLA-4 Ig to CD80-Ig yielded a K_d of 3.17 and 0.8 μM, respectively (data not shown). Competitive binding assays were performed by injecting a mixture of CD28-Ig (3.2 μM) or CTLA-Ig (0.8 μM) at a constant concentration together with increasing concentrations of CD28 peptides as analytes over immobilized CD80-Ig. Representative sensograms of the competition between CD28-Ig or CTLA-4Ig and EL-CD28 are shown in Fig. 1. The top curve represents the interaction of CD28-Ig (Fig. 1*A*) or CTLA-4Ig (Fig. 1*B*) alone with the CD80-Ig. In Fig. 1*A*, the response level gradually decreases as the concentration of peptide mimic increases, indicating that the EL-CD28 peptide competes with CD28-Ig for binding CD80-Ig. No appreciable decrease in response units was observed when EL-CD28 peptide at 51.6–804 μM was competed with CTLA-4-Ig for binding the CD80-Ig (Fig. 1*B*). A similar response was observed with RI-CD28 peptide (23). However, a decrease of 168 RU was observed when EL-CD28 at 1237 μM or 3 mg/ml was competed with CTLA-4Ig (Fig. 1*B*). The RI-CD28 peptide also exhibited a decrease of 189 RU at a 1237 μM concentration (23), suggesting that at very high concentrations, both the EL-CD28 and the RI-CD28 peptides may compete weakly with the CTLA-4 fusion protein for binding the B7-1 ligand.

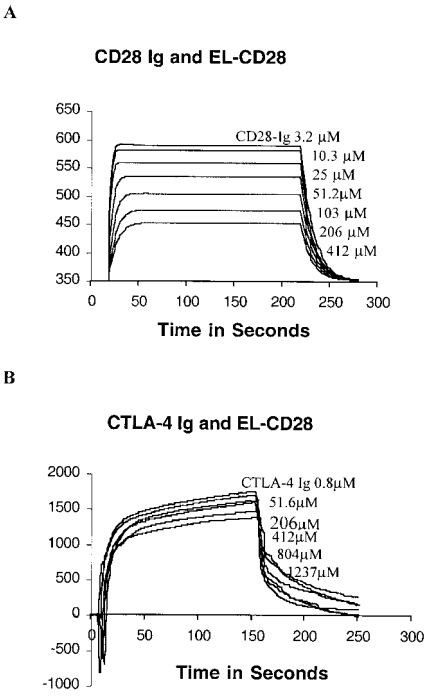

FIGURE 1. Competition between EL-CD28 and CD28Ig or CTLA-4 Ig for binding CD80-Ig. Overlay of sensograms obtained from injection of a mixture of (*A*) CD28-Ig (3.2 μM) or (*B*) CTLA-4 Ig (0.8 μM) and EL-CD28 peptide at varying concentrations (10.3–1237 μM) at 5 μl/min over a flow cell with bound CD80-Ig. In both sensograms, the top curve represents the binding of the respective fusion protein alone in the absence of the competing peptide. The response of (*A*) CD28-Ig binding decreases with increasing concentrations of EL-CD28 (10.3–412 μM). The response of (*B*) CTLA-4 Ig binding decreases appreciably (168 RU) only at the highest concentration (1237 μM) of EL-CD28 peptide.

Costimulatory blockade in vivo by CD28 peptides suppresses clinical EAE in B10.PL mice

To evaluate the biological activity of synthetic CD28 peptide mimics during Ag priming, B10.PL mice were immunized with MBP and received i.v. a single injection of EL-CD28, RI-CD28, control CD28 peptides, or PBS on the day of immunization. The vehicle-treated and control (L-CD28, RL-CD28, and D-CD28) peptide-treated mice exhibited maximum disease incidence of 100, 100, 91.5, and 91.5% and mean cumulative scores of 50.2, 47.6, 44.9, and 52.3, respectively (Table II). In contrast, significant suppression of EAE was observed in mice treated with EL-CD28 and RI-CD28 peptides with a mean cumulative score of 29.2 and 19.4, respectively (Fig. 2, *A* and *B*). We also observed a trend of decreased mean maximal score for mice treated with EL-CD28 and RI-CD28 peptides compared with controls (Table II). The RI-CD28 peptide produced greater protection that lasted for the duration of the observation period (Fig. 2).

Synthetic CD28 peptide treatment ameliorates established EAE

We assessed the ability of CD28 peptide mimics to suppress ongoing established clinical disease. Mice immunized with MBP in CFA and showing clinical signs of disease by 14 days postimmunization were randomly distributed into six groups, such that the mean clinical score for each group was approximately the same. Groups of mice were injected i.v. with PBS or 500 μg of EL-CD28, RI-CD28, or control CD28 peptides. The mean clinical score of vehicle-treated mice continued to increase, reaching a maximum of 3.4 on day 20. Similarly the disease continued to

Table II. *EAE clinical signs in CD28 peptide-treated mice treated on the day of MBP immunization*

Group	No. of Animals	Mean Cumulative Score	Mean Score Per Day[a]	Mean Maximal Score[b]
PBS	11	50.23 ± 4.71	1.93 ± 0.18	4.05 ± 0.17
L-CD28	6	47.6 ± 2.02	1.83 ± 0.09	4.2 ± 0.2
EL-CD28	10	29.2 ± 7.27[c]	1.12 ± 0.28[c]	2.55 ± 0.47
RI-CD28	9	19.39 ± 6.57[d]	0.74 ± 0.25[d]	2.05 ± 0.55
RL-CD28	6	44.9 ± 3.62	1.73 ± 0.14	3.8 ± 0.2
D-CD28	6	52.25 ± 9.5	2.01 ± 0.37	3.5 ± 0.67

[a] Average of the cumulative clinical score of each animal divided by the total number of days.
[b] Average of the highest score of all mice in each group.
[c] $p < 0.05$ by ANOVA.
[d] $p < 0.01$ by ANOVA.

progress in mice treated with control CD28 peptides, viz, L-CD28, RL-CD28 and D-CD28, reaching a mean maximal clinical score of 3.8 (day 18), 3.4 (day 16), and 3.7 (day 16), respectively (Table III). In contrast, mice treated with EL-CD28 or RI-CD28 peptides showed clinical improvement from day 16 throughout the observation period (26 days postimmunization) (Fig. 3, *A* and *B*). These results demonstrate that blockade of CD28 costimulation can attenuate the progression of ongoing disease in EAE.

CD28 peptides suppress Ag-specific proliferation and IL-2 production by MBP-primed T cells

To determine the effect of costimulatory blockade in vivo by CD28 peptides, we assessed the proliferation of MBP-primed T cells upon restimulation in vitro. Splenocytes from mice with EAE treated with PBS, EL-CD28, RI-CD28, or control CD28 peptide analogs on the day of immunization were collected 10 days postimmunization and stimulated in vitro with MBP. The proliferative response to MBP, but not an irrelevant Ag, tetanus toxoid, was significantly decreased in splenocytes from mice treated with EL-CD28 or RI-CD28 peptide as compared with PBS-treated mice (Fig. 4, *A* and *B*). We observed a relatively high degree of variance in the MBP-specific proliferative response of spleen cells from D-CD28 peptide and L-CD28 peptide-treated mice, although this variance was not observed in the response from RL-CD28-treated mice (Fig. 4, *A* and *C*). Splenocytes from mice treated with RI-CD28 14 days after MBP immunization also exhibited reduced proliferation in response to MBP as compared with controls (Fig. 4*C*). Separate groups of animals were sacrificed 26 days after immunization and MBP-specific proliferative responses remained suppressed (data not shown).

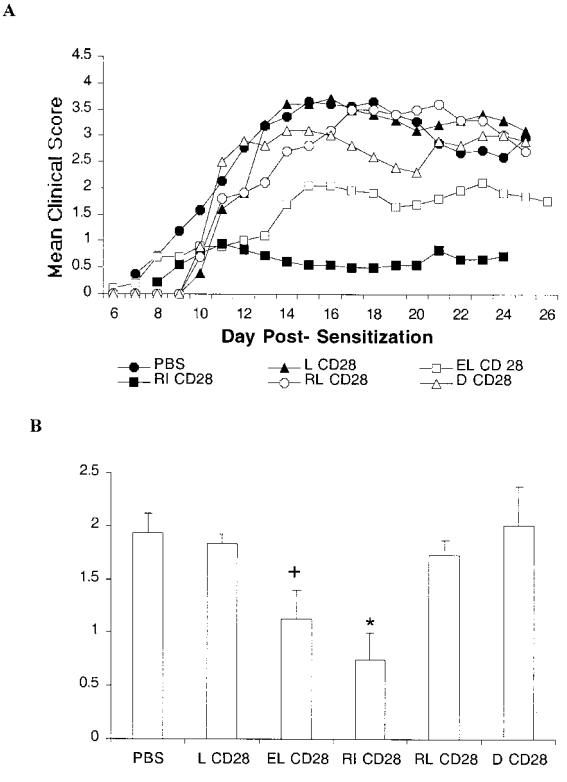

FIGURE 2. CD28 peptide treatment inhibits EAE. B10.PL mice were immunized for EAE as described in *Materials and Methods*. *A*, The data are presented as the mean clinical score per group over time. *B*, The severity of EAE is depicted as the mean score per day, which is the cumulative score for each animal divided by the number of days that animal was observed. The mean of these values was calculated for each group. EL-CD28- and RI-CD28 peptide-treated mice had significantly reduced scores (+, $p < 0.05$; ∗, $p < 0.01$ by ANOVA) as compared with control mice. No significant differences were observed between PBS- and control CD28 peptide-treated mice. Data represents pooled values from two experiments.

We evaluated the effect of synthetic CD28 peptides on T cell cytokine secretion. Mice were treated with CD28 peptides on the day of immunization and ELISPOT was used to assess in vitro cytokine production by LNC upon restimulation with MBP. The frequency of IL-2-secreting LNC decreased significantly with EL-CD28 and RI-CD28 treatment as compared with PBS and control peptide treatment (Fig. 4*D*). The frequency of IFN-γ secreting LNC and splenocytes at this time was equivalent in all cultures (data not shown). Perrin et al. (12) have also reported similar findings of decreased IL-2 and unaltered IFN-γ production after costimulatory blockade of MBP-specific T cells.

In vivo blockade of CD28 costimulation induces apoptosis in Ag-specific T cells

Our previous studies demonstrated that MBP NAc1-11 specific Vβ8.2$^+$ T cells showed decreased proliferation when stimulated in vitro with MBP peptide in the presence of CD28 peptides (23). One possibility to explain this in vitro observation and the suppression of EAE observed in this study is that peptide treatment induces apoptosis of disease-relevant T cells. We measured the DNA strand breaks in CD4$^+$ lymphocytes from peptide-treated mice using enzymatic labeling of nicked DNA. A significantly higher percentage of CD4$^+$ T cells were apoptotic in mice treated on the day of immunization with RI-CD28 peptide (16%) as compared with PBS-treated mice (8.7%) or control peptide-treated mice (10.2%) (Fig. 5*A*). Similarly, an increase in the percent of apoptotic cells was observed in mice treated with RI-CD28 peptide (8.3%) during acute disease as compared with vehicle (6%) and control peptide-treated mice (2.6%), although the difference did not reach statistical significance (Fig. 5*B*). Fig. 5, *C* and *D*, are representative histograms showing increased apoptosis of LNC from mice treated with RI-CD28 peptide on days 0 and 14, respectively. This suggests that the CD28 peptide mimic engages the

Table III. *EAE clinical signs in CD28 peptide-treated mice treated during acute disease*[a]

Group	No. of Animals	Mean Cumulative Score	Mean Score Per Day[b]	Mean Maximal Score[c]
PBS	12	37.56 ± 3.1	3.13 ± 1.04	4 ± 0.25
L-CD28	6	17.06 ± 2.67	2.84 ± 1.5	4.17 ± 0.17
EL-CD28	12	19.24 ± 2.82[d]	1.92 ± 0.98[d]	2.92 ± 0.34
RI-CD28	11	19.38 ± 2.47[d]	1.76 ± 0.87[d]	3.23 ± 0.35
RL-CD28	5	12.82 ± 3.97	2.56 ± 0.91	3.7 ± 0.3
D-CD28	5	13 ± 3.96	2.6 ± 0.92	3.9 ± 0.4

[a] Fourteen days postimmunization; disease scores included beginning from time of treatment initiation.
[b] Average of the cumulative clinical score of each animal divided by the total number of days.
[c] Average of the highest score of all mice in each group.
[d] $p < 0.05$ by ANOVA.

B7-ligands on the APC and effectively blocks the costimulatory signal required for sustained activation and long-term survival of $CD4^+$ T cells.

Treatment with CD28 peptides down-regulates the CD80:CD86 ratio in $CD4^+$ T cells and $F4/80^+$ macrophages

Previous studies on the in vivo expression of B7 during the course of EAE have shown that CD80 is up-regulated on spleen cells during clinical disease (29). To investigate whether the observed protection following treatment with CD28 peptides is a result of reduced costimulation, we analyzed the expression of CD80, CD86, and CD28 on T cells and APCs in the spleen.

Consistent with previous reports, lymphoid cells from naive B10.PL mice express higher levels of CD86 than CD80 (30–32). Immunization with MBP results in the up-regulation of CD80 on both $CD4^+$ LNC and spleen cells. However, CD86 expression is down-regulated on $CD4^+$ spleen cells from mice with EAE as

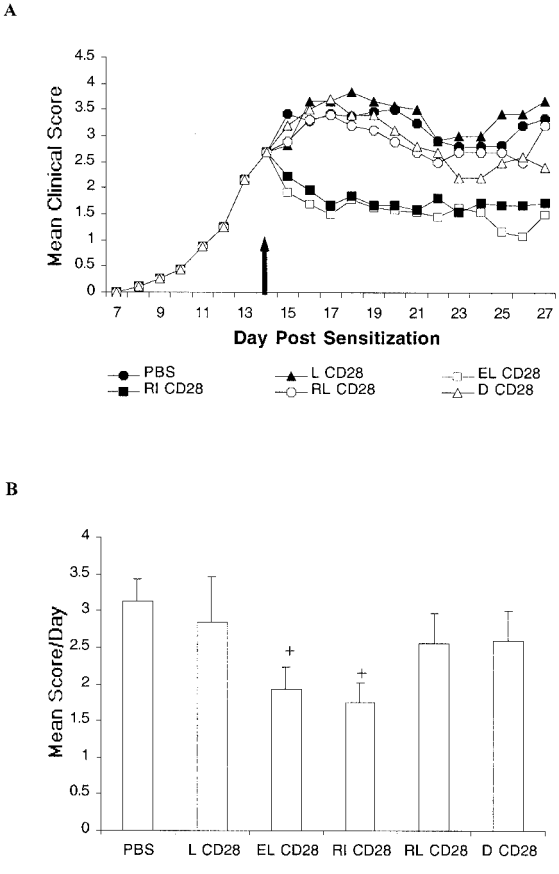

FIGURE 3. CD28 peptide mimics attenuate established EAE. B10. PL mice were immunized for EAE as in Fig. 2. *A,* Mice that received EL-CD28 or RI-CD28 peptide 14 days postimmunization showed significant clinical improvement. *B,* The clinical severity was significantly reduced in EL-CD28- and RI-CD28- (+, $p < 0.05$ by ANOVA) treated mice as compared with PBS-treated mice. There were no significant differences between control peptide and PBS-treated mice. Data represents pooled values from two experiments.

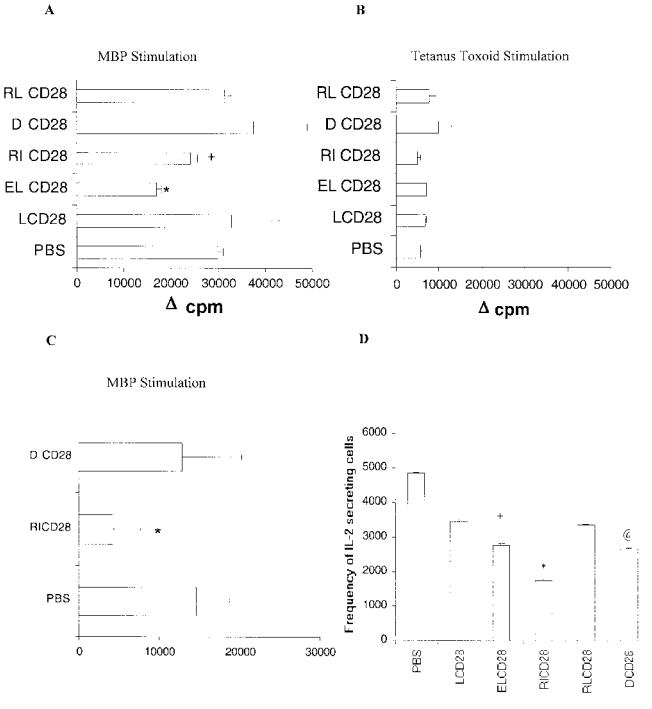

FIGURE 4. Proliferative responses and IL-2-secreting cells are reduced in mice treated in vivo with CD28 peptides. *A,* Splenocytes collected 10 days postimmunization from mice treated with RI-CD28 and EL-CD28 peptide on the day of immunization showed significant inhibition of proliferation in response to MBP. +, $p < 0.05$ as compared with PBS-, RL-CD28-, and L-CD28 peptide-treated mice; ∗, $p < 0.01$ compared with PBS-treated and all control peptide-treated mice by ANOVA. *B,* Splenocytes from peptide-treated mice showed no differences in response to tetanus toxoid. *C,* Splenocytes collected 16 days postimmunization from mice treated with RI-CD28 peptide during acute disease showed significant inhibition of proliferation in response to MBP compared with cells from PBS- or control peptide-treated mice. *D,* The frequency of IL-2-producing cells (as measured by ELISPOT) in response to MBP stimulation is decreased in mice treated on the day of immunization with EL-CD28 and RI-CD28 peptide as compared with PBS-treated and L-CD28- and RL-CD28 peptide-treated mice. (+, $p < 0.05$; ∗, $p < 0.01$ compared with PBS-, L-CD28-, and RL-CD28 peptide-treated mice; @, $p < 0.01$ as compared with PBS-treated mice by ANOVA).

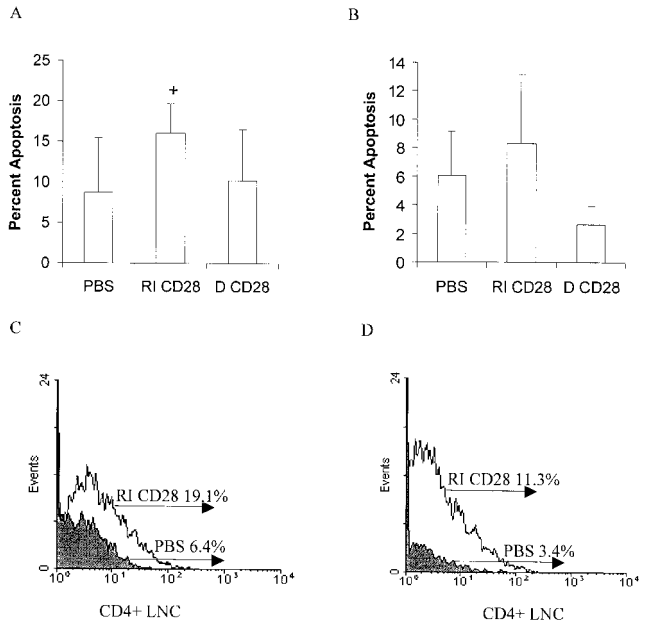

FIGURE 5. CD28 peptide treatment in vivo induces apoptosis in T cells. Apoptosis among $CD4^+$ cells was assayed by TUNEL as described in *Materials and Methods*. *A,* A higher percentage of $CD4^+$ LNC was apoptotic in mice treated with RI-CD28 peptide on the day of immunization (+, $p < 0.05$ by one-way ANOVA) vs PBS-treated mice ($n = 3$). *B,* A greater percent apoptosis was also observed in $CD4^+$ LNC collected 16 days postimmunization from mice treated with RI-CD28 peptide on day 14 ($n = 3$). *C* and *D,* Representative histograms showing an increase in TUNEL-positive cells in mice treated with RI-CD28 peptide on the day of immunization (day 0) (*C*) or after disease development (day 14) (*D*).

compared with naive animals. The CD80 expression is only mildly elevated in $CD4^+$ spleen cells of mice treated with RI-CD28 peptide with CD86 expression at levels similar to that of unimmunized animals (Fig. 6*A*). The difference in the expression of CD80 was less marked between EL-CD28 peptide and control peptide-treated mice. Thus, the CD80/CD86 ratio was significantly reduced in $CD4^+$ spleen cells of RI-CD28 peptide-treated mice relative to control or PBS-treated mice (Fig. 6, *C–F*). A lowered CD80:CD86 ratio was observed in EL-CD28 and RI-CD28 peptide-treated mice even at 26 days postimmunization (data not shown). In general, CD28 expression was lower in $CD4^+$ LNC and splenocytes from mice treated with EL-CD28 or RI-CD28 peptide as compared with PBS-treated mice.

$F4/80^+$ spleen cells from naive mice expressed higher levels of CD86 than CD80, as previously reported (29). Following immunization with MBP, the expression of CD80 was up-regulated on $F4/80^+$ macrophages in all groups of mice. However, the $F4/80^+$ macrophages in the EL-CD28-treated mice had higher CD86 expression than in the control groups. Thus, the CD80/CD86 ratio was significantly reduced in $F4/80^+$ macrophages in the EL-CD28 peptide-treated mice relative to the control peptide or PBS-treated mice (Fig. 6*B*). We performed similar analyses for $CD11c^+$ dendritic cells and $CD19^+$ B lymphocytes. No differences in CD80:CD86 ratios were observed in either cell type (data not shown). Interestingly, we observed a decrease in the number of $CD11c^+$, but not $CD19^+$, spleen cells in the EL-CD28 peptide-treated mice relative to the PBS-treated controls (data not shown).

Discussion

CD28 and CTLA-4 on the T cell surface bind the same ligands CD80 and CD86 on APC. However, CTLA4 has a faster on-rate of binding and higher avidity than CD28 for both ligands (33, 34). We took advantage of the differential binding kinetics in the design of peptides to selectively block B7:CD28 interactions. The synthetic peptides likely produce a steric hindrance preventing the cell surface CD28 from binding the B7 ligands. This likely reduces the degree of CD28 aggregation or "receptor capping" required for signal transduction and T cell activation (35). The cell surface CTLA-4 can still potentially down-regulate the immune response by overriding the competition from the synthetic CD28 peptide due to its higher affinity for the same ligands.

The highly conserved nature of the hydrophobic motif in CD28 and its localization in the solvent exposed CDR3-like region strongly suggests a functional significance (21). Mutagenesis of the "MYPPPYLDN" resulted in loss of binding of CD28 with B7 ligands without affecting the cell surface expression of these moieties (20). Rather than providing a structurally defined complex, the proline-rich regions in cytoplasmic proteins are thought to play a role in bringing proteins together such that subsequent interactions are probable. Typically, these polyproline sequences adopt a polyproline type II helical conformation, an extended structure with three residues per turn (22). Structural characterization by circular dichroism showed that the synthetic CD28 peptide mimics adopt a typical spectrum of a polyproline type II helix (23).

In addition to structural integrity, the biological activity of the peptide mimics depends on the duration of stability. Marini et al. (16) reported that a synthetic CD4 peptide, effective in inhibiting the clinical signs of EAE in the SJL mouse, had a half-life of 45 min (16). Both the EL-CD28 and RI-CD28 peptides, when exposed to mouse serum, were stable in vitro for over 4 wk as determined by HPLC (data not shown).

We have previously shown that the CD28 peptide mimics bind B7-1 with low affinity and fast kinetics (23). Significantly, the synthetic CD28 peptide mimics effectively compete with CD28-Ig to bind B7-1 ligands, indicating that the selected peptide sequence represents a ligand binding epitope of CD28. Competitive kinetic studies showed that the CD28 peptide mimics exhibit a much lower affinity than the CTLA-4 Ig for the B7-1 ligand. Collectively, these results suggest that the CD28 peptides carry a greater potential to selectively block B7:CD28 interactions while maintaining the higher affinity B7:CTLA-4 interactions largely intact.

Treatment with synthetic CD28 mimic at the time of immunization protects B10.PL mice from EAE. The retro-inverso isomer (RI-CD28) inhibited EAE development more effectively, with the treated mice exhibiting lower disease incidence and significantly decreased clinical severity than the end group-blocked parent peptide. This difference in activity may be attributed to a decreased susceptibility of a peptide composed of D-amino acids to proteases in vivo. Furthermore, the lack of protection following treatment with L-CD28 peptide can be attributed to rapid proteolytic cleavage in vivo of peptide acids by amino and carboxyl-peptidases. Importantly, a single administration of synthetic CD28 peptide mimic ameliorated ongoing disease in B10.PL mice. The clinical signs of EAE were dramatically improved within 24–48 h of administration of RI-CD28 or EL-CD28 peptide. All mice treated with the CD28 peptide mimics showed a decrease in clinical score throughout the period of observation, but there was not complete clinical recovery.

If CD80 and CD86 are able to direct T cell differentiation and activation, the outcome of an immune response will depend upon the level of expression of the two molecules on the APC. Our observation of increased expression of B7-1 relative to B7-2 on splenocytes in mice immunized for EAE is consistent with the studies of Karandikar et al. (29) who reported similar findings in

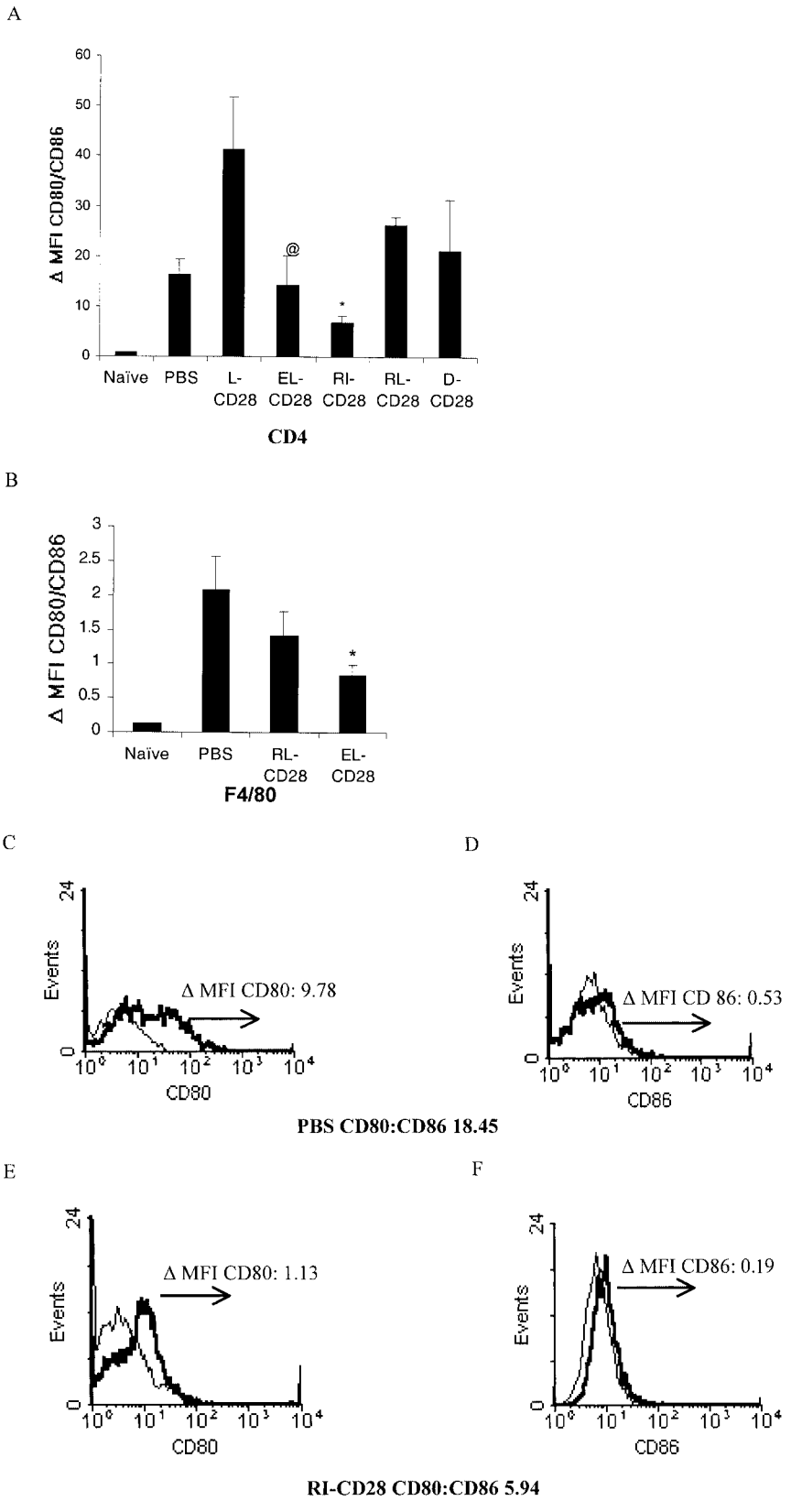

FIGURE 6. CD28 peptide treatment alters the expression of CD80 and CD86 on $CD4^+$ T cells and $F4/80^+$ macrophages following induction of EAE. A significant decrease in the CD80:CD86 ratio is observed in (*A*) $CD4^+$ splenocytes from mice treated with RI-CD28 peptide and (*B*) macrophages from mice treated with EL-CD28 peptide on the day of immunization relative to PBS-treated controls (*, $p < 0.05$). Representative histograms showing CD80 (*C* and *E*) and CD86 (*D* and *F*) expression on $CD4^+$ splenocytes collected 10 days postimmunization from PBS (*C* and *D*) or RI-CD28 peptide-treated mice (*E* and *F*) on the day of immunization. The figures indicate the mean channel fluorescence intensity of the histograms. Δ MFI indicates detectable surface expression above background (light gray line).

proteolipid protein (PLP) peptide-induced EAE. However, in contrast to their studies, we observed a similar trend of increased B7-1 expression in peripheral LNC as well. This difference may be attributed to different genetic backgrounds of the mice or the method of EAE induction (PLP vs MBP). $CD4^+$ splenocytes and LNC of mice treated with the RI-CD28 peptide exhibited significantly lower B7-1 expression relative to B7-2 during both the initial and late effector phases of EAE. These data, together with a significant decrease in the frequency of IL-2-secreting cells and cell surface expression of CD25 (data not shown), suggest that there are fewer activated encephalitogenic T cells in the periphery of mice treated with RI-CD28 peptide. Collectively, our findings can be viewed as

supportive of the hypothesis proposed by Cross et al. (30) that T cells expressing B7-1 preferentially migrate to the CNS during acute EAE.

Infiltration of T cells alone into the CNS is not sufficient for induction of clinical EAE (36). It has been suggested that the infiltrating T cells recruit peripheral macrophages, which eventually cause tissue damage. The number of infiltrating macrophages correlates well with disease and the depletion of peripheral macrophages prevents EAE (36–38). Although CD86 is constitutively expressed on a variety of APCs, the expression of both CD80 and CD86 is elevated upon activation (2). Increased expression of CD80 on APCs is correlated with disease progression in EAE (30, 31). Consistently, we observed elevated levels of CD80 on macrophages in all groups of mice. However, CD86 expression was not down-regulated on F4/80$^+$ macrophages in the EL-CD28 peptide-treated mice, resulting in a significantly decreased CD80:CD86 ratio in these mice. In this context, it is pertinent to note that in mice, CTLA-4 has been shown to preferentially bind CD86 (39). Furthermore, CTLA-4 ligation on activated CD4$^+$ T cells has been shown to induce apoptosis (40). These observations, together with the data presented in this study, suggest that the CD80:CD86 ratio on CD4$^+$ T cells and macrophages, rather than the expression of each molecule alone, is a dynamic factor that plays a significant role in determining the pathogenic cell response following Ag stimulation.

Previously, Kearney et al. (41) have shown that in vivo treatment with a combination of anti-B7-1 and anti-B7-2 mAbs blocked clonal expansion with subsequent loss of these cells presumably due to programmed cell death. Our observation of increased apoptosis of CD4$^+$ T cells from the lymph nodes of mice treated with RI-CD28 peptide suggests clonal deletion as the mechanism of protection against EAE. This is supported by the absence of up-regulation of CD25 and CD28 on CD4$^+$ T cells from the peripheral lymphoid organs of CD28 peptide-treated mice as compared with the activated phenotype of CD4$^+$ T cells from vehicle-treated mice.

The therapeutic potential of CD28 blockade in autoimmune diseases is exemplified by the fact that within a decade of demonstration of its potential immunosuppressive effects, CTLA4-Ig has entered phase I clinical trials for the treatment of psoriasis (42). Perrin et al. (14) have shown that specific blockade of CD28 signaling induced less severe disease in PLP-induced EAE in PL $\times$ SJL F_1 mice. Whereas systemic administration of CTLA-4 Ig had minimal effect, local CNS delivery of CTL4-Ig using a nonreplicative adenoviral vector has been shown to ameliorate ongoing EAE (12, 43). Taken together, our data and those of others suggest that short-term CD28 blockade may provide a means to ameliorate an established autoimmune disease. The advantage of small peptide-based therapeutics over mAbs lies in their lack of immunogenicity with the potential for use over long periods. In addition, the peptides have a substantially lower m.w. than the Abs or fusion proteins and perhaps have greater accessibility to the tissues of the CNS.

In conclusion, we have integrated the results of mutagenesis experiments, binding kinetics, molecular modeling, and structural characterization of T cell costimulatory molecules together with peptidomimetics in the design of two biologically active peptide mimics that selectively block CD28 costimulation in vivo. The potential therapeutic application of these peptides can be extended to most T cell-mediated autoimmune disorders and graft-vs-host disease where the aim is to down-regulate T cell responses and accelerate recovery.

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