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Suppression of Immune Responses in Collagen-Induced Arthritis by a Rationally Designed CD80-Binding Peptide Agent

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Objective. **The CD80/CD86–CD28/CD152 costimulatory pathways transmit signals for CD4+ T cell activation and suppression and are critically involved in the pathogenesis of rheumatoid arthritis (RA). A significant number of CD4+ T cells and macrophages in the rheumatoid synovium express elevated levels of CD80, increasing the potential for costimulation in trans of naive T cells. To determine the effect of blockade of this costimulatory axis in RA, we designed novel CD80-binding peptides and evaluated their therapeutic potential in collagen-induced arthritis (CIA), an animal model of RA.**

Methods. **The conserved MYPPPY motif of CD152 adopts a polyproline type II (PPII) helical conformation in the CD80–CD152 complex. The pairing preferences of the critical residues at the CD80–CD152 interface and their propensity to form PPII helices were integrated to design peptides with optimum PPII helical content that selectively block CD80–receptor interactions. The clinical efficacy was tested in DBA/1LacJ mice that were administered the CD80 blocking agents, called CD80-binding competitive antagonist peptides (CD80-CAPs), at the time of immunization with bovine type II collagen or 3 weeks after immunization.**

Results. **A single administration of select CD80-CAPs significantly reduced the clinical, radiologic, and histologic disease severity in CIA. Importantly, administration of CD80-CAPs during activated immune response significantly suppressed disease development by reducing mononuclear cell infiltration in the joints and mediating peripheral deletion of activated CD4+ T cells.**

Conclusion. **A rationally designed CD80-binding peptide both prevents and suppresses CIA, suggesting a potential application in RA. Apoptosis of activated CD4+ T cells following in vivo blockade suggests that the effects of CD80-CAPs may be long-lasting.**

Over the years, the development of effective therapies for rheumatoid arthritis (RA) has advanced, with better understanding of the pathogenesis of the disease. Activated CD4+ T cells play a central role in the joint inflammation and destruction characteristic of RA (1). Interactions between the CD28/CD152 costimulatory receptors on T cells and the CD80/CD86 (B7) ligands on antigen-presenting cells (APCs) are critical in the activation and termination of immune response (2). The importance of targeting this pathway for immunomodulation is exemplified by the fact that, within a decade of its development, CD152-Ig, a fusion protein that blocks the CD80/CD86–CD28/CD152 interactions, is in clinical trials for multiple autoimmune diseases, including RA (3). Recently, efforts have been directed toward developing small molecule inhibitors to selectively block activated T cells (4,5).

Rational modification of the binding interfaces of

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Dr. Srinivasan developed the CD80-binding functional pseudoreceptor for which Indiana University holds a provisional patent. Dr. Blum developed a peptide for which Indiana University has a patent pending.

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known receptor–ligand complexes is an attractive approach for designing antagonists of protein–protein interactions (6). Although both B7 molecules bind the CD28/CD152 receptor through the conserved ^{99}MYPPPY^{104} motif, only in the complex with CD80 does the Pro^{101} of CD152 adopt a unique polyproline type II (PPII) helical conformation, a structure commonly observed in transient intermolecular interactions (7,8). Furthermore, the Pro^{102} is tightly packed against the Tyr^{28} of CD80 (8,9). This geometric orientation is more frequently observed for functionally important proline residues in binding interfaces (10). Similar structural information derived from the CD152–CD80 complex, together with the residue preferences for interface localization and PPII helix formation, was adopted in the design of peptide antagonists of CD80–receptor interactions. Significant advantages of peptides as therapeutic agents include the ease of synthesis and the amenability to modifications to mimic the topology of the binding epitope without losing functional efficacy (6).

The rationally designed peptides called CD80-binding competitive antagonist peptides (CD80-CAPs) have low molecular mass and optimum PPII helical content for better intermolecular fit with the CD80 ligand. Kinetic studies suggested that select CD80-CAPs inhibit interactions between the cell surface B7 ligands and the CD28/CD152 receptors. The clinical efficacy of CD80-CAPs was evaluated in collagen-induced arthritis (CIA), an animal model for human RA. Significantly, treatment with select CD80-CAPs prevented and suppressed ongoing immune responses in CIA. The reduced clinical disease was accompanied by increased apoptosis of the draining CD4+ lymph node cells (LNCs) and decreased infiltration of CD4+ T cells and macrophages in the joints. These observations suggest that the peripheral deletion of activated CD4+ T cells is one mechanism of protection following selective blockade of CD80–receptor interactions.

MATERIALS AND METHODS

Peptide synthesis. CD80-CAPs and the control peptide were synthesized on Rink amide resin at the Biochemistry and Biotechnology facility at Indiana University School of Medicine, as previously described (11). The free NH_2-terminal group of the NH_2-terminal residues of the CD80-CAPs was acetylated. The peptides were purified by semipreparative reverse-phase high-performance liquid chromatography, and the identity of the purified peptide was confirmed by mass spectrometry.

Binding experiments. Cultured Raji cells (American Type Culture Collection, Manassas, VA) that expressed both CD80 and CD86 on their surface were used to evaluate competitive binding (5). Initially, the affinity of human CD152-Ig and CD28-Ig (R&D Systems, Minneapolis, MN) for cell surface B7 ligands was determined as 0.68 μM and 5.1 μM, respectively. For competitive binding, Raji cells were incubated with human CD28-Ig (5 μM) or CD152-Ig (0.7 μM) (R&D Systems) in phosphate buffered saline (PBS)/0.1% bovine serum albumin (BSA)/0.1% sodium azide and increasing concentrations (3.13–200 μM) of individual CD80-CAPs, for 30 minutes at 4°C. After washing, the cells were stained with phycoerythrin-conjugated anti-mouse IgG2a for 30 minutes. The cells were washed, fixed in 2% paraformaldehyde, and analyzed immediately using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) (12). Mean fluorescence intensity was representative of the amount of cell-bound fusion proteins, and the data were evaluated by Scatchard plot analysis. The dissociation constant (K_d) was estimated from the negative reciprocal of the slope of the fitted line (12).

Induction and assessment of CIA. CIA was introduced into female DBA/1LacJ mice (6–8 weeks old; The Jackson Laboratory, Bar Harbor, Maine) by immunizing with 100 μg bovine type II collagen (CII) emulsified in an equivalent amount of Freund's complete adjuvant (4 mg/ml; Chondrex, Redmond, WA) (13). Mice were monitored daily for signs of arthritis and graded using a scale of 0–4, where 0 = normal, 1 = mild swelling with erythema, 2 = significant joint swelling, 3 = severe swelling and digit deformity, and 4 = maximal swelling with ankylosis. Each joint was scored, with a maximum possible score of 16 per mouse (11).

Treatment protocols. To evaluate the preventive potential of CD80-CAPs, mice received 500 μg of CD80-CAP or control peptide intravenously on the day of CII immunization. The control peptide used in disease protocols and subsequent functional studies was composed of a scrambled sequence of CD80-CAP and was predicted to have no PPII helical content. In order to evaluate suppressive potential, CD80-CAP (500 μg) was administered intravenously 21 days after immunization, at which time no mice exhibited clinical signs of disease (14,15).

Measurement of anticollagen antibody levels. Serum samples were collected on days 11, 18, and 45, and levels of anti-CII antibodies were measured by enzyme-linked immunosorbent assay (ELISA) (16). Briefly, ELISA plates were coated overnight at 4°C with 500 ng of CII in PBS. After washing and blocking with 5% BSA/PBS, the plates were incubated with serum samples in serial dilutions from 1:10^2 to 1:10^5, for 2 hours at room temperature. The plates were then washed and incubated with biotin-conjugated goat anti-mouse IgG, IgG1, or IgG2a, followed by the addition of avidin–peroxidase, and subsequently were developed using avidin–biotinylated enzyme complex. The optical density at 450 nm was measured with a microplate reader. The standard sample consisted of a mixture of sera from arthritic mice in serial dilution, and a standard curve was generated with arbitrary relative units of total IgG, IgG1, and IgG2a anti-CII antibodies.

Histologic analysis. At the end of the experiments, the mice were killed, and the hind paws were dissected, fixed in formalin, decalcified in 5% EDTA, embedded in paraffin, and

stained with hematoxylin and eosin. Changes in the interphalangeal joints were scored as mild (mild synovial hyperplasia), moderate (moderate inflammatory cell infiltration, pannus formation, and erosion), or severe (severe bone and cartilage erosions and loss of joint architecture) (17).

Three-dimensional (3-D) microfocal computed tomography (micro-CT). Paws were fixed in formalin and scanned with a SkyScan 1072 microtomography system (SkyScan, Aartselaar, Belgium), using a cone beam configuration with standardized x-ray tube settings of 35 keV and 75 mA (22). All interphalangeal joints were scanned at an isotropic voxel resolution of 14.83 μm^3 with an exposure time of 1.3 seconds and a rotation step of 0.90°. Regions of interest in the captured images extended 5 mm on the proximal or distal end of the bones constituting the middle interphalangeal joints for all digits but the thumbs, where the proximal interphalangeal joints were analyzed. Morphologic measurements of the trabecular structure were performed using standard SkyScan software (18).

Collagen-specific in vitro culture. Mice were killed 56 days after CII immunization. Single-cell suspensions of draining LNCs were cultured at 1×10^6 cells/ml in complete RPMI 1640 (Sigma, St. Louis, MO) in the presence or absence of 20 μg/ml CII, as previously described (17). Supernatants were collected after 24 hours and 48 hours and stored at −20°C until further analysis. Proliferation assays were performed in parallel cultures for 72 hours, including a final 18-hour pulse with tritium-labeled thymidine. Cultures were harvested onto glass-fiber mats and counted by liquid scintillation using a Betaplate (LKB, Stockholm, Sweden). Results are expressed as the stimulation index (SI) (counts per minute CII-activated cells/cpm unstimulated cells in medium) (19).

Culture of synoviocytes. Mononuclear cells from the inflamed joints were prepared as previously described, with modifications (20). The hind paws were dissected away from the ankle, the skin was removed, and the joints were cut into small pieces. After washing thoroughly, the pieces of the joints were incubated with complete RPMI 1640 containing collagenase A (1 mg/ml), Dispase (2.4 mg/ml), and DNase I (100 μg/ml) (all enzymes from Roche, Mannheim, Germany), for 1 hour at 37°C. The digested tissue was passed through a nylon mesh, and the cells were washed and cultured in triplicate at 0.5×10^6 cells/ml for cytokine and proliferation assays as described above.

Flow cytometry. Single-cell suspensions of draining LNCs or synoviocytes (0.5×10^6) were incubated with 1 μg of phycoerythrin-conjugated anti-CD80 (clone 16-10A1) or anti-CD86 (clone GL-1) and fluorescein isothiocyanate (FITC)–conjugated anti-CD4 (clone RM4-5) for 30 minutes at 4°C. For macrophages, a 3-step staining procedure was used (21). Synoviocytes were incubated with 1 μg of anti-F4/80 (clone C1: A3-1; R&D Systems), followed by washing and incubation with FITC-conjugated anti-rat IgG2b (clone A95-1). All monoclonal antibodies (unless specified) were purchased from BD PharMingen (San Diego, CA). Labeled cells were washed, resuspended in 1% paraformaldehyde, and analyzed using a FACSCalibur flow cytometer.

Cytokine analysis. The levels of cytokines in serum and culture supernatants were determined by ELISA, using OptEIA mouse interleukin-2 (IL-2), IL-6, IL-10, IL-12, and interferon-γ (IFNγ) kits, according to the manufacturer's recommendations (BD PharMingen).

Detection of apoptosis. The TUNEL assay was performed as previously described (22). Draining LNCs (10^6) were stained with phycoerythrin-labeled anti-mouse CD4 (L3T4, clone RM 4-4; BD PharMingen), fixed, permeabilized, and then incubated with FITC-labeled terminal deoxynucleotidyl transferase (TdT) reaction mixture according to the manufacturer's instructions (Roche). A positive control consisted of cells that were incubated in DNase (1 μg/ml) before incubation with TdT. A negative control consisted of cells that were incubated without TdT. Cells were then washed, resuspended in PBS, and analyzed by flow cytometry.

Statistical analysis. For the mean clinical score, proliferation and cytokine assays, micro-CT analysis, cell surface expression of CD4 and F4/80, and TUNEL assays, one-way analysis of variance with Tukey's post hoc comparison was performed to determine the differences between the groups. P values less than 0.05 were considered significant.

RESULTS

Design of CD80-CAPs. The T cell costimulatory receptors CD28 and CD152 share a highly conserved hydrophobic motif with 3 consecutive prolines (^{101}PPP^{103}) in the ligand-binding region (23). In the complex with CD80, Pro^{101} of CD152 is restricted to a PPII helical conformation and Pro^{102} is in edge-to-face contact with the conserved Tyr^{28} of CD80 (8,9). These structural features of CD152, in the context of the residues at the binding pocket of CD80 and the amino acid preferences for interface localization, pairing, and PPII helix formation, were integrated in the design of peptide mimics for binding CD80 (10,11). The rationally designed mimics that exhibited optimum PPII helical content, low deviation, and greater similarity upon superimposition with the CD152–CD80 complex were referred to as CD80-CAPs (11). In silico docking with CD80 showed that Pro^3 of CD80-CAP1 (M^{1}QPPGC^6) is in edge-to-face contact with the Tyr^{28} of CD80, and the backbone of Gln^2 and Cys^6 (not shown) are in close proximity with Glu^{81} of CD80, suggesting a structural mimic of the ligand-binding region of CD152 (Figure 1A).

Competitive kinetics of CD80-CAPs for interaction with cell surface B7 ligands. Cultured Raji cells expressing CD80 and CD86 on their surface were used to evaluate competitive binding of CD28-Ig/CD152-Ig in the presence of serial dilutions of different CD80-CAPs, by flow cytometry (5,12). The affinity of CD152-Ig for the cell surface B7 ligands was 0.68 μM (data not shown). This interaction was completely abrogated in the presence of CD80-CAP1 (Figure 1B). The affinity of the interactions between CD28-Ig and the cell surface B7 ligands was similar in the presence (K_d = 7.8 μM) (Figure 1C) or absence (K_d = 5.1 μM) (data not shown)

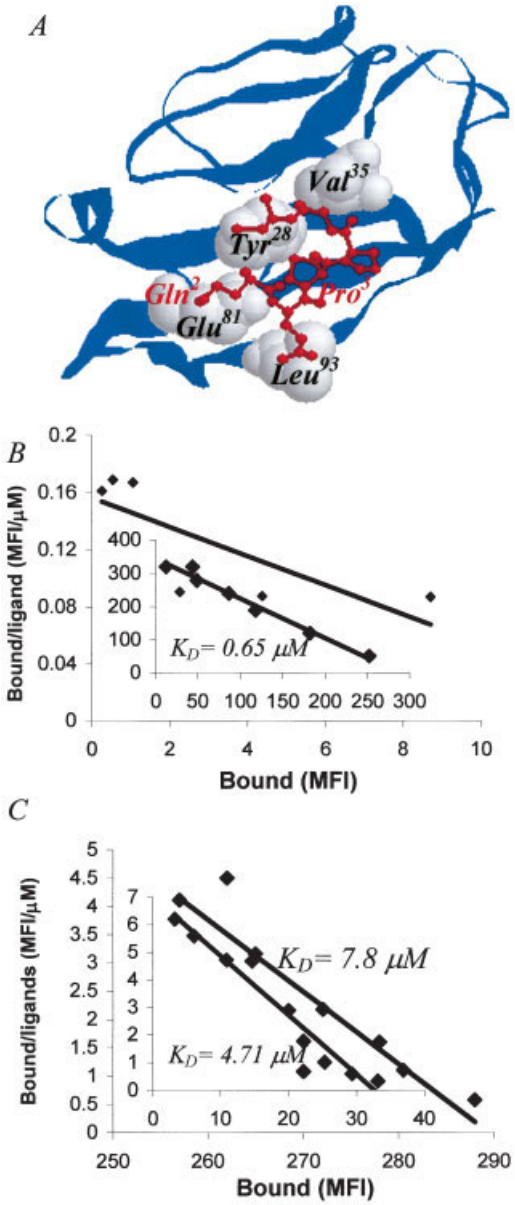

Figure 1. A, Molecular model of human CD80 extracellular domain (blue) in complex with CD80-binding competitive antagonist peptide (CD80-CAP) (red). The residues at the binding pocket of CD80 (Tyr^{28}, Val^{35}, Leu^{93}, and Glu^{81}) (gray) in close proximity with the CD80-CAP residues (Gln^2 and Pro^3) are highlighted. **B** and **C,** Binding of CD80-CAPs to cell surface B7 ligands. **B,** Competitive binding between a constant concentration of human CD152-Ig and serial dilutions (3.3–200 μM) of CD80-CAP1 (inner plot) or CD80-CAP3 (outer plot) to the B7 ligands on Raji cells, as evaluated by flow cytometry. **C,** Competitive binding between a constant concentration of human CD28-Ig and serial dilutions (3.3–200 μM) of CD80-CAP1 (inner plot) or CD80-CAP3 (outer plot) to the cell surface B7 ligands. Mean fluorescence intensity (MFI) represents the amount of cell-bound fusion proteins. Data are expressed as linear regression lines in Scatchard plots of binding. The affinity constants for the interactions are shown. Results are representative of 2 separate experiments.

of CD80-CAP1. A second CD80-CAP with significant PPII helical content, CD80-CAP3, did not exhibit inhibitory potential. The affinity of CD152-Ig and CD28-Ig for the cell surface B7 (CD80/CD86) ligands in the presence of CD80-CAP3 was 0.65 μM (Figure 1B) and 4.71 μM (Figure 1C), respectively. Collectively, these results suggest that CD80–CAP1 adopts an optimum structure for binding the cell surface B7 ligands. Hence, CD80–CAP1 was selected for evaluation as a potential therapeutic agent in CIA and is henceforth referred to as CD80-CAP.

Ability of synthetic CD80-CAP to prevent and suppress CIA. Previous studies have shown that administration of CD152-Ig or anti-B7 antibodies prevents CIA (24). We evaluated the ability of CD80-CAP (500 μg intravenously), administered on the day of disease induction, to prevent CIA. The mean arthritis score decreased more significantly in CD80-CAP–treated mice than in the control peptide–treated or vehicle-treated mice (Figure 2A). The CD80-CAP–treated mice continued to be protected even 72 days after immunization with CII (data not shown). No mice died during the period of observation or exhibited signs of toxicity at death. A BLAST search did not yield an exact match with known protein sequences for either CD80-CAP or the control peptide. The increase in clinical disease symptoms observed in mice treated with the control peptide is attributed to an unknown effect of the latter on T cell responses.

Increased numbers of CII-specific CD4+ T cells have been observed in the lymph nodes and blood of mice 10 days to 2 weeks after CII immunization (14,15). The number of synovial and circulating CII-specific T cells decreases shortly after the first clinical signs of disease (14). Here, the DBA/1LacJ mice exhibited the first clinical signs 28 days or more after CII immunization. Hence, in order to assess the ability of CD80-CAP to suppress ongoing immunopathology in CIA, groups of mice were distributed randomly 3 weeks after CII immunization and administered PBS, CD80-CAP, or control peptide. None of the animals exhibited clinical signs of disease at this time. The appearance of clinical signs was delayed in mice that received CD80-CAP (day 35) compared with those that received PBS (day 29) or control peptide (day 27) (Figure 2B). Furthermore, continued observation for 41 days showed that the mean arthritis score was significantly lower in the mice that received CD80-CAP than in the mice treated with vehicle or control peptide (Figure 2B). These results suggest both preventive and suppressive potential for CD80-CAP in CIA.

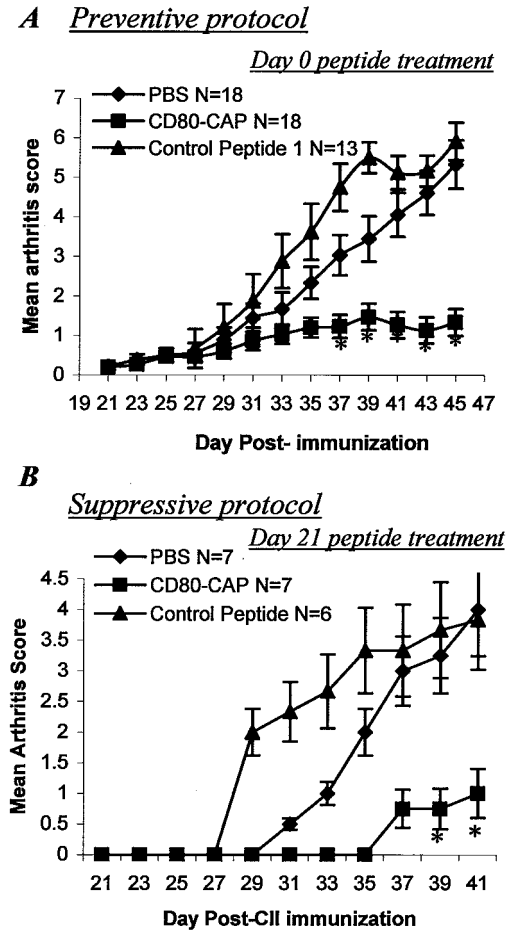

Figure 2. Treatment with CD80-binding competitive antagonist peptide (CD80-CAP) prevents and suppresses collagen-induced arthritis. DBA/1LacJ mice were immunized with 100 μg bovine type II collagen (CII) in Freund's complete adjuvant. Groups of mice were administered 500 μg CD80-CAP, control peptide, or phosphate buffered saline (PBS) intravenously, according to preventive (day 0) (**A**) or suppressive (day 21) (**B**) treatment protocols. Severity of arthritis was evaluated by assigning a score of 0–4 per paw based on the degree of inflammation in each limb, with 0 = no arthritis and 4 = severe arthritis, for a maximum possible score of 16 per mouse. The number of mice in each treatment group is indicated. Results are representative of 3 different experiments and are the mean ± SEM. ∗ = $P < 0.05$ versus PBS– or control peptide–treated mice.

Micro-CT and histologic assessment following CD80-CAP treatment in CIA. The effect of CD80-CAP treatment on synovium and bone destruction was assessed by micro-CT and histologic analysis (17,18). Representative 3-D tomograms of paws of mice treated with vehicle, CD80-CAP, or control peptide, obtained 8 weeks after CII immunization, are shown in Figures 3A–C. Three-dimensional bone volume of the affected distal interphalangeal joints and trabecular thickness were significantly higher in mice treated with CD80-CAP than in those treated with vehicle or control peptide (Figure 3G and data not shown, respectively), suggesting reduced bone destruction after treatment with CD80-CAP (18).

Sections from the hind paws were histologically graded for the degree of inflammation 4 weeks after the onset of arthritis (17). For direct comparison between groups, mice whose arthritis index equaled the average index of their respective groups were selected for histopathologic analysis. Untreated mice and mice treated with control peptide exhibited increased inflammatory cell infiltration, synovial hyperplasia, multinucleated giant cells and osteoclasts, bone erosion, and some pannus formation (Figures 3D and F). Paws of CD80-CAP–treated mice exhibited low inflammatory cell infiltration, mild synovial hyperplasia, and no cartilage or osseous changes (Figure 3E).

Effect of CD80-CAP treatment on anti-CII IgG1 and IgG2a and serum IL-6. The Th1 cytokine IFNγ is known to mediate IgG2a production, while Th2 cytokines regulate the abundance of other circulating IgG

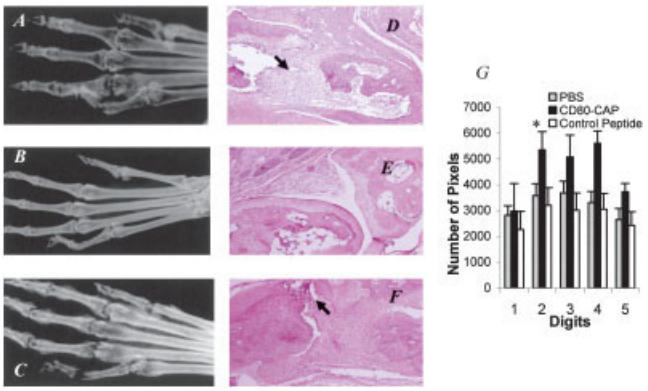

Figure 3. A–F, Radiographic and histologic appearance and 3-dimensional microarchitecture 56 days after immunization with bovine type II collagen, in mice treated on day 0 with phosphate buffered saline (PBS) (n = 7) (**A** and **D**), CD80-binding competitive antagonist peptide (CD80-CAP) (n = 6) (**B** and **E**), or control peptide (n = 6) (**C** and **F**). **Arrows** in **D** and **F** indicate sites of inflammatory cell infiltration. Hematoxylin and eosin–stained sections of hind paws from mice treated with PBS or control peptide exhibited synovial hyperplasia, inflammatory infiltration, and osseous changes, whereas CD80-CAP–treated mice exhibited mild synovial hyperplasia. Results shown are representative of all mice in each treatment group. **G,** Three-dimensional trabecular bone volume of the distal interphalangeal joint of the thumb (designated 1) and the middle interphalangeal joints of all other digits (designated 2–5). Values are the mean and SEM number of pixels. ∗ = $P < 0.05$ versus PBS– or control peptide–treated mice.

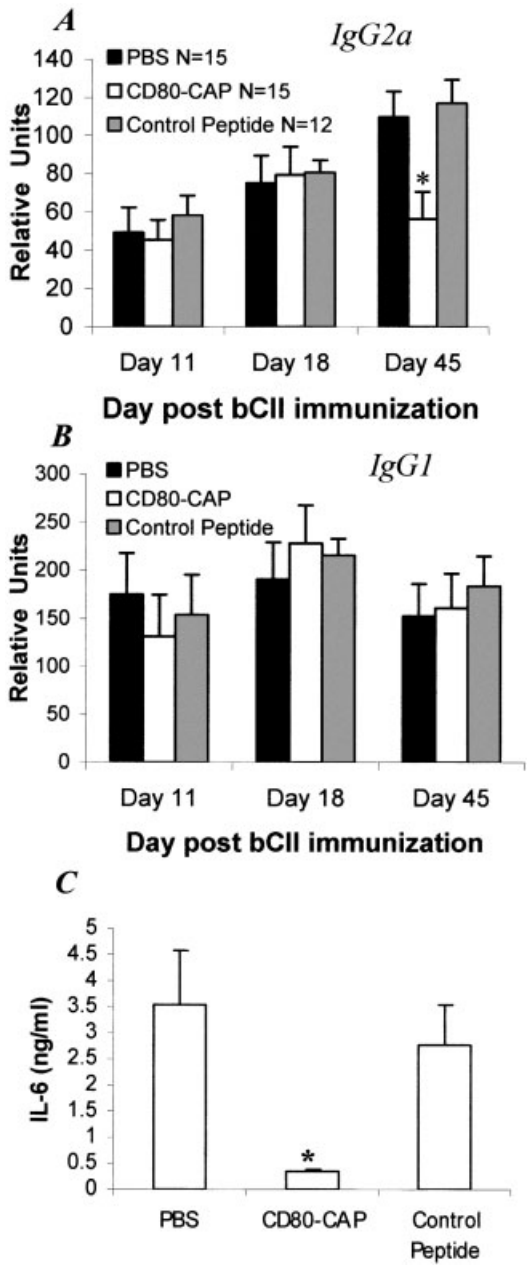

Figure 4. Effect of CD80-binding competitive antagonist peptide (CD80-CAP) on serum anticollagen antibody titers and interleukin-6 (IL-6) levels in mice with collagen-induced arthritis. **A** and **B,** Serum titers of **A,** anti–bovine type II collagen (bCII) IgG2a and **B,** anti-bCII IgG1 were measured by enzyme-linked immunosorbent assay (ELISA) at the indicated time points. The numbers of animals analyzed in each group are indicated in **A.** Data are expressed in relative units based on the titers from standard serum from arthritic mice. **C,** Levels of IL-6 in serum collected 11 days after immunization with CII, as measured by ELISA. IL-6 levels were significantly reduced in CD80-CAP–treated mice ($P < 0.05$ versus phosphate buffered saline [PBS]– or control peptide–treated mice). Values are the mean and SEM.

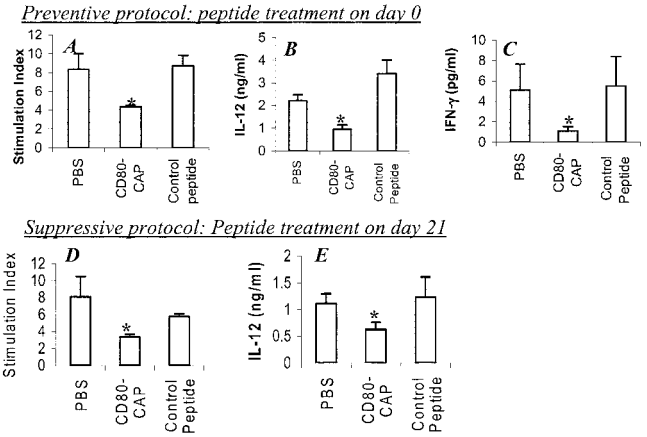

Figure 5. Significant inhibition of cytokine secretion and proliferation of lymph node cells and synoviocytes, in mice with collagen-induced arthritis treated with CD80-CAP in the preventive and suppressive protocols. Synoviocytes (**A, D,** and **E**) or lymph node cells (**B** and **C**) isolated from bovine CII–immunized DBA/1LacJ mice after preventive (**A–C**) or suppressive (**D** and **E**) treatment protocols were cultured with 20 μg/ml CII. For cytokine analysis, levels of IL-12 (**B** and **E**) and interferon-γ (IFNγ) (**C**) in culture supernatants collected at 24 and 48 hours were measured by ELISA. For proliferation assays (**A** and **D**), similar cultures were incubated for 72 hours, including a final 16-hour pulse with tritium-labeled thymidine. Data are expressed as the stimulation index, determined by calculating the ratio of cell proliferation with antigen relative to that with medium alone (cpm). The mean and SEM cpm of synovial cells in medium was 8,958 ± 1,353 in the preventive protocol and 5,617 ± 719 in the suppressive protocol. * = $P < 0.05$ versus vehicle (PBS) or control peptide–treated mice. See Figure 4 for other definitions.

subtypes (25). As an indirect measure of the effect of CD80-CAP treatment on Th1 subset development, the relative abundance of anti-CII IgG1 and IgG2a in the serum was measured 11, 18, and 45 days after CII immunization. Although serum anti-CII IgG2a levels increased in all groups of mice initially, the relative amount of this Ig subtype was significantly lower in CD80-CAP–treated mice 45 days after immunization (Figure 4A). No significant difference was observed in serum anti-CII IgG1 levels in the different groups of mice (Figure 4B).

Serum IL-6 levels. Elevated levels of IL-6 in serum are observed in both CIA and RA (19). The CD80–CD28 interaction is known to induce IL-6 secretion by murine dendritic cells (26). The ability of the costimulatory blockade by CD80-CAP to modulate serum IL-6 in CIA was measured by ELISA. Serum IL-6 measured 11 days after CII immunization was significantly lower in mice treated with CD80-CAP than in mice treated with control peptide or vehicle (Figure 4C).

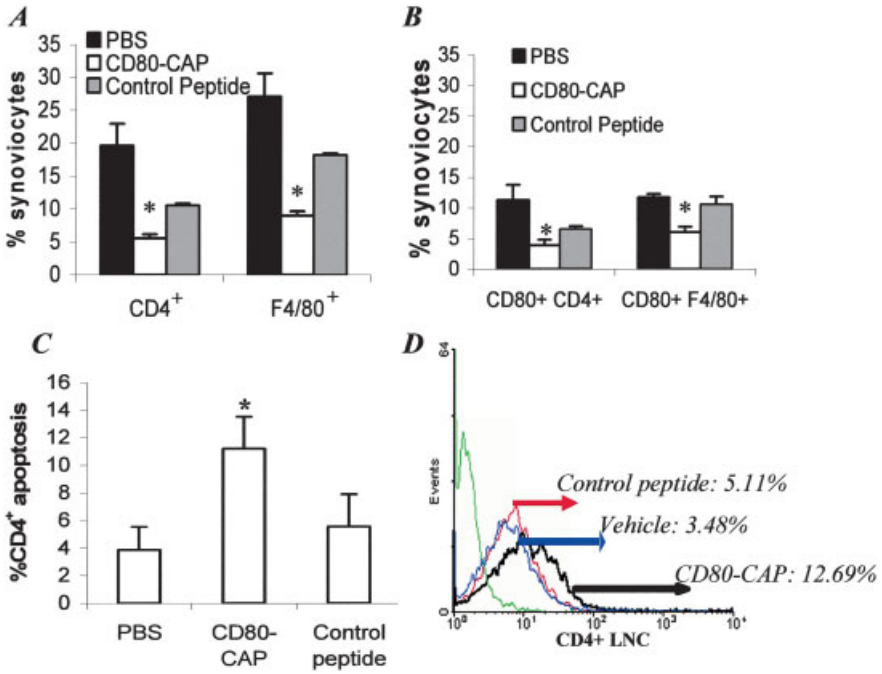

Figure 6. CD80-binding competitive antagonist peptide (CD80-CAP) treatment suppresses ongoing immunopathology in collagen-induced arthritis (CIA) by reducing mononuclear cell infiltration and mediating apoptosis of CD4+ T cells. DBA/1LacJ mice with CIA were treated with vehicle (phosphate buffered saline [PBS]), CD80-CAP, or control peptide (n = 3 per group). Single-cell suspensions of draining lymph node cells (LNCs) and synoviocytes were obtained on day 23 and characterized by flow cytometry. None of the mice exhibited clinical signs of arthritis on the day they were killed. **A** and **B,** Percentage of CD4+ and F4/80+ synoviocytes (**A**) and CD80+,CD4+ and CD80+,F4/80+ double-positive synoviocytes (**B**). **C,** Apoptosis in draining LNCs, measured by TUNEL assay. Values in **A–C** are representative of 2 independent experiments and are presented as the mean and SEM. $* = P < 0.05$ versus PBS- or control peptide–treated mice. **D,** Representative histogram showing an increase in TUNEL-positive cells in mice treated with CD80-CAP. Green line represents the isotype control. Color figure can be viewed in the online issue, which is available at http://www.arthritisrheum.org.

Modulation of T lymphocyte immune responses in vivo by CD80-CAP treatment. The effect of costimulatory blockade in vivo by CD80-CAP on T cell responses was measured by proliferation assay. Draining LNCs and synovial cells from mice with CIA treated on day 0 with CD80-CAP, control peptide, or PBS were isolated 56 days after immunization and stimulated with CII/ovalbumin peptide in vitro. The proliferation of synovial cells, measured in terms of SI (4.38), from CD80-CAP–treated mice was significantly lower than in mice treated with control peptide (8.71) or vehicle (8.38) (Figure 5A). The SI of LNCs was also significantly lower in the CD80-CAP–treated mice (data not shown). No significant difference was observed in the proliferative response to an irrelevant antigen among the different treatment groups (data not shown). These results suggest that CD80-CAP diminishes T cell activation in vivo.

To determine the ability of the CD80-CAP treatment to suppress T cell activation, groups of mice were administered PBS, CD80-CAP, or control peptide 21 days after CII immunization, and synovial cells that were isolated 2 days later (day 23) were assessed for proliferative responses to CII. Synovial cells from the CD80-CAP–treated mice exhibited a significantly decreased SI (3.36) compared with that in cells from the control peptide–treated (5.75) or vehicle-treated (8.09) mice (Figure 5D).

Cytokine analysis. Since Th1 cytokines have been linked to the development and progression of CIA (1), the effect of CD80 blockade by CD80-CAP on proin-

flammatory cytokine secretion was assessed. Production of IL-12 and IFNγ was significantly lower in cultures of LNCs from mice treated with CD80-CAP (0.95 ng/ml and 1.08 pg/ml, respectively) than from mice treated with control peptide (3.4 ng/ml and 5.5 pg/ml) or vehicle (2.2 ng/ml and 5.1 pg/ml) (Figures 5B and C). No significant difference was observed in the levels of the Th2 cytokines IL-4, IL-5, and IL-10 in any of the treatment groups (data not shown). Similar results have been observed following costimulatory blockade in CIA (24,27). This may be due to the effect of CD86–receptor interactions in mediating Th2 cytokine secretion and/or the complex effects of blocking CD80 on both APCs and CD4+ T cells (28,29). Importantly, synovial cells from the mice that were administered CD80-CAP during the ongoing immune process produced significantly lower levels of IL-12 (0.62 ng/ml) than did cells from mice administered control peptide (1.23 ng/ml) or vehicle (1.11 ng/ml) (Figure 5E).

Association of CIA suppression with reduced inflammatory cell infiltration and peripheral deletion of activated CD4+ T cells. Increased numbers of lymphocytes and macrophages constitute the pathognomonic features of inflamed joints in CIA (1,30). The degree of long-term inflammatory cell infiltration in the joints during disease development was determined, in order to evaluate the suppressive potential of CD80-CAP in CIA. Mice were administered PBS, CD80-CAP, or control peptide 21 days after CII immunization. Single-cell suspensions of synoviocytes isolated 2 days later were characterized by flow cytometry. The synovial infiltrates of CD80-CAP–treated mice exhibited fewer CD4+ T cells and F4/80+ macrophages than those of mice treated with vehicle or control peptide (Figure 6A). Variations in the susceptibility and molecular pathology within groups of animals with CIA are attributed to the observed reduction in CD4+ T cells in mice treated with control peptide, which otherwise does not exhibit protective potential. A significantly lower percentage of CD4+ synovial T cells and F4/80+ macrophages from CD80-CAP–treated mice expressed CD80 compared with those from mice treated with vehicle or control peptide (Figure 6B). These findings suggest that CD80-CAP treatment suppresses long-term inflammatory cell infiltration and reduces joint pathology.

To delineate the mechanism of action of CD80-CAP in CIA, draining LNCs isolated on day 23, 2 days after the administration of CD80-CAP, control peptide, or PBS to CII-immunized mice, were measured for apoptosis by the TUNEL assay. Significantly higher percentages of CD4+ LNCs from the CD80-CAP–treated mice were apoptotic compared with vehicle- or control peptide–treated mice (Figures 6C and D). These results suggest that costimulatory blockade by CD80-CAP protects against CIA by mediating apoptosis of activated CD4+ T cells in the periphery.

DISCUSSION

Targeting T cell costimulation has been extensively validated as an effective method of immunomodulation in multiple autoimmune diseases (31). Interactions between the B7 ligands on APCs and the CD28/CD152 costimulatory receptors on T cells are critically involved in the initiation and expansion of autoreactive T cells and survival in the target tissue (2). In active RA, class II major histocompatibility complex (MHC)–positive synovial T cells coexpress CD28 and functional CD80 molecules, enhancing the potential for autocrine stimulation that contributes to persistent synovitis (32,33). Furthermore, a soluble form of CD80 present in the synovial fluid may contribute to this process (34). Hence, blockade of CD80–receptor interaction has significant therapeutic potential in RA. To successfully treat RA, the biologic agents may need to enter the synovial fluid at relatively high concentrations and remain effective in the synovial compartment (35). The development of small molecule antagonists of receptor–ligand interactions offers an attractive strategy.

Although the CD152 receptor binds both B7 ligands via the same polyproline motif, it has higher affinity for binding CD80 than CD86 (2). The local environment of the CD80 binding pocket, together with the PPII helical conformation of the critical proline of CD152 at the binding interface, may contribute to the differences in the strengths of interactions between the CD152 and the CD80/CD86 ligands (11). Structural data, together with the pairing preferences of residues at the CD80–CD28/CD152 interface, were critically considered in the design of novel CD80-binding peptides such as CD80-CAP (11). Previously, short peptides as conformational mimics of proline-rich sequences were shown to specifically interact with the binding domain, inhibit protein–protein interactions, and exhibit significant therapeutic potential in humans and in animal models (6,36,37).

Binding studies by competitive ELISA showed that CD80-CAP inhibited CD80–CD28/CD152, but not CD86–CD28/CD152, interactions (11). In the present study, this was confirmed by flow cytometry analysis using APCs. These experiments measured the combined affinities of CD28-Ig/CD152-Ig for both B7 ligands at

the cell surface. The observed weak inhibition of the CD28-Ig cell surface B7 ligands by CD80-CAP could be attributed to the higher affinity of CD28-Ig for the cell surface CD86 or to the inherent design of the CD80-CAP derived from the structure of the CD80–CD152 complex.

CIA is often used as a preclinical means of evaluating the efficacy of antirheumatic therapeutic agents (38). While $CD28^{-/-}$ mice are either resistant to or develop minimal disease in CIA, blockade with CD152-Ig suppressed clinical disease in both CIA and RA (3,24,27,39). Although not all animal models share features of CD4+ T cell and/or class II MHC restriction in CIA, here we show that CD80-CAP treatment at the time of immunization protects DBA/1LacJ mice against CIA (38). This is in contrast to the observation that anti-CD80 antibody was not protective in CIA (24). The lower molecular weight and the optimal structure in relation to the binding cavity of CD80 likely facilitate greater tissue permeability and blocking efficacy of CD80-CAP, accounting for its protective effect. Importantly, a single administration of CD80-CAP at the time of heightened immune response (elevated serum IL-6, increased infiltration of CD4+ T cells in the joint) resulted in significantly less clinical disease in CII-immunized mice. Although onset was delayed, the disease was not completely suppressed following CD80-CAP treatment.

Preactivated T cells are thought to be continuously recruited to the rheumatoid synovium, thus perpetuating the inflammatory process (1). In the present study, administration of CD80-CAP at the time of maximum immune response reduced synovial hyperplasia and suppressed inflammatory cell infiltration in the joints. Previously, the degree of synovial macrophage infiltration has been correlated with the radiologic progression of joint destruction in RA (40). In addition, the efficacy of conventional antirheumatic therapy correlates with the down-regulation of functions of the mononuclear phagocyte system (35). Here we show that there were fewer activated (CD80+) macrophages infiltrating the synovium and no or minimal osseous change following CD80-CAP treatment.

Several studies suggest involvement of the CD80/CD86–CD28/CD152 costimulatory pathway in T cell clonal deletion (41,42). B7-dependent costimulation is required for optimum T cell survival and clonal expansion, while blockade with CD152-Ig induces apoptosis of activated CD4+ T cells (41,43). Recently, Gao et al (44) have shown that the survival of autoreactive T cells is regulated by costimulation, and mice with a targeted mutation of CD28 have fewer peripheral autoreactive T cells. Thus, it has been suggested that the costimulatory blockade may mediate protection in autoimmune disease models by reducing the burden of autoreactive T cells in the periphery (44). Our observation of increased apoptosis of CD4+ T cells from the lymph nodes of mice treated with CD80-CAP suggests that peripheral deletion of activated T cells may be one potential mechanism of protection against CIA.

One of the major goals in the generation of novel therapies for the treatment of RA is specific inhibition of the inflammatory disease process without global immunosuppression. Although specific arthritogenic T cells are associated with the initiation of the inflammatory process, its progression and persistence in the synovium are attributed to the continuous recruitment of nonspecifically activated T cells that mediate inflammation and tissue damage (30). Hence, strategies that selectively target and delete activated T cells from the periphery over a relatively short time have potential benefits in the treatment of RA. Selective blockade of CD80 signaling by CD80-CAP suppresses complete activation of primary T cells, mediates apoptosis of activated T cells in the periphery, and reduces inflammatory cell infiltration into the joints, thus protecting against CIA. The beneficial effects of CD80-CAP may be extended to other autoimmune diseases in which chronic inflammation is mediated by both antigen-specific and nonspecific T cells.

AUTHOR CONTRIBUTIONS

Dr. Srinivasan had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Drs. Srinivasan, Eri, and Blum.

Acquisition of data. Drs. Srinivasan and Eri.

Analysis and interpretation of data. Drs. Srinivasan, Eri, and Zunt, Mr. Summerlin, and Dr. Blum.

Manuscript preparation. Drs. Srinivasan and Blum.

Statistical analysis. Dr. Srinivasan.

Model development and purification/supply of arthritis induction reagents. Dr. Brand.

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