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Therapeutic Effect of Urocortin on Collagen-Induced Arthritis by Down-Regulation of Inflammatory and Th1 Responses and Induction of Regulatory T Cells

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Objective. **To investigate the potential therapeutic action of the immunomodulatory neuropeptide urocortin (UCN) in an experimental model of rheumatoid arthritis (RA).**

Methods. **After disease onset, DBA/1J mice with collagen-induced arthritis (CIA) were treated with UCN, and the incidence, severity (clinical score), and joint histopathology were evaluated. The inflammatory response was determined by measuring the levels of different mediators of inflammation (cytokines and chemokines) in the joints and sera. The Th1-mediated autoreactive response was evaluated by determining the proliferative response and cytokine profile of draining lymph node cells stimulated with the autoantigen and by assaying the content of serum autoantibodies. The number of regulatory CD4+,CD25+ T cells and their capacity to suppress self-reactive Th1 cells were determined in joints and lymph nodes.**

Results. **UCN treatment significantly reduced the incidence and severity of CIA, completely abrogating joint swelling and cartilage and bone destruction. The therapeutic effect of UCN was associated with a striking reduction of the 2 deleterious components of the disease: the Th1-driven autoimmune response and the inflammatory response. UCN also induced the generation and/or activation of efficient interleukin-10/transforming growth factor β1–producing Treg cells in arthritis with the capacity to suppress the autoreactive response and to restore immune tolerance, thus playing a pivotal role in the therapeutic effect of UCN.**

Conclusion. **Our findings provide a powerful rationale for assessing the efficacy of UCN as a novel multistep therapeutic approach to the treatment of RA in humans.**

Rheumatoid arthritis (RA) is an autoimmune disease of unknown etiology that leads to chronic inflammation in the joints and subsequent destruction of the cartilage and erosion of the bone. The initial stages of RA involve multiple steps, which can be divided into 2 main phases: the initiation and establishment of autoimmunity and the later events associated with the evolving immune and inflammatory responses. Although the contribution of Th1 responses in RA is still a matter for debate, several studies in animal models indicate a pathogenic role of Th1-derived cytokines (1,2). Th1 cells reactive to components of the joint infiltrate the synovium, release proinflammatory cytokines and chemokines, and promote macrophage and neutrophil infiltration and activation. Excessive levels of inflammatory mediators, such as cytokines and free radicals, that are produced by infiltrating inflammatory cells play a critical role in joint damage (2,3).

In contrast, the induction of antigen-specific tolerance is essential to maintaining immune homeostasis, controlling autoreactive T cells, and preventing the onset of autoimmune diseases, and peripheral regulatory mechanisms are required to protect against self-directed immune responses. The crucial role of CD4+,CD25+ regulatory T cells in the control of self-reactive T cells and in the induction of peripheral tolerance has recently been demonstrated in both mice and humans (4–10). Failures in the functioning of the Treg compartment may therefore be responsible for the

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development of RA, and enhancing this function may represent a therapeutic strategy.

Although available therapies based on immunosuppressive agents inhibit the inflammatory component of RA and either reduce the relapse rate or delay disease onset, they have multiple effects, some of which are undesirable, and over the long term, they do not suppress the progression of clinical disability (11). This illustrates the need for novel therapeutic approaches for preventing the inflammatory and autoimmune components of the disease and for promoting the restoration of immune tolerance.

Urocortin (UCN) is a neuropeptide that is structurally related to corticotropin-releasing factor (CRF) (12). Although UCN was initially recognized by its cardiovascular protective activity (13), there is evidence to suggest that it could act as an endogenous immunomodulatory factor, with predominantly antiinflammatory effects. UCN and its receptors are detected in several types of immune cells, including macrophage/monocytes and T cells, and their expression increases under inflammatory conditions (12–14). UCN has been reported to inhibit experimental autoimmune encephalomyelitis (15), endotoxin-induced tumor necrosis factor α (TNFα) production by Kupffer cells (16), inflammatory bowel disease (17), and sepsis-induced death (18). In addition, levels of UCN present in the mucosa of patients with *Helicobacter pylori*–induced gastritis increase during the stage of eradication and amelioration of inflammation, whereas in patients resistant to treatment, UCN levels remain low (19). In contrast, UCN and its receptors are significantly increased in RA patients, and UCN in the synovium reaches maximum levels during the active stage of RA inflammation (20,21), suggesting an important role of UCN as an autocrine and/or paracrine regulator of synovial inflammation in RA.

The aim of this study was to investigate the potential therapeutic action of UCN in an experimental model of RA. Administration of UCN demonstrated great benefit at the clinical and pathologic levels. The therapeutic effect of UCN was exerted at multiple levels and was associated with the down-regulation of both inflammatory and Th1-mediated autoimmune components of the disease and with the emergence of Treg cells that had suppressive effects on autoreactive T cells.

MATERIALS AND METHODS

Arthritis induction and treatment. Animal experimental protocols were reviewed and approved by the Ethics Committee of the Spanish Council of Scientific Research (CSIC). To induce collagen-induced arthritis (CIA), 7–10-week-old DBA/1J mice (The Jackson Laboratory, Bar Harbor, ME) were injected subcutaneously into the base of the tail with 200 μg of chicken type II collagen (CII; Sigma, St. Louis, MO) emulsified in Freund's complete adjuvant (CFA) containing 200 μg of *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI). On day 21 after primary immunization, mice were given a subcutaneous booster injection of 100 μg of CII in CFA.

UCN or CRF (American Peptides, Sunnyvale, CA) treatment consisted of the intraperitoneal administration of 2 nmoles (6.6 μg per mouse per day; or other doses when indicated) on 5 consecutive days or on a unique pulse dosing regimen starting on day 25 postimmunization, when all mice showed established arthritis (clinical score >2). In each experiment, a control group of mice was injected intraperitoneally with phosphate buffered saline (PBS; untreated controls).

For adoptively transferred CIA, draining lymph node (DLN) cells were obtained on day 35 postimmunization from untreated or UCN-treated (2 nmoles) arthritic mice, purified, and stimulated in vitro for 72 hours with inactivated CII (20 μg/ml). After stimulation, CD4 T cells were isolated and depleted of CD25+ cells by immunomagnetic selection (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's recommendations, and then injected intravenously (5×10^6 cells) into mice with CIA on day 24 postimmunization. In some experiments, UCN-treated mice with CIA received intravenous injections of neutralizing anti–interleukin-10 (anti–IL-10) polyclonal antibody and/or neutralizing anti–transforming growth factor β (anti-TGFβ) monoclonal antibody or preimmune rat IgG as control Ig (50 μg of antibody per mouse) on 5 alternate days. For CD25+ T cell depletion, DBA/1J mice were treated intravenously with 1 mg of anti-CD25 antibody (clone PC61) 3 days before immunization with CII. Depletion of CD4+,CD25+ T cells in the spleen was >98% at 72 hours and at 7 days, as determined by flow cytometry.

Assessment of arthritis. Two independent examiners (AC and EG-R) who were blinded to the experimental group examined the mice every other day and monitored them for signs of arthritis according to 2 clinical parameters: paw swelling and clinical score. Paw swelling was assessed by measuring the thickness of the affected hind paws with the use of 0–10 mm calipers. Clinical arthritis was graded on a scale of 0–4 according to the following system: grade 0 = no swelling, grade 1 = slight swelling and erythema, grade 2 = moderate swelling and edema, grade 3 = extreme swelling and pronounced edema, and grade 4 = joint rigidity. Each limb was scored, yielding a maximum possible score of 16 per animal.

Histologic analysis. For histologic analysis, mice were killed by cervical dislocation at day 45 after primary immunization and the paws from 4 to 6 animals were randomly collected by two independent experimenters, then fixed in 4% buffered-formaldehyde, decalcified in Bouin liquor DC, embedded in paraffin, and 4-μm sections were stained with H&E or Masson-Goldner trichrome stain. Histopathologic changes were scored in a blinded manner based on cell infiltration, cartilage destruction, and bone erosion parameters as described previously (22).

Neutrophil infiltration into the joint was determined by measuring myeloperoxidase (MPO) activity as previously de-

scribed (23). MPO activity was measured in joint extracts isolated on day 35 postimmunization (see below).

Cytokine determinations. For determination of cytokine levels in joints, protein extracts were isolated by homogenization of joints (50 mg of tissue/ml) in 50 m*M* Tris HCl, pH 7.4, with 0.5 m*M* dithiothreitol and 10 μg/ml of a cocktail of proteinase inhibitors containing phenylmethylsulfonyl fluoride, pepstatin, and leupeptin (Sigma). Samples were centrifuged at 30,000*g* for 20 minutes and stored at −80°C until cytokine determination. Serum samples were collected on day 35 postimmunization, and the levels of anti-CII IgG, IgG1, and IgG2a antibodies were measured by enzyme-linked immunosorbent assay (ELISA) as described previously (22).

Cytokine and chemokine levels in the serum and joint protein extracts prepared 35 days after primary immunization were determined by specific sandwich ELISAs using capture/biotinylated detection antibodies from BD PharMingen (San Diego, CA) and PeproTech (London, UK) according to the manufacturers' recommendations.

Assessment of T cell autoreactive response. On day 30 after primary immunization, the spleen, DLNs, and synovial membrane were harvested. Single-cell suspensions were obtained from spleen and DLNs. Synovial cells were isolated by enzymatic digestion of knee joint synovium as previously described (22). Antigen-presenting cells (APCs) were prepared by T cell depletion of DBA/1J spleen cells using anti-CD3 monoclonal antibody and complement-mediated lysis as described elsewhere (24). T cells were isolated from DLNs and synovial membrane by immunomagnetic selection (Miltenyi Biotech) according to the manufacturer's recommendations.

DLN cells and synovial cells (10^6/ml) were stimulated in complete medium (RPMI 1640 containing 10% fetal calf serum, 2 m*M* L-glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin) with different concentrations of heat-inactivated CII for 48 hours (for cytokine determination) or for 72 hours (for proliferative response). Cell proliferation (expressed as absorbance at 450 nm) was evaluated by using a cell proliferation assay (bromodeoxyuridine) obtained from Roche Diagnostics (Mannheim, Germany).

The cytokine content in culture supernatants was determined by specific sandwich ELISAs as described above. For intracellular analysis of cytokines in DLN and synovial cells, the cells were stimulated with inactivated CII (10 μg/ml) for 8 hours, in the presence of monensin, and then stained with peridinin chlorophyll A protein (PerCP)–labeled anti-CD4 monoclonal antibody for 30 minutes at 4°C, washed, fixed/saponin permeabilized, stained with 0.5 μg/sample of fluorescein isothiocyanate (FITC)–conjugated or phycoerythrin (PE)–conjugated anticytokine-specific monoclonal antibody (BD PharMingen), and analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). To distinguish between monocyte/macrophage and T cell sources, intracellular cytokine analysis was done exclusively in the PerCP-labeled CD4 T cell population.

Alternatively, synovial cells (10^6 cells/ml) isolated from mice with CIA on day 30 after primary immunization were stimulated with inactivated CII (10 μg/ml) in the absence or presence of different concentrations of UCN or CRF. Cytokine levels were determined in supernatants after 48 hours of culture.

To determine the suppressive capacity of regulatory T cells, autoreactive T cells (4×10^5 cells/well) were isolated from DLN cells obtained from mice with CIA as described above and then stimulated with spleen APCs (10^5 cells/well) and inactivated CII (10 μg/ml) in the absence or presence of DLN T cells (5×10^4 cells/well) isolated from untreated or UCN-treated mice with CIA. After 72 hours, we measured the proliferative response and cytokine production of the autoreactive T cells as described above.

Flow cytometric analysis. Synovial and DLN cells isolated on day 30 postimmunization were incubated with various monoclonal antibodies (FITC-labeled anti-CD25, PE-labeled anti-CD45RB, PerCP-labeled anti-CD4; final concentration 2.5 μg/ml) at 4°C for 1 hour. After extensive washing, cells were fixed/saponin permeabilized, incubated for 45 minutes at 4°C with PE-labeled anti–forkhead box P3 (anti-FoxP3), PE-labeled anti-TGFβ, and/or FITC-labeled anti–IL-10 monoclonal antibody (0.5 μg/sample) diluted in 0.5% saponin, and analyzed on a FACSCalibur flow cytometer. We used isotype-matched antibodies as controls, and we used IgG block (Sigma) to avoid nonspecific binding to Fc receptors.

Statistical analysis. All values are expressed as the mean ± SD. The differences between groups were analyzed by Mann-Whitney U test and, if appropriate, by Kruskal-Wallis analysis of variance test.

RESULTS

Decreased incidence and severity of CIA after treatment with UCN. CIA is a murine experimental disease model induced by immunization with CII that shares a number of clinical, histologic, and immunologic features with RA, and it is used as a model system to test potential therapeutic agents (3). In this study, we used the CIA model to examine the effects of UCN.

UCN treatment of mice with established clinical signs of arthritis resulted in a progressive decrease in the incidence and severity of CIA as compared with untreated mice (control), as assessed by the clinical score and paw swelling (Figure 1A). Daily administration of 2 nmoles of UCN for 5 days offered the best protection against disease, although a single injection was enough to significantly ameliorate the pathologic signs of arthritis (Figure 1A). In contrast, the UCN-related peptide CRF did not protect from CIA (Figure 1A).

The therapeutic effect of UCN was dose-dependent, showing an optimal effect with a dosage of ~6 μg per mouse per day (Figure 1B). Because we observed few differences between the 2- and 10-nmole doses, all further experiments used 5 intraperitoneal injections of the 2-nmole dose on consecutive days.

Histopathologic analyses of joints showed that UCN treatment completely abrogated the chronic inflammation of synovial tissue (infiltration of inflammatory cells into the joint cavity and periarticular soft

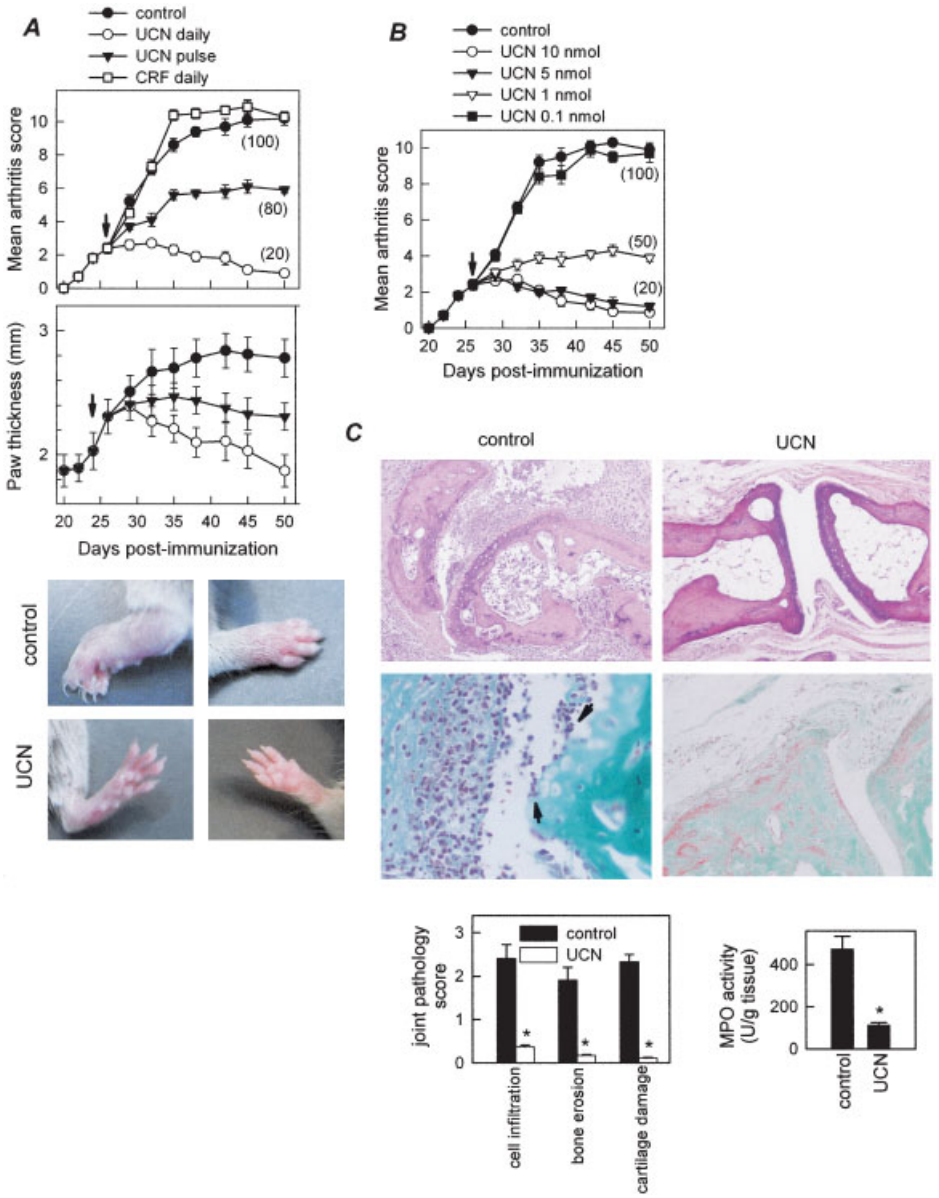

Figure 1. Decreased incidence and severity of collagen-induced arthritis (CIA) in DBA/1J mice after treatment with urocortin (UCN). **A,** Severity of arthritis, as assessed by clinical scoring and paw thickness measurements, in mice with established CIA injected intraperitoneally (arrow) with either phosphate buffered saline (PBS; control), UCN, or corticotropin-releasing factor (CRF) (2 nmoles per mouse) daily for 5 days or with UCN on a unique pulse regimen. Numbers in parentheses represent the incidence of arthritis (percentage of mice with an arthritis score >2 on day 50). Values are the mean ± SD of 8–16 mice per group. Differences were significant at $P < 0.001$ for UCN (both regimens) versus controls after day 32. Representative examples of paw swelling in mice from the control and daily UCN treatment groups are shown at the bottom. **B,** Clinical scores (arthritis incidence) in mice with CIA injected intraperitoneally either with PBS (control) or with different doses of UCN daily for 5 days. Values are the mean ± SD of 8 mice per group. Differences were significant at $P < 0.001$ for 1, 5, or 10 nmoles of UCN versus controls after day 32. **C,** Histologic features of hematoxylin and eosin–stained (top) and trichrome-stained (bottom) sections of joints from mice with CIA treated with PBS (control) or UCN (2 nmoles per mouse; daily for 5 days). Note the osteoclasts destroying bone **(arrows)**. Scoring of inflammation, cartilage damage, and bone erosion, as well as myeloperoxidase (MPO) activity (indicating neutrophil infiltration into the joints) in paws from untreated (control) and UCN-treated mice with CIA are shown at the bottom. Values are the mean and SD of 5 mice per group. $* = P < 0.001$ versus controls.

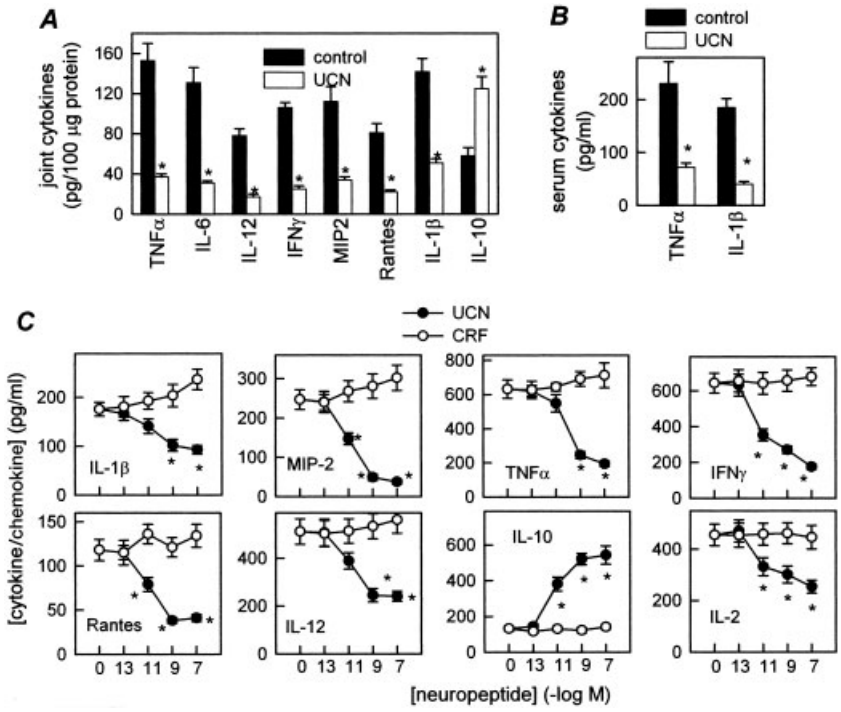

Figure 2. Inhibition of the inflammatory response in DBA/1J mice with collagen-induced arthritis (CIA) by treatment with urocortin (UCN), assessed by systemic and local expression of mediators of inflammation in untreated (control) or UCN-treated mice with CIA on day 35 postimmunization. **A,** Cytokine and chemokine contents in joints. A paw from a nonimmunized mouse was analyzed simultaneously for assessment of the basal response. Values are the mean and SD of 6 mice per group. $* = P < 0.001$ versus controls. TNFα = tumor necrosis factor α; IL-6 = interleukin-6; IFNγ = interferon-γ; MIP-2 = macrophage inflammatory protein 2. **B,** TNFα and IL-1β levels in serum. Values are the mean and SD of 6–8 mice per group. $* = P < 0.001$ versus controls. **C,** Cytokine/chemokine production by synovial membrane cells from mice with CIA activated with type II collagen in the absence or presence of different concentrations of UCN or corticotropin-releasing factor (CRF). Values are the mean ± SD of 4 mice per group (n = 3 experiments, each performed in duplicate). $* = P < 0.001$ versus controls.

tissue, consisting of lymphocytes, plasma cells, macrophages, and neutrophils), pannus formation, cartilage destruction, and bone erosion that are characteristic of CIA (Figure 1C). The UCN-mediated inhibition of neutrophil infiltration was confirmed by the decrease in joint MPO activity. In addition, UCN treatment inhibited the osteoclast-inducing activity observed in the mice with CIA by recruitment of osteoclasts in basic multicellular units that produced focal subchondral bone erosion (arrows in Figure 1C).

No reduction of the therapeutic effects was observed 3 weeks after cessation of UCN treatment (Figure 1A), indicating that after a short period of UCN treatment, no additional neuropeptide is necessary to maintain protection from CIA. In addition, throughout our study, we did not observe any overt toxicity or lethality caused by daily injection of this peptide.

Inhibition of inflammatory response in mice with CIA after treatment with UCN. We next investigated the mechanisms underlying the decreased incidence and severity of CIA following UCN treatment. Several studies have shown a wide array of cytokines and chemokines involved in joint inflammation and arthritis progression (1,2). Since it was recently suggested that UCN is a potent antiinflammatory factor (14–19), we evaluated the effect of UCN on the production of mediators of inflammation that are mechanistically linked to the severity of CIA.

UCN treatment dramatically reduced the production of inflammatory cytokines (TNFα, interferon-γ [IFNγ], IL-6, IL-1α, IL-1β, and IL-12) and chemokines (RANTES and macrophage inflammatory protein 2 [MIP-2]) in the joints of arthritic mice (Figure 2A). In addition, the joints of UCN-treated mice showed in-

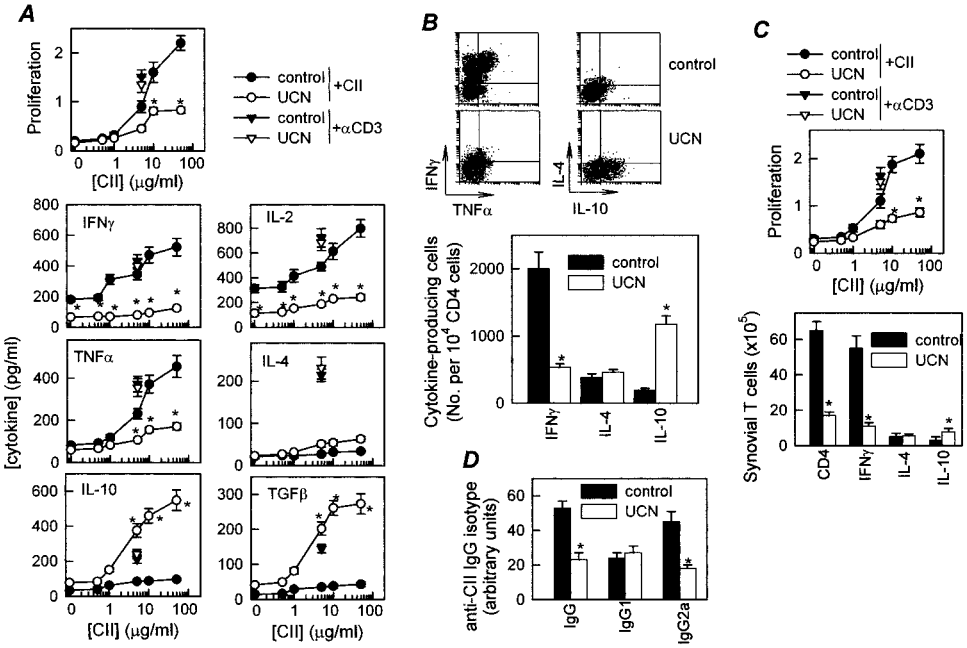

Figure 3. Down-regulation of the Th1-mediated response in DBA/1J mice with collagen-induced arthritis (CIA) by treatment with urocortin (UCN). **A,** Proliferative response of, and cytokine production by, draining lymph node (DLN) cells isolated on day 30 from untreated (control) or UCN-treated mice with CIA and stimulated in vitro with different concentrations of type II collagen (CII). Stimulation of DLN cells with anti-CD3 antibodies (αCD3) was used to assess nonspecific stimulation. A pool of DLN cells from 3 nonimmunized mice was used to assess the basal response. Values are the mean ± SD of 5 mice per group. $* = P < 0.001$ versus controls. IFNγ = interferon-γ; IL-2 = interleukin-2; TNFα = tumor necrosis factor α; TGFβ = transforming growth factor β. **B,** Number of CII-specific cytokine-producing T cells in DLN cells from untreated (control) or UCN-treated mice with CIA. Cells were restimulated in vitro with CII (10 μg/ml) and analyzed for CD4 and intracellular cytokine expression by flow cytometry. Dot plots (top) show representative double staining for IFNγ/TNFα or IL-4/IL-10 expression in gated CD4 T cells. Numbers of T cells expressing IFNγ, IL-4, and IL-10 relative to 10^4 CD4+ T cells are shown at the bottom. Values are the mean and SD of 3 mice per group and are pooled from 2 independent experiments. $* = P < 0.001$ versus controls. **C,** CII-specific proliferative response (top) and number of cytokine-producing CD4+ T cells (bottom) in synovial membrane cells derived from untreated (control) or UCN-treated mice with CIA and then stimulated in vitro with CII (10 μg/ml) for 48 hours. Values are the mean ± SD of 3 mice per group and are pooled from 3 animals per group. $* = P < 0.001$ versus controls. **D,** Levels of CII-specific IgG, IgG1, and IgG2a in serum collected on day 35 from untreated (control) or UCN-treated mice with CIA. Values are the mean and SD of 8–12 mice per group. $* = P < 0.001$ versus controls.

creased levels of the antiinflammatory cytokine IL-10 (Figure 2A). The broad antiinflammatory activity of UCN in the inflamed joint was accompanied by down-regulation of the systemic inflammatory response. UCN decreased CIA-induced serum levels of the proinflammatory cytokines TNFα and IL-1β (Figure 2B).

The decrease in mediators of inflammation observed in the UCN-treated mice with CIA could be a consequence of the diminished infiltration of inflammatory cells in the inflamed joints. However, UCN inhibited the production of proinflammatory mediators by synovial cells isolated from mice with CIA upon restimulation with CII in vitro (Figure 2C). In contrast, CRF slightly stimulated the synovial inflammatory response (Figure 2C). This suggests that, in addition to the reduction in inflammatory infiltration, UCN administration may deactivate the inflammatory response of infiltrating/proliferating synovial cells.

Down-regulation of Th1-mediated autoreactive response in mice with CIA after treatment with UCN. The crucial process underlying disease initiation is the induction of autoimmunity to collagen-rich joint components; later events involve a destructive inflammatory process (1–3). Progression of the autoimmune response

involves the development of autoreactive Th1 cells, their entry into the joint tissues, and future recruitment of inflammatory cells through multiple mediators. In fact, CIA is considered an archetype of Th1-type cell-mediated autoimmune disease (2,3). Therefore, UCN could ameliorate CIA by reducing autoreactive T-cell responses and/or migration to the joints. We therefore determined the proliferation and cytokine profile of DLN cells isolated from UCN-treated arthritic mice in response to CII in vitro.

DLN cells obtained from mice with CIA showed marked CII-specific proliferation and effector T cells that produced high levels of Th1-type cytokines (IFNγ, IL-2, and TNFα) and low levels of Th2-type cytokines (IL-4 and IL-10) (Figure 3A). In contrast, DLN cells from UCN-treated mice proliferated much less and produced low levels of Th1 cytokines and high levels of regulatory cytokines (IL-10 and TGFβ1); levels of the Th2-type cytokine IL-4 were not significantly affected (Figure 3A). This suggests that UCN administration during disease progression partially inhibits clonal expansion of autoreactive Th1 cells.

To determine whether the decrease in Th1 cytokine production induced by UCN treatment is a consequence of either the down-regulation of cytokine release or the inhibition of Th1 cell differentiation, as well as which cells (macrophages or CD4 T cells) are the source of IL-10, we determined the intracellular expression of these cytokines by flow cytometry in sorted CD4 T cells. UCN significantly decreased the number of TNFα/IFNγ-producing Th1 cells and increased the number of IL-10–producing CD4 T cells in DLN cells (Figure 3B). Thus, UCN administration to mice with CIA regulates the generation/differentiation of autoreactive/inflammatory Th1 cells and, presumably, IL-10–secreting Treg cells. We observed a similar effect on synovial cells, where synovial cells from UCN-treated mice with CIA proliferated less than those from untreated animals in response to antigen (Figure 3C, top), and showed fewer IFNγ-producing CD4 Th1 cells and more IL-10–producing T cells (Figure 3C, bottom).

High levels of circulating antibodies directed against collagen-rich joint tissue invariably accompany the development of RA and CIA, and their production is a major factor in determining susceptibility to the disease (25). UCN administration resulted in reduced serum levels of CII-specific IgG, particularly autoreactive IgG2a antibodies, which are generally reflective of Th1 activity (Figure 3D). These data provide further evidence that UCN administration during CIA reduces the Th1 autoreactive responses both in the joint and in the DLNs.

Induction of the emergence of regulatory CD4+,CD25+ T cells in mice with CIA after treatment with UCN. Several studies have indicated that Treg cells confer significant protection against CIA by decreasing the activation and joint homing of autoreactive Th1 cells (4–10). Because UCN treatment also inhibited events in the inflammatory phase of CIA following the activation of antigen-specific Th1 cells and induced the generation of IL-10/TGFβ1–producing T cells, there exists the possibility that UCN induces Treg with suppressive activity during the progression of the disease. UCN-treated mice with CIA had significantly higher percentages and numbers of CD4+,CD25+ cells in both DLNs and synovium compared with control mice with CIA (Figures 4A and B). UCN-induced CD4+,CD25+ cells additionally exhibited a Treg phenotype, i.e., CD45RB^{low}, FoxP3+ (Figures 4A and B).

When stimulated, Treg cells suppress the proliferation of, and IL-2 production by, antigen-specific effector T cells. Several mechanisms of Treg function have been identified, such as surface expression of CTLA-4 and TGFβ, costimulatory blockade, and release of IL-10 and/or TGFβ (26). To determine whether T cells isolated from UCN-treated mice with CIA function as suppressive Treg cells, T cells isolated from DLNs obtained from arthritic mice treated with PBS or UCN were cocultured with autoreactive T cells isolated from DLNs obtained from mice with CIA in the presence of APCs and antigen (CII).

Arthritic mice treated with PBS did not show any suppression of the proliferation of autoreactive T cells and showed a slightly up-regulated production of IL-2 and IFNγ in response to CII (Figure 4C). In contrast, arthritic mice treated with UCN showed suppression of autoreactive T cell proliferation, inhibition of IL-2 and IFNγ production, and increased levels of the regulatory cytokines IL-10 and TGFβ (Figure 4C). The suppression increased with the number of arthritic mice treated with UCN, being effective even at a ratio as low as 1 arthritic mouse treated with UCN to 8 autoreactive T cells (Figure 4D). Flow cytometric analysis of the DLN cells from UCN-treated mice with CIA indicated that ~70% of the IL-10–producing CD4 T cells induced by UCN are FoxP3+ and TGFβ+ (Figure 4E). These results demonstrate that UCN administration during arthritis development induces the generation and/or activation of IL-10/TGFβ1–producing CD4+,CD25+,FoxP3+ Treg that efficiently suppress autoreactive CD4 T cells.

Because UCN seems to induce the in vivo expan-

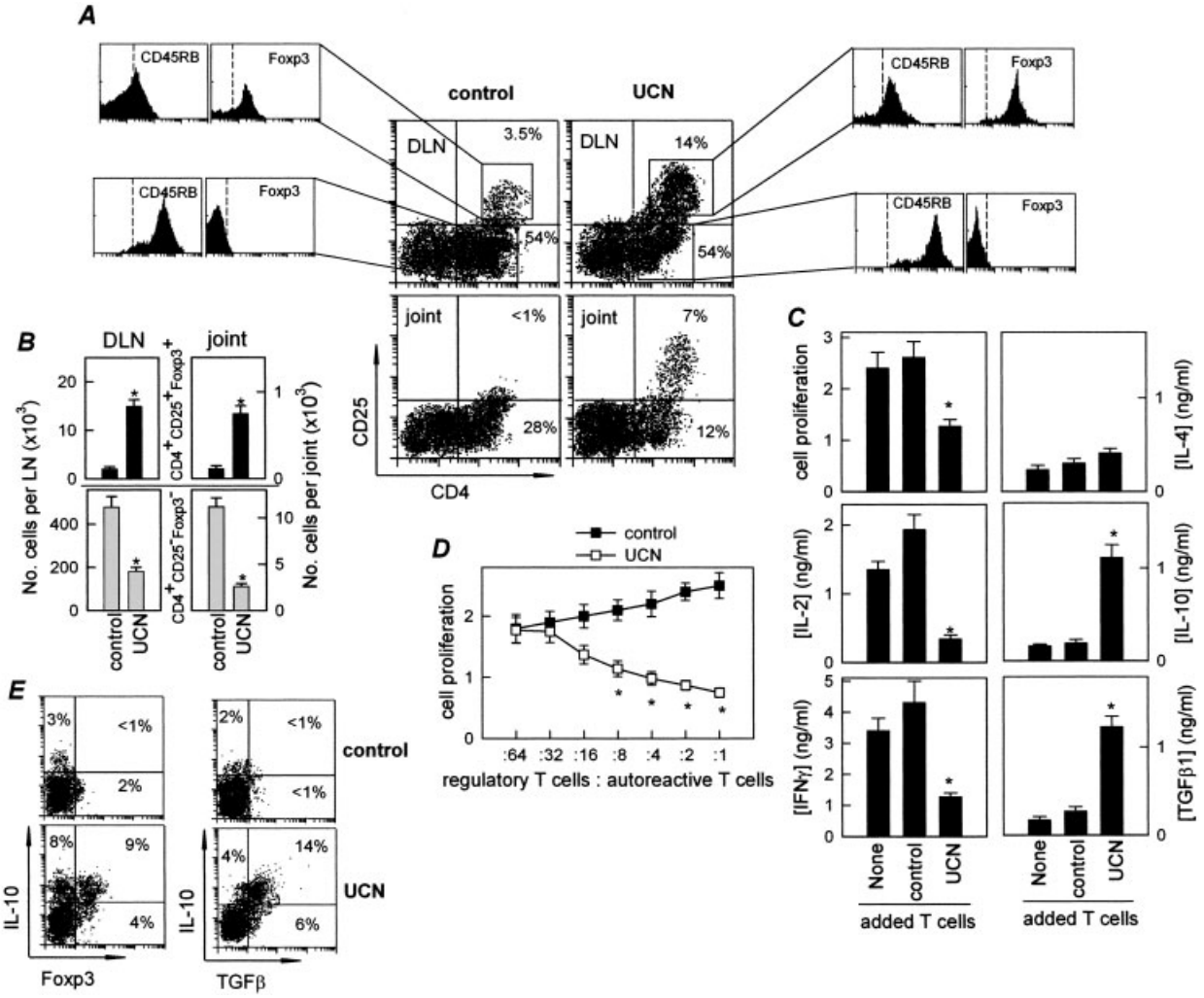

Figure 4. Induction of the emergence of regulatory CD4+,CD25+ T cells in DBA/1J mice with collagen-induced arthritis (CIA) by treatment with urocortin (UCN). **A,** Flow cytometric analysis of draining lymph node (DLN) and synovial (joint) CD4+,CD25+ cells isolated on day 30 from untreated (control) or UCN-treated mice with CIA. Percentages of CD4+,CD25– and CD4+,CD25+ cells are shown in the dot plots. Histograms show the expression of CD45RB and forkhead box P3 (FoxP3) in gated CD4+,CD25– and CD4+,CD25+ cells from DLNs. Similar profiles were observed for synovial cells. Broken vertical lines in histograms indicate antibody isotype controls. Data are representative of 5 mice per group. **B,** Number of CD4+,CD25+,FoxP3+ cells (solid bars) and CD4+,CD25–FoxP3– cells (shaded bars) per DLN and synovial membrane (joint) isolated from untreated (control) and UCN-treated mice with CIA. Values are the mean and SD of 6 mice per group. $* = P < 0.01$ versus controls. **C,** Proliferative response and cytokine production by autoreactive T cells isolated from mice with CIA and then stimulated with spleen antigen-presenting cells (APCs) and type II collagen (CII) (10 μg/ml) in the absence (none) or presence of DLN T cells isolated from untreated (control) or UCN-treated mice with CIA. Values are the mean and SD of 5 mice per group (n = 3 experiments, each performed in duplicate). $* = P < 0.01$ versus controls. IL-2 = interleukin-2; IFNγ = interferon-γ; TGFβ1 = transforming growth factor β1. **D,** Proliferative response of autoreactive T cells (4×10^5 cells) isolated from mice with CIA and then cocultured with increasing numbers of DLN T cells (regulatory T cells) from untreated (control) or UCN-treated mice with CIA (ratios range from 1:64 to 1:1) and then stimulated with CII (10 μg/ml) and splenic APCs. Values are the mean $\pm$ SD of 5 mice per group (n = 3 experiments, each performed in duplicate). $* = P < 0.01$ versus controls. **E,** Flow cytometric analysis of DLN CD4+,IL-10+,FoxP3+ and CD4+,IL-10+,TGFβ+ cells isolated on day 30 from untreated (control) or UCN-treated mice with CIA. Percentages of cells are shown in each quadrant. Data are representative of 4 mice per group.

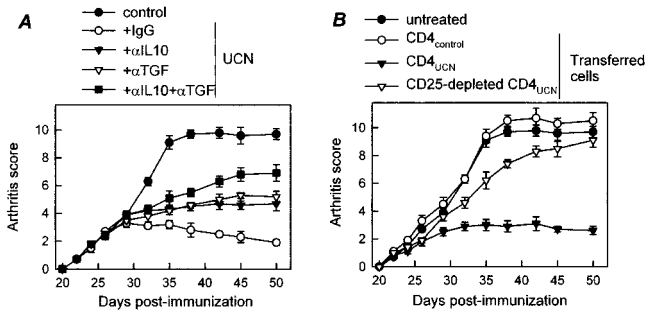

Figure 5. Involvement of T regulatory cells in the therapeutic effect of urocortin (UCN) in DBA/1J mice with collagen-induced arthritis (CIA). **A,** Mice with CIA were treated with medium (control) or with UCN (2 nmoles/day) for 5 days and then were treated with control Ig, anti–interleukin-10 (anti–IL-10 [αIL-10]), anti–transforming growth factor β (TGFβ [αTGF]), or a combination of the 2 antibodies on alternate days starting with the first administration of UCN. Severity of arthritis was assessed by scoring clinical features. Values are the mean ± SD of 5–7 mice per group. Differences were significant at $P < 0.001$ for anti–IL-10 and/or anti-TGFβ treatments versus control Ig treatment after day 37. **B,** CD4+ cells were isolated from draining lymph nodes obtained from mice with CIA treated with medium ($CD4_{control}$) or UCN ($CD4_{UCN}$) at the peak of disease and stimulated in vitro with CII. Some samples were depleted of CD25+ cells before transfer. Cells (5×10^6) were injected intravenously into arthritic mice 2 days after disease onset. Untreated mice with CIA were used as arthritic controls. Values are the mean ± SD of 4–6 mice per group. Differences were significant at $P < 0.001$ for CD4+ T cells from mice treated with UCN versus untreated controls after day 32, as well as for CD4+ T cells from mice treated with UCN versus CD25-depleted CD4+ T cells from mice treated with UCN cells after day 35.

sion of IL-10/TGFβ–producing CD4+,CD25+ Treg cells in mice with CIA, we further examined the role of these cells in the therapeutic action of UCN in arthritis. In vivo blockade experiments showed that treatment with antibodies against IL-10 and/or TGFβ, which are major mediators of Treg function, significantly reversed the therapeutic effect of UCN on arthritis (Figure 5A), suggesting the involvement of Treg cells. In addition, we

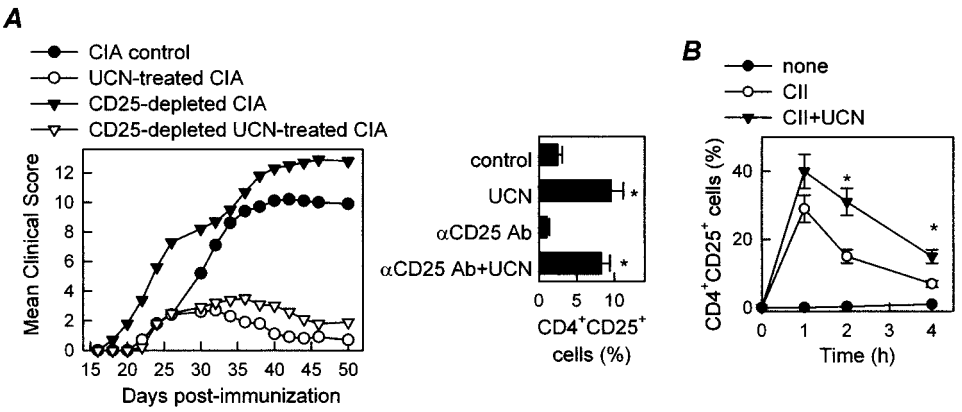

Figure 6. Urocortin (UCN)–induced expansion of peripheral CD4+,CD25+ T regulatory cells from CD4+,CD25– T cells derived from DBA/1J mice with collagen-induced arthritis (CIA). **A,** Clinical scores in mice with CIA. Mice were injected with anti-CD25 antibodies to eliminate CD25+ T cells and, 10 days later, were immunized with type II collagen (CII). UCN treatment was started on day 22 postimmunization. Values are the mean of 4–7 mice per group. Percentages of CD4+,CD25+ T cells in the spleen on day 28 are shown at the right. Values are the mean and SD of 4 mice per group. αCD25 Ab = anti-CD25 antibody. **B,** Numbers of CD4+,CD25+ cells in mice with CIA. Draining lymph node CD4+,CD25– cells isolated from mice with CIA at the peak of disease were cultured with medium (none) or were stimulated with CII (20 μg/ml) in the absence or presence of UCN ($5 \times 10^{-8} M$). Percentages of CD4+,CD25+ cells were determined by flow cytometry at the time points indicated. Values are the mean ± SD of 4 mice per group (n = 4 experiments, each performed in duplicate). $* = P < 0.001$ versus samples treated with CII alone.

assessed the ability of these cells to affect CIA after disease onset. Mice with CIA were treated 2 days after disease onset with CD4+ T cells isolated from arthritic mice treated with medium or UCN. In contrast to CD4+ T cells from arthritic mice treated with medium, injection of CD4+ T cells from mice treated with UCN prevented the development of arthritis, and depletion of CD25+ cells from CD4+ T cells from mice treated with UCN significantly abolished their therapeutic effect (Figure 5B).

CD4+,CD25+ Treg cells can be generated peripherally from CD4+,CD25– T cells (4). To determine whether UCN–induced increase in CD4+,CD25+ Treg cells during CIA is due to the expansion of the existing naturally occurring CD4+,CD25+ Treg cells or to newly generated Treg cells from CD4+,CD25– T cells, mice with CIA were depleted of CD4+,CD25+ T cells by treatment with anti-CD25 antibody before inoculation with UCN. Depletion of CD25+ T cells prior to CIA induction resulted in a more severe disease than that in controls, with earlier onset and higher clinical scores (Figure 6A). In contrast, CD25+ depletion did not affect the beneficial effect of UCN (Figure 6A). At the time of maximum clinical score in the control group (day 35), mice with CIA depleted of CD25+ cells and treated with UCN possessed almost the same number of splenic CD4+,CD25+ T cells as did mice that were not depleted of CD25+ cells but were treated with UCN (10 and 12%, respectively) (Figure 6A, right). This is in contrast to the control arthritic mice that were not treated with UCN (2.3% CD4+,CD25+ cells).

These experiments suggest that UCN could induce the peripheral generation of CD4+,CD25+ Treg cells from the CD4+,CD25– compartment. To further confirm this hypothesis, CD4+,CD25– cells isolated from DLNs obtained from mice with CIA at the peak of disease were stimulated in vitro with CII in the absence or presence of UCN. Subsequent incubation with UCN significantly increased the percentage of CD4+,CD25+ cells (Figure 6B).

DISCUSSION

Certain therapeutic approaches address the autoimmune component of CIA and RA, complementing existing antiinflammatory therapies. In this study, we found that the neuropeptide UCN provides a highly effective therapy for CIA by strikingly reducing the 2 deleterious components of the disease: the autoimmune response and the inflammatory response. UCN treatment decreased the presence of autoreactive Th1 cells in the peripheral circulation and in the joint. In addition, UCN treatment strongly reduced the inflammatory response during CIA progression by down-regulating the production of several mediators of inflammation, such as various cytokines and chemokines in the joints. As a consequence, UCN reduced the frequency of arthritis, ameliorated symptoms, and prevented joint damage. From a therapeutic point of view, it is extremely important to consider that delayed administration of UCN had the ability to ameliorate ongoing disease and that initial treatment with the peptide prevented recurrence of the disease, which fulfills an essential prerequisite for an antiarthritic agent, since in humans, treatment is started after the onset of arthritis.

The capacity of UCN to regulate a wide spectrum of mediators of inflammation might offer a therapeutic advantage over neutralizing antibodies and receptor antagonists directed against a single mediator. Chemokines are responsible for the joint infiltration and activation of various leukocyte populations that contribute to the pathology of CIA (1,2,27). The fact that UCN treatment reduced the expression of a plethora of chemokines could partly explain the absence of inflammatory infiltrates in the joint tissues of UCN-treated mice, being especially relevant for such chemokines as MIP-2 (chemotactic for neutrophils) and RANTES/MIP-1α (for macrophages and T cells), which are involved in the pathogenesis of CIA (28,29).

In addition to regulating cell recruitment into the joint, UCN also regulates inflammatory cell activation and cytokine production. Thus, UCN down-regulated the production of the proinflammatory/cytotoxic cytokines TNFα, IFNγ, IL-6, IL-1β, and IL-12 in the inflamed joint, and increased the levels of the antiinflammatory cytokines IL-10 and TGFβ, which ameliorate the disease (29,30). The decrease in mediators of inflammation could be the consequence of a diminished infiltration of inflammatory cells into the synovium. However, the fact that UCN inhibited the production of proinflammatory mediators by synovial cells from mice with CIA upon in vitro stimulation with CII suggests that, in addition to reducing inflammatory cell infiltration, UCN deactivates the inflammatory response. Recent studies demonstrated that UCN acts as a macrophage-deactivating factor by down-regulating the production of a wide range of mediators of inflammation (16–18). Therefore, it is plausible that deactivation of resident and infiltrating macrophages is a major mechanism involved in the antiinflammatory action of UCN in CIA.

Although macrophages and neutrophils are the major sources of mediators of inflammation, CD4 T cells

play a key role in the initiation and perpetuation of CIA by producing IFNγ, a potent inducer of the inflammatory response (1,2). Our results demonstrate that the administration of UCN to arthritic mice results in a decreased CII-specific Th1-mediated response. It appears that the inhibition of the Th1 response is caused by a direct action on synovial and DLN cells, since synovial and DLN cells obtained from UCN-treated animals were refractory to Th1 cell stimulation. In contrast to the production of IFNγ and TNFα, UCN increased the production of IL-10 and TGFβ1. The inhibition of Th1 cytokines by UCN seems to be independent of its action on macrophages, since UCN directly affected T cells in the absence of APCs. Thus, we have found that UCN inhibits IFNγ production by activated mouse spleen lymphocytes as well as by peripheral blood lymphocytes isolated from healthy subjects and from patients with RA (45%, 50%, and 65% inhibition in mouse lymphocytes, control human lymphocytes, and lymphocytes from arthritic subjects, respectively) (data not shown).

However, the fact that UCN increased the production of IL-10, but not IL-4, in DLN and synovial CD4+ T cells is evidence against a shift toward Th2 responses. IL-10 has recently been recognized as a signature cytokine for a subset of CD4 T cells that exert regulatory functions (31). CD4+,CD25+ Treg cells have been reported to play a critical role in the regulation of autoimmune diseases, including RA and CIA (4–10). Of physiologic relevance is the fact that UCN administration in mice with CIA induced the appearance of CD4+,CD25+,FoxP3+ cells with a Treg phenotype in the DLNs and joints. In addition, the unfractionated CD4 cells isolated from UCN-treated mice showed an important therapeutic effect on CIA, which was significantly abrogated when they were depleted of CD25+ cells. Furthermore, in vivo blockade of the Treg mediators IL-10 and TGFβ partially reversed the therapeutic effect of UCN. This suggests the partial involvement of IL-10/TGFβ–producing CD4+,CD25+ Treg cells in the action of UCN on CIA and shows that delayed administration of UCN inhibits events in the inflammatory phase of CIA following the activation/differentiation of antigen-specific effector Th1 cells.

There are, however, debates about the function of Treg cells in the suppression of established arthritis, indicating the importance of Treg functions. Frey et al (32) reported that Treg was unable to counteract established arthritis. However, Morgan et al (5) demonstrated that adoptive transfer of Treg cells to mice with signs of arthritis prevented the progression of CIA, although they did not affect antigen-specific T cell and B cell responses. The efficiency of inhibition of established arthritis by UCN and UCN-induced Treg cells could be explained in the fact that in addition to expanding the CD4+,CD25+ population, UCN also induces a very efficient Treg population, both in terms of cytokine secretion and suppressive activity. The UCN-induced Treg cells produced high levels of IL-10 and TGFβ1, and on a per cell basis, they were very strong suppressors of responder autoreactive T cell proliferation, particularly at low regulatory T cell to autoreactive T cell ratios. UCN-induced generation of Treg cells during CIA correlates with our data showing that UCN treatment increased the production of IL-10 and TGFβ1, 2 of the major mediators of Treg cells.

The mechanisms involved in the generation/activation of Treg cells by UCN during CIA are not fully understood. Whether UCN acts directly on T cells to induce the generation or expansion of CD4+,CD25+ Treg cells remains to be established. However, the present study shows evidence that they can be peripherally generated from the CD4+,CD25– compartment, since UCN treatment prevented CIA progression in CD25-depleted mice and restored the number of CD4+,CD25+ Treg cells. In addition, UCN converts in vitro–activated antigen-primed CD4+,CD25– cells to CD4+,CD25+ cells. Because TGFβ and IL-10 have been found to be critical for the peripheral generation of CD4+,CD25+ Treg cells (33,34), the UCN-induced increase in both factors could participate in the induction of Treg cells by this neuropeptide. Therefore, the Treg cells induced by UCN in the DLNs and spleen can apparently migrate to the joints and induce local suppression of the self-reactive response.

At this point, the obligatory question is, How does UCN regulate such a wide spectrum of inflammatory and immunomodulatory mediators? The answer to this question may lie in the fact that the cAMP/protein kinase A pathway is the major intracellular signal involved in most of the effects of UCN, including down-regulation of inflammatory mediators (16). Agents that induce cAMP have been found to be potent antiinflammatory factors, inhibitors of Th1 response, and inducers of Treg function (35). They act by down-regulating the activation of the transcription factor NF-κB, a factor essential for the transcriptional activation of most of the inflammatory cytokines, chemokines, and costimulatory factors. We have recently shown the inhibitory effect of UCN in the NF-κB pathway on activated macrophages (Gonzalez-Rey E, et al: unpublished observations).

UCN is structurally related to CRF and is an endogenous ligand of CRF receptors. Depending on the

site of secretion, both proinflammatory and antiinflammatory actions have been attributed to CRF. Both physical and psychological stresses induce the central and peripheral expression of CRF and UCN. Central secretion of CRF activates the hypothalamus–pituitary–adrenal axis that ultimately induces the release of corticosteroids, which mediate the antiinflammatory effects of central CRF (36,37). In contrast, peripherally secreted CRF is largely proinflammatory, especially in the gastrointestinal tract (38–40). In contrast to CRF, the antiinflammatory effect of UCN on systemic inflammation and experimental autoimmune encephalomyelitis is corticosteroid-independent (16,41). The present study and other evidence suggest that UCN at sites of inflammation may counteract the proinflammatory effects of CRF. Interestingly, UCN preferentially binds to the type 2 CRF receptor with higher affinity than does CRF, and the distribution of UCN, but not CRF, correlates with the distribution of the type 2 CRF receptor in the immune cells (12,13).

Of physiologic relevance is the observation that the expression of UCN is increased in activated inflammatory cells, especially under several inflammatory conditions, including RA (14,19–21,41). Therefore, it is attractive to speculate that the body responds to an exacerbated inflammatory response by increasing the peripheral production of endogenous antiinflammatory factors, such as UCN, in an attempt to restore the inflammatory homeostasis. It is important to note that UCN has been tested in human subjects (42,43). Therefore, UCN should be well tolerated at doses similar to those that are able to prevent CIA. In addition, the fact that UCN does not completely block the inflammatory/immune response and does not induce a general immunosuppressive state is an advantage over other agents used to treat RA, which show immunosuppressive-associated side effects.

In summary, this study identified UCN as an immunomodulatory factor with the capacity to deactivate the inflammatory response in vivo at multiple levels and to maintain immune tolerance. Our findings provide a powerful rationale for studies assessing the efficacy of UCN as a novel therapeutic approach to the treatment of RA and other chronic inflammatory diseases and autoimmune disorders.

AUTHOR CONTRIBUTIONS

Drs. Gonzalez-Rey and Delgado had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Drs. Gonzalez-Rey and Delgado.

Acquisition of data. Drs. Gonzalez-Rey, Chorny, Varela, O'Valle, and Delgado.

Analysis and interpretation of data. Drs. Gonzalez-Rey and Delgado.

Manuscript preparation. Drs. Gonzalez-Rey and Delgado.

Statistical analysis. Dr. Varela.

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