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Dendritic cells (DCs) have a dual ability to either stimulate or suppress immunity, which is primarily associated with the expression of costimulatory molecules. Ag-loaded DCs have shown encouraging clinical results for treating cancer and infectious diseases; however, the use of these cells as a means of suppressing immune responses is only recently being explored. Here, we describe the induction of RNA interference through administering short interfering RNA (siRNA) as a means of specifically generating tolerogenic DCs. Knockdown of CD40, CD80, and CD86, prior to loading DCs with the arthritogenic Ag collagen II, led to a population of cells that could effectively suppress onset of collagen-induced arthritis. Maximum benefits were observed when all three genes were concurrently silenced. Disease suppression was associated with inhibition of collagen II-specific Ab production and suppression of T cell recall responses. Downregulation of IL-2, IFN-γ, TNF-α, and IL-17 and increased FoxP3+ cells with regulatory activity were observed in collagen-induced arthritis mice treated with siRNA-transfected DCs. Collectively, these data support the use of ex vivo gene manipulation in DCs using siRNA to generate tailor-made tolerogenic vaccines for treating autoimmunity. The Journal of Immunology, 2010, 184: 6457–6464.

Treatment of Autoimmune Arthritis Using RNA Interference-Modulated Dendritic Cells

Xiufen Zheng,*†‡ Motohiko Suzuki,*†‡ Thomas E. Ichim,§ Xusheng Zhang,*†‡ Hongtao Sun,*†‡ Fei Zhu,¶ Aminah Shunnar,*†‡ Bertha Garcia,*†‡ Robert D. Inman,§ and Weiping Min*†‡¶§#

Dendritic cells (DCs) offer an ideal platform for Ag delivery given their natural role in acting as a gatekeeper for controlling the adaptive immunity. In physiological situations, DCs reside in an immature state, presenting Ags to T cells in absence of costimulation, therefore maintaining tolerance (1). In conditions associated with inflammation or tissue damage, DC maturation occurs, which is characterized by upregulation of costimulatory molecules and activation of T cells recognizing the Ag presented by DCs (2). This balance between tolerance and immunity has allowed researchers to use DCs for various types of immunotherapy. For example, tumor Ags delivered to DCs in the form of RNA (3), DNA (4), peptides (5), or even protein transduction domain peptides have been used to induce tumor immunity in both mice and humans (6). Phase III clinical trials are currently underway for DCs pulsed with PAP-GM-CSF fusion protein (7), and in Sweden, autologous DCs have been used to induce tumor immunity in both mice and humans (6). Despite the clinically advanced use of DCs for therapeutic induction of immunity, relatively little work has been performed in the area of Ag-specific immune regulatory therapy. Perhaps one reason for this is the relative ease of endowment of DCs with immune stimulatory properties versus generation of immune inhibitory DCs.

Creation of immunosuppressive DCs has been achieved by chemical, tissue culture, and genetic engineering methods (9, 10). One common feature of immune regulatory DCs appears to be reduced expression of costimulatory molecules. NF-κB–inhibited DCs, whether by proteasome inhibition (11), decoy oligonucleotides (12), or IKK suppression (13), all inhibit immune responses and are all associated with reduced costimulatory molecule expression. Another means of DC manipulation is treatment with 1α,25-dihydroxyvitamin D3 (calcitriol), which has been demonstrated to cause immune modulation by generating DC populations with an immature phenotype and ability to activate regulatory T cells (Tregs) (9, 14). Mechanistically, calcitriol also has been shown through mediate effects through inhibition of NF-κB (15). We have previously demonstrated that RNA interference may be evoked in DCs with short interfering RNA (siRNA) without induction of DC maturation or alteration of viability (16–18). These findings show that suppression of genes such as IL-12 p35, CD40, and RelB could endow DCs with the ability to inhibit immune responses both in vitro and in vivo (17, 18). For example, administration of DCs pulsed with the model Ag OVA followed by subsequent silencing of IL-12 p35 led to a Th2 shift in Ag-specific recall response (16). Thus, the possibility of using siRNA to silence selective genes on Ag-bearing DCs could be an ideal method of generating cellular therapies for immune modulation.

We and others have previously reported that the state of transplantation tolerance is associated with DCs expressing low levels of costimulatory molecules resembling an immature state (13, 19). Although there are data supporting CD80 and CD86 in terms of the qualitative state of T cell activation, the role of CD40 is widely recognized as essential for stimulating T cell-mediated immunity. The suppression of these molecules is believed to inhibit T cell activation and in many cases will instead stimulate Tregs to inhibit immune responses in an Ag-specific manner (13, 19).
Rheumatoid arthritis (RA) is an example of a condition in which modulation of the T cell compartment may be highly beneficial. This notion is supported by studies in which depletion of Tregs accelerates disease progression in the collagen-induced arthritis (CIA) model (20) as well as clinical data in which responses to anti–TNF-α therapy are associated with augmentation of Tregs (21). Thus, it would be ideal to generate Tregs that act in an Ag-specific manner. Some previously attempted methods included vaccination with modified peptides, i.e. administration of Ag, or mucosal delivery (22). Because we have previously used immature DCs to inhibit CIA progression (23), we sought to extend these findings to a system in which expression of specific costimulatory molecules may be knocked down. We observed that gene silencing of CD40, CD80, and CD86 achieved optimal protection from disease and induced Ag-specific Tregs in CIA mice. Collectively, the data presented here support the feasibility of gene-silenced DCs as a means of suppressing immune responses in autoimmunity.

Materials and Methods

Animals

Male DBA/1 LacJ and BALB/c mice (The Jackson Laboratory, Bar Harbor, ME), 5 wk of age, were kept in filter-top cages at the Animal Care and Veterinary Services Facility at the University of Western Ontario according to the Canadian Council for Animal Care Guidelines. Mice were fed by food and water ad libitum and allowed to settle for 2 wk before initiation of experimentation, which had ethical approval from the university review board.

CIA model

DBA/1 LacJ mice, 7 wk of age, were intradermally immunized (day 0) at several sites into the base of the tail with 200 μg bovine type II collagen (CII) (Sigma-Aldrich, St. Louis, MO) dissolved in 100 μl 0.05 M acetic acid and mixed with an equal volume of CFA (Sigma-Aldrich, St. Louis, MO). CII was dissolved at a concentration of 2 mg/ml by stirring overnight at 4°C. On day 21 after priming, the mice received an i.p. booster injection with 200 μg CII in an equal volume (100 μl) of PBS. Mice were examined visually three times per week for the appearance of arthritis in the peripheral joints, and the arthritis score index for disease severity was given as follows: 0, no evidence of erythema and swelling; 1, erythema and mild swelling confined to the midfoot; 2, erythema and mild swelling extending from the ankle to the midfoot; 3, erythema and moderate swelling extending from the ankle to the metatarsals; 4, erythema and severe swelling encompassing the ankle, foot, and digits. Scoring was performed by two independent observers, without knowledge of the experimental and control groups.

Generation of bone marrow-derived DCs

DCs were generated from bone marrow progenitor cells, as previously described (24). Briefly, bone marrow cells were flushed from the femurs and tibias of DBA/1 LacJ mice (The Jackson Laboratory) and then washed and cultured in six-well plates (2 × 10^6 cells per milliliter) in 4 ml complete medium: RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg streptomycin, 50 μmol/l 2-ME, and 10% FCS (all from Invitrogen Life Technologies, Burlington, Ontario, Canada), supplemented with 10 ng/ml recombinant GM-CSF (PeproTech, Rocky Hill, NJ) and 10 ng/ml recombinant mouse IL-4 (PeproTech). All of the cultures were incubated at 37°C in 5% humidified CO2. Nonadherent granulocytes were removed after 48 h of culture, and fresh medium was added. DCs were cultured for 6 d (immature DCs) or for 8 d after being activated with LPS (100 ng/ml) for 24 h (mature DCs).

siRNA synthesis and transfection

siRNA sequences targeting CD40, CD80, and CD86, respectively, were selected in accordance with the method described by Elbashir et al. (25). The sequences of siRNA were as follows: CD40, 5'-TGTTCACTGGGCGTGAA-3'; CD80, 5'-TATTGATGGCCAGAATCTC-3'; CD86, 5'-ATAATTGATCCGTTGCGG-3'. siRNA was synthesized and annealed by the manufacturer (Dharmacon, Lafayette, CO). siRNA specific to the Luciferase gene GL2 Duplex (Dharmacon) was used as a sham silencing control. Transfection was carried out as described previously (16). Briefly, 60 pmol annealed siRNA was incubated with 5 μl GeneSilencer (Gene Therapy Systems, San Diego, CA) in a volume of 100 μl RPMI 1640 (serum-free) at room temperature for 30 min. This then was added to 400 μl DC culture, as described above. After 4 h of incubation, an equal volume of RPMI 1640, supplemented with 20% FCS, was added to the cells. A total of 24–48 h later, transfected DCs were washed and used for subsequent experiments.

Quantitative RT-PCR

Total RNA was extracted from cells using TRIzol (Invitrogen). A total of 3 μg total RNA was used to synthesize the cDNA with oligo(dT) and reverse transcriptase (Invitrogen) in a reaction volume of 20 μl. Primers used for the amplification of murine CD40, CD80, CD86, and GADPH were as follows (26): CD40, 5'-GATTGGCGACGCCAGGAAGC-3' (forward) and 3'-CCCTGAGTGTTGACACGTTC-3' (reverse); CD80, 5'-GCCTCCGT-TTCTTTGTTGTG-3' (forward) and 5'-TATGGCGCGGATTCCGTG-3' (reverse); CD86, 5'-GATGTCGACAAGGCGCAGATC-3' (forward) and 5'-TAAAAACAAAGCGAGACCAATAGA-3' (reverse); GADPH, 5'-TGAT-GACATCAAGAAGGGTGTAAG-3' (forward) and 5'-TGAGGATGGAAATTTGTGAGGAGAT-3' (reverse). Quantitative PCR (qPCR) was performed on a MX 4000 PCR Instrument (Stratagene, Cedar Creek, TX) with SYBR Green as per the manufacturer (Stratagene). qPCR data were normalized to GADPH.

CII-specific T cell response

T cell proliferative responses to CII in subsequent groups of mice were measured with a standard microtiter assay. After CII immunization, the proliferative responses could be detected for several weeks. Immune cells from either draining lymph node or spleen T cells collected from the mice treated with CII and siRNA-silenced DCs or control DCs at 5 × 10^6 cells per well were seeded into a 96-well flat-bottom microplate (Corning Glass, Corning, NY) in triplicate and mixed with serial dilutions of CII with concentrations ranging from 5 to 50 μg per well. After 72 h incubation, 1 μCi [3H]thymidine (Perkin Elmer, Woodbridge, Ontario, Canada) was added to each well for 18 h. With an automated cell harvester, the cells were collected onto glass microfiber filter, and the radioactive labeling incorporation was measured by a Wallac Betaplate liquid scintillation counter.

CD4*CD25* cell isolation

For splenic and lymph node T cell isolation, a nylon strainer with a pore size of 0.2 mm was used for mechanical dissociation. Mononuclear cells were purified by Ficoll-Paque gradient separation (Amersham Pharmacia Biotech, Quebec City, Quebec, Canada). CD4*CD25* T cells were isolated using a CD4*CD25* Regulatory T cell Isolation Kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instructions.

Inhibitory T cell response

CD4*CD25* T cells were isolated from spleens and lymph nodes of CIA mice and added at increasing numbers to a CII-specific T cell response, as described above. The culture was continued for 3 d at 37°C with 5% CO2. H-labeled thymidine was added to the culture at the last 18 h. With an automated cell harvester, the cells were collected onto a glass microfiber filter, and the radioactive labeling incorporation was measured to assess T cell proliferation by a Wallac betaplate liquid scintillation counter. The inhibitory function of Treg cells was measured by the reduction of T cell proliferation.

Anti-CII Ab measurement

CII-specific Abs were evaluated using a standard indirect ELISA in which 500 ng CII was absorbed to each well of a 96-well microtiter plate. After blocking and washing steps, serial dilutions of immune serum were added to the appropriate wells in duplicate and incubated overnight at 4°C. Dilutions of serum were 1:100–1:10,000. To develop the ELISA, HRP-conjugated goat anti-mouse IgG Fc and ortho-phenylenediamine dihydrochloride substrate buffer (Sigma-Aldrich) were used. Finally, an OD in each well was measured at a wavelength of 490 nm in an ELISA plate reader.

Cytokine quantification

T cells isolated from the CIA mice treated with gene-silenced DCs were cultured in the presence of Ag CII for 48 h. The supernatants were collected and assessed for T cell cytokines (IFN-γ, IL-2, IL-17, and IL-4) by ELISA. Cytokine-specific ELISA (Endogen, Rockford, IL) was used for detecting cytokine concentrations in culture supernatants according to the manufacturer’s instructions using a Benchmark Microplate Reader (Bio-Rad, Hercules, CA). TNF-α from serum was detected by ELISA using mouse TNF-α Ready-Set-Go kit as per the manufacturer’s instructions (eBioscience, San Diego, CA).

Histology

Paws from experimental and control groups of freshly dissected mice were removed, and joint tissues were immersion-fixed in 10% w/v neutral buffered formalin in 0.15 M PBS (pH 7.4). After decalcification by...
immersion in Decalcifier I solution (Surgipath, Winnipeg, Manitoba, Canada) overnight and subsequent dehydration in a gradient of alcohols, tissues were rinsed in running water. The specimens were processed for paraffin embedding in paraplast (ThermoFisher Scientific, Waltham, MA) as a routine procedure. Serial paraffin sections throughout the joint were cut at a thickness of 5 μm on a microtome, heated at 60°C for 30 min, and deparaffinized. Hydration was done by transferring the sections through the following solutions: xylene for 6 min three times, then 100% ethanol for 2 min twice, 95% ethanol, and 70% ethanol. Sections were stained with H&E and mounted on glass slides.

**Flow cytometry**

DCs were collected and stained with specific Abs to CD11C, CD40, CD80, CD86, programmed death 1 ligand (PD1-L), and inducible T cell costimulator-ligand (ICOS-L) for 30 min at 4°C. All of the Abs were purchased from eBioscience. The cells were washed and analyzed by flow cytometry (BD Biosciences, San Jose, CA).

T cells were stained with Cy5-, PE-, or FITC-conjugated anti-mouse CD4, CD25, and Foxp3 (eBioscience) mAb, respectively. Foxp3 expression was assessed by intracellular staining using a cell permeabilization kit (eBioscience). All of the flow cytometric analyses were performed using appropriate isotype controls (Cedarlane Laboratories, Hornby, Ontario, Canada).

**Statistical analysis**

Data are expressed as mean ± SEM. Differences between different groups of mice were compared using one-way ANOVA for nonparametric data and linear regression for the disease score data. A p value < 0.05 was considered significant.
Results
Validation of gene silencing in DCs

To validate the efficacy of gene silencing, we used bone marrow-derived DCs cultured for 6 d and transfected them with siRNA specifically targeting CD40, CD80, and CD86 molecules using GeneSilencer. Twenty-four hours after gene silencing, expression of CD40 (Fig. 1A), CD80 (Fig. 1B), and CD86 (Fig. 1C) was evaluated at the transcription level using quantitative RT-PCR (left panel) and at the protein level by FACS (right panel). Although CD40 was almost completely knocked down, CD80 and CD86 were partially inhibited by siRNA. Additionally, the cell viability was not affected by gene silencing (data not shown). Knocking down of CD40, CD80, and CD86 on DCs did not affect gene expression of other costimulatory molecules such as ICOS-L (Fig. 1D) or PD1-L (Fig. 1E). The costimulatory molecules CD40 and CD80/86 are known to act as secondary signals for activating naïve T cell responses. To assess alterations of APC function of DCs after gene silencing, we performed a MLR using CD40 or CD80/CD86 gene-silenced DCs as stimulators. DCs treated with CD40 or CD80/CD86 siRNA exhibited significantly decreased allostimulatory activity as compared with that of control siRNA-silenced DCs (Fig. 1F), suggesting that gene-silenced DCs possess impaired APC function. Collectively, these data demonstrate that siRNA inhibition of costimulatory molecules is feasible with bone marrow DCs and correlates with suppressed allostimulatory activity.

Treatment of CIA by gene-silenced DCs

CIA is a well-established animal model of autoimmune arthritis that displays similar onset, progression, and pathology to clinical RA. Additionally, the autoantigen in CIA is known to be CII, and T cell responses to this protein are essential for disease progression. We have previously reported a clinically applicable intervention for CIA by adoptive transfer of tolerogenic DCs generated in vivo in which costimulatory molecules are expressed at low levels as a result of treatment with an IKK inhibitor (23). Given that chemical manipulation may induce various nonspecific effects on DCs, we sought to extend these observations of tolerance induction by reducing costimulatory molecule expression using siRNA. To test this, mouse CIA was established by two injections of CII Ag, were administrated before clinical disease was apparent. As shown in Fig. 2A, mice treated with control siRNA-transfected DCs or mature DCs developed severe arthritis, whereas mice treated with immature DCs showed mild disease. Simultaneous silencing of CD40 and CD80/86 achieved a synergistic effect of treatment, as demonstrated by the lower clinical score observed in the group treated with multigene-silenced DCs compared with that of the group treated with single-gene–silenced DCs (Fig. 2A).

We also assessed arthritic inflammation by measuring the circumference of joints with arthritis. Although CIA mice show inflammatory reactions with joint effusions, treatment with gene-silenced DCs significantly decreased the intensity of swelling (Fig. 2B).

To confirm the therapeutic effects of gene-silenced DCs, we further sought to examine microscopic histological differences after treatment of CIA mice with the gene-silenced and CII-pulsed DCs. Animals treated with siRNA-silenced DCs or control DCs were sacrificed 4 wk following the onset of arthritis, and joints were examined by serial sectioning. We observed that control mice possessed severe bone erosion, pannus formation, and synovitis (Fig. 2C). A marked neutrophil and mononuclear cell infiltration was seen. In contrast, joint histology of the mice treated with gene-silenced DCs revealed a significant attenuation in morphological changes and cellular infiltration, and normal cartilage structure was preserved (Fig. 2C).

Reduced production of Ab response to CII and inflammatory cytokines in arthritic mice

The importance of CII-specific Abs in the development of CIA pathology is well-known (23). However, to our knowledge, control
of Ab responses with DCs has not been previously examined in a therapeutic sense. Tolerogenic DCs may directly block Ab production through inhibition of BLYs and APRIL, factors that the DCs use to directly induce Ig production and class switching in B cells (27, 28). Alternatively, tolerogenic DCs may indirectly prevent Ab production through the inhibition of T cell helper function. We assessed Ab responses to CII in animals 4 wk after arthritis onset. With a titration experiment, we compared the serum levels of anti-CII Ig from control mice with CIA and the mice treated with DCs after single-silencing of CD40, CD80/86, or in combination of siRNA. In contrast to the control CIA mice, which were treated with control siRNA-transfected DCs or mature DCs and showed high levels of CII-Ab, the treatments with gene-silenced DCs or immature DCs resulted in remarkable suppression of CII-specific Ab production (Fig. 3A).

TNF-α plays an important role in RA pathogenesis (29, 30). Because we have observed that the mice treated with CD40/CD80, CD86-silenced DCs demonstrated less inflammation in the joints (Fig. 2B), we next determined inflammatory cytokine TNF-α secretion in the blood. The levels of TNF-α in blood from the mice treated with CD40-silenced DCs, CD80/86-silenced DCs, or combination were significantly decreased in comparison with those of control mice (Fig. 3B).

**Immunomodulation induced by gene-silenced DCs**

We have previously demonstrated that silencing the expression of IL-12 p35 in DCs leads to in vivo immune modulation of T cell response (16). More recently, we showed that silencing the RelB gene in DCs results in maturation arrest and a decreased expression of costimulatory molecules, subsequently inducing immune tolerance in a transplantation model (17). Thus, we postulated that silencing costimulatory molecules may induce DC-mediated Ag-specific suppression of autoimmune response. To test this, CII-specific T cell responses were determined using splenocytes (Fig. 4A) or lymph node cells (Fig. 4B) from the CIA mice. A strong CII-specific T cell response was present 4 wk after CIA onset. Inhibited recall responses were observed in mice treated with CD40- or CD80/86-silenced DCs (Fig. 4A). Furthermore, multi-gene silencing with a mixture of siRNAs demonstrated an additive effect in inhibiting T cell responses.

**Inhibition of Th1 and Th17 by gene-silenced DCs**

It has been demonstrated that DCs are the key determinant of Th polarization (10). Fully mature DCs that provide strong costimulation to T cells initiate a Th1 response (31). We postulated that knockdown of costimulatory molecules using siRNA may affect DC-mediated Th1/Th2 differentiation. To assess this, we detected IFN-γ, IL-2, and IL-4 produced by the T cells in response to CII from CIA mice. The levels of IFN-γ (Fig. 5A) and IL-2 (Fig. 5B) in the T cells from the CIA mice that were treated with CD40, CD80/86, or combination siRNA were decreased, as compared with those of the T cells from control CIA mice. In contrast, IL-4, a Th2 cytokine, was increased in the T cells from the CIA mice treated with gene-silenced DCs (Fig. 5C).

Th17 differentiation is associated with autoimmune arthritis (32). It has been demonstrated that inhibition of IL-17 or upstream cytokines such as IL-21 is effective at preventing CIA (33) and that both TGF-β and IL-21 are required for development of the pathogenic Th17 phenotype (34). To determine whether gene-silenced DCs may interrupt Th17 differentiation, we detected the levels of IL-17 produced by T cells isolated from CIA mice reconstituted in vitro with CII. Although T cells from control CIA mice produced high levels of IL-17, mice treated with gene-silenced DCs showed inhibited IL-17 secretion from T cells stimulated with CII Ag in vitro (Fig. 5D). These data suggested that costimulatory gene-silenced DCs suppress Th1 and Th17 cytokine production, which may contribute to the therapeutic effects in autoimmune arthritis.

**FIGURE 3.** Inhibition of CII-specific Ab production and suppression of TNF-α in arthritic mice following injections with siRNA-transfected DCs. A, Inhibition of CII-specific Ab production by gene-silenced DCs. Blood was taken 4 wk after arthritis onset from control CIA mice or from the mice treated with DCs in which CD40 or CD80/86 was silenced alone or in combination. Serum levels of anti-CII Ig Fc were determined using ELISA. *p < 0.05; **p < 0.01 versus groups treated with control siRNA-transfected DCs or mature DCs.

**FIGURE 4.** CII-specific T cell responses in arthritic mice treated with siRNA-transfected DCs. A and B, Four weeks after arthritis onset, splenocytes (A) and lymph node cells (B) were isolated from control CIA mice or from the mice treated with CD40- or CD80/86-silenced DCs. CII-specific T cell responses were performed as described in Materials and Methods. T cells were restimulated in vitro with different concentrations of CII, and [3H]-labeled thymidine incorporation was measured. Results represent one of three similar experiments (n = 4 per group per experiment). *p < 0.05; **p < 0.01 versus groups treated with control siRNA-transfected DCs or mature DCs.
Generation of Tregs by gene-silenced DCs

Our previous studies showed that immature DCs with low expression of costimulatory molecules (19) had an ability to generate Tregs. We hypothesized that knockdown of costimulatory molecules CD40 or CD80/86 using siRNA might promote generation of Tregs. To test this hypothesis, we used a previously established in vitro Treg generation system (19) by coculturing siRNA-silenced DCs with allogeneic T cells for 7 d. CD40- or CD80/CD86-silenced DCs generated more Tregs in comparison with the control siRNA-silenced DCs (Fig. 6A). Siencing costimulatory molecules CD40 or CD80/86 resulted in synergistic effects for generating Tregs, highlighting that gene-silenced DCs may promote Treg generation in vivo.

To test the inhibitory function of Tregs, we isolated CD4+CD25+ cells and added them into a CIA-specific T cell response. The T cell reaction was inhibited by CD4+CD25+ cells but not by CD4+CD25- cells in a dose-dependent manner (Fig. 6B).

Discussion

Ag-specific immunotherapy offers the possibility of selectively inhibiting pathological T cell clones while sparing the patient the adverse effects associated with systemic immune suppression. Although current proteomic/serological techniques offer novel means of identifying autoantigens (36, 37), relatively few methodologies exist for inducing Ag-specific tolerance once the autoantigen is defined. Previous attempts to induce tolerance in autoimmunity using oral delivery of Ags, or i.v. tolerance, have been marked by successes in Phase II trials but failure in double-blind Phase III trials (22). We sought to expand on our previous studies in which chemical manipulation of DCs was able to generate a cellular population capable of suppressing initiation and onset of CIA when pulsed with CII (23). The previous studies used DCs treated with LF 15-0195, a small molecule inhibitor of IKK (13), which blocks DC maturation and upregulation of costimulatory molecules. In the current studies, we used siRNA to selectively inhibit costimulatory molecule expression on DCs prior to pulsing with Ags. We observed that concurrent inhibition of CD40, CD80, and CD86 was able to induce maximal suppression of DC immunogenicity as well as delay onset of joint pathology. Results were comparable, if not superior, to those of our previous publication using LF 15-0195–inhibited DCs (23) in terms of pathology, as well as suppressing T cell recall and Ab responses.
These data support the feasibility of using siRNA as a means to create a population of DCs capable of eliciting a therapeutic effect. Manipulation of DCs for immune regulation is conventionally considered difficult because immature DCs tend to mature upon exposure to a variety of factors that are difficult to control. For example, factors associated with ongoing inflammation or autoimmunity such as mild temperature elevation (38), acidity (39), uric acid production by dying cells (40), or extracellular matrix fragments (41, 42) have all been demonstrated to induce DC maturation and augment immunogenic functions. Thus, any or all of these factors could theoretically exacerbate an autoimmune response instead of inhibiting it in an in vivo situation. In our studies, we demonstrated that administering siRNA-modified Ag-pulsed DCs led to generation of cells exhibiting a Treg-like phenotype. Conceptually, these cells may go on to establish a self-promoting immune regulatory feedback loop in which the generated Tregs inhibit DC maturation. We originally observed this phenomenon in cardiac transplant tolerance achieved by 2 wk cover of concurrent T cell and DC manipulation (19). The ability of Tregs to inhibit DC maturation, thus shifting the systemic APC compartment toward tolerance, has been demonstrated in several other systems. In the nonobese diabetic mouse model, DCs have been shown to remain in a systemically immature state in the presence of Tregs, a state that can be reversed by removal of the Tregs or ligation of CD40 on the DCs (43). Subsequent studies using two-photon laser-scanning microscopy provided visualization of Tregs “swarming” the DCs and blocking the DCs from activating Th cells (44). A murine model of autoimmune encephalomyelitis also demonstrated this Treg inhibition of DC–Th cell interaction (45). Thus, it may be possible to prevent subsequent maturation of DCs in RA by promoting generation of Tregs.

Various “tolerogenic adjuvant” approaches may enhance the observed anti-autoimmune effects. For example, anti–TNF-α–based therapies have been demonstrated to reduce the systemic and local inflammatory responses observed in RA (46). It is theoretically possible that by reducing the “danger signal” threshold, anti-TNF agents may synergize with siRNA-modified DCs to stimulate Treg production. Supporting this notion are observations that patients responding to infliximab have more Tregs subsequent to treatment (21, 47). It may be that one of the natural tendencies of the immune response is resetting itself to a state of self-tolerance through generation of Tregs; however, ongoing inflammation inhibits this protective pathway. Theoretically, the suppression of inflammation by agents such as TNF-α blockers would allow for an ideal therapeutic window for administration of Ag-specific immunomodulatory therapies. However, inflammatory cytokines including IL-6 are decreased after TNF-α blockade (46). It is known that IL-6 and TGF-β signaling is involved in the generation of Th17 cells, whereas blocking IL-6 leads to increased Treg production (48). Th17 cells are believed to be major effectors in a variety of autoimmune disorders (49). We observed a reduction of IL-17 secretion in T cells purified from mice treated with siRNA-modified DCs. The possibility that Treg-mediated inhibition of inflammation is occurring as a result of the reduction in IL-17 that occurred is a question we are currently investigating in our laboratory.

Although the current approach presented offers the possibility of reprogramming immune responses to selectively inhibit ongoing synovial damage, the problem remains in the clinical situation that the damage has already occurred by the time the patient is eligible for therapeutic intervention. Accordingly, an interesting synergistic approach would be stimulation of regenerative processes by administration of mesenchymal stem cells (MSCs). Given that MSCs are inherently immunosuppressive, studies have shown that monotherapy with these cells is sufficient to inhibit onset of autoimmunity and trigger conditions favorable for Treg generation (50). In fact, allogeneic MSCs have been demonstrated to inhibit the onset of CIA as well as induce therapeutic effects (51). Given the advanced state of MSC therapy for articular injuries (52), it will be interesting to see whether synergies between immunomodulatory and regenerative therapies will be harnessed.

In conclusion, we demonstrated Ag-specific immunotherapy using gene-silenced and Ag-pulsed DCs that are capable of inhibiting both onset and progression of CIA. Mechanistically, knockdown of the costimulatory molecules CD40, CD80, and CD86 on Ag-pulsed DCs seemed to elicit an inhibited recall response, possibility associated with an increased Treg population. Currently, we are optimizing conditions for induction of disease regression, which will allow for clinical translation.

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Disclosures
The authors have no financial conflicts of interest.

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