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Immune Modulation by Silencing IL-12 Production in Dendritic Cells Using Small Interfering RNA1,2

Jonathan A. Hill,3† Thomas E. Ichim,3‡§ Kornel P. Kusznieruk,‡ Mu Li,‡§ Xuyan Huang,§ Xiaotao Yan,‡§ Robert Zhong,‡§¶ Ewa Cairns,* †§¶ David A. Bell,* †¶ and Wei-Ping Min4§¶

RNA interference is a mechanism of posttranscriptional gene silencing that functions in most eukaryotic cells, including human and mouse. Specific gene silencing is mediated by short strands of duplex RNA of ~21 nt in length (known as small interfering RNA or siRNA) that target the cognate mRNA sequence for degradation. We demonstrate here that RNAi can be used for immune modulation by targeting dendritic cell (DC) gene expression. Transfection of DC with siRNA specific for the IL-12 p35 gene resulted in potent suppression of gene expression and blockade of bioactive IL-12 p70 production without affecting unrelated genes or cellular viability. Inhibition of IL-12 was associated with increased IL-10 production, which endowed the DC with the ability to stimulate production of Th2 cytokines from allogenic T cells in vitro. Furthermore, siRNA-silenced DC lacking IL-12 production were poor allostimulators in MLR. IL-12-silenced and KLH-pulsed DC polarized the immune response toward a Th2 cytokine profile in an Ag-specific manner. These data are the first to demonstrate that RNA interference is a potent and specific tool for modulating DC-mediated immune responses. The Journal of Immunology, 2003, 171: 691–696.

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to-transcriptional gene silencing is a mechanism that functions to inhibit viral replication in many eukaryotic organisms (1–3). This process is mediated by dsRNA and can evoke many cellular reactions, including the nonspecific inhibition of protein synthesis seen in the IFN response of mammalian cells (4). It has recently been discovered that short sequences of RNA that are 21 nt in length (known as small interfering RNA or siRNA) can bypass the broad suppression of the IFN response and can lead to the specific degradation of cognate mRNA (5, 6). This process, known as RNA interference (RNAi), is immensely specific, since a single substitution in the 21-nt sequence can abrogate its effects, and extremely efficient, since the siRNA is incorporated into an enzymatic complex that conducts multiple rounds of target mRNA degradation (7). As such, RNAi provides a powerful tool for inhibiting endogenous gene expression and could provide a means to effectively modulate immune responses.

Dendritic cells (DC) serve as a junction between the innate and adaptive immune system (8). Being the most potent APC, the DC is endowed with the unique ability to stimulate and polarize naive T cells to either Th1 or Th2 phenotypes (9). In addition, the DC plays a critical role in the maintenance of self-tolerance by curtailting T cell responses directly or indirectly through the generation of T regulatory cells (10–12). The difference between DC subsets that stimulate and those that suppress immune responses seems to reside in the expression of costimulatory molecules and cytokines (13, 14). Generally, expression of IL-12 seems to stimulate Th1 activation (15), whereas production of IL-10 by DC stimulates Th2 activation (16) and in some cases regulatory T cell generation (17, 18). Understanding this duality in function has led to DC-based immunotherapies, which have been used to potentiate T cell responses (in the case of cancer vaccines) or diminish them (in autoimmune disorders and transplantation) (19–21). A potential barrier to DC immunotherapy is the plasticity of these cells in an in vivo environment. Therefore, the ability to generate DC with a specific phenotype and function would be advantageous. It is in this context that RNAi would be a powerful tool for modulating DC-initiated immune responses. However, to our knowledge, the utility of RNAi for immune modulation has never been reported.

We describe here the induction of RNAi in DC using siRNA specific for IL-12 p35 (siRNA-IL12p35). We show that bioactive IL-12 p70 production in bone marrow-derived DC is inhibited after stimulation with LPS plus TNF-α and is accompanied by an increase in IL-10 production. Moreover, when siRNA-IL12p35-treated DC were cultured with allogenic T cells, a Th2 polarization was observed since T cell expression of IFN-γ was reduced while IL-4 was increased. Inhibiting IL-12 production using siRNA-IL12p35 was associated with suppressed DC allostimulatory function. In vivo, initiation of Ag-specific Th2 responses was observed when DC treated with siRNA-IL12p35 were pulsed with keyhole limpet hemocyanin (KLH) and used for immunization experiments. These results provide the first evidence that RNAi can be
induced in DC and that siRNA is a potent tool for modulating DC function and subsequently T cell polarization.

**Materials and Methods**

**Generation of bone marrow-derived DC**

DC were generated from bone marrow progenitor cells as previously described (2). Briefly, bone marrow cells were flushed from the femurs and tibias of C57BL/6 mice (The Jackson Laboratory, Bar Harbor ME), washed, and cultured in 24-well plates (2 x 10⁶ cells/ml) in 2 ml of complete medium (RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg of streptomycin, 50 μg 2-ME, and 10% FCS (all from Life Technologies, Ontario, Canada) supplemented with recombinant GM-CSF (10 ng/ml; PeproTech, Rocky Hill, NJ) and recombinant mouse IL-4 (10 ng/ml; PeproTech). All cultures were incubated at 37°C in 5% humidified CO₂. Nonadherent granulocytes were removed after 48 h of culture and fresh medium was added. After 7 days of culture, >90% of the cells expressed characteristic DC-specific markers as determined by FACS. DC were washed and plated in 24-well plates at a concentration of 2 x 10⁶ cells/well in 400 μl of serum-free RPMI 1640.

**siRNA synthesis and transfection**

siRNA sequences were selected according to the method of Elbashir et al. (23). The siRNA sequences specific for IL-12p35 (AACCUGCCUGA AGUUGGUGC), IL-12p40 (AAAGUG AUAUCUGGCAUCC), and IFN-γ (AAGTGGCAAAAGGGATGGTAC) were synthesized and annealed by the manufacturer (Dharmacon, Lafayette, CO). siRNA for IFN-γ was used as a control since bone marrow-derived DC generated by the conditions described above did not produce IFN-γ after stimulation. Transfection efficiencies were determined using unlabeled and fluorescein-labeled siRNA luciferase GL2 duplex (Dharmacon). Transfection was conducted as described previously (23). Briefly, 3 μl of 20 μM annealed siRNA were incubated with 3 μl of GenePorter (Gene Therapy Systems, San Diego, CA) in a volume of 100 μl of RPMI 1640 (serum free) at room temperature for 30 min. This was then added to 400 μl of DC cell culture as described above. Mock controls were transfected with 3 μl of GenePorter alone. After 4 h of incubation, an equal volume of RPMI 1640 supplemented with 20% FCS was added to the cells. Twenty-four to 48 h later, transfected DC were washed and used for subsequent experiments.

In the transfection by phagocytosis, bone marrow DC progenitors at day 4 of culture were incubated in a final concentration of 60 pM FL-siRNA-Luc. Cells remained in culture with GM-CSF and IL-4 as described above. At day 8 of culture, cells were activated with LPS/TNF-α and incorporated FL-siRNA-Luc was assessed by flow cytometry on day 9.

**DC activation and MLR**

Transfected DC (1 × 10⁶ cells) were plated in 24-well plates and stimulated with LPS (10 ng/ml; Sigma-Aldrich, St. Louis, MO) plus TNF-α (10 ng/ml; PeproTech) for 48 h, at which point supernatants were used for ELISA and RNA was extracted from the cells for RT-PCR. For MLR, T cells were purified from BALB/c splenocytes using nylon wool columns and used as responders (1 x 10⁶/well). siRNA-treated DC (5 x 10⁵ from C57BL/6 mice) were used as stimulators. Seventy-two-hour MLR was performed and the cells were pulsed with 1 μCi [³H]thymidine for the last 18 h. The cultures were harvested onto glass fiber filters (Wallac, Turku, Finland). Radioactivity was counted using a Wallac 1450 Microbeta liquid scintillation counter and the data were analyzed with UltraTerm 3 software (Microsoft, Seattle, WA).

**Flow cytometry**

Phenotypic analysis of siRNA-treated DC was performed on a FACScan (BD Biosciences, San Jose, CA) and analyzed using CellQuest software (BD Biosciences). The following FITC-conjugated anti-mouse mAbs were used: anti-I-Ab, anti-CD11c, anti-CD40, and anti-CD86 (BD PharMingen, San Diego, CA). The annexin V-propidium iodide method of determining apoptosis/necrosis was used as previously described (22). All flow cytometric analyses were performed using appropriate isotype controls (Cedarslane Laboratories, Hornby Ontario, Canada).

**RT-PCR**

Total RNA from siRNA-treated DC (10⁶ cells) or from T cells purified from MLR (10⁶ cells) was isolated by TRIzol reagent (Life Technologies, Grand Island, NY) according to the manufacturer’s instructions. First-strand cDNA was synthesized using a RNA PCR kit (Life Technologies) with the supplied oligosdT16 primer. One micromole of reverse transcription reaction product was used for the subsequent PCR. The primers used for IL-12p35 and IL-12p40 flanked the sequences targeted by siRNA (IL-12p35, forward primer 5'-GCCAGGTGTCTTCGCCACGTCC-3', reverse primer 5'-GTCCTCCTTTTGTTGGAGAAG-3'; IL-12p40, forward primer 5'-ATCTTTTGTCTGGTTCTC-3', reverse primer 5'-CTTGGTG GCAGTGGACTGG-3'). In addition, IL-10, IFN-γ, IL-4, and GADPH (internal control) primers were used as previously described (24). The PCR conditions were: 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and PCR was done for 35 cycles. PCR products were visualized with ethidium bromide on 1.5% agarose gel.

**ELISA**

siRNA-treated DC (10⁵, C57BL/6 origin) were cultured with the allogenic T cells (1 x 10⁵) for 48 h. The supernatants were harvested and assessed for DC cytokines (IL-12p70, IL-10) and T cell cytokines (IFN-γ, 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).

**Immunization of mice with peptide-pulsed DC**

Day 7 bone marrow-derived DC were transfected with siRNA-IL-12p35, or transfection reagent alone as described above, and pulsed with 10 μg/ml KLH (Sigma-Aldrich) for 24 h. DC were then activated with LPS plus TNF-α for 24 h, washed extensively, and used for subsequent transfection experiments. Ag-pulsed DC (5 x 10⁵ cells/mouse) were injected s.c. into syngeneic mice. Mice were sacrificed after 10 days and cell suspensions were prepared from the draining lymph nodes. These cells were cultured in 96-well plates at a concentration of 4 x 10⁵ cells/well in the presence or absence of Ag for 48 h, at which point culture supernatants were used for analyzing cytokine production by ELISA.

**Statistical analysis**

One-way ANOVA followed by the Newman-Keuls test was used to determine the significance between groups for cytokine production and MLR. Differences with p < 0.05 were considered to be significant.

**Results**

DC are efficiently transected with siRNA

To establish a protocol for RNAi in DC, we first assessed the siRNA transfection efficiency. Although many studies have shown a limited ability of DC to be transfected with DNA, RNA transfection has proven to be efficient. To determine the transfection efficiency, we synthesized fluorescein-labeled siRNA specific for luciferase (FL-siRNA-Luc), a gene that does not exist in mammalian cells and thus does not affect cellular function. siRNA lacking fluorescein (siRNA-Luc) was used as a nonlabeled control. FL-siRNA-Luc and siRNA-Luc were transfected by GenePorter into bone marrow-derived and cultured DC. After 24-h siRNA transfection, the percentages of DC that had incorporated FL-siRNA-Luc were quantified by flow cytometry. As seen in Fig. 1, FL-
siRNA-Luc had been successfully incorporated into 88% of the cells, as analyzed by flow cytometry.

It is known that immature DC are highly phagocytic and can internalize a variety of molecules, including nucleic acids. Thus, we asked whether immature DC are able to internalize naked siRNA. To assess this, immature DC on day 4 were cultured with FL-siRNA-Luc in the absence of transfection reagent and assessed for siRNA internalization by flow cytometry on day 9 of culture. Despite the long incubation period, 19% of DC still contained incorporated siRNA (Fig. 1), suggesting that naked siRNA may be used for transfection of DC.

siRNA transfection does not alter DC viability, maturation or phenotype

One of the major concerns for gene transfection is that transfection reagents may affect cellular function or viability. Although we have demonstrated a high level of transfection efficiency using the GenePorter method, we needed to establish whether siRNA or the transfection procedure itself altered the viability of the DC. Thus, day 7 bone marrow-derived DC were treated with transfection reagent (GenePorter) alone, siRNA-IL12p35 alone, or the combination of transfection reagent and siRNA-IL12p35. After 24 h of transfection, apoptosis and necrosis were assessed using annexin V and propidium iodine staining, respectively. Compared with untreated DC, neither the transfection protocol alone nor the siRNA affected cell viability (Fig. 2).

Next, we addressed whether the siRNA or the transfection procedure affected DC maturation. DC were transfected with siRNA following activation with LPS and TNF-α. DC maturation was assessed by flow cytometry to analyze expression of MHC II, CD40, and CD86 or the DC-specific marker CD11c. It can be seen that neither treatment with siRNA nor mock transfection altered DC maturation in response to LPS and TNF-α (Fig. 3A).

An additional concern associated with transfecting DC with nucleic acids is induction of maturation. Since long dsRNA (poly(I):poly(C)) has previously been shown to induce maturation and activation of immature DC (25), we wished to determine whether or not siRNA had the same effect. Thus, immature DC were treated with siRNA-IL12p35 for 24 h and cell surface maturation markers were assessed by FACS. Fig. 3B illustrates that siRNA treatment alone failed to up-regulate MHC II, CD40, or CD86 on immature DC. Although these experiments used a concentration of 60 pM siRNA-IL12p35, higher concentrations of siRNA-IL12p35 (up to 10-fold) were also assessed, with no alteration in viability or differentiation (data not shown). These data indicate that transfection of DC with siRNA-IL12p35 affects neither the viability nor phenotype.

siRNA induces specific gene silencing in DC

The gene specificity of siRNA targeting of immune-associated molecules in T cells has been previously demonstrated (26). However, since DC exhibit distinct cellular features from T cells, we first chose to confirm the specificity of siRNA-induced gene silencing in DC. The specificity of siRNA gene inhibition was investigated by transfecting DC with siRNA-IL12p35 and siRNA
targeted to the p40 component of IL-12 (siRNA-IL12p40). Transcripts of IL-12 p35 and IL-12 p40 were detected by RT-PCR using primers flanking the siRNA-targeted sequence. Specific inhibition was demonstrated at the transcript level: siRNA-IL12p35 exclusively suppressed p35 transcripts while siRNA-IL12p40 suppressed only p40 transcripts (Fig. 4). In addition, both siRNA-IL12p35 and siRNA-IL12p40 failed to affect transcripts of the housekeeping gene GAPDH. These data suggested that siRNA-mediated gene silencing is specific in DC.

siRNA-IL12p35 inhibits IL-12 expression in DC

We next wished to verify whether siRNA-IL12p35 can block production of IL-12 protein. Since IL-12p35 is critical for the formation of the IL-12 p70 heterodimer, we assessed production of this cytokine in the supernatant of LPS/TNF-α-activated DC using ELISA. DC transfected with siRNA-IL12p35 were stimulated with LPS plus TNF-α for 48 h to induce maturation and cytokine expression. To confirm specificity of gene silencing, siRNA specific for IFN-γ (siRNA-control) was used since this cytokine is not expressed in bone marrow-derived DC. Additionally, negative controls included DC transfected with GenePorter alone (mock-transfected) and unmanipulated DC (untreated control). As shown in Fig. 5A, siRNA-IL12p35 reduced IL-12p70 heterodimer production (as determined by ELISA) by 85–90% compared with untreated or mock-transfected DC. More important, this effect was specific since no significant difference in IL-12p70 production was seen in DC treated with the IFN-γ siRNA-control. In addition, we tested for levels of IL-10 production since a reciprocal relationship between IL-12 production has been previously reported (27). IL-10 production in DC treated with siRNA-IL12p35 was significantly and specifically up-regulated compared with controls (Fig. 5B).

siRNA-IL12p35 suppresses DC allostimulatory activity

DC function can be characterized in part by their ability to stimulate alloreactive T cells in the MLR (8). To determine whether siRNA-IL12p35 affected DC allostimulatory activity, MLR was performed using DC transfected with siRNA-IL12p35, siRNA-control, mock-transfected, or untreated controls. Allogenic T cells were cultured with siRNA-transfected DC for 48 h, at which point allostimulation was determined by proliferation. Although the control DC groups all showed similar allostimulatory activity, DC transfected with siRNA-IL12p35 significantly suppressed this response (Fig. 6).

siRNA-IL12p35-treated DC promote Th2 differentiation

Since IL-12p70 is a key cytokine responsible for polarizing T cells toward an IFN-γ-producing or Th1 phenotype (28), we assessed whether allostimulation with DC that were transfected with siRNA-IL12p35 could alter cytokine production from responding T cells. As shown in Fig. 7, 60 pM siRNA-IL12p35 (siRNA-IL12p35) significantly increased IL-10 production (as determined by ELISA) by 24 h. DC transfected with 60 pM siRNA-IL12p35 produced IL-12p70 in the supernatant of LPS/TNF-α-activated DC, while DC transfected with 60 pM siRNA-control produced only a trace amount of IL-12p70 (siRNA-control). In addition, we tested for levels of IL-12p70 production since a reciprocal relationship between IL-12 production has been previously reported (27). IL-10 production in DC treated with siRNA-IL12p35 was significantly and specifically up-regulated compared with controls (Fig. 5B).

siRNA-IL12p35 specifically blocks IL-12 and up-regulates IL-10. DC (1 × 10⁶) were unmanipulated (control), transfected with GenePorter alone (mock transfected), transfected with 60 pM siRNA-IL12p35, or 60 pM siRNA-IFN-γ (siRNA control). The transfected DC were activated with 10 ng/ml LPS and 10 ng/ml TNF-α for 24 h. A, Supernatants were harvested from cultures and analyzed for IL-12p70 production using ELISA. B, Supernatants were harvested from cultures and analyzed for IL-10 production using ELISA. Data represent mean ± SD and are representative of three experiments (*, p < 0.01, by one-way ANOVA and Newman-Keuls test).

siRNA-IL12p35 transfection inhibits DC allostimulatory ability. C57BL/6-derived DC (1 × 10⁶) were untreated (untransfected, ○), transfected with GenePorter alone (mock transfected, □), transfected with 60 pM siRNA-IFN-γ (Control siRNA, △), or transfected with 60 pM siRNA-IL12p35 (●) for 24 h. Allogenic (BALB/c) T cells (2 × 10⁵/well) were incubated with siRNA-treated DC at the indicated numbers for 72 h. Proliferation was determined using [³H]thymidine incorporation. Data are representative of three independent experiments (*, p < 0.01, by one-way ANOVA and Newman-Keuls test).
T cells. Mock-transfected DC stimulated high IFN-γ and low IL-4 mRNA transcripts from responding T cells; however, stimulation with siRNA-IL12p35-treated DC resulted in low IFN-γ and high IL-4 transcripts (Fig. 7A). To confirm these results at the protein level, we assayed for IFN-γ and IL-4 from MLR culture supernatants using ELISA. The T cells incubated with siRNA-IL12p35-treated DC produced low levels of IFN-γ (Fig. 7B) and high levels of IL-4 (Fig. 7C). In contrast, T cells incubated with untransfected DC, GenePorter-transfected DC, or DC transfected with control siRNA showed a cytokine profile of high IFN-γ and low IL-4. These data suggest that siRNA-IL12p35-treated DC have the ability to polarize naive T cells along the Th2 pathway.

Modulation of Ag-specific response in vivo using siRNA-IL12p35-treated DC

Although a shift from Th1 cytokine production to Th2 is seen when naive T cells are incubated with siRNA-IL12p35-treated DC, we wondered whether this effect could also be obtained in vivo. To accomplish this, we pulsed siRNA-IL12p35-treated or mock-transfected DC with KLH and used these modified DC as immunogens in vivo by injecting into syngeneic hosts. Ten days after immunization with KLH-pulsed control DC, a Th1 recall response was evident when draining lymph node cells from recipient mice were challenged with KLH in vitro, as determined by up-regulated IFN-γ and down-regulated IL-4 production (Fig. 8). Under the same conditions, the siRNA-IL12p35-treated DC promoted a Th2 shift in the recall cytokine response, showing increased IL-4 production and suppressed IFN-γ. These results suggest that Ag-pulsed and siRNA-modified DC can be used to modulate the Th1 vs Th2 balance in vivo during a primary immune response.

Discussion

We have demonstrated that RNAi can be successfully used for immune modulation of DC by targeting expression of the Th1-polarizing cytokine IL-12. Using a siRNA sequence specific for the p35 subunit of IL-12, we have successfully blocked the expression of bioactive IL-12p70. Suppression was noted at the transcript and protein level and endowed the DC with higher IL-10 production, inhibited DC allostimulatory activity but allowed preferential Th2 differentiation in vitro and in vivo. This study is, to our knowledge, the first to report that RNAi can be successfully used for the purpose of DC immune modulation.

Although many techniques have been used to block specific molecules in vitro and in vivo, such as antisense oligonucleotides (29) and mAbs (30), RNAi provides several distinct advantages. First, mRNA degradation by siRNA is extremely efficient as only a few copies of dsRNA are necessary to activate the RNA-induced silencing complex (31). Once the RNA-induced silencing complex is activated, it can conduct multiple rounds of gene-specific mRNA cleavage. Second, RNAi is specific, in that only sequences with known function since siRNA sequences from predicted exon regions should be effective in inducing a knock-down phenotype. Thus, RNAi may help to elucidate much of the genomic data that is presently being accumulated.
Interestingly, DC silenced by siRNA-IL12p35 showed decreased allostimulatory capacity in this study. This is in contrast to results reported using DC generated from IL-12 knockout mice that possess normal allostimulatory activity (33, 34). We attribute this discrepancy to compensatory immunological mechanisms that may have arisen in the lifetime of the IL-12 knockout mice. This is suggested by studies that have demonstrated the importance of IL-12 in MRL. First, IL-12 production by APC was demonstrated to be critical for MLR proliferative response since addition of anti-IL-12 Abs resulted in suppression of proliferation (35). Second, overexpression of IL-12 in DC results in increased allostimulatory function (36). Another possible explanation for suppressed MLR in siRNA-IL12p35-transfected DC is that the increased IL-10 production may act as an inhibitor of T cell proliferation (37, 38).

Other studies examining naturally occurring Th2-promoting DC have shown that these cells have a reduced allostimulatory function and reduced IL-12 production (39, 40). The combination of Th2-promoting properties as well as poor allostimulation suggests that siRNA-IL12p35-transfected DC may possess the phenotype of a “tolerogenic” DC and thus may be useful for treatment of Th1-mediated autoimmune diseases and transplant rejection.

Manipulating immune response at the level of DC is attractive since the DC plays a critical role in both the initiation and suppression of immune responses. We and others have used DC-modifying approaches for induction of tolerance or treatment of autoimmunity (22, 41). Although antisense manipulation of DC has previously been reported (42), low transfection efficiency of DNA into DC presents a problem for widespread use of this approach (43). In contrast, transfection of DC with siRNA is quite efficient and therefore may offer the possibility of treating immune-based diseases in a specific and effective manner. The successful utility of Ag-pulsed siRNA-modified DC to shift the Ag-specific immune response from Th1 to Th2 (Fig. 8) suggests that this approach could be used for Ag-specific immune modulation. Future work will focus on these avenues of in vivo immune modulation and may expand the armamentarium of strategies available for immunotherapy.

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References
CORRECTIONS

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In Materials and Methods, under the subheading siRNA synthesis and transfection, two of the sequences in the second sentence are incorrect. The sentence should read as shown below.

The siRNA sequences specific for IL-12p35 (AACCUGCUGAAGACCACAGAU), IL-12p40 (AAGAUGACAUCACGUGCACC), and IFN-γ (AACTGGCAAAAGGATGGTGAC) were synthesized and annealed by the manufacturer (Dharmacon, Lafayette, CO).