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Induction of RNA Interference in Dendritic Cells

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Abstract

Dendritic cells (DC) reside at the center of the immunological universe, possessing the ability both to stimulate and inhibit various types of responses. Tolerogenic/regulatory DC with therapeutic properties can be generated through various means of manipulations in vitro and in vivo. Here we describe several attractive strategies for manipulation of DC using the novel technique of RNA interference (RNAi). Additionally, we overview some of our data regarding yet undescribed characteristics of RNAi in DC such as specific transfection strategies, persistence of gene silencing, and multi-gene silencing. The advantages of using RNAi for DC genetic manipulation gives rise to the promise of generating tailor-made DC that can be used effectively to treat a variety of immunologically mediated diseases.

Key Words

Dendritic cells
Gene silencing
RNA interference
siRNA
Cytokines
Gene therapy

Introduction

Successful intervention in disease states requires identification of primary causative factors. Taking this idea into immunology, modification of immune responses is dependent on manipulating key cells involved in immunity. Over the past several years it has become apparent that dendritic cells (DC) act as major regulators of immune responses. Originally identified by Steinman as a “novel class of lymphoid cells with dendritic morphology” (1), the DC was quickly recognized as the most potent activator of T cell responses amongst all of the antigen presenting cells (APC) of the immune system (2). Presently, it is accepted that the stimulation and inhibition of T cell (3), B cell (4), natural killer (NK) cell (5,6) and natural killer T (NKT) cell (7) functions are coordinated directly and indirectly by the DC. Control of immunity by DC is based on the ability of these cells, after maturation, to provide three signals to T cells during activation: Signal 1, the antigenic signal that is communicated via the high expression of MHC molecules found on DC (8); Signal 2, the “costimulatory” signal, comprising membrane-bound molecules such as CD40, CD80/86, and OX-40L (9), which are essential for T-cell expansion and escape from anergy; and Signal 3, soluble cytokines that induce T-cell differentiation into a Th1 phenotype (interleukin-12 [IL-12]) or Th2 phenotype (IL-10) (10).

When any of these three signals are inhibited, the T cell can undergo apoptosis, anergy, Th2 differentiation, or under the right circumstances, differentiate into a T regulatory cell (Treg) (11). DC capable of suppressing immune responses are termed tolerogenic DC (Tol-DC) (12,13) or regulatory DC (14,15). Functionally, DC isolated from tolerant mice possess the ability to inhibit MLR as well as to give rise to Treg through establishment of a

tolerogenic feedback-loop (16). Therapeutic manipulations for induction of Tol-DC have included transfection with various immune suppressive cytokines (17), alteration of culture conditions (18), or utilization of specific chemical inhibitors that block the T-cell stimulatory function of DC (19–21). Genetic modification has been used for generating Tol-DC by either gene transfection or gene inhibition. Thomson’s group demonstrated that retroviral transfection of bone marrow (BM)-derived myeloid DC with transforming growth factor- β (TGF- β) resulted in a population of cells that possessed inhibited allostimulatory potential, inhibitory activity to CD8 T cells, and protected cardiac allografts from rejection (22). Transfecting DC with FasL resulted in generation of “killer DC” that prolonged allograft survival in murine heart-transplant models (23). On the other hand, Tol-DC can be generated by inhibiting the expression of genes associated with DC maturation. For instance, generation of Tol-DC was described after inhibition of the NF- κ B pathway using chemical or genetic manipulations (19,24). Administration of antisense oligonucleotides to DC has been used successfully to inhibit expression of CD80 and CD86. DC manipulated in this manner were able to enhance survival of allografts, by causing deviation to Th2 and apoptosis of donor-reactive T cells (25).

Despite the attractiveness of using the DC as target for immune modulation, several factors impede the wide-spread use of this approach. Ex vivo manipulation of culture conditions does not lead to gene-specific effects. Additionally, the manipulated phenotype has the potential of reversing once the cells are re-introduced in vivo (17). Chemical and antisense approaches have the disadvantages of inhibiting not only the target gene but also other genes nonspecifically. Additionally, owing to the protein and gene redundancy, these approaches possess nonspecific and

unpredictable biological effects. Administration of antibodies against immune stimulatory molecules found on DC may cross-react to the antigens found on other cells. This problem became apparent during clinical trials of the anti-CD40L monoclonal antibody (MAb), which was associated with thrombosis owing to the expression of CD40L on platelets (26). In order to overcome these obstacles, we started to investigate the feasibility of using RNA interference (RNAi) as a means of selectively silencing genes encoding immunostimulatory molecules in DC. In this article, we will review the field of RNAi and describe varying methods through which this potent means of gene silencing can be applied to immunology.

What is RNA Interference?

RNAi is a cellular defence mechanism against viral double-stranded RNA (dsRNA). It has been observed that the host cell selectively inactivates viral dsRNA through cleavage by the enzyme DICER. The initial suggestion that dsRNA may also possess the ability to inhibit the endogenous mRNA transcripts that are homologous to exogenous dsRNA came from work in *Petunias*, in which plasmid-mediated overexpression of the gene responsible for purple pigmentation actually caused the flower to lose its endogenous color (27). This phenomenon was termed “co-suppression” because both the inserted gene transcript and the endogenous transcript were suppressed. Subsequently, in 1998, Fire et al. demonstrated that injection of *Caenorhabditis elegans* with a combination of both sense and antisense RNA specific to endogenous genes led to a more potent suppression of gene expression than when sense or antisense were administered individually. Inhibition of gene expression was so potent that approx 1–3 molecules of duplexed RNA per cell were effective at

knocking down gene expression. Interestingly, suppression of gene expression was capable of migrating from cell to cell and the inhibitory effect was transferred to progeny cells. This was the first description of RNAi (28). One problem arising from the initial description of RNAi was that in order to induce RNAi, long pieces of 200–800 base pairs of dsRNA had to be used. This was impractical for therapeutic uses owing to the sensitivity of long RNA to cleavage by plasma and intracellular RNases. In addition, long pieces of dsRNA induced a panic response in eukaryotic cells, part of which includes nonspecific inhibition of gene transcription, which was associated with production of interferon- α (IFN- α) (29). In 2001, it was demonstrated that following the entry of long dsRNA duplexes into the cytoplasm, a ribonuclease III-like enzymatic activity cleaved the duplex into smaller 21–23 base pairs, which are active in blocking endogenous gene expression. These small pieces of RNA, termed small interfering RNA (siRNA) are capable of blocking gene expression in mammalian cells without triggering the nonspecific panic response (30).

Therefore, there are two pathways of inducing RNAi. The first is the naturally occurring pathway that is triggered when viral or long double-stranded RNA enters a cell. Upon crossing the membrane, the dsRNA activates several enzymatic components: (1) (2',5')oligoadenylate synthetase, an enzyme that initiates a biochemical cascade leading to inhibition of protein synthesis (31); (2) protein kinase R (PKR), which also results in nonspecific shut-down of cellular activity (32); and (3) DICER, a nuclease that cuts the dsRNA into 21–23 base pairs that are active in blocking endogenous gene expression (33) (Fig. 1A). Gene silencing by long dsRNA is not advantageous for therapeutic or experimental purposes owing to the nonspecificity of effects. Another pathway of

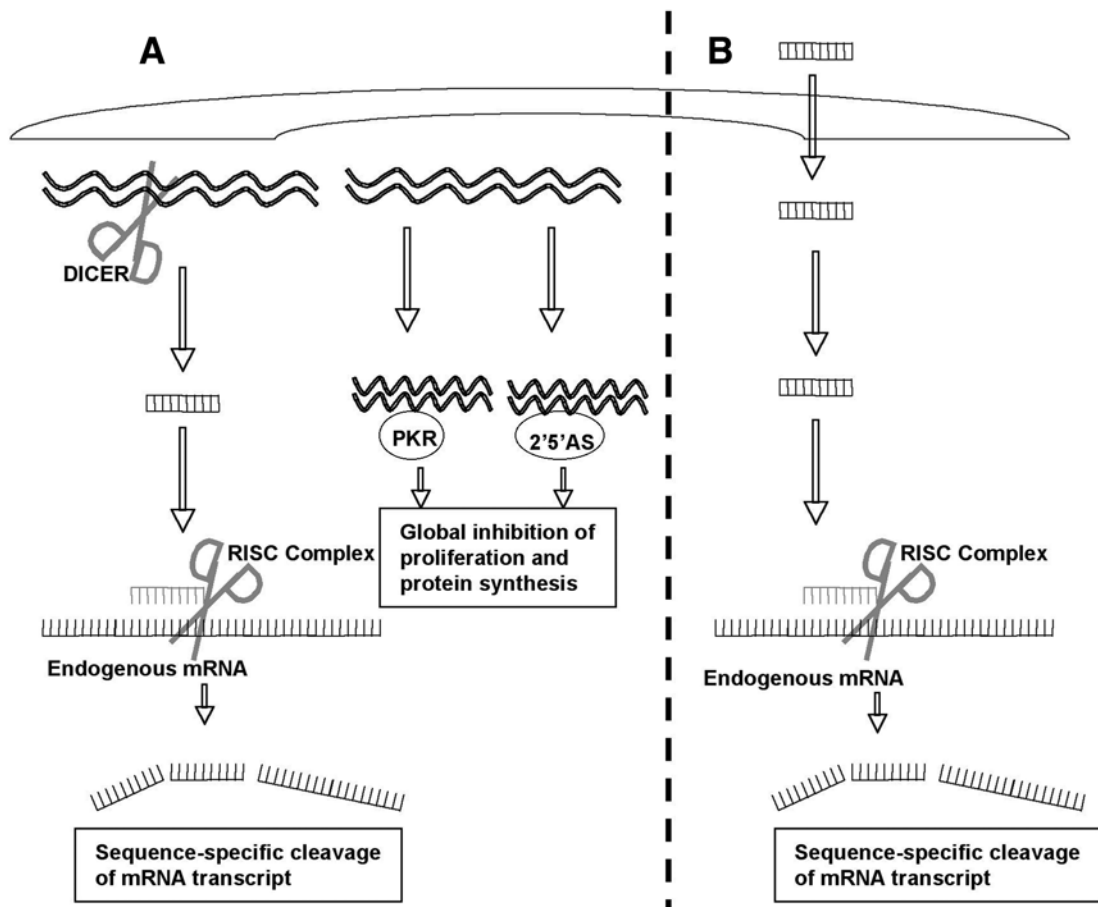


Fig. 1. Two pathways of RNA interference. (A) Natural gene silencing by long dsRNA. In the naturally occurring pathway, long dsRNA enters the cell and activates 2'5 oligosynthetase, which induces IFN response and blockade of cellular activities. Once the long dsRNA is recognized by the type III endonuclease DICER, there is a sequential cutting of the DNA that results in 21 nucleotide double-stranded fragments. These fragments then associate with the RISC complex and induce cleavage of endogenous mRNA transcripts in a sequence- and length-specific manner. (B) Artificial gene silencing through siRNA. In order to obtain gene silencing alone in the absence of other cellular responses, synthetic dsRNA of 21 nucleotides are transfected into the cell. This siRNA is not recognized by DICER or 2'5 oligosynthetase, but instead directly binds the RISC complex that subsequently induces selective silencing of endogenous transcripts.

RNAi induction is initiated by synthetic siRNA of 21–23 nucleotide base-pairs (34). The siRNA forms and guides the RNA induced silencing complex (RISC) to dock with endogenous mRNA transcripts. RISC subsequently cleaves the mRNA transcript in a sequence and a length-specific manner (Fig.

1B). This approach is highly selective in that only endogenous mRNA transcripts with strict homology to the siRNA are destroyed. Furthermore, because siRNA does not activate (2',5')oligoadenylate synthetase or PKR, the cell maintains normal protein synthesis and function.

Silencing gene expression through siRNA is superior to conventional gene- or antibody-mediated blocking approaches owing to the following advantages: (1) blocking efficacy is more potent (35); (2) targeting gene expression is more specific (36); (3) inhibitory effects can be passed vertically for multiple generations (37); (4) in vitro transfection efficacy is high and can be expressed in a stable manner (38); (5) in vivo use is more practical and safer owing to the low concentration needed (picomolar levels are biologically effective), and there is no neutralizing antibody against siRNA; (6) tissue- or cell-specific gene targeting is possible using a specific promoter vector (39,40) or a specific antibody-conjugated liposome; (7) simultaneously silencing multiple genes or multiple exons is possible for increasing efficacy (41).

How to Induce RNAi?

The simplest method of inducing RNAi is through administration of presynthesized dsRNA of 21-nucleotide base pairs termed siRNA. Presynthesized siRNA can be purchased from many commercial sources, disadvantages include cost, lack of constitutive expression, and inability to determine a priori whether or not the synthesized duplex will possess inhibitory activity (42). In an attempt to overcome the uncertainty associated with target selection, long dsRNA specific to various target genes was generated through in vitro transcription and degraded into siRNA using RNase III extracted from *Escherichia coli*. A stronger inhibition of gene expression was observed using the in vitro generated siRNA as compared to chemically synthesized siRNA (41). It was reported that *E. coli*-derived RNase III generates primarily siRNA of 12–15 nucleotides (43). However, because the optimal siRNA effect occurs at 21–23 nucleotide base pairs, attempts have been

made to use recombinant human DICER for siRNA generation in vitro. In a recent study, silencing of both exogenous puromycin-resistance gene and endogenous expression of *H-ras*, *c-jun*, and *c-fos* was performed using this approach (44). Although enzymatically generated siRNA overcomes the problem of sequence-specificity, a great deal of technical sophistication is required during RNA expression and purification after cleavage. Furthermore, enzymatically generated siRNA does not possess the advantage of varying backbone chemistries that are available with chemically synthesized siRNA for increasing in vivo half-life (42).

Once free siRNA is added to mammalian cells, the silencing effect lasts up to approx eight cell divisions (45). Therefore, a stable gene-silencing effect is needed in order to produce “knocked-down” cell lines. To circumvent the problem that presynthesized siRNA only achieves transient gene inhibition, methods of generating siRNA intracellularly using plasmid-based expression methods have been devised (46). The first method involves tandem expression of sense and antisense RNA from a DNA template using independent promoters and terminators. Once the construct is internalized, two single-stranded RNA transcripts are hybridized intracellularly into dsRNA that in turn induces the gene-silencing effect. This approach has been used successfully to induce a 4-log reduction in HIV transcripts from a permanently transfected cell line (47). An alternative method to induce RNAi involves using a DNA plasmid that forms an RNA hairpin loop upon transcription. Because siRNA is double-stranded, the transcription of a hairpin loop yields a double-stranded portion of RNA, which is recognized by the cellular-processing machinery and cleaved into siRNA (48). Owing to the early termination associated with conventional RNA pol II promoters, it was discovered that driving

hairpin loop expression by RNA pol III promoters is ideal for hairpin-loop production (49). Additionally, because RNA pol III promoters U6 or H1 both cause termination after the second uridine, the product of transcription resembles the siRNA that is naturally formed after cleavage of long dsRNA by DICER. This siRNA contains two 3' overhanging T or U nucleotides that are necessary for gene-specific silencing (45). Comparison between tandem- and hairpin loop-expressed siRNA suggested a stronger *in vivo* silencing efficacy using the hairpin approach. In a study by Kobayashi et al., siRNA specific to the green fluorescent protein (GFP) plasmid was administered to mice using the hydrodynamic method of transfection. Superior silencing efficacy and longer inhibitory effect were observed using hairpin-expressed siRNA compared to the tandem approach (50).

Using a selection marker, stable knock-down of several genes has been performed both in cell lines and at the embryonic stem-cell level, giving rise to animals progeny in which every cell was silenced for the gene of interest (51). Mass-screen for a desired phenotype has been successfully performed using siRNA vectors in order to determine cellular phenotypic alterations following silencing of 50 de-ubiquitinating enzymes. This study revealed that a specific enzyme encoded by the familial cylindromatosis tumor-suppressor gene possesses NF- κ B activating functions through de-ubiquitination of an upstream protein NEMO. This finding was used as the basis for proposing the use of aspirin derivatives for restoring growth control in patients afflicted with the genetic disease familial cylindromatosis (52). Elucidation of other important biological properties using stable-transfected siRNA plasmids has been performed for knocking down genes associated with apoptosis (53), metastasis (54), cellular senescence (55), and neurogenesis (56). Fur-

thermore, siRNA can be directed to distinct anatomical locations using tissue-specific promoters in transgenic animals (57).

In contrast to transfection of cell lines in which selectable markers are used, transfection of primary cells requires a high level of efficiency. Stable transfection with siRNA has been performed using a variety of viral vectors. Retroviral transfection of siRNA targeting p53 was successful in both cell lines and primary fibroblasts. Inhibition of p53 mRNA and protein expression was effective under basal conditions and following etoposide-induced DNA damage (58). Because the authors were able to successfully target the viral vector using a cell-specific surface marker (CD4), it is anticipated that tissue-directed gene silencing might be possible using such vector-mediated approaches. Adenoviral-based siRNA approaches have been performed both *in vitro* and *in vivo*. Using a cytomegalovirus (CMV)-promoter to drive hairpin siRNA formation, it was demonstrated that adenoviral delivery can not only inhibit marker-gene expression *in vivo*, but can also decrease formation of pathological polyglutamine-mediated cellular aggregation (59). This demonstrates the feasibility of adenoviral-vector approaches for therapeutic siRNA delivery. Retroviral and adenoviral vectors both possess varying degrees of dependency on proliferation of target cells for maximal incorporation. This problem has been overcome through utilization of lentiviral vectors that can incorporate with high efficiency in nondividing cells. *In vitro* silencing of GFP using lentiviral-delivered siRNA was of great potency, lasting up to 25 d in culture (60). When used as therapy, lentiviral delivery of siRNA was capable of inhibiting HIV production from primary human T cells (61) and macrophages (62) *in vitro*. *In vivo* administration of siRNA using lentiviral vectors has demonstrated gene silencing in transgenic

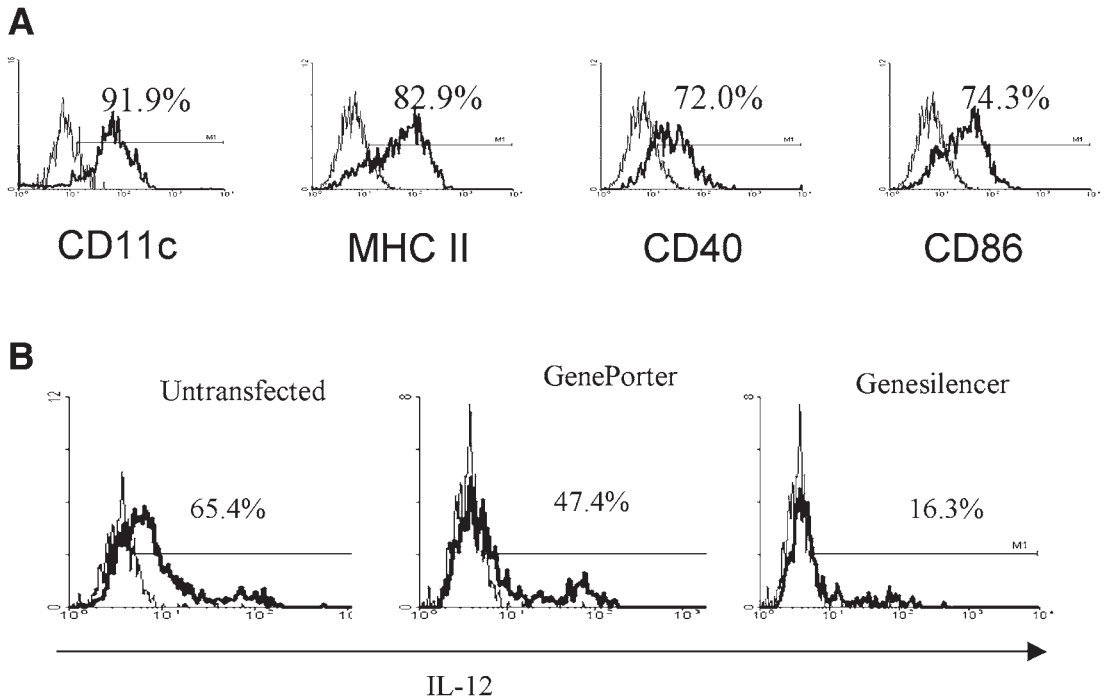


Fig. 2. Silencing DC by transfection with siRNA. **(A)** Phenotypic analysis of DCs. BM-derived DCs were cultured in the presence of GM-CSF and IL-4 for 7 d. DCs were activated with 10 ng/mL of TNF- α and LPS for 24 h. Phenotypic analysis was performed by flow cytometry after staining with the indicated FITC-conjugated MAbs. **(B)** Silencing of the IL-12 gene in DC. Seven-day cultured DCs were transfected with 60 pMol siRNA specific for IL-12p35 by GenePorter and Genesilencer, respectively. Forty-eight hours after gene silencing, IL-12 expression was assessed by flow cytometry following intracellular staining with PE-conjugated antimouse IL-12 MAb.

mice (63), indicating the in vivo applicability of this approach.

What Are the Optimal Methods to Deliver siRNA to DC?

Methods of incorporating nucleic acids into cells include electroporation (87), viral (64), and liposomal-based systems (65). Electroporation is associated with significant damage to cells, although Ledarche et al. have reported successful transfection of human DCs by electroporation with minimal cellular damage (87). On the other hand, viral

vector could inadvertently lead to activation of DCs. Therefore, we have focused our interest on liposomal-based systems for delivering siRNA to DCs.

The standard BM-derived DC generation strategy (23) comprises culturing C57/BL6 (Jackson Labs, Bar Harbor, ME) BM cells for 7 d in the presence of IL-4 and granulocyte macrophage colony stimulating factor (GM-CSF). This results in a population in which >90% of the cells express characteristic DC-specific markers as determined by fluorescence-activated cell sorting (FACS) (Fig. 2A). The BM-derived DC were used for optimization

siRNA transfection. siRNA specific to IL-12p35 (IL12-siRNA; AACCGCUGAAGACCACA-GAU) was synthesized by Dharmacon Inc. (Lafayette, CO). The standardized transfection protocol was performed as described elsewhere (66). The siRNA was transfected by two liposome reagents, GenePorter and GeneSilencer (Gene Therapy Systems, San Diego, CA). Briefly, 60 picomoles (pmol) of IL12-siRNA was incubated with 3 μ L of GenePorter or 3 μ L of Genesilencer in a volume of 100 μ L of RPMI-1640 at room temperature for 30 min. This was then added to 400 μ L of DC cell culture. Mock controls were transfected with 3 μ L of nonspecific siRNA that does not match any sequences of known genes. After 4 h of incubation, an equal volume of RPMI-1640 supplemented with 20% fetal calf serum (FCS) was added to the cells. Twenty-four to forty-eight hours later, transfected DCs were washed and activated by stimulation with lipopolysaccharide (LPS) (10 ng/mL) and tumor necrosis factor α (TNF- α) (10 ng/mL). The efficiency of gene silencing was determined by flow cytometry following intracellular staining. Although transfection with GenePorter caused an inhibition of IL-12 expression by 27.5%, GeneSilencer-mediated transfection showed more effective, which reduced IL-12 expression by 75.1% (Fig. 2B). Using either of the transfection reagents did not induce DC maturation, loss of DC viability, or alteration in DC maturation capacity subsequent to LPS/TNF- α activation (data not shown). Therefore, the liposomally based GeneSilencer reagent seemed optimal for transfection of DC.

Can DC Be Silenced by Endocytosis?

DC are highly phagocytic in the immature state. The role of the DC as a “watchguard” against foreign antigens is facilitated by its ability to sample their environment. Receptors, such as DEC-205 or CD36, are used by the DC for internalizing bacterial products or

apoptotic cells (67). The DC are also able to acquire components shed by live cells using scavenger receptors such as SRA (68). In addition to receptor-mediated endocytosis, direct acquisition of environmental components surrounding the immature DC occurs through pinocytosis (69). This high endocytic capacity is illustrated by experiments assessing immature DC in which FITC-dextran is constitutively acquired by immature but not mature DC (70). It is known that intradermal administration of naked DNA plasmids using either injection or gene-gun approaches leads to preferential gene expression in DC (71), suggesting that DC may take up genetic materials efficiently. Furthermore, the capacity of mRNA-pulsed DC to transcribe and express the encoded antigens demonstrates that DC can actively sequester and utilize exogenous RNA instead of leading it to cytoplasmic destruction. This approach is considered to be a valuable method for in vivo “DC vaccination” to be used in the treatment of cancer patients (72). These findings led us to hypothesize that administration of naked siRNA or siRNA-expression plasmids to DC could induce gene silencing in the absence of transfection reagents. In support of this hypothesis, siRNA can be administered in vivo in the absence of transfection media through the “hydrodynamic” technique. This essentially involved administration of naked siRNA in 1–1.5 mL of saline into the tail vein of mice in a time frame of less than 10 s (73). This technique has been subsequently used by other groups in the therapeutic sense for inhibition of hepatitis through silencing either Fas receptor or caspase-8 in the liver (74,75).

To examine the efficacy of naked siRNA incorporation by phagocytosis, a fluorescein-labeled siRNA (Fl-siRNA) specific for the luciferase gene was added to immature DCs on d 5 under a conditioned culture system. As a control, DCs were also transfected with

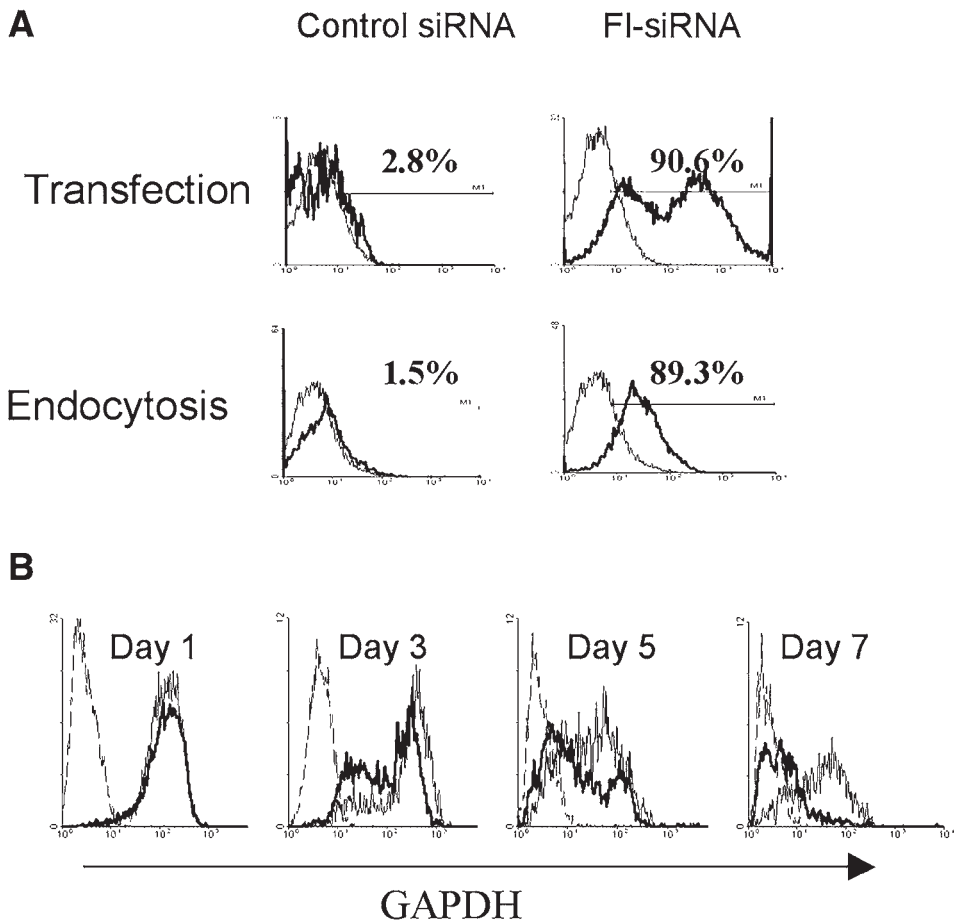


Fig. 3. Silencing DC by endocytosis of siRNA. **(A)** Transfection efficacy of siRNA in DC. DCs were cultured from BM progenitors in the presence of GM-CSF and IL-4. 1×10^6 of 7-d cultured DCs were liposomely transfected with unlabeled (control siRNA), or fluorescein-labeled (Fl-siRNA) siRNA specific for luciferase sequence at indicated concentration (upper panel). Alternately, these siRNAs were added to DC culture without transfection reagent at d 5. DCs were activated with LPS/TNF- α on d 8 and the transfection efficacy assessed by flow cytometry on d 9. The efficacy of siRNA incorporation was assessed using flow cytometry on the FL1-channel. **(B)** Silencing DCs by phagocytosis of siRNA. DCs were cultured for 5 d in six-well plate. siRNA specific for GAPDH was added in the culture without transfection reagents. DCs were collected at indicated times and stained intracellularly with anti-GAPDH MAb. The expression of GAPDH was analyzed using flow cytometry and compared between gene silenced DCs (bolded lines) and nonsilenced DCs (fine lines). The isotype control is shown as a dotted line.

Fl-siRNA using liposomal transfection reagent. Effective incorporation of Fl-siRNA as high as 89% was observed by flow cytometry (Fig. 3A). This high level of transfection was substantially similar to that observed using

GeneSilencer (90.6%). The ability to directly transfect DCs with siRNA may open possibilities, such as topical administration or perfusion of organs with siRNA-bearing solutions, which are currently under investigation.

How Long Does the Gene Silencing Last in DC?

Gene silencing in plants and nematodes can pass from cell to cell, as well as be amplified across cellular generations (76). In contrast, gene silencing in mammalian cells is a transient phenomenon unless siRNA is constitutively expressed using a plasmid-based system. This discrepancy is accounted for by the differing mechanisms of gene silencing. In mammalian cells, gene silencing occurs at the level of mRNA cleavage, whereas in lower organisms gene silencing takes place through modification of the transcriptionally active DNA (77). Despite these significant interspecies differences, intercellular differences in terms of siRNA efficacy have been noted. For example, gene silencing was reported to be less effective in neuronal cells compared to other cell types (78). The persistence of the siRNA effect in DCs has never been investigated.

Owing to the limited lifespan of cultured primary DC, we silenced DC by adding siRNA in the early stage of DC culture. The siRNA specific for a housekeeper gene GAPDH (GAPDH-siRNA) was added to DCs on d 5 in a conditioned culture system. After the addition of GAPDH-siRNA, the expression of GAPDH was assessed by flow cytometry following intracellular staining with anti-GAPDH antibody. Gene silencing was observed to last until the end-point of the DC cultures, i.e., 7 d after transfection (Fig. 3B). When assessed at the protein level, the silencing effect was detectable on d 3, stronger on d 5, and maximal on d 7 after siRNA transfection.

It has been suggested that proliferating cells may possess a dilutive effect of the cellular machinery needed for maintaining the siRNA efficacy, which may diminish the gene-silencing effect in a time-dependent manner (79). In contrast to this, we observed a persistent effect of gene silencing. A possi-

ble explanation could be that the target cells in this study are DCs that are nondividing after maturation.

Can Multiple Genes Be Simultaneously Silenced in DC?

McManus et al. proposed that competition occurs between multiple siRNA molecules for the cellular machinery that performs RNAi (79). Indeed, it is known that siRNA efficacy is dependent on members of the RISC complex that hypothetically could be temporarily depleted if silencing activity was taking place at an extraordinary rapid rate. However, in contrast to this, Martin-Lluesma et al. have shown that it is possible to simultaneously silence *Hec1* and *Mad2* genes in the various mammalian cell lines (80). The feasibility of multigene silencing was also demonstrated in *Drosophila* by studies examining silencing of the alcohol dehydrogenase gene together with the *polycomb* gene. Efficient post-transcriptional gene silencing was observed for the expression of both genes in a noncompetitive manner (81).

Although the aforementioned studies used proliferating cell lines as targets, we questioned whether such an effect could be present in DCs to prevent multigene silencing. DC function is dependent on concordance of immune stimulatory molecules providing antigenic, costimulatory, and differentiation signals to T cells. Multiple gene silencing would facilitate the generation of tolerogenic DC for immune modulation, but it has not been tested yet. To test such a possibility, DCs were transfected with IL-12-siRNA and GAPDH-siRNA, simultaneously. Forty-eight hours after gene silencing, the expression of both IL-12 and GAPDH molecules was significantly inhibited (Fig. 4). Subsequently, we succeeded in inducing triple-gene silencing in

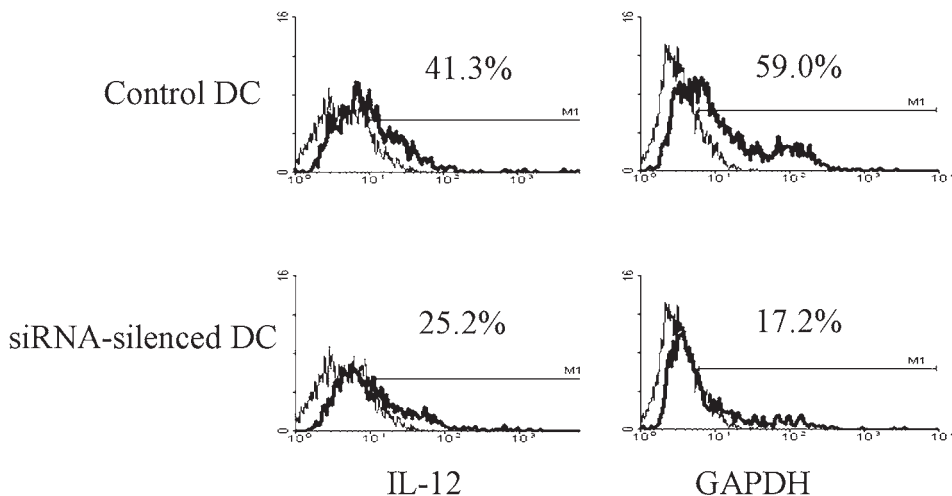


Fig. 4. Multigene silencing in DC. siRNAs specific for IL-12p35 and GAPDH were co-transfected to 7-d cultured DCs by GeneSilencer. Forty-eight hours after gene silencing, the control siRNA transfected DC (control DCs, upper panel) and siRNA transfected DCs (lower panel) were intracellularly stained with anti-mouse IL-12 and GAPDH (bolded lines), respectively. The fine lines indicate isotype controls.

DCs by simultaneously silencing MHC II, IL-12, and GAPDH (data not shown).

Can Immune Modulation Be Achieved Through Gene-Silenced DC?

Because siRNA silencing is long-lasting, can be performed with several genes simultaneously, and is not cytopathic to DC, we have initiated studies using siRNA-modified DCs as a type of “tolerogenic vaccine” by pulsing the manipulated DCs with antigen. The first target selected for silencing was IL-12, the potent Th1-promoting component expressed by DCs. IL-12 is comprised of two subunits, p35 and p40, which heterodimerized to form bioactive IL-12p70 (82). Because the p40 subunit also can homodimerize to form a biologically active antagonist to IL-12, or heterodimerize with p19 to form IL-23, we decided to target p35 to inhibit specifically bioactive IL-12 but not other cytokines (83). The initial investigations

into silencing efficacy of IL-12siRNA demonstrated >95% suppression of bioactive p70 production by as little as 60 picomolar concentration of siRNA (84). When IL-12siRNA-silenced DC were pulsed with the nominal antigen KLH, a potent Th2 deviation of antigen-specific recall response was observed, suggesting that: (1) siRNA treatment does not adversely affect the *in vivo* biology of DC after *ex vivo* manipulation; (2) siRNA treatment does not impair or alter antigen-processing abilities of the DC; and (3) targeting immune stimulatory genes on DC using siRNA is an effective method of inducing antigen-specific immune modulation (84).

In addition to antigen-specific modulation *in vivo*, we further determined whether the IL12p35-siRNA affected DC allostimulatory activity. The rationale for this is that the response to alloantigen, as in the case of transplantation, is more robust and accelerated as compared to single-antigen responses.

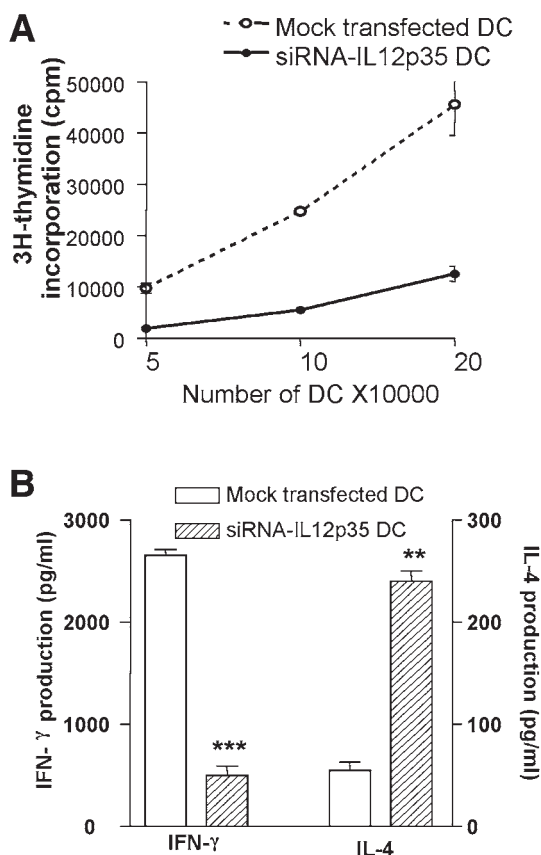


Fig. 5. Immune modulation by siRNA-silenced DCs. BM-derived DCs (C57/BL6) were cultured for 7 d as described in Fig. 3. DCs were transfected with 10 pMol IL-12p35-siRNA by GeneSilencer or transfection reagent alone for 4 h and subsequently activated with LPS+TNF- α for 24 h. Gene-silenced and control DCs were mixed with 2×10^5 allogeneic (BALB/c) T cells in a 96-well plate and subsequently incubated for 72 h. (A) The proliferation of T cells was determined using tritiated thymidine incorporation assay. (B) The supernatants were collected, and the IL-4 and IFN- γ proteins were assessed by ELISA.

In order to assess allostimulatory activity. Allogeneic T cells were cultured with IL12p35-siRNA-silenced DCs or mock transfected DCs for 72 h. The capacity of allostimulation was determined by proliferation assay in MLR. A dose-dependent stimulation of T cells was observed with both IL12p35-siRNA-silenced DCs and control DCs. However, a potent inhibition of T-cell proliferation was seen after stimulation with IL12p35-

siRNA-silenced DCs but not with controls (Fig. 5A). The diminished alloreactivity in absence of IL-12 is consistent with a previous report in an IL-12 knockout model (85).

Because IL-12 is a key cytokine responsible for polarizing T cells to an IFN- γ producing or Th1 phenotype (86), we assessed IL12p35-siRNA-silenced DCs for their ability to manipulate Th differentiation of alloreactive T cells. Allogeneic T cells were cultured with IL12p35-

siRNA-silenced DCs or mock transfected DC for 48 h. IFN- γ (Th1) and IL-4 (Th2) protein production was determined by enzyme-linked immunosorbent assay (ELISA). IL12p35-siRNA-silenced DCs significantly inhibited the production of IFN- γ and augmented IL-4 (Fig. 5B), suggesting a Th1>Th2 shift in cytokine production. These results suggest that IL12p35-siRNA-silenced DC can shift T-cell polarization from Th1 to Th2.

Recently, Laderach et al. utilized siRNA to silence the p50 component of NF- κ B in human monocyte-derived DCs (87). Using presynthesized siRNA oligonucleotides transfected through electroporation, the authors demonstrated gene-specific inhibition at the transcript and protein level. Furthermore, silencing of p50 was associated with suppressed IL-12 production, but did not alter expression of costimulatory molecules or MHC II (87). Because suppression of NF- κ B using chemical inhibitors is known to block IL-12, costimulatory signals, and MHC II expression, the findings of Laderach et al. suggest that individual components of NF- κ B complex possess distinct biological activities in DCs. Because siRNA is more specific than chemical inhibitors, it could be used as a potent tool for dissecting the cell-specific signaling events, which have been difficult to investigate using conventional methods.

The utility of siRNA for DC signaling studies was also demonstrated in a study in which the expression of the neuronal protein

plexin-A1 on DCs was silenced by siRNA (66). Targeting the plexin-A1 transcript in BM-derived DCs resulted in suppression of T-cell activation. Interestingly, the suppression was not mediated by inhibition of peptide loading or costimulatory molecules. This novel finding took advantage of siRNA to demonstrate that DC–T-cell interactions use similar molecular mechanisms as those used by neuron–neuron interactions.

In conclusion, the novel technique of siRNA-mediated gene silencing seems to be particularly suited for manipulation of DC immunological function. The findings that siRNA can become incorporate into DCs using the endocytic pathway in the absence of transfection reagent suggests this technology can be widely used in immunologic research. Furthermore, the possibility of multigene silencing makes siRNA a very promising therapy for clinical use.

Acknowledgments

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