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Cholesterol Modification of p40-Specific Small Interfering RNA Enables Therapeutic Targeting of Dendritic Cells

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Small interfering RNA (siRNA)–based therapies allow targeted correction of molecular defects in distinct cell populations. Although efficient in multiple cell populations, dendritic cells (DCs) seem to resist siRNA delivery. Using fluorescence labeling and radiolabeling, we show that cholesterol modification enables siRNA uptake by DCs in vitro and in vivo. Delivery of cholesterol-modified p40 siRNA selectively abolished p40 transcription and suppressed TLR-triggered p40 production by DCs. During immunization with peptide in CFA, cholesterol-modified p40 siRNA generated p40-deficient, IL-10–producing DCs that prevented IL-17/Th17 and IFN-γ/Th1 responses. Only cholesterol-modified p40-siRNA established protective immunity against experimental autoimmune encephalomyelitis and suppressed IFN-γ and IL-17 expression by CNS-infiltrating mononuclear cells without inducing regulatory T cells. Because cholesterol-modified siRNA can thus modify selected DC functions in vivo, it is intriguing for targeted immune therapy of allergic, autoimmune, or neoplastic diseases. The Journal of Immunology, 2015, 195: 2216–2223.

The RNA interference (RNAi) technology is established as efficient treatment strategy for various gene-targeted therapies. Because RNAi delivery to target cells has to be effective and safe, nonviral delivery systems and biochemical modifications are coming into major focus for targeting gene expression in vivo. The in vivo application of small interfering RNA (siRNA) requires structural modifications to improve serum stability and target tissue delivery. For instance, siRNA encapsulated by lipid nanoparticles facilitates siRNA delivery to hepatocytes followed by sequence-specific knockdown. Recently published clinical trials demonstrated first results on the efficacy of RNAi therapy in humans to correct metabolic disorders (1, 2). In contrast, the in vivo use of RNAi technologies for the therapy of inflammatory autoimmune diseases still faces major hurdles. For the successful delivery of RNAi in autoimmunity, the constructs should preferentially target immune cells without inducing major off-target effects. In addition, the constructs should not be immunogenic to minimize the risk for activating TLR and to avoid the induction of proinflammatory responses. One possibility to minimize off-target effects is the selection of a target gene preferably expressed by immune cells. Therefore, we decided to focus on sequences that silence p40 (Il12b). As part of IL-12 and IL-23, p40 is mainly produced by dendritic cells (DCs) (3). DCs producing IL-12 and IL-23 are of central importance for the activation of autoreactive T cells toward pathogenic Th1 and Th17 cells (4). Both of these Th cell subsets are crucially involved in the pathogenesis of numerous autoimmune diseases such as multiple sclerosis or psoriasis (5, 6). Notably, the spontaneous uptake of siRNA by DCs is very limited and typically requires the use of transfection reagents (7), which cannot be applied in vivo. Also, the use of naked siRNA is not ideal, because naked siRNA is less stable and does not penetrate the cell membrane easily. Therefore, we decided to use siRNA with a chemical modification. Cholesterol modification of siRNA has been introduced to deliver specific RNAi to liver and other scavenger receptor–expressing tissue (8–12). Because DCs express scavenger receptors (13), we aimed to generate cholesterol-modified siRNAs (chol-siRNA) specific for p40. To demonstrate that p40-specific chol-siRNAs are functional in DCs in vivo, we used these constructs in the mouse model of experimental autoimmune encephalomyelitis (EAE), where the key role of p40-expressing DCs is well established (14, 15).

We first identified p40-specific sequences and analyzed the uptake of p40-targeting chol-siRNA by DCs in vitro and in vivo. Furthermore, we studied the effects of p40-specific chol-siRNA on the DC phenotype, and demonstrate its potential as an RNAi-based therapeutic to induce type II DCs and to silence autoimmune inflammation in the model of EAE.

Materials and Methods

Mice

SJL mice, 6–8 wk of age, were purchased from Janvier and maintained in the animal care facilities of the University Medical Center Tübingen. Animal experiments were approved by the Institutional Animal Care and Use Committee of the Regierungspräsidium Tübingen (HT01/08 and HT10/13).

Experimental autoimmune encephalomyelitis

EAE was induced by s.c. immunization of female SJL mice with 37.5 μg PLP139–151 peptide (EMC Microcollections) in CFA (Difco) and i.p. injection of 200 ng pertussis toxin (Calbiochem) (5). The clinical EAE score was followed and rated by the following scale: 0 = no disease; 1 = limp
Tail: 2 = hind-limb weakness or partial paralysis; 3 = complete hind-limb paralysis; 4 = forelimb and hind-limb paralysis; 5 = moribund state (5).

RNA isolation and gene expression

Total RNA was purified from cultured cells or from ex vivo–isolated cells and reverse transcribed into cDNA using commercially available kits (Bioryn). Relative gene expression levels were determined by quantitative real-time PCR using TaqMan probes (TM; ThermoFisher Scientific) for β-actin, β2-microglobulin, IL-12p40, Ifn-γ, Ifn-β, Il10, Il17, Il23p19, and Foxp3 and the LightCycler480 system (Roche). The relative expression of the indicated genes was calculated relative to the expression of β-actin as an internal control (ΔΔCT method). Detection of the exogenous sequences was performed with the LightCycler480 system (Roche). The relative expression of the indicated genes was calculated relative to the expression of β-actin as an internal control (ΔΔCT method).

Cytokine analysis and flow cytometry

Commercially available ELISA kits were used for the quantification of the cytokines IL-12p40, IL-23p19, IL-10, and IL-6, from serum or cell culture supernatants. Cells were isolated from draining lymph nodes on day 3 or 7 as indicated or from the CNS on day 16 after immunization and analyzed for cytokine production by quantitative real-time PCR or flow cytometry. Intracellular cytokine staining was performed after stimulating cells with LPS (100 ng/ml) and PE-conjugated Abs directed against IL-12p40, IL-12p70, IL-23, IL-10, and IL-6, from serum or cell culture supernatants. Cells were isolated from draining lymph nodes by magnetic cell sorting. Equal cell numbers of CD4+ T cells were cocultured with PLP-pulsed DCs. On day 3, [3H]thymidine (0.25 μCi/well; PerkinElmer) was added for 16 h before harvest. Incorporation of radioactivity was determined by scintillation counting.

Statistical analyses

Statistical analyses were performed by paired or unpaired t test or by ANOVA, followed by Dunnett’s multiple comparison test using GraphPad Prism 5 software. The p values <0.05 were considered significant.

Results

Construction and identification of a chol-siRNA specific for IL-12/IL-23p40

We first constructed a series of siRNA sequences for specific silencing of the IL-12/IL-23 subunit p40 (Supplemental Fig. 1A). To evaluate the functional activity of each sequence, we transfected immature in vitro–generated DCs with different siRNA constructs by electroporation. After electroporation, DCs were activated with LPS (16). Only two of the siRNA constructs tested, siRNA4 and a previously published sequence (17) (siRNA6), reproducibly diminished LPS-triggered IL-12p70 protein production down to 10–20% of control DCs (Supplemental Fig. 1B). To test the potential biological activity of siRNA4 and siRNA6 in vivo, we cholesterol-modified the constructs (chol-p40-siRNA) as described previously (8, 9) (Supplemental Fig. 1C).

Following an application protocol that allows efficient prevention of EAE using naked siRNA against T-bet (18, 19), we tested whether this chol-p40-siRNA could interfere with IL-12 production by APCs. We injected mice i.v. with chol-p40-siRNA or PBS, challenged the mice with LPS, and determined serum concentrations of p40, IL-10, and IL-6 after 6 h. In PBS-treated mice, all three cytokines were readily detectable in the sera, albeit IL-10 at very low levels (Fig. 1A). Sera from mice treated with chol-p40-siRNA contained significantly reduced concentrations of p40, whereas IL-10 protein was increased. IL-6 levels remained unaffected by chol-p40-siRNA treatment, indicating selectivity and specificity of the chol-p40-siRNA (Fig. 1A). These in vivo results were surprising, as LPS induces rapid IL-12 production by DCs, a cell population that is thought to resist transfection with siRNA under in vitro and in vivo conditions (7, 20, 21).

The data suggest that, in contrast with the reported resistance of APCs to spontaneously take up naked siRNA under in vitro conditions (22), DCs are capable of incorporating biologically
efficient amounts of chol-p40-siRNA in vivo. We therefore tested the spontaneous uptake of chol-p40-siRNA and nonmodified p40-siRNA by DCs.

**Spontaneous uptake of chol-p40-siRNA by DCs in vitro and ex vivo**

We generated a FAM-labeled chol-p40-siRNA (FAM-chol-p40-siRNA) and first performed functional analysis. Immature DCs were incubated with medium alone, or with medium and either nonmodified p40-siRNA or chol-p40-siRNA, and activated with LPS for 1 h (Fig. 1B). Whereas in the absence of LPS activation p40 and Il6 were undetectable at mRNA level, LPS stimulation readily induced their expression. Only chol-p40-siRNA significantly inhibited LPS-mediated p40 expression, whereas nonmodified p40-siRNA did not affect p40 mRNA (Fig. 1B). In line with the in vivo data, the level of Il6 remained unaffected by either

![Image of Figure 1](http://www.jimmunol.org/)
Chol-p40-siRNA suppresses the development of Th1 and Th17 cells

As DC is the key APC that primes naive T cells and determines the differentiation fate of CD4+ T cells during this priming period, we investigated whether the treatment with chol-p40-siRNA interferes with the polarization of naive CD4+ T cells in vivo. To address this question, we injected SJL mice with chol-p40-siRNA or p40-siRNA and immunized the mice for active EAE, a protocol that strongly induces Th1 and Th17 cells (25). The immunization for EAE induced the DC cytokines p40, l10, and l110 on day 3 and the Th1 and Th17 cytokines on day 7 in draining lymph node cells. On day 3 after immunization, treatment with chol-p40-siRNA significantly suppressed p40 expression but induced l110 mRNA without affecting l10 expression (Fig. 3A). On day 3, nonmodified p40-siRNA also impaired p40 expression under...
conditions of in vivo immunization, although significantly less than chol-p40-siRNA (p = 0.0013; Fig. 3A). Importantly, this slight impairment had no further consequences on the EAE-specific immune response and is best explained by a discrete unspecific siRNA uptake (see later) as observed with other naked siRNA (26). On day 7 after immunization, the expression of Il17, Ifnγ, and Foxp3 mRNA (B), as well as IL-17, IFN-γ, and TNF protein by CD4+ T cells (C), was determined. Before flow-cytometric analysis, cells were stimulated with PMA/ionomycin. Data of three independent experiments (n = 12) are shown. (D) Mice received a single i.v. injection of [33P]-p40-siRNA or [33P]-chol-p40-siRNA. After 2 h the indicated tissues were isolated and analyzed for [33P] by liquid scintillation counting. (E) Mice were treated as in (D). The uptake of 33P-labeled siRNA by isolated CD11c+ or CD11c- cell fractions from spleen and lymph nodes was determined by liquid scintillation counting. Burs in (D) and (E) represent mean ± SEM of four independent experiments, n = 8. *p < 0.05, **p < 0.01, ***p < 0.001.

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FIGURE 3. Chol-p40-siRNA accumulates in lymphoid tissue and impairs Th1 and Th17 development in vivo. (A–C) Mice were treated with PBS, p40-siRNA, or chol-p40-siRNA and immunized for EAE. Expression of Il12p40, Il10, and Il6 in draining lymph nodes was determined on day 3 after immunization (A). Data of three independent experiments (n = 9) are shown. Data were normalized to β-actin, and cytokine expression of control mice was set as 1.0. On day 7 after immunization, the expression of Il17, Ifnγ, and Foxp3 mRNA (B), as well as IL-17, IFN-γ, and TNF protein by CD4+ T cells (C), was determined. Before flow-cytometric analysis, cells were stimulated with PMA/ionomycin. Data of three independent experiments (n = 9) are shown. (D) Mice were treated with PBS, p40-siRNA, or chol-p40-siRNA and immunized for EAE. Expression of Il12p40, Il10, and Il6 in draining lymph nodes was determined on day 3 after immunization (A). Data of three independent experiments (n = 9) are shown. Data were normalized to β-actin, and cytokine expression of control mice was set as 1.0. On day 7 after immunization, the expression of Il17, Ifnγ, and Foxp3 mRNA (B), as well as IL-17, IFN-γ, and TNF protein by CD4+ T cells (C), was determined. Before flow-cytometric analysis, cells were stimulated with PMA/ionomycin. Data of three independent experiments (n = 9) are shown. (D) Mice received a single i.v. injection of [33P]-p40-siRNA or [33P]-chol-p40-siRNA. After 2 h the indicated tissues were isolated and analyzed for [33P] by liquid scintillation counting. (E) Mice were treated as in (D). The uptake of 33P-labeled siRNA by isolated CD11c+ or CD11c- cell fractions from spleen and lymph nodes was determined by liquid scintillation counting. Burs in (D) and (E) represent mean ± SEM of four independent experiments, n = 8. *p < 0.05, **p < 0.01, ***p < 0.001.

siRNA constructs (9, 27). More importantly, separate analysis of CD11c+ cells and of CD11c- cells showed only a significant enrichment of [33P]-chol-p40-siRNA in DCs of lymphoid tissue. In sharp contrast, CD11c+ cells showed no preferential uptake of the nonmodified [33P]-p40-siRNA (Fig. 3E). This explains the modest inhibition of p40 by p40-siRNA in vivo (Fig. 3A) and the strong, biologically significant impact that had exclusively the chol-p40-siRNA on p40, Th1, and Th17 cells (Fig. 3A–C). So far, only CpG-conjugated siRNA constructs or siRNA encapsulated in polysaccharides have been reported to target macrophages in vivo. Yet, these constructs are not suitable for the generation of type II DCs (21, 28). In vivo treatment with chol-p40-siRNA provided the unique possibility to induce type II DCs that severely impaired the generation of autoreactive Th1 and Th17 cells (Fig. 3B, 3C). Therefore, we speculated that chol-p40-siRNA might protect against EAE.

Only chol-p40-siRNA protect from severe EAE, as nonspecific chol-siRNA and nonmodified p40-siRNA do not show clinical benefit

To study the effects of chol-p40-siRNA on EAE, we immunized SJL mice for EAE induction and treated the mice with either PBS, chol-p40-siRNA, or chol-luc-siRNA. PBS-treated mice and chol-luc-siRNA–treated mice developed severe EAE and paraplegia within
Vaccination with chol-p40-siRNA in vivo protects from EAE.

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FIGURE 4. Vaccination with chol-p40-siRNA in vivo protects from EAE. (A–C) Mice treated with PBS or the indicated siRNA constructs were immunized for EAE and followed for clinical signs. Dots represent mean EAE score ± SEM from two (A and C) or four (B) independent experiments with five to nine mice per group. (D) Mice were treated and immunized as in (B) and (C). On day 16 after immunization, shortly before the maximal intensity of encephalomyelitis was achieved, CNS-infiltrating mononuclear cells were quantified. Data were normalized to β-actin, and gene expression of CNS-infiltrating cells from the PBS-treated group was set as 1.0. All data are shown as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

Discussion

In vivo, siRNA allows selective targeting and manipulation of metabolic functions in a variety of cells such as microglia, endothelia, tumor cells, or hepatocytes (8, 9, 28). In sharp contrast, in vitro data suggest that macrophages and DCs largely resist siRNA uptake. To circumvent this problem, we followed different strategies. For instance, siRNA was encapsulated with liposomes, lipoplexes, polymers, and peptides, or conjugated to Ab complexes or CpG as siRNA carriers (28–30). Yet, none of these approaches seems to be suitable for treatment of inflammatory autoimmune diseases, because they cause major problems related to direct activation of APCs (28, 31), pharmacokinetics (21), toxicities, and target cell delivery. We show that in vitro and in vivo chol-siRNA allow specific silencing of genes and relatively precise targeting of cells where lipoprotein-bound conjugates can be internalized by receptor-mediated processes with predictable pharmacodynamics (8, 9). So far, only one report suggested using chol-siRNA for silencing gene expression by DCs, confirming the feasibility of our approach (32). Pinocytosis/endocytosis of chol-siRNA depends on the binding to lipoprotein receptors and transmembrane proteins like scavenger receptor class B type I or...
CD36 (9). CD11c+ DCs typically express such receptors (33) and are therefore ideal targets for chol-siRNA constructs (9). By using two methodologies, FAM-labeling and [3H]-radiolabeling, we could demonstrate for the first time, to our knowledge, the efficient uptake of chol-siRNA by DCs in vitro and in vivo. Although Cpg-conjugated siRNA is an elegant alternative approach to target DCs, the use of this strategy is not feasible in the setting of inflammatory autoimmune diseases like EAE. In contrast, by using chol-p40-siRNA, we could show that cholesterol modification of siRNA had no stimulatory effects on the DC phenotype or their maturation status in vitro and in vivo (data not shown). An additional advantage of chol-siRNA is the prolonged stability compared with nonmodified constructs (8). As reported, cholesterol modifications help to reduce the sensitivity of siRNA to serum nucleases. This explains that only the cholesterol-modified p40-siRNA, but not the nonmodified p40-siRNA construct, effectively suppressed Th1/Th17 responses and improved EAE. Interestingly, chol-p40-siRNA treatment not only suppressed p40 expression but simultaneously promoted IL-10 production, a phenomenon reported previously in mice deficient in p40 signaling (34, 35). Thus, chol-p40-siRNA induced a type II phenotype in DCs with low IL-12, low IL-23, and high IL-10 levels. Such type II DCs can prime T cells to suppress a type II phenotype in DCs with low IL-12, low IL-23, and high IL-10 levels. Such type II DCs can prime T cells to suppress autoimmunity (5, 36).

Moreover, p40 as target gene/sequence seems to be advantageous over approaches using Abs against IL-10 levels. Such type II DCs can prime T cells to suppress a type II phenotype in DCs with low IL-12, low IL-23, and high IL-10 levels. Such type II DCs can prime T cells to suppress autoimmunity (5, 36).

We could demonstrate for the first time, to our knowledge, the efficient uptake of chol-siRNA by DCs in vitro and in vivo.


Supplemental FIGURE 1  Identification and cholesterol-modification of p40-targeting siRNAs. (A) Sequences of p40-targeting siRNAs. (B) Immature bone marrow-derived DC were electroporated with a panel of 6 cholesterol modified siRNA constructs (A) in vitro. DC were then activated with LPS for 18 h before analyzing the cytokine production from supernatants by ELISA. Bars represent mean ± SEM. (C) Structure of the cholesterol modification conjugated to the 3’-end of siRNA constructs.
**Supplemental FIGURE 2** Treatment with chol-p40-siRNA reduces T cell infiltration and myelin loss within the CNS. Fluoro-myelin-staining and immunostaining of CD3+ CNS-infiltrating lymphocytes in brain tissue sections. Tissue samples were prepared from naïve mice, mice immunized for EAE (control) and mice treated with the indicated siRNA constructs before immunization for EAE on day 14-16 after immunization. Cryosections were stained with anti CD3 (red), fluoromyelin (green) and Dapi (blue).
Supplemental FIGURE 3 Treatment with chol-p40-siRNA inhibits Th1 and Th17 cell development in the CNS. (A) Mice were injected with PBS, chol-p40-siRNA, chol-luc-siRNA or p40-siRNA. On day 7 after immunization, CD4+ T cells were purified from draining lymph nodes and co-cultured with DCs and PLP peptide. T cell proliferation was assessed by [3H]thymidine incorporation (B-E) Mice were treated as in Fig. 3B. On day 16 after immunization CNS-infiltrating mononuclear cells were isolated and stimulated with PMA and ionomycin. Cells were stained for CD4 and intracellular cytokines or FoxP3. Dot plots are representative for two independent experiments with mice treated with either PBS, p40-siRNA or chol-p40-siRNA. (E) The absolute numbers of CNS-infiltrating IL-17+, IFN-γ+ or IL-4+ CD4+ T cells of each group were assessed by intracellular cytokine staining. Pooled cells from 5 mice per group.*p < 0.05, **p < 0.01.
Supplemental FIGURE 4 Chol-p40-siRNA injections delay disease severity in mice with EAE. Mice were immunized with PLP peptide. When mice developed EAE with a score of ≥1 they were injected on three consecutive days with either PBS, chol-luc-siRNA, chol-p40-siRNA or p40-siRNA (↓↓) and followed for clinical signs. The dots represent mean EAE score ± SEM from 2 independent experiments (*p < 0.05).