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# Cationic lipids enhance siRNA-mediated interferon response in mice

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**Abstract**

RNA interference mediated by small interfering RNAs (siRNAs) shows promise as a powerful research tool for gene function studies. However, controversy exists over the potential of siRNA-induced interferon response in vitro and in vivo. In this study, we showed that although intravenous administration of siRNA alone is essentially inert, injection of siRNA complexed with cationic liposomes resulted in a potent induction of both type I and type II interferon responses. Furthermore, i.v. administration of cationic lipid/siRNA complexes led to activation of STAT1. This study suggests cautions in data interpretation and the potential toxicity with in vivo use of siRNA, particularly when delivered via a cationic lipid vector. This study also suggests the potential of siRNA as an immunostimulatory agent for immunotherapy.


*Keywords:* RNA interference; siRNA; Cationic liposome; Interferon response; Innate immunity

RNA interference (RNAi) is a natural cellular process that effects gene silencing in eukaryotic systems at transcriptional, posttranscriptional, and/or translational levels [1–3]. Small interfering RNA (siRNA) molecules are the key intermediaries in this process which when exogenously administered can potentially inhibit the expression of any given target gene. Due to its high reliability and high efficiency siRNA has recently become the most widely used nucleic acid-based sequence-specific gene silencing molecule. SiRNA molecules hold great promise as biological tools and as potential therapeutic agents for targeted inhibition of disease-causing genes.

Despite the demonstrated sequence-specific inhibition of target genes in many studies, siRNA has recently been shown in several studies to induce the silencing of unintended genes in cultured cells [4–8]. In addition, intracellular delivery of siRNA has been shown to activate interferon (IFN) response which appears to be responsible for the "off-target" effect observed in some studies [4–6]. Interestingly, intravenous (i.v.) administration of siRNA has been shown to induce minimal, if any, IFN response in mice [9]. While it remains to be determined what is responsible for the differences in those studies, it is apparent that cationic lipids were used in the transfection of siRNA in cultured cells while siRNA siRNA alone was used in the animal studies. It has been previously shown by us and others that cationic lipids significantly enhance the CpG DNA-mediated immune response in cultured cells and in intact mice [10–13]. We hypothesized that cationic lipids may similarly enhance the siRNA-mediated immune response. In this study, we showed that although i.v. administration of siRNA alone is essentially inert, injection of cationic lipid/siRNA complexes resulted in a potent induction of both type I and type II IFN response. Furthermore, i.v. administration of cationic lipid/siRNA complexes led to activation of STAT1. This study suggests caution in data interpretation and the potential toxicity with in vivo use of siRNA, particularly when delivered via a cationic lipid vector.

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## Materials and methods

*Reagents.* 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) and cholesterol were purchased from Avanti Lipids (Alabaster, AL). CpG oligodeoxynucleotide (ODN) 1668 was synthesized by MWG Biotech (High point, NC). All double-stranded and single-stranded siRNAs were synthesized from Dharmacon RNA Technologies (Lafayette, CO).

*Preparation of liposomes.* Liposomes containing DOTAP in a 55:45 molar ratio with cholesterol were prepared as follows. Lipid mixtures in chloroform were dried as a thin layer in a 25-ml round-bottomed flask, which was further dried under vacuum for 2 h. The lipid film was hydrated in water to give a final concentration of 10 mg DOTAP/ml. Small unilamellar lipid vesicles were then prepared by extrusion through 0.2 μm polycarbonate membranes.

*Preparation of DOTAP/siRNA complexes.* SiRNA was mixed with DOTAP liposome in a charge ratio (+/−) of 4:1 in 5% dextrose. The mixture was allowed to stand at room temperature for 10 min before use. Complexes were injected into mice through the tail vein (200 μl/mouse).

*Animals and in vivo experiments.* Female C57BL/6 mice, 20 g in body weight, were purchased from Charles River Laboratories (Wilmington, MA). All mice were housed in accordance with Institutional Guidelines. All experiments were conducted under approved Guidelines of the Animal Ethics Committee at the University of Pittsburgh. For challenge experiment, groups of three mice were i.v. injected with siRNA (50 μg/mouse), liposome, DOTAP/CpG (50 μg CpG DNA/mouse), and DOTAP/siRNA complexes (50 μg siRNA/mouse), respectively. At 2 (for TNF-α) or 4 (for IFN-α and IFN-γ) h following the injection, blood samples were collected and examined for the cytokine levels by ELISA. For siRNA multiple injection experiment, mice were injected with siRNA dissolved in 5% dextrose daily for 2 or 3 days. Serum was collected at 2 or 4 h following the last injection and subjected to cytokine analysis.

*ELISA.* Mouse TNF-α and INF-γ ELISA sets were purchased from BD Bioscience Pharmingen (San Diego, CA). Sandwich ELISA was performed according to manufacturer's instructions. Serum IFN-α levels were determined with a specific ELISA kit for mouse IFN-α (R&D, Minneapolis, MN).

*Western blotting.* Female C57BL/6 mice were i.v. injected with siRNA, DOTAP liposome or DOTAP/siRNA complexes, and lungs and spleen were collected 4 or 6 h following the injection. Tissues were lysed in lysis buffer containing 1% Triton X-100, 100 mM NaCl, 20 mM Tris–HCl, pH 7.6, and protease inhibitor cocktail (Calbiochem, San Diego, CA). Protein was analyzed on a 10% Tris–HCl polyacrylamide gel (Bio-Rad) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked in 5% non-fat milk, 0.05% Tween 20 in PBS for 1–2 h at room temperature, and incubated overnight with primary antibody followed by a 1-h incubation at room temperature with anti-rabbit horseradish peroxidase (Cell Signaling Technology, Beverly, MA). Immunoblotting was performed using ECL (Amersham Pharmacia Biotech, Piscataway, NJ). Phosphor-STAT1 antibody was purchased from Upstate Cell Signaling Solutions (Lake Placid, NY). β-Actin antibody was obtained from Sigma (St. Louis, USA).

## Results and discussion

A previous study by Heidel et al. [9] showed that i.v. administration of siRNA was associated with minimal cytokine response in mice. Similar results were observed in our study (Fig. 1A). Only a low level of IFN-α was observed in the serum of mice at 4 h following i.v. injection of TLR4-siRNA and there was no significant increase in IFN-α response following repeated dosing (Fig. 1A). TLR4-siRNA is a siRNA that is specific for mouse TLR4. Delivery of TLR4-siRNA to cultured mouse macrophages has been shown to downregulate TLR4 expression and inhibit the ICAM-1 response following a subsequent challenge by LPS (Ma et al., unpublished data). No IFN-γ was detected in mouse serum at 4 h following i.v. administration of TLR4-siRNA (Fig. 1B). We then similarly examined the serum levels of IFN-α and IFN-γ following i.v. administration of TLR4-siRNA that was complexed with DOTAP cationic liposomes. As shown in Fig. 1, i.v. administration of DOTAP/TLR4-siRNA complexes led to a potent immune response as demonstrated by a drastic increase in the serum levels of both IFN-α (Fig. 1A) and IFN-γ (Fig. 1B). Injection of DOTAP liposomes alone was only associated with a low level of IFN response (Figs. 1A and B).

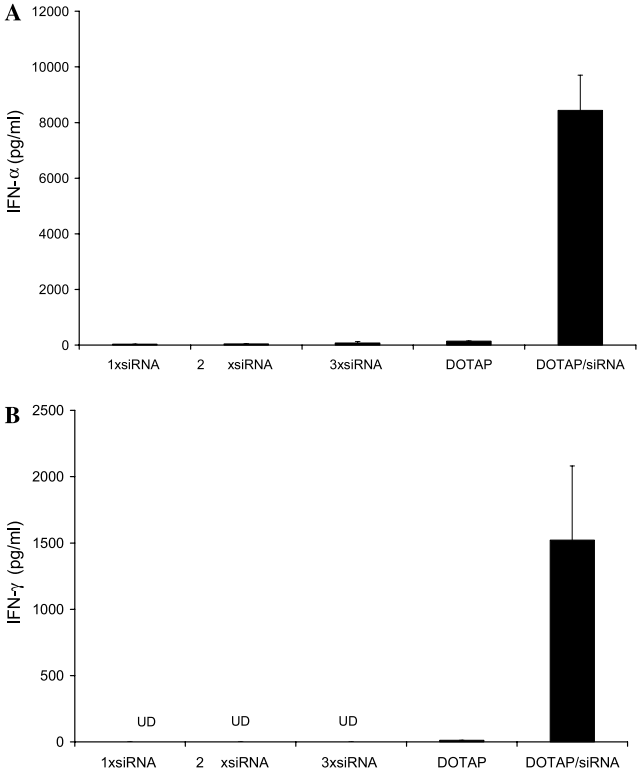


Fig. 1. Intravenous injection of DOTAP/TLR4-siRNA complexes led to a potent IFN response. Mice were i.v. injected with siRNA alone, DOTAP liposomes alone or DOTAP/TLR4-siRNA complexes. Serum levels of IFN-α (A) and IFN-γ (B) were evaluated, respectively, at 4 h following the injection ($n = 3$). UD, undetectable.

We then investigated whether the complex-induced IFN response was due to the specific sequence of TLR4-siRNA. Thus, we similarly examined the serum levels of IFN-α and IFN-γ following i.v. administration of DOTAP liposomes complexed with three other siRNAs (Table 1), respectively. As shown in Fig. 2, a potent IFN response was observed for all siRNAs