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Immune Activation and Target Organ Damage Are Consequences of Hydrodynamic Treatment but Not Delivery of Naked siRNAs in Mice

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Short-interfering RNAs (siRNAs), key mediators of RNA interference comprise a promising therapeutic tool, although side effects such as interferon (IFN) response are still not perfectly understood. Further, delivery to target organs is a major challenge, possibly associated with side effects including immune activation or organ damage. We investigated whether immune activation as a consequence of double-stranded RNA induced IFN response (Jak/STAT pathway activation or cytokine production) or target organ damage is induced by *in vivo* low-volume (LV) or high-volume (HV) hydrodynamic delivery or treatment with naked siRNA. NMRI mice were injected with naked siRNAs or saline by hydrodynamic injection (HDI) and positive control mice received polyinosinic–polycytidilic acid (poly I:C). LV (1 mL/mouse) and HV (10% of body weight) HDI were compared. After LV HDI, STAT1 and OAS1 gene expression inflammatory cytokine plasma levels and target organ injury were assessed. LV HDI induced slight alanine aminotransferase elevation and mild hepatocyte injury, whereas HV HDI resulted in high ALAT level and extensive hepatocyte necrosis. STAT1 or OAS1 was not induced by LV siRNA; however, HV saline led to a time-dependent slight increase in gene expression. Inflammatory cytokine plasma level and organ histology and functional parameters demonstrated no damage following LV HDI with or without siRNA. Our data demonstrate that naked siRNAs may be harnessed, without the induction of IFN response or immune activation, and that LV HDI is preferable, because HV HDI may cause organ damage.

Introduction

RNA interference (RNAi) is a recently described, potent, nucleic acid-based antiviral defense mechanism (Napoli et al., 1990). It may be potentially utilized in human therapy by target sequence-specific inhibition of cellular protein synthesis. Cellular entrance of a double-stranded (ds) short-interfering RNA (siRNA) leads to the enzymatic degradation of complementary messenger RNA, thus silencing protein synthesis. Thus, a wide range of diseases including viral infections and malignancies may be treated with siRNA. RNAi is also an important research tool, reviewed by Racz and Hamar (2006).

One of the main difficulties for clinical application of siRNAs is delivery into target organs and the cytosol of target cells. Under experimental conditions in rodents, this is often achieved by hydrodynamic injection (HDI). As HDI has been proposed for clinical application (Sawyer et al., 2009), its possible side effects should be also considered. Originally, high-volume (HV) HDI is a high-pressure tail vein injection wherein injection volume equals 10% of the body weight (2.5 mL for a 25 g mouse), which is injected into the vena cava system through the tail vein within a short time (10 seconds). Such HV causes reversible and irreversible morphological changes in the liver (Budker et al., 2006). The sudden pressure increase leads to pore openings of the parenchymal cells of organs in the vena cava system (liver, spleen, and kidney) (Liu et al., 1999; Kobayashi et al., 2001). Consequently, parenchymal cells of these organs take up injected oligonucleotides, such as siRNAs that guide gene silencing. However, irreversible cell damage may also occur because of the sudden pressure overload. Because of its liver-damaging effects, we (Hamar et al., 2004) and others (Zheng et al., 2006) used a low-volume (1 mL saline per 25 g mouse) HDI. Although it has been demonstrated that transfection efficiency is highly dependent on the injected volume, 1 mL/25 g BW has been demonstrated to be effective (Liu et al., 1999) for siRNA uptake and silencing in target organs without irreversible organ damage (Zhang et al., 1999).

Intracellular administration of alien RNA molecules may activate the interferon response, a possible side effect of siRNA injection. Interferons (IFNs) (Isaacs and Lindenmann,

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1957) are involved in antiviral defense and immune activation (Goodbourn, Didcock et al., 2000), especially type I IFNs, such as IFN-α/β, which are secreted in direct response to dsRNAs produced during the life cycle of viruses. dsRNAs trigger the Janus activated kinase/signal transducer and activator of transcription (Jak/STAT) signaling pathway (Li and Hassel, 2001), inducing the transcription of IFN-α/β–inducible genes (Stark et al., 1998). Further, it has been suggested that Jak1 is required for STAT activation (Yang et al., 2005).

Another downstream pathway from dsRNA recognition is the activation of the oligoadenylate synthase (OAS) enzyme, which polymerizes ATP into oligoadenylate polymers that activate the latent RNaseL enzyme, which cleaves single-stranded RNAs (Dong and Silverman, 1995), leading to the elimination of alien RNA molecules (Castelli et al., 1998) as part of the intrinsic cellular antiviral defense (Boo and Yang, 2010). IFN-α/β production can be initiated also through receptor-mediated recognition of siRNA by toll-like receptors (TLRs). TLR3 recognizes dsRNA such as siRNAs (Marques and Williams, 2005) and a synthetic dsRNA: polyinosinic–polycytidilic acid (poly I:C) (Alexopoulou et al., 2001). As part of the antiviral cellular response, TLR3 recognition of dsRNA is followed by immune-cell activation and IFN-α, tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6) cytokine production (Sioud, 2005). Thus, poly I:C evokes the production of IFNs (Garcia-Sastre and Biron, 2006). In the present study, we treated mice with poly I:C as a positive control to induce an IFN response and related immune activation. Also, TLR7 and TLR8 recognize RNA viruses (Marques and Williams, 2005) accompanied by inflammatory cytokine and IFN production (Heil et al., 2004).

Initially, it was held that short, 19–21-mer dsRNA do not induce IFN response (Dahlgren et al., 2006). However, this thesis has been questioned *in vitro* (Sledz et al., 2003) and *in vivo*. To investigate whether short dsRNA may induce an IFN response, we systemically administered 2 different naked siRNAs in mice via low-volume (LV) HDI (Hamar et al., 2004) and investigated IFN response gene expression, cytokine plasma levels, and morphology in target organs of HDI.

Materials and Methods

Mice

Male NMRI (Naval Medical Research Institute) mice, weighing 25–30 g, were purchased from ToxiCoop. Animals were kept on a regular rodent chow and water *ad libitum*. All animal experiments were carried out according to the institutional regulations (Semmelweis University Board 53/2001 [V.31.] ET) and the Hungarian law on animal care and protection (1998/XVIII, 243/1998[XII.31]).

siRNAs

Naked, lyophilized siRNAs were purchased from Dharmacon. Sequences were for mouse Fas (*mFas*: 5-pGUG CAAGUGCAAACCAGACdTdT-3 [sense] and 5-pGUCUG GUUU GCACUUGCACdTdT-3 [antisense]) and green fluorescent protein (*GFP*: 5-pGGCUACGUCCAGGAGCGCACC-3 [sense] and 5-pUGCGCUCCUGGACGUAGCCUU-3 [antisense]). siRNAs were dissolved in diethylpyrocarbonate (DEPC)-treated water for stock solutions, and the solutions were prepared by diluting 50 μg naked siRNA in 1 mL (LV HDI) or 2.5 mL (HV HDI) sterile physiological salt solution (SalsolA; HUMAN) right before use.

Injections

siRNAs were delivered *in vivo* using a modified HDI method. Briefly, 50 μg siRNA dissolved in 1 mL of physiological salt solution was rapidly injected (within maximally 10 seconds) into one of the dilated side tail veins of mice ($n = 24$), and the point of injection was pressed for 30 seconds after needle removal, to prevent backflow of the injected fluid. Physiological saline-treated mice were injected with 1 mL (LV) saline ($n = 24$) to control for the hydrodynamic process *per se*. To reproduce liver-damaging effect of HV HDI reported in the literature, we injected an HV corresponding to 10% of body weight (2.5 mL in average; HV) saline into the tail vein of mice, within 10 seconds.

Further, 5 mice were harvested without any treatment using the same harvest protocol (see later) as otherwise to serve as negative controls.

To stimulate an IFN response, positive controls received 50 μg poly I:C (P9582; Sigma) dissolved in 0.1 mL SalsolA physiological salt solution (500 μg/mL) intraperitoneally ($n = 24$) (Kuhn et al., 1995).

siRNA detection

To demonstrate the cellular uptake and intracellular localization of siRNAs, we injected fluorescently labeled siRNA, BlockiT Alexa Fluor Red Fluorescent Oligo (4750-100; Invitrogen), into the tail vein of mice. Ten to 30 minutes after injection, small pieces of kidney, liver, and spleen of mice were dissected, embedded into OCT compound (01480; Surgipath Medical, Inc.), and snap frozen in liquid nitrogen. Five-micrometer-thick sections were cut and mounted with Mounting Medium for Fluorescensce with DAPI (S3023; Dako, Biomarker). Samples were observed using a Leica DMR microscope (Leica Microsystems) coupled to a Leica DC500 CCD camera controlled by the Leica IM50 software.

Harvest

Mice were harvested at 6 and 24 hours after treatment. A separate set of animals with similar treatment were harvested at 1.5, 2, 6, and 9 hours after LV HDI for detection of organ functional parameters and plasma cytokine levels. Harvest began with ether anesthesia (08640-1-01-65; REANAL) and heparinization (4958; Heparin Biochemie 5000NE/mL) of mice. Blood was taken from the vena cava superior and plasma was separated by centrifugation of heparinized blood at 4°C and 2000 rpm for 10 minutes. Mice were perfused with 20 mL of ice-cold Hank's buffered salt solution (H9394; Sigma-Aldrich) administered through the left ventricle using a 20-mL syringe and an 18G needle. Kidney, liver, and spleen were excised and small pieces of these organs were placed in microtubes for further RNA analysis.

Measurement of alanine aminotransferase, aspartate aminotransferase, and blood urea in plasma and whole blood

Alanine aminotransferase (ALAT; U/L), aspartate aminotransferase (ASAT; U/L), and blood urea (mg/dL) levels

were evaluated in a Reflotron Plus laboratory machine with enzymatic reagents (Boehringer Mannheim) from 32 μL plasma or heparinized whole blood of hydrodynamically treated mice at 1, 3, 6, 16, and 24 hours after injection.

RNA isolation

Total RNA was isolated from harvested mouse kidney, liver, and spleen using TRIzol (15596-018; Invitrogen) according to the manufacturer's instructions (www.invitrogen.com/content/sfs/manuals/15596018%20pps%20Trizol%20Reagent%20061207.pdf). Briefly, 1 mL of ice-cold TRIzol was added to 30–50-mg frozen tissue pieces, which were then homogenized for 40–100 seconds (according to tissue structure) in a FastPrep homogenizer. Samples were phase separated after centrifuging in the presence of 0.2 mL chloroform (C2432; Sigma-Aldrich). RNAs within the aqueous phase were precipitated by adding 0.5 mL isopropyl alcohol (I9516; Sigma-Aldrich). After centrifugation, RNA pellets were washed with 75% ethanol and briefly air dried. Pellets were dissolved in 50–250 μL DEPC-treated water according to pellet size. Isolated RNAs were stored at -80°C until use. Prior to reverse transcription, 36 μL RNA sample was digested with 4 U of TURBO DNase (2238; Ambion) to eliminate genomic DNA contamination using the manufacturer's protocol.

cDNA synthesis

Equal amounts (1 μg) of RNA samples were reverse transcribed using SuperScript III (1808-093; Invitrogen) and random hexamers (Integrated DNA Technologies) according to the manufacturer's instructions. Briefly, 1 μg of DNA-free RNA sample was mixed with 1 μL of 10 mM dNTPs (18427-013; Molecular Bioprobes) and 1.2 μL random hexamers, heated to 65°C for 5 minutes, and placed on ice for 1 minute. After adding 4 μL of 5×first-strand buffer, 1 μL of 0.1 M dithiothreitol (DTT), 1 μL RNase Out Recombinant Ribonuclease Inhibitor (10777-019; Invitrogen), and SuperScript III reverse transcriptase (18080-044; Invitrogen) (200 U/μL) or nuclease-free water (for no reverse transcriptase reaction) into the tubes, they were centrifuged and incubated in water bath at 50°C for 1 hour. The reaction was inactivated by heating at 70°C for 15 minutes. cDNAs were stored at -20°C until real-time polymerase chain reaction (PCR).

Quantitative real-time-PCR

Real-time PCR was performed on 1 μL of cDNA using Platinum Taq Polymerase (10966-018; Invitrogen) and an IQ5 Bio-Rad iCycler (Bio-Rad) as described elsewhere (Palliser et al., 2006). Briefly, reactions were performed in 25 μL reaction mixtures and in triplicates. The following primers were used:

GAPDH: Forward, 5′-TTCACCACCATGGAGAAGGC-3′
Reverse, 5′-GGCATGGACTGTGGTCATGA-3′
STAT1: Forward, 5′-TTTGCCCAGACTCGAGCTCCTG-3′
Reverse, 5′-GGGTGCAGGTTCGGGATTCAAC-3′
OAS1: Forward, 5′-TATGCCCTGCCTCCCTTCCATC-3′
Reverse, 5′-CTGTTGGGCTTTGGGCACCTTC-3′

SYBR Green (67563; Molecular Probes) was used to detect PCR products. PCR parameters consisted of 5-minute Taq activation at 95°C, followed by 40 cycles of 95°C for 20 seconds, 60°C for 20 seconds, and 65°C for 1 minute. Relative gene expression of STAT1 and OAS1 were calculated with the aid of GADPH as a housekeeping gene. Specificity was verified by melt curve analysis.

Histologic staining and evaluation

Small pieces (0.5×0.5 cm) of liver, spleen, and kidney harvested at 6 and 24 hours after LV HDI were fixed in paraformaldehyde (4.5%, phosphate buffered) for 1 day and then were dehydrated and embedded in paraffin. Four- to 6-μm-thick sections were deparaffinized, rehydrated, and stained consecutively with hematoxylin and eosin and periodic-acid Shiff. Samples were examined in a blinded fashion. All photomicrograms were taken with a Leica DC500 microscope.

Plasma enzyme-linked immunosorbent assay measurements

Plasma IL-6, IL-12 (88-7064, 88-7120; eBioscience), TNF-α (MTA00; R&D Systems), and IFN-beta (IFN-β) levels were quantitatively measured ($n = 4$) by commercial enzyme-linked immunosorbent assay (ELISA) (42400-1; PBL Interferon Source) according to manufacturer's instructions. In brief, after coating plates, 100 μL of standards and 100 or 50 μL samples were added into each well and incubated overnight at 4°C (IL-6 and IL-12) or at room temperature for 2 hours (TNF-α). Then, the wells were washed 5 times. One hundred microliters of detection antibody was added and incubated for 1 hour. After 5 washes, avidin–HRP solution was added into the wells (100 μL/well), and further incubated at room temperature for 30 minutes. The wells were washed 5 times, and TMB substrate solution was added (100 μL/well) and allowed to stay at room temperature for 15 minutes. Optical density was determined within 5 minutes at 450 nm (Perkin Elmer Victor 3 1420 Multilabel counter).

Statistical analysis

Results are presented as average ± SEM of n independent experiments. Multiple comparison statistical analysis was carried out by one-way ANOVA followed by a post hoc Tukeys HSD test (with Statistica8 program). $P < 0.05$ was considered statistically significant.

Results

Effective siRNA uptake and silencing

LV HDI of fluorescently labeled siRNAs, BlockiT Alexa Fluor Red Fluorescent Oligo (BlockiT), demonstrated successful cellular siRNA uptake within the kidney (Fig. 1A), liver (Fig. 1B), and spleen (Fig. 1C) after hydrodynamic tail vein injection. Injected siRNAs appeared in the cytoplasm at 10–15 minutes after injection.

In our previous experiment, mFas-specific siRNA sequence effectively silenced FAS expression in mouse kidneys as demonstrated by real-time-PCR compared with untreated, saline-treated, or indifferent GFP-silencing siRNA-treated mice (Hamar et al., 2004).

LV HDIs

LV HDI induced slight STAT1 and OAS1 expression not further elevated by siRNA. In positive control poly I:C LV

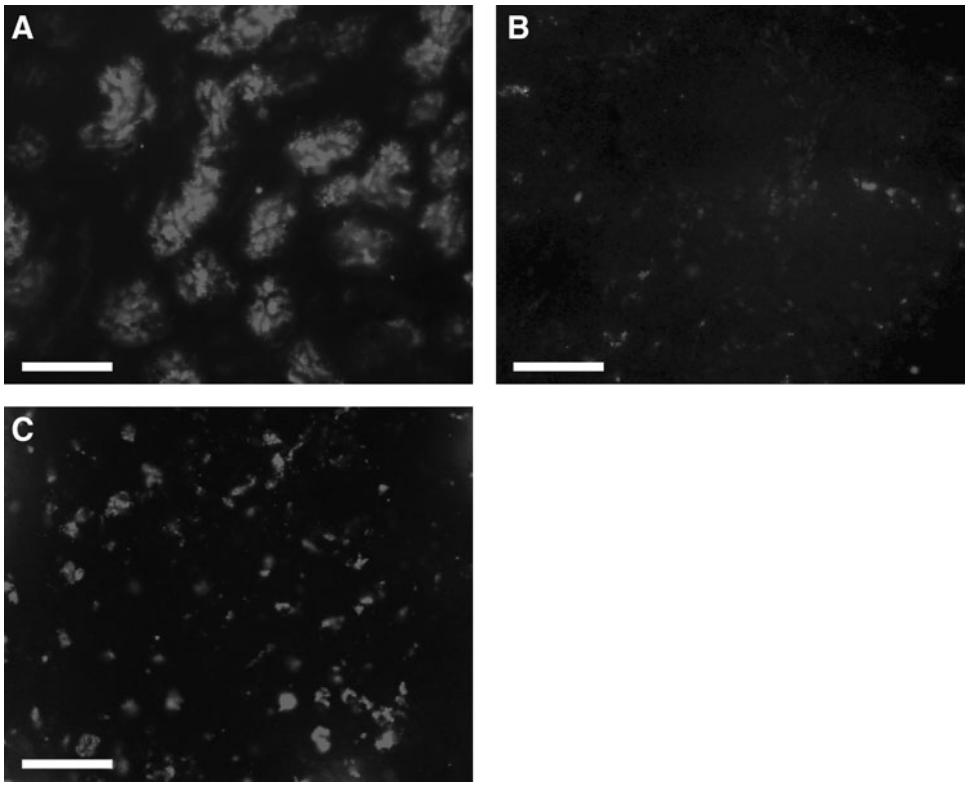

FIG. 1. Intracellular uptake of siRNA in kidney **(A)**, liver **(B)**, and spleen **(C)** (400×) at 30 minutes after HDI of BLOCK-iT Alexa Flour Red Fluorescent Oligo. Photos taken with N2.1 filter cube (excitation range: 515–560 nm) of a Leica DC500 microscope. Scale bar represents 37.5 μm. HDI, hydrodynamic injection; siRNA, short-interfering RNAs.

HDI-treated mice, significant STAT-1 and OAS1 organ gene expression was induced as revealed by real-time-PCR at 6 and 24 hours after injection in all investigated target organs (kidney, liver, spleen) (Table 1). On the contrary, slight increases were seen in STAT1 and OAS1 gene expressions after LV HDI with physiologic saline, compared with untreated control animals, in the liver and kidney but not in the spleen, 6 hours postinjection. OAS1 expression further slightly increased at 24 hours postinjection in the kidney (Table 1). On the other hand, no significant difference was observed between saline- and

Table 1. Gene Expression Changes of STAT1 and OAS1 After Hydrodynamic Treatment

Hours postinjection	*Gene expression*	*siRNA treatment*	*Kidney*	*Liver*	*Spleen*
6	STAT1	mFas	13.4 ± 4.1^{a}	0.94 ± 0.1	3.5 ± 3.5
6	STAT1	GFP	15.9 ± 5.5^{a}	1.3 ± 0.2	2.5 ± 0.5
6	STAT1	Saline	14.6 ± 3.7^{a}	1.21 ± 1.0	2.7 ± 1.3
6	STAT1	Poly I:C	7.24 ± 4.8^{b}	115.8 ± 3.5^{b}	287.1 ± 72^{b}
6	OAS1	mFas	4.4 ± 3.8	3.1 ± 1.5	7.1 ± 1.7
6	OAS1	GFP	6.5 ± 2.2	5.5 ± 3.8	9.4 ± 5.5
6	OAS1	Saline	3.8 ± 0.8	8.5 ± 2.2^{a}	9.5 ± 6.6
6	OAS1	Poly I:C	19.5 ± 2.0^{b}	24.7 ± 3.9^{b}	32.1 ± 4.0^{b}
24	STAT1	mFas	14.09 ± 2.9^{a}	2.1 ± 1.1	1.9 ± 2.1
24	STAT1	GFP	17.1 ± 6.0^{a}	2.0 ± 1.1	3.0 ± 2.2
24	STAT1	Saline	16.2 ± 5.2^{a}	1.7 ± 0.7	4.3 ± 3.5
24	STAT1	Poly I:C	43.5 ± 0.7^{b}	67.7 ± 1.7^{b}	125.1 ± 88.1^{b}
24	OAS1	mFas	$8.8 \pm 5.8^{a,c}$	10.7 ± 6.0^{a}	4.81 ± 9.6
24	OAS1	GFP	$11.1 \pm 9.0^{a,c}$	12.6 ± 6.0^{a}	8.55 ± 5.2
24	OAS1	Saline	$10.7 \pm 3.5^{a,c}$	13.3 ± 7.3^{a}	7.93 ± 7.2
24	OAS1	Poly I:C	28.9 ± 4.1^{b}	33.1 ± 2.8^{b}	28.35 ± 6.0^{b}
0	STAT1	No treatment	10.3 ± 2.6	0.8 ± 3.7	2.2 ± 2.4
0	OAS1	No treatment	3.4 ± 1.3	3.5 ± 1.3	4.2 ± 1.9

Gene expression (%) relative to mGAPDH at 6 and 24 hours after hydrodynamic treatment of mice. Data are the average of 3 real-time-PCRs for all samples ± SD.

[a] $P < 0.05$ vs. no treatment.

[b] $P < 0.01$ vs. no treatment.

[c] $P < 0.05$ vs. corresponding 6-hour result.

GFP, green fluorescent protein; mFas, mouse Fas; PCR, polymerase chain reaction; OAS, oligoadenylate synthase; poly I:C, polyinosinic–polycytidilic acid; siRNA, short interfering RNA.

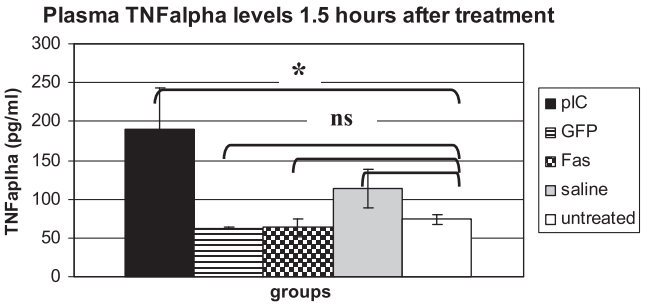

FIG. 2. Plasma TNF-α levels at 1.5 hours after LV HDI of mFas siRNA (Fas), GFP siRNA (GFP), saline, and poly I:C (pIC). Bars represent groups; error bars represent standard deviation. $^{*}P < 0.05$ vs. untreated. mFas, mouse Fas; pIC, poly I:C; GFP, green fluorescent protein; LV, low volume; TNF-α, tumor necrosis factor alpha; poly I:C, polyinosinic–polycytidilic acid; ns, not significant vs. untreated.

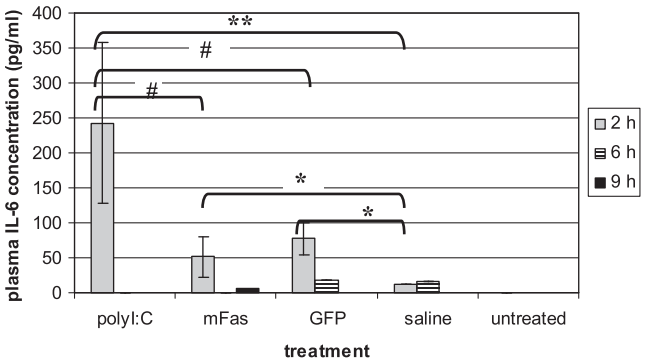

FIG. 3. Plasma IL-6 levels at 2, 6, and 9 hours after HDI of mFas siRNA (mFas), GFP siRNA (GFP), saline, and poly I:C (pIC). Bars represent groups; error bars represent standard deviation. $^{*}P < 0.05$ vs. saline treatment; $^{**}P < 0.01$ vs. saline treatment; $^{\#}P < 0.05$ vs. poly I:C treatment.

siRNA-containing saline-treated animals at any time point, any organ, or any siRNA sequence. Thus, the addition of siRNA to saline LV HDI did not result in further upregulation of STAT1 or OAS1 expression.

LV HDI did not induce significant systemic inflammatory cytokine (IFN-β, TNF-α, IL-6, and IL-12) elevation. Cytokine ELISAs detected baseline IFN response-related cytokine (TNF-α, IL-6, IL-12) levels in untreated negative control mice. In poly I:C-treated positive control mice, these cytokines were stimulated. However, siRNA treatment did not induce cytokine responses comparable to poly I:C treatment.

Plasma IFN-β level increased at 2 hours after poly I:C treatment (210.85 ± 164.28 pg/mL), whereas samples of all the other groups contained unmeasurably low amount of IFN-β.

TNF-α was only stimulated at 1.5 hours after poly I:C treatment (Fig. 2) and returned to undetectable levels in all samples of untreated and treated mice at 6 and 24 hours after injection.

Plasma IL-6 concentration was undetectable in untreated mice. Hydrodynamic treatment with poly I:C induced a major IL-6 response at 2 hours after treatment, diminishing thereafter. In siRNA-treated mice, a small peak of IL-6 was observed at 2 hours, but this returned to normal level at 6 and 9 hours after LV HDI (Fig. 3). IL-6 levels in siRNA- and saline-treated mice were significantly lower than in poly I:C-treated positive control mice (Fig. 3).

Plasma IL-12 levels were significantly higher in poly I:C-treated positive control compared with saline-treated mice ($P = 4.32\text{E-}08$), peaking at 2 hours. siRNA injection did not significantly elevate plasma IL-12 levels compared with either untreated or saline-treated mice (Fig. 4).

Poly I:C induced an IFN response accompanied by organ damage

Intraperitoneal injection of poly I:C induced severe histopathologic changes in mouse kidney samples.

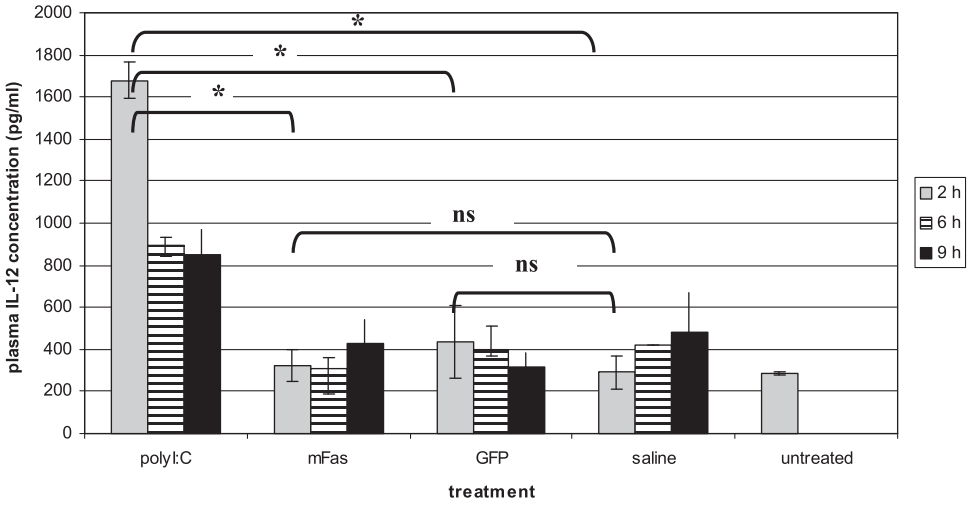

FIG. 4. Plasma IL-12 levels at 2, 6, and 9 hours after HDI of mFas siRNA (mFas), GFP siRNA (GFP), saline, and poly I:C (pIC). Bars represent groups; error bars represent standard deviation. $^{*}P < 0.05$ vs. poly I:C treatment; ns, not significant vs. saline.

Table 2. Whole-Blood Urea and Aspartate Aminotransferase Measurements at 2, 6, 9, and 24 Hours After Low-Volume Hydrodynamic Injection of 50 mg Mouse Fas siRNA, Green Fluorescent Protein Short-Interfering RNAs, Polyinosinic–PolyCytidilic Acid, or 1 mL Saline

Harvest time point	*Groups compared*	*Urea (mg/dL)*	*ASAT (U/L)*
2	mFas siRNA	47.7 ± 6.5 (0.1)	167.5 ± 13 (1.0)
	GFP siRNA	46.1 ± 2.6 (0.1)	174.5 ± 11.4 (0.5)
	Saline	41.8 ± 5.1 (0.4)	167.3 ± 22.1 (0.5)
	Poly I:C	46.7 ± 1.8 (0.1)	258.1 ± 78.1 (0.01)[a]
6	mFas siRNA	41.6 ± 13.7 (0.9)	167.2 ± 10.3 (0.7)
	GFP siRNA	40.6 ± 14.8 (0.8)	161.4 ± 73.6 (0.9)
	Saline	42.5 ± 4.1 (0.5)	164.9 ± 13.8 (0.6)
	Poly I:C	48.1 ± 4.4 (0.01)[a]	290.5 ± 118.7 (0.1)[a]
9	mFas siRNA	39.8 ± 7.3 (0.2)	165.6 ± 14.1 (0.1)
	GFP siRNA	48.1 ± 4.4 (0.3)	153.3 ± 13.6 (0.5)
	Saline	45.3 ± 4.9 (0.5)	144.1 ± 30.06 (0.4)
	Poly I:C	53.5 ± 2.7 (0.01)[a]	503 ± 195.7 (0.01)[a]
24	mFas siRNA	46.9 ± 2.2 (0.8)	165.5 ± 3.5 (0.7)
	GFP siRNA	47.2 ± 17.4 (0.9)	159.5 ± 6.4 (0.9)
	Saline	46.1 ± 2.9 (0.3)	156.5 ± 30.4 (0.9)
	Poly I:C	57.9 ± 6.3 (0.01)[a]	550.7 ± 56.2 (0.01)[a]
-[b]	Untreated	43.6 ± 1.3	158.6 ± 22.8

Numbers represent average ± SD of each group ($n = 6$/group).
[a]P values ($P < 0.05$) (in brackets) are vs. saline-treated group.
[b]Untreated (-) control urea and ASAT data are in the last row.
ASAT, aspartate aminotransferase.

Glomerular damage included mesangial cell necrosis appearing as homogenous, eosinophilic material in glomerular mesangial areas, widening of the mesangial compartment, and compression of glomerular capillaries.

Further, tubular necrosis appeared affecting both the proximal and distal tubules at 6 and 24 hours after treatment, with a time-dependent increase in severity and deterioration of both glomerular and tubular function (Table 2).

The number of megacaryoblasts increased in the spleen. Degenerative hepatocytes with picnotic nuclei were observed in the liver (Fig. 5).

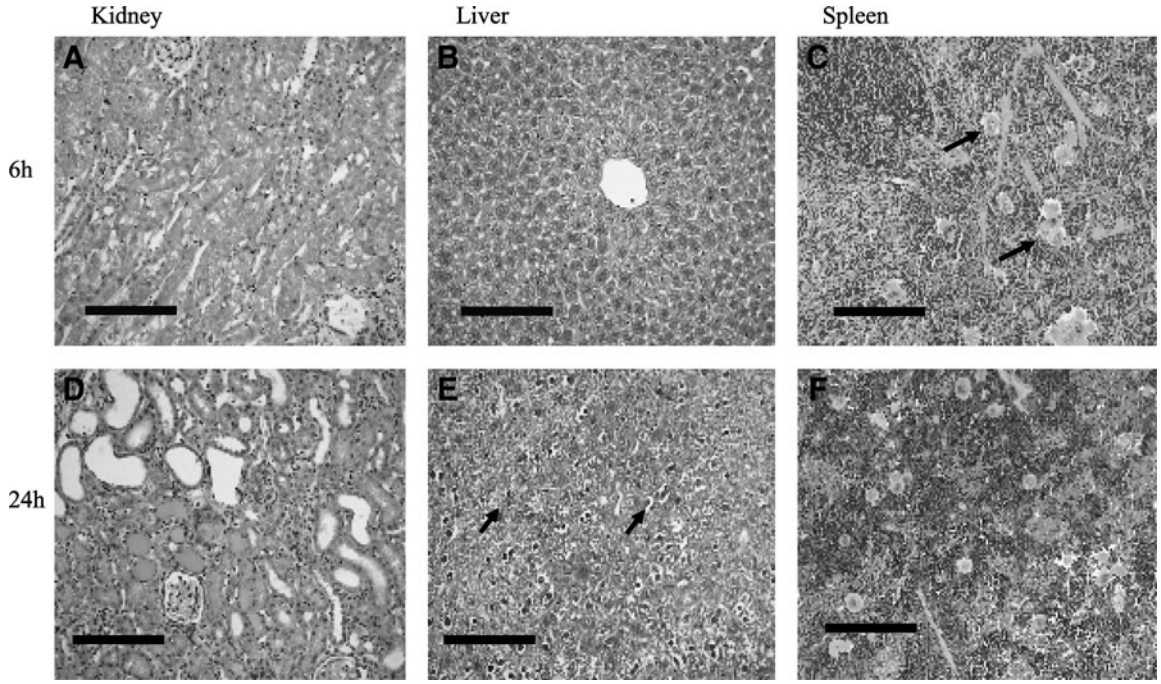

FIG. 5. Organ damage of poly I:C-treated mice after HDI. Poly I:C treatment induced glomerular damage, tubular necrosis, hepatocyte apoptosis (*arrows* on **E** show apoptotic hepatocytes with pycnotic nuclei), and increase in the number of megacaryocytes in the spleen (*arrows*, **C**). Six hours after treatment, these alterations were mild **(A–C)** and they were further aggravated by 24 hours in kidney **(D)**, liver **(E)**, and spleen **(F)**. Hematoxylin and eosin; 200×magnification. Scale bars represent 25 μm.

No deterioration of organ function and histology after LV siRNA injection

LV siRNA-treated mice had normal kidney, liver, and spleen histology at both 6 and 24 hours postinjection (Fig. 6). Renal glomerular, tubular, and interstitial structure was normal in all mice treated with LV HDI (with or without siRNA).

Normal renal histology was supported by renal glomerular and tubular function. Both blood urea and ASAT (Table 2) levels were similar to untreated negative control. Hepatocytes were arranged in hepatic plates in a radian position with normal structure of portal triads without any sign of inflammation or destruction. Completely normal histology was observed in all kidney, liver, and spleen samples at 1, 3, 6, 16, and 24 hours after LV HDI.

Deleterious effects of HV HDIs

Liver damage was severe after HV HDI but was only slight and transient after LV HDI. The level of plasma ALAT, a parenchymal liver enzyme deliberated from damaged hepatocytes, was 13.33 ± 0.85 mM in untreated mice. ALAT was elevated to 1 order of magnitude after LV HDI (1 mL) compared with untreated mice (Fig. 7A) and was reduced to

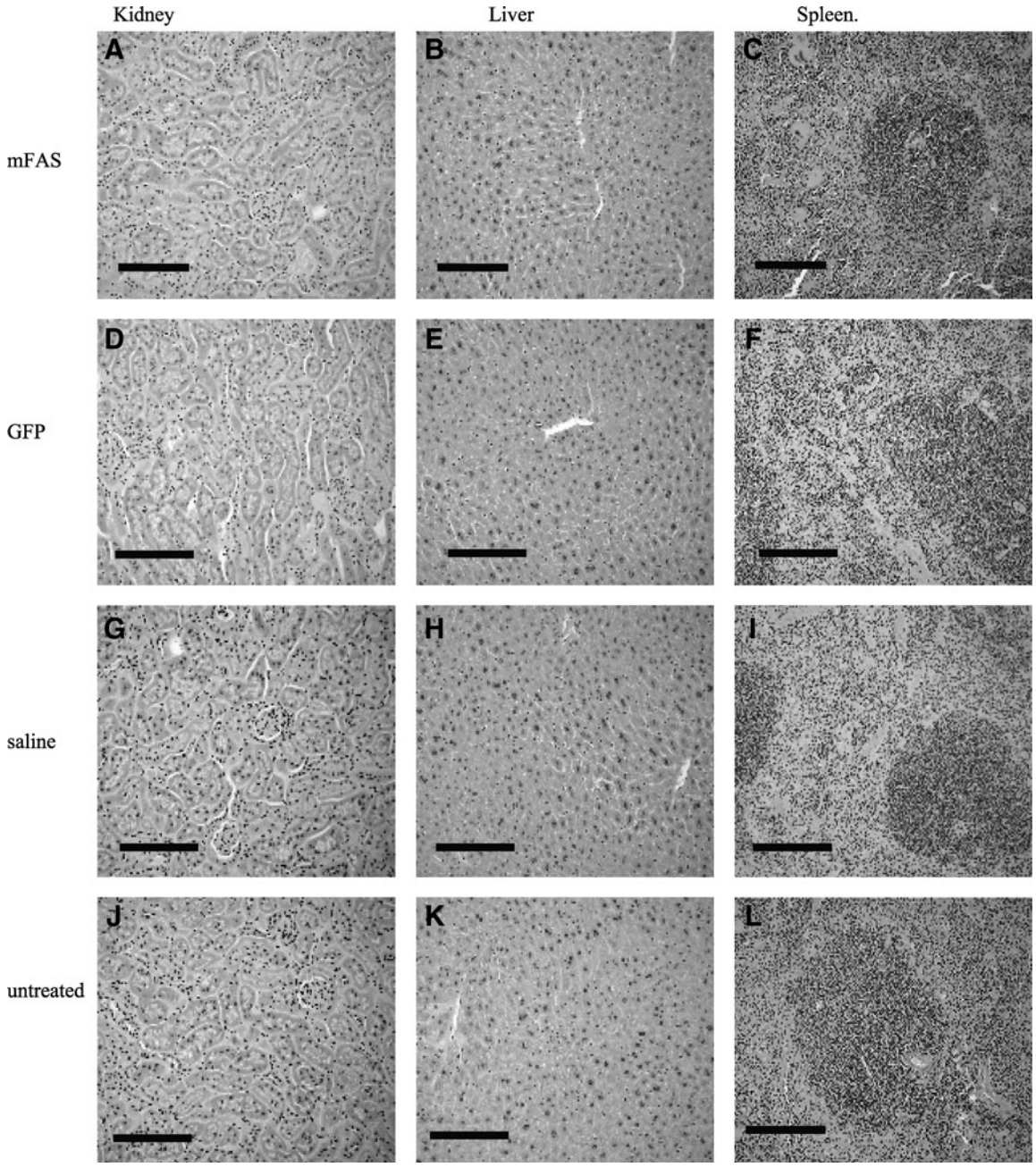

FIG. 6. Normal histology of kidney, liver, and spleen samples of mFas siRNA **(A–C)**, GFP siRNA **(D–F)**, physiological salt solution-treated **(G–I)**, and untreated **(J–L)** mice at 24 hours after LV HDI treatment. Hematoxylin and eosin; 200×magni- magnification. Scale bars represent 25 μm.

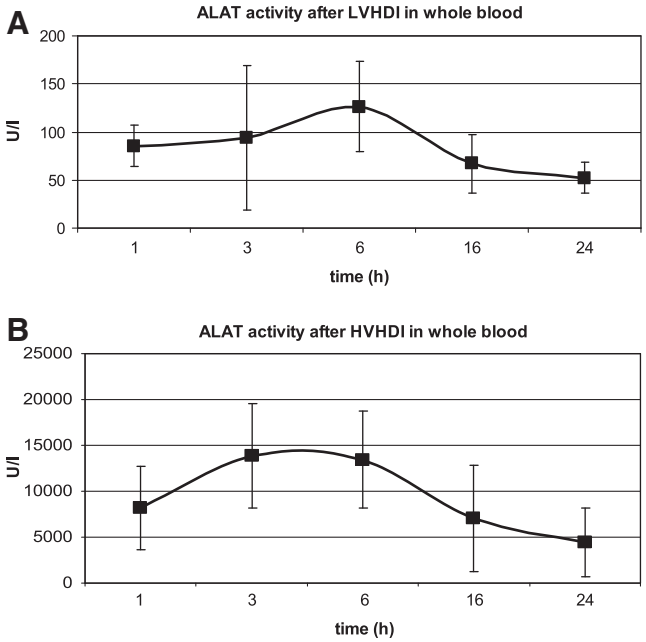

FIG. 7. Time course of liver damage after low **(A)** and HV hydrodynamic treatment **(B)** between 1 and 24 hours. ALAT values of physiological salt-treated mice after injection with LV HDI. ALAT, alanine aminotransferase; HV, high volume.

reference values within 24 hours. On the other hand, in HV HDI (2.5 mL)-treated mice, ALAT elevation was 2 orders of magnitude higher. ALAT levels peaked at 3 hours postinjection in both the LV HDI and HV HDI groups, but ALAT levels were elevated to 2 orders of magnitude at 24 hours post-HV HDI (Fig. 7B).

HV HDI caused severe liver damage histologically manifested as diffuse, confluent hepatocyte necrosis, with dissolved plasma membrane, eosinophilic cytoplasmic material, and pycnotic nuclei (Fig. 8).

Discussion

Currently, the number of clinical trials utilizing siRNAs is increasing (www.clinicaltrials.gov/ct/action/GetStudy). Despite the fast progress of gene silencing, many nonspecific and toxic effects of siRNAs remain undiscovered. Besides, delivery remains the main obstacle for human therapy. In rodent models, HDI is the most common form of *in vivo* siRNA therapy, and human application has been suggested, even though the method itself entails side effects.

HDI is a stressful intervention for the whole body; several side effects had been already reported in relation to this procedure. Hepatocyte disruption had been demonstrated by basic histologic and electron microscopic studies and the elevation of hepatic intracellular enzyme ALAT within the first 20 minutes after injection normalized only in 4 days (Liu et al., 1999; Rossmanith et al., 2002). However, liver damage is probably only temporary, as liver regeneration after necrosis was suggested because of an increased number of mitotic hepatocytes (Budker et al., 2006). To clarify the possible side effects of HDI *per se*, we compared the effects of low and HV HDI in mice.

In the background of ALAT elevation, we hypothesize that the low-grade, transient increase of ALAT after LV HDI may have been due to reversible pore openings on hepatocytes and cannot be compared with high levels of ALAT in previous experiments of HV tail injections wherein hepatocyte necrosis was also observed. This hypothesis is supported by the transient, mild histologic damage observed in LV HDI compared with the severe and lasting necrosis in HV-injected mice. Heidel and colleagues demonstrated the same pattern of ALAT elevation after HV HDI. However, they also observed a mild ALAT elevation after LV injection, despite a very low (0.2 mL/mouse) injection volume (Heidel et al., 2004).

On the other hand, STAT1 and OAS1 gene expression elevation in hydrodynamically treated mice suggests that hydrodynamic treatment *per se* may induce inflammation-related genes, such as IFN response genes. We hypothesize that IFN-regulated genes may be upregulated by such small cellular and subcellular damage without apparent morphologic changes. We observed mild elevation of interferon stimulated gene (*ISG*) expression in the kidney and liver, but not in the spleen, and thus, small-scale damage may occur in the liver and the kidney, but not in the spleen, during the hydrodynamic procedure. The slight STAT1 and OAS1 expression elevation was significantly lower than STAT1 and OAS1 expression elevation in positive control poly I:C-treated mice, suggesting that the observed STAT1 and OAS1 expression elevation in saline- and siRNA-treated mice was part of a nonspecific innate immune activation, not comparable to the massive IFN response induced by poly I:C treatment.

The IFN response as a possible side effect of siRNA treatment had been first described by Sledz et al. (2003), who demonstrated the activation of *ISGs* and Jak/STAT pathways

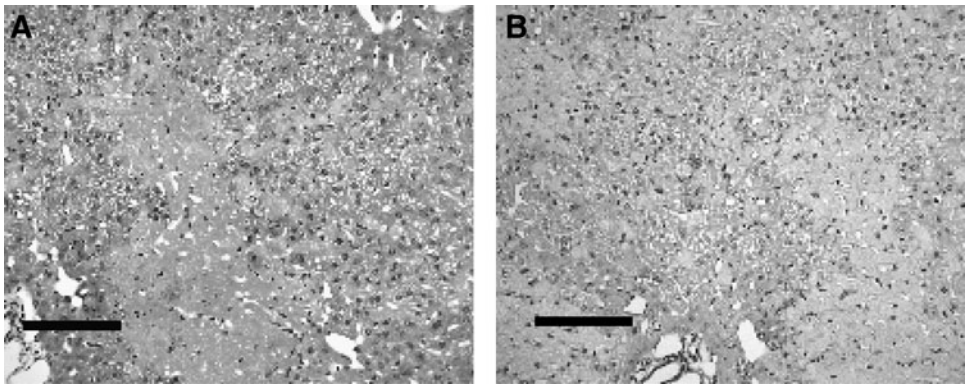

FIG. 8. Histology of confluent hepatocyte necrosis at 24 hours after HV HDI. **(A)** Hematoxylin and eosin– and **(B)** periodic-acid Shiff–stained specimens (200×). Scale bars represent 25 μm.

upon introduction of siRNAs into cells *in vitro*. shRNA-mediated RNAi has also induced OAS1 and IFN-β genes in fetal fibroblasts (Stewart et al., 2008). *In vivo* activation of IFNs or *ISGs* had been also reported following siRNA delivery with different vectors, such as cationic liposomes (Ma et al., 2005), polycations (Judge et al., 2005), siRNA expression shRNA lentiviral vectors (Bridge et al., 2003), or T7 RNA polymerase (Pebernard and Iggo, 2004). Yokota et al. (2007) demonstrated that administration of liposome-encapsulated siRNAs into rodents induced IFN production. It has been also suggested that IFN response after lentiviral expression occurs only in case of 21 or more complementary nucleotides below which there is no IFN response (Fish and Kruithof, 2004). Activation of the innate immune system (IFN-α and γ, TNF-α, and IL-6) by tail vein injection of liposome–siRNA complex had been demonstrated to be strong and sequence independent (Kim et al., 2007). It was reported that unmodified 19-mer siRNAs can evoke dose-dependent IFN-α and TNF-α production in peripheral blood mononuclear cells (PBMCs) (Zamanian-Daryoush et al., 2008). However, conflicting data had been also published: chemically synthesized siRNAs did not induce IFN production, whereas *in vitro* transcribed siRNAs initiated it (Kim et al., 2004). No significant elevation of IFN-α and IL-6 was detected by Wesche-Soldato et al. (2005) after systemic delivery of Fas and caspase-8 siRNAs. In summary, the observed induction of IFNs may have been a consequence of the delivery vector and not so much a direct effect of the siRNA *per se*. Thus, it remains unclear how the whole body reacts to systemic injections of naked siRNA. In our experiment, we investigated siRNA-induced IFN response following LV HDIs in mice. TNF-α—a marker of systemic inflammation—unlike in poly I:C-treated mice, was not significantly elevated by systemic siRNA injection in our study, suggesting that LV HDI with naked siRNA did not induce systemic inflammatory response *in vivo*. The time point for TNF-α measurement was chosen to be 1.5 hours after injection, as our and previous experiments demonstrated that TNF-α peaks at 1.5–2 hours after stimulation and returns to normal by 4–6 hours (Wang et al., 2002). In our experiment, TNF-α was elevated only at 1.5 hours after poly I:C treatment and was undetectable from 6 hours postinjection.

Heidel and colleagues demonstrated no significant increase in the plasma concentration of IFN-α and IL-12 (suggesting no IFN response) after HV injection of naked siRNAs (2.5 mg/kg) compared with poly I:C-injected positive control mice (Heidel et al., 2004). Our data confirmed Heidel's observation that significant IL-6, IL-12, and IFN-β elevation occurred only in poly I:C-treated animals at 2 hours after treatment. IFN-β production was slightly induced by mFas or GFP siRNA treatment only at 2 hours, which is much less extent compared with poly I:C-treated positive controls, and disappeared by 6 and 9 hours after treatment.

Although naked siRNAs might cause cytokine induction, complexation may enhance this effect. Sioud and colleagues demonstrated that marked IL-6 production was induced at 6 hours after tail vein injection of mice with cationic liposome–siRNA complexes (Sioud and Sorensen, 2003). Thus, use of naked siRNAs should be favored.

Our data add evidence that the addition of naked siRNA to the hydrodynamic solution does not induce a significant increase of either STAT1 or OAS1 gene expression when compared with saline-treated animals in any major target organ, and thus, the IFN response is an unlikely side effect of siRNA treatment *in vivo*.

In conclusion, siRNAs did not induce the IFN response in our *in vivo* experiment. However, under experimental conditions utilizing the hydrodynamic delivery of 10% of body weight, one must take into account the possibility of sustained and severe liver damage, which might alter gene expression and induce inflammatory cytokine production, whereas on injecting lower volumes, tissue damage appears to be transient with a low-scale IFN response induction. Thus, side effects due to the delivery method should be also considered besides siRNA-related side effects.

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Author Disclosure Statement

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