Chemical modifications on siRNAs avoid Toll-like-receptor-mediated activation of the hepatic immune system in vivo and in vitro

Ruth Broering†*, Catherine I. Real†*, Matthias J. John‡, Kerstin Jahn-Hofmann‡, Ludger M. Ickenstein‡, Kathrin Kleinehr†, Andreas Paul§, Kathrin Gibbert¶, Ulf Dittmer¶, Guido Gerken† and Joerg F. Schlaak†

†Department of Gastroenterology and Hepatology, University Hospital of Essen, Hufelandstrasse 55, Essen, North Rhine-Westphalia 45122, Germany
‡Roche Kulmbach GmbH, Fritz-Hornschuch-Strasse 9, Kulmbach, Bavaria 95326, Germany
§Department of General, Visceral and Transplantation Surgery and
¶Institute of Virology, University Hospital of Essen, Hufelandstrasse 55, Essen, North Rhine-Westphalia 45122, Germany

Correspondence to: J. F. Schlaak; E-mail: joerg.schlaak@uni-due.de

*Both authors contributed equally to this work.

Received 24 October 2012, accepted 11 July 2013

Objectives: The therapeutic application of small interfering RNAs (siRNAs) is limited by the induction of severe off-target effects, especially in the liver. Therefore, we assessed the potential of differently modified siRNAs to induce the hepatic innate immune system in vitro and in vivo.

Methods: Primary isolated liver cells were transfected with siRNAs against apolipoprotein B1 (APOB1), luciferase (LUC) or galactosidase (GAL). For in vivo use, siRNAs were formulated in lipid nanoparticles (LNPs) and administered intravenously to C57BL/6 mice. Liver tissue was collected 6–48 h after injection and knock-down efficiency or immune responses were determined by quantitative reverse-transcription-linked PCR.

Results: Unmodified GAL siRNA transiently induced the expression of TNF-α, IL-6, IL-10, IFN-β and IFN-sensitive gene 15 in vivo, whereas a formulation of 2′-O-methylated-LUC siRNA had no such effects. Formulation of unmodified APOB1-specific siRNA suppressed APOB1 mRNA levels by ~80% in the liver 48 h after application. The results were paralleled in vitro, where transfection of liver cells with unmodified siRNAs, but not with chemically modified siRNAs, led to cell-type-specific induction of immune genes. These immune responses were not observed in MYD88-deficient mice or in chloroquine-treated cells in vitro.

Conclusions: Our data indicate that siRNAs activate endosomal Toll-like receptors in different liver-derived cell types to various degrees, in vitro. LNP-formulated siRNA selectively leads to hepatic knock-down of target genes in vivo. Here, off-target immune responses are restricted to non-parenchymal liver cells. However, 2′-O-methyl modifications of siRNA largely avoid immune-stimulatory effects, which is a crucial prerequisite for the development of safe and efficient RNA-interference-based therapeutics.

Keywords: hepatocytes, innate immunity, lipid nanoparticles, non-parenchymal liver cells, siRNA

Introduction

The phenomenon of RNA interference (RNAi) was first discovered by Fire et al. and describes post-transcriptional gene silencing of cognate target sequences, mediated by double-stranded (ds) RNAs (1). Chemically synthesized small interfering RNAs (siRNAs) of 19–24 nucleotides in length provide the means to bypass endogenous RNA-processing steps and thus to induce RNAi in eukaryotic cells (2, 3). Meanwhile, as RNAi is widely accepted as a promising tool for the development of new therapeutic concepts, piloting studies have already been performed in clinical trials (4, 5).

Still, the safe and efficient delivery of RNAi-based therapeutics remains an important challenge for their clinical development. Because siRNA is highly negatively charged, the molecule cannot permeate the cell wall. Potential siRNA-based therapeutic drugs against liver diseases such as viral hepatitis thus require a delivery system for improved cellular uptake. siRNAs modified by lipophilic moieties enhance the uptake of siRNA via a receptor-mediated mechanism or by an increased membrane permeability (6). By using lipid nanoparticle (LNP) formulations, the siRNA doses necessary to achieve sufficient target knock-down in...
vivo can be substantially reduced (7). Following intravenous injection, LNPs naturally accumulate in the liver and spleen (8). In vivo, gene silencing can therefore relatively easily be achieved in hepatocytes, which represent the largest population of cells in the liver, and even the simultaneous down-modulation of several hepatocyte targets is feasible (9).

So far, the immune-stimulatory properties of siRNAs have largely been investigated using human PBMCs. These immune-competent cells respond to transfection of siRNA with a sequence-specific induction of IFN-α and TNF-α, which was proposed to be Toll-like receptor 7/8 (TLR7/8) dependent (10, 11). Treatment with siRNAs exhibiting different ribose backbone modifications results in a lack of such immune-stimulatory effects in PBMCs (12). These modified siRNAs also contribute to reduced liver toxicity in vivo. LNPs-siRNA formulations cause target-specific knock-down in the liver of non-human primates without increasing serum levels of aspartate aminotransferase (AST) or alanine aminotransferase (ALT) (13).

However, our knowledge of the pathogenesis of siRNA-mediated side-effects in the liver is far from complete. Hepatocytes represent about two-thirds of all liver cells; the remaining cell population consists of non-parenchymal liver cells (NPCs) including Kupffer cells (KCs), hepatic stellate cells (HSCs) and liver sinusoidal endothelial cells (LSECs). ‘All of these cells are part of the local innate immune system’, as reviewed by Broering et al. (14, 15), and ‘are potential candidates’ that might mediate side-effects that are in turn induced by siRNA treatment. We therefore investigated the capacity of differently designed and modified siRNAs to activate innate immune responses in parenchymal and non-parenchymal murine liver cells both in vitro and in vivo. As the side-effects in mice may not be predictive for use in humans, we further analysed the immune stimulation of enriched primary human liver cell populations in vitro.

### Methods

**Materials**

The LNPs and the siRNAs (Table 1) targeting apolipoprotein B1 (APOB1), luciferase (LUC) and galactosidase (GAL) mRNAs were provided by Roche Kulmbach GmbH (Kulmbach, Germany). Recombinant murine and human IFN-α were purchased from Sigma (Heidenheim, Germany). Chloroquine was obtained from Invivogen (Toulouse, France).

**Animals**

C57BL/6 wild-type mice, MYD88-deficient mice and TLR3-deficient mice were bred at the University Hospital of Essen and maintained under 12-h dark/light cycles. The animals were fed ad libitum and received humane care according to the criteria outlined in the ‘Guide for the Care and Use of Laboratory Animals’, prepared by the National Academy of Sciences and published by the National Institutes of Health.

**Isolation and culture of murine hepatocytes and NPCs**

Primary murine hepatocytes and NPCs were prepared as described previously (16, 17). The purity of these cell types was determined regularly and ranged between 92 and 99% (data not shown).

**Isolation and culture of human hepatocytes and NPCs**

Primary human hepatocytes and NPCs were prepared from liver tissue obtained after surgical tumour resection. This study was approved by the hospital's ethics committee. A more detailed description of materials and methods is given in the Supplementary material, available at International Immunology Online.

### Table 1. siRNA sequences and modifications

<table>
<thead>
<tr>
<th>Target of siRNA</th>
<th>Sequence</th>
<th>Characteristics</th>
<th>Modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>APOB1 Sense</td>
<td>5’-GUC AUC ACA CUG AAU ACC AAU-3’</td>
<td>Single overhang of 21 bp</td>
<td>—</td>
</tr>
<tr>
<td>Anti-sense</td>
<td>5’-AUU GGU AUU CAG UGU GAU GAC AC-3’</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>APOB1 2’-O-Me Sense</td>
<td>5’-GUC AUC AcA cuG AAu Acc AAu-3’</td>
<td>Single overhang of 21 bp</td>
<td>2’-O-methylation</td>
</tr>
<tr>
<td>Anti-sense</td>
<td>5’-AUU GGu AUU cAG UGU GAu GAc AC-3’</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>LUC Sense</td>
<td>5’-CUU ACG CUG AGU ACU UCG ATTsT-3’</td>
<td>Double overhang of 19 bp</td>
<td>—</td>
</tr>
<tr>
<td>Anti-sense</td>
<td>5’-UCG AGG UACU CAG CGU AAG TTS-3’</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>LUC 2’-O-Me Sense</td>
<td>5’-CUu AcG cuG AGu ACu uCG ATsT-3’</td>
<td>Double overhang of 19 bp</td>
<td>2’-O-methylation</td>
</tr>
<tr>
<td>Anti-sense</td>
<td>5’-UCG AGG uACU cAG CGU aAG TsT-3’</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>GAL Sense</td>
<td>5’-CUA CAC AAA UCA GGC AUU UCC AUG U-3’</td>
<td>Blunt end of 25 bp</td>
<td>—</td>
</tr>
<tr>
<td>Anti-sense</td>
<td>5’-ACA UGG AAA UCG CUG AUU UGU GUA G-3’</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>GAL 2’-O-Me Sense</td>
<td>5’-CuA cAc AAA uCA GGC Auu uCc AuG U-3’</td>
<td>Blunt end of 25 bp</td>
<td>2’-O-methylation</td>
</tr>
<tr>
<td>Anti-sense</td>
<td>5’-AcA UGG AAA UCG CUG AUU UGU GuA G-3’</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Small caps = 2’-O-methylation; s = phosphorothioate; T (bold T) = deoxyT (deoxy thymidine).
Synthesis of siRNAs and manufacturing and characterization of LNPs

Materials and methods for siRNA synthesis and generation of primary LNP formulation (LNP01) are described in the Supplementary material, available at International Immunology Online.

siRNA transfection

Human and murine primary isolated liver cells were transfected at an siRNA concentration of 25 nM using the Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) or at an siRNA concentration of 100 nM using the HiPerFect transfection reagent (Qiagen, Hilden, Germany), respectively. The characteristics of the siRNAs are presented in Table 1. APOB1-specific, LUC 2′-O-Me and GAL siRNAs were added to the cells and the culture medium was changed after 8 h. The cells were cultured for 16 h in the presence or absence of 50 U ml⁻¹ IFN-α. For chloroquine pre-treatment, cells were cultured with 100 nM chloroquine for 30 min, the medium was changed and the cells were transfected with siRNAs as described above. Total RNA was extracted 24 h after transfection and the expression of IFN-β, interferon-sensitive gene 15 (ISG15), interferon-induced protein with tetratricopeptide repeats 1 (IFI-T1) and TNF-α were determined by quantitative RT–PCR. In addition, cell culture supernatants were harvested to quantify protein secretion of IFN-β and TNF-α.

In vivo application of LNP-formulated siRNAs

LNP-formulated (LNP01) formulations of APOB1, LUC 2′-O-Me and GAL siRNAs were administered intravenously at an siRNA dosage of 4 mg kg⁻¹ body weight by tail vein injection to 7-week-old male C57BL/6 mice. Mice were sacrificed at various time intervals and tissues from various organs were prepared 6–48 h after injection. Total RNA was isolated and the knock-down efficiency (for APOB1) as well as gene induction (for IFN-β, ISGs, TNF-α, IL-6 and IL-10) were determined by quantitative RT–PCR.

Cytokine array

LNP01 siRNAs were injected via the tail vein into 7- to 9-week-old C57BL/6 mice. After 6 h, total blood was collected by orbital sinus puncture under narcosis, centrifuged and serum was stored at −80°C. The sera of three mice were pooled and 150 μl of total serum was used for analysis with the murine cytokine Proteome Profiler Panel A Array Kit (R&D Systems, Abingdon, UK) according to the manufacturer’s instructions.

RNA isolation and quantitative RT–PCR

Total RNA was isolated and purified using Qiazol™ solution (Qiagen) and the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. Quantitative RT–PCR was performed with the QuantiTect SYBR Green RT–PCR Kit (Qiagen) using 0.1–0.3 μg of total RNA. Human or murine IFN-β, ISG15, IFI-T1, TNF-α, IL-6 and IL-10 expression levels were detected using commercially available primer sets (QuantiTec Primer Assay, Qiagen). The calculated copy numbers were normalized to the following housekeeping genes: human β-actin: sense 5′-TCCCTGGAGAAGAGCTACGA-3′ and anti-sense 5′-AGC AATGTGTTGCCTACAG-3′; murine β-actin: sense 5′-AAAT CGTGCGTACATCAA-3′ and anti-sense 5′-CAAGAAGQGA GGCTGGAAA-3′; murine glyceraldehyde 3-phosphate dehydrogenase (GAPDH): sense 5′-AAATTCAAGGGCCACAGT CAA-3′ and anti-sense 5′-TCTCCATGTTGGTGAAAGACA-3′.

ELISA

Cell culture supernatants from human hepatocytes, LSECs, HSCs and NPCs were harvested to quantify protein secretion of IFN-β and TNF-α concentrations were determined by ELISA according to the manufacturer’s instructions [TNF-α: R&D Systems (Wiesbaden, Germany), IFN-β: PBL Biomedical Laboratories (New Brunswick, NJ, USA)].

Statistical analysis

Data are expressed as mean ± SEM. Differences between any two groups were determined by the Wilcoxon test. A value of P < 0.05 was considered to be statistically significant.

Results

Chemical modifications on differently designed siRNAs avoid the induction of immune genes in PBMCs

Although siRNAs have been developed and optimized for both in vitro and in vivo applications within the past decade, knowledge about the pathogenesis of siRNA-mediated side-effects in the liver is far from complete. Therefore, we have previously selected a set of differently designed and modified siRNAs for this study (Table 1). Sequences, chemical modifications and induction of immune response were derived from the literature (6, 11, 13). The set of siRNAs was tested in a human PBMC assay. PBMCs were treated with 100 nM of siRNA for 24 h. RNA was extracted and the expression of immune genes was analysed. Significant induction of IFN-β (P < 0.01), ISG15 (P < 0.01) and TNF-α (P < 0.05) expression in response to unmodified APOB1 and GAL siRNAs could be shown. In comparison, unmodified LUC siRNA only marginally induced IFN-β expression (P < 0.01). Interestingly, the 2′-O-methyl modification on the siRNA backbones of these siRNAs (APOB1 2′-O-Me, GAL 2′-O-Me and LUC 2′-O-Me) totally abrogated the induction of these immune genes (Supplementary Figure SM1A–C, available at International Immunology Online). The knock-down efficiency for the differently modified APOB1-specific siRNAs was determined in primary murine hepatocytes transfected with 100 nM siRNA for 24 h. Both siRNAs led to similar suppression of APOB1, compared with untreated and non-silencing control-treated (LUC, LUC 2′-O-Me) cells (Supplementary Figure SM1D, available at International Immunology Online). To characterize siRNA-mediated side-effects in the liver, immunostimulatory siRNAs against APOB1 and GAL were chosen for further analysis, whereas LUC 2′-O-Me siRNAs were used as negative control.

Differently modified siRNAs induce cell-type-specific immune responses in primary isolated liver cells in vitro

In the past decade, siRNAs have been developed and optimized for in vitro and in vivo applications. In this study, we tested the capacity of differently modified siRNAs to activate...
the hepatic innate immune system in vitro and in vivo. Primary human hepatocytes, HSCs, LSECs and a mix of the remaining NPCs, which mostly contained KCs but also natural killer cells and T lymphocytes, were prepared using liver samples obtained after tumour resection. These cell populations were transfected with differently modified siRNAs at a concentration of 25 nM each. The expression levels of IFN-α, ISG15 and TNF-α genes were determined by quantitative RT–PCR 24 h after transfection (Table 2). Whereas HSCs responded exclusively to transfection with GAL siRNA, the hepatocytes, LSECs and the remaining NPCs exhibited an immune activation pattern after treatment with APOB1. In contrast, LUC 2′-O-Me siRNAs induced, if any, only a weak expression of IFN-β, ISG15 or TNF-α in these cell types. These findings were confirmed at the protein level by the detection of IFN-β and TNF-α in the cell culture supernatants.

The results also suggest that cell-type-specific immune responses are independent of the silencing effect. Transfection with GAL led to strong induction of IFN-β in all four cell populations. The most potent induction of IFN-β was observed in NPCs treated with GAL siRNA (1910.8 ± 49.9 pg ml⁻¹). Transfection with APOB1-specific siRNA led to weak but significant, immune activation in HSCs, LSECs and NPCs. LUC 2′-O-Me siRNA led to no or only marginal induction of IFN-β (Fig. 1A). Similar results were obtained for secretion of TNF-α (Fig. 1B). Here LSECs were the most potent producers of TNF-α (586.1 ± 7.0 pg ml⁻¹) after GAL transfection and they responded to both control and APOB1 siRNAs. Similar immune responses were obtained in isolated murine liver cell populations. GAL siRNA treatment led to strong up-regulation of IFN-β expression in KCs (fold change, FC: 2641.5 ± 4.1) and TNF-α (FC: 20.69 ± 2.44) in LSECs (Supplementary Figure 2A and B, available at International Immunology Online).

Chloroquine pre-treatment abolishes siRNA-mediated immune response in vitro

To elucidate the mechanisms of siRNA-mediated immune responses, the involvement of pathogen recognition receptors in endosomes was assessed. Primary isolated human and murine hepatocytes were pre-treated with chloroquine, an inhibitor of endosomal acidification. After transfection with siRNAs at a concentration of 25 nM for 24 h, RNA was extracted and the differences in mRNA levels before and after the treatment were determined. In both human and murine hepatocytes, chloroquine pre-treatment minimized the expression of IFN-β (Fig. 2A) and TNF-α (Fig. 2B) in response to APOB1 and GAL siRNAs. The murine data set is shown in Supplementary Figure 2C (available at International Immunology Online).

**Table 2. Induction of immune genes by siRNA in different liver cell populations**

<table>
<thead>
<tr>
<th>Genes</th>
<th>siRNA</th>
<th>Fold change ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hepatocytes</td>
</tr>
<tr>
<td>IFN-β</td>
<td>w/o</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>APOB1</td>
<td>22.4 ± 3.4</td>
</tr>
<tr>
<td></td>
<td>LUC 2′-O-Me</td>
<td>2.1 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>GAL</td>
<td>64.2 ± 11.3</td>
</tr>
<tr>
<td>ISG15</td>
<td>w/o</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>APOB1</td>
<td>274 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>LUC 2′-O-Me</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>GAL</td>
<td>42.3 ± 4.1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>w/o</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>APOB1</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>LUC 2′-O-Me</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>GAL</td>
<td>4.5 ± 0.2</td>
</tr>
</tbody>
</table>

Primary human hepatocytes, HSCs, LSECs and remaining NPCs were transfected with siRNAs and cultured for 6 h. Increases in expression levels of IFN-β, ISG15 and TNF-α were determined by quantitative RT–PCR. Fold changes (mean ± SEM) were calculated after normalization against β-actin and comparison with untreated control (w/o, without treatment). Fold changes >2 units are given in bold numbers.

siRNA-activated hepatocytes do not respond to exogenous IFN-α in vitro

The development of siRNA-based therapeutic strategies, especially in viral hepatitis, raised the question whether siRNA-mediated activation of hepatocytes can be strategically used and whether these cells additionally respond to stimulation with or exogenous interferons. We therefore stimulated the cells 8 h after siRNA transfection with 50 U ml⁻¹ IFN-α, and RNA was extracted 16 h after IFN-α treatment. Significant interferon-mediated induction of ISGs was only detectable in untreated hepatocytes (ISG15: 9.93 ± 1.66 FC; IFI-T1: 7.57 ± 1.68 FC) and in LUC 2′-O-Me-siRNA-treated hepatocytes (ISG15: 8.99 ± 0.08 FC; IFI-T1: 8.84 ± 1.66 FC). In comparison, siRNA-induced interferon responses to APOB1 (ISG15: 27.43 ± 0.57 FC; IFI-T1: 34.62 ± 1.55 FC) and GAL (ISG15: 42.26 ± 4.99 FC; IFI-T1: 50.90 ± 1.74 FC) were much more potent, but additional treatment with IFN-α had no further effect on ISG expression (Fig. 2C).

LNP01-formulated siRNAs suppress hepatic gene expression in vivo

For in vivo applications of siRNAs, parameters such as knock-down efficiency, bio-distribution, cell-specific targeting and biocompatibility have to be optimized. We therefore evaluated differently modified siRNAs formulated in LNP01 in terms of their knock-down efficacy against hepatic targets as well as their toxicity as determined by AST/ALT serum levels following systemic administration. We evaluated the knock-down efficacy of APOB1 siRNA against the liver-specific marker
Modified siRNAs avoid hepatic immune responses

APOB1, as an indicator for successful delivery to hepatocytes, C57BL/6 mice received LNP01–APOB1 siRNA at a dosage of 4mg kg⁻¹ via tail vein injection. Mice were sacrificed at various time points and RNA was extracted from different liver lobes. APOB1 mRNA levels were determined by quantitative RT–PCR. APOB1 mRNA levels decreased by 33% 6h after administration and reached a knock-down level of 83% 48h after injection (Fig. 3A). There was no difference in knock-down efficiency between different liver lobes at 48h, indicating an even distribution of LNP–siRNA within the liver (Fig. 3B).

Differently modified siRNAs specifically activate hepatic immune responses in vivo

C57BL/6 mice received LNP01-formulated siRNAs at a dosage of 4mg kg⁻¹ body weight via tail vein injection. After 6h, total blood was collected by orbital sinus puncture, the mice were sacrificed and RNA was extracted from the left liver lobe. The concentrations of a subset of chemokines (C–C motif ligands: CCL2, CCL5 and CCL12; and C–X–C motif ligands: CXCL9, CXCL10), complement component (C5a), granulocyte colony stimulating factor (G-CSF) and IL-1 receptor antagonist (IL-1 Ra) were found to be up-regulated in the serum of LNP01-GAL-siRNA-treated mice 6h after tail vein injection, indicating a systemic immune response. By contrast, LNP01–LUC 2′′-O-Me siRNA treatment had no such effect (Fig. 3C and D).

The hepatic gene expression of IFN-β (Fig. 4A), ISG15 (Fig. 4B) and TNF-α (Fig. 4C) was determined by quantitative RT–PCR in siRNA-treated animals. All three markers were significantly induced (P < 0.01) following administration of LNP01-formulated GAL siRNAs, whereas LNP01–APOB1 only caused a significant increase in TNF-α expression.

---

Fig. 1. Differently modified siRNAs induce cell-type-specific immune responses in primary isolated liver cells in vitro. Primary isolated human hepatocytes (Heps), LSECs, HSCs, and a mix of remaining NPCs were transfected with 25nM of different siRNAs (APOB1, LUC 2′′-O-Me or GAL) using the Lipofectamine RNAiMAX transfection reagent (w/o indicates untreated control). Then, 24h after transfection, cell culture supernatants and total cell lysates were collected. Secretion of cytokines (A) IFN-β and (B) TNF-α was quantified by ELISA (mean values ± SEM). Asterisks indicate significant results (*P < 0.05; **P < 0.01; ***P < 0.001).
Modified siRNAs avoid hepatic immune responses

In contrast, LNP01-formulated LUC 2’-O-Me siRNAs did not induce significant changes. Histological staining of liver specimens at 6, 72 and 96 h after injection revealed no differences between LUC 2’-O-Me- and GAL-siRNA-treated mice. Neither signs of necrosis nor infiltration of immune cells were observed. In accordance with the histological data, there was no significant difference of liver serum markers among the untreated, LUC 2’-O-Me- or GAL-siRNA-treated mice (Supplementary Figure 3, available at International Immunology Online).

siRNA-induced immune responses are largely mediated by MYD88-dependent signaling pathways

We further examined whether the siRNA-activated hepatic immune system can be activated synergistically by endogenous interferon induced by polynucleosidopolyphosphoric acid (poly(I:C)). This artificial dsRNA is frequently used as a surrogate for infections by viruses, including the hepatitis C virus (HCV), and activates interferon pathways independently of the siRNA. C57BL/6 mice were injected with poly(I:C) at a dosage of 4 mg kg⁻¹ body weight 3 h after administration of LUC 2’-O-Me and GAL siRNAs formulated in LNP01. Animals were sacrificed 6 h after poly(I:C) injection. Poly(I:C) induced significant expression of IFN-β (P < 0.05), ISG15 (P < 0.01) and TNF-α (P < 0.001) in untreated or LNP01–LUC 2’-O-Me-siRNA-treated mice (P < 0.05), whereas no additional induction was observed in LNP01-GAL-pre-treated animals (Fig. 4D–F).

The siRNA-activated hepatic immune system does not respond to poly(I:C) in vivo

We investigated TLR-dependent signaling pathways in vivo. LNP01-formulated LUC 2’-O-Me and GAL siRNAs were injected into MYD88⁻/⁻ and TLR3⁻/⁻ knockout mice. Mice were sacrificed after 6 h, RNA was extracted from the liver and expression levels of IFN-β, IL-6 and IL-10 were determined. IFN-β expression significantly increased in GAL-siRNA-treated wild-type and TLR3⁻/⁻ mice, but not in MYD88⁻/⁻ mice.

Fig. 2. Chloroquine pre-treatment abrogates siRNA-mediated immune responses in vitro. Primary isolated human hepatocytes, pre-treated with chloroquine for 30 min, were transfected with 25 nM of different siRNAs (APOB1, LUC 2’-O-Me or GAL) using the Lipofectamine RNAiMAX transfection reagent. Then, 24 h after transfection, changes in gene expression of (A) IFN-β and (B) TNF-α were determined by quantitative RT-PCR. Primary isolated human hepatocytes were (C) additionally stimulated with IFN-α (50 U ml⁻¹) 8 h after transfection of siRNAs. Changes in gene expression of ISG15 and IFI-T1 were determined by quantitative RT-PCR 16 h later. Fold changes were calculated by normalization to 100 000 copies of β-actin and comparison with the untreated control (mean values ± SEM). Asterisks indicate significant results (* P < 0.05; ** P < 0.01; *** P < 0.001).
Modified siRNAs avoid hepatic immune responses

Similar results were obtained regarding the siRNA-induced expression of the pro-inflammatory cytokine IL-6 (Fig. 5B) and the anti-inflammatory cytokine IL-10 (Fig. 5C). The 2′-O-methylation-bearing LUC siRNAs did not induce any of these cytokines in the mouse models tested, independent of the genetic background.

The hepatic immune response to siRNA in vivo is determined by NPCs

To further analyse the cellular response to treatment with LNP01-formulated siRNAs in vivo, we isolated hepatocytes and a mixed population of hepatic NPCs from C57BL/6 mice 6h after treatment with LNP01-formulated LUC 2′-O-Me siRNA or GAL siRNA. The two different cell fractions were lysed immediately, RNA was extracted and gene expression of IFN-β, ISG15, TNF-α, IL-6 and IL-10 was determined. IFN-β expression was significantly increased in NPCs, but not in hepatocytes, isolated from GAL-siRNA-treated animals (Fig. 6A). However, both cell populations responded to IFN-β, produced by the NPCs in vivo, with an increased expression of ISG15. The expression level in NPCs was higher (FC: 55.0±20.7) compared with that in hepatocytes (FC: 4.0±1.1). Interestingly, although IFN-β was not significantly induced in animals treated with LUC 2′-O-Me siRNA, ISG15 expression was slightly increased in isolated hepatocytes (Fig. 6B). The expression of inflammatory cytokines TNF-α, IL-6 and IL-10 significantly increased in NPCs isolated from GAL-, but not in LUC 2′-O-Me-siRNA-treated animals (Fig. 6C–E). In comparison, IL-6 increased only slightly but significantly in hepatocytes isolated from GAL-siRNA-treated animals.

Discussion

The development of RNAi-based therapeutics has become a promising approach to treat human diseases with unmet clinical needs. With respect to the treatment of liver diseases such as
Modified siRNAs avoid hepatic immune responses

Infection with HCV, hepatitis B virus (HBV) or hepatocellular carcinoma, systemic delivery of siRNA conjugates or liposomal formulations to the liver is a prerequisite (18, 19). However, the balance between potent lipidoid delivery of the siRNA drug candidates to the liver and an acceptable safety profile has to be carefully reviewed, for example in mice, rats and non-human primates (7, 20). For certain liposomal formulations, it was demonstrated that dexamethasone co-treatment suppressed inflammatory responses in mice and had a positive effect on the induced toxicities (21).

Previously, we have investigated the interplay of different human and murine liver cell populations in orchestrating the innate immune system and have demonstrated the importance of cell-type-specific expression and activation of TLRs (22). In this study, we investigated the ability of a set of differently modified siRNAs to activate the hepatic innate immune system.

In vitro, isolated human liver cells responded to transfected siRNAs similarly to the response of human PBMCs. GAL siRNAs were the strongest inducers of ISGs, whereas APOB1-specific siRNAs were intermediate stimulators. Interestingly, the degree of activation was comparable to that of IFN-α treatment. The 2′-O-Me-modified LUC siRNAs, however, led to minimal or no activation of the interferon pathways. Hepatocytes and NPCs were more sensitive to siRNA stimulation than HSCs and LSECs were. The immune-stimulatory effects were sensitive to chloroquine treatment of the cells, which clearly points towards the involvement of endosomal receptors such as TLRs. The methods for cell isolation from human and mouse liver are complex and may lead to artifacts in vitro. We observed, for example secretion of TNF-α for all transfected siRNAs in LSECs. However, there was no sign for a general activation of interferon pathways as judged by ISG expression. The transfection
Modified siRNAs avoid hepatic immune responses

process or transfection reagent itself might be able to activate TNF-α in an siRNA-independent way.

In order to confirm our findings in vivo, lipid-based LNP01 formulations were used to determine the siRNA-mediated hepatic immune activation in C57BL/6 mice. The formulation of an APOB1-specific siRNA was found to be distributed evenly throughout the liver and it suppressed APOB1 mRNA levels in a target-specific manner by >80%. Liposomal formulation induced very little liver toxicity, as judged by histological sections and blood parameters (Supplementary Figure 2, available at International Immunology Online). Mice treated with the unmodified APOB1-specific siRNA only exhibited low levels of hepatic immune stimulation, i.e. a slight increase in TNF-α expression 6h after injection. In contrast, administration of an LNP01 formulation of unmodified GAL siRNAs strongly induced the hepatic expression of IFN-β, ISG15 and TNF-α. Animals treated with this siRNA additionally showed an initial, transient increase in serum levels of chemokines, resulting in attraction and differentiation of monocytes, granulocytes and lymphocytes (23, 24), which may orchestrate the NPC-mediated immune response in the liver. This immune induction did not further increase after additional treatment with interferon-inducing poly(I:C). It can be speculated that the lack of an additive effect by poly(I:C) was mediated by a tolerance induction due to an initial activation of the hepatic innate immune system by the siRNA.

The data obtained in knockout mice suggest that the siRNA-mediated immune responses in vivo are independent of TLR3 but involve MYD88-dependent pathways. Previous studies demonstrated the involvement of TLR3 in siRNA recognition in mice, irrespective of chemical modification patterns or sequence of the siRNA. Only a minimal length of 21 nucleotides per single strand of RNA was determined as the key requirement for immune stimulation by systemic and topical application of naked siRNA, resulting in inhibition of blood vessel growth (25, 26). At least for systemic application of LNP01-formulated siRNA, we conclude that no activation of TLR3 is detectable in vivo at efficient siRNA doses. Although we only used one exemplary 2′-O-methyl-carrying LUC siRNA, it has been shown that the use of chemical
Modified siRNAs avoid hepatic immune responses

44

modifications allows selection of virtually any siRNA with silencing efficiency and no activation of the innate immune system. The induction of the interferon response is rather dependent on TLR7, as shown previously by several authors (27, 28). The immune response induced by LNP01-GAL siRNA was mediated by the NPCs because hepatocytes isolated from GAL-siRNA-treated mice did not produce interferon but responded to endogenously expressed interferon. Similar to the in vitro findings, LNP01–LUC 2′-O-Me siRNAs did not activate a hepatic immune response in vivo. Some aspects of the immune-activating properties of unmodified GAL siRNAs described in the present report confirm the findings previously presented by Zamanian-Daryoush et al. using human PBMCs (11). These immune-competent cells were reported to respond to transfection with unmodified GAL siRNA in a retinoic acid-inducible gene 1 (RIG-I)-independent induction of IFN-α and TNF-α, and it was proposed that this effect is dependent on TLR7/8. The authors further concluded that this siRNA-induced immune response was caused by sequence motifs and did not depend on the final conformation of the siRNAs (11). PBMCs treated with 2′-O-Me-modified siRNA do not respond with increased expression of IFN-α and TNF-α (12). In contrast to these findings, siRNA transfection of a human glioblastoma cell line led to JAK-STAT activation and expression of ISGs mediated by dsRNA-dependent protein kinase (PKR; (29)). Immune activation in different cell types by transfection with siRNA is apparently mediated by different receptor molecules. The reasons could stem from differences in the experimental set-ups but could also be explained by the different immunological properties of the cell types used in these studies.

Fig. 6. siRNA-induced hepatic immune responses are restricted to NPCs in vivo. Seven-week-old, male C57BL/6 mice received 200 µl LNP01-formulated siRNA (LUC 2′-O-Me or GAL; 4 µg g⁻¹) by tail vein injection. Mice were sacrificed after 6 h and primary hepatocytes and a mix of NPCs were prepared after liver perfusion and digestion. RNA was extracted from these cells and changes in gene expression of (A) IFN-β, (B) ISG15, (C) TNF-α, (D) IL-6 and (E) IL-10 were determined by quantitative RT–PCR. Copy numbers were normalized to 100 000 copies of GAPDH (mean values ± SEM). Asterisks indicate significant results (* P < 0.05; ** P < 0.01; *** P < 0.001).
RNAi represents a promising therapeutic approach in particular for diseases caused by ‘non-druggable’ factors, which cannot be targeted by conventional therapeutics. Frank-Kamenetsky et al., for example have developed cross-reactive siRNAs for murine, rat, non-human primate and human pro-protein convertase subtilisin/kexin type 9 (PCSK9). Suppression of PCSK9 in the liver by LNP-formulated siRNA reduced low-density lipoprotein (LDL) cholesterol serum levels and may represent a therapeutic strategy to treat hypercholesterolemia (13). Apart from host factors, viruses have been targeted by siRNAs in vitro and in vivo for prophylactic or therapeutic applications. Recent studies have demonstrated the efficacy of siRNAs in inhibiting HBV and HCV. HBV X-gene-specific siRNA with 5′-end triphosphate modification showed significantly stronger inhibitory effects on HBV replication than the comparable but unmodified siRNAs in vitro. This was accompanied by RIG-I activation, resulting in higher expression of type I interferons, ISGs and pro-inflammatory cytokines (30). Morrissey et al. identified a potent siRNA specific for the HBV surface antigen (HBsAg) region, which efficiently suppressed levels of HBV DNA, HBV RNA and HBsAg in both HBV cell culture and an HBV mouse model. Chemical modification and delivery by LNPs decreased the siRNA concentrations used and increased efficacy in vivo (31,32). In the case of HCV, siRNAs targeting host as well as viral gene expression have been evaluated using sub-genomic and infectious cell culture systems. Combinations of siRNAs targeting the HCV core, non-structural protein 3 (NS3) or NS5B resulted in effective suppression of HCV replication (33). Targeting viral genes harbours the risk of resistance; therefore, a combination of these siRNAs with those specific for host factors, efficient for viral replication, may increase the anti-viral effects and may overcome development of resistance. A combination of siRNAs against the HCV envelope protein 2 and HCV entry factors (CD81, LDL receptor) efficiently suppressed HCV replication in vitro (34). Another promising target to suppress HCV replication is the host factor IISG15, which directly promotes viral replication and additionally abrogates an efficient interferon response (35).

In conclusion, we have shown that chemical modifications of the siRNA backbone have an impact on the degree of hepatic off-target effects. LNP-formulated siRNAs, which are used to target the hepatic system in vivo, comprise a powerful tool to deliver siRNAs, specific for either host or viral factors. In addition, the choice of siRNA design offers the possibility of targeted activation as well as inhibition of the hepatic innate immune system. The clinical usefulness of such additional effects for example in viral hepatitis or malignant diseases remains to be determined. Still, these findings will improve the development of promising RNAi-based therapeutics against a broad range of liver diseases.

**Supplementary data**

Supplementary data are available at *International Immunology* Online.

**Funding**

Deutsche Forschungsgemeinschaft (SCHL 377/2-4 and SCHL 377/6-2, TRR60).

**Acknowledgements**

The authors would like to thank Prof. H. Baba and Mrs D. Möllmann (Institute for Pathology, University Hospital of Essen) for excellent technical support in the histology staining process. K.J.H., L.M.I. and M.J.J. have performed this work as employees of Roche Kulmbach.

**References**

Modified siRNAs avoid hepatic immune responses


