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Dicer-substrate siRNA inhibits tumor necrosis factor alpha secretion in Kupffer cells in vitro: In vivo targeting of Kupffer cells by siRNA-liposomes

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Abstract

Tumor necrosis factor alpha (TNF- α) plays a major role in the pathogenesis of many inflammatory diseases. Neutralizing TNF- α by antibodies or antisense oligodeoxynucleotides, alleviate disease symptoms. In this study, we introduce the new generation of gene-silencing molecules, namely the small interfering RNAs (siRNAs) to reduce TNF- α . Although siRNAs of 19–21 base pairs are commonly used, it is reported that longer siRNAs have much higher efficacies. Here, we report the identification of a 27-mer Dicer-substrate siRNA (DsiRNA) against TNF- α mRNA. Primary cells of rat Kupffer cells were transfected with five 27-mer siRNA constructs (si27-1, si27-2 si27-3, si27-4 and si27-5) for 24 h, following which, TNF- α secretion was induced by exposure to LPS (0.1 μ g/ml) for 2 h. TNF- α released to the medium was measured by ELISA. Of the five si27 constructs, si27-3 had the highest inhibitory effect on TNF- α secretion. At 10 nM, si27-3 inhibited TNF- α secretion by 80% compared to a 60% inhibition by a 21-mer (SSL3). Following encapsulation in anionic liposomes, si27-3 at 100 μ g/kg body weight, on two successive days by intravenous administration, inhibited the secretion of TNF- α by 50%. These data demonstrate the identification of a highly efficacious siRNA formulation, which can be used in the treatment of TNF- α mediated diseases.

Keywords

TNF alpha; siRNA; Kupffer cells; lipopolysaccharide; liposomes

1. INTRODUCTION

Tumor necrosis factor alpha (TNF- α), a cytokine produced by many cell types, including macrophages, monocytes, lymphocytes and fibroblasts, is a growth promoter under normal physiological conditions. It exhibits pleiotropic effects on various cell types [1]. Excessive amounts of the cytokines such as TNF- α and interleukins are released into circulation in response to infection, inflammation and other environmental insults, often resulting in tissue injury [2]. An overproduction of TNF- α has been associated with the development of alcoholic liver injury [3–5], rheumatoid arthritis [6], inflammatory bowel disease [7] and septic shock [8] and many other diseases such as atherosclerosis, psoriasis, diabetes, obesity [9] and tumor promotion [10].

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Kupffer cells, the resident macrophages in the liver, play an important role in the pathogenesis of liver injury. It has been shown that obliteration of Kupffer cells prior to their activation by hepatotoxins prevents liver damage [11–13]. Kupffer cells are the major producers of TNF- α in the liver following exposure to lipopolysaccharide (LPS), the bacterial endotoxin [14]. Antibodies against TNF- α neutralize the effects of TNF- α in conditions such as ischemia reperfusion injury [15] and experimental liver damage induced by chronic alcohol consumption [16]. From the above, it is concluded that any approach to mitigate the excessive production of pro-inflammatory cytokines, such as TNF- α , would be of therapeutic value in the treatment of various inflammatory diseases. Further, because they are the major source of cytokines in the liver, targeting Kupffer cells would have to be the primary objective in any intervention in the treatment of liver disease.

Earlier studies from our laboratory have shown that anionic liposomal formulations are an effective delivery system for targeting Kupffer cells with antisense oligodeoxynucleotides (AsODNs) and other organs such as spleen and lungs, which are enriched in macrophages [17, 18]. Although, theoretically, AsODNs can be used to target any of the disease-causing genes, in practice, there are limitations to its application due to off-target effects [19, 20]. Against that background, the discovery of RNA interference (RNAi) is of significance. The discovery, that the administration of short interfering RNA (siRNA) can activate an endogenous cellular process for gene-specific silencing, has led to an explosion of interest in this field [21]. Under appropriate conditions, short (21–23 nucleotides) double-stranded RNA (siRNA) molecules are generated from a double-stranded parent molecule by an endonuclease called the ‘Dicer’, an RNase III enzyme [22]. In turn, the siRNAs are incorporated into an RNA-induced silencing complex (RISC), which unwinds the double strand and is guided by the antisense strand to the homologous mRNA for degradation [22–24]. Earlier studies by Brummelkamp et al., [25] and Bertrand et al., [26] suggest that siRNAs are highly sequence specific and are much more sensitive compared to AsODNs. The extraordinary success in gene-silencing realized by the RNAi system has also led to several *in vivo* applications [27, 28].

The commonly used *in vivo* delivery systems include, adenoviral vectors coding for hairpin RNA molecules (shRNA) that generate siRNA in the cell, cationic lipids, hydrodynamic injection, electroporation and also the ‘naked’ form of administration [27]. While the choice of the delivery system depends upon the target, it is generally observed that delivery of oligonucleotides in the ‘naked’ form is the least efficient one. In a recent study, we reported the identification of a 21-mer siRNA duplex, SSL3, against rat TNF- α mRNA, and its successful delivery *in vivo* using an anionic liposomal delivery system [29]. Although for gene-silencing studies, most researchers employ synthetic RNA duplexes that are 19–21 bases long, in 2005, it was demonstrated that the use of a slightly longer siRNAs, which are substrates for the Dicer enzyme, show higher potency than the traditional 21-mer siRNAs [30, 31]. Typically, these are 27 bp siRNAs, and are referred to as Dicer-substrate siRNAs (DsiRNAs). Inside the cell, these longer siRNAs are processed by Dicer into 21-mer siRNAs in a predictable manner. The principle behind DsiRNA design and their function have been recently reviewed by Amarzguoui and Rossi [32]. In the current study, we describe the identification of potent DsiRNA against TNF- α mRNA and also characterize an anionic pH-sensitive liposomal delivery system for the *in vivo* delivery of DsiRNA to Kupffer cells.

2. MATERIALS AND METHODS

2.1 Animals

Unless indicated otherwise, experiments were primarily carried out in barrier maintained male Sprague Dawley rats purchased from Harlan (Indianapolis, IN). Animals were acclimatized for at least 1 week following their arrival in our facility. The body weights of

the animals used ranged from 250–300 g. All animals were maintained on lab-chow until used. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University, Philadelphia, which is an AAALAC-accredited facility.

2.2 Chemicals

Cholesterol and cholesteryl hemisuccinate (CHEMS) were purchased from Sigma-Aldrich (St. Louis, MO). Phosphatidyl ethanolamine (PE) (transphosphatidylated from egg lecithin) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Dicer-substrate siRNA duplexes (DsiRNA) with 27-bp, targeted to rat TNF- α mRNA as well as si27-3 mismatched duplexes (si27M and si27R) for the in vitro studies, were synthesized from Integrated DNA Technologies (IDT) Inc. Coralville, IA. Fluorescent-labeled DsiRNA (FAM-DsiRNA) was obtained from Dharmacon Inc. Large-scale synthesis of siRNA was carried out either by Bioneer Inc., (Alameda, CA; DsiRNA) or by Qiagen Inc., (Valencia, CA; siRNA, SSL3). The sequences of the individual siRNAs are as follows: **Si27-1**, Sense - 5'-P-C-A-A-C-U-A-C-G-A-U-G-C-U-C-A-G-A-A-C-A-C-A-dC-dG -3' Antisense 3'-U-U-G-U-U-G-A-U-G-C-U-A-C-G-A-G-U-C-U-U-U-G-U-G-U-G-C -5'; **Si27-2**, Sense 5'-P-G-G-A-C-C-A-G-C-C-A-G-G-A-G-G-G-A-G-A-A-C-A-G-dC-dA -3', Antisense 3'-U-C-C-C-U-G-G-U-C-G-G-U-C-C-U-C-C-C-U-C-U-U-G-U-C-G-U-5'; **Si27-3**, Sense 5'-P-C-C-A-U-G-A-G-C-A-C-G-G-A-A-A-G-C-A-U-G-A-U-C-dC-dG -3' Antisense 3'-G-U-G-G-U-A-C-U-C-G-U-G-C-C-U-U-U-C-G-U-A-C-U-A-G-G-C -5'; **Si27-4**, Sense 5'-P-C-C-A-A-C-A-A-G-G-A-G-G-A-G-A-A-G-U-U-C-C-C-A-dA-dA -3', Antisense 3'-A-G-G-G-U-U-G-U-U-C-C-U-C-C-U-C-U-U-C-A-A-G-G-G-U-U-U-5'; **Si27-5**, Sense, 5'-P-G-G-G-A-A-U-U-G-U-G-G-C-U-C-A-G-G-G-U-C-C-A-A-dC-dT-3' Antisense 3'-U-U-C-C-C-U-U-A-A-C-A-C-C-G-A-G-U-C-C-C-A-G-G-U-U-G-A-5'; **Si27-M**, Sense, 5'-P-C-C-A-U-G-A-G-C-U-U-A-C-A-A-A-G-C-A-U-G-A-U-C-dC-dG, Antisense G-U-G-G-U-A-C-U-C-G-A-A-U-G-U-U-U-C-G-U-A-C-U-A-G-G-C -5'; **Si27-R**, Sense, 5'-P-C-C-G-U-C-C-A-A-G-C-A-G-G-A-U-G-A-G-U-A-G-U-C-dC-dG -3' Antisense 3'-G-U-G-G-C-A-G-G-U-U-C-G-U-C-C-U-A-C-U-C-A-U-C-A-G-G-C -5'; **SSL3**, Sense, 5'-U-C-U-C-A-A-A-A-C-U-C-G-A-G-U-G-A-C-A-U-U-3' Antisense 5'-P-U-G-U-C-A-C-U-C-G-A-G-U-U-U-U-G-A-G-A-U-U -3'.

Transfection reagent, RNAiMax™, RNA extraction kit, RNeasy® Mini Kit were purchased from Invitrogen and the reverse transcription kit, Qunatitect® was purchased from Qiagen Inc. (Valencia, CA). Taqman Universal PCR master mix (Cat # 4304437) and TaqMan MGB probe was purchased from Applied Biosystems (Foster City, CA);, forward and reverse primers for rat TNF- α (Cat # Rn00562055_m1) and the forward and reverse primers for rat β -actin (Cat # Rn 00667869_m1) were purchased from IDT Inc., All other chemicals used were of reagent grade, purchased either from Sigma-Aldrich (St. Louis, MO) or from Fisher Scientific (Pittsburgh, PA).

2.3 Preparation of Kupffer cells

Kupffer cells were prepared as previously described by Ponnappa et al [17], which is a modification of the procedure originally described by Bautista et al, [33]. Briefly, following anesthesia (Isoflurane machine), the liver was pre-perfused *in situ* with Ca²⁺ - and Mg²⁺ - free Hank's Balanced Salt Solution (HBSS, GIBCO-BRL, Gaithersburg, MD) for 20 min followed by a sequential perfusion with pronase (Sigma Cat # P-5147) and collagenase (Sigma Cat # C-5138) in HBSS (Ca²⁺ - and Mg²⁺ -reconstituted). All the solutions used in the perfusion were maintained at 37°C. After the perfusion, the liver was carefully cut into small pieces and placed in 90 ml of HBSS containing a mixture of collagenase and DNase (Sigma Cat # D-4527). The contents were shaken in an Orbital Shaker (Forma Scientific) at 37°C and 250 rpm for 15 min. The digested liver suspension was passed through a mesh to separate the dissociated cells from the undigested tissue. The heavier hepatocytes were

separated from the non-parenchymal cells by centrifugation at 50 g for 2 min. The pellet was washed three times with HBSS for maximum recovery of the non-parenchymal cells into the supernatant. The pooled supernatant was further centrifuged at 430 g for 6 min to pellet the non-parenchymal cells, washed twice and finally resuspended in 10 ml of in Gey's Balanced Salt Solution (GBSS) for centrifugal elutriation. Kupffer cells were separated from other cell-types by centrifugal elutriation (J2-MC centrifuge, JE-6B elutriator rotor, Beckman Coulter, Inc., Fullerton, CA). Purity of the Kupffer cell fraction (>85% pure) was routinely monitored by peroxidase staining. Kupffer cells stain brown and endothelial cells stain blue. The Kupffer cell fraction was further washed and resuspended in PBS. Viability, as determined by trypan blue exclusion, was generally greater than 90%.

2.4 Tissue culture

Freshly isolated Kupffer cells were routinely plated in 12-well plates at a density of $1-1.2 \times 10^6$ cells/well in a culture medium consisting of 1640 RPMI supplemented with 10% fetal bovine serum (FBS), 30 mM Hepes buffer, 1 mM pyruvate and antibiotics. Cells were cultured at 37°C in 5% CO₂ atmosphere and allowed to attach for 1 h, after which, the medium was changed. Routinely, Kupffer cells were cultured for 4–5 days before transfection with siRNAs. The transfected cells were exposed to bacterial lipopolysaccharide (E. Coli 055-B5, Sigma-Aldrich, USA) for the induction of TNF- α release as per details described in legends to figures representing specific experiments. Tissue culture media containing TNF- α were stored at –80° C until assayed using ELISA kit from eBioscience, (San Diego, CA), USA. TNF- α values from various treat conditions were normalized to the protein content of Kupffer cells.

2.5 Real-time RT-PCR

Total RNA was isolated from Kupffer cells using RNeasy® Mini Kit. After genomic DNA elimination, RNA was reverse-transcribed using QuantiTect® Reverse Transcription Kit according to manufacture's instructions. For real-time PCR, 10 ng of the reverse transcriptase product was used for the amplification of TNF- α or β -actin genes using respective primers and TaqMan MGB probe (6-FAM dye-labeled) together with TaqMan Universal PCR Mix. The plate was run on the ABI 7000 Sequence Detection System. Standard curves were generated and the relative quantity of target gene mRNA in each sample was normalized to β -actin mRNA. All reactions were done in triplicate.

2.6 Preparation of siRNA-encapsulated liposomes

Liposome-encapsulated siRNA was prepared by the reverse phase method of Szoka and Papahadjopoulos [34] with following modification for the encapsulation of siRNAs. A mixture (25 mg) of lipids (PE, CHEMS and cholesterol in a molar ratio of 8:2.5:2,) was dissolved in 4.5 ml of chloroform. Two to three mg of the siRNA was dissolved in 0.4 ml of Tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 8) and diluted to 1.5 ml with a hypotonic buffer (made up of 1:9 diluted phosphate buffered saline (PBS) supplemented with 25 mM sodium phosphate, pH 7.4). The aqueous siRNA solution was added to the lipid mixture in chloroform and sonicated in a bath-type sonicator for 5 min. The organic solvent was evaporated at room temperature using a rotary-type evaporator (BUCHI Rotovapor R-134, Flawil, Switzerland). The resultant liposomal suspension was diluted with the hypotonic buffer and centrifuged at $100,000 \times g$ for 45 min to separate the liposomes from the medium. The liposomal pellet was washed twice with modified PBS (PBS supplemented with 25 mM phosphate buffer, pH 7.4) and resuspended in a volume not exceeding 1 ml of modified PBS. Empty liposomes, used as the placebo in the *in vivo* studies, were prepared using the same proportion of buffers and lipid mixture but without the siRNA. The concentration of encapsulated siRNA was determined spectrophotometrically at 260 nm, after extracting siRNA from an aliquot of the liposomal preparation. Routinely, the amount

of siRNA encapsulated by the liposomes ranged from 10–14% of the total siRNA taken for encapsulation.

2.7 pH-sensitivity of siRNA liposomes

Aliquots of DsiRNA-encapsulated liposomes were exposed to varying pH conditions in the range of pH 4 to 7.4 at 37°C for 15 min. At the end of the incubation, the suspensions were centrifuged at 100,000g for 45 min at 4°C to separate the supernatant from the liposomal pellet. The amount of liposome-associated DsiRNA at each pH condition was expressed as percentage of the total liposomal DsiRNA at pH 7.4

2.8 Size determination, plasma stability and shelf-life of the siRNA-liposomes

Dynamic light scattering was performed with a DynaPro-MSXTC dynamic light scattering instrument (Wyatt Technology Corporation) using a 10 sec acquisition time at 25°C. The laser power was adjusted to keep the intensity between 300,000 and 400,000 counts/sec. The results were then processed with DYNAMICS™-Version 6.7.6 software program. The radii and the size distribution of the liposomes were calculated with the algorithm provided by this software. From the above data, the average diameters of the liposomal particles were calculated.

The stability of the liposomal-siRNA preparations in plasma was determined essentially as described earlier for liposome-encapsulated oligonucleotides [18]. Briefly, aliquots of siRNA-encapsulated liposomes were mixed with heparinized rat plasma and incubated at 37° C for 60 min. The final concentration of plasma was at least 90% and the concentration of liposomal lipid ranged from 0–4 mg per ml. In the control group, plasma was replaced by PBS. At the end of the incubation period, all samples were diluted 10-fold with PBS and centrifuged at 100,000 × g for 45 min to separate the liposomes from the medium. The amount of siRNA associated with the liposomal pellet was determined spectrophotometrically as described above.

To determine the stability of the liposomes upon storage at 0–4° C, at various intervals of time up to 6 months, the liposomal suspensions were recentrifuged at 100,000 × g for 45 min and the amount of siRNA associated with the liposomal pellets were determined.

2.9 *In vivo* targeting of Kupffer cells by liposomal siRNA - Fluorescence microscopy

For *in vivo* targeting, fluoresce (FAM)-labeled DsiRNA was encapsulated in liposomes and administered i.v. by tail vein and after 90 min, liver was perfused to obtain total hepatic cells as well as purified Kupffer cell fractions. A sample of the hepatic cells was subjected to peroxidase staining and cross-stained with methylene green, to identify peroxidase-negative endothelial cells from the other hepatic cells and the contaminating erythrocytes. Hepatic cells and purified Kupffer cells were fixed in 2% paraformaldehyde prior to fluorescent microscopy.

2.10 *In vivo* delivery of liposomal-siRNA

The *in vivo* efficacy of si27-3, was determined using the liposomal delivery method that was successfully used in our laboratory for antisense oligonucleotides [18,19]. Briefly, liposome-encapsulated si27-3, prepared as described above was injected intravenously (in a volume of PBS not exceeding 1 ml) into the animal by the tail vein route. Rats were injected with 0.1 mg/kg body weight of si27-3 each day for two successive days. On the fourth day, the animals were injected with LPS (0.1mg/Kg body wt., i.v.) to induce TNF- α secretion and sacrificed 90 min later. Venous blood was collected and allowed to coagulate at room temperature. Following centrifugation, serum was collected and stored at –80°C until TNF- α levels were determined by ELISA. Animals injected with empty liposomes were treated as

‘control’. In all of the experiments, the concentration of the liposomal lipid injected was maintained below 10 mg/kg body weight to minimize non-specific effects of the lipids on LPS-induced TNF- α secretion [18].

2.11 TNF- α assay

TNF- α was assayed by ELISA using a kit from eBioscience as per manufacturer’s specifications. Serum or cell culture media, treated with LPS, were diluted appropriately to make sure that TNF- α values were within the range of the standard curve.

2.12 Protein assay

Following exposure to LPS, after the media were taken out for TNF- α assay, Kupffer cells were rinsed with ice-cold PBS and solubilized in 0.5 N NaOH and assayed for total protein content using Micro BCA™ protein assay kit (Kit # 23235, Pierce, Rockford, IL) using bovine serum albumin as the standard.

2.13 Statistical analysis

Where indicated, values are expressed as the means $\pm$ S.E. of (n) determinations. Statistical differences were evaluated between two samples by ‘Student’s paired t test’ using the Microsoft Excel program. *P* values of < 0.05 were considered statistically significant.

3. RESULTS

3.1 *In vitro* studies

3.1.1 Efficacy and specificity of anti-TNF- α siRNA—The inhibitory effects of five DsiRNA constructs targeted against rat TNF- α mRNA were tested in primary cultures of rat Kupffer cells by assaying the LPS-induced secretion of TNF- α . As shown in Fig. 1, among the five DsiRNA constructs tested, si27-3 showed the highest efficacy inhibiting LPS-induced TNF- α release from Kupffer cells by 80%. Mismatches to the si27-3 sequence (si27-M and si27-R) confirmed that the inhibitory effect of si27-3 against was sequence-specific (Fig. 2). Also, as expected, the 27-bp dicer-substrate siRNA, si27-3, was more effective against TNF- α than SSL3, the 21-bp anti-TNF- α siRNA (Fig. 2). Further, RT-PCR studies (Fig. 3) confirmed that the inhibitory effect of si27-3 was due to the down-regulation of TNF- α mRNA, the targeted mRNA.

3.1.2 Dose-response studies—In the studies described above (Figs. 1–3), the efficacies of DsiRNAs were tested at 10 nM, a recommended starting concentration for newly synthesized siRNAs. However, DsiRNA constructs have been reported to demonstrate high degree of sensitivity at lower concentrations [30,31]. Therefore, we tested the dose-dependent effects of si27-3 in the range of 0.5 –20 nM. As shown in Fig. 4, significant inhibition (52%, $P < 0.05$) of TNF- α secretion was observed at 0.5 nM, increasing to 70% at 1 nM and peaking at 82% inhibition ($P < 0.01$) at 10 nM concentration of si27-3. However, in time-course studies, we observed that at lower (< 5 nM) concentrations, the inhibitory effect of si27-3 was lost at 96 h after transfection (data not shown). Therefore, in most studies, siRNA was used at the stable 10 nM concentration. Above 10 nM, the inhibitory effect of the siRNA was variable, suggesting non-specific effects.

3.1.3 Time-course studies—The temporal effects of si27-3 against TNF- α was determined by exposing cultured Kupffer cells to the si27-3 (10nM) for up to 96 h, after which, they were stimulated with LPS to induce TNF- α secretion. For purposes of comparison, we also included the SSL3, the 21-bp siRNA. Data in Fig. 5 show that the inhibitory effects of both si27-3 and SSL3 on TNF- α production, persisted for at least 4

days, the maximal duration studied. Once again, the higher (70–80%) inhibitory effect of si27-3 compared to that of SSL3 (50–60%) was maintained over the entire duration of 96 h.

3.2 *In vivo* studies

3.2.1 Characterization of the *in vivo* liposomal delivery system—The mean diameter of the liposomal preparation was 317 ± 21 nm ($n=4$). The liposomes were stable up to pH 6.2 and when the pH transitioned between 6 and 5, almost 80% of the liposomal contents were released into the medium, fulfilling the criteria of pH sensitivity (Fig. 6). When stored at 0–4° C (pH 7.4), greater than 90% of the siRNAs remained encapsulated in the liposomes for up to 6 months, demonstrating long shelf-life. Further, the stability of DsiRNA-liposomes in plasma depended on the concentration of the lipid. Similar to our previous studies using liposomal-AsODNs [18], the liposomal stability decreased with increased lipid concentration, losing 35% and 20% of the liposomal contents at 4 and 2 mg lipid/ml plasma respectively, after 60 min incubation at 37° C (data not shown). However, since the final concentration of liposomal lipid used in our *in vivo* studies is less than 0.5 mg/ml of blood, it is a reasonable assumption that there would be minimal loss of liposomal contents before they are sequestered by macrophages.

3.2.2 Targeting Kupffer cells with liposomal siRNA—The primary objective of using the liposomal delivery system was to achieve a high degree of specificity with regard to targeting Kupffer cells. Specific targeting of Kupffer cells, as opposed to other hepatic cells, was demonstrated by fluorescence microscopy using FAM-DsiRNA-encapsulated liposomes. As shown in Fig. 7, 90 min after liposome administration, fluorescence was detected primarily in Kupffer cells and not in other cell types, such as hepatocytes, endothelial cells or red blood cells. These observations confirm that in the liver, following intravenous administration, similar to AsODN-encapsulated liposomes [17,18], siRNA-liposomes are also preferentially sequestered by Kupffer cells, the primary source of TNF- α in the liver.

3.2.3 *In vivo* efficacy of si27-3—The *in vivo* efficacy of si27-3 was determined using the liposomal delivery system formulated as described above. Rats were intravenously injected either with liposome-encapsulated si27-3 or with empty liposomes, used as the control. Conforming to the established protocol from our previous studies (with AsODNs) [18], rats were injected with liposome-encapsulated siRNA (0.1 mg/kg body wt/day) for 2 days and challenged with LPS on the fourth day. Data presented in Fig. 8 show that si27-3-liposomes inhibited TNF- α secretion by 50% ($P < 0.005$) compared to the control group.

4. DISCUSSION

Macrophages are the primary source of TNF- α [14] and as reported earlier, an overproduction of TNF- α has been associated with many inflammatory diseases [3–9]. Therefore, developing a delivery system to target macrophages would be of therapeutic value. We have previously demonstrated that liposomes, being small particles in the size range above 200 nm in diameter, are primarily sequestered by Kupffer cells and splenic macrophages [17] and therefore, it is possible to selectively target a population of cells, such as these macrophages, which constitute a very small percentage ($< 0.2\%$) of the body weight but are major producers of TNF- α . Such an approach not only provides a high degree of targeting, but off-target effects, if any, would be confined to a very small population of cells. Previous studies from our laboratory have shown a 5–10-fold advantage of the liposomal delivery system versus the delivery of the AsODNs in the free form [19], a clear indication of the advantage of using the liposomal delivery system for the *in vivo* targeting of Kupffer cells and other macrophages.

The discovery of RNA interference, with the associated high sensitivities of siRNAs as gene-silencing agents, has provided newer opportunities in the treatment of many diseases [27,28]. Having successfully used the liposomal delivery system to down-regulate TNF- α using AsODNs, it was our objective to identify highly effective siRNAs against TNF- α , so as to be able to achieve *in vivo* efficacy at as low a concentration of the nucleotide as possible. To that end, in a recent study [29] using a 21-mer siRNA (SSL3) against TNF- α and the same liposomal delivery system, we demonstrated that siRNA was an order of magnitude more sensitive than the single stranded anti-TNF- α AsODN [29]. The observation that Dicer-substrate siRNAs are even more potent than the traditional 21-mer siRNAs [30,31], gave us the opportunity to explore the therapeutic potential of DsiRNAs against TNF- α using the liposomal delivery system. Among the five DsiRNA constructs, DsiRNA si27-3, was the most potent one against rat TNF- α , demonstrating greater than 80% inhibition at 10 nM; inhibition was still substantial (70%) at 1 nM. Under similar conditions (10 nM), SSL3 inhibited TNF- α production by only 60%, thus confirming the relative advantage of using DsiRNAs versus the 21-mer siRNAs. However, we would like to mention that these two classes of siRNAs are targeted to different regions of the mRNA, and hence, their relative potencies can not be compared in the same way as that described by Kim et al [30], in which the 21-mer siRNA is derived from the same DsiRNA. It is also possible that a traditional 21-mer siRNA, targeted to one region of the mRNA molecule, could be almost as potent as a DsiRNA targeted to a different region.

Nevertheless, our data show the obvious advantage of using DsiRNA to suppress TNF- α production in Kupffer cells. Four mismatches (si27-M) or random mismatches (si27-R) to the si27-3 sequence almost completely abolished the inhibitory effects of the DsiRNA, suggesting that si27-3 mediated effect on TNF- α was sequence specific. Further confirmation of RNAi-mediated mechanism of action of si27-3 came from real-time -PCR studies, in which, the reduction in mRNA levels paralleled the extent of inhibition of TNF- α secretion in Kupffer cells.

Time-course studies in primary cultures of rat Kupffer cells showed that the inhibitory effect of si27-3 remained relatively constant (70–80%) for at least 4 days, the longest duration studied. Interestingly, SSL3 also showed a similar trend but at as expected, at reduced (50–60%) levels. Taken together, our data confirm the identification of an anti-TNF- α DsiRNA construct, which can be potentially used in the treatment of inflammatory diseases.

Similar to other drugs, the major challenge facing siRNA therapeutics is in their *in vivo* efficacy, which requires an efficient delivery system. In most cases, siRNAs are delivered in the ‘naked’ form, which has the inherent problem of being subject to degradation in plasma and poor cellular uptake. To overcome such a problem, several chemical modifications to siRNAs have been attempted but off-target effects are a major concern [18, 27]. Against that background, an ideal delivery system was considered to be the one, in which, siRNA could be delivered to the target cells without interference from plasma components. Our previous studies show that pH-sensitive liposomes are ideally suited for plasma stability and delivery of oligonucleotides to Kupffer cells *in vivo* [18]. However, since the original liposomal delivery system formulated in our laboratory was optimized for the release of the single-stranded AsODNs [18], and since siRNAs are double stranded and larger, it was necessary to reformulate the preparation so that most of the liposomal siRNA was readily released below pH 6 (Fig. 6). This would allow the encapsulated siRNAs to remain stable within the liposomes while in circulation (pH 7.4) and release the contents from the endosomes following sequestration by Kupffer cells and other macrophages. Following phagocytosis the pH within the endosomes drops to pH 6 within minutes [35].

Fluorescence microscopy of hepatic cells after the intravenous administration of FAM-DsiRNA-encapsulated liposomes showed that in the liver, the liposome-encapsulated DsiRNAs are almost exclusively sequestered by Kupffer cells (Fig. 7). A preferential uptake by the Kupffer cells/macrophages is facilitated by the fact that the average diameter of the liposomes (317 nm) was much bigger than the size of the fenestrations (~150 nm) in the liver sinusoids.

The *in vivo* efficacy of the DsiRNA was assessed by measuring LPS-induced serum levels of TNF- α following delivery of liposome-encapsulated si27-3. We observed that si27-3 inhibited LPS-induced TNF- α secretion by 50% (Fig. 8), which is slightly better than the *in vivo* efficacy of SSL3, the 21-mer siRNA, which showed similar efficacy at a 40% higher concentration [29]. Our studies also show that siRNAs or DsiRNAs targeted against TNF- α are at least ten times more potent than the anti-TNF- α AsODNs used in an earlier study [19]. These data clearly suggest that DsiRNAs can be successfully used *in vivo* with a high degree of efficacy. It is also to be pointed out that in other models using the 'naked' form, it was necessary to administer multiple doses of a much higher siRNA concentration for *in vivo* efficacy [27].

The primary focus of the current study has been to target Kupffer cells. However, it is to be pointed out that spleen, by way of splenic macrophages, also has a high capacity to produce TNF- α in response to LPS stimulation [19]. In that study, we observed that intravenous administration of an anti-TNF- α AsODN inhibits LPS-induced TNF- α secretion by similar degrees in plasma, liver and spleen. More interestingly, splenectomy completely prevents LPS-induced liver damage in ethanol-fed animals [36]. These observations reinforce the need to simultaneously target splenic macrophages, a process that is also facilitated by the liposomal delivery system [17,18]. Although not included in the current study, the possibility that liposomes are also sequestered by the macrophages in the lung, has also been observed [17]. Thus, the liposomal delivery system provides an efficient system for the delivery of antisense drugs not only to Kupffer cells but to macrophages in multiple organs that are in direct contact with blood circulation.

5. Conclusions

In summary, studies described here provide data on the identification of a DsiRNA which has high efficacy against rat TNF- α mRNA. The DsiRNA shows reasonable *in vivo* efficacy at a very low concentration when administered as a liposomal formulation. With future options to incorporate sequence modifications, it may be possible to enhance the sensitivity and biological half-life of the DsiRNA. With these observations, we believe that liposomal-DsiRNAs have major potential as therapeutic formulations in the treatment of diseases such as alcoholic liver disease, septic shock and rheumatoid arthritis.

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Fig. 1. Effect of multiple DsiRNA constructs against TNF- α

Freshly isolated rat Kupffer cells from male Sprague-Dawley rats were cultured in 12 well-plates for 4 days. Cells were transfected (RNAiMax™) with five separate Dicer-substrate siRNAs (10 nM) targeted to various regions of rat TNF- α mRNA for 24 h. Cells exposed to transfection reagent alone (none) were included as controls. Twenty four hours after the transfection, Kupffer cells from all treatment groups were exposed to LPS (0.1 μ g/ml) for 2 h to induce TNF- α secretion. TNF- α secreted into the medium was assayed by ELISA and expressed as pg/ μ g protein. Data points are the means $\pm$ S.E. (n=4). In the absence of LPS treatment, there was no detectable secretion of TNF- α into the medium (data not shown).

Fig. 2. Sequence specificity of si27-3

The sequence specificity of the si27-3 was assessed by comparing its efficacies with four (si27-M) and multiple (si27-R) mismatches to its sequence. In addition, another 21-mer siRNA against TNF- α mRNA, SSL3 was also included. Primary cultures of rat Kupffer cells were transfected with 10 nM each of the siRNAs their efficacies were determined 24 h post transfection as described in legend to Fig. 1. Cells exposed to transfection reagent alone (plus LPS) were included as controls. Bars indicate values of TNF- α secretions which are the means $\pm$ S.E. of 4–6 determinations. ** $P < 0.01$ between cells treated with siRNA and transfection reagent alone.

Fig. 3. Inhibitory effect of si27-3 on TNF- α gene expression: Determination of mRNA levels by real-time RT-PCR

Freshly isolated Kupffer cells were cultured for 4 days followed by transfection with specific (si27-3) and irrelevant (si27-R) siRNAs (10 nM) along with SSL3 the 21-mer anti-TNF- α siRNA for 24 h as described in legend to Fig. 1. The cells were then exposed to LPS for 1 h and the total RNA was extracted and reverse-transcribed to cDNAs. Ten ng (cDNA) aliquot of each sample was taken for the determination of quantitative real-time PCR. The rates of TNF- α mRNA synthesis in various treatment groups were normalized to rates of synthesis of β -actin, included as the internal control. Bars indicate relative rates of TNF- α mRNA expression which are the averages ($\pm$ S.D.) of three determinations.

Fig. 4. Effect of si27-3 treatment on TNF- α secretion in Kupffer cells: Dose-response studies
 Primary cultures of rat Kupffer cells were transfected with various concentrations (0.5 –20 nM) of si27-3 for 24 h. The efficacy of the DsiRNA to inhibit TNF- α , secretion at each of the concentrations was determined following LPS stimulation as described in legend to Fig. 1. Data points indicate TNF- α values which are the means $\pm$ S.E. of n=4 determinations. * P < 0.05, ** P < 0.01 compared to controls treated with transfection reagent alone (None).

Fig. 5. Comparison of si27-3 and SSL3, on TNF- α secretion in Kupffer cells: Time-course studies

Freshly isolated Kupffer cells were cultured for 4 days followed by transfection with 10 nM of each of the siRNAs. At 24, 48, 72 and 96 h post transfection, TNF- α secretion was induced by LPS for 2 h. Data points indicate TNF- α values which are the means $\pm$ S.E. (n=3–8). Bars indicate TNF- α values secreted by siRNA- treated samples expressed as percent of that secreted by cells treated with transfection reagent alone. * $P < 0.05$ between si27-3 and SSL3- treated cells.

Fig. 6. Effect of pH on stability of liposomes

si27-3 siRNA-encapsulated liposomes were prepared as described in Methods and the stability of liposomes was determined at various pHs ranging from pH 4 to 7.4. Liposomes were incubated at 37° C for 15 min and centrifuged at $100,000 \times g$ for 45 min to separate the medium from the liposomes. The liposomal content of siRNA at each pH was expressed as percent of liposomal siRNA at pH 7.4. Data points are means $\pm$ S.D of 4–6 determinations.

Fig. 7. *In vivo* targeting of Kupffer cells with siRNA

Fluorescent-labeled siRNA was encapsulated in a pH-sensitive liposomal preparation and intravenously injected into a rat by way of tail vein. Liver was perfused with collagenase 90 min later for the isolation of hepatic cells. Kupffer cells were separated from the rest by centrifugal elutriation. Purified cell preparations were fixed in 4% paraformaldehyde and subject to fluorescence microscopy. Top panel: Fluorescent, transmitted and merged images of hepatic cells (upper) and Kupffer cells (lower). Bottom panel: Magnified image of an siRNA- targeted Kupffer cell (left) and the evidence of preferential targeting of liposomal siRNA to Kupffer cells vs hepatocytes (right). Hepatocytes, *brown arrow*; Kupffer cells, *orange arrow*; Endothelial cells, *blue arrow*; Red blood cells, *green arrow*.

Fig. 8. *In vivo* efficacy of si27-3

The *in vivo* efficacy of si27-3, the anti-TNF- α DsiRNA, was determined following administration of liposome-encapsulated si27-3. Littermate pairs of male Sprague Dawley rats were intravenously injected with either liposomal si27-3 (0.1 mg/kg body wt) or empty liposomes as the lipid control, daily for two days. On the fourth day, TNF- α secretion was induced by LPS (0.1mg/kg body wt) administration and blood was collected at 90 min post LPS. Serum levels of TNF- α were determined by ELISA and expressed as pg/ml. Bars indicate serum TNF- α values, which are the means $\pm$ S.E. of (n=11) rats per group. *** $P < 0.005$ between animals treated with si27-3 and empty liposomes.