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Protection against TNF α -Dependent Liver Toxicity by Intraperitoneal Liposome Delivered DsiRNA Targeting TNF α *In Vivo*

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Abstract

Tumor necrosis factor alpha (TNF α) is a classic proinflammatory cytokine implicated in the pathogenesis of several autoimmune and inflammatory diseases including viral encephalitis. Macrophages being major producers of TNF α are thus attractive targets for *in vivo* RNA interference (RNAi) mediated down regulation of TNF α . The application of RNAi technology to *in vivo* models however presents obstacles, including rapid degradation of RNA duplexes in plasma, insufficient delivery to the target cell population and toxicity associated with intravenous administration of synthetic RNAs and carrier compounds.

We exploited the phagocytic ability of macrophages for delivery of Dicer-substrate small interfering RNAs (DsiRNAs) targeting TNF α (DsiTNF α) by intraperitoneal administration of lipid-DsiRNA complexes that were efficiently taken up by peritoneal macrophages and other phagocytic cells. We report that DsiTNF α -lipid complexes delivered intraperitoneally altered the disease outcome in an acute sepsis model. Down-regulation of TNF α in peritoneal CD11b⁺ monocytes reduced liver damage in C57BL/6 mice and significantly delayed acute mortality in mice treated with low dose LPS plus D-galactosamine (D-GalN).

INTRODUCTION

Tremendous progress has been made in exploiting the RNAi pathway for silencing gene expression *in vitro* and *in vivo*. The availability of small interfering RNAs (siRNAs) that are highly efficient for gene silencing is routine, thanks in large part to the development of new algorithms for optimal target identification [1, 2]. *In vivo* application of RNAi is a

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promising approach for silencing disease genes, interfering with replication of invading pathogens and deciphering gene function in mice [3–7]. Factors that need to be considered for *in vivo* siRNA studies have been reviewed in detail [2] and include the target tissue, route of administration, delivery vehicle and chemical modifications to confer nuclease resistance while minimizing off-target effects and non-specific immune responses. Over recent years, a variety of increasingly complex liposomal and nanoparticle delivery systems have been described for *in vivo* use of siRNA in both animal model research systems and in therapeutic applications [8–11]. Significant effort has been expended on improving the efficiency and toxicity of delivery tools that can carry a nucleic acid cargo *in vivo*. Much of the toxicity of these tools arises from complications encountered with intravenous (IV) administration. For some applications, however, intraperitoneal (IP) administration can be equally efficacious and fewer adverse toxic events are encountered. In fact, we have observed that some of the same cationic lipid emulsions employed for cell culture work *in vitro* can be safely employed using IP administration *in vivo*.

We showed previously that resistance to lethal herpes simplex virus encephalitis (HSE) is dependent on TNF α signaling. Specifically, intraperitoneal treatment with the phagocytic cell toxin clodronate or DsiRNA targeting TNF α (DsiTNF α) encapsulated in liposomes or administration of a soluble TNF α p55 receptor (TNFR1) or a monoclonal antibody specific for TNF α by the same route during ocular HSV infection dramatically increased susceptibility of the normally resistant C57BL/6 (B6) mice [12, 13]. The increased susceptibility to HSV mortality of B6.TNF $\alpha^{-/-}$ mice relative to wild type control mice affirms the protective role of TNF α on the B6 genetic background and we further demonstrated this using *in vivo* DsiRNA [14]. Intraperitoneal delivery of siTNF α in DOTAP liposomes has previously been used to limit LPS-induced septic shock in a mouse model [15] although the end-point employed was subjective in that euthanized mice were included in the mortality analysis as opposed to being censored. We extended these studies, assessing the effect of a high potency DsiRNA targeting TNF α in a different sepsis model: treatment with low dose LPS plus D-galactosamine. Additionally, we present data on efficiency of DsiTNF α uptake and demonstrate an optimal amount of DsiTNF α allowing targeting of TNF α with a minimum of non-specific activation of peritoneal monocytes after DsiTNF α -lipid complex administration.

We initially studied a set of DsiRNAs targeting TNF α *in vitro* in the murine RAW264.7 macrophage cell line to validate reagents and methods and achieved highly efficient knockdown of TNF α following LPS stimulation. We next optimized *in vivo* intraperitoneal delivery of the DsiRNA to achieve potent knockdown of TNF α in CD11b-positive peritoneal cells in the absence of non-specific immune responses and demonstrated protection against liver damage induced by treatment of mice with D-GalN plus low dose LPS (D-GalN is a sensitizing agent making hepatocytes more susceptible to TNF α -induced apoptosis). These results suggest that targeting appropriate macrophage genes with DsiRNAs delivered intraperitoneally may be an effective strategy to modulate diseases involving macrophages that occur in tissues outside of the peritoneal cavity. The peritoneal cavity is a reservoir for macrophages and delivering immune-response modifying reagents via an IP route can result in systemic effects for a variety of inflammatory disease processes.

MATERIALS AND METHODS

Cells, mice, virus and LPS

RAW264.7 cells were maintained in DMEM supplemented with 5% low endotoxin serum (Omega Scientific, Tarzana, CA), 4% mM L-glutamine, 100U/ml Penicillin G and 100 mg/ml Streptomycin. Cells were passaged using trypsin-free Dissociation Buffer (Gibco-BRL) and a rubber policeman. 10 mg/ml stocks of *E. coli* LPS O55:D5 (Sigma) were sonicated

until clear and stored at -20°C . Low dose LPS (200 ng/mouse) with D-galactosamine (20 mg/mouse) were the lowest concentrations for reproducible induction of liver damage (data not shown). C57BL/6 mice were purchased from Jackson Labs and bred in the City of Hope vivarium. All animal experiments were done according to protocols approved by the City of Hope Research Animal Care Committee in compliance with federal regulations for animal welfare.

***In vitro* neutralization of TNF α**

RAW264.7 cells were plated at 6.0×10^4 cells per 24-well in DMEM with 5% low endotoxin FBS (Omega Scientific, Tarzana, CA) without antibiotics and incubated overnight. Only wells that were 70–80% confluent after incubation were used in assays. For transfections, the manufacturer's protocols were followed. Briefly, for each well to be transfected, 47.5 microliters of serum-free medium (Invitrogen, Carlsbad, CA) and 2.5 microliters of TransIT-TKO (MirusBio, Madison, WI) were mixed and incubated at room temperature for 10 minutes. After incubation, DsiRNA was added at a 5X concentration, gently vortexed and incubated a further 10 minutes at room temperature. Following the preparation of the DsiRNA-lipid complexes, the medium in each 24-well was aspirated and replaced with 200 microliters DMEM-5%FBS to which the 50 microliter complex mix was added. Cells were then incubated overnight (16–18 hours) before used in LPS stimulation assays and flow cytometry analysis. RAW264.7 cells transfected with varying concentrations of DsiTNF α were treated with 3ng/ml LPS (*E. coli* O55:B5; Sigma, St. Louis, MO), for 6 hours, previously determine to result in half-maximal TNF α production (data not shown). One hour after the start of LPS treatment, 10 microgram/ml final concentration of Brefeldin A was added to trap newly synthesized TNF α . These cells were then harvested for intracellular staining for TNF α and flow cytometry analysis.

***In Vivo* Neutralization of TNF α**

The DsiRNA duplexes specific for TNF α and the control (eGFP-targeting) duplex used in this study followed established design rules [16, 17] and are shown in Table 1. Peritoneal administration was performed as previously described in detail [2, 14]. Briefly, 5 μg DsiTNF α complexed to TransIT TKO in a total volume of 200 μl PBS was injected into the peritoneal cavity of mice. To determine the level of DsiRNA uptake *in vivo*, DsiTNF α preparations were spiked with a Cy3-conjugated non-targeting control DsiRNA (the same control DsiRNA, eGFP-Cy3, used in the *in vitro* optimizations) at 5% of the total amount of DsiTNF α . High dose LPS treatment to assess non-specific activation from intraperitoneal siRNA treatment was achieved by intraperitoneal injection of 100 mg LPS on day 0; DsiTNF α was given on day 0, 1, and 2 and peritoneal exudate cells were collected on day 3 and analyzed by flow cytometry. The high dose LPS was only used to optimize knockdown of TNF α production with minimal non-specific activation of peritoneal monocytes *in vivo*.

***In vivo* sepsis model using D-GalN and LPS to induce acute liver toxicity**

The acute liver toxicity model for sepsis was performed as previously described [18]. Briefly, mice were injected intraperitoneally with 20mg D-GalN and 100ng LPS to induce TNF α -dependent hepatic failure. Following this injection, DsiTNF α was administered as described above to assess its efficacy in this stringent *in vivo* model.

Flow cytometric, histological and microscopic analysis

RAW264.7 cells transfected with DsiTNF α and treated with LPS and Brefeldin A as described above were fixed with 2% formaldehyde and stained for intracellular TNF α . Briefly, cells were permeabilized for 30 minutes with 1% saponin in PBS with 2% FBS and

stained with antibody to TNF α , washed with PBS-2% FBS and re-fixed with 2% paraformaldehyde before flow cytometry analysis.

Peritoneal exudate cells were re-stimulated *ex vivo* with 3 ng/ml LPS in the presence of Brefeldin A for 6 hours followed by intracellular staining for TNF α (as described above for RAW264.7 cells) and surface staining for MHC II, CD11b and CD11c (BD Pharmingen, San Diego, CA). All antibodies were used at 1:500–1:800 dilution of manufacturer's stocks (0.5 mg/ml). Samples were analyzed on a FACSCalibur and data processed using FlowJo software.

5 micron horizontal sections of the right liver lobe were stained with hematoxylin/eosin and tissues were examined by a blinded observer familiar with liver morphology under a light microscope for pathological changes.

Fluorescence microscopy was performed using an inverted Olympus BH2 Microscope equipped with a DVC Digital Video Camera; images were captured using XCap Lite image processing software from EPIX Inc. In order to visualize faint fluorescence in some cell to allow for correlation with flow cytometric analysis, the exposure times and gain settings were increased. Without this adjustment, all images except those where DsiTNF α was used at 5 or 25 nM concentrations would be nearly or completely black and thus not be useful in the analysis.

Statistical analysis

Data were analyzed using Prism 5.0a for Macintosh using LogRank and Student's t-test statistics where appropriate to determine differences between groups of mice and judged significant if $p < 0.05$. All experiments in this study were performed two-four times.

RESULTS

In vitro optimization of DsiTNF α designs using the RAW 264.7 macrophage cell line

We used the *TransIT*TKO transfection reagent (Mirus Bio, Madison, WI) for all studies reported here. Transfection of the DsiRNAs into macrophages showed exceptionally high efficiency without obvious adverse effects on cellular viability during the initial *in vitro* screening phase. Specific uptake of a Cy3-labeled control DsiRNA (eGFP-Cy3) specific for GFP was assessed in transfected RAW 264.7 cells by monitoring Cy3 fluorescence using flow cytometry as shown in Figure 1A. The complete population shift at even the lowest siRNA concentrations is suggestive of transfection efficiency approaching 100%, which we confirmed with fluorescence microscopy (Figure 1B). We observed similarly high transfection rates (~100%) with other cell types, including HL60 suspension cells that are notoriously difficult to transfect (not shown).

Rationally designed anti-TNF α 27-mer DsiRNAs showed superior activity relative to the corresponding 21-mer designs in our hands [2]. To test the efficacy of various designs of anti-TNF α DsiRNA reagents, we developed an intracellular staining assay for TNF α production after overnight DsiRNA transfection followed by short-term *in vitro* LPS stimulation of RAW 264.7 cells (Figure 2A). Briefly, TNF α expression by mock-transfected cells not stimulated with LPS was used as baseline and mock-transfected cells exposed to a dose of LPS (3ng/ml) that results in half-maximal TNF α production was used to normalize the read-out to 100%. DsiRNA at three sites in the murine TNF α gene were studied and the most potent site was further optimized (data not shown). At the best site (Site1, DsiTNF α S1), anti-TNF α RNA duplexes were studied for potency as triggers of RNAi comparing a blunt 1st generation Dicer-substrate design [16] with newer asymmetric designs [19]. In this study, the duplex “DsiTNF α S1v1” is a blunt 27mer while the “DsiTNF α S1v2.1” and

“DsiTNF α S1v2.2” duplexes correspond to the asymmetric “Left” and “Right” processed DsiRNA designs as described in Rose et al. [19]. Similar to results reported by Rose, the asymmetric “Right” processed DsiRNA (DsiTNF α S1v2.2) was most potent and was the reagent used in subsequent *in vivo* studies. At a concentration of 5 nM, greater than 50% of the cells no longer produced TNF α (Figure 2B) as determined by intracellular staining. In all *in vitro* experiments, a parallel Cy3-siRNA control (eGFP-Cy3) was performed to confirm high transfection efficiencies were attained (not shown).

Optimization of DsiTNF α dose for *in vivo* use

We speculated that the lipid to nucleic acid complexation ratio of the DsiRNA-liposome complexes would affect the uptake efficiency *in vivo* and therefore tested different ratios of siRNA to liposome in both RAW cells and peritoneal cells *in vivo* (Figure 3). The complexation ratio influences both particle size and transfection efficiency [20]. Significantly higher cellular uptake of the dye-labeled RNA (i.e., visible fluorescence signal) was seen using high complexation ratios (1:60) than using low complexation ratios (1:10) (Figure 3A). A comparison of the uptake of dye-labeled RNA by RAW264.7 cells and peritoneal exudate cells (PECs) using FACS analysis is shown in Figure 3B. The relative fluorescence patterns are similar between these two cell populations, indicating that the optimization work performed *in vitro* using RAW264.7 macrophages directly correlated with behavior of peritoneal mononuclear cells, which are the intended *in vivo* target population. The “1:20” ratio of DsiTNF α :TKO was employed in subsequent *in vivo* experiments.

To use DsiTNF α for *in vivo* experiments, the dose employed must be effective in suppressing TNF α without causing non-specific activation of the transfected cells, for example induction of MHCII on monocytic cells. This is a particular concern since our target cell populations are immune cells, the target is an inflammatory cytokine, and the system studied is a pathological inflammatory state. We titrated the dose of DsiTNF α from 2 to 50 mg *in vivo*, administering the DsiRNA intraperitoneally over a period of three days (given in three separate doses, as 20–40–40%), to determine the lowest dose giving maximal *in vivo* TNF α knockdown while minimizing non-specific activation of peritoneal CD11b⁺ monocytic cells. Mice were sacrificed on day 4 and the peritoneal exudate cells (PECs) were collected by lavage and incubated *ex vivo* in the presence of LPS for 6 hours as was done for the RAW cell assay in Figure 2. Cells were stained for CD11b, CD11c, MHCII and intracellular TNF α and analyzed by flow cytometry. TNF α and MHCII staining of peritoneal cells pre-gated to be CD11b⁺ CD11c[–] is shown in Figure 3C. Compared to mock transfected cells, a 2 mg DsiTNF α -s1 dose reduced TNF α production and a 10 mg was even more effective resulting in a 4-fold reduction in TNF α -positive cells without an increase in MHCII expression. In contrast, non-specific activation, evidenced by increased MHC II and TNF α expression, was seen with the 50 mg dose (Figure 3C). A 10mg dose of DsiRNA delivered intraperitoneally over a 3 day period was therefore adopted as optimal for *in vivo* studies in mice. It is well established that synthetic dsRNAs can trigger immune responses from cells through interaction with specific cellular receptors, such as TLRs 3, 7, and 8, RIG-I, and others [21], especially when using cationic lipid-based reagents to facilitate transfection. The degree of immune stimulation is influenced by the length, sequence composition, structure, and chemistry of the synthetic RNA trigger. This response is also sensitive to dose. The 2 and 10 mg doses of the DsiTNF α reagent were high enough to elicit an anti-TNF α RNAi effect while being under the threshold necessary to trigger an immune response, whereas at the 50 mg dose an immune response occurred which resulted in an increase in TNF α levels in the face of co-existing suppressive RNAi effects. It is often possible to reduce or eliminate unwanted immune-activating effects of dsRNA through use of chemical modifications, particularly through substitution of 2'-modified sugar groups

(such as 2'-*O*-methyl RNA) which act as a competitive inhibitor of TLR7 activation [22]. Although a wide variety of modification options exist that work well with siRNA *in vivo* [23], we elected to employ unmodified DsiRNA for our *in vivo* studies and used the 10 mg dose, which was shown to effectively suppress TNF α production while at the same time being below the threshold to trigger an immune response.

Next, we repeated these studies using the Cy3-conjugated control DsiRNA and stained PECs for the presence of lymphocytic (CD4/CD8 pool mAbs) or monocytic (CD11b/F480) cells to determine that the DsiRNA was taken up by CD11b-positive cells (>85% Cy3-positive vs. <3% in the lymphocyte gate) in the peritoneal cavity (Figure 3D) as implied by results in Figure 3A and B). This difference in uptake likely reflects the differences between phagocytic monocytes relative to lymphocytes.

***In vivo* DsiTNF α ameliorates the severity of induced liver damage and prolongs survival**

Mice sensitized with D-GalN, become highly susceptible to fatal LPS-induced liver failure, which occurs rapidly within 9 h post-induction [24]. Since, liver damage in this model is dependent on LPS-induced TNF α production from peritoneal macrophages, it is a useful model to evaluate the capacity of *in vivo* delivered DsiTNF α to moderate liver damage. Mice were untreated or treated intraperitoneally with a control DsiRNA (eGFP-Cy3) or DsiTNF α S1v2.2a and immediately thereafter with D-GalN/LPS. As an independent control, DsiTNF α for another location within TNF α (Site 3, DsiTNF α -S3v2.2) was included (in this experiment only). Mice were sacrificed 6 hours after treatment, livers were processed for histology and PECs were analyzed by flow cytometry to estimate *in vivo* transfection efficiency. Figure 4A shows the efficiency of Cy3-DsiRNA transfection of peritoneal cells (left side panels), representative of two mice per treatment group, while the right side shows H/E stained liver sections from two individual mice in four groups: No D-Gal/LPS (**I, II**), D-Gal/LPS with irrelevant DsiGFP (**III, IV**), DsiTNF α (**V, VI**), and DsiTNF α design 2 (**VII, VIII**). Tissue damage (visualized by interstitial bleeding) and cellular infiltrates (arrows) were reduced only in the DsiTNF α treated groups as compared to the eGFP-Cy3 control. Since the DsiTNF α was highly effective in preventing liver damage in the D-GalN/LPS model we next evaluated its ability to reduce mortality. Monitoring mice for survival in a separate experiment revealed that DsiTNF α was capable of delaying, but not preventing mortality in this model when administered at the time of D-GalN/LPS injection (n=5, MOCK n=3), but had no effect when given 16 h prior to D-GalN/LPS treatment (n=3) (Figure 4B). We interpret this to mean that the peritoneal cells transfected with the pretreatment are not the same as those that respond to the subsequent D-GalN/LPS treatment. One possibility is that the D-GalN/LPS injection recruits circulating monocytes that are only the target of transfection if the DsiTNF α liposome complex is administered simultaneously. The delay in mortality of approximately 2 hours in this model was statistically significant (p=0.0101) as mice typically succumb to acute liver failure within six to nine hours as did our control group. This delay demonstrates how local intraperitoneal administration of a DsiRNA can have systemic effects, altering disease outcome in the liver.

DISCUSSION

TNF α is a proinflammatory cytokine that is primarily made by activated macrophages [25, 26]. It is a necessary immune factor involved in host defense against a wide variety of pathogens. At the same time, inappropriate TNF α expression is associated with a wide range of autoimmune pathologic states, including rheumatoid arthritis, ankylosing spondylitis, psoriasis, and the inflammatory bowel diseases. As a result, therapeutics that suppresses TNF α expression are now widely employed to treat autoimmune diseases; however, these

treatments are often complicated by an increased incidence of infection resulting from impaired innate defenses. High levels of TNF α are also associated with gram negative sepsis or endotoxemic states. TNF α production is a normal host response to the presence of these foreign antigens/pathogens, yet the resulting over-expression of TNF α results in severe complications for the host. High TNF α levels are an integral part of the 'cytokine storm' that accompanies overwhelming infection, leading to cardiovascular collapse and often premature death. Many groups have tried to use inhibitors of TNF α as a therapy in models of sepsis; while such interventions do transiently improve clinical status, in most cases the animals still succumb to the infection or inflammatory process [27], probably because TNF α is not the only important mediator of inflammation involved in the pathophysiology of sepsis or other experimental endotoxemic states. Such interventions may eventually find a therapeutic niche, or at least their study can be informative about the biology and pathology of TNF α . More importantly, the therapeutic intervention did alter the expected time course of the disease, demonstrating positive effects at a distal site when using local peritoneal administration of a DsiRNA.

The same anti-TNF α DsiRNA sequence employed in the present work has also been used in several animal models to study the role of TNF α in other disease states. We previously employed the DsiTNF α reagent (also using IP administration with the TransIT-TKO cationic lipid) to modify host response to CNS HSV-1 infection (herpes encephalitis) [2, 14]. In this case, CNS effects were achieved with a therapy given by intraperitoneal injection. It is unlikely that the DsiRNAs directly transited the blood-brain barrier but rather were taken up by peritoneal macrophages that, as a mobile cell population, were subsequently recruited to the site of inflammation in the CNS, carrying the RNAi cargo with them.

A group from the University of Aarhus, Denmark, led by Jørgen Kjems and Ken Howard, employed IP administration of a chitosan nanoparticle delivery system with the same anti-TNF α DsiRNA in yet other disease models [28]. In one example, the chitosan/DsiTNF α was demonstrated to moderate the severity of murine collagen-induced arthritis [29]. In a second example, the chitosan/DsiTNF α was demonstrated to entirely prevent development of radiation fibrosis following high dose gamma irradiation of a hind limb [30]. In all four of these examples (herpes encephalitis, collagen-induced arthritis, radiation-induced fibrosis, and the present hepatic necrosis following endotoxin administration), the anti-TNF α compound (DsiTNF α) was administered by IP injection and was demonstrated to modify an inflammatory process at a distant site, presumably by targeting resident peritoneal macrophages which are recruited to the distal sites of inflammation while carrying the transfected RNA cargo, thereby blunting the severity of the local inflammatory response at that site.

The intraperitoneal delivery route is commonly used in research settings where such injections are convenient in rodents but is employed far less frequently in medical therapeutics. IP administration of chemotherapeutic agents, however, is gaining acceptance as a standard treatment for certain cancers with metastatic spread in the peritoneal cavity, and physicians are gradually becoming more comfortable with these kinds of treatments. It is attractive to consider the possibility that human inflammatory disorders might one day be treated using IP injection of agents which alter the ability of macrophages to secrete inflammatory cytokines, such as TNF α . To be practical, sustained release formulations would need to be employed which would permit long term effects to be realized from a single injection. Unfortunately, small nanoparticles are rapidly cleared from the peritoneal cavity and are absorbed into the lymphatic circulation or cleared in the spleen [31]. For RNAi-based therapeutics to succeed using this route, a functional RNA/nanoparticle delivery complex will probably need to be formulated in a matrix that permits high loading

with slow release over time yet is not an irritant that promotes fibrosis or formation of adhesions.

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Figure 1. TransIT-TKO liposome transfection efficiency
A. RAW 264.7 cells were transfected as described in **Materials and Methods** using 0.2–25 nM eGFP-Cy3 duplex and monitored for cellular Cy3 fluorescence by flow cytometry. **B.** Samples in **A.** were photographed under a fluorescence microscope.

Figure 2. Efficiency of rationally designed DsiTNF α duplexes

A. RAW 264.7 cells were transfected as described in **Materials and Methods** using 0.2–25 nM DsiTNF α .S1v2.2 duplexes and stained for intracellular TNF α production after LPS stimulation. Gated population indicates TNF α -negative cells. **B.** Gated populations in the histograms in **A.** were graphed as the percentage TNF-negative cells using the LPS only control as the common origin. Statistically significant values showing improvement compared to DsiTNF α .S1v1 are indicated as * $p < 0.05$ and ** $p < 0.01$.

Figure 3. Transfection optimization for *in vitro* and *in vivo* DsiRNA delivery

A. Varying ratios of eGFP-Cy3 and TransIT-TKO affects transfection efficiency and is confirmed both visibly (left panels) and fluorescently (right panels). **B.** The complexation ratios shown in **A.** transfect RAW 264.7 and peritoneal exudate cells with parallel and variable efficiency when analyzed by flow cytometry. **C.** PECs transfected *in vivo* with different amounts of DsiTNF α S1v2.2a following high dose LPS *in vivo* demonstrate an optimum concentration of DsiRNA to target TNF α without non-specific stimulation as judged by staining for MHCII and intracellular TNF α . **D.** PECs stained for T-cell (CD4 and CD8 pooled) and monocyte (CD11b) markers are shown in a dot plot (left panel) and cell populations positive for either stain are shown in the histogram (left) identifying the level of eGFP-Cy3 uptake after *in vivo* transfection.

Figure 4. DsiTNF α abrogates disease development in the D-GalN/LPS model

A. Mice were either untreated (top row microscope and panels I and II) or given D-GalN/LPS and control eGFP-Cy3 (2nd row and III/IV), DsiTNF α -S1v2.2 (3rd row and V/VI) or DsiTNF α -S3v2.2 (4th row and VII/VIII). **B.** Survival following D-GalN/LPS and either eGFP-Cy3 (squares), 16h pre-treatment with DsiTNF α -S1v2.2 (circles) or concomitant DsiTNF α -S1v2.2 treatment (triangles). P-value in panel **B.** refers to comparison between the MOCK and DsiTNF α -S1v2.2 groups.

Table 1
DsiRNA duplexes used in this study

RNA nucleotides are shown in upper case letters, DNA nucleotides are shown in lower case letters

Duplex name	Oligo strand 1 (5' -> 3')	Oligo strand 2 (3' -> 5')
TNF α -S1v1	GCCUAUGUCUCAGCCUCUUCUCAUUUU	CGGAUACAGAGUCGGAGAAGAGUAAUU
TNF α -S1v2.1 ^a	gcCUAUGUCUCAGCCUCUUCUCAUUC	CGGAUACAGAGUCGGAGAAGAGUAAp
TNF α -S1v2.2 ^b	pGUCUCAGCCUCUUCUCAUUCUGct	UACAGAGUCGGAGAAGAGUAAGGACGA
TNF α -S3v2.2	pGGUCUACUUUGGAGUCAUUGCUCtg	GUCCAGAUGAAACCGAGUAACGAGAC
eGFP-Cy3 (control)	Cy3-AAGCUGACCCUGAAGUUAUCUGCACC	UUCGACUGGGACUUCAAGUAGACGUGG

p = phosphate, and Cy3 = Cyanine-3 fluorophore.

^av2.1 = "Left" design and v2.2 = "Right" design from Rose et al.

^b"DsiTNF α " primarily used in present study.