Supporting Information (SI)

Supportive Data for the Manuscript

Peritoneal Macrophage-Specific TNFα Gene Silencing in LPS-Induced Inflammation Model using CD44 Targeting Hyaluronic Acid Nanoparticles

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Results

In Table 1 SI, is shown the siRNA encapsulation efficiency. Including HA-PEI in the formulation likely promoted the siRNA encapsulation therefore high values of 99% efficiency were measured. In the main paper, we showed the efficacy benefit of encapsulating chol-siTNFα into HA-PEI: HA-C6: HA-PEG at 3:2:1 ratio. The polymer was provided by the Northeastern University, Department of Pharmaceutical Sciences lab [1]. The formulation appeared to be consistent from batch to batch with respect to particle size, distribution, and siRNA encapsulation efficiency. These characteristics were tested routinely for the new formulations.

In Table 2 SI, are shown the average normalized concentrations of the active strand RNA taken up in peritoneal cells and tissues. The anti-sense RNA concentrations, measured by qPCR technique, indicated that the naked chol-siRNA was taken up in equally or more in tissues, as compared to the chol-siRNA in HA polymer. Plausibly, the uptake of the HA encapsulated chol-siTNFα was CD 44 mediated in the resident tissue macrophages, but there could have been a nonspecific uptake of the naked chol-siTNFα in tissues, due to cholesterol delivering properties.
In the peritoneal macrophage cells the concentration of the active strand RNA, was at least 14 folds lower during the first couple of hours, and dropped to 127 fold lower at 48 hours, in cells extracted from animals treated with naked chol-siTNFα as compared to the treated ones with chol-siTNFα encapsulated in HA. It is arguable that the HA nanoparticle would remain available to continuous cell uptake in the peritoneal cavity for up to 48 hours. Therefore the fairly consistent active RNA concentration in nanoparticle treated cells starting from 4 hours post IP injection up until 48 hours post injection indicates a stability increase dedicated to the chol-siTNFα encapsulation. Likely, the stable nanoparticle constantly releases the siRNA into the cells, enabling a fairly consistent siTNFα concentration within the cell. This data also suggested that the concentration of the active RNA in peritoneal cells extracted from animals treated with naked chol-siTNFα, was progressively decreasing within the cell, which plausibly indicates that the engulfed siRNA, gets degraded more rapidly in a naked form.

The concentrations of active RNA measured in the kidney tissue of animals suggested that the naked chol-siRNA started clearing as early as 2 hours post injection. The concentration

of the active RNA in the kidneys of naked chol-siRNA treated animals was higher during the early hours, and then remained fairly consistent until 48 hours. In the kidney tissues of the nanoparticle treated animals the active strand RNA concentration was stable throughout the experiment time points, which indicated consistent siRNA clearance.
The data suggested that there were measurable active RNA concentrations in the plasma. This was the compartment which exhibited large margin of variability. It is likely that the chol-siRNA either naked or encapsulated got into plasma through local lymph nodes into the blood stream. The concentrations of the active RNA in plasma dropped quickly 2 hours following the IP injection.

In this study peritoneal macrophages were targeted. The quantitative data from biodistribution studies indicated the advantage of HA-chol-siTNFα over the naked chol-siTNFα, in delivering more active RNA within the peritoneal cells, where macrophages were recruited with use of thioglycollate, prior to siTNFα injection.

In Figure 1 SI a), is shown the effect of LPS injection in TNF-α level of peritoneal macrophages, in naïve animals versus LPS treated animals. The LPS at 0.5 mg/kg dosing increased the level of TNF-α by two folds at 24 hours after its injection. The amplification of TNF-α expression between 0 and 24 hours after LPS injection, was not measured. It is reported that the TNF-α peak in LPS treated macrophages is reached at 6 hours post challenging[2].

In Figure 1 SI b), is shown the result for the group of mice treated with scramble siRNA encapsulated in HA polymer, versus the LPS treated and empty polymer treated. This data indicated that the polymer injected in mice did not affect the level of the TNF-α.

In Figure 2 SI, is shown that the LPS induction at 0.5 mg/kg upregulated the TNF-α gene expression in peritoneal macrophages in mice, but also caused systemic immune response illustrated by upregulation of a panel of cytokines in the blood stream of animals.

In Figure 3 SI a), is shown the sequence order of the siRNA used with the *in-vivo* studies. In Figure 3 SI b) displays the principle of quantitative qPCR method used for quantifying the active strand of siRNA in tissues and cells. In Figure 3 SI c), is shown the designation process of the stem loop oligo, primers, and probe for this qPCR protocol.

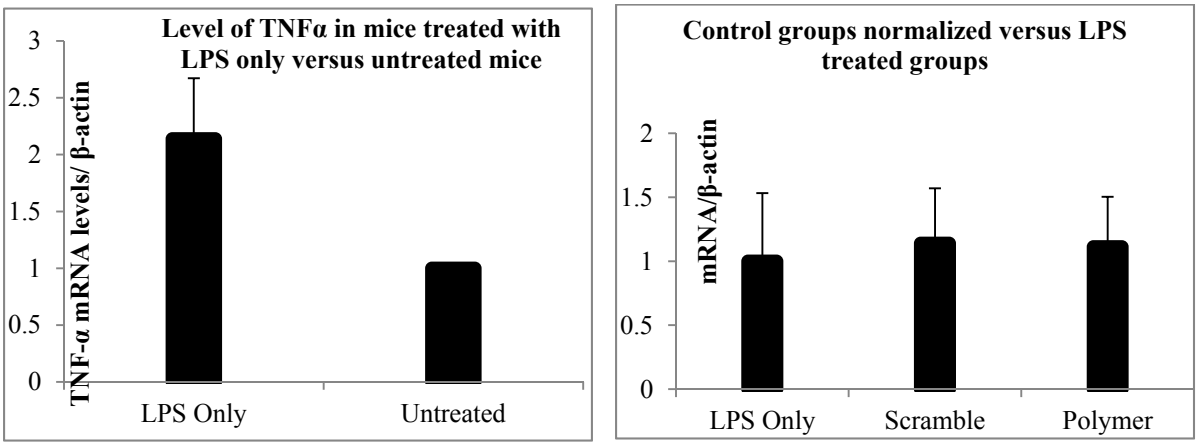
In Table 3.1 and 3.2 SI are respectively shown the amounts of siRNA and polymers administered via IP in 6 groups of animals (n=4), and the level TNF-alpha and IFN-γ measured in their blood serum as an indication for drug adverse effect. The data indicated that the level of the two markers were lower than the limit of detection of the Milliplex method, same as for the naïve animals. Therefore, it was concluded that no adverse effect was triggered by the chol-siRNA or HA polymer.

Table 1.SI. Encapsulation efficiency measured by RiboGreen assay, after dialysis on 25KDa membranes for a set of formulations which were screened for silencing efficiency. RFU represents relative fluorescence units (RFU); PAA represents Poly Acrylic Acid used for nanoparticle disruption; TRIT represents triton used for disruption hydrophobic interaction between cholesterol on siRNA and hexyl group on the HA polymer.

Sample	RFU NO PAA	RFU + PAA	RFU _ PAA+TRIT	Free siRNA %	Encapsulation Efficiency %
BLK	0.7	8.3	4.6		
C6:PEI:PEG Chol siRNA3:2:1	1.1	60.2	93.1	0.9	99.1
C6:PEI:PEG Chol siRNA 5:2:1	1.1	77.9	116.3	0.6	99.4
C6:PEI:PEG Chol siRNA6:1:0.5	1.2	52.8	85.4	1.1	98.9
C6:PEI:PEG Chol siRNA 1:3:1	1.1	93.4	115.3	0.6	99.5
C6:PEI:PEG Chol siRNA 2:3:1	1.1	104.3	128.1	0.4	99.6
C6:PEI:PEG Chol siRNA 2:5:1	0.9	36.9	63.7	0.9	99.1
C6:PEI:PEG Chol siRNA2:7:1	0.9	59.1	67.8	0.5	99.5
PEI:PEG 10:1 Chol siRNA	1	33.6	40.6	1.3	98.7
PEI:PEG 10:1 Unmodified siRNA	1	21.1	20.9	2.56	97.4
C6:PEI:PEG 1:1:1 Unmodified siRNA	0.9	30.9	32.1	1.1	98.9

Table 2.SI. Average normalized concentrations of active RNA measured by qPCR in peritoneal cavity tissues and cells following naked siRNA IP injection, versus siRNA HA encapsulated IP administration. *Unmeasurable* was considered a result which fell below the limit of detection of the qPCR method. *Inconclusive* was considered the result from a group of animals with a large error bar.

	Peritoneal cells		Liver Tissue		Spleen Tissue		Plasma		Kidney	
Time point (Hours)	Naked siRNA (µg/mg total cell protein)	Nano-particle siRNA (µg/mg protein)	Naked siRNA (ng/g tissue)	Nano-particle siRNA (ng/g tissue)	Naked siRNA (ng/g tissue)	Nano-particle siRNA (ng/g tissue)	Naked siRNA (ng/mL plasma)	Nano-particle siRNA (ng/mL)	Naked siRNA (ng/g tissue)	Nano-particle siRNA (ng/g tissue)
2	$0.21*10^{-1}$ $\pm0.05*10^{-1}$	0.3 ±0.2	2.18 ±0.3	2.61 ±0.5	48.61 ±20.2	21.39 ±10	Inconclusive	1784.34 ±1438	4.68 ±2	0.27±0.1
4	Unmeasurable	0.973 ±0.3	1.96 ±1	7.02 ±1.16	12.82 ±4.1	2.91 ±0.8	87.6±40	5.04 ±2	0.63 ±0.2	0.36 ±0.1
24	$0.13*10^{-1}$ $\pm0.08*10^{-1}$	0.65 ±0.2	0.51 ±0.1	1.57 ±0.4	0.95 ±0.03	24.15 ±10	0.9 ±0.13	0.35 ±0.14	0.05 ±0.01	0.13 ±0.07
48	$0.87*10^{-2}$ $\pm0.1*20^{-2}$	1.105 ±0.15	2.21 ±0.6	2.28 ±0.4	0.36 ±0.06	3.38 ±1	0.17 ±0.01	0.17 ±0.07	0.04 ±0.01	0.46 ±0.1



(a) (b)

Figure 1.SI. Control groups: a) Effect of LPS injection in TNF-α level in peritoneal macrophages from mice treated only with 0.5 mg/kg LPS as compared to naïve untreated mice. LPS triggered a TNF-α elevation in peritoneal macrophages of at least two folds, and 48 hours after LPS IP injection. b) Comparison among LPS treated mice, scramble siRNA sequence treated mice, and HA polymer empty particle treated mice. The level of TNF-α in peritoneal macrophages extracted from mice of three groups was not significantly different at α=0.05, p-value=0.33, after 48 hours of LPS/ scramble siRNA injection. This allowed use the TNF-α level to compare the cholesterol siRNA silencing effect in peritoneal macrophages from chol-siRNA treated mice versus scramble siRNA treated mice. In can be noticed that polymer itself did not exhibit upregulating effect on the TNF-α level in macrophages.

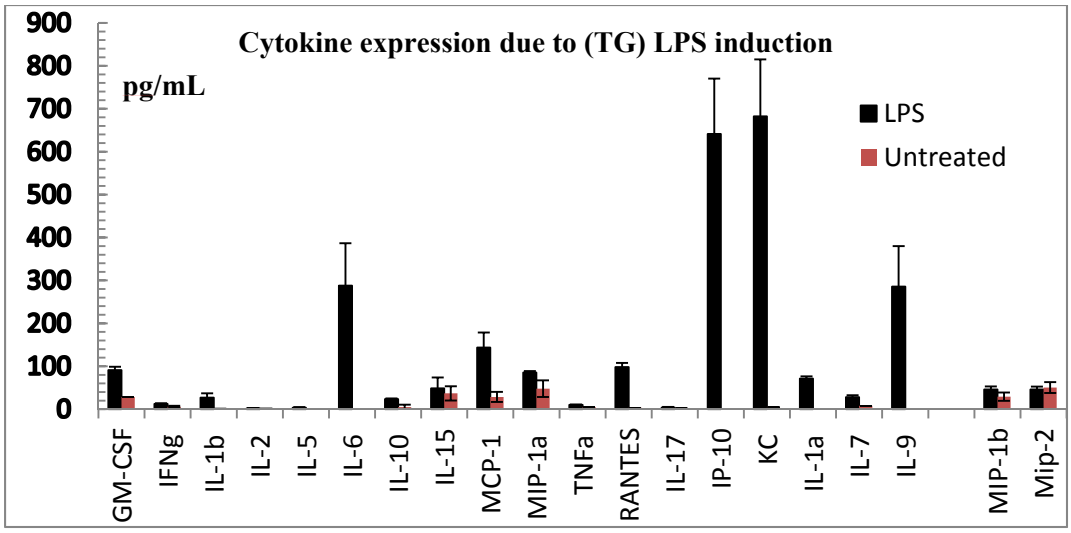
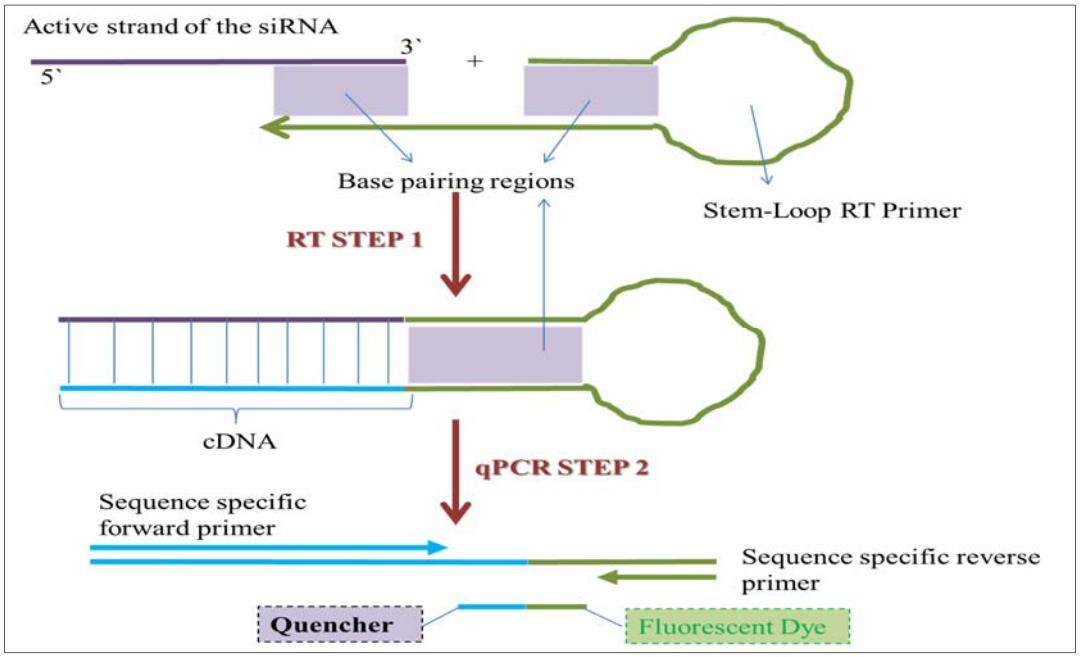
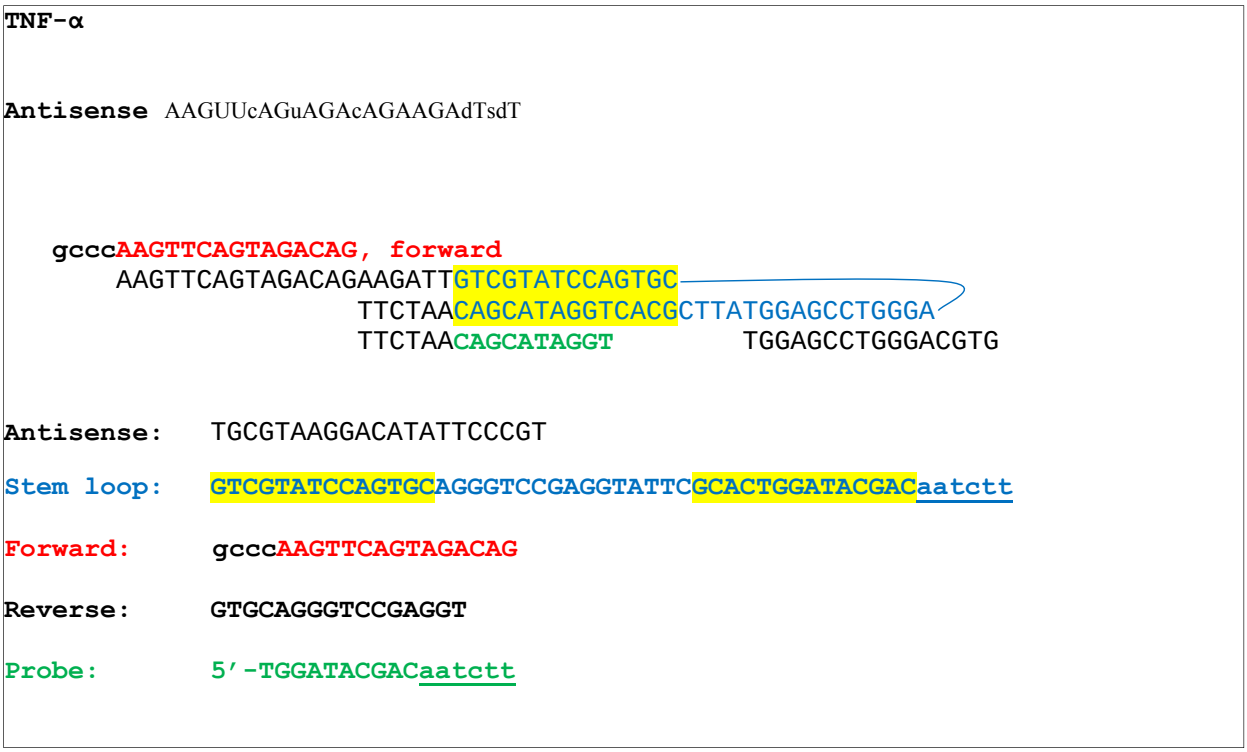


Figure 2 SI. Effect of LPS IP injection in cytokines level measured in blood serum of treated mice with 0.5 mg/kg LPS, versus serum level in naïve untreated animals.

Sense strand: 5`[ucuucuGucuAcuGAAcuudTsdT] 3`Chol
Anti-sense Strand:5` [AAGUUcAGuAGAcAGAAGAdTsdT]3`
(a)



(b)



(c)

Figure 3 SI. a) The sequence order of single strands for the TNF-α siRNA, used in this study. The order of bases for the siRNA was maintained the same to the one published by previous study [3]. As explained in the main paper, there was one cholesterol molecule conjugated at the 3` end of the sense strand. b) Principle of quantitative qPCR method tracing the active strand of the siRNA, in tissues and cells. Step 1) Stem-loop RT primer binds to the 3` end of the active RNA Step 2) RT product quantified: forward primer, reverse primer, sequence specific dye-labeled probe [4]. c) Scheme of designation for the stem loop oligo sequence specific to TNF-α active strand (antisense) siRNA; forward and reverse oligo fragments; and the probe oligo sequence for qPCR on tissues and cells.

Table 3.1. Amount of siRNA/HA polymer administered via IP route in 6 groups of animals (n=4). The dosing in the formulated chol-siRNA (group 1-4) is expressed in siRNA amounts.

Animal group (n=4)	Article injected	Dosing (mg/kg)
Naïve	PBS	
1	Chol-siRNA HA	2
2	Chol-siRNA HA	5
3	Chol-siRNA HA	10
4	Chol-siRNA HA	30
5	Naked chol-siRNA	30

6	Unloaded HA Polymer	85.5

Table 3.2. Level of TNF-alpha and IFN-γ (pg/mL) in blood serum of animals measured by Milliplex technology. The Minimum Detectable Concentration (MinDC) determined by StatLIA® Immunoassay Analysis Software from Brendan Technologies was MinDC+2SD TNF-α=1.8 pg/mL; and MinDC+2SD IFN-γ=4 pg/mL. Overnight protocol was performed with this experiment as specified in manufacturer`s manual. The result reported by the software as OOR< indicates: Out of Range Below

Group	IFN-γ Instrument reading	Interpretation in (pg/mL)	TNF-alpha	Interpretation (pg/mL)
Naïve	OOR<	<4	OOR<	<1.8
1	OOR<	<4	OOR<	<1.8
2	OOR<	<4	OOR<	<1.8
3	OOR<	<4	OOR<	<1.8
4	OOR<	<4	OOR<	<1.8
5	OOR<	<4	OOR<	<1.8
6	OOR<	<4	OOR<	<1.8

Table 3.3. Level of TNF-α and TNF-γ measured in the control solution during the assay, along with the experimental samples. Expected ranges are shown, as specified by the manufacturer. Suitability of the assay was confirmed.

Control / Cytokine	IFN-γ (pg/mL)	Range IFN-γ (pg/mL)	TNF-α (pg/mL)	Range TNF-α (pg/mL)
Control 1	122.49	(101-209)	111.47	(100-208)
Control 2	560.82	(595-1236)	679.12	(601-1344)

References

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