Evaluation of Efficacy, Biodistribution, and Inflammation for a Potent siRNA Nanoparticle: Effect of Dexamethasone Co-treatment

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Despite recent progress, systemic delivery remains the major hurdle for development of safe and effective small inhibitory RNA (siRNA)–based therapeutics. Encapsulation of siRNA into liposomes is a promising option to overcome obstacles such as low stability in serum and inefficient internalization by target cells. However, a major liability of liposomes is the potential to induce an acute inflammatory response, thereby increasing the risk of numerous adverse effects. In this study, we characterized a liposomal siRNA delivery vehicle, LNP201, which is capable of silencing an mRNA target in mouse liver by over 80%. The biodistribution profile, efficacy after single and multiple doses, mechanism of action, and inflammatory toxicity are characterized for LNP201. Furthermore, we demonstrate that the glucocorticoid receptor (GR) agonist dexamethasone (Dex) inhibits LNP201-induced cytokine release, inflammatory gene induction, and mitogen-activated protein kinase (MAPK) phosphorylation in multiple tissues. These data present a possible clinical strategy for increasing the safety profile of siRNA-based drugs while maintaining the potency of gene silencing.

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INTRODUCTION

Therapeutic small inhibitory RNAs (siRNAs) are an emerging modality for unmet medical needs because of their specificity, potency, reversibility, and large number of disease-relevant targets which are unreachable by conventional small molecules or monoclonal antibodies. siRNA approaches exploit the endogenous RNA interference (RNAi) machinery, which is present in all eukaryotic cell types and can be harnessed to induce sequence-specific mRNA silencing for almost any gene.[1] Preclinical validation of therapeutic siRNAs has been achieved for indications ranging from cancer[2] and viral infection[3] to cardiovascular disease.[4] Clinical evaluation is ongoing for both local and systemic administration of siRNA, and is most advanced for ocular diseases where topical administration eliminates the requirement for delivery technologies.[5] Despite this progress, major challenges for potent and safe systemic delivery of siRNA-based therapeutics remain. These challenges include sufficient protection from circulating nucleases, tissue targeting, cellular uptake, endosomal escape, and avoidance of immune and inflammatory responses.[6]

Liposomes, lipoplexes, or lipid nanoparticles have been approved by US Food and Drug Administration as drug delivery systems for several cancer chemotherapeutics[7] and antifungals,[8] and have been extensively studied for delivery of plasmid DNA and siRNA.[5] In addition to protecting the drug payload from serum components, liposomal encapsulation can be used to improve its pharmacokinetic properties; this is often accomplished by employing "stealth" strategies such as addition of PEGylated lipids to decrease opsonization.[9] For many drug targets, another benefit of encapsulation is the preferential uptake of nanoparticles into tissues with relatively leaky vasculature, such as liver and tumor, due to their size and physical properties.[10] Finally, there is the exciting potential to develop specific tissue targeting strategies by attaching antibodies, peptides or carbohydrate-based ligands to the liposomal surface.[11] Recently, liposome-delivered siRNA targeting the liver genes *APOB* and *PCSK9* have been shown to have efficacy and duration in nonhuman primates comparable or exceeding that of conventional cholesterol-lowering drugs.[4,12] Nonliposomal siRNA delivery strategies are also being pursued.[13]

A major liability of liposome-based drugs is their potential to cause inflammatory toxicities through activation of the innate immune response.[14] Innate immunity is triggered by recognition of nonself particulate matter by macrophages and other hematopoietic cell populations through engagement of pattern recognition receptors. For example, bacterial endotoxins activate toll-like receptor 4 (TLR4) on the surface of Kupffer cells in the liver and other macrophages of the reticuloendothelial system.[15] TLR engagement promotes phagocytosis and release of inflammatory intermediates such as chemokines and cytokines. This inflammatory response leads to tissue infiltration of monocytes and neutrophils, which ultimately causes tissue necrosis, hypotension, and other potentially severe sepsis-like toxicities. In addition, liposomes have also been shown to activate components of

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the complement pathway in blood, which increases the risk of anaphylactic shock.[16] In contrast to these known risks, it has been reported that a cationic liposome formulation currently in clinical evaluation, SNALPs (stable nucleic acid delivery particles), can deliver effective doses of modified siRNA *in vivo* with minimal cytokine induction.[17,18] The mechanism by which SNALPs appear to evade an innate immune response is unclear, as is their therapeutic window. Therefore, despite recent advances, there are still liabilities which create a potential roadblock to development of novel liposome-encapsulated drugs.

In the current study, we demonstrate that a liposomal RNA delivery vehicle, LNP201, causes sustained silencing of the murine *Ssb* gene in the mouse liver after single or multiple doses through an RNAi mechanism. LNP201 caused an acute inflammatory response, inducing expression of numerous genes involved in innate immunity as well as mitogen-activated protein kinase (MAPK) phosphorylation in tissues. We present an example where siRNA base modifications are insufficient to mitigate inflammation in the context of a PEGylated cationic liposome, therefore inviting similar analysis of other novel delivery vehicles. LNP201-induced inflammatory response was inhibited by the glucocorticoid receptor (GR) agonist dexamethasone (Dex), without sacrificing siRNA efficacy. These data suggest that a potential clinical path forward for some siRNA liposomes is to administer them in combination with a widely used anti-inflammatory drug such as Dex.

RESULTS

LNP201 is a liposome assembly for systemic delivery of siRNA. LNP201 is composed of the cationic lipid CLinDMA, cholesterol, and the helper lipid polyethylene glycol-dimyristoylglycerol at a molar ratio of 50:44:6 (**Figure 1a**). A chemically stabilized siRNA targeting the *Ssb* gene was incorporated into LNP201 during the assembly process at an N:P ratio[19] of three, and a lipid to siRNA mass ratio of 13. Ssb encodes the La antigen, a protein involved in transfer RNA maturation,[20] and was selected for these studies because of its ubiquitous expression pattern in tissues. The RNA chemistry includes *O*-Me, F, and deoxyribose modifications at the 2′ position of the ribose, as well as abasic caps on the passenger strand[18] to promote incorporation of the guide strand into the RNA-induced silencing complex.[1] The sequences and chemical modification schemes are presented in **Figure 1b**. The mean particle diameter as measured by dynamic light scattering was 140 ± nm, and the RNA encapsulation efficiency was 82%.

To determine the biodistribution profile of LNP201 in mice, a modified stem-loop reverse transcription–PCR (RT-PCR) method[21] was employed to measure the siRNA concentration in tissues 2 hours after systemic administration (**Figure 1b**). A single dose of 9 mg/kg LNP201, based on the siRNA mass, was administered by tail vein injection. Although similar concentrations of Ssb siRNA, normalized to the control small RNA *Rnu6b*, were detected in liver and spleen, significantly less siRNA was detected in kidney, lung, muscle, and adipose tissue. These data identified liver and spleen as the primary target organs of LNP201 in mice. Next, a pharmacokinetic analysis was performed in liver and spleen, in which [siRNA] was measured at time points ranging from 2 hours to 1 week. siRNA clearance rates were similar in liver and spleen, with ~10,000-fold less siRNA detected in both tissues 1 week after dosing relative to the earliest measurement (**Figure 1c**).

To investigate whether siRNA uptake in the liver and spleen led to the predicted pharmacodynamic effect of gene silencing, Ssb mRNA levels were measured by quantitative RT-PCR after systemic

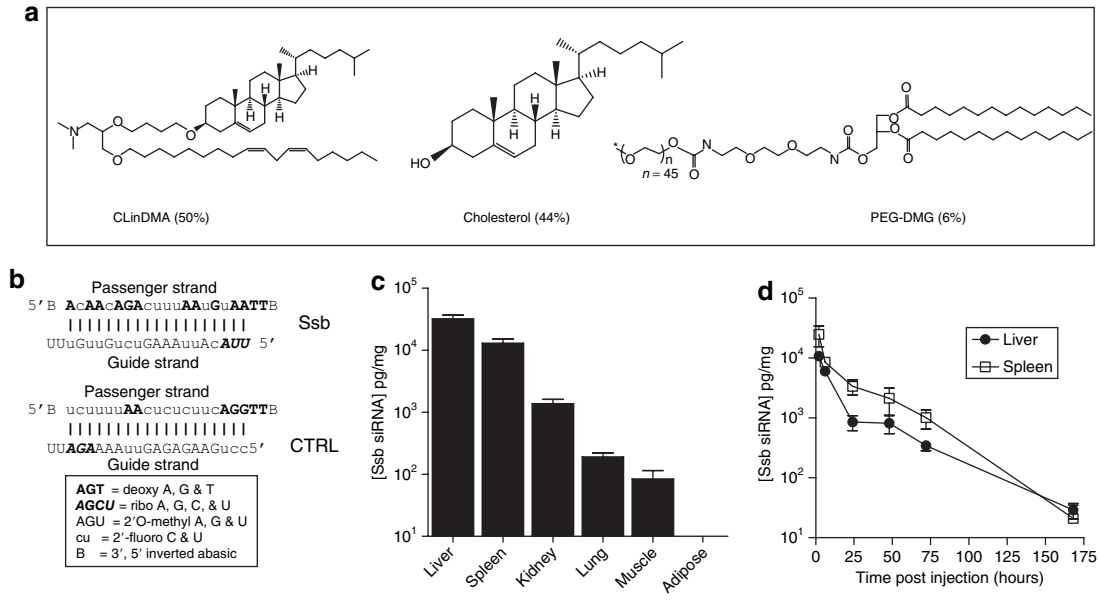

Figure 1 Lipid composition, siRNA design, and mouse biodistribution profile of LNP201. (**a**) Lipid components and ratio of the LNP201 assembly. (**b**) Sequences and chemical modification schemes for Ssb and control siRNAs. (**c**) Biodistribution of siRNA in mice. CD-1 mice (five/group) were dosed intravenously with LNP201/Ssb siRNA; tissues were collected 2 hours later and [Ssb siRNA] was measured by quantitative PCR as described in Materials and Methods. Values on the chart are reported as mean pg [siRNA] per mg of tissue ± SEM. (**d**) siRNA kinetics in mouse tissues after intravenous administration of LNP201. Liver and spleen tissue from mice (5/group) was collected at time points ranging from 2 hours to 1 week postdose; [Ssb siRNA] was measured as described. siRNA, small inhibitory RNA.

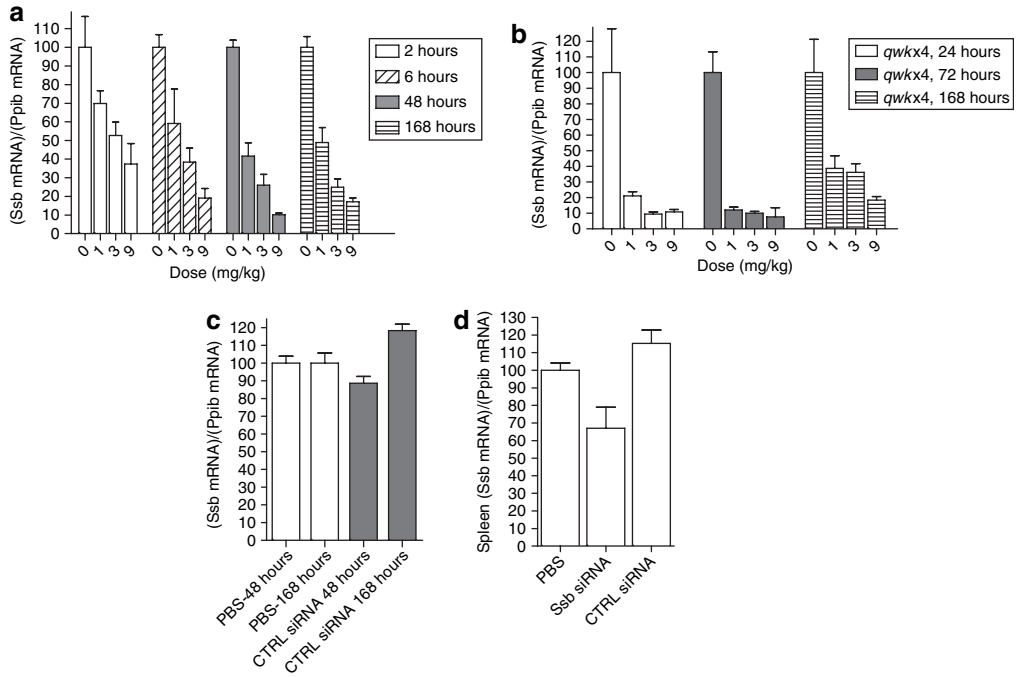

Figure 2 Administration of LNP201 *in vivo* results in target mRNA silencing. (**a**) Reduction of target mRNA in liver after a single intravenous dose of LNP201. Mice were treated with LNP201/Ssb siRNA at the indicated siRNA doses. At the indicated time points, the medial lobe of the liver was sampled and total RNA was isolated. quantitative PCR was performed to measure Ssb mRNA expression relative to a housekeeping gene, *Ppib*. Values from mice treated with vehicle only (PBS) were fixed to 100. Values are presented as the mean (five/group) ± SEM. (**b**) LNP potency increases after multiple weekly doses. Mice were treated with LNP201/Ssb siRNA once weekly for 4 weeks. At the indicated time points after the final of 4 doses, liver was collected and [mRNA] was measured as described above. (**c**), LNP201 loaded with a control siRNA sequence does not cause changes in the target mRNA. Mice were treated with either vehicle only ("PBS") or LNP201/Control siRNA ("CTRL") for either 48 hours or 1 week, and relative [Ssb mRNA] was measured as described above. (**d**), Activity of LNP201 in the spleen. Spleens were collected from mice (five/group) 48 hours after LNP201/Ssb siRNA or LNP201/CTRL siRNA dosed at 9 mg/kg, and mRNA was measured as described above. LNP, lipid nanoparticle; mRNA, messenger RNA; PBS, phosphate buffered saline; siRNA, small inhibitory RNA.

administration. After a single treatment, liver Ssb knockdown was observed at all three dose levels tested (1–9 mg/kg) in a dose-dependent manner (**Figure 2a**). A maximum liver [Ssb mRNA] reduction of 89%, relative to vehicle-treated subjects, was detected 48 hours after a single dose of 9 mg/kg LNP201. Knockdown was detected as early as 2 hours post dose, peaked at 48 hours, and only decreased slightly after 1 week. The long duration of knockdown is comparable to other siRNA liposomes,[12] and shows that activity persists even after the majority of tissue [siRNA] has cleared. Furthermore, we also tested the effect of repeated administration of LNP201 in mice. It was observed that a multiple dosing regimen of once-weekly for 4 weeks (*qwk*x4) increased the potency of LNP201 relative to a single treatment. **Figure 2b** shows that near-maximum Ssb knockdown was achieved at a dose of only 1 mg/kg, measured 24–72 hours after the final dose of the *qwk*x4 schedule. Significantly, the siRNA efficacy was sequence dependent, as a matched nontargeting siRNA control had no effect on Ssb mRNA (**Figure 2c**).

Significantly, less mRNA silencing was observed in the spleen compared to the liver. In spleen, a 33% mRNA reduction relative to phosphate-buffered saline was observed 48 hours after a dose of 9 mg/kg, compared to 89% in the liver (**Figure 2d**). Therefore, siRNA uptake in mouse tissues (**Figure 1b**) does not always correlate with efficacy. This observation indicates that the ability of LNP201 to deliver its payload to the cytosol after cellular internalization, where the siRNA is accessible to RNA-induced silencing complex, is tissue-dependent.

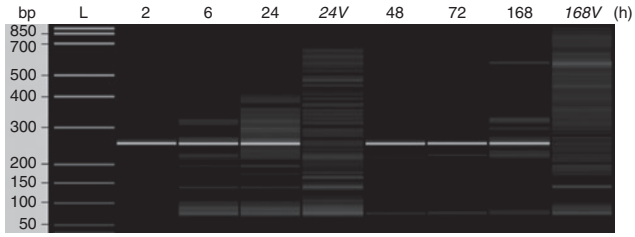

Figure 3 Silencing of target mRNA occurs through an RNA interference mechanism. Liver tissue was collected at the indicated time points after dosing mice with LNP201/Ssb siRNA or vehicle only (labeled "*V*"). Pooled total liver RNA from (five/group) was subjected to 5′ RACE analysis at each time point indicated. As described in Materials and Methods, 5′ RACE primers were designed to detect the RNA interference cleavage product of the target Ssb mRNA, which is expected to create a species in which the 5′ terminus is complementary to the 11th nucleotide of the siRNA guide strand. Following ligation of a RACE adapter oligonucleotide and s amplification, the resulting DNA products were analyzed via Agilent Bioanalyzer. The pseudogel image is shown. The presence of cleaved Ssb mRNA is confirmed by detection of a 287-bp DNA fragment. bp, base pairs; L, DNA ladder; siRNA, small inhibitory RNA.

RNAi causes mRNA knockdown through a well-characterized pathway, in which the final event is endonucleolytic cleavage of the target mRNA.[1] Invariably, this Ago2-mediated mRNA cleavage occurs at the position which is complementary to the phosphodiester

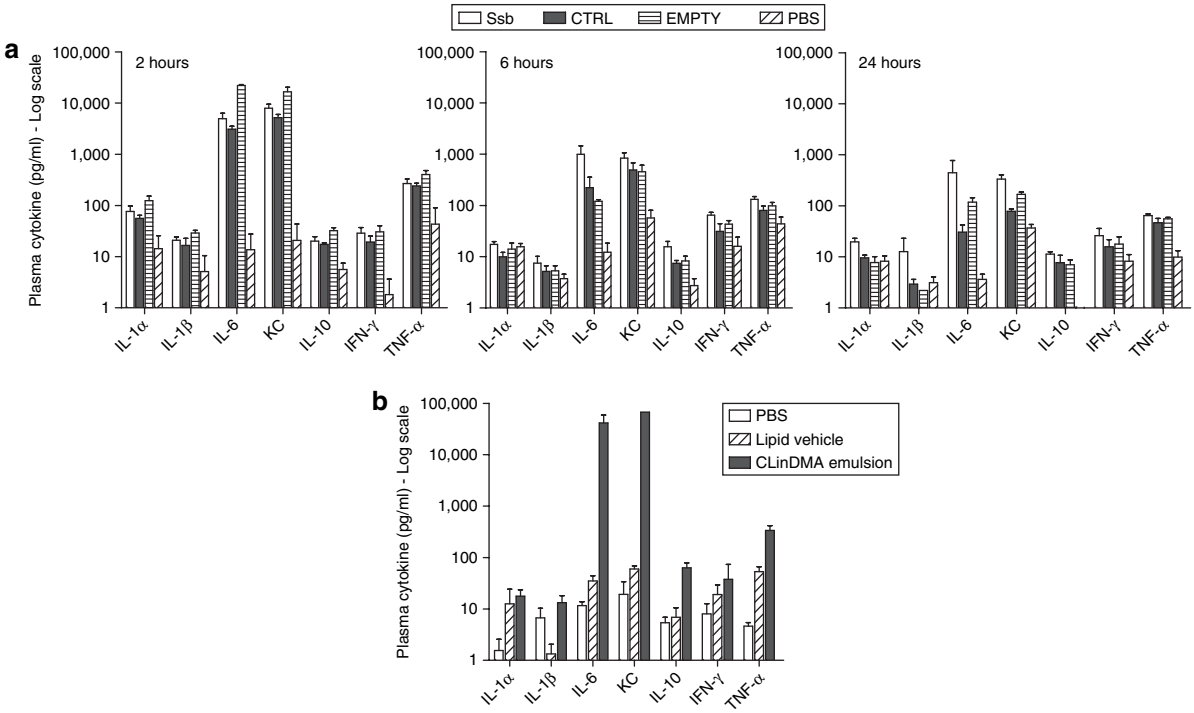

Figure 4 LNP201 and CLinDMA cause an acute inflammatory response after systemic administration, independent of the siRNA payload. (**a**) Plasma was collected 2, 6, or 24 hours after intravenous dosing of LNP201 containing Ssb siRNA, control siRNA, or empty liposomes as indicated. The dose was 3 mg/kg based on siRNA mass, or in the case of empty liposomes a comparable dose of 39 mg/kg based on lipid mass. (**b**) Plasma was collected 2 hours after intravenous dosing of CLinDMA emulsion (95 mg/kg CLinDMA), or its matched vehicle (0.25% polyethylene glycol-dimyristoylglycerol, 5% sucrose, 9 mmol/l sodium citrate pH 4). A multiplexed enzyme-linked immunosorbent assay was used to measure 12 cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, keratinocyte-derived cytokine, TNF-α, and interferon-γ) at 2, 6, or 24 hours postdose as labeled. Data are shown as mean plasma cytokine concentrations ± SEM, $n = 5$. Only data for those cytokines which were induced in response to LNP201/siRNA or CLinDMA emulsion are shown. IL, interleukin; siRNA, small inhibitory RNA; TNF, tumor necrosis factor.

bond between nucleotides 10 and 11, counting from the 5′ end of the siRNA guide strand. To confirm that the decrease in Ssb mRNA observed in the liver after dosing LNP201 was due to RNAi-mediated gene silencing, 5′ RACE (rapid amplication of complementary DNA ends) analysis was performed.[4] An RNAi mechanism of action was demonstrated by the detection of a specific truncated species of Ssb mRNA, with a 5′ terminus complementary to base 10 of the siRNA guide strand (**Figure 3**). A PCR-amplified complementary DNA corresponding to this Ssb mRNA cleavage product was detected at all time points for which [Ssb mRNA] knockdown was observed, from 2 hours to 1 week postdose. A RACE product was not observed in RNA isolated from control subjects.

The therapeutic window of nanoparticle drug formulations may be limited by inflammatory toxicities associated with uptake by the macrophages of the reticuloendothelial system.[14] Immunostimulatory lipids are recognized by TLR2 and TLR4 on the cell surface of macrophages and other cells; whereas immunostimulatory nucleic acids are recognized by TLR8 and TLR9 on the surface, and TLR3 and TLR7 in endosomes.[22] Downstream events triggered by engagement of these pattern recognition receptors ultimately initiates proinflammatory transcriptional programs, including induction of many cytokine and chemokine genes. We measured cytokine concentrations in plasma after dosing mice with LNP201 for 2, 6, or 24 hours (**Figure 4a**). Induction of interleukin-1α (IL-1α), IL-1β, IL-6, keratinocyte-derived cytokine, IL-10, interferon-γ, and tumor necrosis factor (TNF-α) was observed, with all except interferon-γ peaking at 2 hours. Numerous lipid nanoparticles (LNPs) in the same structural class as LNP201, tested negative for the presence of endotoxin, showed similar levels of cytokine induction (data not shown). The induction of both proinflammatory cytokines such as IL-6 and TNF-α, as well as anti-inflammatory cytokines such as IL-10 is typical of an unregulated or underregulated innate immune response; the most severe form of which is referred to as a cytokine storm.[23] The cytokine levels were comparable when animals were dosed with LNP201 containing Ssb siRNA, control siRNA, or no siRNA, suggesting that the majority of the inflammatory is induced by the lipid components (**Figure 4a**). These data also suggest that there is a likely secondary contribution from the payload, because IL-6 and mouse keratinocyte-derived cytokine levels for LNP201 containing control siRNA return closer to baseline values by 24 hours than LNP201 containing Ssb siRNA or empty particles. This limited effect of the siRNA on cytokine induction is consistent with recent observations using dioleoyl trimethyl ammonium propane-Cholesterol siRNA liposomes.[24]

To further investigate whether the cationic lipid CLinDMA is the primary contributor to the inflammatory response, we treated mice with CLinDMA lipid emulsions, containing only CLinDMA and polyethylene glycol-dimyristoylglycerol (as a surfactant) in a 10:1 weight ratio (**Figure 4b**). Details about the assembly of CLinDMA emulsions are listed in Materials and Methods. At comparable doses, the CLinDMA emulsions caused a response which

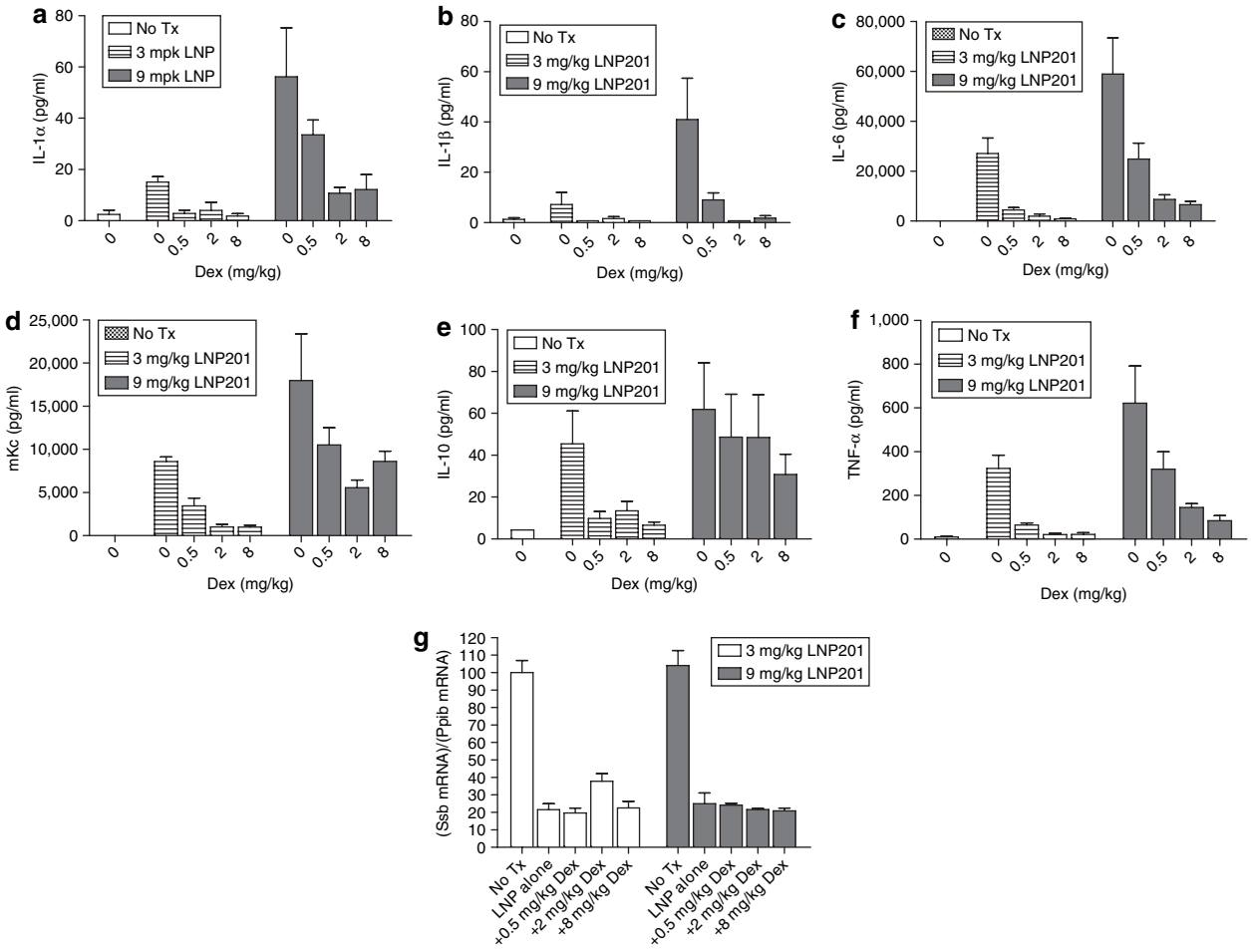

Figure 5 LNP201/siRNA induced cytokine response is mitigated by dexamathasone (Dex) pretreatment. Mouse plasma was obtained 3 hours after intravenous dosing. Subjects were pretreated with phosphate-buffered saline or 0.5–8 mg/kg 1 hour prior to lipid nanoparticle (LNP) administration. (**a**) IL-1α, (**b**) IL-1β, (**c**) IL-6, (**d**) mouse keratinocyte-derived cytokine, (**e**) IL-10, and (**f**) TNF-α were measured in plasma by multiplexed ELISA. (**g**) [mRNA] of the siRNA target, Ssb, was measured (as in **Figure 2**) 24 hours after LNP dosing ± Dex pretreatment. Values are presented as mean ± SEM (n = 5). IL, interleukin; siRNA, small inhibitory RNA; TNF, tumor necrosis factor.

was very similar in cytokine signature and magnitude to LNP201 with or without siRNA. These data suggest that CLinDMA, and not the other lipid components or the nucleic acid component, is primarily responsible for this innate immune response.

We reasoned that pretreating the subjects with Dex, a widely used anti-inflammatory agent, might increase the therapeutic window of LNP201. Dex is a full agonist of the GR, a cytoplasmic nuclear hormone receptor. Activation of the GR in tissues inhibits the transcriptional activity of the TLR effectors nuclear factor κB NFκB and activator protein-1, and also functions directly as a transcription factor by binding to palindromic glucocorticoid-response elements on the promoters of many genes.[25] To test this hypothesis, mice were treated by intraperitoneal injection with either phosphate-buffered saline or increasing doses of Dex 1 hour before dosing LNP201/siRNA (**Figure 5**). The levels of all six cytokines whose induction peaked at 2 hours were reduced by the Dex pretreatment in a dose-dependent manner. At the LNP dose where near-maximum liver mRNA knockdown was achieved (3 mg/kg), Dex reduced the plasma cytokine response to near-baseline levels (**Figure 5a–f**). Importantly, Dex caused little or no decrease in LNP201 liver mRNA knockdown efficacy at any of the doses tested (**Figure 5g**). These data demonstrate that the liposome-induced innate immune response can be mitigated by pharmacological intervention of inflammatory signaling.

To further characterize the LNP201 inflammatory response and the inhibitory effect of Dex, tissues were harvested 2 hours after LNP201/siRNA administration and analyzed for expression of known proinflammatory mRNAs (**Table 1**). Among a panel of 93 known proinflammatory genes, including cytokines and chemokines, LNP201/siRNA caused at least tenfold upregulation (relative to a vehicle control) of 19 genes in liver, 12 in spleen and 3 in lung. Each of these three tissues had a unique inflammatory response, reflecting differences in tissue-specific immune cell populations. In addition to induction of cytokine and chemokine genes such as *Il6*, *Tnf*, *Ccl2*/MCP-1, and *Cxcl10*/IP-10, we also observed induction of genes involved in oxidative stress response (Nos2/iNOS), prostaglandin signaling (Ptgs2/COX-2), platelet activation (Selp/P-selectin) and other inflammatory pathways. Many of the LNP201-induced genes are known to be responsive to bacterial endotoxin, wounds and viral infections.[26,27] There is

Table 1 Inflammatory mRNAs are induced by LNP201/siRNA in tissues, and partially inhibited by Dex pretreatment

	Dex pretreatment (mg/kg)			
Gene symbol	**0**	**0.5**	**2**	**8**
Liver				
Ccl2	364.3	288.8	74.5	72.6
Ccl3	123.3	90.5	30.1	23.9
Csf1	12.8	5.9	3.6	3.9
Csf3	2,000.8	1,287.2	871.8	1,056.5
Ctla4	225.0	36.4	4.7	105.4
Cxcl10	426.2	161.7	33.5	65.3
Cxcl11	15.0	5.2	0.8	1.4
Hmox1	11.3	8.8	1.9	1.5
Ifng	129.4	24.5	10.8	1.6
Il1b	24.6	13.4	3.0	2.0
Il6	13.4	11.9	1.7	1.9
Nfkb2	15.5	10.4	5.0	4.0
Nos2	5,777.8	574.5	22.1	18.4
Ptgs2	906.9	301.9	60.3	17.4
Sele	1,442.4	801.6	171.7	153.5
Selp	81.4	37.8	9.8	6.2
Socs1	116.5	34.2	7.5	7.6
Tnf	38.2	17.9	4.5	3.6
Vcam1	11.3	9.2	2.6	2.5
Spleen				
Ccl2	119.5	78.3	45.7	31.3
Ccl3	13.6	8.1	7.1	6.5
Csf3	775.3	283.2	266.5	195.8
Cxcl10	17.0	16.2	13.8	14.4
Cxcl11	10.6	17.7	7.7	12.2
Il10	27.8	15.8	37.8	26.6
Il17	24.7	0.3	2.7	0.5
Il1b	11.0	4.8	4.3	2.2
Il6	1,215.2	456.8	253.1	163.6
Ptgs2	361.4	256.8	122.1	50.6
Sele	111.8	22.3	11.3	5.5
Socs1	15.7	10.5	10.5	10.5
Lung				
Csf3	81.2	16.4	18.2	24.7
Cxcl10	15.9	15.2	2.3	2.5
Sele	12.1	9.2	2.5	1.6

Subjects were treated with 3 mg/kg LNP201/Ssb siRNA ± Dex pretreatment as in **Figure 5**. Tissue RNAs from 5 mice/group were pooled and subjected to quantitative PCR analysis for a panel of 93 immune and inflammatory response mRNAs and 3 control/housekeeping mRNAs. A list of all 96 gene symbols contained on the panel is found at http://www.appliedbiosystems.com/support/mouse_immune_panel.xls. Shown on the table are those genes which were induced >10× by LNP201. Values represent the mean fold induction relative to phosphate-buffered saline-treated subjects. Data points in which Dex reduced the response by >3× are highlighted, with darker shading for points in which Dex reduced the response by >10×.

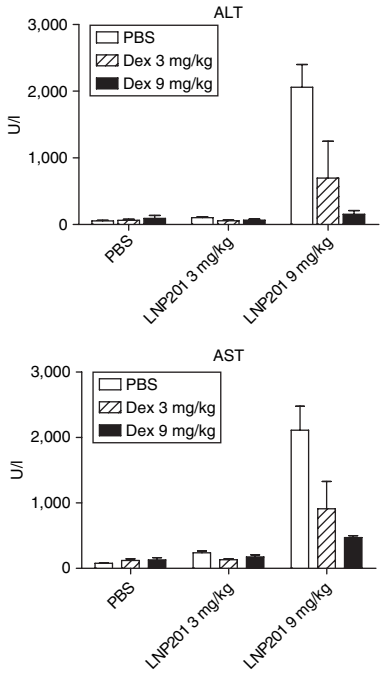

Figure 6 Effect of Dex pretreatment on serum ALT/AST levels. 5 mice/group were treated with indicated doses of LNP201/siRNA, with or without (phosphate-buffered saline) pretreatment with 3 or 9 mg/kg Dex 1 hour before LNP201 administration. AST/ALT levels were measured 24 hours after LNP201 dosing using standard clinical chemistry methodologies. Values are presented as mean ± SEM. ALT, alanine aminotransferase; AST, aspartate amintransferase; Dex, dexamethasone; siRNA, small inhibitory RNA.

also significant overlap with the inflammatory signature of human fibrosarcoma cells transfected with unencapsulated siRNA mixed with a cationic lipoplex, suggesting that some of these effects of can be reconstituted *in vitro*.[28] Among the overlapping set of 34 total genes induced in response to LNP201 in liver, spleen, or lung, Dex pretreatment reduced the level of induction by at least threefold for 26 mRNAs (76%) (**Table 1**). These gene expression data demonstrate that the inhibition of cytokine secretion observed by Dex is due primarily to suppression of proinflammatory gene transcription in multiple tissues.

Unchecked upregulation of inflammatory effectors such Nos2 can cause tissue damage through release of reactive oxygen species by macrophages and neutrophils.[29] To assay for biomarkers of liver damage, we measured the levels of alanine aminotransferase (ALT) and aspartate amintransferase in serum 24 hours after LNP201 administration (**Figure 6**). Although the efficacious dose of 3 mg/kg did not cause an increase in serum levels of these tissue damage markers, a higher dose of 9 mg/kg caused a significant induction. The induction of liver enzymes was transient, as ALT and aspartate amintransferase levels returned to those of naive subjects by 1 week postdose (data not shown). Pretreatment with Dex caused a dose-dependent reduction in LNP201-mediated aspartate amintransferase and ALT induction. For ALT, pretreatment with 9 mg/kg Dex was sufficient to reduce the serum concentration back to baseline levels. These data further illustrate the potential of anti-inflammatory treatment to positively affect the therapeutic window of siRNA liposomes.

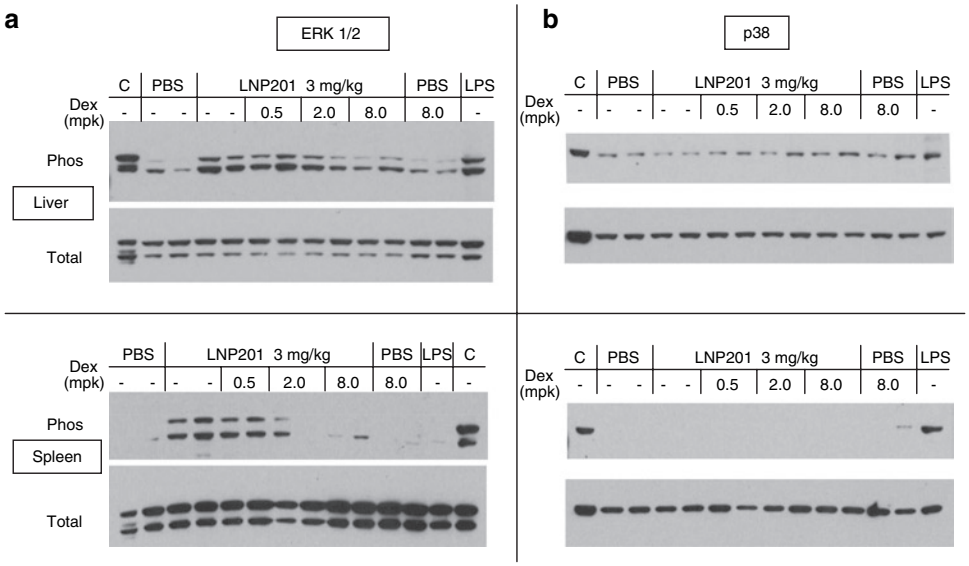

Figure 7 ERK1/2, but not p38 MAPK, is phosphorylated in response to LNP201/siRNA, and inhibited by Dex pretreatment. For two subjects/group treated with 3 mg/kg LNP201/Ssb siRNA ± Dex, total protein from liver and spleen was analyzed by western blot for phosphorylated and total (**a**) ERK1/2 and (**b**) p38. Dex, dexamethasone; MAPK, mitogen-activated protein kinase; siRNA, small inhibitory RNA.

MAPKs, namely extracellular signal-regulated kinase (Erk), p38, and c-Jun-N-terminal kinase, are pathway intermediates which use sequential protein phosphorylation events to transmit signals from activated membrane receptors to nuclei. MAPK phosphorylation occurs in tissues in response to mitogenic, stress response, apoptosis, and inflammatory pathways.[30] In TLR2/4-mediated signaling, receptor engagement by lipids or endotoxin causes recruitment of the MyD88 adapter protein on the cytoplasmic side, which in turn recruits IL-1 receptor-associated kinases and other kinases, ultimately leading to activation of the Erk1/2 MAPKs.[31] Erk1/2 activates activator protein-1, a heterodimer of Fos and Jun transcriptional activators, which initiates a proinflammatory transcriptional program which has yet to be fully characterized in macrophages.[31] **Figure 6** shows that a single 3 mg/kg dose of LNP201 induces phosphorylation of Erk1/2, but not p38 MAPKs in both liver and spleen. In addition, a pretreatment of 2–8 mg/kg Dex lead to near-complete inhibition of Erk1/2 phosphorylation in the liver, and complete inhibition in the spleen. These data suggest that the LNP201-induced inflammatory response and the inhibitory activity of Dex is, in part, mediated through Erk1/2. Interestingly, LPS-induced robust p38 phosphorylation, indicating a difference in inflammatory signaling induced by endotoxins compared to liposomes that should be explored further. These data present the possibility that, in addition to glucocorticoids, MAPK inhibitors may also be effective in improving the therapeutic window of siRNA liposomes.[32]

DISCUSSION

Therapeutic siRNAs have potential to make major contributions toward unmet medical needs in the coming years due to their virtually unlimited target space. Effective systemic siRNA delivery to the liver is an important advance, allowing for treatment of diabetes, hepatitis C infection, hepatacellular carcinoma and liver metastases. The exciting potential of liposomes as siRNA nanocarriers is balanced by their propensity to cause inflammatory toxicities. Although several reports show a lack of cytokine induction at efficacious doses for one cationic lipid-based vehicle containing modified siRNA, termed SNALPs,[12,17,18] further evaluation at supra-efficacious doses is necessary to determine the therapeutic window. It is still a matter of continuing investigation to determine why there is an apparent difference in inflammatory response between SNALPs and LNP201, but possibilities include differences in the lipid structures, the presence of a phospholipid in the SNALP formulation, pharmacokinetics, or even differences in the methods used to determine cytokine levels.[33]

This study has demonstrated that the synthetic glucocorticoid Dex significantly inhibits the inflammatory response of a novel effective siRNA delivery vehicle, LNP201. In a very recent report, it was also shown that Dex pretreatment is also a promising strategy to alleviate toxicity from adenoviral gene therapy vectors in a preclinical model.[34] Liposomal drugs which have been approved by the regulatory agencies, including liposomal doxorubicin or danorubicin for multiple myeloma and solid tumors[35] and amphotericin B for fungal infection,[36] are often administered in combination with glucocorticoids, providing precedent for this proposed clinical strategy for siRNA. It should be noted, however, that these pretreatments are often administered to prevent infusion-related reactions rather than severe sepsis-like responses. Even with the option of Dex pretreatment, further lead optimization efforts will be necessary to minimize the toxicities associated with LNP201.

Although the clinical benefits of synthetic glucocorticoids have been known for decades, so have their liabilities. The GR is expressed in almost all tissues, and its activation has far-reaching effects on the global transcriptome.[37] Chronic dosing of glucocorticoids has been associated with adverse effects on bone density, glucose metabolism, and susceptibility to infection.[38] Novel nonsteroidal GR-agonists which may retain the anti-inflammatory effects of glucocorticoids with reduced toxicity profile have been identified[39] and are the focus of drug discovery efforts. Another strategy to limit the adverse effects of glucocorticoids

is to encapsulate the anti-inflammatory agent in the liposome formulation along with an active pharmaceutical ingredient, thereby limiting its biodistribution to only those tissues which internalize the nanoparticles. Indeed, this approach has been shown to reduce circulating cytokines after administration of plasmid DNA liposomes in mice.[40]

The severity of inflammatory toxicity is also likely to be dependent on the target organs of the nanoparticle. The tissue mRNA profiling data in this study (**Table 1**) identify the tissue sources of the cytokines which enter circulation shortly after LNP201 treatment. For example, the majority of IL-6 originates from the spleen, whereas the majority of TNF-α is secreted by the liver. This is consistent with previous findings that depletion of resident liver macrophages (Kupffer cells) inhibits adenovirus-induced plasma TNF-α but not IL-6 induction in mice.[41] A more recent report identified the spleen as a major site of adenovirus vector-induced inflammation, and demonstrated that modifying the vector surface to reduce spleen uptake reduced circulating IL-6 and markers of liver damage (aspartate amintransferase and ALT).[42] Because LNP201 distributes equally to both tissues (**Figure 1**), but it is far more efficacious in the liver (**Figure 2**), the safety of siRNA liposomes would likely be improved by employing liver targeting strategies to avoid spleen uptake. For example, targeting of siRNA liposomes specifically to hepatocytes may be achievable by modifying the surface with galactose or apolipoproteins.[43]

In addition to cytokines, Dex significantly reduced the upregulation of genes involved in leukocyte infiltration (**Table 1**). Most notably, Sele (E-selectin), Selp (P-selectin), and Vcam1 (vascular cell adhesion molecule 1) are expressed on the surface of activated endothelial cells and promote adhesion followed by migration of neutrophils and monocytes from circulation into tissues.[44] Leukocyte infiltration is a well-characterized process in innate immunity, contributing to tissue damage by releasing proteases, histamines, leukotrienes, prostaglandins, and reactive oxygen species into the surrounding tissue. In addition to localized tissue damage, release of such effector molecules often has detrimental systemic effects, including hypotension.[45] Inhibition of selectins using monoclonal antibodies reduces LPS-induced endotoxemia, underscoring the important role of the endothelium in inflammatory toxicity.[46] Another Dex-sensitive category of LNP-induced genes, chemokines such as Ccl2/MIP-1 (**Table 1**) and mouse keratinocyte-derived cytokine (**Figure 5**), have a clearly defined role in neutrophil infiltration.[47] It has been reported that induction of chemokines by dioleoyl trimethyl ammonium propane liposomes is prevented by an ERK1/2 inhibitor or siRNA, but not a p38 inhibitor,[48] consistent with our conclusion that ERK1/2 is likely to be a key inflammatory mediator of siRNA liposomes *in vivo* (**Figure 7**). Therefore, it is likely that inhibition of tissue infiltration is a key mechanism by which Dex prevents tissue damage and induction of downstream effectors such as Nos2/iNOS and Ptgs2/COX-2.

In summary, this study provides proof-of-concept for the use of a common anti-inflammatory treatment to increase the safety margin of therapeutic siRNAs. More specific pharmacological agents to block MAPK activation[32] or nuclear factor κB activation[49] can also be considered in a pretreatment strategy, however such agents may not provide the necessary coverage against multiple inflammatory pathways that glucocorticoids offer. In addition to the pretreatment option explored in this study, understanding the key mechanisms of toxicity will facilitate identification siRNA-liposome formulations with optimized safety profiles.

MATERIALS AND METHODS

Animals. Female Crl:CD-1/ICR mice were obtained from Charles River (Wilmington, MA) and were between 6 and 10 weeks old at time of study (25–30 g). All studies were performed in Merck Research Laboratories' AAALAC-accredited West Point, PA animal facility using protocols approved by the Institutional Animal Care and Use Committee.

Liposome assemblies, siRNAs, and reagents. siRNA Lipid Nanoparticles were assembled using a process involving simultaneous mixing of the lipid mixture in an ethanol solution with an aqueous solution of siRNA, followed by stepwise diafiltration. Specifically, the siRNA was prepared in a citrate buffer at pH 4 using a lipid-to-RNA weight ratio of 13, and assembled into the particle by continuous mixing with the lipids through a T-junction at 40 ml/min, regulated by an high performance liquid chromatography pump. The ethanol was removed using tangential flow filtration, followed by buffer exchange so that the particle is in 100% phosphate-buffered saline. The lipids included in the mixture are as follows: CLinDMA (2-{4-[(3β)-cholest-5-en-3-yloxy]butoxy}-*N*,*N*-dimethyl-3-[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yloxy] propan-1-amine), cholesterol, and polyethylene glycol-dimyristoylglycerol (alpha-[8′-(1,2-dimyristoyl-3-propanoxy)-carboxamide-3′,6′-dioxaoctanyl] carbamoyl-omega-methyl-poly(ethylene glycol)$_{2000}$) at a molar ratio of 50:44:6. Particle size was determined by dynamic light scattering using a ZetaSizer (Malvern Instruments, Worcestershire, UK), and encapsulation efficiency was determined by RiboGreen assay (Invitrogen, Carlsbad, CA). Dex sodium phosphate was purchased from Phoenix Pharmaceuticals as a 4 mg/ml solution (St Joseph's, MO). RT-PCR primers and probes were synthesized by Applied Biosystems (Carlsbad, CA). Chemically modified siRNAs[18] were synthesized by Merck/Sirna Therapeutics. The guide (antisense) sequences of the siRNA strands were as follows: Ssb 5′-ACAACAGACUUUAAUGUAA-3′; control, 5′-UCUUUUAACUCUCUUCAGG-3. In addition, the passenger strands contain a 3′ TT overhang and the guide strands contain a 3′ UU overhang. The 5′ and 3′ terminal nucleotides of the passenger strands contain inverted abasic caps (**Figure 1b**). For the CLinDMA emulsions referenced in **Figure 4**, CLinDMA was mixed with polyethylene glycol-dimyristoylglycerol at a 10:1 weight ratio, then vortexed and sonicated for 10 minutes. After adding 10 mmol/l sodium citrate pH 4, the sample was then vortexed and sonicated for an additional 15 minutes followed by readjustment to pH 4 as necessary with 1 N HCl. The sample was extruded using a Lipex lipid extruder (Northern Lipids, Burnaby, British Columbia, Canada) by passing the sample ten times through two stacked 100 nm filters at 60 °C. Sucrose (50 mg/ml) was then added followed by filtering through 0.45 μm and 0.2 μm Supor syringe filters (Pall, East Hills, NY).

[siRNA] assays. siRNA concentration in tissues was determined using a modified stem-loop RT-PCR protocol.[21] Samples preserved in RNAlater (Qiagen, Valencia, CA) were homogenized in Trizol buffer (Qiagen) at 20 μg/μl in a bead mill. The homogenates were diluted 1:100 and subjected to a reverse transcription reaction using 200 nmol/l of an Ssb-specific stem-loop primer (5′-GTCGTATCCAGTGCAGGGTCCGAG GTATTCGCACTGGATACGACAAACAACAGA-3′) in an RT reaction mix (Applied Biosystems). A stem-loop RT primer for the nonspecific small RNA RNU6B was also included as a normalization control (Applied Biosystems, cat. no. 4373381). For each experiment, a standard curve was generated using Ssb siRNA serially diluted and spiked with tissue homogenate from naive subjects to match the buffer composition of the samples. Following the RT step, one-third of the reaction volume was used as the complementary DNA input for real-time PCR using TaqMan FAST Universal Master Mix (Applied Biosystems, cat. no. 4352042). The primer and probe sequences used for detection of Ssb siRNA were as follows: forward

primer (900 nmol/l), 5′-CGCGCGTTACATTTAAGTC-3′; reverse primer (900 nmol/l),5′-GTCCAGGGTCCCAGGT-3′;probe(250 nmol/l),5′-6FAM-TCGCACTGCATACGACAAACAACA-3′. An additional one-third of the complementary DNA reaction volume was used for detection of the control RNA, RNU6B, using a commercial primer-probe set. quantitative PCR reactions were performed using standard cycling conditions in an ABI7900 Sequence Detection System. Normalized C_t values were transformed into tissue concentrations using a linear equation derived from the standard curve.

[mRNA] assays and low density array profiling. [Ssb mRNA] was measured using standard quantitative RT-PCR methods. 50 ng total RNA was subjected to one-step RT-PCR using Applied Biosystems Universal Fast Enzyme Mix and recommended PCR cycling conditions for detection in ABI7500 or ABI7900 thermocyclers. The primer and probe sequences for Ssb mRNA detection were as follows: forward primer (900 nmol/l), 5′-TGGAAGGAGGACAAGATTTGATG-3′; reverse primer (900 nmol/l), CTTATGCTTTGATGCGGGTTCT-3′; probe (250 nmol/l), 5′-6FAM-TCGTCGTGGACCAATGAAAAGAGGAAGA-3′. For normalization purposes, Ppib mRNA was measured in separate reactions for all samples as housekeeping gene (Applied Biosystems, cat. no. Mm00478295_m1). Acute inflammatory response profiling was performed using TaqMan Mouse Immune Panel low density arrays (Applied Biosystems, cat. no. 4367786) according using the recommended protocols.

5′ RACE analysis. 5′ RACE analysis to detect the siRNA-cleaved mRNA product was performed as described,[50] using RNA ligase-mediated PCR and GeneRacer reagents (Invitrogen). The primer sequences used were as follows: Ssb outer, 5′-TTGACCCCTTAAATGTTTTGTGTAATGTTCTT CTCA-3′; Ssb inner, 5′-ATTCAGTATTTGGCCTTTATCGTCTAGCCAT TC-3′; 5′ RACE adapter primer, GGACACTGACATGGACTGAAGGAG TAGAAA-3′, and nested primer 5′-GGACACTGACATGGACTGAAGG AGTAGAAA-3′.

Cytokine assays and phosphoprotein analysis. Cytokine assays were performed using 1:10-diluted plasma as the input the 12-plex Searchlight ELISA (Pierce, Rockford, IL). For phosphoprotein analysis, snap-frozen liver and spleen samples were homogenized in radio immunoprecipitation assay buffer containing protease and phosphatase inhibitors (Sigma, St Louis, MO). 20 µg of protein was loaded onto 4–12% Bis-Tris gels for sodium dodecyl sulfate–polyacrylamide gel electrophoresis. In addition, 10 µg of phospho-enriched whole cell lysates (Santa Cruz Biotechnology, Santa Cruz, CA, cat. nos. 24820, 24817) were included as positive controls. Western blots were performed using rabbit anti-mouse total/phospho-p38 (Cell Signaling Technologies, Beverly, PA, cat. nos. 9212/9211) and rabbit anti-mouse total/phospho-erk1/2 (9102/9101) primary antibodies diluted 1:1,000, and standard chemilumenescent detection.

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