Rational design and *in vitro* and *in vivo* delivery of Dicer substrate siRNA

Mohammed Amarzguioui[1], Patric Lundberg[2], Edouard Cantin[2], James Hagstrom[3], Mark A Behlke[4] & John J Rossi[5]

[1]The Biotechnology Centre of Oslo, Gaustadalleen 21, 0349 Oslo, Norway. [2]Division of Virology, Beckman Research Institute of the City of Hope, 1450 East Duarte Road, Duarte, California 91010, USA. [3]Mirus Bio Corporation, 505 S. Rosa Road, Suite 104, Madison, Wisconsin 53719-1267, USA. [4]Integrated DNA Technologies, 1710 Commercial Park, Coralville, Iowa 52241, USA. [5]Division of Molecular Biology and Graduate School of Biological Sciences, Beckman Research Institute of the City of Hope, 1450 East Duarte Road, Duarte, California 91010, USA. Correspondence should be addressed to M.A. (moamarzg@biotek.uio.no) or J.J.R. (jrossi@bricoh.edu).

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RNA interference is a powerful tool for target-specific knockdown of gene expression. The triggers for this process are duplex small interfering RNAs (siRNAs) of 21–25 nt with 2-bp 3′ overhangs produced in cells by the RNase III family member Dicer. We have observed that short RNAs that are long enough to serve as Dicer substrates (D-siRNA) can often evoke more potent RNA interference than the corresponding 21-nt siRNAs; this is probably a consequence of the physical handoff of the Dicer-produced siRNAs to the RNA-induced silencing complex. Here we describe the design parameters for D-siRNAs and a protocol for *in vitro* and *in vivo* intraperitoneal delivery of D-siRNAs and siRNAs to macrophages. siRNA delivery and transfection and analysis of macrophages *in vivo* can be accomplished within 36 h.

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INTRODUCTION

Existing approaches to siRNA design

Small interfering RNA (siRNA)–mediated RNA interference has quickly become the method of choice for functional genomics research in mammals[1]. Delivery of chemically synthesized siRNAs results in highly sequence-specific and robust silencing of the expression of the corresponding endogenous gene[1]. In the first stage of RNA interference (RNAi), the RNase III enzyme Dicer processes longer double-stranded RNA to yield products that are 21 nt long with 2-nt 3’ overhangs. siRNAs of this configuration have therefore been most widely used[1,2]. These require no further processing from Dicer and are primed to be incorporated directly into the RNA-induced silencing complex (RISC), mediating antisense strand selection (when rationally designed to ensure proper asymmetry[3,4]), target recognition and cleavage. This property was originally considered to be beneficial, as it bypasses one step in the pathway and enters closer to the effector stage.

One obvious advantage of conventional 21-mers is that their pervasive design has been the basis for generation of all siRNA sequence-based rational design algorithms. Numerous minor changes to this basic design through site-specific chemical modification, with the goal of increasing stability, have been reported[5–12]. These modifications are important for *in vivo* efficacy[8] but are properly to be considered as elaborations that are likely to be applicable to multiple siRNA designs; detailed discussion of their properties is therefore outside the scope of this work. Chemical modification is, however, an integral part of what seems to be a different siRNA design, commercially available from Invitrogen under the brand name of Stealth siRNA. These 25-nt duplexes of undisclosed chemical composition are supposed to increase chemical serum stability. Because the design is based on proprietary criteria, the design algorithm itself is not generally available to investigators.

The use of longer siRNA was initially discouraged by the nonspecific effects generally encountered in mammalian cells in response to the presence of double-stranded RNA longer than approximately 30 bp. Recent reports, however, suggest that this initial thinking may have been flawed in this respect and that longer RNAi effectors that are able to undergo processing by Dicer can often have superior activity[13–15]. We will describe the rational design of these effectors in more detail in the following section.

Dicer substrate siRNA (D-siRNA) design

The groups of John Rossi and Greg Hannon have both reported improved efficacy of longer-than-standard RNAi effectors. During an investigation by Rossi and colleagues of cellular interferon induction caused by *in vitro*–transcribed siRNAs, limiting concentrations of some 25- to 27-nt siRNAs seemed to have greater potency than all of the synthetic 21-nt siRNAs that could potentially be generated from the larger duplex[13]. Hannon and colleagues reported a similar phenomenon for small hairpin RNA: synthetic small hairpin RNAs with 29-bp stems and 2-bp 3′ overhangs were more potent inducers of RNAi than were shorter hairpins[15]. Their studies further demonstrated that *in vitro* processing by Dicer is directional, starting predominantly from the open end of the stem and generating a mixture of 21- and 22-nt cleavage products. In both of the above cases, increased potency could be confidently attributed to Dicer processing, which is thought to promote more efficient incorporation into RISC through physical association of Dicer with the Argonaute proteins, the effectors of RNAi. This interpretation is supported by biochemical evidence in *Drosophila melanogaster*, indicating a role for Dicer in the initial stages of RISC assembly[16], and by recent reports that Dicer-mediated processing of microRNA (miRNA) precursors in human cells is functionally coupled to miRNA-specific RISC assembly and improves subsequent silencing[17,18].

Although Dicer processing is generally beneficial, the composition and potency of the processing products is also of importance for overall efficacy. Dicer processing of unmodified 27-nt duplexes is largely unpredictable, sometimes resulting in the generation of

siRNAs of poor activity, thereby reducing the activity of the 27-mer to below that of an optimal 21-mer within its target sequence. Consequently, there is no guarantee that a randomly designed 27-mer will be more efficacious than the best of the potential 21-mers within its target sequence. The problem of making D-siRNA processing predictable, thereby enabling rational design on the basis of published design algorithms, now seems to have been solved. The new optimal design introduces directionality and uniqueness of processing into the Dicer cleavage step by mimicking the relevant structural features of pre-miRNAs, which are naturally occurring Dicer substrates with bulged stem-loop structures with 2-nt 3′ overhangs. Recent reports suggest that the overhangs in the open end of the stem in such structures are bound by Dicer and determine the direction of processing and preferential strand selection[15,19]. In a natural Dicer substrate, the other end of the duplex is closed by a loop, precluding binding of Dicer to that end. This feature can be mimicked in D-siRNA by blunting the corresponding duplex end and introducing two DNA nucleotides in the sense strand in the blunt end of the duplex[14] (**Fig. 1**). The incorporation of a 3′ overhang in one end introduces a preference for processing to start from that end, while the DNA nucleotides in the opposite blunt end enforce this asymmetry and block processing events involving the terminal two phosphodiester linkages. This results in the predictable production of a single or major 21-nt processing product starting from the overhang terminus, sometimes accompanied by a minor 22-nt product resulting from processing from the same end. This mixture is similar to that previously reported to result from Dicer cleavage[2,15], and this flexibility in Dicer processing may reflect some level of sequence preference near the putative cleavage site.

The abovementioned D-siRNA configuration has in our hands resulted in improved efficacy compared to the corresponding conventional 21-mer for four randomly designed sites targeting two different genes[14]. Additional data presented below for a D-siRNA targeting tumor necrosis factor (TNF)-α provide further substantiation. From our limited data set, it seems that highly active 21-mers show relatively less improvement in activity than do less active 21-mers. This tentative conclusion has been supported by additional experiments in which corresponding D-siRNAs were compared to the most efficacious experimentally validated siRNA among sets of 12–14 siRNAs targeting two different genes (M.A., unpublished observations). The siRNA pairs were compared in cotransfection experiments with dual luciferase-based reporter vectors (psiCheck2, Promega) at gradually limiting concentrations of siRNA. In one case, a moderate improvement equivalent to a twofold difference in apparent IC_{50} value was observed, whereas no significant differences were observed for the other pair. If this is generally true, less dramatic improvements in activity would be expected for rationally designed siRNA, which would be expected to be enriched for highly efficacious siRNA.

The same 21-mer can be generated from D-siRNAs of slightly different sequence and opposite 'polarity': one in which the passenger strand carries the 3′ overhang, and processing proceeds from right to left (L form); and another in which the overhang is on the guide strand, and processing proceeds from left to right (R form; **Fig. 1**). Although processing of the two forms of D-siRNA produces the same 21-nt siRNA species (confirmed by mass spectrometry data), when considering that the strand with the 2-nt 3′ overhang is antisense to the target mRNA, the R forms are consistently more efficacious than the L forms. This comparison was performed for nine different pairs of D-siRNAs targeting four different genes. In seven of nine cases, the R form was superior to the L form[14]. We hypothesized that preferential binding to the 3′ overhang by Dicer during processing favors incorporation of the strand bearing the overhang. Thus, sense target silencing is more efficient with the R form as it is the configuration in which the guide strand bears the overhang. This effect can be demonstrated experimentally by cotransfection experiments with dual luciferase reporters (psiCheck2) in which the target gene is cloned in both orientations in the 3′ untranslated region (UTR) of a reporter gene[14] (**Fig. 2a**). Silencing of the sense reporter is a measure of guide strand incorporation, whereas silencing of the antisense reporter is a measure of passenger strand incorporation. Comparison of the relative silencing of the two reporters by the two different forms of D-siRNA and their corresponding 21-mer revealed superior antisense reporter silencing by the L form, consistent with preferential passenger-strand incorporation (**Fig. 2b**). The relatively weaker antisense reporter silencing by R-form D-siRNA suggests that this configuration has the added advantage of reduced passenger strand–mediated off-target effects.

siRNA design algorithms

Because a substantial fraction (variously estimated at 50–80%) of randomly designed siRNAs are non- or poorly functional, taking full advantage of the D-siRNA design requires a combination of predictable Dicer processing and rational design of 21-mers of superior efficacy or higher likelihood of functionality. The creation of highly successful design rules has been helped by the realization that miRNAs—naturally occurring RNAi effectors that structurally resemble siRNAs and use a similar, if not identical, silencing complex—show some characteristic sequence biases that are also reflected in functional siRNAs[3,4]. This suggests that the sequence of an siRNA is its single most important determinant of functionality. Based on this assumption, statistical analyses of increasingly larger sets of sequences have resulted in the identification of design rules and procedures that substantially improve the success rate of siRNA design[20–22]. A brief description of three of the published algorithms is given below.

1. The algorithm of Reynolds *et al.*[22] is based on statistical analysis of duplex-region motifs of 180 21-nt siRNAs targeting two genes. The following motifs were found to be positively correlated with functionality: (i) 30–52% GC content; (ii) at least three A/U bases in positions 15–19; (iii) absence of internal repeats; (iv) A or U in position 19 (W19); (v) A in position 3; (vi) U in

Target	cDNA	5′	ttgttgaa[cttgaatcagaagatgaagtc]aaattgg
21–mer	siRNA	5′	CUUGAAUCAGAAGAUGAAGUC
		3′	UUGAACUUAGUCUUCUACUUC
25/27-R	siRNA	5′	CUUGAAUCAGAAGAUGAAGUCAA**at**
		3′	UUGAACUUAGUCUUCUACUUCAGUUUA
27/25-L	siRNA	5′	GUUGAACUUGAAUCAGAAGAUGAAGUC
		3′	c**a**ACUUGAACUUAGUCUUCUACUUC

Figure 1 | Comparative design of conventional 21-mer and R and L forms of asymmetrical D-siRNA. Ribonucleotides are in upper case and deoxyribonucleotides in boldface underlined lower case. Target sequence area is boxed.

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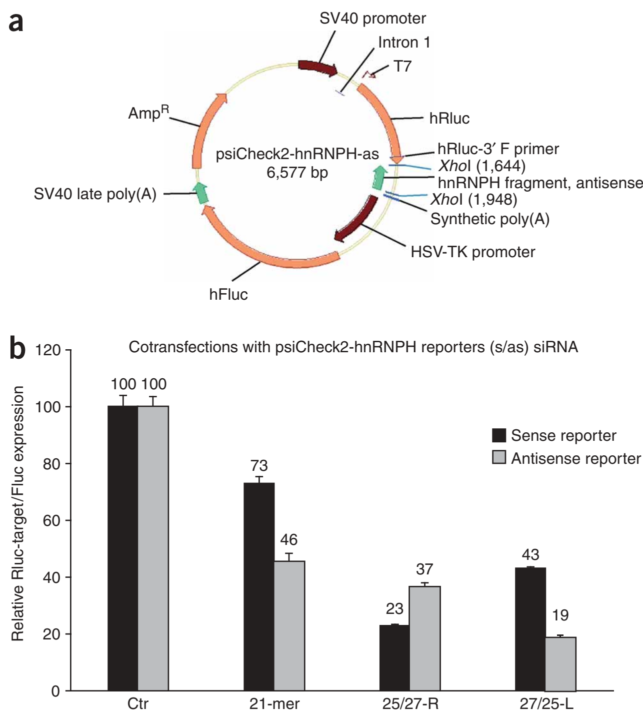

Figure 2 | psiCheck2 dual luciferase-based reporter cotransfections for monitoring sense and antisense D-siRNA strand selection. (**a**) Vector diagrams of strand-specific reporters. Arrows indicate direction of transcription and polarity of target RNAs at the 3′ UTR of the *Renilla* luciferase transcription unit. hRluc and hFluc indicate humanized versions of *Renilla* and firefly luciferases, respectively. hnRNPH is the target gene cDNA. (**b**) Comparative knockdowns of sense- and antisense (AS)-specific hnRNPH-lac reporter with 21-mer siRNA and R-form and L-form D-siRNA. Cells were cotransfected in 24-well plates with 100 ng of reporter, 2 nM (sense reporter) or 0.4 nM (antisense reporter) siRNA and 0.5 µl of Lipofectamine 2000 per well. Target-specific *Renilla* luciferase expression (Rluc) was normalized to firefly luciferase expression (Fluc; internal control) for all replicates and samples. Average expression ratio for control samples (Ctr; cells transfected with irrelevant control siRNA) was set to 100%, and relative expression levels for other samples were calculated accordingly. Error bars indicate standard deviations.

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position 10; and (vii) absence of G in position 13. All correlated features were incorporated into the design algorithm.

2. The algorithm of Amarzguioui and Prydz[20] is based on statistical analysis of duplex-region motifs of 80 21-nt siRNA duplexes targeting five genes. The algorithm incorporates the following features: the A/U differential for the three terminal base pairs at each end of the duplex; the positively correlated motifs W19 (A or U at position 19 of sense strand), S1 (G or C at position 1) and A6; and the negatively correlated motifs U1 and G19.

3. The algorithm of Huesken *et al.*[21] is a neural network–derived algorithm that was trained on the full 21-nt guide sequence of 2,182 distinct siRNAs targeting 34 genes. Because this algorithm is based on machine learning, no distinct design rules can be formulated, and it operates as a black box. This algorithm is currently the state of the art in siRNA target-site prediction.

Another useful link for calculating the thermodynamic end stabilities of siRNAs is available on the Rossi lab website (http://www.cityofhope.org/Researchers/RossiJohn/RossiJohnResearch.htm).

Web-based siRNA design tools

All of the above design algorithms are freely available for academic use in automated form through siRNA Calculator v1.0 beta (http://proteas.uio.no/siRNAbeta.html), BIOPRED*si* (http://www.biopredsi.org/start.html) and Dharmacon (http://www.dharmacon.com/sidesign/default.aspx). The BIOPRED*si* algorithm, developed by Novartis, has been licensed to Qiagen Sciences and is not intended for commercial use. In addition to the above resources, proprietary design algorithms are offered for siRNA design by suppliers such as Invitrogen, Ambion and Integrated DNA Technologies. These are, however, used on ordering and are not available as stand-alone tools.

Some web-based search engines combine features from multiple design algorithms and may allow the user to vary the weight assigned to existing design criteria or to add user-defined design criteria (for example, http://www.idtdna.com/Scitools/Applications/RNAi/RNAi.aspx and http://i.cs.hku.hk/~sirna/software/sirna.php). The output of these online search engines is generally in the form of standard 21-nt sequences, but fully automated R-form D-siRNA design is also available (http://www.idtdna.com/Scitools/Applications/RNAi/RNAi.aspx).

To increase the chances of success, it is preferable to use several design algorithms, of which one should be BIOPRED*si* (if application is consistent with the limited license granted to users), and to choose targets that score highly in multiple or all of the algorithms.

Determination of siRNA specificity

Published data suggest that although near-complete inactivation of siRNA by a single mutation is possible, multiple mutations are generally necessary to ensure that the siRNA will be inactive[5,23–26]. When targeting the 3′ UTR of a transcript, it is also important to consider the possibility that mismatches with the target may abrogate cleavage but can still function in translational repression. Although sequence context, mismatch type and mismatch position all influence the impact of mutations[5,23,27], making it difficult to devise clear rules, some general guidelines can be formulated:

1. Ideally, the selected siRNA should have multiple mismatches to all nontarget mRNA sequences.

2. Mismatches located near the cleavage site or within the seed region (positions 2–11 of the putative guide strand and 9–18 within the target) are more disruptive than mismatches within the 5′ end of the target site. Full matches within positions 9–18 of the 'off-targets' should be avoided even if there are multiple mismatches in the 5′ end.

3. Extensive matches to the self-complement of the target sequence are less crucial, as they would be targeted by the passenger strand, whose incorporation into RISC is designed to be minimal. For these sequences, the duplex-region mismatch sensitivity is reversed, such that mismatches within positions 2–11 of the query are more important.

Specificity has traditionally been determined using BLAST searches. Recent data, however, have cast serious doubt on the value of BLAST searches for general siRNA specificity determination[27]. Experimentally determined siRNA off-target effects were shown to correlate strongly with matches between positions 2 and 8 within the guide strand (the seed sequence) and sequences in the 3′ UTRs of affected genes[27]. These seed matches are too short to be confidently detected by BLAST, so BLAST searches are only useful

for identifying near-perfect matches. A web-based search tool is available for identification of all possible seed matches for any given siRNA (http://www.dharmacon.com/seedlocator/default.aspx). Despite the significant correlation of seed matches with off-targets, the predictive value of such a list is at present limited, as only a small fraction of seed matches result in actual off-target effects. Thus, although BLAST searches can be used to weed out the poorest candidates, and seed match searches can help in deciding among sequences on the basis of the number of potential off-targets, there seems for the foreseeable future to be no substitute for experimental determination of specificity, preferably by genome-wide gene expression profiling. For functional genomic studies, verification of phenotypes by a combination of multiple active target-specific siRNAs and inactivated or irrelevant control sequences is of paramount importance. Because D-siRNAs are processed to predictable 21- and 22-nt sequences, the number of off-target effects is not expected to be substantially different from those resulting from their corresponding 21-mers. Examples of D-siRNAs resulting from this procedure are shown in **Figure 3**.

Potential nonspecific effects of D-siRNA

An emerging property of siRNAs is their potential immune-stimulatory effects *in vitro* and *in vivo* resulting from engagement of members of the Toll-like receptor family after liposome-mediated endosomal trafficking[28–32]. However, no induction of interferon or activation of dsRNA-dependent protein kinase was observed in HEK 293 cells after delivery of 27-nt siRNA[13]. A recent study appears to cast some doubt on the generality of these conclusions, as siRNA duplexes longer than 19 bp showed greater toxicity and interferon stimulation than did conventional 21-mers. In addition, the immune response of cell type dependent, as no stimulation was seen in HeLa and HEK 293 cells. It should, however, be noted that the above studies were done with first-generation blunt 27-mers and not with D-siRNA of the current asymmetrical DNA-modified design. While blunt-ended 27-mers do indeed trigger interferon through RIG-1 pathways in some cells, D-siRNAs of the design described here largely escape this effect[33]. This effect is completely abrogated by limited selective 2′-*O*-methyl modification of the D-siRNA (M.A.B., unpublished observations), a strategy similar to that described for disruption of immune stimulation by conventional 21-mers *in vivo*[34]. Thus, although D-siRNA may in some cases be associated with a slightly increased potential for nonspecific interferon induction, these effects are likely to be easily circumvented. For the cautious and less advanced user, however, conventional 21-mers may be the safest option, particularly when immune stimulation is likely to confound interpretation of observed phenotypes.

Screening of siRNA for efficacy

Because of the variable efficacy of even rationally designed siRNAs, it is advisable to design multiple D-siRNAs targeting different sites and to titrate their concentrations to determine the optimal sequences and concentrations for adequate silencing. Irrelevant control siRNA(s) should be included at all concentrations tested. Lipid-siRNA or D-siRNA complexes should be prepared in one batch for all different treatments of the same siRNA, and the complexes should be diluted appropriately to their final concentration[25]. Adherent cells can be transfected while either adherent or in suspension after trypsin-mediated detachment[35]. The latter procedure is recommended for its greater flexibility and robustness (confluency of cells is not an issue). For difficult-to-transfect adherent cells, the methodology is frequently also associated with improved silencing[35].

In vitro and *in vivo* application of D-siRNA

Efficient delivery of D-siRNA into cells is essential for effective downregulation of gene expression both *in vitro* and *in vivo*. Here we describe the use of a transfection reagent and protocols that are highly effective in delivering D-siRNA both in tissue culture and in mice. The procedure is largely scaleable but may require some minor optimization for substantial changes in scale. The procedures below are described for two different transfection reagents, *Trans*IT-TKO and Lipofectamine 2000, but are essentially compatible with other cationic liposome–based transfection agents, although the optimal D-siRNA–to–lipid complex ratios may differ. Steps 7–14, which are the most labor intensive, can typically be completed in 60–90 min. For peritoneal macrophages and macrophage cell lines *in vitro*, we prefer to use the *Trans*IT-TKO reagent because of its high transfection efficiency and low toxicity. Full turnover time, from the start of an experiment until results are available, can range from 2 days to a week, depending on the properties of the specific target gene and the chosen method of knockdown evaluation.

Although we describe D-siRNA delivery in this protocol, the methodology can also be used to deliver conventional siRNA. *In vitro*, we used RAW 264.7 macrophages, although we have successfully applied this method to suspended HL60 cells with similar success (P.L., J.H., M.A.B. and E.C., unpublished data). *In vivo* transfection, accomplished by intraperitoneal injection of D-siRNA complexed with a transfection reagent, has proven useful in our studies on macrophage trafficking and activation in a mouse model

Finalized design of GAP117si

```
Target cDNA    111   ggtgaaggtcggagtcaacggatttggtcgtattgg

GAP117/21                  GGUCGGAGUCAACGGAUUUGG
                         UUCCAGCCUCAGUUGCCUAAA

GAP117/27R                 GGUCGGAGUCAACGGAUUUGGUCgt
                         UUCCAGCCUCAGUUGCCUAAACCAGCA
```

Finalized design of GAP677si

```
Target cDNA    671   gcccctccgggaaactgtggcgtgatggccgcgggg

GAP677/21                  CCGGGAAACUGUGGCGUGAUG
                         GAGGCCCUUUGACACCGCACU

GAP677/27R                 CCGGGAAACUGUGGCGUGAUGGCcg
                         GAGGCCCUUUGACACCGCACUACCGGC
```

Finalized design of GAP1268si

```
Target cDNA    1261  gggagccgcaccttgtcatgtaccatcaataaagt

GAP1275/21                  GCACCUUGUCAUGUACCAUCA
                          GGCGUGGAACAGUACAUGGUA

GAP1275/27R                 GCACCUUGUCAUGUACCAUCAAUaa
                          GGCGUGGAACAGUACAUGGUAGUUAUU
```

Figure 3 | Finalized designs of GAPDH R-form D-siRNA (27R) and their respective 21-mers. Excerpt of target region cDNA and composition of the respective siRNA duplexes are shown. Ribonucleotides are in upper case and deoxyribonucleotides in boldface underlined lower case.

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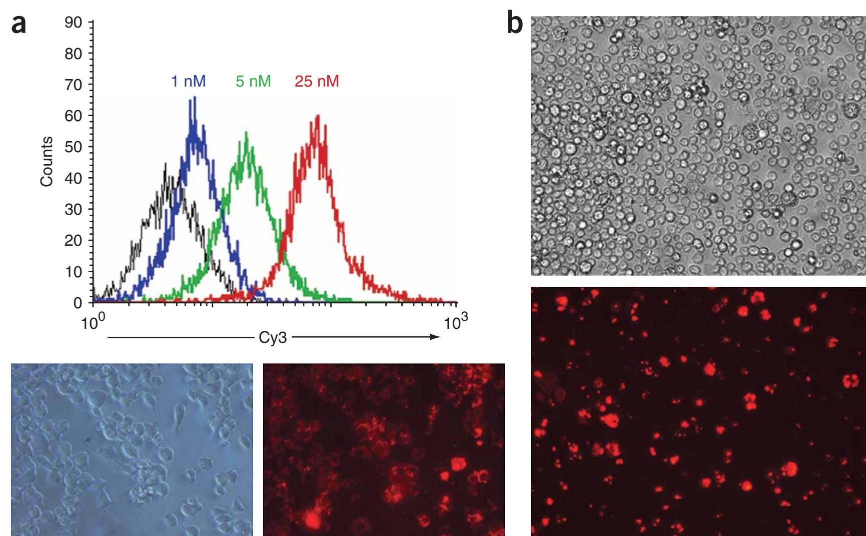

Figure 4 | Transfection efficiency using *Trans*IT-TKO and siRNA-Cy3. (**a**,**b**) Quantitation of transfection efficiency by Cy3 fluorescence in RAW 264.7 cells *in vitro* (**a**) and peritoneal exudate cells *in vivo* (**b**) after 16 h transfection. Histogram overlay in **a** shows the FL-2 fluorescence of RAW 264.7 cells that were transfected with 1 (blue), 5 (green) or 25 (red) nM Cy3-labeled siRNA relative to untransfected cells (black population). Below the histogram panel are shown corresponding visible light (left) and red fluorescence (right) fields of view from the 5 nM transfection. In (**b**) are shown visible light (top) and the corresponding red fluorescence (bottom) fields of view of peritoneal cells that were transfected *in vivo* for 16 h with 0.2 μg siRNA.

of virus infection. We describe the protocol for macrophage delivery and show transfection efficiency *in vitro* and *in vivo* using a Cy3-labeled siRNA.

These protocols have been successfully used for delivery of D-siRNA targeting TNF-α both *in vivo* (**Fig. 4**) and *in vitro* (**Fig. 5**). For *in vitro* studies using this assay, cells are plated on day 1, treated with RNAi on day 2 and stimulated on day 3, after which the cells are analyzed for differential responses to RNAi treatment.

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MATERIALS

REAGENTS

- Mice (optional) ▲ CRITICAL Users must comply with national regulations concerning use of animals.
- Low-endotoxin FBS (Omega Scientific, cat. no. FB-02) ▲ CRITICAL This is used to avoid terminal differentiation into macrophages.
- Serum-free medium (OPTI-MEM I; Invitrogen Gibco, cat. no. 31985-070)
- Cells to be transfected, in suspension (this protocol has been shown to be compatible with a wide range of adherent cells, and choice of cells should therefore be dictated by relevance to downstream functional assays)
- RAW 264.7 cells (American Type Culture Collection, cat. no. TIB71)
- Cells for screening and titration of siRNA: the cell lines chosen should express the target gene and be readily transfectable; there is no generic cell line recommended for this
- Distilled water ▲ CRITICAL Diethylpyrocarbonate treated glass distilled water or commercially available "nuclease free glass distilled water" should be used.
- Transfection agent: *Trans*IT-TKO (Mirus, cat. no. MIR 2150) or Lipofectamine 2000 (Invitrogen, cat. no. 11668-019) (see REAGENT SETUP)
- Complete medium for RAW 264.7 cells: DMEM (Cellgro, cat. no. 15-013-CV) supplemented with 5% low-endotoxin FBS (see above), 2 mM L-glutamine (Cellgro, cat. no. 25-005-CI) and 10 mM HEPES (Irvine Scientific, cat. no. 9319)

EQUIPMENT

- Multiwell plates: from any tissue culture supplier

REAGENT SETUP

Transfection agent *Trans*IT-TKO (Mirus, cat. no. MIR 2150) or Lipofectamine 2000 (Invitrogen, cat. no. 11668-019) ▲ CRITICAL The protocol below is based on the manufacturer's protocol for assembly of the reagents when using *Trans*IT-TKO. We have tried other transfection reagents, but only the *Trans*IT-TKO reagent gives us a 100% transfection rate and gene knockdown without toxicity in these cells. Note that the optimal ratio of D-siRNA to lipid complex may differ for other transfection agents.

PROCEDURE

Rational design of D-siRNA

1| Query the UCSC genome browser (http://genome.ucsc.edu/cgi-bin/hgGateway) with the appropriate term (for example, human GAPDH) and identify the most up-to-date mRNA sequence (NM_002046 in the case of GAPDH).

2| Base the 21-nt target selection on the whole mRNA sequence. Our experience is that the 3′ UTR is as suitable for targeting as the coding region, so there is no reason to exclude it. In fact, because the 3′ UTR is the primary target region of naturally occurring miRNAs, the RNAi machinery may function more efficiently for targets in this region. Less is known about the accessibility of the 5′ UTR, but again, there is no reason to disqualify this region *a priori*, although more emphasis may be placed on the open reading frame and 3′ UTR where equally suitable candidate sequences are available.

3| Use the BIOPRED*si* algorithm (http://www.biopredsi.org/start.html) and at least one other freely available web-based search tool for the design. In this case, we used the siRNA Calculator (http://proteas.uio.no/siRNAbeta.html), but this can easily be substituted for other algorithms. The results from the second design tool should be used only to select among the best of the sites recommended by BIOPRED*si*. Run the BIOPRED*si* algorithm with an output of 10 sequences, and run the siRNA Calculator with the recommended range (0.3–0.6) of preferred GC content and with absolute design criteria disabled to allow listing of scores for all sequences. Select the top five scoring siRNAs from the siRNA Calculator design that are also included in

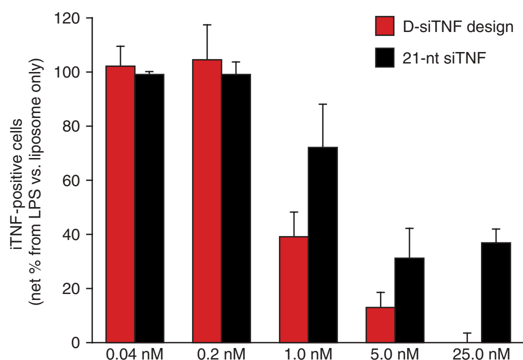

Figure 5 | Comparison of gene knockdown using D-siRNA and conventional 21-nt siRNA against TNF-α in RAW 264.7 cells. Serial dilutions of D-siTNF and 21-nt siTNF from 0.04 to 25 nM were used to interfere with TNF-α protein production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Cells were plated and treated with RNAi overnight (16 h), as described in the protocol text, and then stimulated with a dose of LPS resulting in half-maximal TNF-α production from mock-transfected control cells. Percent inhibition was normalized to baseline TNF-α production (0% indicates no LPS induction) and TNF-α production from mock-transfected cells with LPS induction (100%). TNF-α protein production was quantitated using intracellular flow cytometry and gated on live cells, as judged by forward and side scatter (70–75% of all events collected, or 50,000, fell within this gate). Results are representative of three experiments, and error bars are shown for three replicate wells per treatment group. iTNF positive cells refers to cells staining positive for intracellular TNF protein by flow cytometric analysis.

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the top 10 list from the BIOPRED*si* algorithm design for further evaluation. The properties of the selected sequences for the GAPDH example are summarized in **Table 1**.

4| Analyze the target sequences of the siRNA candidates for specificity using the siRNA Seed Locator (http://www.dharmacon.com/seedlocator/default.aspx). Use the sense sequence of the duplex region as query sequence. As a rough measure of potential off-target effects, compare number of reference sequences with multiple seed matches. The number of hits for the five candidates is shown in **Table 1**.

5| Select the three sequences combining high scores in two independent algorithms with the least number of hits from the siRNA Seed Locator for conversion to the D-siRNA format. Extend the sequence of the core 21-mer toward the 3′ end of the target such that the top strand contains 25 nt and the bottom guide strand contains 27 nt, maintaining target complementarity and Watson-Crick base-pairing. Substitute the last two 3′ nucleotides of the top strand with deoxynucleotides. The completed design for our selected target sites is shown in **Figure 3**.

Screening and titration of siRNA

6| Carry out a titration series of the various D-siRNAs to identify the most effective duplexes and the lowest possible concentration that still generates the desired level of knockdown. The subsequent steps describe suspension transfection of cells with siRNA at three different concentrations (50, 15 and 5 nM) in six-well plates, in a final volume of 2.5 ml per well. We previously found[13] that D-siRNAs could in some cases be titrated to subnanomolar concentrations and still elicit potent knockdown.
▲ **CRITICAL STEP** High concentrations of the D-siRNAs can elicit more off-targeting. Thus, it is strongly advised to carry out these titrations to obtain the lowest possible amount of D-siRNA that gives the desired target knockdown. The most appropriate control for knockdown specificity of observed phenotypes is a second D-siRNA to another sequence in the same target mRNA.

7| Calculate the total amount of siRNA required for the three different treatments with the same siRNA according to the following formula, which includes a 5% safety margin: $n = (50 + 15 + 5 \text{ nM}) \times 2.5 \text{ ml} \times 1.05$. This equals 1.83 nmol, or 9.2 μl of 20 μM siRNA.

8| Dilute siRNA approximately 10-fold (100 μl) in Opti-MEM.
▲ **CRITICAL STEP** Opti-MEM or serum-free medium, and not the complete medium, should be used for forming the complexes.

TABLE 1 | Algorithm-based scoring of GAPDH siRNA candidates for efficacy and specificity.

Target sequence (sense, duplex region)	Start position	BioPred*si* score	siRNA Calculator score	SeedLocator hits
GUCAUGUACCAUCAAUAAA	**1275**	**0.845**	**5**	**1,675**
GGUCGGAGUCAACGGAUUU	**117**	**0.844**	**5**	**37**
CCUCUGACUUCAACAGCGA	950	0.826	3	—
CCGGGAAACUGUGGCGUGA	**677**	**0.802**	**5**	**534**
CGUCAUGGGUGUGAACCAU	**495**	**0.781**	**4**	**1,041**
GCAUUGCCCUCAACGACCA	1001	0.776	3	—
UGUCAUGUACCAUCAAUAA	1274	0.841	2	—
GCACCUUGUCAUGUACCAU	**1268**	**0.806**	**4**	**435**
AUGUACCAUCAAUAAAGUA	1278	0.800	1	—
AGGUGGUCUCCUCUGACUU	941	0.771	3	—

Shown are the top ten scoring siRNA candidates recommended by the BIOPRED*si* algorithm, together with their scores from BIOPRED*si* and an alternative web-based search tool (siRNA Calculator). A measure of specificity, the potential number of 3′ UTR seed matches to irrelevant targets, is shown for the top five candidates. The top 5 candidates using the siRNA calculator are shown in bold.

TABLE 2 | Calculations of reagent volumes for four mice given 0.2 μg D-siRNA in 200 ul.

siRNA	*Trans*IT-TKO	dH_2O	Est. vol after vacuum	Add 10× dH_2O-PBS	Adjust to 1× PBS w/ H_2O	1 μg μl^{-1} siRNA	Dilute with PBS
0.85 μg	3.4 μl	3.4 μl	3.5 μl	0.7 μl	2.6 μl	0.85 μl	+0.85 ml

To administer 0.2 μg siRNA per mouse, a small excess (0.85 μg) of siRNA is prepared. This amount requires 3.4 μl of *Trans*IT-TKO reagent to achieve a ratio of 4 μl *Trans*IT-TKO per 1 μg siRNA. To remove the 100% ethanol that *Trans*IT-TKO is shipped in, add an equal volume of RNase-free, pyrogen-free water to the *Trans*IT-TKO and then vacuum-concentrate the mixture for a brief period of time (see text). After the ethanol has evaporated, add 0.1 volume equivalent (relative to the final combined *Trans*IT-TKO–water mix; in this case, 6.8 μl) of RNase-free, pyrogen-free 10× PBS (6.8 μl × 0.1 = 0.7 μl in this example). Finally, add RNase-free, pyrogen-free water to bring the solution to 1× PBS, which here corresponds to 2.6 μl. The resulting 6.8 μl of *Trans*IT-TKO in 1× PBS is now ready to be complexed with siRNA right before intraperitoneal injection (add 0.85 μg; that is, 0.85 μl of a 1 μg μl^{-1} siRNA stock). Incubate as described in the text and dilute with the equivalent of 200 μl of 1× RNase-free, pyrogen-free PBS per mouse (in this example, 0.85 μg total siRNA ÷ 0.2 μg per mouse × 200 μl per mouse = 850 μl) before injection.

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9| Prepare a batch dilution of Lipofectamine 2000 in Opti-MEM (Lip/Opti) sufficient for all (*N*) complexes to be formed (for example, $N = 4$ for irrelevant control siRNA and three target-specific siRNAs) according to the following formula: Lip/Opti = 100 × *N* μl basal medium + 7.5 × *N* μl Lipofectamine 2000. Incubate at room temperature (here and throughout, the exact room temperature is not crucial) for 5–10 min.
! CAUTION The amount of Lipofectamine 2000 in the formula was calculated to ensure a complex ratio of liposome (volume) to siRNA (weight) of 2.5:1. While this is a good starting point, the optimal complex ratio and transfection efficiency may vary with cell line, confluency (if transfection is performed to adherent cells) and absolute amount of liposomes.

10| Mix Lip/Opti (100 μl; see Step 9) with diluted siRNA (100 μl; see Step 8) by gentle pipetting and leave at room temperature for 30 min to allow complexes to form.
! CAUTION This incubation time can be varied between 20 and 45 min with little effect on silencing efficiency but should ideally not vary greatly between samples. To avoid substantial differences in incubation time between samples, it may be appropriate to process samples in staggered intervals to reduce handling time.

11| During complex assembly, detach cells, resuspend them in complete medium and pellet by gentle centrifugation at 400*g* for 5 min at room temperature. Resuspend the cells in 10 ml of complete medium. Calculate the amount of cell suspension to use per well (ml) according to the formula $V = 10 \times A \times B \div (C \times D)$, where *A* is the area of the well (10 cm^2 for a six-well plate), *B* is the desired confluency equivalent of cells at plating (30–70%, depending on growth rate of cells and desired time of collection), *C* is the area of the original cell culture dish or plate and *D* is its confluency (typically 90–100%) at detachment. For a typical experiment starting from a confluent T-75 flask ($C = 75$, $D = 100$) and plating of cells per well in six-well plates equivalent to 50% surface coverage ($B = 50$), this amounts to 665 μl of cell suspension per well.
! CAUTION The cell suspension should not sit for extended periods of time in 15- or 50-ml plastic tubes. It is preferable to start preparing the cell suspension immediately after initiation of complex formation.

12| Pipette aliquots of each complex into three different tubes according to the formula $V = A \times B \div C$, where *A* is amount of siRNA for each sample (for example, 50 nM × 2.5 ml), *B* is the total complex volume (210 μl in this case) and *C* is the total amount of siRNA in each batch of complex.

13| Dilute aliquots of complexes with medium and cell suspension (see Step 11) to a final volume of 1.0 ml. Mix by gentle pipetting.

14| Add the cell-complex mixture to 1.5 ml of complete medium in a six-well plate. Shake well to ensure even distribution of cells in the well. Incubate until desired time of collection.
! CAUTION There is no need for medium replacement on the day of transfection. If cells are incubated for longer than 1 d before collection, medium should be replaced the day after transfection.

15| Assess knockdown by northern blot, real-time RT-PCR or western blot analyses, and determine the optimal combination of siRNA sequence and concentration.

In vitro and *in vivo* application of D-siRNA

16| D-siRNAs can be transfected *in vitro* (A) or *in vivo* (B).

(A) *In vitro* transfection of RAW 264.7 cells with D-siRNA

(i) Plate cells at 6.0×10^4 cells/well in the afternoon. Cells must be 60–80% confluent at the time of transfection and should be grown in antibiotic-free medium.
▲ **CRITICAL STEP** It is crucial that the serum used for the RAW medium has a very low endotoxin content to avoid terminal differentiation of cells into mature macrophages. We have had success with serum from Omega Scientific; however, each source of serum should be tested to ensure that it does not promote differentiation of RAW 264.7 cells.

Macrophage cell lines tend to respond less well over time and eventually senesce if subjected to continuous low levels of endotoxin stimulation.

(ii) Add 1.0 ml of prewarmed medium containing RAW 264.7 cells to each well.

(iii) Rock plate in north-to-south direction, pause and then rock in east-to-west direction to generate an even monolayer.
▲ **CRITICAL STEP** Do not swirl plate. Swirling causes the cells to gather in the centers of the wells, making the monolayer uneven. The rocking method ensures a more uniform cell distribution.

(iv) Incubate overnight (16–20 h; the exact time is not critical).

(v) Check the density of cells using an inverted microscope.

(vi) Cell confluency should be 60–80%, as estimated visually.

(vii) Aspirate the medium in each well and immediately add 200 μl of fresh complete medium (with serum) to each well.

(viii) Add 45 μl of serum-free medium to nuclease-free, standard 1.5-ml microcentrifuge tubes.

(ix) Add 2.5 μl of *Trans*IT-TKO transfection reagent to the serum-free medium and mix thoroughly by vortexing. Incubate at room temperature for 10 min.

(x) Add D-siRNA (in 2.5 μl), mix gently by pipetting up and down and flicking the tube, and incubate at room temperature for 5–10 min. This is the siRNA complex. Use 0.2–25 nM D-siRNA (0.2, 1, 5 or 25 nM). Transfection of Cy3-labeled 21-nt siRNA is used here to monitor efficiency of siRNA internalization.
! CAUTION Cellular internalization (which may be largely endosomal) does not necessarily equate with cytoplasmic release or silencing in transfected cells. Caution should therefore be used in interpreting such transfection data.

(xi) Add the 50 μl of siRNA complex to each well and gently rock the plate back and forth and from side to side with both hands.

(xii) Incubate for 16–18 h in a humidified incubator at 37 °C in 5% CO_2 before checking transfection efficiency. (We have successfully used a transfection period as short as 4–6 h and still achieved nearly 100% efficiency; **Figure 4a** and P.L. and E.C., unpublished observations).

(B) *In vivo* transfection of peritoneal cells with siRNA

(i) Empirically determine the amount of D-siRNA to be given to the mouse by testing a twofold dilution series, starting at 0.4 μg per treatment and increasing the dilution until there no longer is an effect (approximately to 0.05 μg). Simultaneously, test the transfected cells for nonspecific activation, such as MHCII upregulation on peritoneal macrophages or an equivalent marker suited to the specific model system in which the methodology is applied. We have used up to 0.4 μg of D-siRNA without discernable nonspecific immune stimulation of resident peritoneal cells, as determined by assaying for activation markers and secretion of cytokines. We routinely resuspend the 21- or 27-nt siRNAs to approximately 60 μM to get a 1 μg μl^{-1} stock solution, as this simplifies subsequent calculations.
▲ **CRITICAL STEP** The siRNA transfection complex should be prepared immediately before injection.

(ii) Prepare 4 μl of *Trans*IT-TKO reagent per 1 μg of D-siRNA (the total amount of *Trans*IT-TKO volume is henceforth referred to as '1×'). Because the transfection reagent is stored in 100% ethanol, it is important to exchange the lipid preparation into an aqueous buffer. To do this, add an equal volume (1×) of diethylpyrocarbonate-treated, glass-distilled sterile water or commercially available nuclease-free water to the *Trans*IT-TKO and vacuum-concentrate the mixture until about half of the volume has evaporated. Stop the vacuum concentration periodically and check that the volume has not evaporated to dryness. The lipids will be suspended in the water and the solution will therefore be slightly opaque.
▲ **CRITICAL STEP** It is important not to completely evaporate the liquid in this step, as it is not possible to subsequently uniformly resuspend desiccated lipids into the aqueous buffer.

(iii) Add 0.2× volume of tissue culture–grade 10× PBS and then restore the lipid suspension to 2× using diethylpyrocarbonate-treated, glass-distilled sterile water. The PBS-suspended *Trans*IT-TKO is now in a 150 mM monovalent cation suspension at half the original concentration; that is, for each microgram of siRNA, 8 μl of 1× PBS–*Trans*IT-TKO is needed. See **Table 2** for an example of how to prepare the injection volume for four mice given 0.2 μg D-siRNA each. For larger or smaller amounts of siRNA or D-siRNA, or for a greater or smaller number of mice, scale the calculations appropriately, maintaining a complex ratio of 1 μg siRNA or D-siRNA to 4 μl of *Trans*IT-TKO (8μl of PBS–*Trans*IT-TKO).
▲ **CRITICAL STEP** Cationic transfection reagents such as *Trans*IT-TKO will effectively form complexes with nucleic acids in the absence of salt. However, for transfection purposes, the inclusion of salt (such as that in the PBS) is needed to make the solution of injectable complex isotonic with the cells with which it comes in contact. In addition, the salt limits the compaction of the transfection complexes, and the overall size of the siRNA-reagent complexes are larger when they are formed in the presence of salt. For *in vitro* transfections, larger complexes (up to a certain point, ∼500 nm) are desirable for obtaining more efficient delivery.

(iv) Transfer the desired amount of D-siRNA into a new tube, add the 1× PBS–*Trans*IT-TKO suspension and incubate at room temperature for 10 min. Immediately after mixing, the complex formation initiates and turns the suspension milky white. This is normal and expected.

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(v) Before intraperitoneal injection, add the desired volume of RNase-free buffer to the complex suspension (we have used 0.2–1.0 ml with equal success). This larger volume is required for practical handling of the transfection suspension and to allow for more uniform distribution of the suspension in the peritoneum.

(vi) Inject the suspension into the peritoneal cavity of the mouse and massage the peritoneum for 15–20 s to promote even distribution of the injected suspension.

(vii) Collect peritoneal exudate cells the next day by lavage using 10 ml of Hank's balanced salt solution. Quantitate the transfection efficiency by flow cytometry and/or visually by fluorescence microscopy as shown in **Figure 4b**. Using these protocols, we get excellent results both *in vitro* and *in vivo*, and we have confirmed that we can completely prevent inducible production of TNF-α protein (**Fig. 5**). However, despite efficient knockdown of the targeted gene, the efficacy with which the phenotypic readout in a specific disease model is achieved will depend on the subsequent biodistribution of the cells transfected in the peritoneum.

ANTICIPATED RESULTS

Well-designed RNAi-mediated target knockdowns can result in greater than 95% reduction in the target RNA and protein[13,14] (**Fig. 5**). It is important to have some indicator of transfection efficiency for the cells being tested. Most often, investigators use just one measure for knockdown, such as quantitative RT-PCR or western blotting. Whenever possible, it is best to use two separate measures to validate the level of target reduction. Our experience with the D-siRNAs is that they often enhance target knockdown from a few fold up to an order of magnitude relative to the corresponding 21-nt siRNA. The key to increased effectiveness is to link the Dicer cleavage to the transfer of the product siRNA to the Argonaute proteins. If the D-siRNAs are going to be used in immune cells, it is important to avoid known immunostimulatory motifs[28,29] and to monitor for Toll-like receptor activation and type I interferon responses. Successful application of the D-siRNAs should result in strong target knockdown without off-target or other nonspecific effects.

Intraperitoneal injection of liposome-encapsulated siRNAs or D-siRNAs should result in rapid uptake by macrophages and other phagocytic cells. Targeting transcripts involved in inflammatory responses, such as inflammatory cytokines, may be applicable to the treatment of sepsis or infections that are exacerbated by macrophage-specific inflammatory responses. The protocols provided in this study should be of use to investigators wishing to study the role of inflammation in various diseases.

In vivo targeting of inflammatory cytokines as reported here can produce results within varying time frames, from minutes to days, depending on the model system. For each target, sampling of cells or monitoring of inflammatory responses should be tested empirically to find the optimal time frame for monitoring RNAi-mediated inhibition. In our hands, the experiments using intraperitoneal injection of siRNAs or D-siRNAs targeting TNF-α gave a readout within 24 to 36 h. The TNF-α knockdown response is quite rapid and may not be generalizable for other cytokines using these methods. For other targets, we recommend carrying out empirical testing over a time course of hours to a few days.

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