

Chapter 2

Monitoring Innate Immune Recruitment by siRNAs in Mammalian Cells

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Abstract

The use of small interfering RNAs (siRNAs) in human therapy may be hindered by the recruitment of nonspecific effects such as the activation of innate immune responses. Recently, several innate immune receptors have been implicated in the detection of siRNAs. This chapter provides a brief overview of the current knowledge of siRNA-induced innate immunity, as well as protocols for the rapid identification of siRNAs with innate immune stimulatory activity.

Key words: Innate immunity, RNA interference, siRNA, RIG-I, TLR7, TLR8

1. Introduction

During viral infection, mammals rely on an early detection of foreign ribonucleic acids to mount a rapid antiviral response. While this phenomenon has been known for more than four decades, insights into the molecular identity of components of the response have been gained only recently (1). Two detection pathways have been identified in blood immune cells as directly involved in innate immune activation by exogenous RNAs. The cells orchestrating the initiation of this antiviral response sense viral RNAs through Toll-like receptors (TLRs) or retinoic acid inducible gene I (RIG-I)-like receptors (1).

Originally thought to be too small to be recognized by the sensors of the innate immune system, small interfering RNA (siRNA) activation of a strong innate immune response is now well established (2). To date, four main characteristics of siRNAs have been associated with the recruitment of innate

immunity and subsequent cytokine production: a) Secondary structure, which is detected by TLR3; b) uridine content, detected by TLR 7/8; c) end terminal structure of blunt-end siRNA from 21-27 nt detected by RIG-I; and 25 nt duplexes bearing a 5' or 3' monophosphate, also detected by RIG-I (3–11).

We have established different protocols that allow for rapid discrimination among different siRNAs for their capacity to recruit TLR7/8 and RIG-I (12, 13). Whether or not the ability of an siRNA to induce immunostimulation through these receptors is the desired outcome (14), these systems are a useful starting point prior to further validation in peripheral blood mononuclear cells (PBMCs) from animal models.

In this chapter, we describe two protocols allowing for the evaluation of mouse TLR7 (and per se, also human TLR7) and human TLR8 recruitment by siRNAs. We also describe a simple real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) protocol, based on human T98G cells (adapted from Marques et al. (8)).

2. Materials

2.1. Cell Culture

1. RAW 264.7: ATCC reference TIB-71. T98G cells: ATCC reference CRL-1690.
2. Ficoll-Paque Plus (GE Healthcare)
3. Lithium-heparin sterile tubes (Sarstedt, Nümbrecht, Germany).
4. Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen Corporation) supplemented with 10% sterile fetal bovine serum (FBS; ICPBio Ltd, Auckland, New Zealand) and 1× antibiotic/antimycotic (Invitrogen Corporation) (referred to as complete DMEM medium).
5. Roswell Park Memorial Institute medium (RPMI) 1640 plus L-glutamine medium (Invitrogen Corporation) complemented with 1× antibiotic/antimycotic and 10% FBS (referred to as complete RPMI 1640).
6. Dulbecco's Phosphate-Buffered Saline (PBS, Invitrogen Corporation).
7. TrypLE™ Express Stable Trypsin (Invitrogen Corporation).
8. Sterile tissue culture-treated microtest™ 96-well plates (Falcon)
9. Sterile, tissue culture-treated 48-well plates (JET BIOFIL, Guangzhou, China).

10. Human TLR8 and mouse TLR7 agonist: CL75 (Invivogen, San Diego, USA).
11. *N*-[1-(2,3-Dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate (DOTAP) (Roche).
12. Opti-MEM® (Invitrogen Corporation).
13. Lipofectamine 2000 (Invitrogen Corporation).
14. siRNAs: synthesized by Integrated DNA Technologies (IDT) as single-stranded RNAs; resuspended in filter-sterilized duplex buffer (100 mM potassium acetate, 30 mM HEPES, pH 7.5) in UltraPure™ DNase/RNase-Free Distilled Water (referred to as RNase-free H₂O, Invitrogen Corporation) to a concentration of 80 μM. Each duplex is annealed at 92°C for 2 min and left for 30 min at room temperature before being aliquoted and frozen at -80°C. siControl is a nontargeting 21 nucleotide siRNA (siControl 1, Ambion).

**2.2. Tumor Necrosis
Factor α (TNF- α)
Enzyme-Linked
ImmunoSorbent Assay
(ELISA)**

1. OptEIA ELISA sets (BD Biosciences).
2. PBS 10×: NaCl 8% (w/v), KCl 0.2% (w/v), Na₂HPO₄ 1.22% (w/v), KH₂PO₄ 0.2% (w/v) in ddH₂O – pH 7.4 (all reagents are from Sigma-Aldrich).
3. PBS-tween (PBST): 1× PBS diluted in H₂O complemented with 0.05% tween 20 (Sigma-Aldrich).
4. Pharmingen Assay Diluent (BD Biosciences Pharmingen).
5. F96 maxisorp plates (nunc, Roskilde, Denmark).
6. Tetramethyl benzidine substrate (TMB, Sigma-Aldrich)
7. Sulfuric acid 2 N (Sigma-Aldrich).
8. Plate reader with 450 nm absorbance filter.

**2.3. RNA Extraction/
cDNA Synthesis/Real
Time**

1. NucleoSpin RNA II kit (MACHEREY-NAGEL, Düren, Germany). Supplement RAI buffer with 1% v/v 2-mercaptoethanol (Bme) (Sigma-Aldrich) immediately before adding to the cells.
2. Superscript III Reverse Transcriptase – includes 5× first strand buffer and 0.1 M dithiothreitol (DTT), 10 mM deoxy-nucleotides triphosphate (dNTPs), Oligo(dT)₂₀ Primer, and RNaseOUT™ (all from Invitrogen Corporation).
3. SYBR GreenER™ qPCR SuperMix for iCycler® instrument (Invitrogen Corporation).
4. IQ5 Multicolor Biorad i-cycler.
5. Optical Tape (Bio-Rad).
6. Multiplate 96-well clear (Bio-Rad).

3. Methods

3.1. Sequence-Specific Recruitment of TLR7 and 8

First and foremost, the ability of siRNAs to recruit the innate immune system is highly dependent on the cell type considered. Plasmacytoid dendritic cells and macrophages/monocytes are the main detectors of TLR7/8 agonists amongst other immune blood cells (1). Because the route of siRNA delivery in vivo is intrinsically related to a potential recruitment of immune blood cells, it is important to assess the detection of siRNAs by TLR7/8 when selecting appropriate siRNA candidates for in vivo delivery. Although uridine-based motifs within small RNA sequences have been found to be important for TLR7/8 activation (4–7), *in silico* prediction of the overall immunostimulatory potency of an siRNA remains highly inaccurate. We and others have found that single-stranded RNAs bearing uridine motifs that induce strong immunostimulation in human PBMCs can be completely masked when present in a double-stranded siRNA structure (6, 13). For this reason, direct measurement of the immunogenicity of a novel siRNA sequence is currently the most accurate method of evaluating recruitment of TLR7/8 by siRNAs.

While both human TLR7 and TLR8 (hTLR7/8) have been implicated in sequence-specific sensing of small RNAs, the murine homolog of TLR8 is not able to detect RNA on its own (12, 15, 16). Rather, sequence-specific sensing of RNAs relies exclusively on TLR7 in the mouse (12, 15). It has recently been shown by us and others that hTLR7 and hTLR8 recognize different RNA sequences, thus the immunogenicity of some sequences preferentially recognized by hTLR8 is not conserved between human and mouse (12, 15, 16). Nevertheless, our observations based on a large panel of oligoribonucleotides have led us to the conclusion that sequence-specific sensing of small RNAs by TLR7 is conserved between human and mouse (12) (see Fig. 1). Here, we describe two protocols allowing for the evaluation of mouse TLR7 (and per se, also human TLR7) and human TLR8 recruitment by siRNAs. For mouse TLR7 recruitment, we rely on the induction of mouse TNF- α (mTNF- α) by a macrophage-like cell line (RAW 264.7) (5, 12). Making use of the conservation of TLR7 sensing between human and mouse avoids using a costly human interferon- α (IFN- α) ELISA and yet captures most of the hTLR7-driven IFN- α response observed in human PBMCs (see Fig. 1). It is noteworthy that when a sequence is found not to trigger TNF- α induction in RAW 264.7 cells, no conclusion can be drawn regarding its innate immune activating potential in human blood without further validation of hTLR8 activity via human TNF- α (hTNF- α) production in human PBMCs (see Fig. 1).

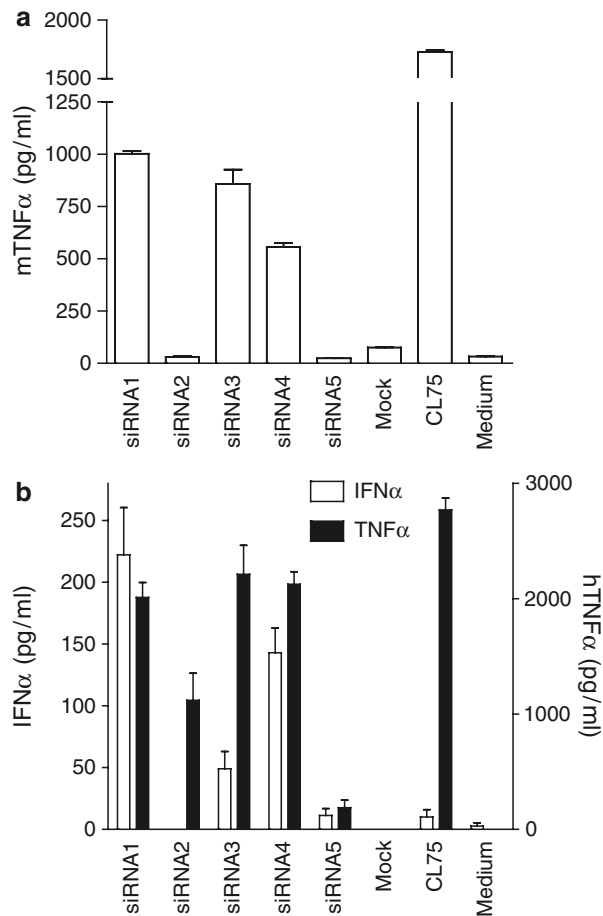


Fig. 1. siRNA-induced TNF- α in human and mouse macrophages. **(a)** Mouse RAW 264.7 cells and **(b)** human PBMCs were treated as presented in Subheading 3.1 with 750 nM of siRNAs complexed with DOTAP for 18 h. Each treatment was carried out in biological triplicate and the data is from one representative experiment for both **(a)** and **(b)**. The error bars represent the standard error of the mean (SEM). In this example, the mouse macrophage cell line data **(a)** indicates that siRNA1, 3 and 4 are immunostimulatory (through mouse TLR7), whereas siRNA2 and 5 are not. While a similar observation can be made in human PBMCs **(b)** when looking at IFN- α (indicative of human TLR7 recruitment), we find that siRNA2 is a good inducer of TNF- α (indicative of human TLR8 recruitment) but not IFN- α . However, siRNA5 appears to be a very low inducer of both IFN- α and TNF- α in PBMCs and would therefore be considered here as very poorly immunostimulatory

3.1.1. Preparation of Mouse RAW Cells for TLR7 Activation

Plate RAW 264.7 cells passaged on surface-treated plasticware to a confluency of ~80,000 cells per well of a 96-well plate in 150 μ L complete RPMI medium in the morning of the TLR stimulation (see Note 1). Incubate the cells at 37°C in 5% CO₂ for a minimum of 4 h prior to treatment with the TLR agonists.

*3.1.2. Preparation
of Human PBMCs
for hTLR8 Activation*

1. Collect blood from healthy volunteers in heparin-treated tubes (see Note 2) and mix with pure RPMI medium (no FBS, no antibiotics) in a 1:1 (v/v) ratio. Very gently, deposit the resulting RPMI-blood solution onto the surface of a Ficoll-Paque Plus layer in a 50 mL sterile tube, while avoiding any perturbation of the Ficoll-Paque Plus. A 1:1.2 ratio of Ficoll-Paque Plus to RPMI-blood volume is used. Centrifuge the 50-mL tubes at $1,000\times g$ for 22 min at 4°C , using reduced break if possible. Following this gradient separation, discard the upper phase by gentle suction until the “white” interphase is reached. Transfer the PBMC-containing interphase to a new 15 mL sterile tube, taking care not to disturb the underlying Ficoll phase. Add RPMI medium to the collected interphase, up to a final volume of 10–12 mL, before spinning at $600\times g$ for 7 min at 4°C . Following centrifugation, a pellet of cells should be visible. Discard the supernatant, wash the cell pellet with 10 mL of RPMI medium, and pellet again at $350\times g$ for 7 min. Resuspend the cell pellet in 2 mL of complete RPMI and count using a hemacytometer.
2. Seed an average of 130,000–200,000 PBMCs in 150 μL of complete RPMI medium in each well of a 96-well plate (see Note 3). Rest the cells for a minimum of 1 h at 37°C in 5% CO_2 prior to stimulation.

*3.1.3. TLR Stimulation
of Human PBMCs
and Mouse RAW Cells*

Both cell types are treated the same way. Perform each treatment in biological triplicate: the amounts of the reagents given here are sufficient for three wells of a 96-well plate.

1. In sterile microcentrifuge tubes, aliquot 63.8 μL of pure RPMI. Dilute 11.2 μL of 40 μM siRNA into each tube (resulting in 75 μL per tube).
2. In a separate tube, mix 21 μL DOTAP with 54 μL pure RPMI (a mastermix conserving this ratio can be made). Mix the tube by gentle tapping, then incubate at room temperature for 5 min.
3. Add 75 μL of DOTAP/RPMI mix to each diluted siRNA, mix gently, then incubate the tubes for a further 10 min at room temperature.
4. Add 50 μL of the DOTAP-siRNA mixture to each well of plated cells (three wells per condition) to give a final volume of 200 μL and a final siRNA-DOTAP concentration of 750 nM (see Note 4). Incubate the plate overnight at 37°C for 14–18 h.
5. The following morning, inspect the cells using inverted microscopy. In all conditions using DOTAP+RNA complexes, some small cell debris/dots should be visible between the cells. Collect 100 μL of supernatant and dilute 1:2 with

OPti-EA buffer if the cells are PBMCs (there is no need to dilute the RAW cell supernatants). Freeze the supernatants at -80°C and keep until cytokine analysis by ELISA.

3.1.4. Cytokine Production Analysis by ELISA

A TNF- α ELISA is performed to assess the sequence-specific recruitment of mouse/human TLR7 and human TLR8. The same procedure is used for both the human and mouse TNF- α ELISA, with the exception of step 3.

1. The day before the assay (or a few days before), coat a maxisorp 96-well plate with 100 μL of capture antibody diluted 1:500 in coating buffer, and leave sealed with tape at 4°C . The morning of the assay, rinse the plate three times with PBST and block for 1 h at room temperature with 130 μL Assay Diluent per well, with rocking.
2. Following blocking, wash the plate three times with PBST. Prepare the TNF- α standard curve following the Analysis Certificate leaflet from the kit, to give a concentration range from 1,000 to 15.6 pg/mL (7 points). Add 75–100 μL of diluted/neat supernatant to each well of the ELISA plate, and incubate for 2 h at room temperature, with rocking.
3. Wash the plate four times with PBST and prepare the diluted capture antibody.
 - (a) For human TNF- α , dilute both detection antibody and streptavidin-horseradish peroxidase (SAv-HRP) to 1:500 in Assay Diluent. Incubate for 10 min before adding 100 μL per well, and further incubate for 1 h at room temperature.
 - (b) For mouse TNF- α , first dilute the detection antibody 1:500 in Assay Diluent. Apply 100 μL per well and incubate for 1 h at room temperature, with rocking. After four PBST washes, add 100 μL of 1:500 diluted SAv-HRP and incubate for 30 min at room temperature.
4. Following five to seven PBST washes, perform the enzymatic assay. Add 100 μL of prewarmed TMB (at $25\text{--}37^{\circ}\text{C}$) per well and stop the reaction with 50 μL sulfuric acid (see Note 5). Read the absorbance in a plate reader within 30 min at 450 nm (correction using absorbance at 570 nm can be applied) (see Note 6).

3.2. RIG-I Recognition of siRNAs

Originally thought to be exclusive to blunt-end siRNAs (8), recent insights into the mechanisms of RIG-I activation have led to the conclusion that other structural features of siRNAs permit innate immune recruitment. First, it was discovered that the presence of a 5'-triphosphate motif on single-stranded RNAs was a trigger for RIG-I activation of innate immunity (10, 17).

In accordance with previous claims for a role of 5'-triphosphate from bacteriophage synthesis of siRNA duplexes in the activation of the IFN pathway (9), these reports imply that all in vitro transcribed siRNAs have the potential to recruit and activate RIG-I. A recent study by Poeck et al. made use of RIG-I activation by 5'-triphosphate siRNA to synergize with the silencing efficacy of a pro-apoptotic siRNA to provoke increased apoptosis in tumor cells (14). Second, a recent publication from Takahasi et al. demonstrated that all synthetic double-stranded RNAs (blunt or with 2 nt overhangs) as short as 25 nt could bind RIG-I and activate it, provided they possess at least a 5' or 3' monophosphate (11). With the growing number of siRNA synthesis options/scaffolds in the past 5 years, an increase in siRNAs found to activate RIG-I should be anticipated.

Whether it is to avoid or intentionally recruit RIG-I activation, siRNAs for use in animal work should be validated in a cell model responsive to RIG-I. Here, we describe a simple real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) protocol based on human T98G cells (adapted from Marques et al. (8)).

3.2.1. Treatment of T98G Cells with siRNA

This protocol relies on reverse transfection of the T98G cells – meaning that the cells are passaged and plated when the siRNAs are to be transfected. Using the volumes indicated here, each transfection mix will give three biological replicates in a 48-well plate format (see Note 7).

1. Prepare a mix of transfection reagent by adding 1.5 μL of Lipofectamine 2000 to 75 μL of Opti-MEM and incubate for 5 min at room temperature.
2. In a separate tube, dilute 1.875 μL of 4 μM siRNA in 75 μL of Opti-MEM. Slowly add the transfection mix to the diluted siRNA, gently agitate, then incubate at room temperature for 20–30 min.
3. While incubating the siRNA/Lipofectamine 2000 mix, trypsinize an 80% confluent flask of T98G cells with TrypLE-Express and neutralize with pure DMEM (no FBS, no antibiotics). Count the cells using a hemacytometer, and dilute the volume required to obtain 90,000 cells in a final 600 μL of pure DMEM.
4. When ready, add 50 μL of siRNA/Lipofectamine 2000 mix to the bottom of the wells of a 48-well plate (using three wells for the same siRNA mix). Gently swirl the plate to cover most of the well surface. Then add 200 μL of cells in pure DMEM to each well (giving 250 μL at 10 nM), avoiding agitation (to keep the cells well dispersed). Incubate the 48-well plate at 37°C, in 5% CO_2 .

5. After 4 h of incubation, aspirate the transfection mix and rinse each well in 200 μ L of fresh DMEM complete. Some cells will detach, but most cells should remain stuck at the bottom of the wells. Incubate the 48-well plate for another 16–20 h at 37°C, in 5% CO₂.
6. Following incubation, the cells should be ~50% confluent, with the majority stuck at the bottom of the wells. Discard the medium (do not wash the cells with PBS) and add 100 μ L of RA1-Bme solution directly to each well. Store the plate at –80°C for further RNA purification, or process directly.

3.2.2. RNA Extraction Using Nucleospin RNA II Columns

Purify the RNA following the manufacturer's protocol, with the following modifications.

1. Slowly thaw the samples, if frozen, at room temperature for ~20 min.
2. Use 100 μ L of 75% EtOH in combination with 100 μ L of filtered lysates before applying the samples to Nucleospin columns.
3. Extend the DNase I treatment to 30 min to minimize genomic DNA contamination.
4. Perform the elution with 40 μ L of RNase-free H₂O only. Place the samples on ice for direct processing or keep at –80°C.

3.2.3. cDNA Synthesis Using Superscript III

For each RNA sample, synthesize cDNA using the following procedure.

1. Add 1 μ L of 10 mM dNTP mix and 0.5 μ L of Oligo(dT)₂₀ to 8.5 μ L of RNA. Incubate at 65°C for 5 min, then place on ice for 2 min.
2. Add 4 μ L of 5× first strand buffer, 1 μ L of DTT, 0.5 μ L of RNase OUT, and 0.2 μ L Superscript III to each sample with RNase-free H₂O up to 20 μ L. Incubate for 50 min at 50°C and denature for 5 min at 85°C. Store the resulting 20 μ L of cDNA at –80°C until real-time PCR analysis.

3.2.4. Real-Time PCR of Human Interferon-Induced Protein 56 (P56): IFIT1

We have been using SYBR-GreenER on an IQ5 Multicolor i-cycler using GAPDH as a housekeeping gene with the primer pairs specified in Table 1. However, any other chemistry can be applied here (such as Taqman gene assay for IFIT1: Hs00356631_g1 – Applied Biosystems). Any significant induction of P56 between the sample and the control siRNA denotes an upstream activation of RIG-I (8) (see Fig. 2).

Prepare a mastermix with 10 μ L of SYBR-GreenER and 8.2 μ L of RNase-free H₂O per sample. Add 0.4 μ L of both forward and reverse primers at 5 μ M per sample. Aliquot this mix

Table 1
The sequences of DNA primers and siRNA

DNA primer sequence name (human)	5'-3' sequence
GAPDH-FWD	CATCTTCCAGGAGCGAGATCCC
GAPDH-REV	TTCACACCCATGACGAACAT
P56-FWD	TCACCAGATAGGGCTTTTGCT
P56-REV	CACCTCAAATGTGGGCTTTT
<i>RNA duplex name</i>	5'-----3' 3'-----5'
siGFP27+0 ^a	5'-AAGCUGACCCUGAAGUUCAUCUGCACC-3' 3'-UUCGACUGGGACUUCAAGUAGACGUGG-5'
siGFP27+2 ^a	5'-GCUGACCCUGAAGUUCAUCUGCACCACUU-3' 3'-UUCGACUGGGACUUCAAGUAGACGUGGUG-5'

^aThe single-stranded RNAs used to create these duplexes do not bear any 5' or 3' monophosphate groups

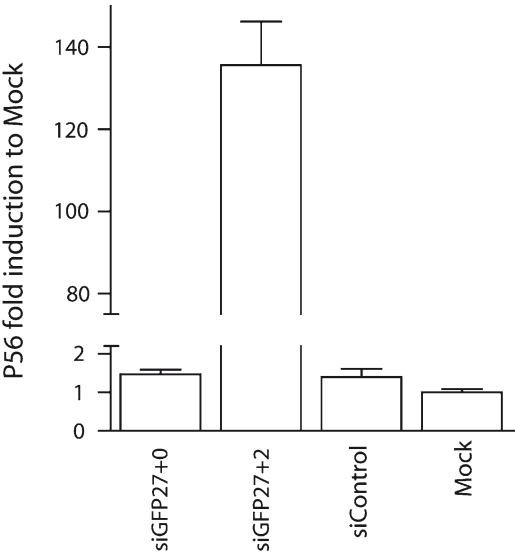


Fig. 2. siRNA-induced P56 mRNA in human T98G cells. 10 nM of each indicated siRNA was transfected into human T98G cells following the procedure presented in Subheading 3.2 and incubated for 20 h. The expression of P56 and GAPDH was measured by real-time RT-PCR using a standard curve for each target gene. P56 expression was normalized to GAPDH levels for each sample, and the values were further reported to the average P56 expression of the Mock (Lipofectamine 2000 only) condition. The data is from one experiment, which is representative of three independent experiments, in biological triplicate. The error bars represent the SEM. While the siGFP27+0 blunt siRNA is a very strong inducer of P56, its variant siGFP27+2 and the 19+2 control siRNA (siControl) appear not to affect the RIG-I pathway

into a 96-well Multiplate and add 1 μ L of cDNA. Seal the plate with optical tape, centrifuge briefly at $300\times g$ to pellet the reaction mix, and run the PCR cycle (using T_m of 58°C) – 2 min at 50°C , 10 min at 95°C , 40 cycles of: 95°C 15 s, 58°C 15 s, 60°C 45 s (Acquire). Perform the melting curve analysis cycle following the manufacturer's guidelines.

4. Notes

1. The RAW cells can be cultured on surface-treated plasticware – enhancing adherence – or in nontreated flasks/dishes. If using surface-treated plasticware, collect the cells with TrypLE-Express for 5 min at 37°C . If using RAW cells on surface-treated plastics, keep the passage number of the cells under 15 from the time they are defrosted to avoid differentiation.
2. As a guide, 18 mL of blood will provide enough PBMCs to plate 60 wells of a 96-well plate – however, there are important variations among blood donors.
3. The number of cells plated is not critical for the assay, although the more cells the higher the cytokine levels produced. The cell confluency should be greater than 50% – however, even 30–40% cell confluency will give good cytokine production.
4. Positive and negative controls should be included for each experiment. CL75 (1 $\mu\text{g}/\text{mL}$) is a strong activator of mouse TLR7 and human TLR8, and therefore conveniently suits both assays (12). CL75 TNF- α induction levels should be at the higher end of the standard curve (and possibly above the linear range of the standard curve), i.e., $\sim 1,000$ – $2,000$ pg/mL . A Mock control, with DOTAP and 11.2 μL of duplex buffer only, together with a Medium-only (50 μL of pure RPMI per well) control should be used. The concentration of TNF- α , obtained for both Mock and Medium controls, gives an idea of the baseline production of TNF- α by the cells. While it should be relatively low for the assay to be relevant, in most cases it averages between 0 and 50 ng/mL .
5. The reaction should be stopped when a blue coloration for each standard of the standard curve is visible or when the intensity of the blue coloration of the samples is more intense than the 1,000 ng/mL control of the standard curve. Importantly, if saturation is reached for some standards, they should be discarded as they will not be within the linear range of the enzymatic reaction.

6. When an siRNA sequence induction of TNF- α is significantly higher than the baseline level over a minimum of two independent experiments in duplicate, it can be concluded that it is immunostimulatory through both mouse and human TLR7 if RAW cells are used, and through hTLR8 only or both hTLR7 and hTLR8 together if human PBMCs are used (see Fig. 1). However, it should be noted that the concentration of siRNAs used here, even if very high, might not be sufficient to activate TLR7 and 8 in vitro. It is possible that a sequence that does not induce any significant TNF- α production in either assay will still promote a low immunostimulation in vivo. Ultimately, this should be assessed in vivo by measuring cytokine production in the blood of the animal treated.
7. The assay should always include one positive control (such as the blunt siRNA siGFP27+0) and one negative control (such as siGFP27+2, with 2 nt overhangs and no monophosphate), in addition to the treated samples. An alternative positive control is to use an in vitro synthesized single-stranded RNA. Of note, it is preferable to use synthetic RNAs from the same source (such as Integrated DNA Technology).

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RNA Interference

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