Video Article

Stimulation of Cytoplasmic DNA Sensing Pathways *In Vitro* and *In Vivo*

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Abstract

In order to efficiently stimulate an innate immune response, DNA must be of sufficient length and purity. We present a method where double stranded DNA (dsDNA) which has the requisite characteristics to stimulate the cytoplasmic DNA sensing pathways can be generated cheaply and with ease. By the concatemerization of short, synthetic oligonucleotides (which lack CpG motifs), dsDNA can be generated to be of sufficient length to activate the cytosolic DNA sensing pathway. This protocol involves blunt end ligation of the oligonucleotides in the presence of polyethylene glycol (PEG), which provides an environment for efficient ligation to occur. The dsDNA concatemers can be used, following purification by phenol/chloroform extraction, to simulate the innate immune response *in vitro* by standard transfection protocols. This DNA can also be used to stimulate innate immunity *in vivo* by intradermal injection into the ear pinna of a mouse, for example. By standardizing the concatemerization process and the subsequent stimulation protocols, a reliable and reproducible activation of the innate immune system can be produced.

Video Link

The video component of this article can be found at https://www.jove.com/video/51593/

Introduction

DNA is a vital component of all organisms and cells, which eukaryotes keep in specific compartments. One function of the innate immune system is to identify when such compartmentalization breaks down or when foreign material is introduced into the organism *via* a breach of physical, external barriers. In the human body there are a number of proteins that exist to help degrade small amounts of DNA which may escape the correct compartmentalization; DNAse I, II, and III break down DNA in the plasma, endosome, and cytosol, respectively. However, it has been shown that mice lacking DNAse II die from severe anemia caused by excessive production of interferons[1] suggesting that the innate immune system responds to extra-nuclear DNA. This response occurs even in mice which are entirely deficient in toll-like receptor signaling capacity. Numerous studies have now shown that stimulator of interferon genes (STING)[2] and TANK-binding kinase 1 (TBK1)[3] are involved in the detection of DNA in the cytoplasm and that this signaling pathway activates interferon regulatory factor (IRF)-3[4]. The subsequent IRF3-dependent transcription of cytokine, chemokine, and interferon genes is characteristic of an innate immune response to either a pathogen or danger associated molecular pattern (PAMP or DAMP)[5].

The desire to study intracellular nucleic acid sensing pathways has led to the requirement to develop robust and reproducible methods for stimulating cells by introducing DNA or RNA into cells without activating other innate immune responses. One method by which the STING/TBK1/IRF3 pathway can be stimulated is by using cationic lipid transfection reagents to deliver nucleic acid into the cytoplasm where it can be accessed by DNA sensors such as DNA-PK, IFI16, and cGAS[6-8].

The characteristics of DNA which are currently understood to allow efficient activation of the cytoplasmic innate immune response without stimulation of other pathways are its length, mass, purity, and lack of CpG motifs[4,7,9]. Synthetic oligonucleotides are highly suitable for the purpose of generating an innate immune response as they allow the DNA sequence to be optimized and prepared as a pure chemical species. A 45 base pair immuno-stimulatory DNA (ISD) sequence was identified by Stetson *et al.*[4] as being a suitable, CpG-free sequence for this purpose. This dsDNA sequence is otherwise random and has no specific features beyond its lack of CpG motifs. We have found that by concatemerizing the ISD oligonucleotide into significantly longer strands increases the magnitude of the stimulation[7]. Generation of concatemerized ISD is therefore useful for stimulating a cytoplasmic innate immune response. Here we present protocols for preparing this reagent and how it can be used to activate the DNA sensing pathway *in vitro* as well as *in vivo*.

NOTE: All animal studies are performed under approved institutional protocols and animal care guidelines.

Protocol

1. Concatemerization

1. Resuspend primers Fw and Rv (see Table of Materials for primer sequences) to a concentration of 10 µg/µl in molecular biology grade H_2O. Then, mix 5 µl of Fw and 5 µl of Rv primer in a 1.5 ml microcentrifuge tube.
2. Anneal primer mix by heating to the correct annealing temperature (75 °C for the primers described here) for 15 min. Leave on bench to cool down at room temperature (RT). Prepare the 60% PEG8000 during this incubation by dissolving in ddH_2O.
3. Add 50 µl of 60% PEG8000 (to achieve a final concentration of 30%), 10 µl of 10x polynucleotide kinase (PNK) buffer, 27 µl of H_2O, and 3 µl of PNK to the primer mix. Incubate at 37 °C for 2 hr.
4. Cool down at RT and add 11 µl of DNA ligase buffer. Then add 3 U of DNA ligase and incubate at 37 °C overnight.

2. DNA Purification and Analysis

1. Add 300 µl of H_2O to increase volume of the ligation mix. Note: Proceed in a fume hood due to toxicity of phenol:chloroform vapor.
2. Add 1 volume (400 µl) of phenol:chloroform and mix by shaking vigorously.
3. Centrifuge at max speed for 1 min in a tabletop microcentrifuge. The aqueous and solvent layers will separate with the aqueous layer on top and a white layer of precipitated protein between the two layers.
4. Transfer top layer to a new tube by careful pipetting (avoid transferring middle, white layer)
5. Repeat steps 2.2 and 2.3 at least twice or until there is no precipitated protein after centrifugation.
6. Add 1 volume (400 µl) of chloroform and repeat steps 2.3-2.4.
7. Add 2 volumes (800 µl) of 100% prechilled ethanol and incubate at -20 °C for at least 1 hr or overnight. NOTE: this step precipitates the DNA concatemers.
8. Spin down at max speed and aspirate supernatant. Wash once by adding 1 ml of 70% ethanol and allow to air dry in fume hood.
9. Resuspend DNA in 50 µl of endotoxin free ddH_2O. Note: The use of endotoxin free reagents is critical to generating a DNA-specific innate immune response in cells and *in vivo*.
10. Remove a 2 µl aliquot of the concatemerized DNA into a fresh tube and add 1 ml DNA gel loading buffer.
11. Dissolve 1% agarose in TAE buffer by heating on full power in a microwave oven for 2 min (or time which is appropriate to the power of the oven and volume).
12. Pour gel into casting apparatus, add 4 µl of fluorescent DNA chelating dye, insert a comb, and leave at room temp for approximately 20 min for the gel to set.
13. Once set, remove the comb and place gel into electrophoresis tank containing TAE buffer. Load 3 µl of DNA sample or size markers into wells.
14. Run gel electrophoresis for 30-40 min at 90 V with constant current. Visualize the DNA under ultra-violet light to observe concatemerization (**Figure 1A**).
15. Measure the DNA concentration in the remainder of the sample by UV absorbance spectroscopy. Blank the spectrometer with the same ddH_2O that was used to resuspend the DNA and measure the absorbance spectrum from 200-300 nm. NOTE: DNA concentration can be calculated using the Beer-Lambert law, A=e*c*l, where A is the absorbance, e the extinction coefficient, and l the distance of the light path. For measuring DNA concentrations the absorbance at 260 nm should be measured and an extinction coefficient of 0.027 $(\mu g/ml)^{-1}$ cm^{-1} used. The light path distance, l, will vary depending on the spectrophotometer. While measuring the concentration, take note of the 260/280 nm absorbance ratio as an indication of DNA purity; a value for this ratio of above 2.00 is recommended.

3. Transfection of dsDNA Concatemers *In Vitro*

1. Seed cells in a tissue culture dish or plate to be 70% confluent at time of stimulation.
2. Calculate the mass of DNA required for stimulation of cells using a final concentration of 1-10 µg/ml. The required mass of DNA will be noted as X below. For example if stimulating cells in a single well of a 6-well plate with 2 ml of medium at a concentration of DNA of 5 µg/ml then a mass of 2*5 = 10 µg of DNA is required.
3. Add 50*X µl of serum free medium into a suitable sized sterile tube. Pipette carefully into the center of the medium, without touching the side of the tube, 2*X µl of cationic lipid transfection reagent and leave for 5 min.
4. Add X µg of DNA to the tube and vortex briefly. Leave at RT for at least 20 min. For *in vitro* transfection add this mixture directly to cells to stimulate cytoplasmic DNA sensing pathways.

4. Transfection of dsDNA Concatemers *In Vivo*

1. Prepare transfection mix as described in 3.1-3.5 using 1 µg DNA, 2 µl lipid transfection reagent, and 18 µl serum free medium per mouse ear to be stimulated. Prepare a sterile 20 µl Hamilton syringe, cap with a sterile 30 G needle, and load with the DNA transfection mix.
2. Anesthetize a C57BL/6 mouse using 1-2% isoflurane. Confirm anesthesia by loss of movement and constant respiratory rate and by pinch reflex test if necessary. Monitor animal's eyes during anesthesia and use ointment as required to avoid excessive dryness.
3. Using sterile technique, inject with care into the ear pinna 10 µl of the transfection mix so that a single 'bubble' of liquid is formed in between the dermal layers of the ear. Note: Use a rubber thimble on your thumb or index finger, stretch the ear over it, and inject at an acute angle to ensure the needle penetrates only the first layer of skin of the ear pinna.
4. Reintroduce mouse back into the cage and regularly monitor post-anesthetic recovery. Use a heating lamp to avoid excessive cooling of the anesthetized mice. Do not leave mice unattended until they have regained sufficient consciousness to maintain sternal recumbency.

5. Flash freeze the whole ear pinna in a microcentrifuge tube by immersing in liquid nitrogen for 2 min.

5. Processing of DNA Stimulated Samples and Analysis by Quantitative PCR (qPCR)

1. From cell culture: aspirate medium with a pipette and discard waste.
2. Add 1 ml of sterile PBS. Repeat 5.1 and 5.2 twice and proceed to step 5.3
3. From ear tissue: grind tissue under liquid nitrogen using sterile ceramic pestle and mortar, transfer ground tissue to a microcentrifuge tube using a sterile spatula, and proceed to step 5.4
4. Add 350 µl lysis buffer and proceed with commercial RNA extraction kit protocol. Elute RNA from extraction kit purification column in 40 µl ddH_2O. Note: The commercial RNA extraction kit (see materials/equipment table) works very well; other techniques have been tested with subpar results.
5. Quantify RNA by UV spectroscopy as described for DNA above (section 2.13). Note: the extinction coefficient for RNA is 0.025 $(\mu g/ml)^{-1}$ cm^{-1}.
6. Add to a sterile PCR tube 1 µl of oligo(dT) primer, 1 ul of 10 mM dNTP mix, and 250 µg RNA. Make up the volume to 11 µl with sterile ddH_2O.
7. Incubate for 5 min at 65 °C. Then add 4 µl First Strand Buffer, 1 µl 0.1 M dithiothreitol, 0.25 µl reverse transcriptase and 1.75 µl ddH_2O to the tube. Incubate at 50 °C for 1 hr followed by 72 °C for 15 min. Optionally, dilute cDNA product 1:5 using ddH_2O.
8. For quantitative PCR use 2 µl cDNA, 2 µl ddH_2O, 5 µl qPCR master mix, and 1 µl each of 10 uM forward and reverse primer stocks to amplify gene(s) of choice. Mix in a 96-well plate and analyze using a quantitative PCR machine (**Figures 1B** and **1C**).

Representative Results

The results below indicate that concatemerized DNA can be generated with relative ease and is capable of stimulating a robust innate immune response in cells and in mice. With the use of PEG8000, it can be clearly seen that the length of DNA being generated is significantly longer and equally capable of inducing transcription of Cxcl10. As seen by agarose gel analysis, the standard concatemerization has lengths of DNA of up to 800 base pairs (bp), whereas for the sample with PEG8000, the length of DNA includes strands in excess of 10,000 bp (**Figure 1A**). Transfection of these DNAs into murine embryonic fibroblasts (MEFs) or mice induces a robust transcription of cytokines and chemokines which can be measured by qPCR indicating stimulation of the innate immune DNA sensing machinery (**Figures 1B** and **1C**).

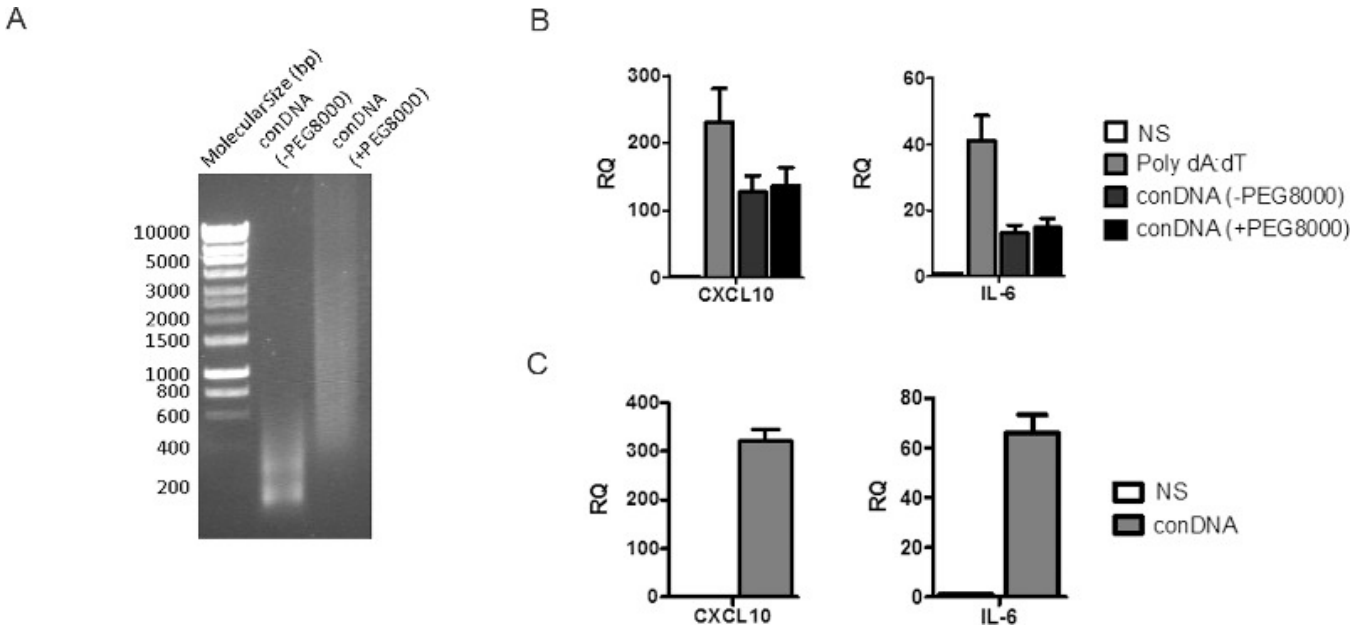

Figure 1. Representative data indicating the production of concatemerized ISD DNA (conDNA) and stimulation of fibroblasts and mice. (**A**) A sample of concatemerized DNA produced either with or without PEG8000 analyzed by agarose gel electrophoresis. (**B**) MEFs were stimulated with various DNAs at 10 µg/ml and the levels of CXCL10 and IL-6 mRNAs measured by qPCR 6 hr later. (**C**) Mice were injected in the ear pinnae with conDNA and the levels of CXCL10 and IL-6 mRNAs measured by qPCR 12 hr later. Data is shown as fold increase relative to the level of HPRT (RQ) and error bars are +/- SEM (n=3).

Discussion

The protocols described here are used to generate dsDNA to stimulate the cytosolic innate immune response with reproducible results in a wide range of cell types and *in vivo*. Since the main substrate reagent used is a synthetic oligonucleotide, there is flexibility in this system for using alternative DNA sequences or chemical modifications. It is possible, for instance, to replace the forward strand oligonucleotide described in this protocol with one that contains a fluorescent label or a biotin moiety to track localization or assess interactions with other biomolecules.

One of the main advantages of DNA concatemerization is the production of long strands of dsDNA in a manner that the sequence can be controlled. There is one limitation here which is that the DNA length cannot be precisely monitored so the product of this reaction is always a mixture of DNA of different lengths (similar to the commercial RNA analogue poly(I:C)). Another advantage of this technique is that it allows the

production of large mass quantities of immuno-stimulatory DNA (up to mg amounts). The concatemerization step can be scaled up easily as required, although it should be noted that subsequent purification steps should then be carried out in aliquots of the scale outlined in the protocol.

Compared to other methods of concatemerizing DNA, the main addition is the use of PEG8000 to the ligation mix to improve ligation efficiency. Note that PEG8000 may be difficult to dissolve to make a 60% (w/v) stock but can be vortexed and gently heated to help solubilization. The ligation can be left to incubate for longer periods than indicated, but this does not appear to affect the length of DNA being generated. Some care needs also be observed when handling phenol and chloroform as these can be carcinogenic and corrosive. In our hands the genes upregulated by other synthetic DNAs (such as commercial poly(dA:dT)) are different from those upregulated by concatemerized ISD. However, others have not found such differences[10], which may reflect variation between cell types being analyzed. Notwithstanding such conflicts, the concatemerized ISD is significantly cheaper to generate when compared to purchasing poly(dA:dT) from a commercial source and allows greater flexibility *via* choice of sequence and modifications.

One aspect of DNA sensing which is under-studied is the *in vivo* response to stimulation. We have shown that this protocol can be used to generate DNA suitable for *in vivo* experiments[7]. The dsDNA product is pure and of high enough concentration to allow injection into mice and subsequent measurement of innate immune signaling processes. In the protocol described here, care must be taken to ensure the water used to dissolve the final DNA pellet is endotoxin, RNase, and DNase free. Injection of free DNA or DNA-lipid complexes results in an interferon response as expected of DNA sensing processes and, as such, the concatemerized DNA can be used to probe multiple aspects of DNA sensing *in vivo*.

Disclosures

The authors have nothing to disclose.

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