

## Development of CpG ODN Based Vaccine Adjuvant Formulations

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### 1 Introduction

The innate immune system responds to the presence of pathogens by sensing “pathogen associated molecular patterns” (PAMPs) expressed by infectious microorganisms [1]. Pathogen-derived nucleic acids represent a critical group of PAMPs that are sensed by a plethora of nucleic acid sensing receptors expressed in immune cells [2]. This recognition initiates a robust innate immune response that enables the host to control the initial spread of infection and subsequently generate sterilizing adaptive immunity. One type of nucleic acid PAMP is the unmethylated CpG motifs present at high frequency in bacterial DNA (but rare in mammalian DNA due to CG suppression and CG methylation) [3]. Unmethylated CpG DNA is recognized by TLR9 expressed by B lymphocytes, dendritic cells (DC), and macrophages. Synthetic oligodeoxynucleotide (ODN) containing unmethylated CpG motifs duplicate the ability of bacterial DNA to stimulate the innate immune system via TLR9 [4].

The immune stimulatory effects of CpG ODNs variegate on the basis of their subcellular distribution, backbone modification, length, and formation of secondary and tertiary structures [5]. Based on their differential activation of immune cells, four major classes of synthetic CpG ODNs have been defined: (a) A or D-type CpG, (b) B or K-type CpG, (c) C-type CpG, and (d) P-type CpG ODNs (Reviewed in ref. 6). In general, K class ODNs are potent B cell activators and induce TNF- $\alpha$  secretion from plasmacytoid dendritic cells (pDC) but not interferon- $\alpha$ . In contrast, D-, C-, and P-class ODNs are capable of stimulating variable amounts of IFN $\alpha$  secretion from pDCs. Of the latter three ODN classes, D ODNs are the most potent IFN $\alpha$  inducers but have the drawback

of forming multimers, and random concatamers complicating their clinical grade manufacturing process. In fact, to date, only three clinical trials tested the vaccine adjuvant and/or immunotherapeutic activity of D class CpG ODN [7–9]. All three studies harnessed a stabilized version of this ODN class following packaging into virus like particles consisting of the bacteriophage Q $\beta$  coat protein.

In this chapter, we describe two alternative methods of preparing CpG ODN-based vaccine adjuvant formulations that can induce a robust IFN $\alpha$  response from human peripheral blood mononuclear cells. Method 1 details a protocol to stabilize D-type CpG ODN in cationic liposomes. Labile bioagents are protected following liposome encapsulation [10]. This mild approach relies on the dehydration–rehydration technique, does not involve detergents or organic solvents and the encapsulation yield is much higher than most other widely accepted liposome generation methods [11–13].

Method 2 describes a simple strategy of anionic bioactive agent stabilization following complexation with cationic peptides [14–16]. Peptide-mediated multimerization of a K-type ODN devoid of IFN $\alpha$  stimulating activity into stable nuclease-resistant nanostructures (i.e., nanorings) with type I interferon inducing activity is only achieved through the use of a short and non-flexible ODN (K23) and the HIV-derived peptide Tat<sub>(47–57)</sub> at a specific ODN–peptide molar ratio (1:16).

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## 2 Materials

### 2.1 CpG ODN Sequences (Alpha DNA, Canada: Bases Shown in Capital Letters Are Phosphorothioate; Lower Case Letters Indicate Phosphodiester Backbone)

D35 (D-type ODN used in Method 1): GGtgcatcgatgcaggggGG  
 D35 flip (Control D-ODN with no immunostimulatory activity): GGtgcatcgatgcaggggGG  
 K23 (K-type ODN used in Method 2): TCGAGCGTTCTC  
 K23 flip (Control K-ODN with no immunostimulatory activity): TCGAGGCTTCTC

### 2.2 Lipids Used in Liposome Preparation (Avanti Polar Lipids, Alabaster, AL)

Dimethylaminoethanecarbamol-cholesterol (DC-Chol), dioleoyl phosphatidylethanolamine (DOPE), and polyethylene glycol2000-phosphatidylethanolamine (PEG-PE).

### 2.3 Cationic Peptides (AnaSpec Inc., USA)

LL-37: LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES  
 HIV-Tat<sub>(47–57)</sub>: YGRKKRRQRRR

#### **2.4 CpG ODN Loaded Liposome Preparation (Method 1)**

1. 50 ml round-bottom flask (Pyrex, vacuum resistant).
2. Rotary evaporator with a water bath attachment (Heidolph, Laborota, Germany, or any brand).
3. Argon cylinder tank (without O<sub>2</sub>).
4. Cup Horn Vibra Cell Sonicator (Sonics and Materials, Danbury, CT, USA, or any brand).
5. Freeze-drier (Heto-Holten, Maxi-Dry Lyo, Denmark, or any brand).
6. LiposoFast extruder equipped with polycarbonate filters (Avestin, Ottawa, Canada).
7. Sterile glass vials (5 ml).

#### **2.5 Demonstration of Complexation between CpG ODN and Cationic Peptides Using Agarose Gel Electrophoresis (Method 2)**

1. Agarose, loading dye, O'GeneRuler 100 bp DNA Ladder (Thermo Scientific, USA), and nucleic acid stain suitable for gel electrophoresis.
2. Agarose gel electrophoresis: for 150 ml of 1.0 % agarose gel, use 1.5 g of ultrapure agarose (electrophoresis grade) with 150 ml of 1× TAE. Prepare 1 l of 10× TAE stock solution in ultrapure water with 48.4 g of Tris base, 3.72 g disodium EDTA, and adjust to pH 8.5 with glacial acetic acid. Include ethidium bromide (1 mg/ml) before pouring the gel.
3. Gel documentation system.

#### **2.6 Assessment of Vaccine Adjuvant Formulations for Their IFN $\alpha$ Triggering Activities Using Human Peripheral Blood Mononuclear Cells (hPBMC)**

1. Ficoll-Paque PLUS density gradient medium (GE Healthcare Biosciences, Sweden).
2. Centrifuge with swing bucket clinical rotor.
3. 96-well tissue culture plates.
4. RPMI-1640 cell culture medium containing 10 % FBS, 50  $\mu$ g/ml penicillin/streptomycin, 10 mM HEPES, 0.11 mg/ml Na pyruvate, 2 mM L-glutamine, 1× nonessential amino acids (from a 100× stock solution), and 0.05 mM 2-mercaptoethanol.

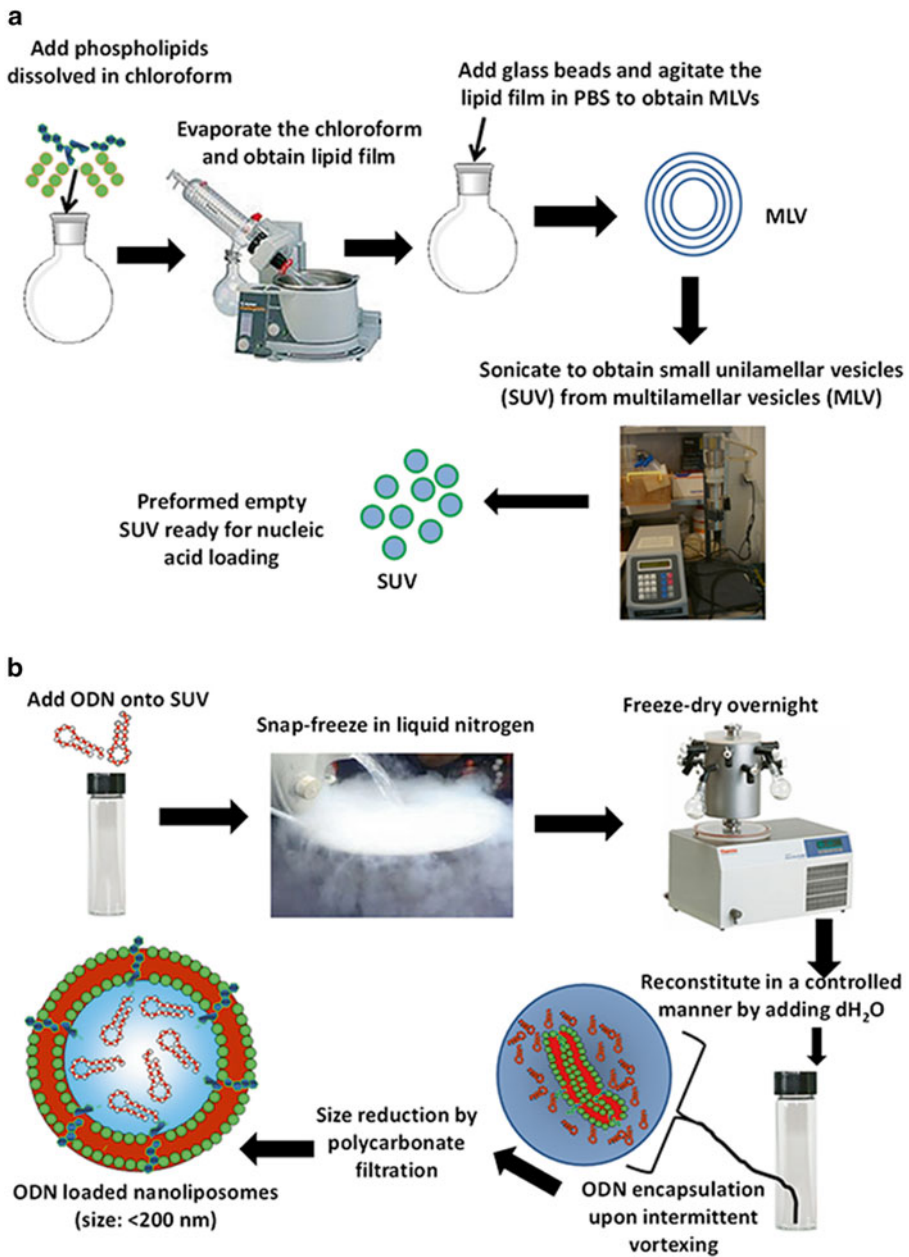
#### **2.7 Cytokine Measurement from Culture Supernatants**

1. Immulon 2B plates (Thermo LabSystems, USA).
2. Human IFN- $\alpha$ 2 ELISA development kit (ALP) from Mabtech, Sweden.
3. ELISA blocking buffer: Phosphate buffered saline (PBS; 10 mM phosphate buffer, pH 7.4, 150 mM NaCl) containing 5 % bovine serum albumin and 0.025 % Tween 20.
4. ELISA washing buffer: PBS containing 0.05 % Tween 20.
5. Detection antibody diluent: PBS containing 5 % FBS and 0.025 % Tween 20.
6. SIGMAFAST p-Nitrophenyl phosphate (p-Npp) substrate tablets.
7. 96-well multi-plate reader equipped with a 405 nm filter.

### 3 Methods

#### 3.1 Preparation of Cationic Liposome Stabilized D-Type CpG ODN (See Scheme 1a, b)

1. Prepare lipid stocks in chloroform (10 mg/ml) and store under argon gas at  $-20^{\circ}\text{C}$  until use.
2. For the preparation of 20  $\mu\text{mol}$  cationic stealth liposomes, pipette lipids from corresponding lipid stocks at a 4:6:0.06 molar ratio (DC-Chol-DOPE-PEG-PE) into a 50 ml round bottom flask.
3. Complete the volume to 2.0 ml by adding chloroform and connect the flask to a rotary evaporator.
4. Set the evaporator rotation speed to maximum (the temperature of the water bath should be set to  $37^{\circ}\text{C}$ ).
5. Evaporate the chloroform in the round bottom flask for 20 min.
6. Solvent-free thin lipid film should appear in the inner wall of the round bottom flask at the end of this process.
7. Remove the flask from the rotary evaporator and purge with argon for 30–60 s. Make sure all residual chloroform is removed from the flask and argon purging will remove residual oxygen remained in the flask (*see Note 1*).
8. Seal the round bottom flask with a glass cap and continue the following steps under laminar hood. Transfer 30–40 sterile glass beads (300  $\mu\text{m}$  average size, from Sigma) into flask.
9. Add 1.0 ml sterile phosphate-buffered saline (PBS) onto beads, and shake the solution in a circular motion until lipid film disappears from the flask wall. This motion helps the lipid film to be removed by the abrasive force of the glass beads and leads to the generation of empty, large multilamellar liposomes.
10. Collect the resulting milky solution from the flask and transfer into a glass vial.
11. In order to generate small unilamellar vesicles (SUVs), sonicate the liposome solution five cycles (30 s/cycle) with an amplitude of 70 % and a second set of five cycles with an amplitude of 50 % on ice. Keep the vial on ice for 15 s in between sonication cycles to prevent excessive heating.
12. For a 20  $\mu\text{mol}$  SUV liposome solution (1.0 ml in PBS) add 1 mg CpG ODN solution (1 mg/ml ODN solution) and mix gently by vortexing. Total volume is 2.0 ml at this stage.
13. Remove the vial cap and seal the vial mouth with a Parafilm. Using a syringe needle, punch 6–8 holes on the Parafilm. This will let air out during the lyophilization step.
14. Immediately freeze the liposome/ODN solution in liquid nitrogen for 1 min.



**Scheme 1** Preparation of cationic liposome stabilized D-type CpG ODN. (a) Method for the preparation of preformed, unloaded small unilamellar vesicles. (b) Method to generate CpG ODN loaded liposomes

15. Place the frozen liposome/ODN mixture in a freeze-dryer and lyophilize overnight (*see Note 2*).
16. Remove the vial from the lyophilizer. At this stage there should be a white powder in the vial.
17. Add 1:10 volume of ddH<sub>2</sub>O (200  $\mu$ l ddH<sub>2</sub>O) on to the liposome powder and vortex vigorously for 15 s.
18. Continue vortexing for 15 s every 5 min for the total duration of 30 min. This will allow the ODN to dissolve in ddH<sub>2</sub>O and diffuse into the liposome bilayer while liposomes are swelling in the aqueous environment.
19. Add 200  $\mu$ l PBS on to the liposome solution, gently vortex, and set aside for 10 min.
20. Complete the volume to 1.0 ml by adding 600  $\mu$ l PBS. This generates the CpG ODN loaded liposome stock.
21. To reduce the size of the loaded liposomes, assemble the LiposoFast extruder, and gently transfer the liposome solution into the glass syringe provided with the extruder. Filter ten times through the 1.0  $\mu$ m cut-off polycarbonate filter. Replace the filter with the 500 nm polycarbonate filter and filter 10 more times. Finally, replace 500 nm filter with the 200 nm filter and repeat 10 more filtrations.
22. Transfer the extruded nanoliposomes encapsulating the CpG ODN into a sterile vial.

### 3.1.1 Determination of ODN Encapsulation Efficiency

1. Remove 50  $\mu$ l of the liposome aliquot into a microcentrifuge tube.
2. Centrifuge for 1 h at 16,100 $\times g$  in an Eppendorf centrifuge.
3. Gently collect the clear supernatant into a clean microcentrifuge tube.
4. Determine the non-encapsulated ODN concentration in the supernatant by recording the OD at 260 nm using NanoDrop<sup>®</sup> ND-100 (NanoDrop Technologies, USA).
5. Determine ODN encapsulation efficiency indirectly by subtracting the amount of non-encapsulated ODN from the original input amount and then divide it to the original input ODN amount that was initially mixed with empty SUVs before freeze-drying. Multiply by 100 (*see Note 3*).

### 3.2 Preparation and Testing of K-Type CpG ODN/Cationic Peptide Complexes (Method 2)

#### 3.2.1 Preparation of CpG ODN/Cationic Peptide Complexes

1. Prepare stock solutions of CpG ODNs (K23 and K23 flip) in DNase-free ddH<sub>2</sub>O (final concentration of 1 mg/ml).
2. Prepare stock solutions of cationic peptides in ddH<sub>2</sub>O (final concentration of 5 mg/ml).
3. Mix the ODNs and peptides at different molar ratios (1:1, 1:2, 1:4, 1:8, 1:16) as detailed in Table 1 (*see Note 4*).
4. Incubate complexes for 30 min at RT and proceed to confirmation of complexation with agarose gel electrophoresis.

**Table 1**  
**Concentrations of K-type CpG ODN and cationic peptides required to form complexes of various molar ratios**

Samples	Molar ratio	K23 ( $\mu\text{M}$ )	Peptide ( $\mu\text{M}$ )	K23 ( $\mu\text{g}$ )	Peptide ( $\mu\text{g}$ )	K23 ( $\mu\text{l}$ ) stock 1 $\lambda$	Peptide ( $\mu\text{l}$ ) stock 5 $\lambda$	H <sub>2</sub> O ( $\mu\text{l}$ )
K23	–	80	–	19.2	–	19.2	–	40.8
K23/LL37	1:1	80	80	19.2	21.54	19.2	4.2	36.6
K23/LL37	1:2	80	160	19.2	43.14	19.2	8.4	32.4
K23/LL37	1:4	80	320	19.2	86.16	19.2	16.8	24
K23/LL37	1:8	80	640	19.2	172.2	19.2	34.2	6.6
K23/Tat	1:2	80	160	19.2	15	19.2	3	37.8
K23/Tat	1:4	80	320	19.2	30	19.2	6	34.8
K23/Tat	1:8	80	640	19.2	60	19.2	12	28.8
K23/Tat	1:16	80	1280	19.2	120	19.2	24	16.8

**3.2.2 Demonstration of Complexation Using Agarose Gel Electrophoresis**

1. To confirm that CpG ODN formed complexes with the cationic peptides, mix 20  $\mu\text{l}$  of each complex (concentration based on ODN amount) with 4  $\mu\text{l}$  of 6 $\times$  loading dye and load the wells of a 1 % agarose gel containing 1 mg/ml ethidium bromide with the samples.
2. Apply uncomplexed CpG ODN (1.6  $\mu\text{g}$ ) to one well as the negative control.
3. Apply the 100–1000 bp range DNA ladder as a marker (3  $\mu\text{g}$ /well).
4. Carry out agarose gel electrophoresis using 1 $\times$  TAE buffer at 70 V for 60 min.
5. Visualize the gels under a UV transilluminator (*see Note 5*).

**3.2.3 Testing of IFN $\alpha$ -Inducing Activity of Vaccine Adjuvant Formulations Using hPBMC**

1. Collect blood samples (10 ml) from healthy donors into anti-coagulant containing (sodium citrate, EDTA, or heparin) tubes (Note that blood collection from healthy donors requires ethical approval).
2. Dilute to 20 ml with 1 $\times$  PBS.
3. Pipette 10 ml of Ficoll-Paque PLUS density gradient medium into a 50 ml conical tube and carefully layer the diluted blood on top of the gradient medium without disturbing the layers.
4. Centrifuge samples at 400 $\times g$  for 30 min with the break off at room temperature.
5. Using a sterile pipette collect the cloudy PBMC layer that resides at the interphase of the uppermost plasma and the clear density gradient medium and transfer to a new tube.

6. Wash the cells two times using 50 ml RPMI medium and centrifugation at  $400 \times g$  for 10 min.
7. Resuspend the resultant cell pellet in 1 ml of RPMI, count the cells using a hemocytometer and adjust the working cell concentration to  $4 \times 10^6$  cells/ml.
8. For testing of the CpG ODN/cationic peptide complexes, stimulate cells in a 96-well tissue culture plate (400,000 cells/well) in a total volume of 200  $\mu$ l using three different doses (0.3, 1, and 3  $\mu$ M) of uncomplexed or complexed CpG ODNs and their flip controls for 24 h at 37 °C and 5 % CO<sub>2</sub> (*see Note 6*).
9. Collect culture supernatants at the end of this incubation period.

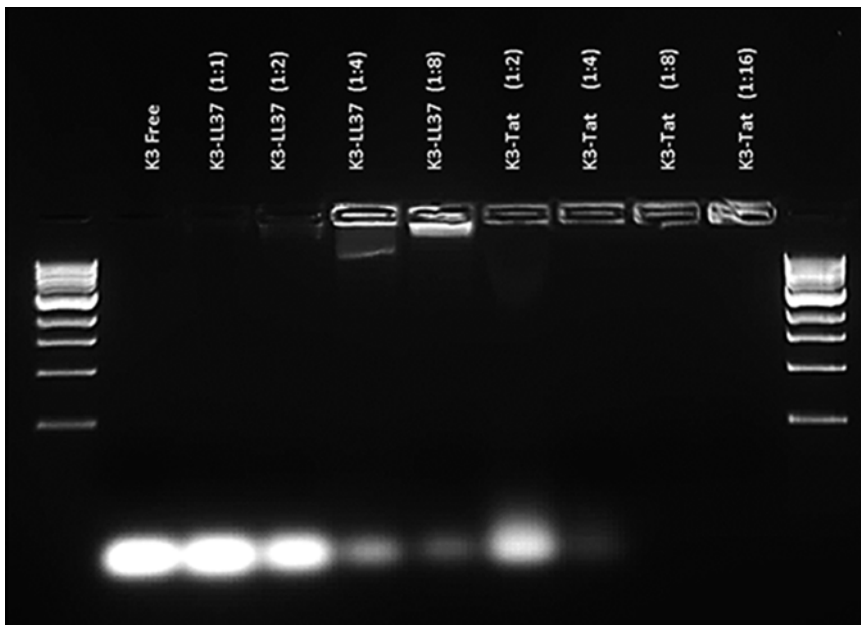
### 3.2.4 Cytokine ELISA

1. Coat a 96-well Immunol II plate using 50  $\mu$ l of anti-human coating antibody in PBS (5  $\mu$ g/ml).
2. Tap the plates to ensure uniform spreading and incubate at RT for 4 h or at 4 °C overnight.
3. Remove the coating solution by inverting the plates, add blocking buffer (200  $\mu$ l) and incubate at RT for 2 h.
4. Decant the blocker, wash plates with ELISA wash buffer five times (immerse plates into a container filled with wash buffer to fill all wells and incubate for 5 min before decanting).
5. Rinse plates with ddH<sub>2</sub>O and dry wells by tapping over an absorbent tissue paper.
6. Distribute 50  $\mu$ l of supernatants and the provided cytokine standard in triplicate (250 ng/ml highest concentration; serially diluted twofold in PBS to make up a standard curve of 12 different concentrations) and incubate for 2–3 h at room temperature or overnight at 4 °C.
7. Wash plates as described above (**steps 4 and 5**).
8. Add 50  $\mu$ l of 1:1000 diluted (dilution in detection antibody diluent) biotinylated-secondary antibody solution into wells and incubate 2–3 h at room temperature or overnight at 4 °C.
9. Wash plates as described above (**steps 4 and 5**).
10. Distribute 50  $\mu$ l of 1:5000 diluted (dilution in detection antibody diluent) streptavidin-alkaline phosphatase solution to each well (*see Note 7*) and incubate 1 h at RT.
11. Wash plates as described above (**steps 4 and 5**).
12. To develop the plates, dissolve a p-Npp At in 4 ml ddH<sub>2</sub>O and 1 ml p-Npp buffer and transfer 50  $\mu$ l of this solution to each well.
13. Follow color development at 405 nm over time using a 96-well multiplate reader until recombinant cytokine standards reach a four-parameter saturation and yield an S-shaped curve. Determine cytokine concentration of each sample using the standard curve (*see Note 8*).



## 4 Notes

1. This step is critical. Argon purging eliminates both residual chloroform and also replaces the oxygen present in the flask. O<sub>2</sub> gas facilitates lipid peroxidation, so it is vital to remove all oxygen in the flask via argon purging.
2. At this stage, there is no encapsulation of ODN within the liposome. The encapsulation will be achieved during the dehydration–rehydration step).
3. Expected encapsulation efficiency for the D ODN should be at least 80 % or higher. The activity of as such prepared liposomes can be tested as described in Subheading 3.2.2 prior to mixing with an antigen of choice for vaccination experiments.
4. Preparation of complexes in salt containing buffers compromises complexation efficiency. Final volume of the solution in which complexes are formed should not exceed 60  $\mu$ l. Table 1 details the optimal volumes and concentrations of reagents to be used for the most efficient complexation.
5. Expected results are demonstrated in Fig. 1.



**Fig. 1** A constant amount of K-ODN (80  $\mu$ M) was incubated with increasing amounts of cationic peptides for 30 min at room temperature in a final volume of 60  $\mu$ l ddH<sub>2</sub>O. CpG ODN or its complexes (1.6  $\mu$ g/well) were subjected to agarose gel electrophoresis. Uncomplexed CpG ODN demonstrates a bright signal at the bottom of the gel whereas this signal disappears following successful complexation. DNA ladder with 100–1000 bp range was used as a marker (3  $\mu$ g/well)

6. For example, for the 3  $\mu\text{M}$  final ODN concentration, mix 48.6  $\mu\text{l}$  of formed complex with 275.4  $\mu\text{l}$  RPMI medium and add 50  $\mu\text{l}$  of this onto 150  $\mu\text{l}$  cells.
7. The streptavidin-alkaline phosphatase solution must be prepared at least 2 h prior to its use to ensure uniform color development.
8. We found that K23:Tat (1:16; 1  $\mu\text{M}$ ) triggered an interferon-alpha response that was equivalent to levels obtained with 3  $\mu\text{M}$  D ODN stimulation. LL-37-incorporating aggregates elicited a substantially lower response.

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