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Innate Immune Activation by cGMP-AMP Nanoparticles Leads to Potent and Long-Acting Antiretroviral Response against HIV-1

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HIV-1 evades immune detection by the cGAS-STING cytosolic DNA-sensing pathway during acute infection. STING is a critical mediator of type I IFN production, and STING agonists such as cGMP-AMP (cGAMP) and other cyclic dinucleotides elicit potent immune response and antitumor response. In this article, we show that administration of cGAMP, delivered by a ultra–pH-sensitive nanoparticle (NP; PC7A), in human PBMCs induces potent and long-acting antiretroviral response against several laboratory-adapted and clinical HIV-1 isolates. cGAMP-PC7A NP requires endocytosis for intracellular delivery and immune signaling activation. cGAMP-PC7A NP-induced protection is mediated through type I IFN signaling and requires monocytes in PBMCs. cGAMP-PC7A NPs also inhibit HIV-1 replication in HIV* patient PBMCs after ex vivo reactivation. Because pattern recognition receptor agonists continue to show more clinical benefits than the traditional IFN therapy, our data present important evidence for potentially developing cGAMP or other STING agonists as a new class of immune-stimulating long-acting antiretroviral agents.

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Abbreviations used in this article: ART, antiretroviral therapy; cGAMP, cGMP-AMP; Dn, hydrodynamic diameter; DLS, dynamic light scattering; IFN-I, type I IFN; IRF3, IFN regulatory factor 3; ISG, IFN-stimulated gene; NP, nanoparticle; PRR, pattern recognition receptor; qRT-PCR, quantitative RT-PCR; RTase, reverse transcriptase; THF, tetrahydrofuran; UPS NP, ultra–pH-sensitive nanoparticle.

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self-assembly of amphiphilic block copolymers (12). These NPs can serve as carriers for therapeutic cargos and achieve cytosolic delivery of protein Ags for the activation of tumor-specific T cells (13). In this study, we evaluated the use of multiple UPS NP compositions to enhance cytosolic delivery of cGAMP into cells, and discovered that cGAMP-PC7A NP elicits potent and long-acting inhibition of multiple HIV-1 isolates in human PBMCs. We also elucidated the mechanism of cGAMP-PC7A NP-mediated immune protection and demonstrated its efficacy in PBMCs isolated from HIV+ individuals.

Methods and Materials

Cells and viruses

PBMCs from anonymous healthy donors were isolated from blood purchased from Carter Bloodcare, a local blood bank, usually on the same day of blood draw. Peripheral immune cells were isolated using Ficoll Plaque Centrifugation at 1700 rpm for 30 min, and lysis of contaminating RBCs was removed by resuspension of PBMCs in ACK lysing buffer (Life Technologies) for 15 min on ice. PBMCs were maintained without stimulation in RPMI 1640 medium supplemented with 10% (v/v) FBS and 2 mM t-glutamine (Sigma-Aldrich), 10 mM HEPES (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich), 100 U/ml penicillin (Sigma-Aldrich), and 100 mg/ml streptomycin (Sigma-Aldrich) at 37 °C and 5% CO2. Consenting HIV+ patients were recruited according to Institutional Review Board–approved protocols, and their PBMCs were similarly isolated and cultured at the Department of Pathology. Human studies are approved by the University of Texas Southwestern Medical Center Institutional Review Board.

Cell viability was determined using Fixable Red Dead Cell stain kit (Life Technologies) using 0.5 μl of dye to stain cells in 1 ml of PBS for 5 min in the dark. These were then washed twice with PBS and measured on FACSCalibur. As a positive control for dead cells, PBMCs were boiled at 95 °C for 20 min and also stained with the same procedure to determine the peak of dead cells.

CD4+ T cells, B cells, and monocytes were positively selected using Miltenyi Biotec human microbeads against CD4+ (130-045-101), CD19+ (130-050-301), and CD14+ (130-050-201) according to the manufacturer’s recommendations. The labeled cells typically had purity >90%. For depletion experiments, the same isolation protocol was used, but now the flow-through PBMCs were taken; we regularly achieved >95% depletion for monocyte and B cells.

To analyze HIV receptor and coreceptor expression, we isolated CD4+ T cells and CD14+ monocytes by positive selection using microbeads (Miltenyi). These cells were then treated with 2 μg/ml cGAMP-PC7A for 2 d. Before treatment, the cells were collected, fixed, and stained with CCR5, CD4, CXCR4 Abs. Flow cytometry was used to determine the mean fluorescence intensity compared with PC7A alone treatment.

HIV strains (Bal, IIIB) were propagated in U937 cell lines, concentrated using Amicon Ultra-15 centrifugal filter units (Millipore), and titrated by quantitative PCR–based product-enhanced reverse transcription assay described later. The HIV LAI strain was obtained from NIH AIDS reagent program (catalog no. 2522), amplified in PBMCs, and concentrated using LentIX concentrator (Clontech) according to the manufacturer’s recommendation.

HIV-1 tier quantitative PCR–based product-enhanced reverse transcriptase assay

As was previously described (14, 15), this assay uses reverse transcriptase (RTase) activity in the sample to power a quantitative RT-PCR (qRT-PCR), with the quantity of accumulated product serving as a proxy for viral concentration. Viral particles in the supernatant are lysed by mixing 1:1 with lysis buffer for 10 min at room temperature. The reaction is quenched by adding nuclease-free water (nine times lysate volume) to lysate. This diluted lysate (1 μl) is added to a MicroAmp fast 96-well reaction plate (Applied Biosystems) along with 1:10 diluted Roche MS2 RNA (1 μl), 10 μM Sigma MS2 Fwd (1 μl) and Rev primers (1 μl), 1:10 diluted Invitrogen RnaseOUT (1 μl), and Bio-Rad Sybr green (10 μl) for each sample. All dilutions were done in nuclease-free water (Ambion). These samples are then vortexed and spun down, before running on a AB 7500 Fast Real Time PCR System using the following program: 1) 42 °C for 20 min; 2) 95 °C for 5 min; 3) 95 °C for 30 s; 4) 60 °C for 30 s; and 5) repeat from step 3, 39 times. To normalize measurements across different plates, we included aliquots from one HIV-1 stock in all plates, and its measurement was used to divide the values in the experimental conditions.

Syntheses of PEG-b-PR block copolymers

PEG-b-PR copolymers were synthesized by atom transfer radical polymerization following similar procedures previously reported (16). The dye-free copolymers were used in polymer characterizations. PEG-b-PC7A is used as an example to illustrate the procedure. First, C7A-MA (1.48 g, 7 mmol), PMDETA (21 μl, 0.1 mmol), and MeO-PEG2OeBr (0.5 g, 0.1 mmol) were charged into a polymerization tube. Then a mixture of 2-propanol (2 ml) and DMF (2 ml) was added to dissolve the monomer and initiator. After three cycles of freeze-pump-thaw to remove the oxygen, we added CuBr (14 mg, 0.1 mmol) into the polymerization tube under nitrogen atmosphere, and the tube was sealed in vacuo. The polymerization was carried out at 40 °C for 10 h. After polymerization, the reaction mixture was diluted with 10 ml of tetrahydrofuran (THF) and passed through a neutral Al2O3 column to remove the catalyst. The THF solvent was removed by rotovap. The residue was dialyzed in distilled water and lyophilized to obtain a white powder. After synthesis, the polymers were characterized by 1H nuclear magnetic resonance and gel permeation chromatography.

Preparation of micelle NPs

cGAMP-loaded micelles were prepared following a solvent evaporation method. In the example of PEG-b-PC7A, both the copolymer (10 mg) and cGAMP (1 mg) were first dissolved in 1 ml of methanol and then added into 4 ml of distilled water dropwise under sonication. The mixture was filtered four times to remove THF using the microultrafiltration system (molecular mass = 100 kDa). The filtrate was collected and cGAMP was quantified by UV-Vis spectrometer at 258 nm. After micelle formation, the NPs were characterized by dynamic light scattering (DLS), Malvern MicroV model; He-Ne laser, λ = 632 nm) for hydrodynamic diameter (Dh). The control unloaded NPs were prepared with same protocol as described earlier, except the addition of cGAMP into the methanol. All NPs were directly added into the culture medium for delivery into cells.

To evaluate the loading stability, we stored the micelle NPs in 4 °C for 1 week. The size and cGAMP encapsulation efficiency were measured by DLS and ultrafiltration method, respectively.

To study PC7A NP uptake mechanism, we labeled PC7A NP with Cy5 dye (PC7A-Cy5). PBMCs from healthy donors were pretreated with endocytosis or endosomolytic inhibitors for 1 h and then treated with PC7A-Cy5 for 4 h. The cells were then collected, washed extensively, and fixed. Activation of Cy5 fluorescence signals within cells was quantified by flow cytometry as an indication of NP dissociation. Similarly, PBMCs from healthy donors were pretreated with endocytosis or endosomolytic inhibitors for 1 h and then treated with 2 μg/ml cGAMP-PC7A for 6 h. The cells were then collected in TRIzol; RNA was isolated for qRT-PCR for induction of immune response genes.

qRT-PCR, Milliplex, and Western blots

Cytokine mRNA was extracted from cells using TRI Reagent (Sigma-Aldrich), and cDNA was generated using iSCRIPT master mix (Bio-Rad). Expression of the cytokines was quantified by real-time quantitative PCR (qRT-PCR) using specific primers (Bio-Rad) and normalized relative to Gapdh mRNA. HIV patient cytokine mRNA profiles were determined using Prime PCR custom plates (Bio-Rad) using their recommended primers against our genes of interest.

Proteins of select cytokines in the culture supernatant were collected and measured using Luminex xMAP technology and a human Milliplex kit (Millipore).

For Western blots, primary Abs were used to detect TNF-α (Cell Signaling), rabbit anti-TNF-α (Cell Signaling), rabbit anti-IL-6 (Cell Signaling), rabbit anti-phospho TBK (Ser172) clone D52c2 (Cell Signaling), rabbit anti-HMGB2 (Abcam), and mouse anti-tubulin (Sigma-Aldrich) according to blotting procedures previously described (17). All sample loadings were normalized to total protein measured by the BCA assay.

Statistical analysis

Statistical significance was determined using a two-tailed Student t test analysis available on GraphPad Prism 6. The p values <0.05 were considered significant.

Results

Formulation and characterization of cGAMP UPS NPs

Cytosolic delivery of cGAMP is challenging due to its membrane-impermeable property as a result of dual negative charges. In this study, we used the UPS NP with exquisite pH response to the endosomolytic environment to deliver cGAMP into cells (Fig. 1A,
Table I. UPS-PC7A NPs allowed for >2-fold higher loading of cGAMP over the other UPS copolymers with linear side chains (Table II), potentially because of the more favorable hydrophobic interactions (i.e., guanidine and adenine bases with cyclic alkyl groups on the tertiary amines) and internal salt-bridge (i.e., cGAMP phosphates to ammonium groups in the UPS copolymer). The resulting cGAMP-NPs were stable during storage (no cGAMP release over 7 d) (Table II). At pH 7.4, UPS NPs were present as self-assembled micelles with a diameter of 30–70 nm and a spherical morphology (Fig. 1B, 1C). Micelle dissociates into unimers at pH values less than pKa, resulting in efficient cGAMP release.

cGAMP-PC7A NP elicits potent antiretroviral response against clinical HIV-1 isolates in human PBMCs

To determine whether activation of STING signaling by cGAMP-NPs could induce protective antiviral response against HIV-1 infection, we performed the HIV-1 spreading assay using human PBMCs isolated from healthy donors. We first infected PBMCs with HIV-BaL, HIV-IIIB, or HIV-LAI (Fig. 2A). The antiretroviral effect of cGAMP-NPs was observed in a dose-dependent manner (Fig. 2B).

**FIGURE 1.** cGAMP-PC7A NPs elicit potent antiretroviral response against HIV-1 in human PBMCs. (A) Structures of the UPS copolymers. (B) Size changes of cGAMP-loaded micelle NPs in buffers at pH greater than or less than pKa (n = 3). (C) Transmission electron micrograph images of cGAMP-loaded PEG-b-PC7A NPs at pH 7.4 and 6.7. Scale bars, 100 nm. (D) A schematic diagram of HIV-1 spreading assay in PBMCs. (E) HIV-BaL replication in PBMCs treated with indicated reagents (right). All NPs were used at 1 µg/ml. (F) HIV-BaL replication in PBMCs treated with increasing amount of cGAMP-NP (PC7A, same below). Two representative donors are shown. (G) HIV-BaL replication in PBMCs treated with the indicated reagents (x-axis). cGAMP was used at 1 µg/ml. cGAMP-NP was used at 1 µg/ml, and equivalent amount of NP alone was used as control. Each data point represents an individual donor. (H and I) HIV-IIIB (H) or HIV-LAI (I) replication in PBMCs treated with Nev or cGAMP-NP. Assay performed as illustrated in (D). Data are representative of at least three independent experiments with independent donors. Error bars represent SEM. ***p < 0.005, Student t test. Nev, nevirapine (4 µM, same throughout).
with a replication-competent HIV-BaL strain in bulk for 5–7 d. Then, infected PBMCs were washed (to remove free viruses) and split into several conditions where we added uninfected naive PBMCs from the same donor (“reseeding”) to allow HIV spreading (Fig. 1D). Different treatments were administered at the point of reseeding (day 0). We then followed the growth of HIV-1 by measuring HIV-1 RTase activity in the media (see Materials and Methods). HIV-BaL titer increased steadily in PBMCs after reseeding for approximately 2 wk before starting to decline (Fig. 1E). As a positive control, RTase inhibitor nevirapine completely inhibited HIV-BaL spreading in PBMCs. When we compared cGAMP-NPs releasing at different pHs ranging from 4.38 to 6.97, only cGAMP-PC7A NP (transition pH 6.97) inhibited HIV-BaL spreading (Fig. 1E), but not with other copolymers with less endosomolytic ability for cytosolic delivery of encapsulated cargos (16). Neither PC7A NP alone nor naked cGAMP inhibited HIV-BaL replication. We also found that cGAMP-PC7A NP (we will call cGAMP-NP later) induced protection against HIV-BaL in a dose-dependent manner in PBMCs isolated from multiple donors (Fig. 1F, 1G). cGAMP-NP did not induce appreciable cytoxicity in PBMCs at the dose needed for full protection (Supplemental Fig. 1A). We also tried to deliver cGAMP-complexed with lipid NPs (Lipofectamine 2000), and observed similar dose-dependent protection against HIV-BaL in PBMCs without cytoxicity (Supplemental Fig. 1B, 1C), although a much higher amount of cGAMP-Lipo is needed to achieve a similar dose-dependent protection against HIV-BaL in PBMCs, and that cGAMP-mediated antiretroviral response can achieve similar potency as RTase inhibitors in vitro.

cGAMP-NP–induced protective immune response against HIV-1 mediated through IFN-I signaling

We next compared cGAMP-NP with TLR3 agonist poly(I:C), TLR7/8 agonist R848, and TLR9 agonist oligodeoxynucleotides containing CpG motifs in the HIV-1 spreading assay. Many of these TLR agonists induce potent inflammatory responses, which we confirmed in PBMCs by qRT-PCR (data not shown). We found that none of the TLR agonists elicited detectable protection against HIV-BaL replication in PBMCs (Fig. 2A). To determine whether cGAMP-induced protection is mediated through soluble factors, we next compared cGAMP-NP with extracellularly versus intracellularly delivered poly(I:C) (complexed with Lipofectamine), activates the cytosolic RIG-like receptor pathway in the “conditioned media” experiment (Fig. 2B). We first treated PBMCs with cGAMP-NP, poly(I:C) alone, or poly(I:C)-Lipo, and washed cells extensively 2 d later to remove excess agonists that were not taken up by cells. We then incubated stimulated PBMCs in fresh media for 3 d to collect secreted soluble factors. We added the conditioned media together with HIV-BaL to fresh PBMCs to examine whether they can confer protection against HIV-BaL replication. Conditioned media from cGAMP-NP–treated cells strongly inhibited HIV-BaL replication, suggesting that soluble factors are largely responsible for cGAMP-NP-mediated protection (Fig. 2C). Media from poly(I:C)-treated cells did not confer

Table II. Characterization of cGAMP-loaded USP NPs in water

<table>
<thead>
<tr>
<th>NPs</th>
<th>Dₐ (nm)ᵃ</th>
<th>EE%ᵇ</th>
<th>Dₐ (nm)</th>
<th>EE%ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>cGAMP-PC7A NP</td>
<td>34.5 ± 5.0</td>
<td>69.7</td>
<td>32.8 ± 5.5</td>
<td>69.5</td>
</tr>
<tr>
<td>cGAMP-PEPA NP</td>
<td>31.8 ± 3.4</td>
<td>33.4</td>
<td>32.7 ± 3.3</td>
<td>33.4</td>
</tr>
<tr>
<td>cGAMP-PDBA NP</td>
<td>42.3 ± 5.3</td>
<td>26.3</td>
<td>43.7 ± 6.9</td>
<td>26.2</td>
</tr>
<tr>
<td>cGAMP-PD5A NP</td>
<td>63.3 ± 2.4</td>
<td>38.5</td>
<td>64.6 ± 4.1</td>
<td>38.5</td>
</tr>
</tbody>
</table>

ᵃSize was measured using DLS, mean ± SD.
ᵇEncapsulation efficiency (EE) was measured by the ultrafiltration method.
any protection, and media from poly(I:C)-Lipo–treated cells conferred only partial protection. We also analyzed cytokines secreted by PBMCs treated with poly(I:C), poly(I:C)-Lipo, cGAMP-NP, or nevirapine (Fig. 2D). As expected, poly(I:C) induced a predominant inflammatory response and poly(I:C)-Lipo induced a predominant IFN response. In contrast, cGAMP-NP induced strong response in both IFN and inflammatory cytokines. We next investigated the role of IFN-I signaling in cGAMP-mediated protection against HIV-BaL. HIV-1 spreading assay was performed as in Fig. 1D. Indicated reagents were added at day 0. Data are representative of at least three independent experiments with independent donors.

cGAMP-induced protective immune response in human PBMCs is dependent on monocytes

We next tried to determine the immune cell type in PBMCs that is responsible for cGAMP-induced protection against HIV-1 infection. We first characterized the signaling kinetics activated by cGAMP in positively selected immune cell populations. cGAMP induced robust IFN-β expression in CD19+ B cells and CD14+ monocytes during the first 12 h followed by gradual decline, likely because of cell-intrinsic mechanisms to prevent excessive IFN activation (Fig. 3A). In contrast, CD4+ T cells presented a much slower kinetics of IFN-β expression after cGAMP stimulation, with IFN-β mRNA becoming detectable after 6 h and continued to increase up to 24 h. All immune cell types express similar levels of endogenous STING, TBK1, and IRF3 proteins analyzed by Western blot (normalized to total protein) (Fig. 3B). HIV-1 replicates in CD4+ T cells in PBMCs. We next depleted B cells, monocytes, or NK cells with magnetic beads from whole PBMCs and evaluated HIV-1 replication with and without cGAMP-Lipo or cGAMP-NP stimulation (Fig. 3C, 3D, Supplemental Fig. 3A–D). We found that cGAMP-mediated protection was completely lost in monocyte-depleted PBMCs, regardless of Lipo or NP delivery, whereas B cell or NK cell depletion had little or no effect (Fig. 3C, 3D). We confirmed the depletion by FACS (Supplemental Fig. 3D). We also analyzed STING signaling activation in PBMCs or monocytes by immunoblots (Supplemental Fig. 3E). The kinetics of TBK1 phosphorylation in both cell cultures are similar, consistent with monocytes being the primary mediators of cGAMP-induced protection against HIV-1.

We next compared cGAMP with another STING against c-di-GMP (both delivered by Lipo) at stimulating immune gene expression in PBMCs and a monocyte cell line THP-1 cells. cGAMP binds to STING at a much higher affinity compared with c-di-GMP (19). We found that cGAMP also stimulated more robust immune response compared with c-di-GMP in PBMCs and THP-1 cells (Fig. 4). Collectively, these data suggest that monocytes are the key immune cell population in PBMCs that are mediating cGAMP-induced protection against HIV-1.

cGAMP-NP elicits long-acting antiretroviral response against HIV-1 replication and spreading

One of the challenges facing current ART is patient adherence. Most conventional antiretroviral regimens require daily dosing of a
combination of antiretroviral drugs, and lapses in treatment often lead to rapid rebound of viral load. Thus, long-acting ARTs represent a major need for HIV/AIDS. Several candidates based on slow-release formulation of current and new ARTs are in advance development (20). To determine whether cGAMP-NP can elicit long-acting inhibition of HIV-1 replication and spreading by activating sustained immune responses, we performed the extended HIV-1 spreading assay interrupted by a "media wash" that simulates treatment interruption and clearance of the initial antiretroviral drug (Fig. 5A). The first stage of the assay is identical to the HIV-1 spreading assay described earlier, where HIV-BaL−infected PBMCs were cocultured with naive PBMCs in the presence or absence of various treatments. After 7 d, we washed the cells and changed fresh media (to remove drugs and viruses), at which time we also added more naive PBMCs and followed HIV-1 spreading for three more weeks. We found that both nevirapine and cGAMP-NP inhibited HIV-1 replication before treatment interruption (media wash; Fig. 5B). After treatment interruption, viral load in both untreated and nevirapine-treated samples increased quickly, rising to the level similar to the untreated condition before treatment interruption (Fig. 5B). This rapid viral load rebound after ART treatment interruption closely resembles the clinical observation of HIV−infected patients experiencing a lapse in treatment. Remarkably, cGAMP-NP−treated samples remained inhibited with low viral load up to 3 wk after media wash in PBMCs isolated from multiple healthy donors (Fig. 5B, 5C). We also measured cytokines produced during the course of “long-acting” experiments. HIV−1 infection with and without nevirapine treatment in PBMCs produced several inflammatory cytokines (e.g., IL-6, IL-8, G-CSF, TNF-α), but not IFNs (IFN-α2, IFN-γ) in the media (Supplemental Fig. 4A). In contrast, HIV−1−infected PBMCs treated with cGAMP-NP produced IFN and inflammatory cytokines. IFN-α2 and MIP-1α (an ISG) are also continuously being produced at high levels in cGAMP-NP−treated cells more than 2 wk after the single treatment (Supplemental Fig. 4B). We also found that coadministration of B18R abolished cGAMP-NP−mediated long-acting protection (Fig. 5D) and no evidence of excessive cytotoxicity during the entire course of cGAMP treatment in multiple donors (data not shown). These data suggest that cGAMP induces a durable protective immune response in PBMCs against HIV−1 infection and replication that is largely dependent on IFN-I.

cGAMP-NP inhibits HIV-1 replication in HIV patient PBMCs ex vivo

We next evaluated cGAMP-NP in HIV patient PBMCs ex vivo. We recruited HIV+ individuals who have detectable viral load (RNA copy > 50/ml) and CD4 T cells (count > 100/ml), treatment-naïve or treatment-experienced, but have not been on any antiretroviral medication for a minimum of 30 consecutive days before specimen collection. We first measured basal immune gene expression in PBMCs isolated from four HIV+ patients and three healthy controls and how they respond to cGAMP-NP stimulation. All healthy control and HIV+ patient PBMCs express low levels of IFN genes (e.g., IFN-α4, IFN-β1), ISGs (e.g., CXCL10, IFIT1, OASL, etc.), and inflammatory genes (e.g., TNF-β, IL-6, IL-8) (data not shown). cGAMP-NP induced comparable immune gene expression profiles in healthy control and HIV patient PBMCs (Fig. 5A).

We expect a substantial amount of HIV patient PBMCs to be latently infected, and would produce HIV-1 after PHA/IL-2 activation. We performed two assays (Fig. 6B, 6C). In the first assay, we waited 7 d after PHA/IL-2 activation to allow sufficient HIV production before treating cells with cGAMP-NP or antiretrovirals. In the second assay, we treated cells with cGAMP-NP or antiretrovirals immediately after PHA/IL-2 activation. We then
measured HIV replication after 1–2 wk. Antiretroviral drugs reduced HIV-1 replication overall in both assays, but not complete elimination, indicating that a substantial amount of latently infected cells in HIV patient PBMCs are producing HIV after reactivation. cGAMP-NP inhibited HIV replication at a similar efficacy as antiretrovirals when PBMCs were actively producing viruses, and cGAMP-NP potently inhibited HIV replication when cGAMP-NPs were administered immediately after reactivation (Fig. 6B, 6C). Together, given the distinct antiretroviral mechanism and long-acting benefit through stimulating the innate immune response, cGAMP-NP could be a desirable candidate as a new category of PRR-based long-acting ART.

Discussion
Recent advances in understanding HIV-1 immune evasion mechanisms highlighted the importance of the cytosolic DNA-sensing cGAS-STING pathway in host immune response to HIV-1 infection. The same signaling pathway can be triggered by a small-molecule cGAMP that directly activates STING. We showed in this study that cGAMP-NP induced potent antiretroviral response against clinical HIV-1 isolates in PBMCs. Intracellular delivery of cGAMP is critical for inducing the protective immune response, as demonstrated by the lack of protection conferred by naked cGAMP. Among the NPs we tested, PCTA NP conferred the best antiretroviral activity over other NP compositions or lipofectamine. In...
addition to the optimal pH transition (6.97) that targets the early endosomal pH for cGAMP release and the cyclic amine structure that facilitates the membrane disruption for cytosolic release (16), the direct binding of PC7A to the cytosolic domain of STING may also contribute to STING activation in addition to cGAMP. Indeed, recent studies in cGAS−/− knockout animals showed the cGAS-independent STING activation by PC7A NP alone in mice (13), although with a lesser magnitude compared with cGAMP. We did not observe antiretroviral activity in human PBMCs by PC7A NP alone. The combined cytosolic delivery of cGAMP and cGAS-independent STING activation by PC7A could confer a potential synergistic activation of STING to achieve maximal protection against HIV-1 infection.

Our study represents the first example, to our knowledge, of evaluating the potential of cGAMP as an ART for HIV-1. Because STING is a critical mediator of IFN production, STING agonists such as cGAMP and other cyclic dinucleotides are being developed as vaccine adjuvant to elicit potent immune response. STING signaling also elicits a strong antitumor response by boosting host immune recognition of tumor Ags (7, 10, 21, 22). We showed that cGAMP-NP induces potent antiretroviral response in human PBMCs, and the effect is long-acting. Several oral and injectable long-acting agents based on reformulation of ARTs are in advanced development with exciting potentials as pre-exposure prophylaxis or treatment (20, 23). The mechanism of cGAMP-NP–mediated antiretroviral activity is different from traditional ARTs or long-acting ART formulations, because cGAMP-NP acts by inducing a broad and sustained antiretroviral innate immune response. We defined that cGAMP-NP–mediated antiretroviral immune response in PBMCs is mediated by monocytes and IFN-I. Although we did not examine plasmacytoid dendritic cells in our depletion experiments, plasmacytoid dendritic cells are main producers of IFN-Is, and thus are likely to also contribute to the overall antiretroviral response elicited by cGAMP-NP. We also did not observe antiretroviral response from TLR agonists in the PBMC HIV-BaL spreading assay. One major difference between TLRs and STING signaling is that TLR expression is restricted to DCs and macrophages, whereas STING is ubiquitously expressed in almost all cell types. TLR- and STING-mediated signaling profiles are also different, which could contribute to their differential antiretroviral capacity. Interestingly, TLR agonist enhances latent HIV reactivation in vitro (24, 25), whereas we found cGAMP-NP inhibits HIV replication and possibly reactivation in PBMCs isolated from HIV+ individuals.

Because PRR-based agonists are becoming more favorable therapeutics than IFN with less toxicity and better immune response profile, our study presents an important proof of principle for harnessing cGAMP-mediated innate immune response for HIV-1 therapy. A recent study using aptamer-mediated siRNA knockdown of TREX1 in humanized mice, which activates the cGAS-cGAMP-STING–mediated IFN response, demonstrated robust protection against HIV-1 challenge in vivo (26). Published evidence from vaccine and cancer studies also showed that cGAMP can induce potent B cell– and T cell–mediated adaptive immune response in mice (7, 10). Further experiments are needed to examine the efficacy of cGAMP-NP in protection against HIV-1 infection in humanized mice models.

We envision cGAMP-NP as a novel class of immune-stimulating ART that can be used for treating HIV as a long-acting agent. As more STING agonists and formulations enter clinical trials for antitumor therapy, we expect many of the concerns of adverse effects to be addressed, as well as more exciting opportunities for parallel development of these reagents for HIV therapy. Using cGAMP-NP or other STING agonists as adjuvants for HIV vaccines is another exciting possibility for future investigation.

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Disclosures
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References


