 DOI: 10.1002/eji.201445133 Eur. J. Immunol. 2015. 45: 1170–1179

European Journal of Immunology

Enhanced immunostimulatory activity of cyclic dinucleotides on mouse cells when complexed with a cell-penetrating peptide or combined with CpG

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Recognition of pathogen-derived nucleic acids by immune cells is critical for the activation of protective innate immune responses. Bacterial cyclic dinucleotides (CDNs) are small nucleic acids that are directly recognized by the cytosolic DNA sensor STING (stimulator of IFN genes), initiating a response characterized by proinflammatory cytokine and type I IFN production. Strategies to improve the immune stimulatory activities of CDNs can further their potential for clinical development. Here, we demonstrate that a simple complex of cylic-di-GMP with a cell-penetrating peptide enhances both cellular delivery and biological activity of the cyclic-di-GMP in murine splenocytes. Furthermore, our findings establish that activation of the TLR-dependent and TLR-independent DNA recognition pathways through combined use of CpG oligonucleotide (ODN) and CDN results in synergistic activity, augmenting cytokine production (IFN-α/β, IL-6, TNF-α, IP-10), costimulatory molecule upregulation (MHC class II, CD86), and antigen-specific humoral and cellular immunity. Results presented herein indicate that 3′3′-cGAMP, a recently identified bacterial CDN, is a superior stimulator of IFN genes ligand than cyclic-di-GMP in human PBMCs. Collectively, these findings suggest that the immune-stimulatory properties of CDNs can be augmented through peptide complexation or synergistic use with CpG oligonucleotide and may be of interest for the development of CDN-based immunotherapeutic agents.

Keywords: Arginine peptide (nona-arginine) · cGAMP · CpG ODN · Cyclic-di-GMP · Immunostimulation

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Introduction

Detection of pathogen-derived nucleic acids by innate immune cells is critical for the initiation of protective responses against bacterial, viral, and fungal pathogens. Pathogen-derived nucleic acids are sensed based on their sequence, structure, nucleotide modifications, and their intracellular localization. So far, several nucleic acid sensors have been identified, including the endosomal TLRs TLR3, TLR7/TLR8, and TLR9 that recognize dsRNA, ssRNA, and CpG-containing DNA; cytosolic dsRNA sensors RIG-I, MDA5, LGP2; and a plethora of cytosolic receptors dedicated for dsDNA recognition (DAI, AIM2, RNA polymerase III, IFI16, DEAD-box helicase DDX41) [1]. Signaling in response to cytosolic DNA depends on the expression of the adaptor protein STING (stimulator of IFN genes, also known as TMEM173, MPYS, MITA and ERIS) and proceeds through the TBK1–IRF3 axis,

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culminating in type I IFN production [2, 3]. STING itself is not a DNA-recognition molecule. However, it can directly recognize cyclic dinucleotides (CDNs) such as the bacteria-derived molecule cyclic-di-GMP (c-di-GMP) [4]. Although CDNs were thought to function as universal secondary signaling molecules only in bacteria, recent discoveries suggest that a new type of CDN called cyclic AMP-GMP (cGAMP) plays an important role in mammalian cells [5]. Specifically, cytosolic DNA has been shown to trigger the synthesis cGAMP from ATP and GTP by an enzyme called cGAMP synthetase (cGAS), leading to STING–TBK1–IRF3 dependent induction of IFN response [6, 7].

C-di-GMP has interesting immune-stimulatory properties [8] and numerous studies explored its in vivo immunotherapeutic potential [9, 10]. In one such study, pretreatment of mice with c-di-GMP resulted in a striking protective effect against subsequent systemic or mucosal bacterial challenge with *Staphylococcus aureus*, confirming the stand-alone immune-protective potential of this molecule [11].

Myeloid dendritic cells (DCs) respond to c-di-GMP stimulation by upregulating costimulatory molecule expression and secreting proinflammatory cytokines TNF-α, IL-12, IFN-γ, IP-10, and IL-8 [8]. c-di-GMP was also shown to trigger IL-6 and IFN-β secretion in various cell types including mouse peritoneal exudate cells, RAW 264.7 (Mouse leukemic monocyte macrophage cell line), and myeloid DCs [8, 12]. Similar cytokine secretion profiles were also observed in hPBMCs and mouse splenocytes [12]. Vaccine adjuvant studies conducted with c-di-GMP demonstrated favorable antigen-specific IgG responses comparable to those seen with potent vaccine adjuvants (such as LPS and CpG oligonucleotide (ODN)). The elicited response was reportedly skewed toward IgG2a, indicating a TH1-dominated response [8, 13]. C-di-GMP was also tested as a mucosal vaccine adjuvant for the inactivated influenza H5N1virosomal vaccine and was shown to induce local and systemic H5N1-specific humoral and cellular immune responses in mice [14].

Despite the favorable immune-stimulatory properties of c-di-GMP, the molecule contains two negatively charged phosphate groups that restrict its passage through the plasma membrane. Therefore, to benefit from its favorable immunostimulatory properties the CDN has to be used at relatively high concentrations or must be transfected to the cytosol [12, 15]. Here, we explored the possibility of using two different strategies that would improve the intracellular delivery and/or boost the immunostimulatory activity of c-di-GMP. Specifically, we show that complexation with the cationic peptide nona-arginine improves cellular uptake and enhances the vaccine adjuvant activity of the STING ligand. Moreover, we demonstrate that c-di-GMP shows synergistic immunostimulatory activity when used in combination with the TLR9 ligand, CpG oligodeoxynucleotide (CpG ODN). Our results further demonstrate that c-di-GMP is less effective in human cells than in mouse but the response can be greatly improved by replacing this CDN with another bacteria-derived second messenger, 3′3′-cGAMP. These findings may be of interest for the development of CDN-based vaccine adjuvants.

Results

Complexing c-di-GMP to arginine(9) peptide improves delivery to murine cells and immunostimulation

The physicochemical properties of native c-di-GMP prevent its free passage across cellular membranes [12]. Since the target receptor STING resides on the ER membrane, the ligand (c-di-GMP) has to be transfected into the cytosol in order to trigger an observable response at low doses [15]. To bypass the need of transfection, we evaluated the feasibility of a simple complexation strategy that would enhance the cellular entry of the molecule. To this end, we chose various cationic peptides/polymers, Arg(9), Lys(9), and polyEthyleneImine (pEI) and $Tat_{(47–57)}$, as complexation agents since these molecules are well-known membrane transduction agents. For example, Arg(9) (or nona-arginine) is a positively charged small peptide, with known cell-penetrating properties [16, 17]. Lys(9) and pEI are also positively charged and can act as transfection reagents for their associated cargo [18, 19]. To evaluate whether these molecules could form complexes with the anionic c-di-GMP, fluorescently labeled form of the cyclic di-nucleotide (2′-Fluo-AHC-c-di-GMP; abbreviated as FAM-c-di-GMP) was mixed with different molar ratios (1:1, 1:2) of Arg(9), Lys(9), or pEI and complexation was followed on an agarose gel. Assessment of complex formation was done based on band shifts of the samples relative to the reference sample (FAM-c-di-GMP alone). Results showed that of the three cationic molecules tested, only Arg(9) caused a significant band shift, indicative of complex formation (Fig. 1A, upper gel image).

C-di-GMP is capable of forming bimolecular, tetramolecular, and octamolecular structures in the presence of monovalent cations, such as Na^+ and K^+ [20]. To assess whether c-di-GMP/Arg(9) complexation may be further enhanced in the presence of such ions, c-di-GMP was mixed with arginine at molar ratios of 1:1, 1:2, and 1:4 either in the presence or absence of KCl. Furthermore, to investigate the contribution of the arginine residues during complexation, another arginine-rich cationic cell-penetrating peptide $Tat_{(47–57)}$ was tested. Tat peptide is an HIV Tat protein-derived 11-mer peptide with a net charge of +8 and has the following sequence: YGRKKRRQRRR [21]. Being rich in arginine residues, it was included in the study to see whether it would reproduce the same effect observed with nona-arginine. The gel image of one representative study is shown in Figure 1A (lower gel image). Compared to Arg(9), $Tat_{(47–57)}$ formed inefficient complexes even when used at the highest ratio of 1:4. On the other hand, K^+ ions had no effect on the complexation since comparable amounts of band shifts took place with or without K^+ (Fig. 1A).

Next, cellular internalization properties of the Arg(9) complexes and the free ligand were tested in mouse splenocytes using the FAM-labeled CDN. Antigen-presenting cells (APCs) were stained and gated based on their MHC class II expression, and percentage of APCs internalizing the CDN was determined prior to and following Trypan blue quenching in order to enable discrimination between the surface bound and internalized and

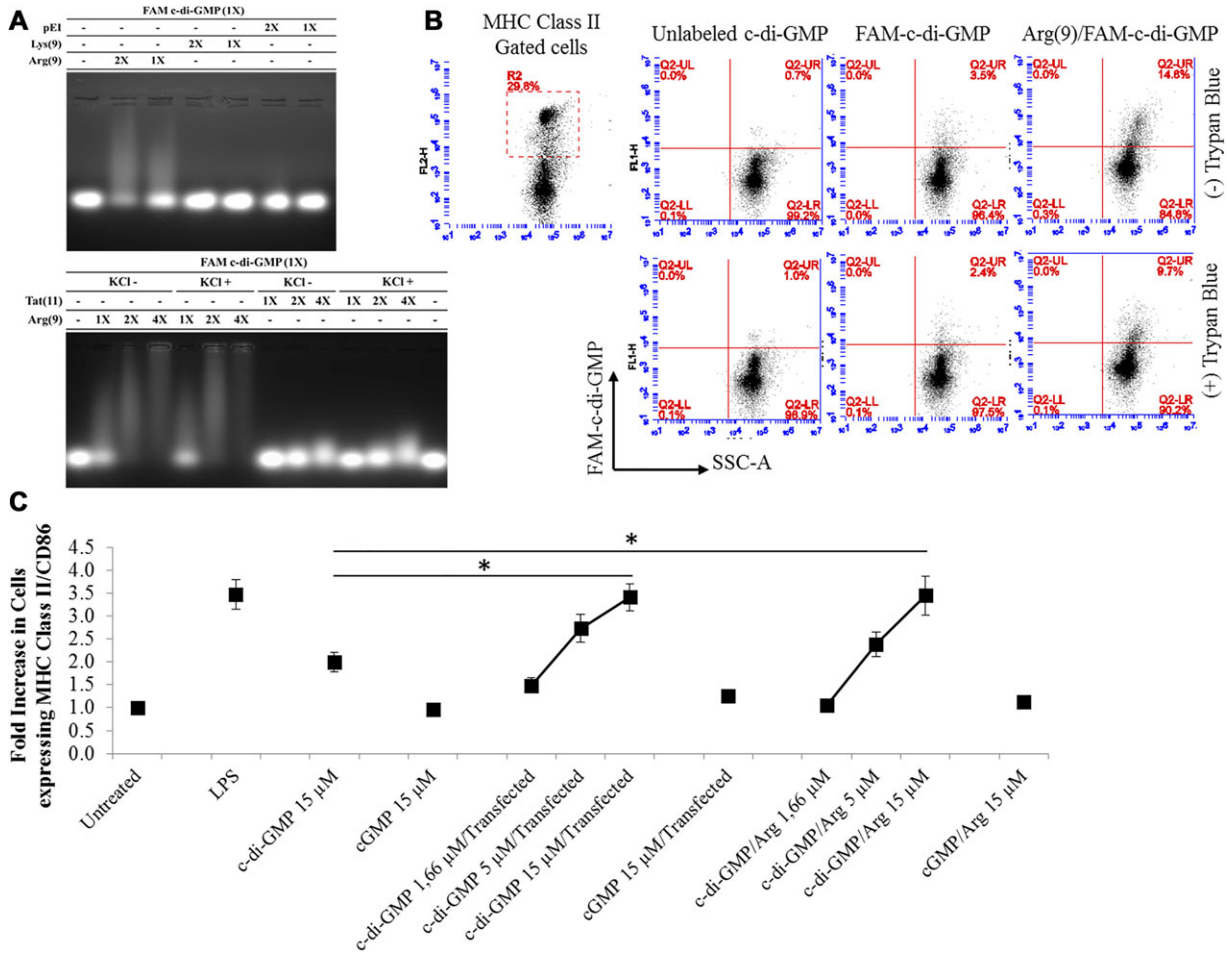

Figure 1. Intercalation properties, cellular internalization patterns, and immunostimulatory activities of c-di-GMP and its arginine(9) peptide complexes. (**A**) FAM-labeled c-di-GMP (0.67 µg) was mixed with increasing molar ratios of intercalating agents (1:1, 1:2, and/or 1:4) in the absence or presence of KCl (15 mM). The resulting c-di-GMP/cationic peptide or polymer complexes were loaded onto 2% agarose gels and resolved at 70 V for 30 min. The gel images are representative of three independent experiments, each giving similar results. (**B**) Free FAM-c-di-GMP (15 µm) or an equivalent amount of its complex with Arg(9) was incubated with splenocytes (1×10^6 cells/mL) at 37°C for 2 h. MHC class II positive mouse splenocytes were gated as indicated and cells positive for cell-surface bound+internalized FAM-labeled CDNs (no Trypan blue, upper panels) or internalized only CDNs (+Trypan blue, lower panels) were analyzed by flow cytometry. Data are representative of two independent experiments. (**C**) Indicated doses of CDNs (free or transfected with Lipofectamine 2000) were cultured with mouse splenocytes (2×10^6 cells/mL) for 24 h. Cells were stained for MHC class II and CD86 expression and analyzed by flow cytometry. Stimulation indexes were calculated according to fold increase in percentage of double-positive cells over unstimulated control. Results are shown as mean ± SD of five individual mice. Data shown are representative of two independent experiments. Statistical comparisons performed with two-tailed Student's *t* test. $^*p < 0.05$.

the internalized signal only. Analysis of surface binding plus internalization (no Trypan blue quenching) revealed that Arg(9) complexation improved cellular association when compared to the free ligand (Fig. 1B, 14.6 vs. 3.5%, respectively). Similarly, percentage of cells that internalized (with Trypan blue quenching) Arg(9)/c-di-GMP was fourfold more when compared to c-di-GMP alone (Fig. 1B, 2.4 vs. 9.7%, respectively), suggesting that the interaction with Arg(9) improved the cellular delivery of the ligand. The amount of FAM-labeled CDNs associated with $CD11b^+$, $B220^+$, or $CD11c^+$ cells was also quantified (based on the mean fluorescence intensity (MFI) of CDNs in each population), revealing all three populations to be the targets of the free and Arg(9)-associated CDNs (Supporting Information Fig. 1). The amounts of Arg(9)/c-di-GMP associated with $CD11b^+$, $B220^+$, and CD11c+ cells were found to be 7.5-, 10-, and 4.2-fold more when compared to the signal from free c-di-GMP in these populations (Supporting Information Fig. 1), suggesting that complexation enhanced the cellular delivery of the CDN to all APCs.

In order to assess whether Arg(9) complexation could improve the immunostimulatory activity of c-di-GMP, mouse splenocytes were stimulated with various doses of Arg(9)/c-di-GMP and the ability to induce APC maturation (as evidenced by an increase in MHC class II/CD86 double-positive cells) was compared to samples stimulated with the free ligand or those that were transfected with Lipofectamine 2000. cGMP, which is not a ligand of STING but structurally similar to c-di-GMP, was also included as a negative control. Results presented in Figure 1C and Supporting Information Figure 2A show that c-di-GMP/Arg(9)

complexes (highest dose) significantly increased the percentage of MHC Class II/CD86 double-positive cells relative to the same concentration of free c-di-GMP ($p < 0.05$). Of note, a 3× lower dose (5 μM) of c-di-GMP/Arg(9) showed equivalent stimulatory activity observed with 15 μM dose of the free drug (Fig. 1C and Supporting Information Fig. 2A). The immunostimulatory activity of c-di-GMP/Arg(9) was comparable to Lipofectamine 2000 transfected groups, suggesting that the active drug was efficiently transferred to the cytosol (Fig. 1C and Supporting Information Fig. 2B). Of note, the majority of cells that upregulated MHC Class II and CD86 were $B220^+$ (Supporting Information Fig. 2C).

The negative controls (cGMP, lipofectamine 2000 transfected cGMP, and cGMP/Arg(9)) did not change the expression levels of the cell-surface markers, indicating that the response was specific to the CDN and not to the complexation agents. Similarly, Arg(9)-associated c-di-GMP stimulated significantly higher levels of IL-12 and TNF-α production from mouse splenocytes when compared to the free drug ($p < 0.05$, Supporting Information Fig. 3). Neither the free ligand nor its Arg(9) complexes induced detectable IL-6 secretion (Supporting Information Fig. 3). Of note, complexes prepared using a 1:4 molar ratio did not differ from those prepared at 1:2 ratio in terms of immune activation (data not shown) and were not cytotoxic (1% cell death) whereas transfection of c-di-GMP with Lipofectamine 2000 resulted in 10% cell death (Supporting Information Fig. 4). Collectively, these findings show that complexation with Arg(9) peptide increases the cytosolic availability and the immunostimulatory activity of c-di-GMP and may replace the need to transfect the drug through the use of cytotoxic lipid-based transfection reagents.

C-di-GMP synergizes with CpG ODN to upregulate costimulatory markers and proinflammatory cytokines

Emerging evidence suggests that to induce an effective immune response, use of multiple PRR ligands can result in synergistic effects that modulate the innate and the adaptive immunity [22]. Based on this information, we explored the possibility of synergism between c-di-GMP and CpG ODN mediated immune activation. To evaluate this, mouse splenocytes were treated with increasing concentrations of free c-di-GMP plus a fixed concentration of CpG ODN (optimum dose of 1 μM) and 24 h later, percentage of MHC class II/CD86-expressing cells were compared to those stimulated with single ligands. Flow cytometric analyses showed that CpG ODN alone induced 5.5% of MHC class II/CD86 double-positives as opposed to 2.1% observed with the unstimulated control (Fig. 2A, upper panels). Cells stimulated with three different doses of c-di-GMP alone showed a dose-dependent increase in the number of double-positive cells (Fig. 2A; middle panels; 3.1, 5.4, and 7.3%). C-di-GMP synergized with CpG-ODN to induce significantly elevated levels of double-positive cells (Fig. 2A; lower panels; 5.3, 9.3, 19.6%). The combination of 15 μM c-di-GMP and 1 μM CpG ODN improved marker upregulation approximately ninefold and ~3.5-fold when compared to unstimulated or CpG ODN stimulated samples, respectively. Consistently, IL-6 and TNF-α secretion was drastically increased with combined use of c-di-GMP and CpG ODN (Fig. 2B, $p < 0.05$), whereas IL-12 levels remained unchanged. Synergism for TNF-α production was evident in the MHC class II/CD11c double-positive DC population, whereas CpG ODN stimulated IL-12-producing cell percentage was not affected by the addition of c-di-GMP (Supporting Information Fig. 5). These results suggest that combined use of STING and TLR9 agonists may augment the magnitude of innate immune responses except for IL-12 production. CpG-ODN induced IL-12 production was previously shown to be independent of MAPK, PI3K, and IRF signaling pathways [23]. It is conceivable that STING-dependent TBK1–IRF3 activation would therefore be ineffective in augmenting the magnitude of CpG ODN induced IL-12 production.

In vivo vaccine adjuvant activities of Arg(9)/c-di-GMP, and combined c-di-GMP+CpG

To test the in vivo immunostimulatory activities of Arg(9)/c-di-GMP and the CDN/CpG combination, C57BL/6 mice were immunized intraperitoneally with ovalbumin (OVA), or OVA plus the adjuvants on days 0 and 14, and OVA-specific antibody responses were determined 2 weeks after the booster injection. To enable assessment of a possible in vivo synergistic activity in the CDN/CpG adjuvanted group, a low dose of CpG ODN was administered to mice (i.e 10 μg/mouse). For the antibody subclasses to be measured, IgG1 and IgG2c were chosen since class switching to these subtypes depends on the type of T-cell help provided to B lymphocytes (Th2 vs. Th1, respectively). Sera of individual mice were serially diluted and OD versus reciprocal serial dilution curves were generated (Supporting Information Fig. 6). Response of individual mice from each group was compared using a serum dilution of 1/32 768 (Fig. 3A). Both c-di-GMP and its complexes increased OVA-specific IgG1 levels ($p < 0.05$). IgG1 response of c-di-GMP/Arg group was significantly higher than the free c-di-GMP group ($p < 0.05$, Fig. 3A). CpG ODN alone or its combination with c-di-GMP had no significant effect on this subclass. Since c-di-GMP augments the OVA-specific IgG1 production only when used alone but not when combined with CpG ODN, the response in the synergy group may have been directed toward IgG2c class switching. Consistent with this idea, combined use of CpG ODN and c-di-GMP induced significantly elevated IgG2c levels when compared to single-ligand adjuvants ($p < 0.05$ for both groups, Fig. 3A). C-di-GMP/Arg(9) did not improve OVA-specific IgG2c response when compared to the free cyclic nucleotide, suggesting that complexation only augmented Th2-dependent IgG1 responses but had no effect on Th1-dependent IgG2c production.

To assess the extent of OVA-specific cell-mediated immunity generated by vaccination, immunized mice were challenged subcutaneously on day 51 with the OVA expressing mouse thymoma cell line, EG.7. Tumor volumes were then measured every second day for 14 days. As seen in Figure 3B, all c-di-GMP adjuvanted groups demonstrated significant reduction in tumor growth

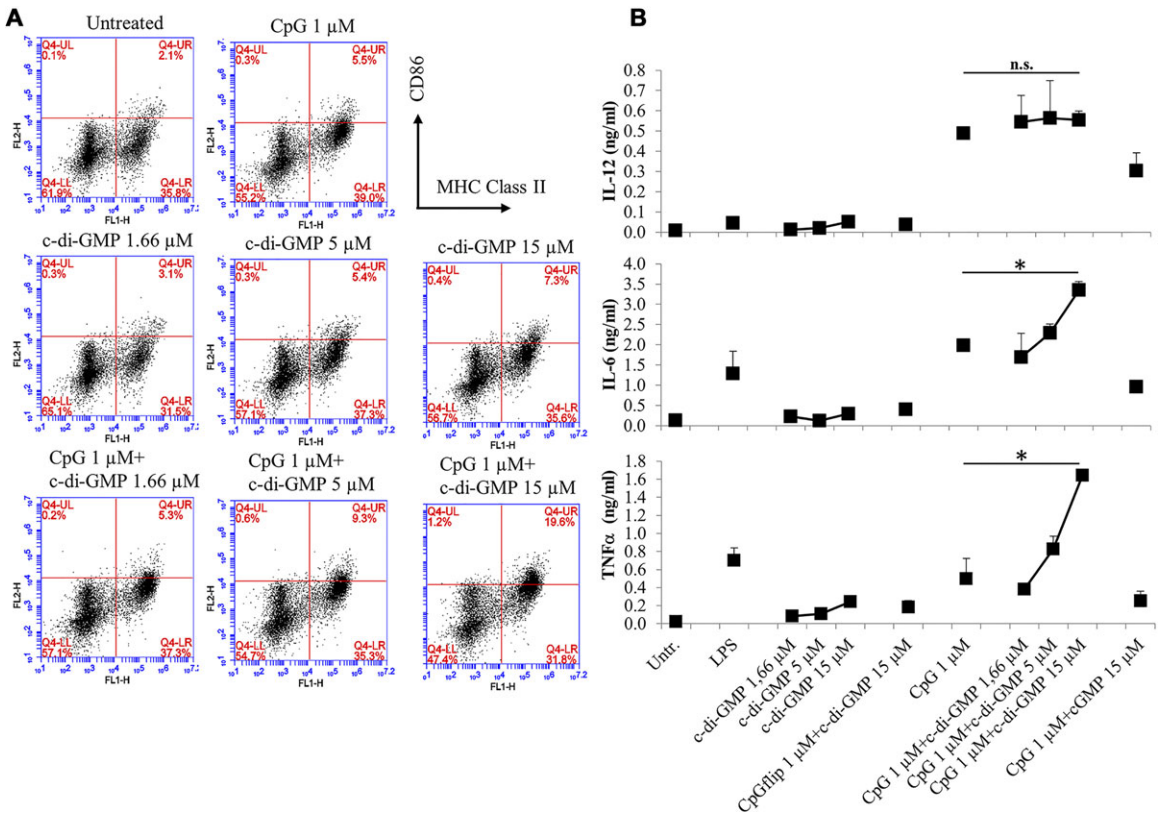

Figure 2. Combined use of CpG ODN and c-di-GMP promote synergistic activation of immune cells. (**A**) Indicated doses of CDN and CpG ODN were cultured with mouse splenocytes (2×10^6 cells/mL) for 24 h. Cells were stained for MHC class II and CD86 expression and analyzed by flow cytometry. Data are representative of four independent experiments, each giving similar results. (**B**) Culture supernatants from stimulated cells (as in A) were quantified for cytokine production by ELISA. Data are shown as mean ± SD of four independent experiments, each performed with two replicates. Significance among groups was tested using one-way ANOVA and post hoc Bonferroni method. $^*p < 0.05$.

($p < 0.05$). Based on individual tumor volume measurements (Fig. 3C, day 12), the most potent formulations that triggered an antitumor response were the Arg(9)/c-di-GMP and the CDN/CpG adjuvanted groups. The sizes of tumors in these groups were negligible when compared to single-ligand adjuvants (Fig. 3D). Consistent with the ability of c-di-GMP, Arg(9)/c-di-GMP, and the c-di-GMP/CpG to induce antitumor immunity, ex vivo restimulation of spleen cells from immunized mice with the SIINFEKL peptide resulted in significantly higher levels of peptide-specific IFN-γ production when compared to OVA alone, suggesting increased tumor-specific CD8 T-cell responses (Supporting Information Fig. 7, $p < 0.05$).

3′3′-cGAMP is a more effective STING ligand than c-di-GMP in both human and mouse cells

To determine whether human cells also responded similarly to c-di-GMP stimulation, preliminary experiments were conducted on human PBMCs using identical doses of the CDN that showed activity in mouse cells. However, under these conditions, we observed a very modest response in terms of IL-6 and TNF-α production or for MHC Class II/CD86 upregulation (data not shown). To determine if this low response was directed only to c-di-GMP, we stimulated hPBMCs with the classical (c-di-GMP) and the alternate CDN 3′3′-cGAMP, with or without transfection and assessed the levels of cytokines produced (IL-8, IFNα, IL-6, IL-10, IL-1β, and TNF-α) using cytometric bead array. Similar to our preliminary findings, c-di-GMP failed to trigger substantial levels of cytokine production even when transfected (Fig. 4A). In contrast, 3′3′-cGAMP was very effective in stimulating the secretion of all tested cytokines even without transfection (Fig. 4A).

Synergism between 3′3′-cGAMP and CpG ODN was also tested comparative to response generated using c-di-GMP. Human $CD14^+$ peripheral blood monocytes did not produce any IP-10 in response to c-di-GMP (Fig. 4B). In contrast, CpG ODN and 3′3′-cGAMP triggered similar amounts (Fig. 4B, ~9 and 11% of cells, respectively; Supporting Information Fig. 8 MFIs of 2284 ± 1250 and 5058 ± 2260, respectively) of IP-10 production from monocytes. Interestingly, c-di-GMP suppressed CpG-ODN mediated chemokine production (Fig. 4B, from 8.8 to 4.4% of cells; Supporting Information Figure 8 MFI decreased from 2284 ± 1250 to 1314 ± 1028). The amount of IP-10 produced by cells increased by 2.5-fold (Supporting Information Fig. 8 MFI of 13 015 ± 3297) for the combined group (cGAMP+CpG-ODN) comparative to cGAMP alone. Further examination of IL-6 and IFN-α

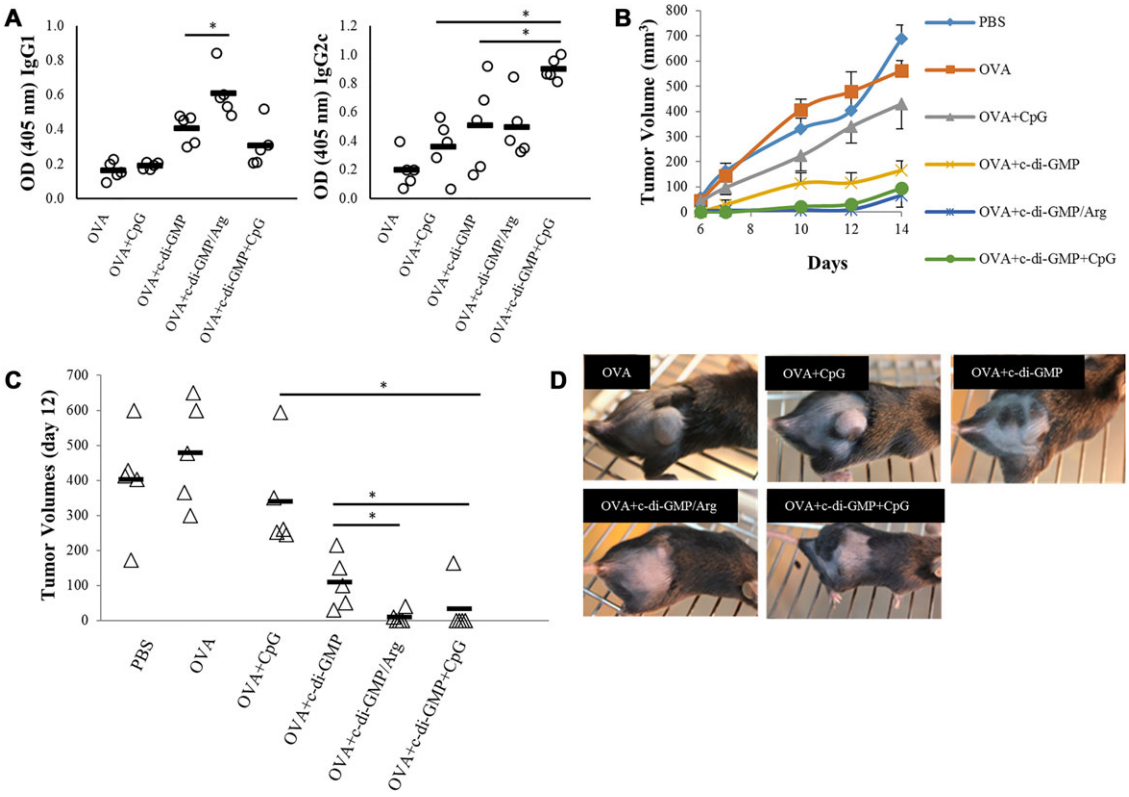

Figure 3. OVA-specific humoral and cellular immune responses generated in immunized mice. C57BL6 mice were immunized intraperitoneally on days 0 and 14 with OVA (7.5 μg/mouse) or OVA mixed with free or Arg(9)/c-di-GMP (15 μg/mouse) or CpG ODN (10 μg/mouse). (**A**) OVA-specific IgG1 and IgG2c responses (OD values at 1/ 32 768 serum dilution) were determined by ELISA from sera collected on day 42. Each symbol represents an individual mouse and bars represent means. Significance among groups was tested using one-way ANOVA and post hoc Bonferroni method. $^{*}p < 0.05$. (**B–D**) On day 51, immunized mice were challenged with subcutaneous E.G7-OVA (4×10^6/mouse) thymoma cells. (**B**) Tumor volumes were measured using a caliper every second day and recorded as length × width × height. Data are shown as mean ± SEM of five mice from a single experiment. (**C**) Tumor volumes of individual mice on day 12 are shown, with bar representing mean. Significance among groups was based on the nonparametric Mann–Whitney *U* test and $^{*}p < 0.05$. (**D**) Representative images of tumors present in indicated groups. (**B–D**) Data shown are from a single experiment.

production from human cells showed no measurable response to c-di-GMP or its combination with CpG-ODN (Fig. 4C). However, 3′3′-cGAMP stimulated human peripheral blood cells to secrete IL-6 and IFN-α in a dose-dependent manner and this response was modestly augmented in the presence of CpG ODN although this increase was not statistically significant ($p < 0.05$, Fig. 4C). Mouse spleen cells upregulated MHC class II and CD86 in response to free c-di-GMP but did not secrete measurable amounts of IFN-α/β (Fig. 4C). Consistent with our previous results, co-administration with CpG ODN showed synergistic activity and amplified the aforementioned responses. When used alone, 3′3′-cGAMP was more effective than c-di-GMP in inducing type I IFN production (Fig. 4C) and synergized with CpG ODN to generate higher levels of cytokine production and costimulator molecule upregulation.

Although the vaccine adjuvant activity of 3′3′-cGAMP was not tested in vivo, in vitro the mouse cells responded well to this CDN. Collectively, data presented here suggest that both mouse and human cells respond to 3′3′-cGAMP stimulation and 3′3′-cGAMP could be a more effective immunostimulatory agent than c-di-GMP for human use.

Discussion

CDNs produced by bacteria act as PAMPs and elicit an innate immune response that is critical for effective host defense against infection. Seminal work by Karaolis et al. demonstrated that c-di-GMP-induced proinflammatory cytokine/type I IFN production from immune cells provided protection against extracellular bacterial pathogens and elicited a Th1-biased antibody response to co-injected antigen [8, 9]. Further work provided evidence for c-di-GMP's role in promoting cellular and humoral immunity in vaccinated mice [14, 24–26]. These studies are of great interest for the development of CDN-based vaccine adjuvants for human use. Although CDNs have great potential as novel immunostimulatory agents, strategies to improve their resistance to digestion by phosphodiesterases and/or enhance their delivery to the cytosol are still required. For example, attempts to increase the intracellular half-life through synthesis of mono- and bisphosphorothioate analogs resulted in attenuation of the elicited inflammatory response [27], warranting adoption of alternate approaches. To overcome this challenge, we evaluated the feasibility of a simple cell-penetrating peptide and CDN complexation strategy that

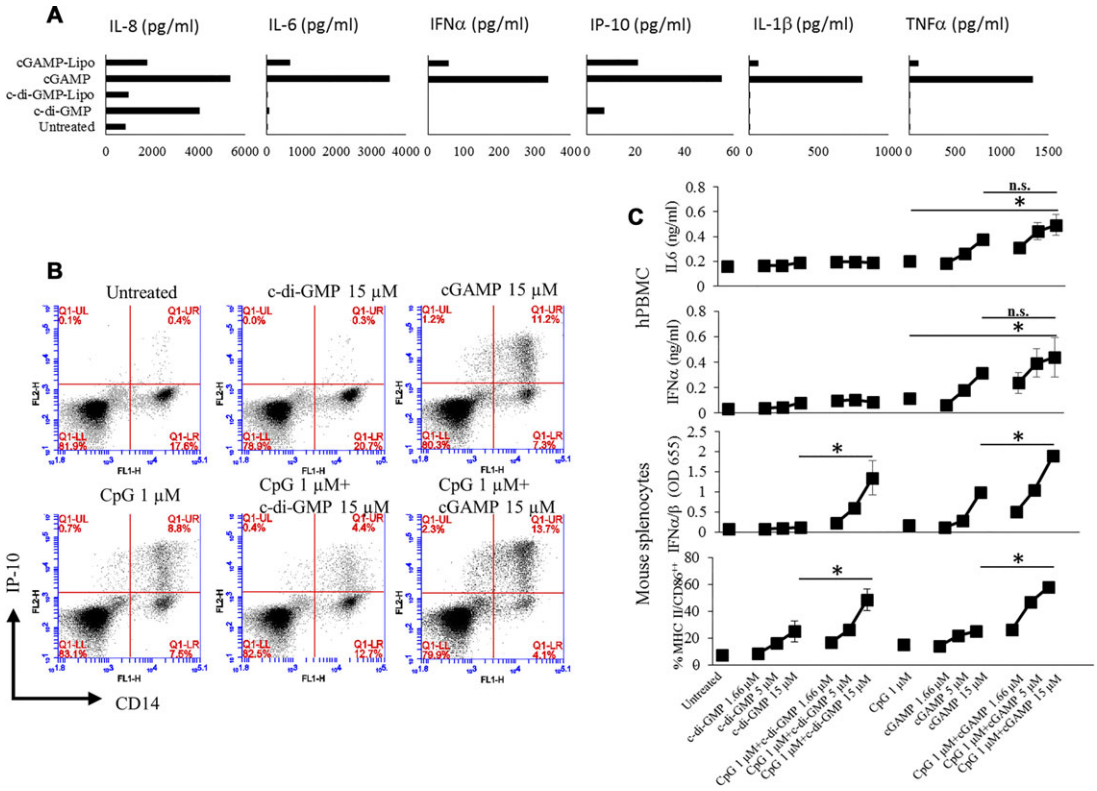

Figure 4. Immunostimulatory activities of c-di-GMP, 3′3′-cGAMP, and their combination with CpG ODN in human PBMCs and mouse splenocytes. Indicated doses of CDN and CpG ODN were cultured with human PBMCs or mouse splenocytes (1–2 × 10^6 cells/mL) for 24 h. (**A**) Cytokine production from human PBMCs was evaluated by cytometric bead array. Representative plots (of three different individuals) show internal fluorescence intensity of capture beads (IL-8, IFN-α, IL-6, IP-10, IL-1β, and TNF-α) versus cytokine levels (analyte intensity). (**B**) Indicated doses of CDN and CpG ODN were cultured with PBMCs (2 × 10^6 cells/mL) for 7 h and brefeldin A (10 μg/mL) was added for the last 2 h of stimulation. IP-10-producing $CD14^+$ cells were evaluated by flow cytometry. Data are representative of three different individuals. (**C**) Cytokine secretion patterns of human PBMCs and mouse splenocytes were evaluated by ELISA. Bioactive mouse IFN-α/β was assessed using the B16-Blue IFN-α/β reporter cells (Invivogen). Data are shown as mean ± SD of three independent experiments, each performed with two replicates. Significance among groups was tested using one-way ANOVA and post hoc Bonferroni method. $^*p < 0.05$.

would enhance the cellular delivery of the molecule. C-di-GMP is known to form supramolecular aggregates stabilized by G-quadruplexes especially in the presence of intercalators [28, 29]. Of the cationic peptide or polymer intercalators tested herein, only the 9-mer arginine peptide aggregated c-di-GMP into different polymorphs. Our results suggest that c-di-GMP/Arg(9) complexation is not a simple electrochemical attraction process and might involve specific interaction of the CDN with the positively charged guanidinium group of Arginine [30]. This protonated form of guanidine is an excellent anion binder and can form two sets of double-hydrogen bonds with phosphate anion residues in what is known as the "arginine fork" binding mode [31]. C-di-GMP possesses two phosphate groups and thus would be considered as an ideal molecule for "arginine fork" binding. Of interest, Arg is the only amino acid with this functional group, and the absence of the guanidino group in Lys(9) may explain lack of complex formation even if the net charges of both peptides are identical (+9).

The finding that Arg(9) uniquely forms complexes with c-di-GMP is an interesting one and is in support of published results showing the importance of Arginine 231/232 in STING-mediated response to the cyclic nucleotide [32]. Absence of covalent bonding between c-di-GMP and Arg(9) is of benefit since the complexes would be readily dissociable in cellular compartments and the ligand may then interact with its receptor. Furthermore, Arg(9) would also be well suited for c-di-GMP delivery since it can bypass endosomal barriers, and transfer its cargo into the cytosol [33]. Data presented herein demonstrate that c-di-GMP/Arg(9) complexes are internalized approximately fourfold more efficiently than the free molecule and the overall potency of the CDN is increased.

Combined activation of multiple PRRs can result in synergistic effects, triggering robust adaptive immune responses [34–36]. Following this concept, we chose to activate the TLR-dependent and TLR-independent DNA recognition pathways through combined use of CpG ODN and CDNs. In contrast to A-class CpG ODN that induce type I IFN in pDC via TLR9-MyD88-IRF7 pathway, recognition of conventional CpG-ODN (Class B) by endosomal TLR9 initiates a signaling cascade where MyD88-dependent recruitment of IRAK-4/IRAK-1/TRAF6 leads to the activation of TAK1 (TGF-β-activated kinase). TAK-1-dependent activation of NF-κB (through IKKα/β/γ) and MAP kinases results in the production of proinflammatory cytokines [37, 38]. Conversely, all pathways of cytosolic

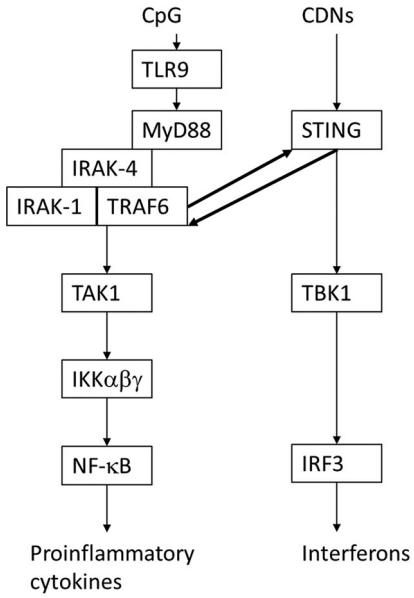

Figure 5. Proposed mechanism of synergism between CpG ODN and CDN dependent signaling pathways. CpG-ODN initiates a TLR9-mediated signaling cascade where MyD88-dependent recruitment of IRAK-4/IRAK-1/TRAF6 leads to the activation of TAK1 (left). TAK1 phosphorylates IκB kinase, which in turn activates NFκB leading to proinflammatory cytokine production. STING recognizes CDNs, recruits and activates TBK1 (right). TBK1 in turn phosphorylates and activates IRF3, leading to IFN gene transcription. TRAF6 may also be recruited to the STING–TBK1 complex, facilitating the activation of NF-κB and IRF-3. Since TRAF6 has a role in activation of both CpG ODN and CDN induced signaling, when cells are stimulated with both ligands, the triggered pathways may synergize through a TRAF6-dependent process.

DNA recognition and CDN sensing converge on STING. Activated STING recruits TBK1 and IRF-3, which in turn results in transcription of IFN-β and proinflammatory cytokines [7, 12]. Recent evidence suggests that TRAF6 may be recruited to the STING–TBK1 complex, facilitating the activation of NF-κB and IRF-3 (through TBK1) [39]. TRAF6 is an important molecule that converges signals from various PRRs, affecting DC maturation and induction of cytokine production [40]. Our results established that combined use of CpG ODN and CDNs promotes synergistic activation of immune cells. TRAF6 appears to be essential for the activation of CpG ODN and CDN-induced downstream signaling cascades. It is therefore conceivable that the endosomal and the cytosolic DNA detection pathways synergize through a TRAF6-dependent process (Fig. 5).

Our data revealed 3′3′cGAMP to be a more effective STING ligand than c-di-GMP. Whether this is due to a difference in membrane permeabilities of the ligands or their affinity to STING remains to be determined.

Vaccine adjuvants are essential for enhancing and directing the adaptative immune response to antigens. Yet, most vaccines are still supplemented with the Th2-skewing adjuvant alum. In this context, CDNs constitute an important class of molecules with a potential to improve vaccine efficacy. Here, we showed that cellular delivery and immune-stimulatory properties of CDNs can be augmented by either Arg(9) peptide complexation, or synergistic use with CpG ODN, suggesting that such strategies may be of value for further clinical development.

Materials and Methods

Reagents and cell lines

Cyclic diguanosine monophosphate (c-di-GMP), 2′-*O*-(6-[Fluoresceinyl] aminohexyl carbamoyl)-cyclic diguanosine monophosphate (FAM-c-di-GMP), and guanosine-2′, 3′-cyclic monophosphate (cGMP) were purchased from Biolog. 3′3′-cGAMP (cyclic [G(3′,5′)pA(3′,5′)p] was from Invivogen. CpG ODN K3 ATCGACTCTCGAGCGTTCTC and its flipped control were from Alpha DNA. LPS (Sigma) and polyinosidicacid:cytidilic acid (polyI:C, Amersham) were used as positive controls. Lipofectamine 2000 was purchased from Invitrogen, and used according to the manufacturer's protocol. Nonamer arginine peptide (Arg(9)), polylysine peptide (Lys(9)), $Tat_{(47–57)}$, and OVA were obtained from Anaspec. B16-Blue IFN-α/β cells that allow the detection of bioactive murine type I IFNs were obtained from Invivogen and used according to the manufacturer's protocol.

Visualization of complexes by agarose gel electrophoresis (AGE)

Complexes were prepared by mixing 0.67 μg of 2′-*O*-(6-[Fluoresceinyl] amino hexylcarbamoyl)-cyclic diguanosine monophosphate (FAM-c-di-GMP) with cationic peptides Arg(9), Lys(9), $Tat_{(47–57)}$, or pEI at 1:1, 1:2, and 1:4 molar ratios. Samples were incubated for 30 min at room temperature, in order to allow for the complexation to proceed and then were loaded onto a 2% agarose gel. Gels were resolved at 70 V for 30 min.

Cells and culture conditions

All hPBMC experiments were conducted following approval from the Bilkent University Human Studies Ethical Committee and with the informed consent of all participants. Human PBMCs were isolated from healthy volunteers using density gradient centrifugation. E.G7-OVA cells (derived from C57BL/6 (H-2b) mouse lymphoma), PBMCs, and single-cell spleen suspensions were cultured in RPMI 1640 medium containing 5% FCS, 50 U/mL penicillin, 50 μg/mL streptomycin, 0.3 μg/mL L-glutamine, 1 μM nonessential amino acids, 1 μM sodium pyruvate, 10 mM HEPES, and 10^{-5} M 2-ME.

Analysis of cellular internalization of c-di-GMP and its complexes

Complexes of c-di-GMP/Arg (molar ratio of 1:2) were prepared using either free c-di-GMP or FAM-c-di-GMP. Splenocytes

(1×10^6/mL) were incubated for 2 h at 37°C with 15 μM of the stimulants. Following incubation, cells were washed and analyzed using an Accuri C6 Flow Cytometer. To detect internalized CDN, surface-bound ODN-FAM signal was quenched using 0.2% Trypan blue (mixed 1:1 with the sample).

Determination of in vitro stimulatory activity of c-di-GMP and cGAMP

Splenocytes or hPBMCs (4×10^5 cells/well) were incubated with various concentration of c-di-GMP, CpG, or cGAMP with appropriate controls in a 96-well plate for 24 h. Culture supernatants were collected and analyzed by ELISA using capture and detection antibodies for mIL6, mIL12p40, mTNF-α, hIL6 (Biolegend), or hPANα (Mabtech). In some experiments, cytokines were detected using the BD Biosciences Cytometric Bead Array Human Flex Set (IL-8, IFN-α, IP-10, IL-6, IL-1β, and TNF-α). For intracellular cytokine staining, cells were stimulated in the presence of brefeldin A (10 μg/mL). For flow cytometric analysis, cells were fixed (and permeabilized where necessary) and stained with conjugated antibodies (1μg/mL final concentration) PE-mI-A/I-E, Alexa Fluor 647-mCD86, PE-hIP10, and/or FITC-hCD14 (Biolegend).

Immunization studies

All animal studies were conducted with prior approval of the animal ethics committee of Bilkent University. Six- to eight-week-old C57BL/6 mice were immunized intraperitoneally on days 0 and 14 with 100 μL OVA (7.5 μg/mouse) or OVA mixed with free or Arg(9)/c-di-GMP (15 μg/mouse) or CpG ODN (10 μg/mouse). To follow OVA-specific antibody responses, sera were collected on day 42 and antibody levels were determined by ELISA. Briefly, Immulon 1B plates were coated overnight with 50 μL of OVA (10 μg/mL) at 4ºC and blocked with PBS-5% BSA. Eight-time-diluted mouse sera were serially diluted fourfold and specific antibodies were detected using goat anti-mouse IgG1 or IgG2a alkaline phosphatase conjugate (1:3000 dilution) followed by PNPP substrate addition. Color development was followed at OD 405 nm using a microplate reader. Thirty-four days after the booster injection (day 51), mice were challenged with subcutaneous E.G7-OVA (4×10^6/mouse) thymoma cells. After the tumors reached a palpable size (~50 mm^3, ~1 week after tumor initiation), tumor volumes were measured using a caliper every other day and recorded as length × width × height.

Statistical Analysis

Data were statistically analyzed with the IBM SPSS v22.0 software using two-tailed unpaired Student's *t*-test, one-way ANOVA with Bonferroni posttest or Mann–Whitney *U* test. For all comparisons, 95% confidence intervals were used and *p*-values <0.05 were considered significant. Statistical tests used to determine significance are provided in individual figure legends.

Acknowledgments: We thank Ihsan Cihan Ayanoglu for technical assistance with statistical analyses. M.G. and I.G. received partial funding from TUBITAK projects 113S207, 111S151, and 113S305.

Conflict of interest: M.G. and I.G. are among the co-inventors of patents concerning the activity of CpG ODN, including their use as vaccine adjuvants. The rights to all such patents have been transferred to the US government. All other authors declare no financial or commercial conflict of interests.

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Abbreviations: **AIM2**: Absent in Melanoma 2 · **c-di-GMP**: cyclic-di-GMP · **CDN**: cyclic dinucleotide · **cGAMP**: cyclic GMP-AMP · **DAI**: DNA-dependent activator of IFN-regulatory factors · **DEAD**: D-E-A-D (asp-glu-ala-asp) · **ERIS**: Endoplasmic Reticulum Interferon Stimulator · **FAM-c-di-GMP**: 2′-Fluo-AHC-c-di-GMP · **IFI16**: Interferon-Inducible myeloid differentiation transcriptional activator · **IRF**: Interferon regulatory factor 3 · **LGP2**: Laboratory of Genetics and Physiology 2 · **MDA5**: Melanoma Differentiation-Associated protein 5 · **MITA**: Mediator of IRF3 Activation · **MPYS**: M-P-Y-S (met-pro-tyr-ser) · **ODN**: oligonucleotide · **pEI**: polyethyleneimine · **RIG-I**: Retinoic Acid Inducible Gene-I · **STING**: stimulator of IFN genes · **TMEM173**: Transmembrane Protein 173

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See accompanying article:
http://dx.doi.org/10.1002/eji.201445132

Received: 15/8/2014
Revised: 12/12/2014
Accepted: 7/1/2015
Accepted article online: 9/1/2015