Bacterial c-di-GMP Is an Immunostimulatory Molecule

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Bacterial c-di-GMP Is an Immunostimulatory Molecule


Cyclic diguanylate (c-di-GMP) is a bacterial intracellular signaling molecule. We have shown that treatment with exogenous c-di-GMP inhibits Staphylococcus aureus infection in a mouse model. We now report that c-di-GMP is an immunodulator and immunostimulatory molecule. Intramuscular treatment of mice with c-di-GMP 12 and 6 h before S. aureus challenge gave a protective effect and a 10,000-fold reduction in CFUs in tissues (p < 0.001). Intraperitoneal injection of mice with c-di-GMP activated monocyte and granulocyte recruitment. Human immature dendritic cells (DCs) cultured in the presence of c-di-GMP showed increased expression of costimulatory molecules CD80/CD86 and maturation marker CD83, increased MIC class II and cytokines and chemokines such as IL-12, IFN-γ, IL-8, MCP-1, IFN-γ-inducible protein 10, and RANTES, and altered expression of chemokine receptors including CCR1, CCR7, and CXCR4. c-di-GMP-matured DCs demonstrated enhanced T cell stimulatory activity. c-di-GMP activated p38 MAPK in human DCs and ERK phosphorylation in human macrophages. c-di-GMP is stable in human serum. We propose that cyclic di-nucleotides like c-di-GMP can be used clinically in humans and animals as an immunomodulator, immune enhancer, immunotherapeutic, immunoprophylactic, or vaccine adjuvant. The Journal of Immunology, 2007, 178: 2171–2181.
Materials and Methods

c-di-GMP and chemicals used

The c-di-GMP (Fig. 1) used in these studies was synthesized and prepared as previously described (19–24). Zymosan A (Saccharomyces cerevisiae), poly(I:C) (dsRNA), and LPS (Escherichia coli K12) were purchased from Sigma-Aldrich. Synthetic lysylated lipopeptide N-palmitoyl-cysteine-serine-lysine (PAM,C5K) was purchased from Bachem. Flagellin was purified from Salmonella typhimurium. R848 (resiquimod) was purchased from GL Synthesis. Oligonucleotide CpG-A (GGTGCGATCGATGCGAGGG GGG) used to stimulate monocyte-derived dendritic cells (DCs) was purchased from InvivoGen. The CpG-A used in activating plasmacytoid DCs (pDCs) was obtained from Invitrogen Life Technologies and is called oligodeoxynucleotide (ODN) 2216 (25).

LPS endotoxin (Limulus amebocyte lysate assay) test

A dose of 500 μM c-di-GMP was tested for the presence of LPS endotoxin using the Limulus amebocyte lysate assay (Charles River Laboratories).

Pretreatment prophylactic studies in the mouse mastitis model

The well-characterized mouse model of S. aureus-induced mastitis (for review, see Ref. 26) was used to evaluate the capacity of c-di-GMP to act as a prophylactic agent. The protocol used here is similar to that described in a previous study (21), except that the c-di-GMP molecule was administered before infection of mammary glands, instead of after, to evaluate its prophylactic capacity. Briefly, 12 and 6 h before bacterial inoculation, saline or 50 or 200 nmol of c-di-GMP was administered into the fourth pair of abdominal mammary glands of lactating CD-1 mice. Administration of 50 and 200 nmol of c-di-GMP corresponded to a dose of 1.0 and 4.1 mg of compound per kg of body weight, respectively, considering an average weight of 35 g for a mouse. For inoculation, 100 CFU of S. aureus Newbold 305 (ATCC 29740) were injected into each gland and the coloniza- tion was allowed for 10 h. Raw bacterial CFU counts obtained after plating serial logarithmic dilutions of mammary gland homogenates were trans- formed in base 10 logarithm values. The experiment was repeated and data combined for a total of nine mice (18 glands) per group. Since values passed the normality test, bacterial counts were analyzed for statistical sig- nificance using the one-way ANOVA test combined with the Tukey posttest (GraphPad Instat, version 3.06). The institutional ethics committee on animal experimentation of the Faculté des Sciences of Université de Sherbrooke approved these experiments and were conducted following the guidelines of the Canadian Council on Animal Care.

Vaccine adjuvant studies in mice

To evaluate the capacity of c-di-GMP to increase Ab production in the mouse, the cyclic dinucleotide was used as adjuvant for vaccination with S. aureus clumping factor A (CfA) recombinant protein (a surface adhesion protein of S. aureus binding fibrinogen), as previously done for CfA alone without adjuvant (27). Two groups of eight CD-1 female mice were vac- cinated i.m. with 100 μl of saline containing 25 μg of CfA. For one of the groups, the solution injected also contained 200 nmol of c-di-GMP. A volume of 50 μl was then injected into both quadriceps. This procedure was repeated 2 wk after, for a second injection. Twelve days later, i.e., 26 days after the initial injections, blood samples were taken and incubated at 35°C for 1 h to allow coagulation and then centrifuged at 13,000 × g for 10 min at 4°C. Sera were harvested and kept at −20°C until tested. The institutional ethics committee on animal experimentation of the Faculté des Sciences of Université de Sherbrooke approved these experiments and were conducted according to the guidelines of the Canadian Council on Animal Care.

Ab assays

Total IgGs. ELISA were used to determine the presence of IgG Abs against the CfA Ag in the mouse serum as previously described (27). Polysine Maxisorp Nunc 96-well plates (Nalge Nunc International) were coated for 2 h with 50 μl of recombinant CfA (domain A) protein at a concentration of 10 μg/ml in carbonate/bicarbonate buffer at pH 9.6. Follow- ing saturation of the wells with a solution of powdered milk in PBS (5% w/v), overnight at 4°C, 4-fold dilutions of sera (1/1000–1/64000) were added and incubated for 2 h at 37°C and then overnight at 4°C. Biotinyl- ated anti-mouse IgG (1/1000) was added and incubated for 2 h at 37°C. After 1 h of incubation with streptavidin-HRP (Amersham Pharmacia Bio- tech) diluted 1/500, 100 μl of Sure Blue Tetrathymethylbenzidine Peroxidase Substrate (Kirkegaard & Perry Laboratories) was added. The enzyme reaction was stopped by the addition of 50 μl of 1 N HCl after 6 min of incubation. Between each step, three washes with PBS-0.05% Tween 20 were conducted. The OD was read on a plate reader (Bio-Tek Instruments) at 450 nm. Each sample was tested in triplicate and the OD of the negative control wells that did not contain Ag was subtracted from the CfA-coated test wells.

IgG isotypes. ELISA were conducted as for the total IgG assay but the secondary Ab was either mouse anti IgG1-HRP or mouse anti-IgG2a-HRP (BD Pharmingen), and 4-fold dilutions of sera from 1/250 to 1/16,000 were assayed. Quantitation of mouse Abs was achieved as for the total IgG assay but the development of coloration was allowed for 10 min. Each sample was tested in triplicate and the OD of the negative control wells that did not contain Ag was subtracted from the CfA-coated test wells. For the statistical analysis of ELISA results, the OD data for mice vaccinated with the c-di-GMP molecule as adjuvant were matched up to the corresponding data (isotype and dilution) where mice were injected with saline instead of c-di-GMP. One-way ANOVA was used with the Bonferroni post hoc test to account for multiple comparisons (GraphPad InStat software, version 3.06). For all isotypes and dilutions analyzed, statistical significance was found to be p < 0.001 between the groups.

c-di-GMP activates monocyte and granulocyte recruitment in vivo

C57BL/6 mice were injected with a volume of 500 μl containing 200 nmol of c-di-GMP. Cervical dislocation was performed after 12 h, after which with 10 ml of cold PBS was injected and the fluid was withdrawn and centrifuged at 1400 rpm at 4°C. Fluid was incubated in PBS/BSA/azide plus 24G2 (anti-Fc) for 15 min at 4°C, washed in PBS, then stained and analyzed by FACS analysis (PBS/BSA/azide, 30 min 4°C). Cells were stained with directly coupled fluorescent mAbs combinations in 200 ml of FACS buffer and further collected on a FACSCalibur cytofluorometer (BD Biosciences). We purchased the following mAbs: fluorescein (FITC)-cou- pled M1/70 (anti-CD11b; BD Pharmingen), PE-coupled I2A (anti-LY-6G; BD Pharmingen), 7/4 (Caltag Laboratories), and alophycocyanin-coupled F4/80 (Caltag Laboratories). Cells were gated according to size and scatter to eliminate dead cells and debris from analysis.

Murine DC isolation

Murine splenic DCs were isolated from spleens of C57BL/6 mice. Briefly, spleens were dissected into small pieces and incubated at 37°C in complete RPMI 1640. Cell suspension was obtained by vigorous pipetting and pas- sage through a 70-μm nylon mesh filter. After RBC lysis, CD11c+ DCs were isolated using CD11c microbeads according to the manufacturer’s instructions (Miltenyi Biotech). The cells were >95% CD11c+ as measured by FACS analysis.

Preparation and treatment of human monocyte DCs

Cytokine, chemokine, and chemokine receptor analysis. Buffy coats were obtained from healthy volunteers and fractionated over Histopaque 1077. The PBMC layer was recovered and erythrocyte depleted by incu- bation in RBC lysis buffer for 5 min at room temperature. PBMCs were cultured in complete medium (RPMI 1640, 1% l-glutamine, 1% penicillin/streptomycin, and 10% low endotoxin FCS) for 2 h in 75 flasks (Corning). Following incubation, nonadherent cells were removed by three washes with 100 ml PBS (Invitrogen Life Technologies). The remaining adherent cells were then cultured in complete medium supplemented with GM-CSF and IL-4 (50 ng/ml each). Human and murine IL-4 and GM-CSF were purchased from PeproTech. On days 2 and 4, the DC cultures re- ceived an additional dose of GM-CSF and IL-4 (50 ng/ml each). On day 5, nonadherent DCs were harvested by gentle pipetting and plated in fresh medium containing GM-CSF and IL-4 (50 ng/ml each). On day 6, some DCs were matured by addition of 100 ng/ml LPS or 10–400 μM c-di-GMP for 1–24 h.
**p38 MAPK signaling studies.** Human PBMCs were isolated by Ficoll density gradient centrifugation from leukopacks supplied by the Department of Transfusion Medicine (Clinical Center, National Institute of Health, Bethesda, MD). Monocytes were purified (>95%) from human PBMCs with a MACS CD14 monocyte isolation kit (Miltenyi Biotec) according to the manufacturer’s instruction. DCs were generated as described previously (28). In brief, DCs were generated by incubating purified monocytes at 2 × 10⁶/ml in RPMI 1640 containing 10% FBS, 2 mM glutamine, 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 ng/ml recombinant human GM-CSF (PeproTech), and 50 ng/ml recombinant human IL-4 (PeproTech) at 37°C in a CO₂ (5%) incubator for 7 days. On days 3 and 5 of the incubation, half of the culture medium was replaced with prewarmed (37°C) fresh GM-CSF- and IL-4-containing medium. On day 7, DCs were collected, washed three times with PBS, and serum-starved for 8 h by incubating in serum-free RPMI 1640 containing GM-CSF and IL-4 (2 × 10⁵/ml) at 37°C in a CO₂ (5%) incubator. Subsequently, DCs (2 × 10⁶/sample) were incubated at 37°C in the absence or presence of c-di-GMP or GMP (Sigma-Aldrich) at concentrations specified for 5 or 30 min. At the end of the incubation, the stimulation was immediately stopped by the addition of large amounts of ice-cold PBS (10-fold). The cells were centrifuged at 1500 × g for 5 min at 4°C, washed with cold PBS, and lysed by sonication for 10 s to shear DNA, boiled for 5 min, and cooled down on ice.

**Preparation and treatment of human pDCs**

Venipuncture was performed on consenting adults using approved protocols. pDCs were prepared, as previously described (29), using a two-step procedure involving Percoll density centrifugation followed with selection for blood DC Ag 4-positive cells (Miltenyi Biotec). The capacity of c-di-GMP to induce IFN-α from these cells was assessed using cell culture and ELISA protocols also previously described (29) and was simultaneously compared with the IFN-α produced by pDCs in response to cells stimulated with CpG oligodeoxynucleotide (ODN) 2216 (25).

**Preparation and treatment of human M-CSF-induced monocyte-derived macrophages**

Preparation of macrophages was performed as previously described (30). Briefly, fully differentiated macrophages from human PBMCs were isolated from leukapheresis preparations obtained by the Blood Bank, Clinical Center, National Institutes of Health. The leukocyte-rich preparation was overlaid on Accu-Prep in 50-ml tubes and the tubes were centrifuged at 800 × g for 20 min at room temperature. PBMC fractions were collected, washed once with PBS at room temperature and twice with complete medium at 4°C, and resuspended in the same medium. Monocytes were further purified by using iso-osmotic Percoll gradient (Amersham Biosciences). At this stage, the purity of monocytes was higher than 90%. One and half million cells were placed on a 12-well plate in 1.5 ml of RPMI 1640 containing 50 ng/ml human M-CSF. On day 4, 1 ml of the medium was replaced with 1 ml of fresh medium containing 50 ng of human M-CSF. On day 7, nonadherent cells were washed out and adherent cells were used as macrophages.

**Quantitative PCR (QPCR)**

Total RNA was extracted using the RNeasy kit according to the manufacturer’s protocol (Qiagen). Briefly, after DNase I (Invitrogen Life Technologies) treatment, 1 μg of total RNA from each sample was used as template for the reverse transcription reaction. Fifty microliters of cDNA was synthesized using oligo(dT)15, random hexamers, and M-MLV reverse transcriptase (Applied Biosystems). All samples were reverse transcribed under the same conditions (25°C for 10 min, 48°C for 30 min) and from the same reverse transcription master mix to minimize differences in reverse transcription efficiency. All oligonucleotide primers for QPCR were designed using Primer Express software 1.0 (PE Biosystems) and synthesized by Invitrogen Life Technologies. The 25-μl QPCR contains 2 μl of cDNA, 12.5 μl of 2 × SYBR Green master mix (Stratagene), and 250 nmol of sense and antisense primer. The reaction conditions were as follows: 50°C for 2 min, 95°C for 10 min, then 40 cycles of 95°C for 1 s and 60°C for 1 min. Emitted fluorescence for each reaction was measured during the annealing/extension phase, and amplification plots were analyzed using the MX4000 software version 3.0 (Stratagene). Quantity values (i.e., copies) for gene expression were generated by comparison of the fluorescence generated by each sample with standard curves of known quantities. Next, the calculated number of copies was divided by the number of copies of GAPDH.

**Quantitation of murine cytokines by ELISA**

Murine IL-8 and TNF protein levels in the DC culture supernatant were measured by sandwich ELISA (R&D Systems).

**T cell proliferation assay**

Immature DCs and c-di-GMP-treated DCs were washed three times, diluted in fresh complete medium, and used as allogeneic stimulators. Cells were seeded in 96-well round-bottom culture plates with APC serial dilutions, ranging from 20,000 to 400 DCs/well, and were mixed along with freshly purified CD8+ T cells (100,000/well). After 5 days of incubation, cells were pulsed with 1 μCi of [³H]thymidine/well for 18 h and were harvested on filter paper. Proliferative responses were measured as [³H]thymidine incorporation by an automatic beta counter. Tests were performed in triplicates, and results were expressed as the mean cpm.

**Flow cytometry**

Surface expression of various markers was assessed using CellQuest analysis software on a FACSCalibur (Becton Dickinson) flow cytometer. Surface expression was determined using the following FITC- and PE-conjugated Abs: CD86-FITC (Research Diagnostics), CCR7-PE (R&D Systems), and CD80-FITC, CD83-FITC, HLA-DR-FITC (BD Pharmingen). The isotype control Abs were used accordingly in all experiments and were purchased from BD Pharmingen. Human DCs were incubated in 1% human AB serum/PBS and incubated with rat anti-CD16/CD32 (BD Pharmingen) to block nonspecific binding.

**SDS-PAGE and Western blot**

DCs. The lysates were loaded (15 μl/lane) and separated on a 4–12% NuPAGE Bis-Tris gel (Invitrogen Life Technologies) using 1 × NuPAGE MES SDS running buffer (Invitrogen Life Technologies) as the electrode buffer. SeeBlue Plus2 (Invitrogen Life Technologies) was used as molecular size marker. After electrophoresis, proteins in the gel were electrotransferred (25 V constant for 1 h) onto a piece of Immobilon membrane (Millipore) using 1 × NuPAGE transfer buffer (Invitrogen Life Technologies). The membrane was sequentially washed, blocked for 1 h at room temperature, washed, and incubated at 4°C overnight in the presence of a 1/1000 dilution of rabbit anti-phospho-p38 MAPK Ab (Cell Signaling Technology). On the next day, the membrane was washed and incubated with a 1/2000 dilution of HRP-conjugated anti-rabbit IgG (Cell Signaling Technology) for 1 h. After washing, incubation with a working solution of ECL Plus Western Blotting Detection System (Amersham Biosciences) for 5 min at room temperature, the membrane was exposed to a piece of BioMax x-ray film (Kodak). The x-ray film was developed using an automatic processor (Kodak X-OMAT 200A). The same membrane was stripped and probed for p38 MAPK protein essentially in the same manner except rabbit anti-p38 Ab (Cell Signaling Technology) as the primary Ab was used.

**Macrophages.**

Western blot analysis of macrophages was performed as previously described (31). Briefly, prepared macrophages were starved in RPMI 1640 without FCS for 10 h. Cells were rinsed three times with PBS and treated with cyclic dinucleotide for various times. Cells were lysed on ice for 20 min in a buffer containing 50 mM NaCl, 20 mM Tris-HCl, 50 mM sodium fluoride, 30 mM Na₃P₂O₇, 5 mM EGTA, 3 mM sodium orthovanadate, 1% Triton X-100, 1 mg/ml leupeptin, 1 mg/ml PMSF, 1 mg/ml aprotinin, 1 mg/ml pepstatin A, and 100 μM sodium orthovanadate pre-treated with H₂O₂. The lysates were spun in a microcentrifuge for 20 min and the supernatants were collected. Proteins were analyzed on 12% polyacrylamide gels by SDS-PAGE and transferred electrothermally to nitrocellulose membranes at 150 mA for 1 h in a semidyne system. The membranes were incubated with an Ab against either phosphorylated or nonphosphorylated p38 MAPK or ERK, followed by an appropriate secondary Ab coupled with HRP. Peroxidase activity was visualized using LumiGLO (Cell Signaling Technology).

**TLR and nucleotide-binding oligomerization domain (Nod) studies**

For TLR studies, the human embryonic kidney (HEK) cell line HEK 293 (CRL-1573) from American Type Culture Collection was used. HEK 293 cell clones stably expressing TLRs were generated by transfection of TLR cDNA, G418 antibiotic selection, and FACS sorting. HEK 293 cells were cultured in DMEM supplemented with 10% FBS. Cells were plated in 24-well tissue culture plates (1 × 10⁵ cells/well) and maintained in the above medium for 24 h. The following day, cells were either left untreated or incubated with the indicated amount of TLR ligand. After the 3-h treatment period, cells were harvested for total RNA using the Qiagen RNeasy
kit per the manufacturer’s protocol. The total RNA was converted to cDNA and qPCR was performed to determine the number of copies of IL-8. For Nod studies, experiments examining the synergistic activation of NF-κB by c-di-GMP in cells overexpressing Nod1 or Nod2 were conducted as previously described (32). Briefly, HEK 293 T cells were transfected overnight with 30 ng of Nod1 or Nod2 plus 75 ng of Igκ luciferase reporter plasmid. Ten nanograms of a constitutive Resilla luciferase reporter was also transfected into cells to adjust for transfection efficiency. At the same time, 200 μM c-di-GMP, N-acetylmuramyl-t-Ala-γ-D-Glu-meso-DAP (Nod1 ligand) or muramyl dipeptide (Nod2 ligand) were added and the synergistic NF-κB-dependent luciferase activation was then measured following 24 h of coinubcation. NF-κB-dependent luciferase assays were based on two independent experiments performed in duplicate.

Stability of c-di-GMP in human serum

Commercially supplied pooled human serum (100 μl; Cambrex) was diluted in ion-exchanged water. An aliquot of the resulting human serum solution was subjected to HPLC analysis (conditions: COSMOSIL 5C18-AR-II column (4.6 × 200 mm); buffer A; buffer B, 80% acetonitrile in water; gradient, 0–10 min A (100%), 10–60 min A:B = 100:0 to A:B = 40:60 in 50 min; detection 254 nm, flow rate, 1.0 ml/min, temperature 40°C). Separately, a 100 μM c-di-GMP solution in human serum was quenched activity of the enzymes. The resulting test sample was subjected to HPLC analysis after a 24-h treatment. Peaks that appeared in both analyses were compared. Results are based on three independent experiments.

Results

Prophylactic pretreatment of mice with c-di-GMP inhibits infection in vivo

We previously reported that intramammary injections of c-di-GMP significantly decreased the colonization of the mouse mammary glands by S. aureus when the cyclic dinucleotide was administered twice, at the time of the bacterial challenge, and at 4 h after inoculation (21). In this study, we further investigated whether c-di-GMP had any prophylactic effect when provided hours before the bacterial inoculation of the mouse mammary glands. We first performed an in vivo study to address the following questions: is c-di-GMP stable in tissues at the site of infection and will c-di-GMP stimulate a host immune response? In this study, c-di-GMP was given −12 h and −6 h before bacterial challenge. Even though the mammary glands were full of milk (~250 μl) at the time of injection with infectious bacteria, Fig. 2 shows that pretreatment with c-di-GMP 12 and 6 h before bacterial challenge produces a significant prophylactic effect with a 1.5 and 3.8 log (~10,000-fold) reduction of the mean bacterial CFU in tissues using a 50- and 200-nmol dose, respectively, compared with the untreated control (p < 0.05 and p < 0.001, respectively). Had c-di-GMP only inhibited biofilm formation, as previously shown in our in vitro (19) and in vivo models (21), we would not have expected to find such dramatically reduced numbers of bacterial cells between pretreated and nontreated mice. These results suggest that c-di-GMP might stimulate the innate immune response.

c-di-GMP has adjuvant properties

Based on our previous in vivo results suggesting that c-di-GMP stimulates the host response and inhibits infection, we performed additional experiments to address basic fundamental questions on the effects of c-di-GMP on the host immune response. To evaluate the possibility that c-di-GMP could also act as an adjuvant, the
cyclic dinucleotide was coinjected into mice with the recombinant ClfA Ag, a surface adhesion protein of S. aureus (33). Following vaccination with two i.m. injections of a mixture of ClfA and c-di-GMP, serum samples analyzed 12 days after the last injection (i.e., day 26) showed significantly higher anti-ClfA IgG Ab titers \((p < 0.001)\) compared with injections of ClfA alone (Fig. 3). ELISA showed optical densities for total IgG and for the IgG1 and IgG2a isotypes that were above or around 0.5 at serum dilutions of 1/64,000 and 1/16,000, respectively, for the c-di-GMP-treated group, whereas OD densities were below 0.5 for serum dilutions >1/1,000 for the group vaccinated with ClfA without c-di-GMP (Fig. 3D). In Fig. 3, C and D, for each particular type of Ab assay and dilution tested, OD results for mice injected with c-di-GMP vs saline are statistically different from each other \((p < 0.001)\), as found by one-way ANOVA used in conjunction with the Bonferroni post hoc test. Thus, in terms of OD values at a serum dilution of 1/1000, c-di-GMP coinjection increased total IgG by 7.7 times, IgG1 by 3.6 times, and IgG2a by 208.9 times (Fig. 3B). The relative increase in production of IgG2a in the presence of c-di-GMP indicates activation of the Th1 pathway. Although we cannot confirm Ag-specific cytokine production without testing, the other results suggest this. In any case, the data clearly demonstrate that c-di-GMP improves Ab production.

c-di-GMP activates monocytes and granulocyte recruitment in vivo

In support of the immunostimulatory activity mediated by c-di-GMP, studies in naive mice injected i.p. with c-di-GMP show that c-di-GMP induced the recruitment of F4/80high LY-6Gmed (monocytes) and F4/80mod LY-6Ghigh (granulocytes). Fig. 4 shows the results of studies in which FACS analysis using F4/80, LY-6G, and CD11b Ab was used to identify monocytes and granulocytes in peritoneal lavage at 18 h following i.p. injection of 200 nmol of c-di-GMP and 50 mg of LPS (positive control). The in vivo recruitment of monocytes and granulocytes into the peritoneal cavity in response to c-di-GMP is likely the outcome of local induction of certain chemokines (such as MCP-1) and the enhancement of adhesion molecules on either monocytes or endothelial cells.

**FIGURE 4.** Intraperitoneal injection of c-di-GMP activates monocytes and granulocyte recruitment. Peritoneal fluid was analyzed by FACS analysis 18 h after injection of 200 nmol of c-di-GMP or 50 mg of LPS. A, Contour plot of F4/80/LY-6G and CD11b/LY-6G showing recruitment of F4/80high LY-6Gmed (monocytes) and F4/80mod LY-6Ghigh (granulocytes). B, Histogram showing recruitment of macrophages and granulocytes. These results are representative of three independent experiments with three animals per group. Difference in relation to controls are denoted by an asterisk for \(p < 0.05\).

**FIGURE 5.** c-di-GMP activates murine DCs. A, Murine splenic DCs were stimulated with 200 mM c-di-GMP for 24 h and stained with PE- or FITC-conjugated Abs specific for CD80 and CD86. B, The concentrations of the cytokines IL-8 and TNF in the above supernatants were measured by ELISA. The results shown are representative of three similar experiments.
c-di-GMP matures murine DCs

We decided to test whether c-di-GMP could also induce maturation of DCs. DCs are central in the immune response as they sense infection and respond appropriately to induce T cell immunity and promote a Th1 immune response. Maturation of DCs and their ability to become potent APC is critical for initiation of immune responses.

Primary murine CD11c<sup>+</sup> DCs were isolated from the spleen of C57BL/6 mice and treated with 200 μM c-di-GMP for 24 h. We observed an increase in the surface expression of the costimulatory molecules CD80 and CD86 (Fig. 5). LPS-treated DCs were used as a positive control in this study and also induced a mature phenotype. To test whether c-di-GMP could induce the production of proinflammatory cytokines and chemokines, we analyzed the supernatants of c-di-GMP-treated DCs. We observed an increase in TNF and IL-8 protein in the supernatant of c-di-GMP-treated murine DCs (Fig. 5).

c-di-GMP matures human DCs

We decided to also test whether c-di-GMP could induce the maturation and activation of human DCs. We used an in vitro culture system for the production of human monocyte-derived DCs in which adherent human monocyte-derived DCs prepared from adherent PBMCs cultured in IL-4 and GM-CSF for 5 days were used in these experiments. Immature DCs (defined by FACS analysis in which DCs expression no CD14 or CD83, but high levels of CD11c) were treated for 24 h with 5–500 μM c-di-GMP. Fig. 6A shows a representative histogram comparing the increased expression of CD83 in DCs treated with 200 μM c-di-GMP (net mean fluorescent intensity (MFI) = 63.34) compared with that of untreated cells (net MFI = 4.89) or cells treated with LPS (net MFI = 141.22) used as negative and positive controls, respectively. A dose-dependent increase in surface CD83 expression was observed on cells treated with a wide range of c-di-GMP concentrations, with significant increases beginning at 32 μM and spiking at 500 mM, the highest concentration tested (Fig. 6A). We also observed an increase in the expression of other maturation markers (CD80, CD86, CCR7, and MHC class II) on DCs treated with 200 μM c-di-GMP (Fig. 6C).

Cytokine, chemokine, and chemokine receptor expression in c-di-GMP-induced DC maturation

To examine the potential of c-di-GMP-treated DCs in activating and/or recruiting other effector cells to sites of infection, we measured cytokine, chemokine, and chemokine receptor expression is altered in DCs treated with c-di-GMP. c-di-GMP stimulated mRNA expression of the regulatory cytokine IFN-α, the proinflammatory cytokines IL-1β and TNF, as well as the Th1 cytokines IFN-γ and IL-12p40 (Fig. 7A). We found no evidence for the induction of Th2 cytokines (IL-4, IL-5, IL-10, IL-13) by c-di-GMP-treated human DCs. To demonstrate that increases in IL-8 and IFN-γ-inducible protein 10 mRNA levels were accompanied by protein production, ELISA was performed on the supernatants of DCs stimulated with c-di-GMP stimulated for 24 h. These data were consistent with the mRNA results (data not shown). Fig. 7B shows that treatment with c-di-GMP caused an increased mRNA expression of several chemokines in DCs, notably IL-8/CXCL8, monokine induced by IFN-γ (MIG)/CXCL9, IFN-γ-inducible protein 10/CXCL10, IFN-inducible T cell α chemomtactant/CXCL11, MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, and RANTES/CCL5.

The migration of DCs from the periphery, where they encounter and take up foreign Ag, to the T cell area of the lymph nodes is an important aspect of immunity. To determine the potential c-di-GMP
has in affecting DCs trafficking, we measured expression of chemokine receptors in cells stimulated with c-di-GMP. We found that the chemokine receptors CCR1 and CCR5 were down-regulated, whereas CXCR4 and the lymph node homing receptor CCR7 were up-regulated on DCs following c-di-GMP treatment (Fig. 7C).

To rule out the possibility that the immunostimulatory effects of c-di-GMP were not due to the presence of LPS endotoxin contamination in the synthetic c-di-GMP preparation, we performed a Limulus amebocyte lysate assay. We found that the highest dose of c-di-GMP (500 μM) used in our experiments did not contain LPS (<3 pg/ml LPS).

**Effect on human pDCs**

Since pDCs represent a separate DC lineage compared to those of myeloid origin, we did test whether c-di-GMP might also activate these cells for cytokine secretion, namely, IFN-α secretion. In two independent experiments done in duplicate, c-di-GMP was negative for inducing this type I IFN. In contrast, both pDC preparations produced IFN-α in response to stimulation with the type A CpG oligodeoxynucleotide ODN-2216 (data not shown).

**c-di-GMP enhances T cell stimulatory activity**

Another feature of DC maturation is an enhanced ability to stimulate T cells due to increased MHC and costimulatory receptor expression. Therefore, to determine whether c-di-GMP-treated DCs have an enhanced ability to stimulate T cells, a T cell proliferation assay was performed. Immature DCs, LPS-treated DCs, and c-di-GMP-treated DCs were cocultured with T cells for 6 days, and allospecific T cell proliferation was measured by radiolabeled thymidine incorporation. Fig. 8 shows that T cell proliferation was...
markedly up-regulated (4- to 7-fold) in cocultures receiving DCs pretreated with c-di-GMP, and this was identical to that seen in cocultures receiving LPS stimulated DCs.

c-di-GMP activation of p38 MAPK in human DCs

To determine whether c-di-GMP could activate intracellular signaling, we investigated the effect of c-di-GMP on the activation of p38 MAPK. As shown in Fig. 9A, c-di-GMP did not activate NF-κB (since it did not activate IkBα degradation), but activated p38 MAPK (as indicated by the increase in the phospho-p38 at both 5- and 20-min treatment). LPS and GMP were used as positive and negative controls, respectively. In addition, c-di-GMP did not activate either ERK (data not shown). Since TNF expression is regulated by NF-κB and we only examined up to 30 min after the addition of c-di-GMP, we cannot rule out activation of NF-κB at some later time point. These results were reproduced in four independent experiments using different donor-derived DCs.

Phosphorylated ERK signaling in human macrophages

Based on three independent experiments, although there was no phosphorylation of p38 MAPK detected under the conditions tested, we found that c-di-GMP (10 and 100 μM) activates ERK phosphorylation in human M-CSF-induced monocyte-derived macrophages (Fig. 9B). The magnitude of c-di-GMP activation of ERK phosphorylation was weaker than that by LPS under the conditions tested. ERK phosphorylation peaked at 5 min and was absent at 20 min, which was earlier than that compared with LPS-induced ERK phosphorylation.

c-di-GMP-induced cell activation is TLR and Nod independent

It has been previously shown that lipopeptides, LPS, dsRNAs, flagellin, ssRNAs, and CpG DNA induce cellular activation via TLRs and that these pathogen-associated factors can induce DC maturation. Because various nucleotide structures have been shown to stimulate DCs through TLRs, we hypothesized that c-di-GMP may also induce cellular activation through a TLR-dependent mechanism. To test this hypothesis, HEK cells, which are normally unresponsive to TLR ligand stimulation, were stably transfected with TLR1/2, TLR3, TLR4/MD2, TLR5, TLR2/6, TLR7, TLR8, and TLR9. As expected, these cell lines responded to TLR ligands according to their TLR expression profile (i.e., TLR3:dsRNA, TLR4/MD2:LPS); however, none of the TLR-expressing cell lines were activated by c-di-GMP (Fig. 10). In addition to our earlier results showing no LPS contamination by the Limulus amebocyte lysate assay, by failing to confer TLR responsiveness to c-di-GMP (i.e., c-di-GMP failed to stimulate the LPS-sensitive MD2/TLR4-HEK cell line), the data confirm that the immune-enhancing activity of c-di-GMP is not due to any LPS contamination. Furthermore, we found that neither Nod1 or Nod2 responded to c-di-GMP (data not shown). These results suggest that c-di-GMP immunostimulation does not involve any known TLR or Nod.

c-di-GMP is stable in human serum

HPLC analysis of the stability of c-di-GMP in human serum after 24 h at 37°C showed only one eminent peak due to a nucleotide at the same retention time, which is identical to the retention time of monomeric c-di-GMP (data not shown). These results indicate that 100 μM c-di-GMP underwent no change in size and two-dimensional structure. Similar results were obtained with 500 μM c-di-GMP in which the ratio of the peaks due to c-di-GMP and the

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**FIGURE 9.** c-di-GMP induces signaling in DCs and macrophages. A. Activation of p38 MAPK in human DCs. Immature DCs starved overnight were incubated in the absence or presence of c-di-GMP or GMP at specified concentrations (micromolar) for 5 or 30 min. Similar results were found in three independent experiments. B. Activation of ERK in human macrophages. Macrophages were exposed to c-di-GMP for 5 min. Following exposure to c-di-GMP, DCs and macrophages were harvested to make cell lysates. Identical amount of cell lysates was separated on a 4–12% gradient PAGE gel, transferred onto a piece of polyvinylidene difluoride membrane, and analyzed by Western blot with the use of Abs.

**FIGURE 10.** TLRs fail to respond to c-di-GMP. HEK cells stably transfected with TLR proteins were stimulated for 3 h with 200 μM c-di-GMP. Cells were harvested for RNA and IL-8 transcripts were quantified by QPCR.
against with c-di-GMP has a significant protective and prophylactic effect in the mouse model of mastitis (21).

We have explored the extracellular biological effects of c-di-GMP and its potential use as a therapeutic agent. We have recently shown that extracellular c-di-GMP specifically inhibits *S. aureus* cell-cell interactions, biofilm formation, and adherence to HeLa epithelial cells in vitro (19). Furthermore, we have shown that c-di-GMP treatment significantly attenuates *S. aureus* infection in a mouse mastitis model (21).

In the current study, we demonstrate that pretreatment of mice with c-di-GMP has a significant protective and prophylactic effect against *S. aureus* infection when c-di-GMP was administered 12 and 6 h before bacterial inoculation in the mouse mammary glands. *S. aureus* infections such as bovine mastitis are difficult to treat and relapsing infections frequently occur (34). The ability of *S. aureus* to enter and survive in phagocytic and nonphagocytic cells has been recognized and often correlates with the high frequency of relapse after antimicrobial therapy (35–37). Also, to date it has been difficult to develop a highly protective vaccine for *S. aureus* (38, 39) and no vaccine that generates both humoral and cell-mediated responses would be adequate for intracellular pathogens (40–42) is available although DNA-based vaccines are promising (43). Not only does c-di-GMP have a protective effect against *S. aureus* infection but when given along with an Ag as part of a vaccine strategy, c-di-GMP promotes an Ag-specific immune response (adjuvant effect). This immunostimulatory effect is perhaps largely due to the capability of c-di-GMP to induce phenotypic and functional maturation/activation of myeloid DCs, but not pDCs.

Although our results show that DCs release MCP-1 in response to c-di-GMP, we do not believe that DC-derived MCP-1 is recruiting monocytes since MCP-1 release from activated macrophages is a more natural occurrence. As our data suggested that i.p. injection of c-di-GMP can activate monocyte and granulocyte recruitment, we propose that c-di-GMP might activate resident peritoneal macrophages to produce or release monocyte chemoattractants, such as MCP-1.

Consistent with an ability to act as an immunostimulator and immune enhancer, our results show that c-di-GMP stimulates both mouse and human DC maturation and differentiation, Ag-presenting capacity, and enhanced T cell stimulatory activity, leading to an overall Th1 response. The immunostimulatory effect of c-di-GMP is more specific and not as overwhelming as that seen with LPS. Supporting an immune enhancer effect, it is known that IL-8 production results in enhanced migration of DCs and macrophages. The increase in MIG/CXCL9 (a chemoattractant for activated T cells) suggests possible antitumor activity. The ability of c-di-GMP to activate mouse and human DCs is consistent with our findings that pretreatment with c-di-GMP has a significant protective effect in the mouse model of mastitis and it has significant adjuvant properties when administered with an Ag.

Activation of the MAPK pathways, including p38, ERK, and JNK, are important for the differentiation/maturational DCs and the NF-κB transcription factor is linked to the production of proinflammatory cytokines by DCs and has substantial impact on innate and adaptive immunity (44–48). The activation of p38 MAPK has been shown to play a critical role in the activation of DCs in response to a variety of stimuli (49–53). In this study, we tested the hypothesis that the action of c-di-GMP on the host immune response involves modulation of cell signaling pathways. Since c-di-GMP did not activate ERK and JNK in DCs under the conditions tested, the exact mechanism by which c-di-GMP activates p38 MAPK awaits further investigation. However, the ability of c-di-GMP to activate p38 MAPK in DCs is consistent with its ability to induce the maturation of DCs, as evidenced by the up-regulation of DC surface costimulatory molecules, cytokines, chemokines, and chemokine receptors, and its capacity to stimulate allogeneic MLR.

Our results showing that mouse i.p. injection of cyclic dinucleotide activates recruitment of monocytes and granulocyte recruitment suggested that c-di-GMP-activated resident macrophages may produce and release monocyte chemoattractants, such as MCP-1 (MCP-1/CCL2) (54). MCP-1 plays a major role in the recruitment of monocytes into inflammatory sites and its production is dependent on activation of p38 MAPK (55, 56). Based on the ability of c-di-GMP to activate monocytes and granulocytes in vivo, we examined whether c-di-GMP could activate human monocyte-derived macrophages in vitro by evaluating the phosphorylation of two MAPKs, ERK and p38 MAPK, in response to c-di-GMP. However, although we detected a transient phosphorylation of ERK in macrophages, we detected no obvious change in p38 MAPK phosphorylation in macrophages under the conditions tested. One possible explanation for this difference is that macrophages (p38 MAPK is not induced) and DCs (p38 MAPK is induced) respond differently to c-di-GMP. Because we found no effect on p38 MAPK in macrophages, our results suggest that the in vivo recruitment of monocytes, granulocytes, and macrophages by c-di-GMP does not result from production of MCP-1.

One primary host mechanism for detection of pathogens or microbial products ("danger signals") is mediated by plasma membrane-bound pathogen recognition receptors called TLRs. TLRs interact with various microbe-associated molecules, and subsequent activation induces up-regulation of costimulatory molecule expression, production of antimicrobial effector molecules, and secretion of proinflammatory cytokines and chemokines. However, it is also possible that instead of innate immune signaling at the cell surface, signaling might occur in the cytosol in a TLR-independent manner. An emerging family of pathogen recognition receptors, called Nods, also appear to be involved in the recognition of bacterial products and can trigger an innate immune response. Nods are cytosolic proteins involved in innate immune defense through pathways that are likely to be independent of TLR signaling. Although c-di-GMP is an immunostimulatory molecule derived from bacteria, our TLR and Nod tests suggest that innate activation does not appear to involve TLRs 1–9 or Nods 1 and 2. In addition, the pDC data also indicate that TLR9 is not likely involved. Therefore, although the exact receptor-mediated mechanism of c-di-GMP immune activation is not yet known, it does not act through any currently known TLR or Nod.

If cyclic dinucleotides (like c-di-GMP) are to be used clinically, it is important to know their stability in vivo since serum might contain mammalian phosphodiesterases that could potentially cleave and degrade the molecule. Our results clearly demonstrate...
that c-di-GMP is stable in pooled human serum and is an advantageous property for potential clinical use.

The data presented in the current study show that bacterial c-di-GMP is an immunostimulatory molecule and we have previously demonstrated that c-di-GMP can inhibit bacterial infection (19, 21) and has potential in cancer treatment (20). Due to its microbial origin and its effect on the host response, c-di-GMP can be considered as a danger signal or novel pathogen-associated molecular pattern. Our findings with c-di-GMP have some features in common with other immunostimulatory molecules. Bacterial DNA containing unmethylated CpG motifs is also known to stimulate production of polyclonal Ig and Th1-associated immunomodulatory cytokines including IFN-γ, IL-12, and TNF-α, which provide some protection against intracellular pathogens (39–41). CpG-containing oligodeoxyribonucleotides, which act as TLR9 agonists, are recognized as adjuvants modulating mucosal immune responses (57). Like CpG, c-di-GMP itself can stimulate an immune response; however, the immunostimulatory ability of c-di-GMP is TLR independent. Our results show that c-di-GMP also enhances a Th1 response: 1) pretreatment of mice with c-di-GMP inhibits bacterial infection, 2) c-di-GMP preferentially promotes the Ag-specific IgG2a over IgG1, 3) c-di-GMP up-regulates IL-12, not IL-10, and 4) c-di-GMP preferentially stimulates p38 MAPK in DCs.

A potential mechanism of action is that c-di-GMP may interact with a putative receptor, trigger an intracellular signal transduction cascade resulting in the up- and down-regulation of genes leading to the mobilization and activation of monocytes and granulocytes and DCs, and hence the inhibition of infection and enhancement of Ag-specific immune responses. Our data do indicate the triggering of intracellular signal transduction cascades. However, the exact receptor (membrane or intracellular) c-di-GMP utilizes is currently under investigation; our data rule out the involvement of all known TLRs and Nod.

In human and animal vaccine development, a major drawback to the development of novel vaccines has been the lack of safe, yet effective adjuvants. For example, biphasic lipid vesicles have been proposed for the delivery of oligo/polynucleotides (58). The demonstration that the c-di-GMP acted as an adjuvant for vaccination with S. aureus CIIA Ag when coinjected i.m. indicates good bioavailability without the requirement of specific delivery systems or formulations. In addition to its use alone in preventing infection, it will be interesting to see whether c-di-GMP coadministered with other Ags or as part of a vaccine preparation improves protection against subsequent challenge.

The inhibition against experimental S. aureus infection in mice clearly establishes a potential clinical use for the protective effect of c-di-GMP against infection (prevention). Another potential use is for increasing the immunity status of individuals or a population either at known risk of developing disease to reduce infection (“metaphylaxis”) or during periods of known disease susceptibility or immune suppression (“immune restoration”). Since c-di-GMP targets the host immune response, these molecules would also be protected from most of the bacterial resistance mechanisms found in microorganisms that affect specific classes of antibiotics. We propose that cyclic dinucleotides, such as c-di-GMP, represent a new class of immunotherapeutic molecules.

In conclusion, this study constitutes a novel investigation into the biological effects of c-di-GMP on the immune response and its potential clinical use. Our findings from various in vivo models (infection, adjuvant, and monocyte and granulocyte recruitment) using different routes of administration (intramammary, i.m., and i.p.) demonstrate that c-di-GMP is an immunostimulatory molecule triggering innate and adaptive immune responses. We also show, using in vitro models, that c-di-GMP activates human monocyte-derived DCs and acts like an adjuvant. We propose that cyclic dinucleotides like c-di-GMP have broad activity and can be used clinically in humans and animals as an immune enhancer, immunotherapeutic and immunoprophylactic agent, or as a vaccine adjuvant to inhibit infection and disease.

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Disclosures
D. K. R. Karaolis has three related patents: method for attenuating virulence of microbial pathogens and for inhibiting microbial biofilm formation (PCT/US04/23498), method for stimulating the immune, inflammatory, or neuroprotective response (U.S. 11/079, 886; PCT/US05/08447), and method for inhibiting cancer cell proliferation or increasing cancer cell apoptosis (U.S. 11/079,779; PCT/US05/08448).

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