c-di-GMP Enhances Protective Innate Immunity in a Murine Model of Pertussis

Abstract

Innate immune responses represent the first line of defense against invading pathogens in the respiratory tract. c-di-GMP is a ubiquitous second messenger that regulates biofilm formation, motility and virulence in a diverse range of bacterial species with potent immunomodulatory properties. In the present study, c-di-GMP was used to enhance the innate immune response against pertussis, a respiratory infection caused by Bordetella pertussis. Intranasal treatment with c-di-GMP resulted in the induction of robust innate immune responses to infection with B. pertussis characterized by enhanced recruitment of neutrophils, macrophages, natural killer cells and dendritic cells. The immune responses were associated with an earlier and more vigorous expression of Th1-type cytokines, as well as an increase in the induction of nitric oxide in the lungs of treated animals, resulting in significant reduction of bacterial numbers in lungs of infected mice. These results demonstrate that c-di-GMP is a potent immune stimulatory molecule that can be used to enhance protection against bacterial respiratory infections.

Keywords

Chiapas; Diarrheagenic E. coli; Llaveoz; Water purification system

Abbreviations

Ptx: Pertussis toxin; SS Medium: Stainer-Scholte Medium; OD: Optical Density; PBS: Phosphate Buffered Saline; i.n.: intranasally; i.c.: intra-cerebral; APMSP: 4-Amidinophenyl-Methanesulfonyl Fluoride; RT: Room Temperature; BSA: Bovine Serum Albumin; mBD3: mouse Beta Defensin 3; CT: Cycle Threshold; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; PG1: Protegrin 1; BD-3: Beta Defensin 3; BD-4: Beta Defensin 4; AMPs: Antimicrobial Peptides; Pw: Peritoneal macrophages following immunization with whole cell vaccine; NO: Nitric Oxide

Introduction

Pertussis is one of the most serious childhood diseases responsible for hundreds of thousands of deaths every year. The disease is caused by infection with the Gram-negative bacterium Bordetella pertussis and is characterized by severe respiratory symptoms including paroxysmal cough and apnea. The infection is mediated via inhalation of aerosol droplets and typically remains localized in the respiratory tract [1,2]. However, in immunosuppressed subjects the disease can progress to generalized bacteremia [3].

Over the past two decades, a resurgence of pertussis in infants and a shift of cases from children to adolescents/adults have been reported worldwide [4,5]. The immune response against B. pertussis relies on both specific and innate immune responses. Although specific correlates of protection have not yet been established, the use of vaccines over decades has proven to be highly effective in reducing the disease. Both acellular and whole-cell vaccines are currently available and various studies have demonstrated the importance of vaccine-induced antibodies against B. pertussis virulence factors including pertussis toxin (Ptx), fimbria (fim 2 and fim 3) and pertactin [6-10]. However, it has been difficult to establish a direct correlation between antibody titers and protection from disease [11,12]. Cell-mediated immunity, and in particular CD4+ T cells are involved in protection, especially when using whole-cell pertussis vaccines [13,14]. Other reports have shown that the secretion of IFN-g is critical for effective control of bacterial spread in the lung. For example, IFN-g/− or IFN-g defective mice developed disseminating lethal infections following challenge [2,15]. Furthermore, protection may also be associated with the induction of IL-12 [16] and TNF-a required for effective phagocytosis of the bacteria [17,18]. More recently, Th-17 cells and the secretion of IL-12 have also been implicated in cell-mediated protection from B. pertussis [19].

The innate immune response against pertussis involves various immune cells including macrophages, neutrophils, dendritic cells, γδ-T cells, NK cells and NKT cells [20]. The two main phagocytic cell populations that constitute pulmonary innate immunity are resident alveolar macrophages and recruited neutrophils [21]. In addition, local and rapidly recruited lung dendritic cells have been demonstrated to internalize bacteria and promote the expression of type 1 cytokines by NK cells, T
cells, and NKT cells [22–26], which then directly impact specific immune responses. Here, we evaluated the potential of cyclic di-guanylate (c-di-GMP; 3',5'-cyclic di-guanylate) to act as innate immune stimulator.

c-di-GMP was first identified in *Glucanocetobacter xylinum* and *Agrobacterium tumefaciens*. The molecule is involved in various intracellular signaling pathways including cellulose biosynthesis [27–29], bacterial growth, cellular adhesion, cell-surface interactions and biofilm formation [30–34]. c-di-GMP is found at concentrations of 5–10 μM in many bacterial species, but not in eukaryotes [35–37]. Recently, a number of studies have shown that the innate immune response elicited by c-di-GMP is a potent immunomodulator for the treatment of bacterial infections and can act as an immune modulator and immunostimulatory molecule [33,38,39]. For example, in transmammary administration of c-di-GMP has been shown to prevent colonization and biofilm formation by *Staphylococcus aureus* in a murine model of mastitis [40]. In another study, intranasal or subcutaneous administration of c-di-GMP stimulated a robust innate immune response characterized by increased recruitment of immune cells into the lung which resulted in protection against intratracheal challenge with *Klebsiella pneumonia* in mice [33]. Intraperitoneal administration of c-di-GMP in mice enhanced monocyte and granulocyte recruitment [38]. Interestingly, stimulation of human immature dendritic cells with c-di-GMP resulted in an increase in the expression of costimulatory molecules CD80/CD86 and maturation marker CD83, MHC classII, cytokines, chemokines and chemokine receptors [38]. More recently, STING has been identified as a direct detector of cyclic dinucleotides, provides insight into the fundamental mechanisms by which the innate immune system can detect bacterial infection [41]. Here, we demonstrate that intranasal administration of c-di-GMP in mice resulted in induction of protective immune responses against bacterial challenge with *B. pertussis*. These results also confirm previous observations that innate immunity plays an important role in disease protection against *B. pertussis* infection.

**Materials and Methods**

**Mice**

Female specific pathogen free BALB/c mice were obtained at 5–7 weeks of age from the Charles River Institute. All animals were maintained under specific pathogen free conditions within the animal care unit of VIDO until the day of sacrifice.

c-di-GMP

The c-di-GMP used in our study was chemically synthesized and prepared as described previously [42]. The purity of the c-di-GMP used in this study was >98% and was confirmed by HPLC, 31P-NMR, and ESI-TOF Mass Spectophotometrical analysis. The peptides used in this study were free of endotoxin contamination as determined by the Limulus assay. Control c-GMP (Sigma) or c-di-GMP was reconstituted at a concentration of 200μM in 30μl of sterile saline for treatment of mice.

**Bacterial culture**

Bacterial suspensions of strain Tohama I were stored at -70 °C in Casamino acid plus 10% glycerol. Organisms were grown on the surface of Charcoal agar (Becton Dickinson and Company, USA) containing 10% (vol/vol) defibrinated sheep blood and 40mg/ml of Cephalexin (Sigma-Aldrich, USA) at 37 °C for 48hr. Bacteria were harvested from plates by scraping off and resuspending bacteria in Stainer-Scholte (SS) medium. Bacteria were washed by centrifugation at 2500g for 10 min. The pellets were resuspended in phosphate buffered saline (PBS; pH 7.2) and adjusted to the indicated optical density (OD) at 600 nm using a spectrophotometer (Ultrspec 3000, Pharmacia Biotech, UK). The bacterial suspension was kept on ice until it was used for the challenge. The corresponding viable counts of these suspensions were determined by plating serial dilutions of the bacterial suspension onto Charcoal agar plates and incubation at 37 °C for 4–5 days.

**Respiratory challenge**

Mice were lightly anaesthetized while a single drop of 30μl c-di-GMP, the nucleotide control c-GMP or PBS (0.1M, pH 7.2) was administered intranasally (i.n.) 24 hr prior to bacterial challenge. For challenge mice were anaesthetized and an inoculum containing 1·3×10^8 *B. pertussis* was carefully placed on the top of each nostril, and allowed to be inhaled [43]. After the challenge at days 2, 4 and 6, lungs were removed from mice and placed in SS medium or PBS (0.1M, pH 7.2) containing 0.4mM of irreversible serine protease inhibitor APMSF (4-Aminophenylmethanesulfonyl fluoride; Roche Diagnostics GmbH, Germany).

Whole lung homogenization for bacterial and cytokine determination: The whole lung from each mouse was homogenized in 2ml of SS medium. Bacterial counts were assessed by plating serial dilutions of the homogenate onto Charcoal agar plates and incubation at 37 °C for 4–5 days. For assessment of cytokine levels the whole lungs were homogenized in 2ml of PBS containing APMSF for cytokine determination and flow cytometric analysis. The lung homogenates in PBS with protease inhibitor were then centrifuged at 12000xg for 10 min. Supernatants were collected and stored at -20 °C for measurement of cytokine levels.

Detection of cytokine levels by ELISA: Concentrations of murine IFN-γ, TNF-α, IL-4, IL-6, IL-12, IL-17, IL-23 and MCP-1 were measured in serum and lung homogenate supernatants using an Endogen®, Optea® or capture Duoset® ELISA development system. Immunolon® 2-HB 96-well plates were coated with anti-mouse IFN-γ (4µg/ml), anti-mouse TNF-α (0.8µg/ml), anti-mouse IL-4 (4µg/ml), anti-mouse IL-6 (2µg/ml), anti-mouse IL-12p70 (4µg/ml), anti-mouse IL-12p70 (2µg/ml), anti-mouse IL-23 (2µg/ml; R & D Systems), anti-mouse IL-17 (2µg/ml; eBioscience) and anti-mouse MCP-1 (1:250; BD Biosciences) overnight at 4 °C. Prior to use, the plates were blocked with PBS and 1% bovine serum albumin (BSA) for 1 h at room temperature (RT). Samples were added to the wells in a volume of 50µl plus 50µl of PBS-1% BSA and incubated for 2h at RT. The reaction was amplified with biotinylated monoclonal antibodies to murine IFN-γ (0.4µg/ml), TNF-α (0.2µg/ml), IL-4 (0.4µg/ml), IL-6 (0.2µg/ml), IL-8 (25µg/ml), IL-10 (50µg/ml), IL-12p70 (0.4µg/ml), IL-23 (Quantikine kit; all from R & D Systems),...
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**Results**

**c-di-GMP pretreatment reduces bacterial load in lungs of infected mice**

Balb/c mice were either treated intranasally with c-di-GMP (200nM) or PBS and 24hr later intranasally challenged with 1-3X10^7 CFU B. pertussis; strain Tohama I, Bacterial counts were determined at days 2, 4 and 6 post challenge. As shown in Figure 1, intranasal treatment with c-di-GMP resulted in a significant reduction of bacterial counts in lungs of infected mice at 2, 4 and 6 days post challenge (p<0.0001).

**c-di-GMP treatment increases cell recruitment to the lung**

To address whether treatment with c-di-GMP enhanced...
recruitment of immune cells to the lung, mice were intranasally treated with either c-di-GMP or equal concentrations of nucleotide control c-GMP. 24 hr post treatment but prior to challenge their lungs was removed and cell recruitment into the lung was assessed by histology using H&E staining. As shown in Figure 2, administration of c-di-GMP resulted in increased numbers of neutrophils and macrophages recruited into alveolar walls and spaces, as well as perivascular lymphoid infiltrations. Flow cytometry at 1, 2, 4 and 6 days post challenge revealed that c-di-GMP administration resulted in a fivefold increase of CD11c+ cells and about a fivefold increase of NK cells expressing DX5 compared to control mice (Table 1). No differences in the number of total aß-T cells or gδ-T cells were observed between the two groups (data not shown).

Enhanced cytokine and chemokine production following treatment with c-di-GMP

To define the potential mechanisms of enhanced dendritic and NK cell recruitment, we assessed the induction of cytokines and chemokines following treatment with c-di-GMP and challenge infection with B. pertussis. Administration of c-di-GMP resulted in significant production of IFN-g, TNF-a, IL-12p70 and IL-6 in the lungs of mice 24 hr post treatment and prior to challenge (P<0.008) and also at days 2, 4 and 6 post challenge (P<0.004; Figure 3). In contrast, pretreatment with c-di-GMP reduced IL-4 production (P<0.003) at days 2, 4 and 6 post infection, but did not significantly alter the expression of either IL-17 or IL-23 (Figure 3 and data not shown). However, pre-treatment with c-di-GMP significantly enhanced the production of MCP-1 prior to and at 24 hr post infection (P<0.004; Figure 3) as well as the expression of MIP-2, mRNA levels (>3 fold increase; Figure 4) at days 2 and 4 post challenge (P<0.005 and P<0.05 respectively). No changes in the expression of beta defensin 3 (BD-3) and beta defensin 4 (BD-4) were observed.

c-di-GMP treatment enhances the production of nitric oxide in vivo

Nitric oxide has been shown to be a necessary component of effective innate immunity against bacterial and fungal agents [44,46]. To investigate whether the enhanced clearance of bacteria observed in c-di-GMP treated mice was associated, in part, to increased production of NO, we assessed the presence of nitrite; the stable metabolite of NO, in c-di-GMP treated or control mice at 24 and 48 hr post intranasal infection with B. pertussis. As shown in Figure 5, mice treated with c-di-GMP produced significantly more NO in their lungs at 24 hr post treatment (P<0.005) and at 24 hr post challenge as compared to control animals (P<0.034).

Disease protection is not mediated by direct antimicrobial activity

To determine whether c-di-GMP has any direct inhibitory effects on the microorganism, the direct inhibitory effects of c-di-GMP were compared with known antimicrobial peptides (AMPs) in vitro. AMPs have both direct, broad-spectrum antimicrobial activity and the ability to modulate immune responses against a wide range of pathogens [47,48]. The antimicrobial activity of c-di-GMP against B. pertussis was tested and compared with PG1 and LL-37, previously tested in our lab, using inhibition assays. c-di-GMP was used at 5 and 150 µg/ml and PG1 and LL-37 were used at 5 and 50 µg/ml respectively. No differences in the number of CFU/ml were observed.
Figure 3: Cytokine (TNF-α, IFN-γ, IL-4, IL-6 and IL-12p70 and IL-17) and chemokine (MCP-1) concentrations in whole lung homogenates following c-di-GMP treatment and intranasal challenge infection with *B. pertussis*. Mice were treated with c-di-GMP (black bars) or control c-GMP (white bars) at 24hr prior to challenge infection. Whole lungs were removed and cytokine concentrations determined in lung homogenate supernatants at 24hr post treatment (24PT) and days two, four and six post challenge (Day 2-6). The results shown are the SD of cytokine and chemokine concentrations detected by ELISA from 3 separate experiments.

were used at concentrations of 5-10µg/ml. As shown in Figure 6, *B. pertussis* was completely resistant to killing by c-di-GMP while both the porcine PG-1 and the human cathelicidin LL-37 displayed strong antimicrobial activities resulting in 1000 to 10,000,000 fold reduction of bacterial numbers (p<0.009). Interestingly, even higher concentrations of c-di-GMP as well as prolonged incubation did not increase its inhibitory effect against *B. pertussis* (data not shown), indicating that c-di-GMP has no direct inhibitory effects on *B. pertussis* in vitro.

**Discussion**

In the present study we demonstrated that intranasal treatment of mice with c-di-GMP resulted in the induction of strong respiratory immune responses against infections with *B. pertussis*. These responses were characterized by early expression of MIP-2, a potent neutrophil recruiter, and MCP-1, a chemokine involved in the recruitment of monocytes, NK cells and iDCs and B cells. The immune responses were skewed towards a Th1-type response, which was further enhanced by challenge infection with *B. pertussis*. In contrast, c-di-GMP treatment reversed the induction of IL-4 production after bacterial infection. These findings are in line with previous studies indicating that Th1-helper T cells and their cytokines are necessary for effective clearance of *B. pertussis* [2,20,47,49-51]. These results are also consistent with a recent study in mice showing that treatment with c-di-GMP stimulates protective innate immune response and provides protection against *Klebsiella pneumoniae* challenge infection [33]. c-di-GMP was also proven efficacious in enhancing bacterial clearance of *S. aureus* in mice [34].

Treatment with c-di-GMP resulted in increased recruitment and influx of neutrophils and macrophages into the lung of c-di-GMP treated animals and induced significant production of MCP-1, which may have contributed to enhanced monocyte, NK cell and DC recruitment. These cells have all been implicated in providing protection against bacterial pathogens including *B. pertussis* [51]. For example, NK cells are potent producers of innate IFN-g in response to extracellular signals including the cytokines IL-12 and TNF-a [52]. NK cells provide resistance to *B. pertussis* by activating IL-12-mediated production of IFN-g, which enhances the antibacterial activity of monocytes/macrophages, but also promotes the differentiation of Th1 cells [20]. *B. pertussis* infection is more severe in IFN-g-/- mice [2], whereas *B. pertussis* disseminates from the lungs and causes organ failure in IFN-g R-/ mice [15]. Therefore, our findings suggest that NK cells through...
secretion of IFN-γ might play a significant role in bacterial dissemination, pathology and bacterial clearance in mouse lungs, possibly via activated macrophages [2,15]. In addition, other studies have shown that NK cells confer resistance to *B. pertussis* by IL-12 mediated production of IFN-γ, suggesting a role for IFN-γ early in infection [20]. Moreover, a defective TNF-α response has been shown as a potential risk factor for infection with *B. pertussis* [18]. Taken together, these results indicate that clearance of infection is dependent on a combination of innate immune responses mediated by IFN-γ, IL-12, TNF-α and other Th1 type cytokines but not IL-23 or IL-17.

Treatment with c-di-GMP also resulted in enhanced recruitment and activation of DCs and macrophages, possibly through increased release of chemoattractants such as MCP-1. Furthermore, the induction of enhanced type 1 cytokines in response to c-di-GMP treatment indicates that c-di-GMP can act directly on DCs which is in agreement with recent studies showing that c-di-GMP stimulated DC mediated immune responses by induction of cytokines, chemokines and increases the cell surface expression of maturation markers, leading to an overall Th1 type response [38].

The recruitment and/or activation of innate immune cells into the lungs prior to challenge infection likely contribute to improved bacterial clearance in c-di-GMP treated animals. Both macrophages and neutrophils have been shown to kill *B. pertussis in vitro*, however their mechanisms of killing, which may involve oxygen or nitrogen intermediates, are still not known [51]. It has been shown that NO is produced in *vitro* by alveolar macrophages stimulated with *B. pertussis* and by peritoneal macrophages following immunization with whole cell vaccine (Pw) or by alveolar macrophages following respiratory infection with *B. pertussis* [53-55]. IFN-γ can augment NO production by macrophages infected with *B. pertussis* [56]. Purified pertussis toxin has been shown to stimulate IFN-γ secretion through direct activation of T cells and subsequent NO production [57]. Moreover, production of NO by peritoneal macrophages from mice immunized with Pw has been shown to correlate with efficacy in the intra-cerebral (i.c.) challenge model of pertussis [58]. In addition, protection induced with Pw is compromised in NO synthase-deficient mouse [59], which indicates reactive nitrogen intermediates may function in intracellular killing of *B. pertussis*. Therefore, enhanced production of nitric oxide in the treated mice with c-di-GMP, possibly by alveolar macrophages, and type 1 cytokines such as IFN-γ, as inducer of iNOS may play a protective role in clearance of *B. pertussis*.

In the present study, we demonstrated that chemically derived c-di-GMP, while having no direct antimicrobial activity against *B. pertussis*, acted as an innate immune stimulator resulting in the expression of various cytokines and chemokines and activation of monocytes, granulocytes and DCs. This resulted in enhanced Th1 type immune response and improved clearance of bacteria from the lung. We suggest that c-di-GMP with such a broad biological activity can be utilized for clinical purposes in human and animals as an immunomodulator, immunoenhancer or vaccine adjuvant. Our study also highlights the importance of the innate immune response and Th1 type cytokines in induction of protection against *B. pertussis* infection.

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**References**


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