CpG Oligodeoxynucleotides Enhance Host Defense during Murine Tuberculosis

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ABSTRACT

Oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs activate immune cells to produce cytokines. CpG ODNs protect mice against infections with intracellular bacteria by the induction of a T helper 1 response. To determine the effect of CpG ODNs in pulmonary tuberculosis, mice were treated with CpG or control ODNs at the time of intranasal infection. CpG ODNs reduced mycobacterial outgrowth up to 5 weeks after *Mycobacterium tuberculosis* infection, which was associated with a decrease in inflammation in lung tissue. CpG treatment was associated with elevated levels of gamma interferon (IFNγ) and decreased levels of interleukin (IL)-4 in the lungs, and an increased capacity of splenocytes to secrete Th1 type cytokines. CpG ODNs given 2 weeks after infection were still able to reduce mycobacterial outgrowth and to enhance a Th1 response 5 weeks postinfection. Administration of CpG ODNs to IFNγ-/- mice failed to reduce mycobacterial outgrowth. These data suggest that CpG ODNs improve host defense during pulmonary tuberculosis by an IFNγ-dependent mechanism.
INTRODUCTION

Despite optimism after the introduction of antituberculous agents in the 1950s, tuberculosis (TB) remains the leading cause of death among infectious diseases, accounting for 2 million deaths annually (1). In addition to the increasing incidence of TB, there is a global emergence of drug-resistant strains, posing a threat to existing therapeutic possibilities (2). The case fatality rate for multi-drug resistant TB is 40-60%, which equals untreated TB (3). Hence, new therapeutic strategies are required for the control of TB.

Unmethylated CpG dinucleotides within bacterial DNA or synthetic oligodeoxynucleotides (ODNs) can activate immune cells (4). These sequence motifs are underrepresented in vertebrates (5), and it has been proposed that immune activation by CpG DNA has evolved as a result of evolutionary selections, contributing to host defense mechanisms that recognize invading microbial agents (6). CpG motifs can stimulate B cells, NK cells, T cells and macrophages to secrete cytokines (7). A number of studies indicate that CpG can switch on T helper 1 (Th1) immunity, with production of IgG2a immunoglobulins (8-10) and a Th1 dominated cytokine profile (7, 11-13). Indeed, protective Th1 biased immune responses could be induced by administration of CpG ODNs in animal models of Listeria and Leishmania infections (13-15).

It is well known that a Th1 immune response conveys protection against infection with M. tuberculosis (16, 17). Therefore, in this study, we investigated the effect of CpG ODNs in a murine model of pulmonary TB. Our results demonstrate that CpG ODNs protect against infection with M. tuberculosis by inducing a Th1 response.

METHODS

Mice

All experiments were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center. Balb/c mice (female, 7-8 weeks old, Harlan Sprague Dawley Inc., Horst, the Netherlands) were used. Each experimental group consisted of 8 mice per time point. In some experiments, IFNγ gene deficient (IFNγ-/-) mice (Jackson Laboratory, Bar Harbor, Maine) back-crossed to a Balb/c background were used.
**Experimental infection**

A virulent laboratory strain of *Mycobacterium tuberculosis* (H37Rv) was grown in liquid Dubos medium containing 0.01% Tween 80 for 4 days. A replicate culture was incubated at 37 °C and stirred gently, harvested at mid-log phase and stored in aliquots at -70 °C. Before each experiment, a vial was thawed and washed twice with sterile saline to clear the mycobacteria of medium. Mice were anaesthetized by inhalation with isoflurane, and lung infection was induced by intranasal inoculation with mycobacteria (10^5 CFU in 50 μl NaCl) as described previously by our and other laboratories (18-21). The inoculum was plated immediately after inoculation to determine viable counts. After 2 and 5 weeks mice were anaesthetized by FFM (fentanyl citrate 0.079 mg/ml, fluanisone 2.5 mg/ml, midazolam 1.25 mg/ml in H2O; of this mixture 7.0 ml/kg intraperitoneally (i.p.).

**Oligodeoxynucleotides (ODNs)**

Phosphorothioate ODNs, which are resistant to nucleases, were obtained from Eurogentec (Seraing, Belgium). The immunostimulatory CpG ODN had the sequence 5’ TCCATGACGTTCTGATGCT 3’. The control ODN in which the CpG motif was inverted had the sequence 5’ TCCATGAGCTTCCTGATCC 3’. 30 μg CpG or control ODNs was dissolved in 200 μl NaCl and i.p. injected 2 h prior and 6 hours after *M. tuberculosis* infection. This treatment schedule was based on a regimen that was found to be protective during murine leishmaniasis (13). For the postponed treatment experiment mice received 40 μg CpG or control ODNs i.p. 2 weeks post-infection and were sacrificed 5 weeks post-infection.

**Enumeration of mycobacteria**

The lungs and livers were harvested and homogenized in sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). 10-fold serial dilutions were plated on Middlebrook 7H11 plates containing OADC enrichment (Difco, Braunschweig, Germany) and incubated at 37 °C in sealed bags. Colonies were counted after 3 weeks.

**Preparation of lung tissue for ELISA**

Homogenates of lungs were diluted 1:1 with lysis buffer (0.5% Triton X-100, 150 mM NaCl, 15 mM Tris, 1 mM CaCl and 1 mM MgCl, pH 7.40) at 4° C for 20 minutes. Homogenates were then centrifuged at 14,000 rpm for 10 min. to remove cell debris, after which the supernatants were stored at -20° C. IFNγ, IL-4 (both R&D Systems,
Abingdon, United Kingdom) and tumor necrosis factor-α (TNFα; Genzyme, Cambridge, MA) were measured by ELISA according to the instructions of the manufacturer. Detection limits of the assays were 31 pg/ml.

**Spleen cell stimulation assays**

Single cell suspensions were obtained by crushing spleens through a 40 μm cell strainer (Becton Dickinson, Mountain View, CA). Erythrocytes were lysed with ice-cold isotonic NH₄Cl solution (155 mM NH₄Cl, 10 mM KHCO₃, 100 mM EDTA, pH 7.4), and the remaining cells were washed twice. Splenocytes were suspended in medium (RPMI 1640 (Bio Whittaker, Verviers, Belgium), 10% fetal calf serum, 1% antibiotic-antimycotic (GibcoBRL, Life Technologies, Rockville, MD)). Round bottom plates were coated overnight with anti-CD3 (clone nr. 145.2c11) and washed with sterile PBS. Splenocytes were seeded in 96-well round bottom culture plates at a cell density of 1×10⁶ cells in triplicate diluted with RPMI containing anti-CD28 (Pharmingen, San Diego, CA). Splenocytes were also stimulated with 20 μg/ml tuberculin purified protein derivative (PPD; Statens Seruminstitut, Copenhagen, Denmark). Supernatants were harvested after a 48-h incubation at 37°C in 5% CO₂, and cytokine levels were analyzed by ELISA.

**Histological Analysis**

Lung tissue samples were fixed in 10% neutral buffered formalin. After paraffin embedding, 4 μm sections were stained with hematoxylin and eosin or Ziehl-Neelsen (ZN) stain for acid fast bacilli. All slides were coded and semi-quantitatively scored for the total area of inflammation (% of surface of the slide) and granuloma format by a pathologist. Granulomas were defined as collections of 10 or more macrophages and lymphocytes widespread in the peripheral lung parenchyma (22).

**Statistical analysis**

Differences were compared using the Mann-Whitney-U test. For comparison of survival curves Kaplan-Meier analysis with a log rank test was used. *P*<.05 was considered statistically significant.
RESULTS

*CpG ODNs reduce mycobacterial outgrowth*

The inoculations with CpG ODNs were not associated with adverse effects. Treatment with CpG at the time of infection resulted in a reduction of the mycobacterial burden in lungs when compared to controls, which was present at 2 weeks (P=.001), and lasted until at least 5 weeks after infection (P<.001, Figure 1). The infection disseminated to the liver in both treatment groups. At 2 weeks, the mycobacterial load in livers of CpG-treated and control mice were similar (respectively 2.1 ± 2 × 10^3, 4.3 ± 1.8 × 10^3). At 5 weeks, CpG-treated mice displayed less *M. tuberculosis* CFUs in their livers than control mice, although this difference did not reach statistical significance (respectively 309 ± 139 x 10^3, 859 ± 317 x 10^3, P=.059).

*Cytokine concentrations in lung tissue*

A number of studies have demonstrated the Th1 stimulatory effect of CpG motifs (7, 11-13). IFNγ is considered the main Th1 cytokine, responsible for activation of macrophages and early T cell activation. To obtain insight in mechanisms contributing to the inhibitory effect of CpG on mycobacterial outgrowth, IFNγ as the principal Th1 cytokine and IL-4 as the principal Th2 cytokine were measured in lung homogenates of the mice used for bacterial counts in this study (Figure 2). CpG treated mice had higher IFNγ concentrations in the lung than mice treated with control ODNs. This difference was most obvious at 2 weeks (P<.05) and was diminished at 5 weeks (P=.06). IL-4 concentrations were lower in lungs of CpG treated mice when compared to controls (P<.05). Again, this difference appeared early in the course of infection with *M. tuberculosis* and had disappeared at 5 weeks. Therefore, the protective effect of CpG in
murine pulmonary tuberculosis seems to be associated with an enhanced Th1 and relatively diminished Th2 response at the site of infection. Macrophages are important sources of TNFα, which is essential for the formation of granulomas (23). Therefore, we measured TNFα levels in the lungs of mice. *M. tuberculosis* induced high levels of TNFα, but no differences between CpG treated mice and mice receiving control ODNs were found (data not shown).

**Figure 2**

CpG ODN treatment enhances IFNγ concentrations in lungs, and increases the IFNγ production capacity of splenocytes. Mice were treated with 2×30 μg CpG ODNs or control ODNs 2 hours before and 6 hours after intranasal infection with 10^5 CFU *M. tuberculosis*. Left panels: IFNγ and IL-4 concentrations in lung homogenates. Right panels: IFNγ and IL-4 concentrations in supernatants of 10^6 splenocytes stimulated *ex vivo* with anti-CD3/CD28. Data are mean ± SEM of 8 mice per group for each time point. *P<.05 versus control.*

**CpG increases the capacity of splenocytes to produce a Th1 cytokine profile**

Th1 response was further studied by harvesting splenocytes during *M. tuberculosis* infection, and the ability to release cytokines after stimulation with the non-antigen specific T cell stimulator anti-CD3/CD28 was analyzed. Splenocytes of CpG treated mice produced more IFNγ compared to splenocytes from controls after 2 weeks (*P<.05), but not after 5 weeks (Figure 2). Levels of IL-4 tended to be lower in splenocytes of CpG treated mice after 2 weeks, which did not reach statistical significance due to a large variation (*P=.11*). This suggests that CpG results in an enhancement of the ability of splenocytes to mount a Th1 response.

**CpG administration results in less inflammation in lungs of *M. tuberculosis* infected mice**

Two weeks after *M. tuberculosis* infection, control mice developed small well-defined granulomas composed primarily of lymphocytes and macrophages throughout the lung parenchyma (Figure 3). Moreover, peribronchiolar and perivascular lymphocytic infiltrates were present. CpG treated mice displayed less lymphocytic infiltrates. In
accordance with the degree of inflammation, acid-fast positive bacilli were much more abundant in control mice than in CpG-treated animals. After 5 weeks, the lungs of both groups showed more inflammation with confluent granulomas composed primarily of macrophages, granulocytes and lymphocytes. Still CpG-treated mice displayed less extensive inflammation of the lung parenchyma (50%) than untreated mice (70%).

![Image](attachment:figure1.jpg)

**Figure 1.** Histopathology of lung tissue. a and c, original magnification x110 and five weeks (c and d, original magnification x70) after *M. tuberculosis* infection. Mice treated with CpG (b and d) show less inflammation than control ODNs treated mice (a and c). Slides are representative for at least 5 mice per group for each time point.

**Survival experiments**

To determine whether CpG had an effect on survival of *M. tuberculosis* infection, 10 mice injected with CpG and 10 mice injected with control ODNs were followed for 4 months. During this observation period, 50% of the CpG treated mice died versus 60% of the control mice (nonsignificant, Figure 4). Considering that the enhancement of a protective Th1 response after single CpG treatment was diminished after 5 weeks when compared to the more profound effect after 2 weeks, another survival experiment was carried out in which CpG administration was repeated two weeks after infection with *M. tuberculosis*. After 4 months, 40% of control mice had died, whereas all CpG treated mice were still alive (*P*=.03, Figure 4).
Protection by CpG is abrogated in IFNγ gene deficient mice

Protection conferred by CpG against *M. tuberculosis* was associated with elevated levels of lung IFNγ. Considering that IFNγ is essential for host defense against TB (17, 24), we wished to determine whether this protection was mediated by IFNγ. Thus, TB was induced in IFNγ-/− mice, which were sacrificed 2 weeks after infection. As has been shown before (17, 24), IFNγ-/− mice had higher numbers of mycobacteria in their lungs compared to wild types (P<.001, Figure 5).

Consistent with the previous results, wild-type mice receiving CpG had lower numbers of mycobacteria in their lungs than mice receiving control ODNs (P<.001). However, in
IFNγ/- mice, the difference in mycobacterial outgrowth between mice receiving CpG or control ODNs was abolished, indicating that protection by CpG is IFNγ dependent.

Postponed treatment with CpG still enhances host defense

To determine the effects of CpG as a possible therapeutic agent, mice received 40 μg CpG ODNs or control ODNs i.p. 2 weeks postinfection, and were sacrificed 5 weeks postinfection. At this time point, the lungs of CpG-treated mice contained 6-fold less viable mycobacteria when compared with the lungs of control ODN-treated animals (P=.027) (Figure 6). To examine the influence of postponed treatment with CpG on the development of Th1/Th2 responses, splenocytes harvested from these mice at 5 weeks postinfection were stimulated ex vivo. In these experiments we also evaluated the ability of CpG treatment to enhance an antigen-specific Th1 response using PPD to stimulate splenocytes. As shown in figure 7, splenocytes from CpG-treated mice released more IFNγ in response to either PPD or anti-CD3/28 Abs (P=.027 and P=.046). IL-4 was not detectable in supernatants of splenocytes stimulated with PPD, but tended to be lower in supernatants of anti-CD3/CD28 stimulated splenocytes from CpG-treated animals compared to control ODN-treated mice (nonsignificant). These data suggest that CpG ODNs given 2 weeks after the infection were still able to promote a protective Th1 response.

DISCUSSION

In this study, administration of CpG ODNs to mice infected intranasally with M. tuberculosis resulted in enhanced survival and a reduction in mycobacterial burden in the
pulmonary compartment. The beneficial effect of CpG, which was associated with a Th1 type immune response, was mediated by IFNγ, as indicated by the absence of protection in IFNγ−/− mice.

The present data are in line with previous reports on the ability of bacterial DNA containing unmethylated CpG motifs or synthetic CpG ODNs to protect against intracellular microorganisms (13-15). Of interest is also the observation that mice vaccinated with BCG and CpG ODNs have an increased Th1 response and a reduced mycobacterial outgrowth after infection with *M. tuberculosis* (25). It should be noted that CpG-induced protection against different types of microorganisms shows kinetic differences. In infection with *Listeria monocytogenes*, at least 48 hours was required between the time of CpG administration and the time of injection of the pathogen, presumably reflecting the time necessary to upregulate the innate immune responses needed to control this rapidly replicating microorganism (14). In contrast, CpG exerted protective effects against the more indolent pathogen *Leishmania major* even when its administration was delayed until 20 days after infection (13). We add to these data that CpG reduced the growth of *M. tuberculosis* in mouse lungs even when the administration of CpG was delayed for 2 weeks postinfection, and that this postponed treatment was associated with an antigen-specific Th1 response. In accordance, the same CpG ODNs as used in the present investigation directed an antigen-specific immune response toward a Th1 phenotype in mice with *Leishmania major* infection (13). Together these findings confirm
the notion that CpG ODNs can enhance both nonspecific innate immune defense mechanisms and antigen-specific immune responses (4). Recent evidence indicates that CpG activates immunocompetent cells via Toll-like receptor 9, and that mice deficient for this receptor cannot mount a Th1 response upon exposure to CpG (26).

The CpG mediated protection we observed was associated with elevated levels of IFNγ in the lung, which presumably are produced by both stimulated T cells and NK cells. In addition, protection was related to an increase in the IFNγ-producing capacity of splenocytes, both in an antigen and a non-antigen specific manner. The disappearance of the protective effect of CpG in IFNγ-/- mice indicates that CpG-induced protection against TB was dependent on IFNγ. This was also found in mouse models of asthma (12) and listeriosis (14). Although not studied, a protective effect of CpG ODNs on cells of the innate immune system can also be expected in our experiments. Several studies have been shown that CpG motifs can activate macrophages (27, 28), dendritic cells (29, 30) and are able to indirectly activate NK cells (31).

Protection by a single administration of CpG lasted up to 27 days in murine leishmaniasis (13) and for 2 weeks in infection with L. monocytogenes (15). We found that the protective effect of CpG during TB lasted at least 5 weeks. This difference may be explained by the lower replication time of mycobacteria compared to other pathogens. When the time course of protection is coupled to the time course of bacterial growth, protection by CpG may be the result of activation of cells of the innate immune system, which limits mycobacterial replication until an antigen-specific T cell response has developed. Interestingly, in the experiments in which CpG ODNs were given simultaneously with M. tuberculosis, Th1 cytokine levels in the lung as well as in supernatants of stimulated splenocytes were elevated 2 weeks, but not 5 weeks after infection. Apparently, an early protective Th1 response is sufficient to reduce mycobacterial burden at later time points. However, survival was improved only when CpG administration was repeated after 2 weeks. This latter finding is in line with earlier reports demonstrating that repeated administration of CpG ODNs can extend the duration of protection against certain pathogens (4). Importantly, repeated administration of CpG was without overt toxicity, confirming previous studies (4, 13, 32). Further studies are warranted to establish the CpG regimen that confers optimal protection against M. tuberculosis.

In previous studies, exogenous administration of IFNγ has been shown useful as an adjuvant therapy for TB (33, 34). CpG treatment results in endogenous production of
IFNγ, which is subject to regulatory pathways of the innate immune response. Presumably, this may be associated with less toxicity compared to exogenous IFNγ administration. Another promising feature of CpG treatment is its long-term protection, requiring infrequent dosing. It should be noted, that CpG may initiate inappropriate immune responses and has been reported to induce septic shock in mice, and to prime mice for the Shwartzmann reaction (35, 36). However, current insights indicate that CpG ODNs can be given relatively safely at doses that increase host defense mechanisms (4). Therefore, the present data may provide the rationale for further development of CpG as a new adjunctive therapy for TB.

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REFERENCES


