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THERAPY

Effect of Calcium Carbonate Encapsulation on the Activity of Orally Administered CpG Oligonucleotides

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Synthetic oligodeoxynucleotides containing unmethylated CpG motifs (CpG ODNs) stimulate immune cells via Toll-like receptor 9 (TLR9). Because oligodeoxynucleotides (ODNs) are susceptible to gastric degradation, clinical trials designed to evaluate their therapeutic utility have relied solely on parenteral routes of administration. A strategy to improve the activity of orally delivered ODNs by reducing their susceptibility to gastrointestinal (GI) digestion via encapsulation in calcium carbonate nanoparticles (ODNcaps) was recently described. This study compares the in vitro and in vivo activity of encapsulated (ODNcaps) versus free CpG ODNs delivered orally or parenterally. ODNcaps mirrored the ability of free ODNs to stimulate splenic B cells and macrophages in vitro. ODNcaps activated immune cells in the Peyer's patches and mesenteric lymph nodes after oral delivery. Their effect on GI immunity was evaluated in studies of dextran sulfate sodium (DSS)-induced colitis and enteric infection, whereas systemic immunity was examined by monitoring their effect on lipopolysaccharide (LPS)-induced cytokine production and systemic pathogen challenge. Results indicate that orally delivered CpG ODNs predominantly induce GI rather than systemic immunity, and that calcium carbonate encapsulation does not significantly alter this behavior.

INTRODUCTION

The innate immune system uses Toll-like receptors (TLRs) to sense evolutionary conserved pathogen-associated molecular patterns.[1–3] For example, TLR9 recognizes unmethylated CpG motifs that are abundant in bacterial and viral, but not eukaryotic, DNA.[2,4–6] Murine cells that express TLR9 include B lymphocytes, dendritic cells, and macrophage.[7–11] When stimulated with CpG DNA, these cells are activated to produce immunoglobulin (Ig) and/or pro-inflammatory cytokines that support the induction of an adaptive immune response.[7,12,13] Synthetic oligodeoxynucleotides containing unmethylated CpG motifs (CpG ODNs) mimic this stimulatory activity and are under clinical evaluation for the prevention or treatment of infection, allergy, and cancer.[14–16] CpG ODNs activate immune cells within minutes, increase host resistance to infection within days, and improve anti-tumor immunity over weeks.[7,13,17–19] Therapy is most effective when initiated early and delivered repeatedly.[20,21] Treatment

would be simplified if these TLR9 agonists could be delivered orally rather than by injection. Unfortunately, CpG ODNs are susceptible to degradation by the harsh conditions present in the gastrointestinal (GI) tract (low pH and nucleases). Because oral delivery significantly reduces oligodeoxynucleotide (ODN) activity,[22] their utility in phase I–III trials has been evaluated only after parenteral administration (intravenously [i.v.], intraperitoneally [i.p.], subcutaneously [s.c.], or intramuscularly [i.m.]).[16]

Yet, orally delivered CpG ODNs can affect systemic immunity. For example, mice fed CpG ODNs showed increased resistance to bacterial infection[23] and mounted stronger responses to co-administered Ag.[24] To improve the bioavailability of ODNs after oral delivery, Wang et al.[22] encapsulated ODNs in calcium carbonate nanoparticles (ODNcaps). The resultant ODNcaps were much more stable and retained significantly greater in vitro activity than free ODNs after incubation in simulated gastric fluid. Following oral delivery, ODNcaps were taken up by macrophage in the Peyer's patches (PP) and were found to alter host susceptibility to topically induced dermatitis.[22] Yet, Wang et al.[22] did not compare the activity of ODNcaps with unencapsulated ODNs delivered either orally or parenterally. This work addresses that issue by evaluating the activity of free versus encapsulated CpG ODNs under multiple conditions. Results from in vitro and in vivo studies indicate that calcium carbonate encapsulation does not improve the stimulatory activity of orally delivered CpG ODNs.

RESULTS

Comparative Activity of Free versus Encapsulated CpG ODNs In Vitro

To examine whether calcium carbonate encapsulation alters the activity of CpG ODNs, we compared their ability to stimulate BALB/c spleen cells to secrete interleukin (IL)-12, interferon γ (IFNγ), IL-6, and tumor necrosis factor alpha (TNF-α) (widely accepted metrics of CpG activation) with that of free ODNs in vitro. Consistent with
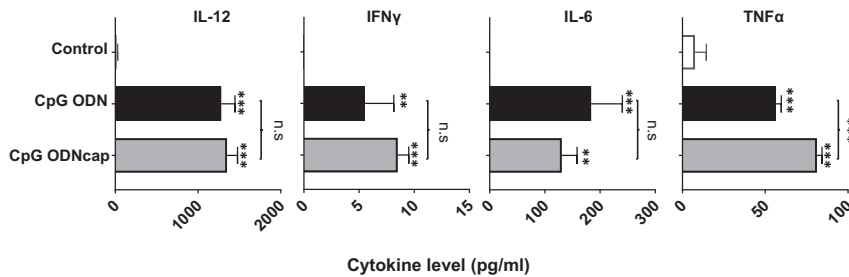
Figure 1. In Vitro Response of Spleen Cells to CpG ODNcaps

Splenocytes from BALB/c mice (n = 4 independently analyzed donors) were stimulated with 6 μg/mL free or encapsulated CpG ODN for 48 hr. All ODNs used in all studies were phosphorothioate modified. Cytokine levels in culture supernatants were measured by ELISA. Both CpG ODNs and CpG ODNcaps increased the production of IL-12, IFNγ, IL-6, and TNF-α. **p < 0.01; ***p < 0.001 versus unstimulated controls. There was no significant difference in IL-6, IFNγ, or IL-12 levels stimulated by CpG ODNs versus CpG ODNcaps, although TNF-α levels were higher in the CpG ODNcap treatment group (p < 0.05).

previous reports, free CpG stimulated splenocytes to increase their production of pro-inflammatory cytokines (p < 0.01 versus unstimulated controls; Figure 1).[7,13] CpG ODNcaps had equivalent effects on the secretion of IL-12, IFNγ, and IL-6 and induced moderately higher levels of TNF-α when compared with free ODN (Figure 1).

To identify the cells responding to CpG ODNcaps, we stained splenocytes to identify TLR9-expressing B cells (CD19+) and macrophage (F4/80+) and TLR9-negative T cells (CD3+). B cells cultured with free or encapsulated ODNs increased their production of IL-6 and IgM by ≈ 5-fold (p < 0.05; Figure 2). Macrophages responded with a 2- to 4-fold increase in IL-12 and IL-6 production following either type of stimulation (p < 0.01; Figure 2). The response of both cell types to free versus encapsulated CpG ODNs was equivalent. In contrast, T cells (which do not express TLR9) failed to respond to stimulation with either form of CpG ODN (Figure 2).

Localization of the In Vivo Response to Orally Administered CpG ODNcaps

Wang et al.[22] reported that IFNγ production increased in the PPs following 3 days of oral CpG ODNcap administration. To verify that observation and determine whether other sites were also responding, 10 μg of CpG ODNcap (the amount used by Wang et al.[22]) was delivered by gastric gavage to BALB/c mice for 3 or 10 days. Cells were harvested from the PPs, mesenteric lymph node (LN), and spleen and stained to detect intra-cytoplasmic cytokines. Administering CpG ODNcaps for 3 days triggered cells in the PPs to secrete IL-6, IL-12, and IFNγ (p < 0.05; Figure 3). When continued for 10 days, CpG ODNcap treatment stimulated cells in the mesenteric LN to secrete cytokine as well. No change in spleen cell activation was detected after 3 or 10 days of ODN administration (Figure 3).

Effect of Orally Delivered CpG ODNcaps on DSS-Induced Colitis

The above findings confirm that orally administered CpG ODNcaps stimulate immune cells that reside in gut-associated lymphoid tissue (GALT). A murine model of dextran sulfate sodium (DSS)-induced colitis was used to assess whether this influenced the development of GI inflammation. DSS disrupts the colonic epithelium, thereby facilitating the invasion of intestinal microbes through the mucosa, resulting in inflammation characterized by weight loss, diarrhea, and rectal bleeding.[25–27] Previous studies showed that systemically

delivered CpG ODNs exacerbated the severity of DSS-induced colitis by enhancing T helper 1 (Th1) responses.[28–31]

Inflammation was elicited by delivering 2% DSS in drinking water to C57/Bl6 mice for 8 days. Animals were also treated with 25 μg of free or encapsulated CpG ODNs every other day for 2 weeks, a dose slightly higher than that which increased disease when delivered systemically.[28–31] During the inductive phase of disease (days 3–8), DSS-induced colitis was significantly worsened by the co-delivery of free CpG ODNs (p < 0.01; Figures 4B and 4C). CpG treatment also slowed recovery during the resolution phase of disease (days 9–12, note the difference in recovery slope; p < 0.01; Figure 4C). Consistent with this longer and more severe course of disease, colon length was decreased significantly in CpG ODN-treated mice when compared with mice that received DSS alone (p < 0.05; Figure 4D). There was no difference in weight loss, disease severity, or colon length between mice treated orally with free versus encapsulated ODNs.

Effect of Orally Delivered CpG ODNcaps on the Systemic Inflammation Induced by LPS

Orally delivered ODNcaps did not activate spleen cells or increase serum cytokine levels (Figure 3 and data not shown), suggesting that their effect might be limited to the GALT. Yet, orally delivered ODNcaps might alter host sensitivity to systemic immune challenge, an effect previously observed in mice treated parenterally with free CpG ODNs.[32,33] To examine this possibility, we delivered free or encapsulated ODNs to mice 3 hr before lipopolysaccharide (LPS) challenge.

Consistent with earlier reports, systemic delivery of 50 μg of CpG ODNs significantly increased the cytokine response elicited by LPS (p < 0.05; Figure 5).[32,33] In contrast, neither free nor encapsulated ODNs had any effect on LPS-induced TNF-α or IL-6 production after oral delivery (Figure 5).

Effect of Orally Delivered CpG ODNcaps on Protection from Infection

A highly sensitive means of assessing the in vivo activity of CpG ODNs involves their ability to protect the host from infectious challenge. Many groups have shown that as little as 20 μg of parenterally administered CpG ODNs protects mice from a diverse array of
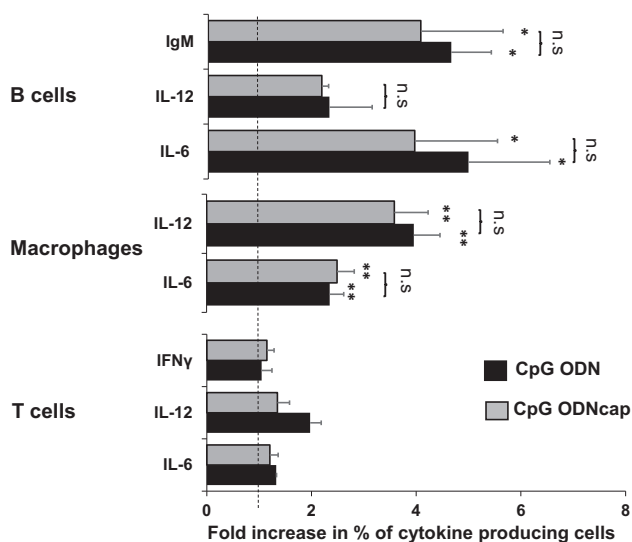
Figure 2. In Vitro Response of Lymphocytes and Macrophage to CpG ODNcaps

BALB/c spleen cells were cultured with 1 μM CpG ODN or CpG ODNcap for 24 hr. Data show the fold increase in percentage of IL-6-, IL-12-, and/or IgM-producing cells compared with unstimulated controls (dashed line). Results represent the mean + SD of two to four independent experiments. Note that free and encapsulated CpG ODNs were equally effective at stimulating cytokine and IgM production by B cells and macrophage. *p < 0.05; **p < 0.01 versus unstimulated cells.

pathogens including *Listeria monocytogenes.*[17,21,34,35] Ray and Krieg[23] demonstrated that higher doses of orally administered CpG ODNs (100–200 μg) also protected against systemic and enteric Listeria infection. Building on those results, mice were treated with 100 μg of free or encapsulated CpG ODNs and challenged 3 days later either systemically (by i.p. injection) or enterically (by gavage) with *L. monocytogenes.* Bacterial burden in the liver (the dominant site of Listeria replication) was monitored 4 days later.

Consistent with previous reports, parenteral treatment with CpG ODNs reduced bacterial load following systemic Listeria challenge by nearly four orders of magnitude (p < 0.0001; Figure 6A). A significant reduction in pathogen burden was also observed in mice treated orally with free CpG ODNs (p < 0.05), although that route provided less protection than i.p. treatment (p < 0.01). There was no significant difference in the protection conferred by free versus encapsulated CpG ODNs after oral delivery (Figure 6A).

A different outcome was observed when CpG-treated mice were challenged with Listeria by the oral route. Consistent with evidence that gastric administration preferentially activates GI immunity, oral CpG ODNs matched the efficacy of parenteral delivery at improving host resistance to enteric pathogen challenge (Figure 6B). As above, free and encapsulated ODNs were equally protective (Figure 6B).

Previous studies indicate that repeated parenteral administration of CpG ODNs can increase both the duration and the magnitude of

host resistance to infection.[21] To determine whether repeated oral delivery of CpG ODNs would duplicate this effect, we treated mice with free or encapsulated CpG ODNs by gavage for 7 days and then challenged i.p. with *L. monocytogenes.* As seen in Figure 6C, repeated oral treatment failed to achieve the level of protection conferred by a single parenteral dose of CpG ODNs (p < 0.05), and encapsulation did not change this outcome.

DISCUSSION

Immunomodulatory ODNs have been used successfully to treat infectious diseases, allergy, and cancer in animal models.[17–20,36] Beneficial outcomes were optimized by treating early and repeatedly in the disease process.[21] For uses ranging from pre-exposure anti-bacterial prophylaxis to tumor immunotherapy, oral administration would simplify ODN-based treatment. Yet, ODNs are highly susceptible to degradation in the GI tract, requiring one to two orders of magnitude higher doses to achieve systemic stimulation comparable with that elicited by parenteral delivery.[23,24,37,38] One strategy to overcome this limitation was described by Wang et al.,[22] who reported that ODNs encapsulated in calcium carbonate nanoparticles could resist acid and nuclease degradation by the GI tract.

In vitro studies show that free and encapsulated CpG ODNs both stimulated cells that express TLR9, including B lymphocytes and macrophage (Figure 2).[7,13] Wang et al.[22] documented that 3 days of oral treatment with CpG ODNcaps triggered cells in the PPs to secrete IFNγ. Current findings confirm and extend that result by demonstrating that CpG ODNcaps trigger cells in the mesenteric LN, as well as PPs, to produce an array of pro-inflammatory cytokines (Figure 3). Consistent with a generalized effect on GI immunity, oral delivery of CpG ODNs increased the severity of DSS-induced colitis while reducing susceptibility to enteric pathogen challenge (Figures 4 and 6). Yet, ODNcaps were no more effective than free CpG ODNs in mediating these outcomes.

Oral administration of ODNcaps did not affect serum cytokine levels or induce spleen cell activation. These findings suggested that oral delivery of ODNcaps might induce GI-restricted, rather than systemic, immune responses. Additional studies were performed to evaluate whether encapsulation improved the ability of orally delivered CpG ODNs to alter systemic immunity. In this context, Kitagaki et al.[37] reported that although oral CpG ODNs did not stimulate a systemic Th1 response, it could reduce allergen-mediated eosinophilic airway inflammation. Similarly, Wang et al.[22] reported that 70 days of CpG ODNcap treatment aggravated chemically induced atopic dermatitis. Our findings show that CpG ODNcaps did not enhance cytokine responses in an LPS challenge model (Figure 5). In a highly sensitive pathogen challenge model, encapsulation did not improve the protection conferred by orally delivered free CpG ODNs (Figure 6).

These findings should not be attributed to technical problems in ODNcap formulation because ODNcap activity was equivalent to that of free ODNs in vitro (where GI-mediated degradation was not a factor). Similar in vitro activity was also observed in preliminary
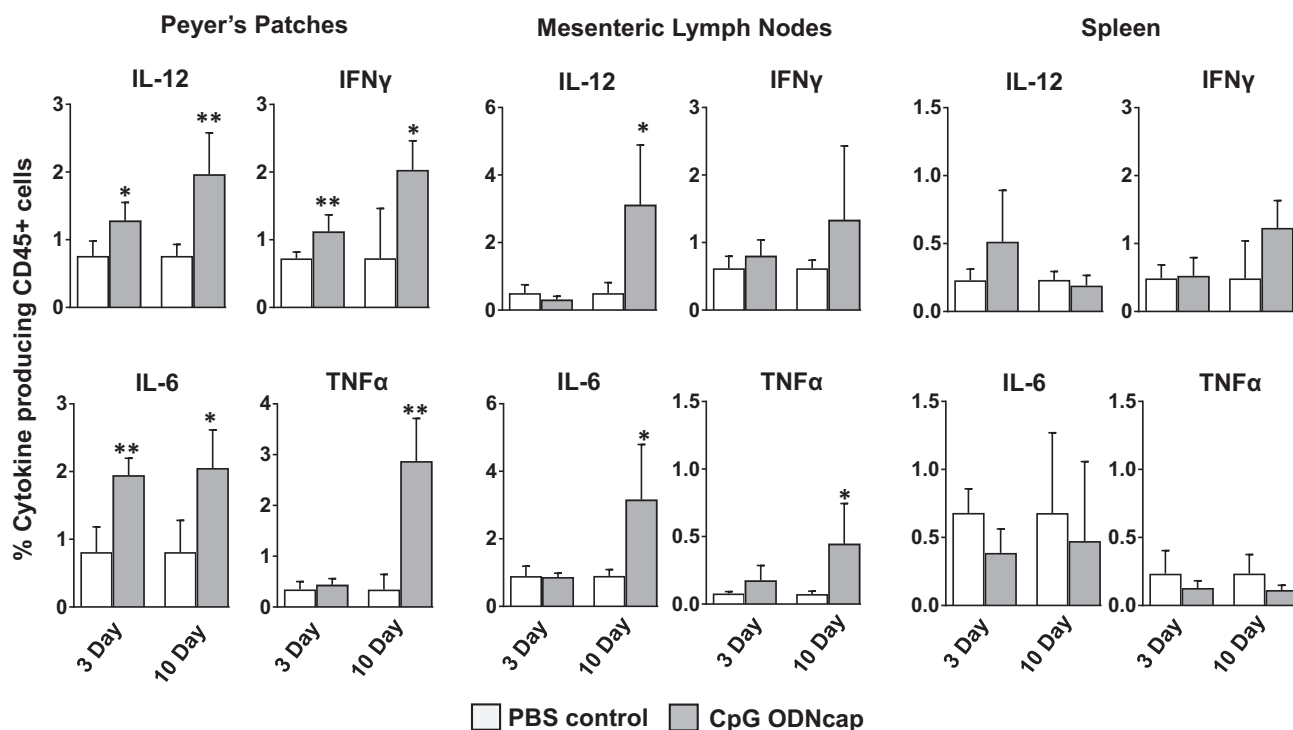
Figure 3. In Vivo Response to CpG ODNcaps

10 µg of CpG ODNcap or PBS was delivered by gastric gavage to BALB/c mice for 3 or 10 consecutive days. The percentage of CD45^{+} cells secreting IL-12, IFNγ, IL-6, and TNF-α in the (left) Peyer's patches, (middle) mesenteric lymph nodes, and (right) spleen is shown. Data represent the mean + SD of three to eight mice per group from three independent experiments. *p < 0.05; **p < 0.01 versus controls.

studies comparing the activity of free versus encapsulated ODNs encoding suppressive and control ODNs, and comparative studies evaluating the in vitro and in vivo activation induced by CpG ODNcaps formulated in-house versus those kindly provided by Wang et al.[22] (see Acknowledgments). Thus, current findings indicate that (1) calcium carbonate encapsulation does not improve the activity of orally delivered CpG ODNs, and (2) orally administered free or encapsulated CpG ODNs preferentially modulate GI rather than systemic immunity. Further innovation is needed to identify a means of modifying ODNs to enable oral delivery that can effectively substitute for parenteral administration in the treatment of cancer, allergy, and systemic infection.

MATERIALS AND METHODS

Study Approval
All rodent experiments were reviewed and approved by the Animal Care and Use Committee of the National Cancer Institute (NCI)-Frederick.

Reagents
Phosphorothioate CpG ODN 1555 (5′-GCTAGACGTTAGCGT-3′) were synthesized at the Center for Biologics Evaluation and Research core facility. CpG ODNcaps were synthesized using these phosphorothioate ODNs under endotoxin-free conditions following the protocol provided by Wang et al.[22] In brief, 2 mg of ODN was dissolved in

1 mL of endotoxin-free water. 53 mL of 1 M $CaCl_2$ was added under constant stirring, and the mixture was incubated at 37°C for 20 min followed by the addition of 345 mL of DMEM-Hi glucose. After 20-min incubation at 37°C, the solution was centrifuged at $3,000 \times g$ for 20 min and the amount of non-encapsulated ODNs measured in the supernatant, yielding an encapsulation efficiency of ≈70%. The pellet of encapsulated ODNs was re-suspended to a concentration of 1 mg/mL, aliquoted, and stored at −80°C until use.

Cell Culture
Single-cell suspensions were prepared from BALB/c spleens (NCI) and cultured at 10^6 cell/mL in 24-well plates (Corning) in RPMI 1640 (Lonza) supplemented with 5% heat-inactivated fetal calf serum, 100 U/mL penicillin, 100 mg/mL streptomycin, 25 nmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mmol/L sodium pyruvate, non-essential amino acids (NEAAs), and 0.0035% 2-mercaptoethanol (2-ME) at 37°C in a 5% CO_2 in air incubator. Cells were stimulated with the indicated concentrations of CpG ODNs or CpG ODNcaps for 24–48 hr.

Detection of Cytokine-Producing Cells
BALB/c splenocytes were cultured with 1 µM CpG ODN or CpG ODNcap for 24 hr with brefeldin A (GolgiPlug; used 1:1,000 diluted, according to the manufacturer's instructions; BD Biosciences) added
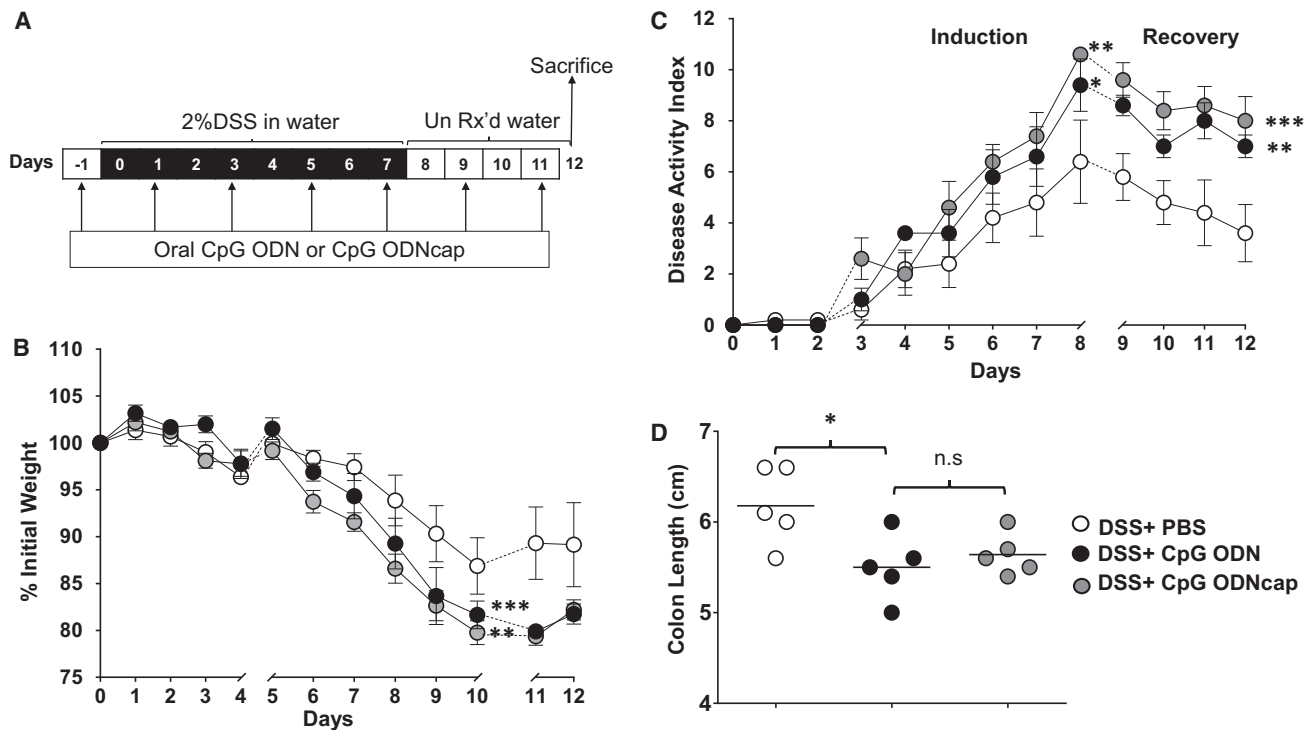
Figure 4. Effect of CpG ODNcaps on DSS-Induced Colitis

(A) C57BL/6 mice received 2% DSS for 8 days followed by regular water for 4 days. Mice were treated with 25 μg of CpG ODNcap or free CpG ODN by gavage every other day. (B and C) Animals were monitored for weight loss (B) and disease activity (C) over time. (D) Colon lengths were measured from the ileocecal junction to rectum on day 12. Data represent the mean ± SE of five mice per group. *p < 0.05; **p < 0.01, ***p < 0.001 versus DSS-treated controls. This experiment was repeated using 1% DSS administration and yielded similar results. There was no difference in any disease parameter between mice treated with CpG ODNcaps versus free CpG ODNs in either experiment.

for the last 5 hr. Cells were then incubated with Fc Block (BD Biosciences) and stained with labeled Ab specific for CD45 (Clone 30-F11; BioLegend), CD19 (Clone 6D5; BioLegend), CD3e (Clone 145-2C11; BD Biosciences), or F4/80 (Clone BM8; BioLegend). Cells were then fixed and permeabilized using the BD Cytofix/Cytoperm kit and stained with PE or PE-Cy7-labeled Ab specific for IL-6 (Clone MP5-20F3; BioLegend), IgM (Clone R6-60.2; BD Biosciences), IL-12 (Clone C17.8; eBioscience), IFNγ (Clone XMG1.2; BioLegend), or appropriate isotype controls.

In Vivo Administration of CpG ODNcaps

10 μg CpG ODNcap in 100 μL of PBS was administered by gastric gavage to BALB/c mice for 3–10 consecutive days. Mice were then sacrificed, and PPs, mesenteric LNs, and spleens were harvested. Single-cell suspensions were prepared from each organ and cultured in six-well plates at a density of 5×10^5 cell/mL in the presence of brefeldin A for 12 hr. Cells were then incubated with Fc Block (BD Biosciences), stained with labeled anti-CD45 Ab, fixed, permeabilized, and counter-stained with cytokine-specific Abs or isotype controls as described above.

Acute Colitis Model

9- to 10-week-old female C57/B6Ncr mice (Jackson Laboratories) received 2% DSS (molecular mass 36,000–50,000 kDa; Gojira Fine

Chemicals) in drinking water for 8 days. 25 μg of CpG ODN, CpG ODNcap, or PBS in 400 μL was administered by gastric gavage on day −1 and every other day until the study ended. Free ODN was prepared in 1.5% sodium bicarbonate buffer, whereas CpG ODNcap was prepared in PBS. There was no difference in fluid consumption between groups.

Assessment of Disease Activity and Colon Length

Mice were monitored daily for weight loss, stool consistency, and rectal bleeding. These parameters were used to assess disease activity (modified from Cooper et al.[25]): weight loss: 1%–5% = 1, 5%–10% = 2, 10%–15% = 3, >15% = 4; stool consistency: normal stool = 0, loose stool = 2, watery diarrhea = 4; rectal bleeding (assessed by Hemoccult SENSA): negative = 0, faint blue = 1, strong blue = 2, visible blood on feces = 3, gross blood around anus = 4. The disease activity index (DIA) represents the sum of all three parameters. Colon length on day 12 was measured from the ileocecal junction to the rectum.

LPS-Induced Inflammation Model

50 μg of free or encapsulated CpG ODN was administered by gavage or i.p. injection in 200 μL to BALB/c mice. 3 hr later, the mice were challenged i.p. with 15 μg of LPS (from *E. coli* serotype O55:B5; Sigma), and serum cytokine levels were measured 3 hr after challenge.
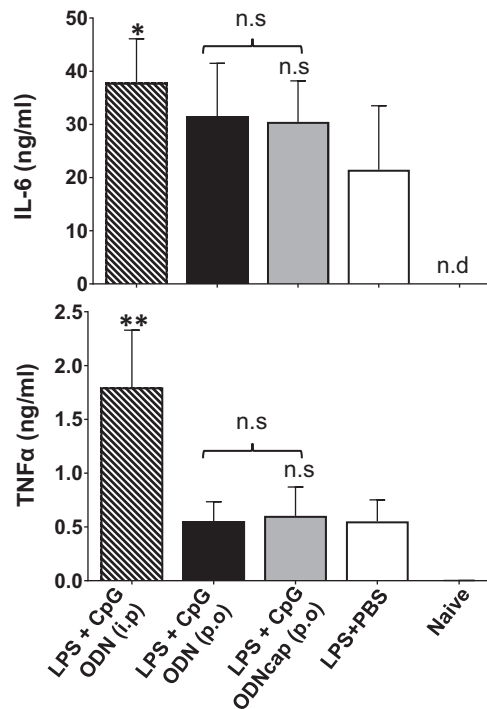
Figure 5. Effect of Oral CpG ODNcaps on LPS-Induced Cytokine Production In Vivo

50 μg of CpG ODN or CpG ODNcap was delivered i.p. or by gavage to BALB/c mice. 3 hr later, the mice were challenged i.p. with 15 μg of LPS. Data represent the mean + SD of serum cytokine levels of five mice per group performed 3 hr after LPS injection. *p < 0.05; **p < 0.01.

Figure 6. Effect of Oral CpG ODNcaps on Host Resistance to Local and Systemic Listeria Challenge

BALB/c mice were treated parenterally (i.p.) or orally with 100 μg of free or encapsulated CpG ODN. (A–C) Animals were challenged i.p. with 5×10^3 CFUs of *L. monocytogenes* (A and C) or orally with 6×10^9 CFUs (B). (C) Mice were treated once with parenteral ODNs but orally for 7 consecutive days before challenge. Data show the mean + SD of bacterial counts (CFUs) per gram of liver harvested 4 days after challenge from four to five mice per group. *p < 0.05; **p < 0.01; ****p < 0.0001 versus PBS-treated controls (A and B) or i.p. CpG ODNs (C).

Cytokine ELISA

Cytokine levels in serum and culture supernatants were measured by ELISA as previously described.[39] In brief, Immulon H2B plates were coated with Ab against IL-6, IL-12, IFNγ (BD Biosciences), or TNF-α (R&D Systems). The plates were blocked with PBS/2% BSA, incubated with cytokine-containing samples, and developed using biotin-labeled secondary Ab followed by alkaline phosphatase (AKP) streptavidin (BD Biosciences) and p-nitrophenyl phosphate (pNPP) substrate (Southern Biotech). Known amounts of recombinant purified cytokine were included for quantification.

Bacterial Challenge

L. monocytogenes EGD strain was grown to log phase in brain heart infusion (BHI) medium (BD Bacto) in a shaking incubator at 37°C, 180 rpm. Bacteria were frozen in BHI medium with 20% glycerol at −80°C and thawed immediately prior to use. Mice were treated with 100 μg of free or encapsulated CpG ODNs either i.p. or orally. They were challenged i.p. with 3–5 × 10^3 colony-forming units (CFUs) or orally with 2–6 × 10^9 CFUs in 200 μL of PBS 3 days after a single ODN treatment or 1 day after seven daily treatments. Bacterial counts in the liver were examined 4 days after challenge by serial 10-fold dilutions of liver suspensions on BHI agar plates (BD Difco).
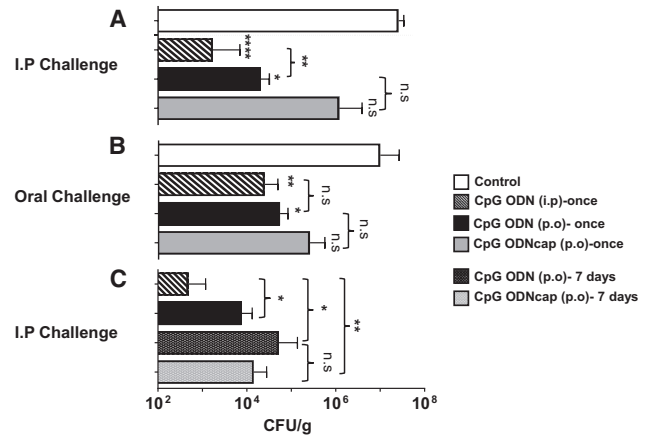
Statistics

Statistical analyses were performed using GraphPad Prism 7.01 and R. Differences between CpG-treated and control groups were assessed by ANOVA followed by Tukey's tests to control for type I errors when more than two groups were compared. A Student's two-tailed t test was used when exactly two groups were being compared. For Listeria challenge studies, statistical analyses were performed on log-transformed data. Longitudinal DIA and weight loss data were analyzed using a linear mixed-effects repeated-measures regression model that takes into account within-mouse correlated responses over time. For those analyses, the F-statistic (from ANOVA) was used to determine treatment group differences.

AUTHOR CONTRIBUTIONS

Conceptualization, N.K. and D.M.K.; Formal Analysis, W.G.A.; Methodology, N.K. and J.S.; Investigation, N.K. and J.S.; Writing, N.K. and D.M.K.; Supervision, D.M.K.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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