# CpG Oligodeoxynucleotides as Vaccine Adjuvants in Primates<sup>1</sup>

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Synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG motifs act as immune adjuvants in mice, boosting the humoral and cellular response to coadministered Ags. CpG ODN that stimulate human PBMC are only weakly active in mice. Thus, alternative animal models are needed to monitor the activity and safety of "human" CpG ODN in vivo. This work demonstrates that rhesus macaques recognize and respond to the same CpG motifs that trigger human immune cells. Coadministering CpG ODN with heat-killed *Leishmania* vaccine provided significantly increased protection of macaques against cutaneous *Leishmania* infection. These findings indicate that rhesus macaques provide a useful model for studying the in vivo activity of human CpG motifs, and that ODN expressing these motifs act as strong immune adjuvants. *The Journal of Immunology*, 2002, 168: 1659–1663.

S ynthetic oligodeoxynucleotides  $(ODN)^4$  containing unmethylated "CpG motifs" are broadly immunostimulatory in mice (1–4). They activate B cells and dendritic cells (DC), and trigger an immune cascade that includes the production of cytokines, chemokines, and IgM (1, 2, 5–8). In mice, CpG ODN boost the protective efficacy of vaccines against bacterial, viral, and parasitic pathogens (9–14).

Due to evolutionary divergence in CpG recognition between species, ODN that are highly active in rodents are poorly immunostimulatory in primates, and vice versa (15–17). Extensive studies involving human PBMC identified two distinct classes of immunostimulatory CpG ODN (17, 18). "K" type ODN have phosphorothioate backbones, encode multiple T<u>CG</u>TT and/or T<u>CG</u>TA motifs (CpG motif is underlined), trigger the maturation of plasmacytoid DC, and stimulate the production of IgM and IL-6 (17, 19). "D" ODN have mixed phosphodiester/phosphorothioate backbones and contain a single hexameric purine/pyrimidine/<u>CG</u>/ purine/pyrimidine motif flanked by self-complementary bases that form a stem-loop structure capped at the 3' end by a poly G tail (17). D ODN trigger the maturation of APC and preferentially induce the secretion of IFN- $\alpha$  and IFN- $\gamma$  (Refs. 17 and 18 and M. Gursel, unpublished observations).

There is considerable interest in evaluating the safety and activity of CpG ODN planned for human use in a relevant animal model. Although Davis and colleagues (20–22) showed that K ODN increased the seroconversion rate and Ab response of orangutans and aotus monkeys immunized with hepatitis B vaccine and malaria proteins, that group was unable to document improved protection against infection by challenge studies. Moreover, no studies have compared the activity of K ODN with the recently discovered D class of ODN in primates.

This work examines whether rhesus macaques provide a useful model for assessing the activity of CpG ODN in vivo. In vitro studies established that PBMC from rhesus macaques responded to the same panel of K and D ODN that were highly active on human PBMC. Building on results from murine studies (23, 24), CpG ODN were coadministered with a mixture of OVA plus alum. The ODN significantly boosted the Ag-specific IgG response of macaques, with D being superior to K ODN. A cutaneous *Leishmania* infection model was used to examine whether CpG ODN could boost protective immunity in primates. The nature, severity, duration, and histopathology of the cutaneous disease caused by *Leishmania major* in macaques is quite similar to that in humans (25, 26). Results indicate that D ODN significantly improve the protection conferred by coadministered heat-killed *Leishmania* vaccine (HKLV).

## **Materials and Methods**

### Rhesus monkeys

Healthy 3-year-old female rhesus macaques (*Macaca mulatta*) were obtained from the Food and Drug Administration colony in Morgan Island, SC. All studies were approved by the Center for Biologics Evaluation and Research Animal Care and Use Committee, and were conducted in an American Association for the Accreditation of Laboratory Animal Careaccredited facility. Animals were monitored daily by veterinarians. No systemic or local adverse reactions to CpG ODN, OVA, or HKLV immunizations were observed. Treatments were administered and peripheral blood samples obtained from ketamine-anesthetized animals (10 mg/kg, Ketaject; Phoenix Pharmaceuticals, St. Joseph, MD).

#### Vaccination groups and protocol

Two in vivo studies were conducted: 1) three monkeys per group were immunized s.c. and boosted 12 wk later with a mixture of 4  $\mu$ g of OVA, 250  $\mu$ g of ODN, and 125  $\mu$ g of alum (Rehydragel HPA; Reheis, Berkeley Heights, NJ); 2) five to six monkeys per group were immunized s.c., and boosted 4 wk later with 250  $\mu$ g of GMP-grade HKLV (Biobras, Montes Claros, Brazil) plus 125  $\mu$ g of alum, as previously described (27). The HKLV was administered alone, or combined with 500  $\mu$ g of ODN. Pre-liminary studies established that this dose of ODN was active in vivo and well-tolerated. Animals were exposed to nonviable *Leishmania amazonensis* metacycle promastigotes on wk 8, a treatment that induced no disease

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<sup>&</sup>lt;sup>4</sup> Abbreviations used in this paper: ODN, oligodeoxynucleotide; SLA, soluble Leishmania Ag; DC, dendritic cell; HKLV, heat-killed Leishmania vaccine.

and no change in Ab titer or proliferative response to *Leishmania* Ags when compared with control animals. Animals were challenged on the forehead on wk 14 with  $10^7$  viable *L. major* (WHOM/IR/-/173) metacyclic promastigotes intradermally. The monkeys developed a typical self-limited in situ lesion characterized by erythema, induration, and ulceration. Lesion size, which reflects the severity of infection (25, 26), was measured weekly.

#### Oligodeoxynucleotides

ODN (Table I) were synthesized by the Center for Biologics Evaluation and Research Core Facility. All ODN had <0.1 EU of endotoxin per milligram of ODN as assessed by a *Limulus* amebocyte lysate assay (QCL-1000; BioWhittaker, Gaithersburg, MD).

#### Mononuclear cell preparation

Human and monkey mononuclear cells were isolated by density gradient centrifugation of PBMC over Ficoll-Hypaque as described (17). Cells were washed three times and cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 1.5 mM L-glutamine, and 100 U/ml penicillin/streptomycin at  $5 \times 10^5$  cells/well in the presence of 3  $\mu$ M ODN. Supernatants were collected after 72 h and tested by ELISA for cytokine and Ab levels.

#### ELISA

Ninety-six-well microtiter plates (Millipore, Bedford, MA) were coated with Abs that cross-reactively recognized human and macaque IL-6 (R&D Systems, Minneapolis, MN), IFN- $\alpha$  (PBL Biomedical Laboratories, New Brunswick, NJ), and IgG (Boehringer Mannheim, Mannheim, Germany). The plates were blocked with PBS-5% BSA (17). Culture supernatants from PBMC cultures were added, and their cytokine content quantitated by the addition of biotin-labeled anti-cytokine Ab followed by phosphatase-conjugated avidin and phosphatase-specific colorimetric substrate. Standard curves were generated using known amounts of recombinant human cytokine or purified Ig. All assays were performed in triplicate. Titers of Abs to OVA in sera were assayed on OVA-coated plates.

### ELISPOT

The number of PBMC secreting IFN- $\gamma$  in response to soluble *Leishmania* Ag (SLA) was determined by ELISPOT as described (28). Briefly, Millipore 96-well filtration plates (Millipore, Bedford, MA) were coated overnight at 4°C with 1  $\mu$ g/ml of anti-human IFN- $\gamma$  Abs (clone GZ4; Alexis, San Diego, CA) in PBS and then blocked with PBS-5% BSA for 2 h. The plates were overlaid with 5 × 10<sup>5</sup> cells/well (1–2 series per monkey) and incubated at 37°C in a humidified 5% CO<sub>2</sub> in air incubator for 18 h in the presence of 25  $\mu$ g of SLA. The plates were then washed with water-0.025% Tween and overlaid with biotin-conjugated anti-human IFN- $\gamma$  (clone 76-B-1; Mabtech, Nacka, Sweden). After 2 h, the plates were washed again and then overlaid with alkaline phosphatase-conjugated streptavidin. Spots were visualized by the addition of 5-bromo-4-chloro-3-indolyl phosphate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and counted using the KS ELISPOT Imagine System (Carl Zeiss, Thornwood, NY).

#### Cell proliferation assay

A total of  $10^5$  PBMC/well were incubated with 3  $\mu M$  of ODN for 68 h, pulsed with 1  $\mu Ci$  of [^3H]thymidine and harvested 4 h later. All assays were performed in triplicate.

## Statistical analysis

Statistically significant differences were determined using a two-tailed nonparametric ANOVA with Dunnett's post test analysis. Differences in lesion

Table I. Sequence and backbone of D, K, and control ODN<sup>a</sup>

ODN	Sequences
D19	GG <b>TGCAT<u>CG</u>ATGCAGG</b> GGGG
D29	GG <b>TGCAC<u>CG</u>GTGCAGG</b> GGGG
D35	GG <b>TGCAT<u>CG</u>ATGCAGGGG</b> GG
D122	GG <b>TGCAT<u>TG</u>ATGCAGG</b> GGGG
K3	AT <u>CG</u> ACTCT <u>CG</u> AG <u>CG</u> TTCTC
K123	T <u>CG</u> TT <u>CG</u> TTCTC
K23	T <u>CG</u> AG <u>CG</u> TTCTC
K163	T <u>TG</u> AG <u>TG</u> TTCTC
AA3M	GG <b>GCAT<u>GC</u>ATGG</b> GGGG

<sup>a</sup> CpG dinucleotides are underlined. Bases in bold are phosphodiester.

sizes were tested by repeated measures ANOVA using the Proc Mixed procedure from the statistical analysis system.

### Results

# *Response of PBMC from human and nonhuman primates to K and D ODN*

Previous studies established that human PBMC respond to two structurally distinct classes of CpG ODN (17). D-type ODN triggered the secretion of IFN- $\alpha$  and IFN- $\gamma$  (17), whereas K ODN induced human PBMC to proliferate and secrete IL-6 and IgM (Fig. 1, Ref. 17, and data not shown). Analysis of several hundred CpG ODN identified several D and K ODN that strongly activated human PBMC (17). These ODN were tested for their ability to stimulate PBMC from rhesus macaques.

In this study, the response of rhesus PBMC to D ODN was evaluated on the basis of IFN- $\alpha$  production. Results show that macaque PBMC are activated by the same D ODN that stimulate human PBMC (p < 0.002, Fig. 1). In contrast, neither K ODN, nor control ODN that are structurally similar to D but lack the critical CpG dinucleotide have this effect.

Proliferation and IL-6 secretion were used to compare the response of macaque and human PBMC to K ODN (Fig. 1). PBMC from both species were stimulated by K ODN to proliferate (p < 0.002) and secrete IL-6 (p < 0.01), whereas controls of the same structure as K ODN, but lacking the critical CpG motif, failed to trigger immune stimulation. These findings demonstrate that the pattern of reactivity of PBMC from rhesus macaques (n = 20) and humans (n = 8-20) to K and D ODN is quite similar.



**FIGURE 1.** Response of primate PBMC to K and D ODN. PBMC from 8–20 normal human donors and 20 rhesus macaques were stimulated for 72 h with 3  $\mu$ M of K, D, or control ODN (in which the critical CpG motifs were inverted or replaced with TpG). IL-6 and IFN- $\alpha$  levels in culture supernatants were determined by ELISA, while cell proliferation was assessed by [<sup>3</sup>H]thymidine uptake. Note that D ODN induce the secretion of IFN- $\alpha$  while K ODN induce cell proliferation and IL-6 production. All assays were performed in triplicate. Statistical significance was determined by ANOVA of log normalized data. \*, p < 0.05; \*\*, p < 0.01.

Ongoing studies in our lab indicate that individual humans and monkeys vary in their response to specific K and D sequences. Indeed, no single D or K motif is optimally stimulatory in all donors (Ref. 29 and C. Leifer, unpublished observations). However, mixtures of ODN were identified that strongly stimulated PBMC from all human donors. These mixtures were tested on PBMC from macaques and found to be uniformly active (Fig. 2). Subsequent in vivo studies were conducted with these ODN mixtures.

## Adjuvant activity of CpG ODN in vivo

Previous studies in mice showed that CpG ODN could boost the immune response to a coadministered protein Ag (such as OVA). This effect was amplified by adding alum to the mixture of CpG ODN plus Ag (23, 30, 31). Building on these results, macaques were immunized and boosted with a mixture of OVA, alum, and ODN. Animals immunized with mixtures containing D ODN increased their IgG anti-OVA response 470-fold after primary (p < 0.05) and 600-fold after secondary (p < 0.01) immunization (Fig. 3). By comparison, K ODN boosted the IgG Ab response 7-fold after primary, and 35-fold after secondary immunization when compared with pretreatment values (p < 0.05). Macaques immunized with OVA plus control ODN generated only a 4-fold increase in anti-OVA titer. These findings indicate that D ODN are particularly effective at boosting the Ag-specific humoral response to a coadministered Ag.

# Effect of CpG ODN on the immunogenicity and protective efficacy of HKLV

Previous human clinical trials showed that HKLV was safe, but poorly immunogenic (26). Building on evidence that HKLV combined with alum and IL-12 induces short-term protective immunity in rhesus macaques (27), and that CpG ODN plus alum increased the immune response to the hepatitis B vaccine in cyalomongus monkeys (20), we immunized and boosted macaques with a mixture of HKLV, alum, and CpG ODN. PBMC from these animals were isolated 10 days postboost and restimulated in vitro with



**FIGURE 2.** Macaque PBMC respond to CpG ODN mixtures optimized for human use. PBMC from rhesus macaques (n = 12-20) were stimulated in vitro for 72 h with a mixture of D19, D29, and D35 (1  $\mu$ M each) or K3 and K123 (1.5  $\mu$ M each). D122 and K163 were used in the control ODN mixture. Levels of IL-6 and IFN- $\alpha$  in culture supernatants were measured by ELISA, while proliferation was measured by [<sup>3</sup>H]thymidine uptake. Statistical significance was determined by ANOVA of the normalized data. \*\*, p < 0.01.



**FIGURE 3.** Antibody titers in macaques immunized with OVA plus ODN. Macaques (three per group) were immunized s.c. with a mixture of 4  $\mu$ g of OVA plus 125  $\mu$ g of alum. A total of 250  $\mu$ g of D19 + D29, K3 + K23, or control (AA3 M) ODN was added to this mixture. Monkeys were boosted with the same material 12 wk later (black arrow). Serum IgG anti-OVA titers were determined by ELISA. Values represent the geometric mean titer ± SEM. Note that the anti-OVA IgG titers in the group that received D ODN are significantly increased over that of OVA plus alum alone (p < 0.01).

*Leishmania* Ag for 18 h. As seen in Fig. 4, both K and D ODN significantly increased the number of PBMC triggered to secrete IFN- $\gamma$  (p < 0.05). In contrast, animals immunized with alumadsorbed HKLV alone showed no increased IFN- $\gamma$  production when compared with unimmunized controls.

The critical measure of an Ag/adjuvant combination is its ability to induce protective immunity. Vaccinated animals were therefore challenged with  $10^7 L$ . *major* metacyclic promastigotes. Animals vaccinated with HKLV-alum alone developed typical cutaneous lesions with a peak surface area of  $300 \pm 60 \text{ mm}^2$  26 days after challenge (Fig. 5). Monkeys vaccinated with HKLV-alum plus K ODN developed lesions of similar size, although the peak lesion formation was slightly delayed. Animals immunized with HKLV-alum plus D ODN had significantly smaller lesions (maximal size  $80 \pm 13$ mm<sup>2</sup>, p < 0.05), consistent with a reduced parasite burden (32).

## CpG ODN safety

All animals treated with CpG ODN, either alone or with Ag, remained healthy and active throughout the study. No hematologic



**FIGURE 4.** IFN- $\gamma$  production by PBMC from macaques immunized with alum-adjuvanted HKLV plus ODN. Rhesus macaques were immunized and boosted with 250  $\mu$ g of HKLV-alum alone (n = 6), or combined with 500  $\mu$ g of a mixture of D (D19, D29, and D35; *n* = 5) or K (K3 and K123; *n* = 5) ODN. PBMC from these animals were incubated with 25  $\mu$ g of SLA and analyzed in vitro for IFN- $\gamma$  production by ELISPOT assay. Animals immunized with HKLV plus K or D ODN had significantly more IFN- $\gamma$ -secreting cells than unvaccinated controls as determined by a one-way ANOVA (*p* < 0.05).



**FIGURE 5.** Cutaneous lesions in macaques vaccinated with alum-adjuvanted HKLV plus ODN. Rhesus macaques were primed s.c. with 250  $\mu$ g of alum-adjuvanted HKLV alone (n = 6) or combined with 500  $\mu$ g of a mixture of ODN (D19, D29, and D35; n = 5) or (K3 and K123; n = 5) and boosted 4 wk later. On wk 14, the monkeys were challenged with 10<sup>7</sup> metacyclic promastigotes. The average size of the lesions on the forehead (the site of challenge) is shown as the mean area (calculated as mean diameter/2 ×  $\pi$ ). Note that macaques immunized with HKLV plus D ODN had significantly smaller lesions (p < 0.01).

or serologic abnormalities were observed 3 days or 9 mo after injection, and no weight loss or change in behavior was detected following administration of CpG ODN at therapeutic doses.

## Discussion

CpG ODN that activate human immune cells in vitro are only weakly immunostimulatory in mice. To expedite preclinical testing of the safety and activity of human ODN requires the identification of a suitable animal model. This report documents that rhesus macaques provide a relevant model for examining the in vivo activity of CpG ODN planned for human use. PBMC from macaques mirrored the response of human PBMC in their response to both K and D ODN. At the immunostimulatory doses used in this study, neither type of ODN triggered any adverse events. In vivo, broadly immunostimulatory mixtures of K and D ODN boosted Ag-specific IgG responses in macaques immunized with OVA and increased IFN- $\gamma$  production in animals vaccinated with HKLV. Of greater importance, D ODN significantly increased the protective response elicited by a coadministered HKLV vaccine.

Several previous reports examined whether K ODN could act as immune adjuvants in nonhuman primates (20–22). Studies by Davis and colleagues (20–22) demonstrated that K ODN boosted the Ag-specific serum IgG response to alum-adjuvanted hepatitis B vaccine, and to a peptide from the circumsporozoite protein of malaria in orangutans and aotus monkeys. This was consistent with results from earlier studies in mice showing that CpG ODN plus alum synergize to boost immunity to Ag (23, 30, 31, 33). Yet these experiments did not establish whether the resulting immune response conferred protection against infection. The current experiments confirm that K ODN boost the Ab response to a coadministered protein (OVA). They further document that D ODN are significantly more effective in this role, boosting Ab production by >500-fold over pretreatment levels and >100-fold over OVA plus alum (Fig. 3).

Cutaneous infection of macaques with *L. major* provides a means for testing the protective efficacy of CpG ODN vaccine combinations. The nature, severity, and duration of the cutaneous disease caused by *L. major* in macaques is quite similar to that in humans (25). The leading *Leishmania* vaccine candidate (HKLV) has proven safe but poorly immunogenic in clinical trials (26).

Coadministration of both D and K ODN with this alum-adjuvanted HKLV vaccine significantly increased the number of PBMC triggered to secrete IFN- $\gamma$  when stimulated with *Leishmania* Ag in vitro. However, the critical test of any vaccine/adjuvant combination is its ability to induce protective immunity. Results show that the cutaneous lesions of macaques vaccinated with HKLV plus D ODN were significantly reduced when compared with HKLValum alone. Previous studies established that a reduction in lesion size correlates with a reduced parasite load (Ref. 32 and R. A. Seder, unpublished observations). These findings suggest that the ability of D ODN to stimulate IFN- $\alpha$  and IFN- $\gamma$  production while promoting the maturation of APC may be particularly useful for the induction of a protective response against *Leishmania* (17, 18).

K and D ODN have unique structural properties. Optimally active K ODN have a phosphorothioate backbone and express multiple TCGTT and/or TCGTA motifs. D ODN have a mixed phosphodiester/phosphorothioate backbone, express a single selfcomplementary purine/pyrimidine/CpG/purine/pyrimidine motif, and are capped by a 3' poly G tail. These two types of ODN trigger human and rhesus PBMC to mount distinct responses. K ODN stimulate B cells to proliferate and secrete IgM, plasmacytoid DC precursors to mature and secrete IL-8, and monocytes to produce IL-6 (Fig. 1 and Refs. 17, 18, 34, and 35). By comparison, D ODN trigger plasmacytoid DC to produce large amounts of IFN- $\alpha$ , and directly or indirectly trigger NK cells to secrete IFN- $\alpha$ , and myeloid DC to mature (Fig. 1, Refs. 17-19, and M. Gursel, unpublished observations). We postulate that D ODN may be superior vaccine adjuvants when a Th1-dependent immune response is required, whereas K ODN may excel at the induction of proinflammatory responses.

It is likely that differences in the recognition, uptake, and/or processing of K and D ODN underlie their distinct functional properties. It was recently established that Toll-like receptor 9 plays a critical role in CpG ODN-mediated activation of human and murine immune cells (35, 36). Using HEK 293 cells transfected with human Toll-like receptor 9, our lab confirmed that the recognition of K-type ODN was mediated by this receptor (37). However, our ongoing studies indicate that these transfected cells do not respond to D ODN, suggesting that a second type of receptor may be involved in D ODN-mediated immune activation.

Clinical trials exploring the utility of CpG ODN as vaccine adjuvants, immunotherapeutic agents, and anti-allergens have been initiated (38). Current results suggest that rhesus macaques may be a useful model for evaluating the safety and activity of these agents in vivo. In this context, neither local nor systemic adverse reactions to K or D CpG ODN were detected in any of the animals studied. Moreover, although K ODN similar to those currently in human clinical trials were found to be active in vivo, our results indicate that D may be superior vaccine adjuvants, improving the humoral response and protective efficacy to certain coadministered vaccines.

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