Prevention and Treatment of Cutaneous Leishmaniasis in Primates by Using Synthetic Type D/A Oligodeoxynucleotides Expressing CpG Motifs

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Oligodeoxynucleotides (ODN) containing CpG motifs mimic microbial DNA and are recognized by toll-like receptor 9 on immune cells. The resulting response limits the early spread of infectious organisms and promotes the development of adaptive immunity. In this regard, CpG ODN show promise as immunoprotective agents and as vaccine adjuvants. Previous studies of nonhuman primates showed that administration of CpG ODN type D (also known as type A) at the site of infection 3 days before and after a challenge with Leishmania major enhanced host resistance and reduced the lesion’s severity. In this study, we show that systemic administration of D/A ODN limits the size of lesions following an intradermal infection with L. major. Importantly, the reduced morbidity was not associated with a reduction in long-term immunity, as such treated macaques were still protected following a secondary challenge. Finally, administration of D/A ODN to macaques that had established cutaneous lesions reduced the severity of the lesions, suggesting a potential role for CpG ODN in L. major treatment. Together, these findings support the development of clinical studies to assess the use of CpG ODN types D/A as immunoprotective and therapeutic agents.

Toll-like receptors (TLR), which recognize conserved microbial determinants, activate the cells of the innate immune system to limit the early spread of pathogens while promoting the development of antigen-specific immunity (21). Toll-like receptor 9 (TLR9)-bearing B cells and dendritic cells (DC) recognize and respond to unmethylated CpG motifs present at high frequency in bacterial, but not vertebrate, DNA, triggering an immune cascade characterized by polyclonal-B-cell activation, improved antigen uptake/presentation by antigen-presenting cells, and the secretion of chemokines and proinflammatory cytokines that foster a strong Th1 response (16). Synthetic oligodeoxynucleotides (ODN) expressing CpG motifs mimic the immunostimulatory activity of bacterial DNA (18). Recent work with murine models indicates that activation of the innate immune system using CpG ODN reduced the severity and time course of infection and facilitated the clearance of virus (herpes simplex virus), bacteria (Listeria monocytogenes, Francisella tularensis, or Klebsiella pneumoniae), and parasites (Leishmania major) (5, 6, 9, 17, 29, 30).

Due to evolutionary divergence, the tissue distribution of TLR9 and the responses to specific CpG ODN sequences are different in rodents and primates or humans. Such differences have potentially important ramifications for translating findings in mice to applications in humans. In this regard, nonhuman primates should provide a better model for approximating the effects of CpG ODN in people (3, 26). Known immunostimulatory sequences for primates include CpG ODN type D (also known as type A [19]), which has a single PuPyCpGPuPy motif, a mixed phosphorothioate-phosphodiester backbone, and a poly(G) tail on the 3′ end (26). Type D/A ODN induce human and nonhuman primate plasmacytoid dendritic cells (pDC) to secrete alpha interferon (IFN-α), monocytes to mature into functionally active DC, and NK cells to secrete IFN-γ (8, 19, 26). D/A ODN do not activate B cells directly (26). This distinguishes them from CpG ODN type K (also known as type B) and type C, which induce polyclonal-B-cell activation, higher levels of interleukin 6 (IL-6) and IL-10, and lower secretion of IFN-α (10, 19, 20, 26). While all CpG ODN types have demonstrated some adjuvant activity in primates (11), the immunoprotective effects of CpG ODN administered alone have so far been demonstrated only in a macaque model of cutaneous leishmaniasis using CpG ODN type D/A (25).

Leishmania major is the causative agent of cutaneous leishmaniasis. While the majority of primary infections are self-limited and eventually resolve, such lesions, depending on the location, can be disfiguring and in some cases persistent. However, once healed, primary infection confers lifelong immunity to reinfection. Moreover, it is thought that the persistence of parasite antigen is critical for mediating such immunity. In this regard, the gold standard for immunization against cutaneous leishmaniasis has been live attenuated L. major. While effective, this type of vaccine still elicits local reactivity. Thus, limiting the morbidity of this type of vaccine but not eliminating all the parasites has the potential to sustain immunity.

Using this model, our work showed that administration of D/A ODN intradermally (i.d.) at the precise site of a challenge with Leishmania major 3 days before and 3 days after infection significantly reduced the severity of the ensuing lesion. Since such a treatment schedule would be impractical outside a very controlled setting, in this study we investigate whether systemic, rather than in situ, administration of CpG ODN type...
D/A can confer protection against *L. major* infection. We further determine whether the animals challenged with *L. major* and treated with D/A ODN develop long-lasting cellular immune responses and are protected against reinfection to the same degree as untreated animals.

**MATERIALS AND METHODS**

Rhesus monkeys. Healthy 2- to 3-year-old female macaques (*Macaca mulatta*) weighing 2 to 3.5 kg were obtained from the Food and Drug Administration colony in South Carolina. All studies were approved by the Institutional Animal Care and Use Committee and were conducted in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility. The animals were monitored closely by veterinarians. No changes in weight, erythrocytopenia, appetite, or demeanor were evident during treatment. No CpG-associated lymphadenopathies or splenomegaly were observed regardless of route. Treatments were administered and peripheral blood samples were obtained from ketamine-anesthetized animals (Ketaject; 10 mg/kg of body weight; Phoenix Pharmaceuticals, St. Joseph, MD).

Oligodeoxynucleotides. ODN were synthesized by the Center for Drug Evaluation and Review Core Facility. The sequences were as follows: phosphodiester bases are in capital letters, phosphorothioate bases are in lowercase, and the unmethylated cytidine-guanidine dimer in each motif is underlined: D19, ggTGCATCGATGCAGGGG; D35, ggTGCATCGATGCAGGGGgg; and D29, ggTGCATCGATGCAGGGGg. All ODN had less than 0.1 endotoxin unit of endotoxin per mg of ODN as assessed by a *Limulus* amebocyte lyase assay (QCL-1000; Nina Laboratories, Burlingame, CA) as previously described (4, 14). Macaques were randomly assigned to treatment groups and then challenged i.d. in the forehead at one site with 10⁷ parasites or at two sites 3 cm apart with 10⁶ parasites. Monkeys inoculated with live metacyclic promastigotes developed a typical self-limited in situ lesion characterized by erythema, induration, and ulceration that resolved in 9 to 11 weeks.

Treatment groups and protocol. To assess the ability of D/A ODN to limit lesion development during primary infection with *L. major*, four groups of Asian rhesus macaques (*M. mulatta*; *n* = 5/group) were challenged on the forehead on day zero with 10⁷ *L. major* (WHOM/MR/175) metacyclic promastigotes intra- dermally (i.d.) as previously described (14, 27). A control group of six animals were infected but remained untreated. The remaining two groups received two doses of CpG ODN type D/A: one dose 3 days before and one dose 3 days after the challenge. The macaques received the D/A ODN i.d. (500 μg) at the site of the challenge as previously described (25), subcutaneously (s.c.) in the interscapular space (0.5 mg/kg), or intramuscularly (i.m.) in the right quadriceps (0.5 mg/kg) with a single dose of D/A ODN 15 days after the infectious challenge and treated with a single dose of D/A ODN 10 days after the infectious challenge. A second group (*n* = 4) of naive monkeys was used to assess the effect of systemic administration of CpG ODN on macaques with ongoing infections. This group was infected at the time of the rechallenge described above—and therefore shared the untreated controls—and was treated systemically with a single dose of D/A ODN 15 days after the infectious challenge (0.5 mg/kg i.d.).

**Parasite load.** The parasite load was estimated as described previously (14). Briefly, 4-mm² biopsy specimens were taken and treated with 1 mg/ml liberase A (Sigma, St. Louis, MO) for 2 h at 37°C, homogenized, filtered, and serially diluted in a 96-well flat-bottom microtiter plate containing biphasic medium prepared using 50 μl of NNN medium containing 30% defibrinated rabbit blood and overlaid with 50 μl of medium 199. The number of viable parasites in each lesion was determined from the highest dilution at which promastigotes could be grown out over 7 days of incubation at 26°C.

**Mononuclear cell preparation.** Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation over Ficoll-Hypaque as described previously (26). The cells were washed three times and cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 1.5 mM L-glutamine, and 100 U/ml of penicillin-streptomycin at 5 × 10⁶ cells/well in the presence of 3 μM ODN. The supernatants were collected after 72 h and tested by enzyme-linked immunosorbent assay for cytokine and antibody levels.

**Enzyme-linked immunosorbent assay.** Ninety-six-well microtiter plates (Millipore Corp., Bedford, MA) were coated overnight at 4°C with 1 μg/ml of anti-human IFN-γ antibodies (Clone GZ4; Alexis, San Diego, CA) in PBS and then blocked with PBS-5% bovine serum albumin for 2 h. The plates were overlaid with 2 × 10⁵ cells in 100 μl of antibody-coated plate and incubated at 57°C in a humidified 5% CO₂ incubator for 18 h in the presence of 25 μl SLA. The plates were then washed with water-0.025% Tween and overlaid with biotin-conjugated anti-human IFN-γ (clone 76-B1-1; Mabtech, Sweden). After 2 h, the plates were washed again and then overlaid with alkaline phosphatase-conjugated avidin and phosphatase-specific colorimetric substrate.

**ELISPOT assays.** The number of PBMC secreting IFN-γ in response to soluble *Leishmania* antigen (SLA) was determined by enzyme-linked immunospot (ELISPOT) assay as described previously (27). Briefly, 96-well filtration plates (Millipore Corp., Bedford, MA) were coated overnight at 4°C with 1 μg/ml of anti-human IFN-γ antibodies (Clone GZ4; Alexis, San Diego, CA) in PBS and then blocked with PBS-5% bovine serum albumin for 2 h. The plates were overlaid with 2 × 10⁵ cells in 100 μl of antibody-coated plate and incubated at 57°C in a humidified 5% CO₂ incubator for 18 h in the presence of 25 μl SLA. The plates were then washed with water-0.025% Tween and overlaid with biotin-conjugated anti-human IFN-γ (clone 76-B1-1; Mabtech, 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 (Kürkäged and Perry Laboratories, Gaithersburg, MD) in low-melt agarose (Sigma, St. Louis, MO) and counted using the KS ELIspot Imagine System (Carl Zeiss, Inc., Thornwood, NY).

**Statistical analysis.** Differences in lesion sizes were tested by repeated-measures analysis of variance (ANOVA) using the Proc Mixed procedure from the Statistical Analysis System. Differences in parasite loads were tested using Kruskal-Wallis one-way analysis of variance on ranks. One-way ANOVA was used to test differences in proliferation and IFN-γ-secreting cells.

**RESULTS**

Systemic administration of CpG ODN type D/A reduces the severity of *L. major* lesions. Asian rhesus macaques (*M. mulatta*) infected i.d. with *L. major* develop a self-limited nodular skin lesion at the site of inoculation similar to those observed in human cutaneous leishmaniasis (1). Intradermal administration of CpG ODN type D/A in situ 3 days before and 3 days after the challenge was shown to reduce the severity of the lesion (25). To determine whether systemic administration of D/A ODN induces similar levels of protection, macaques (six per group) were treated with CpG ODN s.c. (interscapular space) or i.m. 3 days before and after an i.d. challenge with *L. major* on the right forehead. As shown in Fig. 1, macaques challenged i.d. with 10⁷ metacyclic *L. major* promastigotes developed typical cutaneous lesions (1) that peaked on day 24 with a surface area of 70.7 ± 10 mm². *Leishmania* lesions in animals treated in situ (500 μg i.d./dose) or systemically (0.5 mg/kg/dose s.c. or i.m.) with D/A ODN tended to peak earlier in lesion development during primary infection with *L. major*, four groups of Asian rhesus macaques (*M. mulatta*; *n* = 5/group) were challenged on the forehead on day zero with 10⁷ *L. major* (WHOM/MR/175) metacyclic promastigotes intra-dermally (i.d.) as previously described (14, 27). A control group of six animals were infected but remained untreated. The remaining two groups received two doses of CpG ODN type D/A: one dose 3 days before and one dose 3 days after the challenge. The macaques received the D/A ODN i.d. (500 μg) at the site of the challenge as previously described (25), subcutaneously (s.c.) in the interscapular space (0.5 mg/kg), or intramuscularly (i.m.) in the right quadriceps (0.5 mg/kg). To assess whether the challenged animals were protected from *L. major*, 4 months after the primary lesions had resolved, the macaques were rechallenged with two inoculations, 3 cm apart, of 10⁵ metacyclic promastigote parasites i.d. in the contralateral forehead. A group of naive macaques (*n* = 6) were infected at the same time and left untreated to serve as controls. The lesion size, which reflects the severity of infection, was measured weekly. Twenty days after rechallenge, skin biopsy specimens (4-mm punch) were taken from the site of one of the *L. major* inoculations to assess the parasite burden at the site of infection. The other site was left untouched to assess lesion development. In a study performed in parallel with the rechallenge, an additional group of four naive monkeys were infected with *L. major* and treated with a single dose of CpG ODN type D/A to assess whether a single CpG ODN (500 μg i.d.) treatment at the time of infection was immunoprotective.

Lastly, to determine whether CpG ODN could limit the lesion severity if administration occurred during an established infection, two additional studies were done. In the first, a group of six macaques were infected with *L. major* in parallel with the first challenge described above (10⁷ parasites/macaque i.d.) and were treated with a single dose of D/A ODN (500 μg i.d.) at the site of infection 10 days after the infectious challenge. A second group (*n* = 4) of naive monkeys was used to assess the effect of systemic administration of CpG ODN on macaques with ongoing infections. This group was infected at the time of the rechallenge described above—and therefore shared the untreated controls—and was treated systemically with a single dose of D/A ODN 15 days after the infectious challenge (0.5 mg/kg i.d.).

**Parasite load.** The parasite load was estimated as described previously (14). Briefly, 4-mm² biopsy specimens were taken and treated with 1 mg/ml liberase A (Sigma, St. Louis, MO) for 2 h at 37°C, homogenized, filtered, and serially diluted in a 96-well flat-bottom microtiter plate containing biphasic medium prepared using 50 μl of NNN medium containing 30% defibrinated rabbit blood and overlaid with 50 μl of medium 199. The number of viable parasites in each lesion was determined from the highest dilution at which promastigotes could be grown out over 7 days of incubation at 26°C.
(days 10 to 18) and were significantly smaller (23.1 ± 13, 28 ± 3, and 25.6 ± 12 mm², respectively; \( P < 0.01 \)) than those in untreated macaques, indicating that systemic or local CpG ODN treatment around the time of infection results in reduced disease severity.

**IFN-γ response to Leishmania antigens in macaques challenged with L. major and treated with CpG ODN.** Effective immune responses in mice against *L. major* are associated with type 1 cytokine responses characterized by IL-12-dependent production of IFN-γ. CpG ODN type D/A is known to elicit high levels of IFN-α by pDC and IFN-γ production by NK cells, but no IL-12 (26). To determine whether the reduced pathology in CpG ODN-treated macaques was associated with an enhanced *L. major*-specific IFN-γ response, fresh PBMC from each group of macaques were tested by ELISPOT analysis 7, 14, 21, and 24 days after infection for their capacity to secrete IFN-γ. Among untreated macaques, higher numbers of IFN-γ-secreting cells were present in animals with bigger lesions (\( r^2 = 0.49 \)); however, no significant differences were evident among treatment groups (Fig. 2a and data not shown). These results suggested that the reduction in lesion severity in macaques treated with systemic or local D/A ODN is not directly correlated with the frequency of IFN-γ-secreting cells from the peripheral blood at these time points. Whether there are differences at earlier time points and/or at the site of infection remains an open question for future study.

Four months after the lesions from the primary infection had resolved, PBMC were collected and restimulated in vitro with SLA to assess the memory response in monkeys that had been exposed to *L. major* and treated with D/A ODN. PBMC from all previously infected macaques had increased numbers of IFN-γ-secreting cells in response to SLA compared to those from uninfected naïve macaques (Fig. 2b) (\( P < 0.01 \)). Of note, the IFN-γ response was higher in monkeys that had been left untreated during the primary infection (\( P < 0.05 \)) than in the treated groups. However, no significant difference was evident between the routes of CpG ODN inoculation.

**Primates challenged with L. major and treated with CpG ODN are resistant to reinfection with L. major.** Previous studies had shown that, like humans, macaques develop smaller lesions when reinfected with *L. major* (2). To assess whether the reduction in lesion size in macaques treated with CpG ODN at the time of the primary infection with *L. major* was detrimental to the maintenance of long-term memory, the macaques were rechallenged with *L. major* 4 months after the lesions from the primary infection had resolved. A group of six infected and untreated naïve macaques served as controls. Upon challenge with live parasites (\( 2 \times 10^9 \) metacyclic *L. major* promastigotes on the left forehead), macaques previously infected with *L. major* developed significantly smaller lesions than the naïve animals regardless of whether they had been treated with CpG ODN (Fig. 3).

The reduction in lesion size could be attributed to a reduced
inflammatory response and/or a diminished local parasite burden. In vitro restimulation of PMBC 2 weeks after reinfection showed a similarly high number of cells secreting antigen-specific IFN-γ for all groups preexposed to L. major compared to the naïve animals (Fig. 4). Skin biopsies performed 3 weeks after the rechallenge showed live parasites in five of six naïve macaques (Fig. 5). In contrast, live parasites were detected in the skin biopsy of only one of the untreated macaques previously challenged (no D/A ODN; \( P < 0.05 \)). No significant differences were found among groups of macaques that had been previously exposed to L. major, regardless of treatment. Importantly, even among the macaques that had detectable parasite loads, the lesion size was smaller than in naïve monkeys. These results suggest that CpG ODN administration during primary infection to reduce morbidity does not impair the capacity to have reduced lesion severity upon reinfection.

**Effect of D/A ODN treatment in situ at the time of challenge.**

Several studies of mice had shown that preactivation of the immune system with CpG ODN is required to achieve protection (15). To assess whether administration of CpG ODN prior to infection was necessary for protection, a group of four rhesus macaques were challenged with L. major and treated one time with D/A ODN i.d. immediately following the challenge. As shown in Fig. 6, CpG ODN treatment at time zero resulted in a 3-week delay in the appearance of lesions and overall smaller lesions than in untreated controls (95.2 ± 9 mm² compared to 44.9 ± 9 mm², respectively). Of note, the lesions resolved at the same time as those in untreated macaques, suggesting that the development of an adaptive immune response to the parasite was not accelerated by the treatment.

**Postexposure administration of CpG ODN reduces the severity of L. major lesions.** Previous studies of BALB/c mice infected with L. major had shown that administration of CpG ODN as late as 20 days after challenge redirected a lethal Th2 response to a Th1-type immune response that resulted in survival (30). To determine whether CpG ODN could reduce the severity of lesions in primates that had ongoing infections, two independent studies were conducted. In the first (done in conjunction with the primary challenge shown in Fig. 1), rhesus macaques (\( n = 6 \)) were challenged with 10⁷ live metacyclic promastigotes i.d. Ten days after infection, when the macaques had developed cutaneous lesions, 500 μg of CpG ODN type D/A was inoculated i.d. at the site. As shown in Fig. 7a, administration of the ODN in situ reduced the lesion size (35 ± 7 versus 70.7 ± 11 mm²) compared with infected macaques that were not treated. In a second experiment (performed in parallel with the rechallenge of CpG ODN-treated macaques), four naïve macaques were challenged with 2 × 10⁶ parasites i.d. Two weeks later, the macaques were treated with CpG ODN s.c. (0.5 mg/kg) (Fig. 7b). Macaques that received the CpG ODN s.c. had reduced lesion size (34 ± 11 versus 95.2
FIG. 5. Local parasite loads in monkeys after rechallenge with *L. major*. Rhesus macaques that had been challenged with *L. major* and left untreated or treated with D/A ODN i.d. in situ (500 μg), s.c., or i.m. (500 μg/kg) 3 days before and after the challenge were rechallenged with live parasites. Twenty days after rechallenge, the skin lesions were biopsied and the parasite loads were assessed. Shown are the parasite loads of individual monkeys. The biopsy procedure and estimation of parasite numbers were as described in Materials and Methods. Statistical differences among groups (*P* < 0.06) were tested using nonparametric Kruskal-Wallis one-way analysis of variance on ranks. ns, not significant.

± 9 mm²), showing that CpG ODN administered systemically could limit lesion development. However, biopsies conducted 1 week after treatment (3 weeks postinfection) showed that systemic administration of CpG ODN did not significantly reduce the parasite load, as only two of four macaques had low or undetectable parasite loads (data not shown).

FIG. 6. Transient protection from lesions in macaques treated with D/A ODN at the time of infection. A group of rhesus macaques (n = 4) was treated with CpG ODN i.d. in situ (500 g/macaque) immediately following the infectious challenge (2 × 10⁶ metacyclic promastigotes). The area of the lesion developed was measured weekly. Note that the development of the lesions was delayed and reduced compared with macaques infected at the same time but left untreated (n = 6). SD, standard deviation.

**DISCUSSION**

The direct activation of the innate immune system by Toll-like receptor ligands represents a compelling strategy to improve the response to a broad spectrum of pathogens. In previous studies, we had shown that administration of CpG ODN type D/A reduced the severity of *Leishmania* lesions when applied prior to infection and at the site of the challenge (25). This report extends the previous study by showing that systemic administration of CpG ODN type D/A confers protection in both prophylactic and postexposure strategies. The protection conferred appears to be due to systemic effects arising from the activation of the immune system, as shown by reduced lesion severity upon challenge at a site distant from the site of CpG ODN treatment. Despite the milder pathology during the primary infection, macaques established an effective adaptive and anamnestic immune response and were protected from reinfection to a degree similar to that in untreated animals.

Several studies had shown that CpG ODN can be used in primates to improve the immune response to vaccines for hepatitis B, malaria, and *L. major* infection (11, 12, 27, 28). Indeed, clinical trials are under way to assess the safety of K-type ODN when administered together with a vaccine for hepatitis B. However, despite multiple studies with mice showing that CpG ODN are effective in prophylactic and postexposure strategies to prevent or ameliorate infection by a wide variety of pathogens (6, 13, 17, 25, 29, 30), the evidence that CpG ODN can act as immunoprotective agents in primates is, to our knowledge, limited to a single study of *L. major* (25). As mentioned above, in that study, macaques received CpG ODN 3 days before and after the challenge at the site of infection. While important as a proof of concept, this form of treatment is impractical outside a research setting. The present study demonstrates that direct administration at the site of challenge is not required for the immunoprotective effects of CpG ODN. Indeed, systemic administration of CpG ODN (i.m. or s.c.) reduced the severity of the skin lesions to the same degree as in situ inoculation. This is unlikely to have been caused by CpG ODN directly reaching the forehead draining lymph nodes.

As in previous primate studies, the mechanism of protection against *Leishmania* is not clear. Unlike murine leishmaniasis, where IL-12 and IFN-γ production are needed to direct a strong Th1 response that controls parasite growth and high levels of IL-12 have been associated with reduced lesion size (24), in this study, the correlation between the IFN-γ responses in peripheral blood and the size of *L. major* lesions in primates is less evident (2, 7, 27). These data are consistent with a prior report by Gicheru et al. showing that vervet monkeys vaccinated with IL-12 and killed *L. major* developed antigen-specific IFN-γ levels similar to those observed among convalescent monkeys but were not protected from infection (7).

Although lesion size has been associated with parasite load, it is determined in large part by the inflammatory response mounted to the pathogen. Since CpG ODN type D/A induce pDC to secrete high levels of IFN-α, which stimulates monocytes to mature into active DC and activates NK cells to secrete IFN-γ, it is possible that these innate responses mediate the
reduced lesion severity. This would explain why CpG ODN type K (which induces strong proinflammatory-cytokine and B-cell activation but low or no IFN-\(\gamma\)) secretion failed to protect macaques from \(L.\ majore\) (25). Studies utilizing the newly developed type C CpG ODN, which induce “K-like” proinflammatory properties and “A-like” IFN-\(\gamma\) secretion, may provide some insight into the role of IFN-\(\gamma\) in CpG ODN-mediated protection against \(L.\ major\). It is possible, however, that changes in IFN-\(\gamma\) or IFN-\(\alpha\) occurring immediately after treatment or locally at the site of infection would not be reflected in the frequency of antigen-specific IFN-\(\gamma\)-producing cells in peripheral blood weeks after infection. Further studies to assess whether the administration of CpG ODN results in activation of the innate immune system at the site of the challenge are being undertaken to better elucidate the mechanism of protection.

As suggested by murine studies, the time of administration of the CpG ODN relative to the challenge appears to influence the effect of the CpG ODN. Figures 1 and 6 suggest that while administration of CpG ODN consistently resulted in smaller lesions, macaques that received CpG ODN prior to infection had accelerated development of skin lesions, which peaked and resolved sooner than those in untreated macaques. In contrast, those that received CpG ODN in situ at the time of the challenge had delayed lesion development. This suggests that preadministration of the CpG ODN (shown to be critical in the control of rapidly dividing pathogens [15, 16]) preactivates the innate immune system, resulting in earlier inflammation at the site of the challenge. In contrast, CpG ODN administration at or after the time of the challenge appears to curtail lesion development (Fig. 6 and 7).

An important limitation in the use of CpG ODN as an immunoprotective agent was the apparent need to administer it before the time of infection. In this model, we have shown that type D/A CpG ODN can also function as therapeutic agents, diminishing the severity of established infections. Although results from animal studies cannot be directly extrapolated to human disease, these findings raise the possibility that systemic or intralesion administration of D/A ODN, alone or in combination with other antiparasitic agents, may accelerate the healing of the cutaneous leishmaniasis lesion in infected patients. Further studies will be needed to confirm this possibility.

There are an estimated 12 million cases of leishmaniasis worldwide. Despite numerous trials, leishmanization, the controlled induction of disease with a few live parasites, is the only successful prophylactic vaccination strategy employed so far (23). However, this type of immunization has serious limitations, including the risk of developing full-blown disease, that have led most countries to stop the practice. Nonetheless, given its proven efficacy, leishmanization might be readopted in certain regions where leishmaniasis is endemic and applied on a broader scale if the size and duration of the cutaneous lesions could be moderated without compromising its ability to
confers strong and durable immunity. Studies of mice had suggested that CpG ODN could be used in concert with leishmanization to improve the immune response and limit lesion development without sterilizing immunity (22). The present study supports the idea that local administration of ODN might be of use to control the primary inoculation with live parasites without interfering with long-lasting protection. Of concern, upon reinfection, 4 of the 12 macaques that received CpG ODN systemically had high levels of parasites. However, this elevated parasite count was not associated with enlarged lesions. Together, these findings suggest that CpG ODN may be used to reduce the lesions induced during leishmanization, making it safer without loss of protective efficacy.

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