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Cutting Edge: CpG Oligodeoxynucleotides Trigger Protective and Curative Th1 Responses in Lethal Murine Leishmaniasis

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Synthetic oligodeoxynucleotides containing CpG dinucleotides (CpG-ODN) mimic the immunostimulatory qualities of bacterial DNA. We asked whether immunostimulation by CpG-ODN predisposes for a commitment toward a Th1 vs a Th2 response in *Leishmania major* infection, a model for a lethal Th2-driven disease, in BALB/c mice. CpG-ODN induced Th1 effector T cells in vitro and conveyed protective immunity to disease-prone BALB/c mice in vivo. Conversion to a Th1-driven resistant phenotype was associated with IL-12 production and maintained the expression of IL-12R β_2-chains. Most strikingly, CpG-ODN were even curative when given as late as 20 days after lethal *L. major* infection, indicating that CpG-ODN revert an established Th2 response. These findings imply an important role of bacterial DNA and CpG-ODN in the instruction of adaptive immune responses. They also point to the therapeutic potential of CpG-ODN in redirecting curative Th1 responses in Th2-driven disorders. ***The Journal of Immunology*, 1998, 160: 3627–3630.**

Uptake of bacterial DNA that is rich in unmethylated CpG motifs causes brisk activation of immune cells (1–5). Certain synthetic oligodeoxynucleotides (ODN)[3] displaying unmethylated CpG motifs in a given base context mimic the immunostimulatory effects of bacterial DNA (1, 6) and activate APCs to up-regulate the expression of the membrane proteins B7-1 and B7-2 (7) and secrete cytokines such as TNF, IL-1, IL-6, and high concentrations of IL-12 (4, 8, 9). In vivo, the injection of CpG-containing ODN (CpG-ODN) together with Ag skewed Ag-specific IgG-isotypes toward IgG2a and promoted the induction of Ag-specific $CD8^+$ cytolytic T cells (7), suggesting that CpG-ODN might favor Th1 development (7, 10). Therefore, we tested the capacity of CpG-ODN to instruct a Th1-mediated adaptive response and to prevent and treat infection with *Leishmania major*, a lethal Th2-mediated disease, in BALB/c mice (11–13).

Materials and Methods

Animals and infection

We purchased 8- to 10-week-old female BALB/c mice from Harlan Winkelmann (Borchen, Germany). *L. major* promastigotes (strain MHOM/IL/81/FE/BNI; a kind gift from Dr. W. Solbach, University of Lübeck, Lübeck, Germany) were grown in Click/RPMI 1640 supplemented with 10% FCS on Novy-Nicolle-MacNeal agar and washed twice in PBS before infection. Promastigotes (2×10^5 or 2×10^6) were injected into the right hind footpad of BALB/c mice. The swelling of the footpad was measured weekly with a metric caliper, and the uninfected footpad served as an internal control. The percent increase of footpad swelling was calculated from both values.

Media, mAbs, and reagents

Cells were cultured in Click/RPMI 1640 supplemented with 10% FCS (Biochrom, Berlin, Germany). Phosphothioate-modified CpG-ODN were custom synthesized by MWG (Munich, Germany). The sequence used here was 5′-TCC AT**G ACG TT**C CTG ATG CT-3′. (the bold letters indicate the proposed active motif). As a control, an ODN with an inverted CG motif was used (5′-TCC ATG A**GC** TTC CTG ATC CT-3′). IgG-subtype-specific mAbs were purchased from PharMingen (Hamburg, Germany). Staphylococcal enterotoxin B (SEB) came from Toxin Technologies (Sarasota, FL). L-N^6-(1-iminoethyl)-lysine (NIL) was purchased from Alexis (Läufelfingen, Switzerland).

Animal treatment

CpG-ODN were injected in quantities of 40 μl per footpad or in 200 μl i.p. *L. major* Ag (LmAg) was prepared as described previously (14). NIL was diluted in the drinking water at 4.5 mM and given ad libidum.

In vitro cell culture

$CD4^+$ lymphocytes (>95% pure) from BALB/c mice were negatively selected using Biotex T cell columns (TEBU, Frankfurt, Germany). Cells (3×10^4) were stimulated in 96-well flat-bottom tissue culture plates with 10 μg/ml SEB, 5×10^5 T cell-depleted and irradiated (30 Gy) spleen cells (APCs), 5 U/ml rIL-2, and anti-IL-4 mAb (clone 11B11) (10 μg/ml) or

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[3] Abbreviations used in this paper: ODN, oligodeoxynucleotides; CpG-ODN, CpG-containing oligodeoxynucleotides; LNC, lymph node cell; NO, nitric oxide.

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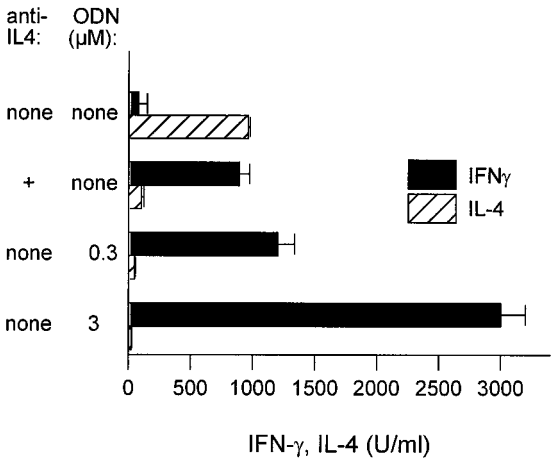

FIGURE 1. Induction of Th1 development by CpG-ODN in vitro. $CD4^+$ T cells were stimulated with SEB, rIL-2, and the additives indicated. After 3 days, the cultures were extensively washed, replated, and expanded further with rIL-2. On day 10, the cells were washed and restimulated again. The 24-h supernatants were tested for IFN-γ by ELISA or for IL-4 content by bioassay.

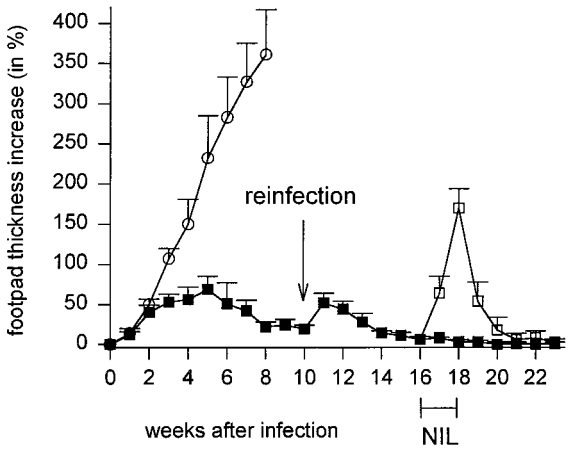

FIGURE 2. Course of infection with *L. major* in CpG-ODN-treated BALB/c mice. BALB/c mice were injected with 2×10^6 *L. major* promastigotes into the right footpad. A total dose of 10 nmol CpG-ODN were given 2 h before and 10 h after infection (5 nmol i.p. and 5 nmol at the site of infection). The mean percent increase (plus SD) of footpad thickness is given (six mice per group). Filled squares indicate the CpG-ODN-treated group, and open circles indicate the untreated control group. After 10 wk, the CpG-ODN-treated mice were reinfected with *L. major*. After 16 wk, one-half of the mice were treated for 2 wk with L-NIL in the drinking water (open squares).

CpG-ODN as described previously (15). After 3 days, cells were transferred into 24-well tissue culture plates and expanded with fresh medium containing 50 U/ml rIL-2. On day 10, cells were washed twice and restimulated with SEB. The 24-h culture supernatant was analyzed for IFN-γ content using an ELISA and analyzed for IL-4 using IL-4-responsive CT4.S cells (a kind gift from Dr. W. Paul) (National Institutes of Health, Bethesda, MD). To restimulate lymphocytes from *L. major*-infected mice, 5×10^5 nonadherent lymph node cells (LNCs) were incubated with LmAg (equivalent to 2×10^5 parasites) and syngeneic APCs (5×10^4 cells, 15 Gy-irradiated) in 200 μl. After 48 h, the supernatant was analyzed for IL-4 and IFN-γ content by ELISA.

PCR analysis

Total RNA was isolated from spleen and lymph nodes using the RNeasy total RNA extraction kit (Qiagen, Hilden, Germany), and 2 μg of RNA were reverse transcribed (Life Technologies, Eggenstein, Germany). IL-12Rβ_2 primers (sense: 5′-CAT CGC TAT CAT CAC GGT GG-3′, position 2079–2098; antisense: 5′-AGT AGC CTT GGA ATC CTT GGC 3′, position 2383–2363) and β-actin primers (sense: 5′-GAT GAC GAT ATC GCT GCG CTG-3′, position 84–104; antisense: 5′-GTA CGA CCA GAG GCA TAC AGG-3′, position 523–503) were synthesized as phosphodiesters by MWG. PCR reaction products were separated on a 2% agarose gel and documented with Stratagene Eagle Eye System (La Jolla, CA).

IgG subtype ELISA and cytokine assays

Microtiter plates were coated with 10 μg/ml soluble LmAg (16) and then blocked with BSA. Serum samples were diluted twofold in PBS. IgG subtypes were detected with alkaline phosphatase-labeled subtype-specific mAb. Sera were titrated to background levels, from which the end-point titer was calculated. IL-12 p40 content was detected in sera using a commercially available ELISA (PharMingen).

Results and Discussion

To investigate the capability of CpG-ODN in promoting Th1 development in vitro, freshly isolated murine $CD4^+$ T cells were stimulated with SEB, syngeneic APCs (15), and various concentrations of CpG-ODN. As shown in Figure 1, $CD4^+$ T cells stimulated under these conditions developed a Th phenotype, producing IL-4 and low amounts of IFN-γ (15). As expected, stimulation of T cells in the presence of neutralizing anti-IL-4 mAb abolished IL-4 production and slightly enhanced IFN-γ secretion. The addition of CpG-ODN promoted dose-dependent, strong IFN-γ production, while IL-4 secretion was reduced.

The fact that CpG-ODN promote Th1 development in vitro (Fig. 1) prompted us to evaluate effect of CpG-ODN in a Th2-driven disease model, i.e., BALB/c mice infected with *L. major* (11, 12). As shown in Figure 2, untreated BALB/c mice developed severe leishmaniasis and had to be killed after 8 wk, while CpG-treated mice developed resistance (Fig. 2). An initial increase in footpad thickness was followed by a decline of lesions, which then remained stable for at least 16 wk postinfection (Fig. 2). Control ODN without the active CG motif failed to influence the course of infection (data not shown). Reinfection of ODN-treated mice after 10 wk resulted in only a slight and transient increase in footpad thickness (Fig. 2).

Nitric oxide (NO) generated by the inducible NO synthase is crucial for the control of *L. major* persisting in resistant mice (17). Inhibition of inducible NO synthase by L-N^6-iminoethyl-lysine (L-NIL) leads to reactivation of the cutaneous lesions (18). To investigate whether CpG-ODN-treated BALB/c mice also control the infection by a NO-dependent mechanism, we administered L-NIL to *L. major*-infected BALB/c mice at 16 wk after reinfection (Fig. 2). Within 2 wk, the footpad thickness increased. The infection was under control again after L-NIL was withdrawn (Fig. 2). Thus CpG-ODN-treated BALB/c mice control *L. major* infection similar to resistant C57BL/6 mice.

To directly demonstrate that CpG-ODN induce *Leishmania*-specific Th1 development, we analyzed the *L. major*-specific T cell responses in vivo and in vitro. LNCs from ODN-treated BALB/c mice produced high amounts of IFN-γ but no IL-4 upon stimulation with LmAg in vitro (Fig. 3, *A* and *B*). In contrast, LNCs from untreated BALB/c mice produced high amounts of IL-4 but no IFN-γ (Fig. 3, *A* and *B*). In addition, the IgG isotype pattern of *L. major*-specific Abs was typical for a Th1 response in CpG-ODN-treated BALB/c mice (Fig. 3*C*). IgG1 was reduced, while IgG2a and IgG2b was markedly enhanced. To obtain direct evidence for Th1 development in vivo, we determined IL-12Rβ_2 mRNA expression on isolated LNCs and spleen cells 10 days postinfection;

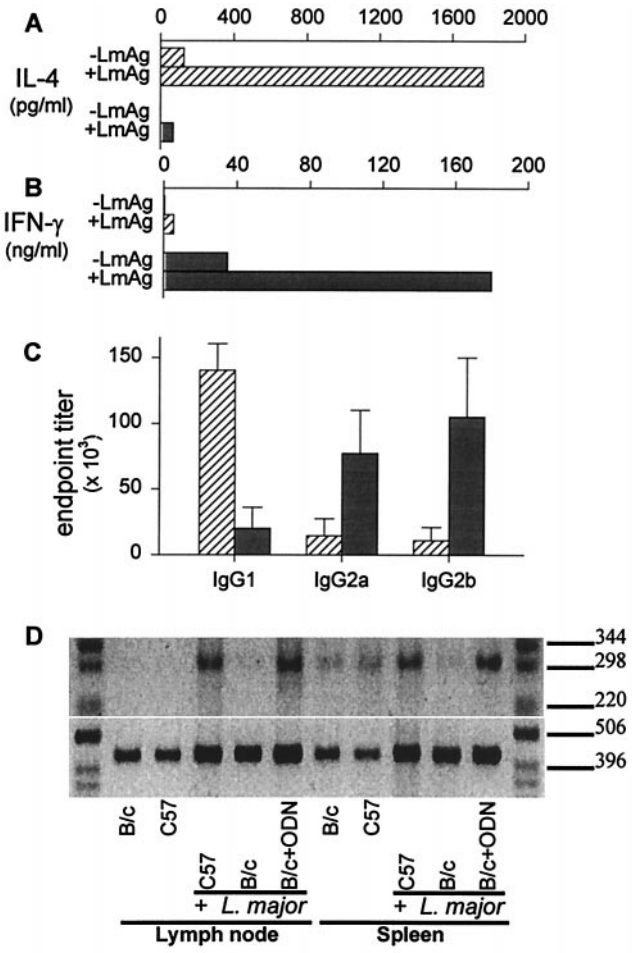

FIGURE 3. Induction of the Th1 phenotype in *L. major*-infected mice by CpG-ODN. BALB/c mice were infected with 2×10^6 *L. major* promastigotes into the right footpad (three mice per group). One group was treated with CpG-ODN 2 h before and 10 h after infection as detailed in Figure 2. After 6 wk, mice were sacrificed, and blood serum and LNCs were recovered (gray bars, ODN-treated mice; hatched bars, control mice). In *A* and *B*, lymphocytes from the draining lymph nodes were restimulated in vitro with LmAg for 48 h. Thereafter, the IL-4 and IFN-γ content of the culture supernatants was analyzed by ELISA. In *C*, the serum was analyzed for *L. major*-specific Abs of the IgG1, IgG2a, and IgG2b isotype. In *D*, total mRNA of draining LNCs and spleen cells was isolated (day 10 after infection) and analyzed by PCR for the expression of IL-12Rβ_2 transcripts (*upper panel*). The internal housekeeping gene β-actin served as a control (*lower panel*).

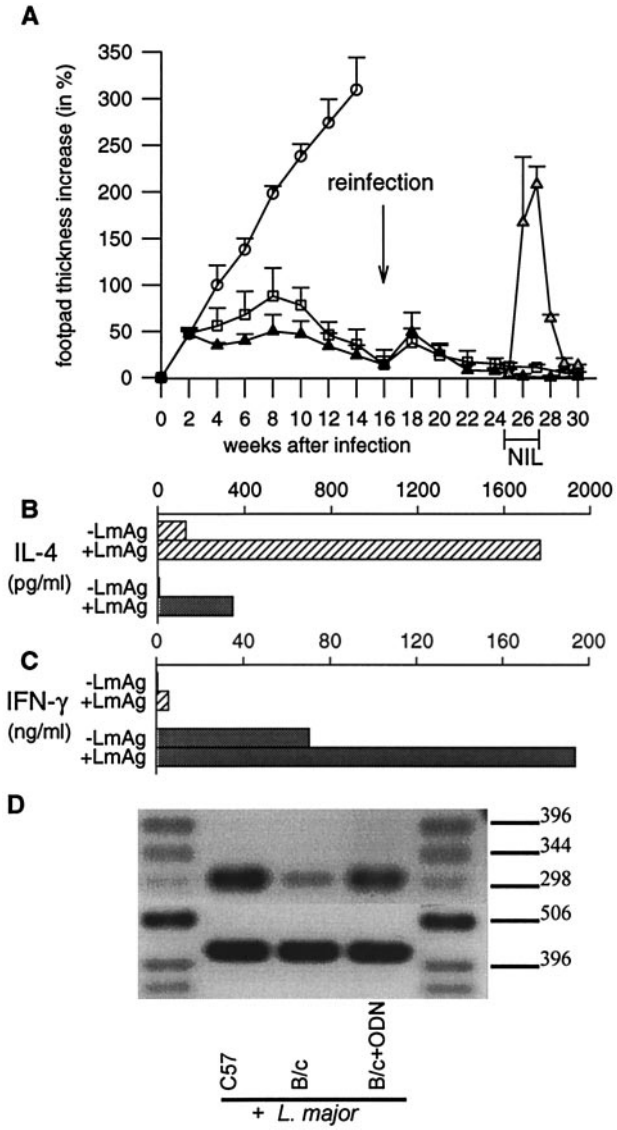

FIGURE 4. Cure of established murine leishmaniasis by CpG-ODN. *A*, Mice were infected with 2×10^5 parasites and received three injections (5 nmol i.p. and 5 nmol at the site of infection) of CpG-ODN every 5 days starting at day 15 (triangles) or day 20 (squares) (cure of infection was observed in five of six mice). Open circles indicate the untreated control group. After 16 wk, the ODN-treated mice were reinfected with *L. major*. After 25 wk, the drinking water of half of the ODN-treated (day 15) mice was supplemented with L-NIL for 2 wk (shaded triangles). *B*, *C*, and *D*, Mice were challenged with LmAg at 6 wk after reinfection, and lymphocytes from the draining lymph nodes were recovered 4 days later (gray bars, ODN-treated mice; hatched bars, control mice). In *B* and *C*, the nonadherent fraction was restimulated in vivo with LmAg, and the supernatants were analyzed for IL-4 (b) or IFN-γ content by ELISA 48 h later. Infected BALB/c mice that were restimulated 6 wk after infection served as a control (hatched bars). In *D*, the expression of IL-12Rβ2 mRNA was analyzed as detailed in Figure 3 (*upper panel*, IL-12β2 transcripts; *lower panel*, β-actin).

this expression is known to be abolished in Th2 cells (19). Following CpG-ODN treatment, IL-12Rβ_2 mRNA expression in lymphocytes from *L. major*-infected BALB/c mice was similar to that observed in resistant C57BL/6 mice (Fig. 3*D*). In contrast, IL-12Rβ_2 mRNA was not detectable in untreated BALB/c mice (Fig. 3*D*). Th1 development may be explained by the ability of CpG-ODN to antagonize *L. major*-mediated suppression of IL-12 production (20, 21). To directly support this hypothesis, we determined IL-12 serum levels in *L. major*-infected BALB/c mice. CpG-ODN-treated mice had 10-fold higher serum levels of IL-12 than *L. major*-infected control mice 16 h postinfection (data not shown). Thus CpG-ODN directed the anti-*L. major* immune response toward a Th1 phenotype.

While Th2 development can be prevented by either anti-IL-4 (13, 22) or IL-12 (23, 24) in the first days after infection, established Th2 responses are not susceptible to these regulatory mechanisms. Treatment with anti-IL-4 or IL-12 following infection with *L. major* is effective only within the first 3 days; at later time points, even multiple injections of IL-12 fail to influence the course of infection (25). Since CpG-ODN effectively stimulate NK cells to produce IFN-γ (26), and since there is evidence that IFN-γ can redirect Th2 responses in vitro and in vivo (27, 28), we tested the curative effect of CpG-ODN in *L. major*-infected BALB/c

mice. Single injections of CpG-ODN were curative when given during the first 8 days of infection but failed when given later (data not shown). Because multiple combined injections of IL-12 plus the anti-leishmanial drug pentostam are capable of curing 85% of diseased animals at 3 wk postinfection (25), we subsequently evaluated multiple CpG-ODN applications during the late phase of infection. BALB/c mice were infected with *L. major* and then treated with three consecutive doses of CpG-ODN starting on day 15 or 20 postinfection (Fig. 4*A*). This regimen reduced the lesion size, and five of six mice ultimately controlled the infection (Fig. 4*A*). Reinfection of mice after 16 wk resulted in only a slight and transient increase in footpad thickness (Fig. 4*A*). The administration of L-NIL led to an exacerbation of the lesions; however, these lesions disappeared again after the withdrawal of L-NIL (Fig. 4*A*). Hence ODN-cured mice control infection by a NO-dependent mechanism (Fig. 4*A*). To obtain additional evidence for Th1 development in ODN-cured mice, we determined IL-12Rβ2 mRNA expression and cytokine production profiles. Lymphocytes from ODN-cured mice produced high amounts of IFN-γ (Fig. 4*C*) but little IL-4 (Fig. 4*B*) upon restimulation with LmAg in vitro. In addition, they expressed IL-12Rβ2 mRNA similar to resistant C57BL/6 mice (Fig. 4*D*). In contrast, normal BALB/c mice only showed weak expression (Fig. 4*D*). Thus, CpG-ODN therapy in BALB/c mice not only prevents the development of Th2 responses causing lethal leishmaniasis but also reverts an already established Th2 polarization toward a protective Th1 response.

As $CD4^+$ T cells in *L. major*-infected BALB/c mice lose IL-12Rβ_2 expression and consequently lose IL-12 responsiveness within the first 3 days of infection (21), it is likely that the protective and curative effects of CpG-ODN are mediated by different pathways. During the early phases of *L. major* infection, CpG-ODN-induced IL-12 production in APCs may be sufficient to shift Ag-reactive $CD4^+$ T cells toward Th1 responsiveness. In late phase disease, additional effects of CpG-ODN might be operational. IFN-γ has been shown to restore IL-12 responsiveness in Th2 cells (28). In addition, successful therapy of leishmaniasis with IFN-γ and pentostam depended upon continued endogenous IL-12 production (29). Thus, during late infection, the curative effect of CpG-ODN may depend upon the ability of CpG-ODN to restore IL-12 responsiveness by promoting IFN-γ production in NK cells (26) and in Ag-reactive T cells (Fig. 1); this effect may also depend upon the capacity of CpG-ODN to trigger endogenous IL-12 production.

In summary, our findings indicate that CpG-ODN influence the course of Th1 vs Th2 development in vitro and in vivo. By the same token, the data reveal a therapeutic potential of CpG-ODN in shifting an established Th2-driven disease toward a protective Th1 response. The capacity of CpG-ODN to promote and redirect Th1 development may have a price if potentially autoreactive T cells become activated (30) or if overshooting cytokine release leads to conditions for septic shock (4, 5). Recent results indicate the possibility of segregating "dangerous" ODN, which preferentially induce TNF-α from potentially curative ODN that trigger IL-12 (9). Understanding the molecular action of ODN will continue to promote progress in engineering therapeutically useful ODN in the treatment of Th2-driven disease.

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