Skin-Derived Macrophages from *Leishmania major*-Susceptible Mice Exhibit Interleukin-12- and Interferon-γ-Independent Nitric Oxide Production and Parasite Killing After Treatment with Immunostimulatory DNA

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Co-administration of CpG-containing immunostimulatory oligodeoxynucleotides and parasite antigen protects susceptible BALB/c mice from otherwise progressive infection with *Leishmania major*. Although the protective effect of CpG-containing immunostimulatory oligodeoxynucleotides is clearly dependent on endogenous interleukin-12 and interferon-γ production, the source of these Th1-promoting cytokines in infected mice is unknown. In contrast to macrophages from *Leishmania*-resistant C57BL/6 mice, macrophages from susceptible BALB/c mice are hyporesponsive to stimulation with lipopolysaccharide and interferon-γ. While studying interactions of various antigen-presenting cells with *Leishmania*, we found that BALB/c inflammatory skin macrophages, whether *Leishmania*-infected or uninfected, produced large amounts of interleukin-12 when treated with CpG-containing immunostimulatory oligodeoxynucleotides. Like lipopolysaccharide, CpG-containing immunostimulatory oligodeoxynucleotides induced production of interferon-γ and release of nitric oxide by skin macrophages. Studies using skin macrophages from interleukin-12- and interferon-γ-deficient BALB/c mice demonstrated that nitric oxide release was not dependent on interleukin-12 and interferon-γ production. Approximately 44% and 27% of intracellular *Leishmania major* amastigotes were killed by infected skin macrophages within 72 h upon stimulation with CpG-containing immunostimulatory oligodeoxynucleotides and lipopolysaccharide, respectively. Parasite killing by macrophages was independent of endogenous interferon-γ production, but was strongly enhanced by exogenous interferon-γ. Parasite elimination was dependent on the induction of nitric oxide, however. *In vivo*, injection of CpG-containing immunostimulatory oligodeoxynucleotides into lesional skin reduced the parasite burden ≈50-fold within the first 5 d of infection prior to full generation of a Th response. These results suggest that skin macrophages, constituting the principal reservoir of parasites in infected susceptible mice, produce Th1-promoting cytokines in response to CpG-containing immunostimulatory oligodeoxynucleotides. In addition, CpG-containing immunostimulatory oligodeoxynucleotides may also act locally on skin macrophages to facilitate *Leishmania* clearance by inducing nitric oxide production. ***Key words: CpG motif/cytokines/Leishmania/monocytes/macrophages/nitric oxide. J Invest Dermatol 119:621–628, 2002***

Murine *Leishmania major* infection provides an excellent experimental system in which to study factors that regulate Th development and represents a useful model of an important human disease (cutaneous leishmaniasis). Resistant (e.g., C57BL/6) mice mount Th1-predominant immune responses and control parasite growth, whereas susceptible BALB/c mice exhibit Th2 immunity and succumb to progressive disease (Reiner and Locksley, 1995; Locksley *et al*, 1999). Manipulations that block production of Th1-promoting cytokines like interleukin-12 (IL-12) or interferon-γ (IFNγ), or that enhance development of Th2 responses, convert resistant animals into susceptible ones. Conversely, treatment of BALB/c mice with IL-12 or anti-IL-4 prevents disease, and targeted mutation of the genes that encode IL-4 or the α chain of the IL-4 receptor confers partial protection (Mohrs *et al*, 1999).

Murine skin macrophages (MΦ) rapidly ingest *L. major* promastigotes after dermal inoculation by sand flies. Initially, it was suggested that skin MΦ might be the principal source of Th1-promoting IL-12 early in infection (reviewed in Scott, 1996). Subsequently, it was determined that infected skin MΦ did not release IL-12 and, in addition, parasitized MΦ were hyporesponsive to inflammatory stimuli (Carrera *et al*, 1996). Prior results from our laboratory together with several other studies implicate dendritic cells (DC) rather than MΦ as early, and important, sources of IL-12

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Abbreviations: CpG-ODN, oligodeoxynucleotide containing CpG-motifs; DC, dendritic cell; MΦ, macrophage; ODN, oligodeoxynucleotide.

production in parasitic infections (Reis e Sousa *et al*, 1997; Gorak *et al*, 1998; von Stebut *et al*, 1998). Studies on interactions of *Leishmania* with skin DC from susceptible BALB/c and resistant C57BL/6 mice subsequently revealed that BALB/c DC ingested organisms, upregulated major histocompatibility complex (MHC) and costimulatory molecules, and produced as much, or more, IL-12 than C57BL/6 DC (von Stebut *et al*, 2000). Other accessory cells (particularly skin MΦ) do exhibit strain-dependent differences in response to several inflammatory stimuli, however (Liew *et al*, 1991; Alleva *et al*, 1998; Mills *et al*, 2000).

Escherichia coli lipopolysaccharide (LPS) is a prototypic agonist of the innate immune system and strain-dependent differences in LPS responsiveness have been described previously (Alleva *et al*, 1998; Mills *et al*, 2000). Studies with various MΦ populations indicated that, with regard to IL-12 production, BALB/c-derived cells are generally hyporesponsive to LPS (or LPS and IFNγ) compared with analogous populations from C57BL/6 mice (Belkaid *et al*, 1998; von Stebut and Udey, unpublished observations). We examined immunostimulatory DNA as an alternative stimulus of innate immune responses because it has unique immunomodulatory properties and immunostimulatory oligodeoxynucleotides (ODN) have therapeutic efficacy in the prevention and treatment of experimental murine cutaneous leishmaniasis.

The immunity-enhancing activities of immunostimulatory DNA result from unmethylated CpG dinucleotides that are frequent in bacterial DNA and that activate natural killer cells, B cells, MΦ, DC, and T cells (Ballas *et al*, 1996; Stacey *et al*, 1996; Yi *et al*, 1996; Sparwasser *et al*, 1998; Jakob *et al*, 1999; Bendigs *et al*, 1999). CpG dinucleotide sequences in appropriate motifs (CpG-ODN) comprise the adjuvant component of plasmid DNA vaccines, and are largely responsible for the strong Th1 bias of immune responses that are initiated via this approach (Carson and Raz, 1997; Klinman *et al*, 1999). The ability of CpG-ODN to influence the outcome of immune responses is illustrated in studies in which CpG-ODN promote development of Th1-predominant immunity to model protein antigens in normally Th2-biased BALB/c mice (Kline *et al*, 1998; Zimmermann *et al*, 1998; Walker *et al*, 1999). Results of experiments in which CpG-ODN switched established Th2 responses that cause bronchospasm in mice to Th1 responses (Kline *et al*, 1998), and those in which otherwise lethal bacterial and parasitic infections of mice were aborted (Zimmermann *et al*, 1998; Walker *et al*, 1999), suggest that CpG-ODN may have clinical utility.

The ability of CpG-ODN to convert ineffective anti-*Leishmania* vaccines into vaccines with efficacy, and to abort murine cutaneous leishmaniasis, has been documented in several laboratories, including ours (Zimmermann *et al*, 1998; Stacey and Blackwell, 1999; Walker *et al*, 1999). The anti-*Leishmania* activity of CpG-ODN is dependent on endogenous IL-12 and IFNγ production (Walker *et al*, 1999). Because previous studies indicated that CpG-ODN activated skin DC *in vivo* as well as *in vitro* (Sparwasser *et al*, 1998; Jakob *et al*, 1999), we inferred that DC were an important source of IL-12 in CpG-ODN treated, *L. major*-infected BALB/c mice. Results described herein demonstrate that BALB/c skin MΦ also produce bioactive IL-12, IFNγ, and nitric oxide (NO) in direct response to CpG-ODN alone. These results suggest that, although the anti-*Leishmania* effects of CpG-ODN in susceptible mice are mediated through a variety of immune and inflammatory cells (DC, T cells, and MΦ), direct actions of CpG-ODN on skin MΦ may be important determinants of CpG-ODN efficacy. We suggest that CpG-ODN-mediated protection against *Leishmania* infection in BALB/c mice is mediated, at least in part, via induction of Th1-promoting cytokines as well as enhanced NO-dependent killing of parasites by lesional skin MΦ.

MATERIALS AND METHODS

Animals Six- to 8-wk-old female BALB/CAnNCr were purchased from the National Cancer Institute Animal Production Program (Frederick, MD). BALB/c with targeted mutations of genes encoding IL-12 p40 (Magram *et al*, 1996) and IFNγ (BALB/c-*Ifng*^{tm1Ts}) (Dalton *et al*, 1993) were kindly provided by Drs. Brian Kelsall (NIAID, NIH) and Alan Sher (NIAID, NIH), respectively. All animals were housed and used in experiments in accordance with institutional guidelines.

Cells Inflammatory murine skin-derived MΦ were elicited by subcutaneous injection of sterile polyacrylamide beads (Fauve *et al*, 1983; Belkaid *et al*, 1998; von Stebut *et al*, 1998). Infiltrates were harvested after 3–4 d and cells were separated from beads by filtering through 70 μm nylon mesh. MΦ were resuspended in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Biofluids, Rockville, MD), and enriched to near homogeneity (based on reactivity with monoclonal antibody F4/80 and anti-CD11b) using a 4 h plastic adherence step followed by vigorous washing. Adherent cells were released by incubation in 2 mg per ml glucose in phosphate-buffered saline on ice, counted, and replated in medium at the indicated densities before fluorescence-activated cell sorter analysis or treatment with agonists.

Antibodies and flow cytometry Anti-CD3e (145-2C11), anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD16/CD32 (2.4G2), anti-I-A^{b,d}/I-E^{d} (2G9), anti-I-A^{d} (AMS-32.1), anti-CD11b (M1/70), anti-CD40 (3/23), anti-CD54 (3E2), anti-CD80 (1G10), anti-CD86 (GL1), anti-NK1.1 (PK136), and anti-Pan natural killer cells (DX5) were purchased as biotin- or phycoerythrin-modified monoclonal antibodies (PharMingen, Burlingame, CA). Biotin-conjugated anti-F4/80 was obtained from Serotec (Raleigh, NC) and phycoerythrin-streptavidin was from Tago (Burlingame, CA). Cells were stained for surface Ag expression and analyzed using a FACScan flow cytometer equipped with Cell Quest software (Becton Dickinson, Mountain View, CA) as previously described (von Stebut *et al*, 1998). Propidium-iodide-permeable cells were excluded from analyses. Neutralizing anti-IL-12 (C17.8) was prepared as described previously (Walker *et al*, 1999) and anti-IFNγ (R4–6A2) monoclonal antibody was purchased from PharMingen.

DNA preparations, LPS, and cytokines ODN with nuclease-resistant phosphorothioate backbones were synthesized by Oligos Etc. (Wilsonville, OR). The ODNs used were selected because of their activity in prior studies (Jakob *et al*, 1999; Walker *et al*, 1999). Sequences of the 20-mer CpG-ODN and the corresponding composition-matched control ODN used were as follows: CpG-ODN 1826, 5′-TCCATGACGTTCCTGACGTT-3′; and ODN 1911, 5′-TCCAGGACTTTCCTCAGGTT-3′. Sodium salts of ODN were stored at −20°C and diluted in phosphate-buffered saline before use. LPS contamination in ODN preparations was undetectable with the Limulus amebocyte lysate (LAL) assay (Bio-Wittaker, Walkerville, MD). *E. coli* K235-derived LPS was a kind gift from Dr. Stefanie Vogel (Uniformed Services, University of the Health Sciences, Bethesda, MD). Recombinant murine IFNγ was purchased from PeproTech (Rocky Hill, NJ). For stimulation, isolated cells were subcultured in their basal medium at 2×10^5 per ml or 1×10^6 per ml as indicated in the figure legends; LPS (100 ng per ml), IFNγ (1000 U per ml), and ODN (0.1–100 μg per ml) were added as indicated.

Quantitation of cytokine and NO production Cytokine release into 18 or 72 h cell supernatants was measured using enzyme-linked immunosorbent assay (ELISA) kits for murine IL-12 p40, IL-12 p70, and tumor necrosis factor α (TNFα) (PharMingen, San Diego, CA). IFNγ ELISA kits were purchased from R&D Systems (Minneapolis, MN). Seventy-two hour supernatants were assayed for the presence of nitrite (a stable derivative of NO) as described previously (Stuehr and Nathan, 1989). Briefly, supernatants were incubated with equal volumes of Griess reagent, and nitrite content was quantitated spectrophotometrically. Sodium nitrite (Sigma Chemical, St. Louis, MO) was used to standardize the assay.

Assessment of leishmanicidal activity *in vitro* and *in vivo* Metacyclic promatigotes of *L. major* clone VI (MHOM/IL/80/Friedlin) were prepared as described previously (Belkaid *et al*, 1998). Isolated parasites were opsonized with 5% normal mouse serum and washed prior to infection. Levels of LPS in parasite stock preparations were below the limit of detection (< 1 pg per ml) in the LAL assay. Skin MΦ were plated at 3×10^5 per well in Permanox microchamber slides (Nalgene, Naperville, IL). Parasites were added (five organisms per cell) for 4 h. Subsequently, free parasites were washed away and cells were treated in basal medium with agonists as indicated. In some experiments, NO synthase inhibitory N^{G}-monomethyl-L-arginine (L-NMMA) or inactive N^{G}-monomethyl-D-arginine (D-NMMA) (Alexis Biochemicals, San Diego, CA) was added at 250 μM together with agonists. Cells were stained with DiffQuick for assessment of intracellular parasites. A

Table I. CpG-ODN-induced release of IL-12 from BALB/c skin MΦ is not inhibited in *L.* major-infected cells[a]

	BALB/c		C57BL/6	
	Uninfected	*L. major*	Uninfected	*L. major*
IL-12 p40				
Control	11 ± 9	7 ± 7	6 ± 6	6 ± 6
LPS/IFNγ	194 ± 79*	283 ± 193	3934 ± 1,403*	2300 ± 1,284*
CpG-ODN 1826	1853 ± 1,309*	1920 ± 1,634*	523 ± 260	401 ± 264
ODN 1911	14 ± 14	14 ± 14	14 ± 14	n.d.
IL-12 p70				
Control	58 ± 44	69 ± 69	22 ± 15	112 ± 82
LPS/IFNγ	118 ± 46	181 ± 111	462 ± 114*	280 ± 173
CpG-ODN 1826	390 ± 174*	455 ± 339	59 ± 32	15 ± 15
ODN 1911	72 ± 72	72 ± 72	130 ± 103	n.d.

[a]Skin M^{Φ}(n = 5) were incubated with *L. major* promastigotes (five organisms per cell). Four hours later, LPS (100 ng per ml), IFNγ (1000 U per ml), CpG-ODN 1826 (10 μg per ml), or control ODN 1911 (10 μg per ml) were added as indicated. Cytokine levels in 18 h supernatants were determined by ELISA. Values represent pg per ml of cytokine produced by 2×10^5 cells per ml in 18 h (mean ±SEM).
*p > 0.05 compared with untreated control. n.d., not done.

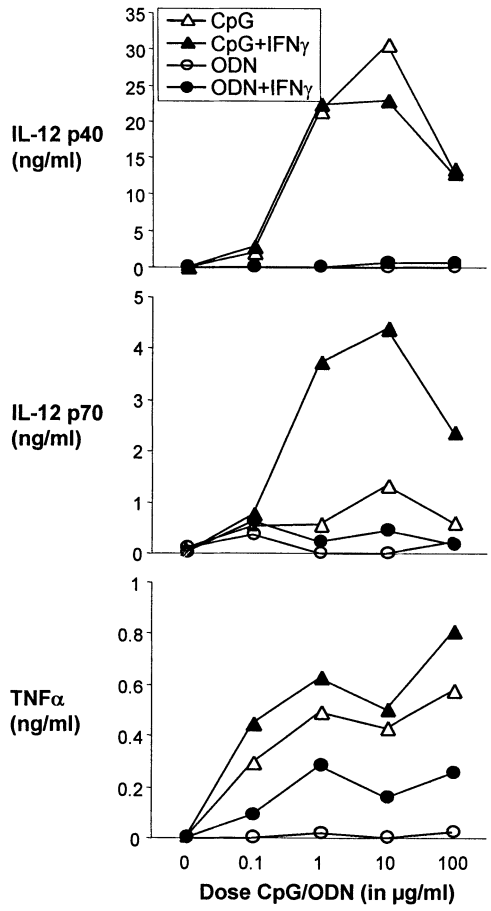

Figure 1. CpG-ODN are strong inducers of IL-12 p70 in BALB/c skin-derived MΦ. Polyacrylamide-bead-elicited skin-derived BALB/c MΦ were incubated with various concentrations of CpG-ODN 1826 or control ODN 1911 (0.1–100 μg per 2×10^5 cells per ml) with or without IFNγ (1000 U per ml) as indicated. Cytokine levels were determined in 18 h supernatants by ELISA. Data from one of two experiments with similar results are shown.

minimum of 200 cells per condition were counted by an investigator blinded to the experimental design using a light microscope. The number of intracellular amastigotes per 100 cells was quantitated and the change compared to untreated control MΦ (= 100%) was calculated.

For *in vivo* experiments, 2×10^5 metacyclic promastigotes were injected into ventral sides of the ears in volumes of 10 μl (day 0). On day 1, CpG-ODN or ODN 1911 (10 μg per 10 μl) were injected locally. After 5 d, ears were harvested and the parasite load was determined as described previously (Belkaid *et al*, 2000).

To assess the IL-12-producing capacity of *L. major*-infected skin MΦ *in vivo*, skin MΦ were infected *in situ* by local injection of 5×10^6 metacyclic promastigotes 3 d after initiation of polyacrylamide granulomas. After 2 h, CpG-ODN 1826 or ODN 1911 were injected locally (10 μg per 50 μl). Cells were harvested after an additional 2 h, enriched by plastic adherence as described above, and replated at 1×10^6 cells per ml in Dulbecco's modified Eagle's medium/5% fetal bovine serum. A fraction of cells was cytospun and stained with DiffQuick for determination of infection rates by light microscopy. Supernatants were harvested after 18 h and IL-12 p40 was determined by ELISA.

Statistics Statistical analysis was performed using the unpaired Student's *t* test.

RESULTS

Induction of IL-12 release from murine skin-derived MΦ by CpG-ODN Previous studies demonstrated that CpG-ODN that exhibit anti-*Leishmania* activity stimulate skin DC *in vitro* and *in vivo* (Zimmermann *et al*, 1998; Jakob *et al*, 1999; Walker *et al*, 1999). One consequence of activation is IL-12 production, and DC-derived IL-12 may facilitate the development of protective Th1-predominant immunity. We have reported previously that skin DC from both *Leishmania*-susceptible (BALB/c) as well as resistant (C57BL/6) mice are similarly responsive to *L. major* and inflammatory stimuli (von Stebut *et al*, 1998; 2000). In particular, skin DC from BALB/c and C57BL/6 mice were comparable with respect to their ability to produce bioactive IL-12 in response to *L. major* amastigotes, CpG-ODN, LPS, or LPS/IFNγ. In this study we examined a variety of accessory cells isolated or propagated from *Leishmania*-resistant and susceptible mice. Although BALB/c peritoneal exudate and bone marrow MΦ as well as bone-marrow-derived DC all released 2–6-fold less IL-12 than C57BL/6 cells in response to LPS ± IFNγ (data not shown), BALB/c skin MΦ were most dramatically deficient, producing ≈20-fold less IL-12 p40 (**Table I**). This result is consistent with flow cytometry data reported previously (Belkaid *et al*, 1998). Strain-dependent differences in the release of other cytokines (TNFα, IL-6, IL-10, IL-4) by skin MΦ were not detected (data not shown).

Extension of these studies to infection with *Leishmania* revealed that, as expected, skin MΦ elicited in BALB/c and C57BL/6 mice by subcutaneous injection of sterile polyacrylamide beads failed to produce IL-12 in response to *Leishmania* infection (**Table I**, and Belkaid *et al*, 1998). Prior infection of C57BL/6 skin MΦ with *L.*

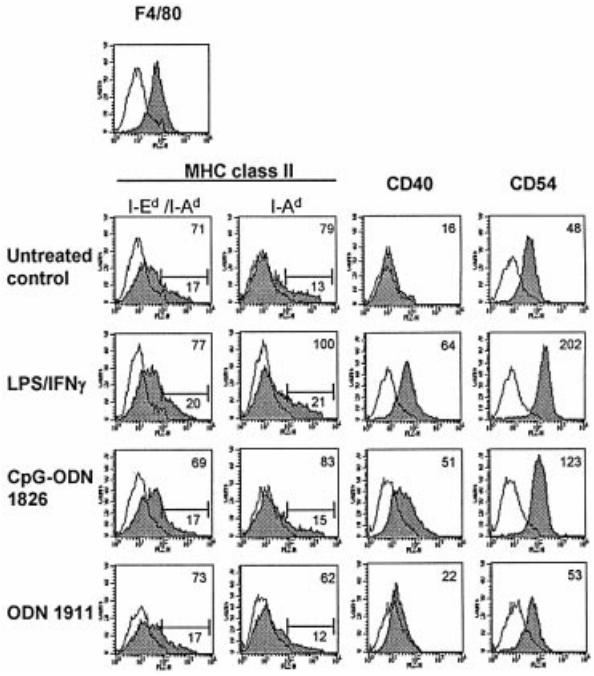

Figure 2. Activation of skin MΦ with CpG-ODN induces upregulation of costimulatory molecules. Skin-derived BALB/c MΦ were incubated with LPS (100 ng per ml), IFNγ (1000 U per ml), or oligonucleotides (10 μg per ml) for 48 h as indicated. Plastic adherent skin MΦ were released from culture dishes and serially incubated with biotin-rat monoclonal antibody and phycoerythrin-streptavidin, and analytical flow cytometry was performed. *Solid line*, isotype staining; *shaded area*, antibody of interest. Only propidium-iodine-impermeable cells were studied. Numbers in upper right corner represent mean fluorescence intensity. Numbers underneath bar indicate percentage positive cells. Data from one of three similar experiments are shown.

major also blunted their response to subsequent challenge with high concentrations of LPS and IFNγ with regard to IL-12 production (Carrera *et al*, 1996). C57BL/6 skin MΦ treated with CpG-motif-containing ODN 1826 released only small amounts of IL-12 p40 compared to LPS/IFNγ stimulated cells. In contrast, treatment of BALB/c skin MΦ with CpG-ODN 1826 resulted in the release of considerable IL-12 p40 and p70 relative to cells treated with non-CpG-containing ODN 1911 (**Table I**, **Fig 3*A*** later) or LPS plus IFNγ. Interestingly, prior infection of BALB/c skin MΦ with *L. major* did not inhibit cytokine production in response to CpG-ODN 1826 (**Table I**). Although additional stimulation of BALB/c skin MΦ with IFNγ together with CpG-ODN 1826 had no effect on IL-12 p40 release, combined treatment resulted in production of high levels of IL-12 p70 (**Fig 1**). To determine the activating potential of CpG-ODN 1826, we analyzed the release of pro-inflammatory TNFα from BALB/c skin MΦ treated with either CpG-ODN 1826 (± IFNγ) or IFNγ alone. High concentration of CpG-ODN (100 μg per ml) appeared to be supra-optimal. Using trypan blue exclusion, however, no apparent toxicity of CpG-ODN for skin MΦ was observed over the period of 72 h compared to untreated medium controls. Interestingly, as the combination of CpG-ODN together with IFNγ led to increased IL-12 p70 release whereas CpG-ODN alone did not to the same extent, we speculate that IL-12 p35 might be upregulated upon additional stimulation with IFNγ.

CpG-ODN, unlike LPS, appeared to be potent inducers of IL-12 release by infected MΦ from *Leishmania*-susceptible mice. As this might be particularly relevant for therapeutic purposes, we subsequently focused on studying the effects of CpG-ODN on BALB/c skin MΦ.

Effects of CpG-ODN on other parameters of BALB/c skin MΦ activation Although CpG-ODN induced release of significant amounts of IL-12 from BALB/c skin MΦ, only modest changes in cell surface antigen expression were observed (**Fig 2**). CpG-ODN as well as LPS induced a 3–4-fold increase in CD54 and 2.5–4-fold increase in CD40 expression after 48 h. Cell surface alterations were even less marked when studying other surface markers (e.g., CD80, CD86) or at earlier time points (data not shown). In all cases, the changes in cell surface marker expression caused by CpG-ODN were less dramatic than those induced by LPS/IFNγ. Only small amounts of MHC class II were detected on BALB/c skin MΦ independent of the antibody used for detection. Additionally, only minimal upregulation of MHC class II antigens was detected after stimulation with LPS/IFNγ or CpG-ODN. This is consistent with previous observations and reflects the relative immaturity of MΦ elicited in polyacrylamide-bead-injected skin compared to MΦ from other sources (bone-marrow-derived MΦ, thioglycollate-elicited MΦ) (Belkaid *et al*, 1998; von Stebut *et al*, 1998). Skin MΦ, however, as well as other MΦ preparations were uniformly positive for lineage markers such as F4/80 (**Fig 2**) and CD11b (data not shown).

Induction of NO and IFNγ production in BALB/c skin MΦ by CpG-ODN and LPS Skin-MΦ-derived NO and IFNγ production may augment innate responses and might play important roles in the initiation of protective immune responses in murine cutaneous leishmaniasis (Zimmermann *et al*, 1998; Walker *et al*, 1999). Exposure of BALB/c skin MΦ to CpG-ODN alone resulted in the production of NO (measured as nitrite accumulation) in 72 h supernatants that was comparable to cells stimulated with LPS/IFNγ or CpG-ODN/IFNγ (**Fig 3*B***). In contrast, addition of the composition-matched control ODN 1911 or *Leishmania* parasites (parasite to cell ratio 3:1) was without effect (data not shown). Surprisingly, although LPS induced only modest upregulation of IL-12 release and surface antigen expression, NO production by LPS-treated BALB/c skin MΦ was comparable to that induced by LPS/IFNγ or CpG-ODN 1826 ± IFNγ.

Induction of NO in MΦ after IFNγ exposure constitutes the major effector mechanism that facilitates Th1-cell-dependent killing of *Leishmania* parasites (Reiner and Locksley, 1995). To determine if production of NO induced by CpG-ODN or LPS stimulation alone was dependent on endogenous IFNγ production from skin MΦ (previously described for other MΦ populations: Gessani and Belardelli, 1998; Munder *et al*, 1998), we assayed 72 h supernatants of CpG-ODN- or LPS-treated skin MΦ for the presence of IFNγ. As shown in **Fig 3**, BALB/c skin MΦ released IFNγ after treatment with immunostimulatory DNA as well as LPS.

To determine if production of IFNγ and NO by BALB/c skin MΦ was dependent on the production of IL-12 and/or IFNγ (Munder *et al*, 1998) (**Fig 3*A***), skin MΦ prepared from BALB/c IL-12 p40 or IFNγ knockout mice were stimulated with LPS or CpG-ODN (± IFNγ) and mediator release was quantified (**Fig 3*D***). Surprisingly, NO production by cytokine-deficient MΦ compared to wild-type MΦ was not reduced after stimulation with CpG-ODN or LPS, suggesting that neither CpG-ODN- nor LPS-induced NO production by skin MΦ was dependent on IL-12 or IFNγ synthesis. These results are in agreement with the results of experiments carried out with wild-type BALB/c skin MΦ and neutralizing anti-IL-12 and/or anti-IFNγ antibodies (data not shown).

CpG-ODN induces parasite killing via NO induction in BALB/c skin MΦ We next wanted to determine if NO induction by CpG-ODN (or LPS) was sufficient to mediate parasite killing in *Leishmania*-infected cells. Skin MΦ of wild-type BALB/c mice were infected with metacyclic promastigotes of *L. major in vitro* and stimulated with CpG-ODN or LPS in the presence or absence of IFNγ (**Fig 4**). The number of intracellular amastigotes was determined after 72 h. The infection rate of untreated control skin MΦ in these experiments was 32 ± 6% and the number of amastigotes per 100 cells was 70 ± 20 (n = 11).

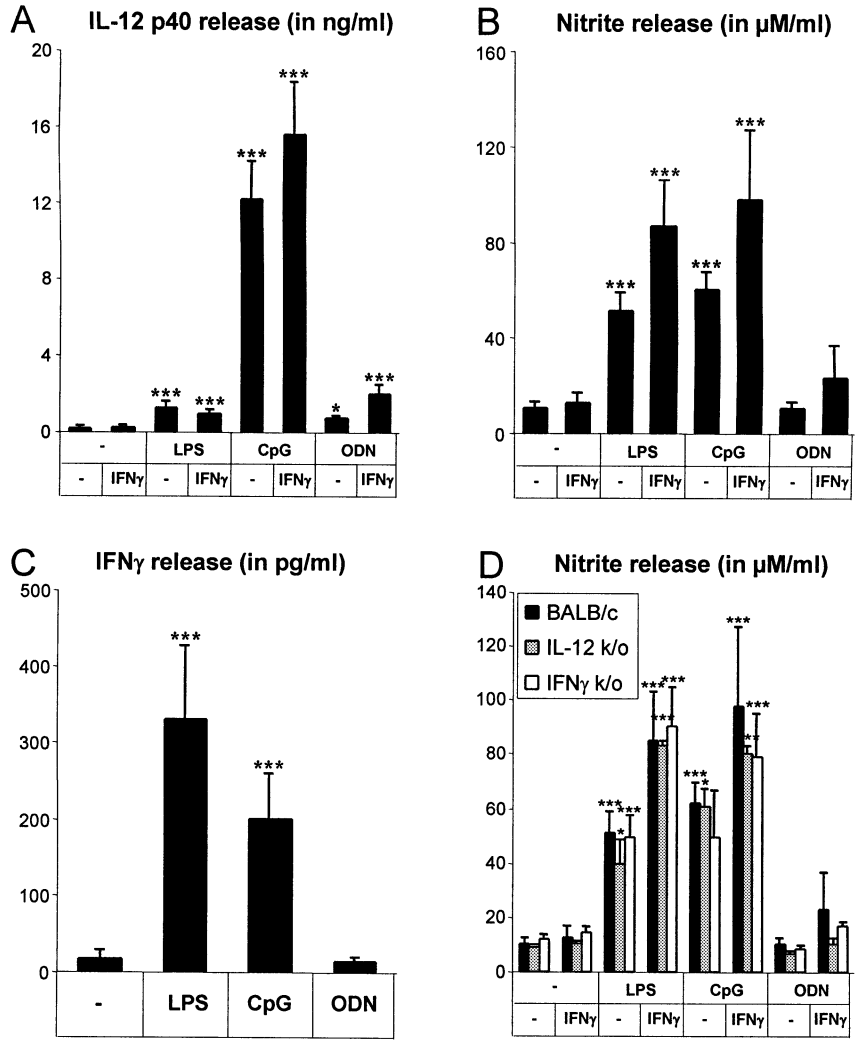

Figure 3. Immunostimulatory DNA is a potent agonist of IL-12, NO, and IFNγ production by skin MΦ from *Leishmania*-susceptible BALB/c mice. Skin MΦ (2×10^5 per 200 μl) from wild-type BALB/c ($n \geq 5$), IL-12-deficient ($n = 2$), or IFNγ-deficient ($n = 4$) BALB/c mice were incubated with LPS (100 ng per ml), CpG-ODN 1826, or control ODN 1911 (10 μg per ml) with or without IFNγ (1000 U per ml) for 72 h as indicated. Nitrite concentrations were measured using the Griess reagent and cytokine levels were determined in supernatants by ELISA. Statistical significance compared to control treatment was calculated using Student's *t* test for unpaired samples (*$p < 0.05$, ***$p < 0.005$).

Upon treatment with either CpG-ODN or LPS, skin MΦ eliminated 44 ± 7% and 27 ± 6% of intracellular amastigotes, respectively, whereas control ODN-treated skin MΦ did not kill parasites. As expected, addition of exogenous IFNγ to the cultures (**Fig 4*A***) strongly increased parasite killing up to 72 ± 5% (CpG-ODN/IFNγ) and 75 ± 5% (LPS/IFNγ). We observed no apparent toxicity of CpG-ODN or LPS treatment over the time period studied (data not shown). As suggested from the results shown in **Fig 3(*D*)**, elimination of parasites from infected cells was no different in IFNγ-deficient skin MΦ, but was strongly inhibited upon addition of NO-inhibiting L-NMMA (but not inactive D-NMMA) to the cultures (**Fig 4*B***). These data demonstrate that CpG-ODN-induced killing of *L. major* parasites by skin MΦ is dependent on the production of NO and can be enhanced by exogenous IFNγ, but it is not dependent on endogenous IFNγ production.

Local administration of CpG-ODN facilitates parasite killing independent of Th1 cells and induces release of IL-12 from lesional MΦ To extend our *in vitro* findings, we tested whether CpG-ODN have the capability to induce parasite killing locally at very early times after infection before full generation of Th1 immunity is observed. Groups of five BALB/c mice were infected intradermally with 2×10^5 metacyclic promastigotes of *L. major*, and 10 μg of CpG-ODN 1826 or composition-matched ODN 1911 was injected locally 18 h later. Five days after infection, ears were harvested and parasite load was determined in limiting dilution assays. As shown in **Fig 5(*A*)**, administration of CpG-ODN reduced the number of lesional parasites ≈50-fold compared to ODN 1911 suggesting that CpG-ODN might contribute directly to early parasite elimination independent of IFNγ-producing Th1 cells.

CpG-ODN were also able to induce IL-12 production from skin MΦ *in vivo* in the presence or absence of infection (**Fig 5*B***). BALB/c skin MΦ were infected *in vivo* by injection of metacyclic promastigotes into polyacrylamide-gel-induced granulomas (Belkaid *et al*, 1998). After 2 h, 10 μg CpG-ODN or ODN 1911 was injected locally, and after additional 2 h skin MΦ were harvested and placed in medium for 18 h. The infection rates of MΦ were determined on cytospins stained with DiffQuick. The infection rates were 8.9 ± 0.7% and 12.2 ± 1.3% for CpG-ODN and ODN 1911-treated MΦ (Belkaid *et al*, 1998), which is comparable to the number of infected MΦ in lesional tissues (Belkaid *et al*, 2000). Analysis of supernatants for IL-12 p40 content revealed that only skin MΦ from CpG-ODN-injected granulomas released significant amounts of IL-12 ($p < 0.002$, **Fig 5*B***). This effect was independent of infections with *L. major*.

DISCUSSION

This study demonstrates that CpG-ODN with anti-*Leishmania* activity *in vivo* are potent and direct agonists of IL-12, IFNγ, and NO release by BALB/c skin-derived MΦ. Our results reinforce the notion that CpG-ODN can act on a variety of cells, including skin MΦ and skin DC (Jakob *et al*, 1999), to produce a number of

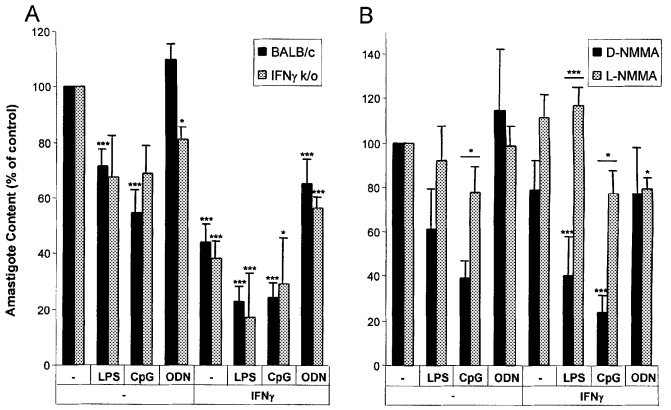

Figure 4. CpG-motif-containing DNA induces leishmanicidal activity in skin MΦ from BALB/c mice that is dependent on NO but independent of IFNγ induction. Skin MΦ (2×10^5 per 200 μl per well) from wild-type BALB/c or BALB/c IFNγ knockout mice were infected with metacyclic *L.* major promastigotes (parasite to cell ratio 5:1) for 4 h. Free parasites were removed and cells were incubated with LPS (100 ng per ml), CpG-ODN 1826, or control ODN 1911 (10 μg per ml) with or without IFNγ (1000 U per ml) as indicated. L-NMMA and inactive D-NMMA were added at 250 μM. After 72 h, slides were fixed and stained with DiffQuick. Intracellular amastigotes were counted in a minimum of 200 cells per condition; the mean number of amastigotes per 100 cells was calculated as percentage of untreated control. Infection rates of untreated controls were 32 ± 6% (BALB/c, $n = 11$), 24 ± 13% (IFNγ knockout, $n = 3$), 29 ± 9% (L-NMMA, $n = 4$), and 34 ± 11% (D-NMMA, $n = 4$). Statistical differences compared to control treatment were calculated by Student's *t* test (*$p < 0.05$, ***$p < 0.005$).

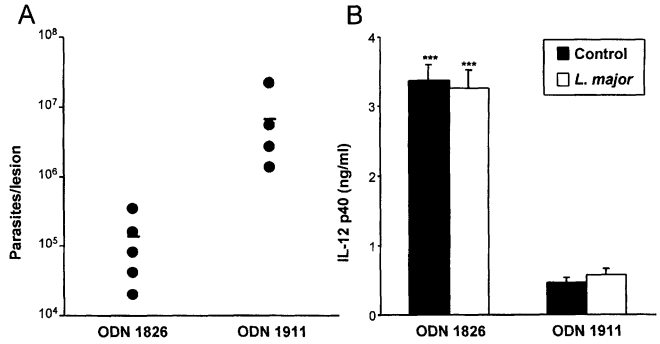

Figure 5. CpG-ODN induce local parasite killing *in vivo* and induce release of IL-12 from lesional MΦ. (*A*) Groups of five BALB/c mice were infected on day 0 by injection of 2×10^5 metacyclic *L.* major into ear skin. On day 1, 10 μg of CpG-ODN or control ODN 1911 were injected locally. On day 5, parasite content was determined by limiting dilution assay. Dots represent number of parasites in ear skin of a single animal; bars show the arithmetic mean of all mice per group. (*B*) Skin MΦ from BALB/c mice were infected *in vivo* with metacyclic *L. major* promastigotes and stimulated *in vivo* 2 h later by local injection of CpG-ODN 1826 or control ODN 1911 (10 μg per 50 μl) as indicated. After an additional 2 h, cells were harvested and were plated at 1×10^6 per ml medium, and supernatants were assayed for IL-12 p40 production by ELISA (ng per ml per 18 h). Statistical differences between groups were calculated by Student's *t* test (***$p < 0.005$).

soluble factors that collectively abrogate disease in an animal model of an important human disease.

In this study we confirmed that accessory cells from BALB/c mice, particularly skin MΦ, are hyporesponsive to inflammatory mediators such as LPS compared with their C57BL/6-derived counterparts with regard to the production of Th1-promoting IL-12 (Oswald *et al*, 1992; Alleva *et al*, 1998; Mills *et al*, 2000). To the extent that these skin-derived MΦ resemble the inflammatory MΦ population in *Leishmania* lesions, our findings may be particularly relevant. Strain differences in MΦ may contribute to the increased susceptibility of BALB/c mice to *L. major* infection. Whether the strain differences that we and others observed are intrinsic to these leukocytes, or reflect strain-dependent variations in cutaneous microenvironments or receptor expression (TLR4 for LPS *versus* TLR9 for CpG signaling), remains to be determined. Our observations that BALB/c skin MΦ were strikingly more responsive to LPS and CpG-motif containing ODN relative to MΦ from resistant C57BL/6 mice when IFNγ and NO release were quantitated (data not shown and Zhu *et al*, 2001), however, and that other BALB/c MΦ populations are also LPS hyporesponsive with regard to IL-12 production (compare also Alleva *et al*, 1998; Mills *et al*, 2000), argue that these differences do not simply reflect alterations in MΦ activation stage or the degree of maturation (Sunderkötter *et al*, 1993).

Our data demonstrate that CpG-ODN alone induce a variety of soluble mediators such as IL-12, TNFα, IFNγ, and NO in BALB/c skin MΦ, and may explain some of the immunomodulatory effects seen with CpG-ODN treatment in cutaneous leishmaniasis. Skin MΦ are the first cells that are parasitized after dermal *L. major* inoculation, and they constitute the principal parasite reservoir in cutaneous leishmaniasis. Initial stages of cutaneous *L. major* are noninflammatory, or perhaps even anti-inflammatory (Carrera *et al*, 1996), allowing expansion of parasites in early lesions. CpG-ODN are potent stimulators of BALB/c skin MΦ *in vitro* as well as *in vivo*, even if cells are infected with *L. major*. Induction of skin MΦ IL-12 and IFNγ production may facilitate the development of Th1-predominant immunity that is observed in CpG-ODN-treated *Leishmania*-infected mice. The direct action of CpG-ODN on skin MΦ that results in IFNγ and NO synthesis may also contribute to early parasite elimination (MacMicking *et al*, 1997; Gessani and Belardelli, 1998) by reducing the dose of antigen and facilitating preferential Th1 education (Nabors *et al*, 1995).

The ability of CpG-ODN to induce significant NO release from skin MΦ in the absence of IL-12 or IFNγ is also of interest. Several groups have reported that exogenous IFNγ was required for immunostimulatory DNA-induced NO production by RAW 264.7 cells and MΦ propagated from CD1 mouse bone marrow (Sweet *et al*, 1998). Others detected release of nitrite after priming with IFNγ and subsequent stimulation with CpG motifs (Ohashi *et al*, 2000; Sester *et al*, 2000; Shoda *et al*, 2001). Recently, Gao and coworkers noted that immunostimulatory DNA did not induce NO release by RAW 264.7 cells in the absence of LPS, and that synergy between LPS and bacterial DNA was IFNγ independent in this system (Gao *et al*, 1999). Puddu *et al* (1997) previously demonstrated that resident murine peritoneal MΦ produced IFNγ in response to IL-12 and IL-18 whereas RAW 264.7 cells did not. The results that we obtained with skin MΦ are not entirely concordant with these studies. The discrepancies between results reported previously and the data presented here may reflect the varying sources (and states of activation) of the MΦ studied. In any case, the data shown in this study suggest that local administration of CpG-ODN might lead to the release of both IFNγ and NO from skin MΦ.

TNFα participates in the induction of NO production and MΦ activation leading to the elimination of intracellular pathogens (Green *et al*, 1990; Taylor and Murray, 1997; Jüttner *et al*, 1998; Wilhelm *et al*, 2001), although its leishmanicidal capacity is still controversial as mice deficient for TNF-R are able to control parasite replication *in vivo* (Nashleanas *et al*, 1998). In this study we observed that skin MΦ release large amounts of TNFα in response to CpG-ODN 1826. Thus, CpG-ODN-induced TNFα production not only might contribute to the recruitment of inflammatory cells to the site of infection (Murray *et al*, 2000), but also may facilitate NO-dependent parasite elimination by local resident MΦ. NO is the principal effector molecule for MΦ-mediated cytotoxicity of intracellular *Leishmania* amastigotes (Green *et al*, 1994; Murray and Nathan, 1999). Although IFNγ regulated NO

production, IFNγ was not required for parasite killing in our studies. Therefore, IL-12- and IFNγ-independent induction of NO by CpG-ODN in BALB/c skin MΦ may contribute to the local elimination of *Leishmania* parasites in the skin.

CpG-ODN hold considerable promise as adjuvants that may be useful in vaccination protocols against tumors and infectious agents, and/or as immunomodulators that may be useful in the treatment of autoimmune or allergic diseases. Current treatment of leishmaniasis is expensive and toxic, and an efficient vaccine has not been described. Thus, an inexpensive CpG-ODN-based therapy might be of value. In this study we demonstrated that CpG-ODN with activity *in vivo* may have important effects on both local inflammatory and systemic immune responses. In murine cutaneous leishmaniasis, CpG-ODN might contribute to efficient development of protective immunity by reducing the initial parasite burden (by induction of NO production in local skin MΦ) and also by allowing for the development of Th1-predominant immune responses via induction of IL-12 from local skin MΦ and DC.

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