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Contribution of Nitric Oxide to CpG-Mediated Protection against *Listeria monocytogenes*

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Immunostimulatory CpG oligodeoxynucleotides (ODN) improve host resistance to listeriae. CpG ODN trigger immune cells to produce gamma interferon and "prime" host cells to secrete nitric oxide in response to bacterial exposure. CpG treatment does not protect inducible nitric oxide synthase 2 knockout mice, indicating that NO is critical to CpG-mediated protection against listeriae.

CpG motifs present in bacterial DNA trigger lymphocytes, dendritic cells, and macrophages to produce a variety of immunoprotective cytokines, chemokines, and antibodies (Abs) (1, 9, 14, 16, 21). Synthetic oligodeoxynucleotides (ODN) containing CpG motifs mimic the ability of bacterial DNA to protect the host from infection (5, 8, 11, 12, 15, 18, 23). For example, CpG ODN significantly reduce host susceptibility to *Listeria monocytogenes*. This immunoprotective effect peaks several days after CpG ODN administration and persists for several weeks (5, 10, 12, 15).

CpG-activated macrophages produce various immunoprotective factors, including large amounts of nitric oxide (NO) (6, 7). Macrophage-derived NO contributes to the clearance of listeria infection (2, 3). Mice lacking inducible nitric oxide synthase 2 (NOS2) are highly susceptible to listeriae (17), consistent with NO playing an important role in host control of this pathogen.

To examine whether NO contributes to CpG-mediated protection against listeriae, 6- to 8-week-old C57BL/6 mice (NCI, Frederick, MD) were treated intraperitoneally (i.p.) with 50 μg of CpG (GCTAGACGTTAGCGT) or control (GCTAGAGC TTAGGCT) ODN. Peritoneal cells were isolated 3 days later by lavage and cultured in vitro with 2×10^5 CFU/ml of heat-killed listeriae (HKL). Nitrite levels in culture supernatants were quantified using the Griess reagent (Sigma, St. Louis, MO) and comparison to a standard curve generated using $NaNO_2$.

As seen in Fig. 1A, CpG ODN had no effect on basal levels of NO production. However, cells from CpG-treated mice produced significantly more NO when cultured with HKL than cells from mice treated with control ODN ($P < 0.001$). This increased response developed within 1 day of ODN administration, peaked on day 3, and persisted through day 7 (Fig. 1B) ($P < 0.01$), mirroring the kinetics of CpG-induced protection against listeriae.

Mice treated with CpG (but not control) ODN survived challenged with 10 50% lethal doses (LD_{50}s) of listeriae (Fig. 2) ($P < 0.001$) (5, 12, 15). The role of NO was further defined by comparing the rates of survival of NOS2 knockout (KO) and wild-type (WT) mice (Jackson Laboratory, Bar Harbor, ME). NOS2 KO mice were more susceptible to listeria infection than WT mice (1 $LD_{50} = 10^3$ CFU in NOS2 mice but 10^4 CFU in WT mice). Moreover, CpG ODN treatment failed to protect NOS2 KO mice from listeria challenge (Fig. 2) ($P < 0.001$).

CpG treatment also activates host cells to secrete gamma interferon (IFN-γ) and increases serum IFN-γ levels, as deter-

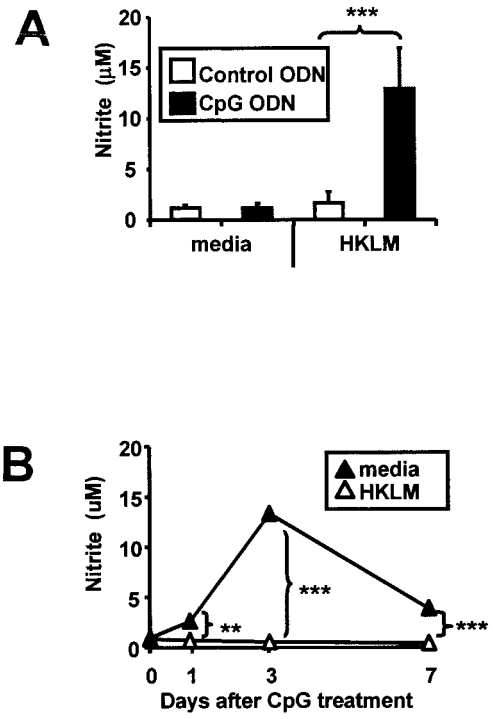

FIG. 1. CpG ODN "prime" host cells to produce NO when exposed to heat-killed *L. monocytogenes* (HKLM). C57BL/6 mice were injected i.p. with 50 μg of control or CpG ODN. Peritoneal cells isolated 3 (A) or 0 to 7 (B) days later were cultured with 2×10^5 CFU/ml of HKL for 48 h. NO production was measured using the Griess reagent. Data represent the means ± standard deviations (SD) of values for more than seven independently studied mice in two independent experiments. **, $P < 0.01$; ***, $P < 0.001$ (two-tailed Student's *t* test).

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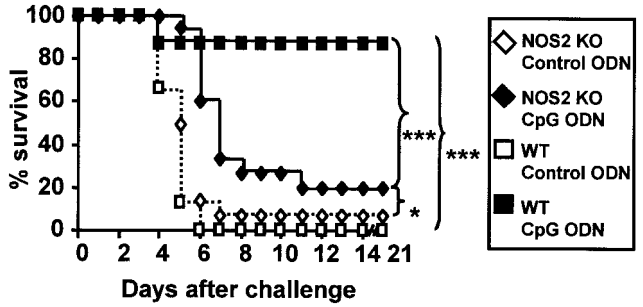
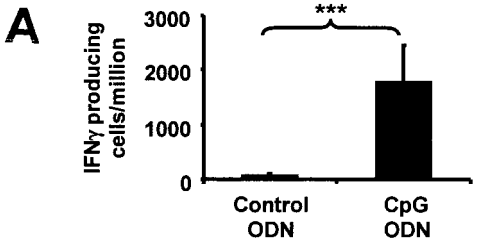


FIG. 2. CpG ODN promote survival following listeria challenge. C57BL/6 or NOS2 KO mice were injected i.p. with 50 μg of control or CpG ODN (n = 10 to 15/group). Three days later, mice were challenged with 10 LD_{50} of *L. monocytogenes*. *, $P < 0.05$; ***, $P < 0.001$ (Wilcoxon rank sum test).

mined by enzyme-linked immunosorbent assay and enzyme-linked immunospot assay ($P < 0.001$) (Fig. 3; methods used to obtain the results in this figure are described in reference 13). The importance of IFN-γ to NO production was examined by culturing peritoneal cells from CpG-treated mice with HKL plus 0.5 ng/ml of neutralizing Abs against IFN-γ, tumor necrosis factor alpha, interleukin-12, or interleukin-18 (R & D Systems, Minneapolis, MN). As seen in Fig. 4A, only anti-IFN-γ Abs significantly reduced NO production ($P < 0.001$).

To confirm the importance of IFN-γ to NO production, CpG ODN were administered to IFN-γ and NOS2 KO mice



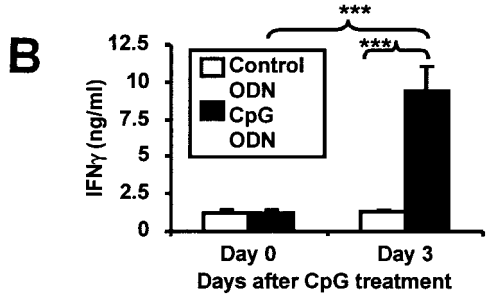
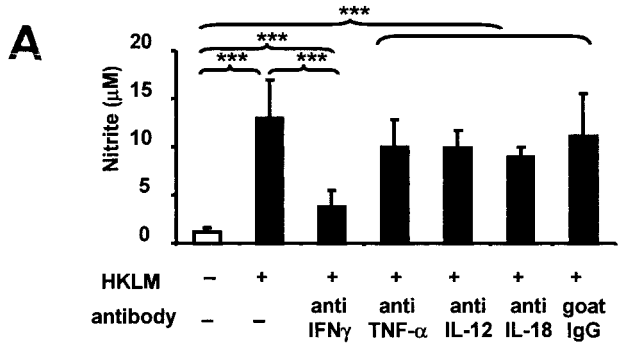


FIG. 3. CpG ODN treatment induces IFN-γ production. C57BL/6 mice were injected i.p. with 50 μg of CpG ODN. (A) IFN-γ-secreting peritoneal cells were quantified on day 3 by enzyme-linked immunospot assay. (B) Serum IFN-γ levels were determined on day 0 and day 3 by enzyme-linked immunosorbent assay. Data represent the means ± SD of values for five to seven independently studied mice in three independent experiments. ***, $P < 0.001$ (two-tailed Student's t test).



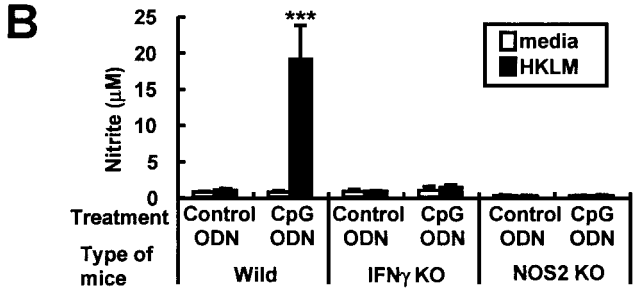


FIG. 4. IFN-γ-dependent NO production following CpG treatment. C57BL/6 and IFN-γ KO mice were treated with 50 μg of CpG ODN. Peritoneal cells were isolated 3 days later and stimulated with HKL. (A) Effect of cytokine-specific neutralizing Abs on NO production. (B) Comparison of cells from WT, IFN-γ KO, and NOS2 KO mice. Data represent the means ± SD of values for six to nine independently studied mice in more than two independent experiments. ***, $P < 0.001$ versus values for all other groups (Student's t test). TNF-α, tumor necrosis factor alpha; IL-12, interleukin-12; IL-18, interleukin-18.

(Jackson Laboratory). Whereas CpG treatment "primed" immune cells from normal mice to produce NO when exposed to HKL, no such effect was observed on cells from either KO strain (Fig. 4B) ($P < 0.001$). Moreover, CpG ODN treatment failed to protect IFN-γ KO mice from listeria infection (Fig. 5).

CpG ODN stimulate a complex immunomodulatory cascade

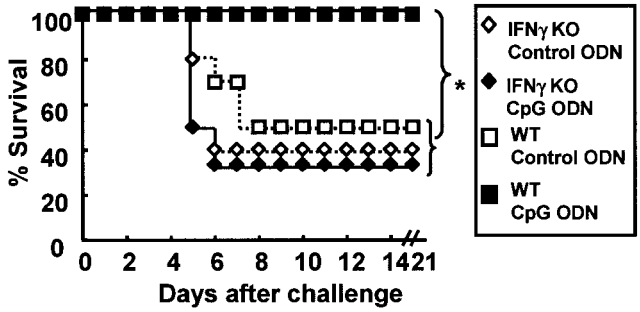


FIG. 5. CpG ODN fail to protect IFN-γ KO mice from listeria infection. WT and IFN-γ KO mice were injected i.p. with 50 μg of control or CpG ODN (n = 5 to 6/group). Three days later, mice were challenged with 1 LD_{50} of *L. monocytogenes* (WT, 10^4 CFU; IFN-γ KO, 0.5×10^3 CFU). *, $P < 0.05$ (Wilcoxon rank sum test).

involving multiple cell types, cytokines, and chemokines (1, 9, 14, 16, 21). This complexity impeded efforts to identify the contribution of specific factors to CpG-mediated host protection. Current findings establish that IFN-γ-dependent NO production is a critical component of host resistance to listeriae elicited by CpG ODN.

Intracellular bacteria take up residence in host macrophages to avoid immune surveillance/destruction (19, 20, 22). These pathogens are cleared when the macrophages are activated to produce bactericidal factors, such as NO. Evidence that IFN-γ-dependent NO contributes to protection against listeriae is provided by Fig. 2, showing that CpG treatment protects WT but not NOS2 KO mice from infection (Fig. 2) ($P < 0.001$). Similarly, Datta et al. recently showed that NO contributes to CpG-mediated resistance against leishmania (4). The possibility of a role for IFN-γ in the regulation of NO production is supported by the finding that anti-IFN-γ Abs selectively blocked NO secretion by CpG-stimulated cells (Fig. 4A). In addition, cells from CpG-treated IFN-γ KO mice failed to produce NO in response to HKL (Fig. 4B). These findings confirm and extend previous results indicating that CpG ODN up-regulate the production of IFN-γ, which in turn modulates the host's ability to produce NO in response to infection (4).

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