CpG DNA Induces Sustained IL-12 Expression In Vivo and Resistance to *Listeria monocytogenes* Challenge

Arthur M. Krieg, Laurie Love-Homan, Ae-Kyung Yi and John T. Harty

*J Immunol* 1998; 161:2428-2434; ;
http://www.jimmunol.org/content/161/5/2428

---

**Why The JI?**

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

**References**  This article cites 32 articles, 16 of which you can access for free at: [http://www.jimmunol.org/content/161/5/2428.full#ref-list-1](http://www.jimmunol.org/content/161/5/2428.full#ref-list-1)

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at: [http://jimmunol.org/subscription](http://jimmunol.org/subscription)

**Permissions**  Submit copyright permission requests at: [http://www.aai.org/About/Publications/JI/copyright.html](http://www.aai.org/About/Publications/JI/copyright.html)

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at: [http://jimmunol.org/alerts](http://jimmunol.org/alerts)
CpG DNA Induces Sustained IL-12 Expression In Vivo and Resistance to Listeria monocytogenes Challenge

Arthur M. Krieg, Laurie Love-Homan, Ae-Kyung Yi, and John T. Harty

Vertebrates have evolved innate immune defense mechanisms that recognize and respond to structural patterns that are specific to microbial molecules. One such pattern recognition system is based on unmethylated CpG dinucleotides in particular sequence contexts (CpG motifs); these motifs are common in bacterial DNA but are under-represented (“CpG suppression”) and methylated in vertebrate DNA. Mice that are injected with bacterial DNA or synthetic oligodeoxynucleotides (ODNs) containing CpG motifs respond with a rapid production of IL-12 and IFN-γ. The serum levels of IL-12 were increased for at least 8 days after a single injection of CpG ODNs, but IFN-γ levels returned to baseline within 24 h. This Th1-like cytokine response to CpG motifs induces a state of resistance to infection by Listeria monocytogenes in susceptible specific pathogen-free BALB/c mice. Resistance developed within 48 h of pretreatment with CpG ODNs, persisted for at least 2 wk, and was dependent upon IFN-γ secretion. These data support the hypothesis that CpG DNA motifs are a “danger signal” that activates protective innate immune defenses and may have therapeutic potential.


M or than a decade has passed since the first report that microbial DNA causes NK cell activation, but vertebrate DNA does not (1). Unmethylated CpG dinucleotides are common in bacterial DNA, but are under-represented (“CpG suppression”) and methylated in vertebrate DNA. Thus, this difference in the content of unmethylated CpG dinucleotides between vertebrate and microbial DNA provides a structural characteristic through which vertebrate leukocytes may detect and respond to infection. Indeed, we have recently reported that immune activation by microbial DNA results from its content of unmethylated CpG dinucleotides in certain immune stimulatory base contexts (CpG motifs) (2).

DNA containing CpG motifs (CpG DNA) triggers humoral immunity by inducing B cell activation, resistance to activation-induced apoptosis, and IL-6 and IgM secretion (2–5). CpG DNA also directly activates monocytes and macrophages to secrete cytokines, especially IL-12, TNF-α, and IFN-αβ (6–9). In contrast, CpG DNA does not directly stimulate highly purified NK cells. Instead, the cytokines produced by macrophages in response to CpG DNA act on NK cells to induce lytic activity and IFN-γ secretion (7, 10). However, the presence of CpG DNA markedly enhances the levels of IFN-γ that are produced by highly purified NK cells in response to IL-12, suggesting that NK cells can also detect CpG DNA (10). The IFN-γ that is secreted in response to CpG DNA promotes B cell activation and Ig secretion (11). In addition to murine cells, human B cells, monocytes, and NK cells are strongly activated by CpG DNA (6, 12), although the optimal flanking bases and the spacing of the CpG motifs are slightly different (our unpublished observations).

Overall, CpG DNA induces a predominantly Th1 pattern of immune activation. These potent and rapid immune-activating effects indicate that CpG DNA may be a “danger signal” that activates innate immune defenses (13). Although this defense system presumably evolved in eukaryotes to protect against infection, it may also be possible to use CpG DNA as an immune modulator for therapeutic applications. For example, CpG motifs are reportedly required for the effectiveness of DNA vaccines (14). Furthermore, we and others have recently reported that CpG DNA acts as an immune enhancer when combined with an Ag to promote an Ag-specific response (15–17). On the other hand, excessive or inappropriate exposure to CpG DNA may contribute to the pathogenesis of the sepsis syndrome (7, 18) and to pulmonary inflammation (19).

Cytokine networks and cellular effectors of innate immunity to infection have been identified in several mouse models of infection. Studies with Listeria monocytogenes, a Gram-positive intracellular bacterium that can be a human pathogen, reveal that innate immunity to infection is mediated by the recruitment and activation of phagocytes (20). Interestingly, L. monocytogenes infection elicits a similar spectrum of cytokine production by macrophages and of IFN-γ production by NK cells as does CpG DNA. These cytokine responses are critical for resistance to primary L. monocytogenes infection. For example, mice that lack the IFN-γR (21) or the IFN-γ structural gene (22) become highly susceptible to L. monocytogenes. Conversely, pretreatment with IFN-γ can render susceptible BALB/c mice resistant to L. monocytogenes (23). NK cells appear to be the source of IFN-γ during the early response to L. monocytogenes infection (24). IL-12 is also a coinducer of IFN-γ secretion, and a depletion of IL-12 exacerbates primary L. monocytogenes infection (25). It has also been demonstrated that TNF-α has a protective role in innate resistance to L. monocytogenes, probably by activating macrophages and facilitating the IFN-γ response or by facilitating the recruitment of neutrophils to
sites of infection (26–28). In the murine model of *L. monocytogenes* infection, i.p. inoculation with viable organisms is followed by an incomplete clearance of the bacteria by phagocytes and other leukocytes (23). The remaining *L. monocytogenes* organisms grow exponentially in the spleen and liver over the following days. Studies with T cell-deficient mice suggest that the innate immune response limits bacterial replication until the development of specific T cell-mediated immunity (29). Since CpG DNA induces the secretion of cytokines that are critical in the innate immune response, we decided to test the possible protective effects of exposure to CpG DNA against *L. monocytogenes* infection. Our results demonstrate that CpG DNA activates the innate immune response and heightens the resistance of mice to *L. monocytogenes* infection.

**Materials and Methods**

**Mice and *L. monocytogenes* infection**

Female BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME) at 4 to 8 wk of age and bred and maintained under specific pathogen-free (SPF) conditions in the University of Iowa Animal Care Unit except as noted. Mice that were maintained under non-SPF conditions often became seropositive for the mouse hepatitis virus. Mice with a BALB/c genetic background in which the IFN-γ gene had been inactivated were also obtained from The Jackson Laboratory.

In a typical experimental design mice were injected i.p. with PBS, CpG DNA, or non-CpG DNA. After 48 h, the mice were challenged i.p. with a lethal dose of 10^5 CFU (−10 LD_{50}) of *L. monocytogenes* strain 10403s. The bacterial stocks were stored in aliquots that were maintained at −70°C, and the titers were checked for each experiment to confirm the number of viable bacteria injected. At 72 h postchallenge, the level of infection in the various experimental groups was determined by enumerating *L. monocytogenes* CFU in liver and spleen organ homogenates as described previously (22). Briefly, spleens and livers from individually challenged mice (three to four mice per experimental group) were homogenized in sterile water with 0.2% Nonidet P-40 and then cultured in triplicate serial 10-fold dilutions on tryptic soy broth with agar and streptomycin (50 μg/ml) at 37°C overnight. The bacterial colonies were counted, the number of organisms per spleen was calculated for each challenged mouse, and then the mean and SD were determined for each group. This assay was used rather than mortality assays to comply with institutional animal care guidelines.

**Cytokine assays**

In some experiments, mice were bled once by retroorbital puncture, and serum cytokine levels were then determined by ELISA at the indicated timepoints. The ELISAs were performed essentially as described previously (3, 4) using anti-IL-12 Abs (that detect both p70 and p40) and an IL-12 standard that were purchased from Genzyme (Cambridge, MA). ELISAs for IFN-γ and IL-6 were performed as described previously (3, 7). The sensitivity of the ELISAs was always <50 pg/ml and was usually <10 pg/ml.

**Oligodeoxynucleotides (ODNs) and DNA**

Nuclease-resistant phosphorothioate ODNs were obtained from Oligos Etc. (Wilsonville, OR). The immunostimulatory CpG ODN that was used in the experiments shown, 1758, has the sequence TCTCCAGCGTGCGCCAT, but other CpG ODNs gave essentially identical results (data not shown). ODNs without immunostimulatory CpG motifs had no effect under these experimental conditions. *Escherichia coli* (EC) DNA (strain B) and calf thymus (CT) DNA were purchased from Sigma (St. Louis, MO). All DNA and ODNs were purified by extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and/or ethanol precipitation. EC and CT DNA were single-stranded before use by boiling for 10 min and then cooling on ice for 5 min. The LPS level in ODNs was undetectable (<1 ng/mg), and the EC and CT DNA contained <2.5 ng of LPS/mg of DNA by Limulus assay.

**Results**

**Bacterial DNA-mediated protection against *L. monocytogenes* infection**

Treating BALB/c mice with CpG DNA leads to the secretion of IFN-γ and IL-12 within 4 h; this secretion returns to background levels by ~24 h (4). Prior studies have shown that the resistance of mice to *L. monocytogenes* challenge was increased if they were pretreated with IFN-γ at 48 h before challenge (23). Therefore, we pretreated susceptible BALB/c mice i.p. with EC DNA, CT DNA, CpG ODNs, control non-CpG ODNs, or PBS and challenged the mice 48 h later with a lethal dose of 10^5 CFU of *L. monocytogenes* strain 10403s.

In initial experiments, we found that a single 200-μg dose of EC DNA that was administered i.p. caused little reduction in the number of organisms that were recovered from the spleen and liver at the end of the experiment (Fig. 1A). However, there was nearly a 2 log reduction in the number of recovered organisms when only 100 μg of EC DNA was given at this timepoint (Fig. 1A). As expected, the administration of CT DNA at either dosage did not substantially alter the recovery of viable organisms (Fig. 1A). Dose-response studies with EC DNA subsequently showed that the optimal dose for protection against *L. monocytogenes* growth was between 10 and 30 μg; slightly lower protection was afforded by either higher or lower doses (Fig. 1B and data not shown).

**CpG ODN-mediated protection against *L. monocytogenes* infection**

To avoid the possibility that the protective effects of EC DNA may be mediated by endotoxin or some other contaminant or a combination of contaminants, we performed additional studies using a synthetic nuclease-resistant phosphorothioate ODN, 1758, that contains two CpG dinucleotides. In previous studies, we found that ODN 1758 was a potent inducer of macrophage IL-12 and NK cell IFN-γ secretion and induced a strong activation of NK cell-mediated immunity; control ODNs that have no CpG motifs or methylated CpG motifs lack this immune activity (4–7, 30). At doses from 3 to 100 μg, ODN 1758 treatment consistently caused an ~2 log reduction in the number of *L. monocytogenes* organisms that were cultured from the liver homogenates of infected mice (Fig. 1A and data not shown).

**Kinetics of CpG ODN-mediated protection against *L. monocytogenes* infection**

To determine the kinetics of CpG DNA-induced protection, the timing of the CpG DNA treatment was varied. No reduction in *L. monocytogenes* colonies was seen in mice that had been injected with CpG DNA at 4 to 24 h before *L. monocytogenes* challenge (data not shown). There was an ~2 to 3 log reduction in the number of organisms that were recovered in mice that had been treated with CpG DNA at 48 to 96 h before challenge (Fig. 2A). Surprisingly, this high level of resistance persisted for at least 2 wk after the CpG pretreatment (Fig. 2B). In a single preliminary experiment to determine the duration of resistance, we found a full resistance to challenge at 27 days after CpG treatment (data not shown). These results indicate that CpG DNA pretreatment induces a relatively long-lived state of resistance to *L. monocytogenes* challenge in susceptible BALB/c mice.

**In vivo induction of a sustained serum IL-12 response to CpG DNA**

In previous kinetic studies of the in vitro spleen cell cytokine responses to CpG DNA, we reported a rapid induction of IL-6, IL-12, and IFN-γ secretion that returned to baseline within 8 to 24 h.

---

1 Abbreviations used in this paper: SPF, specific pathogen-free; ODN, oligodeoxynucleotide; EC, *Escherichia coli*; CT, calf thymus; wt, wild-type.
The striking and surprisingly persistent resistance to *L. monocytogenes* challenge in CpG DNA-treated mice led us to re-examine systemic cytokine levels in mice that had been injected with CpG DNA. Therefore, we examined the kinetics of the serum cytokine responses to CpG DNA, including TNF-α, IL-6, IL-12, IFN-γ, and TNF-α (Table I and data not shown) in accord with the results of prior studies (3, 4, 8). However, all cytokines returned to basal levels within 24 h, except for IL-12. In wild-type (wt) mice, serum IL-12 levels remained markedly elevated for at least 8 days after a single injection of CpG DNA; however, these levels were not increased in mice that had been injected with an ODN in which the cytosines of the CpG motifs were replaced with 5-methylcytosine (mCpG) (Fig. 3). The persistent IL-12 induction appeared to be dependent upon IFN-γ, since mice that were genetically deficient in IFN-γ had only an initial burst of IL-12 secretion in response to CpG DNA and lacked the sustained response seen in wt mice (Table I). Consistent with our previous inability to detect a prolonged splenic secretion of IL-12 protein following CpG DNA injection (4), we were unable to detect IL-12 p40 mRNA in the spleen for longer than 12 h following CpG DNA injection (data not shown).

**Dependence of the CpG protective effect upon IFN-γ**

IL-12 is known to be a potent inducer of IFN-γ expression by NK cells. Moreover, we have recently shown that NK expression of IFN-γ in response to IL-12 is markedly enhanced by the addition of CpG DNA (10). To determine whether protection against *L. monocytogenes* in the CpG DNA-treated mice was mediated by IFN-γ, the challenge experiment was repeated using BALB/c mice in which the IFN-γ gene had been deleted by homologous recombination. Because of the much greater sensitivity of these mice to *L. monocytogenes* (LD50; 10 CFU (22)), the infectious challenge dose was reduced to 10^3 CFU. Not only did CpG treatment of the IFN-γ-deficient mice fail to protect against *L. monocytogenes* challenge, but these mice actually had increased spleen and liver *L. monocytogenes* colonies compared with untreated control mice (Fig. 4A).
Despite the reduced challenge dose used in these experiments, it remained possible that the ability of CpG DNA to induce protective innate immune defenses may still have been overwhelmed; this possibility would become apparent using an even lower challenge dose. Therefore, the experiment was repeated with lower challenge doses of $2 \times 10^2$ and $2 \times 10^1$ CFU. Despite these very low doses, the CpG-primed, IFN-γ-deficient mice still tended to show increased spleen and liver L. monocytogenes colonies compared with untreated control mice (Fig. 4B).

### Table I. Kinetics of the serum IL-12 response to CpG in wt and IFN-γ deficient mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Treatment</th>
<th>1 h</th>
<th>4 h</th>
<th>12 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>PBS</td>
<td>66</td>
<td>121</td>
<td>109</td>
<td>93</td>
</tr>
<tr>
<td>wt</td>
<td>CpG</td>
<td>175</td>
<td>3482</td>
<td>1340</td>
<td>1591</td>
</tr>
<tr>
<td>GKO</td>
<td>PBS</td>
<td>735</td>
<td>792</td>
<td>542</td>
<td>489</td>
</tr>
<tr>
<td>GKO</td>
<td>CpG</td>
<td>1638</td>
<td>1388</td>
<td>135</td>
<td>465</td>
</tr>
</tbody>
</table>

*IL-12 levels are given in picograms per milliliter and are measured by ELISA on serum from BALB/c mice (two to four mice/group) with (wt) or without (GKO) a functional IFN-γ gene at the times specified following the indicated treatment. The SD of the triplicate wells was <5%.

**Spontaneous resistance to L. monocytogenes challenge and lack of protection by CpG DNA in non-SPF mice**

For most of our studies, we used mice that had been maintained continuously under SPF conditions until the time of L. monocytogenes challenge. However, in some experiments, we used BALB/c mice that had been housed for several weeks in a conventional mouse facility, in which mouse hepatitis virus and other pathogens are endemic. These experimental results were noteworthy for several reasons. First, the level of L. monocytogenes growth after challenge was frequently, but not always, reduced by 1 to 2 logs compared with that seen in SPF conditions.
mice that had been challenged with the same dose (Fig. 5). Second, there was often minimal or no protection of these “dirty” mice with any dose of CpG DNA (Fig. 5). However, these results were highly variable, as full protection was seen in some experiments using these mice. Thus, although we speculate that the environmental microbial exposures in these mice were responsible for the occasional spontaneous resistance to infection, it has not been possible to study further the factors responsible for these results.

Discussion

Immune defenses against infection can be divided into acquired immunity, such as that conferred by specific Abs and CTLs, and innate immunity. A common theme in innate immune defenses is the use of pattern recognition molecules that bind certain molecular structures that are present in microbes but not in host cells. For example, macrophages contain molecules that are able to bind LPS, high mannose proteins, and certain dsRNA structures. The ability to respond to such microbial structures may contribute to optimal resistance against infection. We have hypothesized that the immune recognition of CpG DNA may also be important in mediating innate immune defenses (13).

In the present study, we provide the first direct evidence that immune activation by CpG DNA confers resistance to infection. SPF BALB/c mice are normally highly susceptible to infection by...
longed in vivo are degraded more slowly by lymphocytes and have a greatly pro-
perform with nuclease-resistant phosphorothioate ODNs, which mice for the sepsis syndrome (7, 18). Most of our studies were consistent with previous studies in which we and others demonstrated CpG DNA can be beneficial in increasing disease resistance, ex-
reduced protection. These data suggest that although the immune experiments.
able, since partial protection or even full protection was observed in some experiments.

**L. monocytogenes.** However, pretreating these mice with a single injection of CpG DNA provides a >2 log reduction in the number of infectious *L. monocytogenes* organisms that were recovered from the mice at 72 h after a normally lethal challenge infection. Such reductions in bacterial growth in vivo are highly correlated with recovery from infection (22).

This CpG DNA-induced protection against *L. monocytogenes* challenge is strongly dependent upon the dose of CpG DNA ad-
ministered. The optimal dose of an effective CpG ODN or EC DNA was just 10 to 30 μg, and higher doses actually afforded reduced protection. These data suggest that although the immune activation resulting from exposure to a low or moderate amount of CpG DNA can be beneficial in increasing disease resistance, ex-
cessive immune activation is deleterious. This hypothesis is con-
sistent with previous studies in which we and others demonstrated that high doses of CpG DNA (several hundred micrograms) prime mice for the sepsis syndrome (7, 18). Most of our studies were performed with nuclease-resistant phosphorothioate ODNs, which are degraded more slowly by lymphocytes and have a greatly pro-
longed in vivo $t_{1/2}$ compared with unmodified DNA (31, 32). This greater stability may enhance the duration of the CpG effect.

For mice to be protected against *L. monocytogenes* challenge, they had to be pretreated with CpG DNA at least 48 h before the challenge. This indicates that the protective mechanism induced by CpG DNA is not simply the induction of serum cytokines, which occurs much more rapidly (within 1 h). Rather, this lag period may be explained by a need to induce cellular effector mechanisms to clear the bacterial challenge.

The CpG-induced protection against *L. monocytogenes* per-
formed for at least 2 wk after priming. To identify the possible mechanisms for this sustained activation of innate immunity follow-
CpG injection, we investigated the kinetics of serum cy-
tokine expression, including IL-12, TNF-α, and IFN-γ, which are known to contribute to innate resistance to *L. monocytogenes* challenge (23, 25, 27). Our studies showed that a single injection of CpG DNA induces a transient systemic expression of TNF-α and IFN-γ that returns to baseline within 24 h; the sustained expression of the serum IL-12 was observed for at least 8 days. This does not mean that TNF-α and IFN-γ play no role in the sustained resis-
tance to *L. monocytogenes* challenge. Since IL-12 can promote IFN-γ secretion, this sustained IL-12 expression following CpG DNA exposure may improve the ability of effector cells to rapidly up-regulate IFN-γ expression, thereby improving resistance to in-
fec tion. In our previous studies using enzyme-linked immunospot assays, we were unable to detect spleen cell IL-12 production for longer than 12 h after the injection of CpG DNA (4); in our recent experiments, we also have been unable to detect a sustained spleen expression of IL-12 mRNA. Further studies will be required to identify the tissue and cellular source of the sustained IL-12 re-
sponse to CpG DNA.

*C. pneumoniae* growth in IFN-γ-deficient mice is thought to be completely unchecked and to proceed at the fastest possible rate. Therefore, we were surprised to find that the knockout mice that had been pretreated with CpG DNA consistently had an $\sim 10$-fold increase in the number of *L. monocytogenes* colonies that were recovered from the spleen and liver (Fig. 4). This increase was not simply the result of an excessive challenge dose, since mice that were challenged with greatly reduced doses still showed no benefit from pretreatment with CpG DNA (Fig. 4B). A potential clue to the mechanism responsible for the loss of CpG-mediated protection in IFN-γ deficient mice comes from our finding that although these mice showed increased serum IL-12 in the first 4 h following CpG injection, these levels rapidly fell back to baseline by 12 h (Table I). Thus, in contrast to wt mice, serum IL-12 levels are not persistently elevated in CpG-injected, IFN-γ deficient mice. This observation is compatible with our hypothesis that sus-
tained IL-12 elevation is important for the protective effects of CpG DNA.

CpG-induced IFN-γ secretion is itself dependent upon the prior production of IL-12 (4, 10). We hypothesize that the initial IFN-γ burst that is induced by CpG DNA in wt mice may feedback-
amplify CpG-induced IL-12 expression, as demonstrated previ-
ously in other systems. The lack of this IFN-γ burst in the deficient mice would consequently lead to a weaker and more transient pro-
duction of IL-12, especially since CpG DNA also induces the pro-
duction of IL-10, which opposes IL-12 production (33).

The increased proliferation of *L. monocytogenes* in IFN-γ defi-
cient mice demonstrates not only the essential role of IFN-γ in mediating the protection induced by CpG DNA but also that the immune effects of CpG DNA may actually be deleterious and pro-
mote the growth of infectious organisms under certain circum-
stances. It is noteworthy that although IFN-γ is required for the protective effect, serum levels of IFN-γ return to baseline by 12 h after CpG DNA injection. The precise mechanism(s) responsible for these effects await further study.

The protective effects of treatment with CpG DNA were most apparent in SPF mice. Our results suggest that mice housed under “conventional” conditions, in which they are infected with minor pathogens, may intermittently have a level of activation of innate immune defenses that renders them partially resistant to infectious challenge. This basal level of immune activation may be due to environmental exposure to CpG DNA or to other microbial prod-
ucts alone or in combination with CpG DNA.

---

Most attention in the treatment of infectious disease has focused on antimicrobial agents and on measures to induce specific immune defenses. The data in the present report demonstrate the possible beneficial effects of activating innate non-Ag-specific defenses, and suggest that CpG DNA may have a role in the prevention of infectious disease.

Acknowledgments

We thank Vickie McCauley and Tilese Arrington for expert assistance in the preparation of this manuscript.

References


