CpG DNA Prevents Liver Injury and Shock-mediated Death by Modulating Expression of Interleukin-1 Receptor-associated Kinases*5

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Tumor necrosis factor-α (TNF-α) produced by macrophages in response to CpG DNA induces severe liver injury and subsequent death of d-galactosamine (d-GalN)-sensitized mice. In the present study we demonstrate that mice pre-exposed to CpG DNA are resistant to liver injury and death induced by CpG DNA/d-GalN. CpG DNA/d-GalN failed to induce TNF-α production and hepatocyte apoptosis in the mice pre-exposed to CpG DNA. In addition, macrophages isolated from the CpG DNA-pretreated mice showed suppressed activation of MAPKs and NF-κB and production of TNF-α in response to CpG DNA, indicating that the CpG DNA-mediated protection of CpG DNA/d-GalN-challenged mice is due to the hyporesponsiveness of macrophages to CpG DNA. CpG DNA pretreatment in vivo inhibited expression of interleukin-1 receptor-associated kinase (IRAK)-1 while inducing IRAK-M expression in macrophages. Suppressed expression of IRAK-1 was responsible for the macrophage hyporesponsiveness to CpG DNA. However, increased expression of IRAK-M was not sufficient to render macrophages hyporesponsive to CpG DNA but was required for induction of the optimal level of macrophage hyporesponsiveness. Taken together, reduced expression of IRAK-1 and increased expression of IRAK-M after CpG DNA pretreatment resulted in the hyporesponsiveness of macrophages that lead to the protection of mice from hepatic injury and death caused by CpG DNA/d-GalN.

The innate immune response is initiated by recognition of evolutionarily conserved molecular motifs (pathogen-associated molecular patterns) found in a variety of microorganisms by pattern recognition receptors present in the host innate immune cells (1, 2). Among the pattern recognition receptors, Toll-like receptors (TLRs) are the most extensively investigated. TLRs are a group of structurally related proteins that contain amino-terminal leucine-rich repeats that are responsible for binding to pathogen-associated molecular patterns, a transmembrane domain, and a carboxyl-terminal Toll/interleukin-1 receptor domain that is responsible for signaling. The TLR family now consists of at least 13 members (TLR1-TLR13) in the mouse and 11 members in humans. Among the different TLRs, TLR9 has been identified as a receptor for bacterial DNA, double-stranded viral DNA, and synthetic oligodeoxynucleotides containing an unmethylated CpG motif (CpG DNA) (3–5). Ligand (CpG DNA)-bound TLR9 recruits the adaptor molecule, myeloid differentiation factor 88 (MyD88), to its intracellular Toll/interleukin-1 receptor domain. MyD88 in turn recruits interleukin-1 receptor-associated kinase-4 (IRAK-4) and IRAK-1 to the TLR9/MyD88 signaling complex. IRAK-1 becomes rapidly phosphorylated by IRAK-4, leaves the receptor complex, and then associates with tumor necrosis factor-α receptor-associated factor 6 (TRAF6). Binding of TRAF6 to IRAK-1 ultimately results in activation of signaling cascades that lead to the activation of mitogen-associated protein kinases (MAPKs) and NF-κB and subsequent expression of proinflammatory cytokines, chemokines, and oncoproteins (6).

The TLR-mediated production of proinflammatory mediators and cytokines by innate immune cells is necessary for efficient control of growth and dissemination of invading pathogens. However, uncontrolled, excessive, and prolonged activation of innate immunity can be detrimental to the host. To prevent such undesirable outcomes as septic shock-like syndrome and chronic inflammatory diseases, the innate immune system might employ regulatory mechanisms that ensure an

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‡2 The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Figs. 1–6.

‡3 The on-line version of this article contains supplemental Fig. 7.

4 The abbreviations used are: TLR, Toll-like receptor; TNF-α, tumor necrosis factor-α; MyD88, myeloid differentiation factor 88; IL, interleukin; IRAK1, IL-1 receptor-associated kinase 1; TRAF6, TNF-α receptor-associated factor 6; DN, dominant negative; MAPK, mitogen-activated protein kinase; SHIP1, Src homology 2-containing inositol-5′-phosphatase; LPS, lipopolysaccharide; SOCS, suppressors of cytokine signaling; PBS, phosphate-buffered saline; iCpG, inhibitory CpG; KC, Kupffer cells; RT, reverse transcription; JNK, c-Jun NH2-terminal kinase; ERK, extracellular signal-regulated kinase; ChIP, chromatin immunoprecipitation; sh, small hairpin interfering.
initial pathogen response of an appropriate magnitude while avoiding excessive responses to multiple waves of pathogenic stimuli. Chronic exposure to endotoxin or lipopolysaccharide (LPS) causes a transient increase in the threshold to endotoxin challenge. Neutrophils and monocytes isolated from septic patients and experimental animals with sepsis have decreased phagocytic ability, reduced bactericidal activity, attenuated proinflammatory cytokine production, and decreased activation of transcription factor NF-κB in response to ex vivo LPS stimulation (7–9). This phenomenon, known as immunoparalysis, endotoxin tolerance, or macrophage hyporesponsiveness, may have developed to minimize damage from severe inflammatory reactions and to prevent chronic inflammatory illness. Like LPS, other bacterial products, such as lipoteichoic acid, mucin-like glycoprotein, flagellin, R-848, poly(IC), and CpG DNA, induce macrophage hyporesponsiveness similar to endotoxin tolerance (10–12). Studies with these TLR ligands indicate that each specific TLR ligand induces macrophage hyporesponsiveness by a different mechanism involving modulation of the expression, modification, or function of TLR-signaling molecules (11–17). In addition, anti-inflammatory cytokines, suppressors of cytokine signaling (SOCS) proteins, IRAK-M, SHIP1, A20, and cylindromatosis expressed in macrophages after initial stimulation with TLR ligands have also been demonstrated to play a critical role in the induction of macrophage hyporesponsiveness to microbial products (18–27). Careful studies of the roles of each TLR-pathway signaling proteins and the contribution of pro- and anti-inflammatory cytokines and mediators to the induction of macrophage hyporesponsiveness are necessary to fully understand the regulatory mechanisms that prevent severe inflammatory reactions and chronic inflammatory illness during the course of microbial infection.

D-Galactosamine (D-GalN), a hepatotoxic agent that inhibits biosynthesis of RNA, proteins, glycoproteins, and glycogen in hepatocytes, treatment increases sensitivity to the lethal effects of LPS, CpG DNA, and other microbial products several thousand-fold in mice (28–30). This microbial product-induced shock-mediated death of D-GalN-sensitized mice is due to massive hepaticocyte apoptosis caused by TNF-α secreted from activated monocyte cells (30–32). Therefore, D-GalN-sensitized mice are an ideal model in which to study the factors that affect host susceptibility to inflammatory responses and liver injury associated with sepsis. In this study we investigated whether CpG DNA induces hyporesponsiveness of macrophages to subsequent CpG DNA challenge in vivo using the D-GalN-sensitized mice model system and explored a part of the host negative feedback mechanisms that prevent excessive proinflammatory responses against multiple waves of pathogenic stimuli.

**MATERIALS AND METHODS**

**Oligodeoxynucleotides and Reagents**—Nuclease-resistant phosphorothioate oligodeoxynucleotides (S-ODN) 1826 (CpG DNA), 1982 (non-CpG DNA), and inhibitory CpG DNA (iCpG DNA: 2088) were purchased from Operon (Alameda, CA) and Coley Pharmaceutical Group (Kanata, Ontario, Canada) and further purified by ethanol precipitation. S-ODN had no detectable endotoxins by Limulus assay. The sequences of S-ODN used are previously reported (33). LPS (Salmonella minnesota Re 595) and d-galactosamine (d-GalN) were purchased from Sigma. Purity of LPS was confirmed by the lack of ability to induce IL-6 production in macrophages isolated from LPS-non-responder C3H/HeJ mice.

**Mice**—BALB/c mice at 4–5 weeks of age were obtained from The Frederick Cancer Research and Development Center, NCI, National Institutes of Health (Frederick, MD) and used within 3 weeks. All animal care and housing requirements were followed, and animal protocols were reviewed and approved by the University of Tennessee Animal Care and Use Committee.

**Experimental Protocol**—Mice were injected intraperitoneally with PBS, CpG DNA (10–40 μg), control non-CpG DNA (30 μg), or LPS (10 μg) in the presence or absence of a TLR9 antagonist iCpG DNA (60 μg). Twenty-four h later mice were challenged intraperitoneally with a lethal dose of CpG DNA (20 μg) plus D-GalN (20 mg). Viability of mice was observed over 120-h time periods. In some experiments mice were anesthetized and then killed before the CpG DNA plus D-GalN (CpG/D-GalN) challenge, and then peritoneal lavage fluid, spleens, and livers were removed for macrophage isolation. In some experiments mice were anesthetized at designated time periods after CpG/D-GalN challenge, blood samples were obtained to prepare serum, mice were then killed, and the livers were removed. Liver lobes were excised and then either fixed by submersion in 10% neutral buffered formalin (Fisher Diagnostics, Middleton, VA) for histopathological analysis or kept at −80 °C for further analysis. In all experiments, 5–10 mice were used for each condition.

**Preparation of Peritoneal Macrophages, Splenic Macrophages, and Kupffer Cells (KCs)**—Twenty-four h after injection with PBS, CpG DNA (30 μg) or control non-CpG DNA (30 μg) mice were killed. Peritoneal cells were collected by peritoneal lavage and washed three times with ice-cold PBS. To obtain macrophage-enriched cell preparations, the peritoneal cell suspensions were incubated for 1 h in Dulbecco’s modified Eagle’s medium at 37 °C to allow macrophages to adhere to plates. Non-adherent cells were removed by washing cells three times with pre-warmed PBS. Splenic macrophages were isolated from splenic cell suspensions by MACS using a macrophage negative selection kit (Invitrogen) according to the manufacturer’s protocol. KCs were isolated as previously described (34). The purity of macrophage preparations was assessed by flow cytometry after staining with biotin-conjugated anti-mouse CD11b (Mac-1) antibody or anti-mouse F4/80 and streptavidin-PE (BD Biosciences).

**Cell Lines and Culture Conditions**—RAW264.7 cells (ATCC, Rockville, MD) and primary macrophages were cultured at 37 °C in a 5% CO2, humidified incubator and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 1.5 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. All culture reagents were purchased from Invitrogen.

**Histopathology**—Liver specimens fixed in 10% neutral buffered formalin were embedded in paraffin and sectioned at a thickness of 5 μm before staining with hematoxylin and eosin for light microscopic examination.
Aspartate-specific Cysteine Protease (Caspase) Assays and DNA Fragmentation Analysis—Activities of the selected caspases in cytosolic extracts of liver tissue were measured using a Caspase-Glo assay kit (Promega Co., Madison, WI), and DNA fragmentation in liver cells was detected as previously described (30).

Cytokine-specific Enzyme-linked Immunosorbent Assay (ELISA) and Reverse Transcriptase (RT)-PCR—Concentrations of selected cytokines (TNF-α, IL-6, IL-10, IL-12p70) in serum and culture supernatants were analyzed by cytokine-specific ELISA, and mRNA levels of selected cytokines and TLR-signaling modulators in liver and macrophages were analyzed by RT-PCR. All recombinant murine cytokines and antibodies specific for murine cytokines were purchased from BD/Pharmingen. The sequences of RT-PCR primers are either previously published (12, 30) or are listed in supplemental Table 1. All RT-PCR primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA).

Electrophoretic Mobility Shift Assay and Western Blot Analysis—To detect DNA binding activity of the transcription factor NF-κB, nuclear extracts (3 μg/lane) were analyzed by electrophoretic mobility shift assay using 32P-labeled double stranded oligodeoxynucleotides containing the NF-κB binding sequence as a probe (35). To detect the presence or phosphorylation status of a specific protein, equal amounts of whole cell lysates or cytoplasmic extracts were subjected to electrophoresis on a 10% polyacrylamide gel containing 0.1% SDS, and then Western blots were performed as previously described (34). Actin and/or the indicated specific total protein was used as a loading control. The density of each protein band was quantitated by densitometry (GS-700 Imaging Densitometer, Bio-Rad) and normalized to the density of the actin band or indicated specific total protein in the same sample. Numbers represent the -fold induction from the normalized densitometric value of each protein band in the unstimulated control sample. Antibodies specific for IκBα, p38, MyD88, TLR4, TRAF6, JNK, p38, ERK, or actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies specific for the phosphorylated form of ERK, JNK, or p38 were purchased from Cell Signaling (Danvers, MA). Anti-IRAK-1 antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Antibodies specific for IRAK-2, IRAK-M, or IRAK-4 were purchased from Chemicon International (Temecula, CA). Anti-TLR9 was purchased from IMGENEX (San Diego, CA).

Plasmids and Generation of IRAK-1 Knockdown Macrophages and IRAK-M-expressing Macrophages—To construct a vector expressing FLAG-tagged IRAK-M, the coding region of IRAK-M was amplified by PCR using murine spleen cDNA as a template and then inserted into a pIREs-hrGFPII (Stratagene, La Jolla, CA) vector. The DNA sequence of cloned IRAK-M was confirmed by DNA sequencing analysis and was identical with the previously reported sequence. To construct a vector expressing a gene-specific small hairpin interfering (sh) RNA, a pair of complementary 64-mer oligodeoxynucleotides containing Caenorhabditis elegans luciferase- or murine IRAK-1-specific target sequences were inserted between the BglII site and the HindIII site of pSUPER.retro.neo/GFP following the manufacturer’s instructions (OligoEngine, Seattle, WA). The authenticity of the gene-specific shRNA constructs was confirmed by DNA sequencing. To generate stable transfectants that constitutively express FLAG-tagged IRAK-M, luciferase-shRNA, IRAK-1-shRNA, or FLAG-tagged IRAK-M and IRAK-1-shRNA, RAW264.7 cells were transfected with pIREs-hrGFPII, pIREs-hrGFPII-IRAK-M, pSuper.Retro.neo.GFP-luciferase-shRNA, pSuper.Retro.neo.GFP-IRAK-1-shRNA, or pIREs-hrGFPII-IRAK-M and pSuper.Retro.neo.GFP-IRAK-1-shRNA using Lipofectamine and Plus transfection reagents (Invitrogen) according to the manufacturer’s instructions. Stable transfectants were selected and maintained in selection medium containing G418 (1 mg/ml). All primers for cloning and gene-specific shRNA targeting were purchased from Integrated DNA Technologies, Inc., and sequences of primers are listed in supplemental Table 1.

Transient Transfection and Reporter Gene Assays—Stable transfectants (1.2 × 106 cells/well) were plated into 6-well culture plates and then incubated for 24 h to reach ~80% confluence. Cells were transfected with reporter gene NF-κB-luciferase (1 μg) plus pRL-TK-luciferase (0.1 μg) and then incubated for 3 h. The transiently transfected cells were pooled and washed 3 times with culture medium and then rested 6 h. Cells (1 × 105 cells/well in 96-well plate) were stimulated with medium, CpG DNA (3 μg/ml), or LPS (50 ng/ml) for 12 h. Luciferase activities in cell extracts were analyzed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol. Luciferase activity was normalized using pRL-TK-luciferase activity (Renilla) in each sample.

Chromatin Immunoprecipitation (ChIP) Assay—Cells (1 × 106) were stimulated as indicated for 1 h and then cross-linked for 10 min at 37 °C by adding formaldehyde directly to the tissue culture medium to a final concentration of 1%. Cross-linked cells were then washed twice with cold PBS (with protease inhibitors), scraped, pelleted, resuspended in 200 μl of ChIP lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0, and protease inhibitors) and incubated for 10 min on ice. The lysates were then sonicated with a Sonic dismembrator (Fisher) at power setting 2 for 3 cycles of 10 s each. After sonication, the samples were centrifuged, and the supernatants were diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 1 mM EDTA, 20 mM Tris, pH 8.0, 150 mM NaCl, and protease inhibitors). Total DNA used for controlling the amount of DNA/sample (input control using β-actin) was purified from one-tenth of the lysate. Each sample was precleared by incubating with 60 μl of salmon sperm DNA/GammaBind plus Sepharose 50% gel slurry (GE Healthcare) for 30 min at 4 °C. The resulting precleared cross-linked chromatin samples were incubated overnight with 4 μg of antibody specific for NF-κB p65 or isotype control antibody (IgG) in a total volume of 1 ml at 4 °C. Antibody-protein-DNA complexes were isolated by immunoprecipitation with 60 μl of salmon sperm DNA/GammaBind. The immunoprecipitates were washed once with each buffer in the following order: low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl); high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl); LiCl wash buffer (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1); TE (10
CpG DNA pretreatment protects mice from shock-mediated death induced by CpG DNA/D-GalN through a TLR9-dependent pathway. A, BALB/c mice (7–9 mice/group) were injected intraperitoneal with PBS or various doses of CpG DNA (10–40 μg/mouse). B, BALB/c mice (7 mice/group) were injected intraperitoneal with PBS, CpG DNA (30 μg/mouse), or control non-CpG DNA (30 μg/mouse). C, BALB/c mice (7 mice/group) were injected intraperitoneal with PBS or CpG DNA (30 μg/mouse) in the presence or absence of a TLR9 antagonist iCpG DNA (60 μg/mouse). D, BALB/c mice (7 mice/group) were injected intraperitoneal with PBS or LPS (10 μg/mouse) in the presence or absence of TLR9 antagonist iCpG DNA (60 μg/mouse). Twenty-four hours later all mice were challenged intraperitoneal with a lethal dose of CpG DNA (20 μg/mouse) and D-GalN (20 mg/mouse). Viability of mice was observed over 120-h time periods. Statistical difference from PBS-pretreated and then CpG DNA/D-GalN-challenged control was indicated (*, p < 0.05; **, p < 0.005).

**FIGURE 1.** CpG DNA pretreatment protects mice from shock-mediated death induced by CpG DNA/D-GalN—TNF-α produced by macrophages in response to the TLR4 ligand LPS and the TLR9 ligand CpG DNA induces massive apoptosis of hepatocytes and subsequent shock-mediated death of mice sensitized with D-GalN (30, 32). Previously, we have demonstrated that murine macrophage RAW264.7 cells pre-exposed to LPS or CpG DNA show substantially reduced production of cytokines in response to later CpG DNA challenge (12). This led us to investigate whether pre-exposure of mice to CpG DNA prevents the shock-mediated death induced by CpG DNA/D-GalN. BALB/c mice were injected intraperitoneally with various doses of CpG DNA. Twenty-four hours later mice were challenged with a lethal dose of CpG DNA/D-GalN, and then viability of the mice was observed. The survival rate of the CpG DNA-pretreated group was markedly improved in a dose-dependent manner compared with that of the PBS- or non-CpG DNA-pretreated control groups (Fig. 1, A and B). To ensure that this CpG DNA-mediated protection of mice from the CpG DNA/D-GalN-induced death was not due to action of any contaminants, such as LPS, mice were injected with CpG DNA in the presence or absence of a TLR9 antagonist iCpG DNA (36). Twenty-four hours later mice were challenged with a lethal dose of CpG DNA/D-GalN, then viability of mice was observed. As shown in Fig. 1C, pretreatment of mice with iCpG DNA alone did not protect mice from shock-mediated death induced by CpG DNA/D-GalN, indicating that iCpG DNA treatment in vivo 24 h before the CpG DNA/D-GalN challenge does not have inhibitory effects on the action of CpG DNA administrated 24 h later. When mice were injected with CpG DNA and iCpG DNA simultaneously, CpG DNA failed to protect mice from shock-mediated death induced by CpG DNA/D-GalN. Like CpG DNA, LPS pretreatment also protected mice from shock-mediated death caused by lethal challenge with CpG DNA/D-GalN (Fig. 1D). However, iCpG DNA failed to inhibit the ability of LPS to protect mice from shock-mediated death induced by CpG DNA/D-GalN, indicating that the inhibitory effect of iCpG DNA is specific for the action of CpG DNA. Taken together, these results demonstrate that CpG DNA, in a sequence-specific and dose-dependent manner, has the ability to induce changes in the intact animal that result in prevention of the shock-mediated death of mice caused by the CpG DNA/D-GalN lethal challenge.

**RESULTS**

**CpG DNA Pretreatment Protects Mice from Shock-mediated Death Induced by CpG DNA and D-GalN—** We further investigated whether CpG DNA pretreatment also prevents acute liver injury in CpG DNA/D-GalN-challenged mice. As demonstrated in Fig. 2A, severe hepatic injury (including steatosis, massive hemorrhage, and massive apoptosis of hepatocytes characterized by shrinkage and hypereosinophilia of cytoplasm and nuclear condensation and fragmentation) after CpG DNA/D-GalN challenge was found in mice pretreated with PBS, non-CpG DNA, or iCpG DNA. In addition, CpG DNA/D-GalN challenge induced activation of caspases 8, 9, and 3/7 and fragmentation of DNA in liver cells in these mice (Fig. 2, B–E). In contrast, no evidence of hepatic injury after CpG DNA/D-GalN challenge was found in mice pretreated with CpG DNA or LPS (Fig. 2A). CpG DNA/D-GalN-induced liver injury after CpG DNA/D-GalN challenge was found in mice pretreated with CpG DNA or LPS (Fig. 2A). CpG DNA/D-GalN-induced liver injury after CpG DNA/D-GalN challenge was found in mice pretreated with CpG DNA or LPS (Fig. 2A).
GalN-induced activation of caspases 8, 9, and 3/7 and subsequent DNA fragmentation in liver cells were also prevented in mice pretreated with CpG DNA against liver injury and shock-mediated death of d-GalN-sensitized mice (30, 37–41). Therefore, we investigated whether CpG DNA pretreatment alters systemic and/or liver expression of these cytokines in mice challenged with CpG DNA/d-GalN. CpG DNA/d-GalN induced systemic production of cytokines TNF-α and IL-6 in mice pre-exposed to PBS or non-CpG DNA (Fig. 3, A and B, data not shown). In contrast, CpG DNA pretreatment completely inhibited systemic production of TNF-α and IL-6 in mice in response to CpG DNA/d-GalN. Systemic production of IL-10, IL-12, and interferon γ in response to CpG DNA/d-GalN was not detected under our experimental conditions, probably due to different kinetics of these cytokines production after CpG DNA/d-GalN challenge (data not shown). Similar to the systemic production of cytokines, CpG DNA/d-GalN-induced expression of cytokines TNF-α, IL-6, IL-10, and IL-12p40 in livers of mice pre-exposed to PBS or non-CpG DNA (Fig. 3C). In contrast, CpG DNA pretreatment almost completely inhibited CpG DNA/d-GalN-induced expression of these cytokines in liver. Taken together, these results indicate that CpG DNA-induced protection of CpG DNA/d-GalN-challenged mice against liver injury and shock-mediated death is due to its ability to inhibit systemic and liver expression of critical proinflammatory cytokines, especially TNF-α, in these mice. These results also suggest that CpG DNA pretreatment in vivo may render cytokine-producing cells, such as splenic macrophages and KCs, to become hyporesponsive to CpG DNA in mice.

CpG DNA Failed to Induce Expression of TNF-α in Liver Resident Macrophages Isolated from the Mice Pre-exposed to CpG DNA—It is known that KCs (liver resident macrophages) are a major source of systemic cytokines, and TNF-α produced by KCs plays an indispensable role in concanavalin A- and LPS/d-GalN-induced liver injury (42–44). Because our results suggest that CpG DNA pretreatment may render cytokine-producing liver cells hyporesponsive to CpG DNA in mice, we investigated whether KCs isolated from mice pre-exposed to CpG DNA are hyporesponsive to later CpG DNA challenge ex vivo. As demonstrated in Fig. 4, CpG DNA induced both mRNA and protein expression of TNF-α in KCs isolated from mice pre-exposed to PBS or non-CpG DNA. In contrast, CpG DNA-mediated
TNF-α expression was completely suppressed in KCs isolated from mice pre-exposed to CpG DNA. Of note, we also found greatly suppressed TNF-α production in peritoneal macrophages and peritoneal macrophages isolated from mice pre-exposed to CpG DNA (supplemental Fig. 1). These results demonstrate that CpG DNA pretreatment in vivo induces hyporesponsiveness of KCs (as well as splenic and peritoneal macrophages) to CpG DNA in mice.

**CpG DNA-mediated Activation of NF-κB and MAPKs in KCs Isolated from Mice Pre-exposed to CpG DNA Is Substantially Suppressed**—Activation of MAPKs and NF-κB plays an indispensable role in CpG DNA-mediated expression of various cytokines, including TNF-α, in macrophages (23, 34, 35). It has previously been demonstrated that activation of stress-responsive transcription factors, such as NF-κB and AP1, in macrophages is required for LPS/β-GalN-mediated liver injury and shock-mediated death in mice (31). In addition, we found that CpG DNA/β-GalN failed to induce activation of NF-κB and ERK in the liver in mice pretreated with CpG DNA (supplemental Fig. 2). Therefore, we investigated whether CpG DNA-mediated activation of MAPKs and/or NF-κB is suppressed in KCs isolated from mice pre-exposed to CpG DNA. CpG DNA strongly induced activation of all three MAPKs (JNK, ERK, and p38), degradation of IκBα, nuclear DNA binding activity of NF-κB, and NF-κB binding to the TNF-α promoter region in KCs isolated from mice pre-exposed to PBS or non-CpG DNA (Fig. 5). Because of prolonged effects of CpG DNA pretreatment, KCs isolated from mice pre-exposed to CpG DNA showed some background level of activation of MAPKs and degradation of IκBα. However, CpG DNA failed to induce activation of these MAPKs, degradation of IκBα, nuclear DNA binding activity of NF-κB, and NF-κB binding to the TNF-α promoter region above the background levels in KCs isolated from the mice pre-exposed to CpG DNA. These results indicate that CpG DNA pretreatment in vivo induces hyporesponsiveness of KCs to subsequent CpG DNA challenge for activation of MAPKs and transcription factors.
NF-κB. These results also suggest that CpG DNA-induced protection of CpG DNA/d-GalN-challenged mice against the liver injury and shock-mediated death may be due to the inhibited activation of MAPKs and NF-κB that is critical for the expression of proinflammatory cytokines, such as TNF-α, in macrophages in these mice.

**In Vivo Effects of CpG DNA on KC Expression of Signaling Modulators in the TLR9-signaling Pathway**—CpG DNA-induced hyporesponsiveness of KCs to later CpG DNA challenge could be due to alterations in the expression level or function of TLR9 and/or signaling molecules in the TLR9-signaling pathway. Therefore, we investigated whether CpG DNA treatment can affect the expression of TLRs and/or their downstream signaling modulators in KCs in vivo. As shown in Fig. 6, CpG DNA treatment in vivo did not significantly alter expression of TLR4, TLR9, Myd88, IRAK4, and TRAF6 at both mRNA and protein levels in KCs. CpG DNA treatment in vivo induced expression of its negative regulator IRAK-M at both mRNA and protein levels in KCs. In contrast, CpG DNA induced the disappearance of IRAK-1 protein and message in KCs. We did not observe any significant alterations in the expression of these signaling modulators in KCs isolated from mice treated with non-CpG DNA compared with those in KCs isolated from PBS control mice. Of note, we observed similar results in peritoneal macrophages and splenic macrophages isolated from the mice (supplemental Fig. 3). These results suggest a possibility that hyporesponsiveness of macrophages to CpG DNA/d-GalN in mice pre-exposed to CpG DNA is due to combinational effects.
of suppressed expression of IRAK1 (a positive regulator of TLR
signaling) and increased expression of IRAK-M (a negative regu-
lar) in macrophages, including KCs.

**IRAK-M Expression Is Dispensable for the CpG DNA-me-
diated Induction of Macrophage Hyporesponsiveness to CpG
DNA**—To determine whether IRAK-M is the sole responsible
factor for CpG DNA-induced macrophage hyporesponsiveness to
CpG DNA, we generated RAW264.7 cells that constitutively
overexpress IRAK-M (IRAK-M-RAW). IRAK-M (#1) and (#2) are two different stable clones. The presence of mRNA (A) and protein (B) for the indicated gene in each sample was detected by RT-PCR and Western blot, respectively. C, Con-RAW cells were stimulated with PBS, CpG DNA, or LPS for 12 h, and luciferase activity was analyzed. Data are the mean ± S.D. of triplicates. G, cells were stimulated with PBS, CpG DNA, or LPS for 6 h. TNF-α was measured by ELISA. Data are the mean ± S.D. of triplicates. All experiments were done more than three times with similar results.

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signaling) and increased expression of IRAK-M (a negative regu-
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**IRAK-M Expression Is Dispensable for the CpG DNA-me-
diated Induction of Macrophage Hyporesponsiveness to CpG
DNA**—To determine whether IRAK-M is the sole responsible
factor for CpG DNA-induced macrophage hyporesponsiveness to
CpG DNA, we generated RAW264.7 cells that constitutively
overexpress IRAK-M (IRAK-M-RAW). Expression of IRAK-M
mRNA and protein was confirmed in IRAK-M-overexpressing
macrophages (Fig. 7, A and B). The level of IRAK-M expression
in IRAK-M-RAW cells was comparable with that induced by
CpG DNA in the control cells (Con-RAW; RAW264.7 cells sta-
bly transfected with empty vector) (Fig. 7C). Activation of
NF-κB and MAPKs and production of TNF-α in response to
CpG DNA in Con-RAW and IRAK-M-RAW cells were ana-
lyzed. In contrast to our expectation, constitutive overexpres-
sion of IRAK-M suppressed neither activation of MAPKs and
NF-κB nor production of TNF-α in RAW264.7 cells in response
to CpG DNA or LPS (Fig. 7, D–G). Of note, our preliminary study
showed that transient overexpression of IRAK-M in RAW264.7
cells ablated CpG DNA-mediated NF-κB activation, indicating
IRAK-M expressed by cloned vector is functional (supplemental
Fig. 4). These results suggest that although IRAK-M is a negative
regulator in TLR-signaling, induction of IRAK-M expression
alone may not be sufficient to inhibit CpG DNA-mediated macro-
phage activation and that IRAK-M expression is a dispensable fac-
tor for CpG DNA-mediated macrophage hyporesponsiveness to
CpG DNA and other TLR ligands.

**IRAK-1 Knockdown Macrophages Are Hyporesponsive to
CpG DNA**—Because KCs as well as peritoneal and splenic mac-
rophages hyporesponsive to CpG DNA show substantially sup-
pressed expression of IRAK-1 (Fig. 6 and supplemental Fig. 3), we hypothesized that CpG DNA pretreatment induces macrophage hyporesponsiveness to later CpG DNA challenge by suppressing IRAK-1 expression. To test this hypothesis, we generated IRAK-1 knockdown macrophages (IRAK-1shRNA-RAW). The effectiveness of IRAK-1-specific shRNA to silence IRAK-1 expression at both the mRNA and protein level without affecting expression of other IRAK family members or TLR signaling modulators in IRAK-1 knockdown macrophages was confirmed (Fig. 8, A and B). Activation of NF-κB and MAPKs and production of TNF-α in response to CpG DNA in vector control cells (Luc-shRNA; RAW264.7 cells stably expressing C. elegans luciferase-specific shRNA) and IRAK-1shRNA-RAW cells were analyzed. As expected, CpG DNA strongly activated NF-κB and all three MAPKs and induced expression of TNF-α in vector control cells (Luc-shRNA; RAW264.7 cells stably expressing C. elegans luciferase-specific shRNA) (Fig. 8, C–G). Neither CpG DNA- nor LPS-mediated activation of p38 and ERK was suppressed in IRAK-1 knockdown macrophages (Fig. 8, C and D). In contrast, CpG DNA-mediated activation of JNK was completely inhibited in IRAK-1 knockdown macrophages. In addition, CpG DNA-mediated activation of NF-κB was partially inhibited in IRAK-1 knockdown macrophages (Fig. 8, E). Furthermore, NF-κB binding to the TNF-α promoter region, a ChIP assay was performed (F). IP, immunoprecipitation. Messenger RNA levels of TNF-α were determined by RT-PCR (G). Concentrations of TNF-α in the culture supernatants were measured by ELISA (H). Data presented are the mean ± S.D. of triplicates. Statistical difference from CpG DNA- or LPS-treated Luc-shRNA cell control was indicated (*, p < 0.05; **, p < 0.005). All experiments were done more than three times with similar results.

FIGURE 8. CpG DNA-mediated activation of MAPKs and NF-κB and production of TNF-α are substantially suppressed in IRAK-1 knockdown macrophages. A, total RNA was prepared from RAW264.7 cells stably transfected with vectors expressing C. elegans luciferase-specific shRNA (Luc-shRNA) or IRAK-1-specific shRNA (IRAK-1shRNA). IRAK1sh (#1) and (#2) are two different stable clones. The presence of mRNA for the indicated gene in each sample was detected by RT-PCR. B, whole cell extracts were prepared from Luc-shRNA and IRAK-1shRNA cells. Equal amounts of cell lysates were subjected to SDS-PAGE, and then Western blots were performed. C, cells were stimulated with PBS (P), CpG DNA (C, 6 μg/ml), or LPS (L, 10 ng/ml) for 1 h. Equal amounts of whole cell lysates (15 μg/lane) were subjected to SDS-PAGE, and then Western blots were performed. D, quantitation of panel C by densitometry. E, cells were transiently transfected with NF-κB-luciferase plus pRL-TK-luciferase and stimulated with PBS, CpG DNA, or LPS for 12 h, and then luciferase activity in cell extracts was analyzed. Data present the mean ± S.D. of triplicates. F–H, cells were stimulated with PBS, CpG DNA, or LPS for 1 h (F and G) or 6 h (H). To detect NF-κB binding activity on TNF-α promoter region, a ChIP assay was performed (F). IP, immunoprecipitation. Messenger RNA levels of TNF-α were determined by RT-PCR (G). Concentrations of TNF-α in the culture supernatants were measured by ELISA (H). Data presented are the mean ± S.D. of triplicates. Statistical difference from CpG DNA- or LPS-treated Luc-shRNA cell control was indicated (*, p < 0.05; **, p < 0.005). All experiments were done more than three times with similar results.

IRAKs in CpG DNA Tolerance in Vivo
expression of IRAK-1 protein and message after CpG DNA pre-treatment in macrophages, including KCs.

CpG DNA-induced Macrophage Hyporesponsiveness to CpG DNA Challenge Is Due to Combinational Effects of Suppressed Expression of IRAK-1 and Increased Expression of IRAK-M—Even though increased expression of IRAK-M alone is not sufficient to inhibit CpG DNA responses in macrophages, it is possible that increased expression of IRAK-M in macrophages that have little or no IRAK-1 can contribute to further suppression of CpG DNA responses. To determine whether IRAK-M expression has further suppressive effects on CpG DNA-mediated IRAK-1 knockdown macrophage activation, we generated IRAK-M-overexpressing IRAK-1 knockdown macrophages (IRAK-1-shRNA&IRAK-M). Constitutive overexpression of IRAK-M and suppressed expression of IRAK-1 without affecting expression of other genes were confirmed in IRAK-1-shRNA&IRAK-M macrophages (Fig. 9, A and B). Effects of increased IRAK-M expression and suppressed IRAK-1 expression on CpG DNA-mediated macrophage activation were investigated. CpG DNA failed to induce activation of JNK, ERK, and p38 in IRAK-1-shRNA and IRAK-M macrophages (Fig. 9C). In addition, CpG DNA-mediated activation of NF-κB and binding of NF-κB to the TNF-α promoter region were substantially, if not completely, inhibited in IRAK-1-shRNA and IRAK-M macrophages (Fig. 9, E–F). Furthermore, expression of TNF-α mRNA and protein in response to CpG DNA was almost completely suppressed in IRAK-1-shRNA&IRAK-M macrophages (Fig. 9, G–H). The degree of suppression of CpG DNA responses in IRAK-1-shRNA&IRAK-M macrophages was greater than that in IRAK-1 knockdown macrophages (compare Figs. 8 and 9). In contrast, LPS-mediated activation of all three MAPKs in IRAK-
**IRAKs in CpG DNA Tolerance in Vivo**

1-shRNA&IRAK-M macrophages was comparable with that in control macrophages (Fig. 9, C and D). However, LPS-mediated NF-κB activation, NF-κB binding to the TNF-α promoter region, and TNF-α mRNA and protein expression were substantially inhibited in IRAK-1-shRNA&IRAK-M macrophages (Fig. 9, E–H). Of note, interferon-γ-mediated activation of NF-κB and production of TNF-α in IRAK-1-shRNA&IRAK-M macrophages were comparable with those in control macrophages (data not shown). These results demonstrate that in addition to the suppressed expression of IRAK-1, increased expression of IRAK-M is necessary for the optimal induction of macrophage hyporesponsiveness to CpG DNA and suggest that CpG DNA-mediated protection of CpG DNA/d-GalN-challenged mice from acute liver injury and subsequent death is due to the combinational effects of suppressed IRAK-1 expression and increased IRAK-M expression induced after CpG DNA treatment in macrophages.

**DISCUSSION**

In this study, using d-GalN-sensitized mice that undergo shock-mediated death due to massive liver damage induced by TNF-α secreted from activated mononuclear cells, we have investigated a molecular mechanism by which CpG DNA, the TLR9 ligand, induces hyporesponsiveness of macrophages to subsequent CpG DNA challenge in vivo. Our results demonstrated that mice pre-exposed to CpG DNA show decreased levels of IRAK-1 and increased levels of IRAK-M in macrophages. This altered expression of IRAK family proteins in macrophages renders them hyporesponsive to later CpG DNA challenge. As a result, mice pre-exposed to CpG DNA become resistant to CpG DNA/d-GalN-induced liver injury and death.

CpG DNA induces production of not only pro-inflammatory cytokines and modulators but also anti-inflammatory cytokine IL-10 and intracellular cytokine signaling inhibitor SOCS proteins (18, 19, 23). It has been demonstrated that SOCS1+/− mice and SOCS1−/− macrophages are hyper-responsive to LPS, indicating SOCS1 is one of the negative regulators in TLR-mediated pro-inflammatory responses (20, 22). In addition, SOCS1 and SOCS3 expressed after CpG DNA stimulation have been shown to inhibit cytokine-induced phosphorylation of STAT proteins (19). However, no increase in SOCS protein expression in KCs, peritoneal macrophages, and splenic macrophages after CpG DNA stimulation in vivo was detected (data not shown), indicating that SOCS proteins may not be critical contributors to CpG DNA-induced macrophage hyporesponsiveness in vivo. Previous studies showed that IL-10 suppresses CpG DNA-mediated, as well as LPS-mediated IL-12 production (23, 46), and IL-10 produced after CpG DNA stimulation contributes to the suppression of TNF-α expression in response to later challenge with LPS in RAW264.7 cells (18). Therefore, it is possible that IL-10 produced after the first exposure to CpG DNA contributes to the hyporesponsiveness of macrophages to a second CpG DNA challenge. We found that neutralization of IL-10 produced in RAW264.7 cells in response to CpG DNA pretreatment partially prevented the suppressed production of IL-6 in response to CpG DNA challenge (supplemental Fig. 5C). However, neutralization of IL-10 produced in RAW264.7 cells in response to CpG DNA pretreatment did not enhance the production of TNF-α, IL-10, and IL-12 in response to second CpG DNA challenge (supplemental Fig. 5B and data not shown). In addition, neutralization of IL-10 produced in response to CpG DNA pretreatment failed to revert suppressed activation of JNK in response to a second CpG DNA challenge (supplemental Fig. 5D). These results suggest that IL-10 expressed in response to CpG DNA stimulation may attenuate the amplification of cytokine responses induced after CpG DNA challenge to some extent, and hence, contribute to the prevention of exaggerated inflammation but may not be the central player (that directly blocks TLR9 signaling) in CpG DNA-induced macrophage hyporesponsiveness.

CpG DNA-induced macrophage hyporesponsiveness in vivo could be due to alterations in expression or function of TLR9 or its downstream signaling molecules in macrophages. Effects of TLR ligands on expression of various TLRs were studied by several groups. However, the effects of CpG DNA or LPS on expression of TLRs and TLR4 co-receptors are controversial (47). We found that neither TLR9 nor TLR4 expression was significantly changed in KCs, peritoneal macrophages, and splenic macrophages after CpG DNA treatment in vivo (Fig. 6 and supplemental Fig. 3), indicating that macrophage hyporesponsiveness induced by CpG DNA in vivo might not be due to alterations in TLR9 expression. Previous studies (26, 27) have demonstrated that LPS and CpG DNA induce expression of SHIP1 in bone marrow-derived macrophages (BMDMs) and that LPS and CpG DNA fail to induce hyporesponsiveness to second LPS or CpG DNA challenge in SHIP1−/− BMDMs. These studies suggest that SHIP1 plays a pivotal role in the LPS- or CpG DNA-mediated hyporesponsiveness of macrophages. In addition, recent studies (48, 49) showed that LPS induces tyrosine phosphorylation of TLR4, which is required for TLR4 signal transduction in human macrophages. TLR4 phosphorylation by Src is blocked in LPS-pretreated macrophages, and the unphosphorylated TLR4 preferentially binds to Myd88 and IRAK-1c (differential splicing variant of Myd88 and IRAK-1, respectively) that results in cessation of TLR4 signal transduction (49). These studies suggested that inhibited phosphorylation of TLR4 in LPS-pretreated human macrophages may be related to the increased expression of SHIP1 after LPS pretreatment and may contribute to the induction of hyporesponsiveness of macrophages to LPS second challenge. In our study, although expression of SHIP1 was increased in peritoneal and splenic macrophages, we could not detect increased expression of SHIP1 in KCs isolated from CpG DNA-pretreated mice (data not shown). At this point it is yet to be clarified whether increased SHIP1 expression in peritoneal and splenic macrophages in CpG DNA-pretreated mice is responsible for hyporesponsiveness of macrophages to CpG DNA in vivo and/or contributes to the protection against CpG DNA/d-GalN-mediated liver injury and death of mice. It is of great interest whether CpG DNA induces tyrosine phosphorylation of TLR9 that is critical for TLR9 signal transduction and whether SHIP1-overexpressing macrophages are hyporesponsive to CpG DNA and/or LPS.

Previously, we have shown that both CpG DNA and LPS pretreatment greatly attenuates activation of MAPKs and transcription factors and production of cytokines in RAW264.7
cells in response to subsequent CpG DNA or LPS challenge. However, dependent on the type of second stimulus, the degree of suppression is different (12). Recently, Dalpke and co-workers (15) demonstrated that CpG DNA pretreatment does not protect mice from LPS/d-GalN-induced death. Similarly, we found that CpG DNA pretreatment attenuated and delayed, but did not prevent, LPS/d-GalN-mediated hepatocyte apoptosis, liver injury, and death of mice (supplemental Fig. 6 and data not shown). In contrast, LPS pretreatment protected mice from CpG DNA/d-GalN-mediated as well as LPS/d-GalN-mediated hepatocyte apoptosis, liver injury, and death (Figs. 1 and 2). TLR4 and TLR9 share the MyD88 a downstream signaling pathway. However, compared with TLR9 signaling, which utilizes only one adaptor molecule, MyD88, TLR4 signaling uses four adaptor molecules and is much more complex. These results indicate that CpG DNA pretreatment induces macrophage hyporesponsiveness by at least partially suppressing an MyD88 downstream signaling step commonly shared by CpG DNA and LPS and that LPS induces macrophage hyporesponsiveness through more complex mechanisms that can block both MyD88- and TRIF-dependent signal transductions. IRAK family proteins and TRAF6 are MyD88 downstream signaling effectors. Involvement of IRAK-1 and IRAK-M in TLR signal transduction and TLR-mediated induction of macrophage hyporesponsiveness has already been reported. Overexpression of the dominant negative (DN) form of IRAK1 inhibits CpG DNA-mediated NF-κB and AP-1 activation in macrophages (33, 45). LPS- and R848 (TLR7 ligand)-mediated production of proinflammatory cytokines and activation of MAPKs and NF-κB are partially impaired in IRAK-1−/− macrophages, and CpG DNA-mediated type I interferon production is completely impaired in IRAK-1−/− plasmacytoid dendritic cells (50). Inhibited IRAK-1 kinase activity in LPS-tolerant macrophages has been observed (51). Expression of several differential splicing variants of IRAK-1 that act as DN-IRAK-1, including IRAK-1c, has been found in LPS- or CpG DNA-pretreated human macrophages (52). In our study differential splicing variants of IRAK-1 were not detected in macrophages isolated from mice pre-exposed to CpG DNA (data not shown). Instead, we found substantially decreased expression of IRAK-1 (at both mRNA and protein levels) in macrophages isolated from mice pre-exposed to CpG DNA (Fig. 6 and supplemental Fig. 3). In addition, activation of MAPKs and NF-κB and production of TNF-α in response to CpG DNA were substantially inhibited in IRAK-1 knockout macrophages (Fig. 8). These findings indicate that induction of CpG DNA-mediated macrophage hyporesponsiveness in vivo may be at least partially due to the suppressed expression of IRAK-1 and that suppressed expression of IRAK-1 is necessary and sufficient to render macrophages hyporesponsive to CpG DNA. Unlike other IRAK family members, expression of IRAK-M is induced in macrophages upon TLR ligand stimulation and has been shown to act as a negative regulator of TLR signaling by binding to the MyD88/IRAK-4/IRAK-1 complex and preventing dissociation of IRAK-1 from the complex (21). Production of inflammatory cytokines after bacterial infection is increased in IRAK-M−/− mice (21, 25). IRAK-M−/− macrophages and IRAK-M knockout macrophages are hyper-responsive to TLR ligands, including LPS and CpG DNA (15, 21). In addition, macrophages pre-exposed to peptidoglycan, LPS, or CpG DNA that are hyporesponsive to these TLR ligands express high levels of IRAK-M (15, 53–55). Moreover, LPS and CpG DNA pretreatment-induced hyporesponsiveness is significantly reduced in IRAK-M−/− macrophages and IRAK-M knockout macrophages (15, 21). These studies demonstrate that IRAK-M is a key negative regulator of TLR signaling. In agreement with these previous findings, transient overexpression of WT-IRAK-M inhibited CpG DNA-mediated activation of NF-κB in RAW264.7 cells (supplemental Fig. 4). However, neither CpG DNA- nor LPS-mediated activation of MAPKs and NF-κB and production of TNF-α was suppressed in macrophages that constitutively overexpress IRAK-M at a level comparable with that induced by CpG DNA pretreatment (Fig. 7). These results suggest that although IRAK-M is a negative regulator in TLR-signaling, induction of IRAK-M expression alone may not be sufficient to inhibit CpG DNA- or LPS-mediated macrophage activation. Interestingly, constitutive overexpression of IRAK-M in IRAK-1 knockout macrophages showed substantially inhibited activation of MAPKs and NF-κB and almost completely suppressed production of TNF-α in response to CpG DNA (Fig. 9). Degrees of suppressed responses to CpG DNA were greater in IRAK-M overexpressing IRAK-1 knockout macrophages compared with IRAK-1 knockout macrophages (compare Figs. 8 and 9). These results demonstrate that in addition to the suppressed expression of IRAK-1, increased expression of IRAK-M is necessary for the optimal induction of macrophage hyporesponsiveness to CpG DNA and suggest that CpG DNA-mediated protection of CpG DNA/d-GalN-challenged mice from acute liver injury and subsequent death is due to the combinational effects of suppressed IRAK-1 expression and increased IRAK-M expression in macrophages after CpG DNA treatment in vivo. Of note, although TNF-α production was substantially inhibited, LPS-mediated activation of all three MAPKs in IRAK-1-shRNA&IRAK-M macrophages was comparable with that in control macrophages, and LPS-mediated NF-κB activation was only partially inhibited in IRAK-1-shRNA&IRAK-M macrophages (Fig. 9). This result explains why CpG DNA pretreatment attenuates and delays, but does not prevent, LPS/d-GalN-mediated hepatocyte apoptosis, liver injury, and death of mice. Our results indicate that in addition to increased expression of IRAK-M and suppressed expression of IRAK-1, additional factors are required for macrophages to be hyporesponsive to LPS and that because of the complexity of TLR4 signaling transduction, the mechanism by which LPS induces macrophage hyporesponsiveness to LPS and other TLR ligands might be more complex than that by CpG DNA.

In summary, CpG DNA treatment induces suppressed IRAK-1 expression and increased IRAK-M expression in macrophages, including KCs, in vivo. This reduced expression of IRAK-1 and increased expression of IRAK-M in macrophages leads to suppressed activation of MAPKs and NF-κB and production of cytokines, including TNF-α, in response to CpG DNA (or CpG DNA/d-GalN) second challenge. As a result, CpG DNA/d-GalN-induced acute liver injury and shock-mediated death are prevented in mice pre-exposed to CpG DNA.
IRAKs in CpG DNA Tolerance in Vivo

Our study provides evidence that suppressed expression of IRAK-1 in macrophages after CpG DNA stimulation is one of the key events that causes macrophages to be hyporesponsive to subsequent CpG DNA challenge.

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