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Mannose Receptor 1 Mediates Cellular Uptake and Endosomal Delivery of CpG-Motif Containing Oligodeoxynucleotides

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Recognition of microbial components is critical for activation of TLRs, subsequent innate immune signaling, and directing adaptive immune responses. The DNA sensor TLR9 traffics from the endoplasmic reticulum to endolysosomal compartments where it is cleaved by resident proteases to generate a competent receptor. Activation of TLR9 by CpG-motif containing oligodeoxynucleotides (CpG ODNs) is preceded by agonist endocytosis and delivery into the endolysosomes. The events that dictate this process remain largely unknown; furthermore, it is unclear whether the receptors involved in mediating uptake of exogenous DNA are conserved for both naturally derived pathogenic DNA and synthetic ODNs. In this study, we report that peritoneal macrophages from a wild-derived inbred mouse strain, MOLF/Ei, are hyporesponsive to CpG ODN but are fully responsive to bacterial DNA, thus implying that microbial recognition is not fully recapitulated by a synthetic analog. To identify the gene responsible for the CpG ODN defect, we have performed genome-wide linkage analysis. Using N2 backcross mice, we mapped the trait with high resolution to a single locus containing *Mrc1* as the gene conferring the trait. We show that mannose receptor 1 (MRC1; CD206) is involved in CpG ODN uptake and trafficking in wild-derived MOLF/Ei peritoneal macrophages. Furthermore, we show that other strains of wild-derived mice also require MRC1 for CpG-induced cytokine responses. These findings reveal novel functions for MRC1 and demonstrate that wild-derived mice are important and indispensable model for understanding naturally occurring regulators of inflammatory responses in innate immune pathways. ***The Journal of Immunology*****, 2013, 191: 5615–5624.**

Microbial component ("patterns") activators of TLRs, such as LPS, are exclusively present in prokaryotes, whereas others, such as CpG-motif containing oligodeoxynucleotides (CpG ODNs) are synthetic and are often used to mimic the immunostimulatory properties of bacterial DNA to elicit innate immune responses (1). Cell surface TLRs sense molecular components exposed on microbes, whereas endosomal TLRs, such as TLR9, recognize pathogen-derived nucleic acids and are thus intracellular sensors of microbes (2). TLR localization is critical to their function because it ensures proper activation of the receptor upon ligand recognition (3, 4).

Although CpG was known to activate immune responses long before the cloning of TLR9, the mechanisms of TLR9 trafficking into endosomes was much better understood than the events regulating CpG uptake and endosomal delivery. Specifically, it is known that TLR9 associates with UNC93B1 (5, 6) to traffic from the endoplasmic reticulum to endolysosomal compartments, where it undergoes proteolytic processing by proteases (7) such as cathepsins to render the receptor signal competent (8). In addition to the cleavage of TLR9, different lysosomal sorting proteins such as AP-3, BLOC-1, and BLOC-2 are required to permit TLR9 signaling (9).

In contrast, there was a lack of definitive studies that examine the events regulating DNA processing and delivery into endosomes. In the absence of identified receptors, it was assumed that CpG DNA is taken up through nonspecific endocytosis (10) and traffics from early to late endosomes. This dogma remained unchallenged until a soluble cofactor, granulin, was identified (11) to assist in the uptake and endosomal delivery of CpG DNA, and more recently, DEC-205 was identified as a CpG ODN receptor (12). Despite these findings, there remain many unknowns with regard to these processes. For example, published reports have only examined the role of these receptors in the context of CpG ODN capture and delivery, and it remains unknown whether these receptors are relevant for TLR9 activation by DNA of microbial origin.

Cellular uptake of synthetic CpG ODNs is thought to be sequence independent but influenced by the thio-substitution of the oxygen in phosphobonds. Phosphorothioate linkages have been

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The microarray data presented in this article were deposited in the GEO database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE50682) under accession numbers GSM259766 and GSM259767.

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Abbreviations used in this article: B6, C57BL/6; CpG ODN, CpG-motif containing oligodeoxynucleotide; IMQ, Imiquimod; LXB, Loxoribine; MEF, mouse embryonic fibroblast; MRC1, mannose receptor 1; NA, numerical aperture; ODN, oligodeoxynucleotide; pfCpG, *Plasmodium falciparum* CpG DNA oligodeoxynucleotide; shRNA, short hairpin RNA.

used to substitute phosphodiester linkages in synthetic CpG ODN because phosphorothioate linkages are less susceptible to DNase degradation. It is also believed that length of DNA is critically important for efficient uptake and immunostimulatory activity (13). Although granulin and CD205 (DEC-205) have been identified as receptors of CpG ODN, there lack studies that determine whether these are receptors are influenced by ligand structure and length (14). Furthermore, the features of CpG ODN that bind granulin and DEC-205 are unknown.

In this paper, we provide additional insight on the mechanism of activation of innate response to CpG. Specifically, we show that mannose receptor (MRC1; CD206) is involved in the process of endosomal delivery and trafficking of CpG. In a genetic screen for responses to TLR agonists, we identified peritoneal macrophages from the wild-derived mouse strain, MOLF/Ei (*Mus musculus molossinus*), to be hyporesponsive to CpG. The trait was mapped to a single locus on mouse chromosome 2 that contains Mrc1.

Unexpectedly, despite being hyporesponsive to CpG ODN, MOLF macrophages were fully responsive to bacterial DNA, thereby challenging another dogma, according to which unmethylated CpG ODN mimic hypomethylated bacterial DNA, thus allowing TLR9 to distinguish noninfectious self from infectious nonself (15).

Finally, we extended our findings to other wild-derived strains and showed that *Mus musculus castaneus* and *Mus spretus* subspecies require MRC1 for effective CpG responses. Thus, *M.m. domesticus* (to which classical inbred mice belong) appears to be the only mouse subspecies that do not use MRC1 for CpG trafficking and endosomal delivery. These findings strongly suggest that studies in classical inbred mice have to be complemented with studies in other mouse subspecies.

These results also raise questions as to whether CpG ODNs are relevant representative ligands for TLR9 and suggests that ligand structure and length are key features that can discern receptor activation. In addition to the previously identified genes, MRC1 is another component of the network of genes that specifically regulate innate responses in wild-derived mice. Thus, using wild-derived strains in forward genetic analysis of the traits should be further supported.

Materials and Methods

Mice and isolation of primary cells

C57BL/6 (B6), SPRET/Ei, CZECH/Ei, MOLF/Ei, MSM/Ms, and CAST/Ei mice were obtained from The Jackson Laboratory. $TLR9^{-/-}$ mice were provided by Dr. A. Marshak-Rothstein (University of Massachusetts, Worcester, MA). $MRC1^{-/-}$ were provided by Dr. S. Levitz (University of Massachusetts).

Peritoneal macrophages were isolated from mice 6–10 wk of age. Cells were obtained by injection with 1 ml 3% thioglycollate, 3–5 d after injection, the peritoneal cavity was lavaged with cold PBS, and cells were suspended in DMEM with 10% FBS (Atlas Biologicals) and 1% penicillin/streptomycin (Invitrogen). Cells were plated and incubated overnight at 37˚C and 5% CO_2 before phenotypic analysis. Bone marrow cells were isolated by washing femurs with cold RPMI 1640 medium, cells were then centrifuged for 10 min at 1000 rpm. Cell pellets were resuspended in L1 conditioned medium (RPMI 1640 medium with L-glutamine, 20% FBS, 28% L1 conditioned medium, and 1% penicillin/streptomycin) to mature bone marrow–derived macrophages (BMDMs) or J558-GM-CSF–conditioned medium (RPMI 1640 medium with L-glutamine, 10% FBS, 3.3% J558 conditioned medium, and 1% penicillin/streptomycin) to mature bone marrow–derived dendritic cells. Mouse embryonic fibroblasts (MEFs) were isolated from 13.5-d mouse embryos by trypsinization after head and liver removal.

Cell lines

HEK 293T cells and L1 cells were obtained from Dr. R. Isberg (Tufts University, Boston, MA) and grown in DMEM, 10% FBS, and 1% penicillin/streptomycin. RAW264.7 mouse macrophage cell line was purchased from American Type Culture Collection and grown in DMEM, 10% FBS, and 1% penicillin/streptomycin.

TLR agonists

Salmonella minnesota Re595 LPS was purchased from Sigma-Aldrich. Poly(I:C) was obtained from GE Healthcare. Loxoribine (LXB), Imiquimod (IMQ) (R837), CL097, endotoxin-free *Escherichia coli* DNA were all purchased from InvivoGen. CpG ODNs were synthesized from Integrated DNA Technologies, including Alexa Fluor–labeled oligos. *Plasmodium falciparum* CpG DNA ODN (pfCpG) were provided by Dr. K. Fitzgerald (University of Massachusetts).

TLR response phenotyping

Cytokine protein concentrations were measured using ELISA kits purchased from R&D Systems: IL-6 (DY406), TNF-α (DY410), and CXCL1/KC (DY453).

Cytokine mRNA expression levels were measured using real-time PCR. Cells were lysed with TRIzol, and RNA was extracted according to the manufacturer's instructions (Invitrogen). cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase, RNase inhibitor, random primers 9, and 2′-deoxynucleoside 5′-triphosphates (New England BioLabs). cDNA was analyzed for mRNA expression levels using SYBR green and TaqMan probe–based gene expression analyses (Applied Biosystems). *Gapdh*-specific primer sets were used to normalize the expression value for the genes of interest.

Genetic mapping and analysis

Peritoneal macrophages were elicited from 6- to 12-wk-old mice and phenotyped as described above. Genomic DNA was isolated from the tails of all mice using DirectPCR (Tail) lysis protocol (Viagen), according to the manufacturer's protocol. Genome-wide genotyping was performed using two to three known polymorphic microsatellite markers per chromosome using primers obtained from The Jackson Laboratory Mouse Genome Informatics (http://www.informatics.jax.org) Web site. Genotyping reactions were amplified using JumpStart Red Taq (Sigma-Aldrich), and PCR products were resolved on a 3% agarose gel to determine the haplotype. Quantitative trait loci analysis was performed by inputting the genotypic and phenotypic data into the QTX software (http://www.mapmanager.org).

DNA sequencing

Genomic DNA or cDNA was amplified using Phusion High- Fidelity DNA polymerase (New England BioLabs) and gel extracted (Qiagen). DNA was sequenced from gene-specific or plasmid-specific primers using an ABI 3130XL DNA sequencer at the Tufts University Core Facility.

Plasmids

Short hairpin RNA (shRNA) plasmids were purchased from Open Biosystems and are in the pLKO.1 vector. The *Mrc1* targeting shRNA vector used in all the studies was clone ID TRCN0000054797. The *Ctsl* targeting shRNA vector was clone ID TRCN0000030582. A GFP targeting shRNA hairpin was used as a control, addgene number 12273.

Lentiviral transduction

Lentiviral particles were generated by transfecting the shRNA expression construct (pKLO.1 vector) along with packaging constructs psPAX2 (lentivirus packaging) and pMD2.G (VSV G) into HEK293-T cells using X-tremeGENE9 DNA transfection reagent or FUGENE 6 (Roche). Supernatants were collected 48 and 72 h after transfection and pooled together before 0.45 μM filtered. Cells were transduced by incubation with lentiviral supernatant for 24 h. BMDMs were infected on day 4 of maturation and puromycin selection began on day 6 at a 3 μg/ml concentration for 24 h. Following selection, cells were recovered for 48 h before assays were conducted.

Luciferase promoter assay

The *Mrc1* promoter region was amplied from genomic DNA to include the GT repeat deletion area from the indicated mouse strains and cloned into the pGL4.20 luciferase vector (Promega). RAW264.7 cells were stably transfected with the constructs using FUGENE 6 transfection reagent (Roche). Cells were activated with CpG for 2 h, and luciferase was quantified as a measure of promoter activity.

Microarray analysis

Total RNA was harvested from peritoneal macrophages that were unstimulated and activated with 200 nM CpG for 4 h using TRIzol. Total RNA

was then quantified and 100 ng was used for GeneChip analysis with Affymetrix GeneChip Mouse Gene 1.0 ST array chips (catalog number 520558; Affymetrix). Chips were labeled in accordance to the manufacturer's instructions. Briefly, RNA was reverse transcribed to produce cDNA from which antisense RNA was produced. After purification, antisense RNA was used to generate sense strand cDNA. The cDNA was then fragmented, labeled, and hybridized to the chips. Microarray data were analyzed using GeneSpring GX software. The microarray data are available at National Center for Biotechnology Information Gene Expression Omnibus http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE50682.

Abs

Phosphorylated and total p38, ERK, JNK, p105, and IKKα/β Abs were purchased from cell signaling. EEA-1 and LAMP-1 Abs were purchased from Abcam. Mannose receptor Ab was a gift from the U. von Andrian laboratory. Alexa Fluor–conjugated secondary Abs and Alexa Fluor–conjugated streptavidin Abs were purchased from Molecular Probes.

FACS analysis

For CpG uptake analysis, cells were incubated with biotinylated or Alexa Fluor–labeled CpG for 30 min at 37°C. Cells were washed with cold PBS three times and then lightly fixed in 0.5% PFA and resuspended in PBS with 5% FBS.

Western blot analysis

Following TLR activation cells were washed with cold PBS three times and lysed on ice with cytoplasmic lysis buffer (50 mM Tris [pH 8], 150 mM NaCl, 2 mM EDTA, 1% Triton-X100, 1 mM sodium vanadate, 10 mM NaF, and protease inhibitor mixture) for 10 min. Lysates were then transferred to microcentrifuge tubes and centrifuged for 10 min at 4°C at 13,000 rpm. Supernatants were transferred and mixed with Laemmli sample buffer and boiled for 10 min. Protein lysates were resolved on a 4–12% gradient Bis-Tris SDS gel (Novex; Invitrogen) using MOPS buffer and transferred onto a nitrocellulose membrane. Membranes were incubated with protein-specific Abs and detected using chemiluminescence ECL substrate (Pierce).

Confocal imaging

Images were collected with an Olympus Fluoview BX50WI inverted microscope and ×10/0.4 numerical aperture (NA), ×20/0.5 NA, or ×60/1.42 NA objectives. Images were analyzed using Volocity software (Improvision) and Photoshop CS3 (Adobe). Colocalization quantitation was analyzed using Imaris software (Bitplane).

Results

MOLF peritoneal macrophages are hyporesponsive to CpG

In a continuous effort to use wild-derived mice in genetic analysis of innate immune responses, we examined peritoneal macrophages from several wild-derived inbred mouse strains against the classical laboratory strain B6 for cytokine production upon activation with CpG ODN 1668 DNA (B-type CpG). Although peritoneal macrophages from all other mouse strains produced high levels of TNF-α in response to CpG activation, cytokine production was not detected from MOLF/Ei macrophages after 4 h of stimulation (Fig. 1A). The CpG defect was specific for the MOLF/Ei strain as macrophages from other wild-derived strains such as MSM, CZECH, SPRETUS, and CAST exhibited high levels of TNF-α.

According to our earlier report, MOLF macrophages are responsive to TLR2/TLR4-specific agonists, thus suggesting that there is no global defect for TLR-induced cytokine production in these mice (16, 17). To confirm that the CpG defect extends to the level of cytokine transcript expression, we compared levels of proinflammatory cytokine mRNA for TNF-α, IL-6, and IL-10 by means of Northern blot analysis. MOLF/Ei macrophages did not upregulate TNF-α, IL-6, or IL-10 in response to CpG DNA stimulation, whereas cytokine responses were robust following LPS stimulation in a time course–dependent manner (Fig. 1B). In contrast, MOLF/Ei peritoneal macrophages were hyperresponsive in comparison with B6 upon LPS because of the preferential upregulation of the proinflammatory isoform of IRAK2 in MOLF, which was previously examined by our laboratory (16). These results indicate that MOLF/Ei peritoneal macrophages are not globally defective for TLR-induced cytokine responses but rather the CpG defect is agonist specific and not because of an inability to induce proinflammatory cytokines.

To determine whether MOLF/Ei peritoneal macrophages are unresponsive to all classes of CpG DNA ODNs, we activated macrophages with A-type (ODN 1585), C-type (ODN 2395) and another B-type CpG (ODN 1826). In addition, we included CpG ODN designed from CG dinucleotide regions in the *P. falciparum* (pfCpG) genome (Fig. 1C). MOLF/Ei peritoneal macrophages were hyporesponsive to all classes of CpG ODNs, including pfCpG ODN. Furthermore, activation with higher concentrations of CpG DNA ODNs (3 μM) did not induce cytokine responses, dismissing the notion that the hyporesponse may be due to low agonist concentration. The results described thus far show that MOLF peritoneal macrophages are hyporesponsive to all classes of CpG DNA ODNs, whereas cytokine responses to LPS are normal.

It is possible that MOLF/Ei peritoneal macrophages are hyporesponsive to CpG ODN because of a defect that resides within the general endosomal pathway. One possibility is that ligand uptake and delivery through the endosomal network is defective in MOLF/Ei macrophages, thus affecting CpG DNA responses. Another possibility is that endosomal TLRs themselves are not properly processed from the endoplasmic reticulum through the Golgi and finally to endolysosomes. We reasoned that if the defect lies within either of these pathways then activation of all endosomal TLRs (TLR3, TLR7, TLR8, and TLR9) would be affected. IL-6 production by peritoneal macrophages was measured in response to several agonists: poly(I:C) (TLR3 agonist), IMQ and LXB (TLR7 agonists), CL097 (TLR7 and TLR8 agonist), and *E. coli* DNA (TLR9 agonist) to assess whether the deficiency is restricted to endosomal TLRs. IL-6 cytokine response was robust following activation by all endosomal TLR agonists tested with the exception of CpG DNA (Fig. 1D). Quite unexpectedly, MOLF macrophages exhibited high levels of the cytokine in response to *E. coli* DNA. These results indicate that MOLF/Ei peritoneal macrophages are fully responsive to all other endosomal TLR agonists, including *E. coli* DNA, with the notable exception of CpG ODN and dismiss the possibility that the CpG defect is the result of a global endosomal TLR defect. Peritoneal macrophages from $TLR9^{-/-}$ mice were not activated in response to *E. coli* DNA, thus dismissing possible contamination of the bacterial DNA with LPS. In addition, *E. coli* DNA responses were abrogated following DNase treatment of the ligand (Supplemental Fig. 1A). The observation that MOLF/Ei peritoneal macrophages are activated in response to *E. coli* DNA indicates that MOLF/Ei TLR9 is functional. This notion is further supported by similar cytokine responses measured following CpG activation in peritoneal macrophages from F_1 (B9 $^{TLR9-/-}$ × MOLF/Ei) and F1 (B6 × MOLF/Ei) (Supplemental Fig. 1B).

The observation that MOLF/Ei peritoneal macrophages can discriminate between synthetic and bacteria-derived CpG DNA as a TLR9 activator is quite remarkable and leads to many questions as to whether CpG DNA ODN is a physiologically relevant TLR9 agonist (1, 18). Furthermore, to our knowledge, this study is the first to report that a primary mouse peritoneal macrophage can distinguish between a natural and synthetic TLR9 ligand.

The CpG hyporesponse in MOLF/Ei macrophages is due to defective delivery of CpG into endosomes

Activation of TLR9 by CpG ODN takes place in endolysosomal compartments, which is preceded by a cellular uptake followed by endocytosis. We therefore analyzed uptake of CpG ODN into

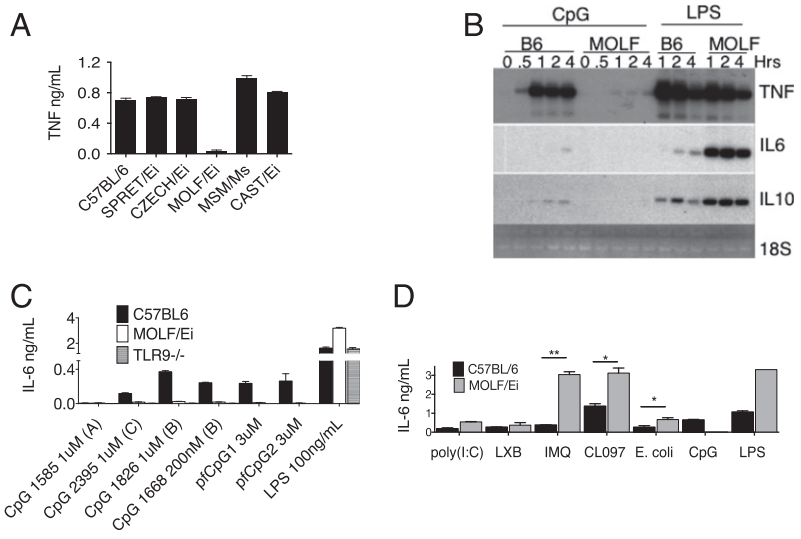

FIGURE 1. MOLF/Ei macrophages are hyporesponsive to CpG DNA ODN. (**A**) Peritoneal macrophages were assayed for CpG-induced TNF-α production by ELISA. Cells were activated with 200 nM CpG ODN for 4 h. (**B**) Peritoneal macrophages were activated with 200 nM CpG and 100 ng/ml LPS for indicated times, and Northern blot analysis was performed with TNF-α–, IL-6–, and IL-10–specific probes. Ethidium bromide staining for 28s and 18s RNA was used as a loading control. (**C** and **D**) Analysis of IL-6 cytokine production by peritoneal macrophages in response to various TLR agonists. Cells were activated with various CpG DNA ODNs at indicated agonist concentrations for 6 h and 100 ng/ml LPS for 4 h. Cells were activated with poly(I:C) (10 μg/ml), LXB (1 mM), IMQ (5 μg/ml), CL097 (1 μg/ml), *E. coli* DNA (10 μg/ml), and CpG (200 nM) for 6 h and 100 ng/ml LPS for 4 h. IL-6 cytokine production was measured by ELISA. ELISA data shown is the mean ± range from triplicate wells and is representative of at least three independent experiments. Northern blot data are representative three independent experiments. $*p < 0.05$, $**p < 0.001$.

endosomes in peritoneal macrophages. To investigate whether MOLF/Ei peritoneal macrophages are capable of internalizing CpG ODN DNA, we incubated macrophages with Alexa Fluor–labeled CpG ODN and observed that CpG DNA uptake was relatively similar between B6 and MOLF/Ei macrophages (Fig. 2A).

Following internalization, CpG DNA is rapidly moved into endolysosomal compartments, where subsequent acidification is critical for TLR9 activation. We used confocal microscopy to examine the compartmentalization of intracellular CpG. In MOLF/Ei peritoneal macrophages CpG DNA does not efficiently colocalize to either EEA-1–positive early endosomes or LAMP-1 positive late endosomes (Fig. 2B). In addition, CpG DNA appears undistributed within MOLF/Ei peritoneal macrophage compartments rather than in the punctate pattern expected for endolysosomal localization, as seen in B6 macrophages. To dismiss the possibility that the observed differences in CpG localization are influenced by the expression of endosomal markers in MOLF macrophages, we took several representative images from multiple experiments and saw equivalent levels of LAMP-1 and EEA-1 staining in macrophages from both strains (Supplemental Fig. 2A). In addition, MOLF/Ei peritoneal macrophages properly take up and process a control substrate (dextran) through their endolysosomes demonstrating that the CpG defect in MOLF is not a consequence of a more general problem with endosomal maturation and trafficking (Supplemental

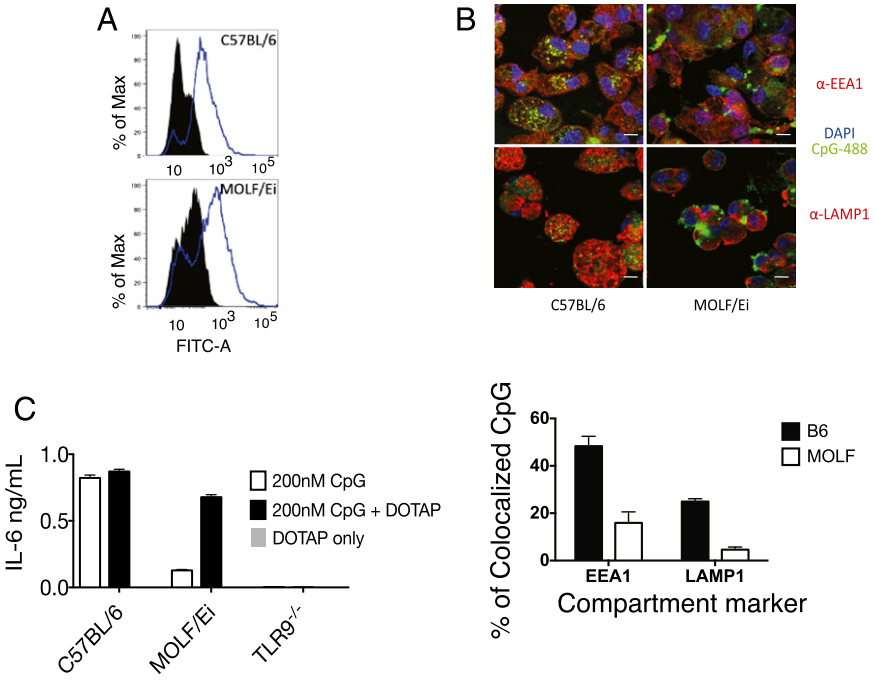

FIGURE 2. MOLF/Ei peritoneal macrophages do not traffic CpG ODN. (**A**) Peritoneal macrophages were treated with 200 nM Alexa Fluor 488–labeled CpG ODN for 1 h at 37°C and then examined for uptake (blue) using FACS analysis, and cells incubated on ice with labeled CpG were used as a negative control (black). (**B**) Peritoneal macrophages were incubated with 200 nM Alexa Fluor 488 CpG for 20 min and then fixed for 10 min in 4% PFA. Colocalization was visualized by labeling endosomal compartments with EEA-1 and LAMP-1 Abs. DAPI staining was used to label nuclei. Scale bars, 7 μM. Colocalization quantitation was measured using ImarisColoc software. (**C**) Peritoneal macrophages were treated with 200 nM CpG transfected with DOTAP. Twenty-four hours after transfection, IL-6 cytokine was measured by ELISA.

Fig. 2B). Additional FACS analysis using two colors of labeled CpG ODN indicated that CpG is internalized by MOLF/Ei peritoneal macrophages and not localized on the outside of the cells (data not shown). Collectively, these data suggest that the MOLF CpG hyporesponse is because of improper intracellular trafficking in peritoneal macrophages.

CpG ODN complexed with cationic liposomes composed of DOTAP can enforce endosomal translocation of these complexes to activate TLR9 (19–21). We reasoned that if ligand trafficking to endosomal compartments is responsible for the hyporesponse then DOTAP delivery of CpG DNA should rescue the cytokine response. We treated MOLF/Ei peritoneal macrophages with CpG ODN complexed with DOTAP to bypass endolysosomal trafficking of CpG and directly deliver CpG DNA to TLR9. Although MOLF/Ei peritoneal macrophages treated with CpG DNA alone produce minimal amounts of IL-6, macrophages stimulated by CpG DNA complexed with DOTAP produce IL-6 cytokine levels comparable to B6 (Fig. 2C).

Genetic analysis of the CpG hyporesponsive phenotype identifies a deletion in Mrc1 of MOLF mice

To examine the pattern of inheritance for the CpG hyporesponsive trait, an absolutely fundamental component of genetic analysis, we took a global approach and compared gene expression changes upon CpG stimulation in F1 (B6 × MOLF/Ei) and B6 peritoneal macrophages using microarray analysis. Analysis of the F1 phenotype can establish the genetic character of the trait as well as its mode of inheritance. We found that the trait was dominantly inherited for MOLF with respect to several genes, such as CXCL1, PTGS2, and TNF, whereas for some others, such as IL6, CXCL2, SAA3, it was dominantly inherited for B6 allele (Fig. 3A).

This difference in inheritance for different cytokines was further confirmed by ELISA, in which we compared cytokine production in response to CpG stimulation in F_1 hybrids (B6 × MOLF/Ei) to each of the parental strains. For the three cytokines analyzed, we observed a similar mode of inheritance for both mRNA expression and protein. Specifically, TNF and Cxcl1/KC responses to CpG in F_1 (B6 × MOLF/Ei) were undetectable, recapitulating the phenotype observed in the MOLF/Ei parental response. However, when we measured IL-6 production, F_1 mice produced an intermediate cytokine response (Fig. 3B, Supplemental Fig. 1C). We reasoned that IL-6 was detected following CpG stimulation in F_1 mice because of the contribution of the MOLF/Ei proinflammatory IRAK2 allele, which preferentially induces IL-6 production. Given B6 contributes half of the genome of F_1 hybrids, any gene(s) involved in CpG sensing or signaling would contribute to a permissive phenotype that is more readily measured by IL-6 production. Furthermore, we have observed a skewing of F1 CpG responses in the signaling pathway upstream of cytokine production. Peritoneal macrophages from B6 and F_1 mice were stimulated with CpG in a time course, and MAPK activation was analyzed by Western blot. We observed that ERK and JNK phosphorylation did not occur in

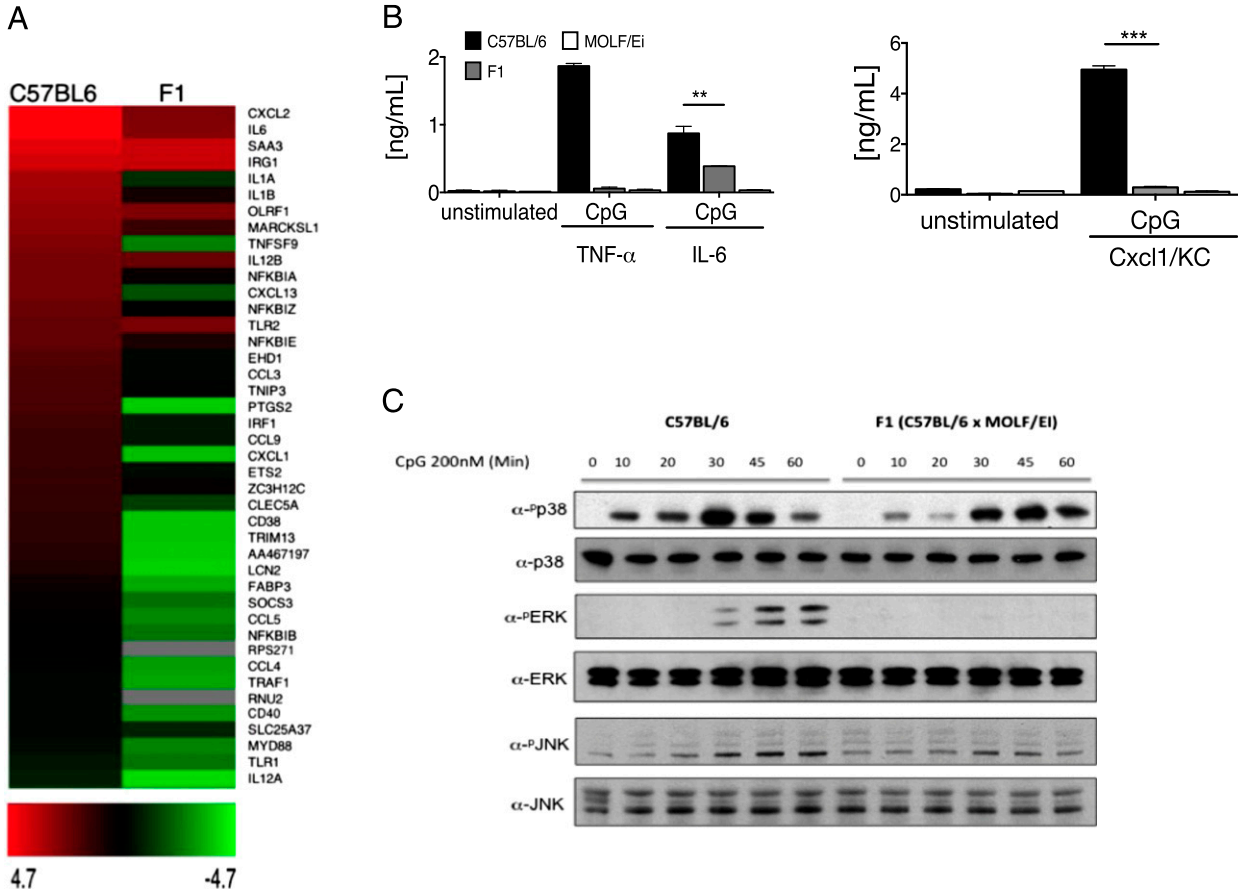

FIGURE 3. Inheritance analysis of the CpG hyporesponse trait in F_1 (B6 × MOLF/Ei). (**A**) Peritoneal macrophages were activated with 200 nM CpG DNA for 4 h, and RNA was harvested for microarray analysis using Affymetrix Gene 1.0 ST chips; the microarray data were deposited in the GEO database under accession numbers GSM259766 and GSM259767. (**B**) Peritoneal macrophages were activated with 200 nM CpG for 6 h and Cxcl1, TNF-α, and IL-6 cytokine production was measured by ELISA. $^{**}p < 0.001$, $^{***}p < 0.0001$. (**C**) MAPK and NF-κB pathways were examined in protein lysates from B6 and F_1 (B6 × MOLF/Ei) peritoneal macrophages after activation with 200 nM CpG in a time course–dependent manner. Data are representative of three independent experiments.

F_1 mice upon CpG activation, but p38 phosphorylation was detected (Fig. 3C). These data supported our previous observation that IRAK2 specifically hyperactivates p38 MAPKs but not ERK and JNK, leading to IL-6 hyperproduction. The data also show that in addition to IL-6, some other cytokines were upregulated significantly higher in MOLF than in B6 mice, probably because of common transcriptional activators. Collectively, the gene expression and cytokine secretion data confirmed that the CpG defect is a genetic trait that is transmitted to the level of F_1 hybrids.

We therefore used a forward genetics approach to analyze the CpG response in N2 (F_1 (B6 × MOLF/Ei] × MOLF/Ei) mice. In choosing different strategies for mapping of the trait, we considered a possibility that CpG-activated F_1 macrophages may have inherited an intrinsic instability for some of the cytokines thus showing low levels of corresponding mRNAs. This would result in a *cis*-acting contribution from such a locus into the trait leading to so-called incomplete penetrance. We therefore decided to use IL-6–induced cytokine response as our phenotypic readout. Following TLR stimulation IL-6 is hyperproduced in MOLF mice, and this trait is inherited at the F_1 level as well. Therefore, we generated N2 mice by backcrossing F_1 hybrids to the MOLF/Ei parental strain to generate progeny with phenotypic variation.

Genome-wide quantitative trait linkage analysis revealed linkage to a locus on chromosome 2 that conferred the CpG hypo-response with a logarithm of odds score of 4.56 (Fig. 4A). Homozygosity at this locus, between D2Mit2 and D2Mit361, was concordant with the MOLF/Ei parental phenotype for CpG hyporesponsiveness (Fig. 4B). To increase the resolution of the mapping, we expanded our analysis by producing additional meioses and increasing the density of markers in the critical area. We narrowed the trait to ~1.5 cM genomic interval between the markers D2Mit429 and D2Mit360.Haplotype analysis of 187 mice at this locus led to the identification of our first candidate gene, *Mrc1* (mannose receptor C type 1) within this 1 Mbp interval (Fig. 4C). We scanned this interval to identify a candidate gene and *Mrc1* was chosen as our likely candidate gene because of its known function as a receptor involved in endocytosis and intracellular trafficking. In addition to *Mrc1*, the region contained several other candidates, most notably zinc-finger family proteins; however, the meioses that recombined after the D2Mit361 marker helped to narrow the critical area and dismiss most of the candidates except MRC1. The genes encoding zinc-finger proteins were also dismissed as they fell outside of this interval. We therefore focused on MRC1.

We measured MCR1 mRNA expression levels in peritoneal macrophages from parental strains and F1 mice, as well as expression levels in BMDMs. The levels of MRC1 mRNA expression in B6, MOLF/Ei and F_1 mice mirrored our CpG phenotyping: MOLF peritoneal macrophages express very low levels of Mrc1, whereas F_1 mice exhibit a moderate level of expression (Fig. 4D). Accordingly, BMDMs from both strains express high levels of MRC1 as M-CSF, used to generate our BMDMs, can induce mannose receptor expression (29). To confirm the mRNA analysis data, we compared mannose receptor protein levels in different macrophages from B6 and MOLF. Using confocal microscopy we stained macrophages with mannose receptor and found similar levels of staining in B6 and MOLF BMDMs (Supplemental Fig. 3A). B6 peritoneal macrophages exhibited somewhat lower levels than BMDMs, whereas MOLF peritoneal macrophages showed no noticeable expression of MRC1.

If *Mrc1* were the gene that confers the trait, low levels of MRC1 in MOLF is likely the result of a *cis*-regulatory element proximal to *Mrc1*. To confirm such a regulatory pattern, we analyzed regulation of B6 and MOLF *Mrc1* alleles in peritoneal macrophages of F1 (B6 × MOLF) hybrid mice using massive parallel RNA-sequencing data from quiescent and LPS-activated F_1 macrophages (Supplemental Table I). For all the single-nucleotide polymorphisms that span most of the exons of MRC1, we observed strong allelic bias toward expression of B6 allele of *Mrc1*, thus confirming that genetic lesion responsible for the low levels of MRC1 mRNA in MOLF is in proximal position to *Mrc1*.

We next examined a proximal area of the promoter region of *Mrc1* to determine whether there were genetic polymorphisms unique to MOLF/Ei that would account for the low expression levels. We sequenced up to 800 bp upstream of the transcription

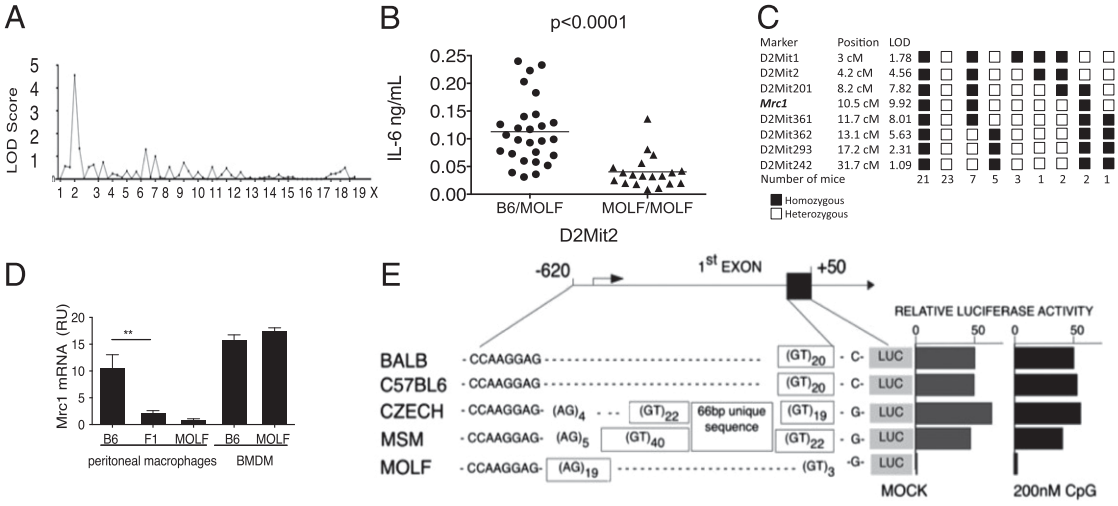

FIGURE 4. Genetic analysis identifies a genetic lesion in *Mrc1* to confer the CpG hyporesponse. (**A**) Genome-wide QTL analysis from N2 mice (F_1 [B6 × MOLF/Ei] × MOLF/Ei) shows linkage to a locus on chromosome 2 using the QTXb20 software program. (**B**) Peritoneal macrophages from 46 independent N2 mice were measured for CpG-induced IL-6 production and plotted by genotype at D2Mit2. (**C**) Haplotype analysis from 187 N2 mice. The number of offspring inheriting each type of chromosome is listed below each column. Marker centimorgan distance and logarithm of odds score are listed, including the location of the *Mrc1* gene. (**D**) *Mrc1* mRNA expression levels were assayed using real-time PCR in peritoneal and bone marrow derived. $^{**}p < 0.001$. (**E**) Schematic of the *Mrc1* promoter region identifying genetic polymorphisms and the unique (GT) repeat deletion in MOLF/Ei. Reporter gene assay was analyzed in RAW264.7 cells stably transfected with constructs containing the (GT) repeat deletion area from various strains fused to luciferase. Cells were stimulated with CpG for 2 h, and luciferase was quantified as a measure of promoter activity. All data are representative of at least three independent experiments.

initiation start site for Mrc1 in three classical inbred strains in addition to three wild-derived strains. We found a significant deletion of GT repeats that was unique to the MOLF/Ei wild-derived strain at the 3′ end of the analyzed alignment. The 5′ end had AG repeats specific for the wild-derived strains but MOLF had significantly larger number of these repeats than CZECH and MSM, probably because of conversion of the GT into GA repeats. Because GT-repeats are known to be transcriptionally active, we hypothesized that such a deletion/conversion might be the reason of low levels of MRC1 mRNA in MOLF. We therefore sought to determine whether this deletion was the genetic lesion in the luciferase reporter assay. We cloned the Mrc1 promoter region from B6, MSM, MOLF/Ei and CZECH and transfected RAW264.7 cells with these constructs. Promoter-driven luciferase activity was used to measure promoter function. We saw that the GT deletion in the *Mrc1* promoter region for MOLF/Ei accounted for the low expression levels of MRC1 (Fig. 4E) in quiescent as well as in the CpG-activated RAW macrophages.

In conclusion we identified *Mrc1* as the likely gene conferring the trait because of the mapping data and because of its differential expression between the strains in peritoneal but not BMDMs. We also identified a genetic lesion in MRC1 promoter of MOLF that was responsible for a disruption of constitutive expression of MRC1.

Mrc1 is involved in CpG responses in MOLF/Ei macrophages but not B6

It is well established that there are cell type–specific differences in CpG responses. We therefore wanted to examine whether the CpG hyporesponse can be observed in other myeloid cell populations or is restricted only to peritoneal macrophages. We matured macrophages and dendritic cells from B6 and MOLF bone marrow and activated them with TLR agonists. MOLF BMDMs and bone marrow–derived dendritic cells produce cytokines in response to CpG and other TLR stimulation (Fig. 5A, 5B).

If differences in levels of Mrc1 mRNA expression contribute to the opposing phenotypes observed in the CpG response in peritoneal macrophages versus BMDMs, then downregulation of MRC1 in BMDMs should affect their CpG response. To test this hypothesis, we used a lentiviral system to introduce Mrc1 (mRNA) targeting shRNA hairpins. BMDMs from B6 and MOLF/Ei were matured in M-CSF–conditioned medium, and cells were transduced with control shRNA and Mrc1 shRNA containing lentiviral particles. Following lentiviral transduction, Mrc1 mRNA expression levels were measured by real-time PCR, which showed macrophages transduced with Mrc1 shRNA exhibited 90% less Mrc1 mRNA expression compared with control shRNA samples (Supplemental Fig. 3B). BMDMs were then activated with CpG to assess the contribution of Mrc1 expression on cytokine responses. Mannose receptor knockdown in MOLF/Ei BMDMs reduced the amount of IL-6 produced in response to CpG, while knockdown in B6 BMDMs did not alter their CpG response (Fig. 5C). MEFs are another primary cell population that is CpG responsive in MOLF/Ei mice. Mrc1 knockdown in MEFs (Fig. 5D) recapitulated the phenotype we observed in BMDMs further supporting a role for MRC1 in MOLF/Ei CpG-induced cytokine response. In addition to CpG ODNs, the MRC1 knockdown affected responses to bacterial DNA in MOLF but not in B6 BMDMs (Supplemental Fig. 3C) thus suggesting that the requirement for MRC1 in MOLF is preserved for different classes of TLR ligands. Finally, we used MRC1-deficient mice to show that their macrophages exhibit comparable CpG-induced IL-6 production to the wild-type macrophages and therefore do not require MRC1 for their CpG response. Genetic complementation of the $Mrc1^{-/-}$ allele with MOLF Mrc1 allele results in normal CpG responses in F_1 animals, thus supporting a unique role of MRC1 for the CpG response in MOLF mice (Supplemental Fig. 3D). In conclusion, MRC1 is essential for CpG-induced cytokine responses in MOLF but not in B6 macrophages. Our data show that MOLF/Ei mice acquired a mannose receptor dependent role for CpG responses across different lineages of cells, whereas B6 macrophages are able to internalize CpG ODN in a mannose receptor–independent manner.

Mannose receptor facilitates cellular uptake and endosomal delivery of CpG ODN in MOLF/Ei macrophages

We observed that peritoneal macrophages from MOLF/Ei mice are able to take up CpG ODN but do not traffic CpG ODN properly into endolysosomal compartments (Fig. 2A, 2B), presumably because of scant mannose receptor expression. In MOLF/Ei BMDMs and MEFs, knockdown of Mrc1 mRNA expression affected subsequent CpG responses leading us to hypothesize that the mannose receptor may be functioning as a CpG uptake and/or delivery receptor in MOLF macrophages. To investigate this hypothesis, we transduced MOLF/Ei BMDMs with control shRNA and Mrc1 shRNA lentivirus and assessed CpG uptake. Macrophages were incubated with FAM-labeled CpG ODN to measure uptake by FACS analysis. In Mrc1 knockdown MOLF BMDMs, there was a ~50% reduction in mean fluorescence intensity for CpG ODN uptake compared with control shRNA samples (Fig. 6A).

To extend our observation that mannose receptor knockdown attenuates CpG uptake in BMDMs, we examined the intracellular localization of CpG following mannose receptor knockdown using confocal microscopy. Lentiviral delivery of control and Mrc1

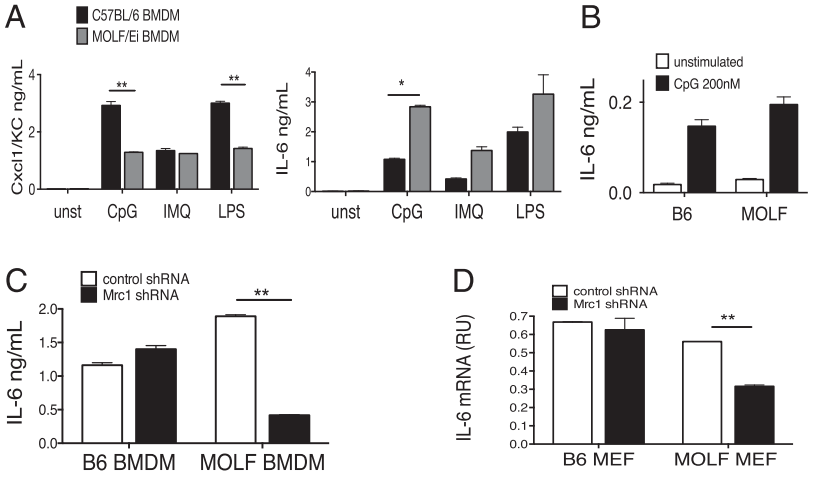

FIGURE 5. Mrc1 is involved in MOLF/Ei CpG-induced cytokine production. (**A**) B6 and MOLF BMDMs were activated with CpG (200 nM), IMQ (5 μg/ml), and LPS (100 ng/ml) for 6 h, and Cxcl1 and IL-6 cytokine production was measured by ELISA. $*p < 0.05$, $**p < 0.01$. (**B**) B6 and MOLF BMDCs were activated with CpG for 6 h, and IL-6 production was measured by ELISA. (**C** and **D**) BMDM and MEF *Mrc1* mRNA knockdown were achieved using lentiviral shRNA hairpins. Knockdown efficiency was examined in Supplemental Fig. 3. Cytokine levels were measured after 6 h of activation with 200 nM CpG for ELISA and 4 h for real-time PCR. Knockdown data are representative of three independent experiments. $**p < 0.01$.

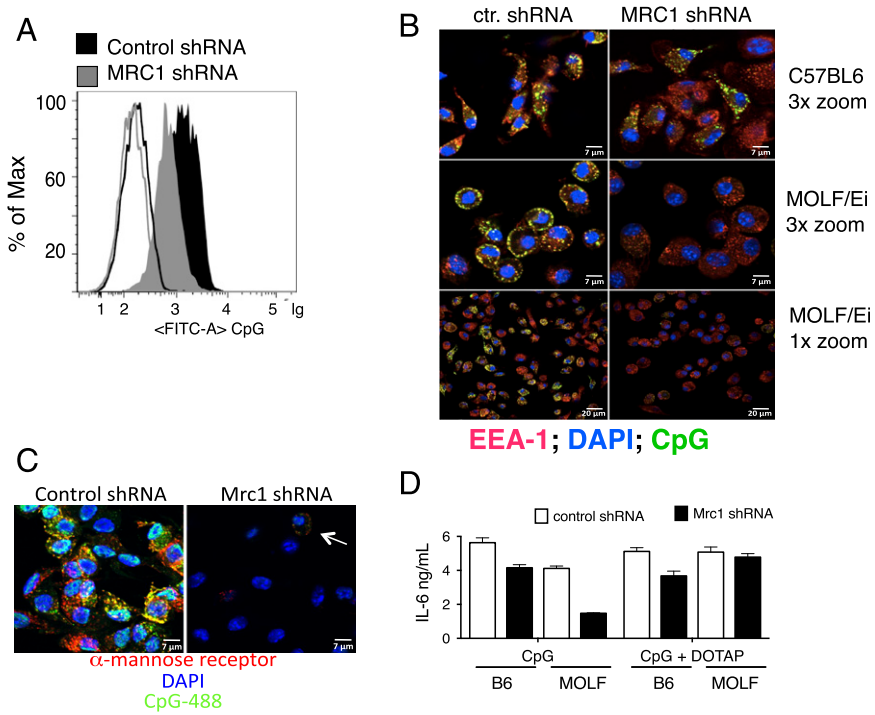

FIGURE 6. Mrc1 is a receptor that binds and delivers CpG ODN in MOLF/Ei macrophages. (**A**) CpG uptake was analyzed in MOLF/Ei BMDMs with *Mrc1* shRNA knockdowns (gray) compared with control shRNA (black) following incubation with 200 nM FAM-labeled CpG ODN for 30 min either at 37°C (filled) or on ice (line) as a negative control. FACS analysis was used to quantify uptake by mean fluorescence intensity (MFI). (**B**) B6 and MOLF/Ei BMDMs were lentivirally transduced with control and Mrc1 shRNA hairpins, and localization of intracellular CpG was examined by confocal microscopy. Macrophages were incubated with 200 nM Alexa Fluor 488–labeled CpG for 20 min and fixed in 4% PFA. Cells were stained with EEA-1 (red) to label early endosomes. DAPI (blue) stain was used to visualize nuclei. Magnification is represented by the scale bars. (**C**) MOLF/Ei BMDMs transduced with control and Mrc1 shRNA hairpins and incubated with Alex Fluor 488–labeled CpG as described above. Macrophages were stained with mannose receptor (red) and DAPI (blue). (**D**) B6 and MOLF BMDM *Mrc1* mRNA knockdown was achieved using lentiviral shRNA hairpins. A total of 200 nM CpG was transfected with DOTAP, and 24 h posttransfection, IL-6 production was measured by ELISA. Data are representative of three independent experiments.

shRNA hairpins were used to knockdown Mrc1 mRNA expression in B6 and MOLF/Ei BMDMs. Following transduction, macrophages were incubated with Alexa Fluor 488–labeled CpG oligos and then stained for the early endosome marker, EEA-1, to label intracellular compartments. In B6 BMDMs with mannose receptor knockdown, intracellular CpG localization was comparable to control samples (Fig. 6B). In contrast, there was almost no detectable intracellular CpG in MOLF BMDMs with Mrc1 knockdown even at the lower ×1 zoom magnification. Because this phenotype was so striking, we additionally stained samples with an MRC1 Ab to verify our level of knockdown at the protein level in these samples. We confirmed that mannose receptor knockdown was sufficiently achieved at the protein level, thus handicapping MOLF macrophages from CpG internalization (Fig. 6C). Remarkably, in the few cells that Mrc1 was not efficiently knocked down, intracellular CpG is observed in those MOLF BMDMs (white arrow). Interestingly, we observed that despite low mannose receptor expression, MOLF/Ei peritoneal macrophages take up CpG ODN; however, intracellular trafficking of CpG is defective leading to CpG hyporesponsiveness. In the context of MOLF/Ei bone marrow macrophages, knockdown of mannose receptor leads to a nearly complete absence of CpG ODN uptake therefore affecting CpG-induced cytokine responses.

We reasoned that if mannose receptor is involved in the trafficking of CpG, and knockdown of the gene affects the proper intracellular ligand localization, then delivery of CpG directly into endolysosomes should rescue CpG responses. We assessed IL-6 cytokine response in Mrc1 knockdown BMDMs that were treated with DOTAP-transfected CpG DNA. MOLF/Ei BMDMs with Mrc1 knockdown showed restored IL-6 responses with DOTAP delivery of CpG (Fig. 6D). Collectively, these data show that the mannose receptor is a critical component in CpG ODN–induced TLR9 activation. Mannose receptor functions as a CpG ODN receptor that facilitates ligand uptake and delivery through the endolysosomal network to activate TLR9 in MOLF/Ei macrophages. In sum, mannose receptor plays a prominent role in CpG ODN uptake and is critical for CpG ODN intracellular trafficking in both peritoneal and bone marrow MOLF/Ei macrophages.

In our previous reports, we observed that differences in the regulation of the innate immune responses observed between wild-derived and classical inbred mice are usually a shared feature among several wild-derived strains compared with the classical inbred mice (such as B6). We therefore hypothesized that the requirement for MRC1 in CpG responses could be extended to mice that belong to other subspecies. To investigate this, we have chosen to silence MRC1 in BMDMs from Castaneus and Spretus mouse strains. Castaneus and MOLF/Ei mice share genomic contributions from the *M.m.castaneus* subspecies, whereas *M. spretus* belongs to a completely different mouse subspecies. To look for the effect of MRC1 silencing, we used BMDMs activated with CpG and several other TLR agonists. In agreement with our data for MOLF, CpG responses from Spretus and Castaneus BMDMs were significantly affected by MRC1 knockdown, whereas other agonists such as LPS or IMQ were not (Fig. 7A, 7B). These data support the notion that there is a universal requirement for MRC1 in CpG-induced cytokine responses for several subspecies of mice with the notable exception of *M.m. domesticus*.

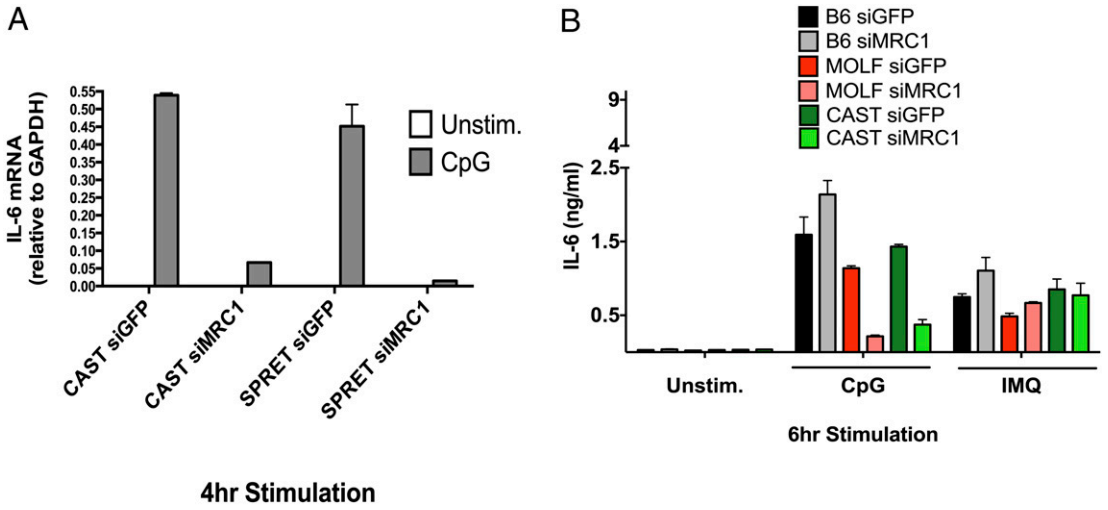

FIGURE 7. Mrc1 is involved in CpG responses in *M.m. castaneus* and *M. spretus* mouse subspecies. (**A** and **B**) BMDM *Mrc1* mRNA knockdown was achieved using lentiviral shRNA hairpins. A total of 200 nM CpG-induced IL-6 production was measured by real-time PCR and ELISA. Data are representative of two independent experiments.

Discussion

The studies presented in this paper describe a novel function for the mannose receptor in CpG uptake, trafficking and delivery for TLR9 activation (21). Using linkage analysis and high-resolution mapping, we identified *Mrc1* as the gene candidate that confers CpG hyporesponse in MOLF macrophages. MRC1-knockdown experiments in BMDMs recapitulated the CpG hyporesponse observed in MOLF/Ei peritoneal macrophages with regard to minimal cytokine production, decreased CpG internalization, and trafficking. Furthermore, when *Mrc1* knockdown MOLF/Ei BMDMs were activated with DOTAP-transfected CpG, cytokine production was restored, thereby showing a specific involvement of MRC1 in the endosomal delivery of CpG.

Sequence comparison of the MRC1 promoter between MOLF and other CpG-responsive wild-derived strains including MSM, SPRETUS, and CZECH was instrumental because it helped to identify a unique genetic lesion in MOLF (Fig. 4) that was responsible for low levels of expression of MRC1. Functional effect of the deletion was further confirmed in the reporter gene assay. In the past, a similar strategy of using several wild-derived strains in genetic screens has helped us to identify correlating phenotypes with molecular defects, thus leading to positional cloning of other genes involved in the innate network of wild-derived mice (22). Unlike previously cloned genes, MRC1 initially emerged as one of several candidate genes in the critical area forcing us to produce additional meioses and increase resolution of mapping to dismiss all other genes except MRC1. Thus, despite a high degree of polymorphism and its complex character, the trait was possible to successfully map because of its complete penetrance. Our studies support the use of wild-derived strains in genetic analysis of innate immune traits and allow for several conclusions.

First, although MOLF/Ei peritoneal macrophages cannot be activated by CpG ODN they were responsive to *E. coli* DNA thus suggesting that there are different requirements for sensing bacterial DNA and its synthetic analog, which is widely used to mimic the immunostimulatory features of pathogenic DNA. Two factors, specifically, the length of DNA and methylation efficiency may not fully represent those found in bacterial DNA. This phenomenon raises questions as to whether CpG ODNs are relevant representative ligands for studies that examine the events and processes that lead to TLR9 activation. The data presented in our studies suggest that ligand structure and length are key features that can discern receptor activation, intracellular uptake and trafficking. This is not too surprising given that in the wild, mice do not encounter CpG ODNs but rather pathogen-derived DNA. Furthermore in vivo, innate cells are most likely to encounter pathogen-derived DNA through intracellular infection. Therefore, the route of DNA uptake and delivery to TLR9 are additional contributing factors that regulate receptor activation (23). Despite the fact that there are many undiscovered discerning key features between bacterial DNA and CpG ODN, the use and study of synthetic CpG ODN remains important, particularly in the context for its use as a vaccine adjuvant.

Second, MOLF peritoneal macrophages are fully responsive to other endosomal TLR agonists, suggesting that there could be different trafficking and endosomal delivery receptors used by poly(I:C), IMQ, CL097, and other stimuli. For some TLR ligands, CD14 was reported to contribute to the formation of complexes that are internalized and delivered to endosomes more efficiently. The involvement of MRC1 in CpG internalization and trafficking suggests the possibility that the mannose receptor is critical for ligand delivery and subsequent activation of TLR9. Alternatively, serum proteins (e.g., soluble mannose receptor or granulin as a partner to TLR9) and other surface proteins may be involved in binding CpG and subsequently engage the mannose receptor and then deliver this ligand to TLR9. Along these lines, a synergistic effect between the mannose receptor and TLR9 has been described in dendritic cells stimulated by *Cryptococcus neoformans* mannoproteins and CpG (24–26).

Third, although MOLF peritoneal macrophages are refractory to CpG, their BMDMs and MEFs are fully CpG responsive, which correlates with the relatively high levels of MRC1, suggesting that all cells in MOLF mice require MRC1 for a normal CpG response. The effect of MRC1 on CpG responses was confirmed by MRC1 knockdown in BMDMs and MEFs. Thus, the requirement for MRC1 in MOLF is preserved across different cell lineages. We hypothesize that BMDMs and MEFs exhibit high levels of MRC1 because they overcome the deletion in the MRC1 promoter because of some degree of genetic reprogramming and epigenetic changes caused by in vitro differentiation of BMDMs and developmental changes induced in MEFs. Although we tried to rescue CpG response by incubating MOLF peritoneal macrophages with M-CSF, the effect of the exogenous growth factor on CpG responses was incremental. In view of these data, the heterogeneity of macrophage populations is underappreciated, and our studies highlight the phenotypic differences observed among these populations. These

differences in gene expression and phenotype observed between MOLF peritoneal and BMDMs emphasizes that although bone marrow–derived myeloid cell populations are undoubtedly useful for a variety of experimental studies, these cells cannot provide the breadth of phenotypically diverse myeloid cell populations observed in vivo.

Fourth, MRC1 is dispensable for CpG sensing in B6 mice. Specifically, in all of our mechanistic studies, we saw a strain-specific role for the mannose receptor in MOLF macrophage CpG responses but not for B6 macrophages. This observation ultimately led us to examine the CpG response in B6-*Mrc1*$^{-/-}$ peritoneal macrophages that were fully CpG responsive. This observation means that multiple receptors can be involved in CpG trafficking and internalization and there is redundancy in the process. In order to address this possibility, we used gene-silencing approaches to investigate whether other members of the MRC1 family such as Ly75 (12), Pla2r1 and Mrc2 were involved in CpG responses. After multiple attempts, we did not observe any effect of the shRNA knockdown of these other genes on the CpG-induced cytokine responses. In contrast with these data, in an earlier published report (12), Ly75 (DEC-205) was shown to regulate CpG uptake, B cell activation, and dendritic cell maturation in response to CpG. To reconcile these conflicting data, it would be interesting in future studies to examine the role of MRC1 in other cell lineages. Recent studies have shown that an intronic microRNA, miR511, is encoded by human and mouse MRC1 genes (27, 28). To investigate whether the expression of this miRNA has consequential effects on CpG-induced cytokine responses, we amplified its expression in quiescent and activated macrophages from B6 and MOLF. We observed almost undetectable amounts of miR511 in MOLF cells as compared with steady levels in B6 samples (Supplemental Fig. 3E). Given these data and posttranscriptional level of gene regulation by miRs, we believe that miR511 does not play any role in the MOLF CpG defect. Although the differential regulation of miR511 in MOLF is worth pursuing, we think that it should be a subject of a separate inquiry. Regardless of the reason for differences in sensitivity, MOLF macrophages are a unique model that allows not only discrimination between a natural and synthetic TLR9 agonist but also identification of a gene that is required for trafficking and delivery of CpG ODN.

Inflammatory cytokine production in response to the identification of pathogen-associated molecules by TLRs is a basic component of the innate immune response. Although seemingly straightforward, the pathways and signaling components involved in receptor activation and cytokine initiation are complex and tightly regulated. The studies presented herein together with our genetic analysis and previously published phenotypic studies reveal that because of selective pressures, MOLF/Ei have evolved to develop a divergent network of genes activated in response to pathogens and their microbial components. Some of the wild-derived mice have 5 million years of separation from the classical inbred progenitors, which is quite comparable with 50 million years between humans and mice. The genetic diversity of these mice combined with their ability to breed with the classical inbred strains makes them attractive genetic models for the study of host-pathogen interactions.

Disclosures

The authors have no financial conflicts of interest.

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