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THE PROTEASOME REGULATES BACTERIAL CpG DNA-INDUCED SIGNALING PATHWAYS IN MURINE MACROPHAGES

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Abstract

Our previous work has provided strong evidence that the proteasome is central to the vast majority of genes induced in mouse macrophages in response to lipopolysaccharide (LPS) stimulation. In the studies presented here, we evaluated the role of the macrophage proteasome in response to a second microbial product CpG DNA (unmethylated bacterial DNA). For these studies, we applied Affymetrix microarray analysis of RNA derived from murine macrophages stimulated with CpG DNA in the presence or absence of proteasome inhibitor, lactacystin. The results of these studies revealed that similar to LPS, a vast majority of those macrophage genes regulated by CpG DNA are also under the control of the proteasome at 4 h. In contrast to LPS stimulation, however, many of these genes were induced much later than 4 h, at 18 h, in response to CpG DNA. Lactacystin treatment of macrophages completely blocked the CpG DNA-induced gene expression of TNF- α and other genes involved in production of inflammatory mediators. These data strongly support the conclusion that, similar to LPS, the macrophage proteasome is a key regulator of CpG DNA-induced signaling pathways.

INTRODUCTION

Bacterial CpG DNA, which contains unmethylated CpG motifs, can act alone or synergistically with other microbial products in its interaction with mouse macrophages, and *in vivo*, may cause an overproduction of cytokines that can lead to sustained hypotensive shock and, ultimately, death (1–5). Our earlier studies to define CpG-DNA induced genes were carried

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out using RAW 264.7 cells, after a 6 h period of stimulation (6,7). Our data indicated that 69 genes were significantly induced or repressed and included genes that encode cytokines, chemokines, cell-surface receptors, enzymes, intracellular signaling proteins, transcription factors, and proteins related to cell proliferation and differentiation. In contrast to these results, LPS induced and repressed a significantly greater number of genes in macrophages, relative to those activated in response to CpG DNA in 6 h, yet all the CpG DNA-regulated genes represented a subset of those modulated by LPS. While these early studies were carried out prior to the discovery of the MyD88-dependent vs. MyD88-independent signaling pathways, these findings were interpreted at the time to indicate that LPS induced additional signaling pathways apart from those shared by CpG DNA. We now know that CpG DNA signals from the endosomes via Toll-like receptor (TLR) 9, utilizing MyD88 as the sole adapter molecule (8,9). In contrast, LPS uses MyD88 and MyD88 adapter-like (Mal)/TIRAP to induce MyD88-dependent signaling, leading primarily to activated NF- κ B, and MyD88-independent signaling, via TRAM and TRIF, to activate IRF-3 (10). In response to LPS, activation of the MyD88-dependent pathway elicits expression of highly pro-inflammatory genes such as TNF- α , IL-1 β , and IL-8, while the TRIF/TRAM pathway leads to the activation of IFN- β and IFN- β -dependent genes such as iNOS. In contrast, CpG DNA-stimulated macrophages produce iNOS (and release NO) only when simultaneously stimulated with exogenous IFN- γ (11). Thus, the mechanism by which CpG DNA induces iNOS and other IRF-3 or IFN- β -dependent inflammatory mediators in macrophages is currently not entirely understood.

The proteasome is a well-recognized and important cytoplasmic organelle of almost all mammalian cells. We have recently shown that the ubiquitin-proteasome pathway plays a central role in regulation of LPS-induced signal transduction in macrophages (12–15). In this respect, lactacystin pretreatment of macrophages blocks LPS-induced expression of multiple genes including those encode for TNF- α , interleukin-6 (IL-6), interleukin-12 (IL-12), inducible nitric oxide synthase (iNOS), cyclo-oxygenase-2 (COX-2), CD14, toll-like receptor-4 (TLR-4), and TLR-2, all of which are linked to development of inflammation (12–15). We have more recently examined the role of the proteasome in CpG DNA and peptidoglycan (PG) - mediated signaling pathways in macrophages. We have demonstrated that lactacystin pretreatment of macrophages also results in inhibition of the CpG DNA- and PG-induced TNF- α secretion and expression inflammatory genes that encode TNF- α , IL-1 β , and iNOS. Moreover, we also found that lactacystin pretreatment prevents phosphorylation of the mitogen activated protein kinases (MAPK), ERK, thus suggesting that the proteasome is likely to be important for CpG- and PG-mediated signal transduction in macrophages as well (14).

Since CpG DNA signals macrophages through TLR9 using similar, although not identical, signaling pathways to those used by LPS, such as other MyD88-dependent TLRs, we examined the question of the extent to which the proteasome pathway of signaling was unique to LPS or if it would extend to CpG DNA as well. To gain a comprehensive appreciation for the contribution of the proteasome in CpG DNA-mediated signaling, we evaluated the role of the proteasome of the murine macrophages in response CpG DNA (type B) by using an Affymetrix microarray analysis of newly synthesized RNA derived in the presence or absence of the relatively selective proteasome inhibitor, lactacystin. The results of this study provide evidence that a vast majority of the genes regulated by CpG DNA are under the control of ubiquitin-proteasome pathway. These data strongly support the conclusion that ubiquitin-proteasome pathway is a key regulator of CpG DNA-induced signaling pathways in macrophages

MATERIALS AND METHODS

Reagents

The following oligonucleotide (ODN; phosphorothioate backbone) CpG ODN, no.1826, 5'-TCCATGACGTTCCCTGACGTT-3', was purchased from Coley Pharmaceutical Group

(Kanata, ON, Canada). This CpG DNA is of the B type, which is a strong inducer of TNF- α , but not type 1 interferons in macrophages (16). These ODN were endotoxin-tested by the company have been reported to be below detectable limits by the LAL assay. Lactacystin was purchased from Boston Biochem, (Cambridge, MA). Dulbecco's Modified Eagle Medium (DMEM), heat-inactivated fetal bovine serum (FBS), and gentamycin were purchased from Cambrex (Walkersville, MD). NE-PER nuclear & cytoplasmic extraction kits, M-PER Mammalian protein extraction kits and BCA protein assay kits were purchased from PIERCE, Inc. (Rockford, IL).

Macrophage Culture and CpG DNA stimulation

Thioglycollate-elicited mouse peritoneal macrophages were prepared from C3HeB/FeJ female mice, 6–8 weeks old, purchased from The Jackson Laboratory (Bar Harbor, ME) as described previously (15). Viable cells (6×10^6 /10 ml/well) were cultured in round tissue culture dishes (100×20 mm) and were preincubated with lactacystin ($5 \mu\text{M}$) or DMEM for 1 h, before being stimulated with CpG ($30 \mu\text{g/ml}$) or DMEM. All samples contained the same final concentration of vehicle, DMSO (0.02%), as contained in the final concentration of lactacystin. Duplicates were carried out for each experimental point, medium, CpG DNA (4 h), CpG DNA plus lactacystin, lactacystin alone and CpG DNA (18 h) as described previously for LPS (15). Cell death was monitored using the MTT assay and there was no significant cell death with lactacystin at 5h. After exposure for 4 h and at 18 h, cells were lysed in $600 \mu\text{l}$ of Buffer RLT/mercaptoethanol, and harvested for total RNA isolation using the RNeasy kit (vendor, location) as described in the manufacturer's directions (15).

Sample preparation and microarray analysis

Reverse transcription and PCR was conducted using a 1-step RT-PCR (Qiagen) according to the manufacturer's instruction. Eight micrograms of the total RNA were converted to cDNA according to the manufacturer's instructions for the Affymetrix GeneChip system (Santa Clara, CA). Double-stranded cDNA was then purified by phase lock gel (Eppendorf, Westbury, NY) with phenol/chloroform extraction. The purified cDNA was used as a template for *in vitro* transcription reactions for the synthesis of biotinylated cRNA using RNA transcript labeling reagent (Affymetrix, Inc., Santa Clara, CA). The biotin-labeled cRNA were then fragmented and the quality of these cRNA in each experiment was evaluated by both gel electrophoresis and hybridization (fraction of the sample) onto test-3 microarray as a measure of quality control before hybridizing onto the Affymetrix Gene Expression Arrays (data not shown) as described previously (15).

The labeled fragmented cRNAs were then hybridized onto the Affymetrix GeneChip mouse genome 430 2.0 (Affymetrix) microarrays again, according to manufacturer's instructions as described previously (15). Briefly, appropriate amounts of fragmented cRNA and control oligonucleotide B2 were added to the hybridization buffer, along with control cRNA (BioB, BioC, BioD), herring sperm DNA, and bovine serum albumin (BSA) as described previously (15). The hybridization mixture was heated at 99°C for 5 min, followed by incubation at 45°C for 5 min before injecting the sample into the GeneChip. The hybridization was carried out at 45°C for 16 h with mixing. After hybridization, the solution was removed and arrays were washed and stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR). After washes, probe arrays were scanned using the Affymetrix GeneChip system confocal scanner at the Mayo Clinic (Rochester, MN) as described previously (15).

Data analysis and Network and Pathway Analysis

Gene expression data were first imported into the GENESpring program (Agilent, Palo Alto, CA) and the data corrected for any difference in the arrays and scaled to a factor of 500 (default) during the data extraction process. CpG/medium, CpG + lactacystin/medium, and lactacystin/

medium log ratio values were normalized to a scale of 0 (instead of 1, which shows decimals), and expression values of up-regulated genes were indicated by positive numbers, whereas down-regulated genes were defined by negative numbers (termed normalized ratios, a log ratio of 2 is equivalent to a 4-fold change). These ratios were imported into the Ingenuity Pathways Analysis software (Ingenuity Systems, Mountain View, CA). A Fischer exact test was used for the normalization ratio as described previously. Activated genes were categorized in different pathways and networks available in the database and ranked by score. Statistically significant genes were analyzed and mapped into different pathways and networks as described previously (15).

RESULTS

Lactacystin pretreatment of murine macrophages resulted in changes in CpG-induced signaling pathways and are manifested through expression of several categories of genes (Table 1) in immune response, hematological system development, cell-cell signaling, immune and lymphatic system, cellular movement, behavior, cancer, hematological disease, cellular growth and proliferation and the nervous system. The numbers represent the relative expression of the genes as modulated by various treatments. In the category of immune response, 257 genes were either up- or down-regulated in response to CpG DNA, whereas a pretreatment with lactacystin followed by CpG DNA resulted in modulation of only 42 of these genes. Similar results were obtained in all other categories, such as cell-cell signaling, behavior, cancer and nervous system as well. In contrast, there were other categories of gene specific response such as hematological disease and cellular growth and proliferation where pretreatment with lactacystin followed by CpG DNA resulted in modulation of more genes than CpG DNA alone. Lactacystin alone modulated much fewer genes than CpG DNA treatment in all categories of high level functions. These data provide evidence that the proteasome regulates some, but not all, of the genes involved in multiple signaling pathways in macrophages in response to CpG DNA.

CpG-DNA induced and proteasome-dependent genes

Although >5594 genes were found to be modulated by CpG DNA, we focused our attention on the most significant 85 genes that were positively regulated by CpG DNA with a normalization ratio of >5.3. Fifty-five of the 85 identified genes were identified as being up regulated by CpG DNA at the 4 h time point and were down-regulated at least 20% when a prior treatment with lactacystin (lactacystin-sensitive) was included. These genes are listed in Table 2. These include many of the inflammation-linked genes such as chemokine ligands, (*e.g.*, CXCL3, CXCL10); cytokines, (*e.g.*, IL-6, IL-12 β , TNF- α , IL-1 β , IL-1 α , IL-12 α); endoperoxide synthase 2, cyclo-oxygenase 2 (COX2); suppressor of cytokine signaling 3 (SOCS3); adhesion molecules, such as intercellular adhesion molecule (ICAM1), and vascular cell-adhesion molecule (VCAM1); and Toll-like receptor 2 (TLR2), as has been observed earlier with LPS (15). Validation of these results for TNF- α and IL-1 β by RT-PCR is shown in Figure 1. Gene expression of both CpG DNA-induced TNF- α and IL-1 β was down regulated >90% with pretreatment of murine macrophages with lactacystin. In contrast, there was no major change in gene expression β -actin. Expression of the gene for TLR2 in response to CpG DNA gene expression was also down-regulated following a pretreatment with lactacystin. Some genes were profoundly down-regulated, while others were down regulated ~20%. Interestingly TNF- α was among those that were profoundly down-regulated with a pretreatment with lactacystin. This observation is distinctly different from our previous results with LPS-treated C3H/FeJ macrophages where gene expression of TNF- α was found to be only partially inhibited by lactacystin pretreatment, followed by LPS (15). A total of 30 genes that were up-regulated by CpG DNA, but were either lactacystin-insensitive and/or further up-regulated in the presence of lactacystin, these are listed in Table 3. There are also ~30 genes induced by CpG DNA whose expression was not significantly altered by lactacystin. These

included chemokine ligand CXCL2, polymerase (POL); ribosomal protein SA, (LAMR1); G-protein-coupled receptor, lysophosphatidic acid receptor 1 (LPAR1); DNA (Cytosine-5) methyl transferase 1 (DNMT1); and NCAM1, neural cell adhesion molecule 1 (Table 3).

In addition to the CpG DNA-induced genes (85 genes), levels of expression of some 40 genes were found to be repressed by CpG DNA at 4 h and their normalization values ranged from -7.0 to -24.9 and genes whose levels of expression were suppressed by CpG DNA treatment was reversed by lactacystin pretreatment (Table 4). These included genes involved in signaling such as MAPK10, transcription regulator aryl hydrocarbon receptor nuclear receptor (ARNT), nuclear receptor co-repressor 2 (NCOR), and cytokines such as leukemia inhibitory factor (LIF).

Genes up-regulated/down-regulated by CpG DNA at 18 h

In a previous study, we demonstrated that CpG DNA induces a relatively limited number of genes after addition to cultures of RAW 264.7 cells for 6 h (6,7). Early and late time points were not considered in that study. In the present study, we explored both the early and late induced genes. To obtain a more complete picture of the CpG DNA-modulated genes, we have listed the 188 genes with normalization ratios of $6.8 - 475$ that are induced at 18 h after treatment (Table 5). These genes are profoundly induced by CpG DNA at 18 h, as compared to 4 h. Some of these genes were also LPS-inducible early, at 4 h, but inducible by CpG DNA at 18 h (15). The genes not marked with an asterisk denote those that are inducible with LPS treatment at 4 h (Table 5). These genes include the formyl peptide receptor 1,2 (FPR1, FPR2), haptoglobin (HP), interferon-induced protein 1–3 (IFIT1-3), arginase type 2 enzyme (ARG2), guanylate-binding 2 and 4 (GBP2 and 4), serine peptidase (CORIN); matrix metalloproteinase 9,14, and 2 (MMP9, MMP14, MMP2); prostaglandin E synthase (PTGES), suppressor of cytokine synthesis (SOCS3), IL-1 α , complement component 3 (C3), and toll-like receptor 1 (TLR1). We have also listed several genes with normalization ratios of -20.6 to -7.0 whose expression was down regulated by CpG DNA at 18 h (Table 6). These genes include mediator complex subunit 13 like (THRAP2), T cell lymphoma invasion and metastasis (TIAM1), high mobility group AT-hook 2 (HMGA2), and superoxide dismutase (SOD1).

Signaling genes up-regulated /down-regulated by CpG DNA

CpG DNA-signaling almost exclusively involves one pathway in macrophages, as shown schematically in Figure 1. CpG DNA up-regulates expression of macrophage genes that encode TLR1, TLR2, and TLR3 (Table 7). However, the genes that encode MyD88 and IRAK1 and IRAK2 were not significantly affected by stimulation with CpG DNA. In contrast, expression of IRAK3 or IRAK4 (inhibitors of IRAK 1 and 4) are up-regulated, as we have observed previously with LPS. The gene encoding TRAF1 is significantly up-regulated, but not those that encode TRAF2 and TRAF3 while the gene for TRAF6 is actually down-regulated. Levels of expression of IRF1 and IRF7, as well as those of STAT1 and STAT2, are up-regulated. However, levels of expression of inducible NOS2A were found not significantly to be affected, whereas those of NOS1 are up-regulated at 18 h.

DISCUSSION

In our manuscript, we provide clear cut evidence to support the conclusion that CpG DNA-mediated modulation of gene expression, like LPS, is in large part mediated via the ubiquitin-proteasome pathway. In the present study, we first analyzed the macrophage response to CpG DNA in terms of proteasome-dependent and -independent gene expression, globally. Pretreatment of primary mouse macrophages with lactacystin resulted in a significant modulation of levels of expression of CpG DNA induced/repressed genes. These results are similar to our previously obtained results with LPS treatment, where most of the macrophage

genes involved with inflammatory processes were found to be proteasome-dependent (15). However, there were some unanticipated findings. Several genes whose levels were not upregulated by LPS at 3 h were robustly up-regulated by CpG DNA treatment of macrophages and were blocked by pretreatment with lactacystin. These genes included CXCL3, RASSF4, PTX3, FPR2, etc. (Table 2). These results strongly suggest that major differences likely exist in the CpG DNA- and LPS-induced pathways in macrophages. Second, in our previous study, levels of LPS-induced gene expression of TNF- α mRNA were only partially (20%) inhibited by lactacystin (5 μ M) pretreatment, while, in contrast, CpG DNA-induced expression of TNF- α mRNA was found to be blocked by >90% with lactacystin pretreatment (15). This again suggests that the gene for TNF- α may well be induced by LPS via other pathways (MyD88/TIRAP and TRIF/TRAM), but only by one pathway in response to CpG DNA (MyD88), the latter of which is totally proteasome-dependent. Third, there were twice as many genes induced by CpG DNA after 18 h, relative to 4 h and many of these have earlier been shown to be readily induced by LPS within 3 h. (We could not use lactacystin as an 18 h treatment because it causes cell death after a prolonged exposure since it is an irreversible inhibitor of the proteasome, no cell death was observed at 4–5 h after CpG DNA/lactacystin treatment). These data suggest that the CpG DNA-mediated gene responses are delayed compared to that observed with LPS and is perhaps due to the fact that most of the CpG DNA-dependent signaling occurs within the endosomes and, compared to those observed with LPS, this is not a particularly potent stimulating agonist (17). Some of the genes that are highly induced after 18 h include genes involved in signaling and cell growth: formyl peptide receptor 1, haptoglobin, lipocalin 2, IFIT3, IFIT1, EPHA4 kinase, BOC arginase 2, and metalloproteinase, MMP9. Those proteins induced by CpG DNA on early may actually contribute to a later wave of stimulated gene-expression at 18 h. Many of these genes are induced early with LPS at 3 h (15). These results show that CpG DNA may be inducing increased levels of gene expression both at early and late times. However, the TLR9-mediated signaling may contribute to the cytokines seen in Gram negative sepsis initially set off by TLR4 (LPS)-mediated signaling, then followed up by TLR9 (CpG-mediated signaling).

The collective published observations regarding what is known about the CpG-DNA- and LPS-induced pathways are summarized in Figure 2. It has been well established that CpG DNA operates in macrophages via TLR9 localized to endosomes (17,18). CpG DNA induces the MyD88 pathway via an NF- κ B dependent mechanism, leading to the gene expression of TNF- α (19–25). NF- κ B normally exists in cell cytoplasm as a p50 and p65 heterodimer complexed with I κ B. Upon activation with agonists I κ B is ubiquitinated and subsequently cleaved by the proteasome. Lactacystin pretreatment blocks this pathway, first by blocking the formation of p50 from its precursor p105 by the proteasome (26). Moreover, it also blocks the degradation of the phosphorylated and ubiquitinated I κ B that is necessary for the translocation of NF- κ B to the nucleus and followed by transcription of genes (27). Therefore, CpG DNA-induced gene expression of TNF- α via MyD88-dependent pathway is blocked >90% by lactacystin (5 μ M, a concentration that blocks primarily the chymotrypsin-like activity of the proteasome) pretreatment. By contrast, our previous data suggest that low doses of lactacystin blocks LPS-induced TNF- α only by 20%, suggesting that either high doses of lactacystin which block all activities of the proteasome (e.g. chymotrypsin-like, trypsin-like and the caspase-like or post-glutamase activities) are required for this and also LPS- induced upregulation of TNF- α gene expression is via the MyD88-independent pathway (15). Our previous results have suggested that high doses of lactacystin 10–20 μ M are effective for completely blocking LPS-induced TNF- α (12). High doses of lactacystin pretreatment of cells may be blocking both the MyD88 and the MyD88-independent pathways induced by LPS.

LPS-induced TNF- α is not only dependent on the MyD88-dependent pathway via TLR4, but is also activated through the TRIF/TRAM (MyD88-independent) pathway via TLR4 (10). Induction of TNF- α gene expression by LPS was originally found to be solely MyD88-

dependent (28), and that TIRAP served as a “bridging adapter”; however, others have suggested that TNF- α is also expressed through signaling by the TRIF/TRAM pathway that leads to a more delayed induction of NF- κ B (29,30). Interestingly, it has been shown that TNF- α can be induced in a TIRAP-independent, but MyD88-dependent fashion through TLR2 (31,32). In contrast, CpG DNA-induced, TLR9-mediated signaling is solely MyD88-dependent in macrophages. Interestingly, results of recent studies suggest that blockade of proteasomal activity by proteasome inhibitors such as lactacystin, blocks the MyD88 pathway, but stabilizes the LPS-induced TRIF/TRAM pathway, via IRF3 and RIP1 (which are ubiquitinated and degraded by the proteasome), that contribute to gene expression of TNF- α .

Several signal transduction proteins such as IRAK1, TRAF6, RIP1, IKK γ etc. are all regulated and/or degraded by the ubiquitin-proteasome pathway (32). These signaling proteins are either ubiquitinated by a K48-linked ubiquitin and are degraded by the proteasome; or by a K63-linked ubiquitin to facilitate regulatory functions and activate other signaling mediators. Additionally, the MAP kinases [also regulated in levels of expression by the proteasome (12, 14, and 15)] may be involved in LPS-induced TNF- α expression. We have previously shown for example that ERK and JNK are involved in gene expression of TNF- α and others have shown that p38 plays a role in regulating both TNF- α message stability and protein expression (33,34). Thus LPS-induced pathways to TNF- α are very complex.

Collectively, these experiments provide strong evidence that a blockade of proteasome activity serves to inhibit production of proinflammatory mediators induced by CpG DNA, similar to that earlier observed with LPS. We now demonstrate that much of the CpG DNA- induced signaling occurs much later than 4 h, thus the proinflammatory genes expressed later during the development of septic shock may, in part, be due to CpG DNA. The genes induced by CpG DNA treatment are almost all MyD88-dependent, and most of the pro-inflammatory ones are also proteasome-dependent. Proteasome inhibitors may, therefore, be relevant in treating diseases such as SIRS, sepsis, and septic shock, where the inflammatory response becomes exaggerated and leads to death. Our previously published results have provided strong evidence that proteasome inhibitors can attenuate multiple signaling pathways activated by different TLRs. We have also recently shown that a combination of proteasome inhibitor as an adjunct to antibiotic therapy helps attain a higher degree of therapeutic efficacy (35).

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Figure 1. Effect of lactacystin on the LPS-induced TNF- α and IL-1 β gene expression in murine macrophages

C3HeB/FeJ thioglycollate-elicited peritoneal macrophages were treated with lactacystin for 1h and then challenged with CpG or medium for 4h, and RNA was extracted from the cells and analyzed by RT-PCR. Lane 1, M, medium; lane 2, L, lactacystin 5 μ M; lane 3, lactacystin plus CpG DNA 30 μ M/ml; and lane 4, CpG DNA 30 μ M/ml, as described under the methods section. This RT-PCR was carried out with the samples that were subjected to microarray analysis for validation purposes.

Figure 2. Comparison of CpG DNA- and LPS-induced signal transduction pathways

TABLE 1

High level functions affected by CpG DNA

HIGH-LEVEL FUNCTIONS	CpG (4h)	LACT/CpG	LACT	CpG (18 h)
Immune response	257 ^a	42	26	192
Hematological System Development	240	53	35	190
Cell-Cell signaling	219	48	38	149
Immune and lymphatic system	208	44	25	147
Cellular movement	145	27	13	131
Behavior	138	0	2	0
Cancer	72	37	49	57
Hematological Disease, Cellular growth & Proliferation	56	69	24	55
Nervous System	41	24	24	19

^aThese data represent the expression of total number of genes induced/repressed by the various treatments: CpG DNA, 30 µg, 4 h; pretreatment of lactacystin for 1h, (LACT, 5µM), followed by CpG DNA, lactacystin alone and CpG DNA for 18 h. These experiments were carried out in thioglycollate-elicited murine macrophages. The RNA was extracted from the treated cells and their gene expression was compared using the Affymetrix microarray analysis and Ingenuity Pathway Analysis.

TABLE 2

Genes up-regulated by CpG DNA^a and proteasome-dependent

	CpG 4	CpG +L	L	CpG 18	DESCRIPTION	FUNCTION
Gm1960A	417.0	41.9	1.7	173.5	Chemokine (C-X-C motif) ligand 3	Chemokine
CXCL3*	145.7	82.0	1.7	54.0	Chemokine (C-X-C motif) ligand 3	Chemokine
IL6	81.8	7.1	-2.1	13.4	Interleukin 6	Cytokine
VCAM1	75.4	5.8	4.1	59.5	Vascular cell adhesion molecule	Inflammation
SOCS3	64.1	25.7	2.4	92.6	Suppressor of cytokine signaling 3	Anti-inflammation
PTGS2 (COX2)	62.6	6.6	4.3	19.0	Prostaglandin-endoperoxide synthase 2	Enzyme
EDAR	49.1	19.2	50.2	20.2	Ectodysplasin A receptor	Transmembrane receptor
IL12B	48.7	16.3	1.9	10.8	Interleukin 12 B	Cytokine
TNF	44.5	4.6	1.2	7.8	Tumor necrosis factor	Cytokine
FPR1	40.7	2.6	-1.3	474.6	Formyl peptide receptor 1	G-protein- coupled receptor
IL1B	39.7	2.2	1.7	10.9	Interleukin 1 beta	Cytokine
NF-κBIZ	30.5	9.1	-1.9	22.6	Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor z	Transcription regulator
RASSF4*	26.3	6.9	-4.83	15.9	Ras association	signaling
CDC42EP2*	23.5	1.2	-2.8	39.9	CD42 effector	other
ITK*	23.4	3.8	29.4	3.5	IL-2 inducible T-cell kinase	Kinase
PTX3	22.7	3.6	-1.9	13.3	Pentraxin-related gene induced by IL-1	other
PFR2*	22.1	1.7	1.1	79.4	Formyl-peptide receptor-like 2	G-protein- coupled receptor
NRK*	21.6	16.2	23.5	5.2	NIK related kinase	kinase
COL9A3	21.4	8.9	3.4	3.9	Collagen, type IX, alpha3	other
MEIS1	20.2	-4.43	-14.9	6.3	Myeloid ecotropic	Transcription regulator

	CpG 4	CpG +L	L	CpG 18	DESCRIPTION	FUNCTION
					viral integration site	
ICAM1	20.0	2.6	1.6	21.1	Intercellular adhesion molecule 1	Transmembrane receptor
RRAD	19.8	15.4	6.5	45.7	Ras-related associated with diabetes	enzyme
DTN*	18.6	10.0	3.6	12.2	Dystrobrein, alpha	other
FABP2	18.8	11.04	2.2	11.1	Fatty acid binding protein 2, intestinal	Transporter
TNFRSF5	17.6	6.5	2.5	10.2	CD40 molecule	Transmembrane receptor
HP2	16.4	1.5	-2.7	248.3	Haptoglobin	Peptidase
PDE4B	15.8	-4.0	-2.6	14.1	Phosphodiesterase 4B	Enzyme
TLR2	11.0	5.9	-1.5	15.2	Toll-like receptor 2	Transmembrane receptor
DSCAM*	10.7	-2.1	4.5	2.5	Down syndrome adhesion molecule	other
TNFSF9	10.5	1.6	2.2	1.6	TNF soluble factor 9	cytokine
GTF2E*	10.5	1.6	2.0	1.6	General transcription factor IE, polypeptide 1 alpha, 56 kD	Transcription regulator
TTR*	10.4	1.6	4.7	-8.2	transthyretin	Transporter
HLAE1	10.2	1.9	2.7	9.1	Major histocompatibility complex, class I E	Transmembrane receptor
CD4*	10.2	1.6	2.6	5.1	CD4 antigen	Transmembrane receptor
APPBP-2*	10.0	2.0	6.5	9.0	Amyloid beta precursor	other
MAML1	9.6	2.0	-2.6	2.3	Mastermind-like 1	Transcription regulator
IL12A	9.2	2.9	2.1		Interleukin 12A	cytokine
CD69*	9.2	3.7	1.8	15.0	CD69 antigen	Transmembrane receptor
NFKBI	9.0	3.0	-1.5	8.5	Nuclear factor of kappa light polypeptide gene	other
CEBPδ	8.5	-1.2	-2.8	6.0	CCAAT/enhancer binding protein CEBPδ	Transcription regulator
UTF1*	8.4	4.6	8.1	1.6	Undifferentiated embryonic cell transcription factor 1	Transcription factor

	CpG 4	CpG +L	L	CpG 18	DESCRIPTION	FUNCTION
IGH-1a*	8.0	3.0	-7.0	5.2	Immunoglobulin heavy chain 1a-IgG2a	Immunoglobulin
CXCL10**	7.9	1.7	1.4	11.8	Chemokine (C-X-C motif) ligand 10	Chemokine
CACNB*	7.0	2.7	1.9	2.5	Calcium channel, voltage-dependent, beta 1 subunit	Ion channel
MAFF	6.9	3.9	-1.0	4.2	v-maf	Transcription regulator
COPS3*	6.6	2.2	1.2	-7.3	COP9 constitutive Photomorphogenic Homolog subunit 3	other
SULT1D1*	6.5	2.1	8.4	6.1	Sulphotransferase family 1D, member 1	enzyme
AZ12*	5.7	2.2	7.1	5.3	5-azacytidine induced 2	other
CIASI	5.7	1.5	-1.4	4.9	NLR family, pyrin domain containing 3	other
PTPRJ	5.7	2.1	-4.1	4.3	Protein tyrosine Phosphatase, receptor type	phosphatase
PTPRD*	5.7	-5.8	7.7	3.9	Protein tyrosine Phosphatase, receptor type D	phosphatase
TRPC6	5.6	-1.8	7.0	12.2	Transient receptor potential cation channel, subfamily C, member 6	Ion channel
TBX1*	5.5	2.5	1.4	1.4	G1 to S phase transition 2	Translation regulator
CD38	5.5	1.9	-1.1	44.3	CD38 molecule	enzyme
IL1A	5.3	2.3	1.1	9.5	Interleukin 1 alpha	cytokine

^aMurine macrophages were treated as described in the legend to Table 1. The gene expression values are reported as average normalization ratios. The gene identifiers were uploaded into the Ingenuity Pathways Analysis and a ratio cutoff of 5.3 was set to identify the most significant genes. In addition to that, this Table only includes genes whose expression was down-regulated $\geq 20\%$ with CpG/LACT as compared to CpG DNA alone.

* Asterisk denotes genes that were not up-regulated by LPS at 4h.

TABLE 3Genes up-regulated by CpG DNA, and proteasome-independent ^a

	CpG 4	CpG + L	L	CpG 18	DESCRIPTION	FUNCTION
CXCL2	85.4	118.6	13.3	35.5	Chemokine (C-X-C motif) ligand 2	Chemokine
POL	16.7	13.9	6.0	10.8	Polymerase (DNA directed), epsilon	enzyme
LAMR1	14.9	18.5	3.7	13.0	Ribosomal protein SA	Transmembrane receptor
SIM2	13.8	14.4	6.0	2.3	Single-minded homolog 2	Transcription regulators
DOKS	12.7	13.1	2.0	2.8	Docking protein S	Docking protein
SALL1	11.8	12.5	1.9	4.3	Sal-like 1	other
EDG	10.7	11.3	8.7	37.1	Endothelial differentiation Lysophosphatidic acid Acid G-protein-coupled Receptor, 2	G-protein coupled receptor
LPAR1	10.6	11.7	8.7	37.1	Lysophosphatidic acid receptor 1	G-protein coupled receptor
ROCK	9.2	9.4	2.0	1.7	Rho-associated, coiled-coil containing protein kinase 1	kinase
PHACTF	9.2	8.2	19.7	14.0	Phosphatase and actin regulator	other
KCND	8.6	12.0	11.2	14.1	Potassium voltage-gated channel Shal-related subfamily, member 3	Ion channel
CASR	8.5	10.4	2.2	1.9	Calcium-sensing receptor	G-protein coupled regulator
PAX2	8.4	11.1	4.9	6.0	Paired box 2	Transcription regulator
PDE3A	8.3	10.4	-7.6	12.5	Phosphodiesterase 3A	enzyme
MAL2	8.2	15.7	4.5	7.1	Mal, T-cell differentiation protein 2	other
SLC7A11	7.8	10.3	3.1	26.7	Solute carrier family 7	Transporter
ADM	7.2	8.9	1.9	5.8	Adrenomedullin	other
ORM1	6.9	43.8	1.1	88.2	Orosomucoid 1	other

	CpG 4	CpG +L	L	CpG 18	DESCRIPTION	FUNCTION
NTF3	6.9	8.0	4.3	1.9	Neurotrophin 3	Growth factor
RORB	6.9	7.4	1.3	3.5	RAR-related orphan receptor B	Ligand dependent Nuclear receptor
FGF8	6.7	9.4	2.9	2.1	Fibroblast growth factor 8 (androgen-induced)	Growth factor
ITGA4	6.5	7.4	11.7	7.0	Integrin, 4 (antigen CD49D alpha 4 subunit of VLA-4 receptor)	other
DNMT1	6.4	7.4	2.5	2.7	DNA (cytosine-5-) methylase transferase 1	enzyme
RELB	CD	8.1	1.3	9.7	V-rel reticuloendotheliosis viral oncogene homolog B	Transcription regulator
GPRI43	CD GO	7.9	11.0	5.0	G-protein coupled receptor	G-protein coupled receptor
EIF3S3	CD GO	13.5	2.3	7.5	Eukaryotic translation factor 3, subunit H	Translation regulator
CASP4	CD	11.1	3.0	8.0	Caspase 4	Peptidase 4
BOC	6.1	10.7	4.2	30.7	Boc homolog	other
TRPM1	5.8	6.0	-1.7	-1.4	Transient receptor potential Cation channel, subfamily M, member 1	Ion channel
NCAM1	5.6	7.7	5.6	4.6	Neural cell adhesion molecule 1	other

^a Murine macrophages were treated as described in the legend to Table 1. The gene expression values are reported as average normalization ratios. The gene identifiers were uploaded into the Ingenuity Pathways Analysis and a ratio cutoff of 5.3 was set to identify the most significant genes. In addition to that, this Table only includes genes whose expression was not down-regulated $\geq 20\%$ with CpG/LACT as compared to CpG DNA alone.

TABLE 4Genes down-regulated by CpG DNA^a at 4 h and proteasome-dependent

	CpG 4	CpG +L	L	CpG 18	DESCRIPTION	FUNCTION
ARNT	-24.9	1.89	-1.9	2.0	Aryl hydrocarbon receptor nuclear translocator	Transcription regulator
GPR73	-18.0	-1.7	-1.5	2.3	Prokineticin receptor 1	G-protein coupled receptor
MAPK10	-15.8	2.3	-3.3	5.0	Mitogen-activated protein kinase 10	kinase
DFNB3	-14.19	1.9	5.1	4.6	Deafness, autosomal recessive 31	other
INPP5	-13.9	1.2	-1.8	1.1	Inositol polyphosphate-5-phosphatase	phosphatase
RORA	-13.9	2.4	-1.4	1.8	RAR-related orphan receptor A	Ligand dependent Nuclear receptor
ADAM10	-12.9	-1.2	-2.0	-1.9	ADAM metalloproteinase domain 10	peptidase
NDUFS	-12.1	-3.0	1.1	-1.7	NADH dehydrogenase e	enzyme
ZNF63	-11.9	4.4	2.4	4.5	Zinc finger protein 638	other
MUF1	-11.8	1.4	-3.3	1.6	Leucine rich repeat containing 41	other
NUMB	-11.6	1.7	-1.3	1.6	Numb homolog	other
KCN	-11.5	-2.5	-1.2	1.1	Potassium voltage-gated Channel, Isk-related family member 2	Ion channel
MASTP1	-11.3	5.8	4.2	5.9	Mannan-binding lectin serine peptidase 1	peptidase
PDH	-11.0	4.5	2.7	2.5	Pyruvate dehydrogenase Complex, component X	enzyme
NCOR	-11.0	-1.4	-1.3	-1.2	Nuclear receptor co-repressor 2	Transcription regulator
STON2	-10.9	-1.6	-1.1	1.2	Stonin 2	other
AQP4	-10.9	5.0	3.3	1.8	Aquaporin 4	transporter
ACDC	-10.9	-1.4	-3.6	2.0	Adiponectin	Other
CCRS	-10.7	-2.2	-2.0	2.2	chemokine (C-C motif) receptor 5	G-protein coupled receptor

	CpG 4	CpG +L	L	CpG 18	DESCRIPTION	FUNCTION
SAMD4	-10.3	1.7	1.5	2.3	Sterile alpha domain containing 4A	other
PMM1	-8.0	1.3	-1.7	1.6	Phosphomannomutase 1	enzyme
DPE2	-8.0	-1.3	-2.3	1.9	Polymerase (DNA directed), epsilon 2	enzyme
EPHA7	-8.0	1.9	-5.7	-3.7	EPH receptor A7	Kinase
REP	-8.0	-1.3	-1.2	2.1	PALBP1 associated Ep domain containing 2	other
TXNRD	-7.9	-1.6	-4.5	-2.4	Thioredoxin reductase 2	enzyme
GZMC	-7.7	-1.1	-1.4	1.7	Granzyme C	peptidase
ABCD	-7.7	1.7	-5.8	-5.2	ATP-binding cassette, Sub family D member 3	transporter
MYC	-7.7	2.5	1.3	1.9	V-myc myelocytomatosis viral related oncogene, neuroblastoma-derived	Transcription regulator
SKP	-7.7	1.5	-2.2	1.8	s-phase kinase-associated protein 2	other
ODF	-7.6	5.6	5.3	4.5	Outer dense fiber of Sperm tail	other
VPS39	-7.6	-1.7	-1.9	1.3	Vacuolar protein sorting 39	transporter
CHEK	-7.3	3.0	2.5	8.5	CHK1 checkpoint	kinase
RASSF1	-7.3	3.5	2.4	2.2	Ras association (RalGDS/AF-6 domain family 1	other
ACTN	-7.3	2.6	-2.7	-2.0	Actinin, alpha	Transcription regulator
LIF	-7.3	4.2	-2.3	2.4	Leukemia inhibitory factor	cytokine
CKLF	-7.2	3.0	-1.8	2.3	Chemokine-like factor	cytokine
GJA4	-7.2	2.5	1.9	1.0	Gap junction protein, alpha 4, connexin 37	transporter
RYR2	-7.2	-2.4	-1.7	-4.5	Ryanodine receptor 2	Ion channel
DSG1	-7.2	2.0	3.5	2.4	Desmoglein 1	Other
SOD	-7.1	4.2	-3.4	-13.4	Superoxide dismutase 1, soluble	enzyme

^aMurine macrophages were treated with the compounds mentioned in the footnote to Table 1. The values reported have been corrected for differences in the arrays. The gene expression values are reported as average normalization ratios. The gene identifiers were uploaded into the Ingenuity Pathways Analysis and a ratio cutoff of -7.0 to -24.9 was set to identify the most significant downregulated genes with CpG

DNA. Most of the genes listed were not upregulated even after an 18 h treatment with CpG DNA. In addition to that, this Table only includes genes whose expression was altered at least 20% with CpG/LACT as compared to CpG DNA alone.

TABLE 5

Genes up-regulated by CpG DNA at 18 h ^a

	CpG 4	CpG +L	L	CpG 18	DESCRIPTION	FUNCTION
FPR1 *	40.8	2.5	-1.2	474.6	Formyl peptide receptor 1	G-protein coupled
HP	16.5	1.5	-2.7	248.3	Haptoglobin	Peptidase
SOC3	64.1	25.7	2.4	92.6	Suppressor of cytokine signaling	other
IFIT2	3.15	2.0	-7.48	88.8	Interferon-induced Protein 2	other
ORM1	6.9	43.8	1.1	88.2	Orosomucoid 1	other
FPR2	22.06	1.7	1.1	79.4	Formyl peptide receptor 2	G-protein coupled
MARCO	2.0	-1.2	2.14	73	Macrophage receptor with collagenous structure	Transmembrane
ZBP1	1.95	1.9	1.7	67.7	Z-DNA binding protein	other
RRAD	19.7	15.4	6.5	45.6	RAS related associated With diabetes	Enzyme
CD38	5.5	1.9	-1.2	44.2	CD38 molecule	Enzyme
CDC42EP2	23.4	1.1	-2.7	39.9	CDC42 effector protein (Rho GTPase binding) 2	other
LPAR1	10.6	11.7	8.7	37.1	Lysophosphatidic acid receptor 1	G-protein-coupled
LCN	1.3	-2.1	-12.0	34.9	Lipocalin 2	Transporter
IFIT3	1.8	-2.6	-3.5	33.8	Interferon-induced Protein 3	other
IFIT1	3.7	1.1	-1.7	33.4	Interferon-induced Protein 1	other
EPHA4	5.0	8.1	-6.8	30.9	EPH receptor A4	Kinase
BOC *	6.1	10.7	4.2	30.7	Boc homolog	other
BF	2.0	1.4	-1.1	28.6	Complement factor B	Peptidase
CNKSR2 *	13.0	1.08	37.2	28.4	Connector enhancer of kinase suppressor of RAS2	other
ARG2	8.2	3.2	1.3	27.9	Arginase type 2	Enzyme
SLC7A11	7.8	10.26	3.1	26.7	Solute carrier family 7	Transporter

	CpG 4	CpG +L	L	CpG 18	DESCRIPTION	FUNCTION
GBP2	8.3	-1.2	-1.7	26.2	Guanylate binding protein 2, interferon inducible	Enzyme
GBP4	11.8	2.5	-1.3	25.1	Guanylate binding protein 4	Enzyme
CCL8A	1.3	1.9	2.1	24.2	Chemokine C-C motif 8	Chemokine
TRIB1*	1.4	3.5	3.9	23.6	Tribbles homolog 1	Kinase
CORIN*	-1.0	1.9	3.3	21.3	Corin, serine peptidase	Peptidase
ICAM1	20.0	2.6	1.6	21.1	Intercellular adhesion molecule	Transmembrane
MMP9	1.2	1.7	-1.2	20.5	Matrix metalloproteinase 9	Peptidase
PTGES	4.5	2.7	-2.4	19.3	Prostaglandin E synthase	Enzyme
MMP14	3.8	-2.0	-5.7	18.9	Matrix metalloproteinase 14	Peptidase
USP18	1.0	1.0	-1.9	18.9	Ubiquitin specific peptidase 18	Peptidase
RSAD2	2.5	4.3	1.6	18.6	Radical S-adenosyl methionine domain containing 2	Enzyme
HSD17B2*	1.6	13.4	19.1	17.5	Hydroxysteroid (17-beta) dehydrogenase 2	Enzyme
MYO1B*	2.8	-2.3	2.4	17.5	Myosin 1B	other
NARF*	-1.8	2.6	2.0	17.3	Nuclear prelamin A recognition factor	Enzyme
MMP2*	-2.7	1.9	1.6	17.1	Matrix metalloproteinase 2	Peptidase
GBP1	7.8	1.2	-2.1	16.9	Guanylate binding protein 1, interferon-inducible, 67 kDa	Enzyme
LDB2*	1.75	-4.11	-1.7	16.9	LIM domain binding 2	Transcription regulator
KCNA1	4.18	2.3	2.9	16.7	Potassium voltage-gated channel, shaker-related subfamily, member 1	Ion channel
CXCL6	5.5	2.5	-1.3	16.6	Chemokine ligand 6	Cytokine
IL4R*	1.7	2.7	1.9	16.0	Interleukin 4 receptor	Transmembrane
STK23*	2.0	2.2	11.6	16.0	SFRS protein kinase 3	Kinase

	CpG 4	CpG +L	L	CpG 18	DESCRIPTION	FUNCTION
ADORA2A	4.4	1.2	-1.5	15.9	Adenosine A2a receptor	G-protein-coupled
TRAF1	12.8	6.9	-7.2	15.9	TNF receptor associated Factor 1	other
TIMELESS	-4.1	-3.7	-3.6	15.8	Timeless homolog	other
XRNI *	4.9	1.7	4.2	15.7	5' -3' exonuclease 1	other
IFI44 *	1.4	1.2	-1.3	15.6	Interferon-induced protein 44	other
GPC3 *	1.5	15.9	5.2	15.5	Glypican 3	other
BDH1	2.4	5.9	2.8	15.4	3- hydroxybutyrate Dehydrogenase, type 1	Enzyme
PTPRU *	2.2	1.4	7.6	15.3	Protein tyrosine Phosphatase, receptor type U	Phosphatase
GIP2	1.5	1.5	1.0	15.3	ISG15 ubiquitin-like modifier	other
TLR2	10.9	5.8	-1.5	15.2	Toll-like receptor 2	Transmembrane
CCL2	3.8	1.7	-1.2	15.1	Chemokine C-C motif ligand 2	Cytokine
SEMA3E *	4.8	4.9	11.4	15.0	Sema domain IG short basic domain, secreted 3E	other
CD69	9.1	3.7	1.8	14.9	CD69 molecule	Transmembrane
IGHM *	-10.1	-6.5	-6.3	14.6	Immunoglobulin heavy constant mu	other
KCND3 *	8.6	12	11.2	14.1	Potassium voltage-gated Channel, Shal-related Subfamily, member 3	Ion channel
FOXP4 *	5.2	2.1	1.2	13.8	Forkhead box P4	Transcription regulator
DYSF *	4.6	2.1	3.7	13.6	Dysferin, limb girdle muscular dystrophy 2B	other
TDO2 *	1.5	8.0	5.8	13.6	Tryptophan 2,3-dioxygenase	Enzyme
DOC1 *	4.9	2.8	-1.2	13.4	Filamin interacting protein	other
ASAMA *	1.9	2.4	1.2	13.3	Adipocyte specific adhesion molecule	other
MX1	1.5	1.3	1.3	13.1	Myxovirus resistance 1	Enzyme

	CpG 4	CpG +L	L	CpG 18	DESCRIPTION	FUNCTION
ACPP8	1.9	7.6	5.8	12.7	Acid phosphatase, prostate	Phosphatase
RADSIL1*	3.6	10.0	16.1	12.6	RADS1-like 1	Enzyme
FGFR4	3.5	4.3	5.2	12.6	Fibroblast growth factor receptor	Kinase
PDE3A*	8.3	10.4	-7.6	12.5	Phosphodiesterase 3A	Enzyme
TLR1	3.9	-1.4	-3.8	12.4	Toll-like receptor 1	Transmembrane
SYTL4*	2.7	9.5	2.1	12.3	Synaptotagmin-like 4	Transporter
PILRA*	1.6	1.0	-1.5	12.2	Paired immunoglobulin-like type 2 receptor alpha.	other
ATP1A1	7.1	4.9	9.6	12.2	ATPase	Enzyme
C3	1.5	1.3	-1.4	11.0	Complement component 3	other
MMRN1*	2.6	7.0	6.3	10.9	Multimerin 1	other
OPRL1*	5.8	-1.5	3.7	10.9	Opiate receptor-like 1	G-coupled protein
SLAMF6*	1.7	2.3	1.6	10.9	SLAM family member 6	Transmembrane
ARGBP2*	4.0	7.6	3.7	10.6	Sorbin and SH3 domain containing 2	other
A2BP1*	2.0	8.3	2.8	10.6	Ataxin 2-binding protein 1	other
IER3	5.2	8.0	3.3	10.6	Immediate early-response 3	other
MYH7*	2.7	1.2	2.0	10.3	Myosin, heavy chain 7	other
SPON1*	2.5	5.7	3.3	10.3	Spondin 1, extracellular Matrix protein	other
TTN	-1.8	11.8	2.5	10.3	titin	Peptidase
MAD	1.3	4.5	-3.6	10.3	MAX dimerization protein 1	Transcription regulator
CBFA2T1*	3.5	5.6	-2.9	10.2	Runt related transcription Factor 1 translocated to 1 (cyclin D-related)	Transcription regulator
CAMP*	1.15	1.7	1.8	10.1	Cathelicidin antimicrobial protein	other
IL7*	1.9	6.4	3.5	10.0	Interleukin 7	Cytokine
GAS7	1.7	1.7	-2.4	9.9	Growth arrest specific 7	Transcription regulator
PDE10A*	1.9	5.2	1.9	9.8	Phosphodiesterase 10A	Enzyme

	CpG 4	CpG +L	L	CpG 18	DESCRIPTION	FUNCTION
ABCG5	3.0	3.2	6.1	9.7	ATP-binding cassette Member 5	Transporter
RELB	6.4	8.1	1.3	9.7	V-rel reticuloendotheliosis viral oncogene homolog B	Transcription regulator
IL-1 A	5.2	2.2	1.0	9.5	Interleukin 1 alpha	Cytokine
ADRA1A *	1.4	1.9	5.7	9.4	Adrenergic, alpha-1A-, receptor	G-protein coupled
SMARCB1	3.7	3.8	2.3	9.4	SW1/SNF related, matrix associated, actin dependent regulator of chromatin	other
ALDOC	-1.6	8.3	-2.3	9.4	Aldolase C, fructose-bisphosphate	Enzyme
VEZATIN*	-1.7	2.7	8.6	9.3	VeZatin, adherens junctions Transmembrane protein	other
IRAK3	2.5	-1.7	-1.9	9.3	Interleukin-receptor associated kinase 3	Kinase
PRSS11	2.8	1.5	-1.6	9.3	HtrA peptidase 1	Peptidase
TNNT2 *	-4.1	1.8	7.2	9.2	Troponin T type 2	other
SOD2	4.3	3.9	1.3	9.1	Superoxide dismutase 2, mitochondrial	Enzyme
SYTI *	5.4	-3.2	3.4	9.1	Synaptotagmin 1	Transporter
SCN7A *	3.2	5.3	2.2	9.1	Sodium channel, voltage gated, type VII, alpha	Ion-channel
G6PD *	5.2	7.1	8.5	9.1	Glucose-6-phosphate dehydrogenase	Enzyme
ATXN2 *	1.6	-1.9	-2.1	9.0	Ataxin 2	other
HSPAIL	4.4	4.8	1.9	9.0	Heat shock 70 kDa protein 1-like	other
SEMG2 *	-7.3	-2.5	6.6	9.0	Semenogelin II	other
SRC	1.7	1.8	-2.0	8.9	v-Src sarcoma	Kinase
FGFI2	-4.9	-4.3	8.1	8.9	Fibroblast growth factor 12	Growth factor
ANK2 *	-3.9	8.0	-4.7	8.8	Ankyrin 2	other
POU2AF1 *	-1.0	1.2	-1.0	8.8	Pou class 2 associating factor 1	other

	CpG 4	CpG +L	L	CpG 18	DESCRIPTION	FUNCTION
NEFL*	1.1	3.9	2.0	8.8	Neurofilament, light polypeptide	other
GEM	5.0	12.7	6.3	8.7	GTP binding protein	Enzyme
DLG7*	2.4	13.1	2.7	8.7	Dises, large, homolog associated protein S	other
BRD4*	-1.3	2.6	1.7	8.7	Bromodomain containing 4	Transcription regulator
BARD1*	1.3	6.8	6.1	8.6	BRCA1 associated RING domain	Transcription regulator
GSTM3*	2.7	4.4	6.0	8.6	Glutathione S-transferase mu 3	Enzyme
CAPH7*	3.3	1.9	-2.1	8.5	Calpain 7	Peptidase
CHEK1*	-7.3	3.0	2.5	8.5	CHEK1	Kinase
IFI16Δ*	3.6	1.8	-1.4	8.5	nterferon gamma inducible Protein 16	other
IL18BP*	2.0	1.5	-2	8.5	Interleukin 18 binding protein	other
NRCAM*	1.7	3.7	2.8	8.4	Neuronal cell adhesion molecule	other
TAFI*	1.5	2.2	-1.4	8.4	TAFI RNA polymerase II	Transcription regulator
RBM9*	3.6	5.1	1.8	8.2	RNA binding motif Protein 9	Transcription regulator
SLC12A1*	4.8	7.1	6.5	8.3	Solute carrier family 12, Member 1	Transporter
FBX07*	2.7	5.7	6.3	8.2	F-box protein 7	Enzyme
PTK6*	2.6	6.8	3.4	8.2	PTK6 protein tyrosine kinase 6	Kinase
HNF4A*	2.2	2.1	2.1	8.1	Hepatocyte nuclear factor 4 alpha	Transcription regulator
NFIB*	-6.1	-9.4	-5.5	8.1	Nuclear factor 1B	Transcription regulator
ENAH*	3.9	6.5	4.7	8.0	Enabled homolog	other
RBMY1A1*	4.7	7.6	4.7	8.0	RNA binding motif protein	other
CASP4	6.2	11.1	3.0	8.0	Caspase 4	Peptidase
ADH7*	1.4	4.0	4.3	8.0	Alcohol dehydrogenase	Enzyme

	CpG 4	CpG +L	L	CpG 18	DESCRIPTION	FUNCTION
LBP	3.3	6.3	3.5	8.0	LPS binding protein	Transporter
AHRR *	4.3	5.1	-1.4	8.0	Aryl-hydrocarbon receptor repressor	other
HSPA1B	2.6	129.8	100	8.0	Heat shock 70 kDa protein 1B	other
SUP16H *	4.8	1.8	2.4	7.9	Suppressor of Ty 16 homolog	Transcription regulator
TERT *	3.9	4.1	6.4	7.9	Telomerase reverse transcriptase	Enzyme
ETS2	4.2	3.2	-3.5	7.9	V-ets erythroblastosis virus E26 oncogene homolog	Transcription regulator
IGKC *	1.8	-2.2	-1.6	7.9	Immunoglobulin kappa constant	other
TNFRSF11B *	4.0	13.0	9.9	7.9	Tumor necrosis factor Superfamily 11b	Transmembrane
POLR2E *	-1.4	3.5	-2.9	7.8	Polymerase (RNA) II	Enzyme
BCLN3 *	4.5	6.7	1.5	7.7	Cerebellin 3 precursor	other
PRKD2 *	2.8	4.9	7.05	7.8	Protein kinase D2	Kinase
IKBKE	3.4	-1.5	-4.4	7.8	Inhibitor of kappa light polypeptide gene enhancer	Kinase
HSPBAP1 *	-3.1	-1.6	-1.5	7.7	HSPB (heat shock Associated protein 1)	other
MME *	-3.4	4.6	2.6	7.7	Membrane metalloendopeptidase	Peptidase
KLRA16 *	-2.7	-5.9	2.78	7.7	Killer cell lectin-like receptor, subfamily A, member 16	other
CLSPN *	4.8	5.0	2.5	7.6	Claspin homolog	other
PTPRK *	2.4	3.0	3.9	7.6	Protein tyrosine Phosphatase, receptor type K	Phosphatase
PCP4 *	2.7	1.3	2.8	7.6	Perkinje cell protein 4	other
NR5A1 *	3.0	1.4	2.4	7.6	Nuclear receptor subfamily 5, group A, member 1	Ligand-dependent
SERPINB *	3.5	2.3	1.7	7.5	Serin peptidase inhibitor, clade (ovalbumin)	other

	CpG 4	CpG +L	L	CpG 18	DESCRIPTION	FUNCTION
CRABPI *	2.1	1.4	2.5	7.5	Cellular retinoic acid binding protein 1	Transporter
BUBIB	1.6	2.3	1.4	7.5	Budding uninhibited by benzimidazoles 1 homolog beta (yeast)	Kinase
CCT8 *	3.8	8.9	1.6	7.4	Chaperonin containing TCP1, subunit 8 (beta)	Enzyme
CCL7	2.0	2.2	-2.1	7.4	Chemokine ligand 7	Cytokine
SLC31A2 *	3.5	3.6	-1.3	7.4	Solute carrier family 31 Member 2	Transporter
PPAP2A *	1.7	-2.6	-3.1	7.3	Phosphatidic acid phosphatase type 2A	Phosphatase
CYP2C39 *	2.5	1.9	4.2	7.3	Cytochrome P450 family 2, subfamily C, polypeptide 39	other
RAB11FIP2 *	-2.9	4.4	-1.4	7.3	RAB11 family interacting protein 2	other
CD8A *	1.7	5.3	3.8	7.2	CD8a	other
BMF	5.5	3.5	1.8	7.2	Bcl2 modifying factor	other
SLC26A3 *	4.2	6.2	2.2	7.2	Solute carrier family 26, member 3	Transporter
PACAP *	-1.3	2.1	1.9	7.2	Hypothetical protein MGC29506	other
PARD6B	-1.6	2.4	4.8	7.2	Par-6 partitioning Defective 6 homolog beta (C. elegans)	other
CDC25C *	1.9	2.9	3.7	7.1	Cell division cycle 25 homolog C	Phosphatase
NCK2 *	-1.8	1.5	3.3	7.1	NCK adaptor protein 2	Kinase
DCK	-1.1	-2.2	-4.0	7.1	Deoxycytidine kinase	Kinase
CAMK2A *	2.7	6.2	8.9	7.1	Calcium/calmodulin-dependent protein kinase II alpha	Kinase
COX 8B *	-1.2	3.2	4.0	7.1	Calcium/calmodulin-dependent protein kinase II alpha	Enzyme
MYCBP *	-1.4	19.3	9.6	7.1	c-myc binding protein	other

	CpG 4	CpG +L	L	CpG 18	DESCRIPTION	FUNCTION
ADORA2B	3.9	-1.6	-2.6	7.1	Adenosine A2b receptor	G-protein-coupled
DDX4*	1.8	4.9	-1	7.1	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4	Enzyme
CX36*	2.3	4.5	4.7	7.0	Gap junction protein, delta 2, 36 kDa	other
IFIH1	1.7	1.5	-1.3	7.0	Interferon, gamma-inducible protein 16	Transcription regulator
SLC1A3*	2.6	2.6	6.2	7.0	Solute carrier family 1 (glial high affinity Glutamate transporter), Member 3	Transporter
HERC5	-1.1	1.5	-1.3	7.0	Hect domain and RLD5	other
SLC18A3*	1.4	7.9	2.8	7.0	Solute carrier family 18 (vesicular acetylcholine), member 3	Transporter
F13B*	-1.3	2.8	1.8	7.0	Coagulation factor XIII, B polypeptide	Enzyme
ITGA4	6.5	7.4	11.7	7.0	Integrin, 4 (antigen CD49D alpha 4 subunit of VLA-4 receptor)	other
STAT2	-2.0	2.0	-2.1	7.0	Signal transducer and activator of transcription 2	Transcription regulator
PBSN*	-1.4	1.8	-1.1	7.0	Probasin	Transporter
CREB1*	-3.3	4.2	-3.0	6.9	cAMP responsive element binding protein 1	Transcription regulator
CDK2*	-3.8	-5.1	-2.9	6.9	Cyclin-dependent kinase 2	Kinase
GRIA2*	2.3	2.2	1.6	6.8	Glutamate receptor, ionotropic AMP2	Ion channel
CDH1*	-1.8	4.3	4.9	6.8	cadherin	other
BIRC2*	5.2	3.0	-1	6.8	Baculoviral IAP repeat-containing 2	other
QSCN6*	-1.7	2.9	2.7	6.8	Quiescin Q6 sulphhydryl Oxidase 1	Enzyme
TRP10*	2.0	2.1	-1.1	6.8	Thyroid hormone receptor interactor 10	other

	CpG 4	CpG +L	L	CpG 18	DESCRIPTION	FUNCTION
RARA *	-2.2	3.3	1.5	6.8	Retinoic acid receptor, alpha	Ligand-dependent Nuclear receptor

^aMurine macrophages were treated as described in the legend to Table 1. The gene expression values are reported as average normalization ratios. The gene identifiers were uploaded into the Ingenuity Pathways Analysis and a ratio cutoff of 6.8 was set to identify the most significant genes. In addition, this Table only includes genes whose expression were up-regulated by CpG DNA treatment for 18 h.

* Asterik denotes genes that were not up-regulated by LPSat4h.

TABLE 6

Genes down regulated by CpG DNA at 18 h^a

	CpG 4	CpG +L	L	CpG 18	DESCRIPTION	FUNCTION
THRAP2	1.3	1.7	-4.2	-20.6	Mediator complex subunit 13.1 like	other
TIAM1	-1.8	-1.5	-2.7	-14.4	T-cell lymphoma invasion and metastasis 1	other
HMGGA2	-1.8	1.8	2.09	-14.1	High mobility group AT-hook2	other
FARP2	-2.4	-8.3	-7.7	-14.1	FERM, RhoGEF and pleckstrin domain protein 2	other
MPP6	-1.35	-1.5	-8.3	-13.8	Membrane protein	kinase
SOD1	-7.1	4.2	-3.4	-13.5	Superoxide dismutase 1, soluble	enzyme
KLRC1	-1.6	-1.1	1.2	-9.6	Killer cell lectin-like receptor subfamily C, member 1	Transmembrane receptor
FABP1	-2.7	2.3	-1.6	-9.4	Fatty acid binding protein 1, liver	transporter
PHKA2	-1.4	1.5	2.9	-9.1	Phosphorylase kinase, Alpha 2 (liver)	kinase
CD47	-1.4	-2.2	-2.6	-8.8	CD47 molecule	other
BRAP	-1.8	-3.9	-1.9	-8.7	BRCA1 associated protein	enzyme
KCNQ5	2.5	2.2	2.7	-8.3	Potassium voltage-gated Channel, KQT family, 5	Ion channel
TTR	10.43	1.6	4.7	-8.2	transthyretin	transporter
GABRG2	-2.0	1.2	1.7	-8.0	GABA A receptor, gamma2	Ion channel
SMYD1	2.0	3.1	1.9	-7.9	LIM and SH3 protein 1	other
PTPN9	2.4	-8.6	5.0	-7.7	Protein tyrosine Phosphatase, non-receptor Type 9	phosphatase
COPS3	6.5	2.2	1.3	-7.3	COP9 constitutive Photomorphogenic homolog subunit 3	other
DRP2	-2.8	1.2	1.2	-7.1	Dystrophin related Protein 2	other

^a Murine macrophages were treated as described in the legend to Table 1. The gene expression values are reported as average normalization ratios. The gene identifiers were uploaded into the Ingenuity Pathways Analysis and a ratio cutoff of -7.0 was set to identify the most significant genes. In addition, this Table only includes genes whose expressions were down regulated by CpG DNA treatment for 18 h.

TABLE 7

Signaling gene expression altered by CpG DNA / lactacystin at 4 and 18 h ^a

	CpG 4	CpG +L	L	CpG 18	DESCRIPTION	FUNCTION
TLR1	3.9	-1.4	-3.8	12.4	Toll-receptor 1	Transmembrane
TLR2	11.0	5.9	-1.5	15.2	Toll-receptor 2	Transmembrane
TLR3	-1.9	-2.8	-4.3	4.1	Toll-receptor 3	Transmembrane
TLR4	2.1	-4.7	-7.9	2.0	Toll-receptor 4	Transmembrane
TLR9	1.0	2.0	1.2	1.3	Toll-receptor 9	Transmembrane
MyD88	1.5	1.4	-1.2	2.2	Myeloid differentiation Primary response gene	Adaptor
IRAK1	-1.5	1.4	-1.5	-1.5	Interleukin receptor Activated kinase 1	Kinase
IRAK2	1.1	1.7	1.2	1.9	Interleukin receptor Activated kinase 2	Kinase
IRAK3	2.5	-1.7	-1.9	9.3	Interleukin receptor Activated kinase 3	Kinase
TRAF1	12.8	6.9	-7.2	15.9	TNF receptor-associated factor 1	Enzyme
TRAF2	1.1	2.7	1.7	2.5	TNF receptor-associated factor 2	Enzyme
TRAF3	2.2	4.7	1.9	2.3	TNF receptor-associated factor 3	Enzyme
TRAF6	-2.8	3.2	3.1	2.3	TNF receptor-associated factor 6	Enzyme
TRAF4	-3.3	-2.0	-2.0	1.7	TNF receptor-associated factor 4	Enzyme
NFKB1A	9.0	3.2	-1.5	8.5	Nuclear factor of kappa light chain gene enhancer in B cells 2	Transcription factor
NFKB1Z	30.5	9.1	-1.9	22.6	Nuclear factor of kappa light chain gene enhancer in B cells, inhibitor beta	Transcription factor
IRF1	2.6	1.4	-1.4	3.5	Interferon regulatory Factor 1	Transcription regulator
IRF2	1.1	1.7	-3.0	-5.4	Interferon regulatory Factor 2	Transcription regulator

	CpG 4	CpG +L	L	CpG 18	DESCRIPTION	FUNCTION
IRF7	-1.0	-1.0	-1.2	6.1	Interferon regulatory Factor 7	Transcription regulator
STAT1	1.6	1.6	-2.1	5.4	Signal transducer and Activator of Transcription 1	Transcription regulator
STAT2	-1.9	-2.0	-2.0	7.0	Signal transducer and Activator of Transcription 2	Transcription regulator
NOS2A	-1.2	1.5	1.2	1.6	Nitric oxide synthase-2 inducible	Enzyme
NOS1	-1.3	3.3	-1.3	5.8	Nitric oxide synthase-1 (neuronal)	Enzyme

^aMurine macrophages were treated as described in the legend to Table 1. The gene expression values are reported as average normalization ratios. The gene identifiers were uploaded into the Ingenuity Pathways Analysis and this Table only includes CpG DNA signaling genes relevant to Figure 1.