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Colony-Stimulating Factor-1 Suppresses Responses to CpG DNA and Expression of Toll-Like Receptor 9 but Enhances Responses to Lipopolysaccharide in Murine Macrophages[1]

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During bacterial infections, the balance between resolution of infection and development of sepsis is dependent upon the macrophage response to bacterial products. We show that priming of murine bone marrow-derived macrophages (BMMs) with CSF-1 differentially regulates the response to two such stimuli, LPS and immunostimulatory (CpG) DNA. CSF-1 pretreatment enhanced IL-6, IL-12, and TNF-α production in response to LPS but suppressed the same response to CpG DNA. CSF-1 also regulated cytokine gene expression in response to CpG DNA and LPS; CpG DNA-induced IL-12 p40, IL-12 p35, and TNF-α mRNAs were all suppressed by CSF-1 pretreatment. CSF-1 pretreatment enhanced LPS-induced IL-12 p40 mRNA but not TNF-α and IL-12 p35 mRNAs, suggesting that part of the priming effect is posttranscriptional. CSF-1 pretreatment also suppressed CpG DNA-induced nuclear translocation of NF-κB and phosphorylation of the mitogen-activated protein kinases p38 and extracellular signal-related kinases-1/2 in BMMs, indicating that early events in CpG DNA signaling were regulated by CSF-1. Expression of Toll-like receptor (TLR)9, which is necessary for responses to CpG DNA, was markedly suppressed by CSF-1 in both BMMs and thioglycolate-elicited peritoneal macrophages. CSF-1 also down-regulated expression of TLR1, TLR2, and TLR6, but not the LPS receptor, TLR4, or TLR5. Hence, CSF-1 may regulate host responses to pathogens through modulation of TLR expression. Furthermore, these results suggest that CSF-1 and CSF-1R antagonists may enhance the efficacy of CpG DNA in vivo. ***The Journal of Immunology,*** **2002, 168: 392–399.**

The ability of the host to respond to a bacterial challenge is conferred by cells of the innate immune system, which detect bacterial products such as LPS, CpG DNA, peptidoglycan, and bacterial lipoproteins (1). Recognition of these products by macrophages results in secretion of cytokines and small molecules that mediate the inflammatory response. Both the site of challenge and the magnitude of the response dictate outcome; local infections trigger a controlled response in which the infection is contained and resolved, whereas systemic infection can lead to dysregulated cytokine production that can ultimately result in multiple organ failure and mortality. Although the effects of LPS and CpG DNA on macrophages are very similar, their toxicities in mice differ greatly: LPS is highly toxic (2, 3), whereas CpG DNA alone is not toxic (4), unless administered to D-galactosamine-sensitized mice (5). This important difference has a major implication for the therapeutic potential of CpG DNA. However, the reason for this difference is still unclear.

Toll-like receptors (TLRs)[3] are an evolutionarily conserved family that share homology with the IL-1R family in the cytoplasmic domain. Mammalian TLRs are critical in instigating responses to bacterial products. C3H/HeJ LPS nonresponder mice contain an inactivating point mutation in the *TLR4* gene (6, 7), and TLR4-deficient mice do not produce inflammatory cytokines in response to LPS (8). TLR2-deficient mice are still LPS responsive but fail to respond to bacterial lipoproteins or peptidoglycan (9), and TLR9-deficient mice are incapable of responding to CpG-containing DNA (10). Engagement of TLRs triggers signaling through at least NF-κB and the mitogen-activated protein kinase (MAPK) family members, extracellular signal-related kinase (ERK)-1 and -2, p38, and c-Jun N-terminal kinase, and results in transcription of proinflammatory genes (1, 11).

The macrophage response to bacterial products is also regulated by a variety of endogenous cytokines. Both IFN-γ and GM-CSF (primarily T cell products) can prime the inflammatory response, whereas IL-4, IL-10, and TGF-β are able to suppress macrophage activation. CSF-1, a cytokine that regulates growth, differentiation, and function of macrophages, is readily detectable in peripheral blood in the steady state and is further induced in vivo after infection (12) or challenge with LPS (13). Priming of macrophages with CSF-1 can enhance LPS-induced IL-1, IL-6, and TNF-α production (14). CSF-1 treatment in vivo increased levels of LPS-induced TNF-α and IL-6 (15), and CSF-1-deficient *op/op* mice have enhanced resistance to LPS shock (16). Furthermore, CSF-1 primed human monocytes for enhanced responses to LPS (17).

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[3] Abbreviations used in this paper: TLR, Toll-like receptor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-related kinase; BMM, bone marrow-derived macrophage; TEPM, thioglycolate-elicited peritoneal macrophage; AO-1, activating oligonucleotide-1; NAO-1, nonactivating oligonucleotide-1; HPRT, hypoxanthine phosphoribosyl transferase; f, forward; r, reverse; p, probe.

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These effects of CSF-1 might be due to regulation of LPS recognition via the CD14-TLR4-MD2 complex or at subsequent levels (MyD88, TNFR-associated factor 6, IL-1R-associated kinase) that appear to be shared with other microbial agonists such as CpG DNA (18, 19). To distinguish these alternatives, we compared the effect of CSF-1 on the response to LPS and CpG DNA in primary murine bone marrow-derived macrophages (BMMs). We report in this work that CSF-1 enhanced macrophage responses to LPS but suppressed expression of TLR9 and responses to CpG DNA. These findings provide a mechanism for the differential cytotoxic effect of LPS and CpG DNA and may lead to novel therapeutic strategies against bacteria-induced shock.

Materials and Methods

Cell culture and reagents

RPMI 1640 medium (Life Technologies, Paisley, U.K.) containing 10% FCS, penicillin/streptomycin, and glutamine (complete medium) was used for culture of BMMs. BMMs were derived from the femurs of adult BALB/c mice (Harlan Olac, Bichester, U.K.). In some experiments, adult CD1 outbred mice were used for preparation of BMMs with similar results. Briefly, femurs were flushed with complete medium and bone marrow cells were plated out in complete medium containing 10^4 U/ml (100 ng/ml) recombinant human CSF-1 (a gift from Chiron, Emeryville, CA) on 10-cm bacteriological plastic plates (Bibby Sterilin, Staffordshire, U.K.) for 7 days in a 37°C incubator containing 5% CO_2. Thioglycolate-elicited peritoneal macrophages (TEPMs) were obtained by injecting BALB/c mice i.p. with 1 ml of 10% thioglycolate broth followed by peritoneal lavage with 10 ml of PBS 5 days later. LPS from *Salmonella minnesota* (Sigma-Aldrich, St. Louis, MO) was used at a final concentration of 100 ng/ml in all cell culture experiments. The synthetic tripalmitoylated lipopeptide Pam3CysSerLys4 (Roche, Basel, Switzerland) was used at a final concentration of 10 ng/ml in cell culture. Phosphodiester oligodeoxynucleotides (Sigma-Genosys, Cambridge, U.K.) were used at a final concentration of 3 μM in cell culture. Oligodeoxynucleotides used were activating oligonucleotide-1 (AO-1) (5′-GCTCATGACGTTCCTGATGCTG-3′) and nonactivating oligonucleotide-1 (NAO-1) (5′-GCTCATGAGCTTCCTGATGCTG-3′) (20). IL-3 (a gift from Dr. A. Hapel, Australian National University, Canberra, Australia) stored at a concentration of 10^4 U/ml at −20°C was used at 10^3 U/ml in cell culture. PMA (Sigma-Aldrich) was stored as a stock solution (10 mg/ml) in DMSO at −70°C and used at a final concentration of 100 ng/ml.

In vitro treatment of cells and ELISAs

For all in vitro experiments, BMMs were plated out in 24-well plates at 5×10^5 cells per well in 1 ml of complete medium with or without CSF-1 (10^4 U/ml) overnight. The next morning, cells were stimulated with 100 ng/ml LPS, 3 μM CpG-containing oligonucleotide (AO-1), 3 μM control oligonucleotide (NAO-1), 10 ng/ml Pam3CysSerLys4, or medium. After 24 h (unless otherwise stated), supernatants were collected and stored at −20°C until ELISAs were performed. ELISAs were conducted using paired Abs (BD PharMingen, San Diego, CA).

Immunoblotting

BMMs (2×10^6) were plated on 60-mm dishes (Corning Life Sciences, Acton, MA) in 5 ml of medium or 5 ml of medium plus CSF-1 (10^5 U/ml) for 18 h. Culture medium was reduced to 2 ml and cells were treated as described in the figures. Cell monolayers were lysed with boiling 66 mM Tris-Cl (pH 7.4)/2% SDS/1 mM sodium vanadate/1 mM sodium pyrophosphate/1 mM sodium molybdate/10 mM sodium fluoride. Equal amounts of total protein in cell extracts were resolved by SDS-PAGE with 10% polyacrylamide seperating gels, transferred to Immobilon-P (Millipore, North Ryde, New South Wales, Australia), blocked, and probed with the anti-phospho p42/p44 MAPK rabbit polyclonal Ab (1:1000) (New England Biolabs, Beverly, MA), washed, and incubated with HRP-linked anti-rabbit IgG (1:2000) (New England Biolabs). Blots were washed and detected using ECL Plus reagents (Amersham Pharmacia Biotech, Piscataway, NJ) and Hyperfilm-ECL (Amersham Pharmacia Biotech). Membranes were then sequentially stripped with 63 mM Tris-Cl (pH 6.7)/2% SDS/100 mM 2-ME and reprobed with rabbit anti-phospho p38 (New England Biolabs), rabbit anti-p42/p44 MAPK (New England Biolabs), and rabbit anti-p38 (New England Biolabs).

Nuclear extract preparation and gel shift assays

The methods used for preparation of nuclear extracts and gel shift assays have been described previously (21). For nuclear extract production, BMMs were pretreated overnight with medium or CSF-1 (5×10^4 U/ml) and then stimulated for 45 min with AO-1 (0.3 μM) or LPS (10 ng/ml). For gel shift assays, a double-stranded oligonucleotide corresponding to an NF-κB site from the murine TNF-α promoter (5′-CAAACAGGGGGCTT TCCCTCCTC-3′) (21) was end-labeled with [γ-^{32}P]ATP using polynucleotide kinase and separated on a NAP-5 column (Amersham Pharmacia Biotech).

Total RNA isolation and quantitative PCR

Total RNA was prepared using RNAzol B (Biogenesis, Poole, U.K.) according to the manufacturer's instructions. RNA was treated with DNase 1 (Ambion, Austin, TX) and reverse transcribed to cDNA using Superscript reverse transcriptase (Life Technologies). Negative control samples (no first strand synthesis) were prepared by performing reverse transcription reactions in the absence of reverse transcriptase. cDNA levels of murine IL-12 (p40), IL-12 (p35), TNF-α, hypoxanthine phosphoribosyl transferase (HPRT), TLR1, TLR2, TLR4, TLR5, TLR6, and TLR9 were quantitated by real-time PCR using an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Amplification was achieved using an initial cycle of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 50°C for 1 min. cDNA levels during the linear phase of amplification were normalized against HPRT controls. Determinations were made in triplicate and mean ± SD was determined. Primers (f, forward; r, reverse) and 5′-6-carboxy-fluorescein-labeled/3′-6-carboxy-tetramethyl-rhodamine-labeled probes (p) used to detect expression of the corresponding murine genes were as follows: IL-12 (p40) (f, 5′-GGAATTTGGTCCACTGAAATTT TAAA-3′; r, 5′-CACGTGAACCGTCCGGAGTA-3′; p, 5′-ACAAGAC TTTCCTGAAGTGTGAAGCACCAAAT-3′); IL-12 (p35) (f, 5′-AAGA CATCACACGGGACCAAA-3′; r, 5′-CAGGCAACTCTCGTTCTTGTG TA-3′; p, 5′-CAGCACATTGAAGACCTGTTTACCACTGGA-3′); TNF-α (Applied Biosystems); TLR1 (f, 5′-TGGATGTGTCCGTCAGCACTA-3′; r, 5′-AGAGCAGCCCTGGTCTTCAA-3′; p, 5′-CACACACTTGATGTTAGA CAGTTCCAAACCGAT-3′); TLR2 (f, 5′-AAGATGCGCTTCCTGAAT TTG-3′; r, 5′-TCCAGCGTCTGAGGAATGC-3′; p, 5′-CGTTTTTACCA CCCGGATCCCTGTACTG-3′); TLR4 (f, 5′-AGGAAGTTTCTCTGGA CTAACAAGTTTAGA-3′; r, 5′-AAATTGTGAGCCACATTGAGTTTC-3′; p, 5′-GCCAATTTTGTCTCCACAGCCACCA-3′); TLR5 (f, 5′-GCA CGAGGCTTCTGCTTCA-3′; r, 5′-GCATCCAGGTGTTTGAGCAA-3′; p, 5′-CATTCTGTGCCCATTCAAAGTCTTTGCTG-3′); TLR6 (f, 5′-CTCGGAGACAGCACTGAAGTCA-3′; r, 5′-CGAGTATAGCGCCTCC TTTGAA-3′; p, 5′-ATGATAGAGCACGTCAAAAACCAAGTGTTCCT C-3′); TLR9 (f, 5′-AGGCTGTCAATGGCTCTCAGTT-3′; r, 5′-TGAA CGATTTCCAGTGGTACAAGT-3′; p, 5′-TGCCGCTGACTAATCTGC AGGTGCT-3′); HPRT (f, 5′-GCAGTACAGCCCCAAAATGG-3′; r, 5′-AACAAAGTCTGGCCTGTATCCAA-3′; p, 5′-TAAGGTTGCAAGCTT GCTGGTGAAAAGGA-3′).

PCR/Southern hybridization

Total RNA was treated with DNase 1 (Ambion), reverse transcribed to cDNA using Superscript reverse transcriptase (Life Technologies), and used as a template for semiquantitative PCR. No reverse-transcriptase negative controls were performed for all samples. Primers used were as follows: TLR9 (f, 5′-CTACAACAGCCAGCCCTTTA-3′, r, 5′-GCTGAG GTTGACCTCTTTCA-3′); TLR4 (f, 5′-AGAGAATCTGGTGGCTGT GG-3′; r, 5′-TCAACCGATGGACGTGTAAA-3′); and HPRT (f, 5′-GTTGGATACAGGCCAGACTTTGTTG-3′; r, 5′-GAGGGTAGGCTGG CCTATAGGCT-3′). PCR cycling conditions were as follows: 94°C for 30 s, 54°C for 30 s, 72°C for 60 s (21 cycles for HPRT, 21 cycles for TLR4, 21 cycles for TLR9 with BMM cDNA, or 24 cycles for TLR9 with TEPM cDNA). PCR products were separated on 1.8% agarose gels, transferred to zetaprobe nylon membrane (Bio-Rad, Hercules, CA), and subjected to Southern hybridization using cDNA probes for TLR9, TLR4, and HPRT. Probes were labeled by random priming (Amersham Pharmacia Biotech).

Results

Differential effects of CSF-1 on LPS- and CpG DNA-induced cytokine production from BMMs

To compare the effects of CSF-1 on macrophage responses to LPS and CpG DNA, we used primary BMMs, which respond well to both agents (20), and measured production of inflammatory cytokines by ELISA. Fig. 1 demonstrates that overnight pretreatment

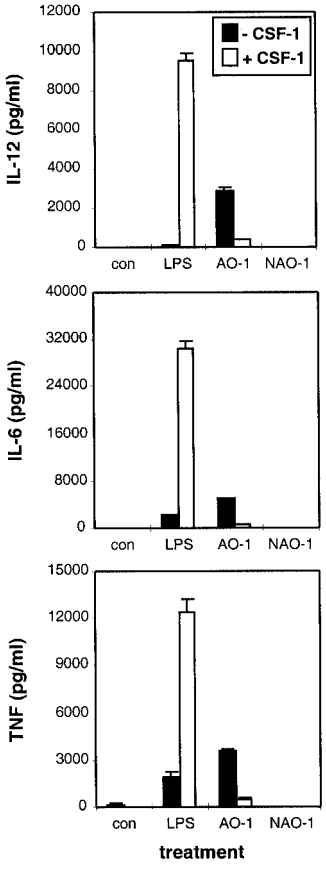

FIGURE 1. Effect of CSF-1 on BMM responses to LPS and CpG DNA. BMMs were pretreated with CSF-1 (10^4 U/ml) or were left untreated overnight. The next morning, cells were treated with medium, LPS (100 ng/ml), AO-1 (3 μM), or NAO-1 (3 μM) for 24 h. IL-12, IL-6, and TNF-α levels in supernatants were assayed by ELISA. Data are mean of triplicates ± SD. IL-6 was not detected in supernatants from control- or NAO-1-treated cells. Similar results were obtained in six independent experiments.

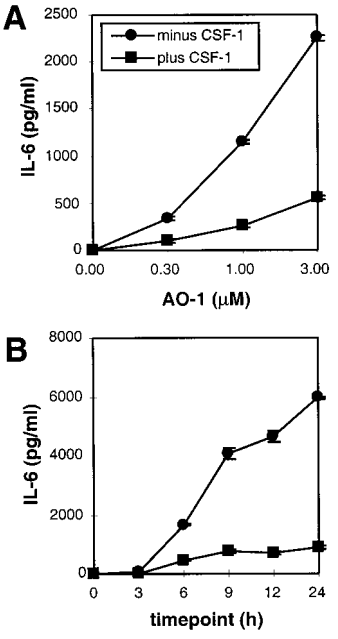

FIGURE 2. Effect of CSF-1 on dose response and time course of CpG DNA-induced IL-6 production. *A*, BMMs were pretreated overnight with medium or CSF-1 (5×10^4 U/ml) and stimulated the next day with the indicated concentrations of AO-1 for 24 h, and IL-6 levels were determined. Data points represent the mean of triplicates ± SD, and similar results were obtained in two independent experiments. *B*, BMMs were pretreated overnight with CSF-1 (10^4 U/ml) and were stimulated the next day with AO-1 (3 μM) for the period indicated. IL-6 levels were estimated by ELISA (mean of triplicates ± SD).

of BMMs with CSF-1 enhanced levels of LPS-induced IL-6, IL-12, and TNF-α protein release into the medium by 15-, 72-, and 6-fold, respectively. In direct contrast, CSF-1 pretreatment suppressed CpG DNA-induced IL-6, IL-12, and TNF-α by 10-, 8-, and 7-fold, respectively. We analyzed the effect of CSF-1 on CpG DNA responses in more detail. Fig. 2*A* shows that CSF-1 downregulated induction of IL-6 by CpG DNA in BMMs over a concentration range, whereas Fig. 2*B* shows that this effect was apparent over the length of a 24-h time course. Because IFN-γ can prime macrophage responses to both LPS and CpG DNA (21, 22), we assessed whether IFN-γ could overcome the suppressive effect of CSF-1 on the DNA response. As expected, IFN-γ pretreatment enhanced levels of LPS-induced IL-6 and IL-12 in BMMs (Fig. 3). This priming effect was not as striking in CSF-1-pretreated macrophages, suggesting that CSF-1 and IFN-γ might provide similar priming signals. Levels of CpG DNA-induced IL-6 and IL-12 were also enhanced by IFN-γ priming, but priming with IFN-γ did not overcome the suppressive effect of CSF-1 on the CpG DNA response (Fig. 3).

Next we assessed whether the effect of CSF-1 on LPS and CpG DNA responses was selective to CSF-1 or was a manifestation of its growth-stimulating activity. IL-3 and PMA can both trigger BMM proliferation (23, 24), and IL-3 is known to prime macrophage responses to LPS (25, 26). Therefore, IL-3, PMA, and CSF-1 were compared for their ability to regulate IL-6 production in response to LPS and CpG DNA. Fig. 4 shows that only CSF-1 was able to enhance the LPS response and suppress the CpG DNA response. IL-3 pretreatment primed responses to both LPS and CpG DNA, whereas PMA did not affect the LPS response but suppressed the CpG DNA response slightly (1.5- to 2-fold). Hence, the ability of CSF-1 to enhance LPS responses and suppress CpG DNA responses is unlikely to be related to its ability to trigger macrophage proliferation.

Regulation of LPS- and CpG DNA-induced TNF-α and IL-12 mRNA by CSF-1

To determine the level at which CSF-1 differentially regulates the LPS and CpG DNA response, we assessed mRNA levels of IL-12 p40 and p35 and TNF-α in response to LPS and CpG DNA with or without CSF-1 priming (Fig. 5). Priming with CSF-1 enhanced LPS-induced IL-12 (p40) mRNA levels at 4 h by ~10 fold but did not alter LPS-induced IL-12 (p35) and TNF-α mRNAs. Whereas CSF-1 was selective in priming LPS-induced cytokine mRNAs, levels of IL-12 (p40), IL-12 (p35), and TNF-α after 4 h of CpG DNA were all suppressed by priming with CSF-1 (6-, 4.5-, and 3-fold, respectively). This suppressive effect was also apparent at 2 h post-CpG DNA treatment (data not shown).

Effect of CSF-1 on LPS-induced and CpG DNA-induced NF-κB, p38, and ERK-1/2 MAPK activation

NF-κB and p38 MAPK activation are early events in triggering both LPS and CpG DNA-induced gene expression (21, 27–29). Nuclear translocation of NF-κB in response to CpG DNA but not LPS was inhibited by CSF-1 pretreatment (Fig. 6*A*). Similarly, phosphorylation of p38 in response to CpG DNA was suppressed by pretreatment with CSF-1 from the earliest time point examined (Fig. 6*B*). To determine whether CSF-1 altered the ligand dose-response curve (sensitivity) or the maximal response, the effect of

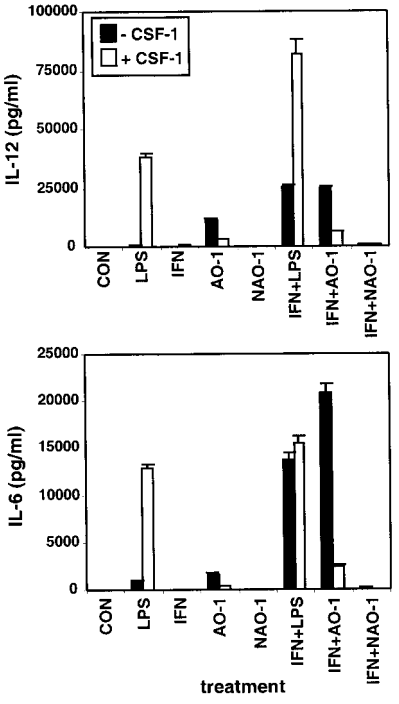

FIGURE 3. IFN-γ does not overcome the inhibitory effect of CSF-1 on CpG responses. BMMs were pretreated with CSF-1 (10^4 U/ml) or were left untreated overnight. The next morning, appropriate wells were pretreated with IFN-γ (50 U/ml) for 30 min before stimulation with LPS (100 ng/ml), AO-1 (3 μM), or NAO-1 (3 μM). Supernatants were collected after 24 h, and IL-6 and IL-12 levels were estimated by ELISA (values are mean of triplicates $\pm$ SD). IL-6 and IL-12 were not detected in supernatants from control- or NAO-1-treated cells.

CSF-1 pretreatment on p38 phosphorylation over a range of CpG DNA doses was determined at 30 min poststimulation. Fig. 7*A* demonstrates that 10-fold higher concentrations of CpG DNA were required to induce p38 phosphorylation in CSF-1-pretreated

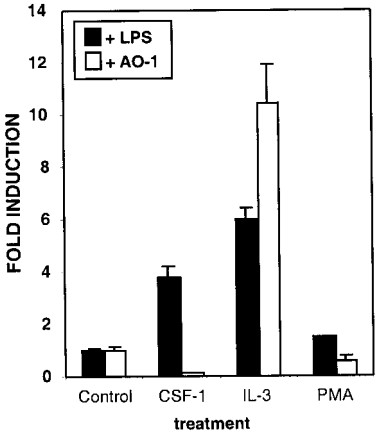

FIGURE 4. Comparison of the effects of CSF-1, IL-3, and PMA on IL-6 production in response to LPS and CpG DNA. BMMs were pretreated with CSF-1 (10^5 U/ml), IL-3 (1000 U/ml), or PMA (100 ng/ml), or were left untreated overnight. The next morning, cells were treated with medium, LPS (100 ng/ml), or AO-1 (3 μM) for 24 h. IL-6 levels in supernatants were assayed by ELISA. Results (mean of triplicates $\pm$ SD) were expressed as fold increase compared with IL-6 levels in unprimed supernatants treated with LPS (1125 $\pm$ 52 pg/ml) or AO-1 (331 $\pm$ 49 pg/ml). IL-6 was not detectable in supernatants of cells primed with medium alone, CSF-1, IL-3, or PMA and triggered with medium only (data not shown). Similar results were obtained in two experiments.

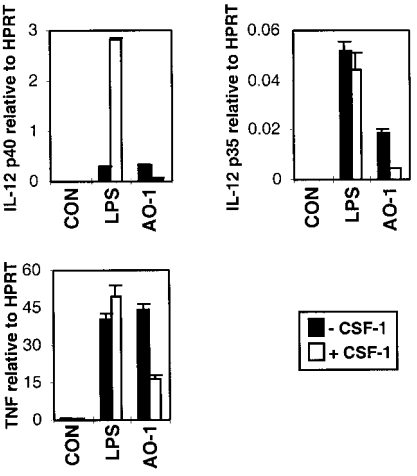

FIGURE 5. Effect of CSF-1 pretreatment on LPS- and CpG DNA-induced IL-12 (p40), IL-12 (p35), and TNF-α mRNAs. BMMs, pretreated overnight with CSF-1 (10^4 U/ml) or left untreated, were stimulated for 4 h with LPS (100 ng/ml), AO-1 (3 μM), or medium. Total cellular RNA was isolated, cDNAs were prepared, and levels of IL-12 (p40), IL-12 (p35), and TNF-α mRNA relative to HPRT were estimated by quantitative PCR (n = 3 $\pm$ SD). Similar results were obtained in two independent experiments.

cells. In contrast, LPS-induced p38 phosphorylation was not affected by CSF-1 pretreatment (Fig. 7*B*), indicating that enhancement of LPS responses by CSF-1 occurred independently of p38 activation. Phosphorylation of ERK-1/2 is also triggered by LPS and CpG DNA in BMMs (20), and we assessed the effect of CSF-1 pretreatment on CpG DNA- and LPS-triggered ERK-1 and -2 phosphorylation (Fig. 7). Because CSF-1 itself triggers sustained phosphorylation of ERK-1 and -2 in BMMs (30), basal levels of phosphorylated ERK-1 and -2 were much higher in CSF-1-pretreated BMMs than in untreated BMMs. Nonetheless, CSF-1 pretreatment blocked the ability of CpG DNA to enhance levels of phosphorylated ERK-1 and -2 over the concentration range examined (Fig. 7*A*), whereas LPS was still able to activate ERK-1/2 even in the presence of CSF-1 (Fig. 7*B*). Furthermore, the extent of the LPS-mediated ERK-1/2 phosphorylation was not altered by CSF-1 pretreatment, despite the elevated basal activation state (Fig. 7*B*). These data suggest that CSF-1 alters an early stage

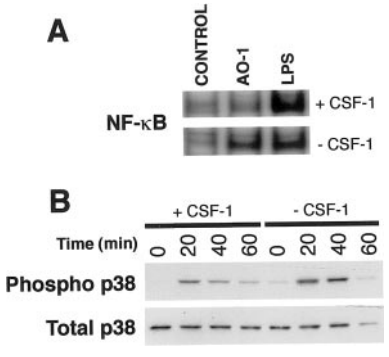

FIGURE 6. Effect of CSF-1 on CpG DNA-induced NF-κB and p38 MAPK activation. *A*, BMMs were pretreated with medium or CSF-1 overnight and were stimulated the next morning with AO-1 or LPS for 45 min. Levels of NF-κB in nuclear extracts were assessed by gel shift assay. *B*, BMMs, pretreated overnight with medium or CSF-1, were stimulated with AO-1 (3 μM) for the time interval indicated. Cell extracts were prepared, and phosphorylated p38 levels were assessed by Western blotting. Blots were stripped and reprobed for total p38 as a loading control. Similar results were obtained in two independent experiments.

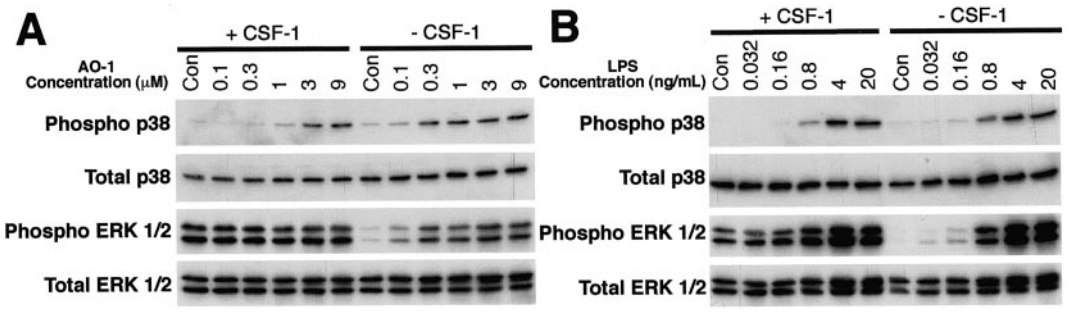

FIGURE 7. Effect of CSF-1 pretreatment on CpG DNA- and LPS-induced MAPK p38 and ERK-1 and -2 phosphorylation. *A*, BMMs, pretreated overnight with medium control or CSF-1, were stimulated with AO-1 over the concentration range indicated for 30 min. Cell lysates were prepared and levels of phosphorylated p38, total p38, phosphorylated ERK-1/2, and total ERK-1/2 were assessed by Western blotting. *B*, BMMs, pretreated overnight with medium control or CSF-1, were stimulated with a range of LPS concentrations for 30 min. Extracts were analyzed as in *A*. Similar results were obtained in two independent experiments.

of CpG DNA recognition but acts more distally to activate LPS responses.

The effect of CSF-1 on expression of TLR family members

Mice deficient for TLR9 are unable to respond to CpG-containing phosphorothioate DNA (10). Whether TLR9 is required for uptake of DNA, directly recognizes CpG DNA, or lies downstream in the recognition pathway is yet to be determined (31). The diminished CpG responsiveness observed above hints at a reduction in receptor expression or affinity in response to CSF-1. Therefore, we assessed the effect of CSF-1 on expression of TLR9 mRNA in BMMs. Indeed, CSF-1 treatment resulted in a 20-fold reduction in TLR9 mRNA levels in BMMs (Fig. 8*A*). To assess the specificity of this response, we examined the effect of CSF-1 on expression of other TLR family members, because these receptors are instrumental in triggering cellular responses to other bacterial products, including LPS, peptidoglycan, and bacterial lipoproteins. Overnight treatment with CSF-1 did not significantly affect mRNA levels of the LPS receptor TLR4 (Fig. 8*B*). Levels of TLR5 mRNA were also unaffected by CSF-1, but mRNA levels of TLR1, TLR2, and TLR6 were all suppressed by CSF-1 treatment (2.4-, 4.8-, and 4-fold, respectively). Hence, CSF-1 has a selective effect on expression of different TLR family members. The effect of CSF-1 was most pronounced on TLR9 expression (20-fold or greater reduction), but the moderate suppression of TLR2 and TLR6 mRNA levels by CSF-1 (4- to 5-fold) implies that responses to bacterial lipopeptides, peptidoglycan, and other TLR2 agonists might also be modulated by CSF-1. Therefore, we tested the ability of CSF-1 to regulate IL-6 production from BMMs in response to a synthetic lipopeptide, Pam3CysSerLys4, that is known to act through TLR2 (32). Fig. 8*C* shows that although CSF-1 pretreatment clearly suppressed IL-6 production in response to CpG DNA, it had no effect on the response to Pam3CysSerLys4. Hence, although CSF-1 can down-regulate the level of TLR2 mRNA, the effect appears to be insufficient to modulate IL-6 production in response to a TLR2 agonist.

Regulation of TLR9 expression in macrophages by CSF-1

Next, we analyzed the effect of other agents that cause BMM proliferation on TLR9 expression. Levels of TLR9 mRNA were assessed in BMMs treated overnight with medium, CSF-1, IL-3, or PMA. Fig. 9*A* demonstrates that only CSF-1 was able to dramatically regulate TLR9 expression; IL-3 did not alter levels of TLR9 mRNA, and PMA, which down-regulated CpG responses ~1.5- to 2-fold (Fig. 4), had a similar effect on TLR9 expression. To assess the time course of CSF-1 down-regulation of TLR9 expression, BMMs starved overnight of CSF-1 were treated with CSF-1 over a 20-h time course. Maximal suppression of TLR9 expression occurred between 4 and 8 h, and an effect was apparent by 1 h post-CSF-1 (Fig. 9*B*).

Although the effects of CSF-1 on BMMs are clearly dissociated from its growth-promoting effects, these cells are unusual in that mature macrophages in vivo are generally not actively proliferating. Therefore, we analyzed the effect of CSF-1 on expression of TLR9 in TEPMs, which are postmitotic. We have found that, although freshly isolated TEPMs respond well to CpG DNA, they rapidly lose responsiveness to CpG DNA but retain LPS responses when cultured ex vivo on either tissue culture or bacterial plastic (data not shown). In keeping with this pattern, TLR9 mRNA levels were 20- to 50-fold less in cultured TEPMs than CSF-1-starved BMMs (data not shown). Nonetheless, this low basal level of TLR9 expression was still regulated by CSF-1. Overnight treatment of TEPMs with CSF-1 further down-regulated expression of TLR9 but did not alter levels of TLR4 compared with control cells (Fig. 9*C*).

Discussion

LPS and CpG DNA have differing toxicities in vivo. Administration of LPS can lead to fever, shock, and multiorgan failure resulting in death. There are no reports of toxicity of bacterial DNA alone, although pretreatment of mice with *Escherichia coli* DNA can enhance the toxicity of LPS in vivo (33), probably by the induction of IFN-γ. Even phosphorothioate-stabilized CpG oligonucleotides have minimal toxicity (4). The relative nontoxicity of CpG DNA is an attractive feature for its use as a vaccine adjuvant and for other therapeutic strategies.

Analysis of macrophage gene expression in vitro has suggested that the differing toxicities of LPS and CpG DNA may be due to both qualitative and quantitative differences in cytokine gene induction. For example, CpG DNA was a relatively poor stimulus for IL-1β (21), and LPS but not CpG DNA stimulated NO production from macrophages without IFN-γ priming (21, 22). However, the situation in vivo is likely to be more complex due to the presence of many other cytokines, which will modify the responses to LPS and CpG DNA. Here, we have found that CSF-1, which is present constitutively in vivo and is a macrophage growth and survival factor, differentially affects the responses to LPS and CpG DNA. In the presence of CSF-1, the LPS response was elevated and the CpG DNA response suppressed so that LPS became far more effective than CpG DNA at stimulating IL-6, IL-12, and TNF-α production from BMMs (62-, 27-, and 23-fold, respectively).

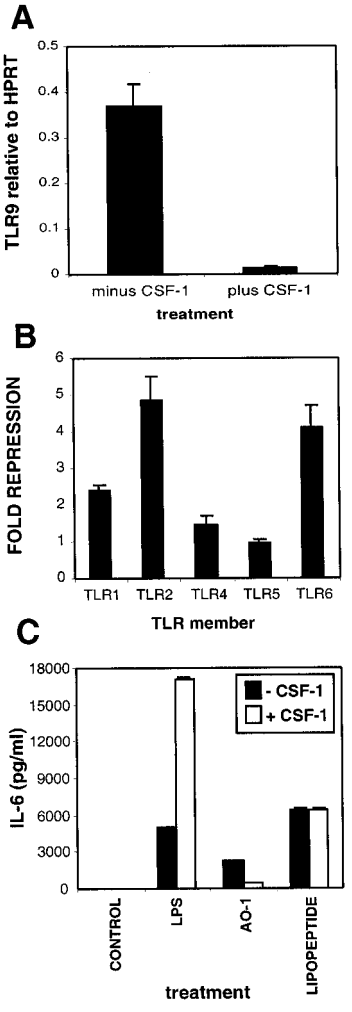

FIGURE 8. Effect of CSF-1 on expression of TLRs in BMMs. *A*, BMMs were treated overnight with medium or CSF-1. Sixteen hours later, total cellular RNA was isolated, cDNAs were prepared, and levels of TLR9 mRNA relative to HPRT were estimated by quantitative PCR ($n = 3 \pm$ SD). Results are representative of two experiments. *B*, cDNAs were prepared as in *A*, and levels of TLR1, 2, 4, 5, and 6 relative to HPRT were determined. Data from two experiments were pooled, and results ($n = 6 \pm$ SEM) are expressed as fold repression in response to CSF-1. *C*, BMMs were pretreated overnight with medium or CSF-1 (5×10^4 U/ml) and then stimulated for 24 h with LPS (10 ng/ml), AO-1 (3 μM), or Pam3CysSerLys4 (10 ng/ml). IL-6 levels in culture supernatants were assessed by ELISA (mean of triplicates $\pm$ SD). Similar results were obtained in two experiments.

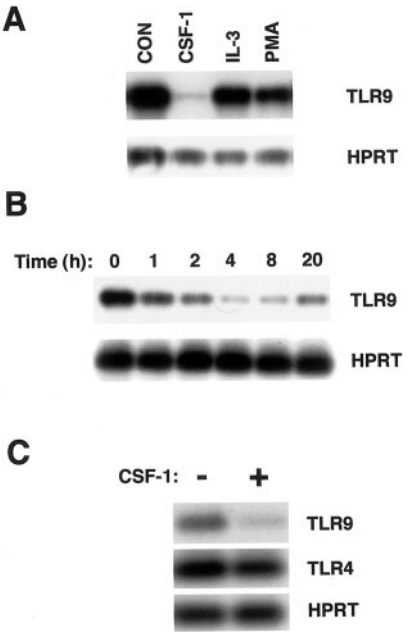

FIGURE 9. Regulation of TLR9 expression in macrophages. *A*, BMMs were treated for 18 h with medium, CSF-1 (5×10^4 U/ml), IL-3 (10^3 U/ml), or PMA (100 ng/ml). Total RNA was prepared and levels of TLR9 and HPRT mRNA were assessed by PCR/Southern hybridization. *B*, BMMs were starved of CSF-1 overnight and then treated with CSF-1 (5×10^4 U/ml) for the indicated times. Expression levels of TLR9 and HPRT were assessed as in *A*. *C*, TEPMs were cultured for 20 h with CSF-1 (5×10^4 U/ml) or medium alone. Levels of TLR9, TLR4, and HPRT mRNAs were estimated by PCR/Southern hybridization in total RNA from these cells. Similar results were obtained in two independent experiments.

The ability of CSF-1 to selectively enhance the LPS and suppress the CpG DNA response of BMMs is unlikely to be related to its activity as a growth factor. IL-3 and PMA did not have selective effects on LPS and CpG DNA responses; IL-3 pretreatment primed BMMs for enhanced IL-6 production in response to both LPS and CpG DNA, whereas PMA pretreatment did not have significant effects on LPS-induced IL-6 and modestly suppressed CpG DNA-induced IL-6 synthesis. Furthermore, IL-3 and PMA had little effect on TLR9 expression, whereas CSF-1 markedly suppressed expression of this molecule. IL-3 was actually more effective at priming CpG DNA responses than LPS responses. Although the ability of IL-3 to synergize with LPS for macrophage activation has been documented (25, 26), its ability to regulate responses to CpG DNA has not been reported. Given that CpG DNA drives strong Th1 responses in vivo and that IL-3 is a product of activated T cells, IL-3 may be involved in amplification of responses to CpG DNA in vivo, as has been suggested for IFN-γ (22).

Because concentrations of CSF-1 are markedly and rapidly enhanced in serum, spleen, liver, lung, and kidney after LPS administration (13) and during infection (12, 34), macrophages recruited to the site of infection during a bacterial challenge would be expected to have an impaired response to CpG DNA. The implications of this are not obvious, because the role of bacterial DNA in an infection is not clear and will remain so until experiments using bacterial infection models in *TLR9* gene-targeted mice are performed. Intact bacterial pathogens do not display their DNA, and detection of CpG DNA during a bacterial challenge may imply that the host has successfully destroyed the invading organism. In this case, CSF-1 may be important in dampening down inappropriate inflammatory responses to bacterial DNA. In contrast, both LPS and CpG DNA rapidly down-regulate cell surface expression of the CSF-1R in BMMs (20). Hence, macrophages present at the site of infection will have already encountered bacterial cell wall products such as LPS and are unlikely to be CSF-1 responsive. Such cells therefore may be hypersensitive to the effects of CpG DNA. Consistent with this model, we have found that LPS and CpG DNA can synergize for IL-6 and IL-12 production from BMMs (data not shown).

TLR9 is an essential component of the DNA response (10), and the ability of human and murine TLR9 to confer species-specific responses to different CpG-containing phosphorothioate-stabilized CpG oligonucleotides is suggestive of a role in direct recognition (35). The expression of TLR9 was profoundly reduced by CSF-1, which provides a clear explanation for the suppression of bacterial DNA responses by CSF-1. CSF-1 also moderately suppressed TLR1, 2, and 6 mRNA levels in BMMs, but surprisingly did not

regulate IL-6 production in response to the TLR2 ligand Pam3CysSerLys4 (Fig. 8*C*). This may be because the level of down-regulation of these TLR family members by CSF-1 is not sufficient to regulate responses to their ligands. Another possibility is that stimulation with TLR2 agonists may up-regulate TLR2 expression, which can in turn overcome the suppressive effect of CSF-1. In this respect, we have found that CpG DNA does up-regulate TLR9 mRNA levels in BMMs, but only in the presence of CSF-1 (data not shown), although this autocrine regulation is apparently insufficent to overcome the suppressive effect of CSF-1 on CpG DNA responses. It would be interesting to assess the effect of TLR2 ligands on TLR2 expression in macrophages in the presence and absence of CSF-1. Indeed, a variety of inflammatory stimuli, including LPS, selectively up-regulated TLR2 but not TLR4 expression (36, 37). The differential regulation of TLR members implies that, as in drosophila, different kinds of pathogens could elicit different outcomes. In keeping with this view, Sing et al. have reported that Gram-negative organisms induce IFN-β, whereas Gram-positive bacteria lack this activity (38).

Although the ability of CSF-1 to prime murine macrophage responses to LPS has previously been reported for IL-6 and TNF-α production (14, 15), its effect on LPS-induced IL-12 production has not been documented. In the case of IL-6, synergy between CSF-1 and LPS might be partially due to CSF-1-induced GM-CSF production (39). We have not fully investigated the mechanism by which CSF-1 augments LPS responses of BMMs. We did find that CSF-1 did not affect levels of LPS-induced TNF-α mRNA, despite having a marked effect on TNF-α protein secretion, implying that posttranscriptional mechanisms are responsible for this effect. In support of this, p38 phosphorylation and nuclear translocation of NF-κB in response to LPS were not altered by CSF-1 pretreatment. In the case of IL-12, LPS-induced p40, but not p35, mRNA was enhanced by CSF-1 priming. Whether the effect of CSF-1 on LPS-induced IL-12 (p40) mRNA is due to enhanced transcription or enhanced mRNA stability has not yet been investigated. One possibility is that CSF-1 and LPS synergize at the level of transcription because CSF-1 triggers sustained phosphorylation and activation of Ets-2 (30) and Ets-2 is necessary for full activation of the IL-12 promoter in response to LPS (40, 41).

The majority of our findings used BMMs as a primary macrophage model. We have found that TEPMs cultured ex vivo retain responsiveness to LPS but lose responsiveness to CpG DNA. Because of this decline in CpG DNA responsiveness ex vivo, we have been unable to address the effect of CSF-1 pretreatment on CpG responses in TEPMs. Nonetheless, the low basal expression of TLR9 in TEPMs was further down-regulated by overnight treatment with CSF-1 (Fig. 9), implying that this phenomenon is likely to occur with all CSF-1-responsive macrophage populations. In summary, CSF-1 reprograms macrophage responses to different microbial stimuli. To our knowledge, this is the first report of a cytokine or growth factor that has differential effects on macrophage responses to LPS and CpG DNA, and it highlights the importance of regulated expression of TLRs. Discordant regulation of TLRs may underlie different toxicities of TLR agonists in vivo and may have relevance for the role of CSF-1 during bacterial infections. Furthermore, CSF-1 and CSF-1R antagonists may enhance the efficacy of CpG DNA in therapeutic strategies and/or increase its toxicity in vivo.

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