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Bacterial/CpG DNA Down-Modulates Colony Stimulating Factor-1 Receptor Surface Expression on Murine Bone Marrow-Derived Macrophages with Concomitant Growth Arrest and Factor-Independent Survival

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Unmethylated CpG motifs within bacterial DNA constitute a pathogen-associated molecular pattern recognized by the innate immune system. Many of the immunomodulatory functions of bacterial DNA can be ascribed to the ability to activate macrophages and dendritic cells. Here we show stimulatory DNA, like LPS, caused growth arrest of murine bone marrow-derived macrophages proliferating in CSF-1. Stimulatory DNA caused selective down-modulation of CSF-1 receptor surface expression. Flow cytometric analysis of CSF-1-deprived bone marrow-derived macrophages revealed that in contrast to the synchronous reduction of CSF-1 receptor upon CSF-1 addition, activating DNA (both bacterial DNA and CpG-containing oligonucleotide) caused rapid removal of receptor from individual cells leading to a bimodal distribution of surface expression at intermediate times or submaximal doses of stimulus. Despite causing growth arrest, both stimulatory DNA and LPS promoted factor-independent survival of bone marrow-derived macrophages, which was associated with phosphorylation of the mitogen-activated protein kinase family members, extracellular-regulated kinase 1 and 2. CSF-1 receptor down-modulation may polarize the professional APC compartment to the more immunostimulatory dendritic cell-like phenotype by suppressing terminal macrophage differentiation mediated by CSF-1. The Journal of Immunology, 1999, 163: 6541–6550.

Recognition by the innate host defense systems of conserved bacterial products including LPS, peptidoglycan, and lipoteichoic acids, or viral products such as dsRNA, results in an acute response that serves as a first line of defense against an invading pathogen. Such recognition of pathogen-associated molecular patterns serves to prime the adaptive immune response by activating professional APC function (1, 2). DNA has recently been identified as another pathogen molecule recognized by the innate immune system (3). Sequences present in bacterial DNA containing a core unmethylated CpG dinucleotide in particular sequence contexts are immunostimulatory (4). The frequency of these sequences is substantially reduced in the mammalian genome, and, where present, the CpG motif is preferentially methylated, rendering it inactive (3, 5).

In monocytes, macrophages, and dendritic cells (DC),4 stimulatory DNA induces a range of cytokine genes such as TNF-α (6, 7), IL-1β (7), IL-6 (6, 8), and IL-12 (9). In spleen cell culture, the macrophage production of IL-12 leads to rapid production of IFN-γ by NK cells and T lymphocytes (9, 10). IFN-γ, in turn, primes murine macrophages to respond to activating DNA by enhancing a number of responses, thereby creating a self-amplifying loop with NK cells and T cells (11). A similar augmentation of responses in B lymphocytes has been attributed to NK and T cell-derived IFN-γ in mixed cultures (12).

When stimulatory DNA is administered in vivo, the nature of cytokines induced causes a general polarization of development of T cells toward the Th1 phenotype. This has opened the possibilities of using CpG-containing oligonucleotides (CpG DNA) as immunotherapy for conditions such as allergy (13–15) and as a Th1-promoting adjuvant for vaccinations (16, 17). The adjuvant effect of CpG DNA promotes the production of the Th1-associated Ig isotype IgG2a and strong cytotoxic T lymphocyte responses in vivo (8, 18–22). Stimulatory DNA has also been shown to enhance APC function in DC models through up-regulation of MHC class II and costimulatory molecules (21, 23).

Internalization of DNA into an acidified endosomal compartment appears to be a prerequisite for the recognition of and response to stimulatory DNA. Chloroquine and related compounds that interfere with endosomal acidification and function selectively block the actions of stimulatory DNA on B cells and macrophages without preventing activation by LPS (24, 25). Signaling, presumably initiated from an unidentified intracellular receptor, has been shown to activate the mitogen-activated protein kinases (MAPK) p38 kinase and c-Jun N-terminal kinase (JNK) (26, 27), as well as...
inducing or activating a range of transcription factors including NF-κB (6, 7, 25, 28), the AP-1 component Jun (26, 27, 29), ATF-2 (26, 27), Ets-2, and C/EBP-β and 6 (30). The induction of these transcription factors and signaling molecules as well as the cytokine profile induced by bacterial DNA is similar to the response to LPS.

B cells, like myeloid cells, respond to stimulatory DNA. B cells treated with bacterial DNA or CpG DNA exhibit enhanced proliferation, polyclonal Ig secretion, and IL-6 production (4). DNA is also cytoprotective, preventing spontaneous and Fas-dependent cell death of primary B cells (29, 31) and apoptosis induced by IgM cross-linking in a B lymphoma cell line (28, 32, 33). The mitogenic effect of stimulatory DNA on murine B cells is another response in common with LPS. By contrast, in macrophages LPS is an inhibitor of cell proliferation and causes rapid down-regulation of cell-surface binding of the major growth factor for the macrophage lineage, M-CSF, otherwise known, and referred to here, as CSF-1 (34–36). CSF-1 elicits its effects through a single high-affinity receptor (CSF-1 receptor) the product of the c-fms protooncogene. CSF-1 is required for survival, proliferation, differentiation, and inducible gene expression in cells of the macrophage lineage. Its absence in the op/op mouse, a natural mutant, leads to gross deficiencies in a subclass of macrophages (37, 38). CSF-1-deficient op/op mice have relatively normal numbers of myeloid DC (39, 40), and there is substantial evidence that down-modulation of CSF-1 receptor and loss of CSF-1 signal is important to commitment to DC-like Ag-presenting phenotype (41, 42). In this paper, we have compared the effects of LPS and stimulatory DNA on macrophage cell-surface CSF-1 receptor levels, CSF-1-induced proliferation, and survival. As a model system, we have utilized macrophages derived from cultivation of murine bone marrow in recombinant CSF-1. Bone marrow-derived macrophages (BMM) have a mature macrophage phenotype and have been used extensively in studies of CSF-1 signaling (34, 38, 43, 44). Our results suggest that the presence of pathogen DNA during an infection could enhance priming of the adaptive immune response by promoting the survival of APC and changing the course of differentiation of cells of the mononuclear phagocyte lineage.

**Materials and Methods**

**Culture conditions and reagents**

BMM were prepared by cultivation of CD1 outbred mouse femoral bone marrow cells in recombinant CSF-1 (a gift from Chiron, Emeryville, CA) and harvested after 6–8 days as described previously (45). Elicited peritoneal macrophages were obtained by injecting CD1 mice i.p. with 1 ml of 10% thioglycollate broth followed by peritoneal lavage with 5–10 ml of calcium-magnesium-free PBS 4 days later. Cells were cultured in phenol red-containing or phenol red-free RPMI 1640 medium (Life Technologies, Gaithersburg, MD), supplemented with 10% heat-inactivated FCS (Serum Supreme; BioWhittaker, Walkersville, MD), 2 mM L-glutamine (Glu-tamax-1, Life Technologies), and 20 μM penicillin and 20 μg/ml streptomycin (Life Technologies).

*Escherichia coli* DNA (ICN Pharmaceuticals, Costa Mesa, CA), calf thymus DNA (Sigma, St. Louis, MO), and salmon sperm DNA (Sigma) were treated with RNase, followed by repeated phenol-chloroform extraction and ethanol precipitation. Phosphodiester oligodeoxynucleotides, activating ODN-1, and nonactivating ODN-1 were purchased from Pacific Oligos (Lismore, Australia). *Salmonella minnesota* Re 595 LPS (Sigma) was prepared as described previously (30). For digestion of DNA with DNase I, 200–500 μg of DNA at a final concentration of 1 ng/ml was incubated with 30 U of RNase-free DNase I (Boehringer Mannheim, Mannheim, Germany) in 10 mM Tris-Cl, 10 mM MgCl2, and 1 mM DTT at 37°C for 6–10 h. Complete digestion was confirmed with agarose gel electro-

**EFFECTS OF BACTERIAL/CpG DNA ON MACROPHAGES**

- **MTT assay for cell viability/proliferation**
  - A measure of the number of viable cells was obtained by incubation with MTT, which is cleaved to an insoluble blue product by the mitochondrial enzyme succinate dehydrogenase as detailed previously (45).

**Cell cycle analysis**

To assess cell cycle stage, 10° cells were treated as indicated, harvested, and processed as outlined in Vadiveloo et al. (47). Briefly, cells were resuspended thoroughly in 50 μL PBS, and 1 ml of ice-cold 70% ethanol was added. Cells were left overnight at 4°C, pelleted, and resuspended in 0.5 ml of a staining solution containing 69 μM propidium iodide (PI) and 5 μg/ml RNase A in 38 mM sodium citrate. Stained cells were analyzed using a FACScalibur (Becton Dickinson, San Diego, CA) flow cytometer. All data analysis was conducted using CellQuest (ver. 3.1) (Becton Dickinson).

**Immunocytochemistry**

BMM were harvested, cultivated overnight on coverslips in the absence of CSF-1, treated with desired stimuli, and fixed with methanol for 5 min at −20°C. Fixed cells were permeabilized with 0.1% Triton X-100 in PBS, washed three times with PBS, then blocked with 0.5% BSA in PBS for 15 min. Coverslips were incubated with anti-CSF-1 receptor mAb AF598 for 90 min, then Cy3-labeled F(ab′)2 goat anti-rat IgG secondary Ab (The Jackson Laboratory, Bar Harbor, ME) with washing in PBS between each step.

**Flow cytometric analysis of cell-surface molecules**

BMM or thioglycollate-elicited peritoneal macrophages (TEPM) (2 × 10⁶) were harvested after indicated treatments and washed once in ice-cold PBS containing 0.1% BSA and 0.1% NaN₃. Cells were then blocked with 0.1% goat serum for 15 min, followed by incubation with primary Ab for 30 min. Cells were then washed with PBS containing 0.1% BSA and 0.1% NaN₃, and secondary Abs were added and incubated with secondary Ab for 30 min. Cells were washed with PBS containing 0.1% BSA and 0.1% NaN₃, resuspended, and analyzed by flow cytometry using a FACScalibur (Becton Dickinson).

**Apoptosis analysis**

BMM (1–2 × 10⁶) were washed and plated in 3 ml phenol red-free RPMI 1640 in 60-mm bacteriological plastic dishes (Greiner, Nuringen, Germany) and starved of CSF-1 for 18 h. At 18 h, BMM were treated with effectors for a further 48 h and assayed for cell viability. The proportion of apoptotic cells was determined using a previously described method that differentiates apoptotic from necrotic cells (48) based on differential kinetics of PI uptake (49). Cells were incubated in RPMI 1640 media supplemented as described earlier, containing 40 μg/ml PI for 2 h at 37°C, then were harvested and analyzed by flow cytometry using a FACScalibur (Becton Dickinson).

**Immunoblotting**

BMM (10⁶) were starved of CSF-1 for 18 h and then treated with effectors as described in the figure legends. Cell monolayers were rinsed twice with ice-cold 66 mM Tris-Cl (pH 7.4) 1 mM sodium vanadate and then lysed with boiling 66 mM Tris-Cl (pH 7.4)/2% SDS/1 mM sodium vanadate. Cell extracts were then homogenized by repeated passage through a 26-gauge hypodermic needle, centrifuged, and supernatants were stored at −70°C. Proteins were resolved by SDS-PAGE with 10% polyacrylamide-resolving gels, transferred to Hybond C nitrocellulose (Amersham Pharmacia Biotech, Piscataway, NJ), blocked and probed with the anti-phospho p42/44 MAPK mAb E10 (1:1000)(New England Biolabs, Boston, MA), washed, and incubated with HRP-linked anti-mouse IgG (1:2000) (New England Biolabs). Blots were washed and detected using an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech) and Hyperfilm-ECL (Amersham Pharmacia Biotech). Membranes were
stripped with 63 mM Tris-Cl (pH 6.7)/2% SDS/100 mM 2-ME then re-probed with a rabbit polyclonal Ab against p42/p44 MAPK (New England Biolabs), washed and incubated with HRP-linked anti rabbit IgG (1:2000) (New England Biolabs), washed, and detected.

Results

E. coli DNA mimics LPS by causing growth inhibition

E. coli DNA, like LPS, is growth inhibitory for murine BMM proliferating in the presence of CSF-1, as demonstrated by a colorimetric-based cell viability/proliferation assay (Fig. 1A). Growth inhibition was specific for E. coli DNA, as vertebrate DNA such as that from calf thymus or salmon sperm was not active (Fig. 1A). The growth inhibitory effect of the E. coli DNA was not due to the presence of contaminating LPS, as DNase I digestion abolished activity. Direct counting of cell numbers confirmed results obtained using the colorimetric assay (Fig. 1B). To extend these observations, cell-cycle analysis was conducted on BMM synchronized in G_0 by CSF-1 deprivation for 18 h and treated with either LPS (100 ng/ml) or E. coli DNA at 10 μg/ml (EC DNA), DNase I-treated EC DNA at 10 μg/ml (DNased EC DNA), salmon sperm DNA at 10 μg/ml (SS DNA), or calf thymus DNA at 10 μg/ml (CT DNA) for 24 h. Cells were then harvested for cell-cycle analysis as described in Materials and Methods. A, Treatment of murine BMM with E. coli DNA results in growth inhibition. A, BMM (2 × 10^5) were grown in the presence of CSF-1 (10^4 U/ml) for 18 h then left to proliferate with no further addition (Control) or treated with either E. coli DNA at 10 μg/ml (EC DNA), calf thymus DNA at 10 μg/ml (CT DNA), salmon sperm DNA at 10 μg/ml (SS DNA), DNase I-treated EC DNA at 10 μg/ml (DNased EC DNA), or LPS (100 ng/ml). After treatment for 48 h, cellular proliferation was measured using a MTT assay as outlined in Materials and Methods. Data is represented as the mean and SD of triplicates and is representative of three experiments performed. B, BMM (1 × 10^5) cells were grown in the presence of CSF-1 (10^4 U/ml) for 18 h and then treated as outlined in A. Proliferation was measured by cell counting at times indicated. Data represents the mean and SD of triplicates and is representative of two independent experiments.

FIGURE 1. Treatment of murine BMM with E. coli DNA results in growth inhibition. A, BMM (2 × 10^5) were grown in the presence of CSF-1 (10^4 U/ml) for 18 h then left to proliferate with no further addition (Control) or treated with either E. coli DNA at 10 μg/ml (EC DNA), calf thymus DNA at 10 μg/ml (CT DNA), salmon sperm DNA at 10 μg/ml (SS DNA), DNase I-treated EC DNA at 10 μg/ml (DNased EC DNA), or LPS (100 ng/ml). After treatment for 48 h, cellular proliferation was measured using a MTT assay as outlined in Materials and Methods. Data is represented as the mean and SD of triplicates and is representative of three experiments performed. B, BMM (1 × 10^5) cells were grown in the presence of CSF-1 (10^4 U/ml) for 18 h and then treated as outlined in A. Proliferation was measured by cell counting at times indicated. Data represents the mean and SD of triplicates and is representative of two independent experiments.

FIGURE 2. Stimulatory DNA blocks BMM from entering S phase of the cell cycle. A, BMM (2 × 10^6) synchronized in G_0 by CSF-1 deprivation for 18 h were treated with CSF-1 (10^4 U/ml) alone (Control) or CSF-1 and either LPS (100 ng/ml), E. coli DNA at 10 μg/ml (EC DNA), DNase I-treated EC DNA at 10 μg/ml (DNased EC DNA), calf thymus DNA at 10 μg/ml (CT DNA), or salmon sperm DNA at 10 μg/ml (SS DNA) for 24 h. Cells were then harvested for cell-cycle analysis as described in Materials and Methods. B, Quantification of cells in S or G_2/M phases of the cell cycle after treatment with effectors. This experiment was repeated three times with similar results.
Stimulatory DNA down-modulates CSF-1 receptor surface expression

The ability of LPS to reduce CSF-1 surface binding to macrophages is well documented (34–36). The availability of a high-affinity mAb directed toward the extracellular domain of the murine CSF-1 receptor allowed the investigation of the effects of CSF-1, stimulatory DNA, and LPS on CSF-1 receptor localization. BMM deprived of CSF-1 for 18 h exhibited surface expression of CSF-1 receptor concentrated on the leading edge of labeled cells (Fig. 3). Treatment with CSF-1, *E. coli* DNA, or LPS resulted in internalization of the CSF-1 receptor to a perinuclear region within the cell (Fig. 3). CSF-1 treatment resulted in rapid polar spreading of cells, with individual cells commonly producing a single extension many cell diameters in length. Both DNA and LPS also caused profound spreading. With time, *E. coli* DNA and LPS treatment resulted in many cells spreading to resemble “fried eggs” distinct from the polar spindle-like morphology exhibited by CSF-1-treated cells.

To investigate receptor down-modulation by stimulatory DNA and LPS further, flow cytometric analysis of CSF-1 receptor surface expression levels on BMM was conducted. Fig. 4 confirms high-level surface expression following CSF-1 starvation and down-modulation by *E. coli* DNA, LPS, and CSF-1 after treatment for 1 h. Vertebrate DNA or DNase-treated *E. coli* DNA did not affect surface expression of CSF-1 receptor (Fig. 4). Down-modulation by *E. coli* DNA was specific for the CSF-1 receptor in that

**FIGURE 3.** Internalization of CSF-1 receptor after treatment with stimulatory DNA and LPS. BMM were seeded onto coverslips and deprived of CSF-1 overnight. Cells were then left untreated (control), treated with either CSF-1 (10⁴ U/ml), *E. coli* DNA (10 μg/ml), or LPS (100 ng/ml) for 1 h. Cells were then permeabilized and immunostained for the CSF-1 receptor as outlined in Materials and Methods.

**FIGURE 4.** Stimulatory DNA causes down-modulation of CSF-1 receptor surface expression in BMM. A total of 2 × 10⁶ BMM were deprived of CSF-1 for 18 h (Control), followed by treatment with either CSF-1 (10⁴ U/ml), *E. coli* DNA (EC DNA) at 10 μg/ml, LPS at 100 ng/ml, DNase I-treated *E. coli* DNA (DNased EC DNA) at 10 μg/ml, calf thymus DNA (CT DNA) at 10 μg/ml, or salmon sperm DNA (SS DNA) at 10 μg/ml for 1 h. Cells were harvested and stained for CSF-1 receptor surface expression and then analyzed by flow cytometry as described in Materials and Methods. Shaded histograms display treatments, while dashed histograms display respective secondary Ab only controls. Bold lined unshaded histograms represent expression levels of CSF-1 receptor on untreated cells for comparison. Similar results were obtained in four separate experiments.
neither the complement receptor 3 component CD11b nor the macrophage-restricted glycoprotein F4/80 surface expression were affected (Fig. 5).

**Stimulatory DNA and LPS down-modulation of CSF-1 receptor at the single-cell level occurs in an all-or-nothing manner**

CSF-1 treatment resulted in a continual decrease of CSF-1 receptor levels on individual cells within the population, as reflected by a synchronous decrease in the mean fluorescence intensity of the entire population with time (Fig. 6). By contrast, treatment with *E. coli* DNA resulted in a bimodal distribution of surface receptor expression (Fig. 6), indicative of an “all-or-nothing” response at the level of the individual cell. The actions of bacterial DNA on macrophages can be mimicked by CpG DNA, oligodeoxynucleotides (ODN) containing activating CpG motifs. In a separate experiment the specificity and bimodality of this response for defined stimulatory motifs was clearly evident using activating (CpG) phosphodiester ODN (Fig. 7), while no response to nonactivating ODN (GpC) was observed. The bimodal regulation of CSF-1 receptor surface expression was also evident upon treatment with substimulatory doses of CpG ODN or LPS, where increasing the dose resulted in a greater proportion of cells with down-modulated surface expression (data not shown).

The extent and speed of CSF-1 receptor down-modulation in response to *E. coli* DNA, CpG ODN, or LPS varied between experiments, with some showing complete down-modulation of receptor to background staining levels (Fig. 7) or to levels similar to those observed after treatment with CSF-1 (Figs. 4 and 6). The source of this variation is unknown; it may be that in some experiments cells proceed to a differentiation state in which receptors are no longer susceptible to down-modulation. CSF-1 treatment consistently resulted in a down-modulation of receptor to a level 5- to 10-fold above background, a steady-state situation in which there is continual recycling of the receptor to the surface balanced by ligand-mediated internalization (37).

To address the possibility that macrophages can progress to a state where they become CpG DNA insensitive, CSF-1 receptor down-modulation was investigated using TEPM as a model. The levels of CSF-1 receptor on these cells were somewhat lower than on BMM. CSF-1 and LPS both caused down-modulation of surface CSF-1 receptor levels (Fig. 8). By contrast, down-modulation on TEPM in response to stimulatory DNA was only observed at...
FIGURE 7. Temporal bimodal surface expression of the CSF-1 receptor after treatment with activating CpG DNA. BMM (2 x 10⁶) were deprived of CSF-1 for 18 h (0 min) and then treated with either 3 μM CpG containing AO-1 or 3 μM GpC containing NAO-1 for indicated times. Cells were harvested and stained for CSF-1 receptor surface expression and analyzed by flow cytometry as described in Materials and Methods. Shaded histograms display treatments, while dashed histograms display respective secondary Ab only controls. Bold lined unshaded histograms represent expression levels of CSF-1 receptor on untreated cells. Results are representative of three experiments.

Sustisory DNA mimics CSF-1 with phosphorylation of extracellular signal-related kinases (ERK)-1 and -2

The phosphorylation and activation of the MAPK ERK-1 and -2 by CSF-1 and LPS has previously been characterized (43, 50–53), and there is evidence implicating the ERK MAPK pathway in maintenance of viability of mammalian cells (54–56). Others have reported that CpG-containing ODN activates the MAPK family members JNK and p38 kinase, but not ERK (26). We re-examined this issue in BMM, where it is possible to establish a low level of ERK phosphorylation following CSF-1 deprivation. In our system using CSF-1-deprived BMM, ERK-1/ERK-2 phosphorylation induced by treatment with E. coli DNA was easily demonstrated (Fig. 11A). While E. coli DNA induced strong phosphorylation of ERK at 15 min, DNase I-treated E. coli DNA did not, excluding the possibility of ERK phosphorylation induced by contaminating LPS (Fig. 11A). Calf thymus DNA did not induce phosphorylation of ERK-1/ERK-2 (Fig. 11A), whereas the effect was mimicked by the CpG-containing ODN, AO-1, but not its GpC counterpart, NAO-1 (Fig. 11B). Low-level phosphorylation of ERK-1/ERK-2 was noted at 10 min with near maximal phosphorylation occurring at 15 min after treatment with E. coli DNA (Fig. 11C), whereas similar levels of phosphorylation were obtained with LPS after 5 min (data not shown). The delayed response to E. coli DNA compared with LPS and CSF-1 most probably reflects the requirement for internalization and possibly processing of DNA, whereas LPS and CSF-1 can act directly at the cell surface by engagement of their respective receptors.

Discussion

We have described the effects of bacterial DNA on the regulation of several aspects of CSF-1 signaling in murine macrophages. Like LPS, E. coli DNA was growth inhibitory for macrophages proliferating in the presence of CSF-1 (Fig. 1), due in part to a block of cells entering S phase (Fig. 2). Macrophage growth arrest observed with LPS was previously associated with a reduction in CSF-1 binding activity at the cell surface (34–36), but it was not previously clear whether this response was due to an altered binding state of the receptor or removal from the cell surface. Immunofluorescence studies on the CSF-1 receptor localization using CSF-1-deprived BMM revealed treatment with stimulatory DNA or LPS mimicked CSF-1 by causing CSF-1 receptor removal from the cell surface to a perinuclear region (Fig. 3). Removal of the CSF-1 receptor from the surface is sufficient to explain the block at the G₁-S boundary by LPS and CpG DNA, although direct effects on other aspects of cell-cycle regulation are not excluded. For example, Vadiveloo et al. (57) have made the puzzling observation that LPS, at least, can induce the expression of the G₁ cyclin, cyclin D2. CSF-1 receptor down-modulation in response to stimulatory DNA was also observed in TEPM (Fig. 8), but when compared with BMM, TEPM required much higher amounts of E. coli DNA or ODN to elicit down-modulation. The mechanism underlying this insensitivity is unclear. It could be due to increased degradation of DNA, given the higher degradative activity of TEPM compared with BMM (58). Alternatively, the CpG-specific ...

very high concentrations of ODN, but CpG specificity was still retained. A concentration of 3 μM CpG containing ODN (AO-1), which caused complete down-modulation in BMM at 50 min (Fig. 7), had no effect on CSF-1 receptor levels on TEPM at 1 h (our unpublished observations).

As measured by a colorimetric cell viability/proliferation assay, E. coli DNA and LPS promoted survival of CSF-1-starved BMM for at least 48 h, whereas vertebrate DNA did not (Fig. 9A). The number of cells is higher with CSF-1 treatment as CSF-1 promoted survival and proliferation, whereas DNA and LPS promoted only survival. This response was also due to specific CpG motifs in that a CpG containing phosphodiester ODN (AO-1) prevented loss of viable cells following CSF-1 deprivation while an ODN with a GpC inversion (NAO-1) had no effect (Fig. 9B). The nature of the response to stimulatory DNA was examined using a previously described assay for differential detection of apoptotic and necrotic cells (48), based on the kinetics of PI uptake due to loss of plasma membrane integrity (49). PI-low cells represent viable cells while PI-high staining is indicative of necrotic cells, with intermediate staining occurring for cells undergoing apoptosis (Fig. 10A). Analysis was conducted by gating for viable cells or nonviable cells (those apoptotic or necrotic). In the absence of CSF-1, ~40% of cells detected were either apoptotic or necrotic, while treatment with either E. coli DNA or LPS prevented this cell death to the same extent as CSF-1 (Fig. 10). Calf thymus DNA did not prevent cell death at 48 h, and the possibility of contaminating LPS in the E. coli DNA sample was again excluded as DNase I treatment removed anti-apoptotic activity (Fig. 10).
detection system may be differentially regulated in postproliferative macrophages. Down-modulation of CSF-1 receptor surface expression by stimulatory DNA and LPS was specific in that neither agonist altered surface expression levels of either the complement receptor 3/Mac-1 component CD11b or the macrophage-restricted cell-surface glycoprotein F4/80 (Fig. 5). Mac-1 has been implicated in oligonucleotide uptake by neutrophils (59), but the absence of any effect of DNA on Mac-1 surface levels argues against a role for this integrin in DNA uptake by macrophages. CSF-1 receptor down-modulation induced by stimulatory DNA or LPS differs mechanistically from that promoted by CSF-1 treatment. Treatment of cells with CSF-1 caused a slow decrease in the level of detectable receptor on all the cells within the population (Fig. 6), presumably reflecting progressive ligand-dependent receptor dimerization, and internalization (37). By contrast, at the single-cell level, down-modulation by stimulatory DNA was rapid and essentially quantal, as manifest by a dose- and time-dependent bimodal distribution of CSF-1 receptor surface expression (Fig. 6 and our unpublished observations). The bimodal distribution implies that the response occurs in an all-or-nothing manner, determined by a threshold of receptor occupancy and/or signaling that, once reached, results in the cell removing effectively all receptor from the cell surface within only a few minutes. Hence, increasing the dose of LPS and CpG DNA act primarily to increase the number of cells in which the CSF-1 receptor has been completely down-modulated rather than the level of receptor per cell. All-or-nothing activation at a single-cell level has also been observed for many other responses of macrophages to activating stimuli, including HIV-1 long terminal repeat-driven gene expression (60), inducible NO synthase expression (61), and induction of TNF-α in human monocytes (62).

LPS, and CpG DNA (R. Himes and D.A.H., unpublished observations), not only cause acute down-modulation of cell-surface CSF-1 receptor expression but also inhibit transcription of the CSF-1 receptor gene, c-fms (63). The ability of stimulatory DNA and LPS to down-modulate CSF-1 receptor and subsequently prevent the actions of CSF-1 may contribute to the immunomodulatory effects of these agents in vivo. Although CSF-1 can prime monocyte/macroage effector functions in vitro, including anti-
microbial activity, anti-fungal activity, cytocidal activity, and expression of certain cytokines (64–68), it is profoundly immuno-suppressive for Ag-specific and mitogen-induced T lymphocyte proliferation (69). Administration of CSF-1 to mice significantly reduced the proliferative response of splenic cells in response to T cell mitogens (70, 71). Recombinant CSF-1 appears to block macrophage stimulation of T lymphocytes in allogenic MLR by secretion of a soluble factor distinct from TGF-β or IL-10 (72). CSF-1 levels rise after infection (73, 74), or after LPS administration (75), presumably increasing the numbers of monocytic precursors for macrophage/DC. CSF-1 receptor down-modulation and transcriptional repression could be a mechanism to block macrophages responding to and mediating the immunosuppressive actions of CSF-1. Given that the inhibitory effects of CSF-1 are dominant over the MHC class II (Ia)-inducing effects of GM-CSF on BMM (76), and that DC can be generated from monocytic precursors depending on the cytokine microenvironment (42), the generation of cells with an immunostimulatory DC phenotype may be enhanced by the ability of DNA and LPS to suppress the pathway of terminal macrophage differentiation mediated by CSF-1 (77). Such a situation may contribute to polarization of the APC compartment toward the more immunostimulatory DC-like phenotype during infection in vivo. Further studies by Palucka et al. (78) suggest that priming with LPS, or CSF-1 and LPS, can reduce the potential of monocytes to develop into DC following subsequent culture in GM-CSF and IL-4. However, if a signal initiating DC differentiation (such as GM-CSF and IL-4) is given concomitantly with LPS priming, no reduction of differentiation of monocytes to DC is observed upon subsequent culture in GM-CSF plus IL-4 (78). These studies did not address the effect of LPS on DC differentiation when both macrophage (CSF-1) and DC (GM-CSF) differentiation stimuli are present simultaneously.

CSF-1 is required for macrophage survival (38), so loss of the CSF-1 receptor might be expected to cause cell death. However,
stirulatory DNA and LPS both prevented apoptosis of CSF-1-deprived macrophages to a similar extent to CSF-1 for at least 48 h after treatment (Fig. 9). Hence, both agonists can potentially block CSF-1 action on inducible genes such as urokinase plasminogen activator (44) and the scavenger receptor (79) without causing cell death due to loss of signal required for viability. This parallels the anti-apoptotic effect of stimulatory DNA and LPS on B lymphocytes except that in these cells both stimuli are themselves mitogens. To address the mechanism by which this anti-apoptotic effect may be mediated in macrophages, we investigated signaling events triggered by stimulatory DNA. Stimulatory DNA in the form of bacterial DNA or activating CpG containing phosphodiester oligonucleotides induced the phosphorylation of the MAPK members ERK-1/ERK-2. Yi et al. (26) reported that using the macrophage-like cell line, J774, ERK-1/ERK-2 were not phosphorylated in response to activating ODN, but these cells are not factor dependent for growth or survival and transforming events may have altered the regulation of ERK-1/ERK-2.

Taking our observations with those published, the main families of MAPK, the ERKs, JNKs, and p38 kinases, can all be activated in response to CpG DNA (26, 27) and LPS (53, 80, 81). Activation of the JNK and p38 pathways is associated with several forms of stress and is proposed to be proapoptotic (54, 81), while MAPK/ERK kinase (MEK1)-dependent ERK activity has anti-apoptotic effects in a variety of cell models including neuronal PC-12 cells (54) and kinase-defective epidermal growth factor receptor-bearing cells after treatment with epidermal growth factor (55). Others have shown that LPS is anti-apoptotic for a factor-dependent DC-like cell line after factor withdrawal (56). Moreover, this anti-apoptotic effect of LPS, but not conditioned media, was dependent on the ERK pathway and was inhibited by the MEK inhibitor PD980590 (56). Similarly, in BMM, PD980590 causes cell death even in the presence of CSF-1 (43). Thus, it seems likely that the ERK-1/ERK-2 activation observed in response to stimulatory DNA, and LPS, and CSF-1 is a cell survival signal.

Cell survival can be an important regulator of the immune response (82, 83). An anti-apoptotic effect exerted on APC by pathogen-associated molecular patterns could increase time of residency in the secondary lymphoid organs, increasing the probability of activating specific T lymphocytes from the recirculating pool. These anti-apoptotic effects may also serve to protect effector macrophages against cytotoxic effects of toxic microbial products and cytotoxic agents such as O2− and NO.

In summary, we have shown both stimulatory DNA and LPS cause internalization of the CSF-1 receptor and inhibit CSF-1-dependent macrophage proliferation while maintaining cell viability possibly through a ERK-1/ERK-2 mediated pathway. We propose that in the case of infection these changes may result in polarization away from the immunosuppressive mature macrophage toward the more effective immunostimulatory APC.

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References

EFFECTS OF BACTERIAL/CpG DNA ON MACROPHAGES


