Lipopolysaccharide and Monophosphoryl Lipid A Differentially Regulate Interleukin-12, Gamma Interferon, and Interleukin-10 mRNA Production in Murine Macrophages

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Monophosphoryl lipid A (MPL) is a nontoxic derivative of the lipid A region of lipopolysaccharide (LPS) that is being developed as both an adjuvant and prophylactic drug for septic shock. We compared the ability of LPS and MPL to induce interleukin-10 (IL-10), IL-12 p35, IL-12 p40, gamma interferon (IFN- γ), glucocorticoid receptor (GR), IL-1 receptor antagonist (IL-1ra), and inducible nitric oxide synthase mRNA expression in murine peritoneal macrophages. These genes were chosen for their ability to positively or negatively regulate the host immune response and thus for their potential involvement in MPL-induced adjuvanticity or in its ability to protect against sepsis. LPS was a more potent inducer of IL-12 p35, IL-12 p40, and IFN- γ mRNA, as well as of IL-12 protein, than MPL. In contrast, MPL induced higher levels of IL-10 mRNA than did LPS from 1 to 1,000 ng/ml. In general, MPL was not a more potent inducer of negative regulatory genes, since MPL and LPS induced similar levels of GR and IL-1ra mRNA. Addition of anti-IL-10 antibody to cultures increased the induction of MPL-induced IL-12 p35, IL-12 p40, and IFN- γ mRNA, suggesting that the enhanced production of IL-10 by MPL-stimulated macrophages contributes to decreased production of mRNA for IL-12 (p35 and p40) and IFN- γ . Conversely, the addition of exogenous IL-10 to LPS-treated macrophages reduced the mRNA expression of these cytokine genes. These studies suggest that enhanced production of IL-10 by MPL-stimulated macrophages may contribute to the reduced toxicity of MPL through its negative action on induction of cytokines shown to enhance endotoxicity.

Administration of lipopolysaccharide (LPS), specifically the lipid A moiety, to humans or experimental animals results in many of the physiological changes observed during gram-negative bacterial infection, including fever, hypotension, hypoglycemia, disseminated intravascular coagulation, and shock (7). In contrast to its role in the pathogenesis of gram-negative bacterial infection, LPS can, with careful dosing, induce a variety of beneficial effects, such as adjuvanticity, radioprotection, and increased nonspecific resistance to infection and malignancy (3, 46, 50, 62). Despite these potentially beneficial properties, the pyrogenicity and toxicity of LPS have precluded its development as a therapeutic agent in humans.

Monophosphoryl lipid A (MPL) is a detoxified derivative of LPS that lacks many of the endotoxic properties of LPS yet retains both its adjuvant and immunostimulatory activities (2, 9, 34, 45, 61). MPL, commercially available as MPL immunostimulant, was developed both as an adjuvant for human vaccines and as a prophylactic drug for septic shock (reviewed in reference 50 and 62). The adjuvant activity of MPL is related, at least in part, to its ability to induce the release of cytokines that promote the generation of a specific immune response. In vitro, MPL induces the production of proinflammatory cytokines from macrophages (34) and IFN- γ and IL-2 from lymphocytes (9). Many of these cytokines also are detected in the serum of humans and experimental animals following treatment with MPL (2, 29, 33, 34). Results from phase I clinical trials demonstrated that the addition of MPL to recombinant herpes simplex virus type 2 and hepatitis B vaccines resulted in

higher seroconversion rates and larger antibody (Ab) titers (reviewed in reference 62), illustrating the efficacy of MPL as an adjuvant. The ability of MPL to promote nonspecific resistance to infection and to induce a state of endotoxin tolerance analogous to that produced by LPS (2, 10, 33, 42) has provided the rationale for the development of MPL as a prophylactic agent for septic shock. Pretreatment of experimental animals with MPL promotes improved survival rates following peritonitis, infection with either gram-negative or gram-positive bacteria, and shock induced by LPS, staphylococcal enterotoxin B, and tumor necrosis factor alpha (TNF- α) (1, 3, 4, 10). These protective effects have been largely attributed to the ability of MPL to induce a state of tolerance to subsequent endotoxin challenge that results in an attenuated systemic response to LPS (chills, fever, and tachycardia), ameliorated production of proinflammatory cytokines (TNF- α , interleukin-6 [IL-6], and IL-8), and inhibited development of disseminated intravascular coagulation (2–4, 30, 33, 66).

While MPL can be a potent immunostimulant and is capable of inducing tolerance to LPS (2, 10, 33, 42), it is 100 to 10,000 times less toxic than LPS (61). The basis for its lack of toxicity has been attributed to its reduced ability (relative to LPS) to induce proinflammatory cytokines like TNF- α , IFN, and IL-6 in vivo (33). Similarly, macrophages cultured in vitro produce lower levels of TNF- α and IL-1 β mRNA following stimulation with MPL than with LPS (34), suggesting that MPL may be a less potent stimulus for macrophages, cells with a central role in the host response to LPS (23, 52). It is not known if some of the differences in the sensitivity of macrophages to LPS and MPL might be related to the relative balance in the production between immune system-amplifying and -deactivating cytokines and other regulatory molecules that could subsequently affect the cytokine production by macrophages. In vivo, im-

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mune system-amplifying cytokines like IL-12 and IFN- γ have been associated with enhanced lethality to LPS (17, 39, 65), while administration of glucocorticoids and negative regulatory cytokines, like IL-10 and IL-1 receptor antagonist (IL-1ra), dampen the host immune response to LPS and promote survival (21, 25–27, 63, 67). In vitro, LPS-stimulated macrophages are potent producers of IL-12, IL-10, and IL-1ra (11, 55, 57) and also increase their glucocorticoid receptor (GR) number in response to LPS (53). Moreover, recent studies have demonstrated that macrophages are capable of producing IFN- γ in response to LPS and other stimuli (16, 24, 54). Using a model of macrophage depletion, we recently reported that macrophages were major contributors to the in vivo production of IL-1ra, IL-10, and IL-12 p40 mRNA following LPS challenge (52).

The purpose of this study was to compare the ability of MPL and LPS to regulate the expression of a variety of positive and negative immune system regulatory genes in murine macrophages. The genes that were examined were chosen on the basis of their potential to be involved either in the differential toxicity observed between LPS and MPL, their ability to contribute to tolerance induction, and/or their possible role in adjuvant activity. Specifically, we assessed, in peritoneal exudate macrophages, the LPS- and MPL-mediated induction of IL-12 p40, IL-12 p35, and IFN- γ mRNA (cytokines involved in immune amplification), as well as of mRNA for IL-10, IL-1ra and GR (genes that are often associated with negative regulation of the immune response). In addition, we compared the ability of LPS and MPL to regulate inducible nitric oxide synthase (iNOS), a LPS-inducible enzyme that produces nitric oxide. Nitric oxide contributes to LPS-induced hypotension (41), negatively regulates TNF- α production by LPS-stimulated macrophages (18), and contributes to endotoxin tolerance in vitro (20). Key findings from this study indicate that LPS is a more potent inducer of IL-12 p35, IL-12 p40, and IFN- γ mRNA, as well as IL-12 protein, than MPL. Conversely, MPL is a more potent inducer of IL-10 mRNA than LPS. The counterbalancing roles of IL-10 and IFN- γ in the response to LPS or MPL are suggested by our findings that (i) addition of exogenous recombinant IL-10 (rIL-10) shifted the LPS gene induction pattern to one that is more “MPL-like,” whereas (ii) depletion of endogenous IL-10 production (with anti-IL-10 Ab) from MPL-treated macrophages shifted the response to a more “LPS-like” pattern of gene expression and (iii) exogenous rIFN- γ down-regulated the expression of IL-10 mRNA induced by either LPS or MPL. These results suggest that the reduced toxicity of MPL may be due not only to its diminished ability to induce immune system-amplifying cytokines, like IL-12 and IFN- γ , but also to its enhanced ability to induce IL-10 production from macrophages.

MATERIALS AND METHODS

Reagents. Protein-free *Escherichia coli* K235 LPS was obtained by phenol-water extraction (44). MPL was a kind gift of RIBI ImmunoChem Research, Inc. (Hamilton, Mont.). Murine rIFN- γ and murine rIL-10 were provided by Genentech, Inc. (South San Francisco, Calif.), and DNAX (Palo Alto, Calif.), respectively. Anti-IL-10 Ab (SXC.2) and isotype-matched control Ab were kindly provided by Thomas Wynn (National Institutes of Health, Bethesda, Md.).

Mice. C3H/OuJ mice (5 to 6 weeks old) were obtained from Jackson Laboratories (Bar Harbor, Maine). The mice were housed in cages with filter tops in a laminar-flow hood and fed standard laboratory chow and acid water ad libitum.

Macrophage cultures and cytokine assays. Peritoneal exudate macrophages were obtained by lavage 4 days after intraperitoneal injection with 3 ml of sterile 3% thioglycolate broth. The cells were washed, resuspended, and plated in RPMI 1640 containing 2% heat-inactivated fetal calf serum and standard supplements as previously described (43). After incubation overnight, cell monolayers were washed to remove nonadherent cells and incubated with either medium, LPS, or MPL for the indicated period prior to analysis of gene induction, cytokine secretion, or nitrite production. The purity of macrophage cultures was

approximately 98%. In some experiments, LPS- and MPL-treated macrophages were simultaneously treated with rIFN- γ (10 U/ml), rIL-10 (100 U/ml), and either anti-IL-10 Ab (10 μ g/ml) or isotype-matched control Ab (10 μ g/ml). Nitric oxide was assessed by measuring the release of nitrite, the stable end product of nitric oxide synthesis, into 24-h culture supernatants (2×10^5 cells in 0.2 ml) as previously described (43). For cytokine assays, supernatants were harvested after 24 h from 4×10^6 macrophages treated with 2 ml of medium, LPS, or MPL. Total IL-12 (p40 monomer, p40 dimer, and p70), IL-12 p70, and IL-10 were measured by enzyme-linked immunosorbent assay (ELISA) (Genzyme, Cambridge, Mass.) as specified by the manufacturer. The lower limits of detection were 10, 5, and 15 pg/ml for total IL-12, IL-12 p70, and IL-10, respectively.

Analysis of mRNA. Total RNA was isolated, and the relative quantities of mRNA for GR, hypoxanthine-guanine phosphoribosyltransferase (HPRT), IFN- γ , IL-1ra, IL-10, IL-12 p35, IL-12 p40, and iNOS were determined by coupled reverse transcription-PCR as described previously (43). The probes and primers for all genes except GR have been published previously (24, 43, 55, 59, 64). For GR, the sense primer was 5'-GGGCCCGTTTATTGCCAGG, the antisense primer was 5'-GCAGAGTTTGGGAGGTGGTCC, and the probe was 5'-CTGGAATGAGACCAGATGTGAG (14). The number of PCR cycles for each gene was as follows: GR, 23; HPRT, 23; IFN- γ , 32; IL-1ra, 19; IL-10, 28; IL-12 p35, 25; IL-12 p40, 28; and iNOS, 27. Amplified products were electrophoresed and transferred to Hybond N+ membranes (Amersham, Arlington Heights, Ill.) in $10 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) by standard Southern blotting techniques. DNA was cross-linked by exposure to UV light, baked onto the nylon membrane, and hybridized with an internal oligonucleotide probe. Labeling of the probe and subsequent detection of bound probe was carried out with an enhanced chemiluminescence system (Amersham) as specified by the manufacturer. Chemiluminescent signals were quantified with a scanner (Datacopy GS plus; Xerox Imaging Systems, Sunnyvale, Calif.). To determine the magnitude of the change in gene expression, cDNA from a sample known to be positive for the transcript of interest was used to generate a standard curve by serial dilution of the positive control and simultaneous amplification. The signal of each band in the standard curve was plotted and subjected to linear regression. The equation from this line was used to calculate the fold induction in test samples. Data were individually normalized for the relative quantity of mRNA by comparison to HPRT. Means are expressed relative to medium-treated controls.

Statistics. The results were analyzed by Student's paired *t* test for comparisons between two groups or analysis of variance for repeated measures by Fisher's protected least significant difference. *P* < 0.05 was accepted as the level of significance. All experiments were repeated at least three times with similar results.

RESULTS

Kinetics of IL-12, IFN- γ , and IL-10 mRNA expression induced by LPS and MPL. In initial studies, we characterized the regulation of IL-12 (p35 and p40), IFN- γ , and IL-10 mRNA expression following incubation of C3H/OuJ peritoneal macrophage cultures with 200 ng of either LPS or MPL per ml. As shown in Fig. 1, the time required to induce peak steady-state mRNA levels following LPS treatment differed for each gene. IL-10 mRNA peaked by 2 h after LPS treatment, while IL-12 p35 and IL-12 p40 mRNA levels both peaked at 6 h. The IFN- γ mRNA level peaked by 12 h after LPS treatment. While the time required to induce peak mRNA levels following MPL treatment was similar to that observed with LPS, substantial differences in the levels of peak mRNA induction were observed between LPS and MPL treatments. Specifically, significantly higher (*P* < 0.05) peak levels of steady-state IL-12 p40 (6 h) and IFN- γ (12 h) mRNA were observed after LPS treatment. Conversely, significantly higher (*P* < 0.05) peak levels of steady-state IL-10 mRNA were observed after MPL treatment. Finally, differences in the decline in steady-state mRNA from peak levels also were observed, with higher levels of LPS- than MPL-induced IL-12 p35 and p40 mRNA at both 24 and 48 h after treatment.

Dose response of IL-12 (p35 and p40), IFN- γ , and IL-10 mRNA expression induced by LPS and MPL. To assess the sensitivity of C3H/OuJ peritoneal exudate cells to LPS and MPL, a dose-response analysis was performed. In these experiments, macrophage monolayers were treated with medium and increasing concentrations of either LPS or MPL. Total cellular RNA was harvested at a time adequate to induce peak

FIG. 1. Kinetics of LPS- and MPL-induced expression of IL-12 (p35 and p40), IFN- γ , and IL-10 mRNA. Macrophage cultures were treated with 200 ng of LPS (□) or MPL (■) per ml for the indicated times. Data are expressed as the mean $\pm$ standard error of the mean (SEM) from four or five independent experiments. When not visible, SEM bars are smaller than the symbol. Asterisks indicate that differences between LPS and MPL treatments were statistically significant ($P < 0.05$).

mRNA expression for the gene of interest (i.e., IL-10, 2 h; IL-12 p35 and p40, 6 h; and IFN- γ , 12 h). Data from a representative experiment is shown in Fig. 2A, and the combined results from multiple experiments are shown in Fig. 2B. LPS induced significantly higher levels of both IL-12 p40 and IFN- γ mRNA than did MPL over a wide range of concentrations (0.1 to 1,000 ng/ml). The range of concentrations over which LPS was a more effective inducer than MPL of IL-12 p35 mRNA was limited to 0.1 to 10 ng/ml. Conversely, MPL induced significantly more IL-10 mRNA than did LPS at concentrations ranging from 1 to 1,000 ng/ml.

Secretion of IL-12 and IL-10 by LPS- and MPL-stimulated macrophages. We next assessed whether LPS and MPL differentially induced the secretion of IL-12 and IL-10 protein. Since IL-12 heterodimer (p70) is the biologically active form of IL-12 and its activity is inhibited by IL-12 p40, which acts as a receptor antagonist (40), we assessed both total IL-12 (p40 monomer, p40 dimer, and p70) and IL-12 p70. As shown in Table 1, LPS was a more potent inducer of IL-12 p70 and IL-12 p40 secretion than was MPL. In fact, MPL-induced IL-12 secretion never achieved the peak levels of IL-12 secretion observed following LPS treatment. The concentration of IL-10 protein in culture supernatants following treatment with either LPS or MPL was either undetectable or only slightly above the level of assay sensitivity and was not reproducible between experiments (data not shown). The inability to detect IL-10 protein despite the presence of a high level of IL-10 mRNA has been observed for other activators of macrophage activity (28). In a previous study, Fultz et al. (24) reported that while LPS-stimulated macrophages produce intracellular IFN- γ (detected by immunofluorescent staining), secreted IFN- γ protein could not be detected. Therefore, secretion of IFN- γ into culture supernatants was not assessed in this study.

Induction of GR and IL-1ra mRNA expression by LPS and MPL. Because we found that MPL was a more potent inducer of the gene encoding the anti-inflammatory cytokine, IL-10, we next sought to evaluate if MPL was, in general, a more potent inducer of genes involved in the negative regulation of the host response to LPS. To do this, the abilities of LPS and MPL to induce mRNA expression for two other negative regulators of cytokine gene expression, GR and IL-1ra, were compared. In initial studies, we characterized the kinetics of GR and IL-1ra mRNA expression following incubation of C3H/OuJ peritoneal exudate macrophages with 200 ng of either LPS or MPL per ml. Both LPS and MPL significantly induced ($P < 0.05$) GR mRNA by 4 h, with peak GR mRNA levels being observed at 6 h (data not shown). Following treatment with either LPS or MPL, IL-1ra mRNA also was significantly induced ($P <$

FIG. 2. Dose responses of LPS- and MPL-induced expression of IL-12 (p35 and p40), IFN- γ , and IL-10 mRNA. Based on the data obtained in the experiment in Fig. 1, macrophage cultures were treated with the indicated concentrations of LPS or MPL for a time adequate to induce peak mRNA expression (IL-12 p35 [6 h], IL-12 p40 [6 h], IFN- γ [12 h], and IL-10 [2 h]). (A) Representative Southern blots of reverse transcription-PCR-amplified products from a single experiment. The Southern blot of HPRT corresponds to the samples obtained from the dose response for IL-12 (both p35 and p40) and is representative of those obtained for both IFN- γ and IL-10. (B) Mean $\pm$ SEM from five to seven experiments. Asterisks indicate that differences between LPS (□) and MPL (■) treatments were significant ($P < 0.05$).

TABLE 1. Production of IL-12 by LPS- and MPL-stimulated macrophages

Treatment ^a and dose (ng/ml)	IL-12 (pg/ml) production ^b	
	p40/p70	p70
Medium		
0	64 ± 20	≤5.0
LPS		
1	20,769 ± 5,283 ^c	46.5 ± 14.0 ^c
10	27,773 ± 766 ^c	48.6 ± 1.4 ^c
100	17,074 ± 1,040	15.5 ± 4.4
1,000	19,922 ± 1,407	20.8 ± 7.8
MPL		
1	24 ± 3	≤5.0
10	5,382 ± 216	≤5.0
100	15,084 ± 1,604	9.1 ± 2.2
1,000	15,944 ± 731	13.8 ± 8.9

^a Macrophage cultures were stimulated with medium, LPS, or MPL, and supernatants were harvested at 24 h.

^b Total IL-12 (p40/p70) detected in this ELISA represents p40 monomer, p40 dimer, and p70. Data are the mean ± SEM from three separate experiments.

^c Data from LPS-stimulated macrophages were significantly higher ($P < 0.05$) than from MPL-stimulated macrophages.

0.05) over untreated controls by 2 h, with peak IL-1ra mRNA expression observed at 12 h (data not shown). No differences in the kinetics of LPS- and MPL-induced GR or IL-1ra mRNA expression were observed at 4, 6, 12, 24, or 48 h. To assess the sensitivity of macrophages to LPS and MPL for GR and IL-1ra mRNA expression, dose-response analyses also were performed. Total cellular RNA was harvested at a time corresponding to peak mRNA expression for GR (6 h) and IL-1ra (12 h). Results of multiple experiments are shown in Fig. 3. As little as 0.1 ng of LPS per ml induced a significant increase ($P < 0.05$) in both GR and IL-1ra mRNA levels over medium-treated controls, while a significant increase ($P < 0.05$) in MPL-induced GR and IL-1ra mRNA levels was observed only when macrophages were treated with ≥ 1 ng/ml. Additionally, the relative fold induction of GR and IL-1ra mRNA by LPS and MPL was substantially lower than that observed for the other genes examined in this study.

Regulation of iNOS mRNA and nitrate production. In macrophages, nitric oxide is produced by the enzyme iNOS. Nitric oxide has been shown to regulate TNF- α production negatively in LPS-stimulated macrophages (18) and to contribute to the induction of endotoxin tolerance in macrophages restimulated with LPS in vitro (20). Since higher doses of MPL than of LPS are required to induce a state of endotoxin tolerance in macrophages cultured in vitro (34), we examined the ability of LPS and MPL to induce iNOS mRNA. Following treatment with 200 ng of LPS or MPL per ml, iNOS mRNA was induced by 2 h, peaked at 6 h, and remained at a heightened level for 48 h (data not shown). No difference in the kinetics or magnitude of iNOS mRNA expression was observed between LPS and MPL treatment at this concentration. A dose-response analysis for iNOS mRNA induction (6 h) and nitrite release into culture supernatants (24 h) after LPS or MPL treatment is shown in Fig. 4. LPS was a more potent inducer of both iNOS mRNA (Fig. 4A) and nitrite production (Fig. 4B) than was MPL.

A hallmark of nitric oxide production is the synergistic inducing capacity of stimulatory agents. In vitro, IFN- γ and LPS synergize to induce nitrite production from macrophages (43). Therefore, we examined whether MPL was as effective as LPS in synergizing with IFN- γ (10 U/ml) for nitrite accumulation in

culture supernatants. As shown in Fig. 4C, 0.1 and 1 ng of LPS, but not MPL, per ml synergized with IFN- γ for nitrite release, whereas at ≥ 10 ng/ml, both LPS and MPL synergized with IFN- γ to induce similar levels of nitrite production from macrophage cultures. A similar pattern of nitrite production was observed when macrophages were treated with LPS or MPL (0.1 to 1,000 ng/ml) in combination with 0.5, 1, and 5 U of IFN- γ per ml (data not shown).

Regulation of IFN- γ and IL-12 (p35 and p40) mRNA expression by endogenous and exogenous IL-10. IL-10 has been shown to inhibit IFN- γ and IL-12 production from peripheral blood mononuclear cells (13). To assess whether the reduced level of IL-12 (p35 and p40) and IFN- γ mRNA induced by MPL was due to the endogenous production of IL-10 and whether interfering with the availability of endogenous IL-10 could make an MPL response more LPS-like, macrophage cultures were treated with LPS alone or with MPL and either medium, control antibody, or anti-IL-10 Ab. Total cellular RNA was harvested at a time adequate to induce peak mRNA expression (IL-12 p35 and IL-12 p40, 6 h; IFN- γ , 12 h). Depletion of endogenous IL-10 by addition of anti-IL-10 antibody to macrophage cultures treated with 100 ng of MPL per ml (Fig. 5) led to significantly increased ($P < 0.05$) levels of IFN- γ , IL-12 p40, and IL-12 p35 mRNA over those in MPL-treated controls (either MPL alone or MPL plus control Ab). Similar results also were obtained for IL-12 p40 mRNA expression in cells treated with 10 ng of MPL per ml and anti-IL-10 antibody. Moreover, IFN- γ , IL-12 p35, and IL-12 p40 mRNA expression were similar in LPS-treated and MPL-plus-anti-IL-10 Ab-treated cells.

Next, macrophage cultures were treated with MPL, LPS, or both LPS and rIL-10 (100 U/ml) to assess if addition of exogenous IL-10 could shift LPS-induced IFN- γ , IL-12 p40, and IL-12 p35 mRNA expression to a more MPL-like response. As shown in Fig. 6, exogenous IL-10 significantly reduced the

FIG. 3. Dose responses of LPS- and MPL-induced GR and IL-1ra mRNA. Macrophages were treated with the indicated concentrations of LPS (□) or MPL (■) for a time adequate to induce peak mRNA expression (GR [6 h] and IL-1ra [12 h]). Data are the mean ± SEM from four to six experiments. Asterisks indicate that differences between LPS and MPL treatments were significant ($P < 0.05$).

FIG. 4. Differential regulation of iNOS mRNA (A) and nitrite production by LPS and MPL (B and C). Macrophage cultures were treated with the indicated concentrations of LPS or MPL in the presence or absence of IFN- γ (10 U/ml). (A) mRNA was harvested 6 h after LPS or MPL treatment. (B) Supernatants were harvested at 24 h and nitrite production was quantified after the addition of Griess reagent. Data are the mean $\pm$ SEM from three separate experiments.

levels of LPS-induced IFN- γ , IL-12 p40, and IL-12 p35 mRNA to the same as or lower than those observed for MPL treatment alone.

Addition of exogenous IFN- γ negatively regulates LPS-induced IL-10 mRNA expression. Just as IL-10 regulates IFN- γ gene expression negatively, IFN- γ , in a counterregulatory fashion, inhibits IL-10 production from LPS-stimulated monocytes (11). Therefore, we compared the ability of IFN- γ to down-regulate MPL- and LPS-induced IL-10 mRNA production (Table 2). While both MPL- and LPS-induced IL-10 mRNA production were sensitive to negative regulation by IFN- γ , higher concentrations of IFN- γ were required to inhibit MPL-induced IL-10 mRNA to the levels observed for LPS-induced IL-10 mRNA. In fact, IL-10 mRNA levels in macrophages treated with 10 ng (per ml) of MPL and IFN- γ were comparable to those in macrophages treated with 100 ng (per ml) of LPS and IFN- γ .

DISCUSSION

The immunostimulant MPL was developed both as a prophylactic treatment for sepsis (50) and as an adjuvant for vaccines (62). In this study, we compared the ability of macrophages to respond to MPL and LPS with increased mRNA

expression for a panel of genes that regulate inflammation. These genes were chosen for their potential involvement in the differential toxicity between LPS and MPL, their ability to contribute to tolerance induction, and/or their potential role in the adjuvanticity of MPL. The reduced toxicity of MPL has been attributed to its reduced capacity to induce cytokines like TNF α , IL-1, and IL-6, both in vitro and in vivo (29, 33, 34, 42). Results from this study indicate that in vitro, MPL is also a less potent inducer of IL-12 mRNA and protein, as well as IFN- γ mRNA, from peritoneal exudate cells than is LPS. Typically, between 10 and 100 times more MPL was required to induce IL-12 (p35 and p40) and IFN- γ mRNA. Although not typically considered to be IFN- γ producers, macrophages have been shown previously to make IFN- γ mRNA and/or protein in response to LPS and other stimuli (16, 24, 54). IFN- γ mRNA has been detected in LPS-stimulated peritoneal exudate cells from anti-NK 1.1-treated C3H/OuJ mice and Thy 1.1-depleted macrophage cultures (24), as well as in IFN- γ -stimulated peritoneal exudate cells from anti-asialo-treated nude mice (16). Studies by Sarawar et al. (54) with bronchoalveolar lavage cells

FIG. 5. Depletion of endogenous IL-10 augments the production of IFN- γ (A) and IL-12 (p35 and p40) (B and C) mRNA in MPL-treated macrophages. Macrophage cultures were stimulated with LPS alone or with MPL in the presence of medium, isotype-matched control Ab, or anti-IL-10 Ab. mRNA was harvested at 6 h for IL-12 p35 and IL-12 p40 and at 12 h for IFN- γ . Asterisks indicate that a significant difference ($P < 0.05$) was observed between cells incubated with both MPL and either medium or control Ab and cells incubated with both MPL and anti-IL-10 Ab. Data are the mean $\pm$ SEM from four separate experiments. Symbols: $\square$, 0; $\square$, 1; $\square$, 10; $\blacksquare$, 100 ng of either LPS or MPL per ml.

FIG. 6. Exogenous rIL-10 down-regulates LPS-induced IFN- γ (A) and IL-12 (p35 and p40) (B and C) mRNA. Macrophage cultures were treated with the indicated concentrations of MPL ($\square$), LPS ($\square$), or both LPS and IL-10 (100 U/ml). mRNA was harvested as described in the legend to Fig. 5. Data are the mean $\pm$ SEM from four separate experiments.

from CD4- and CD8-depleted influenza virus-infected mice demonstrated that a majority (~70%) of IFN- γ mRNA-producing cells were phagocytic (latex positive). Based on these studies from our laboratory (24) and others (16, 54), it is likely that macrophages contributed, at least in part, to IFN- γ mRNA production. However, NK and T cells were not rigorously depleted from macrophage cultures in these experiments, and therefore their potential contribution to IFN- γ mRNA production cannot be eliminated.

In this study, LPS and MPL were more potent inducers of IL-12 p40, which acts as a receptor antagonist (40), than of bioactive IL-12 (p70). This has been observed by other investigators both in vitro from LPS-stimulated human monocytes (58) and in the serum of LPS-challenged mice (32). Excess IL-12 p40 in serum during endotoxemia, however, was not sufficient to prevent IL-12-induced circulating IFN- γ (32). Thus, it is likely that bioactive IL-12 secretion from macrophages contributed to IFN- γ mRNA induction in this study.

These studies demonstrate the important role that IL-10 plays in regulating the response to MPL. In striking contrast to IL-12 (p35 and p40) and IFN- γ , IL-10 mRNA was more strongly induced by MPL than by LPS. Enhanced production of IL-10 by MPL-stimulated macrophages resulted in a pattern of gene expression in which IFN- γ and IL-12 mRNAs were suppressed. This was demonstrated by the ability of anti-IL-10 Ab to increase IFN- γ and IL-12 (p35 and p40) mRNA expres-

sion to levels that more closely resembled levels induced by LPS. Conversely, the addition of exogenous IL-10 to LPS-stimulated macrophages decreased IFN- γ and IL-12 mRNA expression, shifting the pattern of gene expression to one that more closely resembled that induced by MPL. The observation that anti-IL-10 Ab increased IFN- γ and IL-12 (p35 and p40) mRNA expression also indicates that bioactive IL-10 protein was present in culture supernatants, despite our inability to detect it reproducibly by ELISA. These data also suggest that secreted IL-10 protein may be rapidly utilized by cells in culture. Previous studies have shown that endogenously produced IL-10 negatively regulates a variety of proinflammatory mediators from LPS-stimulated macrophages, including IL-1 α , IL-1 β , IL-6, IL-8, and TNF- α (15, 56). The reduced production of many of these mediators upon MPL stimulation of macrophages (compared to LPS) also is probably attributable to feedback inhibition secondary to enhanced production of endogenous IL-10 by MPL.

The enhanced production of endogenous IL-10 in MPL-stimulated cultures is clearly one of the primary mechanisms that accounts for the lower levels of IL-12 (p35 and p40) and IFN- γ mRNA induced by MPL. The addition of anti-IL-10 antibody to MPL-stimulated cultures, however, increased IL-12 (p35 and p40) and IFN- γ mRNA levels only when higher concentrations of MPL (10 and/or 100 ng/ml) were used, suggesting that another mechanism(s) must also account for the differential induction of these mRNAs at concentrations ≤ 10 ng/ml. Other possible mechanisms include the overexpression of other negative regulators of gene expression not measured in this study, such as IL-4 and transforming growth factor- β (54), and/or the absence of positive regulators of gene expression underexpressed following MPL stimulation.

Given previous observations that MPL and LPS induce the same panel of genes and that the action of both is inhibitable by the LPS structural antagonist *Rhodobacter sphaeroides* diphosphoryl lipid A (35), one would presume that LPS and MPL engage the same signaling apparatus. The finding that some genes (e.g., the IL-10 and TNFR2 genes [34]) are preferentially induced by MPL in the face of similar levels of other genes (e.g., the IL-1 α and GR genes) and reduced levels of most genes (e.g., the IL-1 β , TNF- α , and IL-12 genes, etc.) suggests that this may not be the case. Although it is likely that LPS and MPL share a principal signaling pathway, CD-14-independent pathways have been either suggested or demonstrated (12, 37, 38) and may engage LPS preferentially, resulting in a pattern of gene expression distinct from that elicited by

TABLE 2. Negative regulation of LPS- and MPL-induced IL-10 mRNA by IFN- γ

Amt of IFN- γ (U/ml)	Fold induction ^a by:				
	Medium	LPS		MPL	
		10 ng/ml	100 ng/ml	10 ng/ml	100 ng/ml
0	1.0	18.7 $\pm$ 1.0	26.8 $\pm$ 3.7	28.4 $\pm$ 3.0 ^b	45.0 $\pm$ 4.8 ^b
0.1	1.2 $\pm$ 0.1	11.8 $\pm$ 0.4	14.9 $\pm$ 2.5	14.3 $\pm$ 1.4	21.3 $\pm$ 1.1 ^b
0.5	1.4 $\pm$ 0.2	8.4 $\pm$ 1.7	7.0 $\pm$ 0.8	11.1 $\pm$ 0.3	10.8 $\pm$ 1.1 ^b
5.0	1.3 $\pm$ 0.1	3.9 $\pm$ 0.5	2.4 $\pm$ 1.1	5.6 $\pm$ 1.2	3.7 $\pm$ 0.5

^a Fold induction represents the increase over the values for medium-treated controls, which were normalized to a value of 1 as described in Materials and Methods. Data represent the mean $\pm$ SEM from three to five separate experiments.

^b The increase in IL-10 mRNA levels in macrophages treated with MPL (with or without IFN- γ) were significantly greater ($P < 0.050$) than that in macrophages treated with LPS and comparable concentrations of IFN- γ .

MPL. Further studies are required to distinguish between these possibilities.

It is likely that the observed reduction in MPL-induced production of IL-12 and IFN- γ , in association with the enhanced production of IL-10 from macrophage cultures, also contributes to the reduced toxicity of MPL *in vivo*. Endogenous production of IFN- γ enhances mortality in several models of endotoxicity (8, 17, 31, 36). IFN- γ production, in turn, modulates LPS toxicity by up-regulating the expression of a variety of genes including the gene encoding TNF- α (8, 51), one of the primary mediators of LPS-induced lethality (6). *In vivo*, MPL is a less effective inducer of both circulating IFN- γ and TNF- α than is LPS (29, 34). More recently, IL-12 production *in vivo* has been shown to be proximal to IFN- γ production. By using anti-IL-12 Ab, it was demonstrated that endogenous IL-12 production following LPS administration was required for the production of circulating IFN- γ and was a lethal mediator of endotoxic shock (32, 47). Moreover, recent studies have shown that macrophages are the primary producers of LPS-induced IL-12 p40 mRNA *in vivo* (52). These observations, in conjunction with our data demonstrating the reduced ability of MPL to induce IL-12 p35 and p40 mRNA expression *in vitro*, suggest that MPL may also be less efficacious than LPS at inducing endogenous IL-12. In contrast to IL-12 and IFN- γ , which amplify the host inflammatory response during endotoxin shock, IL-10 dampens the immune response to LPS. Endogenous IL-10 production has been shown to prevent uncontrolled production of TNF- α , IL-1 α , IFN- γ , and IL-12 in serum and to protect against death due to endotoxin shock and peritonitis (5, 27, 63). We had previously reported that *in vivo*, macrophages are the primary cellular source of hepatic IL-10 mRNA (52). Whether MPL is more efficacious at inducing hepatic IL-10 mRNA and/or circulating IL-10 and is less efficacious at inducing IL-12 *in vivo* is currently under investigation.

While MPL was a more potent inducer of IL-10 mRNA *in vitro*, this was not the case for all genes involved in the negative regulation of the host response to LPS, since GR and IL-1 α mRNA induction by MPL and LPS were similar at concentrations of ≥ 1 ng/ml. These data also demonstrate that differential gene expression between MPL- and LPS-treated macrophages (≥ 1 ng/ml) is not always observed. As little as 0.1 ng of LPS per ml but not of MPL, induced GR and IL-1 α mRNA expression, providing further evidence that at very low concentrations, MPL may be less effective at binding the LPS receptor and transducing intracellular signalling pathways. Previously, LPS had been shown to increase macrophage GR number in both primary macrophages and the macrophage cell line RAW 264.7 (53). Both LPS and MPL induced similar increases in GR mRNA levels, as well as ~ 25 and 50% increases in GR number (at 4 and 24 h, respectively) in RAW 264.7 cells (data not shown), confirming that increased steady-state mRNA levels were associated with increased GR number.

In vitro, tolerance to endotoxin develops after exposure to LPS and is characterized by attenuation of cytokine production from macrophages. Studies with macrophages have suggested a role for nitric oxide, IL-10, and transforming growth factor- β as mediators of LPS tolerance *in vitro* (20, 48). We demonstrated that at concentrations of MPL required to induce tolerance in macrophages (≥ 100 ng/ml [34]), IL-10 mRNA production was heightened, perhaps accounting for the ability of MPL to induce tolerance *in vitro*. The mechanisms of tolerance induced *in vitro* in macrophages and *in vivo* appear to differ. While IL-10 can potentiate the process of tolerance induction, studies with IL-10 knockout mice have shown that LPS-induced tolerance was not dependent upon IL-10 (5). Adrenalectomized mice, which lack endogenous glucocorti-

coids, do not become LPS tolerant, and administration of exogenous glucocorticoids restores tolerance in these mice (19). The finding that both MPL and LPS up-regulate macrophage GR suggests a potential mechanism whereby macrophage sensitivity to circulating glucocorticoids might be enhanced. More recently, cardiovascular tolerance to endotoxin has been linked to reduced iNOS production in the lung due to heightened production of endogenous glucocorticoids (60).

Release of local cytokines in response to antigen and adjuvant is necessary for the initiation of an immune response following vaccination. MPL has been shown to induce low levels of the proinflammatory cytokines IL-1 and TNF- α (2), as well as to induce both IFN- γ and IL-2 from lymphocyte cultures (9); these cytokines are important for T-cell activation and antibody production. Numerous studies have demonstrated the importance of IL-12 in driving IFN- γ production from T cells and NK cells (13), and MPL-induced production of IL-12 from macrophages may contribute to IFN- γ production in these systems. *In vitro* studies have demonstrated that IL-10 is an important B-cell differentiation factor (49), which synergizes with IL-2 to induce Ab production (22). This synergy is derived by IL-10 up-regulation of high-affinity IL-2 receptors on B cells (22). Thus, production of IL-10 and IL-12 from MPL-stimulated macrophages very probably plays an important role in the efficacy of MPL as an adjuvant and its ability to produce higher seroconversion rates and antibody titers in vaccine clinical trials (62).

In summary, we have demonstrated that MPL and LPS can differentially regulate the production of IL-10, IL-12, and IFN- γ . Specifically, LPS was a more potent inducer of IL-12 (p35 and p40) and IFN- γ mRNAs, while MPL was a more potent inducer of IL-10 mRNA. Enhanced production of IL-10 in MPL-stimulated macrophage cultures contributed to the suppressed production of IL-12 and IFN- γ mRNAs. Thus, these studies suggest that the difference in the ability of MPL and LPS to stimulate IL-10 production by macrophages may contribute to the reduced ability of MPL to induce proinflammatory cytokines in the serum and to its diminished toxicity.

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