Lipopolysaccharide-induced Selective Priming Effects on Tumor Necrosis Factor α and Nitric Oxide Production in Mouse Peritoneal Macrophages

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Summary

Preculture of thioglycollate-elicited C3HeB/FeJ mouse peritoneal macrophages in vitro with subthreshold stimulatory concentrations of lipopolysaccharide (LPS) can induce hyporesponsiveness (desensitization) to both tumor necrosis factor α (TNF-α) and nitric oxide (NO) production when these cells are subsequently stimulated with 100 ng/ml of LPS. We have established, however, that the primary dose of LPS required for inducing downregulation of NO production is significantly lower than that required for inducing downregulation of TNF-α production. Further, when LPS-pretreated macrophages become refractory to subsequent LPS stimulation for NO production, the secondary LPS-stimulated TNF-α production is markedly enhanced, and vice versa. These results indicate that LPS-induced TNF-α and NO production by macrophages are differentially regulated, and that the observed desensitization process may not reflect a state in which macrophages are totally refractory to subsequent LPS stimulation. Rather, our data suggest that LPS-pretreated macrophages become selectively primed for differential responses to LPS. The LPS-induced selective priming effects are not restricted to LPS stimulation, but extend as well to stimuli such as zymosan, *Staphylococcus aureus*, and heat-killed *Listeria monocytogenes*.

LPS, a major component of the Gram-negative bacteria cell wall, is well recognized for its ability to activate the host immune system and elicit a wide array of pathophysiological effects, which can result in the development of the syndrome known as endotoxic shock in both human beings and animals (1). When stimulated in vitro by LPS, macrophages produce a number of cytokines and other inflammatory mediators, including TNF-α and nitric oxide (NO), both of which contribute directly to the ability of macrophages to kill invading bacteria and tumor cells and to the pathogenesis of septic shock (2–4).

Although it has been well established that LPS can, under appropriate conditions, induce a state of hyporesponsiveness to its own effects (5), the cellular and molecular changes that contribute to LPS-induced tolerance remain to be fully defined. Two phases of "endotoxin tolerance" have been recognized. First, an early-phase tolerance to the lethal activity of LPS, which is lipid A dependent and develops within a few hours after a single injection of LPS; and second, a late-phase tolerance, which has O antigen specificity and is dependent upon the development of specific anti-LPS antibodies in the circulation. There is increasing evidence to suggest that the induction of the early tolerance to LPS by LPS pretreatment is mediated primarily by macrophages or monocytes (6–8). Recently it has been reported that PGE₂ and the protein kinase C (PKC) activator, PMA, both of which are known to be involved in the LPS-stimulated macrophage activation, act synergically to induce the LPS refractory state of macrophages, at least as assessed by TNF-α production (9). Therefore, studies of the LPS-induced desensitization of macrophages may provide important information on the biochemical mechanisms by which LPS activates macrophages in vitro, as well as on the mechanisms of LPS-induced tolerance in vivo.

LPS-induced tolerance appears to be a complex process and may vary considerably in different experimental models (10). A recent comprehensive study by Mathison et al. (11) has confirmed that subthreshold stimulatory concentrations of LPS could cause downregulation of the macrophage TNF-α response when such cells were subsequently stimulated by an activating dose of LPS either in vivo or in vitro. In another study, however, the LPS-dependent expression of IL-1 was shown not to be inhibited by preexposure of macrophages to low doses of LPS under conditions in which the LPS-stimulated TNF-α production was markedly suppressed (12). Elevation of intracellular [Ca²⁺] in response to LPS was also not suppressed by LPS pretreatment (13). Therefore, to more

fully understand the LPS pretreatment–induced tolerance, an analysis of additional macrophage responses to LPS stimulation may prove fruitful. In this manuscript, production of two macrophage-derived immune mediators, TNF-α and NO, after LPS pretreatment and/or challenge, has been investigated. The specificity of the LPS pretreatment effects has been evaluated. Our results demonstrate that LPS pretreatment does not result in a true desensitization per se, but rather selectively reorients the macrophage toward a specific response that is manifest upon subsequent stimulation with either LPS or, other macrophage activators.

Materials and Methods

Animals. Female C3HeB/FeJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and used at 6–20 wk of age.

Thioglycollate-elicited Peritoneal Macrophages. 1.5 ml 4% (wt/vol) Brewer thioglycollage (Difco Laboratories, Inc., Detroit, MI) was injected intraperitoneally into each mouse. 5 d later, cells were harvested by peritoneal lavage with 5 ml of RPMI 1640 (with glutamine and 25 mM Hepes; Whittaker M. A. Bioproducts, Walkersville, MD) containing 10% heat-inactivated FCS (Intergen Co., Purchase, NY), 4 mM additional glutamine (JRH Biosciences, Lenexa, KS), 200 U/ml penicillin, and 200 U/ml streptomycin (JRH Biosciences). The cells were washed once with the same medium, and 5 × 10⁵ peritoneal cells in the same culture medium were then added to each well of 24-well cluster plates (Costar Corp., Cambridge, MA). The cells were incubated overnight at 37°C in a humidified 5% CO₂ incubator to allow macrophages to adhere to the plates. The plates were then washed twice with 0.5 ml HBSS without Ca^{2+} and Mg^{2+} (JRH Biosciences) to remove nonadherent cells.

Stimulation of Macrophages. *Escherichia coli* 0111:B4 smooth LPS (S-LPS) was prepared as the lipid A–rich fraction II of phenol-extracted *E. coli* 0111:B4 in our laboratory (14). The LPS stock solution prepared in double-distilled water at 1–5 mg/ml was sonicated for 3 min, using a sonicator (W385; Heat-System Ultrasonics Inc., Farmingdale, NY) with output control at microtip limit 8 before being diluted into working solutions with the cell culture medium.

Various concentrations of LPS were added to the macrophage cultures as indicated. The cells were then incubated at 37°C in a humidified 5% CO₂ incubator for 18 h. Supernatants from three identically treated wells were pooled and assayed for the presence of TNF-α and NO. In all experiments in which the desensitization phenomenon was investigated, macrophages were pretreated with various subthreshold stimulatory concentrations of LPS for 6 h, washed twice with 0.5 ml HBSS, then stimulated with the effective stimulatory concentration of 100 ng/ml LPS for 18 h. In some experiments, macrophages were also stimulated with 50 μg/ml of zymosan (Sigma Chemical Co., St. Louis, MO), heat-killed *Staphylococcus aureus* at 50 or 250 bacteria per macrophage (generously provided by Dr. Chia Y. Lee, Kansas University Medical Center), or HKLM at 10⁵ to 10⁷ bacteria/ml (generously provided by Dr. Judith L. Pace, Kansas University Medical Center).

Cytotoxicity Assay for TNF-α Production. The amount of TNF-α was quantitated by assessing the extent of killing of the TNF-α-sensitive cell line L929 (15). Briefly, L929 cells were pretreated with 5 μg/ml of actinomycin D (Merck Sharp & Dohme, St. Louis, MO) for 3 h. Macrophage culture supernatants were then serially diluted into the L929 cell cultures in 96-well plates (Costar Corp.), and the plates incubated overnight at 37°C in a humidified 5% CO₂

incubator. The viability of the L929 cells was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co.) assay (16). 1 U of TNF-α activity was defined by the amount of TNF-α required to lyse 50% of the L929 cells in the assay.

Analysis of Nitric Oxide (NO). The presence of NO in macrophage culture supernatants was determined by measuring the amount of nitrite, a metabolic product of NO (17). Briefly, 100 μl of macrophage culture supernatant was mixed with 100 μl of Griess reagent (1:1, [vol/vol] of 0.1% N-[1-naphthyl] ethylenediamine dihydrochloride [NED; Sigma Chemical Co.] in H₂O/1% sulfanilamide [Sigma Chemical Co.] in 5% H₂PO₄) in round-bottomed 96-well microtiter immunoassay plates (Dynatech Labs. Inc., Chantilly, VA), and the absorbance at 570 nm was measured on a microplate reader (MR700; Dynatech Labs. Inc.). The nitrite amount was calculated from a NaNO₂ standard curve.

Endotoxin Level in the Culture System. The endotoxin levels in each component of complete culture medium were negative at the working dilution by limulus amebocyte lysate kit (sensitivity, 0.5 EU/ml; Pyrotell, Woods Hole, MA).

Results

Dose-Response Profiles for LPS Stimulation of Mouse Peritoneal Macrophages. To characterize the properties of primary LPS-induced downregulation of subsequent LPS-stimulated macrophage responses, it is important to define both the minimum and the optimal concentrations of LPS required for TNF-α and NO production. *E. coli* 0111:B4 S-LPS was used to stimulate thioglycollate-elicited C3HeB/FeJ peritoneal macrophages. TNF-α and NO production in the same culture supernatants were assessed at 18 h, and the results are shown in Fig. 1. Preliminary experiments indicated that TNF-α production was optimal after 2–4 h of LPS stimulation, whereas NO production was optimal after 12–18 h. Since TNF-α levels in the culture supernatants were still readily detectable at 18 h, assays were routinely carried out with the 18-h culture supernatants, so that both TNF-α and NO could be determined in the same culture supernatants. This representative experiment indicates that a concentration of 10 ng/ml of S-LPS is the threshold for effective stimulation of macrophages as assessed by either TNF-α or NO production, whereas significant responses were always observed with ∼100 ng/ml S-LPS. For all of the experiments described in this manuscript, 100 ng/ml of S-LPS has been defined as the effective stimulatory dose for macrophage activation in terms of TNF-α and NO production.

Different Concentrations of Primary LPS Are Required for Selective Regulation of Subsequent LPS-stimulated TNF-α and NO Production. According to Mathison et al. (11), LPS-induced hyporesponsiveness to subsequent LPS-stimulated TNF-α production can be established. In those experiments, downregulation could be detected within 6 h, and reached a maximum after 9 h of exposure of procaine-elicited peritoneal exudate rabbit macrophages to the primary LPS. A similar time course of desensitization for TNF-α and NO production was observed with thioglycollate-elicited mouse peritoneal macrophages in our experiments (data not shown).
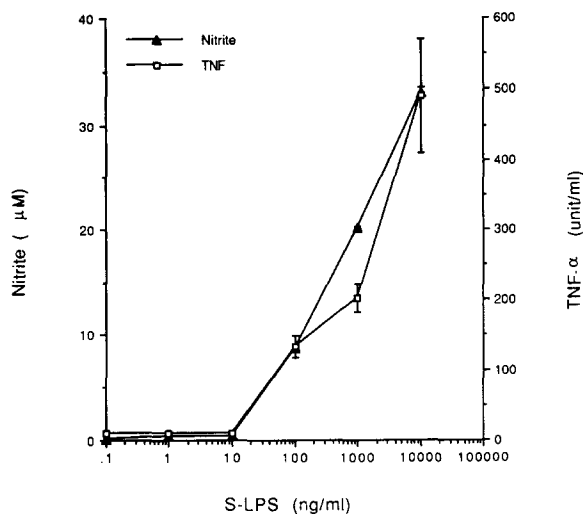
To assess the relationship between LPS-dependent NO and

Figure 1. Dose responses of LPS stimulation. Different concentrations of *E. coli* 0111:B4 S-LPS were added to thioglycollate-elicited C3HeB/FeJ peritoneal macrophage cultures as indicated. After an 18-h stimulation, the macrophage culture supernatants were collected. Supernatants from three identical treatments were pooled. The results represent three similar experiments. For TNF-α production, the data shown are the average of duplicates; bar indicates range. The amounts of NO in the same macrophage supernatants were measured by the presence of nitrite as described in Materials and Methods. Data are triplicates ± SEM.
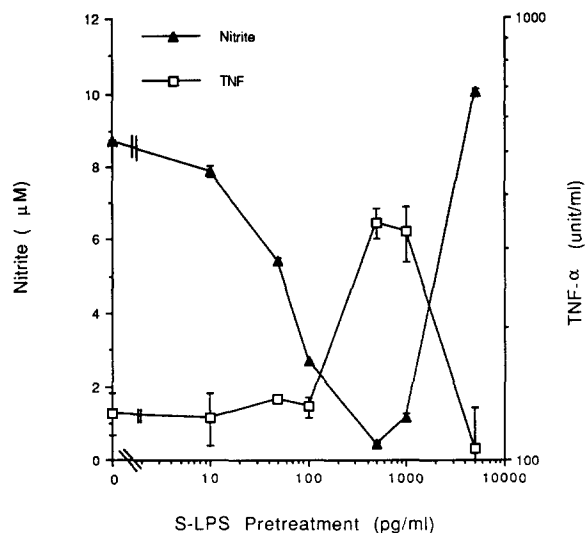
Figure 2. Different concentrations of primary LPS are required for selective regulation of subsequent LPS-stimulated TNF-α and NO production. Various concentrations of *E. coli* 0111:B4 S-LPS were added to the macrophage cultures as indicated. 6 h later, each LPS-primed macrophage culture was washed twice with HBSS, then stimulated with 100 ng/ml *E. coli* 0111:B4 S-LPS for 18 h. The amounts of both TNF-α and NO in the culture supernatants were assessed as described in Materials and Methods. Data for TNF-α production are the average of duplicates; bar indicates range. Data for NO production are triplicates ± SEM. The results are representative of three similar experiments.

TNF-α production as a function of LPS pretreatment doses, we carried out experiments in which macrophages were pretreated with different subthreshold stimulatory concentrations of S-LPS for 6 h. The cells were then washed twice with HBSS, challenged with the effective stimulatory concentration (100 ng/ml) of S-LPS for 18 h, and NO and TNF-α production from same macrophage culture supernatants were then assessed. The results of one such experiment are shown in Fig. 2. The primary dose of LPS required for the induction of downregulation of subsequent LPS-stimulated NO production was 10-fold lower than those required for the induction of downregulation of the TNF-α response. The most important observation in these experiments, however, is that when the subsequent LPS-stimulated NO production was maximally reduced as a result of pretreatment with subthreshold stimulatory concentrations of LPS, the subsequent LPS-stimulated TNF-α production in the same macrophage cultures was optimally enhanced, and vice versa. These results indicate that the macrophages pretreated with LPS are not truly hyporesponsive to the subsequent LPS stimulation; rather, they become selectively primed to respond differentially to the subsequent LPS challenge as assessed by either TNF-α or NO production.

The LPS-induced Selective Priming Effects on Macrophage Responses Are Not Restricted to LPS Stimulation. To evaluate the specificity of the LPS-induced selective priming effects, macrophages were preexposured to different subthreshold stimulatory concentrations of primary LPS for 6 h and subsequently stimulated with 50 μg/ml of zymosan for 18 h. As shown in Fig. 3 *a*, LPS pretreatment also modulated zymosan-

stimulated TNF-α and NO production. Production of TNF-α was initially enhanced and then downregulated, whereas NO production was initially suppressed and then enhanced. These results are essentially the same as noted in the earlier experiments in which LPS was used as the subsequent challenge stimuli (Fig. 2). The effect of LPS pretreatment on *S. aureus*–mediated stimulation of macrophages was also assessed, and the results are shown in Fig. 3 *b*. Of interest, *S. aureus*, by itself, did not induce any NO production at any of the concentrations tested. However, preexposure of the macrophages to subthreshold stimulatory concentrations of LPS primed the cells such that they became capable of generating NO when subsequently stimulated with *S. aureus*. LPS pretreatment also enhanced *S. aureus*–induced TNF-α production at doses equivalent to those observed earlier for LPS stimulation (Fig. 2). Stimulation of macrophages with heat-killed *Listeria monocytogenes* (HKLM), another Gram-positive bacterium, after pretreatment of these cells with the subthreshold stimulatory concentrations of S-LPS for 6 h, also resulted in the similar enhancement/inhibition profiles (data not shown). Therefore, these results demonstrate that the LPS pretreatment–induced priming effects on macrophage-derived TNF-α and NO production are not restricted to subsequent LPS stimulation.

Discussion

Preexposure of macrophages/monocytes to subthreshold stimulatory doses of LPS has been known to cause down-
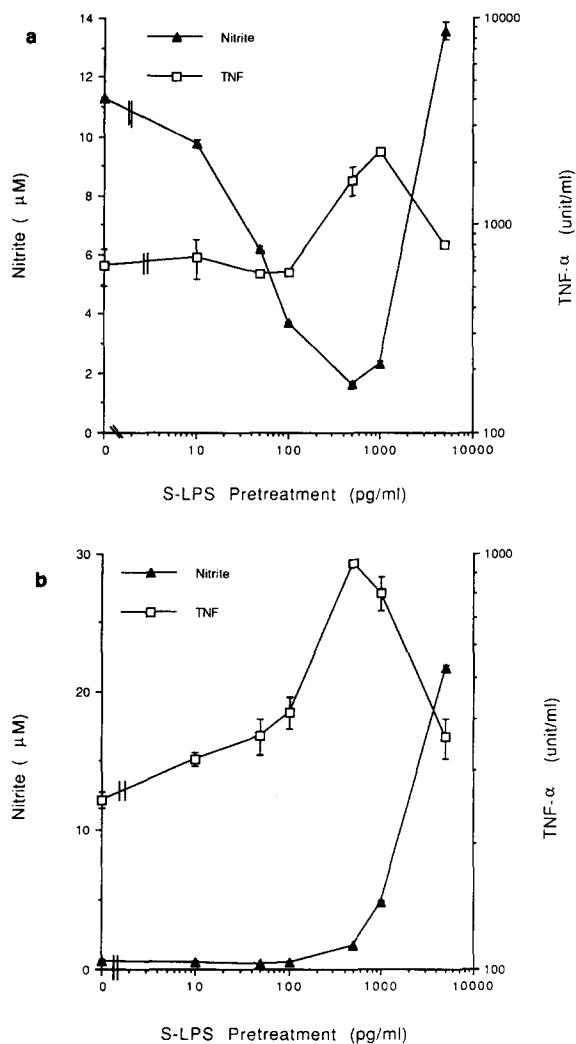
Figure 3. The LPS-induced selective priming effects are not restricted to LPS stimulation. Various concentrations of *E. coli* 0111:B4 S-LPS were added to the macrophage cultures as indicated. 6 h later, each LPS-primed macrophage culture was washed twice with HBSS, then stimulated with either 50 μg/ml zymosan (*a*) or 50 bacteria per macrophage of *S. aureus* (*b*) for 18 h. The amounts of both TNF-α and NO in the culture supernatants were assessed as described in Materials and Methods. Data for TNF-α production are the average of duplicates, bar indicates range. Data for NO production are triplicates ± SEM.

regulation of LPS-dependent responses when a single macrophage response was subsequently assessed (7, 8, 11, 18, 19). This phenomenon has been termed "desensitization." However, by simultaneously studying the effects of LPS pretreatment on two of the LPS-stimulated macrophage responses, i.e., TNF-α and NO production, using a wide range of primary LPS doses, we have shown that macrophage-derived TNF-α production can be enhanced at the LPS primary dose coincident with a suppression of NO production. Therefore, pretreatment of macrophages with low doses of LPS does not necessarily result in the macrophages becoming totally refractory to subsequent stimulation. Rather, our data allow a somewhat different view of the traditional concept of LPS

desensitization: upon exposure to the subthreshold stimulatory doses of LPS, macrophages become selectively primed or reoriented for the subsequent stimulation. We suggest, therefore, that a more appropriate terminology to define this phenomenon would be "LPS-induced selective priming effects." The primary dose of LPS appears to be critical for macrophages to select among the potential subsequent responses. The subthreshold stimulatory dose required for the upregulation of TNF-α is much lower than that required for the downregulation of TNF-α. The concentration of primary LPS used by other investigators previously in desensitization studies may not have been low enough to observe the enhancement effect on the TNF-α production.

Both TNF-α and NO are important immune effector molecules for macrophage-mediated early nonspecific host responses to nonself. We have previously shown that a G protein inhibitor, pertussis toxin (PT), can enhance LPS-dependent TNF-α production, while under the same conditions, inhibit LPS-induced NO production (20). Those results suggest that production of TNF-α and NO is differentially regulated in LPS-stimulated macrophages. Additional experimental evidence summarized in this manuscript would support this hypothesis. In this respect, we have shown that the primary LPS doses required for downregulation of TNF-α and NO production are ~10-fold different. Further, when macrophages are primed by pretreatment with LPS so as to increase TNF-α production in response to subsequent stimulation, NO production is coordinately suppressed, and vice versa. It would, therefore, be interesting to speculate that macrophages have mechanisms to select different potential strategies in response to nonself when they are exposed to different concentrations of LPS. Upon exposure to low levels of LPS, the initial macrophage response would favor TNF-α production. When the LPS level increases, the macrophage response may be switched to generate more NO at the expense of TNF-α production.

Primary LPS treatment can modulate zymosan-initiated as well as two types of Gram-positive bacteria-stimulated macrophage responses in a similar manner as that of LPS stimulation. Similar results have recently been reported (21). Therefore, the LPS-induced selective priming effects would appear not to be mediated directly by specific receptors for the stimuli used in the subsequent challenge. Rather, pretreatment of macrophages with primary LPS may initiate changes in the intracellular portion of the biochemical pathways selective for TNF-α or NO production. It is well established that LPS-induced downregulation of LPS-dependent TNF-α production can occur at the transcriptional level (9, 11, 12, 19), and that NF-κB is known to be one of the transcriptional factors required for initiation of TNF-α gene expression (22, 23). Haas et al. (9) have recently made the interesting observation that Mono-Mac-6 cells pretreated with subthreshold stimulatory doses of LPS were still able to induce NF-κB in the nucleus upon subsequent LPS challenge, although the LPS-stimulated TNF-α mRNA level was, nevertheless, dramatically reduced. Considering this observation, and the results described in this manuscript, it is reasonable to speculate that the primary LPS may initiate some common steps in signal transduction, and

selectively modulate nonself-stimulated macrophage responses at a level subsequent to activation of transcriptional factors, such as NF-κB.

It has been reported that IFN-γ and TNF synergize to induce NO production, and that anti-TNF antibodies will partially suppress NO production induced by costimulation of murine peritoneal macrophages with IFN-γ and LPS (24). These results indicate that endogenously produced TNF is involved in the induction of NO. However, Ding et al. (3) have tested 12 cytokines for their capacity to generate NO and showed that only IFN-γ can induce substantial NO production on its own. TNF-α does not stimulate NO synthesis by itself, although this cytokine could enhance IFN-γ-induced NO production (3, 24). Further, experiments carried out in our laboratory have shown that combination of

TNF-α and IFN-β also did not induce NO production by mouse peritoneal macrophages (X. Zhang and D. C. Morrison, unpublished observation). These results suggest that either IFN-γ or LPS, in addition to TNF-α, is required for macrophages to produce NO. Data described here have demonstrated that pretreatment of macrophages with subthreshold stimulatory concentrations of LPS enhanced subsequent LPS-stimulated NO production while the TNF-α production was downregulated. Therefore, macrophage-derived endogenous TNF-α seems not to play a role in priming macrophages in terms of NO production. These results support the concept that different mechanisms may be involved in priming and stimulating macrophages to produce NO. Further studies need to be carried out to clarify the regulatory functions of TNF-α and IFNs in these processes.

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