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Transforming Growth Factor β1 and Gamma Interferon Provide Opposing Signals to Lipopolysaccharide-Activated Mouse Macrophages

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Bacterial lipopolysaccharides (LPS) are potent inducers of macrophage activation, leading to the production of a number of proinflammatory mediators. Although several cytokines that prime macrophages for enhanced LPS-triggered responses have been identified, far less is known regarding the role that cytokines play in down-regulating macrophage responses to LPS. This study was designed to determine the effects of recombinant transforming growth factor β1 (rTGF-β1) on macrophage activation by LPS. Pretreatment of either mouse peritoneal macrophages or cells of the RAW 264.7 macrophage-like cell line with rTGF-β1 inhibited their ability to produce both tumor necrosis factor alpha (TNF-α) and nitric oxide (NO) in response to LPS. These inhibitory effects were reversed by increasing the concentration of LPS or by priming cells with optimal concentrations of recombinant gamma interferon (rIFN-γ). Pretreatment of cells with rTGF-β1 had only a modest inhibitory effect on the expression of TNF-α mRNA. By contrast, the expression of mRNA for the inducible form of nitric oxide synthase (iNOS), which is responsible for NO production in activated macrophages, was significantly inhibited by rTGF-β1 pretreatment. Thus, rTGF-β1-dependent suppression of macrophage TNF-α biosynthesis was manifest at a posttranscriptional level, whereas the inhibition of NO production correlated with a direct effect on iNOS gene expression. Importantly, both of these suppressive effects of rTGF-β1 were reversed by exposing the cells to priming concentrations of rIFN-γ. As with NO production, immunocytochemical analysis of iNOS expression in LPS-stimulated macrophages revealed that rIFN-γ and rTGF-β1 had antagonistic effects, with the former increasing, and the latter reducing, the number of iNOS-expressing cells induced by LPS. These data suggest that a balance between the priming effects of IFN-γ and the inhibitory effects of TGF-β1 can determine the overall level of macrophage activation induced by LPS.

Macrophage responses to bacterial endotoxic lipopolysaccharides (LPS) include the production of a number of potent proinflammatory mediators. While extensive studies have demonstrated that many cell-derived factors, including cytokines, can enhance LPS-initiated macrophage activation, much less is known about negative regulation of these responses. Recent attention has focused on the cytokine transforming growth factor β1 (TGF-β1) as a potential inhibitor of macrophage activation (2, 31), and evidence supporting a role for TGF-β1 includes the recent finding that transgenic mice lacking the ability to produce TGF-β1 constitutively express the TNF-α gene (35). Treatment of macrophages or macrophage-like cell lines in vitro with TGF-β1 has been reported to inhibit their ability to initiate a respiratory burst (37, 38), to produce cytokines (3, 5, 8) and reactive nitrogen intermediates (7, 39), and to kill intracellular microbes (9, 22, 24, 36) or extracellular tumor cells (12, 14, 29) following activation. Experiments with neutralizing anti-TGF-β1 antibodies (1, 7, 38) have further suggested a role for this cytokine in macrophage activation. Observations regarding the pathogenesis of multifocal inflammatory disease in TGF-β1-deficient transgenic mice (16, 35) are consistent with the findings from these in vitro studies that indicate that TGF-β1 regulates inflammatory responses. Furthermore, cytokine genes that are normally expressed at high levels by lymphocytes and macrophages only upon cell activation are constitutively expressed in TGF-β1 knockout transgenic mice (35). In contrast to these observations, a clear role for TGF-β1 in host responses to bacterial endotoxin has not been firmly established. Moreover, although the mechanisms by which TGF-β1 inhibits responses to cytokines (5, 7, 24, 36, 39) or phorbol esters (36) are becoming better defined, less attention has been paid to the mechanisms by which TGF-β1 inhibits macrophage responses induced by LPS (3).

Important proinflammatory mediators produced by LPS-activated mouse macrophages include tumor necrosis factor alpha (TNF-α) and nitric oxide (NO). Evidence indicates that the biosyntheses of these two macrophage products are regulated independently (43, 44), suggesting that the induction of their synthesis does not necessarily involve a common biochemical activation pathway. For this reason, we have examined whether TGF-β1 inhibits both LPS-induced TNF-α and NO responses and whether the mechanisms of inhibition are the same in both cases. Because gamma interferon (IFN-γ) is a potent priming agent for macrophage activation by LPS and significantly potentiates the production of both TNF-α and NO (17, 34), we also determined whether TGF-β1 suppressed responses to LPS by recombinant IFN-γ (rIFN-γ)-primed cells.

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MATERIALS AND METHODS

Reagents. Mouse rIFN-γ (specific activity, 5.2×10^6 U/mg), human rTNF-α (specific activity, 6.27×10^7 U/mg), and human

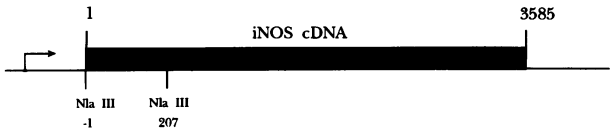

FIG. 1. Construction of a probe specific for mouse macrophage iNOS. An *Nla*III restriction fragment corresponding to the first 207 bp of the coding region of the mouse iNOS gene, which is not present in mouse neuronal or endothelial NOS (33, 40) genes, was subcloned from a 4,110-bp mouse iNOS cDNA (17, 19). This 208-bp *Nla*III fragment was cloned into the *Sph*I site of the pGEM-3Z vector, yielding plasmid pNOS207Ts. Labeled antisense transcripts were generated by transcription with SP6 RNA polymerase in vitro.

rTGF-β1 (protein concentration, 73 μg/ml) were obtained from Genentech Inc. (South San Francisco, Calif.). A rabbit antibody to the inducible form of mouse nitric oxide synthase (iNOS) was prepared by immunizing rabbits with a synthetic peptide specific for mouse iNOS (18). The antiserum was then affinity purified by adsorption to immobilized iNOS peptide conjugated to bovine serum albumin. Rabbit immunoglobulin G (IgG) was purchased from Chemicon International Inc. (Temecula, Calif.). Phenol-extracted LPS from *Escherichia coli* O111:B4 was obtained from List Biological Laboratories (Campell, Calif.).

Mice. Female C3HeB/FeJ mice (6 to 8 weeks old) were purchased from Jackson Laboratory (Bar Harbor, Maine) and were maintained in the Laboratory Animal Resources Center at the University of Kansas Medical Center. All animal experimentation protocols were approved by the Medical Center Animal Care and Use Committee.

Cells and cell culture. Mouse peritoneal macrophages were elicited by injecting 2 ml of Brewer's thioglycolate into the peritoneal cavities of C3HeB/FeJ mice. Four days later, cells were harvested by lavage, washed, resuspended in complete medium (RPMI 1640 medium containing 2 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% fetal bovine serum [JRH Bioscience, Lenexa, Kans.]), and dispensed into 24- or 96-well tissue culture plates. After 2 to 4 h to allow for macrophage adherence, the monolayers were washed three times with warm Hanks' balanced salt solution (JRH Bioscience) and cultured in complete medium. The mouse macrophage-like cell line RAW 264.7 was kindly provided by Stephen Russell (University of Kansas Medical Center, Kansas City). All cultures were maintained at 37°C in 5% CO_2–95% air. All reagents used in tissue culture, when tested at their final concentrations, contained less than 5 pg of LPS per ml, as determined by the *Limulus* amebocyte lysate assay (Whittaker Bioproducts, Walkersville, Md.).

Assays for inflammatory mediators. The concentration of TNF-α in macrophage culture media was determined by the L929 cell bioassay as previously described (27), using human rTNF-α (Genentech) as a standard. Nitrite in macrophage culture fluids was measured by using the Griess reagent (11). Optical densities of the assay samples were then measured on a Dynatech MR700 plate reader at 570 nm and compared against a standard curve prepared with sodium nitrite.

cRNA probes. A full-length iNOS cDNA, ligated in the *Eco*RI site of a Bluescript M13 vector, was obtained from Stephen Russell (Cancer Center, University of Kansas Medical Center) (17, 19). An *Nla*III fragment corresponding to the first 207 bp of the iNOS 5′ coding region (Fig. 1) was subcloned into a pGEM-3Z vector (Promega, Madison, Wis.) to generate plasmid pNOS207Ts. To synthesize specific iNOS antisense probes, pNOS207Ts was linearized with *Eco*RI and transcribed with SP6 RNA polymerase (Promega). A similar construct containing a 300-bp *Pvu*II fragment of cDNA corresponding to the coding region of the mouse TNF-α gene was obtained from Genentech (28). This fragment had been subcloned into the *Sma*I site of pSP64. For probe synthesis, this plasmid was linearized with *Eco*RI and transcribed with SP6 RNA polymerase. A pGEM construct containing the 1,600-bp cDNA insert coding for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was also generously provided by Stephen Russell. This construct was linearized with *Hin*dIII, and RNA complementary to the G3PDH sequence was transcribed by T7 RNA polymerase (Promega). Probes were synthesized for high specific activity as described by Melton et al. (20). The transcription mixtures contained 3.5 μCi of [α-^{32}P]CTP (3,000 Ci/mmol) per μl, 0.5 mM ATP, GTP, and UTP, and 0.012 mM CTP.

RNA extraction and Northern (RNA) blot analysis. Total cellular RNA was isolated from peritoneal macrophages, bone marrow culture-derived macrophages, and RAW 264.7 cells by a modification of the guanidine isothiocyanate method described previously (6). Cells were first lysed on ice with 4 M guanidine thiocyanate–25 mM sodium citrate (pH 7)–0.5% sarcosyl–100 μM β-mercaptoethanol. The lysates were then combined with 1/10 volume of 2 M sodium acetate (pH 4) and extracted with an equal volume of phenol (equilibrated with 20 mM sodium acetate [pH 5.2]) containing 0.1% 8-hydroxyquinoline. Phase separation was accomplished by mixing with 1/3.5 volume of chloroform-isoamyl alcohol (50:1). After an additional chloroform-isoamyl alcohol extraction, the RNA was precipitated with isopropanol. A second ethanol precipitation was performed for isolating peritoneal macrophage RNA. The precipitated RNA was then dissolved in 0.5% sodium dodecyl sulfate (SDS) for 15 min at 65°C and mixed on a vortex mixer for 20 min at room temperature. Prior to gel electrophoresis, 6- to 8-μg samples of RNA were boiled for 2 min in sample loading buffer (57% formamide, 7.4% formaldehyde in 1× morpholinepropanesulfonic acid [MOPS] buffer [20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0], 200 μg of freshly added ethidium bromide per ml) and then chilled on ice.

For Northern blot analysis, samples were electrophoresed with 1× MOPS buffer–7.4% formaldehyde in 1% agarose gels prepared with 1× MOPS buffer–5% formaldehyde. They were then transferred overnight to Gene Screen nylon membranes (DuPont, Boston, Mass.) by capillary blotting. The membranes were dried and baked for 2 h at 80°C. Prehybridization of the membranes was performed for at least 2 h at 42°C in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50 mM potassium phosphate) (pH 6.5)–5× Denhardt's reagent (0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone)–250 μg of denatured salmon sperm DNA per ml–0.5% SDS. The membranes were then hybridized at 55°C for 10 to 18 h in the same solution containing the respective ^{32}P-labeled RNA probe at a concentration of 50 ng/ml. Blots were then washed twice for 5 min at room temperature in 2× SSC and twice at 72°C for 5 min in 0.15% SDS–0.1× SSC (15). The membranes were exposed to X-ray films (Konica, Newark, N.J.) at −70°C, using intensifying screens. After probing with either the iNOS- or the TNF-α-specific probe, the membranes were stripped for 20 min at 95°C in 10 mM Tris-HCl (pH 8)–1 mM EDTA–1% SDS and reprobed to assess G3PDH mRNA expression as a means of ensuring equal loading and transfer of RNA samples in each lane. As an additional control for even sample loading and RNA degradation, 1/10 of each sample was loaded on an analytical agarose gel containing 450 ng of ethidium bromide

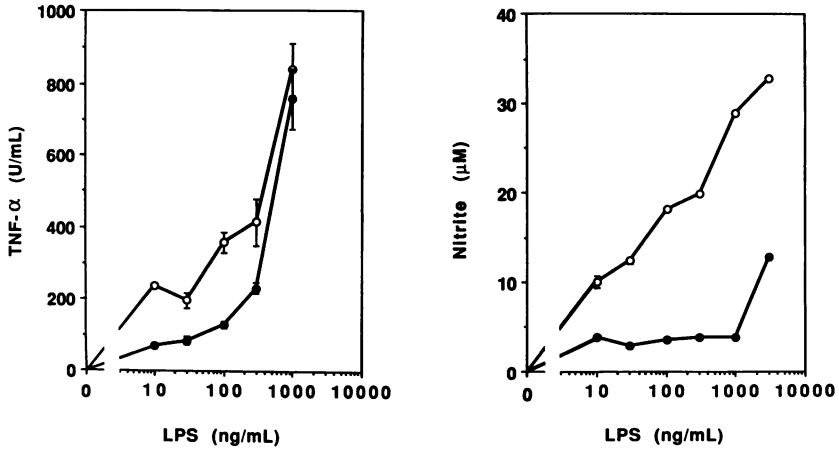

FIG. 2. Effects of rTGF-β1 on TNF-α and NO production by LPS-activated mouse peritoneal macrophages. Thioglycolate-elicited peritoneal mouse macrophages were pretreated for 6 h with medium (○) or 4 ng of rTGF-β1 per ml (●), the indicated concentrations of LPS were added, and the macrophages were incubated for an additional 18 h. Cell-free culture fluids were collected and assayed for TNF-α and nitrite. The data shown are mean responses ± 1 standard deviation. Values for standard deviations were always less than 10% of the means. The experiment was performed twice with similar results.

per ml and 1× TAE buffer (40 mM Tris acetate, 15 mM EDTA, pH 8.4), and the relative stainings of 18S and 28S rRNA for each sample were compared.

Quantitation of Northern blot results was performed with a scanning densitometer (EC Apparatus Corp., St. Petersburg, Fla.). All values obtained with the specific TNF-α or iNOS probes were corrected for slight differences in RNA sample loading by normalizing to the values obtained with the G3PDH probe. For each experiment, arbitrary densitometry units were then calculated by assigning a value of 100 to the reading obtained with maximally activated cells that were not exposed to rTGF-β1.

Immunocytochemistry. Immunocytochemical identification of iNOS-positive cells was performed with a horseradish peroxidase kit (Zymed Laboratories, South San Francisco, Calif.). Briefly, 1.75×10^5 peritoneal mouse macrophages were allowed to adhere in each chamber of eight-chamber Lab-Tek culture slides (Nunc Inc., Naperville, Ill.). Adherent macrophages were then cultured with either medium, LPS, rIFN-γ, or LPS plus rIFN-γ. Fifteen hours later, the monolayers were washed three times with RPMI 1640, air dried, fixed in acetone, air dried overnight, and rehydrated with phosphate-buffered saline. Nonspecific binding sites were blocked with 10% goat serum. Slides were then incubated with either rabbit anti-iNOS (5 μg/ml) or nonimmune rabbit IgG (5 μg/ml) for 60 min at 37°C. Bound specific antibody or control IgG was detected with the commercial kit, using biotinylated goat anti-rabbit IgG as a second antibody and streptavidin-conjugated horseradish peroxidase–3-amino-9-ethyl carbazole as the peroxidase substrate. Endogenous cellular peroxidase was inactivated by exposure of the monolayers to 3% H_2O_2. Care was taken to maintain moisture on the cell monolayers throughout the procedure. Following immunocytochemical staining, the cells were counterstained with hematoxylin and mounted with Crystal/Mount (Biomeda Corp., Foster City, Calif.) followed by Permount (Fisher Scientific, St. Louis, Mo.).

RESULTS

Recombinant TGF-β1 inhibits LPS-induced TNF-α and NO production by mouse macrophages and RAW 264.7 cells. The first goal of this study was to determine the effects of rTGF-β1 on TNF-α and NO production by LPS-stimulated mouse macrophages. Initial experiments determined that maximum effects were seen with rTGF-β1 at a concentration of 4 ng/ml when cells were pretreated for at least 6 h. Therefore, we used these conditions throughout this study, unless otherwise indicated.

As shown in Fig. 2, rTGF-β1 inhibited TNF-α and NO production by peritoneal macrophages that were activated with 10 to 300 ng of LPS per ml. However, responses to higher concentrations of LPS were not as effectively inhibited by rTGF-β1 pretreatment, and rTGF-β1-treated cells activated with high concentrations of LPS produced significant quantities of TNF-α and NO. Similar results were obtained with resident and peptone-elicited peritoneal macrophages, bone marrow culture-derived macrophages, and cells of the RAW 264.7 and J774.1 macrophage-like cell lines (data not shown).

rIFN-γ enhances and rTGF-β1 suppresses the production of NO and TNF-α by LPS-activated mouse peritoneal macrophages and RAW 264.7 cells. The interferons, particularly IFN-γ, are known to prime macrophages for enhanced responses to LPS, with regard to production of both TNF-α and NO (17, 26, 34, 41). Therefore, we next determined whether a relationship existed between the ability of rIFN-γ to augment, and rTGF-β1 to suppress, LPS-induced responses in these cells. The results shown in Fig. 3 confirm earlier reports that responses to 0.5 to 1.0 ng of LPS per ml are greatly enhanced by the addition of rIFN-γ in the concentration range from 0.01 to 3 U/ml. Pretreatment of the cells with rTGF-β1 inhibited these responses by both peritoneal macrophages and RAW 264.7 cells; however, this inhibition was most apparent at the lower priming concentrations of rIFN-γ (i.e., <1 U/ml). Cells that had been pretreated with rTGF-β1 and then stimulated with LPS and >1 U of rIFN-γ per ml produced significant quantities of both TNF-α and NO and often showed responses that approximated those of activated cells that had not been exposed to rTGF-β1. This was particularly apparent in the case of RAW 264.7 cells. Thus, rTGF-β1 inhibited LPS–rIFN-γ-activated cells, but the effects of the cytokine were readily reversed simply by increasing rIFN-γ concentrations.

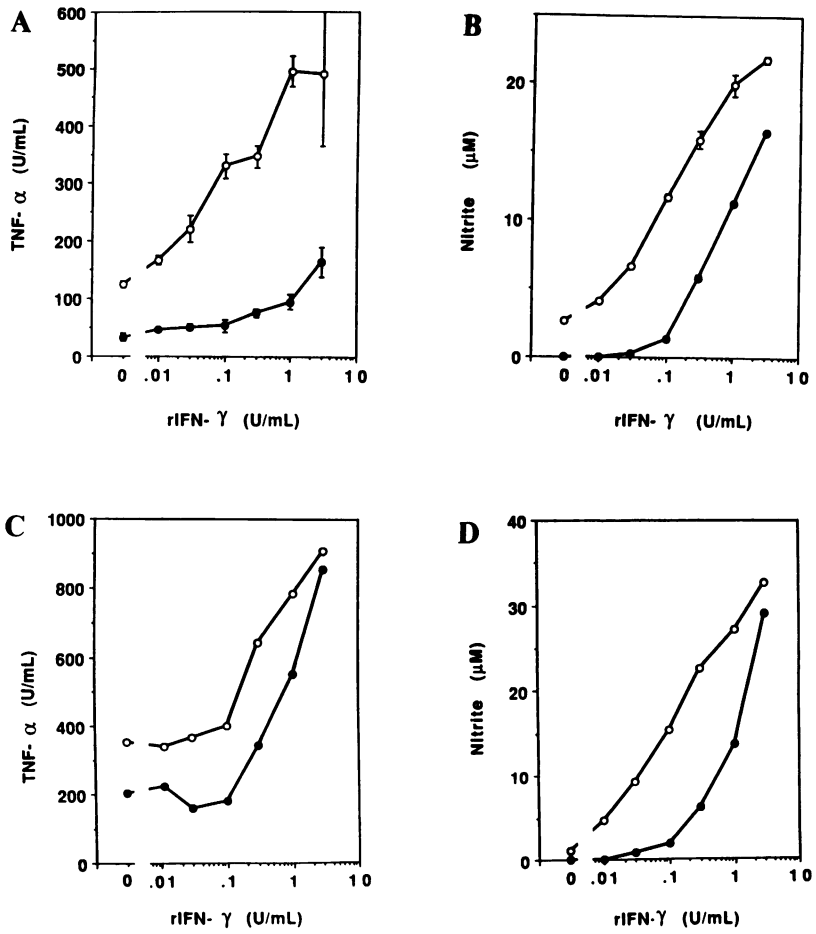

FIG. 3. Effects of rTGF-β1 on production of TNF-α and NO by mouse peritoneal macrophages and RAW 264.7 cells activated by LPS and IFN-γ. Peritoneal mouse macrophages (A and B) or RAW 264.7 cells (C and D) were cultured in the presence of medium (○) or 4 ng of rTGF-β1 per ml (●) for 6 h. They were then stimulated with either 0.5 (A and B) or 1 (C and D) ng of LPS per ml and the indicated concentrations of rIFN-γ. Eighteen hours later, culture fluids were collected and assayed for TNF-α and nitrite. The data shown are representative of four experiments and include mean responses ± 1 standard deviation. With the exception of a single datum point in panel A, standard deviation values were always less than 15% of the mean values.

Effects of rTGF-β1 and rIFN-γ on TNF-α and iNOS gene expression in LPS-stimulated cells. iNOS plays a key role in the regulation of NO biosynthesis by activated mouse macrophages (21). To determine whether rTGF-β1 inhibited NO synthesis by regulating iNOS gene expression, RAW 264.7 cells, peritoneal macrophages, or bone marrow culture-derived macrophages were pretreated with either medium or rTGF-β1 and then activated with LPS as described above. Then TNF-α and iNOS mRNA levels were measured by Northern analysis on total cellular RNA prepared 1 to 3 and 12 h, respectively, after stimulation of the cells with LPS. Membranes were then stripped and reprobed with the G3PDH probe as a control for RNA loading and transfer. In RAW 264.7 cells, rTGF-β1 showed only marginal effects on TNF-α mRNA levels (Fig. 4A). By contrast, the LPS-dependent activation of iNOS gene expression was significantly inhibited by pretreating the cells with rTGF-β1. These effects are most apparent when one compares the densitometric scans of the autoradiographs shown in Fig. 4C. The intensity of hybridization of the G3PDH probe was not related to the degree of activation of the cells, whereas the G3PDH signal did correlate well with ethidium bromide staining of 18S rRNA. Similar results were obtained with thioglycolate-elicited peritoneal macrophages activated with either 1 or 100 ng of LPS per ml (Fig. 4B and D) as well as bone marrow culture-derived macrophages (data not shown). Thus, there was a clear difference in the level at which the biosynthesis of these two macrophage inflammatory mediators was regulated by rTGF-β1 in LPS-activated cells. Whereas rTGF-β1 significantly reduced LPS-induced iNOS mRNA expression, the inhibition of TNF-α biosynthesis was not accompanied by a significant reduction in TNF-α mRNA levels.

Similar results were obtained with cells stimulated with LPS plus rIFN-γ. Peritoneal macrophages cultured with 1 ng of LPS per ml expressed high levels of TNF-α mRNA (Fig. 5), and the amount of the transcript was increased approximately threefold by the addition of rIFN-γ. A significant augmentation in secreted TNF-α in macrophage culture fluids accompanied this change. Pretreatment of the cells with rTGF-β1 only slightly altered the pattern of TNF-α mRNA expression (Fig. 5B). In contrast to the results obtained with TNF-α, the same cells expressed very low levels of iNOS mRNA when stimulated with 1 ng of LPS per ml alone, but this response was greatly enhanced by the addition of rIFN-γ (0.03 to 3 U/ml). Again,

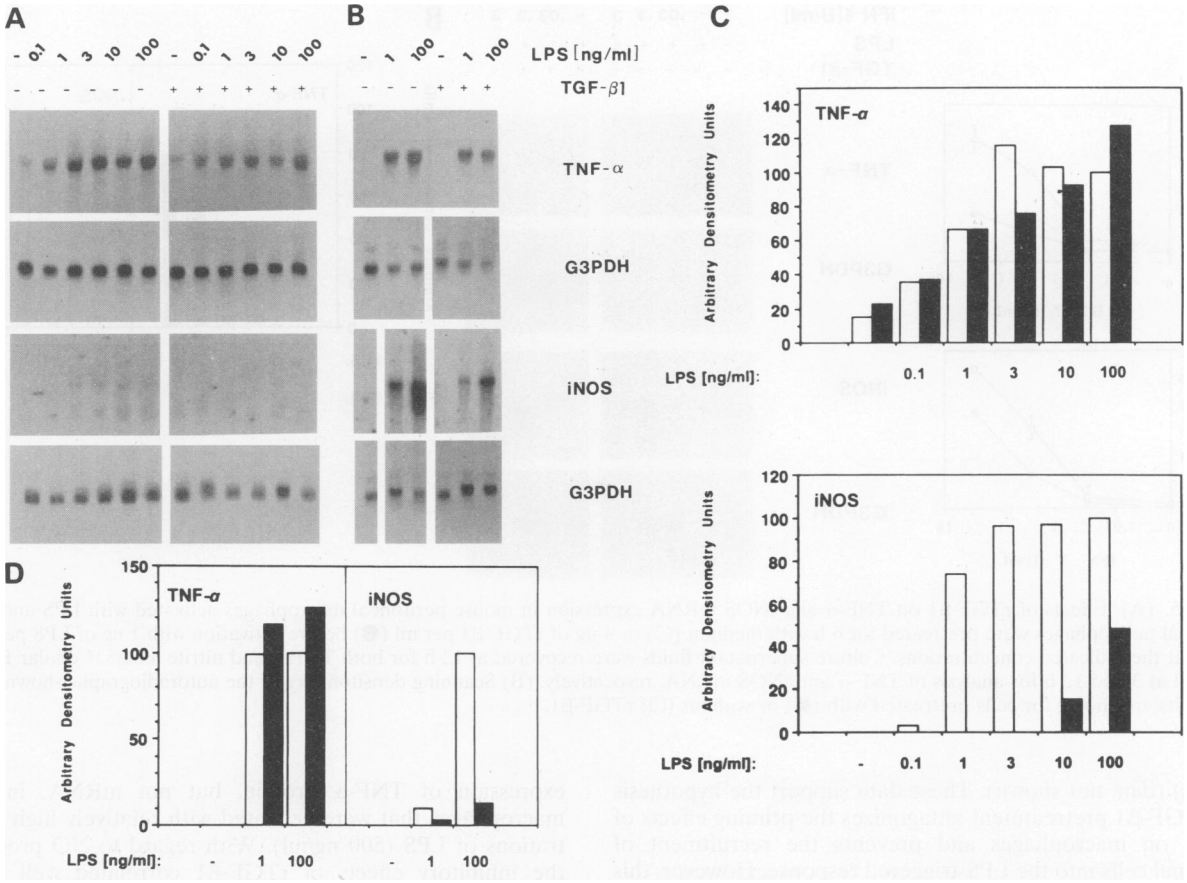

FIG. 4. Northern blot analysis of TNF-α and iNOS gene expression in rTGF-β1-treated, LPS-activated cells. RAW 264.7 cells (A) or peritoneal macrophages (B) were cultured in the presence or absence of 4 ng of TGF-β1 per ml for 6 h and stimulated with the indicated concentrations of LPS for 1 h (TNF-α) or 12 h (iNOS). Total RNA was then isolated and analyzed by Northern blot hybridization. The hybridization was first performed with either a radiolabeled TNF-α or radiolabeled iNOS probe, and then the membranes were stripped and reprobed with a radiolabeled G3PDH probe as an internal control. This experiment was repeated two times with similar results. (C) Scanning densitometry of the autoradiographs shown in panel A. Results are shown for cells pretreated with (■) or without (□) rTGF-β1. (D) Scanning densitometry of the autoradiographs shown in panel B.

pretreatment of the cells with rTGF-β1 significantly inhibited LPS–rIFN-γ-induced iNOS gene expression (Fig. 5B), as was observed for cells stimulated with LPS alone (Fig. 4D). However, it should be noted that the inhibitory effects of rTGF-β1 on iNOS mRNA expression were much less evident at the higher concentrations of rIFN-γ (3 U/ml; Fig. 5B). Similar results were obtained with RAW 264.7 cells (data not shown). Thus, in many ways the opposing effects of rTGF-β1 and rIFN-γ on iNOS gene expression paralleled those observed for NO production. Taken together, these findings indicate that rIFN-γ and rTGF-β1 have distinct and contrasting effects on LPS-activated mouse macrophages. Although the production of TNF-α and NO appears to be regulated by different mechanisms, the antagonism between rIFN-γ and rTGF-β1 in regulating these responses is readily apparent in each case.

Regulation by rTGF-β1 of iNOS protein expression at the cellular level. We next determined what effect rTGF-β1 had on intracellular iNOS protein expression in activated macrophages. Because it has been reported that rIFN-γ increases the proportion of LPS-stimulated macrophages that express iNOS protein (25, 40), we predicted that rTGF-β1 treatment should be able to prevent this recruitment effect. To measure the expression of iNOS, immunocytochemical analyses were performed with a monospecific anti-iNOS antibody. Peritoneal macrophages were pretreated with rTGF-β1 as described above and then stimulated with activating concentrations of LPS plus rIFN-γ. Fifteen hours later, the cells were fixed and treated with either anti-iNOS antibody or nonimmune rabbit IgG.

The results shown in Table 1 demonstrate several important points. As reported earlier (25), peritoneal macrophages activated with LPS alone showed a very low frequency of iNOS-positive cells, as was also the case for cells treated only with 0.3 or 3 U of rIFN-γ per ml (data not shown). The addition of rIFN-γ to LPS-stimulated cultures greatly enhanced iNOS expression, with nearly all of the cells scoring iNOS positive at an rIFN-γ concentration of 3 U/ml. Cells pretreated with rTGF-β1 showed a distinctly different pattern of staining that correlated well with both the pattern of NO production and iNOS mRNA expression in these cells. Pretreatment with rTGF-β1 followed by activation with LPS plus 0.3 U of rIFN-γ per ml resulted in only a small proportion of the cells scoring positive (Table 1), a pattern similar to that seen with cells not exposed to rTGF-β1 but stimulated with LPS. When the concentration of rIFN-γ was increased to 3 U/ml, the frequencies of iNOS-positive cells in the two treatment groups were comparable. No staining was detected when nonimmune rabbit IgG was used instead of antibody, regardless of the activating

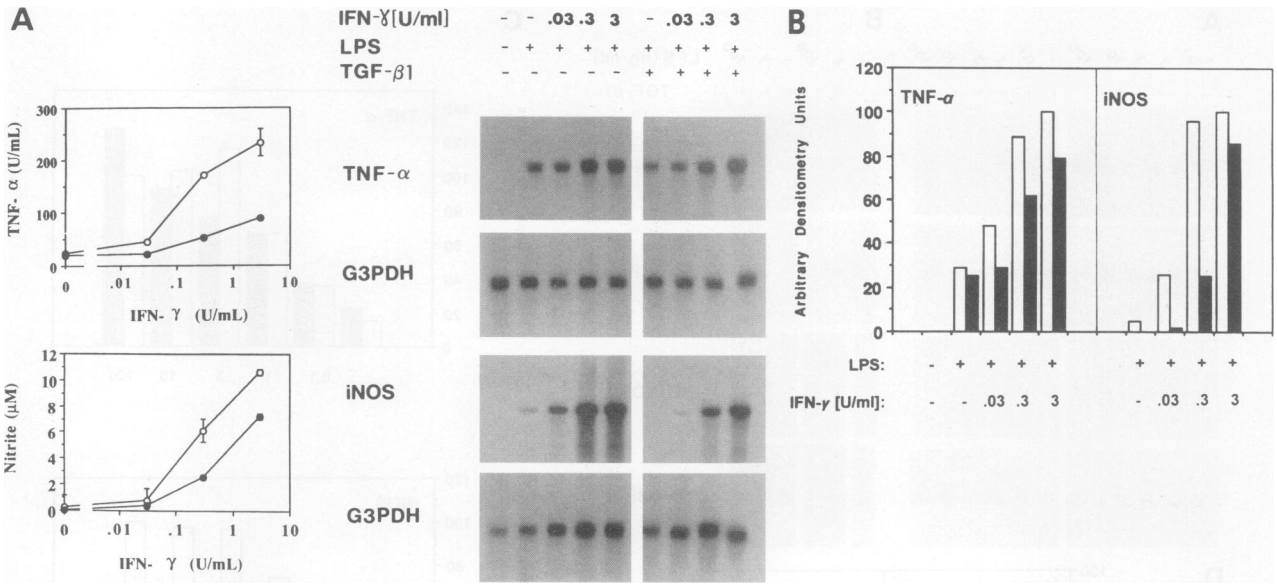

FIG. 5. (A). Effects of rTGF-β1 on TNF-α and iNOS mRNA expression in mouse peritoneal macrophages activated with LPS and rIFN-γ. Peritoneal macrophages were pretreated for 6 h with medium (○) or 4 ng of rTGF-β1 per ml (●) before activation with 1 ng of LPS per ml plus rIFN-γ at the indicated concentrations. Culture supernatant fluids were recovered at 12 h for both TNF-α and nitrite assays. Cellular RNA was prepared at 3 and 12 h for analysis of TNF-α and iNOS mRNA, respectively. (B) Scanning densitometry of the autoradiographs shown in panel A. Results are shown for cells pretreated with (■) or without (□) rTGF-β1.

agent(s) (data not shown). These data support the hypothesis that rTGF-β1 pretreatment antagonizes the priming effects of rIFN-γ on macrophages and prevents the recruitment of additional cells into the LPS-triggered response. However, this effect was readily overcome by higher concentrations of rIFN-γ.

DISCUSSION

The results reported here provide strong support for the hypothesis that rTGF-β1 inhibits the production of both TNF-α and NO by LPS-activated mouse macrophages. Of particular significance is the finding that the mechanisms of inhibition of these two mediators by rTGF-β1 are distinctly different. The biosynthesis of TNF-α appears to be regulated at a posttranscriptional level by rTGF-β1 since only very modest reductions in TNF-α mRNA levels were seen in rTGF-β1-pretreated cells. Our results confirm and extend those reported by Bogdan et al. (3), who found that rTGF-β1 inhibited the expression of TNF-α protein, but not mRNA, in mouse macrophages that were activated with relatively high concentrations of LPS (500 ng/ml). With regard to NO production, the inhibitory effects of rTGF-β1 correlated well with its suppression of iNOS protein and mRNA expression, suggesting direct inhibition by rTGF-β1 of iNOS gene transcription. Preliminary analyses of iNOS mRNA half-lives in rTGF-β1-treated macrophages are consistent with this conclusion. It is noteworthy that these differences between the regulation of TNF-α and NO biosynthesis were observed regardless of the cell type tested or whether the macrophages were activated with LPS alone or substimulatory concentrations of LPS in the presence of priming concentrations of rIFN-γ.

TABLE 1. Effects of rTGF-β1 on iNOS protein expression in activated peritoneal macrophages

Pretreatment with rTGF-β1	Activator		% iNOS$^+$ cells[a]	Nitrite concn (μM)
	LPS (1 ng/ml)	rIFN-γ (U/ml)		
−	−	0	0	0.2
−	+	0	0.8	2.0
−	+	0.3	70.0	10.6
−	+	3.0	94.0	16.6
+	−	0	0	0.4
+	+	0	0	0.7
+	+	0.3	10.6	4.9
+	+	3.0	84.0	12.7

[a] Five hundred cells were scored for each entry.

The results of this study, however, contrast with earlier published findings of Ding et al. (7), who reported that LPS by itself was a poor inducer of NO production in mouse macrophages. In these studies, mouse peritoneal macrophages were instead activated with rIFN-γ alone at concentrations (>50 U/ml), which were substantially higher than those used in our study. The authors reported that under these conditions, the inhibitory effects of rTGF-β1 were seen over a 100-fold range in rIFN-γ concentrations and that concentrations as high as 1,000 U/ml failed to reverse the inhibition of macrophage NO production caused by rTGF-β1. Similar results were obtained with macrophages that were activated with a combination of rIFN-γ and rTNF-α. In contrast, in the studies reported both here and in an earlier report (29), rTGF-β1 inhibited mouse macrophage responses to LPS and LPS plus rIFN-γ, but this effect was reversed by simply increasing the concentration of either LPS or rIFN-γ. Thus, there appears to exist a fundamental difference between the effects of rTGF-β1 on macrophages that are activated with a cytokine(s) alone versus those cells that are exposed to low concentrations of LPS plus rIFN-γ.

Pretreatment of macrophages with low concentrations of LPS can significantly modify subsequent responses by the cells

to activating concentrations of LPS (4). Because our experimental protocol involved pretreating cells with rTGF-β1 for 6 h, we wished to exclude the possibility that inhibition of subsequent LPS-initiated responses was due to the desensitizing effects of contaminating LPS in our rTGF-β1 preparations. Two observations argue against this interpretation. First, in the *Limulus* amebocyte lysate assays, we were unable to detect the presence of LPS in these samples at concentrations above 5 pg/ml. Significant changes in macrophage responses to activating stimuli require pretreatment with at least five times this concentration of LPS (43). Second, on the basis of the studies of Zhang and Morrison (43), one would not anticipate that pretreatment of macrophages with rTGF-β1 would lead to an inhibition of production of both NO and TNF-α in response to subsequent LPS challenge if the effects of the rTGF-β1 were due to a minor LPS contaminant. These investigators clearly showed that such pretreatment leads to a divergence in these two responses, with one response being inhibited while the other is potentiated.

A noteworthy finding from this study was the ability of rTGF-β1 to inhibit macrophage responses to low, but not high, concentrations of activating agents. This was true not only when cells were activated with LPS alone but also when cells were primed with various concentrations of rIFN-γ and triggered with a suboptimal concentration of LPS (1 ng/ml). Likewise, both resident and elicited peritoneal macrophages, bone marrow culture-derived macrophages, and cells of the RAW 264.7 and J774.1 lines showed this effect. Thus, it would appear that there exists an antagonism between TGF-β1 and IFN-γ in the regulation of LPS-activated mouse macrophages. A similar relationship may also exist between TGF-β1 and IFN-α/β, as there is increasing evidence indicating that IFN-β can also prime mouse macrophages for LPS responses (26). Among the findings supporting this conclusion is a recent report that anti-IFN-β can inhibit LPS-induced NO production by mouse macrophages (42).

The ability of a given cytokine to synergize (10, 14, 24) or antagonize (2, 7, 13, 24, 29, 32, 36) macrophage activation by a second cytokine is well established. In the present study, antagonism between rTGF-β1 and rIFN-γ was apparent at the levels of iNOS mRNA accumulation, iNOS protein biosynthesis, and NO production. Antagonistic relationships have been shown to exist not only between TGF-β and IFN-γ (1, 22, 23, 29) but also between TGF-β and TNF-α (1, 7, 13, 30). A spontaneous, high-level expression of both the TNF-α and IFN-γ genes has been reported to occur in TGF-β1-deficient transgenic mice (35), suggesting that TGF-β1 may be required to maintain low-level constitutive expression of these genes in normal animals.

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