Suppression of Proinflammatory Cytokines in Monocytes by a Tetravalent Guanylhydrazone

By Marina Bianchi,[§] Ona Bloom,[*] Tobias Raabe,[§] Pamela S. Cohen,[‡§] Jason Chesney,[§] Barbara Sherry,[§] Helena Schmidtmayerova,[§] Thierry Calandra,[§] Xini Zhang,[§] Michael Bukrinsky,[§] Peter Ulrich,[§] Anthony Cerami,[§] and Kevin J. Tracey[*§]

From the [*]Laboratory of Biomedical Science, Department of Surgery, North Shore University Hospital; the [‡]Department of Pediatrics, North Shore University Hospital; and [§]The Picower Institute for Medical Research, Manhasset, New York 11030

Summary

An overproduction of proinflammatory cytokines by activated macrophages/monocytes mediates the injurious sequelae of inflammation, septic shock, tissue injury, and cachexia. We recently synthesized a tetravalent guanylhydrazone compound (CNI-1493) that inhibits cytokine-inducible arginine transport and nitric oxide (NO) production in macrophages, and protects mice against lethal endotoxemia and carrageenan-induced inflammation. During these investigations we noticed that CNI-1493 effectively prevented lipopolysaccharide (LPS)-induced NO production, even when added in concentrations 10-fold less than required to competitively inhibit L-arginine uptake, suggesting that the suppressive effects of this guanylhydrazone compound might extend to other LPS-induced responses. Here, we report that CNI-1493 suppressed the LPS-stimulated production of proinflammatory cytokines (tumor necrosis factor [TNF], interleukins 1β and 6, macrophage inflammatory proteins 1α and 1 β) from human peripheral blood mononuclear cells. Cytokine suppression was specific, in that CNI-1493 did not inhibit either the constitutive synthesis of transforming growth factor β or the upregulation of major histocompatibility complex class II by interferon γ (IFN-γ). In contrast to the macrophage suppressive actions of dexamethasone, which are overridden in the presence of IFN-γ, CNI-1493 retained its suppressive effects even in the presence of IFN-γ. The mechanism of cytokine-suppressive action by CNI-1493 was independent of extracellular L-arginine content and NO production and is not restricted to induction by LPS. As a selective inhibitor of macrophage activation that prevents TNF production, this tetravalent guanylhydrazone could be useful in the development of cytokine-suppressive agents for the treatment of diseases mediated by overproduction of cytokines.

Proinflammatory cytokines, nitric oxide (NO),[1] and other macrophage products mediate the injurious sequelae that define a spectrum of diseases including lethal septic shock, tissue injury, cachexia, hemorrhagic shock, vascular leakage syndromes, transplant rejection, rheumatoid arthritis, and other inflammatory states (1–4). This understanding led to the development of experimental therapies based on directly inhibiting cytokines in a number of diseases (e.g.,, monoclonal anti-TNF antibodies, IL-1 receptor antagonists, and TNF-receptor fusion proteins) (5–9). More recently, there has been renewed interest in the development of novel therapeutic strategies to suppress the production of proinflammatory cytokines and NO by monocytes (10–14). The development of effective compounds in this regard has provided a better understanding of the biology of the activated macrophage, and indicates that it may be feasible to treat cytokine-mediated toxicity by suppressing the excessive production of cytokines.

In the course of investigating a strategy to inhibit NO production in macrophages without simultaneously inhibiting endothelium-derived relaxing factor activity, we recently designed and developed a tetravalent guanylhydrazone compound (15). Termed "CNI-1493," *N,N'*-bis [3,5-bis [1(aminoiminomethyl) hydrazono]ethyl]phenyl]decanediamide tetrahydrochloride (CAS Reg. No. 164301-

[1] *Abbreviations used in this paper:* FBS, fetal bovine serum; GADPH, glyceraldehyde-3-phosphate dehydrogenase; IC_{50}, 50% inhibitory concentration; iNOS, inducible nitric oxide synthase; LDH, lactate dehydrogenase; L-NMA, N^G-methyl-L-arginine; MIP-1α, macrophage inflammatory protein 1α; MTT, 3-[4, 5-Dimethy/Thiazol-2-yl]-2, 5-diphenyltetrazolium bromide; NO, nitric oxide; TSST-1, toxic shock syndrome toxin.

51-3) was developed as a competitive inhibitor of cytokine-inducible (but not constitutive) L-arginine uptake in macrophages activated with LPS and IFN-γ (15). Administration of this compound to mice prevented the production of NO in stimulated resident peritoneal macrophages, and conferred protection against both carrageenan-induced inflammation and lethal endotoxemia (15). While studying the mechanism by which CNI-1493 inhibited L-arginine transport in nonactivated (quiescent) macrophages before LPS exposure, we discovered that 10-fold less CNI-1493 concentrations effectively antagonized the induction of NO synthesis in naive cultures (15). These macrophage-suppressive activities at very low concentrations of CNI-1493 were independent of extracellular L-arginine, and could not be attributed to competitive inhibition of L-arginine uptake. Since increased arginine uptake and stimulated NO production are hallmarks of the LPS-induced macrophage activation response that also includes the production of proinflammatory cytokines, we reasoned that the suppressive effects of CNI-1493 might include antagonizing other LPS-induced responses, including cytokine synthesis.

In this study, we report that CNI-1493 effectively suppresses several functional components of the activated macrophage phenotype, including the induction of pro-inflammatory cytokines, inducible nitric oxide synthase (iNOS) and L-arginine uptake in LPS-stimulated monocytes/macrophages. The mechanism of cytokine suppression by CNI-1493 treatment was independent of extracellular L-arginine content and NO production. CNI-1493 suppressed the production of proinflammatory cytokines, but not the anti-inflammatory cytokine TGF-β. Moreover, CNI-1493 did not suppress the upregulation of MHC class II antigen expression induced by IFN-γ. When administered to mice receiving lethal doses of LPS, CNI-1493 blocked the appearance of TNF in serum.

Materials and Methods

Cell Isolation and Culture. For studies of murine macrophage-like cells, RAW 264.7 cells were obtained from The American Type Culture Collection (Rockville, MD) and seeded into 6- or 24-well tissue culture plates (10^6 cells/ml RPMI with 10% fetal bovine serum [FBS]) as required in different experiments. For studies of human monocytes, buffy coats were obtained by elutriation from normal individual donors to the Long Island Blood Bank Services (Melville, NY). PBMC were isolated by density gradient centrifugation through Ficoll (Ficoll-Paque® PLUS, endotoxin tested; Pharmacia, Piscataway, NJ); typically one preparation yielded 200×10^6 adherent cells. These were cultured in 24-well plates (2×10^6 cells/ml RPMI with 10% normal human serum). Nonadherent cells were removed by changing the media after 18 h.

Cytokine Induction. Working stock LPS (*Escherichia coli* 0111: B4; Sigma Chemical Co., St. Louis, MO) solutions (100 μg/ml in PBS, pH 7.4) were sonicated for 10 min before use. For induction of monocytes, LPS was diluted in individual wells to a final concentration of 100 ng/ml. IFN-γ (25 U/ml) was coadministered with LPS; recombinant m-IFN-γ (Genzyme Corp.; Cambridge, MA) was used for induction of RAW cells, recombinant h-IFN-γ (Boehringer Mannheim, Mannheim, Germany) was used for induction of human PBMC. In independent experiments, we found that the simultaneous addition of these concentrations of LPS and IFN-γ were maximally effective in stimulating the release of TNF (data not shown). Toxic shock syndrome toxin (TSST-1; Toxin Technology; Sarasota, FL) was used for TNF induction in RAW cells at a final concentration of 2 μg/ml.

Cytokine Assays. TNF concentrations in murine serum and in the supernatants of stimulated RAW cells were determined by ELISA (Genzyme Corp.) performed in 96-well microtiter plates (minimum detectable concentration [MDC], 10 pg/ml). Human cytokines were measured by other commercially available ELISA kits according to the package instructions. MDCs were the following: TNF (R&D Systems, Inc., Minneapolis, MN), 4.4 pg/ml; IL-1β (R&D Systems, Inc.), 0.3 pg/ml; IL-6 (R&D Systems, Inc.), 0.7 pg/ml; and TGF-β (Genzyme Corp.), 50 pg/ml. Macrophage inflammatory protein 1α (MIP-1α) and MIP-1β concentrations were determined by an in-house ELISA (MDC, 10 pg/ml). Where indicated in some experiments, TNF (murine and human) bioactivity was assayed by a standard L929 cell cytotoxicity bioassay; specificity was verified by addition of neutralizing anti-TNF antibodies. Separate experiments (not shown) revealed that CNI-1493 did not interfere with the L929 bioassay, as identical standard curves (prepared using rmTNF and rhTNF) were obtained in the absence and presence of added CNI-1493.

CNI-1493 Additions. CNI-1493 was synthesized and purified as previously described (15). The purity was >99% as estimated by melting point, nuclear magnetic resonance, elution from HPLC, and elemental analysis. Working stock solutions (1 mM) were prepared fresh in sterile-filtered deionized water. For individual experiments, aliquots of the stock solution were added directly into individual tissue culture wells, yielding the final concentrations indicated. In all experiments, control wells received an equal volume of sterile deionized water (vehicle) only. In agreement with previous results, neither CNI-1493 nor vehicle in the concentrations used has any significant effect on cell viability as assessed by morphology, conversion of 3-[4, 5-Dimethy/Thiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) to formazan, or the release of lactate dehydrogenase (LDH) into the media as tested for RAW 267.4 cells, murine fibroblast L929 cells, and human PBMC.

RNA Isolation and RNase Protection Analyses. Total cytoplasmic RNA was isolated from RAW 264.7 cells (typically from 10-cm tissue culture plates) at various time points after LPS/IFN-γ stimulation (100 ng/ml LPS and 25 U/ml IFN-γ) essentially as described (16) except that 200 mM ribonucleoside–vanadyl complex (10 μl, Sigma Chemical Co.) was added to the cells before lysis with detergent (300 μl, 0.5% NP-40, 0.14 M NaCl, and 1.5 mM Tris, pH 8.6). mRNAs for mTNF and murine glyceraldehyde-3-phosphate dehydrogenase (GADPH) were detected by RNase protection analysis performed as described (16). Briefly, antisense mRNAs were transcribed in vitro in the presence of [^{32}P]CTP from vectors containing cDNA templates coding for the above cytokines cloned behind the bacterial promoters T3, T7, or SP6. Radiolabeled antisense mRNAs were then purified by electrophoresis through a urea polyacrylamide gel and eluted in 0.1% SDS, 0.3 M sodium acetate, pH 5.0, and precipitated with ethanol. Hybridization of antisense probe to 1 μg of total cytoplasmic RNA was in 80% formamide at 45°C for 8–12 h. Hybridization mixes were then digested with 40 μg/ml RNase A and 200 U/ml RNase T1 for 45 min at 30°C followed by proteinase K digestion. The digested RNAs were separated by electrophoresis on a sequencing-type urea gel. Murine TNF mRNA

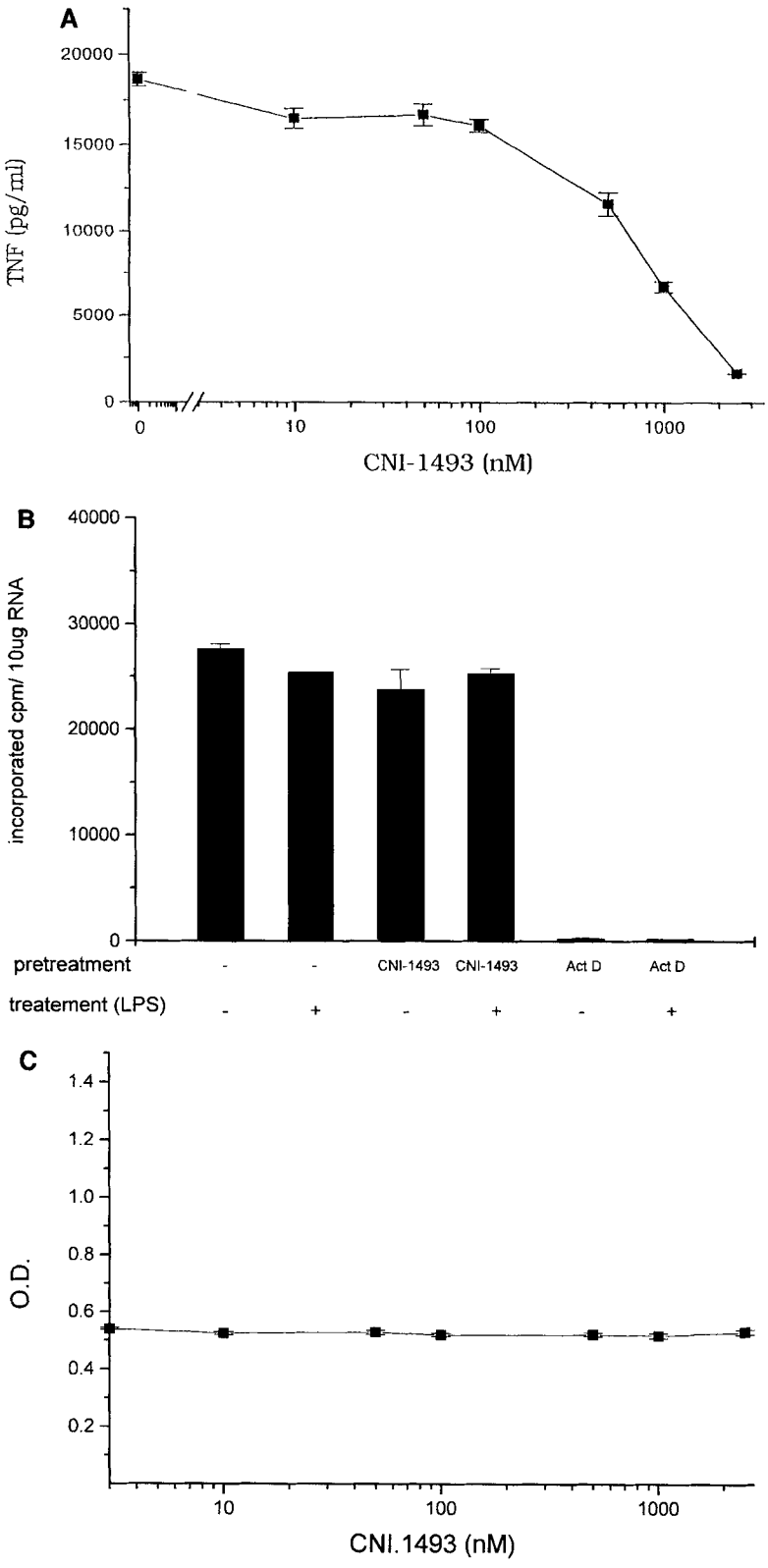

Figure 1. (*A*) Dose–response study of the inhibitory effects of CNI-1493 on immunodetectable TNF produced by LPS plus IFN-γ–stimulated RAW 264.7 cells. Cultured cells were pretreated with CNI-1493 for 1 h, stimulated with LPS plus IFN-γ, and media harvested 4 h later as described in Materials and Methods. TNF levels in the conditioned media were determined by ELISA. Data shown are mean ± SE, each in triplicate. (*B*) Effect of CNI-1493 on RNA synthesis in LPS-stimulated RAW 264.7 cells. Cultured cells were pretreated with 10 μM CNI-1493 for 1 h, then stimulated with LPS. 4 h later, the incorporation of [^{14}C]uridine into RNA was measured by adding the radiolabeled nucleotide for a 3-h period. The RNA fraction was then isolated, as described in Materials and Methods, and the radioactivity determined in a Beckman scintillation counter. Data shown are mean ± SE, each in triplicate. (*C*) Effect of CNI-1493 on cellular respiration (MTT assay) in RAW 264.7 cells. RAW cells were pretreated with CNI-1493 for 1 h, stimulated with LPS plus IFN-γ, and 4 h later, an MTT-based assay of cellular respiration was performed as previously described (27). Data shown are mean ± SE, each in triplicate.

was detected with a 212-nucleotide RNA probe derived from the EcoRI linearized template plasmid pGEM3Zf$^+$ mTNF; this probe is complementary to the native 5′-end of the transcript and yields a protected fragment of 169 nucleotides. Mouse GADPH mRNA was detected with a 135-nucleotide RNA probe derived from a linearized plasmid purchased from GIBCO BRL (Gaithersburg, MD). This probe is complementary to an internal region of human GADPH and yields a protected fragment of 95 nucleotides. However, because human and mouse GADPH are not completely identical, incomplete hybridization leads to the gener-

ation of a shorter protected fragment of about 50 nucleotides as measured by gel electrophoresis.

Construction of Plasmids. All plasmids contained a bacteriophage promoter to direct in vitro RNA synthesis of antisense RNA used for RNase protection analysis. pGEM3Zf + TNF was constructed by cloning a RT-PCR product with EcoRI and BamHI ends into the multiple cloning site of pGEM3Zf+ (Promega Corp., Madison, WI). The PCR product (185 bp) was obtained using total cytoplasmic RNA from LPS-stimulated RAW 264.7 cells as template and the primers 5'-GGGGAATTCA-GAAGGCTCCCTC-3' and 5'-GGGATCCGGTGTCTTTTC-TGG-3'. A plasmid coding for GADPH was obtained linearized from GIBCO BRL.

Determination of RNA Synthesis. RAW 264.7 cells (10^6 cells/ml) were allowed to adhere in petri dishes (10 ml) for 18 h, in RPMI with 10% FBS. The media were then replaced with media containing either CNI-1493 in the concentrations indicated, or actinomycin D (50 μg/ml) as a positive control. After 1 h, the cells were stimulated with LPS (100 ng/ml) and 4 h later, they were washed twice with PBS and exposed to [^{14}C]uridine (1 μCi/plate, specific activity 521 mCi/mmol; NEN-DuPont, Boston, MA) for 3 h. The RNA was extracted with RNAzol (RNAzol B; TEL-TEST, Inc., Friendswood, TX) and the radioactivity incorporated into RNA was measured in a scintillation counter (model LS 7800; Beckman Insts., Fullerton, CA).

Determination of Protein Synthesis. RAW 264.7 cells were allowed to adhere in 6-well plates (2×10^6 cells/ml RPMI with 10% FBS) for 18 h, washed with methionine-free DMEM, and then incubated for 1 h in methionine-free DMEM with 10% dialyzed FBS. [^{35}S]Methionine (8 mCi/ml, NEN-DuPont) was added to individual wells (50 μCi/well) simultaneously with or without stimulating agents (LPS and IFN-γ). CNI-1493 (5 μM) was added 1 h before the stimulating agents where indicated. At the indicated time points after addition of the stimulating agents, supernatants were collected and radiolabeled cells washed in ice-cold PBS, then lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris-HCl, pH 7.5, and 2 mM EDTA). Intracellular proteins were precipitated by addition of TCA, the pellets collected by centrifugation, and radioactive counts determined.

Results

Effects of CNI-1493 on the Production of TNF. When activated by exposure to LPS and IFN-γ, cells of the macrophage/monocyte lineage produce copious amounts of TNF. We initially examined the direct effects of CNI-1493 on TNF production in activated cells of the murine macrophage-like cell line RAW 264.7. CNI-1493 effectively inhibited LPS and IFN-γ–stimulated RAW cell production of TNF as measured by ELISA (Fig. 1 *A*). The 50% inhibitory concentration (IC_{50}) was ~500 nM; >90% suppression occurred with concentrations ≥1 μM. Western blotting of concentrated supernatants obtained from LPS and IFN-γ–stimulated RAW cells indicated that CNI-1493 inhibited the production of immunodetectable TNF (not shown). We also measured TNF bioactivity in a standard L929 cell cytotoxicity assay, and observed a similar IC_{50} for CNI-1493 inhibition of bioactive TNF (500 nM).

Previous observations demonstrated that TNF transcription and translation are upregulated within minutes after LPS stimulation (17, 18). We performed a time course study to assess the effects of CNI-1493 added to monocytes at different stages of LPS and IFN-γ–induced activation. TNF protein production was completely suppressed when CNI-1493 was added to quiescent monocytes before stimulation with LPS and IFN-γ (Table 1), but when added to monocytes just before or after their activation by LPS and IFN-γ, CNI-1493 was less effective in suppressing TNF production. This loss of cytokine-suppressive activity suggests that CNI-1493 inhibits macrophage activation at an early stage in the intracellular signaling pathway.

Additional experiments were performed to address whether the cytokine-suppressive effects of CNI-1493 were reversible. Cells were pretreated with CNI-1493 (2.5 μM) for 1 h; CNI-1493 was then removed by culturing under fresh media devoid of CNI-1493 for 30 min, and LPS and IFN-γ were added. We observed no difference in TNF produced over the subsequent 2 h in controls (25 ± 1 ng TNF/10^6 cells) or in CNI-1493–treated and washed cells (24 ± 2 ng TNF/10^6 cells). Thus, the cytokine-suppressive effects of CNI-1493 are readily reversible, suggesting that monocytes exposed to this guanylhydrazone are not permanently impaired from future immunological responsiveness.

We addressed the possibility that CNI-1493 exerted its effects by inhibiting RNA synthesis. The RNA synthesis inhibitor actinomycin D (50 μg/ml) was used as positive control. As shown in Fig. 1 *B*, we did not observe any change in the incorporation of [^{14}C]-uridine into RNA in the cells treated with 10 μM of CNI-1493 and LPS (25,290 ± 97 cpm) vs. the controls receiving LPS alone (25,370 ± 70 cpm). Thus, the cytokine-suppressive actions of CNI-1493 are not the result of nonspecific inhibition of de novo transcription. Addition of pharmacologic quantities of CNI-1493 to LPS plus IFN-γ–stimulated RAW cell cultures was not toxic, as assessed by LDH release into the media, MTT assay (as shown in Fig. 1 *C*), cell counting, and cell morphology by light microscopic inspection.

Table 1. *Kinetic Study of the Inhibitory Effects of CNI-1493 on TNF Production in LPS and IFN-γ–stimulated RAW 264.7 Cells*

Time CNI-1493 added	TNF (ng/10^6 cells)
min	
−60*	<0.4
−30	<0.4
−5	17 ± 1
+5	16 ± 3
+30	16 ± 1
Control (no CNI-1493)	25 ± 1

*LPS plus IFN-γ were added at time zero and CNI-1493 (2.5 μM) added at the relative time indicated.
TNF in supernatants collected 2 h after LPS and IFN-γ were assayed by ELISA. Data are mean ± SE from three experiments, each in triplicate.

CNI-1493 Inhibits TNF Production In Vivo. Because TNF produced in vivo occupies a pivotal role in the mediation of endotoxin lethality (5, 19), we next assessed whether CNI-1493 inhibited peak serum TNF levels in mice given a lethal dose of LPS. CNI-1493 attenuated the LPS-induced increases in serum TNF in a dose-dependent manner (Fig. 2), suggesting that the previously described protective effects of CNI-1493 against LPS lethality in mice (15) are in part accounted for by inhibition of the systemic TNF response. This effective suppression of TNF by CNI-1493 in an animal model of acute cytokine overproduction indicates that it may be feasible to prevent cytokine toxicity in vivo with this agent.

CNI-1493 Inhibits Expression of iNOS. The observation that CNI-1493 suppressed TNF production both in cultured monocytes and in vivo prompted us to investigate whether it inhibited the induction of iNOS, another characteristic element of the murine macrophage activation response to LPS and IFN-γ (13, 20). We previously reported that CNI-1493 is not a direct inhibitor of iNOS activity when added to enzyme preparations (15), but in the present experiments we assessed whether pretreatment with CNI-1493 inhibited the induction of iNOS activity. Accordingly, we measured iNOS activity in cell lysates of LPS plus IFN-γ–activated RAW cells pretreated with CNI-1493 (Table 2). In agreement with others (13, 20), LPS plus IFN-γ in control cultures induced a robust increase in iNOS activity. CNI-1493 effectively inhibited the induction of iNOS.

Effects of CNI-1493 Are Specific. We next investigated the possibility that the suppressive effects of CNI-1493 were secondary to a generalized suppression of protein synthesis. In a protein synthesis assay, CNI-1493 (5 μM) did not significantly inhibit the total incorporation of [^{35}S]methionine into TCA-precipitable proteins from lysates of RAW 264.7 cells that had been stimulated with LPS plus IFN-γ during a 4-h pulse of [^{35}S]methionine (controls, 1.5 × 10^5 cpm, vs. CNI-1493, 1.3 × 10^5 cpm). Cycloheximide (60 μg/ml), used as a positive control, significantly inhibited incorporation of radioactivity (8.3 × 10^2 cpm). When lysate proteins were separated by SDS-PAGE and visualized by autoradiography, no differences were noted in either the pattern or the quantity of labeled proteins synthesized (not shown).

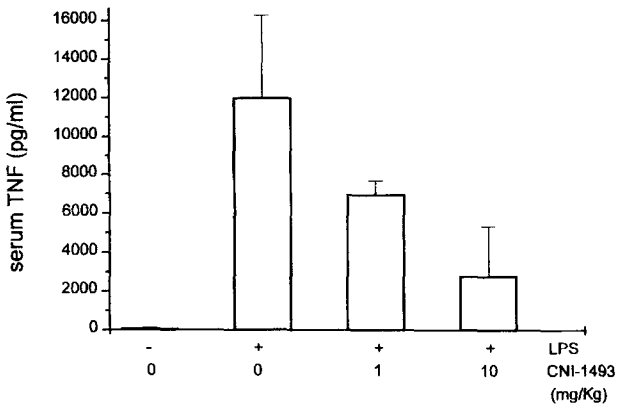

Figure 2. CNI-1493 inhibits serum TNF levels in endotoxemic mice. BALB/c mice received CNI-1493 by intraperitoneal injection at the dose indicated, and 90 min later received LPS (*E. coli* 0111:B4) by intraperitoneal injection (13.75 mg/kg). After 90 min, blood was collected by cardiac puncture, and serum TNF determined by ELISA. Data shown are mean ± SE, n = 6–8 animals per group shown.

Table 2. *CNI-1493 Prevents the Induction of iNOS Activity*

[CNI-1493]	LPS/IFN-γ	Total NOS activity (*pmol/mg/min*)
μm		
0	−	2.8 ± 0.14
0	+	28.2 ± 1.31
1	+	18.2 ± 0.71*
5	+	4.1 ± 0.17*

RAW 264.7 cells were plated and induced with LPS plus IFN-γ as described in Materials and Methods. CNI-1493 was added 1 h before inducing agents. Cell lysates were prepared 8 h after activation and total NOS activity was determined as previously described (9). Data are mean ± SE of three different experiments, each in triplicatae.
*<0.05 vs. LPS/IFN-γ–induced cells without CNI-1493.

TNF mRNA Levels. The mechanism of action of CNI-1493 was studied by testing its ability to inhibit LPS plus IFN-γ–stimulated TNF mRNA levels. In agreement with previous observations, TNF mRNA levels determined by RNase protection analysis were increased in RAW 264.7 cell cultures after addition of LPS plus IFN-γ; levels peaked 2 h after exposure to stimulating agents (17, 18). Pretreatment with CNI-1493 depressed peak steady-state TNF mRNA levels in a dose-independent manner (Fig. 3). We observed significant suppression of TNF mRNA levels when CNI-1493 was present in concentrations >1 μM, indicating that at these concentrations the agent either inhibits transcription or accelerates degradation of TNF mRNA. As noted above, we observed that CNI-1493 inhibited 50% of TNF protein with <1 μM concentrations, suggesting that CNI-1493 independently suppressed translational or posttranslational processing steps in TNF biosynthesis.

Inhibitory Effects of CNI-1493 Are Independent of NO. CNI-1493 is an inhibitor of NO production by activated murine monocytes (15), and NO has previously been implicated in the signaling pathway leading to TNF production (21). Thus, inhibition of NO production may be one explanation for the cytokine-suppressive effects of CNI-1493. We addressed this possibility by measuring the production of TNF and NO in RAW cells treated for 24 h with LPS plus IFN-γ and pretreated with either CNI-1493 or a competitive inhibitor of NOS, N^G-methyl-L-arginine (L-NMA) (Fig. 4). A concentration of CNI-1493 (5 μM) that resulted in a 70% suppression of NO production also resulted in >95% suppression of TNF production. This differed significantly from the results observed with L-NMA,

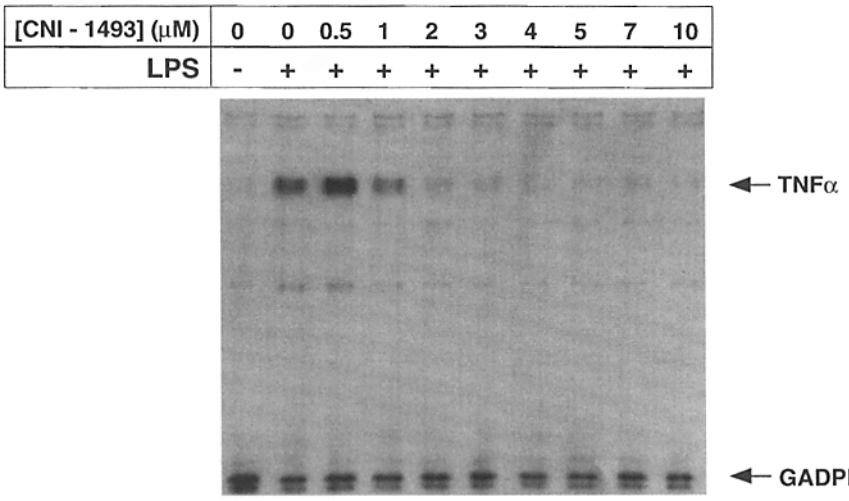

Figure 3. Expression of TNF mRNA in LPS and IFN-γ–stimulated RAW cells. RAW 264.7 cells were exposed to CNI-1493 for 1 h at the concentrations shown, then stimulated with LPS and IFN-γ for 2 h. Total RNA was isolated and RNase protection analysis performed as described in Materials and Methods. Note that TNF mRNA levels are maintained even though TNF protein is inhibited by 0.5–1 μM concentrations of CNI-1493.

for which an L-NMA concentration (1 mM) resulting in a 60% suppression of NO did not suppress TNF production from control values. This suggests that the cytokine-suppressive effects of CNI-1493 do not depend upon inhibition of NO.

Inhibitory Effects of CNI-1493 Are Not Restricted to Induction by LPS. To address the possibility that CNI-1493 acts by inhibiting an early step in LPS signaling, we measured the effect of this agent on TNF production from macrophage cultures activated with an independent stimulus, toxic shock syndrome toxin (TSST-1). As shown in Fig. 5, CNI-1493 suppressed TNF production in TSST-1–treated cells, giving evidence that the cytokine responsive-inhibiting activity of CNI-1493 is not restricted to LPS-induced macrophage activation signals.

Effects of CNI-1493 on Human Monocytes. Because of the potential therapeutic implications for an experimental agent that suppresses production of proinflammatory cytokines in monocytes and in vivo, the cytokine-suppressive effects of

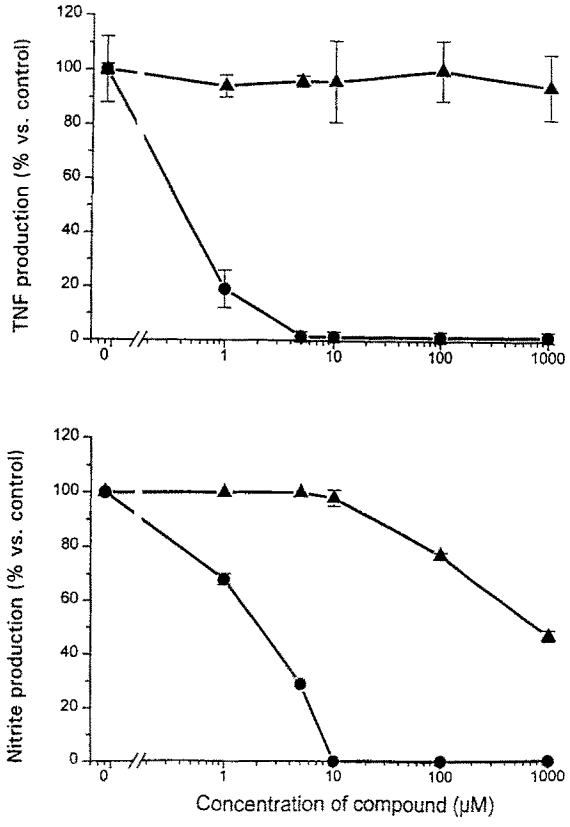

Figure 4. Inhibition of NO does not mediate decreased TNF production in LPS plus IFN-γ–stimulated RAW cells. RAW 264.7 cells were exposed to either CNI-1493 (*circles*) or L-NMA (*triangles*) in the concentrations shown for 1 h, and then stimulated with LPS plus IFN-γ as described in Materials and Methods. After 24-h culture, media were harvested for determination of TNF by ELISA (*A*), and nitrites (*B*) using the Greiss reagent (20). Data shown are mean ± SE, each in triplicate. The absolute value for nitrite production in absence of CNI-1493 was 51 ± 4 nmol/24 h per 10^6 stimulated cells.

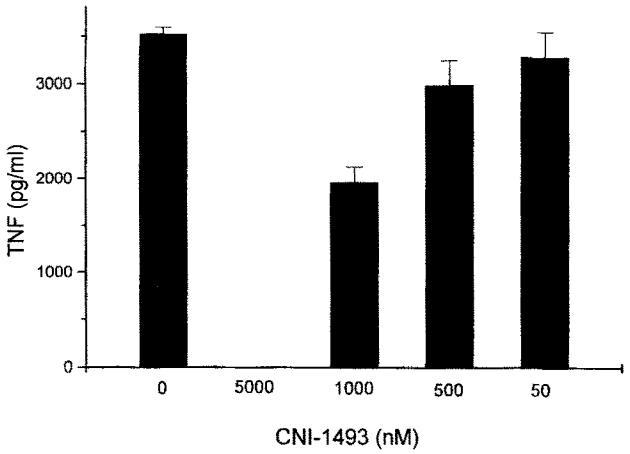

Figure 5. Dose–response study of the inhibitory effects of CNI-1493 on immunodetectable TNF produced by TSST-1–stimulated RAW 264.7 cells. Cultured cells were pretreated with CNI-1493 at the indicated doses for 1 h, then stimulated with TSST-1 (2 μg/ml) for 15 h. Supernatants were collected and analyzed for TNF by ELISA. Data shown are mean ± SE, in triplicate.

CNI-1493 were assessed in LPS-stimulated human monocytes. As shown in Fig. 6, CNI-1493 was a potent and selective inhibitor of proinflammatory cytokine production by human monocytes. It was ~10-fold more potent in preventing TNF and IL-1 production in human monocytes (with an estimated IC_{50} of 30–70 nM) as compared to the murine RAW 264.7 cells (IC_{50}, 500–750 nM). The estimated IC_{50} for CNI-1493 in preventing the production of other proinflammatory cytokines (MIP-1α, MIP-1β, and IL-6) by human PBMC was also in the nanomolar range (IC_{50}, 125–175 nM). In separate experiments, we observed nearly identical dose–response curves when human monocytes were coinduced with rhIFN-γ (25 U/ml) plus LPS (100 ng/ml) (data not shown). Thus, the inhibitory actions of CNI-1493 on proinflammatory cytokine production are not overridden by IFN-γ.

Specificity of CNI-1493's effects on monocytes was evaluated by assay for the production of TGF-β and the expression of MHC class II antigens. Even high concentrations of CNI-1493 (2.5 μM) failed to inhibit the con-

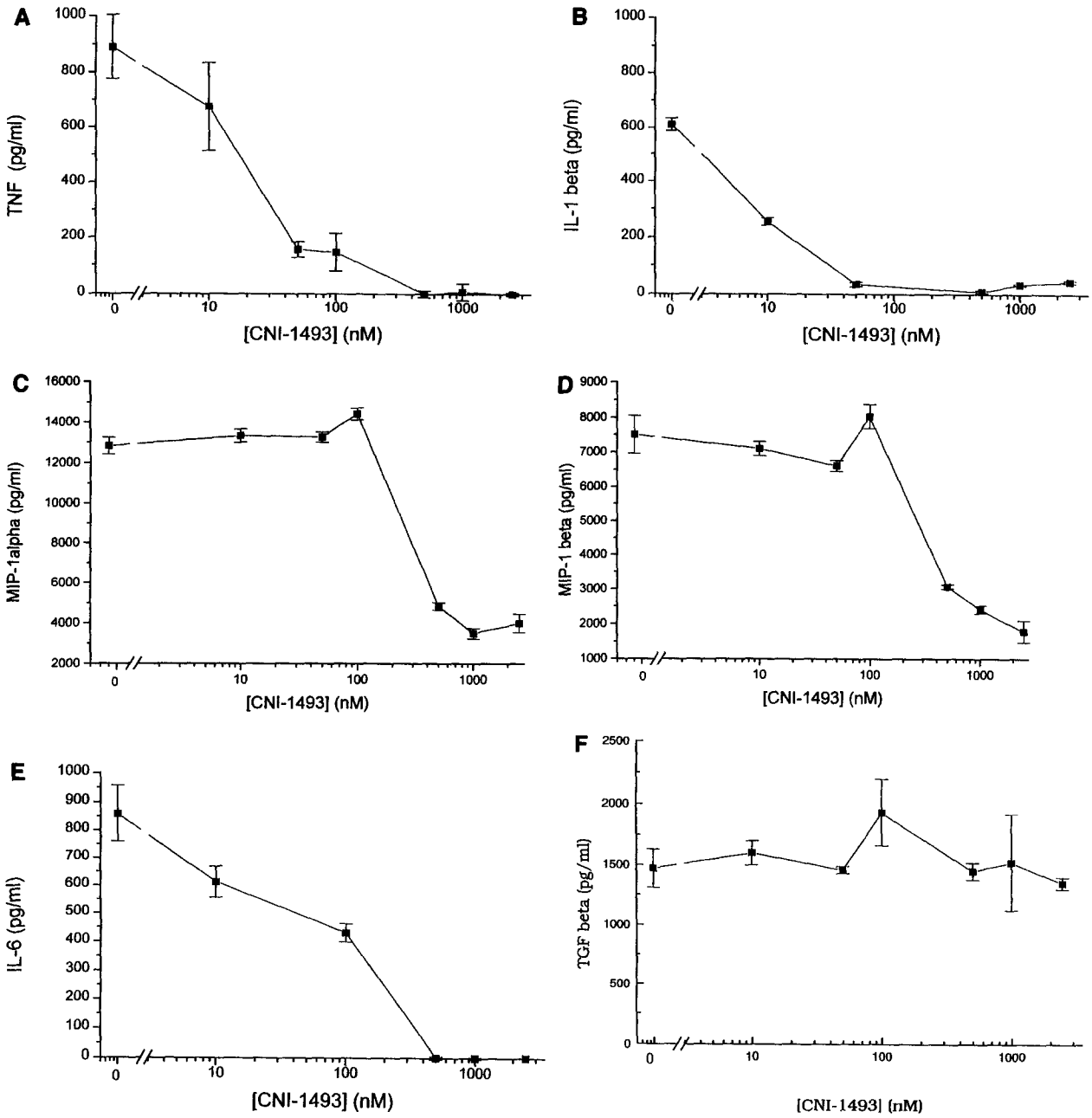

Figure 6. CNI-1493 inhibits proinflammatory cytokine production in human monocytes. Human PBMC were isolated, allowed to adhere, and washed as described in Materials and Methods. Cells were exposed to CNI-1493 at the concentrations indicated for 1 h, stimulated with LPS (100 ng/ml), media harvested at 4 h, and cytokine levels measured by ELISA. Data shown are mean ± SE, $n = 6$. *A*, TNF, *B*, IL-1 IL-Iβ; *C*, MIP-1α; *D*, MIP-1β; *E*, IL-6; and *F*, TGF-β.

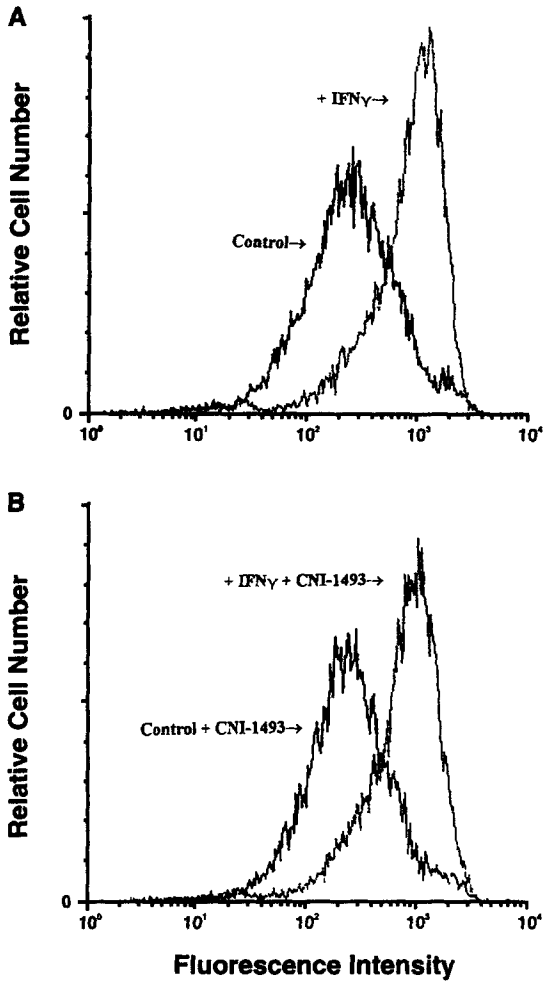

Figure 7. CNI-1493 does not inhibit IFN-γ–mediated upregulation of MHC class II expression on human monocytes. Human PBMC were dispersed by treatment with cold 0.2% ethylene diamine tetraacetic acid in PBS, pH 7.2. Cell suspensions were washed twice in PBS containing 1% BSA and 0.1% sodium azide (FACS® media; Becton Dickinson & Co., Mountain View, CA). The cells were then resuspended in 25 μl of diluted FITC-conjugated anti-HLA-DR or an isotype standard antibody (PharMingen, San Diego, CA) and incubated on ice for 20 min. The cells were washed twice and at least 10,000 cells analyzed on a FACScan® instrument (Becton Dickinson & Co.). Cells shown in *A* were exposed to IFN-γ dose for 4 h; cells shown in *B* were exposed to CNI-1493 (2.5 μM) plus IFN-γ (25 U/ml) for 4 h.

stitutive production of the "anti-inflammatory cytokine" TGF-β by adherent human monocytes (Fig. 6 *F*). Additional evidence of selectivity is given by the observation that CNI-1493 did not inhibit IFN-γ–mediated upregulation of MHC class II expression on human monocytes (Fig. 7). Since CNI-1493 was originally designed as a direct, competitive inhibitor of cytokine-inducible L-arginine transport, we also investigated the possibility that the cytokine-suppressive mechanism in human monocytes was dependent upon extracellular L-arginine availability. As shown in Table 3, we observed no relationship between L-arginine media concentrations and the inhibition of LPS-induced TNF responses in human monocytes, suggesting that the cytokine-suppresive effects did not depend upon L-arginine availability. In separate studies using a related but structurally distinct trivalent guanylhydrazone, 2,2′-[5-[[[[[4-[1-[2-(aminoiminomethyl)-hydrazono]ethyl]phenyl]amino]carbonyl]amino]-1,3-phenylene]diethylidyne]-bis(hydrazinecarboximidamide) trihydrochloride (CNI-993), we observed similar cytokine-suppressive activity (not shown), suggesting that other members of this structural class of guanylhydrazone compounds also possess cytokine-suppressive activity.

Discussion

The tetravalent guanylhydrazone CNI-1493 was initially developed as a competitive inhibitor of cytokine-inducible L-arginine transport in activated monocytes, but we found it to be more effective in preventing the induction of NO production in quiescent monocytes (15). The present results now reveal that compounds of this structural class effectively inhibit the production of TNF and other proinflammatory cytokines by human monocytes (Table 4). These cytokine-suppressive effects are not mediated by inhibition of NO or L-arginine transport or by generalized suppression of protein or RNA synthesis, and do not lead to inhibition of either TGF-β release or IFN-γ–mediated upregulation of MHC class II expression.

The molecular target of these guanylhydrazone compounds in activated monocytes has yet to be determined, but the present studies suggest important differences from another class of anti-inflammatory agents that inhibit cytokine production. Glucocorticoid hormones have been studied extensively for their cytokine-suppressive effects, which have been linked to the anti-inflammatory properties of these steroids.

When added to monocytes before LPS stimulation, dexamethasone is an inhibitor both of TNF gene transcription (with resultant suppression of mRNA accumulation), and of mRNA translation (together leading to a >95% suppression of TNF protein synthesis) (18, 22). IFN-γ can completely override the cytokine-suppressive effects of the glucocorticoids, enabling LPS plus IFN-γ–stimulated monocytes to produce robust quantities of TNF despite glucocorticoid treatment (23). In contrast to the glucocorticoids, CNI-1493 was not overridden by IFN-γ treatment, because CNI-1493 effectively inhibited cytokine production by LPS and IFN-γ–costimulated murine and human monocytes. This may be particularly relevant to anti-inflammatory strategies in vivo, since IFN-γ is nearly ubiquitous in acute and chronic inflammatory states, and serves to limit the utility of glucocorticoids in clinical settings when effective cytokine-suppressive compounds are most critically needed. Additional evidence for the divergent actions of dexamethasone and CNI-1493 in activated monocytes is found in the observation that CNI-1493 prevented induction of both L-arginine uptake and iNOS, whereas dexamethasone suppresses only iNOS and leaves the induction of L-arginine transporters unaffected (13).

The present findings give evidence that CNI-1493 inhibits TNF production at the translational or posttransla-

Table 3. *The Cytokine-suppressive Effects of CNI-1493 Do Not Respond to Increasing L-arginine Availability*

	TNF (IU/ml)		
[L-arginine]	[CNI-1493] = 0	[CNI-1493] = 1	[CNI-1493] = 5
μM		μM	μM
10	543 ± 31	17 ± 3	<0.3
100	543 ± 22	23 ± 2	<0.3
1,000	743 ± 19	23 ± 3	<0.3

Human PBMC were prepared as described in Materials and Methods and incubated in defined media (RPMI) containing L-arginine in the concentrations shown. CNI-1493 was added 1 h before LPS, and conditioned media were harvested 4 h after activation. TNF bioactivity was determined by L929 bioassay. (Note: the physiological range of serum L-arginine concentration is 100–150 μM.) Data are mean ± SE of three different experiments, each in triplicate.

Table 4. *LPS-induced Monocyte Responses Inhibited by CNI-1493*

L-arginine transport
NO production
TNF
IL-1β
IL-6
MIP-1α
MIP-1β

tional level, because TNF protein synthesis is reduced by lesser concentrations of CNI-1493 than are required to suppress steady-state mRNA levels. Although the molecular target(s) of CNI-1493 remains undefined, it will be of interest to measure TNF mRNA stability, transcription rates, and the activity of LPS-induced kinases in CNI-1493–treated monocytes. These studies might enable a direct comparison between the CNI-1493 mechanism of action, and that of other TNF inhibitors. For instance, Lee et al. (12) recently showed that pyridinyl imidazoles that target mitogen-activated kinase inhibit TNF production in human monocytes, primarily by blocking translational or posttranslational processing. Another strategy to inhibit TNF has been proposed by Moreira et al. (11), who reported that thalidomide is a selective inhibitor of TNF production, which acts primarily by enhancing the degradation of TNF mRNA. Because CNI-1493 inhibits a spectrum of LPS-induced activation responses (Table 4), it was reasonable to hypothesize that its principle site of action could be at a proximal stage in LPS signaling. The result that CNI-1493 inhibited the TSST-1–induced TNF response suggests that the target is not restricted to the LPS signaling pathway in macrophages. Both LPS and TSST-1 induce the activation of mitogen-activated protein kinases and nuclear factor κB (24), which stimulate macrophage activation and translational regulation of cytokine production.

A relative overproduction of TNF occurs in patients with septic shock syndrome, HIV infection, meningitis, allograft rejection, hemorrhagic shock, and other inflammatory states (4). Although excessive TNF levels in critically ill patients may be lethal, lesser quantities of TNF occupy an important role in host defense, especially, for instance, against intracellular pathogens (25, 26). Therefore, an ideal cytokine-suppressive agent would act reversibly, in order to enable the monocytes to recover their ability to mount a protective TNF response after the drug is cleared. In addition, an ideal cytokine-suppressive agent would not interfere with expression of MHC class II molecules on the cell surface, in order to maintain immunological effectiveness in host defense. Our studies now show that a tetravalent guanylhydrazone selectively and reversibly inhibits proinflammatory cytokine synthesis in vitro, and attenuates in vivo TNF production and endotoxemic death. It is possible that guanylhydrazone compounds of this structural class may be useful in the treatment of diseases mediated by an overproduction of pro-inflammatory cytokines. Studies are in progress to identify the molecular target of CNI-1493 in antagonizing the activation of macrophages. It is hoped that these will provide additional insight into the signals regulating the macrophage activation phenotype.

We thank Kirk Manogue and Richard Bucala for helpful discussion.

These studies were supported in part by National Institutes of Health grant RO1 DK49283 (K. Tracey), by a Faculty Fellowship Award from the American College of Surgeons (K. Tracey); by a grant from Cytokine Network, Inc., and by Institutional Funding from the Picower Institute.

Address correspondence to Dr. K.J. Tracey, The Picower Institute for Medical Research, 350 Community Drive, Manhasset, NY 11030.

Received for publication 25 May 1995 and in revised form 8 November 1995.

References

1. Parrillo, J.E. 1993. Pathogenetic mechanisms of septic shock. *N. Engl. J. Med.* 328:1471–1477.
2. Nathan, C. 1992. Nitric oxide as a secretory product of mammalian cells. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 6:3051–3064.
3. Cobb, J.P., R.E. Cunnion, and R.L. Danner. 1993. Nitric oxide as a target for therapy in septic shock. *Crit. Care Med.* 21:1261–1263.
4. Tracey, K.J. 1992. The acute and chronic pathophysiological effects of TNF: mediation of septic shock and wasting (cachexia). *In* Tumor Necrosis Factors: The Molecules and Their Emerging Role in Medicine. B. Beutler, editor. Raven Press, Ltd., New York. 255–273.
5. Tracey, K.J., Y. Fong, D.G. Hesse, K.R. Manogue, A.T. Lee, G.C. Kuo, S.F. Lowry, and A. Cerami. 1987. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature (Lond.).* 330:662–664.
6. Gershenwald, J.E., Y. Fong, T.F. Fahey III, S.E. Calvano, R. Chizzonite, P.L. Kilian, S.F. Lowry, and L.L. Moldawer. 1990. Interleukin 1 receptor blockade attenuates the host inflammatory response. *Proc. Natl. Acad. Sci. USA.* 87:4966–4970.
7. Mohler, K.M., D.S. Torrance, C.A. Smith, R.G. Goodwin, K.E. Stremler, V.P. Fung, H. Manani, and M.B. Widmer. 1993. Soluble tumor necrosis factor (TNF) receptors are effective therapeutic agents in lethal endotoxemia and function simultaneously as both TNF carriers and TNF antagonists. *J. Immunol.* 151:1548–1561.
8. Abraham, E., R. Wunderink, H. Silverman, T.M. Perl, S. Hasraway, H. Levy, R. Bone, R.P. Wenzel, R. Balk, R. Allred, et al. 1995. Efficacy and safety of monoclonal antibody to human tumor necrosis antibody to human tumor necrosis factor α in patients with sepsis syndrome. *J. Am. Med. Assoc.* 273:934–941.
9. McNamara, M.J., J.A. Norton, R.J. Nauta, and H.R. Alexander. 1993. Interleukin-1 receptor antibody (IL-1rab) protection and treatment against lethal endotoxemia in mice. *J. Surg. Res.* 54:316–321.
10. Sampaio, E.P., E.N. Sarno, R. Galilly, Z.A. Cohn, and G. Kaplan. 1991. Thalidomide selectively inhibits TNF-α production by stimulated human monocytes. *J. Exp. Med.* 173: 699–703.
11. Moreira, A.L., E.P. Sampaio, A. Zmuidzinas, P. Frindt, K.A. Smith, and G. Kaplan. 1993. Thalidomide exerts its inhibitory action on TNF-α by enhancing mRNA degradation. *J. Exp. Med.* 177:1675–1680.
12. Lee, J.C., J.T. Laydon, P.C. McDonnell, T.F. Gallagher, S. Kumar, D. Green, D. McNulty, M.J. Blumenthal, J.R. Heys, S.W. Landvatter, et al. 1995. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature (Lond.).* 372:739–746.
13. Baydoun, A.R., R.G. Bogle, J.D. Pearson, and G.E. Mann. 1991. Selective inhibition by dexamethasone of induction of NO synthase, but not of induction of L-arginine transport, in activated murine macrophage J774 cells. *Br. J. Pharmacol.* 110: 1401–1406.
14. Novogrodsky, A., A. Vanichkin, M. Patya, A. Gazit, N. Osherov, and A. Levitzki. 1994. Prevention of lipopolysaccharide-induced lethal toxicity by tyrosine kinase inhibitors. *Science (Wash. DC).* 264:1319–1322.
15. Bianchi, M., P. Ullrich, M. Meistrell III, G.A. Zimmerman, H. Schmidtmayerova, M. Bukrinsky, T. Donnelly, R. Bucala, B. Sherry, K.R. Manogue, et al. 1995. An inhibitor of macrophage arginine transport and nitric oxide production (CNI-1493) prevents acute inflammatory and endotoxin lethality. *Mol. Med.* 3:254–266.
16. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Book 1. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 7.12–7.15.
17. Jue, D.-M., B. Sherry, C. Leudke, K.R. Manogue, and A. Cerami. 1990. Processing of newly synthesized cachectin/tumor necrosis factor in endotoxin-stimulated macrophages. *Biochemistry.* 29:8371–8377.
18. Beutler, B., J. Han, V. Kruys, and B.P. Giroir. 1992. Coordinate regulation of TNF biosynthesis at the levels of transcription and translation—patterns of TNF expression in vivo. *In* Tumor Necrosis Factors: The Molecules and Their Emerging Role in Medicine. B. Beutler, editor. Raven Press, New York. 561–574.
19. Tracey, K.J., B. Beutler, S.F. Lowry, J. Merryweather, S. Wolpe, I.W. Milsark, R.J. Hariri, T.F. Fahey III, A. Zentella, J.D. Albert, et al. 1986. Shock and tissue injury induced by recombinant human cachectin. *Science (Wash. DC).* 234:470–474.
20. Vodovotz, Y., N.S. Kwon, M. Pospischil, J. Manning, J. Paik, and C. Nathan. 1994. Inactivation of nitric oxide synthase after prolonged incubation of mouse macrophage with IFN-gamma and bacterial lipopolysaccharide. *J. Immunol.* 152:4110–4118.
21. Van Dervort, A.L., L. Yan, P.J. Madara, J.P. Cobb, R.A. Wesley, C.C. Corriveau, M.M. Tropea, and R.L. Danner. 1994. Nitric oxide regulates endotoxin-induced TNF-alpha production by human neutrophils. *J. Immunol.* 152:4102–4109.
22. Beutler, B., V. Tkacenko, I. Milsark, N. Krochin, and A. Cerami. 1986. Effect of γ interferon on cachectin expression by mononuclear phagocytes. Reversal of the lpsd (endotoxin resistance) phenotype. *J. Exp. Med.* 164:1791–1796.
23. Leudke, C.E., and A. Cerami. 1990. Interferon-γ overcomes glucocorticoid suppression of cachectin/tumor necrosis factor biosynthesis by murine macrophages. *J. Clin. Invest.* 86:1234–1240.
24. Trede, N.S., E. Castigli, R.S. Geha, and T. Chatila. 1993. Microbial superantigens induce NF-kappa B in the human monocytic cell line THP-1. *J. Immunol.* 150:5604–5609.
25. Pfeffer, K., T. Matsuyama, T.M. Kundig, A. Wakeham, K. Kishihara, A. Shahinian, K. Wiegmann, P.S. Ohashi, M. Kronke, and T.W. Mak. 1993. Mice deficient for the 55 kD tumor necrosis factor receptor are resistant to endotoxin shock, yet succumb to *L. monocytogenes* infection. *Cell.* 73: 457–467.
26. Titus, R.G., B. Sherry, and A. Cerami. 1989. Tumor necrosis factor plays a protective role in experimental murine cutaneous Leishmaniasis. *J. Exp. Med.* 170:2097–2104.
27. Hansen, M.B., S.E. Nielsen, and K. Berg. 1989. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J. Immunol. Methods.* 119:203–206.