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CNI-1493 inhibits monocyte/macrophage tumor necrosis factor by suppression of translation efficiency

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ABSTRACT **Tumor necrosis factor (TNF) mediates a wide variety of disease states including septic shock, acute and chronic inflammation, and cachexia. Recently, a multivalent guanylhydrazone (CNI-1493) developed as an inhibitor of macrophage activation was shown to suppress TNF production and protect against tissue inflammation and endotoxin lethality [Bianchi, M., Ulrich, P., Bloom, O., Meistrell, M., Zimmerman, G. A., Schmidtmayerova, H., Bukrinsky, M., Donnelley, T., Bucala, R., Sherry, B., Manogue, K. R., Tortolani, A. J., Cerami, A. & Tracey, K. J. (1995) *Mol. Med.* 1, 254–266, and Bianchi, M., Bloom, O., Raabe, T., Cohen, P. S., Chesney, J., Sherry, B., Schmidtmayerova, H., Zhang, X., Bukrinsky, M., Ulrich, P., Cerami, A. & Tracey, J. (1996) *J. Exp. Med.*, in press]. We have now elucidated the mechanism by which CNI-1493 inhibits macrophage TNF synthesis and show here that it acts through suppression of TNF translation efficiency. CNI-1493 blocked neither the lipopolysaccharide (LPS)-induced increases in the expression of TNF mRNA nor the translocation of nuclear factor NF-κB to the nucleus in macrophages activated by 15 min of LPS stimulation, indicating that CNI-1493 does not interfere with early NF-κB-mediated transcriptional regulation of TNF. However, synthesis of the 26-kDa membrane form of TNF was effectively blocked by CNI-1493. Further evidence for the translational suppression of TNF is given by experiments using chloramphenicol acetyltransferase (CAT) constructs containing elements of the TNF gene that are involved in TNF translational regulation. Both the 5′ and 3′ untranslated regions of the TNF gene were required to elicit maximal translational suppression by CNI-1493. Identification of the molecular target through which CNI-1493 inhibits TNF translation should provide insight into the regulation of macrophage activation and mechanisms of inflammation.**

Following the activation of monocytes and macrophages, the rapid production of proinflammatory cytokines [such as tumor necrosis factor (TNF), interleukins 1 and 6 (IL-1 and IL-6) and others] mediates a wide variety of pathologic states, including lethal shock and tissue injury, acute and chronic inflammation, and cachexia (1). We recently described a tetravalent guanylhydrazone compound (CNI-1493) that suppressed lipopolysaccharide (LPS)-stimulated macrophage synthesis of proinflammatory cytokines [TNF, IL-1, IL-6, macrophage inflammatory proteins 1α and 1β (MIP-1α and MIP-1β], inhibited the synthesis of inducible nitric oxide synthase (iNOS), and protected against lethal endotoxemia and carrageenan-induced inflammation in mice (2, 3). Previous evidence indicates that these macrophage-suppressive effects were selective. For instance, concentrations of CNI-1493 that completely suppressed TNF protein synthesis did not suppress total cellular RNA transcription as measured by incorporation of labeled uridine into total RNA and did not suppress total cellular protein synthesis as measured by incorporation of labeled methionine into total protein (3). Moreover, these concentrations of CNI-1493 did not inhibit enhanced expression of major histocompatibility complex (MHC) class II antigens mediated by γ interferon (IFN-γ) (3). By contrast with glucocorticoids, another class of inhibitors that suppresses proinflammatory cytokine production, CNI-1493 retained its TNF-suppressive effects even in the presence of IFN-γ.

TNF synthesis in monocytes/macrophages is stimulated by LPS and other invasive stimuli (1). This production is mediated by increases in the transcription and translation of the *TNF* gene (4). Recent evidence suggests that discrete signal transduction pathways regulate the transcriptional and translational regulatory events (Fig. 1). The p38 MAP kinase signal transduction pathway, whose intermediates include cdc42, MEKK, MKK3/MKK4, and p38 MAP kinase (5–7), has been implicated in the enhancement of TNF translation efficiency after LPS stimulation (8–10). Transcriptional activation is mediated by pathways culminating in nuclear translocation of the transcriptional regulator NF-κB. In these cascades, LPS stimulates the release of I-κB from cytoplasmic NF-κB, resulting in translocation of NF-κB from the cytoplasm to the nucleus, where it binds κB consensus binding sites in the promoters of target genes including *TNF* and genes of other proinflammatory cytokines (11–13). In the present investigation, we determine the mechanism by which CNI-1493 suppresses TNF protein synthesis. The results now indicate that CNI-1493 acts by inhibiting TNF translational efficiency and not by inhibiting the nuclear translocation of NF-κB in LPS-stimulated macrophages.

MATERIALS AND METHODS

Cell Isolation and Culture. For studies of human monocytes, buffy coats were obtained by elutriation from normal individual donors to the Long Island Blood Bank Services. Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation through Ficoll/Hypaque (Pharmacia); typically one preparation yielded 200×10^6 adherent cells. These cells were cultured in 100-mm plates at 2×10^6 cells per plate in 5 ml of RPMI 1640 medium containing 10% (vol/vol) normal heat-inactivated human serum, antibiotics, and L-glutamine. Cells were allowed to adhere overnight in the presence of 5% CO_2 in a humidified incubator at 37°C. Nonadherent cells were washed away twice with phosphate-buffered saline (PBS), and adherent cells (monocytes) were then treated with CNI-1493 at 1 μM

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Abbreviations: PBMC, peripheral blood mononuclear cell; CMV, cytomegalovirus; TNF, tumor necrosis factor; LPS, lipopolysaccharide; CAT, chloramphenicol acetyltransferase; IL-1 and IL-6, interleukins 1 and 6; MAP kinase, mitogen-activated kinase; MIP, macrophage inflammatory protein; iNOS, inducible nitric oxide synthase; IFN-γ, γ interferon; MHC, major histocompatibility complex; UTR, untranslated region.

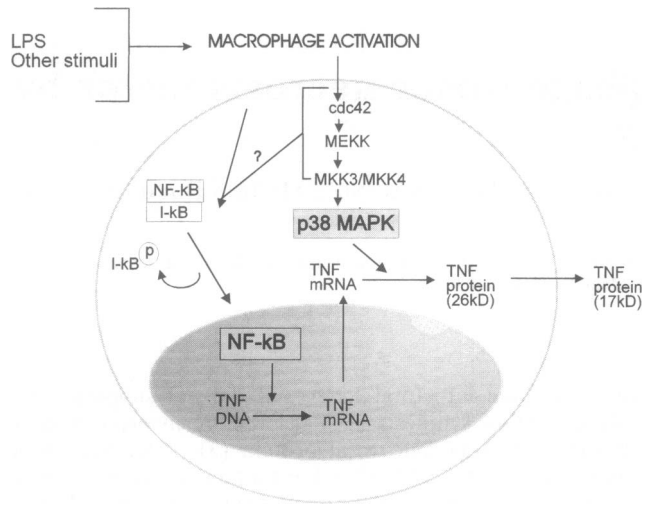

FIG. 1. Schematic illustration of putative signal transduction pathways regulating the macrophage activation-mediated production of TNF. MAPK, mitogen-activated protein (MAP) kinase.

for various periods of time, followed by stimulation with LPS (*Escherichia coli* 0111:B4, 100 ng/ml; Sigma) for either 2 hr (for RNA studies) or 4 hr (for protein studies). The murine macrophage-like RAW 264.7 cells [obtained from the American Type Culture Collection (ATCC), Rockville, MD] were maintained in RPMI 1640 medium containing 10% fetal calf serum (FCS), antibiotics, and L-glutamine. Cells were allowed to adhere for 4 hr prior to use in experiments.

Total RNA Isolation and Quantitation of TNF mRNA Levels. Total RNA was prepared from primary PBMCs treated with LPS at 100 ng/ml with or without 1 μM CNI-1493 and/or 5 μg of actinomycin D per ml for 0, 20, or 40 min by using the RNAzol B reagent (Tel-Test, Friendswood, TX) per the manufacturer's instructions. Competitive reverse transcription (RT)–PCR methods were used to quantitate TNF mRNA levels (14). Briefly, one set of oligonucleotide primers was used to amplify both endogenous TNF cDNA and exogenously added artificial TNF-MIMIC DNA of known concentration. MIMIC template was constructed with the use of a PCR-MIMIC construction kit (Clontech) following the manufacturer's instructions. Primer pairs used for MIMIC synthesis were 5′-ACTGAAAGCATGATCCGGGACCGCAAGTGAAATCTCCTCCG-3′ and 5′-TCACAGGGCAATGATCCCAAATCTGTCAATGCAGTTGTGAG-3′. Primers used for RT–PCR of both TNF-MIMIC and endogenous TNF mRNA were: 5′-ACTGAAAGCATGATCCGGGAC-3′ (spanning TNF base pairs 92–112) and 5′-TCACAGGGC-AATGATCCCAAA-3′ (spanning TNF base pairs 766–787), which yielded a 695-bp fragment from endogenous TNF mRNA and a 450-bp TNF-MIMIC fragment (15). RT–PCR was performed by using the Perkin–Elmer RT–PCR kit as per the manufacturer's instructions with the following modifications: (*i*) 0.35 μg of RNA was used per reaction, (*ii*) the RT reaction was primed with random hexamers, (*iii*) the PCR reaction was amplified for 30 cycles, and the (*iv*) PCR reaction was adjusted to contain 2.5 mg of $MgCl_2$ per ml. The PCR cycle consisted of 95°C for 15 sec, 55°C for 30 sec, and 65°C for 60 sec. After the final amplification, the reaction was elongated for 7 min at 72°C. Twenty microliters of each reaction mixture was separated by electrophoresis by using 1.5% agarose gels, followed by staining with ethidium bromide. The quantity of TNF cDNA in a given reaction sample was measured by extrapolating from serial dilutions of TNF-MIMIC in the same reaction.

Electrophoretic Mobility Shift Assay. RAW 264.7 cells were treated with CNI-1493 at various concentrations and stimulated with 100 ng of LPS per ml for 15 min. Cells were then rinsed in ice-cold PBS and harvested. Extracts were prepared essentially as described (16) and incubated with ^{32}P-labeled oligodeoxynucleotides corresponding to the NF-κB consensus binding site (Promega). Binding reactions and electrophoresis were performed as described (16).

TNF Pulse Labeling. Newly synthesized proteins were radiolabeled by using a modified version of previously described methods (17, 18). Briefly, human PBMCs isolated by elutriation and purified by adherence were incubated overnight in Dulbecco's modified Eagle's medium containing 10% FCS, antibiotics, and L-glutamine without methionine and were treated with CNI-1493 in concentrations shown. After 1 hr, the medium was replaced with fresh methionine-free medium containing 0.2 mCi of [^{35}S]methionine (NEN) and 100 ng of LPS per ml for 1 hr. The pulse-labeling period was terminated by removing the culture medium, washing the cells twice with PBS, and adding methionine-containing medium. Cells were lysed in buffer [50 mM Tris·HCl, pH 7.4/100 mM NaCl/1% Triton X-100/5 mM EDTA/0.02% sodium azide/100 μM phenylmethylsulfonyl fluoride (PMSF)/1 μM pepstatin]; 50 μl of 10× lysis buffer was added per 500-μl supernatants. Cell lysates were immunoprecipitated with polyclonal rabbit anti-human TNF (from Barbara Sherry, Picower Institute), collected over protein A-agarose beads, and analyzed by SDS/PAGE electrophoresis and autoradiography.

Chloramphenicol Acetyltransferase (CAT) Assay. Plasmid constructs containing the CAT gene and either elements of the human TNF 3′ untranslated region downstream (3′ UTR), the 5′ untranslated region (5′ UTR), both 5′ and 3′ fragments (5′ UTR–3′ UTR), or the cytomegalovirus (CMV) promoter were generous gifts of Bruce Beutler (University of Texas Southwestern Medical Center, Dallas). Through use of these reporter constructs, both the 3′ and 5′ UTR regions of the TNF gene have been shown to contribute to the regulation of TNF biosynthesis at the translational level (19–21). CAT construct (2–10 μg of DNA) was cotransfected into RAW 264.7 cells with 2 μg of plasmid pCH110 containing the β-galactosidase gene under the control of the simian virus 40 promoter (Pharmacia), which was included as an internal control for transfection efficiency. Transfections were performed by a calcium phosphate precipitation method (22). Cells were washed 24 and 48 hr later with medium, treated with 1 μM CNI-1493 for 1 hr where indicated and then exposed to LPS at 1 μg/ml. After four hours, cell lysates were harvested for determination of both β-galactosidase and CAT activity. β-galactosidase activity was photometrically assayed by measuring the conversion of *o*-nitrophenyl β-D-galactopyranoside (23). CAT assays were performed by using 25 unit equivalents of β-galactosidase per TLC assay according to the method of Gorman and others (24). CAT results were imaged and quantitated on a phosphorimager (Packard Instantimager); TLC plates were also exposed to Kodak XAR film. All experiments were performed in triplicate.

RESULTS

CNI-1493 Does Not Suppress Increased Expression of TNF mRNA. In agreement with previous results (2), we found that addition of 1 μM CNI-1493 to PBMCs suppressed 95% of secreted TNF protein measured by ELISA in supernatants collected 4 hr after LPS stimulation (data not shown). To evaluate whether CNI-1493 inhibited TNF via suppression of transcriptional regulation, steady-state TNF mRNA levels were measured in LPS-activated PBMCs by using the method of quantitative RT–PCR. TNF mRNA was observed to increase nearly one million-fold within 2 hr after LPS activation (Fig. 2). Addition of CNI-1493 in quantities that suppressed 95% of secreted TNF protein (1 μM) in

these experiments did not significantly reduce the increased expression of TNF mRNA (Fig. 2). We observed a modest reduction in peak steady-state level of TNF mRNA, which was attributable to decreased TNF mRNA stability, measured by treating PBMCs with LPS and CNI-1493 in the presence of actinomycin D (Fig. 2). Thus, these data give evidence that the suppressive effect of CNI-1493 on TNF protein production cannot be due to suppression of TNF mRNA expression.

CNI-1493 Does Not Inhibit Nuclear Translocation of NF-κB During Early Macrophage Activation. Because CNI-1493 did not interfere with LPS-induced up-regulation of TNF mRNA, these results predicted that CNI-1493 would not suppress the early activation of NF-κB, a transcriptional activator of TNF (13, 25, 26). Accordingly, the electrophoretic mobility-shift assay was utilized to address this hypothesis. Addition of CNI-1493 at concentrations that inhibit 50% and 95% of TNF protein (0.5 and 1 μM, respectively) did not inhibit the nuclear translocation of NF-κB following LPS stimulation (Fig. 3). Thus, CNI-1493 failed to inhibit the early NF-κB signal transduction pathways activated within 15 min after LPS treatment and allowed increased expression transcription of TNF mRNA.

CNI-1493 Inhibits Synthesis of Membrane-Bound TNF (26 kDa). To address directly CNI-1493 inhibition of TNF protein translation, we used [^{35}S]methionine labeling and immunoprecipitation to determine the effect of CNI-1493 on the translation of the 26-kDa membrane TNF prohormone (17). CNI-1493 effectively suppressed the synthesis of the 26-kDa prohormone (Fig. 4). Because the 26-kDa prohormone is the precursor to mature 17-kDa TNF, inhibition of 26-kDa protein translation thereby prevented appearance of the 17-kDa form as well (Fig. 4). Since LPS stimulation normally increases the translational efficiency of the 26-kDa TNF (17), these results indicate that CNI-1493 suppresses TNF production by inhibiting the translational activation of 26-kDa TNF.

CNI-1493 Suppresses TNF Translation Through Both 5′ and 3′ UTRs of TNF. It has previously been shown that elements in both the 5′ and the 3′ UTRs of the *TNF* gene

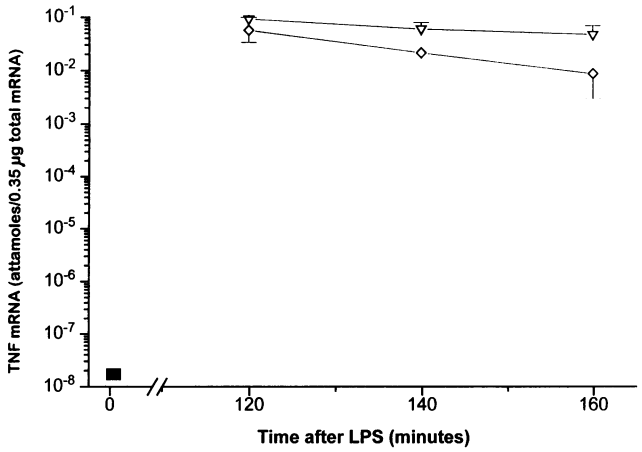

FIG. 2. Quantitation of steady-state TNF mRNA levels in human PBMCs. Human PBMCs were harvested for total mRNA, and TNF mRNA levels were quantitated by using competitive RT–PCR as described in text. TNF mRNA was assayed in the unstimulated state (■), after 2 hr of LPS treatment at 100 ng/ml (▽) and after 1-hr pretreatment with 1 μM CNI-1493 followed by 2 hr of LPS treatment (◇). Half-life studies were performed by treating PBMCs as above with addition of actinomycin D at 5 μg/ml prior to mRNA harvest. Time points shown are after addition of LPS; actinomycin D was added at 120 min. Data are expressed as the mean ± SD (n = 3 experiments); where error bars are not visible, they lie within the symbol itself.

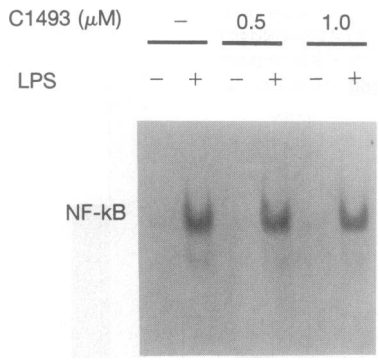

FIG. 3. Effect of CNI-1493 on LPS-induced NF-κB activation in RAW 264.7 cells. RAW 264.7 cells were treated with CNI-1493 at various concentrations for 1 hr as indicated. Cells were stimulated with LPS at 100 ng/ml for 15 min. Whole-cell lysates were prepared and analyzed for NF-κB binding activity as described in text.

contribute to the enhanced translational efficiency of TNF mRNA in monocytes/macrophages after LPS stimulation (19, 20, 27). We used these same CAT plasmid constructs to address whether the suppressive effect of CNI-1493 on TNF protein translation depended on the presence of either or both of these elements. In agreement with previous work (19, 20, 27), transfection of RAW 264.7 cells with the 3′ UTR, 5′ UTR, and 5′ UTR–3′ UTR construct suppressed expression of CAT as compared with transfections with CMV-CAT constructs not containing *TNF* elements (data not shown), and addition of LPS significantly stimulated CAT reporter activity (Fig. 5). Addition of 1 μM CNI-1493 suppressed CAT reporter biosynthesis in LPS-treated cells containing either the 5′ UTR, the 3′ UTR, or the 5′ UTR–3′ UTR constructs (Fig. 5). Average suppression of LPS-mediated CAT biosynthesis for the different constructs we observed was: 5′ UTR (24 ± 2%, n = 3), 3′ UTR (24 ± 17%, n = 3) and 5′ UTR–3′ UTR (74 ± 10%, n = 2). Expression of CAT in CMV-CAT control transfectants was detected under all conditions and was not responsive to the addition of either LPS or CNI-1493 (data not shown). Note that maximal suppression by CNI-1493 was observed only when both elements were present, suggesting that suppression of TNF translation by CNI-1493 is dependent upon synergy between the 5′ UTR and 3′ UTR elements of TNF mRNA.

DISCUSSION

CNI-1493 is a potent inhibitor of monocyte/macrophage TNF synthesis. The present data now give direct evidence that pharmacological quantities of CNI-1493 inhibit the

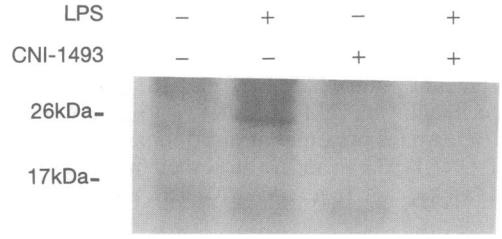

FIG. 4. Inhibition of 26-kDa TNF protein synthesis by CNI-1493 in human PBMCs. Human PBMCs were incubated in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of 2.5 μM CNI-1493 and in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of LPS and [^{35}S]methionine for 1 hr. Cell lysates were prepared and immunoprecipitated with anti-TNF antisera and were analyzed by electrophoresis and autoradiography. The positions of the TNF immunoreactive bands are identified by their apparent molecular weights on the left.

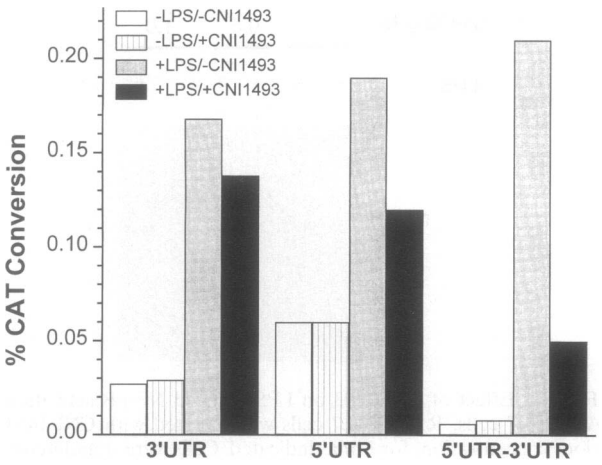

FIG. 5. Effects of CNI-1493 on LPS-induced CAT expression in RAW 264.7 cells transfected with CAT-TNF constructs. CNI-1493 (1 μM) was added to transfected RAW 264.7 cells for 1 hr prior to addition of LPS at 1 μg/ml or to uninduced controls as indicated. CAT assay was performed on all transfected cells 4 hr after addition of LPS. Results shown are representative of three experiments. Data are expressed as the percentage of total CAT conversion.

translation of membrane 26-kDa TNF, which is normally stimulated by LPS. CNI-1493 did not inhibit early nuclear translocation of NF-κB after LPS stimulation (Fig. 3) and thereby enabled significant increases of TNF mRNA, as normally occurs after LPS stimulation (Fig. 2). The translation inhibition was observed to be dependent upon both the 3′ and 5′ UTR elements of the TNF mRNA (Fig. 5). We have previously shown that inhibition of TNF synthesis is specific, because total cellular synthesis of RNA and protein were unaffected by quantities of CNI-1493 that effectively suppress TNF synthesis (3).

Previous work suggests that after macrophage activation, discrete signaling pathways regulate the p38 MAP kinase-dependent increases of TNF translation and the NF-κB-dependent enhancement of TNF transcription (Fig. 1). Raf-1 activation has been implicated in the stimulation of an NF-κB pathway in NIH 3T3 cells, but this pathway has not been confirmed in monocyte/macrophages (8, 9). It is possible that these signaling pathways diverge upstream of MEKK, the raf-1 homolog implicated in activation of JNK/p38 MAP kinase family (5–7). The present results place the TNF-suppressive activity of CNI-1493 in the p38 cascade because it inhibits translation of the 26-kDa prohormone but does not inhibit the translocation of NF-κB. Although the molecular target of CNI-1493 remains unproved, the inhibitory effects of CNI-1493 are not restricted to LPS-activated signaling pathways because it suppressed TNF synthesis in monocytes activated by exposure to TSST-1 (3), a stimulating agent that is independent of LPS. Thus, the target of CNI-1493 may represent a shared signaling point where LPS- and TSST-induced cascades converge. Finally, it will be of interest to determine whether the site of action is proximal or distal to p38 MAP kinase and whether CNI-1493 can inactivate other members of the p38 MAP kinase cascade (e.g., cdc42, MEKK, and MKK3/MKK4) (5–7).

TNF occupies a proximal position in amplifying the proinflammatory cytokine cascade in LPS-activated macrophages. For instance, neutralization of TNF with monoclonal antibody during overwhelming infection suppressed subsequent increases of serum IL-1 and IL-6 despite persistent bacteremia (28). It is plausible that by inhibiting TNF synthesis, CNI-1493 might prevent subsequent release of these secondary cytokines. In support of this hypothesis, we previously showed that CNI-1493 inhibited proinflammatory cytokines (including IL-1, IL-6, MIP-1α, MIP-1β), iNOS, and cytokine-inducible L-arginine transporter functions in LPS-stimulated macrophages (2, 3). It will be of interest to determine whether CNI-1493 suppressed these proinflammatory responses as an indirect consequence of TNF blockade or by a direct effect that suppressed a shared regulatory mechanism common to these proinflammatory mediators. CNI-1493 is also an effective macrophage cytokine suppressor in the presence of IFN-γ, suggesting that it will retain its effectiveness in clinical conditions where IFN-γ is ubiquitous (29). Because IFN-γ can override the suppression of TNF in the C3H/HeJ mice, which normally fail to produce TNF in response to LPS, it will be of interest to use CNI-1493 to further characterize the molecular basis for this genetic defect (29, 30). It is hoped that these studies will further understanding of the mechanism of macrophage activation and cytokine synthesis and lead to the development of therapeutic strategies to prevent the manifestation of macrophage-mediated disease.

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