Cooperative Signaling by Tumor Necrosis Factor Receptors CD120a (p55) and CD120b (p75) in the Expression of Nitric Oxide and Inducible Nitric Oxide Synthase by Mouse Macrophages*

(Received for publication, December 29, 1997, and in revised form, May 5, 1998)

David W. H. Riches,Edward D. Chan,Elizabeth A. Zahradka,Brent W. Winston,Linda K. Remigio, and Fiona R. Lake

From the Division of Basic Science, Department of Pediatrics, National Jewish Medical and Research Center, Denver, Colorado 80206 and the Department of Biochemistry, Biophysics and Genetics, the Division of Pulmonary Sciences, Department of Medicine, Department of Immunology, and Department of Pharmacology, University of Colorado Health Sciences Center, Denver, Colorado 80220 and the Departments of Medicine, Biochemistry, and Molecular Biology, Division of Critical Care Medicine, University of Calgary, Calgary, Alberta T2N 4N1, Canada

Tumor necrosis factor-α (TNFα) is recognized by the cell-surface receptors CD120a (p55) and CD120b (p75). In the present study, we have investigated the role of these receptors in the expression of NO2−, a stable metabolite of nitric oxide, and inducible nitric oxide synthase (iNOS) by mouse macrophages. Specific antibody-mediated aggregation of CD120a (p55) induced NO2− accumulation in culture supernatants and iNOS mRNA expression in macrophage lysates, whereas cross-linking of CD120b (p75) had a minimal effect. In contrast, simultaneous cross-linking of both receptors led to a marked augmentation in NO2− and iNOS mRNA expression. Antibody-mediated blockade of CD120a (p55) completely inhibited NO2− expression in response to TNFα, whereas blockade of CD120b (p75) reduced NO2− accumulation by ~50%. Specific ligation of CD120a (p55) with either (i) human TNFα or (ii) by incubation with mouse TNFα following pretreatment of macrophages with blocking concentrations of anti-CD120b (p75) antibody resulted in a similar reduction in NO2− production in response to TNFα. Quantification of iNOS mRNA, protein, and NO2− expression during independent and coligation of CD120a (p55) and CD120b (p75) indicated that iNOS mRNA and protein expression was transient in nature when CD120a (p55) was cross-linked alone but was prolonged when both receptors were simultaneously cross-linked. In addition, cross-linking both receptors also led to a potentiation of NO2− accumulation in culture supernatants that was more pronounced at later time points. These findings suggest that while cross-linking of CD120a (p55) is necessary and sufficient for iNOS mRNA and NO2− expression, CD120b (p75) participates by (i) increasing the sensitivity of the cells to TNFα, probably by “passing” ligand to CD120a (p55), and (ii) initiating a signaling event that results in a more sustained induction of iNOS mRNA and protein and thereby augments the production of nitric oxide.

Nitric oxide (NO) is a major effector of macrophage-mediated cytocidal activity against both tumor cells and obligate and facultative intracellular parasites such as Toxoplasma gondii (1), Leishmania major (2, 3), Mycobacterium leprae (4), and Listeria monocytogenes (5, 6). The production of NO is controlled by inducible nitric oxide synthase (iNOS) that catalyzes the oxidation of the guanidino nitrogen of L-arginine to form NO and L-citrulline (7). However, in contrast to constitutive nitric oxide synthases of brain and endothelium (8–10), macrophage iNOS expression results in NO production for prolonged periods and at high levels of output. The expression of iNOS is stimulated by a variety of exogenous stimuli including lipopolysaccharide (11) which act to stimulate the transcription of the iNOS gene. However, in many situations it is the cytokines IFNγ and TNFα produced during the host inflammatory and immune responses that act cooperatively to induce iNOS expression (12).

TNFα interacts with macrophages and other cell types through two distinct, although related, cell-surface receptors with molecular masses of 55 kDa (CD120a (p55)) and 75 kDa (CD120b (p75)) (13–16). With the aid of monoclonal and polyclonal antibodies that can function both as mimics and antagonists of TNFα actions, it has become clear that most functions elicited by TNFα are mediated by cross-linking of CD120a (p55). These functions include cytotoxicity against L1 cells (16), acute phase protein synthesis by hepatoma cells (17), and synthesis of the fibroblast progression-type growth factor insulolin-like growth factor I by macrophages (IGF-I) (18). Antibody-induced cross-linking of CD120b (p75) has also been shown to induce certain restricted functions such as thymocyte proliferation (16) and granulocyte/macrophage colony-stimulating factor secretion (19). However, an important function of this receptor appears to be the enhancement of functions initiated by cross-linking of CD120a (p55). Two concepts have been proposed to address the mechanism by which ligation of CD120b (p75) may assist the functions of CD120a (p55). First, by virtue of its higher affinity and more rapid association and dissociation...
tion rate constants than CD120a (p55), CD120b (p75) has been proposed to regulate the rate of interaction of TNFα with CD120a (p55) thereby enhancing signaling by CD120a (p55) (20, 21). Second, cross-linking of CD120b (p75) may generate a signal that may or may not synergize with signals produced during ligation of CD120a (p55). Since both concepts are not mutually exclusive, cross-linking of CD120b (p75) may contribute to the functions elicited by ligation of CD120a (p55) although may not necessarily, by itself, stimulate a cellular response. In this study we have investigated the roles of CD120a (p55) and CD120b (p75) in the induction of NO₂⁻ (a stable oxidation product of NO) and iNOS mRNA expression by mouse macrophages and the mechanisms underlying their involvement. Our findings show that whereas cross-linking of CD120a (p55) is necessary and sufficient for iNOS and NO₂⁻ expression, additional cross-linking of CD120b (p75) and signaling by this receptor are required for the optimal and prolonged induction of iNOS and NO₂⁻.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium was obtained from Hazard Laboratories, Denver, PA, and was supplemented immediately before use with 2 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin. Fetal bovine serum was purchased from Irvine Scientific, Santa Ana, CA. 24-Well tissue culture plates and 100-mm tissue culture dishes were purchased from Costar, Cambridge, MA. [a-32P]deoxyctydine 5'-triphosphate (≥3000 Ci/mmol) and [γ-32P]ATP (≥3000 Ci/mmol) were obtained from NEN Life Science Products. Recombinant IFNγ was kindly provided by Dr. Gary Johnson, National Jewish Center under specific pathogen-free conditions. Recombinant GST-c-Jun was pur chased from Genzyme Diagnostics, Cambridge, MA. All other reagents were of the highest possible purity. C3H/HeJ mice were bred at the Biological Resource Center at National Jewish Medical and Research Center under specific pathogen-free conditions. Recombinant GST-c-Jun was kindly provided by Dr. Gary Johnson, National Jewish Medical and Research Center, Denver, CO. Anti-mouse iNOS antibody was purchased from Alexis Biochemicals, San Diego, CA.

Isolation and Culture of Mouse Macrophages—Mouse bone marrow-derived macrophages were obtained by culturing femoral and tibial bone marrow from C3H/HeJ mice in growth medium that comprised Dulbecco’s modified Eagle’s medium supplemented with 2 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 10% (v/v) heat-inactivated fetal calf serum, and 10% (v/v) L-cell conditioned medium as a source of macrophage colony-stimulating factor as previously reported (24). CD120α (p55) and rabbit anti-mouse CD120b (p75) antibodies were generously donated by Genentech, Inc., San Francisco, CA. Human TNFα was purchased from Hazleton Laboratories, Denver, PA, and was supplemented immediately before use with 2 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin. Fetal bovine serum was purchased from Irvine Scientific, Santa Ana, CA. 24-Well tissue culture plates and 100-mm tissue culture dishes were purchased from Costar, Cambridge, MA. 24-Well tissue culture plates and 100-mm tissue culture dishes were purchased from Costar, Cambridge, MA. All other reagents were of the highest possible purity. C3H/HeJ mice were bred at the Biological Resource Center at National Jewish Medical and Research Center under specific pathogen-free conditions. Recombinant GST-c-Jun was kindly provided by Dr. Gary Johnson, National Jewish Center under specific pathogen-free conditions. Recombinant GST-c-Jun was kindly provided by Dr. Gary Johnson, National Jewish Medical and Research Center, Denver, CO. Anti-mouse iNOS antibody was purchased from Alexis Biochemicals, San Diego, CA.

Isolation and Culture of Mouse Macrophages—Mouse bone marrow-derived macrophages were obtained by culturing femoral and tibial bone marrow from C3H/HeJ mice in growth medium that comprised Dulbecco’s modified Eagle’s medium supplemented with 2 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 10% (v/v) heat-inactivated fetal calf serum, and 10% (v/v) L-cell conditioned medium as a source of macrophage colony-stimulating factor as previously reported (24, 25, 26). The cells were incubated with stimuli for 24 h. Each experiment was conducted in triplicate and was conducted a minimum of three times. The results presented are the mean ± S.D. of three experiments.

Analysis of iNOS mRNA and Protein Expression—The expression of iNOS mRNA was determined by Northern analysis. The extraction, purification, electrophoresis, and transfer of the RNA to nylon membranes was carried out as described (22, 23). Briefly, macrophage mono-

layers were extracted with 4 ml guanidine isothiocyanate (24), and the RNA was purified by centrifugation through 5.7 ml cesium chloride at 100,000 × g for 18 h. Fifteen μg of total RNA were electrophoresed under denaturing conditions through a 1.0% (w/v) agarose-formaldehyde gel and then transferred to Nitran membranes. The blots were hybridized with 5 × 10⁶ dpm of 32P-labeled iNOS cDNA probe (8), and autoradiograms were prepared by exposure to Kodak XAR-5 film at −70 °C. The mouse iNOS cDNA probe was kindly provided by Dr. Charles Lowenstein, Johns Hopkins University School of Medicine, Baltimore. For the measurement of iNOS protein, macrophages were lysed in 500 μl of Nonidet P-40 lysis buffer (50 mM Tris/HCl buffer, pH 8.0, containing 137 mM NaCl, 1% (v/v) glycerol, 1% (v/v) Nonidet P-40, 1 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 μg/ml Na3VO4, and 1 μg/ml phenylmethylsulfonyl fluoride). Samples were then separated by SDS-PAGE through a 7.5% gel, electroblotted onto nitrocellulose membranes, and immunoblotted with anti-mouse iNOS antibody as described previously (26).

Analysis of c-Jun-NH2-terminal Kinase (JNK) Activation—The activation of JNK was quantified using a solid phase in vitro kinase assay employing a glutathione S-transferase-c-Jun fusion protein (GST-c-Jun) bound to glutathione-Sepharose beads as substrate in the presence of [γ-32P]ATP as described previously (25, 26). Briefly, macrophage monolayers were stimulated and then lysed at 4 °C with 500 μl of an ice-cold lysis buffer (50 mM Tris/HCl buffer, pH 8.0, containing 137 mM NaCl, 1% (v/v) glycerol, 1% (v/v) Nonidet P-40, 1 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 μg/ml Na3VO4, and 1 μg/ml PMSF) (25). JNK activity in each sample of lysate was bound to 15 μl of 1:1 slurry of lysis buffer:GST-c-Jun-Sepharose beads and incubated at 4 °C for 2 h. The beads were then washed twice with lysis buffer and twice with washing buffer (10 mM Hepes buffer, pH 7.2, containing 30 mM β-glycerophosphate, 10 mM β-nitrophenylphosphate, 10 mM MgCl₂, 0.5 mM diithiothreitol, and 50 μM Na3VO4). JNK activity was detected in an in vitro kinase assay in the presence of [γ-32P]ATP (10 μCi/sample) before being subjected to SDS-PAGE, transferred onto nitrocellulose membranes, and detected by autoradiography.

RESULTS

NO₂⁻ Production by IFNγ and TNFα—The production of NO₂⁻ by mouse bone marrow-derived macrophages was similar to that previously reported for elicited mouse peritoneal macrophages (12) in that both IFNγ and TNFα were either unable or poorly capable of stimulating NO₂⁻ when presented alone but induced abundant levels of NO₂⁻ when presented together. As can be seen in Fig. 1, NO₂⁻ production was initially detected at a TNFα concentration of 0.1 ng/ml, whereas maximal production was achieved at concentrations of TNFα of 30 ng/ml or more.

Agonistic Effects of Anti-TNF Receptor Antibodies on NO₂⁻ Production—The independent roles of CD120a (p55) and CD120b (p75) in NO₂⁻ production were initially addressed by incubating macrophage monolayers with increasing concentrations of polyclonal antibodies directed against each receptor type in the presence and absence of IFNγ. As can be seen in Fig.

![Fig. 1. Stimulation of NO₂⁻ expression by mouse macrophages after incubation with increasing concentrations of TNFα in the presence (○) but not in the absence (■) of IFNγ (10 units/ml). The cells were incubated with stimuli for 24 h.](image-url)
when macrophages were incubated with anti-CD120a (p55) antibody in the presence of IFNγ (10 units/ml), a concentration-dependent increase in NO₂ production was observed when added at between 0.5 and 1.0 μg/ml antibody. Further increments in the concentration of the anti-CD120a (p55) antibody were found to result in a decline in NO₂ production until at 10 μg/ml NO₂ production was not detected. By contrast, incubation of macrophages with anti-CD120b (p75) antibody either in the presence or absence of IFNγ did not result in any significant production of NO₂ (Fig. 2). However, when macrophages were simultaneously challenged with equivalent amounts of both anti-CD120a (p55) and anti-CD120b (p75), antibodies in the presence of IFNγ, an augmentation in NO₂ production was observed compared to when each antibody was added alone (Fig. 2). Exposure of macrophages to either antibody in the absence of IFNγ failed to stimulate NO₂ production (data not shown). Furthermore, incubation of mouse macrophages with non-immune rabbit IgG in the presence or absence of IFNγ did not result in the induction of NO₂ production (data not shown). These data suggest that although ligation of CD120a (p55) is sufficient for NO₂ production, cross-linking of CD120b (p75) augmented the response initiated by the antibody-mediated cross-linking of CD120a (p55).

**Antagonistic Effects of Anti-CD120a (p55) and Anti-CD120b (p75) Antibodies on TNFα-induced NO₂ Production**—Given the specificity of the anti-TNF receptor antibodies, it seemed reasonable to assume that the interaction with their respective antigens will result in homologous cross-linking of each receptor type. Thus, the data presented above suggests that during antibody-mediated cross-linking, CD120b (p75) enhanced the signal generated by cross-linking of CD120a (p55). Furthermore, since TNFα was not required in these experiments, hence eliminating any possibility of “ligand-passing,” it seemed likely that cross-linking of CD120b (p75) initiated or augmented an independent signaling event. To determine if this conclusion also applied to TNFα itself, we independently blocked CD120a (p55) and CD120b (p75) with inhibitory concentrations of the anti-receptor antibodies (10 μg/ml) and quantified the effect on the TNFα-mediated stimulation of NO₂ production. Macrophage monolayers were pretreated with either anti-CD120a (p55) antibody or anti-CD120b (p75) antibody for 30 min prior to stimulation with an optimal concentration of TNFα (10 ng/ml) for 24 h in the presence of IFNγ (10 units/ml). Fig. 3 shows that under these conditions anti-CD120a (p55) antibody completely inhibited NO₂ production. In addition, anti-CD120b (p75) antibody partially, although significantly (p < 0.05), blocked NO₂ production by TNFα.

Neither antibody stimulated the production of NO₂ when added in the absence of IFNγ (Fig. 3). Furthermore, non-immune rabbit IgG (10 μg/ml) had no effect on the production of NO₂ in response to challenge with IFNγ (10 units/ml) and TNFα (10 ng/ml) (data not shown).

To explore further the possibilities that (i) CD120b (p75) may function to “pass” TNFα to CD120a (p55) and/or (ii) that CD120b (p75) may initiate a signal to enhance the response of CD120a (p55), macrophage monolayers were pretreated with an inhibitory concentration of anti-CD120b (p75) antibody (10 μg/ml) for 30 min prior to stimulation with IFNγ and increasing concentrations of TNFα (0.1–30 ng/ml) to span the entire concentration response curve for NO₂ production. If CD120b (p75) was functioning to “pass” ligand to CD120a (p55), the inhibitory effect of the anti-CD120b (p75) antibody should be overcome by increasing the concentration of TNFα since the ligand passing phenomenon is based on the higher affinity of CD120b (p75) for TNFα compared with CD120a (p55). By contrast, if CD120b (p75) was required in a signaling capacity, the inhibitory effect of the anti-CD120b (p75) antibody would not be overcome by excess TNFα under conditions in which both CD120a (p55) and CD120b (p75) were saturated with ligand. As can be seen in Fig. 4, blocking CD120b (p75) function with an inhibitory concentration of the specific antibody resulted in an almost complete cessation of NO₂ production at low concentrations of TNFα. However, importantly, the inhibitory effect of the anti-CD120b (p75) antibody was not overcome with increasing concentrations of TNFα and remained significantly blocked by almost 50% (p < 0.05) when the cells were exposed to a concentration of TNFα (30 ng/ml) that maximally stimulated NO₂ production (Fig. 4). These findings suggest that although CD120b (p75) may function to pass TNFα to CD120a (p55) at low ligand concentrations, it directly contributes to TNFα-mediated signaling under saturating ligand concentrations.

**Effect of Human TNFα on NO₂ Production**—The results obtained above were confirmed by determining the effect of human TNFα on the production of NO₂ since previous studies have shown human TNFα to bind to mouse CD120a (p55) but not to mouse CD120b (p75). Mouse macrophage monolayers were incubated with increasing concentrations of human or mouse TNFα (0.01–500 ng/ml) in the presence of IFNγ (10 units/ml) for 24 h. As can be seen in Fig. 5, incubation of mouse macrophages with human TNFα resulted in a concentration-dependent increase in NO₂ production that was identical to that seen earlier (Fig. 1). However, the induction of NO₂ expression in response to human TNFα was different in two respects as follows: (i) human TNFα was ineffective at low concentrations...
Effect of blockade of CD120b (p75) on the responsiveness of mouse macrophages to TNFα. Cells were preincubated with anti-CD120b (p75) antibody (10 μg/ml) for 30 min prior to stimulation with increasing concentrations of TNFα (0.1–30 ng/ml) in the presence of a fixed concentration of IFNγ (10 units/ml) for 24 h. ■, macrophages preincubated with anti-CD120b (p75) antibody; □, macrophages exposed to TNFα in the absence of antibody.

Effect of blockade of CD120b (p75) on the responsive-ness of mouse macrophages to TNFα. Cells were preincubated with anti-CD120b (p75) antibody (10 μg/ml) for 30 min prior to stimulation with increasing concentrations of TNFα (0.1–30 ng/ml) in the presence of a fixed concentration of IFNγ (10 units/ml) for 24 h. ■, macrophages preincubated with anti-CD120b (p75) antibody; □, macrophages exposed to TNFα in the absence of antibody.

Effect of Anti-TNF Receptor Antibodies on iNOS mRNA and Protein Expression—To investigate further the roles of CD120a (p55) and CD120b (p75) in iNOS induction, we quantified the time course of induction of iNOS expression during independent and concurrent ligation of both receptors to determine the contribution of each receptor to iNOS induction. The results shown in Fig. 6 indicate that ligation of CD120a (p55) alone for 3 h stimulated iNOS mRNA expression to a level that was not different to that seen during concurrent ligation of both receptors. However, by 9 and 24 h post-stimulation, it was apparent that although the level of iNOS mRNA expression was relatively sustained in macrophages in which CD120a (p55) and CD120b (p75) were co-ligated with either the combination of anti-CD120a (p55) and anti-CD120b (p75) antibodies, or with mouse TNFα, the level of expression progressively declined at these later time points in cells in which CD120a (p55) alone was ligated. Thus, while at 3 h, the level of expression of iNOS mRNA was 98.0 and 99.2 densitometer units for macrophages exposed to anti-CD120a (p55) antibody alone or to both CD120a (p55) and CD120b (p55) antibodies, respectively, the levels at 24 h were 76.1 and 33.3 densitometer units, respectively. These findings were also reproduced at the level of iNOS protein expression. Lysates from macrophages stimulated with mouse or human TNFα in the presence of IFNγ (10 units/ml) for time intervals up to 24 h were analyzed for the presence of iNOS protein by SDS-PAGE followed by immunoblotting with an anti-iNOS antibody. As can be seen in Fig. 7, incubation with mouse TNFα, a ligand for both CD120a (p55) and CD120b (p75), led to a progressive increase in the expression of iNOS protein that peaked at 9 h and that remained elevated for the remainder of the time course. In contrast, while incubation with human TNFα, a selective ligand for CD120a (p55), led to a similar rate of increase in iNOS protein during the first 6 h, the response peaked at 9 h but then began to decline. Consistent with both the mRNA data shown in Fig. 6, the level of iNOS protein detected at 24 h was approximately half that seen when both receptors were ligated. It can also be seen in Fig. 7 that while the amount of NO₂ detected in culture supernatants was similar at early time points in response to both mouse and human TNFα, the accumulation was markedly reduced at later time points in cells in which CD120a (p55) had been ligated with human TNFα in the absence of ligation of CD120b (p75). Thus, ligation of CD120a (p55) alone resulted in a transient expression of iNOS mRNA and protein, whereas co-ligation of CD120a (p55) and CD120b (p75) resulted in a more prolonged induction of the enzyme.

Effect of Ligation of CD120a (p55) and CD120b (p75) on JNK Activation—To begin to define how co-ligation of CD120a (p55) and CD120b (p75) may augment and prolong the expression of iNOS, we investigated the role of these receptors in the activation of JNK. In previously reported work, we have shown that specific members of the mitogen-activated protein kinase family, i.e. p42<sup>mapk</sup>/erk2, p38<sup>mapk</sup> and p46<sup>mapk</sup>/nar, are activated by TNFα (26–28). Although CD120b (p75) plays no apparent role in p42<sup>mapk</sup>/erk2 and p38<sup>mapk</sup> activation, other than by the phenomenon of ligand passing at low concentrations of TNFα (27, 28), the role of CD120b (p75) in the activation of JNK in macrophages has not been previously investigated. Therefore, to determine the relative role of each receptor in the activation of JNK, we utilized the differential binding properties of human and mouse TNFα to CD120a (p55) and CD120b (p75) to...
selectively ligate CD120a (p55) alone or to ligate both receptors, respectively, as described earlier. Mouse macrophages were stimulated with increasing concentrations of human or mouse TNFα (0.1–100 ng/ml) for 10 min and lysed, and the level of JNK activation was quantified using a solid phase in vitro kinase assay. As can be seen in Fig. 8, ligation of both CD120a (p55) and CD120b (p75) with mouse TNFα led to a concentration-dependent activation of JNK. However, although ligation of CD120a (p55) alone with human TNFα was capable of activating JNK, the responses differed between the two ligands in that the macrophages were (i) approximately 10-fold less sensitive to human TNFα than to the mouse cytokine at low concentrations of ligand, and (ii) there was a markedly reduced amplitude of the maximal response to human TNFα compared with mouse TNFα at high concentrations of ligand.

**DISCUSSION**

With the recognition that TNFα binds to cells through a binary system of receptors, a significant emphasis has been placed on determining the relative roles of each receptor in the initiation of specific cellular responses. Initial reports showed that each receptor was capable of signaling independent responses in some cell systems. For example, cross-linking of CD120a (p55) with polyclonal antibody was found to stimulate cytotoxicity in mouse LM cells (16), whereas polyclonal anti-CD120b (p75) antibody was shown to stimulate the proliferation of both mouse thymocytes and the cytolytic T-cell line, CT-6 (16). The results of the present study show that cross-linking of CD120a (p55) by either homotrimeric TNFα or by stimulatory concentrations of specific antibodies resulted in an augmentation and prolongation of the expression of iNOS mRNA, protein, and NO2.

The conclusion that cross-linking of CD120a (p55) was necessary for iNOS and NO2 expression was supported by three lines of evidence. First, polyclonal antibodies directed against CD120a (p55) were found to stimulate the expression of iNOS mRNA and NO2 in the presence of IFNγ. Second, human TNFα, which binds to CD120a (p55) but not CD120b (p75) (29),
was able to stimulate iNOS protein and NO<sub>2</sub> production, albeit to a lesser extent. Third, blockade of anti-CD120a (p55) function at high (antibody excess) concentrations of anti-receptor antibody resulted in an almost complete inhibition of NO<sub>2</sub> production in response to TNFα. The anti-TNF receptor antibodies were less efficient agonists of iNOS mRNA and NO<sub>2</sub> production than TNFα itself. Moreover, additional cross-linking with goat anti-rabbit IgG F(ab')2 fragments did not increase the level of NO<sub>2</sub> production (data not shown). The reason(s) for this finding is unclear although possible explanations include the following: (i) possible steric hindrance by the large IgG molecule leading to inefficient receptor aggregation, (ii) the formation of smaller receptor aggregates (e.g., receptor dimers), or (iii) receptor cross-linking with antibody may not fully mimic all events necessary for nitric oxide synthesis.

Stimulation of NO<sub>2</sub> expression by anti-CD120a (p55) antibody or by the combination of anti-CD120a (p55) and anti-CD120b (p75) antibodies was quite strikingly different from other systems in which these antibodies have been applied, e.g., L929 cell cytotoxicity (30, 31), in that the concentration-response curve for NO<sub>2</sub> expression was bell-shaped. Thus, the pattern of anti-TNF receptor antibody-mediated NO<sub>2</sub> production was similar to that observed in other cell systems in which receptor aggregation is an important element of cell signaling, e.g., the high affinity IgE receptor of mast cells. Although speculative, it is conceivable that this difference in the concentration-response curves for L929 cytotoxicity and macrophage NO<sub>2</sub> expression may relate to the requirement for recruitment of additional signaling molecules to the intracellular domain of CD120a (p55) for the initiation of iNOS and NO<sub>2</sub> expression. Moreover, IFNγ, which is required for iNOS expression but not for L929 cytotoxicity, may participate in this putative event. Direct support for this notion has been provided by the finding using deletional mutants of the CD120a (p55) intracellular domain that the membrane proximal region of the intracellular domain is essential for NO<sub>2</sub> production but is not necessary for L929 cytotoxicity (31).

In addition to the essential role of CD120a (p55) in signaling, our results show that CD120b (p75) acts cooperatively with CD120a (p55) in two distinct ways to stimulate optimal induction of iNOS and NO<sub>2</sub> expression. At low concentrations (pg/ng) of TNFα, mouse TNFα was a more efficient stimulus of NO<sub>2</sub> production than human TNFα. Moreover, selective blockade of CD120b (p75) function with a specific antibody substantially inhibited the capacity of low concentrations of mouse TNFα to stimulate NO<sub>2</sub> production to an extent that resembled the dose-response curve for human TNFα. These findings are consistent with the ligand passing concept originally developed by Tartaglia and Goeddel (20, 21) and which was recently shown to result in the ligand-dependent formation of a short-lived heterocomplex between CD120a (p55) and CD120b (p75) (32).

However, in addition to ligand passing, a second form of cooperation between CD120a (p55) and CD120b (p75) was observed at higher concentrations of TNFα and which was characterized in three ways. First, the maximum (plateau) level of NO<sub>2</sub> production seen in response to human TNFα was only about 50% the level detected in response to mouse TNFα. Second, blockade of CD120b (p75) function with specific antibody inhibited the production of NO<sub>2</sub> in response to mouse TNFα to the same level as that observed in response to human TNFα. Moreover, in both situations, the deficit in NO<sub>2</sub> production could not be overcome by increasing the concentration of the cytokine as would be predicted if CD120b (p75) acted solely to pass ligand to CD120a (p55). Third, in the absence of TNFα, stimulation of mouse macrophages with agonistic concentra-
(p75) dramatically enhances CD120a (p55)-mediated cytotoxicity in HeLa cells, also through a TRAF2-dependent mechanism; and Kalb and colleagues (39) provided data to suggest that co-ligation of both receptors enhanced fibroblast proliferation over that seen in response to ligation of CD120a (p55) alone. The results of the present study provide new insights into how these receptors may also interact to regulate the expression of inducible nitric oxide synthase and have clear implications with respect to macrophage defense against obligate intracellular pathogens such as T. gondii.  

Acknowledgments—We thank Drs. David Goeddel, Richard Weber, and Mike Rothe at Genentech and Tularik, Inc., South San Francisco, for providing the anti-TNF receptor antibodies used in this study and for helpful discussions. We acknowledge the excellent photographic work by Nadia de Stackleberg, Barry Silverstein, and Leigh Landskroner.

REFERENCES

1. Adams, L. B., Hibbs, J. B., Taintor, R. R., and Krahenbuhl, J. L. (1990) J. Immunol. 144, 2725–2729
2. Green, S. J., Melzer, M. S., Hibbs, J. J., and Nacy, C. A. (1990) J. Immunol. 144, 578–83
3. Liew, F. Y., Li, Y., Moss, D., Parkinson, C., Rogers, M. V., and Moncada, S. (1991) Eur. J. Immunol. 21, 3009–3014
4. Adams, L. B., Pranzblau, S. G., Vavrin, Z., Hibbs, J. J., and Krahenbuhl, J. L. (1991) J. Immunol. 147, 1642–1646
5. Beckerman, K. P., Rogers, H. W., Corbett, J. A., Schreiber, R. D., McDaniel, M. L., and Unanue, E. R. (1993) J. Immunol. 150, 888–895
6. Bermudez, L. E. (1993) Clin. Exp. Immunol. 91, 277–281
7. Kwon, N. S., Nathan, C. F., Gilker, C., Griffith, O. W., Matthews, D. E., and Stuehr, D. J. (1996) J. Biol. Chem. 271, 1908–1913
8. Liew, F. Y., Li, Y., Moss, D., Parkinson, C., Rogers, M. V., and Moncada, S. (1991) Eur. J. Immunol. 21, 3009–3014
9. Lyons, C. R., Orloff, G. J., and Cunningham, J. M. (1992) J. Biol. Chem. 267, 6370–6374
10. Xie, Q.-W., Cho, H. J., Calaycay, J., Mumford, R. A., Swiderow, K. M., Lee, T. D., Ding, A., Troso, T., and Nathan, C. (1992) Science 256, 225–228
11. Lorsbach, R. B., Murphy, W. J., Lowenstein, C. J., Snyder, S. H., and Russell, S. W. (1993) J. Biol. Chem. 268, 1908–1913
12. Ding, A. H., Nathan, C. F., and Stuehr, D. J. (1988) J. Immunol. 141, 2407–2412
13. Hohmann, H. P., Remy, R., Brockhaus, M., and van Loon, A. P. G. M. (1989) J. Biol. Chem. 264, 14927–14934
14. Brockhaus, M., Schoenfeld, H., Schaeger, E., Hunziker, W., Lesslauer, W., and Loetscher, H. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 3127–3131
15. Smith, C. A., Davis, T., Anderson, D., Solam, L., Beckmann, L. P., Jerzy, R., Dower, S. K., Cosman, D., and Goodwin, R. G. (1990) Science 248, 1019–1023
16. Tartaglia, L. A., Weber, R. F., Figari, I. S., Reynolds, C., Palladino, M. A., and Goeddel, D. V. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9292–9296
17. Baumann, H., Morella, K., and Wong, G. H. W. (1993) J. Immunol. 151, 4248–4257
18. Lake, F. R., Noble, P. W., Henson, P. M., and Riches, D. W. H. (1994) J. Clin. Invest. 93, 1661–1669
19. Vandenabeele, P., Declere, W., Vercaemen, D., Van De Cramen, M., Grooten, J., Loetscher, H., Brockhaus, M., Lesslauer, W., and Fiers, W. (1992) Exp. Med. 176, 1615–1624
20. Tartaglia, L. A., and Goeddel, D. V. (1992) Immunol. Today 13, 151–153
21. Tartaglia, L. A., Pennica, D., and Goeddel, D. V. (1993) J. Biol. Chem. 268, 18542–18548
22. Riches, D. W. H., and Underwood, G. A. (1991) J. Biol. Chem. 266, 2478–24792
23. Noble, P. W., Lake, F. R., Henson, P. M., and Riches, D. W. H. (1993) J. Clin. Invest. 91, 2368–2377
24. Chirgwin, J. M., Przybyla, R. J., MacDonald, R. J., and Rutter, W. J. (1979) Biochemistry 18, 5294–5299
25. Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993) Genes Dev. 7, 2135–2148
26. Chan, E. D., Winston, B. W., Jarpe, M. B., Wynes, M. W., and Riches, D. W. H. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13169–13174
27. Winston, B. W., and Riches, D. W. H. (1995) J. Immunol. 155, 1525–1533
28. Winston, B. W., Chan, E. D., Johnson, G. L., and Riches, D. W. H. (1997) J. Immunol. 159, 4491–4497
29. Lewis, M., Tartaglia, L. A., Lee, A., Bennett, G. L., Rice, G. C., Wong, G. H. W., Chen, E. Y., and Goeddel, D. V. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2830–2834
30. Tartaglia, L. A., and Goeddel, D. V. (1992) J. Biol. Chem. 267, 4304–4307
31. Tartaglia, L. A., Merrill Ayres, T., Wong, G. H. W., and Goeddel, D. V. (1993) Cell 74, 845–853
32. Pincard, J. R., Sheehan, K. C. F., and Schreiber, R. D. (1997) J. Biol. Chem. 272, 10784–10789
33. Reinhard, C., Shamon, B., Shyamala, V., and Williams, L. T. (1997) EMBO J. 16, 1080–1092
34. Natoli, G., Costanzo, A., Ianni, A., Templeton, D. J., Woodgett, J. R., Balsano, C., and Leverer, M. (1997) Science 275, 200–203
35. Hsu, H., Shu, H.-B., Pan, M.-G., and Goeddel, D. V. (1996) Cell 84, 299–308
36. Rothe, M., Wong, S. C., Henzel, W. J., and Goeddel, D. V. (1994) Cell 78, 681–692
37. Xie, Q.-W., Kashiwabara, Y., and Nathan, C. (1994) J. Biol. Chem. 269, 4705–4708
38. Weiss, T., Grell, M., Heschli, K., Bourteele, S., Muller, G., Scheurich, P., and Wajant, H. (1997) J. Immunol. 158, 2388–2404
39. Kalb, A., Bluthmann, H., Moore, M. W., and Lesslauer, W. (1996) J. Biol. Chem. 271, 28097–28104.