Role of the 75-kDa TNF Receptor in TNF-Induced Activation of Neutrophil Respiratory Burst

Pietro Dri, Elvira Haas, Rita Cramer, Renzo Menegazzi, Chiara Gasparini, Roberta Martinelli, Peter Scheurich and Pierluigi Patriarca

*J Immunol* 1999; 162:460-466; http://www.jimmunol.org/content/162/1/460

---

**References**
This article *cites 45 articles*, 32 of which you can access for free at: http://www.jimmunol.org/content/162/1/460.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

---

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 1999 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Role of the 75-kDa TNF Receptor in TNF-Induced Activation of Neutrophil Respiratory Burst

Pietro Dri, Elvira Haas, Rita Cramer, Renzo Menegazzi, Chiara Gasparini, Roberta Martinelli, Peter Scheurich, and Pierluigi Patriarca

The exclusive role of the 55-kDa TNF receptor (TNF-R55) as the signaling receptor in TNF-induced activation of respiratory burst by human polymorphonuclear leukocytes residing on biologic surfaces has been inferred from results obtained with receptor-specific monoclonal and polyclonal Abs. In this work, we confirm this assumption by a more direct approach, i.e., by using receptor-specific TNF mutants (p55TNF and p75TNF) and, as a novel contribution, we show that cooperation of the 75-kDa TNF receptor (TNF-R75) is required for a full blown response to the cytokine. This conclusion stems from three sets of data: 1) none of the TNF-R55-specific agonists used, i.e., mAbs or p55TNF, induced a respiratory burst comparable with that induced by TNF; 2) selective down-modulation of TNF-R75 resulted in a diminished response to TNF but not to TNF-R55-specific agonists or to the chemotactic peptide FMLP; and 3) mAbs that either block or stabilize binding of TNF to TNF-R75 inhibited the response to the cytokine, suggesting that cooperation requires not only TNF binding to the receptor but also an appropriate dissociability from it. The inhibitory effect of the Abs increased as the cytokine concentrations decreased, indicating that cooperation by TNF-R75 becomes more relevant at low TNF doses. Such a cooperation does not seem to rely on the activation of a TNF-R75-linked signaling pathway independent of TNF-R55, since the response to p55TNF and p75TNF given in combination was not higher than the response to p55TNF alone. The possible mechanisms of cooperation are discussed. The Journal of Immunology, 1999, 162: 460–466.

Tumor necrosis factor is an important regulator of inflammation and immunity, two interrelated processes that are essential for resistance to infection, healing, and repair, and is also of crucial relevance in the pathology of many diseases (1, 2). Polymorphonuclear leukocytes (PMN)3 play a prominent role in all these processes, and their functions are markedly influenced by this cytokine, which acts via membrane receptor binding. Two types of high affinity TNF receptors have been described, a 55-kDa receptor (TNF-R55) and a 75-kDa receptor (TNF-R75), which are expressed at low levels on most cell types including PMN (3–5). The absence of homology in the cytoplasmic domains of the two receptors led to the prediction that they would activate different signaling pathways (6). Indeed, in several studies it has been shown that the two receptors mediate distinct cell responses (7–9). In other studies, however, a redundancy of the responses induced by the two receptors has been reported (10–13). Most of the activities of TNF are reported to be mediated by TNF-R55 (8, 10, 14–16) whereas only a few examples have been described of TNF-R75-mediated responses (9, 17). PMN make no exception to this rule as demonstrated by studies from our and other laboratories that have established that TNF-R55 is the receptor that signals for all neutrophil responses thus far studied. Thus, this receptor has been shown to mediate PMN adherence, β2 integrin expression, production of superoxide anion (O2−) (18–21), priming for FMLP-induced O2− production (22), potentiation of Fc-mediated phagocytosis (21), chloride ion efflux (23), and shedding of both TNF-Rs (19). Very little is known about the specific function of TNF-R75 in these PMN responses. Inhibition by anti TNF-R75 ligand-competing mAbs of TNF-induced O2− production (19, 20) led to the conclusion that both receptors are involved in this function. In another study, based on a similar approach, it has been hypothesized that TNF-R75 might control β2 integrin expression and potentiation of Fc-mediated phagocytosis by regulating the binding of TNF to the signaling receptor TNF-R55 (21). In the present study, we sought to define the functional interactions of the two receptors in TNF-induced activation of the respiratory burst by combining the usual approach that uses anti-receptor monoclonal and polyclonal Abs with the use of receptor type-specific TNF mutants and selective down-regulation of TNF-R75. Our results prove that TNF-R55 is the exclusive signaling receptor for this PMN response and show that cooperation by TNF-R75 is essential for an optimal response to the cytokine.

Materials and Methods

Reagents

Cytochrome c, type VI, from horse heart; elastase, type VI, from porcine pancreas; BSA Fraction V, endotoxin tested and cell culture tested; and FMLP were purchased from Sigma (St. Louis, MO). Iodo-Gen (1,3,4,6-tetrachloro-3a,6a-diphenylglycouril) was from Pierce Chemical (Rockford, IL). NaCl123I was from Amersham International (Little Chalfont, U.K.). Percoll was from Pharmacia AB (Uppsala, Sweden). All solutions were made in endotoxin-free water for clinical use.
Abs and cytokines

Purified mouse mAbs specific for TNF-R55 (mAb htr-9, an IgG1, and mAb H398, an IgG2a) or TNF-R75 (mAb uto-l, an IgG1, and mAb uto M2, an IgG1) were either a generous gift of Dr. M. Brockhaus, Hoffmann La Roche, Basel, Switzerland (mAbs htr-9 and uto-l) (3), or prepared as described elsewhere (mAbs H398 and 80 M2) (14, 24). M80, a purified rabbit polyclonal Ab specific for TNF-R75 was prepared as described (24). Fab fragments derived from the polyclonal M80 were prepared using the Immunopure Fab preparation kit (Pierce). Pure recombinant human TNF, expressed in yeast, was obtained from Bissendorf (Hannover, Germany). p55TNF and p75TNF, human TNF mutants with exclusive specificity for TNF-R55 and TNF-R75, respectively (25), were generously donated by Dr. H. Loetscher (Hoffmann La Roche). p55TNF carries an Arg→Trp substitution at position 32 and a Ser→Thr substitution at position 86. p75TNF carries an Asn→Gln substitution at position 143 and an Ala→Arg substitution at position 145.

Neutrophil isolation

Blood was drawn from healthy volunteers and anticoagulated with 4 mM EDTA. Neutrophils were isolated according to a single-step separation procedure (26) with slight modifications. Blood (4 ml) was layered over a two-step Percoll gradient formed by 4 ml of 7% isotonic Percoll (75% Percoll, 10% PBS 10×, 15 ml of 3 M H2O2, density (d) 1.10 g/ml, 30–310 mOsM) and 4 ml of 62% isotonic Percoll (62% Percoll, 10% PBS 10×, 38% H2O, d 1.078, 300–310 mOsM) in 15-ml conical test tubes. After centrifugation for 25 min (10 min at 200 × g and 15 min at 400 × g) at 20°C, the neutrophils, located at the interface between the two Percoll solutions, were collected, diluted in Ca2+- and Mg2+-free HEPES-buffered saline solution containing BSA (HBS-BSA, 145 mM NaCl, 5 mM HEPES, 5 mM glucose, 0.2% BSA, pH 7.4), and centrifuged for 7 min at 200 × g. After elimination of contaminating erythrocytes by a brief hypotonic treatment (10-s incubation with 3 parts of a 1 M sodium phosphate buffer solution, pH 7.4, followed by addition of 7 parts of 1.3% NaCl solution in 1 mM sodium phosphate buffer, pH 7.4, to restore isotonicity) and centrifugation, the cells were suspended in HBS-BSA. The resulting cell population contained 94–98% neutrophils, 2–5% eosinophils, and 1–2% mononuclear cells. The procedure was conducted at room temperature and in the absence of divalent cations to prevent neutrophil aggregation and activation. All subsequent experiments were conducted in HBS-BSA supplemented with 1 mM CaCl2 and 1 mM MgCl2 (HBS-BSA).

Assay of O2− production

Production of O2− by neutrophils residing on fibronectin-coated microtiter wells was measured by the superoxide dismutase-inhibitable cytochrome c reduction method, as described (18, 27).

Radiodiociation of TNF

The Iodo-Gen method was used. Iodo-Gen, the oxidizing reagent, was dissolved in chloroform at a concentration of 50 μg/ml and 50 μl were added to a glass test tube. Just before the iodination was started, after the solvent was evaporated by a gentle stream of nitrogen, the tube was washed twice with PBS, and 2.5 μg of TNF in 25 μl of Ca2+- and Mg2+-free PBS were deposited at the bottom of the tube together with 2 μl of Na125I (0.2 mCi). The reaction was left to proceed for 8 min under gentle shaking and was stopped by the addition of 100 μl of a solution containing 100 μCi/mg tyrosine in 1 mM NaI. After the addition of 200 μl of PBS containing 0.3% BSA, iodinated TNF was separated from free Na125I by gel filtration over a 5-ml column filled with Bio-Gel P-6 desalting gel (Bio-Rad, Richmond, CA). The two iodinations performed gave a TNF-TNF with estimated specific activities of 33 and 45 μCi/μg, respectively.

Receptor binding studies

Saturation binding experiments were conducted at 0–4°C to block release (shedding) or internalization of the receptors (28–30). PMN (5–10 × 105 cells/ml) in HBS-BSA were exposed to varying [125I]TNF concentrations (10–800 pM) in the absence and in the presence of an excess of cold TNF (200 times), to determine the nonspecific binding. To measure TNF-R55, mAb uto-l at a concentration of 10 μg/ml was included in the incubation mixture to block binding of TNF to TNF-R75 (3, 31). To measure TNF-R75, mAb H398 was included in the reaction mixture to block binding of TNF to TNF-R55 (14). After 2.5 h of incubation on a rocking platform, unbound TNF was removed by centrifugation of the cell suspension for 30 s at 12,000 × g in a microfuge, followed by two washings with 0.75 ml of ice-cold HBS-BSA. The bottom parts of the conical microfuge tubes were cut off and counted in a gamma counter. A nonlinear regression analysis of the results was performed as described (32) by fitting the data to the equation of a binding isotherm (Langmuir isotherm) using the computer program Graphpad PRISM, version 2.0.

Determination of soluble TNF-R55 and soluble TNF-R75

A commercially available kit was used for these determinations (Amer sham). The kit is based on an ELISA sandwich assay in which mAbs are used as capturing reagents and peroxidase-conjugated polyclonal Abs to detect binding to the capturing monoclonals.

Statistical analysis

Data were analyzed according to Student’s t test for paired data.

Results

TNF-induced O2− production is under the exclusive control of TNF-R55 but requires the cooperation of TNF-R75 to be optimal

The expression on PMN of both types of TNF receptors, with TNF-R75 outnumbering TNF-R55, has been reported in most published studies (3, 5, 18, 29), but their binding characteristics in these cells are not known. Table I shows that PMN express more than twice as much TNF-R75 as TNF-R55, thus confirming the data of the majority of previous studies, and that the affinity of TNF-R75 for TNF is higher than that of TNF-R55, in our experimental conditions, in agreement with what has been reported in other cell systems (4, 6, 33, 34). In spite of its lower degree of expression, TNF-R55 is considered the exclusive signaling receptor in the TNF-induced activation of respiratory burst and other PMN responses. This notion derives from the results of studies with receptor-specific mAbs showing that anti-TNF-R55 mAbs are on their own agonistic and that their Fabs inhibit the responses to the cytokine, while anti-TNF-R75 mAbs do not exert any effects (18–20). To verify this conclusion more directly, we studied the effects that recombinant mutants of TNF, which specifically recognize TNF-R55 (p55TNF) or TNF-R75 (p75TNF), exert on the respiratory burst of PMN adherent to fibronectin-coated surfaces, assayed as O2− production. Fig. 1 shows that: 1) p55TNF, but not p75TNF, stimulates O2− production by the adherent PMN; 2) the response to p55TNF is lower than that to TNF; and 3) p55TNF and p75TNF given in combination do not induce a response greater than that induced by p55TNF alone. The response to p55TNF is dose dependent and plateau at concentrations above 15 ng/ml. The lower response to p55TNF with respect to TNF cannot be attributed to differences in affinity for the receptor since TNF and p55TNF have a similar KD for TNF-R55 (25). Concentrations of p75TNF 3 times as high as those used in Fig. 1 were also ineffective (data not shown), making it unlikely that the lack of response to p75TNF is due to an affinity for the receptor lower than that of TNF (25). Table II shows that both a monoclonal (uto-l) and a polyclonal (M80) TNF-R75-specific and ligand-competing Ab inhibited the production of O2− induced by TNF suggesting that TNF-R75 is required for maximal response to TNF. Table II also shows that a similar inhibition is observed using the Fab fragments.

Table I. Number and dissociation constants of TNF receptors in PMN

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Kd (pM) a</th>
<th>No. of Receptors/PMN a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Both TNF-Rs</td>
<td>230 ± 95</td>
<td>1155 ± 155</td>
</tr>
<tr>
<td>TNF-R75</td>
<td>100 ± 50</td>
<td>802 ± 122</td>
</tr>
<tr>
<td>TNF-R55</td>
<td>704 ± 400</td>
<td>344 ± 33</td>
</tr>
</tbody>
</table>

a The dissociation constants (mean ± SEM) were obtained by fitting to the equation of a binding isotherm (32) for the data of equilibrium binding from three independent experiments.

b The number of receptors is given in mean ± SEM of seven experiments.
of M80, indicating that the inhibitory effect of the Abs is not due to receptor cross-linking. Table III shows that neither mAb utr-1 nor the polyclonal Ab M80 and its corresponding Fab fragments influence the response induced by TNF-R55-specific agonists, i.e., p55TNF and mAbs htr-9 and H398, indicating that their inhibitory effect is not due to interference with TNF-R55 signaling, and hindrance of TNF binding remains the more likely possibility. Another TNF-R75 specific mAb (M80 80M2), which at variance with the anti-TNF-R75 Abs utr-1 and M80 does not compete for TNF binding also inhibits the response to TNF but not the response to the TNF-R55-specific agonists p55TNF and mAb H398 (Table IV). This mAb increases the affinity of TNF-R75 for TNF by more than one order of magnitude by strongly decreasing the dissociation rate (K_{diss}) of TNF (35). These results suggest that cooperation by TNF-R75 requires not only TNF binding to the receptor but also an appropriate dissociability from it.

Cooperation by TNF-R75 is more relevant at decreasing TNF doses

The results of the previous section show that TNF-R75 cooperates with TNF-R55, the signaling receptor, for maximal response to TNF. Fig. 2 shows that inhibition of the O_2^- production by PMN exposed to varying TNF doses by the TNF-R75-specific and ligand-competing mAb utr-1 becomes more prominent at decreasing TNF concentrations, indicating that the contribution of TNF-R75 might be particularly important for the response to physiologic TNF concentrations. It might be argued that the lower inhibition observed at increasing TNF doses is due to an ineffective competition of mAb utr-1 with TNF for binding to TNF-R75. Two sets of data speak against this possibility: 1) the strength of binding of utr-1 for TNF-R75 appears much greater than that of TNF as indicated by experiments showing that TNF bound to the receptor is easily removed by a brief acid wash at pH 3, while mAb utr-1 is not (data not shown); and 2) utr-1 concentrations 2 or 3 times higher than those used in the experiments of Fig. 2 gave an inhibition of the TNF-induced respiratory burst comparable with that observed at lower concentrations (data not shown). The data obtained with mAb utr-1 are further supported by the results of experiments with mAb 80 M2 which, as already noted before, does not compete for TNF binding to TNF-R75 but decreases its dissociation from the receptor (Fig. 3). Fig. 3 in fact shows that, even with this mAb, inhibition of the respiratory burst increases by decreasing TNF concentrations.

Table III. Effect of a TNF-R75-specific mAb (utr-1), and of a polyclonal Ab (M80) and its corresponding Fab fragments on the respiratory burst of PMN exposed to TNF-R55-specific agonists (mAbs htr-9 and H398, and p55-TNF)a

<table>
<thead>
<tr>
<th>mAb</th>
<th>O_2^- /10^6 PMN/60 minb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting</td>
<td>1.34 ± 1.5</td>
</tr>
<tr>
<td>htr-9</td>
<td>29.80 ± 5.6</td>
</tr>
<tr>
<td>htr-9 + utr-1</td>
<td>29.75 ± 9.0 (NS')</td>
</tr>
<tr>
<td>H398</td>
<td>25.46 ± 7.2</td>
</tr>
<tr>
<td>H398 + utr-1</td>
<td>27.93 ± 6.3 (NS)</td>
</tr>
<tr>
<td>H398 + M80</td>
<td>27.82 ± 1.1 (NS)</td>
</tr>
<tr>
<td>p55TNF</td>
<td>29.90 ± 2.8 (NS)</td>
</tr>
<tr>
<td>p55TNF + utr-1</td>
<td>33.15 ± 3.6</td>
</tr>
<tr>
<td>p55TNF + M80</td>
<td>34.30 ± 4.7 (NS)</td>
</tr>
<tr>
<td>p55TNF + M80 Fab</td>
<td>34.52 ± 4.6 (NS)</td>
</tr>
<tr>
<td>p55TNF + M80 Fab</td>
<td>35.20 ± 6.4 (NS)</td>
</tr>
</tbody>
</table>

a PMN suspensions (1.5 × 10^6/ml in HBS) were equilibrated for 10 min at 37°C, and 50-μl aliquots were added to the assay for O_2^- production (0.15 ml final volume) in the absence (resting) or in the presence of TNF (2.5 ng/ml), utr-1 (5 μg/ml), M80 (5 μg/ml), or M80 Fab (20 μg/ml).

b Data are means ± SD of the results of five experiments.

c p < 0.001 vs TNF-treated cells in the absence of Abs.

d NS, not significant (difference between utr-1-, M80, and M80 Fab-treated cells and their corresponding control).

Table IV. Effect of the anti-TNF-R75 mAb 80M2 on the respiratory burst of PMN exposed to TNF and TNF-R55-specific agonistsa

<table>
<thead>
<tr>
<th>mAb</th>
<th>O_2^- /10^6 PMN/60 minb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting</td>
<td>1.37 ± 1.5</td>
</tr>
<tr>
<td>80M2</td>
<td>1.90 ± 0.3</td>
</tr>
<tr>
<td>TNF</td>
<td>41.10 ± 11.7</td>
</tr>
<tr>
<td>TNF + 80M2</td>
<td>30.70 ± 5.4a</td>
</tr>
<tr>
<td>htr-9</td>
<td>32.86 ± 5.6</td>
</tr>
<tr>
<td>htr-9 + 80M2</td>
<td>33.57 ± 8.1 (NS')</td>
</tr>
<tr>
<td>H398</td>
<td>26.15 ± 7.2</td>
</tr>
<tr>
<td>H398 + 80M2</td>
<td>28.60 ± 8.0 (NS)</td>
</tr>
<tr>
<td>p55TNF</td>
<td>29.62 ± 3.6</td>
</tr>
<tr>
<td>p55TNF + 80M2</td>
<td>28.47 ± 4.0 (NS)</td>
</tr>
</tbody>
</table>

a PMN suspensions (1.5 × 10^6/ml in HBS) were equilibrated for 10 min at 37°C, and 50-μl aliquots were added to the assay for O_2^- production (0.15 ml final volume) in the absence (resting) or in the presence of TNF (2.5 ng/ml), htr-9 (5 μg/ml), H398 (5 μg/ml) and p55-TNF (15 ng/ml). The concentrations of the anti-TNF-R75 Abs were 5 μg/ml for utr-1 and M80 and 20 μg/ml for M80 Fab.

b Data are means ± SD of the results of four experiments.

c NS, not significant (difference between utr-1-, M80, and M80 Fab-treated cells and their corresponding control).

d p < 0.001 vs TNF-treated cells in the absence of Ab.
Selective down-modulation of TNF-R75 decreases $O_2^-$ production in response to TNF but not to TNF-R55-specific agonists or FMLP.

Given the results of the previous sections, it is reasonable to predict that cells with reduced expression of TNF-R75 would mount a lower response to the cytokine. To this end we used elastase, an enzyme known to specifically cleave TNF-R75 from neutrophils (5). Fig. 5 shows that, indeed, elastase down-modulates the expression of TNF-R75 (Fig. 5A), that this is accompanied by release of the soluble form of the receptor (Fig. 5B), and that this effect is specific for TNF-R75 since expression of TNF-R55 as well as release of its soluble form remain unaffected. Fig. 6 shows that elastase-treated PMN, on exposure to TNF, produce less $O_2^-$ than control PMN. The difference in response between elastase-treated cells and control cells is observed at all TNF concentrations, although statistical significance could be demonstrated only at 0.5 and 5 ng/ml TNF. The lack of effect of the elastase treatment on the response to 20 and 40 ng/ml TNF is not surprising if one considers that the cooperation of TNF-R75 to TNF-R55 signaling is more pronounced at low cytokine doses (see previous paragraph) and that elastase-treated cells have lost only part of TNF-R75 (Fig. 5), which may not be sufficient to alter the response to high TNF doses.

We can exclude that the observed inhibition of $O_2^-$ production might be accounted for by a modification of the expression of $\beta_2$ integrins or the major neutrophil sialoprotein CD43, both known to be involved in the TNF-induced activation of respiratory burst (36, 37). In fact, $\beta_2$ integrin expression has been shown not to be affected by elastase treatment (38–40) (our data not shown), and CD43 has been shown to be totally insensitive to the porcine pancreatic elastase, that has been used in our experiments (41, 42). Fig. 7 shows that elastase treatment does not affect the $O_2^-$ generation induced by TNF-R55-specific agonists, i.e., p55TNF and mAb H398, or FMLP, indicating that the enzyme did not harm the TNF-R55 and the FMLP receptor or the signaling cascade downstream of the two receptors or the $O_2^-$-generating enzyme complex.

**FIGURE 2.** Inhibition by mAb utr-1 of $O_2^-$ production by PMN stimulated with varying TNF doses. PMN suspensions (1.5 x 10^6/ml) were equilibrated at 37°C for 10 min, and 50-μl aliquots were added to the wells for the assay of $O_2^-$ containing mAb utr-1 (5 μg/ml) and TNF at the indicated concentrations. The data are means ± SD of the results of four experiments. Controls $O_2^-$ production, i.e., $O_2^-$ production in the absence of mAb utr-1, were (in nanomols/10^6 PMN/60 min ± SD) 18.9 ± 9.3, 24.1 ± 7.5, and 31.7 ± 9.1 with 0.5, 2.5, and 25 ng/ml TNF, respectively.

**FIGURE 3.** Inhibition by mAb 80M2 of $O_2^-$ production by PMN stimulated with varying TNF doses. PMN suspensions (1.5 x 10^6/ml) were equilibrated at 37°C for 10 min, and 50-μl aliquots were added to the wells for the assay of $O_2^-$ containing mAb 80M2 (1 μg/ml) and TNF at the indicated concentrations. The data are means ± SD of the results of four experiments. Controls $O_2^-$ production, i.e., $O_2^-$ production in the absence of mAb 80M2, were (in nanomols/10^6 PMN/60 min ± SD) 18.9 ± 9.3, 24.1 ± 7.5, and 31.7 ± 9.1 with 0.5, 2.5, and 25 ng/ml TNF, respectively.

**FIGURE 4.** Production of $O_2^-$ by PMN exposed to TNF, TNF + utr-1, and p55TNF. PMN suspensions (1.5 x 10^6/ml in HBS) were equilibrated for 10 min at 37°C, and 50-μl aliquots were added to the wells for the assay of $O_2^-$ containing TNF and p55TNF at the indicated concentrations. The concentration of mAb utr-1 was 5 μg/ml. Data are means of the results of three experiments.

**FIGURE 5.** Effect of elastase on expression (A) and solubilization (B) of TNF receptors. PMN suspensions (5 x 10^6/ml in HBS) after 10 min of equilibration at 37°C were incubated without and with elastase (5 μg/ml) for an additional 15 min. After cooling at melting ice temperature, the cells were centrifuged at 400 x g for 7.5 min, and the soluble receptors were measured in the supernatants by using an ELISA assay (see Materials and Methods). Receptor expression was evaluated by measuring cell-associated radioactivity after incubating the cell suspensions with 125I-TNF and receptor-specific competing mAbs as described in Materials and Methods.
Our results clearly show that TNF-R75 cooperation is required for an optimal response to TNF to take place. In fact, 1) none of the TNF-R55-specific agonists (p55TNF and mAbs) were able to induce a respiratory burst comparable with that induced by TNF, 2) the response to TNF was decreased either by blocking its binding to TNF-R75 (Table II) or by increasing its strength of association with the receptor (Table IV) using monoclonal or polyclonal Abs, and 3) PMN with a decreased expression of TNF-R75 showed a diminished response to the cytokine (Figs. 5–7).

The cooperative role of TNF-R75 is more remarkable at low TNF doses, as indicated by the observations that inhibition by TNF-R75-specific mAbs increases by decreasing the concentrations of the cytokine (Figs. 2 and 3) and that the diminished response of elastase-treated PMN is particularly evident at low TNF concentrations (Fig. 6). This suggests that TNF-R75 might play an important modulatory role in TNF-induced respiratory burst at physiologically relevant concentrations of the cytokine.

Our results strongly argue against the possibility that the cooperation between the two receptors might take place at the signaling level, i.e., that occupancy of the TNF-R75 might activate a signaling pathway independently of TNF-R55. In fact, the combination of p55TNF and p75TNF does not result in a response greater than that induced by p55TNF alone (Fig. 1); neither was the response to p55TNF increased by p75TNF in the presence of mAb 80 M2, which strengthens the interaction of p75 TNF with TNF-R75 (data not shown).

Two explanations have been offered thus far in the literature for the cooperation between the two TNF receptors observed in other experimental sets: 1) formation of TNF-R55/TNF-R75 heterocomplexes; and 2) the ligand passing model.

TNF-induced formation of heterocomplexes consisting of both TNF-R55 and TNF-R75 has been shown to occur in vitro in cultured and primary murine cells (44). It has also been demonstrated that one of the signal-transducing proteins that interact with TNF-R75, i.e., TNF-R75-associated factor 2 (TRAF2) can also interact with TNF-R55-associated death domain protein (TRADD) bound to the intracellular moiety of TNF-R55 (45). Therefore, it is conceivable that the TNF-R55/TNF-R75 complexes potentiate signaling via TNF-R55 by facilitating the association of TNF-R75 and TNF-R55 signal transducers, through juxtaposition of intracellular tails of the receptors. We tried to verify the heterocomplex hypothesis by comparing the respiratory burst of PMN in which the two TNF receptors were cross-linked separately, or jointly. As expected, addition of a second anti-mouse polyclonal Ab to PMN preincubated with the Fab fragments from an anti-TNF-R55 mAb (H398), but not to PMN preincubated with an anti-TNF-R75 mAb (utr-1) stimulated O$_2^-$ production. When the second Ab was added to PMN preincubated with both the anti-TNF-R55 Fabs and the anti-TNF-R75 mAb, the response was lower than that induced by cross-linking the TNF-R55 alone (data not shown). These findings seem to speak against a cooperation between the two TNF receptors based on heterocomplex formation, although they cannot be taken as a conclusive evidence. In fact, the heterocomplexes formed after Ab cross-linking may contain the two TNF receptors in proportions different from those found in the presence of TNF, which would preclude any direct comparison between the two conditions.

According to the ligand passing model which has been proposed on the basis of binding data obtained at 0–4°C (46), TNF-R75, the higher affinity receptor (4), would catch TNF and deliver it to TNF-R55 and in this way enhance binding to and signaling through TNF-R55. This model encounters some difficulties with the thermodynamics. How can a ligand move from a high affinity to a low affinity receptor? Why should TNF pass to the low affinity receptor? Why should TNF-R75 cooperate with TNF-R55?
receptor rather than reassociate with the same receptor? Where does the energy required come from? Recent published data showing that at 37°C, at variance with what observed at 0–4°C, TNF-R55 is the higher affinity receptor (47) appear to overcome some of the above difficulties. However, even in this case, a question remains open and that is whether TNF-R-75, the low affinity receptor, may bind enough TNF to explain its cooperation with TNF-R55, according to the passing model. Reasoning only in terms of affinity is not helpful in this respect since the concept of affinity refers to equilibrium conditions and does not take into account the dynamics of the interaction, which may be critical for subsequent events to occur. Thus, on the basis of the data obtained at 37°C (47) and of the equations reported in Ref. 32, we have determined some kinetic parameters i.e., $K_{obs}$, $t_{1/2(ass)}$, and $t_{1/2(diss)}$ and receptor saturation ($R_{eq}$) for a TNF concentration of 0.5 ng/ml (10–11 M). This concentration was chosen because cooperation by TNF-R75 is more prominent at low TNF doses. What the calculated effective association rate constants ($K_{obs}$) tell us is that TNF associates 20 times more rapidly with TNF-R75 ($K_{obs} = 0.645$ min$^{-1}$) than with TNF-R55 ($K_{obs} = 0.032$ min$^{-1}$). Association to TNF-R55 is rather slow ($t_{1/2(ass)} = 21.6$ min) and reaches 34% saturation at equilibrium ($R_{eq} = 0.34$). For TNF-R75, equilibrium is more rapid ($t_{1/2(ass)} = 1.07$ min) reaching, however, only 2.3% saturation ($R_{eq} = 0.023$). Accordingly, these few TNF-R75 complexes should dissociate quite rapidly to feed significantly the TNF-R55 pool. Given that $t_{diss}$ for dissociation of TNF-R75 complexes is 1.1 min, it turns out that in ~10 min 184 TNF molecules should exchange (802 TNF-R75/cell, 2.3% saturation, 10 times an exchange). Since this figure accounts for ~50% of the available TNF-R55 (see Table I), it is reasonable to believe that ligand passing may significantly contribute to the response. Once formed, TNF/TNF-R55 complexes appear to have an extraordinary stability ($t_{1/2(diss)} = 33.2$ min) which would favor the association with the cytoplasmic tail of the oligomerized receptor of transducing molecules known to be involved in signaling via this receptor (48).

The ligand passing model does not exclude that receptor heterocomplexes may form, which could represent a potential intermediate in the passing process.

In conclusion, we have shown that the participation of TNF-R75 is necessary for an optimal TNF-induced activation of the respiratory burst and discussed the possible mechanisms of action. Whether TNF-R75 only serves as a modulator of the respiratory burst and discussed the possible mechanisms of action. Whether TNF-R75 only serves as a modulator of the respiratory burst and discusses the possible mechanisms of action.
(TNF) receptors is independently regulated, and both receptors mediate activation of the transcription factor NF-κB: TNFα is not needed for induction of a biological effect via TNF receptors. J. Biol. Chem. 265:22409.


