Tumor Necrosis Factor (TNF)–α–Induced Interleukin-8 in Human Blood Cultures Discriminates Neutralization by the p55 and p75 TNF Soluble Receptors

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The dose-dependent increase in mortality in patients with sepsis who are treated with tumor necrosis factor (TNF) p75 soluble receptor Fc conjugate (p75-Fc) remains unexplained. In this study, neutralization of TNF-α–induced interleukin (IL)–8 by p75-Fc in whole human blood exhibited a U-shaped inhibition curve, whereas the TNF-soluble p55 receptor, linked to polyethylene glycol (p55-PEG), exhibited a dose-dependent inhibition. Native soluble p75 increased TNF-α–induced IL-8, versus a 61% reduction by native p55. Spontaneous IL-8 production was increased by p75-Fc or native p75 but not by p55-PEG or native p55. Unexpectedly, TNF-α–stimulated IL-1 receptor antagonist was suppressed by p75-Fc but not by p55-PEG. Studies of binding to TNF trimers revealed that p75-Fc has an affinity 40-fold lower than that of p55-PEG and a faster off rate. Native and p75-Fc pass TNF-α to membrane receptors more readily than does native or p55-PEG, which may partly explain the increased mortality in patients with sepsis who are treated with p75-Fc.

Use of neutralizing antibodies or soluble (extracellular domain) receptors of tumor necrosis factor (TNF) to treat patients with sepsis has been reported in several large, placebo-controlled double-blind trials, in which >6000 patients with sepsis have been enrolled. In addition, >2500 patients with sepsis have received interleukin (IL)-1 receptor antagonist (IL-1Ra) in similar controlled trials [1–3]. In each trial, there was a consistent small reduction in 28-day all-cause mortality, in comparison with placebo-treated patients. A meta-analysis of studies involving >10,000 patients with sepsis who were treated with anti-inflammatory mediators concluded that, with the exception of glucocorticoids and the TNF soluble receptor p75 linked to the Fc segment of IgG1 (p75-Fc), anticytokine therapy resulted in a small but consistent reduction in mortality [4]; however, the administration of p75-Fc was associated with an unexplained dose-dependent increase in mortality [5]. By comparison, when the p55 TNF receptor, which is also fused to the Fc segment of IgG1, was tested in 2 large clinical trials involving 1840 patients, there was no evidence of increased mortality, although there was a small increase in 28-day mortality with a smaller dose during an interim analysis [6]. Nevertheless, these 2 trials suggest that the soluble TNF receptor p55-Fc fusion construct is not associated with the dose-dependent increase in mortality characterized in the p75-Fc study.

The concept that soluble receptors can stabilize and hence retain the biologic activity of TNF-α was described by Aderka et al. [7]. Although ligand passing of TNF-α to the cell surface TNF receptor for native p75 is well-documented in vitro, use of various cell line assays for neutralization of TNF-α by p75-Fc is highly effective and does not explain the increase in deaths associated with the p75-Fc versus other anti-TNF strategies in patients with sepsis. In contrast to neutralization of TNF-α in cell lines, we used whole human blood cultures for cytokine production, which provide naturally occurring proteins and conditions found in the circulation.

When used to assess the possible effect of a particular cytokine, antibody, soluble receptor, or pharmacologic agent, whole blood cultures may reflect conditions not present in cell cultures or in nonhuman animals. Naturally occurring proteins in the human circulation (e.g., α2–macroglobulin or the third component of complement) can bind and affect the biologic effect of cytokines when added to tissue culture media [8]; however, these and nearly all human plasma proteins are present in whole blood cultures, where they may affect cytokine interactions. In addition, the presence of red blood cells, also absent in tissue culture conditions, may affect cytokine binding and quenching. For example, red blood cells bind chemokines, which does not occur under...
tissue culture conditions. In patients receiving high-dose IL-2 therapy, measurement of the circulating chemokine IL-8 by red blood extraction of whole blood revealed greater and more prolonged levels than those in plasma [9]. Hence, whole blood assays affect the assessment of agents intended for human therapeutic use. The limitation of whole blood assays is the lack of control in the change in concentration of an agent, as there is no renal or hepatic removal.

Whole blood cultures are useful for assessing the efficacy of a therapeutic agent administered in vivo. In human volunteers receiving IL-10 parenterally, ex vivo blood culture revealed the duration of the suppression of cytokine synthesis by IL-10 [10, 11]. The ability of interferon-α administration to induce IL-1Ra and to suppress IL-1β-induced IL-8 production was also shown in whole blood cultures [12]. Whole blood cultures have also shown that production of endotoxin-induced IL-1β and TNF-α was suppressed in human subjects 24 h after a single injection of IL-11, compared with placebo-injected subjects [13]. In the present study, we used whole blood cultures to assess differences in the ability of the p55 and p75 extracellular TNF receptors to neutralize TNF-α-induced IL-8. We examined the native forms of these 2 soluble receptors and the modified forms of the receptors currently being used in patients.

Materials and Methods

Reagents. Recombinant human TNF-α was obtained from Peptech (Rocky Hill, NJ) and was reconstituted in RPMI 1640 (Cellgro, Herndon, VA) containing HEPES (25 mM), l-glutamine, penicillin, and streptomycin (100 U/mL and 100 µg/mL, respectively; Gibco/BRL, Grand Island, NY). Fetal calf serum (FCS) and trypsin/EDTA were also purchased from Gibco. The F12K culture medium for the A549 cells was purchased from Cellgro.

Soluble receptors. The p75-Fc (Etanerecept; Immunex, Seattle) was purchased and reconstituted in the buffer supplied by the manufacturer at the recommended concentration of 25 mg/mL. We made 50-µL aliquots in polypropylene Eppendorf tubes, which were frozen at −70°C. A single frozen aliquot was thawed, diluted, and used once for each experiment. The p55-polyethylene glycol (PEG) form of the p55 TNF receptor was used as described elsewhere [14]. It was reconstituted in sterile water for injection at 15 µg/mL, stored in 50-µL aliquots, and kept at −70°C; frozen aliquots were thawed, diluted, and used once for each experiment. The recombinant human forms of the soluble p75 and p55 receptors (monomeric and unmodified and termed “native” in this report) were purchased from R&D Systems (Minneapolis). These were reconstituted at 50 µg/mL in RPMI, aliquoted, and frozen as described above.

Whole blood cultures. Whole blood for in vitro studies was obtained from healthy volunteers by venipuncture. The blood was placed in 50-mL conical tubes containing heparin (Elkin-Sinn, Cherry Hills, NJ), at a final concentration of 20 U/mL. Whole blood (0.5 mL) was added to 6 mL (75 mm) of sterile snap-capped polypropylene tubes (Becton-Dickinson, Franklin Lanes, NJ). Soluble TNF receptors were diluted in RPMI, and 100 µL was added and mixed with the blood. This was followed by the addition of 400 µL of TNF-α (final concentration in whole blood, 12.5 ng/mL), resulting in a culture volume of 1.0 mL per tube. The optimal concentration of TNF-α that induced IL-8 in whole blood was determined in separate experiments (data not shown). Unstimulated control cultures consisted of 500 µL of RPMI plus 500 µL of blood. To TNF-α-stimulated control cultures, 100 µL of RPMI was added in place of 100 µL of TNF soluble receptors. The tubes were capped, inverted several times, and incubated upright at 37°C in 5% CO₂. After incubation, the tubes were mixed, and we added 50 µL of Triton X-100 (10% v/v; Sigma, St. Louis), for a final concentration of 0.5% v/v. The tubes were mixed again to lyse the cultures. The lysates were frozen at −70°C until assayed by electrochemiluminescence (ECL) assays for either IL-8 or IL-1Ra, as described elsewhere [12, 15]. The limit of quantification was 40 pg/mL (range, 40–25,000 pg/mL) for each cytokine. Duplicates for ECL cytokine assays varied <10%.

A549 cultures. A549 cells, a human pulmonary epithelial carcinoma cell line with type II alveolar epithelial cell differentiation, were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in F12K medium containing penicillin and streptomycin with 10% FCS. When confluent, cells were disaggregated with trypsin-EDTA at room temperature for 5 min. Cells were then seeded into 24-well flat-bottom polystyrene tissue culture plates (Falcon, Lincoln Park, NY) at a density of 5 × 10⁴ cells/well in a volume of 1 mL and were incubated at 37°C in 5% CO₂. After reaching confluence (48 h), 950 µL of medium was removed and was replaced with 100 µL of RPMI 1640 medium (without FCS) or 100 µL of soluble TNF receptor. Cells were stimulated with TNF-α (final concentration, 1 ng/mL) in a final volume of 0.5 mL. Unstimulated control wells received 500 µL of medium alone. After the cells were incubated at 37°C in 5% CO₂ for 24 h, we added 25 µL of Triton X-100 (10% v/v), for a final concentration of 0.5% v/v, and assayed for IL-8 by ECL.

Cell-free binding assays of native TNF-α. We performed bioluminescence-based analysis (BIA; model 2000; BIAcore, Uppsala, Sweden). The BIAcore sensor chips (CM5) were equilibrated by molecular interaction analysis (BIA; model 2000; BIAcore, Uppsala) using 1:1 Langmuir binding modes (BIAevaluation software version 3.0; BIAcore). We processed the raw data by subtracting the differences in binding events. The resulting sensorgrams were analyzed by 1:1 Langmuir binding modes (BIAevaluation software version 3.0; BIAcore).

Cell-free binding assays of covalently linked TNF-α trimers. Because of the instability of the native TNF-α trimer, covalently linked TNF-α trimers were prepared. Native TNF-α was covalently linked into multimers by using ethylene glycolbis succinimidyl succinate (sulfo-EGS; Pierce Chemical, Rockford, IL). In brief, a high concentration of TNF-α in PBS was subjected to 4 treatments (30 min each) of a 10-fold molar excess of sulfo-EGS at room temperature. The cross-linked material was purified by SDS-PAGE to obtain the homogeneous trimer. Residual SDS was removed by
the Extracti-Gel process (Pierce). The purified trimers were assayed for biologic activity by L929 assay [16] and were then used in the BIACore assay with TNF receptors immobilized to the sensor chip, as described above. Using the covalently linked TNF-α trimers, we evaluated the binding of p75-Fc or p55-PEG with 1:1 Langmuir binding modes (BIACore software version 3.0; BIACore) [17].

Statistical analyses. Data for analysis were converted to percentage of change from either control (spontaneous) or TNF-α-stimulated values. Donors were selected at random. For each donor, the spontaneous or TNF-α-stimulated value was set at 100% (hatched bar); mean % change (± SE) is shown for each concentration of soluble TNF receptor. In each culture containing a soluble receptor, there was a statistically significant reduction in TNF-α-induced IL-8 production (P < .001). *** P < .001, p75-Fc vs. p55-PEG.

Results

TNF soluble receptors suppress TNF-α–induced IL-8 from A549 cells. As shown in figure 1, the p55-PEG and p75-Fc soluble receptors suppressed TNF-α–induced IL-8 synthesis. From 320 to 5160 ng/mL, the suppression was nearly complete (90%) for both receptors. However, at concentrations of 40–160 ng/mL, suppression by p55-PEG was less effective (60% suppression; P < .001). These results demonstrate that both receptors effectively neutralize TNF-α–induced IL-8 in this human epithelial cell line.

Suppression of TNF-α–induced IL-8 by soluble TNF receptors in whole human blood cultures. Soluble TNF receptors were added to fresh whole human blood and were stimulated with 12.5 ng/mL TNF-α. After 6 h at 37°C, the cultures were lysed and were assayed for IL-8 concentration. Similar to the data shown in figure 1, p75-Fc was effective in reducing TNF-α–induced IL-8 at 320 ng/mL (90%; P < .001). However, when the concentration of p75-Fc was increased to 1280, 2560, or 5160 ng/mL, there was less suppression than at 320 ng/mL. As shown in figure 2, the effect of p75-Fc results in a U-shaped dose-response curve, whereas the p55-PEG in blood from the same 6 donors resulted in a dose-dependent inhibition. At 5120 ng/mL, the p75-Fc form inhibited TNF-α–induced IL-8 by 38%, whereas the p55-PEG suppressed IL-8 by 87% in the same donors’ cultures (P < .001).

These experiments were repeated in 3 additional donors, in whom the concentrations of p75-Fc and p55-PEG began as shown in figure 2 but then increased to 10,000 and 20,000 ng/mL. Suppression by the p55-PEG form at 10,000 and 20,000 ng/mL was 93% and 98%, respectively, whereas the suppression by the p75-Fc form (~60%) remained unchanged from 1280 to 20,000 ng/mL (P < .001, p55-PEG vs. p75-Fc).

Effect of native forms of p55 and p75 TNF soluble receptors on TNF-α–induced IL-8 in whole blood cultures. The native forms

![Image](https://academic.oup.com/jid/article-abstract/182/6/1722/915442/1542)
and were assayed for IL-8. IL-8 induced by TNF-α (12.5 ng/mL, final concentration). After 6 h at 37 C, cultures were lysed and were assayed for IL-8. IL-8 induced by TNF-α from each of 4 donors was set at 100%. Figure shows % change in IL-8 production (± SE) for TNF-α plus native p55 receptors. Concentration of native p55 is below the horizontal axis, in ng/mL. The reduction in TNF-α-induced IL-8 production for each concentration of native p55 receptor was statistically significant (P < .001; inset). Soluble native p75 receptors were added to whole blood at 5000 ng/mL (final concentration) and were challenged with TNF-α (12.5 ng/mL, final concentration). After 6 h at 37 C, cultures were lysed and were assayed for IL-8. IL-8 induced by TNF-α from each of 4 subjects was set at 100%. Mean % change in IL-8 production (± SE) for TNF-α plus native p75 is shown. *P < .05.

Figure 3. Effect of native p55 and p75 on tumor necrosis factor (TNF)-α–induced interleukin (IL)-8. Native p55 in increasing concentrations was added to whole blood and was stimulated with TNF-α. After 6 h at 37°C, cultures were lysed and were assayed for IL-8. IL-8 induced by TNF-α from each of 4 donors was set at 100%. Figure shows % change in IL-8 production (± SE) for TNF-α plus native p55 receptors. Concentration of native p55 is below the horizontal axis, in ng/mL. The reduction in TNF-α-induced IL-8 production for each concentration of native p55 receptor was statistically significant (P < .001; inset). Soluble native p75 receptors were added to whole blood at 5000 ng/mL (final concentration) and were challenged with TNF-α (12.5 ng/mL, final concentration). After 6 h at 37°C, cultures were lysed and were assayed for IL-8. IL-8 induced by TNF-α from each of 4 subjects was set at 100%. Mean % change in IL-8 production (± SE) for TNF-α plus native p75 is shown. *P < .05.

The increase in TNF-α–induced IL-8 by native p75 is reduced by the presence of native p55. To assess the possibility that the increase in TNF-α–induced IL-8 by native p75 is caused by a “ligand passing” mechanism, native p75 was added to TNF-α–stimulated blood with and without the presence of native p55. As shown in the inset to figure 3, native p75 soluble receptor at a concentration of 5000 ng/mL increased IL-8 production to 166% (P < .05) above that effected by TNF-α alone. On the other hand, native p55 reduced TNF-α–induced IL-8 in a dose-dependent fashion, similar to that observed with the p55-PEG construct. As shown in figure 3, at 5000 ng/mL, the native p55 receptor suppression reached 90% and increased further to 95% and 99% at 10,000 and 20,000 ng/mL, respectively.

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Spontaneous IL-8 production in whole blood cultures is enhanced in the presence of p75-Fc. In whole blood cultures, low concentrations of IL-8 are synthesized during a 24-h incubation. The effect of increasing concentrations of either soluble TNF receptor on spontaneous IL-8 production was assessed. In 12 donors, the mean spontaneous production of IL-8 was 206 ng/mL (range, 82–363 ng/mL). This spontaneous IL-8 production from each donor was set at 100%, and IL-8 production in the presence of either the p75-Fc or the p55-PEG soluble receptor was calculated as percentage of change. As shown in figure 5, at 2560 (P < .01) and 5120 (P < .001) ng/mL of the p75-Fc form, there was enhanced spontaneous production of IL-8 of 170% and 220%, respectively. In contrast, no increased IL-8 production was observed at the same concentrations of p55-PEG.

The enhanced spontaneous production of IL-8 was also observed when we used the native form of p75. As shown in figure 6A, at 1280 and 2560 ng/mL, the native form of the p75 soluble...
Figure 5. Effect of soluble tumor necrosis factor (TNF) receptors on spontaneous interleukin (IL)-8 production in whole blood. Whole blood was cultured in absence or presence of soluble TNF receptors (p75-Fc or p55-PEG [polyethylene glycol]), at concentrations indicated below horizontal axis. After 24 h at 37°C, cultures were lysed with Triton-X and were assayed for IL-8. Spontaneous amount of IL-8 produced from each of 12 donors was set at 100% (hatched bar). Mean % change (± SE) in IL-8 production is shown for each concentration of soluble TNF receptor. ** P < .01; *** P < .001, p75-Fc vs. spontaneous production.

Figure 6. Effect of native p75 and p55 on spontaneous interleukin (IL)-8 production in whole blood cultured in absence or presence of native p75 or p55 at concentrations shown below the horizontal axis. After 24 h at 37°C, cultures were lysed with Triton-X and were assayed for IL-8. Spontaneous production of IL-8 from each donor group was set at 100% (hatched bar). Mean % change (± SE) is shown for each concentration of (A) native p75 soluble receptor ( ), * P < .05, native p75 vs. spontaneous production) and (B) native p55 receptor ( , n = 4).

Effect of p75-Fc or p55-PEG on TNF-α-induced IL-1Ra in whole blood cultures. Similar to circulating IL-6 levels, circulating IL-1Ra levels in persons with rheumatoid arthritis can be used as an indicator of the severity of disease [18]; furthermore, in placebo-controlled trials of rheumatoid arthritis, blocking of TNF activity with a monoclonal antibody to TNF-α resulted in a rapid decrease in IL-1Ra levels. Therefore, the effect of the p75-Fc and p55-PEG on TNF-α-stimulated IL-1Ra production was measured in whole blood cultures. As shown in figure 7, there was no suppression of TNF-α-induced IL-1Ra by the p55-PEG soluble receptor. However, the p75-Fc construct dose-dependently suppressed IL-1Ra production. At 2560 ng/mL, the reduction was 72% (P < .01). At 5180 ng/mL, p75-Fc reduced IL-1Ra production by 87% (P < .001).

Figure 8. Comparison of p75-Fc and p55-PEG dissociation by BIAcore kinetics. As shown in figure 8A and 8B, respectively, p75-Fc has faster off kinetics than p55-PEG. The off rate of 620 s for p75-Fc, compared with 865 s for p55-PEG, was surprising, because p75-Fc is a dimeric molecule that was thought to have higher avidity. Figure 8C and 8D show the respective on-off rates of the native p75 and p55. These curves also reveal a difference in the 2 native forms of the soluble receptors. The curve-fitting programs supplied by the manufacturer were applied to the sensorgrams. The interaction between p55-PEG or p75-Fc with TNF-α is not a simple one-to-one binding. Because the analyte (TNF-α) is a trimeric molecule, there are no simple mathematical programs that fit perfectly with the sensorgrams. Therefore, the data were analyzed by a 1:1 Langmuir binding model (table 1). The results show that the affinity of p75-Fc to TNF-α is possibly stronger, if stronger at all, than that of p55-PEG (121 vs. 498 pM). As shown in the sensorgrams (figure 8), a faster off rate was observed for p75-Fc than for p55-PEG or dimeric p55-PEG. The faster off rate of p75-Fc was also shown elsewhere, by measurement of the exchange rate of TNF-α and TNF receptors [19].

Because of the intrinsic instability of the native TNF-α trimer, covalently linked TNF-α trimers were generated (see Materials and Methods). By use of the p55-PEG and the p75-Fc soluble receptors immobilized on the sensor chip, covalently
linked TNF-α trimers were exposed to the chip. As shown in table 1, the $K_d$ value for the TNF-α trimer binding to p75-Fc was $2.50 \times 10^{-9}$ M versus $6.25 \times 10^{-11}$ M for p55-PEG (a 40-fold difference). These results indicate that the stable TNF-α trimer has a greater affinity for the p55-PEG than for the p75-Fc soluble receptor.

**Discussion**

The production of IL-8 from TNF-α-stimulated human whole blood cultures was used to assess the neutralizing differences between 2 forms of TNF soluble receptors, p55 and p75. The native forms of these receptors and their modified constructs, which are currently administered to humans with rheumatoid arthritis, were evaluated. TNF-α–induced IL-8 was studied, because this chemokine has a clear pathologic role in several inflammatory diseases [20]. In the epithelial cell line, p75-Fc impressively reduced TNF-α–induced IL-8 in a dose-dependent fashion. In contrast, the addition of the same soluble receptor to whole blood cultures resulted in a U-shaped dose-response curve of TNF-α–induced IL-8.

As the concentration of p75-Fc added to whole blood cultures increased above 1 µg/mL, its ability to neutralize TNF-α diminished from 90% reduction at 320 ng/mL to 38% reduction at 5120 ng/mL. Even at 20,000 ng/mL p75-Fc, the reduction was 40%, compared with 99% for p55-PEG. According to the package insert, patients with rheumatoid arthritis who receive twice-weekly subcutaneous 25 mg doses of p75-Fc for 6 months have a median blood level of 3.0 µg/mL (range, 1.7–5.6 µg/mL), within the range tested in the whole blood cultures. In the sepsis trial, patients with the highest mortality received a 30-min intravenous infusion of 1.5 mg/kg (~100 mg) of p75-Fc, and it is likely that their blood levels of p75-Fc reached 5 µg/mL. Of considerable relevance, there was a dose-dependent increase in the level of circulating IL-1β in patients with sepsis who were treated with p75-Fc [5]. Unexpectedly, not only did the native p75 not reduce TNF-α–induced IL-8, it increased its production. Also unexpectedly, spontaneous IL-8 production in whole blood of 12 donors was enhanced in the presence of p75-Fc (>200%) or the native p75 form (1600%). In contrast to these findings, no enhancement of spontaneous IL-8 production was observed in the presence of the p55-PEG or native p55. These data demonstrate that the differential effects observed between the 2 receptors on spontaneous IL-8 production in whole blood are not due to any structural change secondary to the modifications themselves.

The dose-dependent increase in TNF-α–induced IL-8 by native p75 in whole blood cultures was reduced by adding native p55 to the cultures. These observations support the concept that the p75 TNF receptor functions as a “ligand passer” in whole blood cultures and not as a direct stimulant of IL-8 production. In studies by Tartaglia et al. [21], the p75 receptor could “regulate” the rate of TNF association with the p55 receptor, thereby increasing the local concentration of TNF available at the cell surface for receptor binding. Others have also demonstrated the “ligand passing” ability of the p75 receptor in the induction of nitric oxide and for gene expression of inductive nitric oxide synthase [22]. Similar results have been reported for the p75 receptor with human immunodeficiency virus type 1 activation [23]. In cell lines expressing high numbers of p75 receptors, the dissociation of TNF-α was rapid, compared with the dissociation of cells expressing a high number of p55 receptors [24]. Similarly, soluble native p55 was more potent than soluble native p75 in inhibiting TNF-α cytotoxicity [24, 25]. The ability of cell-bound p55 receptor to induce cytotoxicity is synergistic with activation of the p75 receptor, likely a ligand passing event [26].

The kinetics of radiolabeled TNF-α binding to the 2 soluble TNF receptors fused to Fc are dramatically different. The soluble p75-Fc to TNF-α exchange rate was ~60-fold faster than that of the p55-Fc to TNF-α [19]. The findings of [19], using the p55-Fc or p75-Fc receptors, are consistent with the present findings of off rates for the p55-PEG and p75-Fc constructs. The $T_1$ of p75-Fc is 620 s, and that of p55-PEG is 865 s. Concanavalin A–induced CD4+ T cell–dependent experimental hepatitis in mice is, in part, mediated by TNF-α. Mice deficient in TNF-α are resistant to liver damage in this model [27]. However, transgenic mice overexpressing the p75 receptor have enhanced hepatotoxicity [27]. Others [28] have reported that the...
p75 receptor plays a role in suppressing TNF-mediated inflammatory responses.

In this study, we observed another difference between the 2 soluble receptors. Whereas p75-Fc completely suppressed TNF-α–induced IL-1Ra production, p55-PEG did not. This was a particularly unexpected observation, because the p55-PEG suppressed TNF-α–induced IL-8 production at the same concentrations in the same cultures from the same 6 donors (figure 2). In an additional 6 blood donors, we again failed to observe suppression of TNF-α–induced IL-1Ra by the concentrations of p55-PEG tested. In contrast, the p75-Fc in these 12 donors consistently and dose dependently suppressed TNF-α–induced IL-1Ra. This dose-dependent suppression of TNF-α–induced IL-1Ra (figure 7) was not expected, because inhibition of TNF-α–induced IL-8 by p75-Fc exhibited a U-shaped dose-response curve in the same cultures (figure 2).

The greatest discrepancy between inhibition of TNF-α–induced IL-1Ra and IL-8 is seen at higher concentrations, at which nearly all TNF-α–induced IL-1Ra production is inhibited by p75-Fc, compared with no inhibition by p55-PEG. If one assumes that TNF-α induces IL-1Ra primarily via the p75 membrane receptor and not the p55 membrane receptor, then ligand passing to the p55 would not result in IL-1Ra production. On the other hand, ligand passing to the membrane p75 by the soluble p75 receptor can be assumed to be weak and ineffective, compared with ligand passing to the membrane p55 receptor. If TNF-α–induced IL-1Ra is preferential via the membrane-bound p75 receptor, then neutralization by p75-Fc

### Table 1. Kinetics of tumor necrosis factor (TNF) receptors by biomolecular interaction analysis.

<table>
<thead>
<tr>
<th>TNF receptor</th>
<th>$K_a$ (1/M)</th>
<th>$K_d$ (1/s)</th>
<th>$K_{app}$ (M)</th>
<th>$T_1/2$ (s)</th>
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<tr>
<td>p55-PEG $^a$</td>
<td>$1.61 \times 10^6$</td>
<td>$8.01 \times 10^{-2}$</td>
<td>$4.98 \times 10^{-10}$</td>
<td>865</td>
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<tr>
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<td>$9.26 \times 10^6$</td>
<td>$1.12 \times 10^{-2}$</td>
<td>$1.21 \times 10^{-10}$</td>
<td>620</td>
</tr>
<tr>
<td>Native p55 $^b$</td>
<td>$1.36 \times 10^6$</td>
<td>$5.77 \times 10^{-4}$</td>
<td>$4.24 \times 10^{-10}$</td>
<td>1201</td>
</tr>
<tr>
<td>Native p75 $^c$</td>
<td>$2.80 \times 10^6$</td>
<td>$4.13 \times 10^{-2}$</td>
<td>$1.48 \times 10^{-10}$</td>
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<td>$6.25 \times 10^{-10}$</td>
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</tr>
<tr>
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<td>$1.77 \times 10^{-2}$</td>
<td>$2.50 \times 10^{-10}$</td>
<td>390</td>
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</tbody>
</table>

**NOTE.** PEG, polyethylene glycol; $T_1/2$, half-life.

$^a$ Kinetic analysis of p55-PEG, p75-Fc, native p55, and native p75 surfaces interacting with native human TNF-α, using a biomolecular interaction analyzer (BIAcore 2000; BIAcore, Uppsala, Sweden). Off rates ($K_{off}$) were obtained by globally fitting the 1:1 Langmuir binding model to the sensorgram data (see figure 8). Hypothetical half-lives of the binding event were derived from the off rates with the equation $T = 0.693/K_{off}$.

$^b$ Kinetic analysis of p55-PEG and p75-Fc surfaces interacting with covalently linked TNF-α trimers.

Figure 8. Binding characteristics of p75-Fc, p55-PEG (polyethylene glycol), native p75, and native p55 tumor necrosis factor (TNF) soluble receptors with TNF-α. Soluble human TNF-α, in diluted buffer (see Materials and Methods), was passed over immobilized p75-Fc (A), p55-PEG (B), native p75 (C), or native p55 (D). p75-Fc and p55-PEG were exposed (from top to bottom) to 1000, 500, 250, 125, 62.5, 31.25, 15.63, 3.90, and 1.95 nM TNF-α. Native p75 and native p55 were exposed (from top to bottom) to 250, 125, 62.5, 31.25, and 15.63 nM TNF-α.
would be observed, despite ligand passing to membrane p55. This hypothesis is testable in mice deficient of the p75 receptor.

In support of the above findings, in the presence of TNF-α, immunoprecipitated receptors contain both p55 and p75, and ligand passing occurs at the membrane level [29]. The hetero-complexes of p55 and p75 are also observed with the soluble receptors of p55 and p75 [29]. In addition, persons pretreated with the p75-Fc construct before experimental endotoxemia have a dramatic decrease in circulating IL-1β and IL-1Ra but not in IL-10 or macrophage inflammatory protein–1α [30]. In addition, low-dose but not high-dose p75-Fc reduces endotoxin-induced plasma cortisol [30]. Similar to the findings in rheumatoid arthritis patients given p75-Fc, fever and other systemic effects of endotoxemia (increased cardiac index, systemic vascular resistance, and heart rate) were unaffected [31].

p75-Fc has been administered to patients before administration of OKT3 for treatment of acute renal syndrome. As seen in cell line bioassays, sera from these patients contained no biologically active TNF-α in vitro but had markedly higher serum TNF-α antigenic levels [32]. Despite reduced TNF activity of sera in cell cultures, persistent OKT3 side effects were observed in patients receiving p75-Fc [32]. In a randomized, double-blind placebo-controlled trial, p75-Fc was administered to patients being treated with high-dose intravenous IL-2 to reduce systemic toxicity. Despite evidence of in vitro neutralization of TNF-α functional activity, p75-Fc did not reduce the clinical toxicity associated with high-dose IL-2 therapy [33]. Thus, it appears that p75-Fc does not reduce systemic inflammation.

There is no doubt that p75-Fc is effective in treating patients with rheumatoid arthritis [34] or Wegener’s granulomatosis [35]. Although a report on long-term use of p75-Fc in 782 patients with rheumatoid arthritis supports its safety [36], a package insert warns that persons receiving p75-Fc appear to be more vulnerable to systemic sepsis and death when harboring infections and caution against its use in patients with local infections. The presence of the Fc segment itself does not explain the difference in mortality between the 2 soluble receptors in sepsis trials. Administration of a p55-Fc construct to 498 patients with sepsis resulted in a nonsignificant reduction in 28-day all-cause mortality [6]. This reduction in mortality was confirmed in a large phase II trial of 1342 patients (E. Abraham, personal communication). In contrast, p75-Fc administered to patients with similar sepsis resulted a dose-dependent increase in mortality [5]. Hence, the p75-Fc results in studies of patients with sepsis remain unexplained, as assessed in a large meta-analysis of anticytokine trials in patients with sepsis [4].

Suppression of host defense mechanisms and increased mortality associated with various agents used to reduce the biologic activity of TNF are well established in various animal models; moreover, the decrease in host defense is not unique to p75-Fc. It is observed with antibodies to TNF-α and in mice lacking the p55 TNF receptor. In humans, assessment of 28-day mortality in the treatment arm of 2 separate sepsis trials revealed that anti-TNF antibodies increased mortality [37, 38]. A lower dose of p55-Fc yielded increased mortality during the interim analysis [6]. Nevertheless, the dose-dependent increase in mortality in patients with sepsis who are treated with p75-Fc [5] is consistent with, but may not fully explain, the U-shaped dose-response curve of p75-Fc in whole blood cultures. Since there was less inhibition of TNF-α-induced IL-8 at the higher concentrations of p75-Fc, the increased mortality in patients with sepsis treated with p75-Fc may be relevant to the agonist role of IL-8 in sepsis [20]. Neutralizing monoclonal antibody against IL-8 attenuated the hypotensive and tachyphylactic effects of lipopolysaccharide, reduced free radical production, and increased survival after lethal endotoxic shock in rabbits [39]. In humans, IL-8 is downstream of TNF and IL-1. It is not known whether the U-shaped dose-response curve of p75-Fc on TNF-α-induced IL-8 in whole blood takes place in the systemic circulation of patients with sepsis.

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References