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ROLE OF 55- AND 75-kDa TNF RECEPTORS IN THE POTENTIATION OF Fc-MEDIATED PHAGOCYTOSIS IN HUMAN NEUTROPHILS

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Summary: Human neutrophils respond with an increased phagocytosis when exposed to TNF. Two types of TNF receptors have been identified, namely 55 kDa (TR55) and 75 kDa (TR75). We addressed the problem of the role of these receptors in the priming effect of TNF. By using monoclonal antibodies (MoAbs) directed either against TR55 or TR75, we have shown that 1) only TR55 is the signaling receptor for the potentiation of Fc-mediated phagocytosis and up-regulation of β2-integrin CD11b/CD18; 2) TR75 may control the function of TR55 by regulating the binding of TNF to the signaling receptor.

Treatment of neutrophils with TNF markedly increases (primes) the Fc-mediated phagocytic activity, both in terms of the number of cells participating in the process, and the number of particles ingested per cell (1,2).

TNF interacts with the target cells through two receptors, TR55 and TR75 (3). Among the cellular responses ascribed to the TR55 are cytolysis of cancer cells (4-8), proliferation of fibroblasts (5-7), induction of ICAM-1 expression (9), activation of NF-kB (10), apoptosis (11,12), activation of the respiratory burst in adherent neutrophils (13,14), and priming the FMLP-stimulated secretion and respiratory burst in neutrophils (15). The TR75 has been reported to mediate the proliferation of thymocytes and a cytotoxic T-cell line (16), aggregation of T-LAK cells (17), apoptosis (12), and, in some cases, cytolysis (18).

By using monoclonal antibodies htr-9 and H398 specifically directed against the TR55 (anti-55 MoAbs), and utr-1 specifically directed against the TR75 (anti-75 MoAb), whose agonistic activity has previously been reported (5,13,14,19,20), we show here that only the selective activation of TR55 is signaling for the priming effect of TNF on Fc-mediated phagocytosis, while TR75 controls the function of the cytokine by regulating its binding to TR55.

MATERIALS AND METHODS

Reagents. Tumor necrosis factor-α (TNF) was kindly donated by Bachem. Inc. Purified mouse IgG1 MoAb htr-9 (anti-55 MoAb) and MoAb utr-1 (anti-75 MoAb) were a generous

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gift of Dr.M.Brockhaus (Hoffmann-La Roche, Basel, Switzerland). Purified mouse IgG2a MoAb H398 (anti-55 MoAb) and its Fab fragment were kindly provided by Dr.P.Scheurich (Institute for Cell Biology and Immunology, Stuttgard, Germany). MoAb OKM1, an IgG2b that recognizes the CD11b subunit of the CD11/CD18 antigen complex, and MoAb 5E9, an IgG1 that recognizes the transferrin receptor, were purified from ascitic fluids of mice injected with hybridoma cell lines obtained from the American Type Culture Collection (ATCC), Bethesda, MD.

Neutrophil isolation. Neutrophils (95% pure) were isolated from healthy donors and suspended in Hank's balanced salt solution (HBSS) as previously described (21).

Preparation of opsonized erythrocytes (E-IgG). Sheep erythrocytes were prepared and opsonized with subagglutinating concentrations of rabbit anti-sheep red blood cells IgG as previously described (22).

Phagocytosis assay. 0.2ml of a neutrophil suspension (10^7/ml) was incubated at 37°C in a shaking water bath with TNF or with anti-TNF-receptor MoAbs at the concentrations and for periods of time indicated in the figure legends and then challenged with E-IgG at a neutrophil/erythrocyte ratio of 1:20 for 10 min. Phagocytosis of E-IgG was evaluated by microscopic examination at x1500 magnification as previously described (22).

Immunofluorescence flow cytometry. Neutrophils (10^7/ml) were incubated with TNF or with anti-TNF-receptor MoAbs in the same conditions employed to study the phagocytosis. At the time indicated in the figure legends, 0.1 ml neutrophil suspension ($1x10^6$ cells) was withdrawn and incubated at 4°C for 30 min with 10% human serum and subsequently with 10 μg/ml of MoAb OKM1 for a further 30 min at 4°C. The cells were then washed twice and incubated with a fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG $F(ab')_2$ (Cappel laboratories, Cochranville, PA) for 30 min at 4°C. After two washing, the cells were suspended in PBS and analyzed by a flow cytometer (Becton-Dickinson).

Binding of ^{125}I-TNF. PMN ($20x10^6$/ml) were preincubated with or without anti-75 MoAb (utr-1, 10 μg/ml), anti-55 MoAb (htr-9, 10 μg/ml) or both MoAbs for 30 min at 4°C. After this time, duplicate aliquots of 0.2 ml were incubated with chloramine T-labeled ^{125}I-TNF in the absence or the presence of 100-fold excess of unlabeled TNF. After 1 hour of incubation in ice, cells were washed three times with ice cold PBS and the cell-associated radioactivity counted with a gamma counter. Specific binding was defined as total binding minus the binding that occurred in excess of unlabeled TNF.

RESULTS AND DISCUSSION

In neutrophils treated with 6 ng/ml TNF for 15 minutes and subsequently challenged with E-IgG, the phagocytic index increased from 103 ± 17 to 225 ± 25 (Fig. 1). Higher doses of TNF did not increase the effect. In order to determine through which receptor TNF primed the phagocytosis, we triggered each TNF receptor separately using monoclonal antibodies htr-9 (anti-55 MoAb) and utr-1 (anti-75 MoAb). Only the anti-55 MoAb was able to mimic the effect of TNF increasing the phagocytic index to 177 ± 15 (Fig.1), while anti-75 MoAb was ineffective. Treatment of neutrophils with both anti-55 and anti-75 MoAbs did not enhance the effect observed with the anti-55 MoAb alone.

The priming by anti-55 MoAb was dose-dependent, being detectable with concentrations as low as 0.3 μg/ml, and reaching a maximum at 1.25 μg/ml . No effect of anti-75 MoAb was observed up to 20 μg/ml (data not shown).

In time course experiments the maximal priming of phagocytosis by anti-55 MoAb htr-9 was reached after 10 minutes of neutrophil treatment (data not shown).

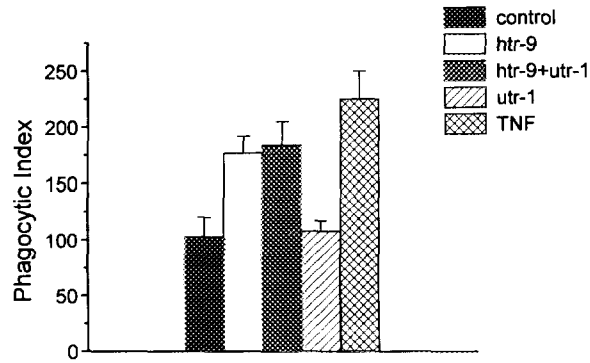

Fig.1. Effect of anti-55 MoAb (htr-9) and anti-75 MoAb (utr-1) on Fc-mediated phagocytosis. Neutrophils (10^7/ml) were preincubated with htr-9 (5 μg/ml), utr-1 (5 μg/ml), htr-9 and utr-1 together or with TNF (6 ng/ml) for 15 min at 37°C and then challenged with E-IgG at a neutrophil/erythrocyte ratio of 1:20 for 10 min. Control samples were preincubated with the 5E9 anti-transferrin receptor MoAb (5 μg/ml). Data are the means ± S.D. of five experiments.

TNF upregulates the CD11b/CD18 β2-integrin (CR3) on neutrophil surface (23) and this would play a relevant role in the potentiation of Fc-mediated phagocytosis (24). We investigated whether the anti-55 MoAb was able to increase CR3 expression and the data reported in Fig.2 show that this was the case. The increase of phagocytosis (Fig.2A) and CR3 expression (Fig.2B) following treatment with anti-55 MoAb or with TNF were measured in parallel in neutrophils prepared from the same donors. The maximal effect for both responses was reached using 1.25 μg/ml of anti-55 MoAb. Simultaneous preincubation with anti-55 and

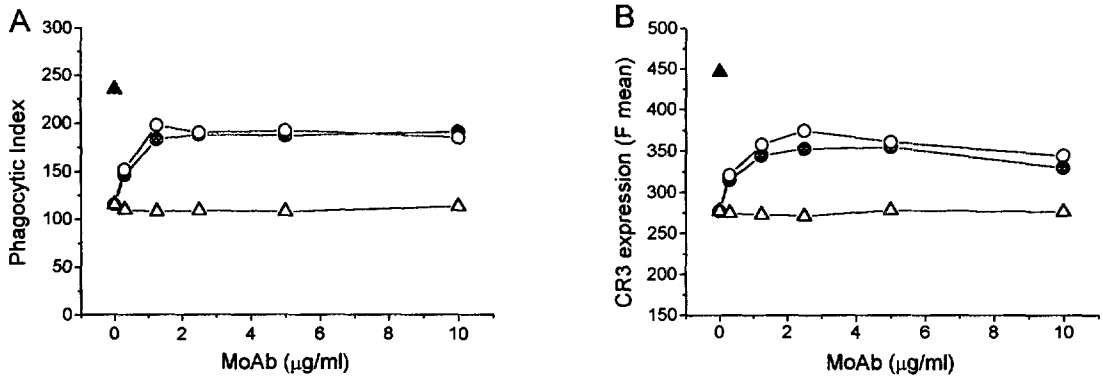

Fig.2. Effect of different doses of anti-55 MoAb (htr-9) and anti-75 MoAb (utr-1), on the Fc-mediated phagocytosis (A) and on the up-regulation of CR3 (B). PMN (10^7/ml) were preincubated with htr-9 (●), utr-1 (△), htr-9 and utr-1 together (○), or with 6 ng/ml TNF (▲) for 15 min at 37°C and then stimulated with E-IgG. Before the addition of opsonized particles, 0.1 ml of the cell suspension was withdrawn to analyze the CR3 expression on neutrophil surface (see Materials and Methods). Phagocytosis was measured after 10 min as described in the Fig.1 legend. Data are from one experiment representative of three.

anti-75 MoAbs did not enhance the effect observed with the anti-55 MoAb alone. Moreover, anti-75 MoAb neither potentiated phagocytosis nor CR3 expression.

Further evidence that TR55 is responsible for the priming was obtained by using another anti-55 MoAb H398 and its Fab fragment. The data reported in Fig.3A show that MoAb H398 primed the phagocytosis while H398 Fab was ineffective. Interestingly, the pretreatment of neutrophils with H398 Fab blocked the effect of TNF. This finding, beside to confirm that TR55 is signaling for priming, adds a further proof that the binding of TNF to TR75 is not able to trigger signals for potentiation of phagocytosis. Fig.3B reports the effect of anti-55 MoAb H398 and its Fab fragment on CR3 expression. It can be seen that, while MoAb H398 increased the expression of this β2 integrin, the Fab fragment alone was ineffective but completely prevented the activity of TNF.

It has been shown that in different cell types the blockade of TR75 by specific MoAbs partially inhibits TNF responses induced by TR55 agonists (5,6,8,14). These inhibitory effects, generally observed at low doses of TNF, have been explained with the ligand-passing model proposed by Tartaglia and Goeddel (3,8) in which TR75 facilitates the association of TNF to TR55. To clarify the functional role of TR75 we examined whether the block of TR75 by pretreating neutrophils with a specific MoAb could influence the priming by TNF or by anti-55 MoAb. The results reported in Fig.4 show that when TR75 was occupied by MoAb utr-1 the potentiation of phagocytosis by high concentrations of TNF (6.0 and 12.0 ng/ml) was unchanged, while that by low doses of TNF was depressed. This depression was inversely

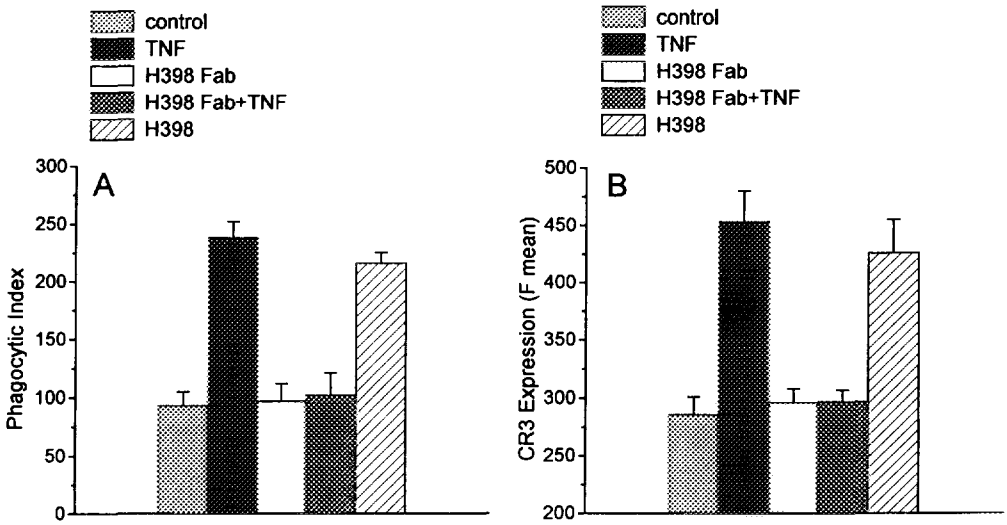

Fig.3. Effect of Fab fragment of the anti-55 MoAb H398 on the potentiation of phagocytosis (A) and on the up-regulation of CR3 (B) induced by TNF. Neutrophils (10^7/ml) were stimulated for 10 min at 37°C with 6 ng/ml TNF or 10 μg/ml MoAb H398 and then assayed for phagocytosis and CR3 expression as described in the Fig.2 legend. In separate tubes, neutrophils were pretreated with MoAb H398 Fab (20 μg/ml) for 10 min at 37°C before the stimulation with 6 ng/ml TNF. Data are the means ± S.D. of three experiments.

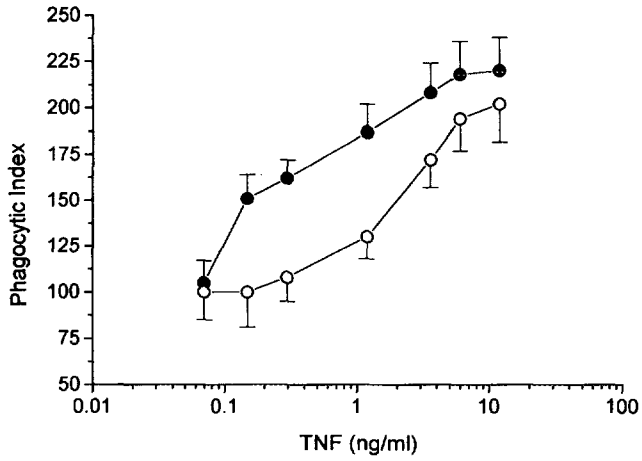

Fig.4. Effect of anti-75 MoAb (utr-1) on the potentiation of phagocytosis by TNF. Neutrophils (10^7/ml) were preincubated for 10 min at 37°C with (O) or without (●) 10 μg/ml MoAb utr-1 followed by treatment with the indicated concentrations of TNF. The cells were then stimulated with E-IgG for 10 min. Data are the means ± S.D. of four experiments.

correlated with the concentration of TNF. Interestingly, anti-75 MoAb utr-1 did not exert any inhibitory effect on the phagocytosis potentiated by anti-55 MoAb htr-9 (data not shown). The finding that the block of TR75 did not inhibit the priming by high doses of TNF and by the anti-55 MoAb indicate that TR75 does not trigger inhibitory signals, in agreement with the data recently shown for other responses (14).

The fact that the occupancy of TR75 with the specific MoAb inhibited the effect of low doses of TNF could be due to an inhibition of the binding of TNF to TR55 according to the ligand-passing model (3,8). We have investigated this problem by measuring the specific binding of different concentrations of ^{125}I-labeled-TNF in the absence of anti-TNF-receptor MoAbs (binding to both receptors) or in the presence of anti-75 MoAb or anti-55 MoAb (binding to TR55 and TR75, respectively). In preliminary experiments we have demonstrated the validity of this procedure showing that the pretreatment of neutrophils with anti-55 and anti-75 MoAbs completely inhibited the binding of ^{125}I-labeled-TNF (data not shown). The results of Fig.5, show that at all the concentrations of TNF the occupancy of TR55 did not influence the binding to TR75, while the occupancy of TR75 caused a decrease of the binding to TR55 which was inversely proportional to the concentrations of the cytokine. From the data of Fig.5 one can calculate that the lack of the binding of TNF to TR75, due to the presence of anti-75 MoAb, caused an inhibition of the binding to TR55 of 16%, 29%, 40%, 57%, 79% at the concentrations of 3.6, 1.2, 0.6, 0.3, 0.15 ng/ml of TNF, respectively. It is worth pointing out that the decrease of priming by TNF caused by the lack of the binding of the cytokine to TR75 (Fig.4) correlated with the decrease of the binding to TR55 (Fig.5) confirming that the binding of TNF to TR75 regulated that of the cytokine to TR55 (3,8).

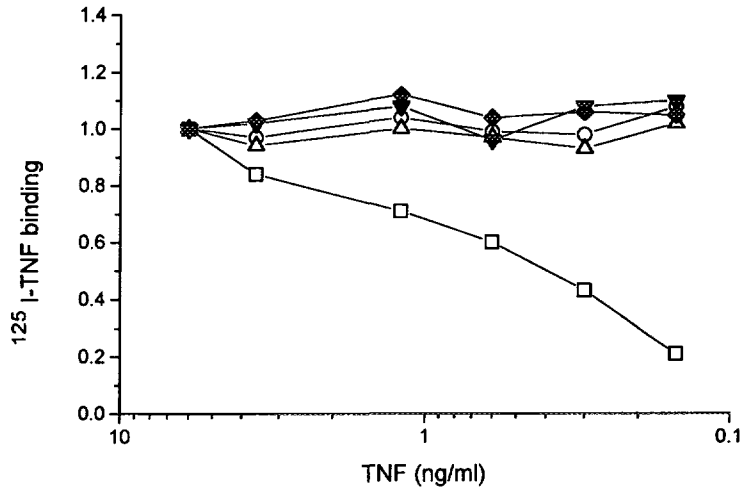

Fig.5. Effect of anti-75 MoAb (utr-1) and anti-55 MoAb (htr-9) on the binding of ^{125}I-TNF to TR55 and TR75, respectively. The values are expressed as ratios TNF bound/TNF free at different concentrations of TNF in the absence or the presence of anti-55 or anti-75 MoAb. The real values of the ratios (see below *) at the maximal concentration of TNF (6ng/ml) in all the conditions (i.e. in the presence or absence of MoAbs) have been made equal to 1.0. (O) Total binding in the absence of blocking MoAbs (* 0.0057 = 6.85×10^3 cpm bound/1.2×10^6 cpm free) ; (△) binding to TR75 in the presence of anti-55 MoAb (* 0.0033); (□) binding to TR55 in the presence of anti-75 MoAb (* 0.0011); (◆) theoretical binding to TR75 as difference between the binding in the absence of blocking MoAbs and the presence of anti-75 MoAb (* 0.0046) ; (▼) theoretical binding to TR55 as difference between the binding in the absence of blocking MoAbs and the presence of anti-55 MoAb (* 0.0023). Data are from one experiment representative of four.

CONCLUSION

The present study allows us to conclude that in human neutrophils TR55 is responsible for the priming effect of TNF on Fc-mediated phagocytosis, while TR75 does not transduce any signal for this priming but it is involved in the function of TR55. Several findings support this conclusion: 1) the priming effect of TNF was reproduced by treatment of neutrophils with the anti-55 MoAbs (htr-9, H398) and not with the anti-75 MoAb (utr-1); 2) the binding of TR55 with MoAb H398 Fab fragment did not reproduce the priming, but completely prevented the agonistic activity of TNF; 3) the occupancy of TR75 with the specific MoAb inhibited the priming effect of low doses but not of high doses of TNF; 4) this inhibition of the priming effect of low doses of TNF when TR75 was blocked by specific antibody correlated with the depression of the binding of TNF to the signaling receptor TR55, in agreement with the cooperative activity of TR75 described by the ligand-passing model (3,8).

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