Clin Investig (1992) 70:22–27

Original Article

Clinical Investigator

Elevated TNF receptor plasma concentrations in patients with rheumatoid arthritis

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Summary. Two types of tumor necrosis factor receptors have been characterized, both capable of transmitting the signal and exerting the biological functions of TNF and lymphotoxin. We measured the plasma concentrations of two types of TNF binding proteins (sTNFR-A and sTNFR-B) in patients with rheumatoid arthritis (RA) and spondylarthropathies (SpA) using an enzyme-linked binding assay. In normal controls ($n=43$), mean plasma concentrations were 1030 ± 55 and 1461 ± 59 pg/ml for sTNFR types A and B, respectively. In 67 patients with moderate RA, mean levels were 1422 ± 82 pg/ml (type A) and 2088 ± 109 pg/ml (type B); in 34 patients with severe RA, 2588 ± 279 pg/ml and 4494 ± 550 pg/ml, respectively, were measured ($P<0.0001$ compared to normal controls). Concentrations of both type A and type B sTNFR were highly correlated in severe RA ($R^2=0.7$) but not in SpA or normal controls. T lymphocytes in synovial fluid of patients with RA expressed predominantly type A TNF receptors on their surface; in some patients a weaker expression of type B receptors was also detectable. Soluble TNF binding proteins in patients with RA were able to neutralize TNF in a cytotoxiity assay, demonstrating their ability to act as "TNF-inhibiting factors". We conclude that both types of TNF receptors are parameters of disease activity in RA and may also act as TNF antagonists.

Key words: Rheumatoid arthritis – Spondylarthropathies – TNF receptors

In rheumatic diseases, cytokines like tumor necrosis factor (TNF), interleukin-1(IL-1), and interleukin-6 (IL-6) have been suggested as mediators of pathophysiologic events [8, 10, 11, 13, 14, 16, 21, 28]. TNF is a pleiotropic cytokine with important regulatory functions in inflammation and immune response [18, 24]. TNF receptors (TNFR) are differentially expressed on macrophages, granulocytes, activated T and B lymphocytes, and various other cell lines [1]. Proteins inhibiting the effects of TNF have been isolated from human urine and plasma [5, 19, 23]. It has been argued that these substances may play a role as antagonists or neutralizing agents of TNF. Recently it has become clear that the "TNF inhibitors" are soluble forms of TNF receptors. Two types of cell surface receptors, a 75-kD molecule called type A or TNFR-A and a 55-kD called type B or TNFR-B, have been characterized biochemically and by reactivity with two sets of monoclonal antibodies (mAbs) [2, 6, 12]. Cloning and sequencing of the cDNAs of the two types of TNFR revealed a homology of the 75-kD receptor with the B-cell-associated antigen CD 40 and with the nerve growth factor receptor [15, 22, 25]. We found that in patients with rheumatoid arthritis (RA), the plasma concentrations of both types of soluble TNF binding proteins are elevated as compared to normal controls and to patients with spondlyarthropathies (SpA). The TNF binding proteins in patients with RA are functionally active and can neutralize the cytotoxic effects of TNF in bioassays. The disease activity is correlated with TNFR plasma concentrations.

Abbreviations: TNF = tumor necrosis factor; TNFR = tumor necrosis factor receptor; sTNFR = soluble tumor necrosis factor receptor; RA = rheumatoid arthritis; SpA = Spondylarthropathy; ELIBA = Enzyme linked binding assay; mAb = monoclonal antibody

Patients, materials, and methods

Patients

The rheumatology outpatient unit of the Department of Internal Medicine V of the University of Heidelberg characterized 101 patients as having rheumatoid arthritis (RA) according to the ARA

criteria. Patients who were found to suffer severe RA had a Ritchie joint index >25, vasculitic lesions, or pulmonary rheumatic disease. Patients deemed to have moderate RA did not match these criteria. EDTA plasma and synovial fluids were stored at −20° C until use. For the TNF cytotoxicity assay, serum was stored at −20° until use. Plasma of 43 healthy volunteers was stored and assayed under the same conditions.

Assays for type A and B TNF receptors

The concentrations of sTNFR were measured by an enzyme linked binding assay (ELIBA) with a TNFα horseradish peroxidase conjugate as detecting agent. The assays were based on a similar protocol with 125 I-TNFα described previously [4]. The details of the assay will be presented elsewere (H. Gallati, M. Brockhaus: manuscript in preparation). Briefly, microtiter plates were coated with either the noninhibitory mAb utr-4 directed against type A TNFR (75 kD) [2] or with the noninhibitory mAb htr-20 against type B TNFR (55 kD) [4]. In a single-step reaction the plasma was incubated together with TNFα enzyme conjugate in the microtiter wells. After removal of the unbound reagent, the bound TNFα enzyme was measured enzymatically with tetramethyl-benzidene as substrate. The TNFα enzyme conjugate was present in surplus; moreover, the addition of up to 40 ng/ml of recombinant TNFα had no influence on the TNFR assays. The assays had a range of 0.1–5 ng/ml and a sensitivity of 100 pg/ml sTNFR-A and sTNFR-B.

ELISAs for TNF

Plasma concentrations were measured using the Quantikine TNF assay (Biermann GmbH, Bad Nauheim, FRG) according to the manufacturer's instructions and analyzed with an ELISA reader at 492 nm. Recovery of 50 pg/ml recombinant TNF was less than 40% in the presence of the patients' plasma. This may indicate an interfering plasma factor. Recovery of higher TNF concentrations (>500 pg/ml) was better but did not reach 100%.

Analysis of surface TNF receptors on synovial T lymphocytes

Lymphocytes from heparinized synovial fluid of patients with RA were separated with Ficoll-Hypaque. Lymphocytes were analyzed after staining with anti-TNFR mAbs UTR-1, HTR-5, and HTR-9 (all IgG1) and IgG1 control mAbs. The generation and characterization of anti-TNFR antibodies UTR-1 HTR-5 and HTR-9 is described in [2]. UTR-1 binds to a 75-kD TNFR; HTR-5 and HTR-9 to a 55-kD TNFR. 10^6 Cells were incubated with 10 μg/ml mAbs in PBS with 0.1% BSA at 4° C for 30 min. Cells were washed with PBS+ 0.1% BSA, and then 1:50 diluted Biotin F(ab)2 goat anti-mouse IgG (Dianova, Hamburg, FRG) was incubated for 30 min at 4° C. After 30 min incubation with 1:50 PBS-diluted streptavidin phycoerythrin (Dianova, Hamburg, FRG) cells were washed again and fixed with 1% formalin (Merck, Darmstadt, FRG). Indirect immunofluorescence was measured using a FACScan flow cytometer (Becton Dickinson). Staining with CD 3, CD 20 (pan B marker), CD 4, and CD 8 mAbs revealed that more than 90% of synovial lymphocytes were T cells.

TNF cytotoxicity assay

Cells of the TNF-sensitive 24T2.5 mouse fibrosarcoma cell line [27] were kindly provided by H. Schreiber, University of Chicago. Human recombinant TNF with a biological activity of 8×10^6 U/ml was generously provided by BASF AG, Ludwigshafen, FRG. The 24T2.5 cells were cultured for 72 h in 1640 RPMI, 5% heat-inactivated fetal calf serum, 2 mM L-glutamine, and penicillin/streptomycin. Sera from a normal donor, two RA patients, and two SpA patients were 1:5 diluted in PBS and equilibrated at room temperature for 4 h with 10 μg/ml HTR-5 mAb and 10 μg/ml UTR-1 mAb, or as a control with 20 μg/ml of unrelated IgG1 mAb. The 1:5 diluted sera equilibrated with HTR-5, UTR-1, or control IgG1, were supplemented with TNF to an final concentration of 0.01 U/ml–100 U/ml TNF and kept for 2 h at 20° C before being added to the 24T2.5 cultures. Viability of the 24T2.5 cells was determined after 72 h with the MTT assay [17].

Results

Plasma concentrations of TNF binding proteins in patients with RA and SpA compared to normal controls

Plasma of healthy donors and patients with RA and SpA were collected, aliquotted, and frozen at −20° C. Each sample was thawed only once. The plasma concentrations of sTNFR types A and B are shown in Table 1. Mean plasma concentrations were 1030±55 pg/ml for sTNFR-A and 1461±

59 pg/ml for sTNFR-B in normal controls ($n=43$). In patients with moderate RA ($n=67$), mean plasma concentrations of both TNF binding proteins were significantly elevated as compared to the control group ($P<0.01$ and $P<0.001$ for TNFR types A and B, respectively). In patients with SpA ($n=51$), both TNF binding proteins also ranged significantly higher than in normal controls (1415±91 pg/ml and 1779±81 pg/ml). Since a similar increase was found in patients with SpA, the differences between patients with moderate RA and SpA are negligible, although a trend toward higher levels of type B TNFR was seen in patients with moderate RA. In patients with severe RA ($n=34$), the highest plasma concentrations for both types of sTNFR were measured. The mean values of this group were significantly elevated when compared to normal controls ($P<0.0001$ for both TNFR) or compared to the patients with moderate rheumatoid disease ($P<0.001$). TNF could not be detected by ELISA in any of the patients' plasma.

Table 1. Plasma concentrations of soluble TNF receptors (sTNFR) in patients with rheumatoid arthritis (RA) and spondylarthropathies (SpA)

Group (n)	Type A sTNFR pg/ml (P*)	Type B sTNFR pg/ml (P*)
Controls (43)	1030±55	1461±59
moderate RA (67)	1422±82 ($P<0.01$)	2088±109 ($P<0.001$)
severe RA (34)	2588±279 ($P<0.0001$)	4494±550 ($P<0.0001$)
SpA (51)	1415±91 ($P<0.01$)	1779±81 ($P<0.05$)

* Determined by Mann Whitney U-test

Correlation of plasma concentrations of both types of TNF binding proteins in RA patients

Linear regression analysis was performed to study the correlation of type A and type B sTNFR plasma concentrations in the patients with RA and SpA and normal controls. Figure 1 demonstrates that the concentrations of both types of sTNFR are correlated in RA patients ($R^2=0.458$ and $R^2=0.708$ for moderate and severe RA). Plasma concentrations of both types of sTNFR are not correlated in SpA patients and normal controls.

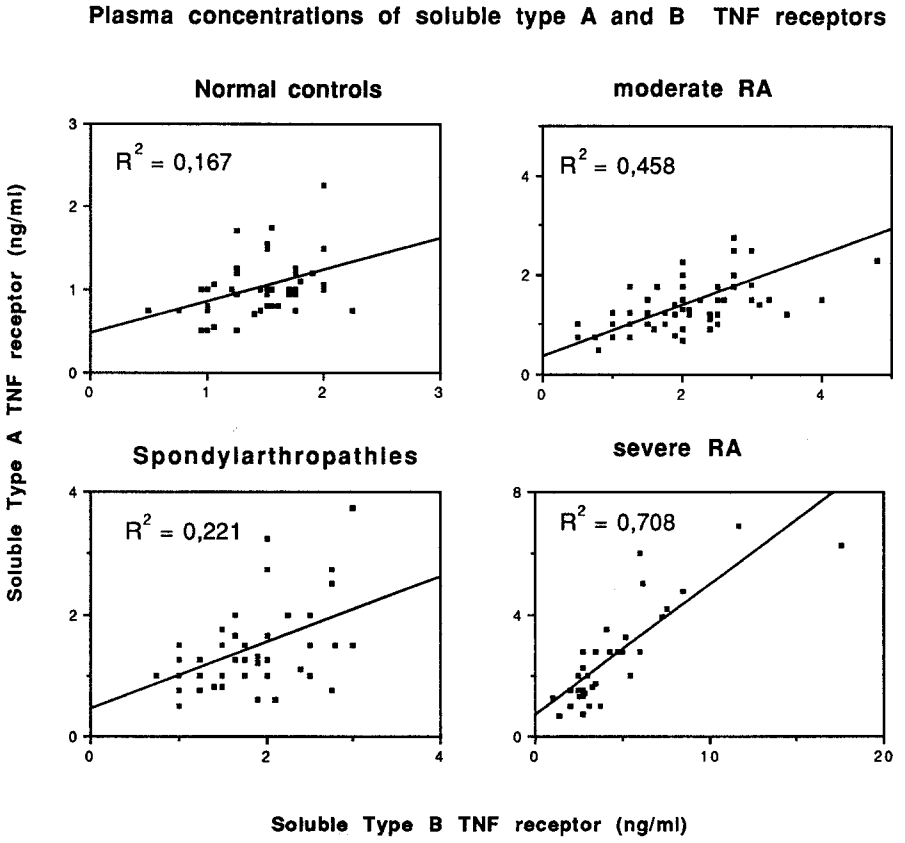

Fig. 1. Correlation of sTNFR-A and sTNFR-B in plasma of patients with moderate and severe RASpA, and normal controls

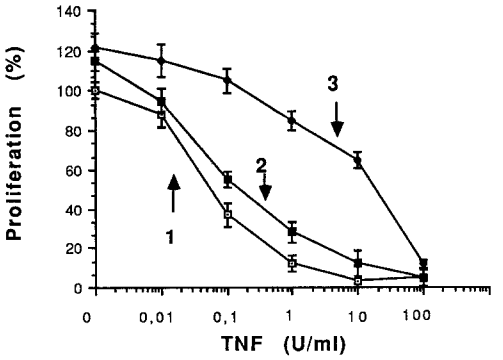

Fig. 2. Proliferation of TNF-sensitive 24T2.5 fibrosarcoma cells. 24T2.5 cells were cultured in medium (curve 1) containing 20% human serum of a patient with high sTNFR concentrations. The human serum was incubated previously with anti-TNF receptor antibodies UTR-1 and HTR-5 to block binding sites for TNF (curve 2) or with a control Ig (curve 3)

Functional activity of plasma TNF binding proteins in a TNF-induced cytotoxicity assay

Using the TNF-sensitive murine fibrosarcoma line 24T2.5, we demonstrated that sTNFR inhibit the TNF-induced cytotoxicity in the 24T2.5 assay (Fig. 2). As documented in curve 1, 24T2.5 cells are killed dose-dependently by TNF. Serum of a patient with RA equilibrated with mAb UTR-1, an inhibitory antibody specific for type A TNFR (75 kD), and mAb HTR-5, which is specific for type B TNFR (55 kD) [2], does not inhibit TNF cytotoxicity (curve 2). The two anti-TNFR antibodies block the binding of TNF to the two types of sTNFR and therefore keep TNF available to work its cytotoxic effects on the 24T2.5 target cells. If serum is equilibrated with a murine IgG1 control antibody instead of the anti-TNFR antibodies, the sTNFR receptors exhibit TNF-neutralizing capacity in the 24T2.5 assay (curve 3).

Expression of TNFR on synovial T lymphocytes

Freshly isolated synovial lymphocytes of 6 patients with RA were analyzed with TNFR types A- and B-specific mAbs. Figure 3 shows data on patients 1–3. T lymphocytes predominated in all patients' synovial fluids. B cells amounted to $<7\%$ of the total. The synovial lymphocytes expressed in all cases type A TNFR. In two cases weak expression of type B TNFR was found also. In contrast, freshly isolated peripheral blood T lymphocytes of these 6 patients and of 5 healthy donors did not express significant amounts of either TNFR (not shown).

Soluble TNF receptors in synovial fluid of patients with RA. Type A and type B TNFR were measured

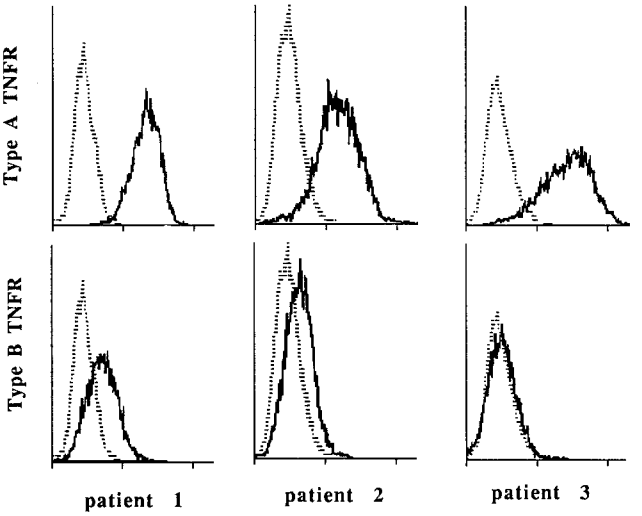

Fig. 3. Expression of type A and B TNF receptors on synovial lymphocytes. Synovial lymphocytes (more than 93% T cells) were stained with UTR-1 (type A TNFR) or with HTR-5 (type B TNFR) or control mAb (dotted lines) using indirect immunofluorescence measured with a FACScan flow cytometer. The individual histograms represent the fluorescence intensity of synovial lymphocytes in three patients with RA

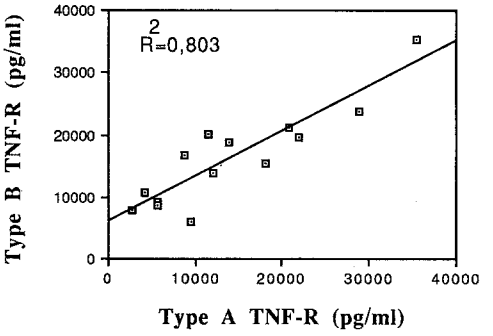

Fig. 4. Correlation of sTNFR-A and sTNFR-B concentrations in synovial fluids of patients with RA

in the synovial fluids of 14 patients with RA. The mean concentrations are 14221 ± 2596 pg/ml for type A and 16228 ± 2098 pg/ml for type B TNFR, significantly elevated compared to the plasma concentrations. The concentrations of both types of receptors were highly correlated (Fig. 4).

Discussion

TNF has been suggested as a possible mediator of the inflammatory events in RA and as an important factor in cartilage and bone destruction [10, 13, 21, 28]. Recent evidence suggests that high amounts of TNF binding proteins are found in severe inflammatory responses like sepsis. These proteins act as specific inhibitors of TNF in var-

ious biological assays, and they may influence the homeostasis of TNF-mediated inflammatory events. In order to gain more information on TNF and endogenous TNF binding proteins in RA and SpA, we measured the plasma concentrations of sTNFR. TNF was not detectable in any of the patients, but all plasma specimens contained TNF binding proteins (TNFR-A and -B). In RA patients the concentrations of both types of receptors were significantly elevated as compared to normal controls. A minor elevation was found in patients with SpA. Patients with severe rheumatoid disease had higher plasma levels than patients with moderate RA. In contrast to our results, others have measured elevated TNF serum concentrations in RA patients using different methods [21]; standardized assays will be needed to compare results. Furthermore, the increased amounts of both types of sTNFR we found in the plasma of RA patients may influence TNF measurements. In vivo TNF application in human cancer patients seems to induce high plasma levels of sTNFR (our own unpublished observation). It may even be that the elevated sTNFR concentrations in RA patients reflect the counterregulation of increased TNF biosynthesis, which itself is not detectable because of its short half-life. Interestingly, the plasma concentrations of both types of TNFR are correlated in RA patients but not in SpA patients. It has been reported that polymorphonuclear cells shed both types of TNFR from their cell surface upon activation in vitro [20], leading to a ratio of approximately 1:2 for sTNFR-A and sTNFR-B. Activated lymphocytes express predominantly type A TNFR (75 kD) [3, 9, 25] and are a possible source of sTNFR-A (our own unpublished data). Although the cellular origin of elevated sTNFR-A and sTNFR-B in RA has not been identified, it remains an intriguing hypothesis that these proteins reflect the state of polymorphonuclear cells and lymphocyte activation in disease. Indeed, in 6 out of 6 cases we found T lymphocytes in the synovial fluid of RA patients expressing type A TNFR, and in 2 out of 6 cases, type B TNFR-positive T cells. Peripheral T cells did not express TNFR. The highest concentrations of both types of soluble TNFR were found in the synovial fluid of patients with RA. This suggests that in RA patients, activated T cells located in the inflammatory joints are predominantly a source of type A TNFR, whereas type B TNFR might be shed by another compartment, perhaps by granulocytes or endothelial cells. The pathophysiological function of the TNF binding proteins is still unclear. Elevated concentrations of TNF binding proteins are also seen in malignant diseases [4, 7] and in patients under the influence of endotoxin [26]. Since soluble TNFR neutralize TNF, they may play a role as a local TNF antagonist at the sites of inflammation as well as an inhibitor of generalized TNF toxicity in the periphery. Further investigations are needed to dissect the homeostasis and balance of the TNF/TNFR system.

Acknowledgement. We thank H. Lötscher and W. Lesslauer for providing us with recombinant TNFR used as standards in the ELIBAs. We also thank I. Pracht for excellent technical assistance.

References

1. Aggarwal BB, Essalu TE, Hass PE (1985) Characterization of receptors for human tumor necrosis factor and their regulation by γ interferon. Nature 318:665–668
2. Brockhaus M, Schoenfeld HJ, Schlaeger EJ, Hunziker W, Lesslauer W, Loetscher H (1990) Identification of two types of tumor necrosis factor receptors on human cell lines by monoclonal antibodies. Proc Natl Acad Sci USA 87:3127–3132
3. Dembic Z, Loetscher H, Gubler U, Pan Y, Lahm H, Gentz R, Brockhaus M, Lesslauer W (1990) Two human TNF receptors have similar extracellular, but distinct intracellular, domain sequences. Cytokine 2:231–237
4. Digel W, Porzsolt F, Lesslauer W, Brockhaus M (1992) Evidence for soluble receptors for tumor necrosis factor in the serum of cancer patients – high levels in B-cell malignancies (in press)
5. Engelmann H, Aderka D, Rubinstein M, Rotman D, Wallach D (1989) A tumor necrosis factor-binding protein purified to homogeneity from human urine protects cells from tumor necrosis factor toxicity. J Biol Chem 264:11974–11980
6. Espevik T, Brockhaus M, Loetscher H, Nonstad U, Shalaby R (1990) Characterization of binding and biological effects of monoclonal antibodies against a human tumor necrosis factor receptor. J Exp Med 171:415–420
7. Gatanaga T, Hwang C, Kohr W, Cappuccini F, Lucci JA, Jeffes EWB, Lentz R, Tomich J, Yamamoto RS (1990) Purification and characterization of an inhibitor (soluble tumor necrosis factor receptor) for tumor necrosis factor and lymphotoxin obtained from the serum ultrafiltrates of human cancer patients. Proc Natl Acad Sci USA 87:8781–8784
8. Guerne PA, Zuraw BL, Vaughan JH, Carson DA, Lotz M (1989) Synovium as a source of interleukin 6 in vitro: contribution to local and systemic manifestations of arthritis. J Clin Invest 83:585–592
9. Heilig B, Mapara M, Brockhaus, Krauth K, Dörken B (1991) Two types of TNF-receptors are expressed on human normal and malignant B-lymphocytes. Clin Immunol Immunopathol 61:260–267
10. Henderson B, Pettipher ER (1987) Arthritogenic actions of recombinant IL 1 and TNF α in the rabbit: evidence for synergistic actions between cytokines in vivo. Clin Exp Immunol 75:306–310
11. Hirano T, Matsuda T, Turner M, Mijasaka N, Buchan G, Tand B, Sato K, Shimizu M, Maini R, Feldmann M, Kishimoto T (1988) Excessive production of interleukin 6/B cell stimulatory factor-2 in rheumatoid arthritis. Eur J Immunol 18:1797–1801

12. Hohmann HP, Remy R, Brockhaus M, van Loon AP (1989) Two different cell types have different major receptors for human tumor necrosis factor (TNF alpha). J Biol Chem 264:14927–14933
13. Hopkins SJ, Meager A (1988) Cytokines in synovial fluid. II. The presence of tumour necrosis factor and interferon. Clin Exp Immunol 73:88–92
14. Husby G, Williams RC (1988) Synovial localization of tumor necrosis factor in patients with rheumatoid arthritis. J Autoimmunol 1:363–371
15. Loetscher HR, Pan YE, Lahm HW, Gentz R, Brockhaus M, Tabuchi H, Lesslauer W (1990) Molecular cloning and expression of the human 55 kD tumor necrosis factor receptor. Cell 61:351–357
16. Miossec P (1987) The role of interleukin 1 in the pathogenesis of rheumatoid arthritis. Clin Exp Rheumatol 5:305–308
17. Mosman T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. J Immunol Methods 65:55–60
18. Old LJ (1985) Tumor necrosis factor (TNF). Science 230:630–632
19. Olson I, Lantz M, Nilsson E, Peetre C, Thysell H, Grubb A, Adolf G (1989) Isolation and characterization of a tumor necrosis factor binding protein from urine. Eur J Haematol 42:270–275
20. Porteu F, Nathan C (1990) Shedding of tumor necrosis factor receptors by activated human neutrophils. J Exp Med 172:599–607
21. Saxne T, Palladino MA, Heinegard D, Talal N, Wollheim FA (1988) Detection of tumor necrosis factor α but not tumor necrosis factor β in rheumatoid arthritis synovial fluid and serum. Arthritis Rheum 31:1041–1045
22. Schall TJ, Lewis M, Koller KJ, Lee A, Rice GC, Wong W, Gatanaga T, Granger GA, Lentz R, Raab H, Kohr WJ, Goeddel DV (1990) Molecular cloning and expression of a receptor for human tumor necrosis factor. Cell 61:361–367
23. Seckinger P, Isaaz S, Dayer JM (1989) Purification and characterization of a specific tumor necrosis factor alpha inhibitor. J Biol Chem 264:11966–11973
24. Sherry B, Cerami A (1988) Cachectin/tumor necrosis factor exerts endocrine, paracrine and autocrine control of inflammatory responses. J Cell Biol 107:1269–1275
25. Smith CA, Davis T, Anderson D, Solam L, Beckmann MP, Jerzy R, Dower SK, Cosman D, Goodwin RG (1990) A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. Science 248:1019–1023
26. Spinas GA, Bloesch D, Kaufmann M, Keller U, Dayer J (1990) Induction of plasma inhibitors of interleukin 1 and TNFa by endotoxin administration to normal humans. J Clin Invest 990:R993–997
27. Urban JL, Shepard HM, Rothstein JL, Sugarman BJ, Schreiber H (1986) Tumor necrosis factor: a potent effector molecule for tumor cell killing by activated macrophages. Proc Natl Acad Sci USA 83:5233–5238
28. Yocum DE, Esparza L, Dubry S, Benjamin JB, Volz R, Scuderi P (1989) Characteristics of tumor necrosis factor production in rheumatoid arthritis. Cell Immunol 122:131–137

Received: August 1, 1991
Accepted: November 4, 1991

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