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Multifaceted inhibition of anti-tumour immune mechanisms by soluble tumour necrosis factor receptor type I

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SUMMARY

Soluble tumour necrosis factor receptor type I (sTNFRI) is a potent inhibitor of TNF with the potential to suppress a variety of effector mechanisms important in tumour immunity. That sTNFRI influences tumour survival *in vivo* is suggested by results from human clinical trials of Ultrapheresis®, an experimental extracorporeal treatment for cancer. While the considerable clinical benefit provided by Ultrapheresis® is correlated with the removal of plasma sTNFRI, there is no direct evidence that sTNFRI inhibits immune mechanisms which mediate tumour cell elimination. To evaluate formally the ability of sTNFRI to inhibit these mechanisms, we have engineered sTNFRI production into the TNF-sensitive murine fibrosarcoma cell line, L929. Soluble TNFRI-secreting L929 cells display increased resistance to direct lysis by TNF, and to lysis by syngeneic lymphokine-activated killer cells and cytotoxic T cells. These findings confirm the suggestion that sTNFRI inhibits immunological mechanisms important in tumour cell eradication, and further support a role for sTNFRI in tumour survival *in vivo*. In addition, these observations suggest the development of methods for more specific removal and/or inactivation of sTNFRI as promising new avenues for cancer immunotherapy.

INTRODUCTION

Ultrapheresis®[1] is a promising experimental cancer therapy that involves extracorporeal fractionation of plasma components by ultrafiltration. Ultrapheresis® selectively removes plasma components within a defined molecular size range, and it has been shown to provide significant clinical benefit to patients presenting with a variety of tumour types. Ultrapheresis® induces pronounced inflammation at tumour sites, often in less than 1 h post-initiation. This rapidity suggests a role for preformed chemical and/or cellular mediators in the elaboration of this inflammatory response, and it probably reflects the removal of naturally occurring plasma inhibitors of that response. One such inhibitor previously shown to be removed by Ultrapheresis® is soluble tumour necrosis factor receptor type I (sTNFRI).[2–4]

Soluble TNFRI[4–8] represents the extracellular domain of the membrane-associated TNF receptor type I which is shed from the cell surface by proteolytic cleavage. It retains the ability to bind TNF with high affinity and, thus, to antagonize the binding of TNF to cell surface receptors.[4–8] The manifestations of this inhibition in tumour-bearing hosts are likely to be profound, considering the critical role of TNF in a variety of anti-tumour immune responses.[9] TNF directly induces apoptosis in tumour cell targets by binding to the type I membrane-associated TNF receptor, CD120a.[10–12] Moreover, vascular endothelial cells are induced to apoptosis by TNF binding, destroying the circulatory network serving the tumour and further contributing to tumour cell death.[13–15] Critical roles for TNF in lymphokine-activated killer (LAK) cell-[16,17] and cytotoxic T-lymphocyte (CTL)-mediated[18–20] cytolysis have also been documented. Inhibition of any or all of these effector mechanisms by sTNFRI has the potential to enhance tumour survival dramatically.

Nevertheless, direct evidence that sTNFRI regulates anti-tumour immunity is not currently available. To examine formally the effects of sTNFRI on immune mechanisms important in tumour cell elimination, we have engineered the murine fibrosarcoma cell line, L929, to produce sTNFRI. Herein, we demonstrate that secretion of sTNFRI by L929 cells renders them more resistant to three immunological mechanisms with fundamental roles in anti-tumour immunity.

MATERIALS AND METHODS

Cell lines and reagents

The C3H/HeN murine fibrosarcoma cell line, L929, and the human histiocytic lymphoma cell line, U937, were obtained from the American Type Culture Collection (ATCC; Rockville, MD). Clone K is a TNF-sensitive subclone of L929 which was isolated by limiting dilution cloning in our laboratory. L929 and U937 cells were maintained in Eagle's modified essential medium (MEM) and RPMI-1640, respectively, supplemented with 10% (v/v) fetal bovine serum and penicillin/

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streptomycin, each at 100 μg/ml. All cells were cultured at 37° in a humidified atmosphere containing 6% CO_2. *Escherichia coli*-derived murine TNF-α and human sTNFRI, as well as polyclonal goat anti-human sTNFRI antibodies, were purchased from R & D Systems (Minneapolis, MN). Biotinylated-goat anti-human sTNFRI antibody was produced in our laboratory using IMMUNOPURE NHS-LC-Biotin II (Pierce, Rockford, IL) according to the manufacturer's specifications.

Mice

Eight- to ten-week-old female C3H/HeN mice were purchased from Harlan Sprague Dawley (Indianapolis, IN) and housed in a specific pathogen-free environment at the Colorado State University Laboratory Animal Resources facility. Mice were given food and water *ad libitum*, and all procedures were conducted in accordance with federal guidelines for animal experimentation.

Construction of the sTNFRI expression vector

The gene encoding a soluble form of the human type I TNF receptor was isolated using reverse transcription–polymerase chain reaction (RT-PCR) in a manner analogous to that previously described.[21,22] Total RNA was prepared from U937 cells using acidified guanidinium–phenol–chloroform[23] and mRNA was reverse transcribed using avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI) and an antisense strand primer with the following sequence: 5′ CAGGGTGGGGGTGAAGCC 3′. The resulting cDNA was amplified using oligonucleotide primers designed to amplify sequences encoding the extracellular domain of the type I TNF receptor. The sense strand primer, 5′ CATAAGCT-TGCCGCCAGCCATGGGCCTCTCCACC 3′, anneals to sequences encoding the initiator methionine and five additional residues of the leader sequence. This primer includes a consensus ribosome binding site, appropriately positioned immediately upstream of the initiator codon, and a *Hin*dIII restriction site to facilitate cloning. The antisense strand primer, 5′ AGTCCTAGGTTATCAATTCTCAATCTGGGGTAG 3′, contains an *Avr*II restriction site and two in-frame stop codons positioned to terminate translation at Asn 172 (numbering according to ref. 21), the C-terminal residue of *bona fide* sTNFRI isolated from human urine and serum.[4] Truncation of the TNFRI gene at this position deletes the transmembrane domain and results in the secretion of the soluble form of the TNFRI. PCR was conducted for 40 cycles with the following profile (1 min each): 95° denaturation, 60° annealing and 72° extension. The resulting 637-base pair PCR fragment was gel-purified and cloned into the pCRII TA cloning vector (Invitrogen, Carlsbad, CA). The resulting pool of recombinant pCRII plasmids was digested sequentially with *Hin*dIII and *Not*I to liberate a 727-base pair fragment which was ligated to *Hin*dIII/*Not*I-digested pcDNA3, a eukaryotic expression plasmid (Invitrogen, Carlsbad, CA). The fidelity of the recombinant plasmids was verified by restriction endonuclease digestion and PCR amplification.

Generation of sTNFRI transfectants

The sTNFRI expression plasmid was introduced into cultures of L929 clone K cells using lipofectamine (Gibco-BRL, Gaithersburg, MD). Cells were seeded at 7×10^5 cells per 60-mm tissue culture dish (approximately 80% confluence) and allowed to adhere overnight. Five micrograms of the DNA, linearized by cleavage of the unique *Sca*I site in the *bla* gene of the vector, was added to the manufacturer's suggested volume of liposomes and incubated with the cells for 4 h. A cloned transfectant cell line, clone 39, was isolated by limiting dilution in the presence of Geneticin (0·5 mg active drug/ml) (Gibco-BRL, Gaithersburg, MD).

sTNFRI ELISA

The production of sTNFRI by the transfectants was evaluated by capture enzyme-linked immunosorbent assay (ELISA). Wells were coated with goat anti-human sTNFRI antibody and blocked with 2% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Duplicate wells were incubated with serial dilutions of recombinant sTNFRI standard or with media conditioned by clone K transfectants or by control cultures. Bound sTNFRI was detected by the sequential addition of biotin-labelled goat anti-sTNFRI antibody and streptavidin–alkaline phosphatase. Conversion of the colorimetric substrate, ρ-nitrophenylphosphate, was quantified at 405 nm.

TNF bioassay

The TNF-sensitivities of clone K cells and of clone K transfectants were determined using *in vitro* bioassays as described previously.[24] Individual cell lines were seeded in 96-well plates at $2{\cdot}25 \times 10^4$ cells/well and allowed to adhere overnight. Actinomycin D (1 μg/ml) (Sigma, St Louis, MO) and varying concentrations of recombinant murine TNF-α were diluted in complete growth medium and added to triplicate wells. Control wells received complete growth medium containing actinomycin D alone. After 24 hr, media were aspirated and viable cells were stained with 1% aqueous crystal violet for 5 min, washed extensively with water, and cell-associated dye was solubilized in 33% glacial acetic acid. Optical density at 550 nm (OD_{550}) was determined to quantify relative cell survival, and percentage cytotoxicity was calculated for each concentration of TNF according to the formula:

$$\% \text{ cytotoxicity} = \frac{(\text{mean absorbance of cells} + \text{TNF})}{(\text{mean absorbance of cells} - \text{TNF})} \times 100\%$$

LAK cell assays

The cytotoxic activity of murine LAK cells on L929 targets was determined using *in vitro* assays. Splenocytes from C3H/HeN mice were cultured for 6 days in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics (DMEM complete medium), supplemented with 25% conditioned LAK supernatant. This conditioned supernatant was derived from *ex vivo* stimulation of human peripheral blood mononuclear cells (PBMC) with 1500 U/ml of recombinant interleukin-2 (IL-2). This supernatant contains considerable residual IL-2, as well as numerous other cytokines secreted by the PBMC[25], and it is superior to IL-2 alone for LAK cell generation. LAK effectors were co-cultured, in quadruplicate, with clone K and clone 39 targets, previously labelled with TRAN^{35}S-LABEL (ICN, Irvine, CA) as described,[26] at various effector : target (E : T) ratios for 5 hr at 37°. Lysis was correlated with release of radiolabel into the culture media as determined by liquid scintillation.

Percentage specific lysis was calculated according to the formula:

$$\% \text{ specific lysis} = \frac{\begin{array}{c}(\text{c.p.m. test sample}) - \\ (\text{c.p.m. spontaneous release})\end{array}}{\begin{array}{c}(\text{c.p.m. 2\% Triton sample}) - \\ (\text{c.p.m. spontaneous release})\end{array}} \times 100\%$$

CTL assays

The cytotoxic activity of L929-reactive CTL was determined using *in vitro* assays. Syngeneic C3H/HeN mice, previously primed intraperitoneally with 1×10^7 clone K cells, were sacrificed and their spleens were harvested. Red blood cells were lysed with 0·83% ammonium chloride, and the remaining cells were washed extensively with DMEM complete medium. Splenocytes were restimulated *in vitro* through co-cultivation with clone K monolayers in 24-well tissue culture plates (3×10^6 splenocytes and $1{\cdot}5 \times 10^5$ clone K cells per well) for 6 days. The resulting CTL effectors were added, in quadruplicate, to radiolabelled clone K and clone 39 targets, and % specific lysis was determined as described above.

RESULTS AND DISCUSSION

The L929 transfectant, clone 39, produces sTNFRI

Secretion of sTNFRI by clone 39, an L929 clone K-subclone derived by transfection with the sTNFRI expression plasmid, was quantified by ELISA (data not shown). Production of sTNFRI by clone 39 was estimated at 20 ng/ml, whereas untransfected clone K and the control transfectant, clone P, produced no detectable sTNFRI. Quantification of sTNFRI secreted by clone 39 was based on an *E. coli*-derived recombinant sTNFRI standard and an antibody preparation reactive with the *E. coli*-derived protein. Thus, this assay may under estimate sTNFRI secretion by clone 39 cells, since sTNFRI produced by eukaryotic cells is glycosylated,[4,21] and it is unlikely to be bound by the capture and detection antibodies with the same stoichiometry as the *E. coli*-derived standard. Nevertheless, stable transformation of L929 clone K cells with the expression construct directs the synthesis and secretion of sTNFRI in the nanomolar range, a level which is comparable to that produced by naturally occurring tumours,[27–29] and similar to levels detected in the circulation of tumour-bearing hosts.[27,30–33]

sTNFRI inhibits direct lysis by TNF

Unmodified clone K cells and sTNFRI-secreting clone 39 cells were cultured in the presence of varying concentrations of recombinant murine TNF-α, and percentage cytotoxicity was calculated (Fig. 1). Targets secreting sTNFRI were found to be 10-fold more resistant to TNF-mediated lysis than unmodified targets; the ED_{50} of clone 39 cells was 60 pg/ml while that of clone K cells was 6 pg/ml. Additional bioassays were conducted to ensure that the enhanced TNF-resistance of clone 39 cells resulted from their secretion of sTNFRI, and not from the isolation of a clone K subclone which is inherently TNF-resistant. In these assays, the ability of clone 39 culture supernatant to transfer TNF-resistance to TNF-sensitive clone K targets was evaluated. Specific lysis of clone K cells

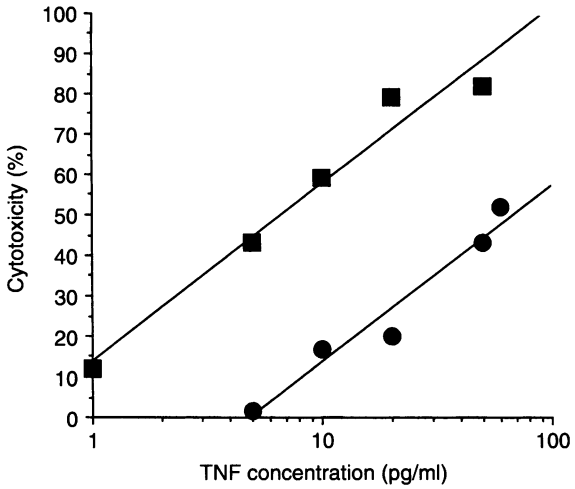

Figure 1. Soluble TNFRI inhibits direct lysis of target cells by TNF. Clone K (■) and clone 39 (●) cells were cultured with the indicated concentrations of murine TNF-α and cytotoxicity was determined as described in the Materials and Methods. Standard errors of the mean absorbance for all samples were less than 18%.

by 10 pg/ml TNF was 43% when assayed in clone K-conditioned medium, however, specific lysis was reduced to just 2% when assayed in medium conditioned by clone 39 cells (data not shown). Further, the TNF-resistance transferable by clone 39 supernatant was completely abrogated by prior incubation with Sepharose-immobilized goat anti-human sTNFRI antibodies (data not shown). These findings confirm that sTNFRI secretion by clone 39 cells confers the observed TNF-resistance, and they are consistent with previous reports which demonstrate high-affinity binding of human sTNFRI to murine TNF.[34]

sTNFRI inhibits LAK cell-mediated lysis

The effect of sTNFRI on LAK cell-mediated lysis was evaluated using 5-h *in vitro* assays (Fig. 2). Soluble TNFRI-secreting clone 39 targets were virtually resistant to LAK-cell mediated cytolysis. In contrast, clone K targets, which do not secrete detectable sTNFRI, were effectively lysed by LAK cells, with specific lysis of up to 36%. Thus, sTNFRI production by target cells inhibits LAK cell-mediated lysis, an observation consistent with the demonstrated role for TNF in LAK cell cytolysis.[16,17] While the present data address only the involvement of TNF in cytolysis by LAK cells, previous reports[35,36] indicate that TNF promotes LAK cell differentiation and proliferation as well. Thus, sTNFRI production by target cells *in vivo* may inhibit not only cytolysis, but also *in situ* activation of LAK cells, further diminishing their effectiveness in tumour cell elimination.

sTNFRI inhibits CTL-mediated lysis

The effect of sTNFRI on CTL-mediated lysis was also examined (Fig. 3). Clone 39 targets were lysed by L929-reactive CTL less effectively than clone K targets. At an E:T ratio of 25:1, specific lysis of clone 39 targets was 24% compared to 54% for clone K targets. At an E:T ratio of 1:1, clone 39 targets were unlysed, while specific lysis of clone K targets

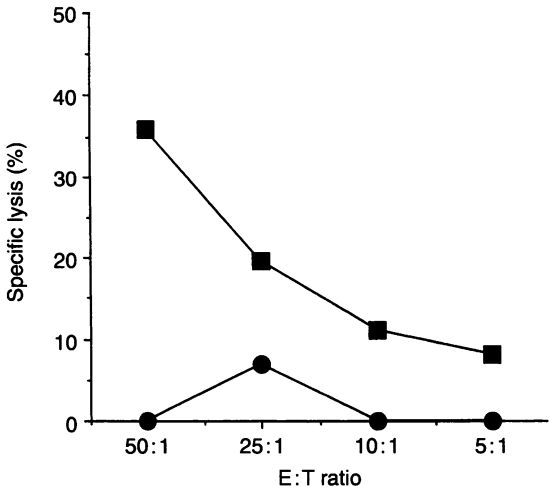

Figure 2. Soluble TNFRI inhibits target cell lysis by LAK cells. Clone K (■) and clone 39 (●) cells were co-cultured with murine LAK cells at the indicated E:T ratios and specific lysis was determined as described in the Materials and Methods. Standard errors of the mean c.p.m. for all samples were less than 10%.

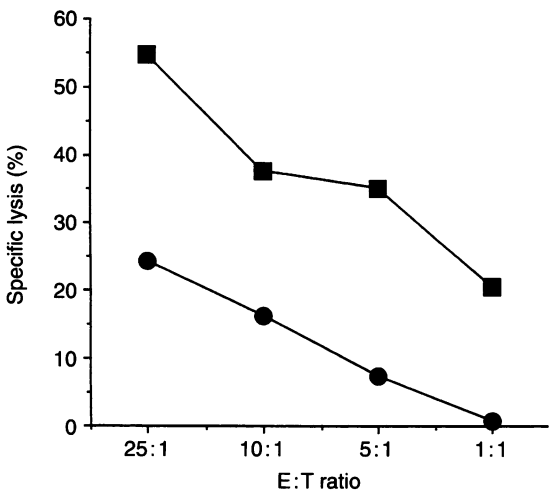

Figure 3. Soluble TNFRI inhibits target cell lysis by CTL. Clone K (■) and clone 39 (●) cells were co-cultured with L929-reactive CTL at the indicated E:T ratios and specific lysis was determined as described in the Materials and Methods. Standard errors of the mean c.p.m. for all samples were less than 10%.

was 20%. Thus, CTL-mediated lysis is reduced as a consequence of sTNFRI production by target cells, in agreement with the documented role for TNF in CTL-mediated cytolysis.[18–20] Cytolysis was not inhibited completely, however, since lytic mechanisms other than TNF contribute substantively to target cell elimination by CTL.[37] Nevertheless, because the differentiation and proliferation of CTL, like that of LAK cells, is enhanced by TNF,[38] sTNFRI production by tumour cells *in vivo* may inhibit CTL activation, including even those cells which employ lytic mechanisms that are independent of TNF.

The involvement of TNF in natural killer (NK) cell- and CTL-mediated lysis has been somewhat controversial. Certain investigators have demonstrated a role for TNF in short-term (e.g. 4–6 hr) *in vitro* assays,[18–20] while others report that the effects of TNF are observable only in 18-hr assays.[16,17,19] Typically, however, these data derive from studies employing a variety of different target cells and, thus, they may reflect differential target cell sensitivity to TNF. The effective LAK cell- and CTL-mediated lysis readily demonstrated in the present report may result from the exquisite TNF-sensitivity of L929 clone K. Moreover, the prior studies have used TNF-neutralizing antibodies to assess the involvement of TNF in cellular cytolysis. In contrast, the present studies have employed sTNFRI, a natural regulator of TNF activity and, thus, they are expected to be more physiologically relevant. Furthermore, the present studies demonstrate the effects of sTNFRI directly synthesized by LAK and CTL targets, circumstances that are expected to reflect, more faithfully than anti-TNF antibodies, the effects of a TNF antagonist on target cell cytolysis. In assays *in vitro*, neutralizing antibodies are present at concentrations that are static, if not declining, with increasing culture time, conditions which cannot estimate the influence of a TNF antagonist continuously replenished at the target cell surface. This distinction is particularly important since sTNFRI has been shown either to stabilize or to antagonize TNF activity, depending upon the biodistribution and rate of decay of TNF, and on the rates of clearance of TNF and sTNFRI.[39] Thus, the present studies reasonably duplicate, *in vitro*, the effects of sTNFRI on target cell lysis, and formally reveal mechanistic bases by which sTNFRI may prevent tumour rejection *in vivo*. The influence of sTNFRI on tumour growth *in vivo* now can be evaluated, in a physiologically relevant manner, by using this sTNFRI-secreting cell line in mouse transplantation models.

Numerous observations suggest that sTNFRI aids tumour survival *in vivo*. Nanomolar concentrations of sTNFRI are synthesized by a variety of tumour types.[27–29] In addition, circulating sTNFRI levels often are elevated significantly in cancer patients,[27,30–33] decline during remission and increase during advanced stages of tumour development[27,30–32] and, when present at high levels, correlate with poorer treatment outcomes.[30] As stated above, data from Ultrapheresis® trials also provide correlative evidence that sTNFRI is operative *in vivo*.[1] It is noteworthy that the levels of sTNFRI circulating in tumour-bearing hosts are increased in response to the infusion of supraphysiological levels of TNF,[40] and of IL-2[41–43] which indirectly stimulates TNF production by NK/LAK cells.[44] Increased production of sTNFRI is considered to be a mechanism for restoring immunological homeostasis,[45] further supporting a role for sTNFRI as a natural regulator of TNF. The production of sTNFRI in response to infusional cytokines may contribute to the limited clinical success reported for these immunotherapeutic strategies.[46,47] These observations, in conjunction with data in the present report, suggest a radically different approach to cancer immunotherapy – one which shifts the cytokine balance to favour tumour cell destruction through the depletion of naturally occurring sTNFRI, as opposed to TNF augmentation.

The therapeutic utility of manipulating sTNFRI levels *in vivo* has been demonstrated, though these prior studies have focused primarily on the inhibition of immune responses. Increases in the levels of circulating sTNFRI, achieved by infusion of recombinant proteins, dramatically reduce the systemic effects of endotoxaemia/sepsis.[48–51] Soluble TNFRI-

induced immune suppression also lessens the clinical manifestations associated with a variety of autoimmune diseases,[52–54] and it has been shown to prolong allograft survival.[55] These findings confirm that sTNFRI effectively inhibits immune responses *in vivo*, and demonstrate that its modulation is a legitimate therapeutic avenue. We, therefore, propose the development of methods and/or reagents capable of specifically removing sTNFRI, or antagonizing its effects *in situ*, as unconventional, yet promising, strategies for cancer immunotherapy.

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REFERENCES

1. Lentz M.R. (1989) Continuous whole blood UltraPheresis procedure in patients with metastatic cancer. *J Biol Response Modif* **8,** 511.
2. Gatanaga T., Lentz R., Masunaka I. *et al.* (1990) Identification of TNF-LT blocking factor(s) in the serum and ultrafiltrates of human cancer patients. *Lymphokine Res* **9,** 225.
3. Gatanaga T., Hwang C.D., Kohr W. *et al.* (1990) Purification and characterization of an inhibitor (soluble tumour necrosis factor receptor) for tumour necrosis factor and lymphotoxin obtained from the serum ultrafiltrates of human cancer patients. *Proc Natl Acad Sci USA* **87,** 8781.
4. Schall T.J., Lewis M., Koller K.J. *et al.* (1990) Molecular cloning and expression of a receptor for human tumour necrosis factor. *Cell* **61,** 361.
5. Olsson I., Lantz M., Nilsson E. *et al.* (1989) Isolation and characterization of a tumour necrosis factor binding protein from urine. *Eur J Haematol* **42,** 270.
6. Seckinger P., Isaaz S. & Dayer J.-M. (1989) Purification and biologic characterization of a specific tumour necrosis factor-α inhibitor. *J Biol Chem* **264,** 11 966.
7. Engelmann H., Aderka D., Rubinstein M., Rotman D. & Wallach D. (1989) A tumour necrosis factor-binding protein purified to homogeneity from human urine protects cells from tumour necrosis factor toxicity. *J Biol Chem* **264,** 11 974.
8. Engelmann H., Novick D. & Wallach D. (1990) Two tumour-necrosis factor-binding proteins purified from human urine. *J Biol Chem* **265,** 1531.
9. Beutler B. (1990) The complex regulation and biology of TNF (cachectin). *Crit Rev Oncogen* **2,** 9.
10. Tartaglia L.A., Weber R.F., Figari I.S., Reynolds C., Palladino M.A. Jr. & Goeddel D.V. (1991) The two different receptors for tumour necrosis factor mediate distinct cellular responses. *Proc Natl Acad Sci USA* **88,** 9292.
11. Tartaglia L.A., Rothe M., Hu Y.-F. & Goeddel D.V. (1993) Tumour necrosis factor's cytotoxic activity is signaled by the p55 TNF receptor. *Cell* **73,** 213.
12. Helson L., Green S., Carswell E. & Old L.J. (1975) Effect of tumour necrosis factor on cultured human melanoma cells. *Nature* **258,** 731.
13. North R.J. & Havell E.A. (1988) The antitumor function of tumour necrosis factor (TNF): analysis of the role of endogenous TNF in endotoxin-induced hemorrhagic necrosis and regression of an established sarcoma. *J Exp Med* **167,** 1086.
14. Robaye B., Mosselmans R., Fiers W., Dumont J.E. & Galand P. (1991) Tumour necrosis factor induces apoptosis (programmed cell death) in normal endothelial cells *in vitro*. *Am J Pathol* **138,** 447.
15. Sato N., Goto T., Haranaka K. *et al.* (1986) Actions of tumour necrosis factor on cultured vascular endothelial cells: morphologic modulation, growth inhibition, and cytotoxicity. *J Natl Cancer Inst* **76,** 1113.
16. Ortaldo J.R., Mason L.H., Mathieson B.J., Liang S.M., Flick D.A. & Herberman R.B. (1986) Mediation of mouse natural cytotoxic activity by tumour necrosis factor. *Nature* **321,** 700.
17. Wright S.C. & Bonavida B. (1987) Studies on the mechanism of natural killer cell-mediated cytotoxicity. *J Immunol* **138,** 1791.
18. Tite J.P. (1990) Evidence of a role for TNF-α in cytolysis by $CD4^+$, class II MHC-restricted cytotoxic T cells. *Immunology* **71,** 208.
19. Ratner A. & Clark W.R. (1993) Role of TNF-α in $CD8^+$ cytotoxic T lymphocyte-mediated lysis. *J Immunol* **150,** 4303.
20. Monastra G., Cabrelle A., Zambon A. *et al.* (1996) Membrane form of TNF-α induces both cell lysis and apoptosis in susceptible target cells. *Cell Immunol* **171,** 102.
21. Himmler A., Maurer-Fogy I., Kronke M. *et al.* (1990) Molecular cloning and expression of human and rat tumour necrosis factor receptor chain (p60) and its soluble derivative, tumour necrosis factor-binding protein. *DNA and Cell Biol* **9,** 705.
22. Gray P.W., Barrett K., Chantry D., Turner M. & Feldmann M. (1990) Cloning of human tumour necrosis factor (TNF) receptor cDNA and expression of recombinant soluble TNF-binding protein. *Proc Natl Acad Sci USA* **87,** 7380.
23. Chomczynski P. & Sacchi N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* **162,** 156.
24. Matthews N. & Neale M.L. (1987) Cytotoxicity assays for tumour necrosis factor and lymphotoxin. In *Lymphokines and Interferons, a Practical Approach* (eds. M. J. Clemens, A. G. Morris & A. J. H. Gearing), p. 221, IRL Press, Oxford.
25. Branda R.F., O'Neill J.P., Jacobson-Kram D. & Albertini R.J. (1992) Factors influencing mutation at the *hprt* locus in T-lymphocytes: studies in normal women and women with benign and malignant breast masses. *Environ Mol Mutagen* **19,** 274.
26. Stedman K.E. & Campbell P.A. (1989) An [^{35}S] methionine cytotoxicity assay requiring small numbers of effector and target cells. *J Immunol Meth*, **119,** 291.
27. Aderka D., Englemann H., Hornik V. *et al.* (1991) Increased serum levels of soluble receptors for tumour necrosis factor in cancer patients. *Cancer Res* **51,** 5602.
28. Adolf G.R. & Apfler I. (1991) A monoclonal antibody-based enzyme immunoassay for quantitation of human tumour necrosis factor binding protein I, a soluble fragment of the 60 kDa TNF receptor, in biological fluids. *J Immunol Meth* **143,** 127.
29. Neuner P., Klosner G., Pourmojib M., Trautinger F. & Knobler R. (1994) Selective release of tumour necrosis factor binding protein II by malignant human epidermal cells reveals protection from tumour necrosis factor-α mediated cytotoxicity. *Cancer Res* **54,** 6001.26.
30. Kalmanti M., Karamolengou K., Dimitriou H. & Tosca A. (1993) Serum levels of tumour necrosis factor and soluble interleukin-2 receptor as markers of disease activity and prognosis in childhood leukemia and lymphoma. *Int J Hematol* **57,** 147.
31. Elsasser-Beile U., Gallati H., Weber W., Wild E.D., Schulte Monting J. & von Kleist A. (1994) Increased plasma concentrations for type I and type II tumour necrosis factor receptors and IL-2 receptors in cancer patients. *Tumour Biol* **15,** 17.
32. Gadducci A., Ferdeghini M., Fanucchi A. *et al.* (1996) Serum levels of soluble receptors for tumour necrosis factor (p55 and p75 sTNFr) in endometrial cancer. *Anticancer Res* **16,** 3125.

33. Digel W., Porzsolt F., Schmid M., Herrmann F., Lesslauer W. & Brockhaus M. (1992) High levels of circulating soluble receptors for tumour necrosis factor in hairy cell leukemia and type B chronic lymphocytic leukemia. *J Clin Invest* **89,** 1690.
34. Smith R.A., Kirstein M., Fiers W. & Baglioni C. (1986) Species specificity of human and murine tumor necrosis factor. *J Biol Chem* **261,** 14871.
35. Ostensen M.E., Thiele D.L. & Lipsky P.E. (1987) Tumour necrosis factor-α enhances cytolytic activity of human natural killer cells. *J Immunol* **138,** 4185.
36. Naume B., Shalaby R., Lesslauer W. & Espevik T. (1991) Involvement of the 55- and 75-kDa tumour necrosis factor receptors in the generation of lymphokine-activated killer cell activity and proliferation of natural killer cells. *J Immunol* **146,** 3045.
37. Kagi D., Vignaux F., Ledermann B. *et al.* (1994) Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science* **265,** 528.
38. Ranges G.E., Figari I.S., Espevik T. & Palladino M.A. Jr. (1987) Inhibition of cytotoxic T cell development by transforming growth factor-β and reversal by recombinant tumour necrosis factor-α. *J Exp Med* **166,** 991.
39. Aderka D., Engelmann H., Maor Y., Brakebusch C. & Wallach D. (1992) Stabilization of the bioactivity of tumour necrosis factor by its soluble receptors. *J Exp Med* **175,** 323.
40. Lantz M., Malik S., Slevin M.L. & Olsson I. (1990) Infusion of tumour necrosis factor (TNF) causes an increase in circulating TNF-binding protein in humans. *Cytokine* **2,** 402.
41. Belldegrun A., Pierce W., Sayah D. *et al.* (1993) Soluble tumour necrosis factor receptor expression in patients with metastatic renal cell carcinoma treated with interleukin-2-based immunotherapy. *J Immunother* **13,** 175.
42. Landmann R., Keilholz U., Scheibenbogen C. *et al.* (1994) Relationship between soluble tumour necrosis factor (TNF) receptors and TNF-α during immunotherapy with interleukin-2 and/or interferon-α. *Cancer Immunol Immunother* **38,** 113.
43. Miles D.W., Aderka D., Englemann H., Wallach D. & Balkwill F.R. (1992) Induction of soluble tumour necrosis factor receptors during treatment with interleukin-2. *Br J Cancer* **66,** 1195.
44. Lazenby A.W., Roth J.A., Owen-Schaub L.B. & Grimm E.A. (1992) IL-1 synergy with IL-2 in the generation of lymphokine activated killer cells is mediated by TNF-α and β (lymphotoxin). *Cytokine* **4,** 479.
45. Van Zee K.J., Kohno T., Fischer E., Rock C.S., Moldawer L.L. & Lowry S.F. (1992) Tumour necrosis factor soluble receptors circulate during experimental and clinical inflammation and can protect against excessive tumour necrosis factor-α *in vitro* and *in vivo. Proc Natl Acad Sci USA*, **89,** 4845.
46. Sidhu R.S. & Bollon A.P. (1993) Tumour necrosis factor activities and cancer therapy – a perspective. *Pharmacol Ther* **57,** 79.
47. Maas R.A., Dullens H.F. & Den Otter W. (1993) Interleukin-2 in cancer treatment: disappointing or (still) promising? A review. *Cancer Immunol Immunother* **36,** 141.
48. Lesslauer W., Tabuci H., Gentz R. *et al.* (1991) Recombinant soluble tumour necrosis factor receptor proteins protect mice from lipopolysaccharide-induced lethality. *Eur J Immunol* **21,** 2883.
49. Ashkenazi A., Marsters S.A., Capon D.J. *et al.* (1991) Protection against endotoxic shock by a tumour necrosis factor receptor immunoadhesion. *Proc Natl Acad Sci USA* **88,** 10535.
50. Bertini R., Delgado R., Faggioni R., Gascon M.P., Ythier A. & Ghezzi P. (1993) Urinary TNF-binding protein (TNF soluble receptor) protects mice against the lethal effect of TNF and endotoxic shock. *Eur Cyt Network* **4,** 39.
51. Evans T.J., Moyes D., Carpenter A. *et al.* (1994) Protective effect of 55- but not 75-kD soluble tumour necrosis factor receptor-immunoglobulin G fusion proteins in an animal model of gram-negative sepsis. *J Exp Med* **180,** 2173.
52. Selmaj K., Papierz W., Glabinski A. & Kono T. (1995) Prevention of chronic relapsing experimental autoimmune encephalomyelitis by soluble tumor necrosis factor receptor I. *J Neuroimmunol* **56,** 135.
53. Mori L., Iselin S., De Libero G. & Lesslauer W. (1996) Attenuation of collagen-induced arthritis in 55-kDa TNF receptor type I (TNFRI)-IgG1-treated and TNFRI-deficient mice. *J Immunol* **157,** 3178.
54. Hunger R.E., Carnaud C., Garcia I., Vassalli P. & Mueller C. (1997) Prevention of autoimmune diabetes mellitus in NOD mice by transgenic expression of soluble tumour necrosis factor receptor p55. *Eur J Immunol* **27,** 255.
55. Eason J.D., Wee S.L., Kawai T. *et al.* (1995) Recombinant human dimeric tumour necrosis factor receptor (TNFR:Fc) as an immunosuppressive agent in renal allograft recipients. *Transplan Proc* **27,** 554.