DISSERTATION

THE ROLE OF SOLUBLE TUMOR NECROSIS FACTOR RECEPTOR TYPE I IN TUMOR SURVIVAL

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY CHERYL L. SELINSKY ENTITLED "THE ROLE OF SOLUBLE TUMOR NECROSIS FACTOR RECEPTOR TYPE I IN TUMOR SURVIVAL" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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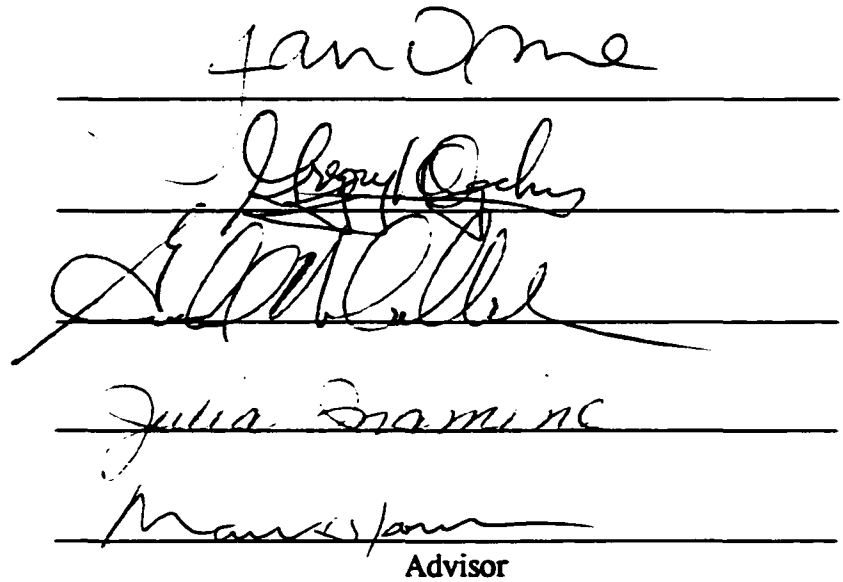

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ABSTRACT OF DISSERTATION

THE ROLE OF SOLUBLE TUMOR NECROSIS FACTOR RECEPTOR TYPE I IN TUMOR SURVIVAL

Direct lysis of tumor cells by tumor necrosis factor (TNF) is an important mechanism of anti-tumor immunity. Nevertheless, tumors often persist in mammalian hosts despite significantly elevated serum levels of TNF. One possible explanation for this paradox is that corresponding increases in the levels of soluble tumor necrosis factor receptor type I (sTNFRI), a potent inhibitor of TNF, interfere with TNF-mediated tumor elimination. To formally evaluate the ability of sTNFRI to inhibit anti-tumor mechanisms, we have engineered sTNFRI production into the TNF-sensitive murine cell line, L929. In *in vitro* analyses, sTNFRI-secreting L929 cells displayed increased resistance to direct lysis by TNF, and to LAK cell- and CTL-mediated cellular cytolysis when compared to the parental cell line which does not secrete sTNFRI. These findings suggest that , *in vivo*, sTNFRI may contribute to tumor development and persistence. A formal role for sTNFRI in tumor survival was demonstrated by comparing the growth of sTNFRI-secreting L929 cells with that of the unmodified parental fibrosarcoma cell line in an *in vivo* mouse transplantation model. L929 cells are non-tumorigenic in syngeneic recipients, yet they produce fibrosarcomas in sub-lethally irradiated animals, indicating that tumor growth in unirradiated animals is prevented by immunological mechanisms. The secretion of sTNFRI by L929 cells, however, markedly enhanced their tumorigenicity in irradiated, as well as

immunocompetent, recipients. Immunization with sTNFRI to induce antibodies which neutralize the binding of sTNFRI to TNF abrogated this tumorigenicity. Collectively, these data formally demonstrate that sTNFRI directly influences tumor growth and persistence *in vivo*, and they justify the development of methods to selectively inactivate sTNFRI in the circulation of tumor-bearing hosts. Toward this end, we have produced monoclonal antibodies that neutralize the binding of sTNFRI to TNF and have tested their efficacy in our mouse transplantation model.

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Dedication

To the memory of Paul and Barbara Selinsky. Their hard work and sacrifice have been the secret of my success.

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CHAPTER 1
INTRODUCTION

As the death toll attributed to infectious diseases has declined in developing countries, cancer has become the second leading cause of death, surpassed only by heart disease. Current estimates predict that in the United States one in three people will develop cancer, and one in five will die from cancer.[1] These grim predictions underscore the limitations of current cancer treatments including surgery, chemotherapy, and radiotherapy. First, due to the inaccessibility of primary tumors, and the inability to accurately detect and resect secondary metastases, surgery is not a viable therapeutic strategy for all tumor types. Second, non-specific targeting of dividing cells by chemotherapy and radiotherapy render these strategies equally toxic to malignant and normal cells. The inherent lack of specificity of first generation cancer therapies has prompted investigators to seek out cancer treatments which specifically target malignant cells without deleteriously affecting normal cells. In so doing, a new generation of cancer treatments has been developed which utilizes the specificity of the host's immune system.

Immunologically, cancer may be viewed as altered self-cells that deviate in normal growth-regulating mechanisms. In normal tissues, homeostasis is maintained by regulatory mechanisms of cellular proliferation and cellular death. If either is in imbalance, malignant transformation may ensue. Although the malignant transformation of cells is a multi-step process, the events leading to the conversion of a normal cell to a cancerous cell may be simplified into two phases. First, chemical carcinogens such as DNA-

alkylating agents and/or physical agents such as ultraviolet or ionizing radiation, initiate changes in the cell's genome. This first phase of initiation, in itself, does not result in the transformation of the cell to a malignant phenotype. During the second phase, however, these genomic changes are amplified by the aberrant expression of growth factors, cellular oncogenes, tumor suppressor genes, and/or apoptotic suppressor genes. The net result is a stimulation of cell division which transforms a normally regulated cell to a disregulated cancer cell.

The Immune Surveillance of Cancer

If, indeed, cancer cells represent altered-self cells, then an obvious question is why is there not a vigorous immune response to them? In the 1900s, Paul Ehrlich suggested that there is an immune response to cancer cells. He proposed the immune surveillance theory which postulated that cancer cells arise, but the host recognizes them as foreign and they are eliminated by the immune system. Some 50 years later, Lewis Thomas supported this theory and further proposed that the cell-mediated branch of the immune system actually evolved for the purpose of patrolling the body to eliminate cancers. According to these theories, tumors arise only when cancer cells are able to escape immune surveillance or when the immune system is impaired.

Among the first observations which seemed to support the immune surveillance theory was the increased incidence of cancer in transplantation patients. The correlation between the use of immunosuppressive drugs and the increased incidence of cancers in these individuals confirmed a role for the immune system in the elimination of tumors.

Further findings, however, were difficult to reconcile within the context of Ehrlich's theory. While patients on immunosuppressive drugs do exhibit an increased incidence of immune system cancers, other common cancers (e.g. lung, breast, and colon) are not increased in these individuals, as would be predicted by the immune surveillance theory. Likewise, the fact that the cancer incidence in the immune-deficient "nude" strain is comparable to that of immunocompetent mice suggests that impairment of the immune system does not necessarily predispose hosts to cancer. Although nude mice lack a functional thymus, and consequently lack mature, functional T cells, they are no more prone to tumors than their fully immunocompetent cohorts.[2]

The inconsistencies of the immune surveillance theory may be attributed, in part, to its presumption that normal cells and tumor cells exhibit qualitative differences. Actually, many types of tumors do not express tumor-specific antigens which sufficiently distinguish them from normal cells such that they induce an immune response capable of eliminating the tumor. Such antigens, if they exist, represent altered cellular proteins which do not occur on normal cells in the body (i.e. tumor-specific antigens) or proteins that are expressed on normal cells during fetal development when the immune system is incapable of responding to them, but are expressed at much higher levels on tumor cells (i.e. tumor-associated antigens). It is these quantitative differences in antigen production which may dictate a tumor's immunogenicity and the extent to which the host immune system recognizes and destroys some tumors.

The Discovery of TNF

Although cancer cells are notoriously poor immunogens, circumstantial evidence that the immune system plays a role in limiting their progression in cancer patients derives from the observations that many tumor cells down regulate their expression of major histocompatibility complex (MHC) class I[3-5] and secrete high levels of immunosuppressive substances such as transforming growth factor-beta (TGF-β).[6] The inference is that tumor cells must have developed a means of evading or escaping an existing, and apparently effective, host immune response. It follows, then, that in the absence of such evasive maneuvers, these tumors should be destroyed by an intact immune system. Indeed, anecdotal reports of the spontaneous regression of tumors persist in the clinical literature.[7] An understanding of the immune mechanisms responsible for this spontaneous regression may allow investigators to capitalize on them therapeutically.

For almost a century, tumor immunologists have searched for the immune-mediators responsible for tumor regression. The "catch" is that if the immune-mediators are effective, tumors disappear or are never manifested clinically; therefore, patients with strong anti-tumor immunity escape analysis. However, in the late 1800s, one cohort of cancer patients undergoing tumor regression were analyzed and found to have concurrent bacterial infections. In hopes of duplicating the tumor regression observed in these patients, clinicians induced infections in patients with advanced malignant disease using bacterial extracts, early on with Coley's concoction of *Streptococcus pyogenes* and *Serratia marcescens* (Coley's toxins)[8], and later with admixtures of irradiated tumor cells

with adjuvants including *Corynebacterium parvum*[9] and *Mycobacterium bovis* Bacillus-Calmette Guerin (BCG).[9,10] Extensive animal studies to test the clinical efficacy of BCG and *C. parvum* revealed that adjuvant-injected mice did display heightened resistance to challenge with transplantable tumors, as well as more rapid rejection of allogeneic skin grafts and more resistance to bacterial and viral infections.[11] It was generally concluded that these adjuvants were acting to non-specifically enhance immune responses, an activity thought to be perfectly suited for the purpose of cancer therapy. Unfortunately, clinical trials of BCG and *C. parvum* in the treatment of cancer patients were generally disappointing. Nevertheless, tumor immunologists continued to test BCG's immunotherapeutic activities in mice, but now in conjunction with other bacterial products. Finally, in one group of BCG-treated mice they observed one of the most dramatic and reproducible phenomena in tumor biology: the hemorrhagic necrosis of tumors following the injection of filtrates from Gram-negative bacteria.

Shear[12] identified the active component in these filtrates as a lipopolysaccharide (LPS), also known as endotoxin, a bacterial pyrogen and major cell-wall component of Gram-negative bacteria. Subsequent experiments documented that the serum of endotoxin-treated animals could promote the hemorrhagic necrosis of established tumors.[13,14] Interestingly, LPS was implicated also as the agent responsible for cachexia, the severe wasting syndrome observed in patients with bacterial sepsis[15] and in animals with chronic parasitic diseases.[16] Thus, with the discovery of endotoxin, a single agent was implicated as the mediator of processes associated with cancer and bacterial pathogenesis that, ostensibly, seemed unrelated. Because the disturbances elicited by

microbial pathogens are generally acute while those of cancer are more chronic and progressive, investigators did not suspect that the two were connected through a common immune-mediated event. Yet, for a long time it was assumed that invasive entities, whether microbial agents or neoplastic cells, exert their pathological effects in the form of tissue injury. Therefore, through a mass effect or through the production of toxins, tumors and bacteria were thought to elicit the same types of metabolic disturbances manifested in the two very disparate activities of LPS (necrosis and cachexia). It wasn't until Carswell *et al.*[17] described the existence of a peptide hormone, tumor necrosis factor (TNF), present in the serum of BCG-injected mice treated with endotoxin that investigators appreciated that the effects of LPS on tumors were indirect. The serum of BCG/LPS-injected mice, but not the serum from mice injected with either agent alone, caused the hemorrhagic necrosis of LPS-sensitive tumors.[17] It became clear that two consecutive events occur to produce the necrotizing activities observed in these mice: (1) sensitization of the reticuloendothelial system; and (2) "elicitation" of the cytotoxic factor. Many agents including BCG and *C. parvum* are capable of sensitizing TNF-producing cells, but LPS was found to be unique in its elicitation of TNF production.[18]

Although the cellular origin of TNF was uncertain, the observation that macrophages when nonspecifically activated by agents such as BCG or endotoxin become hyperplastic and selectively toxic to malignant cells[19-22], implicated them as the major source of TNF. Multiple investigators demonstrated that mouse macrophages produced a factor that is cytotoxic to L cells (the standard TNF assay cell), and that the level of this factor in the serum of mice is increased by LPS administration.[23-25] Investigators who

observed the cytotoxicity of transformed cell lines *in vitro* and the hemorrhagic necrosis of established subcutaneous tumors *in vivo* following exposure to this macrophage-derived factor hailed TNF as the "cure for cancer".

The Destruction of Tumors by TNF

Despite the excitement surrounding TNF, questions regarding its potentially deleterious effects remained. Once attributed to the direct actions of LPS, it had subsequently been shown that most, if not all, of the metabolic disturbances associated with endotoxin administration are mediated by endogenous cytokines. For example, one of the hallmarks of endotoxemia is disseminated vascular coagulation with increased vascular bed permeability. The loss of membrane integrity causes leakage of fluids and macromolecules into the tissue spaces. TNF is operative in most of these derangements including the direct cytotoxicity of vascular endothelial cells via apoptosis or programmed cell death[26-28], the increase in tissue factor procoagulant activity, and the suppression of cofactor activity for the anticoagulant protein C pathway.[29,30] In addition, TNF stimulates the production of interleukin-1 (IL-1) which has also been shown to enhance tissue factor-like procoagulant activity on the endothelial cell surface.[29, 31-33] Thus, TNF effectively promotes the formation of blood clots in the vascular bed, an activity that, when sufficiently intense or prolonged, may inflict injury on the host in the form of hypotension, organ failure, and even death.

Hoping to dissect the necrotizing properties of TNF on tumors from its potentially detrimental effects on vascular endothelium, investigators tested the ability of TNF to promote the regression of subcutaneous tumors in mice when injected intra-tumorally.[17] Unlike the deleterious systemic effects of TNF during endotoxemia, local administration of TNF in cancer patients resulted in significant therapeutic benefit. TNF binding to vascular endothelial cells promoted the destruction of the circulatory network which supplied the tumor with blood resulting in the hemorrhagic necrosis of the tumor. Histological analysis revealed only a thin ring of viable tumor cells surrounding the tissue mass. Furthermore, unlike the mass inflammatory responses generated by endocrine-acting TNF, administration of TNF at paracrine levels limited the inflammation to the tumor site, effectively killing the tumor without deleteriously altering the surrounding normal tissue.

In an attempt to duplicate and further enhance these dramatic effects, investigators searched for other naturally-occurring sources of TNF. In so doing, several immune cells which employ TNF in the effector phase of their responses to tumors were identified. The following sections describe the effector cells which possess direct anti-tumor activities, the way in which they recognize tumor targets, and the role that TNF plays in their ability to eliminate tumors.

Macrophages - Numerous observations suggest that activated macrophages are key players in the development of strong anti-tumor immune responses. For example, they are a major component of the lymphoreticular infiltrate of tumors, often observed to cluster around malignant cells, and their presence at the tumor site directly correlates with tumor

regression.[34] Macrophage activation is primarily dependent upon interferon-gamma (IFN-γ) provided by local helper T lymphocytes, and secondarily by autocrine acting TNF. Once activated, macrophages lyse tumor targets in an MHC-unrestricted manner. They express receptors for the Fc portion of immunoglobulins which allow them engage in antibody-dependent cell-mediated cytotoxicity of antibody-coated tumor targets, but much of their lytic capabilities are manifested through the production of cytotoxic molecules. While cytolytic enzymes and reactive oxygen and nitrogen intermediates play a role in their cytotoxicity, TNF is the primary mediator of tumor lysis by macrophages. Lysis may involve direct cell-to-cell contact through the interaction of membrane-anchored TNF, but most tumor targets are lysed following the engagement of specific cell surface receptors by secreted TNF.

$CD8^+$ Cytotoxic T Lymphocytes - A number of tumors have been shown to induce tumor-specific cytotoxic T lymphocytes (CTLs). Rosenberg *et al.*[35] demonstrated that *ex vivo* expansion of these so-called tumor-infiltrating lymphocytes (TILs) in the presence of irradiated tumor cells and interleukin-2 (IL-2) produces lymphocytes with exquisite specificity and anti-tumor activities. Reinjection of activated TILs as a form of active immunotherapy has shown significant clinical benefit in mammals with advanced malignant disease.[36,37]

The induction of reactive CTLs from precursor T cells, however, is dependent upon several interdependent factors. First, antigenic peptide is presented in the context of MHC class I directly by the tumor cell, but more frequently by local antigen presenting

cells, to reactive CTLs. Second, costimulatory signals are provided by the interaction of cell surface molecules on the antigen presenting cell and the T cell (other than MHC and T cell receptor interactions), the most important of which is the interaction between B7-1 (CD80) and/or B7-2 (CD86) on the antigen presenting cell and CD28 on the T cell.[38] Without this second signal, T cells fail to differentiate into cytotoxic effectors. Third, soluble cytokine factors produced by $CD4^+$ helper T lymphocytes stimulate the proliferation of tumor-specific $CD8^+$ T cells and enhance their lytic capabilities. The cytokines of most importance to the generation of cell-mediated responses include IL-2, interleukin-3, lymphotoxin (LT), granulocyte-macrophage colony stimulating factor, and IFN-γ, cytokines of the T-helper cell type 1 phenotype.

Defining the precise mechanisms by which activated $CD8^+$ CTLs lyse tumor targets has been controversial. The difficulty is that none of the suggested mechanisms can fully explain the pattern of lysis observed for CTLs. Clearly CTLs can lyse tumor targets via Fas- Fas ligand interactions[39], perforin-induced pore formation[40], and granzymes (serine proteases)[41], yet none of the mechanisms are mutually exclusive. For example, the granule-exocytosis model implicates the latter two in the acute lysis of targets. Upon appropriate adherence of effector cells to targets, preformed cytoplasmic granules are released at the junction of the interacting cells. This degranulation event releases, among other things, perforin which polymerizes to form ring-like pores in the target cell membrane. These pores permit water and granzymes to enter the cell further contributing to cell swelling and cellular degradation.[40,42,43] However, several investigators have shown, using agents such as EGTA and cyclosporin A, that in acute cytolysis, degranulation and

killing are dissociable events.[44-47] Moreover, isolation of CTL clones which lack measurable intracellular perforin kill as well as CTL clones that have large quantities of endogenous perforin.[48-50] These experiments provided strong evidence for the existence of perforin- and granzyme-independent mechanisms of CTL lysis. Due to their previously defined cytotoxic properties, TNF, and its close relative LT, were potential candidates.

TNF and LT, both in the secreted and membrane-bound forms, play a pivotal role in CTL-mediated lysis of tumor targets. CTL clones incubated with syngeneic targets, even for as little as 90 minutes, secrete a material into the culture media which lyses TNF-sensitive targets in a slow lytic assay (i.e. 18 hours).[51] Preincubation of the culture media with anti-TNF antibody neutralized this cytotoxicity. Furthermore, treatment of these reactive CTLs with cyclosporin A did not significantly inhibit their cytolytic potential, suggesting that membrane-anchored TNF can substitute for secreted TNF in mediating cellular cytolysis. Likewise, studies by Perez *et al.*[52] using TNF mutants deficient in proteolytic processing showed that the membrane form, like the secreted form, can induce cytotoxicity. Surface LT is expressed on activated T cells[53] and, thus, may function as a contact-dependent mediator of immune responses.

Natural Killer Cells - Since the early 1970s, natural killer (NK) cells have been recognized as a functionally distinct subset of lymphocytes with a unique and spontaneous ability to target and destroy tumor cells while sparing normal tissue cells.[54,55] Recent evidence has shown that NK cells are not only involved in the resistance to, and control of, cancer spread, but that their presence and state of activation may influence the outcome of the

disease. Convincing evidence exists that low NK activity in mammals is associated with a higher risk and frequency of cancers. Genetic defects which result in the impairment of NK cells, such as the beige mutant mouse strain and the Chediak-Higashi syndrome in humans, predispose these hosts to certain cancers.[56-57] Analogously, patients with advanced metastatic disease often have abnormalities in NK cell-function and/or NK cell numbers.[57] These observations underscore the importance of NK cells in the immunosurveillance of cancer and prompted investigators to identify methods of enhancing their anti-tumor activities.

While sensitizing lymphocytes to tumor antigens by the method necessary to generate TILs, Rosenberg *et al.* discovered that in the presence of high concentrations of IL-2 and in the absence of additional tumor antigens, large numbers of activated lymphoid cells were generated that specifically killed freshly isolated tumor cells without harming normal cells.[58] These so-called "lymphokine activated killer (LAK) cells" represent a mixed population of activated NK cells and natural cytotoxic cells, the relative distribution of which depends upon the source of the lymphocytes and the conditions of IL-2 activation. Adoptive transfer of LAK cells in conjunction with IL-2 to patients with such malignancies as renal cell carcinoma, melanoma, colorectal carcinoma, and non-Hodgkins lymphoma has resulted in significant clinical benefit.[58-59]

NK cells do not have antigen-specific receptors which would allow them to detect transformed cells; thus the mechanism by which NK cells target and destroy tumor cells without affecting normal cells has been controversial. However, the recent identification of a unique co-receptor signaling model provided some insight into the mechanism by

which NK cells recognize and destroy tumor targets. NK cells appear to discriminate malignant cells from normal cells by at least two interactive receptor systems. First, the NKR-P1 receptor allows NK cells to discern oligosaccharide moieties on abnormal cells. The interaction of NKR-P1 with aberrant patterns of glycosylation found on malignant cells triggers NK cells to kill.[60] This killing can be prevented, however, by a signal from the second receptor system, Ly49.[61] Binding of Ly49 to class I MHC molecules on the surface of the target transmits a negative signal which overrides the first, NKR-P1 signal. Thus, when malignant cells reduce their levels of MHC expression, the Ly49 signal is also reduced allowing the NKR-P1 signal to induce killing of the abnormal cell.

The lysis of tumor targets by NK cells, like that of CTLs, is mediated by perforin, granzymes, and TNF, however a role for LT remains controversial. Certain investigators demonstrate the expression of LT on activated NK cells[52,53], while others suggest that it does not contribute significantly to their lytic activities.[62] Nevertheless, there is a defined role for TNF as an autocrine-acting cytokine in the generation and activation of NK cells, particularly in the mobilization of the lytic mediators perforin and granzymes.[63,64] Differentiation of NK cells is dependent upon TNF in that signals transduced by agonist-acting monoclonal antibodies specific for TNF receptors enhances the NK activity against NK-susceptible K562 targets.[65] While NK cells are capable of lysing tumor targets without prior sensitization, preincubation with IL-1 and/or IL-2 enhances both the proliferation and cytotoxicity of activated LAK cells.[66] This synergy between IL-1 and IL-2 in the generation of potent LAK immune responses is highly dependent upon TNF

and LT such that neutralization of the cytokines with specific antibodies greatly diminishes NK cytotoxicity. These observations further support a role for TNF in the generation and promotion of NK cell activity and in the ability of NK cells to effectively eliminate tumors.

Clearly, TNF is important not only in the generation of potent anti-tumor immune responses, but also in the direct destruction of tumor cells by specific effector cells. Its intriguing property of selective cytotoxicity against tumor cells provided a new modality for the treatment of cancer, one which capitalized on the specificity of the host's immune system. Therefore, the discovery of TNF fueled a generation of tumor biologists. The gene expression and regulation, as well as the biosynthesis, of TNF have since been under intense scrutiny in hopes of harnessing its potency for cancer immunotherapy.

Tumor Necrosis Factor (TNF)

Genetics of TNF - The gene encoding TNF is located on chromosome 6 in man[67,68] and chromosome 17 in the mouse.[69,70] In both species, it is located within the major histocompatibility complex.[68,69] Likewise, in humans and in mice, the TNF gene lies directly upstream from the gene encoding LT, a related cytokine which is produced by mitogen-stimulated lymphocytes and which, as discussed previously, is thought to play a major role in lymphocyte-mediated cytotoxicity.[71] TNF and LT share approximately 50% amino acid sequence homology (28% identity)[67] as well as common receptors and overlapping biological activities including lysis of L929 cells (an L cell successor used in bioassays of TNF cytotoxicity) and the ability to cause hemorrhagic necrosis of tumors *in vivo*.

Biosynthesis of TNF - The biosynthesis of TNF appears to be tightly regulated both at the transcriptional and post-transcriptional levels. Synthesis of TNF mRNA occurs at very low levels in resting macrophages. Moreover, TNF mRNA, which is generally unstable, is not efficiently translated, since no TNF can be detected in cultured cells or in the surrounding media, nor is TNF detectable in the plasma of normal individuals. However, following the stimulation of cultured macrophages with endotoxin or other stimuli, TNF mRNAs increase 3-fold within 15-60 minutes and detectable levels of TNF are secreted within 15-20 minutes post-exposure.[72-74] This endotoxin-stimulated production of TNF can be suppressed by dexamethasone which blocks TNF gene transcription when added at any time relative to LPS, and inhibits mRNA translation when added prior to LPS challenge.[75] The transcription of the TNF gene also may be under the control of a short-lived repressor molecule acting on as yet uncharacterized promotor/enhancer elements upstream of the gene.[76] In addition, other cytokines including IFN-γ and TGF-β influence the production of TNF. IFN-γ augments both the transcriptional and post-transcriptional activities of LPS[76,77], while TGF-β diminishes TNF biosynthesis through unknown mechanisms.[78]

Post-transcriptional regulation of TNF gene expression involves, in part, the presence of an unusual, but highly conserved, AU-rich sequence located in the 3'-untranslated region of TNF mRNA.[79] This sequence is not only observed in TNF mRNA, but also in the mRNAs of a variety of inflammatory mediators including LT, IFN-α, and IL-1α and IL-1β, and in the mRNAs of certain protooncogenes such as *c-myc*

and *c-fos*.[79] It has been suggested that these AU-rich sequences represent critical regulatory elements which control the half-life of the mRNA molecules and the efficiency with which these mRNAs are translated.[79,80] Beutler *et al.* have described a nuclease present in the lysates of certain mammalian tissues, including macrophages, that selectively degrades mRNAs that possess these sequences.[81] Investigators suspect that tagging the 3'-untranslated region of TNF mRNAs marks them for rapid degradation, limiting the amount of biologically active, and highly potent, TNF.

The amino acid sequences of murine, human, and rabbit TNF have been determined from complementary DNA (cDNA) clones.[82-85] The mature mouse protein is 156 amino acids in length[82], while the mature proteins of humans and rabbits are 157 amino acids, due to an insertion of an additional histidine residue[86], and 154 amino acids, due to the absence of two amino-terminal residues[83], respectively. In all three species, however, TNF is initially expressed as a 233-amino-acid membrane-anchored protein which is proteolytically cleaved at several discrete sites during the processing of the mature, 17 kD secreted hormone.[79,82,86] While the enzyme mediating the processing of mature TNF is yet to be fully characterized, it is well documented that in leukocytes a specific matrix metallo-proteinase cleaves the precursor protein to release mature TNF.[87] Metalloproteinase inhibitors prevent the secretion of TNF from macrophage cultures[88] and protect mice from lethal doses of endotoxin.[89]

The amino acid sequences of the propeptide and the mature peptide are highly conserved between mice and humans with approximately 86% and 79% similarity[82,84,90], respectively. The fact that the propeptide sequence is conserved between species suggests

that it plays an integral role in the cytokine's biological activities, although this role has not been formally demonstrated. Three distinct species of precursor proteins, produced through the differential cleavage of the propeptide sequence, are secreted by LPS- and IFN-γ-stimulated monocytes. The 20, 23, and 25 kD species are all recognized by polyclonal anti-sera raised to TNF, but vary in their biological activity.[91]

Mature TNF is produced as 17 kD subunits which non-covalently oligomerize into dimeric, trimeric, or pentameric forms with the trimeric 51 kD form being the most prevalent and eight-fold more active than the other forms with respect to receptor binding.[92] All three species contain two cysteine residues per subunit which form an intrachain disulfide bridge on the surface of the molecule.[93] Reduction of the disulfide bonds or amino acid substitution of the cysteine residues has no effect on the conformation or bioactivity of the molecule.[94,95] Likewise, the N-glycosylation of the mature mouse protein with sialic acid and galactosamine[96] appears to have no effect on hormone action since TNF produced recombinantly in *E. coli* causes hemorrhagic necrosis of mouse tumors and displays the same pattern of cytotoxic activity for mouse cells as the fully glycosylated form.[97]

TNF Immunotherapy

Cytokines regulate the immune reactivity and direct maturation, activation, and migration of inflammatory cells. Observations in animal models suggest that local cytokine production, either by direct injection or by gene delivery, can induce strong anti-tumor immune responses that provide long-lived immunity and, in some cases, regression

of established tumors. In the early attempts at enhancing tumor immunity with cytokines, TNF was undoubtedly the best candidate. Serum containing TNF could promote the necrosis of some tumors *in vivo* and was cytotoxic and/or cytostatic to other tumor cells *in vitro*[17,98,99] Sufficient quantities of TNF for use in clinical trials became available through the cloning and expression of the cDNA in *Escherichia coli.* [85,86,90] Phase I and phase II clinical trials of TNF, usually in conjunction with IL-2, appeared promising. Cures and partial regression of tumors were reported in some human breast[100] and ovarian[101] cancer patients following injections of TNF. However, the effects were not reproducible and the side-effects of TNF administration were devastating.

The limited efficacy of TNF as a therapeutic treatment for cancer is due to the toxicity inherent to systemic administration. Because most cytokines act as local hormones whose accumulation in the serum is prevented by their short half-lives, it was suggested that local administration (i.e. at the tumor site) may enhance TNF's therapeutic benefit. However, several attempts to stimulate immune responses to pre-existing tumors by repeated injections of TNF at the tumor site failed to induce tumor regression or to establish immunological memory.[102-104] It was suggested that delivery of TNF by this method did not sufficiently approximate physiological levels of the cytokine requisite for optimal induction of anti-tumor responses.

Therefore, an alternative to local administration of TNF through injection was to introduce genes encoding the cytokine into tumor cells and use them as vehicles of vaccination. Tumor cells engineered to produce TNF have been used as vaccines in various murine tumor models.[105,106] In general, two observations can be taken from these

studies: (1) tumor cells secreting low levels of TNF failed to produce tumors in syngeneic mice; and, (2) animals rejecting the cytokine-secreting tumors were protected from subsequent challenge with the parental, unmodified tumor cells. However, the excitement for TNF-secreting tumor cells as vaccines for cancer waned when it was discovered that, depending on the tumor model, not all modified tumors were rejected, nor did all of them induce systemic immunity.[107] Based on these observations, immunotherapeutic trials of TNF in the treatment of cancer patients were ceased.

It was clear that TNF had not lived up to its billing as the cure for cancer. Tumor biologists offered many rationalizations to explain why such a potent cytokine, with the intriguing ability to selectively kill malignant cells without harming normal cells, failed to promote the regression of tumors in cancer patients. They first suggested that, although murine tumors underwent TNF-mediated regression, human tumors displayed, at best, a differential sensitivity to TNF. This theory was quickly dispelled. While certain tumor types are refractory to the effects of TNF, virtually all cultured human cancer cell lines exhibit some susceptibility to the cytostatic or cytotoxic properties of this molecule.[108-110] Alternatively, investigators suggested that the dose of exogenous TNF required to promote the regression of tumors exceeded the threshold for toxicity in humans. In *in vitro* analysis, however, tumor cells isolated from human patients were susceptible to TNF-mediated cytolysis at TNF concentrations well below those administered *in vivo*. The only explanation that investigators could reasonably turn to, then, was that the activities of TNF were somehow inhibited in cancer patients. In the response of a cell to TNF, the proximal event is binding of TNF to its receptors. Therefore, in hopes of

elucidating the manner in which TNF may be inhibited in cancer patients, investigators focussed much of their attention on characterizing the receptors which mediate TNF's biological effects.

TNF Receptors

The biological effects of TNF are initiated by binding to two species of high affinity cell receptors: TNF receptor type I (TNFRI), a 55 kD receptor, and TNF receptor type II (TNFRII), a 75 kD receptor.[111] The pluripotent activities of TNF are due to the ubiquitous nature of these receptors. Virtually every cell type analyzed expresses one, and in most cases, both of these receptors[112,113], and both receptors bind TNF and LT, albeit with differing affinities.[114] The extracellular portion of TNFRI and TNFRII share significant homology and are characterized by a repetitive amino acid sequence pattern of four cysteine-rich domains with a constrained and highly conserved quaternary structure.[115] Signaling occurs when a TNF trimer binds two or three receptors in an extracellular complex which permits the aggregation and activation of the cytoplasmic domains. Despite the similarities in their TNF-binding extracellular domains, the complete absence of homology between the intracellular domains suggests that the two receptors utilize distinct signaling pathways. The 55 kD receptor has been implicated as the mediator of much of the cytotoxic properties of TNF[116,117], while the 75 kD receptor is responsible for the induction of cellular proliferation and differentiation in a variety of cell types.[65,118]

Soluble forms of these receptors (sTNFRI and sTNFRII) can be generated by a proteolytic cleavage event which liberates the extracellular domains. These truncated receptors retain the ability to bind TNF with high affinity and, thus, to act as cytokine antagonists *in vitro* and *in vivo*.[119-124] Since soluble TNF receptors represent naturally-occurring inhibitors of TNF and analogs to cell surface receptors, their relative distribution may affect the course and outcome of immune responses in a variety of disease states.

Soluble TNFRs originally were identified in the urine of febrile patients.[124] Due to the potent, and potentially harmful, systemic effects of TNF, investigators were searching for naturally occurring blocking factors which could down regulate TNF-mediated responses. Other investigators had already identified soluble extracellular fragments of receptors for IL-1[125], IL-2[126], and interleukin-6[127] in human biological fluids. The intimate relationship between IL-1 and TNF in the generation of fever suggested that a soluble form of the receptor for TNF also might be found. Several investigators subsequently identified and purified both sTNFRI and sTNFRII from human serum and urine.[120,121,124,128]

Following the identification of sTNFRs in febrile patients, elevated levels of sTNFRs were also reported in humans during sepsis[129], meningitis[130], transplant rejection[131], autoimmune diseases[132-134], and human immunodeficiency virus infection.[135] All of these disease states are characterized by significant elevations in circulating TNF which are followed by concomitant increases in the levels of circulating sTNFRs. These correlations have led to the hypothesis that production of sTNFR is a mechanism for restoring immunologic homeostasis.[136]

The ability of sTNFRs to modulate the activities of TNF during disease states has been demonstrated experimentally in mouse models. In most of these experimental systems, chimeric molecules comprised of sTNFRI fused to a sequence encoding the Fc portion and hinge region of a mouse IgG heavy chain[137] were used to evaluate the effects of sTNFR on TNF-mediated disease processes. In models of bacterial sepsis, intravenous administration of sTNFR-IgG chimeras was shown to protect mice from the lethal effects of endotoxin-induced shock.[138] To demonstrate that the diminishing level of circulating TNF caused by its binding to sTNFR was responsible for this protection, mice were challenged with intracellular pathogens whose containment is dependent upon TNF. Mice were injected with sTNFR-IgG chimeras and then challenged with *Listeria monocytogenes* and *Leishmania major*[138], or *Mycobacterium tuberculosis*[139] exhibited an enhanced susceptibility to and exacerbation of disease. These models were among the first to demonstrate a complete, or partial, impairment of TNF by sTNFR.

Soluble TNFR-induced immune suppression has been demonstrated also in models of autoimmune disease. Mice treated with sTNFR-IgG chimeras exhibited a significantly reduced incidence and severity of collagen-induced arthritis.[140] Likewise, in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, administration of sTNFR-IgG chimeras completely prevented the clinical signs and pathological changes including demyelination and inflammatory cell infiltration.[141] Collectively, these experimental models indicate that sTNFR controls the bioactivity of TNF, thus protecting individuals from the potentially harmful effects of systemic TNF.

The Role of sTNFR in Tumor Survival

While sTNFR-mediated immune suppression ultimately benefits hosts with certain bacterial infections or autoimmune diseases by limiting the degree of inflammation-induced pathogenesis, inhibition of TNF by sTNFR in cancer patients may prevent the desired immunomodulatory and cytotoxic effects of TNF paramount to tumor rejection. Indeed, sTNFR inhibits the necrotizing properties of TNF when co-injected directly into tumors grown as subcutaneous transplants in mice.[128] In addition, several lines of circumstantial evidence suggest that sTNFR actually contributes to tumor survival *in vivo*. First, cells of various tumor-derived lines produce nanomolar concentrations of sTNFR spontaneously in culture.[142-144] Second, sTNFR is elevated in the serum of cancer patients[142,145-147] and the levels correlate with the stage of malignant disease: sTNFR levels are significantly elevated during advanced stages of cancer, decline during stages of remission, and when present at high levels, are prognostic of poorer treatment outcomes.[126,142,145-147] It is noteworthy that infusion of TNF[148] and IL-2[149,150] increases the levels of sTNFR in cancer patients, and that IL-1 and IL-2 induce the secretion of sTNFR from NK/LAK cells.[66] These concomitant increases of sTNFR following cytokine immunotherapy is suggested to reduce the therapeutic value of cytokine infusion.

Further evidence that sTNFR contributes to tumor survival *in vivo* is provided by data from human clinical trials of Ultrapheresis[151], a promising experimental cancer treatment (Figure 1.1). Unlike conventional cancer biotherapy which attempts to enhance anti-tumor immune responses through the addition of biological response modifiers such

as cytokines, Ultrapheresis proposes to boost immune responses through the removal of immunosuppressive substances found in the blood of tumor-bearing hosts. The removal of these immunosuppressive substances is accomplished using extracorporeal ultrafiltration of the patient's blood.

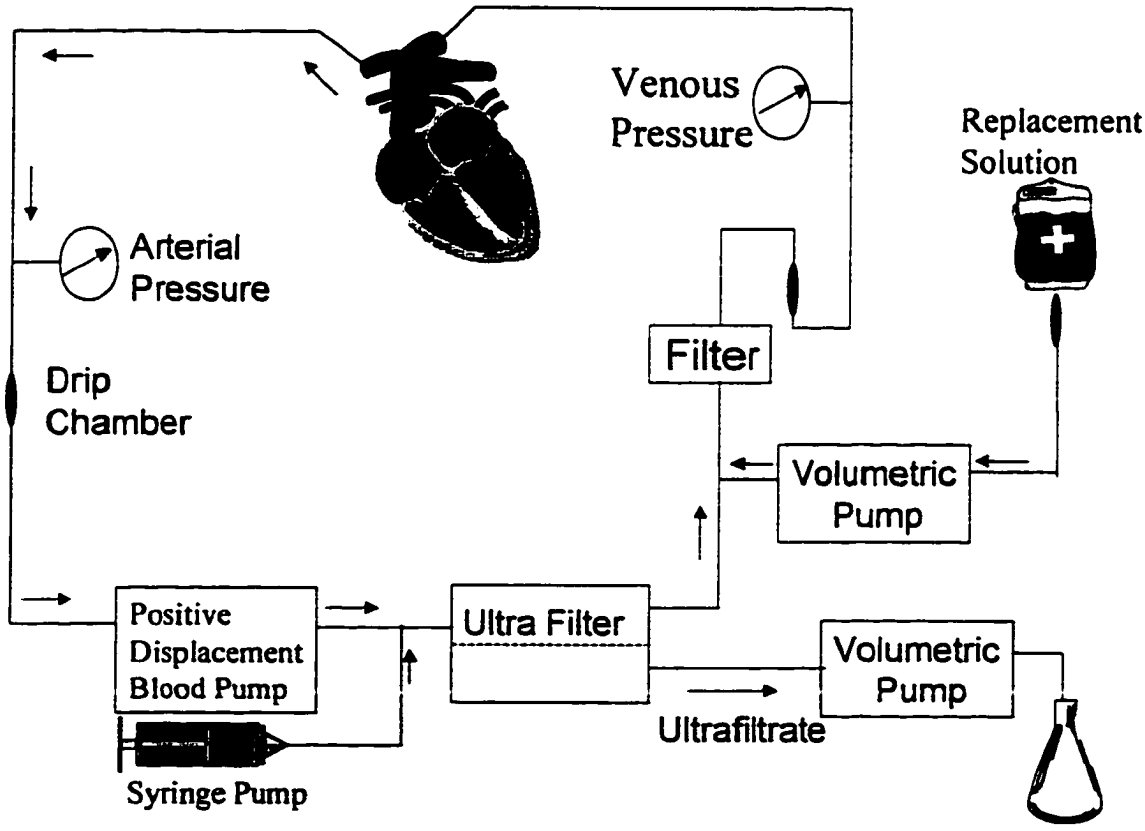

Figure 1.1. Schematic of Ultrapheresis, an extracorporeal treatment for cancer.

Arterial blood is removed through a catheter and cycled through a device similar to that used for hemodialysis. The blood passes by an ultrafilter and, by virtue of a slight pressure differential, fluid is forced through the membrane filter. This fluid (called the "ultrafiltrate") contains plasma-borne molecules capable of passing through the pores of the filter (molecular weight cutoff = 100 kD). Consequently, immunosuppressive substances found in the circulation of cancer patients are retained in the ultrafiltrate, resulting in the "unblocking" of pre-existing chemical and/or cellular mediators of anti-tumor immunity. Ultrapheresis provides rapid and significant clinical benefit to patients presenting with a variety of tumor types. Inflammation occurs at the tumor site within minutes to hours post-initiation, consistent with the removal of pre-existing inhibitors of this response.

One potent inhibitor in the ultrafiltrates removed from cancer patients is sTNFRI. Originally, a protein fraction of ultrafiltrate was identified which accelerated both the tumor growth and the development of metastases in mice and suppressed NK cell activity *in vitro*.[153-154] Ultimately, these activities were ascribed to sTNFRI which was purified from these protein fractions. The fact that sTNFRI is removed by Ultrapheresis, combined with the clinical benefit associated with the treatment, provides further correlative evidence that sTNFRI is operative in tumor survival. However, no direct evidence exists to indicate that sTNFRI provides a growth advantage to tumors *in vivo*.

Therefore, the present work was intended to formally evaluate the importance of sTNFRI in tumor survival. As described in Chapter 2, a TNF-sensitive murine fibrosarcoma cell line was genetically engineered to produce sTNFRI. In so doing, we

created a physiologically relevant model to assess the effects of sTNFRI secretion on the lysis of this fibrosarcoma by immune mechanisms known to be important in the elimination of tumors. *In vitro* assays indicated that sTNFRI not only inhibits the direct lysis of tumor targets by TNF, but that sTNFRI is also a potent inhibitor of cellular cytolysis by NK cells and CTLs. Thus, sTNFRI inhibits three immune mechanisms with preeminent roles in tumor eradication. These findings support the hypothesis that sTNFRI may contribute to tumor persistence *in vivo*. In Chapter 3, we demonstrated that secretion of sTNFRI by tumor cells dramatically enhanced their tumorigenicity and persistence in syngeneic hosts. Further, by immunizing mice with sTNFRI prior to tumor challenge, we induced antibodies which neutralize the binding of sTNFRI to TNF and which abrogate the survival benefit previously shown for sTNFRI-secreting tumors. These findings justified the development of immunotherapeutic agents for the selective inactivation of sTNFRI in tumor-bearing hosts. Chapter 4 describes the development of a neutralizing monoclonal antibody specific for sTNFRI and our initial evaluation of the antibody's therapeutic utility.

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CHAPTER 2
MULTIFACETED INHIBITION OF ANTI-TUMOR IMMUNE MECHANISMS BY SOLUBLE TUMOR NECROSIS FACTOR RECEPTOR TYPE I

ABSTRACT

Soluble tumor necrosis factor receptor type I (sTNFRI) is a potent inhibitor of TNF with the potential to suppress a variety of effector mechanisms important in tumor immunity. That sTNFRI influences tumor 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 tumor cell elimination. To formally evaluate the ability of sTNFRI to inhibit these mechanisms, we have engineered sTNFRI production into the TNF-sensitive murine fibrosarcoma cell line, L929. sTNFRI-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 immunologic mechanisms important in tumor cell eradication, and further support a role for sTNFRI in tumor 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 tumor types. Ultrapheresis induces pronounced inflammation at tumor sites, often in less than one hour post-initiation. This rapidity suggests a role for preformed chemical and/or cellular mediators in the elaboration of this inflammatory response, and it likely reflects the removal of naturally occurring plasma inhibitors of that response. One such inhibitor previously shown to be removed by Ultrapheresis is sTNFRI.[2-4]

sTNFRI[4-8] represents the extracellular domain of the membrane-associated TNF receptor type I which is shed from the cell surface by proteolytic cleavage. sTNFRI 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 tumor-bearing hosts are likely to be profound, considering the critical role of TNF in a variety of anti-tumor immune responses.[9] TNF directly induces apoptosis in tumor cell targets by binding to the type I membrane-associated TNF receptor, CD120a.[10,11,12] Moreover, vascular endothelial cells are induced to apoptosis by TNF binding, destroying the circulatory network serving the tumor and further contributing to tumor cell death.[13,14,15] Critical roles for TNF in LAK cell-[16,17] and CTL-mediated [18-20] cytolysis also have been

documented. Inhibition of any or all of these effector mechanisms by sTNFRI has the potential to dramatically enhance tumor survival. Nevertheless, direct evidence that sTNFRI regulates anti-tumor immunity is not currently available. To formally examine the effects of sTNFRI on immune mechanisms important in tumor 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 immunologic mechanisms with fundamental roles in anti-tumor immunity.

MATERIALS AND METHODS

Cell Lines and Reagents - The C3H/HeN murine fibrosarcoma cell line, L929 (CCL 1), and the human histiocytic lymphoma cell line, U937 (CRL 1593), were obtained from the 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 MEM and RPMI-1640, respectively, supplemented with 10% (v/v) fetal bovine serum and penicillin/streptomycin, each at 100 μg/mL. All cells were cultured at 37°C in a humidified atmosphere containing 6% CO_2. *E. coli*-derived murine TNF 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 accord 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 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 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'-CATAAGCTTGCCGCCAGCCATGGGCCTCTCCACC-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 *Hind* III 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 carboxy-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 minute each): 95°C denaturation, 60°C annealing, and 72°C 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 *Hind* III and *Not* I to liberate a 727 base-pair fragment which was ligated to *Hind* III/*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. Further, the nucleotide

sequence of the sTNFRI gene in this construct was determined to be identical to that previously reported.[4,21]

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 x 10^5 cells per 60 mm tissue culture dish (approximately 80% confluence) and allowed to adhere overnight. Five μg 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 hours. 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 ELISA. Wells were coated with goat anti-human sTNFRI antibody and blocked with 2% (w/v) BSA in 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-labeled 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 2.25 x 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 were diluted in complete growth medium and added to triplicate wells. Control wells received complete growth medium containing actinomycin D alone. After twenty-four hours, media were aspirated and viable cells were stained with 1% aqueous crystal violet for 5 minutes, washed extensively with water, and cell-associated dye was solubilized in 33% glacial acetic acid. OD_{550} was determined to quantify relative cell survival, and percent cytotoxicity was calculated for each concentration of TNF according to the formula:

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

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 six 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 (PBMCs) with 1500 U/mL of recombinant IL-2. This supernatant contains considerable residual IL-2, as well as numerous other cytokines secreted by the PBMCs[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 labeled with TRAN^{35}S-LABEL (ICN, Irvine, CA) as described[26], at various effector:target (E:T) ratios for five hours at 37°C. Lysis was correlated with release of radiolabel into the culture media as determined by liquid scintillation.

Percent specific lysis was calculated according to the formula:

$$\text{Percent specific lysis} = \frac{(\text{cpm test sample}) - (\text{cpm spontaneous release})}{(\text{cpm 2\% Triton sample}) - (\text{cpm spontaneous release})} \times 100\%$$

CTL Assays - The cytotoxic activity of L929-reactive CTLs 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 1.5×10^5 clone K cells per well) for six days. The resulting CTL effectors were added, in quadruplicate, to radiolabeled clone K and clone 39 targets, and percent specific lysis was determined as described above.

RESULTS AND DISCUSSION

The L929 Transfectant, Clone 39, Produces sTNFRI - Cultures of L929 were transfected with the sTNFRI/pcDNA3 expression construct (Figure 2.1).

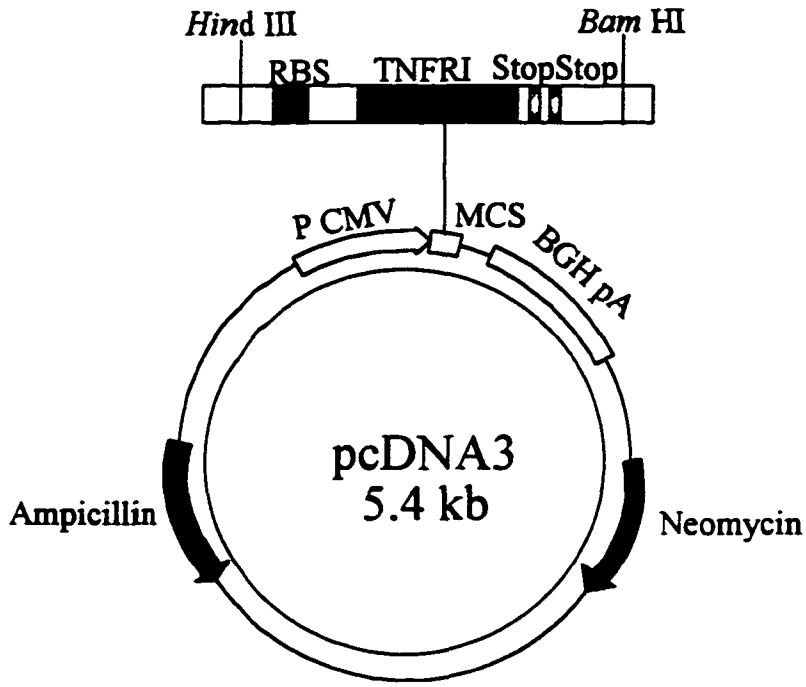

Figure 2.1. Schematic of the pcDNA3 eukaryotic expression construct. Expression of the sTNFRI gene, cloned into the multiple cloning site (MCS) of pcDNA3 as a *Hind* III/*Bam*HI fragment, is under the control of the cytomegalovirus immediate early promoter. Translation is terminated by a strong bovine growth hormone polyadenylation signal (BGH pA). Locations of the ribosome binding site (RBS) and the two in-frame termination codons (stop) are indicated. The vector contains ampicillin and neomycin resistance genes for the selection of prokaryotic and eukaryotic transformants, respectively.

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 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 tumors[27-29], and similar to levels detected in the circulation of tumor 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, and percent cytotoxicity was calculated (Figure 2.2). 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.

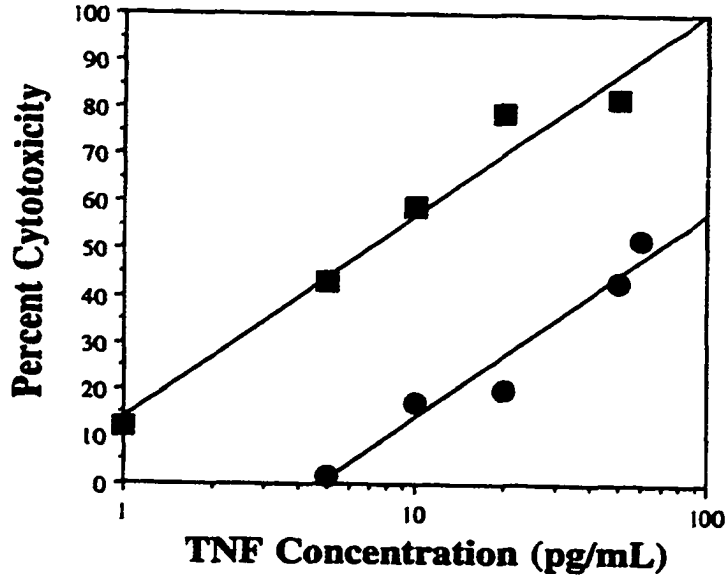

Figure 2.2. sTNFRI inhibits direct lysis of target cells by TNF. Clone K (■) and clone 39 (●) cells were cultured with the indicated concentrations of murine TNF and cytotoxicity was determined as described in Materials and Methods. Standard errors of the mean absorbance for all samples were less than 18%.

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 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-hour *in vitro* assays (Figure 2.3). sTNFRI-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%.

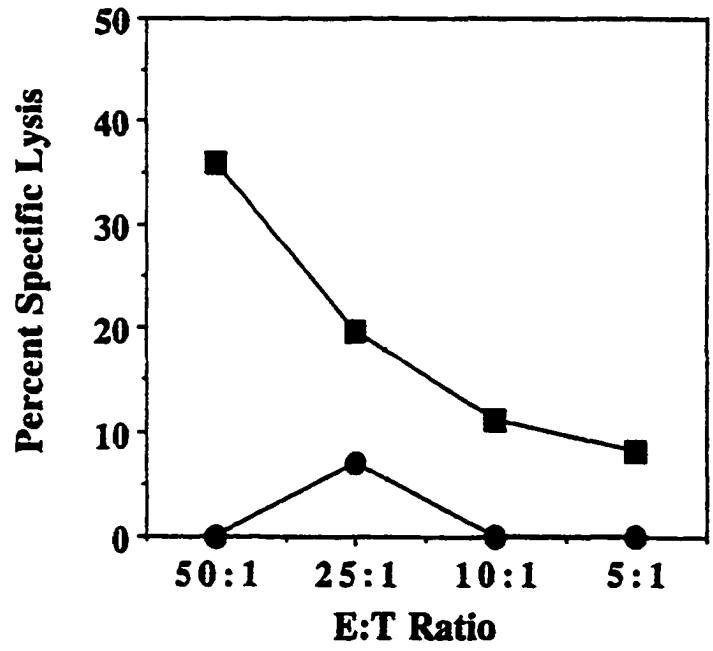

Figure 2.3. sTNFRI inhibits target cell lysis by LAK cells. Clone K (■) and clone 39 (●) cells were co-cultured with murine LAK cells at the indicated E:T ratios and specific lysis was determined as described in Materials and Methods. Standard errors of the mean cpm for all samples were less than 10%.

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 tumor cell elimination.

sTNFRI Inhibits CTL-Mediated Lysis - The effect of sTNFRI on CTL-mediated lysis also was examined (Figure 2.4). Clone 39 targets were lysed by L929-reactive CTLs 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 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 CTLs.[37] Nevertheless, because the differentiation and proliferation of CTLs, like that of LAK cells, is enhanced by TNF[38], sTNFRI production by tumor cells *in vivo* may inhibit CTL activation, including even those cells which employ lytic mechanisms that are independent of TNF.

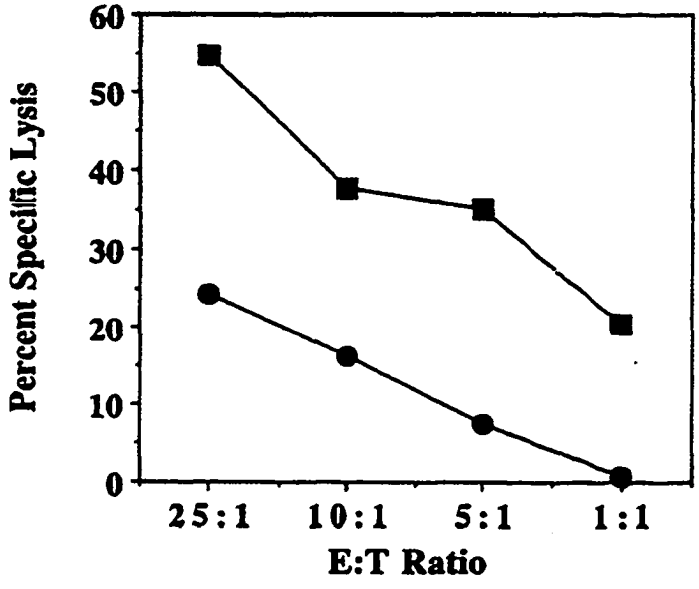

Figure 2.4. sTNFRI inhibits target cell lysis by CTLs. Clone K (■) and clone 39 (●) cells were co-cultured with L929-reactive CTLs at the indicated E:T ratios and specific lysis was determined as described in Materials and Methods. Standard errors of the mean cpm for all samples were less than 10%.

The involvement of TNF in 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 hour) *in vitro* assays[18-20], while others report that the effects of TNF are observable only in 18-hour 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. Further, 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 *in vitro* assays, 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 tumor rejection *in vivo*. The influence of sTNFRI on tumor growth *in vivo* now can be evaluated, in a physiologically relevant manner, by using the sTNFRI-secreting cell line described herein in mouse transplantation models.

Numerous observations suggest that sTNFRI aids tumor survival *in vivo*. Nanomolar concentrations of sTNFRI are synthesized by a variety of tumor 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 tumor 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 tumor-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 immunologic 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 favor tumor 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 endotoxemia/sepsis.[48-51] sTNFRI-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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CHAPTER 3
SOLUBLE TUMOR NECROSIS FACTOR RECEPTOR TYPE I ENHANCES TUMOR DEVELOPMENT AND PERSISTENCE *IN VIVO*

ABSTRACT

In vitro assays have demonstrated that the mouse fibrosarcoma, L929, engineered to secrete soluble tumor necrosis factor receptor type I (sTNFRI), displays increased resistance to direct lysis by TNF, and to LAK- and CTL-mediated cellular cytolysis. These findings suggest that, *in vivo*, sTNFRI may contribute to tumor development and survival by inhibiting immunologic mechanisms which are preeminent in the eradication of tumors. To formally evaluate this hypothesis, we have compared the growth of sTNFRI-secreting L929 cells with that of the unmodified parental fibrosarcoma cell line in an *in vivo* mouse transplantation model. Previous studies have shown L929 cells to be non-tumorigenic in syngeneic recipients, yet they produce fibrosarcomas in sub-lethally irradiated animals, indicating that tumor growth in unirradiated animals is prevented by immunological mechanisms. In the present studies, the secretion of sTNFRI by L929 cells is shown to markedly enhance their tumorigenicity in irradiated, as well as in immunocompetent, recipients. That sTNFRI was responsible for the acquired tumorigenicity was demonstrated by immunizing mice with sTNFRI prior to tumor challenge. Significant titers of neutralizing anti-sTNFRI antibody, present in the serum of immunized mice, correlated with abrogation of tumorigenicity. These data formally

demonstrate that sTNFRI directly influences tumor growth and persistence *in vivo*, and suggest the development of reagents for the selective removal and/or inactivation of sTNFRI as promising new avenues for cancer immunotherapy.

INTRODUCTION

TNF is a pluripotent cytokine originally identified by its ability to induce hemorrhagic necrosis and subsequent regression of established subcutaneous tumors in mice.[1] Since the original demonstration of its cytostatic and cytotoxic effects on tumor cells, TNF has been found to regulate a large number of biological phenomena including inflammation, cell proliferation, and various immunoregulatory and antiviral responses.[2] These biological effects are initiated by binding of TNF to the type I, membrane-associated TNF receptor (TNFRI) which is expressed nearly ubiquitously on mammalian cells.[3,4] Given the myriad activities of TNF and its exquisite potency ($K_a = 10^{-10}$ M), it is not surprising that there exist regulatory mechanisms to modulate its biological activity. One such mechanism is a naturally occurring "blocking factor" of TNF that originally was identified in the urine of patients in renal failure[5] and in the urine of febrile patients.[6] Purification of this protein[7-11], and the subsequent cloning and sequencing of the respective cDNA[12-13], revealed that this inhibitory factor corresponded to the extracellular domain of the intact, membrane-associated TNF receptor Type I.[14] This soluble receptor (sTNFRI) results from a proteolytic cleavage event which liberates the extracellular domain from the transmembrane and intracellular domains. sTNFRI retains the ability to bind to TNF with high affinity, and thus to prevent the binding of TNF to cell surface receptors.[7-9,11,13]

The levels of sTNFRI in biological fluids are found to be increased in a variety of conditions which are characterized by an antecedent increase in TNF. These include sepsis/endotoxemia[15], meningitis[16], autoimmune diseases[17-19], transplant rejection[20], and

human immunodeficiency virus infection.[21] In each of these disease states, TNF production contributes directly to the pathogenesis, and the production of sTNFRI is postulated to be a mechanism for restoring immunologic homeostasis.[22]

sTNFRI levels are reported to be increased also in tumor bearing hosts[23-27], presumably in response to a burst of TNF production driven by the presence of the tumor. The production of sTNFRI in this scenario likely is intended to reduce the localized, as well as systemic, toxicity associated with elevated TNF levels in a manner analogous to that which is operative in those inflammatory conditions described. Yet in tumor bearing hosts, the production of sTNFRI may have the unintended consequences of protecting tumors from immunological destruction, thus, facilitating their growth *in vivo*.

Several lines of correlative data suggest that elevated levels of sTNFRI enhance tumor survival. First, cell lines derived from various tumors produce sTNFRI spontaneously in culture[28,29], and malignant cells have a greater tendency to produce and to shed their cell surface receptor than do non-malignant cells.[30] Second, the levels of sTNFRI in the circulation of tumor-bearing hosts are significantly elevated during advanced stages of disease and decline during stages of remission.[23,24,26] Third, data from human clinical trials of Ultrapheresis correlate the removal of sTNFRI from the circulation of cancer patients with remarkable clinical benefit.[31] Fourth, we have shown using *in vitro* assays that sTNFRI protects transformed cells from the direct cytotoxic effects of TNF and from cellular cytolysis mediated by natural killer/lymphokine activated killer cells and cytotoxic T cells.[32] Collectively, these observations indicate that sTNFRI may contribute to the survival of tumors *in vivo* by inhibiting anti-tumor immune mechanisms which

employ TNF. Formal proof of this hypothesis, however, currently is unavailable. The present studies, therefore, were designed to evaluate the role that sTNFRI plays in tumor development and progression. Herein, we demonstrate: 1) that secretion of sTNFRI by tumor cells markedly enhances their tumorigenicity and promotes their persistence in syngeneic hosts, and; 2) that this survival benefit is abrogated by antibodies which neutralize the binding of sTNFRI to TNF.

MATERIALS AND METHODS

Cell Lines and Reagents - The L929 murine fibrosarcoma cell lines used in these studies, and their *in vitro* cultivation, have been described.[32] Briefly, clone K is a TNF-sensitive subclone of L929 and clone 39 is an sTNFRI-secreting transfectant of L929 clone K. *E. coli*-derived murine TNF and human sTNFRI, as well as polyclonal goat anti-human sTNFRI antibodies were purchased from R & D Systems (Minneapolis, MN). Biotinylated rat anti-mouse TNF monoclonal antibody was purchased from Pharmingen (San Diego, CA).

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 accord with federal guidelines for animal experimentation.

Radiation - Radiation was administered at the Colorado State University Veterinary Teaching Hospital using a 6 MV clinical linear accelerator. Mice were restrained in plastic containers with bolus material above and below the animals. Radiation treatments were administered at the indicated dosages using SSD geometry.

Growth of Clone K and Clone 39 in C3H/HeN Mice - C3H/HeN mice, syngeneic to the L929 fibrosarcoma, were used in all *in vivo* transplantation studies. Tumor growth was evaluated in both irradiated and unirradiated mice. Groups of 10 mice were injected

subcutaneously in the dorsal lumbar region with 10^7 L929 clone K or clone 39 cells in 0.5 mL phosphate buffered saline (PBS). Tumor development was monitored visually and by palpation each day. Mice were sacrificed by CO_2 asphyxiation on day 13 post-injection. Tumors were resected and a portion of each was fixed in 10% neutral buffered formalin, sectioned, and stained with hematoxylin eosin for histological analysis. The statistical significance of differences between clone K and clone 39 tumor incidence was determined by Fisher's Exact Test.

Induction of Neutralizing Anti-sTNFRI Antibodies by Immunization - Each of the five mice was immunized with 10 μg of sTNFRI (produced and purified as described in Chapter 4) emulsified 1:1 with complete Freund's adjuvant. Each mouse received 4, 50 μl subcutaneous injections. Two groups of 5 control mice were immunized similarly; one group received subcutaneous injections of complete Freund's adjuvant (CFA) emulsified 1:1 with PBS, and the second group received PBS alone. Four weeks later, mice were boosted as above with 10 μg of sTNFRI emulsified 1:1 with incomplete Freund's adjuvant (IFA), adjuvant emulsion, or PBS. Six weeks later, mice were boosted intraperitoneally with 10 μg of sTNFRI in 0.5 mL of PBS or with PBS alone. Two days later, mice were exposed to 3.0 Gy of ionizing radiation, rested overnight, and challenged with 10^7 clone 39 cells. Irradiation, tumor challenge, monitoring of tumor growth, and histological and statistical analyses were performed as described above.

Quantification of Neutralizing Anti-sTNFRI Antibody - Sera from immunized mice were collected at sacrifice and neutralizing antibody to sTNFRI was quantified by capture ELISA. Wells of a 96-well plate were coated with goat anti-human sTNFRI antibody at 2 μg/mL and blocked with 2% bovine serum albumin (BSA) in PBS. Recombinant human sTNFRI was added to the wells at 0.5 μg/mL. Wells were incubated with serial dilutions of mouse sera and sequentially with 1 μg/mL of recombinant mouse TNF, 1.5 μg/mL biotinylated rat anti-mouse TNF and streptavidin-alkaline phosphatase. Conversion of the colorimetric substrate, para-nitrophenylphosphate, was quantified at 405 nm. Titers of immune and control sera were defined as the reciprocal dilution at which absorbances were equivalent to that of maximum TNF capture obtained in the absence of mouse serum.

RESULTS AND DISCUSSION

Growth of Clone K and Clone 39 in Irradiated Mice - Our initial studies were conducted in irradiated mice, an experimental design guided by prior reports concerning the tumorigenicity of L929 cells.[33] These prior studies have shown L929 cells to be non-tumorigenic in syngeneic recipients, yet to produce fibrosarcomas in sub-lethally irradiated (4.25 Gy) mice. Therefore, to permit the growth of both of our L929-derived cell lines, thereby maximizing the potential for observing differences in tumorigenicity between the two, we first evaluated their growth in irradiated animals. Injection of clone K cells into mice previously exposed to 3.0 Gy of whole-body irradiation, produced tumors in 3 of 10 mice beginning on day 6 post-injection (Figure 3.1A). All 3 of these tumors regressed by day 10 post-injection. Injection of clone 39 cells produced tumors in 7 of 10 irradiated mice, also beginning on day 6 post-injection. In contrast to the results observed with clone K, however, all of these tumors persisted to day 13 (the time of necropsy) (Figure 3.1A). Thus, sTNFRI-secretion significantly enhances the tumorigenicity of L929 cells ($p = 0.089$), and also allows those tumors which develop to persist longer than those produced by the parental cell line.

The growth of clone K-derived tumors in the present studies generally is consistent with prior reports regarding the tumorigenicity of L929 cells in irradiated recipients, though some important differences must be noted. The parental L929 cell line, from which we derived clone K, is reported to produce tumors in 64% of syngeneic mice, but only when those mice previously were exposed to 4.25 Gy of radiation.[33]

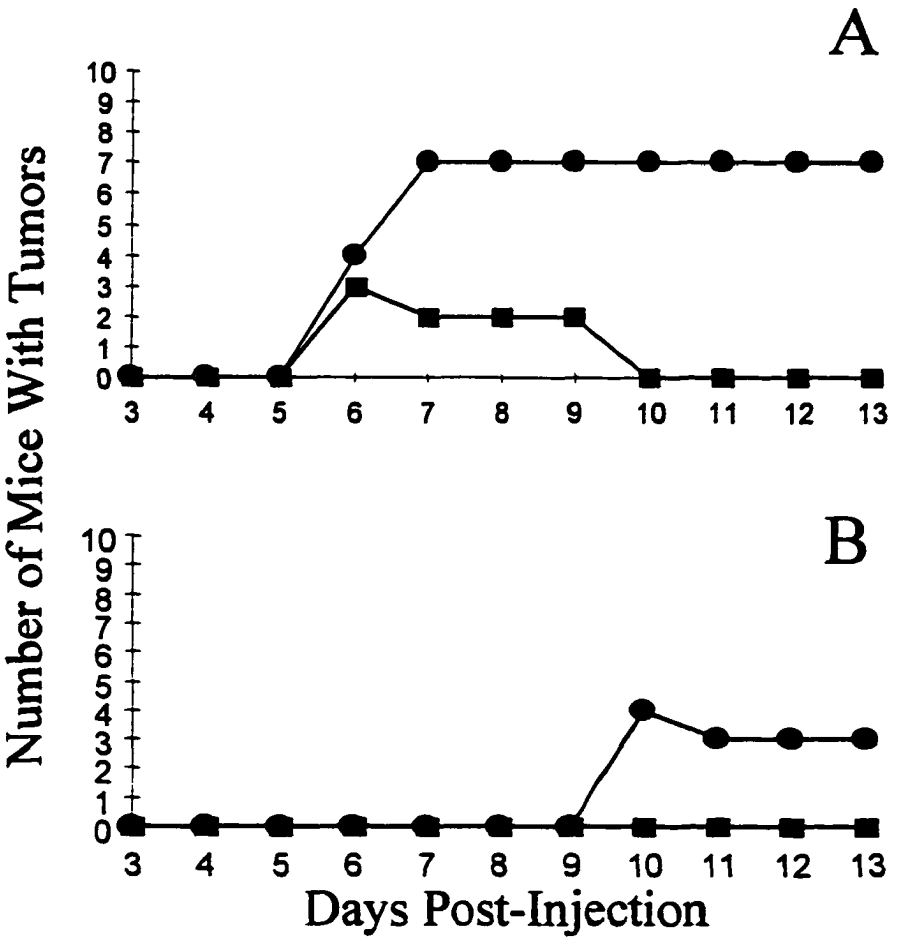


Figure 3.1. sTNFRI secretion enhances the tumorigenicity and persistence of fibrosarcomas *in vivo*. Clone K (■) and clone 39 (●) cells were injected subcutaneously into sublethally irradiated (3.1A) or unirradiated (3.1B) syngeneic mice. Data shown represent one of two identical experiments. The statistical significance of the difference between clone K and clone 39 tumor incidence in irradiated ($p = 0.089$) and unirradiated ($p = 0.025$) was determined by Fisher's exact test.

In the present studies, clone K produced tumors in only 30% of the recipient mice, yet these mice received only 3.0 Gy of irradiation prior to inoculation. This decrease in tumor incidence likely reflects the decreased radiation dose. This assertion is supported by our studies wherein a dose of 5.0 Gy permitted tumor development in 100% of mice inoculated with clone K (data not shown). It is likely that our use of an inoculum of 10^7 cells also reduced tumor incidence relative to the prior studies which used an inoculum of 3×10^7 cells.[33] Nevertheless, our observations confirm previous reports that there is no inherent defect in clone K which prevents its growth *in vivo;* rather, its growth is influenced by the immune status of the host. Further, not only does host immunocompetence influence tumor incidence, but also tumor persistence. Previous studies which used 4.25 Gy[33], as well as our studies which used 5.0 Gy (data not shown), gave rise to L929 tumors that persisted for 15 and 5 weeks, respectively. Yet, mice receiving 3.0 Gy in the present studies developed clone K tumors that spontaneously regressed in only 4 days. Thus, while the lower dose of radiation immunosuppressed recipient mice sufficiently to permit the establishment of the tumor mass, it likely permitted a more rapid regeneration of immunocompetence which led to the rejection of the tumors.

The extent of hemopoietic regeneration following radiation has been well documented.[34-42] Although the radiosensitivity of hemopoietic progenitor cells is influenced by radiation dose, fractionation, rate, and distribution[38], even in mice exposed to radiation doses as high as 5.0 Gy, the recovery of independent populations begins soon after exposure.[35] Lymphocytes are among the most radiosensitive of hemopoietic cells,

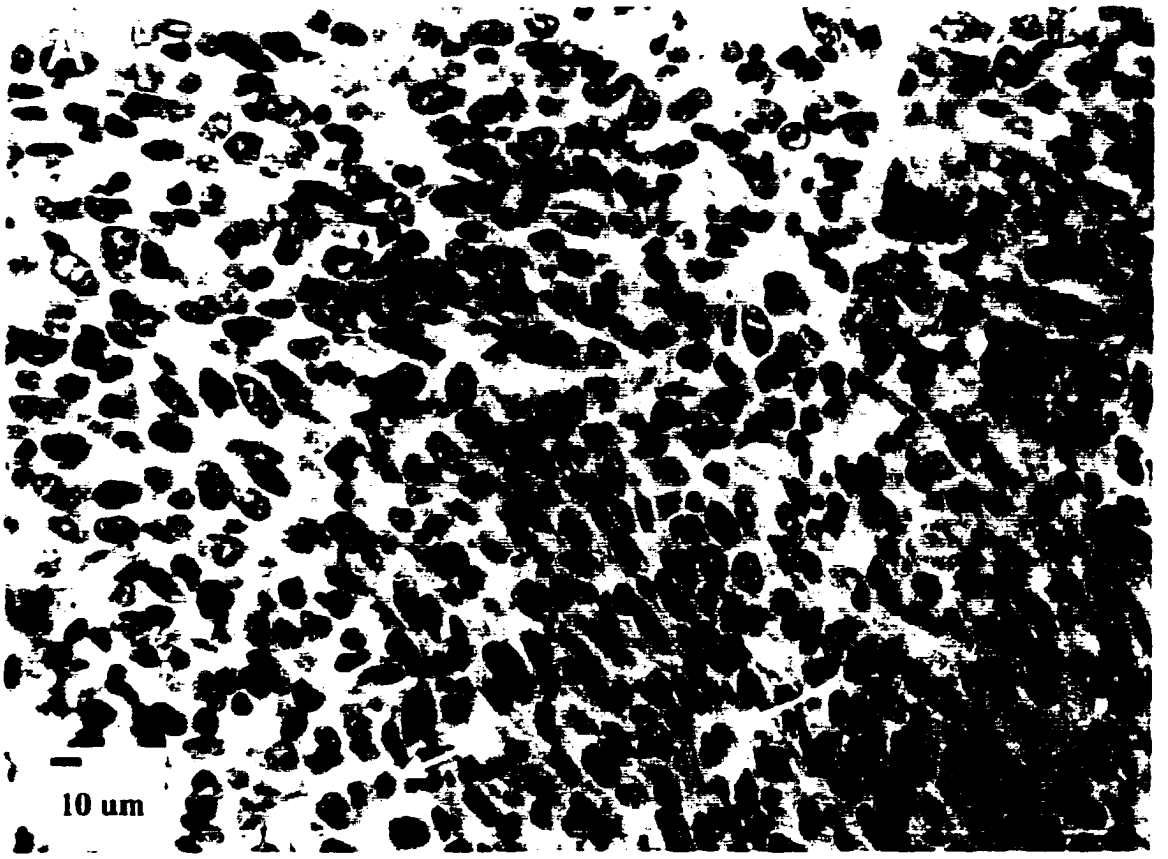
yet responsiveness of T lymphocytes to antigens such as pokeweed mitogen and sheep erythrocyte antigen fully returns to normal by four weeks following a dose of 3.0 Gy.[39] Likewise, recovery of antibody responses begins in the first week, and is largely complete by two weeks after exposure.[40,41] Auxiliary cells, such as macrophages and polymorphonuclear cells, are depressed in number due to the destruction of hemopoietic stem cells; nevertheless, their phagocytic and cytotoxic functions remain intact following kilorad doses of radiation.[42] Thus, it is likely that the rejection of clone K tumors, initially established in the absence of a fully competent immune system, reflects the restoration of the immune system during the two week course of these studies. Importantly, clone 39 fibrosarcomas persisted despite this reconstitution, suggesting that sTNFRI complements the radiation-induced immunosuppression and, thus, further facilitates tumor growth.

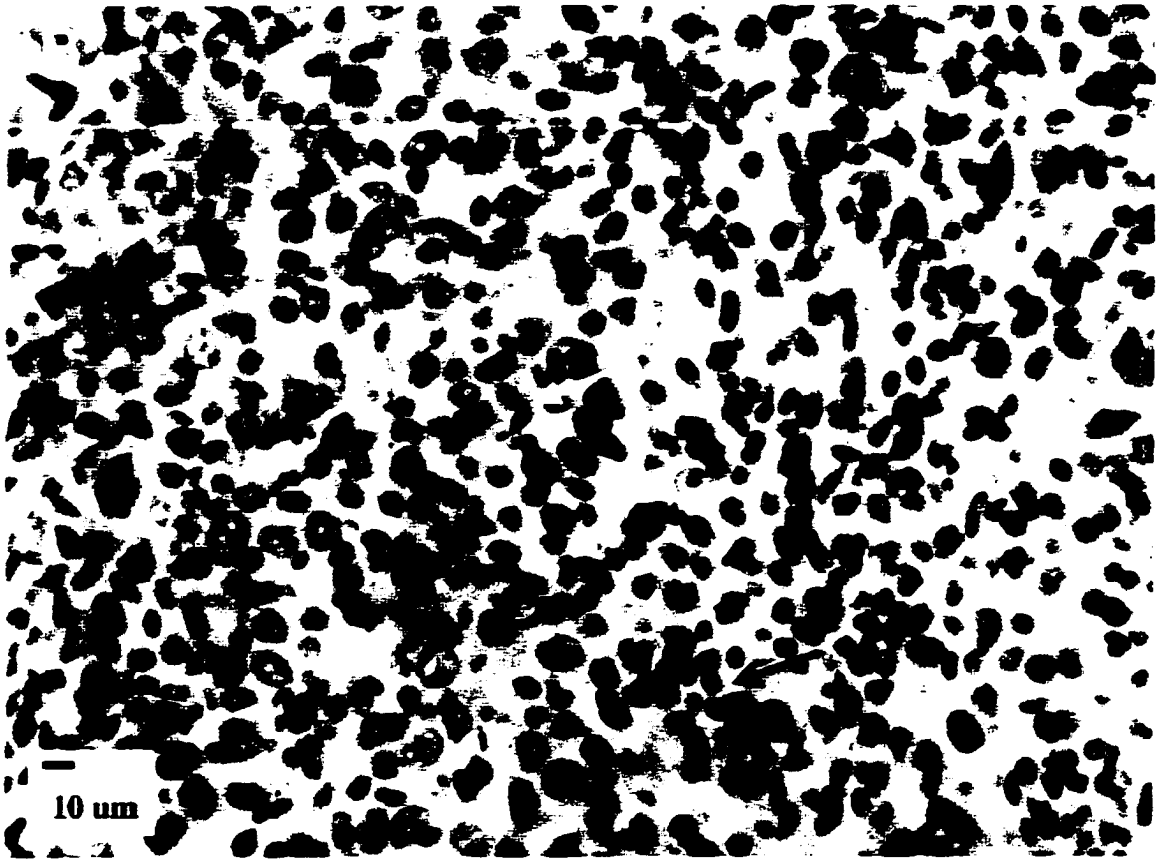
Tumor Growth of Clone K and Clone 39 in Unirradiated Mice - Whereas sTNFRI-secreting clone 39 cells exhibit enhanced tumorigenicity relative to unmodified clone K cells in irradiated animals, we evaluated whether or not sTNFRI secretion would enhance tumor survival in fully immunocompetent mice. Unirradiated mice were injected with clone 39 and clone K cells, and tumor development was monitored (Figure 3.1B). Ten days post-injection, 4 of the 10 mice injected with clone 39 cells developed tumors, 3 of which persisted to day 13. In contrast, none of the 10 mice injected with clone K cells developed tumors (Figure 3.1B), an observation consistent with lack of tumorigenicity for L929 reported by others.[33] Thus, even in the presence of a fully functional immune system the tumorigenicity of sTNFRI-secreting L929 cells is significantly increased ($p = 0.025$).

Not surprisingly, the overall incidence of clone 39 tumors in unirradiated mice was less than that observed in irradiated mice, and the day of onset was delayed by three days. These changes support the notion that the tumorigenicity and persistence of L929 cells reflect the balance between immunocompetence and immune suppression, though in this case the suppression results from the actions of sTNFRI. Immunosuppression by sTNFRI has been demonstrated recently in studies of allograft rejection[43], where it was shown that grafts engineered to secrete sTNFRI resisted rejection, whereas unmodified grafts were readily rejected.

Histological Analysis of Clone 39-Derived Tumors - Tissues were resected, formalin-fixed, sectioned, and stained with hematoxylin and eosin (Figure 3. 2). Histological analysis revealed that the masses were tumors of connective tissue origin, likely fibrosarcomas. In addition, and of greater importance, the histological analysis revealed that the clone 39-derived fibrosarcomas were persisting in the presence of a demonstrable leukocyte infiltrate. The clone 39 cells continued to proliferate, as evidenced by the 1-2 mitotic figures per high power field (i.e. 430x), despite the accumulation of leukocytes in multiple foci surrounding the tumor (Figure 3.2A). Though the nature of this leukocyte infiltrate has yet to be fully characterized, morphological evaluation (Figure 3.2B) indicated that two cell types predominated: (1) cells with multi-lobed, horseshoe-shaped nuclei and diffuse cytoplasm sprinkled with azurophilic granules which are most likely neutrophils, and (2) cells whose morphology included darkly staining nuclei and scant cytoplasm devoid of granules which is typical of lymphocytes.

Figure 3.2. sTNFRI-secreting fibrosarcomas survive *in vivo* despite a demonstrable leukocyte infiltrate. Tumor sections were prepared as described in the Materials and Methods and stained with hemotoxylin and eosin. Proliferative activity of the tumors is indicated by the presence of mitotic figures (arrow in 3.2A). Leukocytes infiltrating the tumor mass include neutrophils (open arrow in 3.2B) and lymphocytes (closed arrow in 3.2B). Two high powered fields (430x) are shown.





Reversal of Clone 39 Tumorigenicity Through the Induction of Antibodies that Neutralize sTNFRI - To confirm that the growth of clone 39 *in vivo* results from the inhibition of immune mechanisms by sTNFRI secreted by this cell line, sTNFRI-neutralizing antibodies were induced by active immunization and the growth of clone 39 cells in the presence of these antibodies was evaluated. The fact that the sTNFRI secreted by clone 39 is human sTNFRI made it possible to induce such antibodies in murine hosts. Quantities of human sTNFRI sufficient to immunize mice were produced recombinantly in *E. coli* and purified as described in Chapter 4. Immunized mice were irradiated with 3.0 Gy to maximize tumor incidence and then were challenged with clone 39 cells. Neutralizing antibody titers were determined as illustrated in Figure 3.3. As shown in Table 3.1, mice immunized with sTNFRI developed neutralizing antibody titers ranging from greater than 2,500 to greater than 10,000. Clone 39 failed to produce tumors in any of these sTNFRI-immunized mice, although the tumor incidence for these experimental conditions in the absence of immunization is predicted to be 70% (Figure 3.1A). A tumor incidence comparable to 70% was observed in control mice which had been immunized with adjuvant or PBS alone; clone 39 tumors developed in 4 of 5 mice in each of these two groups. As expected, none of the animals in these two groups possessed significant sTNFRI-neutralizing antibodies (Table 3.1). Thus, the binding of neutralizing antibodies to the sTNFRI secreted by clone 39, in effect, "inhibits the inhibitor" and prevents it from suppressing immune mechanisms that normally mediate the elimination of the tumor.

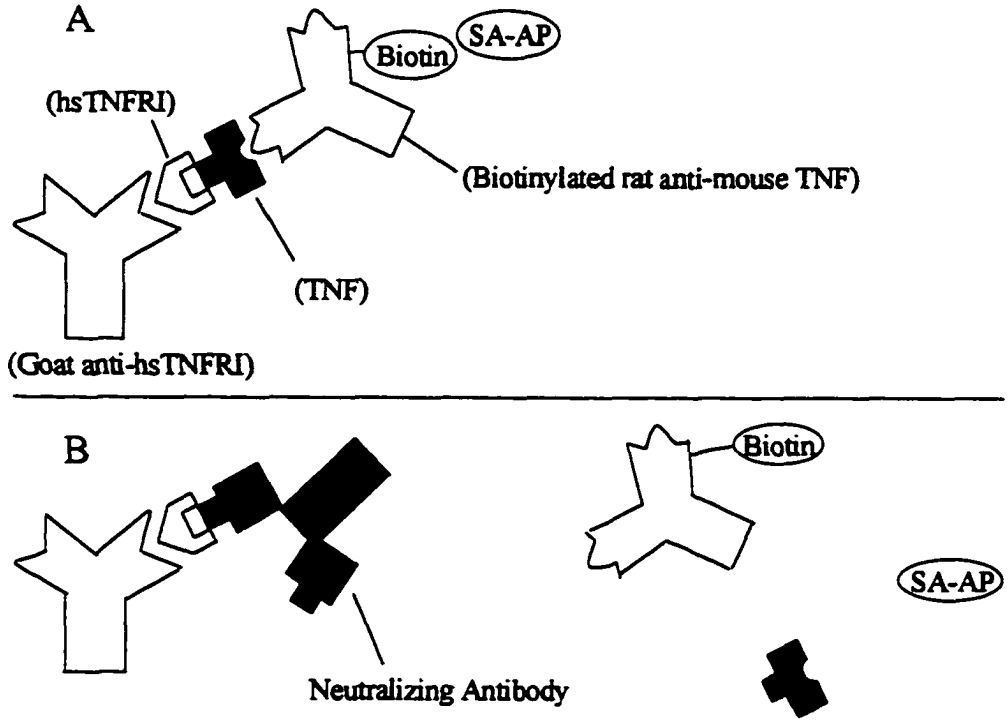


Figure 3.3. Schematic of the capture ELISA used to detect neutralizing anti-sTNFRI antibody. **(A)** Recombinant human sTNFRI was captured using goat anti-human sTNFRI antibodies. Subsequent capture of TNF by human sTNFRI was detected by the sequential addition of biotinylated rat anti-mouse TNF, streptavidin alkaline phosphatase, and the colorimetric substrate, p-nitrophenylphosphate. **(B)** Inhibition of TNF binding to captured sTNFRI by neutralizing antibody.

Table 3.1. The presence of serum anti-sTNFRI neutralizing antibodies correlates with decreased clone 39 tumor incidence. Mice were immunized and boosted with sTNFRI in adjuvant, adjuvant alone, or PBS alone prior to irradiation and challenge with clone 39. Neutralizing antibody titers in the sera of these mice were quantified by capture ELISA as described in the Materials and Methods and as illustrated in Figure 3.3. The statistical significance of the difference between immune and control mice ($p = 0.014$) was determined by Fisher's exact test. n.d., not determined.

Immunization	Tumor Incidence	Titer of α-sTNFR Neutralizing Antibody
PBS alone	4/5	<5
		<5
		<5
		<5
		<5
Adjuvant alone	4/5	<5
		<10
		<20
		<5
		<20
sTNFRI	0/5	>10,000
		>10,000
		> 2,500
		>10,000
		n.d.

It could be argued that the rejection of clone 39 by immune mice results rather from the induction of immune responses to human sTNFRI, which by virtue of its production by this mouse fibrosarcoma may in effect serve as a tumor-associated antigen (TAA). In this context, it is possible that immune responses induced to this TAA mediated the rejection of clone 39 without the requirement for neutralization of sTNFRI. Careful consideration of the various mechanisms induced by sTNFRI immunization and of the ways in which those mechanisms kill tumors, however, suggest that this is unlikely to be the case. Antibody-dependent cellular cytotoxicity (ADCC), for example, has been implicated in tumor lysis[44], yet for ADCC to be operative in the present model would require the association of sTNFRI with the membrane of the clone 39 target cell so that anti-sTNFRI antibody binding could facilitate the attachment of F_c receptor-bearing cytotoxic effectors to the target cells. Alternatively, class I MHC-restricted $CD8^+$ cytotoxic T cells, specific for sTNFRI, may contribute to the elimination of clone 39 cells in immunized mice, since the induction of TAA-specific $CD8^+$ cytotoxic T cells by immunization has proved effective at reducing the tumorigenicity of a subsequent challenge.[45,46] Those studies, however, have used transfectant cell lines engineered to produce cytosolic, endogenous TAAs to achieve their effects. In the present studies, immunization with purified sTNFRI, an exogenous antigen, in complete Freund's adjuvant is not predicted to be presented by class I MHC and, thus, will be ineffectual at eliciting $CD8^+$ T cell responses. Rather, class II MHC-restricted $CD4^+$ T cell responses are likely to represent the predominate T cell response induced by this immunization regimen. sTNFRI-specific $CD4^+$ effectors are unlikely to be directly responsible for the elimination of clone 39 cells, however, since

these fibrosarcoma cells lack class II MHC. sTNFRI secreted by clone 39 may be acquired by professional APCs and, thus, stimulate sTNFRI-specific $CD4^+$ T cells to enhance natural killer cell activity through the production of IL-2 and IFN-γ. While we cannot completely exclude that this mechanism contributes to the elimination of clone 39 in sTNFRI-immunized mice, the fact that clone 39 is largely resistant to NK/LAK-mediated lysis (Chapter 2) minimizes the importance of this mechanism in the rejection of clone 39 tumors. Rather, the most likely interpretation of these studies is that neutralizing antibody is responsible for the effects. Thus, these findings correlate the inhibition of sTNFRI with the reversal of clone 39 tumorigenicity, formally implicating sTNFRI production by clone 39 cells as the primary basis for the conversion of clone K cells from a non-tumorigenic to a tumorigenic phenotype.

The *in vivo* studies described in this chapter, as well as the *in vitro* studies described in Chapter 2, support the interpretation that sTNFRI is responsible for the differential tumorigenicity of clone K and clone 39. Interpretation of transfection experiments must be guarded, however, since it is possible that single cell cloning of transfectants selects for cells which are inherently different from the parental cell line for reasons other than the production of the heterologous gene. For example, clone 39 might represent an isolated variant of clone K which inherently is more tumorigenic by virtue of replicating ter and/or being resistant to immunological destruction without regard to the production of sTNFRI. This appears unlikely, however, since the growth rates of clone K and clone 39 cells are virtually identical with doubling times of 23.25 ± 2.7 hours and 25.23 ± 2.2 hours, respectively. If a difference does exist, it is that clone 39 grows more

slowly than clone K. Moreover, while clone 39 does exhibit enhanced resistance to direct lysis by TNF and to cellular cytolysis by CTLs and NK cells, clone 39 is not completely resistant to immunological destruction as evidenced by the elimination of clone 39 cells once injected into mice with anti-sTNFRI antibodies (Table 3.1). Therefore, the enhanced tumorigenicity of clone 39 cells cannot be explained by an inherent difference in their cell cycling nor can it be attributed to an inherent defect in their susceptibility to immunological destruction. Rather, it clearly reflects the role that sTNFRI plays in preventing tumor cell elimination; tumorigenicity was conferred upon a non-tumorigenic fibrosarcoma cell line by inducing it to produce sTNFRI. Confirmation that sTNFRI production was responsible for the conversion from a non-tumorigenic to a tumorigenic phenotype was demonstrated by the reversal of the acquired tumorigenicity by antibodies which neutralize sTNFRI. These findings are completely complementary and, together, illustrate the importance of sTNFRI in tumor growth and persistence.

The reversal of tumorigenicity by neutralizing anti-sTNFRI antibodies suggests that the inhibition of sTNFRI *in vivo* may be a viable therapeutic strategy for cancer. This was made possible in our experimental model since the sTNFRI produced by the transformed mouse cell line was of human origin. Direct implementation of an analogous strategy in mammals with spontaneous tumors, however, is anticipated to be problematic. Immunization with sTNFRI of the subject is not likely to induce significant antibody responses since the sTNFRI necessarily would be of self origin. Were neutralizing antibodies to be induced, for example through the use of a strong adjuvant, some of those antibodies certainly would cross-react with the membrane form of the TNFRI. Certain of

those antibodies would be expected to be TNF agonists and, thus, would cause profound systemic toxicity similar to that associated with elevated plasma TNF levels. Thus, while immunization with sTNFRI is not likely to be useful, it may be possible to passively infuse reagents which have been derived *ex vivo* and which neutralize the binding of sTNFRI to TNF and which do not bind to the membrane form of the TNFRI.

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CHAPTER 4
MONOCLONAL ANTIBODIES REACTIVE WITH SOLUBLE TUMOR NECROSIS FACTOR RECEPTOR TYPE I AS POTENTIAL THERAPEUTIC AGENTS

ABSTRACT

Soluble tumor necrosis factor receptor type I (sTNFRI) has been shown *in vitro* to inhibit multiple anti-tumor immune mechanisms, including direct lysis by TNF as well as cellular cytolysis mediated by lymphokine activated killer cells and cytotoxic T lymphocytes. Furthermore, *in vivo*, sTNFRI has been demonstrated to augment the tumorigenicity and persistence of transformed cell lines. These observations suggest the inactivation of sTNFRI in tumor-bearing hosts, through the infusion of neutralizing antibodies, as a possible immunotherapeutic strategy for cancer. Toward that end, we have produced a monoclonal antibody that neutralizes the binding of sTNFRI to TNF, and describe its initial evaluation as an immunotherapeutic agent in a mouse transplantation model.

INTRODUCTION

One form of cancer biotherapy involves the enhancement of anti-tumor immune mechanisms through the addition of biological response modifiers. The infusion of supraphysiological levels of TNF, alone or in combination with other cytokines (e.g. interferon-α or IL-2), has resulted in the partial regression of some transplanted human tumors in mice.[1-4] Despite this limited success, the administration of high doses of recombinant TNF has failed to consistently induce complete responses, either through the direct lysis of tumors and the associated vascular endothelium, or through the enhancement of anti-tumor immune responses.[5,6] Similarly, human clinical trials of infusional TNF have not met the expectations engendered by preclinical studies.[7] Consequently, it has been suggested that the delivery of TNF by intravenous methods does not sufficiently approximate the physiological levels requisite for optimal induction of long-lived anti-tumor immunity.

The exquisite regulation of TNF *in vivo* was first suggested when investigators isolated naturally-occurring inhibitors of the cytokine in the urine and serum of humans.[8-10] These inhibitors are soluble receptors for TNF (sTNFRs), which represent the proteolytically cleaved extracellular domains of the membrane-associated TNF type I and type II receptors. Subsequently, sTNFRI[11-12] and sTNFRII[12-13] (R. Lentz personal communication) have been found in the plasma and in the ultrafiltrates of cancer patients. Production of sTNFRs in cancer patients increases during advanced stages of malignancy and declines during stages of remission.[12-15] Moreover, the levels of sTNFRs in the

circulation of tumor-bearing hosts increase in response to the infusion of TNF and IL-2 which may explain the limited clinical success of these immunotherapeutic strategies.[16-19]

Since these sTNFRs represent receptor analogs which can compete with cell surface receptors for TNF binding, and because TNF is a critical player in anti-tumor immunity, the distribution of sTNFRs relative to TNF likely affects the course and outcome of malignant disease. Indeed, purified sTNFR type I (sTNFRI) suppresses the cytotoxic/cytostatic activities of TNF *in vitro*[11,20-22] and inhibits the necrotizing properties of TNF when co-injected directly into tumors grown as subcutaneous transplants in mice.[21] In addition, sTNFRI suppresses CTL- and NK/LAK cell-mediated cytolysis of tumor targets *in vitro*.[22] Collectively, these observations suggest that *in vivo* sTNFRI disarms multiple immune mechanisms essential to the eradication of tumors.

Recently we have developed an *in vivo* mouse transplantation model to formally evaluate the importance of sTNFRI in tumor survival and persistence. A mouse fibrosarcoma cell line was engineered to secrete human, rather than mouse, sTNFRI.[22] The unmodified fibrosarcoma is non-tumorigenic in syngeneic hosts, however secretion of sTNFRI by the engineered fibrosarcomas dramatically enhances their tumorigenicity and persistence *in vivo*. The tumorigenicity imparted to these modified fibrosarcomas, however, is reversed by neutralizing antibodies induced by immunization with sTNFRI prior to tumor challenge. These data suggest that neutralization of sTNFRI *in situ* unblocks once inhibited, but effective, mediators of tumor rejection. Therefore we propose that the specific inactivation of sTNFRI, rather than the infusion of additional TNF, may sufficiently shift the cytokine balance in the tumor microenvironment to favor

TNF-mediated tumor destruction. In the present studies, we describe the development of a monoclonal antibody that neutralizes sTNFRI binding to TNF and the initial evaluation of this antibody as an immunotherapeutic agent for the specific inactivation of sTNFRI in tumor-bearing hosts.

MATERIALS AND METHODS

Mammalian Cell lines, Bacterial Strains, and Reagents - Clone K is a TNF-sensitive subclone of the C3H/HeN fibrosarcoma, L929, and clone 39 is a transfectant of L929 clone K that secretes human sTNFRI. The generation of these cell lines and their *in vitro* cultivation has been described.[22] The Balb/c plasmacytoma, SP2/0 (CRL 8006), and the anti-TNP hybridoma, 1B7.11 (TIB 191), were obtained from the American Type Culture Collection (ATCC; Rockville, MD). These cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum and penicillin/streptomycin, each at 100 μg/mL (complete DMEM growth medium).

The prokaryotic expression plasmid, pet24a, which permits the production of fusion proteins with a carboxy-terminal $(His)_6$ tag, and the corresponding *E. coli* host strain, BL21(λ)DE3, were obtained from Novagen (Madison, WI). *Escherichia coli*-derived murine TNF 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). Alkaline-phosphatase conjugated, goat anti-mouse polyvalent Ig (IgG, IgA, IgM) was purchased from Sigma (St. Louis, MO).

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 accord with federal guidelines for animal experimentation.

Construction of the Prokaryotic sTNFRI Expression Construct - The gene encoding the soluble form of the human type I TNF receptor was isolated by PCR using the pcDNA3/ sTNFRI expression construct (Chapter 2) as template DNA. Oligonucleotide primers were designed to isolate the sTNFRI gene in a form suitable for cloning into the pet24a expression vector. The sense primer, 5'-GCTGGATCCGATAGTGTGTGTCCC-3', anneals to nucleotides encoding amino acid residues 1-5 of the mature polypeptide and, thus, excludes the mammalian leader sequence.[23] This primer also includes a *Bam* HI restriction site (underlined) to facilitate cloning. The antisense primer, 5'-GGAAGCTTATTCTCAATCTGGGGTAG-3', anneals to sequences encoding amino acid residues 156-161 of the sTNFRI polypeptide. This primer contains two additional nucleotides immediately downstream of the sTNFRI coding region to maintain a common reading frame between the sTNFRI gene and the $(His)_6$ tag in the vector. This primer also contains a *Hind* III restriction site (underlined) to facilitate cloning. PCR was conducted for 40 cycles with the following profile (1 minute each): 95°C denaturation, 60°C annealing, and 72°C extension. The resulting 500 base-pair PCR fragment was gel-purified and was cloned without modification into T-tailed[24] pBluescript SK^- (Stratagene, La Jolla, CA). The sTNFRI gene was liberated from a pool of recombinant pBluescript plasmids by digestion with *Bam* HI and *Hind* III, and was ligated to *Bam* HI/*Hind* III digested pet24a expression plasmid. Ligation products were introduced by

were selected on LB-agar plates containing 25 μg/ml kanamycin. The fidelity of the recombinant plasmids was verified by restriction endonuclease digestion and PCR amplification.

Expression and Immunoblot Analysis of Recombinant sTNFRI - The sTNFRI expression plasmid, as well as the non-recombinant pet24a vector, were introduced into the bacterial host strain BL21(λ)DE3 by electroporation, and transformants were selected for kanamycin resistance. To rapidly assess the relative expression of recombinant proteins in this system, lysates of bacteria transformed with the sTNFRI recombinant plasmid and with the vector control were produced as described.[25] Briefly, two milliliter cultures were grown to an OD_{600} of 0.4-0.5. Each culture was split in half, 1 mM IPTG was added to 1mL of each, and all four cultures were grown an additional two hours at 37°C. Following this induction period, bacteria were recovered by centrifugation, and the pellets were washed once with 0.5 mL ice-cold 50 mM Tris-HCl (pH 7.4). Bacterial pellets were resuspended in 25 μL of dH_2O, and 25 μL of 2x SDS-PAGE sample buffer were added. The samples were boiled for 5 minutes, centrifuged, and the supernatants were resolved, in duplicate, by SDS-PAGE in a 17% gel. One set of the samples was visualized by silver staining. The second sample set was transferred electrophoretically to nitrocellulose. The filter was blocked with 2% BSA in PBS, and was incubated with 2 μg/mL of biotinylated goat anti-sTNFRI polyclonal antibody, previously adsorbed against an immobilized wild-type λgt11 lysate to remove *E. coli*-reactive antibodies. Binding of the antibody to the recombinant sTNFRI was detected by subsequent addition of streptavidin-alkaline

phosphatase, and visualized using the colorimetric substrates BCIP (16.5% w/v) and NBT (33% w/v) (Sigma, St. Louis, MO) in 100 mM Tris-HCl (pH9.5), 100 mM NaCl, 5 mM $MgCl_2$.

Large-Scale Production and Purification of Recombinant sTNFRI - Cultures of the sTNFRI transfectant were grown in LB supplemented with 25 μg/mL of kanamycin to an OD_{600} of 0.4-0.5 at 37°C, supplemented with 1mm IPTG and grown an additional 2 hours at 37°C. sTNFRI produced in *E. coli* by this method is sequestered in inclusion bodies. Therefore, denatured sTNFRI was isolated from these inclusion bodies by the method of DeLamarter.[26] Briefly, induced cells were harvested by centrifugation at 5000 x g for 5 minutes and were resuspended in 100 mM Tris-HCl (pH7.5), 50 mM EDTA [100 mL per 30 g of *E. coli* (wet weight)] using a Polytron homogenizer. Cell walls were weakened by osmotic shock through the addition of sucrose (25.6 g per 30 g *E. coli)* and digested with lysozyme (30 mg per 30 g *E. coli)* for 30 minutes at 30°C. Cells were lysed using a French press (8000 psi), and the soluble components were separated from the cellular debris and sTNFRI aggregates by centrifugation at 10,000 x g for 30 minutes. The major membrane proteins and the oligopolysaccharides were removed from the pellet by washing three times in 100 mM Tris-HCl (pH7.5), 50 mM EDTA, 0.75 M guanidine hydrochloride, 1% (v/v) Tween 20. The pellet was washed one final time in PBS.

Inclusion bodies were resuspended in binding buffer [20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, 6 M urea] containing 5 mM imidazole and incubated on ice for 1 hour. Insoluble material was removed by centrifugation at 39,000 x g for 20 minutes, and the

supernatant was filtered through a 0.45 μm filter and loaded on a chelated nickel column pre-equilibrated with binding buffer containing 5 mM imidazole. Extraneous proteins were removed by washing the column in binding buffer containing 20 mM imidazole, and recombinant sTNFRI was eluted from the column with binding buffer containing 200 mM imidazole. The relative purity of the eluted protein was determined by silver stained SDS-PAGE and the identity of the protein was confirmed by immunoblot analysis as described above.

Immunization of Mice with Recombinant sTNFRI and Production of Hybridomas - C3H/HeN mice were immunized subcutaneously with 10 μg of recombinant sTNFRI emulsified 1:1 with CFA. Four weeks later, mice were boosted subcutaneously with 10 μg of recombinant sTNFRI emulsified 1:1 with IFA, followed three weeks later by an intravenous (via the tail vein) boost with 15 μg of recombinant sTNFRI in PBS. The next day, mice were sacrificed and their spleens were harvested. Red blood cells were lysed in 0.83% ammonium chloride, and the remaining cells were washed extensively with serum-free DMEM. Splenocytes were fused with the SP2/0 fusion partner using the methods and medium of Kennett[27], and the hybridomas were selected in HAT medium for 10-14 days prior to screening. After screening, hybridomas were weaned from selection medium and cultured thereafter in complete DMEM growth medium.

Characterization of Monoclonal Antibodies by ELISA - Hybridomas producing monoclonal antibodies reactive to sTNFRI were identified by capture ELISA. Wells of a 96-well plate were coated with 2 μg/mL of goat anti-sTNFRI antibody and blocked with

2% (w/v) BSA in PBS. Wells received one of three different sTNFRI preparations: denatured, prokaryotic protein (50 ng/mL) purified as above; commercial, native, prokaryotic sTNFRI (50 ng/mL), or native, eukaryotic sTNFRI (clone 39 supernatant containing approximately 25 ng/mL). Negative control wells received all reagents except sTNFRI. After the addition of hybridoma supernatants, bound anti-sTNFRI antibodies were detected by the sequential addition of alkaline phosphatase conjugated, polyvalent goat anti-mouse Ig (IgG, IgA, IgM) at 2 μg/mL and ρ-nitrophenylphosphate. Conversion of the colorimetric substrate was quantified at 405 nm, and the background absorbance of the negative control wells was subtracted from that of all test samples. Isotypes of the monoclonal antibodies produced by cloned and uncloned hybridomas were determined using a mouse immunoglobulin isotyping kit (Pharmingen, La Jolla, CA) according to the manufacturer's specifications.

Identification of Neutralizing anti-sTNFRI Monoclonal Antibodies Using TNF Bioassays- Anti-sTNFRI antibodies capable of neutralizing the binding of sTNFRI to murine TNF were identified using *in vitro* bioassays.[28] sTNFRI-secreting clone 39 cells were seeded in 96-well plates at 2.25 x 10^4 cells/well and allowed to adhere overnight. Triplicate wells were incubated with supernatants from individual hybridoma cell lines for 30 minutes at 37°C prior to the addition of actinomycin D (1 μg/mL) (Sigma, St. Louis, MO) and 50 pg/mL recombinant murine TNF. Control wells were incubated with SP2/0 supernatant for 30 minutes at 37°C prior to the addition of actinomycin D and TNF. After 24 hours, media were aspirated and viable cells were stained with 1% aqueous crystal

violet for 5 minutes, 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:

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

Antibodies found to neutralize sTNFRI were purified by protein G column chromatography (Pierce, Rockford, IL) according to the manufacturer's specifications, and were retested in the neutralization bioassay as above except targets were pre-incubated with complete DMEM growth medium alone or growth medium containing varying concentrations of the purified antibody. After a 30 minute incubation at 37°C, actinomycin D and TNF were added, and cytotoxicity was determined as above.

Anti-sTNFRI Antibodies in the Treatment of Clone 39 Tumors - Groups of 20 mice were injected intraperitoneally with 0.5 mg of purified anti-sTNFRI (1D3.B1) or isotype control antibodies (1B7.11) in PBS. An additional group of 10 mice was injected with PBS alone. Four hours later, mice were injected subcutaneously in the dorsal lumbar region with 10^7 clone 39 cells in 0.5 mL PBS. On days 3 and 6 post-injection, mice received an additional 0.25 mg of the appropriate antibody or PBS alone administered intraperitoneally. Tumor development was monitored visually and by palpation each day.

Expression, Purification, and Characterization of the Recombinant sTNFRI - Quantities of sTNFRI sufficient for immunization were produced recombinantly in *E. coli*. The gene encoding sTNFRI was cloned into the pet24a expression plasmid (Figure 4.1) which has several features that make it useful for the production of large quantities of recombinant protein.

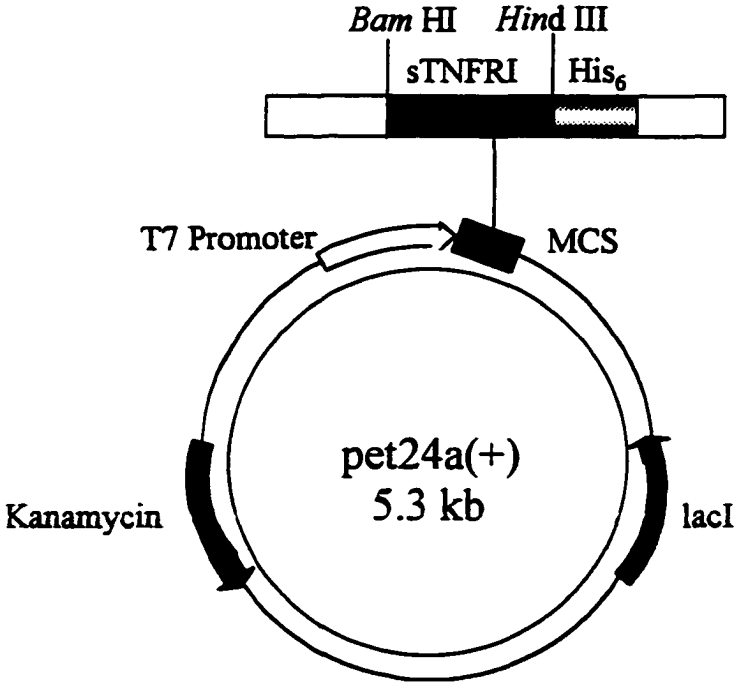

Figure 4.1. Schematic of the pet24a prokaryotic expression plasmid. The sTNFRI gene cloned into the multiple cloning site of pet24a creates a fusion with sequences encoding the $(His)_6$ tag. Expression is under the control of the bacteriophage T7 promoter. The vector contains a kanamycin resistance gene for the selection of transformants.

First, cloning into the multiple cloning site of the pet24a vector creates an in-frame fusion between an initiator methionine, the sTNFRI gene, and residues encoding the carboxy-terminal $(His)_6$ tag. This tag permits recombinant proteins to be purified readily using nickel column chromatography. Second, the target gene is cloned into the vector under the control of the strong bacteriophage T7 promoter. The BL21(λ)DE3 host strain contains a chromosomal copy of the T7 RNA polymerase gene under *lacUV5* control and, thus, its expression can be induced by the addition of IPTG. Induction of T7 RNA polymerase expression results in production of the sTNFRI polypeptide at levels up to 50% of the total cell protein, yields which are not possible when producing foreign proteins in a eukaryotic cell system. These production levels, combined with the significant degree of disulfide bond formation in the cysteine-rich sTNFRI, however, caused much of the sTNFRI produced in *E. coli* to be sequestered in inclusion bodies. Although protein recovered from inclusion bodies is fully denatured, it nevertheless can serve as an immunogen in the generation of antibodies which recognize epitopes exposed on the native receptor.

The production of sTNFRI using the pet24a expression system is illustrated in Figure 4.2. SDS-PAGE of lysates from BL21(λ)DE3 transformed with sTNFRI recombinant plasmid contain a 21 kD protein, the prevalence of which is markedly enhanced upon induction (lane 4). This protein also was present in uninduced cultures due to the "leakiness" on the *lacUV5* promoter (lane 3). The 21 kD protein was not present, however, in either uninduced or induced cultures transformed with the control plasmid (lanes 1 and 2, respectively). This 21 kD protein was fully retained on the chelated nickel

column as evidenced by the absence of this protein in the column effluent (lane 5), and its presence in eluate (lane 6). The purity of the $(His)_6$ tagged protein was estimated to be >95%.

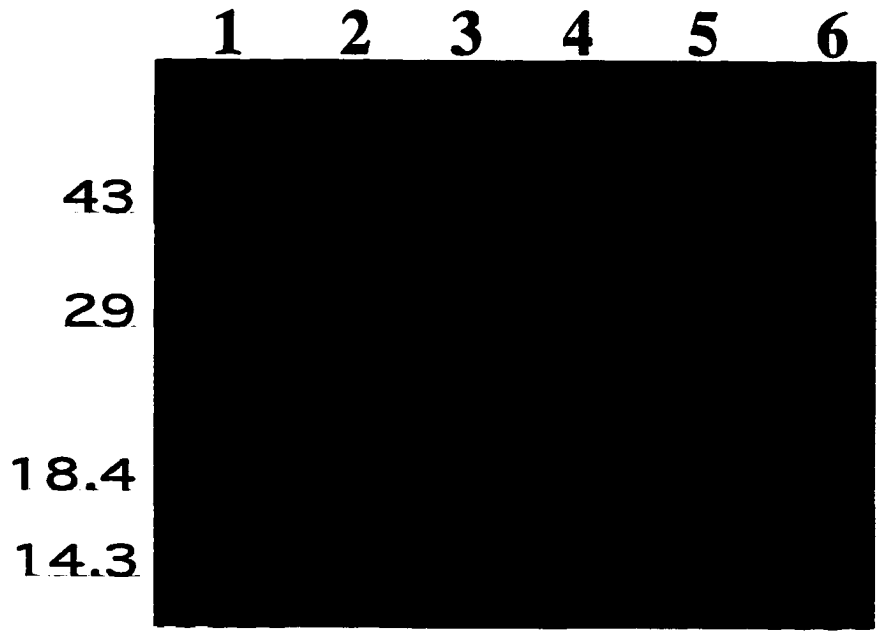

Figure 4.2. SDS-PAGE of lysates produced from bacteria transformed with pet24a expression constructs. Lane 1, pet24a uninduced; Lane 2, pet24a induced; Lane 3, pet24a-sTNFRI uninduced; Lane 4, pet24a-sTNFRI induced; Lane 5, Ni column effluent; Lane 6, Ni column eluate. Mobilities of molecular weight standards (in kD) are indicated.

To confirm that the induced 21 kD protein was in fact sTNFRI, immunoblot assays were performed on SDS lysates of the cultures described above (Figure 4.3). Lysates of induced (lane 3), as well as uninduced (lane 4), cultures of the sTNFRI transfectants contained a 21 kD protein which was reactive with goat anti-sTNFRI antibody, while no reactive proteins were detected in the lysates of pet24a-transfected cultures (lanes 1 and 2). As above, the intensity of reactive protein was increased dramatically by IPTG induction. The 21 kD protein was not present in the effluent (lane 5), but was eluted from the column as evidenced by its reactivity with the goat anti-sTNFRI antibody (lane 6).

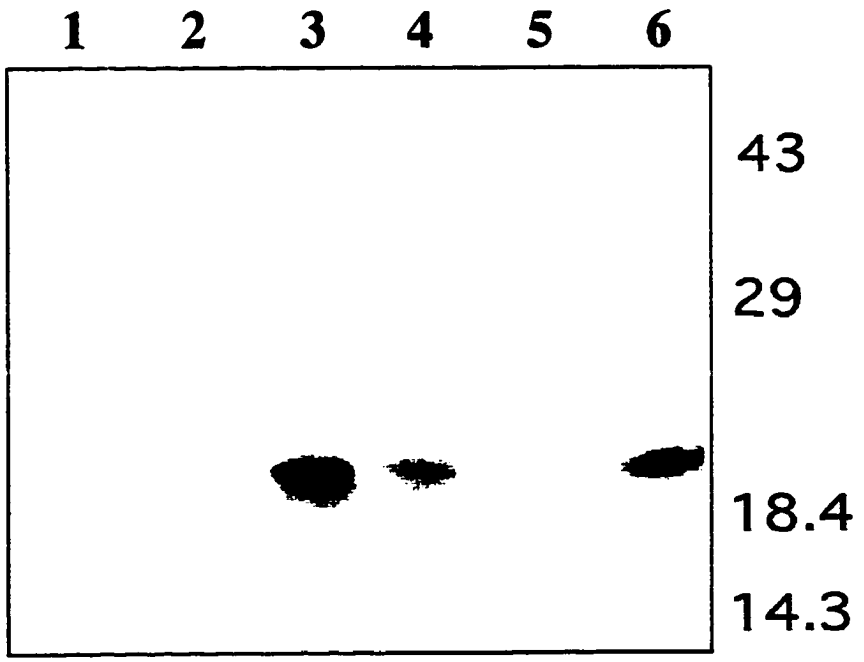

Figure 4.3. Immunoblot of lysates produced from bacteria transformed with pet24a expression constructs. Lane 1, pet24a uninduced; Lane 2, pet24a induced; Lane 3, pet24a-sTNFRI induced; Lane 4, pet24a-sTNFRI uninduced; Lane 5, Ni column effluent; Lane 6, Ni column eluate. Mobilities of molecular weight standards (in kD) are indicated.

Characterization of anti-sTNFRI Monoclonal Antibodies - Capture ELISAs were used to identify the hybridomas that produce monoclonal antibodies reactive with sTNFRI (Table 4.1). Five hybridoma cell lines (1A8, 3B11, 1H7, 3E6, and 1D3) produced monoclonal antibodies that bound specifically to the denatured form of sTNFRI used in the immunizations. In addition, four of these five monoclonal antibodies (1A8, 3B11, 1H7, and 3E6) bound to the commercial preparation of native, prokaryotic sTNFRI. These data indicate that the antibodies bind to linear epitopes of sTNFRI which are exposed on both the denatured and native forms of the protein produced in *E. coli*. Furthermore, despite the potential blockage of epitope sites by glycosylation, four of the five monoclonal antibodies (3B11, 1H7, 3E6, and 1D3) recognized the eukaryotic sTNFRI produced by clone 39.

Table 4.1. Binding characteristics and isotypes of the anti-sTNFRI antibodies. Binding of the anti-sTNFRI monoclonal antibodies to prokaryotic and eukaryotic sTNFRI is indicated. Isotypes were determined using a mouse immunoglobulin isotyping kit as described in the Materials and Methods.

	Antigen			
Hybridoma Designation	Denatured Prokaryotic	Native Prokaryotic	Native Eukaryotic	Isotypes
1A8	+	+	-	IgM, IgA, IgG
3B11	+	+	+	IgM
1H7	+	+	+	IgM
3E6	+	+	+	IgM, IgA
1D3	+	–	+	IgG_1
1D3.B1	+	–	+	IgG_1

It is noteworthy that although monoclonal antibody 1D3 fails to recognize native, prokaryotic sTNFRI, it does bind to the eukaryotic version of the protein. The fact that several of these antibodies bind to sTNFRI derived from mammalian cells indicates that they may serve as inhibitors of sTNFRI produced by tumors *in vivo*.

The immunoglobulin isotypes present in the supernatants derived from these five hybridoma cultures are indicated in Table 4.1. Several of the supernatants contain multiple antibody isotypes, an observation in keeping with the fact that the supernatants tested were produced from uncloned hybridoma lines.

Identification of Neutralizing anti-sTNFRI Monoclonal Antibodies using TNF Bioassays- Monoclonal antibodies were evaluated in *in vitro* bioassays for their ability to neutralize the protective effects of sTNFRI (Figure 4.4). As described in Chapter 2, clone 39 cells are 10-fold more resistant to TNF-induced lysis than the parental line, clone K. Antibodies which bind to the active site of sTNFRI and, thus, prevent its binding to TNF are expected to reduce this protective effect as evidenced by an increase in cytotoxicity at particular TNF concentrations. sTNFRI-secreting clone 39 targets were pre-incubated with culture supernatants from individual hybridomas prior to the addition of TNF. Interestingly, specific lysis of clone 39 targets in the presence of four of these five monoclonal antibodies (1A8, 3B11, 1H7, and 3E6) was less than in the presence of control supernatant from the SP2/0 plasmacytoma (Figure 4.4). While lysis of clone 39 targets incubated with SP2/0 supernatant was 61%, the percent cytotoxicity decreased to 5-29% when targets were pre-incubated with supernatants containing the anti-sTNFRI

monoclonal antibodies. Thus, these four antibodies do not neutralize sTNFRI; rather, they enhance the sTNFRI-mediated protection afforded clone 39 cells.

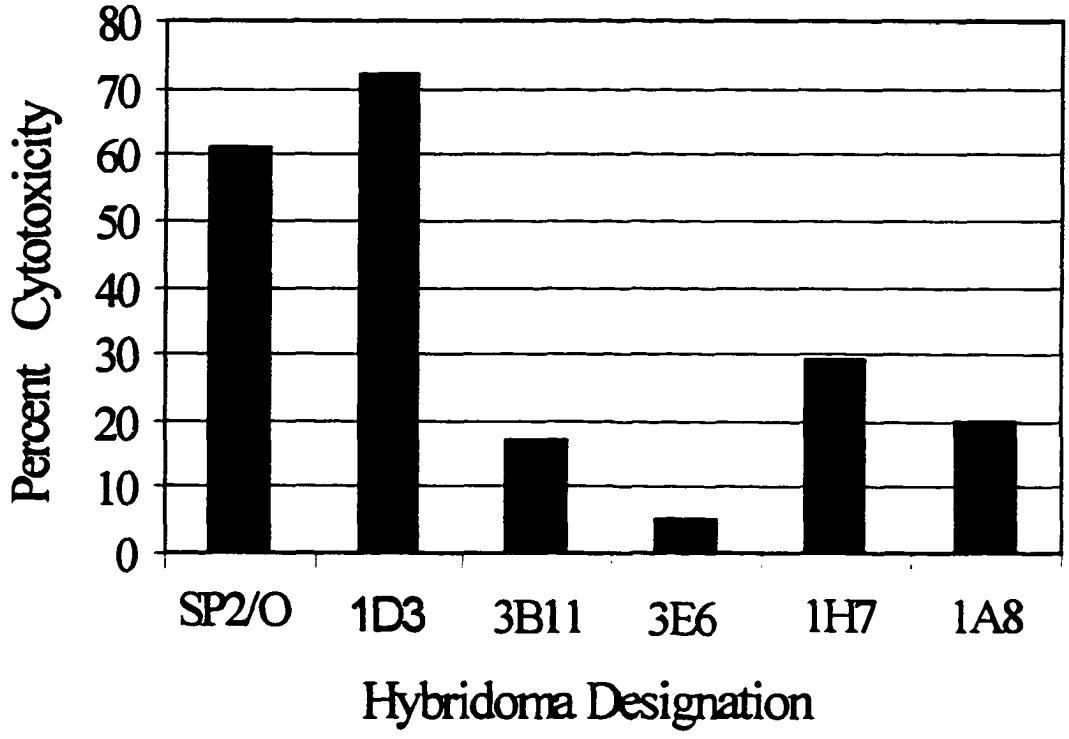

Figure 4.4. Evaluation of sTNFRI neutralization by monoclonal antibodies in *in vitro* TNF bioassays. TNF-mediated cytotoxicity for clone 39 targets was determined in the presence of the indicated hybridoma supernatants. Neutralization of sTNFRI by hybridoma antibodies is indicated by an increase in cytotoxicity.

These observations are consistent with a previous report which demonstrated that monoclonal antibody binding to sTNFRI can enhance its protective activity against TNF toxicity.[29] Non-neutralizing antibodies bind to epitopes outside the active site of sTNFRI resulting in the multimerization of this otherwise monovalent receptor. The binding of multivalent sTNFRI to trivalent TNF is anticipated to stabilize the interaction of sTNFRI and TNF, due to the influence of avidity, which is not operative with monomeric sTNFRI. Nevertheless, these antibodies are certain not be useful as immunotherapeutic agents for the inactivation of sTNFRI *in vivo*.

The 1D3 monoclonal antibody, in contrast, was able to reduce sTNFRI-mediated protection against TNF cytotoxicity. Clone 39 targets incubated with culture supernatant containing 1D3 were rendered more sensitive to TNF-mediated cytolysis relative to control targets incubated with SP2/0 supernatant alone (72% versus 61%) (Figure 4.4). To confirm that this enhanced cytotoxicity was mediated by a monospecific anti-sTNFRI antibody, the 1D3 hybridoma was cloned by limiting dilution, and the clonally-derived antibody was characterized for sTNFRI binding, antibody isotype, and for sTNFRI neutralization in the TNF bioassay. As shown in Table 4.1, antibody produced by clone 1D3.B1 was an sTNFRI-reactive IgG_1 like that of the parent cell line. This 1D3.B1 antibody was purified to homogeneity from culture supernatant using protein G chromatography and the purified antibody was re-tested in the neutralization assay. Specific lysis of clone 39 targets was enhanced, in a dose dependent manner, after pre-incubating targets with purified 1D3.B1 antibody (Table 4.2).

Table 4.2. Neutralization of sTNFRI by the 1D3.B1 monoclonal antibody in *in vitro* TNF bioassays. TNF-mediated cytotoxicity for clone 39 and clone K targets was determined in the presence of purified 1D3.B1 monoclonal antibody. Neutralization of sTNFRI is indicated by an increase in cytotoxicity.

Clone 39 Targets

	Percent Cytotoxicity			
	1D3.B1 Concentration			
TNF (pg/mL)	0 μg/mL	0.1 μg/mL	1 μg/mL	10 μg/mL
0	0	0	0	0
6.25	30	31	40	49
12.5	46	49	53	68
25	60	75	67	83
50	80	92	88	91

Clone K Targets

	Percent Cytotoxicity			
	1D3.B1 Concentration			
TNF (pg/mL)	0 μg/mL	0.1 μg/mL	1 μg/mL	10 μg/mL
0	0	0	0	0
6.25	72	78	79	66
12.5	82	85	87	76
25	94	98	95	90
50	100	100	100	90

When present at 0.1 μg/mL, 1D3.B1 increased specific lysis, but only at higher TNF concentrations (25 or 50 pg/mL). The lack of effect at lower TNF concentrations reflects the net result of two competing equilibrium reactions; the binding of TNF to sTNFRI, and the binding of 1D3.B1 to sTNFRI. When TNF is present at low levels (6.25 or 12.5 pg/mL), its binding to sTNFRI, which is present at approximately 25 ng/mL, is incomplete; that is, free TNF and free sTNFRI both exist. Clearly, some TNF is bound by sTNFRI as evidenced by the differential sensitivities of clone 39 and clone K (Chapter 2). At antibody concentrations as low as 0.1 μg/mL, the quantity of sTNFRI bound by 1D3.B1 is not sufficient to alter the TNF-sTNFRI equilibrium as indicated by the lack of increased cytotoxicity. As antibody concentrations are increased, more sTNFRI is bound by the antibody and, thus, is removed from the pool of sTNFRI capable of neutralizing TNF. Consequently, more TNF is available for binding the membrane TNFR and cytotoxicity increases accordingly. At TNF concentrations of 25 and 50 pg/mL, similar increases in cytotoxicity are observed in the presence of 1D3.B1, though in these samples cytotoxicity is enhanced by as little as 0.1 μg/mL antibody. In this case, the quantity of sTNFRI bound by the antibody is anticipated to be identical to that bound in samples containing 6.25 or 12.5 pg/mL TNF. Yet, at these higher TNF concentrations, the TNF-sTNFRI equilibrium is shifted in favor of complex formation, and the reductions in the free sTNFRI pool that result from antibody binding now are manifested; less sTNFRI is available, consequently a greater fractional part of the TNF remains unbound and cytotoxicity is increased.

The enhancement in TNF cytotoxicity for clone 39 targets by 1D3.B1 was not a result of agonistic binding of the antibody to cell-associated receptors for TNF since the addition of 1D3.B1 antibody without TNF was not cytotoxic either for clone 39 or clone K (Table 4.2). Likewise, the enhanced specific lysis was due to a specific interaction between 1D3.B1 and sTNFRI since the parental clone K cell line from which clone 39 was derived, and which does not secrete sTNFRI, did not exhibit an enhanced susceptibility to TNF-mediated cytotoxicity in the presence of 1D3.B1, even at concentrations as high as 10 μg/mL (Table 4.2). Collectively, these data suggest that 1D3 neutralizes the binding of sTNFRI to TNF and may serve as an immunotherapeutic agent for the inactivation of sTNFRI in tumor bearing hosts.

Growth of Clone 39 Tumors in Mice Infused with 1D3.B1 - The efficacy of 1D3.B1 as an immunotherapeutic agent in the treatment of tumor-bearing mice was evaluated in an *in vivo* transplantation model. Anti-sTNFRI antibodies (1D3.B1) and isotype (IgG_1) control antibodies (1B7.11) were isolated from culture supernatant by protein G chromatography. Purity of each antibody preparation was shown by silver-stained SDS-PAGE to be >95% (data not shown). These antibodies were infused into mice, and their resistance to a subsequent clone 39 challenge was assessed. Eight of the 20 mice treated with the isotype control antibody, 1B7.11, developed tumors. Of the 10 mice treated only with PBS, 4 developed tumors, indicating that the isotype control antibody did not influence clone 39 tumorigenicity in this model. This frequency of tumor growth (40%) is in consistent with that previously demonstrated for clone 39 cells in unirradiated recipients (Chapter 3),

illustrating the reproducibility of clone 39 tumorigenicity in syngeneic mice. Further, the fact that PBS-treated mice developed tumors at the same frequency as mice receiving no prior treatment demonstrates that tumor formation is not influenced significantly by the stress associated with injection and handling. Surprisingly, and much to our chagrin, tumor incidence in 1D3.B1 treated mice also was 40% (8/20 mice) and, thus, was not reduced relative to the control groups. Further, the persistence of tumors in 1D3.B1-treated mice was not decreased.

The inability of 1D3.B1 antibodies to prevent clone 39 tumor development and persistence may result from a number of factors. Foremost is the fact that this antibody appears to have a relatively low affinity for sTNFRI. Although we have not formally determined its affinity, the use of 1D3.B1 as a capture antibody in ELISA assays indicates that it binds sTNFRI weakly; approximately 4- to 8-fold more antibody is required to produce signals comparable to those obtained with the polyclonal goat anti-sTNFRI capture antibody (data not shown). This difference is magnified by the fact that only a fraction of the polyclonal IgG preparation is specific for sTNFRI. Moreover, as shown in Table 4.2, 1D3.B1 fails to neutralize sTNFRI activity in *in vitro* TNF bioassays when antibody and TNF concentrations are limiting, despite the fact that the antibody is present at a 3,000- to 6,000-fold molar excess relative to TNF. This indicates that 1D3.B1 competes poorly with TNF for occupancy of the sTNFRI active site, and it is consistent with low affinity antibody binding to sTNFRI.

The pharmacokinetics of infusional 1D3.B1 also may contribute to the lack of protection against clone 39 challenge. While a total of 1 mg of antibody was injected into

each mouse, it is not certain that this quantity of antibody is adequate to inhibit sTNFRI *in vivo* for a period necessary to completely prevent tumor growth. While many other studies have used milligram quantities of antibodies *in vivo*, most of these have involved the depletion of specific cell populations (e.g., $CD4^+$ or $CD8^+$ T cells), and paradigms established in those models may not apply herein. Once depleted, restoration of the targeted population to functional competence may take several days to weeks, even though the injected antibody is cleared from circulation within a matter of hours or, at most, days. sTNFRI, however, is continuously replenished at the surface of the clone 39 cells once they are injected, and the clearance of 1D3.B1 will result in a complete loss of sTNFRI-neutralization. Also, in cellular depletion studies, the elimination of the targeted population involves host effector mechanisms (e.g., complement and/or ADCC) which are recruited by antibody binding whereas, in the present study, the desired effects are mediated solely by the sTNFRI-neutralizing antibody.

Monoclonal antibodies reactive with TNF have been used as passive therapy and those studies provide more appropriate precedents for the present experimentation. Antibodies reactive with TNF have been administered passively to inhibit the systemic effects of endotoxemia[30] or cutaneous allergic responses[31], or to evaluate the role of TNF in host defenses against infectious agents.[32] As in the present analysis, multiple administrations of 200-500 μg of antibody have been employed in those studies, yet typically they are concluded within 48-72 hours. They, therefore, are unlikely to be influenced by the time-dependent clearance of circulating antibody to the same degree as the present studies which continue for nearly two weeks. Thus, the maintenance of

persistent titers of injected antibody, either through the injection of larger quantities or through more frequent administration, is of considerably greater importance in the present work. This is due to the fact that the goal of the present work is the consistent inhibition of a protein that is continuously and rapidly synthesized as opposed to the one time depletion of a cellular population whose regeneration requires several days, or as opposed to the short-term inhibition of cytokine effects. These considerations suggest that the feasibility of the therapeutic strategy proposed herein ultimately can be proven, through the optimization of antibody infusion regimens which maintain circulating antibody levels adequate to persistently neutralize sTNFRI.

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