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Soluble Tumor Necrosis Factor Receptor Type I Enhances Tumor Development and Persistence *in Vivo*[1]

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Secretion of human soluble tumor necrosis factor receptor type I (sTNFRI) by the mouse fibrosarcoma cell line, L929, previously has been demonstrated to confer resistance to *in vitro* lysis by TNF and to LAK- and CTL-mediated cytolysis. These findings suggest that, *in vivo,* sTNFRI contributes to tumor survival by inhibiting these immunologic mechanisms. To evaluate this hypothesis, we compared the growth of sTNFRI-secreting L929 cells with that of the unmodified parental fibrosarcoma in an *in vivo* mouse transplantation model. Secretion of sTNFRI by L929 cells markedly enhanced their tumorigenicity and persistence in syngeneic recipients. This benefit was abrogated by sTNFRI-neutralizing antibodies induced by immunization prior to tumor challenge. These data demonstrate that sTNFRI directly influences tumor formation and persistence *in vivo* and suggest the selective removal and/or inactivation of sTNFRI as a promising new avenue for cancer immunotherapy.

Key Words: **tumor immunity; cytokines; cytokine receptors; immunomodulators; *in vivo* animal models.**

INTRODUCTION

Tumor necrosis factor (TNF) is a pluripotent cytokine identified by its ability to induce hemorrhagic necrosis and regression of established subcutaneous tumors in mice (1). Since the original demonstration of its effects on tumors, TNF has been found to regulate a number of phenomena including inflammation, cellular proliferation, and various immunoregulatory and antiviral responses (2). These effects are initiated by binding of TNF to the type I, membrane-associated TNF receptor (mTNFRI), which is expressed nearly ubiquitously on mammalian cells (3, 4). Given the pluripotency of TNF, it follows that regulatory mechanisms exist to modulate its activity. One such mechanism is a naturally occurring "blocking factor" of TNF that originally was identified in the urine of febrile (5) and renal failure (6) patients. Purification of this factor (7–11) and the subsequent cloning and sequencing of its cDNA (12, 13) revealed it to correspond to the extracellular domain of the intact mTNFRI (14). This 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 inhibit the binding of TNF to mTNFRI on cell surfaces (7–9, 11, 13).

The levels of sTNFRI in biological fluids are 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 pathogenesis, and sTNFRI production is postulated to be a mechanism for restoring immunologic homeostasis (22).

sTNFRI levels are increased also in tumor-bearing hosts (23–27), presumably in response to an increase in TNF production stimulated by the presence of the tumor. This sTNFRI production likely is intended to reduce localized, as well as systemic, toxicity associated with elevated TNF levels in a manner analogous to that which is operative in the inflammatory conditions described above. Yet, in tumor-bearing hosts, the production of sTNFRI may have the unintended consequences of protecting tumors from immunological destruction and 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 shed their mTNFRI than do

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nonmalignant cells (30). Second, sTNFRI levels in the circulation of tumor-bearing hosts are elevated during advanced stages of disease and decline during remission (23, 24, 26). Third, human clinical trials of ultrapheresis, an experimental cancer therapy involving extracorporeal fractionation of plasma components by ultrafiltration, have correlated the removal of circulating sTNFRI with significant clinical benefit (31–33). Fourth, we have shown that sTNFRI protects transformed cells *in vitro* from the cytotoxic effects of TNF and from cytolysis mediated by natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) (34). Collectively, these observations suggest that sTNFRI contributes to tumor survival *in vivo* by inhibiting antitumor immune mechanisms which employ TNF. Formal proof of this hypothesis, however, currently is unavailable. The present studies were designed to evaluate the role that sTNFRI plays in tumor development and progression. Herein, we demonstrate that secretion of sTNFRI by tumor cells markedly enhances their tumorigenicity and persistence in syngeneic hosts and that this benefit is abrogated by antibodies which neutralize the binding of sTNFRI to TNF.

MATERIALS AND METHODS

Mammalian cell lines, bacterial strains, and reagents. The L929 murine fibrosarcoma cell lines used in these studies and their *in vitro* cultivation have been described (34). Briefly, clone K is a TNF-sensitive subclone of L929 and clone 39 is a transfectant of L929 clone K that secretes human sTNFRI. The prokaryotic expression plasmid, pet24a, which permits the production of fusion proteins with a carboxy-terminal $(His)_6$ tag, and the corresponding *Escherichia coli* host strain, BL21(λ)DE3, were obtained from Novagen (Madison, WI). *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).

TNF bioassays. The TNF sensitivities of clone K cells and of clone 39 cells were determined using *in vitro* bioassays as described (34, 35). Briefly, individual cell lines were seeded in 96-well plates at 2.25×10^4 cells/well and allowed to adhere overnight. Cells were incubated with actinomycin D (1 μg/ml) (Sigma, St. Louis, MO) and 10 μg/mL anti-human sTNFRI monoclonal antibody (Selinsky and Howell, unpublished) for 30 min at 37°C. TNF was added and, 24 h later, cells were fixed and stained and the percentage of cytotoxicity was determined as described (34, 35).

Mice. Eight to 10-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 dosage 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 recipients. 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 PBS. Tumor development was monitored visually and by palpation each day in a blinded manner. The statistical significance of differences between clone K and clone 39 tumor incidence was determined using Fisher's exact test. At sacrifice, tumors were resected and a portion of each was fixed in 10% neutral buffered formalin, sectioned, and stained with hematoxylin and eosin for histological analysis.

Production of the prokaryotic sTNFRI expression construct. The gene encoding human sTNFRI was isolated by PCR. A pcDNA3 expression construct (described in Ref. 34) served 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 (numbering according to Ref. 13) and, thus, excludes the mammalian leader sequence. 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 also contains a *Hind*III restriction site (underlined) to facilitate cloning. PCR products were ligated to *Bam*HI–*Hind*III-digested pet24a, recombinant plasmids were selected, and their fidelity was verified by restriction endonuclease digestion and PCR amplification.

Production of recombinant sTNFRI. sTNFRI production was induced in BL21(λ)DE3, and recombinant sTNFRI was isolated from inclusion bodies by the method of DeLamarter *et al.* (36) and affinity purified using a chelated nickel column. The purity of the eluted protein was determined by silver-stained SDS–PAGE and the identity of the protein was confirmed by

immunoblot analysis using biotinylated goat anti-sTNFRI polyclonal antibody.

Induction of neutralizing anti-sTNFRI antibodies by immunization. Each of five mice was immunized with 10 μg of sTNFRI in 0.1 mL PBS, emulsified 1:1 with CFA. Each mouse received four 50-μl subcutaneous injections. Two groups of five control mice were immunized: one group received subcutaneous injections of CFA emulsified 1:1 with PBS, and the second group received PBS alone. Four weeks later, mice were boosted with 10 μg of sTNFRI emulsified 1:1 with 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 sTNFRI-neutralizing antibody was quantified by capture ELISA. A 96-well plate was coated with goat anti-human sTNFRI antibody at 2 μg/mL and blocked with BSA, and 0.5 μg/mL of recombinant human sTNFRI (R&D Systems) was added. Serial dilutions of mouse sera were added, followed by 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, *p*-nitrophenyl phosphate, 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

Tumor formation by clone K and clone 39 in irradiated mice. Our initial studies were conducted in irradiated mice, a design guided by prior reports concerning the tumorigenicity of L929 cells (37). Those studies have shown L929 cells to be nontumorigenic in syngeneic recipients, yet to produce fibrosarcomas in sublethally irradiated (4.25 Gy) mice. Therefore, to permit the growth of both of our L929-derived cell lines and maximize 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 postinjection (Fig. 1A). All of these tumors regressed by day 10 postinjection, which likely reflects the restoration of immune function documented to begin within days after the administration of 3.0 Gy (38–40). Clone 39 cells produced tumors in 7 of 10 irradiated mice, also beginning on day 6 postinjection. In contrast to clone-K-derived tumors, however, all of these tumors persisted to day 13, the time of necropsy (Fig. 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.

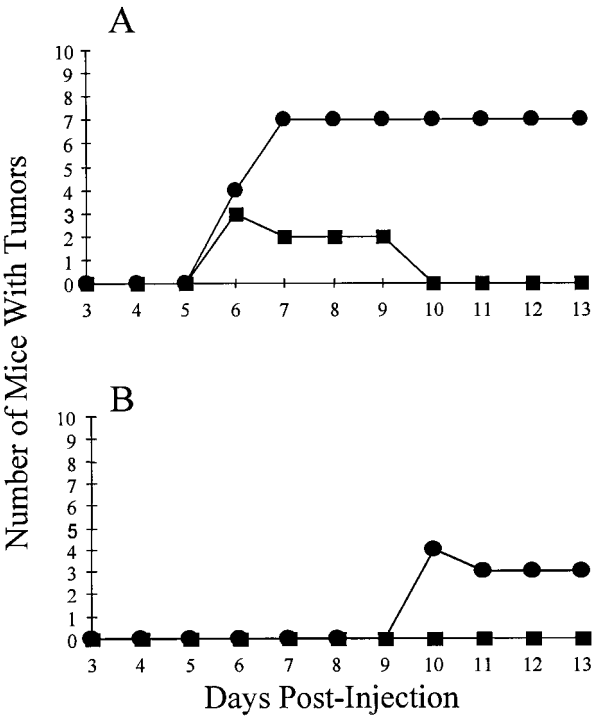

FIG. 1. sTNFRI secretion enhances the tumorigenicity and persistence of fibrosarcomas *in vivo.* Clone 39 (●) and clone K (■) cells were injected subcutaneously into sublethally irradiated (A) or unirradiated (B) syngeneic mice. The statistical significance of the differences between clone K and clone 39 tumor incidence in irradiated (P = 0.089) and unirradiated (P = 0.025) mice was determined by Fisher's exact test.

Tumor formation by clone K and clone 39 in unirradiated mice. Whereas sTNFRI-secreting clone 39 cells were more tumorigenic than unmodified clone K cells in irradiated animals, we evaluated the growth of these cell lines in fully immunocompetent mice. Unirradiated mice were injected with clone 39 and clone K cells, and tumor development was monitored (Fig. 1B). Ten days postinjection, 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 (Fig. 1B), an observation consistent with the lack of tumorigenicity of L929 in unirradiated recipients reported by others (37). 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

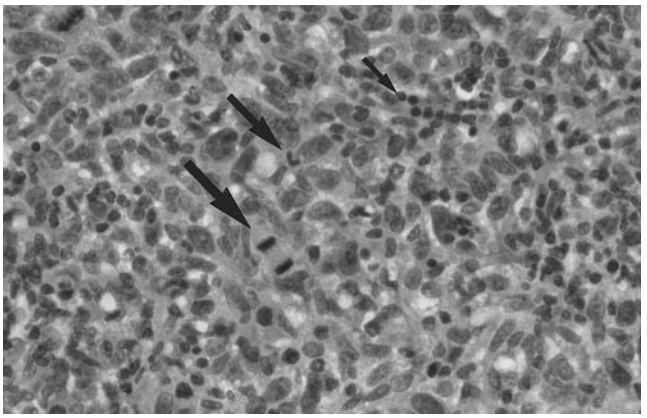

FIG. 2. sTNFRI-secreting fibrosarcomas survive *in vivo* despite a demonstrable leukocyte infiltrate. Tumor sections were stained with hematoxylin and eosin. Proliferative activity of the tumors is indicated by the presence of mitotic figures (large arrow). Leukocytes infiltrating the tumor mass included neutrophils (medium arrow) and lymphocytes (small arrow). Original magnification, 430×.

unirradiated mice was less than that observed in irradiated mice, and the day of onset was delayed by 3 days. These differences support the notion that the tumorigenicity and persistence of L929 cells reflect the balance between immunocompetence and immunosuppression, although in this case, the suppression is effected not by radiation but solely by sTNFRI.

Histology of clone-39-derived tumors. Tumor tissues were resected from unirradiated mice, formalin-fixed, sectioned, and stained with hematoxylin and eosin (Fig. 2). Histology was consistent with a fibroblastoid tumor. In addition, histological analysis revealed that clone-39-derived fibrosarcomas were persisting in the presence of a demonstrable leukocyte infiltrate. Clone 39 cells continued to proliferate, as evidenced by one to two mitotic figures per high-power field, despite the accumulation of leukocytes in multiple foci within the tumor. Although the nature of this leukocyte infiltrate has yet to be fully characterized, morphological evaluation indicated that two cell types predominated: (1) cells with multilobed, horseshoe-shape nuclei and azurophilic granules which most likely are neutrophils and (2) cells with darkly staining nuclei and scant, agranular cytoplasm, which is typical of lymphocytes.

Reversal of clone 39 tumorigenicity through the induction of antibodies that neutralize sTNFRI. To confirm that the growth of clone 39 *in vivo* resulted from its secretion of sTNFRI, sTNFRI-neutralizing antibodies were induced by immunization and the growth of clone 39 cells in immune mice was evaluated. Since the sTNFRI secreted by clone 39 is human sTNFRI, it was possible to induce high-titer antibodies in murine hosts. Mice were immunized with recombinant sTNFRI produced in *E. coli,* irradiated with 3.0 Gy to maximize tumor incidence, and challenged with clone 39 cells. As shown in Table 1, mice immunized with sTNFRI developed neutralizing antibody titers ranging from greater than 2500 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% (Fig. 1A). A comparable incidence was observed in control mice immunized with adjuvant or PBS alone; clone 39 tumors developed in four of five mice in each of these two groups. As expected, none of the animals in these two groups possessed significant sTNFRI-neutralizing antibodies (Table 1). Thus, antibodies which neutralize the binding of sTNFRI to TNF, in effect, "inhibit the inhibitor" and relieve its suppression of immune mechanisms that mediate tumor destruction.

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 Ag (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. 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 cy-

TABLE 1

Neutralizing Anti-sTNFRI Antibodies Inhibit the Development of Clone 39 Tumors

Immunization	Tumor incidence	sTNFRI-neutralizing antibody titer
PBS alone	4/5	<5
		<5
		<5
		<5
		<5
PBS/adjuvant	4/5	<5
		<10
		<20
		<5
		<20
sTNFRI/adjuvant	0/5	>10,000
		>10,000
		>2500
		>10,000
		ND

Note. The presence of 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 cells. Neutralizing antibody titers in the serum of each mouse are indicated. The statistical significance of the differences in tumor incidence between immunized and control mice ($P = 0.014$) was determined by Fisher's exact test.

totoxicity (ADCC), for example, has been implicated in tumor lysis (41), yet for ADCC to be operative in the present model would require the association of sTNFRI with the membrane of clone 39 target cells. Only then would anti-sTNFRI antibody binding facilitate the attachment of F_c receptor-bearing cytotoxic effectors to the target cells. Complement, for similar reasons, is anticipated not to participate significantly in the rejection of clone 39 cells. Alternatively, class I MHC-restricted $CD8^+$ CTLs, specific for sTNFRI, may contribute to the elimination of clone 39 cells in immunized mice, since the induction of TAA-specific $CD8^+$ CTLs by immunization has proved effective at preventing the growth of tumors upon subsequent challenge (42, 43). Those studies, however, immunized and challenged with transfectant cell lines engineered to produce cytosolic, endogenous TAAs which maximizes their presentation in association with class I MHC on the transfectant surface. In the present studies, immunization with purified sTNFRI is unlikely to elicit $CD8^+$ T cells, since the administration of exogenous antigen does not permit effective presentation by class I MHC. Rather, class II MHC-restricted $CD4^+$ T cells likely predominate in the T cell response induced by sTNFRI immunization. Upon challenge, however, these sTNFRI-specific $CD4^+$ effectors are not anticipated to be directly cytolytic for clone 39 cells, 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 which enhance NK cell activity through the production of IL-2 and IFN-γ. While we cannot 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 (34) minimizes its importance. Rather, it is most likely that the neutralization of sTNFRI by antibody binding is responsible for reversing the tumorigenicity of clone 39.

The *in vivo* studies described herein, as well as the *in vitro* studies described previously (34), 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 selects for transfectants which are inherently different from the parental cell line for reasons other than the expression of the heterologous gene. For example, clone 39 might represent an isolated variant of clone K which is more tumorigenic by virtue of replicating faster and/or resisting immunological destruction irrespective of sTNFRI production. This appears unlikely, however, since the doubling times of clone K and clone 39 cells are virtually identical (23.25 $\pm$ 2.7 and 25.23 $\pm$ 2.2 h, respectively). Moreover, while clone 39 is more resistant to *in vitro* lysis by various immune mechanisms (34), this resistance does not result from a loss of TNF susceptibility. Incorporation of an sTNFRI-neutralizing monoclonal antibody into *in vitro* TNF bioassays markedly enhances the TNF susceptibility of clone 39 cells (Fig. 3). Specific lysis of clone 39 cells was increased in the presence of a neutralizing anti-human sTNFRI antibody at all TNF concentrations tested. Specific lysis of clone K cells, predictably, was unchanged by the incorporation of this anti-sTNFRI antibody. Thus, clone 39 retains the TNF sensitivity of the parental cell line, clone K, yet the production of sTNFRI by clone 39 renders it less sensitive to immune mechanisms which employ TNF. The ultimate importance of this decreased sensitivity is illustrated by the *in vivo* studies reported herein. L929 clone K was transformed from a nontumorigenic to a tumorigenic phenotype simply through the production of sTNFRI, findings which affirm the importance of this single molecule in tumor survival.

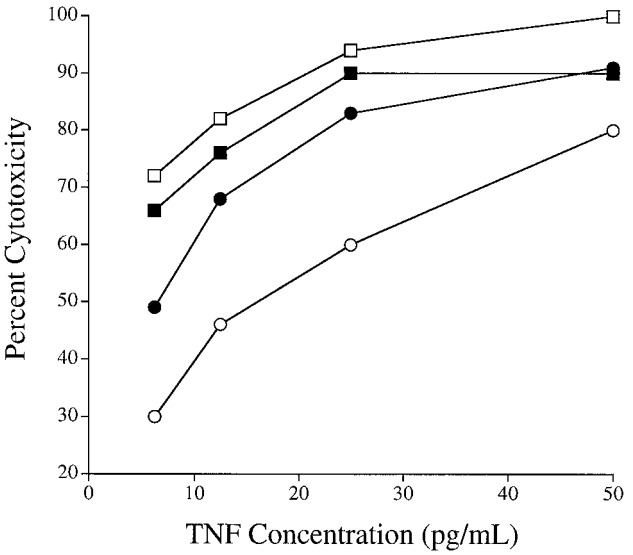

FIG. 3. TNF-mediated cytotoxicity of clone 39 cells is enhanced in the presence of a neutralizing anti-sTNFRI antibody. Clone 39 (●, ○) and clone K (■, □) and cells were cultured with (closed symbols) or without (open symbols) neutralizing anti-sTNFRI monoclonal antibody and TNF at the indicated concentrations. Cytotoxicity was determined as described (34, 35).

Similar findings have been obtained from studies of an allograft rejection model (44). Murine allografts, engineered to secrete human sTNFRI type II fused to the C region of a human IgG heavy chain (dimeric sTNFRII), resisted rejection when transplanted into MHC disparate recipients. Unmodified grafts were rejected readily. The resistance of sTNFRII-producing grafts was abrogated, however, if mice were infused with monoclonal antibody which neutralized the binding of sTNFRII to TNF. These findings confirm the present results, yet they can be distinguished. The present research exploited syngeny between the tumor and recipient and also evaluated monomeric sTNFRI, conditions which more accurately parallel those

present during development of spontaneous tumors in mammals.

Clearly, sTNFRI can suppress the immunological rejection of nonidentical tissues. As demonstrated herein, this suppression is sufficient to permit the establishment and persistence of tumors, findings which implicate sTNFRI as a legitimate target for cancer therapy. Clinical manipulation of sTNFRI levels should be explored, guided by the realization that circulating sTNFRI levels must be diminished to intensify anti-tumor immunity. This is in contrast to traditional cancer biotherapy which has endeavored to boost anti-tumor immunity through the addition of biological response modifiers. Preeminent among these has been the infusion of supraphysiological levels of TNF (45) and of IL-2 (46), which indirectly stimulates the production of TNF. The limited success of these therapies (45, 46) may derive from the fact that each increases the level of circulating sTNFRI (22, 47). Together, these observations suggest a radically different biotherapeutic approach to cancer—one which involves the *removal* of immunosuppressive factors such as sTNFRI, rather than the *addition* of immunostimulatory compounds.

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