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Targeted delivery of Tumor Necrosis Factor-alpha to tumor vessels induces a therapeutic T cell-mediated immune response that protects the host against syngeneic tumors of different histologic origin

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## TARGETED DELIVERY OF TUMOR NECROSIS FACTOR-A TO TUMOR VESSELS INDUCES A THERAPEUTIC T CELL -MEDIATED IMMUNE RESPONSE THAT PROTECTS THE HOST AGAINST SYNGENEIC TUMORS OF DIFFERENT HISTOLOGIC ORIGIN

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### Abstract

Purpose:We sought to demonstrate that a single systemic administration of L19mTNFa (a fusion protein constituted by the scFv L19 specific for the oncofetal ED-B domain of fibronectin and tumor necrosis factor a,TNFa) in combinationwithmelphalan induced complete and long-lasting tumor eradication in tumor-bearing mice and triggered the generation of a specificTcell ^ based immune response that protects the animals from a second tumor challenge, as well as from challenges with syngeneic tumor cells of different histologic origin.

Experimental Design and Results: Treatment with L19mTNFa, in combinationwithmelphalan, induced complete tumor regression in 83% of BALB/c mice withWEHI-164 fibrosarcoma and 33% of animalswith C51coloncarcinoma. All curedmice rejected challenges with the sametumor cells and, in a very high percentage of animals, also rejected challenges with syngeneic tumor cells of different histologic origin. In adoptive immunity transfer experiments, the splenocytes from tumor-curedmice protected naivemice both fromC51colon carcinoma and fromWEHI-164 fibrosarcoma.

S imilar results were also obtained in adoptive immunity transfer experiments using severely immunodepressed mice.Exper iments using depleted splenocytes showed that Tcells play amajor role in tumor rejection.

Conclusions: The results show that the selective targeting of mTNFa to the tumor enhances its immunostimulatory properties to the point of generating a therapeutic immune response against different histologically unrelated syngeneic tumors. These findings predicate treatment approaches for cancer patients based on the targeted delivery of TNFa to the tumor vasculature.

### Introduction

Fibronectin is an extracellular matrix component widely expressed in a variety of normal tissues and body fluids. Different fibronectin isoforms could be generated by the alternative splicing of the fibronectin pre-mRNA, a process that is modulated by cytokines and extracellular pH(1, 2). The complete type III repeat ED-B may be included or omitted in the fibronectin molecule (3). ED-B is highly conserved in different species, having 100% homology in all the mammalians studied thus far (human, rat, and mouse), and 96% homology with a similar domain in chicken. The fibronectin isoform containing ED-B (B-FN) is immunohistochemically undetectable in normal adult tissues, with the exception of tissues undergoing physiologic remodeling (e.g., endometrium and ovary) and during wound healing (4). By contrast, its expression in tumors and fetal tissues is high (4, 5). Furthermore, we showed that B-FN is a marker of angiogenesis (6, 7), and that endothelial cells invading tumor tissues migrate along extracellular matrix fibers containing B-FN (8). The function of B-FN, however, is still unclear. Fukuda et al. (9) generated mice lacking the ED-B exon and showed that, whereas B-FN is highly expressed throughout early embryogenesis, ED-B-deficient mice developed normally and were fertile. We previously reported on the possibility to selectively target tumor vasculature using a human recombinant antibody specific for B-FN, L19(scFv), in both experimental animal models and cancer patients (8, 10–14). This observation paved the way for the antibody's use in vivo for both diagnostic and therapeutic purposes. Indeed, the selective targeted delivery of cytokines to the ED-B domain of fibronectin using L19(scFv) dramatically enhances their anticancer properties (15–19). Building on this finding, the fusion protein L19-interleukin-2 is now in phase I/II clinical trials in patients with different kinds of solid tumors. Tumor necrosis factor-a (TNFa) is a pleiotropic cytokine (20) composed of three noncovalently linked TNFa monomers, f17.5 kDa each, that yield a compact bell-shaped homotrimer (21). TNFa exerts its major antitumor effects mainly via a preferential toxicity for the endothelial cells of the tumorassociated vasculature and through an increase of the antitumor immune response. The toxicity for endothelial cells of the tumor vasculature leads to extensive thrombosis and destruction of tumor vasculature resulting in extensive tumor necrosis (22, 23). Furthermore, TNFa increases vascular permeability (24) and reduces the tumor's interstitial fluid pressure (25), a process pivotal to facilitating the penetration of antitumor agents at the tumor site. Like other primary proinflammatory signals, TNFa promotes the maturation of dendritic cells in vivo and their migration to draining lymph nodes (26), and, in some cases, fosters long-lasting protective immunity (27, 28). Although TNFa is one of the most potent antitumor cytokines, its unacceptable toxic side effects have prevented its systemic administration at therapeutically effective doses. To date, the clinical use of TNFa has been limited to locoregional applications, such as "isolated limb perfusion," in combination with melphalan for the treatment of nonresectable high-grade sarcoma and melanoma (29–31). TNFa is also being evaluated for the therapy of nonresectable liver tumors by isolated hepatic perfusion (32). These results prompted a number of studies aimed at decreasing TNFa toxicity without modifying its antitumor properties, thereby allowing its use not only for locoregional treatments but also for systemic therapy. Resulting strategies include the production of engineered TNFa mutants (33), encapsulation of TNFa in liposomes (34), and the targeted delivery to tumors of TNFa through its coupling to specific ligands (17, 35–38). These last endeavors seem to represent the most promising strategy. We recently generated the fusion protein L19mTNFa (17), consisting of mouse TNFa (mTNFa) and the human antibody fragment L19(scFv) directed to the ED-B domain of fibronectin. When injected i.v. into tumor-bearing mice, this fusion protein selectively accumulates around the tumor vasculature and, 48 hours after injection, the dose of L19mTNFa in the tumor is roughly 35 times higher than the dose achieved with a control fusion protein in which mTNFa is conjugated to an irrelevant scFv (TN11). Here, we show that a single systemic administration of L19mTNFa and melphalan in mice bearing two histologically unrelated syngeneic tumors induces complete and long lasting tumor eradication and triggers the generation of a specific T cell-based immune response that protects the animals from a second tumor challenge, as well as from challenges with syngeneic tumor cells of different histologic origin.

### **Materials and Methods**

Animal tumor models. WEHI-164 mouse fibrosarcoma (3  $\Box$  106 cells; European Collection of Animal Cell Cultures, Sigma-Aldrich, Milan, Italy), C51 mouse colon adenocarcinoma (0.5  $\Box$  106 cells; kindly provided by Dr. M.P. Colombo, Department of Experimental Oncology, Istituto Nazionale Per Lo Studio E La Cura Dei Tumori, Milan, Italy), all of BALB/c origin, were s.c. implanted in the left flank of immunocompetent syngeneic BALB/c mice or of severe combined immunodeficiency (SCID) beige mice. All mice were 8 to 10 weeks old and purchased from Harlan UK (Oxon, United Kingdom). The tumor volume was determined using the following formula: (d)2  $\Box$  D  $\Box$  0.52; where d and D are the short and long dimensions (cm) of the tumor, respectively, measured with a caliper (8). Housing, treatment, and sacrifice of animals followed national legislative provisions (Italian law no. 116; January 27, 1992) for the protection of animals used for scientific purposes. Lung metastases were established in BALB/c mice injecting 75  $\Box$  103 C51 cells in 100 AL PBS [20 mmol/L NaH2PO4, 150 mmol/L NaCl (pH 7.4)] into the tail vein. When respiratory distress was present and/or a 10% weight loss was recorded over a 24-hour period, the

mice were sacrificed and the lungs were infused through the trachea with 15% India ink solution. Only normal lung parenchyma was stained black by the ink solution, whereas the tumor metastases appeared white and could be counted.

**Tumor therapy**. Groups of tumor-bearing mice (when the tumors reached a volume off0.2 cm3) received an injection in their tail vein of the fusion proteins L19mTNFa or TN11mTNFa (the expression, purification, and characterization of the fusion proteins have previously been reported; ref. 17) or of recombinant mTNFa (2 🗆 107 units/mg, kindly provided by Dr. A. Corti, Department of Oncology, Cancer Immunotherapy and Gene Therapy Program, San Raffaelle M. Scientific Institute, Milan, Italy), in 100 AL of PBS. As already reported (17), in the therapeutic protocols with a single compound, 1 pmol/g of L19mTNFa, TN11mTNFa, or mTNFa was used, whereas 0.7 pmol/g was used in combination with other drugs. The group of controls received 100 AL of PBS only. Lyophilized melphalan (Alkeran, Glaxo Smith Kline, Research Triangle Park, NC) was reconstituted (10 mg/mL) in the solvent provided by the manufacturer immediately before use and, after further dilution in PBS, was administered i.p. (4.5 Ag/g in 400 AL). The weight of the animals and the tumor volume were recorded at 24-hour intervals before and after treatments. Toxicity was evaluated on the basis of weight loss, as reported by Borsi et al. (17). The mice were sacrificed when the tumor reached a volume of 1.5 cm3. TNFa cytolytic assays were carried out in the presence of actinomycin D as described by Borsi et al. (17). Quadruplicates were carried out and the results expressed as a percentage of cell viability (average F SD) versus mTNFa (pg/mL).

Adoptive immunity transfer experiments (Winn assay), cell-mediated cytotoxicity, and enzyme-linked immunospot assay. WEHI-164- or C51 tumor–cured mice were given a s.c. booster dose in the contralateral flank with cells derived from the same tumors ( $3 \square 106$ , WEHI-164;  $0.5 \square 106$ , C51) and, after 12 days, the total splenocytes were obtained, following the procedure described by Meazza et al. (39). To establish the amount of effector splenocytes able to protect nai"ve mice against WEHI-164 or C51 tumor, different effector-tumor cell ratios (E/T), from 5:1 to 0.3:1, were calculated. In the adoptive immunity transfer experiments using splenocytes from WEHI-164 tumor–cured mice, an E-T ratio of 1:1 was used with WEHI-164 tumor ( $3 \square 106$  cells) whereas with C51 tumor ( $0.5 \square 106$  cells) an E/T ratio of 5:1 was used. For in vitro depletion, negative magnetic separation (Clin ExVivo Dynabeads, Dynal Biotech ASA, Oslo, Norway) was used, following the manufacturer's instructions. The magnetic beads were coated with anti- CD4 (clone GK1.5, ATCC), anti-CD8 (clone 2.43, ATCC), anti-B (clone RA3-3A1/6.1, ATCC) rat monoclonal antibodies (mAb) or rabbit anti-asialo-GM1 antiserum (Wako Chemicals GmbH, Dusseldorf, Germany). The recovered splenocytes underwent a second specific antibody incubation and a complement-mediated depletion step with 1:10 rabbit complement (Cederlane, Hornby,

Ontario, Canada). Cell depletion was assessed by immunofluorescence and cytofluorimetric analysis by indirect staining of B cell subset (primary mAb, clone RA3- 6B2, Southern Biotech, Birmingham, AL; secondary antibody, FITCconjugated goat F(abV)2 anti-rat IgG; Southern Biotech) and by direct staining for CD4 (FITC-conjugated YTS 191.1.2 mAb; Immunotools, GmbH, Germany), CD8 (PE-conjugated YTS 169.4 mAb; Immunotools) and natural killer (FITC-conjugated DX-5 mAb, Caltag Laboratories, Burlingame, CA) subsets. Isotype-matched mAbs of unrelated specificity were used as controls. Analysis was done on a FACScan (Becton Dickinson, Milan, Italy). Cell-mediated cytotoxicity was evaluated by a standard (4 hour) 51Cr release assay in mixed lymphocyte-tumor cell cultures, using either immune splenocytes obtained from WEHI-164 tumor–cured mice, 12 days after the third WEHI-164 tumor challenge, or splenocytes from nai"ve mice as described by Croce et al. (40). Inhibition test of lysis with concanamycin A (0.2 Ag/mL, Sigma-Aldrich) was done as described by Seki et al. (41). Enzyme-linked immunospot assay was conducted using ex vivo splenocytes from either nai"ve or WEHI-164 tumor-cured mice as described by Croce et al. (40). A >2-fold increase in the number of spots compared with the control was considered a positive response.

### Results

L19mTNFa in combination with melphalan cures different murine tumors. Tumor-bearing mice were treated with a single i.v. administration of L19mTNFa (0.7 pmol/g) in combination with melphalan (4.5 Ag/g), given i.p., as described in Materials and Methods. For control molecules, we substituted L19m TNFa with mTNFa or TN11mTNFa. Two murine experimental models, WEHI-164 fibrosarcoma and C51 colon carcinoma, were used for their different in vitro sensitivities to mTNFa. In fact, as shown in Fig. 1A, in the presence of actinomycin D, WEHI-164 cells are f300 times more sensitive than C51 cells to mTNFa. The treatment with L19mTNFa and melphalan induced complete and irreversible tumor eradication in 83% of mice bearing WEHI-164 fibrosarcoma (74 out of 89 mice treated in different experiments carried out using identical conditions; Fig. 1B) and in 33% (6 out of 18) of mice bearing C51 colon carcinoma (Fig. 1C). On the contrary, using mTNFa (Fig. 1C) or TN11mTNFa (data not shown), in combination with melphalan, no tumor eradication was observed in C51 colon carcinoma-bearing mice (0 out of 9 in both cases), whereas in the case of WEHI-164 fibrosarcoma, eradication was achieved in 54.5% (6 out of 11) of the mice treated with mTNFa and melphalan (data not shown) and in 61% of the mice treated with TN11mTNFa and melphalan (22 out of 36 mice treated in different experiments carried out using identical conditions; Fig. 1B). The therapeutic efficacy on WEHI-164 fibrosarcomabearing mice of L19mTNFa or melphalan alone is reported in Fig. 1B. Only a moderate increase of survival time of the tumorbearing mice was observed in either case. In order to assess whether the combined treatment with L19mTNFa and melphalan induced tumor eradication in immunocompromised mice, we s.c. induced WEHI-164 tumor formation in SCID beige mice. In these animals, the treatment resulted in no tumor eradication but only in tumor growth retardation (Fig. 1D), indicating that the treatment "per se" was not sufficient to cure the tumor, and strongly suggesting that the immune system plays a crucial role in the antitumor activity of L19mTNFa.

Cured mice reject challenges of syngeneic tumors of different histologic origin. To better investigate the immune system's involvement in tumor eradication induced by the treatments, we assessed whether WEHI-164-cured and C51-cured mice were able to reject tumors on challenge. One hundred percent of the cured mice rejected challenges with the same tumor cells. In the case of WEHI-164 fibrosarcoma, the mice resisted challenges with  $15 \square 106$  cells (five times the dose used to induce the tumor in 100% of the mice; Table 1A). Moreover, 100% of C51 colon carcinomacured mice rejected challenges with histologically unrelated syngeneic WEHI-164 fibrosarcoma. (Table 1B). In WEHI-164 tumor-cured mice, the challenge with the histologically unrelated syngeneic C51 colon carcinoma was rejected by 60% of the animals, when the injected number of tumor cells was  $0.5 \square 106$  (a cell dose inducing tumors in 100% of the animals), and by 30% of the mice challenged with 3  $\Box$  106 tumor cells (Table 1A). The ability of WEHI-164 tumor–cured mice to reject challenges with syngeneic tumor cells of different histologic origins increased if the cured mice were first challenged with the same tumor (WEHI-164). In fact, in WEHI-164 tumor-cured mice, after the first homologous tumor challenge, a new challenge with either C51 (3  $\square$  106 cells) or RENCA (renal cell carcinoma,  $1 \Box 106$  cells) tumors was rejected by 100% of the mice (Table 1A). This finding was not restricted to s.c. implants, but also to lung metastasis generated by i.v. injection of the C51 tumor cells (Fig. 2; Table 1A).

**Immunologic correlates of tumor rejection: in vitro and in vivo studies**. To study the contribution of different host cellular effector mechanisms responsible for tumor clearance and for tumor immunity induced by L19mTNFa/melphalan therapy, we evaluated the ability of total spleen cells from several WEHI- 164 tumor–cured mice to kill different tumor cell lines (WEHI-

164 fibrosarcoma, C51 colon carcinoma, RENCA renal carcinoma, C26 colon carcinoma) in vitro in a classical 4-hour 51Cr release assay. Splenocytes, assayed at 12 and 30 days after an in vivo WEHI-164 tumor rechallenge, were restimulated in vitro for 5 days with irradiated WEHI-164 tumor cells. As shown in Fig. 3A, at 12 days, high specific lysis was detected on WEHI-164 target cells and on all other syngeneic tumor cell lines tested. At day 30, a strong specific lysis was still found for WEHI-164 tumor cells and, to a lesser extent, for C51 targets (Fig. 3B). The addition during the test of concanamycin A, a specific inhibitor of perforin-dependent lysis, resulted in a dramatic decrease of specific cells lysis (66% for WEHI-164 and 100% for C51 target cells, respectively; Fig. 3B), indicating that CD8+ CTL effectors play an important role in the killing process. We then evaluated whether effector splenocytes, at 12 days after WEHI-164 rechallenge, presented on restimulation in vitro, a preferential production of IFN-g or of interleukin-4, representative, respectively, of type 1- and type 2-specific cytokines. For this purpose, we analyzed the frequencies of freshly isolated spleen cells by enzyme-linked immunospot assay. As shown in Fig. 4A and B, ex vivo immune spleen cells specifically recognized all four different syngeneic tumor cells tested, and high frequencies of effector cells were detected for both IFN-g (Fig. 4A) and interleukin-4 (Fig. 4B); thus, indicating that the tumor immunity was associated with a strong induction of both T helper 1 and T helper 2 types of responses. We also evaluated mTNFa secretion, upon in vitro tumor stimulation, by total spleen cells at 30 days after WEHI-164 tumor rechallenge. As shown in Fig. 4C, we found that the basal level of mTNFa secretion by unstimulated splenocytes from tumor-rejecting mice was higher compared with naïve splenocytes; moreover, 4 days after in vitro antigen stimulation using WEHI-164 and C51 tumor cells, the amount of mTNFa in the supernatant of the spleen cells of tumor-rejecting mice was more than twice that found in the supernatant of naïve spleen cells. Finally, we investigated by adoptive transfer experiments whether immune splenocytes of WEHI-164 tumor-bearing mice cured with L19mTNFa and melphalan, and rejecting a subsequent homologous tumor challenge, were able to protect naïve animals from tumor formation. Mixtures of tumor cells and splenocytes at different proportions were s.c. coinjected into naïve mice (Winn assay). Results showed that immune splenocytes in the E/T ratio of 1:1 were fully competent in protecting naïve mice (100%, 30 out of 30) from WEHI-164 tumor formation. Moreover, these mice acquired complete resistance to homologous tumor challenges carried out 45 days after the Winn assay using up to  $10 \square 106$ WEHI-164 cells (Fig. 5A). To determine whether immune splenocytes from WEHI-164 tumorcured mice were able to protect naive animals against the histologically unrelated C51 colon carcinoma, a Winn assay was done using 0.5  $\square$  106 C51 tumor cells and an E/T ratio of 5:1. In these conditions, 80% (16 out of 20) of the mice completely rejected the C51 tumor cells (Fig. 5B). Transferred immune splenocytes also induced resistance to WEHI-164 tumor formation (E/T = 1:1) in 78% (seven of seven) SCID beige mice (Fig. 5C) that were able to reject a new tumor challenge up to 3 months after the adoptive transfer. Adoptive transfer experiments were also done using immune splenocytes after in vitro depletion of specific cell subsets. The results shown in Fig. 5D indicate that removal of B cells or natural killer cells did not meaningfully alter the ability of the immune splenocytes to reject WEHI-164 tumor (100% rejection, eight out of eight, in B celldepleted; and 87% rejection, seven out of eight, in natural killer-depleted spleen cells). Also,

removal of CD8+ cells did not determine any dramatic change in the ability of immune splenocytes to reject WEHI tumor: in fact, six out of eight animals were protected. On the contrary, immune splenocytes depleted of CD4+ cells protected only two out of eight animals. These results reveal the fundamental role exerted by T cells, the CD4+ subset in particular, in the adoptive rejection process.

### Discussion

The findings presented in this study show that the therapeutic combination of the fusion protein L19mTNFa and melphalan, given as a single systemic administration, results in a high rate of complete and long-lasting tumor eradication without any apparent adverse side effects (>8 months with no sign of tumor recurrence at the writing of this article) in both the WEHI-164 fibrosarcoma (83%) and the C51 colon carcinoma (33%) models. Treating the tumor-bearing mice with melphalan and mTNFa alone or fused to an irrelevant antibody, we obtained, in the case of the C51 colon carcinoma, no tumor eradication and,

in the case of WEHI-164 fibrosarcoma, tumor eradication at a reduced rate compared with what was achieved with L19mTNFa treatment. The different responses of these two tumors to L19mTNFa/melphalan therapy may be due to one of two reasons: either the different sensitivities of the two tumor cell lines to mTNFa, or the higher immunogenicity of WEHI-164 fibrosarcoma with respect to C51 colon carcinoma. This issue is presently under investigation. We also observed a T cell-mediated immune response able to reject further tumor challenges in the mice cured using melphalan and mTNFa or TN11mTNFa (data not shown). Thus, the combined treatment of tumorbearing mice with melphalan and L19mTNFa, which induces a much higher rate of complete and longer-lasting tumor eradication compared with melphalan combined with mTNFa alone or fused to an irrelevant antibody, enhances the intrinsic anticancer activity of TNFa. The attempt to treat WEHI-164 fibrosarcoma grown in SCID beige mice did not result in any cure, but only in a retardation of tumor growth (Fig. 1D), due to the antitumor effects of melphalan and of TNFa exerted mainly on the angiogenic endothelial cells of the tumor vasculature (22, 23). In addition, all cured mice were resistant to tumor challenge, and the tumor rejection was not limited to the original tumor that was subjected to therapy, but was extended to histologically unrelated s.c. tumors and metastases. Moreover, the results of Winn assays reported here (Fig. 5) show that the splenocytes from cured mice protect naïve animals also from histologically

unrelated syngeneic tumors. Taken together, these findings show that, in addition to the cytotoxic effects of TNFa on the tumor vasculature, the immune system plays a role in the processes leading, first, to tumor cure and, subsequently, to the acquisition of immunologic memory and effector

functions, two traits that are instrumental to the recognition and rejection of tumors. These findings also indicate that the immunologic response is likely directed against tumor-associated antigens (TAA) shared by the different tumors tested. Previous studies by Curnis et al. (36) showed the enhancement of TNFa antitumor immunotherapeutic properties by its targeted delivery to amino peptidase CD13; this approach achieved only sporadic cases of complete cure, however, and rejection of challenges was limited to only few cases of the same tumor from which the animals were originally cured. The enhancement of TNFa immunotherapeutic activity generated by its targeted administration to B-FN may be due to the cytokine's high level of accumulation in the tumor environment, which, in concert with high local concentrations of melphalan (42), induces massive tumor cell killing with high levels of tumor antigens available for antigen-presenting cells. Antigen-presenting cells infiltrating into the tumor site with the contribution of TNFa (that stimulates endothelial cell adhesion of circulating phagocytic cells) subsequently migrate to lymph nodes where they can present TAAs to CD4+ T helper cells (27, 28). Thus, the availability of large amounts of TAAs from the necrotic area of the tumor, in conjunction with an

efficient TAA uptake by antigen-presenting cells, may result in the strong triggering and maintenance of the antitumor immune response observed here. The results of the present investigation reveal the fundamental role of T cells, and especially of the CD4+ subset, as effectors of the antitumor immune response generated by L19mTNFa in combination with melphalan. The role of effector CD8+ CTLs, clearly present as shown by the strong cytolytic response against tumor targets of different histologic origin (Fig. 3), seems to be less important in the in vivo rejection of WEHI-164 tumor in the Winn assay with CD8-depleted immune splenocytes (Fig. 5D). No major role in the effector response seems to be played by either B cells or natural killer cells (Fig. 5D). CD4+ T helper cells are required for the optimal induction of both humoral and cellular effector mechanisms (43). T helper-derived cytokines, particularly T helper 1-type cytokines, are fundamental for the maturation and functional competence of CTLs and B cells, as well as for the activation of antigen-presenting cells (44). Our results also indicate that in L19mTNFa/melphalan tumor-cured mice CD4+ T cells produce large amounts of IFN-g which, in addition to its important effects on the triggering and maintenance of immune effector cells, may exert an antiangiogenic effect and therefore play a role in the inhibition of tumor growth (45). It is noteworthy that the CD4+ T cells of tumor-rejecting mice also produce large amounts of interleukin-4, a T helper 2type cytokine. Although some authors report that polarized T helper 2 responses promote, rather than inhibit, tumor growth and spread (46, 47), other investigators have observed mixed T helper 1/2 immune responses that correlate with the tumor rejection (44, 48). The potent T cell-mediated immune response against the different types of tumors achieved with this treatment indicates that the immune response is directed against TAAs shared by tumors of different histologic origin. In fact, the existence of TAAs shared by different types of tumor has been reported for both mouse and human tumors (49). Furthermore, considering that TNFa has also shown a potent adjuvant activity, L19TNFa could be systemically administered to cancer patients in combination with vaccination approaches. This prospect may represent the rationale for a new therapeutic strategy against human cancer based on the targeted delivery of TNFa to tumor blood vessels.

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