

Targeted Delivery of Tumor Necrosis Factor- α 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 L19mTNF α (a fusion protein constituted by the scFv L19 specific for the oncofetal ED-B domain of fibronectin and tumor necrosis factor α , TNF α) in combination with melphalan induced complete and long-lasting tumor eradication in tumor-bearing mice and triggered 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.

Experimental Design and Results: Treatment with L19mTNF α , in combination with melphalan, induced complete tumor regression in 83% of BALB/c mice with WEHI-164 fibrosarcoma and 33% of animals with C51 colon carcinoma. All cured mice rejected challenges with the same tumor 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-cured mice protected naive mice both from C51 colon carcinoma and from WEHI-164 fibrosarcoma. Similar results were also obtained in adoptive immunity transfer experiments using severely immunodepressed mice. Experiments using depleted splenocytes showed that T cells play a major role in tumor rejection.

Conclusions: The results show that the selective targeting of mTNF α 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 TNF α to the tumor vasculature.

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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 mammals 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- α (TNF α) is a pleiotropic cytokine (20) composed of three noncovalently linked TNF α monomers, ~17.5 kDa each, that yield a compact bell-shaped homotrimer (21). TNF α exerts its major antitumor effects mainly via a preferential toxicity for the endothelial cells of the tumor-associated 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, TNF α 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, TNF α 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 TNF α 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 TNF α 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). TNF α 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 TNF α 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 TNF α mutants (33), encapsulation of TNF α in liposomes (34), and the targeted delivery to tumors of TNF α 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 L19mTNF α (17), consisting of mouse TNF α (mTNF α) 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 L19mTNF α in the tumor is roughly 35 times higher than the dose achieved with a control fusion protein in which mTNF α is conjugated to an irrelevant scFv (TN11).

Here, we show that a single systemic administration of L19mTNF α 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 (3×10^6 cells; European Collection of Animal Cell Cultures, Sigma-Aldrich, Milan, Italy), C51 mouse colon adenocarcinoma (0.5×10^6 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: $(d)^2 \times D \times 0.52$; 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 75×10^3 C51 cells in 100 μ L 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 of ~0.2 cm³) received an injection in their tail vein of the fusion proteins L19mTNF α or TN11mTNF α (the expression, purification, and characterization of the fusion proteins have previously been reported; ref. 17) or of recombinant mTNF α (2×10^7 units/mg, kindly provided by Dr. A. Corti, Department of Oncology, Cancer Immunotherapy and Gene Therapy Program, San Raffaele M. Scientific Institute, Milan, Italy), in 100 μ L of PBS. As already reported (17), in the therapeutic protocols with a single compound, 1 pmol/g of L19mTNF α , TN11mTNF α , or mTNF α was used, whereas 0.7 pmol/g was used in combination with other drugs. The group of controls received 100 μ L 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 μ g/g in 400 μ L). 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.

TNF α 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 \pm SD) versus mTNF α (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 (3×10^6 , WEHI-164; 0.5×10^6 , 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 naive 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 (3×10^6 cells) whereas with C51 tumor (0.5×10^6 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, FITC-conjugated goat F(ab')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 naïve mice as described by Croce et al. (40). Inhibition test of lysis with concanamycin A (0.2 μ g/mL, Sigma-Aldrich) was done as described by Seki et al. (41).

Enzyme-linked immunospot assay was conducted using *ex vivo* splenocytes from either naïve 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

L19mTNF α in combination with melphalan cures different murine tumors. Tumor-bearing mice were treated with a single i.v. administration of L19mTNF α (0.7 pmol/g) in combination

with melphalan (4.5 μ g/g), given i.p., as described in Materials and Methods. For control molecules, we substituted L19m TNF α with mTNF α or TN11mTNF α . Two murine experimental models, WEHI-164 fibrosarcoma and C51 colon carcinoma, were used for their different *in vitro* sensitivities to mTNF α . In fact, as shown in Fig. 1A, in the presence of actinomycin D, WEHI-164 cells are ~300 times more sensitive than C51 cells to mTNF α .

The treatment with L19mTNF α 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 mTNF α (Fig. 1C) or TN11mTNF α (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 mTNF α and melphalan (data not shown) and in 61% of the mice treated with TN11mTNF α and melphalan (22 out of 36 mice treated in different experiments carried out using identical conditions; Fig. 1B). The therapeutic efficacy on WEHI-164 fibrosarcoma-bearing mice of L19mTNF α or melphalan alone is reported in Fig. 1B. Only a moderate increase of survival time of the tumor-bearing mice was observed in either case. In order to assess whether the combined treatment with L19mTNF α and melphalan induced tumor eradication in immunocompromised

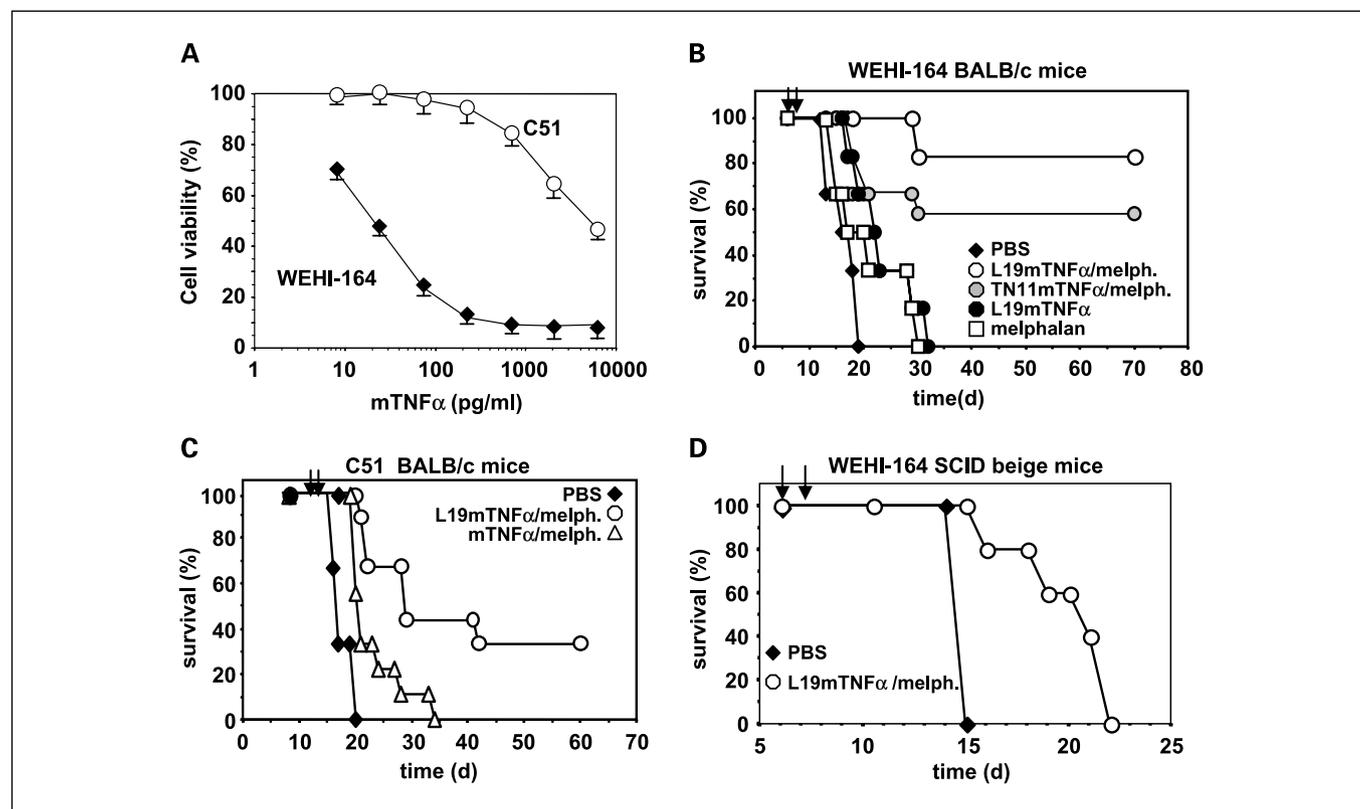


Fig. 1. A, different *in vitro* sensitivities to mTNF α of the WEHI-164 and C51 tumor cells. B, antitumor efficacy of different treatments in WEHI-164 tumor-bearing mice. In the experiment, groups of six animals were treated as reported, at the times indicated (arrows). C, antitumor efficacy of different treatments in C51 tumor-bearing mice. In the experiment, groups of nine C51 tumor-bearing mice were treated as reported, at the times indicated (arrows). D, antitumor efficacy of the combined treatment with L19mTNF α and melphalan (arrows) in a group of six WEHI-164 tumor-bearing SCID beige mice.

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 L19mTNF α .

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 15×10^6 cells (five times the dose used to induce the tumor in 100% of the mice; Table 1A). Moreover, 100% of C51 colon carcinoma-cured 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 0.5×10^6 (a cell dose inducing tumors in 100% of the animals), and by 30% of the mice challenged with 3×10^6 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 (3×10^6 cells) or RENCA (renal cell carcinoma, 1×10^6 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 L19mTNF α /melphalan therapy, we evaluated the ability of total spleen cells from several WEHI-

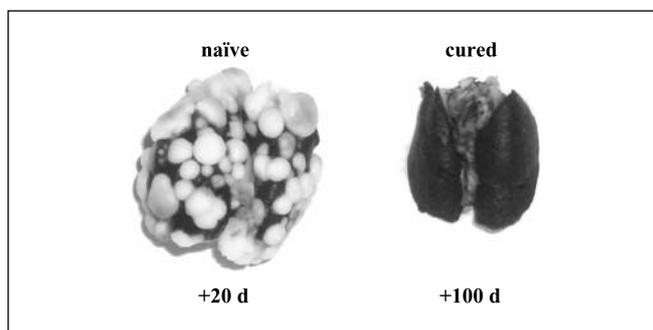


Fig. 2. Left, lungs of one out of five similar control mice sacrificed 20 days after induction of C51 metastasis. Right, lungs of one out of five WEHI-164 tumor-cured mice sacrificed 100 days after i.v. injection of C51 tumor cells.

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 ^{51}Cr 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

Challenge	Tumor	No. of cells	Rejection (%)
(A) Rejection of tumor challenges by WEHI-164 tumor-cured mice			
1	WEHI-164 s.c.	3×10^6	40 of 40 (100)
2	WEHI-164 s.c.	10×10^6	10 of 10 (100)
2	WEHI-164 s.c.	15×10^6	10 of 10 (100)
2	C51 s.c.	3×10^6	5 of 5 (100)
2	RENCA s.c.	1×10^6	5 of 5 (100)
2	C51 i.v.	75×10^3	5 of 5 (100)
1	C51 s.c.	0.5×10^6	6 of 10 (60)
1	C51 s.c.	3×10^6	3 of 10 (30)
2	RENCA s.c.	1×10^6	3 of 3 (100)
(B) Rejection of tumor challenges by C51 tumor-cured mice			
1	WEHI-164 s.c.	3×10^6	3 of 3 (100)
1	C51 s.c.	0.5×10^6	3 of 3 (100)

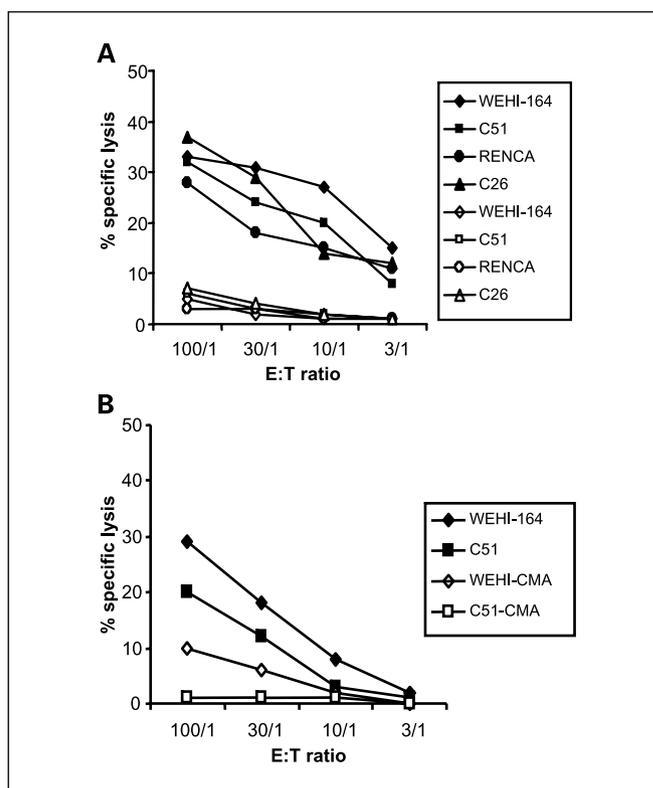


Fig. 3. Tumor rejection and induction of tumor-specific CTLs that cross-react with different syngeneic tumors. Results are representative of three independent ^{51}Cr release experiments with similar results. **A**, Specific cytotoxic activity against the indicated target tumor cells was tested in splenocytes from WEHI-164 tumor-cured mice (black symbols), isolated 12 days after a WEHI-164 tumor rechallenge and cocultured with irradiated WEHI-164 cells for 5 days (see Materials and Methods). As controls, naïve splenocytes were used under the same conditions (white symbols). **B**, Inhibitory effect of concanamycin A (white symbols) on the specific cytotoxic activity against the indicated tumor target cells (black symbols) of splenocytes from WEHI-164 tumor-cured mice, isolated 30 days after a WEHI-164 tumor rechallenge.

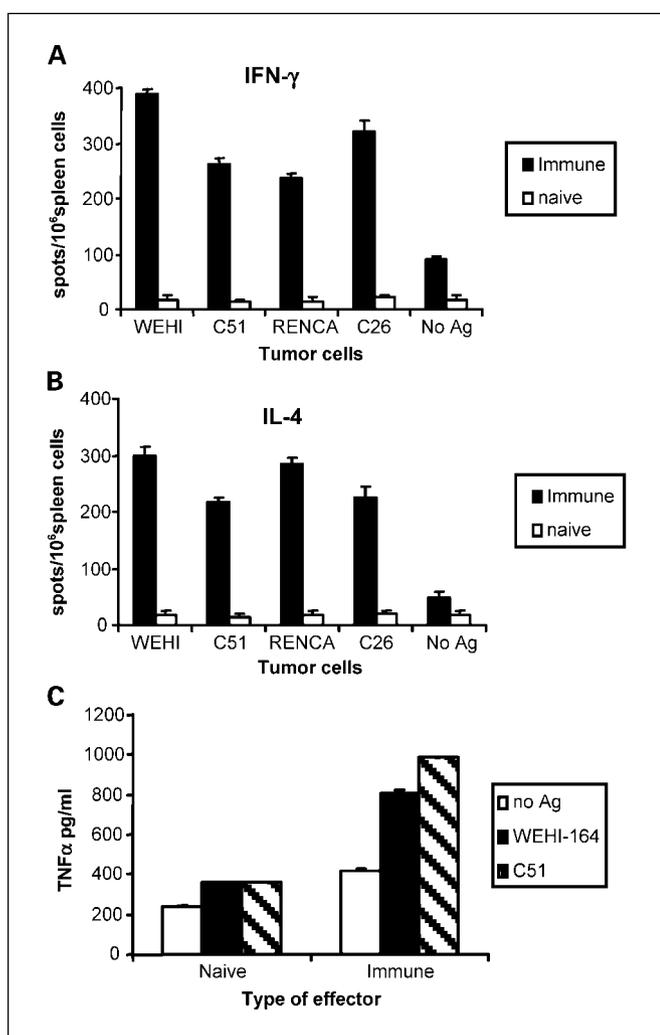


Fig. 4. Effector immune splenocytes of mice resistant to WEHI-164 tumor challenges show both T helper 1 and T helper 2 types of response. *A* and *B*, enzyme-linked immunospot assays were done on splenocytes from naïve mice (*white columns*) or from WEHI-164 tumor-cured mice (*black columns*), 12 days after a second WEHI-164 tumor challenge, to detect the specific release of either IFN- γ (*A*) or interleukin-4 (*B*) in response to the indicated tumor target cells. Columns, representative of three independent experiments with similar results. *C*, splenocytes from naïve and immune mice at 30 days from a second WEHI-164 tumor challenge, were *in vitro* stimulated with WEHI-164 or C51 tumor cells or with no antigen. mTNF α was evaluated in the supernatants after 4 days of stimulation (see Materials and Methods).

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- γ 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- γ (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 mTNF α 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 mTNF α 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 mTNF α 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 L19mTNF α 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 10×10^6 WEHI-164 cells (Fig. 5A).

To determine whether immune splenocytes from WEHI-164 tumor-cured mice were able to protect naïve animals against the histologically unrelated C51 colon carcinoma, a Winn assay was done using 0.5×10^6 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 cell-depleted; 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 L19mTNF α 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 mTNF α alone or fused to an irrelevant antibody, we obtained, in

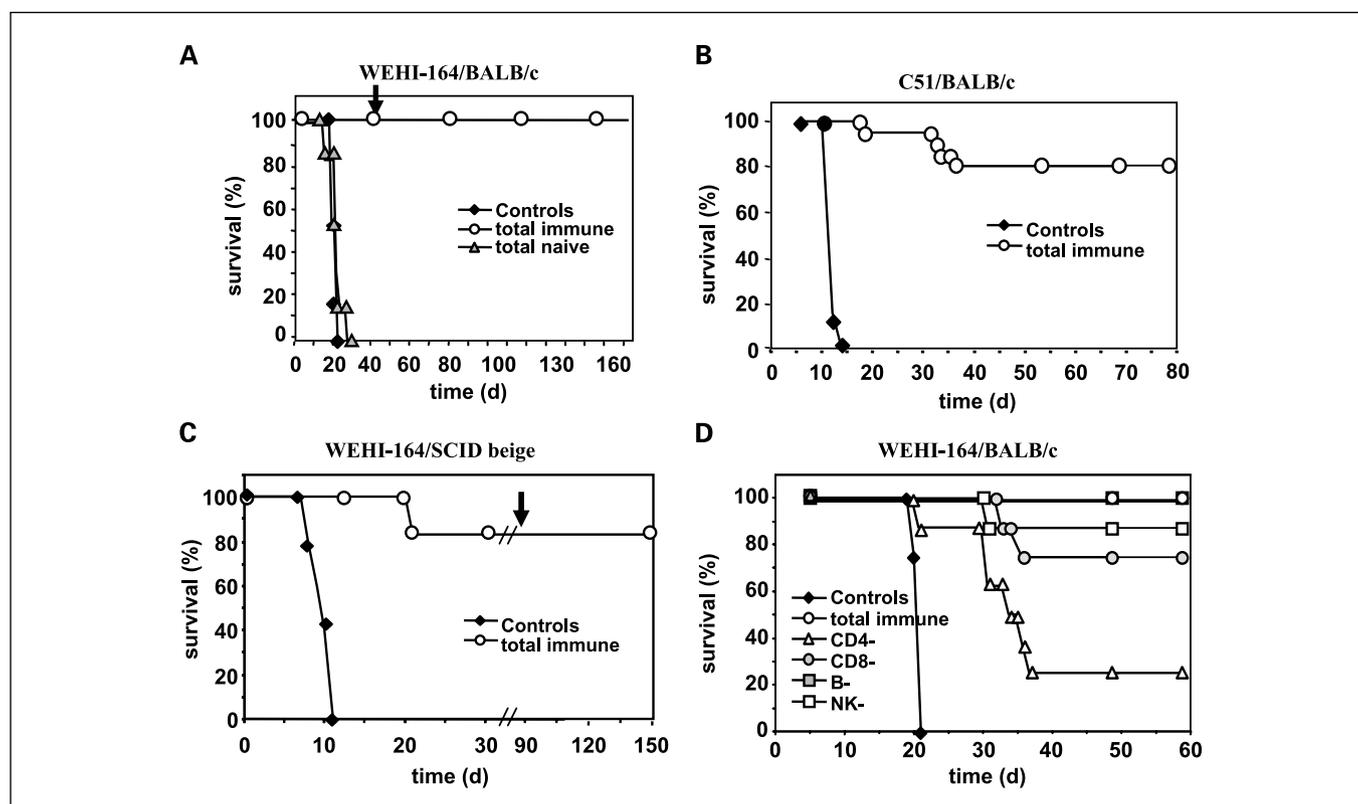


Fig. 5. Winn assay experiments: *A*, total immune splenocytes from WEHI-164 tumor – cured mice induce complete resistance to the same tumor in naïve BALB/c mice (E/T = 1:1; 3×10^6 WEHI-164 tumor cells). In this experiment, groups of six mice were used. On day 45 (arrow), a rechallenge with 10×10^6 WEHI-164 tumor cells was given to tumor-free mice. *B*, Total immune splenocytes from WEHI-164 tumor-cured mice (E/T = 5:1) protect 80% of naïve BALB/c mice (16 of 20) from the histologically unrelated C51 tumor (0.5×10^6 cells). *C*, Total immune splenocytes from WEHI-164 tumor-cured mice (E/T = 1:1) protect 78% (7 of 9) SCID beige mice against the same WEHI-164 tumor (3×10^6 cells). Arrow, a new tumor challenge on day 90 using 3×10^6 WEHI-164 tumor cells. *D*, Winn assay in groups of eight BALB/c mice using *in vitro* depleted immune splenocytes from WEHI-164 tumor – cured mice (see Materials and Methods) and WEHI-164 tumor cells (3×10^6). The E/T ratio was kept at 1:1.

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 L19mTNF α treatment. The different responses of these two tumor cell lines to L19mTNF α /melphalan therapy may be due to one of two reasons: either the different sensitivities of the two tumor cell lines to mTNF α , 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 mTNF α or TN11mTNF α (data not shown). Thus, the combined treatment of tumor-bearing mice with melphalan and L19mTNF α , which induces a much higher rate of complete and longer-lasting tumor eradication compared with melphalan combined with mTNF α alone or fused to an irrelevant antibody, enhances the intrinsic anticancer activity of TNF α .

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 TNF α 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 TNF α 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 TNF α 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 TNF α 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 TNF α (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 L19mTNF α 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 L19mTNF α /melphalan tumor-cured mice CD4⁺ T cells produce large amounts of IFN- γ 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 2-type 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 TNF α has also shown a potent adjuvant activity, L19TNF α 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 TNF α to tumor blood vessels.

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