Synergistic Therapeutic Effects of a Tumor Targeting Antibody Fragment, Fused to Interleukin 12 and to Tumor Necrosis Factor α[1]

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ABSTRACT

The potent antitumor activity of certain cytokines is often achieved at the expense of unacceptable toxicity. One avenue to improve the therapeutic index of cytokines in cancer therapy consists of fusing them to monoclonal antibodies capable of a selective localization at the tumor site. We have constructed fusion proteins of interleukin-12 (IL-12) and tumor necrosis factor (TNF-α) with L19, an antibody fragment specific to the extradomain B of fibronectin which has been shown to target tumors in animal models and in patients with cancer. These fusions display a potent antitumor activity in several immunocompetent murine models of cancer but do not lead to complete remissions of established aggressive tumors. In this article, we have evaluated the tumor-targeting properties and the anticancer activities of combinations of the two antibody-cytokine fusion proteins, as well as of a triple fusion protein between IL-12, L19, and TNF-α. Although all fusion proteins were active *in vitro*, the triple fusion protein failed to localize to tumors *in vivo* and to show significant therapeutic effects. By contrast, the combination of IL-12-L19 and L19-TNF-α displayed potent synergistic anticancer activity and led to the eradication of F9 teratocarcinomas grafted in immunocompetent mice. When cured mice were rechallenged with tumor cells, a delayed onset of tumor growth was observed, indicating the induction of a partial antitumor vaccination effect. Potent anticancer effects were achieved at doses of IL-12-L19 and L19-TNF-α (2 μg + 2 μg/mouse), which were at least 5-fold lower than the maximal-tolerated dose. The combined administration of the two fusion proteins showed only a modest increase in toxicity, compared with treatments performed with the individual fusion proteins. These results show that the targeted delivery of cytokines to the tumor environment strongly potentiates their antitumor activity and that the combination treatment with IL-12-L19 and L19-TNF-α appears to be synergistic *in vivo*.

INTRODUCTION

Systemic administration of cytokines, such as IL[3]-2, TNF-α, granulocyte macrophage colony-stimulating factor, or IL-12 can render some nonimmunogenic tumors immunogenic, activating a protective immunity (1–3). However, systemic administration of cytokines is often associated with severe toxic side effects, which prevent the administration of a curative dose.

One possible way of increasing the therapeutic index of certain cytokines consists of using them for locoregional treatments, in the case of localized tumors. Indeed, the perfusion of an isolated limb with TNF-α (often in combination with IFN-γ and melphalan; Ref. 4) allows reaching therapeutic concentrations of TNF-α, with a therapeutic benefit for patients with in transit melanoma metastases and limb salvage in soft tissue sarcoma patients (5). Consequently, this application of TNF-α was approved for use by the European Agency for the Evaluation of Medicinal Products (6).

Another possible way of increasing the therapeutic index of cytokines consists of fusing them to antibodies, which mediate a preferential accumulation of the cytokine at the tumor site. Indeed, in the past decade, several groups have reported different antibody-cytokine fusions for different tumor-associated antigens (7–10). These novel proteins were shown to retain both antibody and cytokine functions and to show superior anticancer activities as compared with equivalent amounts of free cytokine (and antibody).

We have previously described the targeted delivery of IL-12 and TNF-α to the subendothelial extracellular matrix by fusion of the cytokine to scFv(L19), a high-affinity human antibody fragment specific to the EDB domain of fibronectin, a marker of angiogenesis (11–14).[4]

IL-12-L19 dramatically enhanced the therapeutic index of IL-12 in both s.c. and metastatic murine tumor models (15). However, complete tumor regression could only be observed occasionally. Furthermore, when complete regression was observed, tumors could be reestablished in cured animals upon reinjection of tumor cells, indicating the absence of a lasting vaccination effect.

Similarly, L19-TNF-α exhibited an excellent tumor uptake in murine cancer models because of the vasoactive properties of TNF-α (16, 17)[4] and to the homotrimeric nature of the fusion protein, resulting in a high binding avidity (18). Administration of L19-TNF-α led to a substantial inhibition of tumor growth, which could further be delayed by melphalan. However, complete cures were rarely observed.[4]

The combination of different cytokine-based immunotherapies, showing synergistic rather than only additive antitumor effects, may represent an avenue for additional improvement of therapeutic efficacy. For instance, Reisfeld *et al.* (19) have shown that a partially protective memory T-cell immune response induced by a cellular tumor vaccine (tumor cells genetically engineered to secrete IL-12) could be additionally boosted by administration of a tumor-specific antibody-IL-2 fusion protein. Other groups have reported similar synergistic effects of IL-2 and IL-12 when administering combinations of recombinant IL-12 and IL-2 or when combining gene therapy with administration of recombinant cytokines (20, 21). Comparable enhancement of therapeutic activity has been demonstrated in murine tumor models with combinations of recombinant IL-12 and TNF-α (22) or when combining TNF-α gene-transduced cancer vaccines with administration of recombinant IL-12 (23).

In this study, we have combined the therapeutic activity of the

[3] The abbreviations used are: IL, interleukin; TNF-α, tumor necrosis factor α; EDB, extradomain B of fibronectin; L19, human antibody specific for the EDB domain of fibronectin; IL12-L19, fusion protein of IL-12 and the human antibody fragment L19; L19-TNF-α, fusion protein of the human antibody fragment L19 with TNF-α; ILT, triple fusion protein of IL-12 with the antibody fragment L19 and TNF-α; PHA, phytohemagglutinin.

[4] L. Borsi, E. Balza, B. Carnemolla, F. Sassi, P. Castellani, A. Berndt, H. Kosmehl, A. Siri, P. Orecchia, J. Grass, D. Neri, and L. Zardi. Selective targeted delivery of TNF-α to tumor blood vessels, submitted for publication.

previously described IL-12-L19 and L19-TNF-α fusion proteins. Besides their immunostimulatory properties, both IL-12 and TNF-α display at least part of their antitumor activity at the level of the tumor vasculature, although the mechanisms of action are different. On one hand, IL-12 is believed to be antiangiogenic by its downstream mediator IP-10 (24). On the other hand, the antitumor effects of TNF-α are mainly exerted on the tumor endothelium (25, 26), with increased permeability (17, 27, 28), up-regulation of tissue factor (29, 30), fibrin deposition, and thrombosis in the tumor vasculature, accompanied by a massive destruction of the endothelial cells (4, 5) that can be responsible for the antitumor response (26, 31–34). A fusion of IL-12 and TNF-α to scFv(L19) is likely to potentiate these activities because this antibody has been shown to be capable of efficient localization around the tumor blood vessels in animal models (11, 35–40) and in patients with cancer (41).

In this article, we report on the construction of a triple fusion, comprising IL-12, scFv(L19), and TNF-α, which was shown to retain full activity in *in vitro* assays. However, this large trimeric fusion protein failed to show tumor accumulation in biodistribution studies and displayed a modest therapeutic activity in a syngeneic murine tumor model. By contrast, combination studies of L19-TNF-α fusion protein (22) and IL-12-L19 molecule exhibited a significantly enhanced antitumor activity, compared with administrations of the individual antibody-cytokine fusions. Furthermore, L19-TNF-α selectively enhanced the tumor uptake of IL-12-L19, as demonstrated in biodistribution studies in tumor-bearing mice.

MATERIALS AND METHODS

Tumor Cell Lines and Reagents. The tumor cell line used was the F9 murine teratocarcinoma (42). ScFv(L19; Ref 39), IL-12-L19 (15), and the L19-TNF-α[4] fusion protein have been described elsewhere. The HEK 293 cell line and L-M fibroblasts were obtained from American Type Culture Collection (Manassas, VA).

Gene Constructs. ILT was constructed by performing a PCR assembly between the gene encoding the p40-p35 fusion (IL-12 in a single polypeptide chain format), amplified from IL-12-L19 (15) and the gene encoding L19-TNF-α.[4] The p40-p35 gene was amplified using the primer *sp40backEco* (5′-ccggaattcatgtgtcctcagaagctaaccatc-3′), which anneals to the endogenous secretion sequence of p40 and appends to its 5′ end a restriction site for the endonuclease *Eco*RI, and the primer *linkp35for* (5′-ctcacctccatcagcgcttccggcggagctcagatagcc-3′), which anneals to the 3′ end of p35 and appends the sequence of a short amino acid linker (GSADGG) to link the p40-p35 gene to the 5′ end of the L19-TNF-α gene. The sequence of L19-TNF-α was amplified using the primer *linkL19back* (5′-gccggaagcgctgatggaggtgaggtgcagctgttggagtc-3′), which appends to the 5′ end of L19 the complementary DNA sequence of the short amino acid linker (GSADGG) between p35 and L19, and the primer *TNFforNot* (5′-ataagaatgcggccgcctacagagcaatgactccaaagtagac-3′), which anneals to the 3′ end of TNF-α and introduces a stop codon, as well as a restriction site for the endonuclease *Not*I. The assembled IL-12-L19-TNF-α gene was cloned into the mammalian cell expression vector pcDNA3.1 (+) vector (Invitrogen, Basel, Switzerland), using the *Eco*RI/*Not*I sites of the vector.

Expression and Purification of L19-TNF-α, IL-12-L19, and ILT. HEK 293 cells were transfected with the plasmids, encoding either L19-TNF-α,[4] IL-12-L19 (15), or ILT, and stable transfectants were selected in the presence of G418 (500 μg/ml). Clones of G418-resistant cells were screened for antibody fusion protein expression by ELISA, using the recombinant ED-B domain of human fibronectin as antigen and an anti-TNF-α antibody (Peprotech EC Ltd., London, United Kingdom) or an anti-FLAG M2 antibody (for IL-12-L19; Sigma, Buchs, Switzerland) for detection. The fusion proteins were purified from cell culture medium by affinity chromatography over antigen columns, as previously described (38, 43), and desalted by passage over a preparative PD-10 gel filtration column (Amersham Pharmacia Biotech, Uppsala, Sweden) in PBS. The size of the fusion proteins was analyzed in reducing conditions on SDS-PAGE and in native conditions by fast protein liquid chromatography gel filtration on a Superdex S-200 (Amersham Pharmacia Biotech). Because IL-12-L19 and ILT were stable upon repeated freezing and thawing (no signs of aggregation or oligomerization), different purifications of each fusion protein were pooled to a single batch, aliquoted, and flash frozen in liquid nitrogen. For L19-TNF-α, gel filtration revealed the presence of aggregates after one and more rounds of freezing and thawing. However, the purified protein could stably be stored in solution at 4°C. BIAcore studies were performed with a BIAcore 3000 instrument (Biacore, Uppsala, Sweden) as previously described (40) using biotinylated EDB immobilized on a streptavidin-coated microsensor chip.

IL-12 Bioactivity Assay. IL-12 activity of IL-12-L19 and of the ILT fusion protein was determined by performing a T-cell proliferation assay (44). In brief, resting human peripheral blood mononuclear cells were cultured with mitogen (PHA and IL-2) for 3 days and then incubated in duplicate with serial dilutions of either fusion proteins or commercially available, recombinant, murine IL-12 as a standard (R&D Systems Europe Ltd., Abingdon, United Kingdom). Proliferation was subsequently measured by [^{3}H]thymidine incorporation.

TNF-α Bioactivity Assay. TNF-α activity of L19-TNF-α and of ILT was determined by performing a cytotoxicity assay on murine L-M fibroblasts (45). L-M fibroblasts were grown in DMEM containing 5% FCS and distributed in 96-well plates at 30,000 cells/well in 100 μl of medium. Plates were incubated for 24 h at 37°C, 5% CO_2. Subsequently, 100 μl of DMEM containing 4 μg/ml Actinomycin-D (final concentration of 2 μg/ml; Sigma) and either recombinant TNF-α (R&D Systems Europe Ltd.) or fusion protein at different concentrations (2000–10 pg/ml) were added to each well. After 24 h of incubation at 37°C, 5% CO_2, 10 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazol blue; Sigma; 5 mg/ml in PBS) was added to the wells. Plates were incubated for an additional 2–3 h at 37°C, 5% CO_2 (for blue crystal formation inside vital cells). Supernatants were aspirated and wells filled with 200 μl of DMSO to solubilize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide crystals formed inside the vital cells. Plates were read at 570 nm.

Biodistribution Experiments. The *in vivo* targeting performance was evaluated by biodistribution analysis as described by Tarli *et al.* (39). Briefly, purified IL-12-L19, ILT, or L19-TNF-α fusion protein was radioiodinated and ~2 μg (2 μCi) injected into immunocompetent 129SV mice bearing s.c. implanted F9 murine teratocarcinoma. In all cases, the immunoreactivity of the radiolabeled proteins was >84%, as measured by ED-B affinity chromatography (37, 38). Mice were sacrificed 1, 4, 24, or 48 h after injection. The organs were weighed, and the radioactivity was counted. Targeting results of representative organs are expressed as percent of injected dose/gram of tissue (%ID/g).

Tumor Model. Twelve to 14-week-old female SV129 mice (RCC, Füllingsdorf, Switzerland) received s.c. injections with 1×10^6 F9 murine teratocarcinoma cells. The mice were monitored daily, and tumor volume was measured with a caliper, using the formula volume = length × width^2 × π/6. When tumors had reached the desired size, mice were grouped ($n \geq 4$) and received i.v. injections of saline, IL-12-L19, L19-TNF-α, or combinations of both antibody fusion proteins diluted in a volume of 100 μl of PBS, supplemented with 50 μg/ml mouse serum albumin (Sigma). The αCTLA-4 antibody, clone 9H10, used in the second therapy study, was purchased at eBioscience (San Diego, CA).

In Vitro **IFN-γ Release Assay.** Groups of mice ($n = 3$) bearing a s.c. F9 teratocarcinoma were treated on days 5 and 9 with i.v. injections of either saline, IL-12-L19 (2 μg), L19-TNF-α (2 μg), or combinations of both fusion proteins (2 μg each). On day 10, mice were sacrificed, and spleens of all animals, as well as of tumor-free, untreated control animals, were collected. Splenocytes were harvested by mechanical disruption, using cell strainers (Becton Dickinson, Basel, Switzerland), and splenocytes of the same treatment group were pooled. RBCs were lysed by incubation in ACK lysis buffer [0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA (pH, 7.4)]. After the removal of lysed RBCs by centrifugation, splenocytes were resuspended in RPMI medium, supplemented with 10% FCS, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate (all from Invitrogen Life Technologies, Inc. AG, Basel, Switzerland). Splenocytes were distributed in 96-well plates at 3×10^5 cells/well in 100 μl of medium (3 wells/group of splenocytes). A total of 1×10^5 mitomoycin C-treated F9

teratocarcinoma cells, in 100 μl of medium, were added to each well, as described in Ref. 44). After 24 h of incubation at 37°C, 5% CO_2, levels of IFN-γ in the supernatant were measured by ELISA (HyCult Biotechnology, Uden, the Netherlands).

Histology. Twelve to 14-week-old female SV129 mice (RCC Füllingsdorf) received s.c. injections of 1×10^6 F9 murine teratocarcinoma cells. When tumors reached a volume of 300 mm³, groups of mice ($n = 3$) received i.v. injections of either saline, IL-12-L19 (2 μg), L19-TNF-α (2 μg), or combinations of both antibody fusion proteins (2 μg each). Mice were sacrificed 2 days after treatment and tumors excised and frozen in OCT (cryo-embedding compound; Microm, Walldorf, Germany). In addition to conventional H&E staining and analysis, quantitative immunohistochemical studies of lymphocytic infiltration were performed as described previously (15).

Immunohistochemical analysis of the ILT fusion protein was performed with sections of F9 tumors embedded in OCT essentially as previously described (36), using an anti-TNF-α antibody (Peprotech EC Ltd.). In negative control experiments, the primary ILT antibody was omitted to assess the background of secondary antibody reactivity to the murine tissues and tumor.

Quantitative Analysis of Necrotic Tumor Area. A H&E section along the largest diameter of the tumor was scanned at low power using the computer aided image analysis system Quantimet 600 and Qwin software (Leica, Germany). The total tumor area and necrotic tumor area were subsequently marked by the examiner on the screen. The areas were calculated by the image analysis system, and the ratio tumor necrosis area/total tumor area was given in percentages.

Toxicity Studies. Twelve to 14-week-old female SV129 mice (RCC Füllingsdorf; $n = 3$) were given one single injection of either saline, IL-12-L19, L19-TNF-α, or combinations of both antibody fusion proteins. Concentrations administered were at 5, 10, or 15 μg of one or both fusion proteins and diluted in a volume of 100 μl of PBS. The mice were monitored daily, and toxicity was measured in terms of weight loss and mortality.

RESULTS

Production and Characterization of IL-12-L19-TNF-α (ILT). The production and the antitumor activity of the individual L19-TNF-α and IL-12-L19 fusion proteins, in which TNF-α and IL-12 are appended to scFv(L19) via a polypeptide linker, have been described elsewhere (15).[4] Starting from these constructs (IL-12-L19 and L19-TNF-α), we constructed an antibody double cytokine fusion protein, comprising both IL-12 and TNF-α in a single polypeptide chain. Using recombinant DNA technology, the p40 and p35 domains of IL-12 were sequentially fused via a $(Ser_4Gly)_3$ linker and appended at the NH_2 terminal extremity of the scFv(L19) antibody fragment (15). Additionally, TNF-α was linked to the COOH terminus of scFv(L19). Schematic representations of L19-TNF-α, IL-12-L19, and the double cytokine fusion protein IL-12-L19-TNF-α (ILT) are shown in Fig. 1A.

Stable cell lines expressing either L19-TNF-α, IL-12-L19, or IL-12-L19-TNF-α (ILT) were generated in HEK 293 cells (46). All fusion proteins were purified from the culture medium at yields of 1–2 mg/liter by affinity chromatography on antigen columns, followed by gel-filtration for desalting (Fig. 1B). L19-TNF-α, IL-12-L19, and IL-12-L19-TNF-α (ILT) were fully immunoreactive, as measured by affinity chromatography on antigen columns and by BIAcore (Fig. 1C; Ref. 38). Both L19-TNF-α and ILT displayed a flat dissociation profile, as a result of their high binding avidity. The ability of ILT to recognize the EDB domain within the fibronectin molecule in tumor specimens was confirmed by immunohistochemistry (Fig. 2).

A cytotoxicity assay performed on murine L-M fibroblasts confirmed that both L19-TNF-α and IL-12-L19-TNF-α (ILT) retained full TNF-α bioactivity (Fig. 3A). Furthermore, the IL-12 moiety of the ILT fusion protein was as potent as recombinant murine IL-12 or
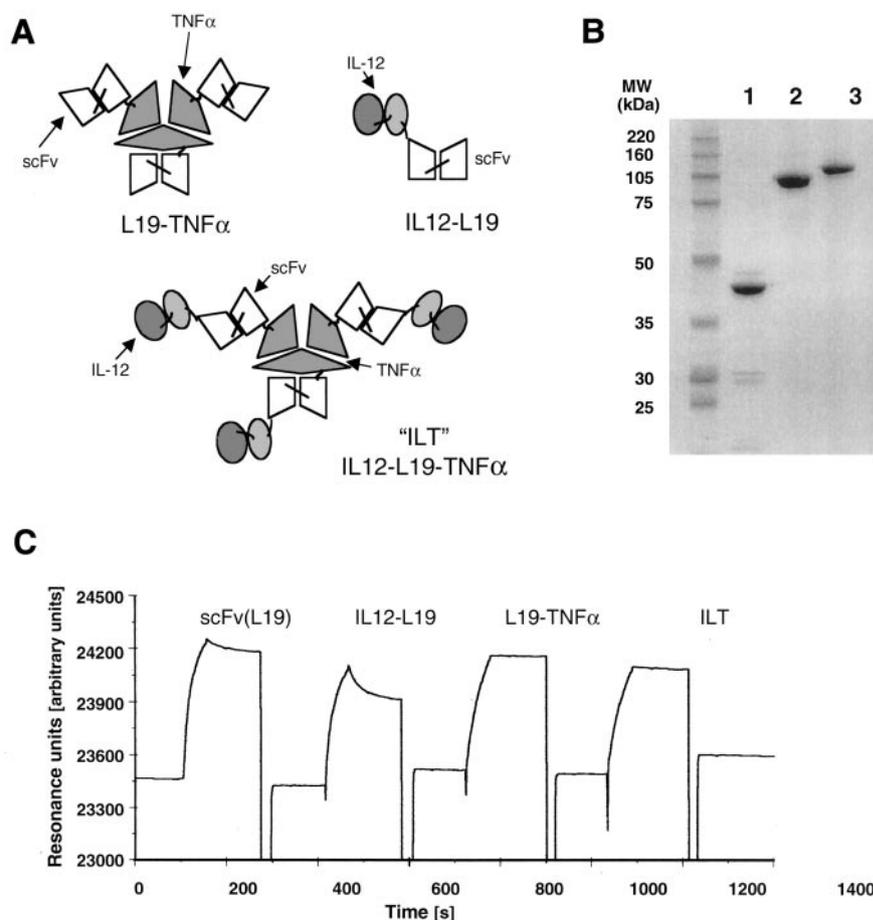
Fig. 1. *A*, schematic representation of the fusion proteins described in this article. L19-TNFα: the TNFα moiety was fused to the COOH terminus of the scFv(L19) antibody by a $(Ser_4Gly)_3$ linker. IL12-L19: the p40 domain of IL-12 was linked to the NH_2 terminus of its p35 domain by a $(Ser_4Gly)_3$ linker and then fused by a six amino acids linker to the NH_2 terminus of scFv(L19). ILT: fusion protein comprising the IL-12 moiety at the NH_2 terminus and the TNF-α moiety at the COOH terminus of the scFv(L19). *B*, SDS-PAGE analysis of purified recombinant of L19-TNFα (*Lane 1*), IL12-L19 (*Lane 2*), and IL-12-L19-TNF-α (*Lane 3*). *C*, BIAcore sensograms of scFv(L19) and scFv(L19)-based fusion proteins binding to an antigen (EDB)-coated chip. L19-TNF-α and ILT feature very slow dissociation profiles by virtue of their trivalent, high avidity binding properties.
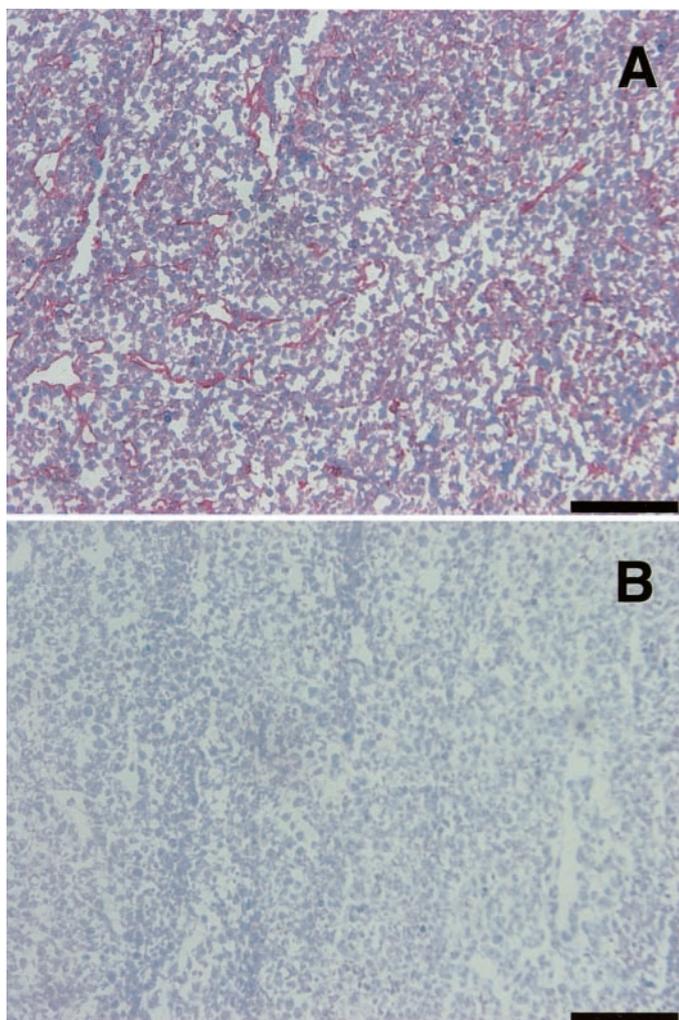
Fig. 2. Immunoreactivity of the ILT fusion protein with tumor neovasculature, detected immunohistochemically (*red staining*) in sections of F9 murine teratocarcinomas Sections were stained in the presence (*A*) and in the absence (*B*) of the ILT primary antibody, using an anti-TNF-α secondary antibody as detection reagent (*bar*, 100 μm).

In this case, the tumor uptake of IL-12-L19 was considerably enhanced (factor 2.5), whereas levels of the fusion protein in normal organs remained similar. This increase in tumor targeting can be attributed to the vasoactive properties of the coinjected L19-TNF-α fusion protein.

Combined Therapeutic effect of IL-12-L19 and L19-TNF-α on Large s.c. Tumors. To investigate whether combined administration of IL-12-L19 with L19-TNF-α would result in enhanced antitumor activity, we performed a therapy experiment in immunocompetent 129SV mice, bearing s.c. grafted F9 teratocarcinoma of ~300 mm^3 size. Groups of mice (five animals) received injections on day 9 after tumor cell implantation either with saline, IL-12-L19, L19-TNF-α, or combinations of the fusion proteins at two different dosages. A single i.v. injection of L19-TNF-α (2 μg) or of IL-12-L19 (2 μg) only resulted in a minimal delay of tumor growth. By contrast, administration of combinations of the antibody cytokine fusion proteins had a much more pronounced antitumor activity; after injection, tumors of mice treated either with high (2 μg of L19-TNF-α + 2 μg of IL-12-L19) or low (2 μg of L19-TNF-α + 0.2 μg of IL-12-L19) mixtures of antibody cytokine fusion proteins initially showed tumor growth arrest or regression for some days (Fig. 5). However, in all mice, tumors eventually started to regrow, indicating that administra-
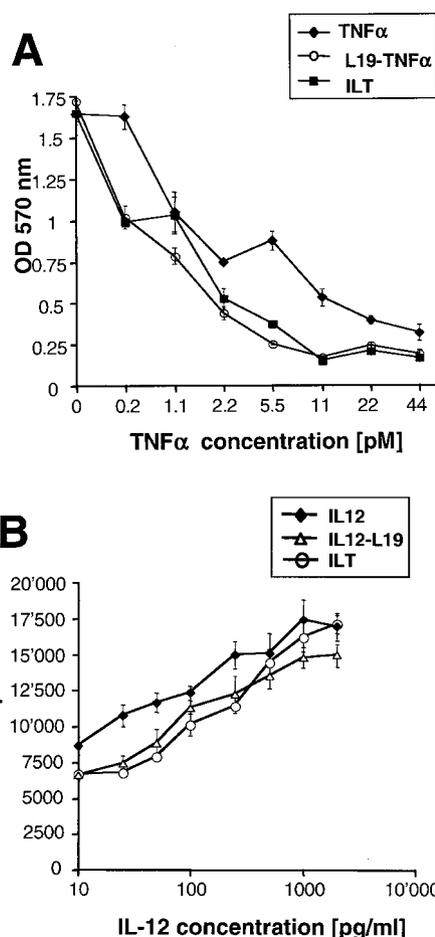

Fig. 3. *A*, the TNF-α biological activity of L19-TNF-α and of ILT was determined by a cytotoxicity assay using murine L-M fibroblasts. After incubation with increasing doses of TNF-α, viable cells were stained with a blue dye and lysed to allow colorimetric evaluation. The experiment was performed three times in triplicate; *error bars* correspond to the SD of the measurements. *B*, bioactivity of IL-12-L19-TNF-α, as compared with recombinant IL-12 and IL-12-L19, was measured by proliferation of PHA-activated human peripheral blood mononuclear cells. The IL-12 activity assay was performed once, using 2 × 3 wells for each concentration data point; *error bars* correspond to the SD of the measurements.

IL-12-L19 in inducing the proliferation of PHA-activated lymphoblasts (Fig. 3*B*).

Biodistribution Studies. The ability of ILT to selectively localize on tumor blood vessels was examined by quantitative biodistribution analysis in immunocompetent 129SV mice bearing a s.c. grafted F9 murine teratocarcinoma. The dose in the tumor, blood, and organs was determined 1, 4, 24, and 48 h after i.v. injection of the radioiodinated fusion protein (Fig. 4*A*). ILT was rapidly taken up and cleared by the liver and the kidneys, but no preferential accumulation in the tumor could be observed at any time point evaluated.

In a second set of biodistribution studies, the tumor-targeting properties of L19-TNF-α and the effect of coadminstration of the latter molecule on the biodistribution of IL-12-L19 were investigated. Fig. 4*B* shows the biodistribution results obtained for L19-TNF-α, featuring a high level of antibody uptake in the tumor and low levels in normal organs and blood. By contrast, the biodistribution of IL-12-L19 (Fig. 4*B*) revealed high values of spleen, liver, and kidney (15). Interestingly, we have observed that the uptake of this fusion protein in normal organs and in blood is considerably higher in immunocompetent mice (which display higher levels of IL-12 receptor-positive cells), compared with biodistributions performed in athymic nude mice (15). In parallel, we performed a biodistribution experiment in which radiolabelled IL-12-L19 was coadministered with L19-TNF-α.
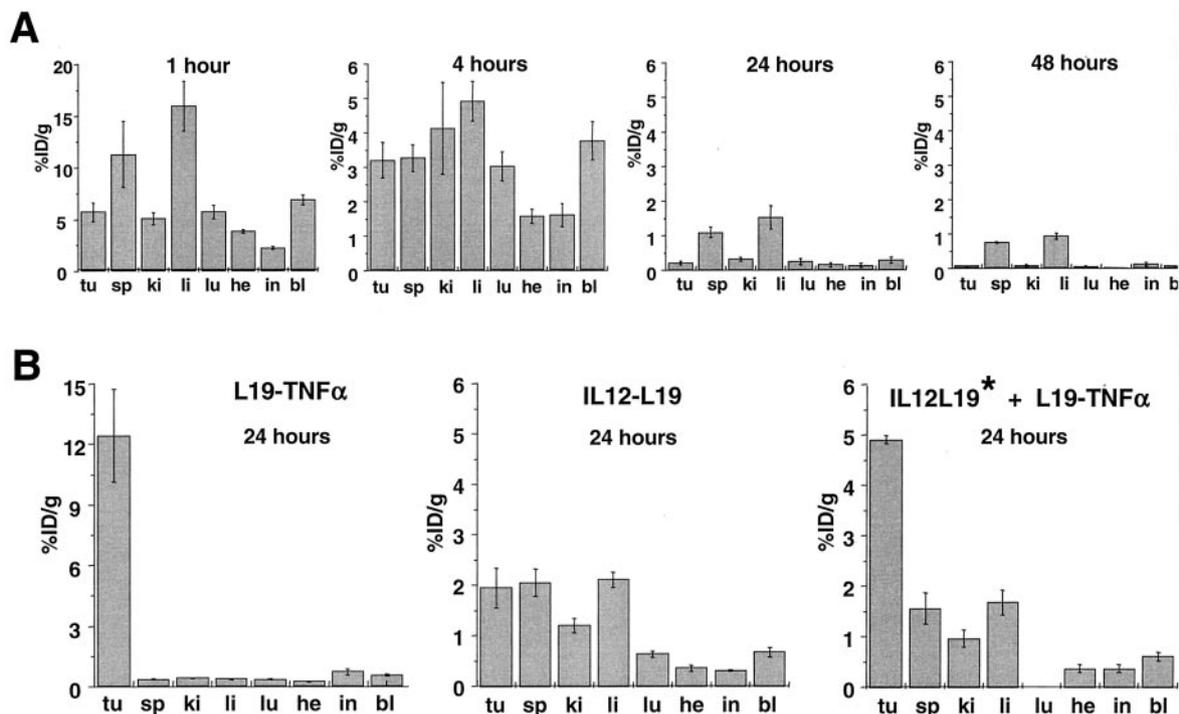
Fig. 4. *A*, biodistribution results obtained with ILT. F9 teratocarcinoma-bearing immunocompetent 129SV mice (groups of four animals) received injections of 2 μg of radioiodinated fusion protein and sacrificed after 1, 4, 24, and 48 h. Targeting results are expressed as percent injected dose/gram of tissue (%ID/g). *B*, biodistribution results obtained in F9 teratocarcinoma-bearing immunocompetent 129 SV mice (groups of four animals) 24 h after injection of 2 μg of L19-TNF-α, IL12-L19, or a combination of radiolabelled IL-12-L19 (2 μg, indicated with *) and unlabelled L19-TNF-α (2 μg). Targeting results are expressed as percent injected dose/gram of tissue (%ID/g) at 24 h.

tion of a single dose at a late point in tumor progression was insufficient to mount a curative immune response.

Combined Therapeutic Effect of IL-12-L19 and L19-TNF-α on Small s.c. Tumors. In a second therapy study, we tried to explore if it was possible to additionally improve the therapeutic performance of combined cytokine immunotherapy by altering the treatment schedule and dose. Treatment was started on day 5 after s.c. tumor cell implantation and repeated on day 9. Therapy groups consisted of mice (five animals/group) receiving either i.v. injections of saline, IL-12-L19, or L19-TNF-α (2 μg/injection) alone or in combination (2 μg
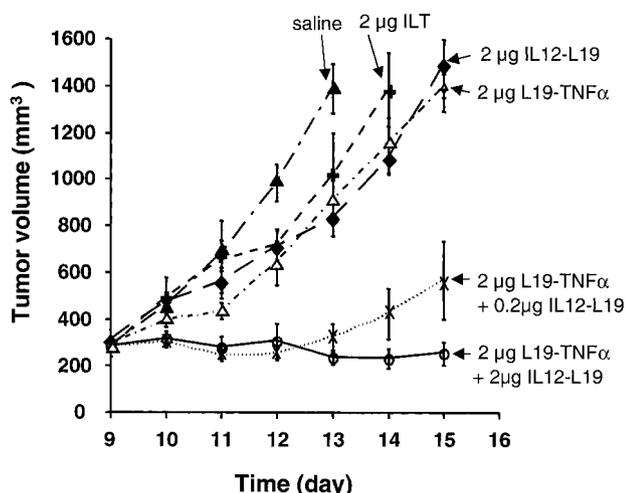

Fig. 5. Therapy studies with large tumors. 129SV mice bearing F9 teratocarcinomas were given a single i.v. injection of either saline, IL-12-L19 (2 μg), L19-TNF-α (2 μg), or combinations of both antibody cytokine fusion proteins at low (2 μg of L19-TNF-α + 0.2 μg of IL-12-L19) high (2 μg of L19-TNF-α + 2 μg of IL-12-L19) doses. Therapy was started 9 days after tumor cell implantation when tumors were large ($\approx$300 mm^3).

each). In addition, two more groups of mice were treated with either 50 μg of a commercially available αCTLA-4 antibody or with a combination of this antibody (50 μg) and IL-12-L19 (2 μg). Anti-CTLA4 antibodies have previously been shown to potentiate the antitumor response and to act synergistically with other antitumor immunotherapies (47, 48). Combining IL-12-L19 treatment with either L19-TNF or with CTLA-4 was clearly more effective than treating mice with saline or with one compound alone (Fig. 6). However, only the combination of IL-12-L19 with L19-TNF-α resulted in complete tumor regression (cure) in four of five mice treated. In all animals treated with IL-12-L19 and CTLA-4, tumors started to regrow within 30 days.

To investigate whether the combined treatment with IL-12-L19 and L19-TNF-α had induced a vaccination effect, the four cured mice were rechallenged on day 30 by s.c. injection of F9 teratocarcinoma cells in the other flank than the previously injected one. At the same time, four age-matched, untreated mice were also injected with F9 cells. Ten days later (day 40 in the graph), all untreated mice presented with large tumors (average of c.a. 460 mm^3), whereas only one of the rechallenged, previously treated mice had developed a tumor of similar size. At this time point, all rechallenged mice received a third injection of the combination treatment (IL-12-L19 and L19-TNF-α, 2 μg each). However, only one of four mice was found to remain without tumor (>100 days).

Toxicity. To investigate whether the synergistic therapeutic activity of IL-12-L19 and L19-TNF-α occurred at the expense of additional toxicity, SV129 mice ($n = 3$) were injected i.v. with either saline or different doses of IL-12-L19, L19-TNF-α or a combination of the two fusion proteins. Treatment with IL-12-L19 was well tolerated at the doses tested (5, 10, and 15 μg/mouse), whereas a transient weight loss (< 10%) was observed for L19-TNF-α and for the combination of IL-12-L19 and L19-TNF-α at the 10-μg dose (Fig. 7). However, at the 15-μg dose, one of three mice treated with
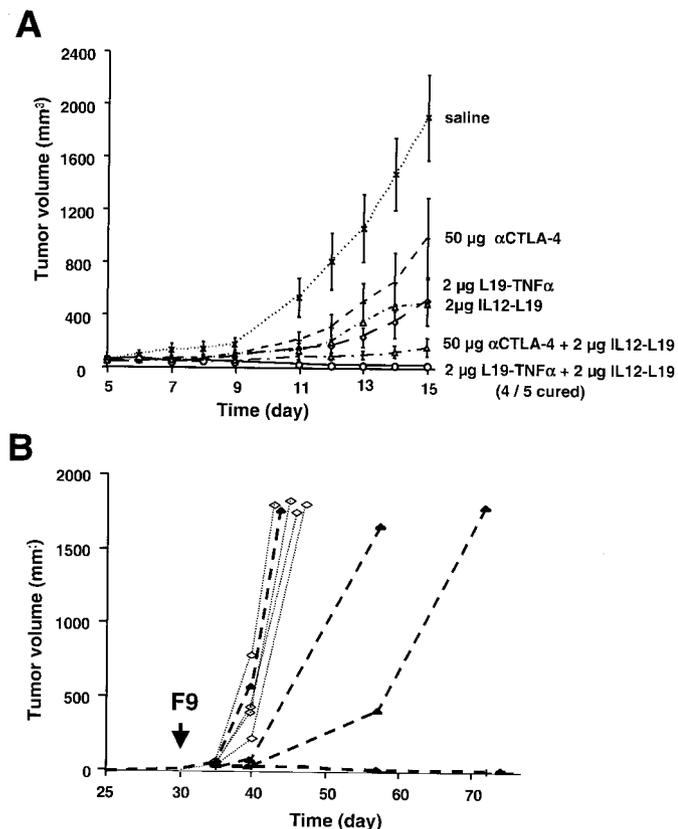
Fig. 6. Therapy studies with small tumors. *A*, 129SV mice bearing F9 teratocarcinomas were treated twice (days 5 and 9) with different doses and/or combinations of antibodies, as indicated in the figure. A complete tumor eradication (four of five mice) could only be observed with the combination of IL-12-L19 (2 μg) and L19-TNF-α (2 μg). *B*, cured mice and control mice were (re)challenged with F9 tumor cells on day 30. On day 40, delayed tumor growth could be observed in three of four mice from the treatment group (thick lines), indicating a partial vaccination effect. Administering one more dose of combination treatment to the four rechallenged mice on day 40 did not prevent tumors growth in three of four mice.

L19-TNF-α and three of three mice treated with the combination of the fusion proteins died.

Overall, the increase in toxicity for the combined treatment appeared to be moderate and occurred at doses >5-fold greater than the ones used in the therapy experiments.

Histology and Mechanistic Analysis. The residual tumor masses of F9 teratocarcinoma from mice (three for each group) 2 days after treatment with saline, IL-12-L19 (2 μg), L19-TNF-α (2 μg) and the combination of the two fusion proteins were analyzed by H&E staining and immunohistochemically for infiltration of lymphocytes, lymphokine-activated killer cells, natural killer cells, and macrophages. An increased infiltration of NK1.1 (+) cells in tumors combination treatment group was observed, whereas other differences with the mice receiving an individual fusion protein were less striking (data not shown).

The percentage of necrotic areas in tumors from mice receiving the combined treatment of IL-12-L19 + L19-TNF-α was significantly higher than the one observed for mice receiving the individual antibody fusions (Fig. 8). Tumors from mice receiving the combination treatment fully developed a histological regression pattern, characterized by necrosis of the tumor vessels themselves and extensive coagulative tumor necrosis, first with a pattern of a geographic necrosis, later with a pattern of a confluent necrosis up to 100% of the tumor area.

Furthermore, to assess if tumor-specific T lymphocytes had been generated, we investigated levels of IFNγ expression by splenocytes from tumor-bearing mice treated with IL-12-L19, L19-TNF-α, and a mixture of the two proteins upon coculture with F9 teratocarcinoma cells. High levels of IFN-γ were produced by splenocytes from mice that had received the combination treatment and (to a slightly lower extent) from animals treated with IL-12-L19. IFN-γ levels from mice receiving the L19-TNF-α were indistinguishable from the negative control groups (Fig. 9).

DISCUSSION

The EDB domain of fibronectin is an oncofetal marker, with 100% sequence identity between mouse and man. This distinctive feature allows to study the tumor targeting performance and the therapeutic activity of antibody-based pharmaceuticals in a syngeneic setting, using immunocompetent tumor-bearing mice. The simultaneous ad-

Fig. 7. Toxicity of fusion proteins. The acute toxicity of IL-12-L19, L19-TNF-α, and their combination was monitored in terms of weight loss of mice that were given injections once with different doses. At the 15-μg dose, one of three mice treated with L19-TNF-α and three of three mice treated with the combination of the fusion proteins died.
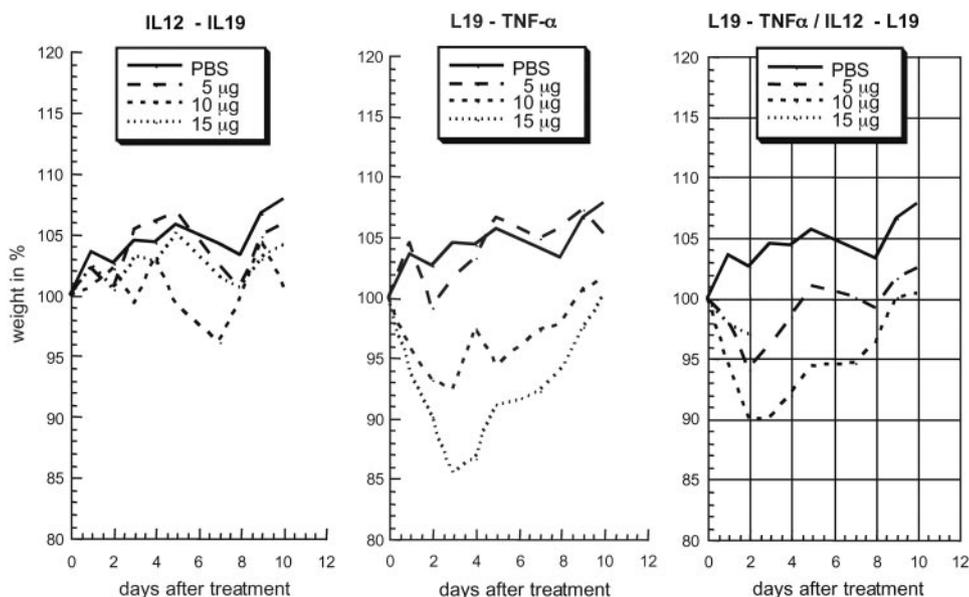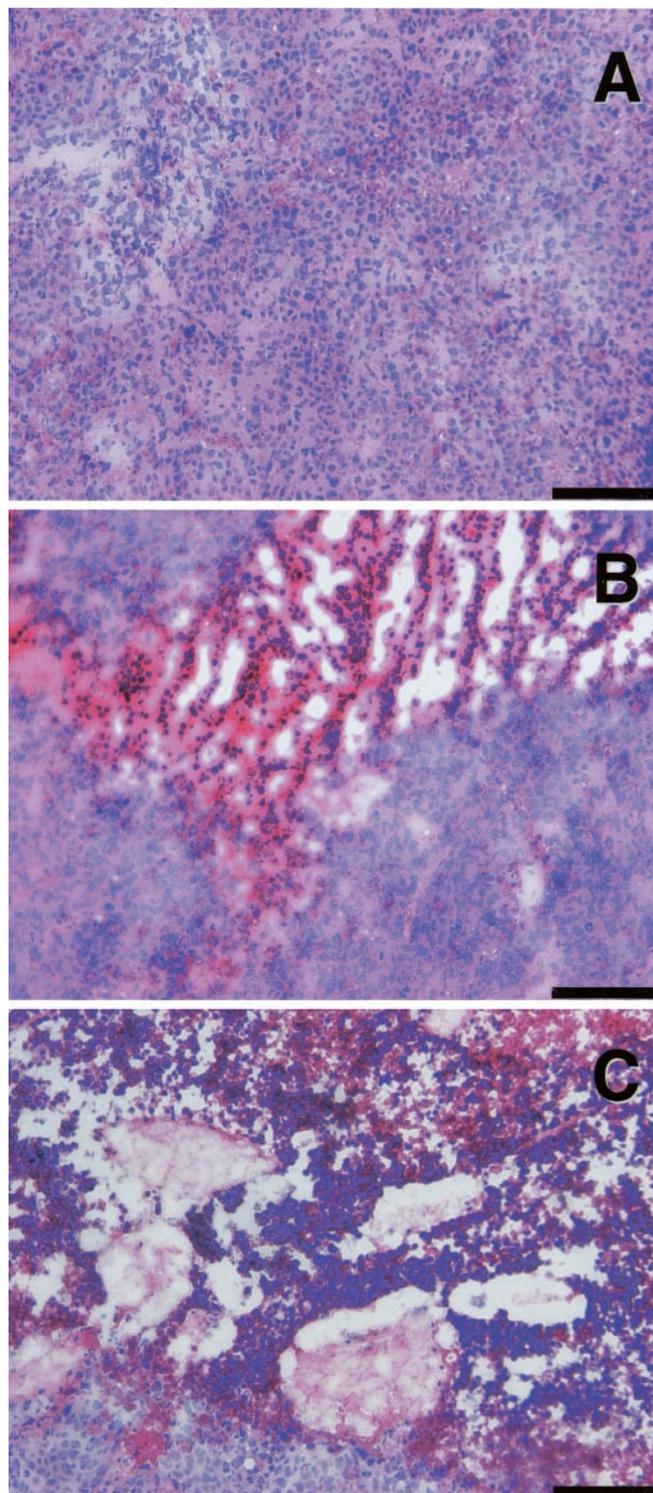
Fig. 8. Histological demonstration of a substantial increase of tumor necrosis observed 2 days after treatment with the combination of IL-12-L19 (2 μg) and L19-TNF-α (2 μg; *C*), compared with the treatment with IL-12-L19 (2 μg) alone (*B*), or the saline control (*A*; H&E, *bar*: 100 μm). The tumor necrosis (*B* and *C*) is characterized by karyopyknosis, loss of tumor cell adhesion, increased eosinophilia of tumor cell cytoplasm, loss of nuclear staining and cellular fragmentation. The average necrotic area, expressed as percentage of the total tumor area, was 4, 28, 49, and 85% for mice treated with saline, IL-12-L19, L19-TNF-α, and the combination of the two fusion proteins, respectively.

tion of the maximal-tolerated dose of doxorubicin (200 μg/mouse) could not eradicate this aggressive tumor (F. Nilsson and D. Neri, unpublished results). Furthermore, the therapeutic results obtained by the coadministration of IL-12-L19 and L19-TNF-α were clearly superior to the ones obtained with the same dose of the individual fusion proteins (2 μg/mouse/injection) and to combinations with an anti-CTLA4 antibody, which was known to stimulate antitumor immunity (47, 48).

The coadministration of L19-TNF-α with IL-12-L19 led to higher uptake of IL-12 in the tumor by virtue of the vasoactive properties of TNF-α. The higher levels of IL-12 account, at least in part, for the improved therapeutic performance observed with the combination treatment. Indeed, we had previously shown that the therapeutic effects of IL-12-L19 are clearly dose dependent (15). Furthermore, the combined treatment with L19-TNF-α and IL-12-L19 led to a more prominent pattern of coagulative necrosis in the residual tumor mass (Fig. 8).

When coadministering therapeutic agents, it is important to ensure that the combination treatment does not lead to additive toxicities. We studied this issue by injecting different groups of mice with increasing single doses of L19-TNF-α, IL-12-L19 and their combination, and monitoring toxic effects in terms of weight loss and mortality. The doses used in the therapy experiments (2 μg/mouse/injection) were well tolerated. A single coadministration of 10 μg of each of the two fusion proteins led to a lower transient weight loss (10%) than the injection of 15 μg of L19-TNF-α, which appeared to be the more toxic component. It appears therefore that the strong synergistic therapeutic action of L19-TNF-α and IL-12-L19 takes place at the expense of an only modest increase in toxicity.

We tried to combine the IL-12, L19 and TNF-α moieties in a single triple fusion protein, encoded by a single polypeptide. The resulting homotrimeric protein was well expressed, could be purified to homogeneity and was fully active *in vitro*. However, this large fusion protein (340 kDa) failed to localize to tumors, as assessed by quantitative biodistribution experiments. In the past, we have successfully used the L19 antibody for the selective targeting of several different agents to the subendothelial extracellular matrix in tumors, including radionuclides, photosensitizers, cytokines, growth factors, procoagulant factors, and drugs (35, 36, 38, 49). However, we had observed that fusion of scFv(L19) to highly charged polypeptides (*e.g.,* calmodulin, VEGF-164, Tat-peptide) completely abrogated the tumor targeting properties of the antibody, possibly as a consequence of the extreme isoelectric point value of the resulting fusion proteins (50). The biodistribution experiments performed with ILT raise the issue of whether an upper barrier exists for the molecular weight of antibody-based pharmaceuticals, which can successfully be used for tumor-
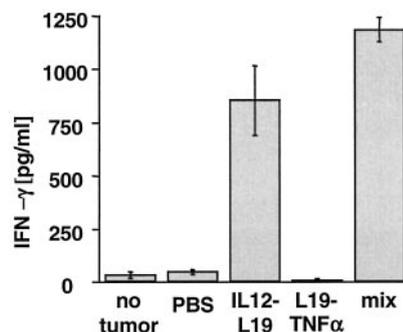

Fig. 9. IFN-γ release assay. Splenocytes were isolated on day 10 after tumor engraftment from F9 teratocarcinoma-bearing mice, which had been treated with fusion proteins (IL-12-L19, L19-TNF-α, or combination of both) or saline on days 5 and 9. Twenty-four h after coculture of splenocytes with mitomycin C-treated F9 teratocarcinoma cells, IFN-γ levels were measured in the culture media by ELISA.

ministration of IL-12-L19 with L19-TNF-α led to the complete cure of F9 teratocarcinomas when treatment was started at early time points (5 days after tumor implantation when the tumor size was 50–100 mm³). In the same experimental conditions, i.p. administra-

targeting experiments. In principle, the combination of several cytokines in the same fusion protein would present advantages for the clinical development because only one compound would have to be manufactured and studied in the clinic. However, the coadministration of different fusion proteins may be advantageous because it allows the dosing of the individual agents.

ScFv(L19), labeled with iodine 123, has been shown to image solid tumors in patients with cancer (41), therefore, validating its potential as a building block for the construction of antibody-based pharmaceuticals. Until now, the therapeutic use of IL-12 in patients with cancer has been hampered by serious toxicity. Similarly, the therapeutic administration of TNF-α is currently limited to locoregional treatments (*e.g.,* isolated limb perfusion; Ref. 6). The antibody-based strategy presented in this article may allow to lower the doses of these cytokines to be used in the clinic, making the corresponding fusion proteins suitable for systemic administration.

ACKNOWLEDGMENTS

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