Self-targeting of TNF-releasing cancer cells in preclinical models of primary and metastatic tumors

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Circulating cancer cells can putatively colonize distant organs to form metastases or to reinfiltrate primary tumors themselves through a process termed "tumor self-seeding." Here we exploit this biological attribute to deliver tumor necrosis factor alpha (TNF), a potent anti-tumor cytokine, directly to primary and metastatic tumors in a mechanism that we have defined as "tumor self-targeting." For this purpose, we genetically engineered mouse mammary adenocarcinoma (TSA), melanoma (B16-F10), and Lewis lung carcinoma cells to produce and release murine TNF. In a series of intervention trials, systemic administration of TNF-expressing tumor cells was associated with reduced growth of both primary tumors and metastatic colonies in immunocompetent mice. We show that these malignant cells home to tumors, locally release TNF, damage neovascular endothelium, and induce massive cancer cell apoptosis. We also demonstrate that such tumor-cell–mediated delivery avoids or minimizes common side effects often associated with TNF-based therapy, such as acute inflammation and weight loss. Our study provides proof of concept that genetically modified circulating tumor cells may serve as targeted vectors to deliver anticancer agents. In a clinical context, this unique paradigm represents a personalized approach to be translated into applications potentially using patient-derived circulating tumor cells as self-targeted vectors for drug delivery.

engineered tumor cells | tumor necrosis factor | vascular damaging agent | endothelial cells | apoptosis

A hallmark of cancer is the dissemination of tumor cells into the vascular system and colonization of distant organs to form metastatic foci. Recent studies showed that circulating cancer cells can traffic from primary or metastatic lesions to the peripheral blood and then back to their tumor of origin through a process termed "self-seeding" (1, 2). This process is proposed to mediate cancer progression by recruiting circulating tumor cells capable of surviving under harsh conditions in the bloodstream, thereby promoting both primary tumor growth and secondary metastatic dissemination (1, 3). Such homing properties may be facilitated by the presence of blood vessels with altered vascular endothelial barrier function in tumors (2, 4) and by the presence of a favorable tumor microenvironment (1).

This unique concept provides a rationale for exploiting cancer cells (5) as vehicles to deliver therapeutic agents to tumors, referred to hereafter as ("tumor self-targeting"). Indeed, oncolytic viruses incorporated within cancer cells have been shown to target lung metastases, being protected from circulating antibodies (5–7). Thus, we reasoned that "armed" tumor cells genetically manipulated to express anticancer cytokines and subsequently administered into the bloodstream should be able to deliver such factors focally to both primary and metastatic lesions as a therapeutic strategy. This delivery method may circumvent toxicity and activation of systemic counterregulatory mechanisms, which currently cause major limitations for cytokine biotherapy against cancer (8). To address this hypothesis experimentally, we engineered murine mammary adenocarcinoma, melanoma, and lung carcinoma cells to produce and release tumor necrosis factor alpha (TNF), a cytokine that damages the tumor vascular endothelia and has anticancer activity (9–11). The rationale for choosing this cytokine is based on our previous work that demonstrated ligand-based delivery of extremely low concentrations of TNF to tumor blood vessels markedly inhibits tumor growth in various xenograft (12–14) experimental mouse tumor models, and in a variety of native tumors in pet dogs (15). We show that systemic administration of several types of TNF-expressing cancer cells in syngeneic and nonsyngeneic tumor-bearing mice inhibits the growth of primary and metastatic cancers. We provide evidence that this effect is due to specific targeting to tumors, whereby injected cells accumulate and locally release TNF, resulting in tumor vasculature damage and tumor cell apoptosis.

Results

TNF-Expressing Tumor Cells: In Vitro and in Vivo Characterization. To exploit the concept of tumor self-targeting for the delivery of TNF to primary and metastatic tumor sites, we transduced murine TSA mammary adenocarcinoma tumor cells, B16-F10 melanoma cells, and Lewis lung cancer (LLC) cells with a lentiviral vector containing the murine TNF gene (pLenti-m*tnf*). The amount of bioactive TNF released in the supernatants of lentivirus-transduced

Significance

A recent paradigm shift has established "tumor cell seeding" as an intriguing biological phenomenon in cancer biology. However, the clinical implications of a bidirectional flow of cancer cells remains largely unexplored. We show that systemic administration of TNF-expressing cancer cells reduces growth of both primary tumors and metastatic colonies defined here as "tumor self-targeting." Our findings support a provocative concept in which circulating tumor cells genetically manipulated ex vivo and readministered into the circulating bloodstream may indeed serve as tumor-targeted cellular vectors in preclinical settings and could potentially open a field of translational investigation.

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cells was evaluated with a sensitive cell-based cytolytic assay (Fig. 1*A*). Through this approach, we generated tumor cell populations that produce levels of TNF that span three to four orders of magnitude, hereafter defined as: $TSA^{tnf\ low}$ and TSA^{tnf} [91.5 and 153 femtograms (fg) per cell per day, respectively], $B16\text{-}F10^{tnf\ low}$ and $B16\text{-}F10^{tnf}$ (2.7 and 23.5 fg per cell per day, respectively), and $LLC^{tnf\ low}$ and LLC^{tnf} (0.015 and 0.178 fg per cell per day, respectively). TNF production did not affect the proliferation index of transduced cells relative to the corresponding parental cells (Fig. 1*B*). Furthermore, escalating doses of exogenous, recombinant TNF (0.5–500 ng/mL) did not exert cytotoxic effects on parental tumor cells (Fig. 1*C*). These results demonstrate that TNF does not affect tumor cell growth and viability in vitro.

Next, the tumorigenic and metastatic potential of TNF-expressing tumor cells was investigated by administering TNF-expressing TSA, B16-F10, or LLC cells to immunocompetent syngeneic mice, either s.c. or i.v. TNF expression levels correlated with reduced growth rates of all s.c.-implanted tumors (Fig. 1*D*), as well as with the number of metastatic colonies in the lungs (Fig. 1*E*), compared with tumor numbers produced by the parental tumor cells. Of note, TNF-expressing TSA tumor cells did not grow at all, presumably as a consequence of the highest levels of secreted TNF (Fig. 1*A*). Furthermore, these mice were protected from subsequent s.c. challenges with parental TSA cells (Fig. 1*F*). Taken together, these results demonstrate that TNF secreted by tumor cells has an antitumor effect on both primary and metastatic tumors in vivo.

TNF-Expressing Cancer Cells Inhibit the Growth of Primary Tumors and Lung Colonies in Mice. To evaluate the potential therapeutic application of TNF-expressing tumor cells, we investigated the

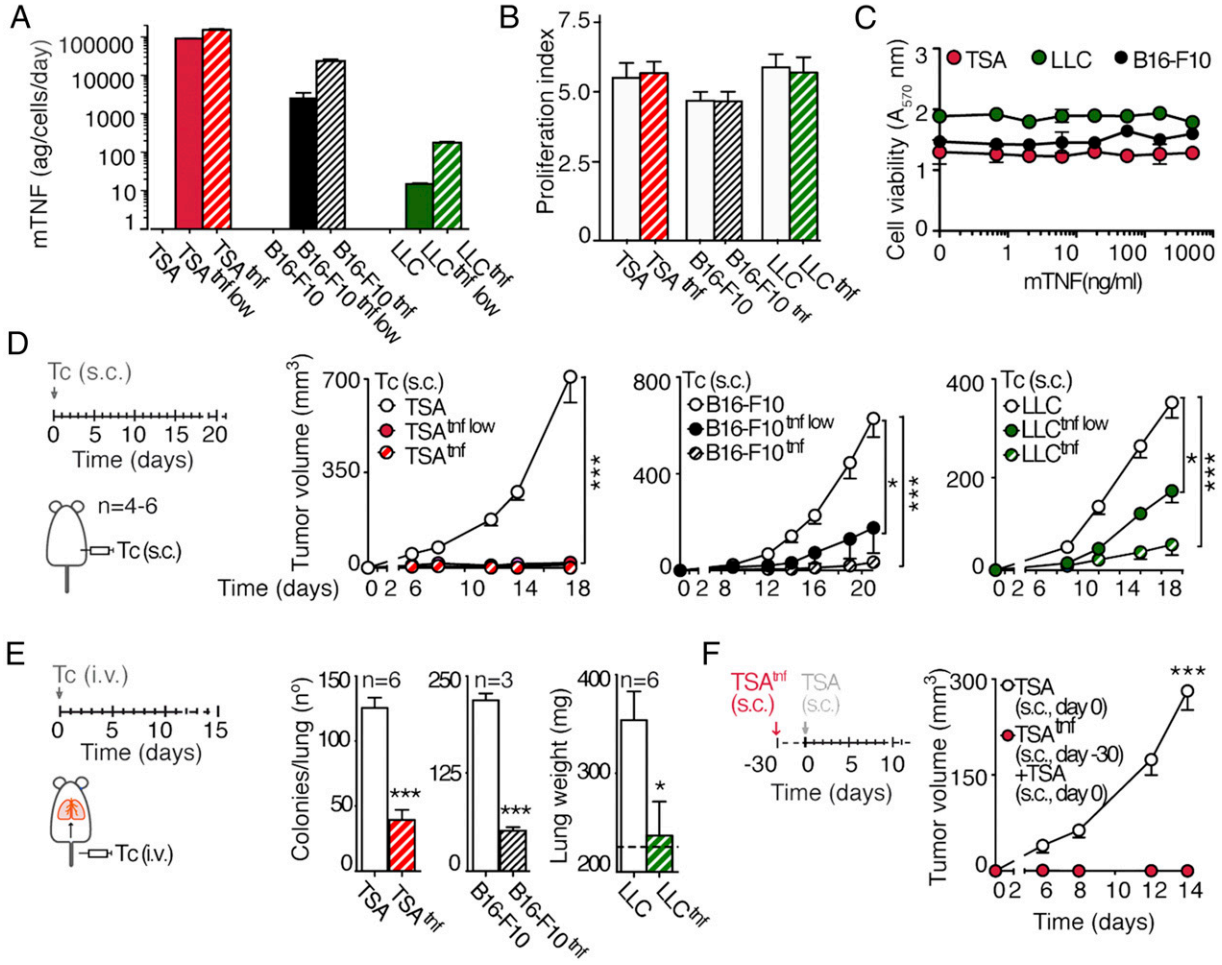

Fig. 1. In vitro and in vivo growth of genetically engineered TSA, B16-F10, and LLC cells that express varying amounts of TNF. (*A*) Amounts of bioactive TNF released per cell per day into the supernatant by different populations of TSA, B16-F10, and LLC cells transduced with pLenti-mTNF (TSA^{tnf}, $B16\text{-}F10^{tnf}$, and LLC^{tnf}), as measured by a cytolytic assay. Each experiment was performed in triplicate, and repeated three times. Representative experiments are shown (SEM) and depicted ± SEM). (*B*) In vitro proliferation of transduced and nontransduced tumor cells as evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining. Two experiments were performed in quadruplicate. One representative experiment is shown (mean ± SEM). (*C*) Effect of TNF on TSA, B16-F10, and LLC cell viability analyzed by a cytotoxicity assay. (*D*) Tumorigenic properties of transduced and nontransduced cells. Mice were administered s.c. with parental or TNF-producing TSA, B16-F10, or LLC cells (Tc, tumor cell). The growth of s.c. tumors was monitored by measuring tumor volumes with a caliper at serial time points. (*E*) Colonies of transduced and nontransduced cells. Parental or TNF-producing TSA, B16-F10, or LLC cells were injected i.v., mice were killed, and the lungs were inspected for the presence of metastasic foci. The numbers of colonies per lung or lung weights are shown (mean ± SEM; $n = 3$–6 as indicated). Dashed line in the LLC bars indicates the average weight of normal lungs. (*F*) TSA^{tnf} cells implanted s.c. in syngeneic mice protect against further challenge with TSA cells. BALB/c mice were injected s.c. with TSA^{tnf} (4×10^5 cells per mouse). No visible tumor formation occurred. Thirty days later, TSA cells were implanted s.c. and tumor growth was monitored. Mice in which TSA^{tnf} cells did not grow were also unaffected by the subsequent challenge with TSA cells. Experimental schedule and tumor volumes (mean ± SEM) of one representative experiment are shown ($n = 3$–4 as indicated). Three experiments were performed. $^*P < 0.05$; $^{***}P < 0.001$ by Student's *t* test, two tailed.

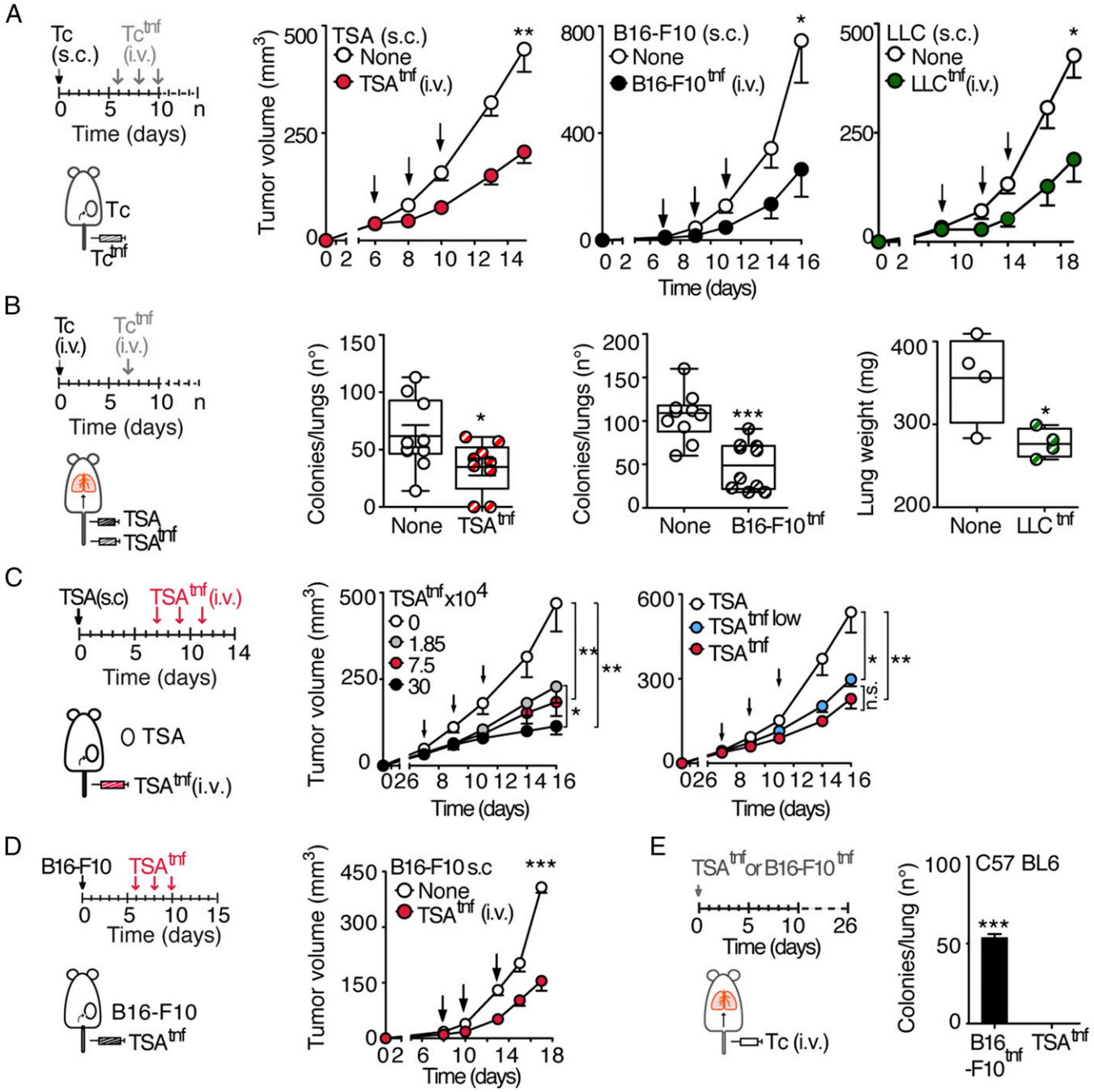

Fig. 2. Systemic cell-dose–dependent administration of TNF-expressing cells reduces the growth of s.c. tumors and the number of lung colonies in syngeneic and nonsyngeneic models. (*A*) Effect of i.v. administration of TSA^{tnf}, B16-F10^{tnf}, or LLC^{tnf} cells on the growth of s.c. TSA, B16-F10, or LLC tumors, respectively. A general treatment schedule is shown (Tc, tumor cell; Tc^{tnf}, TNF-expressing tumor cells). Treatments started when tumor volumes reached ~50 mm^3. Tumor volumes (mean ± SEM) from one representative experiment are shown (n = 4–8 as indicated). Two experiments were performed. (*B*) Effect of i.v. administration of TSA^{tnf}, B16-F10^{tnf}, or LLC^{tnf} on the number of colonies in the lungs of mice receiving i.v. TSA, B16-F10, or LLC cells, respectively. A general treatment schedule is shown. Treatments started 7 d after i.v. administration of parental tumor cells. The number of colonies per lung (mean ± SEM; cumulative data from two experiments, n = 4–5 each) or lung weight (mean ± SEM; one experiment, n = 4) are shown. (*C*, *Left*) Experimental schedule. (*Center*) Effect of three i.v. injections of 0, 1.85, 7.5, or 30 × 10^4 TSA^{tnf} cells on s.c. TSA tumors (n = 4–5, mean ± SEM). (*Right*) Effect of three i.v. injections of TSA^{tnf}, or TSA$^{tnf\ low}$, or TSA cells (7.5 × 10^4 cells per mouse) on s.c. TSA tumors. Tumor volumes (mean ± SEM) are shown (n = 5). (*D*) Effect of i.v. administration of TSA^{tnf} cells (arrows, 7.5 × 10^5) on the growth of B16-F10 tumors implanted s.c in C57/BL6 mice (syngeneic with B16-F10, nonsyngeneic for TSA cells). Tumor volumes (mean ± SEM) are shown as cumulative results from two different experiments (n = 3 each). (*E*) Evaluation of metastatic colonies following B16-F10^{tnf} or TSA^{tnf} i.v. injection in C57BL/6 mice (syngeneic for B16-F10 cells, nonsyngeneic for TSA cells). Lungs were inspected for the presence of visual colonies at day 26 (n = 4, mean ± SEM). *P < 0.05; **P < 0.01; ***P < 0.001 by Student's t test, two tailed.

effect of their systemic administration on the growth of established primary tumors. We administered TSA^{tnf}, B16-F10^{tnf}, or LLC^{tnf} (i.v., three times) to syngeneic mice bearing s.c. implanted TSA, B16-F10, or LLC tumors, respectively. Although TSA^{tnf}, B16-F10^{tnf}, or LLC^{tnf} produce widely ranging levels of TNF in vitro, i.v. administration inhibited tumor growth by 50%, 65%, or 55%, respectively (Fig. 2*A*). When we compared i.v. and s.c. routes of administration, we observed that s.c. injection of TSA^{tnf} cells into the contralateral

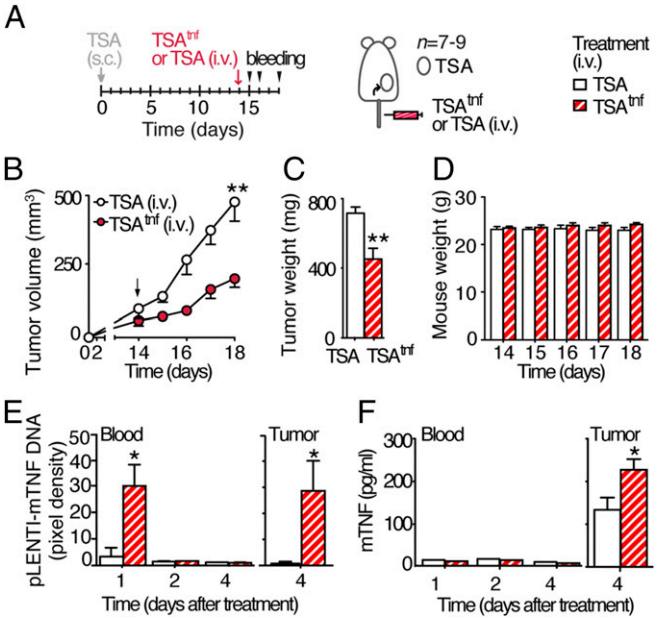

Fig. 3. TSA^{tnf} cells home to TSA tumors and locally release within the primary tumor. (*A*) Experimental schedule. (*B–D*) Effect of i.v. administration of TSA^{tnf} or TSA cells to TSA tumor-bearing mice (s.c.) on (*B*) tumor volume, (*C*) tumor weight, and (*D*) animal weight (n = 7–9, as indicated; mean ± SEM). (*E*) Quantification of pLENTI-m*tnf* DNA, by semiquantitative PCR, in tumors and in the blood after i.v. administration of TSA or TSA^{tnf} cells (n = 6). (*F*) Quantification of TNF levels by ELISA in tumors and in the peripheral blood of mice after i.v. administration of TSA or TSA^{tnf} cells (n = 7–9, as indicated). *P < 0.05; **P < 0.01 by Student's t test, two tailed.

flank was unable to inhibit growth of the established s.c.-implanted TSA tumor (Fig. S1*A*); in contrast, when TSA^{tnf} cells were i.v. administered, tumor growth of the established s.c.-implanted TSA tumor was inhibited (Fig. S1*B*). This intriguing observation indicates that the presence of TNF-expressing tumor cells in the vascular circulation is critical for antitumor activity of the primary tumor.

To determine whether i.v.-administered TNF-expressing cells would limit the growth of metastatic tumors, mice were injected i.v. with TSA, B16-F10, or LLC parental tumor cells and allowed to circulate and form lung colonies. One week later, tumor-bearing mice were injected i.v. with TSA^{tnf}, B16-F10^{tnf}, or LLC^{tnf} tumor cells, respectively. Tumor-bearing mice were killed to quantify the number of metastatic lung colonies at 14 (TSA), 11 (B16-F10), or 28 (LLC) days, post-injection of each of the three tumor types. Remarkably, the number of metastatic colonies in the lungs of mice treated with TNF-expressing tumor cells was significantly reduced compared with control mice (Fig. 2*B*). Taken together, these data demonstrate that systemic administration of TNF-expressing cancer cells partially inhibits the growth of tumors in the primary and metastatic settings.

The Therapeutic Effect of Circulating TSA^{tnf} Cells Is Dependent on the Number of TNF-Expressing Cells. To characterize the antitumor effects of circulating TNF quantitatively, we next analyzed whether this effect is dependent on (*i*) the number of TNF-expressing cells or (*ii*) the amount of TNF. For these refinement studies, we chose the TSA cell line because its tumor-homing properties have been extensively characterized (2) and, the range of expressed TNF was higher in these cells than in the B16-F10 or LLC cells. When we i.v. administered 1.85×10^4, 7.5×10^4, or 30×10^4 TSA^{tnf} cells per mouse (at 153 fg per cell per day) to mice bearing s.c.-implanted TSA tumors, we observed a cell-number–dependent antitumor effect (Fig. 2*C, Left* graph). Moreover, when an equal number of cells (7.5×10^4 cells per mouse) was injected that express either low or high TNF (91.5 or 153 fg per cell per day), we found no significant difference in their antitumor activity (Fig. 2*C*, *Right* graph). These independent results are in line with the data shown in Fig. 2*A* and indicate that the antitumor effect of TNF-expressing cells does not require a large concentration of TNF to inhibit tumor growth. Rather, tumor growth inhibition is proportional to the number of TNF-expressing tumor cells administered.

Irradiated TSA^{tnf} Cells Partially Inhibit Tumor Growth. To assess whether TSA^{tnf} cells retained any antitumor activity in the absence of proliferation, we irradiated them to induce cell cycle arrest (16). Irradiation reduced the cell proliferation index without affecting TNF production (Fig. S2*A*). When these cells were injected into mice bearing s.c. TSA tumors, they showed a nonsignificant tumor volume reduction, indicating the importance of cell proliferation to support their therapeutic effect (Fig. S2*B*).

Systemic Administration of TSA^{tnf} Inhibits the Growth of B16-F10 Tumors. Given that cross-seeding between heterotypic tumors (melanoma and mammary tumors) is experimentally established (1), we next investigated whether the therapeutic effect of TSA^{tnf} cells would be effective in a nonsyngeneic setting using B16-F10 tumors. TSA^{tnf} cells (derived from BALB/c mice) were administered i.v. into C57BL/6 mice bearing s.c.-implanted B16-F10 tumors. We observed antitumor effects similar to those obtained with the syngeneic models described above (Fig. 2*D*). As predicted, because TSA^{tnf} cells were administered into nonsyngeneic mice, they were eventually rejected by the host and did not establish lung colonies (Fig. 2*E*). Nevertheless, our results demonstrated that TSA^{tnf} cells circulate for a sufficient period to exert a therapeutic effect. Collectively these results indicate a potential antitumor approach to deliver cytokines by transducing host tumor cells.

TSA^{tnf} Cells Home to Subcutaneous TSA Tumors and Locally Release TNF. To quantify tumor homing and local release of TNF by circulating TNF-expressing cells, we first administered TSA or TSA^{tnf} cells i.v. into mice bearing s.c. TSA tumors (Fig. 3*A*). The growth rate of TSA tumors was significantly lower in

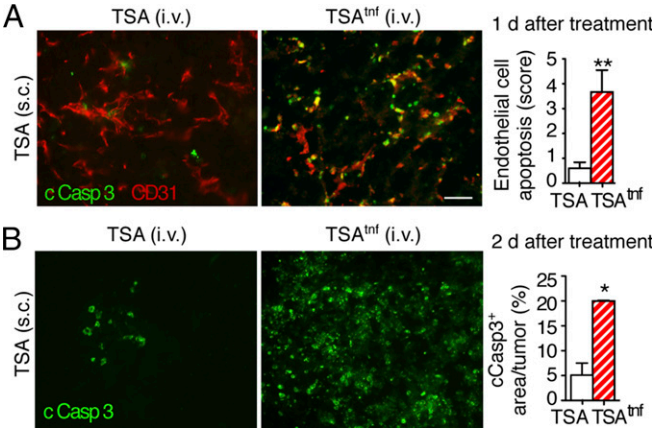

Fig. 4. TSA^{tnf} cells induce endothelial and tumor apoptosis. (*A*) Effect of i.v. injection of TSA or TSA^{tnf} cells on endothelial cell apoptosis in TSA tumors (s.c.). Immunofluorescent costaining for cCasp3 and CD31 in tumor tissue sections obtained 1 d after treatment. The whole section was inspected (one section per tumor, five tumors per group; mean ± SEM). (*B*) Effect of i.v. administration of TSA or TSA^{tnf} cells on cell apoptosis in TSA tumors (s.c.) 2 d after treatment, by immunofluorescence analysis of cCasp3 in tumor tissue sections. Positive cCasp3 areas (mean ± SEM) are shown as percentage of the total area (20 images per section, one section per tumor, three tumors per group). *P < 0.05; **P < 0.01 by Student's t test, two tailed.

TSA^{tnf}-treated mice (Fig. 3 *B* and *C*); a result consistent with our hypothesis. It is noteworthy that injected TSA^{tnf} cells did not cause weight loss in TSA tumor-bearing animals (Fig. 3*D*), which is a typical toxic side effect caused by conventional therapeutic doses of TNF (13). The presence of TSA^{tnf} cells was analyzed both in the peripheral blood (at days 1, 2, and 4 after systemic administration) and in tumors (at day 4) by semiquantitative PCR with primers to amplify a sequence in pLenti-m*tnf*–transduced cells that is absent in the normal mouse genome. We also analyzed the circulating levels and intratumor concentrations of TNF by ELISA (DuoSet, R&D Systems). Notably, pLenti-m*tnf* DNA was detected in the circulation at day 1 after TSA^{tnf} administration, but not at days 2 or 4 (Fig. 3*E*, *Left*), data indicating that TSA^{tnf} cells are cleared from the systemic circulation. In contrast, PCR analysis revealed an accumulation of pLenti-m*tnf* DNA in TSA tumors excised at day 4, confirming that TSA^{tnf} cells home to the tumor, thus corroborating the tumor self-seeding hypothesis (Fig. 3*E*, *Right*) (2). TNF was not detected in the circulation by ELISA even when TSA^{tnf} cells were present (Fig. 3*F*, *Left*). By contrast, increased levels of TNF were detected at day 4 in tumors of mice injected with TSA^{tnf} cells compared with mice injected with TSA cells (Fig. 3*F*, *Right*). These experimental findings support our interpretation that TSA^{tnf} cells are capable of homing to tumors and locally releasing TNF. Furthermore, the absence of detectable TNF in the peripheral blood and the lack of weight loss in mice treated with TSA^{tnf} cells strongly indicate that the therapeutic effect is mediated by local cellular production of TNF and not by systemic release from circulating TSA^{tnf} cells.

Systemic Administration of TSA^{tnf} Cells Induces Vascular Endothelial Damage and Causes Apoptosis in Subcutaneous Tumors. To characterize the mechanism underlying the antitumor activity of TNF-expressing cells, we investigated the effect of TSA^{tnf} cells on the viability of endothelial and tumor cells in s.c. TSA tumors. To this end, we evaluated the presence of apoptotic endothelial cells with antibodies against cleaved caspase 3 (cCasp3) (a marker of apoptosis) and CD31 (a surface marker of endothelial cells) in tumor tissue sections by coimmunofluorescence staining 1 d after TSA^{tnf} injection. A significant increase in apoptotic CD31-expressing cells in TSA^{tnf}-treated mice was observed (Fig. 4*A*), revealing the presence of vascular endothelial cell damage. To assess whether this vascular damage could cause secondary apoptotic responses in tumors, we analyzed cCasp3 expression 2 d after TSA^{tnf} administration. TSA^{tnf}-treated tumors had larger apoptotic areas compared with TSA-treated tumors (Fig. 4*B*), consistent with our experimental hypothesis that tumor growth inhibition is dependent on number of TNF-expressing cells rather than on the amount of TNF expressed per cell. These results indicate that, after homing to tumors, TSA^{tnf} cells release TNF, which in turn, causes local vascular damage, leading to tumor cell apoptosis.

Systemic Administration of TSA^{tnf} Cells Does Not Affect Recruitment of $CD45^+$ Cells in Tumors. Because TNF is an inflammatory cytokine (10), we evaluated whether treatment with TSA^{tnf} cells would affect the inflammatory infiltrates in TSA tumors by FACS analysis. After evaluating different populations of $CD45^+$ cells, including lymphocytes, dendritic cells, and monocytes ($CD8^+$, $CD4^+$, $CD11c^+$, $Gr1^+CD11b^-$, $Gr1^+CD11b^+$, and $Gr1^-CD11b^+$), we found no significant differences between controls or mice treated with TSA^{tnf}, except for a modest increase of $CD4^+$ T cells (Fig. S3). These results confirm that the release of TNF by tumor cells does not change the composition of infiltrating inflammatory cells into s.c.-implanted TSA tumors under our experimental conditions.

Discussion

The present study shows that systemic administration of genetically engineered tumor cells that produce and release TNF—a very potent pleiotropic cytokine (10, 11)—inhibits the growth of primary and metastatic tumors in three different murine models: TSA, melanoma (B16-F10), and LLC. These TNF-expressing cells home to tumors, locally release TNF, induce vascular endothelial cell damage, and subsequently cause tumor cell apoptosis, a biological phenomenon defined here as "tumor self-targeting." Our preclinical findings support a provocative paradigm in which tumor cells manipulated ex vivo and administered into the peripheral bloodstream may serve as carriers for the therapeutic delivery of bioactive cytokines and potentially additional proteins, as tumor-targeted agents.

The efficacy of cytokines in cancer therapy is often reduced by their toxicity and counterregulatory mechanisms. Such limitations may be partially overcome in the case of TNF and IFNγ by a ligand-directed strategy based on cytokine fusion with peptides or antibodies selective for unique surface receptors on tumor endothelial or epithelial cells (12–15, 17). However, administration of targeted TNF or IFNγ close to the maximum tolerated dose still leads to systemic activation of counterregulatory mechanisms that reduce their potential anticancer activity (12). The approach described here represents an alternative and/or additional strategy to deliver cytokines to tumors, which is based on living cells and offers several advantages. First, tumor cells can be engineered to produce and release multiple cytokines into the tumor microenvironment, thus favoring local synergistic interactions that can be achieved with much lower levels of each cytokine. For example, a coexpression of EMAP-II or IFNγ, two cytokines known to enhance TNF activity, may potentially increase the antitumor efficacy of TNF (18, 19). Second, the presence and amounts of such cytokines can be tailored by using inducible systems to optimize spatiotemporal expression, and further limit cytokine-dependent systemic counterregulatory and toxic effects.

Our characterization of the system demonstrates that the anticancer activity of TNF-expressing cells in vivo is dependent on the number of cells administered, rather than on the amount of TNF released per cell (Fig. 2 *A* and *C*). In fact, we show here that TSA, B16-F10, or LLC cells expressing widely different levels of TNF (from 0.015 to 153 fg per cell per day) induced comparable delays in tumor growth rates. We hypothesize that single cells, even low TNF-producing cells can locally release an amount of TNF that is sufficient to cause endothelial damage in tumor blood vessels. Increasing the numbers of administered cells, regardless of the absolute amounts of secreted TNF per cell, are expected to be paralleled by increased numbers of affected vascular loci, and damage of even a single endothelial cell can theoretically lead to impaired perfusion of an entire capillary that may exponentially affect large tumor areas. In support of our hypothesis, we observed apoptosis of endothelial cells in s.c. tumors as early as 1 d after i.v. injection of TNF-expressing cells that was sequentially followed by marked apoptosis of tumor cells after 2 d. This putative mechanism is further confirmed by the finding that TNF-expressing cells inhibit the growth of preexisting tumors when injected i.v., but not when injected s.c. in the contralateral flank. This suggests the antitumor activity of TNF-expressing cells presumably requires homing to the tumor from the circulation, a key event to induce vascular endothelial damage leading to tumor cell death.

Cancer cells engineered to express antitumor agents reduced both primary tumor and metastatic colony growth, but did not abrogate them. Efficacy of our system, however, could be improved by combination therapy. For example, a significant increase of the response to targeted TNF was found with either chemotherapy or immunotherapy, both active and adoptive (20–22). Remarkably, strong synergistic effects were achieved (including tumor eradication), compared with the modest reduction induced by targeted TNF alone (23). Combinatorial therapy could also improve the treatment of larger cancer lesions due to their density in vascularization. Furthermore, metastatic derivatives showed a superior ability to infiltrate parental tumors (1); therefore, engineering

highly metastatic cells may further improve their homing properties and thus efficacy.

Considering the multiple functions of TNF (11), other mechanisms might perhaps contribute to the antitumor effects of TNF-expressing cells, such as direct cytotoxic effects or activation of inflammatory and immune responses. However, we did not observe any detectable effect on the growth and vitality of tumor cells by tumor-cell–released TNF in vitro. Furthermore, analysis of immune cell infiltrates revealed only a modest and not statistically significant increase in $CD4^+$ T cells upon treatment of s.c. TSA tumors with TNF-expressing cells. These data confirm that cell toxicity and inflammation are not likely to be crucial in TNF-mediated inhibition of tumor growth in our experimental models. Interestingly, mice injected s.c. with syngeneic TNF-expressing cells were unable to develop tumors after subsequent challenges with corresponding parental tumor cells. Although further experiments will be needed to clarify this observation, one may speculate that TNF expression causes increased immunogenicity and consequent rejection of cancer cells. Concomitantly or alternatively, TNF-expressing cells may have induced a long-lasting effect at the regional injection site, which negatively impacted VEGF secretion and angiogenesis to create a microenvironment that is no longer permissible for tumor growth.

Finally, although cancer cells may represent a uniquely effective delivery system for cytokines, important safety concerns must be addressed before embarking on translational applications of this work. An obvious concern is the risk that injected tumor cells could give rise to new neoplastic lesions. Our characterization of the tumorigenic and metastatic properties of TNF-expressing tumor cells showed that injection of these cells into the circulation of syngeneic mice, results in lung colonies, even though their tumorigenic and metastatic potential is markedly reduced compared with parental cells. Furthermore, we report that systemic administration of TNF-expressing cells to nonsyngeneic mice inhibited tumor growth to about the same extent as the administration in syngeneic mice, but in the complete absence of lung colonies, representing a further improvement toward addressing the safety concerns. In this case, an immune response likely caused the rejection of the nonsyngeneic cells within a few days, after a period that was sufficient to exert their therapeutic effect. To increase the safety of this approach, and to circumvent any possible escape from the immune system, cytokine-expressing tumor cells could be further engineered to express immunogenic proteins and/or inducible suicide genes (24, 25) with the aim of clearing the injected tumor cells after the desired therapeutic effect is achieved. The observation that the antitumor effects of TNF-releasing cells are similar in nonsyngeneic and syngeneic systems, at least in mice, suggests that a common tumor cell line might be effective in different individuals. This may allow the production of highly standardized and finely controlled cells under good manufacturing conditions and with precise protocols for future translational applications. Extensive studies, however, will be required to demonstrate that the safety issues of this delivery system can be managed in a clinical context.

Materials and Methods

Animal Models. Animal studies were approved by the Institutional Animal Care and Use Committee of The University of Texas, MD Anderson Cancer Center and are described further in *SI Materials and Methods*.

Cell Lines, Recombinant TNF, and Antibodies. Murine TSA mammary adenocarcinoma cells (derived from BALB/c mice) were provided by Giulia Casorati (San Raffaele Institute, Milan). The following cell lines were purchased from the American Type Culture Collection: murine B16-F10 melanoma, murine Lewis lung carcinoma (both on a C57BL/6 strain), and human EAhy.926 endothelial cells. Murine TNF was prepared by recombinant DNA technology and purified from *Escherichia coli* cell extracts as described (12). Rabbit polyclonal anti-cCasp3 and CD31 antibodies were purchased from Cell Signaling Technology.

Preparation of TNF-Expressing Tumor Cells. The coding region for murine TNF in the pET-11b-*tnf* plasmid (12) was PCR amplified and inserted into the pLenti6/V5 DEST Gateway Vector (Invitrogen) with the In-fusion cloning kit (Clontech). The resultant pLenti-m*tnf* vector was transfected into HEK-293FT cells (Invitrogen) by incubation with Lipofectamine 2000 (Invitrogen). Culture medium was replaced after overnight incubation at 37 °C in 5% CO_2, and viral particles were collected 24 and 48 h later and pooled. Supernatants were tested for viral particle content by using Lenti-X GoStix (Clontech). TSA, B16-F10, or LLC cells were transduced with 1–2 mL of virus-containing supernatant. Tumor cells expressing low levels of TNF were selected in the presence of 3 μg/mL blasticidin (Sigma-Aldrich). Derived cell lines were named $TSA^{tnf\ low}$, $B16\text{-}F10^{tnf\ low}$, or $LCC^{tnf\ low}$. Cells expressing higher levels of TNF were selected in the presence of 50 μg/mL blasticidin (for TSA) or by reinfection with the lentivirus (for B16-F10, LCC). These cells were designated as TSA^{tnf}, $B16\text{-}F10^{tnf}$, or LCC^{tnf}.

Additional materials and methods are presented in *SI Materials and Methods*.

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Supporting Information

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SI Materials and Methods

Animal Models. Animal studies were approved by the Institutional Animal Care and Use Committee of The University of Texas, MD Anderson Cancer Center. For the s.c. xenograft model, BALB/c or C57BL/6 mice (8-wk-old females, Charles River Laboratories) were injected into the flank with either the parental (i.e., nontransduced with pLenti-mTNF) or TNF-expressing tumor cells. Unless stated otherwise, BALB/c mice were injected with 4×10^5 TSA or TSA^{tnf} cells per mouse, and C57BL/6 mice were injected with 4×10^5 B16-F10 or $B16\text{-}F10^{tnf}$ cells per mouse, or with 8×10^5 LLC or LLC^{tnf} cells per mouse. Tumor growth was monitored every 2–3 d by measuring tumor sizes with a caliper. For the metastatic model, mice were injected i.v. into the tail vein (same mouse strain/cell line combination as for the s.c. model) with 7×10^4 TSA or TSA^{tnf} cells per mouse, 1.2×10^5 B10-F16, or $B16\text{-}F10^{tnf}$ cells per mouse, or with 3×10^5 LCC or LLC^{tnf} cells per mouse. After 14 (TSA), 11 (B16-F10), or 28 (LCC) days, mice were killed. Lungs were harvested, weighed, and the number of pulmonary colonies counted with a stereomicroscope. For the intervention trials, the following numbers of TNF-expressing cells were injected i.v. when primary s.c. tumors reached 50 mm^3 or after 7 d from tumor onset for the following metastatic models: TSA^{tnf}, 7×10^5 cells per mouse; $B16\text{-}F10^{tnf}$, 2×10^5 cells per mouse; and LLC^{tnf}, 3×10^5 cells per mouse. Other specific mouse strain/cell line combinations and numbers of injected cells are detailed in *Results*.

Detection of pLenti-mTNF-Transduced Cells in the Blood Circulation and in Tumors. To track circulating TNF-expressing cells, mice were bled 1, 2, and 4 d after i.v. injection of TNF-expressing cells. Aliquots of blood (100 μL) were collected in heparinized tubes lysed with Red Blood Cell Lysis Solution (Promega) and DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen). To assess tumor homing of TNF-expressing cells, DNA was directly extracted from tumor masses using the same protocol 4 d after i.v. injection. In both cases, DNA was subjected to PCR with the following primer pair: 5′-TGGAATTCTGCAGATATCAAC-3′ and 5′-CCGGTACGCG-TAGAATCG-3′, designed to specifically amplify a pLenti-mTNF sequence that is not present in the murine genome. PCR products were analyzed by 2% (wt/vol) agarose gel electrophoresis in the presence of ethidium bromide, and band intensities were quantified using Photoshop CS4 software (Adobe).

Immunofluorescence Analysis of Tumor and Endothelial Cell Apoptosis. Cell apoptosis in TSA, B16-F10, and LLC tumors was quantified by immunofluorescence analysis of cCasp3 immunoreactivity from frozen, 10-μm-thick tumor sections. Tissue sections were fixed with 2% (vol/vol) paraformaldehyde in PBS and blocked with 5% (vol/vol) normal goat serum, 1% BSA, and 0.3% Triton X-100 in PBS. Sections were incubated with rabbit polyclonal anti-cCasp3 in 1% normal goat serum, 1% BSA, 0.3% Triton X-100 in PBS, followed by goat anti-rabbit IgG Cy3 antibody (Jackson ImmunoResearch). Black/white images were acquired using an Olympus IX70 inverted fluorescence microscope/MagnaFire software and analyzed using Photoshop CS4 software (Adobe). At least 20 images per tumor were acquired (three tumors per group). Positive cCasp3 areas were selected with the Photoshop "magic wizard" tool and the total number of positive pixels (pp_T) for each image was calculated. To calculate the percentage of apoptotic areas in a tumor section, pp_T was divided by the total number of pixels per tumor section. Similarly, to assess endothelial cell apoptosis, sections were incubated with anti-cCasp3 and CD31 antibodies (Abcam), followed by goat anti-rabbit Alexa Fluor 488 and anti-rat Alexa Fluor 546 (Invitrogen), respectively. Quantification of apoptotic cells was performed on entire tumor sections. Scores were assigned to each section based on the number of cCasp3/CD31 double positive cells: 0 (0 vessels), 1 (1–10 vessels), 2 (11–20 vessels), 3 (21–30 vessels), 4 (31–40 vessels), and 5 (>41 vessels).

Titration of TNF in Cell Supernatants by EAhy.926 Cytolytic Assay. The amounts of TNF secreted by the pLenti-mTNF–transduced cells were quantified in cell supernatants by a cytolytic assay. Thirty thousand EAhy.926 cells were plated per well in a 96-well flat-bottom plate in complete medium and incubated overnight at 37 °C, 5% (vol/vol) CO_2. Fifty microliters of actinomycin-D (positive control, 3 μg/mL final concentration in complete medium), serial dilutions of recombinant murine TNF (standard curve, 0.6–1500 pg/mL final concentration in complete medium), or serial dilutions of supernatants from the TNF-producing cells were added to each well, followed by 24-h incubation at 37 °C, 5% (vol/vol) CO_2. Cells were washed with PBS, fixed with 4% (vol/vol) paraformaldehyde [15 min, room temperature (RT)] and stained with 0.5% crystal violet in 20% (vol/vol) methanol (10 min, RT). Plates were extensively washed with tap water before measuring the absorbance at $\lambda = 570$ nm (EC_{50}: 150 ± 50 fg/mL TNF; detection limit: 6 fg/mL TNF).

Tumor Cell Cytotoxicity Assay. TSA (6×10^4 cells per well), B16-F10 (4×10^4 cells per well), or LLC (6×10^4 cells per well) were plated in a 96-well flat-bottom plate and incubated in complete medium for 24 h at 37 °C, 5% vol/vol CO_2. Murine TNF (0–500 ng/mL) was added to cells in complete medium and incubated for 24 h at 37 °C, 5% vol/vol CO_2. Viable cells were stained with crystal violet, the absorbance was measured at $\lambda = 570$ nm.

Tumor Cell Proliferation Assay. TSA (1.8×10^3 cells per well), B16-F10 (15×10^3 cells per well), or LLC (7.5×10^3 cells per well) cells were seeded per well of 96-well plates and incubated in complete medium at 37 °C, 5% (vol/vol) CO_2. Viable cells were evaluated at day 0 and day 3 by staining with 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (MTT, 10 μL per well, 2 h at 37 °C). Absorbance was measured at $\lambda = 570$ nm in a microtiter plate reader. The proliferation index of each cell population was calculated by dividing the absorbance values at day 3 by those at day 0.

Tumor Cell Irradiation. TSA^{tnf} cells were plated in a 10-cm dish (1×10^7 cells per plate) and irradiated at 35 Gy. Cells were further analyzed for cell proliferation and TNF production as reported.

FACS Analysis of Immune Cells Within Tumors. The number of tumor-infiltrating $CD45^+$ lymphatic and myeloid cells was analyzed by FACS. Tumors were excised, mechanically disaggregated with a 70-μm cell strainer, resuspended in 2% (vol/vol) FCS in PBS, and incubated for 15 min with antibodies as follows: anti-CD45 PE-Cy 7, anti-CD4 PE, anti-CD8b APC, anti-CD11c FITC or CD45 PE-Cy 7, anti-CD11b PerCP-Cy 5.5, and anti-Gr1 FITC (BD Pharmingen). Samples were analyzed with an LSRII FACS equipped with Diva software.

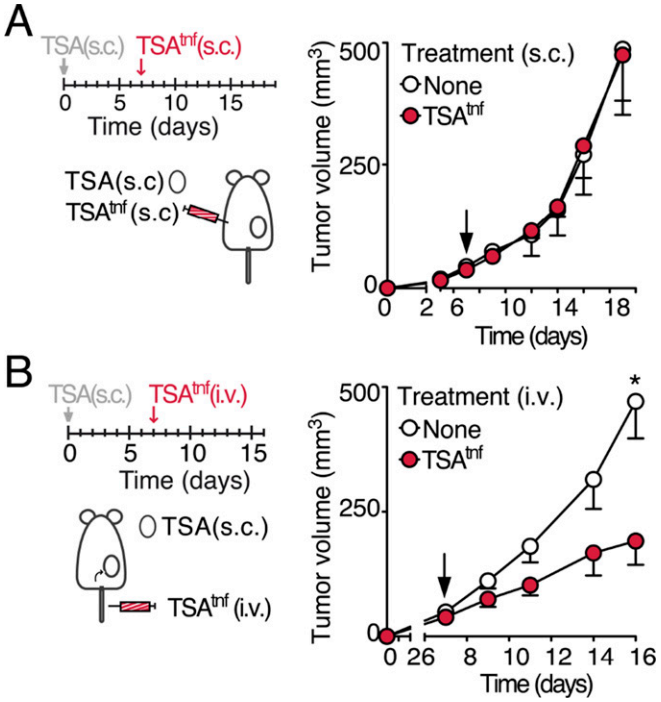

Fig. S1. Intervention trial: intravenous, but not s.c. administration of TSA^{tnf} cells reduces the growth of established s.c. TSA tumors. Mice were injected s.c. with TSA cells (4×10^5 cells per mouse). After 7 d, mice were injected with TSA^{tnf} cells (4×10^5 cells per mouse) s.c. (*A*) or i.v. (*B*). Two experiments were performed ($n = 3$ each). Experimental schedule and tumor volume (mean ± SE) of one representative experiment is shown. *$P < 0.05$ by *t* test, two tailed.

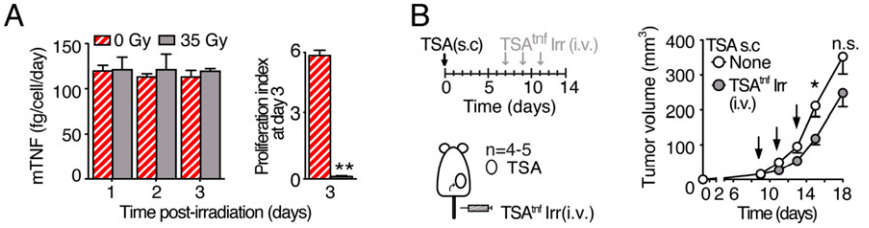

Fig. S2. Irradiation reduces TSA^{tnf} cell antitumor activity. (*A*) Amount of bioactive TNF released in the supernatant by irradiated TSA^{tnf} at days 1, 2, and 3 postirradiation, as measured by a cytolytic assay (mean ± SE). Experiments were performed in triplicate and repeated two times. Proliferation of irradiated TSA^{tnf} cells as evaluated by MTT staining ($n = 4$, mean ± SE). Two experiments were performed, and one representative experiment is shown. (*B*) Effect of i.v. administration of irradiated (Irr) or nonirradiated TSA^{tnf} cells (3×10^5) on the growth of TSA tumors implanted s.c. in syngeneic mice. Tumor volumes (mean ± SEM) are shown ($n = 5$). *$P < 0.05$; **$P < 0.01$ by *t* test, two tailed.

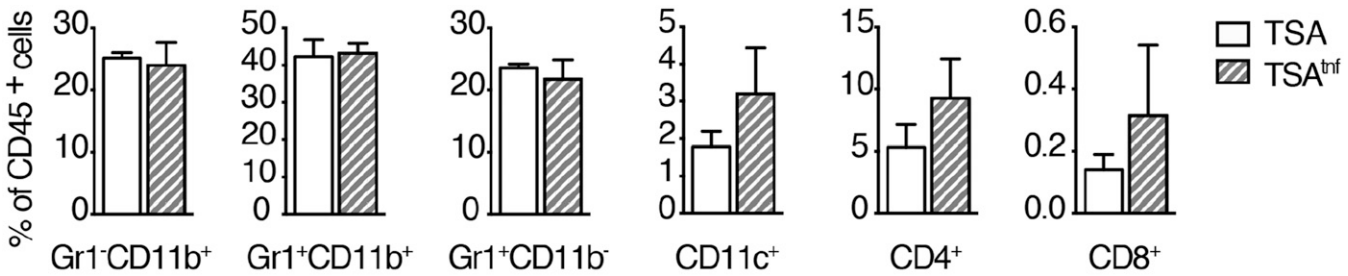

Fig. S3. I.v. administration of TSA^{tnf} cells does not affect recruitment of subpopulations of CD45$^+$ cells to the tumors. Evaluation of CD45$^+$ immune infiltrating cells (Gr1$^-$CD11b$^+$, Gr1$^+$CD11b$^+$, Gr1$^+$CD11b$^-$, CD11c$^+$, CD4$^+$, and CD8$^+$) by FACS. Results are represented as percentage of the amount of CD45$^+$ ($n = 4$–5). None of the markers were significantly different following i.v. administration of TSA^{tnf} in comparison with parental TSA cells.