Targeting TNF-α to Neoangiogenic Vessels Enhances Lymphocyte Infiltration in Tumors and Increases the Therapeutic Potential of Immunotherapy

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Targeting TNF-α to Neoangiogenic Vessels Enhances Lymphocyte Infiltration in Tumors and Increases the Therapeutic Potential of Immunotherapy

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Abnormal tumor vasculature impairs T lymphocyte adhesion to endothelial cells and lymphocyte extravasation into neoplastic tissues, limiting the therapeutic potential of both active and adoptive immunotherapies. We have found that treatment of tumor-bearing mice with NGR-TNF, a Cys-Asn-Gly-Arg-Cys peptide-TNF fusion product capable of altering the endothelial barrier function and improving drug penetration in tumors, associated with the intratumor upregulation of leukocyte-endothelial cell adhesion molecules, the release of proinflammatory cytokines and chemokines, and the infiltration of tumor-specific effector CD8+ T cells. As a result, NGR-TNF enhanced the therapeutic activity of adoptive and active immunotherapy, delaying tumor growth and prolonging survival. Furthermore, we have found that therapeutic effects of these combinations can be further increased by the addition of chemotherapy. Thus, these findings might be relevant for the design of novel immunotherapeutic approaches for cancer patients. The Journal of Immunology, 2012, 188: 000–000.

Growing tumors develop strategies that directly or indirectly impair effector T lymphocyte functions (1). In addition, the hypoxic tumor microenvironment favors the formation of new vessels (i.e., neoangiogenesis) that are disorganized, tortuous, and more leaky than the normal ones. These vascular abnormalities can increase the interstitial pressure, cause heterogeneous permeability, and promote irregular blood flow (2). Furthermore, angiogenic factors such as vascular endothelial cell (EC) growth factors (VEGF) and fibroblast growth factors can cause downregulation of intracellular adhesion molecule-1/2 (ICAM-1/2), VCAM-1, and CD34 on EC, a phenomenon defined as EC anergy (3). Hence, the interaction of leukocytes with the endothelial lining of vessels is reduced, and effector T cells, regardless of being induced in vivo by vaccination or adoptively transferred (4, 5), may be impaired in their migration into tumor sites and cannot exert the antitumor effects necessary to eradicate the tumor (6, 7).

TNF-α is a cytokine produced in the tumor microenvironment mainly by macrophages, but also by endothelial cells and tumor cells (8). This cytokine can exert potent antitumor effects both in animal models and in patients treated by isolated limb perfusion, a regional cancer therapy used to deliver high doses of TNF into the bloodstream of a limb and to avoid severe systemic side effects (9). In this setting and depending on its dose, TNF can cause endothelial cell activation, increased vessel permeability, endothelial cell damage, and massive hemorrhagic necrosis (9). Although the administration of TNF at therapeutic doses is precluded by its severe systemic toxicity, safe and therapeutic systemic administration can be obtained by targeting TNF to angiogenic tumor vessels. For example, TNF has been fused to Cys-Asn-Gly-Ary-Cys (NGR) (10), a tumor-homing peptide that recognizes an aminopeptidase N (CD13) isoform selectively expressed by endothelial cells in tumor vessels (11, 12). The selective recognition of the endothelial lining of angiogenic tumor vessels by this peptide has also been confirmed with peptide-labeled paramagnetic quantum dots and quantitative molecular magnetic resonance imaging in tumor mouse models (13). Systemic administration of low doses of the NGR-TNF fusion protein can induce antitumor effects stronger than those elicited by similar doses of TNF (14). In addition, the delivery of ultra-low doses (e.g., picograms) of NGR-TNF to the tumor vasculature overcomes major counterregulatory mechanisms and increases the penetration of doxorubicin (DOXO) and other chemotherapy agents in murine models of lymphoma, melanoma, and spontaneous prostate cancer (15, 16). While a primary mechanism of NGR-TNF-induced drug penetration is related to disassembly of endothelial VE-cadherin dependent adherence junctions and alteration of endothelial barrier function in tumors (17), other mechanisms might also be brought into play, such as increased tumor perfusion and reduction of interstitial pressure.

NGR-TNF, either alone or in combination with chemotherapy, is currently being tested in various clinical studies in cancer patients (18, 19).

In this study, we investigated whether selective targeted delivery of NGR-TNF to tumor vessels might promote the activation of...
tumor-associated EC and the recruitment of effector T cells, and enhances the local production of immunomodulating cytokines, thereby favoring the extravasation of immune cells and improving therapeutic activity of immunotherapy.

Materials and Methods

Animals, cell lines, and reagents

Wild type C57BL/6 (Charles River Breeding Laboratories; Calco, Italy), heterozygous C57BL6 transgenic adenocarcinoma of the mouse prostate (TRAMP) (20), C57BL/6-Tg(TrapTcrlb) 1100Mjb/J (21) and B6.129S7-Rag1tm1Mom/J mice (22) were housed in a pathogen-free animal facility and treated in accordance with the European Community guidelines. The latter two lines were crossed to obtain RAG-1−/− OTI mice. The in vivo experiments were approved by the Ethical Committee of the Istituto Scientifico San Raffaele. B16-OVA are B16F1 melanoma cells (American Type Culture Collection) expressing the truncated form of OVA lacking the leader sequence (23). RMA is an H-2b Rauscher virus-induced thymoma (24). Cells were maintained in RPMI 1640 with penicillin-streptomycin and 10% heat-inactivated FCS and the medium of B16-OVA cells was supplemented with hygromycin (100 μg/ml). B16F1 melanoma cells were maintained in IMDM with penicillin-streptomycin and 10% heat-inactivated FCS (Euroclone, Pero, Italy). HUVECs (Clonetics, Lonza, Switzerland) were cultured according to the recommended protocols. All experiments were performed with the RHA and TNF and TGF were produced and characterized as described in (14), and they were administered i.p. (5 ng/kg). DOXO was purchased from Pharmacia-Upjohn (Milan, Italy) and was administered i.p. (4 mg/kg). All drugs were diluted with 0.9% sodium chloride, containing 100 μg/ml endotoxin-free HSA (Farma-Biagini, Lucca, Italy), except for DOXO, which was diluted with 0.9% sodium chloride alone.

OTI cells

OVA-specific and in vitro-activated CD8+ T (OTI) cells from RAG-1−/− OTI mice were obtained as previously described (25). Single-cell suspensions (1 × 106 cells/ml) of spleen and lymph nodes cells from RAG-1−/− OTI mice were seeded into six-well plates together with bone marrow–derived dendritic cells (DCs; ratio, 20:1) (26) loaded with 100 nM OVA323-339 (SIINFEKL; Proimmune, Bradenton, FL) in RPMI 1640 supplemented with penicillin-streptomycin, 10 μM HEPES, 10 mM sodium pyruvate, 50 μM 2-ME, 10% heat-inactivated FCS, and IL-12 (3.5 ng/ml; R&D Systems, Minneapolis, MN). On day 3 of culture, cells were harvested and seeded into six-well plates together with culture medium and 50 μM of the IL-2 (R&D Systems). At day 5, cells were labeled with CFSE as described (26), suspended in PBS, and injected into the tail vein of mice.

Immunization procedures

Tag-IV (VYDFKLCK), OVA323-339 (SIINFEKL), and TRP-2180–188 (VYDFKLWVLH) peptides were purchased from Research Genetics (Huntsville, AL). DCs were prepared from bone marrow as described elsewhere (26). On day 7 of culture, DCs were resuspended in PBS at 2 × 106 cells/ml and incubated for 60 min at 37°C with 2 μg/ml of the synthetic peptide. DCs were injected intradermally (i.d.) (5 × 106 cells/mouse) in the right flank.

Tumor implantation, processing, and flow cytometry analyses

Mice were challenged s.c. in the left flank with 2 × 106 B16-OVA or 5 × 106 B16F1 cells. Tumor size was evaluated by measuring two perpendicular diameters with a caliper. In survival experiments, animals were killed when the tumor became ulcerated or as indicated in the figure legends. For tumor EC analysis, 19-d-old melanomas were processed to single-cell suspension and stained with primary anti–VCAM-1, anti–ICAM-2 mAb, and secondary PE-conjugated anti-rat IgG and APC-conjugated anti-CD31 mAb (BD Biosciences). Dead cells were excluded by physical parameters or by the addition of 7-aminoactinomycin D (BD Biosciences) immediately before flow cytometric analysis.

For tumor-infiltrated lymphocytes (TIL) analysis, tumors were collected at day 14; they were disaggregated and digested in collagenase D for 1 h at 37°C to obtain single-cell suspensions. After neutralization of unspecific binding with FcR blocker (BD Pharmingen, Bucinasco, Italy), cells were stained with specific mAb and assessed for phenotype by flow cytometry. Cells were also assessed for intracellular cytokine production after stimulation with PMA/ionomycin or RBM cells pulsed with the relevant peptide as previously described (27). Samples were acquired with a FACScanto system. Analyses were conducted with FlowJo software gating on low physical parameters that select for lymphocytes.

Combined treatments in TRAMP mice and mice bearing B16F1 melanomas

TRAMP mice were sublethally irradiated (60 rad), and the next day they were transplanted i.v. with 1 × 106 viable bone marrow cells via hemato-topoietic stem cell transplantation (HSCT). A donor lymphocyte infusion (DLI) consisting of 6 × 107 splenocytes was provided 2 wk later; these splenocytes were presensitized (pDLI) against the HAg by injection of 5 × 105 syngenic male bone marrow cells (28). The day after, mice were immunized with DCs pulsed with the immunodominant Tag epitope IV (DC-Tag-IV) as described above. Six days after the vaccination, mice were treated i.p. with NGR-TNF or TNF (5 ng/kg) and 2 h later animals were sacrificed. Their urogenital apparata (UGA) were processed for immunohistochemistry and scored on blind-coded samples by an expert pathologist as described by Hess Micheli et al. (28). Anti-CD3 (Serotec) immunodetection was performed according to the manufacturer’s instructions.

CD3 sections were digitally scanned (ScanScope, Aperio) and then analyzed with the Spectrum Plus software (Aperio).

Immunofluorescence

Tumors were embedded in KIlik frozen section medium (Bio-Optica, Milan, Italy) for quick freezing. Cryostatic sections (6 μm thick) were prepared, adsorbed on polylisine-coated slides, fixed for 30 min with PBS containing 4% paraformaldehyde. Detection of endothelial cells was done as follows: tissue sections were incubated with 150 μl PBS containing 1% BSA, 0.1% Triton X-100 (PBS-BT), and 5% normal goat serum for 1 h at room temperature. The solution was then removed and replaced with PBS-BT containing APC-conjugated anti-CD31 mAb MEC-13.3 (1:100) and FITC-conjugated anti-CD8 (1:100) and then incubated for 1 h at room temperature. The slides were rinsed again and incubated for 5 min with PBS containing 0.1 μg/ml DAPI (Sigma) to stain cell nuclei. Sections were examined under a microscope (Carl Zeiss, Axioscope 40FL; original magnification ×630).

Analysis of ICAM-1 expression on HUVEC

Five × 106 HUVEC were detached with trypsin-EDTA, washed, and immediately incubated with TNF or NGR-TNF at various concentrations for 1.5 h on ice. After washing, cells were resuspended in culture medium, incubated for 16 h at 37°C, 5% CO2, and stained with primary mouse anti-human ICAM-1 and secondary goat anti-mouse (BD Biosciences). Samples were acquired with a FACScanto system and DEVA software, and data were analyzed using FlowJo software.

In vitro cytolytic assay

B16F1 cells were cultured in 96-well flat-bottom plates with TNF or NGR-TNF (100 ng/ml, 6 × 105 cells per 100 μl) in DMEM complete medium containing 2 μg/ml actinomycin D (200 μl/well, 9 × 6-well plate). After
20 h at 37°C, 5% CO₂, the cytotoxic activity was quantified by standard MTT assay (29).

**Statistics**

Statistical analyses were performed with unpaired Student t test or Student t test followed by Wilcoxon posttest. Survival curves were compared using the log rank test; p values < 0.05 were considered statistically significant.

**Results**

**NGR-TNF transiently modifies the tumor microenvironment by inducing upregulation of leukocyte-adhesion molecules on endothelial cells, release of cytokines or chemokines, and infiltration by functional CD8⁺ T cells**

TNF activates ECs and increases vessel permeability (9). To investigate the effect of NGR-TNF on ECs, we designed an in vitro assay in which the binding phase is limited to 1.5 h on ice. Then the unbound reagent is washed away and cells are incubated overnight to quantify the effects of bound TNF on cells. Therefore, we incubated CD13¹ HUVECs (30) with NGR-TNF or TNF and measured the cell surface expression of ICAM-1, a molecule involved in leukocyte adhesion and transmigration (31). Both TNF and NGR-TNF induced dose-dependent expression of ICAM-1 on HUVECs (Fig. 1A); however, NGR-TNF was more effective at each dose tested (Fig. 1B).

Tumor cells may express CD13, but they should not express the isoform recognized by NGR-TNF (12). Therefore, we investigated whether B16 melanoma cells expressed CD13 and, more importantly, were targeted by NGR-TNF. As shown in Fig. 1C, B16 melanoma cells expressed CD13, although the commercially available mAb did not allow to identify the specific isoform. When added to the culture medium, both NGR-TNF and TNF did not kill melanoma cells, even at 10 ng/ml, and when combined with DOXO they did not increase the DOXO-mediated cytotoxicity (Fig. 1D). Thus, NGR-TNF does not appear to have a direct effect on tumor cells.

To verify whether NGR-TNF administration can induce the upregulation of adhesion molecules also on ECs of tumor vessels, we exploited the well-characterized (23) and well-vascularized (32) B16-OVA model. Therefore, mice bearing an 8-d-old melanoma (i.e., 126.3 ± 8.6 mm² ± SEM; n = 17) were infused i.v. with in vitro-activated OVA-specific OTI cells to follow the tumor. Consequently, OTI cells in NGR-TNF treated mice, some mice were sacrificed 24 h after treatment. As reported in Supplemental Fig. 2, the absolute numbers of tumor infiltrating CFSE⁺ (Supplemental Fig. 2A) and IFN-γ⁺ (Supplemental Fig. 2B) cells quantified at 2 and 24 h were similar, suggesting that tumor infiltration was a rather precocious and rapid event.

We also evaluated whether the phenomenon we observed in vivo with OTI cells occurred as well for nontransgenic T cells within a spontaneous cancer. We have recently reported that the infusion of alloreactive T cells overcomes tumor-specific T cell tolerance usually found in TRAMP mice (33) with autochthonous prostate cancer (20), allowing tumor debulking and increased overall survival (28). Therefore, 16-wk-old TRAMP mice were subjected to nonmyeloablative total body irradiation (600 rad) and 1 d later transplanted with 1 × 10⁶ bone marrow cells (HSCt) derived from congenic female donors. Two weeks after the HSCt, mice received a DLI of 6 × 10⁷ splenocytes derived from congenic females previously sensitized against male Ag (pDLI) (28). One day after the pDLI, mice were vaccinated with DC–Tag-IV, and 6 d later treated with either NGR-TNF or TNF (Fig. 3A). Immunohistochemical analyses conducted 2 h after the last treatment showed that the infusion of NGR-TNF favored infiltration of donor-derived CD3⁺ cells within the prostate tumors (Fig. 3B). Quantification of prostate tumor sections indicated that the number of CD3⁺ cells in prostate tumors was significantly higher in NGR-TNF-treated mice when compared with TNF-treated ones (Fig. 3C). Thus, targeting TNF to tumor vessel increases T cell infiltration of both transplantable and autochthonous tumors.

**The combination of NGR-TNF and adoptive immunotherapy increases the survival of melanoma-bearing mice**

Having found that activated OTI cells performed better when combined with NGR-TNF (Fig. 2), we wished to investigate whether adoptive immunotherapy and NGR-TNF act in synergy against a growing melanoma. Preliminary experiments were conducted to identify the number of OTI cells that as a single therapeutic agent exerted a measurable effect. Activated OTI cells were tested in mice bearing an 8-d-old melanoma (Fig. 4A). In the range between 1 and 6 × 10⁶ cells, the highest dose gave the best antitumor effect (Fig. 4B and data not shown).

Next, we tested the association between NGR-TNF treatment and adoptive transfer (Fig. 4A). Whereas TNF combined with 6 × 10⁶ OTI cells failed to significantly delay tumor growth and to prolong mouse survival (Fig. 4B, 4C), the administration of NGR-TNF prior to T cell infusion delayed tumor development and provided a survival advantage to tumor-bearing mice (Fig. 4B, 4C). These data indicate that NGR-TNF enhances tumor infiltration by CD8 T cells and the therapeutic potential of adoptive immunotherapy.
We next investigated whether NGR-TNF could also increase efficacy of active immunotherapy (vaccination) either alone or in combination with chemotherapy. To minimize the therapeutic efficacy of chemotherapy or NGR-TNF as single agents, treatment was started at day 14, when tumors had an average size of $\sim 47$ mm$^2$. Vaccination of mice bearing a B16-OVA melanoma was delivered by i.d. injection of DCs (26) pulsed with OVA257–264 and biweekly boosting. NGR-TNF was administered 1 wk after DC-OVA, based on the hypothesis that NGR-TNF favors tumor infiltration by activated CTL at the peak of the vaccine-induced immune response. As expected (23), DC-OVA only modestly prolonged survival of melanoma-bearing mice (Fig. 5A), but its combination with NGR-TNF significantly prolonged overall animal survival (Fig. 5A). Of note, $\sim 50\%$ of NGR-TNF treated mice in combination with DC-OVA were still alive when control mice treated with DC-OVA alone or NGR-TNF alone had to be sacrificed because of tumor overgrowth (Fig. 5A).

Because immunotherapy in several instances could be combined with chemotherapy (34) and NGR-TNF has been shown to enhance tumor permeability to chemotherapeutic agents (15, 16), we next investigated the possibility that NGR-TNF administration might increase the therapeutic efficacy of chemoinmunotherapy. As a result, the DC-OVA vaccine was combined with DOXO given biweekly 2 h after NGR-TNF. The rationale was to boost the vaccine-induced immune response by causing chemotherapy-mediated tumor cell death and release of tumor-associated Ags. To establish the triple combined treatment, we started by associating NGR-TNF with DOXO. NGR-TNF, given alone (Fig. 5B) or provided 2 h before DOXO (Fig. 5B), was largely ineffective in this experimental setting, possibly because of the overt tumor growth. Likewise, the association of DC-OVA with DOXO increased mice survival (Fig. 5C), but to lower extents than those observed in mice receiving the combination of DC-OVA, NGR-TNF, and DOXO (Fig. 5D). Indeed, the triple combination further increased the overall survival of the mice (DC-OVA plus DOXO versus DC-OVA plus NGR-TNF plus DOXO; $p < 0.0001$). Delayed tumor growth in NGR-TNF–treated mice (Fig. 5D) correlated with a higher frequency of CD8$^+$ TIL (Supplemental Fig. 3), and the former was comparable to that observed in mice receiving the combination of DC-OVA, NGR-TNF, and DOXO (Fig. 5D). The triple combination further increased the overall survival of the mice (DC-OVA plus DOXO versus DC-OVA plus NGR-TNF plus DOXO; $p < 0.0001$). Delayed tumor growth in NGR-TNF–treated mice (Fig. 5D) correlated with a higher frequency of CD8$^+$ TIL (Supplemental Fig. 3), and the former was comparable to that of mice receiving DC-OVA and DOXO (Fig. 5B), thus highlighting the importance of the NGR-vascular targeting moiety.

Finally, the combined therapeutic approach was investigated in the context of an immune response specific for TRP-2, a natural tumor-activated OTI cells ($6 \times 10^6$). Two days later, NGR-TNF ($5 \text{ ng/kg}$; $n = 3$), TNF ($5 \text{ ng/kg}$; $n = 3$) or PBS (None; 100 $\mu$l; $n = 3$) was injected i.p. After an additional 2 h, tumors were excised and processed to single-cell suspension for flow cytometric analysis after staining with anti-CD31, anti-VCAM-1, and anti-ICAM-2 mAb. Expression of VCAM-1 (E) and ICAM-2 (F) on live CD31$^+$ cells reported as mean ($\pm$ SD) fluorescence intensity (MFI). Data are representative of three independent experiments. (G) Alternatively, the tumor tissue was homogenized and analyzed for the indicated soluble molecules by Mouse CytokineMAP B version 1.0. The graphs report the concentration of the indicated soluble molecules as a percentage of the concentration found in PBS-treated mice. Data from two independent experiments involving five to six animals per group were aggregated. Statistical analysis was performed using the Student $t$ test: $*0.01 < p < 0.05$, $**0.001 < p < 0.01$, $***p < 0.001$. 

**FIGURE 1.** NGR-TNF treatment induces the upregulation of adhesion molecules on CD31$^+$ cells and cytokine and chemokine release in the tumor microenvironment. HUVECs were detached with trypsin-EDTA, washed, and immediately incubated with TNF or NGR-TNF solutions at various concentrations for 1.5 h on ice. After washing, the cells were resuspended in culture medium, incubated for 16 h at 37°C, 5% CO$_2$, and analyzed by flow cytometry. (A) Fluorescence curves obtained with NGR-TNF, TNF, or no treatments. (B) Quantification of mean fluorescence intensity is reported. Data are representative of at least two independent experiments. (C) B16-OVA cells were stained with primary anti-mouse CD13 and secondary PE-conjugated anti-rat IgG2a (black histogram) or only with secondary PE-conjugated anti-rat IgG2a Ab (white histogram). (D) B16-OVA cells were incubated with TNF or NGR-TNF solutions at 10 ng/ml in DMEM complete medium containing 2 $\mu$g/ml actinomycin D (200 $\mu$l/well, 96-well plate). After 20 h at 37°C, 5% CO$_2$, cell viability was quantified by standard MTT assay. (E–G) Mice were challenged s.c. with B16-OVA cells, and 19 d later they were infused i.v. with activated OTI cells ($6 \times 10^6$). Two days later, NGR-TNF ($5 \text{ ng/kg}$; $n = 3$), TNF ($5 \text{ ng/kg}$; $n = 3$) or PBS (None; 100 $\mu$l; $n = 3$) was injected i.p. After an additional 2 h, tumors were excised and processed to single-cell suspension for flow cytometric analysis after staining with anti-CD31, anti-VCAM-1, and anti-ICAM-2 mAb. Expression of VCAM-1 (E) and ICAM-2 (F) on live CD31$^+$ cells reported as mean ($\pm$ SD) fluorescence intensity (MFI). Data are representative of three independent experiments. (G) Alternatively, the tumor tissue was homogenized and analyzed for the indicated soluble molecules by Mouse CytokineMAP B version 1.0. The graphs report the concentration of the indicated soluble molecules as a percentage of the concentration found in PBS-treated mice. Data from two independent experiments involving five to six animals per group were aggregated. Statistical analysis was performed using the Student $t$ test: $*0.01 < p < 0.05$, $**0.001 < p < 0.01$, $***p < 0.001$. 

**NGR-TNF promotes the efficacy and therapeutic activity of immunotherapy and chemoimmunotherapy**
associated Ag (23). B16 melanoma-bearing mice were sublethally irradiated and infused with splenocytes from TRP-2–sensitized C57BL/6 mice. Seven days later, the mice were vaccinated with TRP-2180–188–pulsed DC and boosted biweekly. NGR-TNF or TNF along with DOXO were provided 1 wk after each immunization. As shown in Supplemental Fig. 4, the survival of NGR-TNF–treated mice was significantly prolonged compared with that of TNF-treated mice. The results of in vivo studies indicate that vascular targeting of TNF is able to act in synergy with active or adoptive immunotherapy or chemotherapy, or both, against cancer.

Discussion

The results show that treatment of tumor-bearing mice with extremely low doses of NGR-TNF (5 ng/kg) is associated with a substantial modification of the cytokine-chemokine milieu of the tumor microenvironment and reverts EC anergy. These rapid and transient modifications favor the selective recruitment within the tumor mass, of fully activated endogenous or adoptively transferred CTL. Indeed, 2 h after NGR-TNF treatment, tumor-specific IFN-γ-producing CD8+ T cells were already found to accumulate in the tumor mass, but not in the blood, spleen, or kidney of tumor-bearing mice. Remarkably, whereas the duration of the measured direct effects of NGR-TNF were short, the beneficial effects induced by NGR-TNF on TIL appeared to last for days. As a direct consequence, the therapeutic efficacy of both active and adoptive immunotherapies were significantly increased. Indeed, NGR-TNF treatment synergized with a DC-based vaccine given alone or in combination with DOXO, and in the context of two models of adoptive immunotherapy. While efficacious, the proposed combined therapies did not cause complete tumor regression, and the mice eventually succumbed to the tumor. Especially in the context of adoptive immunotherapy, the addition of exogenous cytokines, such as IL-2 and IL-15, or preconditioning of the
recipient to favor proliferation of the transferred T cells would likely synergize with NGR-TNF based strategies (5). Further study is warranted to specifically address this issue.

The results of the studies on the mechanism of action show that a comparable dose of TNF was marginally or not active, thereby suggesting the hypothesis of an NGR-mediated targeting mechanism. One may wonder whether a targeting mechanism is indeed necessary for the local activity of TNF, or whether high-dose TNF can exert multiple effects in tumors, all potentially cooperating with the chemoimmunotherapy combination, as we have previously documented that NGR-TNF can promote the penetration of chemotherapeutic drugs in tumors (14, 41). Finally, NGR-TNF treatment can also transiently reduce hypoxic areas of lymphomas (41) and favor TIL proliferation and survival. Thus, NGR-TNF can exert multiple effects in tumors, all potentially cooper-
ating in the synergism with chemoimmunotherapy. Further study is necessary to assess what is predominant.

NGR-TNF is the first TNF derivative to be developed for its delivery to tumors and has shown antitumor efficacy in cancer patients (18, 19). In addition to NGR-TNF, other tumor vessel-homing derivatives of TNF could be exploited, in principle, for the same purpose. For example, TNF-fusion proteins with ACDCRGDCFCG or CisoDGRC peptides (both ligands of \( \alpha_v \beta_3 \) integrins) (42, 43), or with the single chain Fv Ab L19, directed against the extradomain B of fibronectin (44), might serve for the same purpose, because they also recognize target molecules that are expressed by the tumor neovasculature. However, given that the location of the target molecules in tumor vessels and their level of expression are different from that of CD13 (the NGR receptor), further study is necessary to assess this hypothesis.

Lymphocyte infiltration in tumors can also be favored by the use of antiangiogenic compounds, such as anti-VEGFAbs (7, 45–47) that are also able to inhibit the formation of new blood vessels and promote vascular normalization (48). From a conceptual point of view, these strategies are different from that proposed in the current study, which exploits NGR-TNF as an inflammatory-vascular targeting agent that induces vascular activation. Notably, the use of antiangiogenic compounds requires higher doses of drugs and more frequent administration, possibly inducing greater toxic reactions. The observation that extremely low doses of NGR-TNF are sufficient to induce local tumor effects with no signs of systemic toxicity makes NGR-TNF a more attractive agent for its combination with immunotherapy and chemoimmunotherapy. In this regard, it is remarkable that most of the effects induced by NGR-TNF on the vessels of murine tumors (14–16) have been observed also in patients (18, 19). Therefore, association of NGR-TNF with current active and adoptive immunotherapies and chemoimmunotherapies appears to be a safe and efficacious strategy. This combination should concomitantly alter tumor vessel permeability and favor chemotherapeutic drug and leukocyte penetration, tumor cell death, inflammation, and tumor Ag cross presentation in peripheral lymphoid organs and at the tumor site. Furthermore, NGR-TNF might transiently decrease intratumoral hypoxia, rendering the tumor more susceptible to chemotherapy and immune-mediated attack. Our findings identify a novel property of NGR-TNF and could be relevant for the design of novel immunotherapeutic approaches for cancer patients.

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Disclosures

A. Corti is the inventor of a patent on NGR-TNF. The other authors have no financial conflicts of interest.

References

Legends for supplemental figures

Supplemental Figure 1. NGR-TNF favors tumor infiltration by CD8\(^+\) T cells. Mice were challenged \textit{s.c.} with B16-OVA cells, and 12 days later they were infused \textit{i.v.} with activated OTI cells (OTI; 3 x 10\(^7\)). After 2 additional days, animals were treated with PBS; NGR-TNF or TNF as described in the legend to Fig. 1, and 2 h later tumors were excised and frozen for immunofluorescence analysis. The panels depict melanoma sections at immunofluorescence. Green: CD8\(^+\) cells; red: CD31\(^+\) cells, and DAPI: cell nuclei.

Supplemental Figure 2. Effects of NGR-TNF on TIL at different time points. Mice were challenged \textit{s.c.} with B16-OVA cells, and 12 days later they were infused \textit{i.v.} with activated and CFSE\(^+\) labeled OTI cells (3 x 10\(^7\)). After one additional day, animals were treated with NGR-TNF as described in the legend to Fig. 1, and 24 h later the number (A) and effector function (B) of CFSE\(^+\) TIL were quantified by flow cytometry as described in the legend to Fig. 2. Alternatively, NGR-TNF was administered 2 day after adoptive transfer of OTI cells, and TIL were analyzed 2 h later (2h). Data are reported as (A) absolute numbers ± SD of CFSE\(^+\) cells/g within the CD8\(^+\) cells. (B) Quantification of CFSE\(^+\)IFN-\(\gamma\)\(^+\) TIL (gated on CD8\(^+\) cells). Statistical analysis was performed using the Student’s t-test.

Supplemental Figure 3. NGR-TNF favors tumor infiltration by CD8\(^+\) T cells. Mice were challenged \textit{s.c.} with B16-OVA cells, and 7 days later they were treated as described in the legend to Fig. 5. (A) Schematic representation of the treatment schedule. Four days after the end of the first cycle of combined therapy mice were killed and TIL were analyzed by flow cytometry as detailed in the Material and Methods section. (B) Representative plots of TIL for each experimental condition. Numbers refer to the percentage of cells in each quadrant. (C) Percentage ± SD of CD8\(^+\) TIL within the gate of lymphocytes for each experimental condition. Statistical analysis was performed using the Student’s t-test.
Supplemental Figure 4. Adoptive immunotherapy combined with vascular targeting in the B16 melanoma model prolongs overall survival of mice. (A) Schematic representation of the experiment. C57BL/6 mice were challenged s.c. with B16 melanoma cells (5 x 10^4), and 6 days later they were subjected to total body irradiation (TBI; 600 Rad). After an additional day, mice were adoptively transferred with splenocytes (AT; 6 x 10^7/mouse) from syngenic mice previously sensitized against TRP-2 antigen. One week after the AT, mice were randomly assigned to either one of the following treatments (5/group): weekly i.p. injection of PBS (black circles); biweekly i.d. injection of DC-TRP-2 (5 x 10^5/mouse) either alone (open squares), or alternated by biweekly i.p. injection of TNF (5 ng/Kg) followed 2 h later by DOXO (4 mg/Kg; black squares); or biweekly DC-OVA injections alternated by biweekly NGR-TNF (5 ng/Kg) and DOXO treatments (open circles). In addition, one group of mice received only TBI and AT (black triangles), and other mice were treated with TBI followed by DC-TRP2 and the combination of NGR-TNF and DOXO (open diamond). Tumor growth was monitored twice a week for individual mice, and mice were killed when the mean tumor diameter was ≥ 15 mm or when the tumor became ulcerated. (B) Survival curves are reported in a Kaplan-Meyer plot. Log-rank test: TBI vs TBI+AT: p = 0.007; TBI vs TBI+DC-TRP-2+NGR-TNF+DOXO: p = 0.03; TBI vs TBI+AT+DC-TRP-2+NGR-TNF+DOXO: p = 0.0015; TBI vs TBI+AT+DC-TRP-2+TNF+DOXO: p = 0.019; TBI+AT vs TBI+AT+DC-TRP-2+NGR-TNF+DOXO: p = 0.0018; TBI+DC-TRP-2 vs TBI+AT+DC-TRP-2+NGR-TNF+DOXO: p = 0.0027. TBI+DC-TRP-2+NGR-TNF+DOXO vs TBI+AT+DC-TRP-2+NGR-TNF+DOXO: p = 0.004. TBI+AT+DC-TRP-2+NGR-TNF+DOXO vs TBI+AT+DC-TRP-2+TNF+DOXO: p = 0.0072.
Calcinotto et al. Suppl. Figure 1

PBS

NGR-TNF

TNF

Green: CD8
Red: CD31
Blue: DAPI
Calcinotto et al. Suppl. Figure 3

A

B16-OVA  DC-OVA  DOXO  NGR-TNF or TNF Killing

0  7  14  18  days

B

DC+PBS+PBS  DC+NGR-TNF+DOXO  DC+TNF+DOXO

CD8  FSC

% CD8+ Cells

C

% CD8+ cells

p=0.021

DC  PBS  NGR-TNF  DOXO  TNF  DOXO
Calcinotto et al. Suppl. Figure 4

A

B

Survival (%) vs. Day since tumor challenge