Targeted Delivery of IFN-γ to Tumor Vessels Uncouples Antitumor from Counterregulatory Mechanisms

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Abstract

Because of its immunomodulatory and anticancer activities, IFN-γ has been used as an anticancer drug in several clinical studies, unfortunately with modest results. Attempts to increase the response by increasing the dose or by repeated continuous injection often resulted in lower efficacy, likely due to counterregulatory effects. We show here that targeted delivery of low doses of IFN-γ to CD13, a marker of angiogenic vessels, can overcome major counterregulatory mechanisms and delay tumor growth in two murine models that respond poorly to IFN-γ. Tumor vascular targeting was achieved by coupling IFN-γ to GCNGRC, a CD13 ligand, by genetic engineering technology. The dose-response curve was bell-shaped. Maximal effects were induced with a dose of 0.005 μg/kg, about 500-fold lower than the dose used in patients. Nontargeted IFN-γ induced little or no effects over a range of 0.003 to 250 μg/kg. Studies on the mechanism of action showed that low doses of targeted IFN-γ could activate tumor necrosis factor (TNF)-dependent antitumor mechanisms, whereas high doses of either targeted or nontargeted IFN-γ induced soluble TNF-receptor shedding in circulation, a known counterregulatory mechanism of TNF activity. These findings suggest that antitumor activity and counterregulatory mechanisms could be uncoupled by tumor vascular targeting with extremely low doses of IFN-γ. (Cancer Res 2005; 65(7): 2906-13)

Introduction

A large body of evidence suggests that IFN-γ, a pleiotropic cytokine mainly produced by T lymphocytes and natural killer cells (1, 2), could promote antitumor responses (3–10). For instance, IFN-γ could induce antiproliferative and proapoptotic effects on many tumor cell types (11), inhibit tumor angiogenesis (8, 12–14), and activate natural killer cells and macrophages to kill a variety of tumor cell targets (11). IFN-γ is also an important regulator of CD4+ T helper cells (15, 16), is the major physiologic macrophage-activating factor (17–19), and can augment the expression of MHC-I and -II on cancer and endothelial cells (2, 20, 21). Within tumor stroma, IFN-γ can induce cytokine and chemokine secretion, including IP-10, an angiostatic protein and a chemokine secretion, including IP-10, an angiostatic protein and a chemokine. Evidence has been obtained to suggest that IFN-γ produced by tumor-infiltrating macrophages plays a role in tumor blood vessel destruction (22). Combined treatment of endothelial cells with IFN-γ and tumor necrosis factor (TNF)-α results in synergistic cytotoxic effects, likely important for tumor vasculature destruction (23). IFN-γ can also increase the production of TNF by activated macrophages, as well as the expression of TNF-receptors in various cell types (24–26). As a consequence of these effects on tumor vasculature and on cells of the immune system, IFN-γ can activate inflammatory/immune responses against established tumors and inhibit tumor growth (27).

The antiproliferative, angiostatic, and immunomodulatory activity of IFN-γ make this cytokine an attractive anticancer agent. For this reason, several clinical studies have been done with this cytokine. Unfortunately, the response rates observed in early studies, based on doses that approached the maximal tolerated dose, were very low (28–30). Studies in animal models showed that the antitumor activity of IFN-γ exhibits a bell shaped dose-response curve (6). Subsequent studies, carried out in patients, showed that induction of immune-activation markers also exhibit a bell-shaped dose-response curve (31–33). This suggests that optimal biological effects could be induced by doses below the maximum tolerated dose. Interestingly, the results of a phase III study on ovarian cancer patients showed that inclusion of relatively low doses of this cytokine (100 μg) in first-line chemotherapy could prolong progression-free survival (34). Higher response rates were also observed in melanoma patients treated with the low-dose weekly regimen compared with higher doses and more frequent schedules (35). However, no difference in outcome was observed in a recent phase III study in patients with renal-cell carcinoma treated with low doses of IFN-γ (60 μg/m2 once every week), as compared with placebo (36).

The results of preclinical and clinical studies suggest that attempts to increase the antitumor efficacy by increasing the dose and the exposure to IFN-γ could actually result in higher toxicity and lower efficacy, likely because of induction of counterregulatory mechanisms. Therefore, the development of new strategies that overcome the untoward effects of IFN-γ and bypass counterregulatory mechanisms could be of great experimental and clinical value.

The biological effects induced by IFN-γ on tumor stroma and blood vessels provide the rationale for a tumor vasculature targeting approach. To this aim, we have fused, by recombinant DNA technology, the COOH terminus of murine IFN-γ with the NH2 terminus of Gly-Cys-Asn-Gly-Arg-Cys (GCNGRC), a ligand of a CD13 (aminopeptidase N) isoform expressed by angiogenic vessels (37, 38). The GCNGRC peptide, previously identified by in vivo panning of peptide-phage display libraries (39), has proven useful for targeting chemotherapeutic drugs, proapoptotic peptides and TNF to tumor blood vessels (38–45). We show here that targeted delivery of minute amounts (picogram doses) of IFN-γ-GCNGRC conjugate (called IFN-γ-NGR) to tumor vasculature could be a valuable strategy for overcoming major counterregulatory mechanisms and inducing antitumor effects.
Materials and Methods

Cell lines and reagents. EA.hy926 cells (human endothelial cells fused with human lung carcinoma A549 cells) (46) were obtained from Dr. Elisabetta Ferrero (San Raffaele H Scientific Institute, Milan, Italy). EA.hy926 cells and murine WEHI-164 fibrosarcoma cells (Sigma-Aldrich, Milan, Italy) were cultured in DMEM (Euroclone, Milan, Italy) supplemented with 2 mmol/L glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum. Murine RMA lymphoma (47) and B16/F1 melanoma cells were cultured as described previously (48). Monoclonal antibody (mAb) R3-63 (rat anti-mouse CD13; ref. 49) was from Acris Antibodies GmbH (Hiddhenhausen, Germany), goat anti-mouse-FITC secondary antibody was from Sigma-Aldrich, mAb Y-3 (mouse anti-H-2Kb) and mAb 19E12 (rat anti-mouse Th1L) were provided by Dr. Paolo Dellabona (San Raffaele Scientific Institute, Milan, Italy), and mAb V1q (rat anti-murine TNF) was kindly supplied by Dr. Daniela N. Mannel (University of Regensburg, Germany). Recombinant murine IFNγ was from Peprotech, London, United Kingdom. Human TNF and the CNRGC-RN-1TNF conjugate (NGR-TNF) were prepared as described previously (41).

Preparation of IFNγ-NGR and IFNγ-C136S. The cDNA coding for murine IFNγ-NGR (IFNγ-NGR, IFNγfs-135 fused with the NH2 terminus of SCGNGRC) was obtained by reverse transcriptase-PCR on total RNA purified from the splenocytes of C57BL/6 mice (Harlan, Italy). Before RNA extraction, the splenocytes were stimulated for 20 hours with 10 ng/mL IFN-γ and 10% fetal bovine serum. Murine RMA lymphoma (47) and B16/F1 melanoma cells were cultured as described previously (48). Before RNA extraction, the splenocytes were detached by treatment with trypsin-EDTA, washed and resuspended in 138 mmol/L sodium chloride, 2.7 mmol/L potassium chloride, 10 mmol/L sodium phosphate (pH 7.3; PBS) containing 2% fetal bovine serum and 2 µg/mL mAb Y-3 (anti-H-2Kb) and incubated for 1 hour on ice. After washing with PBS, the cells were incubated with goat anti-mouse-FITC secondary antibody (1:100 in PBS containing 2% FCS, 30 minutes on ice), washed, fixed with 4% formaldehyde in PBS, and analyzed by fluorescence-activated cell sorting.

EA.hy926 cell adhesion assay. Polyvinyl chloride microtitre plates (Falcon code #3912, Becton Dickinson, Franklin Lakes, NJ) were coated with 30 µg/mL IFNγ-NGR or IFNγ-C136S [50 µL/well in 150 mmol/L sodium chloride, 50 mmol/L sodium phosphate (pH 7.3), at 4°C overnight]. After washing with 0.9% sodium chloride, each well was filled with DMEM containing 2% bovine serum albumin (45 minutes at 37°C), to block the uncoated surface, and washed again. EA.hy926 cells in DMEM culture media (100 µL) were then added to the plates (3 x 104 cells per well). After incubation (1-1.5 hours) at 37°C, 5% CO2, unbound cells were removed by washing with DMEM. Adherent cells were fixed with 3% paraformaldehyde, 2% sucrose in PBS (pH 7.3), and stained with 0.5% crystal violet (Fluka Chemie, Buchs, Switzerland). Adherent cells were quantified by measuring the absorbance of each well at 540 nm, using a microplate reader.

Soluble tumor necrosis factor receptor assays. Soluble p55-TNF receptor (sTNF-R1) and soluble p75-TNF receptor (sTNF-R2) in animal sera were measured by ELISA as previously described (50).

In vivo studies. Studies on animal models were approved by the Ethical Committee of the San Raffaele H Scientific Institute and done according to the prescribed guidelines. C57BL/6 mice or BALB/c (Harlan), 8 weeks old, were challenged with s.c. injection in the left flank of 7 x 106 RMA or 106 WEHI-164 living cells, respectively; 6 to 10 days later, mice were treated i.p. with IFNγ, IFNγ-NGR, or IFNγ-C136S solutions (100 µL). All proteins were diluted with 0.9% sodium chloride containing 100 µg/mL endotoxin-free human serum albumin (Farma-Biagini SpA, Lucca, Italy). Tumor growth was monitored daily by measuring tumor volumes with calipers as previously described (51). Animals were sacrificed before tumors reached 1.0 to 1.5 cm in diameter. Tumor sizes are shown as mean ± SE (five animals per group).

Results

Production and characterization of IFNγ-NGR and IFNγ-C136S. Murine IFNγ is a homodimeric protein of 136 residues characterized by the presence of two cysteines in the NH2-terminal region (Cys-Tyr-Cys) and one cysteine at the COOH terminus (52, 53). We have fused the COOH terminus of IFNγfs-135 (lacking the NH2- and COOH-terminal cysteines) to the NH2 terminus of (S)GCNGRC by recombinant DNA technology. NH2-terminal cysteines were omitted and Cys136 was replaced with a serine to reduce the risk of disulfide bridge formation with the GCNGRC targeting domain. The final product was called IFNγ-NGR. In parallel, the IFNγfs-135-C136S mutant (called IFNγ-C136S) was also prepared (see Fig. L4 for a schematic representation of these products). Both proteins were purified by hydrophobic interaction chromatography, ion exchange, and gel-filtration chromatography. Only fractions corresponding to dimeric species were collected during gel-filtration chromatography. The purity and identity of both products were characterized by SDS-PAGE, gel-filtration HPLC, and mass spectrometry. Reducing and nonreducing SDS-PAGE of IFNγ-NGR and IFNγ-C136S showed a single band of about 16 kDa, as expected for monomeric subunits (Fig. 1B). The molecular mass of subunits, as measured by electrospray mass spectrometry, was 16,225.2 ± 2.3 and 15,635.0 ± 1.3 Da, respectively, corresponding to products with unprocessed NH2-terminal methionine (expected 16,225.5 and 15,636.8 Da, respectively). Analytic gel-filtration chromatography showed that IFNγ-NGR and IFNγ-C136S were homogeneous and characterized by a hydrodynamic volume of about 30 to 35 kDa, corresponding to dimers (Fig. 1C).

Biological activity of IFNγ-NGR in vitro. The functional properties of effector and targeting domains of IFNγ-NGR, i.e., the IFNγ and GCNGRC domains, were first investigated using in vitro biological assays. To assess whether the IFNγ domain was functional, we compared the effect of various doses of IFNγ-NGR and IFNγ on MHC class I and II expression on murine B16/F1 cells.


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and fusion of IFN-β with IFN-γ tumor-associated vessels (38). Competitive binding experiments inhibit the binding of the anti-human CD13 mAb WM-15 to peptides and drug conjugates containing the CNGRC motif can tochemical analysis of renal cell carcinoma tissue sections, that were also investigated. We have previously shown, by immunohis-

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Figure 1. Schematic representation (A), SDS-PAGE (B), and analytic gel-filtration chromatography (C) of IFN-γ-NGR, IFN-γ-C136S, and IFN-γ, SDS-PAGE was done under reducing (+/Me) and nonreducing (−/Me) conditions. Analytic gel-filtration chromatography was done using a Superdex 75-HR column (Amersham) pre-equilibrated with 0.15 sodium chloride, 0.05 sodium phosphate buffer (pH 7.3); bars on chromatograms represent the elution volume of molecular weight markers.

Fluorescence-activated cell sorting analysis of treated cells showed that both proteins could induce MHC-I expression with similar potency (Fig. 2A and B). IFN-γ-C136S induced MHC-I in a similar manner and similar results were obtained for MHC-II (data not shown). These results indicate that replacement of Cys136 with Ser and fusion of IFN-γ-C136S with GCNGRC do not prevent folding, dimerization, or binding to IFN-γ receptors.

The accessibility and functional properties of the NGR domain were also investigated. We have previously shown, by immunohistoechemical analysis of renal cell carcinoma tissue sections, that peptides and drug conjugates containing the CNGRC motif can inhibit the binding of the anti-human CD13 mAb WM-15 to tumor-associated vessels (38). Competitive binding experiments with IFN-γ-NGR and IFN-γ showed that IFN-γ-NGR, but not IFN-γ, could compete the binding of mAb WM-15 to tumor vessels (data not shown), suggesting that IFN-γ-NGR could interact with CD13 expressed in tumor vessels. Considering that peptides containing the NGR motif can also interact with αvβ3- and αvβ5-integrines with low affinity (54, 55) and inhibit αβ3- and αβ5-integrin-mediated cell adhesion (56) we have also analyzed the effects of IFN-γ-NGR and IFN-γ-C136S in a cell adhesion assay. To this aim, the adhesion of endothelial-epithelial E.Ahy926 hybrid cells to microtiter wells coated with IFN-γ-NGR or IFN-γ-C136S was studied (Fig. 3). In parallel, we also analyzed cell adhesion to CNGRC-TNF (called NGR-TNF), a conjugate with a functional NGR domain (41, 42). As expected, we observed adhesion of E.Ahy926 cells to microtiter plates coated with NGR-TNF or IFN-γ-NGR, but not with TNF or with IFNγ-C136S (Fig. 3). Cell adhesion was not inhibited by mAb WM15 (data not shown), suggesting that this effect was likely mediated by integrins and not by CD13. These results, collectively, suggest that both IFN-γ and CNGRC domains are properly folded and accessible for the interaction with membrane receptors.

Antitumor activity and toxicity of IFNγ-NGR in vivo. The antitumor activity of IFN-γ-NGR, IFN-γ-C136S, or IFN-γ against s.c. RMA lymphoma and WEHI-164 fibrosarcoma was investigated in immunocompetent mice. Various doses of each cytokine, ranging from 0.03 to 5000 ng were given (i.p.) to tumor-bearing C57BL6 (RMA) or BALB/c (WEHI-164) mice. Administration of 0.1 or 0.3 ng of IFN-γ-NGR to RMA tumor-bearing mice, 10 days after tumor implantation, was sufficient to induce significant antitumor effects (Fig. 4A and B). The antitumor effect decreased when the dose was increased to 3 or 300 ng (Fig. 4B) or when the dose was decreased to 0.03 ng (data not shown), indicating that the dose-response curve of IFN-γ-NGR is bell-shaped. Thus, maximal effects were achieved with 0.1 ng (0.005 μg/kg). No loss of body weight was induced by any tested dose (Fig. 4A and B, bottom). This suggests that IFN-γ-NGR could induce antitumor effects without causing major toxic effects. No significant antitumor effects were observed when IFN-γ was given at doses ranging from 0.3 to 300 ng (Fig. 4B, middle) or when IFN-γ-C136S was used (data not shown).

A bell-shaped dose-response curve was also observed in the WEHI-164 model. Again maximal effect was achieved with 0.1 ng of IFN-γ-NGR (Fig. 5A), whereas lower effects were induced by 0.3 or 0.9 ng doses (Fig. 5A). Also in this model, the antitumor effect induced by the 0.1 ng dose was not associated with loss of body weight (Fig. 5A, bottom). The effect of repeated administrations was then investigated. Repeated administration of IFNγ-NGR produced different effects depending on dose and time schedule. For instance, we found that the antitumor effects of daily treatment with 0.03 or 0.1 ng were lower than those of biweekly treatments (Fig. 5B). Of note, whereas the first and the second treatment with 0.1 or 0.03 ng induced an antitumor response, the subsequent daily treatments were not effective and inhibited the antitumor response induced by the first treatment (Fig. 5B, bottom). Apparently, repeated treatment also inhibited the spontaneous transient regression observed in control animals from day 14 to day 16 in this experiment (Fig. 5B, bottom). This phenomenon was also observed in another experiment (data not shown).

When the dose of IFNγ-NGR was increased to 5000 ng (given weekly) no significant effects were observed (Fig. 5C, top). IFNγ was virtually inactive at any tested dose (Fig. 5A and C). Overall, these results of in vivo experiments indicate that IFNγ-NGR is endowed with more potent antitumor activity than IFNγ and that the antitumor activity depends on dose and time schedule.

Role of the aminopeptidase N (CD13) in the antitumor activity of IFNγ-NGR. We have shown previously that a CD13 isoform expressed in tumor vessels could function as the main vascular receptor for NGR-TNF, as most of the antitumor activity of this drug is inhibited by an excess of an anti-murine CD13 mAb (mAb R3-63; refs. 38, 41). To investigate the role of CD13 in the antitumor activity of IFNγ-NGR, we have coadministered this conjugate with an excess of the anti-CD13 mAb R3-63 to RMA and WEHI-164 tumor-bearing mice. This antibody inhibited most of the antitumor effects of different doses of IFNγ-NGR (3 and 0.06 ng) in these models (Fig. 6A and C). In contrast, a control antibody
(mAb 19E12, anti-Thy 1.1) did not affect the antitumor activity of IFNγ-NGR (Fig. 6A). Although we cannot exclude that integrins could also play a role in vascular targeting, the almost complete inhibition observed after CD13 neutralization suggest that CD13 plays a major role.

Role of endogenous and soluble tumor necrosis factor receptors. IFNγ and TNF can exert synergistic cytotoxic effects against tumor and endothelial cells (23, 57, 58). Keeping this in mind, we have studied the role of endogenous TNF in the IFNγ-NGR antitumor activity. To this aim, RMA and WEHI-164 tumor-bearing mice were treated with IFNγ-NGR alone or in combination with a neutralizing anti-murine TNF mAb (V1q). This antibody completely inhibited the antitumor effects of IFNγ-NGR in both models, whereas it was inactive when given alone (Fig. 6B and D). Also in this experiment, the control mAb 19E12 did not inhibit the antitumor activity of IFNγ-NGR. These results suggest that endogenous TNF is critical for the antitumor activity of IFNγ-NGR.

Given that soluble p55 and p75 TNF receptors (sTNF-R1 and sTNF-R2, respectively) could inhibit endogenous TNF and consequently inhibit the antitumor activity of IFNγ-NGR, we have addressed the hypothesis that induction of soluble TNF receptors by high doses of IFNγ-NGR contributes to inhibiting its activity. Three hundred nanograms of IFNγ-NGR, but not 3 ng, significantly induced sTNF-R1 and sTNF-R2 shedding in the blood of RMA tumor-bearing mice (Fig. 7B). Thus, the release of sTNF-Rs could be one of the counterregulatory mechanisms that contribute to generate the bell-shaped dose-response curve of IFNγ-NGR. This phenomenon is not a peculiarity of high doses of targeted IFNγ, as nontargeted IFNγ (IFNγ-C136S) could also induce sTNF-R2 shedding (Fig. 7B).

Soluble TNF-R2 shedding in mice can be induced by TNF itself (42). To assess whether shedding of sTNF-Rs was indirectly mediated by endogenous TNF or was a direct consequence of IFNγ, IFNγ-induced shedding of sTNF-R2 was studied in mice pretreated with anti-TNF mAb V1q. As shown in Fig. 7B, IFNγ-C136S-induced shedding of sTNF-R2 was not inhibited by V1q, pointing to a direct mechanism.

**Discussion**

We have found that targeted delivery of low doses of IFNγ to CD13, a marker of angiogenic vessels, can delay tumor growth in murine models that respond poorly to IFNγ. Targeted delivery of IFNγ to CD13 was achieved by coupling the COOH terminus of IFNγ to the NH2 terminus of GCNGRC peptide, a CD13 ligand (37). To avoid potential disulfide bridge formation between cysteine residues present in the GCNGRC targeting domain and in the NH2- and COOH-terminal regions of IFNγ (Cys4, Cys3, and Cys136) we deleted the first three residues of murine IFNγ and replaced Cys136 with Ser. The results of biochemical and in vitro biological studies of the IFNγ4-135-C136S-GCNGRC conjugate (called IFNγ-NGR) showed that the final product was homogeneous and characterized by a dimeric structure with accessible and functional targeting and effector domains (i.e., GCNGRC and IFNγ).
The results of studies on the mechanism of action of IFN-NGR suggest that the improved antitumor activity of this conjugate depends on the GCNGRC targeting domain, as originally postulated, and not on the C136S substitution. This view is supported by the following observations: (a) the antitumor activities of IFNγ and IFNγ-C136S (lacking the targeting domain) were similar and very low; (b) the in vivo antitumor activity of IFNγ-NGR was almost completely inhibited by an antibody (mAb R3-63) against CD13, a CNGRC-receptor. These findings, together, support the hypothesis that IFNγ-NGR works via a GCNGRC/CD13-dependent targeting mechanism.

We have previously shown that tumor-associated vessels of human breast carcinoma tissue section and other primary and metastatic tumors could be stained by immunohistochemistry with the anti-CD13 mAb WM15 (38). The finding that IFNγ-NGR can compete mAb WM15 binding to tumor vessels in tissue sections and the notion that RMA tumor cells of our murine model do not express CD13 (41) further support the CD13-mediated vascular targeting hypothesis.

Another important observation of this work is that the dose-response curve of IFNγ-NGR is bell-shaped. Maximal effects were achieved by the administration of 0.1 ng (0.005 μg/kg) of IFNγ-NGR (i.p.) in both RMA-lymphoma and WEHI-164 models, whereas administration of nontargeted IFNγ induced little or no effects over a range of 0.06 to 5000 ng (0.003-250 μg/kg). Attempts to increase the effect of IFNγ-NGR by increasing the dose (up to 250 μg/kg) or by frequent (daily) administration resulted in a decrease of activity.

The bell-shaped dose-response curve is not a peculiarity of targeted IFNγ as a similar behavior has also been reported for nontargeted IFNγ in other animal models and in patients (6, 31–33). However, it is noteworthy that doses of about 2 μg/kg of IFNγ were necessary to induce maximal biological effects in patients and even higher doses (250 μg/kg) were required in mice (6). One explanation for the bell-shaped dose-response curve of IFNγ-NGR is that low doses of this modified cytokine could activate local antitumor effects, by virtue of a targeting mechanism, without activating counterregulatory mechanisms, whereas high doses could induce counterregulatory effects that prevent its potential antitumor activity. The same phenomenon could explain the low response rates observed in animals and in patients treated with IFNγ.

![Figure 4](image1.png)

**Figure 4.** Effect of IFNγ-NGR or IFNγ on tumor growth and animal weight in the RMA model. Animals (five mice per group) bearing RMA-tumors were treated at days 10 and 17 (arrows) with the indicated doses of IFNγ-NGR or IFNγ (i.p.). Tumor volume and animal weight after treatment are reported. A and B represent separate experiments. A (top), ◇ versus ■ (P < 0.005; two-tailed t test at day 18); B (middle), ◇ versus ■ (P < 0.05; two-tailed t test at day 14).

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![Figure 5](image2.png)

**Figure 5.** Effect of IFNγ-NGR or IFNγ on tumor growth and animal weight in the WEHI-164 model. Animals (five mice per group) bearing WEHI-tumors were treated at the indicated times after tumor implantation (arrows) with various doses of IFNγ-NGR or IFNγ (i.p.). Tumor volume and animal weight after treatment are reported. A-C represent separate experiments. A (top), ◇ versus ■ (P < 0.05; two-tailed t test at day 15).
Induction of soluble TNF receptors (sTNF-R) could be one of these counterregulatory mechanisms for the following reasons. Previous clinical studies have shown that treatment of patients suffering from metastasizing renal cell carcinoma with IFN\textsubscript{g} induces the release of endogenous TNF into the serum (59). The known synergism between IFN\textsubscript{g} and TNF in inducing tumor and endothelial cell cytotoxicity and other antitumor effects suggest that TNF could contribute to the antitumor activity of IFN\textsubscript{g} (23, 57, 58, 60–62). Interestingly, administration of a neutralizing anti-murine TNF mAb (mAb V1q) to our tumor-bearing mice inhibited the antitumor activity of low doses of IFN\textsubscript{g}-NGR. Endogenous TNF is, therefore, indeed critical for the antitumor activity of IFN\textsubscript{g}-NGR. However, we have also found that high doses of IFN\textsubscript{g}-NGR (e.g., 300 ng), but not low doses (3 ng), could induce a significant increase of sTNF-R1 and sTNF-R2 in the circulation. Similarly, high doses of nontargeted IFN\textsubscript{g} induced sTNF-Rs. Given that the release of sTNF-Rs is an important counterregulatory mechanism for TNF (63), one possibility is that sTNF-Rs shedding contributes to the bell-shaped dose-response curve of IFN\textsubscript{g}-NGR and to the lack of effect by IFN\textsubscript{g} in our models. Of course, many other cytokines and counter-regulatory mechanisms could be activated by high doses of targeted and nontargeted IFN\textsubscript{g}. Nevertheless, the observation that low doses of IFN\textsubscript{g}-NGR (0.06 and 3 ng) are sufficient to activate a TNF-dependent antitumor mechanism, whereas high doses (300 ng) are necessary to induce sTNF-Rs shedding implies that antitumor and counterregulatory mechanisms could be uncoupled by the low-dose targeting strategy. This finding offers a new rationale for targeted delivery of very low doses of cytokines to tumors.

The results have also pointed out some important limitations of IFN\textsubscript{g}-NGR that deserve to be discussed. As reported for IFN\textsubscript{g} by many investigators, we observed that daily treatments with IFN\textsubscript{g}-NGR was less effective than biweekly or weekly treatments and that, remarkably, repeated treatment inhibited the response induced by the first treatment. Previous studies have shown that the release of endogenous TNF induced by IFN\textsubscript{g} in the serum of patients with renal cell carcinoma is down-regulated by repeated application of the same dose (59). Other studies have shown that prolonged treatment with IFN\textsubscript{g} can induce hyporesponsiveness of natural killer activity (7, 64). It is therefore possible that excessive exposure to IFN\textsubscript{g}-NGR can induce counterregulatory mechanisms (locally and/or systemically) and/or inhibit ongoing antitumor responses. Remarkably, concern was expressed about rapid disease progression in patients with Kaposi’s sarcoma or other tumors repeatedly treated with high doses of IFN\textsubscript{g} in early clinical studies (65). In our models, the spontaneous regression occasionally observed in control groups was apparently inhibited in groups repeatedly treated with targeted IFN\textsubscript{g}, through an unknown mechanism. Therefore, dosage and schedule of

Figure 6. Effect of neutralizing anti-CD13 (R3-63) and anti-TNF (V1q) mAbs on the antitumor activity of IFN\textsubscript{g}-NGR. RMA (A and B) or WEHI-tumor-bearing mice (C and D) were treated at day 10 (RMA) or day 6 (WEHI) with IFN\textsubscript{g}-NGR alone or in combination with mAb R3-63 (anti-murine CD13 mAb), or mAb V1q (anti-murine TNF), or mAb 19E12 (anti-murine Thy 1.1, control antibody) at the doses indicated in each (five mice per group). Each mAb was given 2.5 hours before IFN\textsubscript{g}-NGR; B, \(\Delta\) versus \(\circ\) \((P < 0.05;\) two-tailed \(t\) test at day 14); C, \(\Delta\) versus \(\circ\) \((P < 0.05;\) two-tailed \(t\) test at day 13).

Figure 7. Circulating levels of sTNF-R1 and sTNF-R2 in RMA-tumor bearing mice after treatment with IFN\textsubscript{g}-NGR or IFN\textsubscript{g}-C136S. Animals were treated 10 days after tumor implantation with 0, 3, or 300 ng of IFN\textsubscript{g}-NGR \((n = 4;\) A) or 5 \(\mu\)g of IFN\textsubscript{g}-C136S alone or in combination with anti-TNF mAb V1q (7 \(\mu\)g, given 2 hours before IFN\textsubscript{g}-C136S; \(n = 5;\) B). Animal sera were collected 1.5 hours after treatment and serum levels of sTNF-R1 and sTNF-R2 were measured by ELISA.
administration could also be very critical for the biological effects of targeted IFNα, as previously observed for IFNα. Further work with different schedules of treatment, routes of administration, and combination with other drugs are therefore necessary to further assess the therapeutic potential and limitations of IFNα-NGR.

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