Critical role of indoleamine 2,3-dioxygenase in tumor resistance to repeated treatments with targeted IFNγ

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Abstract

Targeted delivery of IFNγ to tumors has been achieved by fusing this cytokine with GCNGRC, a tumor neovasculature homing peptide. Although the therapeutic efficacy of this protein (called IFNγ-NGR) in animal models is greater than that of IFNγ, frequent administrations of IFNγ-NGR may result in lower efficacy and tumor resistance. We investigated the role of indoleamine 2,3-dioxygenase (IDO), an IFNγ-inducible enzyme that may down-regulate T cells by affecting local tryptophan catabolism in tumor resistance to repeated treatments with IFNγ-NGR. The study was carried out in immunocompetent mice and in *nu/nu* mice bearing RMA lymphoma, B16F melanoma, or WEHI-164 fibrosarcoma and *in vitro* using cultured tumor cells. IDO activity was increased in lymphoma homogenates after multiple treatments with IFNγ-NGR but not after a single treatment. Coadministration of 1-methyl-tryptophan, an inhibitor of IDO, increased tumor responses to multiple treatments in the lymphoma, melanoma, and fibrosarcoma models. No synergism between IFNγ-NGR and 1-methyl-tryptophan was observed *in vitro* in tumor cell proliferation assays or in *nu/nu* tumor-bearing mice, suggesting that the antitumor effect was host mediated. We conclude that IDO is critically involved in tumor resistance to repeated treatments with IFNγ-NGR, likely causing excessive stimulation of tryptophan catabolism and inhibiting antitumor immune mechanisms. Coadministration of IFNγ-NGR with IDO inhibitors could represent a new strategy for increasing its antitumor activity. [Mol Cancer Ther 2008;7(12):3859–66]

Introduction

The immunomodulatory, antiproliferative, and angiostatic activities of IFNγ make this cytokine an attractive anticancer agent (1–4). For instance, IFNγ is the major physiologic macrophage-activating factor (5–7), activates natural killer cells to kill a variety of tumor cell targets (8), augments the expression of MHC-I and MHC-II on cancer and endothelial cells (9, 10), and regulates $CD4^+$ T helper cells (11, 12). This cytokine can also inhibit proliferation of many tumor cell types (8). IFNγ can induce chemokine and cytokine secretion in the tumor microenvironment, including IP-10, an angiostatic protein (8, 13), and can inhibit tumor angiogenesis (3, 13, 14). Because of these effects, IFNγ can activate inflammatory or immune responses against tumors and inhibit tumor growth (15). Unfortunately, the results of preclinical and clinical studies showed that the response rates are very low and that attempts to increase the antitumor efficacy by increasing the dose of IFNγ could actually result in higher toxicity and lower efficacy (16–21).

We have previously shown that targeted delivery of low doses of IFNγ to tumor vasculature can overcome major counterregulatory mechanisms and delay tumor growth in murine models that respond poorly to IFNγ (22). Vascular targeting has been achieved by fusing IFNγ with Gly-Cys-Asn-Gly-Arg-Cys (GCNGRC), a ligand of CD13 (aminopeptidase N) expressed by angiogenic vessels (23, 24). Pharmacologic studies in murine fibrosarcoma and lymphoma models have shown that the dose-response curve of IFNγ-GCNGRC conjugate (called IFNγ-NGR) is bell shaped with maximal effects being induced with a dose of 0.005 μg/kg, whereas nontargeted IFNγ induces little or no effect over a range of 0.003 to 250 μg/kg in these models (22). Evidence was also obtained to suggest that excessive stimulation of IFNγ receptors by high-dose IFNγ or by frequent administration of low doses of IFNγ-NGR can activate counterregulatory mechanisms and inhibit ongoing antitumor responses (22).

A growing body of evidence suggests that induction of indoleamine 2,3-dioxygenase (IDO) represents an important counterregulatory mechanism activated by IFNγ. IDO is an IFNγ-inducible enzyme (25) that catalyzes the first and rate-limiting step in the kynurenine pathway of the tryptophan catabolism (26). The expression of this enzyme in the placenta plays a critical role in preventing rejection of allogenic fetuses (likely by inhibiting T-cell proliferation), which are extremely sensitive to extracellular tryptophan shortage (27). IDO can also play a role in tumor immune escape and systemic inhibition of this enzyme by 1-methyl-tryptophan (1-MT), a substrate

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analogue, can increase the efficacy of therapeutic vaccination in murine tumor models (28).

In this work, we have investigated the role of IDO in tumor resistance to repeated treatments with IFNγ-NGR using mice bearing WEHI-164 fibrosarcomas, B16 melanomas, or RMA lymphomas. We show that IDO activity is increased in tumors after repeated treatments and that inhibition of this enzyme by p.o. administration of 1-MT increases the therapeutic response, suggesting that IDO is indeed critically involved in tumor resistance to multiple treatments with targeted IFNγ.

Materials and Methods

Cell Lines and Reagents

Murine RMA lymphoma (29) and B16/F1 melanoma cells were cultured as described previously (30). Recombinant murine IFNγ was from PeproTech. Murine IFNγ-NGR ($IFN\gamma_{4\text{-}135}$ fused with the NH_2 terminus of SGCNGRC) was prepared by recombinant DNA technology as described previously (22). Murine NGR-TNF [tumor necrosis factor (TNF) fused with the COOH terminus of CNGRCG] was prepared by recombinant DNA technology as described previously (31). Melphalan (Alkeran) was from Glaxo Wellcome. Anti-CD4 monoclonal antibody L3T4 and anti-CD8 monoclonal antibody Ly-2 were from BD Pharmingen.

In vivo Studies

Studies on animal models were approved by the Ethical Committee of the San Raffaele Scientific Institute and done according to the prescribed guidelines. C57BL/6 mice or BALB/c, 8 wk old, were challenged with s.c. injection in the left flank of 7×10^4 RMA or B16F1 living cells; 6 to 10 d later, mice were treated with IFNγ-NGR solutions, i.p. (100 μL), diluted with 0.9% sodium chloride containing 100 μg/mL endotoxin-free human serum albumin (Farma-Biagini SpA). Mice were given 1-MT [Sigma-Aldrich, 5 mg/mL (pH 9.5–10)] in the drinking water of which they drank 3 to 4 mL/d. Tumor growth was monitored daily by measuring tumor volumes with calipers as previously described (32). Animals were sacrificed before tumors reached 1.0 to 1.5 cm in diameter. Tumor sizes are shown as mean ± SE (5 animals per group).

In vitro Proliferation Assay

RMA cells (5×10^3/well) were plated in RPMI 1640 containing 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, IFNγ-NGR, and 1-MT at various concentrations (200 μL/well) and incubated for 4 d at 37°C, 5% CO_2. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (10 μL, 5 mg/mL in PBS) was added to each well and incubated for 2 h. The cells were treated with 10% (w/v) SDS, 50% (v/v) *N*,*N*-dimethylformamide, 0.025 mol/L hydrochloric acid, and 0.35 mol/L acetic acid solution (100 μL/well, 24 h at 37°C). The absorbance of each well at 570 and 650 nm (reference) was then measured using a microtiter plate reader. The same procedure was applied to proliferation assays involving WEHI-164 and B16F1 cells except that in the latter case, the cell culture medium was replaced with 200 μL DMSO after MTT staining and mixed with a pipette before spectrophotometric measurements.

Assay of IDO

IDO activity in RMA tumor extracts was determined colorimetrically essentially as described previously (33). A mixture of 0.8 mmol/L tryptophan, 40 mmol/L ascorbic acid, 20 μmol/L methylene blue, 200 units/mL catalase, 63 μL Dulbecco's PBS without Ca^{2+} and Mg^{2+} (BioWhittaker) was mixed with 63 μL of tissue extract (0.36 g of tissue/mL of PBS), both preincubated at 37°C for 5 min before mixing. After incubation at 37°C for 20 min, the reaction was stopped by adding 25 μL of 30% trichloroacetic acid. The mixture was then incubated at 50°C for 30 min to hydrolyze *N*-formylkinurenine to kynurenine. The product was centrifuged at 12,000 × *g* for 20 min and the supernatant (120 μL) was mixed with 1% (w/v) *p*-dimethylaminobenzaldehyde in 1% acetic acid (120 μL). The mixture was further incubated at room temperature for 15 min and analyzed by measuring the absorbance at 405/655 nm. All samples were analyzed in duplicate (four mice per group).

Results

Oral Administration of 1-MT to RMA Lymphoma-Bearing Mice Enhances the Antitumor Activity of Melphalan

Various studies have shown that 1-MT can affect T-cell function *in vivo* and improve immune response to tumors (28, 34). Various means were used to deliver 1-MT to mice in these studies, including p.o. administration of this inhibitor in the drinking water (5 mg/mL; ref. 28). To verify that this means of delivery can produce biologically active concentration of drug in our models, we took advantage of the notion that 1-MT cooperates with alkylating agents and other cytotoxic drugs to elicit regression of tumors refractory to single-agent therapy (35) and did a preliminary test in which 1-MT was given p.o. to RMA tumor-bearing mice in combination with melphalan, an alkylating agent. Stronger antitumor effects were observed when animals were treated with both drugs compared with melphalan alone (Fig. 1A). This suggests that p.o. administration of 1-MT could produce biologically active levels in RMA-bearing mice. Oral administration of 1-MT was therefore used in all subsequent studies.

1-MT Increases the Antitumor Activity of Repeated Doses of IFNγ-NGR in RMA Lymphoma, B16F1 Melanoma, and WEHI-164 Fibrosarcoma Models

We have previously shown that a single administration of IFNγ-NGR to WEHI-164 fibrosarcoma-bearing mice can induce significant antitumor effects, whereas repeated (daily) administrations of the same drug does not induce antitumor effects at all (22). To assess whether this can also occur in other models, we analyzed the effect of single and multiple treatments with IFNγ-NGR in immunocompetent mice bearing RMA lymphomas. Administration of IFNγ-NGR (0.1 ng) induced a significant delay in tumor growth also in this model (Fig. 1B, *left*), whereas repeated (daily) treatment with the same dose could not induce any therapeutic effect (Fig. 1C, *left*). It is noteworthy that

multiple treatments completely inhibited the response to the first treatment.

To investigate the role of IDO in this phenomenon, we examined the effect of 1-MT on tumor response to single or repeated treatments with IFNγ-NGR. When 1-MT was added to the drinking water (5 mg/mL), no major changes in the antitumor activity of single treatment were observed (Fig. 1B, *right*). In contrast, a marked change was observed in the response to repeated treatments (Fig. 1C, *right*). Interestingly, in this case, the effect of multiple treatments was greater than that of a single treatment.

To assess the role of CNGRC domain in the synergism observed between IFNγ-NGR and 1-MT, we analyzed the antitumor properties of repeated doses (0.1 ng) of normal IFNγ, lacking the NGR domain, in combination 1-MT. IFNγ could not induce a significant delay in tumor growth either in the absence or in the presence of 1-MT (Fig. 1D). This suggests that the NGR domain is critical for the antitumor activity of IFNγ-NGR/1-MT combination, likely for delivering low doses of IFNγ to tumors.

We then investigated whether 1-MT could enhance the response to repeated treatments with IFNγ-NGR in other models and with different administration schedules. We treated B16F1 melanoma-bearing mice with a higher dose of IFNγ-NGR (1 ng), five times, with or without 1-MT. Treatment with IFNγ-NGR alone was totally ineffective and apparently promoted faster growth of tumors (Fig. 2A, *left*). However, IFNγ-NGR could induce significant antitumor effect when animals were concomitantly treated with 1-MT (Fig. 2A, *right*). Similarly, an increase of response to repeated treatment was also observed in the WEHI-164 model (Fig. 2B). These results suggest that IDO plays a critical role in tumor resistance to repeated treatments with targeted IFNγ.

Repeated Administrations of IFNγ-NGR Increase IDO Activity in RMA Lymphoma Tumors

To assess the hypothesis that repeated treatments with IFNγ-NGR lead to induction of IDO in tumors, we measured the levels of IDO activity in RMA tumor extracts after treatment. To this aim, we treated tumor-bearing mice with 100 pg of IFNγ-NGR five times, everyday, and analyzed IDO activity in tumors 2 h after the last treatment. As expected, a significant increase of enzymatic activity was observed, compared with control and single treatment (Fig. 3), supporting the hypothesis that IDO activity is increased after repeated IFNγ-NGR administrations.

The Antitumor Activity of 1-MT/IFNγ-NGR Combination Is Host Mediated

We then addressed the question whether the potentiation of antitumor activity of IFNγ-NGR induced by 1-MT was related to direct effects on tumor cells or required host components. To this aim, we investigated the effect of IFNγ-NGR in combination with 1-MT on the proliferation of RMA, WEHI-164, and B16F1 cells *in vitro*. Previous studies showed that the circulating level of 1-MT in mice treated with 5 mg/mL 1-MT in the drinking water is 205 μmol/L (i.e., ~45 μg/mL; ref. 28).

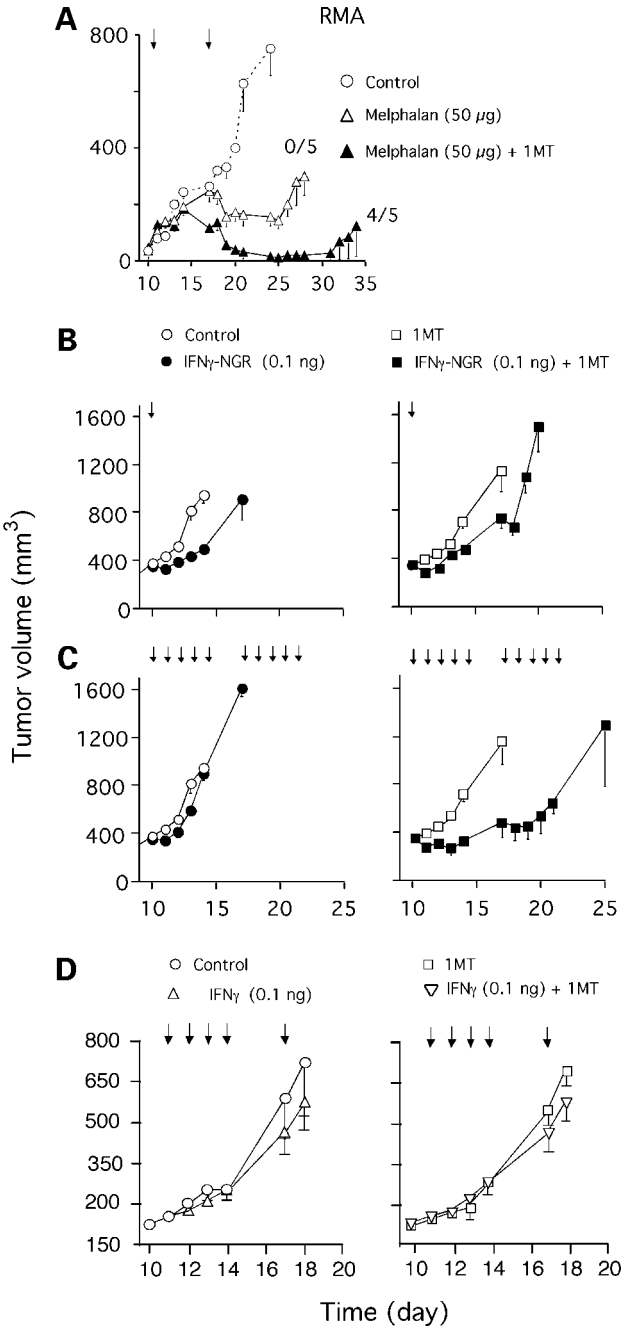

Figure 1. Effect of 1-MT on the antitumor activity of melphalan, IFNγ-NGR, or IFNγ in RMA lymphoma-bearing mice. **A,** animals (5 mice per group) bearing RMA tumors were treated at the indicated day (*arrows*) with melphalan (50 μg in physiologic solution) or with physiologic solution alone (control, i.p.). One group of mice was given melphalan in combination with 1-MT (5 mg/mL in the drinking water) as indicated. Fraction numbers indicate complete responses. Animals (5 mice per group) bearing RMA tumors were treated at the indicated days (*arrows*) with IFNγ-NGR (0.1 ng; **B** and **C**) or IFNγ (0.1 ng; **D**) in physiologic solution containing 0.1 mg/mL human serum albumin or with human serum albumin solution alone (control, i.p.). Mice were given 1-MT (5 mg/mL in the drinking water), as indicated, starting from day 10 to the end of the experiment. Tumor volumes after treatment are reported. *Points,* mean; *bars,* SE. **B,** *left*, ○ versus ● ($P = 0.0004$; two-tailed t test at day 14); **C,** *right,* □ versus ■ ($P = 0.023$; two-tailed t test at day 17).

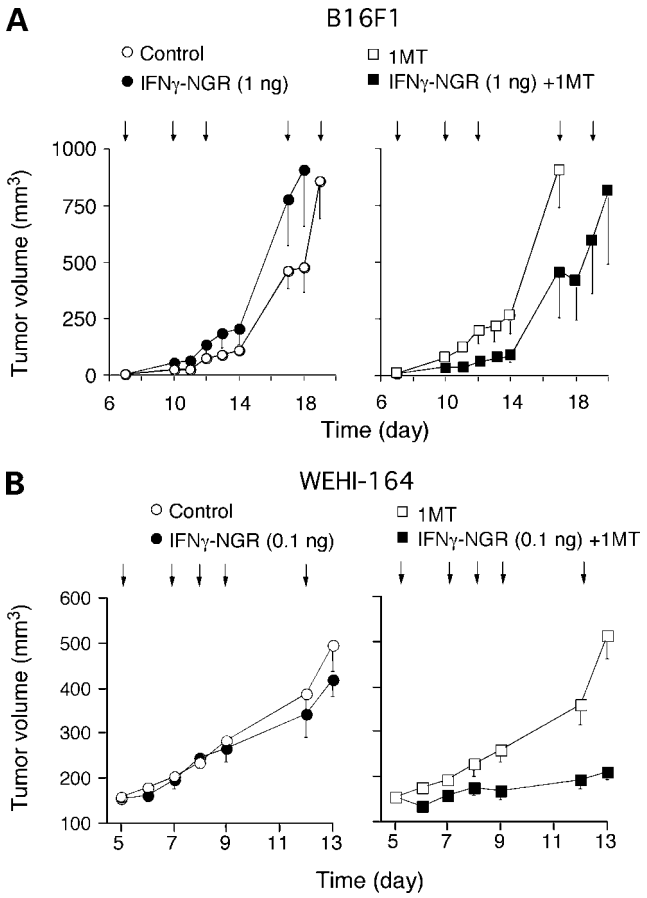

Figure 2. Effect of 1-MT on repeated treatments with IFNγ-NGR in B16F1 melanoma and WEHI-164 fibrosarcoma-bearing mice. Animals (5 mice per group) bearing B16F1-tumors were treated at the indicated days (*arrows*) with IFNγ-NGR (0.1 ng) in physiologic solution containing 0.1 mg/mL human serum albumin or with human serum albumin solution alone (*Control*, i.p.). Mice were given 1-MT (5 mg/mL in the drinking water), as indicated, starting from day 6 (*top*) or day 5 (*bottom*) to the end of the experiment. Tumor volumes after treatment are reported. *Points,* mean; *bars,* SE. **B,** *right*, □ versus ■ (P = 0.0005; two-tailed t test at day 13).

Thus, each cell line was cultured for 4 d in the presence of 0, 8, 40, and 200 μg/mL 1-MT in combination with various doses of IFNγ-NGR (0, 1, 10, and 100 ng/mL). Proliferation of RMA cells was affected by 1-MT alone in a dose-dependent manner, whereas proliferation of WEHI-164 and B16F1 cells was little or not at all affected (Fig. 4, *left*). Conversely, IFNγ-NGR alone could inhibit B16F1 proliferation in a dose-dependent manner; however, it has little or no effect on RMA and WEHI-164 cells (Fig. 4). Whereas a modest additive effect was observed with B16F1 cells treated with a mixture of 200 ng/mL 1-MT and 100 ng/mL IFNγ-NGR, no synergistic effects were observed with any cell line tested and with all combinations. These results suggest that the synergism observed *in vivo* depends on host components.

To investigate the importance of the immune system in the host-mediated response to IFNγ-NGR/1-MT, we tested these drugs in CD1 *nu/nu* RMA tumor-bearing mice, lacking functional T cells. Because tumors grew faster in *nu/nu* mice compared with immunocompetent C57BL6 mice, we started the treatment at day 8 instead of day 10. No significant response was observed even after repeated treatment with IFNγ-NGR and 1-MT, either alone or in combination (Fig. 5A). This result supports the concept that the immune system plays a crucial role in the antitumor response observed in immunocompetent mice treated with this drug combination.

To further test this hypothesis, we performed an additional experiment using BALB/c mice bearing WEHI-164 tumors, pretreated with anti-CD4 and anti-CD8 monoclonal antibodies. This treatment completely depleted CD4 and CD8 T cells (as checked by fluorescence-activated cell sorting analysis of circulating cells with specific antibodies; Fig. 5B), induced a more rapid growth of s.c. tumors (Fig. 5C), and inhibited the antitumor response to repeated treatment with IFNγ-NGR/1MT (Fig. 5C). These results altogether suggest that the inhibitory effect of IDO, caused by repeated treatment with IFNγ-NGR, is primarily related to the T-cell function.

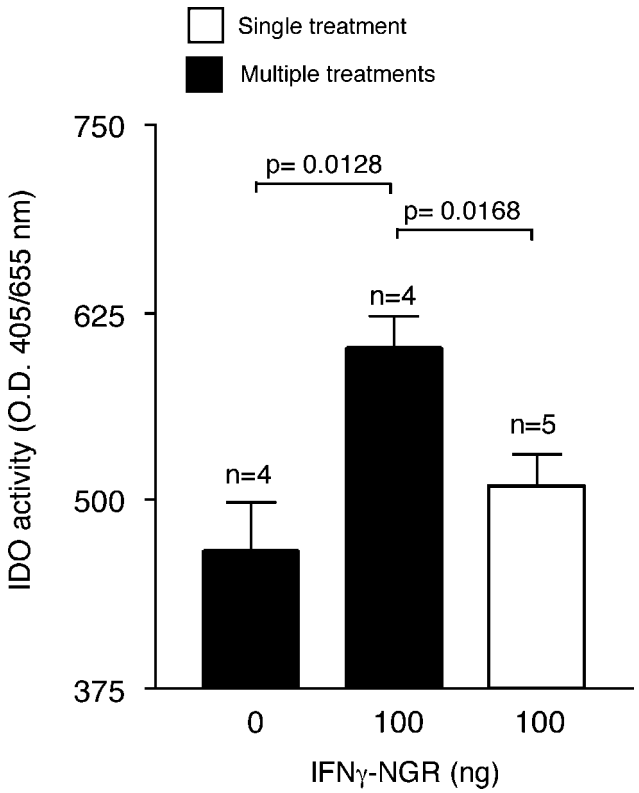

Figure 3. Induction of IDO activity in RMA tumors after repeated treatments with IFNγ-NGR. C57BL6/N mice bearing RMA tumors (4-5 mice per group as indicated) were treated with IFNγ-NGR (0.1 ng) 5 times (at day 10, 11, 12, 13, and 14) or once (at day 14) after tumor implantation. Two hours after the last treatment, animals were sacrificed and tumors were excised. IDO activity was measured in tumor tissue extracts (0.36 g of tissue/mL of PBS) as in Materials and Methods.

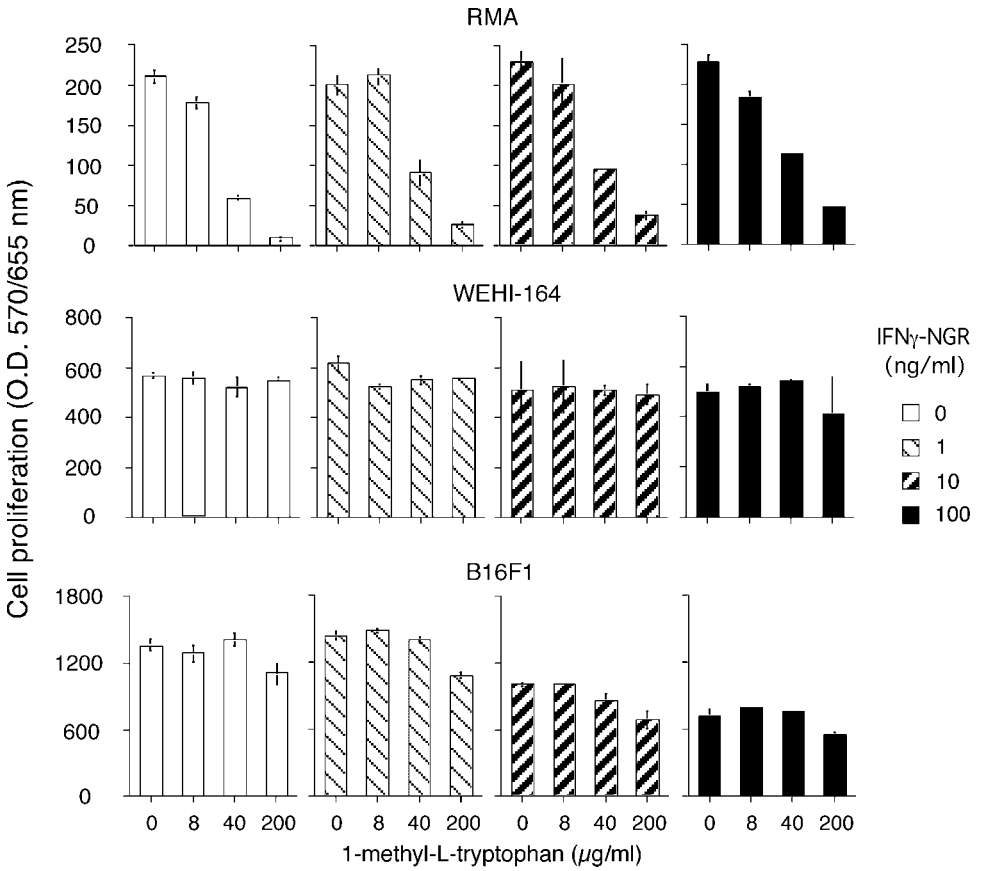

Figure 4. Effect of 1-MT and IFNγ-NGR, alone and in combination, on the proliferation of cultured RMA, WEHI-164, and B16F1 cells *in vitro*. Tumor cell lines were cultured for 4 d in cell culture medium containing various doses of 1-MT and IFNγ-NGR. Cell proliferation was detected by staining with MTT as in Materials and Methods.

Tumor Resistance to Repeated Doses of IFNγ-NGR Is Related to the IFNγ Domain and not to the NGR Domain

IFNγ-NGR is composed of two bioactive domains: the IFNγ domain, which can interact with IFNγ receptor, and the CNGRC targeting domain, which can interact with CD13 (22).

To assess the role of the CNGRC domain in the induction of IDO, we studied the effect of 1-MT on NGR-TNF, a different CNGRC-cytokine conjugate. Repeated treatments of RMA-bearing mice with NGR-TNF significantly delayed tumor growth (Fig. 6). This finding is in line with previous studies showing that low doses of NGR-TNF can affect the growth of this tumor (36). When 1-MT was added to the treatment, no enhancement of the antitumor activity of NGR-TNF was observed, even when NGR-TNF was given daily. This suggests that neither the TNF domain nor the CNGRC domain could activate this counterregulatory mechanism and supports the hypothesis that tumor resistance to IFNγ-NGR was primarily related to the IFNγ domain.

Discussion

We have previously shown that the dose-response curve of IFNγ-NGR in the WEHI-164 murine model is bell shaped and that attempts to increase its antitumor effects by increasing the dose or by frequent administration paradoxically results in decreased activity (22). The results of the present work confirm these findings in three different models, including RMA lymphomas, B16F1 melanomas, and WEHI-164 fibrosarcomas, and provide evidence to suggest that the potential antitumor activity of frequently repeated doses of IFNγ-NGR is overwhelmed by counterregulatory mechanisms. The following observations suggest that one crucial counterregulatory mechanism is related to induction of IDO: First, measurement of IDO activity in tumor extracts after repeated treatment with IFNγ-NGR showed a significant increase of enzyme activity, compared with single treatment; second, coadministration of IFNγ-NGR with 1-MT, an inhibitor of IDO, induced antitumor effects against RMA lymphomas, B16F1 melanomas, and WEHI-164 fibrosarcomas even when the cytokine was given repeatedly. Remarkably, in the presence of 1-MT, the antitumor effects of the repeated treatment schedule were stronger than those obtained with a single treatment.

IDO is an extrahepatic enzyme that catalyzes the initial and rate-limiting step of the catabolism of tryptophan, an essential amino acid. High levels of IDO activity, which is present in many mammalian organs, have been observed in

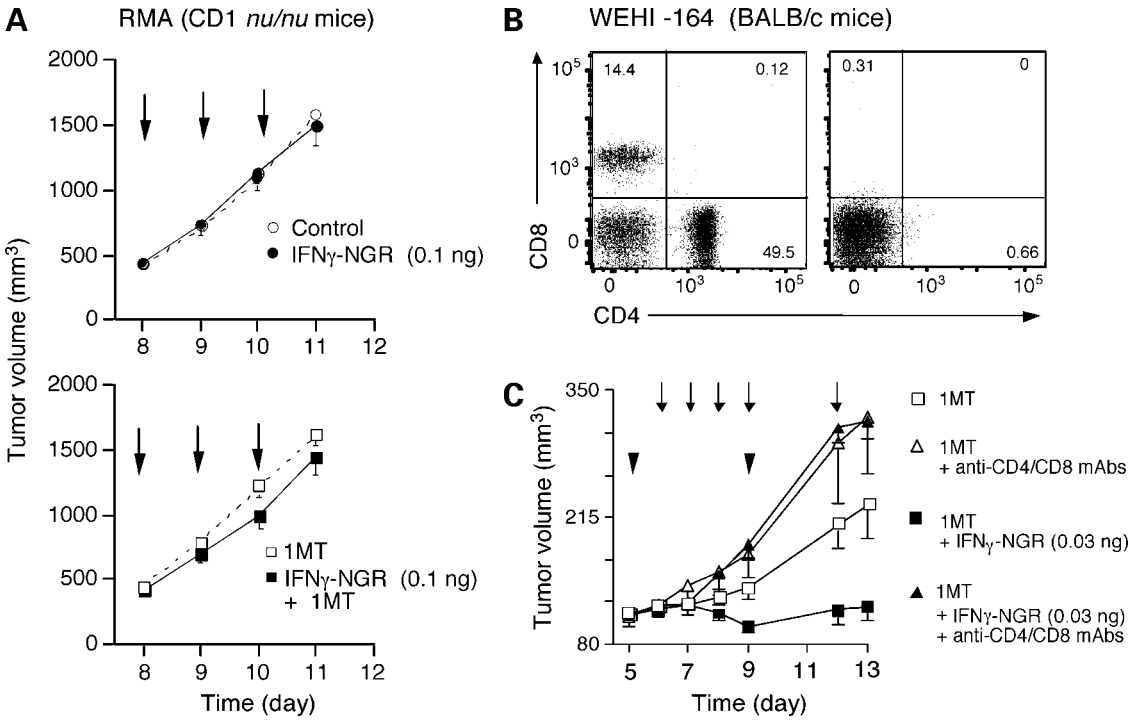

Figure 5. Antitumor effect of 1-MT on repeated treatments with IFNγ-NGR in *nu/nu* mice and in CD4/CD8 T-cell–depleted mice. **A,** CD1 *nu/nu* mice (5 per group) bearing RMA tumors were treated at the indicated days (*arrows*) with or without IFNγ-NGR (0.1 ng). Mice were given 1-MT (5 mg/mL in the drinking water), as indicated, starting from day 7 to the end of the experiment. BALB/c mice (6 per group) bearing WEHI-164 tumors were treated at days 5 and 9 with anti-CD4 (GK1.5) and anti-CD8 (53-6.72) T-cell–depleting monoclonal antibodies (125 μL of ascitic fluid, i.v.; *arrowheads*). **B,** fluorescence-activated cell sorting analysis of circulating cells stained with anti-CD4 (L3T4) and anti-CD8 (Ly-2) specific antibodies 24 h after treatment with (*right*) or without (*left*) antibodies. **C,** at days 6, 7, 8, 9, and 12, mice were treated with IFNγ-NGR (0.03 ng), with or without 1-MT as indicated. Tumor volumes are reported. *Points,* mean; *bars,* SE.

the placenta, epididymis, and other sites of immune privilege (37–39). Because IDO is an IFNγ-inducible protein (40, 41), this enzyme has been historically considered part of the antitumor mechanism of IFNγ, as it may inhibit tumor cell proliferation by causing local tryptophan depletion and by producing toxic catabolites (34, 42–45). However, recent work has shown that IDO is overexpressed in most growing tumors (28). Furthermore, the notion that IDO can play a crucial role in peripheral tolerance suggests that this enzyme can be exploited by tumors to facilitate immune escape (27, 28). According to this view, tryptophan degradation by IDO inhibits accumulation of specific T lymphocytes at the tumor site, likely because T cells are much more sensitive than tumor cells to tryptophan shortage or to cytotoxic effects caused by tryptophan catabolites (28). Remarkably, endothelial cells have also been reported to express IDO in response to stimulation with IFNγ (46). IDO expression in antigen-presenting cells in tumor-draining lymph nodes can also contribute to immune escape (34). Thus, IFNγ is a two-faced cytokine that can induce antiproliferative, angiostatic, and immunomodulatory anticancer effects and may favor tumor immune escape by inducing IDO expression. This ''balance'' is likely regulated by the time of exposure to IFNγ, its site of action, its local concentration, and, possibly, also by the presence or absence of other cytokines, which may vary in different tumors.

Based on these notions and on our finding that 1-MT can enhance the tumor response to repeated treatments with IFNγ-NGR in different murine models, we hypothesize that frequent treatments of tumors with this cytokine can lead to excessive stimulation of tryptophan catabolism, tipping the balance toward inhibition of potential antitumor immune mechanisms triggered by IFNγ-NGR. The hypothesis that multiple treatments with IFNγ-NGR in combination with 1-MT can activate an antitumor immune response is supported by the observation that no response occurred in *nu/nu* mice bearing RMA lymphomas or in immunocompetent BALB/c mice pretreated with anti-CD4 and anti-CD8 T-cell–depleting monoclonal antibodies. The lack of response in these models also suggests that direct cytotoxic effects on tumor cells play a minor role. This view is also supported by the lack of synergism between IFNγ-NGR and 1-MT in *in vitro* tumor cell proliferation assays. Thus, the synergistic antitumor effect observed *in vivo* with immunocompetent mice is likely the result of a complex interplay between IFNγ-NGR/1-MT and host components of the tumor, which may include endothelial cells, myeloid-derived cells, T cells, and, possibly, other stromal cells, rather than direct cytotoxic/antiproliferative effects on tumor cells.

IFNγ-NGR is composed of the IFNγ domain, which can interact with IFNγ receptor, and the CNGRC-targeting

domain, which can interact with aminopeptidase N (CD13). CD13 is a membrane protease expressed by endothelial cells of angiogenic vessels and other cells within the tumor stroma (22, 24, 47). This raises the question as to whether CNGRC plays a role in the synergism with 1-MT. The results of experiments comparing the effect of repeated administration of CNGRC fused to IFNγ or TNF showed that synergism with 1-MT occurs only with IFNγ-NGR. This suggests that induction of IDO-dependent counterregulatory mechanisms was primarily related to stimulation of the IFNγ receptor and not of the CNGRC receptor. On the other hand, the results of experiments carried out with low-dose IFNγ and 1-MT, showing considerably lower activity compared with IFNγ-NGR/1-MT, suggest that the NGR domain plays a critical role in the antitumor activity of this combination. The simplest explanation is that CNGRC is critical for delivering IFNγ to tumors in sufficient amounts for activating antitumor mechanisms.

Another question that deserves to be addressed is how targeting the vasculature in the presence of 1-MT results in an antitumor effect that seems to be almost totally dependent on a functional immune system. One possibility is that low-dose IFNγ-NGR, after interacting with tumor vasculature and/or other CD13-positive cells in the tumor stroma, activates a cascade of events leading to induction of secondary cytokines and activation of an immune response. Alternatively, cells of the immune system are necessary for the production of cytokines or factors that are critical for the pleiotropic activity of IFNγ. These mechanisms are not mutually exclusive.

In conclusion, the results of the present study show that repeated and frequent administrations of IFNγ-NGR to tumor-bearing mice activate counterregulatory mechanisms based on the induction of IDO. This finding provides an explanation for the paradoxical lower response to repeated administration of IFNγ-NGR compared with single administration. Development of IDO inhibitors with high p.o. bioavailability or with different selectivity for different IDO-related enzymes expressed by tumor cells and by dendritic cells is currently pursued for clinical applications (48, 49). Considering that IFNγ can potentially induce a wide variety of favorable anticancer effects together with unfavorable mechanisms, such as induction of IDO, coadministration of IDO inhibitors with IFNγ-NGR could represent a novel strategy for shifting the equilibrium toward favorable effects.

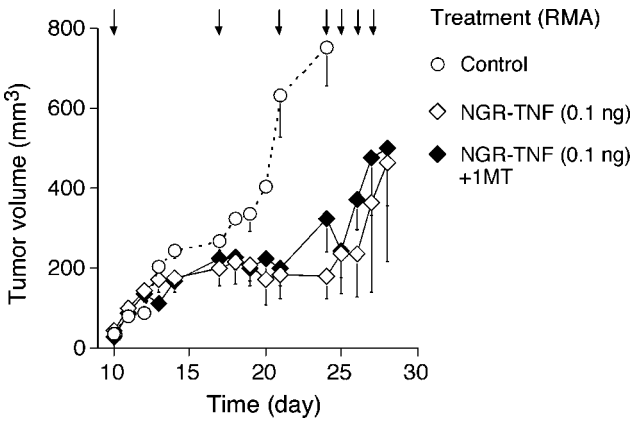

Figure 6. Effect of 1-MT on repeated treatments with NGR-TNF in RMA lymphoma–bearing mice. C57BL6/N mice (5 per group) bearing RMA tumors were treated at the indicated days (*arrows*) with NGR-TNF (0.1 ng) and with or without 1-MT (5 mg/mL in the drinking water). Tumor volumes after treatment are reported. *Points,* mean; *bars,* SE.

Disclosure of Potential Conflicts of Interest

G-P. Rizzardi and C. Traversari are employees of MolMed SpA; A. Corti is a consultant to MolMed SpA for the development of IFNγ-NGR. No other potential conflicts of interest were disclosed.

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