

# Critical role of indoleamine 2,3-dioxygenase in tumor resistance to repeated treatments with targeted IFN $\gamma$

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## Abstract

Targeted delivery of IFN $\gamma$  to tumors has been achieved by fusing this cytokine with GCNGRC, a tumor neovascularization homing peptide. Although the therapeutic efficacy of this protein (called IFN $\gamma$ -NGR) in animal models is greater than that of IFN $\gamma$ , frequent administrations of IFN $\gamma$ -NGR may result in lower efficacy and tumor resistance. We investigated the role of indoleamine 2,3-dioxygenase (IDO), an IFN $\gamma$ -inducible enzyme that may down-regulate T cells by affecting local tryptophan catabolism in tumor resistance to repeated treatments with IFN $\gamma$ -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 IFN $\gamma$ -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 IFN $\gamma$ -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 IFN $\gamma$ -NGR, likely causing excessive stimulation of tryptophan catabolism and inhibiting antitumor immune mechanisms. Coadministration of IFN $\gamma$ -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 IFN $\gamma$  make this cytokine an attractive anticancer agent (1–4). For instance, IFN $\gamma$  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<sup>+</sup> T helper cells (11, 12). This cytokine can also inhibit proliferation of many tumor cell types (8). IFN $\gamma$  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, IFN $\gamma$  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 IFN $\gamma$  could actually result in higher toxicity and lower efficacy (16–21).

We have previously shown that targeted delivery of low doses of IFN $\gamma$  to tumor vasculature can overcome major counterregulatory mechanisms and delay tumor growth in murine models that respond poorly to IFN $\gamma$  (22). Vascular targeting has been achieved by fusing IFN $\gamma$  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 IFN $\gamma$ -GCNGRC conjugate (called IFN $\gamma$ -NGR) is bell shaped with maximal effects being induced with a dose of 0.005  $\mu$ g/kg, whereas nontargeted IFN $\gamma$  induces little or no effect over a range of 0.003 to 250  $\mu$ g/kg in these models (22). Evidence was also obtained to suggest that excessive stimulation of IFN $\gamma$  receptors by high-dose IFN $\gamma$  or by frequent administration of low doses of IFN $\gamma$ -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 IFN $\gamma$ . IDO is an IFN $\gamma$ -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 IFN $\gamma$ -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 IFN $\gamma$ .

## Materials and Methods

### Cell Lines and Reagents

Murine RMA lymphoma (29) and B16/F1 melanoma cells were cultured as described previously (30). Recombinant murine IFN $\gamma$  was from PeproTech. Murine IFN $\gamma$ -NGR (IFN $\gamma$ <sub>4-135</sub> fused with the NH<sub>2</sub> 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  $7 \times 10^4$  RMA or B16F1 living cells; 6 to 10 d later, mice were treated with IFN $\gamma$ -NGR solutions, i.p. (100  $\mu$ L), diluted with 0.9% sodium chloride containing 100  $\mu$ g/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  $\pm$  SE (5 animals per group).

### In vitro Proliferation Assay

RMA cells ( $5 \times 10^3$ /well) were plated in RPMI 1640 containing 10% fetal bovine serum, 100 units/mL penicillin, 100  $\mu$ g/mL streptomycin, IFN $\gamma$ -NGR, and 1-MT at various concentrations (200  $\mu$ L/well) and incubated for 4 d at 37°C, 5% CO<sub>2</sub>. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (10  $\mu$ L, 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  $\mu$ L/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  $\mu$ L 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  $\mu$ mol/L methylene blue, 200 units/mL catalase, 63  $\mu$ L Dulbecco's PBS without Ca<sup>2+</sup> and Mg<sup>2+</sup> (BioWhittaker) was mixed with 63  $\mu$ L 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  $\mu$ L of 30% trichloroacetic acid. The mixture was then incubated at 50°C for 30 min to hydrolyze *N*-formylkynurenine to kynurenine. The product was centrifuged at 12,000  $\times$  *g* for 20 min and the supernatant (120  $\mu$ L) was mixed with 1% (w/v) *p*-dimethylaminobenzaldehyde in 1% acetic acid (120  $\mu$ L). 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 IFN $\gamma$ -NGR in RMA Lymphoma, B16F1 Melanoma, and WEHI-164 Fibrosarcoma Models

We have previously shown that a single administration of IFN $\gamma$ -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 IFN $\gamma$ -NGR in immunocompetent mice bearing RMA lymphomas. Administration of IFN $\gamma$ -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 IFN $\gamma$ -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 CNR domain in the synergism observed between IFN $\gamma$ -NGR and 1-MT, we analyzed the antitumor properties of repeated doses (0.1 ng) of normal IFN $\gamma$ , lacking the NGR domain, in combination 1-MT. IFN $\gamma$  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 IFN $\gamma$ -NGR/1-MT combination, likely for delivering low doses of IFN $\gamma$  to tumors.

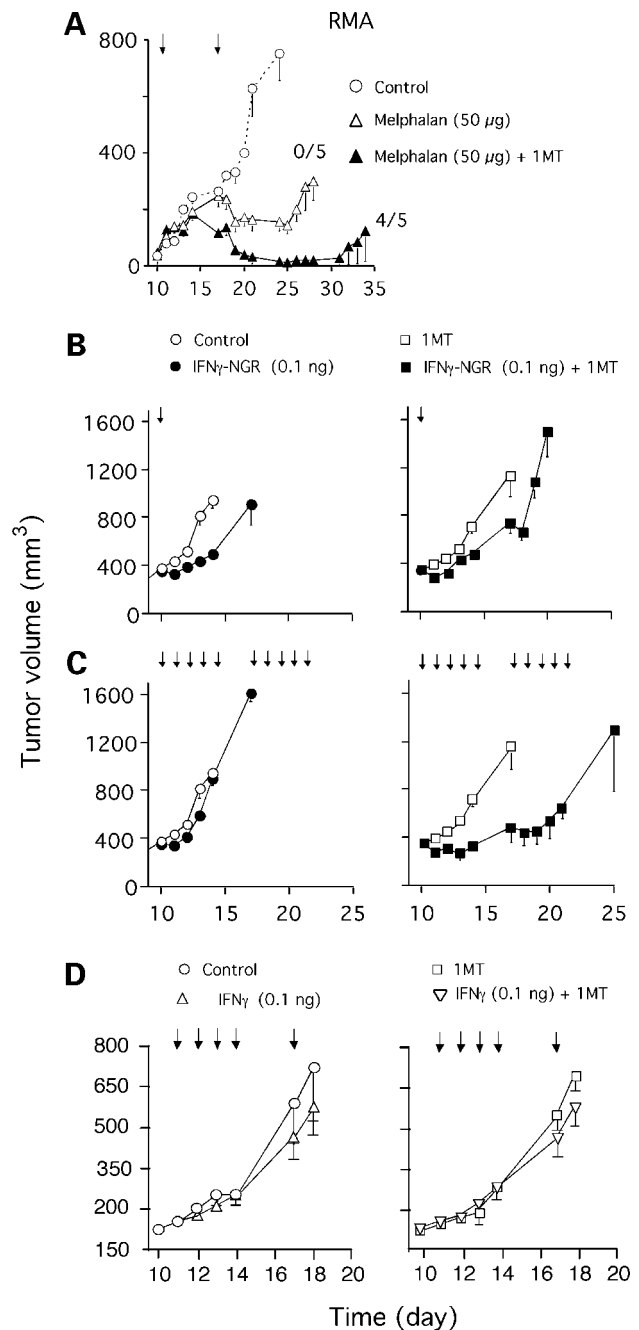
We then investigated whether 1-MT could enhance the response to repeated treatments with IFN $\gamma$ -NGR in other models and with different administration schedules. We treated B16F1 melanoma-bearing mice with a higher dose of IFN $\gamma$ -NGR (1 ng), five times, with or without 1-MT. Treatment with IFN $\gamma$ -NGR alone was totally ineffective and apparently promoted faster growth of tumors (Fig. 2A, left). However, IFN $\gamma$ -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 IFN $\gamma$ .

#### Repeated Administrations of IFN $\gamma$ -NGR Increase IDO Activity in RMA Lymphoma Tumors

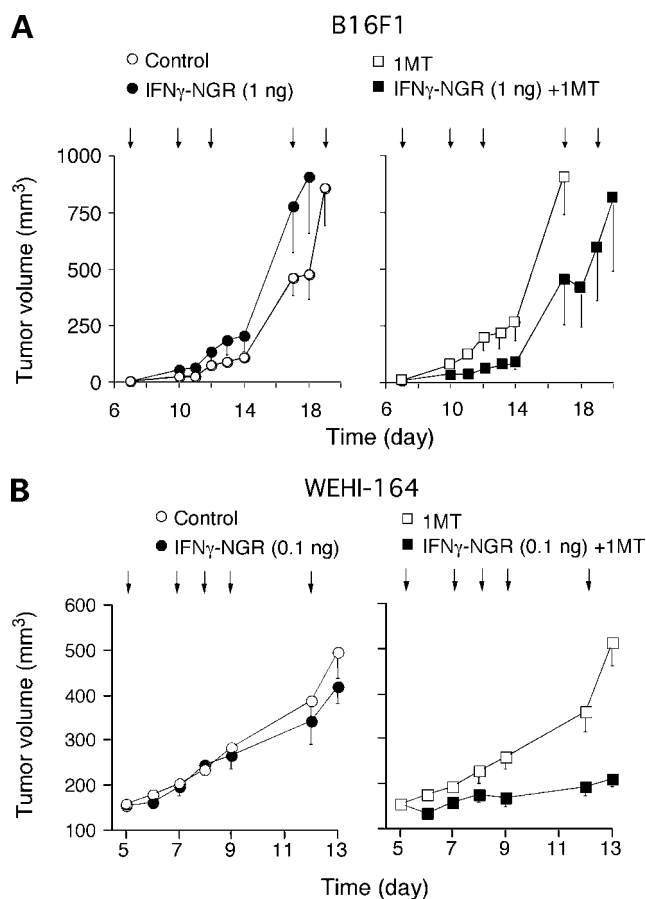
To assess the hypothesis that repeated treatments with IFN $\gamma$ -NGR lead to induction of IDO in tumors, we measured the levels of IDO activity in RMA tumor extracts after treatment. To this aim, we treated tumor-bearing mice with 100  $\mu$ g of IFN $\gamma$ -NGR five times, everyday, and analyzed 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 IFN $\gamma$ -NGR administrations.

#### The Antitumor Activity of 1-MT/IFN $\gamma$ -NGR Combination Is Host Mediated

We then addressed the question whether the potentiation of antitumor activity of IFN $\gamma$ -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 IFN $\gamma$ -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  $\mu$ mol/L (i.e.,  $\sim$ 45  $\mu$ g/mL; ref. 28).



**Figure 1.** Effect of 1-MT on the antitumor activity of melphalan, IFN $\gamma$ -NGR, or IFN $\gamma$  in RMA lymphoma-bearing mice. **A**, animals (5 mice per group) bearing RMA tumors were treated at the indicated day (arrows) with melphalan (50  $\mu$ g 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 IFN $\gamma$ -NGR (0.1 ng; **B** and **C**) or IFN $\gamma$  (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).



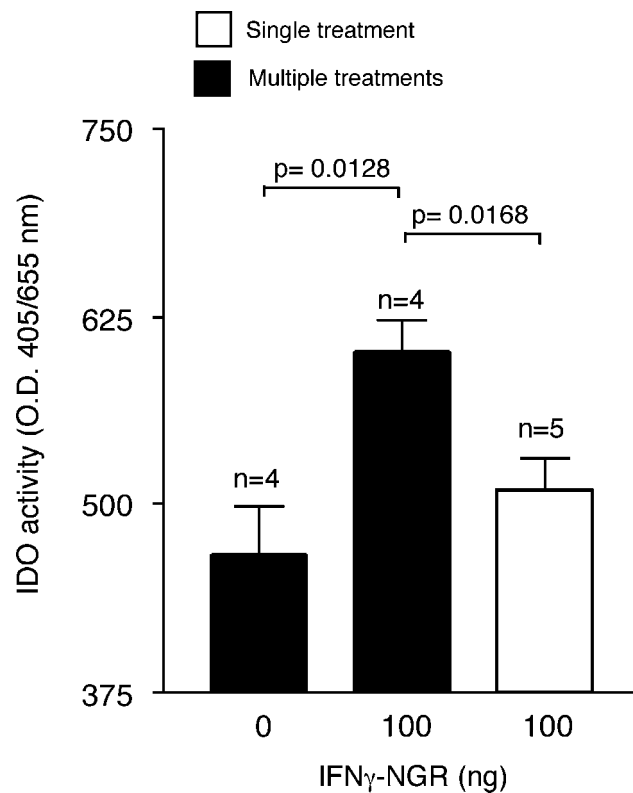
**Figure 2.** Effect of 1-MT on repeated treatments with IFN $\gamma$ -NGR in B16F1 melanoma and WEHI-164 fibrosarcoma-bearing mice. Animals (5 mice per group) bearing B16F1-tumors were treated at the indicated days (arrows) with IFN $\gamma$ -NGR (0.1 ng) in physiologic solution containing 0.1 mg/mL 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**,  $\square$  versus  $\blacksquare$  ( $P = 0.0005$ ; 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  $\mu$ g/mL 1-MT in combination with various doses of IFN $\gamma$ -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, IFN $\gamma$ -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 IFN $\gamma$ -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 IFN $\gamma$ -NGR/1-MT, we tested

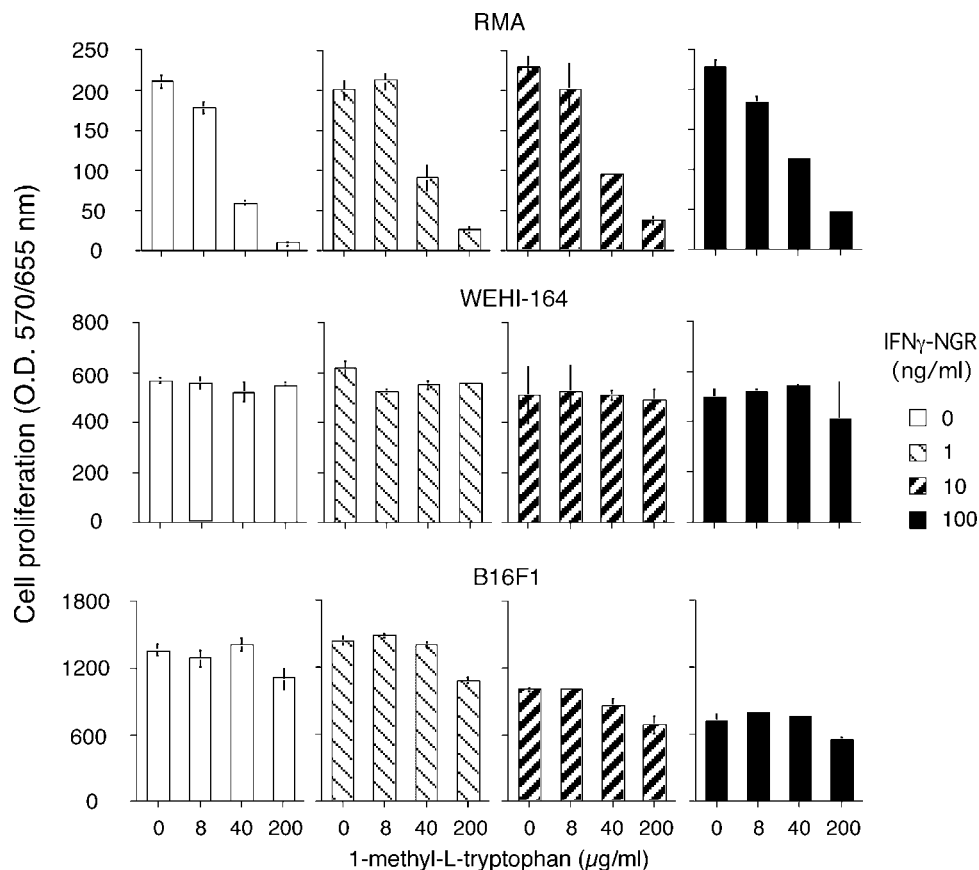
these drugs in CD1 *nu/nu* RMA tumor-bearing mice, lacking functional T cells. Because tumors grew faster in *nu/nu* mice compared with immunocompetent C57BL/6 mice, we started the treatment at day 8 instead of day 10. No significant response was observed even after repeated treatment with IFN $\gamma$ -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 IFN $\gamma$ -NGR/1MT (Fig. 5C). These results altogether suggest that the inhibitory effect of IDO, caused by repeated treatment with IFN $\gamma$ -NGR, is primarily related to the T-cell function.



**Figure 3.** Induction of IDO activity in RMA tumors after repeated treatments with IFN $\gamma$ -NGR. C57BL/6/N mice bearing RMA tumors (4-5 mice per group as indicated) were treated with IFN $\gamma$ -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 IFN $\gamma$ -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 IFN $\gamma$ -NGR. Cell proliferation was detected by staining with MTT as in Materials and Methods.

#### Tumor Resistance to Repeated Doses of IFN $\gamma$ -NGR Is Related to the IFN $\gamma$ Domain and not to the NGR Domain

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

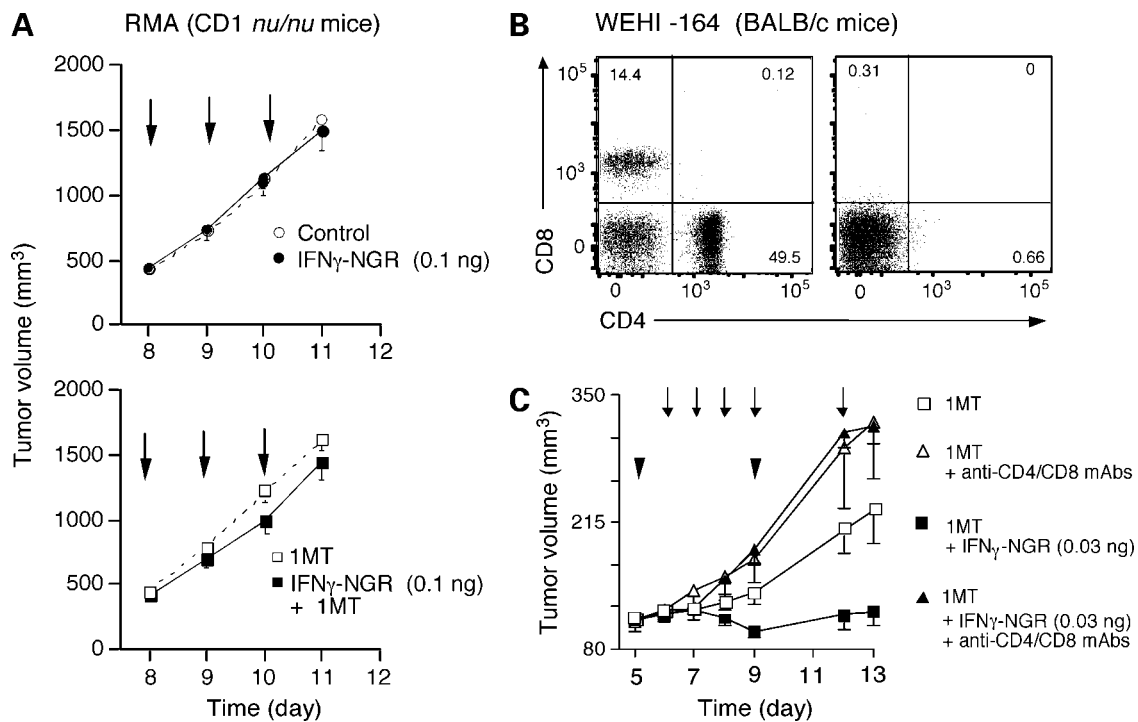
To assess the role of the CNGRC domain in the induction of IDO, we studied the effect of 1-MT on NGR-TNF, a different CNGRC-cytokine conjugate. Repeated treatments of RMA-bearing mice with NGR-TNF significantly delayed tumor growth (Fig. 6). This finding is in line with previous studies showing that 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 IFN $\gamma$ -NGR was primarily related to the IFN $\gamma$  domain.

#### Discussion

We have previously shown that the dose-response curve of IFN $\gamma$ -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 IFN $\gamma$ -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 IFN $\gamma$ -NGR showed a significant increase of enzyme activity, compared with single treatment; second, coadministration of IFN $\gamma$ -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 IFN $\gamma$ -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 IFN $\gamma$ -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  $\mu$ L 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 IFN $\gamma$ -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 IFN $\gamma$ -inducible protein (40, 41), this enzyme has been historically considered part of the antitumor mechanism of IFN $\gamma$ , 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 IFN $\gamma$  (46). IDO expression in antigen-presenting cells in tumor-draining lymph nodes can also contribute to immune escape (34). Thus, IFN $\gamma$  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 IFN $\gamma$ , 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 IFN $\gamma$ -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 IFN $\gamma$ -NGR. The hypothesis that multiple treatments with IFN $\gamma$ -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 IFN $\gamma$ -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 IFN $\gamma$ -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.

IFN $\gamma$ -NGR is composed of the IFN $\gamma$  domain, which can interact with IFN $\gamma$  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 IFN $\gamma$  or TNF showed that synergism with 1-MT occurs only with IFN $\gamma$ -NGR. This suggests that induction of IDO-dependent counterregulatory mechanisms was primarily related to stimulation of the IFN $\gamma$  receptor and not of the CNGRC receptor. On the other hand, the results of experiments carried out with low-dose IFN $\gamma$  and 1-MT, showing considerably lower activity compared with IFN $\gamma$ -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 IFN $\gamma$  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 IFN $\gamma$ -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 IFN $\gamma$ . These mechanisms are not mutually exclusive.

In conclusion, the results of the present study show that repeated and frequent administrations of IFN $\gamma$ -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 IFN $\gamma$ -NGR com-

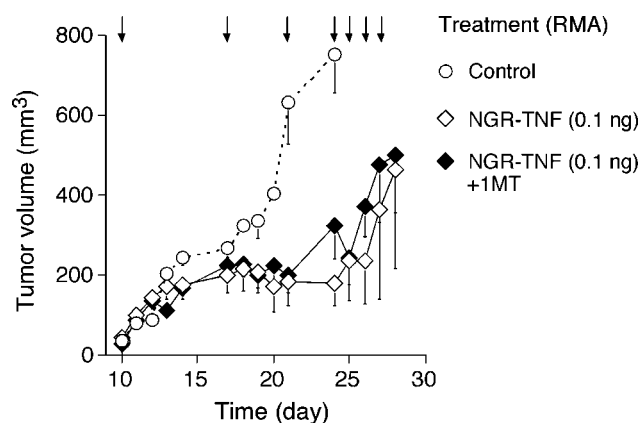
pared with single administration. Development of IDO inhibitors with high p.o. bioavailability or with different selectivity for different IDO-related enzymes expressed by tumor cells and by dendritic cells is currently pursued for clinical applications (48, 49). Considering that IFN $\gamma$  can potentially induce a wide variety of favorable anticancer effects together with unfavorable mechanisms, such as induction of IDO, coadministration of IDO inhibitors with IFN $\gamma$ -NGR could represent a novel strategy for shifting the equilibrium toward favorable effects.

## 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 IFN $\gamma$ -NGR. No other potential conflicts of interest were disclosed.

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**Figure 6.** Effect of 1-MT on repeated treatments with NGR-TNF in RMA lymphoma-bearing mice. C57BL/6/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.

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