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Interferon γ Enhances the Effectiveness of Tumor Necrosis Factor-Related Apoptosis–Inducing Ligand Receptor Agonists in a Xenograft Model of Ewing's Sarcoma

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ABSTRACT

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) induces selective apoptosis in a variety of tumors, including most cell lines derived from Ewing's sarcoma family of tumors, an aggressive sarcoma that afflicts children and young adults. To determine the *in vivo* efficacy of TRAIL receptor agonists in Ewing's sarcoma family of tumors, mice with orthotopic xenografts were treated with anti-TRAIL-R2 monoclonal antibody or TRAIL/Apo2L in a model that can identify effects on both primary tumors and metastases. Administration of either agonist slowed tumor growth in 60% of animals and induced durable remissions in 11 to 19% but did not alter the incidence of metastatic disease. Response rates were not improved by concurrent doxorubicin treatment. Cells recovered from both TRAIL receptor agonist–treated and nontreated tumors were found to be resistant to TRAIL-induced death *in vitro* unless pretreated with interferon (IFN) γ. This resistance coincided with a selective down-regulation of TRAIL receptor expression on tumor cells. *In vivo* treatment with IFNγ increased tumor expression of TRAIL receptors and caspase 8, but did not increase the antitumor effect of TRAIL receptor agonists on primary tumors. However, IFNγ treatment alone or in combination with a TRAIL receptor agonist significantly decreased the incidence of metastatic disease and the combination of TRAIL receptor agonist therapy with IFNγ-mediated impressive effects on both primary tumors and metastatic disease. These data demonstrate that *in vivo* growth favors TRAIL resistance but that TRAIL receptor agonists are active in Ewing's sarcoma family of tumors and that the combination of TRAIL receptor agonists with IFNγ is a potent regimen in this disease capable of controlling both primary and metastatic tumors.

INTRODUCTION

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), a tumor necrosis factor family member, plays an important role in defense against viral and neoplastic disease (1–4). Although systemic toxicity has limited the therapeutic use of other members of this superfamily (Fas and tumor necrosis factor), TRAIL demonstrates a higher degree of tumor specificity, raising the prospect of clinical use of TRAIL receptor agonists as tumor-specific agents. TRAIL is a type II transmembrane protein that, after cleavage from the cell membrane, forms a zinc-coordinated homotrimer that induces caspase-dependent apoptosis through two death domain–containing receptors, TRAIL-R1 (TR1) or TRAIL-R2 (TR2; refs. 5 and 6). A wide variety of tumors are sensitive to TRAIL-mediated apoptosis *in vitro*, including cell lines established from Ewing's sarcoma family of tumors (7–10), rhabdomyosarcoma (11), neuroblastoma (12, 13), osteosarcoma (14), lymphoma (15), leukemia (16), melanoma (17), and colon and breast carcinoma (3). Interestingly, although many tumors demonstrate exquisite sensitivity to TRAIL receptor agonists *in vitro*, preclinical studies using TRAIL receptor agonists as single agents in *in vivo* tumor models have demonstrated more moderate antitumor effects (6, 18). As a result, several agents have been studied for their ability to enhance the antitumor effects of TRAIL receptor agonists *in vivo* (19–21). Treatment of tumor-bearing animals with doxorubicin, etoposide, cisplatin, or ionizing radiation results in increased surface expression of TR1 and TR2 on tumor cells, and these agents have enhanced the *in vivo* effectiveness of TRAIL receptor agonists in several models presumably via this mechanism (8). However, TRAIL resistance can also result from a variety of other changes including decreased caspase 8 expression (7, 12), increased nuclear factor κB activity, and up-regulation of c-FLIP (22), to name a few. Thus, the development of rational combination therapies that can target multiple mediators of the TRAIL receptor signaling pathways is important to increase the efficacy of TRAIL receptor–based therapies.

Interferon (IFN) γ exerts multiple pro-apoptotic effects on cells through JAK1 or JAK2/STAT1 signaling and subsequent induction of IFN-sensitive genes (23). Several points of convergence have been described between IFN-sensitive genes and the TRAIL-mediated apoptosis pathway, including mediators that play a role in the TRAIL resistance described above. For example, IFNγ increases the expression of apical caspases and can sensitize caspase 8–deficient, TRAIL-resistant Ewing's sarcoma family of tumors and neuroblastoma cell lines *in vitro* (12). Furthermore, TR1, TR2, and TRAIL itself are among the many IFN-sensitive genes up-regulated by IFNγ (24, 25). Thus, evidence from a variety of *in vitro* model systems suggests that IFNγ or agents that induce its production (*i.e.*, IL18 or IL12) might enhance sensitivity to TRAIL agonists.

The studies reported herein address the *in vivo* efficacy of TRAIL receptor agonists in Ewing's sarcoma family of tumors, one of several pediatric malignancies that are highly susceptible to TRAIL-based therapies *in vitro* (7–10). Despite exquisite *in vitro* sensitivity, TRAIL receptor agonists as single agents demonstrated only moderate activity *in vivo*. Using explants from Ewing's sarcoma family of tumors xenografts, we demonstrate that tumors growing *in vivo* acquire TRAIL resistance, which is associated with down-regulation of TRAIL receptors but which can be overcome using IFNγ. Furthermore, combining TRAIL receptor agonist therapy with IFNγ enhanced the effectiveness of both agents. These results demonstrate that acquired TRAIL resistance *in vivo* is an important obstacle to the clinical efficacy of TRAIL receptor agonists and that combining TRAIL receptor agonists with therapies that up-regulate intracellular mediators of the TRAIL pathway and the surface expression of death domain–containing TRAIL receptors may be critical to fully realize the clinical potential of this therapy.

MATERIALS AND METHODS

Cell Lines, Flow Cytometry, and Apoptosis Assays. Ewing's sarcoma family of tumors cell lines used in this study include the previously reported lines TC71, TC32, RD-ES, 5838, A4573, EWS-925, NCI-EWS-94, NCI-EWS-95, NCI-EWS-011, and NCI-EWS-021 (26). Cell lines were maintained *in*

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vitro in RPMI 1640 supplemented with 2 mmol/L L-glutamine, penicillin (100 U/mL)-streptomycin (100 μg/mL), and 10% fetal calf serum (Life Technologies, Inc., Gaithersburg, MD). For apoptosis assays, cells were plated for 24 hours to allow adherence before addition of 200 μg/mL Apo2L/TRAIL (Genentech, San Francisco, CA) or 50 μg/mL anti-TR2 monoclonal antibody (mAb; M413; Amgen, Thousand Oaks, CA). Cell growth was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay (Sigma, St. Louis, MO) at 8 to 72 hours after treatment. Viability was determined by trypan blue exclusion and by propidium iodide staining by flow cytometry. Nuclear morphology was shown by staining the cells in 10 mmol/L Hoechst33342 solution (Calbiochem, San Diego, CA) for 5 minutes at room temperature. Photomicrographs were taken on an inverted Nikon phase contrast and fluorescent microscope.

For flow cytometry, adherent cells were removed by treatment with trypsin + EDTA (Life Technologies, Inc.) and washed in fluorescence-activated cell-sorting buffer (PBS containing 2% bovine serum albumin and 0.1% NaN_3). Cells ($1–5 \times 10^5$) were stained with phycoerythrin-conjugated anti-DR4 or anti-DR5 (ebioscience, San Diego, CA), or fluorescein isothiocyanate–conjugated anti-CD99 (Caltag Laboratories, Burlingame, CA). Apoptosis was detected by staining with annexin V fluorescein isothiocyanate and 7AAD or propidium iodide in calcium-containing buffer (Alexis Platform, Läufelfingen, Switzerland). One- or two-color immunofluorescence was performed using a FACSCalibur (Becton Dickinson, Burlington, MA) and standard techniques. A minimum of 10,000 cells were acquired and analyzed using Cell Quest software.

Immunohistochemistry. Xenograft tumor tissues were formalin-fixed, placed in paraffin blocks, and sectioned to make individual slides. Paraffin sections of 5 μm were deparaffinized in xylene and rehydrated in alcohol. Endogenous peroxidase activity was quenched for 10 minutes at room temperature in methanol containing 1.5% hydrogen peroxide. After washing twice with water, sections were subjected to antigen retrieval by incubation in Dako Antigen Retrieval solution (pH 6.2; Dakocytomation, Carpinteria, CA) for 15 minutes in a microwave oven. The sections were washed in PBS and incubated for 1 hour with a blocking solution consisting of 10% normal goat serum (Vector Laboratories, Burlingame, CA) and 0.4% Tween 20 (Roche Diagnostics Corporation, Indianapolis, IN) in PBS at room temperature. The mouse monoclonal anti-caspase 8 antibody (Upstate Biotechnology, Lake Placid, NY) or primary antibodies anti-TR1 (5 μg/mL) and anti-TR2 (5 μg/mL; Immunex/Amgen, Seattle, WA) were applied at 4°C overnight 1:75. After washing, slides were incubated with goat antimouse immunoglobulins conjugated to peroxidase-labeled dextran polymer, developed with 3,3-diaminobenzidine (Dakocytomation) and counterstained with hematoxylin.

Xenograft Studies. Ewing's sarcoma family of tumors tumor cells were expanded *in vitro* to 75% confluence and harvested with trypsin + EDTA, then washed twice in PBS. Two million cells were injected in 100 μL of PBS into the gastrocnemeous muscle of 4- to 12-week-old SCID/bg mice (Taconic, Germantown, NY). A single primary tumor developed in >95% of mice over the ensuing 3 to 4 weeks. Two diameters of the tumor sphere were measured every 1 to 2 days with digital calipers. The tumor volume was approximated using the formula $(D \times d2/6) \times \pi$ (where D is the longer diameter and d is the shorter diameter). Lower extremity volumes without tumor are approximately 50 mm^3. Mice were treated with Apo2L/TRAIL (50 μg/kg/d), anti-TR2 mAb (5 μg/kg/d), or HBSS alone by intraperitoneal injections at either day 7 after tumor injection or when palpable tumor reached 100 mm^3. In selected experiments, higher doses of Apo2L/TRAIL (100 μg/kg/d) and anti-TR2 mAb (10 μg/kg/d) were administered. When tumors reached 1000 mm^3, they were resected as described previously (26), and animals were followed for an additional 3 to 6 months. During this period of minimal residual disease, animals were evaluated for the development of metastases in lung, bone/soft tissue, or abdomen of 75 to 100% of mice. Durable regression was defined as no evidence of tumor for >3 months. All studies were approved by the Animal Care and Use Committee of the National Cancer Institute, and all animal care was in accordance with NIH guidelines.

RNase Protection Assays. The RiboQuant Multi-Probe protection assay system (BD-PharMingen, Hamburg, Germany) was used according to manufacturer's instructions to determine mRNA levels in Ewing's sarcoma family of tumors cell lines at baseline and after treatment with IFNγ (2000 units/mL). An hApo3c probe set containing DNA templates for TRAIL, TR1, TR2, and caspase 8 was used for T7 polymerase direct synthesis of [α^{32}-P]UTP-labeled antisense RNA probes. Probes were hybridized with 5 μg of RNA isolated from Ewing's sarcoma family of tumors cell lines. Samples were then digested with RNase to remove nonhybridizing, single-stranded RNA. Remaining probes were resolved on denaturing 5% polyacrylamide gels.

TRAIL R2 Transfection. The coding sequence of TR2 cDNA was cloned into the expression vector pBSPTR1, which contains a puromycin-resistant selection marker. The expression of TR2 was driven by a tetracycline-repressible cytomegalovirus promoter (27). The TC71 Ewing's sarcoma cell line was transfected with pBSPTR1-TR2 using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). The transfected cells were maintained in 1.5 μg/mL tetracycline and selected in 0.4 μg/mL puromycin until isolated colonies were formed. The colonies were picked and cultured in 1.5 μg/mL tetracycline and 0.4 μg/mL puromycin for expansion. TR2-stable clones were selected by identifying clones with regulation of TR2 expression in the presence and absence of tetracycline (1.5 μg/mL) using both reverse transcription-PCR and flow cytometry.

Statistical Analysis. One-way ANOVA was performed using Graphpad Prism 3.0 software (Graphpad Software, Inc., San Diego, CA). Tumor growth curves were compared with a Bonferroni post-test to reduce the overall chance of a type one error (28). Data were considered statistically significant at $P < 0.05$. Kaplan–Meier survival curves were analyzed using a logrank test.

RESULTS

Cytotoxicity of Apo2L/TRAIL and Anti-TRAIL 2 Monoclonal Antibody *In vitro*. Previous reports have demonstrated that a variety of preparations of recombinant TRAIL constructs induce apoptosis in the vast majority of Ewing's sarcoma cell lines (7–10). As these agents simultaneously signal through TR1 and TR2, we began by establishing whether TR2 signaling alone was sufficient to induce apoptosis in Ewing's sarcoma. All cell lines tested expressed TR2 (Fig. 1*A*; see also ref. 7), and an agonistic mAb to this receptor induced apoptosis in the majority of Ewing's sarcoma family of tumors cell lines *in vitro* to a similar degree to that observed after treatment with Apo2L/TRAIL (Fig. 1*B*). Apoptosis was readily apparent within 1 day after treatment in nine of 11 Ewing's sarcoma family of tumors cell lines treated with either Apo2L/TRAILor anti-TR2 mAb (data not shown). A majority of cells were found to be apoptotic as early as 16 hours after the addition of Apo2L/TRAIL as evidenced by the Hoescht dye staining of TC71, a representative TRAIL-sensitive line (Fig. 1*C*). Therefore, agonistic binding to TR2 alone is sufficient to induce apoptosis in Ewing's sarcoma family of tumors cell lines *in vitro*, and we observed equal susceptibility of Ewing's sarcoma family of tumors cell lines in response to Apo2L/TRAIL (which ligates both TR1 and TR2 receptors) and anti-TR2.

TRAIL Receptor Agonists Impact Tumor Growth *In vivo*. To investigate the antitumor activity of TRAIL receptor agonists *in vivo*, xenograft tumors were established using TRAIL-sensitive Ewing's sarcoma family of tumors lines (TC71 or EWS-925) that routinely generate both primary and spontaneous metastatic tumors after intramuscular injection in SCID/bg mice. A single lower extremity tumor was palpable in each animal by day 21, and mice were randomized to receive Apo2L/TRAIL (50 μg/kg/d), anti-TR2 mAb (5 μg/kg/d), or HBSS alone. Tumor dimensions were measured three times weekly during the 14-day treatment course, and the calculated mean tumor volumes are plotted in Fig. 2*A*. Both Apo2L/TRAIL and αTR2 mAb slowed the growth of these established tumors ($P < 0.01$), and there was no difference observed between the two treatment arms. Of 21 mice treated, Apo2L/TRAIL therapy resulted in four long-term complete responses (19%) and nine temporary responses wherein growth of tumor resumed after withdrawal of treatment (43%), for an overall response rate of 62%. Similar results were observed after anti-TR2 mAb treatment with four of 44 (9%) long-term complete responses and 27 of 44 (61%) temporary responses for an overall response rate of 70% ($P > 0.05$ compared with Apo2L/TRAIL). Overall, TRAIL

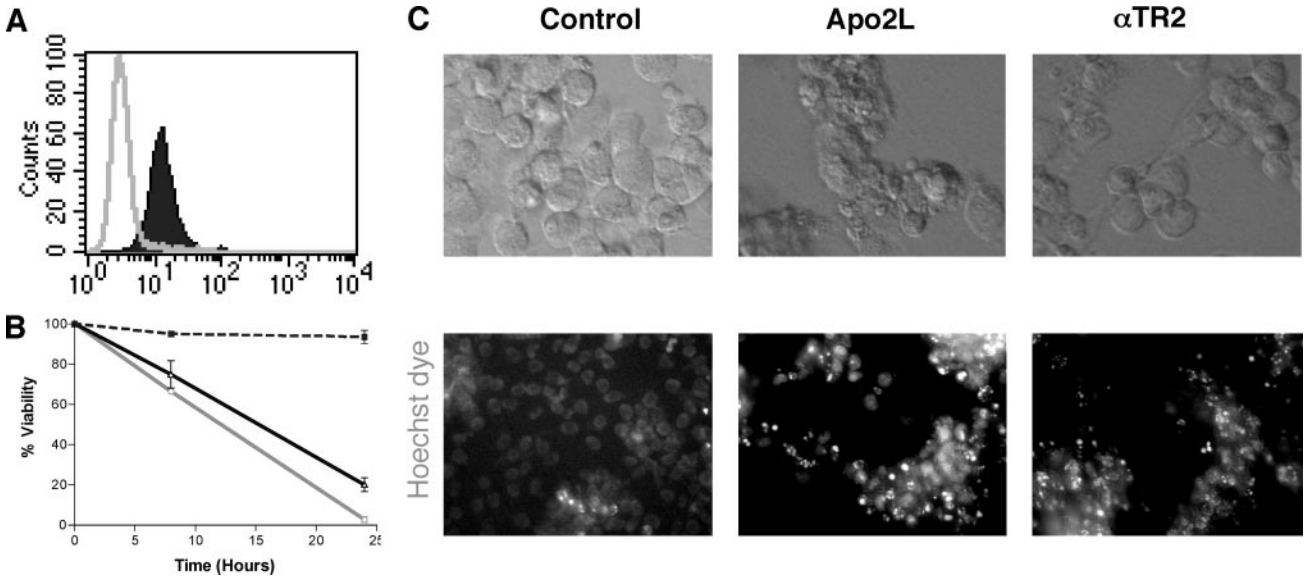

Fig. 1. Ewing's sarcoma family of tumors cells are exquisitely sensitive to both Apo2L and agonistic anti-TR2 (*αTR2*) mAb *in vitro*. Results are representative of a minimum of three individual experiments on eight different TRAIL-sensitive cell lines. *A*, surface expression of TR2 on a representative Ewing's sarcoma family of tumors cell line (TC71) as determined by fluorescence-activated cell sorter staining. *B*, decreased viability of Ewing's sarcoma family of tumors over time after culture with either TRAIL/Apo2L (200 μg/mL; *black solid line*) or anti-TR2 mAb (50 μg/mL; *gray line*) compared with medium alone (*dotted line*) as determined by trypan blue exclusion and confirmed by propidium iodide staining. *C*, phase contrast photomicrographs and Hoescht dye staining of cells cultured for 24 hours in medium alone, Apo2L/TRAIL, or anti-TR2. Magnification, ×100.

receptor agonists as single agents were able to slow tumor growth or cause durable regression in 44 of 65 (67%) of mice with Ewing's sarcoma family of tumors xenograft tumors. Therefore, tumors from Ewing's sarcoma family of tumors are sensitive to TRAIL receptor agonists *in vivo* as well as *in vitro*.

To determine whether TRAIL receptor agonist therapy may be more effective against microscopic primary tumors, TRAIL receptor agonist therapy was initiated before the establishment of a palpable tumor (day 7 after tumor injection). Again, both Apo2L/TRAILand αTR2 mAb significantly decreased tumor growth as measured by mean tumor volume and overall survival (Fig. 2*B*); however, there was no significant improvement compared with experiments in which TRAIL receptor agonists were begun after the establishment of palpable tumor. Furthermore, neither increases in dose nor duration of treatments substantially altered the efficacy of the TRAIL receptor agonists (data not shown). Therefore, although it is clear that TRAIL receptor agonists are active against tumors from Ewing's sarcoma family of tumors *in vivo*, the antitumor activity observed was less than that which would have been predicted based on *in vitro* sensitivity alone.

Several chemotherapeutic agents, including doxorubicin, have been shown to increase TRAIL receptor expression in tumor cells via a

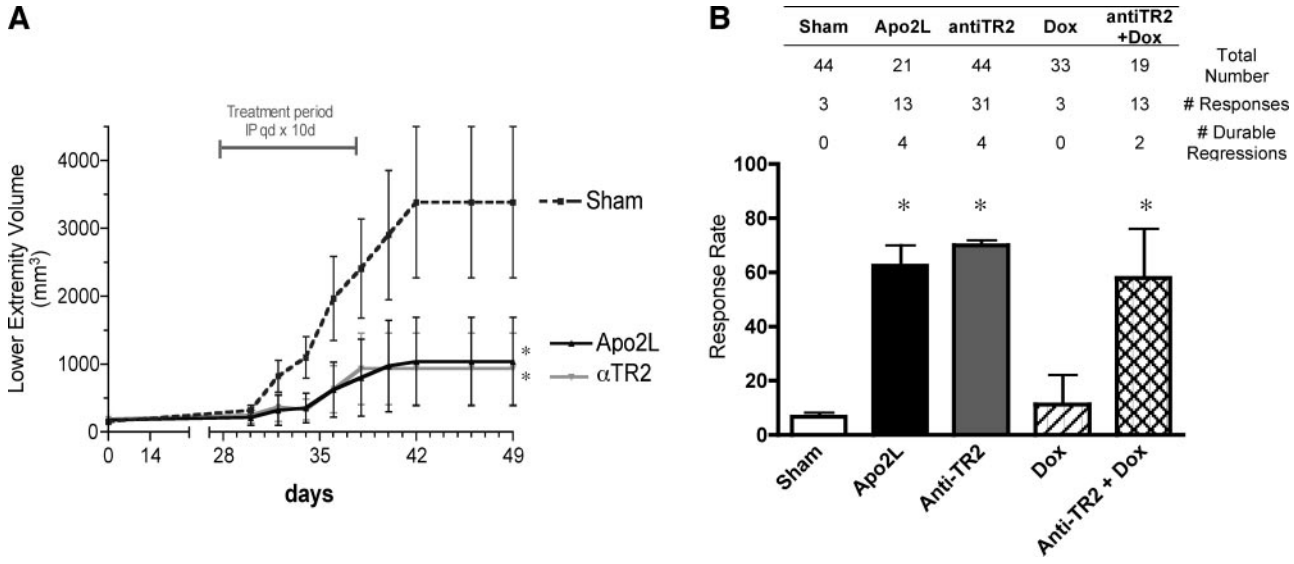

Fig. 2. TRAIL receptor agonists mediate antitumor effects on established Ewing's sarcoma xenografts. Xenograft tumors were established by intramuscular injection of TC71 Ewing's sarcoma family of tumors cells. Mice with palpable tumors were randomized to receive TRAIL/Apo2L (50 μg/kg/d), anti-TR2 (5 μg/kg/d), or HBSS intraperitoneal injections for 10 days. Mice were followed with serial lower leg measurements [lower extremity volume at the site of the tumor was determined using the formula $(D \times d^2/6) \times \pi$, where D is longer diameter and d is shorter diameter]. *A*. Mean tumor volume + SE are plotted over time for a single experiment. *B*, summary of response rates for indicated agents after treatment of established xenograft tumors. Overall response rates are defined as percentage of mice with tumors less than 50% of control mean at end of treatment as averaged across six experiments and two different cell lines. Subtherapeutic doses of doxorubicin (1 mg/kg/week) were administered alone (▨) or in conjunction with anti-TR2 mAb (▨) and compared with responses observed in sham-treated (□), TRAIL/Apo2L (■), or anti-TR2 mAb single agent (▤). *, $P < 0.01$ compared with control.

p53-dependent mechanism (29, 30). Ewing's sarcoma family of tumors cells are generally sensitive to doxorubicin both *in vivo* and *in vitro*. We determined that doxorubicin administered as a single agent at 1 mg/kg/week intraperitoneally in our xenograft model was the optimal dose to slow tumor growth without resulting in a full regression. When used in combination with anti-TR2 or Apo2L, doxorubicin did not increase the number of animals responding to TRAIL receptor agonists or overall survival (Fig. 2*B*; data not shown). These findings were consistent across three experiments and using two different Ewing's sarcoma family of tumors cell lines. Therefore, subtherapeutic doxorubicin did not increase the response rate to TRAIL receptor agonist therapy in this setting.

Because this xenograft model of Ewing's sarcoma family of tumors allows for control of local tumor growth via resection of tumor-bearing limb but reproducibly results in metastases, we also assessed the efficacy of TRAIL receptor agonists against metastatic disease. After removal of the primary tumor mass, untreated mice routinely developed recurrent or metastatic disease to their lungs, chest wall, or abdomen. Mice were randomized to receive treatment with anti-TR2, Apo2L/TRAIL, or sham from day 7 post-amputation to day 21. Metastatic disease was identified by twice weekly physical examinations and confirmed on necropsy as described previously (31). Metastatic disease was evident in all sham-treated mice by 4 weeks after surgery, and treatment with either TRAIL receptor agonist did not significantly decrease the incidence or alter the time to development of metastatic disease (data not shown). Thus, TRAIL receptor agonists appear to be more effective against the primary Ewing's sarcoma family of tumors than in preventing recurrent metastatic disease.

***In vivo* Growth Decreases TRAIL Sensitivity of Ewing's Sarcoma Family of Tumors Xenograft Cells.** Although TRAIL receptor agonists consistently impacted overall tumor growth in this model, it was clear that some tumors in each group were nonresponsive to TRAIL receptor agonist therapy, thus suggesting the emergence of TRAIL resistance during *in vivo* growth. To assess TRAIL sensitivity of cells within xenograft tumors, primary tumors from both TRAIL receptor agonist–treated and nontreated animals were surgically removed, minced, and plated as single-cell suspensions in RPMI +10% fetal calf serum. Five days later, when a semiconfluent layer of explant cell growth was evident, Apo2L/TRAIL was added to explant cultures, and subsequent apoptosis was assessed. Tumor explants prepared in this manner showed diminished sensitivity to TRAIL-mediated apoptosis when compared with the parent cell line that had been exclusively passaged in *vitro* (Fig. 3*A*). The resistance was observed regardless of which TRAIL receptor agonist was administered *in vitro*. Explants were also resistant to death induced by anti-TR2 mAb (data not shown). Similar results were obtained with multiple cell line/xenograft pairs, suggesting that this phenomenon was not cell line specific. Moreover, explant cells from untreated animals were equally resistant to Apo2L/TRAIL-induced apoptosis as cells from TRAIL receptor agonist–treated animals (Fig. 3*A*). This demonstrates that alterations in TRAIL sensitivity were not due to selection pressure by the TRAIL receptor agonist therapy.

To identify the changes that lead to decreased TRAIL sensitivity *in vivo*, we analyzed the expression of TR1 and TR2 on the surface of xenograft explant cells. The surface expression of both TR1 and TR2 were consistently decreased in cells grown from tumor tissue explants compared with parent cell lines that had been solely passaged *in vitro* (Fig. 3*B*). In contrast, expression of other surface markers (*e.g.*, CD99) were unaltered or slightly increased after *in vivo* growth. To determine whether the level of TRAIL receptor expression correlated with tumor size, xenograft tumors spanning a range of sizes were removed for analysis. TR1 and TR2 expression were consistently

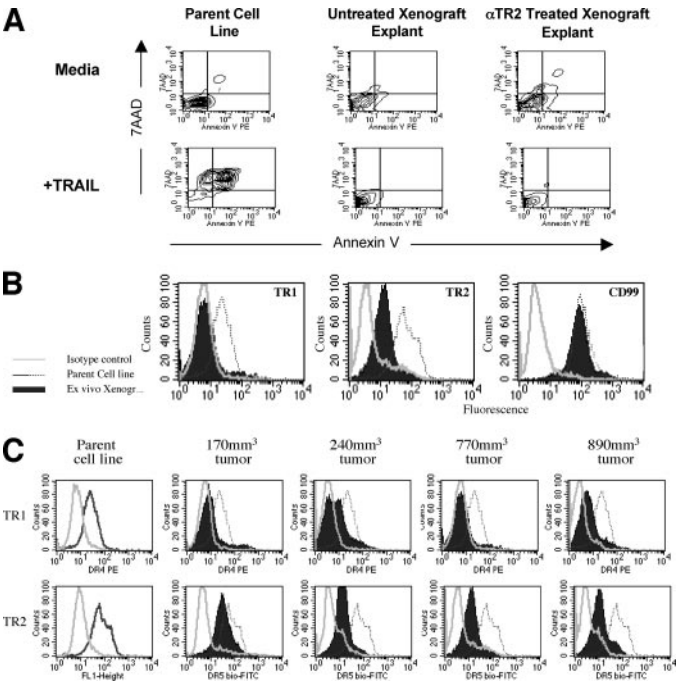

Fig. 3. Explant cells are less sensitive to TRAIL-mediated apoptosis and have decreased surface expression of TR1 and TR2 compared with parent cell lines. *A*. Xenograft tumors were removed from untreated or anti-TR2 mAb–treated mice at a tumor volume of 700 mm^3. Single-cell suspensions of explant cells were cultured with medium alone (*top row*) or 200 μg/mL Apo2L/TRAIL for 16 hours. Apoptosis was measured by annexin V/7AAD staining. *B*. Single-cell suspensions were obtained from untreated xenografts and stained with anti-TR1, anti-TR2, and anti-CD99 analyzed by flow cytometry. Xenograft explant staining (*shaded histogram*) is compared with cell lines that had been exclusively passaged *in vitro* (*dotted lines*). Isotype control is shown with a *solid gray line*. *C*. Xenograft tumors were harvested at indicated sizes and stained as in *B*.

down-regulated independent of tumor size or time *in vivo* (Fig. 3*C*). The down-regulation was reversible because TR1 and TR2 expression returned to baseline after several passages *ex vivo* (data not shown). Taken together, these results demonstrate that the *in vivo* growth of tumors from Ewing's sarcoma family of tumors renders them less sensitive to TRAIL receptor agonists and that *in vivo* TRAIL resistance correlates with TRAIL receptor down-regulation, suggesting that receptor down-regulation is one mechanism by which tumors growing *in vivo* acquire resistance to TRAIL-mediated cell death.

Lack of TR2 expression has been shown to correlate with TRAIL resistance in Ewing's sarcoma family of tumors cells (8) and neuroblastoma (12). To determine whether forced expression of TR2 could restore TRAIL sensitivity in the xenograft/explant system, we transfected TC71 Ewing's sarcoma family of tumors cell lines with a TR2 expression vector driven by a tet-off regulatable cytomegalovirus promoter (Fig. 4*A* and *B*). TR2-transfected and control cell lines were injected into SCID/bg mice as before, and tumor was evident in all experimental groups with similar growth curves (data not shown). Tumors were surgically removed at day 25 and plated in single-cell suspensions. Apo2L/TRAIL was added on day 5 when cells reached approximately 75% confluence. Viability and apoptosis were determined after 16 hours. Explant cells, whether wild-type or TR2 transfected, were significantly less sensitive to TRAIL treatment when compared with parent cell lines (Fig. 4*C*). Therefore, forced expression of TR2 was not able to restore sensitivity of explant cells to TRAIL receptor, suggesting that either down-regulation is mediated by factors other than transcriptional regulation and/or other additional changes in the TRAIL signaling pathway may be involved in the decreased TRAIL sensitivity.

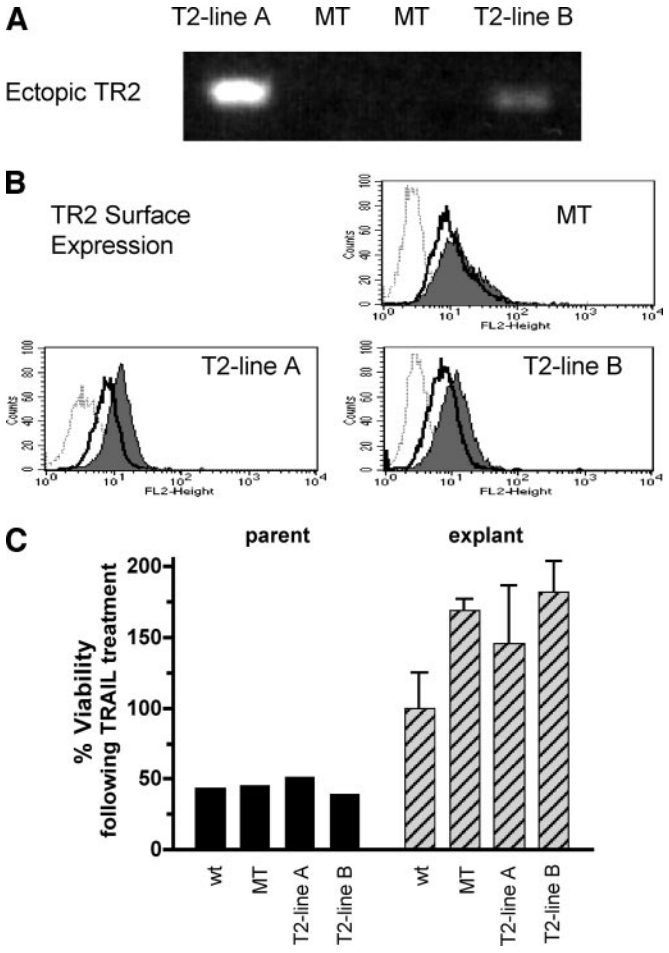

Fig. 4. Transfection of TR2 is not sufficient to restore *ex vivo* TRAIL sensitivity. *A*, PCR evidence of ectopic TR2 plasmid expression in transfected lines *versus* nontransfected lines (*MT*). *B*, fluorescence-activated cell sorter detection of surface TR2 on transfected cells grown in the absence of tetracycline (*shaded histograms*) compared with baseline expression in the presence of tetracycline. Xenograft tumors were established with TC71 cells that were nontransfected (*wt*), mock-transfected (*MT*), or transfected with a TR2 receptor (*T2-line A*, *T2-line B*). Tumors had similar growth patterns and were removed after 25 days of untreated growth. Single-cell suspensions of parent cell lines (■) or explant cells (▨) were grown in the presence or absence of 200 μg/mL Apo2L/TRAIL, and viability was determined at 16 hours by propidium iodide staining. Data represent percent viability after Apo2L compared with duplicate wells that were untreated.

Interferon Restores TRAIL Sensitivity in Explant Cells and Up-regulates Expression of TRAIL-R1 and TRAIL-R2 Expression *In vitro* and *In vivo*. It has been previously reported that TRAIL-resistant Ewing's sarcoma family of tumors cell lines can be rendered sensitive to TRAIL by pretreatment with IFNγ (7). To determine whether IFNγ was able to overcome the changes that rendered explant cells TRAIL resistant, we treated explant cultures with IFNγ for 24 hours before addition of TRAIL/Apo2L. Addition of IFNγ had little effect on the TRAIL-induced apoptosis in parent cell lines but significantly improved TRAIL sensitivity in explant cells (Fig. 5*A* and *B*). In all cases, whether explant cells were derived from treated or untreated mice, IFNγ restored TRAIL sensitivity to levels seen in parental cell lines passaged exclusively *in vitro*. To determine whether TRAIL pathway mediators were up-regulated by IFNγ, Ewing's sarcoma family of tumors cell lines were assessed by RNase protection assay. Although TR1 message levels were unchanged after treatment, TR2, caspase 8, and even the ligand TRAIL itself were up-regulated in Ewing's sarcoma family of tumors cells following with IFNγ treatment (representative line shown in Fig. 5*C*).

Based on the ability of IFNγ to sensitize TRAIL-resistant explants and the ability for IFNγ exposure *in vitro* to up-regulate RNA for TRAIL pathway components, human IFNγ (25,000 IU/day) was added to the treatment regimen during primary tumor growth in the xenograft model. Mice with palpable tumors were treated every 24 hours with IFNγ or carrier alone for 5 days. Sixteen hours after the last dose, tumors were removed and fixed in formalin. Immunohistochemistry was performed on these samples to assess expression of TR1, TR2, and caspase 8. Sections from treated xenograft tumors were compared with tissue sections from nontreated mice. Expression of caspase 8, TR1, and TR2 were minimally detectable in untreated tumors but substantially increased after IFNγ treatment (Fig. 5*D*). Thus, IFNγ modulates at least three elements of the TRAIL receptor signaling pathway that have been reported to limit TRAIL sensitivity in a variety of models. These results provided the rationale to determine whether such modulation via IFNγ would enhance the sensitivity of Ewing's sarcoma family of tumors to TRAIL receptor agonist therapies.

Efficacy of Interferon plus TRAIL Receptor Agonist *In vivo*. Because treatment with IFNγ plus Apo2L/TRAIL was able to induce apoptosis in explant cells and TRAIL-resistant Ewing's sarcoma family of tumors cell lines, we hypothesized that combination treatment *in vivo* could overcome the resistant phenotype and lead to greater antitumor efficacy. Mice received injections of Ewing's sarcoma family of tumors tumor cells, and tumors were allowed to develop for 7 days before initiation of treatment with anti-TR2, IFNγ, or both. Mice were then followed throughout the 2-week treatment period for development of measurable tumor. IFNγ treatment alone was not active in controlling growth of primary tumor as it did not significantly slow tumor growth when compared with control. Combination therapy using TRAIL receptor agonist and IFN was equally effective, with a trend toward more effective compared with anti-TR2 alone at controlling primary tumor growth during treatment because there was a modest increase in the time to reach the target volume for amputation (700 mm^3): day 27 in controls or IFNγ only, day 32 in animals treated with TRAIL receptor agonists alone, and day 38 in animals receiving both TRAIL receptor agonists and IFNγ ($P = 0.07$). Mean tumor volume on day 28 was 153 ± 51 mm^3 in anti-TR2–treated groups *versus* 133 ± 47 mm^3 in TR+IFNγ–treated groups ($P = 0.8$), and the response rate of animals was 60% whether they were treated with single-agent anti-TR2 or combination of TR+IFNγ (Fig. 6). Therefore, there was no definitive evidence for significant improvements in local tumor control when IFNγ was added to the TRAIL receptor agonist treatment regimen.

After cessation of therapy, tumors were allowed to grow until they reached a volume of 1000 mm^3 and were then surgically removed. After resection, no additional treatment was administered, and mice were followed for the development of metastatic disease as determined by serial exams and confirmed by autopsy. Mice treated with anti-TR2 alone had identical metastatic incidence rates as seen in the control groups. However, the group that received anti-TR2 plus IFNγ had a significantly decreased incidence of metastatic disease (Fig. 6). Because metastatic disease was less prevalent in combination-treated mice, there was a significant difference in overall survival (60% *versus* 0% at 5 months for treated *versus* nontreated, respectively). This remarkable difference was borne out in three similar experiments on two separate cell lines. Therefore, combination therapy, at best, modestly increases sensitivity of the primary tumor to TRAIL-mediated apoptosis, but it provides remarkable increases in overall survival through antimetastatic effects.

DISCUSSION

In this study, we demonstrate that ligation of a TRAIL death receptor by either its synthetic ligand (Apo2L) or an agonist mAb

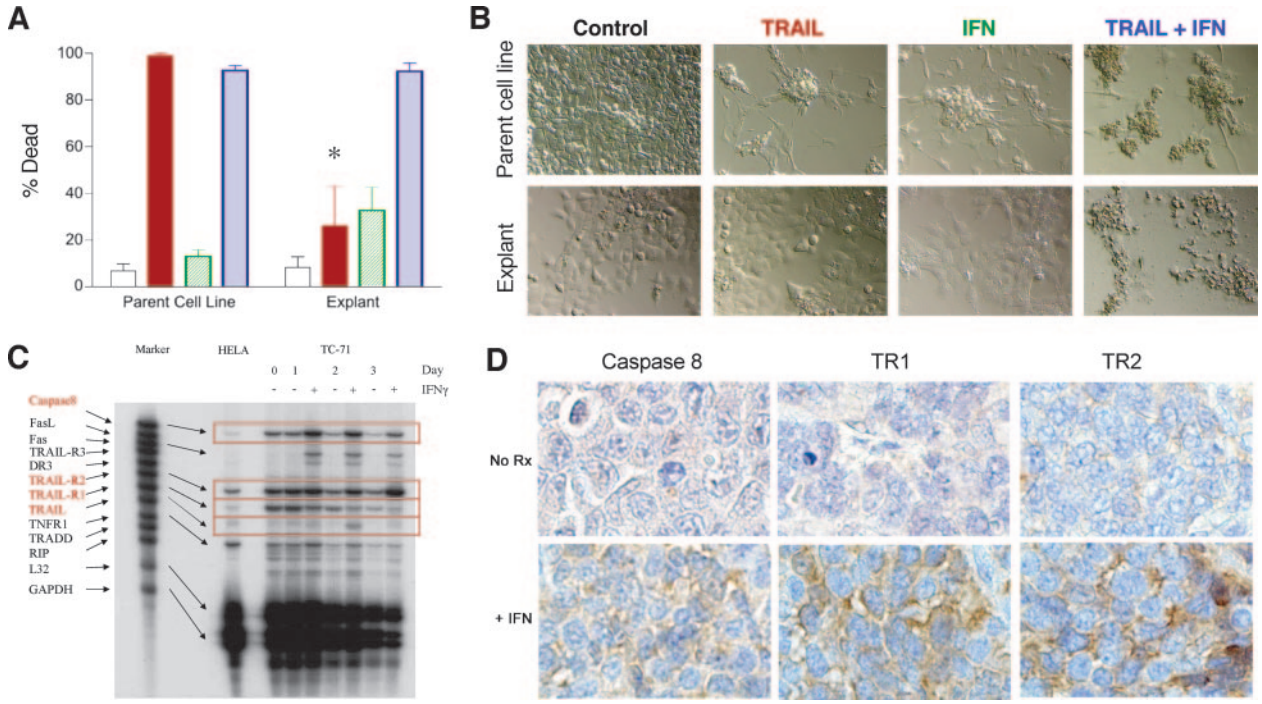

Fig. 5. IFNγ treatment increases susceptibility of explants to TRAIL while increasing caspase 8, TR1, and TR2 expression *in vivo*. *A*. TC71 xenograft tumors were removed after 10 days of sham and plated as single-cell suspensions. Cultures of TC71, which had been passaged solely *in vitro*, were used for comparison. At 70% confluence, IFNγ was added to wells. After 24 hours, TRAIL/Apo2L (200 μg/mL) was added to cultures, and then cells were harvested 8 hours later. Viability as determined by trypan blue exclusion is shown for sham (□), TRAIL only (■), IFN only (■), or TRAIL + IFN γ (■). *, $P < 0.01$ compared with parent cell line. *B*, phase contrast photomicrographs of LG and LG explant cells taken at ×100 magnification after treatment as described in *A*. *C*. Ewing's sarcoma family of tumors cells were cultured with (+) or without (−) IFNγ for 1 to 3 days as indicated and assayed by RNase protection assay to determine levels of mRNA. *D*. LG xenograft tumors were removed after 10 days of IFNγ injections (25,000 IU once daily intraperitoneally). Immunohistochemistry was performed on paraffin sections using the mouse anti-caspase 8 mAb, anti-TR1, or anti-TR2. Slides were counterstained with hematoxylin.

(anti-TR2) induces efficient apoptosis of Ewing's sarcoma family of tumors cells *in vitro*. This activity translates into significant antitumor activity *in vivo* because mice bearing Ewing's sarcoma family of tumors xenografts and treated with Apo2L/TRAIL or anti-TR2 mAb demonstrate slower tumor growth and increased overall survival rates. Although *in vivo* activity of TRAIL receptor agonists has been shown previously in several epithelial cancers (20, 32), this is the first report of *in vivo* efficacy in pediatric sarcomas. These results encourage the pursuit of clinical trials for TRAIL receptor agonists in childhood solid tumors and potentially in sarcomas if these agents are found to have a tolerable safety profile.

Although treatment with either Apo2L/TRAIL or anti-TR2 mAb induced reproducible and significant antitumor effects *in vivo*, the level of response was less than might be predicted by the exquisite sensitivity of these cell lines observed *in vitro* (Fig. 1). Complete responses were occasionally seen, and partial responses were common after TRAIL receptor agonist treatment of established or developing Ewing's sarcoma family of tumors xenografts, but some tumors in each experimental group did not respond to Apo2L or anti-TR2. Similar results have been seen in other *in vivo* models of TRAIL receptor agonist therapies, and chemotherapy has often been coadministered to improve the overall response rate (6, 30). However, concomitant administration of doxorubicin did not improve the *in vivo* response rate of Ewing's sarcoma family of tumors xenografts to TRAIL receptor agonist therapy (Fig. 2*B*).

We postulated that the limited efficacy of TRAIL receptor agonists *in vivo* might be due to the selection pressure of the TRAIL receptor agonist therapy itself through receptor down-regulation or clonal selection. To test this, xenograft explants were assayed *in vitro* to determine their sensitivity to Apo2L. TRAIL resistance was observed after TRAIL receptor agonist treatment *in vivo*, but surprisingly, TRAIL resistance was also equally present in explant cells from nontreated animals. Even when tumors were initiated from a single subclone that demonstrated *in vitro* sensitivity to Apo2L, tumor explants demonstrated TRAIL resistance (data not shown). These results provide the novel observation that the milieu within the tumor microenvironment attenuates signaling via TRAIL death receptors, rendering growing tumors substantially less TRAIL sensitive than is predicted based on studies of cells passaged exclusively *in vitro*.

The mechanisms by which tumors acquire TRAIL resistance *in vivo* are potentially manifold and remain unclear. We observed significant down-regulation of TR1 and TR2 both in TRAIL receptor agonist–treated and untreated mice. Immune surveillance is partially mediated by TRAIL expression on immune cells (33), suggesting that selection pressure by immune elements could result in such effects. However, the SCID/bg mice used in these experiments lack both T cells and NK cells, which would be expected to mediate such immune selection. Furthermore, although antigen-presenting cells present in SCID/bg mice also express TRAIL, TRAIL signaling is species specific (34), and therefore murine TRAIL would not be expected to select against TRAIL-sensitive human Ewing's sarcoma family of tumors cells. Thus, although the mechanism of receptor down-regulation remains unclear, we can conclude that it is not a result of selection pressure by TRAIL receptor agonist therapies, and it appears unlikely to result from immune selection pressure.

As signaling through these TR1 and/or TR2 is critical for initiation of the TRAIL death signal, we postulated that receptor down-regulation contributed to TRAIL resistance. These death pathway receptors are likely to be regulated at both the level of transcription and protein expression. Indeed, TR1 and TR2 have been previously shown to have rapid turnover (35), suggesting that protein trafficking, stability, and/or cleavage mechanisms play key roles in the regulation of their

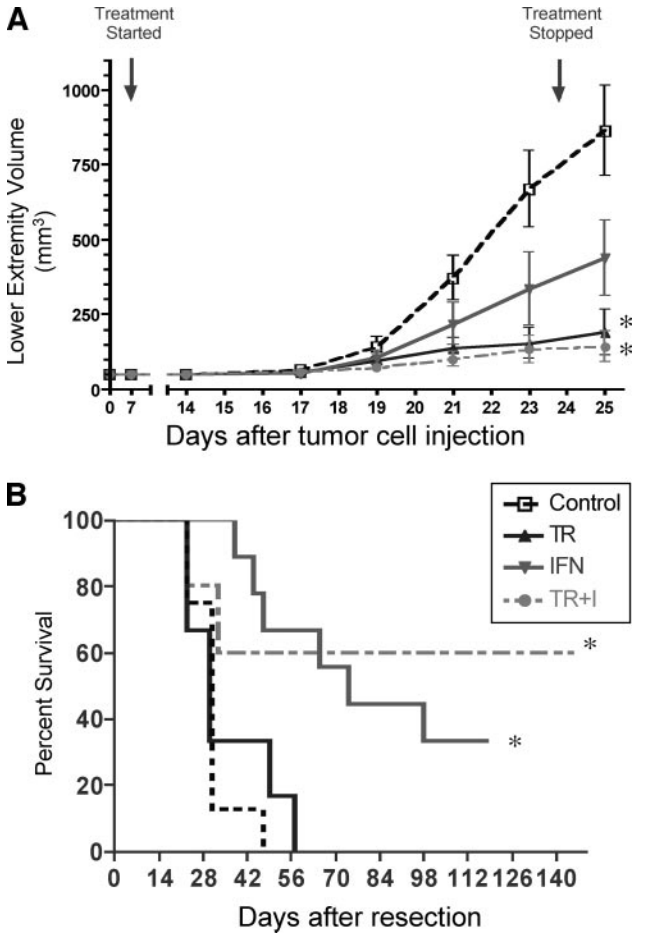

Fig. 6. Antitumor and antimetastatic effects of TRAIL receptor agonists and IFNγ. Ewing's sarcoma tumor cells (2×10^6) were injected into the gastrocnemius of 4- to 12-week-old SCID/bg mice on day 0. After tumor was allowed to establish for 7 days, daily intraperitoneal injections were begun (on 5 days, off 2 days for three cycles) of TRAIL receptor (anti-TR2 at 5 μg/kg/d), IFN (25,000 IU/d), or both (*TR+I*). *A*. Mice were followed for development of palpable tumor with lower leg measurements. Lower extremity volumes without tumor were approximately 50 mm^3. Primary tumors were removed by survival surgery when they reached >1,000 mm^3. *B*. Mice were followed for palpable recurrent tumors or shortness of breath indicative of pulmonary metastases, which were confirmed on necropsy. Overall survival of mice from *A*. *, $P < 0.05$ compared with control.

expression. This is consistent with our results that Ewing's sarcoma family of tumors cells with forced expression of TR2 can still acquire TRAIL resistance after growth *in vivo*. Although this result could be due to other as yet unidentified changes within the TRAIL signaling cascade that may also contribute to TRAIL resistance *in vivo*, post-translational regulation is likely an important component of the mechanisms leading to TRAIL resistance *in vivo*. For example, even in these transfected cells, surface expression of TR2 was variably decreased after *in vivo* growth (data not shown). Ongoing studies are under way to formally dissect the mechanisms responsible for TRAIL receptor down-regulation.

Previous work has demonstrated that *in vitro* exposure to IFNγ can influence several elements of the TRAIL-mediated death pathway, including caspase 8 expression (36), and expression of TRAIL itself (12, 37). TRAIL-resistant Ewing's sarcoma family of tumors and neuroblastoma cell lines are rendered TRAIL sensitive after exposure to IFNγ *in vitro* (7, 12). Based on these reports and the capacity for IFNγ to reverse TRAIL resistance in explants (Fig. 5*A*), we sought to determine whether exposure to IFNγ could up-regulate TRAIL receptor expression and/or render the TRAIL receptor agonist therapy more effective. Indeed, Ewing's sarcoma family of tumors cell lines or Ewing's sarcoma family of tumors xenograft tumor cells both showed an increased expression of TR1, TR2, and caspase 8 compared with untreated cells or tissues (Fig. 5*C* and *D*). Because IFNγ shows exquisite species specificity (38), we conclude that the effects of IFNγ on Ewing's sarcoma family of tumors in this model are mediated by direct signaling of IFNγ in the tumor cells themselves rather than via immune effectors. It is known that IFNγ directly up-regulates caspase 8 in neuroblastoma cells *in vitro* as well as in tumors from patients treated with IFNγ through modulation of IFN-sensitive genes (12). The data presented here confirm that similar increases in caspase 8 occur after *in vitro* or *in vivo* treatment of Ewing's sarcoma family of tumors. We also observed substantial up-regulation of TR2 protein after IFNγ therapy *in vivo* that correlated with an increase in transcription of TR2 after IFNγ treatment *in vitro*. In contrast, TR1 expression was up-regulated *in vivo* without any evidence of transcriptional regulation after IFNγ treatment of Ewing's sarcoma family of tumors cell lines, providing additional evidence that post-translational factors are important in the effects of IFNγ on Ewing's sarcoma family of tumors xenografts. The potential role of intermediaries such as TIMP, which has been reported to diminish metalloproteinase expression and potentially inhibit TRAIL receptor cleavage (39), are currently under study. Although TRAIL itself has been reported to be up-regulated by IFNγ (25) and we observed this effect in Ewing's sarcoma family of tumors cell lines (Fig. 5*C*), IFNγ-induced up-regulation of TRAIL itself seems an unlikely mechanism to explain the increases in TRAIL receptor expression observed in IFNγ-treated animals, because up-regulation of TRAIL on the tumor cells would be expected to select for low TRAIL receptor expression rather than high TRAIL receptor expression as was observed.

Despite the impressive effects of IFNγ on caspase 8, TR1, and TR2, all critical proximal elements of the TRAIL signaling pathway, the addition IFNγ to TRAIL receptor agonists resulted in only marginal, nonsignificant improvements in primary tumor response compared with treatment with TRAIL receptor agonists alone. Importantly, however, the combination treatment was much more effective when the mice were followed for metastatic disease. When IFNγ was administered either as single agent or together with a TRAIL receptor agonist, there was a substantial decrease in incidence of metastases with the best overall survival after combination therapy. Therefore, these results demonstrate an antimetastatic role for IFNγ in addition to modulation of TRAIL receptor signaling elements and sensitization to TRAIL-mediated apoptosis.

In summary, these studies demonstrate *in vivo* activity of TRAIL receptor agonists in Ewing's sarcoma. Although the overall response rate of 67% is impressive, very few mice were cured of their tumors with TRAIL receptor agonist therapy alone, and we routinely observed the acquisition of TRAIL resistance after *in vivo* growth. The mechanisms responsible for *in vivo* TRAIL resistance are not entirely clear, but it is associated with TRAIL receptor down-modulation and can be reversed *ex vivo* using IFNγ. Therefore, combination strategies are likely to be required to optimally exploit TRAIL pathways of programmed cell death in the clinical setting, even in tumors such as Ewing's sarcoma family of tumors that demonstrate exquisite *in vitro* sensitivity. Combining TRAIL receptor agonists with IFNγ appears compelling because IFNγ modulates several elements of the TRAIL signaling pathway that may be responsible for TRAIL resistance, and the group receiving combination therapy was the only one in which effects were observed in both primary tumors and metastatic disease. In summary, our observations show that treatment with TRAIL plus IFNγ is a rationally designed combination therapy, which has significant antitumor efficacy in Ewing's sarcoma family of tumors.

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