Interferon-γ–Dependent Infiltration of Human T Cells into Neuroblastoma Tumors In vivo

Gregor S.D. Reid,1 Xiaochuan Shan,3 Christina M. Coughlin,1 Wiem Lassoued,3 Bruce R. Pawel,2 Leonard H. Wexler,7 Carol J. Thiele,5 Maria Tsokos,5 Jack L. Pinkus,6 Geraldine S. Pinkus,6 Stephan A. Grupp,1,2,4 and Robert H. Vonderheide3,4

Abstract Purpose: To investigate the impact of interferon-γ–mediated upregulation of major histocompatibility complex class I expression on tumor-specific T-cell cytotoxicity and T-cell trafficking into neuroblastoma tumors in vivo. Experimental Design: Restoration of major histocompatibility complex class I expression by interferon-γ treatment enhances killing of neuroblastoma cells. To understand the potential of this approach in vivo, we developed a novel model of neuroblastoma in which NOD/scid/IL2Rγnull immunodeficient mice are engrafted with both human T cells and tumor cells. Results: Here, we show enhanced killing of neuroblastoma cells by patient-derived, tumor-specific T cells in vitro. In addition, interferon-γ treatment in vivo induces efficient upregulation of major histocompatibility complex class I expression on neuroblastoma tumor cells, and this is accompanied by significantly enhanced infiltration of T cells into the tumor. In a pilot clinical trial in patients with high-risk neuroblastoma, we similarly observed augmented T-cell trafficking into neuroblastoma nests in tumor biopsy specimens obtained from patients after 5 days of systemic interferon-γ therapy. Conclusions: Interferon-γ overcomes critical obstacles to the killing of human neuroblastoma cells by specific T cells. Together, these findings provide a rationale for the further testing of interferon-γ as an approach for improving the efficacy of T cell–based therapies for neuroblastoma and other major histocompatibility complex class I–deficient malignancies. In addition, we describe a model that may expedite the preclinical screening of approaches aimed at augmenting T-cell trafficking into human tumors. (Clin Cancer Res 2009;15(21):6602–8)
Although the downregulation of major histocompatibility complex class I expression in cancer, particularly neuroblastoma, is a well-recognized mechanism of escape from cellular immune surveillance. In this study, we show that exposure of human neuroblastoma tumor cells to interferon-γ upregulates major histocompatibility complex class I expression in vitro and in vivo, augments killing of neuroblastoma cells by specific T cells in vitro, and induces significantly enhanced infiltration of human T cells into neuroblastoma tumor-bearing mice and patients with the disease. This work shows the need for further clinical testing of interferon-γ as an approach to augment immune therapy for neuroblastoma and other major histocompatibility complex class I-deficient tumors.

**Translational Relevance**

Lack of major histocompatibility complex class I expression in cancer, particularly neuroblastoma, is a well-recognized mechanism of escape from cellular immune surveillance. In this study, we show that exposure of human neuroblastoma tumor cells to interferon-γ upregulates major histocompatibility complex class I expression in vitro and in vivo, augments killing of neuroblastoma cells by specific T cells in vitro, and induces significantly enhanced infiltration of human T cells into neuroblastoma tumor-bearing mice and patients with the disease. This work shows the need for further clinical testing of interferon-γ as an approach to augment immune therapy for neuroblastoma and other major histocompatibility complex class I-deficient tumors.

**Materials and Methods**

**Patient samples, normal T cells, and cell lines.** For preclinical studies, peripheral blood leukocytes were obtained by leukapheresis from patients after obtaining informed consent based on the Institutional Review Board–approved Children's Hospital of Philadelphia protocols for the treatment of high-risk neuroblastoma (patients with stage 3 or 4 disease with unfavorable biological features). Leukapheresis was done at the time of diagnosis. Paraffin-embedded tumor samples were obtained at the time of diagnosis. Human CD3+ T cells were derived from normal donor blood obtained by phlebotomy or leukapheresis after obtaining informed consent based on protocols approved by the University of Pennsylvania Institutional Review Board. CD3+ T cells that had undergone costimulated activated expansion in culture using anti-CD3/CD28 beads (21) were kindly provided by Dr. Bruce Levine (University of Pennsylvania, Philadelphia, PA). The neuroblastoma cell lines SY5Y, NLF, and CHP-134 were obtained from Dr. Garrett Brodeur (Children's Hospital of Philadelphia); the NSJ3 line was obtained from Dr. Richard Carroll (University of Pennsylvania). Other neuroblastoma lines were from American Type Culture Collection. All cell lines were maintained in RPMI supplemented with 10% fetal bovine serum.

**Clinical trial of interferon-γ in neuroblastoma.** In a previously reported clinical trial (22), patients with relapsed neuroblastoma were enrolled after obtaining informed consent based on the clinical protocol NCI 90-C-0210 approved by the Institutional Review Board of the NCI to investigate the safety and immunologic impact of interferon-γ in patients. Patients were treated s.c. every day for 5 days with recombinant human interferon-γ (0.1 mg/m²). Tumor biopsies were obtained before treatment and immediately or up to 4 days after the last day of IFN-γ therapy, and were evaluated by immunohistochemistry.

**Interferon-γ treatment of neuroblastoma cells.** For in vitro studies, neuroblastoma cell lines were incubated in the presence or absence of 100 U/mL human interferon-γ (Actimmune) for 48 hours before analysis by flow cytometry. For in vivo studies, NOD/scid/IL2Rγnull (NOG) mice were purchased from Jackson Laboratory and housed under specific pathogen-free conditions in microisolators. All experiments were done in accordance with protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Mice were injected s.c. in the flank with 5 × 10⁶ neuroblastoma tumor cells in Matrigel (BD Biosciences). Tumor growth was monitored, and recombinant human interferon-γ injections were initiated when tumors measured >200 mm³. Mice were injected with 30,000 IU interferon-γ s.c. every day for 3 days in peritumoral region, and tumors were harvested on day 4.

**Human T-cell tumor infiltration assay.** NOG mice were first injected s.c. in the flank with 5 × 10⁶ SY5Y tumor cells. One week later, mice were injected through the lateral tail vein with 50 × 10⁶ human CD3+ T cells. Mice were then monitored weekly for T-cell engraftment by flow cytometric detection of human CD45+/CD3+ cells in mouse peripheral blood. Mice with SY5Y tumors of >100 mm³ in which peripheral T cells were detected were randomized to PBS or interferon-γ experimental arms. Treatment consisted of a regimen of 3 days of treatment (30,000 IU interferon-γ or PBS) followed by 3 days rest, repeated three times. Upon completion of treatment, the mice were sacrificed, and the spleen and tumor were harvested for analysis.

**Flow cytometry.** Single cell suspensions were prepared from cell cultures, spleens, and tumors by passage through a 40-μm filter. For analysis of cell surface molecules, all samples were labeled with directly conjugated antibodies at 4°C for 20 minutes. The samples were then washed and resuspended in 1.5% paraformaldehyde and incubated with fluorescent antibodies at 4°C for 20 minutes. The samples were then washed and resuspended in 1.5% paraformaldehyde and analyzed with a FACSCalibur. Antibodies to human CD3, CD4, CD8, and HLA-A, HLA-B, HLA-C were obtained from BD Biosciences. The relevant labeled isotype control antibodies were included in all experiments.

**Expansion and evaluation of survivin-specific T cells.** Patient-derived CD40-activated B cells lines were established and RNA electroporation was performed.
done as previously described (17, 23). Survivin mRNA and enhanced green fluorescent protein mRNA was used at 2 to 5 μg per sample. Electroporated CD40-B were washed in T-cell media (RPMI with 10% human AB serum, 2 mmol/L glutamine, 20 mmol/L HEPES, and 15 μg/mL gentamicin) and plated at 5 × 10^5 cells/well with 2 × 10^5 PBMC/well in 24-well plates. Cultures were supplemented with 500 U/mL rHLA-4 (R&D Systems) and 10 ng/mL rHIL-7 (Sigma) on day 1 and 20 U/mL interleukin 2 (Chiron) on days 2 and 5. T-cell cultures were restimulated with RNA-transfected CD40-B on day 7.

Chromium release assays were done as previously described (23). SD was <5%. Target cells were evaluated for HLA-A2 and major histocompatibility complex class I expression by FACS analysis (BB7.2, Dako NorthAmerica; anti–HLA-ABC, BD Biosciences). In some cases, neuroblastoma cells were pretreat with 100 IU/ml rIFN-γ (R&D systems) for 48 hours.

**Immunohistochemistry.** For immunoperoxidase studies on primary human tissue, paraffin sections of formalin-fixed tissue samples were used. Sections were deparaffinized, and antigen retrieval was done using heat-induced epitope retrieval. Sections were incubated with polyclonal antibody to CD3 (1:400; Dako NorthAmerica), CD8 (1:100; C8/144B Dako NorthAmerica), or monoclonal antibody specific for major histocompatibility complex class I (1:200; clone HC-10; kind gift of Dr. Soldano Ferrone, University of Pittsburgh). Sections were incubated with peroxidase-labeled secondary antibodies and signal localized using 3,3′-diaminobenzidine tetrahydrochloride as the chromogen. Hematoxylin was used as a counterstain. For immunoperoxidase studies on engrafted tissue from NOG mice, paraformaldehyde-fixed, paraffin-embedded tissue from NOG mice, paraformaldehyde-fixed, paraffin-embedded tissue was used. Paraffin sections of high-risk neuroblastoma tumors were stained with anti–major histocompatibility complex class I monoclonal antibody. Results for patient A (stroma-rich tumor) and patient B (stroma-poor tumor) are shown. Black boxes, areas of major histocompatibility complex class I-negative tumor nests (left, x10; magnified on the right); certainstromal elements, blood vessels, and tumor-associated macrophages are major histocompatibility complex class I positive. Similar results were obtained for a total of 26 patients with high risk neuroblastoma.

**Results**

**Major histocompatibility complex class I expression in neuroblastoma tumors.** To evaluate expression of major histocompatibility complex class I in neuroblastoma tumors, we did immunohistochemistry for major histocompatibility complex class I expression. In diagnostic tumor biopsies from 26 patients presenting with high-risk neuroblastoma, we found that neuroblastoma tumor cells in all cases were negative for major histocompatibility complex class I, consistent with previous observations (18–20). Two representative patients are shown in Fig. 1. Major histocompatibility complex class I expression was readily detectable on benign cells, including lymphocytes, macrophages, and endothelial, mesothelial, and epithelial (e.g., adrenal) cells (Fig. 1). Rare large major histocompatibility complex class I-positive cells within the tumor seemed to be tumor-associated macrophages.

**Enhanced T-cell killing of interferon-γ-treated neuroblastoma cells.** To determine whether interferon-γ upregulation of major histocompatibility complex class I expression on neuroblastoma cells enhances specific CTL responses, we first screened a panel of neuroblastoma cell lines for major histocompatibility complex class I expression. In contrast to primary tumors, most neuroblastoma cell lines that we examined (73%; 8 of 11) were found by flow cytometry to express medium or high levels of major histocompatibility complex class I (Fig. 2A). Three of 11 neuroblastoma cell lines (NLF, SY5Y, and CHP-134) were negative for major histocompatibility complex class I by this analysis, but 48 hours of treatment with interferon-γ in vitro upregulated major histocompatibility complex class I expression by each cell line. Interferon-γ further upregulated major histocompatibility complex class I expression on major histocompatibility complex class I-positive neuroblastoma lines (Fig. 2A).

Using major histocompatibility complex class I negative cell lines as targets, we then compared the cytolytic activity of survivin-specific CTL from two neuroblastoma patients against untreated neuroblastoma tumor cells or neuroblastoma cells treated with interferon-γ in vitro. For targets, we used the major histocompatibility complex class I-negative neuroblastoma lines NLF and SY5Y, both of which upregulated major histocompatibility complex class I after 48 hours of treatment with interferon-γ. NLF cells express HLA-A2, but SY5Y cells are HLA-A2 negative; both cell lines express survivin (data not shown).

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http://www.farsight-toolkit.org/wiki/Histopathology
HLA-A2+ patient T cells stimulated with survivin mRNA showed minimal lysis of untreated NLF cells but efficiently lysed NLF following treatment with interferon-γ and upregulation of major histocompatibility complex class I (Fig. 2B). These T cells failed to lyse the HLA-A2–negative SY5Y, either untreated or after interferon-γ treatment. Patient T cells stimulated with green fluorescent protein mRNA showed only minimal lysis of these tumor targets (Fig. 2B). These data support the notion that survivin-expressing neuroblastoma cells may escape immunosurveillance by failing to express major histocompatibility complex class I but that class I expression and susceptibility to T-cell lysis may be restored by exposure to inflammatory cytokines.

To investigate the ability of interferon-γ to enhance major histocompatibility complex class I expression in vivo, we established explants of human neuroblastoma tumor cells in immunodeficient NOG mice. This highly immunodeficient mouse strain (T-cell, B-cell, and natural killer cell deficient) efficiently accepts human tumor xenografts and also has the potential to allow the engraftment and expansion of normal human T cells. NOG mice were injected with major histocompatibility complex class I–negative SY5Y, NLF, or CHP-134 neuroblastoma tumor cells, and when tumors measured >200 mm3, recombinant human interferon-γ or PBS was injected s.c. around the tumor bed daily for 3 days. On day 4, the tumors were harvested, processed to single cell suspensions, and evaluated by flow cytometry for expression of HLA-A, HLA-B, and HLA-C. Although no differences in morphology were observed for tumors from PBS- and interferon-γ–treated mice, major histocompatibility complex class I expression was markedly elevated on all three cell line tumors after interferon-γ treatment compared with tumors from mice given PBS (Fig. 2C).

**T-cell trafficking to tumors in interferon-γ treated mice.** Given the ability of interferon-γ to efficiently enhance major histocompatibility complex class I expression on neuroblastoma tumors in vivo, we hypothesized that the use of this cytokine may increase the infiltration of tumors by T cells recognizing the newly expressed major histocompatibility complex class I antigens. To test this hypothesis, we established explants of SY5Y tumors s.c. in NOG mice. One week later, the mice were given 50 × 106 in vivo expanded allogeneic human CD3+ T cells from a healthy donor. Mice were then followed for T-cell engraftment, measured by the presence of human CD45+/CD3+ cells in peripheral blood, and tumor size. Mice that achieved detectable peripheral human T cells and tumors of >100 mm3 were then treated with PBS or interferon-γ for 18 days on a thrice repeated regimen of 3 days of treatment followed by 3 days rest. Upon completion of treatment, mice were sacrificed, and the spleen and tumor were removed for analysis. There was no significant difference in the sizes of tumors in the two groups at the completion of treatment (data not shown). Although all mice achieved peripherally detectable human T cells, a more accurate measure of systemic engraftment levels was obtained by flow cytometric analysis of the spleen from each animal, showing very high levels of T-cell engraftment in the NOG animals. Of the eight animals used in the study, three PBS-treated mice and four interferon-γ–treated mice achieved >50% human T cells in the spleen, with no significant difference in T-cell engraftment levels between these groups (70±15.2% versus 85±1.4% CD3+ cells among total splenocytes, respectively; P = 0.09; Fig. 3A). One PBS-treated mouse achieved significantly <50% engraftment and, for this reason, was excluded from the analysis of T-cell trafficking.

Single cell suspensions were prepared from tumors and spleens from each animal were analyzed by flow cytometry for the presence of human T cells. A significantly higher percentage of human CD3+ T cells, calculated as percentage of total cells to account for variation in tumor size, was detected in tumors from...
interferon-γ–treated mice compared with PBS-treated mice (7.0%±1.2 versus 1.8%±0.8; P = 0.0013; Fig. 3A). Immunohistochemistry was then done on tumor sections from PBS- and interferon-γ–treated mice to determine the localization of the human T cells (Fig. 3B). Consistent with the flow cytometric data, the total number of CD3+ T cells detected by immunohistochemistry was increased in interferon-γ–treated tumors compared with PBS-treated tumors (45.9±26 per ×400 field versus 11.6±10.5; P < 0.0001). Infiltrating T cells were then evaluated histologically as peritumoral or intratumoral. Increased numbers of peritumoral CD3+ T cells were observed in interferon-γ–treated tumors compared with PBS-treated tumors (18.7±18.3 versus 6.6±8.9; P = 0.0012; Fig. 3C). Strikingly, greater increases in the number of intratumoral CD3+ T cells were achieved in interferon-γ–treated tumors (27.2±21.5 versus 4.9±3.9; P < 0.0001; Fig. 3C). Although CD8+ T cells accounted for only about 25% of the T cells detected by immunohistochemistry, the number of intratumoral and peritumoral CD8+ cells was also increased by interferon-γ treatment (Fig. 3C).

As the increase in of intratumoral T-cell numbers could be the result of either increased trafficking into or proliferation within the tumor, we examined the infiltrating CD3+ T cells for expression of the proliferation marker Ki-67 (Fig. 3D). Although significantly more Ki-67 expressing CD3+ T cells were present in interferon-γ–treated than in PBS-treated tumors (2.66±0.4 versus 0.44±0.25; P < 0.0001), only a small fraction of the T cells from either tumor source were proliferating.

**Interferon-γ effects in a neuroblastoma clinical trial.** We hypothesized that, given the ability of interferon-γ to enhance human T-cell numbers in neuroblastoma tumors in our mouse model, this cytokine may be useful clinically as a means to improve major histocompatibility complex class I expression and T-cell infiltration. To determine whether systemic administration of interferon-γ to patients with neuroblastoma modulates tumor expression of major histocompatibility complex class I, we studied tumor biopsies from five high-risk neuroblastoma patients obtained before and after administration of interferon-γ per NCI protocol NCI-90-C0210. Patients on this study were treated s.c. every day for 5 days with recombinant human interferon-γ (0.1 mg/m² (2 million IU) per day; ref. 22). Tumor biopsies were obtained before treatment and immediately after the 5-day treatment (Fig. 4). As expected, major histocompatibility complex class I expression was not detected on tumor cells at baseline in any patient; however, in two patients, major

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**Fig. 3.** Interferon-γ induces T-cell tumor infiltration. A, spleen and tumors from NOG mice treated with interferon-γ or PBS were evaluated for the presence of CD3+ human T cells by flow cytometry. Although T-cell engraftment measured in the spleen was not significantly different between the two groups (P = 0.09), the percentage of T cells in tumor preparations was significantly higher in interferon-γ treated mice (P = 0.0013). B, representative ×10 magnification fields of tumors from three PBS-treated mice (i–iii) and three interferon-γ–treated mice (iv–vi) showing infiltration of CD3+ T cells. C, cumulative counts of intratumoral and peritumoral CD3+ and CD8+ T cells. At least 10 ×400 magnification fields were counted for each tumor from three PBS– (light bars) and four interferon-γ–treated mice (dark bars). *, P < 0.005; **, P < 0.0005. D, Ki-67 expression by tumor infiltrating T cells. Following dual-labeling with anti-CD3 and anti-Ki-67 antibodies, the percentage of double positive T cells was calculated from eight ×400 fields for each tumor (three PBS treated, four interferon-γ treated). Significantly more double positive T cells were observed in interferon-γ treated tumors (P < 0.0001), but <3% of all CD3+ T cells were proliferating in any case.
histocompatibility complex class I expression was upregulated following interferon-γ treatment, with a subset of neuroblastoma tumor cells clearly expressing major histocompatibility complex class I. Upregulation of major histocompatibility complex class I correlated with the presence of infiltrating CD3+ T cells. At baseline in each patient, CD3+ T cells were rare or absent. After interferon-γ treatment, there was a notable increase in CD3+ T-cell infiltration in the biopsies of the same two patients, both within the tumor nests and in the fibrovascular bundles. In three other patients, interferon-γ administration failed to upregulate major histocompatibility complex class I expression and only very rare CD3+ T cells were observed before and after treatment.

Discussion

Lack of major histocompatibility complex class I expression in cancer, particularly neuroblastoma, is a well-recognized mechanism of escape from cellular immune surveillance. In this study, we show that exposure of human neuroblastoma tumor cells to interferon-γ upregulates major histocompatibility complex class I expression in vitro and in vivo, augments killing of neuroblastoma cells by specific T cells in vitro, and induces significantly enhanced infiltration of human T cells into neuroblastoma tumor-bearing mice and patients with the disease. Interferon-γ-dependent induction of major histocompatibility complex class I expression on neuroblastoma cells in vivo was comparable with that achieved in vitro. Among the multiple defects in the antigen processing machinery that have been well characterized in neuroblastoma (20), none is considered structural but rather reflects abnormalities in regulatory mechanisms involved in the expression of these molecules, including major histocompatibility complex class I heavy chain itself. Interferon-γ induces or upregulates major histocompatibility complex class I expression on most neuroblastoma tumor cell lines in vitro by enhancing the transcription of critical antigen presentation machinery components (24, 25). It seems most likely that the upregulation of major histocompatibility complex class I achieved on neuroblastoma cells in NOG mice and in patient tumors on the clinical trial is achieved through a similar influence on transcription of antigen presentation machinery components.

We have previously reported that functional CTL recognizing the tumor-associated antigen survivin can be isolated from neuroblastoma patients despite the lack of T-cell infiltration into tumors from these patients (17). Here, we observed that interferon-γ treatment of major histocompatibility complex class I–negative neuroblastoma cells resulted in tumor lysis by patient-derived survivin-specific CTL, which otherwise did not kill such neuroblastoma cells. This finding indicates that interferon-γ can augment the presentation of the peptide repertoire that is relevant for these CTL. Thus, the ability of interferon-γ to restore major histocompatibility complex class I expression by neuroblastoma cells in vivo shown in this study may provide a clinical strategy to enhance the impact of endogenous neuroblastoma-specific CTL in patients.

Increasing the susceptibility of neuroblastoma cells to killing by CTL is, however, irrelevant if the CTL never gain access to the tumor. In this regard, our observation that in vivo administration of interferon-γ in mice engrafted with human T cells leads to enhanced infiltration of total CD3+ and CD8+ lymphocytes into established neuroblastoma tumors is encouraging. The expression of Ki-67 by only a small minority of intratumoral T cells supports our conclusion that enhanced trafficking rather than in situ proliferation in response to allogeneic tumor cells is responsible for the increased lymphocyte numbers. The modest number of T cells infiltrating neuroblastoma tumors in patients treated for 5 days with interferon-γ is consistent with observations made in our mouse xenograft model. Although significantly enhanced T-cell infiltration was obtained after our 18-day interferon-γ treatment, such effects were not observed following 3- and 5-day treatment regimens despite the enhanced major histocompatibility complex class I expression on neuroblastoma cells achieved with the shorter interferon-γ treatments (data not shown). These results suggest that longer courses of treatment may be needed to achieve clinical enhancement of T-cell therapy by interferon-γ. Alternatively, the different outcomes may be associated with the proximity of the tumor to the site of interferon-γ administration in the human and mouse experiments. The model described in this study will provide a useful tool for the evaluation of such variables in the optimization of this approach.

The lack of T-cell trafficking into patient neuroblastoma tumors that failed to upregulate major histocompatibility complex class I in response to interferon-γ suggests that the increased antigen expression is a critical component for the T-cell infiltration. This hypothesis is consistent with the observation that restoration of major histocompatibility complex class I restricted
antigen presentation by murine tumor cells through antigen presentation machinery gene transfer is sufficient to enhance tumor infiltration by T cells, perhaps through increased cross-presentation of tumor antigens by dendritic cells (26). However, interferon-γ has been shown to exert several additional effects on the tumor microenvironment that may contribute to enhanced T-cell infiltration, including stimulation of chemokine production and activation of the vasculature (27, 28). The observation that many of the infiltrating T cells in our model were CD8 negative suggests that additional influences of interferon-γ may contribute to the enhanced trafficking. We did not detect any expression of major histocompatibility complex class II antigens on neuroblastoma cells after interferon-γ treatment (data not shown). The xenograft tumor model for human T-cell infiltration developed in this study, with the potential for high-level human T-cell engraftment in these NOG mice, will provide a powerful tool to evaluate the role of each of the interferon-γ–mediated microenvironment changes. This mouse model will be useful for the development and testing of novel approaches to enhance lymphocyte trafficking into human tumors. Such approaches have been reported to improve the efficiency of immune-based therapies in mouse tumor models (14, 15).

In summary, our results show that interferon-γ enhances trafficking of human T cells into neuroblastoma cell tumors in vivo, providing further rationale for using this approach to overcome immune evasion mediated by downregulated major histocompatibility complex class I expression in this tumor. These observations are particularly relevant to neuroblastoma given that recombiant human interferon-γ (Actimmune) has Food and Drug Administration–approved indications for use in children (chronic granulomatous disease and osteopetrosis) with a defined pediatric dose. Further clinical study in neuroblastoma is warranted, particularly because treatment courses of Actimmune longer than that used in the NCI study are tolerated in children.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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