Immunologic and Therapeutic Synergy of IL-27 and IL-2: Enhancement of T Cell Sensitization, Tumor-Specific CTL Reactivity and Complete Regression of Disseminated Neuroblastoma Metastases in the Liver and Bone Marrow


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Immunologic and Therapeutic Synergy of IL-27 and IL-2: Enhancement of T Cell Sensitization, Tumor-Specific CTL Reactivity and Complete Regression of Disseminated Neuroblastoma Metastases in the Liver and Bone Marrow


IL-27 exerts antitumor activity in murine orthotopic neuroblastoma, but only partial antitumor effect in disseminated disease. This study demonstrates that combined treatment with IL-2 and IL-27 induces potent antitumor activity in disseminated neuroblastoma metastasis. Complete durable tumor regression was achieved in 90% of mice bearing metastatic TBJ-IL-27 tumors treated with IL-2 compared with only 40% of mice bearing TBJ-IL-27 tumors alone and 0% of mice bearing TBJ-FLAG tumors with or without IL-2 treatment. Comparable antitumor effects were achieved by IL-27 protein produced upon hydrodynamic IL-27 plasmid DNA delivery when combined with IL-2. Although delivery of IL-27 alone, or in combination with IL-2, mediated pronounced regression of neuroblastoma metastases in the liver, combined delivery of IL-27 and IL-2 was far more effective than IL-27 alone against bone marrow metastases. Combined exposure to IL-27 produced by tumor and IL-2 synergistically enhances the generation of tumor-specific CTL reactivity. Potentiation of CTL reactivity by IL-27 occurs via mechanisms that appear to be engaged during both the initial sensitization and effector phase. Potent immunologic memory responses are generated in mice cured of their disseminated disease by combined delivery of IL-27 and IL-2, and depletion of CD8+ ablates the antitumor efficacy of this combination. Moreover, IL-27 delivery can inhibit the expansion of CD4+CD25+Foxp3+ regulatory and IL-17-expressing CD4+ cells that are otherwise observed among tumor-infiltrating lymphocytes from mice treated with IL-2. These studies demonstrate that IL-27 and IL-2 synergistically induce complete tumor regression and long-term survival in mice bearing widely metastatic neuroblastoma tumors. The Journal of Immunology, 2009, 182: 4328–4338.

Neuroblastoma is the most common extracranial solid tumor in children, with highly variable biologic features and clinical outcome. Among the clinicopathologic features that contribute to existing risk stratification criteria for patients with neuroblastoma, age and stage play a particularly important role in predicting prognosis and clinical outcome (1, 2). Notably, the outcome of patients with high-risk neuroblastoma is poor overall, and this has fueled an intense effort to develop new therapeutic approaches for high-risk patients, including those with widespread metastatic disease. Among the novel approaches under investigation, several studies have evaluated immunotherapeutic agents including cytokines (3–5) or immunocytokines (6–9) in preclinical tumor models and/or early phase clinical studies in children with neuroblastoma. Among these agents, IL-2 (6, 10), tumor-targeted immunocytokines (9), and IL-12 (3, 11) have demonstrated particular efficacy in preclinical neuroblastoma models, and now have been evaluated in the clinical setting as well (7, 12–15). Nonetheless, the clinical antitumor efficacy of single cytokines or immunocytokines has been modest in studies to date (7, 8, 12–15), and can be associated with appreciable side effects at high doses (16).

More recent reports have highlighted the complex regulation of the host immune response (17–19), and suggest that the optimal approach to the initiation, expansion, and maintenance of a successful antitumor immune response will likely necessitate rationally designed combinations of agents with complementary mechanisms of action. For example, although antitumor cytokines such as IL-2 and IL-12 play broad roles in potentiation of an adaptive T cell-mediated antitumor immune response, important contributions are also provided by more recently described cytokines including...
IL-18 (20, 21), IL-23 (22–24), and IL-27 (25–27). IL-27 is a heterodimeric cytokine with structural and functional homology with IL-12, and is produced by APCs including monocyte/dendritic cell populations in response to stimuli that include LPS ≥ IFN-γ and CD40L among others (28–29). In turn, IL-27 potently enhances various aspects of T and/or NK cell function in vitro including enhancement of proliferative responses and the production of IFN-γ (28).

Based on its potent immunoregulatory activity, recent studies have investigated the antitumor efficacy of IL-27 in preclinical tumor models, including murine models of colon carcinoma and neuroblastoma (25–27). We have reported that IL-27 mediates potent antitumor effects against s.c. and orthotopic intradrenal TBJ murine neuroblastoma tumors, resulting in complete durable tumor regression in up to 90% of mice (26). IL-27 can mediate its potent antitumor effects via mechanisms that are dependent on the induction of endogenous IFN-γ production and the activity of CD4+ and/or CD8+ T cell populations in vivo (25–27). In mice bearing TBJ neuroblastoma tumors, IL-27 up-regulates MHC class I expression and enhances the generation of both tumor-specific immune responsiveness and immunologic memory responses in mice cured of their original tumors by IL-27. In turn, we have found that IL-27 mediates overall tumor regression in this model via mechanisms that are critically dependent on CD8+ but not CD4+ T cells or NK cells (26). Despite the broad effects of IL-27 against s.c. and orthotopic primary neuroblastoma tumors, the antitumor efficacy of IL-27 alone is more modest against metastases. In this model, IL-27 mediates complete durable tumor regression in only 40% of mice bearing disseminated TBJ IL-27 tumors.

In light of the difficulty in treating patients with metastatic neuroblastoma, we have investigated approaches to potentiate the immunoregulatory activity and antitumor efficacy of IL-27 in mice bearing metastatic TBJ neuroblastoma tumors. Previous studies have demonstrated that IL-27 synergizes with IL-2 plus IL-12 to enhance IFN-γ production by human NK cells in vitro (28). The present studies were designed to investigate the antitumor activity and mechanisms of interaction by combined delivery of IL-27 and IL-2 in mice bearing disseminated neuroblastoma metastasis. We demonstrate in this study for the first time that combined delivery of IL-27 and IL-2 mediates synergistic antitumor efficacy in mice bearing TBJ neuroblastoma metastases in the liver and bone marrow, and that it does so in conjunction with generation of potent tumor-specific immune responsiveness.

Materials and Methods

Reagents, tumor cells, and mice

Male A/J mice were used in all studies and were obtained from the Animal Production Area (Charles River Laboratories). Mice were maintained in a dedicated specific pathogen-free environment and generally used between 8 and 10 wk of age. The TBJ neuroblastoma cell line syngeneic to A/J mice was used where indicated, and was generously provided by Dr. Morritz Ziegler (Children’s Hospital, Boston, MA). TBJ tumor cells were engineered to overexpress murine IL-27 using the p-FLAG-CMV-1 vector (Sigma-Aldrich) containing a fusion sequence encoding the mature coding sequences for murine EBI3, followed by the synthetic linker GSGSGS GSGSGSGL and the mature coding sequence of mouse p28 as previously described (28). In brief, TBJ neuroblastoma cells were stably transfected with either p-IL-27/FLAG-CMV-1 (TBJ IL-27) or the empty p-FLAG-CMV-1 vector alone (TBJ-FLAG) using the calcium phosphate method, and cells were subsequently selected in G418 (800 μg/ml) (Invitrogen). Individual clones were isolated by limiting dilution cloning, and were screened subsequently for IL-27/FLAG or FLAG gene expression using RT-PCR. The level of IL-27 protein was also confirmed by FLAG immunoprecipitation followed by Western blot as described previously (26). For hydrodynamic experiments, a vector encoding murine IL-27 was codon optimized for expression by altering the nucleotide sequence, without affecting the primary amino acid sequence, and was inserted into pCMV-kan (30), which contains the human CMV promoter without an intron, a bovine growth hormone polyadenylation signal, and a kanamycin resistance gene. IL-27 is produced as hyperkine, which contains the mature EBI3 chain linked to the mature p28. This plasmid was designated AG192/IL-27. Highly purified, endotoxin-free DNA plasmid preparation was obtained by using a Qiagen kit.

The SA-1 murine sarcoma cell line (syngeneic to A/J mice) was generously provided by Dr. Suzanne Ostrand-Rosenberg (University of Maryland, Baltimore, MD). Single chain IL-27 protein generated as previously described (28) was used for in vitro studies. Commercially available recombinant human IL-2 (Chiron) was used for both in vitro and in vivo studies. Animal care was provided in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85–23, 1985).

The effects of combined treatment with IL-27 and IL-2 in proliferative responses and IFN-γ production by murine splenocytes was assessed in vitro. Single cell suspensions were prepared using spleens from normal A/J mice as described above. Cells were suspended in complete medium at 2 × 106 cells/ml in RPMI 1640 containing 5% FCS, 2 mM glutamine, 1 mM pyruvate, 5 × 10−3 M 2-ME, 2 mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES. For proliferation assays, 5 × 105 cells/well were cultured similarly to round-bottom 96-well plates in the presence or absence of IL-2 (50 IU/ml) and/or IL-27 (50 ng/ml) for 72 h. [3H]Thymidine (1 μCi/well) was added 18 h before harvest and [3H]thymidine incorporation was determined using a beta counter and standard techniques. For IFN-γ production, 1 × 106 spleen cells/ml were incubated in complete medium with IL-27 (50 ng/ml) or IL-2 (50 IU/ml) or medium alone for 48 h. Culture supernatants were then harvested and IFN-γ levels were assessed by ELISA (R&D Systems).

In vivo tumor models and treatment regimens

Where indicated, syngeneic male A/J mice were injected i.v. with 1 × 106 TBJ IL-27 or control TBJ FLAG tumor cells/animal on day 0. For survival studies, IL-2 (200,000 IU/injection) in 0.2 ml HBSS containing 0.1% homologous mouse serum or vehicle alone was administered i.p. on days 5–9, 12–16, 19–23, and 26–30 post tumor implantation. To evaluate the effect of IL-27 protein, mice were injected i.v. with 1 × 106 TBJ parental cells on day 0, and IL-27 hydrodynamic delivery was administered by using 10 μg of either AG192/IL-27 vector, or the empty vector BV4, as control in a total volume of 1.6 ml saline solution injected into the tail vein within about a 5 to 15 s period, on day 5 and 15 post tumor injection. Administration of IL-2 was performed as described above. To assess the impact of combined delivery of IL-2 and IL-27 more specifically on liver metastasis, mice bearing metastatic TBJ IL-27 or TBJ FLAG tumors were treated similarly with IL-2 or vehicle alone on days 5–9 and 12–15 post tumor implantation. Cohorts of mice were euthanized on day 16 post tumor implantation. Livers were then removed for inspection and imaging/quantitation of the metastatic disease burden. To quantitatively assess the impact of combined delivery of IL-2 and IL-27 on neuroblastoma metastases in the bone marrow, mice bearing metastatic TBJ IL-27 or TBJ FLAG tumors were treated with IL-2 or vehicle alone on days 5–9, 12–16, 19–23, and 26–30 post tumor implantation. Mice were euthanized as they became moribund, or all the remaining surviving mice were euthanized at day 37 post tumor implantation. At the time of euthanization, tibias and femurs were removed, and bone marrow was isolated and cultured as described below. To assess the frequency of neuroblastoma metastasis via colony assay. In complementary studies, we also evaluated the tumorigenicity of neuroblastoma metastases in the bone marrow of treated mice. In these studies, mice bearing metastatic TBJ IL-27 or TBJ FLAG tumors were treated with IL-2 or vehicle alone on days 5–9 and 12–16, and mice were euthanized on day 19 post tumor implantation. Tibias and femurs were resected and bone marrow was isolated as described below. Using the mouse marrow specimens from the resected TBJ neuroblastoma metastases via colony assay. The subsequent growth of tumors in these mice was then monitored twice per week as an indicator of tumor burden in the respective bone marrow specimens.

To evaluate whether specific immunologic memory responses were generated in mice cured of their original tumors by combined delivery of IL-2 and IL-27 as described above, mice were rechallenged s.c. with TBJ parental tumor cells (1 × 106 cells/animal) where indicated and then monitored for survival. To investigate the specific role of T and/or NK cell...
subsets in the antitumor activity of the IL-27/IL-2 combination, mice were injected i.v. with 1 × 10^7 TBJ-FLAG or TBJ IL-27 tumor cells/animal on day 0. To deplete CD4^+ vs CD8^+ T cell subsets, respectively, in vivo, mice were injected i.p. with rat anti-mouse CD4^+ (GK1.5, diluted 1/2) or mouse anti-mouse CD8^+ (Ly2.2, diluted 1/20) Abs on day −1 and days 2, 5, 7, 9, 12, 14, 16, and 19, and 21 post tumor implantation. To deplete NK cells in vivo, anti-asialoGM1 (diluted 1/20, Wako Pure Chemical) was administered i.p. on day −1 and days 2, 7, 12, 16, 21, and 26 post tumor cell implantation. Single cell suspensions were prepared as described elsewhere in detail (26), and were then treated with IL-2 (200,000 IU/injection) or vehicle alone i.p. on days 10–12 post tumor cell implantation. Mice were then euthanized and tumors were resected for isolation of TIL as described below.

**Generation of immunologic responsiveness:** IFN-γ production and generation of tumor-specific CTL reactivity

The impact of combined delivery of IL-27 and IL-2 on the generation of immunologic reactivity was assessed in mice bearing metastatic TBJ neuroblastoma tumors. Initially, spleens were rescued from mice bearing disseminated TBJ IL-27 or TBJ FLAG tumors at day 15 post tumor injection. Spleen cells (1 × 10^7 cells/ml in complete medium) were cultured in the presence of the corresponding irradiated (4,000 rads) TBJ FLAG or TBJ IL-27 tumor cells (2.5 × 10^7 cells/ml) or with IL-2 (50 IU/ml). The cultures were then incubated for 3 days at 37°C, and supernatants were harvested for determination of IFN-γ concentrations by ELISA as described above. To investigate the impact of combined delivery of IL-27 and IL-2 on the induction of tumor-specific CTL reactivity in mice bearing TBJ neuroblastoma tumors, spleens were resected from mice bearing disseminated TBJ IL-27 or TBJ FLAG tumors at day 15 post tumor injection. Single cell suspensions were prepared as described above and total splenocytes (1 × 10^7 cells/ml) were incubated in the presence of irradiated (6,000 rads) TBJ FLAG or TBJ IL-27 tumor cells (1 × 10^7 cells/ml) for 7–10 (10 IU/ml) in complete medium. Cultures were incubated for 6 days, and the cytolytic activity of these splenic effector cells was assessed using a standard 111Indium-release assay. In brief, 1 × 10^7 TBJ parental or irrelevant syngeneic SA-1 tumor cells were labeled with 10 μCi (C9H6NO3)111In (Amersham Health Medi-Physics) for 15 min at room temperature. Target cells were washed and resuspended at 1 × 10^7 cells/ml in the above medium. Variable numbers of splenic effector cells in complete medium were plated in triplicate in 96-well plate (Costar) and then 1 × 10^6 labeled target cells/well were used to achieve the desired E:T ratio, and plates were then incubated for 18 h at 37°C. At the conclusion of the incubation, cultures supernatants were harvested and counted individually in a gamma counter (Wallac). The percentage of specific lysis was calculated as follows: % of specific lysis = (ER − SR)/100(MR − SR), where ER = experimental release; SR = spontaneous release; MR = maximum release. Minimum release was determined by measuring target cell supernatant alone. Maximum release was determined by exposing the target cells to 1% SDS.

**Imaging of liver metastases**

To image the organ-specific impact of combined delivery of IL-27 and IL-2 on metastatic neuroblastoma tumors in the liver, mice bearing induced metastatic TBJ IL-27 or TBJ FLAG tumors were treated with IL-2 or vehicle alone as described above. Mice were euthanized on day 16 post tumor implantation, and livers were resected and placed in HBSS for imaging of the disease burden using a slit fiber optic illuminated light table (Lighthools Research). Images were taken using a Nikon Eclipse E400 Microscope fitted to a Nikon digital camera DXM1200 (Image Systems).

**Bone marrow colony assay**

To assess the impact of combined delivery of IL-27 and IL-2 on neuroblastoma metastases in the bone marrow, mice bearing IV-induced TBJ IL-27 or TBJ FLAG tumors were treated with IL-2 or vehicle alone as described above. Femurs and tibias were resected from mice in the respective treatment groups, and bone marrow cells were flushed out with 5 ml of RPMI 1640. Bone marrow cells were then spun down at 1,500 rpm for 5 min at 4°C and cells were resuspended at 5 × 10^6 cells/ml in RPMI 1640 containing 5% FCS and 2 mM glutamine. One hundred microliters of bone marrow cell suspension (5 × 10^6 cells) were added to 1.3 ml of methylcellulose medium containing 40% bone Methylocellulose M3134 (Metho-Cult, Stem Cell Technologies) and 60% RPMI 1640 (20% FCS, 4 mM l-glutamine) in a 35-mm petri dish. Plates were incubated for 12–14 days in humidified atmosphere at 37°C with 5% CO2. Colony forming units were scored and the composition of tumor infiltrating lymphocytes (TIL) was determined as described elsewhere in detail (26), and were then treated with IL-2 (200,000 IU/injection) or vehicle alone i.p. on days 10–12 post tumor cell implantation. Mice were then euthanized and tumors were resected for isolation of TIL as described below.

**Flow cytometric analysis of tumor infiltrating lymphocytes**

To investigate the composition of infiltrating lymphocytes within TBJ FLAG or TBJ IL-27 tumors, mice were injected with these tumors on day 0, and treated with 200,000 U of IL-2 on day 7–9. On day 10, tumors were dissected and digested with 200 U/ml collagenase (Invitrogen) and 100 μg/ml DNase I (Boehringer Mannheim), at 37°C for 2 h. After digestion, cell aggregates were removed using a 40-μm nylon cell strainer (BD Biosciences). BD GolgiStop (BD Biosciences) at dilution 1/1000 was used during the digestion and staining steps. Single cell suspensions (1 × 10^7 cells/ml) were incubated on ice for 10 min with rat anti-mouse CD4 (Ly2.2, diluted 1/20) and CD8 (Ly2.48, diluted 1/20), followed by 1% SDS.

**Statistical methods**

Where indicated, mice were monitored for overall survival and tumor growth as assessed by abdominal size twice weekly. Survival studies were analyzed by the log-rank test and Kaplan-Meier curves were plotted for survival comparisons. The relative proportion of mice achieving complete durable and long-term survival were calculated using Fisher’s exact test (FET). Similarly, the proportions of mice rejecting a durable tumor regression and long-term survival were compared by the FET. Proportional comparisons of tumor growth rates were in complete interpretative agreement. The proportion of mice achieving complete durable and long-term survival were compared using FET. Interpretations regarding survival duration and complete response rates were in complete interpretative agreement. The proportion of mice developing s.c. tumors after injection of bone marrow cells containing metastatic neuroblastoma tumor deposits were compared using the FET. For proliferative responses, IFN-γ production, and CTL responses by the murine splenocytes, mean values were determined for the respective condition (duplicate for IFN-γ, triplicate for proliferation and CTL responses), and were compared using the one-way and two-way ANOVA procedure. Follow-up pairwise comparisons were performed by means of the Tukey’s HSD procedure. In all cases, the statistical procedures were performed on log-transformed values of the dependent variables. For all statistical comparisons, pairwise comparisons were performed using the nonparametric Mann-Whitney Wilcoxon test. All p values were two-tailed and were considered significant at p < 0.05.
Results

IL-27 and IL-2 synergistically enhance proliferative responses and IFN-γ production by murine splenocytes in vitro

To investigate whether the combination of IL-27 and IL-2 could interact favorably in the enhancement of immune responsiveness, we evaluated the impact of combined treatment with IL-27 and IL-2 on proliferative responses and IFN-γ production by murine splenocytes in vitro. We found for the first time that the combination of IL-27 and IL-2 can synergistically enhance proliferative responses by murine splenocytes. The proliferation of murine splenocytes treated with the combination of IL-27 and IL-2 was 3.3- to 5.9-fold greater than that observed with splenocytes treated with either single cytokine or medium alone (medium alone: 3694 ± 980.5 cpm, IL-2: 8,936 ± 450.6 cpm, IL-27: 5,035 ± 243.5 cpm, IL-2 plus IL-27: 29,633 ± 1,969 cpm; p < 0.001, IL-2 plus IL-27 vs either IL-2 or IL-27 alone or medium alone) (Fig. 1A). Further, synergistic increases in IFN-γ production were observed when splenocytes were stimulated with the combination of IL-27 and IL-2 compared with either single cytokine or medium alone (medium alone: ≤ 50 pg/ml, IL-2: ≤ 50 pg/ml; IL-27: 62.65 ± 12.6 pg/ml; IL-2 plus IL-27: 502.5 ± 6.5 pg/ml; p < 0.001, IL-2 plus IL-27 vs either IL-2 or IL-27 or medium alone) (Fig. 1B). Thus, the combination of IL-2 and IL-27 appears to synergize with respect to the induction of splenocyte proliferation and IFN-γ production in vitro. It is of note that these effects could be mediated by lymphocytes and NK cells.

Combined delivery of IL-27 and IL-2 mediates synergistic antitumor activity in mice bearing disseminated TBJ neuroblastoma tumors

Based on the ability of IL-2 to synergize with IL-27 in the enhancement of proliferative responses and IFN-γ production by murine splenocytes in vitro, we hypothesized that combined delivery of IL-2 and IL-27 could mediate a more potent antitumor activity than either single agent alone. To test this hypothesis, we used a disseminated model of TBJ neuroblastoma metastasis characterized by the formation of metastasis predominantly in liver, lung, and bone marrow. In this model, we have shown previously that IL-27 alone can mediate complete tumor regression and long-term survival in ∼40% of mice bearing induced neuroblastoma metastases (26).

Systemic administration of IL-2 to mice bearing disseminated TBJ IL-27 tumors resulted in complete tumor regression and long-term survival in nine of ten mice (90%) compared with four of ten mice (40%) bearing TBJ-IL-27 tumors alone and zero of ten mice (0%) bearing TBJ FLAG tumors treated with or without systemic IL-2 alone (p = 0.018, TBJ IL-27 plus IL-2 vs TBJ IL-27 alone; p < 0.0001, TBJ FLAG treated with or without IL-2 vs TBJ IL-27 plus IL-2), (Fig. 2A). Augmentation of the therapeutic effect of systemic cytokine administration may depend on the ability to deliver them in a targeted manner. To assess whether IL-27 protein will result in antitumor responses when combined with IL-2 administration in this tumor model, mice bearing disseminated neuroblastoma tumors were subjected hydrodynamic delivery of plasmid AG192/IL-27 as described in the Materials and Methods section. Nine of ten mice...
showed long term survival in the IL-27 hydrodynamic plus IL-2 combination in contrast to 5 of 10 mice in the IL-27 hydrodynamic alone ($p = 0.0416$). IL-27 hydrodynamics alone vs IL-27 hydrodynamics plus IL-2 (Fig. 2B). No significant antitumor effects were observed by IL-2 alone in this model. Thus, combined, delivery of IL-27 with IL-2 mediates synergistic antitumor effects in mice bearing disseminated neuroblastoma metastases.

**Combined delivery of IL-27 and IL-2 mediates the regression of hepatic TBJ neuroblastoma metastases**

After i.v. administration, TBJ tumor cells form metastases in the liver, lung, and bone marrow. We directly examined the impact of combined delivery of IL-27 and IL-2 on the development of disease burden in the liver and bone marrow. Sixteen days after i.v. tumor cell injection, a marked reduction in the number of liver metastases was noted in mice bearing TBJ IL-27 tumors compared with mice bearing disseminated TBJ FLAG tumors (TBJ IL-27: 19.7 ± 5.4 macrometastases/liver vs TBJ FLAG 270 ± 30 macrometastases/liver; $p = 0.001$) (Fig. 3, A and B). The burden of metastatic disease in the liver of mice bearing TBJ IL-27 tumors was further inhibited by systemic administration of IL-2 (200,000 IU/injection, days 5–9, 12–15) (TBJ IL-27 plus IL-2: 4.7 ± 1.95 macrometastases/liver) ($p = 0.035$; TBJ IL-27 vs TBJ IL-27 plus IL-2). At this time point, a modest but statistically insignificant inhibition of the formation of hepatic metastases was observed by IL-2 treatment in mice bearing TBJ FLAG tumors compared with control mice treated with vehicle alone (TBJ FLAG: 270 ± 30 macrometastases/liver vs TBJ FLAG plus IL-2: 162 ± 41 macrometastases/liver; $p = 0.105$) (Fig. 3, A and B). Thus, combined delivery of IL-27 and IL-2 can mediate potent antitumor effects against TBJ neuroblastoma tumors in the liver.

**Combined delivery of IL-27 and IL-2 completely abrogates bone marrow disease in mice bearing disseminated TBJ neuroblastoma metastases**

To directly investigate the antitumor effects of combined delivery of IL-27 and IL-2 specifically in the bone marrow compartment, mice bearing disseminated TBJ neuroblastoma tumors were harvested individually as they became sick or after long-term survival. Bone marrow cells were isolated as described and the burden of neuroblastoma metastases within the bone marrow compartment was assessed by assay of the formation of tumor colonies grown in methylcellulose in the presence of G418 (1 mg/ml) to provide selective pressure permitting the growth of metastatic tumor cells but not normal bone marrow cells. Remarkably, colonies formed from the bone marrow of eight of nine mice bearing TBJ IL-27, nine of nine mice bearing TBJ FLAG tumors treated with systemic administration of IL-2, and eight of eight control mice bearing TBJ FLAG tumors, while tumor colonies formed from only three of ten mice bearing TBJ IL-27 tumors and treated with IL-2 ($p = 0.0198$, TBJ IL-27 plus IL-2 vs TBJ IL-27 alone) (Fig. 4A).

In complementory studies, we assessed the tumorigenicity of TBJ neuroblastoma metastases in the bone marrow of mice bearing widespread TBJ FLAG or TBJ IL-27 tumors treated with IL-2 or vehicle alone. Bone marrow specimens were isolated from mice in the respective groups, and $1 \times 10^7$ nucleated bone marrow cells were injected s.c. into naive littermate control mice. Tumors formed after injection of bone marrow cells in six of ten mice (60%) bearing TBJ IL-27 tumors, seven of ten mice (70%) bearing TBJ FLAG tumors, and nine of ten mice (90%) bearing TBJ-FLAG tumors treated with IL-2. In marked contrast, none of the ten (0%) mice injected with the bone marrow from mice bearing TBJ IL-27 tumors and treated with IL-2 developed tumors over 85...
Cohorts of mice (10 mice/group) were injected with 1 × 10^6 TBJ-IL-27 or TBJ-FLAG tumor cells on day 0. Mice were concurrently depleted of NK cells, CD4^+ T cells or CD8^+ T cells as described in Materials and Methods. Mice were treated with either IL-2 or medium alone on days 5–9, 12–16, 19–23, or 26–30 post tumor implantation. Mice surviving at last follow-up were tumor free. In complementary studies, IL-27 plus IL-2 vs TBJ IL-27 plus vehicle (Fig. 4A). The data shown represent the proportion of mice developing colonies assay in the presence of G418 (1 mg/ml) as described in Materials and Methods. The data shown represent the proportion of mice developing colonies in each of the respective treatment groups, *p = 0.0198; TBJ IL-27 plus IL-2 vs TBJ IL-27 plus vehicle (A). In complementary studies, cohorts of mice (10 mice/group) were injected with 1 × 10^6 TBJ-FLAG or TBJ-IL-27 cells on day 0, followed by therapy with IL-2 or vehicle on days 5–9 or 12–16 post tumor cell injection. Nineteen days post tumor injection, mice were euthanized and bone marrow cells were isolated as described in Materials and Methods. Single cell suspensions containing both marrow cells and metastatic neuroblastoma tumor cells were prepared and injected s.c. (1 × 10^6 cells/injection) into naive mice to assess the tumorigenicity of contaminating neuroblastoma metastases in the bone marrow. Tumor size was monitored twice a week. The proportion of mice that developed a tumor at any time post injection of bone marrow cells from the respective groups is shown in B. **p = 0.011, TBJ IL-27 plus IL-2 vs TBJ IL-27 plus vehicle.

Contribution of adaptive immunity to the antitumor activity of IL-27/IL-2

Among the mice treated with combined delivery of IL-27 and IL-2, we found that seven of nine mice (77.7%) that were cured of their original tumor rejected a subsequent rechallenge with wild-type parental TBJ tumor cells. In contrast, all mice (10/10) in the naive rechallenge control group died of tumor (data not shown). To directly assess the role of T and/or NK cell subsets in the antitumor mechanisms induced by combined delivery of IL-27 and IL-2, mice bearing disseminated TBJ-IL-27 tumors were treated with IL-2 and concurrently depleted of either NK cells or CD4^+ vs CD8^+ T cells as described above, and then monitored for survival. Among mice bearing TBJ-IL-27 tumors treated with IL-2, complete durable tumor regression was achieved in ten of ten nondepleted mice (100%), eight of ten mice (80%) treated with normal rabbit serum, nine of ten mice (90%) depleted of CD4^+ T cells, and nine of ten mice (90%) depleted of NK cells, but zero of ten mice depleted of CD8^+ T cells (p < 0.0001, nondepleted vs CD8^+ T cells depleted; p = 1.0, nondepleted vs either CD4^+ or NK cells depleted; p < 0.0001, CD8^+ T cell depleted vs either CD4^+ T, or NK cell depleted) (Fig. 5). These findings clearly demonstrate that CD8^+ T cells but not CD4^+ T cells or NK cells are the predominant effectors cells mediating the antitumor effects of IL-27 and IL-2 therapy in the disseminated TBJ neuroblastoma model. Notably, IL-27/IL-2 therapy significantly enhanced the number of CD8^+ cells within the tumor microenvironment (data not shown).

Local delivery of IL-27 inhibits IL-2 induced expansion of CD4^+ CD25^+ Foxp3^+ regulatory T and IL-17 expressing CD4^+ T cells within the microenvironment of TBJ tumors

IL-2 appears to play a critical role in the regulation of CD4^+ CD25^+ Foxp3^+ regulatory T cells within the tumor microenvironment of TBJ tumors (31). In that combined delivery of IL-27 and IL-2 nonetheless mediates potent antitumor activity in vivo, we subsequently investigated whether IL-27 could modulate IL-2-induced alterations in regulatory T cell populations. Studies were performed using orthotopic TBJ Flag or TBJ-IL-27 tumors treated with IL-2 or vehicle alone. In this setting, IL-2 induces an increase by 2.42-fold in the number of regulatory T cells within the microenvironment of TBJ tumors. To further characterize the regulation of regulatory T cells by IL-2 was substantially inhibited by concurrent delivery of IL-27 (IL-27 + IL-2: %CD4^+ CD25^+ Foxp3^+ = 45.95 ± 6.13; vehicle control: %CD4^+ CD25^+ Foxp3^+ = 19.01 ± 4.24; p = 0.0172). Similar results were obtained using metastatic tumors (data not shown). Recently, it was shown that IL-27 can block the production of IL-17, among other cytokines, by activated CD4^+ T cells.
mechanistic effects of IL-2 and IL-27 combined therapy, intracellular cytokine analysis was assessed by flow cytometry. IL-2 induced a marked increase in IL-17 content in tumor infiltrating CD4^+ T cells (%CD4^+IL17^+ cells: 53.24 ± 12.15) but not in CD8^+ or NK cells (data not shown). And this effect was remarkably inhibited by IL-27 (IL-2 IL-27%CD4^+IL17^+ cells: 6.6 ± 5.76). (Fig. 6, B and D). Thus, the potentially deleterious effects of IL-2 regarding the increase of tumor infiltrating regulatory T cells and IL17 producing CD4 cells in this tumor model was counteracted by IL-27.

**Combined delivery of IL-27 and IL-2 synergistically enhances immunologic reactivity: induction of CTL responses**

To more directly investigate whether the combination of IL-27 and IL-2 could influence tumor-specific immunologic reactivity, we

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**FIGURE 6.** IL-27 inhibits IL-2-induced increases in the proportion of CD4^+CD25^+Foxp3^+ T cells within TIL from TBJ neuroblastoma tumors. Mice bearing primary orthotopic TBJ Flag or TBJ-IL-27 tumors treated with vehicle control or IL-2 were euthanized as described in the Materials and Methods section. Tumors were dissected and TIL were isolated and stained with PECy7 conjugated anti-mouse CD4, allophycocyanin conjugated anti-mouse IL17A, and FITC-conjugated anti-mouse Foxp3 as described in Materials and Methods. CD45 was used as marker for leukocytes. The percentage of CD4^+CD25^+Foxp3^+ T cells within the total CD4^+ T cell population is shown A and C. The percentage of CD4^+ IL-17^+ cells within the CD45^+ gate (B) and the percentage of IL-17^+ expression within CD4^+ TILs is shown for tumor-derived samples from different treatment groups (D).

**FIGURE 7.** Combined exposure to IL-27 and IL-2 synergistically enhances immune responsiveness: IFN-γ production. Spleens were resected under sterile conditions from mice bearing disseminated TBJ IL-27 or TBJ FLAG (control) tumors at day 15 post tumor implantation. Single cell suspensions were prepared and splenocytes were then restimulated with the corresponding irradiated TBJ FLAG or TBJ IL-27 tumor cells respectively in the presence (10 IU/ml) or absence of IL-2 for 3 days. Culture supernatants were then harvested and the production of IFN-γ was assayed by ELISA. More than 30-fold greater production of IFN-γ was observed when splenic effector cells from mice bearing TBJ IL-27-expressing tumors were restimulated with irradiated TBJ IL-27 tumor cells in the presence of IL-2 vs medium alone or effector cells from mice bearing TBJ FLAG tumors and restimulated with irradiated TBJ FLAG cells in the presence of IL-2 or medium alone (TBJ FLAG plus medium alone: <9.4 pg/ml; TBJ FLAG plus IL-2: 37 ± 22 pg/ml; TBJ IL-27 plus medium alone: 12 ± 3 pg/ml; TBJ IL-27 plus IL-2: 1301 ± 219 pg/ml) (Fig. 7). These observations demonstrate that the combination of IL-27 and IL-2 can synergistically enhance the production of IFN-γ in mice bearing metastatic neuroblastoma tumors.

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*Combined delivery of IL-27 and IL-2 synergistically enhances tumor-specific immunologic reactivity: induction of CTL responses*

In light of the potent immunologic memory responses generated in mice treated with combined delivery of IL-27 and IL-2, and the ability of IL-27 to suppress IL-2-induced expansion of CD4^+CD25^+Foxp3^+ regulatory T cells within the TIL population, we directly investigated the ability of this combination to modulate immunologic reactivity in vivo. Splenic effector cells from mice bearing disseminated TBJ FLAG or TBJ IL-27 tumors were harvested 15 days post tumor implantation and were restimulated with the corresponding irradiated TBJ FLAG or TBJ IL-27 tumor cells respectively in the presence (10 IU/ml) or absence of IL-2 for 3 days. Culture supernatants were then harvested and the production of IFN-γ was assayed by ELISA. More than 30-fold greater production of IFN-γ was observed when splenic effector cells from mice bearing TBJ IL-27-expressing tumors were restimulated with irradiated TBJ IL-27 tumor cells in the presence of IL-2 vs medium alone or effector cells from mice bearing TBJ FLAG tumors and restimulated with irradiated TBJ FLAG cells in the presence of IL-2 or medium alone (TBJ FLAG plus medium alone: <9.4 pg/ml; TBJ FLAG plus IL-2: 37 ± 22 pg/ml; TBJ IL-27 plus medium alone: 12 ± 3 pg/ml; TBJ IL-27 plus IL-2: 1301 ± 219 pg/ml) (Fig. 7). These observations demonstrate that the combination of IL-27 and IL-2 can synergistically enhance the production of IFN-γ in mice bearing metastatic neuroblastoma tumors.
also investigated the impact of this combination on the generation of tumor-specific cytolytic activity in mice bearing metastatic neuroblastoma tumors using the Indium (C9H6NO)3-In-111-release assay. The design of these studies allowed us to investigate the relative role of IL-27 both in enhancing the in vivo priming of effector cells as well as the effector limb of the immune response when effector cells were re-exposed to irradiated tumor cells ex vivo. Spleens from mice bearing disseminated TBJ FLAG or TBJ IL-27 tumors were resected under sterile conditions at day 15 post tumor implantation, and single cell suspensions of murine splenocytes were expanded in vitro using restimulation with either irradiated TBJ FLAG or TBJ IL-27 cells in the presence or absence of IL-2 (10 IU/ml). At the conclusion of the culture period, effector cells were harvested, and the generation of CTL activity against indium labeled TBJ FLAG tumor cells or irrelevant syngeneic SA-1 tumor target cells was assessed.

Lymphocytes from mice bearing disseminated TBJ FLAG tumors and restimulated ex vivo with irradiated TBJ FLAG tumor cells exhibited very low level CTL activity (percent lysis at 50:1 E:T ratio = 10.3 ± 2.4), and this was increased by coincubation with IL-2 (percent lysis at 50:1 E:T ratio = 24.1 ± 0.8), p < 0.001. In contrast, restimulation of spleen cells from mice bearing disseminated TBJ FLAG tumors with irradiated TBJ IL-27 tumor cells, enhanced tumor specific lysis by 4-fold (percent lysis at 50:1 E:T ratio = 40.0 ± 1.2) and concurrent addition of IL-2 synergistically enhanced the generation of CTL reactivity (percent lysis at 50:1 E:T ratio = 90.3 ± 4.5), p < 0.001. These observations demonstrate that IL-27 and IL-2 can synergistically enhance the generation of CTL reactivity, and that IL-27 can mediate its effects at the effector phase in the generation of this response. Interestingly, restimulation of splenocytes with irradiated TBJ-FLAG tumor cells demonstrates substantially higher CTL reactivity using effector cells from mice bearing disseminated TBJ IL-27 tumors compared with the responses generated using effector cells from mice bearing disseminated TBJ FLAG tumors (percent lysis at 50:1 E:T ratio: TBJ IL-27 in vivo plus TBJ FLAG ex vivo = 38.3 ± 2.3% vs TBJ FLAG in vivo plus TBJ FLAG ex vivo = 10.3 ± 2.4%), p < 0.001 (Fig. 8A). Similar increases in CTL reactivity are observed when splenocytes from mice bearing disseminated TBJ-FLAG tumors are restimulated with irradiated TBJ IL-27 tumor cells compared with those restimulated with irradiated TBJ FLAG tumor cells (% lysis at 50:1 E:T ratio, TBJ FLAG in vivo plus TBJ IL-27 ex vivo = 40.0 ± 1.2% vs TBJ FLAG in vivo plus TBJ FLAG ex vivo = 10.3 ± 2.4% as noted above), p < 0.001 (Fig. 8B). These observations indicate that IL-27 can also mediate important immunoregulatory effects during both the sensitization/priming and effector phases of the immune response for the generation of CTL reactivity. We have also confirmed the specificity of the cytolytic responses observed under these conditions in that negligible reactivity was generated against irrelevant syngeneic SA-1 tumor cells (data not shown). These findings demonstrate that IL-27 in combination with IL-2 can synergistically enhance the generation of specific cytotoxic activity against TBJ neuroblastoma tumors, and that IL-27 can potentiate the generation of CTL reactivity via mechanisms that act both at the initial sensitization phase as well as the effector phase of CTL generation.

**Discussion**

The generation of a productive adaptive immune response is coordinately regulated by potent immunoregulatory cytokines that are generated by monocyte/macrophage and dendritic cell populations during innate immune surveillance mechanisms (33). These include a group of heterodimeric IL-12 related cytokines (IL-12, IL-23, and IL-27) (28, 34, 35), as well as IL-18 (36). Collectively, these cytokines potentiate the initiation, expansion, and effector phases of an evolving adaptive T cell mediated immune response that leads ultimately to the induction of IFN-γ. In turn, these immunoregulatory cytokines including IL-12 (37 and 38), IL-23 (22–24), IL-27 (25–27), and IL-18 (39) can mediate marked antitumor efficacy in preclinical tumor models. Further, we and others have now shown that IL-2 can synergistically enhance the antitumor activity of IL-12 (40–43) and IL-18 (4, 44, 45) in preclinical tumor models. More recently, we have shown that IL-27 alone can mediate complete regression and long term survival in up to 90% of mice bearing s.c. and orthotopic intra-adrenal primary neuroblastoma tumors (26). Nonetheless, IL-27 alone mediates complete tumor regression and long-term survival in only 40% of mice bearing disseminated neuroblastoma tumors. Given the frequent occurrence of metastatic disease in patients with high-risk neuroblastoma, and the poor prognosis of these patients, we have actively investigated approaches to potentiate the therapeutic efficacy of IL-27 in this setting using cytokines such as IL-2.
The present studies provide the first evidence to our knowledge that IL-2 can enhance the antimitotic effects of IL-27 in vivo, with up to 90% of mice bearing disseminated TBJ neuroblastoma metastases achieving complete tumor regression and long-term survival. We also have directly investigated the effects of combined delivery of IL-27 and IL-2 on disease burden within common sites of neuroblastoma metastasis in this model including both the liver and the bone marrow. Interestingly, both IL-27 alone and combined delivery of IL-27 and IL-2 mediate significant regression of metatstatic neuroblastoma tumors in the liver, although the reductions in tumor burden are greatest in the IL-27/IL-2 combination group. Further, there is clear synergy by combined delivery of IL-27 and IL-2 in eliminating bone marrow metastases. Marked reductions in the disease burden (as assessed by bone marrow colony counts) are noted in mice exposed to the combination of IL-27/IL-2 compared with mice treated with either IL-27 or IL-2 alone or untreated control mice. Further, follow-up re-injection of bone marrow cell preparations was also performed to evaluate the tumorigenicity of disease burden within the bone marrow of mice from the respective groups. Complete loss of tumor formation was noted in virtually all mice in the IL-27/IL-2 group, while tumors formed in the majority of mice exposed to IL-27 alone, IL-2 alone, or untreated control groups. The ability of IL-27/IL-2 to mediate potent antitumor effects against metastatic neuroblastoma tumors may be particularly useful therapeutically given the high frequency of bone marrow disease in patients with high-risk neuroblastoma (46–48) and the poor prognosis of these patients. Future studies will also attempt to evaluate whether metastatic neuroblastoma tumor cells can be detected in the peripheral blood of mice bearing TBJ neuroblastoma tumors, and the impact of IL-27/IL-2 on the presence of tumor cells in this compartment. This may be particularly important to address given the frequent use of peripheral blood stem cells as hematologic support in high-risk neuroblastoma patients treated with myeloablative consolidation chemotherapy (49), and the worsened outcome of patients treated with peripheral blood stem cell products contaminated with tumor cells (50).

Consistent with previous in vitro studies using human effector cells (NK cells) (28), we have found that treatment of murine splenocytes with the combination of IL-27 and IL-2 can synergistically enhance proliferative responses and IFN-γ production in vitro. Previous studies have emphasized the functional role of IL-27 in activating naive T cell populations (28, 51), while IL-23 is felt to act predominantly on memory T cell populations (52). We previously demonstrated that IL-27 alone appears to both directly and indirectly up-regulate MHC class I expression on tumor cells in vitro and in vivo, and to enhance the generation of tumor-specific immunologic reactivity and immunologic memory in mice cured of their original TBJ neuroblastoma tumors by IL-27 (26). In the present studies, administration of IL-2 in combination with IL-27 also induces an effective T cell memory response even in mice bearing advanced metastatic disease. Mice cured of their original disseminated neuroblastoma tumors by combined delivery of IL-27 and IL-2 reject a subsequent rechallenge with TBJ parental tumor cells. Consistent with these observations, we have now demonstrated with depletion studies that the antitumor mechanisms engaged by combined delivery of IL-27 and IL-2 are clearly dependent on CD8+ T cells, but not CD4+ T cells or NK cells. Further, the combination of IL-27 and IL-2 also synergistically enhances the generation of immunologic reactivity as evidenced by the production of IFN-γ in response to ex vivo restimulation with irradiated tumor cells. Greater than 30-fold increases in IFN-γ production are seen when splenocytes from mice bearing disseminated TBJ-IL-27 tumors are restimulated ex vivo with the corresponding irradiated TBJ-IL-27 tumor cells in the presence of IL-2 compared with the same effector cells restimulated ex vivo with irradiated TBJ-IL-27 tumors alone or effector cells from mice bearing TBJ-FLAG tumors restimulated ex vivo with the irradiated TBJ-FLAG in the presence or absence of IL-2.

We previously reported that delivery of IL-27 alone can enhance the generation CTL responses to TBJ neuroblastoma tumors (26). Further, others have reported that IL-27 can act directly on naive CD8+ T cells to enhance granzyme B expression and the generation CTL reactivity directed against C26 colon carcinoma (53). In this study, we further demonstrate that IL-27 enhanced the generation of antitumor CTL responses via mechanisms that occur at not only the initial sensitization or priming of effector cells, as previous literature would predict (28, 54), but also at the effector phase when primed effector cells are restimulated with tumor cells ex vivo, an unexpected finding suggesting that the immunoregulatory activity of IL-27 may not be tightly restricted to naive T cell populations in vivo. Further, combined exposure to IL-27 and IL-2 synergistically enhances the generation of CTL responses specific for TBJ but not irrelevant syngeneic SA-1 tumor cells, and the overall antitumor effects were mediated via CD8+ T cells but not CD4+ T cells or NK cells.

The observation that IL-2-deficient mice develop autoimmunity (55) and that in vivo neutralization of IL-2 results in autoimmune disease with a concomitant reduction in the number of CD4+CD25+Foxp3+ regulatory T cells. Administration of systemic IL-2 can increase the frequency circulating CD4+CD25+Foxp3+ regulatory T cells in patients with metastatic melanoma or renal cell carcinoma, leading to the suggestion that IL-2-induced expansion of regulatory T cells could compromise the efficacy of IL-2 therapy in these patients (32). Consistent with these observations, our results demonstrate that IL-2 administration leads to enhancement of the proportion of regulatory T cells within TIL populations in TBJ murine neuroblastoma tumors. Moreover, we demonstrate that IL-2 induced increases in the proportion of CD4+CD25+Foxp3+ regulatory T cells within TILs appeared to be strongly attenuated by concurrent delivery of IL-27. Although the role of this effect in the overall antitumor activity mediated by combined delivery of IL-27 and IL-2 remains to be defined, this is the first report to our knowledge indicating that IL-2-induced increases in tumor-infiltrating regulatory T cells can be inhibited by IL-27 in vivo, and this observation may provide important new insight for the investigation of cytokine combinations in the treatment of solid tumors. Concordantly, in vitro studies indicated that IL-27 inhibits generation of regulatory T cells induced by TGF-β, in part via STAT3 (56, 57). In a recent study, treatment of mice bearing Neuro-2a neuroblastoma cells, with CD25 Ab resulted in reduced levels of CD4+Foxp3+ cells, fostered tumor rejection, and enhanced immune responses in tumor-vaccinated mice (58). The authors pointed out the important role of T regulatory cells in enabling neuroblastoma growth. We have confirmed this finding using our TBJ neuroblastoma cell line, which was derived from Neuro-2a (data not shown).

Our data regarding the suppressive effects of IL-27 on tumor-infiltrating IL-17+CD4+ T cells is in line with reports indicating that IL-27 can attenuate the production of proinflammatory cytokines on activated CD4+ T cells, including IL-17, via STAT3 (59). Concordantly, IL-27 attenuating effects on collagen-induced arthritis correlated with a decrease of IL-17 content in leukocytes from these mice (60). It is of note that previous reports indicated that IL-2 attenuates the production of IL-17 (61, 62), nevertheless, in our in vivo tumor model, IL-2 therapy had a dramatic effect on promoting IL-17 expression by CD4+ tumor-infiltrating leukocytes.
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The present studies demonstrate that combined delivery of IL-27 and IL-2 can mediate potent antitumor effects even in mice bearing disseminated murine neuroblastoma, and that the effects of IL-27/IL-2 may be particularly pronounced against metastatic tumor in the bone marrow, a common site of disease involvement in children with high-risk neuroblastoma. Further, these studies have now provided novel insight into the potent immunoregulatory activity mediated by IL-27/IL-2, in that we have shown that the combination synergistically enhances the generation of tumor-specific CTL responses and that IL-27 potentiates these responses both during the initial sensitization of effector cells as well as during the subsequent effenter limb of the response when effector cells are restimulated with tumor. Collectively, these observations also provide the first evidence that an IL-27-based therapeutic regimen can mediate antitumor effects against bone marrow metastases, and suggest that clinical investigation of IL-27/IL-2 may be warranted in children with high-risk neuroblastoma, particularly those patients with marrow involvement.

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Disclosures

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References


