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A recombinant antibody–interleukin 2 fusion protein suppresses growth of hepatic human neuroblastoma metastases in severe combined immunodeficiency mice

(cytokine fusion protein/hepatic metastases)

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ABSTRACT **A genetically engineered fusion protein consisting of a human/mouse chimeric anti-ganglioside GD2 antibody (ch14.18) and recombinant human interleukin 2 (rhIL-2) was tested for its ability to target rhIL-2 to tumor sites and stimulate immune effector cells sufficiently to achieve effective tumor cell lysis *in vivo*. The ch14.18–IL-2 fusion protein proved more effective than equivalent doses of rhIL-2 in suppressing dissemination and growth of human neuroblastoma in an experimental hepatic metastases model of scid (severe combined immunodeficiency) mice reconstituted with human lymphokine-activated killer cells. The ch14.18–IL-2 fusion protein was also more proficient than equivalent doses of rhIL-2 in prolonging the life-span of these animals. This recombinant antibody–cytokine fusion protein may prove useful for future treatment of GD2-expressing human tumors in an adjuvant setting.**

Cancer immunotherapy with interleukin 2 (IL-2) has found increased use during the past few years because of its stimulatory effects on a wide range of immune effector cells and the availability of large quantities of recombinant human IL-2 (rhIL-2) (1–5). Stimulation of peripheral blood lymphocytes by IL-2 *in vitro* and *in vivo* produced lymphokine-activated killer (LAK) cells, derived mainly from natural killer (NK) cells, that were applied in clinical trials that produced responses in some patients with melanoma and renal carcinoma (1, 2). In pediatric neuroblastoma, systemic therapy with rhIL-2 resulted in only modest regressions of metastases with marginal clinical improvement (6, 7). This treatment did, however, stimulate the potent generation of NK and cytotoxic T cells, as well as functionally active LAK cells (4, 7).

Clinical studies indicated that prolonged partial and complete remissions of pediatric neuroblastoma could be induced by both murine anti-GD2 antibody 14.G2a (8) and its human/chimeric variant ch14.18 (9). Prior preclinical experiments had shown that both of these antibodies could effectively mediate antibody-dependent cellular cytotoxicity (ADCC) by peripheral blood mononuclear cells (10, 11). This ADCC-mediated killing of neuroblastoma cells could be further augmented by $CD56^+$ NK cells that were produced in increased amounts by IL-2 treatment of pediatric neuroblastoma patients (12). Furthermore, therapy of neuroblastoma patients with rhIL-2 and monoclonal antibody (mAb) 14.G2a generated conditions within the peripheral blood of these patients that enabled their own lymphocytes to mediate ADCC sufficiently to effectively kill human neuroblastoma cells *in vitro* (13). Taken together, these findings provided a rationale for evaluating the efficacy of an anti-GD2–IL-2 fusion protein in killing human neuroblastoma cells *in vivo*.

In the studies described here, we used a genetically engineered fusion protein, ch14.18–IL-2, consisting of chimeric anti-ganglioside GD2 antibody (ch14.18) and rhIL-2. This ch14.18–IL-2 fusion protein was shown previously to target IL-2 to tumor cells and to stimulate T-cell-mediated killing of autologous melanoma cells *in vitro* more effectively than equivalent amounts of rhIL-2 (14). In this report, we demonstrate the ability of the ch14.18–IL-2 fusion protein to suppress the dissemination and growth of human neuroblastoma in experimental hepatic metastases of severe combined immunodeficiency (scid) mice in the presence of human LAK cells and to prolong the life-span of these animals. Our results indicate that the ch14.18–IL-2 fusion protein is considerably more proficient in achieving these objectives than equivalent amounts of rhIL-2. These findings support our hypothesis that recombinant mAb–cytokine fusion proteins can specifically target cytokines to tumor sites and stimulate immune effector cells sufficiently to achieve efficient tumor cell lysis.

MATERIALS AND METHODS

Cytokine, Antibody, and Antibody–Cytokine Fusion Protein. rhIL-2 derived from *Escherichia coli* was a gift from Amgen Biologicals. Mouse/human chimeric anti-ganglioside GD2 antibody ch14.18 has been described (10, 15). The ch14.18–IL-2 fusion protein was constructed as reported by fusing a synthetic human IL-2 sequence to the carboxyl end of the human Cγ1 gene (14).

Tumor Cell Line. The human neuroblastoma cell line SK-N-AS was a gift from D. L. Helson (Memorial Sloan–Kettering Cancer Center, New York). These cells were maintained in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine at 37°C in 7.5% CO_2.

Direct Binding Assay with Radioiodinated Proteins. mAb ch14.18 and the ch14.18–IL-2 fusion protein were each iodinated for saturation binding assays with Iodo-Gen (Pierce) as described (16). The specific activity of ^{125}I-labeled ch14.18 and ^{125}I-labeled ch14.18–IL-2 was typically 0.5–1.5 nCi per ng of antibody (1 Ci = 37 GBq). SK-N-AS neuroblastoma cells (3×10^4 cells per well) in serum-free RPMI 1640 medium were plated into 24-well plates and allowed to attach overnight. The plates were placed on ice and wells

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Abbreviations: IL-2, interleukin 2; rhIL-2, recombinant human IL-2; LAK cells, lymphokine-activated killer cells; NK cells, natural killer cells; ADCC, antibody-dependent cellular cytotoxicity; mAb, monoclonal antibody; IU, international units; scid, severe combined immunodeficiency.

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were washed twice with ice-cold binding buffer [RPMI 1640 medium containing 0.1% bovine serum albumin (BSA), 0.02% NaN_3, and 20 mM Hepes (pH 7.5)]. Cells in binding buffer were incubated with either ^{125}I-labeled ch14.18 or ^{125}I-labeled ch14.18–IL-2. Nonspecific binding was determined in the presence of a 200-fold excess of unlabeled ch14.18 and was generally found to be <15% of the total bound radiolabel. After 2 hr of incubation on ice, the plates were washed with phosphate-buffered saline (PBS) containing 0.1% BSA and 0.02% NaN_3. After this step, control wells from each plate were trypsin treated to assess the number of cells remaining and this cell count was used to calculate the number of binding sites per cell. Cell-bound radiolabel in duplicate samples was determined in a γ-scintillation counter and the data were analyzed by the method of Scatchard (17).

IL-2 Assay. IL-2 activity of the ch14.18–IL-2 fusion protein was determined with a standard T-cell proliferation assay by measuring the uptake of [^{3}H]thymidine into the IL-2-dependent mouse cell line CTLL-2 (18). Briefly, after IL-2 depletion for 48 hr, 3×10^4 CTLL-2 cells were added to individual wells of a 96-well microtiter plate in a vol of 200 μl with various concentrations of rhIL-2 or fusion protein normalized for rhIL-2 content. After 24 hr, 0.5 μCi of [*methyl*-^{3}H]thymidine was added to each well and plates were harvested 12 hr later. All samples were tested in quadruplicate.

Human LAK Cells. Peripheral blood mononuclear cells were isolated from the blood of healthy volunteers by density gradient centrifugation. LAK cells were generated by incubation of these cells with rhIL-2. Routinely, rhIL-2 [200 international units (IU)/ml] was admixed with 3×10^6 peripheral blood lymphocytes and incubated for 72 hr at 37°C.

Cytotoxicity Assay. Cytotoxic activity of LAK cells was measured in a 4-hr ^{51}Cr-release assay as described (10). Briefly, ch14.18 or ch14.18–IL-2 was diluted in culture medium to 10 μg/ml (50 μl) and added to wells of 96-well U-bottomed microtiter plates (Corning) containing ^{51}Cr-labeled SK-N-AS cells (5×10^3 cells) and 25×10^3 LAK cells in a total vol of 150 μl. All tests were done in triplicate at a target/effector ratio of 1:5.

Animals. C.B-17 *scid/scid* mice were obtained from the rodent breeding colony of The Scripps Research Institute and 6- to 8-week-old mice were routinely used for metastasis experiments. These animals were housed under specific pathogen-free conditions and all experiments were performed according to National Institutes of Health guidelines for the care and use of laboratory animals.

Hepatic Metastasis Model. All surgical procedures were done under sterile conditions in a laminar flow hood. Scid mice were anesthetized by intraperitoneal (i.p.) injection of 2.5 mg of ketamine. The skin of the animals was prepared for surgery with povidone-iodine and a left subcostal incision was made to expose the spleen. SK-N-AS cells (5×10^5 cells) in 100 μl of PBS was injected with a 27-gauge needle beneath the splenic capsule during a period of 1–2 min. The needle was removed after an additional 2 min, the splenic pedicle was ligated with a 5.0 silk suture, and the incision was closed with staples. Four weeks after the injection of SK-N-AS cells, the mice were sacrificed and their livers were fixed in Bouin's solution and examined under a low-magnification microscope for tumor foci visible on the surface. The statistical significance of the difference in the number of metastatic foci between control and experimental groups of animals was determined with the nonparametric Wilcoxon rank sum test.

Adoptive Immunotherapy Model. One day after intrasplenic injection of 5×10^5 SK-N-AS neuroblastoma cells and induction of hepatic metastasis, a control group received daily i.p. injections of 0.2 ml of PBS. The other animals were each injected i.p. with 4×10^7 human LAK cells and then randomized into experimental groups, each being injected i.p. for 7 days with one of the following: 0.2 ml of PBS, ch14.18, ch14.18 plus rhIL-2, or ch14.18–IL-2 fusion protein containing an equivalent amount of rhIL-2. One month after tumor cell injection, all animals were sacrificed and their livers were examined for neuroblastoma metastases.

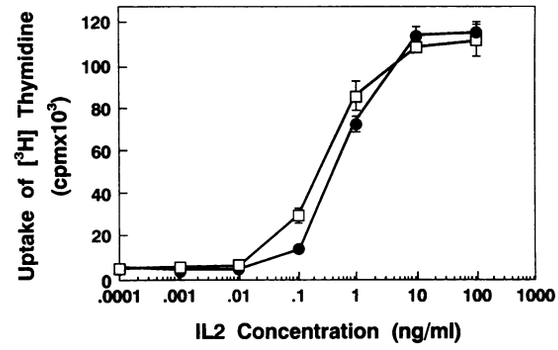

FIG. 1. IL-2 activity of ch14.18–IL-2 fusion protein and rhIL-2. Dilutions of ch14.18–IL-2 (●) and rhIL-2 (□) were compared by [^{3}H]thymidine incorporation into IL-2-dependent mouse CTLL-2 cells. Values were normalized to the content of IL-2 in the fusion protein.

RESULTS

Antigen-Binding Activity of ch14.18–IL-2 Fusion Protein. Both mAb ch14.18 and the ch14.18–IL-2 fusion protein revealed essentially identical binding patterns with human neuroblastoma cells in direct binding assays. Dissociation constants (K_d) and number of binding sites for ^{125}I-labeled ch14.18 and ch14.18–IL-2 were calculated from Scatchard analysis of saturation binding curves obtained with SK-N-AS cells. The average K_d in three independent assays was 1.8×10^{-8} M for ch14.18 and 2.4×10^{-8} M for ch14.18–IL-2. Both ch14.18 and the ch14.18–IL-2 fusion protein bound to the same number of GD2 sites—i.e., 1.1×10^6 per SK-N-AS neuroblastoma cell.

IL-2 Activity. The IL-2 activity of ch14.18–IL-2 was measured as the proliferation of the IL-2-dependent mouse cell line CTLL-2 and compared against that of rhIL-2. Cells were incubated with serial dilutions of rhIL-2 or ch14.18–IL-2 covering the range of 0.1 pg/ml to 1×10^6 pg/ml. The concentration of IL-2 in the ch14.18–IL-2 fusion protein was

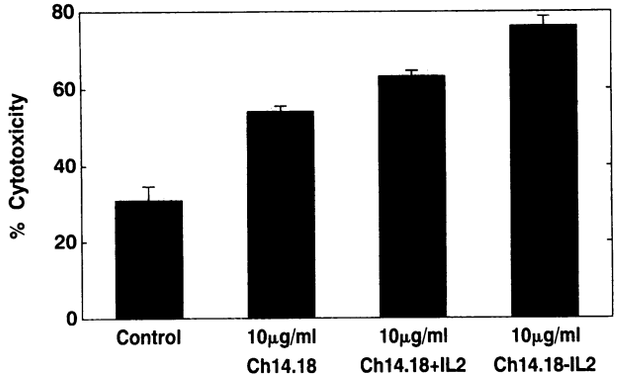

FIG. 2. Enhancement of ADCC activity by pretreatment of LAK cells with ch14.18–IL-2 fusion protein. Cytotoxic activities of the following were determined in a ^{51}Cr release assay: control (without ch14.18), ch14.18 (10 μg/ml), ch14.18 (10 μg/ml) plus 30,000 IU of rhIL-2, and ch14.18–IL-2 fusion protein (10 μg/ml) containing 30,000 IU of rhIL-2. SK-N-AS cells (5×10^3 cells) were added in 50 μl followed by 25×10^3 LAK cells in a total vol of 200 μl. All tests were done in triplicate at a target/effector ratio of 1:5.

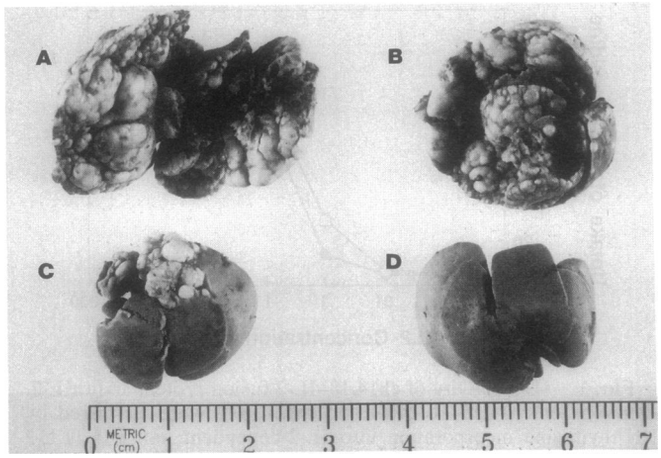

FIG. 3. Hepatic metastasis of SK-N-AS neuroblastoma cells. A representative liver specimen is depicted after completion of the initial adoptive immunotherapy experiments described in Table 1. The therapy regimens were PBS control (*A*), LAK cells plus PBS (*B*), LAK cells plus ch14.18 (*C*), and LAK cells plus ch14.18–IL-2 fusion protein (*D*).

calculated as two molecules of IL-2 per molecule of the fusion protein. As shown in Fig. 1, the IL-2 activity of the ch14.18–IL-2 fusion protein was essentially identical to that of a comparable amount of rhIL-2.

Cytotoxicity Mediated by Human LAK Cells. We have previously reported that ch14.18 was effective in mediating the lysis of human neuroblastoma cells by human effector cells such as granulocytes and NK cells (11). We observed here that the lysis of SK-N-AS neuroblastoma cells by human LAK cells was most effectively potentiated by the ch14.18–IL-2 fusion protein when compared to either ch14.18 or an admixture of ch14.18 and rhIL-2 (Fig. 2). Essentially the same amount of lysis was obtained in three separate experiments done at identical effector/target cell ratios (5:1) with antibody concentrations of 0.1, 1, and 10 μg/ml, respectively (data not shown).

Biodistribution of Human LAK Cells in scid Mice. The biodistribution and time of survival of human LAK cells injected into scid mice was determined to evaluate their potential in mediating *in vivo* lysis of SK-N-AS cells coated with either ch14.18 or ch14.18–IL-2. Human peripheral blood lymphocytes, incubated with rhIL-2 for 72 hr and then labeled with ^{51}Cr, were injected i.p. into scid mice. Approximately 20% of the total radioactivity injected was detected 48 hr later in the liver of these animals, suggesting that injected LAK cells localize, in part, to the major metastasis site in our model (data not shown).

Hepatic Metastasis of SK-N-AS. Upon intrasplenic injection of SK-N-AS cells and excision of the spleen, hepatic metastases were found in 100% of the scid mice 4 weeks postinjection. Lymph node involvement was not detected in any of these animals. The presence of metastatic neuroblastoma in the liver was confirmed by histological examination and demonstrated to be distributed throughout the hepatic parenchyma (data not shown). As depicted in Fig. 3, in the majority of these animals, most of the liver tissue was replaced by tumor.

Suppression of Hepatic Neuroblastoma Metastases in scid Mice. The results of studies designed to critically assess the efficacy of the ch14.18–IL-2 fusion protein in suppressing dissemination and growth of hepatic neuroblastoma metastases in scid mice are summarized in Table 1. There was a lack of a statistically significant difference from controls in the number of hepatic metastases found in scid mice treated with either PBS or LAK cells. Most of these animals presented with a very large number (>500) of metastatic foci in their livers that was not possible to count accurately and exhibited up to 3-fold greater liver weights than untreated animals. Although scid mice treated with LAK cells plus ch14.18 showed a statistically significant decrease ($P = 0.01$) in the number of metastatic foci, all of these animals presented with metastases ranging from 5 to 100 liver foci and had increased liver weights. In contrast, all scid mice treated with the ch14.18–IL-2 fusion protein revealed a complete absence of macroscopic metastatic liver foci ($P = 0.003$) and also presented with normal liver weights. However, when scid mice were treated with a mixture of ch14.18 and rhIL-2 at dose levels equivalent to the ch14.18–IL-2 fusion protein, they also showed a complete lack of macroscopic metastatic liver foci (Table 1). Identical results were obtained when scid mice were treated with high doses (7.5×10^5 IU/day) of rhIL-2 *per se* (data not shown). Since we observed these same effects with both ch14.18–IL-2 and a mixture of ch14.18 and rhIL-2 at these relatively high dose levels—i.e., 250 μg of ch14.18–IL-2—we compared their effect at lower dose levels. As shown in Table 2, as little as 1 μg of ch14.18–IL-2 per injection proved effective in suppressing dissemination and growth of metastasis. Indeed, dose levels of 1, 8, and 16 μg of ch14.18–IL-2 fusion protein were far more capable in suppressing growth of tumor metastasis than equivalent doses of rhIL-2. Moreover, another set of experiments indicated that relatively low dose levels of ch14.18–IL-2 (1 and 16 μg) were also more proficient than equivalent amounts of rhIL-2 (3000 and 48,000 IU) in prolonging the life-span of tumor-bearing scid mice reconstituted with human LAK cells (Table 3).

DISCUSSION

The application of recombinant DNA technology has recently led to the construction of antibody–cytokine fusion proteins designed to achieve optimal biological effectiveness by combining the unique targeting ability of antibodies with the multifunctional activities of cytokines (14, 19, 20). Antibody–cytokine fusion proteins have been proposed for the treatment of solid tumors, including melanoma (14) and

Table 1. Effect of a ch14.18–IL-2 fusion protein on experimental metastasis of SK-N-AS neuroblastoma cells in scid mice reconstituted with human LAK cells

Treatment*	Liver metastases		
	No. of foci†	*P* value	Liver wt,‡ g
Control (PBS)	500 (250, 300, 500, 500, 500, 500, 500, 500, 500, 500, 500, 500, 500)		3.06 ± 1.3
LAK	500 (2, 500, 500, 500, 500, 500, 500)	0.8	3.6 ± 2.7
LAK + ch14.18	43 (5, 10, 26, 36, 50, 55, 70, 100)	0.01	1.63 ± 0.2
LAK + ch14.18 + IL-2	0 (0, 0, 0, 0, 0, 0, 0)	0.008	1.5 ± 0.1
LAK + ch14.18–IL-2	0 (0, 0, 0, 0, 0, 0, 0, 0)	0.003	1.4 ± 0.05

*Daily doses × 7 of ch14.18 and ch14.18–IL-2 were 250 μg and those of rhIL-2 were 7.5×10^5 IU.
†Median.
‡Mean ± SD.

Table 2. Effect of ch14.18–IL-2 fusion protein and equivalent doses of IL-2 on experimental metastases of human neuroblastoma cells in scid mice reconstituted with human LAK cells

Treatment	Dose	No. of foci in liver	*P* value*
Control (PBS)	—	500, 500, 500, 500	
ch14.18–IL-2	1 μg	0, 0, 0, 0, 1, 1	0.0095
IL-2	3,000 IU	0, 0, 76, 80, 500, 500	0.114
ch14.18–IL-2	8 μg	0, 0, 0, 0, 0, 2	0.0095
IL-2	24,000 IU	0, 0, 12, 30, 72, 80, 500	0.024
ch14.18–IL-2	16 μg	0, 0, 0, 0, 0, 0, 0	0.0061
IL-2	48,000 IU	0, 1, 4, 17, 18, 86	0.0095

*Tested by nonparametric Wilcoxon rank sum test.

carcinoma (21, 22). Furthermore, antibody–cytokine fusion proteins may also be used as a cancer vaccine, as has been demonstrated in a model for B-cell lymphoma (23).

Here, we evaluated an antibody–cytokine fusion protein, ch14.18–IL-2, for its ability to suppress dissemination and growth of human neuroblastoma in a preclinical model. Our primary aim was to test the hypothesis that this fusion protein can specifically target rhIL-2 to tumor sites and is more effective than rhIL-2 in achieving efficient tumor cell lysis. We could demonstrate that this was the case in an experimental hepatic metastasis model for human neuroblastoma in scid mice reconstituted with human LAK cells. In this experimental model, the ch14.18–IL-2 fusion protein was also more proficient than equivalent doses of rhIL-2 in prolonging the life-span of these animals.

The choice of our adoptive immunotherapy model in scid mice reconstituted with human LAK cells was based on the following considerations. First, the model was highly reproducible, as hepatic metastases were routinely found in 100% of the scid mice 4 weeks postintrasplenic injection of human neuroblastoma cells. Second, LAK cells were easily and reproducibly available in large numbers by stimulation of human peripheral blood mononuclear cells with rhIL-2 and up to 20% of LAK cells were detected in the liver of scid mice 48 hr after i.p. injection. In this regard, human LAK cells were reported to remain viable in the livers of scid mice for up to 14 days (24). Third, a further rationale for using LAK cells in our model is that treatment of pediatric neuroblastoma patients with IL-2 plus mAb 14.G2a was reported to induce effector cells capable of mediating LAK activity against NK-resistant Daudi target cells and that further IL-2 treatment of these LAK cells greatly enhanced their cytolytic activity against neuroblastoma cells (13).

Although we found that in our experimental model the ch14.18–IL-2 fusion protein effectively activated human LAK cells to suppress neuroblastoma dissemination and growth, the ability of recombinant antibody–IL-2 fusion proteins to activate effector cells is by no means limited to LAK cells. Thus, treatment of neuroblastoma patients with anti-GD2 antibody plus rhIL-2 induced ADCC of the patients' peripheral blood mononuclear cells sufficient to effectively kill neuroblastoma cells *in vitro* (13). In preclinical studies of human melanoma, we also found that antibody–IL-2 fusion proteins can activate tumor-infiltrating lymphocytes (14) as well as other leukocytes bearing FcγRIII and/or high-affinity IL-2 receptors, including NK cells and $CD8^+$-activated T cells (25).

Table 3. Effect of ch14.18–IL-2 fusion protein and equivalent doses of IL-2 on survival of scid mice with experimental metastases of human neuroblastoma

Treatment	Dose	*n*	Median survival, days	Range, days
Control (PBS)	—	6	45	40–78
ch14.18–IL-2	1 μg	6	98	61–128
IL-2	3,000 IU	7	53	40–60
ch14.18–IL-2	16 μg	7	97	78 to >140
IL-2	48,000 IU	7	56	43 to >140

We clearly established that a high dose (250 μg per injection) of ch14.18–IL-2 is more effective than either LAK cells or LAK cells plus ch14.18 in suppressing growth of human neuroblastoma metastasis in scid mice. However, it was particularly impressive that the fusion protein can achieve this effect at very low dose levels (1 μg per injection) and more effectively than equivalent amounts of rhIL-2. These findings are encouraging for two reasons. First, they strongly support our hypothesis that recombinant antibody–cytokine fusion proteins can specifically target cytokines to tumor sites and stimulate immune effector cells sufficiently to achieve efficient tumor cell lysis. Second, the fact that very low dose levels of the ch14.18–IL-2 fusion protein proved more effective than equivalent amounts of rhIL-2 in suppressing tumor growth in scid mice and in prolonging their life-span suggests that it may also be feasible to apply an optimal biological dose of ch14.18–IL-2 for future treatment of pediatric neuroblastoma patients. Third, based on our data, one might anticipate that the potentially lower effective dose levels of ch14.18–IL-2 may produce less toxicity than the relatively high dose levels of rhIL-2 necessary to achieve anti-tumor effects in clinical applications.

In summary, we demonstrated here that the ch14.18–IL-2 fusion protein can effectively suppress dissemination and growth of hepatic metastasis of human neuroblastoma in scid mice reconstituted with human LAK cells. This antibody–cytokine fusion protein was shown to be superior in this regard to an equivalent dose of rhIL-2 and also proved more capable in prolonging the life-span of these animals. Although we realize the limitations of our experimental metastasis model in scid mice in terms of its predictive value for the eventual clinical effectiveness of ch14.18–IL-2, we believe that this recombinant fusion protein may hold some promise for future treatment of human neuroblastoma and other GD2-expressing tumors in an adjuvant setting.

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