Enhanced Activity of Hu14.18-IL2 Immunocytokine against Murine NXS2 Neuroblastoma when Combined with Interleukin 2 Therapy

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ABSTRACT

Established s.c. NXS2 murine neuroblastoma tumors exhibited transient resolution after suboptimal therapy using the hu14.18-IL2 immunocytokine (IC). The hu14.18-IL2 IC is a fusion protein that has linked a molecule of interleukin 2 (IL-2) to the COOH terminus of each of the IgG heavy chains on the humanized anti-GD_2 monoclonal antibody hu14.18. To induce more potent and longer lasting *in vivo* antitumor effects, we tested hu14.18-IL2 IC in a regimen combining it with constant infusion IL-2 in NXS2 tumor-bearing mice. The addition of the constant infusion IL-2 augmented the antitumor response induced by treatment with the hu14.18-IL2 IC in animals with experimentally induced hepatic metastases and in animals bearing localized s.c. tumors. The combined treatment induced prolonged tumor eradication in most animals bearing s.c. tumors and involved both natural killer cells and T cells. The enhanced ability of this combined treatment to prevent tumor recurrence was not observed when a larger dose of hu14.18-IL2 IC, similar in IL-2 content to the IC plus systemic IL-2 regimen, was tested as single-agent therapy. Animals showing prolonged tumor eradication of established tumors after the combined hu14.18-IL2 plus IL-2 regimen exhibited a protective T-cell-dependent antitumor memory response against NXS2 rechallenge.

INTRODUCTION

We have been analyzing the cellular mechanisms of antitumor efficacy of immunocytokines (ICs) and studying the pathways of tumor escape from IC therapy in preclinical murine models. ICs are tumor-reactive monoclonal antibodies (mAbs) genetically linked with cytokines (1). The ch14.18-IL2 IC is comprised of the chimeric 14.18 mAb, which recognizes the GD_2 disialoganglioside expressed on human neuroectodermally derived tumors, including neuroblastoma (NB) and melanoma (2), and a human interleukin 2 (IL-2) molecule linked to the COOH terminus of each human IgG1 heavy chain (3). The chimeric antibody Fab region retains the specificity of the original murine antibody and the effector function of the human antibody. The ch14.18 mAb is ~75% human and 25% murine derived (4).

The next generation of genetically engineered antibodies is humanized. The hu14.18-IL2 IC is identical to the ch14.18-IL2 IC, except the Fab ends involve fully human amino acid sequences for IgG1 heavy and kappa light chains, and the complementarity determining regions correspond to the antigen binding sequences of the murine 14.18 mAb. The resulting hu14.18-IL2 IC is ~98% derived from human genes, yet maintains the specificity of the murine 14.18 mAb (5). In A/J mice bearing the NXS2 tumor, a GD_2^+ murine NB treatment with the hu14.18-IL2 IC provided an effective and potent natural killer (NK)-dependent antitumor response (6, 7). IC treatment given early after tumor establishment at a sufficient dose could promote complete resolution of all detectable tumor with long-term survival for at least some animals (8). As such, hu14.18-IL2 is being evaluated clinically. Phase I trials of single-agent therapy with hu14.18-IL2 in adults with melanoma[6] and in children with NB[7] have recently been completed, and Phase II studies are scheduled to begin in both of these disease settings.

However, if hu14.18-IL2 treatment was provided to mice bearing more established NXS2 tumors, many animals showed initial tumor shrinkage, only to be followed by a recurrence of progressive tumor (7). We have recently reported (7) that these NXS2 tumors that have escaped a NK-dependent antitumor response induced by hu14.18-IL2 IC treatment exhibited a >5-fold increase in MHC class I expression, associated with lowered susceptibility to NK-mediated killing *in vitro*.

We have focused on generating regimens that enable greater antitumor efficacy against well-established tumors using the hu14.18-IL2 IC. Enhanced antitumor efficacy has been

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[6] D. M. King, M. R. Albertini, H. Schalch, J. Hank, J. Gan, J. Surfus, D. Mahvi, J. H. Schilller, T. Warner, K. M. Kim, J. Eickhoff J, K. Kendra, R. Reisfeld, S. D. Gillies, and P. M. Sondel. A Phase I clinical trial of the immunocytokine EMD 273063 (hu14.18-IL2) in melanoma patients. J Clin Oncol. In press 2004.
[7] P. M. Sondel *et al.*, unpublished observations.

demonstrated in mice bearing established tumors by combining IC therapy with cytotoxic chemotherapy (9), with tumor vaccines (10) and with antiangiogenic treatments (11). In this study, we demonstrate that the combination of IC and constant infusion (c.i.) systemic IL-2 resulted in durable resolution of established s.c. NXS2 tumors, which involved both NK and T cells and induced protective T-cell antitumor memory.

MATERIALS AND METHODS

Animals. Female A/J mice (6–8 weeks of age) were obtained from the Jackson Laboratory (Bar Harbor, ME) or from Harlan Sprague Dawley (Madison, WI). All animals were housed in university-approved facilities and were handled according to NIH and University of Wisconsin–Madison Research Animal Resource Center guidelines.

Cell Line and Murine Tumor Models. The murine NXS2 NB cell line was developed and characterized previously (8, 12). Cells were used for tumor induction only if their viability exceeded 95%, as determined by eosin staining.

Experimental hepatic metastases were induced by tail vein injection of 5×10^5 tumor cells in 200 μl of PBS. Mice were sacrificed 28 days later or when they became moribund and evaluated for hepatic tumor burden by wet liver weight and enumeration of hepatic metastatic foci, as described previously (6). Animals with metastases too numerous to count were scored as >200 metastases.

Subcutaneous tumors were induced by injection of 2×10^6 tumor cells in 100 μl of PBS in the lateral flank. Tumor growth was monitored by measuring s.c. tumors one to three times/week with microcalipers and determining tumor volume (width × length × width/2 = mm^3).

Hu14.18-IL2. The hu14.18-IL2 IC was obtained from EMD-Lexigen-Research Center (Billerica, MA) and has been described previously (7, 13). One μg of hu14.18-IL2 IC contains ~3000 IU of IL-2 activity, as determined by *in vitro* proliferative assays with IL-2-responsive human cells expressing the intermediate affinity IL-2 receptor (IL-2R) (Ref. 13 and unpublished data). This observed IL-2 activity of the IC corresponds approximately to the predicted amount of IL-2 activity of the IC based on molar ratios and assuming that the IL-2 within the IC has a specific activity of 15 million IU IL-2/mg of IL-2 within the IC. The hu14.18-IL2 preparation used for these studies was from lot 31916, which was vialed and initially labeled as containing 1.0 mg/ml hu14.18-IL2, which is consistent with protein content assays we have performed on this material. This same lot was also vialed and used for our clinical study.[6] During the course of the clinical study, the concentration of lot 31916 was subsequently determined to be 0.8 mg/ml by the clinical quality assurance lab of EMD. As many of the murine experiments reported in these studies were completed or underway using the IC as originally labeled on the vial, and all dose calculations for the experiments reported here have been based on the original 1.0 mg/ml concentration.

Antitumor Therapy. Mice bearing experimental metastatic or s.c. tumors were treated, beginning on day 9 after tumor inoculation, for 5 consecutive days with 5 μg hu14.18-IL2/day (5 days × 5 μg/day) by 100-μl tail vein injections. In some experiments, modifications in the above dose or timing were used and are indicated.

Systemic IL-2 therapy was initiated on day 7 after tumor inoculation by the surgical implantation of a s.c. osmotic pump (ALZET model 2001 pump; Alza Corporation, Palo Alto, CA) into the dorsal s.c. tissue of each mouse. These pumps delivered 50,000 IU/day of recombinant human IL-2 (TECIN; Hoffmann-La Roche, Inc., Nutley, NJ) for 7 days (unless indicated otherwise). Pumps were surgically removed from anesthetized mice 7 days later.

***In Vivo* Effector Cell Depletions.** NK cells were depleted as previously described (14) by i.p. injection with 50 μl (in a total volume of 1 ml) of anti-Asialo GM1 antisera (Wako Chemicals) on days −2, 4, and 10 posttumor injection.

Mice were depleted of T cells with a mixture of 300 μg of anti-CD4 mAb (GK1.5 hybridoma) and 300 μg of anti-CD8 mAb (2.43 hybridoma) i.p. on days 4, 9, 14, and 19 as described previously (14, 15). Control mice were given 600 μg of rat IgG i.p. according to the same schedule.

Flow Cytometry. Single-cell suspensions of peripheral blood mononuclear cells (PBMCs) or splenocytes were assessed by flow cytometry for expression of murine surface markers as described previously (7, 14). The following reagents were used: rat antimouse CD4 phycoerythrin; rat antimouse CD8 FITC; rat antimouse B220 (pan B cell) phycoerythrin; and rat anti-DX5 (pan NK) FITC (PharMingen, San Diego, CA).

Cytotoxicity Assays. The 4-h ^{51}Cr-release cytotoxicity assay was performed as described previously (14). Target cells were the NK-resistant NXS2 cells (7) or the NK-sensitive YAC-1 cells (16). Hu14.18-IL2 IC (5 μg/ml) was added to some wells before the 4-h incubation. Lytic units/10^6 effectors were determined by testing multiple E:T ratios (usually 100, 30, and 10 to 1), as described previously (17).

Statistics. Comparisons between treatment groups presented in Figs. 3, 4, and 5*A* were performed using a two-tailed *t* test assuming equal variances. *P* values were assessed for individual time points, as indicated in Figs. 3, 4, and 5*A*. In Fig. 3, the proportions of tumor-free mice in each treatment group on day 51 were compared using Fisher's exact test. In Fig. 5*B*, statistical analysis between groups included data for animals that had been previously sacrificed due to large tumors by imputing values for those missing data points using regression imputation techniques. The *P* values for the pairwise group comparisons for the averages over days 13–22 were computed using the nonparametric Wilcoxon signed rank test.

RESULTS

Constant Infusion IL-2 Therapy Enhances Splenic NK Cytolytic Activity against NXS2 Cells. Earlier studies demonstrated the ability of c.i. IL-2 immunotherapy to enhance the *in vitro* cytolytic activity of patient PBMCs toward GD_2^+ human NB cells in the presence of ch14.18-IL2 IC (13). We initially determined the effect c.i. IL-2 treatment had on the *in vitro* cytolytic capacity of A/J splenocytes against GD_2^+ NXS2 NB cells. The s.c. constant infusion of IL-2 by osmotic pump enhanced *in vivo* NK activity when compared with bolus i.p. injection (18). This method corresponds to the continuous i.v. infusion regimen of IL-2 that has been used in the clinical

Table 1 Cytolytic activity of splenocytes from IL-2-treated mice against NXS2 and YAC-1 targets[a]

Mice treated with	YAC-1	NXS2	NXS2 + hu14.18-IL2[b]
c.i. PBS	8 ± 1	8 ± 11	13 + 4
c.i. IL-2	96 ± 23	11 + 1	59 ± 2

[a] Splenocytes were harvested from four A/J mice after 6 days of continuous s.c. infusion of PBS or 50,000 IU/day recombinant human IL-2 and used as effector cells. The 4 h ^{51}Cr release assay was performed in medium alone or medium supplemented with hu14.18-IL2 (5 μg/ml). Results are expressed as the mean lytic unit values ± SE for three experiments.

[b] IL-2, interleukin 2.

setting (19, 20). Our initial toxicity studies of c.i. IL-2 showed that a dose of 150,000 IU/day for 7 days could be administered to A/J mice but with evident IL-2-associated toxicity (ruffled fur and hunched posture), suggesting this may be the maximal tolerated dose by this route and schedule (data not shown). In contrast, mice treated with IL-2 by osmotic infusion pump at one-third of the prior dose (50,000 IU/day for 7 days) did not exhibit any grossly evident cytokine-induced toxicities. Splenocytes from mice harvested after 6 days of c.i. IL-2 (50,000 IU/day) displayed increased NK activity against YAC-1 target cells when compared with splenocytes from PBS-treated mice (Table 1). Splenocytes from IL-2-treated mice showed no enhanced killing of NXS2 cells in media alone but did show augmented killing when hu14.18-IL2 was added to the *in vitro* assay. These results are similar to data reported for killing of GD_2^+ human tumors by PBMCs from patients receiving IL-2 therapy (13, 17).

Combined hu14.18-IL2 IC and c.i. IL-2 Augments Antitumor Activity against Experimental NXS2 Metastases. We initially evaluated IC plus IL-2 combined therapy in mice bearing hepatic metastases. To determine whether c.i. IL-2 treatment provides additional antitumor effects when combined with hu14.18-IL2 therapy, we established suboptimal conditions where hu14.18-IL2 treatment was partially effective in eradicating NXS2 liver metastases. Initiating ch14.18-IL2 therapy (10 μg/day × 5 days) on day 5 after tumor inoculation prevents formation of NXS2 liver metastases (6). Here, mice treated with 5 μg/day × 5 days of hu14.18-IL2 IC beginning on days 3, 5, 7, or 9 after i.v. injection of NXS2 tumor cells displayed a range of antitumor effects (Fig. 1). PBS-treated mice had >200 metastatic liver foci, whereas mice treated with hu14.18-IL2 beginning on day 9 after tumor inoculation showed 50–80 metastatic foci, and mice beginning treatment on days 3 or 5 had relatively few detectable metastases. These results indicate that the hu14.18-IL2 treatment is more effective when initiated early after induction of metastases when there is a smaller tumor burden.

With the hypothesis that prior and concurrent exposure to c.i. IL-2 would boost the hu14.18-IL2-mediated antitumor response by increasing the number and activity of NK cells *in vivo*, we tested c.i. IL-2 treatment combined with suboptimal IC therapy in the NXS2 metastatic model. IL-2 treatment (50,000 IU/day × 7 days) was initiated on day 7 after tumor inoculation, 2 days before starting suboptimal hu14.18-IL2 therapy (5 μg/day × 5 days; Fig. 2*A*). Most PBS-treated mice displayed >200 liver metastases on day 28 when all mice were sacrificed (Fig. 2*B*). Mice treated with hu14.18-IL2 or c.i. IL-2 showed a decrease in metastases compared with the PBS-treated control mice ($P < 0.0001$). Mice receiving hu14.18-IL2 plus c.i. IL-2 showed significantly fewer liver metastases compared with groups receiving either the hu14.18-IL2 or the c.i. IL-2 therapy ($P < 0.0029$ and $P < 0.0168$, respectively), demonstrating that IL-2 treatment provides additional antitumor benefit when combined with IC therapy in this regimen.

Combined IC plus IL-2 Therapy Can Provide Eradication of s.c. NXS2 Tumors. The combined treatment regimen was additionally evaluated in the s.c. NXS2 tumor model using the identical dosing and scheduling schema used with the experimental liver metastases model (Fig. 2*A*). Hu14.18-IL2 therapy was initiated on day 9 after tumor inoculation when all tumors were palpable and had grown to a range in size of 90–120 mm^3. Most animals exhibiting progressive tumor growth were sacrificed by day 28 after tumor inoculation (Fig. 3*A–E*). Mice that received c.i. IL-2 (Fig. 3*B*) or suboptimal IC (Fig. 3*C*) treatment exhibited a transient tumor reduction, com-

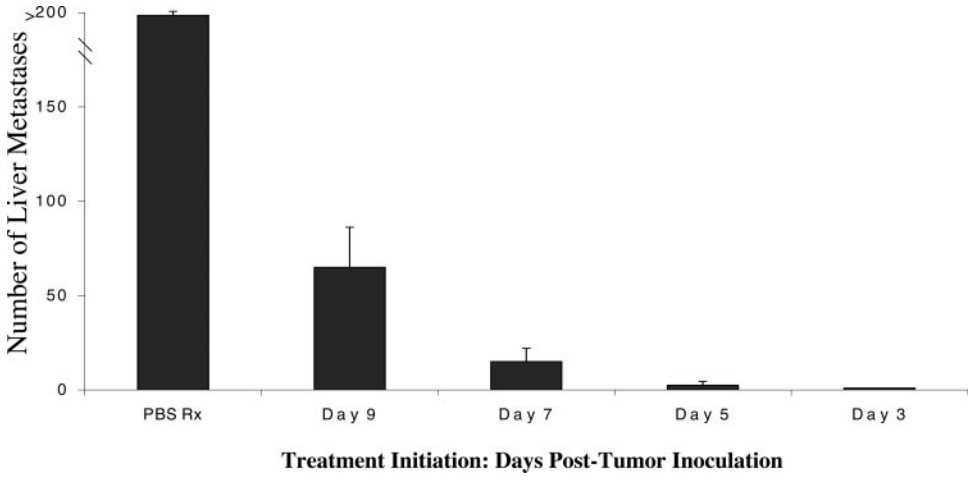

Fig. 1 Efficacy of hu14.18-IL2 therapy is influenced by the timing of its initiation. Groups of four mice were treated i.v. with 5 μg/day × 5 days of hu14.18-IL2 beginning on days 3, 5, 7, or 9 after i.v. injection of 5×10^5 NXS2 tumor cells. Mice were sacrificed on day 28 after tumor injection, and the numbers of liver metastatic foci were enumerated. Control mice received PBS injections. Data are expressed as the group mean values ± SE. A representative experiment of two is shown.

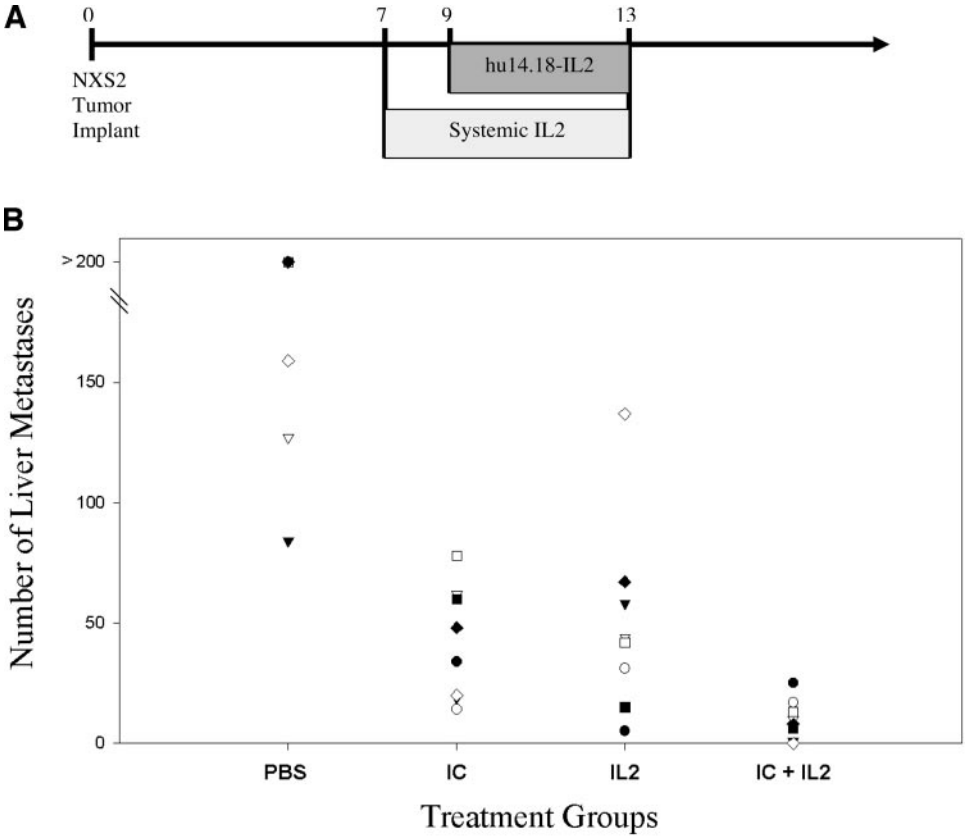

Fig. 2 Systemic interleukin 2 (IL-2) augments the hu14.18-IL2 antitumor response against NXS2 liver metastases. *A*, after i.v. injection of 5×10^5 NXS2 tumor cells, groups of mice were treated with immunocytokine (IC) (5 μg/day × 5 days beginning on day 9 after tumor injection, $n = 7$), recombinant human IL-2 (50,000 IU/day × 7 days beginning on day 7 after tumor injection), or the combined IC and recombinant human IL-2 therapy ($n = 8$). Control mice received PBS injections beginning on day 9 after tumor injection ($n = 5$). *B*, mice were sacrificed on day 28 after tumor injection, and the numbers of liver metastatic foci were enumerated. Each data symbol represents an individual animal. A representative experiment of three is shown. Mice treated with combined hu14.18-IL2 plus systemic IL-2 had significantly ($P < 0.05$) fewer liver metastases compared with groups receiving the hu14.18-IL2 or systemic IL-2 therapy.

pared with control animals (Fig. 3*A*), during and immediately after therapy (day 18: $P < 0.014$ and $P < 0.024$, respectively). With time, most of these treated animals developed recurrent or progressive tumors. As shown, only three animals receiving c.i. IL-2 (Fig. 3*B*) and two receiving IC (Fig. 3*C*) remained tumor free as of day 51. In contrast, the combined IC plus IL-2 regimen resulted in complete and durable tumor resolution in all treated mice, which was significantly better than all other treatment groups [Fig. 3; day 51 evaluation of IC + IL-2 (Fig. 3*E*) compared with PBS (Fig. 3*A*) $P = 0.0002$; IL-2 (Fig. 3*B*) $P = 0.026$; and 5 μg/day IC (Fig. 3*C*) $P = 0.007$].

To test whether the enhanced antitumor response observed with the suboptimal IC plus c.i. IL-2 regimen could be duplicated by increasing the dose of IC to a level that supplies a similar amount of IL-2 as the combined therapy (65,000 IU of IL-2/day), some mice were treated with 22 μg/day IC by daily i.v. bolus on days 9–13 (corresponding to ~66,000 IU of IL-2/day). As seen in Fig. 3*D*, the pattern of s.c. NXS2 tumor growth was similar to that observed in the group receiving the 5 μg/day IC treatment (Fig. 3*C*), suggesting that adding c.i. IL2 to hu14.18-IL2 treatment [Fig. 3. Day 51 evaluation of IC + IL-2 (Fig. 3*E*) compared with 22 μg/day IC (Fig. 3*D*) $P = 0.026$] provides additional antitumor effects not afforded by increased IC dosing alone in this model.

Induction of T-Cell Memory in Mice Receiving Combined Treatment. Those eight mice remaining tumor free after combined therapy (Fig. 3*E*) were rechallenged with another s.c. injection of NXS2 cells on day 120 after their initial NXS2 s.c. tumor implantation. Four of these mice were T-cell depleted, and four were mock depleted to determine whether T-cell memory was induced by the initial antitumor response (Fig. 4). After NXS2 rechallenge, three of four mice that were mock depleted demonstrated a memory response as shown by protection from detectable tumor growth (Fig. 4). In contrast, all four mice that were depleted of $CD4^+$ and $CD8^+$ T cells showed progressive tumor growth (Fig. 3*E* mice rechallenged: T-cell *versus* mock depletion day 17, $P < 0.02$).

Combined IC plus IL-2 Therapy Increases the Number of $DX5^+$ NK Cells in the Blood. We assessed the lymphocyte profile in the peripheral blood for NXS2-bearing mice receiving treatment as in Fig. 2*A*. Blood samples were collected 20 h after the third daily injection of hu14.18-IL2 because prior studies with a separate mAb linked to IL-2 showed peak PBMC NK activity at that time (14). As shown in Table 2, PBMCs from control mice had only 4% $DX5^+$-staining cells, a pan-specific NK cell surface marker (21), whereas treatment with hu14.18-IL2 or c.i. IL-2 induced increases in the $DX5^+$ population (26 and 17%, respectively). Animals treated with hu14.18-IL2 combined with c.i. IL-2 showed 45% $DX5^+$-staining of their PBMCs.

Tumor Regression after Combined IC plus c.i. IL-2 Therapy Involves both T and NK Cells. The *in vivo* control of NXS2 tumors in A/J mice by the ch14.18-IL2 IC is known to be NK dependent (6, 8). Similarly, the antitumor effect observed

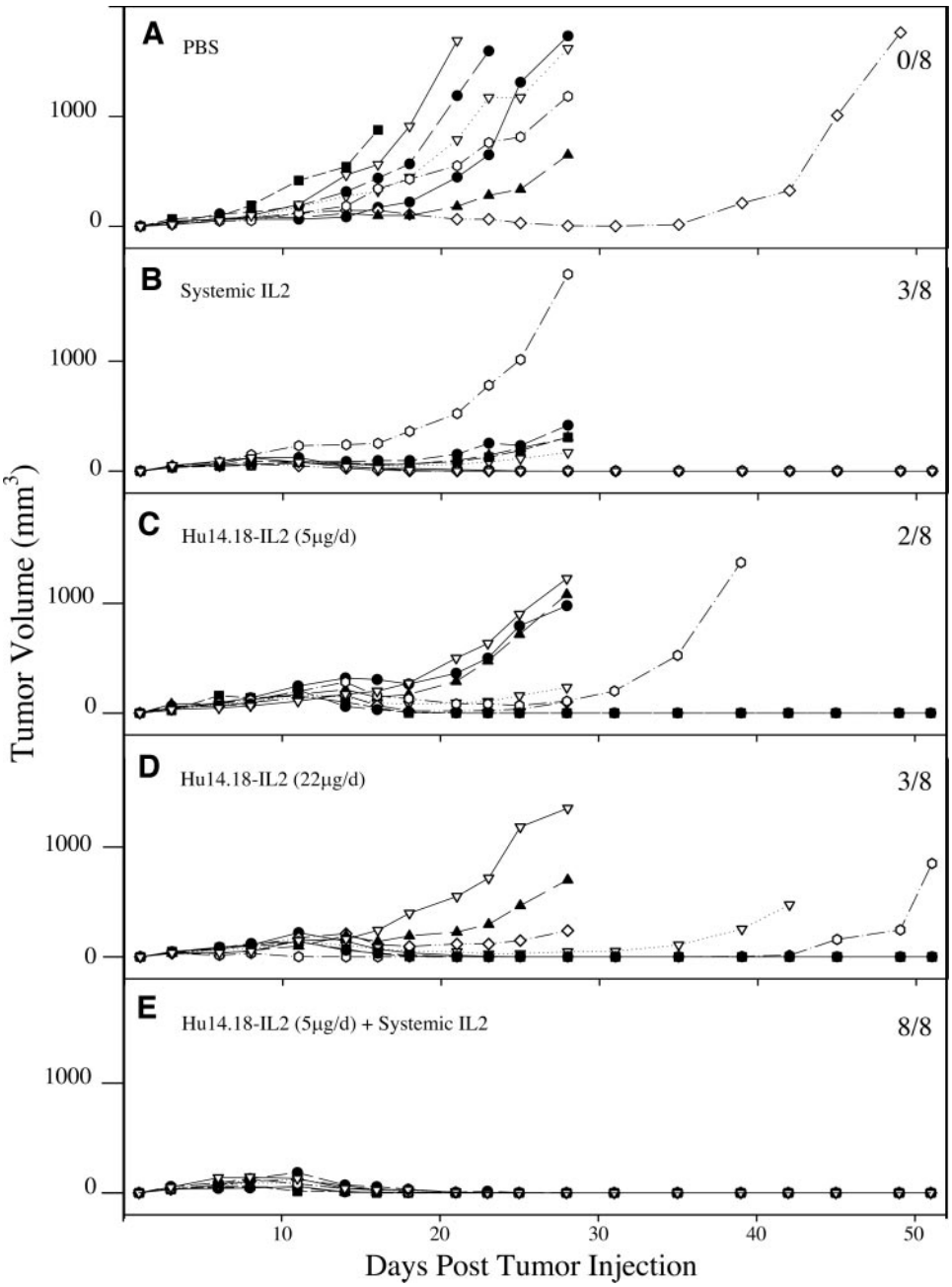

Fig. 3 Combinatorial hu14.18-IL2 plus systemic interleukin 2 (IL-2) therapy can eradicate established s.c. NXS2 tumors. Primary tumors were induced by s.c. injection of 2×10^6 NXS2 tumor cells. Groups of eight mice were treated with recombinant human IL-2 (*B*), 5 μg/day × 5 days of immunocytokine (IC) (*C*) 22 μg/day × 5 days of IC (*D*), or the combined IC and recombinant human IL-2 therapy (*E*), according to the treatment schedule and doses shown in Fig. 2*A*. Control mice (*A*) received PBS injections. Mice with large progressive tumors were sacrificed when tumors exceeded ~1000 mm^3. In addition, in this experiment, tumors that were smaller than 1000 mm^3 but that were growing progressively on day 28 were removed to enable analysis of their surface phenotype (7). The graph presents tumor growth curves for each of eight separate mice in each group. Numbers on the right indicate mice without tumors at the end of the experiment (day 51)/total number of mice/group.

in mice receiving this combined hu14.18-IL2 plus c.i. IL-2 regimen is NK dependent (Fig. 5*A*: IC + IL-2 compared with IC + IL-2 + NK depletion day 22 values, $P < 0.000025$). Depletion of NK cells *in vivo* prevents the combined therapy from inducing an effective antitumor response.

Because T-cell memory to NXS2 NB develops in animals treated with IL-2 + hu14.18-IL2 (Fig. 4), we also assessed the role of T cells in the antitumor effect induced by IL-2 + hu14.18-IL2 combination treatment of animals bearing primary s.c. NXS2 tumors (Fig. 5*B*). Two independent experiments showed that depletion of T cells during the combined therapy also resulted in a diminished antitumor response (Fig. 5*B*). Although mice did not show complete tumor resolution following combined IC plus c.i. IL-2 therapy in these particular experiments, likely reflecting the influence of NXS2 tumor growth variability from experiment to experiment, there was a significant ($P < 0.01$) increase in tumor growth in T-cell-depleted mice when compared with mock-depleted animals.

DISCUSSION

Our preclinical study presented here demonstrates that the antitumor response induced by hu14.18-IL2 IC against the murine NXS2 NB can be enhanced when combined with systemic

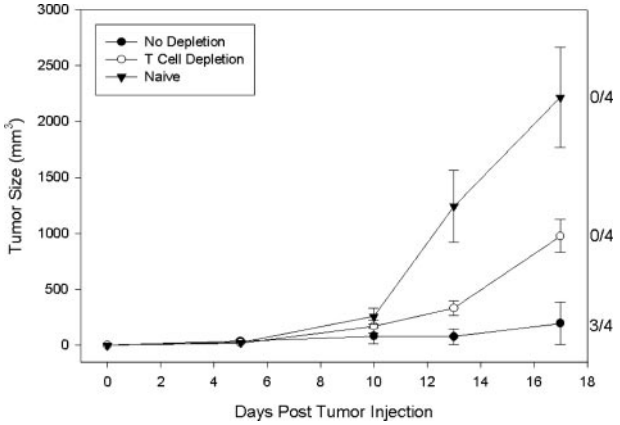

Fig. 4 Immunocytokine (IC) plus systemic interleukin 2 (IL-2)-treated mice develop T-cell-dependent antitumor memory. Mice with resolved tumors and remaining tumor free (Fig. 3*E*) after combined therapy were rechallenged on day 120 with s.c. injection of 2×10^6 NXS2 tumor cells. Groups of four mice received either anti-CD4 + anti-CD8 monoclonal antibodies (T-cell depletion) or rat IgG (no depletion) on days −1, 3, 8, and 13. Naïve mice implanted with s.c. NXS2 tumors served as a positive control. Data are plotted as mean ± SE of four mice/group. Numbers on the right indicate mice without tumors/total number of mice.

Table 2 Profile of PBMCs of NXS2 tumor-bearing mice after 3 days of antitumor therapy

	% positively staining PBMCs[b]			
Treatment group[a]	CD4	CD8	B220	DX5
PBS	49[c]	15	25	4
Hu14.18-IL2	37	8	33	26
IL-2 infusion	39	10	31	17
Hu14.18-IL2 + IL2 infusion	17	7	20	45

[a] Mice bearing s.c. NXS2 tumors were treated with hu14.18-IL2 (5 μg/day × 5 days beginning on day 9 posttumor injection), recombinant human IL-2 (50,000 IU/day × 7 days beginning on day 7 posttumor injection), or the combined hu14.18-IL2 and recombinant human IL-2 therapy and evaluated 20 h after the third-day injection of hu14.18-IL2. Blood samples from four individual mice/group were obtained and pooled for each treatment group. Flow cytometric analysis was performed as described in "Materials and Methods."

[b] PBMC, peripheral blood mononuclear cell; IL-2, interleukin 2.

[c] Numbers reflect the percentage of viable cells staining positive for cell surface markers CD4, CD8, B220, or DX5 from the population of gated lymphocytes.

IL-2 therapy. Treatment of mice bearing 9-day NXS2 tumors with a suboptimal treatment regimen of hu14.18-IL2 demonstrated only partial antitumor effects in the experimental liver metastases model (Figs. 1 and 2) and a transient resolution of measurable s.c. tumors (Fig. 3*C*). Previous studies have shown that IC treatment of NXS2-bearing mice depends on NK cells and not on T cells (6, 8). In those studies, IC therapy was abrogated in NK-depleted or NK-deficient mice but still effective in T-cell-depleted or T-cell-deficient mice; tumor-bearing mice previously treated with IC did not mediate protection from NXS2 rechallenge (6, 8). In contrast, the combination of this same hu14.18-IL2 regimen plus systemic c.i. IL-2 provided enhanced control of metastatic liver disease (Fig. 2*B*) and complete eradication of established s.c. NXS2 lesions in many treated animals (Fig. 3*E*). The augmented antitumor responses observed with the combinatorial hu14.18-IL2 plus systemic IL-2 therapy were associated with an increase in the $DX5^+$ NK population of PBMCs (Table 2). NK cells were requisite in mediating the antitumor effects of this combined hu14.18-IL2 plus systemic IL-2 regimen in the NXS2 model (Fig. 5*A*). Furthermore, animals showing long-term eradication of their primary NXS2 tumors after combined therapy demonstrated

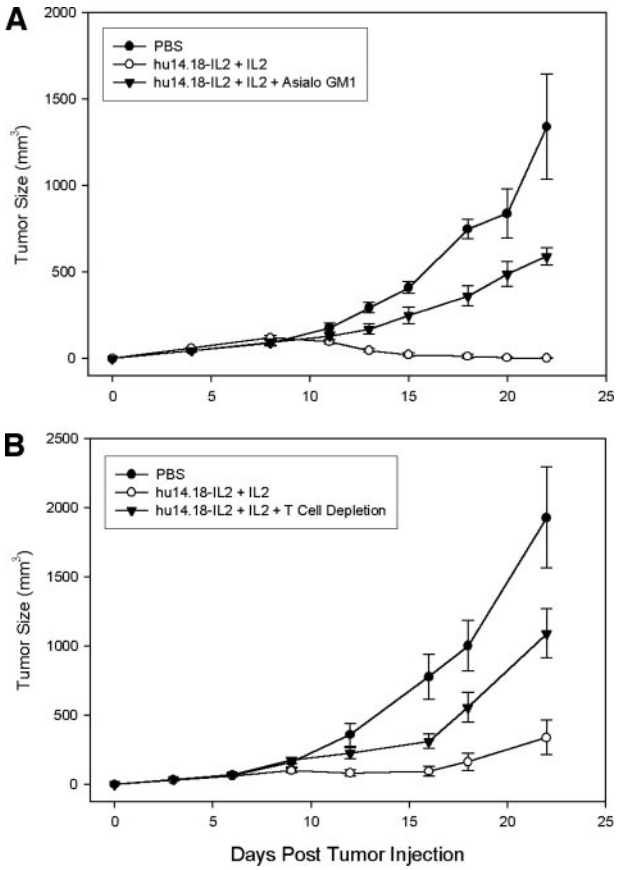

Fig. 5 Combined treatment efficacy depends on both natural killer (NK) and T cells. *A*, mice were implanted s.c. with 2×10^6 NXS2 tumor cells. Groups of four mice were treated with PBS (control) or recombinant human IL-2 + hu14.18-IL2 (according to the schema outlined in Fig. 2*A*). One group of mice receiving IL2 + hu14.18-IL2 treatment was also depleted of NK cells by i.p. injection with 50 μl of anti-asialo GM1 antisera on days −2, 4, and 10. Data shown represent one of two experiments, plotted as mean ± SE. Animals treated with anti-asialo GM1 antibody showed a reduced antitumor response compared with the mice that were not NK cell depleted ($P < 0.000025$). *B*, mice were implanted s.c. with 2×10^6 NXS2 cells. Groups of eight mice were treated with IL2 + hu14.18-IL2. Systemic IL-2 and IC were given according to the schedule shown in Fig. 2*A*. One group of control tumor-bearing mice received no treatment. A mixture of 300 μg of each anti-CD4 monoclonal antibody and anti-CD8 monoclonal antibody (T-cell depletion) or rat IgG (600 μg) was given i.p. every 5 days starting on day 4. Data are plotted as mean ± SE of tumor sizes for one of two experiments with similar results. Control *versus* IL-2 pump + IC: $P < 0.001$; IL-2 pump + IC *versus* IL-2 pump + IC + T-cell depletion: $P < 0.01$.

T-cell memory that protected against a rechallenge of NXS2 cells (Fig. 4). As such, T cells were also shown to play a role against the primary NXS2 tumors in animals receiving combined therapy (Fig. 5*B*).

Because IL-2 is an integral component of the IC molecule, it is unclear why c.i. IL-2 promotes an enhanced antitumor response not achieved by increased IC dosing. Inclusion of systemic IL-2 in a combinatorial regimen with hu14.18-IL2 might appear redundant because the hu14.18-IL2 molecule induces a systemic IL-2 effect in mice as well as in patients (6–10). However, the antitumor effect obtained in a regimen combining 5 μg of hu14.18-IL2/mouse/day with 50,000 IU IL-2/mouse/day was greater than when a similar amount of IL-2 was administered solely as 22 μg of hu14.18-IL2/mouse/day (Fig. 3*D*). Three distinct explanations may, in part, account for the augmented effect of adding c.i. IL-2 to treatment with IC.

First, the augmented effect of adding c.i. IL-2 to IC treatment may relate to pharmacokinetic and administration schedule differences of these two molecules. The half-life of the IC is 3–4 h in mice and in patients (22).[6] The half-life of IL-2 is shorter (~45 min) after a bolus i.v. injection (23–25). However, giving the same dose of IL-2 as a c.i., rather than as a bolus, results in prolonged *in vivo* exposure to IL-2 and greater *in vivo* activation of NK cells (18). Thus, it is possible that administration of higher dose IC as a 24-h c.i. for 5 days, rather than as five daily bolus i.v. injections, might accomplish the same effect as our administration schedule of i.v. bolus IC given together with c.i. IL-2. Unfortunately, s.c. osmotic pump c.i. of IC cannot be tested in mice as the IC molecule is cleaved in 4 h when in mouse tissue or plasma (but not in human plasma), separating the IL-2 component from the IgG antibody component (Ref. 22 and unpublished data). Thus, IC administered by s.c. infusion pump would not circulate into the vascular system as intact IC. Furthermore, administration of 5 days of i.v. c.i. of IC is not feasible in mice.

Second, the IL-2 component of the IC and soluble recombinant human IL-2 may differ from each other in their interactions with murine and human IL-2Rs. The human IL-2 component of the IC molecule and soluble recombinant human IL-2 show similar activation of human and murine cells expressing high-affinity IL-2Rs and of human cells with intermediate affinity IL-2Rs (13). However, murine NK cells with intermediate affinity IL-2Rs (βγ heterodimers) require ~20 times more hu14.18-IL2 than soluble recombinant human IL-2 to achieve similar proliferation *in vitro*.[8] Because NK cells respond to IL-2 predominantly through intermediate affinity receptors, the ability of c.i. IL-2 treatment to enhance this NK-dependent antitumor response against NXS2 tumors in mice may be attributed to increased stimulation of intermediate affinity IL-2R$^+$ effectors by soluble IL-2, which could not be realized with the modest dose increase (from 5 μg up to 22 μg) of ICs (Fig. 3). Therefore, increased IC dosing in patients may potentially achieve similar antitumor responsiveness achieved here in mice by using c.i. IL-2 plus IC therapy.

Third, treatment of mice with a humanized IC induces a neutralizing mouse antihuman antibody detectable after 4 days of IC treatment (14). Somewhat similarly, treatment of patients with the chimeric ch14.18 mAb or with the hu14.18-IL2 IC induces a neutralizing human antichimeric antibody or a neutralizing anti-idiotype antibody in some patients (26, 27). In patients, giving IL-2 before and during treatment with ch14.18 can inhibit the development of the human antichimeric antibody response (26). It is possible that pretreatment of mice with IL-2 in our experiments enables the subsequently injected IC to function longer (and more effectively) by delaying the mouse antihuman antibody response. It is also possible that the strong mouse antihuman antibody response is associated with activation of an inhibitory TH2 response, which may be more pronounced in mice receiving 22 μg of IC/day than in those receiving 5 μg/day. If so, the anticipated boost in antitumor effect expected by increasing the IC dose from 5 to 22 μg/day might be blunted by the inhibitory mouse antihuman antibody or TH2 response.

In the present study, c.i. IL-2 increased the pool of available activated NK effectors. This increase in the spleen (Table 1) and blood NK populations for animals receiving the combined therapy (Table 2) was associated with the augmented antitumor responses in our experimental metastatic (Fig. 2) and s.c. NXS2 tumor models (Fig. 3). The IL-2-activated effector cells might then be recruited by hu14.18-IL2 to the tumor microenvironment. As the anti-GD_2 component of the IC molecule targets the IC to the tumor, both the Fc and IL-2 components of the IC molecule can interact with receptors on NK cells (FcR and IL-2R, respectively), thereby facilitating cellular interactions with the tumor in the tumor microenvironment. In this study, splenocytes from IL-2-treated mice demonstrated enhanced killing of NXS2 cells in the presence of hu14.18-IL2 (Table 1) and suggests that in the NXS2 model prior or concurrent IL-2 treatment increases antitumor responsiveness induced by IC therapy.

The NXS2 murine NB is considered poorly immunogenic, as standard immunization regimens induce poor or undetectable T-cell responsiveness (8). However, protective T-cell memory can be invoked under certain *in vivo* conditions. Mice vaccinated with NXS2 cells transfected to express a single-chain IL-12 (10, 28, 29) or with attenuated bacteria carrying DNA sequences encoding murine tyrosine hydroxylase, a NB tumor-associated antigen (30, 31), develop protective T-cell-mediated immunity to NXS2. Additionally, we have recently reported that Flt3-ligand therapy initiated on day 3 after implantation of s.c. NXS2 tumor could cause tumor rejection and resulted in development of T-cell-dependent antitumor memory (7). In the present murine study, the combination of c.i. IL-2 plus IC therapy evoked T-cell antitumor involvement that participated in resolution of the primary s.c. tumor and establishment of protective antitumor memory.

The mechanisms that account for the antitumor benefit observed with the IC plus c.i. IL-2 regimen require additional clarification. At least three distinct processes may be involved: an increase in tumor reactive effector cells; a modification of the tumor-infiltrating lymphocyte profile; or a decrease in tumor-mediated immunosuppression.

First, an early robust NK-mediated antitumor response, possibly provided by this combined regimen, may shift the

[8] S. Gillies, unpublished data.

balance in promoting tumor-specific T-cell responsiveness (32). As shown for B16 melanoma (33) and influenza viral infection (34, 35), the appearance of NK cells in the early stages of disease are essential for the induction of an effective T-cell-mediated immune response. Infusional IL-2 treatment enhances antiganglioside-targeted antibody-dependent cellular cytotoxicity with patient (17) and murine (Table 1) effectors *in vitro* and may enhance provision of tumor-derived material for processing by antigen-presenting cells at an earlier stage of disease. Furthermore, IL-2 is an important pleotrophic cytokine that influences dendritic cell interactions with various effector cell populations (36). As such, the combined regimen in this study may facilitate greater dendritic cell antitumor involvement with subsequent development of tumor-specific T-cell responses. Treating mice with chimeric-IC at an earlier stage of disease or shortly after NXS2 tumor injection (6, 8) is insufficient to induce demonstrable T-cell-mediated antitumor effects or durable cure, as provided with the c.i. IL-2 plus IC regimen used in this study.

Second, combining c.i. IL-2 with ICs may modify the pattern of immune cells entering into the tumor. We have performed preliminary flow cytometric analyses of lymphocytes infiltrating into NXS2 tumors (data not shown). Tumor-infiltrating lymphocytes from mice treated with IC or with c.i. IL-2 showed an increase in intratumoral $DX5^+/B220^+$ activated NK cells compared with tumor-infiltrating lymphocytes of PBS-treated mice. Tumor-infiltrating lymphocytes from mice receiving the combined regimen showed an enhanced ingress of both NK and $CD8^+$ T cells (data not shown). This may be consistent with the role for T cells (Fig. 5*B*) in the NXS2 tumor response to the combined regimen and the subsequent induction of antitumor T-cell memory (Fig. 4).

Third, gangliosides shed by tumors are able to suppress or prevent T-cell activation. This immune suppression can be attributed, in part, to the ability of gangliosides to bind to the IL-2R, thereby competitively inhibiting the interaction of IL-2 with its receptor (37, 38). *In vitro* studies have shown that exogenously supplied IL-2 is able to overcome this ganglioside-induced T-cell hyporesponsiveness (38, 39). Therefore, in the combined regimen tested in this study, c.i. IL-2 might have subverted some tumor-induced T-cell immunosuppression by providing an excess of IL-2 capable of attenuating a ganglioside/IL-2R interaction.

In summary, our study has demonstrated that systemic c.i. IL-2 treatment augments the hu14.18-IL2 IC-mediated antitumor response in A/J mice bearing either experimentally induced hepatic metastases or localized s.c. NXS2 tumors. In the s.c. NXS2 model, the combined regimen resulted in durable resolution of established tumors, involved both NK and T cells, and resulted in protective T-cell-dependent antitumor memory.

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