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ORIGINAL ARTICLE

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NXS2 murine neuroblastomas express increased levels of MHC class I antigens upon recurrence following NK-dependent immunotherapy

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Abstract We evaluated recurrent NXS2 neuroblastoma tumors that developed following NK- or T-cell–mediated immunotherapy in tumor-bearing mice. Recurrent tumors developed following an NK-dependent antitumor response using a suboptimal dose of hu14.18-IL2, a humanized IL-2 immunocytokine targeted to the GD_2-ganglioside. This treatment initially induced complete resolution of measurable tumor in the majority of mice, followed, however, by delayed tumor recurrence in some mice. These recurrent NXS2 tumors revealed markedly enhanced (> fivefold) MHC class I antigen expression when compared with NXS2 tumors growing in PBS-treated control mice. A similar level of enhanced MHC class I antigen-expression could be induced on NXS2 cells in vitro by culturing with interferon γ, and was associated with reduced susceptibility to both NK-cell–mediated tumor cell lysis and antibody-dependent cellular cytotoxicity in vitro. In contrast, Flt3-ligand treatment of NXS2-bearing mice induced a protective T-cell–dependent antitumor memory response. Recurrent NXS2 tumors that developed following Flt3-L therapy revealed a decreased expression of MHC class I antigens. While NXS2 tumors are susceptible to in vivo destruction following either hu14.18-IL2 or Flt3-ligand immunotherapies, these results suggest that some tumor cells may be selected to survive and progress by expressing either higher or lower levels of MHC class I antigen in order to resist either NK- or T-cell–mediated antitumor responses, respectively.

Keywords Tumor escape · Hu14.18-IL2 · Flt3-ligand · Major histocompatibility complex · GD_2-disialoganglioside

Abbreviations *ADCC* antibody-dependent cellular cyto-toxicity · *Flt3-L* Flt3-ligand · *GD_2* GD_2-disialoganglioside · *IC* immunocytokine · *mAb* monoclonal antibody · *NB* neuroblastoma · *NXS2* transplantable murine neuroblastoma · *s.c.* subcutaneous

Introduction

Tumors are known to utilize diverse mechanisms to evade antitumor immune effects [46, 63]. The majority of immunotherapeutic strategies involve activation of T-cell–dependent antitumor responses [7, 17, 52, 60]. Tumors can evade T cells through various mechanisms. These include a reduction in the level of tumor antigen [2, 59], a decrease in antigen processing by altered transporter associated with antigen processing (TAP) function [10, 14], or a down-regulation in MHC class I antigen expression [1, 49]. Tumors can inhibit T-cell activation by decreased expression of requisite costimulatory molecules such as B7 [8, 13], or suppression of T cells via secretion of immunosuppressive factors, including gangliosides [41, 53], IL-10 [5, 20] or transforming growth factor β [4, 56]. Some tumors are able to directly eliminate tumor-specific T cells through apoptotic death by up-regulating their Fas-L expression [31, 55].

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Other immunotherapies do not rely on T cells. These include strategies that activate NK cells or facilitate recognition of tumors using monoclonal antibodies (mAbs). NK cells express Ly-49 inhibitory [62] and activating [44, 45] receptors, which bind to specific MHC class I antigen determinants. Binding of NK inhibitory Ly-49 receptors with the appropriate MHC class I molecule on tumor targets results in inactivation of the NK cell cytolytic machinery [24]. Targets induced to enhance their MHC class I antigen expression become less susceptible to NK-mediated lysis [23], while down-regulation of MHC class I antigen expression increases their susceptibility to NK-mediated destruction [18, 32]. Thus the level of MHC class I antigen expression on tumor cells can influence both T-cell–dependent and NK-dependent antitumor responses [54].

We have been evaluating [19, 25] the antitumor efficacy of immunocytokines (ICs), which are tumor-reactive mAbs genetically linked with cytokines [15, 48]. The hu14.18-IL2 IC comprises the humanized 14.18 mAb linked to IL-2. The 14.18 mAb recognizes the GD_2-disialoganglioside expressed on certain neuroectodermally derived human tumors, including neuroblastoma (NB) and melanoma [50, 51]. The 14.18-IL2 IC has a human IL-2 molecule linked to the carboxy-terminus of each human IgG1 heavy chain [15]. In A/J mice bearing the NXS2 NB, administration of the chimeric IC (ch14.18-IL2) provides an effective and potent NK-dependent antitumor response [35]. IC treatment provided early after tumor establishment, at a sufficient dose, can promote complete resolution of all detectable tumor, with long-term survival for at least some animals [33]. If IC treatment is provided at a lower dose or against more established tumors, as utilized in the present study, many animals show initial tumor shrinkage, followed by a recurrence of progressive tumor.

Flt3-L is a hematopoietic stem cell growth and differentiation factor that induces in vivo expansion of dendritic cells (DCs) and NK cells. In some murine models, Flt3-L treatment induces complete tumor clearance and the induction of tumor-specific T-cell immunity [38, 39, 42].

In the present study, we have analyzed NXS2 tumors that have developed following immunotherapy with hu14.18-IL2 or Flt3-L, and focused on their modulation of MHC class I and GD_2 antigens.

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, Indianapolis, IN. All animals were housed in university-approved facilities and were handled strictly according to National Institutes of Health and University of Wisconsin-Madison Research Animal Resource Center guidelines and the principles of laboratory animal care (NIH publication No. 85-23, revised 1985).

Cell line and murine tumor models

The NXS2 murine NB cell line was maintained as described previously [33]. Both NK-cell–mediated [33, 35] and T-cell–mediated [34, 36] antitumor responses against NXS2 tumors have been reported. Cells were used for tumor induction only if their viability exceeded 95%, as determined by eosin staining.

Subcutaneous (s.c.) tumors were induced by injection of 2×10^6 tumor cells in 100 μl of phosphate-buffered saline (PBS) in the left lateral flank proximal to the spleen. Tumor growth was monitored by periodically measuring s.c. tumor with microcalipers and determining tumor volume using the formula: tumor size = width × length × width / 2 = mm^3.

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.

Spontaneous hepatic metastases were evaluated in animals which had their primary subcutaneous tumor resected 18 days after implantation. Tumors were surgically excised under aseptic conditions after general anesthesia with i.p. injection of ketamine (100 mg/kg) / xylazine (10 mg/kg). Animals were monitored and tumors were harvested and analyzed for MHC class I and GD_2 antigen expression when animals were noted with distended abdomens or when animals became moribund [33].

Ex vivo tumors (tumors that were freshly harvested from tumor-bearing mice) were mechanically disaggregated in RPMI 1640 medium after careful removal of adjacent nonmalignant tissue. Erythrocytes were removed by hypotonic shock and a single-cell suspension was obtained by filtering through a nylon strainer (Becton Dickinson Labware, Franklin Lakes, NJ). The cells were directly evaluated by flow cytometry or maintained in DMEM as outlined above for the in vitro propagation of NXS2 cells.

Antitumor therapy

Mice bearing s.c. NXS2 tumors were treated, beginning on day 9 post–tumor inoculation, for 5 consecutive days with 5 μg hu14.18-IL2 (Lexigen Pharmaceuticals, Lexington, MA) (5 μg/day × 5 days) in 100 μl of PBS given by daily tail vein injection.

Tumor-bearing mice were treated with human Flt3-L (Immunex, Seattle, WA) at 10 μg/day for 17 days (10 μg/days × 17 days) beginning on day 3 post–tumor inoculation by daily 1-ml i.p. injections.

Flow cytometry

A single-cell suspension of splenic or tumor tissue was assessed by flow cytometry for expression of murine surface markers: CD4, CD8, B220 (pan B-cell), DX5 (pan NK), $H2K^k$, and $H2D^d$ antigen with the appropriate commercially available fluorochrome-conjugated monoclonal antibodies (PharMingen, San Diego, CA). Cell surface expression of GD_2 molecules was determined by incubating with hu14.18-IL2 (1 μg/10^6 cells) and secondary staining with FITC-conjugated polyclonal goat antibody against human IgGs (1 μg/10^6 cells) (Caltag Laboratories, Burlingame, CA) [19]. Flow cytometric analysis was performed as previously described [21].

Some data are shown as specific mean fluorescence intensity (MFI) ratios determined by the formula: specific MFI ratio = test antibody MFI / isotype control MFI [30], which allows direct comparison of the results obtained with different tumors on different days.

Cytotoxicity assay

The 4-h ^{51}Cr-release cytotoxicity assay was performed as described previously [21]. Some targets were pretreated with 25 U/ml of recombinant murine IFN-γ (rmIFN-γ) (Roche Molecular

Biochemicals, Mannheim, Germany) for 48 h prior to use in the cytotoxicity assay. Hu14.18-IL2 (10 μg/ml final concentration) was added to some wells.

NXS2 plus splenocyte cocultures

Activated splenocytes from mice that received rhIL-2 (50,000 IU/day) for 7 days by subcutaneous osmotic pump implant were seeded at 8×10^6 cells/well with 2×10^6 NXS2 cells in a volume of 5 ml of culture media in a 6-well plate in the presence or absence of 5 μg/ml of hu14.18-IL2. Media was not replaced and fresh media was not added in order to preserve the tumor and splenic effector cell interactions, including soluble factors that might be present at or near the target:effector conjugates. Some cocultures with IL-2–activated splenocytes included anti-IFN-γ mAb (R4-6A2: used as purified ascites preparation) at 50 μg/ml. Cells were harvested following 4 days of culture and assessed for $H2K^k$ and $H2D^d$ antigen expression by flow cytometry.

In vivo effector cell depletions

In vivo T-cell and NK-cell depletions were performed using anti-CD4 and anti-CD8 or anti-asialo GM-1 antibody as described previously [21, 57]. To monitor for depletion efficacy, in some experiments, blood from sex- and age-matched control mice that were similarly treated was obtained 2 days following the final in vivo depletion procedure. Flow cytometric analysis of PBMC indicated >95% depletion of the targeted effector cell populations.

Tyrosine hydroxylase RT-PCR

Total RNA was extracted from approximately 5×10^6 tumor cells using the guanidinium method as described previously [6], according to the manufacturer's instructions (Promega, Madison, WI). Primers (Integrated DNA Technologies, Coralville, IA) were designed to specifically amplify tyrosine hydroxylase TH1, an NB selective molecule [33, 35] (upper, 5′-TCTCACTTCTTGAAGGAACG-3′; lower, 5′-CCCCATTCTGTTTACACAGC-3′). The conditions for the PCR were initial denaturation of 4 min at 94°C; 36 cycles of denaturation (94°C for 45 s), annealing (55°C for 45 s) and extension (72°C for 1.5 min); and a final extension at 72°C for 7 min. The PCR product (325 bp) was then evaluated by 1.4% agarose gel electrophoresis in ethidium bromide and visualized by UV light.

Statistics

All recurrent tumors from mice that had subcutaneous NXS2 tumors and received hu14.18-IL2 or Flt3-L immunotherapy over a 14-month period were evaluated for this study, provided that the primary NXS2 challenge tumor had completely (albeit transiently) resolved following immunotherapy and was no longer measurable or palpable (i.e., a complete response). Also included were those subcutaneous NXS2 primary tumors that showed a transient reduction in tumor size following hu14.18-IL2 treatment but not disappearance (i.e., a partial response), followed by progressive tumor growth at the primary site (PR.Primary: Table 1). Wherever shown, means of replicate values and standard deviation values (SD) are presented.

The Wilcoxon-Mann-Whitney test was used to assess the statistical significance of the observed differences in $H2K^k$ and $H2D^d$ antigen MFI values between tumors from PBS-treated mice and recurrent tumors from hu14.18-IL2–treated mice in Fig. 2. The statistical significance of the observed differences between tumor groups presented in Table 1 for GD_2, $H2K^k$, and $H2D^d$ antigen MFI ratios was assessed by the Kruskal-Wallis test and Dunn method for multiple comparisons [12]. Statistical comparisons for H2 ($H2K^k$ and $H2D^d$) specific MFI ratios in Table 1 between PBS- and Flt3-L-treatment tumors and between PBS- and hu14.18-IL2-treatment tumors were performed using the Wilcoxon-Mann-Whitney test. For the Dunn test, *P* values are adjusted *P* values.

Results

Hu14.18-IL2 therapy induces an antitumor effect against established subcutaneous NXS2 tumors

Mice were given 2×10^6 NXS2 cells s.c. on day 0. Tumors were palpable by day 3–4 and were >100 mm^3 by day 9 (Fig. 1) when treatment began. While mice that received PBS treatment exhibited progressively growing tumors, all mice receiving suboptimal hu14.18-IL2 treatment (5 μg/day for 5 days) showed a slowing of tumor growth. Three of four treated mice no longer had palpable tumor by day 22. Previous in vivo studies indicate that the antitumor effects mediated by ch14.18-IL2 treatment against experimental metastatic NXS2 tumors in A/J mice require NK cells but not T cells [35]. In similar studies we found that the antitumor effects induced by hu14.18-IL2 therapy against s.c. NXS2 tumors are also NK-dependent. Two groups of four mice each received 5 days of hu14.18-IL2 treatment starting on day 9 of tumor implantation. Four of four animals that were NK depleted (treated with anti-asialo-GM1 antibody) had large tumors (>200 mm^3) by day 15. In contrast, three of four hu14.18-IL2–treated animals not receiving asialo-GM1 antibody were tumor free at that same time (data not shown).

Recurrent tumors from hu14.18-IL2–treated mice have elevated MHC class I antigen expression

Although the suboptimal hu14.18-IL2 regimen used in Fig. 1 was able to eliminate palpable subcutaneous NXS2 tumors for most mice, many animals developed recurrent disease. As shown in Fig. 1, three of four NXS2-bearing mice that received hu14.18-IL2 therapy had no measurable tumor (a clinical ''complete response'') by day 22 post–tumor inoculation. Two of these three mice that showed a complete response by day 22 subsequently developed recurrent tumors detectable on day 36 at the original site of NXS2 tumor challenge.

Recurrent tumors were harvested, mechanically disaggregated and assessed for $H2K^k$ and $H2D^d$ antigen cell-surface expression. Figure 2 displays MFI values of $H2K^k$ and $H2D^d$ antigen cell-surface staining of five recurrent subcutaneous tumors harvested from 5 hu14.18-IL2–treated mice. These values are compared with those of five separate NXS2 tumors harvested from PBS-treated control mice. The recurrent tumors from hu14.18-IL2–treated mice showed a > sevenfold increase in $H2K^k$ and $H2D^d$ antigen expression, as determined by MFI values, over the primary NXS2

Table 1 NXS2 tumor escapee profile[a]: GD2 and MHC class I

Therapy[b]	Tumor identification	Tumor type[c]	Harvest week[d]	GD_2 ratio[e]	H2 K^k ratio	$H2D^d$ ratio
Cultured	A			22	9	16
Cultured	B			22	7	17
Cultured	C			10	16	15
Cultured	D			nd[f]	16	17
PBS	1	Primary	3	40	38	43
PBS	2	Primary	3	59	13	19
PBS	3	Primary	3	10	13	24
PBS	4	Primary	3	13	12	33
PBS	5	Primary	4	7	15	23
PBS	6	Exp. metastic	4	nd	14	51
PBS	7	Exp. metastic	4	nd	5	16
PBS	8	Exp. metastic	4	7	11	28
PBS	9[g]	Spon. metastic	nd	6	6	32
PBS	10	Spon. metastic	nd	3	12	41
Flt3-L	11	Rec. primary	5	9	4	10
Flt3-L	12	Rec. primary	7	16	17	16
Flt3-L	13	Rec. primary	7	19	8	6
Flt3-L	14	Rec. primary	13	nd	11	26
Flt3-L	15[h]	Rec. metastic	8	92	18	32
Flt3-L	16	Rec. metastic	8	12	3	4
Flt3-L	17	Rec. metastic	8	4	7	7
Flt3-L	18	Rec. metastic	8	17	3	2
Flt3-L	19	ReChal. met[i]	7	10	2	2
Flt3-L	20	ReChal. met	15	88	18	12
hu14.18-IL2	21	Rec. primary	5	11	45	109
hu14.18-IL2	22	Rec. primary	7	4	16	28
hu14.18-IL2	23	Rec. primary	5	24	40	82
hu14.18-IL2	24	Rec. primary	5	7	170	204
hu14.18-IL2	25	Rec. primary	nd	nd	283	332
hu14.18-IL2	26	PR. primary	4	9	112	182
hu14.18-IL2	27	PR. primary	4	7	78	149
hu14.18-IL2	28	PR. primary	4	2	57	120
hu14.18-IL2	29	PR. primary	4	23	199	224
hu14.18-IL2	30	PR. primary	4	4	35	80
hu14.18-IL2	31	Rec. metastic	8	4	32	81
hu14.18-IL2	32	Rec. metastic	9	nd	8	21

[a]Profile reflects cell surface expression levels of GD_2 and MHC class I molecules H2 K^k and $H2D^d$ antigen as determined by flow cytometric analysis described in "Materials and methods"

[b]NXS2 tumor-bearing mice were treated with PBS, Flt3-L, or hu14.18-IL2 as described in "Materials and methods"

[c]Tumors harvested from mice were classified as: progressive growth of primary site subcutaneous tumor lesion (*Primary*), recurrent tumor that developed at the primary site following complete resolution of initial primary tumor lesion (*Rec. Primary*), primary site subcutaneous lesion that had partially regressed in size then continued to progressively grow (*PR. Primary*), internal metastatic lesion that developed following complete resolution of initial primary tumor lesion (*Rec. Metastatic*), metastatic lesion that developed following resolution of secondary NXS2 rechallenge (*ReChal. Met*), tumor from PBS-treated experimental metastatic tumor model (*Exp. Metastatic*), or tumor from PBS-treated spontaneous metastatic tumor model (*Spon. Metastatic*)

[d]Weeks following initial or secondary NXS2 tumor injection until tumor harvest

[e]Specific MFI ratio = test MFI / isotype MFI. Conversion of test MFI into specific MFI ratio allows direct comparison of flow cytometric staining values obtained on different days and from various tumors [47]

[f]*nd* Not determined

[g]Tumor #9 is a spontaneous metastatic tumor that developed in a PBS-treated mouse following resection of the primary tumor #4. Similarly, tumor #10 is a metastatic tumor that developed in a PBS-treated mouse following resection of primary tumor #3

[h]Tumors #15 and #16 are distinct hepatic metastases harvested from a single animal. Similarly, tumors #17 and #18 were harvested from a single animal. All other tumors reported in Table 1 were obtained from distinct animals

[i]Metastatic lesions were obtained from animals that had successfully resolved initial s.c. tumor challenge following Flt3-L therapy (10 g/day × 7 days) and resolved their subsequent s.c. NXS2 rechallenge, followed by development of distant metastases

tumors from PBS-treated mice ($P < 0.0129$ and $P < 0.0148$, respectively).

Flt3-L therapy induces immune destruction of NXS2 tumors

To determine whether recurrent NXS2 tumors uniformly exhibit elevated H2 expression levels following any immunotherapeutic approach, we evaluated NXS2 tumors following Flt3-L therapy. As Flt3-L affects $CD34^+$ progenitor cells [37], its antitumor effects are not realized until after several days of treatment. Therefore, Flt3-L treatment was begun on day 3 in order to induce immune activity before tumors progressed to a size that may be ineffectively managed by this form of immunotherapy. Control mice given PBS treatment showed progressively growing tumors, while mice that received

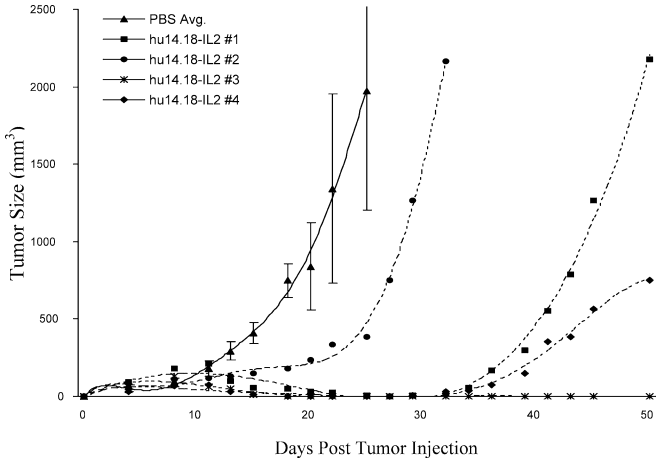


Fig. 1 Hu14.18-IL2 therapy induces resolution of established subcutaneous NXS2 tumors. Female A/J mice, 6–8 weeks of age, were injected subcutaneously with 2×10^6 NXS2 cells and were treated with PBS or hu14.18-IL2 (5 μg/day) for 5 days beginning on day 9 post–tumor inoculation and monitored for tumor growth. Data points represent PBS-treatment *(solid triangle)* group mean ± SD ($n=4$, mice/group) and four individual hu14.18-IL2–treated mice *(solid square, solid circle, star, solid diamond)*

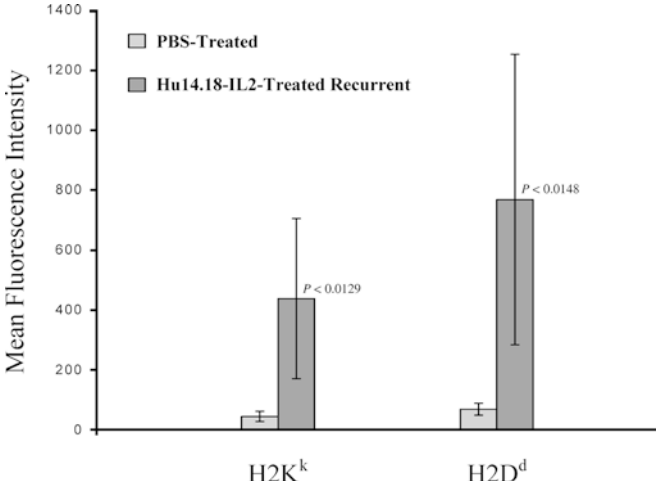


Fig. 2 Recurrent NXS2 tumors from hu14.18-IL2–treated mice have increased MHC class I expression. Mechanically disaggregated subcutaneous NXS2 tumors from PBS-treated mice or recurrent NXS2 tumors harvested from mice that had shown an initial complete response following hu14.18-IL2 therapy, were assessed for $H2K^k$ and $H2D^d$ antigen cell-surface expression by flow cytometric analysis. The data are represented as mean fluorescence intensity (MFI) ± SD of $H2K^k$ and $H2D^d$ antigen cell-surface staining for the average values of five subcutaneous NXS2 tumors harvested from PBS-treated mice *(light-gray bar)*, and five recurrent subcutaneous tumors *(dark-gray bar)* harvested from mice that had resolved their established subcutaneous NXS2 tumors following hu14.18-IL2 therapy. $H2K^k$ and $H2D^d$ antigen cell-surface staining of hu14.18-IL2-treated mice were significantly elevated ($P<0.0129$ and $P<0.0148$, respectively) when compared with staining of PBS-control mice NXS2 tumors

17 days of Flt3-L therapy displayed slowed, but progressive, tumor growth until day 10 post–tumor inoculation (Fig. 3A). At that time, when the Flt3-L treatment group tumors ranged from 40–175 mm^3 in size, tumors began regressing, so that by day 20 post–tumor inoculation most tumors had completely resolved.

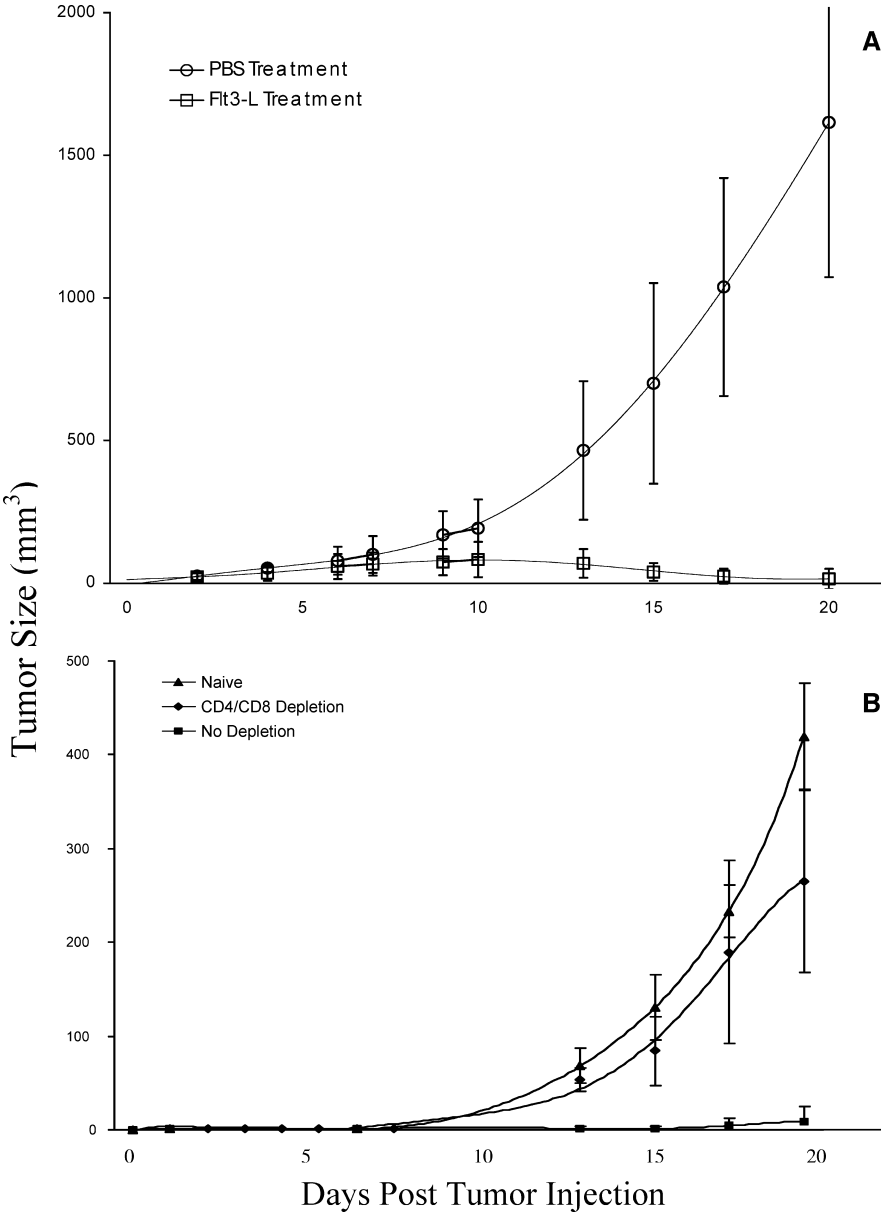
At 10 weeks following the initial NXS2 tumor challenge, six of nine mice from the Flt3-L treatment group for Fig. 3A remained free of any detectible tumor following regression of their subcutaneous tumors, while the other three animals developed late recurring tumors. We rechallenged the six tumor-free Flt3-L–treated mice from the experiment shown in Fig. 3A by injecting 2×10^6 NXS2 cells in the opposing flank and monitored them for tumor growth. To determine whether T cells were involved in any observable antitumor protection, we divided the rechallenged Flt3-L–treated mice into two groups: mice that were depleted of T cells (three of six mice), or were mock-depleted (three of six mice). Mice that resolved their initial NXS2 challenge following Flt3-L therapy were protected against rechallenge and exhibited a T-cell–dependent antitumor memory response, as demonstrated by the lack of tumor growth in the mock-depleted animals and the progressive growth of the NXS2 rechallenge tumor in mice that were depleted of T cells (Fig. 3B). Additional experiments have confirmed that NXS2 tumor-bearing animals that become tumor-free following Flt3-L treatment show protective memory responses against rechallenge with NXS2 tumor (data not shown).

Recurrent NXS2 tumors display polarized MHC class I antigen expression depending on hu14.18-IL2 or Flt3-L immunotherapy

Table 1 presents the complete set of results for MHC class I and GD_2 antigen expression levels for all 32 separate, independently derived, NXS2 tumors obtained between weeks 3 and 15 following tumor injection from 28 individual mice analyzed during a 14 month period for this study.

Following hu14.18-IL2 treatment, 12 recurrent tumors were harvested 4–9 weeks following tumor injection (2–7 weeks following hu14.18-IL2 treatment cessation). They displayed pronounced increases in their H2 class I antigen expression compared with the 10 tumors evaluated from PBS-treated animals (comparison #1, Table 2; $P<0.001$). The means of group-specific MFI ratio values for the 10 tumors that grew following an initial response to hu14.18-IL2 treatment (Table 1: tumor samples 21–30) were 104 for $H2K^k$ antigen and 151 for $H2D^d$ antigen. These values reflect 5.8- and 5.4-fold increases, respectively, in expression relative to $H2K^k$ and $H2D^d$ antigen levels observed on the five subcutaneous tumors (Table 1: tumor samples 1–5) obtained from PBS-treated animals (comparison #2, Table 2: $P<0.041$ and $P<0.045$, respectively).

In contrast, some recurrent tumors from Flt3-L–treated mice (Table 1: tumor samples 11–20) showed a depressed MHC class I antigen expression level when



compared with the PBS control-treated group tumors. When all 10 recurrent tumors harvested from Flt3-L-treated mice are compared with all 10 tumors from the PBS-treatment group for both $H2K^k$ and $H2D^d$ antigen, there is a statistically significant reduction in MHC class I antigen expression (comparison #3, Table 2: $P < 0.004$) by the tumors from Flt3-L–treated animals. This finding is consistent with the decrease in MHC class I antigen expression reported for some tumors in other murine models that have continued to progress following a T-cell–mediated antitumor response [1, 49]. When compared with all 12 recurrent tumors from hu14.18-IL2–treated mice, the 10 Flt3-L–treated recurrent tumors showed 11.5- and 12.6-fold differences in $H2K^k$ and $H2D^d$ antigen expression levels (comparison #4, Table 2: $P < 0.001$ and $P < 0.001$, respectively).

GD2 expression on recurrent tumors

Decreased expression of surface molecules recognized by mAbs is a mechanism allowing tumor persistence following mAb therapy [11, 58]. Some of the individual tumors we harvested, particularly after hu14.18-IL2 treatment, expressed low levels of GD_2 (e.g., tumor 31 in Table 1). We confirmed (data not shown) that these GD_2-low expressing recurrent tumors (i.e., tumor samples 10, 17, 24, and 31 from Table 1), were NBs by their RT-PCR positive signal for tyrosine hydroxylase, while control tumors Yac-1 and the murine colon carcinoma CT26-EpCAM were negative for tyrosine hydroxylase mRNA (data not shown). We thus compared the cell surface level of GD_2 on tumors that grew following in vivo anti-GD2 treatment with the GD_2 levels on all tumors not exposed to anti-GD_2 treatment. The mean

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Fig. 3A, B Flt3-L therapy induces T-cell–mediated anti-NXS2 memory. **A** Flt3-L therapy results in durable resolution of subcutaneous NXS2 tumors. Female A/J mice, 6–8 weeks of age, were injected subcutaneously with 2×10^6 NXS2 cells and were treated with PBS *(open circle)* or Flt3-L *(open square)* (10 μg/day) for 17 days beginning on day 3 post–tumor inoculation and monitored for tumor growth. Data points represent group mean ± SD ($n = 8$–9 mice/group). **B** Flt3-L–treated mice exhibiting durable cure possess T-cell–mediated memory responses against NXS2 rechallenge. Mice that exhibited durable resolution of established subcutaneous NXS2 tumors following Flt3-L (as shown in **A**) were rechallenged with NXS2 tumor 10 weeks following initial NXS2 challenge (the day of rechallenge is designated as day 0 in **B**). Na mice *(solid triangle)* and the six of nine Flt3-L-treated mice which showed a complete and durable cure of their NXS2 tumors (shown in **A**), were injected subcutaneously in the opposing flank with 2×10^6 NXS2 cells on day 0. The six mice that were previously Flt3-L–treated and rechallenged on day 0 were divided into two groups of three mice. One group *(solid diamond)* was T-cell–depleted by intraperitoneal injections of 250 μg anti-CD4 mAb plus 250 μg anti-CD8 mAb, while the other group *(solid square)* was mock-depleted with 1-ml Rat IgG, on days –1, 5, and 11 post–tumor rechallenge. Tumor growth was monitored and is represented as group mean ± SD

specific MFI value for GD_2 was 26.5 for NXS2 cells from animals that did not receive the GD_2-targeted hu14.18-IL2 treatment (Table 1: samples A–C, 1–5, 8–13, and 15–20). In contrast, the 10 recurrent tumors harvested from mice receiving hu14.18-IL2, showed a lower value of 9.5 for the mean specific MFI ratio for GD_2 (comparison #5, Table 2; $P < 0.021$), and suggest that decreased expression of the GD_2 molecule may be an additional mechanism involved in the selection of NXS2 cells that progress to form recurrent tumors following anti-GD_2–targeted IC therapy.

Loss of $H2^{high}$-phenotype by recurrent tumors following culture

To evaluate the stability of the altered MHC class I antigen expression on recurrent NXS2 tumors, we cultured recurrent NXS2 tumors and monitored their $H2K^k$ and $H2D^d$ antigen expression profile (Fig. 4). Tumor sample 24 (Table 1), a recurrent NXS2 tumor that developed after suboptimal hu14.18-IL2 therapy, displayed a high level of MHC class I antigen expression when assessed immediately following harvesting (ex vivo), with MFI values of 595 and 1,020 for $H2K^k$ (Fig. 4A) and $H2D^d$ (Fig. 4D) antigen expression, respectively. These high levels of $H2K^k$ and $H2D^d$ antigen expression dropped substantially following 1 week in culture to near baseline values typically observed on cultured NXS2 cells. These baseline H2 expression levels were maintained through the remainder of the culturing period (7 weeks), suggesting that the development of recurrent tumors following hu14.18-IL2 immunotherapy was not due to the survival and outgrowth of constitutively $H2^{high}$ NXS2 variants present in the tumor inoculum. Rather, our observations suggest that recurrent NXS2 tumors transiently increased their MHC class I antigen expression in response to in vivo hu14.18-IL2 immunotherapy, and returned to a lower-baseline H2 phenotype once removed from the animal and placed in vitro.

Coculture with activated splenocytes plus hu14.18-IL2 increases MHC class I antigen expression on surviving NXS2 cells

To determine whether persistent NXS2 cells increased MHC class I antigen expression in response to NK-mediated antitumor effects, we attempted to simulate the in vivo interaction of tumor cells, effector cells, and hu14.18-IL2 therapy by coculturing normal or IL-2–activated splenocytes with NXS2 cells in the absence or presence of hu14.18-IL2. We have previously shown that splenocytes activated in vivo with IL-2 or IC are able to mediate IC-facilitated antibody-dependent cellular cytotoxicity (ADCC) of tumor cells in a 4 h ^{51}Cr release assay [21]. Culturing NXS2 cells for 4 days with hu14.18-IL2, in the absence of splenocytes, had no direct influence on modulating MHC class I antigen expression on NXS2 cells in vitro (data not shown). The results in

Table 2 Tumor comparisons for statistical analyses. *IC* = Hu14.18-IL2 immunocytokine

Comparison number	Group comparison[a]	Tested parameter	*P* value[b]
1	Post-IC therapy (21−32) vs PBS-treatment control (1–10)	MHC class I ($H2K^k$and$H2D^d$)	< 0.001
2	Post-IC therapy: primary site s.c. lesions (21–30) vs PBS-treatment s.c. lesions (1–5)	$H2K^k$	< 0.041
2	Post-IC therapy: primary site s.c. lesions (21–30) vs PBS-treatment s.c. lesions (1–5)	$H2D^d$	< 0.045
3	Post-Flt3-L therapy (11–20) vs PBS-treatment control (1–10)	MHC class I ($H2K^k$and $H2D^d$)	< 0.004
4	Post-IC therapy (21–32) vs post-Flt3-L therapy (11–20)	$H2K^k$	< 0.001
4	Post-IC therapy (21–32) vs post-Flt3-L therapy (11–20)	$H2D^d$	< 0.001
5	Non-IC treated (A–C, 1–5, 8–13, and 15–20) vs post-IC therapy (21–24 and 26–31)	GD_2	< 0.021

[a] Ex vivo tumors were grouped based on immunotherapy received and location of harvested tumor. The numbers shown in parentheses for each comparison (e.g., *(21–32)* vs *(1–10)* for comparision 1) correspond to the designations of specific tumors listed in Table 1

[b] *P* values were determined on specific MFI ratio values for samples indicated in Table 1using methods of statistical analyses as described in "Materials and methods"

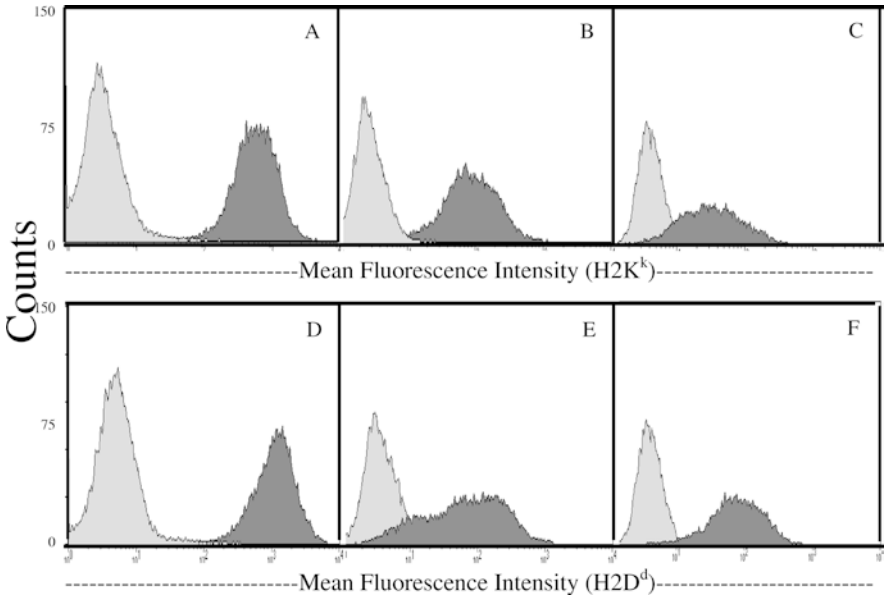


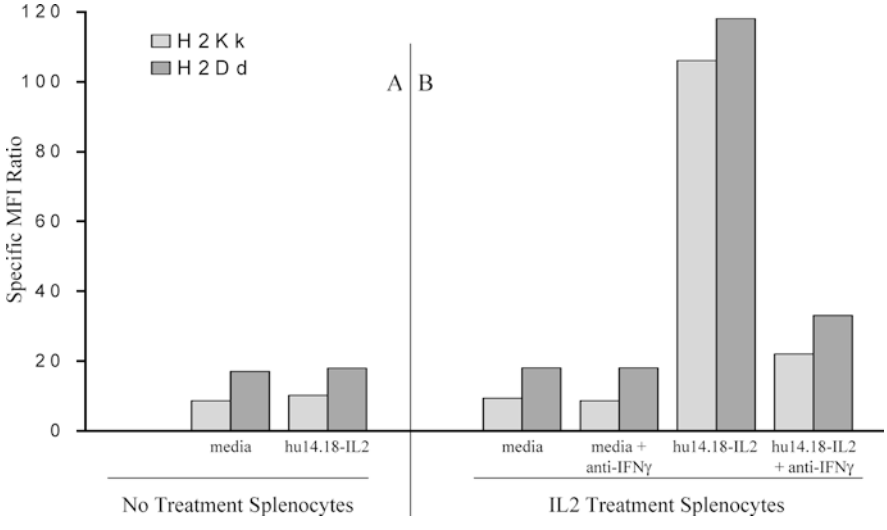
Fig. 4A–F Culturing results in down-regulated MHC class I expression by MHC class I^{high} recurrent NXS2 tumor. Tumor 24 (Table 1) was a recurrent subcutaneous tumor harvested from an animal that had completely resolved its primary subcutaneous NXS2 tumor challenge following hu14.18-IL2 therapy. The tumor was disaggregated and assessed for $H2K^k$ (**A–C**) and $H2D^d$ (**D–F**) antigen cell-surface expression by flow cytometry. Tumor was analyzed directly ex vivo (**A, D**), following 1 week in culture (**B, E**), and after 7 weeks of culture (**C, F**)

Fig. 5A indicate that NXS2 cells that remained viable following the 4 day coculture with normal splenocytes in either the absence or presence of hu14.18-IL2, did not appreciably increase their level of $H2K^k$ or $H2D^d$ antigen expression. In contrast (Fig. 5B), the NXS2 cells that survived the 4 days of ADCC and NK cytolytic conditions induced by coculture with IL-2–activated splenocytes (consisting of 26% activated NK cells according to flow cytometric analysis) in the presence of hu14.18-IL2, exhibited > eightfold increases in $H2K^k$ and $H2D^d$ antigen-specific MFI ratios over control NXS2 cultures. Furthermore, this increase in $H2K^k$ and $H2D^d$ antigen on NXS2 cells could be partially inhibited by the addition of anti-IFN-γ mAbs to the culture (Fig. 5B).

Fig. 5 MHC class I expression is increased on NXS2 cells when cocultured with activated splenocytes in the presence of hu14.18-IL2. Splenocytes were harvested from normal mice (**A**) or mice that had received systemic rhIL2 (50,000 IU/day) treatment for 7 days via subcutaneous osmotic pump implant (**B**). Flow cytometric analysis of splenocytes from normal mice indicated that only 3.8% of the cell population were $DX5^+/B220^+$ activated NK cells, whereas 26.5% of the IL2-activated splenocyte population were activated NK cells (data not shown). Splenocytes were cultured at 8×10^6 cells/well with 2×10^6 NXS2 cells in a volume of 5 ml. Cells were cultured in the absence or presence of 5 μg/ml of hu14.18-IL2 and harvested following 4 days of culture to be assessed for MHC class I expression by flow cytometry as described for Fig. 4. Some cocultures with IL-2–activated splenocytes (**B**) also included the addition of anti-IFN-γ mAb (50 μg/ml) on day 0. Values are expressed as the specific MFI ratio of anti-$H2K^k$- or $H2D^d$-mAb staining

Increased MHC class I antigen expression on NXS2 targets corresponds with reduced susceptibility to in vitro cytolysis

We hypothesized that NXS2 cells exhibiting augmented H2 expression levels are resistant to IC-facilitated NK-



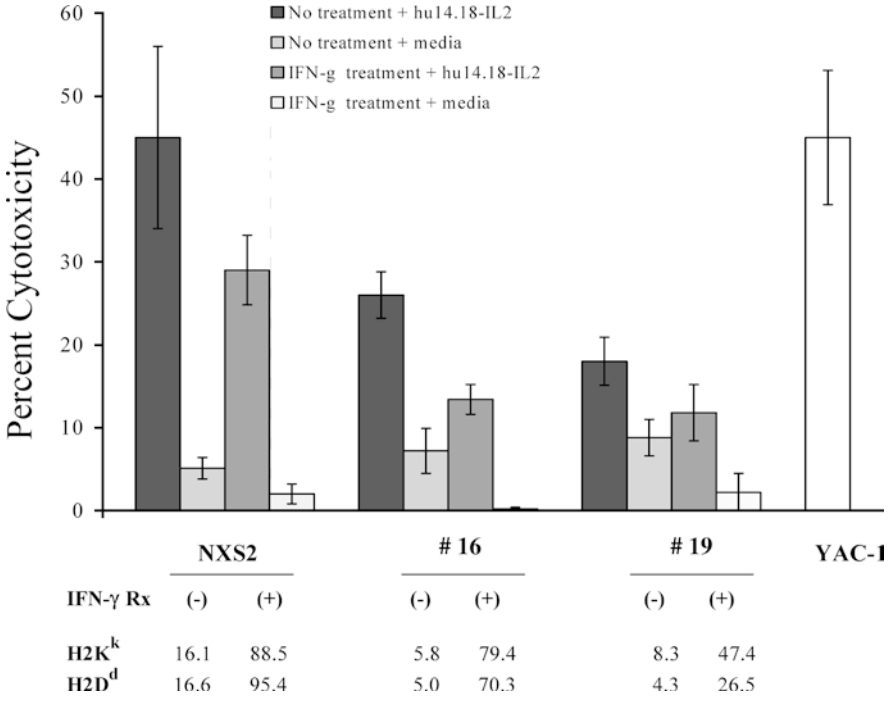


Fig. 6 NXS2 cells with increased MHC class I expression are less susceptible to hu14.18-IL2–facilitated NK-mediated cytolysis. The influence of rmIFN-γ–induced increased MHC class I expression on NK-mediated tumor lysis was tested on NXS2 cells and tumors # 16 and # 19, which were recurrent metastatic tumors harvested from Flt3-L–treated mice that displayed depressed MHC class I expression. Effector cells were the same splenocytes from IL-2–treated mice described in Fig. 5. Effectors were used in a 4-h ^{51}Cr release assay at 100:1 effector-to-target ratio in the presence or absence of hu14.18-IL2 (10 μg/ml final concentration) with nontreated or rmIFN-γ–pretreated tumor targets. Tumor targets were pretreated by a 48-hr culture period in the presence or absence of rmIFN-γ (25 U/ml). Nontreated and rmIFN-γ–pretreated tumor targets were analyzed for their MHC class I expression prior to initiating the cytotoxicity assay. Specific MFI ratios of anti-H2K^k- or H2D^d-mAb staining are reported with or without IFN-γ, below their representative tumors in the figure. Results are expressed as the percent cytotoxicity ± SD. Killing of YAC-1 cells in hu14.18-IL2-supplemented medium was included as positive control for NK efficacy

mediated antitumor effects and tested cultured NXS2 cells and cells from tumors 16 and 19 (from Table 1), which were recurrent metastatic tumors harvested from Flt3-L–treated mice that initially displayed depressed MHC class I antigen expression. These tumor cells were susceptible to killing when evaluated in a 4-h ^{51}Cr-release cytotoxicity assay using the same freshly harvested splenocytes from IL-2–activated mice shown in Fig. 5, in the presence of exogenous hu14.18-IL2 (Fig. 6). As shown in Fig. 6, these NB cells displayed increased H2K^k and H2D^d antigen expression following 48 h of culture with IFN-γ, and reduced susceptibility to killing when compared with NB cells not pretreated with IFN-γ. These results are consistent with data from other models showing that targets expressing elevated MHC class I molecules are less susceptible to direct [16, 28] or ADCC-facilitated [21] NK-mediated lysis.

Discussion

The experiments presented here focused on tumors growing in animals that initially responded to immunotherapy in vivo, as measured by complete or partial shrinkage of palpable tumors. The tumors that subsequently recurred in these animals are the outgrowths of tumor cells that survived and remained viable during the hu14.18-IL2 or Flt3-L immunotherapy that induced complete or partial regression of the primary established subcutaneous tumors. This in vivo resistance to immune attack, followed by tumor outgrowth, has been referred to as escape or evasion.

The ability of tumor cells to escape T-cell–mediated antitumor effects by decreasing their MHC class I antigen expression is well documented [1, 17, 47, 49, 63]. The decreased levels of MHC class I antigen shown here for NXS2 tumors recurring after Flt3-L are consistent with these prior reports. Yet, mechanisms allowing tumors to escape in vivo destruction by NK-mediated antitumor responses remain uncharacterized.

The data presented here demonstrate that NXS2 tumor cells that persisted and generated recurrent tumors after in vivo exposure to IC-facilitated, NK-mediated, antitumor effects, have augmented MHC class I antigen expression (Tables 1 and 2). Our data are consistent with recent reports showing that higher levels of H2 expression on tumor cells correspond to decreased vigor of the induced NK-cell response in vivo [16, 32]. This NK resistance, associated with increased MHC class I antigen expression on tumor cells, may be mediated by activation of inhibitory receptors on NK cells by the MHC molecules themselves [23]. Thus, in vivo blockade of inhibitory Ly-49 receptors is able to

augment NK-mediated antileukemia effects in vivo [26]. These murine data are consistent with the recent observation that metastatic uveal melanoma tumors, unlike most melanomas, expressed relatively high HLA class I antigen levels, which may have facilitated tumor escape from NK-mediated surveillance of hematogenous metastases [22].

The mechanisms of in vivo up- or down-regulation of MHC class I antigen on recurrent tumors in hu14.18-IL2–treated or Flt3-L–treated mice requires further study. Figure 5 documents increased H2 class I antigen on the viable tumor cells remaining following 4 days of coculturing of activated splenocytes with NXS2 cells in the presence of hu14.18-IL2. The induced up-regulation of MHC class I antigen, as opposed to the selection of preexisting $H2^{high}$-expressing variants in vivo, is further supported by the transient nature of the increased MHC class I antigen expression (Fig. 4), and the increased class I antigen expression on the five s.c. NXS2 tumors that exhibited only partial responses to hu14.18-IL2 therapy (Table 1: samples 26–30). This major increase in MHC class I antigen level on these five tumors (mean specific MFI ratio of 151 for $H2D^d$) occurred in partially responsive tumors where a palpable component of the primary tumor had not completely resolved following hu14.18-IL2 treatment, suggesting that intratumoral NXS2 cells had up-regulated their H2 expression in response to some effect not present in Flt3-L- or PBS-treated mice (mean specific MFI of 12 and 31, respectively, for $H2D^d$). Furthermore, the MHC class I antigen increase found on NXS2 tumor cells surviving 4 days culture with IL-2–activated splenocytes and hu14.18-IL2 (Fig. 5B) can be partially inhibited by adding anti-IFN-γ mAb to the culture. IFN-γ is a cytokine that induces up-regulated H2 expression on many tumor cells in vitro, including NXS2 (Fig. 6) and many human NB tumors [9]. These observations suggest that release of IFN-γ in the tumor microenvironment induced by hu14.18-IL2 (but not Flt3-L or PBS treatment), possibly by recruited and activated NK cells, may cause a transient increase in MHC class I antigen expression by tumor cells that persist in the presence of an ongoing NK-mediated antitumor response. If so, this elevated H2 expression is maintained in vivo for several weeks, long after the several hours required to clear the exogenously administrated hu14.18-IL2 from the blood [25].

Established NXS2 NB tumors are eradicated through an NK-dependent response with hu14.18-IL2 (data not shown) or ch14.18-IL2 IC therapy [35]. In contrast, the ch14.18-IL2 IC mediates a T-cell–dependent antitumor response in the B78-D14 murine melanoma model [3]. In this experimental system, we have not characterized the relative roles of Fas-ligand, perforin, or soluble cytotoxic mediators in the in vitro (or in vivo) destruction of NXS2 tumor cells by NK cells from A/J mice [31, 55]. We have reported that the level of MHC class I antigen expression on CT-26 murine colon cancer lines could influence the level of in vitro ADCC and the in vivo antitumor effector mechanism mediated by tumor-targeted IC therapy [21]. Tumor-targeted IC therapy mediated a T-cell–dependent antitumor effect in vivo against a high MHC class I antigen-expressing CT-26 cell line [61], whereas the same IC therapy induced an NK-dependent antitumor effect against a low H2-expressing subclone of CT-26 [21]. Thus the phenotype of the tumor cell, as opposed to some inherent quality of the IC, appears to determine whether the IC-induced antitumor effector response is mediated predominantly by T cells or by NK cells.

This study also demonstrates that some NXS2 tumors that progress following 14.18-IL2 anti-GD_2 treatment have decreased expression of GD_2 (Table 1). The anti-GD_2–directed response may select for variants with transient or permanent lower GD_2 expression from the heterogeneous pool of NXS2 cells comprising the primary tumor mass. In vitro analyses have correlated the sensitivity of human NB cells to ADCC with their level of GD_2 expression [43], although no significant loss of GD_2 expression has been seen in recurrent or refractory tumors in NB patients following 3F8 anti-GD_2 mAb therapy [27]. Apart from GD_2 or H2 expression, many other factors may also be influencing NXS2 "escape" from hu14.18-IL2 treatment [7, 10, 13, 29, 40, 55]. These have not been investigated here, and require further analyses. For example, the strong response to the xenogeneic IC might secondarily induce some in vivo immune suppression that enables tumor escape, and might not be expected in patients treated with humanized IC.

The divergently polar MHC class I antigen expression levels displayed by recurrent tumors following hu14.18-IL2 or Flt3-L therapy suggest that alteration of MHC class I antigen expression may be a common mechanism which facilitates immune selection of NXS2 tumors that persist during an NK- or T-cell–mediated antitumor attack in vivo. Additional studies are required to determine whether modulation of MHC class I antigen expression is involved in other tumor models that evade a NK- or T-cell–mediated immune therapy, and whether this H2 alteration is indeed the mechanism directly responsible for tumor "escape." Our data suggest an in vivo scenario where IC immunotherapy mobilizes activated NK antitumor effectors to the tumor microenvironment. These NK effectors facilitate the destruction of most tumor cells, yet some tumor cells, which rapidly augment their MHC class I antigen phenotype in response to the soluble IFN-γ secreted by the activated NK effector cells, may survive. As recurrent NXS2 tumors from Flt3-L–treated mice (i.e., tumors 16 and 19, Table 1) are still susceptible to hu14.18-IL2 facilitated NK-mediated lysis (Fig. 6), we hypothesize that combined treatment with Flt3-L plus hu14.18-IL2 IC might induce both T-cell– and NK-cell–mediated antitumor effects, and may potentially prevent the development of recurrent NXS2 tumors that occur through modulated expression of their MHC class I antigen molecules.

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