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The Level of MHC Class I Expression on Murine Adenocarcinoma Can Change the Antitumor Effector Mechanism of Immunocytokine Therapy[1]

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ABSTRACT

The huKS1/4-IL2 fusion protein, directed against the human epithelial cell adhesion molecule (huEpCAM) has been shown to induce a strong $CD8^+$ T-cell-dependent, natural killer (NK) cell-independent, antitumor response in mice bearing the huEp-CAM-transfected CT26 colon cancer CT26-EpCAM. Here we investigate the effectiveness of huKS1/4-IL2 against CT26-Ep21.6, a subclone of CT26-EpCAM, expressing low levels of MHC class I. *In vitro* antibody-dependent cellular cytotoxicity (ADCC) assays in the presence of huKS1/4-IL2 demonstrate that murine NK cells from spleen and blood can kill CT26-Ep21.6 significantly better than they kill CT26-EpCAM. NK-mediated ADCC of CT26-EpCAM can be enhanced by blocking the murine NK cell-inhibitory receptor, Ly-49C. A potent *in vivo* antitumor effect was observed when BALB/c mice bearing experimental metastases of CT26-Ep21.6 were treated with huKS1/4-IL2. The depletion of NK cells during huKS1/4-IL2 treatment significantly reduced the antitumor effect against CT26-Ep21.6. Together our *in vitro* and *in vivo* data in the huEp-CAM-transfected CT26 models indicate that the amount of MHC class I expressed on the tumor target cell plays a critical role in the *in vivo* antitumor mechanism of huKS1/4-IL2 immunotherapy. A low MHC class I level favors NK cells as effectors, whereas a high level of MHC class I favors T cells as effectors. Given the heterogeneity of MHC class I expression seen in human tumors and the prevailing T-cell suppression in many cancer patients, the observation that huKS1/4-IL2 has the potential to effectively activate an NK cell-based antitumor response may be of potential clinical relevance.

INTRODUCTION

Prior studies in murine models have demonstrated that Ab[3]-cytokine fusion proteins (immunocytokines) have superior antitumor effects over the combination of Ab plus cytokine (1, 2). Ab-IL-2 fusion proteins have been tested extensively in experimental models. In a murine model for neuroblastoma using the NXS2 tumor cell line in A/J mice, treatment with ch14.18-IL2, a chimeric Ab-IL-2 fusion protein that recognizes the ganglioside GD2, induced absence of detectable metastases, whereas the combination of comparable amounts of anti-GD2 Ab plus soluble IL-2 did not completely clear metastases from mice. *In vivo* $CD4^+$ and $CD8^+$ T cell depletion during the fusion protein treatment did not decrease the striking antimetastatic effect of the ch14.18-IL2 fusion protein in NXS2-bearing A/J mice. In contrast, *in vivo* depletion of NK cells dramatically inhibited the antitumor effect of the ch14.18-IL2 fusion protein (3, 4), clearly showing that the antitumor effect induced by the ch14.18-IL2 fusion protein against the NXS2 tumor is based on NK cell effectors.

In a different murine tumor model using a subline of the CT-26 colon tumor cell line that had been transfected to express the huEp-CAM, immunotherapy with the huKS1/4-IL2 fusion protein that recognizes huEp-CAM antigen, showed a striking antitumor effect, similar in efficacy to that seen with ch14.18-IL2 treatment in the NXS2 neuroblastoma model (3). In contrast to the neuroblastoma model, the response in the CT26-EpCAM model was dependent on T cells and not on NK cells (5). One hypothesis for the discrepancy in the effector cells responsible for these immunocytokine-induced antitumor effects may be a differential in NK cell susceptibility by these tumor cells. This could be regulated by relative differences in the expression of MHC class I molecules on the surface of the two target cell lines. The level of MHC class I on tumor target cells has been shown to influence recognition by effector cells. A down-regulation of MHC class I results in a decreased MHC class I-mediated presentation of peptide antigens to T cells. As a consequence, fewer cytotoxic T cells can be activated to kill the MHC class I down-regulated tumor cells (6). In contrast, MHC class I expression has an important regulatory role on natural killing (7, 8) and ADCC-mediated (9–11) killing by NK cells. Subsets of murine and human NK cells have inhibitory receptors on their surface that are able to bind certain MHC class I determinants. The Ly-49C receptor on NK cells of BALB/c mice was shown to bind to $H\text{-}2^d$ molecules on target cells and thereby down-regulate the killing activity of these NK cells (12–15). Thus, low MHC class I expression on a tumor may make it less susceptible to T-cell killing through decreased T-cell receptor recognition, but more susceptible to NK killing.

In the study presented here, we evaluate how the level of MHC class I expressed on huEp-CAM expressing CT-26 cells regulates the selection of effector cells that mediate tumor destruction *in vivo* after targeted IL-2 therapy with huKS1/4-IL2 fusion protein. For this evaluation, the CT26-Ep21.6 cell line was derived from the CT26-EpCAM line through clonal selection for low MHC class I expression (1). This cell line allowed us to test the influence of MHC class I on fusion protein-mediated killing *in vitro* and *in vivo*. In the experiments presented in this report, we demonstrate that the huKS1/4-IL2 fusion protein is capable of generating a potent antitumor response against the low MHC class I expressing CT26-Ep21.6 tumor *in vitro* and *in vivo*. Furthermore, in contrast to the T-cell response that controls the CT26-EpCAM tumor in huKS1/4-IL2-treated mice, the response in huKS1/4-IL2 treated CT26-Ep21.6-bearing animals is mediated primarily through NK cells.

MATERIALS AND METHODS

Animals. Female BALB/c mice (6–8 weeks of age) were obtained from The Jackson Laboratory (Bar Harbor, ME). All of the animals were housed in University-approved facilities and were handled strictly according to NIH and University of Wisconsin-Madison Research Animal Resource Center guidelines.

Cell Lines. The cloning of the huEp-CAM antigen into the CT26 colon epithelial cell line was described previously (1). The subclone expressing low levels of MHC class I, CT26-Ep21.6, was described previously (1). Briefly, the CT26-EpCAM cell line was subjected to cell sorting to identify low MHC

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[3] The abbreviations used are: Ab, antibody; IL, interleukin; NK, natural killer; huEp-CAM, human epithelial cell adhesion molecule; V region, variable region; ADCC, antibody-dependent cellular cytotoxicity; mAb, monoclonal AB; PBMC, peripheral blood mononuclear cell; MAHA, mouse antihuman Ab.

class I expressing cells, which were then cloned by subsequent limiting dilution. Subclones were tested for stable huEp-CAM expression and low levels of MHC class I expression. CT26-EpCAM and CT26-Ep21.6 cell lines were maintained in DMEM, supplemented with 5% fetal bovine serum, vitamins, L-glutamine, sodium pyruvate, nonessential amino acids, 100 units/ml penicillin/streptomycin, and 1 mg/ml G418 sulfate, at 37°C and 7.5% CO_2. The murine lymphoma cell line YAC-1 was grown in complete RPMI 1640 with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin, at 37°C and 7.5% CO_2.

Fusion Protein. The generation and structure of the huKS1/4-IL2 fusion protein was described previously (1). Briefly, the complementarity-determining V regions of the murine monoclonal KS1/4 Ab, which recognizes the huEp-ÖCAM, were grafted into the framework of human V regions on the protein level and then reverse translated to obtain the genes. The genes for the humanized V regions were then inserted into an expression vector containing the constant regions of the human κ light chain and the human C γ_1 heavy chains that have the sequence for IL-2 linked to their COOH terminus (16). Fusion protein was expressed by transfection of the plasmids into the NS/0 myeloma cell line in selective media. Expressing clones were identified by ELISA and purified by protein A-Sepharose columns (17). Thus, the resulting fusion protein is an intact humanized KS1/4 IgG1 immunoglobulin that recognizes the human Ep-CAM molecule, and has an intact human IL-2 molecule linked to the Fc end of each IgG heavy chain.

Experimental Pulmonary Metastasis. To induce pulmonary metastases in BALB/c mice, $1–4 \times 10^5$ tumor cells in a volume of 100 μl of PBS were injected slowly into the tail vein. After 3 days, when metastases were established, fusion protein treatment or control treatment with PBS was started. All of the animals were killed on the same day in any given experiment. This timing varied and depended on the severity of symptoms of metastatic disease of the untreated, tumor-bearing, control animals. India ink was injected into the lungs *in situ*; the lungs were removed from the animals to destain and bleach in Feketes solution (18), and metastases were counted.

Cytotoxicity Assays. Effector cells from the spleen or blood were obtained from BALB/c mice 24 h after the last of three daily i.v. injections of huKS1/4-IL2 or from untreated control mice. Splenocytes were prepared by mechanical disaggregation and subsequent hypotonic shock treatment to remove erythrocytes. After filtration through a 70 μm cell strainer, the splenocytes were washed, and viable cells counted by eosin exclusion staining. Blood was obtained from mice through either tail vein or retro-orbital bleeding into heparinized tubes. PBMCs were separated from erythrocytes by centrifugation over Histopaque-1083 (Sigma).

Target cells were labeled for 2 h with 250 μCi of ^{51}Cr in 1 ml of culture medium. The target cells were mixed every 15 min to keep them in suspension. Targets were washed twice before adding to the assay. Five $\times 10^3$ target cells per well were used with splenocytes as effectors. Because of limitations in available blood effector cells, 2.5×10^3 target cells per well were used with blood effector cells. To some wells, huKS1/4-IL2 fusion protein was added at a concentration of 10 μg/ml. For the inhibitory receptor (Ly-49C) blocking assay, 50 μg/ml of 5E6 Ab (BD PharMingen, San Diego, CA) was added. After targets and effectors were mixed in 96-well U-bottomed microtiter plates, they were centrifuged at $200 \times g$ for 5 min. The plates were then incubated for 4 h at 37°C. Maximum ^{51}Cr release was measured by lysing target cells with the detergent cetrimide (Sigma). Spontaneous ^{51}Cr release was measured by incubating target cells for 4 h in RPMI-FCS alone. Percentage cytotoxicity values were calculated for each E:T ratio as follows:

$$\% \text{ cytotoxicity} = 100 \times \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}}$$

Results presented are expressed as percentage cytotoxicity or as lytic units per 10^7 effector cells, where 1 lytic unit is calculated as [10^7 effector cells]/[number of effectors required to achieve 20% lysis (LU20) of 5×10^3 targets] (19). All of the conditions in all of the cytotoxicity assays were tested in quadruplet wells.

Flow Cytometry Analysis. Cell surface-expressed huEp-CAMs were detected by using mAb KS1/4 or huKS1/4-IL2 at a concentration of 1 μg/10^6 cells. The secondary Ab for this staining was a FITC-conjugated goat antihuman Ab (Caltag, Burlingame, CA) used at a concentration of 2 μg/10^6 cells. Detection of $H2K^d$ or $H2D^d$ was done by using phycoerythrin-conjugated anti-$H\text{-}2K^d$ or -$H\text{-}2D^d$ mAb (BD PharMingen) at a concentration of 1 μg/10^6.

ELISA. For the MAHA ELISA, plates were coated overnight at 4°C with 0.1 μg/ml hIgG1, diluted in sodium bicarbonate buffer (pH 9.6). After washing 4 times with 100 mM Tris 0.05%-Tween 20 (pH 7.4), plates were blocked for 3 h with PBS-5% milk. After washing four times with 100 mM Tris 0.05%-Tween 20 (pH 7.4), the serum samples were diluted 1:1000 and added to the wells for overnight incubation at 4°C. Wells were then washed four times with 100 mM Tris 0.05%-Tween 20 buffer, and, subsequently, 0.05 μg/ml goat antimouse IgG horseradish peroxidase was added, and plates were incubated for 3 h at room temperature. Plates were then washed four times with 100 mM Tris 0.05%-Tween 20 buffer and 50 μl of tetramethylbenzidine (Dako) one-step substrate were added to each well. The plates were incubated 18–30 min until the desired blue color was reached; then the reaction was stopped by the addition of 50 μl/well of 2 N H_2SO_4. The plates were then read at 450 nm with a 570-nm reference filter. The standard curve reagent used for this assay was obtained by performing these measurements on serial dilutions of mouse antihuman IgG1(BD PharMingen) in concentrations between 5 and 250 ng/ml in 2-fold increments.

The ELISA for the detection of huKS1/4-IL2 in mouse serum samples was done as follows: the plates were coated overnight at 4°C with 2 μg/ml of goat antihuman IgG that was diluted in sodium bicarbonate buffer (pH 9.6). After washing four times with 100 mM Tris 0.05%-Tween 20, the plates were blocked for 3 h at room temperature with PBS-5% milk. After washing four times with 100 mM Tris 0.05%-Tween 20, the serum samples were added at a dilution of 1:5 and incubated overnight at 4°C. Wells were then washed four times with 100 mM Tris 0.05%-Tween 20, and 0.4 μg/ml sheep antihuman IgG1 horseradish peroxidase was added; samples were then incubated for 3 h at room temperature. Plates were washed four times with 100 mM Tris 0.05%-Tween 20, and 100 μl tetramethylbenzidine one-step substrate were added to each well. The readout was performed as described above. The huKS1/4-IL2 fusion protein was used for the standard curve at concentrations between 5 ng/ml and 300 ng/ml

RESULTS

CT26-Ep21.6 Expresses Low Levels of MHC Class I and Is Recognized by huKS1/4-IL2. The mouse colon cancer cell line CT26-EpCAM (5), which expresses MHC class I, was selected for low MHC class I expressing cells by fluorescent cell sorting as described previously (1). The low MHC class I expressing cell fraction was subjected to limiting dilution, and the resulting clones were again tested for MHC class I and huEp-CAM expression. Clone CT26-Ep21.6 was found to have a low expression of MHC class I and a stable expression of huEp-CAM. Fig. 1 shows a comparison of MHC class I (detected by anti-$H2D^d$ Ab) and huEp-CAM expression on CT26-EpCAM and CT26-Ep21.6 cells measured by flow cytometry. Both of the clones show similar expression of huEp-CAM (Fig. 1*B*), whereas MHC class I expression for the CT26-Ep21.6 clone was markedly reduced in comparison with the CT26-EpCAM clone (Fig. 1*A*). Similar results were obtained when using an Ab against $H2K^d$ (data not shown).

***In Vivo* Treatment with huKS1/4-IL2 Activates Blood and Spleen NK Cells.** The cytotoxic activity of NK cells was measured at different time points during and after a 5-day course of *in vivo* huKS1/4-IL2 treatment. NK activation was monitored in the blood and spleen of mice with samples obtained 18 h after the first, third, or fifth i.v. dose of 20 μg of fusion protein. Blood and spleen specimens were also taken on day 8, which was 3 days after the last i.v. treatment. The cytotoxicity assays using NK cell-sensitive, YAC-1 target cells, revealed that a strong up-regulation of NK activity over nontreated control animals occurred in the spleen on days 1, 3, and 5, with peak levels seen on day 3 of huKS1/4-IL2 fusion protein treatment (Fig. 2*A*). By day 8, 3 days after the fusion protein treatment was completed, the NK cell activity was further reduced to background level. The same activation pattern was observed in the blood of fusion

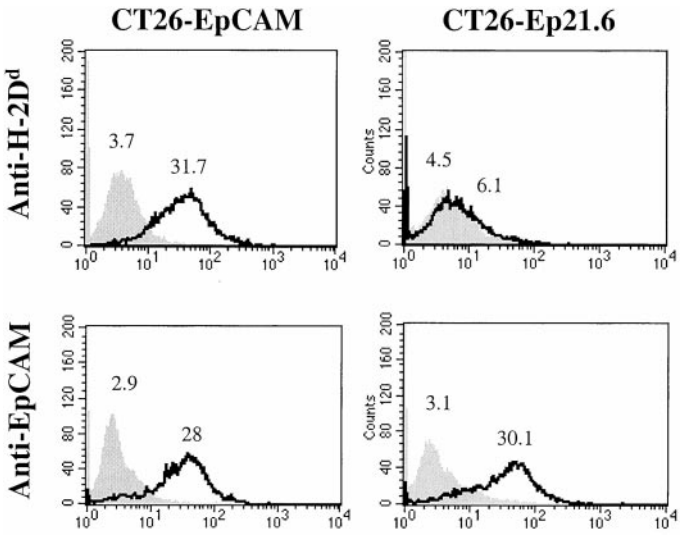


Fig. 1. MHC class I and huEp-CAM expression on CT26-EpCAM and CT26-Ep21.6 tumor cell lines determined by flow cytometry. *Top two panels*, staining of cells with either FITC-conjugated anti-H2D^d Ab (*solid line*) or FITC-conjugated IgG2A isotype control (*gray shade*). *Bottom two panels*, staining of cells with huKS1/4 mAb (primary staining) followed by FITC-conjugated goat antihuman Ab (*solid line*) and staining with secondary Ab without primary huKS1/4-IL2 treatment (*gray shade*). All of the antibodies were used at a concentration of 1 μg/10^6 cells. Live cells (10^4) were analyzed using propidium iodide staining. *Numbers inside graphs*, mean fluorescence intensity values.

spleen of treated animals. This study also shows that NK activity is not continuing to rise after day 3, and that NK activity is waning by day 5 and back to baseline by day 8.

A Strong MAHA Response Is Generated in Mice Receiving the huKS1/4-IL2 Fusion Protein. To identify possible explanations for the down-regulation of NK activity while the fusion protein treatment is still ongoing (*i.e.*, less NK activity on day 5 than on day 3 in Fig. 2), we looked for the development of MAHA in fusion protein-treated mice. Because the KS1/4-IL2 fusion protein is a humanized Ab (~98% human IgG1), it was expected that a 5-day treatment regimen would induce a MAHA response in mice. To measure the development of this MAHA response, we treated non-tumor-bearing mice with 15 μg of huKS1/4-IL2 per day for 5 days beginning on day 1. On days 0, 2, 4, and 7, blood was drawn from these mice and analyzed for MAHA in an ELISA assay. This ELISA assay was designed to detect the MAHA response against the human IgG1 component of the huKS1/4-IL2 molecule. Fig. 3*A* shows that the development of a MAHA response in mice is first detected on day 4 after the third fusion protein injection, with a greater value on day 7, 2 days after the last fusion protein injection. To determine how powerful this MAHA response is in clearing fusion protein given in a second course of huKS1/4-IL2 fusion protein treatment, mice that underwent one 5-day course of fusion protein treatment were subjected to a single bolus of fusion protein 2 weeks after the last injection of the first course. After the bolus injection, blood was drawn at different time points, and the

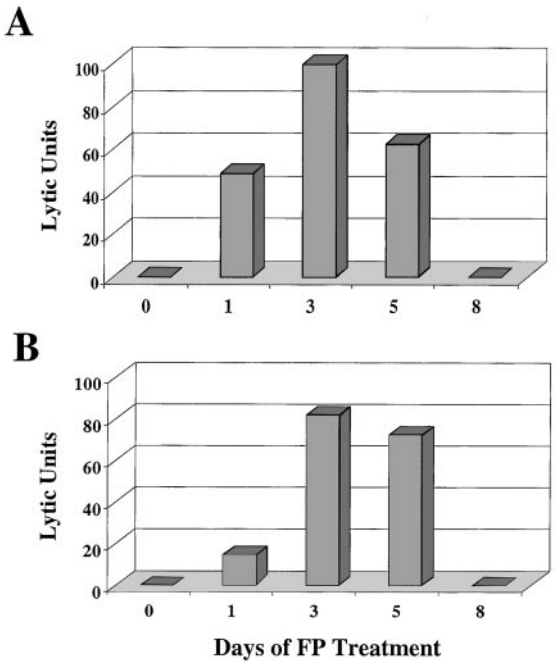


Fig. 2. Activation of NK cell-mediated killing in the spleen and peripheral blood of BALB/c mice during, and 3 days after, huKS1/4-IL2 treatment. PBMC and splenocyte effector cells were taken from untreated control animals or from treated animals 18 h after their first, third, or fifth daily dose, or 3 days after their fifth daily dose of 20 μg huKS1/4-IL2. Mice received one dose of huKS1/4-IL2 per day. Each control and treatment group consisted of two mice. NK-sensitive YAC-1 cells were used as targets; huKS1/4-IL2 was not present in the *in vitro* assay. *A*, killing of YAC-1 cells by effectors from the spleen. *B*, killing of YAC-1 cells by PBMC. The experiment was performed twice with similar results.

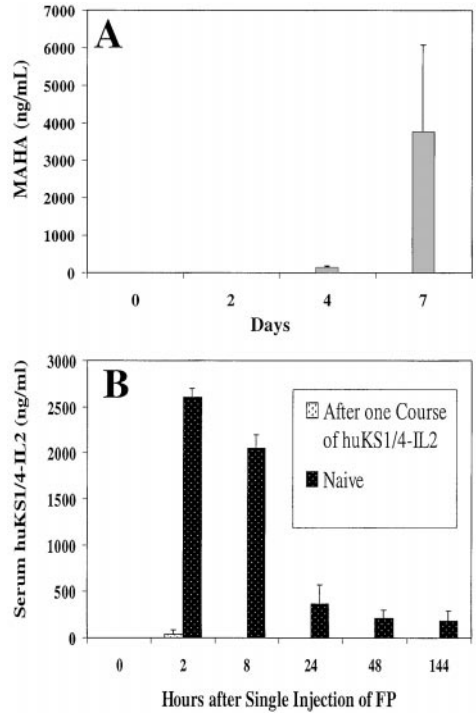


Fig. 3. MAHA response in the serum of naive and preexposed mice. *A*, naive mice received five daily injections of 15 μg of huKS1/4-IL2 fusion protein starting on day 1. Blood was drawn on day 0, 2, 4, and 7. A total of 23 mice was used in this experiment; groups of 12 and 11 mice were bled on day 0 and day 2, respectively. All of the 23 mice were bled on day 4 and day 7. *Each column*, the average with SD corresponding to the number of mice used for this particular time point (11, 12, or 23 samples). The MAHA response on day 0 and 2 was undetectable. In *B*, naive mice (23 animals) and mice that received a 5-day course of fusion protein 3 weeks earlier (23 animals), were given a single i.v. bolus of 15 μg huKS1/4-IL2 fusion protein. From each group, five or six mice were bled for each of the 2-, 8-, 24-, and 48-h time points. All of the 23 mice in each group were bled for the 144-h time point. Sera were then tested for huKS1/4-IL2 levels by ELISA. *Each column*, the average with SD corresponding to the number of mice (5, 6, or 23) used for this particular time point. The values for hour 0, 8, 24, 48, and 144 for the mice that were treated previously with fusion protein were undetectable. The time point 0 in both *A* and *B*, represents a pretreatment control.

protein-treated mice (Fig. 2*B*). Similar results were seen in a separate experiment evaluating NK activity in the spleen and blood of tumor-bearing animals receiving huKS1/4-IL2 treatment (data not shown). The data shown in Fig. 2 indicate that the huKS1/4-IL2 is capable of inducing strong NK cell activation in the circulating blood and in the

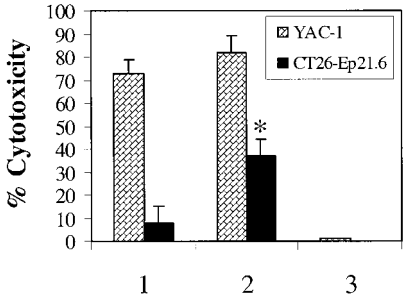


Fig. 4. NK cells mediate killing of CT26-Ep21.6 in the presence of KS1/4-IL2 fusion protein. Splenocytes from either 3-day fusion protein-treated mice (*Lanes 1* and *2*) or 3-day fusion protein-treated and NK-depleted mice (*Lane 3*) were used as effector cells against NK-sensitive target cells YAC-1 or tumor target cells CT26-Ep21.6. NK cells were depleted by a single dose of antiasialo GM-1 antiserum (Wako Chemicals) 1 day prior to starting the 3-day fusion protein treatment. Two mice were used for each NK-depleted and -undepleted group. E:T ratio was 100:1. *Lane 1*, killing without huKS1/4-IL2 fusion protein present in the assay; *Lanes 2* and *3*, killing rates with 10 μg/ml huKS1/4-IL2 fusion protein present in the assay. Killing for the CT26-Ep21.6 target in *Lane 3* was undetectable. Averages with SDs from three different experiments are shown. *, statistical significance (*t* test, $P = 0.01$) when compared with the value in *Lane 1*.

serum was tested for the concentration of circulating huKS1/4-IL2 fusion protein. As can be seen in Fig. 3*B*, naive control animals that were not previously exposed to the huKS1/4-IL2 show the expected pharmacokinetics of an IL-2 fusion protein (20–22). In contrast, in the mice that underwent a 5-day course of fusion protein 2 weeks earlier, fusion protein was detectable 2 h after the injection, but at a level far lower than that seen in the naive mice. At 8 h after the injection, the fusion protein was no longer detectable. Together these data show that during the first 5-day course of fusion protein treatment in mice, a MAHA response is generated and is detectable as early as day 4. Furthermore, when mice are given a second treatment of fusion protein, the existing MAHA response is able to rapidly clear the huKS1/4-IL2 fusion protein from the circulation. These results suggest that the decreased NK activation on day 5 for animals receiving 5 days of fusion protein (Fig. 2) is attributable to the clearance of *in vivo* fusion protein by the MAHA response detected on day 4.

CT26-Ep21.6 Is Killed *in Vitro* by Activated NK Cells in a Fusion Protein Dose-dependent Manner. Previous studies (5, 23) have shown strong antitumor effects of the huKS1/4-IL2 fusion protein *in vivo* and *in vitro* against high MHC class I expressing CT26-EpCAM tumor cells. However, many human cancer patients have tumors expressing a low level of MHC class I (6, 24, 25). It is, therefore, desirable to know whether the huKS1/4-IL2 fusion protein also has an antitumor effect against tumors that are low in MHC class I.

To test this hypothesis *in vitro*, we performed cytotoxicity assays with CT26-Ep21.6 tumor target cells and activated NK cells. On the basis of the data presented in Fig. 2, we chose to use splenocytes from mice after three daily injections of fusion protein as the source of activated NK cells. The effector cells used for these assays were from non-tumor-bearing animals, to exclude prior *in vivo* sensitization of T cells to the CT26-Ep21.6 cells, which might influence results in the cytotoxicity assay. As shown in Fig. 4, these *in vivo* activated splenocytes mediate the potent destruction of YAC-1 cells and the weak killing of CT26-Ep21.6 (Fig. 4, *Lane 1*). Furthermore, splenocytes from fusion protein-stimulated mice mediate potent ADCC because they are able to kill the CT26-Ep21.6 target cells significantly better ($P = 0.01$) in the presence of 10 μg/ml huKS1/4-IL2 in the assay (*Lane 2*), compared with no fusion protein in the assay (*Lane 1*). The killing of the YAC-1 target did not change significantly ($P = 0.1$) when huKS1/4-IL2 was added (*Lanes 1* and *2*). To determine whether NK cells are responsible for the killing of CT26-Ep21.6 *in vitro*, we also tested splenocytes from mice depleted of NK cells during the 3-day fusion protein treatment. As can be seen in Fig. 4, *Lane 3*, this depletion abrogated killing against CT26-Ep21.6 completely, which indicated that NK cells are crucial in the fusion protein-mediated killing of CT26-Ep21.6 *in vitro* by splenocytes from non-tumor-bearing animals that were treated with fusion protein for 3 days.

To test whether the huKS1/4-IL2 fusion protein is effective against CT26-Ep21.6 in a dose-dependent manner, a fusion protein dose escalation cytotoxicity assay was done. We were able to detect marked differences in the killing of CT26-Ep21.6 *versus* CT26-EpCAM by *in vivo* activated NK cells (Fig. 5). Statistical analysis using linear regression revealed that the CT26-Ep21.6 target is killed at significantly higher rates (*t* test, $P < 0.0005$) than CT26-EpCAM at all of the fusion protein concentrations tested, thus indicating that CT26-Ep21.6 is, in fact, more susceptible to fusion protein-induced killing through NK cells than CT26-EpCAM is.

Up-Regulation of MHC Class I on CT26-Ep21.6 Reduces Susceptibility to Killing Mediated by huKS1/4-IL2-activated NK Cells. Low levels of MHC class I expressed on target cells makes them more susceptible to NK cell-mediated killing, because of the "missing self" signal that is transmitted through the family of inhibitory receptors on NK cells (7). This difference in MHC class I expression is the likely explanation for the differential susceptibility to NK-mediated ADCC shown in Fig. 5. To further test the role of MHC class I expression in this model system, we modulated the level of MHC class I expression on the target cells with subsequent testing in cytotoxicity assays. We were able to change MHC class I expression on the target cells by two different means: through prolonged *in vitro* culturing and through induction with IFN-γ. We found that by a-yet-undefined activation mechanism, prolonged culturing of CT26-Ep21.6 cells *in vitro* does increase their MHC class I expression to levels that are comparable with that of CT26-EpCAM cells. This spontaneous up-regulation of MHC class I in prolonged cultures of CT26-Ep21.6 cells (designated CT26-Ep21.6^{high}) allowed us to test the same cell clone expressing different levels of MHC class I in a cytotoxicity assay using NK cells as effectors in the presence of fusion protein. As can be seen in Fig. 6*A*, CT26-Ep21.6 differs markedly from CT26-Ep21.6^{high} in its MHC class I expression. These two cell variants were tested in a cytotoxicity assay as described above. As can be seen in Fig. 6*B*, CT26-Ep21.6^{high} cells were killed by NK cells at a significantly lower rate than were CT26-Ep21.6. To confirm this observation by a different approach, we artificially increased the level of MHC class I expressed on both CT26-EpCAM and CT26-Ep21.6

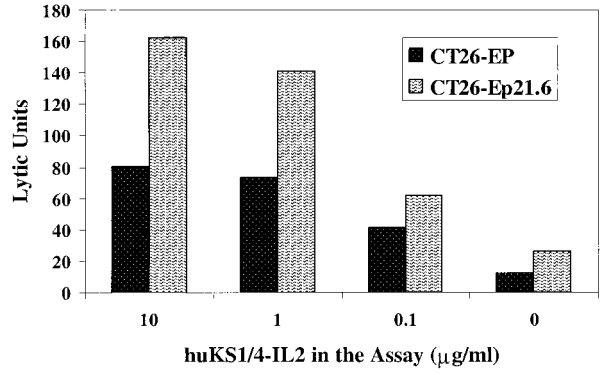


Fig. 5. HuKS1/4-IL2 dose-dependent killing of huEp-CAM-positive tumor cells with different levels of MHC class I expression. Naive mice received three daily i.v. injections of 20 μg huKS1/4-IL2 per mouse to activate splenocytes *in vivo*. Freshly harvested splenocytes were used as effector cells at E:T ratios of 100:1, 50:1, 25:1, and 12.5:1. A representative experiment of three is shown. Statistical analysis was performed using all of the three data sets.

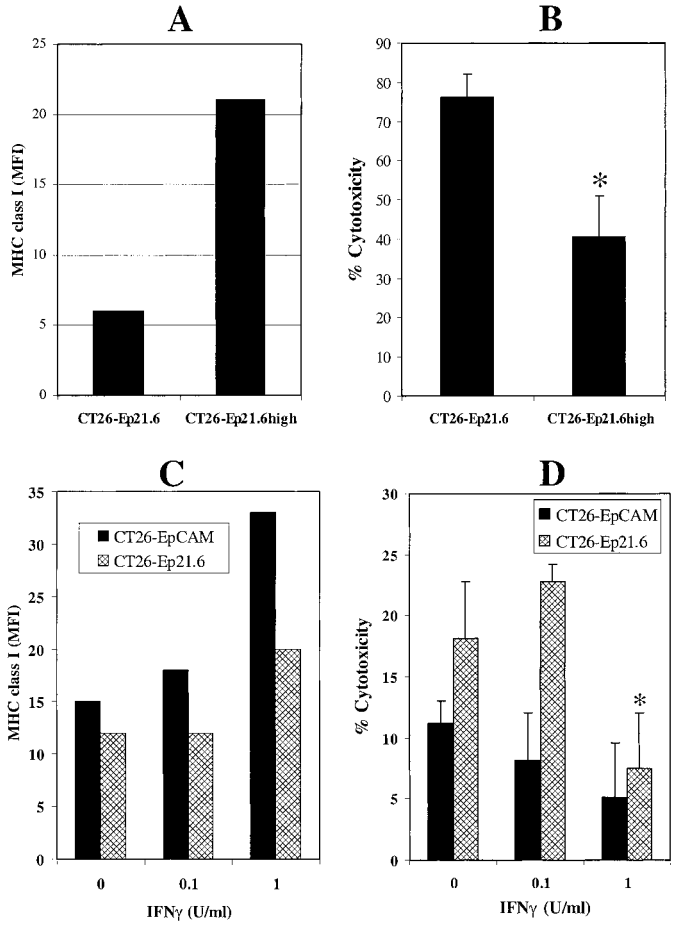


Fig. 6. The level of NK-mediated fusion protein-induced killing varies inversely with the level of MHC class I expression on CT26-Ep21.6 target cells. *A*, flow cytometry assay performed with phycoerythrin-conjugated anti-H2K^d Ab on CT26-Ep21.6 and a variant of this cell line that developed augmented MHC class I expression after prolonged culturing (CT26-Ep21.6^{high}). Values shown are mean fluorescence intensities (*MFI*) for H2K^d. *B*, cytotoxicity assay; effector cells were splenocytes from non-tumor-bearing mice after 3 days of fusion protein treatment. *C*, induction of MHC class I expression on the surface of CT26-EpCAM and CT26-Ep21.6. Cells were cultured at different concentrations of IFNγ for 48 h. Cells were stained with FITC-conjugated anti-H2D^d (BD PharMingen, San Diego, CA). Isotype control was FITC-conjugated IgG2a. Mean fluorescence intensity values are shown. *D*, cytotoxicity assay using targets with different levels of MHC class I expression. Target cell lines CT26-EpCAM and CT26-Ep21.6 were kept at different concentrations of recombinant mouse IFNγ (R&D Systems, Minneapolis, MN) for 48 h and were washed prior to the assay. Splenocytes from 3-day huKS1/4-IL2-treated animals were used as effectors. The E:T ratio used was 100:1. Mean and SDs of two different sets of experiments are shown. *, $P < 0.05$, for the 1.0 units/ml IFN-γ treatment when compared with the values from the 0 unit/ml and 0.1 units/ml IFN-γ treatment groups of CT26-Ep21.6.

cell lines by incubating them at different concentrations of IFN-γ for 48 h. Fig. 6*C* shows the IFN-γ dose-dependent up-regulation of MHC class I (H2D^d) on the surface of CT26-Ep21.6 and CT26-EpCAM. Cells from the different IFN-γ treatment groups were then tested in a cytotoxicity assay using activated splenocytes as effector cells. Fig. 6*D* depicts the influence of MHC class I expression on the target cell on the amount of NK-mediated killing in the presence of the fusion protein. Killing of the CT26-Ep21.6 target is significantly reduced as a result of 1 unit/ml IFN-γ treatment (compared with killing of CT26-Ep21.6 in the absence of IFN-γ), whereas the same IFN-γ treatment did not change the killing of CT26-EpCAM significantly.

Thus we were able to observe that augmenting MHC class I inhibits NK-mediated fusion protein-dependent killing of target cells in three distinct protocols: (*a*) comparing killing of two different clones, CT26-EpCAM and CT26-Ep21.6; (*b*) comparing killing of two variants of the same CT26-Ep21.6 clone; and (*c*) comparing killing of CT26-EpCAM and CT26-Ep21.6 after IFN-γ induction of MHC class I. These independent assays argue that the elevated susceptibility of CT26-Ep21.6 to NK cell-mediated killing is primarily attributable to its relative deficiency in MHC class I rather than some other clonal differences between CT26-Ep21.6 and CT26-EpCAM.

Blocking of Ly-49C on NK Cells Significantly Increases huKS1/4-IL2-mediated Killing of CT26-EpCAM. To further clarify the role of MHC class I expressed on the tumor target cell lines in this model system, we wanted to identify the mechanism through which augmented MHC class I expression can suppress NK cell-induced fusion protein-dependent killing. As mentioned above, this question has been previously addressed and answered in other *in vitro* systems. In particular, blocking the function of Ly-49C receptors (by clone 5E6 mAb) on NK cells was shown to augment the killing of sygeneic target cells by simulating the missing self signal situation (13, 14, 26, 27). We wished to determine whether this same pathway was involved in the fusion protein-dependent killing by *in vivo* activated NK cells. Therefore, we compared 5E6 mAb-pretreated or -nonpretreated effector cells in their ability to kill CT26-EpCAM or CT26-Ep21.6 target cells to determine the role of the Ly-49C inhibitory receptor in fusion protein-facilitated killing of huEp-CAM-expressing target cells. As can be seen in Fig. 7, the fold increase in the killing of CT26-EpCAM target cells by 5E6 mAb-treated effector cells is 1.45 times greater than the killing by nontreated effector cells. The huKS1/4-IL2-facilitated killing of CT26-Ep21.6 target cells by 5E6 mAb-treated effectors is 1.2 times greater than the killing by nontreated effector cells. The 1.45-fold increase for CT26-EpCAM targets was found to be greater than the 1.2-fold increase observed with the CT26-Ep21.6 targets ($P < 0.013$, by *t* test). Thus, blocking the function of the Ly-49 inhibitory receptor allows a greater augmentation in the killing of the high MHC class I expressing target CT26-EpCAM than of the low MHC class I expressing CT26-Ep21.6 target. Together with the results obtained from testing of target cell lines with different levels of MHC class I (Fig. 6), these data clearly prove that one mechanism for the increased killing of CT26-Ep21.6 by NK cells in the presence of the huKS1/4-IL2 fusion protein is based on the reduced level of MHC class I and, therefore, a reduced inhibitory interaction with Ly-49C receptors.

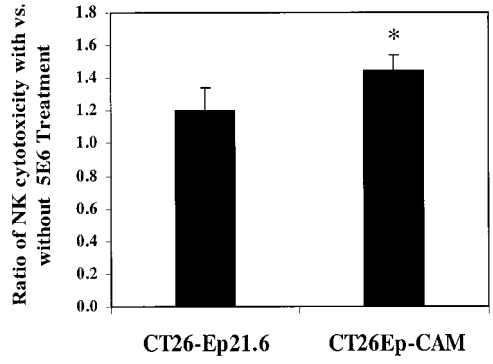


Fig. 7. Effect of blocking inhibitory receptor Ly-49C on NK cells during fusion protein-mediated killing of tumor cells. In this four hours ^{51}Cr-release assay, non-tumor-bearing mice were given a 3-day fusion protein treatment, and splenocytes were used as effectors at an E:T ratio of 100:1. The anti-Ly-49C Ab, clone 5E6 (BD PharMingen), was used at 5 μg/ml and was added to the effectors 30 min prior to the killing assay. HuKS1/4-IL2 was present in the assay at 10 μg/ml. The ratios of killing observed with the 5E6 mAb pretreatment:killing without 5E6 pretreatment are shown. Averages from 5 independent experiments with SDs are shown. The fold increase for CT26-EpCAM and the fold increase for CT26-Ep21.6 were found to be different from each other by *t* test (*, $P < 0.013$).

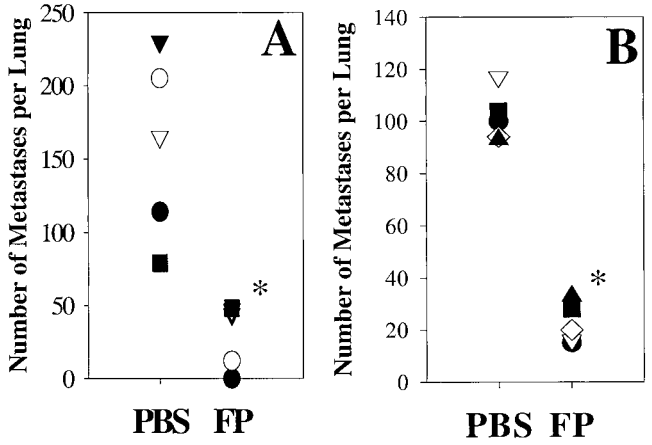


Fig. 8. HuKS1/4-IL2 fusion protein induced antitumor efficacy in low and high MHC class I expressing huEpCAM transfected CT26 cells. *A*, number of metastases detected on day 14 in mice that were given injections of 4×10^5 cells of CT26-Ep21.6 and treated with fusion protein (*FP*) or saline (*PBS*). *B*, number of metastases detected on day 21 in mice that were given injections of 1×10^5 cells of CT26-EpCAM and treated with fusion protein (*FP*) or saline (*PBS*). The fusion-protein treatment started on day 3 after tumor injection and was given at a dose of 20 μg/mouse/day for 5 days. Each treatment group contained five animals. Each point reflects an individual animal. A representative experiment of three is shown. *, $P < 0.003$, when compared with PBS-treated animals.

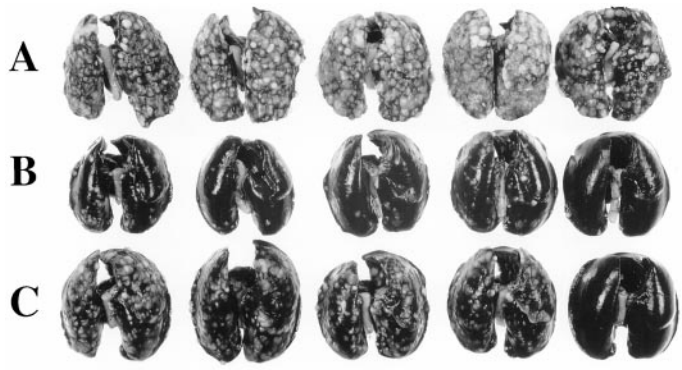
The huKS1/4-IL2 Fusion Protein Mediates Potent Antitumor Effects against CT-26Ep21.6 and CT26-EpCAM Experimental Metastasis in Syngeneic BALB/c Mice. To achieve suitable numbers of experimental metastases in our model systems with the CT26-Ep21.6 or CT26-EpCAM cell lines, we performed tumor inoculum dose escalation experiments. We found that the tumorigenic dose that is needed to induce ≥100 pulmonary metastases in BALB/c mice was 4×10^5 cells for CT26-Ep21.6 and 1×10^5 for CT26-EpCAM (data not shown). In animals receiving 4×10^5 CT26-Ep21.6 or 1×10^5 for CT26-EpCAM cells without any antitumor treatment, this tumor burden leads to death within 30 days (data not shown). We found that killing animals between 14 and 21 days after tumor inoculation was the preferred time to adequately quantitate metastases in treatment and control groups and to avoid significant morbidity of the animals.

To evaluate the effect of huKS1/4-IL2 fusion protein treatment against the CT26-Ep21.6 tumor *in vivo*, different treatment timings and different doses of fusion protein were tested. Similar to earlier observations in the CT26-EpCAM model (5), we were able to observe a dose response against CT26-Ep21.6 metastases using doses of 15, 18, 20, and 30 μg of huKS1/4-IL2 per day, with 20 μg being the optimal dose (data not shown). The optimal timing of the treatment was observed when five daily injections of huKS1/4-IL2 fusion protein were given starting on day 3 (after tumor injection). Fig. 8*A* demonstrates the strong antitumor effect of the huKS1/4-IL2 fusion protein against CT26-Ep21.6 metastases. As a positive control for the huKS1/4-IL2 fusion protein treatment, the antimetastatic effect against the high MHC class I expressing CT26-EpCAM clone was tested in parallel (Fig. 8*B*). The huKS1/4-IL2 treatment groups from both tumors showed a striking decrease in the number of lung metastases compared with their controls (*t* test, $P < 0.003$). These data indicate that the huKS1/4-IL2 fusion protein has similar antitumor effects in mice bearing either high or low MHC class I expressing variants of the same huEp-CAM$^+$ syngeneic tumor.

NK Cells Are Involved in huKS1/4-IL2-mediated Antitumor Effect against CT26-Ep21.6. Mice that are depleted of NK cells are not able to control the NXS2 neuroblastoma when treated with the ch14.18-IL2 fusion protein, a fusion protein similar in structure to the huKS1/4-IL2 (4, 5). In contrast, mice that receive huKS1/4-IL2 therapy against the CT26-EpCAM tumor are able to control this tumor entirely using their T cells; NK cells are not required (5). To evaluate the cellular mechanism of the fusion protein-induced antitumor mechanism in mice bearing the low MHC class I expressing CT26-Ep21.6 clone, NK cell depletions were performed. In experiments aimed at determining the best NK depletion regimen and timing, we found that depleting NK cells before the I.V. injection of tumor cells caused a significant increase in metastases compared with those in nondepleted controls (data not shown). This result indicates that there is a role for NK cell-based innate immunity in regulating metastases of CT26-Ep21.6. To avoid influencing the establishment of metastases immediately after tumor injection, we chose to deplete NK cells from tumor-bearing mice with antiasialo GM1 Ab just prior to and during the actual fusion protein treatment (day 3–7). The effectiveness of this NK cell depletion regimen was tested independently by demonstrating virtually absent *in vitro* cytotoxicity against the NK-sensitive target YAC-1 with splenocytes from NK cell-depleted animals (data not shown). As can be seen in Fig. 9, the depletion of NK cells in huKS1/4-IL2 fusion protein-treated animals (Fig. 9*C*) dramatically inhibits the antitumor effect seen in Fig. 9*B* ($P < 0.01$). This is in contrast to the absence of any NK role in the fusion protein mediated *in vivo* control of the high MHC class I expressing CT26-EpCAM clone (5). It indicates that the expression level of MHC class I can directly influence whether an antitumor response that is induced by the huKS1/4-IL2 fusion protein involves NK cells. Furthermore, these results demonstrate that the huKS1/4-IL2 fusion protein can be an effective tool against huEp-CAM$^+$ tumors, independent of their MHC class I expression.

DISCUSSION

A heterogeneous expression of MHC class I is often seen on human tumors, and it has been shown recently that the level of MHC class I expressed on human carcinoma plays an essential role in regulating NK cell activity (28). Therefore, additional information about the influence of tumor cell MHC class I expression on the *in vivo*



B	163	30	48	75	7
C	>350*	>350*	>350*	>350*	14

Fig. 9. NK cell depletion in fusion protein-treated mice bearing low MHC class I expressing EpCAM positive tumors. Mice were given i.v. injections of 4×10^5 CT26-Ep21.6 cells. Treatment for 5 days with huKS1/4-IL2 or PBS was started on day 3. Animals were killed on day 14, and the lungs were stained with India ink. *A*, control treatment group, tumor-bearing animals received PBS. *B*, treatment group, animals received huKS1/4-IL2 during 5 days at 20 μg/mouse/day. *C*, NK-depletion group; animals received 5 days of huKS1/4-IL2, starting on day 3, NK cells were depleted during huKS1/4-IL2 treatment by giving three injections of antiasialo GM1 Ab (Wako Chemicals) on days 2, 6, and 10 at a concentration of 50 μl (~1 mg). The table represents the number of metastases from the five individual mice in groups B and C used for the statistical analysis proving that the number of metastases in group B is less than that in group C in this experiment (*t* test, $P = 0.009$). *, values set to 350 for the statistical analysis. Two experiments were conducted with identical statistical outcome.

response to huKS1/4-IL2 immunotherapy is of potential clinical importance. The study presented here was designed to: (*a*) test the antitumor effect of the huKS1/4-IL2 fusion protein against low MHC class I expressing huEp-CAM$^+$ adenocarcinoma; and (*b*) determine the role of tumor cell MHC class I expression on the strength and cellular mechanism of huKS1/4-IL2-mediated antitumor effects.

The huKS1/4-IL2 fusion protein can mediate potent *in vitro* killing via NK cells against CT26-Ep21.6 but is significantly less effective in facilitating *in vitro* killing against the high MHC class I-expressing CT26-EpCAM cell line. Blocking of the Ly-49C receptor on NK cells by the 5E6 Ab did significantly increase killing of CT26-EpCAM cells. This indicates that the lower susceptibility of CT26-EpCAM to NK cell-mediated killing is controlled, at least in part, by the Ly-49C inhibitory receptors on NK cells that are engaged through their recognition of MHC class I expressed on the target cell. Although only about 40% of the NK cells in BALB/c mice express the Ly-49C receptor (29), we were still able to detect a significantly increased level of killing of CT26-EpCAM when the effectors were blocked with the 5E6 mAb. We anticipate that blocking the remaining 60% of the NK population with other antibodies specific for other inhibitory receptors, for example Ly-49A, may have further increased killing against the high MHC class I expressing target, as has been shown in other models (30).

When the huKS1/4-IL2 fusion protein was given to naive mice, a very pronounced activation of NK cells was measurable in the spleen and blood on the 3rd day of a 5-day regimen. To our surprise, the cytotoxic activity of NK cells decreased rapidly during the last 2 days of fusion protein treatment and was back to baseline level by day 8. This means that even during the ongoing huKS1/4-IL2 treatment, a down-regulatory mechanism is activated that is able to inhibit the NK cell stimulatory signal that is mediated through the IL-2 component of the fusion protein. This down-regulation was also observed in tumor-bearing mice (data not shown), which implies that this modulatory effect is tumor independent. In studies performed by Gillies *et al.* (31), it was found that splenocytes, taken from BALB/c mice 3 days after a 5-day huKS1/4-IL2 treatment, produced high levels of IL-4. We hypothesize that two different mechanisms might contribute to the down-regulation of NK cell activity in huKS1/4-IL2-treated animals. First, down-regulatory mechanisms acting on NK cells may be engaged by the secretion of IL-4. Second, the strong MAHA response that we have detected by day 4 can clear the fusion protein rapidly from the circulation, thereby preventing the IL-2 component of the fusion protein from maintaining the activation state of NK cells *in vivo*.

The huKS1/4-IL2 fusion protein administration is able to induce a potent antitumor response in mice despite the neutralizing MAHA response that appears as early as 4 days after starting huKS1/4-IL2 treatment (Fig. 3). Experiments done by Gillies *et al.* (31) with a murine KS1/4-IL2 (consisting of murine KS1/4 mAb linked to murine IL-2), in comparison with the huKS1/4-IL2, showed that the murine version of the fusion protein is clearly superior to the humanized version in controlling CT26-Ep21.6 intradermal tumors. This likely reflects the absence of any MAHA response against this murine fusion protein when used to treat mice. Consequently, it is anticipated that the humanized huKS1/4-IL2 fusion protein should be more effective in clinical testing, because human patients are less likely to make a neutralizing Ab to this humanized fusion protein than they would to a murine mAb or fusion protein.

With the depletion experiments in this study, we were able to show that the depletion of NK cells during the fusion protein treatment significantly reduced the antitumor effect of the huKS1/4-IL2 fusion protein in CT26-Ep21.6 tumor-bearing animals. This is in contrast to our earlier findings in CT26-EpCAM tumor-bearing mice in which we demonstrated a strong T-cell dependence of the antitumor effect mediated by the huKS1/4-IL2 fusion protein (5). The combined results from these two studies demonstrate that the same huKS1/4-IL2 fusion protein is effective against tumors of different MHC class I levels in a syngeneic mouse model.

These observations are of potential significance for the translation of these preclinical data to the testing of huKS1/4-IL2 in human cancer patients. First, many human tumors exhibit decreased or heterogeneous levels of MHC class I expression (25, 32–36). In fact, it was shown that the susceptibility to T-cell-mediated killing is directly correlated with the amount of MHC class I expressed on these tumor cells (6). Second, tumor-bearing patients often suffer from severe T-cell dysfunction (37, 38) attributable either to the immunosuppressive effects of the cancer itself or to the immunosuppressive effects of the surgery, radiotherapy, or chemotherapy used to treat the cancer. Nevertheless, even in the face of T-cell deficiency, most patients have the potential to expand and activate NK cells on IL-2 treatment (39–41). Therefore, therapeutic agents are required that can act through different effector cell populations, depending on the target cell phenotype. The data presented here suggest that the huKS1/4-IL2 fusion protein should be able to meet these requirements.

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