Enhanced Killing of Primary Ovarian Cancer by Retargeting Autologous Cytokine-Induced Killer Cells with Bispecific Antibodies: A Preclinical Study


Abstract

Cytokine-induced killer (CIK) cells are ex vivo activated and expanded CD8+ natural killer T cells that have been shown to have antitumor activity. This is the first study exploring cell killing of primary ovarian carcinoma cells with and without bispecific antibodies. Primary cancer cells and autologous CIK cells were collected from women with epithelial ovarian cancer. Bispecific antibodies against cancer antigen-125 (BSAbxC125) and Her2 (BSAbxHer2) were developed using chemical heteroconjugation. On fluorescence-activated cell sorting analysis, the expansion of CIK cells resulted in a significant increase of CD3+CD8+ and CD3+CD56+ T cells. With enhancement by bispecific antibodies, the mean percent lysis in a 51Cr release assay of fresh ovarian cancer cells exposed to autologous CIK cells increased from 21.7 ± 0.3% to 89.4 ± 2.1% at an E:T ratio of 100:1 (P < 0.001). Anti-NKG2D antibodies attenuated the CIK activity by 56.8% on primary cells (P < 0.001). In a xenograft severe combined immunodeficient mouse model, real-time tumor regression and progression was visualized using a noninvasive in vivo bioluminescence imaging system. Four hours after CIK cell injection, we were able to visualize CD8+NKG2D+ CIK cells infiltrating Her2-expressing cancer cells on fluorescence microscopy. Mice that underwent adoptive transfer of CIK cells redirected with BSAbxC125 and BSAbxHer2 had significant reduction in tumor burden (P < 0.001 and P < 0.001) and improvement in survival (P = 0.05 and P = 0.006) versus those treated with CIK cells alone. Bispecific antibodies significantly enhanced the cytotoxicity of CIK cells in primary ovarian cancer cells and in our in vivo mouse model. The mechanism of cytolysis seems to be mediated in part by the NKG2D receptor.

Ovarian cancer is the most lethal gynecologic malignancy in the United States, causing 14,500 deaths annually (1, 2). Although primary ovarian carcinosmas initially respond to platinum-based chemotherapy in up to 80% of women with advanced disease, responses typically are incomplete, recurrence rates are high, and long-term survival is poor (3, 4). Novel biological therapies are warranted for women diagnosed with this deadly disease.

The development of cellular therapy for the treatment of cancer has received considerable attention (5–7). Previous studies have suggested that ovarian cancer patients with greater T-cell infiltration of their tumor have improved outcomes (8). In addition, the finding that regulatory T cells commonly infiltrate ovarian cancer tumors suggests that specific immunity to the malignancy may be attenuated in these patients (9). Given these limitations in ovarian cancer patients, the development of a cellular therapy for ovarian cancer is an attractive alternative. Expanded and activated ex vivo for reinfusion into the patient, cytokine-induced killer (CIK) cells are a biologically feasible treatment strategy. Functionally, CIK cells are like natural killer cells because they do not require priming but also like T cells in that they are CD3+ and rapidly expandable in culture (10). In a randomized clinical trial of hepatocellular cancer patients who underwent surgical resection, those who received adjuvant cellular therapy had a statistically significant improvement in survival compared with controls (11). Recently, we have shown that CIK cells can be readily expanded from relapsed lymphoma patients for clinical application. More importantly, the toxicity was minimal and clinical responses were shown in these heavily pretreated patients (10).
Bispecific antibodies are capable of redirecting effector cells to the cancer targets. A bispecific antibody (BSAbxHer2) with affinity to both CD3 and Her2 has been shown to enhance CIK cell cytotoxicity in an ovarian xenograft mouse model (12). However, based on Group d’Investigateurs Nationaux pour l’Etude des Cancers Ovariens and Gynecologic Oncology Group data, Her2 is overexpressed in only 6% to 16% of ovarian carcinomas (13, 14). On the other hand, cancer antigen-125 (CA125) is a surface molecule associated with >80% of advanced epithelial ovarian carcinomas. Persistent elevation of serum CA125 predicts for residual disease in >95% of women (15). We designed a novel bispecific antibody that binds with high affinity to both CA125 and CD3 (BSAbCA125).

Although established cell lines can provide useful information to study ovarian cancer, these cells typically undergo many manipulations during their development and propagation that can result in nonrandom genomic aberrations (16). Given that cell lines may not accurately represent the original tumors, we performed experiments using fresh ovarian cancer cells and believe that these results may be more readily translated into the clinical setting. A powerful tool for evaluating disease progression in cancer models involves labeling biological processes with reporters that are propagated along with cell proliferation. Using highly sensitive photon counting cameras, our system can detect light emanating from internal bioluminescent reporters of tagged cancer cells. Because photon emission increases in proportion to the number of cell division, the bioluminescence imaging system provides the opportunity for temporal and sequential evaluation of various therapeutic approaches in animal models. In this study, we investigated the ability of novel bispecific antibodies to enhance the cytotoxicity of autologous CIK cells against primary ovarian carcinomas. Furthermore, we proposed to determine the mechanism of CIK cytotoxicity in a xenograft severe combined immunodeficient (SCID) mouse model using bioluminescence imaging system.

Materials and Methods

Generation of CIK cells. Human peripheral blood lymphocytes were obtained from buffy coats of healthy donors or ovarian cancer patients by Ficoll-Hypaque density centrifugation and washed thrice with PBS. The final product was resuspended in RPMI 1640 containing 10% FCS (HyClone, Logan, UT), 100 units/mL penicillin, 100 mg/mL streptomycin, 2 mmol/L sodium phosphate, 0.15 mol/L NaCl (pH 7.2) was reacted with a 4-fold excess of sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Pierce) at room temperature for 1 hour. Both mAbs were purified on PD-10 columns (Pharmacia, Uppsala, Sweden) in PBS to remove unbound cross-linker. The cross-linked mAbs were mixed in equimolar ratios and conjugated at 4°C overnight. Protein quantification was done with a BCA Protein Assay kit (Pierce). The hetero-conjugation product was analyzed using nonreducing SDS-gel electrophoresis using a 2% to 15% gradient gel (OWL Scientific, Woburn, MA) and detected using Coomassie blue staining. Densitometric analysis was done using the Gel Doc 1000 System (Bio-Rad, Hercules, CA). The BSAbCA125 was prepared using the same procedure. BSAbCD3 does not bind to either human or murine CD18 or any known antigens and was used as a control (18–20).

To determine arming concentrations for our studies, we did dose titration studies in which we armed effector cells with 0, 1, 5, 25, 50, 100, or 500 ng bispecific antibody/10^6 effector cells and did 51Cr release assays at an E:T ratio of 50, 25, 12.6, 6.25, and 3.125 against a relevant target cell line. We found that the cytotoxicity is dose dependent with the curve reaching a plateau at 25 ng/10^6 effector cells. For our experiments, we used the next higher concentration after reaching the plateau concentration (i.e., 50 ng/10^6 effector cells) as our optimal arming dose.

Cell lines and fresh tumor cells. Human ovarian carcinoma cell lines CA-OV3, OVCAR-3 and SK-OV3 were obtained from American Type Culture Collection (Manassas, VA). Cell line UCI-101 was generously provided by Drs. P. DiSaia and A. Manetta (University of California-Irvine, Irvine, CA). Consents were obtained from women with primary epithelial ovarian carcinoma for tissue and blood collection under protocols approved by the Stanford University Institutional Review Board. Tumor cells were extracted from primary ovarian masses by finely cutting the tumor in DMEM (HyClone), passing through a sterile cell strainer (BD Bacteriological, Bedford, MA). Remaining fluid was centrifuged at 1,200 rpm for 5 minutes. The pellet was resuspended in RPMI 1640 containing 10% FCS, 100 units/mL penicillin, 100 mg/mL streptomycin, 2 mmol/L L-glutamine, and 50 μmol/L 2-mercaptoethanol and placed in a cell culture flask. Fresh RPMI 1640 (10 mL) containing 100 units/mL penicillin and 100 mg/mL streptomycin was added daily for 3 days. After 3 days, the RPMI 1640 was removed along with any nonadherent cells and 25 mL fresh RPMI 1640 containing 10% FCS, 100 units/mL penicillin, 100 mg/mL streptomycin, 2 mmol/L L-glutamine, and 50 μmol/L 2-mercaptoethanol and placed in a cell culture flask. Tumor cells were grown for another 3 to 10 days in cell culture before use in 51Cr release assays.

Purification of fresh ovarian cancer cells. All specimens were obtained from ovarian cancer patients. Mononuclear cells were isolated from tumor specimens by ficoll-hypaque density centrifugation and washed twice with PBS. The final product was resuspended in RPMI 1640 containing 10% FCS, 100 units/mL penicillin, 100 mg/mL streptomycin, 2 mmol/L L-glutamine, and 50 μmol/L 2-mercaptoethanol and placed in a cell culture flask. Fresh RPMI 1640 (10 mL) containing 100 units/mL penicillin and 100 mg/mL streptomycin was added daily for 3 days. After 3 days, the RPMI 1640 was removed along with any nonadherent cells and 25 mL fresh RPMI 1640 containing 10% FCS, 100 units/mL penicillin, 100 mg/mL streptomycin, 2 mmol/L L-glutamine, and 50 μmol/L 2-mercaptoethanol and placed in a cell culture flask. Tumor cells were grown for another 3 to 10 days in cell culture use in 51Cr release assays.

Transfection of ovarian cell line. Using ClaI and HindIII restriction enzymes, the luciferase gene from pS8-luc+ vector (Promega, Madison, WI) was cloned into a LNCX vector. UCI-101 human ovarian cancer cells were cultured to 80% confluency and transfected by incubation with 3 μg DNA and 30 μL LipofectAMINE (Invitrogen, Carlsbad, CA). Optimal DNA delivery was deduced by adding 0.15 mg/mL luciferin (Biosyn, Naperville, IL) into the cell culture. Stable transfectants (UCI-101Luc) were selected in 200 μg/mL geneticin (Invitrogen). Using an ICCD camera (C2400-32; Hamamatsu Photonics, Hamamatsu, Japan), cultures were screened for luciferase gene expression.

Fluorescence-activated cell sorting analysis for CIK cells. Cells were taken from CIK cultures for staining with mAbs against IgG2 and CD3, respectively, coupled to FITC and mAbs against IgG1 and CD8, respectively, coupled to phycoerythrin (PE; Becton Dickinson, San Francisco, CA) was added to a final concentration of 1,000 units/mL on day 0. On day 1, interleukin-2 (Chiron, Inc., Emeryville, CA) at a final concentration of 300 units/mL and monoclonal antibody (mAb) anti-CD3 (OKT3; Ortho Biotech, Raritan, NJ) at 25 ng/mL were added. Fresh medium and new interleukin-2 were added every 2 days.

Chemical heteroconjugation. OKT3 is an anti-CD3 IgG2a (Ortho Biotech) and trastuzumab is a humanized anti-Her2 IgG1 (Genentech). Chemical heteroconjugation was done as described previously (17). Briefly, OKT3 (1:5 mg) in 50 mmol/L NaCl, 1 mmol/L EDTA (pH 8.0) was reacted with a 5-fold molar excess of Traut’s reagent (2-iminothiolane HCl; Pierce, Rockford, IL) at room temperature for 1 hour. Anti-CA125 IgG1 (generous gift from Dr. Suresh, University of Alberta, Edmonton, Alberta, Canada) in 0.1 mol/L sodium phosphate, 0.15 mol/L NaCl (pH 7.2) was reacted with a 4-fold excess of sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Pierce) at room temperature for 1 hour. Both mAbs were

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Jose, CA). Fluorescence-activated cell sorting analysis was done as described previously (21, 22).

**31Cr release cytotoxicity assays.** Tumor cell lysis by effector cells was quantitated in a 4-hour 31Cr release assay as described previously (21, 23). Briefly, target cells (1 × 10^6) were labeled with 300 μCi sodium chromate (DuPont-NEN, Boston, MA). After washing twice with PBS, the labeled cells were resuspended in RPMI 1640 and plated in 96-well plates at 1 × 10^4 per well in triplicate. Effector cells were added at specified E:T cell ratios (10:1, 20:1, 40:1, and 100:1) and incubated for 4 hours at 37 °C, 5% CO2. The supernatant was removed and radioactivity measured in a gamma counter (Cobra/AII, Packard BioScience, Meriden, CT). The percent cytotoxicity was determined according to the formula: % cytotoxicity = [(sample release) – (spontaneous release)] / (maximum release) – (spontaneous release)].

Spontaneous release was obtained by incubating target cells in medium alone. Maximum release of target cells was measured following treatment with 2% detergent Igepal CA-630 (Sigma, St. Louis, MO).

**NKG2D blocking assays.** In 4-hour 31Cr release assay blocking experiments, mAbs against NKG2D (Amgen, Seattle, WA) were added immediately before the addition of tumor cell targets for 30 minutes before the addition of tumor cell targets for 30 minutes before the addition of tumor cell targets. Furthermore, the UCI-101 cell line has undergone extensive characterization overexpresses both Her2 receptor and CA125 antigen, which allowed us to test both BSAbxCA125 and BSAbxHer2. The UCI-101 cell line was chosen over the other cell lines for the SCID mouse model because it overexpresses both Her2 receptor and CA125 antigen. Because SK-OV3, OVCAR-3, and UCI-101 ovarian cancer cell lines, respectively.

The UCI-101 carcinoma xenograft model using tumor xenografts was initially described in 1993 (26). After i.p. injection of tumor cells, there is a latent period of 5 to 6 days until tumor implantation and 4 to 5 weeks until macroscopic evidence of ascites and disease progression. The expected average animal survival ranges from 6 to 9 weeks.

With our bioluminescence imaging model, the sensitivity of detecting tumor burden based on photon emission limits our ability to follow these animals beyond 6 weeks due to light reduction secondary to tumor necrosis. Animals were anesthetized with isoflurane (1.2 and 2.5 L in the knockdown chamber and the imaging chamber, respectively) before in vivo imaging. An aqueous solution of the substrate luciferin (150 mg/kg; Biosynth) was injected i.p. and mice were then placed in the light-tight chamber of a low-light imaging system equipped with an ICCD camera fitted with a 50 mm F1.2 Nikkor lens (Nikon, Tokyo, Japan; ref. 22). A gray-scale surface reference image was collected under weak illumination. A pseudocolor image representing light intensity was generated using LivingImage software (Xenogen, San Jose, CA). UCI-101 ovarian cancer cells were labeled with purified anti-Her2 antibodies (Genentech) and detected by a secondary goat anti-rabbit Ig antibody conjugated with the Alexa 488 fluorochrome (Molecular Probes, Eugene, OR). Primary and secondary antibodies were diluted in 1:100 and 1:200 concentration in PBS, respectively. Nuclei of CIK cells and tumor cells were stained with 4',6-diamidino-2-phenylindole (Vector, Burlingame, CA). Slides were then washed with 1× PBS (thrice, 3 minutes each) after antibody incubation and 4',6-diamidino-2-phenylindole staining. Fluorescence microscopic evaluation was done using a Nikon microscope (Eclipse, TE 300) with a digital camera system (Spot, Diagnostic Instruments, Sterling Heights, MI).

**Statistical analysis.** To determine differences between experimental groups in vivo, the bioluminescence signals from living animals were measured (photons/s/mouse). Percent signal reduction following immunotherapy was calculated according to the formula: % signal reduction = 100 – [(signal intensity at day 25 after treatment) – (background signal intensity)] × 100 / [(pretreatment signal intensity) – (background signal intensity)]. Data are reported as mean and range. ANOVA was done to show statistically significant differences among experimental groups at the 0.05 level. The t test was then used to analyze the differences between each experimental group and the control group, and their normal Ps are reported. Mice survival was analyzed using the Kaplan-Meier method.

**Results.**

**Characterization of CIK cells.** To characterize the cytotoxic populations of cells in expanded CIK cells, fluorescence-activated cell sorting analyses were used to evaluate cell populations in CIK cells over 21 days. Results indicate that, between days 7, 14, and 21, populations of CD3+CD8+ T cells increased from 24%, 38%, and 56%, respectively. Concurrently, populations of CD3+CD56+ T cells also increased from 7%, 10%, and 14%, respectively.

**Redirection of CIK cell cytotoxicity.** Dose-titration studies at E:T ratios of 10:1, 20:1, 40:1, and 100:1 were done. Increasing E:T ratio correlated directly with mean percent specific cytotoxicity. From six separate experiments, the CIK cell mean percent lysis with an E:T ratio at 100:1 was 76.4 ± 1.3%, 21.6 ± 3.1%, 15.1 ± 1.7%, and 16.8 ± 0.1% in CA-OV3, SK-OV3, OVCAR-3, and UCI-101 ovarian cancer cell lines, respectively. The addition of BSAbxA25 significantly increased the cytotoxicity of CIK cells on CA125-expressing OVCAR-3 and UCI-101 ovarian cancer cells from 15.2% to 30.0% (P < 0.001) and from 16.8% to 25.4 ± 0.9% (P < 0.001). In contrast, the BSAbxA25 did not have an effect on the SK-OV3 (P = 0.30) or CA-OV3 (P = 0.90) cells that do not express CA125. Because SK-OV3, OVCAR-3, and UCI-101 overexpress Her2, the combination of effector cells with the BSAbxA25 significantly enhanced the CIK cell-induced cytotoxicity of SK-OV3, OVCAR-3, and UCI-101 cells with a percent lysis of 21.6% to 60.2 ± 7.3% (P = 0.01), 15.1% to 40.8 ± 1.6% (P < 0.001), and 16.8% to 35.2 ± 0.8% (P < 0.001), respectively. However, BSAbxA25 did not alter the percent lysis of CA-OV3 (P = 0.90), which does not overexpress Her2 (Fig. 1). Adding both BSAbxA25 and BSAbxA2 in combination with CIK cells did not result in a synergistic effect on tumor cell lysis.
**Autologous CIK cell cytotoxicity on fresh ovarian cancer cells.**

Because CIK cells can lyse allogeneic tumor cells in a non-MHC-restricted manner, we also obtained tumor specimens and blood from 17 patients to determine whether similar cytotoxic activity can be achieved in fresh tumor cells exposed to autologous CIK cells. Clinically, these patients presented with advanced stage IIIC ovarian cancer with tumor involving one or both ovaries and peritoneal implants outside the pelvis. The majority of these patients had upper abdominal implants 2 cm in diameter and/or positive retroperitoneal nodes.

From five separate and representative experiments, the mean percent specific lysis of primary ovarian cancer cells with autologous CIK cells was 21.7 ± 0.3% at an E:T ratio of 100:1. The redirection of the effector cells with the BSAbxCA125 and BSAbxHer2 increased the percent lysis to 65.7 ± 1.0% (P < 0.001) and 89.4 ± 2.1% (P < 0.001), respectively (Fig. 2). We have reported on the cytotoxicity of CIK cells with BSAbxCA125 and BSAbxHer2 in conjunction with the individual patient’s tumor characteristics in Table 1. With respect to CA125 expression, the patient with CA125 level >1,000 units/mL had a 37% enhancement of CIK killing with BSAbxCA125. However, in the patient with CA125 expression <200 units/mL, the BSAbxCA125 improved the cytotoxicity by only 22.6%. The BSAbxHer2 also improved the cytolytic activity of the CIK cells independent of Her2 expression on fluorescence in situ hybridization. Nonetheless, CIK cells augmented by BSAbxHer2 had a 14% greater cytotoxicity in tumors expressing Her2 compared with those without Her2 expression.

**NKG2D blocking antibody.** Previous studies have shown that CIK cell-mediated cytotoxicity against several different tumor cell lines proceeds through a NKG2D-directed mechanism (28). To investigate the role of NKG2D-mediated recognition of ovarian carcinoma cell lines and primary cells, a blocking antibody against the NKG2D receptor was employed as a selective inhibitor of CIK cell cytotoxicity. Blocking antibodies to the NKG2D receptor significantly attenuated the cytotoxic activity of CIK cells by 33.4% (P = 0.12), 59.1% (P = 0.001), and 22.3% (P < 0.001) on SK-OV3, OVCAR-3, and UCI-101 cells, respectively (Fig. 3A). In addition, the blocking antibody reduced the overall activity of autologous CIK cells on primary ovarian cancer cells by an average of 56.8% (P < 0.001). In autologous CIK cells enhanced by BSAbxCA125 and BSAbxHer2, the NKG2D blocking antibody significantly reduced cytotoxicity by 33.5% (P < 0.001) and 16.1% (P < 0.001), respectively (Fig. 3B).

**In vivo NKG2D expression on tumor-infiltrating CIK cells in a mouse model.** Immunofluorescent stainings were done on tumor specimens isolated from SCID mice after adoptive immunotherapy with CIK cells. Morphologically, the tumors showed the typical growth pattern of papillary serous ovarian cancers with central necrosis. On the tumor surface, we identified aggregates of activated lymphocytes and mixed inflammatory infiltrates. Using four-color immunofluorescence analysis, we noted a qualitative difference in the distribution of...
CIK cells. CD8-expressing CIK cells accumulated in clusters surrounding the superficial layer of the tumor nodule. In the deep tumor layers, we visualized direct CIK cell contact with Her2-expressing ovarian cancer cells (Fig. 4A, arrows). Interestingly, the effector cells that have infiltrated into the tumor showed pronounced costaining for NKG2D and CD8 (Fig. 4B, arrows). On the other hand, the majority of CIK cells localized on the tumor surface stained positive for CD8 but had weak or negligible NKG2D expression. Although we were able to show a clinical benefit in our in vivo mouse model, the enhancement of the cytolytic activity of CIK cells using bispecific antibodies was not clearly quantified by the extent of CIK cell infiltration under immunofluorescence microscopy (Fig. 4C and D). Our laboratory is currently developing novel strategies on CIK cell trafficking using our bioluminescence imaging system.

**Reduction of tumor burden visualized by bioluminescence xenograft mouse model.** Using bioluminescence imaging, we developed an ovarian cancer xenograft mouse model to explore the in vivo efficacy of CIK cells. After UCI-101 luc tumor cells were injected i.p. into SCID mice, bioluminescence light intensity was detectable immediately after tumor injection and was monitored weekly for 40 days. Control animals received PBS or bispecific antibodies alone without effector cells (some data not shown; Fig. 5A). One course of CIK cell treatment (E:T ratio of 20:1) was given alone or redirected with BSAbxCD18, BSAbxCA125, or BSAbxHer2. Although tumor regression occurred as early as 96 hours after initiation of therapy, a substantial reduction of bioluminescence (tumor burden) was detected after 25 days in mice treated with CIK (mean light reduction, 90.6%; range, 85.3-99.7%; \( P = 0.03 \); Fig. 5B). The mice treated with BSAbxCA125 or BSAbxHer2 redirected CIK cells showed a significant enhancement of CIK [cell cytotoxicity with a mean light reduction of 98.3% (range, 97.8-98.9%; \( P < 0.001 \)) and 99.6% (range, 99.6-99.8%), respectively; \( P < 0.001 \); Fig. 5C and D]. The administration of BSAbxCA125 or BSAbxHer2 alone without CIK cells had no effect on bioluminescence intensity and was similar to the control group injected with PBS. Similarly, CIK cells treated with BSAbxCD18 did not show enhanced cytotoxicity over CIK cells alone (data not shown). The bioluminescence light intensity correlated inversely with the long-term survival of tumor xenografts. The median survival of mice treated with CIK cells alone or with CIK cells with BSAbxCD18 was 18 days (\( P = 0.006 \)) and 25 days (\( P = 0.01 \)) compared with only 11 days in those treated with PBS. Survival of mice treated with CIK cells with BSAbxCD18 was not significantly different from those treated with CIK cells alone (\( P = 0.90 \)). Furthermore, the addition of BSAbxCA125 and BSAbxHer2 to CIK cells significantly increased the overall survival of mice up to 32 days (\( P = 0.05 \)) and 52 days (\( P = 0.006 \)) compared with CIK cells alone (Fig. 5E and F).

**Table 1.** Cytotoxicity of autologous CIK cells with BSAbxCA125 and BSAbxHer2 in conjunction with the individual patient’s tumor characteristics

<table>
<thead>
<tr>
<th>Patients</th>
<th>CA125 levels* (Units/mL)</th>
<th>HER-2 positivity †</th>
<th>% Lysis of CIK cells ‡</th>
<th>% Lysis of CIK cells &amp; BSAbxCA125 ‡</th>
<th>% Lysis of CIK cells &amp; BSAbxHer2 ‡</th>
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<tr>
<td>1</td>
<td>1,170</td>
<td>–</td>
<td>13.0</td>
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<tr>
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<td>304</td>
<td>++</td>
<td>13.7</td>
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<tr>
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*Serum levels at time of diagnosis.
†Fluorescence in situ hybridization.
‡E:T of 40:1.
Discussion

Despite good initial responses to chemotherapy, 75% of women with stage III or IV disease die of complications associated with disease progression. Given the high recurrence rate and poor long-term survival of women with advanced ovarian cancer, there is a strong impetus to investigate new technologies to improve the outcome of women diagnosed with this deadly disease. Immunotherapy has received considerable attention for the treatment of various malignancies. CIK cells have cytotoxic activity in multiple cancers and have been used in clinical trials against lymphoma as well as hepatocellular, renal, and colorectal carcinomas (10, 11, 29).

This is one of the first reports on the cytotoxicity of autologous CIK cells against fresh cancer cells harvested from ovarian cancer patients. Although established cell lines can provide useful information to study ovarian cancer, cell lines undergo many manipulations during their development and propagation that may result in changes that no longer accurately represent the original tumor (16). Thus, results obtained from primary tumor cultures rather than established cell lines may be more readily translated into the clinical setting. However, the culturing of fresh tumor cells is often complicated by contamination or overgrowth of fibroblasts. As such, it is important to purify this heterogeneous mixture of cells derived from patients before performing experiments. Using the MACS beads specific for human epithelial antigen 125, we removed nonepithelial cells and enriched up to 90% tumor purity as confirmed with fluorescence-activated cell sorting analyses. After purification, we were able to show that the addition of bispecific antibodies further enhanced the cytolytic activity of autologous CIK cells against primary ovarian cancer cells by more than four times that of CIK cells alone.

Previously, we have shown that BSAbxHer2 can successfully redirect and enhance the cytotoxicity of CIK cells and anti-CD3 activated T cells (12, 30). Immunotherapy with effector cells and BSAbxHer2 substantially augmented the cytotoxicity of CIK cells. In vivo studies revealed that animals treated with CIK cells redirected with BSAbxHer2 had a significant survival advantage compared with controls ($P = 0.001$). Nevertheless, Her2 is only overexpressed in 6% to 16% of epithelial ovarian carcinomas, whereas CA125 is found in 80% of advanced epithelial ovarian carcinomas (13, 14, 31). As such, we proposed to determine the efficacy of a novel BSAbxCA125 against cell lines and primary ovarian tumors overexpressing CA125.

The use of bispecific antibodies can potentially overcome tumor resistance against immunotherapy and chemotherapy. We found that the BSAbxCA125 significantly enhanced the cytotoxic activity of CIK cells against three epithelial ovarian cancer cell lines and fresh ovarian tumors. Given the possible difficulty in generating sufficient quantities of CIK cells in the clinical setting, bispecific antibodies can potentially overcome this challenge by bridging tumor cells and CIK cells to decrease the numbers of CIK cells required to elicit a therapeutic response. For example, based on our in vitro results in Fig. 2, the amount of CIK cells required to achieve a cytolytic response of 21.7% decreased by 5-fold with the use of BSAbxCA125. By fluorescence microscopy, we showed that CIK cells that overexpress both NKG2D and CD8 surface markers are more capable of infiltrating solid tumor nodules. In addition to their ability to overcome tumor resistance, bispecific antibodies may also trigger T-cell activation, cytotoxicity, and cytokine production. Furthermore, low expression of NKG2D ligands, such as MICA and MICB, on resistant tumor cells may be another mechanism that allows tumor cells to escape from CIK cell-mediated cytolysis (28).
Previously, our laboratory showed that perforin is a major pathway to CIK cell-mediated cytotoxicity and is triggered through a NKG2D-directed mechanism (28). Groh et al. showed that the NKG2D receptor is expressed on natural killer cells, γδ T cells, and CD8αβ T cells (32). The associated NKG2D ligands, MICA and MICB, are overexpressed in epithelial tumors (33–35). Engagement of NKG2D with its ligands triggers calcium influx, cytokine release, perforin-mediated cytotoxicity, and lysis of tumor cells (36). Extrapolating from these findings, we proposed that a blocking antibody against the NKG2D receptor would attenuate the cytolytic activity of CIK cells. In fact, the blocking antibody decreased the tumor cell lysis by 63.3%. However, despite using high doses of NKG2D blocking antibodies, we were unable to completely eliminate the cytotoxic activity of CIK cells and the blocking antibody was even less effective in CIK cells enhanced by bispecific antibodies. Thus, it seems that the mechanism of CIK cytotoxicity and bispecific antibody enhancement is mediated only in part through the NKG2D receptor.

To better visualize the kinetics of tumor cell lysis by CIK cells, we transfected a constitutively expressed reporter gene encoding firefly luciferase into our CA125-expressing UCI-101 cells. The photons of light were transmitted through tissues and detected with our real-time bioluminescent imaging system. Through this process, we did comparative analyses of antibody-directed therapies in living animals. From our immunofluorescent microscopy results, we were able to show a dramatic tumor response that could be observed as early as 4 hours after CIK cell exposure. More importantly, animals treated with CIK cells and BSAbxCA125 (P = 0.05) or BSAbxHer2 (P = 0.006) had significant survival advantages compared with animals treated with PBS or bispecific antibodies without CIK cells. This dual biological cellular therapeutic approach resulted in the rapid eradication of CA125-expressing tumors and improved survival in the SCID mice.

In this current report, we found that the addition of BSAbxCA125 to CIK cells was not more effective than BSAbxHer2. These results were unexpected because we anticipated that the surface antigen CA125 is overexpressed in 80% of ovarian cancer cells. One of potential explanation for this finding is that CA125 antigen can serve as a potent inhibitor of natural killer cell-mediated cytotoxicity (37). Natural killer cells from healthy donors exposed to 15,000 units/mL CA125 showed an attenuation of cytolytic activity by 70%. Furthermore, CA125 was found to be a potent inhibitor of interleukin-2-stimulated natural killer cells. Lastly, in our dose-titration studies to empirically determine the optimal bispecific antibody arming concentrations, we found that the cytolytic activity of CIK cells with BSAbxHer2 is significantly higher than that of BSAbxCA125 at each E:T ratio and at each bispecific antibody dose. These experiments indirectly suggest that the binding affinity of the BSAbxHer2 is greater than that of BSAbxCA125.

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Fig. 5. Visualization of CIK cell cytotoxicity against ovarian cancer xenograft mouse model monitored over 25 days by photon emission of the luciferase reporter gene labeled on human ovarian cancer cells (UCI-101Luc cells). SCID mice were injected i.p. with 1 × 10⁶ UCI-101Luc cells 24 hours before single treatment of (A) PBS, (B) 1 × 10⁷ donor CIK cells, (C) 1 × 10⁷ donor CIK cells redirected with CA125 bispecific antibody, or (D) 1 × 10⁷ donor CIK cells redirected with Her2 bispecific antibody. Four representative images of seven mice per experimental arm. E, light emission was recorded as photons/s/mouse for 25 days post-treatment. Signal intensity over tumor sites with respect to time of each tumor signal is measured and plotted as a geometric mean. F, survival was analyzed using the Kaplan-Meier method. Bars, SE.
To overcome this challenge, we propose to saturate the nonspecific binding of CA125 by using mAbs against CA125 (mAbCA125) before treatments with BSAbxCA125-conjugated CIK cells in our animal model. Furthermore, we may need to escalate the dose of BSAbxCA125 to overcome the nonspecific binding. Lum et al. showed that it is feasible to treat patients with up to 2 × 10^9 bispecific antibody-treated effector T cells/kg without significant toxicity (38). Thus, it may be safe and efficacious to administer large quantities of bispecific antibody in an effort to improve the efficacy of CIK cells.

It is interesting to note that BSAbxHer2 was capable of improving the cytotoxic activity of the CIK cells even in the absence of Her2 expression on fluorescence in situ hybridization. This may be explained by the potential limitations of immunohistochemistry and fluorescence in situ hybridization. For example, nonstandardized, delayed, or excessive fixation of tissue specimen can result in differential expression of protein markers (39). Furthermore, certain epitopes may be masked, poorly retrieved, or absent in tumor samples even if a proven antibody is used (40). Lastly, an activated receptor may mask antibody-epitope recognition while in its phosphorylated state (41). Thus, these factors can contribute to the discordance shown between the lack of Her2 expression on the patient tumor samples and yet enhanced CIK cell cytotoxicity in the presence of BSAbxHer2. Furthermore, there is clinical evidence that trastuzumab (Herceptin) has activity even in patients with Her2-negative cancers (30, 42). In spite of these limitations in our study, those with a positive expression of Her2 in our patient samples had a 14% higher enhancement of CIK cell cytotoxicity with the BSAbxHer2 compared with those without Her2 expression.

Our clinical laboratory has been able to readily expand CIK cells under GMP-compliant conditions in a completely closed system using the Aastrom Replicell device. In a phase I clinical trial, four of nine lymphoma patients with extensive disease responded to treatment using these CIK cells with minimal toxicity. Two had radiographically documented reduction and two had stabilization of disease. Although all clinical responses were transient in these heavily pretreated cohort of patients with extensive disease, one patient had disease stabilization for >1 year (10). Laboratory and clinical data suggest that cellular therapeutics may be more applicable for cancer patients with minimal residual disease. In fact, Takayama et al. randomized 150 postsurgical hepatocellular carcinoma patients to adoptive immunotherapy versus observation. The authors found that the treatment group had a significantly longer recurrence-free survival (P = 0.01) and disease-specific survival (P = 0.04) compared with the control group (11). Thus, these findings will ultimately be considered as we design clinical trials in ovarian cancer patients with minimal residual disease after cytoreductive surgery.

Our results suggest that bispecific antibodies with CIK cells is an attractive antitumor immunotherapy for the treatment of CA125- and Her2-expressing tumors. In vivo activity of CIK cells in conjunction with bispecific antibodies was enhanced compared with CIK cells alone in a SCID mouse model. The mechanism of CIK cell cytolysis seems to be mediated in part by the NKG2D receptor. The data generated from our study can be used to support a clinical trial for the treatment of women with advanced ovarian cancer.

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