Autologous lymphocyte responses to adenovirus-B7-1-transduced human cancer cells

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Transfection of the costimulatory molecule B7-1 (CD80) into murine tumors can increase antitumor immunity and eradicate tumor growth. The purpose of this study was to test autologous lymphocyte responses against freshly resected human cancers infected *in vitro* with an adenovirus vector expressing the B7-1 molecule (AdB7). Resected tumors (sarcomas, adenocarcinomas, melanomas, and multiple myelomas) were disaggregated into single-cell suspensions and divided into three groups: (a) native, noninfected tumor cells (TM); (b) AdB7-infected, B7-1⁺ tumor cells (TMB7); and (c) control Addl70.3-infected, B7-1⁻ tumor cells (TMA_d). B7-1 expression was verified by flow cytometry. Autologous peripheral blood lymphocytes from these patients were tested for proliferative and cytotoxic activity against the three tumor groups. There was an increased lymphocyte-proliferative response against B7-1⁺ tumor cells, particularly in the presence of interleukin-12 (IL-12) or low-dose IL-2. B7-1⁺ tumor cells were also killed more efficiently than B7-1⁻ tumor cells in natural killer cell-mediated cytotoxicity assays, and this was most significant when lymphocytes had been pretreated with IL-12. Human natural killer cells were found to express CD28, a receptor for B7-1. The high efficiency of AdB7-mediated gene transfer and the augmented B7-1-mediated lymphocyte responses suggest that AdB7 vectors may be effective in human cancer immunotherapy.

Key words: Tumor immunology; costimulatory molecules; natural killer cells; human gene therapy; adenovirus vectors.

Recent studies using animal models have demonstrated an important role for B7-1, a costimulatory molecule, in the induction of antitumor immunity *in vivo*.¹⁻¹⁰ B7-1 is normally expressed on activated B cells and other professional antigen (Ag)-presenting cells (APCs).¹¹⁻¹⁵ Without costimulation, recognition of Ag by T cells may result in unresponsiveness, anergy, or programmed cell death.^{12,16} Delivery of both an Ag-specific signal, *via* the T-cell receptor in the context of the major histocompatibility complex (MHC), and a costimulatory signal, resulting from the interaction of T-cell CD28 receptors with B7-1 ligand, is essential for optimal T-cell stimulation.^{17,18} This results in the increased production of several lymphokines, including interleukin-2 (IL-2) and interferon- γ ,¹⁹⁻²¹ leading to the proliferation of CD4⁺ T cells and cytolytic activity of CD8⁺ T cells.^{18,22,23} The transfection of B7-1 into murine tumor cell lines increases antitumor T-cell immunity and leads to tumor regression in many models.¹⁻⁷ Murine cell lines that remain tumorigenic after B7-1 gene transfer fail to present an adequate Ag-specific first

signal (low T-cell receptor ligand density/affinity)^{6-9,24} or fail to present other necessary adhesion molecules, such as intercellular adhesion molecule-1.²⁵

Other recent reports indicate that natural killer (NK) cell activity may also be affected by costimulatory molecules. Depletion experiments involving the administration of monoclonal antibodies (mAbs) *in vivo* indicated that NK cells are involved in the rejection of B7-1-transfected tumor cells in some murine models.²⁴⁻²⁶ *In vitro* cytotoxicity studies also support an enhanced activation of NK cells by B7-1.²⁶⁻²⁸ Geldhof et al showed that B7-1 expression leads to the NK cell-mediated elimination of metastasizing, NK-resistant mouse T lymphoma cells.²⁷ Yeh et al reported that B7-1 enhances NK cell-mediated cytotoxicity and inhibits tumor growth of a poorly immunogenic murine lung carcinoma.²⁶ Chambers et al demonstrated that B7-1 functions as a triggering signal for murine NK cell-mediated cytotoxicity, and that the strength of this response overrides the negative signal mediated by MHC class I molecules.²⁸ Azuma et al described B7-1/CD28-mediated cytotoxic activity by YT2C2, a human NK leukemia cell line.²⁹ It is well established that murine NK cells express CD28,³⁰ and that the human NK leukemia cell line YT2C2 also expresses CD28,²⁹ but it has been reported that adult human NK cells are normally CD28⁻.³¹ We show here that approximately one-third of normal adult human CD56⁺/CD16⁺ NK cells express CD28.

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Figure 1. Surface B7-1 expression on primary human tumor cells (renal cell carcinoma) after infection with AdB7 virus. Cells were incubated with either a murine Ab that recognizes human B7-1 (BB1) or an isotype control Ab (mIgG1), washed, and stained with an FITC-conjugated goat anti-mouse IgG Ab. Cells were gated for live tumor cells using light scatter and EMA fluorescence. Cells were harvested for flow cytometry at 4 days (**A**), 6 days (**B**), and 6 weeks (**C**) postinfection. PBS, tumor cells mock-infected with PBS; Addl, tumor cells infected with Addl70.3 (an E1/E3-deleted virus with no B7-1 cDNA); AdB7, tumor cells infected with AdB7.

Experimental animal models are valuable in the study of tumor immunology and *in vivo* immune mechanisms, but there are limitations in the extrapolation of murine studies to human cancer immunotherapy. First, human APCs process antigenic peptides in the context of human MHC for human T-cell recognition. MHC-binding motifs are species-specific, and antigenic epitopes that contribute to tumor immunogenicity in the mouse may not be significant in humans. Specific immune responses and clinically significant antitumor effects cannot be predicted from animal models alone. Second, most animal models use established cell lines in which tumor Ags are selected over time. Culture conditions may result in the selection of Ags that are not normally

present *in vivo*. Mouse tumor models are inbred, and consequently fail to reflect the heterogeneity seen in human tumors. Humans develop tumors that express a heterogeneous panel of (presumably) weak immunogenic tumor Ags. Human cancer cell lines acquire a selected phenotype in culture, and so freshly resected spontaneous tumors best represent tumors found in the clinical situation. Finally, many animal models have used stably transfected B7-1⁺ tumor cells generated using retroviral vector systems, lipofection, or electroporation. These methods of transduction are inefficient and usually require drug selection of transduced cells in culture. Fresh human cancer specimens are not easily amenable to such means of gene transfer, and require a system that

Figure 2. Flow cytometric analysis showing MHC class I expression on various primary human tumor cells. Cells were incubated with a rat anti-human MHC class I Ab (■) or a rat IgG2a isotype control Ab (□), washed, and stained with an FITC-conjugated goat anti-rat IgG Ab. Cells were gated for live tumor cells using light scatter and EMA fluorescence. **A:** 110SRC1, leiomyosarcoma; **B:** 132MM1, malignant melanoma; **C:** 134SRC5, malignant fibrous histiocytoma (metastatic to lung); **D:** 135SRC6, liposarcoma; **E:** 138MFH3, malignant fibrous histiocytoma; **F:** 153SRC9, liposarcoma; **G:** 158LM4, colon adenocarcinoma (metastatic to liver); **H:** 160SCC3, leiomyosarcoma (metastatic to lung); **I:** 161CRC6, colon adenocarcinoma; **J:** 169RCC5, renal cell carcinoma.

Figure 3. Representative figures showing that MHC class I expression on primary human cancer cells is not affected by adenovirus infection. Cells were incubated with a rat anti-human MHC class I Ab (dark lines) or a rat IgG2a isotype control Ab (light lines), washed, and stained with an FITC-conjugated goat anti-rat IgG Ab. Cells were gated for live tumor cells using light scatter and EMA fluorescence. The panels to the left show flow cytometric results using a tumor expressing high levels of MHC class I (135SRC6, liposarcoma); the panels to the right show results from a tumor expressing low levels of MHC class I (169RCC5, renal cell carcinoma). PBS, tumor cells mock-infected with PBS; Addl, tumor cells infected with Addl70.3 (an E1/E3-deleted virus with no B7-1 cDNA); AdB7, tumor cells infected with AdB7.

is highly efficient, rapid, and does not require host cell proliferation. Hodge et al have constructed a recombinant vaccinia virus containing the murine *B7-1* gene and have shown that infection of tumor cells with this vector leads to rapid and efficient cell surface expression of B7-1.³ We have recently described the construction of an adenovirus-B7-1 (AdB7) expression vector containing human cDNA, and we have shown that it is a rapid and highly efficient means of gene transfer into freshly resected human tumor cells.³²

In the experiments to be described, our goal was to study the immunologic effects of *B7-1* gene transfer into human cancer cells *in vitro*. We have shown previously that B7-1 mediates increased allogeneic T-cell-proliferative responses against WM35 melanoma tumor cells that express MHC molecules (class I⁺II⁺) and antigenic epitopes, but not against MeWo and H125 cells, which

presumably fail to deliver an adequate Ag-specific signal that can be costimulated by B7-1/CD28 interaction.³³ We also showed that human NK cell-mediated cytotoxic responses could be enhanced in a stable cell line system.³³ In the present study, we sought to test the hypothesis that B7-1 expression by human tumor cells could enhance autologous T-cell and NK cell responses *in vitro*. We used freshly resected cancer specimens to reflect the heterogeneous nature of spontaneous tumors found in the clinical situation. These tumor cells were efficiently transduced with the AdB7 expression vector.³² Peripheral blood lymphocytes (PBLs) from cancer patients were tested *in vitro* for both proliferative and cytotoxic activity against three groups of autologous tumor cells: (a) native, noninfected tumor cells (TM); (b) AdB7-infected, B7-1⁺ tumor cells (TMB7); and (c) Addl70.3-infected, B7-1⁻ tumor cells (TMAd). We cor-

Figure 4. Flow cytometric analysis of CD28 expression on CD56⁺ PBLs (NK cells) from a normal human adult volunteer. PBLs were stained with both a PE-conjugated mouse anti-human CD56 Ab and either an FITC-conjugated rat IgG2b isotype control Ab (**A**) or an FITC-conjugated rat anti-human CD28 Ab (**B**). The histogram in **C** shows CD56⁺ PBLs (PE⁺ cells in **B**) stained with anti-CD28-FITC (black line) or isotype control (gray line) Ab.

related lymphocyte responses with the degree of tumor B7-1 expression, tumor MHC class I expression, and tumor pathology.

We also studied the effect of IL-2 and IL-12 on these autologous B7-1-mediated lymphocyte responses. IL-12, a 75-kDa heterodimeric glycoprotein, was first recognized by its ability to augment cytotoxic T lymphocyte (CTL) responses^{34,35} and NK cell activity;³⁶ it was initially isolated from B-lymphoblastoid cell lines and called NK cell stimulatory factor³⁶ and cytotoxic lymphocyte maturation factor.³⁷ IL-12 synergizes with IL-2 in several activities, including interferon- γ production,^{38,39} enhancement of NK cytolytic activity,^{36,40} and proliferation of activated T and NK cells.^{41,42} Kubin et al have shown that IL-12 also synergizes with B7-1/CD28 interaction in inducing efficient proliferation and cytokine production in human T cells.⁴³ IL-12 has been shown to promote T helper type 1-specific immune responses^{44–46} and induce potent antitumor activity in animal models,⁴⁷ acting synergistically with both IL-2^{48–50} and B7-1.^{51,52}

MATERIALS AND METHODS

Patients

Blood and tissue specimens were obtained from patients at Mount Sinai Hospital and Toronto Hospital (Toronto, Ontario, Canada). Ethics committee approval (University of Toronto) was obtained, and patients gave informed consent before donating blood for the study.

PBLs

Peripheral blood was obtained from patients in heparinized vacutainers. Lymphocytes were separated by Ficoll-Hypaque gradient centrifugation, washed twice with phosphate-buffered saline (PBS), counted, and resuspended in culture medium as described below.

Adenoviral expression vectors

The AdB7 vector has been described previously.³² Briefly, the B7-1 cDNA (800 base pairs) resides in the E1 region of an E1/E3-deleted Ad5 virus. A negative control virus, Addl70.3, is an adenovirus vector with the same E1/E3 deletion, but no B7-1 sequence. Techniques for virus growth and purification are described elsewhere.^{53,54} Viral titers were determined by plaque-forming unit assays on 293 cell monolayers.

Human tumor cells

Bone marrow specimens of patients with multiple myeloma were obtained during routine marrow aspirates. Red blood cells were lysed with ammonium chloride. Cells were then washed and resuspended in RPMI 1640 medium (Life Technologies, Grand Island, NY) at a concentration of $2-4 \times 10^6$ cells/mL for viral adsorption (described below).

Freshly resected solid tumors were disaggregated into single-cell suspensions by aseptic mechanical dissociation into 1–4-mm fragments (within 30 minutes of surgical resection), followed by digestion with a collagenase-based enzymatic cocktail: 2 mg/mL collagenase, 0.3 mg/mL deoxyribonuclease I, and either 0.2 mg/mL pronase E (protease type XXV) or 0.3 mg/mL hyaluronidase type V (all from Sigma, St. Louis, Mo) in RPMI 1640 medium. The tissue fragments were exposed to the

enzyme cocktail and stirred vigorously with a sterile magnetic bar for 2–4 hours at room temperature (until most fragments were fully digested). Single cells were obtained by filtration of the mixture through a sterile 44- μ m nylon filter (Small Parts, Miami Lakes, Fla) and centrifugation of the supernatant. Cells were washed and resuspended in RPMI 1640 medium at a concentration of $2-4 \times 10^6$ cells/mL.

Each tumor cell suspension was divided into three groups: (a) native, mock-infected tumor cells (TM); (b) AdB7-infected, B7-1⁺ tumor cells (TMB7); and (c) Addl70.3-infected, B7-1[–] tumor cells (TMAd). Cells were infected with AdB7 or Addl70.3 (negative control) at a multiplicity of infection of 10–20. Control cells were mock-infected with PBS. Viral adsorption was performed at 37°C for 60 minutes. Cells were then washed with RPMI 1640 medium and resuspended in appropriate media (AIM-V (Life Technologies) for proliferation assays; RPMI 1640 medium plus fetal bovine sera (FBS) (Life Technologies) for culture). Cells were maintained in culture at 37°C in RPMI 1640 medium supplemented with 20% FBS (except for breast adenocarcinoma tumor cell cultures, for which only 5% FBS was added to prevent fibroblast overgrowth).⁵⁵ Cell culture viability was maintained best when using Falcon polystyrene tissue culture flasks (Becton Dickinson Labware, Franklin Lakes, NJ).

Immunostaining and flow cytometric analysis

Harvesting tumor cells. Tumor cells were harvested for immunostaining after variable times in culture (24 hours to 6 weeks). Cells were gently scraped off the tissue culture flask, washed, counted, and resuspended in RPMI 1640 medium plus 10% FBS. Cells not used for immunostaining were either used in cytotoxicity assays or returned to culture for later use. The immunostaining of multiple myeloma samples is described elsewhere.⁵⁶

B7-1 expression. Tumor cells were first incubated for 45 minutes at 4°C with either BB1 (a murine immunoglobulin G1 (mIgG1) mAb that recognizes human B7-1) or a mIgG1 isotype control antibody (Ab) (Becton Dickinson, San Jose, Calif) at 5 μ g/mL in RPMI 1640 medium containing 10% FBS. Cells were then washed twice with PBS containing 1% FBS and incubated for an additional 45 minutes at 4°C with fluorescein isothiocyanate (FITC)-conjugated goat F(ab')₂ anti-mouse IgG (Caltag Laboratories, San Francisco, Calif). Cells were again washed twice with PBS plus 1% FBS and subsequently incubated with 0.125 μ g/mL ethidium monoazide (EMA) (Molecular Probes, Eugene, Oreg) on ice for 10 minutes under a fluorescent light source to photoactivate the EMA and covalently link it to the DNA of nonviable cells.³² Next, cells were washed twice with PBS plus 1% FBS and fixed with 1% paraformaldehyde (BDH Inc., Toronto, ON). Stained cells were analyzed on an Epics Elite flow cytometer (Coulter Electronics, Miami, Fla). Cells were gated for live tumor cells using light scatter and EMA fluorescence. Raji cells (American Type Culture Collection (ATCC), Manassas, Va), which are derived from an Epstein-Barr virus-negative human Burkitt's lymphoma that expresses high levels of surface B7-1, were used as positive controls.

Figure 5. Representative, positive *in vitro* NK cell-mediated cytotoxicity ⁵¹Cr release assay: Improved killing of AdB7-infected colorectal adenocarcinoma tumor cells by autologous NK cells. Shaded symbols indicate mock (PBS)-infected target cells (TM). Solid symbols indicate AdB7-infected target cells (TMB7). Open symbols indicate K562 target cells (positive control). **A:** Killing by unstimulated NK cells. **B:** Killing by NK cells pretreated with exogenous IL-2 (100 U/mL). **C:** Killing by NK cells pretreated with exogenous IL-12 (100 U/mL).

MHC class I expression. Cells were first incubated for 45 minutes at 4°C with either a rat mAb to human histocompatibility leukocyte class I Ag (Serotec, Oxford, U.K.) or a rat IgG2a isotype control Ab (Immunotech, Marseille, France) at 10 µg/mL in RPMI 1640 medium containing

10% FBS. Cells were subsequently washed twice with PBS containing 1% FBS and incubated for an additional 45 minutes at 4°C with FITC-conjugated goat anti-rat IgG (Cedarlane Laboratories, Hornby, Ontario, Canada). Cells were again washed twice with PBS plus 1% FBS, incubated

Figure 6. NK cell-mediated cytotoxic responses against autologous tumor cells. Results represent the mean number of LU per million effector cells for each group ($\pm$ SE). Target cells (TM, TMB7, and TMAAd) are described in *Materials and Methods*. **A:** Cytotoxic response of unstimulated NK cells. **B:** Cytotoxic response of NK cells pretreated with exogenous IL-2 (100 U/mL). **C:** Cytotoxic response of NK cells pretreated with exogenous IL-12 (100 U/mL). *, $P = .05$ by analysis of variance, comparing cytolytic activity against TMB7, TM, and TMAAd.

Figure 7. Lymphocyte-proliferative responses to autologous tumor cells. Error bars represent one SE from the mean. *, $P < .02$, comparing PI in response to TMB7, TM, and TMAAd. **, $P < .02$, comparing PI in response to TMB7, with and without BB1 blocking Ab.

with EMA, fixed with 1% paraformaldehyde, and analyzed by flow cytometry as described above.

CD28 expression on normal human adult PBLs. PBLs from normal healthy adult human donors were cultured in the presence or absence of cytokines (IL-2 or IL-12 at 100 U/mL) for 18 hours in AIM-V medium at 5×10^6 cells/mL. Cells were then harvested and incubated for 45 minutes at 4°C with either an FITC-conjugated rat anti-human CD28 Ab (Serotec) or an FITC-conjugated rat IgG2b isotype control Ab (Cedarlane Laboratories) at 2.5 µg/mL in RPMI 1640 medium containing 10% FBS. The PBLs were simultaneously labeled with phycoerythrin (PE)-conjugated mouse anti-human CD56 Ab, PE-conjugated mouse anti-human CD16 Ab, or a PE-conjugated mIgG1 isotype control Ab (Becton Dickinson). Cells were subsequently washed twice with PBS plus 1% FBS and fixed with 1% paraformaldehyde. Stained cells were analyzed on an Epics Elite flow cytometer. Dead cells were excluded for analysis using forward light scatter. The percentage of CD28⁺ cells within both the CD56⁺ and CD16⁺ populations of PBLs (NK cells) was determined by subtraction analysis.

[³H]thymidine uptake T-cell proliferation assays

Patient PBLs were resuspended in sera-free AIM-V culture medium and plated into 96-well flat-bottom microtiter plates (Becton Dickinson Labware) at 10^5 cells/well. TM, TMB7, and TMAAd tumor cells were plated at either 5000 or 100,000 cells/well in a final volume of 200 µL. Negative controls included PBLs with medium alone and each of the tumor cell groups with medium alone. Positive controls included PBLs cultured with phytohemagglutinin (PHA) (Difco, Detroit, Mich). Some groups were cultured with exogenous IL-2 (Sigma); on day 3 of culture, some groups were treated with exogenous IL-12 (Sigma). When sufficient cells were available, extra groups were treated with 5 µg/mL BB1 (a murine mAb that blocks B7-1) or with an azide-free mIgG1 (an irrelevant isotype-matched control Ab from PharMingen, San Diego, Calif) to block the proliferative responses mediated by B7-1. All groups were plated in triplicate. The cells were incubated for 6 days at 37°C and then pulsed with 1 µCi of [³H]thymidine (DuPont New England Nuclear, Boston, Mass). Cells were harvested 16 hours later and counted in a scintillation counter (LS 6000TA; Beckman Instruments, Palo Alto, Calif). The mean counts per minute (cpm) were calculated for each triplicate measurement. Data were analyzed after conversion to proliferation indices (PIs) as follows: $PI = ([cpm \text{ of sample}] / [cpm \text{ of negative control}])$.

Table 1. B7-1 Expression After AdB7 Infection of Freshly Resected Human Cancer Cells

Pathology	% B7-1 ⁺ cells (mean $\pm$ SD)*	Percent of tumors with $\geq 20\%$ B7 ⁺ cells (number)
Solid tumors	56% $\pm$ 28%	88% (57/65)
Sarcoma†	65% $\pm$ 25%	100% (20/20)
Adenocarcinoma	46% $\pm$ 28%	77% (24/31)§
Breast	38% $\pm$ 25%	83% (5/6)
Gastrointestinal tract‡	49% $\pm$ 28%	76% (16/21)
Lung	45% $\pm$ 32%	75% (3/4)
Malignant melanoma	80% $\pm$ 21%	100% (5/5)
Renal cell carcinoma	60% $\pm$ 37%	80% (4/5)
Squamous cell carcinoma	33% $\pm$ 18%	100% (3/3)
Carcinoid	72%	100% (1/1)
Multiple myeloma	24% $\pm$ 18%	40% (6/15)¶

*Most samples were analyzed for B7-1 expression at 3–6 days postinfection. Serial analyses in representative specimens showed that B7-1 expression peaked during this time period and remained elevated for several weeks thereafter.

†Adamantinoma, clear cell sarcoma, Ewing's sarcoma, fibrosarcoma, leiomyosarcoma, liposarcoma, malignant fibrous histiocytoma, neurofibrosarcoma, osteosarcoma, and spindle cell carcinoma.

‡Colorectal, esophageal.

§ $P = .016$ (χ^2), percent successful gene transfer, adenocarcinoma versus other solid tumors.

¶ $P < .0001$ (χ^2), percent successful gene transfer, solid tumors versus multiple myeloma.

|| $P = .0001$ (paired Student's t test), mean percent B7-1⁺ cells, solid tumors versus multiple myeloma.

responder PBLs + stimulator tumor cells] – [cpm of tumor cells in culture alone)]/(cpm of PBLs in culture alone).

⁵¹Cr release NK cell-mediated cytotoxicity assays

Patient PBLs were used as effector cells in standard 4-hour ⁵¹Cr release cytotoxicity assays. For some experiments, PBLs were maintained in culture (AIM-V) for 1–5 days before the cytotoxicity assay. Cells were separated into three groups and plated in flat-bottom, 24-well tissue culture plates (Corning Glass Works, Corning, NY) at 5×10^6 cells/mL. PBLs in the first group were maintained in the absence of exogenous cytokines. PBLs in the second and third groups were maintained in the presence of IL-2 and IL-12, respectively (both at 100 U/mL). These effector cells (IL-2 lymphokine-activated killer (LAK) cells, IL-12 LAK cells, and NK cells incubated in the absence of cytokines) were then tested against autologous TM, TMB7, and TMA⁵¹Cr-labeled target cells (5000 target cells/well; serial effector to target (E:T) ratios of ~50:1, 25:1, 12.5:1, 6.125:1, and $\leq 100:1$ depending upon cell availability). Target cells were labeled by incubating 1×10^6 cells in 200 μ L of RPMI 1640 medium plus 10% FBS with 100 μ Ci of ⁵¹Cr-sodium chromate (DuPont) for 2 hours. The target cells were subsequently washed three times with PBS, resuspended in 20 mL of culture medium, and added in triplicate to the effector cells in 96-well V-bottom microtiter plates (Nunc, Roskilde, Denmark). The plates were spun at 500 revolutions per minute for 7 minutes and subsequently incubated for 4 hours at 37°C. Plates were then centrifuged again, after which 100- μ L aliquots of supernatant were harvested for the measurement of radioactivity in an automated γ -counter (LKB-Wallac ClinGamma 1272, Wallac Oy, Turku, Finland). The spontaneous cpm released by target cells in the absence of PBLs and the maximal cpm released by target cells in the presence of 1% Triton X-100 were also determined. NK-sensitive K562 target cells (ATCC) were used as positive controls. The percentage of specific lysis was calculated as follows: % specific lysis = [(experimental cpm – spontaneous cpm)/(maximal cpm – spontaneous cpm)] $\times$ 100%.

The number of effector cells required for 30% specific lysis (1 lytic unit (LU)) was calculated by linear regression analysis.

The number of LU per 10^6 effector cells was calculated for each group.

Statistics

The means of the percentage of B7-1 expression after AdB7 infection were compared by paired Student's t tests. The effect of tumor pathology on successful B7-1 gene transfer (arbitrarily defined as cell populations with $>20\%$ B7-1⁺ cells after AdB7 infection) was tested using the χ^2 test.

T-cell-proliferative responses (PI) and NK cell cytotoxic responses (LU per 10^6 effector cells) against the three tumor groups were compared by analysis of variance (SAS software). Various response transformations were used when necessary to improve the fit and satisfy model assumptions.⁵⁷ Lymphocyte responses to the three groups of tumor cells were correlated with the degree of tumor B7-1 expression, tumor MHC class I expression, and tumor pathology after adjusting for each of the other covariates (adjusted P values).

RESULTS

Surface expression of B7-1 by AdB7-infected tumor cells

All tumors were tested for B7-1 glycoprotein expression by flow cytometry after infection with the AdB7 virus. Figure 1 represents an example of flow cytometric data from one tumor specimen after mock-infection with PBS, infection with AdB7, and infection with Addl70.3. The results of analyses from a number of different tumors are summarized in Table 1. The spectrum of tumor pathologies included primary and metastatic sarcomas, adenocarcinomas, multiple myelomas, malignant melanomas, renal cell carcinomas, and squamous cell carcinomas. Tumor cells not infected with AdB7 never expressed B7-1. Infected tumor cells expressed B7-1 within 24 hours of infection; B7-1 expression persisted for ≥ 7 days and for ≤ 9 weeks in culture, likely because

Table 2. MHC Class I Expression of Freshly Resected Human Cancer Cells

Pathology	% MHC expression (mean $\pm$ SD)	% MHC expression: Number of tumors per group		
		<33%	33%–66%	>66%
Sarcoma	62.8% $\pm$ 29.3%	4/20	5/20	11/20
Breast adenocarcinoma	71.8% $\pm$ 30.6%	1/6	0/6	5/6
Colorectal adenocarcinoma	76.2% $\pm$ 25.2%	2/19	3/19	14/19
Lung adenocarcinoma	92.2% $\pm$ 7.8%	0/6	0/6	6/6
Malignant melanoma	43.6% $\pm$ 43.3%	3/5	0/5	2/5
Renal cell carcinoma	58.0% $\pm$ 28.9%	1/5	2/5	2/5
Multiple myeloma	65.0% $\pm$ 36.5%	1/4	1/4	2/4
Squamous cell carcinoma	72.7% $\pm$ 23.0%	0/3	1/3	2/3
Carcinoid	60%	0/1	1/1	0/1
Total	68.6 $\pm$ 29.1	12/69	13/69	44/69

of low B7-1 glycoprotein turnover. The proportion of B7-1⁺ cells was greater in AdB7-infected solid tumors than it was in AdB7-infected multiple myeloma specimens (56% $\pm$ 28% B7-1⁺ cells for solid tumors versus 24% $\pm$ 18% B7-1⁺ cells for multiple myeloma cells; $P = .0001$, paired Student's t test). The proportion of samples with >20% B7-1⁺ was also greater for solid tumors: 88% of solid tumors (57 of 65) had >20% B7-1⁺ cells after infection ($\leq 97\%$ B7-1⁺ cells), whereas only 40% (6 of 15) of multiple myeloma specimens had >20% B7-1⁺ cells after infection ($P = .0001$, χ^2). Of the solid tumors, all sarcomas (20 of 20) were efficiently transduced (mean percentage of B7-1⁺ cells, 65% $\pm$ 25%; range, 21–96%). Adenocarcinomas showed relative resistance to AdB7-mediated gene transfer: only 77% (24 of 31) of specimens had >20% B7-1⁺ cells after infection ($P = .016$, χ^2). The mean proportion of the percentage of B7-1⁺ cells among adenocarcinoma specimens was 46% $\pm$ 28% (range, 5–94%).

Expression of MHC class I by fresh human tumor cells

All tumors were tested for class I MHC expression to determine the association between MHC expression and B7-1-mediated lymphocyte responses. Flow cytometry results demonstrated that MHC class I expression was present on 68.6% ($\pm$ 29.1%) of all fresh human cancer cells (Table 2). Thus, on average, approximately one-third of tumor cells did not express MHC class I, in agreement with previous reports.^{58–69} There was significant heterogeneity in MHC class I expression among various tumors within each histology (particularly in malignant melanomas, in which the number of cells expressing MHC class I ranged from 11% to 94%), as

well as among cells within each tumor specimen (Fig 2). MHC class I expression by tumor cells did not change after infection with either AdB7 or Addl70.3, both of which are E1/E3-deficient adenovirus vectors: all tumor samples listed in Table 2 were tested, and all displayed the same pattern (Fig 3).

CD28 expression by adult human NK cells

It is well established that murine NK cells express CD28,²⁹ but it has been reported that adult human NK cells are CD28[−].³¹ Figure 4 shows the flow cytometry analysis of CD28 expression on CD56⁺ PBLs (NK cells) from a normal donor. In this individual, 9.1% of PBLs were CD56⁺, and 42.1% of these cells were CD28⁺. Table 3 summarizes the data from five normal donors. Approximately 13% of PBLs were NK cells, as determined by either CD56 or CD16 staining, and this did not change with exposure to exogenous cytokines (IL-2 or IL-12). Of the cells tested, 38.4% ($\pm$ 11.3%) of CD56⁺ NK cells and 29.5% ($\pm$ 9.3%) of CD16⁺ NK cells expressed CD28; the cytokines IL-2 and IL-12 did not affect CD28 expression.

NK cell-mediated cytotoxicity of autologous tumor cells

Figure 5 shows the results of an NK cell-mediated cytotoxicity experiment that was carried out with tumor cells from a patient with colorectal adenocarcinoma. Flow cytometric analysis showed that 28% of the tumor cells expressed MHC class I Ag; 72% of the TMB7 cell population expressed B7-1. Of the PBLs of the patient, 42% expressed CD28. Figure 5 shows that, despite the low level of MHC class I expression, none of the

Table 3. CD28 Expression by Adult Human NK Cells

Cytokine*	% CD56 ⁺ PBLs	% CD28 ⁺ CD56 ⁺ NK cells	% CD16 ⁺ PBLs	% CD28 ⁺ CD16 ⁺ NK cells
Nil	13.7 $\pm$ 6.9	38.4 $\pm$ 11.3	12.1 $\pm$ 5.4	29.5 $\pm$ 9.3
IL-2	12.8 $\pm$ 6.5	36.2 $\pm$ 13.7	11.4 $\pm$ 4.6	27.5 $\pm$ 7.5
IL-12	12.7 $\pm$ 7.6	37.8 $\pm$ 8.9	11.1 $\pm$ 4.6	27.8 $\pm$ 11.8

*PBLs from five normal donors were incubated at 37°C for 18 hours in the presence of either IL-2 at 100 U/mL, IL-12 at 100 U/mL, or AIM-V medium alone at a concentration of 5×10^6 cells/mL. These cells were subsequently harvested and stained with Abs against CD56, CD16, and CD28 for flow cytometric analysis. Results represent means $\pm$ SD ($n = 5$).

nontransduced TM cells were killed by autologous NK cells. However, 35% of the TMB7 cells were killed by autologous NK cells at an E:T ratio of 62:1. PBLs cultured in the presence of IL-2 for 45 hours prior to the ⁵¹Cr release cytotoxicity assay killed 48% of TM cells and 97% of TMB7 cells at a 56:1 E:T ratio. Similar results were seen using PBLs cultured in the presence of IL-12: 22% of TM cells and 81% of TMB7 cells were killed by IL-12 LAK cells at an E:T ratio of 65:1.

Figure 6 summarizes the results for all 24 patients. Unstimulated NK cells killed autologous B7-1⁺ tumor cells to a greater degree than B7-1⁻ tumor cells, but this difference was not statistically significant ($P = .15$, $n = 24$). NK cells pretreated with IL-12 (100 U/mL) showed a statistically significant increase in the killing of B7-1⁺ tumor cells ($P = .05$, $n = 19$). NK cells pretreated with IL-2 (100 U/mL) had an increased ability to kill all tumor cells, whether B7-1⁺ or not. All effector cells (unstimulated NK cells as well as LAK cells) killed K562 control cells very efficiently, suggesting that these were NK cells and not CTLs. Furthermore, none of the PBLs were stimulated by tumor cells *in vitro* before the chromium release assay. We also showed that high MHC class I expression correlated with decreased LAK-mediated cytotoxic activity ($P < .05$), again arguing in favor of these effector cells being NK cells. These results suggest that B7-1 expression can play a significant role in NK cell-mediated killing of human cancer cells.

Regression analysis showed that, after adjusting for tumor pathology and the degree of MHC class I expression, the degree of B7-1 expression on tumor cells within the TMB7 group was significant and positively correlated with NK cell-mediated cytotoxicity, regardless of whether the NK cells had been pretreated with cytokines ($P = .01$, $.05$, and $.01$, respectively, for unstimulated NK cells, NK cells pretreated with IL-2, and NK cells pretreated with IL-12). High levels of tumor MHC class I expression correlated with decreased NK cell-mediated cytotoxic activity, and this correlation was statistically significant when the NK cells were pretreated with cytokines ($P = .04$ and $.03$ for IL-2-treated and IL-12-treated NK cells, respectively). Tumor pathology did not affect NK cell-mediated cytotoxic activity against freshly resected autologous cancer cells.

T-cell-proliferative responses to autologous tumor cells

T-cell proliferation assays were carried out with tumor cells and PBLs from 66 patients. PBLs from seven patients failed to respond to PHA ($PI < 2.5$), presumably due to generalized immunosuppression from the malignant disease process or its treatment, and these results were not included in subsequent analyses. PBLs from one patient displayed a minor response to PHA ($PI = 4.1$) but failed to respond to 100 U/mL IL-2 ($PI = 1.9$); results from this patient were also excluded from further analysis. Of the remaining 59 patients, proliferation assays were carried out with either 100,000 tumor cells/well (36 patients) or 5000 tumor cells/well (29 patients). Lymphocyte responses in those patients in

which proliferation assays were performed using both tumor cell concentrations ($n = 9$) were not statistically different.

In the first group of studies (using 100,000 tumor cells/well), we found that B7-1⁺ tumor cells induced an enhanced lymphocyte-proliferative response in 6 of 36 patients (17%); however, the proliferative responses to the TM, TMB7, and TMAAd groups were all weak ($PI < 1$), and there was no statistical difference in the mean responses to the three tumor groups (adjusted $P = .67$). The number of tumor cells obtained from 12 of the patients was sufficient to allow for experiments in which PBLs and tumor cells were cocultured in the presence of cytokines (IL-2 and/or IL-12). There was no statistical difference in the lymphocyte-proliferative responses to the TM, TMB7, and TMAAd groups, but an increased degree of B7-1 expression in the TMB7 group was directly correlated with an increased proliferative response in the groups cultured in the presence of low-dose IL-2 (5 U/mL), IL-12 (100 U/mL), or a combination of the two ($P = .003$, $.04$, and $.002$, respectively, after adjusting for pathology and the degree of MHC expression).

In the second group of studies (using 5000 tumor cells/well), there was an increased lymphocyte PI in response to TMB7 tumor cells in 12 of 27 patients (44%). Again, the mean PIs for lymphocytes cultured with the three tumor groups (in the absence of exogenous cytokines) were not statistically different (Fig 7; adjusted $P = .09$). The mean proliferative responses correlated with degree of MHC class I expression (adjusted $P = .03$). Figure 7 shows that, with the addition of high-dose IL-2 (100 U/mL), there was an increase in the lymphocyte PI for all tumor groups, with no significant difference between the three groups (adjusted $P = .12$). However, with the addition of low-dose IL-2 (5 U/mL), there was a statistically significant difference in the lymphocyte response between the three groups (adjusted $P = .001$). A similar difference was noted in responses when lymphocytes were tested in the presence of IL-12 (100 U/mL) (adjusted $P = .015$). When both IL-2 (5 U/mL) and IL-12 (100 U/mL) were present in the culture medium, there was an increase in lymphocyte proliferation in all three groups, with no statistically significant difference between them (adjusted $P = .23$). These data suggest that the requirement for B7-1-mediated costimulation of T-cell proliferation can be circumvented by a high concentration of IL-2 or a combination of IL-12 and a low concentration of IL-2. Differences in T-cell responses to B7-1-expressing tumor cells are seen only in situations in which there is inadequate cytokine costimulation.

Our data also show that the proliferative response elicited by TMB7 cells in the presence of both cytokines was blocked by a murine Ab (BB1) that recognizes human B7-1 ($P = .02$) but not by isotype control mIgG1 Ab (Fig 7). Interestingly, BB1 also caused a reduction in the proliferative response elicited by B7-1⁻ tumor cells. It is possible that, among the PBLs and tumor-infiltrating lymphocytes within the experimental wells, there

were professional APCs (dendritic cells, B cells, and macrophages) that expressed B7-1 and presented tumor-specific (as well as non-tumor-specific) Ag that elicited a minor response in the TM and TMA groups, and that this response was blocked by BB1.

DISCUSSION

In this report, we demonstrate that B7-1 expression on the surface of human cancer cells can play a significant role in autologous lymphocyte responses when combined with the use of cytokines. Other studies have shown that IL-12, even in association with IL-2, has no significant ability to induce proliferation in fresh resting PBLs.^{41,42} However, IL-12 can enhance the T-cell proliferation of 5- to 6-day-old cultures induced by a variety of stimuli (mitogenic lectins, allogeneic cells, anti-CD3 Abs, and phorbol diesters). In conjunction with IL-2, IL-12 can act in an additive, synergistic, or antagonistic fashion depending upon the dose and timing of its use.^{41,42,70} High-dose IL-2 (100 U/mL) has been reported to induce the proliferation of T cells even in the absence of antigenic stimulation.⁷¹ IL-2 at suboptimal concentrations (5 U/mL), however, does not mediate CTL proliferation. IL-12 (50–100 U/mL) can cause the proliferation of IL-2-activated peripheral blood mononuclear cells that have received suboptimal concentrations of IL-2 (3–10 U/mL), but only if the cells have been recently costimulated with Ag or solid-phase α CD3 Ab.⁴¹ Therefore, we had expected to improve both the sensitivity and specificity of detecting antitumor lymphocyte responses by combining these two cytokines *in vitro*. Figure 7 shows that there was an increase in lymphocyte proliferation in response to autologous tumor cells when cocultured in the presence of IL-2 (5 U/mL) and IL-12 (100 U/mL) compared with either cytokine alone, but this response was modest in comparison with the response elicited in the presence of high-dose IL-2 (100 U/mL). Furthermore, the increased cytokine costimulation obviates the dependence upon B7-1 costimulation, because significant B7-1-mediated increases in lymphocyte proliferation were noted only in those groups with suboptimal stimulation with low-dose IL-2 (5 U/mL) or IL-12 (100 U/mL). These results, along with the lack of response noted in the absence of cytokines, suggest that T-cell activation is determined by a predetermined activation threshold,⁷² and that costimulation, either by membrane-bound B7-1 or by soluble cytokines such as IL-2 or IL-12, renders T cells more sensitive to antigenic stimulation.

Our most interesting results are those that show that NK cell-mediated killing of B7-1⁺ tumor cells was greater than that of B7-1[−] tumor cells, and that this difference is most significant when PBLs are pretreated with IL-12 (100 U/mL). Although we did not purify the NK cell population, it is highly unlikely that the observed effects are mediated by CTLs. Whether this effect is mediated by B7-1 interaction with the CD28 receptor (which we show is expressed by approximately one-third

of human NK cells) or with another, yet unidentified, receptor remains to be determined. Chambers et al have recently shown that the triggering of mouse NK cells by B7-1 occurred even in the absence of CD28 and could not be blocked by either anti-CD28 or anti-CTLA-4 Abs, suggesting that the B7-1-mediated cytotoxic activity of NK cells may be due, at least in part, to receptors other than CD28.²⁸

It has been proposed that the final outcome of NK cell-mediated cytotoxicity is a result of the interaction between triggering and inhibitory ligands and receptors, and that the net result of these interactions determines susceptibility to lysis.^{28,73} We show that B7-1 molecules help trigger NK cell cytotoxicity, particularly when PBLs have been pretreated with IL-12; however, further studies are needed to identify the receptors and signaling pathways involved. Our data also provide further support for the hypothesis that MHC class I molecules act as inhibitory signals for NK cell-mediated cytotoxic activity.⁷⁴

Many of our T-cell PIs were negligible (mean PIs of <5). In addition to the experimental conditions that are used to stimulate CTLs *in vitro*, the ability to detect lymphocyte responses also depends upon the antitumor CTL precursor frequency. The number of T-cell precursors specific for any particular tumor Ag in unprimed hosts has been shown to be extremely low.^{75–77} Vose reported frequencies of CTLs against lung and breast tumors ranging from 1/1500 to 1/4700 PBLs.⁷⁵ More recently, Boon and colleagues reported that the frequency of antitumor CTL precursor cells of seven melanoma patients ranged from 1/900 to 1/33,000 PBLs.⁷⁶ Improved stimulation methods in their protocols, involving repeated stimulation with irradiated autologous tumor cells in the presence of IL-2 and IL-4 for a period of 2–4 weeks, may have led to an increased frequency of precursors. Our results are in keeping with these reports and reflect the nature of human tumor immunogenicity in cancer patients.

Our results suggest that the successful immunotherapy of cancer patients could potentially be mediated by genetically modifying tumor cells to express B7-1 and secrete IL-12. We have shown that the AdB7 vector is a rapid and efficient means of gene delivery, particularly in dissociated cells from solid tumors. Because lymphocyte responses are greater when cells express higher degrees of B7-1, patients with solid tumors are likely better candidates for AdB7-mediated gene therapy than patients with multiple myeloma, and patients with adenocarcinomas may not benefit to the same degree as patients with other forms of solid tumors (sarcomas, renal cell carcinomas, and malignant melanomas). To date, our AdB7 vector has only been used to transduce tumor cells *in vitro*, but the vector could also be used to infect tumors directly, *in vivo*. We (F.L.G.) have used other Ad vectors (Ad-IL-4, Ad-IL-2, and Ad-IL-12) in mouse tumor models and have shown efficient cytokine gene transfer after direct injection *in vivo*, with corresponding functional immune responses.^{78–81} Cusack et al have also demonstrated high levels of gene transduc-

tion in human lung tumors after an intralesional injection of recombinant adenovirus.⁸² In the clinical setting, malignant lesions could be treated with percutaneous intratumoral AdB7 injections; these lesions could also be resected, infected *ex vivo*, irradiated, and inoculated into the patients as tumor vaccines. Patients whose tumors are inoperable because of tumor location rather than tumor size might be expected to have better responses, because low tumor burden has been shown to be important for success in animal models. Ad vectors expressing B7-1 and IL-12 have recently been generated (Pützer BM, unpublished observations; Hitt M, Muller WJ, Emtage P, Gauldie J, Graham FL, unpublished observations) and will soon be available for further *in vitro* work and clinical studies in the adjuvant and palliative setting. Plans for clinical trials testing the utility of B7-1-mediated gene therapy in cancer patients are underway.

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REFERENCES

1. Chen L, Ashe S, Brady WA, et al. Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. *Cell*. 1992;71:1093-1102.
2. Townsend SE, Allison JP. Tumor rejection after direct costimulation of CD8⁺ T cells by B7-transfected melanoma cells. *Science*. 1993;259:368-370.
3. Hodge JW, Abrams S, Schlom J, Kantor JA. Induction of antitumor immunity by recombinant vaccinia viruses expressing B7-1 or B7-2 costimulatory molecules. *Cancer Res*. 1994;54:5552-5555.
4. Baskar S, Ostrand-Rosenberg S, Nabavi N, Nadler LM, Freeman GJ, Glimcher LH. Constitutive expression of B7 restores immunogenicity of tumor cells expressing truncated major histocompatibility complex class II molecules. *Proc Natl Acad Sci USA*. 1993;90:5687-5690.
5. Townsend SE, Su FW, Atherton JM, Allison JP. Specificity and longevity of antitumor immune responses induced by B7-transfected tumors. *Cancer Res*. 1994;54:6477-6483.
6. Chen L, McGowan P, Ashe S, et al. Tumor immunogenicity determines the effect of B7 costimulation on T cell-mediated tumor immunity. *J Exp Med*. 1994;179:523-532.
7. Chen L, McGowan P, Ashe S, Johnston JV, Hellstrom I, Hellstrom KE. B7-1/CD80-transduced tumor cells elicit better systemic immunity than wild-type tumor cells admixed with *Corynebacterium parvum*. *Cancer Res*. 1994;54:5420-5423.
8. Li Y, McGowan P, Hellstrom I, Hellstrom KE, Chen L. Costimulation of tumor-reactive CD4⁺ and CD8⁺ T lymphocytes by B7, a natural ligand for CD28, can be used to treat established mouse melanoma. *J Immunol*. 1994;153:421-428.
9. Hodge JW, McLaughlin JP, Abrams SI, Shupert WL, Schlom J, Kantor JA. Admixture of a recombinant vaccinia virus containing the gene for the costimulatory molecule B7 and a recombinant vaccinia virus containing a tumor-associated antigen gene results in enhanced specific T-cell responses and antitumor immunity. *Cancer Res*. 1995;55:3598-3603.
10. Ramarathinam L, Castle M, Wu Y, Liu Y. T cell costimulation by B7/BB1 induces CD8 T cell-dependent tumor rejection: an important role of B7/BB1 in the induction, recruitment, and effector function of antitumor T cells. *J Exp Med*. 1994;179:1205-1214.
11. Schwartz RH. A cell culture model for T lymphocyte clonal anergy. *Science*. 1990;248:1349-1356.
12. Mueller DL, Jenkins MK, Schwartz RH. Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Annu Rev Immunol*. 1989;7:445-480.
13. Liu Y, Linsley PS. Costimulation of T-cell growth. *Curr Opin Immunol*. 1992;4:265-270.
14. Gimmi CD, Freeman GJ, Gribben JG, et al. B-cell surface antigen B7 provides a costimulatory signal that induces T cells to proliferate and secrete interleukin 2. *Proc Natl Acad Sci USA*. 1991;88:6575-6579.
15. Jenkins MK, Taylor PS, Norton SD, Urdahl KB. CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. *J Immunol*. 1991;147:2461-2466.
16. Harding FA, McArthur JG, Gross JA, Raulet DH, Allison JP. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature*. 1992;356:607-609.
17. Moretta A, Pantaleo G, Lopez-Botet M, Moretta L. Involvement of T44 molecules in an antigen-independent pathway of T cell activation. Analysis of the correlations to the T cell antigen-receptor complex. *J Exp Med*. 1985;162:823-838.
18. Thompson CB, Lindsten T, Ledbetter JA, et al. CD28 activation pathway regulates the production of multiple T-cell-derived lymphokines/cytokines. *Proc Natl Acad Sci USA*. 1989;86:1333-1337.
19. Walter H, Schepens S, Van Wauwe J, de Boer M. Ligation of CD28 on resting T cells by its ligand B7 results in the induction of both Th1- and Th2-type cytokines. *Eur Cytokine Netw*. 1994;5:13-21.
20. Lanier LL, O'Fallon S, Somoza C, et al. CD80 (B7) and CD86 (B70) provide similar costimulatory signals for T cell proliferation, cytokine production, and generation of CTL. *J Immunol*. 1995;154:97-105.
21. Damle NK, Klussman K, Linsley PS, Aruffo A. Differential costimulatory effects of adhesion molecules B7, ICAM-1,

- LFA-3, and VCAM-1 on resting and antigen-primed CD4⁺ T lymphocytes. *J Immunol.* 1992;148:1985–1992.
22. Lindsten T, June CH, Ledbetter JA, Stella G, Thompson CB. Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. *Science.* 1989;244:339–343.
23. Norton SD, Zuckerman L, Urdahl KB, Shefner R, Miller J, Jenkins MK. The CD28 ligand, B7, enhances IL-2 production by providing a costimulatory signal to T cells. *J Immunol.* 1992;149:1556–1561.
24. Wu TC, Huang AY, Jaffee EM, Levitsky HI, Pardoll DM. A reassessment of the role of B7-1 expression in tumor rejection. *J Exp Med.* 1995;182:1415–1421.
25. Cavallo F, Martin-Fontecha A, Bellone M, et al. Co-expression of B7-1 and ICAM-1 on tumors is required for rejection and the establishment of a memory response. *Eur J Immunol.* 1995;25:1154–1162.
26. Yeh KY, Pulaski BA, Woods ML, et al. B7-1 enhances natural killer cell-mediated cytotoxicity and inhibits tumor growth of a poorly immunogenic murine carcinoma. *Cell Immunol.* 1995;165:217–224.
27. Geldhof AB, Raes G, Bakkus M, Devos S, Thielemans K, De Baetselier P. Expression of B7-1 by highly metastatic mouse T lymphomas induces optimal natural killer cell-mediated cytotoxicity. *Cancer Res.* 1995;55:2730–2733.
28. Chambers BJ, Salcedo M, Ljunggren HG. Triggering of natural killer cells by the costimulatory molecule CD80 (B7-1). *Immunity.* 1996;5:311–317.
29. Azuma M, Cayabyab M, Buck D, Phillips JH, Lanier LL. Involvement of CD28 in MHC-unrestricted cytotoxicity mediated by a human NK leukemia cell line. *J Immunol.* 1992;149:1115–1123.
30. Nandi D, Gross JA, Allison JP. CD28-mediated costimulation is necessary for optimal proliferation of murine NK cells. *J Immunol.* 1994;152:3361–3369.
31. Nagler A, Lanier LL, Cwirla S, Phillips JH. Comparative studies of human FcR3-positive and -negative NK cells. *J Immunol.* 1989;143:3183–3191.
32. Dessureault S, Graham F, Gallinger S. B7-1 gene transfer into human cancer cells by infection with an adenovirus-B7 (Ad-B7) expression vector. *Ann Surg Oncol.* 1996;3:317–324.
33. Dessureault S, Gallinger S. Allogeneic lymphocyte responses to B7-1-expressing human cancer cell lines. *J Surg Res.* 1996;64:42–48.
34. Gately MK. Synergy between recombinant IL-2 (rIL-2) and IL-2-depleted lymphokine-containing supernatants in facilitating allogeneic human cytolytic T lymphocyte responses in vitro. *J Immunol.* 1986;136:1274–1282.
35. Wong HL, Wilson DE, Jenson JC, Familletti PC, Stremlo DL, Gately MK. Characterization of a factor(s) which synergizes with recombinant interleukin 2 in promoting allogeneic human cytolytic T-lymphocyte responses in vitro. *Cell Immunol.* 1988;111:39–54.
36. Kobayashi M, Fitz L, Ryan M, et al. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J Exp Med.* 1989;170:827–845.
37. Stern AS, Podlaski FJ, Hulmes JD, et al. Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells. *Proc Natl Acad Sci USA.* 1990;87:6808–6812.
38. Chan SH, Perussia B, Gupta JW, et al. Induction of interferon γ production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. *J Exp Med.* 1991;173:869–879.
39. Chan SH, Kobayashi M, Santoli D, Perussia B, Trinchieri G. Mechanisms of IFN- γ induction by NK cell stimulatory factor (NKSF/IL-12): role of transcription and mRNA stability in the synergistic interaction between NKSF and IL-2. *J Immunol.* 1992;148:92–98.
40. Chehimi J, Starr SE, Frank I, et al. Natural killer (NK) cell stimulatory factor increases the cytotoxic activity of NK cells from both healthy donors and human immunodeficiency virus-infected patients. *J Exp Med.* 1992;175:789–796.
41. Gately MK, Desai BB, Wolitzky AG, et al. Regulation of human lymphocyte proliferation by a heterodimeric cytokine, IL-12 (cytotoxic lymphocyte maturation factor). *J Immunol.* 1991;147:874–882.
42. Perussia B, Chan SH, D'Andrea A, et al. NK cell stimulatory factor or IL-12 has differential effects on the proliferation of TCR- $\alpha\beta$ +, TCR- $\gamma\delta$ + T lymphocytes, and NK cells. *J Immunol.* 1992;149:3495–3502.
43. Kubin M, Kamoun M, Trinchieri G. Interleukin 12 synergizes with B7/CD28 interaction in inducing efficient proliferation and cytokine production of human T cells. *J Exp Med.* 1994;180:211–222.
44. Manetti R, Parronchi P, Giudizi MG, et al. Natural killer cell stimulatory factor (interleukin 12 (IL-12)) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. *J Exp Med.* 1993;177:1199–1204.
45. Trinchieri G. Interleukin-12 and its role in the generation of TH1 cells. *Immunol Today.* 1993;14:335–338.
46. Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. Development of TH1 CD4⁺ T cells through IL-12 produced by *Listeria*-induced macrophages. *Science.* 1993;260:547–549.
47. Nastala CL, Edington HD, McKinney TG, et al. Recombinant IL-12 administration induces tumor regression in association with IFN- γ production. *J Immunol.* 1994;153:1697–1706.
48. De Wit D, Flemming CL, Harris JD, et al. IL-12 stimulation but not B7 expression increases melanoma killing by patient cytotoxic T lymphocytes (CTL). *Clin Exp Immunol.* 1996;105:353–359.
49. Pappo I, Tahara H, Robbins PD, et al. Administration of systemic or local interleukin-2 enhances the anti-tumor effects of interleukin-12 gene therapy. *J Surg Res.* 1995;58:218–226.
50. Rossi AR, Pericle F, Rashleigh S, Janiec J, Djeu JY. Lysis of neuroblastoma cell lines by human natural killer cells activated by interleukin-2 and interleukin-12. *Blood.* 1994;83:1323–1328.
51. Coughlin CM, Wysocka M, Kurzawa HL, Lee WM, Trinchieri G, Eck SL. B7-1 and interleukin 12 synergistically induce effective antitumor immunity. *Cancer Res.* 1995;55:4980–4987.
52. Zitvogel L, Robbins PD, Storkus WJ, et al. Interleukin-12 and B7.1 co-stimulation cooperate in the induction of effective antitumor immunity and therapy of established tumors. *Eur J Immunol.* 1996;26:1335–1341.
53. Graham FL, Prevec L. Manipulation of adenovirus vectors. In: Murray EJ, Walker JM, eds. *Methods in Molecular Biology: Gene Transfer and Expression Protocols*. Clifton, NJ: The Humana Press Inc., 1991:109–128.
54. Bett AJ, Haddara W, Prevec L, Graham FL. An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. *Proc Natl Acad Sci USA.* 1994;91:8802–8806.
55. Jaffee EM, Dranoff G, Cohen LK, et al. High efficiency

- gene transfer into primary human tumor explants without cell selection. *Cancer Res.* 1993;53:2221-2226.
56. Prince HM, Dessureault S, Gallinger S, et al. Efficient adenoviral-mediated gene expression in malignant human plasma cells: relative lymphoid cell resistance. *Exp Hematol.* 1998;26:27-36.
 57. Myers RH. *Classical and Modern Regression with Applications.* Boston: Duxbury Press; 1990.
 58. van den Ingh HF, Ruiter DJ, Griffioen G, van Muijen GN, Ferrone S. HLA antigens in colorectal tumours: low expression of HLA class I antigens in mucinous colorectal carcinomas. *Br J Cancer.* 1987;55:125-130.
 59. Moller P, Momburg F, Koretz K, et al. Influence of major histocompatibility complex class I and II antigens on survival in colorectal carcinoma. *Cancer Res.* 1991;51:729-736.
 60. Concha A, Cabrera T, Ruiz-Cabello F, Garrido F. Can the HLA phenotype be used as a prognostic factor in breast carcinomas? *Int J Cancer.* 1991;6:146-154.
 61. Moller P, Mattfeldt T, Gross C, et al. Expression of HLA-A, -B, -C, -DR, -DP, -DQ, and of HLA-D-associated invariant chain (Ii) in non-neoplastic mammary epithelium, fibroadenoma, adenoma, and carcinoma of the breast. *Am J Pathol.* 1989;135:73-83.
 62. Moller P, Hammerling GJ. The role of surface HLA-A,B,C molecules in tumour immunity. *Cancer Surv.* 1992;13:101-127.
 63. Redondo M, Concha A, Oldiviela R, et al. Expression of HLA class I and II antigens in bronchogenic carcinomas: its relationship to cellular DNA content and clinical-pathological parameters. *Cancer Res.* 1991;51:4948-4954.
 64. Wintzer HO, Benzing M, von Kleist S. Lacking prognostic significance of β_2 -microglobulin, MHC class I and class II antigen expression in breast carcinomas. *Br J Cancer.* 1990;62:289-295.
 65. Natali PG, Nicotra MR, Bigotti A, et al. Selective changes in expression of HLA class I polymorphic determinants in human solid tumors. *Proc Natl Acad Sci USA.* 1989;86:6719-6723.
 66. Fleming KA, McMichael A, Morton JA, Woods J, McGee JO. Distribution of HLA class I antigens in normal human tissue and in mammary cancer. *J Clin Pathol.* 1981;34:779-784.
 67. Kabawat SE, Bast RC, Jr, Welch WR, Knapp RC, Bhan AK. Expression of major histocompatibility antigens and nature of inflammatory cellular infiltrate in ovarian neoplasms. *Int J Cancer.* 1983;32:547-554.
 68. Connor ME, Stern PL. Loss of MHC class-I expression in cervical carcinomas. *Int J Cancer.* 1990;46:1029-1034.
 69. Lopez-Nevot MA, Esteban F, Ferron A, et al. HLA class I gene expression on human primary tumours and autologous metastases: demonstration of selective losses of HLA antigens on colorectal, gastric, and laryngeal carcinomas. *Br J Cancer.* 1989;59:221-226.
 70. Zeh HJ, Hurd S, Storkus WJ, Lotze MT. Interleukin-12 promotes the proliferation and cytolytic maturation of immune effectors: implications for the immunotherapy of cancer. *J Immunother.* 1993;14:155-161.
 71. Williams JM, Abbud-Filho M, Kelley VE, Strom TB. Interleukin-2 apparently upregulates its receptor and induces proliferation of various resting mononuclear leukocytes in the absence of antigen. *Cell Immunol.* 1985;94:383-393.
 72. Viola A, Lanzavecchia A. T cell activation determined by T cell receptor number and tunable thresholds. *Science.* 1996;273:104-106.
 73. Lanier LL. Natural killer cells: from no receptors to too many. *Immunity.* 1997;6:371-378.
 74. Karre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defense strategy. *Nature.* 1986;319:675-678.
 75. Vose BM. Quantitation of proliferative and cytotoxic precursor cells directed against human tumours: limiting dilution analysis in peripheral blood and at the tumour site. *Int J Cancer.* 1982;30:135-142.
 76. Coulie PG, Somville M, Lehmann F, Hainaut P, Brasseur F, Devos R, Boon T. Precursor frequency analysis of human cytolytic T lymphocytes directed against autologous melanoma cells. *Int J Cancer.* 1992;50:289-297.
 77. Mazzocchi A, Belli F, Mascheroni L, Vegetti C, Parmiani G, Anichini A. Frequency of cytotoxic T lymphocyte precursors (CTLp) interacting with autologous tumor via the T-cell receptor: limiting dilution analysis of specific CTLp in peripheral blood and tumor-invaded lymph nodes of melanoma patients. *Int J Cancer.* 1994;58:330-339.
 78. Addison CL, Gauldie J, Muller WJ, Graham FL. An adenoviral vector expressing interleukin-4 modulates tumorigenicity and induces regression in a murine breast cancer model. *Int J Oncol.* 1995;7:1253-1260.
 79. Addison CL, Braciak T, Ralston R, Muller WJ, Gauldie J, Graham FL. Intratumoral injection of an adenovirus expressing interleukin 2 induces regression and immunity in a murine breast cancer model. *Proc Natl Acad Sci USA.* 1995;92:8522-8526.
 80. Bramson J, Hitt M, Gallichan WS, Rosenthal KL, Gauldie J, Graham FL. Construction of a double recombinant adenovirus vector expressing a heterodimeric cytokine: *in vitro* and *in vivo* production of biologically active interleukin-12. *Hum Gene Ther.* 1996;7:333-342.
 81. Bramson JL, Hitt M, Addison CL, Muller WJ, Gauldie J, Graham FL. Direct intratumoral injection of an adenovirus expressing interleukin-12 induces regression and long-lasting immunity that is associated with highly localized expression of interleukin-12. *Hum Gene Ther.* 1997;7:1995-2002.
 82. Cusack JC, Spitz FR, Nguyen D, Zhang W-W, Cristiano RJ, Roth JA. High levels of gene transduction in human lung tumors following intralesional injection of recombinant adenovirus. *Cancer Gene Ther.* 1996;3:245-249.