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ORIGINAL ARTICLE

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Antigenicity of human melanoma cells transfected to express the B7-1 co-stimulatory molecule (CD80) varies with the level of B7-1 expression

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Abstract The aim of this study was to compare the antigenicity of human melanoma cells molecularly modified by particle-mediated gene transfer to have transient or stable expression of the B7-1 co-stimulatory molecule (CD80). The unmodified melanoma cells (mel5, m21) had no constitutive expression of B7-1, but 22%–28% of cells had transient B7-1 expression 24 h following transfection with cDNA for B7-1 (mel5-B7, m21-B7). In addition, 85%–90% of cells had stable B7-1 expression following transfection with cDNA for B7-1 and in vitro culture under selection conditions (mel5-B7neo, m21-B7neo). Allogeneic HLA-unmatched normal donor peripheral blood mononuclear cells (PBMC) secreted greater amounts of granulocyte/macrophage-colony-stimulating factor (GM-CSF) when incubated for 3 days with m21-B7neo than did PBMC incubated with m21-B7, which, in turn, secreted greater amount of GM-CSF than PBMC incubated with m21. Similarly, cell-mediated cytotoxicity against unmodified melanoma cells by PBMC co-cultured for 5 days with the modified or unmodified melanoma cells was proportional to the level of B7-1 expression on the stimulating cells. This cytolytic activity had both an HLA-class-I-restricted and an HLA-class-I-unrestricted component. Following 5 days of co-culture, PBMC expression of CD28, the ligand for B7-1, was down-regulated in proportion to the level of B7-1 expression on the stimulating melanoma cells. Thus, particle-mediated gene delivery of cDNA for B7-1 into human melanoma cells increased expression of functional B7-1 and enhanced the antigenicity of the gene-modified cells in proportion to their level of B7-1 expression.

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Introduction

The importance of the T cell response to autologous human melanoma has been suggested for many years [4, 11]. Unfortunately, this response is clearly not effective in patients who develop metastatic disease, but it may be possible to be enhanced by appropriate intervention. Treatment approaches that augment expression of MHC antigens on melanoma cells may make these modified melanoma cells better stimulators of T cell immunity [13]. However, T cell activation also requires a second co-stimulatory signal from the antigen-presenting cell [15]. The best-characterized co-stimulatory molecule is B7-1 (CD80), the T cell ligand of which is the CD28 antigen [14]. The importance of B7-1 in the T cell response to melanoma has been demonstrated in murine melanoma models, in which rejection of murine melanoma cells was mediated by $CD8^+$ T cells following stimulation by melanoma cells stably transfected with B7-1 cDNA [5, 17]. Systemic immunity against non-transfected melanoma cells was induced, as subsequent tumor challenge with non-transfected melanoma cells did not result in tumor formation [17]. It has been suggested that the level of B7-1 expression on the stimulating murine melanoma cells may be an important factor in stimulating an anti-melanoma immune response [6].

There is evidence that human melanoma cells lines stably transfected with B7-1 cDNA are better stimulators of a T cell response against melanoma than are the unmodified parental cells [7, 10, 12, 16, 19]. This enhanced antigenicity has been shown to be partially blocked by addition of anti-B7-1 (CTLA-4) antibody to the cultures of PBMC and tumor cells, indicating the need for the B7-CD28 interaction in activation of the T cell response to the modified tumor cell [7]. In addition,

this cytolytic activity has been shown to be T cell-mediated and HLA-class-I-restricted, as it can be partially blocked by pre-incubating the target cells with anti-CD3 [16], anti-CD8, or anti-HLA [10, 12, 19] blocking antibodies prior to their addition to the cytotoxicity assay.

While prior studies have demonstrated that melanoma cells modified with B7-1 cDNA to express moderately high levels of B7-1 can become more antigenic, further investigation is needed to determine whether the enhanced stimulation is proportional to the level of B7-1 expression on the modified tumor cells. In addition, the ability of transient expression of B7-1 to enhance the antigenicity of human melanoma cells is clinically relevant and has not been reported. We have previously shown that particle bombardment with a "gene gun" can be used to deliver cDNA for B7-1 into human melanoma cells, resulting in transient expression of B7-1 immediately and without selection [1]. In the present study we also cultured these cells under selection conditions to obtain melanoma cells with defined levels of stable B7-1 expression. We examined the ability of these gene-modified $HLA2^+$ and $HLA2^-$ melanoma cells to activate allogeneic peripheral blood mononuclear cells (PBMC) to lyse unmodified melanoma cells. Our findings demonstrate that B7-1-modified melanoma cells can activate enhanced HLA-class-I-restricted and HLA-class-I-unrestricted cytolytic activity by allogeneic PBMC. The enhanced antigenicity of the gene-modified cells was present in melanoma cells with transient and with stable expression of B7-1, and was proportional to the level of B7-1 expression on the modified tumor cells.

Materials and methods

Expression vectors

Human B7-1 cDNA was obtained by the polymerase chain reaction (PCR) from a Raji cell library (human B cell line cDNA library, Clontech catalog no. HL-1002a) using PCR primers based on a published sequence [8]. The PCR product was cloned into Bluescript SK(+), and its sequence was found to be identical to that published by Freeman et al. [8]. Human B7-1 cDNA was excised from the Bluescript vector, blunted with T4 DNA polymerase, and ligated with *Not*I linkers to facilitate insertion into the *Not*I site of pNASS/CMV.

The β-galactosidase control plasmid DNA was obtained by inserting the cytomegalovirus immediate–early promoter into the pNASSβ vector (Clontech, Palo Alto, Calif.). The human cDNA for B7-1 was placed into pNASS/CMV expression vector as previously described [1]. To obtain stable transfectants, a similar vector that also contained cDNA for neomycin phosphotransferase was used.

Particle-mediated gene transfer

The particle-mediated gene transfer procedure for these cell lines has been previously reported [1]. Briefly, plasmid DNA was precipitated onto 0.95 μm (average diameter) gold particles (Degussa Corp., South Plainfield, N.J.) at a ratio of 2.5 μg plasmid DNA/mg gold particles. The DNA/gold particle precipitate was re-suspended at 7 mg gold/ml 100% EtOH and used to coat Tefzel tubing (McMaster-Carr; internal diameter of 2.4 mm). The tubing was cut into 12.5-mm sections for loading into the pulse gun chamber, resulting in 0.5 mg gold (1.25 μg DNA)/shot. For gene transfer, the DNA/gold was accelerated through an expansion chamber by a helium pulse of 300–400 psi. The Accell gene gun (PowderJect, Madison, Wis.) was used for these gene transfer experiments. The target cells (1×10^6) received particle-mediated gene transfer while in suspension in 15 μl RPMI-1640 medium containing L-glutamine, penicillin/streptomycin, HEPES, and 10% fetal bovine serum (medium/FCS). The pulse-gun gene is described in a PCT patent application by Dennis McCabe (publishing date 24 July 1995). B7-1 expression (CD80, Becton Dickinson) was measured 24 h after transfection by flow cytometry as previously described [1].

Cell lines and culture conditions

The M-21 melanoma cell line (m21) was a gift from Dr. Ralph Reisfeld (Scripps Research Institute, La Jolla, Calif.). The SK Mel-5 melanoma cell line (mel5) was obtained from ATCC (cell line HTB70). Both cell lines were characterized for intracellular expression of GP-100-melanoma-associated antigen as well as surface expression of GD2 and GD3, utilizing flow cytometry analysis as previously described [1]. Both cells lines were 98% positive for MHC class I expression by flow cytometry.

All cell lines were maintained in culture in medium/FCS and serially passaged using trypsin/EDTA when confluent. Cells transfected with cDNA for B7-1 (mel5-B7 and m21-B7) were cultured in medium/FCS and used in experiments 24 h after transfection. Stable transfectants (mel5-B7neo and m21-B7neo) were selected and cultured in medium/FCS containing 1 mg/ml G418 (Mediatech, Herndon, Va.). B7-1 expression of the stable transfectants was measured weekly by flow cytometry as previously described [1]. Following 12 weeks of culture, the m21-B7neo cells were sorted by brightness of CD80-positive staining, yielding populations of cells that were 85%–90% (bright) or 26%–30% (dim) positive for B7-1 expression. The dim cells were again sorted into a subpopulation of cells 50% (bright/dim) or 14% (dim/dim) positive for B7-1 expression.

HLA typing of tumor cells and normal donor leukocytes

PBMC from the same four normal donors were used for these experiments. Specific HLA/DR typing of the normal donor PBMC and the two melanoma cells lines was performed in the HLA/DNA laboratory of the American Red Cross (Badger Hawkeye Region, Madison Wis.) using sequence-specific primers to amplify genomic DNA [3]. The HLA types were detected by agarose gel electrophoresis (see Table 1).

Table 1 HLA typing of human melanoma cell lines and normal donor peripheral blood mononuclear cells (PBMC). PBMC from the same four normal donors were used for these experiments. The HLA A/B/DR typing of the normal donor PBMC and the two melanoma cells lines was performed in the HLA/DNA laboratory of the American Red Cross (Badger Hawkeye Region, Madison, Wis.) using sequence-specific primers to amplify genomic DNA. The HLA types were detected by agarose gel electrophoresis

Cell line	HLA locus		
	A	B	DR
Tumor			
m 21	11, 24	15, 35 Bw6	4, 13, 52, 53
mel 5	2, 11	7, 60 Bw6	4, 13, 52, 53
Donor			
A	2, 24	60, 65 Bw6	1, 12, 52
B	2, 26	44, 57 Bw4	7, 11, 52, 53
C	3, 26	7, 18 Bw6	4, 15, 51, 53
D	2, 11	51, 55 Bw6	4, 8, 53

Mixed lymphocyte and tumor cell cultures

Whole blood was diluted 1:1 with phosphate-buffered saline (PBS), and the PBMC were obtained by density centrifugation on a Ficoll gradient (Lymphoprep; Gibco, Gaithersburg, Md.). The cells were washed twice in PBS, counted, and re-suspended at 10×10^6 cells in RPMI-1640 medium containing L-glutamine, penicillin/streptomycin, HEPES, and 10% human serum (medium/HS). Aliquots of wild-type (mel5, m21) and B7-1-transfected cells (mel5-B7, m21-B7, mel5-B7neo and m21-B7neo) were inactivated by irradiation with 260 Gy. The cells were re-suspended in medium/HS at 1×10^6 cells/ml. Samples comprising 1 ml PBMC and 1 ml tumor cells were placed into 25-cm^3 tissue-culture flasks in a final volume of 10 ml at a ratio of 10:1. In some cases, the normal donor PBMC were also cultured with an equal number of irradiated allogeneic PBMC (1:1) in a final volume of 10 ml. All cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 5 days.

Pheotypic analysis of PBMC following culture with transfected and nontransfected melanoma cells

Aliquots of normal donor PBMC from each of the four donors were stained for surface antigens CD3, CD4, CD8, CD56, and CD28 both before and after the 5-day co-culture with mel5, mel5-B7neo, m21 or the subpopulations of m21-B7neo using fluorescein-isothiocyanate (FITC)-conjugated anti-CD4, CD3, CD28 and Phycoerythrin (PE)-conjugated anti-CD8, CD56, and CD3 (Becton Dickenson, San Jose, Calif.). To determine if $CD4^+$ cells would respond differently from $CD8^+$ cells, PBMC from each donor were stained with FITC-conjugated anti-CD28 in combination with PE conjugated with either anti-CD3, CD4 or CD8. Mean channel fluorescence of surface antigen expression was assessed by flow cytometry. Correlations between mean channel fluorescence of CD28 expression in responding PBMC and levels of CD80 expression on stimulating m21 cells were analyzed using SPSS-PC+ 3.1 (SPSS Inc., Chicago, Ill.).

Cytokine production

On day 3 of co-culture, 1 ml supernatant fluid was removed from co-cultures of PBMC with the m21, m21-B7 and m21-B7neo cells. The supernatant was centrifuged at 500*g* for 5 min and then frozen at −20 °C for later analysis of granulocyte/macrophage-colony-stimulating factor (GM-CSF) content. On day 5 of co-culture, all the fluid containing the non-adherent cells was collected from co-cultures of PBMC with each of the mel5 or the m21 cell lines. The fluids were centrifuged at 500*g* for 5 min, and the cell-free supernatants frozen at −20 °C for later analysis of interleukin-2 (IL-2) and GM-CSF content. The levels of GM-CSF in the 3-day supernatants (*n*-12) and the level of IL-2 and GM-CSF in the 5-day supernatants (*n*-4) were determined, using a commercially available enzyme-linked immunosorbent assay (Immunotech, Marseilles, France). Data are reported as mean concentrations (pg/ml) ± SEM. Correlations between the levels of CD80 expression on stimulating m21 cells and levels of GM-CSF in the 3-day supernatant fluids were analyzed using SPSS-PC+ 3.1 (SPSS Inc., Chicago, Ill.). Group differences in levels of GM-CSF in supernatants obtained from m21, m21-B7 and M21-B7neo 3-day co-cultures were analyzed by paired *t*-tests of group means (SPSS) with the α level set at 0.01 to allow for the multiple pairwise comparisons. Only descriptive statistics (mean ± SEM) are reported for the levels of IL-2 or GM-CSF in supernatants from 5-day co-cultures because of the small sample size.

Cytolytic activity

Following collection of the 5-day culture supernatants, the PBMC pellet was re-suspended in medium/HS at 1.5×10^6 cells/ml and the cytolytic activity of normal donor PBMC was assayed by the standard 4 h chromium-release assay as previously described [2]. In brief, PBMC in 150 µl medium/HS were placed in quadruplicate wells of a U-bottom 96-well microtiter plate and serially diluted 1:3. Wild-type unmodified mel5 and m21 were utilized as target cells. Aliquots of the target cells were pelleted and incubated with 0.25 mCi chromium for 2 h, and washed twice in PBS. The target cells were counted and re-suspended at 5×10^4 cells/ml medium, and 0.1 ml was added to all wells of the microtiter plate to obtain effector:target ratios of 30:1, 10:1, and 3:1 in a final volume of 0.2 ml/well. Target cells in medium alone or in Centrimide were used to determine spontaneous and maximum chromium release. The cultures were incubated for 4 h, and the supernatants harvested, dried overnight, and counted in a gamma counter. PBMC from each donor were tested at least twice. For each experiment, PBMC from at least two donors were used and PBMC from each donor were used in at least two experiments. Data are expressed as percentage cytotoxicity and represent the pooled mean of at least two experiments.

To determine if cytolytic activity of the effector PBMC was due to HLA-class-I-restricted or HLA-class-I-unrestricted lysis, PBMC effectors from two donors were evaluated with chromium-labeled K562 [a natural-killer (NK)-susceptible cell line], Daudi (an NK-resistant cell line) as well as mel5 and m21 cells as targets. In additional assays using PBMC effectors from the same two donors, the W6/32 anti-HLA-ABC antibody (Dako, Carpinteria, Calif.), or its isotype control, was added at a final concentration of 10 µg/ml to microtiter plates containing chromium-labeled m21 or K562 target cells. To verify that the antibody could block HLA-class-I-restricted lysis, K562 as well as PBMC incubated with 1 µg/ml PHA-M (sigma, St. Louis, Mo.) for 48 h were labeled with chromium and used as target cells with the addition of the anti-HLA-ABC antibody or its isotype control. The PBMC effectors had been cultured with an equal number of irradiated allogeneic PBMC-stimulating cells for 5 days prior to assay. Data are presented as percentage cytotoxicity and represent findings from one experiment each.

Proliferation assays

Normal donor PBMC were suspended at 1×10^6 cells/ml and 0.1-ml aliquots were placed in quadruplicate wells of a 96-well microtiter plate. Aliquots of irradiated (35 Gy) allogeneic PBMC (10^5/well), or irradiated (260 Gy) mel5, mel5-B7 or mel5-B7neo (10^4/cells well) were added to the cultures, which were then incubated for 6 days. Cultures were pulsed with 1 µCi [^{3}H]thymidine 18 h prior to termination of the cultures, and incorporation of thymidine determined by scintillation counting. Results are expressed as mean counts per minute (cpm) of quadruplicate cultures from one experiment. Similar experiments using the m21 cell lines were not done as baseline uptake of thymidine by the irradiated cells alone could not be reliably shut down by this level of irradiation (260 Gy).

Results

Expression of the B7-1 co-stimulatory molecule on human melanoma cells following stable transfection with B7-1 cDNA

Two established melanoma cell lines (mel5, m21) were evaluated by flow cytometry for constitutive expression of B7-1, and both were negative. The transfected cell lines with transient B7-1 expression (mel5-B7, m21-B7), evaluated for B7-1 expression 24 h after transfection with cDNA for B7-1, were 22%–28% B7-1 positive. The cells with stable B7-1 expression (mel5-B7neo, m21-B7neo) were between 90% and 99% positive for B7-1 expression 1–2 weeks following transfection and subse-

quent culture in medium containing G418. The level of B7-1 expression by the mel5-B7neo line remained consistent for up to 4 months. Levels of B7-1 expressed by m21-B7neo slowly declined to 60%–70% over a period of 12 weeks while the cells were still being maintained in culture under selection. The m21-B7neo cell line was subsequently sorted by brightness of B7 expression, yielding populations of cells 85%, 62%, 50%, 26%, and 14% positive for CD80 (see Fig. 1).

Phenotypic analysis of PBMC effectors

Mean channel fluorescence of surface antigens CD3, CD4, CD8, and CD56 on normal donor PBMC did not change following 5 days of co-culture with wild-type or B7-1-transfected tumor cells. However, mean channel fluorescence of CD28 expression on $CD3^+$ cells declined significantly. When the PBMC were cultured with subpopulations of m21-B7 cells that had been previously sorted by levels of B7-1 expression, the decline in CD28 mean channel fluorescence by the responding $CD3^+$ PBMC was inversely proportional to the level of B7-1 expression by the stimulating m21-B7neo cells ($r = -0.80$, $P = 0.001$; see Fig. 2). When the decline in CD28 expression was examined in the context of PBMC subpopulations of $CD4^+$ and $CD8^+$ cells, the decline in CD28 expression was similar for $CD4^+$ ($r = -0.85$, $P < 0.01$) and $CD8^+$ ($r = -0.77$, $P < 0.01$) cells.

Cytokine production

Culture supernatants from normal donor PBMC co-cultured for 3 days with irradiated m21, m21-B7 or m21-B7neo were assayed for GM-CSF by ELISA. Supernatant fluids from PBMC co-cultured with m21-B7neo contained 173 ± 19.4 pg/ml GM-CSF, which was significantly more than fluids from PBMC cultured with m21-B7 (81 ± 8.7; $P < 0.001$) and m21 (56 ± 5.1; $P < 0.001$), while fluids from PBMC cultured with m21-B7 contained significantly more GM-CSF than fluids from PBMC cultured with m21 ($P = 0.01$). The level of GM-CSF in the supernatant fluids was proportional to the level of CD80 expression on the stimulating cells ($r = 0.76$, $n = 36$, $P < 0.001$).

A similar pattern of cytokine secretion was seen in supernatant fluids from normal donor PBMC co-cultured for 5 days with irradiated mel5, mel5-B7, mel5-B7neo, m21,m21-B7 or m21-B7neo. Supernatants from PBMC exposed to the stably transfected mel5-B7neo or m21-B7neo contained greater levels of GM-CSF (Fig. 3A) and IL-2 (Fig. 3B) than supernatants from PBMC exposed to the wild-type mel5 or m21 parent cells. Culture supernatants from PBMC alone contained no detectable levels of IL-2 or GM-CSF (data not shown).

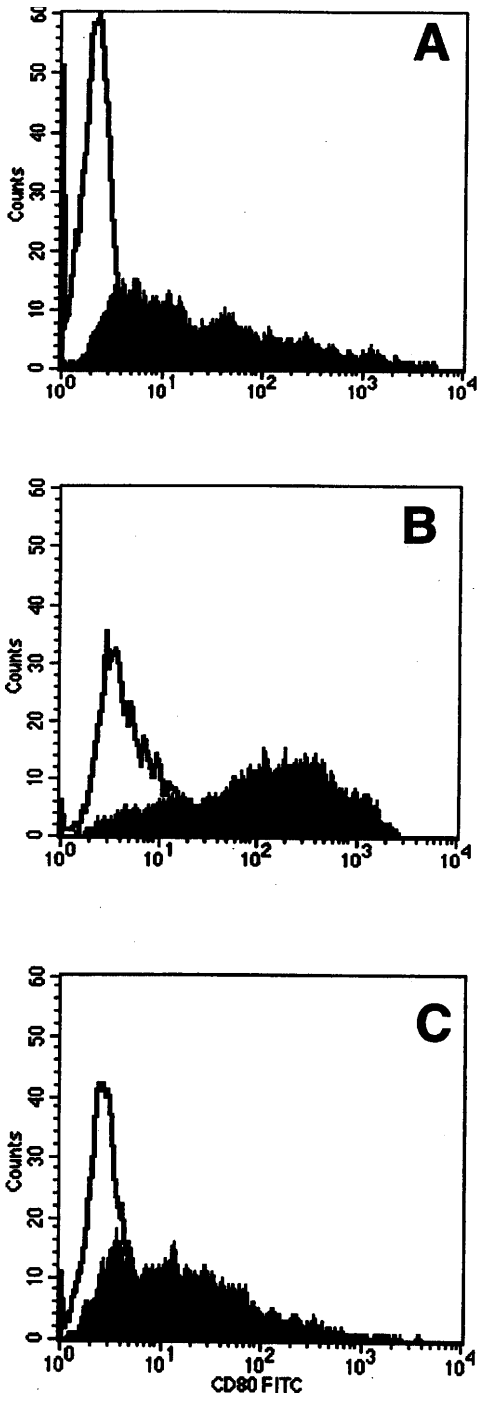

Fig. 1A–C Histograms comparing CD80 expression on (**A**) unmodified m21 cells (*open histogram*) with that of the bulk population of m21-B7neo cells (*shaded histogram*) 12 weeks after transfection with B7-1 cDNA by particle-mediated gene transfer; (**B**) on m21-B7neo-modified cells sorted by flow cytometry into those with high expression (85%–90%; shaded histogram) and those with low expression (26%–30%; *open histogram*) of CD80; and (**C**) low-expression m21-B7neo cells sorted into subpopulations of dim/bright (50%; *shaded histogram*) and dim/dim (14%; *open histogram*) expression of CD80

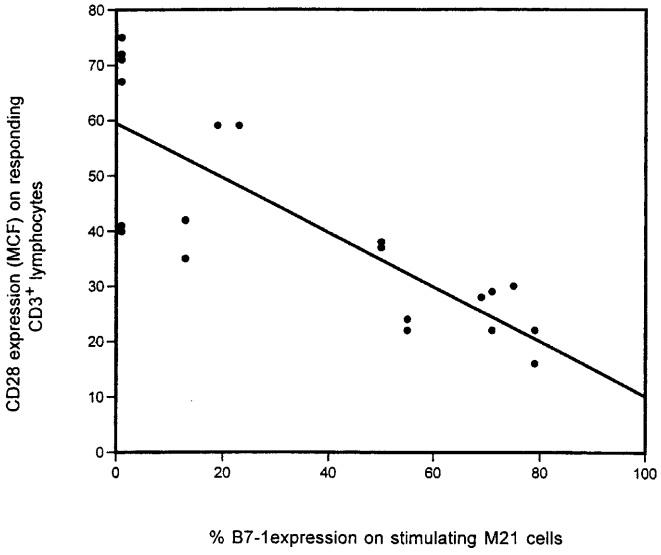

Fig. 2 Normal donor peripheral blood mononuclear cells (PBMC) were co-cultured with the subpopulations of m21-B7neo cells for 5 days. Following co-culture, the PBMC were stained with phycoerythrin-conjugated anti-CD-3 and fluorescein-isothiocyanate-conjugated anti-CD28 monoclonal antibodies and analyzed by flow cytometry. Data points represent mean channel fluorescence of CD28 expression on responding $CD3^+$ PBMC plotted against the level of CD80 expression on stimulating m21 cells

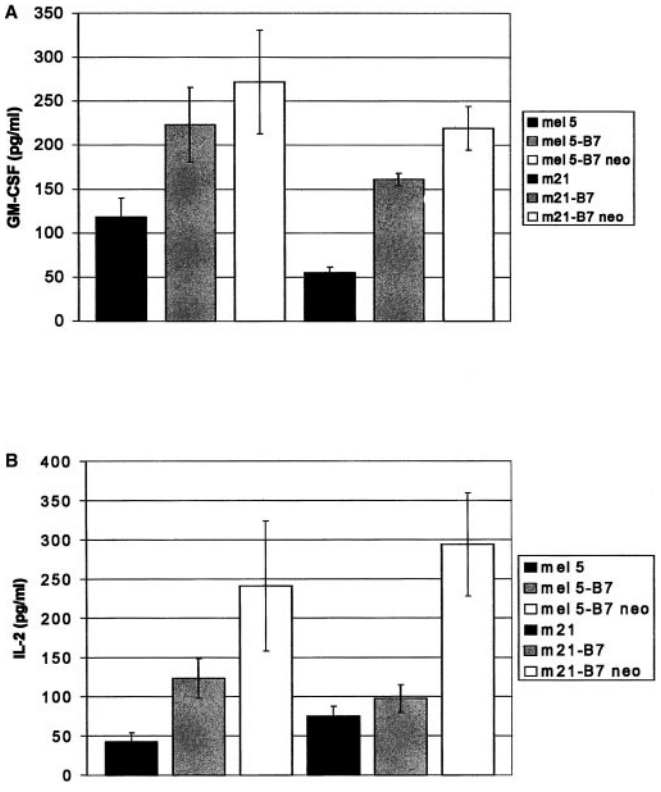

Fig. 3 Normal donor PBMC were co-cultured for 5 days with irradiated mel5, mel5-B7, mel5-B7neo, m21, m21-B7 or m21-B7neo cells at a ratio of 10:1. The cell-free supernatants were analyzed by enzyme-linked immunosorbent assay for levels of granulocyte/macrophage-colony-stimulating factor (*GM-CSF*; **A**), or interleukin-2 (*IL*-2; **B**). Data represent the mean ± SEM of four supernatants tested

Cytolytic activity

Healthy donor PBMC were cultured 10:1 with unmodified, B7-1- or B7-1neo-transfected melanoma cells for 5 days. The PBMC were then tested for cytolytic activity against chromium-labeled target cells (mel5, m21). PBMC from each donor were tested at least twice. As shown in Fig. 4, the cytolytic activity of PBMC co-cultured with B7-1-transfected cells was greater than that of PBMC cultured with unmodified tumor cells when tested against mel5 or m21 targets and this increase in cytolytic activity was proportional to the level of B7-1 expression on the stimulating tumor cells. Cytolytic activity of PBMC against mel5 targets correlated 0.90 ($n = 12$, $P < 0.001$) with the level of B7-1 expression on the stimulating mel5 cells. Cytolytic activity of PBMC against m21 targets correlated 0.86 with level of B7-1 expression on the stimulating m21 tumor cells ($n = 12$, $P < 0.001$). There was no apparent difference in magnitude of cytolytic activity by virtue of the HLA class I or class II phenotypes of the effector PBMC or target cells.

In a subset of experiments, m21-B7neo-stimulated PBMC effectors from two donors were also tested for cytolytic activity against NK-susceptible K562 target cells and against NK-resistant Daudi target cells. In both cases, the lytic activity of the PBMC effectors against these target cells was similar to that seen against the mel5 and m21 target cells (see Fig. 5).

To determine whether the lysis of tumor targets had an MHC-restricted component, anti-HLA-ABC antibody (w6/32) or its isotype control was added at a final concentration of 10 μg/ml to parallel microtiter plates containing chromium-labeled m21 or k562 target cells. As shown in Fig. 6, lysis of K562 targets was not altered by addition of the antibody. Lysis of the HLA-$A2^-$ m21 targets by the HLA-$A2^-$ effector PBMC was reduced 64%, while lysis by HLA-A2+ effector PBMC was reduced 27%. The addition of more antibody (20 μg/ml final concentration) did not yield greater blocking of lytic activity (data not shown). Ability of the antibody to block HLA-class-I- mediated lysis was demonstrated by addition of the anti-HLA-ABC antibody to microtiter plates containing PBMC effectors previously stimulated with irradiated allogeneic PBMC. These effectors were tested against chromium-labeled PHA-stimulated blasts of the same allogeneic donors. As shown in Fig. 6, lysis of the PHA-stimulated blasts was reduced 50% by addition of the antibody at 10 μg/ml or 20 μg/ml. The magnitude of this inhibition was similar to that seen with the m21 target cells.

Proliferation assays

Normal donor PBMC incubated for 6 days in the presence of irradiated mel5-B7neo incorporated more [^{3}H]thymidine than PBMC incubated with wild-type mel5. A similar enhancement effect was not seen with

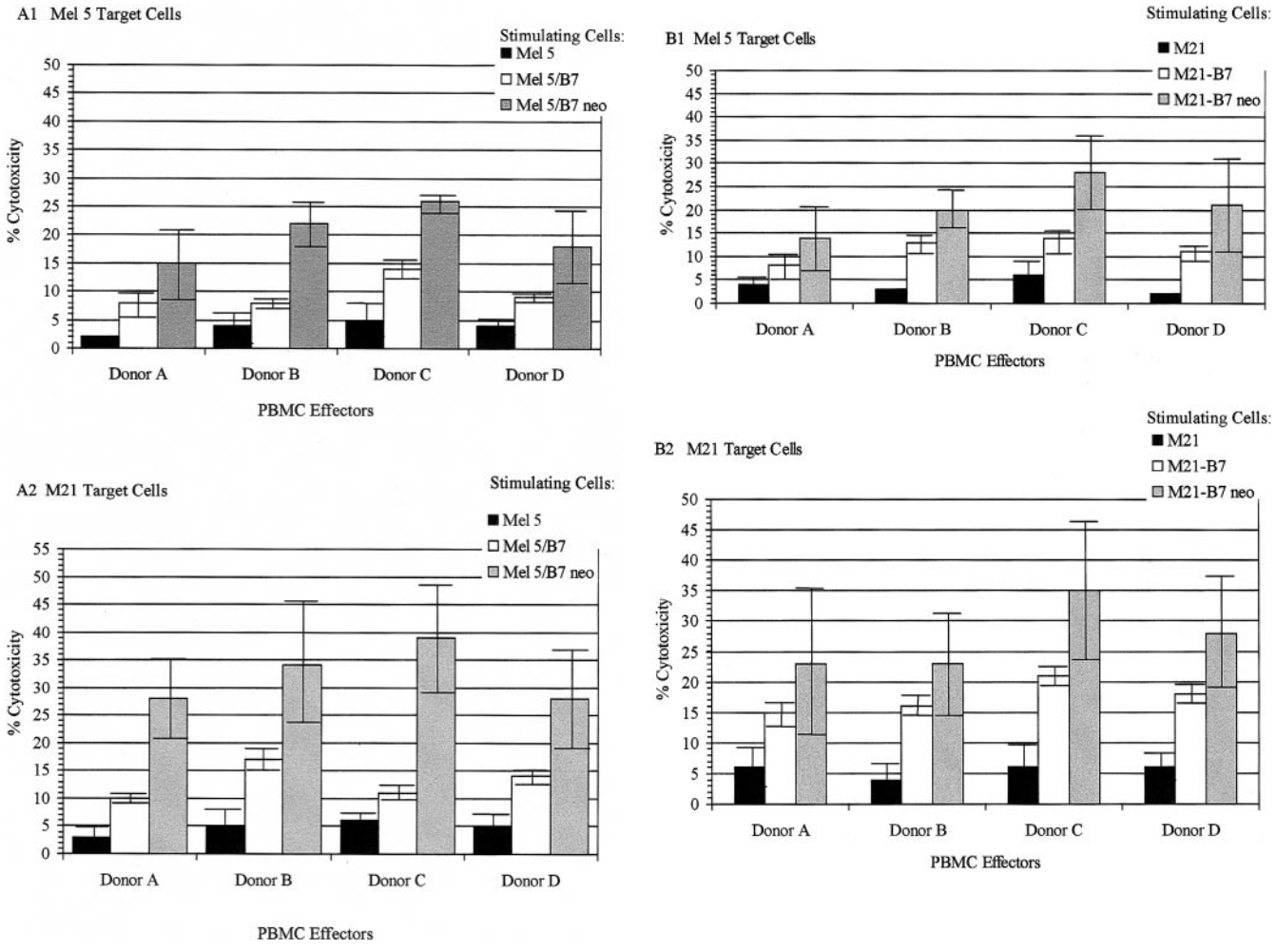

Fig. 4A, B Lytic activity of normal donor PBMC co-cultured for 5 days with irradiated mel5, mel5-B7, mel5-B7neo, m21, m21-B7 or m21-B7neo cells. Effector PBMC were then tested against mel5 or m21 target cells at a ratio of 30:1, 10:1, and 3.3:1. Data are presented as percentage cytotoxicity at the 30:1 ratio. **A** Mel5 target cells (**A2**) were evaluated with mel5-, mel5-B7-, or mel5-B7neo-stimulated PBMC effector cells of donors A, B, C, and D. **B** Mel5 target cells (**B1**) and m21 target cells (**B2**) were evaluated with m21-, m21-B7- or m21-B7neo-stimulated effector PBMC. Data represent the mean and standard deviation of at least two different assays for each donor

PBMC incubated with mel5-B7, compared to that with wild-type mel5. As shown in Fig. 7, the amount of proliferation in PBMC incubated in the presence of the stable transfectants was similar to that seen when PBMC were incubated in the presence of allogeneic PBMC.

Discussion

The necessary components for effective T cell stimulation by antigen include recognition of peptide that is presented by appropriate MHC molecules and interaction with co-stimulatory molecules on the antigen-presenting cell. Transfection of HLA-A2$^+$ human melanoma, which can then stably express moderately high levels of B7-1, has been shown to increase the antigenicity of the melanoma cells, as measured by their ability to activate cytotoxic T cell precursors, T cell proliferation, and cytokine production [7, 10, 12, 16,19]. We previously demonstrated that particle-mediated gene transfer via a gene gun is a rapid, non-viral method of genetically modifying human melanoma cells to express functional levels of B7-1 immediately and without selection [1]. In the present study, we demonstrate that more than 90% B7-1 expression can be achieved in melanoma cells following in vitro particle-mediated gene transfer of cDNA for B7-1 and neomycin phosphotransferase and subsequent in vitro selection with G418. Both HLA-A2$^+$ and HLA-A2$^-$ melanoma cells became better stimulators of cytokine production and lytic activity in allogeneic PBMC following this gene transfer of B7-1 cDNA. Furthermore, the enhanced antigenicity was proportional to the level of B7-1 expression on the stimulating tumor cells.

In the present study, normal donor PBMC co-cultured for 3 days in the presence of the stably transfected m21-B7neo tumor cells secreted greater levels of GM-CSF than PBMC incubated in the presence of the transiently transfected m21-B7 tumor cells. In addition,

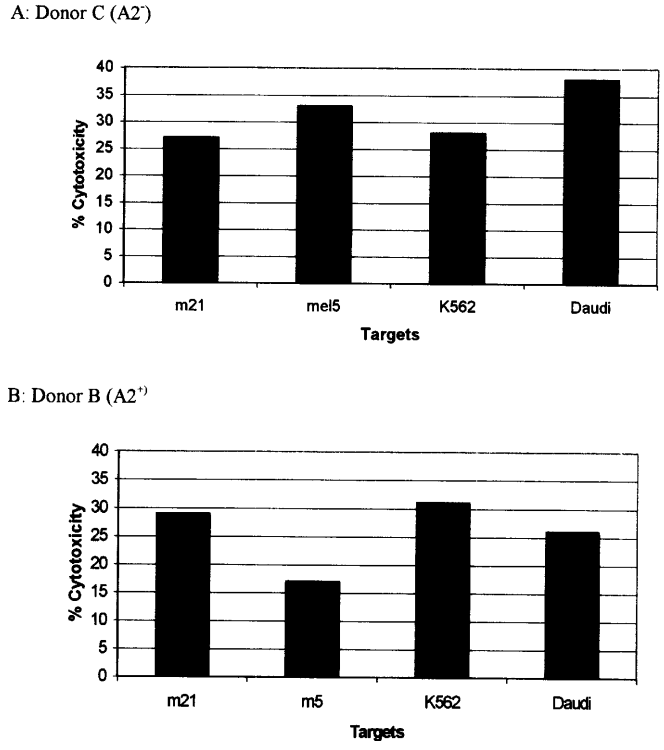

Fig. 5A, B Lytic activity of normal donor PBMC co-cultured for 5 days with irradiated m21-B7neo when tested against mel5, m21, K562 or Daudi target cells. Data represent the results of one experiment for donor C (**A**) and donor B (**B**)

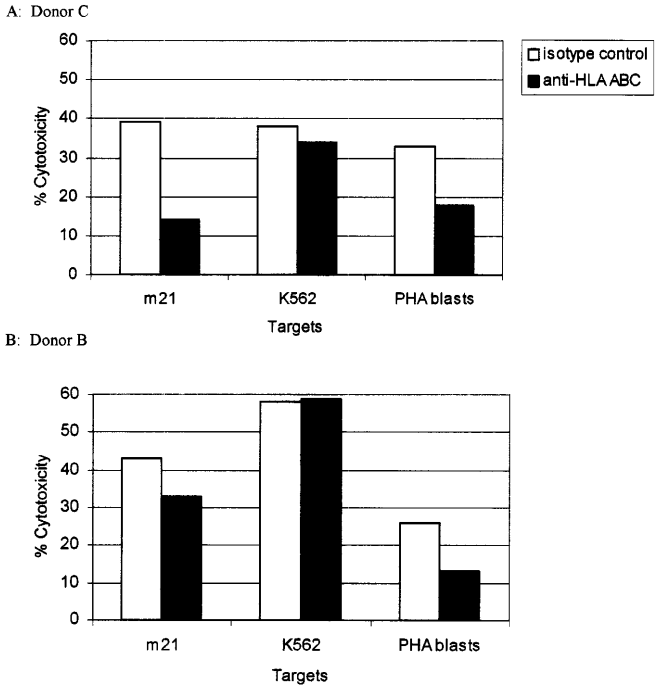

Fig. 6A, B Lytic activity of normal donor PBMC co-cultured for 5 days with irradiated m21-B7neo when tested against m21 or K562 target cells with the addition of 10 μg/ml anti-HLA-ABC antibody or its isotype control and lytic activity of normal donor PBMC cultured for 5 days with irradiated allogeneic PBMC when tested against the allogeneic PBMC that had been incubated for 48 h with 1 μg/ml phytohemagglutinin (PHA) with the addition of 10 μg/ml anti-HLA-ABC antibody or its isotype control. **A** The results of one experiment using donor C; **B** the results of one experiment using donor B

the M21-B7 tumor cells stimulated greater levels of GM-CSF secretion than did the unmodified m21 tumor cells. A similar pattern of GM-CSF secretion was seen in supernatant fluids from 5-day co-cultures of normal donor PBMC and the m21 and mel5 tumor cells. Similar findings have been reported by others when evaluating allogeneic HLA-matched PBMC stimulated by B7-1-modified melanoma cells [7, 16, 19]. The previously reported studies modified the stimulating melanoma cells with cDNA for B7-1 by lipofection [7] or by infection using a potentially antigenic retroviral vector [16, 19] and only HLA-A-matched tumor cells were used as recipient stimulating cells. In the present study, we extend these findings to include HLA-unmatched tumor cells as stimulating cells and use a nonantigenic means of direct gene transfer to modify the stimulating cells.

Uptake of radiolabeled thymidine was greater in PBMC cultured for 6 days with irradiated mel5-B7neo than in PBMC cultured with unmodified mel5 cells. In addition, PBMC expression of CD28 was reduced from control levels following 5 days of culture with irradiated mel5-B7neo or m21-B7neo. In the case of PBMC incubated with subpopulations of m21-B7neo sorted by level of B7-1 expression, the reduction in expression of CD28 by PBMC was inversely proportional to the level of B7-1 expression on the tumor cells and occurred on both CD4 and $CD8^+$ cells. These data are supported by Linsley et al. [9] who found down-regulation of CD28 synthesis of T cells cultured with$B7^+$ Chinese hamster ovary cells. These results demonstrate an interaction involving both CD4 and $CD8^+$ cells with the B7-1-transfected melanoma cells.

The aim of transfecting tumor cell lines with cDNA for B7-1 was to increase the ability of the tumor to activate anti-melanoma T cell cytolytic activity. The results of the present study demonstrate that the cytolytic activity of normal donor PBMC was enhanced when the PBMC were stimulated by melanoma cells transfected to express B7-1. The enhancement in cytolytic activity was proportional to the level of B7-1 expression on the stimulating cells. We found that this cytolytic activity has both an HLA-class-I-restricted and an HLA-class-I-unrestricted component, as it was only partially blocked by addition of anti-HLA ABC antibody. Mogi et al. [12] concluded that the enhanced cytolytic activity induced by melanoma cells expressing B7-1 was predominately exerted by T-cell-receptor-mediated recognition. However, we also observed significant lytic activity against K562 and Daudi target cells, suggesting that a portion of the lytic activity is mediated by nonspecific effector cells such as NK or lymphokine-activated killer cells. In contrast to our results, Fenton et al. [7] and Yang et al. [19] observed very low levels of lytic activity against K562 targets (8% and 11% respectively). The mecha-

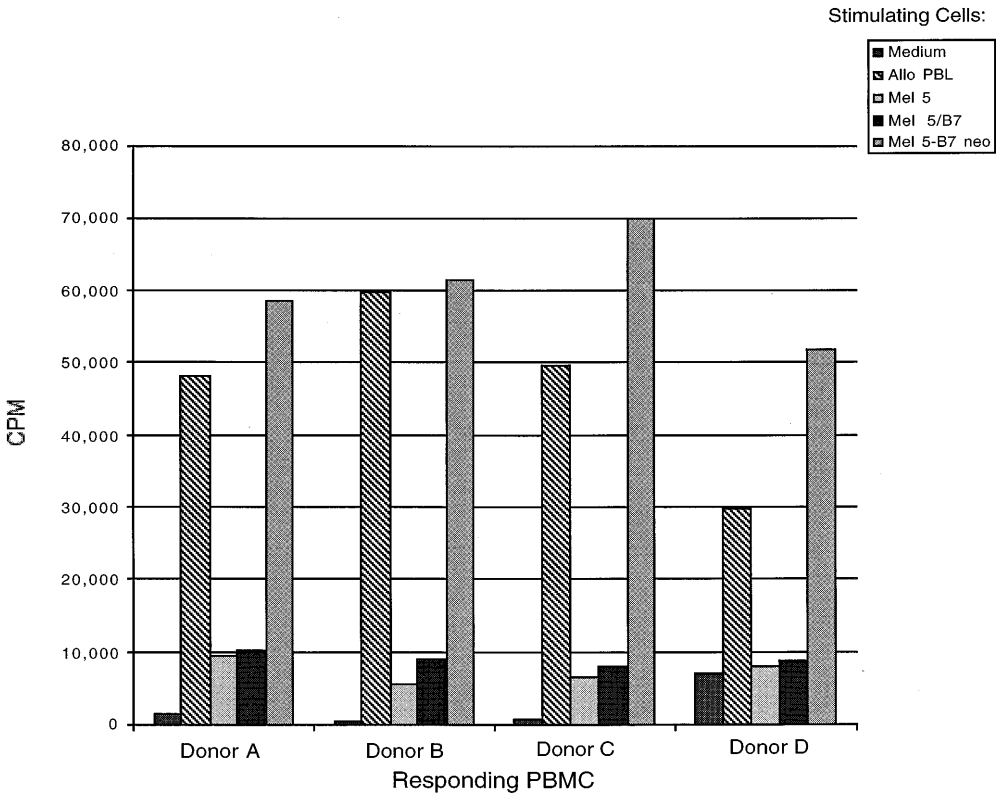

Fig. 7 Thymidine uptake by normal donor PBMC incubated for 7 days in medium alone or with irradiated allogeneic PBMC, mel5, mel5-B7 or mel5-B7neo cells. Data represent the results of one experiment with four donors run simultaneously

nism for the enhanced HLA-class-I-unrestricted cytotoxicity in our study may be secondary to the cytokines, such as IL-2, released by responding cells. Alternatively, there is evidence that, in vivo, the local antitumor response to B7-1-expressing melanoma cells is mediated in part by NK cells [6, 18].

Other studies have reported that B7-1-transfected melanoma cells can elicit potent cytolytic activity by allogeneic, but HLA-A locus-matched PBMC effectors [10, 19]. In the present study, we observed significant lysis of tumor cell lines without the requirement that the PBMC effectors be matched to the tumor cells at the HLA-A locus. We conclude that allogeneic melanoma cells transfected with cDNA for human B7-1 can function as stimulator cells to elicit enhanced cytolytic activity by allogeneic PBMC. Additional investigation is needed to determine the cytolytic activity of PBMC following stimulation by autologous melanoma cells transfected with cDNA for human B7-1. However, results from our allogeneic system demonstrated that the cytolytic activity by PBMC is proportional to the level of B7-1 expression by the stimulating allogeneic melanoma cells and has a component that is MHC-class-I-restricted and a component that is without MHC class I restriction. These results will facilitate investigation of B7-1- modified melanoma cells as a vaccine approach for patients with melanoma.

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