

Induced Expression of B7-1 on Myeloma Cells Following Retroviral Gene Transfer Results in Tumor-Specific Recognition by Cytotoxic T Cells¹

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The aim of this study was to evaluate whether tumor cells from patients with multiple myeloma activate allogeneic and autologous T cells. Results showed that myeloma cells expressed few B7-2 and no B7-1 in six cell lines and primary cells from 11 patients. They expressed substantial levels of HLA class I, CD40, and a set of adhesion molecules. In accordance with the low density of B7 molecules on these cells, they were poor allogeneic CD8⁺ T cell stimulators. Neither IFN- γ plus TNF- α nor CD40 stimulation significantly induced B7-1 or up-regulated B7-2 on human myeloma cell line or primary myeloma cells from six of seven patients. However, such induction was found on autologous bone-marrow nontumoral cells and on autologous dendritic cells following CD40 stimulation. High B7-1 expression was stably obtained on human myeloma cell line using transduction with a B7-1 retrovirus, enabling these cells to stimulate allogeneic CD8⁺, though not CD4⁺, T cell proliferation. For one patient with advanced disease, B7-1 gene transfer made it possible to amplify autologous cytotoxic T cells that killed autologous myeloma cells in an HLA class I-restricted manner, but not autologous PHA blasts. These results suggest that B7-1 gene transfer could be a promising immunotherapeutic approach in multiple myeloma. *The Journal of Immunology*, 1999, 163: 514–524.

Multiple myeloma (MM)³ is a B cell neoplasia affecting the late stage of B cell differentiation. Tumor plasma clones expand following a multistep transformation process (1) in close contact with the bone-marrow stromal cells expressing adhesion molecules and producing cytokines, especially IL-6, which sustains survival and growth of myeloma cells (2, 3).

Although high-dose chemotherapy and autologous stem cell transplantation have improved the rate of complete remission, some myeloma cells escape treatment and all MM patients relapse (4–6). The development of immunotherapy approaches designed to get CTL might help to eradicate or control residual tumor cells. The use of professional APC such as dendritic cells (DC) to generate anti-tumoral response is a promising strategy (7, 8). Vaccination of patients with DC pulsed with monoclonal Ig as tumor Ag has already begun in MM (9, 10). DC pulsed with tumor RNA (11–13) or cell lysates (14) may prove to be useful in obtaining CTL clones against various unidentified Ag. However, this ap-

proach is limited in that there is no guarantee that DC will present the same tumor peptides as those presented by the tumor cells.

Alternatively, anti-tumoral CTL can be obtained by using tumor cells themselves as APC. The in vitro or in vivo stimulation of CTL requires TCR activation by a peptide/MHC complex combined with costimulatory signals, in particular CD28 activation by B7 molecules present on APC (15–17). A high density of B7 molecules is needed to trigger a full T cell response (18, 19). B7-1 (CD80) and B7-2 (CD86) are generally absent or only marginally present on tumor cells but they could be induced upon CD40 ligation or by cytokines such as IFN- γ in some hemopoietic malignancies (20–22). In B cell lymphoma and pre-B acute lymphoblastic leukemia, CD40-mediated B7 induction on tumor cells makes it possible to obtain autologous specific anti-tumoral CTL (23, 24). In contrast, in acute myeloid leukemia, CD40 stimulation fails to induce B7-1 and does not increase allogeneic T cell proliferation (20). Myeloma cells express a set of adhesion molecules as well as CD40 and CD28 (25–27). The myeloma CD28 is able to bind B7 and to activate the phosphatidylinositol 3 kinase (26). Most studies have shown that only a small number of tumor plasma cells express B7-2 and that B7-1 is generally lacking (25–32). A recent report suggests that the weak B7-1 expression can be doubled after cell culture with IL-6, IFN- γ , or TNF- α (31). CD40 stimulation has been shown to induce IL-6 production and to increase growth and survival of MM cells in vitro (30, 32). In other studies, CD40 stimulation was reported to induce growth arrest (28) and apoptosis (33) on a human myeloma cell line (HMCL). These conflicting results could be explained in part by the use of few cell lines that, sometimes, were not true HMCL but EBV-infected cell lines (ARH 77, HS-Sultan, IM9) (34). Furthermore, research on primary myeloma cells should be developed given the marginal induction of B7 molecules reported and the difficulty of clearly distinguishing myeloma cells from late-stage B cells with anti-CD38 mAb labeling (31). Another way to induce B7 expression is by gene transfer. A recent study reported that the transfer of

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³ Abbreviations used in this paper: MM, multiple myeloma; BMMC, bone-marrow mononuclear cells; DC, dendritic cells; HMCL, human myeloma cell line; MFI, mean fluorescence intensity; PCL, plasma cell leukemia; SI, stimulation index. CD40L, CD40 ligand; allo-MLR, allogeneic MLR; rh, recombinant human.

B7 genes using adeno-associated virus vectors in two HMCL enable them to activate and be lysed by a mixture of allogeneic NK and T cells (35). These effects were due in part to NK cells as previously described for the *in vivo* rejection of some B7-expressing animal tumors (36, 37).

To date, no study has demonstrated that B7-1⁺ myeloma cells can be used to obtain anti-tumoral autologous T cells *in vitro*. We show here, using an anti-syndecan-1 mAb that recognizes all viable tumor cells in bone-marrow samples (38–40), that myeloma cells express no B7-1 and only a few B7-2 molecules. This low B7-2 density was responsible for a weak stimulation of highly purified allogeneic T cells by HMCL. B7-1 was not significantly up-regulated on primary myeloma cells activated by CD40 or IFN- γ plus TNF- α for a majority of patients. In contrast, B7-1 was induced by CD40 ligation on autologous nontumoral bone-marrow cells and on autologous DC. Finally, the transduction of B7-1 on HMCL with a B7-1 retrovirus increased their ability to activate allogeneic CD8⁺ T lymphocytes. For one patient with advanced disease, obtaining B7-1⁺ HMCL made it possible to get specific autologous anti-tumoral CTL.

Materials and Methods

Preparation of primary samples

Eleven patients with MM (median age, 66.5 years) were included in this study. There were four IgG λ , five IgG κ , one IgA κ , and one Bence-Jones λ . Bone-marrow aspirates were collected for the determination of plasma cell labeling index, a marker of disease activity (41), and exceeding cells were used for this study after informed consent. Bone-marrow mononuclear cells (BMMC) were isolated by Ficoll-Hypaque centrifugation (BioWhittaker, Walkersville, MD) and either stained with appropriate mAbs or plated in culture for 4 days. For two patients, purification of syndecan-1⁺ tumor plasma cells was performed with the MI15 anti-syndecan-1 mAb (40) and magnetic cell separation system anti-murine IgG microbeads (Tebu, Le Perray-en-Yvelines, France) as previously described (42). Purified cells contained >92% plasma cells.

Cell lines

Five IL-6-dependent (XG-1, XG-2, XG-6, XG-13, and XG-14) and one autonomous (RPMI 8226) HMCL were used in this study. These cell lines were true myeloma cell lines with a plasma cell phenotype, no expression of B cell markers, and no infection with EBV. XG-1, XG-2, XG-6, and XG-13 had been previously established in our laboratory (29, 43), and RPMI 8226 was purchased from American Type Culture Collection (Manassas, VA). They were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, and 5×10^{-5} M 2-ME, and with 3 ng/ml of human IL-6 for XG-1, XG-2, XG-6, and XG-13. XG-14 was cultured in X-VIVO 20 serum-free medium (BioWhittaker) supplemented with 3 ng/ml of human IL-6. L cells stably transfected with CD40 ligand (CD40L) were kindly provided by Sem Saeland (Schering-Plough, Dardilly, France).

Phenotypic analysis

Expression of cell surface molecules on HMCL and B7-1 HMCL was determined using FITC-conjugated anti-CD28, anti-CD54, anti-CD58, anti-CD11a, anti-CD18, anti-HLA-ABC, anti-HLA-DR (Immunotech, Marseille, France), and anti-B7-2 (PharMingen, San Diego, CA) and PE-conjugated anti-B7-1, anti-Fas, and anti-CD40 mAbs (Immunotech). For primary BMMC, double staining of malignant plasma cells was performed using the same mAbs and FITC- or biotin-conjugated anti-syndecan-1 mAb (MI15). All fluorescence analyses were conducted on a FACScan apparatus (Becton Dickinson, San Jose, CA). Negative controls were done with corresponding irrelevant isotypic-matched murine mAbs.

Induction of costimulatory molecules on myeloma cells

HMCL were cultured at 2×10^5 /ml and BMMC and purified syndecan-1⁺ plasma cells at 1×10^6 /ml in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, and 5×10^{-5} M 2-ME, except for the XG-14 cell line, which was cultured in X-VIVO 20 medium. All cultures were performed in the presence of 3 ng/ml of human IL-6. Cells were stimulated with either IFN- γ (100 U/ml; Genzyme, Cambridge, MA) plus TNF- α (20 ng/ml; R&D Systems, Minneapolis, MN) or cocultured with CD40L-transfected L cells (1×10^5 /ml). As a control of the specificity of CD40 stimulation, we

used nontransfected L cells (1×10^5 /ml). After 4 days of culture in these different conditions (IL-6, IL-6 plus IFN- γ plus TNF- α , IL-6 plus L cells, IL-6 plus CD40L-transfectant), cells were harvested and simple (HMCL and purified primary plasma cells) or double (BMMC) stainings were done as described above.

Obtention of HMCL transfected with B7-1 cDNA (B7-1 HMCL)

The full-length human B7-1 cDNA was subcloned into the neomycin-selectable 5192 retroviral vector with *EcoRI/XhoI* cloning sites and transfected into the E293 packaging cell line using Lipofectamine (Life Technologies, Paisley, U.K.). The packaging cell line was also transfected with the empty retroviral vector. The 5192 retroviral vector and the E293 packaging cell line were a gift from Dr. Mehtali (Transgene, Strasbourg, France). Transfected cells were selected by outgrowth with 600 μ g/ml of G418 (Life Technologies) for 10 days. Resistant cells expressing the B7-1 molecule were sorted using a Vantage cell sorter (Becton Dickinson), cultured at a high cell density, and the supernatant was filtered and stored at -70°C . Culture supernatant of G418 resistant clones of E293 packaging cells transfected with the empty vector was also collected, filtered, stored at -70°C , and used as a source of control retrovirus. HMCL were plated at 10^6 cells/well in six-well dishes and exposed to control or B7-1 retroviral supernatant for 2 h in the presence of 8 μ g/ml of polybrene (Sigma, St. Louis, MO). After two cycles of infection, cells were selected with 1200 μ g/ml of G418 over a period of 10 days. Resistant clones obtained with the B7-1 retrovirus were analyzed for surface expression of B7-1 and sorted as described above. The stable transfected HMCL obtained with the B7-1 retrovirus (named B7-1 XG-1, B7-1 XG-6, and B7-1 XG-14) and with the control retrovirus (named Neo XG-1, Neo XG-6 and Neo XG-14) were subsequently maintained with 500 μ g/ml of G418.

Allogeneic MLR (allo-MLR)

Heparinized venous peripheral blood from healthy volunteers was collected after written informed consent. T lymphocytes were purified by depletion of non-T cells with mAbs (Immunotech) and magnetic beads (Dyna, Oslo, Norway) as described (44). Briefly, monocytes and B cells were first depleted using CD14- and CD19-coated beads. Then, non-T cells were removed by incubating with a mixture of CD16, CD56, and HLA-DR mAbs and then goat anti-mouse Ig beads. After two rounds of purification, the purity of resting CD3⁺ cells was always >98%. CD4⁺ and CD8⁺ T cells were further purified by removing CD8⁺ or CD4⁺ cells using anti-CD8 and anti-CD4 mAbs and magnetic beads. The purity of the T cell subpopulations was >98%. Cells were frozen in 50% FCS-10% DMSO at 20×10^6 cells per vial. To evaluate HMCL-induced T cell proliferation, primary allo-MLR was performed using mitomycin C (Sigma)-treated (50 μ g/ml) HMCL or B7-1 HMCL cocultured at 5×10^4 cells/well with 10^5 allogeneic T cells in 96-well round-bottom plates in RPMI 1640 and 5% heat-inactivated human AB serum. When indicated, antagonist (CD28.6) anti-CD28 (provided by Daniel Olive, Marseille, France), anti-HLA-ABC, or anti-B7-2 mAbs or their isotypic controls were added at 10 μ g/ml. After 5 days of coculture, cells were pulsed with 1 μ Ci of [³H]TdR (sp. act. 25 Ci/ml; Amersham, Buckingham, U.K.) for 12 h, harvested, and counted. All microculture tests were conducted in triplicate and stimulation indexes (SI) were calculated as follows: $SI = \frac{\text{cpm}_{(\text{T cells} + \text{HMCL})}}{\text{cpm}_{(\text{T cells})} + \text{cpm}_{(\text{HMCL})}}$. To quantify T cell amplification and activation, bulk cocultures of mitomycin-treated HMCL, neo HMCL, or B7-1 HMCL and purified T cells were performed at a responder:stimulator cell ratio of 3:1. Cells were seeded at 1.5×10^6 cells/ml in RPMI 1640 and 5% AB serum in 6-well plates. At days 2 and 5 of culture, cells were counted and used for phenotypic analysis with FITC-anti-CD25 and FITC-anti-HLA-DR mAbs (Immunotech). At the same time, culture supernatants were collected and stored at -20°C for IL-2 measurement using a commercially available ELISA test (Genzyme).

Generation of DC

For two patients, DC were generated from adherent apheresis cells as described (44). Briefly, apheresis cells were collected after mobilization of hemopoietic precursors with high dose cyclophosphamide (4 g/m²) and recombinant human G-CSF (rhG-CSF, filgrastim, Neupogen; Amgen-Roche, Neuilly-sur-Seine, France; daily injection of 5 μ g/kg) and plated for 2 h at 37°C . Nonadherent cells were discarded and adherent cells were then cultured for 7 days in X-VIVO 15 medium (BioWhittaker) with 2%

Table I. Expression of adhesion and costimulatory molecules on HMCL^a

HMCL	CD54	CD58	CD11a	CD18	HLA-ABC	HLA-DR	B7-1	B7-2	CD28	CD40
XG-1	100 (158)	100 (30)	<0.5	<0.5	100 (416)	<0.5	<0.5	30 (22)	100 (120)	0.5
XG-2	100 (710)	100 (49)	100 (42)	100 (53)	100 (964)	<0.5	<0.5	35 (25)	100 (60)	100 (7504)
XG-6	100 (293)	100 (31)	<0.5	<0.5	100 (1213)	<0.5	<0.5	32 (25)	86 (45)	85 (44)
XG-13	100 (165)	100 (21)	<0.5	<0.5	100 (1100)	<0.5	<0.5	25 (26)	100 (66)	70 (55)
XG-14	100 (210)	100 (54)	<0.5	<0.5	100 (812)	<0.5	<0.5	92 (62)	25 (23)	75 (71)
RPMI 8226	100 (431)	100 (31)	15 (18)	16 (19)	100 (602)	<0.5	<0.5	20 (34)	100 (153)	52 (60)

^a HMCL were harvested during the exponential growth phase, stained with corresponding mAbs, and fluorescence was analyzed on a FACScan apparatus. The data show the percentage of cells expressing Ag and the MFI in parentheses. The MFI obtained with irrelevant matched mAbs were set between 4 and 6. A percentage of positivity <0.5% was considered as negative.

human albumin, 1000 U/ml of rhGM-CSF (Leucomax; Sandoz, Basel, Switzerland) and 500 U/ml of rIL-4 (Genzyme). Cells harvested at day 7 exhibited the typical morphological features of DC and expressed HLA class I, HLA class II, B7-1, B7-2, CD4, CD40, and no CD14 as reported (44). They were cultured at 4×10^5 /ml in the presence of 4×10^4 non-transfected L cells or CD40L-transfected L cells/ml. After 3 days of culture, cells were stained with FITC-anti-B7-1 or control murine mAbs.

Generation of autologous anti-tumoral T cells

For one patient with plasma cell leukemia (PCL), PBMC (comprising 30% CD3⁺ T cells, 40% syndecan-1⁺ tumor cells, and 15% CD16⁺ NK cells) were frozen at diagnosis and an autologous HMCL was established (XG-14) and transduced with control vector (Neo XG-14) and B7-1 retrovirus (B7-1 XG-14). PBMC (1.5×10^6 /ml) were stimulated with mitomycin-treated (50 μ g/ml) HMCL, Neo HMCL, or B7-1 HMCL at an E:T ratio of 2:1 in RPMI 1640 supplemented with 5% human AB serum for 6 days. Dead cells were eliminated by Ficoll-Hypaque centrifugation. Viable cells

were then cultured at 7.5×10^5 /ml and restimulated on days 6, 12, 18, and 24 with mitomycin-treated HMCL, Neo HMCL, or B7-1 HMCL at an E:T ratio of 2:1 in the presence of IL-2 (30 U/ml) and IL-12 (1 ng/ml) (R&D Systems). Cytokines were added on days 7, 13, 19, and 25 of culture. When indicated, IL-7 was added at 5 ng/ml. Cell-mediated toxicity was determined on day 28 using a standard 4-h ⁵¹Cr release (Amersham) assay using E:T ratios of 3:1, 10:1, and 30:1. XG-14 (autologous HMCL), XG-2 (allogeneic HMCL), PHA-blasts (autologous nontumoral cells), and K562 were used as target cells at 5000 cells/well in 96-well plates. All experiments were performed in triplicate. To determine whether the cytotoxicity was restricted by HLA class I, target cells were preincubated with 10 μ g/ml of anti-HLA class I mAb. After a 4-h incubation at 37°C, supernatants were harvested and radioactivity was measured with a γ counter. The percentage of specific lysis was determined as: (experimental ⁵¹Cr release - spontaneous ⁵¹Cr release)/(maximum ⁵¹Cr release - spontaneous ⁵¹Cr release) \times 100. The ratio (spontaneous release/maximum release) was always below 30%.

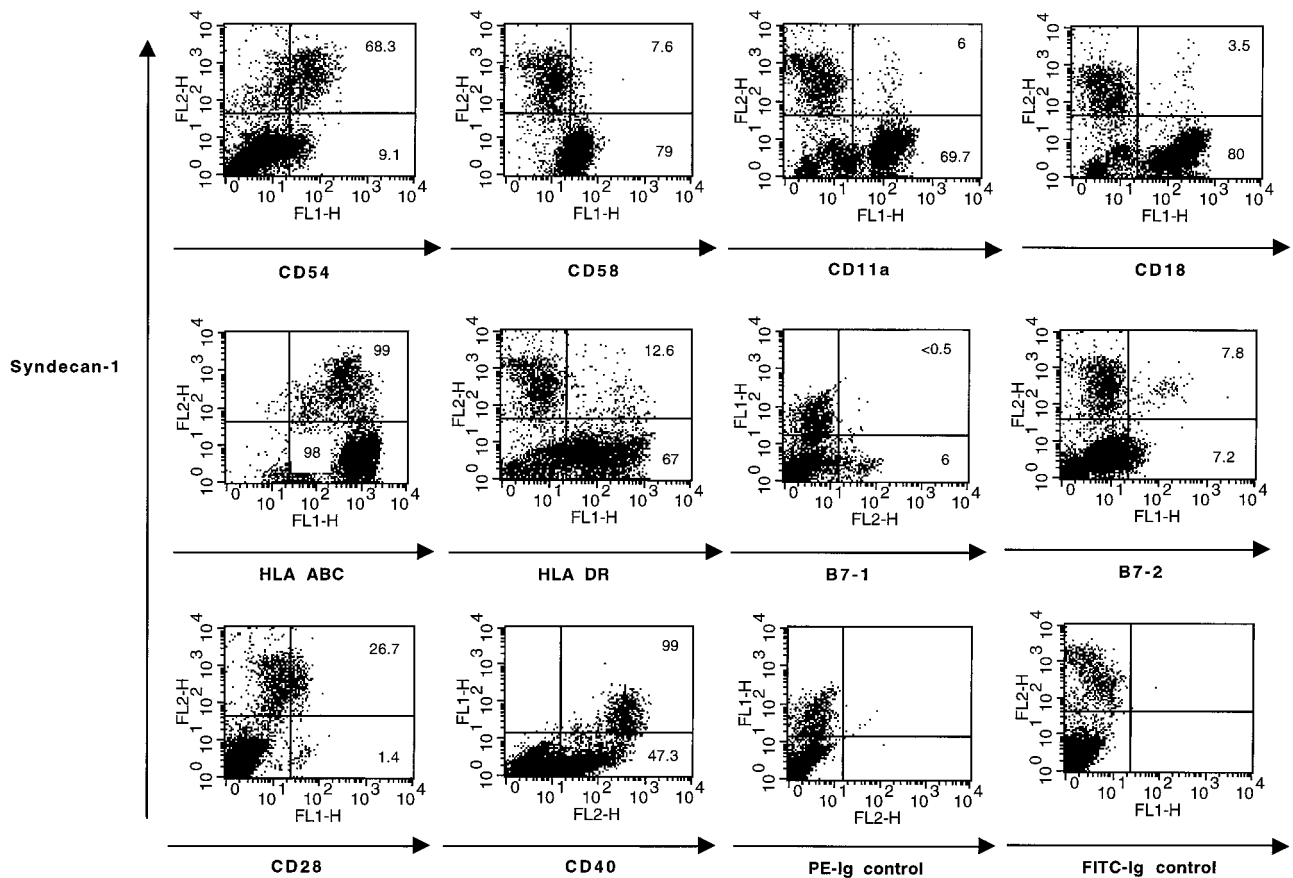


FIGURE 1. Expression of adhesion and costimulatory molecules on primary myeloma cells. BMCL from patient 4 were double-stained either with biotin-MI15 plus PE-streptavidin (FL-2) and FITC-conjugated anti-CD54, CD58, CD11a, CD18, HLA-ABC, HLA-DR, B7-2, CD28 mAbs or with FITC-MI15 (FL-1) and PE-conjugated anti-B7-1, CD40 mAbs. Irrelevant mouse Ig were used as negative controls as shown on the last two plots. On each dot plot, the percentages of syndecan-1⁺ and syndecan-1⁻ cells expressing the cell-surface markers were indicated in the upper right and lower right panels, respectively.

Table II. Expression of adhesion and costimulatory molecules by primary myeloma cells^a

Patient	Stage	Syndecan-1 ⁺ Cells (%)	CD54	CD58	CD11a	CD18	HLA-ABC	HLA-DR	B7-1	B7-2	CD28	CD40
1	II A	10	95 (83)	37 (39)	7.5 (137)	8 (245)	99 (778)	25 (62)	<0.5	20 (150)	10 (43)	100 (129)
2	III A	39	82 (45)	20 (37)	<0.5	<0.5	100 (980)	14 (131)	<0.5	25 (53)	10 (23)	100 (97)
3	III B	60	76 (56)	53 (27)	2.5 (188)	3.4 (205)	100 (2518)	12 (54)	<0.5	80 (46)	6 (37)	90 (157)
4	III A	17	68 (78)	7.6 (57)	6 (144)	3.5 (217)	99 (554)	12.6 (334)	<0.5	7.8 (186)	27 (41)	99 (371)
5	PCL	88	100 (174)	77 (21)	<0.5	<0.5	100 (1353)	1.5 (267)	<0.5	100 (68)	89 (34)	91 (122)
6	III A	60	100 (68)	96 (20)	3.2 (77)	2.6 (94)	100 (928)	2.3 (98)	<0.5	5.3 (97)	<0.5	100 (179)
7	III A	52	36 (49)	30 (52)	<0.5	<0.5	100 (1049)	4.6 (42)	<0.5	2.7 (196)	76 (35)	86 (70)
8	III A	20	100 (103)	62 (32)	7.5 (182)	8.5 (328)	100 (1291)	21 (203)	<0.5	59.5 (76)	100 (70)	99 (263)
9	III B	29	95 (152)	20 (19)	<0.5	<0.5	100 (702)	6 (195)	<0.5	28 (69)	25 (42)	75 (124)
10	III B	72	94 (46)	23 (34)	11.4 (86)	14.5 (131)	100 (692)	3.1 (431)	<0.5	5.2 (38)	7.6 (37)	100 (128)
11	III B	85	70 (112)	91 (52)	<0.5	<0.5	100 (816)	1 (65)	<0.5	91 (198)	15 (45)	86 (60)
Mean %		48.4	83.3	47	3.5	3.7	99.8	9.4	<0.5	38.6	35.1	93.3
± SD		±27.4	±19.9	±30.8	±4	±4.8	±0.4	±8.2		±37.1	±38.1	±8.4

^a BMCL were stained with FITC- or biotin-conjugated MI15 anti-syndecan-1 mAb and with the appropriate FITC- or PE-conjugated antiadhesion or costimulatory molecule mAbs. When necessary, PE-streptavidin was added. The data show the percentage of syndecan-1⁺ cells expressing Ag. The MFI are indicated in parentheses. A percentage of positivity <0.5% was considered as negative.

Results

Expression of adhesion and costimulatory molecules on HMCL and primary myeloma cells

Because the expression of adhesion and costimulatory molecules on APC plays a major role in the activation of T cells, we first investigated it on six HMCL (Table I) and on bone-marrow plasma cells from 11 MM patients. To label patient tumor cells, we used an anti-syndecan-1 mAb that stains all viable myeloma cells in tumor samples (38–40). The phenotype obtained with patient 4's cells is outlined on Fig. 1, and data obtained for the 11 patients are summarized in Table II. Concerning adhesion molecules, CD54 was expressed at a high level and CD58 at an intermediate level by all HMCL (percentage of positive cells, 100%; mean fluorescence intensity (MFI), 158–710 for CD54 and 21–54 for CD58) and primary tumor samples (mean percentage of positive cells, 83.3 ± 19.9% for CD54 and 47 ± 30.8% for CD58). CD11a and CD18 were expressed on 2 of 6 HMCL, on <20% of tumor cells in 6 of 11 fresh samples, and were virtually absent in other samples. HLA class I was largely present on all plasma cells. HLA-DR was absent on HMCL and marginally present on patient tumor cells

(mean percentage of positive cells, 9.4 ± 8.2%). In addition, in keeping with results obtained with HMCL, primary tumor cells expressed no detectable B7-1 and a variable level of B7-2 (mean percentage of positive cells, 38.6 ± 37.1%). It should be noted, as previously reported (25, 27), that the CD28 molecule was present on all cell lines and on 10 of 11 patient tumor samples. CD40 expression was heterogeneous in HMCL but was always present on tumor samples (mean percentage of positive cells, 93.3 ± 8.4%; mean MFI, 155 ± 90). Thus the phenotype of HMCL and patients' myeloma cells was very similar. In particular, myeloma cells expressed CD54 and CD58, HLA class I, CD40, no B7-1, and few B7-2.

B7-1-negative HMCL were poor allogeneic T cell stimulators

When XG-1, XG-6, and XG-14 were used as stimulator cells in primary allo-MLR, a small but significant T cell proliferation was induced with a mean SI of 9 for XG-1 (range, 3–20; *n* = 7), 5 for XG-6 (range, 4–8; *n* = 4), and 8 for XG-14 (range, 7–11; *n* = 3), depending on the various donors of allogeneic T cells (not shown).

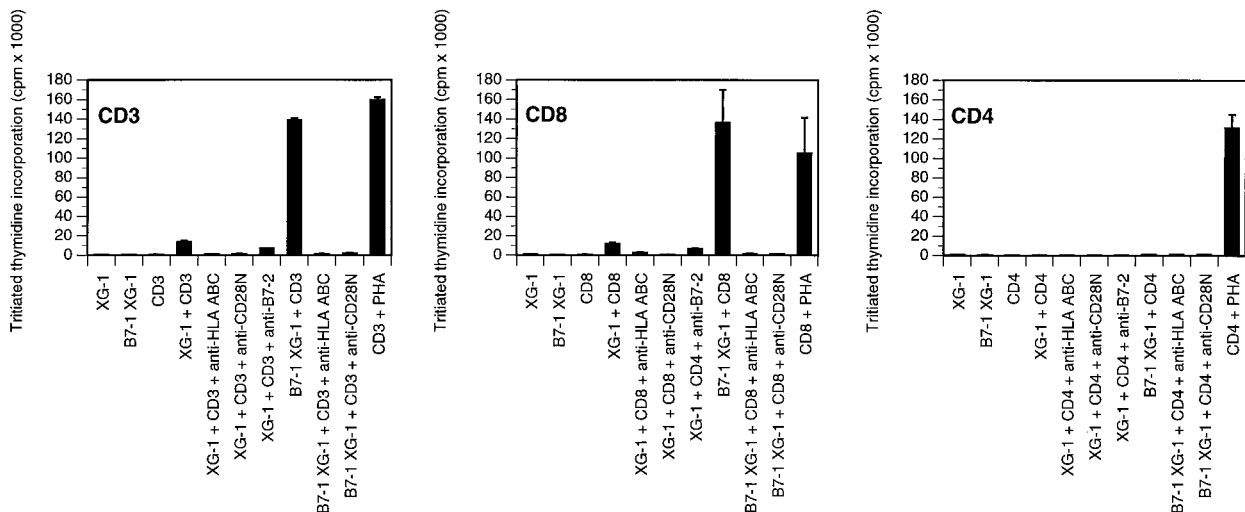


FIGURE 2. Stimulation of allogeneic CD3⁺, CD8⁺, and CD4⁺ T cell proliferation in response to XG-1 and B7-1 XG-1. A total of 5 × 10⁴ mitomycin-treated XG-1 and B7-1 XG-1 were used as stimulator cells for 10⁵ highly purified allogeneic CD3⁺, CD8⁺, or CD4⁺ T cells. When indicated, 10 μg/ml of anti-HLA-ABC, antagonist anti-CD28 (anti-CD28N), or anti B7-2 mAbs were added. Cell proliferation was evaluated by a 12-h pulse with [³H]TdR after 5-day cocultures. No inhibition was found with control mouse mAbs. Data are the mean ± SD of the [³H]TdR incorporations determined on sextuplicate culture wells. In most groups, [³H]TdR incorporation and SD were too small to be visible on the graph.

Table III. Induction of B7-1, HLA-DR, Fas, and CD54 on HMCL by IFN- γ and TNF- α or by the CD40L transfectant^a

HMCL	B7-1			HLA-DR			Fas			CD54		
	Medium	IFN + TNF	CD40L	Medium	IFN + TNF	CD40L	Medium	IFN + TNF*	CD40L	Medium	IFN + TNF*	CD40L
XG-1	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	100 (40)	<0.5	100 (158)	100 (295)	100 (155)
XG-2	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	60 (23)	100 (36)	58 (25)	100 (710)	100 (720)	100 (802)
XG-6	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	75 (26)	100 (32)	72 (22)	100 (293)	100 (351)	100 (299)
XG-14	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	90 (38)	92 (45)	96 (44)	100 (210)	100 (296)	100 (198)
RPMI 8226	<0.5	46 (35)	<0.5	<0.5	100 (730)	<0.5	10 (13)	100 (98)	9 (11)	100 (431)	100 (956)	100 (388)

^a HMCL were cultured in medium alone, with nontransfected L cells, with IFN- γ (100 U/ml) and TNF- α (20 ng/ml), or with CD40L-transfected L cells. Because CD40 stimulation produced no effect on adhesion and costimulatory molecule expression, the data obtained with control L cells, which were similar to those obtained with medium alone, are not indicated in this table. After 48 h, cells were harvested, stained with corresponding mAbs, and analysed on a FACScan. Results are the percentages of labeled cells and, in parentheses, the MFI. The MFI with irrelevant control mAbs was set between 4 and 6. A percentage of positivity <0.5% was considered as negative.

* Significant up-regulation of the percentage and/or of the MFI as evaluated with a nonparametric Wilcoxon test for pairs.

This activation was inhibited by an anti-HLA-ABC mAb, indicating that it was mediated through alloptide presentation by MHC class I molecules. In addition, it was also inhibited by antagonist anti-CD28 or anti-B7-2 mAbs, suggesting that it was mediated in part through the activation of T cell CD28 by the myeloma B7-2 (Fig. 2).

B7-1 was not induced on HMCL nor on primary tumor samples in a majority of MM patients

Stimulation of CD40 is known to induce B7-1 expression on normal B cells and DC as well as in several tumor cells from patients with B cell lymphoma or B cell leukemia (21, 22). IFN- γ and TNF- α may also increase costimulatory molecule expression (20, 31). To enhance the Ag-presenting capacity of myeloma cells, we looked for the effect of IFN- γ plus TNF- α and CD40 stimulation on the expression of costimulatory molecules. As outlined in Table III, the association of IFN- γ and TNF- α induced a low level of

B7-1 and a high level of HLA-DR on the RPMI 8226 cell line. However, these molecules remained undetectable on the other cell lines stimulated by these cytokines. As a control, Fas was up-regulated on HMCL upon IFN- γ plus TNF- α stimulation, and this was associated with an increased sensitivity to Fas-mediated apoptosis for XG-1 and XG-2 cell lines (Table III and data not shown). The density of CD54 was also significantly up-regulated on four of the five HMCL tested, as can be seen in Table III. B7-2, CD11a, CD18, and CD58 were not modulated by IFN- γ plus TNF- α stimulation (data not shown). Independent of their level of CD40 expression, it was impossible to induce B7-1, HLA-DR, or adhesion molecules on HMCL by coculture with CD40L-transfected cells (Table III and data not shown).

We then tested CD40 and IFN- γ plus TNF- α stimulations on seven nonfractionated bone-marrow samples (patients 2, 3, 5, 6, 9, 10, and 11). All the cultures were performed in the presence of IL-6, a survival factor for myeloma cells (2). B7-1 was clearly

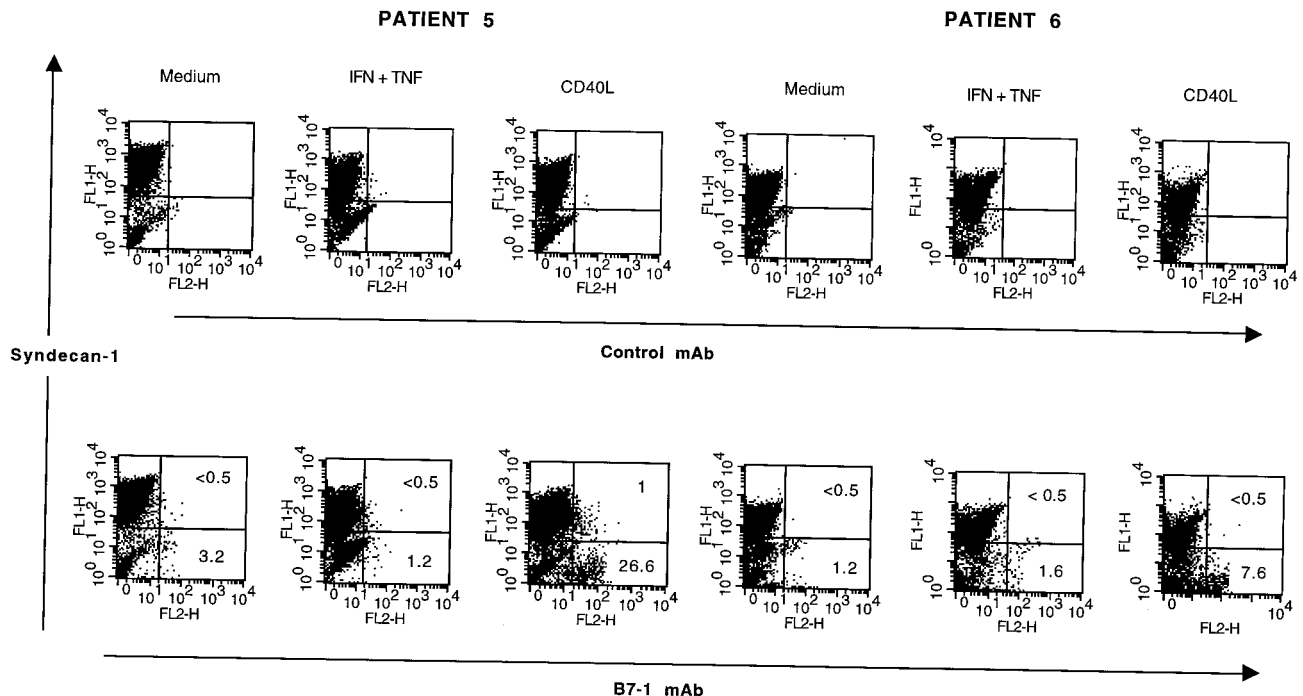


FIGURE 3. Neither IFN- γ plus TNF- α nor CD40 stimulation could induce B7-1 on nonpurified primary myeloma cells. BMMC were cultured at 10^6 /ml in RPMI 1640–10% FCS supplemented with IL-6 (medium) and were stimulated with IFN- γ plus TNF- α or CD40L-transfected L cells. Results obtained with nontransfected L cells were identical with those obtained in medium alone and are not shown. After 4 days, cells were harvested and double-stained as described in Fig. 1. The percentages of B7-1⁺ syndecan-1⁺ and B7-1⁺ syndecan-1⁻ cells are indicated in the upper right and the lower right quadrant, respectively.

Table IV. Induction of costimulatory molecules on nonpurified myeloma cells^a

Patients	% of Syndecan-1 ⁺ B7-1 ⁺ Cells			MFI of Syndecan-1 ⁺ CD54 ⁺ Cells			MFI of Syndecan-1 ⁺ Fas ⁺ Cells		
	Control	IFN + TNF	CD40	Control	IFN + TNF	CD40	Control	IFN + TNF	CD40
2	<0.5	<0.5	3.9	131	211	513	3	9	15
3	1.5	1.1	3.6	125	132	116	20	18	21
5	<0.5	<0.5	1	121	289	374	15	38	25
6	<0.5	<0.5	<0.5	172	232	552	7	29	12
9	1	0.9	1.2	151	168	160	7	15	21
10	<0.5	<0.5	<0.5	63	190	225	49	177	185
11	<0.5	<0.5	12.3	125	459	1940	5	19	33
Mean ± SD	<0.5	<0.5	3.1 ± 4.3	127 ± 34	240 ± 108	554 ± 633	15 ± 16	44 ± 60	45 ± 62

^a BMNC were cultured for 4 days at 1×10^6 /ml in the presence of IL-6 alone, with nontransfected L cells, with IFN- γ + TNF- α , or with CD40L-transfected cells and double-stained as described in Table II. There was no difference between IL-6 alone and nontransfected L cells, and the control column contained the values obtained with IL-6 alone. The molecules up-regulated by the different stimulation systems are shown in bold.

up-regulated by CD40 stimulation on syndecan-1⁻ nontumoral cells, from $3.7 \pm 2.9\%$ in the presence of nontransfected cells to $21.3 \pm 9.2\%$ with CD40L-transfectant (representative results obtained for two patients are shown in Fig. 3). However, this was only marginally the case for syndecan-1⁺ myeloma cells in the same samples (percentage of positive cells always <4%) for six of seven patients (Fig. 3 and Table IV). For patient 11, a low induction of B7-1 and HLA-DR was detected in 12% of myeloma cells (Table IV and data not shown). As for HMCL, the density of CD54 was up-regulated for five of seven patients, and the Fas density for six of seven patients, by IFN- γ plus TNF- α . However, in contrast to cell lines, their expression was also increased by CD40 ligation (Table IV). B7-2, CD11a, CD18, and CD58 were not up-regulated by either method of stimulation. Of note, the association of IFN- γ plus TNF- α induced some tumor cell death as previously described (45, 46) so that the percentage of syndecan-1⁺ cells was 10–20% lower in the presence of these cytokines than with IL-6 alone (data not shown). To further investigate the effect of CD40 stimulation on the induction of B7-1 on the patients' tumor cells, the same experiment was conducted with the purified plasma cells of patients 2 and 5. B7-1 and B7-2 were not induced either by IFN- γ plus TNF- α or by CD40 stimulation on purified myeloma cells. B7-1 results with tumor cells from patient 5 are shown in Fig. 4A. In addition, these CD40L-stimulated purified myeloma cells were poor stimulators of purified allogeneic T cells (results not shown). As shown in Fig. 4B, B7-1 was up-regulated on DC obtained from patient 5, as described for healthy subjects' DC, indicating that the CD40 pathway was selectively nonfunctional for the induction of B7 molecules in myeloma cells from a majority of patients.

B7-1-transduced HMCL were potent allogeneic CD8⁺ T cell stimulators

After transduction of XG-1, XG-6, and XG-14 HMCL with the B7-1 retrovirus, selection, and sorting, we obtained B7-1 HMCL that expressed high levels of the B7-1 molecule without any modification in the pattern of expression of the other costimulatory or adhesion molecules (CD54, CD58, CD11a, CD18, B7-2, CD40; data not shown). Neo HMCL transduced with an empty control retrovirus did not express B7-1 and had a similar phenotype as that described in Table I for parental cells (data not shown). B7-1 HMCL were four to five times more potent for stimulating allogeneic CD3⁺ T cells than parental HMCL with a mean SI of 41 for B7-1 XG-1 (range, 10–99; $n = 7$); 26 for B7-1 XG-6 (range, 10–31; $n = 5$), and 13 for B7-1 XG-14 (range, 9–20; $n = 4$) (data not shown). As detailed in Fig. 2, B7-1 XG-1 was unable to acti-

vate CD4⁺ T cells, in agreement with the absence of HLA class II expression, but it induced a high level of proliferation of purified CD8⁺ T cells, a proliferation inhibited by an anti-HLA-ABC mAb. The level of CD8⁺ T cell proliferation induced by B7-1 XG-1 was not different from that induced by PHA. In addition, B7-1 XG-1 induced purified CD8⁺ T cells but not purified CD4⁺ T cells to express HLA-DR and CD25 (data not shown) and to produce IL-2 (Table V). In contrast, parental XG-1 and Neo XG-1 induced only a weak expression of CD25 and HLA-DR on CD8⁺ T cells with no measurable IL-2 production (data not shown and Table V). Similar data were obtained with XG-6, Neo XG-6, and B7-1 XG-6 as well as XG-14, Neo XG-14, and B7-1 XG-14 HMCL (data not shown).

B7-1 HMCL activated autologous anti-tumoral cytotoxic T cells

For one patient with a PCL at diagnosis, PBMC containing 30% CD3⁺ T cells (15% CD4⁺ and 15% CD8⁺ T cells) and 40% syndecan-1⁺ myeloma cells were harvested and cultured with IL-6. An IL-6-dependent autologous HMCL (XG-14) was obtained and transduced with the B7-1 retrovirus (B7-1 XG-14) and with the empty retrovirus (Neo XG-14). XG-14 cells expressed the same adhesion and costimulatory molecules as the primary patient myeloma cells (Table I and data not shown). As mentioned in Table I, neither IFN- γ plus TNF- α nor CD40 stimulation induced B7-1 on XG-14 HMCL but transduction with B7-1 retrovirus induced high B7-1 expression and strong alloreactivity. We then tested to see whether the transduction of the B7-1 molecule made it possible to generate specific anti-tumoral T cells. Twenty million nonpurified PBMC (containing 6 million CD3⁺ T cells) were cocultured with 10 million irradiated XG-14, Neo XG-14, and B7-1 XG-14. Cells were restimulated on days 6, 12, 18, and 24 with XG-14, Neo XG-14, or B7-1 XG-14. IL-2 and IL-12 were added only after the second stimulation on days 7, 13, 19, and 25. Neither parental XG-14 nor Neo XG-14 supported the growth of autologous T lymphocytes because all the cells died before day 12 (Fig. 5). On the contrary, B7-1 XG-14 allowed a great amplification of CD3⁺ CD8⁺ TCR $\alpha\beta^+$ CD16⁻ cells (Fig. 6). After 28 days of culture, CD8⁺ T cells had expanded by 15-fold. When these cells were used in a standard ⁵¹Cr release assay, they efficiently killed autologous HMCL (XG-14) but not the NK-sensitive K562 cell line nor autologous PHA blasts. The allogeneic XG-2 cell line was only marginally killed, and the lysis of XG-14 was

A. PURIFIED PLASMA CELLS FROM PATIENT 5

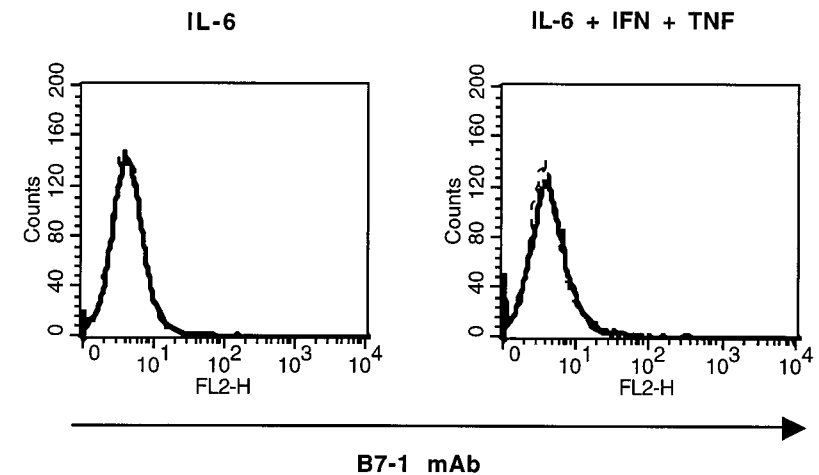
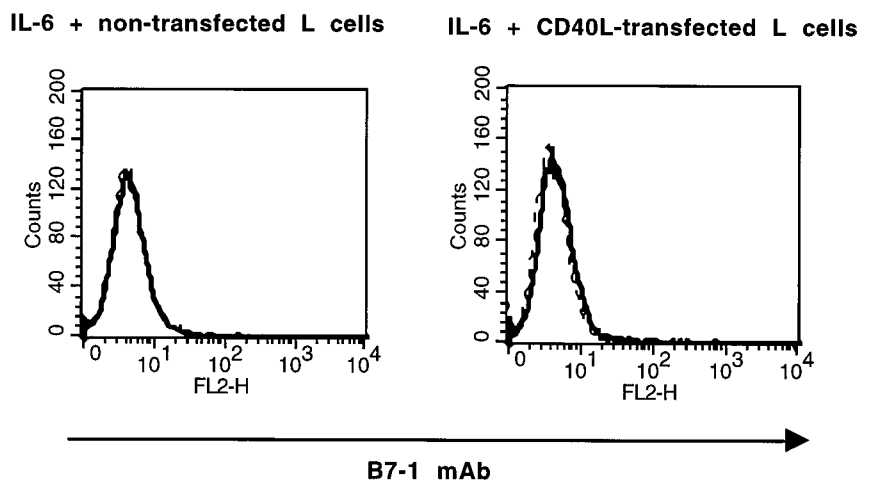
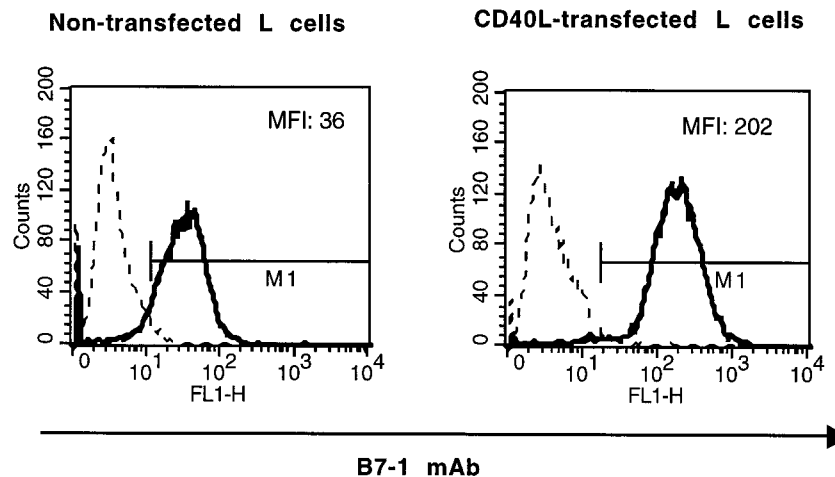


FIGURE 4. Neither IFN- γ plus TNF- α nor CD40 stimulation induced B7-1 on purified primary myeloma cells, unlike autologous DC. *A*, Syndecan-1⁺ purified myeloma cells (patient 5) were cultured at 10⁶/ml in medium alone or in the presence of IFN- γ plus TNF- α , nontransfected L cells, or CD40L-transfected L cells. *B*, Autologous DC (patient 5) obtained after 7 days of culture of adherent apheresis cells in X-VIVO 15 medium supplemented with 2% human albumin, GM-CSF, and IL-4, were cultured at 4 \times 10⁵/ml with nontransfected or CD40L-transfected L cells. Myeloma cells and DC were harvested after 3–4 days of stimulation and were stained with anti-B7-1 mAbs (bold lines) or isotype-matched control murine mAbs (dotted lines). The same data were obtained with patient 2's cells.



B. DENDRITIC CELLS FROM PATIENT 5



blocked by anti-HLA-ABC mAb (Fig. 7), indicating that we have obtained HLA-restricted anti-tumoral cytotoxic T cells for this patient suffering from a very aggressive disease. After 35 days of culture, CD8⁺ T cells could not be further expanded and they progressively died within 15 days. Of note, in our culture condi-

tions, i.e., nonpurified T cells with a great tumoral (40% syndecan-1⁺) and NK (15% CD16⁺) cell contamination, the addition of cytokines at the beginning of the culture or the use of IL-7 led to a proliferation of CD16⁺ NK cells and TCR $\gamma\delta$ ⁺ T cells (data not shown).

Table V. *Allogeneic CD8⁺ T lymphocytes proliferate and produce IL-2 when cocultured with B7-1 XG-1^a*

HMCL		CD3 ⁺ T Cells		CD4 ⁺ T Cells		CD8 ⁺ T Cells	
		No. of cells	IL-2 (pg/ml)	No. of cells	IL-2 (pg/ml)	No. of cells	IL-2 (pg/ml)
XG-1	Day 2	3 × 10 ⁶	<4	2.3 × 10 ⁶	<4	4.2 × 10 ⁶	<4
	Day 5	3.3 × 10 ⁶	<4	1.9 × 10 ⁶	<4	3 × 10 ⁶	<4
Neo XG-1	Day 2	2.6 × 10 ⁶	<4	2.8 × 10 ⁶	<4	4.4 × 10 ⁶	<4
	Day 5	3.1 × 10 ⁶	<4	2.3 × 10 ⁶	<4	3.7 × 10 ⁶	<4
B7-1 XG-1	Day 2	4.2 × 10 ⁶	310	2.6 × 10 ⁶	<4	4.8 × 10 ⁶	340
	Day 5	7.2 × 10 ⁶	160	2.6 × 10 ⁶	<4	11 × 10 ⁶	190

^a CD3⁺, CD4⁺, and CD8⁺ T cells (4.5 × 10⁶ in 3 ml) were cultured with mitomycin-treated XG-1, Neo XG-1, or B7-1 XG-1 (1.5 × 10⁶ in 3 ml). On days 2 and 5, viable T cells were counted and IL-2 was assayed in the culture supernatants by ELISA.

Discussion

The aim of this study was to determine whether myeloma cells could function as APC and whether it was possible to improve their immunogenicity to generate autologous anti-tumoral T cells.

We first analyzed 6 HMCL and 11 bone-marrow tumoral samples for their expression of adhesion and costimulatory molecules. As previously described (25–32), myeloma cells expressed CD28, HLA class I, CD54, CD58, and CD40, and some of them expressed low levels of CD11a and CD18. In addition, primary plasma cells expressed few HLA-DR molecules. With regards to B7 molecules, we found a variable expression of B7-2 (38.6 ± 37.1%) but no detectable B7-1. However, Yi et al. recently reported that 3.5% of patient myeloma cells expressed B7-1 (31). This study was conducted using CD38 Ag as a tumor marker. CD38 is highly expressed on primary myeloma cells (47–49) but it is also expressed on a large proportion of normal BMMC (50). Thus, it cannot be excluded that a minority of the bone-marrow nontumoral cells might express a high level of CD38, especially after 3 days of culture with various activation signals, and therefore account for the very weak B7-1 positivity found by Yi et al. on myeloma cells (<5%). To study the phenotype of primary myeloma cells, we used an anti-syndecan-1 mAb. Indeed, we and others have shown that syndecan-1 is present only on viable myeloma cells, unlike nontumor cells (38–40). We have recently confirmed that all syndecan-1⁺ cells had a plasma-cell morphology using serial histological sections of decalcified bone of MM patients (51). However, as with the study conducted by Yi et al. mentioned above, we could not exclude that a minority of syndecan-1⁺ cells were not myeloma cells; consequently, results below 5% must be considered cautiously. The low-level expression of B7-2 and the lack of B7-1 may explain the poor ability of HMCL and purified primary myeloma cells to induce CD8⁺ allogeneic T cell activation, IL-2 production, and proliferation. Similar results were reported for pre-B acute lymphoblastic leukemia cells (21) and acute myeloid leukemia cells (20) and are in line with the demonstration that B7 density is critical to promote an effective T cell response, especially for CD8⁺ T lymphocytes (18, 19).

To up-regulate B7 expression on myeloma cells, we tested two strategies of activation: by IFN-γ plus TNF-α and by CD40 ligand. Indeed, IFN-γ and TNF-α increase B7-1 expression on normal cells as well as on acute myeloid leukemia cells (20). In addition, Yi et al. reported that B7-1 was induced on 14.3% of patient CD38⁺⁺ bone-marrow cells by IL-6 and on 13.3% by IFN-γ (31). In the present study's culture conditions, i.e., in the presence of IL-6, no B7-1 expression was induced by IFN-γ plus TNF-α stimulation on HMCL, except for the RPMI 8226 cell line on which HLA-DR was induced as well. This result suggests that B7-1 is induced by cytokines in only rare cases. We found that IFN-γ induced apoptosis of primary myeloma cells as previously reported (45, 46), despite the presence of IL-6 and TNF-α, both survival factors for myeloma cells (2, 52). In addition, no B7-1 induction nor B7-2 up-regulation was observed after culture with IFN-γ plus TNF-α on the remaining syndecan-1⁺ viable cells. Thus, the use of IFN-γ plus TNF-α is not an effective strategy for inducing high levels of B7 molecules on the majority of myeloma cells. Of further interest, Fas and CD54 were induced by IFN-γ plus TNF-α on some HMCL and primary tumor samples, as recently reported (53).

We then used CD40 stimulation, known to increase B7-1 expression on several B cell neoplasia (21, 22) as well as on normal B cells (54) and DC (55). As with data reported for the XG-2 cell line (28), we showed that B7 molecules were not significantly induced by CD40 stimulation on myeloma cells from five of five cell lines and primary myeloma cells from six of seven patients. Investigation is needed into whether additional costimulatory signals (provided by cytokines or activation molecules) could overlap the defect of B7-1 induction by CD40 activation. For only one patient, 12% of myeloma cells expressed B7-1 and HLA-DR following CD40 stimulation. In contrast, B7-1 was induced by CD40 stimulation on syndecan-1⁻ nontumoral cells cultured with tumor cells. For two patients, CD40 stimulation also up-regulated B7-1 on DC, whereas it had no effect on purified myeloma cells. No adhesion nor costimulatory molecule could be induced on HMCL by CD40 stimulation. However, CD54 and Fas molecules were up-regulated by CD40 ligation on most primary plasma cells. It is not possible to conclude presently whether this induction of Fas

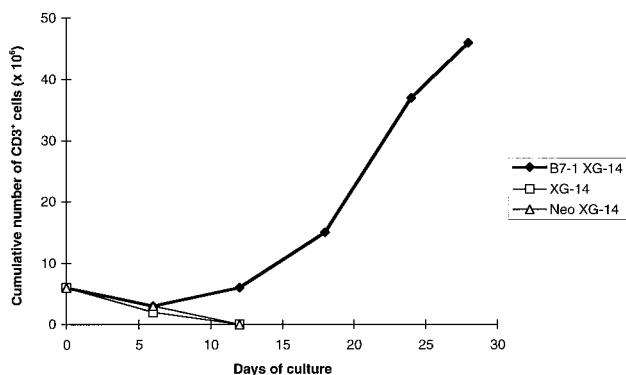


FIGURE 5. B7-1 gene transfer allows the expansion of autologous T cells. PBMC (1.5 × 10⁶/ml) were cultured at an E:T ratio of 2:1 with autologous mitomycin-treated HMCL (XG-14), Neo HMCL (Neo XG-14), or B7-1 HMCL (B7-1 XG-14) in RPMI 1640–5% AB serum. Every 6 days, cells were harvested, counted, and restimulated with the appropriate cell line. IL-2 and IL-12 were added after the second boost.

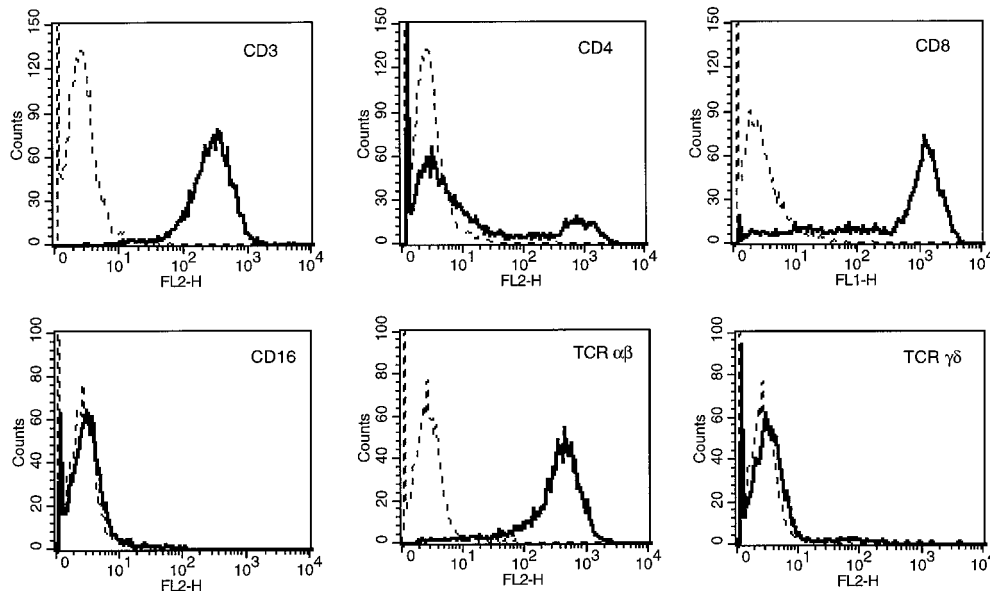


FIGURE 6. Phenotype of autologous T cell line. After 28 days of coculture with B7-1 XG-14 (five stimulations), T cells were stained with anti-CD3, -CD4, -CD8, -CD16, -TCR $\alpha\beta$, and -TCR $\gamma\delta$ mAbs (bold lines). Controls (dotted lines) were isotype-matched irrelevant Abs.

and CD54 is due to a direct activation of myeloma CD40 or to an indirect activation of CD40⁺ nonmyeloma cells and a secondary secretion of inflammatory mediators, especially TNF- α (56, 57).

Transferring the B7-1 gene is an alternative way to obtain myeloma cells expressing a high density of the B7-1 molecule. By using a B7-1 retrovirus, myeloma cell lines with a stable and high expression of B7-1 were obtained. B7-1 myeloma cells efficiently activated allogeneic CD8⁺ cells but failed to activate CD4⁺ T cells because they lacked expression of HLA class II molecules. T cell activation was evidenced by an early induction of activation markers (CD25 and HLA-DR), production of IL-2, and proliferation that was inhibited by anti-CD28 mAb. The production of IL-2 was transient, as previously reported for the activation of CD8⁺ T cells (18). In a recent study, Wendtner et al. showed that the transfer of B7 genes in two HMCL using adeno-associated viral vectors made it possible to activate allogeneic PBMC containing nonpurified preactivated NK and T cells (35). The authors state that this effect was due not only to a specific activation of TCR through allo-peptide presentation by MHC molecules on myeloma cells but also to the activation of allogeneic NK cells. Contrary to this study, we used highly purified resting T cells and showed that their activation was mediated through HLA class I/TCR interaction.

Of major interest in getting B7-1 myeloma cells is the generation of anti-tumoral autologous T cells. For one patient, we were able to get T cells at diagnosis and a myeloma cell line (XG-14) at the terminal stage of the disease. XG-14 could be then transduced with the B7-1 retrovirus (B7-1 XG-14). CD8⁺ TCR $\alpha\beta$ ⁺ cells could be expanded by coculture with B7-1 XG-14 in the presence of both IL-2 and IL-12 added after the second stimulation. No T cell amplification occurred with parental XG-14 or Neo XG-14 as stimulator cells. The resulting T cells were able to lyse XG-14 but neither allogeneic HMCL nor autologous PHA T cell blasts. However, these data need to be confirmed with other patient primary cells before concluding that primary myeloma cells transduced with B7-1 are efficient autologous APC. Such experiments are seriously limited by the difficulty in obtaining large numbers of primary myeloma cells. Indeed, myeloma cell lines can only be obtained from patients with terminal plasma cell leukemia. This difficulty is further compounded by the multiple chemotherapy

regimen and corticoid treatments that most likely delete anti-tumoral T cells. For patients with chronic disease, we have developed a method to purify large numbers of syndecan-1⁺ plasma cells from bone-marrow using B-B4 or MI15 anti-syndecan-1 mAbs (42). In addition, several studies have reported the efficiency of adenovirus-mediated gene transfer in primary myeloma cells (58, 59). If the early harvesting of large bone-marrow samples soon after diagnosis for purifying myeloma cells is deemed as ethical, the development of T cell activation by modified primary myeloma cells will be possible.

Another strategy would be to use DC, which are phenotypically and functionally identical both in MM patients and in healthy individuals (44, 60), and to pulse them with identified tumor Ags (9, 10, 61), cell lysates, or tumor RNA. Even if DC are undoubtedly the best APC, there is no guarantee that they will present the same tumor peptides through MHC class I as those presented by tumor cells. Thus, it is not yet possible to predict which of these two strategies will be the most promising for generating specific anti-tumor CTL in MM patients.

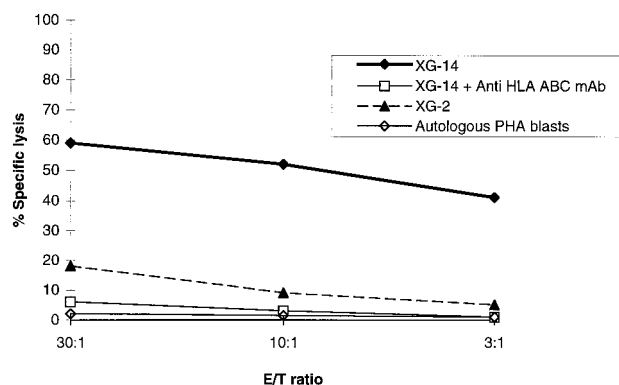


FIGURE 7. Cytotoxicity of CTL expanded by stimulation with autologous B7-1 HMCL. Cytotoxicity was measured at day 28 using a standard 4-h ⁵¹Cr release assay. Autologous XG-14 HMCL, allogeneic XG-2 HMCL, and autologous nontumoral PHA-blasts were used as target. When indicated, cells were preincubated with anti-HLA-ABC mAb at 10 μ g/ml.

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