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ORIGINAL ARTICLE

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Adenoviral-mediated transfer of human wild-type p53, GM-CSF and B7-1 genes results in growth suppression and autologous anti-tumor cytotoxicity of multiple myeloma cells in vitro

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Abstract Multiple myeloma (MM) remains incurable despite the use of high-dose chemotherapy and stem cell transplantation. However, immunotherapy is expected to offer long-term disease control, or even possibly a cure. We have previously demonstrated the suppressive effect of a recombinant adenovirus carrying human wild-type p53, granulocyte–macrophage colony-stimulating factor, and B7-1 genes (Ad-p53/GM-CSF/B7-1) on the growth of laryngeal cancer cells. In the present study, we evaluated the effects of an Ad-p53/GM-CSF/B7-1-modified myeloma cell vaccine strategy aimed to induce apoptosis and to augment the immunogenicity of MM cells. Both MM cell lines and purified primary myeloma cells were infected with Ad-p53/GM-CSF/B7-1. High expression levels of these three genes were confirmed separately by Western blot, enzyme-linked immunosorbent assay (ELISA), and flow cytometry. When wild-type p53, GM-CSF and B7-1 genes were introduced, the growth of MM cells was inhibited via enhanced apoptosis and the immunogenicity of tumor cells was augmented. The combinatorial effect of these three genes on inducing cytotoxic T lymphocytes (CTLs) was more evident than that of p53 individually or any combinations of two (p53 plus GM-CSF or p53 plus B7-1). Furthermore, significant proliferation of autologous peripheral blood lymphocytes (PBLs) and specific cytotoxicity against autologous primary MM cells were induced in vitro. These results suggest that myeloma cell vaccination co-transferred with p53, GM-CSF and B7-1 genes may be a promising immunotherapeutic approach against MM.

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Introduction

Multiple myeloma (MM) is a clonal B cell neoplasia that affects both the immune system and the skeletal system. Little progress in the treatment of this disease has been made despite decades of treatment with various chemotherapy regimens, which have resulted in 5-year survival rates of $< 10\%$ [1, 2]. Although high-dose chemotherapy and stem cell transplantation have improved the rate of complete remission, some myeloma cells escape from treatment and thus all MM patients experience relapse [3, 4]. Additionally, the age of patients, the difficulty of finding HLA-matched donors and the high rate of treatment-related mortality restrict the application of stem cell transplantation in MM therapy. Therefore, new therapeutic techniques are needed for combating this disease.

Immunotherapy may offer long-term disease control, or even a cure, after high-dose chemotherapy by the transfer of immunostimulatory genes into autologous tumor cells for subsequent vaccination. Previous studies have indicated that the ability of myeloma cells to escape from immune surveillance is mainly due to a downregulation of the expression of costimulatory molecules and an inhibition of the induction and maturation of dendritic cells (DCs), rather than an absence of tumor-specific antigens [5–8]. B7-1 (CD80) plays a critical costimulatory role in the activation of TCR-stimulated cytotoxic T lymphocytes (CTLs) by binding to CD28. The failure to induce an effective antitumor T-cell response by myeloma cells appears to be due to lack of the costimulatory molecule B7-1 [5]. GM-CSF has the ability to promote maturation of precursor cells into DCs.

Mature DCs generate tumor antigen epitopes for cross-presentation on HLA-I class to stimulate CD8+ cytotoxic T-cells, or for conventional presentation on HLA-II class to stimulate CD4+ helper T-cells [9, 10] . Recent studies have shown that DCs cultured with apoptotic bodies stimulated significantly greater T-cell proliferation than did MM lysate-plused DCs or MM cells alone [8]. Therefore, a new approach of co-transferring the immunomodulatory genes GM-CSF, B7-1 and apoptosis-inducing gene p53 into plasma cells for tumor vaccine might invoke an autologous immune response to tumor cells and be of potential therapeutic value in MM.

In this study, human wild-type p53, GM-CSF, and B7-1 genes were co-transferred into MM cell lines and primary myeloma cells via a recombinant adenovirus carrying wild-type p53, GM-CSF, and B7-1 genes. Transfer efficiency of the recombinant adenovirus, expression of exogenous genes, growth inhibition and immunogenicity enhancement of modified myeloma cells were assayed systematically. Our results suggest that the tumor cell vaccination modified by human wild-type p53, GM-CSF, and B7-1 genes might be further developed into a potential immunotherapeutic strategy for treating MM.

Materials and methods

Cell lines and cell culture

The 293 cell line (Ad5 E1-transformed human embryonal kidney cells) was purchased from the America Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM—Invitrogen, Carlsbad, CA, USA) plus 10% fetal bovine serum (FBS—Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified incubator with 5% CO_2.

Three human myeloma cell lines (Sko-007, U266 and RPMI8226), as well as the NK cell-sensitive human leukemia cell line, K562, were used in this study and were cultured in RPMI 1640 (SIGMA, St. Louis, MO, USA) supplemented with 10% FBS, 100 U/ml penicillin, and 100μg/ml streptomycin. Sko-007 and U266 were kindly provided by Prof. Bei-Fen Shen (Beijing Institute of Basic Medical Sciences). RPMI8226 and K562 were obtained from the Cell Center of Chinese Academy of Medical Sciences and the America Type Culture Collection, respectively.

Adenoviral vectors

Ad-GFP (recombinant adenovirus expressing green fluorescence protein), Ad-p53 (recombinant adenovirus carrying human wild-type p53 gene), and Ad-p53/GM-CSF (recombinant adenovirus carrying human wild-type p53 and GM-CSF genes) were kindly provided by the Gene Therapy Unit, Baxter Healthcare Corp., USA. Ad-p53/B7-1 (recombinant adenovirus carrying human wild-type p53 and B7-1 genes) and Ad-p53/GM-CSF/B7-1 (recombinant adenovirus carrying human wild-type p53, GM-CSF and B7-1 genes) were expanded in 293 cells. In Ad-p53/GM-CSF/B7-1, the inserted human wild-type B7-1 gene is driven by a Rous sarcoma virus (RSV) promoter, while p53 and GM-CSF genes, linked by an internal ribosome entry site (IRES), are driven by a cytomegalovirus (CMV) promoter. The structure of Ad-p53/B7-1 is the same as that of Ad-p53/GM-CSF/B7-1, but without the GM-CSF gene and IRES. These five kinds of recombinant adenoviruses with high titer and purity were obtained by large-scale amplification in 293 cells and ultra-centrifugating in CsCl step gradient solutions. The infection titers of Ad-GFP, Ad-p53, Ad-p53/GM-CSF, Ad-p53/B7-1, and Ad-p53/GM-CSF/B7-1 used in this study were 1×10^{11} pfu/ml, 2×10^{10} pfu/ml, 4.4×10^{10} pfu/ml, 2.6×10^{10} pfu/ml, and 5×10^{10} pfu/ml, respectively.

Primary MM cells purified by CD138 microbeads and PBLs

Human primary MM cells were separated from bone marrow aspirates harvested from MM patients at General Hospital of Chinese PLA (Beijing, P.R.China). $CD138^+$ MM cells were isolated by magnetically activated cell separation (MACS) as described previously [12] . In brief, the mononuclear fraction was obtained following Ficoll–Paque density gradient centrifugation (Amersham, Sweden), labeled with MACS CD138 microbeads (clone B-B4, Miltenyi, Germany), and separated by MACS MS+ separation columns in the magnetic field of MultiMACS separator. Cells were then labeled by a phycoerythrin (PE)-conjugated monoclonal antibody against CD138 (clone B-B4, Miltenyi) before and after MACS separation to determine their purity. Primary MM cells were cultured in RPMI 1640 supplemented with 10% FBS, 3 ng/ml IL-6, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Peripheral blood lymphocytes (PBLs) of two patients, applied to mixed lymphocytes and tumor cell reaction (MLTR) and cytotoxicity assay, were isolated by Ficoll–Paque separation as described previously [13], and cultured in AIM-V (Invitrogen) or RPMI 1640 medium containing 15% FBS, 5% human AB sera, 50 U/ml IL-2, 100 U/ml penicillin, and 100 μg/ml streptomycin. PBLs of $HLA\text{-}A2^+$ normal donors were isolated and cultured according to the same protocol.

Transfer efficiency of the recombinant adenovirus in MM cell lines and primary MM cells

Sko-007, U266 or RPMI8226 cells were seeded in 12-well tissue culture plates (Corning, Corning, NY, USA) at a density of 1×10^6 cells/1 ml RPMI 1640/well, and

were infected with Ad-GFP at a multiplicity of infection (MOI) of 0, 50, 100, 200 and 400 pfu/cell. After 3 h, 1 ml RPMI 1640 plus 10% FBS was added to each well. Following a 48-h incubation at 37°C, 5% CO_2, cells were collected in phosphate buffered saline (PBS). The infection efficiency was detected by flow cytometry (FACSCalibur—BD Biosciences, San Jose, CA, USA). According to the optimum MOI in MM cell lines, primary MM cells from five patients were infected with Ad-GFP at a MOI of 200 pfu/cell to determine the infection efficiency.

Expression of exogenous B7-1 gene in MM cell lines and primary MM cells detected by flow cytometry

Sko-007, U266, RPMI8226 or primary MM cells from patients were seeded in 12-well plates at a density of 1×10^6 cells/1 ml RPMI 1640/well, and were infected with Ad-p53/GM-CSF/B7-1 at a MOI of 200 pfu/cell. Blank culture medium was used for mock infection. After a 48-h incubation, cells were collected and were labeled with a fluorescein isothiocyanate (FITC)—conjugated monoclonal antibody against human B7-1 (CD80—BD Bioscience Parmingen, Chicago, IL, USA). Flow cytometry was used to determine the expression level of B7-1 gene in MM cells before and after Ad-p53/GM-CSF/B7-1 infection.

Expression of exogenous GM-CSF gene in MM cells detected by ELISA

Sko-007 cells seeded in 12-well plates (5×10^5) were infected with Ad-p53/GM-CSF/B7-1 at a MOI of 200 pfu/cell. The supernatant of MM cells was collected daily for 6 days and frozen at −80°C. The concentration of human GM-CSF in the supernatant of the Ad-p53/GM-CSF/B7-1-infected MM cell cultures was measured by ELISA kit (Jingmei Biotech, P. R. China) according to manufacturer's instructions.

Determination of exogenous P53 protein expression by Western blot

Sko-007 or U266 cells (5×10^6) were infected with Ad-p53/GM-CSF/B7-1 at a MOI of 200 pfu/cell. Blank culture medium was used for mock infection. After a 48-h incubation, cells were collected and washed twice with cold PBS by centrifugation at 500×*g* for 5 min at 4°C. The pellet was resuspended in lysis buffer supplemented with proteases inhibitor and incubated for 1 h at 4°C. The lysate was collected by centrifugation at 14,000×*g* for 20 min at 4°C, and the supernatant was stored at −20°C. For Western blot analysis, 40–50 μg proteins were resolved by 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a nitrocellulose membrane. Immunoblotting was done as described previously [14] . The blot was visualized by chemiluminescence and autoradiography using X-ray film. Rabbit anti-P53 polyclonal antibody (FL-393) was obtained from Santa Cruz Biotechnology Inc., CA, USA. A protein marker (New England Biolabs, Beverly, MA, USA) was run for each gel to identify the P53 band.

Effects of the transduced wild-type p53 gene on MM cell growth

Sko-007, U266 or RPMI8226 cells were seeded in 96-well tissue culture plates at a density of 1×10^4 cells/well and were infected with Ad-p53/GM-CSF/B7-1 or Ad-GFP at a MOI of 200 pfu/cell. Blank culture medium was used for mock infection. Cell proliferation of each treatment group was determined by MTT assay performed every day for 6–7 days after infection. Wells were done in triplicate to verify data.

Detection of apoptotic MM cells

Apoptosis was detected by dual staining with annexin V-FITC and propidium iodide (PI). Briefly, after being infected with Ad-p53/GM-CSF/B7-1 at a MOI of 200 pfu/cell for 24 h, 1×10^6 Sko-007, U266 or RPMI 8226 cells were stained with annexin V-FITC (Caltag, Burlingame, CA, USA) and PI as recommended by the manufacturer. Stained cells were analyzed by flow cytometry. Apoptotic cells were quantified with the Cellquest program. Ten thousand cells were analyzed. Mock-infected cells were detected under the same conditions as for FITC-stained cells without any additional manipulations.

Mixed lymphocyte and tumor cell reaction

Considering the relatively low growth rate of PBLs, proliferation of autologous lymphocytes stimulated by modified myeloma cells was detected by a Cell Proliferation Assay kit rather than MTT assay. This kit requires fewer steps than a MTT assay, thus decreasing experimental errors, which is especially important when dealing with small amounts of cells. Briefly, primary MM cells were infected with Ad-p53/GM-CSF/B7-1 or Ad-GFP at a MOI of 200 pfu/cell. Blank culture medium was used for mock infection. After inactivation with 30 Gy of ^{60}Co gamma-ray irradiation, 1×10^4 MM cells were co-cultured with autologous PBLs at a stimulator: responder cell ratio of 1:5 in RPMI 1640 plus 15% FBS, 5% AB sera, and 50 U/ml IL-2 in 96-well plates for 5 days. PBL proliferation was determined using CellT-

iter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Cytotoxicity assay

Cytotoxicity of CTLs against MM cell lines, K562 cells or autologous MM cells was assessed by a lactate dehydrogenase (LDH) release assay. Briefly, Sko-007 (HLA-A2$^+$) or primary MM cells were infected with Ad-GFP, Ad-p53, Ad-p53/GM-CSF, Ad-p53/B7-1 or Ad-p53/GM-CSF/B7-1 at a MOI of 200 pfu/cell. Blank culture medium was used for mock infection. After 48 h of incubation, cells from each group were inactivated with ^{60}Co gamma-ray irradiation. HLA-A2$^+$ or autologous PBLs were isolated with Ficoll-Paque gradient, and co-cultured with irradiated MM cells of each group at a ratio of 5:1 in 60-mm dishes for 7 days. After two to three rounds of stimulation, the PBLs were recovered and used as effector cells. Target cells (4×10^3 uninfected MM cells or K562 cells) were co-cultured with effector cells, in triplicate, at various ratios in 96-well U-bottom tissue culture plates for 4 h at 37° C. Fifty microlitres of supernatant was then harvested from each well. Cytotoxicity was assessed by a LDH assay using CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega) according to the manufacturer's protocol. Percent specific lysis was calculated as: (experimental—effector spontaneous—target spontaneous)/(target maximum—target spontaneous)×100. For target blocking assays, target cells were added after incubation with a blocking antibody against HLA-class I (clone W6/32, eBioscience, San Diego, CA, USA), HLA-class II (clone TDR31.1, Ancell, Bayport, MN, USA), or isotype control mouse IgG (SouthernBiotech, Birmingham, AL, USA) at a concentration of 10 μg/ml for 30 min at room temperature.

Statistical analysis

SAS software (SAS, USA) was employed to determine the statistical significance of differences in growth of MM cell lines, proliferation of autologous PBLs and cytotoxicity of PBLs using ANOVA analysis and DUNNETT t test. The minimal level of significance was $P<0.05$. Values are given as mean ± standard deviation (SD).

Results

Enrichment of primary MM cells

CD138$^+$ tumor cells were isolated from mononuclear cells of MM patients with MACS beads. The mean purity and yield of myeloma cells of the four patients were 75.60 ± 13.14% and 68.01 ± 28.24%, respectively. Figure 1 shows the representative percentage of CD138$^+$ tumor cells from one patient, before and after, purification.

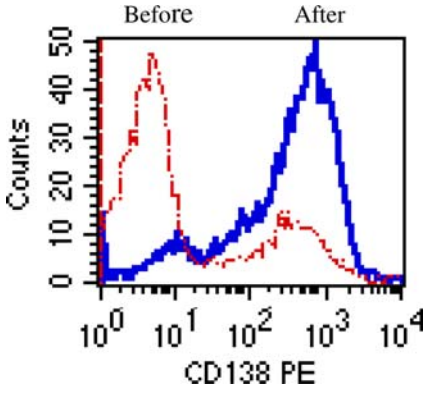


Fig. 1 Purification of CD138$^+$ myeloma cells from a MM patient. The myeloma cells in bone marrow mononuclear fraction were labeled with CD138-microbeads and positively separated by MACS MS+ separation columns. Isolated cells (1×10^6) were labeled with CD138-PE before and after separation. The percentages of CD138$^+$ cells before and after purification were 21.64 and 88.58%, respectively. Results are representative of results gathered from 4 MM patients. *Dotted line* before purification, *continuous line* after purification

Adenovirus-mediated gene transfer efficiency by MM cell lines and primary MM cells

To optimize the adenovirus infection efficiency in MM cells, we infected three MM cell lines with Ad-GFP at different MOI ranging between 0 pfu/cell and 400 pfu/cell. Preliminary titration revealed a high transfer efficiency of the recombinant adenovirus in all three cell lines and an optimal expression at 48 h postinfection. The relationship between the infection efficiency and MOI is shown in Fig. 2a. When cells were infected with Ad-GFP at a MOI of 200 pfu/cell, nearly all of Sko-007, U266 and RPMI8226 cells were GFP-positive, without obvious adenoviral toxicity. Infection efficiencies of the recombinant adenovirus (200 pfu/cell) in primary MM cells from five patients were assayed 48 h after infection and the mean efficiency was 32.60 ± 16.75%. Figure 2b shows the adenovirus-mediated GFP expression by CD138$^+$ cells of one patient before and after infection.

Adenovirus-mediated B7-1 gene expression in MM cell lines and primary MM cells

Ad-p53/GM-CSF/B7-1-infected Sko-007, U266, RPMI8226 cells and primary MM cells from patients and their uninfected counterparts were all stained with FITC labeled anti-B7-1 mAb. Fluorescence analysis was performed to determine the expression levels of the B7-1 gene in MM cells before and after infection. The results

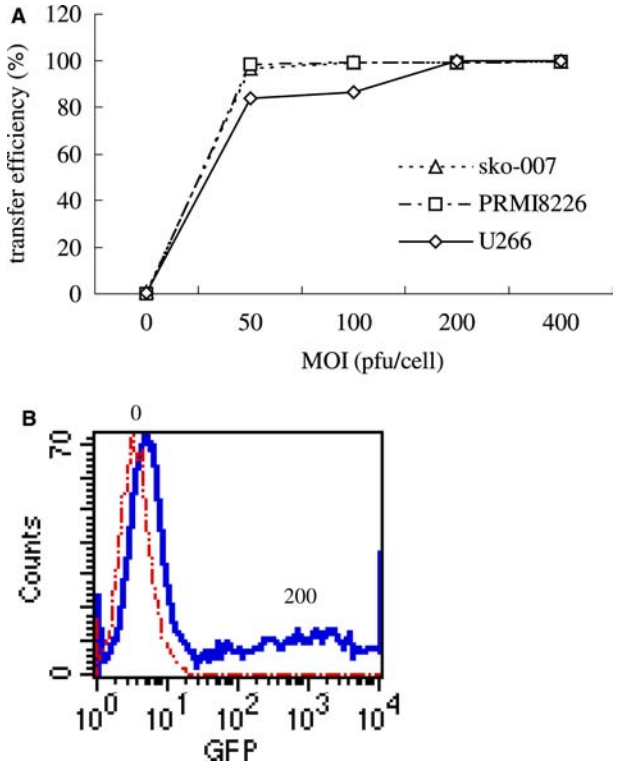


Fig. 2 Ad-GFP infection of MM cell lines and primary MM cells. **a** Sko-007, U266, and RPMI8226 cells were infected with Ad-GFP at a MOI of 0–400 pfu/cell. A dose-dependent infection between 50 pfu/cell and 400 pfu/cell for all cell lines tested was observed. The optimal MOI was 200 pfu/cell, and high transfer efficiency (>99%) was observed without obvious adenoviral toxicity. Results are representative of three independent experiments. **b** Primary MM cells from one patient were infected with Ad-GFP at a MOI of 200 pfu/cell. The transfer efficiency was 33.03%. Results are representative of results gathered from five MM patients. *Dotted line* MOI 0 pfu/cell, *continuous line* MOI 200 pfu/cell

show that few or no B7-1 molecules were expressed in Sko-007, U266, RPMI8226 or primary cells before infection. However, after infection, higher expression levels of B7-1 were detected in all three cell lines (Fig. 3a, b, c), and primary MM cells were also induced to express B7-1 (Fig. 3d). Our results demonstrate that Ad-p53/GM-CSF/B7-1 is able to mediate B7-1 gene expression in myeloma cells.

GM-CSF secretion in adenovirus-infected MM cells detected by ELISA

The concentration of human GM-CSF in the supernatant of the Ad-p53/GM-CSF/B7-1- or mock- infected Sko-007 cell cultures was measured by ELISA. As shown in Fig. 3e, no GM-CSF was secreted by uninfected Sko-007 cells. After infection with Ad-p53/GM-CSF/B7-1 for 24 h, GM-CSF was detected in the cell culture (4.71 ng/ml/10^6 cells) and reached its peak at 48 h (16.88 ng/ml/10^6 cells) postinfection.

High level expression of P53 protein induced by recombinant adenovirus infection

As shown in Fig. 3f, Sko-007 and U266 cells infected with Ad-p53/GM-CSF/B7-1 (lanes 2 and 3) had much higher levels of P53 than those without infection (lanes 1 and 4) as determined by Western immunoblotting.

Suppressive effect of Ad-p53/GM-CSF/B7-1 on growth of myeloma cells

In order to observe the effect of exogenous p53 gene on the proliferation of myeloma cells, three MM cell lines Sko-007, U266 and RPMI8226 were infected with Ad-p53/GM-CSF/B7-1 or Ad-GFP at 200 pfu/cell. Blank culture medium was used for mock infection. The growth of the virus- or mock-infected cells in triplicate was determined by MTT assay every day for six successive days. As shown in Fig. 4, growth of the Ad-p53/GM-CSF/B7-1-infected cells was significantly inhibited compared with mock-infected cells ($P < 0.05$, DUNNET t test, by day 3, 4, 5 and day 6), whereas, growth of Ad-GFP-infected cells was similar to that of mock-infected cells ($P > 0.05$). This suggests that the transferred exogenous genes inhibit the growth of myeloma cells.

Induction of apoptosis of myeloma cells by Ad-p53/GM-CSF/B7-1 infection

Dual staining with Annexin V-FITC and PI was employed to determine whether apoptosis was induced in the Ad-p53/GM-CSF/B7-1-infected MM cells. Three separate experiments showed relatively low percentages of apoptosis of uninfected myeloma cells. However, in the Ad-p53/GM-CSF/B7-1 treated cells there was an increased occurrence of apoptosis (Annexin V-FITC-positive and PI-negative cell fraction) and even death (Annexin V^+ / PI^+ fraction) of all three cell lines in 24 h. Figure 5 shows the result of one of three independent experiments. These results indicate that the observed growth inhibition was largely due to increased apoptosis.

Tumor-specific CTLs induced by Ad-p53/GM-CSF/B7-1-infected myeloma cells

We examined the ability of the virus-infected or uninfected MM cells to induce tumor-specific CTLs. As shown in Fig. 6a, after two weekly stimulations, HLA-$A2^+$ PBLs primed with Ad-p53-, Ad-p53/GM-CSF-, Ad-p53/B7-1- or Ad-p53/GM-CSF/B7-1-infected HLA-$A2^+$ Sko-007 cells showed significant more cytolytic activity than those primed with Ad-GFP- or mock-infected Sko-007 did ($P < 0.05$, DUNNET t test). Among them, Ad-p53/GM-CSF/B7-1-infected cells induced the highest level of cytolytic activity. These data suggest that

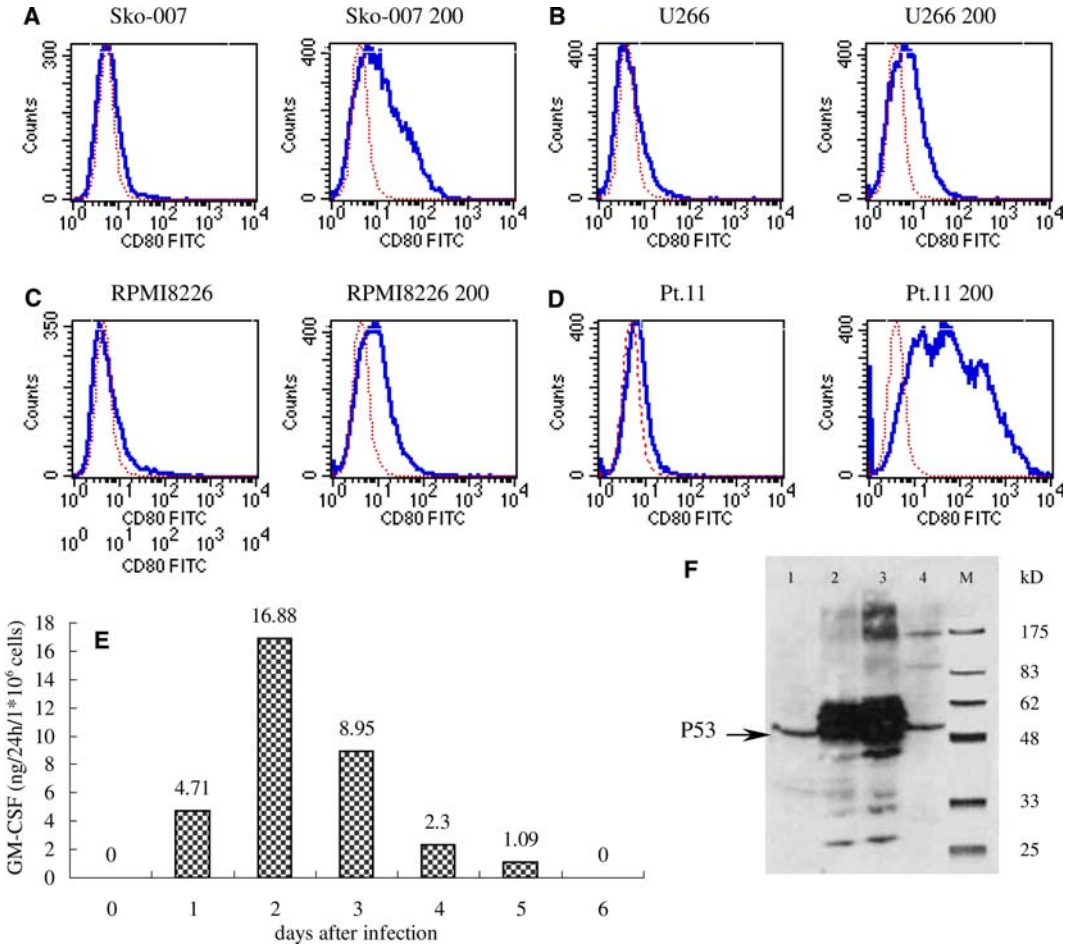


Fig. 3 Expression of B7-1, GM-CSF, and P53 molecules mediated by Ad-p53/GM-CSF/B7-1 in MM cells detected by flow cytometry, ELISA or Western blot. All results are representative of at least two independent experiments. 1×10^6 Sko-007 (**a**), U266 (**b**), RPMI8226 (**c**) and primary MM cells from a patient (**d**) were infected with Ad-p53/GM-CSF/B7-1 at a MOI of 0 or 200 pfu/cell and then labeled with CD80 (B7-1)-FITC or isotype control Ab. Fluorescence analysis showed that higher expression levels of B7-1 were detected in all three MM cell lines in infected versus uninfected cells (Sko-007: 1.76 and 33.7%; U266: 0.90 and 8.7%; RPMI8226: 4.02 and 11.9%). Primary MM cells without B7-1 were also induced to express B7-1 (0.41 and 71.48%, uninfected and infected respectively). Percentages are calculated relative to an isotype-matched control Ab. **e** Sko-007 cells (5×10^5) were infected with Ad-p53/GM-CSF/B7-1 at a MOI of 200 pfu/cell. ELISA analysis revealed that there was no GM-CSF secreted by Sko-007 cells. After infection with Ad-p53/GM-CSF/B7-1, GM-CSF was detected on the first day and reached its peak on the second day. By the sixth day, GM-CSF was no longer detectable. **f** Western blot analysis showed that Sko-007 and U266 cells infected with Ad-p53/GM-CSF/B7-1 at a MOI of 200 pfu/cell (lanes 2 and 3, respectively) had higher levels of P53 than those without infection (lanes 1 and 4, respectively). *Arrow* indicates P53 bands. *M* Protein marker

the delivery of human p53 gene into myeloma cells induces functional CTLs against the parental cells, and GM-CSF and B7-1 genes further strengthen the cytotoxicity. In order to characterize the reactivity of CTLs induced by repeated stimulations with Ad-p53/GM-CSF/B7-1-infected Sko-007 cells, we examined their cytotoxicity against cells other than those used for stimulation. As shown in Fig. 6b, CTLs effectively lysed Sko-007, but not unrelated U266 cells and NK cell-sensitive cell line K562. We then investigated the mechanism of recognition of target cells by incubating Sko-007 with neutralizing antibodies against HLA-class I or class II. Cytotoxicity of CTLs against target cells was significantly inhibited by the anti-HLA class I antibody ($P<0.05$), but not by the anti-HLA class II or control antibodies ($P>0.05$) (Fig. 6c). These results indicate that the cytotoxicity is specific for target cells and restricted to HLA-class I.

Proliferation and tumor-specific cytotoxicity of autologous PBLs induced by Ad-p53/GM-CSF/B7-1 infection

In order to determine whether the virus-infected or parental myeloma cells could induce autologous tumor-specific CTLs, we tested Ad-p53/GM-CSF/B7-1-, Ad-GFP- or mock-infected primary MM cells for their ability to stimulate autologous PBL proliferation by MLTR. As shown in Fig. 7a, the OD490 value of Ad-p53/GM-CSF/B7-1-, Ad-GFP- or control group measured by MTS was 0.45 ± 0.05, 0.35 ± 0.04, and 0.35 ± 0.02, respectively. Ad-p53/GM-CSF/B7-1-infected myeloma cells induced significantly more PBL

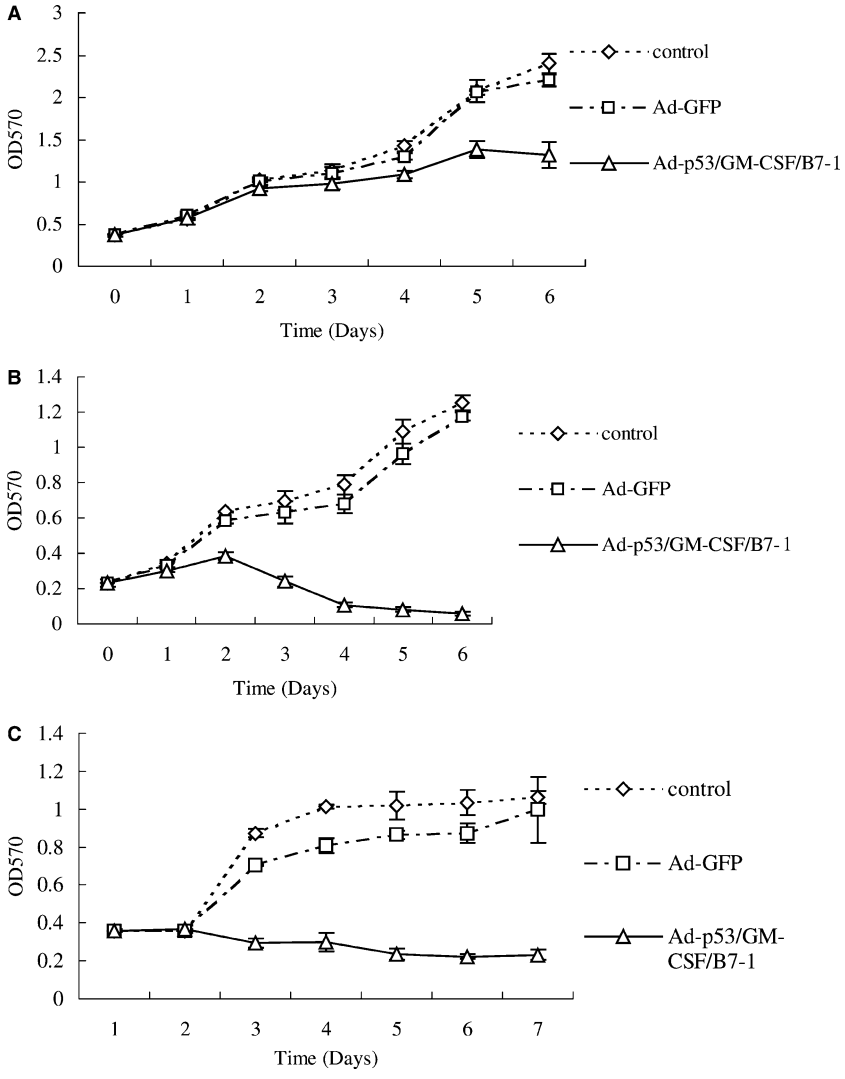


Fig. 4 Growth rate of Ad-p53/GM-CSF/B7-1-, Ad-GFP- or mock-infected MM cells. 1×10^4 Sko-007 (**a**), U266 (**b**) and RPMI8226 cells (**c**) were seeded in 96-well plates and were infected with Ad-p53/GM-CSF/B7-1 or Ad-GFP at a MOI of 200 pfu/cell. Blank culture medium was used for mock infection. Cell growth in each treatment group in triplicate wells was determined by MTT assay once daily for 6–7 successive days after infection. Growth of the Ad-p53/GM-CSF/B7-1-infected cells of these three MM cell lines was significantly inhibited compared with growth of the Ad-GFP- or mock-infected cells ($P<0.05$, day 6). Data shown are Means ± SD. of triplicate wells and representative of three experiments

proliferation than Ad-GFP or control group did ($P<0.05$, DUNNETT t test). Cytotoxicity assays indicated that autologous CTLs established by three weekly stimulations with Ad-p53/GM-CSF/B7-1-infected MM cells showed significantly more cytolytic activity than those primed with mock-infected MM cells (especially at an effector: target cell ratio of 40:1, $P<0.05$). Ad-GFP-infected cells induced a relatively high level of cytolytic activity, which might be related to tumor-nonspecific natural killers (NK)-activated by exogenous proteins (Fig. 7b).

Discussion

The development of immunotherapy using gene-modified autologous tumor vaccines will require myeloma cells to be purified from the bone marrow of MM patients. CD138 (syndecan-1) is present on myeloma cells and it is specifically induced when murine B cells differentiate into plasma cells [15]. Positive or negative selection techniques using anti-CD138 antibodies to isolate plasma cells have been widely described. In this study, we employed a positive procedure using CD138-microbeads [12]. The purity and yield of myeloma cells would be sufficient to generate tumor vaccines. It is worth noting that the expression of CD138 protein is heterogeneous in three MM cell lines of different origins. Both Sko-007 and U266 cells highly expressed CD138, whereas, only a quarter of RPMI8226 cells were CD138 positive, which is consistent with what Ilene et al. [16] found in their study.

Based on the fact that myeloma cells express multiple tumor antigens that can effectively stimulate antitumor T-cells, numerous experiments testing genetically modified myeloma cell vaccinations have been carried out in vitro and in mouse models [5, 8, 17–23]. Candidates for transfection are genes that encode cytokines (IL-2, IL-12, GM-CSF), costimulatory molecules (B7-1 or B7-2), tumor suppressor proteins (P53) and other molecules.

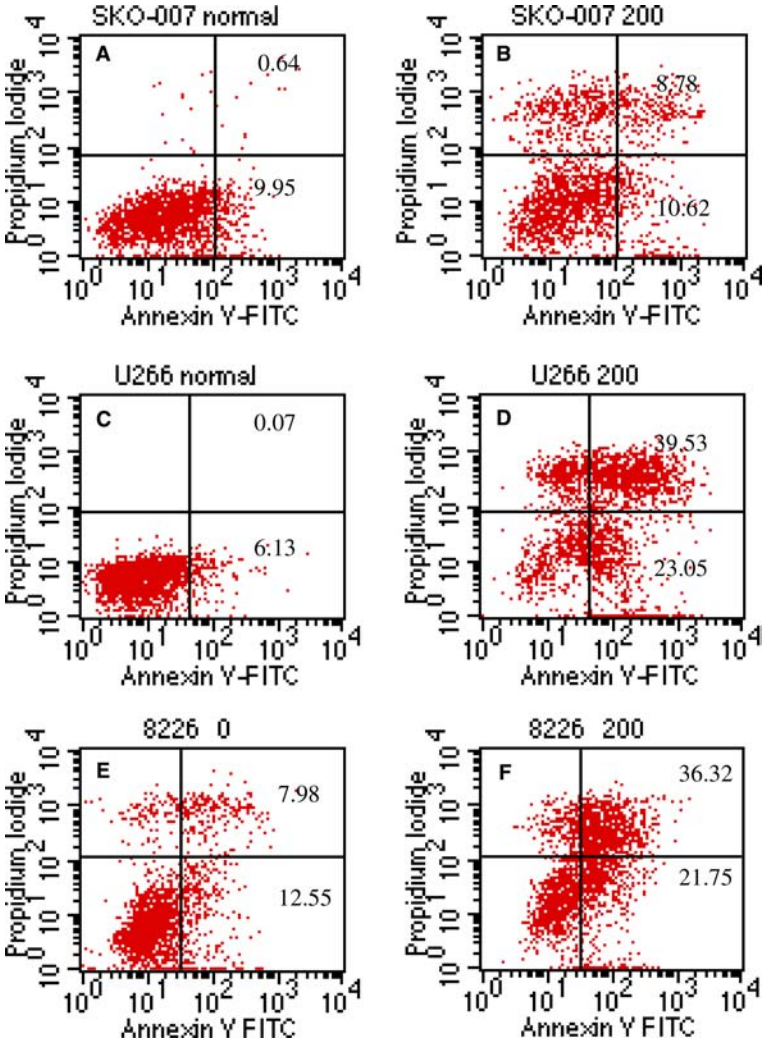


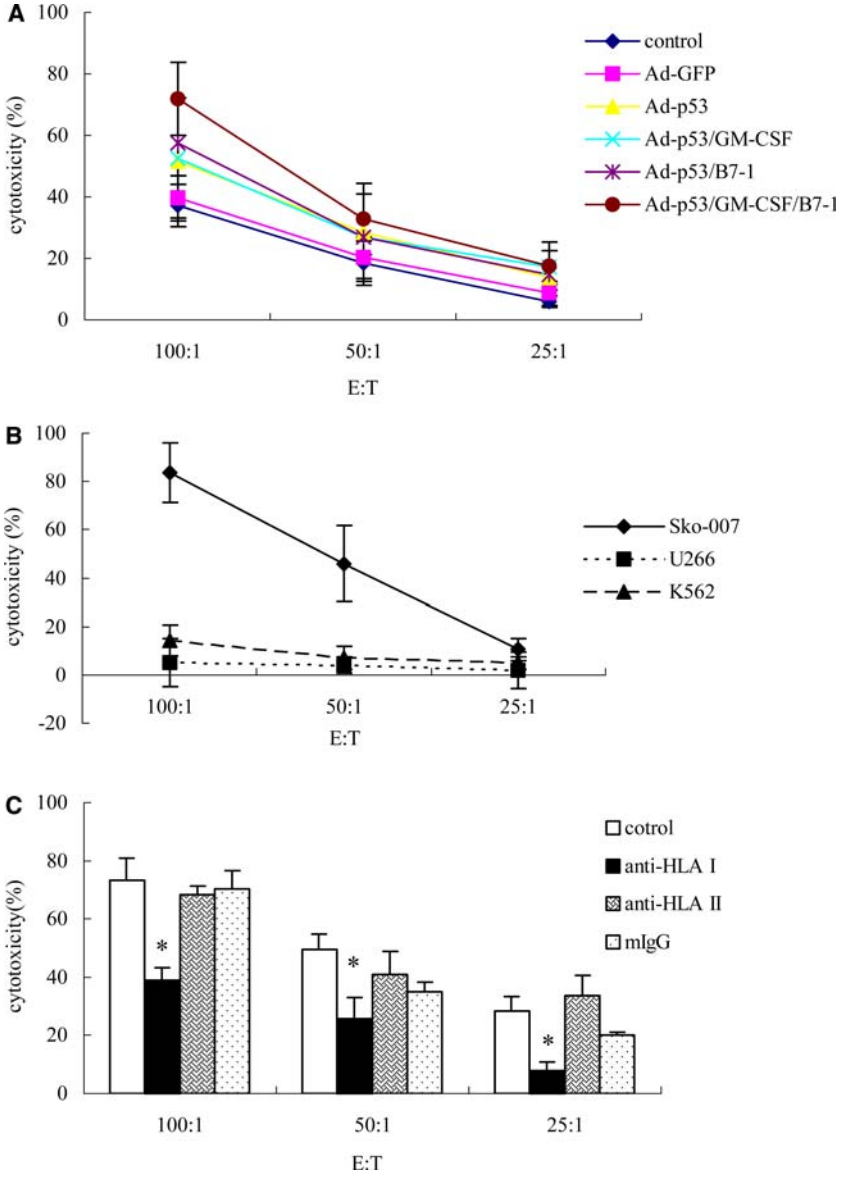
Fig. 5 Induction of apoptosis of myeloma cells by Ad-p53/GM-CSF/B7-1 infection. Dual staining with annexin V-FITC and PI was employed to detect apoptosis as described in the Methods section. Flow cytometry analysis showed that percentages of apoptosis of normal myeloma cells were relatively low, and treatment with Ad-p53/GM-CSF/B7-1 resulted in increased apoptosis and death in all three cell lines. Percentages of apoptosis and death of myeloma cells before and after viral infection are shown on the FACS profiles. Results are representative of three experiments

Tarte et al. [5] demonstrated that allogenetic $CD8^+$ T-cell proliferation was induced after retroviral transduction of MM cells with the B7-1 gene. In one patient with advanced disease, B7-1 gene transfer induced amplification of CTLs that could kill autologous myeloma cells in a HLA-class I-restricted manner. Turner et al. [18] observed that immunization of mice with a murine MM cell line MPC11 and fibroblasts transfected with IL-12 and GM-CSF genes led to tumor rejection in 60% of the mice. Dotti et al. [21, 22] tested the hypothesis that expression of CD40 ligand (CD154) in a myeloma cell vaccine might trigger tumor-specific immunity by recruitment of antigen-presentating cells (APCs). Mice were inoculated with three murine MM cell lines (MPC11, S107, and X-24) mixed with CL7.1 fibroblasts retrovirally transfected with mCD40L, resulting in resistance to the subsequent challenge with parental tumor cells. Liu et al. [23] found that adenovirus-mediated delivery of the p53 gene induced substantial apoptosis in myeloma cells. Hayashi et al. [8] further demonstrated that cultured DCs with apoptotic bodies stimulated significantly greater T-cell proliferation and cytotoxicity than MM lysate-plused DCs or MM cells alone did.

Fig. 6 Tumor-specific CTLs were induced by Ad-p53/GM-CSF/B7-1-infected myeloma cells. Cytotoxicity of CTLs against target cells was assessed by colorimetric LDH assay and results shown are Means ± SD of triplicate wells and representative of two experiments. **a** Ad-GFP-, Ad-p53-, Ad-p53/GM-CSF-, Ad-p53/B7-1-, Ad-p53/GM-CSF/B7-1 or mock-infected Sko-007 cells were inactivated (20 Gy ^{60}Co irradiation) and were cultured with PBLs isolated from HLA-$A2^+$ normal donors in AIM-V medium. After two rounds of stimulation, 4×10^3 Sko-007 (target cells) were cultured with PBLs in triplicate at indicated effector to target (E:T) ratios. The supernatants were harvested and cytotoxicity was assayed. CTLs stimulated with Ad-p53-, Ad-p53/GM-CSF-, Ad-p53/B7-1- or Ad-p53/GM-CSF/B7-1-infected Sko-007 cells showed significantly higher cytolytic activity than other stimuli ($P < 0.05$, DUNNETT t-test). Among them, Ad-p53/GM-CSF/B7-1-infected cells induced the highest cytolytic activity. **b** CTLs induced by stimulations with Ad-p53/GM-CSF/B7-1-infected Sko-007 cells showed significantly higher cytotoxicity ($P < 0.05$) against Sko-007 cells than against U266 cells or K562 cells, assessed with LDH assay at indicated E:T ratios for 4 h at 37°C. **c** HLA restriction of CTLs was examined using target blocking LDH assay. CTLs stimulated with Ad-p53/GM-CSF/B7-1 -infected Sko-007 cells were co-cultured with Sko-007 cells after incubation with blocking antibodies against HLA-class I, HLA-class II, control antibody, or without antibody. Cytotoxicity was significantly inhibited by the anti-HLA class I antibody (*$P < 0.05$)

Although the results of most preclinical investigations are encouraging, the clinical evaluation is less satisfactory [7, 24]. To date only one group has published the results of clinical application of IL-2 modified myeloma cell vaccination. Preliminary results indicate that the approach is feasible and side effects are mild. However, the clinical response is limited and additional observation time is required [25]. Since tumors often contain highly heterogeneous cells, a combination of immune-stimulating genes should be more efficient than any single gene. Our hypothesis was that co-transfer of B7-1, GM-CSF, and p53 genes would produce a synergistic augmentation of myeloma cell immunogenicity. This expectation is consistent with previous work by us and other groups in several solid tumors such as laryngeal cancer, hepatocellular carcinoma, and lung cancer [26–28] .

Effective infection of target cells is generally a prerequisite for a successful gene therapy strategy. The ability of adenoviruses to infect nondividing cells makes them a preferable vector to infect myeloma cells [21–23, 25]. Applying GFP as the reporter gene, several groups have investigated the adenovirus-mediated gene transfer efficiency in myeloma cell lines and primary myeloma cells. In the present study, human wild-type p53, GM-CSF, and B7-1 genes were co-transferred into MM cell lines and primary myeloma cells via the recombinant adenovirus Ad-p53/GM-CSF/B7-1 and high-level expressions of these three exogenous genes were confirmed. Our results demonstrated that Ad-p53/GM-CSF/B7-1 had growth-inhibiting and apoptosis-inducing effects, as well as immunogenicity-enhancing effects on myeloma cells. Consistent with previous research [8], human p53 gene delivery into myeloma cells induced functional CTLs against the parental cells. GM-CSF and B7-1 genes, though not fully characterized in our study, could further strengthen the cytotoxicity. Furthermore, significant proliferation of autologous PBLs



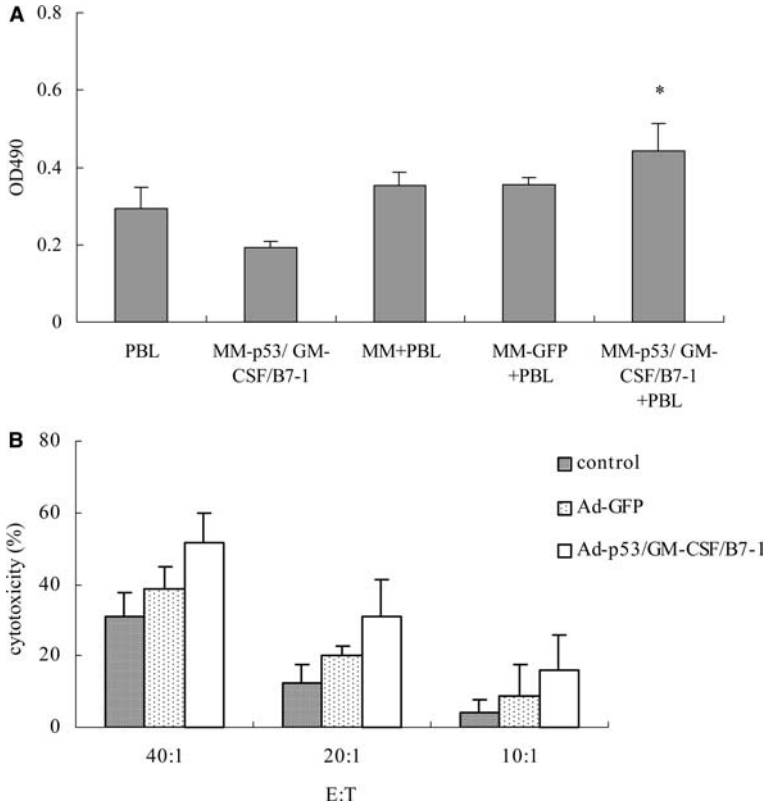


Fig. 7 Proliferation and tumor-specific cytotoxicity of autologous PBLs induced by Ad-p53/GM-CSF/B7-1 infection. **a** Primary MM cells were infected with Ad-p53/GM-CSF/B7-1 or Ad-GFP at a MOI of 200 pfu/cell. Blank culture medium was used for mock infection. After irradiation (30 Gy), the cells (1×10^4) were co-cultured with autologous PBLs at a ratio of 1:5 for 5 days. MTS assay revealed greater proliferation in the PBLs stimulated with Ad-p53/GM-CSF/B7-1-infected MM cells than those stimulated with other stimuli (*$P < 0.05$, DUNNETT t test). Data shown are Means ± SD of triplicate wells and representative of three experiments. **b** Cytotoxicity of CTLs against parental myeloma cells was assessed by LDH assay. Ad-p53/GM-CSF/B7-1-, Ad-GFP- or mock-infected primary MM cells were irradiated (30 Gy) and co-cultured with autologous PBLs at a ratio of 1:5 for 7 days. After three rounds of stimulation, the PBLs were recovered and used as effector cells. The effector cells and target cells (uninfected parental MM cells, 4×10^3 cells/well) were co-cultured at 40:1, 20:1 or 10:1. After incubation for 4 h, the supernatant was harvested and cytotoxicity assay was performed. CTLs primed with Ad-p53/GM-CSF/B7-1-infected MM cells showed significant more cytolytic activity than those primed with mock-infected MM cells (especially at an effector: target cell ratio of 40:1). Ad-GFP-infected cells induced relatively high cytolytic activity. Data shown are Mean ± SD of triplicate wells and representative of three experiments

and specific cytotoxicity against autologous primary MM cells were induced in vitro. The limited number of cells in our study, however, precluded exclusion of tumor-nonspecific NK-activation by GFP. While additional animal experiments and clinical trials are needed to assess the clinical potential of using a modified myeloma vaccination, this study represents a substantial step in characterizing the beneficial synergistic effect of co-transferring three genes to combat multiple myeloma.

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