

Functional B7.2 and B7-H2 Molecules on Myeloma Cells Are Associated with a Growth Advantage

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Abstract Purpose: B7 family molecules expressed on antigen-presenting cells stimulate or inhibit normal immune responses. The aim of this study was to investigate whether functional B7.2 and B7-H2 molecules are expressed on myeloma cells and, if so, whether they are associated with pathophysiology in myeloma.

Experimental Design: The expression of B7.2 and B7-H2 molecules on normal plasma and neoplastic (myeloma) plasma cells was analyzed. The cell proliferation and immunomodulatory function of myeloma cells related to B7.2 and B7-H2 expression were examined.

Results: Human myeloma cell lines commonly expressed B7.2 and B7-H2 molecules. B7.2 expression on plasma cells was more common in myeloma patients ($n = 35$) compared with that in patients with monoclonal gammopathy of unknown significance ($n = 12$) or hematologically normal individuals ($n = 10$). Plasma cells expressing B7-H2 were observed in myeloma patients alone, although rarely. Patients whose myeloma cells showed high B7.2 expression were more anemic and thrombocytopenic than other myeloma patients. The expression of these molecules was induced or augmented by cultivating myeloma cells with autologous stroma cells or tumor necrosis factor- α , a key cytokine in myeloma biology. Cell proliferation was more rapid in the B7.2⁺ and B7-H2⁺ populations compared with the B7.2⁻ and B7-H2⁻ populations, respectively, in the human myeloma cell lines examined. B7.2 and B7-H2 molecules on myeloma cells induced normal CD4⁺ T cells to proliferate and produce soluble factors, including interleukin-10 that stimulate myeloma cell proliferation.

Conclusions: Functional B7.2 and B7-H2 molecules detected on myeloma cells may be involved in the pathophysiology of myeloma.

Multiple myeloma (MM) is a virtually incurable hematologic malignancy characterized by monoclonal growth of plasma cells (myeloma cells). In addition to the well-known deficiency in B-cell immunity, T-cell dysfunction, such as reduced cytotoxic activity (1) and reduced responsiveness to interleukin 2 (IL-2; refs. 2, 3), has been reported, which may weaken antitumor immune responses in MM patients. The interaction

between myeloma cells and bone marrow (BM) stroma cells stimulates the production of a variety of cytokines that are involved in the pathophysiology of MM (4, 5). Those include IL-6, IL-10, and tumor necrosis factor- α (TNF- α), which stimulate myeloma cell growth (6–9). An interesting aspect is that both IL-10 and TNF- α have an immunomodulating function, including inhibition of CTLs (10–12).

The B7 family molecules play an important role in the immune response by costimulating or coinhibiting T cells via antigen–T-cell receptor interactions (13–16). Interactions between B7.1/B7.2 ligands on professional antigen-presenting cells and CD28/CTLA-4 receptors on T cells represent a classic pathway and control antigen-specific T-cell proliferation, energy, and survival. B7-H2 is another B7 family molecule induced by TNF- α (17). The binding of B7-H2 to the inducible costimulatory receptor (ICOS), a counterreceptor, induces T cells to proliferate and secrete both Th1 and Th2 cytokines, such as IFN- γ and IL-4 but not the potent Th1 cytokine IL-2 (18). Furthermore, the B7-H2-ICOS signal induces IL-10 production, which plays an important role in reducing immune responses (19, 20). B7 family molecules are expressed not only on professional antigen-presenting cells but also on some tumor cells and the latter may modulate antitumor immunity in hosts. For example, we detected the expression of B7.2 and B7-H2 molecules on blasts from patients with acute myeloid leukemia and showed that these molecules on acute myeloid

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Translational Relevance

Multiple myeloma (MM) is a virtually incurable hematologic malignancy. Therefore, research that could result in improved MM treatment and/or a breakthrough in our understanding of this disease is very important. This article shows that myeloma cells from a substantial number of MM patients express functional B7.2 or B7-H2 molecules. Furthermore, it provides evidence that these molecules on myeloma cells may be involved in the pathophysiology of the disease. It is anticipated that these data will be translated into a new therapeutic strategy for MM.

leukemia blasts inhibited anti-acute myeloid leukemia immunity *in vitro* and were associated with poor patient prognosis (21). In MM, data on B7 family molecules are lacking. To the best of our knowledge, only one study examined this topic. Pope et al. (22) observed that MM patients whose tumor cells

expressed B7.2 molecules had a poor prognosis. However, they did not examine whether the B7.2 molecules on myeloma cells were functional. Here, we investigated whether functional B7.2 and B7-H2 molecules are expressed on myeloma cells and, if so, whether these B7 molecules are associated with pathophysiology in MM.

Materials and Methods

Cell lines. Eleven human myeloma cell lines (HMCL), KMM-1, KMS-11, KMS-12BM, KMS-12PE, KMS-18, KMS-20, KMS-26, KMS-27, KMS-28BM, KMS-28PE, and KMS-34, were kindly provided by Dr. Otsuki (Kawasaki Medical School, Okayama, Japan). PCM6 cells were obtained from the Riken Cell Bank, and RPMI8226 and U266 cells were from the American Type Culture Collection. PCM6 cells were maintained in McCoy's 5A modified medium (Life Technologies) containing 20% FCS and 3 ng/mL of recombinant IL-6 (Kirin Brewery Co.). The other cells were maintained in complete medium, i.e., RPMI 1640 supplemented with 10% FCS and 2 mmol/L L-glutamine. In experiments examining the effects of cytokines on these cells, TNF- α

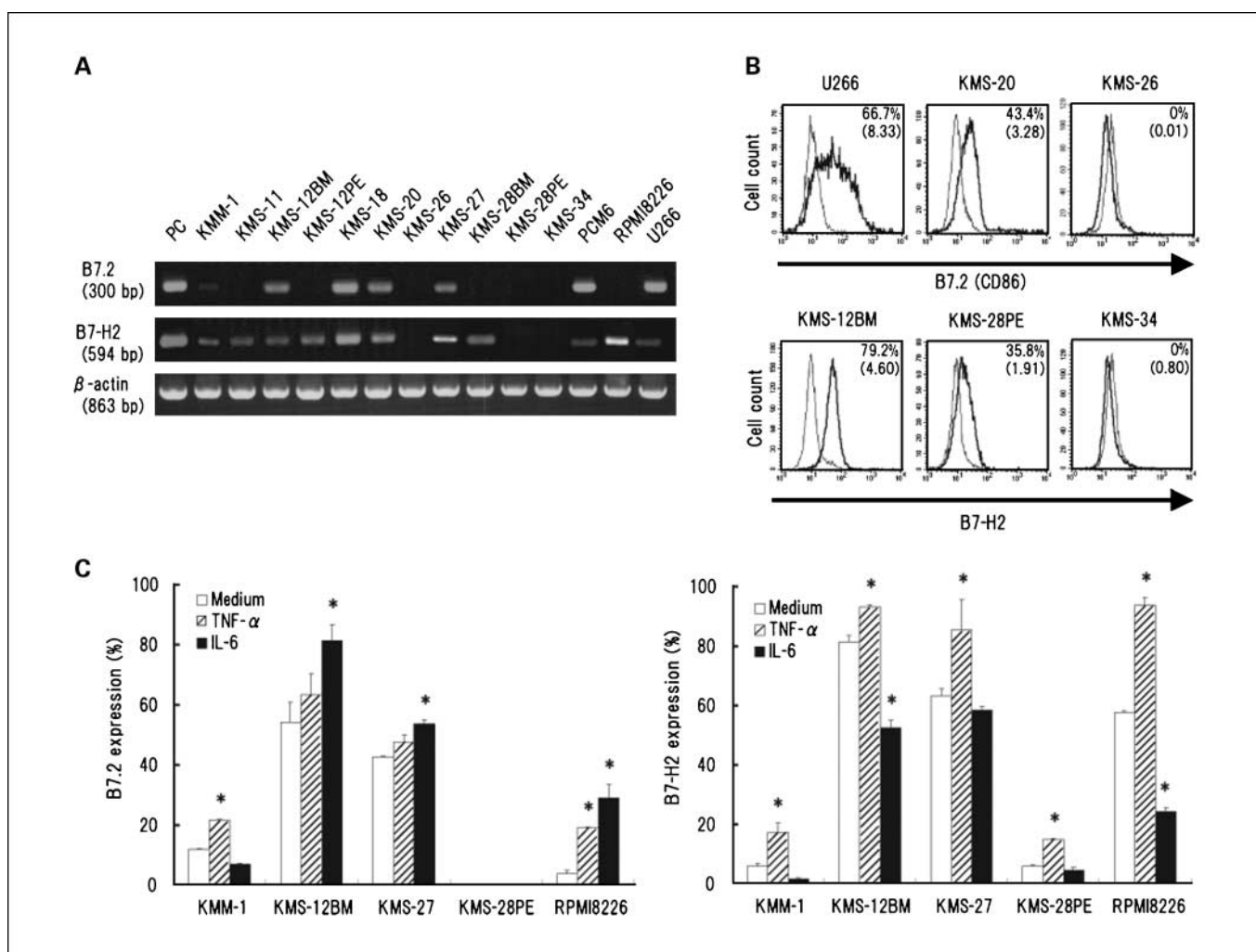


Fig. 1. A, B7.2 and B7-H2 mRNA expression analyzed using reverse transcription-PCR in 14 HMCLs. Equal amounts of cDNA from each cell line were amplified using primers specific for B7.2, B7-H2, and β -actin. B, representative flow cytometry analyses of the B7.2 and B7-H2 expression in HMCLs. Bold curves, staining with anti-B7.2 or anti-B7-H2 mAb; thin curves, staining with isotype-matched control immunoglobulin. Data are expressed in the percentages of positive cells and in relative mean fluorescence intensity (numbers in parentheses). C, effects of TNF- α and IL-6 on B7.2 and B7-H2 expression in HMCLs. Columns, mean of three independent experiments; bars, SD. Medium, no cytokine was added. *, $P < 0.05$, significantly different from Medium.

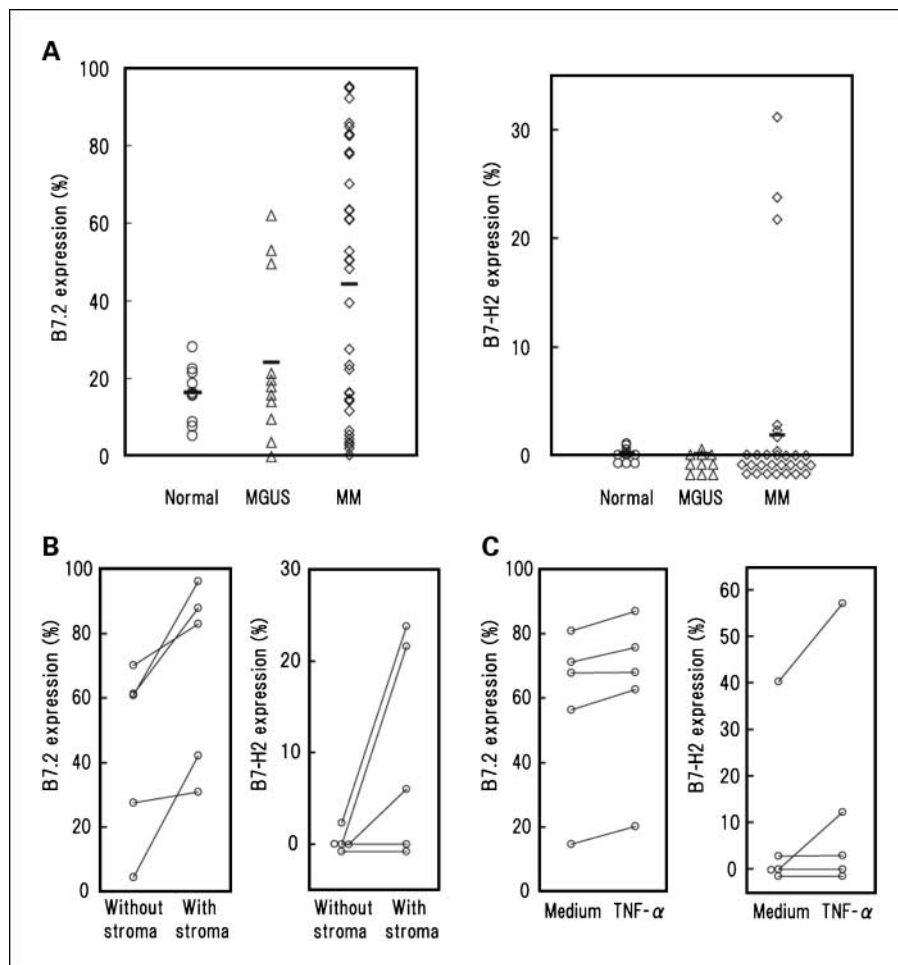


Fig. 2. A, percentages of B7.2⁺ and B7-H2⁺ plasma cells among all plasma cells in 10 hematologically normal individuals and 12 monoclonal gammopathy of unknown significance and 35 MM patients determined using flow cytometry. Horizontal bars, mean. Effects of autologous stroma cells (B) and TNF- α (C) on B7.2 and B7-H2 expression on myeloma cells from patients.

(500 units/mL; PeproTech), IL-10 (10 ng/mL; MBL), or IL-6 (10 ng/mL) were added to the cultures. The cell number was counted using the trypan blue dye-exclusion method.

In some experiments, B7.2⁺, B7.2⁻, B7-H2⁺, or B7-H2⁻ KMS-27 cells were purified with FACSvantage (Becton Dickinson) as described previously (23).

Patients, hematologically normal individuals, and cell preparation. BM samples were obtained from individuals who underwent BM aspiration for diagnostic purposes after obtaining written informed consent. They included 35 MM patients [4 stage I and 31 stage III according to the definition of Durie and Salmon (24)], 12 patients with monoclonal gammopathy of unknown significance, and 10 hematologically normal individuals. All BM samples from MM patients were obtained at the initial diagnosis, except for those from 6 patients, 2 of whose samples showed that they were refractory to conventional chemotherapy and 4 of whose samples were in the plateau phase according to the standard definition. Diagnoses were made according to the WHO classification. Mononuclear cells were separated from BM samples with Histopaque (Sigma) density centrifugation. These cells were used immediately or cryopreserved in liquid nitrogen until use. In cell samples from hematologically normal individuals, CD19-positive cells were enriched from BM mononuclear cells using magnetic cell sorting (Miltenyi Biotec; ref. 23) to ensure plasma cell identification in flow cytometry (25). This study was approved by the institutional review board of Nippon Medical School.

Stroma cells were prepared as follows. BM mononuclear cells from MM patients (2×10^6 /mL) were plated in 6-well plates in complete medium. The cultures were fed weekly by removing 75% of the

medium and adding fresh medium to make up the same volume. After the cultivated cells became adherent, stromal cell shaped, positive for mesenchymal stem cell markers (CD44 and CD90), and negative for hematopoietic markers (CD34, CD45, and CD11b), they were used as stroma cells (26). In experiments inducing B7.2 and B7-H2 expression, mononuclear cells were cultured in complete medium on autologous stroma cells for 3 wk or with TNF- α (500 units/mL) for 2 d.

Reverse transcription-PCR. Total RNA extracted from each HMCL was reverse transcribed with Superscript II Reverse transcriptase (Invitrogen) using random hexamers. PCR amplification was done using the primer sets for B7.2 and B7-H2 and PCR conditions previously described (21).

Flow cytometry. Immunophenotyping was done with FACScan (Becton Dickinson; refs. 21, 27). Briefly, after blocking with human immunoglobulin, patient BM samples were stained with anti-CD38 monoclonal antibody (mAb) labeled with FITC and phycoerythrin-labeled anti-B7.2 (Becton Dickinson) or anti-B7-H2 mAb (e-Bioscience). Plasma cells were identified by a high expression of CD38 molecules (22). We also confirmed that the identified plasma cells expressed another plasma cell marker, CD138 (Becton Dickinson). Examples of flow cytometry analysis are shown in Supplementary Fig. S1. HMCLs were single stained with FITC/phycoerythrin-conjugated mAbs against lymphocyte function-associated antigen-1 (LFA-1), intercellular adhesion molecule-1 (ICAM-1; Beckman Coulter), very late antigen-4, vascular cell adhesion molecule-1 (Becton Dickinson), B7.2, and B7-H2.

Cell cycle analysis. After blocking with human immunoglobulin, HMCLs were stained with purified mouse anti-B7.2 or anti-B7-H2

mAbs. The cells were washed and further incubated with FITC-conjugated antimouse IgG (Biosource). Then, the cells were fixed with 70% ethanol at 4°C for 3 h. The fixed cells were washed and resuspended in 100 μ L of PBS and 1 μ g of RNase (Qiagen) containing 0.1 mg/mL of propidium iodide (Sigma). The cell cycle profiles of B7.2⁺, B7.2⁻, B7-H2⁺, and B7-H2⁻ HMCLs were analyzed using flow cytometry.

Colony-forming assay. Purified B7.2⁺, B7.2⁻, B7-H2⁺, and B7-H2⁻ cells (1×10^3 per culture dish) were cultured in MethoCult H4230 methylcellulose medium (StemCell Technologies; ref. 23). Colonies (aggregates of 50 or more cells) were scored on day 14 of culture.

Mixed lymphocyte-myeloma reaction. CD4⁺ T cells (purity > 95%) were prepared from peripheral blood of healthy volunteers on magnetic cell sorting columns (21). These cells (1×10^5) were cocultured with irradiated (20,000 rad) myeloma cells (1×10^5) expressing both B7.2 and B7-H2 molecules in microtiter wells for 5 d. Antagonistic mAbs (10 μ g/mL) against B7.2 and ICOS (e-Bioscience) were added to the cultures to block the B7.2-CD28 and B7-H2-ICOS pathways, respectively. During the final 18 h of culture, [³H]thymidine (1 μ Ci/well) was added to determine T-cell proliferation. All samples were assayed at least in quadruplicate.

In some experiments, culture supernatants of mixed lymphocyte-myeloma reaction (MLMR) were collected on day 5 of culture. The

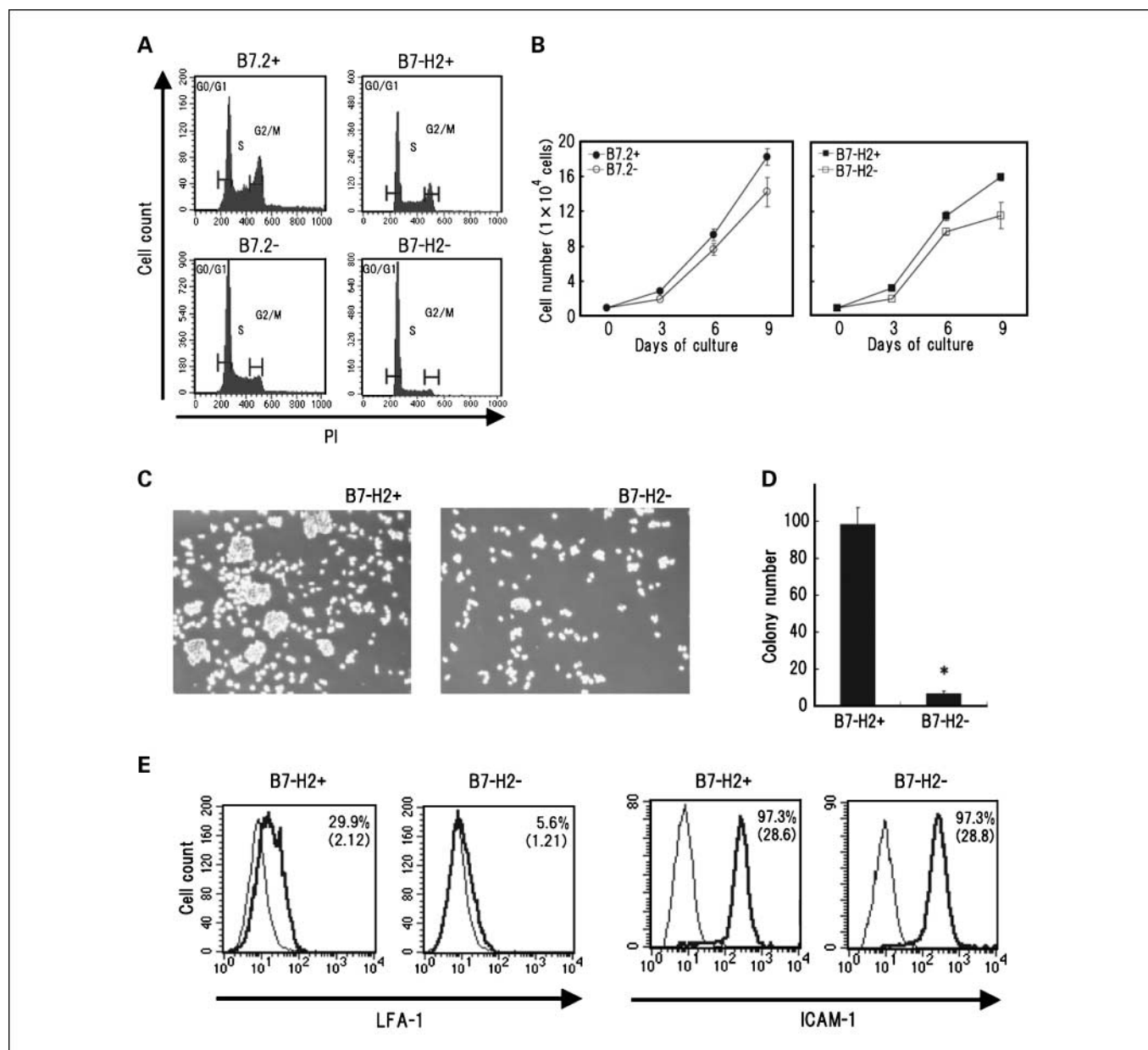


Fig. 3. A, cell cycle analyses of B7.2⁺ or B7.2⁻ KMS-27 cells (left) and of B7-H2⁺ or B7-H2⁻ KMS-27 cells (right). Each cell population was gated and analyzed in flow cytometry. Analysis on at least 10,000 events for each sample. The cell cycle data were reproducible when B7-H2⁺ and B7-H2⁻ KMS-27 cells isolated using FACS Vantage were analyzed (data not shown). B, proliferation of isolated B7.2⁺ or B7.2⁻ KMS-27 cells and B7-H2⁺ or B7-H2⁻ KMS-27 cells. Points, mean of three independent experiments; bars, SD. C, photomicrographs of purified B7-H2⁺ and B7-H2⁻ KMS-27 cells during exponential cell growth in culture. D, number of colonies (defined as aggregates composed of 20 or more cells) formed by purified B7-H2⁺ and B7-H2⁻ cells on day 7 of culture. Columns, mean of three independent triplicate cultures; bars, SD. *, $P < 0.01$ compared with the data of B7-H2⁻. E, representative flow cytometry analyses of LFA-1 and ICAM-1 expression on B7-H2⁺ and B7-H2⁻ KMS-27 cells. Bold curves, staining with anti-LFA-1 or anti-ICAM-1 mAb; thin curves, staining with isotype-matched control immunoglobulin. Data are expressed in percentages of positive cells and in relative mean fluorescence intensity (numbers in parentheses).

concentrations of IFN- γ , IL-2, IL-4, and IL-10 in the supernatants were measured with sandwich ELISA kits (eBioscience).

Coculture using a transwell system. KMS-27 (1×10^4) cells were plated onto a transwell membrane insert (Nunc) placed above the culture containing normal CD4⁺ T cells (1×10^3) with or without irradiated KMS-27 cells (1×10^5). Antihuman IL-10 polyclonal antibody (R&D Systems), anti-B7.2, and/or anti-ICOS mAbs were added to the lower cultures to neutralize their biological activities. After 5 d of culture, KMS-27 cells above the insert were harvested and counted using the trypan blue dye-exclusion method.

Statistical analysis. Differences between two groups of data were determined with the χ^2 test and Student's *t* test for categorical and continuous variables, respectively, unless otherwise stated. The Mann-Whitney *U* test was used for two groups of data with continuous nonparametric variables. A *P* value of <0.05 was considered significant.

Results

Expression and induction of B7.2 and B7-H2 molecules on HMCLs. First, we analyzed the expression of B7.2 and B7-H2 molecules on HMCLs. Seven and 11 of 14 HMCLs expressed high levels of B7.2 and B7-H2 mRNA, respectively (Fig. 1A). These results were consistent with the protein expression analyzed using flow cytometry (Fig. 1B; Supplementary Table S1): The expression of B7.2 and B7-H2 molecules was detected in 7 (50.0%) and 9 (64.3%) HMCLs, respectively. Next, we examined whether cytokines, i.e., TNF- α , IL-6, or IL-10, affect B7.2 and B7-H2 expression in five HMCLs. TNF- α up-regulated the expression of both molecules in almost all cell lines examined. Meanwhile, IL-6 up-regulated B7.2 expression in three of five HMCLs and down-regulated B7-H2 expression in two of five HMCLs (Fig. 1C). IL-10 did not affect the expression of these molecules (data not shown).

Expression and induction of B7.2 and B7-H2 molecules in myeloma patients. Using flow cytometry, we examined the expression of B7.2 and B7-H2 molecules on fresh plasma cells. The percentages of B7.2⁺ cells in plasma cells were much higher in MM patients than those in monoclonal gammopathy of unknown significance patients or in hematologically normal individuals (Fig. 2A, left; MM versus monoclonal gammopathy of unknown significance, *P* = 0.0318; MM versus normals, *P* = 0.0145; Mann-Whitney *U* test). When MM patients were divided into two groups using various cutoff percentages of B7.2 positivity, those in whom >40% of myeloma cells expressed B7.2 (*n* = 18, called B7.2^{high+} MM patients in this article) showed significantly lower levels of hemoglobin and platelets compared with other MM patients (B7.2^{low} MM patients in this article, *n* = 17; Supplementary Table S2). Although there was no difference in survival between the two groups of patients (data not shown), both patients refractory to chemotherapy were in the B7.2^{high+} group and all four patients in the plateau phase were in the B7.2^{low} group. Meanwhile, B7-H2 expression on plasma cells was clearly documented only in three MM patients. The disease of these three patients was intractable: one patient had plasma cell leukemia and the other two had chemotherapy-resistant MM. In our cohort of patients, BM cells from only two MM patients were analyzed for B7.2 and B7-H2 expression in different disease statuses at the initial diagnosis and at the stage of refractory disease. The expression of these molecules on myeloma cells was augmented at the refractory stage in both patients, except for B7-H2 expression in one patient in whom B7-H2 was not detected at either time

point (Supplementary Table S3). All of the above findings support the idea that these molecules may be associated with disease progression in MM, although the clinical evidence remains insufficient for the B7-H2 molecule because of the rarity of B7-H2⁺ positive patients.

Next, we examined whether stroma cells and TNF- α , both important for myeloma cell proliferation *in vivo*, modulate the expression of B7.2 and B7-H2 on myeloma cells from MM patients. B7.2 or B7-H2 expression on myeloma cells from >50% of patients examined was up-regulated after the cells were cultivated with autologous stroma cells or TNF- α (Fig. 2B and C).

Cell cycle and proliferation of myeloma cells based on B7.2 and B7-H2 expression. Based on the above data, we speculated that B7.2 or B7-H2 expression on myeloma cells was associated with their proliferative potential. When KMS-27 cells that did or did not express these B7 family molecules were analyzed, B7.2⁺ and B7-H2⁺ cells had significantly fewer G₀-G₁ phase cells and more G₂-M phase cells compared with B7.2⁻ and B7-H2⁻ cells, respectively (Fig. 3A; Table 1). Consistent with these results, the B7.2⁺ and B7-H2⁺ KMS-27 cells proliferated more rapidly in liquid cultures and formed more colonies in semisolid cultures compared with the B7.2⁻ and B7-H2⁻ KMS-27 cells, respectively (Fig. 3B; Supplementary Table S4). The same growth advantage of myeloma cells expressing B7.2 and B7-H2 molecules was also documented in all other HMCLs examined (Table 1; Supplementary Table S4; Supplementary Fig. S2).

We then examined whether myeloma cells have a growth advantage when the cells are induced to express B7.2 and B7-H2 molecules. When RPMI8226 cells, for which B7.2 and B7-H2 expression is inducible by TNF- α as shown in Fig. 1C, were treated with TNF- α , the cell cycle of the cells was clearly

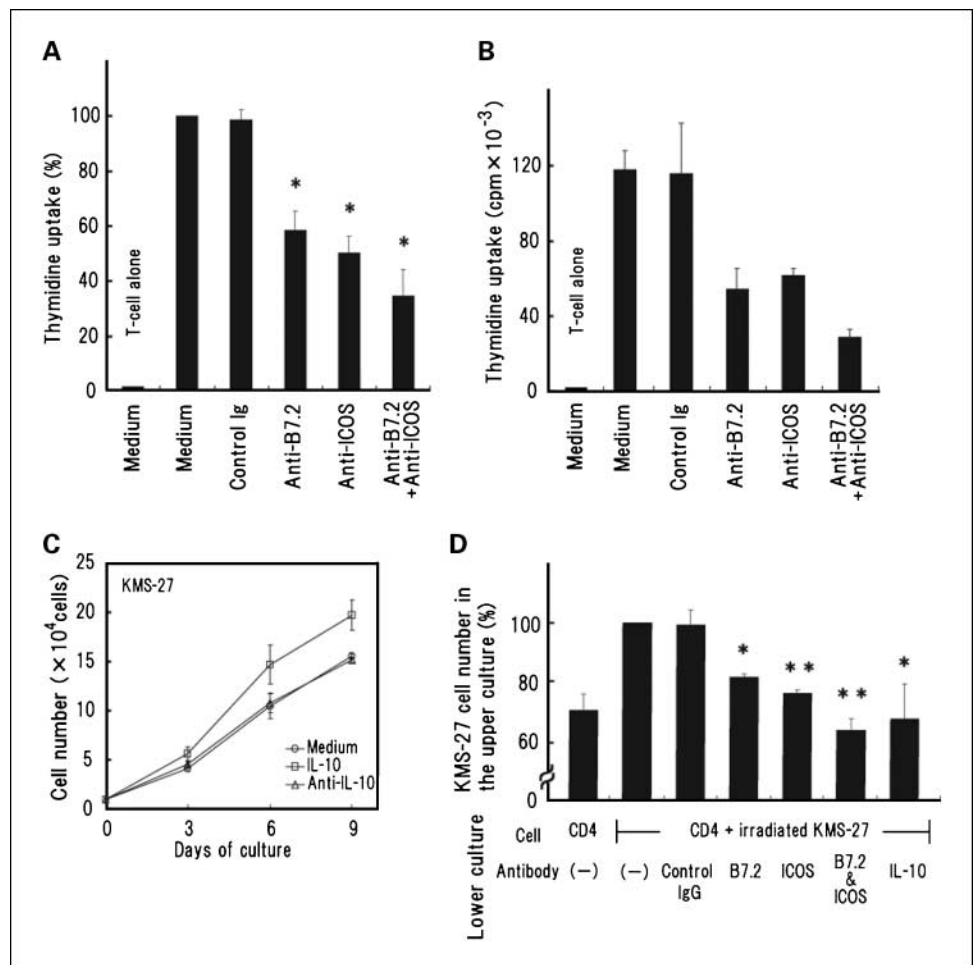
Table 1. Cell cycling of HMCLs as a function of B7.2 and B7-H2 expression

Cell fraction in HMCLs	Cell cycle		
	G ₀ -G ₁	S	G ₂ -M
KMS-27 cells			
B7.2 ⁺	52.8 ± 0.9*	19.7 ± 0.1	27.6 ± 0.9*
B7.2 ⁻	80.8 ± 1.6	16.0 ± 1.5	3.7 ± 0.4
B7-H2 ⁺	53.7 ± 1.4*	18.6 ± 2.2	28.0 ± 0.9*
B7-H2 ⁻	78.5 ± 0.8	17.5 ± 0.8	4.1 ± 1.3
KMS-20 cells			
B7.2 ⁺	42.5 ± 0.7*	20.0 ± 0.1*	37.7 ± 0.8*
B7.2 ⁻	74.2 ± 1.0	13.7 ± 0.6	12.3 ± 0.3
B7-H2 ⁺	43.4 ± 1.1*	26.9 ± 0.4*	30.2 ± 0.6*
B7-H2 ⁻	81.5 ± 0.9	13.4 ± 0.7	4.8 ± 0.3
U266 cells			
B7.2 ⁺	49.5 ± 4.0*	23.7 ± 1.6	25.7 ± 3.8*
B7.2 ⁻	65.0 ± 2.7	21.2 ± 0.5	13.9 ± 1.7
RPMI8226 cells			
B7-H2 ⁺	39.5 ± 0.9*	33.1 ± 2.2*	26.9 ± 4.1*
B7-H2 ⁻	64.4 ± 3.0	25.8 ± 2.4	9.3 ± 2.1

NOTE: Mean ± SD of three independent experiments. B7-H2⁺ and B7.2⁺ fractions in U266 and RPMI8226 cells, respectively, were sparse and thus were not analyzed.

*Significant difference (*P* < 0.05) when data of each cell cycle phase were compared between B7.2⁺ (or B7-H2⁺) and B7.2⁻ (or B7-H2⁻) cell fractions in each HMCL.

Fig. 4. *A*, MLMR. Normal CD4⁺ T cells were cultured with (five columns, right) or without (first column, left) irradiated KMS-27 cells that expressed B7.2 and B7-H2. Effects of anti-B7.2 and anti-ICOS blocking mAbs on CD4⁺ T-cell proliferation were examined in this assay. Columns, mean of three independent triplicate cultures, in which the second column from the left was defined as 100%; bars, SD. Medium, no antibody was added. *, $P < 0.01$ compared with the data for control immunoglobulin. *B*, MLMR done using the same method as described in *A*, except that irradiated patient MM cells expressing B7.2 and B7-H2 were used instead of KMS-27 cells. Columns, mean of triplicate cultures; bars, SD. *C*, kinetics of KMS-27 cell proliferation. The cells were cultured in medium alone or in medium containing anti-IL-10 neutralizing antibody or recombinant IL-10. Points, mean of three independent experiments; bars, SD. *D*, cultures using a transwell system. KMS-27 cells were plated onto a transwell membrane insert, which was placed above the culture containing CD4⁺ T cells alone (first columns, left) or CD4⁺ T cells with irradiated KMS-27 cells (six columns, right). Blocking antibodies to B7.2, ICOS, and/or IL-10 were added to the lower cultures and their effects on KMS-27 cell proliferation in the upper cultures were examined. Columns, mean of three independent triplicate cultures, in which the second column from the left was defined as 100%; bars, SD. *, $P < 0.05$; **, $P < 0.01$ compared with the data for control immunoglobulin.



stimulated (Supplementary Table S5). Furthermore, when 293T cells (a human kidney cell line suitable for efficient transfection experiments) were transfected with either B7.2 or B7-H2 gene or Mock, B7.2 or B7-H2 gene induction induced cell cycle activation (Supplementary Table S6).

We noted that even in liquid culture, B7-H2⁺ KMS-27 cells often formed colonies during exponential cell growth (Fig. 3C): There were many more colonies of B7-H2⁺ cells compared with B7-H2⁻ cells in liquid culture (Fig. 3D). Meanwhile, there was no difference in colony formation in liquid culture between B7.2⁺ and B7.2⁻ KMS-27 cells (data not shown). Therefore, we investigated the expression of adhesion molecules (LFA-1, ICAM-1, very late antigen-4, vascular cell adhesion molecule-1), which might mediate the adhesion of myeloma cells to BM stroma cells and induce drug resistance (28, 29) on B7-H2⁺ and B7-H2⁻ KMS-27 cells. The expression levels of LFA-1 were much higher on B7-H2⁺ KMS-27 cells compared with those on B7-H2⁻ KMS-27 cells, although there was no difference in the expression of the other adhesion molecules (Fig. 3E).

Interaction between B7.2⁺ and B7-H2⁺ myeloma cells and CD4⁺ T cells confers a myeloma growth advantage. In the MLMR, normal CD4⁺ T cells were cultured with KMS-27 cells with or without anti-B7.2 and anti-ICOS mAbs. In a 5-day culture, either the anti-B7.2 or anti-ICOS mAb decreased the proliferation of CD4⁺ T cells and the combined use of these antibodies resulted in the maximum decrease (Fig. 4A). In

other words, both B7.2 and B7-H2 molecules on KMS-27 cells stimulated CD4⁺ T-cell proliferation. The same result was obtained when fresh myeloma cells obtained from a plasma cell leukemia patient and expressing both B7.2 and B7-H2 molecules were used in the MLMR (Fig. 4B).

It was reported that B7.2 enhanced the production of Th1 and Th2 cytokines and that B7-H2 did not contribute to IL-2 induction (18, 19, 30). Consistent with these results, in MLMR using KMS-27 cells and CD4⁺ T cells, the mAb against B7.2 decreased the production of IL-10 as well as IFN- γ and IL-2. Meanwhile, the mAb against ICOS decreased the production of IL-10 and IFN- γ but not that of IL-2 (Supplementary Fig. S3). IL-4 was not detected in the supernatant of MLMR. The finding that both B7.2 and B7-H2 molecules on myeloma cells enhanced IL-10 production is particularly interesting because IL-10 not only reduces the antitumor immune response in general but also is a growth factor for myeloma cells.

Then, we examined whether soluble factors including IL-10 produced by the interaction between myeloma cells and CD4⁺ cells stimulate myeloma cell growth *in vitro*. First, we confirmed that when anti-IL-10 neutralizing antibody or control immunoglobulin was added to the KMS-27 cell culture, cell proliferation was not affected (Fig. 4C; data for control immunoglobulin are not shown). Furthermore, IL-10 was not detected in the supernatant of KMS-27 cell culture when examined using ELISA (data not shown). Therefore, KMS-27

cells themselves did not produce IL-10, but their proliferation was stimulated by exogenous IL-10 (Fig. 4C). Next, we cocultured using a transwell system, in which KMS-27 cells were plated onto a transwell membrane insert placed above the culture containing normal CD4⁺ T cells with or without irradiated KMS-27 cells. The presence of irradiated KMS-27 cells in the lower cultures, compared with their absence, stimulated KMS-27 cell proliferation in the upper cultures (Fig. 4D, *two columns on the left*). Furthermore, this growth-promoting effect resulting from the CD4⁺ T cell-irradiated KMS-27 cell interaction was eliminated partially or completely by adding anti-B7.2 mAb, anti-ICOS mAb, and/or anti-IL-10 neutralizing mAb to the lower cultures (Fig. 4D, *four columns on the right*). These results suggest that B7.2 or B7-H2 molecules on KMS-27 cells enhance CD4⁺ T-cell proliferation and stimulate them to produce soluble factors, one of which, IL-10, enhances KMS-27 cell proliferation.

Discussion

The B7 family molecules stimulate or inhibit immune responses by costimulating or coinhibiting T cells. For example, in a mouse tumor model, B7-H2-expressing tumors increase the proliferation of tumor-specific CTLs (31). Meanwhile, the B7-H2-ICOS signal down-regulates the immune response in experimental animal models of autoimmune disease or acute graft-versus-host disease (32–34).

This study showed that the B7.2 expression levels on plasma cells were higher in MM patients compared with those in monoclonal gammopathy of unknown significance patients and hematologically normal individuals, and B7-H2 expression was detected in three MM patients alone but not in any monoclonal gammopathy of unknown significance patient or hematologically normal individual. Next, the expression of B7.2 and B7-H2 on MM cells was induced or enhanced by coculture with autologous stroma cells or by stimulation with TNF- α . Furthermore, myeloma cells expressing B7.2 and B7-H2 had increased cell cycling and more potential for proliferation. Finally, B7.2 and B7-H2 molecules on myeloma cells enhanced CD4⁺ T-cell proliferation and stimulated them to produce soluble factors, one of which, IL-10, further stimulates the proliferation of myeloma cells.

B7.2 and B7-H2 molecules on myeloma cells were also involved in the production of other cytokines, IFN- γ and IL-2, which are known to enhance antitumor immune responses in general (35, 36). However, we and others observed that the expression of these molecules on acute myelogenous leukemia cells was associated with poor patient prognosis (21, 37). Similarly, lymphoma cells expressing B7.2 are associated with poor prognosis in a mouse lymphoma model (38). The effects of IFN- γ and IL-2, the production of which might be induced by B7.2 and B7-H2 molecules on myeloma cells, on the immunology and clinical behavior of MM should be clarified in further studies.

TNF- α , an immunomodulatory cytokine capable of inhibiting CTLs, is produced by the interaction between BM stroma cells and myeloma cells (39). It was shown that TNF- α directly stimulates myeloma cell growth *in vitro* and that serum TNF- α levels are higher in advanced-stage compared with early-stage MM patients (6, 7, 40). Based on our data presented here, we speculate that the TNF- α -induced growth advantage in

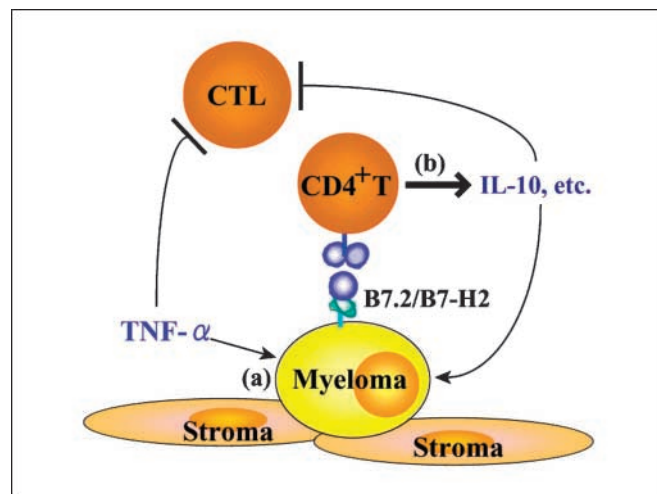


Fig. 5. New insight into myeloma biology based on the present data. *A*, in the BM environment, TNF- α and stromal cell contact induce B7.2 and B7-H2 molecule expression on myeloma cells. B7.2⁺ and B7-H2⁺ myeloma cells gain an intrinsic proliferative advantage. TNF- α also inhibits myeloma-specific CTLs. *B*, the B7.2 and B7-H2 molecules on myeloma cells induce CD4⁺ T cells to produce soluble factors, one of which, IL-10, stimulates myeloma cell proliferation and inhibits myeloma-specific CTLs.

myeloma may be, at least in part, associated with the induction of B7.2 and B7-H2 molecule expression on myeloma cells. The mechanism underlying the finding that myeloma cells expressing B7.2 and B7-H2 show increased cell cycling and greater potential to proliferate remains unknown. To the best of our knowledge, one previous report observed a similar phenomenon. Ghebeh et al. reported that high B7-H1 expression was correlated with high Ki-67 expression in tumor cells in breast cancer patients (41). In addition to this intrinsic advantage in cell proliferation of B7.2⁺ and B7-H2⁺ myeloma cells, these cells may obtain a further growth advantage by inducing the production of the myeloma-stimulatory cytokine IL-10 by CD4⁺ T cells. It is well known that IL-10 inhibits the generation of tumor-specific CTLs (11, 12). We also confirmed that IL-10 inhibited the generation of KMS-27-specific CTLs *in vitro* (data not shown). This IL-10-mediated effect probably contributes further to myeloma cell growth. The above cascade of events is illustrated in Fig. 5. The IL-10 production induced by B7.2 and B7-H2 molecules is not myeloma specific because we observed that that AML cells expressing B7.2 and B7-H2 induced IL-10 production by CD4⁺ T cells *in vitro*. It would be interesting to determine how broadly this mechanism works in a variety of human neoplasia.

The occurrence of immunologic derangement in MM supports the notion that modulation or normalization of this derangement would be beneficial for MM patients. Thalidomide and its analogues (e.g., lenalidomide), which have a variety of immunomodulatory activities, including potent inhibition of TNF- α production and stimulation of Th-1 immunity (42, 43), are effective in the treatment of MM patients. We speculate that full clarification of immunology in MM is the basis on which more specific, targeted therapy will be developed.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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