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 (1×10^5) 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 $B7\text{-}H2^+$ cells had significantly fewer $G_0\text{-}G_1$ phase cells and more G_2-M phase cells compared with $B7.2^-$ and $B7\text{-}H2^-$ cells, respectively (Fig. 3A; Table 1). Consistent with these results, the $B7.2^+$ and $B7\text{-}H2^+$ KMS-27 cells proliferated more rapidly in liquid cultures and formed more colonies in semisolid cultures compared with the $B7.2^-$ and $B7\text{-}H2^-$ 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		
	$G_0\text{-}G_1$	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
$B7\text{-}H2^+$	53.7 ± 1.4*	18.6 ± 2.2	28.0 ± 0.9*
$B7\text{-}H2^-$	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
$B7\text{-}H2^+$	43.4 ± 1.1*	26.9 ± 0.4*	30.2 ± 0.6*
$B7\text{-}H2^-$	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			
$B7\text{-}H2^+$	39.5 ± 0.9*	33.1 ± 2.2*	26.9 ± 4.1*
$B7\text{-}H2^-$	64.4 ± 3.0	25.8 ± 2.4	9.3 ± 2.1

NOTE: Mean ± SD of three independent experiments. $B7\text{-}H2^+$ 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 $B7\text{-}H2^+$) and $B7.2^-$ (or $B7\text{-}H2^-$) cell fractions in each HMCL.

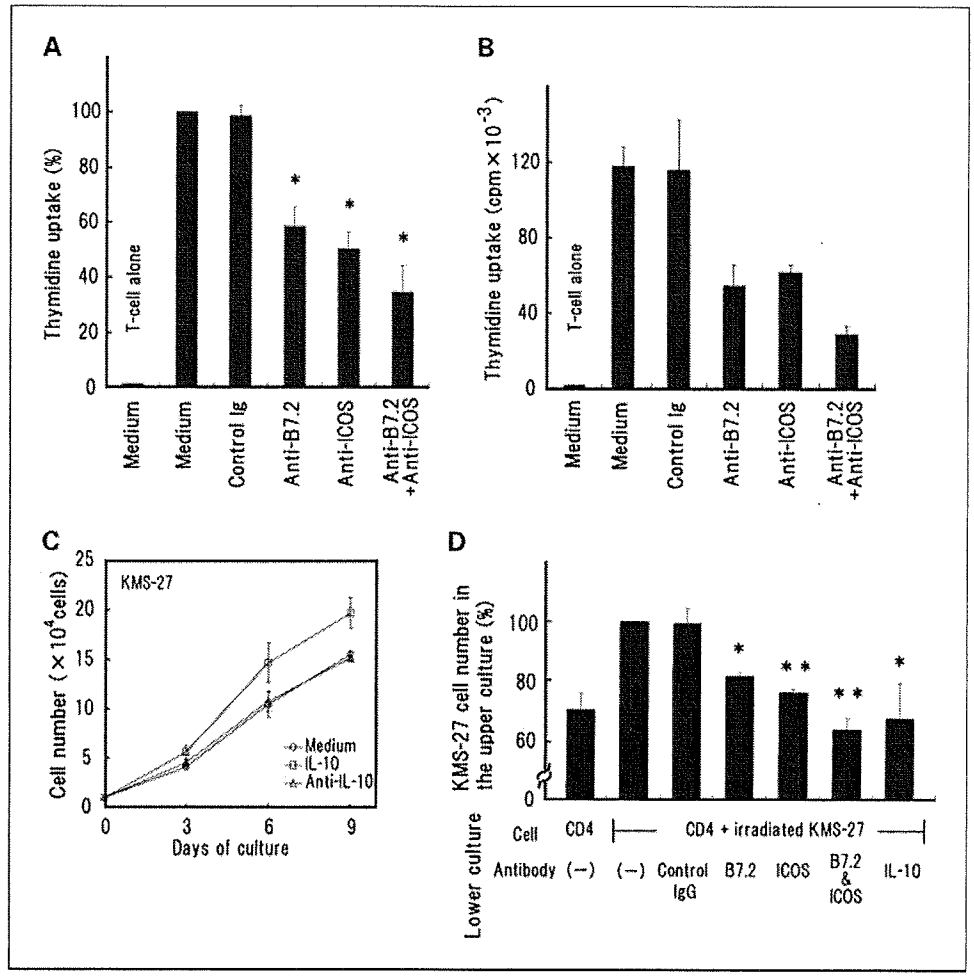

Fig. 4. *A*, MLMR. Normal CD4$^+$ Tcells were cultured with (*five columns, right*) or without (*first column, left*) irradiated KMS-27 cells that expressed B7.2 and B7-H2. Effects of anti-B7.2 and anti-ICOS blocking mAbs on CD4$^+$ T-cell proliferation were examined in this assay. Columns, mean of three 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$^+$ Tcells alone (*first columns, left*) or CD4$^+$ Tcells 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 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 $B7\text{-}H2^+$ 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.

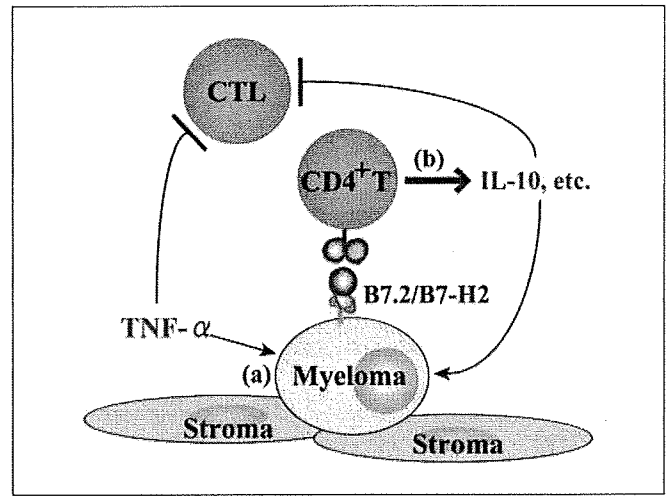

Fig. 5. New insight into myeloma biology based on the present data. *A*, in the BM environment, TNF-α and stromal cell contact induce B7.2 and B7-H2 molecule expression on myeloma cells. $B7.2^+$ and $B7\text{-}H2^+$ myeloma cells gain an intrinsic proliferative advantage. TNF-α also inhibits myeloma-specific 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.

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 imunomodulatory 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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ORIGINAL ARTICLE

A possible mechanism of intravesical BCG therapy for human bladder carcinoma: involvement of innate effector cells for the inhibition of tumor growth

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Abstract Intravesical bacillus Calmette-Guerin (BCG) therapy is considered the most successful immunotherapy against solid tumors of human bladder carcinoma. To determine the actual effector cells activated by intravesical BCG therapy to inhibit the growth of bladder carcinoma, T24 human bladder tumor cells, expressing very low levels of class I MHC, were co-cultured with allogeneic peripheral blood mononuclear cells (PBMCs) with live BCG. The proliferation of T24 cells was markedly inhibited when BCG-infected dendritic cells (DCs) were added to the culture although the addition of either BCG or uninfected DCs alone did not result in any inhibition. The inhibitory effect was much stronger when the DCs were infected with live BCG rather than with heat-inactivated BCG. The live BCG-infected DCs secreted TNF-α and IL-12 within a day and this secretion continued for at least a week, while the heat-inactivated BCG-infected DCs secreted no IL-12 and little TNF-α. Such secretion of cytokines may activate innate alert cells, and indeed NKT cells expressing IL-12 receptors apparently proliferated and were activated to produce cytocidal perforin among the PBMCs when live BCG-infected DCs were externally added. Moreover, depletion of $\gamma\delta$ T-cells from PBMCs significantly reduced the cytotoxic effect on T24 cells, while depletion of CD8β cells did not affect T24 cell growth. Furthermore, the innate effectors seem to recognize MICA/MICB molecules on T24 via NKG2D receptors. These findings suggest the involvement of innate alert cells activated by the live BCG-infected DCs to inhibit the growth of bladder carcinoma and provide a possible mechanism of intravesical BCG therapy.

Keywords Bladder cancer · Dendritic cells · Innate immunity · BCG · NKT cells

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Introduction

Intravesical bacillus Calmette-Guerin (BCG) therapy is considered the most successful immunotherapy against solid tumors in cases of human superficial bladder carcinoma particularly in preventing from its recurrence [1, 4]. Intravesical immunotherapy with live BCG results in a massive local immune response characterized by the secretion of various cytokines in the urine [14, 27] or bladder tissue as well as by the infiltration of granulocytes and mononuclear cells into the bladder wall after repeated treatment with BCG instillation [3, 21], indicating the immunopathological responses induced at the local mucosal compartment may correlate with the BCG-mediated anti-tumor effect. However, neither the precise mechanisms nor the actual effector cells underlying the anti-tumor effect that BCG therapy stimulates remain to be elucidated.

The bladder is a confined mucosal compartment, where BCG is able to be maintained at a high concentration and thus may achieve long-lasting, continuous immune activation, which seems to better stimulate innate local immunity having broad cross-reactivity with less memory rather than acquired systemic immunity with high specificity and memory originated from rearranged genes. Therefore, live

BCG appears to activate various types of innate immune effectors such as γδT lymphocytes [17, 18] and CD1 molecule-restricted lipid/glycolipid antigen-specific T cells including CD1d-restricted natural killer T (NKT) cells [12, 13] via live BCG-infected dendritic cells (DCs). Such DCs express not only peptide antigen-loaded individually restricted class I and II MHC molecules but also species-specific CD1 molecules on their surface to present BCG-derived lipid/glycolipid antigens [15, 20]. Indeed, findings that live BCG-infected DCs can be recognized by CD1 molecule-restricted but not by class I MHC molecule-restricted $CD8^+$ T cells [16] and that the Vγ2Vδ2 T lymphocytes response to BCG by immunization in macaques with live BCG [5] have recently been reported. Moreover, a close relationship between BCG-immunization, and NKT cell activation has also been shown [9]. Therefore, continuous stimulation in the confined bladder space with live BCG may activate those local innate effectors, which may control bladder cancer expansion in vivo.

The cell line T24, a well-known cell for human bladder cancer [19], expresses markedly down-modulated MHC class I molecules on the cell surface in comparison with normal peripheral blood mononuclear cells (PBMCs). Hence, the T24 line is possibly regulated by cells in a class I MHC molecule-unrelated manner rather than by the autologous class I MHC molecule-restricted conventional CD8-positive cytotoxic T lymphocytes (CTLs). Therefore, we co-cultured T24 cells with allogeneic PBMCs pretreated with live BCG to determine the actual cells activated by the BCG for controlling T24 tumor cell proliferation and elimination, and found that innate alert cells such as Vγ2Vδ2 T cells and particularly NKT cells derived from allogeneic PBMCs activated by the live BCG-pretreated DCs appear to inhibit the proliferation of T24 tumor cells as well as eliminate them. The findings shown in the present study strongly suggest the involvement of innate alert effectors in controlling bladder cancer growth and shed light on the actual feature of the mechanisms for the anti-tumor effect of intravesical BCG therapy.

Materials and methods

Cell lines

Human urinary bladder carcinoma T24 cells (ATCC HTB-4) were cultured in McCoy's 5a medium (Invitrogen, Carlsbad, CA) supplemented with 10% FCS (HyClone Laboratories, Logan, UT), 50 U/ml penicillin (Invitrogen), and 50 mg/ml streptomycin (Invitrogen). Human colon cancer derived HCT116 cells (ATCC CCL 247), C1R cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St Louis, MO) supplemented with 10% FCS (HyClone), 50 U/ml penicillin, and 50 mg/ml streptomycin (Invitrogen). Myelogenous leukemia K562 cells, and T lymphoblast Jurkat cells were cultured in RPMI 1640 (Sigma-Aldrich, St Louis, MO)-based complete T-cell medium (CTM) [25] supplemented with 10% FCS, 2 mM L-glutamine (ICN Biomedicals, Aurora, OH), 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM HEPES (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 50 mM 2-mercaptoethanol (2-ME) (Invitrogen).

Infection of DCs with live or heat-inactivated BCG

A lyophilized preparation of BCG, the Tokyo 172 strain (12 mg dry weight per ample) (Japan BCG Laboratory, Tokyo, Japan) was used to carry out the experiments. For the infection experiments, BCG was harvested at a mid-log growth phase, washed, and suspended in RPMI 1640 medium supplemented with 10% FCS. The suspension was passed through a 5-μm pore size filter to obtain single-cell bacteria. The viability of bacteria was constantly >90%. The BCG preparation was divided into two equal aliquots; one incubated for 30 min at 85°C to kill the bacteria and the other left at room temperature as reported recently [16].

Generation of DCs from PBMCs and their treatment with BCG

DCs were obtained from PBMCs as described recently [26]. In brief, PBMCs were freshly isolated with Ficoll-Hypaque (Amersham-Pharmacia Biotech, Uppsala, Sweden) from peripheral blood of healthy volunteers, and $CD14^+$ monocytes were immediately separated by magnetic depletion using a monocyte isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) containing hapten-conjugated antibodies to CD3, CD7, CD19, CD45RA, CD56, and anti-IgE Abs and a magnetic cell separator (MACS, Miltenyi Biotec) according to the manufacturer's instructions, routinely resulting in >90% purity of $CD14^+$ cells. Cells were cultured in 24-well plates for 6–7 days in CTM supplemented with 200 ng/ml GM-CSF (PeproTech, Rocky Hill, NJ), and 10 ng/ml IL-4 (Biosource Intl., Camarillo, CA) to obtain DCs. For the treatment with BCG, 1×10^5 DCs in 1 ml of CTM were incubated overnight with 0.1 mg of either live BCG or heat-inactivated BCG. After being washed three times with RPMI1640 medium, the BCG-treated DCs were further co-cultured with 1×10^6 PBMCs of the same donor to carry out the experiments.

Antibodies and flow-cytometric analysis

Fluorescein isothiocyanate (FITC)-conjugated anti-human monoclonal antibodies (mAbs) to mouse IgG1κ, isotype control (MOP-21), HLA-ABC (G46-2.6), CD3 (H1T3a),

CD161 (DX12), CD80 (B7-1) (L307.4), CD86 (B70/B7-2) [2331(FUN-1)], as well as phycoerythrin (PE)-conjugated mouse IgG1κ, isotype control, CD3, CD56 (B159), and unlabeled anti-human CD3, CD4 (RPA-T4), Vδ2 (B6), and CD161, were all purchased from BD Biosciences (San Diego, CA). Unlabeled anti-human CD8β (2ST8.5H7) mAb was purchased from IMMUNOTECH (Marseille, Cedex, France). Cells were stained with the relevant antibody on ice for 30 min in phosphate-buffered saline (PBS) with 2% FCS and 0.01 M sodium azide (PBS-based medium), washed twice, and re-suspended in the PBS-based medium. Then, the labeled cells were analyzed with a FACScan (BD Biosciences) using CellQuest software (BD Biosciences). Live cells were gated based on propidium iodide gating.

Depletion of cells from PBMCs

To deplete Vδ2-positive cells, PBMCs were incubated with mouse anti-human Vδ2 mAb (B6) for 30 min at 4°C and washed three times to remove free mAb. Then the stained cells were further incubated with magnetic beads-conjugated anti-mouse IgG (Dynabeads Pan Mouse IgG) (DYNAL BIOTECH, Oslo, Norway), and Vδ2-positive cells were eliminated by magnetic device (Perspective Biosystems, Framingham, MA) following the manufacturer's instruction. CD8β, CD3, CD161, and CD4-positive cells were also depleted using the same procedure.

Quantification of cytokine production from BCG-treated DCs by ELISA

Monocyte-derived DCs (1×10^6) were incubated with 1 ml of CTM containing 0.1 mg of BCG in 24-well culture plate for 2–3 days and the culture supernatants were collected and stored at −80°C until the measurement of cytokines. Production of TNF-α, IL-12, IL-10, and IL-4 was measured using the DuoSet ELISA Development Kit (R&D systems, Minneapolis, MN) according to the manufacturer's instructions.

Chromium-51 release assay

The cytotoxicity of BCG-activated cells was measured by a standard 4-h ^{51}Cr-release assay using T-24 human bladder cancer cells or NK-sensitive K562 myelogenous leukemia cells as targets. In brief, various numbers of effector cells were incubated with 3×10^3 ^{51}Cr-labeled targets for 4 h at 37°C in 200 µl of RPMI 1640 medium containing 10% FCS in round-bottomed 96-well cell culture plates (BD Biosciences). After incubation, the plates were centrifuged for 10 min at 330×g, and 100 µl of cell-free supernatant was collected to measure radioactivity with a Packard Auto-Gamma 5650 counter (Hewlett-Packard Japan, Tokyo, Japan). Maximum release was determined from the supernatant of cells that had been lysed by the addition of 5% Triton ×-100 and spontaneous release was determined from target cells incubated without added effector cells. The percent specific lysis was calculated as 100× (experimental release – spontaneous release)/(maximum release – spontaneous release). Standard errors of the means of triplicate cultures were always <5% of the mean. Data are expressed as the mean ± SEM. Each experiment was performed at least three times.

T24 growth inhibition assay

The T24 growth inhibition assay was performed by incubating 1×10^4 T24 cells with 5×10^4 or 1×10^5 freshly isolated allogeneic PBMCs in 200 µl of CTM for 3 days at 37°C in 5% CO_2 based on a recent study [22]. Samples were cultured in triplicate on 96-well U-bottom plates. The cells were then labeled for 16 h with 1 µCi/well of tritiated thymidine (^{3}H-TdR; MP Biomedicals, Morgan, CA), harvested in an automated plate harvester (TomTech, Orange, CT), and counted in a 1,450 Micro Beta TRILUX scintillation spectrometer (Wallac, Gaithersburg, MD). Data are expressed as the mean count per minute (cpm) ± SEM.

RT-PCR for CD1d mRNA in T24 cells

Total RNA was extracted from T24, Jurkat, and HCT cells using the RNeasy Protocol Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA (2 µg) was reverse transcribed with oligo-(dT)18 (Perkin Elmer, Wellesley) priming and Superscript III (Invitrogen) reverse transcriptase in a 20 µg reaction mixture at 50°C for 60 min. A measure of 1 µl (equal to about 200 ng) of the cDNA product was then subjected to 30 cycles of 30 s at 94°C, 1 min at 64°C, and 1 min final extension at 72°C, with a thermocycler (PCR express; Hybaid, Teddington, Middlesex, UK). The amplification was performed in a reaction volume of 20 µl with LA PCR buffer (Takara, Shiga, Japan), composed of 2.5 mM $MgCl_2$, 0.3 nM of each deoxynucleotide triphosphate, 2.5 mM of each primer, and 1 U of LA Taq polymerase (Takara). The following oligonucleotide primers were designed from the published cDNA sequence [6]: GAPDH sense-primer (5′-GCCTCAA GATCATCAGCAATGC-3′) and antisense-primer (5′-AT GCCAGTGAGCTTCCCGTTC-3′), human CD1d (hCD1d) full-length sense-primer (5′-CGGGATCCATGGGGTGC CTGCTGTTTCTG-3′), antisense-primer (5′-ATTTGCGG CCGCCAGGACGCCCTGAT-3′), hCD1d short fragment sense-primer (5′-CTCCAGATCTCGTCCTTCGCCATT-3′), antisense-primer (5′-TTGAATGGCCAAGTTTACCCAA AG-3′).

Measurement of cytotoxicity by BCG-activated innate effctors through NKG2D-receptor against MICA/MICB molecules on T24 tumor cells

Cytotoxicity of innate effectors activated by live BCG-treated DC against T24 cells was investigated using 51-chromium release assay shown above in the presence of various blocking antibodies such as anti-human MICA/MICB (6D4) (BioLegend, San Diego, CA), anti-human NKG2D (CD314)-specific mouse mAbs (1D11) (BioLegend), or isotype-matched control mouse IgG1κ (BD Biosciences). $CD3^+CD56^+$ NKT cells were sorted out with FACS-Vantage SE (BD Biosciences) according to the manufacturer's instruction.

Results

T24 growth inhibition by allogeneic PBMCs activated with live BCG-treated DCs

The bladder cancer cell line T24, a well-known cell for human bladder cancer, expresses markedly reduced levels of MHC class I molecules on the cell surface in comparison with normal PBMCs (data not shown). Thus, the T24 line is possibly regulated by cells in a class I MHC molecule-unrelated manner rather than by the autologous class I MHC molecule-restricted conventional CTLs. Therefore, we used allogeneic PBMCs to gain insight into the actual cells activated by BCG for controlling T24 tumor cell proliferation and elimination.

When 5×10^4 or 1×10^5 freshly isolated allogeneic PBMCs were co-cultured with 1×10^4 T24 cells in the presence of live BCG, strong inhibition of T24 cell proliferation measured with ^{3}H-TdR was observed as compared with BCG-absent control (Fig. 1a). Because the BCG-susceptible cells are thought to be DCs, DCs from the PBMCs were pretreated with (0.1 mg/ml) live BCG for 6 h at 37°C. Then, after confirmation that the addition of live BCG-pretreated DCs alone did not affect the T24 cell proliferation, 1×10^4 T24 cells were incubated with an equal number of the indicated DCs together with 5×10^4 or 1×10^5 allogeneic PBMCs of the same donor. Profound inhibition of T24 cell proliferation was observed when live BCG-infected DCs were co-cultured with PBMCs of the same donor (Fig. 1b). Moreover, the effect of live or heat-inactivated BCG-treated DCs on T24 cell proliferation was also examined. As indicated in Fig. 1c, the addition of heat-inactivated BCG-pretreated DCs resulted in partial inhibition of the proliferation. These results indicated that some cells derived from allogeneic PBMCs activated by the live BCG-pretreated DCs might gain the capacity to inhibit the proliferation of T24 tumor cells.

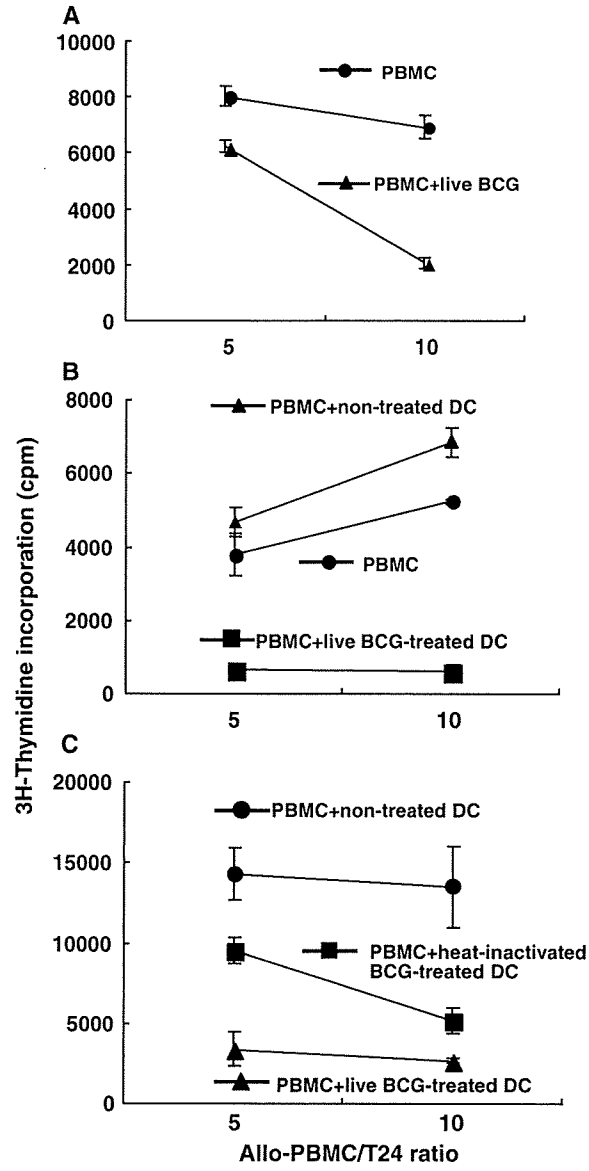

Fig. 1 Inhibition of T24 growth by allogeneic PBMCs activated with live BCG-treated DCs. **a** When 5×10^4 or 1×10^5 freshly isolated allogeneic PBMCs were co-cultured with 1×10^4 T24 cells in the presence of live BCG (*closed triangle*), strong inhibition of T24 cell proliferation measured by ^{3}H-TdR was observed in comparison with the BCG-absent control (*closed circle*). **b** T24 cells (1×10^4) were incubated with 5×10^4 or 1×10^5 allogeneic PBMCs. Profound inhibition of T24 cell proliferation was observed when live BCG-infected DCs were co-cultured with PBMCs (*closed square*). However, no inhibition was observed when T24 cells were co-cultured either with PBMC alone (*closed circle*) or with PBMC plus BCG-uninfected DCs (*closed triangle*). **c** T24 cells (1×10^4) were incubated with 5×10^4 or 1×10^5 allogeneic PBMCs plus live BCG-treated DCs (*closed triangle*), heat-inactivated BCG-treated DCs (*closed square*), or control untreated DCs (*closed circle*). Again, strong inhibition of T24 cell proliferation was observed when PBMCs were co-cultured with live BCG-infected DCs (*closed triangle*) and partial inhibition was seen when they were co-cultured with heat-inactivated BCG-treated DCs (*closed square*)

Kinetics of cytokine secretion by live BCG-treated DCs

Next, the live BCG-treated DCs were compared with the inactivated BCG-treated DCs in terms of the kinetics of cytokine secretion. As demonstrated in Fig. 2a with closed columns, live BCG-treated DCs secreted quite a large amount of IL-12 2–3 days after the incubation, however, heat-inactivated BCG-treated DCs shown with open columns secreted almost no detectable amount of IL-12. As for TNF-α, live BCG-treated DCs secreted more of it than heat-inactivated BCG-treated DCs (Fig. 2b). In contrast, the amount of IL-10 secretion was almost the same between the two (Fig. 2c). Furthermore, the expression levels of co-stimulated molecules, CD80 and CD86, were higher in live BCG-treated DCs (data not shown).

T24 growth inhibition was mainly mediated through CD8β-negative T cells

These findings suggest live BCG-activated DCs to activate effectors from PBMCs to inhibit T24 cell proliferation through the secretion of IL-12 and TNF-α. Therefore, to examine the actual cells that eliminate T24, CD3-positive T cells were eliminated from among the activated PBMCs with live BCG-treated DCs, and the cytotoxicity against ^{51}Cr-labeled T24 targets was measured. The cytotoxicity was significantly reduced by the elimination of the CD3-positive cells (Fig. 3a). The remaining weak cytotoxicity, shown as open circles in Fig. 3a, might be due to the effect of activated non-T cells such as NK cells. Moreover, the cytotoxicity against T24 cells was not inhibited by the elimination of CD8β-positive cells (shown as closed squares) (Fig. 3b). These results suggest that the class I MHC molecule-restricted conventional CD8β-positive CTL do not seem to be involved in this T24-related cytotoxicity.

T24 tumor growth was partially inhibited by Vγ2Vδ2 T cells

Collectively, the cytotoxity against T24 cells mediated through live BCG-treated DCs appeared to be provided by the major effectors of innate immunity; NK cells, NKT cells, and γδ T cells. Thus, we then examined the possible involvement of γδT cell effectors in the elimination of tumor cells. γδ T cells are classified into two distinct types, type-1 expressing Vγ1Vδ1 T-cell receptor (TCR) and type-2 expressing Vγ2Vδ2 TCR, with the majority of cells generated by BCG reported to be the latter type-2 γδ T cells [11]. When the Vδ2-positive type-2 γδT cells were eliminated from live BCG-activated PBMCs, slight inhibition of the cytotoxicity against T24 cells was observed and this was apparent when the Vδ2-positive cells were depleted from PBMCs before co-culturing with BCG-treated DCs

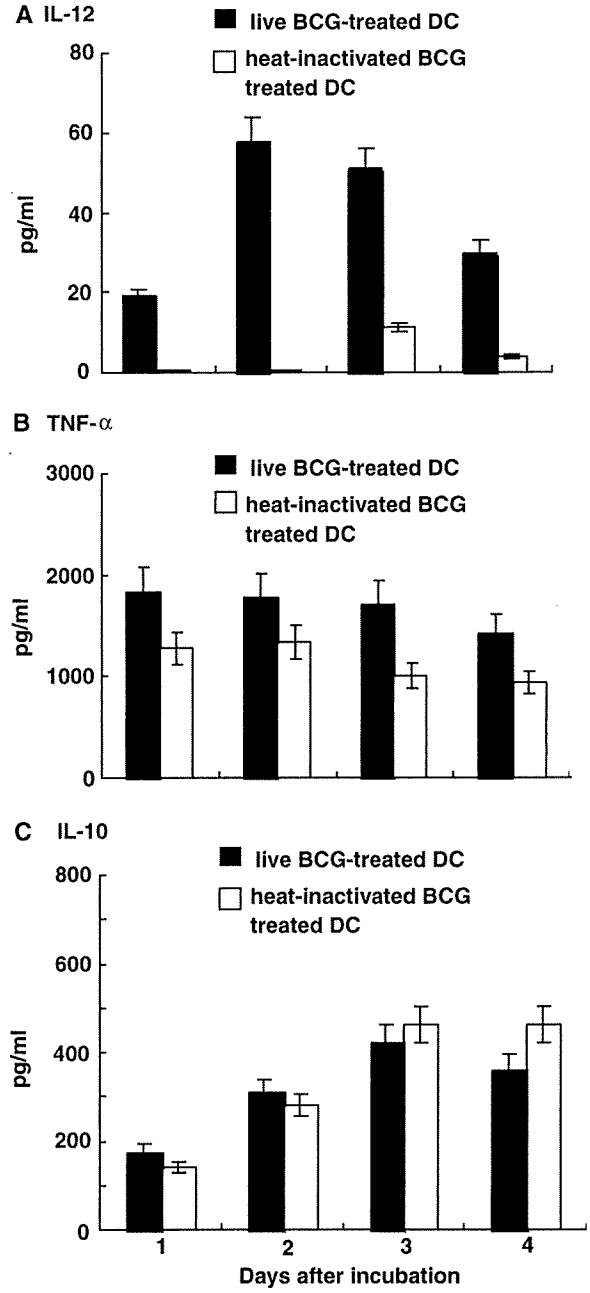

Fig. 2 Measurement of cytokine production by BCG-treated DCs.The difference in the kinetics of cytokine secretion between live BCG-treated DCs and inactivated BCG-treated DCs was compared. Live BCG-treated DCs (*closed column*) secreted predominantly large amounts of IL-12 (**a**) and larger amounts of TNF-α (**b**) than heat-inactivated BCG-treated DCs (*open column*), while the amount of IL-10 secretion (**c**) was lower than in the case of heat-inactivated BCG-treated DCs

(Fig. 3c). Moreover, PBMCs treated with risedronate, an activator of Vδ2 [8], showed strong anti-tumor effect against T24 cells (Fig. 3d). These results indicate the involvement of live BCG-activated Vγ2Vδ2 TCR-expressing type-2 γδT cells in the elimination of T24 tumor cells.

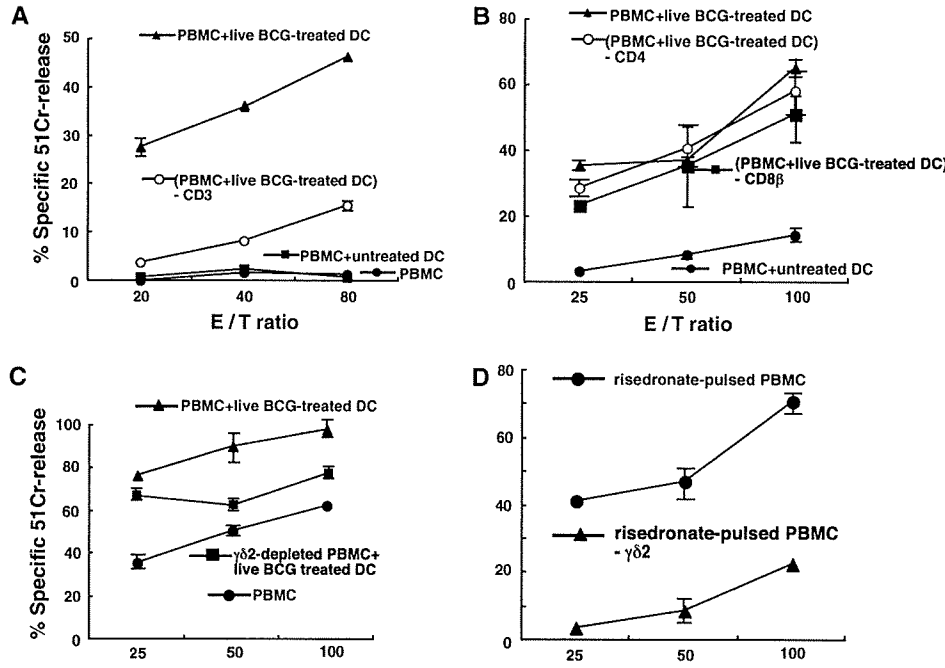

Fig. 3 T24 growth inhibition was partially mediated through CD8β-negative Vγ2Vδ2 T cells. **a** When CD3-positive T cells were eliminated from among the activated PBMCs with live BCG-treated DCs, the cytotoxicity against T24 tumor cells was significantly reduced (*open circle*) almost to the basal level mediated by normal PBMC (*closed circle*) or PBMC plus untreated DCs (*closed square*) in comparison with positive PBMCs activated with live BCG-treated DCs (*closed triangle*) and the remaining weak cytotoxicity might be due to the effect of activated non-T cells such as NK cells. **b** Such cytotoxicity against T24 cells was not abrogated by the elimination of CD8β-positive cells (*closed square*). The results suggest the class I MHC molecule-restricted conventional CD8β-positive CTLs do not seem to be involved in this T24-related cytotoxicity. The elimination of CD4-positive cells (*open circle*) from live BCG-activated PBMCs (*closed triangle*) did not result in any reduction of cytotoxicity against T24 targets in comparison with the basal level mediated by normal PBMC plus untreated DCs (*closed circle*). **c** When the Vδ2-positive cells were depleted from PBMCs before co-culturing with live BCG-treated DCs (*closed square*), the cytotoxicity against T24 was apparently reduced nearly by half in comparison with PBMCs activated with live BCG-treated DCs (*closed triangle*). **d** Moreover, the Vδ2 cell-enriched population from PBMCs co-cultured with risedronate showed a strong anti-tumor effect against T24 cells (*closed circle*). The elimination of type-2 $\gamma\delta$T cells resulted in a significant reduction in the cytotoxicity against T24 cells (*closed triangle*). These results indicate the involvement of live BCG-activated Vγ2Vδ2 TCR-expressing type-2 $\gamma\delta$T cells in the elimination of T24 tumor cells

Effect of depletion of CD161-positive cells on T24 growth

CD161 is known as a marker for NK and NKT cells. Thus, to examine the effect of NK and NKT cells on the elimination of T24 cells, CD161-positive cells were depleted from PBMCs activated by live BCG-treated DCs, and their elimination was confirmed by flowcytometry (Fig. 4a). After confirming that the CD161-positive cells were depleted, the cytotoxicity of the remaining cells against T24 cell was measured by ^{51}Cr-release assay. A profound reduction in the cytotoxity was observed when the CD161-positive cells were eliminated and the reduced cytotoxicity was slightly greater than that of the activated PBMCs co-cultured with live BCG-treated DCs (Fig. 4b). These findings suggest the residual $\gamma\delta$T cells after the elimination of CD161-positive cells to be slightly more cytotoxic than the activated NK cells in PBMCs. Also, as shown in Fig. 4c, that the live BCG-activated PBMCs showed far stronger cytotoxicity than untreated PBMCs against K562 cells that are known to be sensitive to NK cells indicates NK cells to be less cytotoxic to T24 tumor cells than innate $\gamma\delta$T or NKT cells. Taken together, these results suggest the most potent effectors among the live BCG-activated cells against T24 seem to be NKT cells.

Significant production and increase of perforin in the NKT cell population among PBMCs co-cultured with live BCG-treated DCs

Thus, to confirm the actual number and increase of NKT cells among PBMCs co-cultured with live BCG-treated DCs, a flow-cytometric analysis was performed. The results showed that the number of both $CD3^+CD56^+$ cells and $CD3^+CD161^+$ NKT cells but not $CD3^-CD56^+$ or $CD3^-CD161^+$ NK cells apparently increased among those PBMCs activated by live BCG-treated DCs but not by heat-

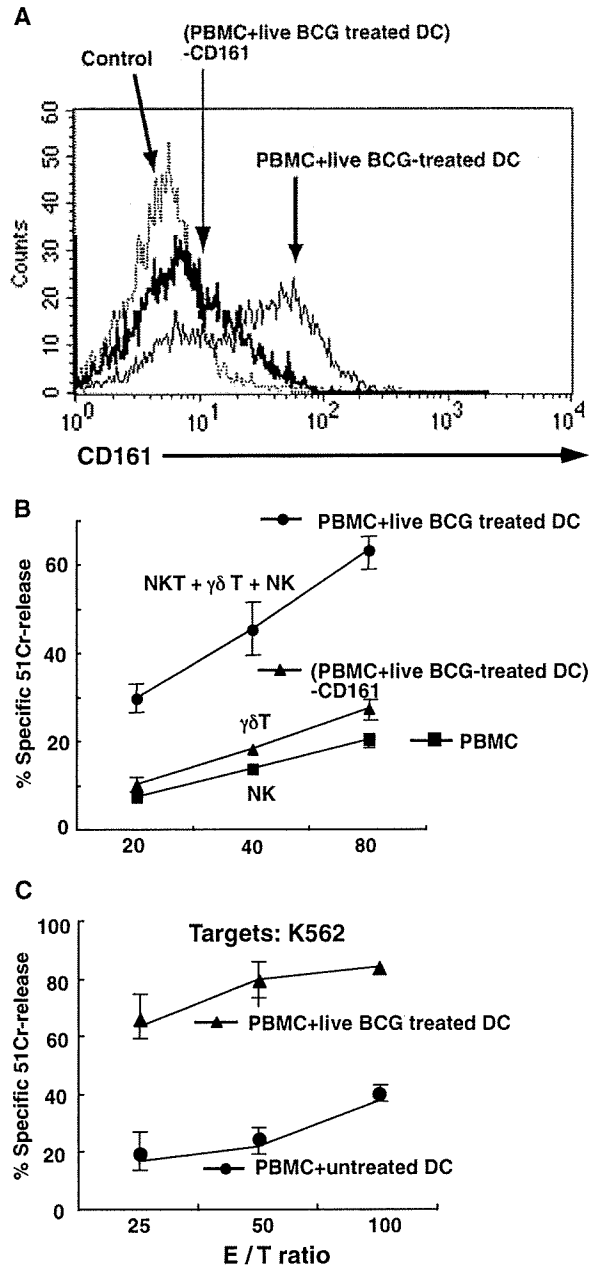

Fig. 4 Effect of CD161 positive cells depletion on T24 growth. **a** The elimination of CD161-positive cells was confirmed by flow cytometry. **b** The remaining cells after the elimination of CD161-positive cells (*closed triangle*) showed a profound reduction of cytotoxicity against T24 cells compared to live BCG-activated PBMCs (*closed circle*). The findings suggest that the residual $\gamma\delta$T cells after the elimination of CD161-positive cells had slightly stronger cytotoxicity than the activated NK cells in PBMCs (*closed square*). **c** PBMCs activated by live BCG-treated DCs (*closed triangle*) showed far stronger cytotoxicity than BCG-untreated PBMCs (*closed circle*) against NK-sensitive K562 cells. These results indicate that NK cells have weaker cytotoxicity against T24 tumor cells than innate $\gamma\delta$T or NKT cells activated by live BCG-treated DCs

inactivated BCG-treated DCs (Fig. 5a). Therefore, the number of NKT cells certainly increased in the live BCG-activated population. Moreover, those live BCG-activated NKT cells actually produced to secrete cytocidal molecules like perforin (Fig. 5b) or granzyme B (data not shown). Also, it should be noted that the live BCG-activated $\gamma\delta$T cells became effector/memory state expressing CD45RO from naïve state expressing CD45RA although the number of $\gamma\delta$T cells did not altered (data not shown).

Increased NKT cells inhibited T24 cells in a CD1d-unrestricted fashion

In general, NKT cells recognize glycolipid antigens presented by CD1d [23]. To clarify whether the live BCG-activated NKT cells see T24 tumor antigens in a CD1d-restricted manner, the expression of CD1d on T24 cells was investigated. Despite careful and intense examination, neither mRNA encoding CD1d nor the surface expression of CD1d was detected in not only untreated T24 cells but also BCG-treated ones (Fig. 6a, b), indicating that T24 will not express CD1d molecules even incorporating BCG into their cellular component.

Therefore, to exclude the possibility of subtle expression for functional CD1d on T24 cells after the BCG treatment, an established human NKT line (HT-AC2) that recognizes α-galactosyl ceramide (α-GalCer) and secretes IL-4 in a CD1d-restricted manner (Shimizu & Takahashi, manuscript in preparation) and C1R/CD1d cells expressing human CD1d gene, we examined whether NKT cells can recognize T24 cells in the presence of α-GalCer. No IL-4 was detected in the supernatant of the NKT cell line co-cultured with α-GalCer-pulsed T24 as well as BCG-treated T24 cells (Fig. 6c). Collectively, NKT cells but not NK cells induced by the live BCG-activated DCs seem to predominantly eliminate or suppress T24 tumor cells in a CD1d-unrestricted, α-GalCer independent fashion.

Possible tumor cell ligands for BCG-activated NKT cell recognition

Because NKG2D expression was observed on the NKT cells or $\gamma\delta$T cells expanded from live BCG-treated PBMCs (data not shown), blocking effect of antibodies for stress-associated tumor cell-specific molecules such as MICA/MICB [2, 10], the counterparts of NKG2D receptor, on the recognition of T24 cells was examined based on the recent finding [28]. As demonstrated in Fig. 7a, significant inhibition of the cytotoxicity mediated by activated NKT cells was seen when anti-MICA/MICB specific antibody was added, although isotype-

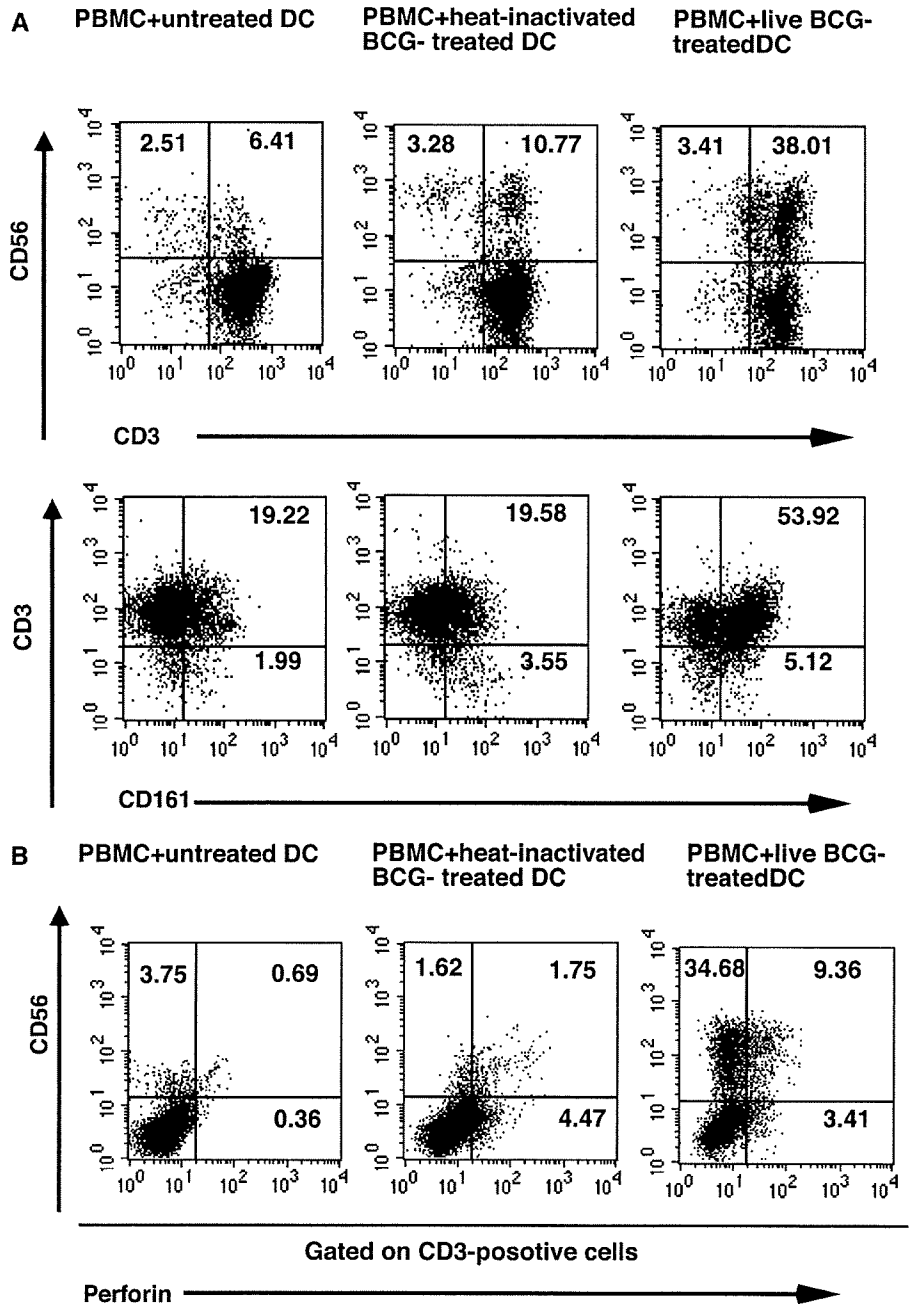

Fig. 5 Significant increase and perforin production in the number of NKT cells among PBMCs activated by live BCG-treated DCs. **a** Flow-cytometric analysis showed that the number of both $CD3^+CD56^+$ cells, and $CD3^+CD161^+$ NKT cells but not $CD3^-CD56^+$ or $CD3^-CD161^+$ NK cells apparently increased among those PBMCs activated by live BCG-treated DCs but not by heat-inactivated BCG-treated DCs. Therefore, the number of NKT cells certainly increased in the live BCG-activated population. **b** The live BCG-activated NKT cells actually produced to secrete cytocidal molecules like perforin, while heat-inactivated BCG-associated NKT did not

matched antibody did not show any inhibition for their cytotoxicity. Moderate inhibition was also seen when the NKG2D receptors blocked by their specific antibody (Fig. 7a). Similarly, cytotoxicity against T24 cells by live BCG-treated PBMCs containing mostly activated NKT cells as well as some $\gamma\delta$T and NK cells was markedly inhibited by anti-MICA/MICB specific antibody (Fig. 7b). Therefore, MICA/MICB molecules on the T24 cells appear to be a possible tumor cell ligands for BCG-activated innate NKT cell recognition.

Discussion

Intravesical BCG therapy is probably the most effective immunotherapy for recurrent superficial bladder cancer. As far as we have examined, the anti-tumor effect does not appear to be due to direct cytotoxicity of BCG itself. In fact, it was recently reported that the treatment of the urothelial carcinoma cell line T24 with BCG did not induce apoptosis, and BCG inhibited camptothecin-mediated apoptosis [7]. Similarly, treatment of T24 cells with BCG

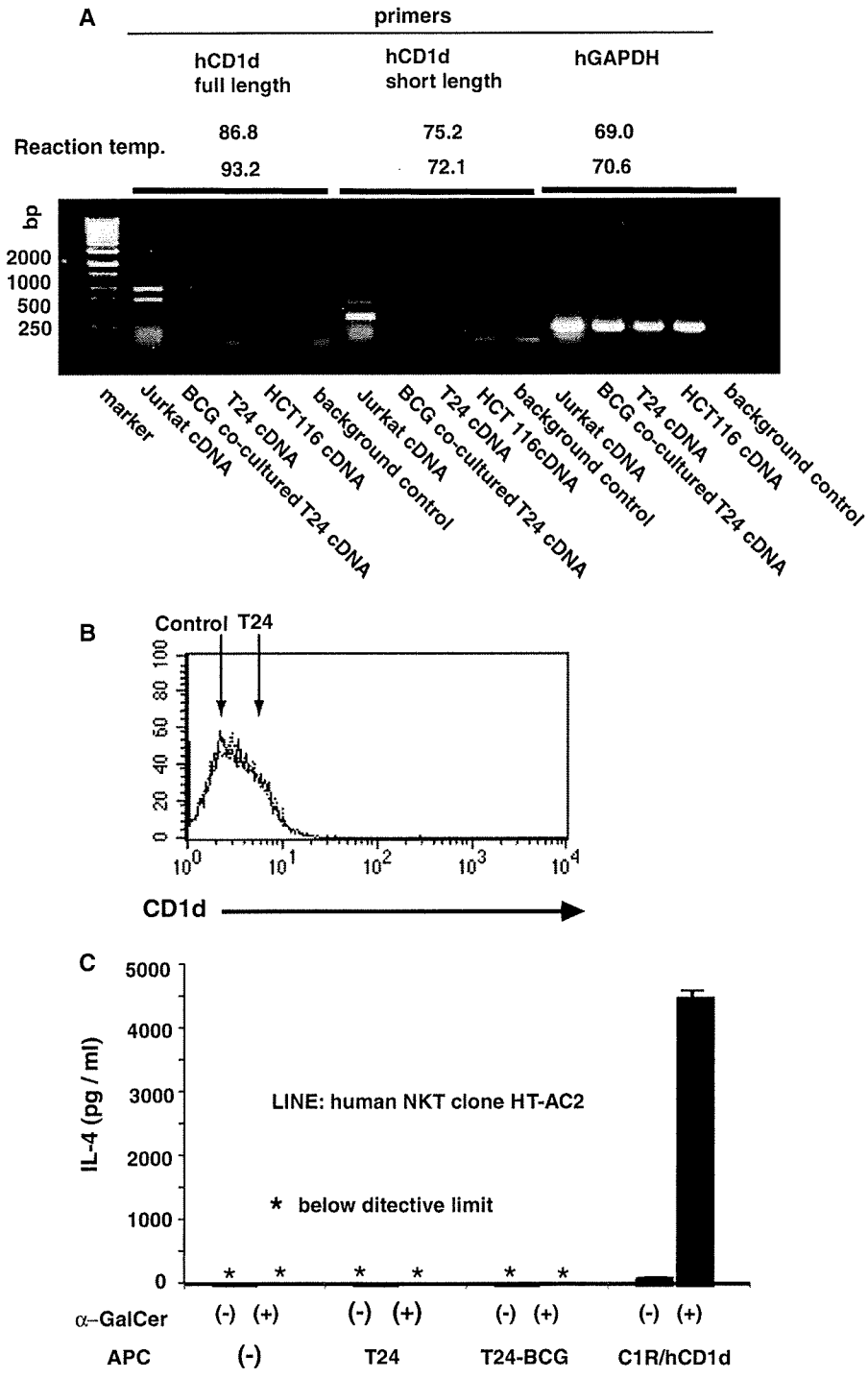

Fig. 6 Inhibition of T24 tumor growth by the BCG-activated NKT cells was mediated in a CD1d-unrestricted manner. NKT cells are usually recognized as antigens in association with CD1d molecules. Thus, **a** both internal mRNA for CD1d expression and **b** external surface expression were examined in T24 tumor cells. However, CD1d expression could not be detected at all even after co-cultured with live BCG. **c** Therefore, using an established human NKT line (HT-AC2) that recognizes α-galactosyl ceramide (α-GalCer) in a CD1d-restricted manner and secretes IL-4 (Shimizu & Takahashi, manuscript in preparation) and CD1d-expressing C1R cells (C1R/hCD1d), we investigated whether NKT cells can recognize live BCG-treated (T24-BCG) or untretaed T24 cells in the presence of α-GalCer. No IL-4 was detected in the supernatant of NKT cells co-cultured with α-GalCer-pulsed those T24 cells. Taken together, NKT cells generated by the live BCG-activated DCs seem to inhibit T24 tumor cell growth in a CD1d-unrestricted manner

did not cause any apoptotic changes as examined with a TUNEL assay [24]. Therefore, BCG itself does not eliminate T24 tumor cells but rather some immune system activated by BCG may indirectly inhibit the growth of these cells or eliminate them.

The body has two distinct immune systems to suppress tumor growth or eliminate tumor cells. One is systemic acquired immunity with highly specific effectors such as class I MHC molecule-restricted $CD8^+$ CTLs, class II MHC molecule-restricted $CD4^+$ T cells, and specific antibodies. These effectors express specific receptors originating from rearranged genes established by periodic stimulation. The magnitude of specific responses will increase synergistically with the number of stimulations.

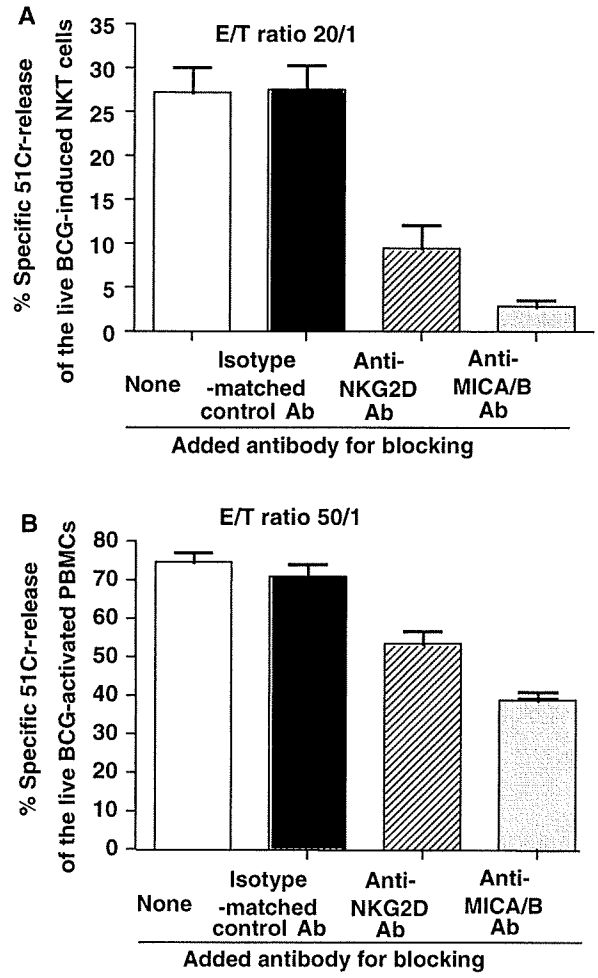

Fig. 7 Possible tumor cell ligands for BCG-activated NKT cell recognition. **a** Effect of antibodies for blocking stress-associated tumor cell-specific molecules such as MICA/MICB, the counterparts of NKG2D receptor, on the recognition of T24 cells was examined. Significant inhibition of the cytotoxicity mediated by activated NKT cells was seen when anti-MICA/MICB specific antibody was added, although isotype-matched antibody did not show any inhibition for their cytotoxicity. Moderate inhibition was also seen when the NKG2D receptors blocked by their specific antibody. **b** Cytotoxicity against T24 cells by live BCG-treated PBMCs containing mostly activated NKT cells as well as some $\gamma\delta$T cells and NK cells was also markedly inhibited by anti-MICA/MICB specific antibody

In contrast, local innate immunity involves toll-like receptors (TLR), $\gamma\delta$ TCR, or invariant NKT–TCR having diverse cross-reactivity without requiring the strict gene-rearrangement seen in the establishment of acquired immune receptors and their activation can be maintained by constant stimulation.

Also, as has been indicated, the bladder cancer cell line T24 expresses markedly down-modulated MHC class I molecules on its surface and the expression did not recover by the treatment with live BCG or live BCG-infected DCs. Thus, the T24 tumor would be recognized in a MHC molecule-unrestricted manner. Hence, we co-cultured the T24 cells with allogeneic PBMCs in the presence of live BCG and found a profound inhibition of tumor growth in vitro. A similar strong inhibition of T24 cell proliferation was observed when live BCG-infected DCs were co-cultured with PBMCs of the same donor. Moreover, the elimination of T24 cells was achieved mostly by CD3-positive innate effectors such as Vγ2Vδ2 TCR-expressing $\gamma\delta$T cells and NKT cells having predominant cytotoxicity, but not by class I MHC molecule-restricted conventional CD8β-positive CTLs, and the innate effectors were activated by live BCG-infected DCs rather than heat-inactivated BCG-treated DCs. Furthermore, the number of NKT cells but not $\gamma\delta$T cells or NK cells certainly increased in the live BCG-activated population.

These results strongly suggest that cells that control T24 tumor growth are not conventional class I MHC molecule-restricted CD8$^+$ CTL in the acquired arm but rather MHC molecule-unrestricted $\gamma\delta$T and NKT cells in the innate arm through the activation of DCs by live BCG. The results are reasonable in that continuous stimulation in the limited confined mucosal compartment of the bladder by a live organism may activate local innate effectors. Although the possible involvement of acquired effectors like CD8$^+$ CTLs in the prevention of surface bladder tumor expansion by intravesical BCG therapy has not be excluded, the data obtained in the present study strongly indicate a dominant effect of innate cells on tumor recurrence at the confined mucosal surface. Moreover, the cytotoxic effect of innate NKT or $\gamma\delta$T cells on T24 tumor cells was mediated thought stress-associated tumor-specific MICA/MICB molecules via their NKG2D receptors but not CD1d molecule-restricted invariant NKT-TCRs, indicating that these invariant TCRs are required mainly for their activation.

If this is the actual reason why intravesical BCG therapy is most successful immunotherapy against solid tumors in terms of preventing recurrence, we must focus on the constant activation of innate immunity for the treatment of other solid tumors and preventing their spread by metastasis. The findings shown in the present study will open the new notion that constant stimulation of innate effectors such as MHC molecule-unrestricted $\gamma\delta$T and NKT cells with live microorganisms like BCG through the activation of local DCs may provide a novel therapeutic way for cancer treatment.

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Quick method of multimeric protein production for biologically active substances such as human GM-CSF (hGM-CSF)

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ABSTRACT

The C-terminal fragment of C4b-binding protein (C4BP)-based multimerizing system was applied to hGM-CSF to induce dendritic cells (DCs) from peripheral blood monocytes (PBMCs), to see whether the C4BP could stimulate immature DCs, since DCs, equipped with pattern recognition receptors such as toll-like receptors (TLRs), are hypersensitive to various immunologically active molecules like LPS. *hGM-CSF* gene was merged to the 3′-terminal region of the *C4BPα-chain* gene, and the transfected human 293FT cells produced sufficient amount of octameric hGM-CSF, which resulted in iDCs with the same phenotype and the same response to a TRL4 ligand, LPS and a TLR3 ligand, poly I:C, as those induced with authentic monomeric hGM-CSF. These results suggest that the C4BP-based multimerizing system could facilitate the design of self-associating multimeric recombinant proteins without stimulating iDCs, which might be seen with the other multimerizing systems such as that using Fc fragment of IgM.

Introduction

Recombinant proteins are generally produced as single chain molecules. In contrast, natural biological ligand–receptor systems are often multimeric, assembling different molecules into complexes and bringing together different functions. Moreover, it is frequently observed that monomeric ligand–receptor interactions at the cell surface are not able to trigger signal transduction or cellular activation. There have only been a few attempts to produce recombinant multimeric molecules mimicking natural biological system, which were usually unsatisfactory mainly due to the undesirable effects of these multimerizing systems such as the immunoglobulin Fc fragment-based multimerizing system that interacts with cell surface receptors and activates complement [1,2]. There are also multimerizing systems based on leucine zippers [3], chemical polyethylene glycol linkage [4], diabodies [5], streptavidin [6], and protein A [7], which use intracellular or foreign proteins. Therefore, they are likely being immunogenic. Chemical linkages using polyethylene glycol are not stable enough *in vivo* and do not mimic natural biological complexes [4].

C4b-binding protein (C4BP) is a spider-like molecule [8,9] involved in the regulation of the complement cluster family and consists of short consensus repeat units (Fig. 1). Five to seven α-chains, that bind C4b and one β chain, which binds protein S, or eight α-chains only are covalently associated together at their C-terminal portion [10]. A multimerizing system was designed based on the C-terminal portion of the *C4BPα chain gene* [11], and the *hGM-CSF* gene was selected as an example of a multimeric recombinant protein to see whether iDCs, which are among the most sensitive cells to immunological stimulation, could be induced from peripheral blood mononuclear cells to confirm that the C4BPα-multimerizing system does not show immunostimulation.

Materials and methods

Cells and medium. 293FT cells (Invitrogen) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma–Aldrich) supplemented with 10% FCS (Moregate, Queensland, Australia), penicillin (50 U/ml), and streptomycin (50 U/ml) (Invitrogen). HepG2 cells were obtained from ATCC (Manassas, VA, USA).

Antibodies for cell staining. The mouse mAbs HI149 (anti-human CD1a), M-T101 (anti-human CD1b), FITC conjugated mouse anti-human mAbs, G46-2.6 (anti-HLA-abc) and G46-6 (anti-HLA-DR), and phycoerythrin (PE)-conjugated mouse mAb HB15e (anti-CD83) and IT2.2 (anti-CD86) were all purchased from BD Pharmingen (San Diego, CA, USA). PE-conjugated goat $F(ab')_2$ antibody to mouse IgG (IM0855) was from Beckman Coulter (Fullerton, CA, USA).

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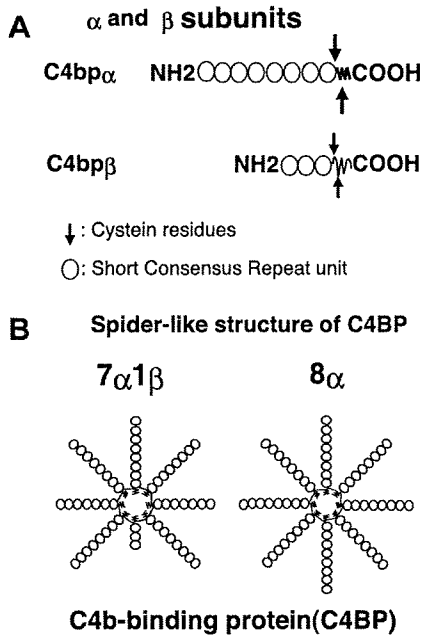

Fig. 1. Spider-like structure of the C4b-binding protein (C4BP). (A) There are two subunits of C4BP: C4BPα consists of eight short consensus repeat (SCR) units and a C-terminal sequence. C4BPβ consists of three SCR units and a C-terminal sequence. (B) C4BP is a high molecular mass (570 kDa) glycoprotein in plasma. The major form is usually composed of seven C4Bpα subunits and one C4BPβ subunit (7α1β) or eight C4Bpα subunits (8α) linked by disulfide bridges. In electron microscope-derived images, C4BP appears as a spider-like structure [9].

DNA constructs. The human *GM-CSF* gene was amplified from the plasmid pCM-hGM (RIKEN DNA Bank, Japan) [12] by PCR using the following primers:

5′-CGCGGATCCATGTGGCTGCAGAGCCTGCT-3′
5′-GGAATTCCTCCTGGACTGGCTCCCAGC-3′

Amplified DNA was digested with BamHI and EcoRI. The digested DNA was subcloned between BamHI and EcoRI sites of plasmid pEF-1a/Myc/6× His/A (Invitrogen) and designated as hGM-CSF/EF1/Myc/His.

Total RNA was extracted from human HepG2 cells using an RNeasy kit (Qiagen, Hilden Germany) and the C-terminal region of the human *C4BPα* gene was cloned by RT-PCR methods using the following primers:

A-1: 5′-CGGAATTCTTCGGGACAGGTCCTGC-3′
A-2: 5′-GCTCTAGATAGTTCTTTATCCAAAG-3′

The amplified C-terminal region of the human *C4BPα* gene was digested with EcoRI and XbaI, subcloned 3′ to the *hGM-CSF* gene in hGM-CSF/EF1/Myc/His between the EcoRI and XbaI sites, and designated as hGM-CSF-C4BPαEF1/Myc/His.

Production of recombinant multimeric GM-CSF. Transfection of 293FT cells with hGM-CSF/EF1/Myc/His or hGM-CSF-C4BPα/EF1/Myc/His was performed using polyethyleneimine as described previously [13,14], and G418-resistant clones were selected. The conditioned culture media of the selected clones were collected, sterilized with a 0.2 μm filter, and quantified using hGM-CSF ELISA kit (R&D Systems, Minneapolis, MN, USA) for the further experiments. As a reference, recombinant hGM-CSF purchased from Peprotech EC (London, Great Britain) was used (see Fig. 2).

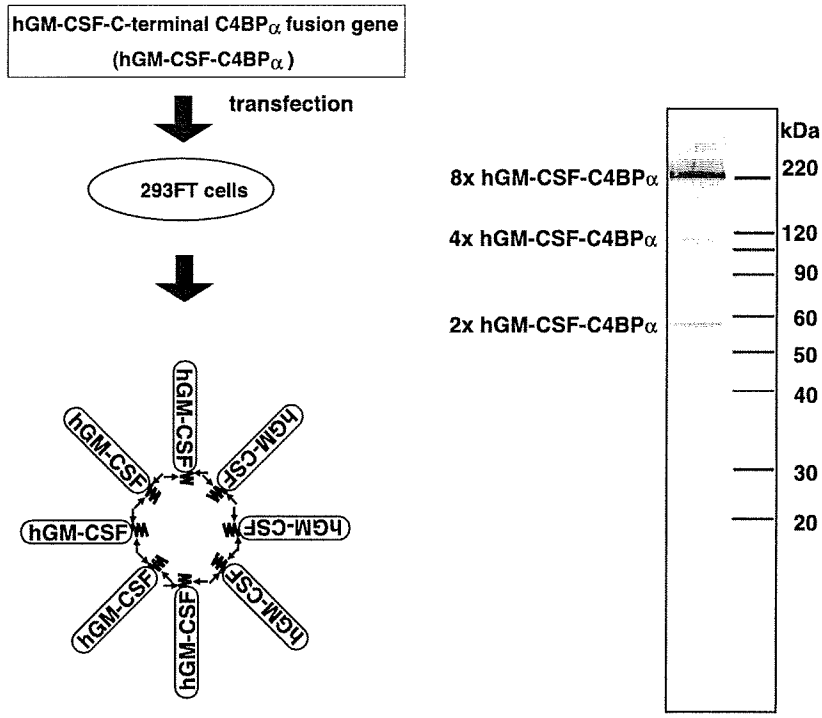

Fig. 2. Production of recombinant octameric hGM-CSF. The hGM-CSF-C4BPα fusion gene was transfected into 293T cells. An immunoblot analysis with anti-myc-epitope antibody revealed a molecular mass of 224 kDa, indicating an octamer of hGM-CSF, as well as small amounts of 112 kDa (tetramer) and 56 kDa (dimer) proteins.

PBMC-derived immature DCs. Immature DCs were obtained from PBMCs as described previously [13]. Briefly, PBMCs were freshly isolated with Ficoll-paque (Amersham-Pharmacia, Uppsala, Sweden) from the peripheral blood of healthy volunteers, and $CD14^+$ monocytes were separated immediately by magnetic depletion using a monocyte isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) containing hapten-conjugated antibodies to CD3, CD7, CD19, CD45RA, CD56, and anti-IgE Abs, and a magnetic cell separator (MACS, Miltenyi Biotec) in accordance with the manufacturer's instructions, routinely resulting in >90% purity of $CD14^+$ cells. The cells were cultured in 24-well culture plates for 6–7 days in complete medium supplemented with 20 ng/ml IL-4 (Biosource Intl., Camarillo, CA, USA) and 50 ng/ml of hGM-CSF obtained from PeproTech EC, or from the conditioned culture medium of 293FT cells transfected with *hGM-CSF* gene or *hGM-CSF-C4BPα* gene, in order to obtain iDCs. After 4–6 days of incubation, fluorescent activated cell sorter (FACS) analysis was performed to analyze the phenotype of the cells.

Immunoblotting. The cells were lysed in triple-detergent lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 0.1% SDS, 100 g/ml phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 1% Nonidet P-40, and 0.5% sodium deoxycholate). The obtained samples were run on a 4–12% NuPAGE Bis–Tris gel (Invitrogen) using MES (morpholine ethanesulfonic acid), SDS (sodium dodecyl sulfate) buffer (1000 mM MES, 1000 mM Tris, 70 mM SDS, 20 mM EDTA) under non-reducing conditions and transferred to a PVDF (polyvinylidene difluoride) membrane (ATTO, Tokyo, Japan). The membrane was incubated with rabbit anti-myc serum (Invitrogen) and immunoblotting was carried out using horseradish peroxidase conjugated-goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA) and a 3,3′,5,5′-tetramethylbenzidine (TMB) substrate kit for peroxidase (VECTOR Laboratory, Burlingame, CA).

Electron microscopy. The conditioned culture medium containing hGM-CSF-C4BPα gene-derived multimeric hGM-CSF was dialyzed against 0.1 M NH_4OAc/0.05 M NH_4HCO_3, pH 7.35, were adsorbed to thin carbon films and were negatively stained with 4.0% uranyl acetate. The photographs were taken at a primary magnification of 40,000 in a Hitachi H-7500 transmission electron microscope, operating at 80 kV.

Results

hGM-CSF produced by 293FT cells could induce iDCs

The first question was whether 293FT cells could produce enough amount of hGM-CSF to prepare iDCs from PBMCs. Human embryonic 293FT cells were transfected with the *hGM-CSF* gene and the hGM-CSF concentration was analyzed by ELISA. As a result, the hGM-CSF concentration in the conditioned medium was always high enough for dendritic cell preparation; between 150 and 200 ng/ml after transient transfection, or with cloned 293FT cells producing hGM-CSF.

The second question was whether the conditioned medium of the *hGM-CSF*-transfected 293FT cells could induce iDCs together with IL-4, because dendritic cells are equipped with pattern recognition receptors such as toll-like receptors that enable DCs to respond to very scarce amounts of stimulants such as LPS or nucleotides. Flow cytometric analysis of DCs prepared with the conditioned culture medium of *hGM-CSF*-transfected 293FT and commercial IL-4 showed the typical phenotype of iDCs derived from PBMCs, suggesting that the conditioned medium of 293FT did not contain any stimulants that induced the maturation of DCs (Fig. 3, right panels).

Production of multimeric hGM-CSF

Next, it was attempted to produce multimeric hGM-CSF using the *hGM-CSF-C4BPα* gene to see whether it could induce iDCs from PBMCs as well as authentic monomeric hGM-CSF. A chimeric gene of *hGM-CSF* fused to *C4BPα* was transfected into 293FT cells and the conditioned culture medium was analyzed by ELISA for hGM-CSF. Immunoblotting was also performed to analyze the *hGM-CSF-C4BPα* chimeric gene product under non-reducing conditions, which was observed as a band with a molecular mass of 220 kDa as well as faint bands with molecular masses of 56 and 112 kDa (Fig. 3). The predicted molecular weight of the monomeric *hGM-CSF-C4BPα* gene product is 28 kDa (Table 1), indicating that the major product of the *hGM-CSF-C4BPα* gene was an octameric protein, but dimer and tetramer proteins were also produced.

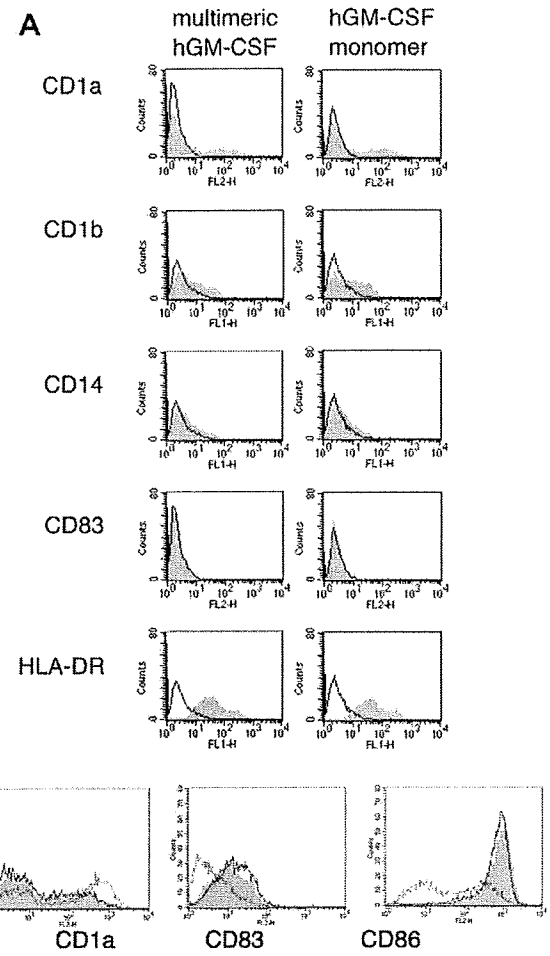

Fig. 3. FACS analysis of the immature dendritic cells. (A) The hGM-CSF gene or the hGM-CSF-C4BPα fusion gene was transfected into 293FT cells and conditioned medium containing hGM-CSF or multimeric GM-CSF was used in combination with IL-4 to induce dendritic cells from peripheral blood monocytes. The resultant DCs either with hGM-CSF (right panels) or multimeric GM-CSF (left panels) showed an identical phenotype each other. The results from the hGM-CSF gene and the hGM-CSF-C4BPα fusion gene are shown as grey lines and negative control data are shown as black lines. (B) The immature DCs induced with multimeric hGM-CSF showed the same response to LPS and polyI:C as that of the iDCs induced with hGM-CSF. Multimeric hGM-CSF-induced iDCs were incubated with LPS (200 ng/ml) (solid line) or poly I:C (100 μg/ml) (filled line) for 48 h and FACS analysis was performed. The iDCs without LPS or poly I:C is shown as dotted line.

Visualization of multimeric hGM-CSF

The purified multimeric hGM-CSF was examined in the electron microscope and a typical field view is shown in Fig. 4A. Several hGM-CSF-C4BPα molecules were examined and the molecules

Table 1
Characteristics of hGM-CSF-C4Bpα monomer with myc and 6× histidine tag.

	Value*
Length	245 amino acids
Molecular weight	27961.0 Da
Isoelectric point	5.07
Net charge at pH 7	−12.3

* The values were calculated using Gene Inspector 1.6 software (Textco BioSoftware, West Lebanon, NH, USA).

exhibited a morphology that resembles the aggregation of globular molecules (Fig. 4B). Human GM-CSF is a flattened globular molecule that is about 4.0 and 2.4 nm thick [15], the size of which corresponds with the electron micrographs of multimeric hGM-CSF.

Multimeric hGM-CSF produced by 293FT cells could induce iDCs

Finally, the induction of iDCs from PBMC using recombinant IL4 and multimeric hGM-CSF was attempted. The conditioned culture medium of 293FT cells transfected with *hGM-CSF-C4BPα* gene and recombinant IL4 were used to make iDCs from PBMC. FACS analysis showed the typical phenotype of iDCs (Fig. 3, left panels). The obtained iDCs were also incubated with either LPS (200 ng/ml for 48 h) or poly I:C (100 μg/ml for 48 h) and the typical phenotypic responses of iDCs to LPS and poly I:C were observed, such as the down-regulation of CD1a surface expression and up-regulation of CD83 and CD86 surface expression (Fig. 3B).

Discussion

For the protein expression, there are several methods such as those using *Escherichia coli*, insect cells, or mammalian cells, but each has their own advantages and disadvantages [16]. First, *E. coli* expression techniques are probably the most popular. The techniques necessary to express sufficient amounts of protein are relatively simple and the amount of time necessary to generate an over-expressing strain is very short. Therefore, *E. coli* is widely used for the expression of commercially important proteins. However, protein expression in *E. coli* does have some disadvantages. Recombinant eukaryotic proteins produced in *E. coli* are not properly modified and often precipitate into insoluble aggregates called "inclusion bodies." The recombinant protein could only be recovered in an active form by solubilization in denaturing agents followed by careful renaturation. Moreover, it is relatively difficult

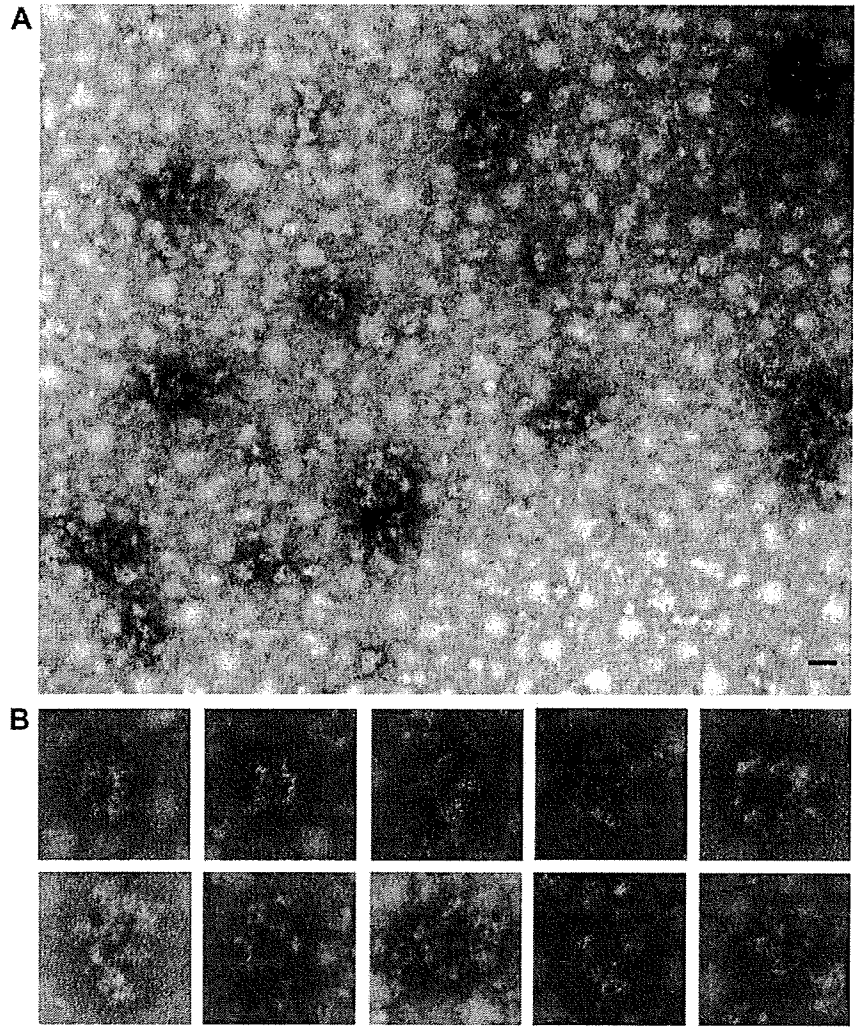

Fig. 4. Electron micrographs of multimeric hGM-CSF. (A) Field view of multimeric hGM-CSF. (B) Selected images demonstrating the aggregation of globular molecules of hGM-CSF. The scale bar in (A) represents 60 nm.

to arrange the secretion of the expressed proteins from *E. coli* if the amount is large. In addition, if the desired recombinant protein was hGM-CSF for DC preparation, the hGM-CSF produced should absolutely be without any trace of LPS, but it is very difficult to obtain recombinant proteins expressed in *E. coli* that are free of LPS. The baculoviral expression system using insect cells also has a number of advantages. With this system, proteins can be expressed at high levels and usually in the proper cellular compartment. For example, membrane proteins are usually localized to the membrane and nuclear proteins to the nucleus in insect cells as well as in mammalian cells, although the proteins expressed in insect cells are not always properly modified. Compared to the above two systems, mammalian expression techniques have certain advantages, especially for the expression of higher eukaryotic proteins. The expressed proteins are usually properly modified and they accumulate in the correct cellular compartment, but it is difficult to perform large-scale expression experiments. To obtain large amounts of recombinant protein using eukaryotic cells, CHO cells are often used because of the high amounts of the desired protein that can be obtained via dihydrofolate reductase (DHFR) [17] based amplification of recombinant genes using increasing concentrations of methotrexate (MTX) [18]. However, the DHFR amplification process is lengthy and may require several months to isolate and characterize a stable, amplified line of CHO cells. Moreover, even after the long amplification period, the amount of product is not always enough for some experimental uses.

This study utilized 293FT cells instead of CHO cells, which were transfected with the *hGM-CSF* gene to see if they could produce sufficient amounts of hGM-CSF to induce iDCs in combination with IL-4 in a laboratory setting. All the procedures took no more than one month from the cloning of the gene, which included subcloning into the vector plasmid, transfection into 293FT cells, production of sufficient amounts of hGM-CSF, and analysis of the quality of the hGM-CSF obtained for the preparation the iDCs. The resultant iDCs showed the same phenotype as iDCs incubated with commercial hGM-CSF and IL-4 as well as the same response to a TLR-4 ligand, LPS, and to a TLR-3 ligand, poly I:C.

Recently, many biologically active substances, such as cytokines and chemokines, have been identified, some of which are often needed for use in further experiments immediately. Therefore, there is a clear need for methods to obtain newly identified biologically substances immediately and without difficulty. The eukaryotic expression system using 293FT cells is a promising candidate because this system can obtain biologically active substances of good quality within a few weeks, and the amount of these substances is usually enough for further experiments because these substances have very high biological activity.

Furthermore, using a C-terminal fragment of the *C4BPα* gene fused to the 3′ end of the *hGM-CSF* gene, the recombinant multimeric hGM-CSF was obtained, which was successfully secreted into the culture medium of the cells despite of its high M_W of 200 kDa, and it was shown that this recombinant hGM-CSF could be used for iDCs preparation from PBMCs, whose phenotype was exactly the same as those iDCs incubated with authentic monomeric hGM-CSF and IL-4. These iDCs, prepared with multimeric hGM-CSF and IL-4, responded to LPS and poly I:C in exactly the same manner as those prepared with authentic monomeric hGM-CSF, which supported the notion that the C4BPα-multimerizing system can be used without the potential risk of immunological stimulation, because iDCs are among the cells that are most sensitive to immunological stimulation. Furthermore, it took no more than one month to obtain multimeric hGM-CSF with the C4BPα-multimerizing system using 293FT cells, which was enough to obtain iDCs from PBMCs.

In conclusion, the present C4BP-based multimerizing system combined with 293FT cells was shown to be the promising quick method to produce sufficient amounts of recombinant multimeric protein with biological activity without any untoward stimulation of the immune system.

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