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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′-GCCTCAAGATCATCAGCAATGC-3′) and antisense-primer (5′-ATGCCAGTGAGCTTCCCGTTC-3′), human CD1d (hCD1d) full-length sense-primer (5′-CGGGATCCATGGGGTGCCTGCTGTTTCTG-3′), antisense-primer (5′-ATTTGCGGCCGCCAGGACGCCCTGAT-3′), hCD1d short fragment sense-primer (5′-CTCCAGATCTCGTCCTTCGCCATT-3′), antisense-primer (5′-TTGAATGGCCAAGTTTACCCAAAG-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.

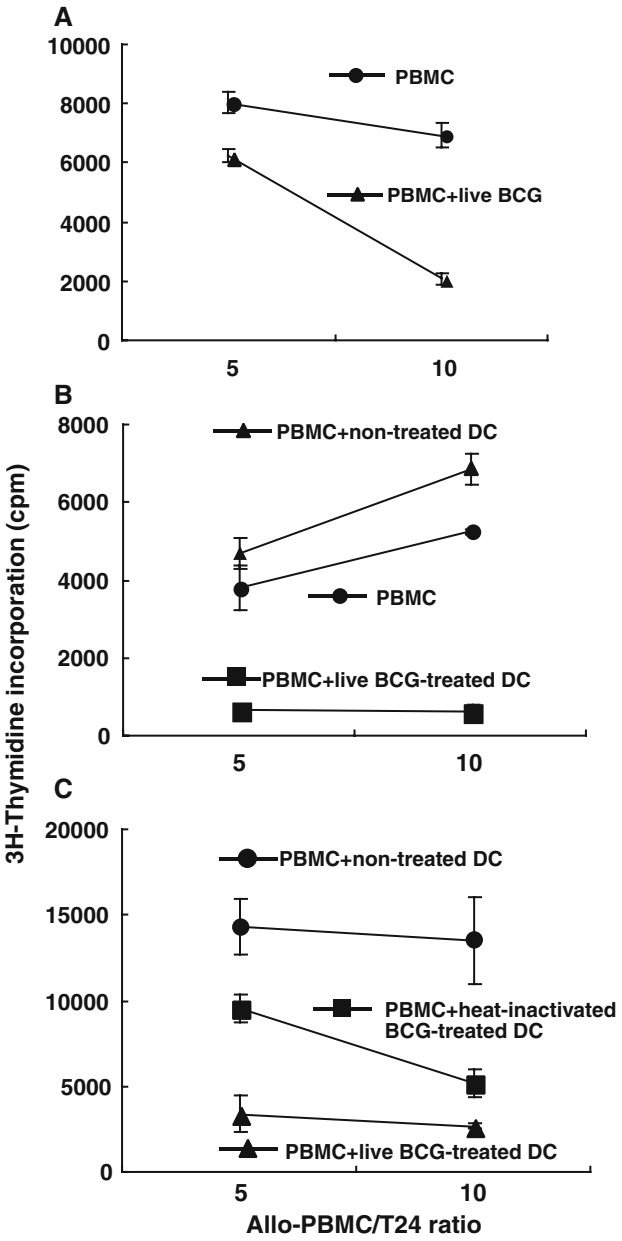

Fig. 1 Inhibition of T24 growth by allogeneic PBMCs activated with live BCG-treated DCs. **a** When 5×10^4 or 1×10^5 freshly isolated allogeneic PBMCs were co-cultured with 1×10^4 T24 cells in the presence of live BCG (*closed triangle*), strong inhibition of T24 cell proliferation measured by ^{3}H-TdR 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 $\gamma\delta$ T cells. Thus, we then examined the possible involvement of $\gamma\delta$T cell effectors in the elimination of tumor cells. $\gamma\delta$ 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 $\gamma\delta$ T cells [11]. When the Vδ2-positive type-2 $\gamma\delta$T 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

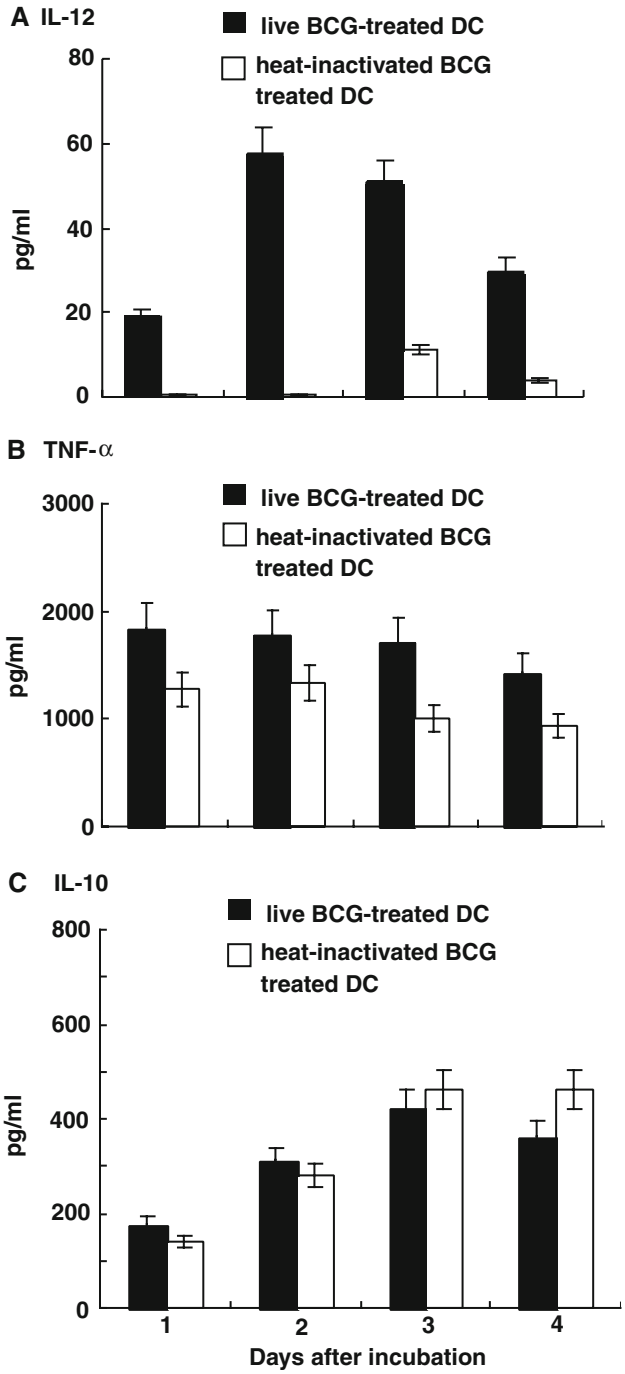

Fig. 2 Measurement of cytokine production by BCG-treated DCs.The difference in the kinetics of cytokine secretion between live BCG-treated DCs and inactivated BCG-treated DCs was compared. Live BCG-treated DCs (*closed column*) secreted predominantly large amounts of IL-12 (**a**) and larger amounts 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 $\gamma\delta$T cells in the elimination of T24 tumor cells.

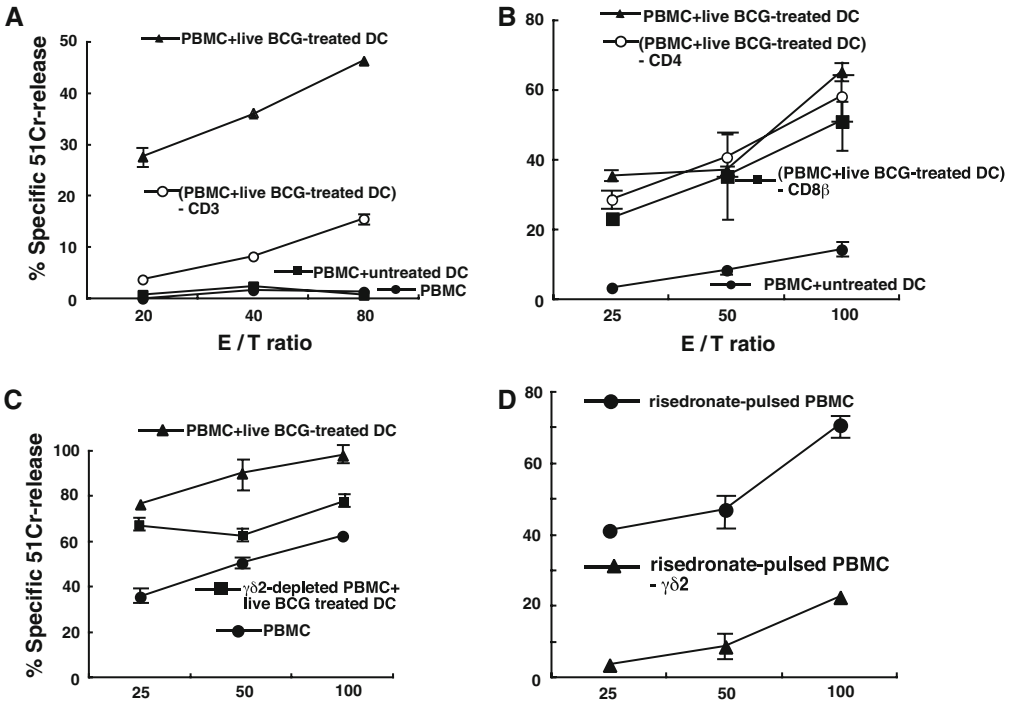

Fig. 3 T24 growth inhibition was partially mediated through CD8β-negative Vγ2Vδ2 T cells. **a** When CD3-positive T cells were eliminated from among the activated PBMCs with live BCG-treated DCs, the cytotoxicity against T24 tumor cells was significantly reduced (*open circle*) almost to the basal level 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-

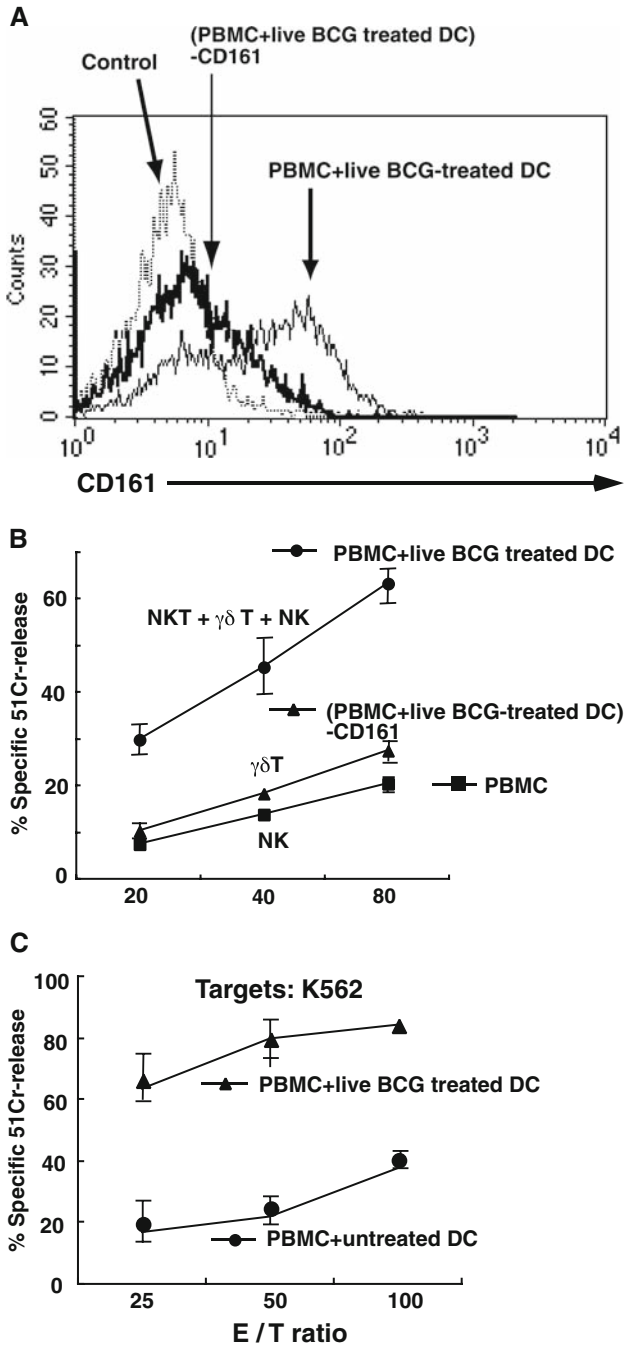

Fig. 4 Effect of CD161 positive cells depletion on T24 growth. **a** The elimination of CD161-positive cells was confirmed by flow cytometry. **b** The remaining cells after the elimination of CD161-positive cells (*closed triangle*) showed a profound reduction of cytotoxicity against T24 cells compared 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-

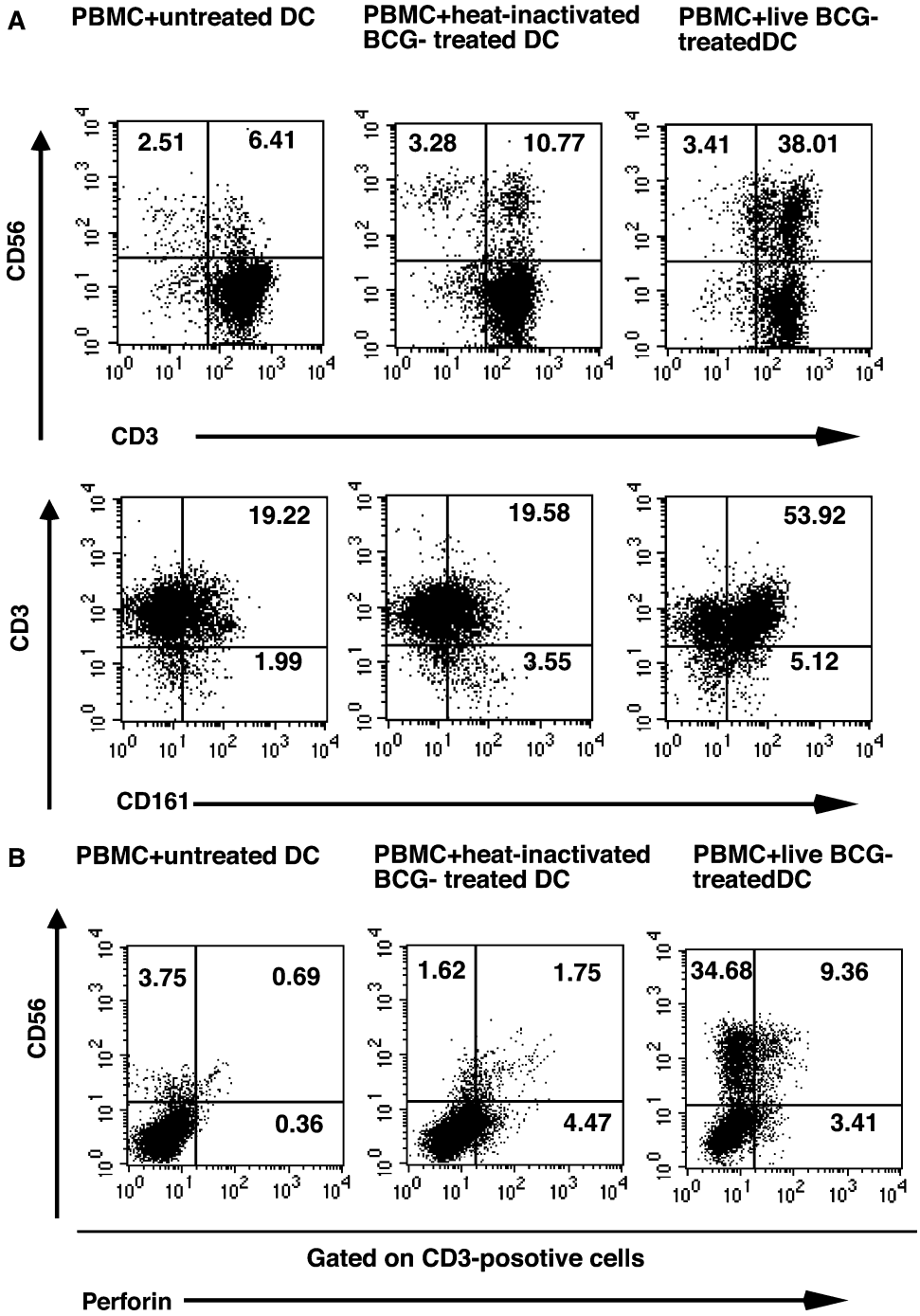

Fig. 5 Significant increase and perforin production in the number of NKT cells among PBMCs activated by live BCG-treated DCs. **a** Flow-cytometric analysis showed that the number of both $CD3^+CD56^+$ cells, and $CD3^+CD161^+$ NKT cells but not $CD3^-CD56^+$ or $CD3^-CD161^+$ NK cells apparently increased 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

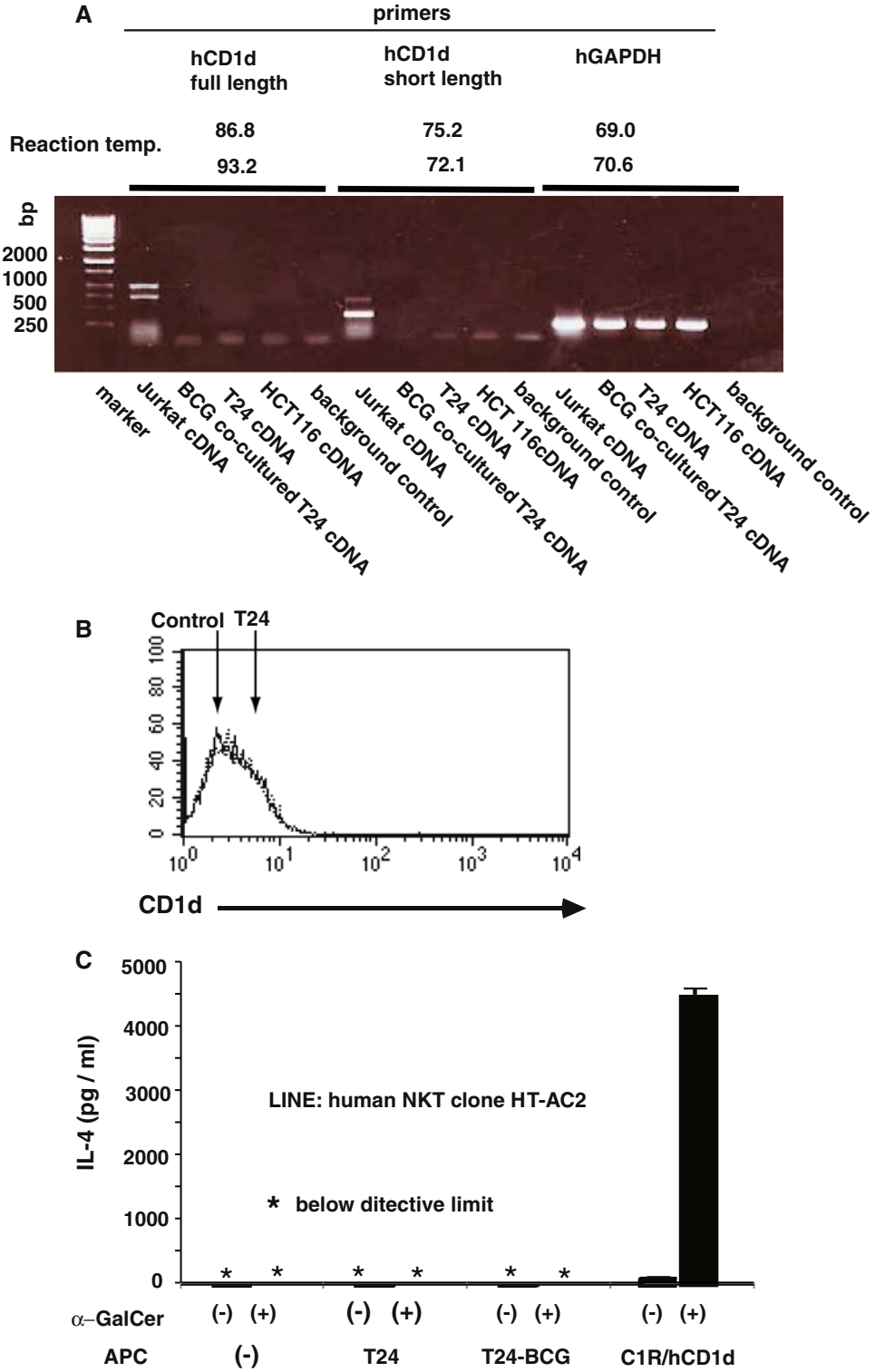

Fig. 6 Inhibition of T24 tumor growth by the BCG-activated NKT cells was mediated in a CD1d-unrestricted manner. NKT cells are usually recognized as antigens in association with CD1d molecules. Thus, **a** both internal mRNA for CD1d expression and **b** external surface expression were examined in T24 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.

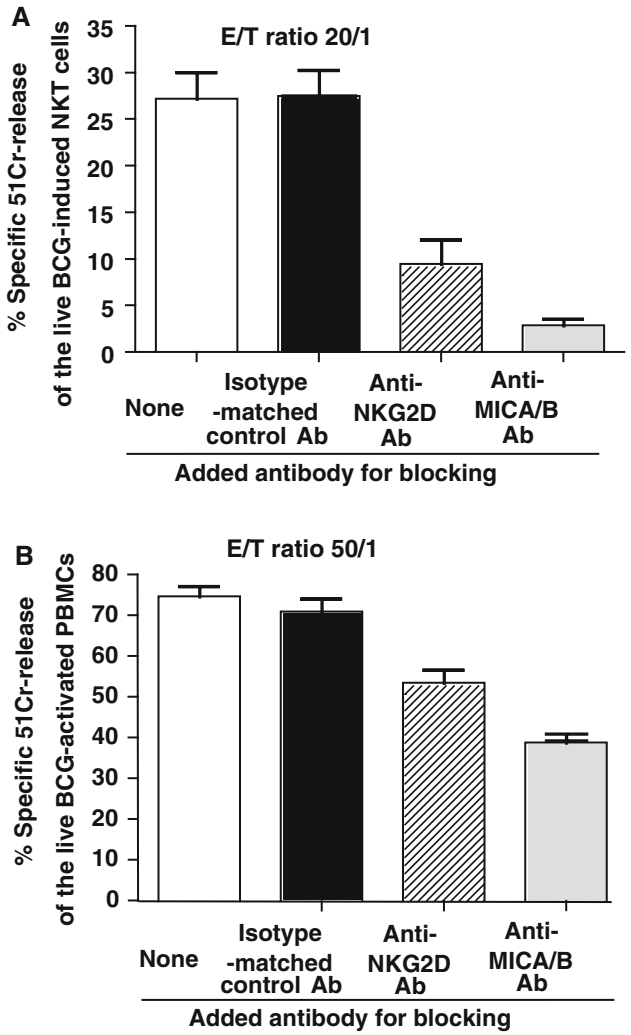

Fig. 7 Possible tumor cell ligands for BCG-activated NKT cell recognition. **a** Effect of antibodies for blocking stress-associated tumor cell-specific molecules such as MICA/MICB, the counterparts of NKG2D receptor, on the recognition of T24 cells was examined. Significant inhibition of the cytotoxicity 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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References

1. Alexandroff AB, Jackson AM, O'Donnell MA, James K (1999) BCG immunotherapy of bladder cancer: 20 years on. Lancet 353:1689–1694
2. Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, Spies T (1999) Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science 285:727–729
3. Bohle A, Gerdes J, Ulmer AJ, Hofstetter AG, Flad HD (1990) Effects of local bacillus Calmette-Guerin therapy in patients with bladder carcinoma on immunocompetent cells of the bladder wall. J Urol 144:53–58
4. Bohle A, Brandau S (2003) Immune mechanisms in bacillus Calmette-Guerin immunotherapy for superficial bladder cancer. J Urol 170:964–969
5. Cairo C, Hebbeler AM, Propp N, Bryant JL, Colizzi V, Pauza CD (2007) Innate-like gammadelta T cell responses to mycobacterium bacille Calmette-Guerin using the public V gamma 2 repertoire in Macaca fascicularis. Tuberculosis (Edinb) 87:373–383
6. Calabi F, Jarvis JM, Martin L, Milstein C (1989) Two classes of CD1 genes. Eur J Immunol 19:285–292
7. Chen F, Zhang G, Cao Y, Payne R, See WA (2007) Bacillus Calmette-Guerin inhibits apoptosis in human urothelial carcinoma cell lines in response to cytotoxic injury. J Urol 178:2166–2170
8. Das H, Wang L, Kamath A, Bukowski JF (2001) Vgamma2Vdelta2 T-cell receptor-mediated recognition of aminobisphosphonates. Blood 98:1616–1618
9. Emoto M, Emoto Y, Buchwalow IB, Kaufmann SH (1999) Induction of IFN-gamma-producing CD4+natural killer T cells by mycobacterium bovis bacillus Calmette-Guerin. Eur J Immunol 29:650–659
10. Groh V, Wu J, Yee C, Spies T (2002) Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. Nature 419:734–738
11. Gumperz JE, Brenner MB (2001) CD1-specific T cells in microbial immunity. Curr Opin Immunol 13:471–478
12. Harada M, Magara-Koyanagi K, Watarai H, Nagata Y, Ishii Y, Kojo S, Horiguchi S, Okamoto Y, Nakayama T, Suzuki N, Yeh WC, Akira S, Kitamura H, Ohara O, Seino K, Taniguchi M (2006) IL-21-induced Bepsilon cell apoptosis mediated by natural killer T cells suppresses IgE responses. J Exp Med 203:2929–2937
13. Ishii R, Shimizu M, Nakagawa Y, Shimizu K, Tanaka S, Takahashi H (2004) In vivo priming of natural killer T cells by dendritic cells pulsed with hepatoma-derived acid-eluted substances. Cancer Immunol Immunother 53:383–390
14. Jackson AM, Alexandroff AB, Kelly RW, Skibinska A, Esuvaranathan K, Prescott S, Chisholm GD, James K (1995) Changes in urinary cytokines and soluble intercellular adhesion molecule–1 (ICAM-1) in bladder cancer patients after bacillus Calmette-Guerin (BCG) immunotherapy. Clin Exp Immunol 99:369–375
15. Kaufmann SH (2004) New issues in tuberculosis. Ann Rheum Dis 63(Suppl 2):ii50–ii56
16. Kawashima T, Norose Y, Watanabe Y, Enomoto Y, Narazaki H, Watari E, Tanaka S, Takahashi H, Yano I, Brenner MB, Sugita M (2003) Cutting edge: major CD8 T cell response to live bacillus Calmette-Guerin is mediated by CD1 molecules. J Immunol 170:5345–5348
17. Lee J, Choi K, Olin MR, Cho SN, Molitor TW (2004) Gammadelta T cells in immunity induced by mycobacterium bovis bacillus Calmette-Guerin vaccination. Infect Immun 72:1504–1511
18. Martino A, Casetti R, Sacchi A, Poccia F (2007) Central memory Vgamma9Vdelta2 T lymphocytes primed and expanded by bacillus Calmette-Guerin-infected dendritic cells kill mycobacterial-infected monocytes. J Immunol 179:3057–3064
19. O'Toole CM, Povey S, Hepburn P, Franks LM (1983) Identity of some human bladder cancer cell lines. Nature 301:429–430
20. Porcelli SA, Modlin RL (1999) The CD1 system: antigen-presenting molecules for T cell recognition of lipids and glycolipids. Annu Rev Immunol 17:297–329
21. Prescott S, James K, Hargreave TB, Chisholm GD, Smyth JF (1992) Intravesical Evans strain BCG therapy: quantitative immunohistochemical analysis of the immune response within the bladder wall. J Urol 147:1636–1642
22. Pryor K, Stricker P, Russell P, Golovsky D, Penny R (1995) Antiproliferative effects of bacillus Calmette-Guerin and interferon alpha 2b on human bladder cancer cells in vitro. Cancer Immunol Immunother 41:309–316
23. Saito N, Takahashi M, Akahata W, Ido E, Hidaka C, Ibuki K, Miura T, Hayami M, Takahashi H (2005) Analysis of evolutionary conservation in CD1d molecules among primates. Tissue Antigens 66:674–682
24. Sasaki A, Kudoh S, Mori K, Takahashi N, Suzuki T (1997) Are BCG effects against urinary bladder carcinoma cell line T24 correlated with apoptosis in vitro? Urol Int 59:142–148
25. Takahashi H, Nakagawa Y, Leggatt GR, Ishida Y, Saito T, Yokomuro K, Berzofsky JA (1996) Inactivation of human immunodeficiency virus (HIV)–1 envelope-specific CD8+cytotoxic T lymphocytes by free antigenic peptide: a self-veto mechanism? J Exp Med 183:879–889
26. Takeuchi J, Watari E, Shinya E, Norose Y, Matsumoto M, Seya T, Sugita M, Kawana S, Takahashi H (2003) Down-regulation of toll-like receptor expression in monocyte-derived Langerhans cell-like cells: implications of low-responsiveness to bacterial components in the epidermal Langerhans cells. Biochem Biophys Res Commun 306:674–679
27. Thalmann GN, Sermier A, Rentsch C, Mohrle K, Cecchini MG, Studer UE (2000) Urinary interleukin-8 and 18 predict the response of superficial bladder cancer to intravesical therapy with bacillus Calmette-Guerin. J Urol 164:2129–2133
28. Wang H, Yang D, Xu W, Wang Y, Ruan Z, Zhao T, Han J, Wu Y (2008) Tumor-derived soluble MICs impair CD3(+)CD56(+) NKT-like cell cytotoxicity in cancer patients. Immunol Lett 120:65–71