Bryostatin-1 Enhances the Maturation and Antigen-Presenting Ability of Murine and Human Dendritic Cells

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ABSTRACT

In this study, we investigated the effect of bryostatin-1 (Bryo-1), an antineoplastic agent, on dendritic cell (DC) maturation, activation, and functions. Murine bone marrow-derived DCs on culture with Bryo-1 alone, Bryo-1 + calcium ionophore (CI), but not CI alone exhibited morphologic changes characteristic of mature DCs and expressed increased levels of CD40, CD80, and CD86. Moreover, Bryo-1 + CI-treated DCs exhibited enhanced antigen-presenting ability to naive and antigen-specific T cells and alloreactive T cells. Bryo-1 + CI-mediated activation of DCs involved protein kinase C (PKC), especially PKC-α, -β, and -δ, and addition of PKC inhibitors impaired their ability to activate T cells. Bryo-1 + CI treatment of DCs did not activate mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase, p38 MAPK, or mitogen-activated protein kinase/c-Jun NH2-terminal kinase pathways. Finally, treatment of DCs with Bryo-1 alone and Bryo-1 + CI, but not CI alone, induced nuclear translocation of nuclear factor κB as studied by confocal microscopy. DCs generated from human peripheral blood monocytes or from human cord blood CD34+ hematopoietic stem cells, when cultured with Bryo-1 + CI, also showed maturation and increased T-cell stimulatory activity. Bryo-1 + CI was more potent in inducing maturation and activation of DCs when compared with other agents such as tumor necrosis factor α, lipopolysaccharide, or phorbol 12-myristate 13-acetate + CI. Collectively, the current study shows for the first time that Bryo-1 alone or in combination with CI may promote the maturation of DCs and therefore may be useful in development of DC-based cancer immunotherapy.

INTRODUCTION

Bryostatin-1 (Bryo-1) is a macrocyclic lactone isolated from the marine bryozoan Bugula neritina (1). The primary interest in Bryo-1 has been initiated by recognition of the potent antiproliferative effects against various tumor cells (2). Such effects have been related to the ability of Bryo-1 to modulate protein kinase C (PKC) activity by activating or degrading certain isoforms of PKC. However, not all of the activities of Bryo-1 are PKC dependent; thus, further studies are necessary to understand its mechanism of action (2). Some studies have shown direct antitumor activity of Bryo-1, whereas others suggested that Bryo-1 modulates cellular response to other anticancer agents by acting as a sensitizer to anticancer drugs. The latter findings are further supported by several clinical trials, in which the effect of Bryo-1 was maximal when Bryo-1 preceded other anticancer drugs (3). Bryo-1 has been shown to activate human hematopoietic progenitor cell growth and promote T-cell activation (4, 5). Another interest in Bryo-1 recently has been stimulated by the observation that Bryo-1 can facilitate the differentiation of myelogenous leukemic cells into monocytic or dendritic-like cells (6).

In the current study, we investigated whether Bryo-1 + calcium ionophore (CI) induces maturation of dendritic cells (DCs) and enhances their antigen-presenting abilities. Unlike other well-known DC stimulators, such as lipopolysaccharide (LPS), tumor necrosis factor α (TNF-α), phorbol 12-myristate 13-acetate (PMA) + CI, and CpG oligonucleotides, Bryo-1 has the advantage that it is in clinical use as an anticancer agent, and if it can activate DCs, it can serve as a novel tool for use in DC-based immunotherapy of cancer. Our data showed that Bryo-1 + CI treatment induced the maturation of DCs and promoted their function to stimulate naive and Ag-specific T cells. Our data also suggested that the ability to activate DCs is PKC dependent and mediated through nuclear factor κB (NFκB) activation without involving mitogen-activated protein kinase (MAPK) or c-Jun NH2-terminal kinase (JNK) pathways.

MATERIALS AND METHODS

Mice. Adult (6 to 8 weeks of age) female C57BL/6 (H-2b) and DBA/2 (H-2b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Dendritic Cell Culture from Murine Bone Marrow. Murine DCs were obtained from bone marrow cells by culturing with murine recombinant granulocyte macrophage colony-stimulating factor (GM-CSF; 5 ng/mL; Pharmingen, San Diego, CA) for 6 days (7). Briefly, bone marrow cells were depleted of CD4+ and CD8+ T cells and B220+ B cells by using specific monoclonal antibodies (mAbs) and complement. The cells (1 × 107/well) were placed in 24-well plates supplemented with GM-CSF. The cultures were fed every 2 days to remove nonadherent monocytes by aspirating 75% of the medium and adding fresh medium with GM-CSF. On day 6 of culture, the attached cells were dislodged by gentle pipetting and applied to 6-well columns of 50% FCS-RPMI 1640, to enrich the aggregated cells, which released a large number of phenotypically characteristic DCs after another day of culture with GM-CSF.

Dendritic Cell Culture from Human Peripheral Blood. Peripheral blood mononuclear cells (PBMCs) were isolated from adult healthy volunteers by centrifugation on Ficoll/hypaque (8). The nonadherent cells were collected and used for isolating T cells, as described below. The adherent cells were cultured with GM-CSF (800 units/mL; Pharmingen) and interleukin 4 (IL-4; 20 ng/mL; R&D System, Minneapolis, MN) for 1 week to generate DCs and were referred to as PBMC-DC.

Dendritic Cell Culture from Human Cord Blood. In some experiments, DCs were generated from CD34+ hematopoietic stem cells (HSCs) isolated from human cord blood provided by St. Louis Cord Blood Bank (SSM Cardinal Glennon Children’s Hospital/St. Louis University School of Medicine, St. Louis, MO). Such DCs were referred to as CD34+ HSC-DC. Briefly, mononuclear cells were enriched by centrifugation on Ficoll/hypaque. Adherent cells were depleted, and CD34+ cells were isolated using MACS technique according to the manufacturer’s recommendations (Miltenyi Biotec, Auburn, CA). The cells then were cultured for 2 weeks with 10% RPMI 1640 supplemented with 5% autologous plasma and cytokines such as GM-CSF (800 units/mL; Pharmingen) and TNF-α (50 units/mL; Pharmingen) as described previously (9).

Treatment of DC with Bryostatin-1 and Other Activating Agents. DCs generated as described previously were cultured for an additional 24 hours in medium with vehicle alone (control DCs), Bryo-1 (10 nmol/L; Biomol, Plymouth Meeting, PA) + CI (A23187, 0.5 μg/mL; Sigma, St. Louis, MO; Bryo-1 + CI DC), Bryo-1 alone (Bryo-1 DC), or CI alone (CI DC). For comparison, we also used DCs cultured with LPS (10 μg/mL; Sigma), TNF-α (50 to 500
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units/mL; PharMingen), or PMA (10 ng/mL; Sigma) + CI (0.5 µg/mL; Sigma) for 24 hours and were referred to as LPS DC, TNF-α DC, and PMA + CI DC, respectively. It should be noted that in our preliminary experiments, we included various concentrations of Bryo-1 and PMA, and based on the results, we selected the optimal concentration of these agents. In particular, it was observed that DCs treated with a high concentration of PMA for 24 hours resulted in a lower yield of viable cells when compared with treatment with Bryo-1.

Cell Surface Antigen Detection with Monoclonal Antibodies Using Flow Cytometry. Phenotypic analysis of DCs was carried out by double-staining with phycoerythrin (PE)-conjugated and FITC-conjugated mAbs as described elsewhere following incubation with Fe-block (PharMingen; ref. 10). The following are the mAbs used against mouse DCs: PE-anti-CD80, PE-anti-CD86, PE-anti-CD40, and FITC-anti-CD11c (PharMingen). For PBMC-DCs and CD34+ HSC-DCs, the following mAbs were used: PE-anti-CD86, PE-anti-CD80, FITC-anti-HLA (PharMingen), and PE-anti-CD83 (Immunotech; Beckman Coulter, Fullerton, CA). The cells were analyzed by flow cytometry (model 752, EPICS V; Coulter Electronics, Miami, FL). The data were depicted as expression indices, which are the products of percentage of marker-positive cells multiplied by the mean fluorescence intensity (% positive × MFI). For comparison, the value obtained for cells exposed to TNF-α or LPS was normalized to 1.

FITC-Dextran Uptake. Murine bone marrow-derived Bryo-1 DCs were harvested, washed, and incubated with FITC-conjugated dextran (1 mg/mL; Sigma) for 30 minutes at either 37°C or 4°C. The cells were harvested, washed, and analyzed by flow cytometry (model 752, EPICS V; Coulter Electronics).

Allogeneic Mixed Lymphocyte Reaction. Murine control DCs, Bryo-1 DCs, CI DCs, CI DCs, Bryo-1 + CI DCs, TNF-α DCs, or PMA + CI DCs were irradiated at 2000 rad and mixed with responder murine splenic T cells to obtain various T cell:DC ratios in 96-well plates. Briefly, DCs were activated with medium (control), Bryo-1, CI, Bryo-1 + CI, TNF-α, or PMA + CI for 24 hours as described previously, and the next day, the cells were thoroughly washed with prewarmed medium, irradiated, and then mixed with allogeneic T cells. After 3 days of culture, the cells were pulsed with [3H]thymidine during the last 8 hours. DNA synthesis was determined by [3H]thymidine incorporation using a liquid scintillation counter.

To study allogeneic mixed lymphocyte reaction (allo-MLR) using human DCs, CD34+ HSC-DCs activated with medium (control), Bryo-1 + CI, LPS, or PMA + CI for 24 hours as described previously were washed and irradiated before mixing with allogeneic T cells (2 × 10^5/well) in 96-well tissue culture plates. The allogeneic T cells were isolated from cord blood by using the MACS technique according to the manufacturer’s recommendations (Miltenyi Biotech), the details of which are described below. After 5 days of incubation, the cultures were pulsed with [3H]thymidine during the last 8 hours. DNA synthesis was determined by [3H]thymidine incorporation using a liquid scintillation counter.

Purification of T Cells. Murine T cells used in allo-MLR were purified by passing the spleen cells of DBA2 mice over nylon wool columns, followed by depletion of B cells and macrophages using specific antibodies + complement, as described previously (11). Thus, the cells purified were >95% CD3+ as determined by flow cytometric analysis. Human PBMC-derived and cord blood-derived T cells were isolated using magnetic beads and columns provided by Miltenyi Biotech. The T cells were >95 to 98% pure as determined by the expression of CD3 using a flow cytometer.

Antigen-Pulsing and Ag Presentation. In antigen-presentation experiments, 18 hours before treatment of DCs with Bryo-1 + CI, the cells were cultured with conalbumin (50 µg/mL). The DCs were washed, irradiated, and used as Ag-pulsed DCs. In experiments aimed at testing the ability of DCs to present tumor-specific antigen, whole LSA tumor cell lysate was prepared by repeated freezing/thawing cycles and was added directly to the murine DC cultures 18 hours before treatment with Bryo-1 or other activating agents. Next, the DCs were washed, irradiated, and used as lymphomasarcoma (LSA)-pulsed DCs.

Antigen-Specific T-Cell Proliferation. To study conalbumin-specific T-cell proliferation, Ag-specific T cells were generated by injecting C57BL/6 mice with 100 µg of conalbumin mixed with complete Freund’s adjuvant (Sigma) in both rear footpads to activate T cells in popliteal lymph nodes (LNs) as described previously (12). Eight to 10 days later, the draining LNs were collected and used as a source of conalbumin-specific T cells. To determine the proliferative responsiveness of murine Ag-specific T cells following contact with DCs, the DCs (Ag pulsed or unpulsed; 1 × 10^5/well) were treated with medium alone or Bryo-1 + CI, irradiated, and then mixed with Ag-specific T cells (3 × 10^5/well). After 72 hours, the cultures were pulsed with [3H]thymidine during the last 8 hours. DNA synthesis was determined by [3H]thymidine incorporation using a liquid scintillation counter.

To study human T-cell responsiveness to tetanus toxoid (TT), PBMC-DCs were cultured with TT (10 µg/mL; Aventis, Bridgewater, NJ) overnight. The next day, the DCs were harvested, washed, and incubated with medium, Bryo-1 + CI, PMA + CI, or TNF-α, and irradiated, and used as TT-pulsed DCs. Various numbers of irradiated PBMC-DCs were mixed in 96-well plates with autologous T cells (1 × 10^5/well) isolated from peripheral blood of same donor. After 5 days, the cultures were pulsed with [3H]thymidine during the last 8 hours. DNA synthesis was determined by [3H]thymidine incorporation using a liquid scintillation counter.

Generation of LSA Tumor-Specific T Cells. LSA tumor-specific T cells were generated as described previously (13). Briefly, LSA tumor cells (1 × 10^6) were injected into syngeneic C57BL/6 mice, and 5 days later, the mice were treated with 1,3-bis(chloroethyl)-1-nitrosourea (50 mg/kg body weight). Three days later, T cells were purified from the spleens and used as LSA-specific T cells.

Cytotoxicity against LSA Tumor Cells. To test whether treatment of Bryo-1 + CI enhances the ability of DCs to generate cytolytic T cells against LSA tumor cells, LSA-specific T cells were obtained as described previously and were incubated for 5 days with either control DC or Bryo-1 + CI DC pulsed with LSA tumor cell lysate as described above. Next, the cytolytic T cells were tested in a 4-hour 51Cr release assay against LSA tumor cells as described previously (13).

Western Blot Analysis. DCs were lysed by repeated freeze/thawing, and protein concentration in the supernatant was determined using the BCA protein assay kit (Pierce, Rockford, IL). Total protein (15 to 40 µg) was separated by SDS-PAGE and transferred to a membrane. Blots were blocked with 5% (w/v) nonfat dry milk in Tris-buffered saline/Tween [0.5 mol/L NaCl, 20 mmol/L Tris-HCl, and 0.1% (v/v) Tween 20 (pH 7.6)] and probed with the primary antibody. Next, blots were washed and incubated with horseradish peroxidase-conjugated secondary antibody and visualized with enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ).

Use of Inhibitors PKC, p44/p42 MAPK, or p38 MAPK. Murine DCs were preincubated with various concentrations of pan-PKCI inhibitor bisindolylmaleimide I (Calbiochem, San Diego, CA), PKC-6 inhibitor Rottlerin (Calbiochem), PD90859 (p44/p42 MAPK inhibitor), or SB203580 (p38 MAPK inhibitor) for 1 hour at 37°C and then treated either with medium (control DC) or Bryo-1 + CI DC for 24 hours as described previously.

Preparation of Nuclear Extract and Electrophoretic Mobility Shift Assay. DCs were incubated for 2 hours with LPS, TNF-α, or Bryo-1 + CI, and nuclear extracts were prepared using commercial nuclear extract kit (Active Motif, Carlsbad, CA). The reactions were started by adding 10 µg of nuclear protein extract to a reaction mixture containing 3 µg of poly(dIdC) (Pharmacia), 4 µL of 5× binding buffer [50 mmol/L HEPES (pH 7.9), 50 mmol/L KCl, 12.5 mmol/L MgCl2, 0.5 mmol/L EDTA, 50% glycerol(v/v), 5 mmol/L A TP, and 5 mmol/L DTT], and [γ-32P]ATP-labeled double-stranded DNA (dsDNA) probe (25 fmol; ref. 14). All of the DNA binding reactions were conducted for 20 minutes at room temperature in a final volume of 20 µL except for supershift, in which the reaction was conducted on ice for 30 minutes. Cold competitor oligonucleotides were added to the reaction mixture before the radiolabeled probe. For supershift assay, protein extracts were incubated with polyclonal anti-p50 (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 minutes on ice, followed by the addition of the radiolabeled probe for another 30 minutes on ice. The entire sample then was loaded on a 5% nondenaturing polyacrylamide gel in Tris-borate-EDTA buffer. After electrophoresis, gels were dried, and separated protein-DNA complexes were visualized by autoradiography using Kodak X-Omat AR film (Rochester, NY). The following dsDNA oligonucleotides were used in electrophoretic mobility shift assay analysis as labeled or competitor probes: NFkB sense, 5′-AGT TGA GGC GAC TTT CCC AGG-3′; NFkB antisense, 5′-GCT CCT GGG AAA GTG CCC C.
Detection of Nuclear Translocation of NFκB by Confocal Microscopy. DCs stimulated for 2 hours with medium alone, LPS, Bryo-1, Bryo-1 + CI, or CI alone were washed and resuspended at 1 × 10⁶ cells/mL, and 100 µL of this cell suspension were layered onto polylysine-coated slides. Cells were allowed to adhere for 30 minutes at 37°C. Next, cells were fixed in 3.7% buffered paraformaldehyde at room temperature for 15 minutes and permeabilized in 0.2% Triton X-100 in PBS for 5 minutes. The Fc receptors were blocked as before. The primary antibodies, goat anti-NFκBp65 (C-19) or rabbit anti-NFκBp50 (C-20; Santa Cruz Biotechnology), were added at appropriate concentrations for 1 hour at room temperature. Cells subsequently were washed twice in PBS and stained with the appropriate fluorescent-labeled antibodies, Cy3 antigoat IgG, or Cy2 antirabbit IgG (Jackson ImmunoResearch, West Grove, PA) at room temperature for 1 hour. Cells were washed twice with PBS, and nuclei were stained with Hoechst 33258 (Molecular Probes, Eugene, OR) for 1 minute before slides were mounted in ProLong Antifade mounting medium (Molecular Probes) and examined using a Zeiss LSM510 META laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany). Images were acquired using thin (~0.8 µm) optical sections through X-Y axis. Images were collected simultaneously and sequentially to rule out bleed-through from one fluorescence channel to another.

Statistical Analysis. The statistical comparisons between different study groups were carried out using Student’s t test, and differences of P < 0.05 were considered to be significant. Each experiment was repeated at least three times.

RESULTS

Effect of Bryo-1 + CI Treatment on DC Morphology and Expression Levels of Costimulatory Molecules. DCs were cultured from mouse bone marrow cells with GM-CSF for 6 days. Next, they were treated either with Bryo-1 + CI (Bryo-1 + CI DCs) or medium alone (control DCs) for 24 hours and observed for morphology. We generally observed larger proportions of cells with dendrites in Bryo-1 + CI DCs when compared with control DCs (Fig. 1A and B). The DCs also were treated with PMA + CI (PMA + CI DCs) or TNF-α (TNF-α DCs; Fig. 1C and D), which served as positive controls. These cultures also showed enhanced numbers of cells with DC morphology when compared with the control culture.

Next, the expression of costimulatory molecules was examined by double-staining the DCs with FITC-CD11c and PE–anti-costimulatory molecules. The CD11c⁺ population was gated, and the expression level of costimulatory molecules was compared between different treatment groups (Fig. 1E). The data showed that there was a significant up-regulation of CD40, CD80, and CD86 molecule expression on Bryo-1 + CI DCs when compared with control DCs. It should be noted that the increased levels of costimulatory molecule expression on Bryo-1 + CI DCs were comparable with those seen on LPS DCs except for Bryo-1 + CI DCs, which expressed higher levels of CD86 and MHC class II molecules than LPS DCs (data not shown).

To further clarify the contribution of Bryo-1 and CI on DC matu-
ration, we also treated DCs with either Bryo-1 alone, CI alone, or the combination of Bryo-1 + CI (Fig. 1E). The data showed that addition of Bryo-1 alone to DCs induced similar up-regulation of costimulatory molecules as treatment of DCs with Bryo-1 + CI. CI alone also failed to induce significant phenotypic changes in DCs. We corroborated the maturation of DCs by Bryo-1 + CI using FITC-dextran uptake (Fig. 2). The data showed Bryo-1 + CI DCs had decreased ability to uptake FITC-dextran when compared with the control DCs, which indicated that treatment of DCs with Bryo-1 + CI induced their maturation.

**Effect of Bryo-1 Treatment on Ability of DC to Stimulate the Proliferative Responsiveness of Allogeneic and Ag-Specific T Cells.** To test the effect of Bryo-1 + CI treatment on functional properties of DCs, we performed allo-MLR (Fig. 3A and B). To this end, responder T cells obtained from DBA/2 mice (H-2^b^) were stimulated with DCs from C57BL/6 mice (H-2^b^). The data showed that Bryo-1 + CI DCs stimulated the proliferative responsiveness of allogeneic T cells stronger than control DCs (Fig. 3A). Furthermore, Bryo-1 DCs were able to stimulate allogeneic T cells similar to Bryo-1 + CI DCs, although to a lesser extent at high stimulator:effector ratios. It also was observed that CI DCs were similar to the control DCs in that they were not effective in further stimulating allogeneic T cells. Thus, in functional studies such as allo-MLR, DCs stimulated with Bryo-1 + CI induced better T-cell responsiveness than Bryo-1 alone, whereas DCs treated with CI alone failed to induce enhanced T-cell activation (Fig. 3A). Thus, in all of the experiments, we used the combination of Bryo-1 + CI to activate DCs. Moreover, the proliferative response of T cells was stronger when Bryo-1 + CI DCs were used for stimulation when compared with the use of TNF-α DCs or PMA + CI DCs (Fig. 3B).

When the DCs were pulsed with conalbumin antigen followed by Bryo-1 + CI treatment and then mixed with conalbumin-specific T cells, Bryo-1 + CI DCs induced stronger Ag-specific T-cell proliferation than control DCs (Fig. 3C and D). Furthermore, when naive T cells were used instead of sensitized Ag-specific T cells, Bryo-1 + CI DCs were able to induce significant proliferative responsiveness of naive T cells to conalbumin stronger than control DCs (Fig. 3E).

We also investigated the proliferative responsiveness of tumor-specific T cells following stimulation with DCs pulsed by tumor cell lysate. The data shown in Fig. 3F indicate that Bryo-1 + CI DCs could induce strong proliferative responsiveness of LSA tumor-specific T cells, whereas control DCs, TNF-α DCs, or PMA + CI DCs were not as effective. It should be noted that LSA tumor-specific T cells did not respond to DCs pulsed with other syngeneic tumor lysates, such as EL-4, as also shown previously (13, 15).
Next, the DCs were pulsed with whole LSA tumor cell lysate and then treated either with Bryo-1 + CI or medium alone and then incubated with LSA tumor-specific T cells for 5 days to activate LSA-specific cytolytic T cells (13, 15). Using $^{51}$Cr-release assay, we observed that the cytolytic activity against LSA targets was enhanced when T cells were cocultured with LSA-pulsed, Bryo-1 + CI DCs compared with similar coculture with control DCs (Fig. 4). Such T cells also failed to mediate significant lysis of a nonspecific tumor, such as EL-4 (data not shown). Collectively, these data showed that Bryo-1 + CI treatment significantly enhanced the DC functions to stimulate naive and Ag-specific T cells and that Bryo-1 + CI DCs are efficient in presenting tumor-derived antigens to tumor-specific T cells.

**Bryo-1 + CI Activated/Degraded Specific PKC Isozymes in DCs.** It is known that Bryo-1 activates and/or degrades particular isoforms of PKC depending on cell types and experimental designs (16). Previous studies also showed that not all of the effects of Bryo-1 were PKC dependent, which suggests that PKC-independent pathways also are involved (17). Therefore, we determined the isoforms of PKC in DCs following Bryo-1 + CI treatment using Western blot analysis (Fig. 5A). There are at least 12 PKC isoforms discovered, and different functions have been attributed to distinct isoyme expression, although precise mechanism of action remains unclear (18). It also is known that Bryo-1 induces two phases of activation/degradation of PKC isoymes, which includes short-term and long-term effects (16). Thus, we included protein extract from the DCs treated with Bryo-1 + CI for a short period (15 or 45 minutes) and similar cells that were treated for longer periods (2, 6, or 24 hours). Among the 12 isoforms, we observed a decrease in PKC-α and -δ and an increase of PKC-ε form with increasing time in Bryo-1 + CI DCs. We also detected expression of PKC-β and -γ, but there was no change in expression level of these isoymes at all of the periods tested (data not shown). However, we could not detect the expression of PKC-ε,-θ, or -η in DCs following Bryo-1 + CI treatment (data not shown). These data suggested that PKC-α, -δ, and -ε activation/degradation may be involved in the Bryo-1 + CI-induced maturation of DCs. Furthermore, when DCs were treated with pan-PKC inhibitor Bisindolylmaleimide I (Bis I) or PKC-δ specific inhibitor Rottlerin before Bryo-1 + CI exposure, 500 nmol/L of Bis I and 1 μmol/L of Rottlerin significantly inhibited the proliferative responsiveness of allogeneic T cells, suggesting that Bryo-1 + CI stimulates DC functions via PKC-dependent pathway (Fig. 5B).

**Effect of Bryo-1 + CI on MAPK and JNK Pathways of Activation and NFκB Nuclear Translocation.** Next, we studied whether there is activation of MAPK or JNK pathways following Bryo-1 + CI treatment. Interestingly, our data showed that Bryo-1 + CI treatment did not induce activation of p42/p44 MAPK, p38 MAPK, or stress-activated protein kinase/JNK pathways (Fig. 6) unlike other known DC stimulators such as LPS or TNF-α (19, 20). To further confirm this, PD 98059 and SB 203580, which are known to be specific inhibitors of p42/44 MAPK or p38 MAPK pathways, were tested as described in Materials and Methods. The data showed that neither impaired the enhancement in proliferative responsiveness of allogeneic T cells induced by Bryo-1 + CI DCs (data not shown).

We also studied the nuclear translation of NF-κB, inasmuch as it is considered to play a critical role in DC survival and maturation (21). As shown in Fig. 7A and B, we observed NF-κB-specific band in nuclear extracts from DCs, which were stimulated with LPS or TNF-α. These bands disappeared when cold or mutant NF-κB oligomers were added. Interestingly, Bryo-1 + CI treatment also induced DNA binding of NF-κB in DCs, which disappeared following cold probe competition or by addition of mutant form of NF-κB oligomers, suggesting that activation of NF-κB might be involved in DC maturation by Bryo-1 + CI treatment. Western blot analysis using nuclear extract from DCs, which were stimulated with different agents for 2 hours, also was undertaken (Fig. 7C). The data showed that there was an increase of RelA, RelB, cRel, and p105/p50 expression in Bryo-1 + CI DCs compared with DCs treated with other activators. Inasmuch as p50 is the predominant subunit of NF-κB dimers, we performed supershift assay using anti-p50 antibodies and showed that there was increased DNA binding activity of NF-κB (Fig. 7D) in
Bryo-1 + CI DCs. Studies also were performed to confirm nuclear translocation of p50 and RelA subunits of NFκB in vehicle, LPS, Bryo-1, CI, and Bryo-1 + CI–treated DCs by staining with the specific antibodies and visualization by confocal microscopy (Fig. 7E). In untreated DCs, RelA and p50 were localized in the cytosolic region of the cells, whereas following treatment with LPS, Bryo-1, or Bryo-1 + CI, there was nuclear translocation and colocalization of both the subunits as depicted by the yellow staining in the last column following merging of the images. Furthermore, CI treatment alone did not lead to nuclear staining for p50 and RelA. It was interesting to note that following treatment with Bryo-1 alone, p50 and RelA were found in the nucleus and cytosol, whereas following treatment with Bryo-1 + CI or LPS almost all of the p50 and RelA translocated to the nucleus.

**Effect of Bryo-1 + CI Treatment on Activation of Human PBMC-DCs.** To study the effect of Bryo-1 on human PBMC-DCs, we carried out several experiments using DCs isolated and cultured from human PBMCs. First, when PBMC-DCs treated with different activators were observed for morphology, we found similar morphologic changes as seen in mouse bone marrow-derived DCs (Fig. 8A). In particular, Bryo-1 + CI treatment induced prominent dendritic processes in PBMC-DCs compared with control DCs or DCs treated with PMA + CI or TNF-α.

It was noted that PBMC-DCs treated with Bryo-1 + CI showed significant up-regulation of HLA-DP/DQ/DR, CD80, CD83, and CD86 compared with the controls (Fig. 8B). For the most part, treatment of PBMC-DCs with Bryo-1 + CI also had a more pronounced effect on the activation of DCs compared with the DCs treated with other well-known DC stimulators, such as PMA + CI or TNF-α.

To test the effect of Bryo-1 + CI on the ability of PBMC-DCs to stimulate the proliferative responsiveness of Ag-specific T cells, the DCs were pulsed with TT antigen, treated with Bryo-1 + CI or various activators, and then mixed with autologous peripheral blood T cells (Fig. 8C). The data showed that Bryo-1 + CI DCs induced T-cell proliferation stronger than other DCs.

**Effect of Bryo-1 + CI Treatment on the Activation of Cord Blood-Derived CD34+HSC-DCs.** Previous studies showed that functional DCs could be generated from CD34+HSC and thereby suggested promising implications of CD34+HSC-DCs in future clinical applications (22). In line with these efforts, we also generated DCs from cord blood-derived CD34+HSC and studied the effect of Bryo-1 + CI on maturation and functional enhancement. We observed an increase in the expression of CD80, CD83, CD86, and HLA on CD34+HSC-DCs treated with Bryo-1 + CI compared with control DCs (Fig. 9A). Moreover, Bryo-1 + CI HSC-DCs expressed CD83 and CD86 stronger than PMA + CI DCs or LPS DCs, suggesting that the mode of action of Bryo-1 + CI might be different compared with other known stimulators of DCs. When Bryo-1 + CI DCs were tested in allo-MLR, dramatic enhancement in T-cell proliferation was observed compared with other treatment groups (Fig. 9B).

**DISCUSSION**

Bryostatins have generated a wide interest because of their antineoplastic activity combined with low toxicity (2). Moreover, Bryo-1 has been shown to induce cell differentiation and increase the sensitivity of tumor cells to cytotoxicity caused by other drugs (3, 23). In the current study, we tested whether Bryo-1 + CI can induce the maturation of DCs and promote their antigen-priming activities to T cells. Our data showed that Bryo-1 + CI could activate the DCs as shown by: (1) up-regulation of costimulatory molecules; (2) decrease in the ability of DCs to uptake FITC-dextran; (3) enhancement of proliferative responsiveness of allogeneic T cells and Ag-specific T cells; and (4) enhancement of cytolytic activity against LSA tumor cells. We also showed that the maturation of DCs by Bryo-1 + CI was PKC dependent, probably via PKC-α, -δ, and -ζ. However, it did not induce any MAPK or JNK pathways unlike other known DC stimulators. NFκB translocation also was detected in DCs followed by Bryo-1 + CI treatment. Collectively, this study suggests a novel use for Bryo-1 + CI in the maturation and functional activation of DCs. Bryo-1 currently is tested in a number of Phase I and II studies sponsored by National Cancer Institute as a single agent or in combination with other chemotherapeutic agents to manage a wide range of cancers (24). Preliminary studies from Phase II trials at Memorial Sloan-Kettering Cancer Center published in 2001 suggested that Bryo-1 treatment enhanced antitumor activity in combination with Taxol® against esophageal cancer (25). Other studies also supported a promising clinical application of Bryo-1 for the management of various human cancers (26, 27). Although most of these clinical studies did not provide the exact mechanism by which Bryo-1 enhanced the antitumor activity, it has been suggested that Bryo-1 acts as a sensitizer to other anticancer drugs. Some studies reported a dose-depen-
dent increase in TNF-α and IL-6 concentrations in plasma following 1-hour intravenous infusion of Bryo-1, whereas others could not detect any increase in such cytokines in plasma (26, 27). In another Phase I study, Bryo-1 was shown to increase IL-2–induced lymphokine-activated killer cell activity and IL-2–induced lymphocyte proliferation (28). However, nothing is known about the effect of Bryo-1 on DCs.

Previous studies showed that various stimuli could initiate the maturation process of DCs in vitro. These include the proinflammatory cytokines such as TNF-α and bacterial products such as LPS (29, 30).

Fig. 7. Nuclear translocation of NFκB and its subunits in murine bone marrow–derived DCs following different modes of stimulation. The nuclear protein from DCs treated with LPS, TNF-α, and Bryo-1 + CI was extracted and incubated with 32P-labeled NFκB, mutant, cold oligonucleotides, or antibody (p50). The specific band of NFκB was indicated with filled arrowhead (A, B, and D). The empty arrowhead depicts supershift of NFκB using anti-p50 antibody (D). The nuclear extracts were prepared from DCs activated by various agents for 2 hours, and Western blot analysis was used to analyze distinct subunits of NFκB (C). E, shows confocal microscopic analysis of vehicle, LPS, Bryo-1, Bryo-1 + CI, or CI-treated DCs by Nomarski differential interference contrast (first column) and following staining with anti-RelA antibody and Cy3 antigoat antibody (second column), anti-p50 antibody and Cy2 antirabbit antibody (third column), nuclear staining with Hoechst (fourth column), and following merging of the images (fifth column). The yellow coloration following merge depicts colocalization of RelA and p50 in the last column.
Ligation of CD40/CD40L and the viral double-stranded RNA also has been shown to stimulate maturation of human DCs (31, 32). Furthermore, it was shown that when ex vivo-generated human DCs were pulsed with tumor-specific peptides or whole tumor cell lysates, they induced antitumor responses in cancer patients, suggesting the promising use of DCs in vaccination and immunotherapy of cancer (33). Unfortunately, despite such possibilities, there are still major limitations in the application of DCs in clinical settings. It is com-

Fig. 8. Effect of Bryo-1 + CI on the activation of human PBMC-DCs. A. The morphology of human PBMC-derived DCs is depicted as seen under a phase contrast microscope. B. The DCs treated with different activators were harvested, washed, and stained with various FITC- and PE-conjugated mAbs. The data are depicted as expression indices, which are the products of percentage of marker-positive cells multiplied by the mean fluorescence intensity (MFI) as described in Materials and Methods. For the sake of comparison, the value obtained for cells exposed to TNF-α has been normalized to 1. Vertical bars represent mean ± SEM of two or three experiments. C. PBMC-DCs were pulsed with TT (10 μg/mL) and either treated with medium alone (control DC) or with various activators. Various numbers of irradiated PBMC-DCs then were mixed with PBMC-derived T cells as described in Materials and Methods. The cultures were incubated at 37°C for 5 days and pulsed with [3H]thymidine during the last 8 hours. Vertical bars represent mean ± SEM of triplicate cultures.

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monly observed that the DCs pulsed with an antigen and injected in vivo are not effective, undergo apoptosis, or sometimes show opposite effects. This is because of, first, the environmental factors that already existed in the immune-deficient host, and, second, the nature of DCs, which can act either as an immune tolerant or immune adjuvant (34–36). Other known DC stimulators, which make them an immune adjuvant, have been reported and shown efficient in vivo, but unfortunately, not all of them can be used in human study (30, 37). CD40L has been considered a promising agent, although no study regarding this has been reported yet in humans (31). On the basis of the current study, we propose that Bryo-1 may serve as a novel and strong DC stimulator with the potential of being used in human clinical trials. This study showed that Bryo-1/H11001 CI treatment induced DC maturation and functional enhancement better than other known DC stimulators, such as LPS, TNF-α, and PMA + CI. Inasmuch as Bryo-1 has already been used in human studies, the current findings provide attractive alternatives for the use of Bryo-1 in DC-based vaccine or immunotherapy.

Bryo-1 is one of the first drugs to target a signaling pathway, although the relationship between its various activities and PKC modulation is not uniformly established. Bryo-1 has been shown to act as PKC stimulator or PKC inhibitor depending on dose, time of treatment, or experimental conditions (38, 39). In the current study, we found that Bryo-1-mediated effect on DCs depended on PKC pathway. PKC is composed of at least 12 isozymes that can be further categorized into subfamilies according to their structures and cofactor regulation (18). The types of PKC isozyme that Bryo-1 activates also are diverse, depending on cell types or treatments. A previous study showed that Bryo-1 substantially regulated PKC-α, -δ, and -ε in NIH mouse fibroblast cells, whereas PMA did not distinguish between these isozymes (40). Other studies showed that Bryo-1 down-regulates PKC-α in epithelial cells and PKC-α and -β in human T cells (41, 16). Bryo-1 also induces a unique biphasic response in PKC-γ, depending on concentrations leading to its degradation in NIH mouse fibroblast cells, B16F10 melanocytes, and HeLa cells (40, 42, 43). PKC-δ recently was discovered, and its exact function is unknown (44). Further studies are required to reveal the relationship between specific PKC isozymes and functions of DCs.

Because NF-κB plays a critical role in DC maturation and survival, observation of the translocation of NF-κB following Bryo-1 + CI treatment is not surprising. Moreover, nuclear extract from Bryo-1 + CI DCs showed enhanced expression of NF-κB subunits compared with DCs treated with other activating agents, suggesting differential regulation of NF-κB by Bryo-1 + CI in DCs, especially at early time points. In the current study, we also noted that Bryo-1 + CI did not induce MAPK or JNK pathways. This is in contrast to

Fig. 9. Bryo-1 + CI treatment up-regulates the expression of surface markers on human CD34+/HSC-DCs and enhances their function to stimulate allogeneic T cells. Human CD34+/HSC-DCs from cord blood were cultured and treated as described in Materials and Methods. A. The DCs treated with different activators were harvested, washed, and stained with various FITC- and PE-conjugated mAbs. The data were plotted as expression indices as described in Fig. 8. Vertical bars represent mean ± SEM of two or three experiments. B. CD34+/HSC-DCs were treated with vehicle alone (control DC) or with various activators. Various numbers of irradiated CD34+/HSC-DCs then were mixed with T cells isolated from allogeneic cord blood (2 × 10^5/well) as described in Materials and Methods. The cultures were incubated at 37°C for 5 days and pulsed with [3H]thymidine during the last 8 hours. Vertical bars represent mean ± SEM of triplicate cultures.
previous studies showing the involvement of MAP/extracellular signal-regulated kinase/MAPK in the differentiation of monocytes or human lymphoid leukemia cells by Bryo-1 (45, 46). Other activators of DCs, such as TNF-α or LPS, also have been shown to activate MAPK or JNK pathways (19, 20). These studies suggest that Bryo-1 + CI may use a distinct signaling pathway to activate DCs compared with the widely tested agents such as LPS or TNF-α. It should be noted that LPS has been shown to act primarily through Toll-like receptor 4 (47). Thus, further studies are necessary to address whether Bryo-1 + CI acts through Toll-like receptors or through independent mechanisms.

In conclusion, this study shows for the first time that Bryo-1 + CI can promote maturation of murine and human DCs and enhance their antigen-presenting properties to activate T cells. Thus, Bryo-1 may exhibit unique properties in activity as an anticancer agent and immunostimulant, which is highly beneficial for the management of cancer.

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