

# IFN- $\gamma$ and T-bet Expression in Human Dendritic Cells from Normal Donors and Cancer Patients Is Controlled through Mechanisms Involving ERK-1/2-Dependent and IL-12-Independent Pathways

Huifen Li, Wojciech Wojciechowski, Chiara Dell'Agola, Natalia E. Lopez, and Igor Espinoza-Delgado<sup>1</sup>

Dendritic cells (DC) play a major role in priming naive T cells and modulating the immune response. We have previously reported that bryostatin-1, a potent immune modulator with antitumor activity, activates monocytes and lymphocytes to produce cytokines. Studies have shown that tumor-bearing hosts have a Th1/Th2 cytokine pattern that is associated with decreased production of IFN- $\gamma$ . We investigated the expression of IFN- $\gamma$  in bryostatin-1-treated human DC. Bryostatin-1 induced both IFN- $\gamma$  and T-bet mRNA expression in a dose- and time-dependent manner. As little as 1 ng/ml bryostatin-1 induced IFN- $\gamma$  and T-bet transcripts within 3 h and protein at 12 h. Treatment of DC with cycloheximide revealed that bryostatin-1-induced T-bet expression requires de novo protein synthesis, but bryostatin-1-induced IFN- $\gamma$  expression is independent of protein synthesis. Furthermore, dexamethasone inhibits bryostatin-1-induced IFN- $\gamma$  mRNA expression but increases bryostatin-1-induced T-bet mRNA expression. Experiments with ERK-1/2 inhibitors demonstrated that bryostatin-1 induction of IFN- $\gamma$  and T-bet was ERK-dependent and IL-12-independent. Similar results were obtained from both normal donors and cancer patients. In summary, our results suggest that bryostatin-1-induced IFN- $\gamma$  expression is T-bet independent. They also suggest for the first time that IFN- $\gamma$  and T-bet can be induced in human DC through an ERK-dependent pathway. Bryostatin-1-induced IFN- $\gamma$  may play a crucial role in the initiation of the immune response, before specific recognition by T cells that could be beneficial in the treatment of cancer. *The Journal of Immunology*, 2006, 177: 3554–3563.

**B**ryostatin-1, a nontumor-promoting protein kinase C (PKC)<sup>2</sup> modulator, is a naturally occurring macrocyclic lactone derived from the marine bryozoan, *Bugula neritina* (1). Bryostatin-1 exhibits a unique pattern of biological activities and has received considerable attention in the past few years because of its antineoplastic and immunomodulatory effects both in vitro and in vivo (2). Bryostatin-1 inhibits the in vitro growth of a variety of tumor cell lines and also acts as a cytotoxic and/or differentiating agent on several tumor types (3, 4). The potential clinical value of bryostatin-1 as an antineoplastic agent is being evaluated in clinical trials (5, 6), yet the mechanisms responsible for the antitumoral activity of bryostatin-1 are poorly understood. The wide ranges of tumors that respond to bryostatin-1 in vitro suggest a direct mechanism of action. However, bryostatin-1 may also exert tumoricidal activity in vivo by indirect mechanisms that are related to its immunomodulatory activity (5, 7). We and others (7–10) have reported that bryostatin-1 activates several types of immune cells in vitro, including monocytes, lymphocytes,

granulocytes, and NK cells. Furthermore, increased levels of IL-6 and TNF- $\alpha$  have been reported in patients receiving bryostatin-1 (5).

IFN- $\gamma$  influences all aspects of the immune system, including the development, maturation, and activation of monocytes, macrophages, NK cells, B cells, and dendritic cells (DC) (11, 12). Consequently, IFN- $\gamma$  plays a pivotal role in the generation of an effective and potent immune response against a variety of targets, including pathogens and tumor cells (11, 13, 14). The importance of IFN- $\gamma$  is underscored by the fact that several mechanisms of cellular immunity are disrupted in both IFN- $\gamma$ <sup>-/-</sup> and in IFN- $\gamma$ R<sup>-/-</sup> mice (15, 16) and also in animals with targeted disruption of STAT-1, a pivotal target of IFN- $\gamma$  signaling (17). It is widely recognized that IFN- $\gamma$  and IL-12 are key to the development of Th1 cells (18, 19), and several studies have indicated that type 1 immune responses are strongly correlated with antitumor activity (20–22). These reports clearly underscore the importance of IFN- $\gamma$  in the immune response against tumor.

IFN- $\gamma$  was originally described as a product of either T cells or NK cells. More recently, the cellular source of IFN- $\gamma$  has expanded and now includes DC. The notion that DC are capable of producing IFN- $\gamma$  was initially taken with enormous reservations; however, several independent groups demonstrated unequivocally that DC are capable of producing IFN- $\gamma$  (23–26). The vast majority of these reports involved IFN- $\gamma$  production by murine DC. Information regarding the mechanisms controlling both IFN- $\gamma$  expression and production by human DC is either limited or absent. We have previously reported that bryostatin-1 activates human monocytes to produce proinflammatory cytokines (9). We also reported that T cells treated with bryostatin-1 in combination with IL-2 produce IFN- $\gamma$  (7). The present study was designed to investigate 1) whether bryostatin-1-treated human monocyte-derived

Section of Hematology-Oncology, Gerontology Research Center, National Institute on Aging, National Institutes of Health, Baltimore, MD 21224

Received for publication September 14, 2005. Accepted for publication June 23, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> Address correspondence and reprint requests to Dr. Igor Espinoza-Delgado, 5600 Nathan Shock Drive, Room 4C10, Baltimore, MD 21224. E-mail address: espinozaig@grc.nia.nih.gov

<sup>2</sup> Abbreviations used in this paper: PKC, protein kinase C; DC, dendritic cell; LGL, large granular lymphocyte; CHX, cycloheximide; DEX, dexamethasone; BI, bisindolylmaleimide I; BDCA, blood DC Ag; iDC, immature DC; mDC, mature DC; RT-qPCR, real-time quantitative PCR.

DC produce IFN- $\gamma$  mRNA and protein, and if so, 2) to elucidate the mechanisms controlling IFN- $\gamma$  mRNA expression. Our results demonstrate that bryostatin-1, through ERK-dependent and IL-12-independent mechanisms, induces expression of IFN- $\gamma$  mRNA in human DC. Induction of IFN- $\gamma$  mRNA occurs rapidly and through mechanisms that do not require de novo protein synthesis. IFN- $\gamma$  mRNA induced by bryostatin-1 leads to IFN- $\gamma$  production. Furthermore, bryostatin-1 induces T-bet mRNA expression with dose-response kinetics similar to that of bryostatin-1-induced IFN- $\gamma$ . Comparable results were observed in DC obtained from normal donors and patients with cancer. These data demonstrate for the first time that the ERK-1/2 pathway is involved in the induction of both IFN- $\gamma$  and T-bet mRNA in primary human DC. It is tempting to speculate that bryostatin-1-induced IFN- $\gamma$  might play a role in the initial stages of the antitumor response before specific recognition of tumor-associated Ags by T cells.

## Materials and Methods

### Human monocyte-derived DC

Peripheral blood leukocytes were obtained from normal healthy volunteers and patients with cancer by leukapheresis using a Fenwall CS-3000 blood cell separator (Fenwall Laboratories). The leukapheresis protocol was approved by the Institutional Review Board of our Institution. Mononuclear cells were separated by density gradient centrifugation on Ficoll-Paque (Amersham Biosciences) and then purified in suspension from the unfractionated mononuclear leukocyte preparation by countercurrent centrifugal elutriation in a Beckman JE-6 elutriation chamber and rotor system (Beckman Instruments) as described elsewhere (7). Cell purity was assessed by flow cytometry using mAbs against CD3, CD14, CD 11c, CD16, CD15, CD56, and CD19 (BD Pharmingen). The purity of the monocyte preparation was  $94 \pm 3\%$ . Other cells present in the monocyte preparation were as follows: 1–3% large granular lymphocytes (LGL), 2–4% lymphocytes, and <1% granulocytes. The purity of the DC preparation was  $95 \pm 4\%$ . Other cells present in the DC preparation were as follows: 1–2% LGL, <2% lymphocytes, and <1% granulocytes. Viability, as determined by the trypan blue exclusion test, was >99%. Monocytes were cultured in RPMI 1640 (BioWhittaker), supplemented with 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine, 20 mM HEPES (Invitrogen Life Technologies), and 10% heat-inactivated FBS (HyClone), hereafter referred to as complete medium. Cells were cultured in a Nunc 150  $\times$  15-mm tissue culture plate (Nalge Nunc International) at  $1 \times 10^6$  cells/ml in complete medium supplemented with recombinant human 500 U/ml GM-CSF and 500 U/ml IL-4 (sp. act. of GM-CSF and IL-4 were  $10 \times 10^6$  and  $5 \times 10^6$  U/mg, respectively; PeproTech) for 5–7 days, and depending on specific experiments, TNF- $\alpha$  (sp. act. of  $>2 \times 10^7$  U/mg, PeproTech) was added for the indicated periods of time. In selected experiments, CD40L (PeproTech) and LPS from *Escherichia coli* (Sigma-Aldrich) were used, after GM-CSF and IL-4, for 48 h.

### Reagents

Clinical grade bryostatin-1 was obtained from Cancer Therapy Evaluation Program, Division of Cancer Treatment, Diagnosis, and Centers, National Cancer Institute, National Institutes of Health. The molar equivalent for bryostatin-1 is 1.0 ng/ml = 0.904 nM. Recombinant human IL-12 was purchased from PeproTech, with a specific activity of  $1 \times 10^6$  U/mg. The inhibitors U0126, bisindolylmaleimide I (GF 109203X, BI), cycloheximide (CHX), and dexamethasone (DEX) were purchased from EMD Biosciences/Calbiochem and dissolved in DMSO. Anti-human IL-12 Ab was purchased from R&D Systems. Anti-human ERK-2 polyclonal Ab and anti-human phospho-ERK-1/2 mAb were obtained from Santa Cruz Biotechnology, HRP-conjugated anti-rabbit and anti-mouse IgG Abs were purchased from Sigma-Aldrich. ECL detection reagent was obtained from Amersham Biosciences, and [ $\gamma$ - $^{32}$ P]dCTP was obtained from PerkinElmer.

### Northern blot analysis

Northern blotting was performed as described previously (7). Briefly, DC were cultured in medium alone or supplemented with the indicated reagents. Total RNA was extracted with TRIzol (Invitrogen Life Technologies) and purified according to the manufacturer's specifications. Fifteen micrograms of total RNA from each sample was electrophoresed under denaturing conditions on 1.2% formaldehyde gel, blotted onto Nytran membrane (Schleicher & Schuell), and cross-linked by UV irradiation. The membranes were prehybridized for 8–10 h at 43°C in hybridization buffer

(Hybrizol; Oncor), followed by overnight hybridization with  $0.5 \times 10^6$  cpm/ml of specific cDNA probe labeled with  $^{32}$ P using High Prime DNA labeling reagent (Roche Diagnostics). The membranes were then washed and exposed to x-ray film (Biomax-MR; Eastman Kodak) and phosphor storage screens. The phosphor screens were scanned using a Storm 840 PhosphorImager and analyzed using ImageQuant image analysis software (Amersham Biosciences). The membranes were stripped and rehybridized with a 18S rRNA probe. The values for IFN- $\gamma$  and T-bet mRNA levels were normalized with respect to 18S rRNA levels, and ratios were plotted. Human T-bet cDNA and human IFN- $\gamma$  cDNA were provided by Dr. L. H. Glimcher (Harvard School of Public Health, Boston, MA) and Dr. H. Young (National Cancer Institute, Frederick, MD), respectively. The 1.6-kb *EcoRI-XhoI* fragment of T-bet cDNA and 1.0-kb *AvaI-HincII* fragment of IFN- $\gamma$  cDNA were used as probes for Northern blot analysis. The pBR322 plasmid containing human 18S rRNA sequence was provided by Dr. D. Radzioch (McGill University, Montreal, Quebec, Canada). The *BamHI-EcoRI* 1.1-kb DNA fragment of the sequence was used as a probe.

### Flow cytometry analysis

Analysis of surface expression Ags by flow cytometry was performed as described previously (7). Following 12 h of treatment with 1 ng/ml bryostatin-1 or medium alone, DC were washed once at 4°C with PBS. Next, the cells were resuspended in FACS buffer (0.5% BSA, 1% heat-inactivated human AB serum, and 0.1% sodium azide in PBS (pH 7.2)) and incubated on ice for 15 min to reduce nonspecific binding. One million cells were resuspended in 100  $\mu$ l of FACS buffer and incubated for 15 min on ice with anti-human Abs conjugated with fluorescein (FITC) or PE. After two washes with FACS buffer, the cells were analyzed using FACScan flow cytometry equipment (BD Biosciences). The data obtained were processed using the WinMDI version 2.8 flow cytometry application (<http://facs.scripps.edu>). Dead cells and cellular debris were gated out from the analysis using forward and side scatter.

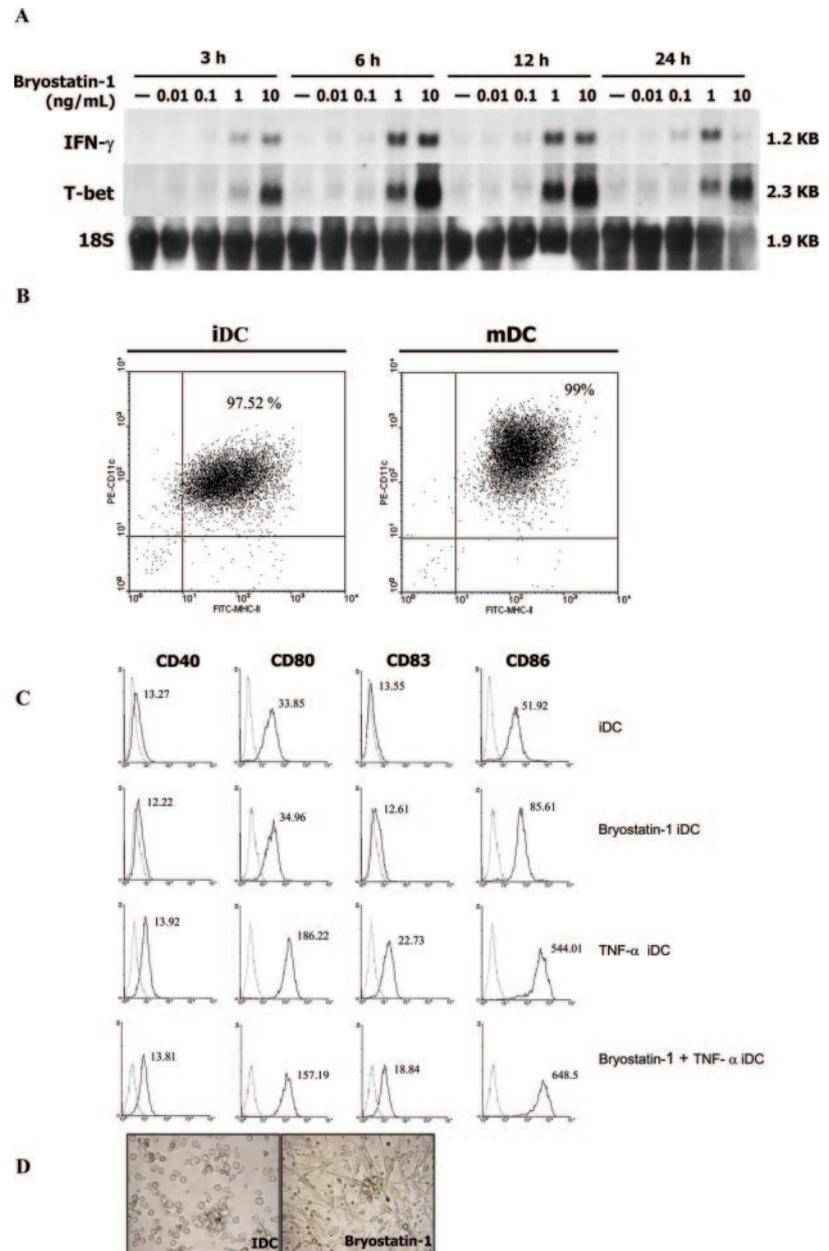
For IFN- $\gamma$  intracellular staining, cells were permeabilized with Cytofix/Cytoperm solution (BD Biosciences) and stained with FITC-mouse-anti-human-IFN- $\gamma$  mAb (BD Pharmingen) according to the manufacturer's instructions. Stained cells were analyzed for two-color immunofluorescence with FACScan flow cytometry equipment (BD Biosciences). A minimum of  $10^4$  cells were analyzed for each sample. Specificity of IFN- $\gamma$  binding was confirmed by using negative staining controls, including isotype control and ligand blocking controls, as per the manufacturer's instructions.

### Western blot analysis

Cells were incubated in the presence or absence of bryostatin-1 at the indicated concentrations and times. Where indicated, inhibitors were added to the cell culture 30 min before bryostatin-1. Cells were harvested and washed twice with cold PBS, and the pellets were collected by centrifugation and resuspended in 100  $\mu$ l of lysis buffer (1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, and PBS), containing PMSF (100  $\mu$ g/ml) and protease inhibitor mixture (500  $\mu$ g/ml) (Roche Diagnostics). The total protein concentration was measured by bicinchoninic acid assay reagent (Pierce). Samples containing 30  $\mu$ g of total protein were heated at 100°C for 5 min in the presence of DTT and then separated on 8–16% gradient Tris-glycine precast gels (Invitrogen Life Technologies). Following electrophoresis, the proteins were electrotransferred (XCell II; Invitrogen Life Technologies) onto polyvinylidene difluoride membranes (Millipore), according to the procedure recommended by the manufacturer. Following overnight blocking at 4°C with 5% nonfat milk in TBS-T buffer (20 mM Tris-HCl (pH 7.5), 0.14 M sodium chloride, and 0.1% Tween 20), the membranes were incubated for 1 h at room temperature with T-bet or phospho-ERK-1/2 primary Abs. Following three 10-min washes with TBS-T, the membranes were incubated for 1 h at room temperature with HRP-conjugated secondary Ab. The signal was visualized using ECL reagent (Amersham Biosciences), and the membranes were exposed to x-ray film. To test the levels of total ERK-2, membranes were stripped with 0.2 N sodium hydroxide and reprobed using anti-ERK-2 Ab.

### Blood myeloid BDCA1 DC isolation

PBMC were purified as described above, and peripheral blood myeloid DC were magnetically sorted with blood DC Ag (BDCA)-1 DC isolation kits (Miltenyi Biotec), according to the manufacturer's instructions. Typically, a total of  $1 \times 10^7$  BDCA-1 DC was isolated from  $2 \times 10^9$  PBMC. The cells were stained with mAbs against BDCA-1, CD3, CD11c, CD14, CD19, CD56, and MHC class II to determine purity, which always was  $\geq 95\%$ . Other cells present in the BDCA1 DC preparation were as follows: <2% lymphocyte, <1% LGL, and <1% monocyte.



**FIGURE 1.** *A*, Bryostatin-1 induces IFN- $\gamma$  and T-bet mRNA expression in human iDC. iDC were stimulated in the absence or presence bryostatin-1 for the indicated dose and times. Total cellular RNA was isolated, and Northern blot analysis for IFN- $\gamma$  and T-bet mRNA expression was performed. The same membrane was rehybridized with 18S template probe to ensure that equal amounts of RNA were loaded in each lane. Data shown are from one representative experiment of four performed. *B*, Monocytes were cultured in complete medium supplemented with 500 U/ml GM-CSF and 500 U/ml IL-4 for 5 days (iDC). mDC were obtained by culturing iDC with 10 ng/ml TNF- $\alpha$  for 2 days. Cells were labeled with PE-CD11c and FITC-MHC class II Abs, and cells were analyzed on a FACScan flow cytometer. Data shown are from one representative experiment of five performed. *C*, iDC were cultured with either bryostatin-1 for 6 h or with TNF- $\alpha$  for 48 h, followed by bryostatin-1 for 6 h. iDC ( $1 \times 10^6$ ) were harvested, washed, and double stained with FITC-conjugated anti-CD11c and PE-conjugated anti-CD40, -CD80, -CD83, and -CD86 mAbs. The CD11c-positive cells, which represent >95% of the entire cell population, was gated, and the expression of CD40, CD80, CD83, and CD86 was investigated. *D*, iDC cultured in the presence or absence of bryostatin-1 were observed under a phase contrast microscopy with a magnification of  $\times 40$ .

#### Real-time quantitative PCR (RT-qPCR) analysis

BDCA-1<sup>+</sup> myeloid blood DC were cultured for 6 h at  $1 \times 10^6$ /ml in the presence or absence of 1 ng/ml bryostatin-1. Cells were then lysed in TRIzol (Invitrogen Life Technologies), and total RNA was purified according to the manufacturer's instructions. Following DNase I (Ambion) treatment, first-strand cDNA synthesis was conducted using the iScript cDNA synthesis kit (Bio-Rad) in a total volume of 20  $\mu$ l containing 0.5  $\mu$ g of total RNA. IFN- $\gamma$  and RLP13A were amplified using specific primers (catalog no. PPH00380A and PPH01020A, respectively) purchased from SuperArray Bioscience. iQ SYBR Green Supermix kit (Bio-Rad) and the Mini Opticon Real-Time PCR System Detector (Bio-Rad) were used to amplify IFN- $\gamma$  and RLP13A cDNA. Protocol for amplification of target genes was performed according to the manufacturer's instructions (SuperArray Bioscience). The cycle was performed as follows: 15 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C. All samples were run in triplicate. The relative amounts of IFN- $\gamma$  transcripts were normalized to RLP13A, and the difference fold among samples was calculated as described in detail in the Real-Time PCR Application Guide (Bio-Rad).

#### IFN- $\gamma$ detection

Cell-free supernatant was collected from DC in the presence or absence of 1 ng/ml bryostatin-1 for 18 h, and IFN- $\gamma$  secretion was evaluated using a

commercially available solid phase sandwich ELISA kits (BioSource International) in accordance with the manufacturer's instructions.

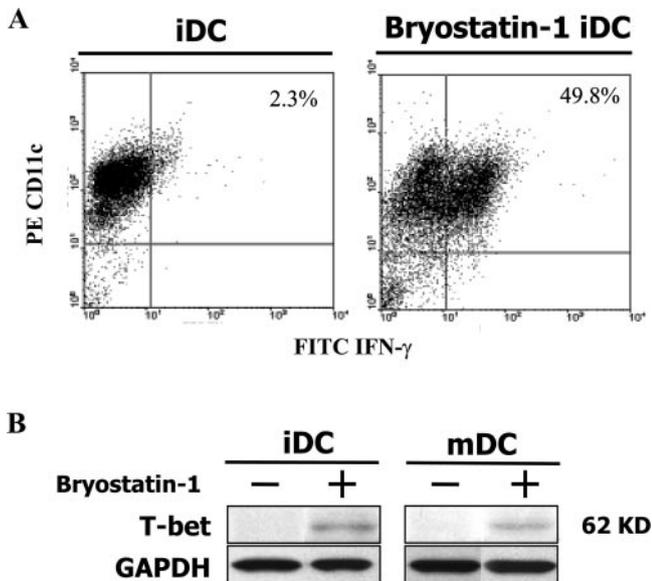
#### IL-12 neutralization

To neutralize endogenous IL-12 in vitro, DC at  $1 \times 10^6$ /ml were incubated with a single dose of 25  $\mu$ g/ml anti-human IL-12 Ab for 1 h (R&D Systems) at 37°C. After incubation, cells were treated with 1 ng/ml bryostatin-1 or medium alone for 6 h. For neutralizing rhIL-12, 25  $\mu$ g/ml anti-h IL-12 Ab was incubated with either 1.5 ng/ml recombinant human IL-12 or medium alone in a total volume of 100  $\mu$ l at 37°C. After 1 h, the mixture of cytokine and Ab was added to the cells for 6 h. Cells were harvested, and total RNA was extracted with TRIzol (Invitrogen Life Technologies) and purified according to the manufacturer's directions.

## Results

### Bryostatin-1 induces both IFN- $\gamma$ mRNA and T-bet mRNA expression in human monocyte-derived DC

To determine whether bryostatin-1 may induce the expression of IFN- $\gamma$  mRNA in human monocyte-derived DC, monocytes were cultured for 5 days with IL-4 and GM-CSF (immature DC (iDC)) and then treated with TNF- $\alpha$  (mature DC (mDC)). Both iDC and



**FIGURE 2.** A, Bryostatin-1 induces IFN- $\gamma$  protein expression in human iDC. iDC were cultured in the absence or presence 1 ng/ml bryostatin-1 for 12 h. CD11c cell surface and IFN- $\gamma$  intracellular staining were performed, and cells were analyzed on a FACScan flow cytometer. B, iDC were cultured as above for 6 h, and total protein was extracted. The levels of T-bet were assayed by Western blot analysis (*upper panel*), and the levels of GAPDH were obtained after stripping and reprobing the membrane (*lower panel*). Data shown are from one of four representative experiments performed.

mDC were treated with increasing concentrations of bryostatin-1 for 3–24 h. Total RNA was extracted, and Northern blot analysis was performed. As shown in Fig. 1A, no expression or very low expression of IFN- $\gamma$  mRNA was detected in medium-treated cells, whereas treatment of DC with bryostatin-1 led to a major induction of IFN- $\gamma$  mRNA. The effect of bryostatin-1 on IFN- $\gamma$  mRNA expression was dose dependent, and as little as 1 ng/ml was sufficient to induce a moderate increase in IFN- $\gamma$  mRNA expression by 6 h while doses of 10 ng/ml were required for maximum expression. The induction of IFN- $\gamma$  mRNA was detected as early as 3 h after bryostatin-1 treatment. Maximum induction of IFN- $\gamma$  mRNA occurred at 6 h and remained elevated for up to 24 h, the last time point tested.

The T-box transcription factor, T-bet, has been shown to play a critical role in the regulation and expression of IFN- $\gamma$  (27). Mice lacking T-bet fail to develop Th-1 cells, and they have a major reduction in IFN- $\gamma$  production (26, 28). To ascertain whether bryostatin-1 affects the expression of T-bet in DC, the blot shown in Fig. 1A was rehybridized with a T-bet probe. As depicted in the *middle panel*, T-bet mRNA expression mimics the expression of IFN- $\gamma$  mRNA but shows a slightly different dose-response pattern. Similar results were observed in mDC (data not shown). The purity of the monocyte-derived DC population was  $95 \pm 4\%$  (Fig. 1B). The phenotype of iDC treated with bryostatin-1 alone, TNF- $\alpha$  alone, or the combination of bryostatin-1 and TNF- $\alpha$  are shown in Fig. 1C. The experiments demonstrate that bryostatin-1 enhances the expression of CD86 but does not appreciably affect the expression of CD40, CD80, or CD83. On the other hand, TNF- $\alpha$ -treated iDC display an increased expression of CD80, CD83, and CD86. Typically, we noticed a larger proportion of cells with dendrites in iDC cells treated with bryostatin-1 compared with control iDC (Fig. 1D). The data suggest that, under our experimental conditions, bryostatin-1 alone is a weak inducer of DC maturation.

To investigate whether the enhanced expression of IFN- $\gamma$  mRNA and T-bet led to an increased protein production, iDC were cultured in the absence or presence of 1 ng/ml bryostatin-1 and analyzed by either flow cytometry for IFN- $\gamma$  production or Western blot analysis for T-bet protein expression. Bryostatin-1-treated cells but not medium-treated cells displayed a major increase in intracellular expression of IFN- $\gamma$  (Fig. 2A) and T-bet total protein expression (Fig. 2B). Similar results were obtained with mDC (data not shown). To evaluate whether the increased intracellular expression of IFN- $\gamma$  caused protein secretion, supernatants were assayed for the presence of IFN- $\gamma$ . iDC were cultured in the presence or absence of bryostatin-1 for 18 h, and their supernatants were analyzed by ELISA. As shown in Table I, iDC treated with medium do not secrete detectable levels of IFN- $\gamma$ , whereas iDC from five donors secreted IFN- $\gamma$  after bryostatin-1 treatment. These experiments indicate that bryostatin-1 induces expression of both T-bet mRNA and IFN- $\gamma$  mRNA, resulting in increased protein expression in human DC.

#### *Protein synthesis is differentially required for IFN- $\gamma$ and T-bet mRNA induction by bryostatin-1*

To establish whether active protein synthesis is needed for the induction of IFN- $\gamma$  and/or T-bet mRNA by bryostatin-1, iDC and mDC were incubated for 6 h in the absence or presence of 1.0 ng/ml bryostatin-1 and in the absence or presence of increasing concentrations of the protein synthesis inhibitor CHX. As shown in Fig. 3A, the addition of CHX to bryostatin-1-treated cells caused a major decrease in the expression of T-bet mRNA. On the other hand, CHX induced only a mild decrease in the expression of IFN- $\gamma$ . These results suggest three things: 1) bryostatin-1-induced T-bet mRNA is dependent on de novo protein synthesis; 2) bryostatin-1-induced IFN- $\gamma$  mRNA is minimally dependent on de novo protein synthesis; and 3) bryostatin-1 induces IFN- $\gamma$  mRNA through a T-bet independent pathway.

#### *Enhanced expression of both T-bet and IFN- $\gamma$ mRNA is PKC and ERK dependent*

Activation of PKC by bryostatin-1 appears to play a significant role in cellular responses to this agent. To ascertain the role of PKC in the induction of either IFN- $\gamma$  mRNA or T-bet mRNA by bryostatin-1, iDC were treated with either medium or bryostatin-1 in the presence of increasing concentrations of the specific PKC inhibitor BI, and the expression of IFN- $\gamma$  mRNA or T-bet mRNA was evaluated. As shown in Fig. 3B, BI inhibits both T-bet and IFN- $\gamma$  mRNA induced by bryostatin-1. The inhibitory effect of BI was more pronounced on T-bet mRNA (56 and 95% inhibition at the doses of 0.1 and 1  $\mu$ M, respectively) than in IFN- $\gamma$  mRNA (20 and 40% inhibition).

Glucocorticoids regulate the expression of several genes (29). Specifically, DEX has been shown to block both NF- $\kappa$ B nuclear translocation and AP-1/CREB interaction with the IFN- $\gamma$  promoter (30). These two nuclear transcriptional complexes are known to

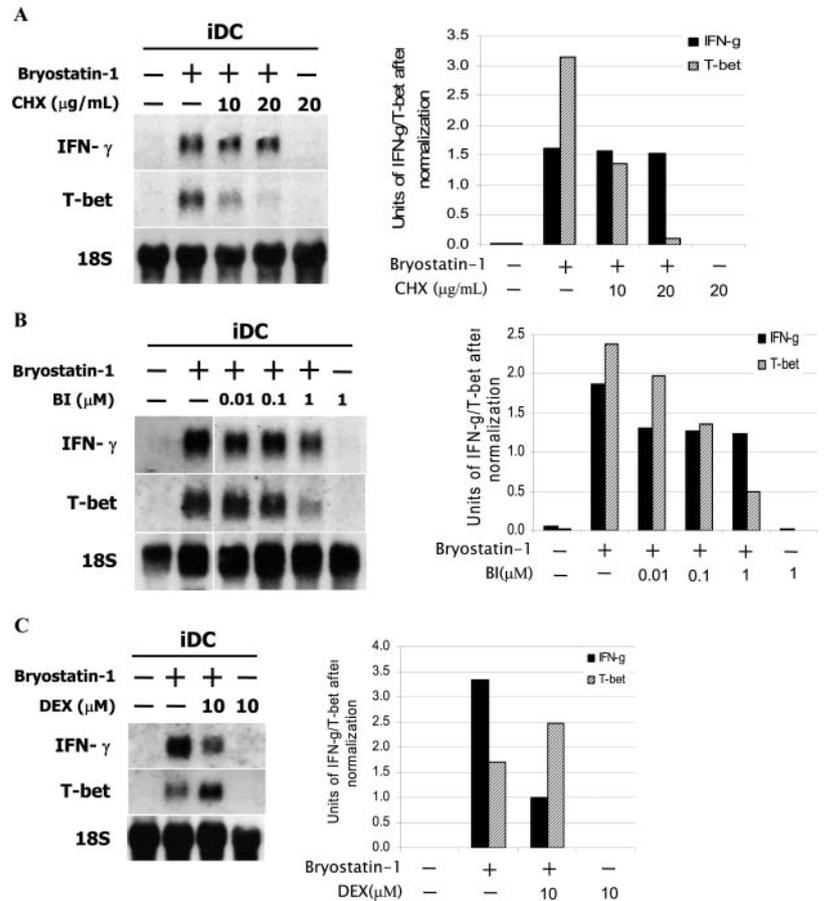
Table I. *Bryostatin-1 induces IFN- $\gamma$  secretion in iDC<sup>a</sup>*

Treatment	IFN- $\gamma$ (pg/ml)				
	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5
Medium	ND <sup>b</sup>	ND	ND	ND	ND
Bryostatin-1 (1 ng/ml)	81	180	190	201	195

<sup>a</sup> Monocyte-derived DC from five normal donors were cultured for 18 h in the presence or absence of 1 ng/ml bryostatin-1. Cell-free supernatants were tested by ELISA for the presence of IFN- $\gamma$ .

<sup>b</sup> ND, not detectable.

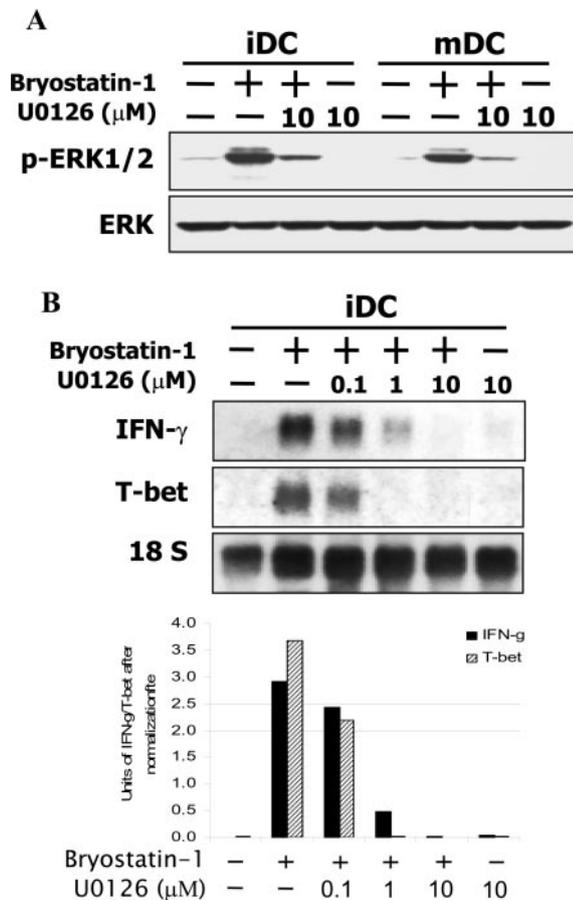
**FIGURE 3.** A, CHX differentially regulates IFN- $\gamma$  and T-bet mRNA expression. iDC were preincubated for 30 min in either medium or CHX and then cultured for 6 h in the absence or presence of 1 ng/ml bryostatin-1. Total cellular RNA was extracted and analyzed by Northern blot for both IFN- $\gamma$  and T-bet mRNA expression. The same membrane was rehybridized with 18S template probe to ensure that equal amounts of RNA were loaded in each lane. Data shown are from one of four representative experiments performed. B, PKC inhibitor decreases IFN- $\gamma$  and T-bet mRNA expression induced by bryostatin-1. iDC were preincubated for 30 min in medium or increasing concentrations of BI and then cultured as above for 6 h. Northern blot analysis for IFN- $\gamma$  and T-bet mRNA expression was performed. Data shown are from one representative experiment of four performed. C, DEX differentially regulates IFN- $\gamma$  and T-bet mRNA expression. iDC were preincubated for 30 min in the absence or presence of DEX and then cultured as above for 6 h. Northern blot analysis for IFN- $\gamma$  and T-bet mRNA expression was performed. Data shown are from one of five representative experiments performed.



play a major role in IFN- $\gamma$  gene transcription. To ascertain the effects of DEX on bryostatin-1-induced expression of IFN- $\gamma$  and T-bet, human DC were cultured with DEX in the presence or the absence of bryostatin-1 for 6 h. As shown in Fig. 3C, DEX inhibits bryostatin-1-induced IFN- $\gamma$  mRNA. In contrast, DEX increases bryostatin-1-induced T-bet mRNA expression. These results suggest that the intracellular events leading to both IFN- $\gamma$  and T-bet mRNA expression in human DC in response to bryostatin-1 occur through different pathways. We and others (31, 32) have previously reported that bryostatin-1 can signal through the ERK-1/2-dependent pathway in tumor cells. To ascertain whether bryostatin-1 could also induce ERK-1/2 phosphorylation in primary cells, human iDC were treated with bryostatin-1 for the indicated times, and Western blot analysis was performed. As shown in Fig. 4A, medium-treated iDC expressed very low basal levels of phosphorylated ERK-1/2. In contrast, bryostatin-1-treated cells displayed high levels of the phosphorylated forms of ERK-1/2. The bryostatin-1-induced ERK-1/2 phosphorylation was blocked by the MEK-1/2 inhibitor U1026. The induction of ERK-1/2 phosphorylation was also observed in mDC. To determine the role of the MEK/ERK pathway in the induction of both T-bet and IFN- $\gamma$  mRNA, we investigated the effects of the U0126 on bryostatin-1-treated DC. iDC were incubated for 30 min with the indicated concentrations of U0126, and then 1 ng/ml bryostatin-1 was added for 6 h and Northern blot analysis was performed. As shown in Fig. 4B, U0126 inhibited the induction of IFN- $\gamma$  mRNA and T-bet mRNA in a dose-dependent manner. As little as 0.1  $\mu$ M U0126 was sufficient to produce a moderate decrease of both transcripts, and a major suppression was observed with 1  $\mu$ M U0126. The decreased expression of IFN- $\gamma$  mRNA and T-bet mRNA was also observed in mDC (data not shown).

#### *Bryostatin-1 induces IFN- $\gamma$ through IL-12-independent pathways*

IL-12 is directly involved in the production of IFN- $\gamma$  by T cells and NK cells (33, 34). It was recently reported that IL-12 may activate murine DC to produce IFN- $\gamma$ ; therefore, it is conceivable that IL-12 could be responsible for bryostatin-1-induced IFN- $\gamma$  mRNA. To ascertain a potential role of IL-12 in the present system, we studied the effects of IL-12 in the phosphorylation status of ERK 1/2 that we previously demonstrated is required for the induction of IFN- $\gamma$  by bryostatin-1. Cells were treated for the indicated periods of time with bryostatin-1 or IL-12 in the presence or absence of U0126, and Western blot analysis was performed. As shown in Fig. 5A, iDC expressed very low levels of phosphorylated ERK 1/2. Bryostatin-1, as expected, induced a major phosphorylation of ERK 1/2. In contrast, IL-12 did not affect the basal levels of phosphorylated ERK 1/2. To further evaluate the role of IL-12, iDC cells were treated with either bryostatin-1 or IL-12 for 6 h in the presence or absence of U0126, and Northern blot analysis was performed. As shown in Fig. 5B, both bryostatin-1 and IL-12 induced IFN- $\gamma$  mRNA. U0126 almost completely blocked bryostatin-1-induced IFN- $\gamma$  mRNA, but it did not inhibit IL-12-induced IFN- $\gamma$  mRNA. Interestingly, bryostatin-1 but not IL-12 induced T-bet mRNA. Finally, we investigated the effects of anti-IL-12 on bryostatin-1-induced IFN- $\gamma$  mRNA. iDC were cultured with either IL-12 or bryostatin-1 in the presence or absence of anti-IL-12 mAb, and Northern blot assay for IFN- $\gamma$  mRNA was performed. As shown in Fig. 5C, IL-12-induced IFN- $\gamma$  mRNA was completely blocked in the presence of anti-IL-12 mAb. On the contrary, anti-IL-12 mAb did not affect the expression of IFN- $\gamma$  mRNA induced by bryostatin-1. These results suggest that IL-12 and bryostatin-1 induce IFN- $\gamma$  mRNA through different pathways.



**FIGURE 4.** A, MEK-1/2 inhibitor suppresses ERK-1/2 activation induced by bryostatin-1. DC were preincubated for 30 min in the absence or presence of 10  $\mu$ M U0126 and then cultured for 1 h in the absence or presence of 1 ng/ml bryostatin-1. Total cellular protein was extracted and analyzed by Western blot for ERK-1/2 activation. The same membrane was reprobbed with anti-ERK-2 Ab to detect the total ERK level. Data shown are from one of four representative experiments performed. B, MEK-1/MEK-2 inhibitor decreases IFN- $\gamma$  and T-bet mRNA expression induced by bryostatin-1. DC were preincubated for 30 min in the absence or presence of increasing concentrations of U0126 and then cultured for 6 h in medium or 1 ng/ml bryostatin-1. Northern blot analysis for IFN- $\gamma$  and T-bet mRNA expression was performed. Data shown are from one of four representative experiments performed.

#### *Bryostatin-1-induced IFN- $\gamma$ mRNA in DC is not compromised in cancer patients*

Cancer patients have been reported to display several immune alterations (35–38). To explore whether bryostatin-1 effects on IFN- $\gamma$  mRNA expression could be compromised in patients with cancer, iDC were obtained from cancer patients and treated with bryostatin-1 *in vitro*. Cells were cultured for 6 h in the presence or absence of bryostatin-1, RNA was extracted, and Northern blot analysis was performed. As shown in Fig. 6A, bryostatin-1-treated iDC or mDC displayed a major induction of IFN- $\gamma$  mRNA. In terms of the ERK-1/2 phosphorylation (Fig. 6B), bryostatin-1-treated iDC or mDC displayed an ERK-1/2 phosphorylation pattern similar to that observed in normal donors. Overall, these results demonstrate that bryostatin-1 is fully capable of inducing IFN- $\gamma$  mRNA in DC obtained from cancer patients. It also suggests that, to the extent the tumor-bearing state may produce functional defects based on inadequate IFN- $\gamma$  levels, bryostatin-1 may overcome some of the functional alterations observed in DC obtained from cancer patients.

#### *Peripheral blood DC and CD40 plus LPS-treated monocyte-derived DC express IFN- $\gamma$ mRNA in response to bryostatin-1*

To investigate whether bryostatin-1 induces IFN- $\gamma$  expression in circulating blood DC, myeloid DC were purified directly from peripheral blood and treated with either medium or bryostatin-1. After 6 h, total RNA was extracted, and RT-qPCR was performed. As shown in Fig. 7A, myeloid blood DC express very low, if any, levels of IFN- $\gamma$  mRNA. In contrast, bryostatin-1-treated cells displayed a 9-fold increase in IFN- $\gamma$  mRNA expression compared with their medium-treated counterpart. The data suggest two things: 1) bryostatin-1 induces IFN- $\gamma$  mRNA in circulating blood DC; and 2) bryostatin-1-induced IFN- $\gamma$  mRNA in monocyte-derived DC is unlikely due to the cytokine pressure that these cells have been subjected *in vitro*.

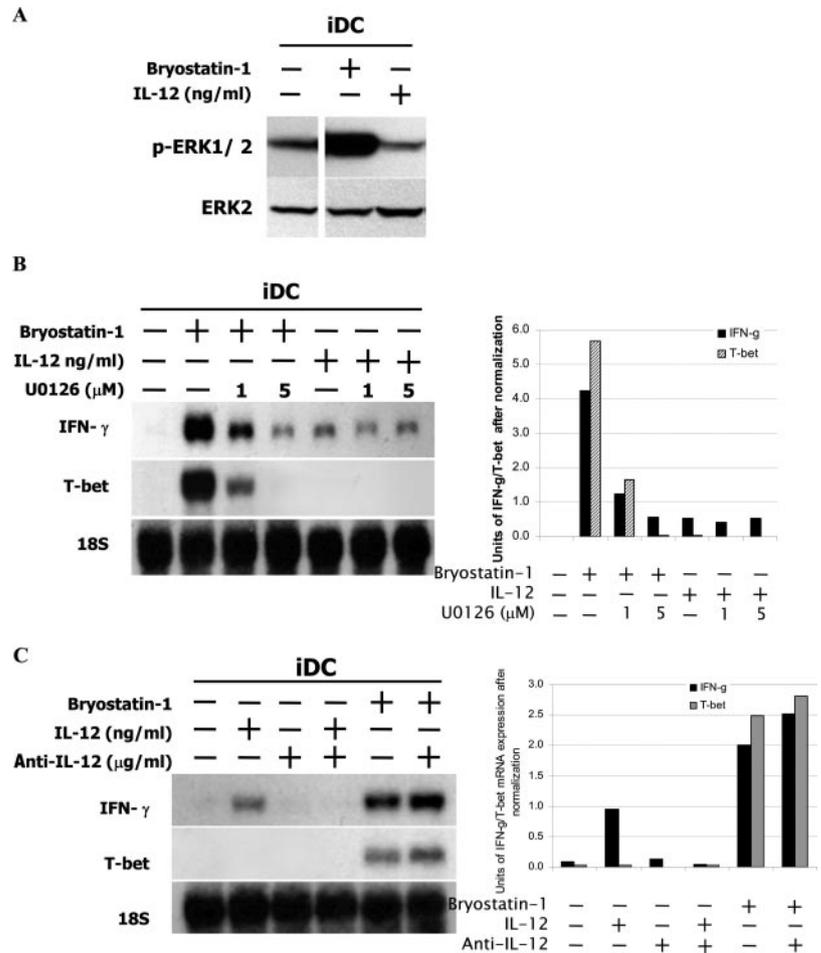
TNF- $\alpha$  is not an optimal stimulus for DC maturation. To ascertain whether bryostatin-1 was capable of inducing IFN- $\gamma$  in fully mDC, iDC were cultured in the presence or absence of CD40 ligand plus LPS. After 48 h, medium or bryostatin-1 was added to the cells. As shown in Fig. 7B, iDC did not express IFN- $\gamma$  mRNA. On the other hand, both bryostatin-1 and, at a lesser degree, CD40 plus LPS-treated cells displayed an increased expression of IFN- $\gamma$  mRNA. Interestingly, bryostatin-1 in combination with CD40 plus LPS synergistically induce the expression of IFN- $\gamma$  mRNA. Overall, the data suggest that bryostatin-1 induces IFN- $\gamma$  in circulating DC and also in monocyte-derived DC independently of their maturation status.

## Discussion

While the contribution of APC in producing IFN- $\gamma$  is well established in murine models (24, 25), data regarding expression and production of IFN- $\gamma$  by human DC are limited and the mechanisms poorly understood. In this investigation, we report for the first time that the antineoplastic and immunomodulating agent bryostatin-1 induces the expression of both T-bet and IFN- $\gamma$  mRNA expression in human monocyte-derived DC. In contrast with T cells, in which bryostatin-1 alone has been reported to be insufficient to induce IFN- $\gamma$  (7, 39), DC produce IFN- $\gamma$  in response to bryostatin-1 alone, suggesting that the mechanisms leading to IFN- $\gamma$  production by these cells are different. Induction of IFN- $\gamma$  mRNA was dose dependent with as little as 0.1 ng/ml bryostatin-1 needed to induce IFN- $\gamma$  expression. Maximum expression was achieved with 10 ng/ml bryostatin-1. The induction of IFN- $\gamma$  occurred within 3 h of treatment was further augmented at 12 h and declined thereafter. The rapid induction of IFN- $\gamma$  mRNA expression suggests a direct response independent of *de novo* protein synthesis. Indeed, the data demonstrate that CHX treatment did not produce a major effect in the early induction of IFN- $\gamma$  by bryostatin-1. This is in sharp contrast with T cells in which CHX up-regulates the expression of IFN- $\gamma$  mRNA in response to treatment with either bryostatin-1 and IL-2 or IL-12 and PHA (7, 40). Bryostatin-1-induced IFN- $\gamma$  mRNA led to protein production and secretion, suggesting that bryostatin-1-driven IFN- $\gamma$  production is, at least in part, controlled at the gene level (Fig. 3A). Interestingly, bryostatin-1-induced IFN- $\gamma$  mRNA levels were consistently higher than those obtained with the potent IFN- $\gamma$  mRNA inducer IL-12.

To gain insights into the biochemical mechanisms leading to the expression of IFN- $\gamma$  by DC in response to bryostatin-1, experiments were designed to investigate whether bryostatin-1-induced IFN- $\gamma$  mRNA expression was affected by either PKC or ERK inhibitors. Bryostatin-1 is a potent PKC modulator, and many of its biological effects are mediated through the activation of PKC (1, 41). However, bryostatin-1 can antagonize several PKC-mediated effects (1, 42, 43), and recent reports suggest that some biological

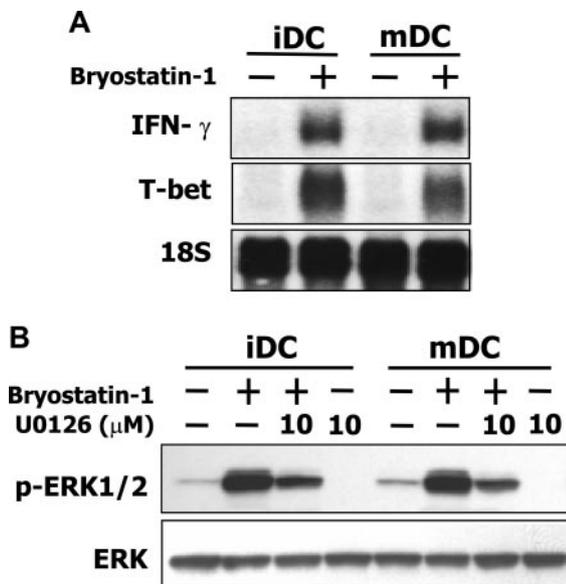
**FIGURE 5.** A, Bryostatin-1 but not IL-12 induces ERK-1/2 activation. iDC were cultured in the absence or presence of 1 ng/ml bryostatin-1 or 1.5 ng/ml IL-12 for 1 h. Total cellular protein was extracted and analyzed by Western blot for ERK-1/ERK-2 activation. The same membrane was rehybridized with anti-ERK-2 Ab to detect total ERK level. B, Bryostatin-1 but not IL-12 induces T-bet mRNA expression. DC were preincubated for 20 min in presence or absence of increasing concentrations of U0126 and then cultured for 6 h in 1 ng/ml bryostatin-1 or 1.5 ng/ml IL-12. Northern blot analysis for both IFN- $\gamma$  and T-bet mRNA expression was performed. Data shown are from one of four representative experiments performed. C, Bryostatin-1 induces IFN- $\gamma$  mRNA through an IL-12-independent pathway. DC were preincubated with either anti-h IL-12 Ab alone or with a premixed combination of IL-12 and anti-human IL-12 Ab for 1 h in the presence or absence of bryostatin-1 for 6 h. Northern blot analysis for both IFN- $\gamma$  and T-bet mRNA expression was performed. Data shown are from one of two representative experiments performed.



effects of bryostatin-1 might be PKC independent (44). In the present study, the specific PKC inhibitor BI, at a dose of 0.1  $\mu$ M, induced a moderate inhibitory effect (33%) on bryostatin-1-induced IFN- $\gamma$  mRNA. These data suggest that other kinases are also involved in the signal transduction pathway leading to IFN- $\gamma$  mRNA expression. We and others (31, 32) have reported that bryostatin-1 induces ERK-1/2 phosphorylation in tumor cells. Confirming and expanding these observations, we demonstrate for the first time that bryostatin-1 can induce ERK-1/2 phosphorylation in primary human DC. Using a specific MEK kinase inhibitor, we also provide evidence that the induction of IFN- $\gamma$  mRNA by bryostatin-1 is MEK/ERK dependent. Using the online-based Genomatrix suite ([www.genomatrix.de](http://www.genomatrix.de)), we identified elements in the promoter of the IFN- $\gamma$  gene capable of binding transcription factors belonging to the Ets family. Some members of this family are activated in an ERK-1/2 dependent fashion (45). Future work in our laboratory will attempt to further define the transcriptional events induced by bryostatin-1 in DC. In contrast with the present results, Do et al. reported that bryostatin-1 enhances the maturation of DC without involving ERK phosphorylation (46). There are several differences between the two experimental systems, most notably: 1) the species used and the source of DC, murine bone marrow cells vs human monocytes; 2) the stimuli used, bryostatin-1 in combination with calcium ionophore vs bryostatin-1 alone; and 3) kinetics, the earliest point at which that Do et al. evaluated ERK-1/2 phosphorylation was 2 h. We found that bryostatin-1 induced ERK-1/2 phosphorylation within 20 min (data not shown). Any of these factors may account for the observed differences.

In an attempt to further explore the molecular mechanisms leading to the induction of IFN- $\gamma$  and T-bet mRNA expression, the

glucocorticoid DEX was used. We demonstrated that DEX differentially regulates the expression of IFN- $\gamma$  and T-bet in response to bryostatin-1. Indeed, DEX strongly inhibits bryostatin-1-induced IFN- $\gamma$  mRNA while it increases bryostatin-1-induced T-bet mRNA expression. Taken together these results suggest that the pathways leading to both IFN- $\gamma$  and T-bet mRNA expression in human DC in response to bryostatin-1 are different. T-bet was initially reported to be expressed mainly in NK cells and T cells. More recently, T-bet has been reported to be expressed in myeloid cells where it is required for optimal production of IFN- $\gamma$  (26, 28). Although the rapid induction of IFN- $\gamma$  mRNA in human DC suggests a direct effect of bryostatin-1 rather than a secondary effect mediated by another bryostatin-1-inducible gene, we investigated the role of T-bet in our experimental system. Similarly to IFN- $\gamma$  mRNA, no basal or very low basal expression of T-bet mRNA was detected in medium-treated cells. Treatment of DC with bryostatin-1 led to a major induction of T-bet mRNA. Dose-response and kinetics of T-bet transcript, as well as protein expression, mimicked that of IFN- $\gamma$  mRNA (Fig. 1). Notably, in our experimental system, bryostatin-1 seems to induce IFN- $\gamma$  and T-bet through different mechanisms. This hypothesis is supported by the following facts: 1) in contrast with IFN- $\gamma$  mRNA, induction of T-bet mRNA is dependent on de novo protein synthesis as CHX completely blocked the induction of T-bet mRNA in DC treated with bryostatin-1 (Fig. 3A); 2) the role of PKC in the induction of T-bet by bryostatin-1 seems to be more prominent than in bryostatin-1-induced IFN- $\gamma$  mRNA (Fig. 3B); and 3) DEX blocked bryostatin-1-induced IFN- $\gamma$  mRNA but not bryostatin-1-induced T-bet mRNA expression. Taken together, our results suggest that in human DC, bryostatin-1 may induce IFN- $\gamma$  mRNA expression

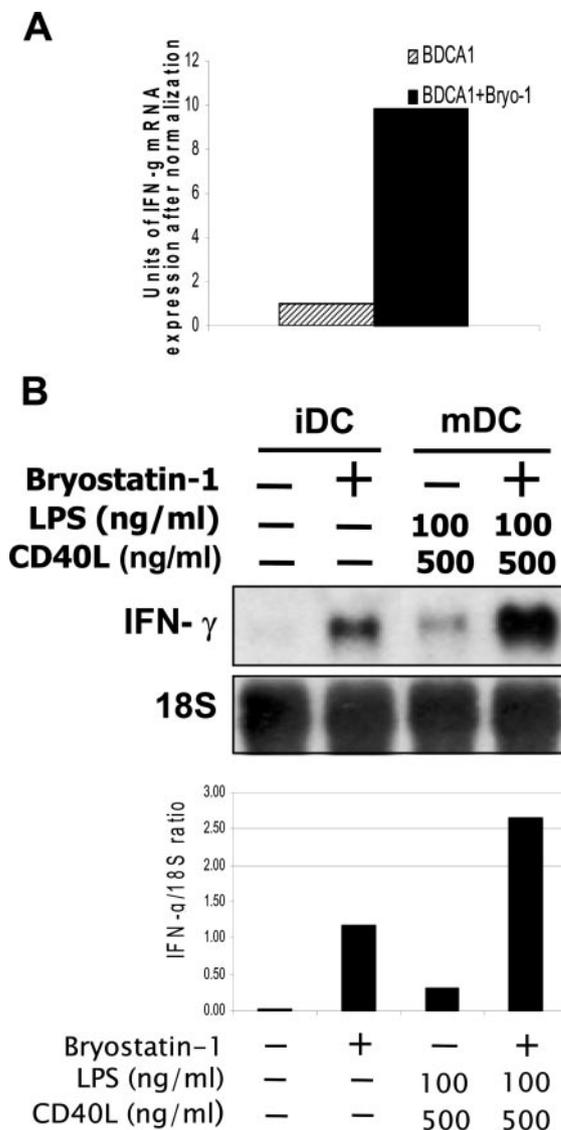


**FIGURE 6.** A, Bryostatin-1 induces IFN- $\gamma$  and T-bet mRNA expression in cancer patients. DC from cancer patients were cultured in the absence or presence of 1 ng/ml bryostatin-1 for 6 h. Total cellular RNA was isolated, and Northern blot analysis for IFN- $\gamma$  and T-bet mRNA expression was performed. Data shown are from one of three representative experiments performed. B, MEK-1/2 inhibitor suppresses ERK-1/2 activation induced by bryostatin-1 in DC derived from a cancer patient. DC obtained from a cancer patient were preincubated for 30 min in the absence or presence of 10  $\mu$ M U0126 and then cultured for 1 h in medium or 1 ng/ml bryostatin-1. Total cellular protein was extracted and analyzed by Western blot for ERK-1/ERK-2 activation. The same membrane was reprobbed with anti-ERK2 Ab to detect the total ERK level. Data shown are from one of three representative experiments performed.

through a T-bet-independent mechanism. These observations confirm and expand previous reports that IFN- $\gamma$  can be induced without T-bet involvement (26). Future work in our laboratory will include the targeting of T-bet with small interfering RNA to provide further insights into the mechanisms leading to IFN- $\gamma$  expression in human DC.

IL-12 produced by DC is a major inducer of IFN- $\gamma$ , so we investigated whether bryostatin-1-induced IFN- $\gamma$  was IL-12 dependent. Having previously shown that phosphorylation of ERK-1/2 plays a pivotal role in the induction of IFN- $\gamma$  mRNA by bryostatin-1, we first examined whether IL-12 could induce phosphorylation of ERK-1/2. In agreement with previous reports (47, 48), IL-12 failed to induce ERK-1/2 phosphorylation (Fig. 5A). Second, bryostatin-1, but not IL-12-induced IFN- $\gamma$  mRNA, was blocked by U0126. Third, bryostatin-1 did not induce IL-12 production by iDC (data not shown). Finally, anti-IL-12 mAb blocked IL-12-induced IFN- $\gamma$  mRNA but not bryostatin-1-induced IFN- $\gamma$  mRNA. Overall, the results strongly suggest that bryostatin-1 induces IFN- $\gamma$  mRNA through an IL-12-independent mechanism. We have not ruled out that other IFN- $\gamma$  inducers such as IL-18 and/or IL-23 (49, 50) could be involved in bryostatin-1-induced IFN- $\gamma$ . It was recently reported that human DC do not produce IFN- $\gamma$  in response to IL-18 (51). Further work will be required to determine whether IL-18 and/or IL-23 are involved in this process.

The generation of effective antitumor immunity involves the production of Th-1 cytokines such as IL-2 and IFN- $\gamma$  that might facilitate the induction and/or activation of tumor Ag-specific CD4 and CD8 cells (52, 53). A fundamental step in the generation of a Th1 type response is the early production of IFN- $\gamma$  by NK and CD4<sup>+</sup> T cells.



**FIGURE 7.** Bryostatin-1 induces IFN- $\gamma$  mRNA expression in both peripheral blood DC and CD40 plus LPS-treated human monocyte-derived DC. A, Myeloid DC were directly purified from blood and cultured in the presence or absence of 1 ng/ml bryostatin-1 for 6 h. Total RNA was isolated, and RT-qPCR for IFN- $\gamma$  expression was performed. Data shown are from one of two representative experiments performed. B, iDC were cultured in the absence or presence of 500 ng/ml CD40 ligand and 100 ng/ml LPS. After 48 h, mDC were treated with either medium or 1 ng/ml bryostatin-1 for 6 h. Total cellular RNA was isolated, and Northern blot analysis for IFN- $\gamma$  mRNA expression was performed. Data shown are from one of two representative experiments performed.

In the present study, we provide evidence that human DC produce IFN- $\gamma$  in response to the antineoplastic agent-bryostatin-1. The effect was observed in DC derived from both normal donors and cancer patients, which may be important because patients with cancer have impaired cell-mediated immunity associated with a switch from Th1 to Th2 (38, 54, 55). It is plausible that the production of IFN- $\gamma$  by DC at the time of Ag presentation could rapidly facilitate the creation of a microenvironment conducive to the generation of a potent cellular antitumor response before the recognition of tumor-associated Ags by T cells. Furthermore, production of IFN- $\gamma$  by DC could facilitate DC maturation through an autocrine loop (56), which in turn might render DC better APC. Our results with DC obtained from cancer patients suggest that

bryostatin-1 may be able to overcome some of the immune alterations described in patients with cancer. They also suggest that bryostatin-1 may have a role in the design of DC-based cancer vaccines.

Several recent reports have substantiated that the optimal response of T cells to DC requires T-bet expression and that sustained T-bet expression facilitates Th2 responses conversion to Th1 responses (26, 57). It is tempting to speculate that bryostatin-1, by inducing both IFN- $\gamma$  and T-bet in DC at the time of Ag presentation, may facilitate the generation of a potent Th1 type of response. Moreover, the production of IFN- $\gamma$  by DC could result not only in enhanced innate immunity, but also it might aid in establishing a link between innate immunity and the evolving adaptive immune response. Further work will be required to determine the functional consequences of such induction.

In summary, we have demonstrated that at doses potentially achievable in a clinical setting, bryostatin-1 induces high levels of both T-bet and IFN- $\gamma$  mRNA and protein secretion by primary human DC. This study is also the first report documenting the involvement of the ERK-1/2 pathway in controlling both T-bet and IFN- $\gamma$  expression in human DC.

## Acknowledgments

We thank Drs. Dan L. Longo, Eric H. Westin, Paritosh Ghosh, and Jill Harper for their critical review of this manuscript. We also thank Dr. Robert P. Wersto for the assistance with flow cytometry experiment, the nurses of the Pheresis Unit of National Institute on Aging for leucopack preparation, and Shannon Marshall for her assistance with monocyte preparation.

## Disclosures

The authors have no financial conflict of interest.

## References

- Ramsdell, J. S., G. R. Pettit, and A. H. Tashjian, Jr. 1986. Three activators of protein kinase C, bryostatins, dioleins, and phorbol esters, show differing specificities of action on GH4 pituitary cells. *J. Biol. Chem.* 261: 17073–17080.
- Hornung, R. L., J. W. Pearson, M. Beckwith, and D. L. Longo. 1992. Preclinical evaluation of bryostatin as an anticancer agent against several murine tumor cell lines: in vitro versus in vivo activity. *Cancer Res.* 52: 101–107.
- Kennedy, M. J., L. J. Prestigiacomo, G. Tyler, W. S. May, and N. E. Davidson. 1992. Differential effects of bryostatin 1 and phorbol ester on human breast cancer cell lines. *Cancer Res.* 52: 1278–1283.
- Mohammad, R. M., A. al-Katib, G. R. Pettit, and L. L. Sensenbrenner. 1993. Differential effects of bryostatin 1 on human non-Hodgkin's B-lymphoma cell lines. *Leuk. Res.* 17: 1–8.
- Philip, P. A., D. Rea, P. Thavasu, J. Carmichael, N. S. Stuart, H. Rockett, D. C. Talbot, T. Ganesan, G. R. Pettit, F. Balkwill, et al. 1993. Phase I study of bryostatin 1: assessment of interleukin 6 and tumor necrosis factor  $\alpha$  induction in vivo. The Cancer Research Campaign Phase I Committee. *J. Natl. Cancer Inst.* 85: 1812–1818.
- Armstrong, D. K., J. A. Blessing, J. Rader, and J. I. Sorosky. 2003. A randomized phase II evaluation of bryostatin-1 (NSC #339555) in persistent or recurrent squamous cell carcinoma of the cervix: a Gynecologic Oncology Group Study. *Invest. New Drugs* 21: 453–457.
- Curiel, R. E., C. S. Garcia, L. Farooq, M. F. Aguerro, and I. Espinoza-Delgado. 2001. Bryostatin-1 and IL-2 synergize to induce IFN- $\gamma$  expression in human peripheral blood T cells: implications for cancer immunotherapy. *J. Immunol.* 167: 4828–4837.
- Philip, P. A., and J. A. Zonder. 1999. Pharmacology and clinical experience with bryostatin 1: a novel anticancer drug. *Expert Opin. Investig. Drugs* 8: 2189–2199.
- Bosco, M. C., S. Rottschaefer, L. S. Taylor, J. R. Ortaldo, D. L. Longo, and I. Espinoza-Delgado. 1997. The antineoplastic agent bryostatin-1 induces proinflammatory cytokine production in human monocytes: synergy with interleukin-2 and modulation of interleukin-2R $\gamma$  chain expression. *Blood* 89: 3402–3411.
- Tilden, A. B., and A. S. Kraft. 1991. The effect of bryostatin 1 on human lymphocyte-mediated cytotoxicity. *J. Immunother.* 10: 96–104.
- Dunn, G. P., A. T. Bruce, H. Ikeda, L. J. Old, and R. D. Schreiber. 2002. Cancer immunoevasion: from immunosurveillance to tumor escape. *Nat. Immunol.* 3: 991–998.
- Uchijima, M., T. Nagata, T. Aoshi, and Y. Koide. 2005. IFN- $\gamma$  overcomes low responsiveness of myeloid dendritic cells to CpG DNA. *Immunol. Cell Biol.* 83: 92–95.
- Park, S. G., H. M. Ryu, S. O. Lim, Y. I. Kim, S. B. Hwang, and G. Jung. 2005. Interferon- $\gamma$  inhibits hepatitis B virus-induced NF- $\kappa$ B activation through nuclear localization of NF- $\kappa$ B-inducing kinase. *Gastroenterology* 128: 2042–2053.
- Kelly, J. M., K. Takeda, P. K. Darcy, H. Yagita, and M. J. Smyth. 2002. A role for IFN- $\gamma$  in primary and secondary immunity generated by NK cell-sensitive tumor-expressing CD80 in vivo. *J. Immunol.* 168: 4472–4479.
- Dalton, D. K., S. Pitts-Meek, S. Keshav, I. S. Figari, A. Bradley, and T. A. Stewart. 1993. Multiple defects of immune cell function in mice with disrupted interferon- $\gamma$  genes. *Science* 259: 1739–1742.
- Huang, S., W. Hendriks, A. Althage, S. Hemmi, H. Bluethmann, R. Kamijo, J. Vilcek, R. M. Zinkernagel, and M. Aguet. 1993. Immune response in mice that lack the interferon- $\gamma$  receptor. *Science* 259: 1742–1745.
- Durbin, J. E., R. Hackenmiller, M. C. Simon, and D. E. Levy. 1996. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell* 84: 443–450.
- Hsieh, C. S., S. E. Macatonia, C. S. Tripp, S. F. Wolf, A. O'Garra, and K. M. Murphy. 1993. Development of TH1 CD4<sup>+</sup> T cells through IL-12 produced by *Listeria*-induced macrophages. *Science* 260: 547–549.
- O'Shea, J. J., and W. E. Paul. 2002. Regulation of T(H)1 differentiation—controlling the controllers. *Nat. Immunol.* 3: 506–508.
- Hu, H. M., W. J. Urba, and B. A. Fox. 1998. Gene-modified tumor vaccine with therapeutic potential shifts tumor-specific T cell response from a type 2 to a type 1 cytokine profile. *J. Immunol.* 161: 3033–3041.
- Nakamori, M., M. Iwahashi, M. Nakamura, K. Ueda, X. Zhang, and H. Yamae. 2003. Intensification of antitumor effect by T helper 1-dominant adoptive immunogene therapy for advanced orthotopic colon cancer. *Clin. Cancer Res.* 9: 2357–2365.
- Tsung, K., J. B. Meko, G. R. Peplinski, Y. L. Tsung, and J. A. Norton. 1997. IL-12 induces T helper 1-directed antitumor response. *J. Immunol.* 158: 3359–3365.
- Murata, Y., T. Ohteki, S. Koyasu, and J. Hamuro. 2002. IFN- $\gamma$  and pro-inflammatory cytokine production by antigen-presenting cells is dictated by intracellular thiol redox status regulated by oxygen tension. *Eur. J. Immunol.* 32: 2866–2873.
- Fukao, T., S. Matsuda, and S. Koyasu. 2000. Synergistic effects of IL-4 and IL-18 on IL-12-dependent IFN- $\gamma$  production by dendritic cells. *J. Immunol.* 164: 64–71.
- Stober, D., R. Schirmbeck, and J. Reimann. 2001. IL-12/IL-18-dependent IFN- $\gamma$  release by murine dendritic cells. *J. Immunol.* 167: 957–965.
- Lugo-Villarino, G., R. Maldonado-Lopez, R. Possemato, C. Penaranda, and L. H. Glimcher. 2003. T-bet is required for optimal production of IFN- $\gamma$  and antigen-specific T cell activation by dendritic cells. *Proc. Natl. Acad. Sci. USA* 100: 7749–7754.
- Szabo, S. J., S. T. Kim, G. L. Costa, X. Zhang, C. G. Fathman, and L. H. Glimcher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100: 655–669.
- Lighvani, A. A., D. M. Frucht, D. Jankovic, H. Yamane, J. Aliberti, B. D. Hissong, B. V. Nguyen, M. Gadina, A. Sher, W. E. Paul, and J. J. O'Shea. 2001. T-bet is rapidly induced by interferon- $\gamma$  in lymphoid and myeloid cells. *Proc. Natl. Acad. Sci. USA* 98: 15137–15142.
- Auphan, N., J. A. DiDonato, C. Rosette, A. Helmborg, and M. Karin. 1995. Immunosuppression by glucocorticoids: inhibition of NF- $\kappa$ B activity through induction of I $\kappa$ B synthesis. *Science* 270: 286–290.
- Cippitelli, M., A. Sica, V. Viggiano, J. Ye, P. Ghosh, M. J. Birrer, and H. A. Young. 1995. Negative transcriptional regulation of the interferon- $\gamma$  promoter by glucocorticoids and dominant negative mutants of c-Jun. *J. Biol. Chem.* 270: 12548–12556.
- Wojciechowski, W., H. Li, S. Marshall, C. Dell'agnola, and I. Espinoza-Delgado. 2005. Enhanced expression of CD20 in human tumor B cells is controlled through ERK-dependent mechanisms. *J. Immunol.* 174: 7859–7868.
- Vrana, J. A., and S. Grant. 2001. Synergistic induction of apoptosis in human leukemia cells (U937) exposed to bryostatin 1 and the proteasome inhibitor lactacystin involves dysregulation of the PKC/MAPK cascade. *Blood* 97: 2105–2114.
- Tominaga, K., T. Yoshimoto, K. Torigoe, M. Kurimoto, K. Matsui, T. Hada, H. Okamura, and K. Nakanishi. 2000. IL-12 synergizes with IL-18 or IL-1 $\beta$  for IFN- $\gamma$  production from human T cells. *Int. Immunol.* 12: 151–160.
- Marcenaro, E., M. Della Chiesa, F. Bellora, S. Parolini, R. Millo, L. Moretta, and A. Moretta. 2005. IL-12 or IL-4 prime human NK cells to mediate functionally divergent interactions with dendritic cells or tumors. *J. Immunol.* 174: 3992–3998.
- Nefedova, Y., M. Huang, S. Kusmartsev, R. Bhattacharya, P. Cheng, R. Salup, R. Jove, and D. Gabrilovich. 2004. Hyperactivation of STAT3 is involved in abnormal differentiation of dendritic cells in cancer. *J. Immunol.* 172: 464–474.
- Gabrilovich, D. I., J. Corak, I. F. Ciernik, D. Kavanaugh, and D. P. Carbone. 1997. Decreased antigen presentation by dendritic cells in patients with breast cancer. *Clin. Cancer Res.* 3: 483–490.
- Ghosh, P., K. L. Komschlies, M. Cippitelli, D. L. Longo, J. Subleski, J. Ye, A. Sica, H. A. Young, R. H. Wiltout, and A. C. Ochoa. 1995. Gradual loss of T-helper 1 populations in spleen of mice during progressive tumor growth. *J. Natl. Cancer Inst.* 87: 1478–1483.
- Pellegrini, P., A. M. Berghella, T. Del Beato, S. Cicia, D. Adorno, and C. U. Casciani. 1996. Disregulation in TH1 and TH2 subsets of CD4<sup>+</sup> T cells in peripheral blood of colorectal cancer patients and involvement in cancer establishment and progression. *Cancer Immunol. Immunother.* 42: 1–8.
- Mohr, H., G. R. Pettit, and A. Plessing-Menze. 1987. Co-induction of lymphokine synthesis by the antineoplastic bryostatins. *Immunobiology* 175: 420–430.
- Chan, S. H., B. Perussia, J. W. Gupta, M. Kobayashi, M. Pospisil, H. A. Young, S. F. Wolf, D. Young, S. C. Clark, and G. Trinchieri. 1991. Induction of interferon  $\gamma$  production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. *J. Exp. Med.* 173: 869–879.

41. Berkow, R. L., L. Schlabach, R. Dodson, W. H. Benjamin, Jr., G. R. Pettit, P. Rustagi, and A. S. Kraft. 1993. In vivo administration of the anticancer agent bryostatin 1 activates platelets and neutrophils and modulates protein kinase C activity. *Cancer Res.* 53: 2810–2815.
42. Kraft, A. S., J. A. Reeves, and C. L. Ashendel. 1988. Differing modulation of protein kinase C by bryostatin 1 and phorbol esters in JB6 mouse epidermal cells. *J. Biol. Chem.* 263: 8437–8442.
43. Hennings, H., P. M. Blumberg, G. R. Pettit, C. L. Herald, R. Shores, and S. H. Yuspa. 1987. Bryostatin 1, an activator of protein kinase C, inhibits tumor promotion by phorbol esters in SENCAR mouse skin. *Carcinogenesis* 8: 1343–1346.
44. Szallasi, Z., L. Du, R. Levine, N. E. Lewin, P. N. Nguyen, M. D. Williams, G. R. Pettit, and P. M. Blumberg. 1996. The bryostatins inhibit growth of B16/F10 melanoma cells in vitro through a protein kinase C-independent mechanism: dissociation of activities using 26-epi-bryostatin 1. *Cancer Res.* 56: 2105–2111.
45. Dittmer, J. 2003. The biology of the Ets1 proto-oncogene. *Mol. Cancer* 2: 29.
46. Do, Y., V. L. Hegde, P. S. Nagarkatti, and M. Nagarkatti. 2004. Bryostatin-1 enhances the maturation and antigen-presenting ability of murine and human dendritic cells. *Cancer Res.* 64: 6756–6765.
47. Visconti, R., M. Gadina, M. Chiariello, E. H. Chen, L. F. Stancato, J. S. Gutkind, and J. J. O'Shea. 2000. Importance of the MKK6/p38 pathway for interleukin-12-induced STAT4 serine phosphorylation and transcriptional activity. *Blood* 96: 1844–1852.
48. Gollob, J. A., C. P. Schnipper, E. A. Murphy, J. Ritz, and D. A. Frank. 1999. The functional synergy between IL-12 and IL-2 involves p38 mitogen-activated protein kinase and is associated with the augmentation of STAT serine phosphorylation. *J. Immunol.* 162: 4472–4481.
49. Strengell, M., S. Matikainen, J. Siren, A. Lehtonen, D. Foster, I. Julkunen, and T. Sareneva. 2003. IL-21 in synergy with IL-15 or IL-18 enhances IFN- $\gamma$  production in human NK and T cells. *J. Immunol.* 170: 5464–5469.
50. Oppmann, B., R. Lesley, B. Blom, J. C. Timans, Y. Xu, B. Hunte, F. Vega, N. Yu, J. Wang, K. Singh, et al. 2000. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 13: 715–725.
51. Yamaguchi, N., Y. Fujimori, Y. Fujibayashi, I. Kasumoto, H. Okamura, K. Nakanishi, and H. Hara. 2005. Interferon- $\gamma$  production by human cord blood monocyte-derived dendritic cells. *Ann. Hematol.* 84: 423–428.
52. Mailliard, R. B., S. Egawa, Q. Cai, A. Kalinska, S. N. Bykovskaya, M. T. Lotze, M. L. Kapsenberg, W. J. Storkus, and P. Kalinski. 2002. Complementary dendritic cell-activating function of CD8<sup>+</sup> and CD4<sup>+</sup> T cells: helper role of CD8<sup>+</sup> T cells in the development of T helper type 1 responses. *J. Exp. Med.* 195: 473–483.
53. Schuler-Thurner, B., E. S. Schultz, T. G. Berger, G. Weinlich, S. Ebner, P. Woerl, A. Bender, B. Feuerstein, P. O. Fritsch, N. Romani, and G. Schuler. 2002. Rapid induction of tumor-specific type 1 T helper cells in metastatic melanoma patients by vaccination with mature, cryopreserved, peptide-loaded monocyte-derived dendritic cells. *J. Exp. Med.* 195: 1279–1288.
54. Sato, M., S. Goto, R. Kaneko, M. Ito, S. Sato, and S. Takeuchi. 1998. Impaired production of Th1 cytokines and increased frequency of Th2 subsets in PBMC from advanced cancer patients. *Anticancer Res.* 18: 3951–3955.
55. Elsasser-Beile, U., S. von Kleist, R. Fischer, M. Martin, U. Wetterauer, H. Gallati, and J. S. Monting. 1993. Impaired cytokine production in whole blood cell cultures of patients with urological carcinomas. *J. Cancer Res. Clin. Oncol.* 119: 430–433.
56. Pan, J., M. Zhang, J. Wang, Q. Wang, D. Xia, W. Sun, L. Zhang, H. Yu, and X. Cao. 2005. Intratumoral injection of interferon- $\gamma$  gene-modified dendritic cells elicits potent antitumor effects: effective induction of tumor-specific CD8<sup>+</sup> CTL response. *J. Cancer Res. Clin. Oncol.* 131: 468–478.
57. Lametschwandtner, G., T. Biedermann, C. Schwarzler, C. Gunther, J. Kund, S. Fassl, S. Hinteregger, N. Carballido-Perrig, S. J. Szabo, L. H. Glimcher, and J. M. Carballido. 2004. Sustained T-bet expression confers polarized human TH2 cells with TH1-like cytokine production and migratory capacities. *J. Allergy Clin. Immunol.* 113: 987–994.