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Enhancement of HLA class II-restricted CD4+ T cell recognition of human melanoma cells following treatment with bryostatin-1

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Abstract

The majority of melanoma cells express detectable levels of HLA class II proteins, and an increased threshold of cell surface class II is crucial for the stimulation of CD4+ T cells. Bryostatin-1, a protein kinase C (PKC) activator, has been considered as a potent chemotherapeutic agent in a variety of in vitro tumor models. Little is known about the role of bryostatin-1 in HLA class II Ag presentation and immune activation in malignant tumors, especially in melanoma. In this study, we show that bryostatin-1 treatment enhances CD4+ T cell recognition of melanoma cells in the context of HLA class II molecules. We also show that bryostatin-1 treatment of melanoma cells increases class II protein levels by upregulating the class II transactivator (CIITA) gene. Flow cytometry and confocal microscopic analyses revealed that bryostatin-1 treatment upregulated the expression of costimulatory molecules (CD80 and CD86) in melanoma cells, which could prolong the interaction of immune cells and tumors. Bryostatin-1 also induced cellular differentiation in melanoma cells, and reduced tumorigenic factors such as pro-cathepsins and matrix-metalloproteinase-9. These data suggest that bryostatin-1 could be used as a chemo-immunotherapeutic agent for reducing tumorigenic potential of melanoma cells while enhancing CD4+ T cell recognition to prevent tumor recurrence.

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Supplementary data

Supplementary figures are included.

Disclosures

The authors have no conflicts of interests.

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Keywords

Melanoma; Bryostatin-1; HLA class II molecules; Matrix metalloproteinase-9; Cathepsins; Costimulatory molecules (CD80/86); CD4+ T cells

1. Introduction

The increase in melanoma incidences among U.S. Caucasians is one of the greatest of any cancer [1–2]. While early, localized tumors are effectively treated with wide excision, metastatic melanomas are largely refractory to cytotoxic chemotherapeutics, and are almost universally fatal [1–4]. While recent advances in melanoma biology offer new strategies for potentially treating this aggressive malignancy, effective cure rates have not been achieved with surgery, radiation or chemotherapy. Thus, targeted immunotherapy of melanoma may show a great promise because of its potential for specificity and limited side effects. Studies suggest that the majority of malignant melanomas constitutively express both HLA class I and II molecules, necessary for tumor antigen (Ag) presentation to T cells [5–7]. Yet, T cell priming and the development of strong memory responses to tumor Ags also require processing and cross-presentation of shed tumor Ags via bystander professional antigen presenting cells (APCs) such as dendritic cells (DC), macrophages, and B cells [8–10]. The abundant expression of adhesion and costimulatory molecules on APCs facilitates prolonged T cell receptor (TCR) engagement by HLA-ligand complexes and enhances cellular activation [8–9,11]. Once T cells are activated, immunological recognition and tumor destruction may be dependent upon the presentation of similar tumor-derived peptides bound to HLA molecules on both malignant cells and professional APCs.

In melanoma, the display of tumor-derived peptides bound to cell surface HLA class II molecules may play a significant role in promoting immunological detection and memory [12–14]. Yet, T cell responses to peptide-class II complexes displayed on melanoma cells are significantly reduced when compared with professional APCs. Potential explanations include reduced HLA class II expression, absence of costimulatory molecules, defects in Ag processing and presentation, and reduced tumor cell responsiveness to immune stimulatory drugs [8–9,11,15]. Recent evidence suggests that cell-based tumor vaccines may be developed based on the idea that tumor cells can be genetically modified to present their own Ags, to directly stimulate T cells. However, due to the self-like nature and low immunogenicity of these Ags, and reduced levels of class II and costimulatory molecules (e.g., CD80 and CD86) on tumor cells, the ineffectiveness of the T cells reactive to these Ags must be addressed and enhanced if immunotherapy is to be clinically effective [14,16–17]. Bryostatin-1, a protein kinase C (PKC) activator, has received considerable attention in the past few years because of its potent and broad antineoplastic activity both in vitro and in vivo [18–19]. Although bryostatin-1 has been shown to influence HLA class II protein expression in professional APC (e.g., DC), its role in melanoma Ag presentation and CD4+ T cell recognition of these tumors, remains unclear [20–21].

The expression of costimulatory molecules such as CD80 and CD86 on tumor cells may also enhance direct presentation of self-Ags, further improving the efficacy of cancer vaccines [6,13–14]. Although CD80 and CD86 molecules are not expressed by the vast majority of human tumors, some melanoma cells express detectable levels of these proteins [16]. Elevation of costimulatory molecules on melanomas may enhance both class I and class II-restricted immune recognition of these tumors. Although bryostatin-1 has been considered as a potent chemotherapeutic agent [22–24], little is known about the role of this drug in the induction of CD80/CD86 molecules in malignant tumor cells. Successful antitumor immunity and long-term immunological memory to prevent tumor metastasis and recurrence

depends on enhanced tumor Ag presentation and activation of T cells. In this study, we show that bryostatin-1 down-regulates tumorigenic potential of melanoma cells while upregulates HLA class II and costimulatory molecules, enhancing CD4⁺ T cell recognition. Understanding the role of bryostatin-1 in the induction of tumor sensitivity to T cell recognition may help develop novel chemo-immunotherapeutic strategies against metastatic melanoma.

2. Materials and methods

2.1. Cell lines

Human melanoma cell lines 1359-mel and SLM2-mel were obtained from Dr. Janice Blum (Indiana University, Indianapolis, USA). HT-144, DM331 and VMM-18 melanoma cell lines were obtained from Dr. Craig L. Slingsluff Jr. (University of Virginia, Virginia, USA). These melanoma lines constitutively express HLA-DR4 (DRB1*0401) molecules as confirmed by flow cytometric analysis using DR4-specific antibody 359-F10. Cells were cultured in complete RPMI containing 5% fetal bovine serum (FBS) (HyClone), 50 U/mL penicillin, 50 µg/mL streptomycin, and enriched with L-glutamate (Mediatech, Inc, Herndon, VA, USA) [5,25–26]. The B cell line, Frev, which constitutively expresses HLA-DR4 molecules were cultured in complete IMDM media containing 10% bovine growth serum (BGS) (HyClone), 50 U/mL penicillin, 50 µg/mL streptomycin as described [5]. T cell hybridoma lines 2.18a, 1.21 and 17.9 specific for Ig κ_{188–203}, κ_{145–159} and HSA_{64–76K} respectively, were cultured in complete RPMI 1640 with 10% FBS, 50 U/mL penicillin, 50 µg/mL streptomycin, and supplemented with L-glutamate and 50 µM β-mercaptoethanol (β-ME) [27–29].

2.2. Ags and peptides

Human IgG *kappa* (Igk) and human serum albumin (HSA) were purchased from Sigma Aldrich (St. Louis, MO, USA). The human IgG immunodominant (κI) peptide κ_{188–203} (sequence KHKVYACEVTHQGLSS), subdominant (κII) peptide κ_{145–159} (sequence KVQWKVDNALQSGNS), HSA_{64–76K} peptide (sequence VKLVNEVTEFAKTK), and tyrosinase_{56–70} peptide (sequence QNILLSNAPLGPQFP) were produced using Fmoc technology and an Applied Biosystems Synthesizer as described [30–31]. Peptide purity (>99%) and sequence were analyzed by reverse phase HPLC purification and mass spectroscopy. Peptides were dissolved in PBS and stored at –20°C until used.

2.3. Ag presentation assays

Melanoma cells HT-144, 1359-mel and SLM2-mel were pretreated with vehicle alone (ethanol) or various concentrations (20, 40 and 60 nM) of bryostatin-1 (Sigma Chemical Co. St. Louis, USA) for 48–72 h, followed by the addition of Igk or HSA Ags for overnight at 37°C in 96-well plate [27,29–31]. Bryostatin-1 (40 nM) treated cells were also incubated with the Ig κI, κII and HSA synthetic peptides (10 µM) for overnight. Cells were then washed three times and co-cultured with the peptide specific T cell hybridomas for 24 h [27,30]. T cell production of IL-2 was measured by ELISA [27,29], and expressed as mean (pg/ml) ± SEM of triplicate wells.

T cell response to melanoma self-protein tyrosinase was also tested using tyrosinase immunodominant peptide (Tyr_{56–70}; sequence: QNILLSNAPLGPQFP) specific CD4⁺ T cells. Human peripheral blood mononuclear cells (PBMC) obtained from HLA-DR4 (DRB1*0401)-positive healthy individual were repeatedly stimulated with Tyr_{56–70} peptide to raise peptide specific CD4⁺ T cells. T cells were tested for peptide specificity and CD4⁺ T cells were separated by MACS® Cell Separation Reagents. The purity of Tyr_{56–70} peptide specific CD4⁺ T cells was >90% as determined by flow cytometry. Melanoma cell lines

HT-144, 1359-mel and SLM2-mel were pretreated with vehicle alone (ethanol) or bryostatin-1 (40 nM) for 48–72 h, and were cocultured with the Try_{56–70} peptide specific T cells for 48 h. Cell supernatants were assayed for IL-2 as described [27,29].

2.4. Enzyme-Linked Immunosorbent Assay (ELISA)

The production of cytokine IL-2 in the supernatants was evaluated by ELISA (R&D Systems) according to the manufacturer's instructions [27, 29]. Anti- IL-2 was purchased from R&D Systems, and IL-2 was purchased from Sigma-Aldrich. The mean IL-2 production was expressed in pg/ml by comparison with values from a standard curve derived from recombinant IL-2 (R&D System). All assays were repeated at least three times.

2.5. MTS cell proliferation assay and trypan blue dye exclusion method

HT-144 melanoma cells (1×10^5 /well) were treated with vehicle alone or bryostatin-1 (40 nM) for 72 h in 96-well flat bottomed plate, and cell viability was measured using the CellTiter 96 AQueous One Solution Cell Proliferation Assay® (MTS; Promega, Madison, WI). Twenty μ l of MTS reagent was added to each well, and the plate was incubated for 2 h at 37°C. Following incubation, absorbance was read at 490 nm. In parallel assays, vehicle- or bryostatin-treated HT-144 cells were detached using Cellgrow stripping buffer (Cellgrow, Cat. No. 25-056-CI, Mediatech, Inc., Manassas, VA), washed, and counted employing the traditional trypan blue dye exclusion method using a hemacytometer. Experiments were repeated at least three times and the data were expressed as percent cell viability $\pm$ SEM of triplicate wells.

2.6. Western blot analysis

Cell lysates obtained from HT-144 and SLM-2 melanoma cells treated with vehicle alone (ethanol) or bryostatin-1 (40 nM), were separated by SDS-PAGE (10%), and analyzed by western blotting for DR $\alpha\beta$ (mAb L243), Ii (mAb PIN 1.1), DM (Santa Cruz), GILT (anti-GILT, Santa Cruz), MMP-9 (Calbiochem) and cathepsins B, D, S and L (Santa Cruz) as described [26–28]. Relative expression levels were quantitated using a Bio-Rad densitometer.

2.7. Flow cytometric analysis

Control and bryostatin-1 (40 nM) treated melanoma cells (HT-144) were stained with mAbs directed against DR (L243) and DR4 (359-F10), CD40, CD80 and CD86, followed by secondary antibodies labeled with FITC (Santa Cruz).^{30,31} The B cell line, Frev, which constitutively expresses class II DR4 and costimulatory molecules, was also stained with those antibodies and used as controls [31–32]. Samples were then analyzed on a FACScan using CellQuest software (BD Bioscience, Mountain View, CA). Background fluorescence was evaluated using irrelevant isotype-matched mAbs as described [27–28].

2.8. RT-PCR

Total RNA was isolated from control and bryostatin-1-treated (40 nM final conc.) melanoma cells, using RNazol reagent (Tel-Test Inc, Friendswood, TX) according to the manufacturer's protocol. Reverse transcription reaction was performed using poly-dT primer and Moloney murine leukemia virus reverse transcriptase (Applied Biosystem) in 25 μ l reaction volume containing total RNA (2 μ g), 1 $\times$ PCR buffer and 2 mM MgCl₂, at 42°C for 15 min followed by 95°C for 5 min. PCR was carried out in a programmed thermal cycler (Applied Bio system) as described [32]. The primer sequences used to amplify glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were 5' CCTACCCCAATGTATCCGTTGTG-3' (sense) and 5'- GGAGGAATGGGAGTTGCTGTTGAA-3' (anti-sense) and for CIITA were 5'-

GGGGAAGCTGAGGGCACG-3' (sense) and 5'-GAAGCTCCAGGTAGCCACCTTCTA-5' (anti-sense). Thermal cycling parameters were 94°C for 3 min, followed by 40 cycles of amplifications at 94°C for 30 sec, 60°C for 1 min, 72°C for 1 min, and 72°C for 5 min as the final elongation step. PCR products were subjected to electrophoresis by using 1.5% agarose gel and visualized by ethidium bromide. The band intensity was analyzed by NIH Image analyzer.

Real-time PCR analysis was performed as described [32]. The quantitative real-time PCR was performed using IQ™ SYBR Green Supermix in an iCycler (iCycler iQ Single – color Real Time PCR detection system; Bio-Rad, Hercules, CA). Relative levels of CIITA mRNA expression were normalized in all the samples analyzed with respect to the levels of GAPDH amplification.

2.9. Immunofluorescent staining and confocal microscopy

Cells treated with or without bryostatin-1 (40 nM final conc.) were seeded on collagen coated glass slides, fixed with 4% freshly made paraformaldehyde (pH 7.0) and permeabilized with Triton X-100 solution (0.2% v/v in PBS) for 10 min. Cells were washed three times with PBS and blocked with 1% BSA in PBS for 30 mins. Primary antibodies against CD80 and CD86 molecules were added to the cells and incubated for 1 hr. Then the slides were washed three times with PBS and probed with the FITC-conjugated secondary antibodies (anti-mouse IgG-FITC, Invitrogen) for 1 hr. After washing three times with PBS, 100 µl of Hoechst (1:1000 in PBS) was added to the slides for nuclear staining. The cells were then examined using a Leica SP5 confocal immunofluorescent microscope system (Leica Microsystems, Heidelberg, Germany) using a 63x oil immersion objective. Blue and green fluorescence was recorded with 405 and 488-nm excitation lasers and emission band-pass settings at 432 and 528 nm, respectively. The acquisition settings were identical in the control and bryostatin-1-treated groups [33].

3. Results

3.1. Bryostatin-1 enhances CD4+ T cell recognition of melanoma cells in the context of HLA class II molecules

The presentation of antigenic peptides by HLA class II molecules to CD4+ T cells is critical for the generation of antitumor immunity [7–9,11,34]. CD4+ T cells can recognize HLA class II-peptide complexes on melanoma cells and provide help for CTL-mediated killing of tumors [34–36]. While most tumor cells constitutively express class I molecules, only a few of them including melanomas express measurable HLA class II proteins [5,13,32]. As metastatic melanoma cells express detectable levels of class II molecules, these tumors could be potential targets for CD4+ T cells. Because bryostatin-1 acts as an anti-neoplastic agent [20,37–39], and recently been shown to induce immune components in DC [40], we investigated whether this drug has any effect on Ag presentation by melanoma cells. Melanoma cell lines HT-144, 1359-mel and SLM2-mel treated with bryostatin-1 (20 and 40 nM) for 48–72 h, showed a significant enhancement of κ epitope presentation and CD4+ T cell stimulation [(1.6–2) times higher in all three cell lines when compared with 40 nM treatment groups, * p <0.05] as measured via IL-2 ELISA (Fig. 1A). Our data also showed that the presentation of IgG κ and HSA peptides was significantly enhanced [(1.5–1.7) folds higher IL-2 production in HT-144, (1.4–1.9) folds in 1359-mel, and (1.7–1.8) folds in SLM2-mel except κ I] when cells were pretreated with 40 nM of bryostatin-1 (Supplemental Fig. 1A). The presentation of κ I κ II and HSA peptides to T cells was highly specific as the production of IL-2 was only detected in matched peptides/hybridoma coculture system (Supplemental Fig. 1B). Cell viability and toxicity were tested at the end of the assay, and no significant cell death was observed after bryostatin-1 treatment (data not shown).

Enhanced Ag/peptide presentation was also observed in two other melanoma cell lines, J3.DR4 and DM331 when treated with bryostatin-1 (data not shown). Taken together, these data suggest that bryostatin-1 positively influences HLA class II-restricted Ag presentation in melanoma cells, enhancing CD4⁺ T cell recognition of these tumor cells.

To investigate whether bryostatin-1-treated melanoma cells also enhance melanoma self-Ag presentation, we generated CD4⁺ T cells against melanoma peptide Tyr₅₆₋₇₀ and tested for class II-restricted immune recognition. HT-144 and DM-331 cells pretreated with or without bryostatin-1 (40 nM) were incubated with Tyr₅₆₋₇₀ peptide and cocultured with the peptide specific CD4⁺ T cells as described in the methods. Interestingly, the direct melanoma Ag presentation by HT-144 and DM-331 cells was significantly enhanced (2.54 folds higher in HT-144, 2.48 folds higher in DM-331, and 2.1 folds higher in SLM2-mel cells) by treatment with bryostatin-1 (Fig. 1B). These data suggest that modulation of protein kinase C by bryostatin-1 may play a role in enhanced Ag presentation and CD4⁺ T cell recognition of melanoma cells.

3.2. Bryostatin-1 treatment upregulates HLA class II molecules and the master regulator of HLA class II proteins, CIITA, in melanoma cells

To investigate whether bryostatin-1-induced enhanced CD4⁺ T cell recognition of melanoma cells was a result of altered HLA class II protein expression, we first measured HLA class II protein levels in melanoma cells by western blotting. Bryostatin-1 treatment (40 nM) of melanoma cells for 24–72 h, significantly increased class II protein levels in HT-144 cells (0.22 vs 0.52 at 24 h; 0.26 vs 0.91 at 72 h) as determined by densitometric analysis of protein bands (Fig. 2A). Treatment of 1359-mel and SLM2-mel cells with 40 nM of bryostatin-1 also showed elevated levels of HLA class II proteins as analyzed by western blotting and densitometry (data not shown). These data suggest that bryostatin-1 upregulates HLA class II proteins in melanoma cells, inducing enhanced CD4⁺ T cell responses via the class II pathway.

Melanoma cells also express invariant chain (Ii) and HLA-DM molecules, which could play important roles in Ag presentation via the class II pathway [5, 14, 25, 32]. Thus, the expression levels of Ii and HLA-DM molecules in bryostatin-treated HT-144 melanoma cells were analyzed. Data showed that Ii and DM proteins were significantly upregulated (0.09 vs 0.16 at 24 h and 0.09 vs 0.38 at 72 h for Ii, and 0.19 vs 0.28 at 24 h and 0.15 vs 0.29 at 72 h for DM) in HT-144 melanoma cells following bryostatin-1 treatment (Fig. 2B). Densitometric analysis confirmed this finding when the values were corrected for actin (Supplemental Figures 2A and 2B), suggesting that this drug influences the major components of the class II pathway. Recent studies also suggest that both constitutive and inducible expression of HLA class II gene requires CIITA, which is the master regulator of HLA class II molecules [41–42]. Thus, we sought to analyze the role of bryostatin-1 in CIITA gene expression by semi-quantitative and real-time PCR. Data showed that the CIITA gene expression in HT-144 melanoma cells was significantly increased (0.38 vs 0.95) following treatment with bryostatin-1 for 72 h (Fig. 2C). Real-time PCR confirmed this data showing that bryostatin-1 markedly increased CIITA mRNA levels in HT-144 cells (Fig. 2D). These data demonstrate that the bryostatin-1-mediated transcriptional up-regulation of CIITA gene contributes to the enhancement of HLA-DR and Ii expression in melanoma cells.

3.3. Bryostatin-1 treatment reduces matrix metalloproteinase-9 (MMP-9) and pro-cathepsins B & D while upregulating surface class II proteins in melanoma cells

Although bryostatin-1 treatment can induce cellular differentiation in different types of malignancies [20–21,43], its role in acidic cathepsins and cell surface class II protein

expression in melanoma cells remains unclear. Treatment of HT-144 melanoma cells with bryostatin-1 for 72 h induced significant morphological changes, and decreased cell growth as observed by microscopic analysis (Fig. 3A). MTS proliferation assay and trypan dye exclusion technique confirmed that bryostatin treatment reduced viability (18–23%) of HT-144 cells (Fig. 3B). It is interesting to note that a shorter 72 h exposure of melanoma cells to bryostatin-1 decreased tumor cell growth as a result of cellular differentiation while upregulated HLA class II protein expression in HT-144 cells (Fig. 2A). Bryostatin-1 treatment also decreased cell growth, and altered morphological features in 1359-mel, DM-331 and SLM2-mel cells (data not shown).

While MMP-9 plays an important role in proteolytic degradation of the extracellular matrix, an aberrant expression of the MMP-9 protein has been shown to contribute to the invasion and angiogenic process of many types of tumors including head and neck carcinomas, astroglomas, gastric cancer, and metastatic melanoma [44–45]. Thus, we examined the effect of bryostatin-1 on MMP-9 protein expression levels in melanoma cells. Western blot analysis showed that bryostatin-1 markedly inhibited MMP-9 protein expression (0.91 vs 0.54 at 24h; 0.89 vs 0.25 at 72h) in HT-144 cells (Fig. 3C). Bryostatin-1-mediated reduction of MMP-9 was also observed in DM-331, SLM2-mel and 1359-mel cells (data not shown). These data suggest that bryostatin-1 has multiple effects on tumor growth, and could be considered as potential drug for inhibition of melanoma invasion and metastasis.

Ag processing involves proteolysis of the Ag yielding peptide fragments that bind to the class II molecules for presentation to CD4⁺ T cells [8–9,11]. Thus, regulation of Ag processing and presentation is the key to the induction of adaptive immune responses. Intracellular proteases, mainly cathepsins (Cats) play important roles in proteolytic processing of the HLA class II and Ii [32,46–47]. Cathepsins can also modulate both endogenous and exogenous Ag processing, epitope generation and presentation via class II molecules. We examined the expression levels of cathepsins B, D, L and S in melanoma cells by western blot analysis. Little to no change in active cathepsins protein expression was observed in HT-144 cells following bryostatin-1 treatment (Supplemental Fig. 2). Interestingly, bryostatin treatment of HT-144 melanoma cells for 72 h, reduced pro-cathepsins B and D protein levels (Fig. 3C), which are normally overexpressed in metastatic tumors [48–51]. While bryostatin-1 may serve as an antineoplastic agent by inducing cellular differentiation and growth inhibition, we found that surface expression of HLA-DR and HLA-DR4 molecules could be significantly increased by treating cells with this agent for 72 h (Fig. 3D). Data also showed that the treatment of HT-144 cells with bryostatin-1 did not significantly alter the value (0.12% vs 0.14% for DR and 0.04% vs 0.02% for DR4) of isotype controls.

3.4. Bryostatin-1 upregulates costimulatory molecules CD80 and CD86 in melanoma cells

While bryostatin-1 may serve as an antineoplastic agent by inducing cellular differentiation and growth inhibition, its role on costimulatory signals and T cell recognition of melanoma cells remains unknown. A number of studies also suggest that an effective T cell response is initiated by two distinct signals: signals 1 and 2 [17, 52]. Signal 1 is Ag specific and transduced by T cell receptor (TcR) interaction with the peptide/HLA complexes on the surface of APC and tumors. Signal 2 is delivered by costimulatory molecules expressed on APC, in an Ag-nonspecific manner. While the presence of these two signals helps T cell activation and differentiation into effector cells, T cell stimulation is abortive and cannot proceed to the stage of efficient effector function in the absence of signal 2 [52–54]. Malignant melanoma cells express little to no costimulatory molecules that need to be increased for optimum T cell stimulation. To investigate whether bryostatin-1 treatment influences costimulatory molecules, we incubated HT-144 melanoma cells with bryostatin-1 for 48 h, followed by immunofluorescence studies to evaluate CD80/CD86 protein

expression. Confocal microscopic analysis showed that bryostatin-1 treatment significantly increased the expression levels of CD80 and CD86 molecules in HT-144 cells (Fig. 4). Flow cytometric analysis also confirmed that the bryostatin-1 treatment upregulated CD80 and CD86 molecules in HT-144 cells (Fig. 5). However, bryostatin-1 treatment only minimally altered the expression of CD40 molecules in HT-144 cells. These results suggest that bryostatin-1 treatment may aid in T cell recognition of melanoma cells by upregulating costimulatory molecules as previously suggested by other groups that increased levels of costimulatory molecules on tumor cells enhance immune-mediated destruction of tumors [54–55].

4. Discussion

Successful antitumor immunity and long-term immunological memory to prevent tumor metastasis and recurrence depends on the recruitment and activation of both CD4+ and CD8+ T cells [56–57]. HLA class II-restricted CD4+ T cells serve cooperative and effector functions in immunity against tumors, influencing antitumor CTL responses and maintaining durable immune memory [19, 58]. In this study, we have demonstrated that the treatment of melanoma cells with bryostatin-1 upregulates both intracellular and cell surface pool of HLA class II proteins, triggering increased CD4+ T cell responses to self-Ags. Mechanistic studies showed that bryostatin-1 treatment upregulates the CIITA gene, with an increased cell surface class II proteins in melanoma cells. The treatment of melanoma cells with bryostatin-1 also increased the expression of CD80 and CD86 costimulatory molecules, which might have helped in the induction of enhanced CD4+ T cell responses to tumors. Bryostatin-1 treatment also attenuated melanoma cell growth and MMP-9 protein expression, two important properties in the regulation of melanoma growth. In addition, pro-cathepsin protein levels in melanoma cells were downregulated following bryostatin-1 treatment. This is promising because cathepsins have been shown to increase metastatic potential by facilitating cell migration and invasiveness. The antineoplastic activity of bryostatin-1 has previously been reported in a number of murine and human tumors and cell lines, including breast, lung, and lymphoma [22–23,59]. Thus, our study suggests that the simultaneous induction of immune components in melanoma cells while restricting tumor growth and invasion by bryostatin-1, may contribute to the design of successful chemo-immunotherapeutics against metastatic melanoma.

While HLA class II molecules are mainly expressed by professional APCs, melanoma cells also express measurable levels of class II proteins. Studies using immunohistochemical analyses of human melanomas have shown that 38–90% of these tumors express HLA class II molecules [25–26]. Although less efficient than professional APCs, these class II-positive malignant tumors can activate Ag-specific CD4+ T cells [27–28], and this could be further enhanced by bryostatin-1 treatment. We and others have previously shown that bryostatin-1 in combination with calcium ionophore influences HLA class II and costimulatory molecules in professional APC such as DC [40]. In this study, we have shown that bryostatin-1 upregulates HLA class II proteins as well as CD80 and CD86 costimulatory molecules in melanoma cells, enhancing CD4+ T cell recognition of these tumor cells. Thus, it is likely that bryostatin-1-mediated upregulation of HLA and CD80/CD86 molecules on both professional APC and tumors would trigger direct as well as cross-presentation of tumor Ags to T cells for the induction of effective antitumor immune responses. Crucial to the stimulation of immune response in humans is the ability to present epitopes on the surface of both APC and tumor cells that are recognizable by CD4+ T cells [20–22]. Epitopes are derived from exogenous and endogenous Ags, which are internalized by APCs as well as tumor cells and then are processed by intercellular proteases, specially the cathepsins [23–26]. While we could not detect any significant changes in active cathepsins B, D and S expression in melanoma cells following bryostatin-1 treatment for 72 h

(Supplemental Fig. 2), longer exposure (>3 days) to this agent reduced pro-cathepsin protein levels, which are implicated in determining the metastatic potential of tumors.

HLA class II molecules are synthesized in the endoplasmic reticulum (ER), processed in the acidic endolysosomal compartments and appear on the cell surface for T cell recognition [28]. HLA class II molecules are initially complexed with a glycoprotein known as the Ii, a molecule necessary for correct folding and function of the molecule as well as serving as a competitive inhibitor of the class II binding site [27, 29]. Epitopes cannot be loaded onto the class II molecules until proper cleavage of the Ii by one of the cysteinyl cathepsins, Cat S. After cleavage of the Ii, the class II associated Ii peptide (CLIP) remains in the class II binding groove, further preventing improper loading of peptides. A non-classical class II protein, HLA-DM, mediates the removal of CLIP and helps the loading of appropriate peptides into the class II binding groove [29–30]. Our present study detected elevated levels of Ii and HLA-DM proteins accompanied with classical class II proteins and costimulatory molecules in melanoma cells following 72 h of bryostatin-1 treatment.

Studies have shown that MMP-9 is involved in melanoma invasion and progression [59–60]. High serum MMP-9 has also been suggested to be linked to hematogenous metastasis in melanoma [44–45]. Studies also suggest that higher levels of MMP protein expression in malignant tumors play multiple roles in various stages of tumorigenesis; including establishment and growth, angiogenesis, invasion, migration, and metastasis. Thus, reduction of these enzymes may halt tumor progression. The present study showed that bryostatin-1 markedly reduced MMP-9 levels in melanoma cells within 72 h of treatment. It is interesting to note that treatment with bryostatin-1 alone elevated class II and costimulatory molecules, while inhibiting MMP-9 and pro-cathepsins in melanoma cells. The elevation of HLA class II proteins in tumor cells treated with bryostatin-1 would facilitate the presentation of melanoma Ag to CD4+ T cells. The interactions between CD80 molecules on APC and/or tumor cells and the counter-receptor CD28 molecules on T cells play a crucial role in inducing cell-mediated immune responses [17,52–53]. However, the melanoma microenvironment could lead to localized T cell tolerance, in part through downregulation of costimulatory molecules. In this scenario, the bryostatin-1 treatment may help in overcoming the immune tolerance by upregulating CD80/CD86 molecules on both melanoma cells and professional APC such as DC. Dual expression of CD80 and CD86 molecules in tumor cells have been reported to produce a tumor vaccine superior to single expression of either molecule [61]. Thus, cell-based cancer vaccines consisting of tumor cells expressing HLA class II and CD80/CD86 molecules may be developed by treating tumor cells with bryostatin-1 alone or in combination with other immunotherapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Kirkwood JM, Tarhini AA, Panelli MC, et al. Next generation of immunotherapy for melanoma. *J Clin Oncol*. 2008; 26(20):3445–3455. [PubMed: 18612161]

2. Nelson AA, Tsao H. Melanoma and genetics. *Clin Dermatol.* 2009; 27(1):46–52. [PubMed: 19095153]
3. Gruber F, Kastelan M, Brajac I, et al. Molecular and genetic mechanisms in melanoma. *Coll Antropol.* 2008; 32 (Suppl 2):147–152. [PubMed: 19138018]
4. Zigler M, Villares GJ, Lev DC, Melnikova VO, Bar-Eli M. Tumor immunotherapy in melanoma: strategies for overcoming mechanisms of resistance and escape. *Am J Clin Dermatol.* 2008; 9(5): 307–311. [PubMed: 18717605]
5. Haque MA, Li P, Jackson SK, et al. Absence of gamma-interferon-inducible lysosomal thiol reductase in melanomas disrupts T cell recognition of select immunodominant epitopes. *J Exp Med.* 2002; 195(10):1267–1277. [PubMed: 12021307]
6. Held G, Neumann F, Sturm C, et al. Differential presentation of tumor antigen-derived epitopes by MHC-class I and antigen-positive tumor cells. *Int J Cancer.* 2008; 123(8):1841–1847. [PubMed: 18688854]
7. Robila V, Ostankovitch M, Altrich-Vanlith ML, et al. MHC class II presentation of gp100 epitopes in melanoma cells requires the function of conventional endosomes and is influenced by melanosomes. *J Immunol.* 2008; 181(11):7843–7852. [PubMed: 19017974]
8. Vyas JM, Van der Veen AG, Ploegh HL. The known unknowns of antigen processing and presentation. *Nat Rev Immunol.* 2008; 8(8):607–618. [PubMed: 18641646]
9. Dickgreber N, Stoitzner P, Bai Y, et al. Targeting antigen to MHC class II molecules promotes efficient cross-presentation and enhances immunotherapy. *J Immunol.* 2009; 182(3):1260–1269. [PubMed: 19155471]
10. Ostrand-Rosenberg S. Immune surveillance: a balance between protumor and antitumor immunity. *Curr Opin Genet Dev.* 2008; 18(1):11–18. [PubMed: 18308558]
11. Haque A, Blum JS. New insights in antigen processing and epitope selection: development of novel immunotherapeutic strategies for cancer, autoimmunity and infectious diseases. *J Biol Regul Homeost Agents.* 2005; 19(3–4):93–104. [PubMed: 16602623]
12. Godefroy E, Scotto L, Souleimanian NE, et al. Identification of two Melan-A CD4+ T cell epitopes presented by frequently expressed MHC class II alleles. *Clin Immunol.* 2006; 121(1):54–62. [PubMed: 16814609]
13. Dissanayake SK, Thompson JA, Bosch JJ, et al. Activation of tumor-specific CD4(+) T lymphocytes by major histocompatibility complex class II tumor cell vaccines: a novel cell-based immunotherapy. *Cancer Res.* 2004; 64(5):1867–1874. [PubMed: 14996751]
14. Bosch JJ, Thompson JA, Srivastava MK, et al. MHC class II-transduced tumor cells originating in the immune-privileged eye prime and boost CD4(+) T lymphocytes that cross-react with primary and metastatic uveal melanoma cells. *Cancer Res.* 2007; 67(9):4499–4506. [PubMed: 17483366]
15. Anichini A, Mortarini R, Nonaka D, et al. Association of antigen-processing machinery and HLA antigen phenotype of melanoma cells with survival in American Joint Committee on Cancer stage III and IV melanoma patients. *Cancer Res.* 2006; 66(12):6405–6411. [PubMed: 16778219]
16. Bernsen MR, Hakansson L, Gustafsson B, et al. On the biological relevance of MHC class II and B7 expression by tumour cells in melanoma metastases. *Br J Cancer.* 2003; 88(3):424–431. [PubMed: 12569387]
17. Linsley PS, Clark EA, Ledbetter JA. T-cell antigen CD28 mediates adhesion with B cells by interacting with activation antigen B7/BB-1. *Proc Natl Acad Sci U S A.* 1990; 87(13):5031–5035. [PubMed: 2164219]
18. Ariza ME, Ramakrishnan R, Singh NP, Chauhan A, Nagarkatti PS, Nagarkatti M. Bryostatin-1, a naturally occurring antineoplastic agent, acts as a toll-like receptor 4 (TLR-4) ligand and induces unique cytokines and chemokines in dendritic cells. *J Biol Chem.* 2011; 286(1):24–34. [PubMed: 21036898]
19. Stone RM, Sariban E, Pettit GR, Kufe DW. Bryostatin 1 activates protein kinase C and induces monocytic differentiation of HL-60 cells. *Blood.* 1988; 72(1):208–213. [PubMed: 2455568]
20. Kudinov Y, Wiseman CL, Kharazi AI. Phorbol myristate acetate and Bryostatin 1 rescue IFN-gamma inducibility of MHC class II molecules in LS1034 colorectal carcinoma cell line. *Cancer Cell Int.* 2003; 3:4. [PubMed: 12787470]

21. Do Y, Mainali E, Nagarkatti PS, Nagarkatti M. Bryostatin-1 in combination with calcium ionophore promotes the maturation of human umbilical cord-blood monocyte-derived dendritic cells capable of activating neonatal alloreactive T cells. *Cell Immunol.* 2004; 231(1–2):8–13. [PubMed: 15919364]
22. Kennedy MJ, Prestigiacomo LJ, Tyler G, May WS, Davidson NE. Differential effects of bryostatin 1 and phorbol ester on human breast cancer cell lines. *Cancer Res.* 1992; 52(5):1278–1283. [PubMed: 1737390]
23. Dale IL, Bradshaw TD, Gescher A, Pettit GR. Comparison of effects of bryostatins 1 and 2 and 12-O-tetradecanoylphorbol-13-acetate on protein kinase C activity in A549 human lung carcinoma cells. *Cancer Res.* 1989; 49(12):3242–3245. [PubMed: 2720677]
24. Hornung RL, Pearson JW, Beckwith M, Longo DL. Preclinical evaluation of bryostatin as an anticancer agent against several murine tumor cell lines: in vitro versus in vivo activity. *Cancer Res.* 1992; 52(1):101–107. [PubMed: 1727368]
25. O'Donnell PW, Haque A, Klemsz MJ, Kaplan MH, Blum JS. Cutting edge: induction of the antigen-processing enzyme IFN-gamma-inducible lysosomal thiol reductase in melanoma cells is STAT1-dependent but CIITA-independent. *J Immunol.* 2004; 173(2):731–735. [PubMed: 15240658]
26. Haque A, Das A, Hajiaghamohseni LM, Younger A, Banik NL, Ray SK. Induction of apoptosis and immune response by all-trans retinoic acid plus interferon-gamma in human malignant glioblastoma T98G and U87MG cells. *Cancer Immunol Immunother.* 2007; 56(5):615–625. [PubMed: 16947022]
27. Younger AR, Amria S, Jeffrey WA, et al. HLA class II antigen presentation by prostate cancer cells. *Prostate Cancer Prostatic Dis.* 2008; 11(4):334–341. [PubMed: 17938645]
28. Haque A, Hajiaghamohseni LM, Li P, Toomy K, Blum JS. Invariant chain modulates HLA class II protein recycling and peptide presentation in nonprofessional antigen presenting cells. *Cell Immunol.* 2007; 249(1):20–29. [PubMed: 18067883]
29. Amria S, Cameron C, Stuart R, Haque A. Defects in HLA class II antigen presentation in B-cell lymphomas. *Leuk Lymphoma.* 2008; 49(2):353–355. [PubMed: 18231926]
30. Haque MA, Hawes JW, Blum JS. Cysteinylation of MHC class II ligands: peptide endocytosis and reduction within APC influences T cell recognition. *J Immunol.* 2001; 166(7):4543–4551. [PubMed: 11254711]
31. Amria S, Hajiaghamohseni LM, Harbeson C, et al. HLA-DM negatively regulates HLA-DR4-restricted collagen pathogenic peptide presentation and T cell recognition. *Eur J Immunol.* 2008; 38(7):1961–1970. [PubMed: 18506881]
32. Goldstein OG, Hajiaghamohseni LM, Amria S, Sundaram K, Reddy SV, Haque A. Gamma-IFN-inducible-lysosomal thiol reductase modulates acidic proteases and HLA class II antigen processing in melanoma. *Cancer Immunol Immunother.* 2008; 57(10):1461–1470. [PubMed: 18343923]
33. Komlosi P, Banizs B, Fintha A, Steele S, Zhang ZR, Bell PD. Oscillating cortical thick ascending limb cells at the juxtaglomerular apparatus. *J Am Soc Nephrol.* 2008; 19(10):1940–1946. [PubMed: 18562570]
34. Godet Y, Bonnin A, Guilloux Y, et al. A new tyrosinase epitope recognized in the HLA-B*4002 context by CTL from melanoma patients. *Cancer Immunol Immunother.* 2009; 58(2):271–280. [PubMed: 18612636]
35. Pandolfi F, Cianci R, Lolli S, et al. Strategies to overcome obstacles to successful immunotherapy of melanoma. *Int J Immunopathol Pharmacol.* 2008; 21(3):493–500. [PubMed: 18831916]
36. Walker EB, Haley D, Petrusch U, et al. Phenotype and functional characterization of long-term gp100-specific memory CD8+ T cells in disease-free melanoma patients before and after boosting immunization. *Clin Cancer Res.* 2008; 14(16):5270–5283. [PubMed: 18698047]
37. Schuchter LM, Esa AH, May S, Laulis MK, Pettit GR, Hess AD. Successful treatment of murine melanoma with bryostatin 1. *Cancer Res.* 1991; 51(2):682–687. [PubMed: 1985785]
38. Tuttle TM, Bethke KP, Inge TH, McCrady CW, Pettit GR, Bear HD. Bryostatin 1-activated T cells can traffic and mediate tumor regression. *J Surg Res.* 1992; 52(6):543–548. [PubMed: 1528028]

39. Thompson CH, Macaulay VM, O'Byrne KJ, et al. Modulation of bryostatin 1 muscle toxicity by nifedipine: effects on muscle metabolism and oxygen supply. *Br J Cancer*. 1996; 73(10):1161–1165. [PubMed: 8630272]
40. Do Y, Hegde VL, Nagarkatti PS, Nagarkatti M. Bryostatin-I enhances the maturation and antigen-presenting ability of murine and human dendritic cells. *Cancer Res*. 2004; 64(18):6756–6765. [PubMed: 15374994]
41. Thompson JA, Dissanayake SK, Ksander BR, Knutson KL, Disis ML, Ostrand-Rosenberg S. Tumor cells transduced with the MHC class II Transactivator and CD80 activate tumor-specific CD4+ T cells whether or not they are silenced for invariant chain. *Cancer Res*. 2006; 66(2):1147–1154. [PubMed: 16424052]
42. Rodriguez T, Mendez R, Del Campo A, et al. Patterns of constitutive and IFN-gamma inducible expression of HLA class II molecules in human melanoma cell lines. *Immunogenetics*. 2007; 59(2):123–133. [PubMed: 17180681]
43. Battle TE, Frank DA. STAT1 mediates differentiation of chronic lymphocytic leukemia cells in response to Bryostatin 1. *Blood*. 2003; 102(8):3016–3024. [PubMed: 12855573]
44. Nikkola J, Vihinen P, Vuoristo MS, Kellokumpu-Lehtinen P, Kahari VM, Pyrhonen S. High serum levels of matrix metalloproteinase-9 and matrix metalloproteinase-1 are associated with rapid progression in patients with metastatic melanoma. *Clin Cancer Res*. 2005; 11(14):5158–5166. [PubMed: 16033831]
45. Vaisanen AH, Kallioinen M, Turpeenniemi-Hujanen T. Comparison of the prognostic value of matrix metalloproteinases 2 and 9 in cutaneous melanoma. *Hum Pathol*. 2008; 39(3):377–385. [PubMed: 18187184]
46. Zhang T, Maekawa Y, Hanba J, et al. Lysosomal cathepsin B plays an important role in antigen processing, while cathepsin D is involved in degradation of the invariant chain in ovalbumin-immunized mice. *Immunology*. 2000; 100(1):13–20. [PubMed: 10809954]
47. Norton DL, Haque A. Insights into the Role of GILT in HLA Class II Antigen Processing and Presentation by Melanoma. *J Oncol*. 2009; 2009:142959. [PubMed: 20016802]
48. Vasiljeva O, Turk B. Dual contrasting roles of cysteine cathepsins in cancer progression: apoptosis versus tumour invasion. *Biochimie*. 2008; 90(2):380–386. [PubMed: 17991442]
49. Podgorski I, Sloane BF. Cathepsin B and its role(s) in cancer progression. *Biochem Soc Symp*. 2003; (70):263–276. [PubMed: 14587299]
50. Roshly S, Sloane BF, Moin K. Pericellular cathepsin B and malignant progression. *Cancer Metastasis Rev*. 2003; 22(2–3):271–286. [PubMed: 12785001]
51. Beaujouin M, Prebois C, Derocq D, et al. Pro-cathepsin D interacts with the extracellular domain of the beta chain of LRP1 and promotes LRP1-dependent fibroblast outgrowth. *J Cell Sci*. 2010; 123(Pt 19):3336–3346. [PubMed: 20826454]
52. Stremmel C, Greenfield EA, Howard E, Freeman GJ, Kuchroo VK. B7-2 expressed on EL4 lymphoma suppresses antitumor immunity by an interleukin 4-dependent mechanism. *J Exp Med*. 1999; 189(6):919–930. [PubMed: 10075975]
53. Chaperot L, Plumas J, Jacob MC, et al. Functional expression of CD80 and CD86 allows immunogenicity of malignant B cells from non-Hodgkin's lymphomas. *Exp Hematol*. 1999; 27(3):479–488. [PubMed: 10089910]
54. Anderson RC, Anderson DE, Elder JB, et al. Lack of B7 expression, not human leukocyte antigen expression, facilitates immune evasion by human malignant gliomas. *Neurosurgery*. 2007; 60(6):1129–1136. discussion 1136. [PubMed: 17538388]
55. Thomas IJ, Petrich de Marquesini LG, Ravanani R, et al. CD86 has sustained costimulatory effects on CD8 T cells. *J Immunol*. 2007; 179(9):5936–5946. [PubMed: 17947667]
56. Srivastava MK, Bosch JJ, Wilson AL, Edelman MJ, Ostrand-Rosenberg S. MHC II lung cancer vaccines prime and boost tumor-specific CD4+ T cells that cross-react with multiple histologic subtypes of nonsmall cell lung cancer cells. *Int J Cancer*. 2010; 127(11):2612–2621. [PubMed: 20473949]
57. Chai JG. Cancer vaccination reprograms regulatory T cells into helper CD4 T cells to promote antitumor CD8 T-cell responses. *Immunotherapy*. 2011; 3(5):601–604. [PubMed: 21554089]

58. Pettit GR, Kamano Y, Herald CL. Antineoplastic agents, 118. Isolation and structure of bryostatin 9. *J Nat Prod.* 1986; 49(4):661–664. [PubMed: 3783162]
59. Ko HM, Kang JH, Jung B, et al. Critical role for matrix metalloproteinase-9 in platelet-activating factor-induced experimental tumor metastasis. *Int J Cancer.* 2007; 120(6):1277–1283. [PubMed: 17187368]
60. Redondo P, Lloret P, Idoate M, Inoges S. Expression and serum levels of MMP-2 and MMP-9 during human melanoma progression. *Clin Exp Dermatol.* 2005; 30(5):541–545. [PubMed: 16045689]
61. Johnson BD, Yan X, Schauer DW, Orentas RJ. Dual expression of CD80 and CD86 produces a tumor vaccine superior to single expression of either molecule. *Cell Immunol.* 2003; 222(1):15–26. [PubMed: 12798304]

Highlights

- We uncover the role of bryostatin-1 in immune recognition of melanoma.
- We investigate changes in immune components by bryostatin-1.
- Enhancement of class II components reduces tumorigenic potential of melanoma.
- Increasing immune responses will enhance immune-mediated elimination of tumors.
- Information obtained will help design better chemo-immunotherapeutics.

Fig. 1. Bryostatin-1 treatment enhances CD4⁺ T cell recognition of human melanoma cells. (A) Melanoma cell lines HT-144, 1359-mel and SLM2-mel were treated with either bryostatin-1 (0, 20 and 40 nM) or vehicle alone (ethanol) daily for 48 h, followed by the addition of Ig κ Ag for another 24 h. Cells were washed and cocultured with the κ peptide specific T cell hybridomas (2.18a and 1.21 T cell hybridomas specific for Ig κ _{188–203}, and Ig κ _{145–159} epitopes respectively) for 24 h. (B) Effects of bryostatin-1 on enhanced melanoma Ag presentation and CD4⁺ T cell recognition. HT-144, 1359-mel and SLM2-mel cells were treated with vehicle alone or bryostatin-1 (40 nM) for 72 h, followed by coculture with the tyrosinase_{56–70} peptide specific CD4⁺ T cells as described in the methods. The production of IL-2 was measured in the cell supernatants and expressed as mean pg/ml $\pm$ SEM of triplicate wells. Data are representative of three separate experiments. *indicates that the data are statistically significant (p<0.05).

Fig. 2. Effects of bryostatin-1 on the modulation of HLA class II molecules in melanoma cells. (A) Western blot analysis of HLA class II proteins in bryostatin-1 treated HT-144 cells. Cells were treated with either bryostatin-1 (40 nM) or vehicle alone for 24 and 72 h, and cell lysates were analyzed by western blotting for HLA class II protein expression. Each lane was loaded with 60 μ g of protein from the cell lysates. The membrane was reprobed with β -actin to confirm equal loading of proteins from the lysates. (B) HT-144 cells treated with bryostatin-1 were also analyzed by western blotting for Ii and HLA-DM protein expression. Actin was used as a loading control. Data are representative of at least three separate experiments. (C) RT-PCR analysis of CIITA mRNA expression in bryostatin-1-treated HT-144 cells. Total RNA extracted from HT-144 was reverse-transcribed, amplified, and separated by electrophoresis. Specific products were detected in these cells by RT-PCR using specific primers to CIITA as described in the methods. The amplification of β -actin was included as a control. (D) CIITA mRNA expression levels in bryostatin-1-treated HT-144 cells were also determined by real-time RT-PCR as described. Relative levels of CIITA mRNA expression were normalized in all the samples analyzed with respect to the levels of GAPDH amplification. CIITA mRNA expression levels were presented as fold increase over control.

Fig. 3.

Bryostatin-1 treatment alters cell morphology, matrix-metalloproteinase-9, acidic cathepsins and surface class II proteins in HT-144 cells. (A) HT-144 cells were treated with vehicle alone or bryostatin-1 (40 nM) for 72 h, and were subjected to microscopic analysis for morphological features. (B) Cells treated with vehicle alone or bryostatin-1 (40 nM) for 72 h, were analyzed by both MTS assay (closed bars) and trypan dye exclusion method (open bars) for determining cell viability. (C) Cells were also subjected to western blotting for detecting MMP-9, Cat B, Cat D, Cat S and Cat L protein expression. Actin was used as a loading control. (D) Flow cytometric analysis of cell surface HLA-DR and HLA-DR4 on HT-144 melanoma cells. HT-144 cells were treated with vehicle alone or bryostatin-1 (40 nM) for 72 h, and were stained with antibodies against DR (L243) and DR4 (359-F10). The B-cell line Frev which constitutively express DR/DR4 was used as a control. NN4 and IN-1 were used as isotype controls. Data are representative of at least three separate experiments.

Fig. 4.

Bryostatin-1 upregulates CD80 and CD86 costimulatory molecules in HT-144 melanoma cells. HT-144 cells were treated with either vehicle alone or bryostatin-1 (40 nM) for 72 h, washed, and incubated with anti-CD80 and CD86 antibodies on glass slides as described in the methods. The slides were washed and incubated with FITC-conjugated secondary antibodies, and analyzed by confocal microscopy. Data are representative of three separate experiments.

Fig. 5.

Effects of bryostatin-1 on the cell surface expression of CD40, CD80 and CD86 in HT-144 melanoma cells. HT-144 melanoma cells were treated with vehicle alone or bryostatin-1 (40 nM) for 72 h, followed by cell surface staining with antibodies against CD40, CD80 and CD86 molecules and matched isotype controls. The B-cell line, Frev, which constitutively expresses CD40, CD80 and CD86 molecules, was used as a control.