

Spectral Flow Cytometry Webinar Series

Watch our webinar series and learn how the ID7000™ system builds on Sony's experience with spectral analysis and simplifies many operations to advance the field of flow cytometry.



Watch Now

SONY



Enhanced Expression of CD20 in Human Tumor B Cells Is Controlled through ERK-Dependent Mechanisms

This information is current as of March 9, 2022.

Wojciech Wojciechowski, Huifen Li, Shannon Marshall, Chiara Dell'Agnola and Igor Espinoza-Delgado

J Immunol 2005; 174:7859-7868; ;
doi: 10.4049/jimmunol.174.12.7859
<http://www.jimmunol.org/content/174/12/7859>

References This article **cites 85 articles**, 44 of which you can access for free at:
<http://www.jimmunol.org/content/174/12/7859.full#ref-list-1>

Why *The JI*? [Submit online.](#)

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
<http://jimmunol.org/subscription>

Permissions Submit copyright permission requests at:
<http://www.aai.org/About/Publications/JI/copyright.html>

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
<http://jimmunol.org/alerts>



Enhanced Expression of CD20 in Human Tumor B Cells Is Controlled through ERK-Dependent Mechanisms

Wojciech Wojciechowski, Huifen Li, Shannon Marshall, Chiara Dell'Agnola, and Igor Espinoza-Delgado¹

Rituximab, a chimeric Ab directed against CD20, induces apoptosis in targeted cells. Although the majority of B cell malignancies express the CD20 Ag, only ~50% of patients will respond to single-agent rituximab. The available data suggest that a decreased CD20 expression could account for the lack of response observed in some patients treated with rituximab. Despite the potential critical role of CD20 in the biology of B cell malignancies, the mechanisms controlling its expression are poorly understood. We evaluated the effect of the immune modulator agent bryostatin-1 on the expression of CD20 in non-Hodgkin's lymphoma cells. Using the B cell lines, DB and RAMOS, as well as tumor cells derived from a chronic lymphocytic leukemia patient, we demonstrated that bryostatin-1 enhanced the expression of both CD20 mRNA and protein. The enhanced expression of CD20 was associated with increased transcriptional activity of the CD20 gene, whereas the stability of CD20 mRNA was not affected. The effect of bryostatin-1 on CD20 expression in non-Hodgkin's lymphoma cells was mediated through the MAPK kinase/ERK signal transduction pathway and involved protein kinase C, but was independent of p38 MAPK and was insensitive to dexamethasone. Cells pretreated with bryostatin-1 were more susceptible to the proapoptotic effect of anti-CD20 Ab. Overall, these data demonstrate for the first time that ERK phosphorylation is required for the up-regulated expression of CD20 on B cell malignancies. The findings also suggest that bryostatin-1 and rituximab could be a valuable combined therapy for B cell malignancies. *The Journal of Immunology*, 2005, 174: 7859–7868.

Rituximab, a human-mouse chimeric mAb that targets CD20, is the first therapeutic Ab approved by the Food and Drug Administration for the treatment of non-Hodgkin's lymphoma (NHL)² patients who have failed standard therapy (1–3). CD20 is a highly phosphorylated, integral membrane protein found on both normal and transformed B lymphocytes (4) and has been reported to be involved in regulation of transmembrane calcium flux (5, 6). The CD20 protein normally does not shed from the cells and is not internalized upon binding by Abs (7–9), which, together with its pattern of expression, makes CD20 an excellent target for therapeutic intervention. The mechanisms responsible for the antitumor activity of anti-CD20 Abs are not fully understood. However, extensive in vitro and preclinical studies suggest that Ab-dependent cell-mediated cytotoxicity (10, 11), complement-dependent cytotoxicity (12–15), and direct effects of transmembrane signaling leading to apoptosis and cell growth arrest (16–18) may be responsible for the observed clinical effects of rituximab. Both in vitro and preclinical data suggest that decreased CD20 expression may be responsible for the lack of response observed in certain patients (12, 19, 20).

Bryostatin-1, a protein kinase C (PKC) modulator (21, 22) with antitumor activity (23, 24), stimulates host immune responses (25–

29) and promotes the production of proinflammatory cytokines (30, 31). Furthermore, bryostatin-1 synergizes with IL-2 to induce the expression and secretion of IFN- γ in human T cells (32). The in vivo mechanisms of antitumor action of bryostatin-1 are not completely understood. However, activation of immune effector cells might play an important role. Cancer patients, including NHL patients, have been reported to have immune dysfunction (33–37). Bryostatin-1, by promoting a Th1-type cytokine response and activating effector populations, may enhance the effectiveness of anti-CD20 therapy.

In this study we report the effect of bryostatin-1 on the expression of CD20 in the well-characterized NHL cell lines, DB and Ramos (38–40). We demonstrate that bryostatin-1 enhances the expression of CD20 on NHL cells through an ERK-1/2-dependent pathway. Up-regulation of CD20 expression by bryostatin-1 occurs rapidly and through mechanisms that are independent of p38 MAPK and de novo protein synthesis and are also insensitive to dexamethasone. Thus, the present work suggests a mechanism by which the ERK-1/2 pathway can be influenced by an immunomodulator-antineoplastic agent and provides a rationale for clinical evaluation of anti-CD20 mAb and bryostatin-1 in patients with NHL.

Materials and Methods

Cells

All cells were cultured in RPMI 1640 tissue culture medium (BioSource International) supplemented with 10% FBS (BioSource International), 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, and 10 mM HEPES, pH 7.3 (Quality Biological), and maintained at 30–80% cell confluence in 5% CO₂ at 37°C. The DB cell line is a human diffuse large cell lymphoma of B cell origin (38). The Ramos cell line was derived from a human B cell Burkitt's lymphoma (40). PBLs were obtained from a chronic lymphocytic leukemia (CLL) patient by leukapheresis using a Fanwell CS-3000 blood cell separator, following the protocol approved by the institutional review board. CLL cells were isolated from PBLs using a negative

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

Received for publication February 11, 2005. Accepted for publication April 12, 2005.

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.

¹ 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

² Abbreviations used in this paper: NHL, non-Hodgkin's lymphoma; Act-D, actinomycin D; BI, bisindolylmaleimide I; Bryo, bryostatin-1; CHX, cycloheximide; CLL, chronic lymphocytic leukemia; GAH, goat anti-human IgG; MEK, MAPK kinase; PKC, protein kinase C.

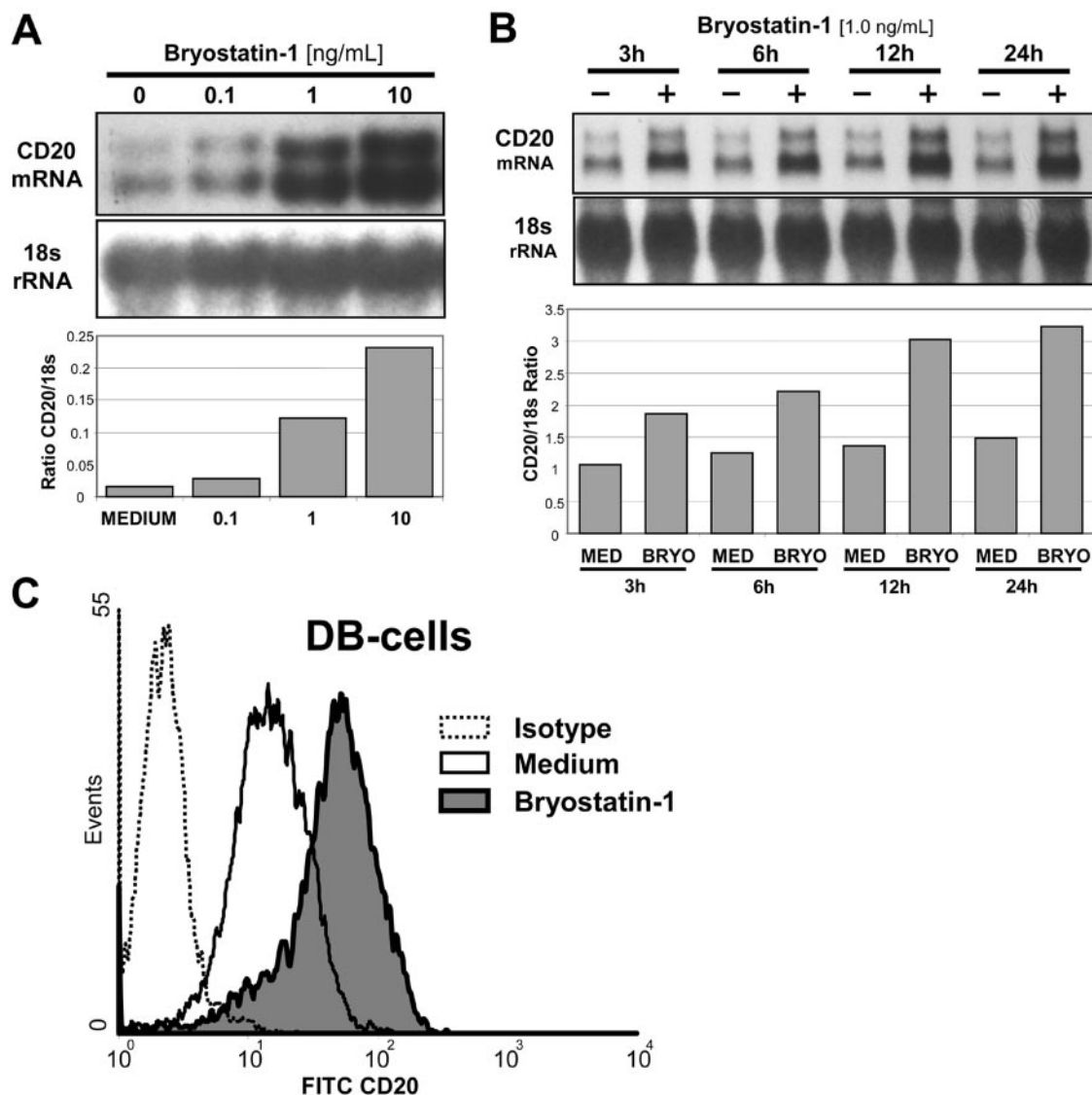


FIGURE 1. Bryostatin-1 enhances CD20 mRNA and protein expression in DB cells. Cells were incubated in the presence or the absence of bryostatin-1 at the indicated concentrations for 12 h (A) or at a single concentration (1 ng/ml) for 3, 6, 12, and 24 h (B). The expression of CD20 mRNA was analyzed by Northern blot as described in *Materials and Methods*, and the normalized levels of CD20 mRNA are presented. Representative results from three experiments are shown. C, DB cells were treated with 1 ng/ml bryostatin-1 for 24 h, labeled with anti-CD20 Abs, and analyzed by flow cytometry as described in *Materials and Methods*. The histograms of CD20-positive, bryostatin-1-treated and control cells are shown.

selection human B cell isolation kit (StemCell Technologies) according to the manufacturer's instructions.

Reagents

Clinical grade bryostatin-1 was obtained from the National Cancer Institute. Rituximab (Rituxan), the chimeric human-mouse anti-human CD20 Ab, was obtained from Genentech. The inhibitors U0126, PD98059, SB203580, and bisindolamide I (GF 109203X) were purchased from EMD Biosciences/Calbiochem and dissolved in DMSO. FITC-conjugated anti-human CD20 Ab and isotype-matched Ab were purchased from BD Biosciences. The anti-human ERK-2 and anti-human phospho-ERK-1/2 Ab were obtained from Santa Cruz Biotechnology. HRP-conjugated anti-rabbit and anti-mouse IgG Abs, actinomycin D (Act-D) and dexamethasone were purchased from Sigma-Aldrich. Goat anti-human IgG Ab was purchased from Pierce, and human IgG fraction control Ab was obtained from Jackson ImmunoResearch Laboratories. ECL chemiluminescence detection reagent was obtained from Amersham Biosciences. The TACS annexin V-FITC apoptosis detection kit was obtained from R&D Systems. The High Prime DNA labeling reagent was purchased from Roche, and [γ -³²P]dCTP was obtained from PerkinElmer.

Northern blot

Northern blotting was performed as previously described (41). Briefly, 5 million cells/sample were plated at a concentration of $0.3\text{--}0.4 \times 10^6$ cells/ml, and after treatment, total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. Ten micrograms of total RNA per sample was separated under denaturing conditions on 1.2% formaldehyde gel and transferred to a nylon membrane (Nytran; Schleicher & Schuell). The RNA was UV cross-linked, and the membranes were prehybridized for 8–10 h at 43°C in hybridization buffer (Hybrizol; Oncor), followed by overnight hybridization with 0.5×10^6 cpm/ml specific cDNA probe labeled with ³²P using High Prime DNA labeling reagent. The membranes were washed and exposed to x-ray films (Biomax-MR; Eastman Kodak) and phosphor storage screens. The phosphor screens were scanned using a Storm 840 PhosphorImager and were analyzed using ImageQuant image analysis software (Amersham Biosciences). The membranes were stripped and rehybridized with an 18S rRNA probe. The values obtained for CD20 mRNA levels were normalized with respective values of 18S rRNA levels, and ratios were plotted. Human CD20 cDNA was provided by Dr. J. Ledbetter (University of Washington, Seattle, WA). The 903-bp *Bam*HI-*Xba*I fragment of CD20 cDNA was used

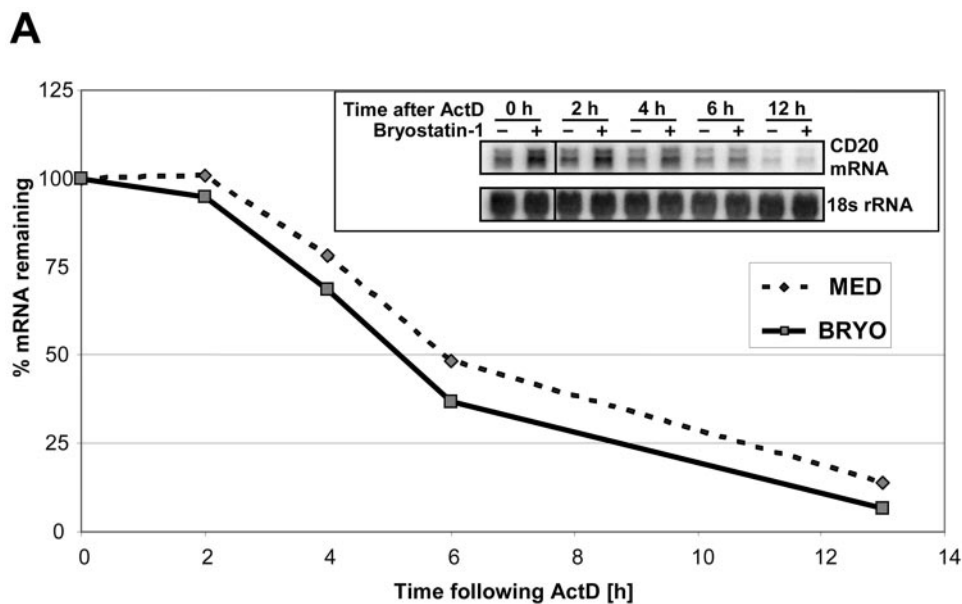
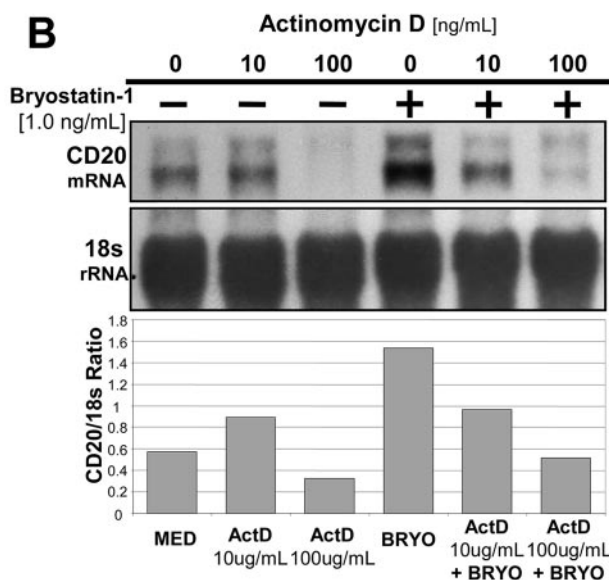


FIGURE 2. Bryostatin-1 regulates CD20 mRNA expression at a transcriptional level. **A**, DB cells were incubated with or without 1 ng/ml bryostatin-1 for 12 h, then 2 μ g/ml Act-D was added to the medium to stop mRNA transcription. The cells were harvested at the indicated time points, and the levels of CD20 mRNA were analyzed by Northern blot. The graph shows the relative amounts of remaining CD20 mRNA normalized with the amounts of 18S rRNA. The results presented are representative of three independent experiments. **B**, DB cells were treated with the indicated amounts of Act-D for 30 min before the addition of 1 ng/ml bryostatin-1 where indicated. CD20 mRNA expression was analyzed by Northern blot 12 h after the addition of bryostatin-1. The results were processed as described in *Materials and Methods*, and the normalized levels of CD20 mRNA are presented.



as a probe for Northern blot analysis (4). The pBR322 plasmid containing human 18S rRNA sequence was provided by Dr. D. Radzioch (McGill University, Montreal, Canada). The *Bam*HI-*Eco*RI 1.1-kb DNA fragment of the sequence was used as a probe. For mRNA synthesis inhibition, Act-D was dissolved in ethanol at 1 mg/ml and used at a final concentration of 2 μ g/ml for the indicated times.

Flow cytometry

Analysis of surface expression of CD20 by flow cytometry was performed as previously described (41). After 24-h treatment with 1 ng/ml bryostatin-1 or medium alone, the cells 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 μ l of FACS buffer and incubated for 15 min on ice with anti-human CD20 Ab conjugated with FITC. After two washes with FACS buffer, the cells were analyzed using FACScan flow cytometry equipment (BD Biosciences). The data obtained was processed using the WinMDI version 2.8 flow cytometry application (www.facs.scripps.edu).

Western blot

Cells were incubated in the presence or the absence of bryostatin-1 at the indicated concentrations for 1 h. Where indicated, inhibitors were added to

the cell culture 30 min before bryostatin-1. The cells were harvested, washed once with ice-cold PBS, and lysed directly in 1 \times Tris-glycine-SDS sample buffer (Invitrogen Life Technologies). The lysates were immediately incubated for 5 min at 100°C. The total protein concentration was measured using bicinchoninic acid protein assay reagent (Pierce). Samples containing 10 μ g of total protein were reheated at 100°C for 5 min in the presence of reducing agent (50 mM DTT), then separated on 4–16% gradient NuPAGE precast gels (Invitrogen Life Technologies) or 10% Criterion XT precast gels (Bio-Rad). After electrophoresis, the proteins were electrotransferred onto polyvinylidene difluoride membranes (Millipore) according to the procedure recommended by manufacturer. After overnight blocking at 4°C with 5% nonfat milk in TBST 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 primary Abs. After three 10-min washes with TBST, 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.

Anti-CD20 cross-linking

Experiments were performed as previously described (17, 18). Briefly, cells were cultured in the presence or the absence of 1.0 ng/ml bryostatin-1

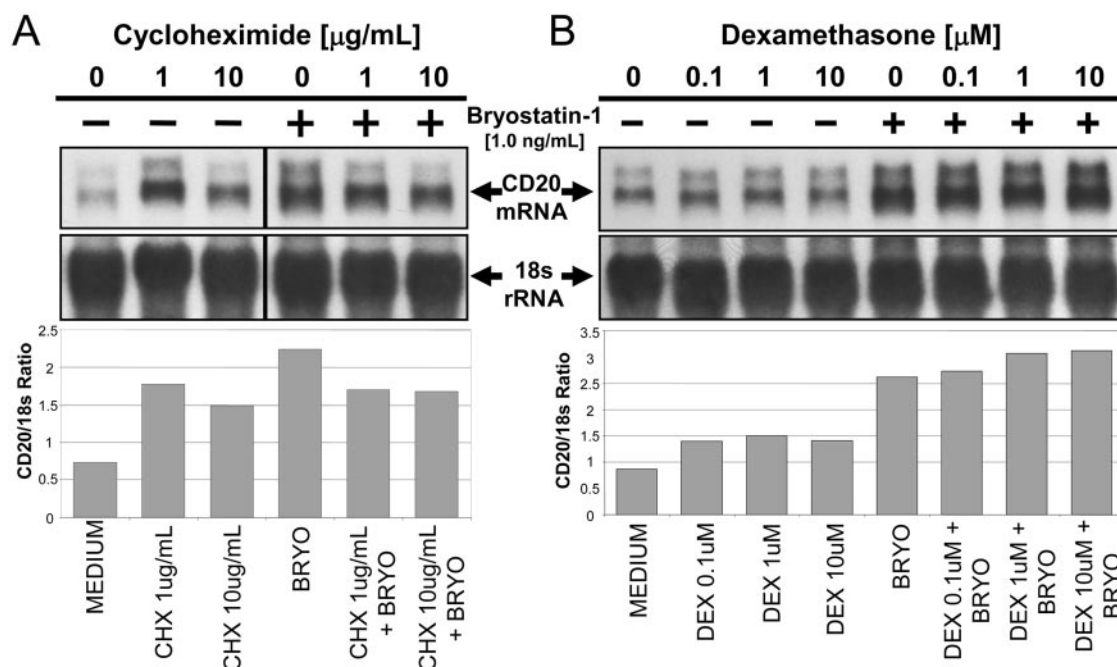


FIGURE 3. The induction of expression of CD20 mRNA by bryostatin-1 does not require new protein synthesis and is not affected by dexamethasone. DB cells were incubated with the indicated amounts of cycloheximide (A) or dexamethasone (B) for 30 min before the addition of 1 ng/ml bryostatin-1. The levels of CD20 mRNA expression were determined by Northern blot 12 h later, as described in *Materials and Methods*. Normalized levels of CD20 mRNA are presented.

for 24 h. Cells were washed with RPMI 1640 containing 10% FBS and incubated for another 24 h with 1 μg/ml rituximab or 10 μg/ml control human IgG alone or in the presence of 5 μg/ml goat anti-human IgG (GAH) cross-linking Ab. Cells were labeled with annexin V and propidium iodine using the TACS Annexin V^{FITC} apoptosis detection kit according to the manufacturer's instructions (R&D Systems). The data were analyzed using the WinMDI version 2.8 flow cytometry application ((www.facs.scripps.edu)).

Results

Bryostatin-1 enhances CD20 mRNA expression in NHL cells

To ascertain the effect of bryostatin-1 on the expression of CD20 in NHL, DB cells were cultured for 12 h in medium alone or in the presence of increasing concentrations of bryostatin-1. As shown in Fig. 1A, low basal expression of CD20 mRNA was detected in medium-treated cells, whereas bryostatin-1-treatment of DB cells led to a significant enhancement of CD20 mRNA expression. The effect of bryostatin-1 on CD20 mRNA expression was dose dependent. As little as 1 ng/ml was sufficient to induce a major increase in CD20 mRNA expression, whereas a dose of 10 ng/ml bryostatin-1 was required for maximum expression. Therefore, 1 ng/ml bryostatin-1 was used in all subsequent experiments. To determine the kinetics of up-regulation of CD20 mRNA by bryostatin-1, DB cells were incubated in medium alone or medium supplemented with 1 ng/ml bryostatin-1 for 3, 6, 12, or 24 h. As shown in Fig. 1B, the increase in CD20 mRNA expression was observed within 3 h after treatment with bryostatin-1. Maximum enhancement of CD20 mRNA expression occurred at 12 h and remained elevated for at least 24 h. To investigate whether the enhanced expression of CD20 mRNA led to increased surface expression, DB cells were cultured in the absence or the presence of 1 ng/ml bryostatin-1 and analyzed by flow cytometry at 24 h. As shown in Fig. 1C, DB cells constitutively expressed basal levels of CD20 surface protein. Upon bryostatin-1 treatment, increased expression of CD20 was observed. Similar results were obtained with Ramos cells and B cells acquired from a CLL patient (data not

shown). These experiments indicate that the bryostatin-1-enhanced expression of CD20 mRNA is associated with an increased expression of CD20 on the surface of these cells.

Transcriptional activity of the CD20 gene in NHL cells is enhanced by bryostatin-1

To investigate whether bryostatin-1-enhanced CD20 mRNA expression was associated with increased CD20 mRNA stability, DB cells were incubated for 12 h with medium alone or supplemented with 1 ng/ml bryostatin-1. After the 12-h incubation period, 2 μg/ml Act-D was added to the cultures for the indicated lengths of time to block further RNA transcription. Northern blot analysis revealed that CD20 mRNA decayed with similar kinetics in medium- and bryostatin-1-treated cells (Fig. 2A). The level of CD20 mRNA in both medium- and bryostatin-1-treated cells decreased by 50% at ~5 h and 30 min. These experiments demonstrated that the enhanced expression of CD20 mRNA by bryostatin-1 is not explained by an increased stability of CD20 mRNA.

To determine whether the enhanced expression of CD20 by bryostatin-1 involved the activation of CD20 gene transcription, DB cells were pretreated for 30 min with 10 ng/ml Act-D (42, 43), then 1 ng/ml bryostatin-1 was added, and the cells were harvested 12 h later. As shown in Fig. 2B, Act-D blocked bryostatin-1-enhanced CD20 mRNA expression. Together, these results demonstrate that bryostatin-1 did not stabilize CD20 mRNA and suggest that the observed increase in CD20 mRNA expression is at least in part the result of enhanced transcription.

Enhanced expression of CD20 mRNA by bryostatin-1 does not require de novo protein synthesis, nor is it affected by dexamethasone

To establish whether bryostatin-1-enhanced expression of CD20 mRNA was dependent upon de novo protein synthesis, DB cells were incubated in the presence or the absence of bryostatin-1 and in the presence or the absence of the protein synthesis inhibitor

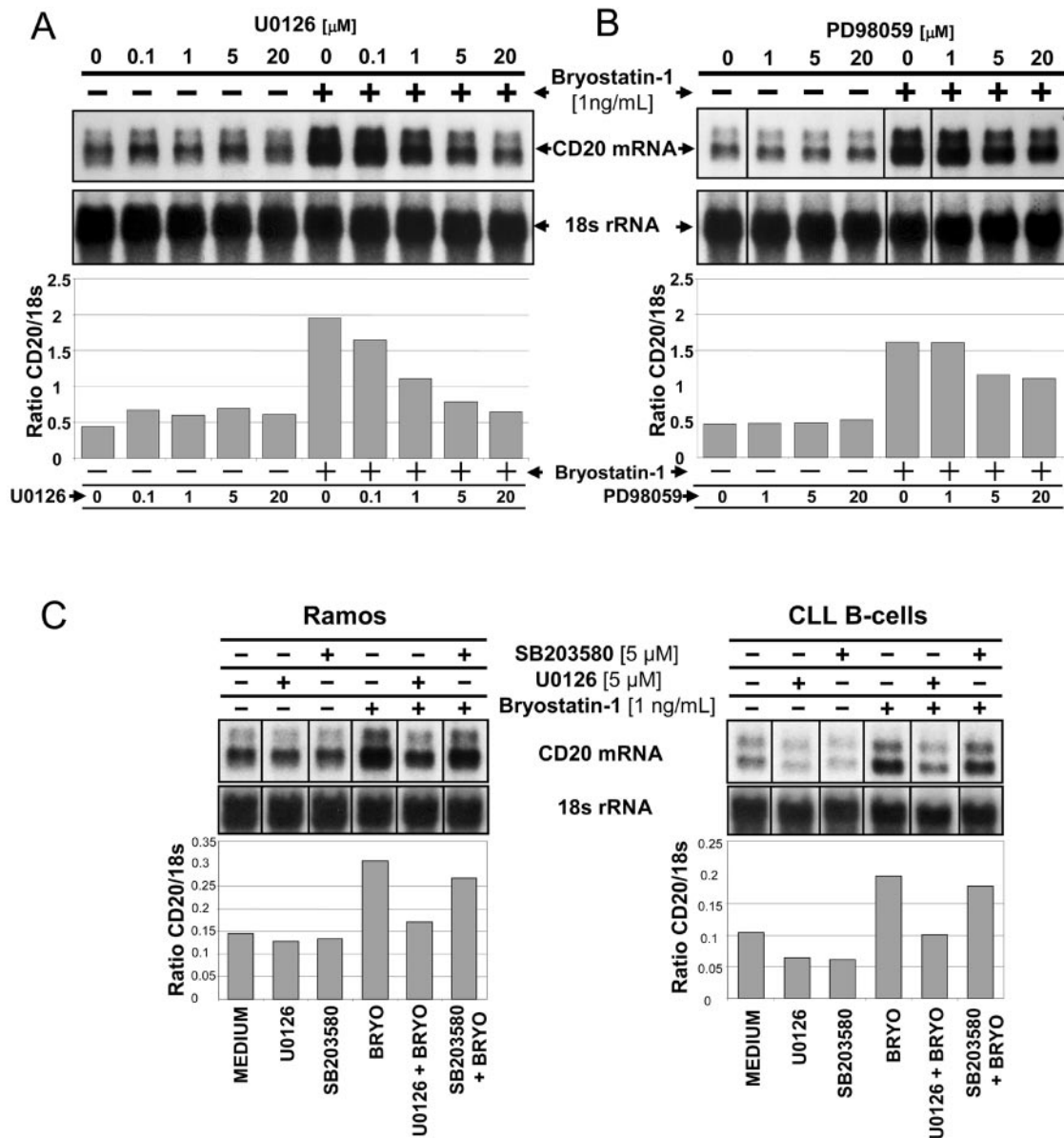


FIGURE 4. The induction of CD20 mRNA expression in malignant B cells by bryostatin-1 is mediated by the MEK1/ERK-1/2 signaling pathway. DB cells were pretreated for 30 min with the indicated amounts of two MEK inhibitors, U0126 (A) and PD98059 (B), before the addition of 1 ng/ml bryostatin-1 for 12 h. Similarly, Ramos and primary B cells isolated from a CLL patient were pretreated with either U0126 or the p38 MAPK inhibitor SB203580 for 30 min before addition of bryostatin-1 for 12 h (C). CD20 mRNA expression was analyzed by Northern blot. Normalized levels of CD20 mRNA are presented.

cycloheximide (CHX) at the indicated concentrations. As shown in Fig. 3A, bryostatin-1-enhanced expression of CD20 was minimally affected by CHX, suggesting that the up-regulation of CD20 mRNA induced by bryostatin-1 is not dependent on de novo protein synthesis. Interestingly, addition of CHX to medium-treated cells caused a major increase in CD20 mRNA expression, implying the presence of a short-lived negative regulator of CD20 mRNA transcription.

Glucocorticoids regulate the expression of several genes (44, 45). Furthermore, they are an integral component of many regimens commonly used in the treatment of B cell malignancies. To investigate the effects of glucocorticoids on bryostatin-1-induced expression of CD20, DB cells were pretreated with the indicated concentration of dexamethasone for 30 min, then incubated with bryostatin-1 for 12 h. As shown in Fig. 3B, dexamethasone did not

decrease bryostatin-1-enhanced expression of CD20 mRNA at any of the doses tested.

Bryostatin-1 enhances the expression of CD20 mRNA in NHL cells through a MAPK kinase (MEK)/ERK-dependent pathway

To determine the role of the MEK/ERK pathway in the enhanced expression of CD20 by bryostatin-1, we investigated the effects of two structurally unrelated chemical inhibitors of MEK1/2, U0126 and PD98059, at concentrations specific for MEK1 inhibition (46, 47). Cells were incubated for 30 min with the indicated doses of U0126 and PD98059, then treated with 1 ng/ml bryostatin-1 or medium alone for 12 h, and Northern blot analysis was performed. As shown in Fig. 4, A and B, both inhibitors decreased bryostatin-1-induced CD20 mRNA expression in a dose-dependent manner. In agreement with previous reports, U0126 appears to be a more

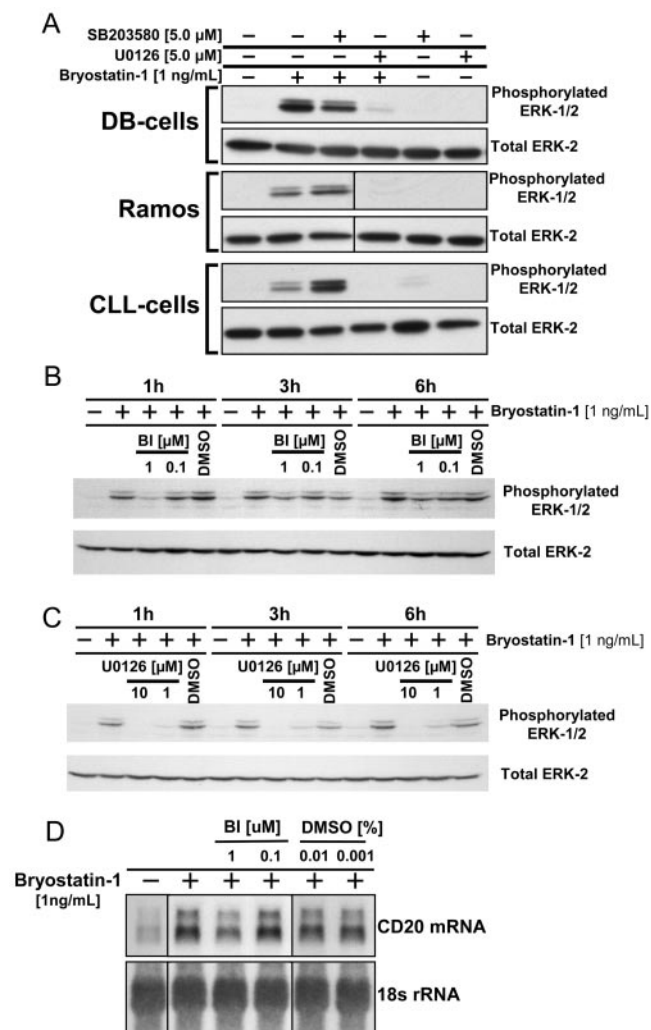


FIGURE 5. The bryostatin-1-induced phosphorylation of ERK-1/2 in DB, Ramos, and CLL B cells depends on PKC, but is not affected by p38 MAPK inhibitor. DB, Ramos, and primary CLL B cells were incubated in the presence of either U0126 or SB203580 for 30 min before a 1-h treatment with 1 ng/ml bryostatin-1 (A). After 30-min pretreatment with the indicated concentrations of either BI (B) or U0126 (C), the DB cells were stimulated with 1 ng/ml bryostatin-1 and harvested after 1, 3, or 6 h. The levels of phosphorylated ERK-1/2 were assessed by Western blot (upper panels), and the levels of total ERK-2 were obtained after stripping and reprobating the membranes (lower panels) as described in *Materials and Methods*. DB cells were pretreated for 30 min with the indicated amounts of the PKC inhibitor BI or with DMSO (diluent) before the addition of bryostatin-1 at a concentration of 1 ng/ml for 12 h (D). CD20 mRNA expression was analyzed by Northern blot.

potent inhibitor of MEK1 than PD98059 (48). The decreased expression of CD20 was also observed in Ramos cells and CLL-B cells (Fig. 4C). In contrast, an inhibitor of p38 MAPK, SB203580, failed to significantly decrease the bryostatin-1-induced expression of CD20 mRNA (Fig. 4C).

Bryostatin-1 induces phosphorylation of ERK-1/2 in a PKC-dependent manner

Given that U0126 inhibited bryostatin-1-enhanced expression of CD20 mRNA, we wanted to confirm that MEK inhibitors were in fact blocking MAPK activation. We investigated phosphorylated ERK expression in NHL cells pretreated with U0126, followed by bryostatin-1 treatment for 1 h. Western blot analysis revealed that

medium-treated cells did not constitutively express phosphorylated ERK-1/2. In contrast, bryostatin-1-treated cells expressed high levels of the phosphorylated forms of both kinases (Fig. 5A). The bryostatin-1-induced ERK-1/2 phosphorylation was blocked by U0126, but not by SB203580, a p38 MAPK inhibitor, or Ly294002, a PI3K inhibitor (data not shown). The induction of ERK-1/2 phosphorylation was observed in both NHL B cell lines, DB and Ramos, and in primary B cells from a CLL patient.

Bryostatin-1 is a potent modulator of several serine-threonine kinases, including PKC (22). Activation of PKC by bryostatin-1 appears to play a significant role in cellular responses to this immunomodulator and antineoplastic agent. Therefore, we tested whether the PKC inhibitor bisindolylmaleimide I (BI) was able to block bryostatin-1-induced phosphorylation of ERK (49). As shown in Fig. 5B, pretreatment of cells with 1 μ M BI blocked phosphorylation of ERK-1/2 in bryostatin-1-treated cells. The inhibitory effect of BI was short-lived. Maximal inhibition was observed at 1 h and decreased thereafter. In contrast, the inhibitory effect of U0126 lasted at least 6 h, the latest point tested (Fig. 5C). To ascertain whether the enhanced expression of CD20 mRNA by bryostatin-1 was associated with PKC modulation, we investigated the effect of BI on CD20 mRNA expression. As shown in Fig. 5D, BI at a concentration of 1 μ M inhibited CD20 mRNA enhancement by \sim 30%. Together, these data suggest that bryostatin-1-enhanced expression of CD20 mRNA is at least partially dependent on the PKC-activated MEK1/ERK-1/2 pathway, but not on p38 kinase.

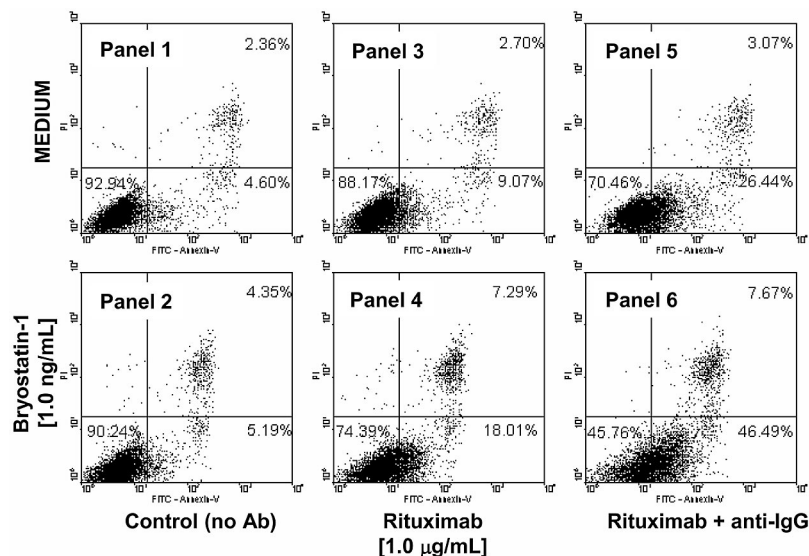
Bryostatin-1 directly increases proapoptotic events induced by anti-CD20 Abs

To determine whether bryostatin-1-enhanced CD20 expression could render NHL cells more susceptible to the proapoptotic effects of anti-CD20 Ab, we investigated the expression of annexin V on DB cells treated with suboptimal doses of bryostatin-1 and rituximab. Cells were pretreated with bryostatin-1, then incubated in the presence or the absence of rituximab with or without GAH Abs. Cells were stained with FITC-labeled annexin V and propidium iodine and were analyzed by flow cytometry. As shown in Fig. 6, panel 2, bryostatin-1 alone induced a minimal increase in the percentage of annexin V-positive cells (5.2%). Similar results were also observed with rituximab treatment (9%). In contrast, pretreatment with bryostatin-1, followed by rituximab, resulted in a 2-fold increase in the percentage of annexin V-positive cells (18%; Fig. 6, panel 4) compared with rituximab alone (9%; Fig. 6, panel 3). As shown in Fig. 6, panel 6, additional increases in the percentage of annexin V-positive cells were observed in cells treated with bryostatin-1 and rituximab, followed by GAH (46%). Treatment with control human IgG, GAH, or human IgG plus GAH did not affect the percentage of annexin V-positive cells (data not shown). Together, these results suggested that bryostatin-1 increases the early apoptotic events induced by rituximab.

Discussion

Targeted therapy has become an important paradigm in oncology. Rituximab, an mAb that targets CD20-expressing cells, is used clinically to treat several B cell malignancies (1, 50–53). One of the potential mechanisms of resistance to CD20-targeted therapy is a decreased expression of CD20 on tumor cells. Indeed, the response of CLL to rituximab reported in the pivotal trial of low-grade lymphoma was 12 vs 58% in those with other histologies (1). CLL cells have the lowest levels of CD20 expression among B cell malignancies, \sim 8,000 molecules/cell compared with 100,000 molecules/cell observed on most malignant B cells (54). Thus, a potential therapeutic approach to increase the rate of responses

FIGURE 6. Treatment of DB cells with bryostatin-1 promotes the proapoptotic effect of the anti-CD20 Ab. DB cells were treated with or without 1 ng/ml bryostatin-1 for 24 h before they were washed and incubated in the presence or the absence of 1 μ g/ml rituximab and/or secondary GAH cross-linking Ab. The cells were labeled with annexin V conjugated with FITC and propidium iodide (PI) and analyzed by flow cytometry as described in *Materials and Methods*. This figure presents the results of a single experiment and includes the percentage of cells positive in a particular quadrant. The results shown are representative of four independent experiments.



could be to enhance the expression of CD20 on the targeted tumor cells.

Recent studies have reported that IL-4, GM-CSF, TNF- α , and IFN- α are able to induce CD20 surface expression on B cells from CLL patients (55–58). However, the mechanisms regulating the expression of CD20 remain poorly understood. Bryostatin-1 is the prototype of a novel family of PKC modulators that has antitumor activity and activates immune effector cells, properties that have encourage its use in early clinical trials as an anticancer agent. The data presented in this study demonstrate for the first time that bryostatin-1 enhances the expression of CD20 in both NHL and CLL cells (Fig. 1). Very low concentrations of bryostatin-1 that might be achievable in vivo (0.1–1 ng/ml) were sufficient to enhance CD20 expression. Bryostatin-1-enhanced CD20 mRNA expression was followed by an increased level of CD20 surface expression, suggesting that bryostatin-1-driven CD20-enhanced protein expression is, at least in part, controlled at the gene level. This effect was not cell line restricted, because the induction was also observed in DB cells, Ramos cells, and primary B cells derived from a patient with CLL. Augmentation of CD20 mRNA expression occurred within 3 h of treatment, and an additional increase was observed through 24 h. The rapid increase in CD20 mRNA expression in NHL cells and the failure of cycloheximide to block this induction suggest a direct effect of bryostatin-1, rather than an effect mediated by another bryostatin-1-inducible factor (59).

Bryostatin-1 may control mRNA expression through both transcriptional and post-transcriptional mechanisms (29, 31, 32, 60). In an attempt to determine whether message stabilization was one of the mechanisms responsible for the enhanced CD20 expression by bryostatin-1, the half-life of the mRNA was studied. After blocking new RNA synthesis with Act-D, CD20 mRNA decayed with a similar half-life in both medium-treated and bryostatin-1-treated cells. These results clearly demonstrated that the increased accumulation of CD20 mRNA in response to bryostatin-1 was not the result of an increased stability of its mRNA. The ability of bryostatin-1 to increase the transcriptional activity of the CD20 gene was suggested by the failure of bryostatin-1 to enhance the expression of CD20 mRNA in the presence of Act-D (Fig. 2A).

CD20 is a B cell-specific gene that lacks many of the common regulatory elements present in other genes expressed by B cells (61). The known functional elements present in the CD20 promoter include an E box, a PU.1/Pip binding site, and a BAT box

(62, 63). The PU.1/Pip binding site can bind transcription factors belonging to the Ets family (63). Several members of this family, including Ets-1 and Elk-1, are activated in an ERK-dependent fashion (64). Using the on-line based Genomatrix suite (www.genomatrix.de), we were able to identify Ets binding sites in the promoters of several bryostatin-1-induced genes, including CD20. It is therefore possible that bryostatin-1-enhanced expression of CD20 is mediated by ERK-activated Ets transcription factors. Future studies will test this hypothesis and identify specific transcription factors involved in the process.

The effects of bryostatin-1 are mediated through numerous pathways, including PKC, p38 MAPK, ERK-1/2, and others (32, 59, 65, 66). It has been shown that bryostatin-1 activates the MEK/ERK pathway (65, 66). Confirming and expanding these observations, we demonstrated that bryostatin-1 induces phosphorylation of ERK-1/2 in NHL cells. Furthermore, using two structurally unrelated chemical inhibitors of MEK kinases, we showed that bryostatin-1-induced expression of CD20 is MEK/ERK-dependent.

Bryostatin-1 shares several biological activities with PMA, including its ability to modulate PKC activity and enhance the expression of CD20 (62). Although many bryostatin-1 biological effects are mediated through the activation of PKC (22, 25, 66), bryostatin-1 may also antagonize PKC-mediated effects (67–69). Recent work has indicated that some bryostatin-1 effects are exerted through PKC-independent mechanisms (70–72), including interaction with phorbol ester receptors such as chimaerins, Ras guanyl nucleotide-releasing proteins, Munc13s, protein kinase D, and 1,2-diacylglycerol kinases (73).

Bryostatin-1 may use the PKC/Raf/MEK pathway during the induction of cell differentiation and/or apoptosis (42, 66, 74). The data presented in this study expand this observation by demonstrating that bryostatin-1-induced phosphorylation of ERK-1/2 can be blocked by BI, a PKC-specific inhibitor. Although BI induces a major decrease in ERK-1/2 phosphorylation (Fig. 5B), its inhibitory effect on CD20 mRNA expression was modest (Fig. 5D). The observed difference could be due to the fact that apparently BI competes with ATP for binding to PKC (75). As a consequence, BI has a short-lived effect on ERK-1/2 phosphorylation (1–3 h) that is overcome after 6–12 h of treatment with bryostatin-1, the time needed for the optimal induction of CD20 mRNA in NHL cells.

Bryostatin-1 can induce gene expression also through p38 MAPK signaling pathway (32). Our results indicated that the effects of bryostatin-1 on CD20 mRNA expression or ERK-1/2 phosphorylation were not mediated by p38 MAPK (Figs. 4C and 5A). Together, these results suggest that p38 MAPK is not directly involved in bryostatin-1-enhanced CD20 expression. Interestingly, treatment of CLL cells with the p38 inhibitor, SB203580, led to minor increase in ERK-1/2 phosphorylation. A nonspecific effect of SB203580 on ERK-1/2 phosphorylation has been reported (76). The increase in ERK-1/2 phosphorylation was observed only in CLL cells, but not in either DB or Ramos cells. Although we do not know the molecular basis of this observation, it is clear that phosphorylation of ERK-1/2 in response to SB203580 does not lead to CD20 mRNA expression. This suggests that ERK-1/2 activation by itself is not sufficient to cause enhancement of CD20 expression.

Cycloheximide might inhibit or induce gene expression in B cells treated with different stimuli (77–80). Our data demonstrated that cycloheximide superinduced CD20 mRNA basal expression and did not have a major effect on bryostatin-1-enhanced CD20 mRNA expression (Fig. 3A). These results indicate that de novo protein synthesis is not required for the constitutive or bryostatin-1-enhanced expression of CD20 mRNA and also suggest that CD20 basal expression may be controlled by an as yet unknown de novo synthesized repressor protein(s).

Glucocorticoids have been reported to regulate gene expression in several cell types, including B cells (81). They are an integral component of many regimens commonly used in the treatment of B cell malignancies. The results presented in this study demonstrate that dexamethasone treatment did not decrease the induction of CD20 mRNA expression (Fig. 3B). These findings are in agreement with previous reports indicating that glucocorticoids did not block the antiproliferative and proapoptotic effects of rituximab (82).

Finally, anti-CD20 Ab cross-linking assays revealed that the pretreatment of DB (Fig. 6) and Ramos (data not shown) cells with bryostatin-1 rendered those NHL cells more susceptible to the proapoptotic effects of rituximab. It is believed that cross-linking of CD20-bound Abs facilitates intracellular signaling resulting in an enhanced apoptosis of target cells (17, 18). Although our studies suggest that the augmented expression of CD20 increases the frequency of proapoptotic events, the mechanism responsible for the enhanced occurrence of these events is unclear.

The data presented in this study provide the first report dissecting the molecular mechanisms and signaling pathways involved in the enhanced expression of CD20 mRNA. We clearly show that through processes not requiring protein synthesis, a mechanism involving transcriptional changes in association with ERK-1/2 phosphorylation is responsible for the enhanced expression of CD20 mRNA in NHL cells treated with bryostatin-1. We propose a model in which bryostatin-1-induced PKC activation is followed by ERK-1/2 phosphorylation and enhanced expression of CD20 mRNA, leading to an increased number of CD20 molecules on the surface of NHL cells, resulting in an increased sensitivity of these cells to the proapoptotic effects of rituximab.

To date, the mechanisms for in vivo resistance to rituximab have not been clearly defined. However, in vitro studies and preclinical data suggest that a decrease in CD20 expression (13, 19), increased expression of complement inhibitors (12, 13, 83), and/or modulation of FcR expression (10) could be responsible for the lack of responses seen in certain patients. The well-characterized dysfunctional immune status observed in tumor-bearing hosts, including patients with NHL and CLL, may also play a role in the lack of effectiveness of anti-CD20 therapy in some patients (33–37). The

current paradigm in the treatment of cancer is targeted therapy. The findings discussed in this study may improve our understanding of the molecular events leading to the expression of CD20. The ability of bryostatin-1 to enhance the expression of CD20 in B cell malignancies together with its immunomodulatory effects, including activation of NK cells, monocytes, and T cells (27, 28, 84, 85), warrant the exploration of the use of bryostatin-1 and rituximab therapy in patients with B cell refractory malignancies.

Acknowledgments

We thank Drs. Dan L. Longo, Eric H. Westin, Darrell R. Abernethy, and Michel Bernier for their critical review of this manuscript. We also thank Dr. Robert P. Wersto and Francis J. Chrest for their assistance with flow cytometry experiments.

Disclosures

The authors have no financial conflict of interest.

References

- Maloney, D. G., A. J. Grillo-Lopez, C. A. White, D. Bodkin, R. J. Schilder, J. A. Neidhart, N. Janakiraman, K. A. Foon, T. M. Liles, B. K. Dallaire, et al. 1997. IDEC-C2B8 (rituximab) anti-CD20 monoclonal antibody therapy in patients with relapsed low-grade non-Hodgkin's lymphoma. *Blood* 90: 2188–2195.
- Maloney, D. G., T. M. Liles, D. K. Czerwinski, C. Waldichuk, J. Rosenberg, A. Grillo-Lopez, and R. Levy. 1994. Phase I clinical trial using escalating single-dose infusion of chimeric anti-CD20 monoclonal antibody (IDEC-C2B8) in patients with recurrent B-cell lymphoma. *Blood* 84: 2457–2466.
- Berinstein, N. L., A. J. Grillo-Lopez, C. A. White, I. Bence-Bruckler, D. Maloney, M. Czuczman, D. Green, J. Rosenberg, P. McLaughlin, and D. Shen. 1998. Association of serum rituximab (IDEC-C2B8) concentration and anti-tumor response in the treatment of recurrent low-grade or follicular non-Hodgkin's lymphoma. *Ann. Oncol.* 9: 995–1001.
- Einfeld, D. A., J. P. Brown, M. A. Valentine, E. A. Clark, and J. A. Ledbetter. 1988. Molecular cloning of the human B cell CD20 receptor predicts a hydrophobic protein with multiple transmembrane domains. *EMBO J.* 7: 711–717.
- Bubien, J. K., L. J. Zhou, P. D. Bell, R. A. Frizzell, and T. F. Tedder. 1993. Transfection of the CD20 cell surface molecule into ectopic cell types generates a Ca^{2+} conductance found constitutively in B lymphocytes. *J. Cell Biol.* 121: 1121–1132.
- Tedder, T. F., and P. Engel. 1994. CD20: a regulator of cell-cycle progression of B lymphocytes. *Immunol. Today* 15: 450–454.
- Anderson, K. C., M. P. Bates, B. L. Slaughenhaupt, G. S. Pinkus, S. F. Schlossman, and L. M. Nadler. 1984. Expression of human B cell-associated antigens on leukemias and lymphomas: a model of human B cell differentiation. *Blood* 63: 1424–1433.
- Press, O. W., A. G. Farr, K. I. Borroz, S. K. Anderson, and P. J. Martin. 1989. Endocytosis and degradation of monoclonal antibodies targeting human B-cell malignancies. *Cancer Res.* 49: 4906–4912.
- Stashenko, P., L. M. Nadler, R. Hardy, and S. F. Schlossman. 1980. Characterization of a human B lymphocyte-specific antigen. *J. Immunol.* 125: 1678–1685.
- Clynes, R. A., T. L. Towers, L. G. Presta, and J. V. Ravetch. 2000. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nat. Med.* 6: 443–446.
- Reff, M. E., K. Carner, K. S. Chambers, P. C. Chinn, J. E. Leonard, R. Raab, R. A. Newman, N. Hanna, and D. R. Anderson. 1994. Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. *Blood* 83: 435–445.
- Golay, J., M. Lazzari, V. Faccinetti, S. Bernasconi, G. Borleri, T. Barbui, A. Rambaldi, and M. Introna. 2001. CD20 levels determine the in vitro susceptibility to rituximab and complement of B-cell chronic lymphocytic leukemia: further regulation by CD55 and CD59. *Blood* 98: 3383–3389.
- Golay, J., L. Zaffaroni, T. Vaccari, M. Lazzari, G. M. Borleri, S. Bernasconi, F. Tedesco, A. Rambaldi, and M. Introna. 2000. Biologic response of B lymphoma cells to anti-CD20 monoclonal antibody rituximab in vitro: CD55 and CD59 regulate complement-mediated cell lysis. *Blood* 95: 3900–3908.
- Di Gaetano, N., E. Cittera, R. Nota, A. Vecchi, V. Grieco, E. Scanziani, M. Botto, M. Introna, and J. Golay. 2003. Complement activation determines the therapeutic activity of rituximab in vivo. *J. Immunol.* 171: 1581–1587.
- Harjunpaa, A., S. Junnikkala, and S. Meri. 2000. Rituximab (anti-CD20) therapy of B-cell lymphomas: direct complement killing is superior to cellular effector mechanisms. *Scand. J. Immunol.* 51: 634–641.
- Mathas, S., A. Rickers, K. Bommert, B. Dorken, and M. Y. Mapara. 2000. Anti-CD20- and B-cell receptor-mediated apoptosis: evidence for shared intracellular signaling pathways. *Cancer Res.* 60: 7170–7176.
- Shan, D., J. A. Ledbetter, and O. W. Press. 1998. Apoptosis of malignant human B cells by ligation of CD20 with monoclonal antibodies. *Blood* 91: 1644–1652.
- Shan, D., J. A. Ledbetter, and O. W. Press. 2000. Signaling events involved in anti-CD20-induced apoptosis of malignant human B cells. *Cancer Immunol. Immunother.* 48: 673–683.
- Davis, T. A., D. K. Czerwinski, and R. Levy. 1999. Therapy of B-cell lymphoma with anti-CD20 antibodies can result in the loss of CD20 antigen expression. *Clin. Cancer Res.* 5: 611–615.

20. Almasri, N. M., R. E. Duque, J. Iturraspe, E. Everett, and R. C. Braylan. 1992. Reduced expression of CD20 antigen as a characteristic marker for chronic lymphocytic leukemia. *Am. J. Hematol.* 40: 259–263.
21. Mutter, R., and M. Wills. 2000. Chemistry and clinical biology of the bryostatins. *Bioorg. Med. Chem.* 8: 1841–1860.
22. Stone, R. M., E. Sariban, G. R. Pettit, and D. W. Kufe. 1988. Bryostatins 1 activates protein kinase C and induces monocytic differentiation of HL-60 cells. *Blood* 72: 208–213.
23. 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.
24. Jones, R. J., S. J. Sharkis, C. B. Miller, E. K. Rowinsky, P. J. Burke, and W. S. May. 1990. Bryostatin 1, a unique biologic response modifier: anti-leukemic activity in vitro. *Blood* 75: 1319–1323.
25. Berkow, R. L., and A. S. Kraft. 1985. Bryostatin, a non-phorbol macrocyclic lactone, activates intact human polymorphonuclear leukocytes and binds to the phorbol ester receptor. *Biochem. Biophys. Res. Commun.* 131: 1109–1116.
26. 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.
27. Drexler, H. G., S. M. Gignac, G. R. Pettit, and A. V. Hoffbrand. 1990. Synergistic action of calcium ionophore A23187 and protein kinase C activator bryostatin 1 on human B cell activation and proliferation. *Eur. J. Immunol.* 20: 119–127.
28. Scheid, C., J. Prendiville, G. Jayson, D. Crowther, B. Fox, G. R. Pettit, and P. L. Stern. 1994. Immunomodulation in patients receiving intravenous bryostatin 1 in a phase I clinical study: comparison with effects of bryostatin 1 on lymphocyte function in vitro. *Cancer Immunol. Immunother.* 39: 223–230.
29. Taylor, L. S., G. W. Cox, G. Melillo, M. C. Bosco, and I. Espinoza-Delgado. 1997. Bryostatin-1 and IFN- γ synergize for the expression of the inducible nitric oxide synthase gene and for nitric oxide production in murine macrophages. *Cancer Res.* 57: 2468–2473.
30. 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 α induction in vivo. The Cancer Research Campaign Phase I Committee. *J. Natl. Cancer Inst.* 85: 1812–1818.
31. Bosco, M. C., S. Rottschäfer, 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 γ chain expression. *Blood* 89: 3402–3411.
32. Curiel, R. E., C. S. Garcia, L. Farooq, M. F. Agüero, and I. Espinoza-Delgado. 2001. Bryostatin-1 and IL-2 synergize to induce IFN- γ expression in human peripheral blood T cells: implications for cancer immunotherapy. *J. Immunol.* 167: 4828–4837.
33. Correa, M. R., A. C. Ochoa, P. Ghosh, H. Mizoguchi, L. Harvey, and D. L. Longo. 1997. Sequential development of structural and functional alterations in T cells from tumor-bearing mice. *J. Immunol.* 158: 5292–5296.
34. Wang, Q., J. Stanley, S. Kudoh, J. Myles, V. Kolenko, T. Yi, R. Tubbs, R. Bukowski, and J. Finke. 1995. T cells infiltrating non-Hodgkin's B cell lymphomas show altered tyrosine phosphorylation pattern even though T cell receptor/CD3-associated kinases are present. *J. Immunol.* 155: 1382–1392.
35. Scrivener, S., R. V. Goddard, E. R. Kaminski, and A. G. Prentice. 2003. Abnormal T-cell function in B-cell chronic lymphocytic leukaemia. *Leuk. Lymphoma* 44: 383–389.
36. de Toter, D., G. Reato, F. Mauro, A. Cignetti, S. Ferrini, A. Guarini, M. Gobbi, C. E. Grossi, and R. Foa. 1999. IL4 production and increased CD30 expression by a unique CD8 $^{+}$ T-cell subset in B-cell chronic lymphocytic leukaemia. *Br. J. Haematol.* 104: 589–599.
37. Kay, N. E., L. Han, N. Bone, and G. Williams. 2001. Interleukin 4 content in chronic lymphocytic leukaemia (CLL) B cells and blood CD8 $^{+}$ T cells from B-CLL patients: impact on clonal B-cell apoptosis. *Br. J. Haematol.* 112: 760–770.
38. Beckwith, M., W. J. Urba, and D. L. Longo. 1993. Growth inhibition of human lymphoma cell lines by the marine products, dolastatins 10 and 15. *J. Natl. Cancer Inst.* 85: 483–488.
39. Cardarelli, P. M., M. Quinn, D. Buckman, Y. Fang, D. Colcher, D. J. King, C. Bebbington, and G. Yarranton. 2002. Binding to CD20 by anti-B1 antibody or F(ab') $_2$ is sufficient for induction of apoptosis in B-cell lines. *Cancer Immunol. Immunother.* 51: 15–24.
40. Klein, G., B. Giovarelli, A. Westman, J. S. Stehlin, and D. Mumford. 1975. An EBV-genome-negative cell line established from an American Burkitt lymphoma: receptor characteristics: EBV infectibility and permanent conversion into EBV-positive sublines in vitro infection. *Intervirology* 5: 319–334.
41. Curiel, R. E., C. S. Garcia, S. Rottschäfer, M. C. Bosco, and I. Espinoza-Delgado. 1999. Enhanced B7-2 gene expression by interferon- γ in human monocytes is controlled through transcriptional and posttranscriptional mechanisms. *Blood* 94: 1782–1789.
42. Wang, X., Q. Wang, W. Hu, and B. M. Evers. 2004. Regulation of phorbol ester-mediated TRAF1 induction in human colon cancer cells through a PKC/RAF/ERK/NF- κ B-dependent pathway. *Oncogene* 23: 1885–1895.
43. Liu, W., N. Reinmuth, O. Stoeltzing, A. A. Parikh, C. Tellez, S. Williams, Y. D. Jung, F. Fan, A. Takeda, M. Akagi, et al. 2003. Cyclooxygenase-2 is up-regulated by interleukin-1 β in human colorectal cancer cells via multiple signaling pathways. *Cancer Res.* 63: 3632–3636.
44. Auphan, N., J. A. DiDonato, C. Rosette, A. Helmsberg, and M. Karin. 1995. Immunosuppression by glucocorticoids: inhibition of NF- κ B activity through induction of I κ B synthesis. *Science* 270: 286–290.
45. De Bosscher, K., W. Vanden Berghe, and G. Haegeman. 2000. Mechanisms of anti-inflammatory action and of immunosuppression by glucocorticoids: negative interference of activated glucocorticoid receptor with transcription factors. *J. Neuroimmunol.* 109: 16–22.
46. Duncia, J. V., J. B. Santella III, C. A. Higley, W. J. Pitts, J. Wityak, W. E. Fritze, F. W. Rankin, J. H. Sun, R. A. Earl, A. C. Tabaka, et al. 1998. MEK inhibitors: the chemistry and biological activity of U0126, its analogs, and cyclization products. *Bioorg. Med. Chem. Lett.* 8: 2839–2844.
47. Alessi, D. R., A. Cuenda, P. Cohen, D. T. Dudley, and A. R. Saltiel. 1995. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase in vitro and in vivo. *J. Biol. Chem.* 270: 27489–27494.
48. Favata, M. F., K. Y. Horiuchi, E. J. Manos, A. J. Daulerio, D. A. Stradley, W. S. Feeser, D. E. Van Dyk, W. J. Pitts, R. A. Earl, F. Hobbs, et al. 1998. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J. Biol. Chem.* 273: 18623–18632.
49. Hauss, P., F. Mazerolles, C. Hivroz, O. Lecomte, C. Barbat, and A. Fischer. 1993. GF109203X, a specific PKC inhibitor, abrogates anti-CD3 antibody-induced up-regulation of CD4 $^{+}$ T cell adhesion to B cells. *Cell. Immunol.* 150: 439–446.
50. Rastetter, W., A. Molina, and C. A. White. 2004. Rituximab: expanding role in therapy for lymphomas and autoimmune diseases. *Annu. Rev. Med.* 55: 477–503.
51. Davis, T. A., A. J. Grillo-Lopez, C. A. White, P. McLaughlin, M. S. Czuczman, B. K. Link, D. G. Maloney, R. L. Weaver, J. Rosenberg, and R. Levy. 2000. Rituximab anti-CD20 monoclonal antibody therapy in non-Hodgkin's lymphoma: safety and efficacy of re-treatment. *J. Clin. Oncol.* 18: 3135–3143.
52. Davis, T. A., C. A. White, A. J. Grillo-Lopez, W. S. Velasquez, B. Link, D. G. Maloney, R. O. Dillman, M. E. Williams, A. Mohrbacher, R. Weaver, et al. 1999. Single-agent monoclonal antibody efficacy in bulky non-Hodgkin's lymphoma: results of a phase II trial of rituximab. *J. Clin. Oncol.* 17: 1851–1857.
53. Piro, L. D., C. A. White, A. J. Grillo-Lopez, N. Janakiraman, A. Saven, T. M. Beck, C. Varns, S. Shuey, M. Czuczman, J. W. Lynch, et al. 1999. Extended rituximab (anti-CD20 monoclonal antibody) therapy for relapsed or refractory low-grade or follicular non-Hodgkin's lymphoma. *Ann. Oncol.* 10: 655–661.
54. Nguyen, D. T., J. A. Amess, H. Doughty, L. Hendry, and L. W. Diamond. 1999. IDEC-C2B8 anti-CD20 (rituximab) immunotherapy in patients with low-grade non-Hodgkin's lymphoma and lymphoproliferative disorders: evaluation of response on 48 patients. *Eur. J. Haematol.* 62: 76–82.
55. Venugopal, P., S. Sivaraman, X. K. Huang, J. Nayini, S. A. Gregory, and H. D. Preisler. 2000. Effects of cytokines on CD20 antigen expression on tumor cells from patients with chronic lymphocytic leukemia. *Leuk. Res.* 24: 411–415.
56. Sivaraman, S., C. G. Deshpande, R. Ranganathan, X. Huang, A. Jajeh, T. O'Brien, R. W. Huang, S. A. Gregory, P. Venugopal, and H. D. Preisler. 2000. Tumor necrosis factor modulates CD 20 expression on cells from chronic lymphocytic leukemia: a new role for TNF α ? *Microsc. Res. Tech.* 50: 251–257.
57. Sivaraman, S., P. Venugopal, R. Ranganathan, C. G. Deshpande, X. Huang, A. Jajeh, S. A. Gregory, T. O'Brien, and H. D. Preisler. 2000. Effect of interferon- α on CD20 antigen expression of B-cell chronic lymphocytic leukemia. *Cytokines Cell. Mol. Ther.* 6: 81–87.
58. Treon, S. P., L. M. Pilarski, A. R. Belch, A. Kelliher, F. I. Preffer, Y. Shima, C. S. Mitsiades, N. S. Mitsiades, A. J. Szczypek, L. Eilman, et al. 2002. CD20-directed serotherapy in patients with multiple myeloma: biologic considerations and therapeutic applications. *J. Immunother.* 25: 72–81.
59. Battle, T. E., and D. A. Frank. 2003. STAT1 mediates differentiation of chronic lymphocytic leukemia cells in response to bryostatin 1. *Blood* 102: 3016–3024.
60. Lilly, M., K. Vo, T. Le, and G. Takahashi. 1996. Bryostatin 1 acts synergistically with interleukin-1 α to induce secretion of B-CSF and other cytokines from marrow stromal cells. *Exp. Hematol.* 24: 613–621.
61. Tedder, T. F., G. Klejman, S. F. Schlossman, and H. Saito. 1989. Structure of the gene encoding the human B lymphocyte differentiation antigen CD20 (B1). *J. Immunol.* 142: 2560–2568.
62. Thevenin, C., B. P. Lucas, E. J. Kozlow, and J. H. Kehrl. 1993. Cell type- and stage-specific expression of the CD20/B1 antigen correlates with the activity of a diverged octamer DNA motif present in its promoter. *J. Biol. Chem.* 268: 5949–5956.
63. Himmelmann, A., A. Riva, G. L. Wilson, B. P. Lucas, C. Thevenin, and J. H. Kehrl. 1997. PU. 1/Pip and basic helix loop helix zipper transcription factors interact with binding sites in the CD20 promoter to help confer lineage- and stage-specific expression of CD20 in B lymphocytes. *Blood* 90: 3984–3995.
64. Dittmer, J. 2003. The biology of the Ets1 proto-oncogene. *Mol. Cancer* 2: 29.
65. Lin, H., C. Chen, X. Li, and B. D. Chen. 2002. Activation of the MEK/MAPK pathway is involved in bryostatin1-induced monocytic differentiation and up-regulation of X-linked inhibitor of apoptosis protein. *Exp. Cell Res.* 272: 192–198.
66. 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.
67. 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.
68. 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.

69. 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.
70. Wang, H., R. M. Mohammad, J. Werdell, and P. V. Shekhar. 1998. p53 and protein kinase C independent induction of growth arrest and apoptosis by bryostatin 1 in a highly metastatic mammary epithelial cell line: In vitro versus in vivo activity. *Int. J. Mol. Med.* 1: 915–923.
71. Spitaler, M., I. Utz, W. Hilbe, J. Hofmann, and H. H. Grunicke. 1998. PKC-independent modulation of multidrug resistance in cells with mutant (V185) but not wild-type (G185) P-glycoprotein by bryostatin 1. *Biochem. Pharmacol.* 56: 861–869.
72. 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.
73. Yang, C., and M. G. Kazanietz. 2003. Divergence and complexities in DAG signaling: looking beyond PKC. *Trends Pharmacol. Sci.* 24: 602–608.
74. Wall, N. R., R. M. Mohammad, and A. M. Al-Katib. 2001. Mitogen-activated protein kinase is required for bryostatin 1-induced differentiation of the human acute lymphoblastic leukemia cell line Reh. *Cell Growth Differ.* 12: 641–647.
75. Toullec, D., P. Pianetti, H. Coste, P. Bellevergue, T. Grand-Perret, M. Ajakane, V. Baudet, P. Boissin, E. Boursier, F. Loriolle, et al. 1991. The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J. Biol. Chem.* 266: 15771–15781.
76. Birkenkamp, K. U., L. M. Tuyt, C. Lummen, A. T. Wierenga, W. Kruijer, and E. Vellenga. 2000. The p38 MAP kinase inhibitor SB203580 enhances nuclear factor- κ B transcriptional activity by a non-specific effect upon the ERK pathway. *Br. J. Pharmacol.* 131: 99–107.
77. Rahmsdorf, H. J., N. Harth, A. M. Eades, M. Litfin, M. Steinmetz, L. Forni, and P. Herrlich. 1986. Interferon- γ , mitomycin C, and cycloheximide as regulatory agents of MHC class II-associated invariant chain expression. *J. Immunol.* 136: 2293–2299.
78. Maffei, A., C. Perfetto, N. Ombra, G. Del Pozzo, and J. Guardiola. 1989. Transcriptional and post-transcriptional regulation of human MHC class II genes require the synthesis of short-lived proteins. *J. Immunol.* 142: 3657–3661.
79. Stordeur, P., L. Schandene, P. Durez, C. Gerard, M. Goldman, and T. Velu. 1995. Spontaneous and cycloheximide-induced interleukin-10 mRNA expression in human mononuclear cells. *Mol. Immunol.* 32: 233–239.
80. Bertoglio, J., J. Dosda, R. Stancou, E. Wollman, and D. Fradelizi. 1989. Expression and regulation of interleukin-1 mRNA and interleukin-1 receptors in human B-cell lines. *J. Mol. Cell. Immunol.* 4: 139–148.
81. Scheinman, R. I., P. C. Cogswell, A. K. Lofquist, and A. S. Baldwin, Jr. 1995. Role of transcriptional activation of I κ B α in mediation of immunosuppression by glucocorticoids. *Science* 270: 283–286.
82. Rose, A. L., B. E. Smith, and D. G. Maloney. 2002. Glucocorticoids and rituximab in vitro: synergistic direct antiproliferative and apoptotic effects. *Blood* 100: 1765–1773.
83. Treon, S. P., C. Mitsiades, N. Mitsiades, G. Young, D. Doss, R. Schlossman, and K. C. Anderson. 2001. Tumor cell expression of CD59 is associated with resistance to CD20 serotherapy in patients with B-cell malignancies. *J. Immunother.* 24: 263–271.
84. Trenn, G., G. R. Pettit, H. Takayama, J. Hu-Li, and M. V. Sitkovsky. 1988. Immunomodulating properties of a novel series of protein kinase C activators: the bryostatins. *J. Immunol.* 140: 433–439.
85. Jayson, G. C., D. Crowther, J. Prendiville, A. T. McGown, C. Scheid, P. Stern, R. Young, P. Brenchley, J. Chang, S. Owens, et al. 1995. A phase I trial of bryostatin 1 in patients with advanced malignancy using a 24 hour intravenous infusion. *Br. J. Cancer* 72: 461–468.