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Chronic Lymphocytic Leukemia and B and T Cells Differ in Their Response to Cyclic Nucleotide Phosphodiesterase Inhibitors[1]

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Abstract

PDE4 inhibitors, which activate cAMP signaling by reducing cAMP catabolism, are known to induce apoptosis in B lineage chronic lymphocytic leukemia (CLL) cells but not normal human T cells. The explanation for such differential sensitivity remains unknown. Here, we report studies contrasting the response to PDE4 inhibitor treatment in CLL cells and normal human T and B cells. Affymetrix gene chip analysis in the three cell populations following treatment with the PDE4 inhibitor rolipram identified a set of up-regulated transcripts with unusually high fold-changes in the CLL samples, several of which are likely part of compensatory negative feedback loops. The high fold-change were due to low basal transcript levels in CLL cells, suggesting that cAMP-mediated signaling may be unusually tightly regulated in this cell type. Rolipram treatment augmented cAMP levels and induced ATF-1/CREB serine 63/133 phosphorylation in both B lineage cell types but not T cells. As treatment with the broad-spectrum PDE inhibitor IBMX induced T cell CREB phosphorylation, we tested a series of family-specific PDE inhibitors for their ability to mimic IBMX-induced ATF-1/CREB phosphorylation. While PDE3 inhibitors alone had no effect, the combination of PDE3 and PDE4 inhibitors induced ATF-1/CREB Ser 63/133 phosphorylation in T cells. Consistent with this observation, PDE3B transcript and protein levels were low in CLL cells but easily detectable in T cells. Combined PDE3/4 inhibition did not induce T cell apoptosis, suggesting that cAMP-mediated signal transduction that leads to robust ATF-1/CREB Ser 63/133 phosphorylation is not sufficient to induce apoptosis in this lymphoid lineage.

Keywords

PDE4; PDE3; CLL; cAMP

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Introduction

Given the potent effects of cAMP signaling on immune system function, many studies have explored the use of family-specific cyclic nucleotide phosphodiesterase (PDE) inhibitors to manipulate the immune response to clinical benefit. While the bulk of such efforts have focused on inhibiting inflammatory pathways regulated by neutrophils, monocytes, and lymphocytes, recent efforts have examined the ability of cAMP PDE inhibitors to induce apoptosis in malignant lymphoid cell populations, either alone or in combination with more traditional therapeutic agents such as glucocorticoids (1-4). Among the 11 currently known families of cyclic nucleotide PDEs, all but three are capable of catabolizing cAMP and at least five PDE families (PDE1-4, PDE7 and PDE8) have been described as expressed in lymphoid cells and regulated by either mitogens or agents that induce cAMP-mediated signaling (5-10). While most studies have focused on identifying active PDE family-specific inhibitors in order to minimize potential side-effects, some studies suggest that inhibition of more than one PDE family may prove to be necessary for some therapeutic indications (11,12).

In B lineage chronic lymphocytic leukemia (CLL) cells, inhibition of the PDE4 family of cAMP-specific PDEs with the relatively family-specific PDE4 inhibitor rolipram (IC_{50} = 1 μM) induces apoptosis while comparable treatment of peripheral blood T lymphocytes has no readily discernable apoptotic effect (13). Rolipram induces a mitochondrial pathway of apoptosis in CLL characterized by cytochrome c release and caspase 9 and 3 activation (14). In addition to protein kinase A (PKA), CLL cells express a second cAMP effector enzyme, the cAMP-activated Rap GDP exchange factor EPAC1 and, consistent with this, rolipram induces Rap1 GDP-loading in CLL cells (15). Rolipram-induced apoptosis appears to be mediated through PKA rather than EPAC signaling pathways. An EPAC-specific agonist is anti-apoptotic in CLL cells while an enantiomeric antagonist of PKA, Rp-8Br-cAMPS, reduces PDE4 inhibitor-mediated apoptosis (15).

PKA alters the function of many enzymes in cells through direct phosphorylation. It also alters the expression of a large number of genes, in part through phosphorylation and activation of a family of three related transcription factors, CREB, CREM and ATF-1 (16,17). While CREB and ATF-1 are widely distributed, CREM expression is largely limited to spermatids (18). However, the *CREM* locus remains important in a wide variety of cell types due to cAMP-mediated up-regulation of ICER, a CREM splice isoform that lacks a transactivation domain, and that serves to repress CREB or ATF-1-mediated transcription as a compensatory negative feedback loop (19). Thus, PKA-mediated apoptotic pathways could be the result of post-transcriptional events or could be mediated through control of transcription of cAMP-regulated "death genes" (20). Of note, PDE4 inhibitors enhance the ability of glucocorticoids to induce apoptosis in CLL cells but not circulating human T cells (21). Strikingly, among all circulating hematopoietic cell types examined, PDE4 inhibitors up-regulate transcript levels of glucocorticoid receptor alpha (GRα) to a large extent only in CLL cells (22).

Despite the range of unique responses of CLL cells to PDE4 inhibitors, it remains unclear at what level such specificity is established. Given that the majority of the studies contrasting CLL cells and normal cells have focused on the responses of CLL and human peripheral blood T cells, it is possible that a subset of such apparently unique "CLL" responses to PDE4 inhibitors are in fact B lineage-specific responses. To clarify at what level of cAMP signal transduction CLL cells differ from other lymphoid cell types and to establish definitively whether CLL cells in fact differ in their response to PDE4 inhibitors from B lineage cells, we carried out a set of studies contrasting the responses of primary CLL, T and B cells to PDE inhibitors at the level of adenylate cyclase expression, cAMP accumulation, ATF-1/CREB Ser 63/133 phosphorylation and gene expression. Our results demonstrate that in some important respects, B lineage cells differ from T lineage cells in their response to PDE4 inhibitors. In

addition, however, CLL cells remain unique in their transcriptional and apoptotic response to this class of drug.

Methods and Materials

Materials

The following reagents were obtained from commercial sources: Rp-8-Br-cAMPS (Biolog Life Science Institute, Bremen, Germany), rolipram (Biomol, Plymouth Meeting PA), forskolin (Sigma, St. Louis MO), IBMX (Sigma), dipyridamole (Sigma), cilostamide (Sigma), DMSO (Sigma), BRL50481 (Tocris, Ellisville MO).

Cell Culture and Isolation

Blood samples were obtained in heparinized tubes with IRB-approved consent from flow cytometry-confirmed B-CLL patients that were either untreated or for whom at least 1 month had elapsed since chemotherapy. Patients with active infections or other serious medical conditions or with white blood cell counts of less than 15,000/μl by automated analysis were excluded from this study. Whole blood was layered on Ficoll-Hypaque (Sigma) and peripheral blood mononuclear cells (PBMC) isolated after centrifugation. PBMC were washed and resuspended in complete media [RPMI-1640 (Mediatech) supplemented with 10% fetal bovine serum (Sigma), 20mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (Mediatech)]. PBMC was found to contain >90% $CD19^+CD5^+$ B-CLL by FACS without additional purification. Normal peripheral B and T cells were obtained from leukopacks (New York Biologics, Southampton NY) or healthy donors, PBMC isolated as above and then subjected to magnetic negative selection per the manufacturer's protocol (Miltenyi, Bergisch-Gladbach Germany) from PBMC. In some cases, normal B cells were obtained with IRB approval from discarded tonsillar tissue from patients undergoing routine tonsillectomy and processed as above after creation of a single cell suspension by mechanical disruption.

Quantitative real-time RT-PCR

Cells were plated at a concentration of 2×10^7 cells/ml in complete media with or without drug treatment as indicated and incubated at 37°C for four hours. Total RNA was obtained using the RiboPure isolation kit (Ambion, Austin TX) per the manufacturer's protocol. RNA purity and quality was determined using both the NanoDrop spectrophotometer (NanoDrop, Wilmington DE) and the Agilent 2100 Bioanalyzer (Agilent, Santa Clara CA). Reverse transcription of 2-5 micrograms of total RNA was carried out with the Superscript III reverse transcription kit (Invitrogen, Carlsbad California) primed with random hexamers. cDNA was diluted 1:50 and qPCR reactions were carried out using Universal PCR Master Mix for TaqMan assays or PowerSYBR for those using SYBR Green chemistry (Applied Biosystems, Foster City CA). Primer and probes were designed with Primer3 (23). Pre-developed Taqman assay reagents (PDAR) for measuring RPLP0 (Applied Biosystems) was used for normalization. Data were expressed relative to an RNA standard made from total white blood cells of a healthy donor or to a commercial human brain RNA standard (Clontech, Mountain View CA). Realtime PCR was carried out on a MX300P instrument (Stratagene, La Jolla CA). All oligonucleotides were purchased from IDT. Primer/probe sets for TaqMan assays were as follows: **ICER-I** Forward - GAGTTGTCTGGCCAGCTTAG, Reverse - CCATCACCACTCCCTGTG, Probe - [FAM]/TGCTGCCACTGGTGACATGC/[TAMRA]; **DUSP4** Forward - TCCCAGTGGAAGATAACCAC, Reverse - TCGATGTACTCTATGGCTTCC, Probe - [FAM]/AGGCCGACATCAGCTCCTGG/[TAMRA]. Primers for SYBR Green assays were as follows: **PDE3B** Forward - TTTATGAGCAGGGAGATGAA, Reverse - CAGCAGCATCATAGGAGTTA; primers for adenylate cyclase isoforms were as previously reported (24). Reaction products were checked for specificity and size using the Agilent 2100 Bioanalyzer according to the manufacturer's protocol.

Affymetrix GeneChip hybridization and expression set generation

Using a poly-dT primer incorporating a T7 promoter, double-stranded cDNA was synthesized from 2 μg total RNA using a double-stranded cDNA synthesis kit (Invitrogen, Carlsbad, CA). Double-stranded cDNA was purified with the Affymetrix sample cleanup module (Affymetrix, Santa Clara, CA). Biotin-labeled cRNA was generated from the double-stranded cDNA template though in-vitro transcription with T7 polymerase, and a nucleotide mix containing biotinylated UTP (3′-Amplification Reagents for IVT Labeling Kit; Affymetrix). The biotinylated cRNA was purified using the Affymetrix sample cleanup module. For each sample, 20 μg of IVT product was digested with fragmentation buffer (Affymetrix, Santa Clara CA) for 35 minutes at 94°C, to an average size of 35 to 200 bases. 15 μg of each fragmented, biotinylated cRNA, along with hybridization controls (Affymetrix), was hybridized to a Human U133 2.0 plus GeneChip for 16 hours at 45 °C and 60 rpm. Arrays were washed and stained according to the standard Antibody Amplification for Eukaryotic Targets protocol (Affymetrix). The stained arrays were scanned at 532 nm using an Affymetrix GeneChip Scanner 3000. The Bioconductor (http://www.bioconductor.org) Affy and Simpleaffy packages for the R statistical environment were used for preprocessing and quality control at the probe level. Arrays were checked by M-A plotting, average background, measures of central tendency and dispersion, and RNA degradation. Expression set data were generated using the Robust Multichip Average (RMA) per the method of Choe *et al.* (25). Probe sets were filtered for minimum intensity of 100 units, interquartile range of > 0.5, and MAS5 present/absent calls. Expression summaries and raw probe-level data were submitted to the NCBI Gene Expression Omnibus (26) under accession number GSE13987.

Microarray statistical analysis and clustering

To assess for differentially expressed genes within each cell type (CLL, normal B, normal T), rolipram treated conditions were compared to DMSO-only vehicle controls using a paired sample CyberT test as previously described (27). To determine if rolipram-induced transcriptional changes varied between cell types, probe sets were tested for rolipram effect via a full factorial mixed effect model (donor, cell type, rolipram treatment, celltype*rolipram) using the F_s-test of Cui *et al* (28). Permutated *p* values of the rolipram effect were adjusted for multiple hypothesis testing using the Qvalue package for R (28). Probe sets with $Q < 0.05$ (614 probe sets) for the rolipram effect were called differentially regulated in all cell types and subjected to hierarchical clustering of genes using the GenePattern software suite (29) with default parameters. Gene lists from each cluster were subsequently imported into Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City CA) for connection analysis. Principal Component Analysis (PCA) was performed using R on both the entire probeset collection and on those probesets identified as significant for the rolipram effect.

Western analysis

Following cell culture, cells were washed with cold PBS and lysed in a 1% Triton-X100 based buffer as previously described (22). Concentrations of soluble proteins in samples of clarified supernatants were determined using Bradford assays. Samples were heat denatured at 100°C for 5 minutes in protein denaturing sample buffer and 50 μg aliquots of denatured protein samples were then subjected to electrophoretic separation through 8-10% SDS-polyacrylamide gels followed by electrotransfer onto Immobilon-P membrane (Millipore). Primary antibodies against PDE3B (Santa Cruz Biotechnology, Santa Cruz CA) diluted 1:500, phospho-CREB/ATF-1 (BD Biosciences) diluted 1:1000, or total CREB (Cell Signaling Technology, Danvers MA) diluted 1:1000 were incubated with the membrane overnight at 4°C. Secondary goat anti-rabbit IgG or rabbit anti-mouse IgG conjugated to horse radish peroxidase (Santa Cruz Biotechnology) were diluted at 1:5000 and incubated for 2 hours at room temperature. Immunocomplexes with HRP activity on membranes were developed using enhanced

chemiluminescent reagent (Pierce) as substrate and imaged on a Kodak ImageStation 4000M (Carestream, New Haven CT). Membranes were blotted with primary monoclonal anti-β-actin (Sigma) to control for equal loading of protein samples on gels and transfer onto membranes.

Phospho-ATF-1/CREB analysis

Phospho-ATF-1/CREB analysis was performed using a protocol modified from Krutzik *et al* (30). Briefly, cells were plated at a concentration of 2×10^6 cells/ml and treated with drugs as indicated. After treatment, cells were rapidly fixed with the addition of paraformaldehyde to a final concentration of 0.5% and incubated for 10 minutes at 37°C, washed in FACS buffer (PBS containing 0.09% NaN_3 and 0.5% BSA), and stained with anti-CD19-PE-Cy5, anti-CD5-PE, and/or anti-CD3-PE (BD Biosciences) for 30 minutes at room temperature. Negative populations were defined by staining with appropriate isotype control antibodies. Cells were then fixed again in 1.6% paraformaldehyde prior to being permeabilized with ice cold ethanol for 15 minutes on ice. After thorough washing, cells were stained with anti-ATF-1/CREB (pS63/133)-Alexa488 (BD, Franklin Lakes NJ). Cells were then washed, and analyzed on a LSR-II flow cytometer (BD). Data were analyzed using FlowJo version 8 (Tree Star, Ashland OR).

Apoptosis assays

PBMC obtained from B-CLL patients or healthy donors were plated at a concentration of one million cells per well, and treated with drug or vehicle as indicated. Cells were incubated for approximately 48 hours at 37°C with 5% CO_2. Cells were harvested, and $DiOC_6(3)$ Molecular Probes (Eugene, OR) was added to a final concentration of 0.1 μM. After incubation at 37°C for 30 minutes, cells were washed once in FACS buffer and incubated with anti-CD19-PE-Cy5, anti-CD5-PE, and/or anti-CD3-PE (BD Biosciences) for 30 minutes on ice. Cells were washed again and analyzed on a LSR-II flow cytometer. Data were analyzed using FlowJo version 8. Comparison analysis demonstrated that this method yields comparable results to Annexin/PI staining in B-CLL cells (data not shown).

Cyclic AMP measurement

Cells were plated at a concentration of 1×10^6 cells/ml and treated with drugs as indicated. After culture, cells were lysed with ice-cold ethanol and extracts concentrated to near dryness by SpeedVac vacuum centrifugation (Savant). The cAMP concentration of extracts diluted into assay buffer was determined by radio immunoassay (RIA) per the manufacturer's instructions using the non-acetylated protocol (PerkinElmer Life Sciences). Results were normalized to exact cell concentration as determined by machine count (Nexcelom Bioscience). Due to a high degree of variability between donors, data from drug treated samples was expressed relative to the DMSO-only vehicle control of the same donor.

Statistical Analysis

Statistical analysis and plotting was performed using Prism version 4 (GraphPad Software, San Diego CA) and SPSS version 12 (SPSS Inc., Chicago, Illinois). The significance of main and interaction effects was determined via repeated measures analysis of variance (ANOVA) tests with significance of subsequent pair-wise comparisons via Bonferroni post-hoc tests. Microarray data were primary processed in the R statistical environment (http://www.rproject.org) as described above.

Results

B-CLL cells show a strong rolipram effect at the global transcriptional level

To assess transcriptional responses to PDE4 inhibition, four CLL, four peripheral blood B cell and four peripheral blood T cell samples were incubated for four hours with 20 μM rolipram or a DMSO-only vehicle control, followed by RNA isolation and Affymetrix gene chip analysis. To better understand how rolipram affects the transcriptional response at a global level in pooled data from all three cell types, we subjected all 54,675 probesets per sample to Principal Component Analysis (PCA). While all samples separated by cell type, without imposing any statistical constraints of the effect of rolipram treatment B-CLL uniquely showed a distinct separation between control and rolipram treated cells. (Figure 1A). To identify genes that were differentially regulated by rolipram treatment globally amongst all three cell types, we performed a mixed model ANOVA on the Affymetrix expression profiles. Probesets with $Q < 0.05$ for the rolipram effect were considered statistically significant (614 genes). No probesets showed a significant drug treatment*cell type interaction effect. A second PCA constrained to significant probesets for drug treatment was performed to assess the similarity of rolipram-induced changes between B-CLL and normal B and T cells. In this case, B-CLL cells demonstrated a very large separation between control and treated cells, while normal B and T cells both demonstrated a far less clear separation (Figure 1B). Since the B-CLL samples demonstrated such a strong rolipram effect *a priori*, we analyzed this subset for rolipram-induced differential gene expression independently and found 1245 genes regulated by rolipram exclusively in the B-CLL subset. To look for enrichment of gene function in this subset, we subjected the dataset to the Ingenuity Pathway Analysis application. A group of 165 genes identified as involved in apoptosis was identified as the most enriched list of this subset ($P < 0.0001$). Together, these data suggest that while rolipram exerts a transcriptional response on both normal and CLL cells, B-CLL cells exhibit a distinct and larger response compared to normal B and T cells.

Rolipram treatment of B-CLL cells results in a unique gene expression pattern characterized by high fold-changes—Genes with $Q < 0.05$ for the rolipram effect across all cell types were subjected to hierarchical clustering to identify specific transcriptional patterns associated with rolipram treatment. CLL cells exhibited a distinct transcriptional response pattern when contrasted with normal B or T cells. The CLL response was distinguished by a large cluster of genes that were expressed at markedly lower levels than normal cells under basal conditions and that rose to fairly high levels when treated with rolipram (cluster 2; Figure 2, Table 1S). The low starting level resulted in a large fold-change of these genes in response to rolipram in CLL cells when compared to normal cells. The converse was also observed in several clusters where genes were more highly expressed in CLL cells and were down-regulated by rolipram, also resulting in a large fold-change.

To determine if any of the identified clusters contained a cAMP signature, gene lists were imported into the Ingenuity Pathway Analysis system and connections culled from peer-reviewed literature between genes in the cluster and cAMP or cAMP-regulated transcriptions factors CREB, CREM, and ATF-1. Cluster 2 showed the highest number of such connections (15 connections from 126 cluster 2 genes) suggesting that it bears a particularly strong cyclic AMP signature, including genes reported to be regulated by cAMP or its downstream transcription factors or genes reported to induce feedback regulation of cAMP signaling (Figure 3). Cluster 2 genes previously reported to be up-regulated following cAMP signaling included amphiregulin, CREM, CYP51, DUSP1, FOSL2, NR3C1, NR4A2, NR4A3, PDE4, PER1, RGS1 and SOCS3 (15,31-37). While these genes have been reported to be cAMP-regulated, including NR3C1 (the glucocorticoid receptor) that is selectively up-regulated in a cAMP-dependent manner in PDE4 inhibitor-treated CLL cells (22), many of the genes in this cluster

have no such published observations. When one such gene, the dual specificity phosphatase DUSP4 was analyzed (see results and further analysis below), we confirmed that DUSP4 was induced by rolipram treatment in a cAMP-dependent manner. Together these data suggest that this cluster of genes, characterized in CLL by extremely low basal expression with a large fold induction upon rolipram treatment, represents a pool of genes whose expression is regulated by cyclic AMP signaling.

B and T lineage cells exhibit differential cAMP responses to PDE4 inhibition— As one of the primary mechanisms by which PKA regulates gene expression is through phosphorylation of the transcription factors CREB and ATF-1, we next assessed such phosphorylation following rolipram treatment of the three purified lymphoid cell populations. To avoid the possibility that manipulation of the cells might alter cAMP signaling, we chose to perform these assays using phospho-flow cytometry with a monoclonal antibody capable of detecting both phosphorylated ATF-1 (Ser63) and CREB (Ser133). This technique has the advantage that cells need not be purified as they can be identified by surface immunophenotyping and their *bona fide* phosphorylation state preserved by the addition of paraformaldehyde to the cultures directly. When the technique was applied to PBMC from a CLL patient, we found that the $CD19^{+}CD5^{+}$ CLL population responded to one hour of rolipram treatment by increasing ATF-1/CREB Ser 63/133 phosphorylation, while the $CD19^{-}CD5^{+}$ T cell population of the same sample failed to show an appreciable difference in such phosphorylation (Figure 4A). In PBMC isolated from a normal donor, the $CD19^{+}CD3^{-}$ normal B cell population responded to rolipram treatment with an increase in ATF-1/CREB phosphorylation while the $CD19^{-}CD3^{+}$ normal T cell population showed only a modest increase in phosphorylation. These studies suggest that, at least as judged by ATF-1/CREB phosphorylation, PDE4 inhibitor treatment induced a significantly greater degree of cAMP signaling in B lineage than in T lineage cells.

To validate the specificity of this assay, protein extracts of CLL cells treated for various lengths of time with rolipram were subjected to western blotting using the identical anti-ATF-1/CREB Ser 63/133 antibody used in the FACS assays. Such Western analysis showed robust rolipram-induced ATF-1/CREB phosphorylation in CLL cells, low levels of rolipram-induced phosphorylation in primary human T cells, and high basal levels of CREB phosphorylation in primary B cells with little change after rolipram treatment. As the phospho-FACS analysis was done on “untouched” B cells while the Western analysis was done on purified B cells, we were concerned that the process of purification might have raised basal levels of B cell ATF-1/CREB phosphorylation. Phospho-FACS analysis of “untouched” and purified B cells confirmed that the process of purification raises levels of basal B cell ATF-1/CREB phosphorylation (data not shown).

The induction of ATF-1/CREB Ser 63/133 phosphorylation as judged by phospho-flow cytometry in rolipram treated B-CLL cells was detectable within 5 minutes and was maintained for at least 4 hours. Furthermore, when CLL cells were pretreated with Rp-8-Br-cAMPS, an enantiomeric PKA antagonist, rolipram-induced ATF-1/CREB phosphorylation was markedly reduced, confirming that rolipram-induced up-regulation of phospho-ATF-1/CREB is mediated by cAMP signaling through PKA (Figure 5A). While rolipram was not sufficient to induce ATF-1/CREB phosphorylation in normal T cells, treatment with isobutylmethylxanthine (IBMX, 50 μg/ml), a PDE inhibitor capable of inhibiting PDEs derived from all 11 families except PDE8 and PDE9, was effective in inducing T cell ATF-1/CREB phosphorylation (Figure 5B).

Given the differential ATF-1/CREB phosphorylation response of T and B lineage cells to PDE4 inhibition, we next sought to determine whether such differences were also reflected in accumulation of intracellular cAMP. When total cAMP levels were measured in the three

populations following a time course of rolipram treatment, the results mirrored those obtained with ATF-1/CREB phosphorylation FACS analyses, with rolipram inducing rapid and sustained cAMP production in both B-CLL and normal B cells but not in normal T cells (Figure 5C). In both the case of ATF-1/CREB phosphorylation and cAMP production, addition of the adenylate cyclase activator forskolin to rolipram proved sufficient to stimulate T cells. Altogether, these data suggest that a large pool of cAMP capable of regulating ATF-1/CREB phosphorylation is largely under the control of PDE4 in B lineage cells, whilst in T cells additional PDEs may play a redundant role that can be overcome through either additional PDE inhibition or by increasing cAMP production.

PDE3B plays a redundant role with PDE4 in T cells but not B cells—To attempt to determine which PDE(s) might act in concert with PDE4 in T lineage cells, peripheral T cells purified from healthy donors were treated for 1 hour with inhibitors of PDE3 (cilostamide, 10 μM), PDE7 (BRL50481, 30 μM), or PDE8 (dipyridamole, 100 μM), alone or in combination with a PDE4 inhibitor (rolipram, 20 μM), followed by assessment of ATF-1/CREB phosphorylation by phospho-flow cytometry. While neither a PDE3 nor a PDE4 inhibitor alone induced phosphorylation, the combination of cilostamide and rolipram yielded ATF-1/CREB phosphorylation nearly comparable to that observed following treatment with IBMX (Figure 6A). Furthermore, when the total microarray data were subjected to unsupervised clustering that separated samples into CLL, normal B, and normal T groups, PDE3B expression was identified *a priori* as highly associated with the normal T cell grouping by marker analysis (data not shown). To validate the microarray data, we performed quantitative real-time RT-PCR that confirmed that T cells express approximately seven times more PDE3B transcript than B-CLL or normal tonsillar B cells (Figure 6B). Western analysis confirmed the selective expression of PDE3B in T lineage cells with easily detectable immunoreactive protein present in normal T cells while the protein was not detected in B-CLL or normal tonsillar B cells (Figure 6C).

B-CLL cells express lower basal levels of rolipram-sensitive genes and the rolipram-induced upregulation appears to be PKA mediated—The Affymetrix analysis identified the cAMP-sensitive transcriptional activator CREM as a strongly downregulated gene under basal conditions in B-CLL yet highly rolipram-sensitive. However, CREM is a complex gene with many transcriptional variants, including short repressor forms (ICER) known to be induced by cAMP (19). Given that the studies above had demonstrated that PDE4 inhibitor treatment leads to a highly non-physiologic prolonged rise in intracellular cAMP levels, we hypothesized that the CREM signal obtained from the Affymetrix analysis was the ICER form of CREM, induced as an apparent compensatory negative feedback loop. To validate the Affymetrix study, we designed real-time RT-PCR assays to measure ICER and DUSP4, a second gene identified as downregulated in B-CLL and sensitive to rolipram-induced up-regulation yet with no published reports as being cAMP-regulated. As expected, B-CLL cells contained significantly less of both transcripts under basal conditions than normal tonsillar B cells or T cells (Figure 7A/B). Rolipram treatment up-regulated ICER and DUSP4 transcript levels in all three lymphoid populations. Pretreatment of the B-CLL cells with Rp-8-Br-cAMPS, an enantiomeric PKA antagonist, reduced the rolipram-induced upregulation of these transcripts by roughly half, suggesting that such up-regulation occurs by a PKA-dependent pathway.

As previously noted, a separate analysis of rolipram-regulated transcripts in CLL cells demonstrated enrichment for apoptosis-associated genes. Unlike those genes identified in cluster 2, this set of CLL-specific apoptotic genes does not necessarily exhibit low basal expression in CLL cells. One such gene, BIM, has been implicated in cAMP-mediated apoptosis of lymphoid cell lines (20). We confirmed by real-time RT-PCR that BIM was significantly up-regulated in B-CLL cells treated with rolipram, and that this up-regulation

could be reversed with Rp-8-Br-cAMPS (Figure 7C). Of note, while the qPCR data confirmed that there was no statistical difference in BIM expression under basal conditions between CLL, normal B, and normal T cells, it also demonstrated significant up-regulation of Bim expression in normal B and T cells.

Finally, the qPCR data also demonstrated that when normal peripheral T cells were treated with the combined PDE3 and PDE4 inhibitors, despite robust ATF-1/CREB phosphorylation, no further augmentation of these rolipram-sensitive transcripts was seen compared to culture with rolipram alone.

Inhibition of PDE3 and 4 does not induce T cell apoptosis

Given the unusual sensitivity of CLL cells to PDE4 inhibitor-induced apoptosis, we questioned if the differences between rolipram-induced ATF-1/CREB phosphorylation and cAMP production amongst the three lymphoid populations would be reflected in the degree to which the cells undergo PDE4 inhibitor-induced apoptosis (13). As assessed by immunophenotyping in combination with measurement of mitochondrial depolarization, within the leukemic PBMC, only the $CD19^{+}CD5^{+}$ B-CLL cells underwent appreciable rolipram-induced apoptosis, while the $CD19^{-}CD5^{+}$ T cells from the same samples failed to show any induction of apoptosis (Figure 8). In contrast to B-CLL cells, $CD19^{+}CD3^{-}$ B cells from a healthy donor also failed to exhibit any appreciable rolipram-induced augmentation of apoptosis above and beyond the high degree of basal apoptosis typically observed in cultured primary B cells (38). Since combined inhibition of PDE3 and PDE4 was required for robust ATF-1/CREB phosphorylation in normal T cells, we also assayed apoptosis of PBMC treated with this PDE inhibitor combination. While the addition of a PDE3 inhibitor to rolipram results in ATF-1/CREB phosphorylation in normal T cells, no significant increase in apoptosis was observed in T cells from leukemic or healthy individuals following such combined therapy. These data suggest that ATF-1/CREB phosphorylation alone is not sufficient to induce apoptosis following PDE inhibition.

Neither adenylate cyclase nor soluble factors appear to account for B lineage sensitivity to PDE4 inhibitors—The results described above in which addition of forskolin restored T cell ATF-1/CREB phosphorylation in response to PDE4 inhibition suggested that T lineage cells have less “flux sensitivity” to PDE4 inhibitors as a result of reduced GPCR signaling or differential adenylate cyclase isoform expression. To determine whether the unique expression of a specific adenylate cyclase isoform might account for B lineage flux sensitivity, we assayed the relative amount of each of the eight known adenylate cyclase (AC) isoforms in B-CLL, normal B and normal T cells by real-time RT-PCR. While in agreement with prior studies, AC7 was shown to be the predominate isoform in lymphoid cells, no appreciable differences in AC expression between cell types were noted (Figure 9A) (39).

As differential stimulation of GPCRs by ligands present in media or produced in an autocrine fashion could account for the apparent B lineage “flux sensitivity” to PDE4 inhibitors, we cultured B-CLL cells in serum-free media overnight, or washed the cells into fresh media 30 minutes prior to the addition of rolipram. In either case, the resulting ATF-1/CREB phosphorylation was nearly identical in magnitude to that of cells cultured under normal conditions with 10% FCS (Figure 9B). These data suggest that differences in the expression of AC isoforms or GPCR-mediated responses to factors present in media do not explain the sensitivity to PDE4 inhibitors exhibited exclusively by B-lineage cells.

Discussion

In this study, we set out to determine why CLL cells differ from T cells with regard to their sensitivity to apoptosis induced by PDE4 inhibitors. While our studies have not succeeded in

identifying the precise reason for such differences in apoptotic sensitivity, they have identified several novel ways in which CLL cells differ from T cells in their response to PDE4 inhibition, some of which are B lineage specific and some of which are unique to CLL.

B-CLL cells appear to exhibit a strong rolipram-induced transcriptional response compared to normal human lymphoid cells as suggested by a distinct separation in the absence of any statistical constraint by PCA analysis. When the PCA was performed only on genes classified as significant for differential expression based on rolipram treatment, the separation distance between control and rolipram-treated B-CLL cells dramatically increased while normal B and T cells continued to exhibit a much more variable pattern of expression. The excessive noise in normal cell expression profiles accounts for the failure to observe any statistically significant interaction effect between the rolipram treatment and cell type terms in a mixed model ANOVA. However, a failure to obtain significant interaction effects does not suggest that B-CLL samples do not exhibit rolipram-induced regulation of a distinct subset of genes. Supporting this hypothesis, when the CLL samples alone are analyzed for differential gene expression with a paired sample Baysean t-test, an additional 1245 genes are found to be rolipram regulated. Interestingly, when these genes are subjected to a functional analysis, the subset was found to be highly enriched for genes involved in apoptosis. This finding is further functionally supported by data suggesting that only B-CLL undergo rolipram-induced apoptosis despite the induction of ATF-1/CREB phosphorylation in normal B cells by rolipram and in T cells by rolipram and cilostamide. Even when the subset of genes found to be rolipram-sensitive across B-CLL, normal B, and normal T cells are analyzed by clustering analysis, the B-CLL samples exhibit a distinct expression pattern categorized by large fold differences in basal expression. Moreover, the cluster of such genes that exhibit dramatically lower basal levels in B-CLL cells appears to be truly cAMP regulated as supported by qPCR studies using a PKA antagonist. Coupled with the observation that B-CLL exhibit a much larger transcriptional response in terms of the number of rolipram-regulated genes, as well as their sensitivity to cAMP-mediated apoptosis, it is possible that B-CLL cells evolved a survival strategy of tightly controlling cAMP signaling accounting for the low basal levels of these apparently cAMP-regulated genes.

Treatment of lymphoid cells with PDE4 inhibitors creates a highly non-physiologic environment in which the compensatory effects of two of the primary mechanisms by which cells adapt to strong tonic cAMP signals are abrogated: PKA-mediated up-regulation of PDE4 activity by activating phosphorylation and ATF-1/CREB-mediated transcriptional up-regulation of PDE4A long forms and PDE4B short isoforms (40). While physiologic GPCR-mediated signals result in either no detectable rise or a transitory elevation in bulk cAMP levels, we detected prolonged elevated cAMP levels in B lineage but not T lineage cells treated with rolipram. Not surprisingly, many of the genes up-regulated following such treatment have previously been identified as regulated by cAMP signaling and in some cases, part of compensatory negative feedback loops that would under normal conditions serve to down-regulate GPCR-mediated cAMP signaling. For example, we observed up-regulation of cAMP phosphodiesterases (PDE4B, PDE4A), RGS proteins that uncouple GPCR-mediated activation of AC (RGS1, RGS2) and two transcriptional repressor ICER splice isoforms of CREM.

The unusually robust up-regulation of ICER in CLL cells is of particular interest given prior studies implicating ICER in the regulation of cAMP-mediated apoptosis. In studies of IPC-81 myeloid leukemia cells which undergo apoptosis within 6-8 hours of treatment with prostaglandin E1 or other agents that raise cAMP levels, ICER was induced by PGE1 treatment but was actually protective against cAMP-induced apoptosis if cells were transfected with ICER prior to challenge with agents that induced cAMP signaling (41). In contrast, ICER has been suggested to be responsible for angiotensin II-mediated apoptosis in cardiac myocytes as a result of a positive feedback loop in which ICER down-regulates myocyte PDE3A, with

consequent up-regulated cAMP/PKA signaling (42). Unfortunately, given the difficulty of altering gene expression in CLL cells by either transfection or siRNA, it was not possible in the current study for us to examine the role of ICER up-regulation in rolipram-mediated apoptosis.

This study demonstrates for the first time that, as judged by phospho-FACS analysis of freshly isolated cells, PDE4 inhibitor-induced ATF-1/CREB phosphorylation differs between B lineage and T lineage peripheral blood lymphocytes. While robust ATF-1/CREB Ser 63/133 phosphorylation is observed in CLL and B cells, comparable activation of T cell ATF-1/CREB phosphorylation requires the additional inhibition of PDE3B. This finding is supported by very clearly different levels of basal PDE3B expression in B and T lineage cells, with no detectable PDE3B in B lymphocytes and substantial amounts evident in bulk T lymphocytes. Our findings are consistent with a prior report by Ekholm et al in which the ratio of PDE4 to PDE3 enzymatic activity in PHA-stimulated peripheral blood cells (a predominantly T cell population), anti-CD19-rosetted B cells and EBV-transformed B cell lines was 1.1, 2.3 and 4.8, respectively (43). Interestingly, Rudensky *et al* have recently reported that PDE3B expression is suppressed by the transcription factor Foxp3 in murine regulatory T cells. It will be of interest to determine whether a comparable process occurs in human regulatory T cells and, if so, whether this renders this T cell subset unusually sensitive to PDE4 inhibitors (44).

Despite the identification of a means by which to induce robust ATF-1/CREB phosphorylation in T lineage cells by combined PDE3 and 4 inhibition, the induction of such phosphorylation in T cells does not alter their insensitivity to PDE inhibitor-mediated apoptosis. This result suggests that the insensitivity of mature T lymphocytes to cAMP-mediated apoptosis is unlikely to lie in a fundamental difference in the cAMP signal transduction pathway that reaches from the cell membrane to augmented CRE-mediated gene transcription. Consistent with this, despite the absence of marked changes in ATF-1/CREB phosphorylation in T lineage cells following PDE4 inhibition, a large number of genes do undergo transcriptional changes in such cells, albeit often at a lower fold-change than CLL cells. The most likely explanation for this finding is that a minor proportion of total cellular ATF-1/CREB undergoes phosphorylation in T lineage cells following PDE4 inhibition but that this degree of ATF-1/CREB signaling is sufficient for regulation of gene expression. The robust augmentation of ATF-1/CREB phosphorylation observed in T cells following combined PDE3/4 inhibition may then reflect a non-physiologic condition in which there has been a loss of the documented ability of PDEs to restrict cyclic nucleotide signaling to specific subcellular domains.

As our studies do identify a number of rolipram-regulated genes that are unique to CLL cells, these results do not rule out the potential contribution of cAMP-induced “death gene” transcription to rolipram-induced CLL apoptosis. As an example, Bim, a well-known pro-apoptotic Bcl2 family member identified as a potentially important cAMP-mediated “death gene” in the S49 murine T cell lymphoma model was up-regulated 2.3-fold in rolipram-treated CLL cells when Affymetrix data were analyzed for each separate lineage but was not significantly altered in B or T cells (20). However, while realtime PCR analysis confirmed up-regulation of Bim in CLL cells following rolipram treatment, up-regulation was also observed in normal B and T cells, suggesting that transcriptional up-regulation of this gene is unlikely to account for the unusual apoptotic sensitivity of CLL cells to PDE4 inhibitors.

Our prior studies have suggested that translocation of the pro-apoptotic Bcl2 family member Bcl-2antagonist of death (BAD) to mitochondria might underlie CLL apoptotic sensitivity to PDE4 inhibitors (14). More recent reports have suggested that CLL cells, in contrast to peripheral blood mononuclear cells, a largely T cell population, are uniquely sensitive to Bad and other BH3-only Bcl2 family members as a result of high basal levels of association of CLL Bcl-2 with the apoptotic activator Bim (“poised for death”) (45). Thus, given our results

demonstrating that despite comparable activation of ATF-1/CREB phosphorylation, normal T cells and B cells remain resistant to PDE inhibitor-mediated apoptosis, it is plausible that pre-sensitization of CLL cells to BH3-only signals such as BAD may contribute to their unusual sensitivity to PDE4 inhibitors.

The observed difference in B and T lineage responses to PDE4 inhibitors is of interest as a variety of studies have demonstrated that use of combined PDE3 and PDE4 inhibitors in animal models of inflammatory disease results in greater control of inflammation than treatment with either agent alone (46). In T cells, combined use of a PDE3 (CI-930) and a PDE4 inhibitor (RO20-1724) yielded greater inhibition of T cell proliferation than either agent by itself (47). Similarly, combined use of the PDE3 inhibitor SK&F 95654 and rolipram reduced PHA-induced human T cell proliferation and IL2 production to a greater degree than either drug alone (48). Such studies have served to support a growing interest in combined PDE3/PDE4 inhibitors that, as a result of the proposed synergy achieved by inhibiting these two classes of PDEs, might be therapeutically active at concentrations that are proportionally lower than those that would be required to achieve comparable clinical efficacy with either drug class alone (49).

The observation that in CLL cells, a variety of genes known to be regulated by cAMP are expressed at unusually low levels under basal conditions suggests the hypothesis that CLL cells are arrested at a developmental stage where cells are unusually sensitive to cAMP-mediated apoptosis. To circumvent this sensitivity, the malignant cells may have undergone secondary changes that reduce basal cAMP-mediated signaling. Alterations in the expression or basal activity of cAMP phosphodiesterases would be an obvious potential mechanism by which CLL cells might achieve this endpoint. Our prior studies have demonstrated that CLL cells express a constitutive 130 kD long form of PDE4A that co-migrates with PDE4A5, as well as a rolipram-inducible 64,000 kDa short inducible form of PDE4B that co-migrates with recombinant PDE4B2 (50). We hypothesize that these or other specific PDE splice isoforms play an important role in reducing basal apoptosis in CLL cells and suggest that this will be an important area for further study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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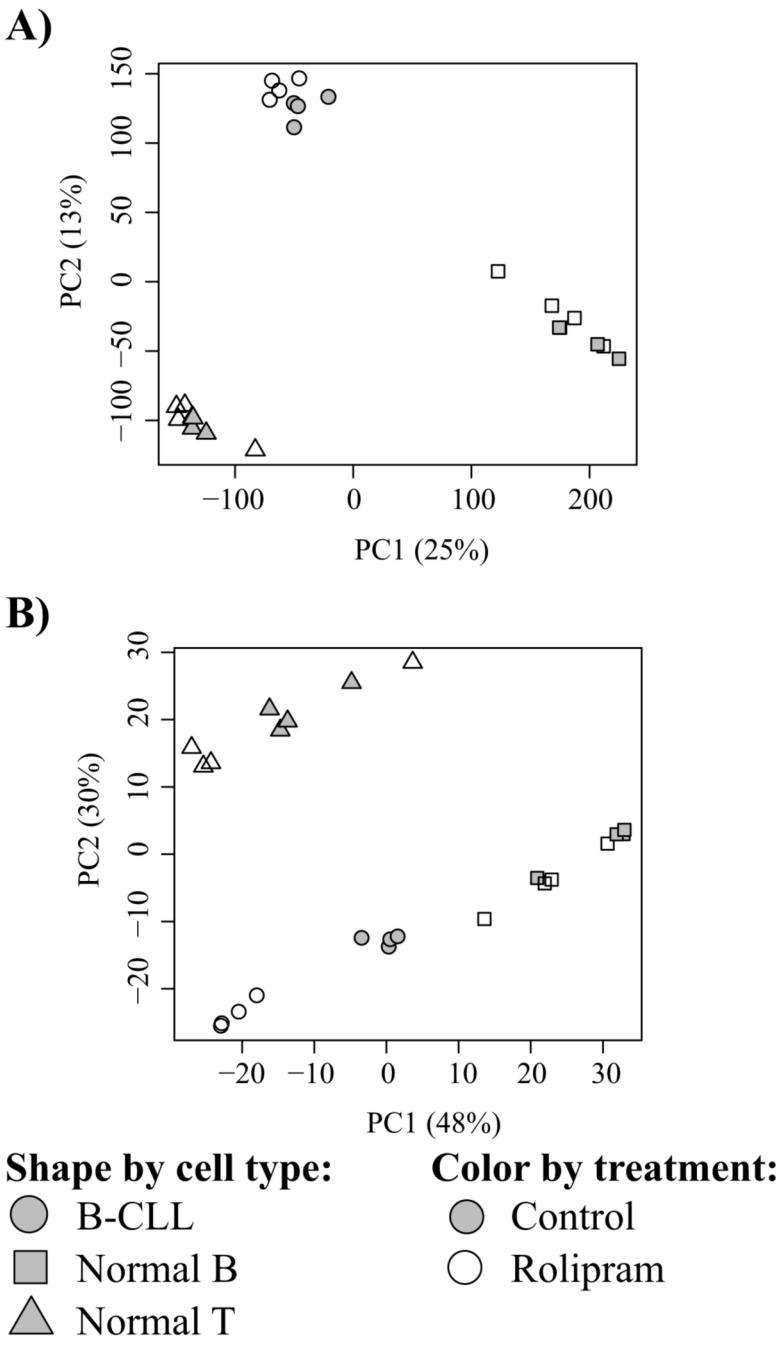

Figure 1. B-CLL cells have the strongest rolipram signature at the global transcriptional level

A) All probesets on the Affymetrix U133A Plus gene chips were subjected to Principal Component Analysis (PCA). **B)** The 614 probesets with significant rolipram effect ($Q < 0.05$ by ANOVA) were subjected to PCA. The fraction of total variation accounted for by each principal component is shown in parentheses.

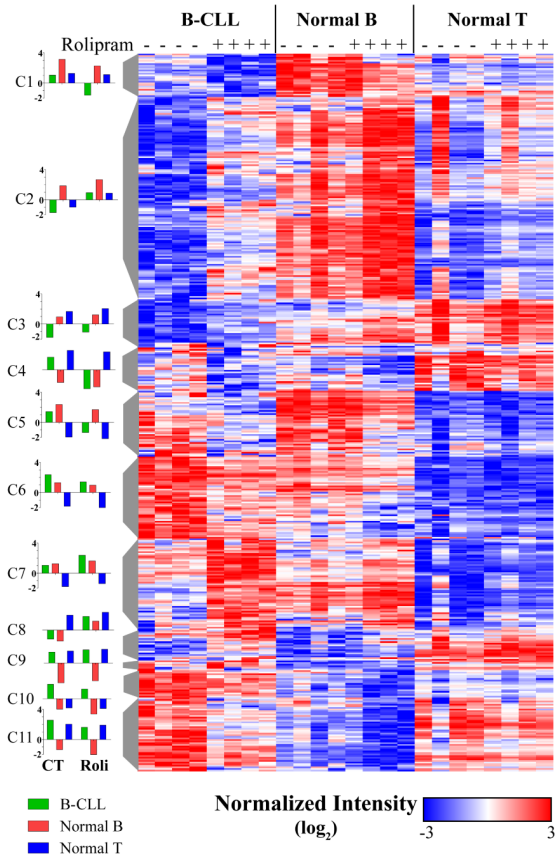

Figure 2. CLL cells exhibit a distinct rolipram transcriptional signature

CLL and normal hematopoietic cells were treated with rolipram (20 μM) or vehicle for 4 hours and RNA subsequently isolated and subjected to Affymetrix analysis. The resulting expression profiles were then analyzed by a mixed effect ANOVA, and genes with $Q < 0.05$ for rolipram effect were further analyzed by hierarchical clustering of genes. Bar graphs on the left indicate mean fold change in expression of each cell type relative to the mean intensity of the entire dataset. All graphs are of equal vertical scale from +4 to -2 fold. Samples were manually ordered.

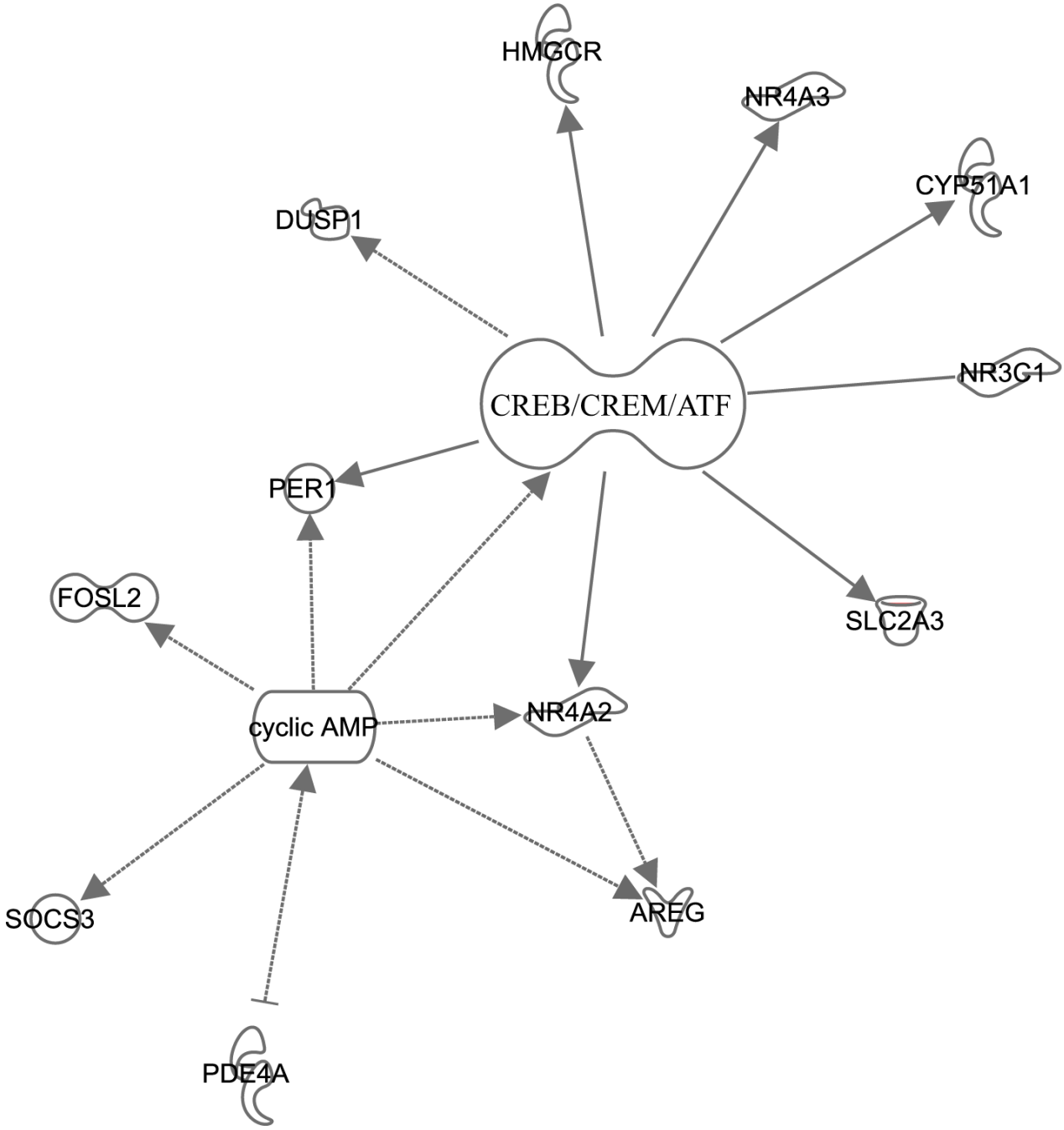

Figure 3. One subset of rolipram-sensitive genes has a cyclic AMP signature

Genes appearing in the second cluster of the rolipram-sensitive ANOVA analysis were connected using Ingenuity Pathway Analysis to cyclic AMP and the cAMP-regulated transcription factors CREM, CREB, and ATF-1. The resulting connections were manually pruned to include genes directly downstream of cAMP, CREB, CREM, or ATF-1. Abbreviations: Amphiregulin (AREG), suppressor of cytokine signaling 3 (SOCS3), dual specificity phosphatase 1 (DUSP1), period homolog 1 (PER1), HMG CoA reductase (HMGCR), orphan nuclear receptor 4A3 (NR4A3), glucose transporter 3 (SLC2A3), glucocorticoid receptor (NR3C1), cytochrome P450 family 51A polypeptide 1 (CYP51A1).

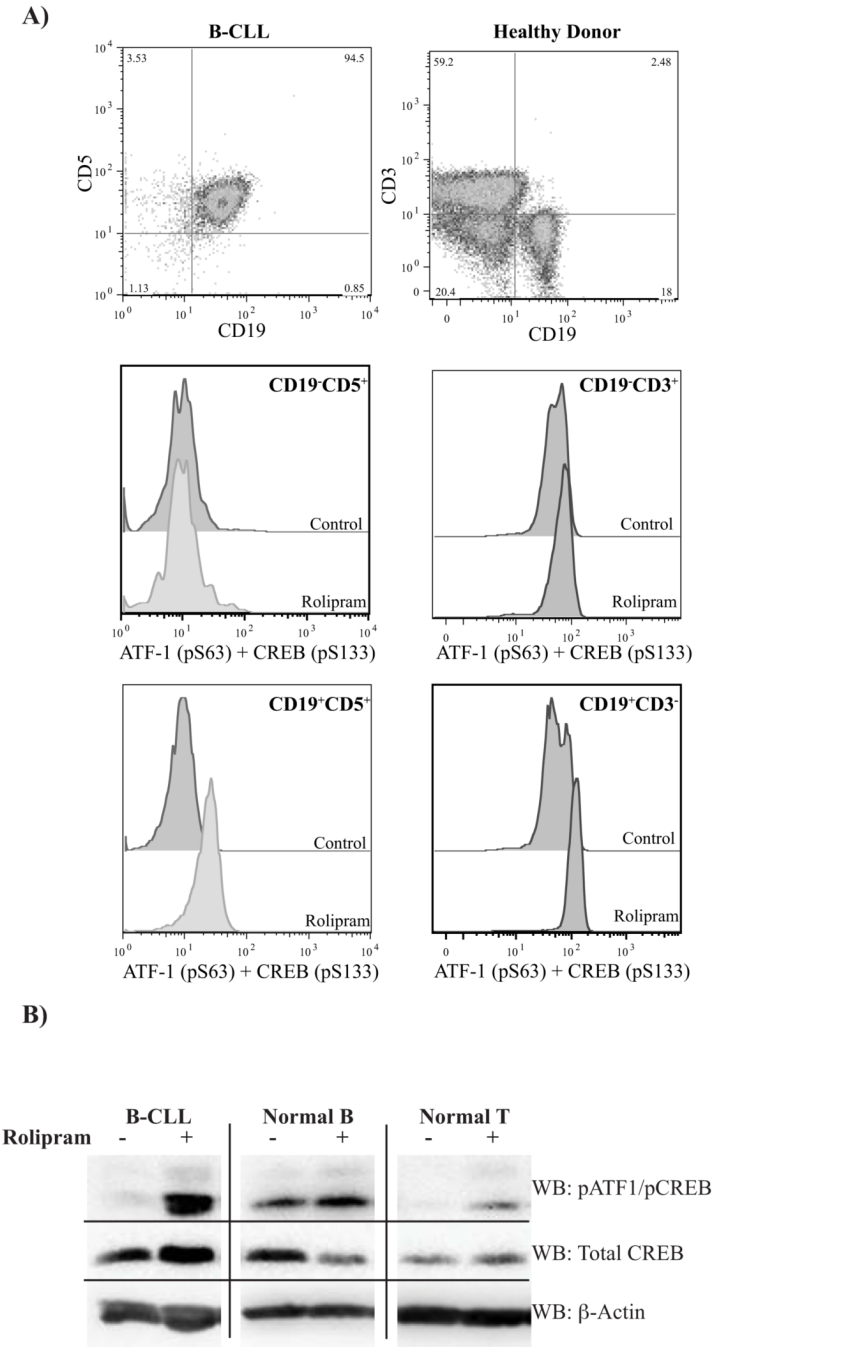

Figure 4. Rolipram treatment induces greater CREB and ATF-1 phosphorylation in B lineage cells than T cells

A) PBMC were isolated from a CLL patient or a healthy donor were treated for one hour with rolipram (20 μM) or vehicle. Cells were then fixed and immunophenotyped prior to permeabilization and intracellular staining with an anti-ATF-1/CREB (pS63/133)-Alexa488 mAb. The histograms represent cells gated on the basis of their surface phenotype. Plot is representative of three normal and leukemic donors. **B)** CLL, normal B, and normal T cells were treated for one hour with rolipram (20 μM) prior to protein extraction and western blotting using the identical anti-ATF-1/CREB (pS63/133) mAb used in (A).

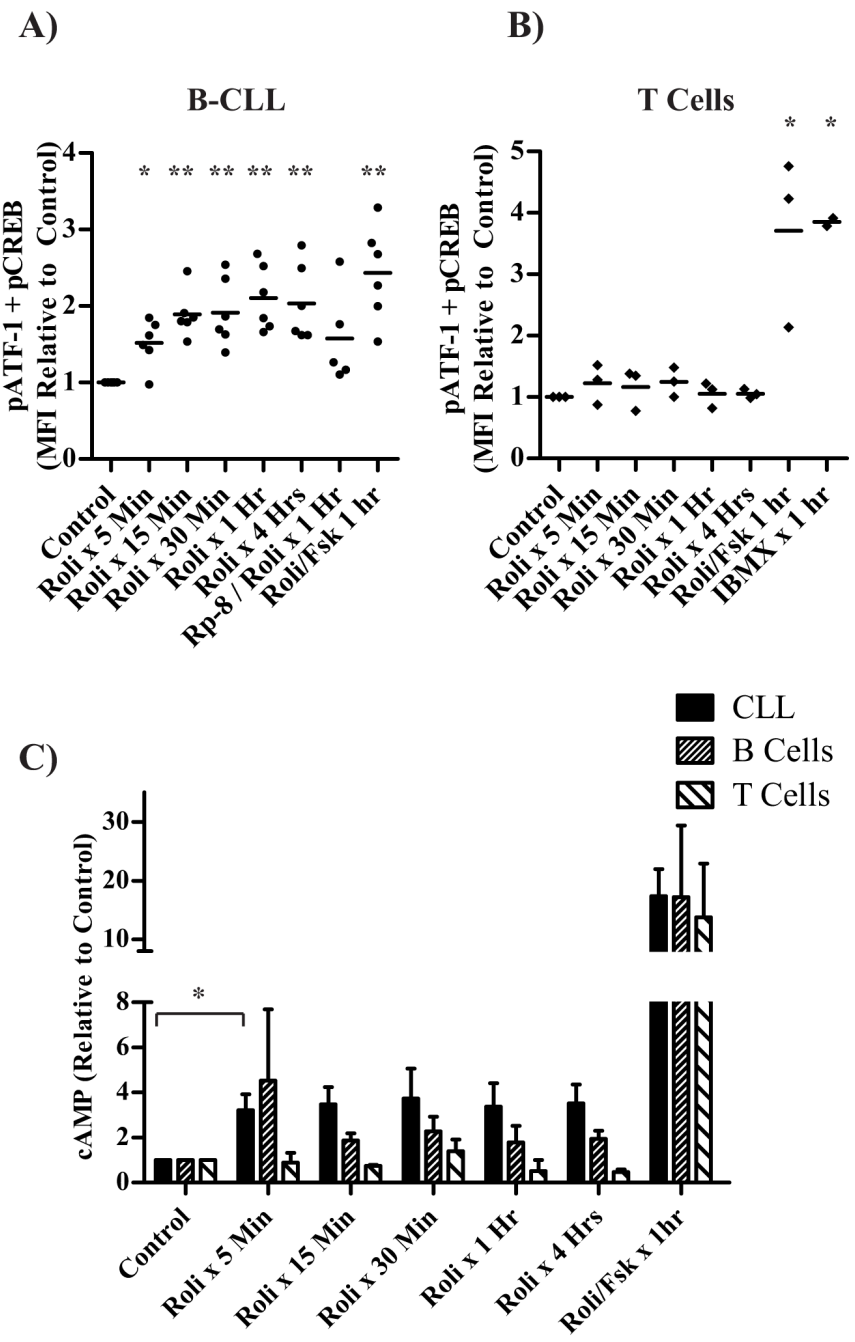

Figure 5. Rolipram induces sustained ATF-1/CREB phosphorylation and cAMP production in B but not T-lineage cells

A) B-CLL and **B)** purified normal peripheral T cells were treated for the indicated period of time with rolipram (20μM), vehicle, IBMX (50 μg/ml), or rolipram and forskolin (40 μM). Where indicated, cells were pretreated with the PKA antagonist Rp-8-Br-cAMPS (1 mM). Cells were then fixed, permeabilized, and stained with anti-ATF-1/CREB (pS63/133)-Alexa488 mAb prior to analysis by flow cytometry. The mean fluorescence intensity (MFI) of each condition was calculated and expressed relative to vehicle treated cells. **C)** B-CLL and purified normal peripheral B and T cells were treated as above prior to lysis and cAMP extraction in cold ethanol. cAMP in the extracts was measured by radio immunoassay (RIA)

and expressed relative to DMSO only treated cells vehicle controls. * = $P < 0.05$, ** = $P < 0.01$.

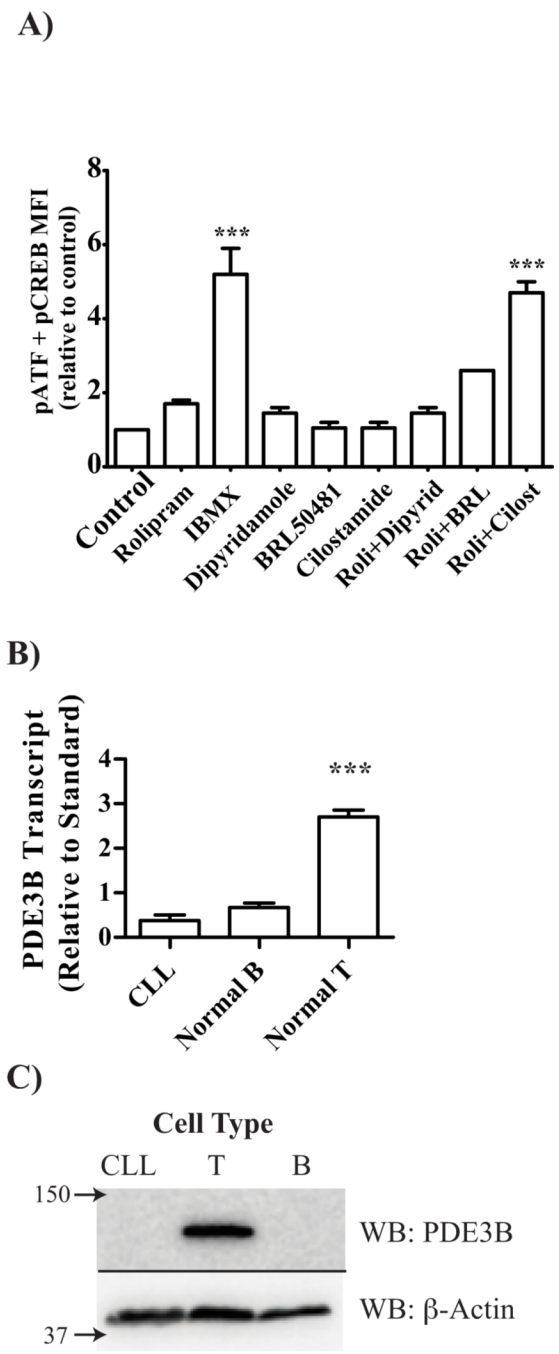

Figure 6. PDE3B is expressed selectively by T cells and acts redundantly with PDE4

A) Purified normal peripheral T cells were treated for one hour with vehicle, rolipram (20 μM), dipyridamole (100 μM), BRL50481 (30 μM), cilostamide (10 μM), IBMX (50 μg/ml), or combinations thereof as indicated. Cells were then fixed, permeabilized, and stained with anti-ATF-1/CREB (pS63/133)-Alexa488 mAb prior to analysis by flow cytometry. The mean fluorescence intensity (MFI) of each condition was calculated and expressed relative to vehicle treated cells. **B)** RNA isolated from B-CLL, purified normal tonsillar B cells, and purified normal peripheral T cells was subjected to quantitative real-time RT-PCR using primers specific for PDE3B. Data are expressed relative to a white blood cell (WBC) RNA standard. **C)** Whole cell lysates from B-CLL, purified normal tonsillar B cells, and purified normal

peripheral T cells were separated by SDS-PAGE, blotted, and probed with an anti-PDE3B antibody. Approximate molecular weights are indicated; β-actin was used as a loading control. *** = $P < 0.001$.

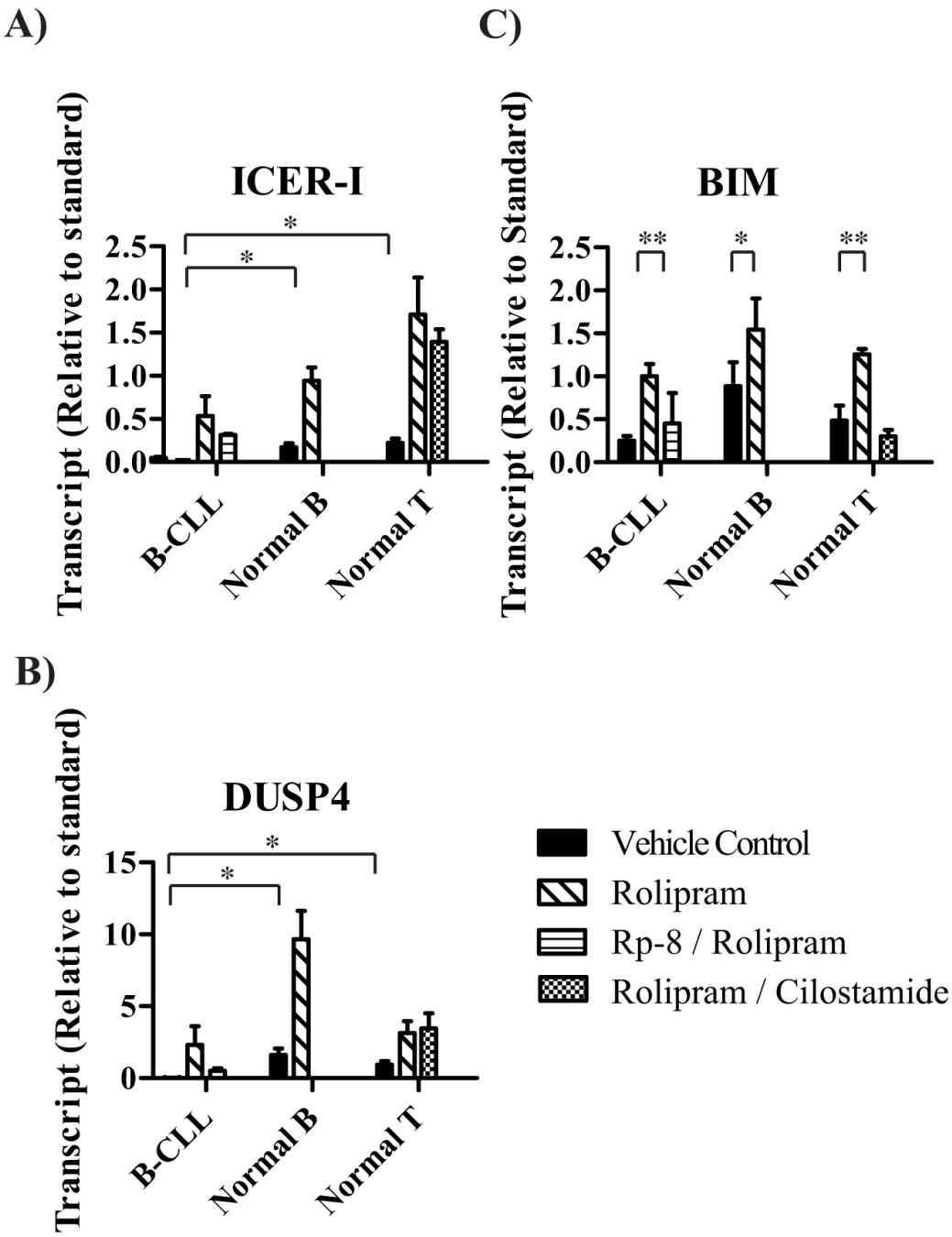

Figure 7. B-CLL expresses low basal levels of ICER and DUSP4, which are upregulated by rolipram in a PKA-dependent fashion while BIM is not differentially expressed under basal conditions

B-CLL cells, purified normal tonsillar B cells, and purified normal peripheral T cells were treated for 4 hours with rolipram (20 μM) or vehicle. Where indicated, B-CLL cells were pretreated with Rp-8-Br-cAMPS and T cells were treated with cilostamide (10 μM). RNA was extracted, purified, and quantitative real-time RT-PCR performed using primer/probes for **A)** ICER-I, **B)** DUSP4, or **C)** BIM. Data are expressed relative to a WBC RNA standard. * = $P < 0.05$.

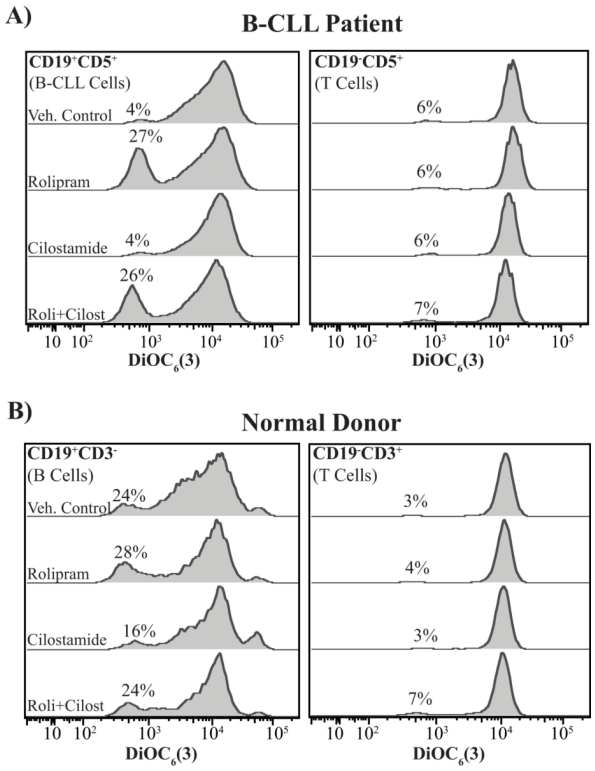

Figure 8. B-CLL cells are uniquely sensitive to rolipram-induced apoptosis

PBMC obtained from **A)** B-CLL patients or **B)** healthy donors were treated for 48 hours with vehicle, rolipram (20 μM), cilostamide (10 μM), or the combination of rolipram and cilostamide. Cells were stained for surface immunophenotype and apoptosis was assessed by mitochondrial depolarization using $DiOC_6(3)$. Histograms were gated to the indicated phenotype. Percentages indicate the extent of the apoptotic peaks. Data are representative of three leukemic and normal donors.

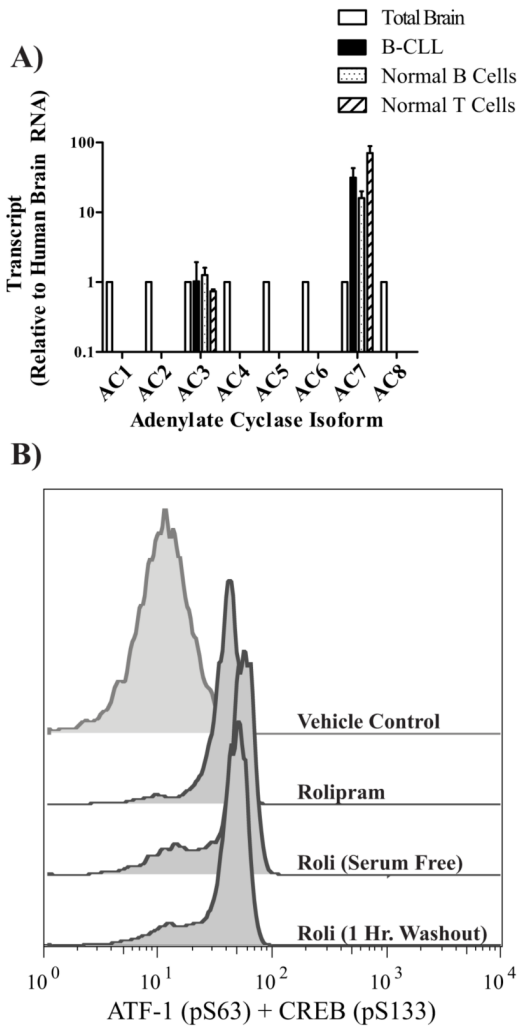

Figure 9. Differential expression of adenylate cyclase or response to factors present in media do not account for B-lineage sensitivity to PDE4 inhibitors

A) RNA obtained from B-CLL, purified normal tonsillar B cells, and purified normal peripheral T cells was analyzed by quantitative real-time RT-PCR for each of the 9 known adenylate cyclase isoforms. Data are expressed relative to a human brain RNA standard. **B)** B-CLL cells were cultured overnight under normal conditions with 10% FCS, serum free media, or washed into serum free media 1 hour prior to treatment with rolipram (20 μM) or vehicle for one hour. Cells were then fixed, permeabilized, and stained with anti-ATF-1/CREB (pS63/133)-Alexa488 mAb prior to analysis by flow cytometry.