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Effect of 5-azacytidine and galectin-1 on growth and differentiation of the human b lymphoma cell line bl36

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Abstract

Background: 5-AzaCytidine (AzaC) is a DNA demethylating drugs that has been shown to inhibit cell growth and to induce apoptosis in certain cancer cells. Induced expression of the galectin1 (Gal1) protein, a galactoside-binding protein distributed widely in immune cells, has been described in cultured hepatoma-derived cells treated with AzaC and this event may have a role in the effect of the drug. According to this hypothesis, we investigated the effect of AzaC and Gal1 on human lymphoid B cells phenotype.

Methods: The effect of AzaC and Gal1 on cell growth and phenotype was determined on the Burkitt lymphoma cell line BL36. An immunocytochemical analysis for detection of Gal1 protein expression was performed in AzaC-treated cells. To investigate the direct effects of Gal1, recombinant Gal1 was added to cells.

Results: Treatment of lymphoid B cells with AzaC results in: i) a decrease in cell growth with an arrest of the cell cycle at G0/G1 phase, ii) phenotypic changes consistent with a differentiated phenotype, and iii) the expression of p16, a tumor-suppressor gene whose expression was dependent of its promoter demethylation, and of Gal1. A targeting of Gal 1 to the plasma membrane follows its cytosolic expression. To determine which of the effects of AzaC might be secondary to the induction of Gal1, recombinant Gal1 was added to BL36 cells. Treated cells displayed growth inhibition and phenotypic changes consistent with a commitment toward differentiation.

Conclusions: Altered cell growth and expression of the cell surface plasma cell antigen, CD138 are detectable in BL36 cells treated by AzaC as well as by Gal1. It seems that AzaC-induced Gal1 expression and consequent binding of Gal1 on its cell membrane receptor may be, in part, involved in AzaC-induced plasmacytic differentiation.

Introduction

DNA methylation is involved in cellular development, differentiation and transformation [1]. In different types of tumours, aberrant methylation of CpG islands in the promoter region has been observed for many differentiation- and cancer-related genes resulting in the silencing of their expression [2]. Therefore, over the past decade, there has been increasing interest in the use of demethylating agents to induce the differentiation or the apoptosis of cancer cells [3,4]. Treatment of the cells with the pyrimidine analogue 5-AzaCytidine (AzaC), which inhibits methylation of cytosine residues during replication in the newly synthesised DNA, has been demonstrated to reactivate the expression of many silenced genes, as well as the expression of the silenced retro viral genomes [5]. Silencing of one of the most important cell cycle regulatory proteins $p16^{INK4a}$ by methylation of the CpG islands in the promoter region has been found to be a common event in tumours [6,7]. Protein p16 suppresses S-phase entry by antagonising the cyclin-dependent kinases CDK4 and CDK6 [8].

Deciphering the molecular mechanisms underlying the phenotypic effects of the treatment with demethylating drugs is a crucial step in understanding what genes may be interesting targets for chemotherapy. The available data on the mechanism of action of these drugs strengthen the idea that it is different from that of agents that act primarily via their cytotoxic effects, such as Arc-C [9]. Several lines of evidence suggest that galectin-1 (Gal-1), a 14 kDa galactoside-binding protein distributed widely in immune cells, could be involved in these mechanisms. Several members of the galectin family have been found to modulate cell differentiation and cell survival [10–15]. Early studies demonstrated that the expression of Gal1 could be induced in cultured hepatoma-derived cells by treatment with AzaC [16]. Chiariotti and co-workers showed that reactivation of the silent Gal1 alleles is accompanied by a transition from a fully methylated to a fully unmethylated state of several CpG dinucleotides in the promoter region [17]. In addition, nonexpressing tissues exhibited highly heterogeneous methylation profiles [18]. Gal is considered to be a typical cytosolic protein, lacking a signal peptide for membrane translocation [19]. However, most of the functions assigned to galectins are confined to the cell surface or extracellular milieu [10,20,21], consistent with evidences for extracellular roles of Gal1 in regulation of cellular differentiation and proliferation. It is clear that Gal1 can be specifically secreted and targeted by an infrequent mechanism [22–24]. The constitutive expression as well as the secretion of Gal1 dramatically depend on cell types [25] and are responsive to developmental events [20,22,23].

An example is found during erythroid differentiation of the K562 human leukaemia cell line. During differentiation induced by erythropoietin and deprivation of granulocyte-macrophage colony-stimulating factor, the cells empty their cytoplasmic content of endogenous Gal1 into the external medium where it is bind to cell surface receptors [24]. The synthesis and secretion of Gal1 by leukocytes are of interest because lactosaminoglycans present at the leukocyte cell surface may be physiologically significant galectin receptors that could mediate autocrine or paracrine functions. Several lines of evidence indicate that Gal1 may function as an autocrine negative growth regulator or as a pro-apoptotic factor [26–28]. We have recently demonstrated that Gal1 binding to Burkitt lymphoma cells results in an intracellular signal, with inhibition of the tyrosine phosphatase activity of CD45 and therefore phosphorylation of Lyn kinase [29,30].

In this work, we study the effect of AzaC treatment on the lymphoma cell line BL36. As $p16^{INK4a}$ gene has been found to be downregulated by hypermethylation at high frequency in different types of tumours [6,7], it is used as a control of AzaC effect. Then, the phenotypic effects of AzaC are compared to those obtained by the addition of exogenous Gal1. The findings that we report here lend further support to a potential role for Gal1 in the AzaC-induced pathway of differentiation in hematopoietic cells.

Materials and Methods

The BL36 B lymphoma cell line [31], a gift from Pr. Lenoir (CIRC, Lyon, France) to Pr. M. Raphael, was maintained in a complete medium of RPMI 1640, containing 10% heat-inactivated foetal calf serum, 2 mM L-Glutamine, 1 mM sodium pyruvate (complete medium). Treatment with AzaC was conducted as follows: 24 hours after seeding 5, 10 or 50 μM of AzaC were added to 5 ml of complete medium [16,32]. The cells were feeded with the appropriate medium during the time of the experiments.

Several independent methods were used to assess the proliferative versus death states of the cells. For direct determination of cell number, at the indicated time point the cells were harvested and counted using a Coulter Counter (Beckman Coulter France, Villepinte, France). Determination of the viable cell numbers used propidium iodide (PI) (Sigma, Saint-Quentin Fallavier, France). To determine the percent cell death including both apoptotic and necrotic cells, AzaC-treated cells were analyzed for AnnexinV binding and PI uptake as described [33] using the ApoDetect AnnexinV-FITC kit (Zymed). Briefly, after washing of cultured cells (3.10^5) with PBS and resuspension in binding buffer, the cells were stained with 10 μl FITC-labelled AnnexinV and 10 μl PI (20 μg/ml). After 5 min of incubation at room temperature in the dark, again 200 μl of binding buffer was added and cells were ana-

lyzed by flow cytometry. The percent cell death was calculated by determining the percent of viable cells:

$$\%\,\text{viable} = \frac{\%\,\text{AnnexinV}^-, \text{P.I.}^-, \text{treated cells} \times 100}{\%\,\text{Annexin V}^-, \text{P.I.}^-, \text{untreated cells}}$$

The MTS assay (CellTiter 96™, Promega, Charbonnières, France) [34] was used to compare the percentage of metabolically active cells in treated cells vs. untreated cells, as previously described [14]. To study the cell cycle, 2.10^6 BL36 cells were pelleted at 1000 g for 5 minutes and the pellet was incubated in a mixture of ethanol-PBS (70/30 v/v). The cells were pelleted another time and suspended in 800 µl PBS. One hundred microliters of 1 mg/ml RNase was added to the cell suspension. One hundred microliters of 400 µg/ml PI was added to the solution to stain the nuclear DNA. The DNA content of the cells was determined by a flow cytometer, FACScan (Becton Dickinson, Mountain View, CA), and the percentages of cells in G0+G1, S, and G2+M phases of the cell cycle were analyzed by a polynomial model (SFIT, Becton Dickinson).

To determine the cell phenotypes, cells were suspended in PBS supplemented with 2% BSA, and incubated in suspension for 30 min with the fluorescently tagged primary antibody or negative control, or incubated with untagged antibody, washed, and incubated again for 30 min with FITC-conjugated second antibody. All incubations were performed at 4°C. Flow cytometry was performed using a Coulter Epics Elite ESP instrument.

Human recombinant Gal1 (rGal1) was obtained as described elsewhere [14]. The protein was purified by affinity chromatography on a lactosyl-divinylsulfone-agarose column. Antibodies against human Gal1 were generated as described in [35] and [36]. The immunolocalization of Gal1 was carried out as described for K562 cells [24].

For the preparation of cell extracts, a total of 17.10^6 cells were solubilized in 1 ml of extraction buffer: 50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% SDS (w/v), 0.5% sodium deoxycholate (w/v), 0.5% Nonidet NP40 (v/v). Finally, 1 tablet of antiprotease cocktail (Roche, Meylan, France) was added to 10 ml of buffer. The cells were sonicated in ice three times. The lysat was centrifuged at 100, 000 g for 15 min at 4°C. The supernatant was collected and stored at -20°C until used. Proteins were resolved by discontinuous SDS-PAGE on 1.5 mm gel (T: 6–18%) according to the method of Laemmli. [37]. For Western blotting, proteins separated by SDS-PAGE were electrotransferred onto Immobilon-P membrane (Millipore). Blots were incubated with 1:50 anti-Gal1 mAb, or 1:500 anti-pl6 mAb (Pharmingen) for 2 h at room temperature. The blots were developed with anti-mouse Ig antibody-HRP diluted 1/10,000, followed by incubation in the Amplified Opti-4CN kit substrate (Bio-Rad). The image of the membranes was acquired from GS-700 Densitometer 4, and analyzed with Molecular Analyst Software (Bio-Rad).

Results

During the four days of treatment with AzaC, cell proliferation appeared to slow (Fig. 1A). When BL36 cells were cultured with AzaC at the high concentration of 50 µM, cell proliferation was strongly inhibited. BL36 cells exposed to 5 µM AzaC also exhibited a significant reduced growth rate but these cells were >95% viable by propidium iodide test even at day 4 (data not shown), and were found metabolically active by MTS assay as described below. According to these results, the following experiments addressing metabolic activity, death, and cell differentiation were performed.

The proportion of metabolically active cells was determined using the MTS test in cultures treated with AzaC in comparison with untreated cells (Fig. 1B). The cellular conversion of MTS to the ultraviolet-absorbing formazan product has been demonstrated to be directly proportional to cellular metabolism resulting in the formation of reducing equivalents such as NADH or NADPH [38]. A drop in the % of metabolically active cells of about 36% was observed over the 24 h period following the addition of 10 µM AzaC, and went up to 42% in cells treated with 50 µM AzaC. Cell death was confirmed by an increase in annexinV/PI staining (Fig. 2). Within 48 h of incubation with AzaC at 10 µM, the number of AnnexinV^+ PI^- apoptotic cells and AnnexinV^+ PI^+ necrotic cells increased. For longer incubation times, induced cell death increased dramatically.

Studies investigating the mechanisms whereby B lymphoma cells are induced to undergo apoptosis demonstrated that an arrest in the cell cycle preceded apoptosis [39]. To determine whether AzaC modified the cell cycle distribution of BL36 cells, the DNA content of AzaC-treated cells was analyzed by PI staining. Cells were exposed to 10 µM AzaC for 2, 4 and 7 days. After 7 days, AzaC-treated cells exhibited a relative increase of cells in G0/G1 (77 %) in comparison with controls (56%). In the same time, the percentage of cells in the S phase compartment drops from 38 to 18%, suggesting an accumulation of cells into the G1 phase of the cell cycle (Fig. 3). However, there was no change in the relative cell cycle distribution at 2 or 4 days after AzaC treatment. By this time, a significant population of cells has undergone apoptosis. This suggests that, at least during the first days of treatment, inhibition of growth is uncoupled with an arrest in the cell cycle.

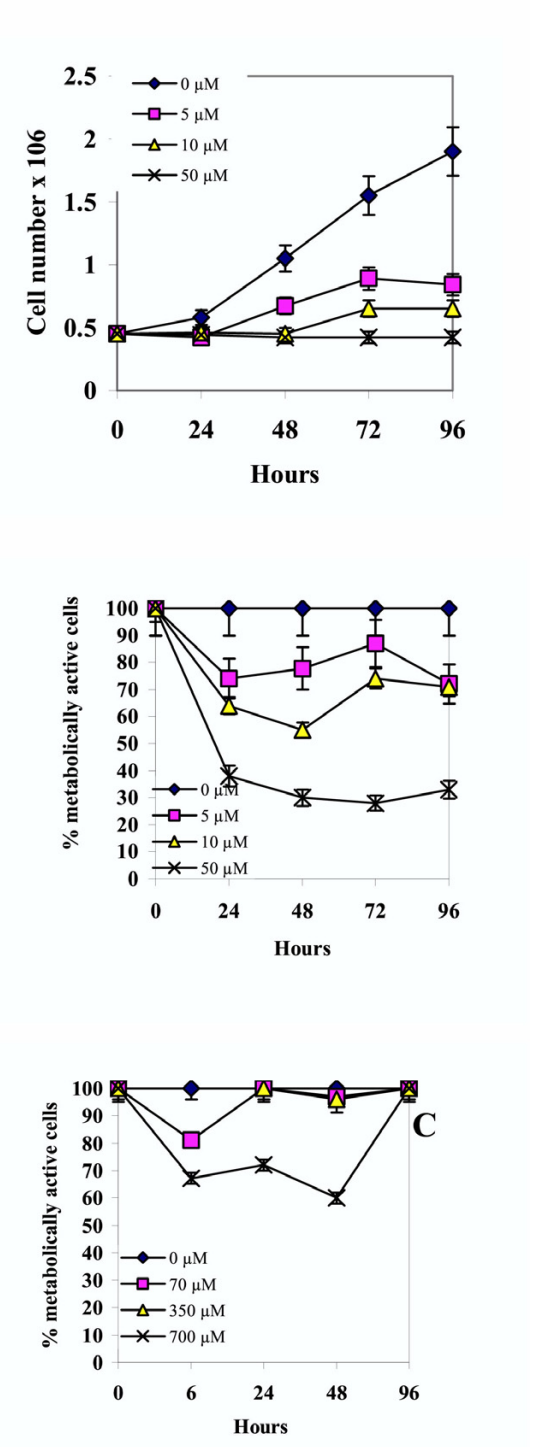

Figure 1
Effect of AzaC and Gal1 on growth and viability of BL36 cells in culture. A. The cells were seeded at 4×10^5/ml and exposed to AzaC at various concentrations for zero to 4 days. Cells were counted every day on a Coulter Counter. B. Cells were cultured in triplicate in the presence or absence of AzaC. Metabolic activity was measured at the indicated times by the MTS assay. Results are given as mean percentage of metabolically active cells (%) in cells grown in the presence of AzaC compared to cells grown in the absence of AzaC. C. Cells grown in the presence of various concentrations of Gal1 were compared to control cells. The data represent the mean of 3 experiments.

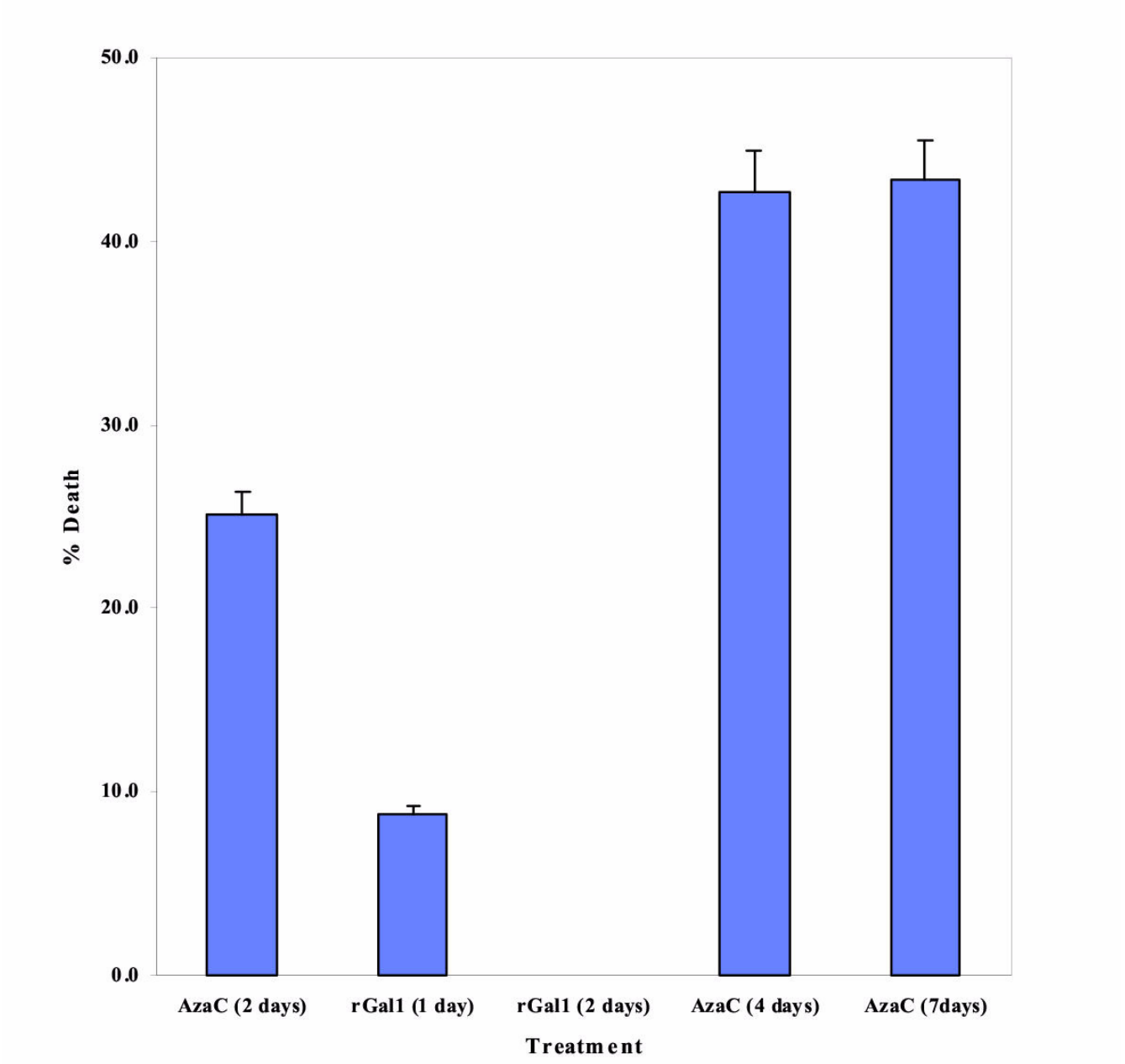

Figure 2
Induction of cell death in cells treated by either AzaC or rGal1. The BL36 cell line was treated with 10 μM AzaC, 700 nM rGal1, or buffer control and the percent cell death was evaluated by annexinV/propidium iodide staining, as described in "Materials and Methods". There is a dramatic increase in annexinV$^+$/propidium iodide$^+$ cells in the BL36 samples treated with AzaC, not seen with the cells treated with Gal1.

The effects of different concentrations of AzaC (5, 10, and 50 μM) on the expression of various surface antigen of BL36 cells were investigated. AzaC increased the cell population that expressed the cell-surface maturation marker CD23, CD30 present on activated B-lymphocytes [40] and CD138 (Syndecan-1). After the treatment with 50 μM

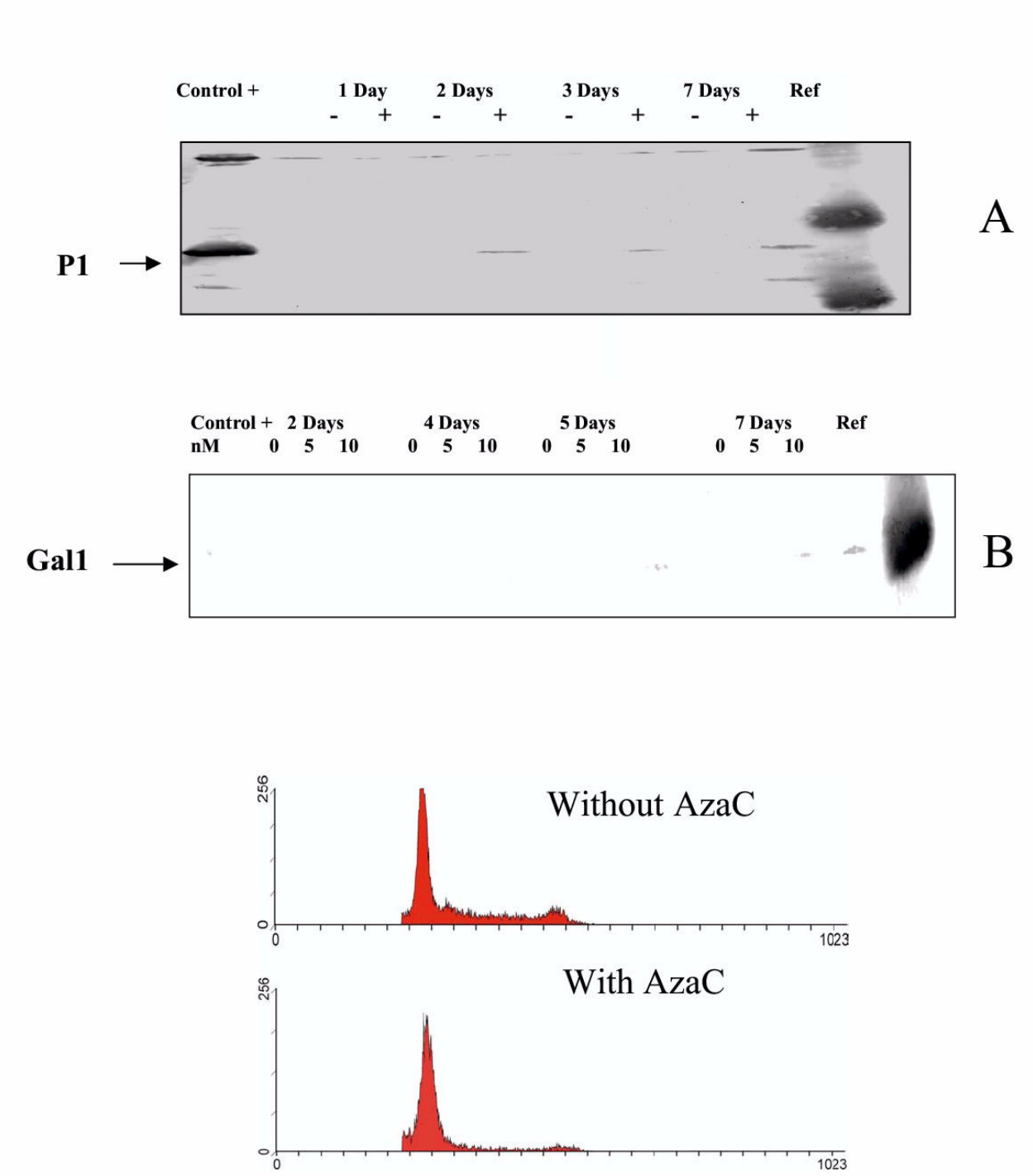

Figure 3
Cell cycle analysis of AzaC-treated and control cells. The BL36 cells were incubated for 7 days in the presence of 10 μM AzaC and the DNA contents were measured.

Table 1: Modulation of urface Markers on BL36 Cells by AzaC

Surface marker	Control	Treatment AzaC = 5 μM	AzaC- 10 μM	AzaC = 50 μM
CD 19	94.9	88.5	86.1	58.1
CD 21	<20	<20	<20	20.3
CD 23	31.5	47.6	45.2	63.8
CD 30	<20	<20	48.4	21.5
CD 45 RA	76.7	74.4	71.2	64.6
CD 45 RO	21.4	35.1	33.3	38.5
CD 71	97.3	94.9	93.8	83.8
CD 138	<20	23.9	23.8	24.8

BL36 cells were exposed with 5 μM, 10 μM and 50 μM AzaC for 4 days. Then cell surface antigens were analyzed by flow cytometry. Values below 20% were considered as negative. In the same conditions, CDs 3 and 10 remain negative, and no variability was observed for CDs 11 a, 18, 25, 38, 44, 48, 54, 58, 77, 80, and 95.

AzaC some changes were observed that were not detected at lower concentrations: an increase of the cell population that expressed CD21, and a decrease of the cell population that expressed CD19 that is lost on maturation to plasma cells [41], and CD71 that is expressed on proliferating cells [42] (Table 1).

P16 is a biochemical marker of cell cycle progression [43] and is also commonly utilized as a marker of demethylation reaction [44]. Consequently, we examined the effects of AzaC treatment on expression of p16 in BL36 cells. As expected, when these cells were treated with AzaC for 48 h, we detected the presence of p16 by Western Blotting (Fig. 4A). Thus, taken together the data showed that AzaC treatment induced an inhibition of cell growth related to an arrest at G0/G1 phase of the cell cycle, and confirmed the efficiency of AzaC treatment for expressing the tumour suppressor gene p16.

To determine whether AzaC is able to induce the expression of Gal1 in B lymphoma cells, protein extracts prepared form cells treated with 5–10 μM AzaC and from untreated cells were separated by SDS-PAGE and analyzed by immunoblotting with anti-Gal1 antibody. Fig. 4B,C shows the rate of variation in Gal1 expression induced by the differentiating agent. The galectin was not detected either in untreated BL36 cells or BL36 cells treated with AzaC for 24 or 48 h. The galectin became detectable in AzaC-treated cells by 96 h. Then, to determine whether the newly synthesised galectin molecules in AzaC-treated BL36 cells are expressed on the cell surface, the binding of anti-Gal1 Ab to untreated and treated BL36 cells was studied by immunocytochemistry. To visualize only extracellular Gal1, cultures of BL36 cells were incubated with anti-Gal1 antibody without permeabilization of their membranes. Over the 4-day time-course, AzaC-induced Gal1 did progressively localise to the cell surface. However, Gal1 did not exclusively localise to the cell surface but was also distributed throughout the cytoplasm (Fig. 5).

Several of the changes in AzaC-treated BL cells might be attributed directly to Gal1 induction, as Gal1 has been implicated in differentiation and growth inhibition [11–13,29,45,46]. A series of experiments were thus performed to address whether changes observed in BL36 were direct AzaC effects and/or secondary effects related to Gal1 expression induced by AzaC treatment. To this end, BL36 was treated with recombinant Gal1 (rGal1). Slower growth rates were confirmed for the rGal1-treated cultures. A drop in the % of metabolically active cells of about 28% was observed over the 24 h period following the addition of 700 nM rGal1. However the proportion of metabolically active cells in cultures treated with rGal1 vs. the proportion in untreated cultures was not modified after 4 days of treatment (Fig. 1C). Moreover the variation of cell death detected in treated-cells relative to the control cultures was discrete (<10%) and limited to the first day after the treatment (Fig. 2). To determine whether the inhibition of proliferation could be correlated to a modified phenotype, the effects of 700 nM rGal1 on the expression of various surface antigens shown to be modified or not by AzaC were investigated. The populations of cells that expressed CD19, CD23, CD45RO and CD45RA were unchanged. On the other hand, 24 h rGal1 treatment increased the cell population expressing CD138 (Fig. 6), a marker for plasma cells, while it decreased the expression of CD71. These modifications were reinforced after 48 h rGal1 treatment (Table 2).

Discussion

In 1994, Chiariotti et al. [16] first reported experimental data showing that the expression of Gal1 can be induced in cultured hepatoma-derived cells by treatment with AzaC. Interestingly, Gal1 and AzaC individually have

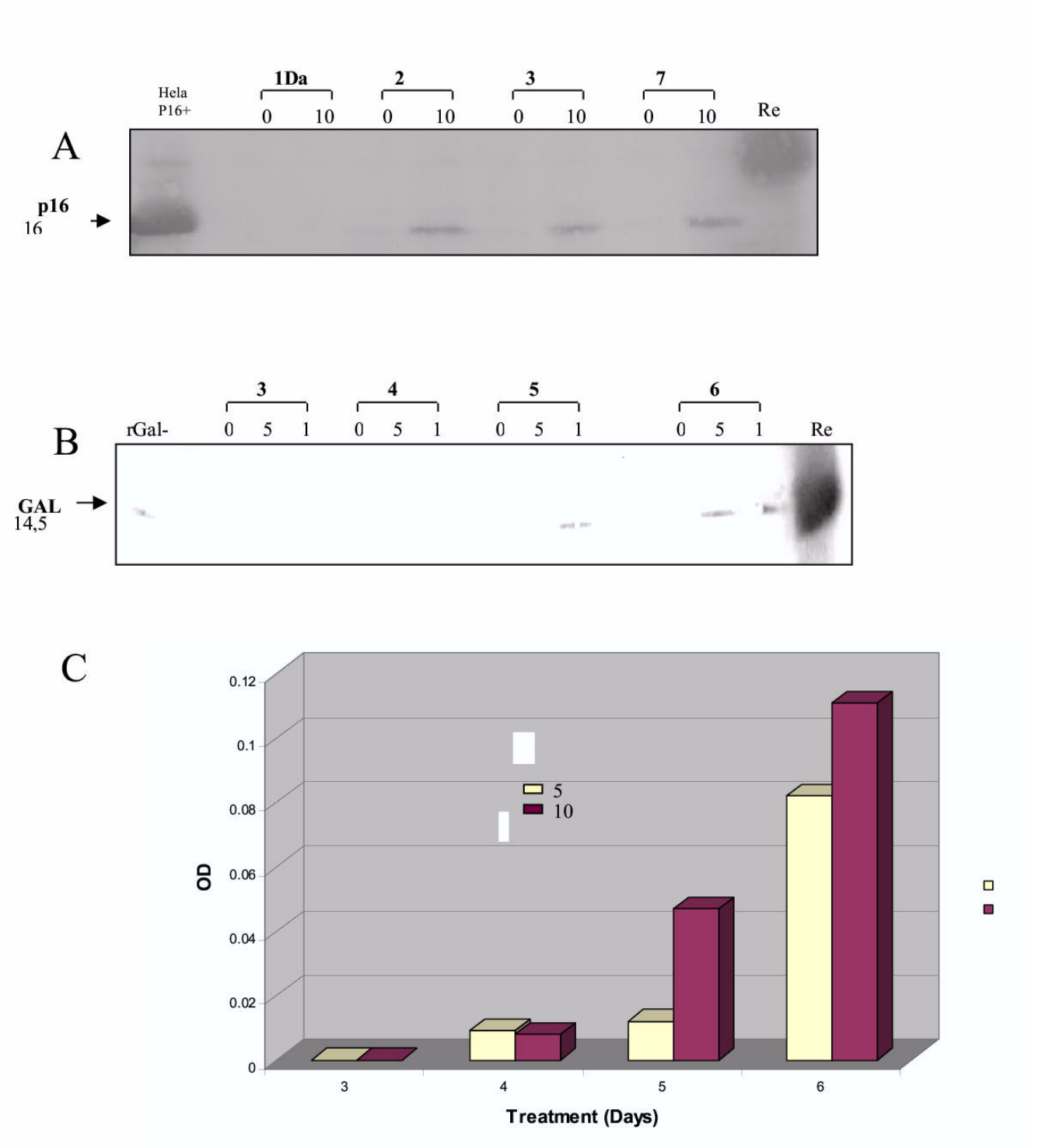

Figure 4
Immunoblotting of p16 and Gal1 from extracts of BL36 treated by AzaC. After different periods of treatment by AzaC, cell extracts were separated by SDS-PAGE, followed by electrophoretic transfer onto Immobilon-P membranes and immunostaining with (A) anti-pl6, or (B) anti-Gal1 monoclonal antibodies. Hela cells extract and rGal1 were used as positive controls for p16 and Gal1, respectively. (C) Representation of the variation of Gal1 expression in cells treated different times with 5 or 10 mM AzaC, using the analysis of the Western blots in Molecular Analyst software.

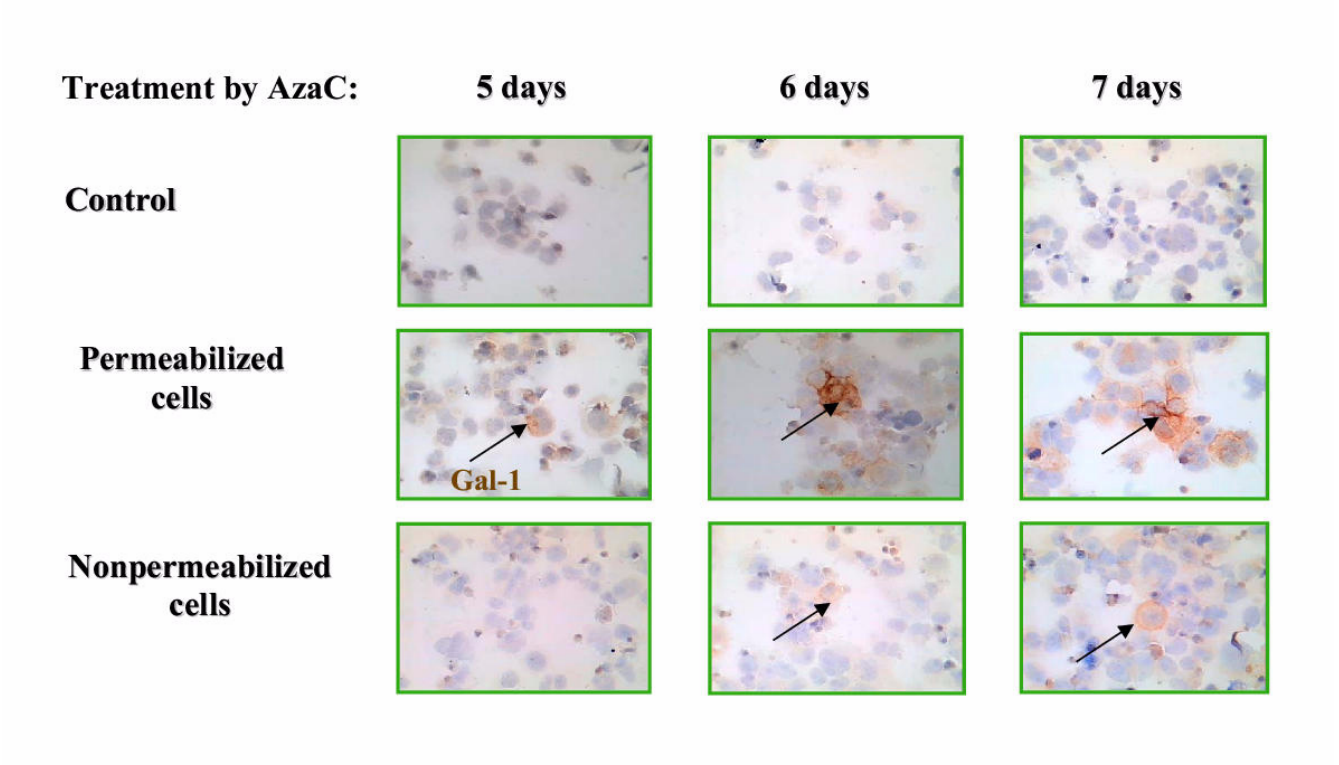

Figure 5
Immunoreactive localization of Gal1 in AzaC-treated BL36 cells. Cytospins were incubated with anti-Gal1 serum and peroxidase-stained (original magnification 1250×). First line: negative controls performed on untreated BL36 cells. No Gal1 was detectable with anti-Gal1 antibody. Second line: permeabilized cells treated cells stained with monospecific anti-Gal1 antibody; arrows indicate the localization of Gal1. Third line: after 144 h, Gal1 was detectable on the cell surface of nonpermeabilized cells. No immunostaining was observed with controls performed with preimmune serum (not shown).

been shown to affect similar cell processes in cancer cells, including differentiation, and growth inhibition [12–15,27,29,46–51]. Available data are consistent with the suggestion that the expression of Gal1, accompanied by its secretion and its binding to cell surface receptors, could be involved in the AzaC observed effects in hematopoietic cells where Gal1 modulates differentiation or apoptosis. However, the mechanisms of these effects on hematopoietic cells are unclear yet.

In the present study, the effects of AzaC, on the cell phenotype, cell differentiation and cell death of BL36 cells were analyzed. The effects on cellular growth were time- and dose-dependent. Five μM AzaC caused significant inhibition on cellular growth, but the cell viability remained practically unchanged. AzaC increased the cell fraction in the G0/G1 phases, suggesting that AzaC inhibits cell division, which may be one of critical mechanisms of cell modulation by AzaC. AzaC-treated cells initially may become arrested at the G1 phase and then may either escape to the cycle arrest or die due to mechanisms leading to programmed cell death.

We also show that incubation of BL36 with AzaC induces expression of Gal1. AzaC is thought to exert its effects as a competitive inhibitor of cytosine methylation, resulting in the expression of silenced genes. The gene for Gal1 is one whose expression is possibly enhanced in this manner. In the results reported here, we found that Gal 1 was detected in cytosol after 120 h of treatment by 10 μM AzaC and then Gal1 was externalized and bound to cell surface receptors 24 h later. A key to understanding the extracellular biological functions of Gal1 is how its secretion appears to be regulated and re-directed during development and differentiation. Gal1 is likely released form vesicles close to the plasma membrane. On the basis of the data, we propose that the released Gal1 be immediately recruited to modulate cell activity. Gal1 may do this by interacting with and modulating cell receptors via its carbohydrate recognition domains because the Gal1-receptor interaction is abrogated by thiodigalactoside [29].

Others and we have previously reported that Gal1 binds to T and B lymphoblastoid cells [29,52,53]. Other studies have demonstrated that galectins are immunosuppressive,

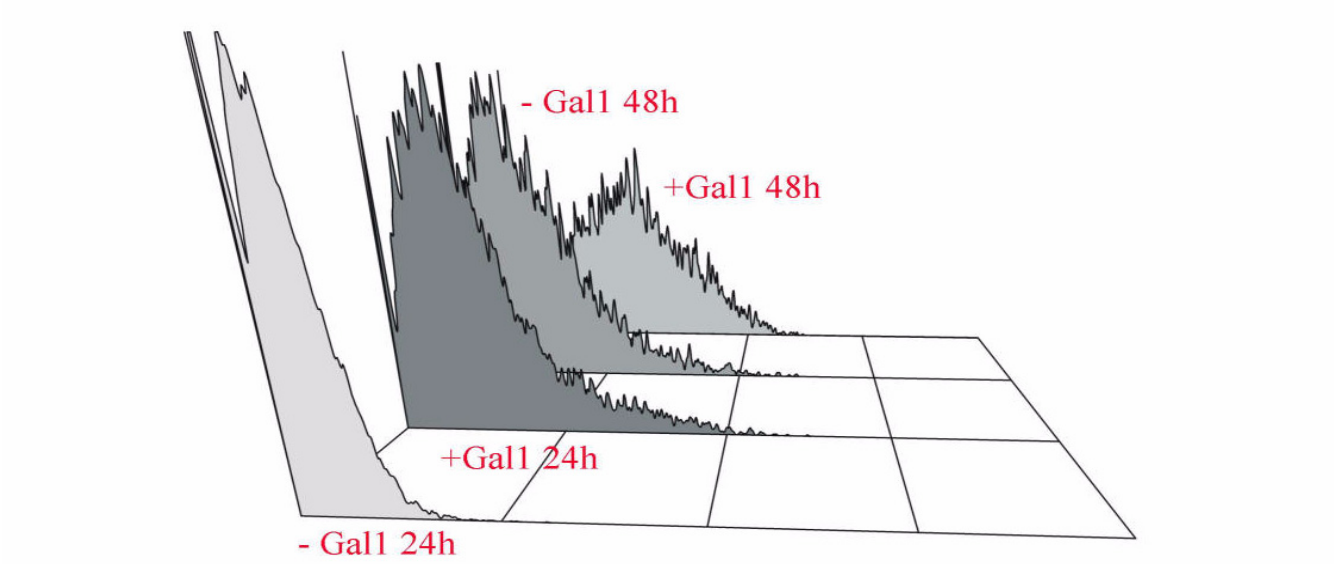

Figure 6
Modification of the expression of CD138 in rGAL1-treated cells. Cells incubated for 24 or 48 h with rGal1 (700 nM) were stained with anti-CD138 antibody and analyzed by flow cytometry. Controls were performed in the absence of rGAL1. Representative-histograms: x-axis, log of the fluorescence intensity; y-axis, number of events.

Table 2: Modulation of Surface Markers on BL36 Cells by rGal1

Surface Marker	Control 24 h	Treatment (% positive) rGal1 – 24 h	Control 48 h	Treatment (% positive) rGal1-48 h
CD71	98.00	94.90	93.00	45.00
CD138	<20	20.20	25.00	70.00

BL36 cells were exposed with 700 nM rGal1 for 1 and 2 days. Then cell surface antigens were analyzed by flow cytometry. Values below 20% were considered as negative.

in animal models of autoimmune diseases [54–56]. Whereas the full role of Gal1 in modulating immune function is not yet understood, the increase in Gal1 expression by AzaC in BL cells suggests that Gal1 may play a role in the behaviour of normal leukocytes and of tumour cells.

What is the underlying mechanism? Although the regulatory machinery triggered by demethylating stimulus and resulting in phenotype modifications is not yet elucidated, it probably involves the stimulation of a signalling cascade that regulates cell proliferation and viability. A recently proposed model for such a cascade suggests the involvement of a cytoplasmic protein, AZ2 [57]. The amino-terminal part of the AZ2 protein is homologous to the previously reported TANK and I-TRAF, which participate in the signal transduction cascade from the TNF-receptor to the transcription factor NFkappaB. Demethylating stimulus may also modify a pathway activated by the membrane-anchored protein-tyro sine phosphatase CD45. Engagement of CD45 is known to regulate Src tyrosine kinases phosphorylation, phospholipase Cγ regulation, inositol phosphate production, diacylglycerol production, PKC activation, and calcium mobilisation [58–60].

Increased synthesis and secretion of Gal1 by the cell could account for part of the phenotypic alterations detected in AzaC treated cells. Gal1-induced dimerisation and/or segregation might inhibit the catalytic site in CD45, thereby

blocking tyrosine phosphates activity. Because Gal1 binding to cell surface receptors results in tyrosine phosphorylation [29,61], it may allow a kinase-dependent signal to be transduced. Several studies have linked Gal1 expression with growth inhibition [27] and cell death [26,62]. However, the reports that some growth inhibitory agents did not induce Gal1 expression indicated that Gal1 expression is not dependent on the cell's growth state in general, through it may be involved in growth suppression [63]. Moreover, it is likely that Gal1 acts in a manner to regulate specific signal transduction processes that is determined by the cell type and by the state of cell differentiation. In this work, exogenous rGal1 added to BL cells inhibited cell growth. Moreover, Gal1 as well as AzaC induced an expression of the cell surface plasma cell antigen, CD138, a phenotypic marker that identify cells with plasmacytic differentiation [64]. This is consistent with the hypothesis that AzaC and Gal1 share similar signals for differentiation, however, since there was a significant difference in the expression of CD19 and CD23 after AzaC or Gal1 treatments it is likely that some pathways are specifically modified by AzaC. The mechanisms involved in these different pathways, important in clinical therapy, remain to be elucidated in the future. Ongoing studies are aimed at identifying as globally as possible the modifications resulting from AzaC treatment by using proteomics [65].

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