Effect of bovine dialyzable leukocyte extract on induction of cell differentiation and death in K562 human chronic myelogenous leukemia cells

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Abstract. Differentiation induction therapy is an attractive approach in leukemia treatment due to the fact that in blast crisis stage, leukemic cells lose their differentiation capacity. Therefore, it has been proposed as a therapeutic strategy to induce terminal differentiation of leukemic blast cells into a specific lineage, leading to prevention of high proliferation rates. The aim of the present study was to demonstrate the potential of cell differentiation and death induced by bovine dialyzable leukocyte extract (bDLE) in the K562 cell line. For this purpose K562 and MOLT-3 human leukemic cell lines and primary human monocytes and murine peritoneal macrophages were exposed to bDLE, phorbol myristate acetate (PMA) and dimethyl sulfoxide for 96 h, and the viability, proliferation and cell cycle were evaluated. To determine the lineage that led to cell differentiation, Romanowsky staining was performed to observe the morphological changes following the treatments, and the expression of the surface markers cluster of differentiation (CD)14$^+$, CD68$^+$, CD163$^+$ and CD42a$^+$, as well as the phagocytic activity, and the production of nitric oxide (NO) (assessed by colorimetric assay), cytokines [interleukin (IL)-1β, IL-6, IL-8 and tumor necrosis factor-α] and chemokines [chemokine (C-C motif) ligand (CCL)2, CCL5 and chemokine (C-X-C motif) ligand 8] in cell supernatants was assessed by flow cytometry. The results of the present study reveal that high doses of bDLE increase the cell death in K562 and MOLT-3 lines, without affecting the viability of human monocytes and murine peritoneal macrophages. Furthermore, low doses of bDLE induce differentiation in K562 cells towards a monocyte/macrophage lineage with an M2 phenotype, and induced moderately upregulated expression of CD42$^+$, a megakaryocytic marker. Cell cycle arrest in the S and G_2/M phases was observed in bDLE-treated K562 cells, which demonstrated similar phagocytic activity, NO levels and cytokine and chemokine production to that of PMA-treated cells. The present study demonstrates that bDLE exhibits an antileukemia effect, suggesting that it may be an effective candidate for leukemia treatment.

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Abbreviations: CML, chronic myelogenous leukemia; DMSO, dimethyl sulfoxide; PMA, phorbol myristate acetate; bDLE, bovine dialyzable leukocyte extract

Key words: leukemia, cell differentiation, cell death, bovine dialyzable leukocyte extract, K562

Introduction

Bovine dialyzable leukocyte extract (bDLE) is a heterogeneous mixture of low-molecular weight substances released from disintegrated leukocytes of homogenized bovine spleen (1). bDLE has been reported to have various biological activities, including induction of cytotoxic effects in several cancer cell lines *in vitro* (1) and in melanoma *in vivo* (2), as well as modulation of the expression of transcription factors, including nuclear factor-κB and activator protein 1 (3), with no effect on normal cells (1). Furthermore, bDLE has demonstrated antioxidant activity (4). bDLE has been used as an immunomodulator and coadjuvant in clinical trials.

Chronic myeloid leukemia (CML) is a malignant hematological disease of hematopoietic stem/progenitor cells caused by the t(9;22)(q34;q11) chromosomal translocation and expression of the Bcr-Abl oncoprotein (1). Leukaemia is the tenth most common cause of cancer-associated mortalities, worldwide, accounting for >265,000 mortalities in 2012 (5). CML incidence increases with age and accounts for 20% of all leukemia cases, with an annual incidence of 1-1.5 cases per

100,000 individuals (5). in 2012. Currently, CML is treated with chemotherapeutics agents and specific inhibitors, such as imatinib or dasutinib. which have demonstrated a high response rate; however, effects are often short-lived and disease progression is common (6). An alternative strategy to treat leukemia, cell differentiation therapy, has been proposed and consists of forcing leukemia cells toward a process of terminal differentiation by using biological or chemical agents (7-9). Certain compounds used with this objective in clinical practice are all-trans retinoic acid (ATRA) (7) and 1,25-dihydroxyvitamin D_3 (7-9). Certain substances used may exhibit selective activity against tumor cells and minimal side effects against normal cells (10). An *in vitro* model for investigating cell differentiation has been established using the human chronic myelogenous leukemia K562 cell line (4), which expresses characteristics of erythrocytes, monocytes and megakaryocytes. Following exposure to phorbol myristate acetate (PMA), the K562 cancer cell line is differentiated toward cells with monocytic and/or megakaryocytic characteristics (2), while treatment with imatinib, butyric acid and haemin cause erythroid differentiation (7,9). The present study investigated the cell death and differentiation activity induced by bDLE in the human CML, using K562 as a model cell line.

Materials and methods

bDLE. bDLE was produced by the Laboratory of Immunology and Virology, Faculty of Biological Sciences, University Autonomous of Neuvo León (UANL) (San Nicolás de los Garza, Mexico). bDLE is a mixture of low-molecular weight substances (cut-off of 10-12 kDa) obtained from the dialysis of disintegrated bovine spleens in water, subsequently lyophilized and determined to be free of pyrogens using the *Limulus amoebocyte* lysate assay (Endotoxin Detection kit; MP Biomedicals, LLC, Santa Ana, CA, USA), and confirmed to be free of bacterial contamination by culturing in various culture media as well as *in vivo* mouse inoculation. bDLE obtained from $75x10^8$ leukocytes is defined as five units (5 U). For the subsequent assays, bDLE was suspended in RPMI-1640 (Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). The suspension was filtered with a 0.2 μm-diameter filter (EMD Milipore, Billerica, MA, USA).

K562 cell treatments. The K562 cell line was originally established from the pleural effusions of a patient with CML in terminal blast crisis. The cell line was obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium supplemented with 10% FBS and 1% antibiotic-antimycotic solution (Gibco; Thermo Fisher Scientific, Inc.), at 37˚C in a humidified incubator with 5% CO_2. To determine the cytotoxic effect and induction of cell differentiation by bDLE in K562 cells, cells were seeded onto 6-well plates at a density of $1x10^5$ cells/well and treated with bDLE (0.07, 0.14, 0.21, 0.28, 0.35, 0.5, 0.75 and 1 U/ml). PMA (10 ng/ml; Sigma-Aldrich; EMD Millipore) and dimethyl sulfoxide (DMSO;1.5% v:v; Sigma-Aldrich; EMD Millipore) were used as positive controls for the induction of cell differentiation in the K562 cell line. All treatments (bDLE, PMA and DMSO) were suspended in RPMI-1640 medium supplemented with 10% FBS, and plates were incubated for 96 h at 37˚C in a 5% CO_2 atmosphere. After trypsinization, adherent and non-adherent cells were collected. The MOLT-3 (T acute lymphoblastic leukemia) cell line was obtained from American Type Culture Collection and was used as a cytotoxic control of the T cell lineage.

Macrophage treatments. A total of 5 male BALB/c mice (6 weeks old; 24-26 g) were obtained from the Laboratory of Immunology and Virology of the University Autonomous of Nuevo León) and maintained in a controlled environment at 25˚C (12 h light/dark cycles) with free access to food and water. The mice were sacrificed by cervical dislocation, and resident peritoneal macrophages were obtained through repeated sterile ice-cold RMPI-1640 lavages within the peritoneal cavity, according to protocols approved by the Institutional Animal Care and Use Committee of the Laboratory of Immunology and Virology of the University Autonomous of Nuevo León. Furthermore, human monocytes were isolated from peripheral blood obtained from normal donors, the blood was diluted with PBS at a ratio of 1:1 (vol/vol) and subsequently the blood was centrifuged on a Ficoll-Paque gradient for 30 min at 500 x *g* at room temperature. The interphase layer consisted of peripheral blood mononuclear cells and was washed three times with culture medium. Murine peritoneal macrophage and human monocyte cells ($2x10^6$ cells/ml) were seeded in 6-well plates and incubated at 37˚C in an atmosphere of 5% CO_2 for 3 h to allow cell adherence to the plastic. Subsequently, the non-adherent cells were removed, and adherent cells were obtained by trypsinization. Cell supernatants were centrifuged at 500 x *g* at 25˚C for 10 min and seeded at $1x10^5$ cells/well. Cells were treated with bDLE (0.5, 0.75 and 1 U/ml) and incubated at 37˚C in a 5% CO_2 atmosphere for 96 h, followed by evaluation of cell death by propidium iodide (PI) staining.

Cell viability assessment by PI staining. To evaluate cell death, cells were centrifuged at 500 x *g* at 25˚C for 10 min and washed with PBS, resuspended in 0.2 ml PBS and PI (50 μg/ml; BD Biosciences, Franklin Lakes, NJ, USA) and incubated for 5 min at room temperature (25˚C) in the dark, followed by analysis with an Accuri™ C6 flow cytometer (BD Biosciences).

Analysis of cell proliferation by trypan blue staining. K562 cells previously treated for 96 h with bDLE, PMA or DMSO were harvested, centrifuged at 500 x *g* at 25˚C for 10 min and resuspended in 0.2 ml PBS. Cell proliferation percentage was estimated by cell counting following trypan blue staining for each group. Furthermore, to assess the proliferative capacity of the differentiated cells, following 96 h of incubation with bDLE, PMA or DMSO, the cells were collected, centrifuged at 500 x *g* at 25˚C for 10 min, washed with RPMI-1640 and seeded at $1x10^5$ cells/well. Cells were subsequently incubated at 37˚C in an atmosphere of 5% CO_2 for 10 days, and the cell count was evaluated by trypan blue staining and proliferation was estimated.

Cell cycle analysis. Cell cycle progression analysis was performed with the Cycle Test™ Plus DNA Reagent kit (BD

Biosciences) according to manufacturer's protocol. In brief, $1x10^5$ cells/well previously treated for 96 h (with bDLE, PMA or DMSO) were washed with PBS and fixed with 1 ml buffer solution (containing sodium citrate, sucrose and DMSO). Subsequently, 250 μl of solution A (containing trypsin in a spermine tetrahydrochloride detergent buffer) was added, and the samples were incubated for 10 min at room temperature. Following incubation, 200 μl of solution B (trypsin inhibitor and RNase buffer) was added, followed by the addition of 200 μl of cold (2-8˚C) solution C (containing propidium iodide staining solution). Samples were gently mixed and incubated for 10 min in the dark on ice. Data acquisition was performed on a BD Accuri C6 flow cytometer (10,000 events were analyzed) and data analysis was performed using ModFit LT version 4.0 software (Verity Software, Inc., Topsham, ME USA).

Morphological characteristics by Romanowsky staining. To determine the effects of treatment on the size and morphological characteristics of K562 cells, the cells were harvested and fixed with 1% formaldehyde for 1 min at room temperature (20-25˚C). Slides were dried, and Romanowsky staining solution was added, rinsed with deionized water, air dried and observed under a Nikon inverted light microscope (Eclipse TE300; Nikon Corporation, Tokyo, Japan) at magnification, x40.

Evaluation of cluster of differentiation (CD)14$^+$, CD68$^+$, CD163$^+$ and CD42$^+$ surface markers. For assessment of monocytic differentiation induced by treatments, K562 cells were harvested and incubated for 30 min at room temperature simultaneously with phycoerythrin-conjugated anti-CD14 (cat. no. 340585; 1:20; Invitrogen; Thermo Fisher Scientific, Inc.), fluorescein isothiocyanate (FITC)-conjugated anti-CD68 (cat. no. 562117; 1:20; BD Biosciences) and allophycocyanin-conjugated anti-CD163 (cat. no. 556018; 1:20; BD Biosciences). To evaluate megakaryocytic lineage the cells were incubated with peridinin chlorophyll protein complex-conjugated anti-CD42a (cat. no. 340537; 1:20; BD Biosciences) in PBS with 1% FBS and 0.1% sodium azide for 30 min at 25˚C. Following incubation, samples were washed and resuspended in PBS and 10,000 events were recorded by flow cytometry (BD Accuri C6 flow cytometer). The CD14$^+$ cell population was gated and the CD68$^+$ and CD163$^+$ populations were evaluated.

Phagocytosis assay. K562 cells previously treated for 96 h (with bDLE, PMA or DMSO) and washed three times with PBS, were incubated with FITC-Dextran (0.1 mg/ml; molecular weight 70,000; Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 60 min. As a negative control, cells were incubated with FITC-Dextran at 4˚C for 60 min. To stop phagocytosis the cells were washed twice with cold PBS supplemented with 1% FBS. Phagocytosis was assessed by flow cytometry (10,000 events were analyzed) and quantified as a percentage of cellular uptake of FITC-Dextran.

Determination of nitric oxide (NO) by colorimetric assay. The supernatants of each treatment were used to determine NO production using the nitrate-nitrite colorimetric assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's protocol. Briefly, 40 μl of supernatants were mixed with 40 μl of assay buffer, 10 μl of enzyme cofactor and 10 μl of nitrate reductase, and incubated at room temperature for 3 h (to allow the conversion of nitrate to nitrite). Following 10 min of incubation in Griess reagent at room temperature, the absorbance was measured at 560 nm in a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Cytokine and chemokine production assessment by flow cytometry. Cytokine production was analyzed using a BD Cytometric Bead Array (CBA) Human Inflammatory Cytokines kit (BD Biosciences). For the evaluation, cell supernatants were harvested following 96 h incubation, and stored at -20˚C until analysis according to manufacturer's protocol. The cytokines evaluated were interleukin (IL)-1β, IL-6, IL-8 and tumor necrosis factor (TNF)-α and the evaluation of chemokine production included: Chemokine (C-X-C motif) ligand (CXCL)8/IL-8, chemokine (C-C motif) ligand (CCL)5/regulated on activation, normal T cell expressed and secreted (RANTES), CXCL9/monokine induced by gamma interferon, CCL2/monocyte chemoattractant protein-1 (MCP-1) and CXCL10/interferon gamma-induced protein 10. Cytokine and chemokine production were measured by flow cytometry (BD Accuri C6) according to the manufacturer's protocol, and CBA analysis was performed using FCAP Array™ software version 1.0 (Soft Flow, Inc., St. Louis Park, MN, USA).

Statistical analysis. All experiments were performed in triplicate, and statistical analysis was performed using analysis of variance followed by post hoc Dunnett's tests. SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for all statistical analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of bDLE on the viability of K562, MOLT-3, murine peritoneal macrophages and primary human monocytes. bDLE decreased the viability of K562 [0.5 U/ml (33.0%), 0.75 U/ml (93.2%) and 1 U/ml (96.8%)] and MOLT-3 [0.21 U/ml (18.8%), 0.28 U/ml (62.0%), 0.35 U/ml (67.1%), 0.5 U/ml (79.8%), 0.75 U/ml (87.5%) and 1 U/ml (94.7%)] leukemia cell lines in a dose-dependent manner ($P=0.03$), demonstrating that bDLE treatment induced a highly cytotoxic effect on the MOLT-3 cell line (Figs. 1 and 2). The relative viability of the primary human monocytes and murine peritoneal macrophages treated with bDLE remained at 75-80% (Fig. 3). PMA treatment weakly affected the viability of K562 cells, and did not affect the MOLT-3 cell line ($P=0.75$). DMSO treatment did not affect the leukemia cell line viability ($P=0.85$; Figs. 1 and 2).

bDLE induces arrest in the S and G_2/M phases of the cell cycle and inhibits cellular proliferation rate in the K562 cell line. bDLE treatments (0.07-0.35 U/ml) induced alterations in cell cycle progression, as shown in Fig. 4 and Table I. bDLE caused S phase arrest at all doses tested [0.07 U/ml (57.28%), 0.14 U/ml (59.62%), 0.21 U/ml (64.45%), 0.28 U/ml (58.68%) and 0.35 U/ml (58.64%)]. PMA or DMSO treatments decreased the percentage of cells in the S phase (16.09 and 45.77%, respectively), compared with the untreated cells (52.34%). bDLE or PMA treatments increased the

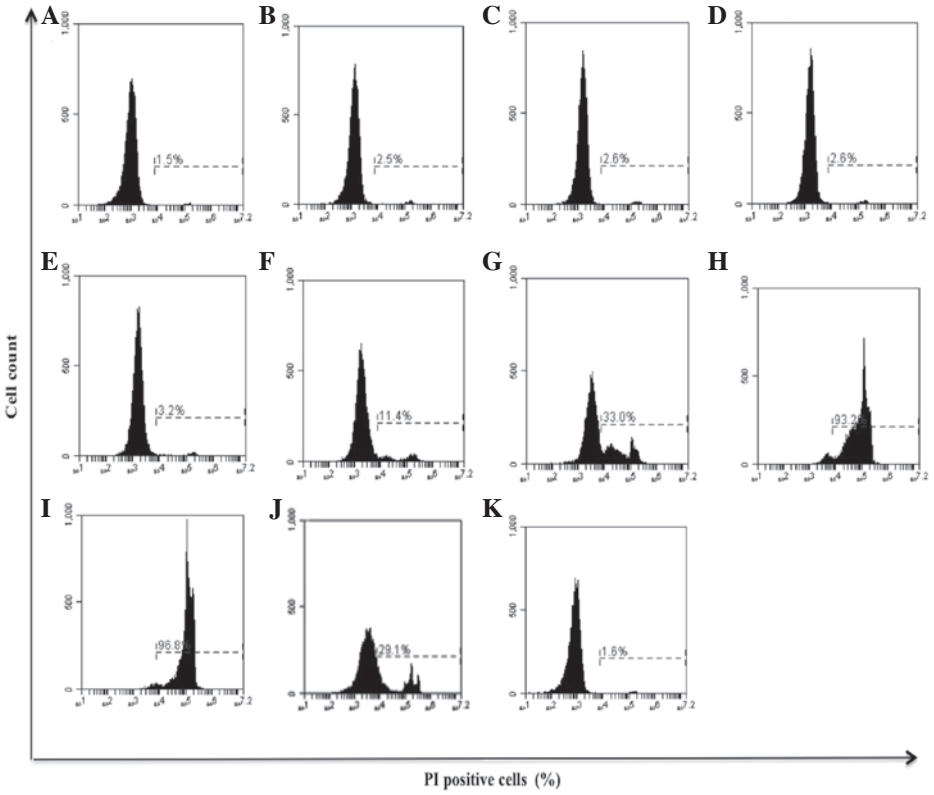

Figure 1. Effect of bDLE on the viability of K562 cells. K562 cells were seeded into plates with or without treatment. (A) Untreated cells, (B) 0.07 U/ml bDLE, (C) 0.14 U/ml bDLE, (D) 0.21 U/ml bDLE, (E) 0.28 U/ml bDLE, (F) 0.35 U/ml bDLE, (G) 0.5 U/ml bDLE, (H) 0.75 U/ml bDLE, (I) 1 U/ml bDLE, (J) 10 ng/ml phorbol myristate acetate or (K) dimethyl sulfoxide (1.5%, v:v) were incubated for 96 h. Cells were harvested and cell death was detected by PI staining and analyzed by flow cytometry. Flow cytometry data show representative results. PI, propidium iodide; bDLE, bovine dialyzable leukocyte extract.

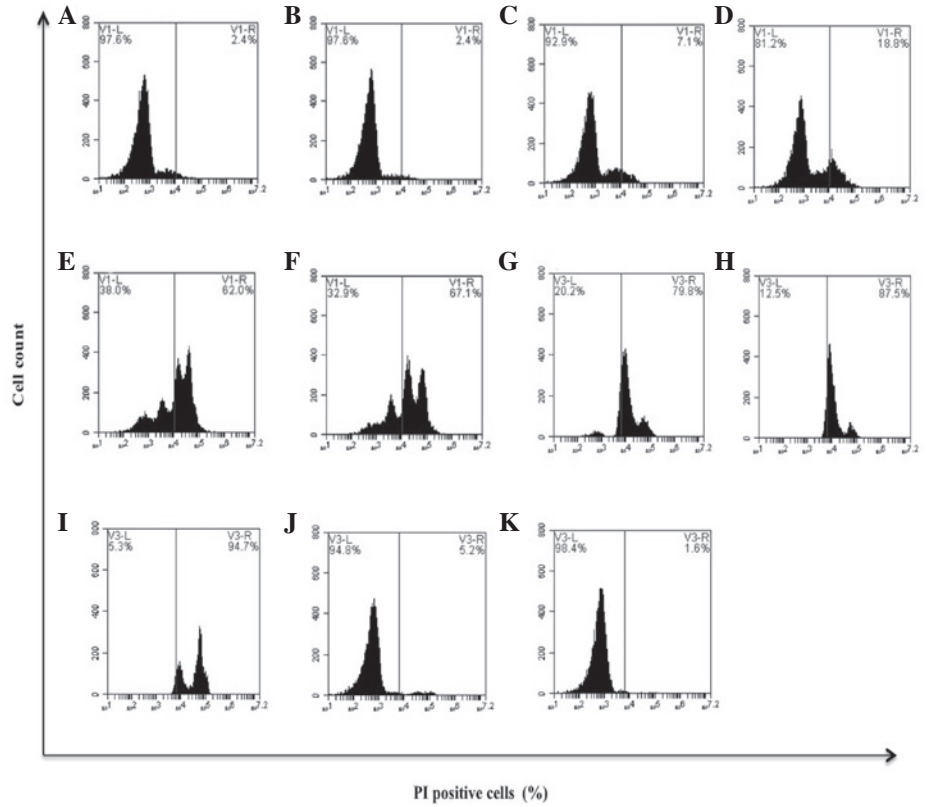

Figure 2. Effect of bDLE on the viability of MOLT-3 cells. MOLT-3 cells were seeded into plates with or without treatments. (A) Untreated cells, (B) 0.07 U/ml bDLE, (C) 0.14 U/ml bDLE, (D) 0.21 U/ml bDLE, (E) 0.28 U/ml bDLE, (F) 0.35 U/ml bDLE, (G) 0.5 U/ml bDLE, (H) 0.75 U/ml bDLE, (I) 1 U/ml bDLE, (J) 10 ng/ml phorbol myristate acetate or (K) dimethyl sulfoxide (1.5%, v:v) were incubated for 96 h. Cells were harvested and cell death was detected by PI staining and analyzed by flow cytometry. Flow cytometry data show representative results. PI, propidium iodide; bDLE, bovine dialyzable leukocyte extract.

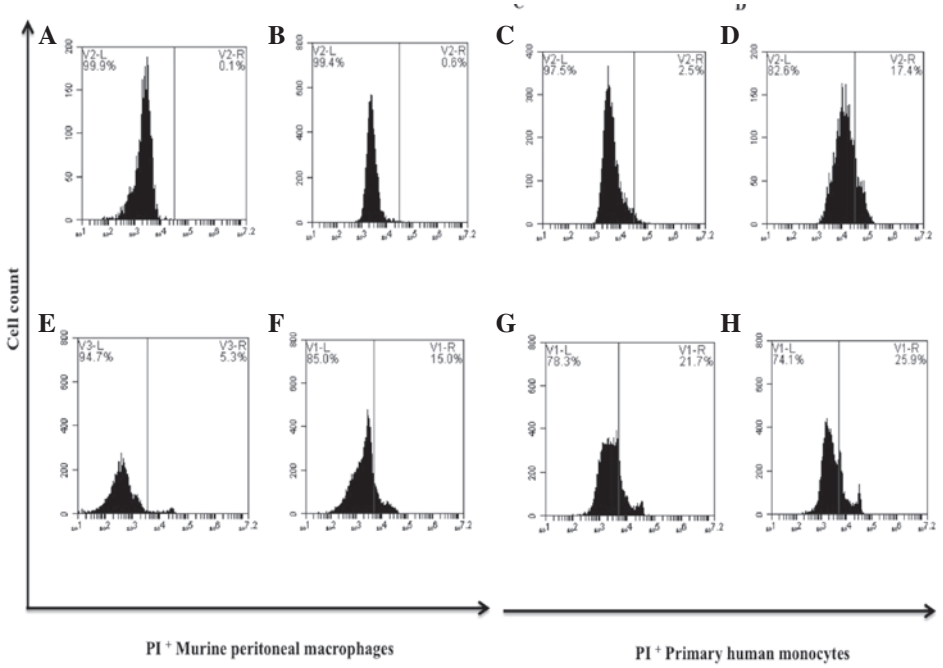

Figure 3. Effect of bDLE on the viability of murine peritoneal macrophages and primary human monocytes. A total of $1x10^5$ cells were seeded into plates with or without treatment. (A) Untreated murine peritoneal macrophages, (B) 0.5 U/ml bDLE, (C) 0.75 U/ml bDLE, (D) 1 U/ml bDLE, (E) untreated human monocytes, (F) 0.5 U/ml bDLE, (G) 0.75 U/ml bDLE or (H) 1 U/ml bDLE, were incubated for 96 h. Cells were harvested and cell death was detected by PI staining and analyzed by flow cytometry. PI, propidium iodide; bDLE, bovine dialyzable leukocyte extract.

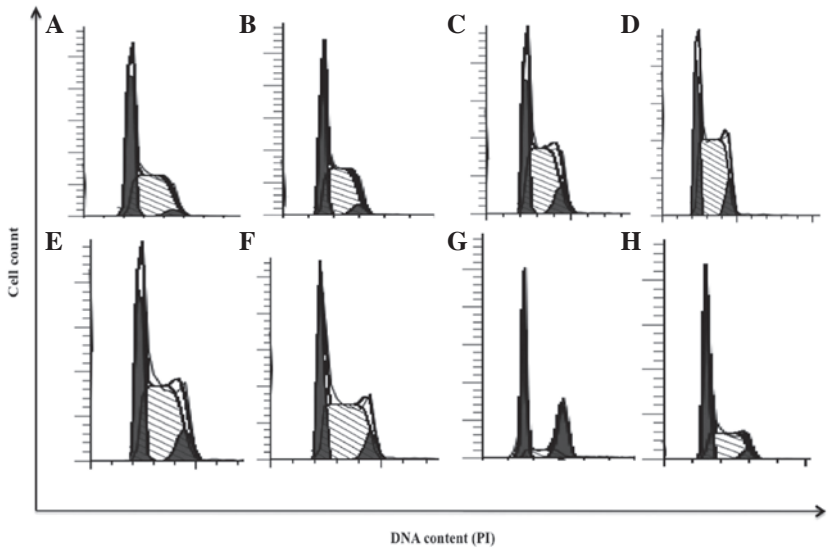

Figure 4. bDLE induces arrest in S and G_2/M phases of the cell cycle in K562 cells. (A) Untreated cells, (B) 0.07 U/ml bDLE, (C) 0.14 U/ml bDLE, (D) 0.21 U/ml bDLE, (E) 0.28 U/ml bDLE, (F) 0.35 U/ml bDLE, (G) 10 ng/ml phorbol myristate acetate or (H) dimethyl sulfoxide (1.5%, v:v) were incubated for 96 h, and cell cycle progression analysis was performed using the Cycle Test™ Plus DNA Reagent kit according to the manufacturer's protocol and ModFit software. PI, propidium iodide; bDLE, bovine dialyzable leukocyte extract.

percentage of cells in the G_2/M phase [0.07 U/ml (5.11%), 0.14 U/ml (9.57%), 0.21 U/ml (8.19%), 0.28 U/ml (9.46%), 0.35 U/ml (8.27%) and PMA (33.07%)]. Untreated cells and cells treated with DMSO showed no affect on the percentage of cells in the G_2/M phase (3.24 and 4.81%, respectively). Furthermore, a significant decrease in the percentage of cells in the G_0/G_1 phase was detected following bDLE treatment [0.07 U/ml (37.61%), 0.14 U/ml (30.80%), 0.21 U/ml (27.36%), 0.28 U/ml (31.86%) and 0.35 U/ml (33.09%)] compared with PMA (50.84%), DMSO (49.42%) and untreated cells (44.43%). Furthermore, bDLE treatment for 96 h significantly inhibited the K562 cell proliferation rate in a dose-dependent manner (P=0.05) [0.07 U/ml (1,550,000 cells/ml), 0.14 U/ml (1,125,000 cells/ml), 0.21 U/ml (775,000 cells/ml), 0.28 U/ml (550,000 cells/ml) and 0.35 U/ml (400,000 cells/ml]. Treatment with PMA (75,000 cells/ml) and DMSO (1,000,000 cells/ml) obtained similar results when compared with untreated cells (1,650,000 cells/ml), in which the cellular proliferation was the highest (P=0.05) (Fig. 5). Subsequently, the K562 cells previously differentiated by treatments with bDLE, PMA

Table I. bDLE induces arrest in the S and G_2/M phases of the cell cycle in K562 cells.

	Percentage of cells, %		
Treatment	G_0/G_1	S	G_2/M
Untreated cells	44.43	52.34	3.24
bDLE 0.07 U/ml	37.61	57.28	5.11
bDLE 0.14 U/ml	30.80	59.62	9.57
bDLE 0.21 U/ml	27.36	64.45	8.19
bDLE 0.28 U/ml	31.86	58.68	9.46
bDLE 0.35 U/ml	33.09	58.64	8.27
PMA	50.84	16.09	33.07
DMSO	49.42	45.77	4.81

Untreated cells and treated cells with bDLE, PMA and DMSO were incubated for 96 h, and cell cycle progression analysis was performed using a Cycle Test™ Plus DNA Reagent kit according to the manufacturer's protocol. The table shows the percentage of cells in each phase of the cell cycle and analysis was performed using ModFit software. bDLE, bovine dialyzable leukocyte extract; PMA, phorbol myristate acetate; DMSO, dimethyl sulfoxide.

Table II. bDLE affects IL-6, IL-8 and TNF-α production in K562 cells.

	Cytokine production, pg/ml			
Treatment	IL-1β	IL-6	IL-8	TNF-α
Untreated cells	0.0	126.03	18.84	830.47
bDLE 0.07 U/ml	0.0	307.83	56.22	1384.69
bDLE 0.14 U/ml	0.0	342.26	54.4	1882.90
bDLE 0.21 U/ml	0.0	293.94	23.22	950.22
bDLE 0.28 U/ml	0.0	213.17	27.06	431.83
bDLE 0.35 U/ml	0.0	115.18	34.95	310.54
PMA	7.17	991.06	17915.32	2347.26
DMSO	0	4.29	2.96	37.96

K562 cells were treated with bDLE, PMA or DMSO, and following 96 h of incubation the supernatants were harvested and prepared according to the manufacturer's protocol (Cytometric Bead Array Human Inflammatory Cytokines kit; BD Biosciences). A total of 10,000 events were analyzed by flow cytometry. bDLE, bovine dialyzable leukocyte extract; IL, interleukin; TNF, tumor necrosis factor; PMA, phorbol myristate acetate; DMSO, dimethyl sulfoxide.

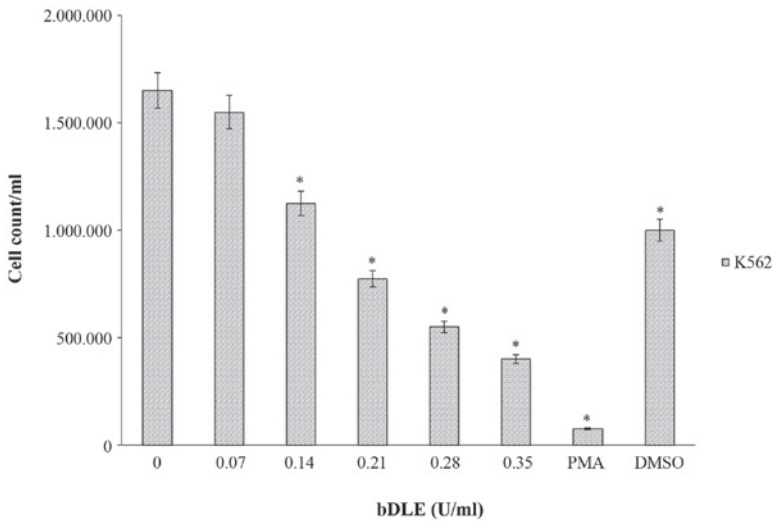

Figure 5. bDLE inhibits the cell proliferation rate of K562 cells. K562 cells were treated with or without bDLE, PMA or DMSO and incubated for 96 h. Following the incubation, the cells were harvested and resuspended in PBS and the cell proliferation percentage was estimated by cell counting for each treatment group by trypan blue staining. *P<0.05 vs. control. bDLE, bovine dialyzable leukocyte extract; PMA, phorbol myristate acetate; DMSO, dimethyl sulfoxide.

or DMSO for 96 h were incubated for 10 days, and it was observed that the bDLE treatment decreased the rate of cell growth in a dose-dependent manner (P=0.03) compared with the control. The cellular growth was significantly decreased following PMA and DMSO treatment (P=0.005) compared with the control (Fig. 6).

bDLE induces monocytic/macrophage and megakaryocytic differentiation in K562 cells. Microscopic examination by Romanowsky staining in the K562 cells treated with bDLE (0.07-0.35 U/ml) for 96 h, revealed morphological changes characteristic of monocytic/macrophage differentiation occurring in a dose-dependent manner. The cells developed pseudopodia extensions, increased cell size and cytoplasm to nuclear ratio, as well as attachment to the culture dishes, compared with untreated cells. These morphological changes were similar to the effects obtained by PMA treatment in terms of induced monocyte/macrophage differentiation in K562 cells (Fig. 7). In order to confirm the monocytic/macrophage differentiation induced by the bDLE and inducer positive controls, the treated cells were monitored for expression of $CD14^+$ monocytic marker. Flow cytometry histograms revealed an increased expression of $CD14^+$ on K562 cells following treatment with bDLE in a dose-dependent manner:

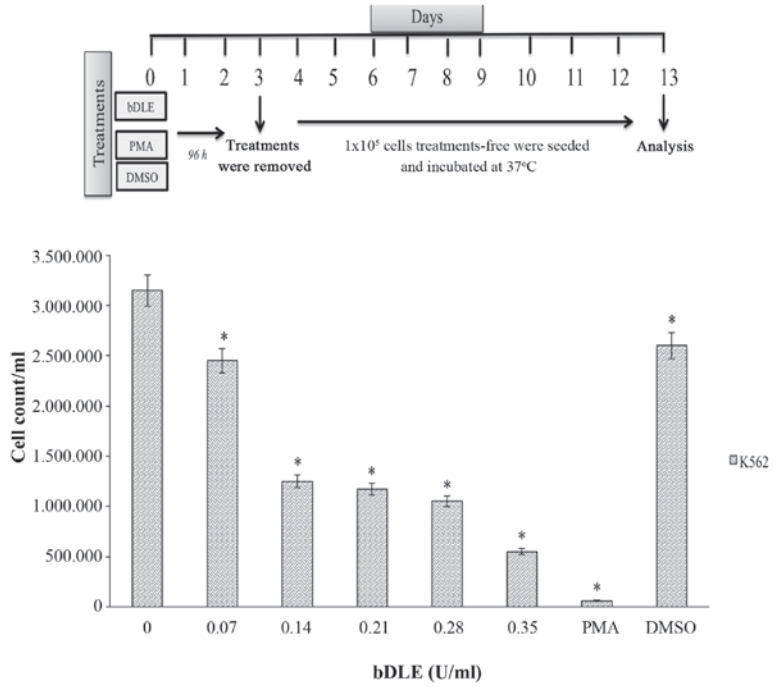

Figure 6. bDLE inhibits the cell proliferation rate following induction of differentiation in K562 cells. K562 cells were treated with bDLE, PMA or DMSO following 96 h of incubation. The cells were harvested, centrifuged and washed with RPMI-1640 medium, seeded at $1x10^5$ cells/well and incubated for 10 days. Cell proliferation was evaluated by trypan blue staining. The timeline of treatments is shown in the upper panel. Data are expressed as the mean ± standard deviation. *P<0.05 vs. control. bDLE, bovine dialyzable leukocyte extract; PMA, phorbol myristate acetate; DMSO, dimethyl sulfoxide.

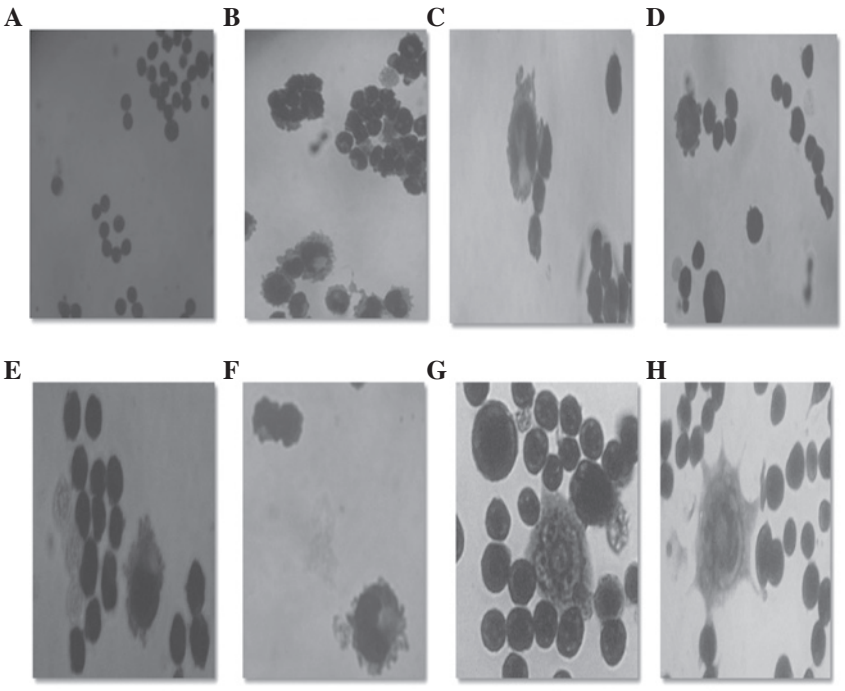

Figure 7. bDLE induces monocytic differentiation in K562 cells. K562 cells were treated with (A) RPMI-1640 (untreated cells), (B) 0.07 U/ml bDLE, (C) 0.14 U/ml bDLE, (D) 0.21 U/ml bDLE, (E) 0.28 U/ml bDLE or (F) 0.35 U/ml bDLE, and positive controls (G) 10 ng/ml phorbol myristate acetate or (H) dimethyl stulfoxide (1.5%, v:v), were incubated for 96 h. Morphological changes were visualized by microscopy (magnification, x40) of Romanowsky staining and the images were photographed. bDLE, bovine dialyzable leukocyte extract.

0.07 U/ml (14.9%), 0.14 U/ml (24.4%), 0.21 U/ml (31.0%), 0.28 U/ml (35.2%) and 0.35 U/ml (39.7%), compared with the untreated cells that expressed 6.1%, DMSO (3.3%) or PMA (29.6%) treatments (Fig. 8). When the $CD14^+$ cell population was gated the surface markers $CD68^+$ (M1-like phenotype) and $CD163^+$ (M2-like phenotype), characteristic of macrophage polarization, were analyzed, it was determined that bDLE treatment at various doses induced cells toward an

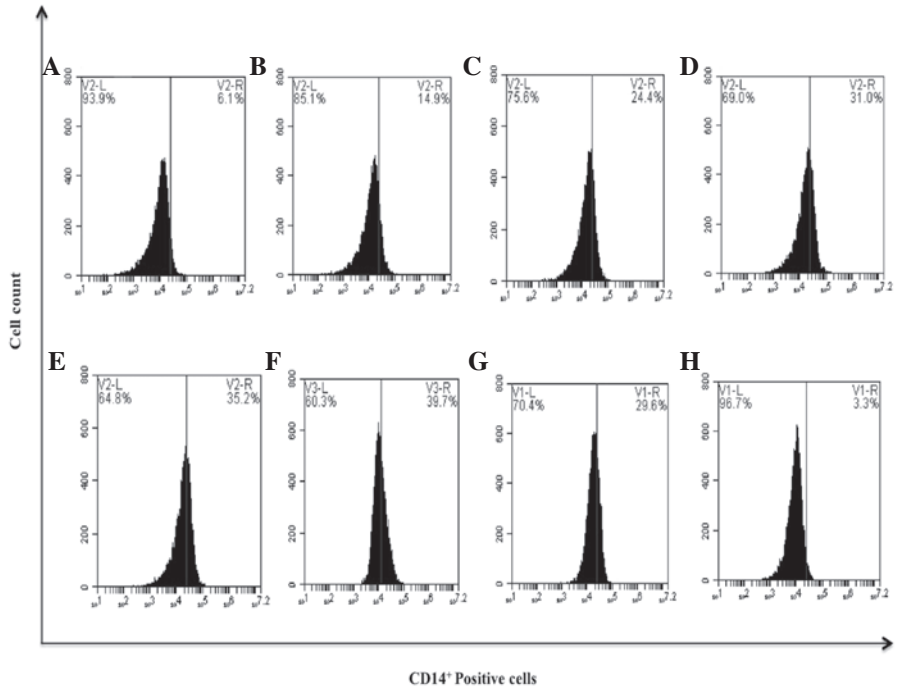

Figure 8. bDLE induces monocytic differentiation in K562 cells assessed by the expression of the CD14+ surface marker. (A) Untreated cells, (B) 0.07 U/ml bDLE, (C) 0.14 U/ml bDLE, (D) 0.21 U/ml bDLE, (E) 0.28 U/ml bDLE, (F) 0.35 U/ml bDLE, (G) 10 ng/ml phorbol myristate acetate or (H) dimethyl sulfoxide (1.5%, v:v) were incubated for 96 h. Cells were harvested and incubated with anti-CD14 phycoerythrin Texas red in PBS with 1% fetal bovine serum and 0.1% sodium azide for 30 min at 4˚C. Samples were washed and resuspended in PBS, and 10,000 events were analyzed by flow cytometry. Flow cytometry data shows representative results from one of three independent experiments. CD, cluster of differentiation; bDLE, bovine dialyzable leukocyte extract.

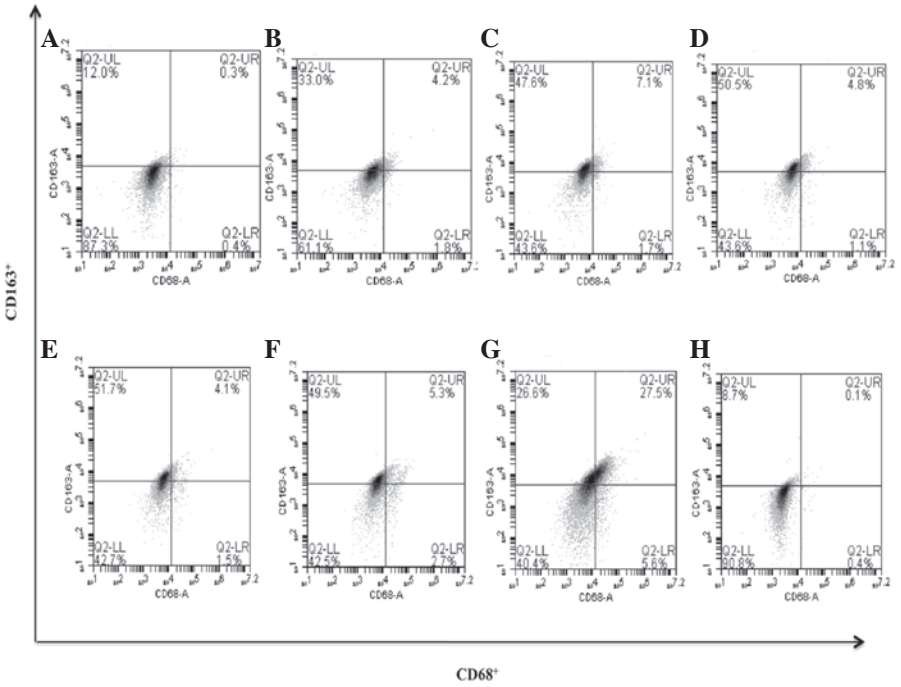

Figure 9. bDLE induces macrophage polarization to M2 assessed by expression of the CD163+ marker in K562 cells. (A) Untreated cells, (B) 0.07 U/ml bDLE, (C) 0.14 U/ml bDLE, (D) 0.21 U/ml bDLE, (E) 0.28 U/ml bDLE, (F) 0.35 U/ml bDLE, (G) 10 ng/ml phorbol myristate acetate or (H) dimethyl sulfoxide (1.5%, v:v) were incubated for 96 h. Cells were harvested and incubated with anti-CD68 and anti-CD163 in PBS with 1% fetal bovine serum and 0.1% sodium azide for 30 min at 4˚C. Samples were washed and resuspended in PBS, and 10,000 events were analyzed by flow cytometry. CD, cluster of differentiation; bDLE, bovine dialyzable leukocyte extract.

M2-like phenotype, increasing the CD163+ surface marker levels in a significant manner (P=0.05) [0.07 U/ml (33.0%), 0.14 U/ml (47.6%), 0.21 U/ml (50.5%), 0.28 U/ml (51.7%), 0.35 U/ml (49.5%) compared with the untreated cells (12.0%), PMA (26.6%) or DMSO (8.7%) treatments]. No difference (P=0.15) was identified between treatments when the CD68+

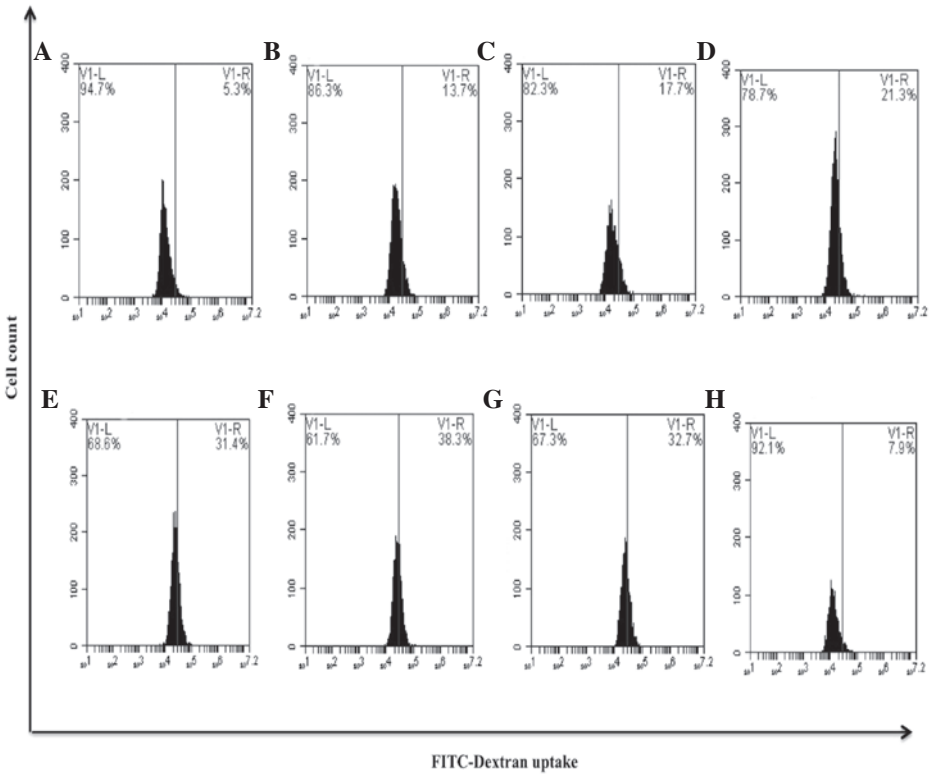

Figure 10. bDLE increases the phagocytic capacity of K562 cells. (A) Untreated cells, (B) 0.07 U/ml bDLE, (C) 0.14 U/ml bDLE, (D) 0.21 U/ml bDLE, (E) 0.28 U/ml bDLE, (F) 0.35 U/ml bDLE, (G) 10 ng/ml phorbol myristate acetate or (H) dimethyl sulfoxide (1.5%, v:v) were incubated for 96 h. Cells were collected and FITC-Dextran was added and incubated for 1 h at 37˚C. Flow cytometry data show representative results from one of three independent experiments. FITC, fluorescein isothiocyanate; bDLE, bovine dialyzable leukocyte extract.

surface marker was evaluated. The double positive population of $CD68^+/CD163^+$ increased slightly with bDLE treatment at doses of 0.07 U/ml (4.2%), 0.14 U/ml (7.1%), 0.21 U/ml (4.8%), 0.28 U/ml (4.1%) and 0.35 U/ml (5.3%), with high levels of expression following PMA treatment (27.5%), and no effect on the percentage of marker-positive cells following DMSO treatment compared with untreated cells (0.3%) (P=0.35) (Fig. 9). It is well-known that differentiated macrophages possess increased phagocytic capacity (11). The present study observed that the bDLE treatments (0.07-0.35 U/ml) significantly increased (P=0.05) in a dose-dependent manner the ability to uptake FITC-Dextran reagent: 0.07 U/ml (13.7%), 0.14 U/ml (17.7%), 0.21 U/ml (21.3%), 0.28 U/ml (31.4%) and 0.35 U/ml (38.3%), similar to PMA treatment (32.7%), when compared with untreated cells (5.3%). DMSO treatment (7.9%) exerted a similar effect to that observed in the untreated control cells (Fig. 10). Furthermore, megakaryocytic differentiation was assessed by the expression of the $CD42a^+$ marker, and it was observed that PMA had the potential to induce the expression of this surface marker in 24.8% of cells and bDLE treatment induced lower expression levels in a dose-dependent manner (P=0.05) [(0.07 U/ml (4.8%), 0.14 U/ml (7.6%), 0.21 U/ml (8.9%), 0.28 U/ml (13.1%) and 0.35 U/ml (13.9%)]. No difference (P=0.38) was observed between the DMSO treated (0.2%) and untreated cells (0.9%). The results of the present study demonstrated that bDLE had the potential to induce monocytic/macrophage differentiation (Figs. 7-10), and had a lower capacity to induce megakaryocytic differentiation (Fig. 11).

Determination of cellular resistance. To determine if the previously differentiated cells remained sensitive to treatment with bDLE, cells were treated with cytotoxic doses. It was observed that treatment at 1 U/ml induced cell death in 84.4% (Fig. 12). Furthermore, murine peritoneal macrophages and primary human monocytes treated with the an identical dose of bDLE demonstrated 17.4 and 27.9% cell death, respectively (Fig. 3).

bDLE decreases NO production and affects the cytokine and chemokine levels in K562 cells. The results of the present study demonstrated that bDLE treatment significantly decreased (P=0.05) NO production in a dose-dependent manner in K562 cells following 96 h of incubation [0.07 U/ml (57.12 M), 0.14 U/ml (50.72 M), 0.21 U/ml (44.32 M), 0.28 U/ml (30.72 M), and 0.35 U/ml (13.12 M)] compared with untreated cells (60.64 M) and PMA treated cells (51.68 M). DMSO treatment significantly decreased NO production (17.2 M), similar to the effects caused by 0.35 U/ml bDLE (Fig. 13). Furthermore, cytokine (IL-1b, IL-6, IL-10, TNF-α and IL-12 p70) and chemokine production (CCL2/MCP-1, CCL5/RANTES and CXCL8/IL-8) were evaluated in the K562 cell line treated with the inducers of differentiation. Untreated cells demonstrated expression of IL-6, IL-8 and TNF-α, and IL-1b and IL-12p70 were not expressed (Table II); all chemokines evaluated in the present study were observed to be expressed in untreated cells (Table III). PMA treatment induced overexpression of the chemokines (Table III) and cytokines (Table II) evaluated, but IL-12p70 was not expressed. bDLE

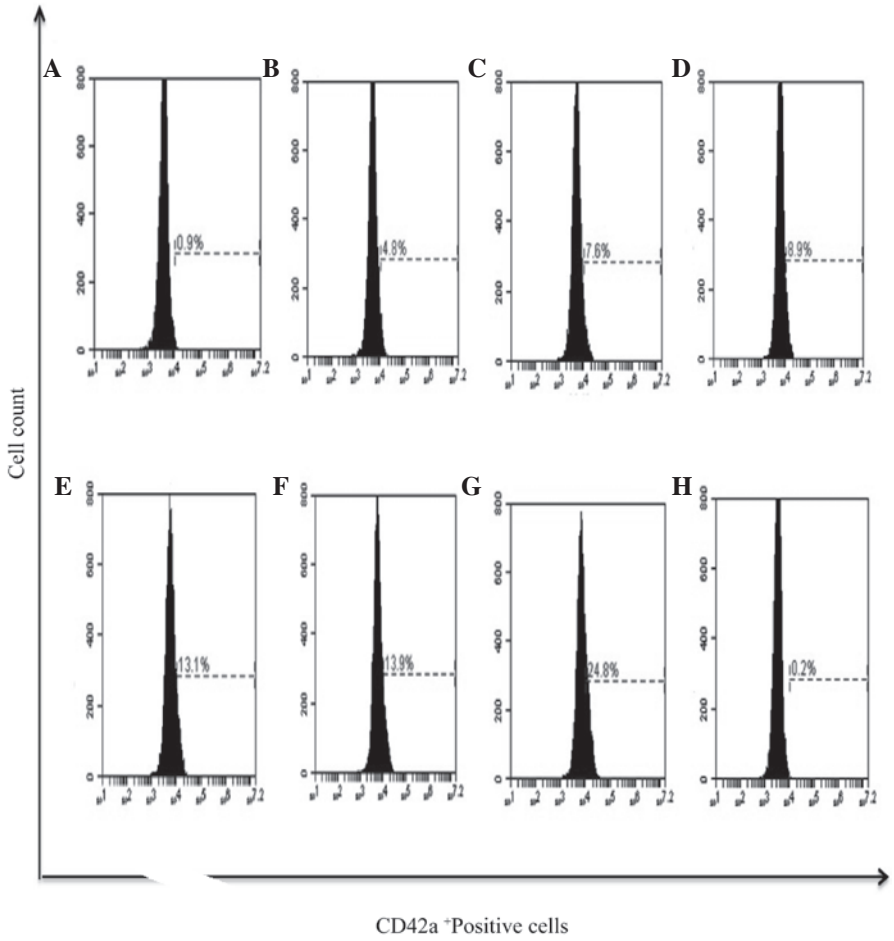

Figure 11. bDLE increases the expression of CD42a+ megakaryocytic marker differentiation in K562 cells. (A) Untreated cells, (B) 0.07 U/ml bDLE, (C) 0.14 U/ml bDLE, (D) 0.21 U/ml bDLE, (E) 0.28 U/ml bDLE, (F) 0.35 U/ml bDLE, (G) 10 ng/ml phorbol myristate acetate or (H) dimethyl sulfoxide (1.5%, v:v) were incubated for 96 h. Cells were harvested and incubated with peridinin chlorophyll protein complex conjugated-anti-CD42a in PBS with 1% fetal bovine serum and 0.1% sodium azide for 30 min at 4˚C. Samples were washed and resuspended in PBS, and 10,000 events were analyzed by flow cytometry. Flow cytometry data show representative results from one of three independent experiments. CD, cluster of differentiation; bDLE, bovine dialyzable leukocyte extract.

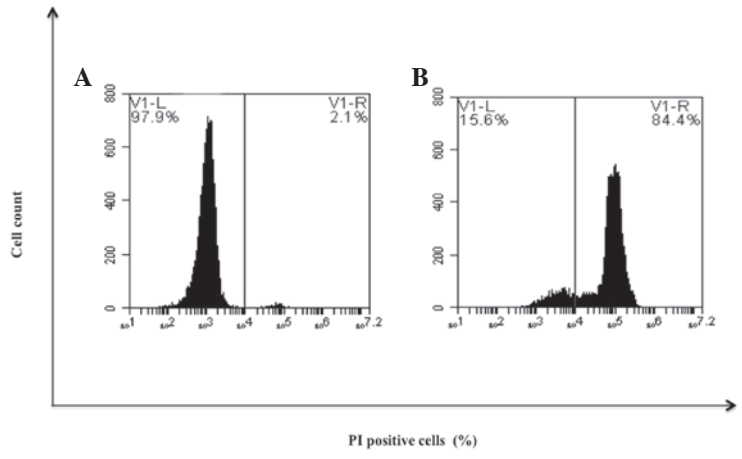

Figure 12. Effect of cytotoxic concentrations of bDLE (1 U/ml) on K562 cells treated previously with bDLE (0.35 U/ml). A total of $1x10^5$ K562 cells were seeded into plates with or without treatment with bDLE (0.35 U/ml) and incubated for 96 h. Cells were harvested, seeded at $1x10^5$ cells/well and incubated with bDLE (1 U/ml) for 96 h. Cell death was detected by PI staining and analyzed by flow cytometry. (A) Untreated cells and (B) bDLE 1 U/ml. PI, propidium iodide; bDLE, bovine dialyzable leukocyte extract.

treatment at doses of 0.07, 0.14, 0.21 and 0.28 U/ml increased the expression of all cytokines evaluated, except IL-1b and IL-12 p70. bDLE at a dose of 0.28 U/ml decreased TNF-α expression. At doses of 0.35 U/ml the expression levels of IL-6 and TNF-α were the lowest compared to the control and PMA treatment groups, and IL-8 expression was elevated.

Table III. bDLE affects chemokine production in K562 cells.

Treatment	Chemokine production, pg/ml		
	CCL2/ MCP-1	CCL5/ RANTES	CXCL8/ IL-8
Untreated cells	25.07	0.7	17.84
bDLE 0.07 U/ml	70.42	4.17	47.35
bDLE 0.14 U/ml	66.07	2.76	46.29
bDLE 0.21 U/ml	74.02	0.7	19.44
bDLE 0.28 U/ml	115.81	0	20.05
bDLE 0.35 U/ml	164.15	0.31	28.23
PMA	9994.53	143.78	12105.23
DMSO	10.22	0	2.58

K562 cells were treated with bDLE, PMA or DMSO. Following 96 h of incubation the supernatants were harvested and prepared according to the manufacturer's protocol (Cytometric Bead Array Human Inflammatory Cytokines kit; BD Biosciences). A total of 10,000 events were analyzed by flow cytometry. bDLE, bovine dialyzable leukocyte extract; PMA, phorbol myristate acetate; DMSO, dimethyl sulfoxide; CCL, chemokine (C-C motif) ligand; RANTES, regulated on activation, normal T cell expressed and secreted; CXCL, chemokine (C-X-C motif) ligand; IL, interleukin.

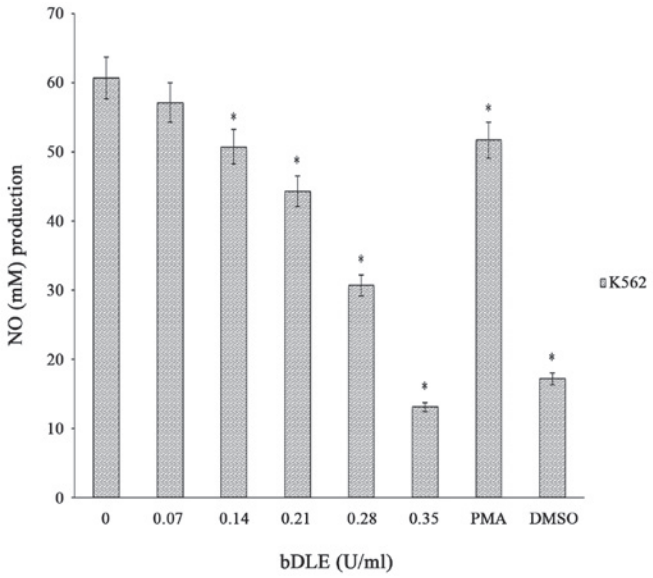

Figure 13. bDLE decreases NO production in K562 cells. K562 cells were treated with or without bDLE, PMA or DMSO and incubated for 96 h. NO production was measured in the supernatants using a commercial kit (nitrate-nitrite colorimetric assay kit according to the manufacturer's protocol. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 vs. control. NO, nitric oxide; bDLE, bovine dialyzable leukocyte extract; PMA, phorbol myristate acetate; DMSO, dimethyl sulfoxide.

bDLE increased the expression of chemokines CCL2/MCP-1 in a dose-dependent manner. At doses of 0.07 and 0.14 U/ml increased expression of CCL5/RANTES was observed, and there was no difference in expression between the doses evaluated compared to the control. All the doses evaluated increased the expression of CXCL8/IL-8 (Table III). DMSO treatment reduced the expression of all cytokines (Table II) and chemokines evaluated (Table III).

Discussion

The discovery of novel compounds with differentiation-inducing activity in CML is required, as the degree of sensitivity of cancer cells to ATRA is not universal (12). Previously, it has been reported that bDLE induces cell death in several cancer cell lines (1). The present study demonstrated a novel antileukemia activity of bDLE, as it exerted cytotoxic effects on K562 and MOLT-3 leukemia cell lines, without affecting the cell viability of monocytes and macrophages. Similar results are observed when K562 cells are treated with Chemlali olive leaf extract (7) or with Huangqi (Hex) extract, which induce K562 and HEL cells to undergo cell differentiation and death in a dose-dependent manner (13). Cell differentiation therapy is a novel proposal that focuses on reducing the adverse effects of chemotherapy and consists of forcing malignant cells to undergo terminal differentiation. This treatment has attracted great interest, particularly for treating leukemia (14). Numerous compounds have been reported to induce differentiation of leukemia cells and some of these are already approved for clinical use (7). The arrest of cell cycle progression allows cells to undergo other processes, including apoptosis and differentiation. By investigating the effect of bDLE on the cell cycle distribution it was demonstrated that bDLE arrested at S and G_2/M phases cell cycle, similar results were obtained by Imen *et al* (15) (2014) using Chemlali olive leaf extract. The present study observed that bDLE treatment for 96 h significantly inhibited the cell proliferation rate in K562 cells, similar to the effects of PMA treatment. When these treated cells were incubated by for 10 days with no treatment, the percentage of cell proliferation was reduced compared with untreated cells, suggesting that K562 cells may have undergone a differentiation process. bDLE treatment induced K562 cell differentiation toward a monocyte/macrophage lineage. This was determined by observation of morphological changes and increased expression of the monocytic differentiation antigens, $CD14^+$ and $CD163^+$. The $CD163^+$ marker has been observed in acute myeloid leukemia with monocytic differentiation (16). Furthermore, the present study determined that K562 cells differentiated with bDLE increased their phagocytic capacity, similar to cells treated with PMA. It is known that K562 cells can be terminally differentiated toward the macrophage lineage using PMA (17). The mechanism of action of bDLE in decreasing NO production may be associated with antioxidant properties (4). There is evidence of certain antioxidants, including grape seed procyanidins, inducing *in vitro* cell differentiation in leukemia cell lines including K562 cells (8). bDLE also induced the expression of the $CD42a^+$ marker, which is characteristic of megakaryocytic differentiation, and is considered to be a classical inductor of megakaryocytic differentiation (18). The macrophage adopts distinct functional phenotypes in response to pathogenic and cytokine signals, leading to the description of two divergent forms of macrophage activation: M1 and M2 (19). In a tumor setting, M1-like macrophages are thought to promote antitumor immunity, whereas M2-like tumor-associated macrophages stimulate angiogenesis and tissue repair (19). Enhanced cytokine and chemokine production by flow cytometry was observed in bDLE and PMA treatments, although this effect

was more enhanced with PMA treatment, suggesting that the production of these molecules is associated with a process of cell differentiation. The fact that bDLE at high doses affected the viability of K562 cells previously differentiated with the identical treatment, and did not affect the viability of murine peritoneal macrophage and human monocytes, may suggest selectivity of action by receptors.

The present study demonstrated for the first time that bDLE exhibits an antileukemia effect on human CML cells. bDLE was shown to inhibit the proliferation of K562 cells and induced differentiation toward the monocyte/macrophage and megakaryocytic lineages through increased expression of molecules associated with differentiation. The present study provides an insight into the underlying mechanism by which bDLE exhibits its antileukemic activity.

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