Akt1 players promote PMA U937 cell line differentiation into macrophage-like cells

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Abstract

Purpose – Monocytes are a leukocytes' subset that plays an important role in immunity. Protein kinase B (AKT) is involved in monocytes' survival, proliferation and differentiation. Using phorbol 12-myristate 13-acetate (PMA) as an inducer for cell line U937 differentiation into macrophage-like cells may be used as a model for cancer cell therapy or other biomedical research studies. The authors investigated the Akt1 signaling pathway's involvement with PMA as a differentiating agent and survival in the U937 cell line.

Design/methodology/approach – PMA was utilized to stimulate the differentiation of the U937 cell line into macrophage-like cells at a concentration of 10 nM. Akt1-phosphorylated Serine 473, Bad-phosphorylated Serine 136 and Caspase9-phosphorylated Serine 196 were tested by flow cytometry for the involvement of the Akt1 signaling pathway during differentiation in addition to the expression of CD14, CD206 and CD83. DNA cell cycle variation analysis was done using PI staining and cell viability and apoptosis detection using Annexin V and PI flow cytometry.

Findings – There was a decrease in phosphorylated Akt1 and Bad activation and an increase in Caspase9 activation, with an increase in surface markers CD14, CD206 and CD83 acquired by PMA-differentiated cells. DNA cell cycle analysis revealed cell accumulation in the G2/M phase and fewer cells in the S phase of PMA-induced U937. Apoptosis induction for Ly294002 or Wortmannin-inhibited cells and part of PMA-induced cells were detected.

Originality/value – These results may be used to create a model for biomedical research studies and advance the understanding of the mechanism involving differentiation of the U937 cell line.

Keywords Caspase 9, Cell survival, Differentiation, G2/M arrest

Paper type Research paper

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Experiments performance: Halla Falih Bakheit.
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Introduction

Monocytes are a type of leukocyte with critical roles in inflammation, pathogen challenge and homeostasis. These immune cells, produced by the myeloid progenitors in the bone marrow, account for about 10% of leukocytes in the bloodstream and ultimately migrate to peripheral tissues (van Furth & Sluiter, 1986). Monocytes are characterized by the high expression of CD14, a lipopolysaccharide-binding protein targeting endotoxins, and demonstrate extensive plasticity and heterogeneity in response to immunological challenges. Monocytes are programmed to undergo apoptosis within 24–48 h without specific survival signals (Cline, Lehrer, Territo, & Golde, 1978; Goyal *et al.*, 2002) or get differentiated into either macrophages or antigen-presenting cells upon cell activation (Lu & Pitha, 2001). Phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway is involved in cell survival and proliferation (Brazil & Hemmings, 2001; Datta, Brunet, & Greenberg, 1999). AKT-1 is a 57 kDa serine/threonine protein kinase, phosphorylating and regulating the function of many cellular proteins involved in metabolism, survival, apoptosis, differentiation and proliferation (Brazil, Yang, & Hemmings, 2004). Thr308 and Ser473 are two distinct phosphorylation sites for Akt activation. Akt is activated by phospholipid binding (PIP3) and activation loop phosphorylation at threonine 308 by PDK1 (phosphoinositide-dependent protein kinase-1) (Dangelmaier *et al.*, 2014), C-terminus phosphorylation at serine 473; this will lead to complete activation of Akt and could be achieved by one of the following: the PI3K-related kinase (PIKK) family, the mTOR/rictor complex (mTORC2), the DNA-dependent protein kinase (DNA-PK) (Fayard, Tintignac, Baudry, & Hemmings, 2005) and the TCR (Yang, Qiao, Ying, Zhang, & Yin, 2010). Constitutive AKT-1 activation is essential for the survival of monocyte differentiated macrophages (Liu, Perlman, Pagliari, & Pope, 2001) and involves downstream effectors such as Bad and Caspase-9 (Datta *et al.*, 1997; Vincent & Feldman, 2002). Immortalized monocyte-like cells, U937 cells, are one of the cell lines mimicking human peripheral blood mononuclear cells (PBMC). The U937 cells were isolated from the histiocytic lymphoma of a 37-year-old male patient (Sundstrom & Nilsson, 1976). U937 cells, depending on the initiators, can adopt a monocytic phenotype and differentiate into either macrophages or dendritic cells. This investigation aimed to explore new signaling pathways during U937 cells' differentiation into macrophage-like cells by PMA, Phorbol 12-Myristate 13-Acetate. Here, we tested the AKT-1 signaling pathway and some of its downstream players, including Bad and Caspase9 activation involvement, in addition to the expression of regulatory cell surface markers CD14, CD206 and CD83. In the present study, we highlighted how AKT signaling appears as a vital regulator of differentiation, survival and surface expression of CD14, CD206 and CD83 in PMA-induced U937 cell differentiation to macrophage-like cells. Furthermore, PMA promoted U937 cell accumulation in the G2M phase of the cell cycle during differentiation. Our findings can provide a valuable model of the molecular mechanism of cell differentiation and survival as it relates to monocyte development.

Material and methods

Cells

U937 cells were purchased from the American Type Culture Collection (ATCC, No. CRL-1593.2, Virginia, USA). Cells were grown in RPMI-1640 medium (American Type Culture Collection, Manassas, VA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco by life technologies corporation, NY, USA), 1% of Penicillin-streptomycin (Gibco by life technologies corporation, NY, USA) and incubated at 37°C, 95% humidity, and 5% CO_2. For cells viability and cells count, trypan blue execution method with 0.4% trypan blue stain using a hematocytometer was carried out. On average, viable cells more than 95% were used for the tests at a cell density of 1×10^6 cells/ml were used per each test.

Antibodies and reagents

All antibodies used for protein expression of CD14, CD206 and CD83, in addition to AKT phosphorylation and annexin V-FITC apoptosis detection kit, were obtained from BD Biosciences, NJ, USA. Antibodies for caspase9 pSer196 and bad pSer136 phosphorylation were from Santa Cruz Biotechnology, Texas, USA. Ly294002 and Wortmannin inhibitors were obtained from Cell Signaling Technology, Massachusetts, USA. PMA was from Abcam Biochemicals, Cambridge, UK.

U937 cell line activation and inhibition

U937 cells were plated in 24-well plates (5×10^5 cells/ml/well). Ly294002 (50 μM) (Cell Signaling Technology) or Wortmannin (1 μM) (Cell Signaling Technology) were used to inactivate the AKT pathway for 1 hour at 37ºC before cell stimulation with PMA (10 nM) (Abcam Biochemicals). Control cells were either stimulated with PMA (10 nM) to induce macrophage differentiation or vehicle control. Cells were first incubated for 24 hours and used for protein phosphorylation detection studies or changed their media with fresh RPMI-1640 complete media, then incubation expanded to more than 48 hours at 37ºC, 95% humidity and 5% CO2 incubator to assess morphological changes, cell cycle analysis, annexin V apoptosis detection and cell surface marker expression. Cells pictures were taken using an Olympic light microscope with magnification using a 40× objective.

Protein phosphorylation detection by flow cytometry

Flow cytometry BD phosflow Protocol III analysis followed the manufacturer's instruction to detect the following protein phosphorylation: phospho-Akt serine 473 (BD Biosciences, Alexa Fluor 647 mouse anti-Akt (pSer473)); Caspase9 pSer196 phosphorylation (Santa Cruz Biotechnology, anti-Caspase 9 (pSer196)); and Bad pSer136 phosphorylation (Santa Cruz Biotechnology, anti-Bad (pSer136)). The BD FACSCalibur system for data acquisition and analysis was done using BD CellQuest Pro software, version v6.0.2.

Cell surface marker expression detection assay by flow cytometry

Expression of CD14 (BD Biosciences, CD14 PerCP-Cy5.5); CD83 (BD Biosciences, CD83 APC); and CD206 (BD Biosciences, CD206 FITC) were measured following 72 h postactivation. Briefly, 20μl of each labeled antibody for every 100μl of cell suspension containing 1×10^6 cells was added and incubated at room temperature protected from light for 15 minutes. Cells were washed with 1X PBS and analyzed by flow cytometry.

DNA staining for cell cycle analysis by flow cytometry

Following U937 activation for 72h, cells were harvested, fixed and permeabilized by cold ethanol 70%. Cells were then washed with 1X PBS and stained with 2.5 μg/ml propidium iodide mixed with 0.5 mg/ml RNase A.

Annexin V apoptosis detection assay by flow cytometry

The Annexin V-FITC apoptosis detection kit (BD Biosciences) was used following manufacturer instructions. Cells were harvested, washed with cold 1X PBS and then resuspended in 1X binding buffer at a cell density of 1×10^6 cells/ml. 100μl (1×10^5cells) were stained with FITC Annexin V and PI stains followed by flow cytometry analysis.

Statistical assessment

For comparison between groups A student *t*-test was used. Statistically significant results were defined as $p \leq 0.05$.

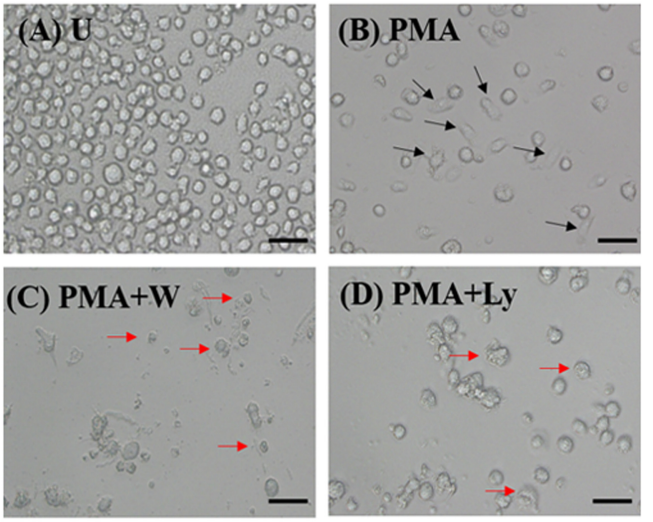
Results

U937 cells show a macrophage-like morphology following PMA differentiation

Microscopic analysis following 72 hours of PMA incubation, U937 cells developed pseudopods, a macrophage-like morphology, and adhesion, as shown in (Figure 1A and B). Dead cells and fragmented cells were observed in Wortmannin-treated cells (Figure 1C) and Ly294002 treated cells (Figure 1D). After treatment with PMA for 72 h, we detected a decrease in the number of cells when compared with untreated cells (Figure 1B). When cotreated with Wortmannin, an inhibitor of PI3 kinase or Ly294002 the number of cells decreased and differentiated cells. This indicates the AKT1 cell signaling pathway is essential for cell differentiation and survival.

AKT controls apoptosis and participates in differentiation after PMA in U937 cells

Wortmannin or Ly294002 are potent inhibitors of the AKT signaling pathway. Cells were incubated for 1 hour with Wortmannin or Ly294002 before activation with PMA (10 nM) for 24 hours. Inhibition of AKT1 by Wortmannin or Ly294002 revealed a decrease in Akt-pSer473 phosphorylation, which triggered induction of apoptosis. Our annexin V and propidium iodide apoptosis detection by flow cytometry indicated a high percentage of apoptotic cells when inhibiting the AKT1 pathway (Figure 7). Following PMA treatment, Akt-pSer473 phosphorylation was also reduced, demonstrating the importance of AKT1 activity in the U937 differentiation process (Figure 2). Earlier research revealed a connection between AKT and PKC in PMA, specifically the activating or inhibiting actions of Akt1-pSer473. According to (Barragan *et al.*, 2006), PMA activates AKT in B-CELL cells, B-cell chronic lymphocytic leukemia cells, without the need of PI3-K but rather through PKCβ leading to an increase in Akt1-pSer473 phosphorylation. On the other hand, according to Liu



Note(s): (A) U: untreated cells, control. (B) PMA treated cells, effect on differentiation of U937 Cells. (C) PMA + Wortmannin treated cells and (D) PMA + Ly294002 treated cells, shows fragmented and apoptotic cells. Apoptotic cells are indicated by red arrows while differentiated cells are indicated by black arrows. Scale bar = 100μM

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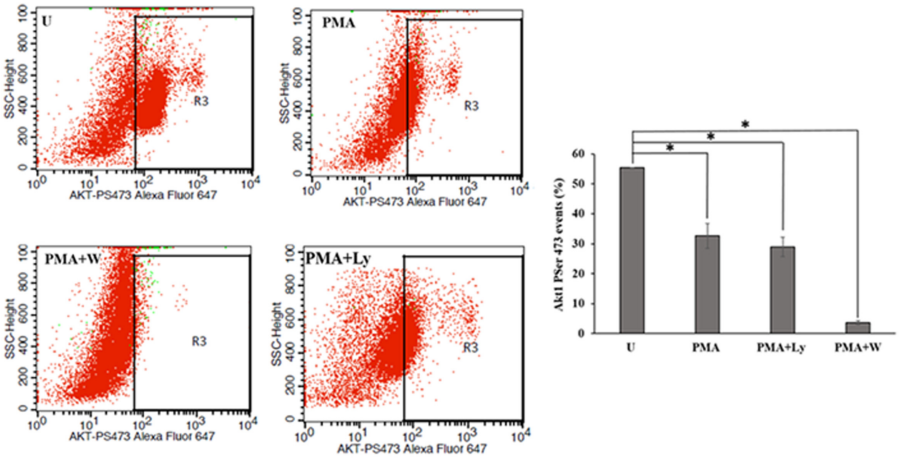
Figure 1.
Morphology of U937 Cells



Note(s): Following a 24-hour treatment with 10nM PMA, U937 cells underwent a protein phosphorylation flow cytometry experiment that revealed a decrease in AKT1 pS473 phosphorylation as the cells differentiated into macrophage-like cells. The AKT pathway was co-treated with PMA, Wortmannin, or Ly294002 to inhibit it, and this resulted in a greater decrease in AKT1 pSer473 phosphorylation. (A) Showing of fluorescent dot blots from flow cytometry. (B) Illustration of the proportion of positive AKT1 Phospho-Serine 473 events, Gate R3. U: untreated cells, PMA: Cells treated with PMA, PMA + Ly: Cells treated with PMA + Ly294002, and PMA + W: Cells treated with PMA + Wortmannin. Average (SD) of two experiments in comparison to untreated cells, $*p \leq 0.05$

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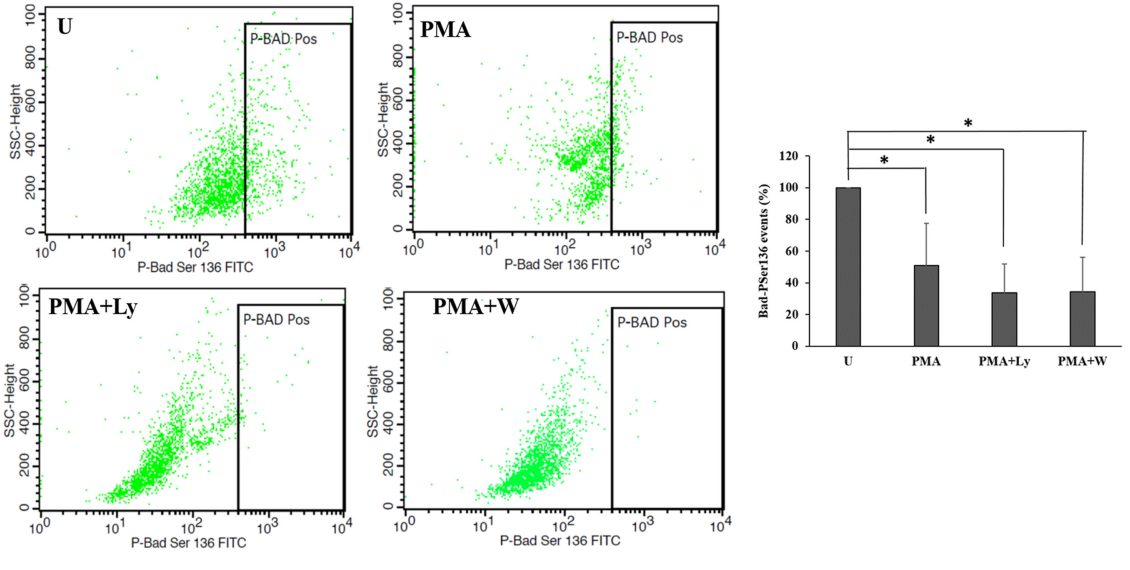
Figure 2.
AKT1 participates in the induction of differentiation by PMA in U937 cells

H.'s 2006 study on myeloid 32D cells (Liu, Qiu, Xiao, & Dong, 2006), PMA can specifically prevent G-CSF from activating A-pSer473 through PKC. PMA's ability to inhibit Akt during G-CSF-induced granulocytic differentiation is linked to a decrease in PKC$^{\varepsilon}$ expression. In our study, PMA led to a decrease in Akt1-pSer473, and this reduction led to the induction of differentiation of U937 cells into macrophage-like cells. The addition of Wortmannin or Ly294002, which are potent inhibitors of AKT, led to an increase in the reduction of Akt-pSer473 and led to apoptosis, which mainly indicates the activation is through the Akt pathway with potential PKC pathway involvement since according to Liu H. findings, PMA reduction of Akt1-pSer473 may be through PKC in previously activated Akt1 U937 proliferating cells.

Inhibition of the AKT pathway led to a reduction in bad phosphorylation

Inhibition of the AKT pathway before PMA U937 cell treatment for 24 h indicated a decrease in phospho-serine136 Bad. Also, PMA treatment of U937 cells for 24 h showed a decrease in phospho-serine136 Bad level (Figure 3) due to a decrease in activated AKT1 upon PMA stimulation. Bad is a proapoptotic protein regulated by phosphorylation of serine-122 and serine-136. Phosphorylation at either site can result in Bad losing its ability to heterodimerize with Bcl-xL or Bcl-2 survival proteins. Serine-136 phosphorylation was shown to be dependent on AKT activation (Datta *et al*., 1997).

Figure 3.
PMA decreases phosphorylation of Ser136 on Bad via AKT in U937 cells



(a) **(b)**

Note(s): After being exposed to PMA for 24 hours, PMA with Ly294002 or Wortmannin activation causes a decrease in the phosphorylation of the protein Bad pSer136 in U937 cells using flow cytometry. (A) U: untreated cells, PMA: PMA-treated cells, PMA + Ly: PMA with Ly294002 treated cells, and PMA + W: PMA with Wortmannin treated cells. Illustration of fluorescent dot blots. (B) Illustration of the proportion of Bad Phospho-Serine 136 successful occurrences. Three experiments' average (SD) with respect to untreated cells, $^{*}p \leq 0.05$

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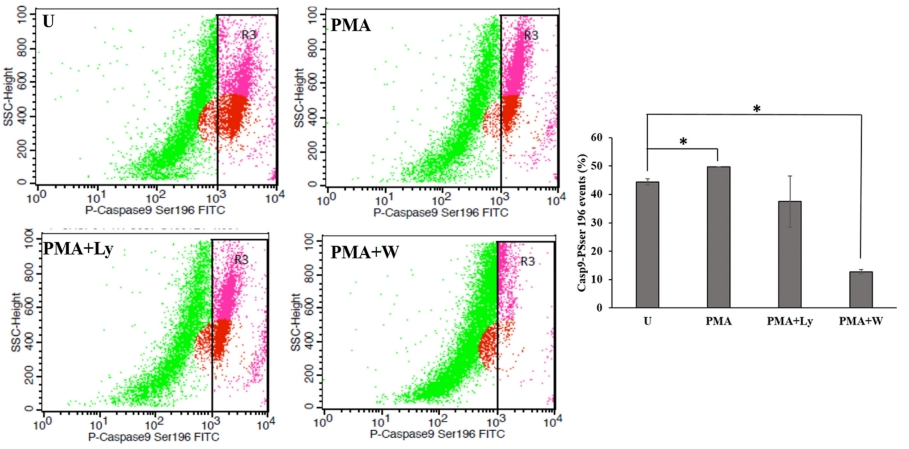
Caspase9 involvement during Ly294002 or Wortmannin induces apoptosis in U937 cells during PMA differentiation

Caspases are intracellular cysteine-aspartic proteases that act as initiators and effectors of apoptosis, as well as have nonapoptotic roles associated with cell proliferation, differentiation, tumor suppression and others (Hart & Vogt, 2011). Caspase-9 is an initiator caspase influenced by the AKT signaling pathway. Caspase 9 can be phosphorylated at multiple sites by different protein kinases, leading to inhibition of caspase 9 activations (Shalini, Dorstyn, Dawar, & Kumar, 2015). The Serine 196 position is the position of activated AKT to inhibit caspase 9. Activated AKT1 induces phosphorylation of Caspase 9 at Ser196 and inhibits its protease activity, therefore inhibiting downstream apoptosis activation. Flow cytometry was performed to confirm the role of caspase-9 phosphorylation at serine 196 by activating AKT1 as the main player in U937 cell survival upon PMA activation. Preincubation with Ly294002 or Wortmannin of PMA-treated U937 cells reduced PMA-induced caspase 9 phosphorylation at serine 196 (Figure 4).

Expression of cell surface markers during U937 cell differentiation

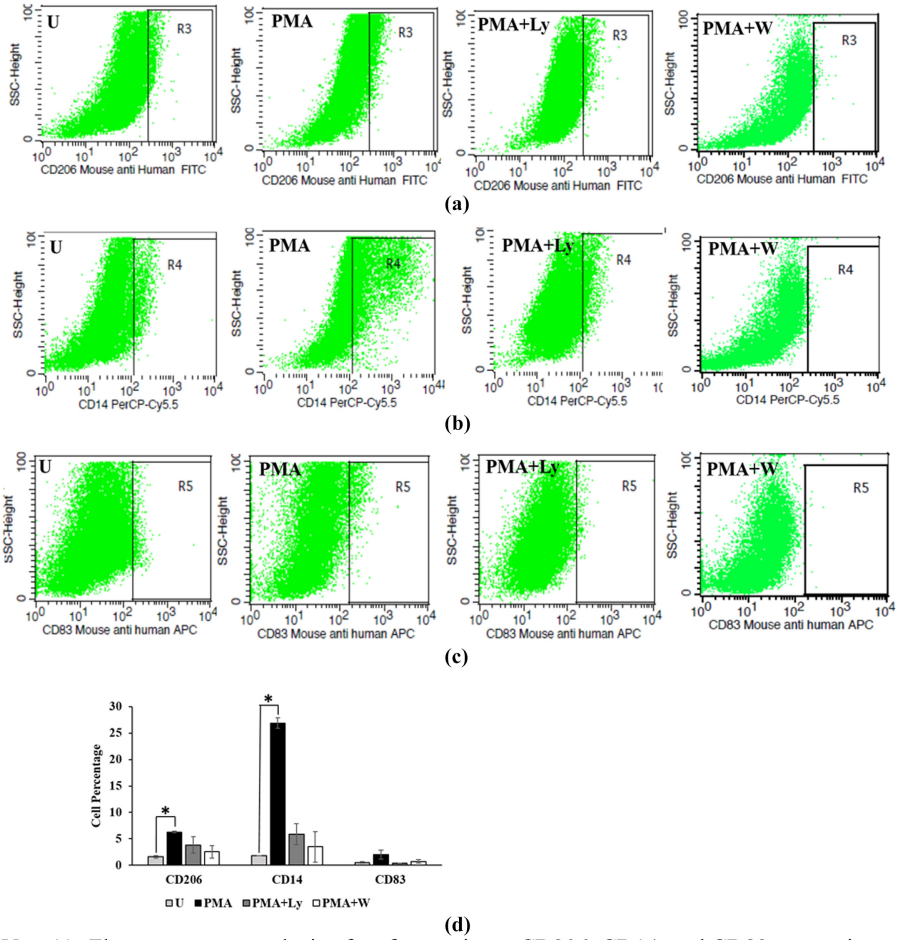
A significant expression of CD14 membrane protein was detected by flow cytometry following the induction of U937 cells with PMA. However, the expression of CD206 and CD83 proteins increased slightly following PMA treatment. Increased CD83 expression on the surface of differentiated cells may confer antigen-presenting cell capability. The inhibition of the AKT pathway by Ly294002 or Wortmannin before cell PMA treatment showed a decrease in CD14, CD206 and CD83 surface marker expression (Figure 5). These findings highlight the role of the AKT1 pathway in the expression of proteins critical to macrophage activity.



Note(s): PMA induces survival of differentiated U937 cells through an increase in Caspase9 pSer196 phosphorylation. (A) U: untreated cells, PMA: PMA-treated cells, PMA + Ly: PMA and Ly294002-treated cells, and PMA + W: PMA and Wortmannin-treated cells. Demonstration of fluorescent dot blots. (B) Caspase 9 Phospho-Serine 196 positive events, Gate R3, are represented as a percentage. The mean (standard deviation) of two experiments $*p \leq 0.05$ compared to untreated cells

Source(s): Figure by Bakheit *et al*. (2023)

Figure 4. PMA upsurges Caspase9 phosphorylation on Ser196 in U937 cells



Note(s): Flow cytometry analysis of surface antigens CD206, CD14, and CD83 expression on U937 cells after 1 hour of AKT pathway inhibition with Ly294002 or Wortmannin followed by 72 hours of PMA activation. (A) Significant increase in CD14 in addition to PMA treatment and reduction when treated by Ly294002, R3 gate. (B) An increase in CD206 following PMA treatment, R4 gate. (C) A rise in CD83 expression following PMA administration, R5 gate. (D) Cell percentage quantification of positive CD206, CD14, and CD83 events. U represents untreated cells, PMA represents PMA-treated cells, PMA + Ly represents cells treated with PMA + Ly294002, and PMA + W represents cells treated with PMA + Wortmannin. The mean (SD) of three experiments was $*p \leq 0.05$ compared to untreated cells

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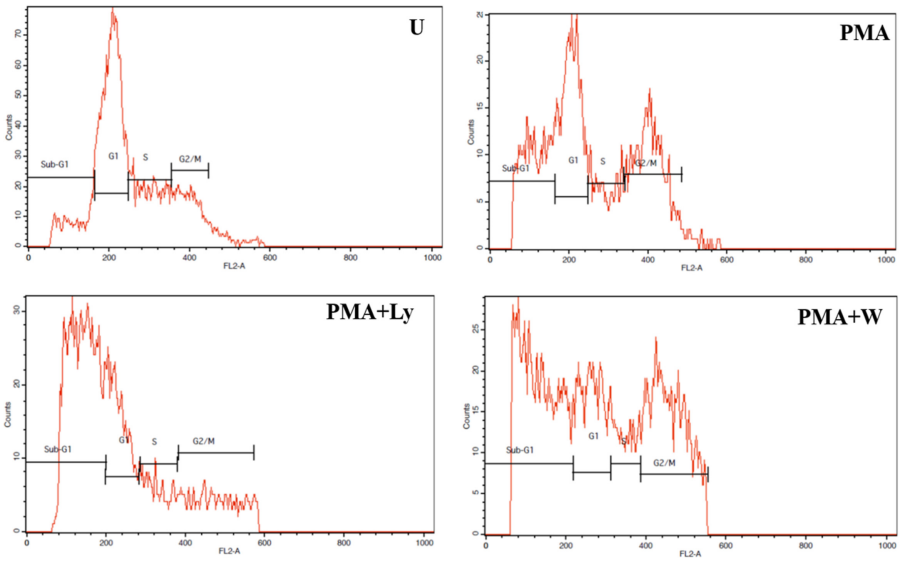
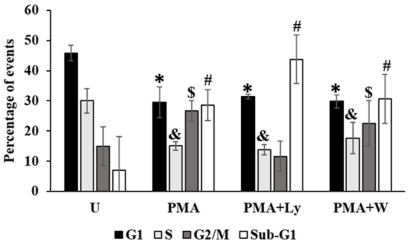
Figure 5.
Surface marker expression in U937 cells following PMA-induced differentiation

PMA induces an increase in G2/M phase during the differentiation of U937 cells

U937 cells treated with PMA for 72 hours showed a decrease in the number of cells found in the G1 and S phases and an accumulation in the G2/M phase of the cell cycle and viability test (Figure 6).



(a)



(b)

Note(s): The DNA content test was performed by flow cytometry using propidium iodide staining for U937cells untreated cells (U), PMA-treated cells (PMA), PMA + Ly294002 - treated cells (PMA + Ly), or PMA + Wortmannin - treated cells. (A) Histogram representation shows PMA induces accumulation of G2M phase and reduction of S phase during differentiation into macrophage-like cells in 72 hours. (B) Calculation of the percentage of PI-positive events in the G1, S, G2/M, and sub-G1 zones. The mean (SD) of the three experiments was $*p \leq 0.05$ compared with untreated cells

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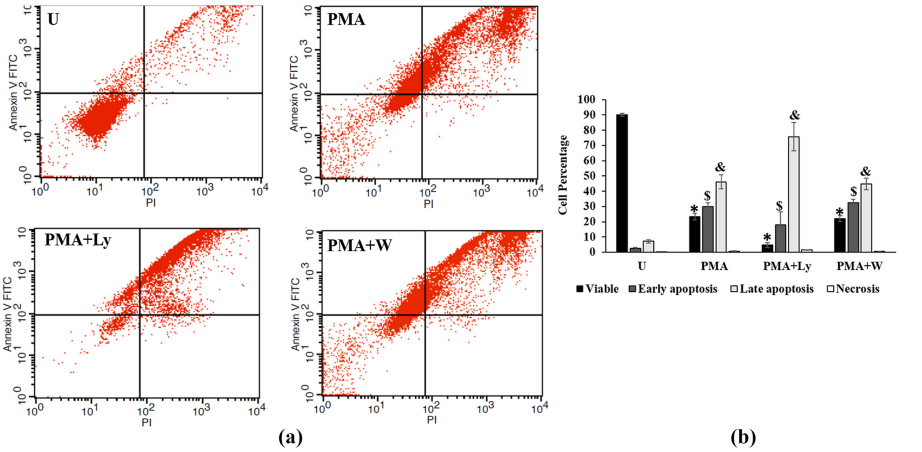
Figure 6.
PMA induces an increase in the G2/M phase during the differentiation of U937 cells

Apoptosis induction for some of the U937-induced PMA cells and the U937-inhibited Akt1 pathway cells

Apoptosis was measured by flow cytometry following PMA stimulation with or without Ly294002 or Wortmannin preincubation. Induction of apoptosis with an increase in early and late apoptosis for cells treated with PMA only, while inhibition of the AKT pathway by Ly294002 or Wortmannin increased the number of apoptotic cells (Figure 7).



Note(s): (A) The flow cytometry analysis of Annexin V and Propidium iodide (PI) dot blots on U937 cells was performed after 72 hours of PMA, PMA + Ly294002, or PMA + Wortmannin stimulation. U: untreated cells. PMA: PMA-treated cells. PMA + Ly: PMA + Ly294002 treated cells. PMA + W: PMA + Wortmannin treated cells. (B) The percentage quantifications of positive events of FITC Annexin V and PI on U937 cells are indicated. Viable cells = Annexin V and PI negative (LL: lower left); Early apoptosis cells = Annexin V positive and PI negative (UL: upper left); Late apoptosis cells = Annexin V and PI positive (UR: upper right); and Necrosis = Annexin V negative and PI positive (LR: lower right). Mean (SD) of the three experiments. $^{*}p \leq 0.05$ comparing viable cells to untreated cells. $^{\$}p \leq 0.05$ comparing early apoptosis cells to untreated cells. $^{\&}p \leq 0.05$ comparing late apoptosis cells to untreated cells

Source(s): Figure by Bakheit *et al*. (2023)

Figure 7.
Apoptosis detection

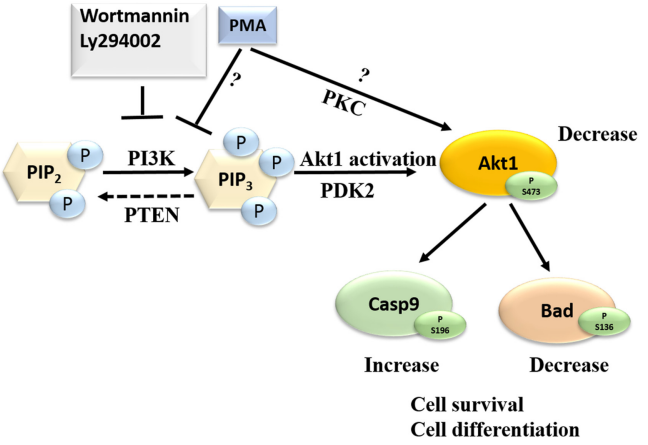
Discussion

U937 cells are one of the cells that can be used as a model to study monocyte/macrophage functions. However, to be used as an in vitro model for macrophages, U937 cells must be differentiated, and several protocols have been tested (Allan & Clarke, 2009), (Prasad *et al.*, 2020), (Scotti *et al.*, 2018). PMA is known as one of the agents that led to the differentiation of U937 cells into macrophage-like cells and other cells such as MO3.13 cells (Damato *et al.*, 2021), HOP 92 cells (Choi, Hyman, & Blumberg, 2006).

The differentiation mechanisms that lead to the PMA-induced U937 differentiation are governed mainly by the balanced activation of kinases and phosphatases. Several pathways are involved, such as the activation of the MAPK/ERK pathway (Olsson, Gullberg, Ivhed, & Nilsson, 1983) with different stimuli, including SET, a nuclear phosphoprotein, which leads to dendritic-like differentiation of U937 cells (Olsson *et al.*, 1983). RhoA/ROCK signaling, induced by PMA (100 ng/ml), was found to be associated with U937 cell-line into differentiation and characterized by the expression of CD68 (Kandilci & Grosveld, 2005). Our study investigates the early involvement of AKT pathway activation in PMA-induced differentiation of U937 cells. One of the significant findings of this study is the hypophosphorylation of AKT1 in U937 cells following PMA activation for 24 hours, which leads to the differentiation by suppression of cell proliferation. The reduced AKT1-phosphorylation plays a role in differentiated cell survival by the reduction of Bad phosphorylation, an Akt-downstream survival mediator. In addition, PMA treatment of U937

cells leads to the increased phosphorylation of caspase-9, promoting cell survival (Figure 8). A study in 2021 demonstrated an increase of Caspase 9 activity during differentiation (Madadi, Akbari-Birgani, Mohammadi, Khademy, & Mousavi, 2021). Inhibition of AKT1 activity by Ly294002 or Wortmannin significantly decreases Bad and Caspase 9 phosphorylation, promoting apoptosis.

Previous studies have indicated that the AKT pathway is necessary for cell survival and differentiation (Yang, Dai, Tang, Le, & Yao, 2017; Abdullah, Hills, Winter, & Huang, 2021). Our results show that AKT1 activity is reduced as PMA induced-U973 differentiation. In addition, Bad phosphorylation reduction, as a consequence of the PMA effect on AKT1 phosphorylation profile, may not be necessary for cell survival; rather, it may be obligatory for U937 cell differentiation induction. PMA suppresses Akt1-PSer473 phosphorylation, possibly via PDK1, and because Bad is downstream of Akt1, as a result, it lessens the downstream activation of Bad pSer136 (Figure 8). In addition, our results showed that cell adhesion in PMA-treated cells led to differentiation compared with untreated rounded cells. In this study, we demonstrated the ability of 10 nM PMA to induce an increase in the expression of cell surface markers such as CD14, CD206 and CD83. The inhibition of the AKT1 pathway by Ly294002 decreased the expression of CD14, CD83 and CD206. Previous research has shown that CD14 expression is dependent on the induction of a protein intermediate, SP1, which is induced by [1,25(OH)2D3] (Busca, Saxena, Iqbal, Angel, & Kumar, 2014). In addition, Baek *et al.* reported that retinoic acid could increase the expression of CD14 in U937 cells following exposure to [1, 25(OH)2D3] (Zamani, Zare Shahneh, Aghebati-Maleki, & Baradaran, 2013). Despite the fact that previous studies of PMA-induced U937 differentiation failed to induce CD14 expression (Baek *et al.*, 2009), our study's indication of CD14 expression may be due to the use of different doses of 10 nM PMA.



Note(s): AKT pathway blockage or promotion of basal-level activated AKT activation is postulated as the mechanism by which PMA induces differentiation of the U937 cell line into macrophage-like cells. The basal-level activation of AKT occurs through PI3K. Upon PMA differentiation, potential inhibition of AKT through PI3K by PDK1 and PDK2 leads to inhibition of activated BAD. On the other hand, potential sustained activation of AKT through the PKC pathway adopted from (Barragan *et al*., 2006) may lead to an increase in activated Caspase 9, leading to cell survival. Dotted lines arrows represent inhibitory action, where solid lines represent activation

Source(s): Figure by Bakheit *et al*. (2023)

Figure 8. Diagram showing potential AKT involvement in the differentiation of PMA-U937 cells

In our study, a slight increase in CD83 expression was found in U937–PMA stimulated cells. CD83, a member of the immunoglobulin superfamily of receptors associated with antigen presentation, is expressed by monocytes, macrophages, and dendritic cells. CD83 expression is transient on activated monocytes and macrophages but stable on activated dendritic cells (Cao, Lee, & Lu, 2005). The slight increase in CD83 expression may allow these cells to gain antigen-presenting cell capabilities (Cao *et al*., 2005). An increase in the expression of CD206 in PMA-differentiated U937 cells was observed in our study. Macrophages express various scavenger receptors, such as CD206, which are upregulated in response to inflammation (Nielsen *et al*., 2020). CD206 is a 175kDa membrane-bound protein, primarily expressed by macrophages and dendritic cells. CD206 is involved in endogenous molecule clearance, antigen presentation and modulation of cellular activity. In addition, CD206 is considered an efficient endocytic receptor that continuously recycles between the cell surface and early endosomal compartments (Gazi & Martinez-Pomares, 2009). Although we did not examine the phagocytic activity of U937 differentiated cells, we provide evidence of differential expression of the protein. Our cell cycle study indicated an accumulation of U937-PMA-treated cells in the G2/M phase with a concomitant reduction in the S and G1 phases after 72 hours. Exit from the cell cycle by G1-phase arrest is one of the indications of U937 cell differentiation (Asada, Yamada, Fukumuro, & Mizutani, 1998). However, others have reported G2/M arrest in differentiated U937 cells (Devlin *et al*., 2003). Apoptosis was estimated by annexin V-FITC (Nagata, Suzuki, Segawa, & Fujii, 2016) and demonstrated that PMA treatment of U937 cells, 23% differentiated cells and 77% were apoptotic cells. PMA combined with Ly294002 U937 cell treatment increased the number of apoptotic cells, indicating the AKT's importance for cell survival.

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