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Down-regulation of superoxide dismutase 1 by PMA is involved in cell fate determination and mediated via protein kinase D2 in myeloid leukemia cells

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ABSTRACT

Myeloid leukemia cells maintain a high intracellular ROS level and use redox signals for survival. The metabolism of ROS also affects cell fate, including cell death and differentiation. Superoxide dismutases (SODs) are major antioxidant enzymes that have high levels of expression in myeloid leukemia cells. However, the role of SODs in the regulation of myeloid leukemia cells' biological function is still unclear. To investigate the function of SODs in myeloid leukemia cell death and differentiation, we used myeloid leukemia cell lines K562, MEG-01, TF-1, and HEL cells for this study. We found that PMA-induced megakaryocytic differentiation in myeloid leukemia cells is accompanied by cell death and SOD1 down-regulation, while SOD2 expression is not affected. The role of SOD1 is verified when ATN-224, a SOD1 specific inhibitor, inhibits cell proliferation and promotes cell death in myeloid leukemia cells without PMA treatment. Moreover, inhibition or silencing of SODs further increases cell death and decreases polyploidization induced by PMA while they were partially reversed by SOD1 overexpression. Thus, SOD1 expression is required for myeloid leukemia cell fate determination. In addition, the knockdown of PKD2 reduces cell death and promotes polyploidization induced by PMA. PMA/PKD2-mediated necrosis via PARP cleavage involves both SOD1-dependent and -independent pathways. Finally, ATN-224 enhanced the inhibition of cell proliferation by Ara-C. Taken together, the results demonstrate that SOD1 regulates cell death and differentiation in myeloid leukemia cells. ATN-224 may be beneficial for myeloid leukemia therapy.

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1. Introduction

Reactive oxygen species (ROS) play many vital roles in myeloid leukemia [1]. This is especially true for chronic myeloid leukemia (CML) cells, over 95% of which express and use BCR-ABL fusion protein to maintain a higher-than-normal level of ROS for survival [2]. These BCR-ABL expressing cells have constitutive active PI3K/AKT pathway under redox regulation that serves as a survival signal for these cells [3]. For this reason, they may be more susceptible to further ROS increases. Consequently, antioxidant enzyme levels are also high in these cells to maintain the delicate ROS balance needed for survival [1]. It has been demonstrated that the increased ROS level caused by the down-regulation of SOD1 leads to cell death in murine thymic lymphoma WEHI7.2 cells [4]. As a matter of fact, unbalanced increases in ROS may also lead to both differentiation and death [5,6]. With the exception of the upstream survival signals, acute myeloid leukemia (AML) cells behave similarly in many aspects to that of CML cells. AML patients with Fms-like tyrosine kinase 3 -internal tandem duplications (FLT3-ITD) mutation constitute about 30% of AML cases and these patients suffer from poor prognosis [7]. FLT3 increase ROS production via the activation of $p22^{phox}$ that maintains a higher ROS level in AML cells [8] and it is essential to their survival. Moreover, inhibition of PDK1/AKT and FLT3 signaling induces AML cell death [9]. Furthermore, ROS level increase has been associated in the differentiation of AML cells [10]. Because both the induction of cell death and differentiation are valuable strategies for leukemia treatment, understanding the regulation of ROS levels in myeloid leukemia cells is vital to our comprehension of myeloid leukemia cell biology.

ROS levels can be maintained via a balance between their biosynthesis and degradation. In myeloid leukemia cells, at least two pathways for the increase in ROS biosynthesis have been identified. First, BCR-ABL increases ROS levels by using NADPH oxidase 4 (Nox4) to inhibit phosphatase 1a (PP1a), a serine threonine phosphatase, and subsequently activates a survival signal via the PI3K/AKT pathway [3]. Moreover, phorbol-12-myristate-13-acetate (PMA) activates $p22^{phox}$, a subunit of NADPH oxidase, in the same system to increase ROS production, which results in megakaryocyte-like differentiation [5]. Second, PMA

Abbreviations: ROS, reactive oxygen species; SOD, superoxide dismutase; PMA, phorbol 12-myristate 13-acetate; PKD2, protein kinase D2; CML, chronic myeloid leukemia; AML, acute myeloid leukemia

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also reduces mitochondrial membrane potential respiratory chain complex IV activity during differentiation [11]. Furthermore, ROS increases may cause mitochondrial damage, leading to cell death [12]. Although ROS catabolism is well-studied, its regulation of cell differentiation and cell death is not.

Superoxide dismutases (SODs) are a major antioxidant enzyme family that transforms two superoxide radicals into hydrogen peroxide and oxygen. There are three SOD isoforms within the family. SOD1 is expressed in the intermembrane space of mitochondria, the nucleus, and the cytosol. SOD2 is located in the mitochondrial matrix, while SOD3 is secreted into the extracellular space [13]. Recently, several reports have suggested that SOD1 may also have signal transduction functions. In breast cancer, SOD2 down-regulation induces SOD1-compensated up-regulation for the removal of excess ROS, activating ERα-dependent unfolded protein response (UPR) in the mitochondria, and promotes ERα transcriptional activity in the nucleus [14,15]. Although the function of SOD1 in the regulation of myeloid leukemia cell death and differentiation has not been reported, the results of studies on other cell types may support this notion. It was reported that in human primary megakaryocyte (MK), the mRNA expression level of SOD1 in $CD41^+$ cells is higher than that in $CD41^-$ cells [16]. Moreover, the down-regulation of SOD1 mRNA induced by PMA during cell differentiation has been reported in acute leukemia cell lines, such as U937, HL60, and HEL cells [17,18]. Furthermore, in murine thymic lymphoma, the inhibition of SOD1 leads to cell death via the nitric oxide-peroxynitrite-dependent pathway [4]. From these observations, we may suggest that SODs level indeed plays a role in the control of cell death and differentiation via the regulation of ROS levels in myeloid leukemia cells. Understanding how SODs expression affects cell fate in myeloid leukemia cells may shed light on the mechanism involved in their survival and its relationship to ROS regulation.

The PMA treatment of myeloid leukemia cells is useful for the study of how ROS balance affects their survival, differentiation, and death. Both TPO and PMA promote MK-like differentiation in myeloid leukemia cell lines, such as K562, HEL and TF-1 cells, via an increase in ROS formation [5], which results in polyploidization and megakaryocytic surface marker (CD41 and CD61) expression [19]. It has long been established that protein kinase C (PKC) isoforms are involved in megakaryocytic leukemia cell differentiation when induced either by thrombopoietin (TPO) or phorbol 12-myristate 13-acetate (PMA). Several PKC isoforms have been reported to be involved in MK differentiation in leukemia cells. PKCβII has been implicated in megakaryocytic proliferation in K562 cells [20]. PKCε activation may also be involved in increases in CD41 expression [21,22]. The down-regulation of PKCε is related to PMA-induced cell death in K562 and TF-1 cells [6].

The role of PKC isoforms in PMA-induced megakaryocytic differentiation has been intensive studied [6,20–23]. However, none of the studied PKC isoforms can account for all of the PMA actions, it is likely that other PMA-activated proteins are also involved. PKD is a novel subfamily of PKC. It consists of three members, PKD/PKD1, PKD2, and PKD3 [24,25]. Notably, SOD2 gene expression was increased under oxidative stress in HeLa cells via PKD1 [26]. PKD2 is also involved in platelet generation [27]. Because PKD2 is the major isoform expressed in leukemia cell lines [28], the question of whether PKD2 regulates myeloid leukemia cell differentiation and whether PKD2 mediates the PMA regulation of ROS requires further study.

In the present study, we studied the role of SOD1 and PKD2 in myeloid leukemia cell death and differentiation. We used the PMA treatment of myeloid leukemia cell lines K562, TF-1, HEL, and MEG-01 for our study. Our results demonstrated that selective SOD1 inhibitor ATN-224 inhibited cell proliferation and promoted cell death in myeloid leukemia cell lines. Moreover, SOD1 was involved in PMA-dependent myeloid leukemia cell death and polyploidization Furthermore, SOD1 expression is down-regulated by PKD2 activation, which also modulates ROS production during myeloid leukemia cell death and polyploidization induced by PMA. Taken together, PMA-induced myeloid leukemia cell death and polyploidization is mediated by PKD2/SOD1 signaling. Targeting SOD1 is important to myeloid leukemia therapy.

2. Materials and methods

2.1. Cell culture

The human erythroleukemia cell lines K562, TF-1, HEL and MEG-01 were purchased from Bioresource Collection and Research Center (Taiwan). The cells were maintained in a humidified 5% CO_2, 37 °C incubator in RPMI medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 Units/ml penicillin, 100 Units/ml streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate (Caisson, Logan, Utah, USA). For TF-1 cells, 2 ng/ml GM-CSF (MDBio, Taiwan) was added. For stable cell line selection, 2 μg/ml puromycin (MDBio) was added. The human embryonic kidney cell line HEK293 was maintained in Dulbecco's modified Eagle's medium (Hyclone, Logan, Utah, USA) supplemented with 10% FBS. For experiments, cells (1.5×10^5 cells per ml) were treated with 25 nM PMA (5 nM for TF-1) to induce megakaryocytic (MK) differentiation.

2.2. Reagents

Annexin V-FITC was purchased from BD Biosciences (San Jose, CA, USA). Propidium iodide (PI) and dihydroethidium (DHE) were obtained from Invitrogen (Carlsbad, CA, USA). Z-VAD-FMK, *N*-acetyl-L-cysteine (NAC), cycloheximide, MG132, polybrene, actinomycin D, and nitroblue tetrazolium chloride (NBT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). ATN-224 was a gift from Professor Ching-Yuh Chen (Department of Applied Chemistry, National Chiayi University, Taiwan (R.O.C.)). Phorbol 12-myristate 13-acetate (PMA), Ara-C, GF109203X, Gö6976, and CID755673 were acquired from Tocris Bioscience (Bristol, UK). Dimethyl sulfoxide (DMSO) and riboflavin were acquired from Merck (Whitehouse Station, NJ). The DMSO in the media was less than 0.1% at the final concentration in order to prevent any effect on activity. All inhibitors were added 30 min before PMA administration at the indicated concentrations.

2.3. Antibodies

PKD1/2 and glycophorin A (GPA) antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). PKD2 antibodies were purchased from Novus. Antibodies against AKT, pAKT (Ser473), $pERK_{1/2}$ (Thr202/Tyr204), pPKD (Ser916), pPKD (Ser744/748), caspase-3, and caspase-9 were obtained from Cell Signaling Technology (Boston, MA, USA). Antibodies against pPKD2 (Ser876) and SOD2 were acquired from Epitomics (Burlingame, CA, USA). Nitrotyrosine was obtained from Bioss (Massachusetts, USA). Anti-CD41-FITC antibodies were purchased from eBioscience. Anti-CD61-PE antibody was acquired from BD. Antibodies against SOD1, GAPDH, and a-tubulin were purchased from GeneTex (California, USA). Anti-PARP-1 antibody was purchased from BD. Goat anti-rabbit IgG HRP and goat anti-mouse IgG HRP were obtained from Jackson ImmunoResearch (Suffolk, UK).

2.4. Small hairpin (sh) RNA lentivirus production

shRNA clones were acquired from the National RNAi Core Facility (Institute of Molecular Biology/Genomic Research Center, Academia Sinica, Taiwan). Lentiviruses were obtained from the RNAi Core Research Center of Clinical Medicine, National Cheng Kung University Hospital. The lentiviruses were prepared in accordance with standard protocols. Briefly, 4×10^6 293 T cells were co-transfected with 5 μg pCMVΔR8.91, 0.5 μg pMD.G, and 5 μg pLKO.1 shRNA using Lipofectamine 2000 (Invitrogen) for 6 h. After 24 h, the supernatants containing viral particles were harvested and filtered through 0.45 μm filters. The cells were infected with lentivirus in the presence of polybrene (8 μg/ml) and treated with

2 μg/ml of puromycin to select puromycin-resistant clones after 48 h. The shRNA target sequences were as follows: scramble shRNA, 5′-AGTTCAGT TACGATATCATGT-3′; SOD1 shRNA, 221566- 5′-CCGATGTGTCTATTGAAG ATT-3′; SOD2 shRNA, 320739- 5′-GCACGCTTACTACCTTCAGTA-3′; PRKD2 shRNA, 1949- 5′-CGTGGCAGTTAAGGTCATTGA-3′, 1950- 5′-CACGACCAACAGATACTATAA-3′, and 342320- 5′-GTTGGGTGGTTCATTA CAGCA-3′.

2.5. Trypan blue exclusion assay

K562, TF-1, HEL, and MEG-01 (1.5×10^5 cells/ml) were harvested and re-suspended with equal volumes of trypan blue. The cells were counted in a hemocytometer chamber. The percentage of cells, excluding the dye, was calculated and expressed as percent viability.

2.6. Flow cytometric analysis

Superoxide production was detected by DHE [29]. The cells were pre-incubated with DHE at a final concentration of 1 μM in phosphate-buffered saline (PBS) for 15 min at RT and analyzed by FASCalibur flow cytometer (BD).

Cell death was determined via propidium iodide (PI) (or FarRed) and Annexin V. The cells were resuspended with Annexin V/PI for 10–15 min at 4 °C and analyzed via FASCan flow cytometer (BD).

Cell differentiation was detected via increases in polyploidization and glycophorin A (GPA), CD41, and CD61 expression levels. In the polyploidization experiments, cells were fixed with 70% EtOH overnight, re-suspended in PBS, and stained with PI solution (0.02 mg/ml PI, 0.1% triton, and 0.2 mg/ml RNase A) for 30 min at RT and analyzed by FASCan flow cytometer (BD). CD41 and CD61 expression levels were measured by incubating the cells with anti-CD41-FITC and anti-CD61-PE antibodies, respectively, for 30 min, and the cells were then re-suspended in PBS. GPA expression levels were measured by fixing, permeabilizing, and incubating the cells with anti-GPA antibody for 60 min, and the cells were then re-incubated with Alexa Fluor 488 goat anti-rabbit (Molecular Probes) antibody for 30 min. Finally, the labeled cells were analyzed by Accuri C6 flow cytometer (Becton Dickinson, San Jose, CA, USA). The data were analyzed with FlowJo software (Ashland, OR, USA).

For intracellular staining, the cells were harvested and fixed with 4% formaldehyde for 20 min. Washed the fixed cells and permeabilized by adding 0.1% triton for 10 min. The cells were stained with antibody overnight and re-incubated with Alexa Fluor 488 goat anti-rabbit for 30 min. Cells were then analyzed within 12 h by FASCalibur flow cytometer (BD).

2.7. Cell lysis and Western blotting

A total of 1.5×10^5 cells/ml were lysed in cell extraction buffer (10 mM Tris.HCl pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.1% SDS, 10% glycerol, and 1% Triton X-100) with protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitors (1 mM PMSF, 20 mM Na_3VO4, and 2 mM $Na_3P_2O_7$). Lysates were centrifuged at 14,500 r.p.m. The supernatants were then collected, normalized by Lowry method, diluted to 1:3 with 4× SDS-PAGE loading buffer (250 mM Tris–HCl pH 6.8, 8% SDS, 0.004% bromophenol blue, and 40% glycerol), and boiled for 10 min. Thirty mg of lysates per well were loaded and electrophoresed on SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes (Pall Corporation, Port Washington, NY, USA), and Western blot reactions were detected using Western Lightning Plus-ECL (PerkinElmer, Santa Clara, California, USA). Membranes were probed with the antibodies and film exposure measurement. Western blot analysis intensity was measured by Image J (Java-based image processing program, NIH).

2.8. In gel SOD1 activity assay

SOD1 activity analysis was performed as previously described [30]. Briefly, the samples were re-suspended using cell extraction buffer without SDS. The resultant cell extracts were electrophoresed directly, without boiling, on a 12% native polyacrylamide gel. After electrophoresis, the gel was stained with gel staining solutions (0.05 M potassium phosphate buffer, pH 7.8, 1 mM EDTA, 0.25 mM nitroblue tetrazolium chloride (NBT), and 0.5 mM riboflavin) for 30 min at room temperature in the dark. After incubation, the gel was exposed to light until transparent bands were observed, the sites of superoxide scavenging.

2.9. Statistical analysis

The data were expressed as means ± S.D. (standard deviation) from at least three experiments. A statistical analysis was performed using Student's t-test. Statistically significant difference was expressed as $^*p < 0.05$ or $^{**}p < 0.01$. Excel or Sigma plot software was used.

3. Results

3.1. ATN-224 inhibits myeloid leukemia cell proliferation and promotes cell death

CML needs to express an increased SOD1 level to counterbalance increased intracellular ROS production [31]. On the other hand, SOD1 level is elevated in acute myelomonocytic leukemia patient sample [32]. Although whether SOD1 activity plays an important role in AML biology is still unreported, the role of SOD1 in AML cells may be very similar to that of CML cells. Therefore, SOD1 may be an important target in myeloid leukemia therapy. ATN-224 is a selective SOD1 inhibitor that has therapeutic potential in the treatment of many types of solid tumors [14,33]. It has been reported that the inhibition of SOD1 induces cell death in B-cell acute lymphoblastic leukemia (B-ALL) cell lines [12]. However, whether the inhibition of SOD1 is equally effective in myeloid leukemia has not been reported. Therefore, we studied whether ATN-224 reduces myeloid leukemia cell proliferation and induces death. The effect of ATN-224 on cell proliferation was studied using trypan blue exclusion assay. As shown in Fig. 1a, ATN-224 dose- and time- dependently inhibited the proliferation of myeloid leukemia cells, K562, TF-1, HEL, and MEG-01. Moreover, ATN-224 also dose-dependently induced K562 and TF-1 cell death, as indicated by Annexin V/PI staining (Fig. 1c and d). These data showed that ATN-224 indeed inhibits myeloid leukemia proliferation and promotes cell death in these cells.

AKT and $ERK_{1/2}$ are survival signal in myeloid leukemia cells which are the downstream of BCR-ABL in CML cells [3,34] while AML cells also use other signaling pathways to activate these survival signals [9]. As reported in literature, pAKT (Ser473) and $pERK_{1/2}$ (Thr202/Tyr204) phosphorylation denote their activation [35,36]. We studied the phosphorylation of AKT and $ERK_{1/2}$ to analyze whether ATN-224 affects these survival signals. As shown in Supplemental Fig. S1, the phosphorylation of AKT and $ERK_{1/2}$ were dose-dependently reduced after ATN-224 treatment in K562, TF-1, and HEL cells.

Peroxynitrite ($ONOO^-$) is produced by the reaction of superoxide and nitric oxide (NO). SOD1 inhibition results in superoxide accumulation and subsequently increases intracellular peroxynitrite level. Peroxynitrite may cause cell death by inducing DNA damage and nitrosylating tyrosine residue in many proteins [4]. Thus, nitrotyrosine is a specific biomarker of peroxynitrite formation [37]. We studied whether ATN-224 increased intracellular superoxide formation. As shown in Supplemental Fig. S2, the expression level of nitrotyrosine was increased after ATN-224 treatment in K562 and TF-1 cells by 2.6 and 2.8 fold, respectively. These results showed that ATN-224 may induce death in myeloid leukemia cells via the increase of peroxynitrite level.

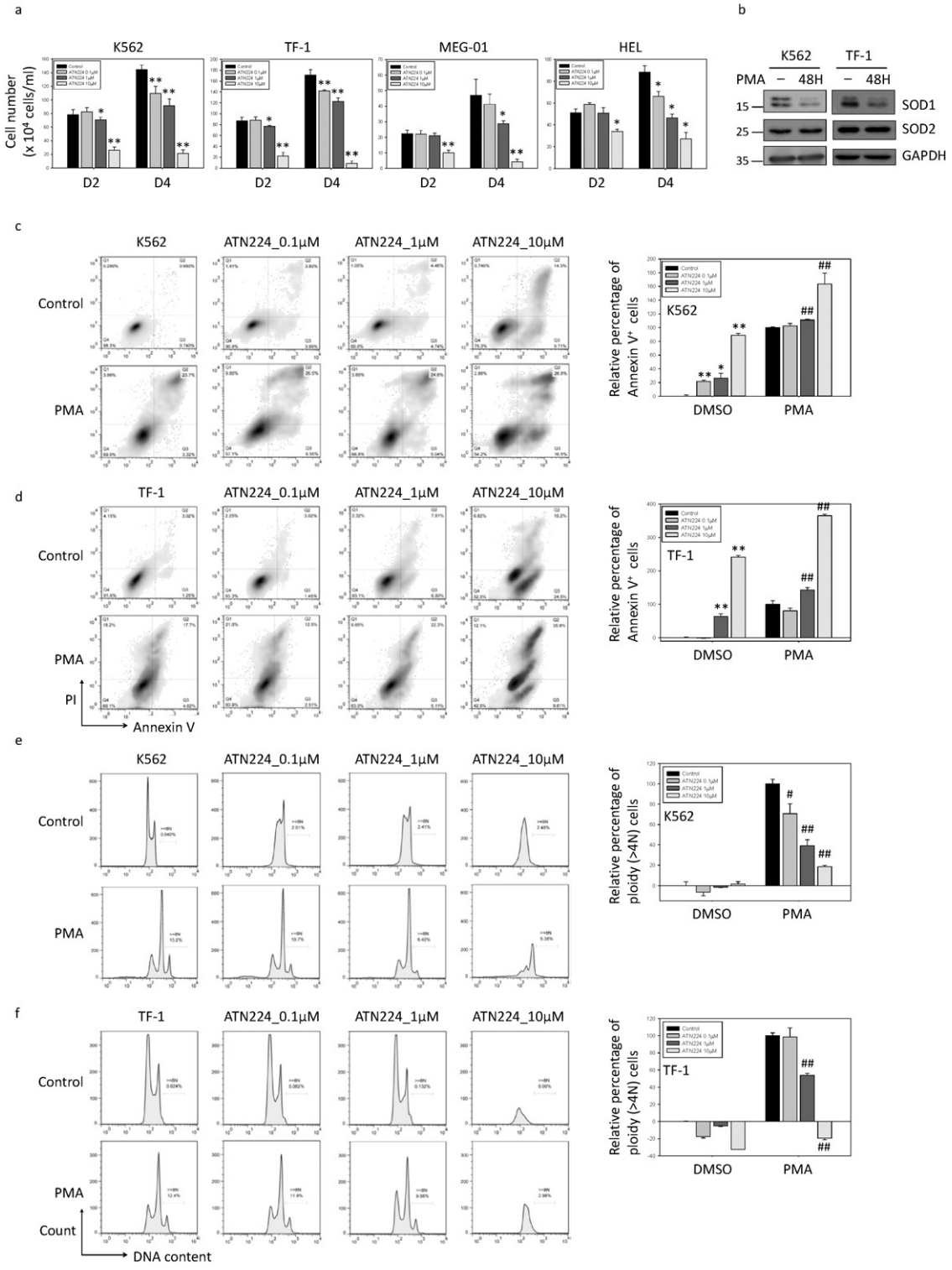

Fig. 1. ATN-224 dose-dependently inhibited cell proliferation and promoted cell death in myeloid leukemia cells. (a) K562, TF-1, HEL, and MEG-01 cells were treated with 0.1, 1, and 10 μM of ATN-224 for 48 and 96 h. The cell number was counted via trypan blue exclusion assay. (b) After 25 nM PMA treatment for 48 h, the cells were lysed and immunoblotted with SOD1 and SOD2. GAPDH was used as an internal control. (c–d) In the presence or absence of ATN-224 pretreatment for 30 min, the cells were harvested and stained with Annexin V/PI after PMA treatment for 48 h. The relative percentage of Annexin V-positive cells was normalized against control cells. (e–f) In the presence or absence of ATN-224 pretreatment for 30 min, the cells were harvested and fixed after PMA treatment for 96 h. DNA content was measured via PI staining assay. The relative percentage of high-ploidy number (≥8 N) cells was normalized against control cells. Similar results were obtained from at least three independent experiments. Values of $^{*}p < 0.05$ and $^{**}p < 0.01$ were indicated for the various treatments in comparison to the non-treated culture. Values of $\#p < 0.05$ and $\#\#p < 0.01$ were indicated for the various treatments in comparison to the PMA-only culture.

3.2. ATN-224 and knockdown of SOD1 reduced polyploidization and enhanced cell death by PMA

PMA activates $p22^{phox}$-dependent NADPH oxidase to increase ROS production and promote myeloid leukemia cell differentiation [5]. Moreover, PMA also induces myeloid leukemia cell death [6]. However, the role of SOD1 in PMA-induced myeloid leukemia cell death and differentiation is still unclear. Therefore, we studied the effect of PMA on SOD1 expression levels via immunoblotting assay. The results were shown in Fig. 1b, PMA down-regulated the protein level of SOD1 but not that of SOD2 in K562 and TF-1 cells. Next, to study the role of SOD1 in PMA-induced myeloid leukemia cell death and differentiation, we used ATN-224 to further inhibit the residual SOD1 after PMA administration. We observed that the further inhibition of SOD1 by ATN-224 dose-dependently increased the percentage of Annexin V-positive cells from 27.02% to 43% and from 21.72% to 45.51% in PMA-treated K562 and TF-1 cells, respectively (Fig. 1c and d).

Moreover, ATN-224 reduced the percentage of polyploidy cells (≥8 N) from 13.2% to 5.36% and from 12.4% to 2.98% in K562 and TF-1 cells, respectively (Fig. 1e and f). Thus, the further inhibition of SOD1 by ATN-224 promoted PMA-induced cell death and reduced differentiation in myeloid leukemia cells.

In order to directly verify the role of SODs, we employed lentiviral shRNA technology for the study. SOD1 and SOD2 were successfully knocked down by their respective shRNAs in K562 and TF-1 cells (Fig. 2a). We measured the intracellular ROS by DHE staining. The data were normalized against cells infected with scrambled shRNA after PMA treatment. As shown in Fig. 2b, SOD1 knockdown of increased intracellular ROS content by 60.9% and by 52.5% in K562 and TF-1 cells, respectively. The knockdown of SOD2 also had similar effects. Moreover, knockdown of SOD1 increased the relative percentage of Annexin V-positive cells by 26.6% and by 31.8% in K562 and TF-1 cells, respectively. Knockdown of SOD2 increased that by 9.7% and by 20.0% in K562 and TF-1 cells, respectively (Fig. 2c). Furthermore, knockdown of SOD1 reduced the relative percentage of ploidy cells by 32.0% (Fig. 2d) in K562 cells. The above data showed that the knockdown of SOD1 amplified the effects of PMA-induced SOD1 down-regulation, including polyploidization inhibition and death promotion. Moreover, the knockdown of SOD2 has the same effect on PMA-induced cell death and polyploidization, while its expression is not regulated by PMA.

3.3. Overexpression of SOD1 enhanced polyploidization and reduced cell death by PMA

To support the notion that SOD1 is involved in PMA-induced myeloid leukemia cell death and polyploidization, we used lentiviral vector to increase SOD1 expression in K562 cells (Fig. 3a). First, we used DHE staining to examine whether SOD1 overexpression reduced ROS formation by PMA. The results showed that SOD1 overexpression significantly reduced PMA-induced ROS formation (Fig. 3b).

It was reported that *N*-acetyl-L-cysteine (NAC) reduced ROS to non-toxic products [38]. In order to study the role of ROS in PMA-induced cell death and differentiation, we used the ROS scavenger NAC for the study. Fig. 3d showed that NAC dose-dependently reduced the relative percentage of Annexin V-positive cells from 94.7% to 57.5%. Next, we studied the effect of SOD1 overexpression on PMA induced cell death. After PMA treatment for 48 h, cells were stained with Annexin V and PI for flow cytometry analysis. SOD1 expression reduced Annexin V-positive cells from 29.29% to 23.02% (Fig. 3c). The relative percentage of Annexin V-positive cells was normalized against control cells and showed that SOD1 overexpression reduced Annexin V-positive cells by 21.4%. We also measured the level of nitrotyrosine by flow cytometry in SOD1 overexpression cells. We observed that compared with vector only cells, the nitrotyrosine level was decreased by 23.5% in SOD1 overexpressing cells (Fig. 3e). These data showed that superoxide are at least partially involved in PMA-induced cell death in K562 cells.

We further examined the effect of SOD1 expression on differentiation markers, polyploidization and CD61 expression (Fig. 3f and g). SOD1 overexpression increased PMA-induced polyploidization by 59.8%. However, the selective MK surface marker CD61 was only slightly increased by 12.9% as compared with control cells (vector only). The

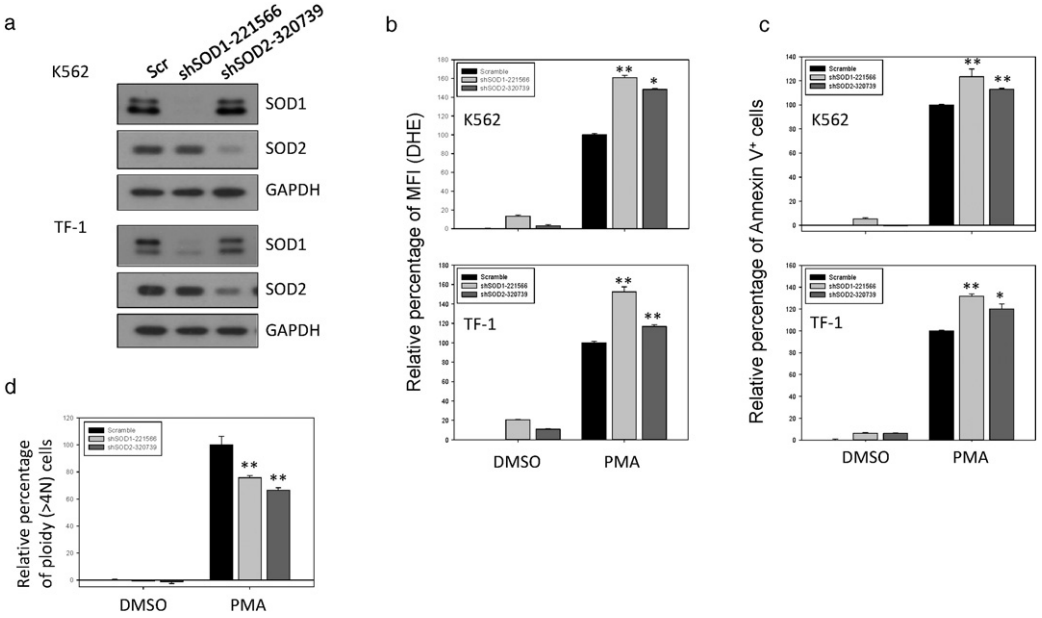

Fig. 2. SOD1 was involved in PMA-induced cell death. (a) K562 and TF-1 cells were infected with lentiviruses encoding for different shRNAs targeting SOD1 and SOD2, as described in "Materials and Methods." Knockdown efficiency was determined by immunoblotting with SOD1 and SOD2 antibody. Vector encoding scrambled shRNA was used as a control. (b–c) After PMA treatment for 48 h, the SOD1 and SOD2 knockdown cells were stained with DHE or Annexin V/PI. The cells were immediately analyzed by flow cytometry. The relative percentage of mean fluorescence intensity (MFI) and Annexin V-positive cells were normalized against control cells. (d) After PMA treatment for 96 h, the selected stable clones of SOD1 and SOD2 knockdown cells were analyzed via flow cytometry. The relative percentage of ploidy (≥8 N) cells was normalized against control cells.

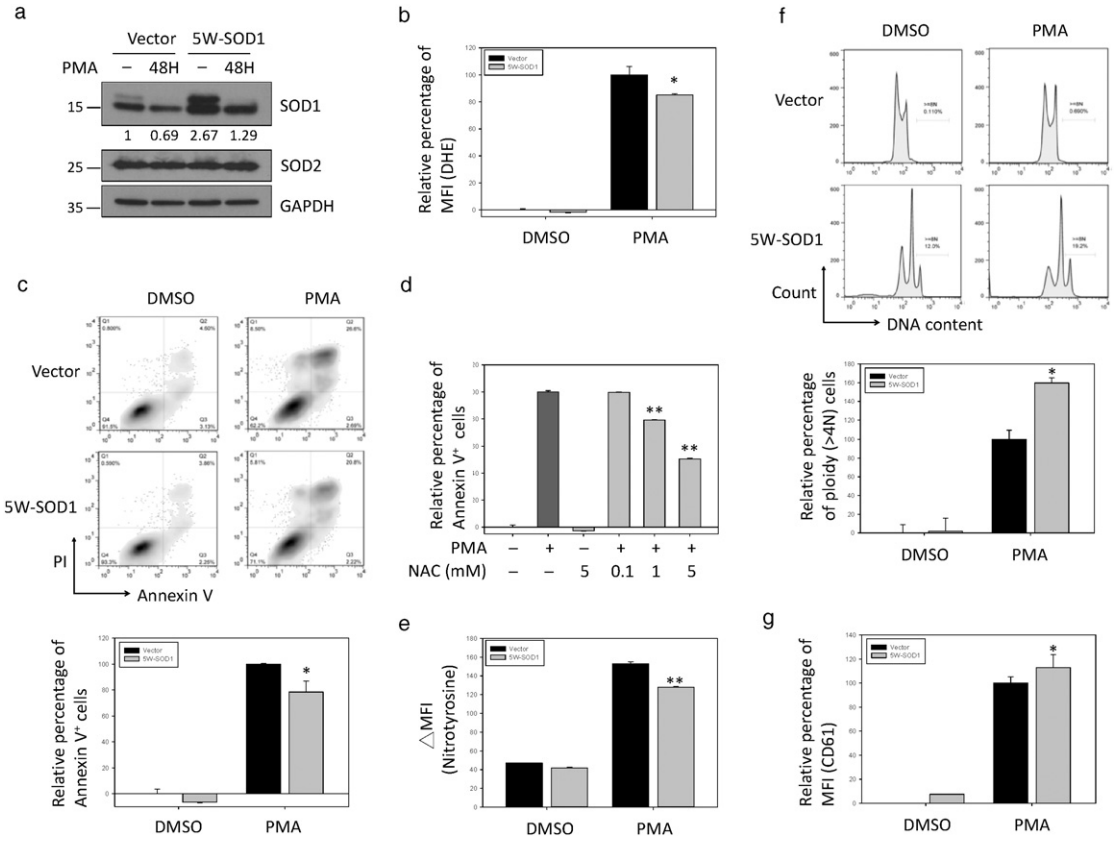

Fig. 3. SOD1 was involved in PMA-induced differentiation. (a) K562 cells were infected with lentiviruses encoding for the full-length cDNA of SOD1. The expression efficiency was determined via immunoblotting with SOD1 antibody. An empty vector was used as a control. (b–c) After PMA treatment for 48 h, the selected stable clones of SOD1-overexpression cells were harvested and stained with DHE and Annexin V/PI. The relative percentage of mean fluorescence intensity (MFI) and Annexin V-positive cells was normalized against the control cells. (d) In the presence or absence of 0.1, 1, and 5 mM NAC pretreatment for 30 min, cells were harvested and stained with Annexin V after PMA treatment for 48 h. The relative percentage of Annexin V-positive cells was normalized against control cells. (e) After PMA treatment for 48 h, the cells were harvested, fixed, permeabilized, and stained with anti-nitrotyrosine antibody. The cells were analyzed by flow cytometry. The relative percentage of △MFI (MFI of nitrotyrosine minus MFI of anti-FITC only) was normalized against control cells. (f–g) After PMA treatment for 96 h, the selected stable clones of SOD1-overexpressing cells were stained with PI solution and CD61-RPE antibody and analyzed via flow cytometry. The relative percentage of ploidy (≥8 N) cells and CD61 expression level was normalized against control cells. Similar results were obtained from at least three independent experiments. Values of $^{*}p < 0.05$ and $^{**}p < 0.01$ were indicated for the various treatments in comparison with the PMA-only culture.

results demonstrate that SOD1 is involved in myeloid leukemia cell death and polyploidization by PMA.

3.4. PMA regulated SOD1 via translational and post-translational control

In order to investigate the mechanism of PMA-induced SOD1 down-regulation, we used transcriptional, translational, and protein degradation inhibitors for the study. Transcriptional inhibitor actinomycin D (ActD) with a concentration up to 100 ng/ml did not reverse SOD1 down-regulation by PMA (Fig. 4a), suggesting that SOD1 down-regulation was not under transcriptional control. Moreover, pretreatment with either protein synthesis inhibitor cyclohexiamide (CHX) (Fig. 4b) or proteasome inhibitor MG132 (Fig. 4c) dose-dependently restored SOD1 protein levels which was down-regulated by PMA. Because SOD1 down-regulation will lead to the accumulation of superoxide, we detected ROS production when K562 cells were treated with these inhibitors by using DHE staining. As shown in Fig. 4d, CHX dose-dependently inhibited ROS production induced by PMA, while MG132 did not (Fig. 4e). Since MG132 itself increases ROS level in K562 cells [39], we were unable to observe ROS level reduction after MG132 treatment. It is possible that PMA regulates SOD1 via post-translational modification that promotes SOD1 degradation. These results indicated that PMA attenuated SOD1 expression via translation and post-translation control.

3.5. PMA down-regulated SOD1 through PKD2 activation

PMA is a potent PKC activator. Next, we identified the PKC isoforms involved in PMA-induced ROS formation by using a classical PKC isoform inhibitor, Gö6976 and a general PKC inhibitor, GF109203x. This approach is commonly used to delineate which PKC families is involved in a biological process [40]. Gö6976 reversed 4.2% of PMA-induced superoxide formation at 5 nM and reduced 55.9% of that at 50 nM, while GF109203x failed to do so (Supplemental Fig. S3). Although Gö6976 inhibits classical PKC isoforms below 10 nM (IC_{50}), it also inhibit PKD isoforms at 20 nM (IC_{50}) [24]. Therefore, PKD isoforms may be involved in PMA-induced superoxide formation and PKC isoforms may not be involved.

PKD isoforms have been reported to be involved in ROS regulation [26]. To confirm the notion that PKD mediates PMA actions in our system, we first studied whether PKD isoforms were activated by PMA and which PKD isoform(s) is/are involved. Mihailovic et al. has shown that myeloid leukemia cells express mainly PKD2 [28]. We used commercially available antibodies to analyze the expression of PKD1 and PKD2 by using 293 T cells as a positive control [28]. We found that K562 cells expressed only PKD2, whereas TF-1 cells expressed both PKD1 and PKD2 (Fig. 5a).

Next, we investigated whether PKD2 is activated by PMA by using phosphorylation antibodies. PKD2 was phosphorylated at various sites, as observed in myeloid leukemia cell lines after PMA treatment.

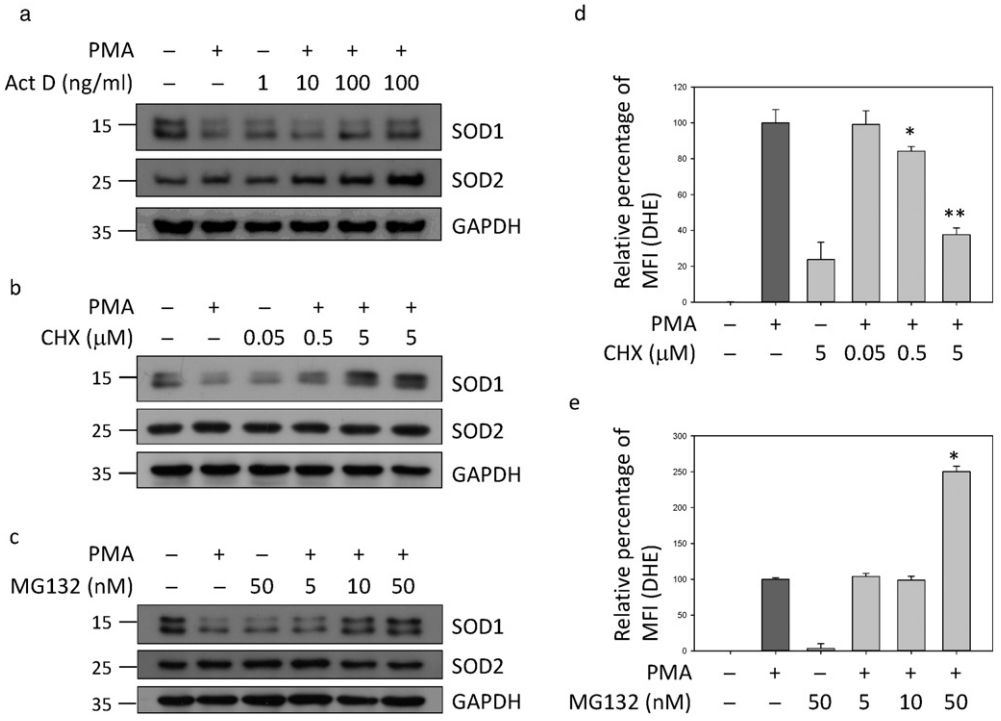

Fig. 4. SOD1 is down-regulated by PMA via translational and post-translational control. In the presence or absence of different concentration of Actinomycin D (Act D), cycloheximide (CHX), and MG132, K562 cells were harvested after PMA addition for 48 h. (a–c) The cell lysates were immunoblotted with SOD1 and SOD2. GAPDH was used as an internal control. (d–e) The cells were harvested, stained with DHE, and analyzed via flow cytometry. The relative percentage of mean fluorescence intensity (MFI) was normalized against control cells. Similar results were obtained from at least three independent experiments. Values of $^{*}p < 0.05$ and $^{**}p < 0.01$ were indicated for the various treatments in comparison with the PMA-only culture.

In K562 cells (Fig. 5b), (1) phosphorylation at Ser876 (which indicates its active state [41]) increased rapidly within 1 min and was sustained for 48 h. (2) After 30 min, a mobility shift was observed using a PKD2-Ser876 (hyper-phosphorylation state [41]) phosphorylation antibody. (3) At auto-phosphorylation site [25], Ser916 exhibited a similar phosphorylation pattern as observed in Ser876. (4) Phosphorylation at Ser744/748 (a unique phosphorylated site catalyzed by PKC [42]) had a shorter period of phosphorylation than that of Ser876. Similar results were observed in PMA-treated TF-1 cells (Fig. 5b, below). Ser876 phosphorylation indicated that PKD2 is activated by PMA. Moreover, PKD2 appears to be phosphorylated by multiple kinases and/or at multiple sites, as shown by its hyper-phosphorylated state after 30 min.

PKD2 has been reported to be involved in BON endocrine cell proliferation/invasion and platelet generation (dense granule secretion) [27,43]. To determine whether PKD2 mediates the PMA regulation of SOD1 expression levels, we used selective PKD inhibitor CID755673 for the study [44]. K562 and TF-1 cells were treated with PMA with or without CID755673 treatment for 48 h. The immunoblotting data showed that CID755673 dose-dependently restored SOD1 expression in both K562 and TF-1 cells from 0.1 μM to 5 μM (Fig. 5c) against PMA down-regulation. The results show that the inhibition of PKD rescued SOD1 expression level after being down-regulated by PMA.

To further confirm whether PKD2 mediates the down-regulation of SOD1 expression by PMA, we employed lentiviral shRNA technology. PKD2 was successfully knocked down by shRNA in both K562 and TF-1 cells (Fig. 5d). After PMA treatment for 24 and 48 h, respectively, we observed that the knockdown of PKD2 reversed both the PMA-induced down-regulation of SOD1 protein level and activity in K562 cells (Fig. 5e). Thus, the data confirmed that PKD2 mediated PMA down-regulation of the SOD1 level.

3.6. Down-regulation of PKD2 enhanced polyploidization by PMA

Because PKD2 knockdown reversed SOD1 down-regulation by PMA, we investigated whether PKD2 mediates PMA-induced polyploidization via PKD2 inhibition. In PMA-treated K562 cells, 12.3% of the cells possessed a high ploidy number ≥8 N. With CID755673 treatment, the population with high ploidy number (≥8 N) increased to 20.1% (Fig. 6a). The relative percentage of high-ploidy-number (≥8 N) cells was normalized against control cells. Both K562 and TF-1 cells with high ploidy numbers (≥8 N) dose-dependently increased from 106.5% to 159.8% and from 97.5% to 145.3%, respectively, after CID755673 treatment from 0.1 μM to 5 μM (Fig. 6a, right and 6e). Thus, PKD is involved in suppressed polyploidization in PMA-treated myeloid leukemia cells.

K562 cells are susceptible to chemically induced differentiation. PMA-induced K562 cells differentiated into megakaryocyte-like cells, while chemicals such as butyric acid induced K562 cells to differentiate into erythroid-like cells [45]. We studied the effect of PKD inhibition on the expressions of PMA-induced MK surface markers CD41 and CD61, and an erythrocyte surface marker, glycophorin A (GPA), was used as a control. We observed that CID755673 had no effect on CD41 or CD61 expression (Fig. 6b), while the basal level of GPA was decreased in cells treated with CID755673 only (Fig. 6b, bottom). These results implied that basal PKD activity might be required for basal level GPA expression in untreated K562 cells, while it does not mediate PMA-induced MK surface markers expression.

To further confirm whether PKD2 was involved in increased polyploidization induced by PMA in myeloid leukemia cell lines, we studied the effect of PKD2 knockdown on the percentage of cells with high ploidy numbers (≥8 N) after PMA treatment. As shown in Fig. 6c, in the two PKD2 knockdown of K562 cells, the percentage of cells with high ploidy numbers (≥8 N) was increased by 51.8% and 56.8%, respectively, when compared to cells infected with scrambled shRNA after

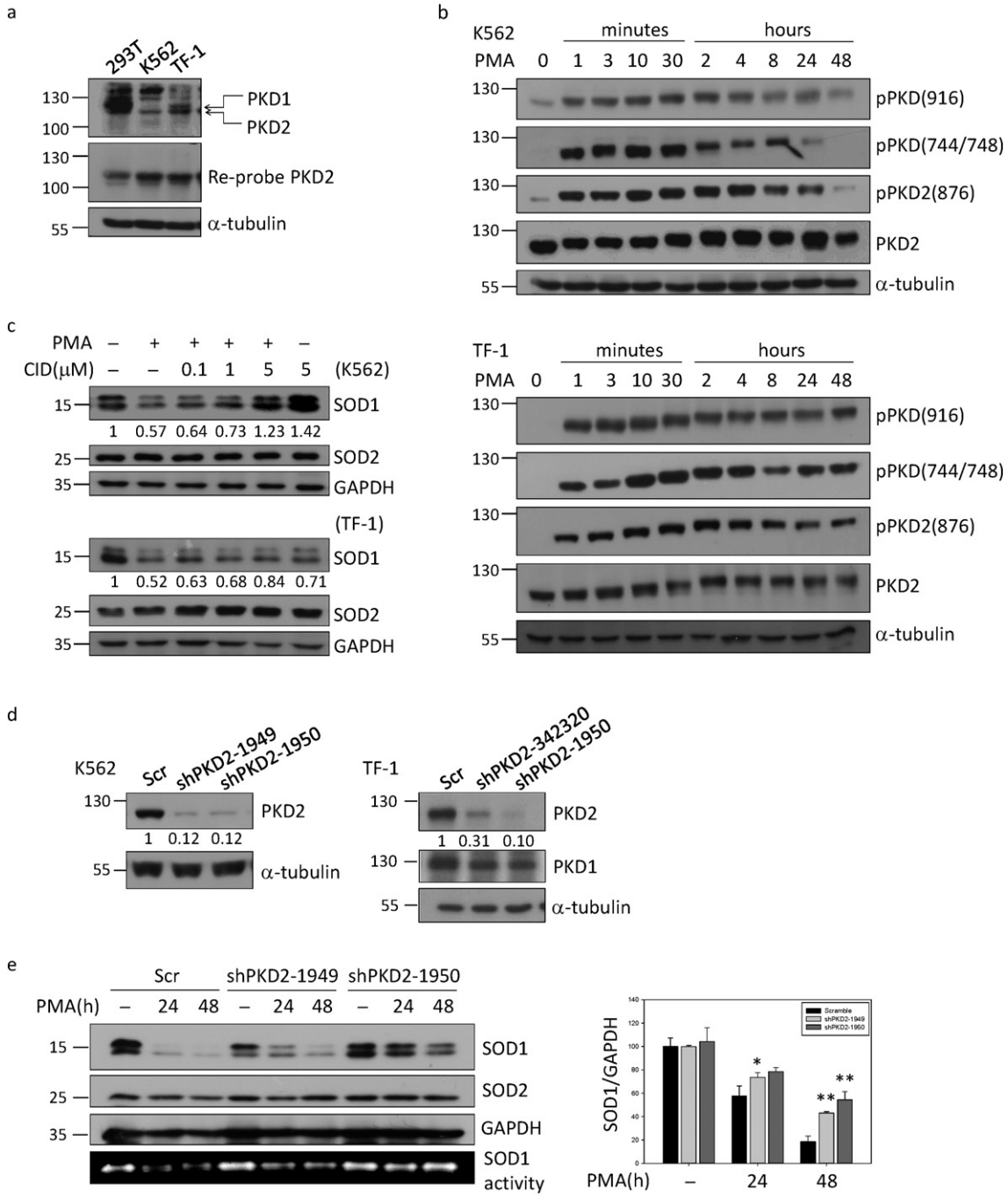

Fig. 5. PMA activated PKD2 and resulted in SOD1 down-regulation. (a) An immunoblot analysis showed that K562 and TF-1 cells expressed PKD isoforms. 293 T cells served as a control. (b) K562 and TF-1 cells were lysed after 25nM PMA treatment for the indicated time and immunoblotted with the following antibodies: pPKD Ser744/748, pPKD Ser876, pPKD Ser916, and PKD2. Alpha-tubulin was used as an internal control. (c) In the presence or absence of 0.1, 1, and 5 μM of CID755673 (CID), the cell lysates were immunoblotted with SOD1 and SOD2 antibodies. GAPDH was used as an internal control. (d) The cells were infected with lentiviruses encoding for various shRNAs targeting PKD2. Knockdown efficiency was determined by immunoblotting with PKD2 antibody. Vector-encoding scrambled shRNA was used as a control. (e) The selected stable clones of PKD2-knockdown cells were harvested after PMA treatment for 24 and 48 h and immunoblotted with SOD1 and SOD2 antibodies, respectively. The relative percentage of SOD1 expression levels was normalized against GAPDH. SOD1 activity was detected by in gel assay (described as "materials and methods"). Similar results were obtained from at least three independent experiments. Values of $^{*}p < 0.05$ and $^{**}p < 0.01$ were indicated for the various treatments in comparison with the PMA-only culture.

PMA treatment. Similar results were also observed in TF-1 cells (Fig. 6f). On the other hand, PKD2 knockdown has no effect on PMA-induced CD41 and CD61 expression (Fig. 6d). Nonetheless, the expression of GPA, an erythrocyte surface marker, was also significantly reduced in PKD2 knockdown cells without PMA treatment (Fig. 6d, bottom), as observed in PKD inhibition. Apparently, PKD2 activity suppressed polyploidization in PMA-treated myeloid leukemia cells. Although it has no effect on PMA-induced CD41 and CD61 expression, it regulates GPA expression in untreated K562 cells. PKD2 is activated by PMA, while PKD2 activation partially attenuated the full-potential polyploidization induced by PMA.

3.7. Down-regulation of PKD2 reversed cell death by PMA

Because PMA-induced PKD2 activation partially suppressed the polyploidization induced by PMA, we investigated the effect of PKD2

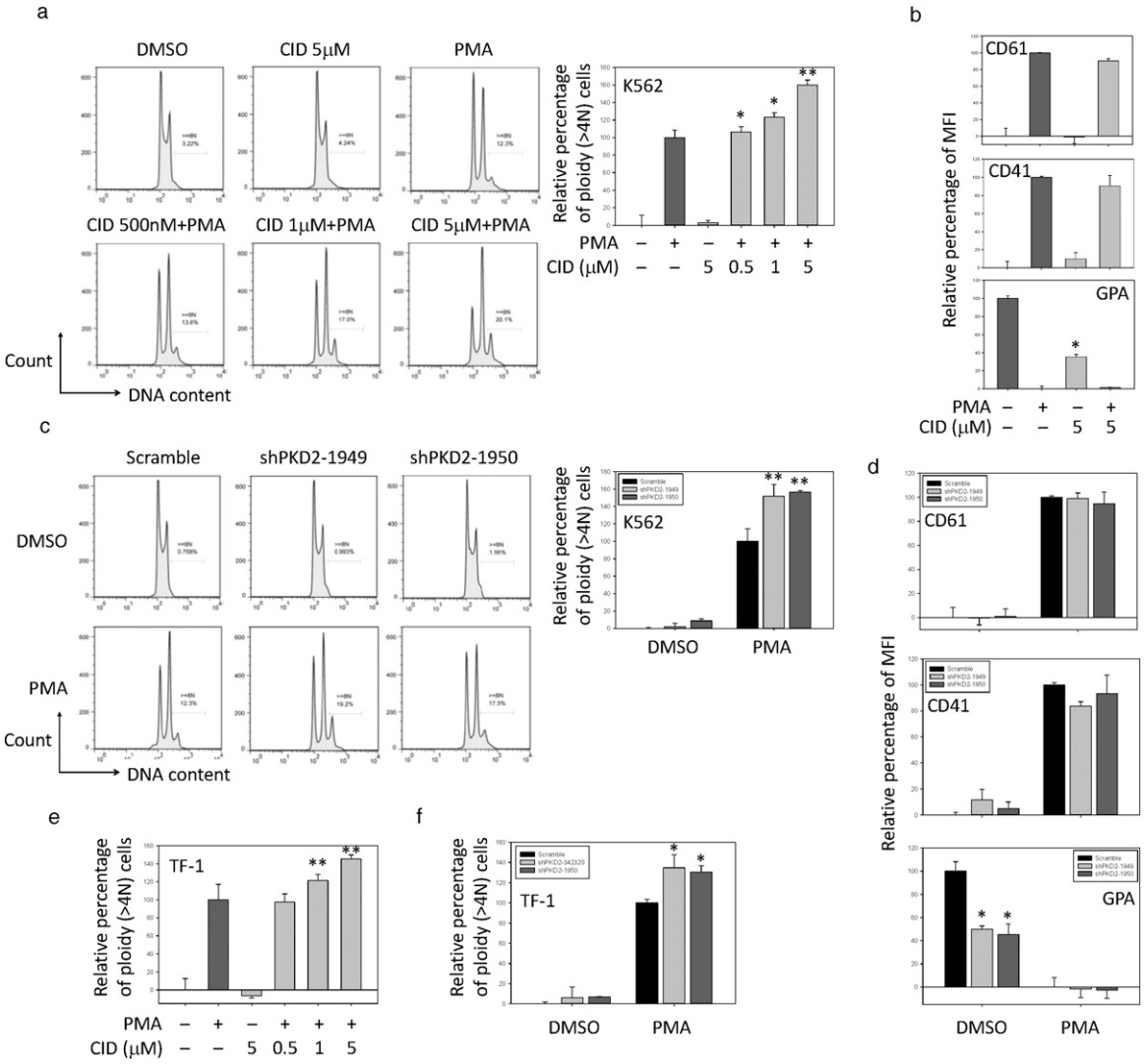

Fig. 6. The inhibition or knockdown of PKD2 promoted PMA-induced polyploidization. (a, e) In the presence or absence of 0.1, 1, and 5 μM of CID755673 (CID), the cells were harvested, stained with PI solution, and analyzed via flow cytometry after PMA treatment for 96 h. The relative percentage of ploidy (≥8 N) cells was normalized against control cells. (b–d) The cells were harvested after PMA treatment for 96 h and staind with CD41, CD61, and GPA antibodies, respectively. The relative percentages of MFI were normalized against control cells (GPA was normalized with PMA-treated cells). (c, f) The selected stable clones of PKD2-knockdown cells were harvested after PMA treatment for 96 h, stained with PI solution, and analyzed by flow cytometry. The relative percentage of ploidy (≥8 N) cells was normalized against control cells. Similar results were obtained from at least three independent experiments. Values of $^{*}p < 0.05$ and $^{**}p < 0.01$ were indicated for the various treatments in comparison with the PMA-only culture.

on PMA-induced myeloid leukemia cell death. First, we characterized the role of PKD in PMA-induced cell death by using Annexin V/PI staining. PKD inhibition by CID755673 showed dose- and time-dependent reductions of PMA-induced K562 cell death (Fig. 7a and b). Similar results were observed in TF-1 cells (Fig. 7b, right). These results suggested that PKD mediates K562 and TF-1 cell death induced by PMA.

Because PKD inhibitor CID755673 reduced cell death induced by PMA, we used PKD2 lentiviral shRNA to further confirm this notion. We examined whether PKD2 knockdown reduced PMA-induced ROS formation by using DHE staining. The results showed that PKD2 knockdown significantly reduced ROS formation by PMA in K562 and TF-1 cells (Fig. 7c). Next, PKD2 knockdown cells were analyzed via Annexin V/PI staining assay after PMA treatment for 48 h. The dot plot data were showed in Fig. 7e. After PMA treatment, the two PKD2 knockdown cells reduced the percentage of Annexin V-positive cells from 38.74% to 17.08% and 19.28%, respectively. Normalized data were showed below. These data were normalized against cells infected with scrambled shRNA after PMA treatment. The two of PKD2 knockdown in K562 cells diminished the percentage of Annexin V-positive cells by 42.1% and 39.2%, respectively. The knockdown of PKD2 in TF-1 cells also showed similar results. The percentage of Annexin V-positive cells was decreased by 37.1% and 21.2%, respectively (Fig. 7f) (shPKD2-342320 has similar effect in K562 cells). We also measured nitrotyrosine level in PKD2 knockdown cells. Compared with control cells (Scramble), nitrotyrosine level was decreased by 55.9% and 57.6% respectively in two PKD2 knockdown cells after PMA treatment (Fig. 7d). The results confirmed that PKD2 positively regulates PMA-induced myeloid leukemia cell death.

3.8. PMA cleaves PARP into active forms via PKD2/SOD1

To examine the mechanism of PMA-induced cell death in myeloid leukemia cell lines, we studied the possible involvement of caspase 3, 8, and 9 [46]. As shown in Fig. 8a, caspase 3 and 8 were not activated after PMA administration in K562 cells. Notably, the cleaved forms (35 kDa and 37 kDa) of caspase 9 were observed after 48 h and were dose-dependently attenuated by PKD inhibitor CID755673. However, pan caspase inhibitor Z-VAD-FMK did not reverse the ROS formation induced by PMA (Fig. 8b). Because caspase 9 may be related to MK

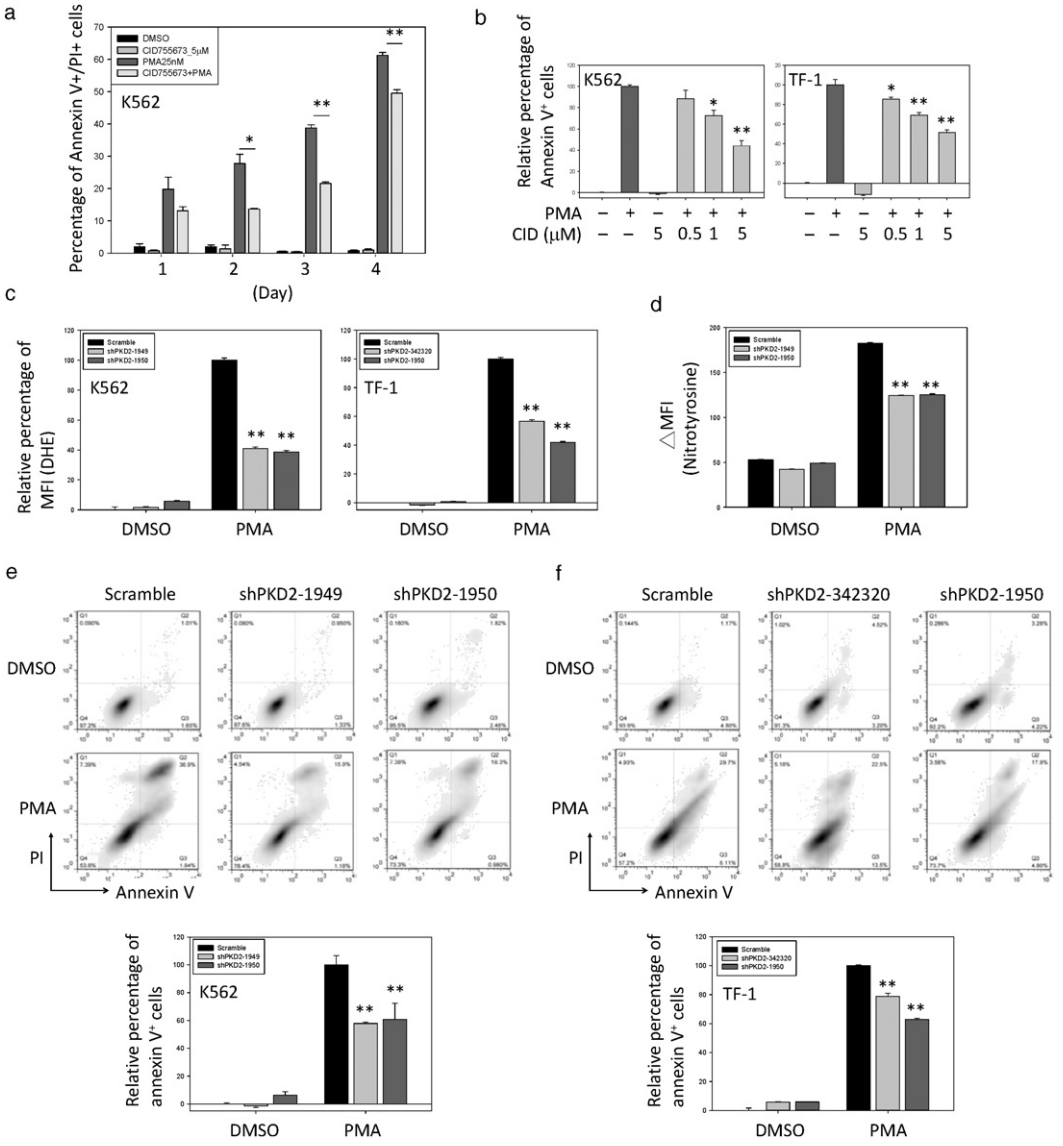

Fig. 7. The inhibition and knockdown of PKD2 reduced cell death by PMA. (a) In the presence or absence of 5 μM CID755673 (CID), the cells were harvested, stained with Annexin V and PI solution, and analyzed via flow cytometry after PMA treatment. The percentage of Annexin V-positive cells at indicated time points was represented as a bar chart. (b) In the presence or absence of 0.1, 1, and 5 μM of CID755673, the cells were stained with Annexin V/PI and analyzed after PMA treatment for 48 h. The relative percentage of Annexin V-positive cells was normalized against control cells. (c) The PKD2 knockdown cells were harvested, stained with DHE, and analyzed via flow cytometry. The relative percentage of mean fluorescence intensity (MFI) was normalized against control cells. (d) After PMA treatment for 48 h, the cells were harvested, fixed, permeabilized, stained with anti-nitrotyrosine antibody, and analyzed by flow cytometry. The relative percentage of △MFI (MFI of nitrotyrosine minus MFI of anti-FITC only) was normalized against control cells. (e) The selected stable clones of PKD2 knockdown cells were harvested and stained with Annexin V/PI after PMA treatment for 48 h. The relative percentage of Annexin V-positive cells was normalized against cells infected by scrambled shRNA. Similar results were obtained from at least three independent experiments. Values of $^{*}p < 0.05$ and $^{**}p < 0.01$ were indicated for the various treatments in comparison with the PMA-only culture.

differentiation, apoptosis signals were not involved in myeloid leukemia cell death induced by PMA [47].

There have been reports suggesting that PMA activates PARP in both thymocytes [48] and human leukemia HL-60 cells [49]. We studied whether PARP activation was involved in PKD2-mediated PMA-induced cell death in K562 cells. As shown in Fig. 8c, we observed that PARP was cleaved into at least three fragments, including both 55 and 44 kDa fragments, after PMA treatment for 48 h. As reported by Gobeil, S. et al., these unique cleavage products of PARP are considered markers of necrosis [50]. The knockdown of PKD2 significantly reduced PMA-induced PARP cleavage. The data suggested that PARP cleavage could be regulated by PKD2 in K562 cells. Therefore, PKD2 is involved in PMA-induced necrosis in K562 cells. Next, we used ROS scavenger NAC to study whether PARP activity was regulated by ROS. The data showed that NAC reduced PARP cleavage in K562 cells (Fig. 8d). We also use SOD1 knockdown cells to confirm the result. As shown in Fig. 8e, PMA-induced cleavage of PARP was enhanced in SOD1 knockdown cells. These indicated that PARP signaling is involved in the PKD2/SOD1-mediated K562 cell death induced by PMA.

3.9. ATN-224 enhances Ara-C-induced inhibition effect on myeloid leukemia proliferation

In order to study whether ATN-224 enhances the therapeutic effect of free-radical generating chemotherapeutic agents, we used cytarabine (cytosine arabinoside or Ara-C) for the study. Ara-C is clinically used for the treatment of myeloid leukemia [51]. To investigate that whether Ara-C increases intracellular ROS level in myeloid leukemia cells, we observed that Ara-C dose increased intracellular ROS in K562, TF-1, MEG-01, and HEL cells by using DHE staining assay (Fig. 9a). Next, we studied whether ATN-224 enhanced the inhibition of cell proliferation by Ara-C. Co-treatment with Ara-C and ATN-224 at different dosage also

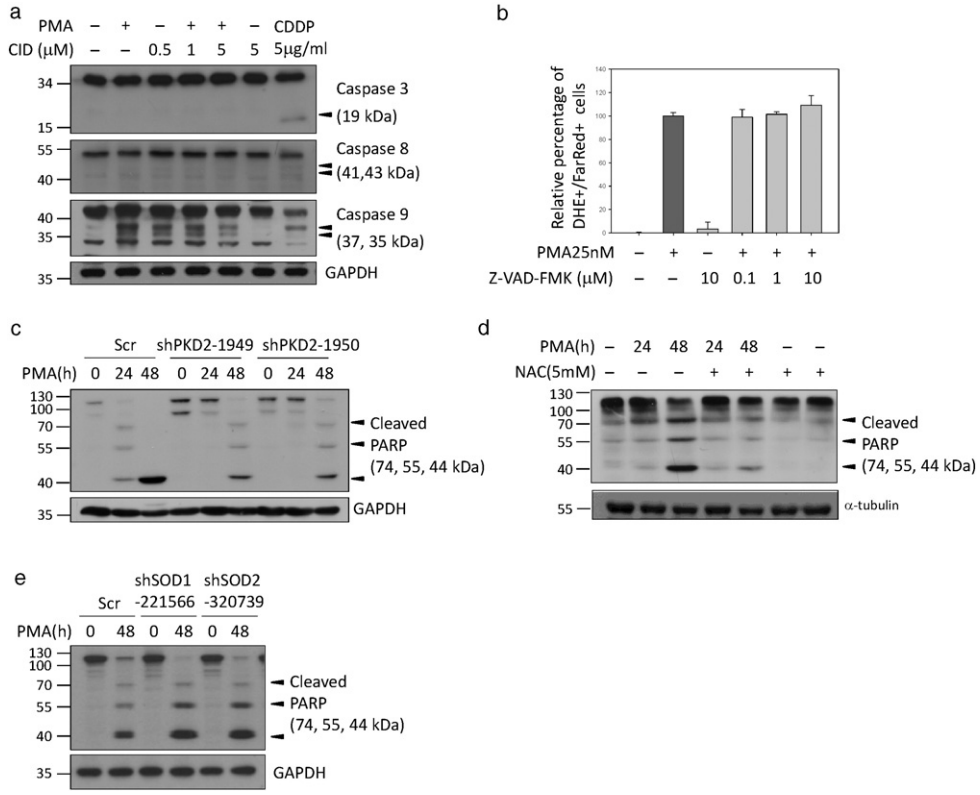

Fig. 8. PMA/PKD2/SOD1-mediated cell death was related to PARP activation and ROS production. (a) In the presence or absence of 0.5, 1, and 5 μM CID755673 (CID), the cell lysates were immunoblotted with antibodies against caspase 3, 8, and 9. GAPDH was used as an internal control. (b) In the presence or absence of 0.1, 1, and 10 μM Z-VAD-FMK, the cells were harvested and stained with DHE and FarRed dye, respectively. The relative percentage of DHE/FarRed double-stained cells was normalized against control cells. (c, e) The cells were harvested after PMA treatment for 24 and 48 h. The cell lysates were immunoblotted with PARP antibody. GAPDH was used as an internal control. (d) In the presence or absence of 5 mM NAC, the cells were harvested and immunoblotted with PARP antibody after PMA treatment for 24 and 48 h. GAPDH was used as an internal control.

synergistically inhibits cell proliferation in these cells by using trypan blue exclusion assay (Fig. 9b). These data indicated that ATN-224 enhances Ara-C-induced inhibition effect on myeloid leukemia proliferation.

4. Discussion

The intracellular ROS level in myeloid leukemia cells is vital to the regulation of cell fate, including cell death and differentiation. Although antioxidant molecules, such as SODs, play a major role in the modulation of ROS levels, their role in the regulation of myeloid leukemia cell death and differentiation is still largely unknown. In this study, we studied the role of SODs in the regulation of cell death and differentiation. Myeloid cell lines K562, TF-1, HEL, and MEG-01, which were treated with PMA, were used as a study model. We discovered that the inhibition of SOD1 reduces cell survival via inhibition of phosphorylation of AKT and $ERK_{1/2}$ and promotes cell death through increasing peroxynitrite formation. Moreover, the inhibition or

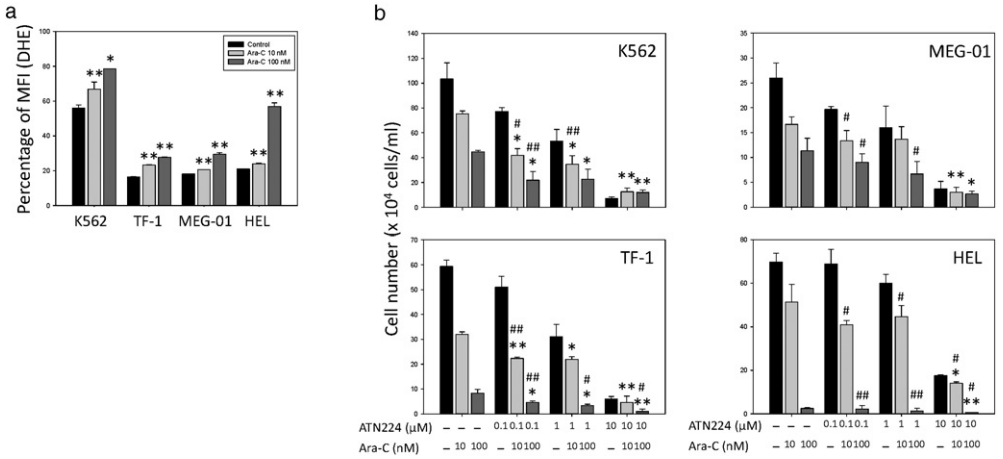

Fig. 9. ATN-224 enhanced the inhibition of proliferation by Ara-C in myeloid leukemia cells. (a) In the presence or absence of 10 and 100 nM Ara-C, the cells were harvested, stained with DHE, and analyzed via flow cytometry. The relative percentage of mean fluorescence intensity (MFI) was normalized against control cells. (b) K562, TF-1, HEL, and MEG-01 cells were co-treated with indicated concentration of ATN-224 and Ara-C for 72 h. The cell number was counted via trypan blue exclusion assay. Results were obtained from at least three independent experiments. Values of $^{*}p < 0.05$ and $^{**}p < 0.01$ were indicated for the various treatments in comparison with the Ara-C-only culture. Values of #p < 0.05 and ##p < 0.01 were indicated for the various treatments in comparison with the ATN-224-only culture.

knockdown of SOD1 promotes myeloid leukemia cell death and inhibits polyploidization induced by PMA. SOD1 can be regulated by PKD2, a PKC isoform, probably via translational and post-translational control. PKD2/SOD1 induced cell death through PARP cleavage and peroxynitrite formation, which is induced by PMA. Finally, we also found that ATN-224 enhances the inhibition of proliferation by Ara-C. These findings help us to understand the physiological functions of SOD1 in myeloid leukemia cell death and differentiation regulation and may also indicate a role for ATN-224 in myeloid leukemia treatment.

ROS play an important role in myeloid leukemia cells cell proliferation, death, and differentiation. ROS level can be regulated via a subtle balance between biosynthesis and degradation. In myeloid leukemia cells, ROS formation can be increased by NADPH oxidase 4 (NOX4) activation, which in turn activates the pro-proliferative molecule Fyn and encourages the survival of myeloid leukemia cells [1]. ROS may also be further increased via the activation of NADPH oxidase subunit $p22^{phox}$, which promotes megakaryocytic differentiation in myeloid leukemia cell lines such as K562 and HEL [5]. Apparently, any further increase in ROS that overwhelms the antioxidant system may result in cell death [52].

Myeloid cells TF-1, K562, and HEL differentiate into MK under PMA treatment that requires ROS increase [5]. Therefore, ROS balance regulation may be the common factor that determines their fate. Although the role of ROS in AML cells are not studied as extensively as that of CML cells, recent studies suggested that ROS regulation is also vital to AML biology [a recent review can be found in [53]]. Our study suggests that ROS increase induced by SODs inhibition or knockdown inhibits proliferation signals such as AKT or $ERK_{1/2}$ in both CML and AML cells. We observed that ATN-224 dose-dependently inhibits cell proliferation in TF-1, HEL, K562, and MEG-01 cells (Fig. 1a). The anti-proliferation effect of ATN-224 is likely due to its ability to inhibit both ATK and $ERK_{1/2}$ activation (Supplemental Fig. S1). Moreover, both SOD1 inhibition and knockdown enhance cell death (Fig. 1c and d).

MG132 also affects NFκB that may have an effect on ROS generation. Since MG132 itself increases ROS level in K562 cells [39], we were unable to observe ROS level reduction after MG132 treatment. We have increased the concentration of MG132 without success while cell death increased. Using another 26S proteasome inihibitor bortezomib, while more selective, it caused serious cell death using the suggested concentrations (data not shown). Similar observation were made by other researchers on K562 cells [39,54]. From the results shown in Fig. 3d and e, it is possible that PMA regulates SOD1 via translation of an unknown protein mediator or through post-translational modification that promotes SOD1 degradation. However, it is not yet conclusive.

SODs are major ROS degradation enzymes that transform superoxide into hydrogen peroxide. SOD1 mRNA down-regulated by PMA has been showed in acute leukemia cell lines (U937, HL60, and HEL) [17,18]. In our study, the expression level of SOD1 was down-regulated in myeloid lukemia cell lines (K562 and TF-1) (Fig. 1b) during differentiation. However, the role of SODs in myeloid leukemia cell differentiation remains unclear. In our study system, PMA plays a role in both inducing death and differentiation, while increasing ROS production via $p22^{phox}$. Thus, PMA may mimic SOD1 down-regulation when it provides additional free radical production. We observed that increased SOD1 expression via infection before PMA treatment actually enhances PMA-induced polyploidization (Fig. 3f). Therefore, the PMA down-regulation of SOD1 expression actually hampers polyploidization. Previous studies have shown that exogenous hydrogen peroxide promotes polyploidization by PMA [16]. Based on our results, reduced SOD1 may attenuate hydrogen peroxide production, which confirms the vital role of hydrogen peroxide in MK cell cycle regulation. This may explain why PMA-induced polyploidization in MK cell lines seldom exceeds $n > 32$.

The localization of SOD1 in the nucleus or the mitochondria may be related to polyploidization. The question of whether SOD1 regulates cell cycle proteins, such as p21, via hydrogen peroxide or is involved in interactions with cyclins through SOD1 nuclear translocation [14,55] requires further study. Recently, Huang R. et al. [11] used cyclosporin A to stabilize mitochondrial membrane potential and promote PMA-induced MK surface marker CD41 and CD61 expression. Because SOD1 is also located in the inter membrane space of the mitochondria, we can expect that SOD1 will degrade the ROS that are produced by respiratory chain complex IV to stabilize the mitochondrial membrane potential and enhance polyploidization.

The physiological functions of SODs in myeloid leukemia cells may be different from those in breast cancer. In breast cancer, SOD1 up-regulation compensated for the down-regulation of SOD2 and removed excess ROS [14,15]. Our data show that the knockdown of either SOD1 or SOD2 increases ROS formation and cell death, and reduces polyploidization induced by PMA (Fig. 2b, c, and d). However, SOD2 is not regulated by PMA (Fig. 1b). The knockdown of either SOD1 or SOD2 did not enhance the expression of their counterparts (Fig. 2a). Moreover, SOD1 overexpression also did not affect SOD2 expression levels (Fig. 3a).

Therapies targeting SOD1 have been studied in some hematological tumors, such as diffuse large B-cell lymphoma and acute T-cell lymphoblastic leukemia [4,12]. Moreover, the knockdown of SOD1 also enhances $CD34^{+}$ K562 cell sensitivity to imatinib treatment [31]. Here, we investigated the effect of ATN-224 on myeloid leukemia cells. We observed that ATN-224 inhibits myeloid leukemia cell proliferation and induces cell death (Fig. 1a, c, and d). Moreover, knockdown of either SOD1 or SOD2 promotes cell death and inhibits polyploidization induced by PMA (Fig. 2c and d). We suggest that the inhibition of both SOD1 and SOD2 may act synergistically in myeloid leukemia treatment.

PKD2 is a PKC isoform that is activated by PMA. PMA is a highly potent PKC activator that activates cPKC, nPKC, and PKD, while it has no action on aPKC, which has no diacylglycerol (DAG) binding site [56]. Jacquel et al. observe that PKCα, β, γ, δ, and ε translocate from the cytosol to the membrane within 15 min after PMA treatment in K562 cells [23]. Thus, all available PKC and PKD isoforms may be activated by PMA simultaneously. In our results, we observed biphasic PKD phosphorylation after PMA treatment, including an early phase and a hyper-phosphorylation phase. Our results suggest that during the early phase of PMA action, PMA simultaneously activates multiple PKC/PKD isoforms within 30 min. In the hyper-phosphorylation phase, a mobility shift was observed from 30 min to 48 h after PMA treatment (Fig. 5b). These activated kinases further interact with their neighboring proteins. Unlike the activation of a single receptor, which has clear cellular objective, the result of PMA treatment is chaotic. The effect of PMA treatment is probably the summation of the effects of all activated PKC/PKD isoforms and their subsequent interactions. Therefore, both differentiation and death signals are observed after the PMA activation of PKC/PKD isoforms.

We also observed that PKD2 plays an important role in myeloid leukemia cell death and differentiation. The lineage control of erythrocyte–megakaryocyte is important for myeloid leukemia differentiation. PKCε has been reported to be involved in regulating megakaryocytic lineage commitment through functional cooperation with GATA-1 in the activation of the CD41 promoter [22]. However, PKCε alone cannot account for other PMA actions in myeloid leukemia cells. In our study, we found that PKD2 is involved in PMA-induced MK differentiation, while PKD2 regulates basal erythroid marker GPA expression (Fig. 6b and d). It has been reported that BCR-ABL phosphorylates PKD2 at Tyr438 [28], while other reports have suggested that Bcr-Abl inhibitor imatinib promotes GPA expression in K562 cells [57]. In this study, we observed that PMA induced the phosphorylation of PKD2 at Ser876 (Fig. 5b). The knockdown of PKD2 decreased the basal level expression of GPA (Fig. 6d). These data suggested the possibility that BCR-ABL may regulate GPA expression via PKD2. We also demonstrated a new function of PKD2 in that it suppressed polyploidization in myeloid leukemia differentiation induced by PMA, while its basal activity maintains GPA expression. To

our surprise, PKCε and PKD2 may play an antagonistic role in the lineage control of myeloid leukemia cells.

PKD2 regulates myeloid leukemia cell death via SOD1. Our data show that endogenous PKD2 may promote SOD1 turnover. Interestingly, PKD2 knockdown reversed 50–60% of cell death induced by PMA, while SOD1 overexpression reversed PMA-induced cell death at a rate of about 20% (Figs. 3c and 7e). The data suggest that PKD2 increased myeloid leukemia cell death induced by PMA and that this may also involve a SOD1-independent pathway. Previous studies have shown that the down-regulation of PKCε relates to PMA-induced K562 and TF-1 cell death [6]. In our data, PKD2 activation is related to PMA-induced K562 and TF-1 cell death. Thus, PKCε and PKD2 may have opposing actions in PMA treatment.

The mechanism of PMA-induced cell death in myeloid leukemia cells was investigated. In our study, PMA caused necrosis through the activation of PKD2, which down-regulates SOD1 and cleaves PARP (Figs. 5e and 8c). Inhibition of SOD1 by ATN-224 increases peroxynitrite levels in K562 and TF-1 cells (Supplemental Fig. S2) indicating an increase in superoxide formation leading to cell death. Moreover, SOD1 knockdown where it increases PMA induced ROS formation (Fig. 2b and c) while SOD1 overexpression attenuates the increase in ROS and peroxynitrite formation by PMA (Fig. 3b and e). Furthermore, PKD2 knockdown reduced superoxide and peroxynitrite formation in PMA pretreated cells (Fig. 7c and d). Therefore, PMA down-regulation SOD1 expression via PKD2 results in increase in ROS and peroxynitrite formation in myeloid leukemia cells. Similar results are seen in K562 cells that are co-treated with hemin and TPA: the degradation of PARP is blocked by the PKC inhibitor bisindolylmaleimide II [58]. The major 55 and minor 44 kDa fragments of cleaved PARP are the active fragments [59]. These fragments amplify PARP activity and result in the depletion of ATP pools. Prolonged ATP depletion inevitably causes necrotic death [60]. The mitochondrial inter membrane space is related to ATP synthesis [14]. In our study, we observed that PMA down-regulates SOD1 expression levels, while PARP was cleaved into three different cleaved forms (Fig. 8c). Thus, we suggested that SOD1 down-regulation reduces the superoxide removal rate, which probably disturbs ATP synthesis efficiency in the electron transport chain.

5. Conclusions

ROS generating chemotherapeutic agents enjoyed some success in myeloid leukemia treatment. In chronic lymphocytic leukemia, the non-selective SOD inhibitor 2-methoxyestradiol enhances arsenic trioxide (a ROS inducer) cytotoxicity [61]. However, in many situations, they suffer from high cytotoxicity to normal cells and drug resistance may be developed. Understanding the ROS metabolic system in myeloid leukemia cells may help to solve this problem. In this study, we showed that SOD1 knockdown increase intracellular ROS level that causes cell death in myeloid leukemia cells (Fig. 2b). Apparently, SOD1 inhibitor may increase the therapeutic effect of ROS generating chemotherapeutic agents to myeloid leukemia cells. This notion is supported by the fact that SOD1 inhibitor ATN-224 is used with ROS generating drug Ara-C inhibits proliferation in myeloid leukemia cells (Fig. 9a and b). ATN-224 is undergoing some clinical trials and was reported as “well-tolerated” [62,63]. Thus ATN-224 may be useful as an adjuvant in treatment of myeloid leukemia.

Conflict of interest

The authors have declared that no competing interests exist.

Author contributions

Yu-Lin Chen performed experiments, analyzed data, and wrote the paper. Wai-Ming Kan designed the study, discussed the results and edited the paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2015.07.025.

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