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Role of diacylglycerol kinase alpha and zeta isoforms in viability and differentiation of two cellular models of Acute Myeloid Leukaemia

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ABSTRACT

Acute Myeloid Leukaemia (AML) is a haematological aggressive and heterogeneous malignancy characterized by different mutations that lead to block of myeloid differentiation and clonal expansion of undifferentiated myeloid progenitors. Despite the advent of some targeted therapies, overall survival remains poor, highlighting the urgent need to identify additional druggable targets. Diacylglycerol kinases (DGKs), a family of ten enzymes, catalysed diacylglycerol (DAG) in phosphatidic acid (PA), two important lipids signalling that act as second messengers. DGKs have been implicated in influencing cell proliferation, survival, and differentiation, and among them, DGKα and DGKζ have been shown to play a role in tumour progression and immune regulation, but their functional importance in AML remains poorly understood. This study aimed to investigate the isoform-specific roles of DGKα and DGKζ in two different AML models representative of distinct myeloid lineages: HEL cells (erythroleukaemia) and THP-1 cells (acute monocytic leukaemia).

In HEL cells, DGKα knockdown significantly impaired cell proliferation and induced late apoptosis, suggesting both cytostatic and cytotoxic effects. In contrast, DGKζ knockdown had no significant effects on HEL viability or morphology, indicating a dispensable role in this cellular context. Moreover, HEL cells were treated with PMA, a DAG analogue, or chemical inhibitors to directly assess DGKα's role in differentiation along the megakaryocytic lineage. PMA induced morphological changes and CD41 expression without affecting CD235a. Similarly, DGK inhibition mimicked effects of PMA treatment, whereas the DGKα-selective inhibitor Amb639752 showing the strongest induction of cell spreading and CD41 upregulation. Additionally, co-treatment with PMA and Amb639752 confirmed that DGKα inhibition does not enhance cell adhesion, suggesting that the effects observed upon DGKα inhibition are not due to altered adhesion capacity.

In THP-1 cells, knockdown of either DGKα or DGKζ did not affect viability, proliferation, or apoptotic rate, suggesting these isoforms may have redundant or non-essential functions in sustaining the survival of this type of AML cells under the conditions tested. However, treatment with isoform-specific inhibitors (ritanserin for DGKα, and DGKζ-IN-4/BAY2965501 for DGKζ) resulted in a marked reduction in viability. Notably, the cytotoxic effects of inhibitors could not be rescued by exogenous PA or LPA, unlike what was observed in other AML models, suggesting either incomplete lipid uptake or the involvement of alternative PA-independent pro-apoptotic pathways, possibly linked to MAPK or survivin/caspase pathways.

This study highlights the isoform- and cell-specific roles of DGKα and DGKζ in AML biology. In particular, focus its attention on DGKα as a critical regulator of HEL cell survival and differentiation, positioning it as a promising dual-action target for drugs that could have a synchronise activity in killing AML myeloblasts and restoring the normal process of hematopoietic cell differentiation. Whereas, concerning THP-1 cells, despite the lack of a cytostatic or cytotoxic effects upon genetic silencing, pharmacological inhibition revealed an appreciable susceptibility to DGK targeting, although the reason for this discrepancy and the underlying mechanisms require further investigation. Future studies should explore the therapeutic benefit of DGKα/ζ co-inhibition/silencing and validate these findings in primary AML samples and in vivo models, as done with other molecules.

INTRODUCTION

Acute Myeloid Leukaemia (AML) is a haematological aggressive and heterogeneous malignancy characterized by the block of myeloid differentiation, and the rapid proliferation of abnormal immature myeloid progenitors (blasts), at different stages of maturation, such as multipotent progenitors, common myeloid progenitors, granulocyte–monocyte progenitors, or megakaryocyte–erythroid progenitors, in the bone marrow (BM) of patients[1,2]. The rapid expansion of myeloblasts in the bone marrow can lead to decreased normal haematopoiesis, leading to neutropenia, and increased susceptibility to infectious agents[3].

While statistically being a relatively rare cancer type (1.1% of all new cancer cases), according to the National Cancer Institute prediction, in 2025, it is estimated that there will be 22,010 new cases of AML and an estimated 11,090 people will die of this disease[4]. AML represents the most prevalent form of leukaemia in the adult population with the average age at diagnosis being 69 years according to NIH (National Institutes of Health) SEER (Surveillance, Epidemiology and End Results) database, and it is associated with an unfavourable prognosis, with incidence rates increasing with age[5]. Young patients with AML tend to have better outcomes compared to older individuals, who generally face a poorer prognosis[6]. This difference is largely attributable to reduced tolerance to intensive chemotherapy, the presence of age-related comorbidities, and mutations that lead resistance to systemic therapies[7]. Multiple factors contribute to the development of AML, including medical comorbidities, exposure to chemical toxins, previous chemotherapy or radiation treatment, genetic predispositions, prior myelodysplastic syndromes, and the overall aging of population[8]. However, in most cases AML presents as a de novo malignant neoplasm in previously healthy individuals, with well-characterized chromosomal translocations such as t(8:21) or t(15:17) which lead to the formation of chimeric proteins, respectively RUNX1-RUNX1T1 and PML-RARE, which alter the normal maturation process of myeloid precursors. In addition, point mutations may be present in isocitrate dehydrogenase 1 or 2 (IDH1/2) or even in FMS-like tyrosine kinase 3 (FLT3)[9,10]. Focus on this genetic heterogeneity, recent efforts have led to a refined classification of AML into two main classes: AML with well-defined genetic abnormalities and AML classified based on the differentiation state[11]. This molecular complexity has also prompted the development of targeted therapies, including inhibitors of FLT3, IDH1, IDH2, and BCL-2, which have opened new avenues for treatment[10,12].

Nevertheless, despite initial responses to chemotherapy, three-quarters of patients will succumb to the disease within 5 years; the 5-year relative survival rate for AML was 32.9% during 2015–2021 in according to the National Cancer Institute[4]. These statistics highlight the urgent need to identify additional druggable targets, as resistance to conventional and targeted therapies remains a major clinical challenge.

DGKs constitute a family of lipid signalling enzymes that attenuate diacylglycerol (DAG) signalling while concurrently generating phosphatidic acid (PA), using ATP as a phosphate donor. While DAG and PA are fundamental structural components of cellular membranes, they also function as bioactive lipids and crucial second messengers, whose intracellular levels are finely regulated in response to both intracellular and extracellular stimuli[13,14]. In addition, several human disorders, including cancer, have been linked to deregulated DAG-PA lipid signalling[15,16].

To fully appreciate the signalling role of DGKs, it is important to consider the broader lipid context involving DAG, PA and its derivative, lysophosphatidic acid (LPA).

DAG is derived from inositol phospholipids by the action of phospholipase C (PLC) upon cell stimulation by growth factors, cytokines, hormones, neurotransmitters, and other agonists. DAG is well known to regulate a wide variety of cellular functions through binding to the C1 domains that are first found in protein kinase C (PKC) which is involved in T cell activation and function, but also in several signalling proteins, such as protein kinase D, contributing to the regulation of growth and differentiation, Unc-13, that plays a role in synaptic transmission by facilitating the release of neurotransmitters, chimaerin, a protein that acts as a GTPase-activating protein for Rac, influencing cytoskeletal dynamics and cell migration and Ras guanyl nucleotide-releasing protein (GRP) which is involved in the activation of Ras, key player in cell growth and survival signalling pathways[14,17].

PA, produced either through DGK-mediated phosphorylation of DAG or de novo via the phospholipase D (PLD) pathway through the hydrolysis of phosphatidylcholine, is not merely a biosynthetic intermediate but a signalling molecule that modulates cellular metabolism and growth through stabilising mTOR activity directly activate mTORC pathways controlling myeloid progenitors proliferation, cell fate determination by MAPK cascade, cytoskeleton dynamics through RhoA/ROCK, vesicular trafficking, cell death through its interaction with specific intracellular effectors such as PI3K/Akt[18–20].

LPA is predominantly produced by phospholipid hydrolysis through a soluble enzyme called autotaxin (ATX) but could also be generated from PA by its deacylation catalysed by phospholipase A (PLA)-type enzymes. It acts predominantly in an extracellular context in a classical autocrine-paracrine way through G protein-coupled seven transmembrane receptors (GPCRs), such as LPA1-5, GPR87 and P2Y5[19,21,22]. LPA is also known to bind other receptors on the plasma membrane, specifically endothelial differentiation gene (EDG)-2, -4, and -7, leading to activation of multiple intracellular signalling pathways, including PLD and ERK1/2[23].

The human genome encodes ten DGK isozymes, many of which are co-expressed, indicating distinct, non-redundant roles. Based on their unique regulatory domains, typically located at the N-terminal region upstream of a conserved catalytic core, DGKs are classified into five subfamilies. This catalytic core includes the ATP-binding site and at least two cysteine-rich domains, structurally homologous to the C1 domain of PKC, which mediates DAG binding. DGKα belong to subfamily I of DGKs and is characterized by the presence of two EF-hand motifs and a recoverin homology domain, conferring calcium sensitivity. In contrast, DGKζ falls in subfamily IV and it is distinguished by the presence of ankyrin repeats, structural motifs known to mediate protein-protein interactions, MARCKS domain, a Ser/Thr rich region phosphorylated by PKCα, important in regulation of DGKζ nuclear and cytoplasmatic localization and PDZ binding motif for interaction with PDZ protein in supramolecular signalling complexes[24–26].

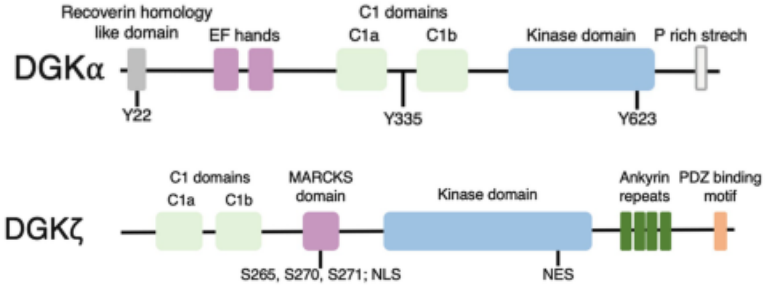

Figure 1: structure of DGKα and DGKζ isoforms[27]

Despite belonging to distinct subfamilies and exhibiting divergent domain architectures, both DGKα and DGKζ act as negative regulators of immune response. They catalyse the phosphorylation and consequent depletion of DAG, attenuating downstream DAG-dependent signalling pathways, and ultimately limiting the anti-cancer activity of lymphocytes and natural killer cells[28,29].

In the context of AML, analysis of gene expression profiles reveals overexpression of several DGK isoforms in AML samples compared to matched normal tissue, as evidenced by TCGA and GTEx datasets, although without a clear correlation to specific subtypes. High expression of DGKα is associated with poor overall survival, whereas elevated DGKγ appears to be linked to a more favourable prognosis. DGKζ, despite being overexpressed in AML, does not show significant correlation with survival and therefore has no prognostic role. Interestingly, in contrast to TCGA data, the Beat-AML dataset revealed that BM cells express all DGK isoforms at very high levels, often exceeding those observed in tumour samples, suggesting that DGK expression may increase during hematopoietic differentiation[30]. Moreover, overexpression of DGKα in human BM AML samples compared to normal samples, has been associated with poor clinical outcomes, in lines with the public datasets[31].

In this context, Gravina and colleagues evaluated the effects of several DGK inhibitors in two AML cell lines. Broad-spectrum DGK inhibitors R59022 and R59949 significantly reduced cell viability, with IC50 values of 32 ± 2 μM and 72 ± 2 μM, respectively, in HL-60 cells, and 49 ± 2 μM and 80 ± 1 μM, respectively, in HEL cells. These compounds also induced alterations in cell cycle progression. However, selective DGKα inhibitors (CU-3 and AMB639752) showed minimal effects on AML cell viability even at high concentrations (100 μM), suggesting that DGKα inhibition alone may not be sufficient to elicit therapeutic responses in these models[30]. Conversely, another DGKα-specific inhibitor, ritanserin, exhibits promising results. In AML cell lines Kasumi-1 and KG-1α, it reduces proliferation in a dose- and time-dependent manner. The reported IC50 values were 29.75 ± 0.47 μM and 25.88 ± 0.11 μM at 72 hours, and 51.01 ± 0.62 μM and 37.7 ± 0.55 μM at 24 hours, respectively. Ritanserin treatment induced caspase-dependent apoptosis both *ex vivo* and in *in vivo* xenograft mouse models, leading to prolonged survival. Systemically, this effect involves negative regulation of sphingosine kinase 1 (SphK1) and inhibition of the Jak-STAT and MAPK signalling pathways. In addition, they showed that the addition of exogenous PA was able to partially rescue the loss of cell viability in Kasumi-1 and KG-1α cells, further confirming that the effects of DGK inhibition are mediated through the depletion of intracellular PA[31].

On the other hand, the relevant role of DGKζ in maintaining the viability of HL-60 cells has been highlighted by Li and colleagues. Silencing of DGKζ led to reduced cell proliferation, G2/M cell cycle arrest, and promoted apoptosis, potentially via modulation of the MAPK/survivin/caspase signalling axis, which suppresses pro-survival pathways and enhances caspase levels. Moreover, DGKζ deletion resulted in the upregulation of key components of stress and apoptosis pathways, including ERK1/2, HSP27, Smad2, p53, p38 MAPK, and SAPK/JNK, underscoring the partial involvement of MAPK signalling in the pro-apoptotic effects induced by DGKζ knockdown[32]. Altogether, these findings highlight the complex, isoform-specific roles of DGKs in AML cell viability.

DGKα and ζ not only plays a central role in AML cell viability and regulation of the immune system but are also key modulators of cell differentiation. Going deeper, both DGKα and DGKζ are expressed at significant levels in megakaryocytes and platelets controlling their activity through modulation of DAG signalling. Indeed, phorbol esters, which mimic the action of DAG, are well known inducers of megakaryocytes differentiation *in vitro* indicating that DAG plays a role in platelet production[24]. Long and colleagues demonstrated that upon phorbol myristate acetate (PMA) stimulation, the rounded floating human erythroleukemia (HEL) cells underwent marked morphological changes, including increased size, adhesion and spreading. PMA treatment induces

also an upregulation of megakaryocyte- and platelet-associated protein, among others glycoprotein IIb (CD41a), platelet factor 4 (PF4 or CXCL4), and thrombospondin, while erythroid CD235a marker remains unchanged[33]. In support of that, Moroi at all demonstrated that DGKζ plays a crucial role in modulating platelets responsiveness whereas Dr A. Bertoni unpublished work indicates DGKα as a negative regulator of megakaryopoiesis and platelet activation by modulation of DAG metabolism[34]. Regarding cell morphology, DGKα is already known to be involved in motility and actin shaping in epithelial, endothelial and T cells controlling several mechanisms. Chianale et all demonstrated that DGKα is involved in growth-factor-induced cell migration and ruffling by controlling activation of the small G protein Rac. In particular, DGKα, upon plasma membrane recruitment and Src kinases dependent activation, catalysed PA formation, enhancing the recruitment of aPKCζ/ι that mediates the release of Rac from the inhibitory complex with RhoGDI, allowing its activation and the consequent cytoskeletal remodelling[20]. In SDF-1a stimulated breast carcinoma cells, DGKα is able to mediate SDF-1a-induced matrix invasion. Also in this case, active DGKα promotes cell protrusions elongation and matrix invasion by the recruitment of atypical PKCs leading to Rac activation, localization of b1 integrin and consequent secretion of MMP-9 metalloproteinase[35]. Rainero et all pointed out that DGKα is also related with invasive migration by controlling RCP-dependent integrin trafficking. Indeed, by producing PA, DGKα promotes RCP mobilization and tethering at the tips of invasive pseudopods and to allow RCP-dependent α5β1 recycling resulting in invasiveness of tumour cells[36]. Thus, several mechanisms link DGKα activity to cytoskeletal remodelling. Highlighting the role of DGKs in cell differentiation, non-selective inhibition of DGKs with R59022 and R59949 drives granulocytes differentiation in HL-60 cells significantly shortening the time required to observe upregulation of differentiation markers such as CD11b and GPI-80, as well as generation of reactive oxygen species (ROS) by 50%. Inhibition of DGKs also perturbs the cell cycle, further supporting their regulatory role in cellular maturation[37]. Conversely, DGKγ is known to be a negative regulator of HL-60 cells differentiation toward macrophages reducing cell attachment[38].

OBJECTIVE OF THE THESIS

Considering the established involvement of DGKs in the regulation of haematopoiesis and the pathogenesis of AML, the aim of this study, conducted as part of a broader research project within our working group, was to investigate the isoform-specific roles of DGKα and DGKζ in two distinct leukemic models: erythroleukemia, using HEL cells, and acute monocytic leukaemia, using THP-1 cells. We selected HEL and THP-1 cell lines as representative models derived from distinct branches of the hematopoietic differentiation hierarchy at the top of which are multipotent hematopoietic stem cells. These stem cells give rise to multipotent progenitors, which further commit to either the lymphoid or myeloid lineage. Along the myeloid axis, progenitors differentiate into either granulocyte-monocyte progenitors, from which THP-1 cells are derived, or megakaryocyte-erythroid progenitors, to which HEL cells can be functionally associated[43].

We aim to understand firstly whether these isoforms had a role on cell viability and more specifically whether their action was isoform-specific or cell-specific in order to propose the appropriate drug treatment due to the right target. Furthermore, since a main feature of AML is the blockage of myeloid differentiation, we wanted to investigate whether DGKs (in particular the α isoform) also play a role in driving cell differentiation from progenitor to mature cell, pointing out another important aspect in drug treatment.

MATERIAL AND METHODS

REAGENT	CONCENTRATION	CAT. NUMBER	PROVIDER
RPMI-GlutaMAX 1640 Medium	-	61870-010	Thermo Fisher
Foetal bovine serum	-	A5256701	Thermo Fisher
Penicillin and Streptomycin	-	A5955	Sigma-Aldrich
rhIL-2	-	-	PeproTech
Trypan blue	0,4%	15250-061	Thermo Fisher
AlamarBlue	1,5 mg/mL in PBS	A50100	Invitrogen
Phosphate buffered saline	-	P4417	SIGMA
Triton X-100	-	04744977001	Roche Diagnostic
Trypsin-EDTA	-	T4174	Sigma-Aldrich
Dimethyl sulfoxide	1%	D2650	Sigma-Aldrich
Phorbol 12-myristate 13-acetate (PMA)	2 µM	524400	Sigma-Aldrich
R59022	10 µM	-	Sigma-Aldrich
R59949	10 µM	-	Cayman
Amb639752	10 µM	-	Ambinter
Cu3	10 µM	-	Vitasmlab
Ritanserin	-	1955	Tocris
BAY 2965501	-	2732902-08-6	MedChemExpress
DGKζ-IN-4	-	HY-156574	MedChemExpress
PA (C8)	25mM	P3591	Sigma-Aldrich
LPA (C6)	17mM	-	Sigma-Aldrich
Annexin V-FITC	-	BMS500FI-300	Invitrogen
7AAD	-	A9400-1MG	Sigma-Aldrich
anti-CD41a	10 µL	557296	BD Biosciences
anti-CD235a	2.5 µL	130-100-258	Miltenyi Biotech
DGKα Rabbit PolyAb	1 mg/mL	11547-1-AP	Proteintech
Rabbit Anti-DGKζ pAb	1 mg/mL	AB105195	Abcam
β-actin Monoclonal Antibody	1 mg/mL	MA1-140	Invitrogen
Rabbit Anti-Mouse IgG	1 mg/mL	A16160	Invitrogen
Goat Anti-Rabbit IgG	1 mg/mL	A16096	Thermo Fisher
PageRuler prestained protein ladder	-	26616	Thermo Fisher
High-Capacity cDNA Reverse Transcription Kit	-	4368814	Thermo Fisher
TaqMan™ Fast Advanced Master Mix	-	4444556	Thermo Fisher

Table 1: reagents used.

Cell culture

Human erythroleukemia cell line HEL, originated in 1980 from the peripheral blood of a 30-year-old man experiencing a relapse of erythroleukemia following treatment for Hodgkin lymphoma[39] and THP-1 cell line, established from the peripheral blood of a 1-year-old male patient with acute monocytic leukaemia[40], were employed as in vitro models of acute myeloid leukaemia. The identity of cell lines was confirmed by the Cell Line Authentication Test (Eurofins Genomics, Ebersberg, Germany and university internal service), and the resulting markers were 100% consistent with those in the ATCC database. The cell lines were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated foetal bovine serum and 1% penicillin-streptomycin following ATCC handling information.

Transfection

In order to silence DGKA and DGKZ, HEL and THP-1 cells were transfected with 80nM of siRNA (table 2) using Neon™ 100µL Kit (MPK10025, Invitrogen) with Neon™ electroporator and the following conditions (table 3).

NAME	TYPE	PRODUCER	CATALOG	ID	SEQ. FORWARD	SEQ. REVERSE
siRNA DGKZ	Custom siRNA	Ambion	AM16104	ACFARCW	GCCGCUUUCGG AAUAAGAUTT	AUCUUAUUCCG AAAGCGGCTG
siRNA1 DGKA	Custom siRNA	Invitrogen	10620310		AUAUUUAGCCA UCUCGCCAUCC UCG	CGAGGAUGGCG AGAUGGCUAAA UAU
siRNA2 DGKA	Custom siRNA	Invitrogen	10620310	494910C11	CGAGGAUGGCG AGAUGGCUAAA UA	AUAUUUAGCCA UCUCGCCAUCC UCG
siRNA cntrl	Silencer select	Invitrogen	465373			

Table 2: siRNA used.

The protocols have been optimized for each cell type according to the manufacture's instructions and ATCC web site. To assess viability and efficiency of the optimized conditions, cells were transfected with BLOCK-iT™ Alexa fluor red 555nm (Thermo Fisher) and analysed by Trypan Blue assay and flow cytometry (BD FACSymphony™ A5). The results are reported below:

	CONDITION	N° OF CELLS	VITALITY (%)	EFFICIENCY (%)
HEL	1350V, 20ms, 2P	760.000	38%	100%
THP-1	1550V, 15ms, 2P	750.000	89%	81%

Table 3: electroporation conditions.

After siRNA transfection, cells were resuspended in RPMI-1640 medium with 10% heat inactivated FBS without streptomycin and penicillin and incubated for 72h at 37°C and 5% CO_2.
Silencing efficiency was evaluated for each experiment by quantitative real-time PCR (qRT-PCR) and Western blotting.

Trypan Blue assay

The Trypan Blue assay was performed on transfected HEL and THP-1 cells to assess cell viability. After 72h of incubation, the trypan blue reagent was added to samples (1:1 v/v) and the number of live/dead cells was automatically recorded by the Automatic counter TC20 (Invitrogen). Each experiment was performed in three technical replicates. The results are reported as the number of total live/dead cells of a representative experiment and as percentage of cell viability of three or more biological replicates. All data were graphed and analysed using GraphPad Prism 8 through one-way ANOVA or unpaired t-test depending on data distribution.

Reverse transcription polymerase chain reaction assay

Transfected cells were lysed in 200µL of nuclease free water supplemented with 2% of thioglycerol. Total RNA was extracted from lysates by Kingfisher automated nucleic acid extractor (Thermo Fisher) with "MVP_2WASH_200_FLEX" protocol and "MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit" (Thermo Fisher) and quantified using NanoDrop (Thermo Fisher).
Then, equal amounts of RNA were retrotranscribed into cDNA using "High-Capacity cDNA Reverse Transcription Kit" (Thermo Fisher) following the manufacturer's instructions.
The relative expression of DGK isoforms (DGKA Assay ID Hs00176278_m1, DGKZ Assay ID Hs05025727_m1) was assessed by qRT-PCR with TaqMan technology. Glucuronidase beta (GUSB Assay ID Hs00939627_m1) was used as internal reference gene.
To perform qRT-PCR, a 384-well plate was prepared following the protocol provided by the "TaqMan™ Fast Advanced Master Mix kit" (Thermo Fisher) with 4µL of mix plus 1µL of cDNA per sample and amplified with Bio-Rad CFX384™ real-time system. The amplification protocol includes an initial denaturation at 95°C for 20s, followed by 50 cycles of denaturation at 95°C for 3s and annealing/extension at 60°C for 30s. The qPCR reactions were performed in technical triplicates. The data were analysed with Bio-Rad CFX Maestro software using GUSB as reference and normalizing DGKα and DGKζ expression to the relative control (siRNA control).

Apoptosis and necrosis assessment

To evaluate cell death due to DGKα and DGKζ silencing in HEL and THP-1 cells, about 500,000 cells per sample, after 72h of transfection, were stained with Annexin V Apoptosis Detection Kit (Invitrogen) according to manufacturer's instructions except using 7AAD instead of propidium iodide.

Analysis was performed by flow cytometry (BD FACSymphony™ A5) with the BD FACS Diva software. Gating strategy: i) FSC-A vs FSC-H plot to exclude cell doublets, ii) FSC-A vs SSC-A plot to separate live cells from debris, iii) 7AAD-A vs Annexin FITC-A to identify the different populations: live (Annexin V- and 7AAD-), necrotic (Annexin- and 7AAD+), early apoptotic (Annexin V+ and 7AAD-) and late apoptotic (Annexin+ and 7AAD+) cells.

Data are shown as the mean ± SD of at least 3 independent experiments analysed by GraphPad Prism 8 through Two-Way ANOVA test (or Mixed Model).

Western blot

Cells were lysed with lysis buffer (HEPES 25mM, NaCl 150mM, EDTA 5mM EGTA 1mM NP40 1%, Glycerol 10%) sodium orthovanadate (1mM, Thermo Fisher) and protease inhibitor cocktail (0.0089mg/mL, Sigma Aldrich) mix. The samples were incubated for 15 minutes at 4°C, under gentle, constant shaking, and then centrifuged at 12,000 × g for 15 min at 4°C. The concentrations of proteins in lysates were quantified by Qubit protein br assay kit (A50669, Thermo Fisher) following manufacturer's protocol. Equal amounts of proteins were separated on SDS–PAGE gels and subsequently transferred to a polyvinylidene fluoride (PVDF) membrane. The membranes were then blocked by incubating with 3% (w/v) BSA for 1 hour at room temperature and subjected to overnight incubation with respective primary antibodies at 4°C with gentle agitation. The following day, membranes were washed 3 times in TBS-T buffer. They were then incubated with alkaline HRP-conjugated rabbit or mouse secondary antibody (1:5000) for 1 hour at room temperature with gentle agitation. After 3 additional washes with TBS-T, the membranes were developed using the Western Chemiluminescence Substrate (PerkinElmer, USA) using the ChemiDoc™ Imaging System (Bio-Rad). Band volumes were analysed by densitometry using Bio-Rad Image Lab 6.1 software and normalised on their respective β-actin and subsequently on their respective siRNA cntrls.

Finally, results were analysed and graphed using GraphPad Prism 8 with one-way ANOVA and unpaired T-test.

HEL cells stimulation and drug treatment

According to Wang et all, HEL cells were treated with PMA to induce their differentiation toward the megakaryocytic lineage[41,42]. In details, HEL cells were plated at the concentration of 500.000 cell/mL in 2mL of complete RPMI and stimulated with PMA (2μM) for 48 hours. For drug induced cell differentiation, HEL cells were plated as mentioned before and treated with DGKα inhibitors 10μM (stock solution 10mM in DMSO) for 48 hours or indicated time to assess cell adhesion. DMSO was used as a vehicle control and stimulation with PMA was used as positive control.

Measurement of HEL cells diameter

To evaluate cells dimensions we measured cell maximum diameter in phase-contrast images. Cells were observed in 6 well plates with a Floyd Evos (Thermo Fisher) microscope and the phase-contrast images were taken at 10X magnification in 3 random fields for each well. The maximum diameter was manually drowned and measured through ImageJ (NIH) for at least 100 cells for sample.

Data were graphed on GraphPad Prism 10 as violin plots showing mean ± SD in Log2 scale and analysed by ordinary one-way ANOVA, Dunnett's multiple comparison test with a single pooled variance.

HEL cells differentiation markers analysis by flow cytometry

To evaluate surface markers, HEL cells were detached from the 6 wells plates through incubation with Trypsin EDTA (37°C per 10 min). $1x10^6$ cells were resuspended in 100μL of PBS 1X and stained with FITC-CD41a (10μL) and PE-Cy7-CD235a (2.5μL) monoclonal antibodies respectively for 20 and 10 minutes at room temperature. Then, cells were washed once in PBS 1X + EDTA (3mM) and resuspended in 200μL of the same buffer before analysis at the flow cytometer "BD FACSymphony A5". Gating strategy: i) FSC-A vs SSC-A plot to separate live cells from debris, ii) SSC-A vs PE-Cy7-A to assess positivity to CD235a (erythroid marker), iii) SSC-A vs FITC-A to assess CD41a positivity (megakaryocyte marker).

All data are expressed as mean ± SD of three independent experiments and were graphed and analysed by ordinary one-way ANOVA or Two-Way ANOVA with GraphPad Prism 10.

HEL cell adhesion measurement

After the indicated time of treatments, HEL cells were washed twice with PBS 1X to allow the detachment of the cells not properly attached to the bottom of a 24 well plate. To measure cell adhesion, each well confluence was analysed at Spark 10M Multimode Plate Reader (Tecan). In details, the instrument takes pictures of them to calculate the spatial arrangement of the cells giving back the percentage of the area of the well occupied by them.

Phosphatidic acid (PA) and lysophosphatidic acid (LPA) treatments

PA (C8) and LPA (C6) stocks, initially dissolved in chloroform, were dried under a stream of nitrogen gas and then resuspended to a concentration of 1mM in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin. In parallel, stock solution of ritanserin (500µM), DGKζ-In-4 (500µM), and BAY 2965501 (750µM) were prepared. To ensure complete solubilization, all solutions were sonicated for 5 minutes at 60°C in a water bath sonicator (SONICA ultrasonic cleaner, Soltec with sweep system) and then vortexed vigorously for 2 minutes. THP-1 cells (50,000 cells/well) were seeded in 96-well plates in a final volume of 100µL and treated with PA or LPA at a final concentration of 100µM, either alone or in combination with the indicated inhibitors (ritanserin 50µM, DGKζ-In-4 50µM and BAY 2965501 75µM or DMSO). Cells were incubated for 24 hours, after which viability was assessed via a 24-hour Alamar Blue assay.

The data are shown as the mean ± SEM of 6 experiments interpolated as [inhibitor] vs % viability and analysed by GraphPad Prism 8 through Two-Way ANOVA test.

Alamar Blue assay

AlamarBlue assay was performed to assess cell viability in presence of chemical inhibitors and PA or LPA treatments. 50,000 cells per well were seeded in a 96-wells plate in a volume of 100µL of complete medium together with 10µL of 10X inhibitor and then incubated for 24h or 72h. Each experimental condition was performed in technical quadruplicates, using cells treated with equal amounts of DMSO (vehicle) as control and medium as background. After incubation, alamarBlue (final concentration of 0.15mg/mL) was added to each well and 24h later samples fluorescence was measured with a Spark 10M Multimode Plate Reader (535nm excitation and 590nm emission wavelength). Thus, for the dose–response curve fitting, fluorescence was normalized as:

$$Viability\ (\%) = \frac{(sample\ mean\ fluorescence)}{(DMSO\ control\ mean\ fluorescence)} * 100$$

Data are the results of at least four independent experiments analysed with GraphPad Prism 8 as [inhibitor] vs. normalized response curve (variable slope, after manual outlier removal).

RESULTS

Role of DGKα and DGKζ on HEL viability

To understand the specific relevance of DGKα and ζ isoforms in AML cell viability, we promote DGKA and DGKZ knockdown in the HEL cell line through siRNA transfection. After 72h, we quantify mRNA and protein levels and we observed that DGKA mRNA levels were reduced by approximately 63% with siRNA1 and 57% with siRNA2, as determined by qRT-PCR. Also at protein level, DGKα was decreased (figure 1A). Similarly, DGKZ silencing led to an mRNA reduction of approximately 61%, which correlated with a marked decrease in DGKζ protein level (figure 1E).

To evaluate the biological effects of reducing DGKα and DGKζ protein on HEL cell viability, we used a Trypan Blue assay at 72h. As reported in figure 1B, DGKA silencing resulted in a significant reduction in the mean live cells percentage from three experiments: a 44% decrease with siRNA1 and 32% with siRNA2. On the right in the same panel, absolute cell number of a representative experiment revealed that only control cells exhibited a net increase in cell density compared to the initial seeding level (dotted line), whereas DGKA-silenced cells remain unchanged in number, indicating a block of proliferation or increased apoptosis.

We further investigated the eventual induction of cell death upon DGKA silencing performing an apoptosis and necrosis assay. As shown in figure 1C and 1D, DGKA silencing elevated total cell death from 6.2% to 11.4% with siRNA1 and to 7.5% with siRNA2. This increase was predominantly due to late apoptosis ($p<0.05$), with minimal contributions from early apoptosis and necrosis.

Conversely, as shown in figure 1F, DGKZ silencing in this cell line does not significant impact mean live cells percentage of three experiments. The absolute cell count of a representative experiment indicates that both control and DGKZ-silenced cells showed comparable increases relative to the initial seeding density (figure 1F, right panel), suggesting that DGKZ does not play a major role in regulating HEL cell survival or proliferation.

Collectively, these results indicate that in HEL cells, DGKα plays a critical role in sustaining cell viability, and its knockdown leads to a strong reduction of cell proliferation and induces apoptotic cell death. In contrast, DGKζ appears to be dispensable for viability under the conditions tested.

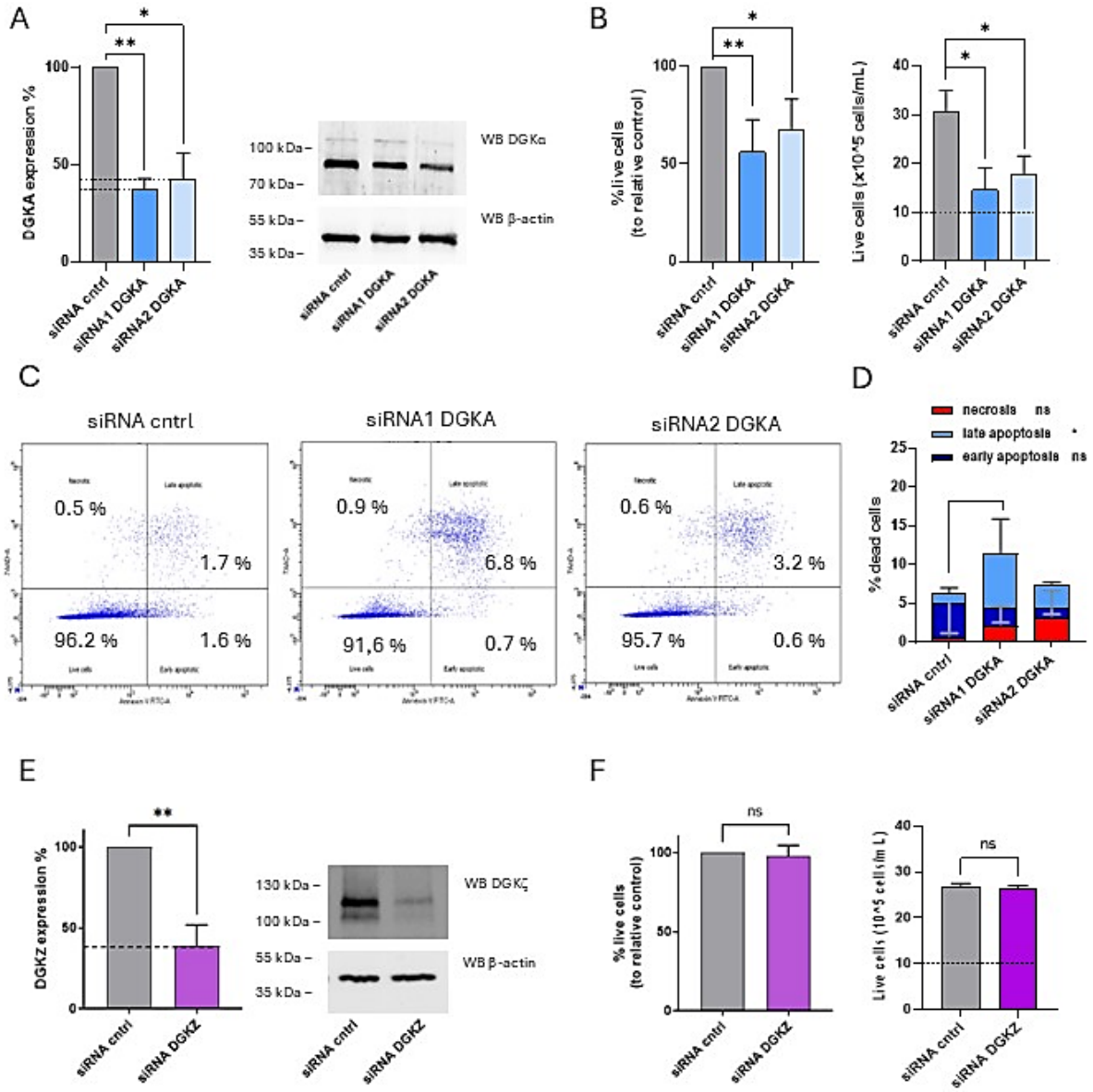

Figure 1: ***Role of DGKα and DGKζ on HEL viability***

HEL cells were transfected with the indicated siRNA and assessed after 72h.

A. Mean ± SEM of three independent experiments showing the silencing of DGKA on HEL cells. Data are presented as percentage of DGKA mRNA expression (left) in comparison with siRNA cntrl transfected cells. Right, a representative western blot for DGKα and β-actin protein.
B. Trypan Blue assay. Mean ± SEM of four independent experiments shown as the percentage of live cells relative to siRNA cntrl (left). Right, a representative experiment showing the number of live cells starting from 10*10^5 cells/ml seeded.
C. Annexin V-7AAD assay analysed by flow cytometry. Data are shown on the left as dots plots of a representative experiment and on the right as the mean ± SD of three independent experiments presenting the percentage of dead cells and the type of cell death.
D. Mean ± SEM of three independent experiments showing the silencing of DGKZ on HEL cells. Data are presented as percentage of DGKZ mRNA expression in comparison with siRNA cntrl transfected cells (left). Right, a representative wester blot for DGKζ and β-actin protein.
E. Trypan Blue assay. Mean ± SEM of four independent experiments shown as the percentage of live cells relative to siRNA cntrl (left). Right, a representative experiment showing the number of live cells starting from 10*10^5 cells/ml seeded.

Two-Way ANOVA or unpaired T test versus siRNA cntrl: p<0.05 *, p<0.01 **, p<0.001 *** and p<0.0001 ****.

Role of DGKα in HEL cells differentiation along the megakaryocytic lineage

DAG accumulation is important for HEL cells differentiation into megakaryocytes in terms of morphology and spreading. Indeed, PMA treatment (DAG analogue) of HEL cells promotes an increase in cell size, cytoplasmic content, nuclear complexity, and a significant increase in CD41 expression, whereas CD235 (glycophorin A) is not affected[33].

Through inhibition of DGKα we expected to promote DAG accumulation thus we intend to verify if that could induce HEL cells differentiation. We suppressed DGK activity with several inhibitors (R59949, R59022, Amb639752 and Cu3) at 10μM for 48 hours or indicated time. These drug doses and exposure times do not compromise cell viability[30]. Cell differentiation was assessed by measuring the maximum diameter after adhesion (figure 2B), the surface expression of both CD41a (increase upon differentiation in megakaryocytic lineage) and CD235a (increases upon erythroid differentiation, figure 2C) and adhesion over time (figure 2D). In figure 2A we can observed that PMA treatment (2μM for 48h) promotes a morphological shift from control floating rounded cells to flat adherent cells. This morphological shift can be quantified as the change of diameter mean from 13 μm in control cells to 33 μm in PMA-stimulated cells (figure 2A, B). Also, cells treated with DGK inhibitors undergo morphology changes, resulting in a heterogeneous population in terms of shape, characterized by larger and some elongated cells. Unlike PMA-treated cells, they do not show the typical spreading respect to DMSO-treated (negative control) cells.

In terms of cell dimensions, notably, Amb639752 and Cu3 promote more elongation than R59022 and R59949. Indeed, based on the observed diameter mean, the most effective inhibitor is Amb639752 (17.7 μm) followed by Cu3 (16.5 μm), R59949 (15.8 μm) and R59022 (15 μm) (figure 2A, B). As expected, PMA treatment promotes a significant increase in the percentage of CD41a+ cells while the percentage of CD235a+ cells remain unchanged (figure 2C). The treatment with DGK inhibitors does not change surface markers apart from Amb639752 that induce a small but significative raise in the percentage of CD41a+ cells.

Those data suggest that the DAG accumulation induced by DGK inhibition is sufficient to drive a partial differentiation of HEL cells with a significant effect on morphology and only for Amb639752 an induction of CD41a. For this, Amb639752 results the most effective inhibitor in driving HEL cells differentiation. Thus, we intent to verify if that could depend on an increased cell adhesion.

We co-treated cells with PMA + Amb639752 to measure cell adhesion at several time points compared to cells treated only with PMA. Evaluating cell confluence data, firstly we observed that there is no difference at basal level among DMSO and Amb639752 treatment and the percentage of

confluence remain constantly low over-time. Concerning PMA treated cells, the percentage of confluence increased from 0 to 6 hours and after remained constant reaching the plateau at 90% without difference with inhibitor co-treatment.

These data confirmed that DGKα is important to drive myeloblasts differentiation along megakaryocytic lineage without altering cell adhesion.

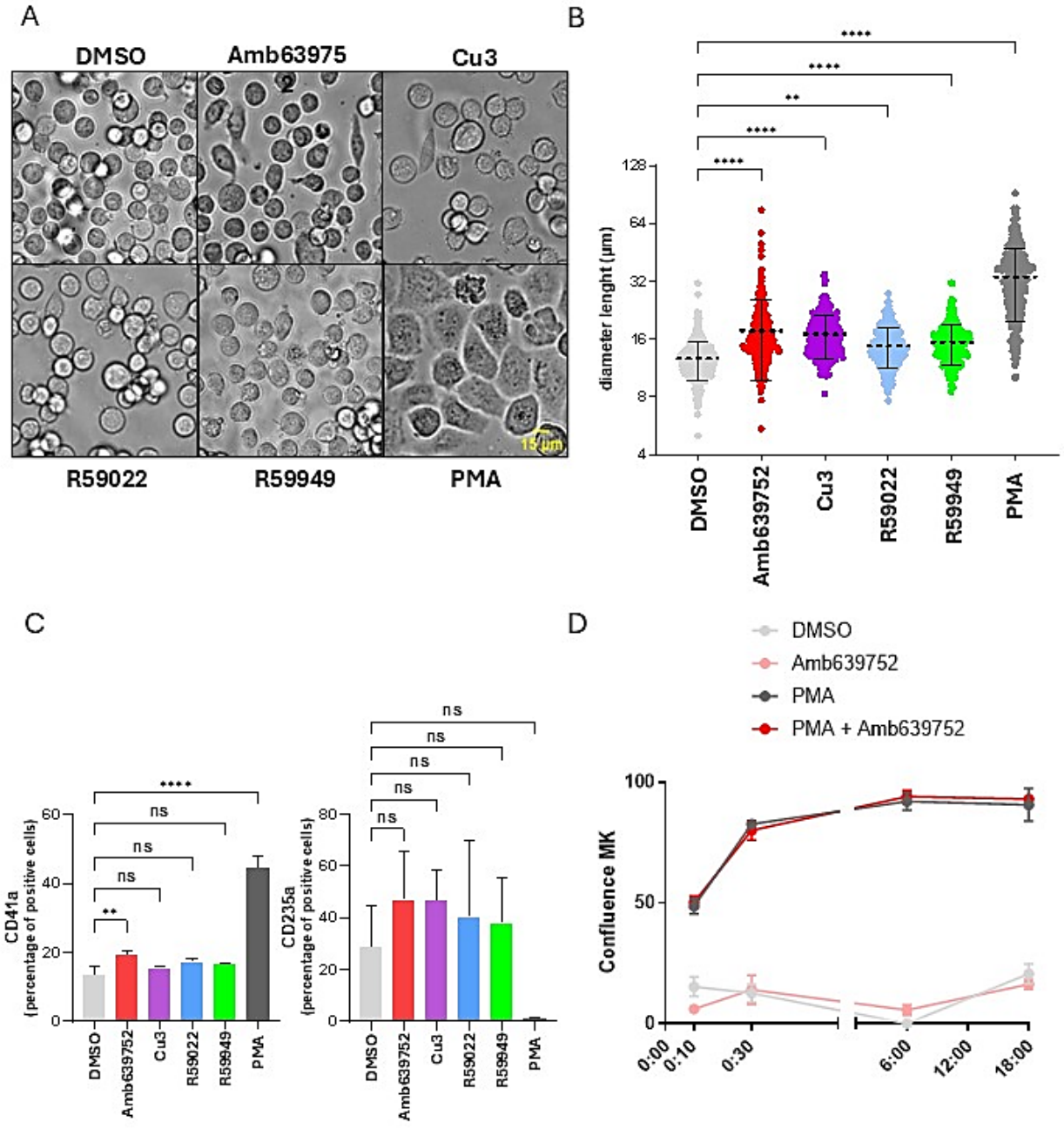

Figure 2: ***Effect of DGKαinhibitors on HEL cells differentiation.***
HEL cells were plated at the concentration of 500.000 cells/mL and stimulated with PMA (2µM) and/or DGK inhibitors (10µM).

A. Representative phase-contrast image after 48 hours.
B. Maximum diameter of >100 cells upon 48h treatment (one representative experiment out of three).
C. Percentage of C41a+ or CD235a+ cells at 48h. Mean with SD of three independent experiments.
D. Mesurement of cell adhesion over-time.

One-way ANOVA versus DMSO: p<0.05 *, p<0.01 **, p<0.001 *** and p<0.0001 ****.

Silencing of DGKA and DGKZ in THP-1 cells does not induce significantly cell death

To investigate the distinct roles of the DGKα and ζ isoforms in another AML cells line, we repeated the experiments in THP-1 cells, a widely used model for AML. We silenced DGKA and DGKZ through siRNA technology and after 72 hours transfection, DGKA mRNA levels were reduced by 30% with siRNA1 and by 32% with siRNA2, while DGKα protein expression decreased by 63% and 60%, respectively (figure 3A). For those regards DGKZ, mRNA levels were reduced by 50%, accompanied by a 79% decrease in protein expression (figure 3C).

To assess the biological impact of reducing DGKα and DGKζ protein on THP-1 cells viability, we performed a Trypan Blue assay. As shown in figure 3B (left panel), cell viability is not affected and the percentage of viable cells, averaged across four independent experiments, remained comparable between either siRNAs and control cells. The right panel displaying the absolute cell number from a representative experiment, indicates that both control and DGKA-silenced cells exhibited an overall increase in cell number relative to the initial seeding density (dotted line).

Similarly, DGKZ silencing did not significantly affect cell viability compared to control conditions (figure 3D, left). The corresponding right panel showing absolute cell counts, confirms that both control and DGKZ-silenced cells experienced a net increase in total cell number relative to the initial seeding density (dotted line), over time.

To confirm that DGKA and DGKZ silencing did not induce cell death in THP-1 cells, we performed apoptosis and necrosis assays. As illustrated in figure 4D and E, control and silenced cells shown a comparable level of apoptosis. Indeed, in a representative experiment, the percentage of apoptotic cells figured: 2.6% in siRNA control-treated cells, 2.8% with DGKA siRNA1, 2.7% with DGKA siRNA2, and 2.2% with DGKZ siRNA. Necrotic cell death remained negligible under all experimental conditions, including siRNA control, DGKA-, and DGKZ-silenced cells.

Collectively, these findings indicate that reduced expression of DGKα and DGKζ does not significantly impair THP-1 cell viability. This suggests that these isoforms may have redundant or non-essential functions in sustaining the survival of this type of AML cells under the conditions tested.

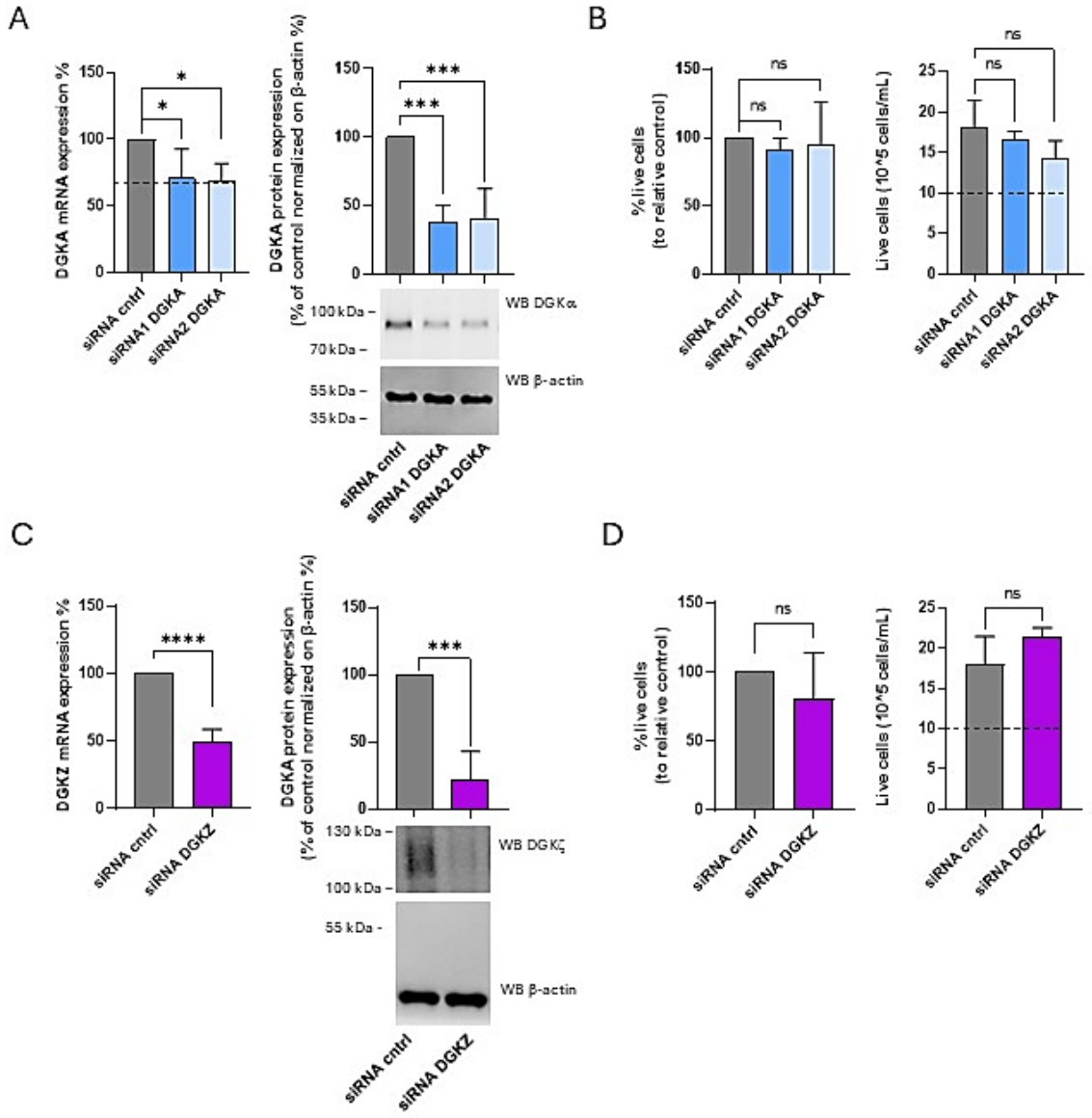

Figure 3: ***Role of DGKα and DGKζ knockdown on THP-1 viability***

THP-1 cells were transfected with the indicated siRNA and assessed after 72h.

A. Mean ± SEM of three independent experiments showing the silencing of DGKA on THP-1 cells. Data are presented as percentage of DGKA mRNA expression (left) or DGKα protein (right) ± SEM in comparison with siRNA cntrl transfected cells. Lower right, a representative wester blot for DGKα and β-actin protein.
B. Trypan Blue assay. Mean ± SEM of four independent experiments shown as the percentage of live cells relative to siRNA cntrl (left). Right, a representative experiment showing the number of live cells starting from 10*10^5 cells/ml seeded.
C. Mean ± SEM of three independent experiments showing the silencing of DGKZ on THP-1 cells. Data are presented as percentage of DGKZ mRNA expression (left) or DGKζ protein (right) in comparison with siRNA cntrl transfected cells. Lower right, a representative wester blot for DGKζ and β-actin protein.
D. Trypan Blue assay. Mean ± SEM of four independent experiments shown as the percentage of live cells relative to siRNA cntrl (left). Right, a representative experiment showing the number of live cells starting from 10*10^5 cells/ml seeded.

Two-Way ANOVA or unpaired T test versus siRNA cntrl: p<0.05 *, p<0.01 **, p<0.001 *** and p<0.0001 ****.

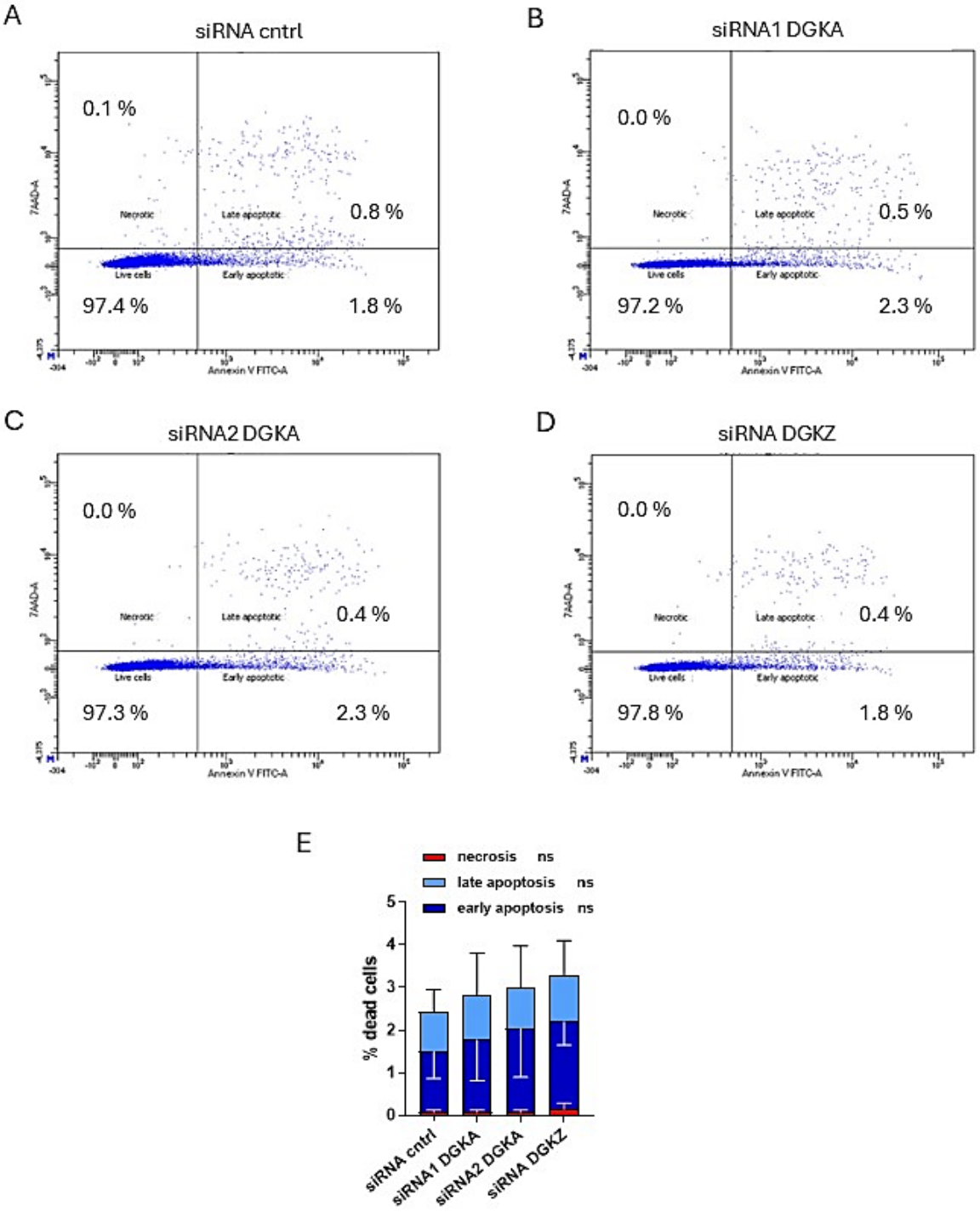

Figure 4: ***Silencing of DGKA and DGKZ in THP-1 cells does not induce significantly cell death***

THP-1 cells silenced for DGKA and DGKZ, stained with "Apoptosis detection Kit Annexin V-FITC" and 7AAD, were analyzed by flow cytometry to detect live (Annexin V- and 7AAD-), necrotic (Annexin- and 7AAD+), early apoptotic (Annexin V+ and 7AAD-) and late apoptotic (Annexin+ and 7AAD+) cell populations.

A. Representative experiment on cells transfected with siRNA control.
B. Representative experiment on cells silenced for DGKA with siRNA1 DGKA.
C. Representative experiment on cells silenced for DGKA with siRNA2 DGKA.
D. Representative experiment on cells silenced for DGKZ.
E. Mean ± SD of three independent experiments presenting the percentage of dead cells and the type of cell death. Paired T test on the total number of dead cells.

Treatment with PA does not rescue cytotoxic effects of DGKs inhibitors

Tan and colleagues demonstrated that ritanserin monotherapy inhibited cell proliferation and promotes apoptosis in AML cells. In addition, they proved that the addition of exogenous PA could rescue this effect in Kasumi-1 and KG-1α cell lines[31]. We intend to explore whether a similar rescue can occur in THP-1 cells treated not only with ritanserin, but also with DGKζ-IN-4 and BAY 2965501 in a concentration correspond to the IC50 previously determined by working group.

In details, 50.000 cells were treated with PA or LPA (as control) at a final concentration of 100 µM either alone or in combination with the indicated inhibitors (at a concentration of 50 µM for ritanserin and DGKζ-IN-4 and 75 µM for BAY 2965501). 24-hour alamarBlue assay revealed that at basal levels, the treatment with PA and LPA alone did not affect cell viability whereas either ritanserin, DGKζ-IN-4 and BAY 2965501 have a strong cytotoxic effect ($p<0.0001$) on THP-1 cells compared to DMSO. However, no rescue effects were observed when PA or LPA was combined with the inhibitors. In the case of DGKA inhibition with ritanserin, LPA showed a greater potential for rescuing cell viability compared to the same treatment with PA, although the effect was not statistically significant when compared to ritanserin treatment alone. For DGKZ inhibition with BAY 2965501, a similar trend was observed, while no appreciable differences were noted with DGKζ-In-4.

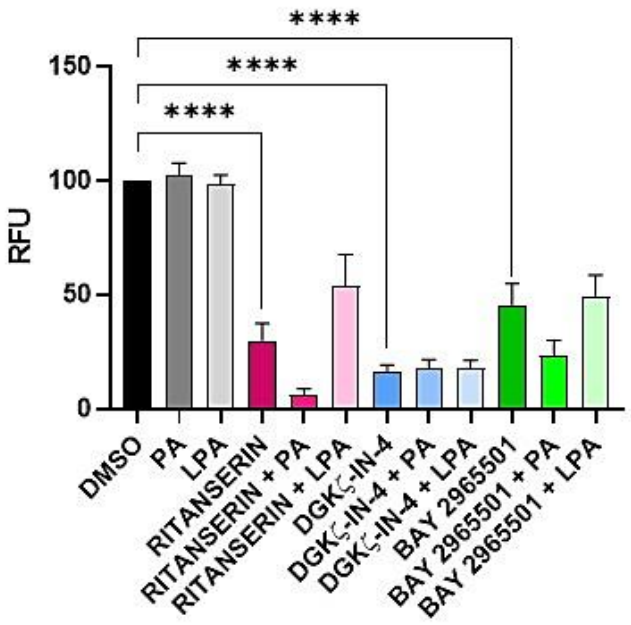

Figure 5: ***Treatment with PA does not rescue cytotoxic effects of DGKs inhibitors.***
THP-1 cells were treated with 100µM of PA or LPA either alone or in combination with 50µM of ritanserin, 50µM of DGKζ-In-4 or 75µM of BAY 2965501 for 24 hours followed by Alamar Blue viability assay for additional 24 hours. Experiments were run in quadruplicates. Data are the mean ± SEM of 6 experiments interpolated as [inhibitor] vs % viability.
Two-Way ANOVA test, $p<0.05$*, $p<0.01$**, $p<0.001$*** and $p<0.0001$****.

DISCUSSION

Due to the relevant of DGK in AML, and in particular the well studied role of α and ζ isoforms, we aimed to investigate the hypothesis that them could be relevant in viability and differentiation using two representative AML cell line models: HEL cells, derived from erythroleukemia, and THP-1 cells, representative of acute monocytic leukaemia.

We observed that, in HEL cells, silencing of DGKA resulted in a significantly reduction (~60%) in DGKA mRNA levels, accompanied by a visible decrease in protein expression. This molecular downregulation correlated with a significant reduction in both the percentage and absolute number of viable cells related to controls. DGKA-silenced cells remain relatively stable over time, showing no expansion beyond the initial seeding density compared to control cells which exhibited normal proliferative capacity. These findings could be indicated a functional dependency of HEL cells on DGKα expression, in particular may suggest a cytostatic effect due to the decrease protein level. Furthermore, apoptosis and necrosis assays revealed an increase in total cell death following DGKA knockdown, primarily attributable to late apoptosis ($p<0.05$). However, since statistical significance was achieved with only one of the two siRNAs tested, the evidence for a cytotoxic effect remains suggestive but not conclusive.

In contrast, silencing of DGKZ, despite achieving a same significantly downregulation at the mRNA level (~60%) and a pronounced reduction in DGKζ protein, did not affect cell viability even in the percentage and in the absolute number of viable cells, compared to controls. DGKZ-silenced cells and control cells exhibit the same proliferative capacity, suggesting a non-essential role of this isoform in HEL cell survival.

Considering that prior data had already indicated a cytostatic effect upon DGKα inhibition, we sought to explore whether this isoform might also play a role in regulating the differentiation process from progenitor cells to mature myeloid cells, since the central role of impaired myeloid differentiation to AML pathogenesis. This hypothesis was supported by transcriptomic analyses from Gravina et all, which showed significant overexpression of DGKα in AML samples compared to $CD34^+$ hematopoietic progenitors, and by unpublished data from Dr A. Bertoni, suggesting that DGKα functions as a negative regulator of megakaryopoiesis: its deficiency enhances megakaryocyte differentiation, thrombopoiesis, and platelet aggregation.

To test this directly, since DGKα plays a role in controlling HEL cells proliferation and this cell line is able to differentiate into megakaryocytes, we treated them in comparison with PMA, a DAG analogue well-established inducer of megakaryopoiesis[33]. As expected, PMA induced a morphological transition from non-adherent, rounded cells to flat adherent cells with an increase in

diameter length, accompanied by increased expression of CD41, a megakaryocyte surface marker, while erythroid CD235a marker remains unchanged, in line with literature[33]. Interestingly, a similar morphological phenotype was observed following DGK inhibition, consistent with intracellular DAG accumulation. All tested inhibitors significantly enhanced cell spreading and elongation; however, the effect was most pronounced with Amb639752, a selective DGKα inhibitor[47], which was also the only compound to significantly upregulate CD41 expression. Should be noted that in any case the effect of inhibitors is smaller than PMA, suggesting a transient or minor DAG accumulation.

Overall, these observations are consistent with the established role of DGKα in regulating cytoskeletal dynamics and cell motility across multiple cell types, including epithelial, endothelial, and T lymphocytes. As demonstrated by Chianale et all, upon recruitment to the plasma membrane and Src-dependent activation, DGKα catalyses the conversion of DAG to PA, which enhancing the recruitment of atypical PKCζ/ι. This leads to Rac activation via release from the RhoGDI complex, ultimately promoting actin cytoskeletal remodelling and cellular migration[20]. Moreover, the modulation of PKC activity, downstream of DAG signalling, has been implicated in the regulation of both erythroid and megakaryocytic differentiation. It may serve as a key determinant in lineage specification, contributing to the selective progression of these pathways during normal haematopoietic development[48]. Thus, inhibition of DGKα may disrupt this axis, contributing not only to altered cell morphology but also to promote the right lineage commitment.

To determine whether the observed phenotypic changes were attributable to increased cell adhesion, we repeated the experiments treating HEL cells with PMA and Amb639752 alone or in combination, choosing only the latter because was the most effective one based on the previous results.

Analysis of cell confluence over multiple time points revealed that, under basal conditions, no significant differences in confluence were observed between DMSO and Amb639752-treated cells, with values remaining consistently low throughout the observation period indicating that DAG accumulation upon Amb639752 treatment is not sufficient for adhesion induction. As expected, PMA induced a marked increase in confluence between 0 and 6 hours, followed by a plateau around 90%, reflecting increased cell adhesion. Importantly, co-treatment with Amb639752 did not further increase this confluence pattern, suggesting that the effects observed upon DGKα inhibition are not due to altered adhesion capacity.

Together, these findings strengthen the hypothesis that DGKα may act as a negative regulator of myeloblast differentiation, particularly along the megakaryocytic lineage, without exerting a direct effect on cell adhesion. Notably, pharmacological inhibition of DGKα appears to partially restore the differentiation, with a particularly pronounced impact on morphological changes associated along this lineage.

Concerning THP-1 cells, silencing of DGKA and DGKZ led to a moderate (~30-50%) decrease in mRNA expression and a more pronounced reduction in protein levels (~60-79%), respectively for DGKα and DGKζ. However, no significant changes in cell viability or proliferation were observed for either isoform, further supported by the fact that both control, DGKA- and DGKZ-silenced populations showed comparable increases in cell number over time, suggesting a lack of cytostatic activity. These results indicate that, under the experimental conditions tested, neither DGKα and DGKζ may be critically required for THP-1 cell survival or proliferative capacity (figure 3). Apoptosis and necrosis assays reinforced these observations, as no significant alterations in apoptotic or necrotic cell death were detected upon silencing of either DGK isoform.
Regarding the role of both DGKα and DGKζ in differentiation of this cell line into macrophages, the research group are still working on. They noticed that also in this case there is a DGK involvement in controlling spreading and morphology.

Therefore, DGKα knockdown impairs proliferation and induces apoptosis in HEL cells, while DGKζ appears to be functionally dispensable in this context. On the other hand, neither DGKα nor DGKζ significantly affect THP-1 cell survival, suggesting that the functional relevance of these isoforms may be tightly linked to the cellular context and stage of myeloid differentiation, and that DGKα and DGKζ may have also a non-redundant role and a cell-specific action. Also evidences in literature supported these data since different DGK isoforms play non-redundant roles depending on the cell type. Among others, Li and colleagues demonstrated that the silencing of DGKζ led to reduced cell proliferation, G2/M cell cycle arrest, and promoted apoptosis in HL-60 cells[32]. In addition, Gravina and colleagues showed that broad-spectrum DGK inhibitors R59022 and R59949 significantly reduced cell viability in HL-60 and HEL cells[30]. Moreover, Tan et all highlighted the DGKα-specific inhibitor, ritanserin, as a promising drug for reducing proliferation of others AML cell line models[31].
Given that DGKA and DGKZ knockdown did not significantly impact THP-1 cell viability, we proceeded to investigate whether pharmacological inhibition of their kinase activity could elicit a more pronounced phenotypic response. This hypothesis was further supported by data from our research group, particularly from studies conducted by Dr Marco Cristiano Cartella.
The cytotoxic response observed in THP-1 cells following treatment with isoform-specific DGK inhibitors, such as ritanserin (DGKα-specific[31]) and DGKζ-IN-4 and BAY2965501 (DGKζ inhibitors[44,45]), may be attributed to their ability to more effectively suppress the protein's function, potentially achieving a more complete inhibition compared to siRNA-mediated silencing. Nonetheless, it remains plausible that these effects arise from off-target interactions, whereby the compounds may interfere with additional signalling pathways beyond DGK inhibition.

Ritanserin (DGKα-specific inhibitor) has previously demonstrated antileukaemic efficacy in AML models, where it was shown to impair proliferation and induce caspase-dependent apoptosis in a dose- and time-dependent manner, both *ex vivo* and *in vivo* xenograft models, ultimately prolonging survival. Systemically, its action has been linked to the depletion of intracellular PA, confirming by the fact that the addition of exogenous PA was able to partially rescue the loss of cell viability, and consequent downregulation of key signalling pathways, including SphK1, Jak-STAT, and MAPK[31].
In alignment with Dr Cartella's findings, we treated THP-1 cells with the same isoform-specific inhibitors at their respective IC_{50} concentrations, to determine whether a comparable mechanism was operative in this AML subtype.
Treatment with ritanserin led to a statistically significant reduction in cell viability ($p<0.0001$) relative to DMSO-treated controls. However, co-treatment with exogenous PA or LPA, included as a control due to the partial metabolic conversion of PA into LPA in cells, failed to rescue cell viability, suggesting that in our model the cytotoxic effects of ritanserin may not be primarily mediated through PA depletion. Although LPA co-treatment produced a modest trend toward increased viability compared to PA; the effect was not statistically significant indicating that also PA hydrolysis in LPA does not contribute to ritanserin-mediated cell death in THP-1 cells.
To assess the role of DGKζ, we employed two distinct specific inhibitors because these compounds differ in their binding sites within the DGKζ catalytic domain and have shown variable efficacy across AML cell lines in prior working group results (Dr Luisa Racca, Dr M. C. Cartella).
Both inhibitors induced a marked decrease in THP-1 viability after 24 hours ($p<0.0001$), confirming that THP-1 cells are sensitive to pharmacological DGKζ inhibition. However, similar to what happens for DGKα inhibition, co-treatment with exogenous PA or LPA failed to rescue the loss of cell viability.
Collectively, these findings confirm that THP-1 cells are susceptible to inhibition of both DGKα and DGKζ isoforms, yet the underlying mechanisms of cell death appear to diverge from the canonical PA-dependent pathways previously described in other AML models[31]. These observations raise the possibility that, beyond PA depletion, alternative pro-apoptotic signalling cascades may mediate the cytotoxic response to DGK inhibition in THP-1 cells. For instance, the silencing of DGKζ has been reported to induce apoptosis potentially via modulation of the MAPK/survivin/caspase signalling axis, suppressing pro-survival pathways and enhances caspase levels. Moreover, DGKζ deletion has been shown to upregulate several key effectors of cellular stress and apoptotic pathways, including ERK1/2, HSP27, Smad2, p53, p38 MAPK, and SAPK/JNK, underscoring the partial involvement of MAPK signalling in the pro-apoptotic effects induced by DGKζ knockdown[32].

An additional consideration is that the lack of rescue observed following exogenous PA supplementation might not necessarily exclude a role for PA, but could instead be attributed to technical limitations in lipid handling; specifically, the poor solubility or bioavailability of PA and LPA under experimental conditions, potentially preventing effective cellular uptake and downstream signalling restoration.

Altogether, these findings underscore the complex, isoform- and cell-specific roles of DGKs in AML cell viability and differentiation and highlight the importance of further elucidating their downstream signalling pathways across distinct AML subtypes.
It should be noted, however, that this study did not include co-inhibition/silencing experiments. Therefore, we cannot exclude the possibility that, upon selective inhibition/knockdown of one isoform (e.g., DGKα), the remaining activity of the other (e.g., DGKζ) may compensate by sustaining sufficient intracellular PA levels to support cell viability, rendering the addition of exogenous PA ineffective, and vice versa. Moreover, pharmacological inhibitors may suppress the protein's function more effectively than siRNA-mediated silencing, which rarely results in complete knockdown.

Whereas our results provide important insights, several questions remain to be addressed.
In particular, the mechanisms by which DGKα regulates cytoskeletal dynamics and lineage commitment merit further investigation, future works should aim to dissect the downstream effectors of DGKα in the context of megakaryocyte differentiation. Furthermore, validating these results in primary AML samples and in vivo models would be essential to assess if could be also a patient-specific activity and subsequent clinical relevance.
From a therapeutic perspective, our findings support the idea that isoform-specific DGK inhibition or silencing, particularly targeting DGKα, could represent a dual-action strategy: not only impairing leukemic cell viability promoting the elimination of AML blasts, but also facilitating the restoration of physiological hematopoietic differentiation limiting the block due to AML.
Future studies could also explore the therapeutic potential of a concentration of DGKα inhibitors able to promote differentiation and at the same time target leukemic stem cell populations.
In addition, the impact of dual inhibition of DGKα and DGKζ warrants exploration to assess potential synergistic effects on AML cell viability and differentiation particularly where the inhibition of a sole isoform did not function. This approach may offer novel opportunities for therapeutic intervention.

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