UNIVERSITÀ DEL PIEMONTE ORIENTALE

SCUOLA DI MEDICINA

Dipartimento di Scienze della Salute

Master Degree in Medical Biotechnology

Role of DGKα and DGKζ inhibition in THP-1 osteoclast differentiation

Relatore:

Prof. Gianluca Baldanzi

Firmato digitalmente da Gianluca Baldanzi
Data: 30.06.2026 18:10:47 CEST
Organizzazione: UNIVERSITA' DEGLI STUDI DEL PIEMONTE ORIENTALE/01943490027

Elaborato finale di:

Bonello Gabriele

N. Matricola: 20047744

Anno Accademico 2025/2026

SUMMARY

ABSTRACT

Osteoclasts are essential for bone homeostasis through bone resorption, and their differentiation is driven by M-CSF and RANKL. The present study was based on evidence indicating that DGKα and DGKζ regulate key signaling pathways involved in cell activation, survival, cytoskeletal remodeling, and differentiation, processes that are essential during macrophage-to-osteoclast transition. We therefore hypothesized that the pharmacological inhibition of DGKα and DGKζ could affect osteoclastogenesis. THP-1 cells were first treated with PMA to induce macrophage differentiation and then stimulated with M-CSF and RANKL to promote osteoclastogenesis. Throughout the experiment, cells were exposed to DMSO, ritanserin, or Bay-2965501 (10 μM), with treatments renewed every 48 hours, to inhibit DGKα and DGKζ, respectively. Cell density, TRAP expression, cell diameter, and number of nuclei per cell were assessed to evaluate osteoclast-like differentiation. Neither inhibitor markedly affected cell density under the tested conditions. By contrast, both compounds impaired morphological maturation, with ritanserin reducing cell spreading and cell size, whereas Bay-2965501 produced a more pronounced phenotype characterized by reduced size and impaired multinucleation. Quantitative analysis confirmed that both inhibitors limited the increase in cell diameter, with a stronger effect for Bay-2965501, while only Bay-2965501 significantly reduced multinucleation at the osteoclast stage. Despite these morphological alterations, TRAP expression remained broadly comparable to control conditions, showing only a slight non-significant decrease, and CTSK expression was not significantly affected. Likewise, DGKα or DGKζ inhibition did not induce consistent changes in the analyzed cytokines, suggesting that these kinases mainly influence morphological maturation rather than basal inflammatory gene expression in this model. These findings suggest that, in PMA-treated THP-1 cells, DGKα and DGKζ mainly regulate morphological maturation rather than osteoclast-like marker induction, with DGKα more closely linked to cell spreading and DGKζ to late multinucleation. This phenotype differs from previous genetic studies, in which DGKα loss enhanced macrophage responsiveness and DGKζ deficiency promoted the formation of larger and more multinucleated osteoclasts, likely reflecting differences between acute pharmacological inhibition in THP-1 cells and constitutive gene deletion in primary murine models. Overall, these findings indicate that DGKα and DGKζ are not major determinants of the osteoclast-like transcriptional program in THP-1 cells, but likely contribute to the morphological remodeling, cell expansion, fusion, and maturation required for osteoclastogenesis. In addition, transcriptional profiling of DGK isoforms in arthritic diseases suggested that DGK family dysregulation may also be relevant in pathological joint remodeling.

INTRODUCTION

The present work started from data collected from multiple studies that have revealed an important role of DGKα and DGKζ in the regulation of main signaling pathways controlling cell activation, survival, cytoskeletal remodeling and differentiation, processes that are essential during macrophage-to-osteoclast differentiation. In this regard, we hypothesized that pharmacological inhibition of DGKα and DGKζ with ritanserin and Bay-2965501 may affect osteoclastogenesis. The inhibition of these two DGK isoforms could disrupt the intracellular balance between diacylglycerol (DAG) and phosphatidic acid (PA), thereby altering DAG-dependent signaling pathways. From this assumption, we performed experiments to evaluate DGKα and DGKζ inhibition on the morphological and functional characteristics of osteoclasts derived from THP-1 cells, which were first treated with PMA to induce macrophage differentiation and subsequently stimulated with M-CSF and RANKL to promote osteoclast formation. Cytokine expression and TRAP levels, a well-established osteoclast-specific marker, were assessed, together with the analysis of transcriptional alterations of DGK family in arthritic diseases, suggesting a potential involvement of these kinases in these pathologies.

1. Osteoclasts

Skeletal bone is maintained by bone remodeling, in which old and damaged bone is replaced with new bone through continuous cellular processes mainly controlled by bone-resorbing osteoclasts and bone-forming osteoblasts. Osteoclasts initiate the process by resorbing portions of old or damaged bone. These resorbed areas are then filled with newly bone matrix produced by osteoblasts. The newly formed bone undergoes mineralization, restoring bone strength and maintaining skeletal integrity. The bone remodeling takes place at sites known as basic multicellular units, which contain all bone cells, including osteoclasts and osteoblasts, and are asynchronously distributed throughout the skeleton (Takegahara et al., 2024). Osteoclasts are giant multinucleated specialized cells that differentiate in the bone marrow and carry out important physiological functions in the regulation of skeletal homeostasis as well as hematopoiesis. Osteoclasts are primarily responsible for bone resorption and remodeling to maintain the normal physiological state of the skeleton. They control bone growth and renewal by binding to the bone surface and releasing acids and enzymes from lysosomes to degrade and dissolve inorganic salts and organic matrices in the bone tissue (Zhu et al., 2026).

1.1 Osteoclast origin: EMP-derived osteoclasts and HSCs-derived osteoclasts

Osteoclast progenitors derive from embryonic yolk-sac erythromyeloid progenitors (EMPs) and fetal hematopoietic stem cells (HSCs) (Yahara et al., 2022). EMP-derived osteoclasts are long-lived, and they are involved in physiological bone remodeling and fracture repair. EMP-derived osteoclast precursors can also migrate through the blood to the site of bone injury and later differentiate into mature osteoclasts. During adulthood, while EMP-derived precursors are gradually replaced by HSC-derived monocyte progenitors, some circulating cells can fuse with the long-lived EMP-derived population to maintain this population (Jacome-Galarza et al., 2019). Additionally, EMP-derived embryonic osteoclasts support the formation of the bone marrow cavity, thereby facilitating the following migration of hematopoietic stem cells and myeloid cells (Yahara et al., 2022). In the classical hematopoiesis model, bone marrow HSCs differentiate into multipotent progenitors (MPPs) which give rise to lineage-restricted precursors. These then give rise to common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). CMPs produce megakaryocyte/erythrocyte progenitors (MEPs) and granulocyte/macrophage progenitors (GMPs). Furthermore, GMPs differentiate into a common macrophage/osteoclast/dendritic cell (DC) progenitor (MODP). These MODPs later give rise to mature osteoclasts through interaction with the receptor activator of the nuclear factor-kappa B (NF-κB) ligand (RANKL) and colony stimulating factor 1 (CSF1), also known as macrophage colony-stimulating factor (M-CSF), essential for the continued differentiation of mature osteoclasts. HSCs migrate from the fetal liver to bone marrow to start definitive hematopoiesis. Under specific microenvironmental conditions, osteoclast precursors mature into pre-osteoclasts, tartrate-resistant acid phosphatase (TRAP)-positive cells that express mRNAs characteristic of osteoclast lineage and, upon further differentiation, fuse to form multinucleated mature osteoclasts (Zhu et al., 2026). TRAP is an enzyme expressed in high amounts by bone resorbing osteoclasts, inflammatory macrophages and dendritic cells. Osteoclasts also possess a specialized proton-pumping system that enables efficient dissolution of mineralized bone. In parallel, they secrete collagenases, various hydrolases and cathepsin K (CTSK), which facilitate degradation of the organic components of the bone matrix (Zhu et al., 2026). CTSK is a lysosomal cysteine protease, able to cleave the triple helix of collagen molecules at multiple locations. Its activity is crucial in bone remodeling processes (Brizuela et al., 2025; Lin et al., 2025).

1.2 Osteoclast multinucleation and fusion dynamics

Osteoclast precursor cells reside in the bone marrow cavity, close to collagen fibers and vascular networks. Following cell-cell fusion with mature osteoclasts, single-nucleated precursors migrate from the bone marrow cavity to sites of resorption on the bone surface (Yahara et al., 2022). Cell-cell fusion of mononuclear osteoclast precursors and multinucleation are essential processes required for osteoclast maturation and its bone resorption ability. Hobolt-Pedersen and colleagues proposed that osteoclast precursors fuse selectively, not randomly, to recognize their fusion partners. This selectivity is based on the intercellular heterogeneity, which reflects differences in the differentiation and functional status of osteoclast precursors, including their maturation stage, migratory capacity, nuclear content, and expression of fusion-related molecules. Fusion preferentially occurs between cells displaying complementary phenotypes, where highly motile precursor cells interact with more mature and fusion-competent osteoclasts (Hobolt-Pedersen et al., 2014; Søe et al., 2020). Mononuclear osteoclast precursors and their fusion partners must migrate toward and adhere to one another to allow multinucleation; in this process, αVβ3 integrin, which localizes to podosomes at the leading edge of osteoclasts, plays a crucial role in cell migration and in the formation of the sealing zone (Nakamura et al., 1999; McHugh et al., 2000).

1.3 Bone remodeling

Bone is continuously remodeled through the coordinated activity of osteoclasts, which resorb bone, and osteoblasts, which form new bone. This tightly regulated process maintains mineral homeostasis and skeletal strength and consists of different sequential phases: initiation, resorption, reversal, formation, and termination. The process is driven by the interaction of osteoclasts, osteoblasts, osteocytes, and bone-lining cells (Yahara et al., 2022). Bone remodeling process is initiated in response to damage or mechanical stress. Osteocytes are the first ones to detect these changes and trigger the release of signaling molecules that promote angiogenesis and recruit osteoclast precursors to start bone resorption. Next, in the resorption phase, osteoclastogenesis is stimulated by RANKL, M-CSF and ligands for immunoglobulin-like receptors, which are produced by osteoblast-lineage cells, including osteocytes. The cytoskeleton of the osteoclast is realigned, a sealing zone is formed, and after completing bone resorption, osteoclasts undergo apoptosis, and the bone resorption phase is terminated. During the early reversal phase, scattered osteoclasts release secreted factors and make direct

cell-cell contacts, allowing a signal to recruit the osteoblast lineage. While mature osteoclasts in contact with mature osteoblasts do not resorb the bone, osteoblasts (especially the DCN high subset) directly suppress osteoclast production by producing osteoprotegerin (OPG). Thus, the number of osteoblast-lineage cells increases, reaching sufficient mass to promote their bone formation activity. Finally, in the formation phase, the resorption lacuna is filled with a new bone matrix and then mineralized to fill the resorption lacuna. During this process, osteoblasts deposit a new bone matrix called the osteoid, which gradually mineralizes and terminally differentiates into osteocytes. The resting bone environment is maintained until the next wave of the remodeling cycle is initiated (Yahara et al., 2022).

1.4 Osteoclast immune and endocrine functions

Osteoclasts are also closely associated with the immune system and can secrete cytokines and growth factors in response to proinflammatory stimuli (Chen et al., 2024). The interaction between osteoclasts and the immune system is also observed in the autoimmune disease rheumatoid arthritis (RA), a form of inflammatory polyarthritis that can lead to joint destruction, deformity and loss of function. Osteoclasts play indeed a role as APCs expressing major histocompatibility complex (MHC) class I and II, CD80, CD86 and CD40, and have the potential to present allogeneic cells, resulting in the activation of both $CD4^+$ and $CD8^+$(cytotoxic) T cells. Thus, osteoclasts can enfold soluble antigens and present them on their cell surfaces. Osteoclast differentiation is accelerated by T cell activation and their differentiation into type 17 helper T (Th17) cells. Th17 cells then stimulate synovial inflammation and produce proinflammatory mediators, such as IL-1 and TNFα from synovial fibroblasts, macrophages and chondrocytes. Furthermore, IL-17A induces RANKL expression in synovial fibroblasts and osteoblasts, which induces osteoclast differentiation and bone destruction associated with RA. Indeed, Th17 cells have been proposed as an osteoclastogenesis-inducer in pathogenic arthritis (Yahara et al., 2022). Moreover, osteoclasts are essential for bone angiogenesis by facilitating endothelial cell migration and stimulating the paracrine secretion of endothelial cells to increase vascular endothelial growth factor (VEGF). On the one hand, osteoclasts control endocrine regulation of calcium and phosphate by regulating their response to parathyroid hormone and calcitonin (Chen et al., 2024).

1.5 Signaling pathways in osteoclast differentiation

Osteoclast differentiation is a highly regulated process driven by the integration of extracellular and intracellular signaling pathways. Following stimulation by RANKL, osteoclast precursors undergo a complex series of molecular events that ultimately lead to the acquisition of a mature bone-resorbing phenotype. RANKL has three isoforms that are differentially expressed in a cell-type specific manner, although the functions of individual isoforms have not yet been clearly defined (Park-Min, 2018). Since RANK lacks intrinsic kinase activity, RANK/RANKL interaction recruits TNF receptor-associated factors (TRAF6) specifically targeting a unique motif [Pro-X-Glu-X-X-(aromatic/acid residue)] within RANK. RANK can interact with multiple TRAF family proteins, such as TRAF1, 2, 3 and 5. Particularly, TRAF6 —a RING-dependent ubiquitin kinase—is indispensable for osteoclastogenesis, as seen in studies revealing that TRAF6-deficient mice exhibit an osteopetrotic bone phenotype (Lomaga et al., 1999). TRAF6 uses a scaffolding protein p62 to interact with PKC or an adaptor protein TGF-β activated kinase 1 binding protein 2 (TAB2) to interact with TGF-β-activated kinase 1 (TAK1), thereby activating IκB kinase 1/2 (IKK1/2). Furthermore, TRAF6 complexes with TAK1 and MKK6 selectively activate p38 (Huang et al., 2006). Beyond TRAFs, RANK interacts with other regulatory molecules such as Gab2, an adaptor protein whose phosphorylation downstream of RANK helps modulate subsequent signaling cascades. Overall, RANK activation triggers the p38, ERK, JNK, AKT, and both canonical and non−canonical NF-κB pathways inducing key transcription factors like c-Fos (Park-Min, 2018). Another essential co-stimulatory pathway for RANKL signaling is mediated by immunoreceptor tyrosine-based activation motif (ITAM), whose engagement activates the protein kinase Syk, downstream PLCγ2 and a signaling complex composed of Btk/Tec kinases and BLNK/SLP-76. This cascade induces calcium oscillations that act synergistically with RANK signaling to robustly drive the expression of nuclear factor of activated T cells c1 (NFATc1), the master regulator of osteoclastogenesis (Park-Min, 2018).

Moreover, Src family kinases phosphorylate ITAM receptors in immune cells. These kinases may have a role in the link between RANK and ITAM signals. Notably, Src-deficient mice exhibit osteopetrotic phenotype with dysfunctional osteoclasts (Lowe et al., 1993).

Instead, M-CSF is a hematopoietic growth factor important for survival, proliferation, differentiation, and mobility of mononuclear phagocyte lineages, including osteoclasts.

Secreted by osteoblasts and bone marrow stromal cells, it binds to its receptor CSF1R on osteoclasts and monocytes/macrophages (Kim et al., 2020). M-CSF promotes monocyte maturation, macrophage activation, and osteoclast progenitor proliferation and differentiation, while also influencing lipid metabolism (Kurzrock et al., 2003). M-CSF binding on CSF1R leads to rapid dimerization and autophosphorylation of CSF1R tyrosines, most of which serve as docking sites for adaptor proteins containing the Src homology 2 (SH2) domain, such as ERK5, SFK/Cbl, Grb2, PI3K and lastly PLCγ, which leads to macrophage differentiation (Mun et al., 2020). Its signaling modulates cell motility and adhesion through different mediators, including c-Src, integrin β3, and DAP12. Integrin β3 is induced by RANKL stimulation and binds to extracellular matrix proteins such as vitronectin, osteopontin, and bone sialoprotein, while DAP12 is an adaptor molecule containing ITAM, necessary for osteoclastogenesis. DAP12 especially is also required for cytoskeletal remodeling in osteoclasts, since it recruits Syk or Zap70 tyrosine kinases through ITAM (Mun et al., 2020). CSF1R signaling is crucial in bone homeostasis, as it promotes RANK in osteoclast precursors and enhances differentiation. The importance of M-CSF/CSF1R is further supported by different studies in osteopetrotic (op/op) mice lacking functional M-CSF. These mice exhibited strong reductions of macrophages and osteoclasts and developed an osteopetrotic phenotype. Importantly, administration of recombinant M-CSF or osteoblast-derived soluble M-CSF was able to rescue osteoclast development and partially restore bone homeostasis (Mun et al., 2020).

1.6 DGKs in macrophages and osteoclasts

Macrophages are highly plastic cells capable of polarizing to a pro-inflammatory phenotype (M1) in response to lipopolysaccharide (LPS) and/or interferon γ (IFNγ), or to an anti-inflammatory phenotype (M2) in response to IL-4 and tissue damage (Mahajan et al., 2020). To investigate the relevance of DGKα in these cells, Manigat et al. focused on DGKα knockout in mice bone marrow-derived macrophages (BMDMs). Firstly, qPCR analysis revealed enhanced induction of macrophage activation and polarization markers such as Nos2 (iNOS encoding gene), Arg1 (arginase encoding gene) and SOCS family genes once stimulated with LPS and IL-4. Baseline expression of these activation markers was not specifically enhanced by DGKα loss, but instead it was the macrophage response to stimulation that was enhanced (Manigat et al., 2021). Moreover, knockdown in J774 macrophage line was also tested, showing

similar patterns of macrophage marker upregulation. Following LPS and IL-4 stimulation, J774 cell expression of Nos2 and Arg1, respectively, was increased in cells that had been transfected with DGKα siRNA significantly more than in cells transfected with negative control siRNA. This was associated with increased PKC activation, likely due to elevated DAG levels, which may amplify macrophage responsiveness. PKCs are DAG-responsive Ser/Thr kinases, key effectors of the DGKα substrate DAG (Manigat et al., 2021) and are involved in the regulation of proliferation, differentiation, migration, survival, and immune responses (Lim et al., 2015). DGKα$^{-/-}$ BMDMs also showed increased proliferation marker Mki67 expression and enhanced MCP-1–driven migration in transwell migration assays. Overall, DGKα knockdown in both primary and cell line models confirms its direct role in restraining macrophage responsiveness. Indeed, the use of pure macrophage cultures excludes the possibility that the effects observed in DGKα-deficient mice were indirectly mediated by other immune cell populations (Manigat et al., 2021).

While DGKα deficiency enhances macrophage responsiveness and migration, studies investigating DGKζ, the most highly expressed DGK isoform in macrophages and osteoclasts (Zamani et al., 2015), have revealed distinct and in some cases opposite effects. In mice DGKζ$^{-/-}$ bone marrow macrophages (BMMs) a defective macrophage polarization to an M1 phenotype was observed, consistent with a reduced production of TNFα, IL-6 and IL-1β. Unlike mice DGKα$^{-/-}$ BMMs, DGKζ deficiency revealed diminished sensitivity and activation to LPS and other TLR ligands and revealed reduced expression of typical M1 markers such as iNOS, CCL5 and IRF5, consistent with the defective M1 polarization. Conversely, IL-4 treatment did not alter M2 polarization. In vivo, DGKζ$^{-/-}$ mice exhibited significantly less swelling. Histological analysis of WT mice ankles of H&E-stained sections showed significant immune infiltration and tissue destruction. Interestingly, despite the presence of inflammatory infiltrates, DGKζ$^{-/-}$ mice were protected from joint and cartilage disruption. qRT-PCR analysis revealed elevated levels of CD11b, a myeloid cell marker expressed in monocytes, and significant upregulation of F4/80, a specific macrophage marker, in the SCW-injected ankles of WT and DGKζ null mice (Mahajan et al., 2020). Moreover, DGKζ has been shown to regulate osteoclast differentiation and function, as in vitro osteoclastogenesis assays demonstrated that DGKζ$^{-/-}$ BMMs generate more numerous, larger, and highly resorptive osteoclasts. These cells exhibited increased size, multinucleation, and functional activity, together with upregulation of osteoclast-associated markers, such as TRAP, NFATc1, c-Fos,

CLCR, DC-STAMP, ATP6V0D2, CTSK, and MMP9 (Zamani et al., 2015). Specifically, loss of DGKζ results in DAG accumulation, leading to increased c-Fos expression, a transcription factor essential for early osteoclast differentiation. Interestingly, M-CSF was identified as a stronger inducer of c-Fos than RANKL, highlighting a previously underappreciated role of the M-CSF–DAG axis in the regulation of osteoclast development. In vivo, the increase in the number of osteoclasts led to osteoporotic phenotype in DGKζ$^{-/-}$ mice. Osteoclasts were seen both in trabecular and in cortical bone (Zamani et al., 2015).

Taken together, these findings indicate that DGK isoforms exert non-redundant functions in the monocyte/macrophage lineage. While DGKα acts predominantly as a negative regulator of macrophage activation, DGKζ appears to promote inflammatory macrophage polarization but restrain osteoclast differentiation and bone resorption. Since these enzymes impact inflammation and bone remodeling, they have been implicated in the development and progression of arthritic diseases. (Zamani et al., 2015; Mahajan et al., 2020; Manigat et al., 2021).

2. DAG-DGK Axis

Phospholipases Cγ (PLCγ) 1 and 2 are highly homologous enzymes that generate inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), key second messengers involved in cell proliferation, motility, angiogenesis, and lipid synthesis (Baldanzi, 2014; Yang et al., 2017). Evidence indicates that DAG exists in three distinct pools with different fatty acid compositions. These pools originate from phospholipase C (PLC)-mediated hydrolysis of PI or PC, phosphatidic acid dephosphorylation, and sphingomyelin synthase activity, respectively (Zamani et al., 2015). In osteoclasts, PLCγ1 generates IP3 and DAG from PIP2, while PLCγ2 plays a critical role in osteoclast differentiation by regulating IP3-mediated calcium oscillations and NFATc1 activation (Yang et al., 2017). Consistently, PLCγ2 deficiency results in an osteopetrotic phenotype due to impaired osteoclast differentiation, while PKCδ loss leads to defective bone resorption and increased bone mass (Cremasco et al., 2012). To regulate these precise spatial and temporal gradients of DAG, cells utilize diacylglycerol kinases (DGKs) as major termination enzymes.

The human genome encodes ten DGK isozymes, often co-expressed with non-redundant functions. DGKs are grouped into five subfamilies based on their N-terminal regulatory

domains, which precede a conserved catalytic region containing an ATP-binding site, accessory subdomains, and C1 domains homologous to the DAG-binding region of protein kinase C (Gravina et al., 2023). All DGKs share a conserved C-terminal catalytic domain of approximately 325 amino acids, composed of a catalytic subdomain (DAGKc) and an accessory subdomain (DAGKa), preceded by two or three tandem C1 domains interrupted by non-conserved sequences (van Blitterswijk & Houssa, 2000).

2.1 DGKs as pharmacological targets

DGKs have emerged as remarkable pharmacological targets since they regulate intracellular balance between DAG and PA, two lipid second messengers critical for cellular homeostasis. Dysregulation of DGK activity is implicated in numerous pathological conditions such as cancer, autoimmune disorders, inflammatory diseases, and metabolic dysfunctions, prompting the development of isoform-selective pharmacological modulators (Racca et al., 2025).

Despite increasing recognition of DGKs as promising therapeutic targets, the development of pharmacological modulators has been significantly limited by various structural and biochemical challenges, such as the complex specific features of each isoform. In particular, the failure to generate a crystal structure of mammalian DGK enzymes has limited the knowledge of their 3D structure, hampering detailed structure-based pharmacodynamics studies, primarily regarding key domains involved in substrate binding and catalysis (Kambayashi et al., 2020; Sánchez-Cabezón et al., 2026). In addition, DGKs isoforms share a high degree of similarity particularly in their catalytic regions, thereby adding further complexity to the selective identification of inhibitors (Riese et al., 2016).

2.2 DGK inhibitors

DGK isoforms can be broadly inhibited by targeting either the substrate-binding site or the ATP-binding cleft. DAG analogues (dioctanoylethylene glycol, 1-monooleoylglycerol) and calphostin C act as substrate-competitive inhibitors, while cochlioquinone A and stemphone block ATP-dependent kinase activity (Racca et al., 2025).

DGKα is one of the first isoforms studied pharmacologically due to its role in immune regulation and cancer signaling (Riese et al., 2016). The first DGKα inhibitors R59022 and R59949 block the catalytic domain near the Mg-ATP site and show antitumor effects, but their

low selectivity and poor stability limit them to in vitro use rather than clinical application (Kambayashi et al., 2020; Liu et al., 2025). A second important step in the development of DGKα-targeting compounds was represented by ritanserin. Ritanserin, originally developed as a 5-HT2A/2C receptor antagonist for the treatment of neurological and psychiatric disorders, was later identified as a selective DGKα inhibitor with an IC_{50} value of 15 μM (Racca et al., 2025). By inhibiting DGKα activity, it enhances TCR signaling and promotes T-cell activation, supporting its potential application in cancer immunotherapy (Liu et al., 2025; Baldanzi, 2022). Ritanserin possesses good pharmacokinetic properties and causes few side effects. It demonstrated its prevalent inhibitory activity against the sole DGKα in a dose-dependent manner, even if higher doses can affect the activity also of other isoforms. Ritanserin exploited its capability to highlight the DGKα role in different malignancies, such as in non-small cell lung cancer, reducing metastasis in vitro and in vivo, in acute myeloid leukemia, where ritanserin led to decreased cell proliferation and increased apoptosis in vitro, as well as demonstrated efficacy as an anti-cancer drug in in vivo models. These effects were associated especially with the negative regulation of Jak-Stat and MAPK signaling pathways, as well as SphK1 expression through DGKα inhibition. Finally, ritanserin has also been proposed for the treatment of glioblastoma, where the mesenchymal subtype was found to be particularly susceptible to this treatment, since DGKα inhibition resulted in the inhibition of geranylgeranyltransferase I, thereby perturbing downstream mediators such as NF-κB (Racca et al., 2025). More selective DGKα inhibitors include CU-3, JNJ-3790339, and AMB639752, which show improved specificity and antitumor or immunomodulatory effects. CU-3 is a potent ATP-competitive inhibitor inducing tumor cell apoptosis and T-cell activation, while JNJ-3790339 and AMB639752 further enhance selectivity, restoring T-cell function and showing activity against cancer and immune disorders (Liu et al., 2016; Granade et al., 2022; Ruffo et al., 2016).

Among selective DGKζ inhibitors, ASP1570 and Bay-2965501 are the most advanced. ASP1570 enhances ERK phosphorylation, IFNγ production, and NK cell activity while promoting DGKζ degradation, increasing DAG signaling and restoring T-cell function, leading to improved antitumor responses in vivo (Racca et al., 2025; Ikeda et al., 2025). Bay-2965501 instead is a highly selective, orally bioavailable DGKζ inhibitor developed by Bayer with a reported IC_{50} value of 4.62 μM. In vitro characterizations have shown that Bay-2965501 does not directly exert cytotoxic or anti-proliferative effects on cancer cells themselves, instead its

therapeutic efficacy is based on its immunomodulatory capacity to rescue tumor-infiltrating lymphocytes (TILs) from exhaustion and anergy (Racca et al., 2025). Moreover, preclinical syngeneic murine models demonstrate a strong therapeutic rationale for combination strategies; when administered alongside anti-PD-L1 antibodies, Bay-2965501 reduces tumor growth more effectively than anti-PD-L1 monotherapy (Liu et al., 2025). The clinical potential of Bay-2965501 is further highlighted by its evaluation into a first-in-human Phase I trial for patients with advanced solid tumors (NCT05614102) such as epithelial, kidney, lung, and stomach, as monotherapy or in combination with Pembrolizumab (anti-PD1 monoclonal antibody) (Racca et al., 2025; Liu et al., 2025). Dual α/ζ DGK inhibitors have been developed for antitumor purposes starting from BMS-684, leading to derivatives such as BMS-502 and BMS-332. These compounds show potent nanomolar inhibition of DGKα, DGKζ, and DGKι, with stronger immune activation in vitro compared to earlier inhibitors like ritanserin and R59949 (Racca et al., 2025).

Most pharmacological studies have focused on DGKα and DGKζ due to their central role in T-cell regulation and immune responses, while other isoforms are less characterized but still targetable (Joshi et al., 2013). DGKγ, DGKδ, and DGKθ can be modulated by compounds such as 3-acetyl indole derivatives, KU-8, 4-hexylresorcinol, U73122, and sodium pyrophosphate respectively, which act as inhibitors or modulators affecting immune, inflammatory, and signaling pathways across different cell types (Racca et al., 2025; Kweon et al., 2014; Tu-Sekine et al., 2010).

MATERIALS AND METHODS

1. Cell culture conditions

The THP-1 cell line identity was verified through a Cell Line Authentication Test conducted by Eurofins Genomics (Ebersberg, Germany). The same cells were used in this study, deriving from the "Acute Myeloid Leukemia: A Key Role of DGKα and DGKζ in Cell Viability (Gorla et al., 2025)" article. THP-1 cells were maintained at a density of 3×10^6 cells/ml in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, cat. no. A5670801) and 1% penicillin–streptomycin (Sigma, cat. no. A5955). The culture medium was refreshed every 2 days to maintain optimal cell density and growth conditions, following the handling protocols provided by ATCC (Manassas, VA, USA).

1.1 Differentiation of THP-1 cells

THP-1 cells were seeded in RPMI 1640 media with 5% heat inactivated FBS to minimize interference with cell adhesion at the concentration of 5×10^5 cells/ml eventually in presence of the indicated treatment. During the whole course of the experiment, the treatments were administered at the concentration of 10μM per condition and replaced every 48 hours. To induce macrophage stage, cells were stimulated with PMA at the concentration of 100 ng/ml for 24 hours to induce cell adhesion and differentiation. THP-1 cells were highly responsive to DAG-dependent signaling, since their differentiation into macrophages was easily induced by phorbol 12-myristate 13-acetate (PMA) exposure, a synthetic DAG analog that directly activates protein kinase C (PKC) and mimics endogenous DAG signaling. DAG is unstable and has a short half-life once administered, while its analog PMA exhibits low enzymatic degradation, conferring a strong and long-lasting signal. Differentiated cells were then maintained in fresh RPMI 1640 medium along the entire experiment. To further induce osteoclast differentiation, cells treated with PMA as indicated, were supplemented with M-CSF and RANKL 50 ng/ml each, renewed every 48 h (for 8–10 days). During osteoclastogenesis, cells progressively increased in size and exhibited multinucleation, consistent with osteoclast maturation. To verify growth rate undifferentiated cells were counted every 48 hours through automated cell counter (TC20™ Automated Cell Counter - BIORAD). At the end of the experiment, to evaluate morphology, cells were stained with NucBlue™ (Live Cell Stain ReadyProbes™ reagent, one drop for 500 μl) for 30 minutes to stain the nuclei and multiple

pictures of every well were acquired with fluorescence microscope (FloydEvos). Subsequently, cell diameters, nuclei count per cell and cell density were measured in the photos. Cell diameters have been measured with FIJI program for at least one hundred cells. The number of nuclei for every cell was manually counted in the same cells as well as cell density (cells/mm^2).

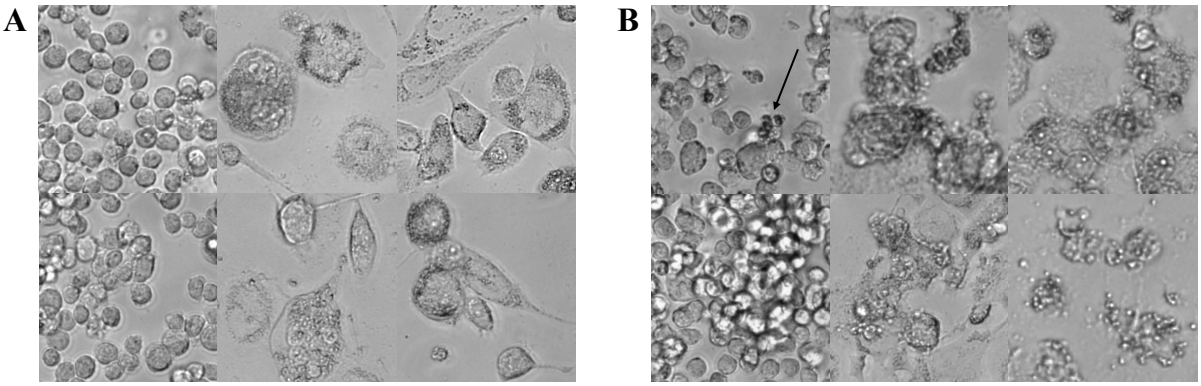

Fig. 1: Inclusion and exclusion criteria in cellular spreading and nuclei count measurement

A) Examples of well-defined fields (clear cells, easily-distincted).
B) Examples of bad-defined fields, especially excluded from measurement (cells agglomeration, debris, amorphous dying cells).

2. Quantitative Real-Time PCR

At the end of the differentiation process cells were lysed in 200 µl of nuclease-free water supplemented with 2% thioglycerol. RNA extraction from lysates was obtained by Kingfisher automated nucleic acid extractor (Thermo Fisher Scientific, Waltham, MA, USA with the MVP_2WASH_200_FLEX protocol and the MagMAXTM Viral/Pathogen Nucleic Acid Isolation Kit (Thermo Fisher). The RNA was then quantified using NanoDrop (Thermo Fisher) and subsequently, equal amounts of RNA were retrotranscribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher) following the manufacturer's instructions. The relative expression of target genes was assessed by quantitative real-time PCR (qRT-PCR) using TaqMan technology. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal reference gene. qRT-PCR was performed in a 384-well plate using the TaqMan™ Fast Advanced Master Mix kit (Thermo Fisher), 4 µl of mix and 1 µl of cDNA and run on a Bio-Rad CFX384TM (Bio-Rad, Hercules, CA, USA) real-time PCR system. The amplification protocol consisted of an initial denaturation at 95 ◦C for 20 s, followed by 50

cycles of denaturation at 95 ◦C for 3 s and annealing/extension at 60°C for 30 s. The qPCR reactions were performed in technical triplicates. Data were analyzed with Bio-Rad CFX Maestro 4.1.2433.1219 software.

The probes used in this study are reported below.

GAPDH (Assay ID: Hs02786624_g1, Applied Biosystems, Thermo Fisher Scientific)
ACP5 (Assay ID: Hs00356261_m1, Applied Biosystems, Thermo Fisher Scientific)
IL-1B (Assay ID: Hs01555410_m1, Applied Biosystems, Thermo Fisher Scientific)
IL6 (Assay ID: Hs00174131_m1, Applied Biosystems, Thermo Fisher Scientific)
IL23A (Assay ID: Hs00372324_m1, Applied Biosystems, Thermo Fisher Scientific)
TNF (Assay ID: Hs00174128_m1, Applied Biosystems, Thermo Fisher Scientific)
TGFB1 (Assay ID: Hs00998133_m1, Applied Biosystems, Thermo Fisher Scientific)
IL10 (Assay ID: Hs00961622_m1, Applied Biosystems, Thermo Fisher Scientific)

3. *DGK Expression Profiling in Arthritic Diseases*

Transcriptomic RNA-Seq datasets encompassing rheumatoid arthritis (RA), osteoarthritis (OA), and knee osteoarthritis (knee OA), along with their respective healthy reference cohorts (normal controls), were retrieved from the OmicSoft Lands within the Ingenuity Pathway Analysis (IPA) ecosystem (QIAGEN Inc., Hilden, Germany, https://www.qiagen.com/us/products/discovery-and-translational-research/next-generation-sequencing/informatics-and-data/interpretation-content-databases/ ingenuity-pathway-analysis/). The downloaded transcriptomic data were represented as log2(FPKM). From these datasets, a curated panel of the DGK family, specifically comprising the transcriptional profiles of DGKA, DGKB, DGKD, DGKE, DGKG, DGKH, DGKI, DGKK, DGKQ, and DGKZ, was selected for subsequent evaluation. For downstream analysis, the expression data were partitioned into three independent contrast matrices to isolate each pathological condition against its matched healthy control group. The final study cohort comprised 264 RA samples, 152 OA samples, and 484 knee OA samples, each comparatively evaluated against 109 normal controls. Statistical comparisons between OA and normal controls, RA and normal controls, and knee OA and normal controls were defined using a two-tailed Wilcoxon rank-sum test, with a significance threshold set at $p<0.05$. Both the Wilcoxon tests and the corresponding boxplots were analyzed within the R statistical computing environment.

RESULTS

To investigate the effect of DGK inhibition on THP-1-derived osteoclast differentiation, a series of experiments was performed using these selective DGK inhibitors. Ritanserin, originally characterized as a serotonin receptor antagonist, has also been identified as a pharmacological inhibitor of DGKα (Boroda et al., 2017). In vitro cytotoxicity assays demonstrated that ritanserin reduced viability of THP-1 leukemia cells in a dose-dependent manner in the micromolar range, with calculated IC_{50} values of approximately 37.0 ± 1.4 μM after 24 h and 32.0 ± 1.5 μM after 72 h, accompanied by increased apoptosis. (Gorla et al., 2025). Bay-2965501 is a selective small-molecule inhibitor of DGKζ developed for preclinical research applications. Bay-2965501 exhibited dose- and time-dependent cytotoxic effects on acute myeloid leukemia cell lines, with the THP-1 monocytic leukemia cells showing an approximate IC_{50} of 67.0 ± 3.5 μM at 24 h and 38.0 ± 3.0 μM at 72 h, accompanied by induction of apoptosis and necrosis in the treated cells (Gorla et al., 2025).

1. Role of DGKα and DGKζ inhibitors on THP-1 cells growth and density

In panel **A**, the growth curve of undifferentiated cells does not show any strong alteration along the eight days treatment. Both ritanserin and Bay-2965501 treatments induced a non-significant reduction in cell numbers compared to DMSO controls, which indicates that those inhibitors do not reduce undifferentiated THP-1 cell viability as previously reported (Gorla et al., 2025).

Panel **B** shows the cell density expressed in nuclei/mm^2 evaluated at the end of the differentiation process, presenting a clear reduction of cell density shifting from undifferentiated cells to the macrophages and osteoclast stage, consistent with cellular enlargement and spreading occurring during differentiation. The trend is similar among the three conditions, showing no statistical significance for each comparison. These findings may indicate that DGKα and DGKζ inhibition does not significantly alter THP-1 cell density under basal conditions or following macrophage/osteoclast differentiation.

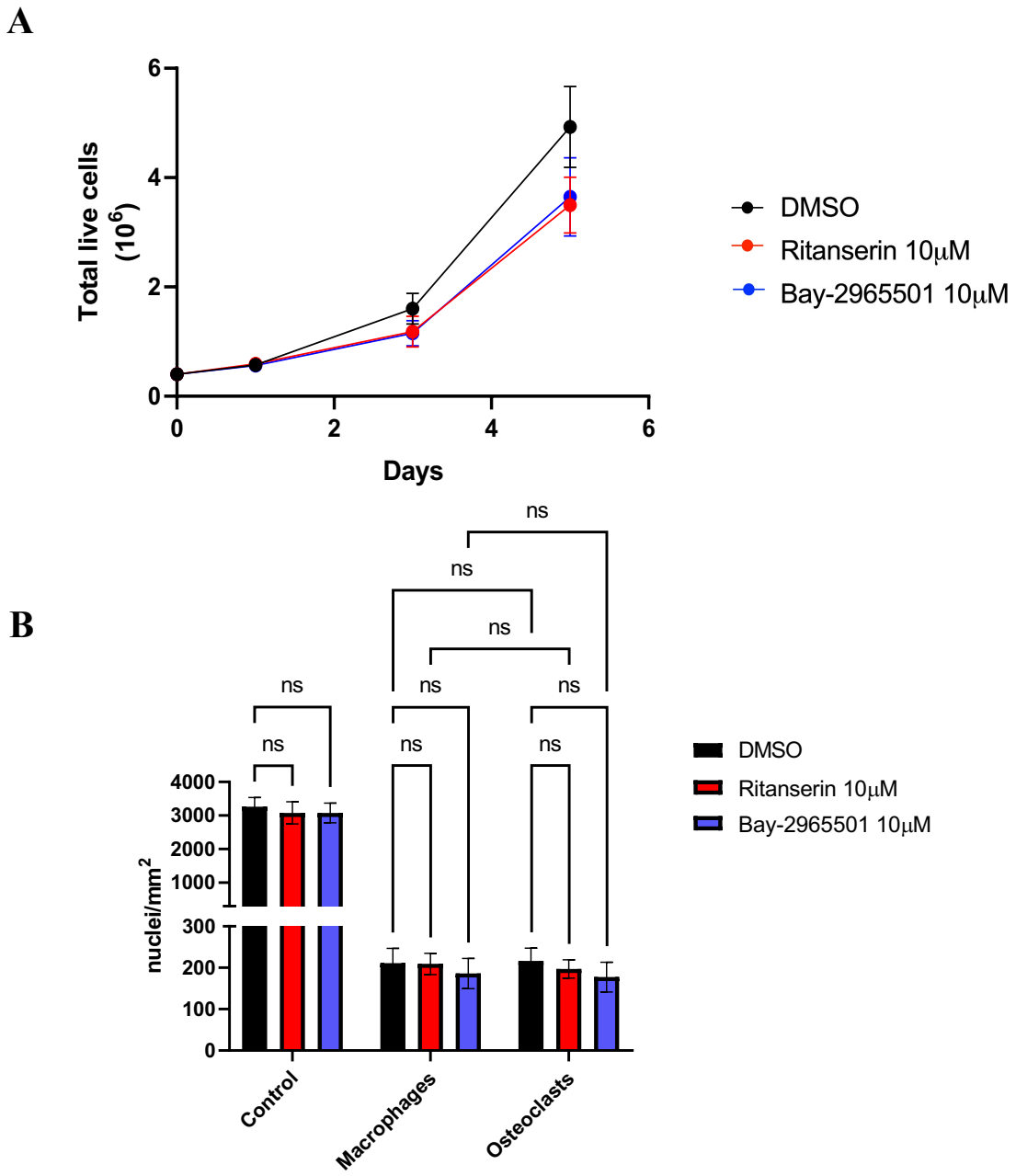

Fig. 2: Role of DGKα and DGKζ in THP-1 growth and density

THP-1 cells were seeded at the concentration of 0.5×10^6 cells/ml and stimulated with PMA (100ng/ml for 24h) to induce cell differentiation into macrophages. After that, M-CSF (50ng/ml) + RANKL (50ng/ml) were added every 48h per 8-10 days to induce osteoclastogenesis. During the whole course of cell differentation, cells were treated with DMSO (as vehicle), Ritanserin (DGKα inhibitor) or Bay-2965501 (DGKζ inhibitor) at the concentration of 10μM and replaced every 48h.

A) Total live cell count of undifferentiated THP-1. Mean of three independent experiments ± SEM.

B) Cell density expressed in nuclei/mm^2 evaluated at the end of the differentiation process. Data are the mean of three independent experiments ± SEM.

2. Role of DGKα and DGKζ inhibitors in THP-1 osteoclast differentiation

Panel **A** illustrates the morphological characteristics of untreated and treated cells. In control samples, undifferentiated cells displayed a normal phenotype, characterized by a round shape and healthy growth conditions. THP-1-derived macrophages were adherent, larger in size and showed expansion of both the cell body and nuclei, with multinucleation readily observable. Osteoclast-like cells exhibited increased spreading, with an enlarged cell body and a higher number of nuclei, reflecting progression toward a fully differentiated phenotype. In undifferentiated cells treated with ritanserin, an increased presence of cellular debris was observed compared to controls, while ritanserin-treated macrophages seemed more contracted. The same features were observable in osteoclasts, where they displayed reduced cell spreading and overall smaller dimensions, with a significant decrease in surface area compared to untreated controls. A comparable trend was observed following treatment with Bay-2965501. Although control cultures showed limited debris, treated cells revealed increased cellular fragmentation. Macrophages treated with Bay-2965501 appeared smaller and less extensively spread, while osteoclasts showed a marked impairment in morphological differentiation, presenting a shrunken phenotype with reduced size and diminished characteristic structural features relative to control cells.

To evaluate a marker of osteoclastogenesis on THP-1 cells, the gene expression of TRAP (tartrate-resistant acid phosphatase) was analyzed by qRT-PCR. TRAP is widely recognized as a hallmark of osteoclast maturation, with increased expression during the acquisition of osteoclast phenotype (Cohen-Karlik et al., 2021). As shown in panel **B**, TRAP expression increased from undifferentiated cells to macrophages and reached higher levels in osteoclasts, consistent with differentiation. Upon ritanserin or Bay-2965501 administration, TRAP expression showed a pattern comparable to the control, with a slight but not significant decrease in osteoclasts.

Thus, under these conditions, neither ritanserin nor Bay-2965501 has a relevant impact on TRAP expression during osteoclastogenesis, suggesting that DGKα and DGKζ do not regulate this marker. In addition, the expression of CTSK, another key osteoclastic marker involved in bone matrix degradation, was also evaluated. However, no significant changes were detected, suggesting that under the applied experimental conditions its expression was not markedly modulated (data not shown).

Panel **C** shows a violin plot representing the maximum cell diameter measured in at least 100 cells per condition, to quantitatively support the observations in panel A. Its analysis showed a progressive increase in cell diameter during differentiation, with DMSO-treated cells increasing from approximately 16 µm in the undifferentiated state to 60–65 µm in macrophages and 80–90 µm in osteoclasts. Ritanserin-treated cells increased from approximately 17 µm to 55–60 µm in both macrophages and osteoclasts, whereas Bay-2965501-treated cells increased from approximately 17 µm to 40–45 µm in macrophages and 55–60 µm in osteoclasts. In control samples, macrophages exhibited increased diameters compared to undifferentiated cells, while osteoclasts showed a further significant increase. Ritanserin treatment resulted in a significant reduction in macrophage diameter, an effect that was also observed with Bay-2965501 administration. Osteoclasts treated with ritanserin showed a general reduction in diameter, although a small subset of cells retained larger sizes, probably reflecting residual multinucleation. Similarly, osteoclasts treated with Bay-2965501 showed a strong and consistent reduction in diameter compared to controls.

Finally, panel **D** highlights the ploidy differences observed in the various experimental conditions. First, in DMSO-treated controls, undifferentiated cells showed only one nucleus per cell, a finding that reflects their mononuclear phenotype. Macrophages had a slight increase in the number of nuclei, while remaining mostly mononucleated, whereas osteoclasts showed a net increase in ploidy, consistent with their differentiation into mature, multinucleated cells. Ritanserin treatment did not significantly alter nuclear number at any differentiation stage: undifferentiated cells, macrophages, and osteoclasts all showed nuclear counts comparable to their respective controls. This indicates that DGKα inhibition does not significantly affect cell fusion under these conditions. Interestingly, treatment with Bay-2965501 produced a significant effect at the osteoclast stage. While treated undifferentiated cells and macrophages remained largely unchanged compared to controls, osteoclasts did not increase in ploidy with respect to macrophages. This suggests that DGKζ inhibition partially impairs the acquisition of multinucleation characteristic in mature osteoclasts.

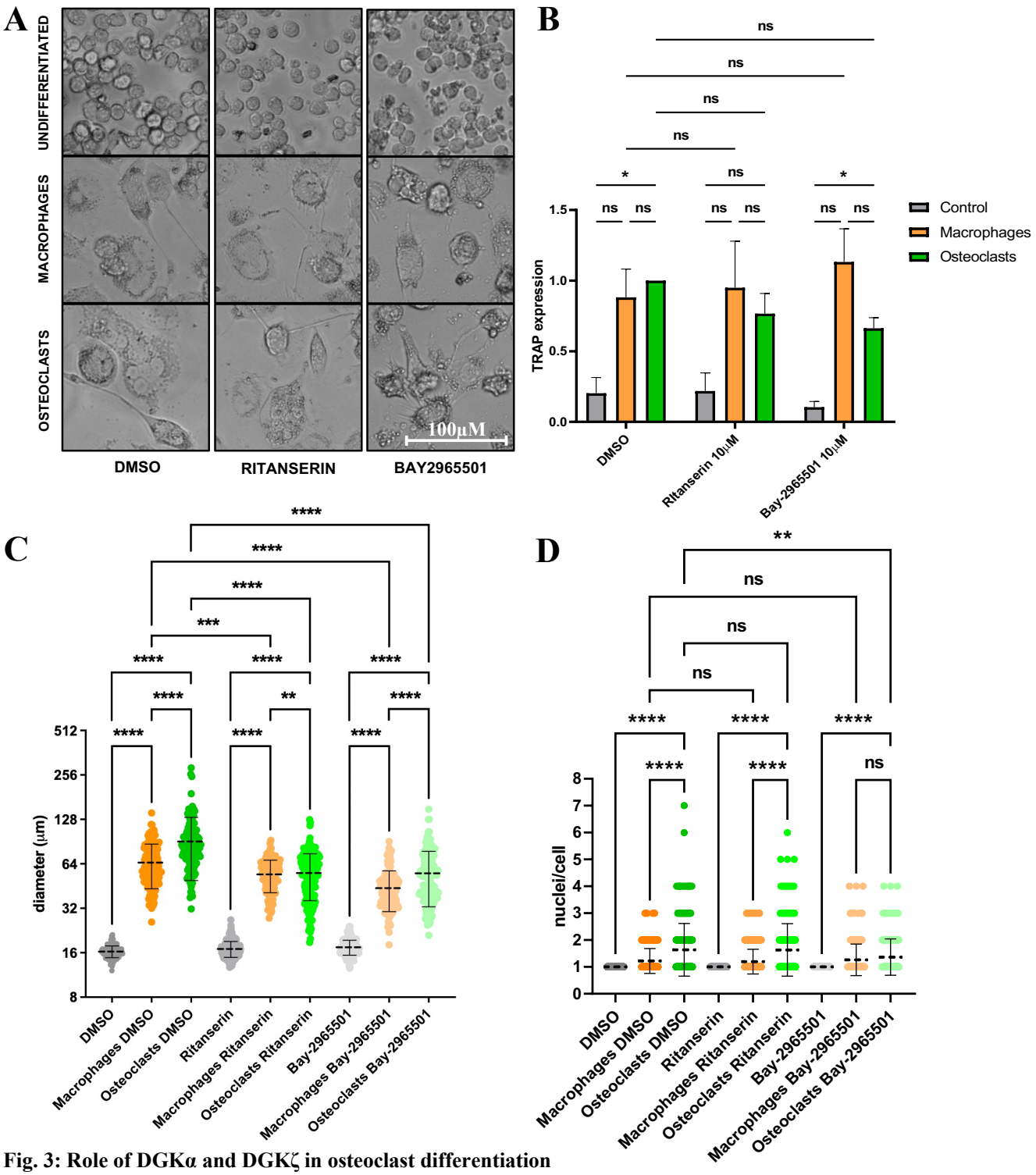

Fig. 3: Role of DGKα and DGKζ in osteoclast differentiation

THP-1 cells were seeded at the concentration of 0.5×10^6 cell/ml and stimulated with PMA (100ng/ml for 24h) to induce cell differentiation into macrophages. After that, M-CSF (50ng/ml) + RANKL (50ng/ml) were added every 48h per 8-10 days to induce osteoclastogenesis. During the whole course of differentation, cells were treated with DMSO (as vehicle), Ritanserin (DGKα inhibitor) or Bay-2965501 (DGKζ inhibitor) at the concentration of 10µM and replaced every 48h.

A) Rapresentative images taken at the end of the differentiation process.

B) Levels of TRAP expression, assessed through qRT-PCR at the end point of the differentiation process at undifferentiated, macrophage and osteoclast stages. Data are normalized on osteoclasts DMSO and expressed as the mean of three independent experiments ± SEM.

C) Measurement of diameter lenght of at least >100 cells per condition. Mean of three independent experiments ± SEM.

D) Each sample was stained with NucBlue to allow nuclei identification. Mean of the total nuclei count of three independent experiments ± SEM.

3. *Evaluation of cytokines expression along THP-1 cells differentiation*

To characterize the immunomodulatory profile during the differentiation of THP-1 cells into macrophages and osteoclasts, the gene expression levels of IL-1β, IL-6, IL-23A, TGF-β, TNF-α, and IL-10 were quantified through qRT-PCR. These cytokines were selected based on their established roles in inflammation and bone remodeling processes associated with osteoclast differentiation. Gene expression was analyzed in control cells and in cells treated with ritanserin or Bay-2965501 to determine whether DGKα or DGKζ inhibition affects cytokine expression during osteoclastogenesis.

IL-1β expression was markedly increased in both macrophages and osteoclasts compared to THP-1 control cells under DMSO conditions, indicating activation of a pro-inflammatory program during differentiation; however, comparisons across treatments were not significant, suggesting limited involvement of DGKα or DGKζ in regulating IL-1β expression. Under DMSO treatment, IL-6 expression was markedly increased in macrophages and moderately increased in osteoclasts. Pharmacological inhibition of DGKα or DGKζ substantially reduced IL-6 levels relative to control cells, although these reductions were not statistically significant when macrophages and osteoclasts were compared within the same treatment condition. IL-23A expression remained overall low, with a slight decrease upon differentiation, none of the comparisons were significant, suggesting again minimal modulation both by differentiation and DGKs inhibition. TGF-β expression appeared increased in macrophages and osteoclasts compared to THP-1 controls, but apart from comparison between control THP-1 and macrophages, all comparisons resulted not significant, indicating high variability and lack of statistical changes.

In contrast, TNF-α expression was highest in THP-1 control cells and significantly decreased upon differentiation into macrophages and osteoclasts, with strong statistical significance under control conditions, supporting a differentiation-associated downregulation. TNF expression was seen to be markedly reduced in ritanserin-treated THP-1 cells, and a similar reduction was observed following treatment with Bay-2965501, while both in treated macrophages and osteoclasts no significant changes were observed. Finally, IL-10 expression showed a trend to increase in differentiated cells compared to THP-1 controls, consistent with acquisition of anti-inflammatory features, although all comparisons were not significant, due to high variability among the conditions.

These data show that basal and induced cytokine expression is dynamically remodeled during THP-1 differentiation with our protocol, reflecting a progressive reprogramming of the inflammatory phenotype from THP-1 toward macrophage-like cells and finally osteoclast-like cells. IL-1β and IL-6 increase in the later stages, suggesting that inflammatory pathways become more active as cells progress toward macrophage and osteoclast-like phenotypes. In parallel, TNF-α decreases markedly, indicating a shift away from the baseline inflammatory state typical of THP-1 cells toward a more specialized cytokine profile which is linked to differentiation. At the same time, the rise in IL-10 suggests that a compensatory, regulatory response is also being established, likely to keep the inflammatory environment under control as differentiation proceeds. IL-23A and TGF-β show only modest and inconsistent changes, indicating that their contribution in this context is relatively minor. Despite these clear differentiation-dependent shifts, inhibition of DGKα or DGKζ does not produce significant or consistent alterations in cytokine gene expression profiles. Across nearly all cytokines analyzed, most comparisons between control and inhibitor-treated conditions remain non-significant. This suggests that, within the experimental setup used, DGK signaling is not a major regulator of basal cytokine transcription during THP-1 differentiation.

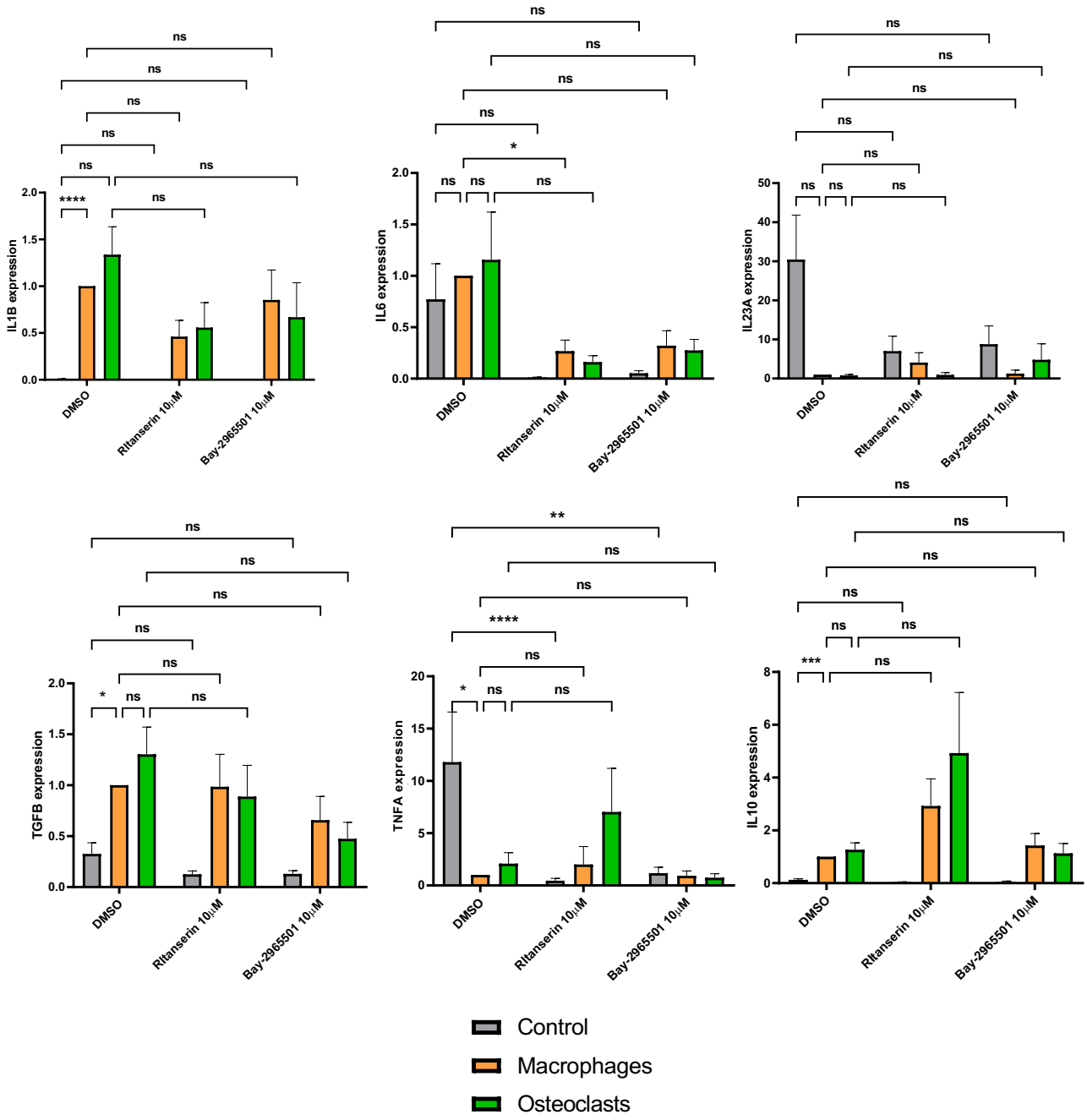

Fig. 4: Role of DGKα and DGKζ inhibition in cytokine expression along THP-1 cells differentiation
THP-1 cells were seeded at the concentration of 0.5×10^6/ml and stimulated with PMA (100 ng/ml) for 24h to induce the differentiation into macrophages, subsequently M-CSF (50ng/ml) and RANKL (50ng/ml) were added every 48h for 8-10 days in order to promote osteoclast differentiation. Cells were also treated with DMSO (vehicle), ritanserin or Bay-2965501 at 10µM. A qRT-PCR was then performed, using control osteoclasts DMSO as reference. Data are the mean of five independent experiments ± SEM.

4. Evaluation of DGK expression profiling in arthritic diseases

To evaluate the transcriptional alterations of the Diacylglycerol Kinase family in arthritic diseases, we requested the assistance of Dr. Teresa Gravina to compare the expression levels of ten DGK isoforms in disease samples against healthy controls (**5,6,7**). Data were extracted from OmicSoft Lands within the Ingenuity Pathway Analysis (IPA) ecosystem. Statistical screening revealed significant shifts in transcript abundance for most of the investigated genes. Specifically, as shown in **Figure 5**, DGKA, DGKD, DGKG, DGKK, DGKQ, and DGKZ exhibited a statistically significant upregulation in osteoarthritis (OA) samples compared to normal controls ($p<0.05$, Wilcoxon rank-sum test). Conversely, a significant downregulation in the OA cohort was observed for DGKE and DGKH ($p<0.05$). No statistically significant variation in transcript levels was detected for DGKB and DGKI. In the context of rheumatoid arthritis (RA), similar to the OA cohort, DGKA, DGKD, DGKG, DGKQ, and DGKZ were significantly upregulated in RA tissues relative to normal controls ($p<0.05$). Conversely, a significant transcriptional downregulation was observed for DGKH and DGKI ($p<0.05$), while DGKB, DGKE and DGKK showed no statistically significant variations. Finally, the transcriptional profiles in knee osteoarthritis (Knee OA) revealed a distinct regulatory pattern that shared features with both generalized OA and RA (**Figure 7**). In Knee OA tissues, a statistically significant upregulation was maintained for DGKD, DGKG, DGKK, and DGKQ ($p<0.05$) compared to normal controls. Conversely, DGKH and DGKI exhibited a significant downregulation ($p<0.05$), showing the specific trend observed in the RA cohort. DGKA, DGKB, DGKE, and DGKZ showed no statistically significant modifications in transcript levels within the Knee OA group.

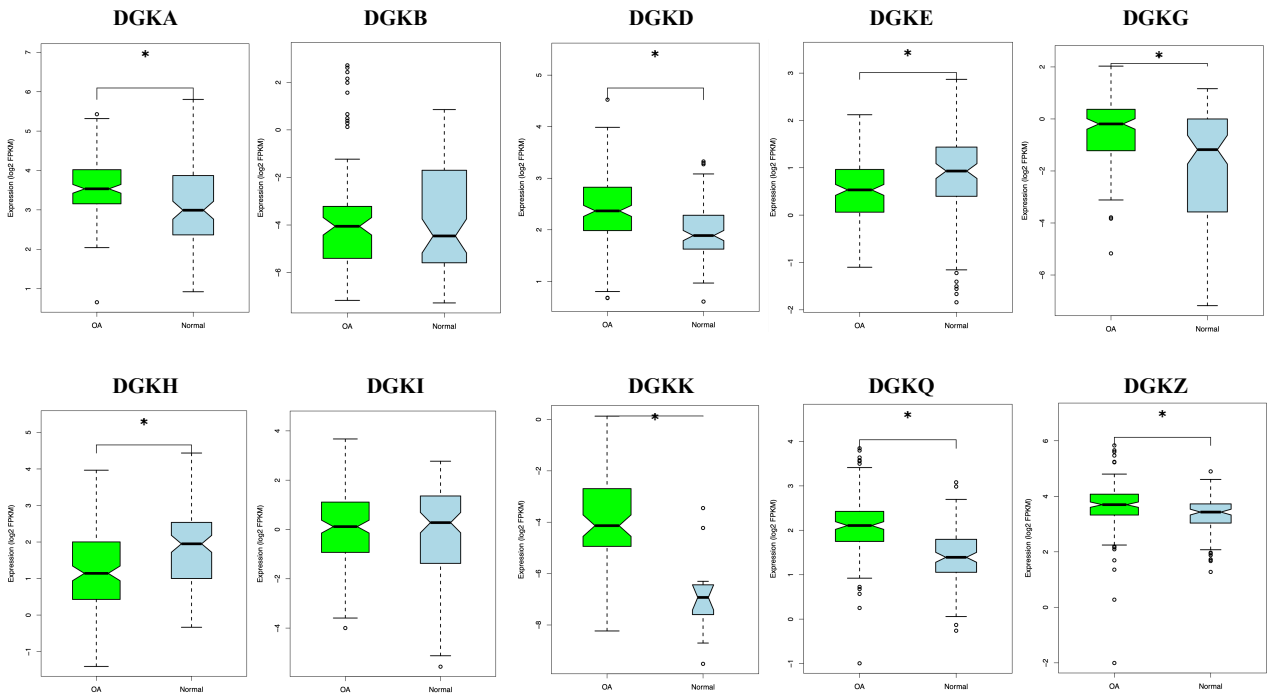

Fig. 5: Transcriptional levels of Diacylglycerol Kinase (DGK) isoforms in osteoarthritis.

Boxplots represent the comparative analysis of transcript abundance (log2FPKM) between osteoarthritis (OA, green) and normal controls (light blue) across DGK genes (*DGKA*, *DGKB*, *DGKD*, *DGKE*, *DGKG*, *DGKH*, *DGKI*, *DGKK*, *DGKQ*, and *DGKZ*). Statistically significant differences ($p<0.05$, determined by a two-tailed Wilcoxon rank-sum test) are highlighted with an asterisk (*).

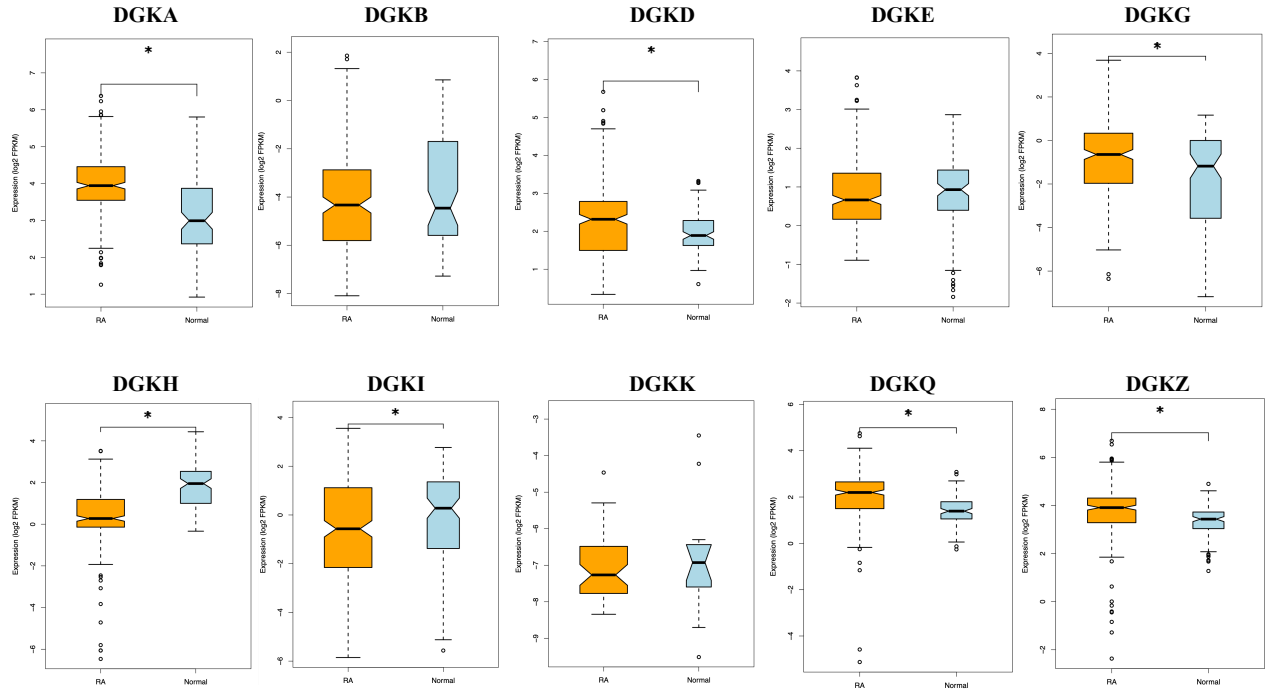

Fig. 6: Transcriptional levels of Diacylglycerol Kinase (DGK) isoforms in rheumatoid arthritis.

Boxplots represent the comparative analysis of transcript abundance (log2FPKM) between rheumatoid arthritis (RA, orange) and normal controls (light blue) across DGK genes (*DGKA*, *DGKB*, *DGKD*, *DGKE*, *DGKG*, *DGKH*, *DGKI*, *DGKK*, *DGKQ*, and *DGKZ*). Statistically significant differences ($p<0.05$, determined by a two-tailed Wilcoxon rank-sum test) are highlighted with an asterisk (*).

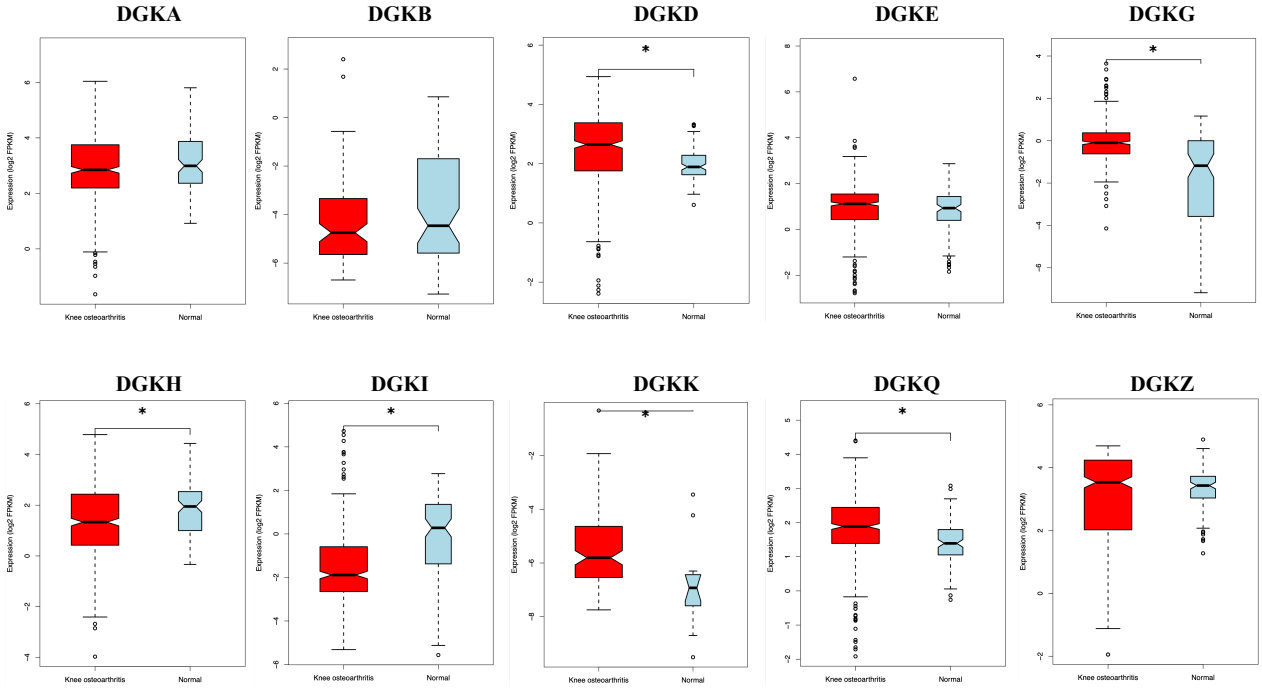

Fig. 7: Transcriptional levels of Diacylglycerol Kinase (DGK) isoforms in knee osteoarthritis

Boxplots represent the comparative analysis of transcript abundance (log2FPKM) between knee osteoarthritis (Knee OA, red) and normal controls (light blue) across DGK genes (*DGKA*, *DGKB*, *DGKD*, *DGKE*, *DGKG*, *DGKH*, *DGKI*, *DGKK*, *DGKQ*, and *DGKZ*). Statistically significant differences ($p<0.05$, determined by a two-tailed Wilcoxon rank-sum test) are highlighted with an asterisk (*).

DISCUSSION

The present study investigated the effects of pharmacological inhibition of DGKα and DGKζ during THP-1-derived osteoclastogenesis. This study was based on the premise that DGKs regulate the intracellular balance between DAG and PA, two lipid second messengers involved in cell activation, survival, cytoskeletal remodeling and differentiation. Because osteoclast differentiation requires coordinated changes in morphology, fusion, inflammatory signaling, and transcriptional activation, the DGK pathway may represent a relevant regulatory node in this process.

The first major finding is that DGKα and DGKζ inhibition did not significantly affect cell density during THP-1 differentiation (**Figure 2**). Both ritanserin and Bay-2965501 produced a non-significant reduction in undifferentiated THP-1 cell number compared with DMSO controls, and cell density followed a similar trend across control and inhibitor-treated conditions during differentiation. This indicates that, under the concentration and timing used in this experimental setting, the inhibitors did not primarily exert a generalized cytotoxic effect, consistent with previous evidence of limited THP-1 sensitivity to ritanserin and Bay-2965501 at concentrations below their reported IC_{50} values (Gorla et al., 2025). Overall, these data suggest that DGKα and DGKζ are unlikely to be major determinants of short-term THP-1 survival under the conditions tested.

The most evident effect of DGK inhibition emerged instead at the morphological level. As shown in **Figure 3**, in control conditions, THP-1-derived macrophages and osteoclast-like cells showed increased size, spreading, and multinucleation during differentiation as expected. By contrast, ritanserin-treated osteoclast-like cells displayed reduced spreading and smaller dimensions, while Bay-2965501-treated cells showed an even more marked impairment, with reduced size, shrunken phenotype, and diminished typical morphological differentiation. Quantitative analysis in panel C confirmed this observation, as both inhibitors limited the increase in cell diameter, with a stronger effect for Bay-2965501, indicating that DGKα and DGKζ contribute to the cytoskeletal and membrane remodeling, required for osteoclast-like maturation.

Notably, the two inhibitors did not produce identical phenotypes. Ritanserin had little effect on nuclear number, suggesting that DGKα inhibition does not strongly impair cell fusion in

this model, but it still reduced cell size and spreading, indicating a role in morphological expansion and cytoskeletal organization. By contrast, Bay-2965501 significantly impaired multinucleation at the osteoclast stage, supporting a more direct involvement of DGKζ in late fusion-dependent maturation.

Although DGK inhibition significantly affected osteoclast-like morphology, it did not result in parallel changes in the classical osteoclast markers TRAP (**Figure 3**, panel **B**). In control cells, TRAP expression increased during differentiation, consistent with the acquisition of an osteoclast-like phenotype, whereas after treatment with ritanserin or Bay-2965501 it remained broadly comparable to control conditions, showing only a slight and non-significant decrease in osteoclast-like cells. Similarly, CTSK expression did not show significant changes under the applied experimental conditions. This represents a limitation of the model, since a more pronounced osteoclast-like differentiation would be expected to associate with a clearer induction of these markers. Taken together, these findings suggest that, in the present system, DGK inhibition mainly affects morphological and fusion-associated maturation rather than the induction of the osteoclast-like transcriptional program. Moreover, these data indicate that DGKα and DGKζ both contribute to osteoclast-like maturation, likely through partially distinct mechanisms.

Although ritanserin did not significantly reduce multinucleation, it still impaired morphological maturation by reducing cell spreading and diameter, suggesting that DGKα may regulate the cellular architecture required for efficient osteoclast-like development. This is consistent with the study by Manigat et al. (2021), which showed that loss of DGKα enhances macrophage responsiveness to LPS, IL-4, and MCP-1 in bone marrow-derived macrophages and in the J774 macrophage cell line, and increases macrophage accumulation in vivo (Manigat et al., 2021). This study does not directly demonstrate altered osteoclast differentiation, but it supports the idea that DGKα regulates macrophage activation, migration, and responsiveness, all of which are relevant to the myeloid precursor context from which osteoclasts arise.

The cytokine expression profile in **Figure 4** further supports the idea that DGK inhibition did not broadly reprogram the inflammatory state of differentiating THP-1 cells. Along the differentiation, IL-1β and IL-6 increased in later stages, TNF-α decreased, and IL-10 showed a trend toward increase, indicating that the transition from THP-1 cells to macrophage-like and

osteoclast-like cells was associated with a dynamic remodeling of inflammatory gene expression. However, inhibition of DGKα or DGKζ did not induce significant or consistent changes among the investigated cytokines. This suggests again that, in this model, DGKα and DGKζ inhibition mainly affects morphological aspects of osteoclast-like differentiation rather than basal cytokine transcription. This finding is particularly important in inflammatory bone diseases, since osteoclasts play a central role in bone resorption, where inflammatory mediators such as IL-1, TNF-α, IL-17, and RANKL contribute to pathological osteoclastogenesis in diseases such as rheumatoid arthritis. However, the present data do not show a strong direct effect of DGKα or DGKζ inhibition on the basal transcription of inflammatory cytokines in THP-1-derived osteoclast-like cells.

To place these findings in a broader pathological context, transcriptomic data retrieved from public disease databases were analyzed, with the assistance of Dr. Teresa Gravina, to compare the expression levels of ten DGK isoforms in arthritic samples and healthy controls. The resulting alterations across **Figures 5, 6, and 7** provide additional biological context for these findings. First, an interesting pattern emerged for DGKD, DGKG, and DGKQ, which were significantly upregulated in osteoarthritis (OA), rheumatoid arthritis (RA), and knee osteoarthritis (kOA), whereas DGKH was consistently downregulated across all three conditions. This could suggest that DGK dysregulation is not random but part of a shared joint disease program. DGKA is significantly increased in OA and RA, but not in kOA, and a similar situation is observed for DGKZ, which is upregulated in OA and RA but not significantly altered in the kOA cohort. In contrast, DGKE is significantly reduced only in the general OA cohort, suggesting a more disease-specific modulation. DGKI instead does not significantly change in OA, but is significantly decreased in RA and knee OA, whereas DGKK is significantly increased in OA and kOA but not in RA. Finally, DGKB does not show significant differences in any of the analyzed conditions. An important point to consider is that this DGK pattern is compatible with chronic immune signaling rather than simple passive tissue damage. Particularly, DGKα and DGKζ are indeed established regulators of DAG-dependent pathways in T-cell, including RasGRP1, PKCθ, and mTOR, so their upregulation can reasonably be viewed as part of a compensatory or maladaptive feedback response to persistent immune activation (Chen et al., 2016). Overall, these transcriptomic findings indicate that DGK family alterations are not uniform, but instead consist of a common disease-associated pattern, alongside isoform-specific changes that may reflect differences in

inflammatory burden, joint site, or tissue remodeling programs.

Isoform	OA	RA	Knee OA	Summary
DGKA	Increased (significant)	Increased (significant)	No significant change	Up in OA and RA only
DGKB	No significant change	No significant change	No significant change	Stable across conditions
DGKD	Increased (significant)	Increased (significant)	Increased (significant)	Consistently upregulated
DGKE	Decreased (significant)	No significant change	No significant change	OA-specific downregulation
DGKG	Increased (significant)	Increased (significant)	Increased (significant)	Consistently upregulated
DGKH	Decreased (significant)	Decreased (significant)	Decreased (significant)	Consistently downregulated
DGKI	No significant change	Decreased (significant)	Decreased (significant)	Down in RA and Knee OA
DGKK	Increased (significant)	No significant change	Increased (significant)	Up in OA and Knee OA
DGKQ	Increased (significant)	Increased (significant)	Increased (significant)	Consistently upregulated
DGKZ	Increased (significant)	Increased (significant)	No significant change	Up in OA and RA only

Fig. 8: **Summary of DGK isoform expression changes in OA, RA, and Knee OA**

In the light of literature, the phenotype observed after Bay-2965501 treatment appears to diverge from previous data on DGKζ genetic deficiency. In the study by Zamani et al. (2015), DGKζ$^{-/-}$ bone marrow macrophages generated more numerous, larger, more multinucleated, and highly resorptive osteoclasts in vitro, and DGKζ$^{-/-}$ mice showed increased osteoclast number in vivo together with an osteoporotic phenotype (Zamani et al., 2015). However, this discrepancy should not necessarily be considered contradictory, since Zamani et al. studied constitutive genetic deletion of DGKζ in primary murine bone marrow macrophages, whereas our work used acute pharmacological inhibition in PMA-treated THP-1 cells subsequently stimulated with M-CSF and RANKL. These two approaches are not biologically equivalent, because constitutive gene deletion may reshape the entire differentiation trajectory from the earliest precursor stages, whereas pharmacological inhibition during an in vitro differentiation protocol may preferentially affect selected steps, such as cytoskeletal remodeling, spreading, and cell-cell fusion.

Another relevant point is that THP-1 cells are an immortalized monocytic cell line and therefore do not fully reproduce primary osteoclastogenesis. However, the key issue here is not simply that THP-1 cells differ from primary precursors, but that the two models may emphasize different stages of the differentiation process. In our setting, Bay-2965501 may mainly affect late fusion-dependent and morphological maturation events, whereas constitutive DGKζ deficiency in primary murine precursors described by Zamani et al. may reshape the osteoclastogenic program from earlier stages.

Moreover, in our model osteoclast-like differentiation occurs in a simplified system without stromal cells, osteoblasts, endothelial cells, lymphocytes, or the broader inflammatory

microenvironment, whereas in vivo osteoclast number and behavior emerge from continuous crosstalk among multiple cell types. In vivo, the number and activity of osteoclasts may depend not only on intrinsic differentiation, but also on precursor recruitment, survival, inflammatory cues, osteoblast-derived signals, and cell-cell communication within the bone niche. For this reason, an increase in osteoclast number in DGKζ$^{-/-}$ mice does not necessarily mean that inhibition of DGKζ in THP-1 cultures must have the same results as in vivo.

A further point concerns the pharmacological nature of our approach. Inhibitor-based perturbation does not always fully replicate genetic deletion, because the outcome can be influenced by timing, concentration, inhibitor selectivity, and the cellular state at the time of treatment administration. The observed effects are compatible with DGKα and DGKζ involvement, but further validation using additional inhibitors or genetic silencing approaches would be necessary to confirm whether the morphological phenotype is fully target dependent. In addition, future analyses of fusion markers such as DC-STAMP, OC-STAMP, ATP6V0D2, and CD44, together with assays of DAG/PA levels, PKC activation, MAPK signaling, NFATc1 activation, actin ring formation, and bone resorption, would help define whether DGKα and DGKζ regulate distinct or overlapping mechanisms during osteoclast-like maturation.

To complement the pharmacological inhibition approach, CRISPR/Cas9-mediated knockout of DGKα and DGKζ was independently performed on THP-1 cells within the same research group, where the resulting phenotype closely mirrored the one observed with inhibitors. This comparison should however be interpreted with caution, since pharmacological inhibition mainly suppresses enzymatic activity, whereas genetic knockout removes not only the catalytic function but also potential scaffolding properties of the protein, which may affect additional signaling interactions beyond lipid kinase activity. Knockout studies revealed that neither approach compromised cell viability, confirming that the observed defects specifically reflect impaired osteoclast maturation rather than cytotoxicity. Also in these studies, a marked reduction in cell spreading and diameter compared to controls was observed, with knockout osteoclasts failing to reach the morphological complexity of control cells. Multinucleation was similarly impaired, particularly at the osteoclast stage, consistent with the defect observed upon DGKζ pharmacological inhibition. Interestingly, knockout cells displayed higher TRAP levels despite their morphological deficit**,** suggesting a partial uncoupling between transcriptional activation and structural maturation. Also in knockout studies, CTSK

expression was not significantly modulated in either experimental setting.

Taken together, our findings support a model in which DGKα and DGKζ both contribute to THP-1-derived osteoclast-like maturation, although through partially distinct mechanisms. DGKα appears to be more closely associated with cell spreading and morphological remodeling, whereas DGKζ appears more directly involved in multinucleation and late fusion-dependent maturation. Therefore, the apparent difference with the literature is better interpreted as evidence of context-dependent DGK biology rather than as a true contradiction. In primary murine cells, chronic absence of DGKζ may enhance osteoclastogenesis through rewiring of the M-CSF, DAG, and c-Fos axis, whereas in PMA-primed THP-1 cells, acute inhibition of DGKα or DGKζ reveals defects in the structural execution of osteoclast-like maturation, with DGKα contributing to cell expansion and morphology and DGKζ contributing more strongly to multinucleation.

BIBLIOGRAPHY

· Takegahara, N., Kim, H., Choi, Y. Unraveling the intricacies of osteoclast differentiation and maturation: insight into novel therapeutic strategies for bone-destructive diseases. Exp. Mol. Med. 2024;56:264-272.

· Zhu, S., Yan, M.Q., Masson, A., Chen, W., Li, Y. Cell signaling and transcriptional regulation of osteoclast lineage commitment, differentiation, bone resorption and diseases. Cell Discov. 2026;12:6.

· Yahara, Y., Nguyen, T., Ishikawa, K., Kamei, K., Alman, B.A. The origins and roles of osteoclasts in bone development, homeostasis and repair. Development. 2022;149:dev199908.

· Jacome-Galarza, C.E., Perçin, G.I., Muller, J.T., Mass, E., Lazarov, T., Eitler, J., Rauner, M., Yadav, V.K., Crozet, L., Bohm, M., et al. Developmental origin, functional maintenance and genetic rescue of osteoclasts. Nature. 2019;568:541-545.

· Halleen, J.M., Tiitinen, S.L., Ylipahkala, H., Fagerlund, K.M., Väänänen, H.K. Tartrate-resistant acid phosphatase 5b (TRACP 5b) as a marker of bone resorption. Clin. Lab. 2006;52:499-509.

· Brizuela, L., Buchet, R., Bougault, C., Mebarek, S. Cathepsin K inhibitors as potential drugs for the treatment of osteoarthritis. Int. J. Mol. Sci. 2025;26:2896.

· Lin, S., Wang, T., Zuo, C. Cathepsin K (CTSK) in inflammatory and immune-mediated diseases. Immunol. Invest. 2026;55:120-142.

· Hobolt-Pedersen, A.S., Delaissé, J.M., Søe, K. Osteoclast fusion is based on heterogeneity between fusion partners. Calcif. Tissue Int. 2014;95:73-82.

· Søe, K. Osteoclast fusion: physiological regulation of multinucleation through heterogeneity, potential implications for drug sensitivity. Int. J. Mol. Sci. 2020;21:7717.

· Nakamura, I., Pilkington, M.F., Lakkakorpi, P.T., Lipfert, L., Sims, S.M., Dixon, S.J., Rodan, G.A., Duong, L.T. Role of αvβ3 integrin in osteoclast migration and formation of the sealing zone. J. Cell Sci. 1999;112:3985-3993.

· McHugh, K.P., Hodivala-Dilke, K., Zheng, M., Namba, N., Lam, J., Novack, D.V., Feng, X., Ross, F.P., Hynes, R.O., Teitelbaum, S.L. Mice lacking β3 integrins are osteosclerotic because

of dysfunctional osteoclasts. J. Clin. Invest. 2000;105:433-440.

· Chen, W., Wang, Q., Tao, H., Lu, L., Zhou, J., Wang, Q., Huang, W., Yang, X. Subchondral osteoclasts and osteoarthritis: new insights and potential therapeutic avenues. Acta Biochim. Biophys. Sin. 2024;56:499-512.

· Park-Min, K.H. Mechanisms involved in normal and pathological osteoclastogenesis. Cell. Mol. Life Sci. 2018;75:2519-2528.

· Lomaga, M.A., Yeh, W.C., Sarosi, I., Duncan, G.S., Furlonger, C., Ho, A., Morony, S., Capparelli, C., Van, G., Kaufman, S., et al. TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. Genes Dev. 1999;13:1015-1024.

· Huang, H., Ryu, J., Ha, J., Chang, E.J., Kim, H.J., Kim, H.M., Kitamura, T., Lee, Z.H., Kim, H.H. Osteoclast differentiation requires TAK1 and MKK6 for NFATc1 induction and NF-κB transactivation by RANKL. Cell Death Differ. 2006;13:1879-1891.

· Lowe, C., Yoneda, T., Boyce, B.F., Chen, H., Mundy, G.R., Soriano, P. Osteopetrosis in Src-deficient mice is due to an autonomous defect of osteoclasts. Proc. Natl. Acad. Sci. U. S. A. 1993;90:4485-4489.

· Kim, J.M., Lin, C., Stavre, Z., Greenblatt, M.B., Shim, J.H. Osteoblast-osteoclast communication and bone homeostasis. Cells. 2020;9:2073.

· Kurzrock, R. Macrophage colony-stimulating factor. In: Kufe, D.W., Pollock, R.E., Weichselbaum, R.R., et al., editors. Holland-Frei Cancer Medicine. 6th ed. Hamilton (ON): BC Decker; 2003.

· Mun, S.H., Park, P.S.U., Park-Min, K.H. The M-CSF receptor in osteoclasts and beyond. Exp. Mol. Med. 2020;52:1239-1254.

· Manigat, L.C., Granade, M.E., Taori, S., Miller, C.A., Vass, L.R., Zhong, X.P., Harris, T.E., Purow, B.W. Loss of diacylglycerol kinase α enhances macrophage responsiveness. Front. Immunol. 2021;12:722469.

· Lim, P.S., Sutton, C.R., Rao, S. Protein kinase C in the immune system: from signalling to chromatin regulation. Immunology. 2015;146:508-522.

· Mahajan, S., Mellins, E.D., Faccio, R. Diacylglycerol kinase ζ regulates macrophage responses in juvenile arthritis and cytokine storm syndrome mouse models. J. Immunol.

2020;204:137-146.

· Zamani, A., Decker, C.E., Cremasco, V., Hughes, L.D., Novack, D.V., Faccio, R. Diacylglycerol kinase ζ (DGKζ) is a critical regulator of bone homeostasis via modulation of c-Fos levels in osteoclasts. J. Bone Miner. Res. 2015;30:1852-1863.

· Baldanzi, G. Inhibition of diacylglycerol kinases as a physiological way to promote diacylglycerol signaling. Adv. Biol. Regul. 2014;55:39-49.

· Yang, Z., Kim, S., Mahajan, S., Zamani, A., Faccio, R. Phospholipase Cγ1 (PLCγ1) controls osteoclast numbers via colony-stimulating factor 1 (CSF-1)-dependent diacylglycerol/β-catenin/cyclin D1 pathway. J. Biol. Chem. 2017;292:1178-1186.

· Cremasco, V., Decker, C.E., Stumpo, D.J., Blackshear, P.J., Nakayama, K.I., Nakayama, K., Lupu, T.S., Graham, D.B., Novack, D.V., Faccio, R. Protein kinase C-δ deficiency perturbs bone homeostasis by selective uncoupling of cathepsin K secretion and ruffled border formation in osteoclasts. J. Bone Miner. Res. 2012;27:2452-2463.

· Gravina, T., Boggio, C.M.T., Gorla, E., Racca, L., Polidoro, S., Centonze, S., Ferrante, D., Lunghi, M., Graziani, A., Corà, D., et al. Role of diacylglycerol kinases in acute myeloid leukemia. Biomedicines. 2023;11:1877.

· van Blitterswijk, W.J., Houssa, B. Properties and functions of diacylglycerol kinases. Cell Signal. 2000;12:595-605.

· Racca, L., Baldanzi, G., Massarotti, A. Modulators of diacylglycerol kinase activity: a review of advances and challenges. Med. Res. Rev. 2026;46:149-175.

· Kambayashi, T., Deshpande, D.A. The role of diacylglycerol kinases in allergic airway disease. Curr. Opin. Pharmacol. 2020;51:50-58.

· Sánchez-Cabezón, J.J., Ávila-Flores, A., Mérida, I. Harnessing lipid metabolism through diacylglycerol kinases: implications for immune modulation and cancer therapy. Adv. Biol. Regul. 2026;99:101129.

· Riese, M.J., Moon, E.K., Johnson, B.D., Albelda, S.M. Diacylglycerol kinases (DGKs): novel targets for improving T cell activity in cancer. Front. Cell Dev. Biol. 2016;4:108.

· Sato, M., Liu, K., Sasaki, S., Kunii, N., Sakai, H., Mizuno, H., Saga, H., Sakane, F. Evaluations of the selectivities of the diacylglycerol kinase inhibitors R59022 and R59949

among diacylglycerol kinase isozymes using a new non-radioactive assay method. Pharmacology. 2013;92:99-107.

· Liu, J., Ma, J., Zhang, Y., Li, J., Yang, Y., Yin, P., Wan, X., Ma, S., Shang, D. DGKs in lipid signaling and disease intervention: structural basis, pathological mechanisms, and emerging therapeutic strategies. Cell. Mol. Biol. Lett. 2025;30:129.

· Baldanzi, G. Immune checkpoint receptors signaling in T cells. Int. J. Mol. Sci. 2022;23:3529.

· Liu, K., Kunii, N., Sakuma, M., Yamaki, A., Mizuno, S., Sato, M., Sakai, H., Kado, S., Kumagai, K., Kojima, H., et al. A novel diacylglycerol kinase α-selective inhibitor, CU-3, induces cancer cell apoptosis and enhances immune response. J. Lipid Res. 2016;57:368-379.

· Granade, M.E., Manigat, L.C., Lemke, M.C., Purow, B.W., Harris, T.E. Identification of ritanserin analogs that display DGK isoform specificity. Biochem. Pharmacol. 2022;197:114908.

· Ruffo, E., Malacarne, V., Larsen, S.E., Das, R., Patrussi, L., Wülfing, C., Biskup, C., Kapnick, S.M., Verbist, K., Tedrick, P., et al. Inhibition of diacylglycerol kinase α restores restimulation-induced cell death and reduces immunopathology in XLP-1. Sci. Transl. Med. 2016;8:321ra7.

· Ikeda, O., Kikuchi, A., Tsuzuki, H., Watanabe, H., Okuyama, K., Seki, Y., Ujihara, S., Matsuda, T., Weng, J., Kawashima, T., et al. Enhanced antitumor immunity by ASP1570, a novel diacylglycerol kinase ζ inhibitor, offers a potential novel immunotherapy for treating cancer. Mol. Cancer Ther. 2025;24:884-895.

· Joshi, R.P., Koretzky, G.A. Diacylglycerol kinases: regulated controllers of T cell activation, function, and development. Int. J. Mol. Sci. 2013;14:6649-6673.

· Kweon, H., Kim, S.G., Choi, J.Y. Inhibition of foreign body giant cell formation by 4-hexylresorcinol through suppression of diacylglycerol kinase delta gene expression. Biomaterials. 2014;35:8576-8584.

· Tu-Sekine, B., Raben, D.M. Characterization of cellular DGK-θ. Adv. Enzyme Regul. 2010;50:81-94.

· Boroda, S., Niccum, M., Raje, V., Purow, B.W., Harris, T.E. Dual activities of ritanserin and

R59022 as DGKα inhibitors and serotonin receptor antagonists. Biochem. Pharmacol. 2017;123:29-39.

· Gorla, E., Cartella, M.C., Borghetti, E., Lovati, G., Racca, L., Gravina, T., Biazzo, G., Bonello, G., Malacarne, V., De Giorgis, V., et al. Acute myeloid leukemia: a key role of DGKα and DGKζ in cell viability. Cells. 2025;14:1721.

· Cohen-Karlik, E., Awida, Z., Bergman, A., Eshed, S., Nestor, O., Kadashev, M., Ben Yosef, S., Saed, H., Mansour, Y., Globerson, A., et al. Quantification of osteoclasts in culture, powered by machine learning. Front. Cell Dev. Biol. 2021;9:674710.

· Chen, S.S., Hu, Z., Zhong, X.P. Diacylglycerol kinases in T cell tolerance and effector function. Front. Cell Dev. Biol. 2016;4:130.