A thesis Submitted to University of Hyderabad for the award of

Doctor of Philosophy (Ph.D.)
by

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University of Hyderabad
(A Central University established in 1974 by act of parliament)
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DECLARATION

I, **Durga Shankar Sharma**, hereby declare that this thesis entitled **"Cannabinoid Receptor 2 Signaling: Role in Megakaryocyte Development and Neuro-immune Regulation"** submitted by me is based on the results of the work done under the guidance and supervision of **Prof. G. Ravi Kumar** at Department of Biochemistry, School of Life Sciences, University of Hyderabad. The work presented in this thesis is original and plagiarism free. I also declare that no part or in full of this thesis has been submitted previously to this University or any other University or Institution for the award of any degree or diploma.

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CERTIFICATE

This is to certify that the thesis entitled "**Cannabinoid Receptor 2 Signaling: Role in Megakaryocyte Development and Neuro-immune Regulation"** submitted by **Durga Shankar Sharma** bearing Reg. No. **15LBPH13** in partial fulfillment of the requirements for the award of Doctor of Philosophy in (Biochemistry) in School of Life Sciences is a bonafide work carried out by him under my supervision and guidance.

The thesis has not been submitted previously in part or in full to this or any other University or Institution for the award of any degree or diploma.

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Part of this thesis have been

A. Published in the following publication:

1- **Sharma D.S.**, Raghuwanshi S, Narasaiah K, Dahariya S, Gautam DK, Paddibhatla I and Gutti RK. Virodhamine, an endocannabinoid induces megakaryocyte differentiation by regulating MAPK activity and function of mitochondria. Journal of Cellular Physiology. 2021 Feb;236(2):1445-1453. doi: 10.1002/jcp.29949.

2- **Sharma D.S.**, Paddibhatla I, Raghuwanshi S, Malleswarapu M, Sangeeth A, Kovuru N, Dahariya S, Gautam DK, Pallepati A, Gutti RK. Endocannabinoid system: Role in blood cell development, neuroimmune interactions and associated disorders. J Neuroimmunol. 2021 Jan 28;353:577501. doi: 10.1016/j.jneuroim.2021.577501.

B. Presented in the following conferences:

1. **Fire-talk Presentation: Durga Shankar Sharma** and Ravi Gutti. Virodhamine: A Cannabinoid Receptor Agonist Induces Megakaryocyte Maturation and Pro-platelet Production from In-vitro Megakaryoblastic Cells. Stem Cell Society Singapore (SCSS) Symposium 2020 jointly organized with Japanese Society of Regenerative Medicine (JSRM), Dec 9 - 11, 2020 (Virtual Meeting)

2. **Poster presentation (P-002): Durga Shankar Sharma** and Ravi Gutti. Virodhamine: A Cannabinoid Receptor Agonist to Enhance the Platelets Production from Megakaryocyte through Attenuation of Mitochondrial Function. Laboratory Medicine Congress & KSLM 60th Annual Meeting, Busan, Korea in Sept 2019.

3. **Poster presentation (P-509): Durga Shankar Sharma**, Usha Gutti and Ravi Kumar Gutti. Cannabinoid receptors and its role in Megakaryocyte development. International Congress of Cell Biology-2018 (The Dynamic Cell- From molecules and networks to form and function)

4. **Poster presentation (P-23): Durga Shankar Sharma** and Ravi Kumar Gutti. **P-23:** Cannabinoid receptors: Role in Megakaryocyte development. BioQuest 2017 – SLS, University of Hyderabad, Hyderabad, India.

Further, the student has passed the following courses towards the fulfilment of the coursework requirement for Ph. D.

Course code	Name	Credits	Pass/Fail
BC 801	Analytical techniques	4	Pass
BC 802	Research ethics, Data analysis, and Biostatistics	3	Pass
BC 803	Lab seminar and Records	5	Pass

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Durga Shankar Sharma.....

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Introduction

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Hematopoiesis

Hematopoiesis is a process by which all blood cells are formed. Hematopoiesis occurs during the embryonic development and throughout adulthood to maintain and replenish blood cells. Hematopoiesis in vertebrates is of two types: primitive hematopoiesis and definitive hematopoiesis (1). Primitive type involves the formation of erythrocyte from erythrocyte progenitor and macrophages during the early embryo development (2). Production of red blood cell is the primary purpose of primitive hematopoiesis which ultimately can facilitate tissue oxygen to developing embryo (3). Definitive hematopoiesis occurs later in the development. In most organism definitive hematopoiesis produces erythroid-myeloid progenitors (EMP). Definitive hematopoiesis through HSC involves in the formation of all type of blood cells in adult organism.

Hematopoiesis begins in yolk sac in humans. Blood cell production involves hierarchical progression of hematopoietic stem cell (HSC). HSCs is the developmental progression becomes primitive progenitor cell, committed precursor cell and lineage committed cells. This lineage committed cell ultimately matures and forms corresponding type of peripheral blood cells. There are multiple growth factors that are required for the proliferation and survival of HSCs at all stages of development. Important growth factors are Fms-like tyrosine kinase 3 (FLT3) ligand, Interleukin-2, Interleukin-3, Interleukin-7 and granulocyte-macrophage colony-stimulating factor (GM-CSF). Each of these cytokines and growth factors support the growth, proliferation and survival of HSCs. Hematopoietic stem cells are responsible for production of matured RBCs, platelets, monocytes, neutrophils, eosinophils, basophils through common myeloid pathway. Common lymphoid cell is ultimately responsible for production of lymphocyte cells such as T-cell, B-cell and natural killer cell (NK cell) (Figure 1) (4).

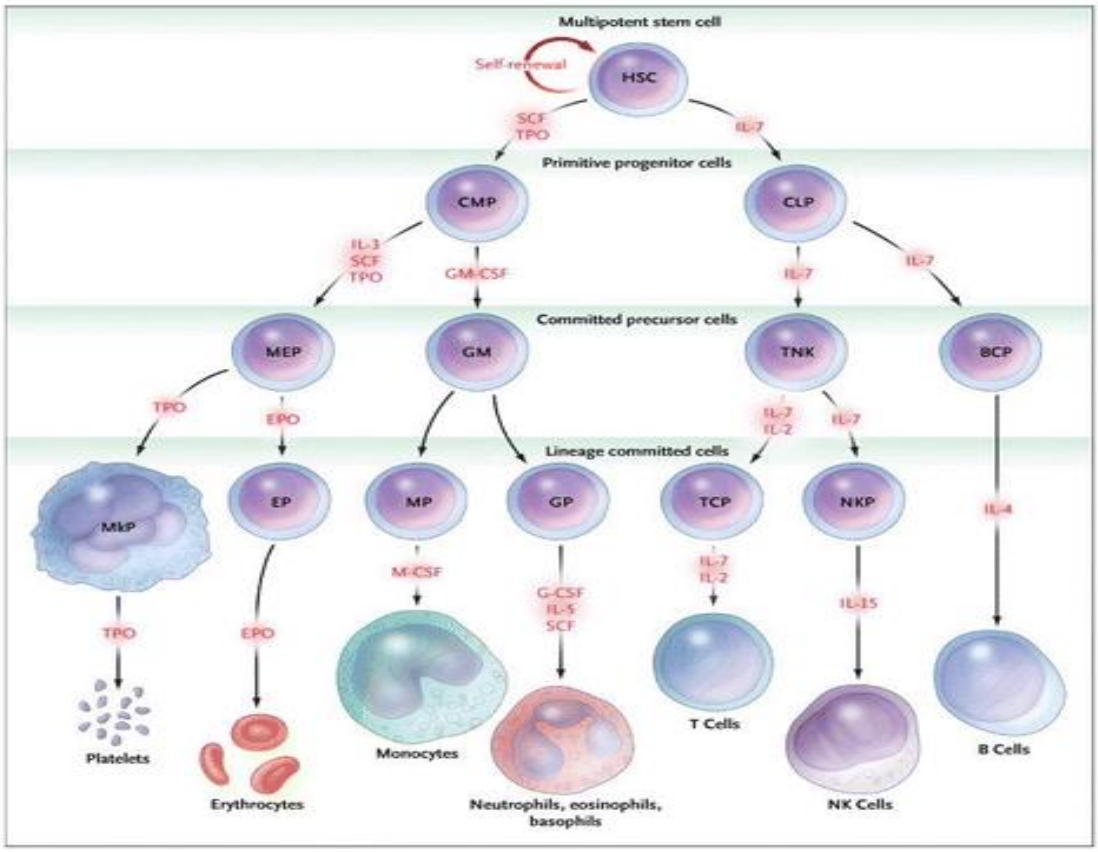


Figure 1. A General Model of Hematopoiesis. Blood-cell development initiates from a hematopoietic stem cell (HSC), which can proceed either self-renewal or differentiation into a multilineage committed progenitor cell: a common lymphoid progenitor (CLP) or a common myeloid progenitor (CMP). These cells then contribute to the production of more-differentiated progenitors, that include T cells and natural killer cells (TNKs), megakaryocytes and erythroid cells (MEPs) and granulocytes and macrophages (GMs). Ultimately, these cells produce unilineage committed progenitors for B cells (BCPs), T cells (TCPs), monocytes (MPs), granulocytes (GPs), NK cells (NKPs), erythrocytes (EPs), and megakaryocytes (MkPs). [Adapted from Kaushansky K. (2006). Lineage-specific hematopoietic growth factors. The New England journal of medicine, 354(19),2034–2045.]

Megakaryopoiesis

Megakaryocytes (MKs) are the largest and rare cell population in the bone marrow (1 in 10,000 cells). MKs are involved in platelet formation and play an important role in thrombosis, hemostasis, vascular biology, inflammation, and immunity (5, 6). MKs come in sizes ranging from 50 to 100μm. MKs produce platelets through a process known as endomitosis (7, 8). Further to provide thrombocyte MKs involves cytoplasmatic maturation and proplatelet formation (7, 8). By this method HSCs are able to provide mature MKs and platelets in

circulation which is termed as megakaryopoiesis (9). Mature MKs have specific characteristics such as big, multilobed, polyploid nucleus and cytoplasmatic protrusions known as proplatelets (10-20 per MK) (8). These cytoplasmic protrusions elongate and branch repeatedly to form proplatelets which are further released in the circulation (8). Maturation of MKs and thrombocyte formation happens in two niches. Maturation of MKs takes place within the osteoblastic niche whereas, thrombocyte release occurs within the vascular niche (10).

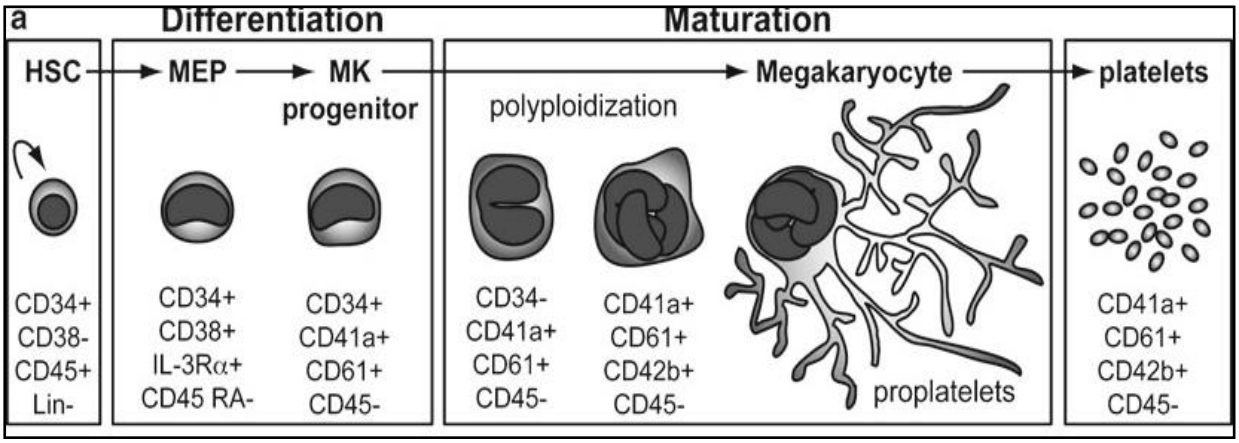


Figure-2 Overview of MK maturation and platelet production. [Adapted from Robert, A., Cortin, V., Garnier, A., & Pineault, N. (2012). Megakaryocyte and platelet production from human cord blood stem cells. Methods in molecular biology (Clifton, N.J.), 788, 219–247.**]**

The cytoplasmatic extensions are shredded by vascular pressure, and platelets are released in the circulation. The discharge of thrombocyte within the circulation is remarked as thrombopoiesis (11-16). Both megakaryopoiesis and thrombopoiesis are regulated by multiple proteins and growth factors. Thrombopoietin is a principal cytokine that regulates both megakaryopoiesis and thrombopoiesis (17) (Figure 2).

Platelets

Platelets are small enucleated cellular fragments of MKs. Size of the platelets ranges from 1-3μM in diameter. Life span of platelets ranges between 7-10 days (18, 19). Despite their smaller stature, platelets are critically important for carrying out essential functions in regulation of several biomolecular processes and for causing various disease conditions. The important

function of platelets is wound healing, hemostasis, thrombosis, immunity, angiogenesis, and inflammation (20-24). Primarily, platelets play an important role in thrombosis (binding to damaged blood vessels) to prevent excessive bleeding (25). Platelets are terminally differentiated cell and the precursor cell for differentiated platelet is MK. MKs through a unique process called endomitosis, give rise to terminally differentiated platelets (26-29). If there is any abnormality in MK differentiation and thrombopoietin signaling, it leads to the production of abnormal platelets in circulation (30, 31). In addition, platelet number associated disorder also can take place via defective thrombopoietin signaling or perturbed MK differentiation (32, 33). Normal range of platelet is 1,50,000 to 4,50,000 platelets/µl of blood. If platelet count is less than the normal count, the condition called thrombocytopenia. If the platelet count is more than the normal platelet count, the condition called thrombocythemia (34-36). Research work presented in this thesis is mainly focused on low platelet count condition called thrombocytopenia.

There are various treatment approaches to treat low platelet count condition such as blood or platelet transfusion, medication such as TPO mimetics (Romiplostim and Eltrombopag) and bone marrow transplantation. Frequent blood or platelet transfusions are difficult because the life span of platelet is very short. In addition, obtaining frequent blood or platelet donor is also difficult. Other treatment approach which includes medications such as TPO mimetics could have side effects if used for longer period. Among these bone marrow transplantation offers good treatment option because it can sustain for longer period (37-41).

Bone marrow transplantation

Bone marrow transplantation (BMT) involves the transplantation of healthy HSCs in patient with attenuated or depleted bone marrow. Imposition of healthy HSCs in patients, helps to augment proper bone marrow functions (42). Administered HSCs can both deplete the

attenuated bone marrow cells and generate active bone marrow cells in order to function properly. Also, it can destroy malignant or tumor cells in the bone marrow (43-46). Bone marrow transplantation are of three types. 1-Autologus bone marrow transplantation, 2-Allogenic transplantation, 3- Umbilical cord blood type of transplantation (44-46).

Autologous type of transplantation can be done using own cells. Patients' bone marrow products are collected, reinfused, purified and used for this type of transplantation. Allogenic type of transplantation can be done by sibling or human leukocyte antigens (HLA) matched donors. Umbilical cord blood type of transplantation can be done using stored cord blood of new born in blood bank (43-46).

After bone marrow transplantation, there are several reports that suggest the involvement of cannabinoids and cannabinoid receptors (CBRs). The circulating endocannabinoid levels has been found to be elevated during BMT. In addition, CBRs has been also found to be regulated during bone marrow transplantation. These circulating endocannabinoids have multiple function such as immunomodulation, regulation of central nervous system, cell differentiation and inflammation (47-49). Due to elevated level of circulating endocannabinoid during BMT, we were further interested to study the role of these endocannabinoids in MK biology.

Cannabinoids

Cannabinoids (CB) are derived from the *Cannabis sativa* plant that are utilized for therapeutic use (50). Cannabinoids can bind and activate CBRs. Recent reports have tested the biological significance of various cannabinoids. Cannabinoid compounds are broadly classified into 3 categories: 1-plant derived cannabinoids (also known as Phytocannabinoids), 2-endogenous cannabinoids (called endocannabinoids), and 3-synthetic cannabinoids (51-53). Phytocannabinoids are chemicals that are found within the genus *Cannabis* which have mind-altering outcome due to their binding to cannabinoid receptors and have clinically beneficial

effects. Example of phytocannabinoid include delta-9-tetrahydrocannabinol (Δ9-THC). Δ9-THC are considered as mind-altering phytocannabinoid compounds which are explored in clinical and biological investigations for their therapeutic potential (51-53). Endocannabinoids (ECs) are synthesized in our body and are endogenous ligands of CBRs. ECs are derived from fatty acids, amides, ethers and esters. Examples of ECs are 2-arachidonoylglycerol (2-AG) and anandamide (54, 55). ECs bind and stimulates G-protein coupled receptor (GPCRs), CB1R and CB2R signaling (56). Various studies have confirmed the regulatory functions of ECs in health and several disease states. These studies have proposed that modulating ECs activity may have therapeutic potential in various disease conditions. Clinical efficacy of cannabinoids was studied enormously in neurological diseases (57). Based on recent literature, cannabinoid ligands have also been considered as the crucial regulators of hematopoietic and immune system (58-61). Various researches have shown that cannabinoids and CBR could be clinically beneficial for various pathological conditions such as Huntington's disease (62), Parkinson's disease (63), Tourette's syndrome, epilepsy (64, 65), Alzheimer's disease (66) and inflammatory disorders (67). In recent years, there is an increasing interest in the medical use of cannabinoids for the treatment of various inflammatory (67), autoimmune (68), hematological disorders (69) and cancers (70). Various scientific committees that studied the therapeutic potential of cannabinoids and its derivatives are House of Lords in Great Britain, Senate Special Committee on Illegal Drugs in Canada and Institute of Medicine in the United States (71). This thesis work includes the investigation of the role of endocannabinoids in MK development.

Types of Cannabinoids

Phytocannabinoids

Phytocannabinoids are naturally occurring chemical compounds, these compounds are abundant in the viscous resin produced by the *Cannabis* plants. The examples of

phytocannabinoids are Δ9-tetrahydrocannabinol (Δ9-THC), cannabigerol (CBG), cannabidiol (CBD), cannabichromene (CBC), tetrahydrocannabivarin (THCV), and cannabinol (CBN) (41). Previous studies have shown that phytocannabinoids have differential binding affinities for CB1R and CB2R receptors (72). In addition to CB1R and CB2R, plant derived cannabinoids are also interacting with other types of receptors such as the serotonin or opioid receptor, G-protein coupled receptor 55 and G-protein coupled receptor 18 (73).

Endocannabinoids

"Endogenous substances, which are capable of binding to CBRs and functionally activating them, are referred to as Endocannabinoids **(**ECs)" (74). The ECs are a large group of fatty acids derived active compounds involved in diverse molecular activities in cell such as cell proliferation, cell differentiation and cell survival (75). Examples are 2-AG, virodhamine, anandamide and arachidonyol 20-chloroethylamide.

First endocannabinoid ligand was isolated from pig brain in 1992 and named as N-arachidonoyl-ethanolamine (AEA, also called anandamide) (76). After Few years another ligand called 2-AG was isolated from canine gut. To date, anandamide and 2-AG are the two most explored ECs, that have been studied in numerous biomolecular processes, such as cell differentiation, proliferation and maturation (74, 76, 77). ECs are synthesized in cells on its requirement. ECs ligands normally binds with either CB1R or CB2R. Following the conventional definition of ECs, several EC-like molecules e.g., DHEA and eicosapentaenoic acid analogs were also identified, but the affinity of these EC-like molecules was observed to be low with CB1R and CB2R. Thus, DHEA, eicosapentaenoic acid analogs and other endocannabinoid-like compounds, such as palmitoyl ethanolamide (PEA) and oleoyl ethanolamide (OEA), are not considered as ECs, but rather as amides (78-79). Recently, other receptors, such as TRPV1, GRP119, GRP18, and GRP55 are also recognized as the part of EC

system. Moreover, there is a growing interest to identify other potential EC ligands which can bind to CBRs or newly recognized EC receptors (TRPV1, GRP119, GRP18 and GRP55). The omega-3 fatty acid derived EC or EC like molecules such as docosahexaenoic acid (DHA) derived N-docosahex-aenoylethanolamide (DHA-EA or DHEA) and eicosapentaenoic acid (EPA) analogs have been recently discovered as the molecule of interest because of their unique bioactivity (80-81). In addition, several other fatty acid amide EC or ECs like molecule such as PEA and OEA are also considered as potential biomolecule that can regulate lipid metabolism and inflammation (82).

Biosynthesis of endocannabinoids

Biosynthesis and degradation of ECs is a tightly regulated process. A distinctive feature of ECs is that they are freshly synthesized upon requirement. This is attainable because of the presence of their precursors in cell membranes, these precursors are cleaved by specific enzymes upon requirement. Metabolism and pharmacology of AEA and 2-AG have been rigorously investigated, and these two cannabinoid compounds are still considered as "major ECs".

Several pathways are proposed to be involved in AEA biosynthesis from their corresponding N-acyl phosphatidyl ethanolamine (NAPE) precursor. The most considered pathway of AEA biosynthesis involve NAPE-phospholipase D (NAPE-PLD), which generates AEA from N-arachidonoyl PE (NArPE) precursor (83). Two alternative pathways are also considered to be involved in AEA biosynthesis, these pathways involve phospholipase-C (PLC)-PTPN22 (84) and αβ hydrolase (αβH4)-GDE1 (85). Although, the functional relevance of these pathways of cannabinoids biosynthesis is yet to be decided, the choice of pathway might depend on precursor's availability and/or the cell and the tissue type.

Three major pathways for 2-AG synthesis have been suggested, the most 2-AG comes from membrane phospholipids: One pathway imply the hydrolysis of precursor di-acyl glycerol

(DAG) via DAG-lipase (GAGL) (86, 87), in turn, the DAG biosynthesis occurs from PIP2 via phospholipase Cβ (PLCβ) (88). The second suggested pathway imply lipid precursor 2-Arachidonoyl-LPA, this precursor converted to 2-AG by the activity of lipid phosphatase (2-LPA phosphatase) (89) and the third pathway utilizes 2-Arachidonoyl-lysophosphatidylinositol (2- Arachidonoyl-LPI) precursor and the activity of lyso-PLC (90).

Biosynthesis of newly identified ECs, such as virodhamine, noladin ether, and N-arachidonoyl dopamine (NADA) is not entirely studied. The enzymes involved in the biogenesis of virodhamine could be phospholipase D which might catalyze fatty acid ethanolamine and arachidonic acid by transphosphotidylation. Earlier studies suggest that the biosynthesis of NADA from arachidonic acid and dopamine or tyrosine shares familiar pathways to those of either arachidonoyl amino acids or of dopamine (91).

Cannabinoid receptors

CBRs are of two types, CB1R and CB2R. To date, only these two CBRs have been discovered and cloned. CB1R and CB2R share 44% amino acid similarity between themselves (92-94). CB1R is highly conserved in vertebrates and also found in some invertebrates (95, 96). The mouse CB1R sequence shows 99% and 97% similarity at the amino acid level to rat and human respectively (97-99). CB2R shows lesser homology between the species as compared to CB1R (100). For instance, human and mouse CB2R shares 82% amino acid similarity (100). Abundant expression of CB1R has been known in the neurons such as amygdala, cortex, striatum, cerebellum, hippocampus, hypothalamus, brain stem, peripheral neurons, and spinal cord of both rodents and human (101). CB2R is principally localized in immune cells and organs, such as mononuclear cells, mast cells, thymus, natural killer cells, lymph nodes and spleen (102, 103). In mammals, CB1R is mostly expressed in central nervous system, whereas, CB2R is not only present in central nervous system but also present in peripheral tissue and

hematopoietic cells (95, 72, 93, 104, 105). Some GPCRs have also been considered to be CBRs; presently, only GPR18 and GPR55 have been demonstrated to be the targets of cannabinoid ligands (72).

Cannabinoid receptor signaling

"CBRs are G-protein coupled receptor (GPCR) and members of the superfamily of seven-transmembrane-spanning (7-TM) receptors". The signaling pathway is commenced by the binding of a cannabinoid ligand to CBRs, involves the coupling of the receptor to the inhibitory G proteins Gi and Go (Gi/o), that consequently inhibits adenylate cyclase (AC) and decrease cAMP levels (74, 104, 105). In other studies, CB1R has been exhibited as one of the activators of mitogen activated protein kinase (MAPK) signaling and phosphoinositide-3 kinase (PI3K)/ Protein kinase B (AKT) (77). CB1R is well-known to regulate MAPK and PI3/AKT signaling in cell type and ligand specific manner (106-109). Intracellular ceramide biosynthesis was also observed to be regulated by the interaction of CB1R with neutral sphingomyelinase (EMN) via interacting protein FAN (factor associated with neutral sphingomyelinase activation) (73). EMN mediates the biogenesis of ceramide from sphingomyelin (EM) in the plasma membrane (110). It is also involved in other cellular functions, such as the control of cell fate and cell survival (110, 59).

As noticed for CB1R, CB2R is also noticed to inhibit AC and decrease cAMP. CB2R have also been reported to activate MAPK pathway (111), specifically extracellular-signal-regulated kinase (ERK) and P38 MAPK cascade (111). Additionally, CB2R observed to activate PI3K/AKT pathway (112). In various studies, CBRs are discussed as the regulators of calcium and potassium channel (113).

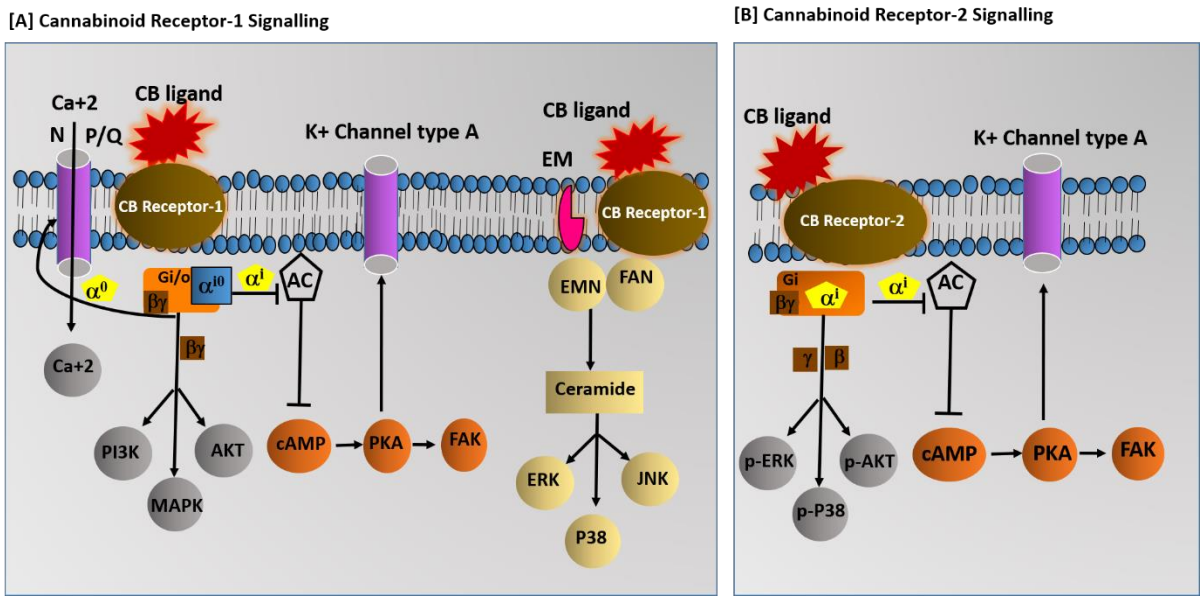


Figure 3. Schematic representation of the signaling pathways associated with cannabinoid receptors-1 and cannabinoid receptor-2. [A] Cannabinoid Receptor-1 signaling. [B] Cannabinoid Receptor-2 signaling. AC, adenylyl cyclase; FAN, factor associated with neutral sphingomyelinase activation; voltage-dependent calcium channels type N, P/Q; PKA, protein kinase A; PKB/Akt, protein kinase B; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; FAK, focal adhesion kinase; PI3K, phosphoinositide-3 kinase.

It has also been observed that various endogenous ligand (ECs) can stimulate transient receptor potential (TRP) ion channels in cell type- and tissue context-dependent manner (114-116). For instance, it was observed that cannabinoid ligands regulate ROS level and target TRPV1 by stimulation of CB2R (117). Additionally, TRPV1 activation may be driven by Ca2+ signaling which is coupled to mitochondrial ROS production (118-120).

Recent article by Paula Morales et al. (2017) elucidates CB1R and CB2R independent mechanisms. There are orphan-GPCR which can induce allosteric binding with cannabinoids called biased signaling (72). These GPCR particularly activates other signaling cascades independent of typical CB1R/CB2R signaling. This response is termed as biased agonism and activates different physiological response (121, 122). Altogether, these development in understanding the EC/CBRs signaling pathways discovered that there are several signaling

pathways which could be stimulated by different ECs interaction with CBRs or orphan-GPCR (Figure 3).

The functional role of endocannabinoids in MK and platelet biology is not well explored. The present study indicates that virodhamine is a positive regulator of megakaryocyte maturation. Our study has two objectives:

1. To study the role of Cannabinoid receptors during megakaryocyte development.

2. Elucidate the molecular mechanism by which Cannabinoid receptors participate in MK differentiation and maturation.

Materials and Methods

Materials and methods

Cell culture

For our studies, we have used DAMI cell line (megakaryoblastic cells, ATCC # CRL-9792). Megakaryoblastic DAMI cell line was derived from the blood of patient with megakaryoblastic leukemia. DAMI cells are suspension cells and shows characteristics of megakaryoblast. They exhibit many biochemical and morphological features of the megakaryocytic lineage. DAMI cells were cultured and grown in "Roswell Park Memorial Institute" (RPMI)-1640 medium (GIBCO), 10% FBS (fetal bovine serum), 1% antibiotic and antimycotic solution (Life Technologies, Inc.) and maintained in 5% CO2 incubator at 37 ^{o}C.

Quantitative analysis of mRNA by Real time-PCR

Total RNA was isolated from cells using the mRNeasy mini kit (Qiagen) following the manufacturers protocol. 1µg of RNA was then reverse transcribed by using ABM superscript first strand cDNA synthesis kit containing a homogenous mixture of oligo (dT). The first strand cDNA obtained by reverse transcription was used as a template for primer (Table 1) specific amplification using SYBR Green Master Mix (Kappa). The thermal cycling parameters were set as follows: an initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s and extension at 72 °C for 30 sec. The results presented are from three individual experiments, in which each sample was assayed in triplicate, normalized to the level of GAPDH, and expressed as relative expression. Relative quantification of genes was calculated by the comparative ΔΔCt method ($2^{\wedge(-\Delta\Delta Ct)}$) and represented as mean ± standard deviation (SD) of three independent experiments. For amplification the following list of primers is used.

Table:1 List of genes with primer sequence

Gene	PCR Primer Sequence (5'- 3')	
CB1 (CNR1)	F: 5'-TTCCCTCTTGTGAAGGCACT-3'	R: 5'-TCTTGACCGTGCTCTTGATG-3'
CB2 (CNR2)	F: 5'-CGTGGCTGTGCTCTATCTGA-3'	R: 5'-CACAGAGGCTGTGAAGGTCA-3'
CD41	F: 5'-GCATGGTTCAACGTGTCCTC-3'	R: 5'-TTGAAGAAGCCGACCTTCCA-3'
CD61	F: 5'-ACCAGTAACCTGCGGATTGG-3'	R: 5'-TCCGTGACACACTCTGCTTC-3'
CD42b	F: 5'-TTCCCCACCAAAGCCCATAC-3'	R: 5'-GGCTTGGGGTTGGTTCAGTA-3'
GATA-1	F: 5'-TGGAGACTTTGAAGACAGAGCGGCTGAG-3'	R: 5'-GAAGCTTGGGAGAGGAATAGGCTGCTGA-3'
GATA2	F: 5'-GCGTCAAGTACCAGGTGTCA-3'	R: 5'-GGCCTTCTGAACAGGAACGA-3'
NFE-2	F: 5'-GTGGAACTGCTGATGGGATT-3'	R: 5'-CCAAACACTTGTTGCCATTG-3'
RUNX-1	F: 5'-AACCTCGAAGACATCGGCAG-3'	R: 5'-GGCTGAGGGTTAAAGGCAGT-3'
IL-6	F:5'-AAGAGGCACTGGCAGAAAA-3'	R: 5'-CAGGGGTGGTTATTGCATCT-3'
IL-10	F: 5'-TGGAGAGAGTGTGGGAACCT-3'	R: 5'-CCACCACCTTCCATGCTTTG-3'
IL-1β	F: 5'-TCCCTAGGAAAAGCTGGG-3'	R: 5'- CACTACCCTAAGGCAGGCAG-3'
TNF-α	F: 5'-CTTCTGAGGCATTTGGAAGC-3'	R: 5'- ACTGGGCGGTCATAGAACAG-3'
RAC1	F: 5'-CAGATTACGCCCCCTATCCT-3'	R: 5'-GGCAATCGGCTTGTCTTTG-3'
CDC42	F: 5'-TGAAGGCTGTCAAGTATGTGGA-3'	R: 5'-CCTTTTGGGTTGAGTTTCCG-3'
NOX4	F: 5'-CAGAAGGTTCCAAGCAGGAG-3'	R: 5'-GTTGAGGGCATTCACCAGAT-3'
TRPA1	F: 5'-ACAGAAGACAAGTCCTGCCG-3'	R: 5'-GAGGGCTGTAAGCGGTTCAT-3'
TRPM8	F: 5'-CCGCCTCAATATCCCCTTCC-3'	R: 5'-GCAATCTCTTTCAGAAGACCCT-3'
TRPV1	F: 5'-ACGGACAGAACACCACCATC-3'	R: 5'-GGCCCTTGTAGTAGCTGTCC-3'
TRPV2	F: 5'-ATGCTGACCGTTGGCACTAA-3'	R: 5'-TCTCGAGAGTTCGAGGGACA-3'
TRPC3	F: 5'-CAGCCAACACGTTATCAGCAG-3'	R: 5'-TGCTTGGCTCTTGTCTTCCA-3'
GAPDH	F: 5'-GGAAGGTGAAGGTCGGAGTC-3'	R: 5'-TGAGGTCAATGAAGGGGTCA-3'

Western Blots

Protein from cultured cells was extracted by using RIPA buffer (Sigma) with protease inhibitor cocktail (invitrogen). Proteins (50 μg) were separated on 12% SDS-PAGE and transferred to nitrocellulose membrane. Blots were blocked using 5% milk powder and incubated overnight with primary antibodies such as CB-2, p-ERK1/2, total ERK, ROCK-1, cdc42, Rac-1, Rho A, cytochrom-C, AIF, BIM, BID, c-IAP, Caspase-9 and PARP (Santa Cruz, Cell Signaling Technologies) and internal control such as GAPDH (Santa Cruz) and β-actin (Cell Signaling Technologies). Detection of the protein bound primary antibody was carried out with appropriate secondary antibodies conjugated with horseradish peroxidase (Santa Cruz) and developed with enhanced Super Signal Chemiluminescent Substrate, femtolucent PLUS-HRP (G-Bioscience). The intensity of protein band was densitometricaly determined by Versa Doc.

Immunofluorescence staining

Cells of different experimental conditions were collected and washed with ice cold (1X) PBS. Cells were fixed in 4% para formaldehyde for 15 minutes, permeabilized with 1% Triton X-100 and blocked with 2% BSA for 60 min. After blocking, cells were incubated for 30 minutes with FITC conjugated phalloidin dye and detected in fluorescence microscopy.

Bright field microscopy

To understand the morphology of the cells, cells were treated with virodhamine for 3 days and morphology of the cells were observed under bright field microscopy. The Cells were harvested on glass slides, dried and subsequently observed under microscope.

Giemsa staining

Giemsa staining was performed to study the nuclear morphology. The cells were harvested on glass slides, dried and stained with 5% May Grünwald-Giemsa (MGG) for 20 min, subsequently washed with distilled water and observed under microscope.

Cell cycle analysis

Cell cycle analysis was performed using Muse™ Cell Analyzer (Millipore). Cells of different experimental conditions were collected and washed with PBS. To calculate the DNA Index, the cells were harvested and stained (1×10^6 cells/mL) with reagents of Muse Cell Cycle Assay Kit (Merck Millipore, USA) according to the manufacturer's protocol. The assay kit comes with propidium iodide (PI) which is detected in the yellow detector. DNA content was analyzed by using a flow cytometer (Muse Cell Analyzer; Merck Millipore, Germany). A histogram plot of DNA content against cell numbers gives the classical DNA profile for a cell culture. The experiments were performed in triplicate and contained a minimum of 5000 events per run.

MAPK assay

MAPK assay was performed by flow cytometer using the Muse MAPK Activation Dual Detection Kit following the manufacturer's protocol. MAPK assay kit includes two directly conjugated antibodies—a phospho-specific anti-phospho-ERK1/2 (Thr202/Tyr204, Thr185/Tyr187)-Phycoerythrin and an anti-ERK1/2-PECy5 conjugated antibody—to measure total levels of ERK. This two-color kit is designed to measure MAPK phosphorylation relative to the total MAPK expression in any given cell population. By doing this, the levels of both the total and phosphorylated protein can be measured simultaneously in the same cell, resulting in a normalized and accurate measurement of MAPK activation after stimulation.

PI3K assay

PI3K assay was performed by flowcytometry using the Muse PI3K Activation Dual Detection Kit following the manufacturer's protocol. To measure total levels of PI3K, two-color kit is designed to measure PI3K phosphorylation relative to the total PI3K expression in any given cell population. By doing this, the levels of both the total and phosphorylated protein can be measured simultaneously in the same cell, resulting in a normalized and accurate measurement of PI3K activation after stimulation.

Oil red O staining

Oil red O stock was prepared by adding 0.5g oil red O in 100% isopropanol. Cells were treated with virodhamine for 72h and were collected and washed with 1X phosphate buffer saline (PBS). Cells were fixed by using 4% paraformaldehyde. After fixing the cells, the fixative was removed by a rinse with PBS, and after those cells were air-dried. Oil red O working solution (6 (oil red):4 (water)) is incubated with cells for 15 minutes and then cells were observed under a bright-field microscope.

Mitochondrial membrane potential

Mitochondrial membrane potential of cells from different experimental conditions was determined by using Muse mitopotential assay kit (Millipore) as per manufacturer's instructions. The assay utilizes the mitopotential dye a cationic lipophilic dye to detect the changes in mitochondrial membrane potential and 7-AAD as indicator of cell death. The cells were observed in muse cell analyser (Millipore).

RT^2 Profiler mitochondrial array

qRT-PCR analysis of mitochondria related genes was performed using RT^2 profiler PCR array Human Mitochondria (PAHS-087ZC-2; Qiagen) and run-on ABI StepOne Plus (Applied

Biosystem). Experiment was performed in triplicate and data analysis was done using Qiagen integrated software package with cycle threshold-based fold change calculation. Sample were assigned as control and group 1 (treated group). Ct value were normalized based on reference gene (ACTB, GAPDH, HPRT1 and RPLP0) provided by manufacturer. Relative quantification of genes was calculated by the comparative ΔΔCt method (2^(-ΔΔCt)) and represented as mean ± standard deviation (SD) of three independent experiments. P-values are calculated by Student's t-test of replicate 2_delta Ct values for each gene in control and treatment group.

Annexin-V assay

Apoptotic event in cells from different experimental conditions was determined by using Muse annexin-V assay kit (Millipore) as per manufacturer's instructions. The assay utilizes the annexin-V dye to detect the changes in phosphatidyl serine translocation to the outer surface of cell membrane as indicator of apoptosis. The cells were observed in muse cell analyzer (Millipore).

Caspase-3 activity assay

For the caspase activation assay 100µg of protein was taken from both control and virodhamine treated cells and volume was made up for 100µl with caspase buffer (20mM HEPES pH 7.4, 10% sucrose, 100mM NaCl, 10mM DTT, 0.1% CHAPS, 1mM EDTA) and 5mM caspase-3 substrate (N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-tri fluoromethylcoumarin) (Sigma) was added. Incubation was performed at 37 °C for 1 h. Samples were then read on multi-well fluorescence plate reader (Molecular Devices) at an excitation of 400 nm, emission wavelength of 450–490 nm and the results were plotted as fluorescence units.

Measurement of intracellular ROS

Intracellular reactive oxygen species (ROS) was determined by using 2', 7' –dichlorofluorescin diacetate (DCFDA). ROS levels were measured by incubating (1 x 10^6 cells) untreated control,

virodhamine treated and decylubiquinone (potent ROS inhibitor) cells with 10µM DCFDA for 40 min at 37 °C. Then after cells were washed three times with 1x PBS and transferred to microplate and intracellular ROS was detected by using fluorescence spectroscopy at ex/em 488 nm/525 nm. The experiments were performed in triplicate.

Intracellular calcium measurement

Intracellular calcium was measured by using Fluo-3 fluorescence probe. The assay was performed as reported elsewhere (Kao JP et al., 1989). The emitted fluorescence intensity of Fluo-3 was recorded on a fluorescence-spectrometer at 25°C (excitation 505 nm, emission 526 nm, slit width 5 nm). Further, Fluo-3/AM (3 µM) loaded cells were also imaged for monitoring the fluorescence intensity using a fluoresce microscope.

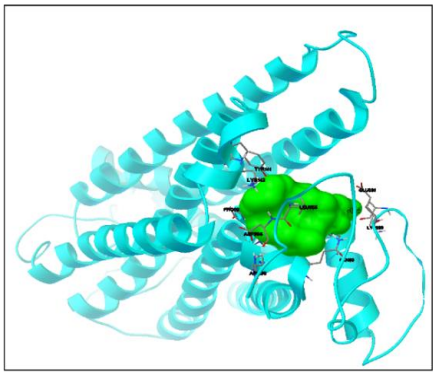
Objective-1

1. To study the role of CBRs during MK development.

Endocannabinoid system comprise of endocannabinoid ligand and CBRs. Endocannabinoids which can bind and activate called CBRs defined as endocannabinoids (74, 75). Here we have used three well known endocannabinoid (Anandamide, nodaline-ether, virodhamine) that are known to regulate several functions such as cell proliferation, differentiation and survival (74-77). These endocannabinoids bind to cannabinoid receptor -2 (CB2R) which are mainly present in immune cells and blood cells (102-105). We have performed virtual screening and docking study using auto dock vina to identify the best suitable endocannabinoid ligand binding to CB2R for our study. Auto dock vina is a tool used to determine the affinity of ligand with receptor.

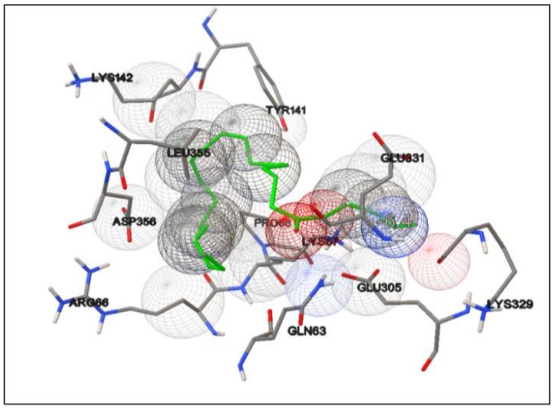
Ligand	Mode	Affinity	RMSD_LB	RMSD_UB	Random Seed
Anandamide	1	-5.9	0	0	-2.4E+08
Noladin_ether	1	-5.7	0	0	-1.7E+09
Virodhamine	1	-6.4	0	0	-8.3E+08

Ligand-Receptor binding site
CB2R sequence length- 340 aa
PDB ID-6KPC



Virodhamine – Auto Dock Vina

Ligand-Receptor binding site amino acid



Binding energy score -6.4

Figure 4. Auto dock vina results table shows the affinity of endocannabinoids (Anandamide, Nodaline-ether and Virodhamine) with cannabinoid receptor-2 and the figure depict cannabinoid receptor-2 and ligand (Virodhamine) receptor interaction with specific amino acids.

Auto-dock vina results suggests that virodhamine is having highest affinity with CB2R (-6.4) and could potentially bind to CB2R (Figure 4). We further used the endocannabinoid Virodhamine, a CB2R agonist, on human cells.

Cannabinoid receptor profiling

CBRs are of two types, cannabinoid receptor-1 (CB1R), and cannabinoid receptor-2 (CB2R). CB1R expression is seen in the central nervous system, whereas, CB2R expression is known in immune and blood cells (102-105). The functional role of CBR signaling in MK and platelet biology is not well explored. To study the expression of both CBRs in MKs, we used a megakaryoblastic Dami cell line model system and to activate CB2R signaling, we used EC Virodhamine, a CB2R agonist.

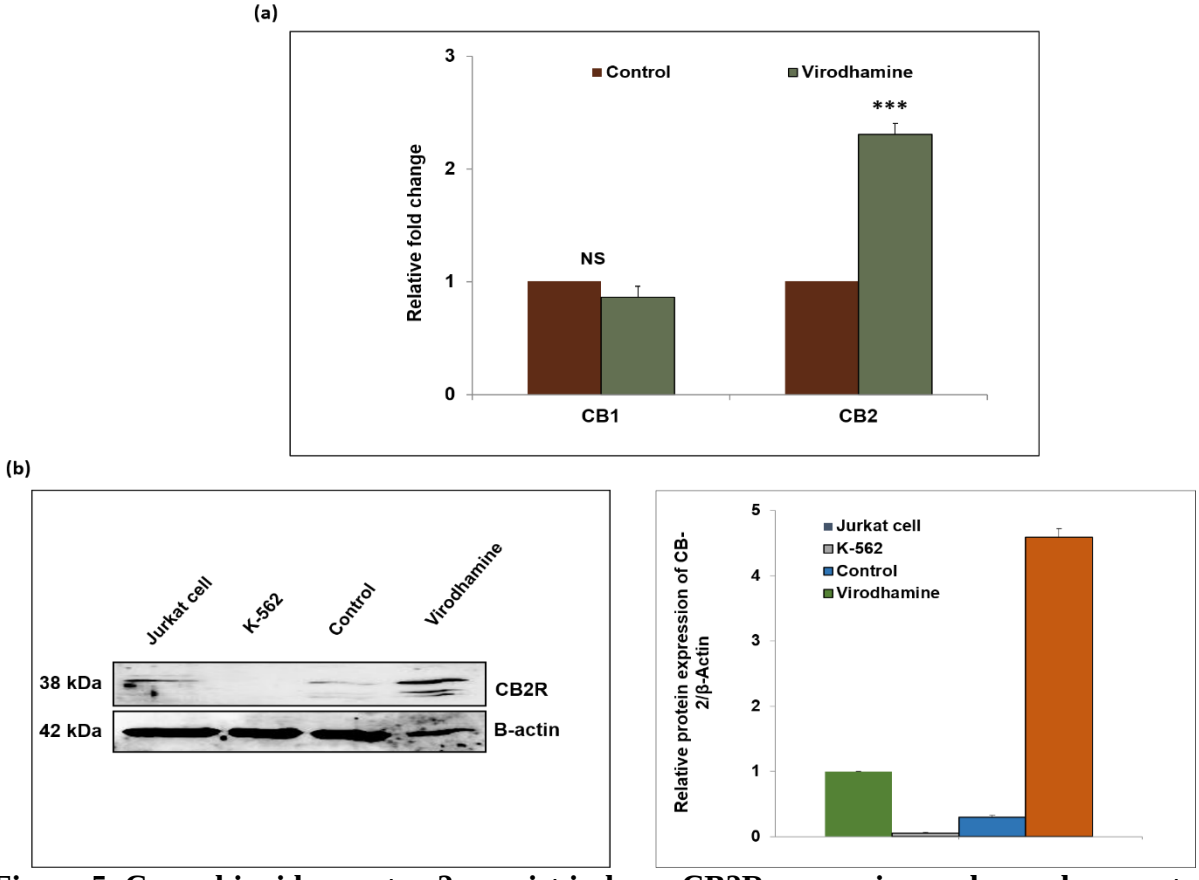


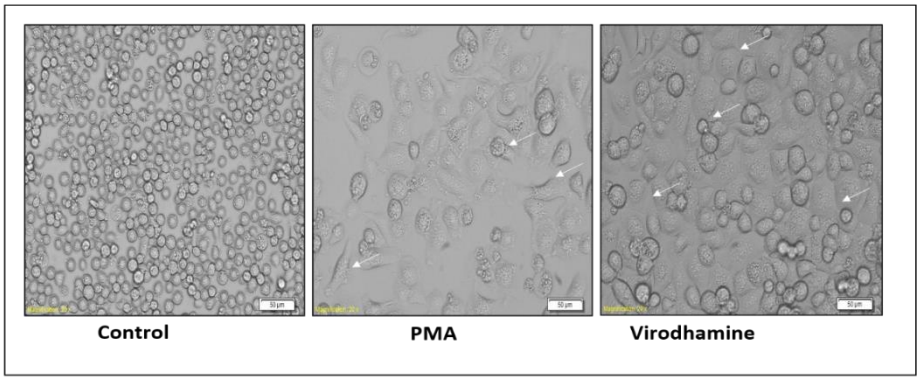
Figure 5. Cannabinoid receptor-2 agonist induces CB2R expression and megakaryocyte maturation (5a) qRT-PCR results show the expression of CB1 and CB2 upon virodhamine treatment as compared to untreated control cells (*n=3*; **p<.02). **(5b)** CB2R expression was analyzed by western blot analysis and relative CB2R protein expression is shown after normalization with loading control β-actin (*n=3*; **p<0.02).

Cells were treated with 20µM of CB2R agonist (Virodhamine) and total RNA was isolated from both untreated control and virodhamine treated cells. 1µg RNA was reverse transcribed and cDNA was prepared. Expressions of both CBRs were measured by qRT-PCR. CB2R expression was found to be higher in virodhamine treated cells compared to untreated control cells, whereas, CB1R expression was not increased in virodhamine treated cells as compared to control (Fig 5a). Similar results were observed in western blot analysis showing higher CB2R expression in virodhamine treated as compared to untreated control cells (Fig 5b). Jurkat cells and K-562 cells were used as positive and negative control respectively for CB2 antibody specificity. Altogether these results confirmed the expression of CB2R in virodhamine treated megakaryoblastic cells.

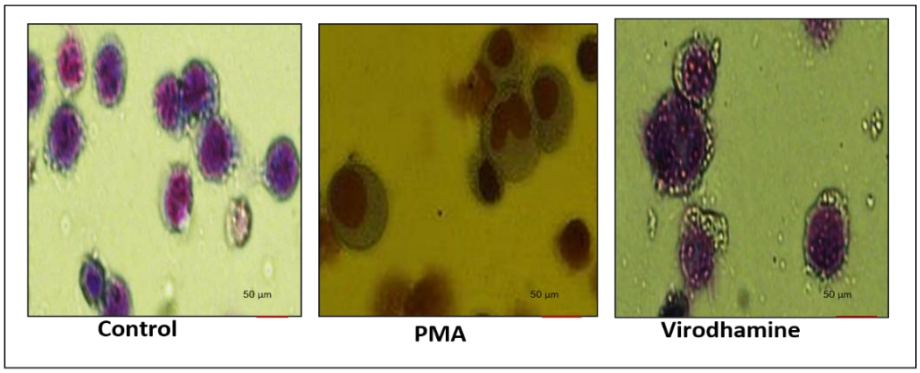
Effect of CB2R agonist in MK maturation

The characteristic features of MK maturation are increase in cell size, multilobed nucleus, adherence, cytoplasmic extension and polyploidy (123). To know the effect of CB2 agonist during MK maturation, Dami cells were treated with virodhamine and were observed in bright field microscope. Our results show an increase in cell size in virodhamine treated as compared to untreated control cells (Fig 6a). Also, increase in adherence and cytoplasmic extensions can be seen in virodhamine treated cells. Virodhamine treated cell shows multi-lobed nucleus, upon staining with Giemsa (Fig 6b). In addition, MK maturation-specific markers were analyzed by qRT-PCR. CD41, CD61 and CD42b are well-known MK maturation-specific markers. CD41 is an early-stage marker, whereas, CD61 and CD42b are late-stage MK maturation-specific markers. CD41, CD61 and CD42b expression were measured by qRT-PCR using specific primers. All the MK maturation markers were found to be increased in virodhamine treated as compared to untreated control cells (Fig 6d).

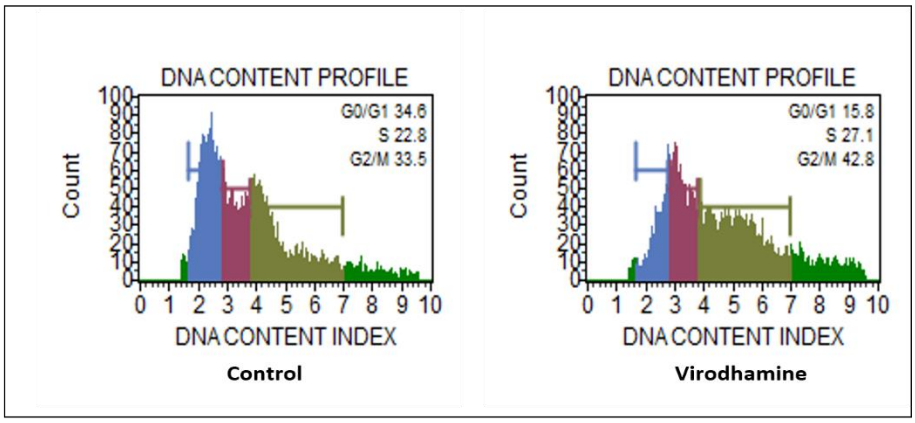
(a)


Control
PMA
Virodhamine

(b)


50 µm
Control
PMA
Virodhamine

(c)


DNA CONTENT PROFILE
G0/G1 34.6
S 22.8
G2/M 33.5
Count
DNA CONTENT INDEX
Control
DNA CONTENT PROFILE
G0/G1 15.8
S 27.1
G2/M 42.8
Count
DNA CONTENT INDEX
Virodhamine

(d)

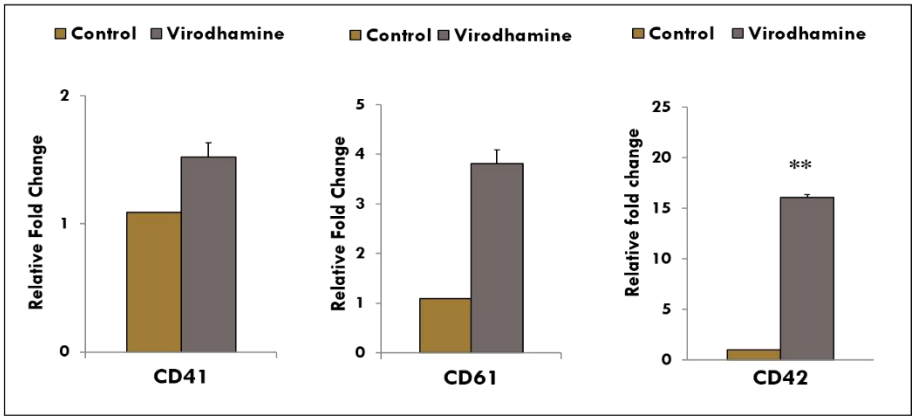

Control Virodhamine
Relative Fold Change
CD41
Control Virodhamine
Relative Fold Change
CD61
Control Virodhamine
Relative fold change
**
CD42

Figure 6. Cannabinoid receptor-2 agonist induce megakaryocyte maturation. (a) Representative image of cells showing megakaryocytic features such as increased cell size, adherence and cytoplasmic protrusions upon virodhamine induction as compared to untreated control cells (72 hrs). **(b)** Cells were stained with Giemsa showing large multilobed nucleus upon PMA or Virodhamine treatment (72 hrs). **(c)** DNA content index was increased due to polyploidy upon virodhamine treatment as compared to untreated control cells (*n=3*). **(d)** qRT-PCR results showing higher fold induction in the expression of CD41, CD61 and CD42b in virodhamine treatment compared to untreated control cells (*n=3*; **p<.02). Bars represent mean ± SD of three independent experiments.

DNA content was found to be induced in virodhamine treated cells which is a typical hallmark of MK maturation. Altogether these results show that virodhamine could induce MK maturation and polyploidy in Dami cells (Fig 6c).

Virodhamine induces MK specific transcription factors

MK maturation is characterized by induction of MK specific maturation markers (CD41, CD61, CD42b) (Fig. 6c), multilobed nucleus, cytoplasmic protrusions and membrane expansion (Fig. 6 a, b) (74). GATA1, GATA2, RUNX1 and NF-E2 are crucial transcription factors essential for MK differentiation (125, 126, 127 and 128). Previous report suggests that RUNX1 is critically important transcription factor that regulates MK differentiation in typical stages of MK development such as polyploidization and cytoskeletal rearrangement. Mutation in RUNX1 gene leads to platelet disorders such as thrombocytopenia [129]. In addition, GATA1 and GATA2 are pivotal transcription factors that regulate hematopoietic lineage and involved in differentiation of MK and erythrocytes [130, 131]. NF-E2, together with RUNX1 and GATA-1 is essential transcription factor involved in regulation of terminal differentiation of MKs [128, 132]. Altogether these studies represent the role of these transcription factors in normal human megakaryopoiesis. Mutation of these transcription factor result in impaired megakaryopoiesis leading to thrombocytopenia.

In present study we have treated the Dami cells (megakaryoblastic cell) with virodhamine (20μM) and analyzed the expression of GATA-1, RUNX1 and NF-E2 by qRT-PCR. Upon

virodhamine treatment, the expression level of these transcription factors was increased (n= 3; Figure 7). Our results are in line with previous findings indicating enhanced expression of these transcription factors during MK differentiation.

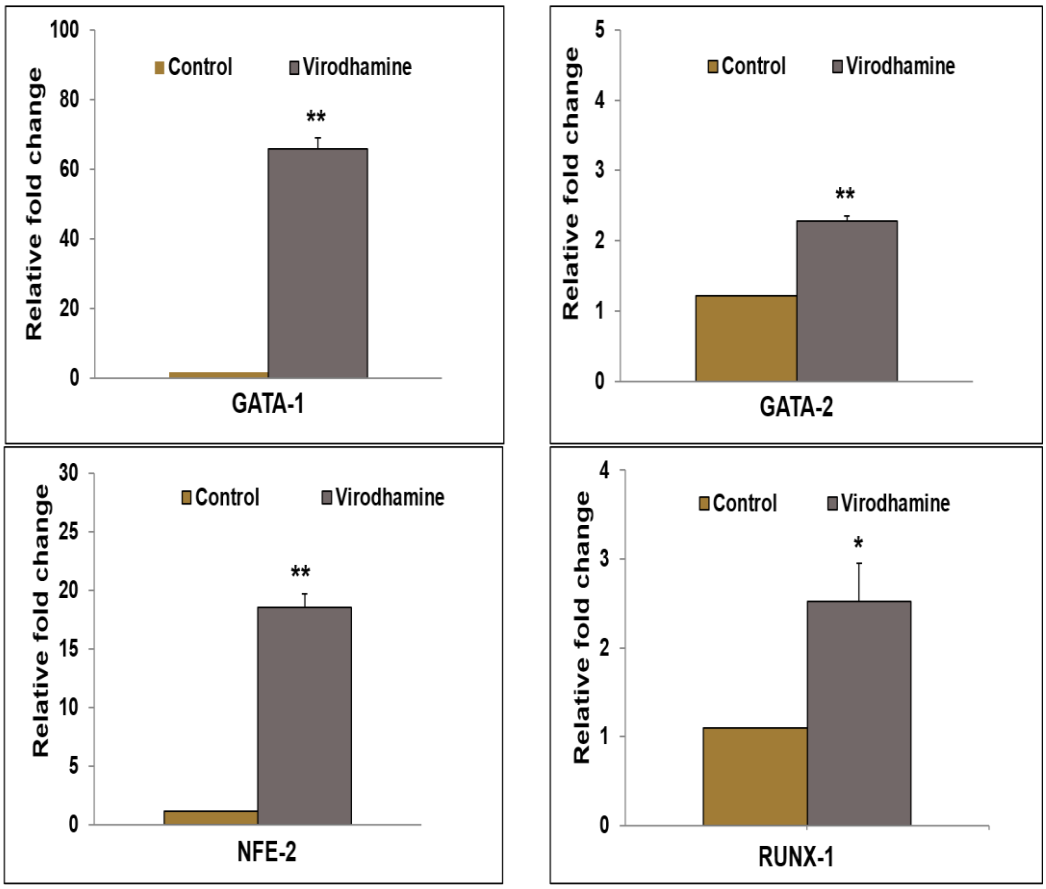


Figure 7. CB2R agonist induces megakaryocyte specific transcription factors (a) qRT-PCR result showing the expression of MK specific transcription factors such as GATA-1, GATA-2, RUNX-1 and NF-E2 (*n=3*; $p<0.05$).

Virodhamine regulates CB2R mediated cytokines

CBRs are known to have immunomodulatory role and are promising therapeutic targets for various disease conditions. CBRs are known to activate several cytokines to induce its immunomodulatory function. Recent report suggests that CB2R signals via G-protein coupled receptor and induces IL-6 and IL-10 cytokine in human primary leukocytes (133).

Interestingly, cytokines such as IL-6, IL-1β and TNFα regulate MK maturation and are critical for MK maturation and platelet production (134).

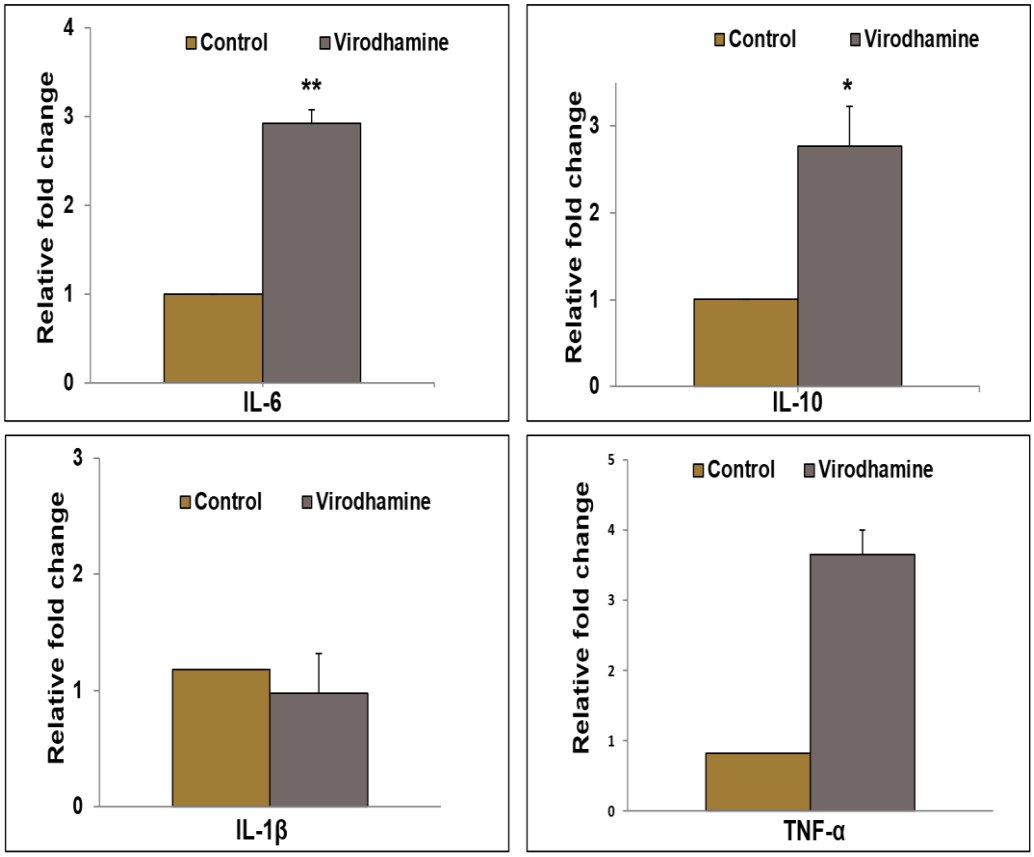


Figure 8. CB2R agonist induces megakaryocyte maturation specific cytokines. qRT-PCR result shows the expression of CB2R mediated cytokine expression which regulates MK maturation such as IL-6, IL-10, IL-1β and TNFα (*n=3*; $p<0.05$).

In order to understand the regulation and expression of cytokines, we have performed qRT-PCR analysis to know the expression of these cytokines in virodhamine induced MK cell. Cells were treated with virodhamine and total RNA was isolated followed by cDNA preparation from both untreated control and virodhamine treated cells. qRT-PCR was performed to know the expression these cytokines by using appropriate primers. IL-6 and IL-10 expression was found to be increased in virodhamine treated cells as compared to untreated control (*n= 3;* Figure 8). These results are in line with previous reports that suggest the regulation of cytokines during MK maturation and CB2R mediated cytokine activation.

Objective 2

Objective-2 Elucidate the molecular mechanism by which Cannabinoid receptors participate in MK differentiation and maturation.

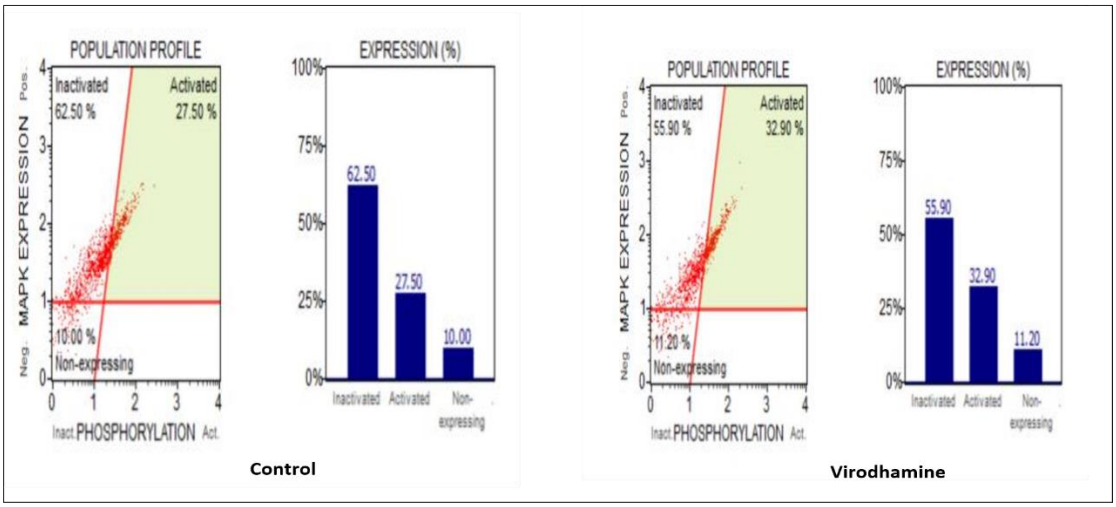
Our results suggest that CB2R agonist virodhamine could be a positive regulator of MK maturation. Further, we wish to elucidate the molecular mechanism by which CB2R agonist can participate in MK differentiation and maturation.

Various cytokines and growth factors promote megakaryopoiesis. Thrombopoietin (TPO) is the principal cytokine which is important for MK maturation (135, 136). TPO dependent signalling involves activation of mitogen activated protein kinase (MAPK) and phosphoinositol-3 kinase (PI3K) during MK maturation (137, 138). Activation of CBRs has been shown to induce megakaryocytic differentiation in Meg01 cells (139). However, the molecular regulation involved in this effect are not completely understood.

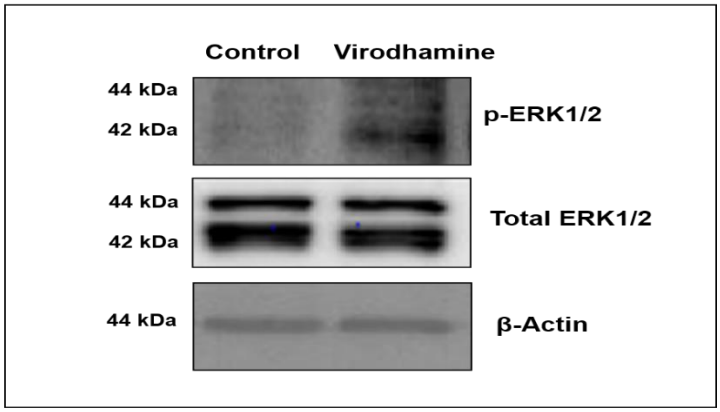
We investigated the activity of MAPK, by Muse analyser using The Muse® MAPK Activation Detection Kit (Millipore Inc.), after CB2 receptor stimulation by Virodhamine. The histogram showing reduced percentage of cells with inactivated MAPK; however, the percentage of cells with activated MAPK was increased upon stimulation by Virodhamine as compared to un-stimulated control (*n=3*; Figure 9a). CB2 receptor is a G protein-coupled receptor that is positively coupled to the mitogen-activated protein kinase (MAPK) (140). Moreover, several recent studies have implicated the activation of MAPK pathway in MK differentiation and endomitosis (141). Further, MAPK expression was measured by western blot analysis. Total protein was isolated from both untreated control and virodhamine treated cells. 100µg protein was loaded on 12% SDS-PAGE gel and was transferred to nitrocellulose membrane, western blot analysis was performed by using p-ERK1/2 and total ERK1/2 antibody. Western blot result suggests the induced expression of p-ERK1/2 compared to control cells, whereas, no change was observed in total ERK1/2 expression (*n=3*; Figure 9b). These results suggest there could

be activation of MAPK during CB2R agonist induced MK maturation. PI3K signaling is involved in proliferation and survival of MK, however, their role in terminal differentiation of MKs is not completely understood (142-144). We investigated the activity of PI3K by Muse analyser using PI3K activation detection kit (Millipore Inc.) after virodhamine treatment. Activated PI3K was observed to be reduced, whereas, inactivated PI3K was increased in virodhamine treated cells (*n=3*; Figure 9c). Typical TPO dependent MK differentiation was observed to stimulate both MAPK and PI3K signaling pathways. Present study reports that CB2R agonist reduces activated PI3K signaling during virodhamine induced MK differentiation.

(a)



(b)



(c)

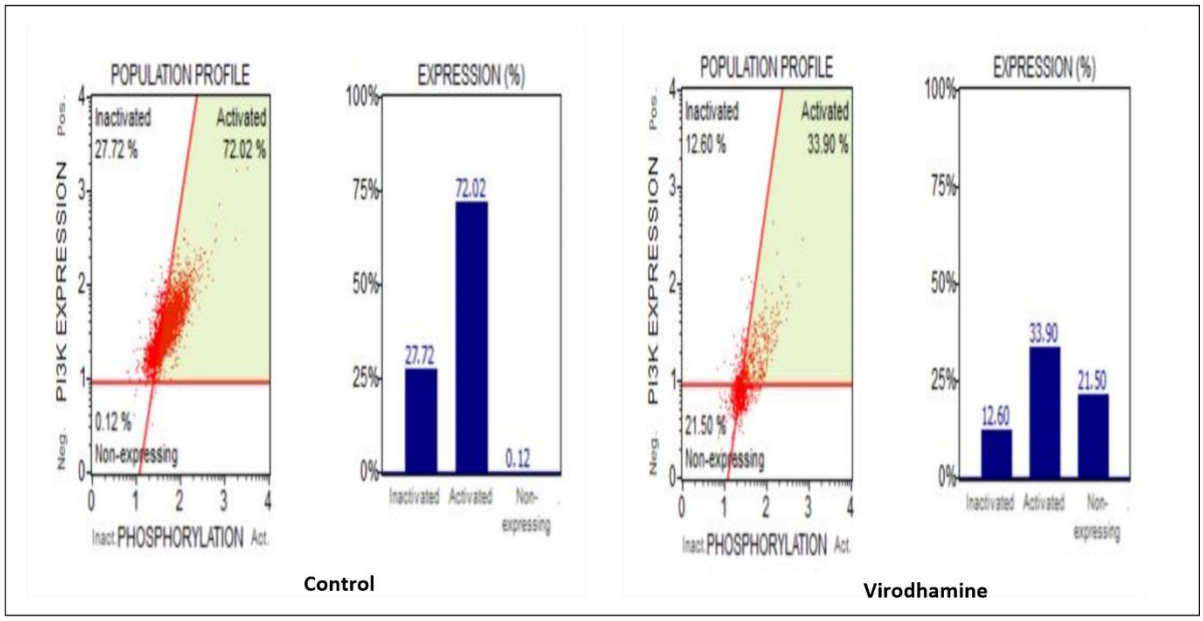


Figure 9. CB2R agonist induce MAPK activity and reduce PI3K activity (a) MAPK activation in virodhamine treated cells as compared to untreated cells as measured by Muse analyzer (*n=3*). **(b)** Western blot analysis showing total ERK1/2 and p-ERK1/2 protein expression (*n=3*). **(c)** Muse flow cytometry analysis showing PI3K inactivation in virodhamine treated compared to untreated control cells (*n=3*).

Virodhamine regulate Rho GTPase family member proteins

Recent evidences suggest the essential role of Rho GTPase family members in MK differentiation and platelet production. Cytoplasmic maturation and demarcation membrane system formation is an important feature of matured megakaryocyte which further forms proplatelet extensions to release platelets into the circulation (145, 146, 147). Rac1 (single) or Rac1/2 (double) deficiency mice study by McCarty OJ et al. (2005) reported normal platelet count but defective lamellipodia formation (148). Similar result was obtained by Pleines I. et al. (2013) which shows defective lamellipodia formation in Pf4-Cre deletion in Rac1 but normal ploidy and proplatelet formation (149). Cdc42 is critically important for proper formation of demarcation membrane system (DMS) during MK differentiation and a study by Pleines I. et al. (2010) shows that Pf4-Cre induced Cdc42 deficiency leads to macrothrombocytopenia in mice. Unlike Rac1 and Cdc42, RhoA and ROCK1 proteins were found to be a negative regulator of proplatelet formation (150, 151, 152).

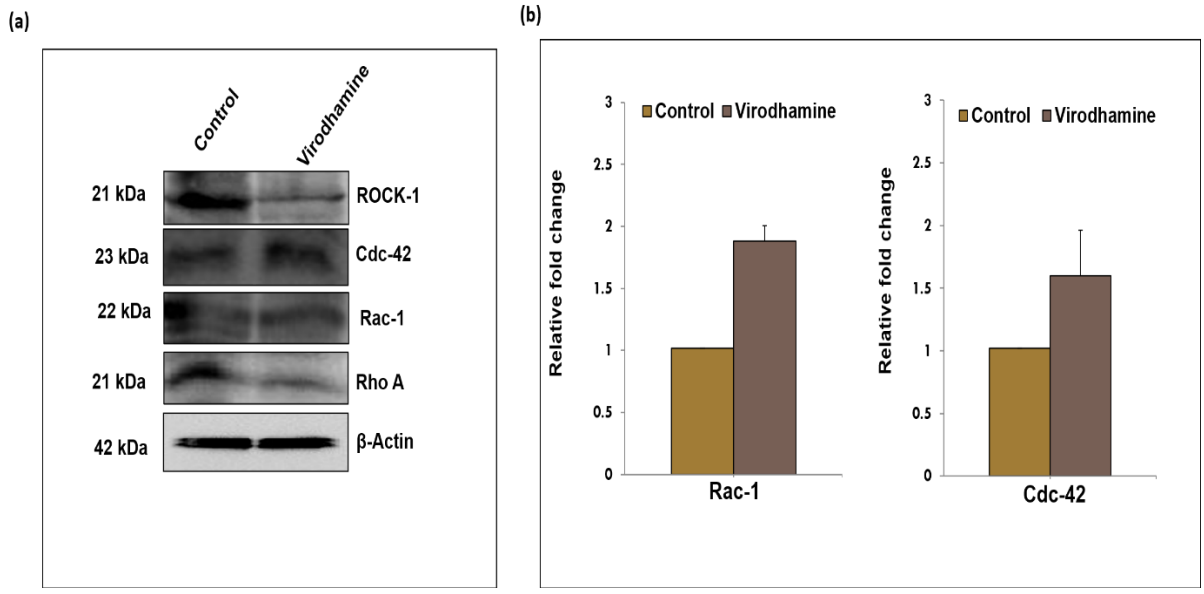


Figure 10. Virodhamine regulate Rho GTPase family member proteins (a) Representative image of western blot analysis showing Rac-1 and cdc42 was upregulation in virodhamine treated as compared to control cells. ROCK-1 and RhoA protein expression levels were reduced in virodhamine treated as compared to untreated control cells. **(b)** qRT-PCR results show the relative fold change in the expression of Rac1 and cdc42 mRNA (*n=3* $p<0.05$). Bar represents ± standard deviation of triplicate experiments.

Studies suggests negative regulation of RhoA expression during proplatelet formation in $CD34^{+}$ derived human MK cells (151, 152). Overexpression of RhoA leads to decrease the proplatelet formation in CD34+ derived MK cells. In line with RhoA negative regulation of proplatelet formation, downstream effector protein ROCK1 inhibition is also observed (151, 152). To study the expression profiles of all the proteins that are involved in cytoplasmic maturation of megakaryocyte development, Dami cells were treated with virodhamine and the expression levels of Rac1, Cdc42, RhoA and ROCK1 proteins was measured by western blot analysis. Our result indicates enhanced expression of Rac1 and Cdc42 in virodhamine treated cells as compared to untreated control cells, whereas, ROCK1 and RhoA expression was found to be reduced in virodhamine treated Dami cells (*n=3*; Figure 10a).

Further, Rac1 and Cdc42 mRNA expression was analysed by qRT-PCR. Enhanced expression of Rac1 and Cdc42 was observed in virodhamine treated as compared to control cells (*n=3*; Figure 10b).

CB2R agonist induces lipid uptake during MK maturation

Membrane expansion and cytoplasmic maturation is observed during MK maturation, which requires lipid transfer to encapsulate individual platelets and to allow MK to form proplatelet (153). To stain the fatty acid levels in virodhamine induced MK cells, we have performed oil red O staining. Dami cells were treated with virodhamine for 72 hour and these cells along with untreated controls were stained with oil red O which stains fatty acids. We observed higher uptake of oil red O by virodhamine treated as compared to control cells (*n=3*; Figure 11). Our result is in line with previous literature which indicates higher expression of fatty acid during MK maturation (153).

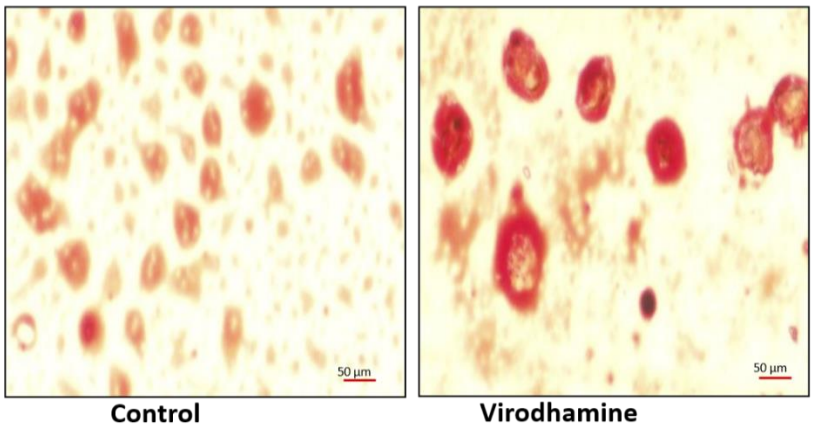


Figure 11. Oil red O staining in virodhamine treated and untreated control cells.

RT^2 profiler mitochondrial array indicates apoptotic pathway is differentially regulated in virodhamine treated MK cells

Various studies have suggested mitochondrial involvement in processes other than energy production, such as ROS generation, Ca^{2+} regulation and apoptosis regulation mechanisms.

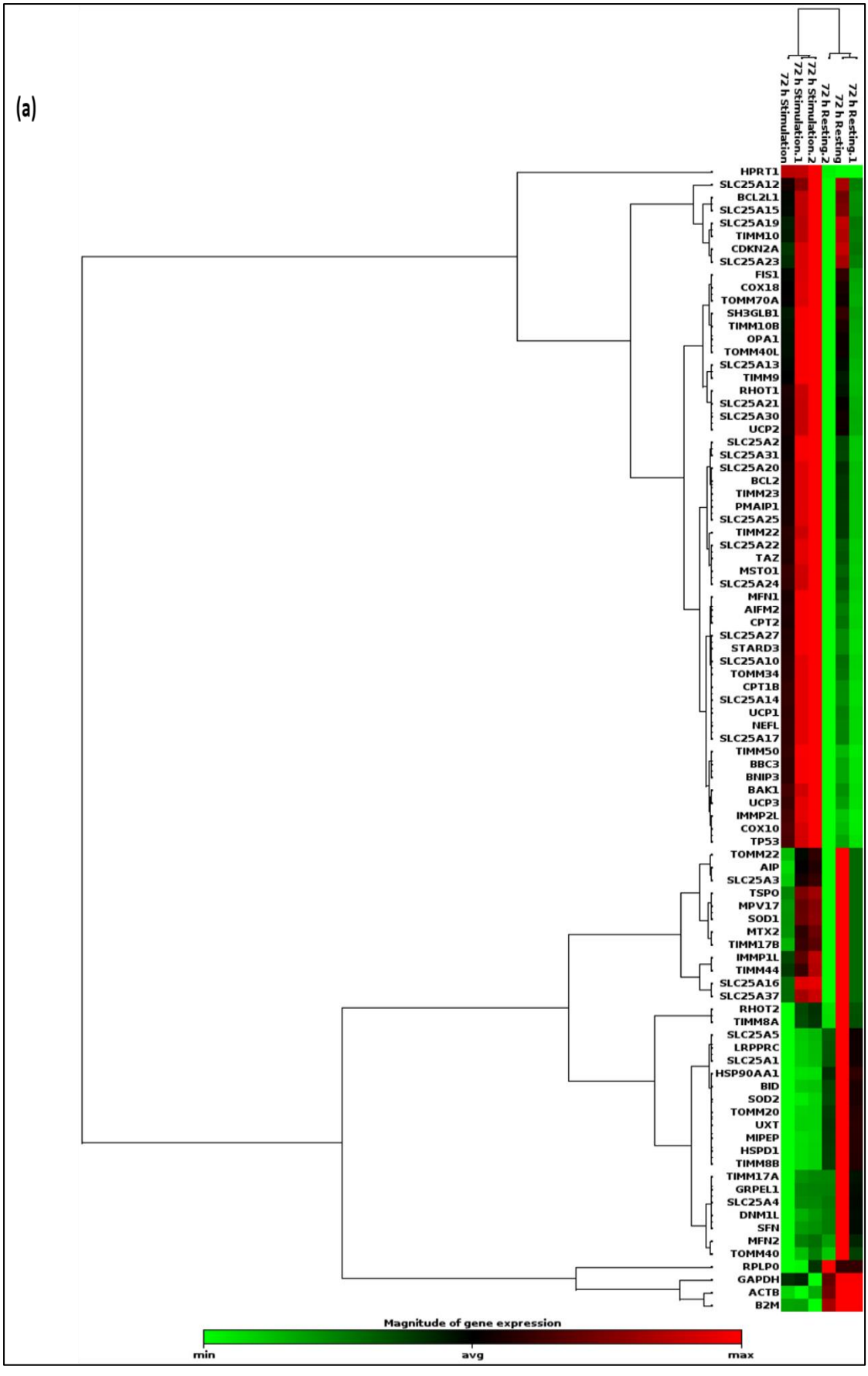
Not only megakaryocyte, but platelets also contain mitochondria. Mitochondria has been known to be involved in variety of processes including metabolism and ATP production, which may facilitate the process of megakaryocyte differentiation. In order to study the effects of virodhamine on mitochondrial regulatory pathways. We have treated the Dami cells with virodhamine which is known to induce MK differentiation. We observed increase in expression of MK specific maturation markers along with increase in cell size and adherence in virodhamine treated cells which are typical hallmark of MK differentiation (Fig. 6 a and d). We isolated total RNA and prepared cDNA. Further we performed RT^2 profiler PCR array Human Mitochondria (PAHS-087ZC-2; Qiagen) to compare virodhamine treated as compared to untreated control cells. This RT^2 profiler PCR array can be used to evaluate the expression of 84 mitochondrial pathway components. Our results showed several mitochondrial pathway components were significantly changed (> 2-fold) when we compared virodhamine treated cells (Group 1) and untreated control group upon normalization (Fig. 12a and b).. Our data analysis using Volcano plot is showing statistically significant differentially expressed genes (Fig. 12c).

Out of 84 genes analysed, which are involved in mitochondrial pathway components in virodhamine treated (Group 1) versus untreated control group, only 45 genes showed statistical significance ($p < 0.05$). Of these differentially regulated genes 33 genes were up-regulated and 12 genes were down-regulated (Fig. 12d). To understand the impact of virodhamine on various signalling pathways associated with mitochondria, KEGG pathway analysis was performed using significantly changed genes.

KEGG pathway analysis revealed that differentially regulated genes were associated with several pathways such as Huntington's disease, p53 signalling, Apoptosis, PPAR signalling and Sphingolipid signalling pathway (Fig. 13b). Among all the suggested pathways, Apoptosis was found to be appealing because in blood circulation platelet survival is regulated by a critical

Table-2: Representation of differentially regulated genes that are involved in various cellular processes such as apoptosis, redox pathway and membrane potential.

Gene Classification	Fold change	Function	Reference
Pro-apoptotic and anti-apoptotic genes			
AIFM2	3.19	AIFM2 gene has homology to NADH oxidoreductases (NOX) and the apoptosis-inducing factor AIF. Previous literature suggests that overexpression of AIFM2 can induce apoptosis.	(154)
PMAIP	2.33	PMAIP gene is known as Noxa. Noxa is a member of Bcl-2 protein family. Bcl-2 family members can act as pro-apoptotic regulators that are involved in various cellular activities.	(155)
BID	-0.35	BID is a member of the Bcl-2 protein family. Bcl-2 family proteins can act as anti- or pro-apoptotic regulators that are involved in various cellular activities.	(156)
BAK1	3.71	BAK1 is member of the Bcl-2 protein family. This protein is found in mitochondria, and functions to induce apoptosis.	(157)
BBC3	4.49	Bcl-2-binding component 3 is a member of Bcl-2 protein family. BBC3 is involves in p53-dependent and -independent apoptosis mechanism. BBC3 interacts with Bcl-2 family members and induces mitochondrial mediated apoptosis.	(158)
BNIP3	4.51	BNIP3 is a member of Bcl-2 protein family. BNIP3 regulates mitochondrial permeability, decrease mitochondrial membrane potential and increase's ROS.	(159)
Redox and antiapoptotic genes			
UCP1	3.24	UCP1 is a member of uncoupling protein family. UCP1 are mitochondrial transporter proteins that create proton leaks across the inner mitochondrial membrane, thus uncoupling oxidative phosphorylation.	(161)
SOD2	-3.06	SOD2 is a member of the iron/manganese superoxide dismutase family. The main function of SOD2 is to clear mitochondrial reactive oxygen species (ROS). SOD2 can play anti-apoptotic role against oxidative stress.	(160)
Membrane polarization and potential			
BAK1	3.71	BAK1 is member of the Bcl-2 protein family. This protein is found in mitochondria, and functions to induce apoptosis through mitochondrial depolarization.	(157)
BNIP3	4.51	BNIP3 is a member of Bcl-2 protein family. BNIP3 regulates mitochondrial permeability, decrease mitochondrial membrane potential and increase's ROS.	(159)
UCP1	3.24	UCP1 is a member of uncoupling protein family. UCP1 are mitochondrial transporter proteins that create proton leaks across the inner mitochondrial membrane, thus uncoupling oxidative phosphorylation.	(161)
UCP3	4.17	UCP1 is a member of uncoupling protein family. UCP3. UCP3 reduces mitochondrial membrane potential in mammalian cells.	(162)


(a)
72 h Stimulation
72 h Stimulation.1
72 h Stimulation.2
72 h Resting
72 h Resting.1
72 h Resting.2
HPRT1
SLC25A12
BCL2L1
SLC25A15
SLC25A19
TIMM10
CDKN2A
SLC25A23
FIS1
COX18
TOMM70A
SH3GLB1
TIMM10B
OPA1
TOMM40L
SLC25A13
TIMM9
RHOT1
SLC25A21
SLC25A30
UCP2
SLC25A2
SLC25A31
SLC25A20
BCL2
TIMM23
PMAIP1
SLC25A25
TIMM22
SLC25A22
TAZ
MSTO1
SLC25A24
MFN1
AIFM2
CPT2
SLC25A27
STARD3
SLC25A10
TOMM34
CPT1B
SLC25A14
UCP1
NEFL
SLC25A17
TIMM50
BBC3
BNIP3
BAK1
UCP3
IMMP2L
COX10
TP53
TOMM22
AIP
SLC25A3
TSPO
MPV17
SOD1
MTX2
TIMM17B
IMMP1L
TIMM44
SLC25A16
SLC25A37
RHOT2
TIMM8A
SLC25A5
LRPPRC
SLC25A1
HSP90AA1
BID
SOD2
TOMM20
UXT
MIPEP
HSPD1
TIMM8B
TIMM17A
GRPEL1
SLC25A4
DNM1L
SFN
MFN2
TOMM40
RPLP0
GAPDH
ACTB
B2M
Magnitude of gene expression
min
avg
max

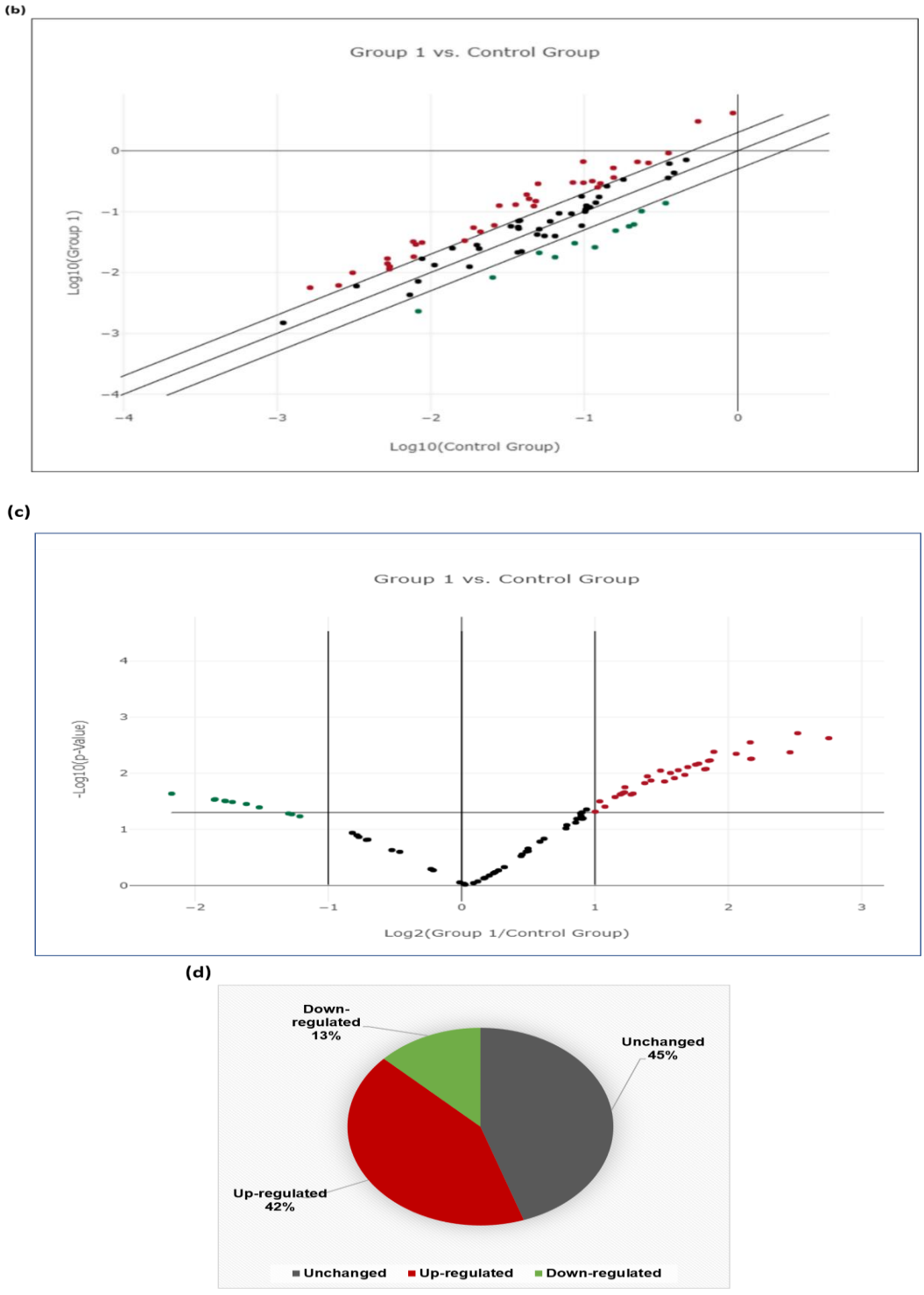


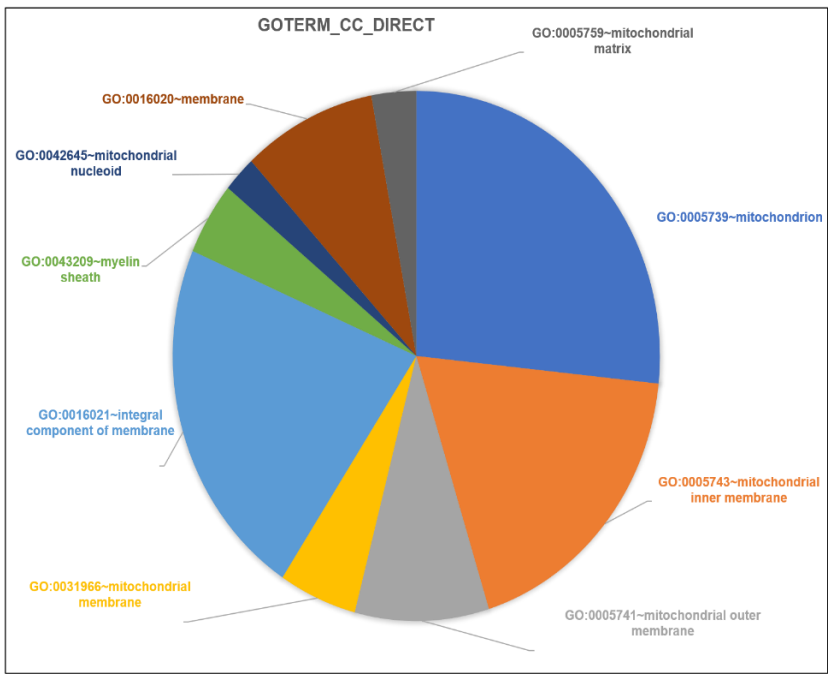
Figure 12. RT^2 profiler PCR Array. (a) cluster gram showing the multitude of expression of differentially regulated mitochondrial genes in control and virodhamine treatment **(b)** Visualization of fold change (log2) of genes using scatter plot (*n=3*). Red circle represents overexpressed genes and green circle represents down-regulated genes in group 1 (virodhamine treated cells) compared to control **(c)** Volcano plot showing fold change in x and y-axis (statistical significant, n = 3; $p < 0.05$). Red circle represents overexpressed genes and green

circle represents down-regulated genes. **(d)** Pie chart represents the percentage of up-regulated, down-regulated and unchanged genes in virodhamine induced Dami cells.

balance between pro-survival (Bcl-xL) and pro-death (Bak and Bax) signals. These pathways during the megakaryocyte maturation and development are not well defined. Studies in mouse showed that MK survival is regulated by Bcl-xL and Mcl-1. We observed majority of the differentially expressed genes are involved in the regulation of apoptosis (Pro-apoptotic and anti-apoptotic), redox pathway and mitochondrial membrane potential (Table 1).

Based on the significance of these pathways on the physiology, these were classified according to their subcellular localization by GO term-cellular component (GO-CC) classification of the DAVID database. Most of these components were localized in mitochondrial matrix, mitochondrial nucleoid, mitochondrial inner membrane, mitochondrial outer membrane, integral component of membrane and myelin sheath (Fig. 13a). Further, we investigated the involvement of mitochondria mediated apoptosis mechanisms in virodhamine treated as compared to untreated cells.

(a)



(b)

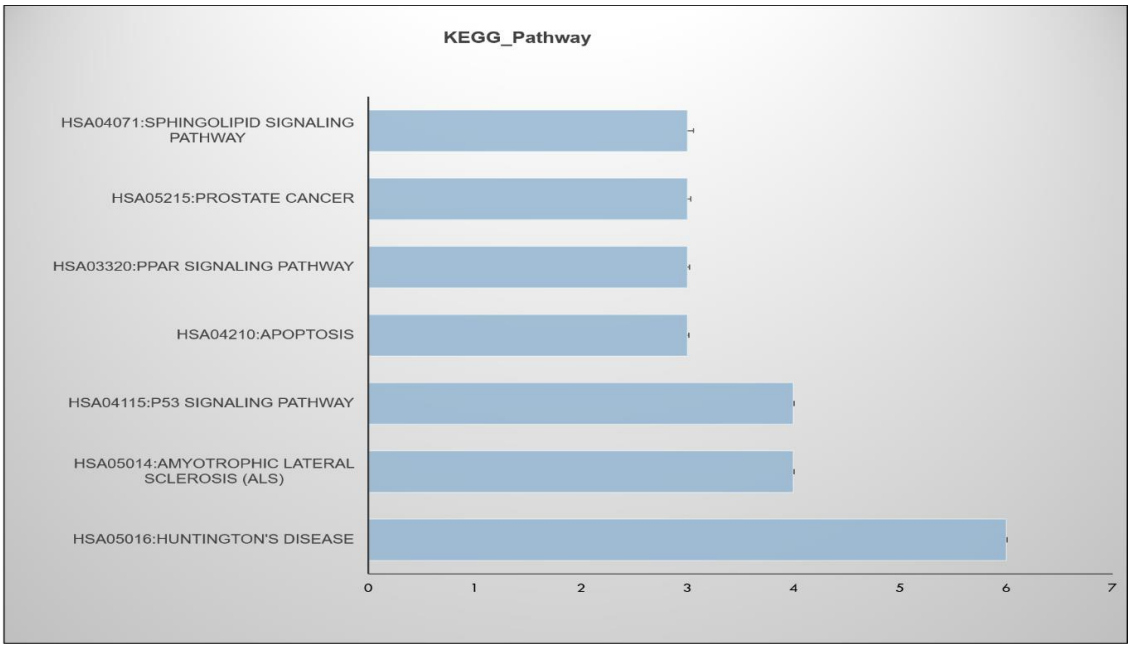


Figure 13. (a) Chart showing the protein localization of differentially regulated genes. Protein localization of differentially regulated genes was identified by GOTERM_CC_DIRECT (DAVID 6.8 Tool). **(b)** KEGG pathway analysis was performed to identify the significantly affected pathways by the differential regulated genes.

Virodhamine induce early apoptotic cells, caspase activity and alters mitochondrial membrane potential in MK cells

Megakaryocytes and platelets depend upon mitochondria mediated intrinsic apoptosis proteins for their viability and development. These proteins help in megakaryocyte clearance after proplatelet formation and shedding of platelets. In order to allow platelet production, pro-apoptosis (BAK/BAX-mediated) intrinsic pathway functions. The apoptosis mechanism in megakaryocytes can be activated in response to stress such as infection or drug treatment. After shedding in circulation, lifespan of platelet is regulated by Bcl group of proteins (163). Signals due to stress regulate the BH3-proteins, which further active prosurvival proteins, leading to oligomerization and insertion into the mitochondrial membrane, leading to permeabilization and release of cytochrome c. This further trigger formation of the apoptosome and causing activation of caspase-9 and the other apoptotic caspase cascades (Figure 14) (164).

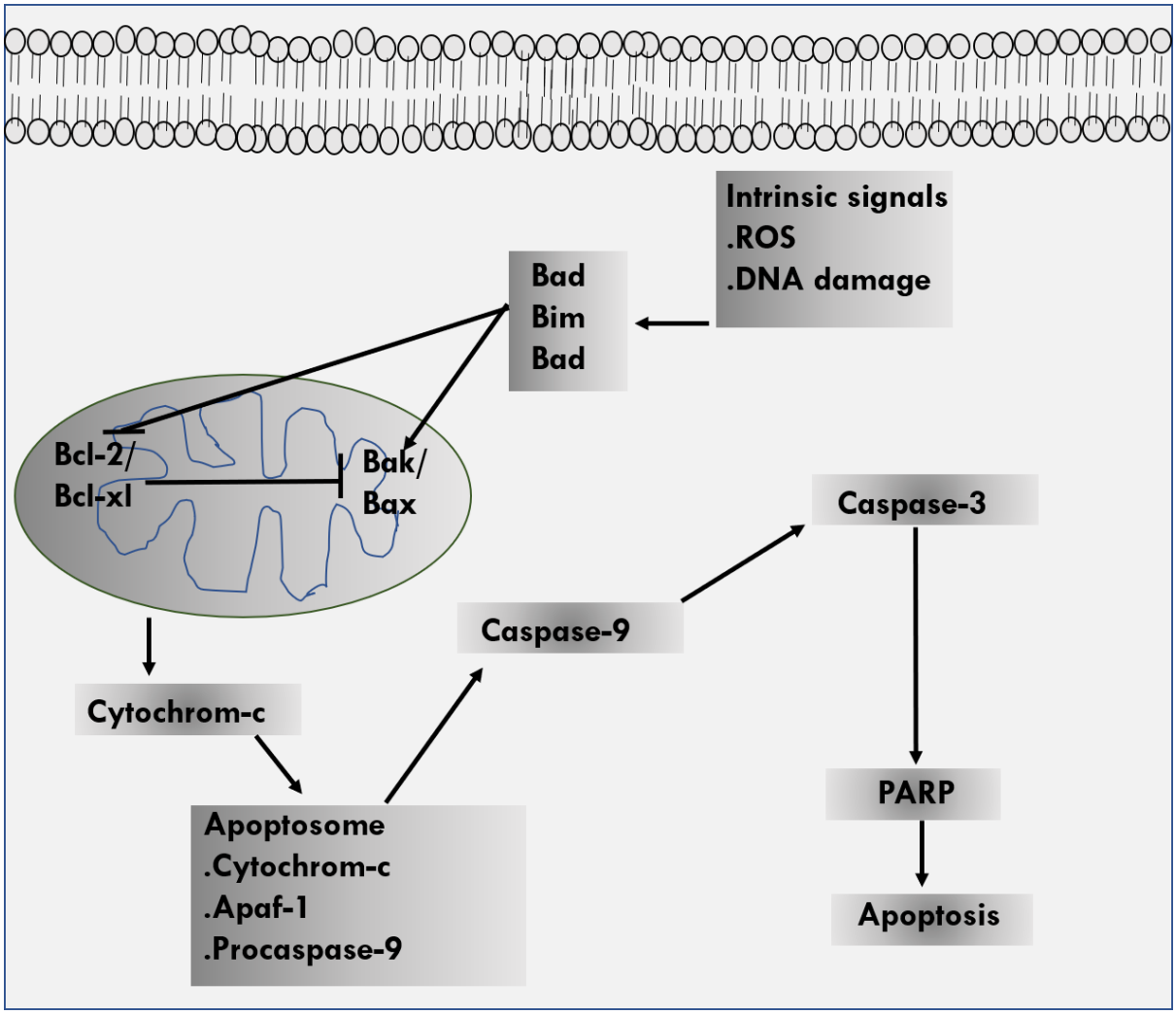
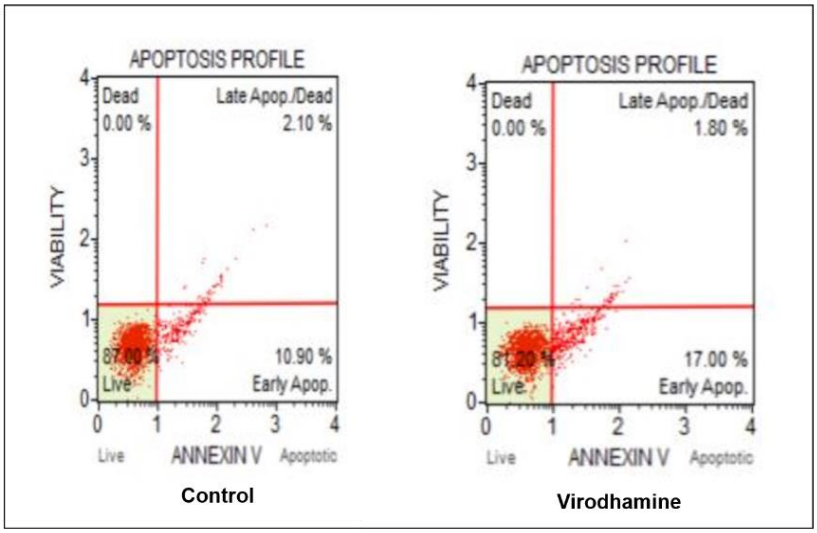


Figure 14. Overview of intrinsic apoptosis pathway.

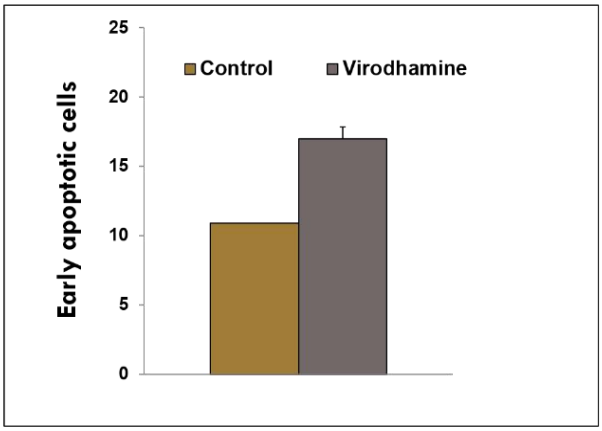
As per our RT^2 profiler mitochondrial array analysis results, mitochondrial genes were significantly altered in virodhamine treatment as compared to untreated control cells. Further KEGG pathway analysis revealed intrinsic mitochondrial mediated apoptotic pathway involvement. Above mentioned reports represent the involvement of active apoptotic pathway during MK maturation and platelet production (165-167). A report by Narasaiah K. et al. (2020) suggest PMA induced apoptosis and caspase activation involvement during MK differentiation (166). In addition, Josefsson EC et al. (2011) also demonstrates that MK possess functional intrinsic apoptotic pathway to produce platelets (165).

In order to know the apoptosis involvement during virodhamine induced MK differentiation, Dami cells were treated with virodhamine and annexin V assay was performed by Muse cell analyser by kit as per manufacturer instructions (Millipore Inc.). Apoptotic profile showed increased early apoptotic cells in virodhamine treated compared to control cells, whereas, live cells were found to be decreased in virodhamine treated cells (*n=3*; Figure 15a (i and ii). Cytochrome-c release in the cytosol is known to initiate caspase cascade, which was measured by western blot analysis. Cytochrome-c level was found to be enhanced in virodhamine treated as compared to the untreated control cells. Apoptosis inducing factor (AIF) expression level was also increased in virodhamine treated cells as compared to control cells.

a (i)



a (ii)



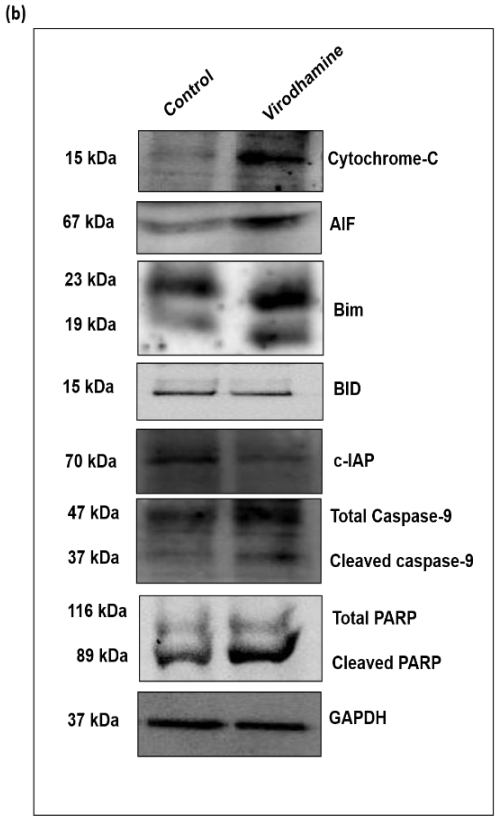


Figure 15. Cannabinoid receptor-2 agonist virodhamine shows induction of apoptotic cells, pro-apoptotic proteins and cleaved caspase product in megakaryocyte cells. 14a (i) Muse flow cytometric analysis for Annexin V shows induction of early apoptotic cells in virodhamine treated compared to control cells. **14a (ii)** Bar graph represents early apoptotic profile of control and virodhamine treated cells. **(b)** Western blot analysis shows increased cytochrome-c, AIF, Bim, cleaved caspase-9 and PARP levels in virodhamine treated cells. Anti-apoptotic protein c-IAP was found to be down-regulated in virodhamine treated cells.

Pro-apoptotic Bim level was found to be increased in virodhamine treated cells, whereas, Bid levels remain unchanged (Figure 14b). In addition, anti-apoptotic (c-IAP) protein level was down-regulated in virodhamine treated cells. Cleavage product of Caspase-9 and its down-stream effector PARP was found to be increased in virodhamine treated compared to untreated control cells by using western blot analysis (*n=3*; Figure 15b). Altogether this data suggests up-regulated pro-apoptotic protein and down-regulated anti-apoptotic protein which confirms the activation cascade of apoptosis during virodhamine induced MK differentiation.

A report by De Botton S. et al. (2002) suggest that cleavage of caspase-3, caspase-9 and PARP in MK differentiation [168-169]. We further measured caspase activity using caspase-3 activity

assay kit (Cat. No. E13184). Caspase-3 activity was enhanced in virodhamine treated as compared to control cells in time dependant manner. (*n=3*; Figure 16a). Taken together, this data suggests that virodhamine increases early apoptotic cells, pro-apoptotic markers and caspase-3 activity in virodhamine treated Dami cells as compared to untreated control cells.

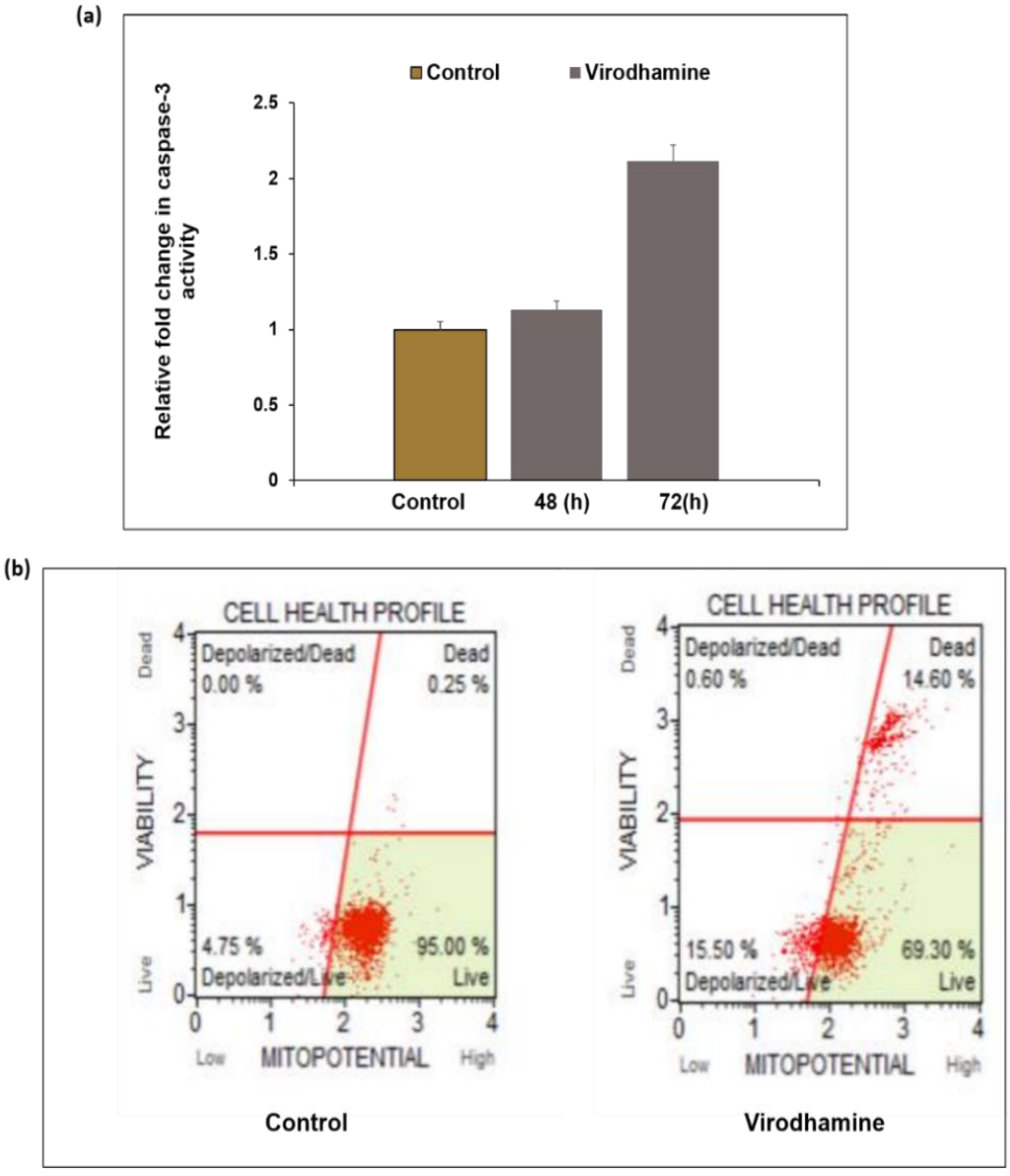


Figure 16. **Cannabinoid receptor-2 agonist virodhamine induce caspase-3 activity and alter mitochondrial membrane potential in megakaryocyte cells. (a)** Bar graph represents increase in Caspase-3 activity in virodhamine treated cells in time dependant manner as compared to untreated control cells. **(b)** Alteration in mitochondrial membrane potential upon virodhamine treatment (*n=3*).

We further tested the effect of virodhamine on the mitochondria health by using Muse mitochondrial membrane potential assay as per manufacturer instructions (Millipore Inc.). Virodhamine treatment caused a decrease in mitochondrial membrane potential, almost 15% cells were depolarized, whereas, untreated control showed 4% depolarized cells (*n=3*; Figure 16b). The depolarization of mitochondrial inner membrane potential indicates that the function of mitochondria is altered. Altogether these results suggest the alteration of mitochondrial function.

Virodhamine induces intracellular ROS, intracellular Ca^{2+}, NOX4 and reduces SOD

Intracellular ROS is produced in megakaryocytes at 2 major sites: in the plasma membrane through the enzyme NADPH oxidase (NOX) and within mitochondria during respiration. This ROS play important role in cellular development and differentiation. It also regulates cytoskeletal dynamics during cellular organelle distribution like mitochondria. ROS is known to influence the cytoskeletal architecture and actomyosin dynamics during wound healing. Mitochondria is the major source of ROS in cells. They create strong impact on ROS production and differentiation of megakaryocyte [170]. These observations led us to study the role of ROS and mitochondrial dynamics in the maturation of MKs.

We have measured the intracellular ROS by fluorescence spectroscopy using DCFDA dye. Dami cells were treated with virodhamine, PMA and decylubiquinone (a potent ROS inhibitor) and intracellular ROS was measured. Treatment with PMA and virodhamine resulted in increase of intracellular level of mitochondrial ROS, whereas, in inhibitor treated cells, intracellular ROS was decreased as compared to PMA and virodhamine alone [*n=3*; Figure 17a].

The antioxidant system in megakaryocytes include enzymes, such as superoxide dismutase

(SOD), glutathione peroxidase (GPX), catalase, and glutashione S-transferases (GST). Superoxide dismutase (SOD) prevent the formation of ROS. As ROS level was found to be elevated, we further checked the activity of SOD. SOD activity was measured by using the inhibition of auto-oxidation of quercetin by the enzyme SOD by spectroscopic measurement at 406nm. SOD activity was reduced in virodhamine treated as compared to control cells [*n=3*; Figure 17b].

Earlier, CB1R and/or CB2R have been reported to either stimulate and/or repress the function of enzymes involved in ROS generation. For instance, CBR activation acts to suppress the cAMP-dependent activation of PKA thereby reducing the expression and/or activity of ROS generating enzymes such as NADPH oxidase (NOX). Alternatively, CBR stimulation can activate MAPK signalling which is reported to be involved in up-regulating the NADPH oxidase expression and/or activity (171).

In the present study, we found that NAPDH oxidase, NOX4 expression was higher in virodhamine stimulated as compared to control cells [*n=3*; $p<.02$; Figure 17c]. It is apparent that in MKs virodhamine could regulate ROS production likely through MAPK/NADPH oxidase axis.

Cross-talk between ROS and Ca^{2+} levels is known to have significance in cell development. Calcium channels are known to be modulated by redox mechanisms and ROS production by NOX and mitochondria may be induced by calcium. To understand this, we measured the intracellular Ca^{2+} levels by using Fluo-3 AM dye. We have treated the cells with virodhamine and found increase of intracellular Ca^{2+} (Figure 17d).

Altogether these results suggest that virodhamine could induce mitochondrial intracellular ROS which could induce intracellular Ca^{2+} during MK differentiation.

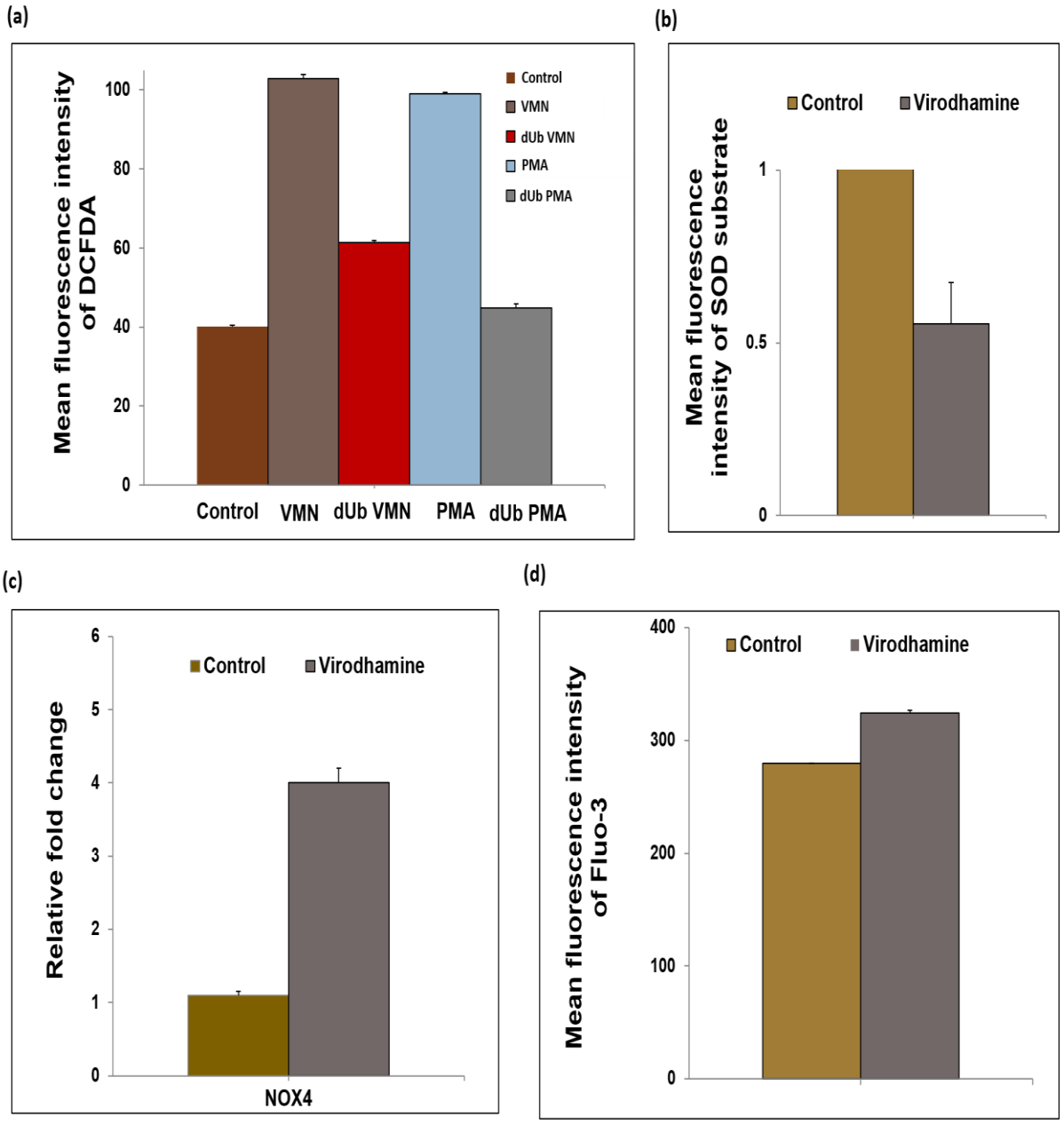


Figure 17. Virodhamine induces intracellular ROS, intracellular calcium and NADPH oxidase levels. **(a)** Intracellular ROS was measured by using DCFDA which can probe intracellular ROS. Fluorescence intensity was measured by fluorescence spectroscopy at 485nm excitation and 535nm emission wavelengths. Graph represents the relative mean fluorescence intensity of control, vehicle control, virodhamine, PMA, dUb+ virodhamine and dUb+ PMA. **(b)** Bar graph represents the superoxide dismutase activity measured by spectroscopy. **(c)** qRT-PCR results show the differential expression of NOX4 upon virodhamine treatment as compared to control (*n=3*; **p<.02). **(d)** Fluo-3 fluorescence dye was utilized to determine intracellular calcium; the emitted fluorescence intensity of Fluo-3 was recorded on a fluorescence-spectrometer (*n=3*). Bar represents the mean ± standard deviation of experiments in triplicate.

Virodhamine induce intracellular calcium levels and ROS mediated calcium channels which helps the translocation of calcium in cells

Ca^{2+} is an important second messenger in most cells, regulating many fundamental cellular processes. In platelets, the Ca^{2+} contributes to cellular activation including reorganization of the actin cytoskeleton, degranulation of integrins and platelet aggregation. Previous studies established that calcium and ROS are necessary for megakaryocytic differentiation and function (172-174). To study the levels of intracellular calcium, a Fluo-3AM dye was used. We observed that the mean fluorescence intensity of Fluo-3 was increased upon virodhamine treatment as compared to control (*n=3*; Figure 17d).

When we visualized cells under confocal microscope, we saw fluorescence distribution of Fluo-3 dye in the positive cells suggesting calcium induction upon virodhamine treatment (*n=3*; Figure 18a).

Calcium is one of the important regulators of mitochondrial function and ROS could stimulate intracellular Ca^{2+} release from endoplasmic reticulum (ER) (175-176). It could be possible that endocannabinoids may regulate ROS levels by targeting alternative ion channels such as TRPV1, TRPA1, TRPM8, TRPV2 and TRPC3.

To check the expression of various calcium channels, Dami cells were treated with virodhamine and expression of various calcium channels we measured by qRT-PCR. Expression of ROS mediated calcium channels (TRPV1, TRPC3) was found to be up-regulated in virodhamine treated cells as compared to control cells (*n=3*; figure 18b). Other calcium channels were not significantly altered. Altogether these study result suggest that virodhamine could alter the ROS mediated calcium channels during MK maturation.

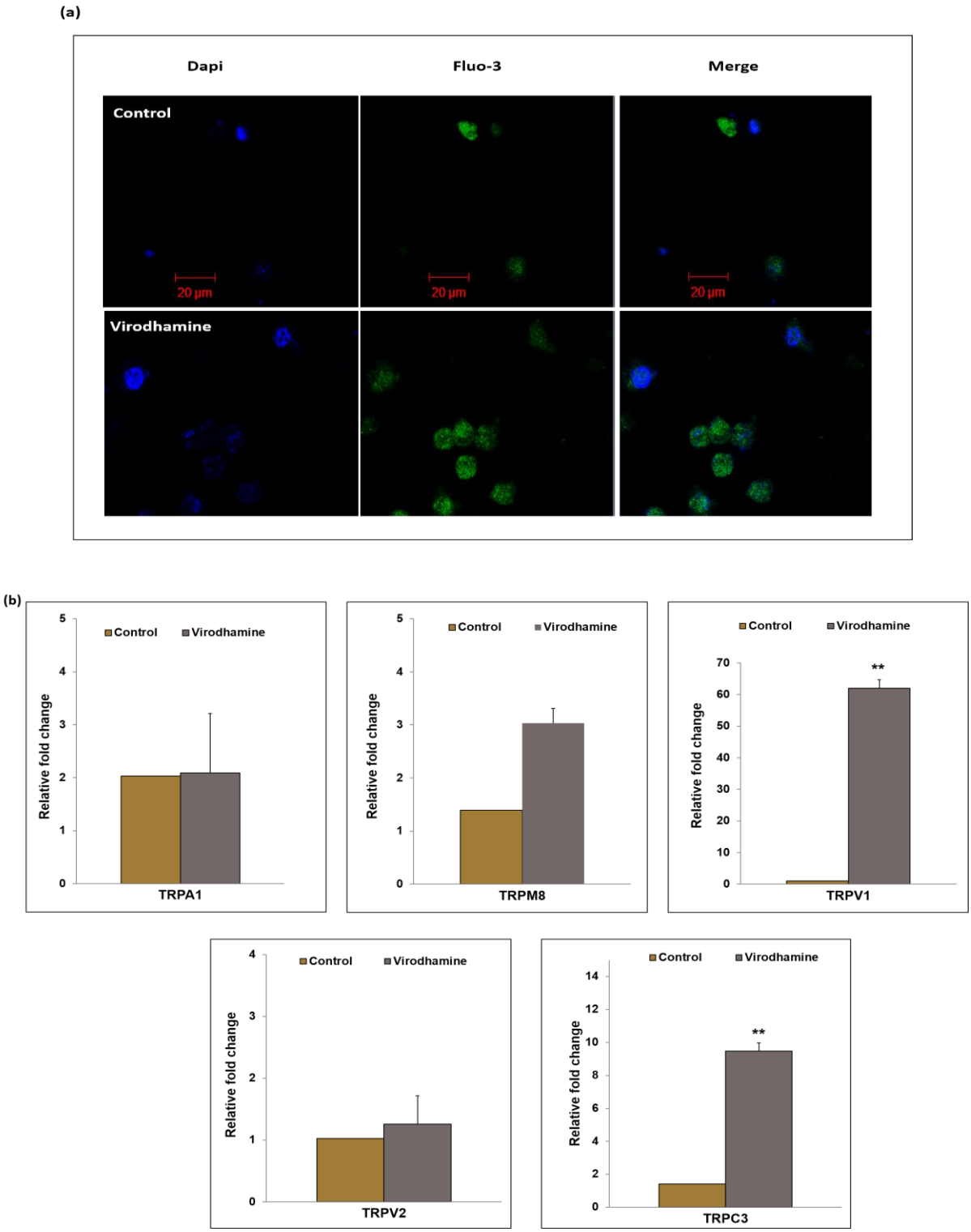


Figure 18. Virodhamine induces intracellular calcium level and ROS mediated calcium cannels (a) Representative immunofluorescence image of Dami cells treated with virodhamine compared to untreated Dami cells. Nuclei stained with Dapi (Blue), Fluo-3, calcium dye (Green). **(b)** qRT-PCR results show the relative fold expression of TRPA1, TRPM8, TRPV1, TRPV2 and TRPC3 in untreated control and virodhamine treated cells (*n=3*; **p<.02). Bars represent mean ± SD of three independent experiments.

Summary

Endocannabinoids (ECs) are endogenous chemical substances, which are capable of binding to CBRs and functionally activating them. ECs are a large group of fatty acids-derived compounds which perform diverse molecular activities in cells by binding to their receptors. CBRs are G-protein coupled receptors (GPCR) and members of the superfamily of seven-transmembrane-spanning (7-TM) receptors. CBRs are of two types, CB1R and CB2R. Endocannabinoids are well explored in neurological disorders, but there exist limited studies in blood cells and the immune system.

Platelets are mainly involved in the metabolism and release of circulating endocannabinoid. In addition, endocannabinoids are known to promote platelet activation. MKs are the immediate precursor of the platelet. MKs are the biggest cells of the bone marrow which undergo a distinctive process called endomitosis and cytoplasmic protrusion formation to generate platelets in circulation. Thrombopoietin (TPO), is an important cytokine which is required for the platelet production. Fluctuation in the platelet counts is a result of the induced consumption or destruction of platelets in various diseases such as HIV, polycythaemia vera, dengue, multiple myeloma and thrombocytopenic purpura. A lower platelet count in blood is called thrombocytopenia, which is reported in various clinical conditions such as viral infections, side effects of chemotherapies and radiotherapies. The widely used treatment strategy of lower platelet count condition (thrombocytopenia) is TPO mimetics and platelet transfusion, but their utilization is limited due to side effects. Here, we wish to explore TPO-independent mechanisms in regulation of MK development.

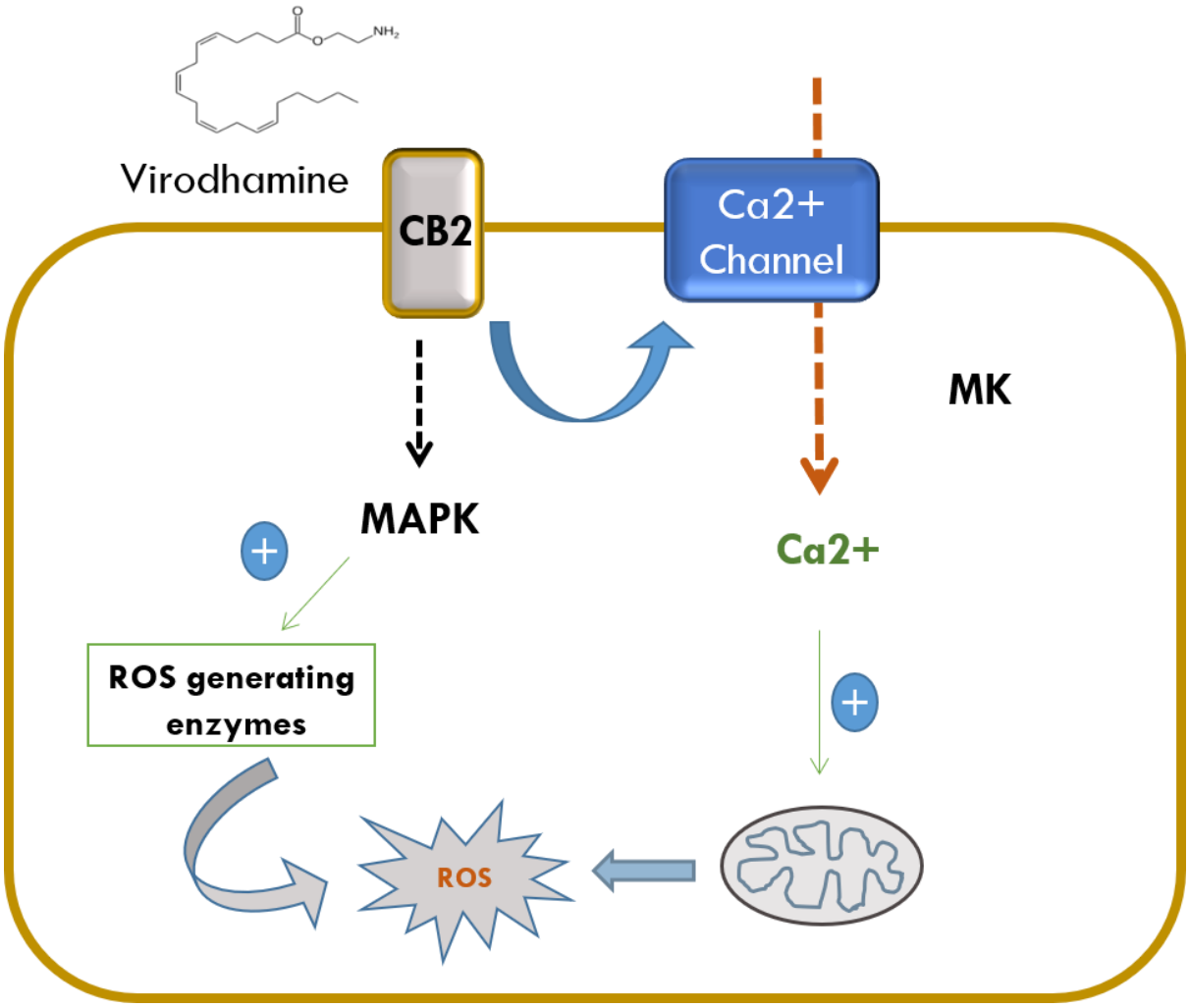
ECs may have an occupational role in MK development and thrombocytopenia, as their metabolism and release are monitored by circulating platelets. In the current study, we investigated the role of EC receptor signaling using an agonist (Virodhamine) in a cell line model system. During this distinctive process, MK loses its proliferative ability and matures

through a unique process endomitosis resulting in a large polyploid multilobed nucleus, further extending the cytoplasmic protrusions and thereby releasing the platelets. The functional role of endocannabinoids in MK and platelet biology is not well explored. The present indicates that virodhamine is a positive regulator of MK maturation.

The functional role of CBR signaling in MK and platelet biology is not well explored, to activate this signaling, we used EC Virodhamine, a CB2R agonist. CB2R expression was found to be more in virodhamine treated cells compared to untreated control cells, whereas, CB1R expression was not increased in virodhamine treated cells as compared to control. The characteristic features of MK maturation are increase in cell size, multilobed nucleus, adherence, cytoplasmic extension and polyploidy. To know the effect of CB2 agonist during MK maturation, Dami cells were treated with virodhamine and observed in bright field microscopy. Our results show an increase in cell size in virodhamine treated cells as compared to untreated control cells. Also, increase in adherence and cytoplasmic extensions can be seen in virodhamine treated cells. Virodhamine treated cell shows multi-lobed nucleus, upon staining with Giemsa. In addition, MK maturation-specific markers were analyzed by qRT-PCR. CD41, CD61 and CD42b are well-known MK maturation-specific markers. All the MK maturation marker was found to be increased in virodhamine treated as compared to untreated control cells. DNA content was found to be induced in virodhamine treated cells which is a typical hallmark of MK maturation. Altogether these results show that virodhamine could induce MK maturation and polyploidy in Dami cells.

Furthermore, we wanted to study the mechanism by which virodhamine could induce MK maturation. We have studied the activity of phosphorylated MAPK and PI3K. FACS analysis suggests that virodhamine induces phosphorylated MAPK. Phosphorylated PI3K activity was found to be downregulated in virodhamine treated cells. Mitochondrial function and intrinsic apoptosis pathway are important for MK maturation and is also critical outcome of the CB2R

signaling pathway. We have measured mitochondrial membrane potential by mitopotential kit using muse analyzer which uses a mitopotential dye to measure the depolarized live cells and 7-AAD to probe dead cells. Virodhamine treated cells shows an enhanced number of depolarized live cells. Dami cells were treated with virodhamine and annexin V assay was performed by using a muse cell analyzer. Early apoptotic cells were found to be increased in virodhamine treated cells. Altogether these results suggest that CB2R agonist could alter mitochondrial function and induce apoptosis. Reactive Oxygen Species (ROS) is reported to play significant role in all important stages of MK maturation. EC ligands are known to regulate ROS production and mitochondrial function. Intracellular ROS was found to be upregulated in virodhamine treated cells. In addition, intracellular calcium was also found to be upregulated in virodhamine treated cells.



To understand the expression of functional key regulators of apoptosis, western blot analysis was performed. Western blot analysis results show that virodhamine could induce the expression levels of pro-apoptotic (AIF, BIM) markers and reduce the protein levels of anti-apoptotic (c-IAP) markers. cytochrome-C levels were found to be elevated in virodhamine treated cells. Altogether these results suggest that virodhamine can induce MK maturation by activation of CB2R that in turn can activate MAPK pathway. In addition, functional mitochondrial mediated intrinsic apoptotic pathway could be involved in virodhamine induced MK maturation.

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List of publications

1- Sharma D.S., Raghuwanshi S, Narasaiah K, Dahariya S, Gautam DK, Paddibhatla I and Gutti RK. Virodhamine, an endocannabinoid induces megakaryocyte differentiation by regulating MAPK activity and function of mitochondria. ***Journal of Cellular Physiology.*** 2020 10.1002/jcp.29949

2- Sharma D.S., Paddibhatla I, Raghuwanshi S, Narasaiah K, Dahariya S, Gautam DK, Pallepati A, Mahesh M and Gutti RK. The Potential Therapeutic Role of Cannabinoids and Endocannabinoid System in Neuro-Immune Disorders. ***Journal of Neuroimmunology.*** 10.1016/j.jneuroim.2021.577501

3- Narasaiah Kovuru, Sanjeev Raghuwanshi, Sharma D.S., Swati Dahariya, Adithya Pallepati and Ravi Gutti. Co-stimulatory effect of TLR2 and TLR4 stimulation on megakaryocytic development is mediated through PI3K/NF-κB and XBP-1 loop. ***Cellular signaling***. 10.1016/j.cellsig.2021.109924

4- Raghuwanshi S, Dahariya S, Sharma DS, Kovuru N, Sahu I, Gutti RK. RUNX1 and TGF-β signaling Cross Talk Regulates Ca2+ Ion Channels Expression and Activity during Megakaryocyte Development. The FEBS Journal 202010.1111/febs.15329

5- Kovuru N, Raghuwanshi S, Sharma DS, Dahariya S, Pallepati A, Gutti RK. Endoplasmic reticulum stress induced apoptosis and caspase activation is mediated through mitochondria during megakaryocyte differentiation. ***Mitochondrion*** 2020; 50:115-120

6- Raghuwanshi S, Sharma DS, Kandi R, Kovuru N, Dahariya S, Musvi SS, Venkatakrishnan AC, Pallepati A, Gutti RK. Pituitary adenylate cyclase- activating polypeptide (PACAP): Differential effects on neonatal vs adult megakaryocytopoiesis. ***Thromb Res.*** 2019; 175:59-60.

7- Raghuwanshi S, Dahariya S, Kandi R, Gutti U, Undi RB, Sharma DS, Sahu I, Kovuru N, Yarla NS, Saladi RGV, Gutti RK. Epigenetic mechanisms: Role in hematopoietic stem cell lineage commitment and differentiation. ***Curr Drug Targets***. 2018;19(14):1683-1695.

8- Bansal P, Dahate P, Raghuwanshi S, Sharma DS, Kovuru N, Gutti U, Yarla NS, Gutti RK. Current Updates on Role of Lipids in Hematopoiesis. ***Infect Disord Drug Targets***. 2018;18(3):192-198.

Conferences and Awards

1- **Fire-talk Presentation: Durga Shankar Sharma** and Ravi Gutti. Virodhamine: A Cannabinoid Receptor Agonist Induces Megakaryocyte Maturation and Pro-platelet Production from In-vitro Megakaryoblastic Cells. *Stem Cell Society Singapore (SCSS) Symposium 2020 jointly organized with Japanese Society of Regenerative Medicine (JSRM), Dec 9 - 11, 2020 (Virtual Meeting)*

2- **Poster presentation (P-002): Durga Shankar Sharma** and Ravi Gutti. Virodhamine: A Cannabinoid Receptor Agonist to Enhance the Platelets Production from Megakaryocyte through Attenuation of Mitochondrial Function. *Laboratory Medicine Congress & KSLM 60th Annual Meeting, Busan, Korea in Sept 2019.*

3- **Poster presentation (P-509): Durga Shankar Sharma**, Usha Gutti and Ravi Kumar Gutti. Cannabinoid receptors and its role in Megakaryocyte development. *International Congress of Cell Biology-2018 (The Dynamic Cell- From molecules and networks to form and function).*

4- **Poster presentation (P-23): Durga Shankar Sharma** and Ravi Kumar Gutti. **P-23:** Cannabinoid receptors: Role in Megakaryocyte development. *BioQuest 2017 – SLS, University of Hyderabad, Hyderabad, India.*

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ORIGINAL RESEARCH ARTICLE

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Virodhamine, an endocannabinoid, induces megakaryocyte differentiation by regulating MAPK activity and function of mitochondria

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Abstract

Endocannabinoids are well-known regulators of neurotransmission by activating the cannabinoid (CB) receptors. Endocannabinoids are being used extensively for the treatment of various neurological disorders such as Alzheimer's and Parkinson's diseases. Although endocannabinoids are well studied in cell survival, proliferation, and differentiation in various neurological disorders and several cancers, the functional role in the regulation of blood cell development is less examined. In the present study, virodhamine, which is an agonist of CB receptor-2, was used to examine its effect on megakaryocytic development from a megakaryoblastic cell. We observed that virodhamine increases cell adherence, cell size, and cytoplasmic protrusions. Interestingly, we have also observed large nucleus and increased expression of megakaryocytic marker (CD61), which are the typical hallmarks of megakaryocytic differentiation. Furthermore, the increased expression of CB2 receptor was noticed in virodhamine-induced megakaryocytic cells. The effect of virodhamine on megakaryocytic differentiation could be mediated through CB2 receptor. Therefore, we have studied virodhamine induced molecular regulation of megakaryocytic differentiation; mitogen-activated protein kinase (MAPK) activity, mitochondrial function, and reactive oxygen species (ROS) production were majorly affected. The altered mitochondrial functions and ROS production is the crucial event associated with megakaryocytic differentiation and maturation. In the present study, we report that virodhamine induces megakaryocytic differentiation by triggering MAPK signaling and ROS production either through MAPK effects on ROS-generating enzymes or by the target vanilloid receptor 1-mediated regulation of mitochondrial function.

KEYWORDS

endocannabinoid, MAPK, megakaryocyte, mitochondria, ROS, virodhamine

1 | INTRODUCTION

Endocannabinoids are endogenous ligand of cannabinoid (CB) receptors and well-known neuromodulators. Endocannabinoids are rigorously studied in neurological disorders such as Alzheimer's and Parkinson's diseases and various other neurological abnormalities. But, there exist limited studies of these endocannabinoids in blood cells. The ability of circulating blood cells to produce

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Review Article

Endocannabinoid system: Role in blood cell development, neuroimmune interactions and associated disorders

Durga Shankar Sharma, Indira Paddibhatla, Sanjeev Raghuwanshi, Mahesh Malleswarapu, Anjali Sangeeth, Narasaiah Kovuru, Swati Dahariya, Dushyant Kumar Gautam, Aditya Pallepati, Ravi Kumar Gutti *

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ABSTRACT

The endocannabinoid system (ECS) is a complex physiological network involved in creating homeostasis and maintaining human health. Studies of the last 40 years have shown that endocannabinoids (ECs), a group of bioactive lipids, together with their set of receptors, function as one of the most important physiologic systems in human body. ECs and cannabinoid receptors (CBRs) are found throughout the body: in the brain tissues, immune cells, and in the peripheral organs and tissues as well. In recent years, ECs have emerged as key modulators of affect, neurotransmitter release, immune function, and several other physiological functions. This modulatory homoeostatic system operates in the regulation of brain activity and states of physical health and disease. In several research studies and patents the ECS has been recognised with neuro-protective properties thus it might be a target in neurodegenerative diseases. Most immune cells express these bioactive lipids and their receptors, recent data also highlight the immunomodulatory effects of endocannabinoids. Interplay of immune and nervous system has been recognized in past, recent studies suggest that ECS function as a bridge between neuronal and immune system. In several ongoing clinical trial studies, the ECS has also been placed in the anti-cancer drugs spotlight. This review summarizes the literature of cannabinoid ligands and their biosynthesis, cannabinoid receptors and their distribution, and the signaling pathways initiated by the binding of cannabinoid ligands to cannabinoid receptors. Further, this review highlights the functional role of cannabinoids and ECS in blood cell development, neuroimmune interactions and associated disorders. Moreover, we highlight the current state of knowledge of cannabinoid ligands as the mediators of neuroimmune interactions, which can be therapeutically effective for neuro-immune disorders and several diseases associated with neuroinflammation.

1. Introduction

Cannabinoids (CB) are chemical compounds, which are well-known to be derived from the *cannabis sativa* plant; this plant is one of the earliest plants that are cultivated for the clinical use (Russo, 2007). Cannabinoids are a class of biologically active compound that can bind and activate cannabinoid receptors (CBRs). A significant number of studies have proven the biological role of different cannabinoids. This class of compounds is broadly classified in three classes based on their source of production: (1) plant derived cannabinoids (also called Phytocannabinoids), (2) endogenous cannabinoids (called endocannabinoids), and (3) synthetic cannabinoids. Phytocannabinoids are a class of the structurally diverse chemical constituents found in the genus *Cannabis*, they interact with our body receptors and initiate different molecular events leading to psychotropic and therapeutic effects. One such example of Phytocannabinoids is delta-9-tetrahydrocannabinol (Δ^9-THC), which is a well-known psychoactive phytocannabinoid compound explored enormously in clinical and biological investigations for its therapeutic potential (Pertwee, 1999; Pertwee and Ross, 2002; Pertwee, 2006). On the other hand, endocannabinoids (ECs) are defined as endogenous ligands of CBRs, they are the lipid-based molecules derived from long-chain polyunsaturated fatty acids, amides, esters, and ethers, e.g., 2-arachidonoylglycerol (2-AG) and anandamide (Fezza et al., 2014; Pisanti et al., 2013). The ECs bind to G-protein coupled receptor (GPCRs), CB1R and CB2R and exert their action (Morales and Reggio, 2017). Several studies have conformed the regulatory functions

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Co-stimulatory effect of TLR2 and TLR4 stimulation on megakaryocytic development is mediated through PI3K/NF-κB and XBP-1 loop

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PI3K

ABSTRACT

Toll-like receptors (TLRs) are a class of proteins (patterns recognition receptors-PRRs) capable of recognizing molecules frequently found in pathogens (that are so-called pathogen-associated molecular patterns-PAMPs), they play a key role in the initiation of innate immune response by detecting PAMPs. Our findings show that the functional effects of TLRs co-stimulation on megakaryocytopoiesis. A single cell may receive multiple signal inputs and we consider that multiple TLRs are likely triggered during infection by multiple PAMPs that, in turn, might be involved in infection driven megakaryocytopoiesis, and the present study provide the evidence for the megakaryocytic effects of TLRs co-stimulation.

We read with interest the recent article by D'Atri et al. [1] which enhances our understanding of the TLR2 and TLR4 signaling importance in megakaryocyte commitment and maturation during the development. Authors analysed TLR2 and TLR4 expression and their functions in human CD34+ cells derived MKs. Though, LPS or Pam3CSK4 had no effect on the growth of CD34+ cells, cell proliferation, megakaryocyte number and maturity was increased when CD34+ cells were costimulated with TLR ligands in the presence of TPO at day 0. They have also described that the release of Interleukin-6 (IL-6) from CD34+ or megakaryocytes was increased upon stimulation with LPS or Pam3CSK4 but not with TPO and this effect was further augmented by LPS or Pam3CSK4 in combination with TPO. Further, they have observed that this effect is suppressed by TLR2/4 or IL-6 neutralizing antibodies or by the inhibitors of PI3K/AKT and NF-kB. The fact is that during infection episodes cells receive multiple signaling inputs in form of multiple PAMPs, therefore it is likely that more than one TLR are activated simultaneously.as it was presented by D'Atri et al. [1]. Further we want to understand the effect of TLR co-stimulation on megakaryocyte maturation, herein we treat the cells with multiple TLR-ligands, LPS (100 ng/ml) for TLR4 and Zymosan (10 μg/ml) for TLR2.

Toll-like receptors (TLRs) are a class of proteins (patterns recognition receptors-PRRs) capable of recognizing molecules frequently found in pathogens (that are so-called pathogen-associated molecular patterns-PAMPs), they play a key role in the initiation of innate immune response by detecting PAMPs [2]. The signals initiated with the activation of PRRs by PAMPs on the immune cells, activate microbicide and pro-inflammatory responses. However, recent studies have shown the expression of TLRs on the surface and internal compartments of platelets and megakaryocytes (MKs) [3]. Previously, we have also noticed that TLR2 induction leads to megakaryocytic differentiation through ROS and cytokine production [4]. Furthermore, in our present study, we have observed that Dami cells (megakaryoblastic cell line) harbors all the TLRs except TLR-8 at transcript levels (Fig. 1A). Thus, considering the functional role of different TLRs in megakaryocytic development [1,4], and the expression of multiple TLRs in megakaryocytes (noticed in present study), we hypothesize that multiple TLRs are likely triggered during infection by multiple PAMPs and that might be involved in infection driven megakaryocytopoiesis.

To study co-stimulatory effects of TLR2 and TLR4 stimulation on megakaryocytic development we used Dami cells (human megakaryoblastic leukemia cell line) as a model system, cells were maintained in RPMI-1640 media with 10% FBS and challenged with commonly known PAMPs, such as lipopolysaccharide (LPS) from gram-negative bacteria and zymosan, a glycan derived from the yeast cell wall. LPS is a potent activator of TLR4, whereas zymosan collaborates with TLR2. The cells treated with zymosan (10 μg/ml), LPS (100 ng/ml), or co-stimulated with zymosan and LPS were analysed with quantitative real-time reverse transcriptase–polymerase chain reaction (qRT-PCR). Herein, we noticed the increased expression of megakaryocytic markers (CD41 and CD61) upon treatment with zymosan or LPS as compared to

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RUNX1 and TGF-β signaling cross talk regulates Ca^{2+} ion channels expression and activity during megakaryocyte development

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Keywords
megakaryocyte; thrombocytopenia; RUNX1; TGF-beta; platelets; calcium; TRP channel

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Thrombocytopenia is characterized by low platelet count and is typically observed among all preterm and low birthweight neonates admitted to the neonatal intensive care unit. Although the underlying cause for this predisposition is unclear, recent studies have proposed that the intrinsic inability of neonatal hematopoietic stem/progenitor cells to produce mature polyploid megakaryocytes (MKs) may result in delayed platelet engraftment. The developmental and molecular differences between neonatal and adult MKs are not yet fully understood. Previously, we had reported that the key MK transcription factor RUNX1, which is crucial for the regulation of MK specification and maturation, is down-regulated in neonatal MKs when compared with adult MKs. In humans, loss-of-function mutations in *RUNX1* cause familial platelet disorder, which is characterized by thrombocytopenia, indicating its crucial role in MK development. However, information about its cross talk with developmentally regulated signaling pathways in MKs is lacking. In this study, we performed a differential gene expression analysis in MKs derived from human cord blood (CB) and adult peripheral blood (PB) $CD34^+$ cells. Further, validation and correlation studies between RUNX1 and transforming growth factor beta (TGF-β) were performed in a differentiating megakaryocytic cell line model. The analysis revealed that TGF-β pathway was the main pathway affected between CB- and PB-MKs. RUNX1 is reported to be a modulator of TGF-β signaling in several studies. The correlation between RUNX1 and TGF-β pathway was analyzed in the PMA-induced megakaryocytic differentiating K562 cells, which exhibit mature megakaryocytic features. The RT^2 profiler PCR array analysis revealed that TGF-β pathway components were up-regulated in the PMA-induced megakaryocytic differentiating cells. Furthermore, our study indicated that human TGF-β1 promotes cytosolic calcium (Ca^{2+}) activity and MK maturation. We noticed that TGF-β1 increased intracellular free Ca^{2+} ($[Ca^{2+}]i$) via reactive oxygen species-mediated activation of transient receptor potential (TRP) ion channels. Moreover, we observed that decreased cytosolic Ca^{2+} activity in the siRUNX1-

Abbreviations
$[Ca^{2+}]i$, intracellular free Ca^{2+}; Ca^{2+}, calcium; CB, cord blood; CD41, platelet glycoprotein (GP) IIb; CD42b, GPIbα; CD61, platelet GP IIIa; ECM, extracellular matrix; GYPA, glycophorin A; GYPB, glycophorin B; H2DCFDA, 2, 7-dichlorodihydrofluoresceindiacetate; HS/PCs, hematopoietic stem/progenitor cells; HSCs, hematopoietic stem cells; MKs, megakaryocytes; NAC, *N*-acetyl-L-cysteine; PB, peripheral blood; PMA, phorbol 12-myristate 13-acetate; pSMAD2/3, phospho-SMAD2/3; ROS, reactive oxygen species; RUNX1, runt-related transcription factor 1; TFs, transcription factors; TGF-β, transforming growth factor beta; TGF-βR, TGF-β receptor; TPO, thrombopoietin; TRP, transient receptor potential; UCP2, mitochondrial uncoupling protein 2.

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Endoplasmic reticulum stress induced apoptosis and caspase activation is mediated through mitochondria during megakaryocyte differentiation

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ABSTRACT

Megakaryocytopoiesis involves the process of the development of hematopoietic stem cells into megakaryocytes (MKs), which are the specialized cells responsible for the production of blood platelets. Platelets are one of the crucial factors for hemostasis and thrombosis. In terminally differentiated MKs, many molecular process such as caspase activation and a massive cytoskeletal rearrangement drive the formation of cytoplasmic extensions called proplatelets. These cytoplasmic extensions packed with granules and organelles are then released from the bone marrow into the blood circulation as platelets. Classically, caspase activation is associated with apoptosis and recent reports suggest their involvement in cell differentiation and maturation. There is no clear evidence about the stimulus for caspase activation during megakaryocyte development. In the current study, we attempted to understand the importance of endoplasmic reticulum stress in the caspase activation during megakaryocyte maturation. We used human megakaryoblstic cell line (Dami cells) as an experimental model. We used PMA (Phorbol 12-myristate 13 acetate) to induce megakaryocytic differentiation to understand the involvement of ER stress and caspase activation during MK maturation. Further, we used Thapsigargin, a non-competitive inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) as a positive control to induce ER stress. We observed larger and adherent cells with the increased expression of megakaryocytic markers (CD41 and CD61) and UPR markers in PMA or Thapsigargin treated cells as compared to control. Also, Thapsigargin treatment induced increased caspase activity and PARP cleavage. The increased expression of megakaryocyte maturation markers alongside with ER stress and caspase activation suggests the importance of ER stress in caspase activation during MK maturation.

1. Introduction

Megakaryocytopoiesis is a tightly regulated developmental process of megakaryocyte (MK) differentiation and maturation from multipotent hematopoietic stem cells (HSCs), this process is govern by various transcription factors and cytokines (Woolthuis and Park, 2016; Raghuwanshi et al., 2018). During maturation process, MK-progenitors undergo successive round of endomitosis and acquires polyploidy, the ploidy varies with the age and the physiological demand of the platelet (Mazzi et al., 2018). Finally, platelets are released into the blood stream from the terminally differentiated MKs (Abd-Elrahman et al., 2013). Recent reports suggest non-apoptotic functions of caspases in myeloid cell differentiation (Solier et al., 2017). Clarke et al. (2003) reported that constitutive formation of the proplatelet-bearing MK is caspase-dependent involving mitochondrial release of cytochrome *c*. Other studies elaborated that both MK frequency and production of functional platelets were significantly reduced by the broad-spectrum caspase inhibitor, zVAD-fmk, these studies implying a role for caspase-directed apoptosis in the formation of proplatelet extensions by MKs and the subsequent production of functional platelets (De Botton et al., 2002).

It is widely accepted that to produce platelets, MKs activate apoptotic pathways (Kaluzhny and Ravid, 2014; Fadeel and Orrenius, 2005; Patel et al., 2005; Siegel, 2006; Galluzzi et al., 2008; Solary et al., 2008). The role of apoptotic pathways in the development and function of the MK and platelets has generated renewed interest in recent years (Kile, 2014). Recently, it was observed that MKs and platelets both depends on mitochondrial apoptotic pathway for their development and viability (McArthur et al., 2018). But, the important question is that how apoptosis is initiated in these cells.

Classically caspase activation and apoptosis occurs in response to any assaults to cells such as potential harm full mutations, viral infection. Primarily apoptosis occurs in two different ways namely, intrinsic

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Letter to the Editors-in-Chief

Pituitary adenylate cyclase-activating polypeptide (PACAP): Differential effects on neonatal vs adult megakaryocytopoiesis

Pituitary adenylate cyclase-activating polypeptide (PACAP), a neuroendocrine hormone of the secretin peptide superfamily, is mainly produced in hypothalamus. PACAP shows diverse effects on CNS, peripheral organs, and some hematopoietic cells by VPAC1 and 2 receptor signaling [1]. Megakaryocytes (MKs) express the stimulatory G protein (Gs)-coupled PACAP/VIP-indifferent VPAC1 receptor. Previously, Peeters et al. [2] reported the inhibitory effects of PACAP receptor on in vitro megakaryocyte differentiation. Recently, it was observed that PACAP contribute to the proliferation of hematopoietic progenitor cells in murine bone marrow by PACAP specific receptor [3]. In our study on human MK development, we found that PACAP receptor-VPAC1 is differentially regulated in MKs through development. Considering differential expression of PACAP receptor-VPAC1 in human MK development, we hypothesized that PACAP may have different effects on MK differentiation from neonatal (CB)- and adults (PB)-derived CD34+ hematopoietic progenitors.

Thrombocytopenia, the deficiency of platelets (platelet count $< 150 \times 10^9$/l) in the blood, is a major clinical problem encountered in infants admitted to Neonatal Intensive Care Unit (NICU); this hemostatic abnormality is more common in all sick, preterm and low birth weight neonates, and play a major role as co-factor in the incidence and severity of neonatal intra-ventricular hemorrhages (IVH) [4], which is a leading cause of poor neurological outcome and mortality in sick neonates [4–6]. The cause for this predisposition is unclear, however we and many other research groups have observed that neonatal MKs are developmentally different from adult's MKs. Specifically, neonatal MK progenitors (MP) are hyper-proliferative and produce smaller and low ploidy MKs as compared to adults. These developmental differences may contribute to this predisposition in neonatal MKs [7–9].

To test the developmental differences in the expression levels of PACAP receptor-VPAC1 in neonatal vs adult's MKs, we cultured human CB- and PB-CD34+ cells ($n = 3$ for each group) in serum-free medium with thrombopoietin (50 ng/mL), as previously described [10]. After 14 day of culture Flow cytometry analysis was performed using human anti-CD42/APC to monitor thrombopoietin stimulated MKs differentiation. We observed that CD34+ cells from CB generated approximately 2-fold more CD42 positive MKs as compared to PB-CD34+ cells ($n = 3$, $P < .02$; Fig. 1A), however the change in CD42 mean fluorescence density was higher in PB-MKs as compared to CB-MKs on day 14 ($n = 3$, $P < .05$; Fig. 1A). Further, we observed the PACAP receptor-VPAC1 mRNA expression in neonatal- and adult-CD34+ progenitor-derived MKs and resulted in approximately 6 fold higher expression levels in CB- as compared to PB-MKs ($n = 3$, $P < .02$; Fig. 1B). However, because of sample limitations, the protein content of PACAP receptor VPAC1 was not measured in current study. Finally, to study the effect of PACAP on in vitro MK differentiation from CB- and PB-CD34+ hematopoietic progenitors, we cultured CB- and PB-CD34+ hematopoietic progenitors for 14 days with or without PACAP (1 μM) (TOCRIS, Cat # 1186) in the presence of TPO (50 ng/mL) in serum-free medium ($n = 3$ for each group). After 14 days, Flow Cytometry analysis was performed using human anti-CD41/FITC and anti-CD42/APC to monitor the number of differentiated MKs. The CD41 and CD42 positive CB- and PB-MK numbers in presence of PACAP were expressed as relative values to their controls (without PACAP) at the same stage of culture. The number of CB-CD41 positive MKs was significantly reduced in the presence of PACAP compared to their control ($n = 3$, $P < .05$; Fig. 1C), whereas PB-CD41 positive MKs were not affected significantly ($n = 3$, $P > .05$; Fig. 1C). Similarly, we also observed about 2 fold decrease in the number of CB-CD42+ MKs in presence of PAPAC as compared to control (n = 3, $P < .05$; Fig. 1D), whereas the decrease in the number of PB-CD42+ MKs was not significant (n = 3, $P > .05$; Fig. 1D).

Over the past two decades, observations from different research groups have proven that human neonatal- and adult-MKs are developmentally different [7–10]. In the same line, we have also observed developmental differences in neonatal vs adult MKs. Current study results are also suggesting that human CB and PB MKs have substantially different biological responses to PACAP. In current study, we observed that PACAP is a negative regulator of MK differentiation, as previously reported [2]. However, the negative effects of PACAP were more drastic on MK differentiation process from CB-CD34+ hematopoietic progenitors as compared to MK differentiation from PB-CD34+ hematopoietic progenitors (Fig. 1C and D). Further, we have also observed developmental differences in the expression levels of PACAP receptor-VPAC1 in neonatal vs adults MKs and found about 6 fold higher expressions in neonatal MKs as compared to adults. Hypothetically, the higher expression levels of PACAP receptor-VPAC1 in neonatal MKs might contribute more to the inhibitory effects of PACAP on neonatal MK differentiation, as we have observed in current study.

In conclusion, our results demonstrate that PACAP is an inhibitor of MK differentiation however the negative effects are more drastic on neonatal MK differentiation as compared to adults. Higher expression of the PACAP receptor-VPAC1 in neonatal MKs possibly contributes to the predisposition of neonatal MKs via PACAP/VPAC1 signaling mechanism.

Conflicts of interest

The authors declare that they have no conflict of interest.

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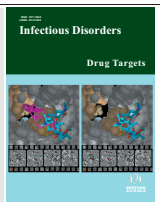
REVIEW ARTICLE

Current Updates on Role of Lipids in Hematopoiesis



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Abstract: Hematopoiesis is the process which generates all the mature blood cells from the rare pool of Hematopoietic stem cells (HSCs). Asymmetric cell division of HSCs provide it dual capacity for self-renewal and multi-potent differentiation. Hematopoiesis is a steady state process in which mature blood cells are produced at the same rate at which they are lost, establishing a homeostasis. HSCs are regulated through their environmental niche, cytokine signalling, and the orchestrated activities of various transcription factors. However, there is very little information available about the signal transduction events that regulate HSC function; in particular, the effects of bioactive lipids and lipid mediators are not well understood. Recent studies have added an important aspect of this process, introducing the role of lipids in cell fate decisions during hematopoiesis. The mechanisms of bioactive lipids and their derivatives have been studied extensively in signal transduction and various other cellular processes.

This review focuses on various categories of lipids and their regulatory mechanisms in HSCs and their comment into different blood cells. Moreover, we also discuss the role of lipid signalling specifically in megakaryocyte and platelets.

Keywords: HSC, Megakaryocyte, Eicosanoids, Phosphatidylinositides, Ceramide, Hematopoiesis.

1. INTRODUCTION

Hematopoiesis is a remarkable process that generates mature blood cells. Hematopoietic stem cells are the common progenitor cells from which different lineages of blood cells arise [1]. This occurs due to finely orchestrated cell signalling networks which involve many factors, including lipids, cytokines and transcription factors. The role of lipid bridges and lipid metabolism in signal transduction of HSCs was not elucidated in detail in past. Until recently, lipids were considered to be a source of energy and components of membranes. Lipids can be converted into derivatives that either complement or antagonize each other's cell signalling function. Research in the past10-15 years have established their role as mediators of inflammatory response and regulators of cell membrane polarity. They are critical for cell fate decisions during stem cell differentiation by inducing apoptosis or sustaining cell survival and polarity [2]. Lipid has always been an important part of the cell since they regulate many vital processes in hematopoiesis. Bioactive lipids like Ceramide-1-Phosphate (C1P) and Sphingosine-1- Phosphate (S1P) are involved in migration and homing of HSPCs in bone marrow niche. Phospholipids like

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REVIEW ARTICLE

Epigenetic Mechanisms: Role in Hematopoietic Stem Cell Lineage Commitment and Differentiation

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Abstract: Major breakthroughs in the last several decades have contributed to our knowledge of the genetic regulation in development. Although epigenetics is not a new concept, unfortunately, the role of epigenetics has not come to fruition in the past. But the field of epigenetics has exploded within the past decade. Now, growing evidences show a complex network of epigenetic regulation in development. The epigenetic makeup of a cell, tissue or individual is much more complex than their genetic complement. Epigenetic modifications are more important for normal development by maintaining the gene expression pattern in tissue- and context-specific manner. Deregulation of epigenetic mechanism can lead to altered gene expression and its function, which result in altered tissue specific function of cells and malignant transformation. Epigenetic modifications directly shape Hematopoietic Stem Cell (HSC) developmental cascades, including their maintenance of self-renewal and multilineage potential, lineage commitment, and aging. Hence, there is a growing admiration for epigenetic players and their regulatory function in haematopoiesis. Epigenetic mechanisms underlying these modifications in mammalian genome are still not completely understood. This review mainly explains 3 key epigenetics mechanisms including DNA methylation, histone modifications and non-coding RNAs inference in hematopoietic lineage commitment and differentiation.

Keywords: Epigenetics, hematopoietic stem cell (HSC), DNA methylation, histones, miRNA, acetyltransferases (HATs), DNA methyltransferases.

1. INTRODUCTION

Blood is a highly regenerative fluid form of connective tissue, with an enormous number of cells produced every day in adult human bone marrow (BM). Hematopoiesis is a complex process of all blood cells formation from HSCs. HSCs are self renewing and can differentiate into multipotent progenitors, which in turn produce all the mature blood cells. During hematopoiesis, HSCs respond to several extracellular signals and differentiate into cells of different lineages. HSCs also respond to different stress conditions, such as blood loss, bacterial and viral infections or cytotoxicity, *via* increasing the population of HSCs and different progenitors to preserve blood homeostasis [1, 2]. HSC functions, including self renewal and differentiation are regulated by cell – intrinsic and –extrinsic factors such as transcription factors (TFs), signaling pathways and niche factors [3]. But, to date, very limited information is available about epigenetic regulatory mechanism and its impact on the regulation of hematopoiesis and blood homeostasis.

Epigenetic mechanisms involve heritable (mitotically and/or meiotically) changes in gene function that are not associated with any alterations in the nucleotide sequence of DNA [4]. Epigenetic regulation of gene function comprises DNA methylation, histones modifications and small non-coding RNA mediated interference [5]. Change in the DNA methylation pattern and histone modification can transform chromatin structure and serve as sites for the recruitment of different regulatory proteins and enzymes [6, 7]. Recent studies have also shown the role of small non-coding RNAs in post-transcriptional fine-tuning of gene expression during hematopoiesis [8, 9].

The chromatin structure and its accessibility are regulated by different chromatin-modifying enzymes which include DNA modifying enzymes, such as DNA methyltransferases (DNMTs) and ten-eleven translocation methylcytosine dioxygenases (TETs); and histones modifying enzymes such as histone acetyltransferases (HATs), histone methyltransferases (HMTs), histone deacetylases (HDACs) and histone demethy-

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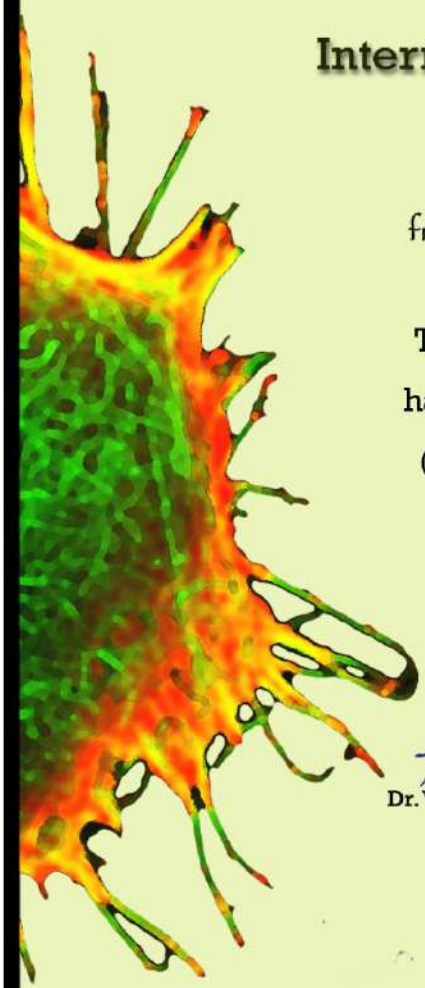

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Cannabinoid Receptor 2 Signaling: Role in Megakaryocyte Development and Neuro-immune Regulation

by Durga Shankar Sharma

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PRIMARY SOURCES

1	Durga Shankar Sharma, Indira Paddibhatla, Sanjeev Raghuwanshi, Mahesh Malleswarapu et al. "Endocannabinoid system: Role in blood cell development, neuroimmune interactions and associated disorders", Journal of Neuroimmunology, 2021 Publication	13%
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