A thesis Submitted to University of Hyderabad for the Award of

# Doctor of Philosophy (Ph.D)

**By**

**Enrollment Number 14LBPH11**


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

I, **Kovuru Narasaiah**, hereby declare that this thesis entitled **"Role of TLR-2 in megakaryocyte maturation and immune modulation"**submitted by me is based on the results of the work done under the guidance and supervision of **Prof. Gutti. 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 ordiploma.

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## CERTIFICATE

This is to certify that the thesis entitled " **Role of Toll-like receptor-2 (TLR2) in Megakaryocyte maturation and immune modulation**" submitted by **Kovuru Narasaiah** bearing registration number **14LBPH11** in partial fulfillment of the requirements for the award of Doctor of Philosophy in the School of Life Sciences is a bonafide work carried out by him under my supervision and guidance.

The thesis is free from plagiarism and 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.

Parts of this thesis have been:

**A. Published in the following publications:**

1. Kovuru N, Raghuwanshi S, Sharma DS, Swati D, Aditya P, Gutti RK. Co-stimulatory effect of TLR-2 and TLR-4 stimulation on megakaryocytic development is mediatedthrough PI3K/NF-κB and XBP-1 loop. ***Cellular Signalling***..2021 :109924.
2. Kovuru N, Raghuwanshi S, Gutti RK. Exosome mediated differentiation of megakaryocytes: a study on TLR mediated effects. ***J Thromb Thrombolysis***. 2019; 48(1):171-173.
3. Kovuru N, Raghuwanshi S, Sharma DS, Swati D, Aditya P, Gutti RK. Endoplasmic reticulum stress induced apoptosis and caspase activation is mediated through mitochondria during megakaryocyte differentiation. ***Mitochondrion***. 2019; 50:115-120.

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1. Conference attended and presented a poster in International Congress Of Cell biology (ICCB) at Hyderabad,2018.
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Further, the student has passed the following courses towards the fulfillment of the coursework requirement for Ph.D.

| Course code | Name | Credits | Pass/Fail |
|---|---|---|---|
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| BC 802 | Research ethics, Data analysis, and Biostatistics | 3 | Pass |
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## Introduction

### Overview of Hematopoiesis

Hematopoiesis is the process of formation of all blood cellular components from the hematopoietic stem cells (1). It occurs in multiple places during the development; however in adults it mainly occurs in the bone marrow, where approximately $10^{11}$–$10^{12}$ new blood cells are produced daily to maintain steady-state levels in the peripheral circulation. Though hematopoiesis occurs mainly in bone marrow in adults, but it begins as early as embryo development well before the birth and it continues throughout the life of an individual. During the first week of the embryo development, the mesoderm of the embryonic yolk sac nourishes the embryo until the placenta is fully developed. As the embryo continues to develop, the hematopoiesis process shifts to the liver and spleen, where hematopoiesis occurs temporarily, and finally it shifts to the bone marrow and creates a niche which become the primary site for hematopoiesis, referred to as definitive hematopoiesis (2).

In infants, it may also continue for some time in the spleen and liver in addition to the bone marrow. Extra-medullary hematopoiesis is increased during the stress (such as infection and spleen) (2). Treatment of mice with Lipopolysacharides (LPS), which mimics an infectious situation, induce the accumulation of hematopoietic stem cells progenitor cells(HSPCs) in the spleen (3). Hematopoiesis is a complex process where all blood cells are produced by proliferation and differentiation of very small populations of multipotent stem cells (HSCs) which also can replenish themselves by self-renewal(4).

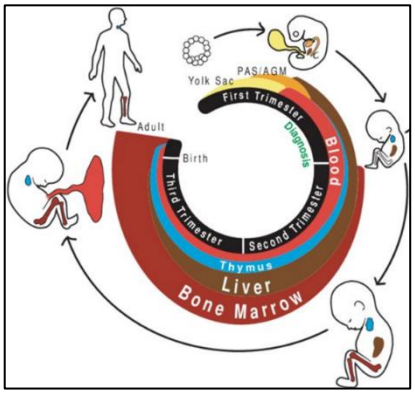


**Fig.1 Schematic representation of ontogeny of hematopoiesis in humans**. Changes in the anatomical location of hematopoiesis during human development are shown on a circular timeline. (Adapted from Muench et al., 2014)

During differentiation, the progeny of HSCs progress through various intermediate stages, generating multipotent progenitors and lineage-committed progenitors, finally forming respective mature cells (5). The first division of the stem cells leads two different daughter cells, out of which one of the daughter cells keeps the stem cell characteristics, whereas, the other daughter cell proceeds to a differentiation pathway (7). These differentiating cells further lose the capacity to self-renew. HSCs are at the apex in the hierarchy of hematopoiesis and are rare cells (ratio is 1 to 10,000 in myeloid tissues), under normal physiological conditions. But a small numbers of hematopoietic stem and progenitor cells (HSPCs) are found in the peripheral blood (PB) (ratio is 1 in 100,000 blood cells), however, the number of these cells in circulation increases during the stress situations such as infections (7).

HSCs give rise to a series of progenitors that gradually lose self-renewal and multipotent nature and produce cells of a given type. For example, multipotent progenitors (MPPs) have the capacity to give rise to either megakaryocyte (MK) and erythrocyte progenitors (MEPs) or granulocyte and macrophage progenitors (GMPs). These progenitors are capable of producing the megakaryocytes, platelets, erythrocytes, eosinophils, basophils,

neutrophils and dendritic cells, whereas, common lymphoid progenitors (CLPs) are capable of producing T, B, and NK-cells and dendritic cells also.

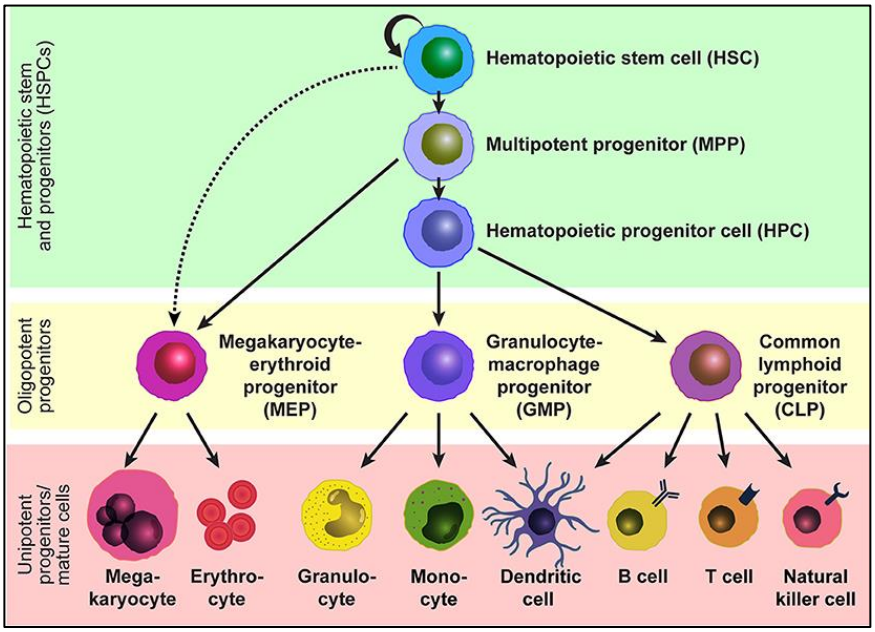


**Fig 2. Schematic representation of hierarchical hematopoiesis.** Hematopoiesis and formation of all blood cells. (Adapted from Hideyuki Oguro et al., 2019).

**Megakaryocyte**

Megakaryocyte constitutes only a small fraction of cells residing in bone marrow-derived from hematopoietic stem cells and releasing daily $10^{11}$ platelets in human adults (8). Megakaryocyte gives rise to circulating platelets (thrombocytes) through the commitment of multipotent stem cell to MK lineage differentiation. This process is characterized by unique process of endomitosis, cytoplasmic maturation followed by expansion of demarcation membrane system leading to cytoplasmic projections as pseudopodia. The release of these cytoplasmic extensions takes place as pro-platelets and platelets into sinusoids(9). Though megakaryocyte are classically known to produce platelets, they additionally, regulate HSC proliferation in both positive and negative manner (10). Moreover, megakaryocytes were shown to influence HSC quiescence via different

cytokines, such as CXCL4(11), Transforming growth factor β1 (TGFβ1)(12) and thrombopoietin(Tpo)(13,14).

Megakaryocytes also express several surface markers related to immune function including members of the TLR family (TLR1, 2, 3, 4, 5, 6, 7, 8 and 9), FcyRIIA (humans only) and CD40L (the ligand of CD40 on immune cells) which suggest their act as immune or inflammatory cells (15). Mature megakaryocytes also present antigens via expression of MHC I(16), which are transferred to platelets thereby increasing their ability to cross-present antigens. Megakaryocytes also contribute to inflammation by packaging cytokines and chemokines into their α-granules and microparticles(17). These released microparticles could contribute to pathogenesis or pro-inflammatory conditions such as systemic lupus and inflammatory arthritis(18). Another way in which megakaryocytes contribute to immunity is through emperipolesis(19). Emperipolesis is a process by which other bone marrow cells, particularly neutrophils are internalized by mature megakaryocytes while staying morphologically intact. The functional significance of this is still unclear.

**Megakaryopoiesis and Thrombopoiesis**

Megakaryocytes are the biggest cells in the bone marrow-derived from hematopoietic stem cells through myeloid lineage. It acts as a progenitor for platelet production, platelets are a nuclear small cytosolic fragments classically known to involved in hemostasis and blood clotting (20). Recent reports suggest that platelets play an important role in inflammation and immunity (21). Single mature megakaryocyte gives 3000-5000 platelets(22). Before producing platelets megakaryocytes undergo unique maturation process which mainly

includes polyploidisation, demarcation membrane formation (DMS) and cytoplasmic maturation(23).

Megakaryocyte acquires polyploidy where cell has more than one set of chromosome. Ploidy ranges from 16N to 128N however mature megakaryocyte could acquires an average ploidy of 16N to 128N during the stress conditions(24). Megakaryocyte acquires polyploidy by a process called endomitosis, where DNA gets replicated due to failure in both karyokinesis and cytokinesis, consequently cells become enlarged with multi lobulated nucleus(25). Megakaryocyte develops a demarcation membrane system (DMS) during maturation which is necessary for the platelet production process (26). Cytoplasmic maturation includes synthesis of proteins and biogenesis of granules such alpha, dense granules and expansion of organelles for production of large number of platelets (27).

During the terminal stage, megakaryocyte forms cytoplasmic extensions (pseudopodia like structures) that transit through the endothelial line of the blood vessels wall and platelets are released into the sinusoids (28). All these processes are under the control of the principal cytokine thrombopoietin (29).

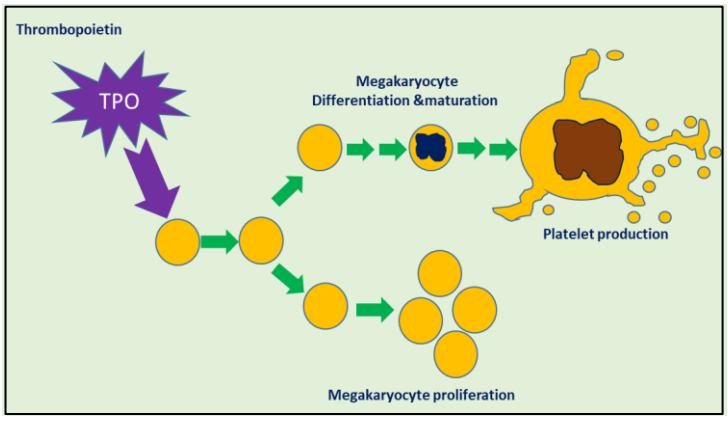


**Fig 3. The four main steps of platelet production**: Thrombopoietin, megakaryocyte progenitor proliferation, megakaryocyte differentiation, maturation, pseudopodia like structure formation and platelet release.

**Platelets**

Platelets are enucleate cytoplasmic fragments derived from the mature megakaryocyte, popularly known to be involved in thrombosis and hemostasis. In healthy individual, platelets counts are in the range of 150,000-450,000/μL of blood(30). Platelet counts more than 450,000/μL is known as thrombocythemia, whereas, platelet count less than 150,000/μL is known as thrombocytopenia (22). In normal conditions, platelets survive 8 to 11 days in circulation and older platelets will be removed by macrophages in spleen and kupffer cells in the liver(30). Although being enucleated, platelets are metabolically active and contain numerous functional organelles such as mitochondria (31), Golgi apparatus and dense tubular system which are orthologous to nucleated cells endoplasmic reticulum. These contain wide array of adhesion molecules, surface receptors and numerous granules. The mRNA present in them helps in synthesizing limited amount of proteins (32). However, a large number of preformed and inherited molecules from megakaryocytes can be released upon stimulation (31). Recent report of platelet proteome analysis suggested that it contain proteins beyond hemostasis (including several proteins not related to the hemostasis).

Platelets play a key role in immunity, angiogenesis and wound healing together with other growth factors. Besides their undiscussed role in hemostasis and thrombosis, platelets are also key effector cells capable of assisting and modulating inflammatory reactions and immune responses(33). Platelets play a sentinel role in immune surveillance by recognizing danger signals from pathogens and cell damage through the expression of toll-like receptors (TLRs) on its surface and internal compartments. Platelets express all 10 TLRs (34) and their signalosome (35), including adaptor proteins and transcription factors. The activation of these receptors in platelets triggers immune thrombosis and inflammatory response which participate in the host's response to bacterial and viral

infections, linking thrombosis with infection and immunity (26). Platelets are able to bind to bacteria, virus particles and their related derivatives proteins and nucleic acids (bacterial DNA, viral double-stranded RNA and single-stranded RNA and un-methylated DNA) which helps in mounting the tightly controlled immunity. Platelets were reported to be involved in the initiation and progression of the platelet-neutrophil interactions which are known to induce the release of neutrophil extracellular traps (NETs) with the help of both platelet soluble factors and adhesion components for the recruitment of the other leucocytes, this enhance the activity of host inflammatory and immune responses(36).

**Signaling pathways involved in Megakaryopoiesis**

Megakaryocyte development is regulated at multiple levels by various cytokines, the most key factor is Thrombopoietin (TPO), upon binding to the c-mpl receptor it induces the HSCs to megakaryocyte commitment and maturation (37). All progenitor cells are primed to become megakaryocytes, including HSCs, CMPs, and MEPs, which express c-mpl receptor. TPO is constitutively produced by the liver and circulates in plasma, where it is sequestered by circulating platelets in a c-mpl–dependent manner. Reduction in platelet counts leads to increased levels of circulating TPO which is free to exert its stimulatory affects on bone marrow HSCs, thus increasing megakaryocyte (and platelet) numbers. When there is sufficient number of platelets in circulation, platelets c-mpl is bound to the TPO, due to which megakaryocyte commitment decrease and hence platelets feedback mechanism (8). TPO signaling results in internalization of the c-mpl and TPO receptor-ligand complex leading to initiation of multiple signal transduction pathways including JAK2 (Janus kinase), STAT3 (signal transducer and activator of transcription)/STAT5, MAPK (mitogen-activated protein kinase)/ERK (extracellular signal-regulated kinases),

and PI3K (phosphoinositide 3-kinase)/AKT (protein kinase B). Specifically, TPO induce phosphorylation of JAK2 which further phosphorylates downstream targets including activation of the transcription factors STAT3/STAT5. Signaling through these pathways cause downstream activation of megakaryocyte-specific transcription factors and regulation of expression of megakaryocyte-specific genes (38). Studies in TPO and c-mpl knockout mice show a global decrease in HSCs re-populating capacity, with particularly drastic reduction in megakaryocytes and platelets. However, TPO and c-mpl knockout mice can make a small number of platelets (39). Besides, humans with a complete loss of functional c-Mpl have a median platelet count of $21\times10^9$/L or below, suggesting that TPO-independent pathways of megakaryopoiesis in mammals exist.

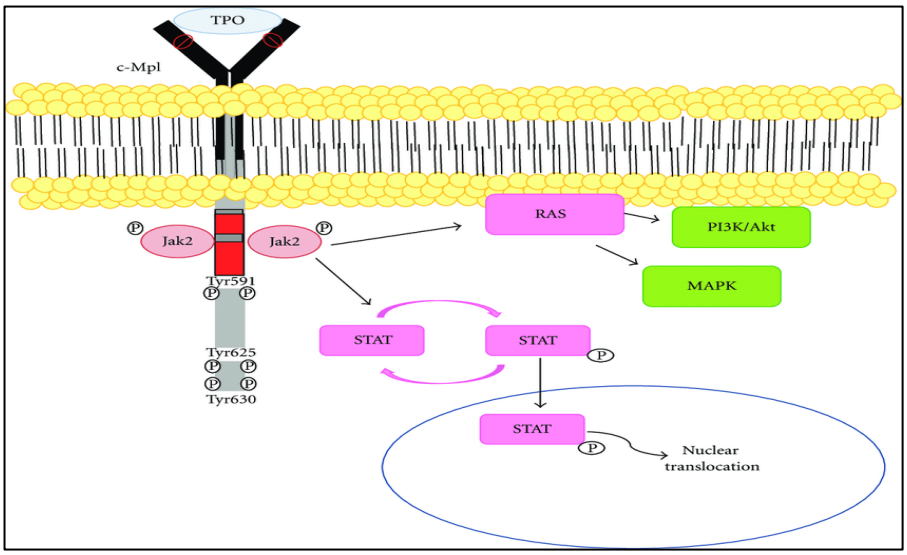


**Fig 4. C-Mpl/TPO signaling pathway**. TPO signals to its receptor, c-Mpl, and induces the downstream signaling cascades: STATs, PI3K, MAPKs and extracellular signal regulated kinases-1 and-2. [**Adapted from Kamonnaree Chotinantakul et al, 2012**].

**Thrombopoietin (TPO) independent pathways of megakaryopoiesis**

Although residual platelet production persists in the absence of TPO signaling (39), TPO-independent regulators of megakaryopoiesis remain elusive. However, some alternative

pathways have been shown to enhance this process. The IL (interleukin) family was found to influence megakaryopoiesis (40). Before the discovery of TPO injections, IL-1β and IL-6 increased platelet counts *in vivo*. However, these cytokines were ultimately found to be associated with increased plasma TPO (41), suggesting that ILs upregulate TPO production, rather than directly stimulating megakaryopoiesis. IL-3 also increases megakaryocyte colony size and numbers *in vitro*, but does not affect megakaryocyte maturation. IL-1α is perhaps the only known IL to have a possible TPO-independent phenotype, as it acts on mature megakaryocytes to cause a rupture and thus shedding of pro-platelets into the bone marrow during the inflammatory stress (42). More recently, other cytokines and chemokines not in the IL family have been identified as megakaryocyte-promoting factors. IGF (insulin-like growth factor)-1 promotes $CD34^+$ differentiation towards the megakaryocyte lineage, a process mediated through AKT signaling with the assistance of steroid receptor coactivator 3 (43). *In vivo* administration of IGF-1 increases, platelet counts in both lethally irradiated mice and c-mpl knockout mice, suggesting a TPO-independent phenotype. Another chemokine, CCL5 (C-C motif chemokine ligand 5 and RANTES (small inducible cytokine A5), has also recently been shown to increase megakaryocyte ploidy, pro-platelet production by suppressing apoptosis *in vitro* (44). Inflammatory cytokines stimulates HSCs towards the megakaryocytic lineage(45). Notch signaling pathway plays an important role as a mediator of megakaryopoiesis (46). Notch signaling is required for murine megakaryocyte commitment from the HSC and increases $CD41^+$ cells, Common myeloid progenitors (CMPs) and megakaryocyte erythroid progenitors (MEPs), potentially via AKT signaling and consequent suppression of FOXO (fork head box O) (47). Downregulation of receptor Notch4 by RUNX1 (runt-related transcription factor) is required for normal

megakaryocyte development in human cells. Specifically, the deletion of the RUNX1-binding site in the intron of NOTCH4 causes an increase in Notch4 expression and inhibition of megakaryocyte differentiation (48). Pharmacological inhibition of Notch signaling enhances human megakaryopoiesis *in vitro*. Overall, megakaryopoiesis appears to be regulated by Notch signaling at different stages of development.

Murine bone marrow treated with LPS also showed an increase in the levels of thrombopoietin and cytokines, which are important factors for thrombopoiesis (42). Thus, it is possible that inflammation and infection can modulate platelet production through TLRs. Megakaryocyte maturation can be monitored by the increase in megakaryocyte specific markers CD41, CD42b and CD61 expression levels (49). TLR4 levels also increased during the development of megakaryocyte (50). In knockout mouse of TLR4, megakaryocytes showed decreased platelet production (51). TLR3 recognizes dsRNA associated with viral infection(52). Thrombocytopenia was seen as a frequent complication of viral infection stimulation of megakaryocytes with two synthetic agonists of TLR3, viz. Poly (I: C) and Poly (A:U), by activating the Nuclear factor-κB (NF-κB), phosphoinositide 3-kinase (PI3K-AKT), extracellular signal-related kinase (ERK1/2) and p38 pathways (53). TLR3-megakaryocyte activation resulted in reduced platelet production *in vitro* and interferon-β release through the PI3K-AKT and NF-κB signaling pathways (54). TLR2 stimulation is also shown to induce megakaryocyte maturation (55).

**Toll-like receptors (TLRs)**

Toll-like receptors are single transmembrane germline-encoded pattern recognition receptors known to play key role in innate immunity. There are 10 toll-like receptors characterized in the humans (56) and 12 TLRs in the mouse(57). TLRs can be generally

divided into two subgroups based on their cellular location and PAMP recognition. The first subgroup is the cell surface receptors composed of TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 which are expressed on the plasma membrane and destined to sense the extracellular pathogens. The second subgroup consists of TLR3, TLR7, TLR8, and TLR9 which are expressed in intracellular compartments like endosomes and recognize bacterial and viral nucleic acids (58). Initially, TLRs were identified in *Drosophila* and later in humans (56). Classically, TLRs are known to be present in sentinel cells (cells which are involved in defense mechanism) which help to distinguish self from non-self by detecting some of the conserved molecular patterns of the diverse microbes or pathogens(59). Each TLR has got its specific ligand or a set of ligands.

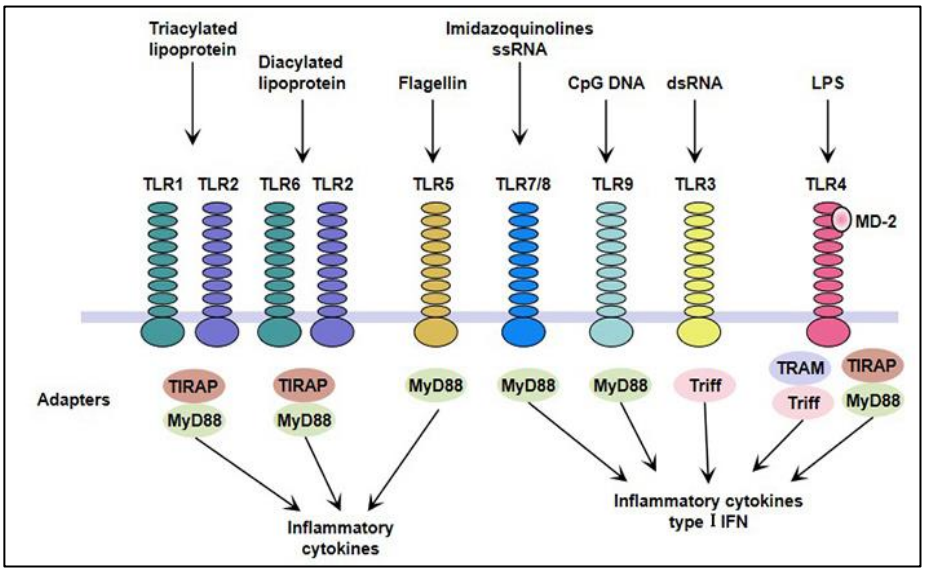


**Fig-5 Toll like receptors (TLRs), its ligands and TLR-mediated immune responses (Adpted from Cusbio.com).**

TLRs consist of three domains in their structure, extracellular leucine rich domain which helps in binding the pathogen-associated molecular patterns (PAMPs), the transmembrane domain and intracellular toll-interleukin 1 receptor (TIR) domain that is required for downstream signal transduction (60). All the toll-like receptors finally converge on the

NF-κB transcription factor which induce the inflammatory cytokines and anti-bacterial proteins and thereby help in tailoring the immunity (61).

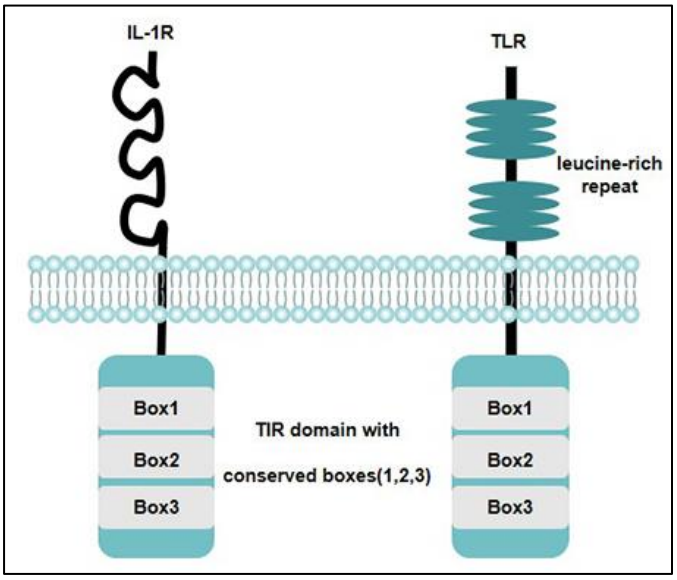


**Fig-6 Structural characteristics of toll-like receptors (Adapted from Cusabio.com)**

**TLRs in hematopoiesis.**

Hematopoietic stem cells HSCs (long-term $LKS^+$ $Flk2^-$ and short-term $LKS^+$ $Flk2^+$ HSCs), as well as lineage-restricted progenitors (CLPs, CMPs, GMPs and MEPs) express TLR4 and its associated accessory molecules, CD14 and/or TLR2 (62). *In vitro* exposure to LPS (a TLR4 agonist) and Pam3CSK4 (synthetic TLR2 agonist), detected by TLR1/TLR2 heterodimers stimulate cells to enter cell cycle and acquire myeloid lineage markers (63). Leukocytes arise from the hematopoietic stem cell (HSC) which are known to self-renew and produce all blood cell types. During homeostasis, the process of HSC self-renewal, as well as the production of lineage-committed progenitors are tightly controlled to maintain daily blood cell production requirements (4). Many cytokines, cell-cell interactions and transcription factors "fine-tune" the proliferation of hematopoietic stem and progenitor cells (HSPCs) and their differentiation into mature myeloid or lymphoid cells. Upon

infection (TLR stimulation), or during other forms of immunological stress, there is an increased demand for leukocytes to help in combating the infection. The cells which are killed by invading microbes or used up during the immune response are replenished to increase immune surveillance(62). The adaptive immune system meets this demand by clonal expansion of T and B-cells. Increased supply of most innate immune cells which have a limited lifespan and must be regularly replenished by increased hematopoiesis, skews towards myeloid lineage which is referred to as emergency myelopoiesis (62). Acute infection usually triggers the mobilization of myeloid cells, in particular neutrophils and monocytes. Studies showed that the effect of infection and inflammation on hematopoiesis can also result in the enhanced production of granulocytes (64) termed emergency granulopoiesis (65). Proliferation and differentiation of HSPCs in the bone marrow takes place to maintain the supply of myeloid cells during most bacterial, viral, and fungal infections. Myelopoiesis becomes the predominant form of cellular production, with the compromised development of other lineages (lymphoid and erythroid). Infection induced myelopoiesis is also commonly accompanied by alterations in the cellular composition and/or functional characteristics of bone marrow HSPCs.

TLRs have both direct and indirect effects on hematopoiesis as HSCs have these receptors that sense the infection and scalps the bone marrow hematopoiesis for the production of needed cells for mounting the effective immunity. TLR induced cytokine by mature cells such as macrophages and neutrophils also drives the TLR mediated hematopoiesis indirectly (62). Myeloid progenitors stimulated by TLR ligands produce monocytes and macrophages, while TLR agonist-stimulated lymphoid progenitors produced DCs (66). TLR-mediated signaling in HSPCs causes changes in the expression of transcription factors consistent with increased myeloid differentiation (62).

**TLRs significance in platelets**

Though the platelets are enucleate cellular fragments that doesn't have nucleus and genome and have limited translational capacity (67). Platelets express numerous receptors including the 10 toll-like receptors as well as pathogen sensors of other nature (Ig- or complement receptors) and their signalosomes contain hundreds of secretory products (68). These receptors together with the secretory products are involved in the platelet functional responses. Platelets to secrete large amounts of cytokines, chemokines and related molecules, which appear intimately related to the importance of the platelets in inflammation (69).

These receptors permit platelets to bind infectious agents and deliver differential signals leading to the secretion of cytokines/chemokines. Platelets play a sentinel role in immune surveillance by recognizing danger signals from pathogens and damaged cells through the expression of Toll-like receptors (TLRs) on its surface and internal compartments(34). Among the responses elicited by the activation of platelet TLRs, are adhesion and aggregation of mixed platelet leukocyte aggregates (70). In atherosclerosis and thrombosis related myocardial infractions conditions, increased TLR1, TLR4 on platelets and a modest increase in TLR2 levels were observed (71). TLR2 agonist Pam3CSK4 and TLR4 agonist LPS showed increased degranulation or surface expression of P-selectin CD61P which is considered as the platelet activation marker (72).

**TLRs during megakaryocyte development**

Megakaryocytes were classically thought to be involved in platelet production which has key role in hemostasis(37), thrombus formation. Platelet number and integrity is crucial for the wellbeing.

Megakaryocytes were thought to be under the control of the Thrombopoietin (TPO). TLRs in megakaryocyte are important for the maintenance of the bone marrow hematopoiesis. Mice without TLR4 had fewer circulating platelets (51) with lower RNA content and were less responsive to the thrombin-activated expression of P-selectin, but were equally sensitive to aggregation or ATP secretion. Treatment of wild-type mice with Pam3CSK4 resulted in a return to normal platelet levels and an increase in megakaryocyte maturation, which did not occur in the $TLR2^{-/-}$ mice(55). Inflammatory conditions are known to increase TPO levels, thereby increasing the number of circulating platelets. IL-6 has been shown to increase TPO levels *in vitro* and *in vivo,* however, current studies suggest that all the 10 TLRs present in humans are also seen on megakaryocytes. TLR2 and TLR4 signaling induce the megakaryocyte maturation through the signaling pathways MAPK and PI3K-AKT pathway along with the transcription factors. Megakaryocyte maturation factors such as GATA-1 and NFE-2 were induced in TLR-2 stimulated megakaryocyte (55). Thrombopoietin induce the increased megakaryocyte commitment, maturation and ploidy from the CD34+ cells in the presence of zymosan and LPS, specific agonists for TLR2 and TLR4 respectively (73). However, the mechanism of the TLR2-mediated megakaryocyte maturation and its mechanism is not clear.

## Materials and methods

### Cell culture

For our studies, we have used DAMI (Megakaryoblastic cells) cell line as a model system. These cells are derived from the peripheral blood of a patient with megakaryoblastic leukemia. They have characteristic of megakaryoblasts or immature megakaryocytes, displaying many of the morphologic and biochemical features of the megakaryocytic lineage. DAMI cells are suspension cells obtained from ATCC (#CRL-9792). These were grown in Roswell Park Memorial Institute (RPMI)-1640 medium (GIBCO), 10% fetal bovine serum (FBS), 1% antibiotic (Anti-anti Life Technologies, Inc.) and maintained in 5% $CO_2$ incubator at 37$^{o}$C. These cells can be induced to differentiate to the megakaryocytic lineage. DAMI cells increase their ploidy and the expression of glycoprotein IIb/IIIa and glycoprotein Ib, both are characteristic markers of the mature megakaryocyte.

### Determination of intracellular ROS

2, 7-dichlorodihydrofluorescein-diacetate (H2DCFDA) is a cell-permeable dye that is used to monitor the intracellular ROS levels from the TLR-stimulated Dami cells ($1\times10^6$ cells/ml). We measured the ROS from stimulation conditions such as TLR2/TLR4 stimulation alone or TLR2 and TLR4 co-stimulation for 24h. Briefly cells were washed with PBS and incubated with H2DCFDA dye (10µM) in PBS for 15 min in dark at room temperature. Green fluorescence of 2, 7-dichlorofluorescein (H2DCF) was measured using flow cytometry (BD LSR FORTEZZA) and mean fluorescence intensity (MFI) represented as bar graphs and as well as by using the fluorimeter and results were represented as fold difference.

**Microscopic analysis**

Dami cells were propagated in RPMI medium containing 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Life Technologies, Inc.). To induce the TLR2 signaling, Dami cells were treated with zymosan (10µg/ml) as per the manufacture's guidelines. LPS (100 ng/ml) was used for TLR4 stimulation and together were used for co-stimulation for 48 hours and further these cells were observed under an inverted light microscope. Matured megakaryocyte cells were counted for upto 15 frames using inverted light microscopy.

**Giemsa stain analysis**

Dami cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Life Technologies, Inc.). To induce the TLR2 signaling, Dami cells were treated with 100µg of zymosan (10µg/mL) for 48 hours and cells/cell-derived extracellular vesicles were harvested and washed with 1xPBS. Giemsa Stain was diluted 1:5 with deionized water, cells were fixed on the coverslip with methanol and incubated for 5–7 min at room temperature. Fixed cells were stained with Giemsa for 20 min followed by rinsing with distilled water. Air-dried slides were observed under an Olympus fluorescence microscope.

**Total RNA isolation**

The RNA extraction was performed by using Qiazol (Qiagen) to lyse the cells. Chloroform (Sigma) was added and the samples were thoroughly mixed followed by centrifugation at 12000g for 15min. This resulted in phase separation and the RNA was obtained in the upper soluble phase. This phase was transferred to a Column (Qiagen) and the RNA was washed with RPE and RWT buffers which were bound to the filter in the Spin Column. Finally the

RNA was eluted using RNase free water. RNA was quantified using a Nano Drop® ND-1000 spectrophotometer (Thermo Scientific).

**C-DNA synthesis**

**Preparation of cDNA from mRNA**

Gene expression can be quantified by measuring the expression of its transcribed mRNA. Before mRNA can be quantified by qRT-PCR (qPCR), the mRNA needs to be transcribed into cDNA. The template for the cDNA synthesis is mRNA and the primers bind either to the poly (A) tail of the mRNA or randomly to the RNA and transcribe it to cDNA. The enzyme reverse transcriptase is used for this.

**Reaction I**

| Sr No | Component | Volume |
|---|---|---|
| **1** | Oligo dT(50uM)/ random hexamers | 1 µl |
| **2** | 10uM dNTPs | 1µl |
| **3** | RNA 10pg-50ug | Variable |

**Table 1: Reverse transcription reaction 1 component for mRNA into cDNA**

After cDNA synthesis, we made up the volume to 13 µl reaction with distilled water and incubated at $65^0$C at 5min.

**Reaction II**

| Sr No | Component | Volume |
|---|---|---|
| **1** | 5x first standard Buffer | 4µl |
| **2** | 0.1M DTT | 1 µl |
| **3** | RNase Out | 1 µl |
| **4** | Super Script III RT | 1 µl |

**Table 2: Reverse transcription reaction 2 components for mRNA into cDNA.**

Collected Reaction-I by brief centrifugation and added 7 µl of Reaction II followed by brief mixing. We Incubated the samples at 50$^{o}$C for 30 min and inactivated the reaction by heating at 70$^{o}$C for 15 min.

**qRT- PCR analysis**

The first-strand cDNA was used as a template in qRT-PCR with SYBR Green Master Mix in ABI StepOne Plus system (Applied Biosystems) to detect the TLRs (TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9), MK-maturation markers (CD41, CD42b, CD61) and inflammatory cytokines (IL-1β, IL2, IL6, IL8, IL10) expression with respective specific primers. The cycling program was set as follows: denature at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 72 °C for 30 s. The specificity of the PCR products was verified by melting curve and agarose gel analyses. Each sample was assayed in triplicate, normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA, and expressed as relative to control (n=3) expression. Analysis of XBP-1 splicing was determined using PCR with an ABI Step One Plus system (Applied Biosystems). Followed by 2.5% agarose gel electrophoresis. (n=3) were normalized against GAPDH, and expressed as relative fold change.

**Immunoblot analysis**

Total protein was extracted from the Dami cells in both control and TLR stimulated cells after 30 minutes for Phospo-proteins and 2 days for Runx-1 using radio immunoprecipitation assay (RIPA) buffer (Sigma) in the presence of a mixture of protease inhibitors (Roche Applied Sciences). The extracts (50 µg) were subjected to separation on a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane (Millipore) by electro-blotting. The membranes were blocked for 1h at room temperature in TBST buffer with 5% non-fat dried milk. These blots were incubated with specific primary antibodies

(1:1000) specific to NF-κB-p65, P-AKT, P-ERK1/2, CD81, CD41, and GAPDH (Sigma) overnight at 4°C. The blots were washed three times for 15 min with TBST buffer and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10000), Goat anti-rabbit IgG and anti-mouse IgG (Cell Signaling) for 1h at room temperature. After washing these blots were developed using enhanced chemiluminescence reagents (ECL, Pierce Biotechnology, Inc., Rockford, IL) and visualized using versadoc instrument (Bio-Rad). To quantify protein expression levels, we used Image-J software and measured the integrated density of the protein signal normalized to GAPDH levels.

**Statistical analysis**

Three independent experiments were carried out. Two-tailed Student T-tests were used to determine the statistical significance of differences between control and TLR2 stimulation. Two way ANOVA was used to compare the multiple groups in co-stimulation studies to compare multiple samples. Data were expressed as mean ± SD. P-value $<0.05$ was considered to be statistically significant.

## Primers List

| Gene | Forward primer | Reverse primer |
|---|---|---|
| CD41 | 5’-GCATGGTTCAACGTGTCCTC-3’ | 5’-TTGAAGAAGCCGACCTTCCA-3’ |
| CD42b | 5’-TTCCCCACCAAAGCCCATAC-3’ | 5’-GGCTTGGGGTTGGTTCAGTA-3’ |
| CD61 | 5’-ACCAGTAACCTGCGGATTGG-3’ | 5’-TCCGTGACACACTCTGCTTC-3’ |
| TLR-1 | 5’-AGATTTCTTGCCACCCTACTG-3’ | 5’-GCTCAACCCCAGAAATTTTAG-3’ |
| TLR-2 | 5’-GGTCTGCCTCGAGTTTCCAA-3’ | 5’-GGAGGAATGAGCAATCAAGCC-5’ |
| TLR-3 | 5’-GCATTTGTTTTCTCACTCTTT-3’ | 5’-TTAGCCACTGAAAAGAAAAAT-3’ |
| TLR-4 | 5’-CGAGGAAGAGAAGACACCAGT-3’ | 5’-CATCATCCTCACTGCTTCTGT-3’ |
| TLR-5 | 5’-AGCTTCAACTATATCAGGACA-3’ | 5’-TGGTTGGAGGAAAAATCTAT-3’ |
| TLR-6 | 5’-CTTCCATTTTGTTTGCTTAT-3’ | 5’-AGCGGTAGGTCTTTTGGAAC-3’ |
| TLR-7 | 5’-AAACTCCTTGGGGCTAGATG-3’ | 5’-AGGGTGAGGTTCGTGGTGTT-3’ |
| TLR-8 | 5’-CTGTGAGTTATGCGCCGAAGA-3’ | 5’-TGGTGCTGTACATTGGGGTTG-3’ |
| TLR-9 | 5’- CAACAGCTCTCAGTCCCCTG-3’ | 5’-CAACCCGTCACTGTTGCTTG-3’ |
| IL-1β | 5’-TCCCTAGGAAAAGCTGGG-3’ | 5’- CACTACCCTAAGGCAGGCAG-3’ |
| IL-2 | 5’- AGGCCTGTCCAAAAGTCCTC-3 | 5-AGGCTAATTACATGCATGGGT-3’ |
| IL-6 | 5’-AAAGAGGCACTGGCAGAAAA-3’ | 5’-CAGGGGTGGTTATTGCATCT-3’ |
| IL-8 | 5’-AGGCCTGTCCAAAAGTCCTC-3’ | 5’-CTAATTACATGCATGGGT-3’ |
| IL-10 | 5’-TGGAGAGAGTGTGGGAACCT-3’ | 5’-CCACCACCTTCCATGCTTTG-3’ |
| Bip | 5’-ACCACCTACTCCTGCGTC-3’ | 5’-TTGGAGGTGACCTGGTTCT-3’ |
| XBP-1 | 5’GAGAACCAGGAGTTAAGACAGCGC3’ | 5’-TCCCAGAGGTCTACCCAGAAGGA-3’ |
| GAPDH | 5’-GGATTTGGTCGTATTGGG-3’ | 5’-GGAAGATGGTGATGGGATT-3’ |

**Aim and Objectives of the Research Work**

Toll Like Receptors (TLRs) are pattern recognition receptors known to play an important role in innate immunity. These TLRs sense specific patterns of pathogens and elicit specific signaling pathways that helps in immune establishment. Megakaryocytes are bone marrow cells derived from Hematopoietic stem cell (HSC) lineage hierarchy, functionally involved in platelet production. These platelets play an essential role in blood clotting and hemostasis, however recent reports have projected that platelets do have a significant role in inflammation and immunity.

Classically megakaryocyte development and platelet production are governed by Thrombopoietin (TPO). Recent publications suggest that TPO receptor, c-Mpl, (myeloproliferative leukemia virus oncogene) is dispensable for platelet production. In addition, inflammatory cytokines IL-6 and IL-1β regulates megakaryocyte maturation. TLRs are involved in innate immunity and induces inflammatory cytokines that modulate immunity, inflammation and maturation of the immune cells. Thus, we checked the available literature and found that both the megakaryocyte and platelets were reported to have TLRs. TLRs on platelets aids in sensing the bacteria pathogens and they get activated and release their internal components which helps in recruiting other cells of the immune system and helps in establishing the immunity, however TLRs role in megakaryocyte maturation is not clearly understood. On the basis of the above literature, we framed following three objectives:

1. Determine the TLR2 regulatory mechanisms during megakaryocyte maturation.
2. Elucidate the effect of TLR2 induced megakaryocyte microparticles (MPs) on its maturation.
3. Study the TLR2 and TLR4 co-stimulation effect on megakaryocyte maturation.

To achieve these objectives we have used the DAMI cell line as a model system for our studies. Zymosan and Lipopolysaccharide (LPS) are used as ligands for TLR2 and TLR4 respectively to prove ours hypothesis.

**Objective 1. Determine the TLR2 regulatory mechanisms during megakaryocyte maturation.**

Toll like Receptor-2 (TLR2) is a single span transmembrane protein belonging to pattern recognition receptors family. TLR2 is a very potent immune-modulatory receptor that can sense the diverse pathogen-associated molecular patterns as ligands(74). Toll-like receptor-2 can dimerize with either TLR1 or TLR6 depending on the nature of the infectious agent (75). Though TLR2 and TLR4 on platelets induce expression of the p-selectin with platelet activation and aggregation with increased platelet count, however the role of the TLR2 in megakaryocyte maturation is not clearly elucidated. In this objective we want to understand the effect of TLR2 on megakaryocyte maturation. We have used the Dami cells (megakaryoblastic cell line) as a model system with zymosan as TLR2 agonist. Dami cells were cultured in RPMI-1640 media with 10% FBS and 1% antibiotic in $CO_2$ incubator under 5% $CO_2$ at 37°C. We initially profiled all the TLRs on megakaryocyte upon stimulation with TLR2 agonist zymosan using qRT-PCR analysis. Upon TLR2 stimulation, increased expression of the TLR2 and its partner's Toll like receptors such as TLR1, TLR6 and TLR4 were increased significantly at transcript level's compared to the untreated control cells. However, there was no significant change in the other non-specific TLRs such as TLR3, TLR5, TLR7 and TLR9 (Fig.1). We further checked the effect of TLR2 stimulation on the protein expression of TLR-2 by flow cytometry. Upon TLR2 stimulation, the expression of TLR2 at protein levels were increased compared to the untreated control cells (Fig.2A), the same was confirmed by immunofluorescence analysis (Fig.2B).

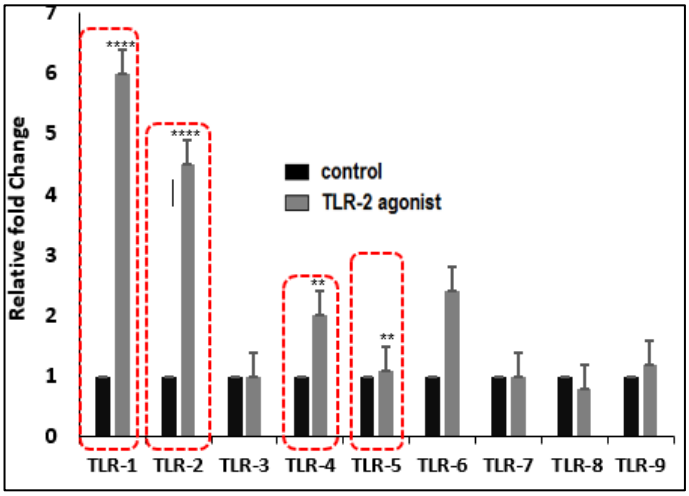


**Figure 1. Profiling of TLRs.** Upon treatment with TLR-2 agonist, TLRs were profiled using the qRT-PCR analysis.

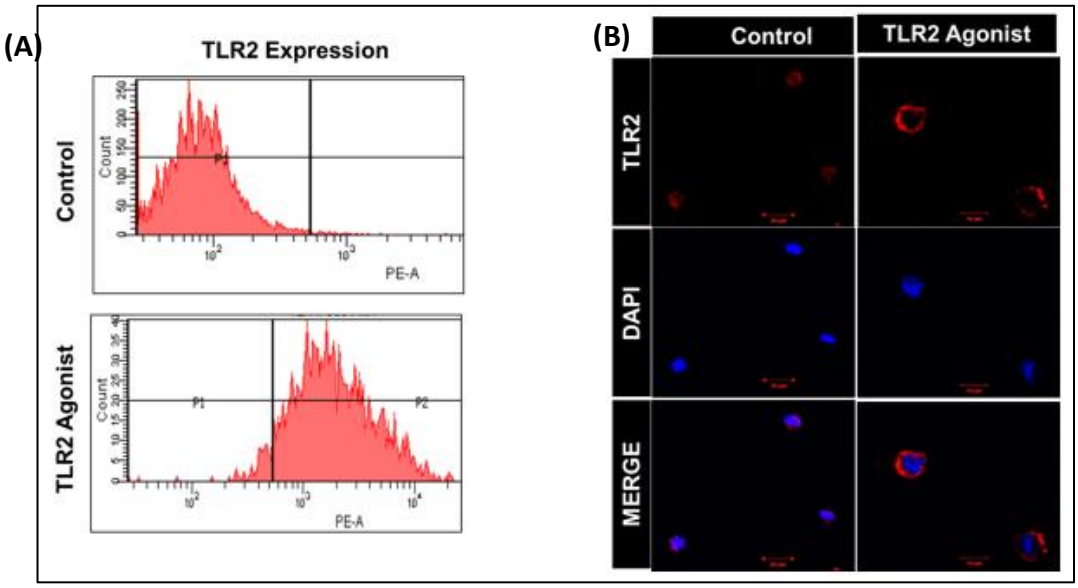


**Figure 2. TLR2 stimulation induces the TLR2 expression on the megakaryocyte membrane**. Dami cells were treated with TLR2 agonist and after 48 h TLR2geneexpression was studied by (A) flow cytometry, (B) confocal microscopy.

Mature megakaryocyte undergo polyploidization, consequently, enlarged cell size (Fig.3) was noticed (76). As megakaryocyte undergoes maturation, they increase its membrane integrin's expression, consequently cells become partially adhered to the substratum which is considered as one of the important feature of mature megakaryocyte. Upon TLR2 stimulation, Dami cells size and adherence to substratum were increased (Fig.3).

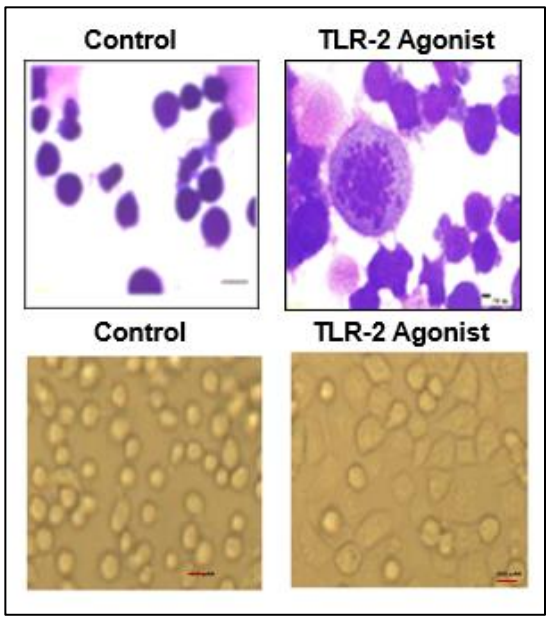


**Figure 3. TLR2 stimulation increases the megakaryocyte cell size and adherence .** Upon TLR2 stimulation cell size and adherence were increased, this increased cell size and adherence are the key phenotypic changes of mature megakaryocyte.

We further checked the integrin's responsible for the megakaryocyte substrate adherence such as CD-41, CD-61, and CD-42b. Dami cells were treated with TLR2 agonist zymosan for 48 hrs. and expression of the integrin's such as CD-41, CD-61, and CD-42b were increased significantly compared to untreated control cells at transcript level by qRT-PCR analysis (Fig.4).

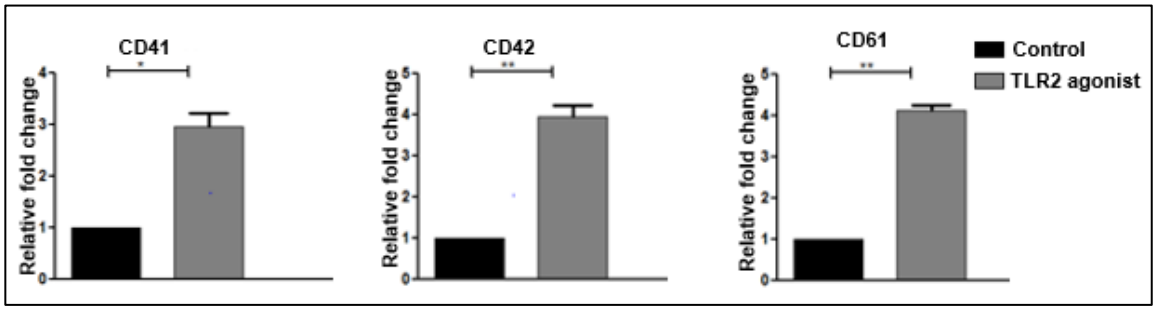


**Figure 4.TLR2 stimulation increases the megakaryocyte maturation markers.** Upon TLR2 stimulation the levels of integrin's CD-41, CD-61 and CD-42b levels go up compared to control untreated cells at transcript levels.

In sentinel cells, TLRs are known to induce transcriptional factor NF-kBp65 which upon translocation to nucleus induce the inflammatory cytokine (77). To investigate the TLR2 effect on NF-kBp65 in megakaryocytes, Dami cells were treated with TLR2 agonist for 30 minutes and total protein was isolated using RIPA buffer with protease and phosphatase inhibitors. Western blot analysis showed increased levels of the NF-kBp65 and pIkB levels in TLR2 stimulated compared to untreated control cells (Fig.5). This increased NF-kBp65 may be playing an important role in inflammation or infection mediated emergency thrombopoiesis.

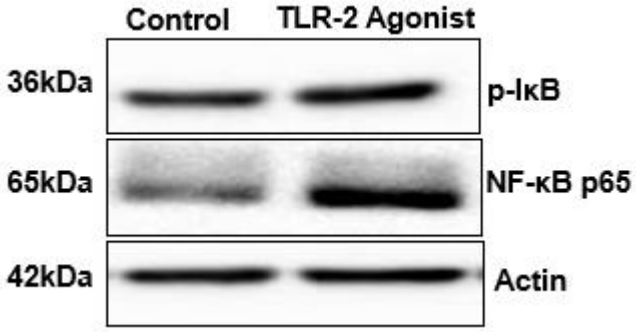


**Figure 5. TLR2 stimulation induces the NF-kBp65 level's in megakaryocyte**. Upon treatment with TLR2 agonist the levels of the phosphorylated NF-kBp65 and pIkB levels were up regulated.

NF-kBp65 is known to act as a transcription factor and induces the inflammatory cytokines in sentinel cells. We therefore investigated the effect of TLR2 stimulation on the cytokine secretion in megakaryocyte. To analyze that Dami cells were treated with TLR2 agonist followed by spent medium collection and Enzyme linked immunosorbent analysis (ELISA) was performed. Increased secretion of IL-2, IL-6 and IL-10 levels were observed, whereas, IL-12 levels were not significantly changed (Fig.6). IL-6 is known to play important role in inflammation mediated megakaryocyte development and platelet production. Increased secretion of the inflammatory cytokines may be important for the

TLR2 induced megakaryocyte maturation. These results are in line with the previous reports suggesting that IL6 has binding site in the Thrombopoietin promoter and thus induce the megakaryocyte maturation by inducing the Thrombopoietin (TPO) (78).

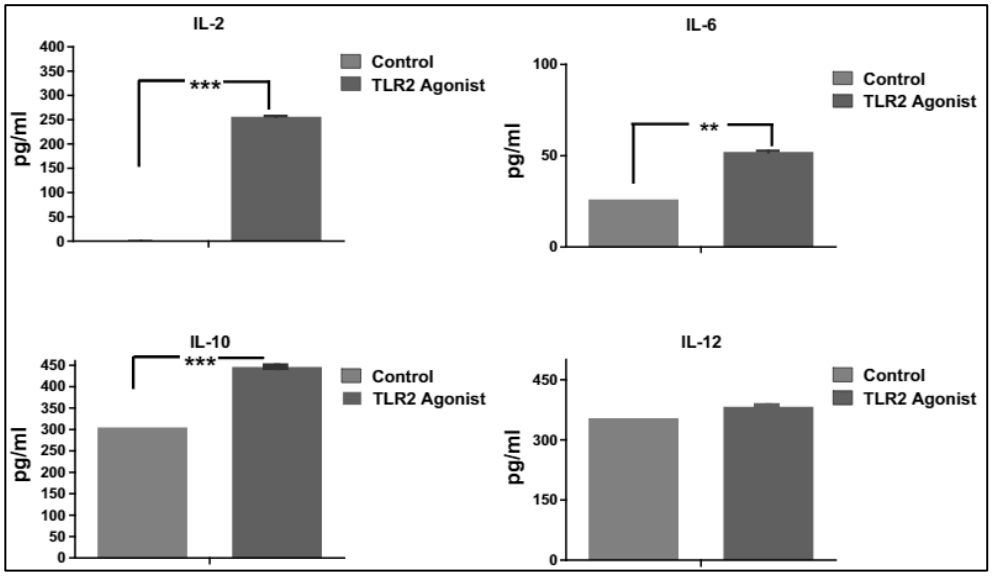


**Figure 6. TLR2 increases cytokine production.** Dami cells were treated with TLR2 agonist and cytokines IL-2, IL-6, IL-10, and IL-12 released into the culture medium was quantified by Quantikine immune assays kits (R&D Systems).

Reactive oxygen species have shown to play an important role in all stages of megakaryocyte maturation (79). Therefore we checked the ROS levels during TLR2 mediated megakaryocyte maturation. To investigate that Dami cells were treated with zymosan for 24 h and the ROS levels were measured by flow cytometry using DCFDA, an increased ROS production was observed with a TLR2 stimulated cells compared to the untreated control cells (Fig.7). An increase in ROS by TLR2 stimulation may be enabling the MKs maturation and this may also help the megakaryocyte as immune cells to clear pathogen.

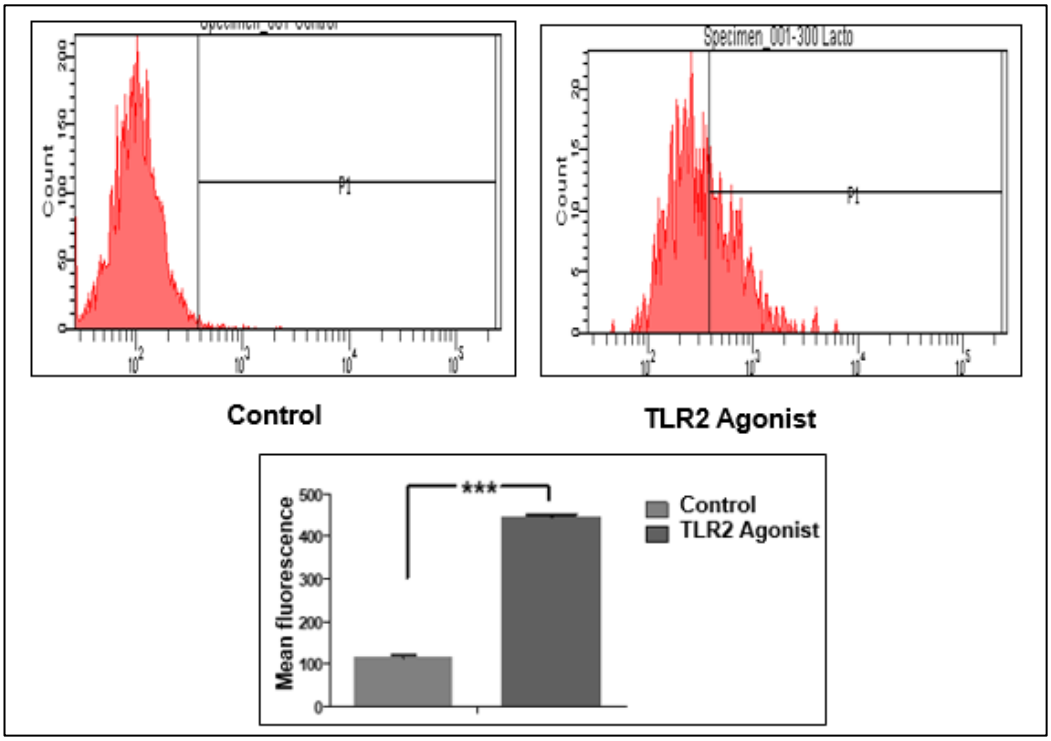


**Figure 7.TLR stimulation induces the ROS production.** ROS production shown by higher staining with 2,7-dichlorofluorescein triacetate for the detection of release of ROS in TLR2 stimulated cells.

Previous reports suggest that TLR2 stimulation results in the activation of Wnt β-catenin signaling (80). Existing reports suggest that both canonical and non-canonical Wnt signaling pathways are functional in megakaryocytes(81). Several Wnt effectors display MK-specific expression. Therefore we further investigated, whether TLR2 has any crosstalk with Wnt signaling during TLR2-induced megakaryopoiesis. To understand the TLR2 crosstalk with Wnt signaling during the megakaryocyte maturation, we treated the Dami cells with TLR2 agonist zymosan for 48 hours and analyzed the FZD4 and wnt signaling components expression by western blot analysis. Zymosan induces the receptors TLR2 and FZD4 expression at transcript levels compared to untreated control cells (Fig.7A). Further immunoblotting analysis suggest that Wnt signaling components such as β-catenin, p-GSK-3β, Dvl2 and LEF1 levels were increased at protein levels alongside

with FZD4 a receptor for wnt signaling pathway. This suggest that TLR2 and Wnt signaling pathways could have possible crosstalk and which helps in megakaryocyte development during infection mediated megakaryocyte maturation.

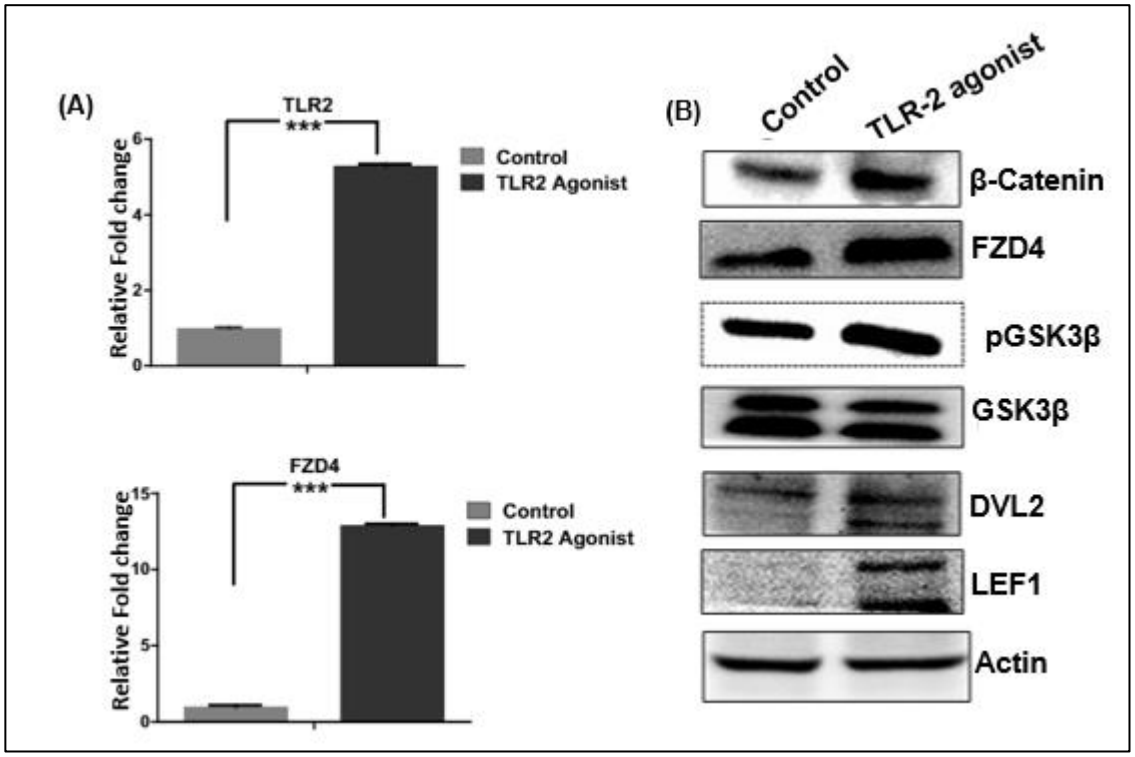


**Figure 7. TLR2 stimulation induces the Wnt signaling.** (A) Dami cells were treated with TLR2 agonist and analyzed for TLR2 and FZD4 expression by qRT-PCR. (B)Immunoblotting showing increase in Wnt β-catenin signaling components upon TLR2stimulation. Actin was used as a loading control.

c-Mpl receptor signaling mediates through downstream PI3K-AKT and MAPK signaling pathways(82) and pharmacological inhibitors of MEK such as PD98059 and UO126 and PI3K/Akt inhibitor LY294002 abrogated the megakaryocyte polyplodization and maturation(83). To understand whether TLR-2 mediated megakaryocyte maturation follows the same pathways and components as TPO mediated megakaryocyte maturation. To check that we treated Dami cells with TLR2 agonist zymosan for 30 minutes followed by total protein isolation using RIPA cell lysis buffer with protease and phosphatase inhibitor. Total protein was analyzed by immunoblotting with specific primary antibodies

and their respective HRP tagged secondary antibodies. P-AKT and P-ERK levels were increased in TLR2 stimulated compared to untreated control cells, whereas, the total AKT and ERK levels were not changed. This suggest that TLR2 mediated megakaryocyte maturation may be sharing the similar pathways and components with TPO mediated megakaryocyte maturation.

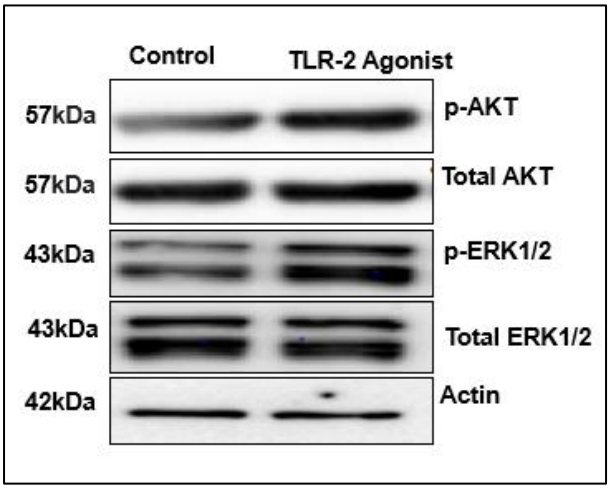


**Figure 8. TLR2 stimulation induces the P-AKT and P-ERK level's in Dami cells**. Upon treatment with the TLR2 agonist, P-AKT and P-ERK levels were increased however total AKT and total ERK1/2 levels levels were not changed, actin was used as loading control.

**Objective 2. Elucidate the effect of TLR2 induced megakaryocyte microparticles (MPs) on its maturation.**

TLR stimulation induces production of a broad range of molecules including cytokines and chemokines, which are essential for host response to infection as well as for the development of an adaptive immune response(84). While cytokines and chemokines were well studied for their roles in mediating cell-cell communication by their secretion into extracellular space, in addition to the cytokine secretion cells also shed complex messengers called extracellular vesicles (EVs) which are involved in carrying the information from one cell to another cells in multicellular organisms. Extracellular vesicles include exosomes, micro particles and apoptotic bodies(85). Exosomes are nano vesicles ranging from 30–100 nm in diameter, micro particles 0.5-1µm in size and apoptotic bodies are in size of 1.5 to 2 microns (86). Extracellular vesicles (EVs) are released by almost all the nucleated cells, enucleate RBCs and platelets. EVs are found in all biological fluids (87). Exosomes are released by all the cells both in resting and activation state's by fusion of intraluminal vesicles in endosomes with the host cell membrane (86). Microparticle (MPs) are released upon activation of cells by pinching of the plasma membrane loaded with cytosolic fraction of the respective cell. EVs carry the cellular macromolecules mRNA, miRNA, proteins and bioactive lipids from one cell to another and these macromolecular cargo reprogram the recipient cells (88, 91). Existing reports suggest that MK-MPs are the most abundant in the circulation (89). Microparticles are heterogeneous membranous vesicles and similar to EVs they carry the macromolecules like nucleic acids, proteins, long non-coding RNA, miRNA and bioactive lipids (90). 70-80% MPs in circulation are contributed by both megakaryocyte and platelets during normal physiological conditions (81-82), which further increases during the infection

conditions. Thus, we wanted to check the significance of TLR-2 stimulated megakaryocyte derived-microparticles during the megakaryocyte maturation and immune modulation. To investigate that we initially checked the whether TLR2 stimulus has any effect on MK-MPs shedding. Dami cells were treated with zymosan for TLR2 stimulation followed by MPs shedding was further analyzed by microscopy analysis. MK derived-MPs were clearly shedding from the cells into the extracellular medium (Fig.1).

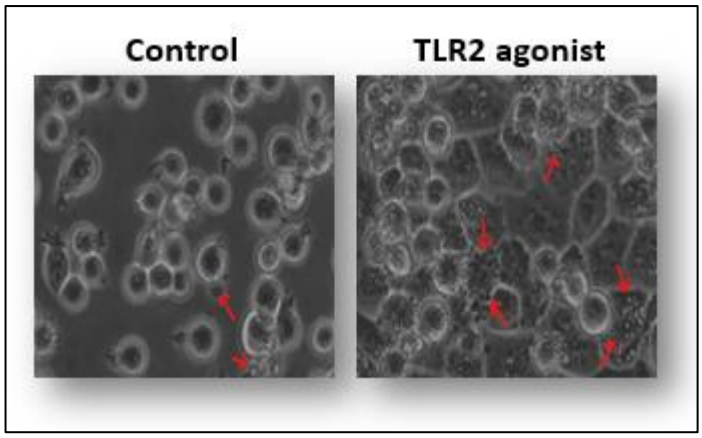


**Figure 1.Microscopic analysis of increased microparticle shedding:** upon TLR2 stimulation Dami cells increased the shedding of the extracellular vesicles and increased cell adherence and size were observed**.**

Further to isolate MPs, we collected the spent media and the MPs were isolated by using the ultra-centrifugation method published by Robert Flaumenhaft *et al*., 2018. Physical characterization of MPs were done using TEM (Transmission electron microscopy) analysis. TEM analysis of MPs suggest that TLR2 stimulated MK derived MPs are around 1µ in size, whereas, the standard exosomes size is 100 nm (Fig.2A). These results were further confirmed by dynamic light scattering (DLS) analysis (Fig.2B). MPs were almost 1µ in size, whereas, exosomes were 100nm in size. MK derived-MP expressed the MK specific marker-CD41 which was analyzed by immune blotting (Fig.2C) (93). CD81, an

exosome specific marker was absent in MP sample and was present in exosome sample (Fig.2C). These results suggest that the isolated EVs were purely MPs in nature.

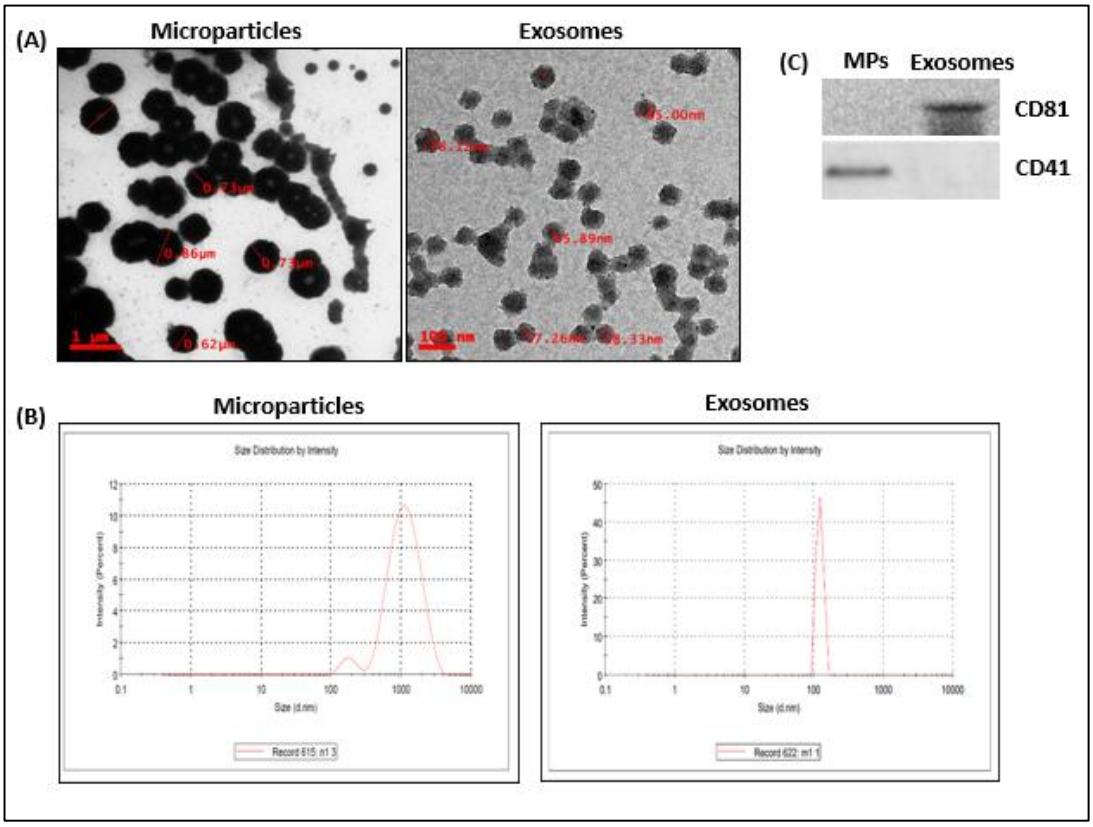


**Figure 2. Characterization of the extracellular vesicles byTEM (Transmission electron microscopy), DLS (Dynamic light scatering) and immunoblotting**. (A) Micro particles were observed under TEM and they were in the size range of 700–1000 nm. (B) Micro particles were 1000 nm whereas exosomes 100 nm in size respectively. (C)Immunoblotting analysis of Molecular marker of exosomes CD-81 and megakaryocytic microparticle marker CD-41.

As MPs are involved in carrying molecular information from one cell to another, we therefore checked the effect of TLR2 stimulated MK derived-MPs affect on the megakaryocyte maturation. To understand that, we treated Dami cells with TLR2 stimulated MK derived MPs for 48 hours and cells size and morphology were analysed by geimsa stianing using inverted light microscope. TLR2 stimulated MK-derived MPs treatment increased the naïve Dami cell size compared to untreated control cells (Fig.3).

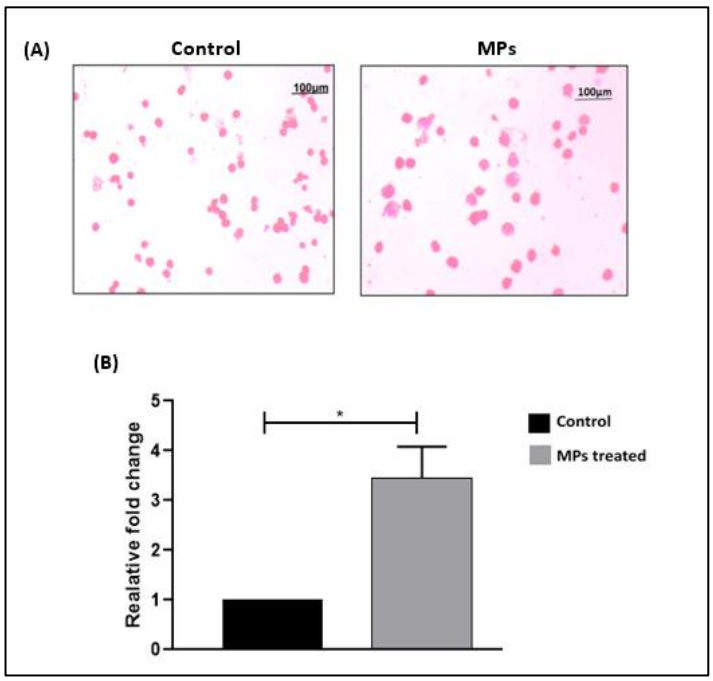


**Figure 3. Morphological analasis with microscopy.**(A)Upon treatment of TLR2 stimulated MK derived-MPs cells size were increased compared to untreated control cells and increased(Fig3A). Cell size was measured by Image J saftware analsyisis(B).

Enlarged cell size is one of the importent feature of mature megakaryocyte. We further investigated the effect of TLR2 stimulated MK-derived MPs effect on megakaryocyte maturation markers using the qRT-PCR analysis. All the MK maturation markers CD41, CD42 and CD61 were increased compared to untreated control cells (Fig 4).

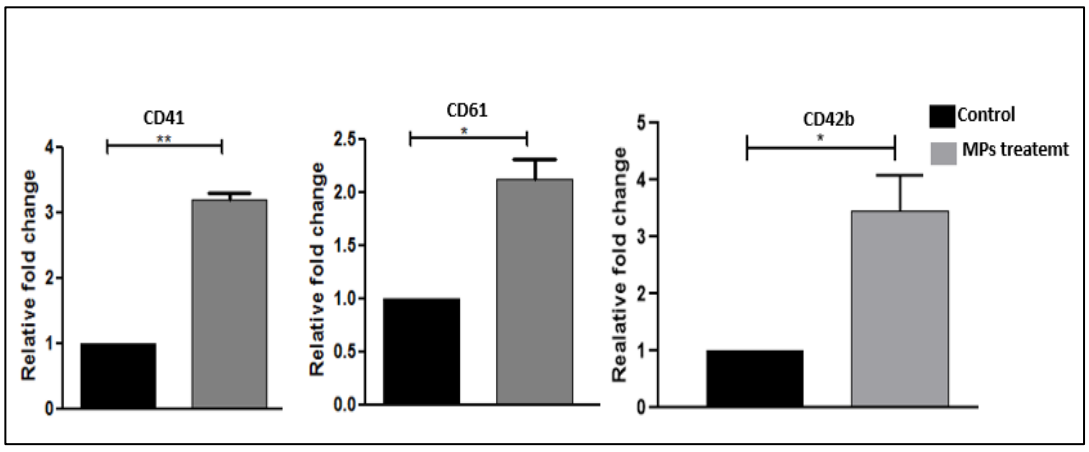


**Fig4. TLR2 induced MPs treatment induces the megakaryocyte maturation**. Upon stimulation of cells with TLR2 derived microvesicles megakaryocyte maturation markers CD41, CD42 and CD61 were significantly increased compared to control cells.

We further checked whether the TLR2 stimulated MK dereived MPs has any effect on TLR2 signaling read outs in recepeint cells. To investigate that naive Dami cells were treated with TLR2 stimulated MK derived-MPs for 40 minutes and cytokines levels were analysed at transcript levels by using qRT-PCR. MPs induces the inflammatory cytokines IL-1β, IL-2, IL-6, IL-8 and chemokines Cox-2, MCP-1 levels were significantly increased at transcript levels compared to untreated control cells(Fig5). This suggest that TLR-2 stimulated MK derived-MPs may recaptulate the TLR-2 signaling in recepient cells.

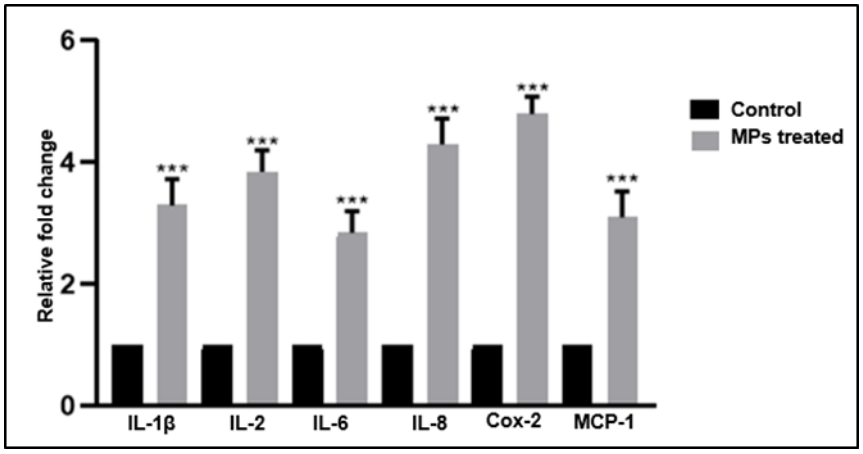


**Figure 5. TLR2 induced MK derived MP treatment induces the cytokines.** Upon stimulation of cells with TLR2 derived MPs in naïve Dami cells inflammatory cytokines IL-1β,IL-2, IL-6,IL-8 and chemokines Cox-2 and MCP-1 levals were significantly increased compared to control cells.

As microparticles are known to carry various macromolecules such as nucleic acids, proteins and lipids, to further understand which component of the TLR2 induced MK derived-MPs is involved in MK maturation. MPs were UV-irradiated for 60 minutes to damage the nucleic acid component. Dami cells were treated with these UV irradiated-MPs for 48 hours and further megakaryocyte maturation markers were analysed by qRT-PCR analysis. Unlike normal MPs, UV irradiation could abrogate the increase of MP-mediated MK maturation markers CD41, CD42 and CD61. This suggest that nucleic

acid component of the MPs could be playing the crucial role in TLR2 stimulated MK derived MPs mediated MK maturation (Fig.6).

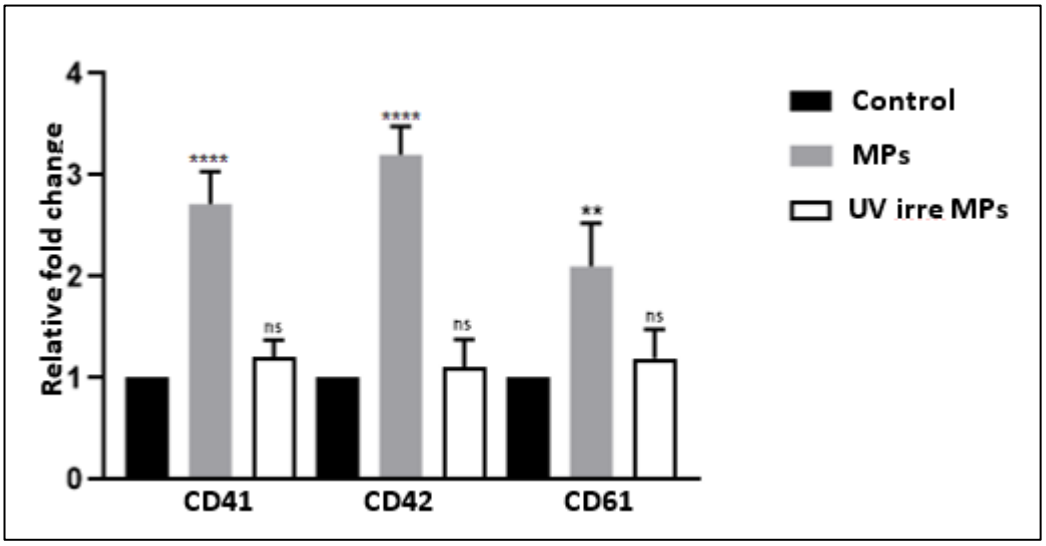


**Figure 6. Ultra violet (UV) irradiation abrogated the the Microparticle (MP) mediated megakaryocyte maturation**. UV irradiated megakaryocyte MPs treatment could not induce the megakaryocyte maturation markers significantly compared to control untreated cell.

We further checked the importance of TLR2 stimulated MK derived-MPs has any effect on megakaryocyte lineage determination of megakaryocyte progenitors such as megakaryocyte erythroied progenitors (MEPs). To check that we treated the bipotent cell line K562 cells with TLR2 stimulated) MK derived-MPs and profiled the megakaryocyte leniage markers CD41, CD42b and CD61 along with erythroid lineage markers Glycophorin-A and Glycophorin-B by using qRT-PCR. Upon treatment with MPs, K562 cells increase the megakaryocyte maturation markers CD41, CD42b and CD61 were increased significantly, whereas, erythroid lineage markers such as glycophorin-A and glycophorin-B levels were decreased at the transcript levels compared to untreated control cells (Fig.7). This suggest that upon receiving the TLR-2 stimulated MK derived-MPs

bipotent cell line could lean towards the megakaryocyte lineage at the cost of the erythroid lineage.

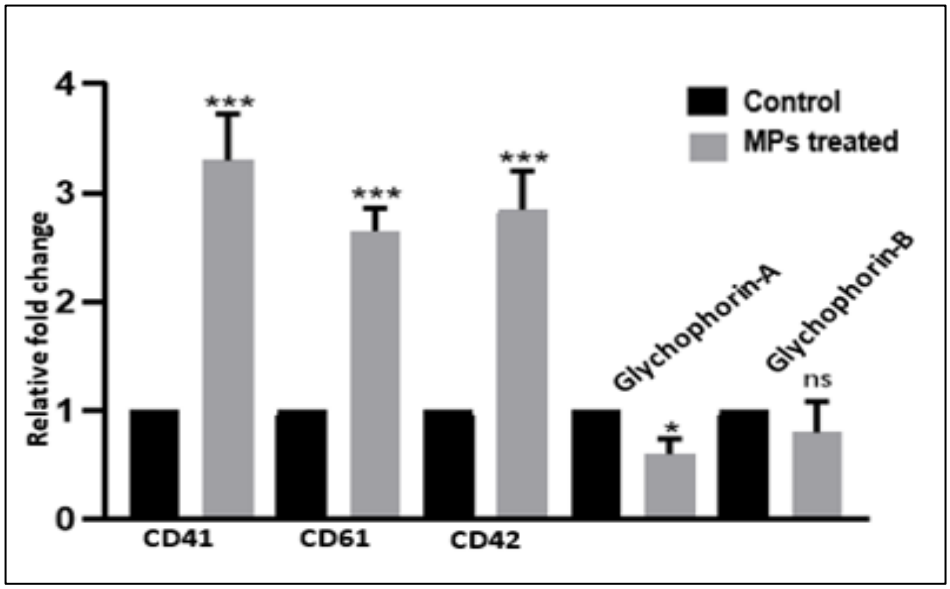


**Figure 7. Microparticle (MP) mediated megakaryocyte lineage determination**. Upon treatment with MPs, MEPs increases the megakaryocyte maturation markers CD41, CD61 and CD42, whereas, erythroid lineage markers glycophorins were decreased.

In physiological conditions, MPs released in circulation are likely to come across multiple blood cells such as neutrophils most abundant cells in circulation, other granulocytes, and lymphocytes such as B-lymphocytes, T-lymphocytes and monocytes. It is more likely that MPs released in circulation could interact with any these blood cells and vascular endothelial cells. Hence, we wanted to further understand the specificity of TLR2 stimulated MK derived-MPs target cells. To understand that human monocytic THP-1 cells were cultured in RPMI media and cells were incubated with 100 μg/ml of TLR2 stimulated MK derived-MPs for 48 hours. THP1 cells are known to gain adherence to substartum and changes in cell shape during its maturation to macrophage. Therefore, we checked whether TLR2 stimulated MK derived-MPs has any effect on monocyte to macrophage maturation. To investigate that morphological analysis was done under

inverted light microscopy and molecular marker analysis was done by qRT-PCR. However, there is no changes in morphology and adherence along with monocyte to macrophage maturation specific molecular marker CD11b compared to untreated control cells (Fig.8). These results suggest that MK derived-MPs may be specifically acting only on MK and its progenitor cells.

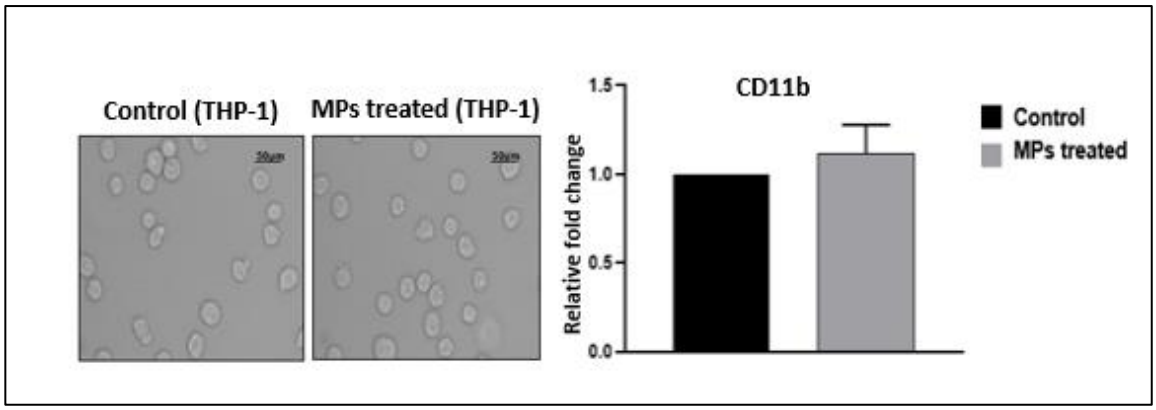


**Figure 8. Megakaryocyte derived MPs doesn't show any effect on monocytes**. TLR2 induced MPs doesn't have any effect on the THP-1 cells morphology. MPs could not affect monocyte marker CD-11b compared to control cells.

**Objective 3. Study the TLR2 and TLR4 co-stimulation effect on megakaryocyte maturation.**

As microbes are called mosaics of antigens, during infection it is likely that more than one TLR may get stimulated in each cell. MKs in humans have all 10 TLRs characterized in the human except TLR10, therefore it is likely that more than one TLR would get activated during infection scenarios (94). Consequently during infection, multiple TLR signaling inputs will be given to cells simultaneously (95), however, the effect of the TLR co-stimulation on megakaryocyte maturation is not known. Therefore to understand the effect of the TLR2 and TLR4 co-stimulation on the megakaryocyte maturation, Dami cells were treated with 10 μg/mL zymosan (Sigma Aldrich) for TLR2 and LPS 100ng/ml for TLR4 stimulation both in isolated, co-stimulation and MK response were studied.

**TLR2 and TLR4 co-stimulation effect on megakaryocyte maturation**

TLR2 and TLR4 co-stimulation effect on megakaryocyte maturation were initially checked by studying the MK maturation markers using qRT- PCR. To understand the TLR co-stimulation effect on the MK maturation, we treated cells with zymosan and LPS for 48 hours and total RNA was isolated, cDNA was prepared and megakaryocyte maturation markers CD41, CD42b and CD61 was analyzed by using the qRT-PCR analysis where GAPDH was used as internal control and results were plotted as fold change. Megakaryocyte maturation markers CD41, CD42b and CD61 were induced in zymosan, LPS stimulated cells significantly compared to untreated control cells. This further increased in co-stimulation (zymosan and LPS co-stimulated cells) compared to the either of the ligand stimulation alone and untreated control cells (Fig 1). This suggest that TLR co-stimulation induces the megakaryocyte maturation effectively.

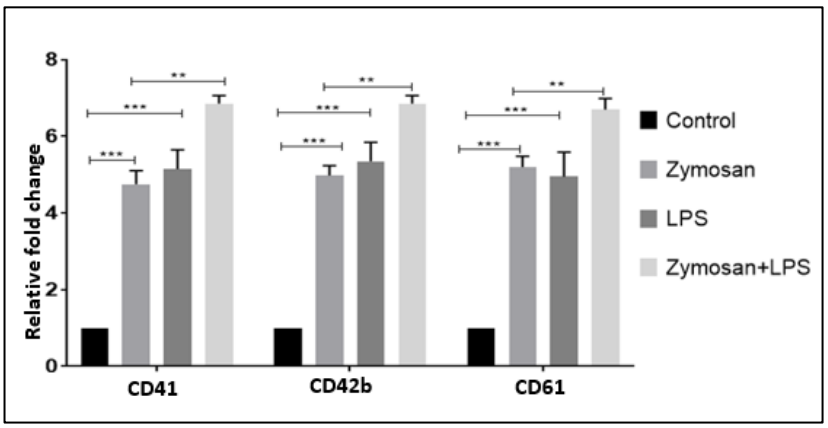


**Figure 1. Zymosan and LPS co-stimulation shows increased megakaryocyte maturation markers at transcript levels.** Upon stimulation of the TLR2, TLR4 by Zymosan and LPS respectively induces the megakaryocyte maturation markers CD-41, CD-42b and CD-61 compared to control cells which are increased further with co-stimulation compared to either of the TLR stimulation alone and untreated control cells**.**

**TLR2 and TLR4 stimulatory effect on the TLRs expression.**

We further checked the effect of TLR2 and TLR4 co-stimulation on the expression of TLRs in Dami cells treated with Zymosan, LPS in isolation and in co-stimulation conditions for 48 hours. Expression of the TLRs was done by qRT-PCR analysis. Though the Toll-like receptors are key players in innate immunity (non-specific), but TLRs respond to ligands specifically and induce their receptors and their partners without impacting other TLRs. Upon treatment with Zymosan TLR 1, 2, 4, 6 were increased at transcript level's which is further increased in co-stimulation of TLR2 and TLR4, whereas, the non-specific endosomal TLRs such as TLR3 and TLR9 were unaffected during both isolated TLR2/TLR4 stimulation and in co-stimulated cells (Fig 2). It suggests that TLRs respond specifically to their cognate ligands, thereby leading to induction of the receptors.

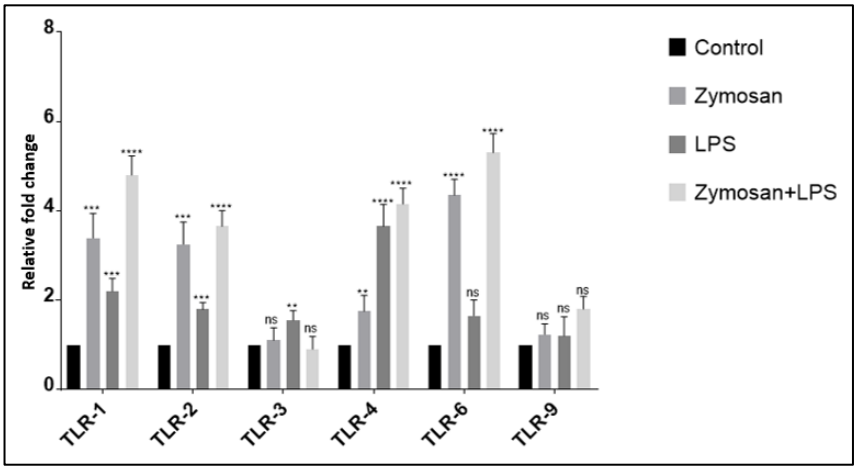


**Figure 2. Stimulation of TLRs with zymosan (TLR2), or LPS (TLR4) induces their specific and partner TLRs**. mRNA expression of the different TLRs was analyzed. TLR1, TLR2, TLR4 and TLR6 were increased upon stimulation with zymosan and LPS which significantly increased further in co-stimulation compared to either of the ligands alone and control cells, whereas TLR3 and TLR9 were unaffected in both the isolated stimulation as well as co-stimulation except for TLR3 in LPS stimulation.

NF-κB is multifaceted protein involved in several physiological functions. TLRs are known to induce the inflammatory cytokines through NF-κBp65 downstream effects. To understand the effect of TLR2 and TLR4 co-stimulation effect on NF-κB p65 levels, cells were treated with zymosan/LPS for 30 minutes in isolated and in co-stimulation. Protein was isolated and NF-κB p65 levels were analyzed by immunoblotting. Upon stimulation of TLR2 and TLR4 in isolation, NF-κB p65 levels were increased compared to untreated control cells which is further increased in co-stimulation (Fig 3).

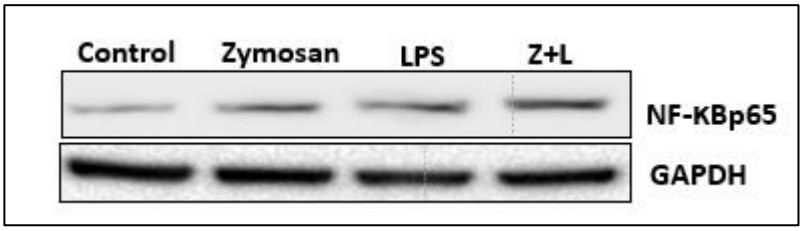


**Figure 3. Immunoblotting analysis of NF-κB p65.** NF-κB levels were increased under co-stimulation compared to either of the ligands, zymosan and LPS stimulation alone and untreated control cells.

Further NF-κB p65 kinetics were analyzed, where Dami cells were stimulated with zymosan/LPS in both isolated and in co-stimulation by incubating the cells for different time intervals as 10, 20, 30, 60, 90 and 120 minutes. Total protein was isolated at respective time intervals and samples were analyzed for NF-κB p65 levels by immunoblotting. In isolated TLR2 stimulation by zymosan, NF-κB p65 levels from 10 minutes to 60 minutes were consistently increasing and further gradually decreased with time (Fig.4). Quantification of NF-κBp65 was done by using densitometry analysis using Image J software.

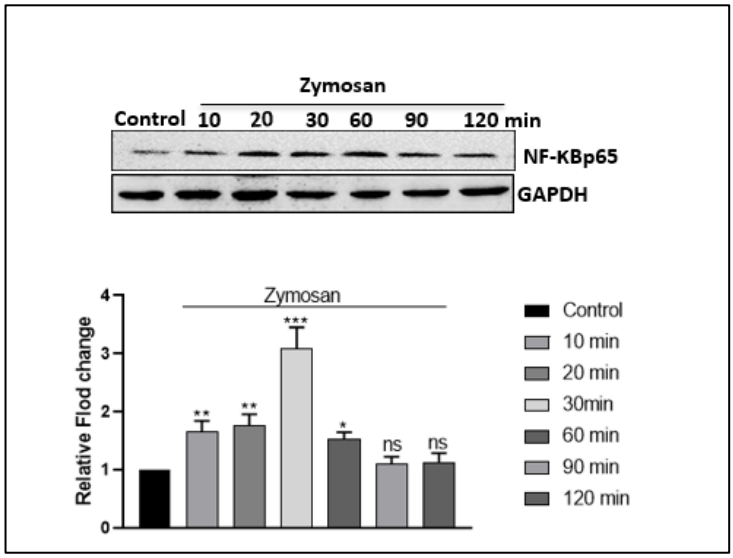


**Figure 4. Time kenetics of TLR2 stimulation effect on NFκBp65.** Upon TLR2 stimulation NF-κB p65 levels significantly increased from 10 to 60 minutes and further gradually decreased.

Similar analysis was done with LPS for TLR4 stimulation at different time intervals using the immunoblotting method. Similar to TLR2, TLR4 stimulation also induce the NFκBp65 levels from 10 to 30 minutes and gradually decreased with time (Fig.5). Quantification of NF-κBp65 was done by using densitometry analysis using Image J software.

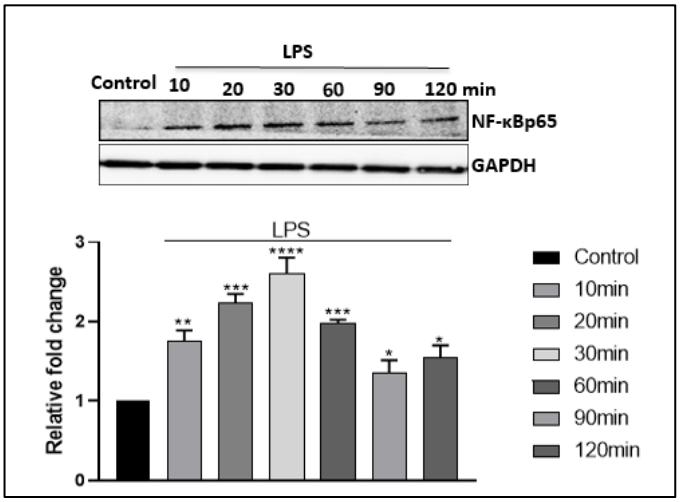


**Figure 5. TLR4 stimulation by LPS induces the NFkBp65 levels.** Upon TLR4 stimulation NFkBp65 levels significantly increased from 10 to 30 minutes and maintains till 60 minutes, which gradually decreased.

In TLR2 and TLR4 co-stimulation, NFkB-P65 levels were not increased at intial time points from 10 to 20 minutes. However, from 30 minutes onwards its levels gradually increased and this increase was maintained upto 120 minutes unlike the isolated TLR2/TLR4 stimulation (Fig.6). Probably this could have sustained expression of the inflammatory cytokines which could help in megakaryocyte maturation.

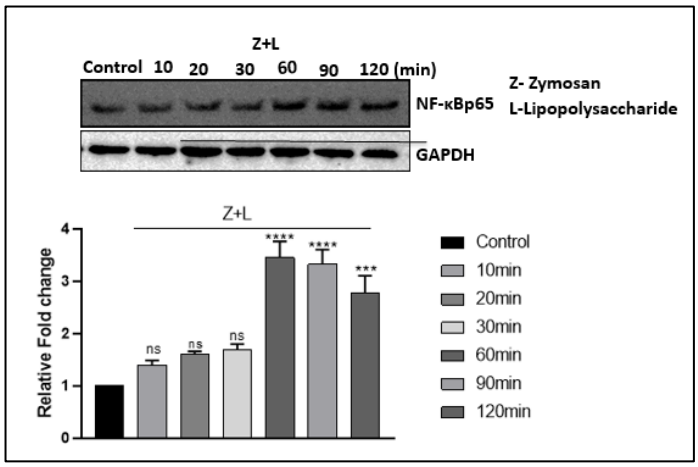


**Figure 6. TLR2 and TLR4 co-stimulation resulted in delayed NFkB-p65 stimulation but maintained consistently for a long time**. NFkB-p65 levels were increased in co-stimulation which is maintained till 120 minutes.

Cytokine signals are crucial for immune responses for intercellular communication. Multiple cytokines are reported to be secreated by megakaryocytes, however the effect of the TLR2 and TLR4 co-stimulation on cytokines expression is not known. To check that Dami cells were stimulated by TLR2/TLR4 agonists in isolation and co-stimulation for 60 minutes. The cytokines expression was analyzed by using qRT-PCR. Cytokines such as IL-1β, IL-2, IL-6, IL-8 and IL-10 were increased more in TLR2 or TLR4 alone stimulated cells compared to untreated cells, which further enhanced in TLR2 and TLR4 co-stimulated compared to either of the TLRs alone or untreated control cells(Fig.7). IL-6 has been shown to enhance the effect of TPO, an essential regulator of megakaryocytes (10). This study suggest that these increased interleukins IL-1β, IL-2, IL-6, IL-8 and IL-10 could be responsible for increased megakaryocyte maturation under co-stimulation.

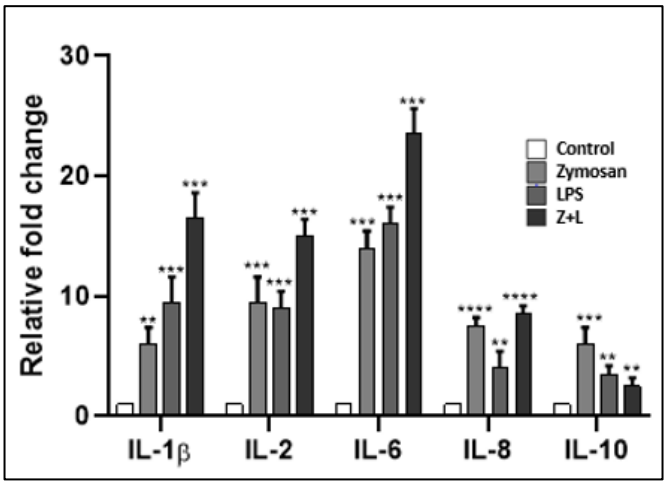


**Figure 7. Cytokine analysis by qRT-PCR upon treatment with TLR2/TLR4 alone and co-stimulation conditions.** Inflammtory cytokines were induced under isolated TLR stimulation and which further increased in co-stimulation compared to either of the TLR stimulation alone and untreated control cells.

XBP-1 plays an important role in the sustained secretion of cytokines and helps in the expansion of the secretory pathway (96). XBP-1splicing is necessary in the plasma cell maturation (97). XBP-1 knockout mice results in increased bacterial burden as XBP-1

is known to be involved in the sustined secretion of cytokines. However, the effect of the TLR2 and TLR4 co-stimulation on XBP-1 splicing is not clearly known.

To understand the effect of the TLR co-stimulation on XBP-1splicing during the megakaryocyte maturation, we treated the Dami cells with Zymosan, LPS, Zymosan and LPS (co-stimulation) for 40 minutes. Total RNA was isolated and analyzed for XBP-1 splicing analysis by PCR. Upon TLR-2 and TLR-4 co-stimulation, expression levels of XBP-1 splicing were increased in co-stimulation compared to either of the TLRs alone and untreated control cells (Fig.8a). This suggests that increased XBP-1 splicing may be a probable reason for the induction of the cytokine more in co-stimulation compared to either of the isolated TLR2/TLR4 stimulation alone. Previous reports suggest that XBP-1 spliced form acts as a transcription factor for the induction of cytokines(98). To understand the XBP-1 and its gene interactions, we performed the XBP-1s transcription factor gene interactions from the TRUST database and analyzed using Cytoscape. XBP-1 spliced transcription factor has binding sites at the promoters of the genes that encode cytokines (Fig.8b).

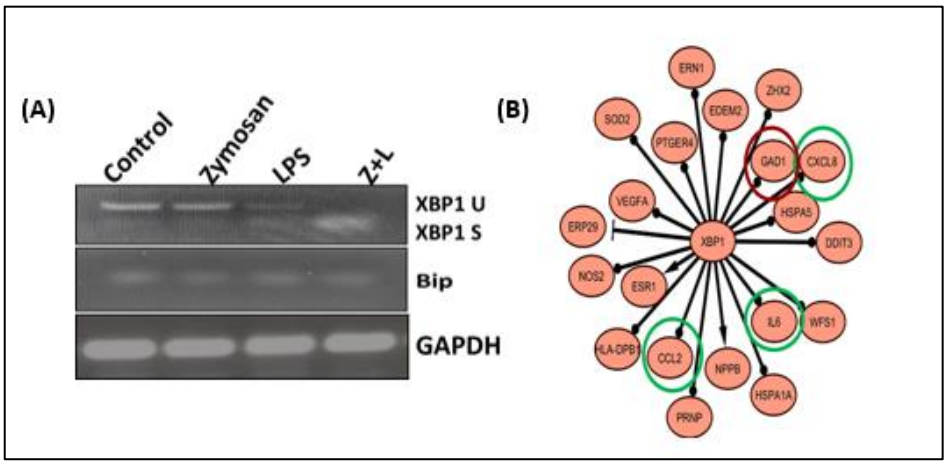


**Figure 8. XBP-1 splicing analysis by agarose gel electrophoresis.** (A) Upon co-stimulation XBP-1 was spliced more compared to isolated TLR stimulation and control cells. Expression levels of the Bip are not changed in all the conditions and GADH was used as the loading control. (B) Cytoscape analysis of the XBP-1s transcription factor.

To further understand the effect of TLR 2 and TLR4 co-stimulation effect on downstream PI3K-AKT and MAPK signaling pathways, Dami cells were treated with zymosan, LPS in isolation and with co-stimulation for 30 minutes. Total protein was isolated and western blot analysis was performed. P-AKT and P-ERK levels were induced in zymosan, LPS stimulated compared to untreated control cells. However, P-AKT levels were increased further marginally, whereas, P-ERK levels were not increased further in co-stimulation (Fig.9).

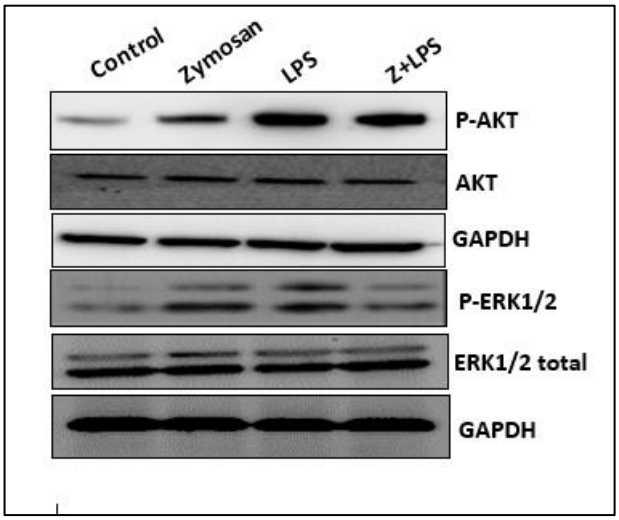


**Figure 9. p-AKT, p-ERK analysis in Zymosan, LPS and co-stimulation (Zymosan and LPS) through Immunoblotting blotting.** Upon treatment with isolated TLR2 or TLR4 stimulation P-AKT and P-ERK levels were increased and which is further increased marginally in case of P-AKT, however, p-ERK1/2 levels were not increased further in co-stimulation of TLR2 and TLR4 with Zymosan and LPS respectively GAPDH was used as loading control. Quantification of p-AKT and p-ERK by ImageJ analysis.

Muse cell analyzer data for activated P-AKT positive cells in co-stimulation further suggest increase of activated P-AKT positive cells compared to either of the TLR2 or TLR4 stimulation alone and control cells (Fig 10).

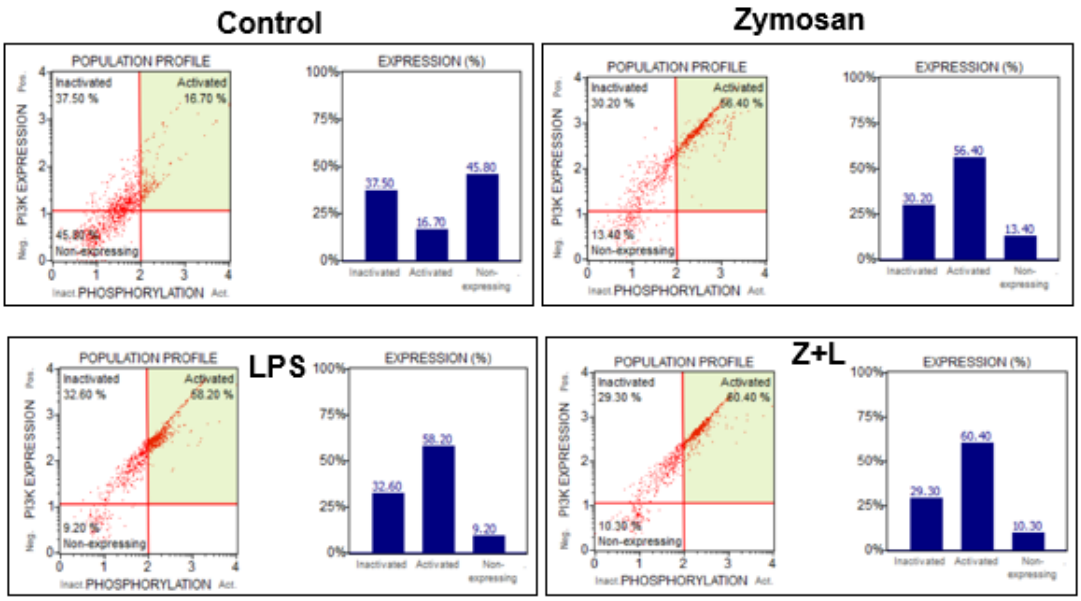


**Figure 10. Flow cytometric analysis of AKT-PI3K analysis**. Upon stimulation of TLR2 with zymosan and TLR4 with LPS, P-AKT positive cells were further increased in co-stimulation.

Reactive oxygen species (ROS) has been known to play an important role in megakaryocyte commitment and maturation. TLRs are also well known to induce the ROS. However, implications of the TLR co-stimulation in ROS production is not clear. To understand the effect of the TLR2 and TLR4 co-stimulation on ROS production, we treated the Dami cells with Zymosan/LPS alone and co-stimulation. ROS levels were measured by DCFDA using the fluorimeter.

ROS levels were induced significantly in TLR2/TLR4 stimulated cells compared to untreated control cells. This increased further in co-stimulation conditions compared to either of the TLR stimulation alone and untreated control cells (Fig.11).

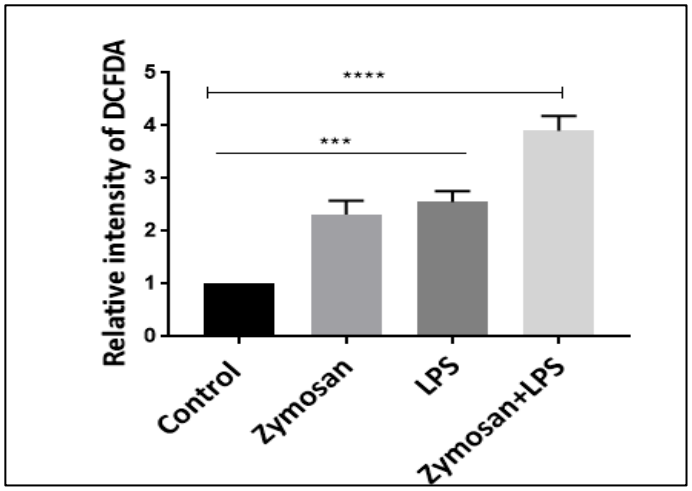


**Figure 11. Estimation of ROS levels using DCFDA.** Upon stimulation of TLR2 by zymosan and TLR4 by LPS, ROS levels were induced which further increased significantly in TLR2 and TLR4 co-stimulation compared to either of the TLR stimulation alone and untreated control cells.

Though there are multiple transcription factors involved in megakaryocyte differentiation and maturation like Fli-1, Gata-1, Gata-2 and RUNX-1 which have been shown to play important role in megakaryocyte maturation (99). However, the importance of RUNX-1 in TLR2 and TLR4 stimulated megakaryocyte maturation is not clear.

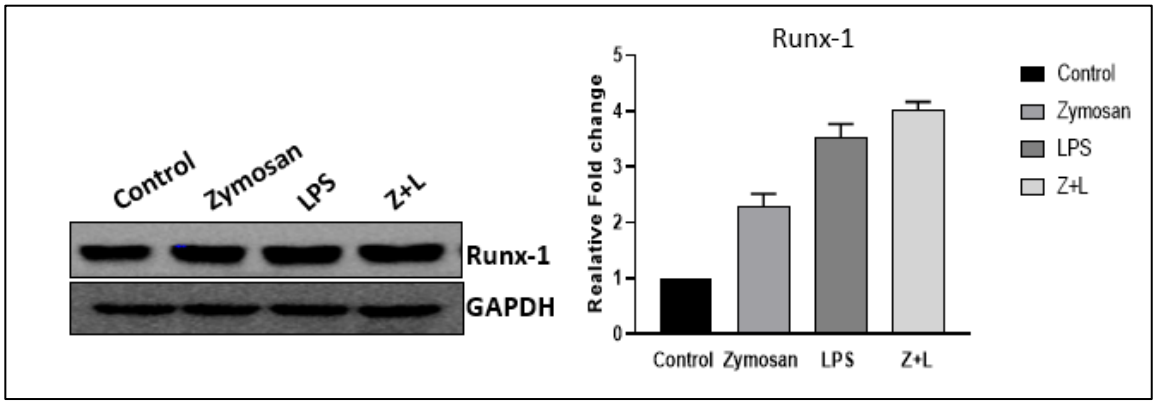


**Figure 12. Zymosan and LPS co-stimulation induce the RUNX-1 transcription factor expression.** Upon stimulation of TLR2/TLR4, Runx-1 levels were increased which are enhanced further in co-stimulated conditions compared to the either of the isolated TLR stimulation alone and untreated control cells.

To understand whether RUNX1 has any role in megakaryocyte maturation, we have stimulated the cells with either TLR2 or TLR4 alone and co-stimulation for 48 hours. Total protein was isolated and analyzed by immunoblotting. Immunoblotting results suggest that upon stimulation of TLR2/TLR4, RUNX-1 expression protein levels were increased, which further enhanced in TLR2 and TLR4 co-stimulated condition compared to either of the TLR stimulation alone and control cells (Fig.12). This suggests that increased levels of the RUNX-1 may be involved in increased megakaryocyte maturation in infection mediated TLR co-stimulation.

**Highlights of the Research**

Our findings are opening the new insights into the TPO-independent molecular mechanisms regulating the megakaryocyte development. TLR2 signaling could induce the megakaryocyte maturation by activation of TLR-2 downstream signaling pathways ways such as PI3K-AKT, MAPK and NF-KB signaling pathways. TLR-2 stimulation is inducing the secretion of cytokines and generation of ROS which plays important role in megakaryocyte maturation. TLR-2 stimulated MK-derived MPs could also induce the megakaryocyte maturation and lineage determination. UV irradiation abrogated the MPs mediated MK maturation and MK-MPs may specifically act on megakaryocyte and its progenitor's cells and involves in feedforward mechanism. TLR-2 and TLR-4 co-stimulation could induce the megakaryocyte maturation more effectively than the either of the TLR2 or TLR4 stimulation alone and control cells.

**Summary**

Toll-like Receptors (TLRs) are pattern recognition receptors known to play an important role in innate immunity. These TLRs sense specific patterns of pathogens and elicit specific signaling pathways that helps in immune establishment. Megakaryocytes are bone marrow cells derived from Hematopoietic stem cell (HSC) lineage hierarchy, functionally involved in platelet production. These platelets play an essential role in blood clotting and hemostasis, however recent reports have projected that platelets do have a significant role in inflammation and immunity.

Classically megakaryocyte development and platelet production are governed by Thrombopoietin (TPO). Recent publications suggest that TPO receptor, c-Mpl, (myeloproliferative leukemia virus oncogene) is dispensable for platelet production. In addition, inflammatory cytokines IL-6 and IL-1β regulates megakaryocyte maturation. TLRs are involved in innate immunity and induces inflammatory cytokines that modulate immunity, inflammation and maturation of the immune cells. Thus, we checked the available literature and found that both the megakaryocyte and platelets were reported to have TLRs. TLRs on platelets aids in sensing the bacteria pathogens and they get activated and release their internal components which helps in recruiting other cells of the immune system and helps in establishing the immunity, however TLRs role in megakaryocyte maturation is not clearly understood. On the basis of the above literature, we framed following three objectives:

1. Determine the TLR2 regulatory mechanisms during megakaryocyte maturation.

2. Elucidate the effect of TLR2 induced megakaryocyte microparticles (MPs) on its maturation.

3. Study the TLR2 and TLR4 co-stimulation effect on megakaryocyte maturation.

**Objective-1**

**Determine the TLR2 regulatory mechanisms during megakaryocyte maturation.**

Profiling was done for all differentially regulated TLRs upon treatment with TLR2 agonist with quantitative real-time PCR (Figure-1a). Stimulation of TLR2 with its agonist zymosan induces the TLR2 and its partner Toll Like receptors TLR1, TLR6 and TLR4 at transcript levels compared to untreated control cells whereas the endosomal TLR were not changed which suggest that though the TLR are part of innate immunity which is non-specific immunity yet TLRs responds specifically to molecular cues that they sense (Figure-1a). We checked the TLR2 at protein levels by using the flow cytometric analysis and it suggest that upon TLR2 stimulation, TLR2 levels were induced at protein levels compared to untreated control cells which were further confirmed with immunofluorescence (Figure-1b). Further to check whether TLR2 has any effect on megakaryocyte maturation we evaluated megakaryocyte maturation markers such as CD-41, CD-42b and CD-61 with quantitative real-time PCR analysis and all of them were upregulated compared to untreated control cells (Figure-1c).

Upon TLR-2 stimulation cells size and adherence were increased compared to untreated control cells which are characteristic features of mature megakaryocyte (Figure-1d). We further checked the TLR-2 effects and found increased polyploidy or DNA content, which is unique marker of mature megakaryocyte (Figure-1i). We further checked the TLR-2 effects on downstream signaling components such as NF-κB, MAPK and PI3K-AKT signaling pathway components. NF-κB-p65, P-ERK and P-AKT levels were significantly increased compared to untreated control cells (Figure-1e).

TLRs are known to induce the various pro-inflammatory and anti-inflammatory cytokines. We checked the effect of TLR-2 stimulation on pro-inflammatory and anti-inflammatory cytokines by using the ELISA which suggest that there is an increase in IL-2, IL-6, IL-10 in TLR2 stimulated cells compared to control cells (Figure-1f).

Reactive Oxygen Species (ROS) is known to play important role in all important stages of megakaryocyte maturation. We checked the status of ROS during TLR-2 stimulated megakaryocyte maturation by using 2′, 7′-Dichlorofluorescin diacetate (DCFDA) with FACS analysis and found elevated levels of ROS in TLR2 stimulated cells compared to control cells (Figure-1g). As most ROS is contributed by mitochondria, analysis of mitochondrial depolarization was done by using muse analyzer; depolarized live cells were more in TLR-2 stimulated cells compared to untreated control cells (Figure-1h).

TLR-2 is also reported to have cross-talk with Wnt signaling which is known to play important roles in megakaryocyte development. We checked the effect of TLR-2 on Wnt signaling components and found that TLR-2 stimulation induces Wnt signaling components.

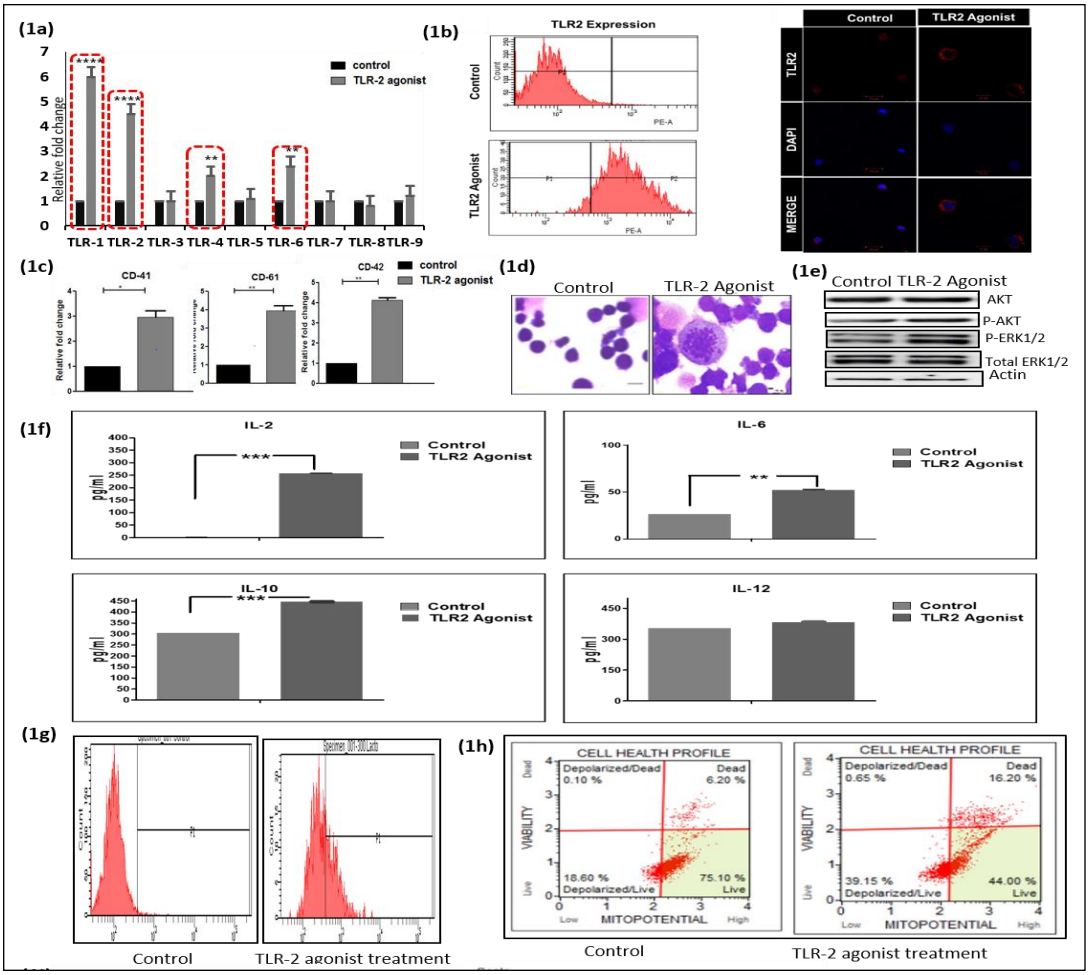


**Figure 1. TLR2 activation lead to increase in TLR2 expression, ROS production and megakaryocyte maturation.** (1a) Analysis of multiple differentially regulated TLRs expression at transcript levels by real time PCR ananalsysis upon treatment with TLR-2 Agonist. Analysis of TLR-2 expression at protein levels (1b) FACS and confocal microscopy. (1c)Analysis of megakaryocyte maturation markers CD-41,CD-42b and CD-61 by Real time PCR analysis. (1d) Morphological analysis of cells upon treatment with TLR-2 agonist by Gemsa stain under brightfeild microscopy. (1e) Analysis of phosphorylated AKT, ERK-1/2 and NF-κB-65 by the western blot analysis. (1f) Dami cells were treated with TLR-2 agonist and cytokines (IL-2, IL-6, IL-10, and IL-12) released into the culture medium was quantified by Quantikine immune assays kits (R&D Systems). (1g) TLR-2 stimulation increases ROS production shown by higher staining with 2, 7 –dichlorofluorescein diacetate (DCFDA) for the detection of release of ROS in TLR-2 agonist treated cells.(1h) Analysis of Mitochondrial depolarization, Depolarized live cells were more in TLR-2 stimulated cells (lower left Quadrant) compared to control cells.

Upon stimulation with TLR-2, megakaryocytes release broad range of components such as pro-inflammatory and anti-inflammatory cytokines whose importance is very much understood in megakaryocyte maturation and immune establishment, however, cells also release complex messengers called extracellular vesicles. For many years these vesicles were considered as cell dust and excretory vesicles that cells release in the process of cleaning their internal environment. However, these vesicles were reported to be involved in transfer of information from one cell to other cells. Extracellular vesicles carry miRNA, lncRNA, transcription factor and bioactive lipids from one cell to other cells (donor cells to recipient cells) therefore upon receiving this component recipient cells undergo reprogramming.

**Objective-2.**

**Elucidate the effect of TLR-2 induced Megakaryocyte microparticles (MPs) on its maturation.**

To study the effect of TLR-2 derived MPs, we treated cells with zymosan (10µg/ml) for 48 hours and analyzed cell morphology by microscopy to check the effect of the TLR-2 on megakaryocyte maturation. Mature megakaryocytes increase cell size, adherence which were found to be more in TLR-2 stimulated cells compared to untreated control cells (Figure-2a), further and we collected the spent medium of TLR-2 stimulated cells to isolate MPs by using ultra-centrifugation. We dissolved the centrifuge pellet in PBS (Phosphate buffer saline $Mg^{+2}$) and filtered using 1µm syringe filter and collected the filtrate. Physical characterization of filtrate was done by Transmission electron microscopy (TEM). The filtrate contained circular (700-1000 nm) size vesicles (Figure-

2b), which were further confirmed by dynamic light scattering (DLS) which give mean particles size. The size of standard exosomes were in the range of 70-100nm in size (Figure-2c).

Megakaryocyte MPs markers CD41, HSP-70 and exosomes marker CD81 were verified by western blotting and these results suggest that these MPs are not contaminated with apoptotic bodies and exosomes (Figure-2d).

To check TLR-2 stimulated megakaryocyte (MK) derived MPs effect on megakaryocyte maturation, we treated the naive Dami cells with TLR-2 stimulated megakaryocyte derived MPs (100µg/ml) for 48 hrs and further checked the cell size by microscopy and maturation markers CD-41, CD-42b and CD-61 by qRT-PCR. These results suggest that upon treatment with TLR-2 stimulated MK MPs, increase in cell size (Figure-2e) and maturation markers was observed (Figure-2f).

As these MPs are derived from TLR-2 stimulated cells we further checked whether these MPs has any effect on the levels of cytokines such as IL-1β, IL-2, IL-6, IL-8, MCP-1 and Cox-2 at transcript level by qRT-PCR. Upon treatment with TLR-2 stimulated-MPs, cells increase the levels of as IL-1β, IL-2, IL-6, IL-8, MCP-1 and Cox-2 compared to untreated control cells (Figure-2g). This suggest that TLR-2 stimulated MK derived MPs may induce the TLR-2 readouts in recipient cells.

Microparticles (MPs) contain various biomolecules such as proteins, nucleic acid and lipids. We investigated which component of TLR-2 stimulated MK specific MPs is important for their effect on megakaryocyte maturation and induction of cytokines. TLR-2 stimulated MK specific MPs were UV irradiated and further we applied this to naive cells, UV irradiation abrogated the TLR-2 MPs driven megakaryocyte maturation markers.

This suggest that nucleic acid component of the TLR-2 stimulated MK specific MPs maybe involved in megakaryocyte maturation (Figure-2h).

We also checked whether TLR-2 stimulated MK specific MPs has any effect on lineage determination of the megakaryocyte progenitors such as a bipotent megakaryocyte erythroid progenitor (MEP) which can either give megakaryocyte or erythroid progenitor depending on the input signal. We choose a K562 cell line (bipotent MEP) to study the effect of TLR-2 stimulated MK specific MPs effect on lineage determination. K562 cells were treated with TLR-2 stimulated MK specific MPs for 48 hrs and megakaryocyte maturation markers were analyzed by qRT-PCR. Megakaryocyte maturation markers (CD41, CD61 and CD42b) were up-regulated at transcript levels, whereas, the erythroid lineage markers (Glycophorin-a and Glycophorin-b) transcript levels were down-regulated compared to untreated control cells (Figure-2i). This suggest that TLR-2 stimulated MK derived MPs induces the bipotent erythro-leukemic cells towards the megakaryocyte lineage.

We further wanted to understand whether TLR-2 stimulated MK derived MPs has any effect on the other lineage blood cells such as monocyte. To check that we treated the TLR-2 stimulated MK derived MPs to the human monocyte THP-1 cells and checked morphology, size and markers of monocyte to macrophage maturation. These results suggest that TLR-2 stimulated MK derived MPs may act specifically on megakaryoblasts and megakaryocyte lineage progenitor and not on other lineages (Figure-2j).

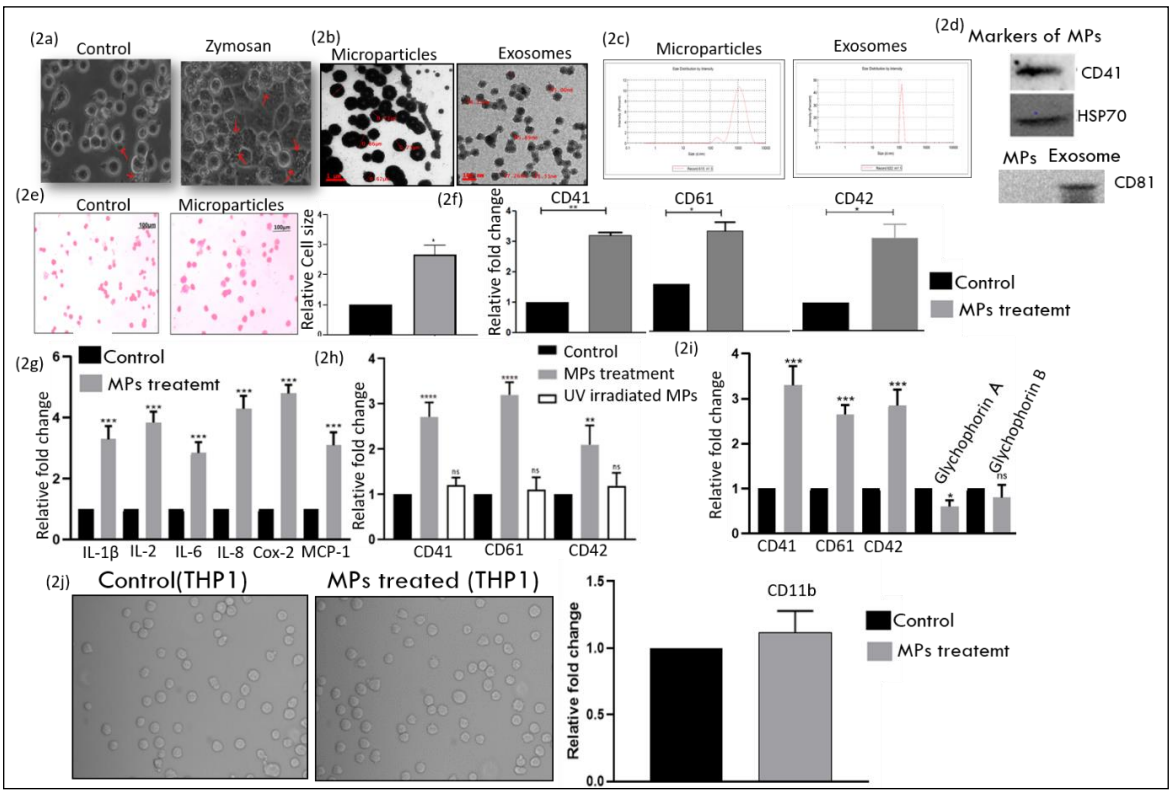


**Figure-2. TLR-2 induced MKEVs induces the megakaryocytic maturation**. (2a) Megakaryocyte maturation (increased cell size and adherence) was observed with Zymosan treatment in Dami cells. (2b) MPs were observed under TEM and they were in the size range of 700–1000 nm. Exosomes were used as size standard. (2c)DLS analysis for microparticle size analysis and were found to be in size range 70–100 nm. (2d) Megakaryocyte micro particle marker CD-41 and HSP70 were analysed by western blot analysis, exosomal marker CD-81 as Control. Microparticle were analysed using DLS and were found to be of size range 700–1000nm and Standard Exosomes are in the range of 70-100nm in size. (2e)Morphological analysis showed megakaryocytic features such as increased cell size upon megakaryocyte micro particles treatment as compared to untreated control cells and cell size was analysed by Image J analysis. (2f) TLR-2 induced megakaryocyte micro particles treatment induce the megakaryocyte maturation markers (CD41,CD-42b and CD-61) expression compared to control cells.(2g)Increased cytokine ( IL-1β,IL-2,IL-6,IL-8,Cox-2 and MCP-1 ) levels were observed in TLR-2 induced MPs treated Dami cells as compared to control.(2h) UV irradiation abrogated the micro particles mediated megakaryocyte maturation.(2i) megakaryocyte micro particles induces the megakaryocyte lineage markers (CD-41,CD-42b and CD-61) and decreases the erythrocyte lineage markers Glychophorin –A and Glychophorin-B from bi-potent progenitor cells.(2j) Cell morphology analysis by microscopy and analysis monocyte marker CD-11b at transcript levels by QRT PCR. (n=3, *P < 0.05).

**Objective-3**

**Study the TLR2 and TLR4 co-stimulation effect on megakaryocyte maturation.**

Cells receive a multitude of signals from the environment, for example during infection. During infection it is likely that more than one TLR gets activated, but how they process simultaneous TLR signalling inputs is not well understood. We therefore examined a scenario involving co-stimulation of TLR4 and TLR2.

To check the effect of TLR-2 and TLR-4 co-stimulation on megakaryocyte maturation we treated the cells with TLR-2 and TLR-4 agonists, both isolated and in combination. We checked megakaryocyte maturation markers by qRT-PCR and results suggest that upon treatment with TLR-2 and TLR-4 agonist stimulation all the maturation markers CD-41,CD-42b and CD61 were significantly upregulated compared to the untreated control cells. This further increased in TLR-2 and TLR-4 co-stimulation compared to the either of the TLR alone and untreated control cells (Figure-3a).

We also checked the TLR-2 and TLR-4 co-stimulation effects on corresponding TLRs and their partner TLRs. Our results suggest that TLR2 and TLR 4 and their partners TLRs such as TLR-6 were induced, whereas, the non-specific TLRs TLR-3 and TLR-9 did not show any change in both isolated TLR stimulation and TLR co-stimulation (Figure-3b). These results suggest that though the TLRs are part of innate immunity which is a non-specific immunity, yet TLRs respond very specifically to the ligands that we provide.

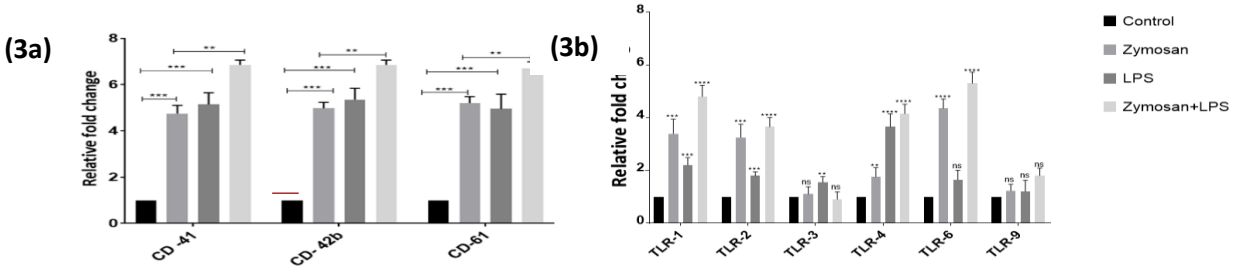


**Figure 3. TLR-2 and TLR-4 co-stimulation increases megakaryocyte maturation markers, corresponding TLR expression beyond their isolated stimulus.** Analysis of megakaryocyte maturation markers CD-41, CD-42b and CD-61 by Real time PCR analysis. Upon treatment with zymosan, LPS, or co-stimulation (Zymosan and LPS) condition and results were expressed as relative to their control levels (3a). Multiple TLR expression analysis at transcript levels upon treatment with zymosan, LPS isolated, or co-stimulation (Zymosan and LPS) condition and the results were expressed as relative to control levels (3b) (n=3, *P<0.05).

Several studies have identified cross talks between TLR signalling and the PI3K/Akt pathway [11-12], hence we checked the effect of TLR-2 and TLR-4 co-stimulation on PI3K/Akt, p-ERK (Figure-4a) and NF-κB p65 pathway by using western blotting analysis. Our results suggest that p-AKT, NF-κB p65 levels were up-regulated in TLR-2 and TLR-4 co-stimulatory conditions (Figure-4b), whereas, p-ERK levels were increased in TLR2 and TLR4 isolated stimulus which is not increased further in TLR-2 and TLR-4 co-stimulation conditions. We analysed the NF-κB kinetics with different time periods, upon Zymosan or LPS stimulation. NF-κB levels were progressively increased till 60 min, while after 60 min incubation its levels was reduced. However, with co-stimulation the levels of NF-κB was further stabilized up to 120 min (Figure-4c). NF-κB plays important role in modulating the inflammatory cytokines, therefore, we further checked the role of TLRs co-stimulation on cytokines expression by using the qRT-PCR method. Cytokines levels were significantly more in co-stimulation as compared to either of the TLRs stimulation alone and untreated control cells (Figure-4d). We further checked the TLR co-stimulation effect on ROS generation by DCFDA method using Fluorimeter, upon treatment with Zymosan or LPS, the ROS levels were up-regulated when compared with untreated control, however, upon TLR-2 and TLR-4 co-stimulation the ROS was more than that observed with either TLR2 or TLR4 stimulation (Figure-4e), which suggest that increased cytokines expression may help the increased megakaryocyte maturation.

**Conclusions**

Our findings are opening the new insights into the TPO-independent molecular mechanisms regulating the megakaryocyte development. TLR2 signalling could induce the megakaryocyte maturation by activation of TLR-2 downstream signalling pathways ways such as PI3K-AKT, MAPK and NF-KB signalling pathways. TLR-2 stimulation is inducing the secretion of cytokines and generation of ROS which plays important role in megakaryocyte maturation. TLR-2 stimulated MK-derived MPs could also induce the megakaryocyte maturation and lineage determination. UV irradiation abrogated the MPs induced MK maturation and MK MPs may specifically act on megakaryocyte and its progenitors. TLR-2 and TLR-4 co-stimulation could induce the megakaryocyte maturation more effectively than the either of the TLR2 or TLR4 stimulation alone.

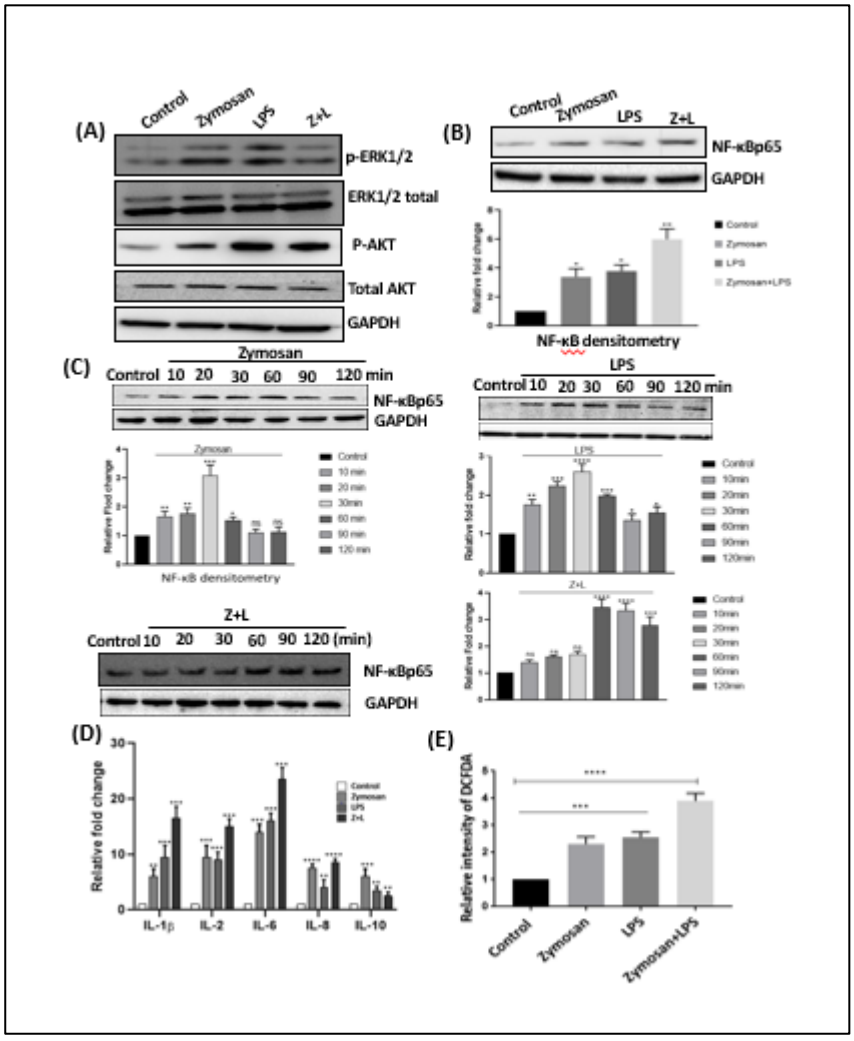


**Figure 4. Functional effects of TLRs co-stimulation on megakaryocytes.** Immunoblotting analysis of p-ERK, p-AKT and their respective total levels (4a) western blotting analysis of NF-κB-p65 levels and quantification by Image J analysis (4b). Time kinetics of NF-κB-p65 levels in Zymosan , LPS isolated stimulus and Zymosan and LPS co stimulation and their quantification by Image J analysis (n=3, *P<0.05) (4c). mRNA expression of multiple inflammatory cytokines was quantified by qRT-PCR upon treatment with zymosan, LPS, or co-stimulation condition, and compared with control (n=3, *P<0.05)(4d). ROS levels were measured by fluorescence spectroscopy using DCFDA, upon treatment with zymosan, LPS, or co-stimulation the DCFDA intensity was increased in TLR-2 or TLR-4 stimulated cells which is increased further in TLR-2 and TLR-4 co-stimulation compared to either of the TLRs stimulation alone and control cells (n=3, *P<0.05).

**Publications**


1. Sharma DS, 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.
2. **Kovuru N**, Raghuwanshi S, Sangeeth A, Malleswarapu M, Sharma DS, Dahariya S, Pallepati A, Gutti RK**.** Co-stimulatory effect of TLR2 and TLR4 stimulation on megakaryocytic development is mediated through PI3K/NF-κB and XBP-1 loop. ***Cell Signal.*** 2021 Jan 11;80:109924.
3. Sharma DS, Raghuwanshi S, **Kovuru N**, Dahariya S, Gautam DK, Paddibhatla I and Gutti RK**.** Virodhamine, an endocannabinoid induce megakaryocyte differentiation by regulating MAPK activity and function of mitochondria. ***J. Cell Physiology.*** 2021 Feb;236(2):1445-1453.
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6. **Kovuru N**, Raghuwanshi S, Gutti RK**.** Exosome mediated differentiation of megakaryocytes: a study on TLR mediated effects. ***J Thromb Thrombolysis***. 2019 Jul;48(1):171-173.
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**Conferences Attended**

1. Indian society of developmental biology (InSDB2017), IISER Pune.
2. International Congress of cell biology (IICB 2018), CCMB Hyderabad.
3. Bio Quest annual meeting 2015-2016 UOH Hyderabad.
4. Bio Quest annual meeting 2016-2017 UOH Hyderabad.
5. Bio Quest annual meeting 2018-2019 UOH Hyderabad.

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