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Original Article

Significance of TLR2 signaling during megakaryocyte development: regulatory cross-talk of miR-125b, cytokine induction, and MAPK pathway during dengue infection

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Abstract: Objective: Dengue is a viral infection endemic in more than 100 countries as per the WHO reports with approximately 5.2 million patients worldwide that spreads from mosquitoes to humans. Severe form of dengue fever can cause serious bleeding (low platelets) and death. Megakaryocytes are the immune cells responsible for the production of platelets. The molecular drivers behind platelet defects are mostly ambiguous. Here, we attempted to understand the distinct pathogen-elicited toll-like receptors (TLRs) functions in megakaryocyte biology. To understand the TLR induction and the molecular events that are governed in the mammalian system during dengue infection and to study TLR2-mediated cellular signaling-associated mechanisms with respect to their dimerization partners during dengue infection. Methods: In this study, we used the human Megakaryoblastic cells, DAMI, and treated them with TLR agonists (LPS and Zymosan) and Dengue virus (DNV-II). Results and Discussion: TLR2 could play an important role by dimerizing with TLR1, TLR4, and TLR6, which we induced for functional characterization. We observed that megakaryocyte maturation markers CD-41 and CD-61 were elevated. This augmentation under the LPS and Zymosan system along with DNV Infection was further confirmed. Our analysis also suggested that activation of miR-125b and MAPK signaling led to lipid droplet elevation. This led us to analyze TLR-mediated consequences and their impact on megakaryocyte development under diverse pathogen-elicited conditions. Conclusion: Pathogenic challenges associated with toll-like receptor system activation could further our understanding of the platelet biogenesis mechanistic pathways under various pathogenic circumstances.

Keywords: Toll-like receptors, MAPK-signalling, inflammasome, lipid droplets, miR-125b

Introduction

Thrombocytopenia is defined as a platelet count <150 × 10^9/L, which reaches levels that are thought to significantly increase the risk of haemorrhage (<50 × 10^9/L). Many factors can cause a low platelet count, such as certain types of cancer (leukaemia or lymphoma), aplastic anaemia, or autoimmune disorders [9, 18], and more importantly certain viruses such as Dengue or chickenpox and certain genetic conditions such as Wiskott-Aldrich and May-Hegglin syndromes.

Megakaryocytes (MKs) are known to be important blood cells and are recently known to be associated with immunity by regulating platelets [12]. These cells undergo endomitosis and thereby regulate the ploidy which is followed by cytoplasmic maturation and demarcation membrane system formation [15] and thereby regulating metabolic components which play a significant role in the maintenance of the platelet counts. Each mature MK produces 4000-5000 platelets and is vital for blood clotting and hemostasias. Although thrombopoietin (TPO) is commonly believed to regulate MK develop-

ment [11], it is unclear how infectious conditions could regulate platelet production. It is largely believed that during infection toll-like receptors (TLRs) could influence the megakaryocyte development and thereby platelets [7].

Toll-like receptors are conserved proteins that facilitate pattern recognition in response to pathogen stimuli and are part of the innate immune system in mammals. Toll-like receptors were identified on a variety of blood cells, including platelets and their precursors known as megakaryocytes. D'Atri et al. (2017) explain the TLR2 and TLR4 signalling importance during megakaryocyte maturation in human $CD34^+$ cells derived-MKs [3]. The release of Interleukin-6 (IL-6) from $CD34^+$ derived megakaryocytes was increased upon stimulation with LPS (TLR4 agonist) or Pam3CSK4 (TLR2 agonist), but not with TPO.

Thrombopoietin (TPO) traditionally governs platelet synthesis and megakaryocyte maturation. It was observed that the knockout of Tpo and its receptor c-mpl is still able to generate a sufficient number of platelets [1]. The platelets are potentially known to play a significant part in immunity and inflammation by modulating the inflammatory cytokines. TLR pathway could lead to the release of inflammatory cytokines that regulate inflammation, immunity and maturation of MKs. Our results in the past suggested that TLR2 were apparently present in both megakaryocytes and platelets [8, 20]. miR-125b functions in innate immune responses [26], against diverse pathogenic diseases related to TLR responses in the host system. It was found to be enhanced in a variety of infectious stimuli (LPS, Zymogen, and Dengue) and identified as a carrier molecule in Exosomes. It was also known as apoptosis associated noncoding RNA. Here, we wish to analyse TLR induction and the molecular events that are governed in the mammalian system during dengue infection. With this conceptual framework, we studied the TLR2-mediated cellular signalling-associated mechanisms with respect to their dimerization partners during dengue infection.

We hypothesize that TLR2 could dimerize with TLR1, TLR4 and TLR6, for regulating downstream functions and correlated metabolic pathway analysis to study the possible consequence of dengue infection. This led us to understand the signalling components including the regulation by non-coding RNA such as miRNA and key kinases which lead to differential gene regulation.

Materials and methods

In vitro culturing of megakaryocytes

Dami (Megakaryoblast cells) cell line was used as a model system. The Megakaryoblastic cell line Dami is derived from the peripheral blood of a patient with Megakaryoblastic leukaemia. These cells have characteristics of megakaryoblasts or immature megakaryocytes, displaying many of the morphologic and biochemical features of the megakaryocytic lineage. These cell lines were grown in Roswell Park Memorial Institute (RPMI)-1640 medium (GIBCO), with 10% fetal bovine serum (FBS), 1% antibiotics and maintained in a 5% CO_2 incubator at 37°C. These cells can be induced to differentiate into megakaryocyte lineage by using phorbol 12-myristate 13-acetate (PMA). These cells increase their ploidy and the expression of CD41, CD42b and CD61 markers in the mature megakaryocyte.

Cell culture and viral propagation for infection

This work made use of the dengue virus serotype 2 (DENV-2) (TR1751) as described previously [1]. The virus was cultivated in C6/36 cells, concentrated, and stored at or below -80°C. Using the D1-4G2-4-15 antibody, the virus was titrated using the focus-forming unit (FFU) test on Vero cells. The L-15 media was used to keep the Aedes albopictus C6/36 cells (CRL-1660, ATCC) at 28°C. Human Megakaryoblastic leukaemia cell line Dami was cultured in antibiotic solution and RPMI medium with 10% FBS and 5% CO_2. Prior to studies, Dami cells were given PMA (50 nM; Sigma), treatment for 48 hours in order to promote further differentiation with Minimal Essential Medium (MEM) (Invitrogen) and also 0.1 moi DENV infection, for 48 hours at 37°C. Cells were then washed with PBS, and resuspended in RPMI-1640 media with 10% FBS/1X antibiotics, for the investigation of viral-induced effects megakaryocyte system.

RNA preparation and RT-q-PCR gene expression analysis

Total RNA was isolated from Dami cells treated with different agonists by using the RNAeasy

Mini Kit (Qiagen Sciences). cDNA synthesis was carried out using Superscript III Reverse Transcriptase (Invitrogen). Quantitative real-time-PCR (qRT-PCR) assay was be performed with a Real-Time PCR Detection System (Applied biosystems). The relative quantities of mRNA for several genes were determined using iTaq universal SYBR green supermix (Bio-Rad). Target-gene transcripts in each sample were normalized to GAPDH or β-actin and expressed as a relative increase or decrease compared with control.

Immunoblotting

Proteins were extracted from the Dami cells after 48 hours of culture using radioimmunoprecipitation assay (RIPA) buffer (G-biosciences) in the presence of a mixture of protease inhibitors (Roche Applied Sciences) and Phosphatase inhibitor. The extracts (50 µg) were subjected to separation on a 10 to 12% SDS-PAGE gel and transferred to a nitrocellulose membrane (Millipore) by electro-blotting. The membranes were blocked for 1 h at room temperature in TBS-T buffer with 5% non-fat dried milk. These blots were incubated with specific primary antibodies, specific to AKT, p-AKT, ERK, p-ERK, NF-Kb, Bcl-2, Bim, Caspase, AIF, PARP, P-21, XIAP, COX-4, and GAPDH (CST) overnight at 4°C. The blots were washed three times for 10 min with TBS-T buffer and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10000), goat anti-rabbit IgG, and anti-mouse IgG (Cell Signalling) for 1 h at room temperature. After washing these blots were developed using enhanced chemiluminescence reagents (ECL, G-bioscience) and visualized using a chemidoc instrument (BioRad).

Measurement of intracellular reactive oxygen species

2,7-dichlorodihydrofluorescein-diacetate (H2DCFDA) is a cell-permeable dye that was used to monitor the intracellular ROS levels from the DNA-stimulated DAMI cell (1×10^6 cells/ml). Here, we measured the ROS from different conditions such as control, DNV, Zymogen, and LPS, along with the combination of both LPS & Zymosan for 48 hrs incubation time period. Briefly, cells were washed with PBS and incubated with H2DCFDA dye (10 µM) 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) was represented as bar graphs by using the fluorimeter. Results were represented as fold difference.

Phase contrast microscopic analysis

As per the previous literature, the human megakaryocyte cell line DAMI was propagated in RPMI medium containing 10% foetal bovine serum (FBS) and 1% antibiotic ant-anti (Life Technologies, Inc.). To induce the Toll-like receptor system, DAMI cells were treated with TLR-2 agonist for 48 hours of stimulation and further, these megakaryocytic maturation features were analysed under an Inverted Light Microscope.

Giemsa staining analysis

DAMI was treated with TLR-2 agonist and Dengue infection was analysed for phenotypic characterisation under 48 hrs incubation period. Cells were harvested and washed with 1×PBS, and 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 (Sigma) for 20 min followed by rinsing with distilled water. Air-dried slides were observed under an Olympus fluorescence microscope.

Oil red staining

The mechanistic pathway involves the formation of proplatelet-like particles from the Megakaryoblast basically by extravesicular signalling molecules which are profoundly formed from Lipid-droplets. These Lipid droplets can reprogram the Megakaryoblast towards proplatelet formation, which are driving particles further to become platelets. To analyse the total lipid droplets deriving under in vitro conditions, oil-red (Sigma) staining was performed as per the previous protocol with minor modification.

Statistical analysis

All data were assessed using the statistical software GraphPad Prism 8.0 version and were expressed as mean ± standard deviation. At least 3 independent experiments were per-

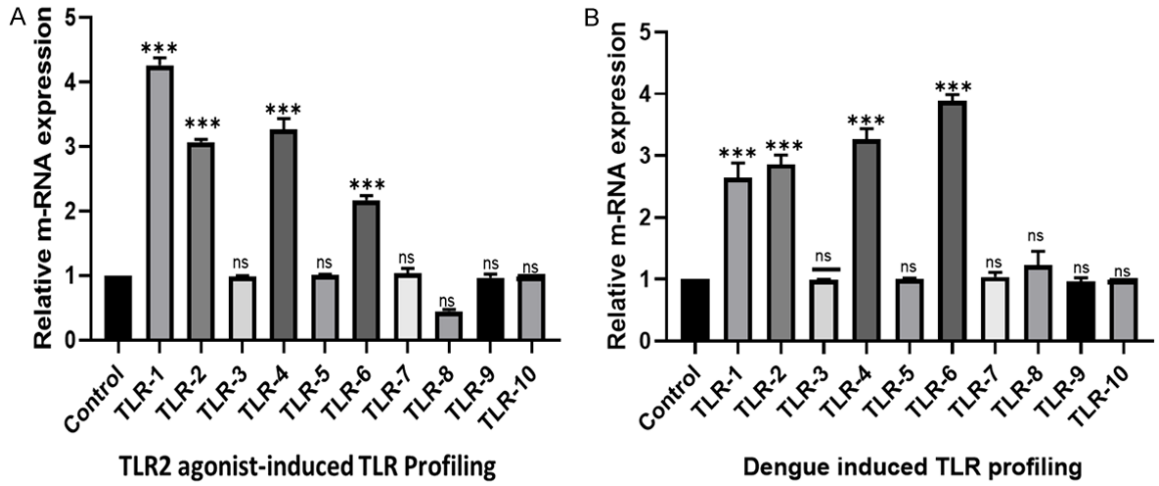

Figure 1. TLR profiling, expression, and regulation. A. TLR1, TLR2, TLR4 and TLR6 show differential expression after being induced by TLR2 agonist (n=3, ***P<0.001). B. TLR1, TLR2, TLR4, and TLR6 show differential expression after being induced by Dengue virus (n=3, ***P<0.001).

formed for each condition, and the statistical analysis was performed by either unpaired student t-test or one-way ANOVA and statistical significance was evaluated by using a GraphPad Prism.

Results

Profiling of toll-like receptor expression alleviated the megakaryocyte maturation

Upon treatment with TLR-2 agonist (Zymosan), TLRs were profiled using the qRT-PCR analysis after induction with TLR2 agonist (Zymosan, 10 μg/ml) (**Figure 1A**). Similar TLR profiling was carried out with dengue infection (**Figure 1A** and **1B**). When cells were treated with a TLR2 agonist, mature megakaryocyte polyploidization, and consequently, enlarged cell size was noticed (**Figure 2A** and **2B**). As megakaryocyte undergoes maturation, they increase its membrane integrin's expression, and consequently cells become partially adhered to the substratum which is considered as one of the important features of mature megakaryocyte. Upon TLR2 stimulation, cells size and adherence to substratum were increased (**Figure 2A** and **2B**). We further checked the integrin's responsible for the megakaryocyte substrate adherence such as CD-41, CD-61, and CD-42b. Expressions 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 (**Figure 2C**).

Similarly, upon TLR2 agonist associated stimulation, increased expression of the TLR2 and its partner's Toll-like receptors such as TLR1, TLR4 and TLR6 were increased significantly at transcript level compared to the untreated control cells (**Figure 1A**). However, there was no significant change in other non-specific TLRs such as TLR3, TLR5, TLR7, TLR8, TLR9 and TLR10 (**Figure 1B**). Interestingly, we also noticed elevated levels of TLR-1, TLR-4 and TLR-6, along with TLR-2 upon treatment with dengue virus (**Figure 1B**).

TLR2 stimulation induces NF-κB p65 along with p-AKT and pERK1/2 levels in megakaryocyte

TLRs are known to induce transcriptional factor NF-κB p65 which upon translocation to nucleus induces the inflammatory cytokine. To investigate the TLR2 effect on NF-κB p65 in megakaryocytes, Dami cells were treated with TLR2 agonist and western blot analysis showed increased levels of the NF-κB p65 and p-IκB levels in TLR2 stimulated compared to untreated control cells (**Figure 3A**) [5], suggesting that increased NF-κB p65 may be playing an important role in inflammation or infection-mediated emergency thrombopoiesis. NF-κB p65 is known to act as a transcription factor and induce inflammatory cytokines in sentinel cells.

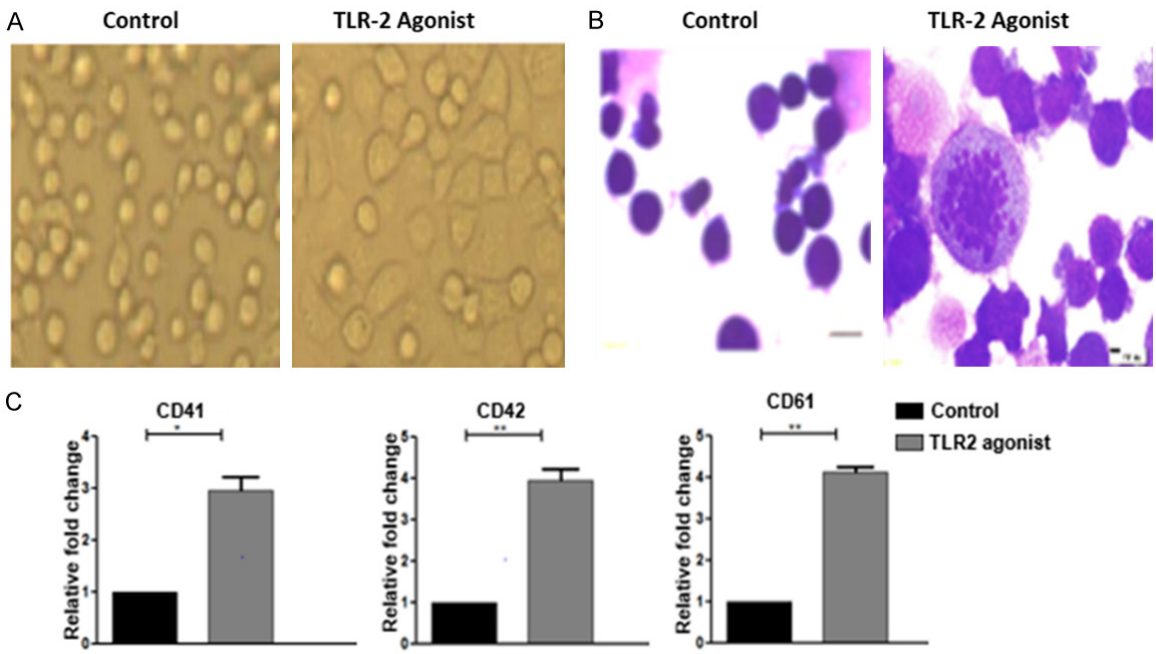

Figure 2. Morphological and molecular characterization of megakaryocyte maturation. A. Increase in the cell size by TLR2 agonist stimulation using microscopy (scale bar-20X, n=3). B. Multilobulated nuclear morphology by TLR2 agonist stimulation using Giemsa staining (scale bar-20X, n=3). C. mRNA expression of megakaryocytic markers (CD41, CD42, and CD61) was quantified by qRT-PCR upon treatment with TLR2 agonist (zymosan) and values are expressed as relative to their levels in control (n=3, *P<0.05, **P<0.001).

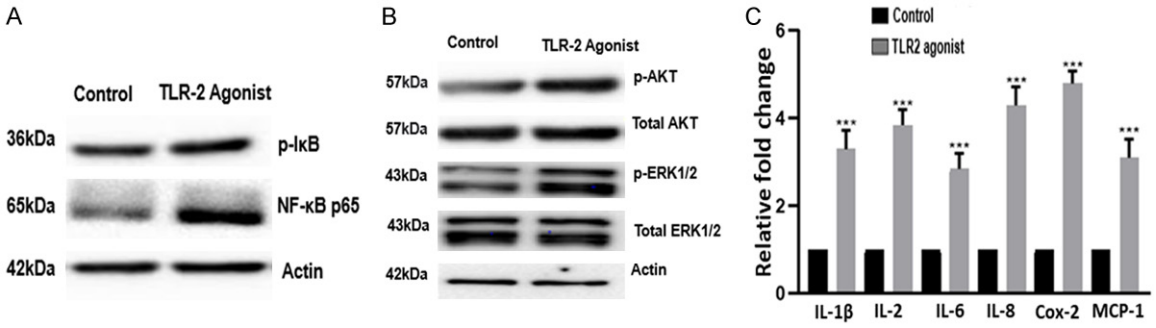

Figure 3. Significance of NFκB, ERK1/2 and cytokines during TLR2 signalling in megakaryocyte development. A. Western blot analysis showing increased levels of the NF-κB p65 and p-IκB levels in TLR2 stimulated compared to untreated control cells. B. p-AKT and p-ERK levels were increased in TLR2 stimulated compared to untreated control cells, whereas the total AKT and ERK levels were not changed. C. 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 (n=3, ***P<0.001).

TLR2 agonist induced cytokine production in megkaryocytes

We investigated the downstream targets of NF-κB pathway, where Dami cells were treated with TLR2 agonist and cytokines levels were analysed at transcript levels by using qRT-PCR. We could induce 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 (**Figure 3C**). These increased cytokines could be involved in megakaryocyte development [2].

Interaction between TLR and miRNA during megakaryocyte development

Extracellular vesicles are well characterised transporters for versatile miRNA, lncRNA, tran-

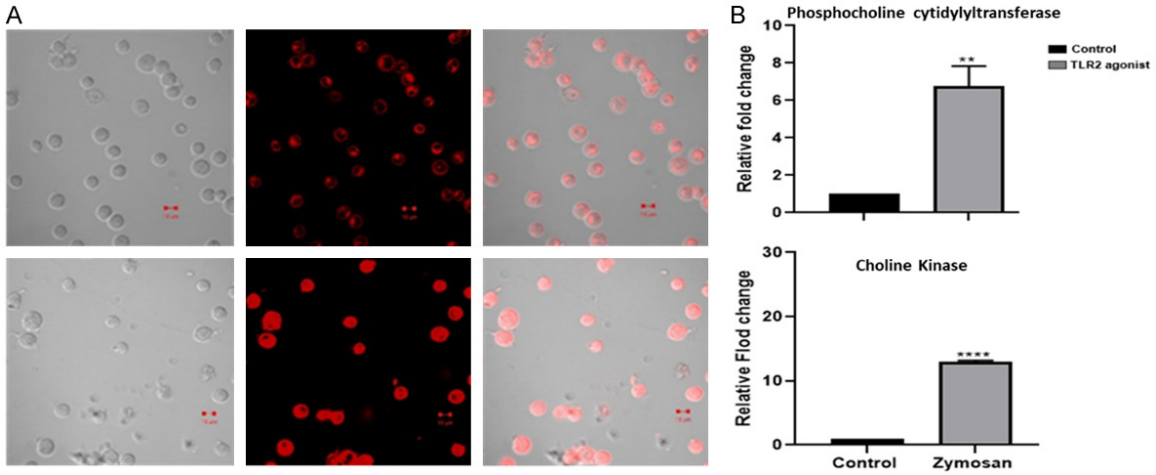

Figure 4. TLR2 signalling induces lipid droplets during megakaryocyte development. A. TLR2 stimulation could induce lipid droplet biogenesis which could supplement the membrane formation (scale bar-10 um, n=3). B. Enzymes involved in phosphatidylcholine (PC) biosynthesis such as choline kinase expression and CTP: phosphocholine cytidylyltransferase levels were increased at transcript levels by using the qRT-PCR (n=3, **P<0.01, ****P<0.0001).

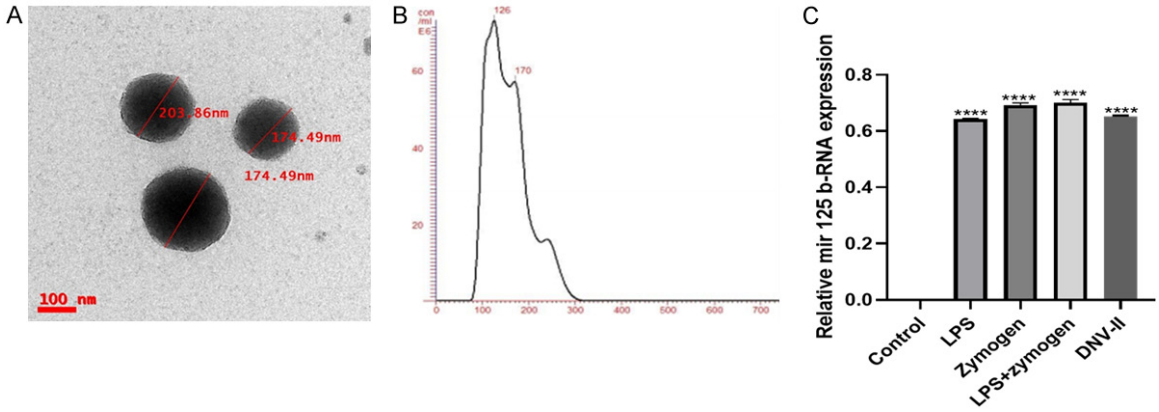

Figure 5. Interaction between TLR and miRNA during megakaryocyte development. A. Circular size exosome vesicles (size-100 nm, n=3). B. Vesicles which were further confirmed by dynamic light scattering (DLS) showing the size of standard exosomes in the range of 150-200 nm. C. miR-125b levels were induced in TLR-2 agonist induced as well as in dengue-induced cells (n=3, ****P<0.0001).

scription factor and bioactive lipids from one cell to other cells (donor cells to recipient cells) and also possibly participate in cellular reprogramming. To study the effect of TLR-2 derived MPs, we treated cells with zymosan (10 µg/ml) for 48 hours and we collected the medium of TLR-2 stimulated cells to characterise the exosome. Physical characterization of filtrate was done by transmission electron microscopy (TEM, **Figure 5A**). The filtrate contained circular size vesicles, which were further confirmed by dynamic light scattering (DLS) which give mean particles size (**Figure 5B**). The size of standard exosomes was identified in the range of 150-200 nm (TEM, **Figure 5A**). Interaction between TLR and non-coding RNAs such a miRNA is known. TLRs are transmembrane proteins belonging to the PRR family. These receptors have interactions with a number of miRNAs. The interactions between miR-125b and TLR have been assessed in megakaryocytes upon TLR2 agonist stimulated exosomes derived from Dami cells and dengue induced Dami cells. miR-125b levels were induced in TLR-2 agonist induced exosomes as well as dengue-induced cells (**Figure 5C**).

Further, bioinformatics analysis using miRwalk (http://mirwalk.umm.uni-heidelberg.de/) revealed strong energy of -24.3 and -24.6 with TLR2 and TLR6, respectively (**Figure 6**).

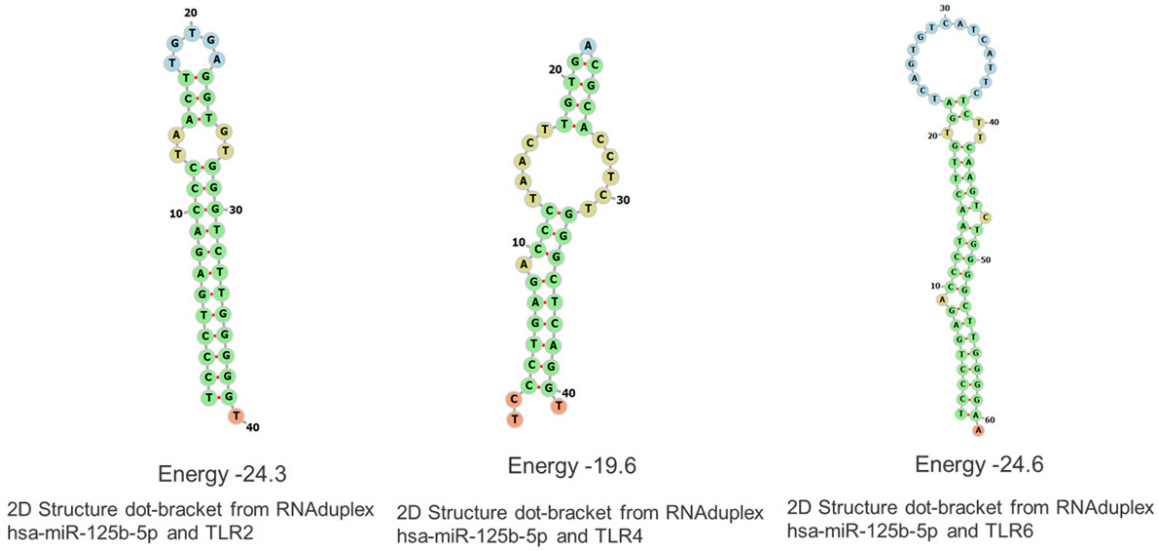

Figure 6. Bioinformatic analysis of TLR2, TLR4 and TLR6 with miR-125b. Bioinformatics analysis using miR-walk revealed strong energy of -24.3 and -24.6 with TLR2 and TLR6 respectively.

Crosstalk between membrane lipids and TLRs

Megakaryocyte develops extensive invaginated membrane system during the maturation which is supported by extensive lipid biogenesis. Therefore, we checked the effect of TLR2 agonist stimulation on lipid droplets in Dami cells. To check that cells were treated with TLR2 agonist for 48 hrs and were stained with Nile red fluorescent dye and imaged under confocal microscopy. TLR2 stimulated cells had increased fluorescence intensity (lipid droplets) compared to untreated control cells (**Figure 4A**).

We further checked the enzymes involved in phosphatidylcholine (PC) biosynthesis such as choline kinase expression and CTP: phosphocholine cytidylyltransferase levels at transcript level were measured by using the qRT-PCR. Upon treatment with TLR2 agonist both enzyme levels were increased at transcript levels compared to untreated control cells (**Figure 4B**). The findings suggest that TLR2 stimulation could induce the lipid droplet biogenesis which could supplement the membrane materials for the demarcation membrane system development [22].

TLR-2 and 4 co-stimulation cause elevation in MAPK signaling and ROS

In silico analysis showed that miR-125b was regulating ten [10] signalling pathways. Among these pathways identified were previously reported to be involved in MK development such as hematopoietic cell lineage MAPK signalling and cytokine-cytokine receptor interaction pathways (**Figure 7A**). Several studies have identified cross talks between TLR signalling and the kinase pathways; hence we checked the effect of TLR-2 and TLR-4 co-stimulation on ERK/p-ERK pathway by using western blotting analysis. Our results suggest that p-ERK1/2 levels were up-regulated in TLR-2 and TLR-4 stimulatory conditions (**Figure 7B**). A recent study suggested activation of TLR2 heterodimers-mediated NF-κB and MAPK signalling pathways triggered inflammatory response *in vitro* in *Vibrio alginolyticus* [21].

NF-κB plays predominant role in modulating the inflammatory cytokines, therefore, we further checked the role of TLRs stimulation by Dengue virus on cytokines expression by using the qRT-PCR method. Cytokines levels of TNF-α, IL-1β, IL-2, IL-6 and IFN-b were significantly increased in dengue as compared to PMA stimulation alone and untreated control cells (**Figure 9**). Significant downregulation of IL-10 was observed. Dengue elevated cellular signalling cascades play a vital role in the defence mechanism against pathogen exposure through releasing inflammatory mediators into the cell during the process of cell survival mechanism. Also, up-regulation in the expression levels of IL-1β, IL-6 and TNF-α in *V. alginolyticus* occurs through NF-κB and MAPK signalling [13].

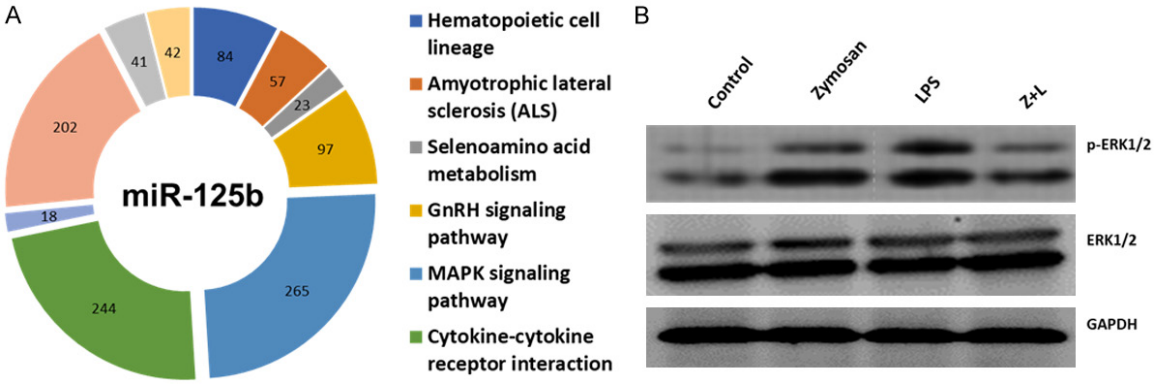

Figure 7. Significance of co-stimulation and TLR2 signalling during dengue infection. A. miR-125b regulated signalling pathways by *in silico* analysis. B. ERK1/2 and pERK1/2 protein levels increased upon zymosan, LPS, or co-stimulation as compared to that in the control (n=3).

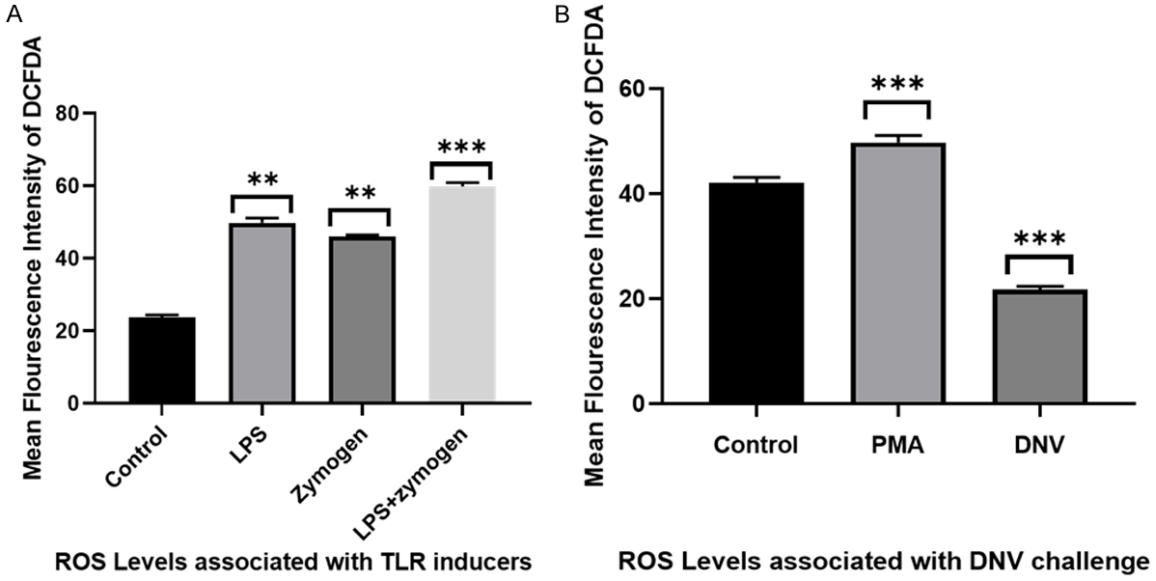

Figure 8. ROS elevation under TLR2 induction and Dengue infection conditions. A. ROS levels were measured by fluorescence spectroscopy using DCFDA. Upon treatment with zymosan, LPS, or co-stimulation, the DCFDA intensity was increased when compared with control (n=3, **P<0.01, ***P<0.001). B. ROS levels were measured by fluorescence spectroscopy using DCFDA. Upon treatment with PMA or dengue virus, the DCFDA intensity was decreased when compared with control (n=3, ***P<0.001).

Dengue infection-induced ROS

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 level was increased more than that observed with either TLR2 or TLR4 stimulation (**Figure 8A**), which suggest that increased cytokines expression may help the increased megakaryocyte maturation. Predominantly NADPH oxidases and the mitochondrial electron transport chain, which are regulated by cytokines and growth hormones, create ROS. However, in dengue infection condition, the ROS levels were downregulated (**Figure 8B**), which possibly affirm that downregulation of ROS possibly deregulates the Megakaryocyte developmental functions.

Dengue thwart megakaryocyte maturation through hampering apoptosis

Apoptosis was found to be a vital tool for the manufacture of platelets from the megakaryo-

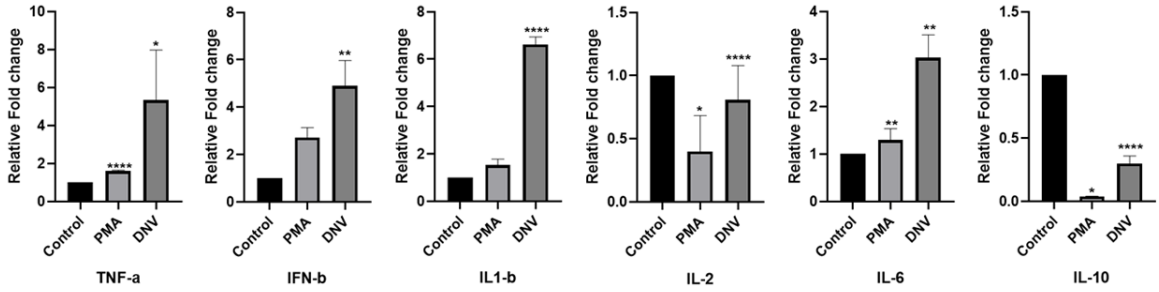

Figure 9. Inflammatory mediator's regulation under TLR2 induction and Dengue infection. Cytokines levels of TNF-α, IL-1β, IL-2, IL-6, and IFN-b were significantly increased in dengue as compared to PMA stimulation alone and untreated control cells (n=3, *P<0.05, **P<0.01, ****P<0.0001).

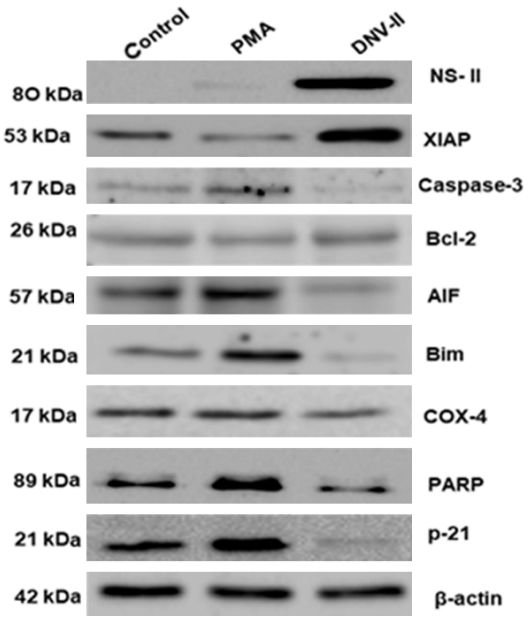

Figure 10. Dengue infection induced apoptosis regulation. Apoptosis-associated protein levels such as Bcl2, AIF, Bim, p21, and PARP were significantly downregulated in dengue as compared to PMA stimulation alone and untreated control cells.

cyte under the physiological conditions. Apoptosis is an important modulator of cellular immune responses during systemic viral infections. Peripheral-blood mononuclear cell (PBMC) apoptosis was frequently seen in dengue fever [19]. Increase in ROS is crucial for the megakaryocyte development and platelet production. All the apoptosis associated protein level such as Bcl2, AIF, Bim, p21, PARP was significantly downregulated in dengue as compared to PMA stimulation alone and untreated control cells (**Figure 10**).

Recent report suggests that activating the TLR2 complex by the agonist Pam3CSK4 in human AML resulted in induction of apoptosis by MAPK-dependent activation and myeloid differentiation in a NF-κB-dependent manner [4]. These results emphasize the significance of apoptosis in megakaryocyte developmental perturbations associated with infectious conditions. In addition, data suggest that viruses such dengue are able to impede megakaryocyte development predominantly by manipulating the apoptosis mechanism.

Discussions

The platelets are known to play a significant part in immunity and inflammation by modulating the inflammatory cytokines. TLR pathway could lead to the release of inflammatory cytokines that regulate inflammation, immunity and maturation of MKs. Interestingly, TLR2 were apparently present in both megakaryocytes and platelets. Here we studied the TLR2-mediated cellular signalling-associated mechanisms with respect to their dimerization partners during dengue infection.

The relevance of TLR-1 and TLR-6 dimerization with TLR-2 was previously investigated as having a potential function in megakaryocyte maturation and platelet generation [8, 20] and TLR4-knockout mice have a lower number of platelets than wild-type mice, showing that TLRs play important role in platelet production and can promote thrombopoiesis [2]. A study showed the activation of TLR2 and TLR6 by Dengue NS1 protein and its significance in dengue virus infection [6]. This suggests the significance of TLR-2 dimerization with TLR-6 as having a potential role in the maturation of megakaryocytes, which legitimately results in platelet production. Dengue-infected and DV NS1 protein-treated TLR6-/- mice have higher sur-

vivability compared to DV-infected and DV NS1 protein-treated wild-type mice [2].

Megakaryocyte development is regulated through c-Mpl receptor signalling, which mediates through downstream PI3K-AKT and MAPK signalling pathways and pharmacological inhibitors of MEK such as PD98059 and U0126, and PI3K/AKT inhibitor LY294002 abrogated the megakaryocyte polyploidization and maturation [16]. We tried to understand whether TLR-2 mediated megakaryocyte maturation follows the same pathways and components as TPO mediated megakaryocyte maturation. Our results show that the 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 (**Figure 3B**). This suggest that TLR2 mediated megakaryocyte maturation may be sharing the similar pathways and components with TPO mediated megakaryocyte maturation.

The cell-associated molecular patterns of inflammatory mediated profile, such as IL-6, IL-1B, MCP-1, COX-2, and IL-8, are known to be crucial for megakaryocyte development and can play a vital role in terminal differentiation. The levels of these were elevated in response to TLR2 agonist (**Figure 3C**). TLR signalling could lead to megakaryocyte maturation, as shown by expression of maturation markers CD-41 and CD-61. It could be anticipated that elevated TLR2/6 levels could control inflammasome levels, which in turn will co-activate MAPK signalling during the development of megakaryocytes.

Previous studies suggest that many miRNA expression depend on the TLR-induced NF-κB and MAPK pathways, where, miRNA expression including miR-146a, miR-155, miR-132, miR-223, miR-147, miR-9, miR-27b, let-7e, miR-21, miR-16, miR-23b, miR-30b, miR-301a, and miR-125b is induced in an NF-κB-dependent manner after TLR stimulus or pathogen infection [6]. Downregulation of miR-125b was reported in TLR-modified colorectal cancer cells (CRC). Kinases regulated miR-125b-5p expression as well as lipogenesis-associated enzyme activities in TLR-modified CRC cells. This upregulation of miR-125b by TLR6-mediated NF-κB activation could reverse drug resistance and reduce EMT in CRC cells [14]. Moreover, the interactions between miR-125b and TLR have been assessed in megakaryocyte development through exosomes participation, including in dengue infectious condition [24].

An interesting aspect of the present study was that, elevation of extracellular vesicle mediated cellular communication in megakaryocyte development. For many years these extracellular released particles/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 and possibly act as cargo for plethora of biomolecules, through which they will be able to reprogram the cellular physiology. In our study, we found the elevation of lipid droplets under TLR associated stimulation. Lipid droplets contain active lipid metabolite derivatives derived from various lipid moieties, such as saturated and unsaturated lipid entities. They could participate in inflammatory pathways in cell-specific manner. The most recent data [23] showed that they can support a variety of cell communication infrastructures and could participate in a wide variety of physiological functions.

Apoptosis was a key aspect in the manufacture of platelets from the megakaryocyte under the physiological conditions and also responsive mechanism against various pathological burdens [25]. We understood differentially regulated apoptosis mechanism which will lead to regulation of megakaryocyte development (**Figure 10**).

Conclusions

In the present study, we investigated the toll-like receptors associated with the dengue virus induction and analysed the pathogen challenges in the generation of platelets from megakaryocyte lineage. It is well known that dengue infection will hinder platelet generation and could result in thrombocytopenia (low platelets) in humans. In assertion, it is necessary to comprehend the receptor mechanisms associated with platelet biogenesis, in addition to the conventional mechanism of thrombopoietin-based megakaryocyte development, towards platelet production. In this study, we attempted to comprehend the diverse roles played by the Toll-like receptor systems under diverse pathogenic

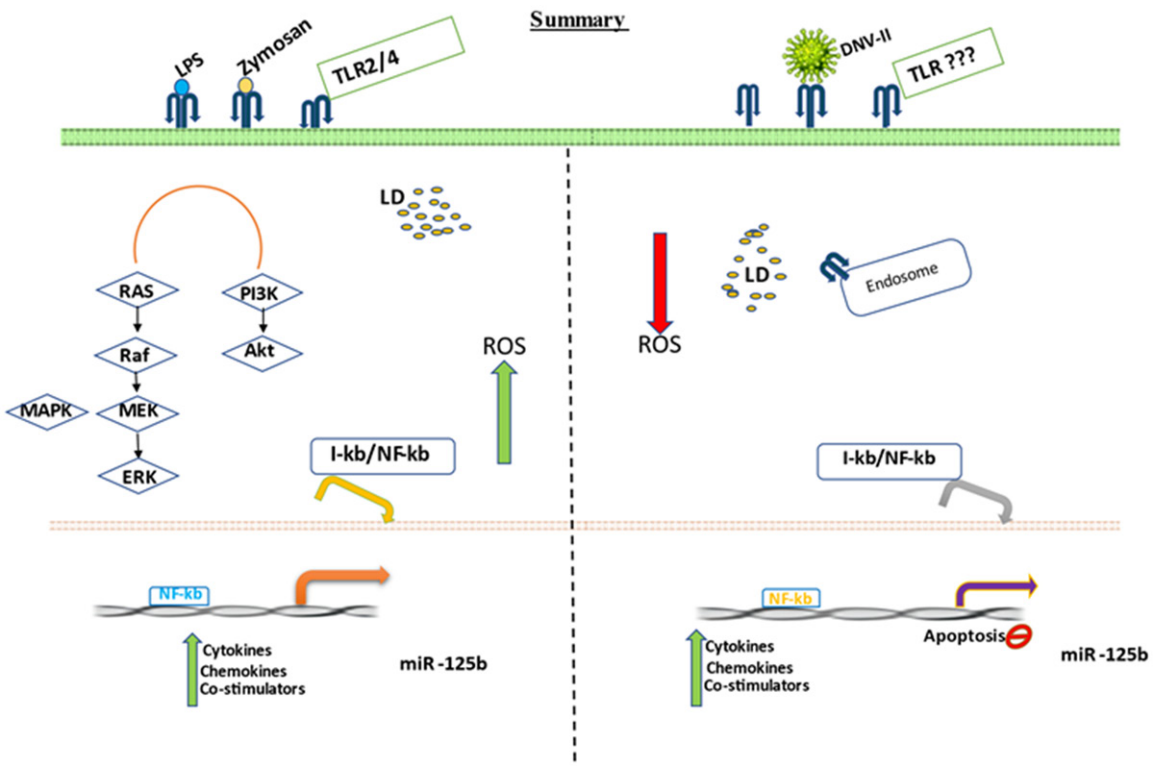

Figure 11. Summary of pathogenic challenges associated with toll-like receptor 2 signalling.

challenges, particularly through the role of TLR-2/4 in megakaryocyte development.

In summary, our findings suggest that pathogenic challenges associated with toll-like receptor system activation would further our understanding of the platelet biogenesis mechanistic pathways under various pathogenic circumstances, and may also serve as a novel therapeutic approach for various immune associated thrombocytopenic disorders (**Figure 11**).

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Disclosure of conflict of interest

None.

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