The Role of Calpain-Myosin 9-Rab7b Pathway in Mediating the Expression of Toll-Like Receptor 4 in Platelets: A Novel Mechanism Involved in α-Granules Trafficking

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Abstract

Toll-like receptors (TLRs) play a critical role in innate immunity. In 2004, Aslam R. and Shiraki R. first determined that murine and human platelets express functional TLRs. Additionally, Andonegui G. demonstrated that platelets express TLR4, which contributes to thrombocytopenia. However, the underlying mechanisms of TLR4 expression by platelets have been rarely explored until now. The aim of this study was to identify the mechanism of TLR4 expression underlying thrombin treatment. The human washed platelets were used in this study. According to flow cytometry and western blot analysis, the surface levels of TLR4 were significantly enhanced in thrombin-activated human platelets and decreased by TMB-8, calpeptin, and U73122, but not Y27632 (a Rho-associated protein kinase ROCK inhibitor) indicating that thrombin-mediated TLR4 expression was modulated by PAR/PLC pathway, calcium and calpain. Co-immunoprecipitation (co-IP) assay demonstrated that the interaction between TLR4 and myosin-9 (a substrate of calpain) was regulated by calpain; cleavage of myosin-9 enhanced TLR4 expression in thrombin treated platelets. Transmission electron microscope data indicated that human platelets used α-granules to control TLR4 expression; the co-IP experiment suggested that myosin-9 did not coordinate with Rab7b to negatively regulate TLR4 trafficking in thrombin treated platelets. In summary, phospholipase Cγ-calpain-myosin 9-Rab7b axis was responsible for the mechanism underlying the regulation of TLR4 containing α-granules trafficking in thrombin-stimulated platelets, which was involved in coagulation.

Introduction

Platelets are non-nucleated cellular elements that play a critical role in the process of homeostasis and also have roles in innate immunity and inflammation [1]. These anucleate megakaryocyte fragments have the ability to rapidly localize to sites of injury and infection where they release mediators that regulate inflammation and immune progression [2]. Platelets contain stores of cytokines and mediators within their α- and dense-granules that are released upon stimulation. Platelets can also bind to and internalize bacteria and viruses through engulfing, endosome-like vacuoles that fuse with platelet α-granules and allow granular proteins access to the pathogen [3]. Toll-like receptors (TLRs), homologs of the Drosophila protein Toll [4], are pattern recognition receptors that mediate cellular responses to a large array of microbial ligands [3]. Currently, more than 10 different TLRs have been identified; among these TLR4 is a receptor for gram-negative bacteria, LPS, and some viruses. TLR4 is expressed in many different cell types, including dendritic cells, neutrophils, macrophages, epithelial cells, keratinocytes, and endothelial cells [6–9]. Recently, both human and murine platelets have been shown to express functional TLR4 [10–12], indicating that the TLR4-mediated signaling pathway may contribute to cellular effects in platelets. An additional in vitro study demonstrates that LPS accelerates thrombin/collagen-induced aggregation in platelets and that this is mediated by TLR4 expression [13]. Data from in vivo assays also show that circulating platelet counts fall precipitously during sepsis and that the degree of thrombocytopenia correlates with the severity of sepsis [10,14]. Furthermore, platelet counts are decreased under septic conditions due to well-established migration into the lungs and liver [15]. Andonegui et al. have demonstrated that TLR4 on platelets is essential for platelet migration into the lungs using adoptive transfer of wild-type or...
TLR4-deficient platelets into wild-type or LPS-treated mice [5]. It had been reported that platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic situation [16]. Interestingly, extracellular histones promote thrombin generation through the TLR4 on platelets [17]. Thus, platelet TLR4 is proposed to have important roles in platelet function, including platelet adhesion and migration, as well as attraction and destruction. Platelets may also mediate inflammation, immune and aggregation progression via TLR4. However, the underlying mechanisms involved in regulation of TLR4 expression on the surface of platelets are still unclear and remain to be explored. Therefore, we used healthy washed human platelets to examine the expression of TLR4 in thrombin-stimulated platelets in this study and explored its underlying mechanisms in vitro.

Materials and Methods

Preparation of Washed Human Platelets
Washed platelets were isolated from health male human whole blood by previously described methods [18,19]. The Institutional Review Board (Taipei Medical University-Joint Institutional Review Board) approved this study, and all volunteers gave written informed consent prior to all procedures. To avoid leukocyte contamination, only the top 75% of the platelet-rich plasma was collected. Briefly, pelleted platelets were washed with HEPES buffer and resuspended in Calcium-free Tyrode's solution. Where indicated, washed platelets (3×10^7 platelets/μL) were pretreated with inhibitors (in 5% CO₂ incubation at 28°C for 60 minutes) before assaying.

Reagents
Thrombin, SFLLRN (a PAR1 thrombin receptor-derived hexapeptide), AYPGKF (a PAR4 thrombin receptor-activating peptide) and U73122 (a phospholipase C inhibitor) were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Calpeptin (a calpain inhibitor), 3,4,5-trimethoxybenzoic acid β-(diethylamino)octyl ester (TMB-8; a calcium antagonist), m-3M3FBS (a phospholipase C activator), Y27632 (a Rho-associated kinase inhibitor) and a calpain activity assay kit were supplied by Merck Co. (Frankfurt, Germany). The monoclonal mouse anti-human TLR4 antibody was obtained from Abcam Co. (Cambridge, UK). The monoclonal rabbit anti-human calpain antibody (clone: HPR3319) was obtained from Epitomics Co. (Burlingame, CA, USA). The monoclonal rabbit anti-human calpain antibody (clone: EP1332Y) and monoclonal mouse anti-human β-actin antibodies (clone: ACTN05/C4) were obtained from GeneTex Co. (Irvine, CA, USA) at 4°C for 1 hr. Precleared lysate was then immunoprecipitated with mouse monoclonal anti-TLR4 antibody (clone: 76B357.1; Abcam, Cambridge, MA, USA) or rabbit anti-myosin heavy chain-9 (myosin-9) antibody (Abcam Co., Cambridge, MA, USA) (2 μg antibody in 1 ml reaction) at 4°C for 16 hrs, and the protein-antibody complexes were then immunoprecipitated by adding 50 μl of 50% protein A sepharose at 4°C for 1.5 hrs. The beads were washed 3 times with extraction buffer. The beads containing immunoprecipitates were then resuspended in 2× SDS-PAGE sample buffer, and the reactions were subjected to western blot analysis using anti-human TLR4 antibody (clone: HTA125) was obtained from Biolegend Co. (San Diego, CA, USA).

Flow Cytometry
Platelets were first fixed with 1% paraformaldehyde for 1 hour at room temperature. Platelets were then stained with phycoerythrin (PE)-labeled monoclonal mouse anti-human TLR4 antibody (clone: HTA125) or a phycoerythrin (PE)-labeled mouse IgG isotype control in the dark. Finally, the platelets were washed with PBS and assayed using a BD FACS Canto II flow cytometer (BD Biosciences, Mountain View, CA, USA) with BD FACS Diva software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). The results were collected from 30,000 events.

Calpain Activity Assay
Calpain activity assays were performed as previously described [20]. This fluorometric assay is based on the detection of cleavage of the calpain substrate Suc-Leu-Leu-Val-Tyr-AMC. Proteolytic hydrolysis of the peptidyl-7-amino bond liberates the highly fluorescent 7-amino-4-methylcoumarin moiety. Quantitation of the 7-amino-4-methylcoumarin (AMC) fluorescence permits the monitoring of enzyme hydrolysis of the peptide-AMC conjugate and can be used to measure enzyme activity.

Membrane and Total Protein Extraction
Total protein were extracted with lysis buffer (0.5 M NaCl, 50 mM Tris, 1 mM EDTA, 0.05% SDS, 0.5% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride) and membrane proteins were harvested in membrane lysis buffer (20 mM Tris-HCl pH 7.5, 137 mM NaCl, 2 mM EDTA, 1% NP-40, 10% Glycerol, 1 mM PMSE, and 1× protease inhibitor cocktail; Sigma St. Louis, MO, USA) [21]. For membrane fraction, the platelets were followed by three cycles of freeze-thaw then centrifuged to remove the cytoplasmic fraction. The pellets were further washed with lysis buffer to prevent cytosolic protein contamination. Finally, the pellets were resuspended in lysis buffer and designated as the membrane protein fraction. The protein concentration was determined using the Bio-Rad Protein Assay Kit (Bio-Rad, Inc., Hercules, CA, USA) with BSA as the standard.

Western Blot Analysis
Approximately 50 μg of protein extract was subjected to polyacrylamide gel electrophoresis and then electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in TBST solution (20 mM Tris-HCl pH 7.5, 138 mM NaCl, and 0.2% NP-40) containing 5% milk powder, and proteins were detected with TLR4 antibody or calpain antibody. Peroxidase conjugated-anti-mouse IgG (Amersham, Arlington Heights, IL, USA) and peroxidase conjugated-anti-rabbit IgG (Amersham, Arlington Heights, IL, USA) were used as secondary antibodies. Peroxidase reactions were carried out and visualized using the chemiluminescence system (Millipore, Bedford, Mass., USA).

Co-immunoprecipitation (Co-IP) Assay
Total protein was extracted from washed platelets. The protein concentration was determined using the Bio-Rad Protein Assay Kit, and approximately 5 mg of protein extract was precleared with 20 μl of 50% protein A suspension (Bio-Rad, Inc., Hercules, CA, USA) at 4°C for 1 hr. Precleared lysate was then immunoprecipitated with mouse monoclonal anti-TLR4 antibody (clone: 76B357.1; Abcam, Cambridge, MA, USA) or rabbit anti-myosin heavy chain-9 (myosin-9) antibody (Abcam Co., Cambridge, MA, USA) (2 μg antibody in 1 ml reaction) at 4°C for 16 hrs, and the protein-antibody complexes were then immunoprecipitated by adding 50 μl of 50% protein A sepharose at 4°C for 1.5 hrs. The beads were washed 3 times with extraction buffer. The beads containing immunoprecipitates were then resuspended in 2× SDS-PAGE sample buffer, and the reactions were subjected to western detection and analysis with mouse monoclonal anti-human TLR4 antibody (clone: 76B357.1; Abcam, Cambridge, MA, USA) or rabbit polyclonal anti-myosin-9 antibody (catalog no: GTX13236; GeneTex Co., Irvine, CA, USA).

Mass Spectrometry
Total protein was extracted from washed platelets and subjected to immunoprecipitation. The immunoprecipitates were eluted with SDS sample buffer, resolved on an SDS-PAGE gel, and stained with Coomassie Brilliant Blue R-250 solution (Sigma, St. Louis, MO, USA). In addition to the TLR4 band, the protein was visualized using the chemiluminescence system (Millipore, Bedford, Mass., USA).
bands common to the anti-TLR4 antibody IP sample but not present in the mouse IgG IP control sample were excised from the gel. The gel pieces were then washed, reduced, alkylated, and digested with trypsin. The tryptic peptides were then analyzed by nano-LC/MS/MS on an LCQ Deca XP Plus ion trap mass spectrometer (Thermo Scientific, San Jose, CA, USA) coupled to an Agilent 1100 HPLC (Agilent Technologies, Inc., San Jose, CA, USA). The MS/MS spectra were searched using Sequest through the Bioworks Browser version 3.3.1 (Thermo Scientific, San Jose, CA, USA) against the NCBI nonredundant protein database.

Immunogold Staining and Transmission Electron Microscopy

Immunogold staining was performed as previously described [22]. In briefly, human platelets were fixed in 4% paraformaldehyde, 0.1% glutaraldehyde, and 0.05% tannic acid in 0.1 M Cacodylate Buffer and embedded in epoxy resin. Prepared 60–90 nm thin sections were detected with rabbit polyclonal anti-TLR4 antibody (catalog no. ab19068, Abcam, Cambridge, MA, USA) and then incubated with 18 nm Colloidal Gold-AffiniPure donkey anti-rabbit IgG secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). At the end, the sections were finally coated with saturated Uranyl acetate and Reynold’s lead citrate solution. Sections were examined with a Hitachi H7000 transmission electron microscope (TEM) (Hitachi Chemical Co., Ltd, Japan) operating at 75 kV under standard operating conditions.

Statistical Analysis

The values were expressed as the mean ± SEM. Statistical evaluation was performed using one-way ANOVA followed by the Dunnett test, with a p value<0.05 being considered significant.

Results

Thrombin Induces TLR4 Expression on the Surface of Platelets via the PAR/PLC Pathway

A previous study demonstrated that expression of TLR4 on the surface of platelets plays an important role in platelet-related immunity [1]. The mechanisms involved in the regulation of TLR4 expression on the surface of resting or activated platelets are as yet unclear and remain to be studied. Flow cytometry using a phycoerythrin (PE)-labeled mouse anti-human polyclonal TLR4 antibody was performed to determine whether surface expression of TLR4 is increased in activated platelets. As shown in figure 1A, TLR4 fluorescence intensity on the surface of platelets was increased (right shift) in the thrombin-activated group compared with the resting naïve group. Furthermore, stimulation with 0.2, 0.3 or 0.4 U/mL thrombin significantly increased the expression of TLR4 in a dose-dependent manner relative to that of the untreated control group (279.56±74.72%, 263.12±79.16% and 263.75±34.07% of control, respectively) (figure 1B). The stimulation of thrombin did not significantly increase the total TLR4 expression in human platelets. The effects caused by thrombin were further supported by western blot analysis of membrane-bound TLR4 proteins (figure 1C). Previous studies using antagonists or antibodies that block PAR1 and PAR4 activation had indicated that PAR1 mediates human platelet activation at low thrombin concentrations, whereas PAR4 contributes to thrombin-induced platelet activation at high thrombin concentrations [23–25]. Thrombin may activate both the PLC and Rho pathways, two major G protein-mediated signaling pathways initiated by Gq and G12, respectively, through G protein-coupled receptors [26].

Thrombin-mediated TLR4 Expression in Platelets is Modulated by Calpain Activity

Previous evidence demonstrated that thrombin can induce calcium mobilization in the cytoplasm of platelets and further increases calpain (calcium-dependent, non-lysosomal cysteine proteases) activation [27]. To confirm the roles of calcium and calpain in thrombin-mediated TLR4 expression and to determine whether the elevation of intracellular calcium can activate calpain and further increased TLR4 expression in platelets, we treated platelets with calpeptin or TMB-8 followed by thrombin or CaCl2 treatment, and the expression levels of membrane-bound TLR4 and cytosolic calpain were assayed. Figure 2A showed that intracellular residual calpain activity was significantly decreased in thrombin-stimulated platelets in a dose-dependent manner indicating that the exhausting of calpain activity. Western blot analysis further confirmed that thrombin treatment markedly decreased intracellular residual level of the active form calpain with a size of approximately 75 kDa in a dose-dependent manner in platelets (figure 2B, upper). Prior treatment cells with calpeptin significantly inhibited thrombin or CaCl2 induced production of the active form calpain; the calpains present here are in inactive form with a size of approximately 80 kDa (figure 2B, upper lane 5 and bottom lane 4). These results indicated that thrombin might induce calcium mobilization firstly, then increased calpain activation, and finally depleted calpain activity in platelets within a short period of time. To investigate whether calcium mobilization and thrombin-induced calpain activation are involved in thrombin-mediated TLR4 expression on the surface in platelets, we treated platelets with TMB-8 or calpeptin prior to thrombin treatment. The data indicated that calpeptin and TMB-8 significantly decreased thrombin-mediated TLR4 expression, as proven by both flow cytometry and western blot (figure 2C and upper panel of 2D). Additionally, CaCl2 treatment markedly increased TLR4 expression relative to the control group; calpeptin pretreatment also significantly inhibited CaCl2 induced TLR4 expression (figure 2D, bottom) indicating that calcium mobilization and thrombin-induced calpain activation are indeed involved in this process. Furthermore, SFLLRN, AYPGKF, and SFLLRN plus AYPGKF treatment significantly increased the expression of TLR4 relative to that of the untreated control group (218.79±12.86% of control, 206.83±27.89% of control, and 196.14±10.12% of control, respectively) (figure 1D) suggesting that thrombin acted through PAR1 and PAR4 to activate downstream effects in platelets. Figure 1E showed that m-3M3FBS treatment significantly increased the expression of TLR4 (266.67±37.58% of control). In contrast, prior treatment of cells with U73122 followed by 0.4 U/mL thrombin strongly inhibited thrombin-dependent expression of TLR4 (127.93±10.30% of control) relative to the thrombin-activated group, whereas prior treatment of cells with the Rho-associated protein kinase ROCK inhibitor Y27632 had no effects (279.18±3.35% of control). These results suggested that thrombin might utilize the PLC pathway, but not the Rho pathway, to regulate thrombin-mediated TLR4 expression on the surface of human platelets.
The Interaction between TLR4 and Myosin-9, A Novel TLR4-Interacting Protein in Platelet, Is Regulated by Calpain

To explore the molecular mechanisms involved in thrombin-mediated TLR4 expression in human platelets, we identified and characterized TLR4-interacting proteins by using IP and mass spectrometry (figure 3A). Untreated wash platelet lysates were subjected to IP with anti-TLR4 antibody-conjugated agarose beads, and mouse IgG IP was used as a negative control. After being resolved by SDS-PAGE, the precipitated proteins were visualized by Coomassie Blue staining. The protein bands present in the anti-TLR4 antibody IP sample (indicated by stars) but not in the mouse IgG IP control sample were excised for further analysis by mass spectrometry (figure 3A). The mass spectrometry demonstrated that myosin-9 was strongly and consistently present as a ~220 kDa band and thus chosen for further characterization. IP-mass spectrometry results demonstrated that TLR4 interact with myosin-9 in platelet. Myosin-9 is a part of myosin IIA protein which plays critical role in platelet internal contraction, maintenance of coagulation, differentiation, and cell motility [28]. Additionally, myosin-9 had been demonstrated to participate in cell migration and receptor segregation [29]. We firstly confirmed the interaction between TLR4 and myosin-9 by using IP-Western assay. As shown in figure 4B, IP of myosin-9 from platelets with an anti-myosin-9 antibody was performed, and the
interaction was analyzed for the presence of TLR4 by western blotting (lane 2). The interaction between TLR4 and myosin-9 was specific because a same result was obtainable when the two antibodies was exchanged (figure 4D). We wander whether the interaction between myosin-9 and TLR4 was involved in the regulation of thrombin-mediated TLR4 expression. Washed platelets were treated with calpeptin and CaCl2 followed by thrombin treatment. The interaction between TLR4 and myosin-9 decreased when the platelets were treated with thrombin and CaCl2 (figures 4B and 4D left bottom lane 3 and 5); these phenomena were reversed when platelets were pretreated by calpeptin (figure 4B and 4D left bottom lane 4 and 6). These results demonstrated that myosin-9 might interact with TLR4 in untreated resting platelets; however this interaction would be disrupted when the platelets were under the activation state derived by thrombin or CaCl2 treatment. We hence suggested that decreased interaction between myosin-9 and TLR4 was positive associated with thrombin-mediated TLR4 expression; the interaction between myosin-9 and TLR4 was regulated by the calcium-calpain axis in thrombin treated platelets.

Myosin-9 Is One of the Substrates of Calpain, and Cleavage of Myosin-9 Enhances TLR4 Expression in Thrombin Treated Platelets

Several reports demonstrate the importance of calpains in many different platelet activation process including spreading, aggregation, granule secretion, and integrin signaling [30]. Calpain is also known to be responsible for the limited proteolysis of a spectrum of cytoskeleton-associated proteins including spectrin, adducin, talin, filamin, vinculin, and cortactin [30]. Previous evidence had indicated that the activation of calpain leads to the proteolysis of platelet cytoskeleton-associated proteins. This phenomena associated with the change of platelet function [31]. We supposed that...
myosin-9 might be also one of the substrates for calpain. We had already found that thrombin treatment significantly induced calpain activation and TLR4 expression. Figure 5A showed that thrombin treatment dramatically decreased the level of myosin-9 in platelet relative to the control. Besides, thrombin treatment was sufficient to stimulate the degradation of myosin-9 because a cleaved form myosin-9 with 95 kDa was significantly present in thrombin treated platelets (figure 5A lane 2) but not in other groups. The degradation of myosin-9 was reversed when platelets were pretreated with calpeptin (figure 5A lane 3) indicating that myosin-9 was indeed one of the substrates of calpain. The data indicated that myosin-9, a TLR4-interacting protein in platelet was one of the calpain substrates; cleavage of myosin-9 by calpain and decreased interaction between myosin-9 and TLR4 were positive associated and enhanced thrombin-mediated TLR4 expression in platelets.

Myosin-9 Did Not Coordinate with Rab7b to Negatively Regulate TLR4 Trafficking in Thrombin Treated Platelets and Enhanced TLR4 Expression in Platelets

In platelets, α-granules containing several growth factors and activation-induced receptors; we predicted α-granules were the major storage compartments for TLR4 in platelets, According to TEM photos, the control platelet had randomly distribution of

Figure 3. TLR4 interacts with myosin-9. (A) Identification of myosin-9 as a TLR4-interacting protein by co-IP and mass spectrometry. Washed platelet lysates were prepared for IP with mouse IgG- or anti-TLR4-conjugated agarose beads. The precipitated proteins were resolved by SDS-PAGE and revealed by Coomassie Blue staining. The stars indicated the protein bands that were pulled down with the anti-TLR4 antibody but not by mouse IgG. The stars indicated myosin-9 that was identified by nano-LC/MS/MS on an LCQ Deca XP Plus ion trap mass spectrometer. doi:10.1371/journal.pone.0085833.g003

Figure 4. Thrombin-mediated TLR4/myosin-9 interaction in human platelets is modulated by calpain activity. Platelets were treated with 5 µg/mL calpeptin followed by 4 U/mL thrombin for 20 minutes or 3 mM of CaCl2 for 30 minutes. (A and C) The interaction of myosin-9 with TLR4 was analyzed using immunoprecipitation. The pre-immune controls IgG were used to confirm the specificities of the TLR4 and myosin-9 antibodies. (B) The total protein extracted from treated platelets was used for immunoprecipitation with anti-myosin-9 antibody and immunoblotting with anti-TLR4 antibody. Immunoblotting with anti-myosin-9 antibody was used as an IP control. (D) The total protein extracted from treated platelets was used for immunoprecipitation with anti-TLR4 antibody and immunoblotting with anti-myosin-9 antibody. Immunoblotting with anti-TLR4 antibody was used as an IP control. The band density was determined using densitometry and was shown in the graph on the right. The data represented the results of three independent experiments (mean ± SD; *p<0.05 was considered significant). doi:10.1371/journal.pone.0085833.g004
Human platelets were pretreated with or without calpeptin at 28°C for 60 min followed by thrombin treatment at 37°C for 20 minutes. (A) The total protein extracted from platelets was used for western blot analysis and immunoblotting with anti-myosin-9 antibody. The β-actin protein served as the loading control. (B) The morphology of platelets were observed using TEM. Control human washed platelets are showed in B-1 and B-2. The platelet with randomly distribution of α-granules (AG) containing immuno-gold conjugated TLR4 (white arrow). Platelets with thrombin treatment at 37°C for 20 minutes were showed in B-3 and B-4. Original magnification of B-1 and B-3 are 40000×, B-2 and B-4 are 100000×. (C) The total protein extracted from platelets was used for immunoprecipitation with anti-Rab7b antibody and immunoblotting with anti-myosin-9 antibody. A pre-immune control IgG was used to confirm the specificity of the Rab7b antibody. Immunoblotting with anti-myosin-9 antibody was used as an IP control.

Figure 5. The calpain-myosin-9-Rab7b axis may regulate TLR4 containing α-granules trafficking in thrombin-stimulated platelets. Human platelets were pretreated with or without calpeptin at 28°C for 60 min followed by thrombin treatment at 37°C for 20 minutes. (A) The total protein extracted from platelets were treated with or without calpeptin at 28°C for 60 min followed by thrombin treatment at 37°C for 20 minutes. (A) The total protein extracted from platelets was used for western blot analysis and immunoblotting with anti-myosin-9 antibody. The β-actin protein served as the loading control. (B) The morphology of platelets were observed using TEM. Control human washed platelets are showed in B-1 and B-2. The platelet with randomly distribution of α-granules (AG) containing immuno-gold conjugated TLR4 (white arrow). Platelets with thrombin treatment at 37°C for 20 minutes were showed in B-3 and B-4. Original magnification of B-1 and B-3 are 40000×, B-2 and B-4 are 100000×. (C) The total protein extracted from platelets was used for immunoprecipitation with anti-Rab7b antibody and immunoblotting with anti-myosin-9 antibody. A pre-immune control IgG was used to confirm the specificity of the Rab7b antibody. Immunoblotting with anti-myosin-9 antibody was used as an IP control. doi:10.1371/journal.pone.0085833.g005

organelles and the immuno-gold-labeled TLR4 were observed in the α-granules (figure 5B-1 and 2). Interestingly, under thrombin treatment, the α-granules with TLR4 had been moved to the cutting edges of the platelet for release (figure 5B-3 and 4) indicating that platelets used α-granules to delivery TLR4. The figure 5A results let us confidently proposed that calpain mediated myosin-9 degradation and decreased interaction between myosin-9 and TLR4 were positive associated and enhanced TLR4 expression in thrombin-treated platelets. Here we try to figure out the mechanism which was responsible for it. Previous reports had demonstrated that Rab7b protein, a lysosome-associated small GTPase negatively regulates TLR4 expression on macrophage surface by promoting lysosomal degradation of TLR4 [32,33]. We think that Rab7b guided lysosomal degradation of TLR4 might be also involved in TLR4 expression regulation in platelet. We postulated that myosin-9 might coordinate with Rab7b and negatively regulated the trafficking of TLR4 in resting platelets to keep TLR4 in a basal level state; however this pathway was disrupted in thrombin-stimulated platelets due to the degradation of myosin-9 by calpain activation, and promoted the expression of TLR4 in thrombin-stimulated platelets. We firstly tested whether Rab7b could interact with myosin-9 in resting platelet. IP of Rab7b from platelets was performed by using an anti- Rab7b antibody, and interaction was confirmed by the presence of myosin-9 in western blotting (figure 5C lane 3). We wonder whether the interaction between Rab7b and myosin-9 decreased in thrombin-treated platelets. The data showed that interaction between Rab7b and myosin-9 indeed significantly decreased after thrombin treatment (figure 5C lane 4) indicating that thrombin treatment prohibited Rab7b guided TLR4 lysosomal degradation in platelet. Furthermore, the interaction was restored when washed platelets were pretreated with 5 calpeptin followed by thrombin treatment (figure 5C lane 5). Calpain-myosin-9-Rab7b axis was responsible for the mechanism underlying the regulation of TLR4 containing α-granules trafficking in thrombin-stimulated platelets. These results supported that Rab7b coordinated with myosin-9 to negatively regulate TLR4 expression in resting platelet by promoting Rab7b guided lysosomal degradation; however thrombin treatment prohibited Rab7b guided TLR4 lysosomal degradation in platelet result from the cleavage of myosin-9 by calpain activation, and promoted the expression of TLR4 on the surface in thrombin-stimulated platelets.

Discussion
Platelet TLR4 expression is associated with sepsis and thrombocytopenia [16]. However, the underlying mechanisms of TLR4 expression by platelets have been rarely explored until now. In figure 6, thrombin-mediated TLR4 expression was modulated by PAR/PLC pathway, calcium and calpain. Additionally, we identified a novel TLR4-interacting protein, myosin-9, which was one of the substrates of calpain; cleavage of myosin-9 enhanced TLR4 expression in thrombin treated platelet. Myosin-9 did not coordinate with Rab7b, a lysosomal-associated small GTPase, to negatively regulate TLR4 trafficking in thrombin treated platelets, and hence enhanced TLR4 expression. Briefly, phospholipase C-Calcium-myosin-9-Rab7b axis was responsible for the mechanism underlying the regulation of TLR4 containing α-granules trafficking in thrombin-stimulated platelets, which was involved in coagulation.

TLR4 is expressed on the cell surface of platelets, which regulate immunity and inflammation. In 2004, Aslam R. and Shiraki R. first determined that murine and human platelets express functional TLRs which are potential regulators of innate and adaptive immunity [34–36]. In 2005, Andonegui G. demonstrated that platelets TLR4 contribute to the accumulation
of platelets in the lungs in response to LPS stimulation [5]. Since then, much work has been devoted to researching the functions and roles of TLR4 in platelets. Platelets express the essential downstream components of the LPS signaling complex, including TLR4/MD2 and MyD88 [13]. Through TLR4-signaling pathway-induced formation of the TLR4/MD2/MyD88 complex, activation of MAP kinase and NF-kB, and production of cGMP, LPS leads to the expression of IL-6, prostaglandin E2, and TNF-α, which is followed by induction of aggregation in platelets [10,13,37]. However, TLR4 also causes LPS-incubated platelets to decrease the expression of RANTES, angiogenin and PDGF-

Figure 6. Mechanisms contributing to thrombin-mediated TLR4 expression in platelets. Thrombin may pass through the PAR1 and PAR4 receptors to activate downstream effectors for the PLC pathway but not the Rho pathway. The PLC pathway further activates calpain via calcium mobilization, and cleavages myosin-9, which decreases the interaction between myosin-9 and TLR4. In the other hand, myosin-9 does not coordinate with Rab7b to negatively regulate TLR4 containing α-granules trafficking in thrombin treated platelets, and leads to the increasing of TLR4 expression in platelets from megakaryocytes.

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cytes [43,46]. In 2009, Tal et al. also showed that myosin-9 (myosin II A) is essential for T cell antigen receptor signaling and immunological synapse stability [26]. Calpain was rapidly activated after calcium ionophore stimulation, and its activity was shown to be essential for the proteolysis of myosin [47]. Myosin-9 consists of four light chains and two heavy chains. The head region of the heavy chains is the major part that interacts with actin to maintain cell movement and shape. Recently more activated after calcium ionophore stimulation, and its activity immunological synapse stability [28]. Calpain was rapidly a many complex mechanisms regulating the exocytosis of myo-granules, which are released in response to stimulation in platelets [48]. There are many complex mechanisms regulating the exocytosis of myo-granules. Previous evidences showed that Rab4 and Rab6 are essential regulators of the vesicle trafficking and myo-granule secretion [49,50]. Rab6, Rab8, myosin II, and myosin VI had been implicated in trans-Golgi network (TGN)-to-membrane antegrade transport [51]. In contrast, Rab7b is required factor for retrograde transport from endosomes to TGN which mediating lysosomal function [52]. Rab7b is a negatively modulator of TLR4 and TLR9 signaling by leading the translocation of TLRs into lysosomes for degradation in mouse macrophage [33,53]. Myosin-9 also interacts with the tail regions of other myosin proteins through its long tail region. Although we have explored that myosin-9 may interact with Rab7b and this interaction may regulate the expression of TLR4 in platelets treated with thrombin, the detailed underlying mechanisms and the binding region on myosin-9 remain to be studied in the future. Our mass spectrometry results also showed that in addition to myosin-9, filamin A strongly interacted with TLR4. Human filamin A is in a widely expressed protein encoded by the FLNA gene [54], and it regulates actin reorganization by interacting with integrins, second messengers, or transmembrane receptors. Recent evidence demonstrated that thrombocytopenia may result from mutations in filamin A [55]. In the future, it will be important to explore the impact of filamin A on TLR4 expression during the process of platelet activation.

Our results highlight the important roles of phospholipase Cγ and calcium-dependent calpain activation in TLR4 expression in thrombin-stimulated platelets. We also explored novel roles for myosin-9 and Rab7b in platelet TLR4 expression through its interaction with TLR4. These primary data indicate a likely mechanism for thrombin-stimulated platelet aggregation and TLR4 expression and also provides a basis for further investigation of TLR4 modulation as a therapeutic strategy for coagulopathy and platelet disorders.

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**Author Contributions**

Conceived and designed the experiments: YWL FLY, CST. Performed the experiments: JCT YWL. Analyzed the data: JCT YWL FLY CYH YHC. Contributed reagents/materials/analysis tools: CST C. Lin YTT CMS C. Lee C. Li NCC. Wrote the paper: FYL YWL.

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