Lipid raft-associated β-adducin participates in neutrophil migration

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Abstract. Previous studies have demonstrated that lipid rafts and β-adducin serve an important role in leukocyte rolling. In the present study the migratory ability and behavior of neutrophils was demonstrated to rely on the integrity of the lipid raft structure. β-adducin was demonstrated to have a critical role in neutrophil migration. Knockdown of β-adducin attenuated the migratory ability of dHL-60 cells and the distribution of β-adducin in lipid raft structures was changed by N-formylmethionyl-leucyl-phenyl-alanine treatment. Furthermore, the findings demonstrated that the tyrosine phosphorylation of β-adducin was required for its relocation. The results of the present study suggested that the lipid raft-associated protein β-adducin may be a novel control point for the excessive infiltration of neutrophils during inflammation.

Introduction

Defense reactions are triggered when the body suffers damage or pathogen infection and is characterized by redness, swelling, pain, fever and functional disorders, and this defensive reaction is known as inflammation (1). When inflammation occurs, the first type of leukocyte to be recruited to the infection site is the neutrophil. Neutrophils, as the first line of defense in the body against external stimuli, have a critical role in the early inflammatory reaction (2,3). The recruitment and function of neutrophils at inflammatory sites requires cell migration (4). The migratory process of neutrophils in inflammatory site requires β2 integrin, a specific type of integrin family exclusively expressed on leukocyte membranes, and the natural ligand is fibrinogen (5). When neutrophils are activated, clustering of β2 integrin occurs and this further promoted the binding of β2 integrin with ligand to regulate neutrophil migration (6).

The lipid raft is a micro-area in the cell membrane, which becomes a large area through diffusion or recruitment under certain stimuli (7). The presence of several types of proteins on the lipid raft structure is the basis of the recruitment of the lipid raft. Among these proteins, adducin is one which is associated with the cellular cortical skeleton (8,9). The cellular cortical skeleton and cellular skeletal network are closely associated with each other, suggesting that the cytoskeletal network has an important role in the function of the lipid raft structure (10,11). Adducin is a type of lipid raft-associated protein that is thought to regulate the assembly of the cell membrane and cell skeletal network through directly connecting with the cellular cortical skeleton and cellular skeleton network (12-15). The adducin family has three members, α-adducin, β-adducin and γ-adducin (16,17). Our previous study demonstrated that β-adducin has an important role in the process of leukocyte rolling and β-adducin as a membrane skeleton protein, acts as a link between the cell membrane and cytoskeleton system. β-adducin can transfer from the lipid raft to the cytoplasm, leading to the cell membrane becoming temporarily disconnected from the cytoskeleton (18).

During the process of neutrophil migration, the formation of filamentous and lamellar pseudopodia requires the cell membrane to become disconnected from the cytoskeleton to promote cell movement (2). Therefore, the cytoskeleton and lipid raft-associated protein β-adducin may have a role in the process of neutrophil migration. In the present study, the function of β-adducin in neutrophil migration was investigated using neutrophils and neutrophil-like differentiated HL-60 cells (dHL-60 cells). The results from immunofluorescence, Transwell assay and the Living cell Imaging System

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Abbreviations: fMLP, N-formylmethionyl-leucyl-phenyl-alanine; MβCD, methyl-β-cyclodextrin

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demonstrated that lipid rafts have crucial role in neutrophil migration. There is a translocation of β-adducin prior to and following N-formylmethionyl-leucyl-phenyl-alanine (fMLP) treatment, and when β-adducin was knocked-down in dHL-60 cells, the ability of neutrophil to migrate was reduced. Notably, the phosphorylation of β-adducin was required for the relocation. The results of the present study are the first to demonstrate, to the best of our knowledge, that the lipid raft-associated protein β-adducin participates in the regulation of neutrophil migration.

Materials and methods

Reagents and antibodies. Methyl-β-cyclodextrin (MβCD), soluble cholesterol, mouse anti-human actin monoclonal antibody (AC-40), anti-phosphotyrosine monoclonal antibody (PY20; P4110), inhibitor of Src-family tyrosine kinase (PP2), non-conjugated F (ab')2 fragment of goat anti-mouse IgG (M0659) and anti-tubulin monoclonal antibody (T4026) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Anti-human β-adducin antibody (SC-376063) and antibody to flotillin-2 (SC-28320) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). AlexaFluor-488-conjugated cholera toxin (CTxB) was obtained from Molecular Probes (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Iscove's modified Dulbecco's medium was from Gibco (Thermo Fisher Scientific, Inc.). The β-adducin truncated mutant plasmid was produced in our laboratory.

Neutrophil isolation. Peripheral blood from randomly selected healthy adult volunteers (age was 25-40 years old, 2 male and 3 female) was drawn into heparinized syringes (10 U/ml). The healthy blood samples were obtained from the Jilin/Changchun Blood Center between January 2014 and December 2017. The collection of samples took place according to the principles of the Declaration of Helsinki. When donors provided blood, they signed informed consent with the Blood Center and the authors also signed an agreement with Blood Center for use of the blood. Peripheral blood (5 ml) and 6% Dextran T-500 balance fluid (1.5 ml) was gently mixed and immobilized at 4˚C for 1 h, then the white blood cells were separated to the top layer and the red blood cells in the bottom layer. The top layer of white cells and lymphocyte separation medium were mixed in a new centrifuge tube at 1:1 ratio. Then the mixture was centrifuged at 500 x g at 4˚C for 20 min and then the supernatant was aspirated and discarded. The sediment was suspended with red blood cell lysis buffer (NH_4Cl 8.29 g/l, EDTA $Na_2.2H_2O$ 37.2 mg/l and potassium bicarbonate 1.0 g/l) and the suspension was immobilized at 4˚C for 10 min, then centrifuged at 200 x g at 4˚C for 5 min. Cell viability was evaluated using trypan blue exclusion test. The cells and 0.4% trypan blue were mixed in equal proportions, and the mixture allowed to incubate at room temperature for 3 min. A drop of the trypan blue/cell mixture was added to a hemocytometer. The hemocytometer was placed on the stage of a binocular microscope and the unstained (viable) and stained (nonviable) cells counted. The morphological alterations were visually assessed using Giemsa-Wright's staining (19,20). The collected cells were resuspended in 1 ml methanolic-acetic acid buffer and fixed for 5 min. A drop of the cell suspension was placed on a low-temperature pre-cooled slide glass, excess liquid removed and the slide allowed to dry naturally. The slides were immersed in a freshly prepared Giemsa-Wright's stain solution: 0.5 g Giemsa stain and 1 g Wright stain were added to 33 ml glycerin and then 500 ml methanol was added once the glycerin was fully dissolved. The slides were immersed for 15-20 min, then rinsed with tap water and allowed to dry. The slide was examined using a bright field microscope (Eclipse80i; Nikon Corporation, Tokyo, Japan).

Differentiation of HL-60. HL-60 cells were purchased from the cell bank of the type culture collection of the Chinese Academy of Sciences (Shanghai, China). The cells were placed into a cell culture bottle (density: $5x10^5$/ml) and 1.3% DMSO was added, then the mixture was incubated at 37˚C in CO_2 incubator for 96 h. Finally, the artificially differentiated HL-60 (dHL-60) cells were obtained (21).

RNA interference. For short hairpin RNA (shRNA) preparation, annealed ds-shRNA oligonucleotides 5'GCAAGATCAGCA GTGTCTA3' and control shRNA oligonucleotides 5'GCTCTA GTACAGAATCGCT3' were cloned into the *HpaI* and *XhoI* cloning sites of the lentiviral pLL3.7 vector (11795; Addgene, Inc., Cambridge, MA, USA). A total of 4 μg β-adducin shRNAs was transfected into $5x10^6$ 293T cells (Cell Bank, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China), together with packaged mix (sPAX2 and pMD2; at a ratio of 4:3:1), to generate the respective lentiviruses. Viral concentrates were prepared and were used to infect dHL-60 cells, according to the manufacturer's protocol (VPK-091; Cell Biolabs, Inc., San Diego, CA, USA). The extent of suppression and specificity for β-adducin were evaluated by western blotting with anti-β-adducin antibody and an anti-actin antibody was used as a control.

Transwell assay. The fMLP treated cells (100 μl IMDM containing $1x10^6$ cells) were placed in 5-μm pore polyester membrane Transwell inserts (Corning Incorporated, Corning, NY, USA) in 24-well plate. IMDM, with 2.5% FCS containing 100 nm fMLP as a chemoattractant, was added to the lower wells. Then the plate was placed in the CO_2 incubator at 37˚C for 90 min. The inserts were removed from the plate and the cells in each well were counted using a hemocytometer under bright field microscopy. (Eclipse80i; Nikon Corporation) The ratio of cells that penetrated into the well to the number of total cells added in the insert was used to determine the migratory ability of cells.

Cell migration assay. Fibrinogen (10 μg/ml) was spread evenly over the central part of a coverslip and immobilized at 4˚C overnight. The neutrophils from human peripheral blood were suspended in migration buffer [1 mM Tris, 0.14 M NaCl, 1 mM Hepes (pH 7.2), 5.4 mM KCl, 1.1 mM $CaCl_2$, 0.4 mM $MgSO_4$, gelatin (1 mg/ml) and 5 mg/ml bovine serum albumin (BSA; Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China). Then, $1x10^6$ dHL-60 cells were suspended in 1 ml migration buffer and added to 22x40 mm coverslips at 37˚C for 10 min. The coverslips were washed with PBS and the cell-covered side of the coverslip was put on the top of a Zigmond chamber. The chamber has two grooves and the migration buffer

was added to one groove, then the migration buffer with 100 nM fMLP was added to the other groove. The Zigmond chamber was put in the Live Cell Imaging System, which comes with its own tracking command (Ultra VIEW VoX, PerkinElmer, Inc., Waltham, MA, USA) and the temperature was set to 37˚C. Images were captured using a Nikon microscope (Eclipse 80i; Nikon Corporation) under bright field with a CCD camera for 4 min at 60 sec intervals. The migratory behavior of the neutrophils was recorded by the Live Cell Imaging System using the tracking command and analyzed by with GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA.

Double-labeled immunofluorescence. fMLP (100 nM) was dropped on one side of a coverslip covered with neutrophils ($1x10^6$), which were then allowed to migrate for a certain time. Then 4% paraformaldehyde was added to the coverslip to fix the cells at room temperature for 10 min. The cells were washed with PBS three times, then 0.2% TritonX-100 was dropped onto the coverslip and the cells were incubated at room temperature for 2 min. The cells were treated with the primary antibody of β-adducin (1:100 dilution) at room temperature for 1 h. Then the cells were treated with the secondary antibody TRITC-conjugated goat anti-mouse secondary antibody (ZF-0313; OriGene Technologies, Beijing, China) at room temperature for 1 h. To stain monosialotetrahexosylganglioside (GM1) (lipid raft maker), the cells were treated with Alexa Fluor 488 conjugated CTx B subunit (2 μg/ml) at 4˚C for 30 min. The treated cells were observed under a laser scanning confocal microscope (FluoView FV1000; Olympus Optical, Tokyo, Japan). The photos were analyzed with Photoshop-CS5 (Adobe Systems, Inc., San Jose, CA, USA).

Immunoprecipitation and immunoblotting. The neutrophils ($1x10^7$ per sample) collected from the peripheral blood of healthy donors were washed twice with PBS. The collection of samples took place according to the principles of the Declaration of Helsinki. The cells were suspended with lysis buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1% NP-40, 2.5 mM $Na_4P_2O_7$•10 H_2O, 1 mM NaF, 1 mM Na_3VO_4, 1 mM β-glycerophosphate and 20 μg/ml aprotin/leupeptin/PMSF]. The lysate was centrifuged at 13,000 x g, 4˚C for 20 min and the supernatant was kept. Determination of protein concentration was performed using the BCA method. The lysates (1 ml) were added to new tubes and then the 2 μg antibody of β-adducin was added. The tubes were incubated on the rotor at 4˚C for 1 h, then 15 μl Protein G Agarose/ Salmon Sperm DNA beads (EMD Millipore, Billerica, MA, USA) were added. Then the tubes were incubated on the rotor at 4˚C overnight. The mixture was centrifuged at 13,000 x g for 1 min at 4˚C, then the beads were washed once with the high-concentration solution (500 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1% NP-40, 2.5 mM $Na_4P_2O_7$•10 H_2O, 1 mM NaF, 1 mM Na_3VO_4, 1 mM β-glycerophosphate and 20 μg/ml aprotin/leupeptin/PMSF), medium-concentration solution (250 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1% NP-40, 2.5 mM $Na_4P_2O_7$•10 H_2O, 1 mM NaF, 1 mM Na_3VO_4, 1 mM β-glycerophosphate and 20 μg/ml aprotin/leupeptin/PMSF) and then the low-concentration solution (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1% NP-40, 2.5 mM $Na_4P_2O_7$•10 H_2O, 1 mM NaF, 1 mM Na_3VO_4, 1 mM β-glycerophosphate and 20 μg/ml aprotin/leupeptin/PMSF) on ice successively. Following centrifugation at 500 x g for 5 min at 4˚C, the beads were suspended with loading buffer and boiled in water bath for 10 min. The supernatant was the solution with the target protein.

The supernatant (25 μl) was added to the wells of an 10% SDS-PAGE gel. At the beginning of the blotting, the voltage was set to 80 V, then the voltage was adjusted to 120 V until the red protein maker appeared. Following protein transfer the nitrocellulose membrane was blocked in 5% BSA-TBST (0.05% Tween-20) on the table concentrator at room temperature for 1 h. The film was placed into the primary antibody dilution and incubated on the table concentrator at 4˚C for 3 h. Then the film was washed 3 times with TBST and incubated with the horseradish-peroxidase-conjugated secondary antibodies at 37˚C for 1 h. Following washing 3 times with TBST, the film was developed with ECL Plus western blotting reagents, according to the manufacturer's protocol (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) and imaged using an image analysis system (Tanon 5500; Tanon Science & Technology Co., Ltd. Shanghai, China). The following primary antibodies were used for analysis: anti-adducin (1:1,000), anti-PY20 (1:2,000), anti-flotillin2 (1:1,000) and anti-tubulin (1:1,000). Anti-β-actin (1:4,000) served as the loading control.

Cholesterol depletion and repletion. Cells ($1x10^6$) were treated with 5 mM MβCD for 30 min at 37˚C. The cell viability was assessed by trypan blue exclusion. To replenish membrane cholesterol, the MβCD treated cells were incubated with 25 mM cholesterol for 30 min at 37˚C.

Sucrose density gradient centrifugation. Cells were collected and washed with PBS ($5x10^7$ dHL-60 or neutrophils) and suspended with IMDM. Then the cells were stimulated with fMLP (100 nM) and washed with PBS. The cells were collected by centrifugation and homogenized with 1 ml cool lysis buffer using tissue homogenizer. The lysate was centrifuged at 12,000 x g at 4˚C for 10 min. The supernatant was kept, and 20 μl of each sample removed to mix with loading buffer, which was then added the wells with 10% SDS-PAGE and Coomassie blue staining performed. The remaining supernatant was mixed with 1 ml 80% sucrose solution. Then the mixture was transferred to a 5 ml ultracentrifugation tube and 2 ml 30% sucrose solution was added, followed by 1 ml 5% sucrose solution. The discontinuous gradient was spun for 18 h at 200,000 x g at 4˚C in Beckman MLS50 rotors (Beckman Coulter, Inc., Brea, CA, USA). Fractions collected from the gradient top were used immediately or kept frozen at -80˚C until use. The tubes were centrifuged at 200,000 x g for 18 h at 4˚C in Beckman MLS50 rotors (Beckman Coulter, Inc.). The tubes were put on ice and 12 layers in each sample were collected. The following primary antibodies were used for analysis: anti-adducin (1:1,000), anti-flotillin2 (1:1,000) and anti-tubulin (1:1,000). The flotillin2 served as a raft marker and tubulin served as the non-raft marker.

Statistical analysis. Data are representative of 3 independent experiments. Data were analyzed by one-way analysis of

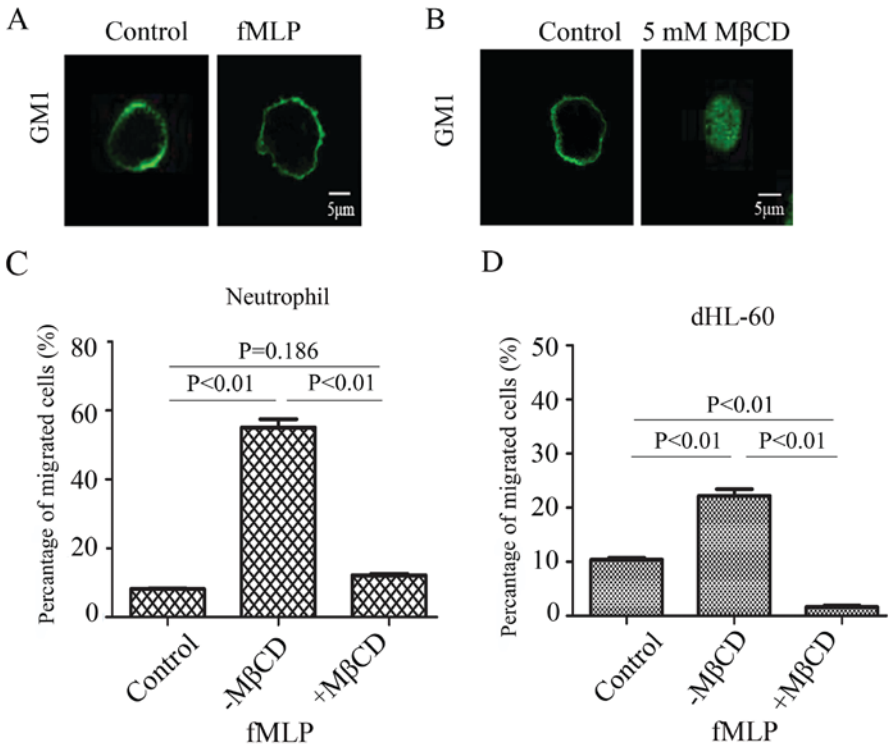

Figure 1. The role of the lipid raft structure in neutrophil migration. (A) Neutrophils were pre-adhered onto the coverslip coated with fibrinogen, and Iscove's modified Dulbecco's medium co-ntaining fMLP was added to one side of the coverslip. Confocal microscopy images showed the distribution of GM1 (lipid raft maker). GM1 was detected using AlexaFluor-488-conjugated CTxB. (B) Neutrophils were treated with 5 mM MβCD, then cells were treated as described above. Confocal microscopy images showed the distribution of GM1 (lipid raft maker). (C) MβCD (5 mM) was used to treat the peripheral blood neutrophils, in a Transwell migration assay. (D) Transwell assay for dHL-60 cells. Cell migratory rate was determined. Migratory rate=the number of migratory cells/the number of total cells. GM1, monosialotetrahexosylganglioside; fMLP, N-formylmethionyl-leucyl-phenyl-alanine; MβCD, methyl-β-cyclodextrin.

variance followed by a Tukey post hoc test using the SPSS statistics version 22 (IBM, Corp., Armonk, NY, USA). Quantitative data are expressed as the mean ± standard deviation. P<0.01 was considered to indicate a statistically significant difference.

Results

Integrity of lipid rafts is required for neutrophil migration. The lipid raft is a specialized micro-area structure in the plasma membrane, which consists of a rich of sheath phospholipids and cholesterol (22,23). It has been reported that lipid rafts can regulate cellular signal transduction and the cell membrane dynamics (18). To investigate whether the integrity of the lipid raft is required for neutrophil migration, cell migratory ability was analyzed with or without integrated lipid raft structure. The peptide chemotaxin fMLP was selected as the inducer of neutrophil migration *in vitro*. The results demonstrated that fMLP could induce deformation of the cell membrane (Fig. 1A), suggesting that fMLP can effectively induce the migration of neutrophils. Based on the existing literature, 5 mM MβCD was chosen as an agent for disrupting the lipid raft structure and GM1 was chosen as a lipid raft marker (24). The results demonstrated that MβCD treatment blurred the boundary of the cell membrane, demonstrating that the integrity of the cell membrane is disrupted (Fig. 1B). To investigate the effect of the disruption of lipid raft integrity on the migration of neutrophils *in vitro*, Transwell assays were performed using fMLP as a chemotaxin. The results demonstrated that when the cells were treated with 5 mM MβCD was their migratory ability was significantly inhibited (P<0.01; Fig. 1C). The same assay was repeated using dHL-60 (Fig. 1D). The results indicated that the migration of neutrophils *in vitro* relies on the integrity of the lipid raft structure.

Migratory behavior of neutrophils is dependent on the integrity of lipid rafts. Subsequently a real-time observation of neutrophil migration was performed in a Zigmond chamber to further confirm the finding that neutrophil migration depends on the integrity of the lipid raft structure. The Live Cell Imaging System was used in this experiment and neutrophil migration was quantitatively analyzed using the Live Cell Imaging System, GraphPad prism 5, and Chemotaxis Tool software. The migratory ability of peripheral blood neutrophils is strong, which is demonstrated by rapid membrane deformation (Fig. 2A). Unlike the cells in the control group, the cells in the MβCD treatment group exhibited a weakly polarized state and the migratory speed was very slow (Fig. 2). When cholesterol was used to recover the integrity of the cell membrane, the cell migratory ability was restored, with regards to deformation velocity and polarization.

Wind-rose plots of the tracked migration paths of neutrophils were plotted using a manual tracking chemotaxis tool plugin (Fig. 2B) and quantitative assessment of migratory behavior, including Euclidean distance, velocity and accumulated distance, was obtained by tracking individual cells. Neutrophils without MβCD treatment migrated a

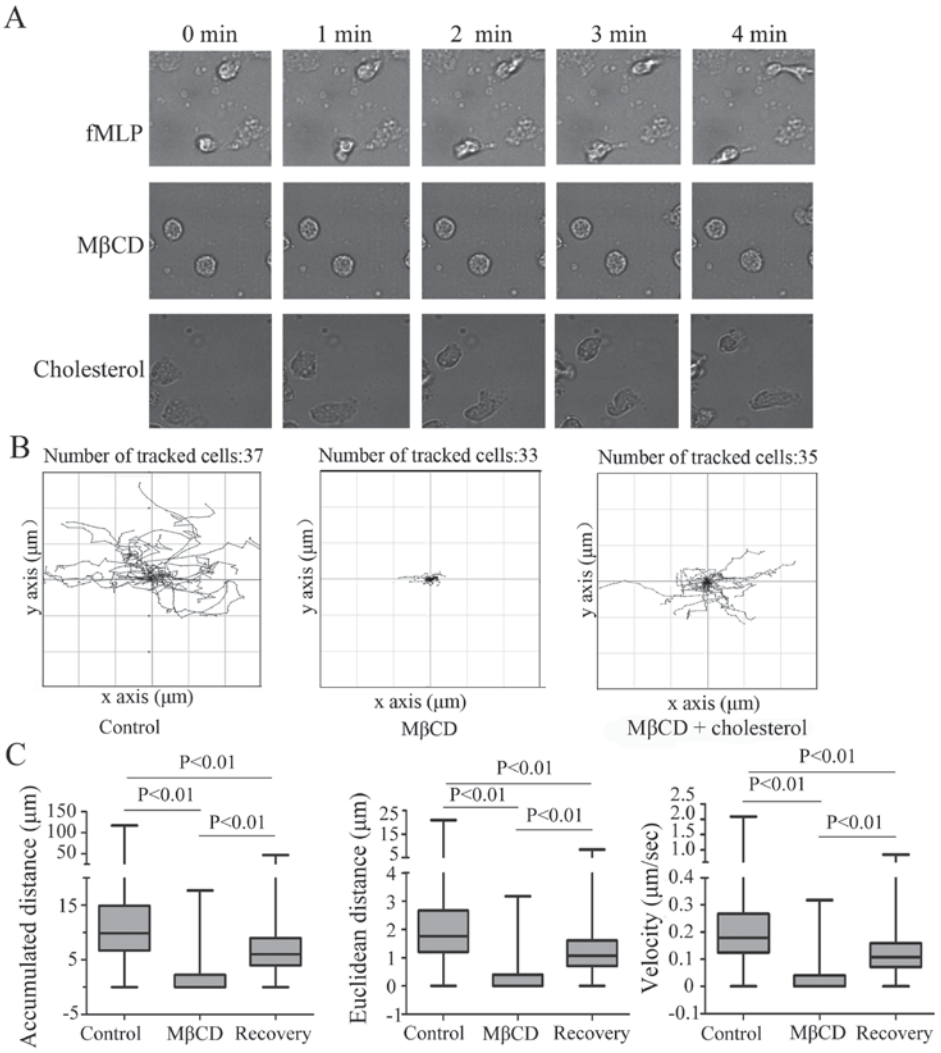

Figure 2. Lipid raft integrity is required for the migratory behavior of fMLP-stimulated neutrophils. Cells were pretreated with MβCD (5 mM) or replenished with cholesterol following MβCD treatment, then stimulated with 100 nM fMLP. (A) Time-lapsed photomicrographs (20x objective) of above different treated neutrophils in a Zigmond chamber were obtained for 4 min at 60 sec intervals. (B) Wind-rose plots of migration paths were obtained over a 4 min observation period and migratory tracks from one experiment are presented. (C) Accumulated distance, Euclidean distance and average velocity were presented as the mean ± standard error from three independent experiments. fMLP, N-formylmethionyl-leucyl-phenyl-alanine; MβCD, methyl-β-cyclodextrin.

significantly greater distance during the 4 min observation period compared with the treatment group and the average accumulated distance, the Euclidean distance and the average velocity were 9.98, 1.76 and 0.18 μm/s, respectively. When treated with MβCD, the migratory capacity of neutrophils was impacted significantly and the accumulated distance, Euclidean distance, and average velocity were reduced compared with that of the untreated neutrophils (P<0.01). When cholesterol was used to recover the integrity of the cell membrane, the migratory capacity of neutrophils was significantly restored (P<0.01; Fig. 2C).

β-adducin, a lipid raft-associated protein participates in neutrophil migration. Our previous study (18) reported that β-adducin, as membrane skeleton protein, has an important role in the process of neutrophil adhesion. In order to determine if β-adducin is involved in the process of neutrophil migration, the distribution of β-adducin was detected using sucrose density gradient centrifugation prior to and following stimulation with fMLP. The results demonstrated that no β-adducin was observed in the lipid raft component in the control cells, but in the fMLP stimulated cells, β-adducin appeared in the lipid raft component (Fig. 3A). The association of β-adducin and lipid rafts was further examined by confocal microscopy. Results demonstrated that, there was no colocalization between lipid rafts and β-adducin when neutrophils were in a resting state (Fig. 3Ba1-a3). Following fMLP stimulation, an obvious colocalization between lipid rafts and β-adducin was observed (Fig. 3Bb1-b3). When MβCD was used to disrupt the structure of lipid rafts, fMLP stimulation did not induce the colocalization of lipid rafts and β-adducin (Fig. 3Bc1-c3). In order to investigate the role of β-adducin in the process of neutrophil migration, the β-adducin was knocked down in dHL-60 cells. The result demonstrated that the β-adducin-shRNA effectively inhibited the expression of β-adducin (Fig. 3C). Transwell experiments were performed to determine the effect of β-adducin interference on the migration of neutrophils. The results demonstrated that, compared with the control group, the migratory rate of the dHL-60 cells decreased by 40%

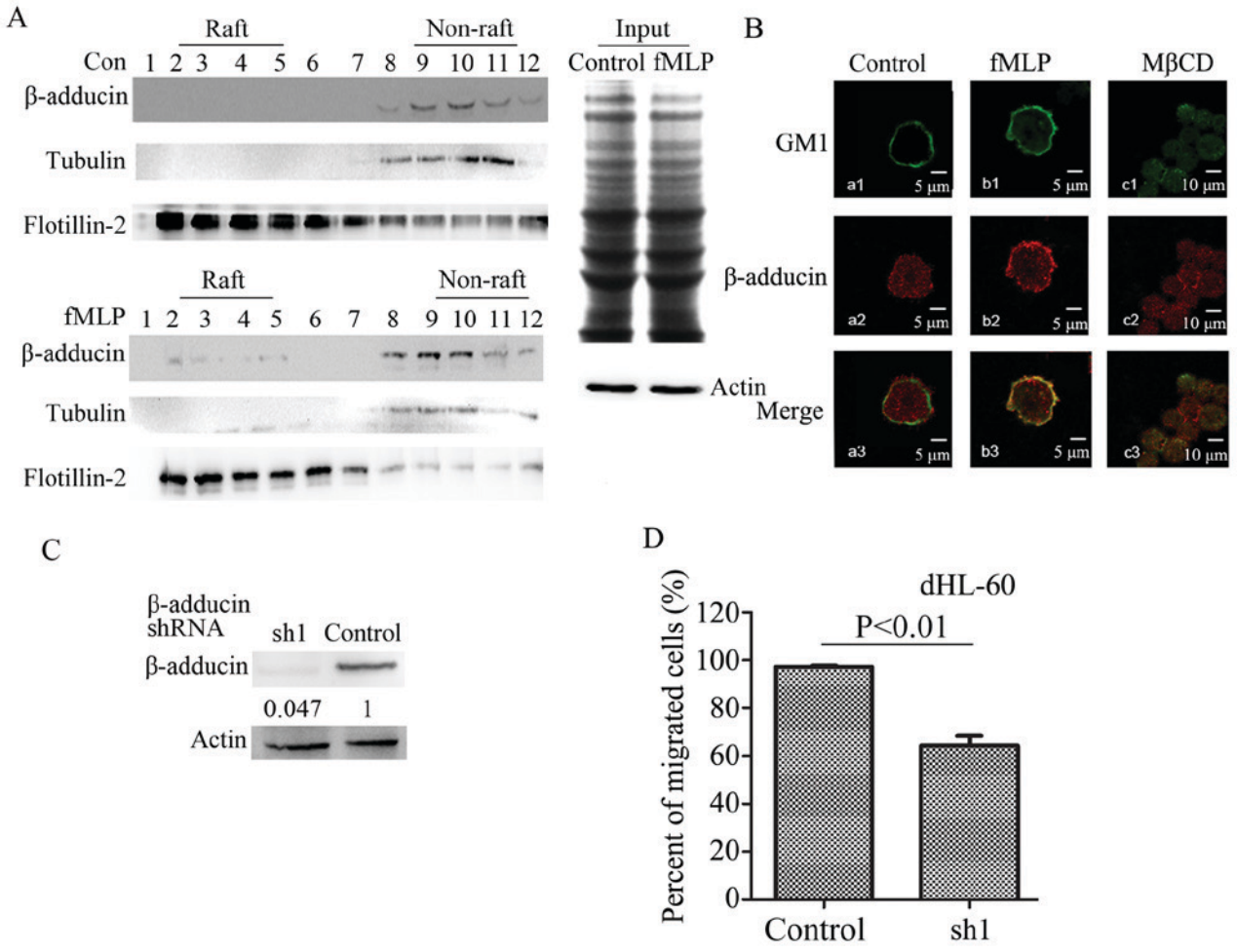

Figure 3. The role of β-adducin in the migration of neutrophils. (A) Sucrose density gradient centrifugation was used to separate the lysates of control dHL-60 cells and fMLP-stimulated dHL-60 cells. β-adducin antibody was used to detect β-adducin in the raft and the non-raft samples by western blotting. Rows 2, 3, 4 and 5 represent raft components, and 9, 10, 11 and 12 represent the non-raft components. The raft marker was flotillin and the non-raft marker was tubulin. The equivalent amount of lysates from control and fMLP treated cells were stained with Coomassie blue and immunoblotted with anti-actin antibody (right). (B) The colocalization between β-adducin and lipid rafts prior to and following fMLP stimulation were observed under a confocal microscope. AlexaFluor-488-conjugated CTxB was used to stain the specific lipid rafts marker GM1 and specific immunofluorescence antibody was used to label β-adducin (red). (C) Nonsense shRNA (control group) and β-adducin shRNA was used to infect dHL-60 cells. The lysates of whole cells following 48 h of culture were collected and analyzed by western blotting, and the β-adducin antibody was used to detect the interference efficiency. (D) The control cells and β-adducin knockdown cells in Transwell experiments. Cell migratory rate was determined by calculating the number of migratory cells/the number of total cells. The migratory rate of the control group (nonsense shRNA sequences) was set as 100%. Con, control; fMLP, N-formylmethionyl-leucyl-phenyl-alanine; MβCD, methyl-β-cyclodextrin; sh, short hairpin RNA.

when β-adducin expression was knocked down (Fig. 3D). The results indicated that β-adducin is involved in neutrophil migration.

Role of β-adducin phosphorylation in neutrophil migration. Subsequently, the expression alterations of β-adducin in dHL-60 cells were detected prior to and following fMLP stimulation. The results demonstrated that fMLP stimulation did not lead to a change of β-adducin expression (Fig. 4A), indicating that β-adducin expression is not the main reason for the inhibition of neutrophil migration. It has been reported that there are four tyrosine residues in the C-terminal of β-adducin and the activity of β-adducin is associated with the phosphorylation of tyrosine residues (25). The phosphorylation of β-adducin prior to and following fMLP stimulation was detected by immunoprecipitation of β-adducin and immunoblotting with anti-phosphotyrosine antibody PY-20. The results demonstrated that fMLP stimulation induced the phosphorylation of β-adducin. The phosphorylation peak appeared at 1 min and was still detectable at 15 min after stimulation (Fig. 4A).

The phosphorylation of β-adducin is dependent on Src-family tyrosine kinases. PP2, an effective inhibitor of Src-family tyrosine kinase, was used in the Transwell assay. The results demonstrated that the inhibition of β-adducin phosphorylation resulted in a decrease in neutrophil migration (Fig. 4B and C), indicating that β-adducin phosphorylation is involved in regulating neutrophil migration.

Discussion

Cell migration is a complex and precisely regulated process, and cellular cortical skeleton and actin cytoskeleton rearrangement have an important role in cell polarization and migration by promoting membrane protrusion and providing the driving force (26,27). Current evidence supports the hypothesis that

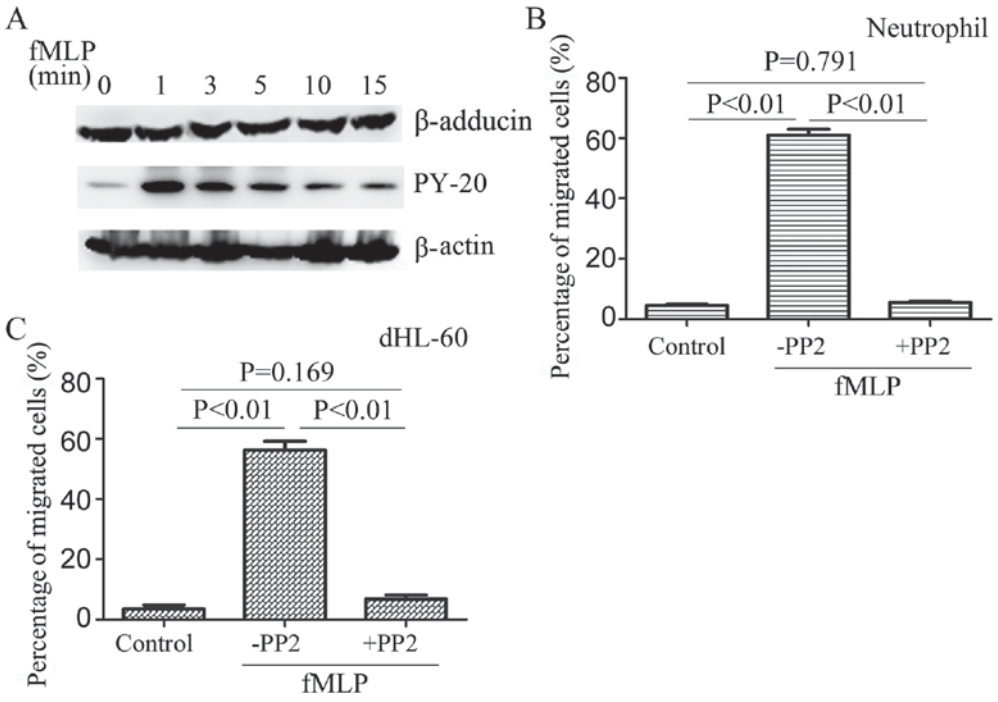

Figure 4. β-adducin phosphorylation is required for neutrophil migration. (A) dHL-60 cells were evenly spread in a culture dish covered with fibrinogen and fMLP stimulation was performed for 1, 3, 5, 10, and 15 min. The cell lysis samples were collected and western blotting was performed with β-adducin antibody. The blots from A were stripped and reprobed with the PY-20 anti-phosphotyrosine antibody. (B) Neutrophils were processed with the Src-family tyrosine kinase inhibitor PP2, then the Transwell experiment was conducted. (C) The Transwell experiment was conducted using dHL-60 cells. Cell migratory rate was determined as the number of migratory cells/the number of total cells. fMLP, N-formylmethionyl-leucyl-phenyl-alanine.

lipid rafts are involved in various types of cell migration processes (28) and the functions of lipid rafts in the neutrophil migration process have been well established (8,10). However, there is no direct, real-time evidence to demonstrate that the integrity of the lipid raft is critical in highly polarized and rapidly moving neutrophils. In the present study the essential role of the integrity of lipid raft in regulating neutrophil migration was demonstrated. The Transwell and time-lapse observation data indicated that treatment with MβCD, a lipid raft disruption agent, impacts membrane protrusion at the leading edge of migratory neutrophils and resulted in a marked impairment of neutrophil migration. On the basis of these results, it was concluded that the migratory behavior of neutrophils is dependent on the integrity of lipid rafts. Thus, the results of the present study have improved upon our previous understanding of how the integrity of lipid rafts affects neutrophil migration.

These results prompted investigation of lipid raft-associated proteins and the mechanism by which the cellular cortical skeleton and the actin cytoskeleton cooperate to regulate the migration of neutrophils. It has been reported that β-adducin is a lipid raft-associated cortical protein and a capping protein of F-actin (14,16,29). β-adducin was demonstrated to be involved in neutrophil migration. The results of the present study demonstrated that when neutrophils were treated with fMLP, β-adducin appeared in the lipid raft component. In addition, fMLP stimulation could not induce the colocalization of lipid rafts and β-adducin when the lipid rafts were disrupted. When β-adducin was knocked down in dHL-60, neutrophil migration was inhibited. Xu *et al* (18) reported that β-adducin can transfer from the lipid raft area to the cytoplasm, which leads to the cell membrane becoming temporarily disconnected from the actin. The results of the present study demonstrated that during neutrophil migration, β-adducin moves from the cytoplasm to lipid rafts. As a capping protein of F-actin, the translocation of β-adducin to the lipid raft may regulate the cluster polymerization of F-actin under the membrane and this may provide a cytoskeleton for the stretching of the membrane during the formation of protruding structures.

Previous studies have demonstrated that β-adducin is phosphorylated during neutrophil rolling and the deformation of the cytoskeleton in erythrocytes (13-16,18,30); however, it is not yet known whether β-adducin regulates neutrophil migration via phosphorylation. The results of the present study demonstrated that tyrosine phosphorylation of β-adducin occurred during neutrophil migration. The phosphorylation of β-adducin is dependent on Src-family tyrosine kinases. Therefore, the function of the Src-family tyrosine kinase was inhibited using PP2, and the data indirectly demonstrated that the phosphorylation of β-adducin is involved in neutrophil migration. Thus, in the present study it was demonstrated that a Src-family tyrosine kinase is a regulator of β-adducin in neutrophil migration.

In conclusion, a novel function of β-adducin in neutrophil migration has been identified. The results of the present study demonstrated that the integrity of lipid rafts and the recruitment of β-adducin to lipid rafts are necessary for membrane protrusion and migration of neutrophils. In addition, it was demonstrated that phosphorylation of β-adducin is a key step in the recruitment of β-adducin to lipid rafts. Following activation, β-adducin transfers to lipid rafts and this may provide a cytoskeletal basis for the membrane protrusion, which further affects the migration of neutrophils.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Author contributions

CY, ZS, TX and WL performed experiments, data analysis, drafted the paper and approved the final version. XZ, XW and CY contributed to the study design and clinical study, drafted the paper, and approved the final version.

Ethics approval and consent to participate

The blood donors signed informed consent with the Blood Center and the authors also signed an agreement with Blood Center for using the blood.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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