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Polysaccharides from *Bupleurum chinense* impact the recruitment and migration of neutrophils by blocking fMLP chemoattractant receptor-mediated functions

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ABSTRACT

Although neutrophils play important roles in host defense, sometimes they contribute to inflammation-related tissue injuries. Thus, restriction of their recruitment to the inflammatory sites is a promising strategy in the amelioration of inflammatory disease. The previous studies reported the anti-inflammatory effects of *Bupleurum chinense*, but the active ingredients and possible mechanism are still unclear. Here, we isolated water-soluble polysaccharides (BCPs) from *B. chinense*, to evaluate its anti-inflammatory effects and possible mechanism. The present results showed that BCPs significantly impaired the *in vivo* neutrophil infiltration, as well as the migration capacity of dHL-60 cells *in vitro*. In addition, BCPs inhibits chemoattractant fMLP-induced activation and clustering of β2 integrin. BCPs impacted fMLP-induced actin polymerization and the activation of cytoskeleton regulatory molecules, Vav1 and Rac1. Together, BCPs significantly impacted recruitment and migration of neutrophils by blocking chemoattractant receptor-mediated functions, and it possesses a potential as novel anti-inflammatory drug.

1. Introduction

Polymorphonuclear neutrophils are essential components of the innate immune system, accounting for 60% of the total peripheral leukocytes (Summers et al., 2010). Neutrophils are rapidly recruited and extravasated from blood flow into infection site in response to chemoattractant gradients during acute inflammatory response, where they destroy invading microorganisms by phagocytosis of pathogens and releasing several antimicrobial chemicals (Sadik, Kim, & Luster, 2011). Although neutrophils play important roles in host defense, under some special circumstances, their participation in the inflammatory response is salutary, the excessive and improper recruitment of neutrophils and their activation contribute to serious injury to host tissues and inflammation-related diseases (Nathan, 2006; Segel, Halterman, & Lichtman, 2011; Tanya, George, & Naotake, 2009). Thus, the destructive potential of neutrophils requires the tight control of their recruitment and migration into tissue compartments, which has been considered as an effective strategy in amelioration of the inflammation-related disease process (Ulbrich, Eriksson, & Lindbom, 2003).

Bupleurum chinense, belongs to the plant family *Bupleurum* spp., is a well-known Traditional Chinese Medicine (TCM) used for over thousand years in China. *B. chinense* widely distributed in the north region of China, and customarily called "Bei chaihu". *B. chinense* was first mentioned as a principal ingredient of an ancient Chinese medicinal formula, *Xiao Chai Hu Tang* (Chang et al., 2007). The clinical practice from ancient plant pharmacy experts showed *B. chinense* possesses many pharmacological functions, such as balancing different organs and energies within the body, strengthen the action of the digestive tract, improve liver and circulatory system function, and relieve liver tension (Xie et al., 2012; Yin, Pan, Chen, & Hua, 2008). In addition, contemporary pharmacological researches indicate *B. chinense* possesses significant anti-inflammatory effects (Ashour & Wink, 2011; Lee, Kim, Oh, & Yune, 2010). However, the active ingredients in *B. chinense* and possible mechanisms for anti-inflammation are still unclear.

More and more studies have confirmed that polysaccharides extracted from medicinal herbs are promising candidates as anti-inflammatory agents (Ramberg, Nelson, & Sinnott, 2010; Volpi, 2011). Natural polysaccharides might affect multiple targets in the inflammatory progression. One of the interests in natural polysaccharides as anti-inflammatory agents is the more and more evidence illustrating their ability to interfere with the migration of leukocytes to inflammatory sites (Jiao, Yu, Zhang, & Ewart, 2011). Therefore, we here isolated water-soluble polysaccharides (BCPs) from *B. chinense*, to evaluate its anti-inflammatory effects and possible mechanism. The present results showed that BCPs significantly impaired the neutrophil infiltration *in vivo* and the migration capacity *in vitro*. In addition, BCPs inhibits

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chemoattractant fMLP-induced activation and clustering of β2 integrin, actin polymerization, and the activation of Vav1 and Rac1. Together, these results showed BCPs exhibits anti-inflammatory effects by blocking chemoattractant receptor-mediated functions, which provide novel mechanistic insights into the poorly understood signaling network that polysaccharide interfere with the migration of leukocytes to inflammatory sites.

2. Materials and methods

2.1. Materials and chemicals

The roots of *B. chinense* were purchased from a local pharmaceutical market, and identified according to the identification standard of Pharmacopeia of the People's Republic of China.

The rabbit polyclonal antibodies against Rac1 and Vav1 were obtained from Santa Cruz Biotechnology. Antibodies for phosphotyrosine (PY20) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Anti-CD18 mAb (IB4) was obtained from Ancell. Activation-dependent anti-CD18 mAbs (m24) was kindly provided by Dr. Martyn Robinson (CellTech, Slough, UK). Alexa-Fluor 488-conjugated goat anti-mouse IgG secondary antibody was purchased from Invitrogen. The negative isotype controls, depending on the species and subclasses of the primary antibodies used, were obtained from Santa Cruz Biotechnology. Chemiluminescent detection reagents ECL Plus Western Blotting and glutathione Sepharose 4B were purchased from Amersham Biosciences. The Immobilon-NC Transfer Membrane was obtained from Millipore. Calcien acetoxymethylester (Calcein-AM) was purchased from Invitrogen. The protease inhibitor cocktail, N-formyl-methionyl-leucyl-phenylalanine (fMLP), TIRTC-conjugated phalloidin were purchased from Sigma. All other chemical reagents used were analytical grade.

2.2. Extraction and isolation

After cleaned and dried, the roots of *B. chinense* were ground, and the powders were extracted with hot water for three times. The whole extract was filtered, concentrated and centrifuged, and then the supernatant was treated with 3 volumes of ethanol at 4 °C overnight. The crude polysaccharide precipitate was collected by centrifugation, and dried under reduced pressure after washed with dehydrated alcohol and diethyl ether. The sample was dissolved in distilled water, then frozen at −20 °C, thawed at room temperature and centrifuged to remove insoluble materials. The supernatant was deproteinated by a combination of proteinase and Sevag method (Staub, 1965). And then, the supernatant was collected, dialyzed and lyophilized to obtain water-soluble *B. chinense* polysaccharides (BCPs). Total carbohydrate contents of BCPs were 97.1% determined by phenol–sulfuric acid colorimetric method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), and the yield of BCPs was 6.3% of dried material. The molecular weight range of BCPs is from 540 to 6.6 kDa, evaluated by HPLC (Shimadzu, Japan) equipped with a TSKGEL G3000 PWXL column and RID-10A Refractive Index Detector.

2.3. Animals

Male BALB/c mice (18–22 g) were purchased from Animal Experimental Center of Jilin University. The mice were housed in plastic cages and kept under standardized conditions at a temperature of 22–24 °C, and 20% humidity with a 12 h light/dark cycle, and free access to tap water and food. They were allowed to acclimatize for 3 days before the experiments started.

2.4. Neutrophil-like differentiation of HL-60

The myeloid cell line HL-60 was grown in IMDM medium supplemented with 10% heat inactivated FCS (fetal calf serum), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2 humidified air at 37 °C. To initiate neutrophil-like differentiation of HL-60 (dHL-60), 5×10^6 HL-60 cells were centrifuged ($100 \times g$) and transferred to new flasks containing 15 ml of IMDM medium supplemented with 10% FCS and 1.25% dimethyl-sulfoxide (DMSO), cultivated for 5 days. Non-viable cells were removed using the following method (Hauert, Martinelli, Marone, & Niggli, 2002). Cell viability was evaluated using trypan blue exclusion and changes in morphology were assessed visually after Giemsa-Wright's stain.

2.5. In vivo neutrophil infiltration assay

Neutrophil infiltration to the peritonitis was carried out as described (Xu et al., 2010) with some modifications. Mice neutrophils were isolated from peripheral blood using a discontinuous Percoll (Pharmacia) gradient. Neutrophils were incubated with 2 μM Calcein-AM at 4 °C for 30 min. After washed, cells were pretreated with BCPs (50 μg/ml) or PBS (vehicle control) at 4 °C for 30 min. After washed three times, cells were resuspended in PBS. For the thioglycollate-induced acute peritonitis model, these treated neutrophils were injected into tail veins of littermates, which were intraperitoneally injected with 2 ml of 3% thioglycolate 2 h earlier. The mice were euthanized 90 min later, and their peritoneal cavities were lavaged with 2 ml of ice-cold PBS. Cells in their peritonea were collected, and fluorescent labeled neutrophils were analyzed by cell counting with fluorescence microscope.

2.6. Transwell cell migration assay

dHL-60 cells, treated with or without BCPs (50 μg/ml), were labeled with 2 μM Calcein-AM for 30 min at 4 °C. After washing to remove free Calcein-AM, the cells were resuspended in IMDM medium. BCPs (50 μg/ml) were also added to the lower wells with cells pretreated with BCPs. And then, the treated cells (0.1 ml) were placed in 3 μm pore Transwell inserts in 24 well plates. IMDM containing 100 nm fMLP as chemoattractants was added to the lower wells, while medium alone was added to the control wells. Cells were allowed to migrate for 120 min at 37 °C. Then the inserts were removed, fluorescence intensity of cells migrated through the inserts was measured on a Molecule Devicer CytoFluor II plate reader. Fluorescence intensity of total cells was also measured allowing determination of the percent of migrated cells.

2.7. Cell populated agarose drop assay

Cell populated agarose drop assay was derived from the method described by Rosello, Ballet, Planus, and Tracqui (2004). dHL-60 cells, treated with or without BCPs (50 μg/ml) at 4 °C for 30 min, were suspended at 20×10^6 cells/ml in IMDM complete medium supplemented with 0.3% agarose (with or without BCPs) and maintained at 37 °C. Drops of 2 μl volume were taken from this cellular suspension and plated in the center of 35 mm culture dishes. The preparation was placed at 4 °C for 15 min to allow the agarose to turn into gel. Then, 100 nM fMLP supplemented medium (contains 0.3% agarose; with or without BCPs) was added to cover the drop. Migration distance was then recorded by fluorescence microscopy after 120 min incubation. Values are normalized to the migration distance of control cells without BCPs treatment. Each bar represents the mean ± SEM from 3 independent experiments.

2.8. Flow cytometry

The quantification of β2 integrin expression and activation on dHL-60 cells was analyzed by flow cytometry. The suspended dHL-60 cells were treated with or without BCPs at 4 °C for 30 min. After being exposed to fMLP (100 nM) for 1 min, dHL-60 cells were fixed with 4% paraformaldehyde for 5 min at RT. Then, the cells were incubated with IB4, m24, or a negative control Ab at RT for 60 min, followed by Alexa-Fluor 488-conjugated anti-mouse Ab. Cells were then washed and resuspended in PBS. Samples were examined on a Beckman FACScan, and data were analyzed by WinMDI software.

2.9. Immunofluorescence and confocal microscopy

dHL-60 cells were treated with or without BCPs (50 μg/ml) at 4 °C for 30 min. After being exposed to fMLP for 1 min, dHL-60 cells were fixed with 4% paraformaldehyde in PBS for 5 min at 37 °C. The cells were washed with PBS, and then incubated with Ab IB4 to β_2 integrin (1:100 dilution in PBS). After incubation for 60 min at RT, the cells were washed and incubated in PBS containing Alexa Fluor 488-conjugated anti-mouse Ab for 60 min at RT. Then cells were washed and recorded by Zeiss confocal imaging system. Clustering of β_2 integrin was analyzed in Image-Pro Plus 6.0.

2.10. F-actin polymerization assay

F-actin content was quantified according to the methods as described (Machesky & Hall, 1997) with some modifications. After treated with or without BCPs (50 μg/ml), dHL-60 cells were stimulated with fMLP (100 nM) for indicated times at 37 °C. After washed with PBS, the cells were fixed with 400 μl fixative containing TIRTC-phalloidin (20 mM KPO_4, 10 mM Pipes, 5 mM EGTA, 2 mM $MgCl_2$, 0.1% Triton X-100, 4% formaldehyde, 2 μM rhodamine phalloidin). After incubated for 1 h at RT on a rotator, cells were pelleted by centrifuge, and the pellets were washed with washing buffer (0.1% saponin, 20 mM KPO_4, 10 mM Pipes, 5 mM EGTA, 2 mM $MgCl_2$). Pellets were then resuspended in methanol and incubated for 1 h to extract TIRTC-phalloidin. The fluorescence intensity of phalloidin in the extract was measured on a Molecule Devicer CytoFluor II plate reader. F-actin content was expressed as a comparison with values obtained for untreated cells processed in parallel. Each time point consists of three independent experiments.

2.11. Immunoprecipitation and immunoblotting

After treatment with or without BCPs (50 μg/ml), dHL-60 cells were stimulated with fMLP (100 nM) for 1 min at 37 °C. The cells were lysed in 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 2.5 mM sodium pyrophosphate, 1 mM NaF, 1 mM Na_3VO_4, 1 mM β-glycerophosphate, and 20 μg/ml aprotin/leupeptin/PMSF. After incubation on ice for 15 min, the lysates were centrifuged at 15,000 × *g* for 25 min. The supernatants were incubated with anti-Vav1 antibody at 4 °C for 1 h, and then 20 μl of protein G-Sepharose beads (50% slurry) was added. After incubation for another 1 h at 4 °C, the immunoprecipitates were washed three times with lysis buffer and resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membranes. The membranes were incubated with 5% nonfat milk in TBST (20 mM Tris (pH 7.5), 50 mM NaCl, 0.05% Tween 20), and then with the indicated primary Abs and the HRP-conjugated secondary antibody at RT for 1 h, respectively. Chemiluminescent detection was performed by using ECL Plus Western blotting reagents according to the manufacturer's protocol (Amersham Pharmacia Biotech). Quantification was performed using the ImageJ software.

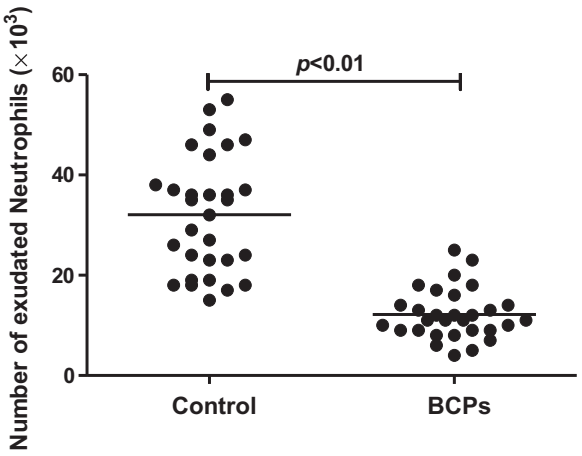

Fig. 1. BCPs inhibited neutrophil recruitment *in vivo*. The Calcein-AM-labeled neutrophils treated with or without BCPs were injected into tail veins of the thioglycollate-induced acute peritonitis model littermates. The fluorescent-labeled neutrophils in their peritonea were analyzed by cell counting under a fluorescence microscope.

2.12. Protein expression and Rac1 activation assay

The GST-PAK1 PBD (amino acids 67–150 from human PAK1) plasmid was kindly provided by Dr. Gary Bokoch (The Scripps Research Institute, California). *E. coli* strain BL-21 (DE3) was transformed with GST-PAK1 PBD plasmid and cultured overnight. GST-PAK1 PBD proteins expression was induced with 0.3 mM IPTG at 37 °C for 3 h. Cells were harvested in lysis buffer (20 mM Hepes pH 7.5, 120 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mg/ml lysozyme, 1 mM PMSF, 10 μg/ml each aprotinin and leupeptin) and homogenized by sonication. Fusion proteins were purified by glutathione-Sepharosed 4B beads from lysates.

Rac1 activation was analyzed using GST pull-down assay (Benard, Bohl, & Bokoch, 1999). The dHL-60 cells were incubated with BCPs (50 μg/ml) for 30 min at 4 °C, and then lysed after stimulated by fMLP (100 nM) for 1 min, and then cell lysates were incubated for 1 h at 4 °C with glutathione-Sepharose beads coated with GST-PAK1 PBD. Beads were washed four times with ice-cold lysis buffer and boiled in Laemmli sample buffer, and the amount of Rac1 protein bound to GST-PAK1 PBD protein, as well as the levels present in whole cell lysates, were analyzed by immunoblotting.

3. Results

3.1. BCPs inhibits neutrophil recruitment and migration by in vivo and in vitro models

We investigated the effect of BCPs on neutrophil recruitment *in vivo* by thioglycollate-induced acute peritonitis model. As shown in Fig. 1, about 3.2×10^4 neutrophils were detected in peritoneal cavities of model mice. When treated with BCPs, only about 1.2×10^4 neutrophils were found in peritoneal cavities. Thus, the results showed that BCPs significantly impaired the *in vivo* neutrophil infiltration in the peritonitis models.

Then, we compared migration capacity of neutrophil-like differentiation of HL-60 cells (dHL-60) cells treated with or without BCPs using Transwell chamber. As illustrated in Fig. 2, ~31.8% of dHL-60 cells migrated through the filters to the lower wells in response to fMLP. However, when treated with BCPs, only ~8.0% of the cells migrated during the incubation time. Further, we evaluated the inhibitory effects of BCPs on dHL-60 cell migration by cell-populated agarose drop assay. dHL-60 cells were fluorescently labeled with Calcein-AM, and then the migration distance between

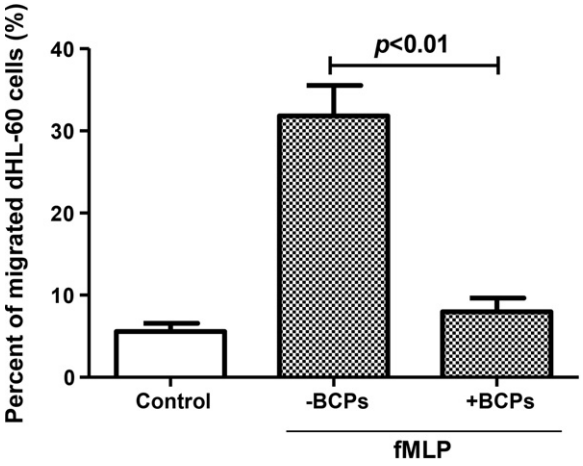

Fig. 2. BCPs inhibited neutrophil migration *in vitro* by Transwell assay. The fluorescent-labeled dHL-60 cells were incubated with or without BCPs before transfer to the upper inserts of Transwell. dHL-60 cells migrated to the lower compartment of Transwell that contained or not (control) 100 nM fMLP were quantified after 120 min. The results are presented as the mean ± SEM from 3 independent experiments.

the edge of the agarose drop and the leading edge of migrating cells was measured under a fluorescence microscope. As shown in Fig. 3, dHL-60 cells without BCPs-treatment migrated a longer distance, and showed much more motile than the treated cells. In contrast, migration of dHL-60 cells out of the agarose drop was significantly reduced when exposed to BCPs. Therefore, the results of Transwell migration assay and cell-populated agarose drop assay confirmed that BCPs could significantly inhibited dHL-60 cell migration.

3.2. BCPs impacted β2 integrin activation and clustering induced by fMLP stimulation

Integrin are of critical functional importance for neutrophil locomotion in extravascular tissue, due to the integrin-mediated adhesion is indispensable for neutrophil to overcome tissue barriers like the endothelial layer. In addition, rapid integrin activation by chemokines or chemoattractants is a critical step for the firm attachment to and the subsequent transmigration through the vascular endothelium. Chemoattractants, such as fMLP, can induce integrin changing their structure from low to high affinity sta-

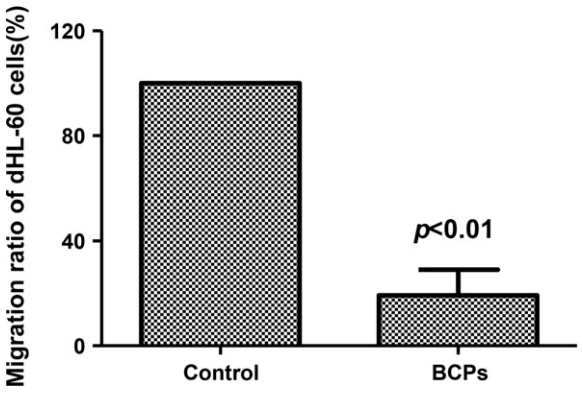

Fig. 3. BCPs inhibited neutrophil migration *in vitro* by cell populated agarose drop (CPAD) assay. The agarose drops with Calcein-AM fluorescent-labeled dHL-60 cells treated with or without BCPs were plated in the center of culture dishes. After 120 min incubation, dHL-60 cells migration out of agarose drops were measured with fluorescence microscopy. Each bar represents the mean ± SEM from 3 independent experiments. Values are normalized to the migration distance of control cells without BCPs treatment.

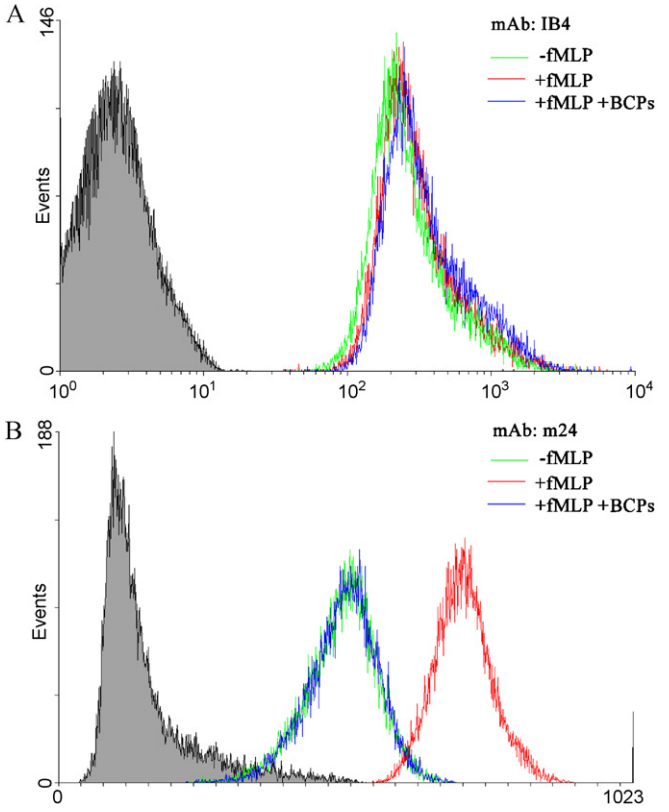

Fig. 4. BCPs impacted β2 integrin activation induced by fMLP stimulation. dHL-60 cells treated with or without BCPs were stimulated with fMLP (100 nM) in suspension at 37 °C for 1 min. After fixed by adding paraformaldehyde, the cells were stained with IB4 (A) for total expression amount of β2 integrin, and with m24 (B) for activated β2 integrin. Cells were analyzed by flow cytometry after staining with FITC-conjugated secondary Abs, and data were analyzed by WinMDI software.

tus, and modulate their binding avidity *via* clustering. We further examined whether BCPs impacted fMLP-induced β2 integrin activation and clustering. As shown in Fig. 4B, under suspension state, addition of chemoattractant fMLP significantly induced β2 integrin activation detected by flow cytometry with the m24 antibody that recognizes the activated β2 integrin. However, the amount of activated β2 integrin was remarkably reduced in BCPs-treated dHL-60 cells. Moreover, the total amount of β2 integrin on the cell surface has not changed regardless treated with or without BCPs (Fig. 4A). We next analyzed the effects of BCPs on the avidity of β2 integrin after stimulated with fMLP. After fixation, cells were allowed to settle on slides for confocal microscope analysis. As shown in Fig. 5A, after stimulated with fMLP, β2 integrin was significantly clustered in control group, approximately 60% of the cells showed clustered. However, clustered appearance of β2 integrin was significantly declined in BCPs-treated dHL-60 cells. Quantitative analysis by counting the cells with β2 integrin clustering showed BCPs-treated dHL-60 cells exhibited ~5–6-fold less clustering than normal control cells (Fig. 5B). In summary, β2 integrin affinity and avidity were significantly impacted in BCPs-treated dHL-60 cells.

3.3. BCPs inhibits fMLP-induced actin polymerization

Neutrophil migration is intimately related to actin polymerization at the leading edge in response to chemoattractant signal (Wang, Chen, & Iijima, 2011). We further evaluated the effects of BCPs on fMLP-induced actin polymerization. After treated with or without BCPs, the dHL-60 cells were stimulated with fMLP for indicated time at 37 °C, and the F-actin content was measured as

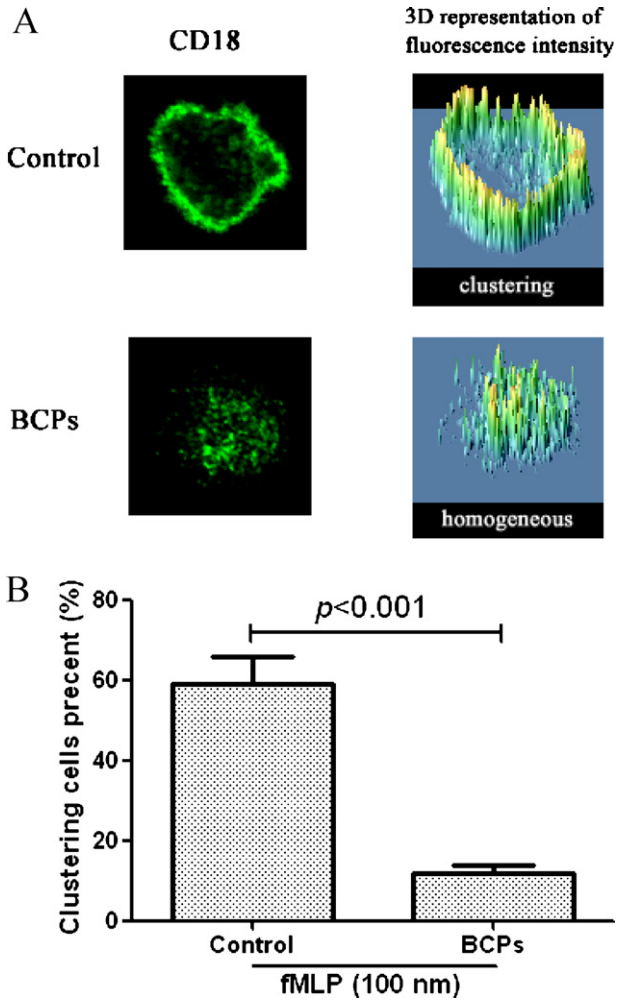

Fig. 5. BCPs impacted β2 integrin clustering induced by fMLP stimulation. (A) After treated with or without BCPs, dHL-60 cells were stimulated with fMLP (100 nM) for 1 min. After fixation, cells were stained with anti-β2 integrin Ab. Fluorescence images were captured by confocal microscopy to show the distribution of β2 integrin. Three-dimensional histograms of fluorescence intensity of β2 integrin distribution are shown in the right panels, analyzed by Image-Pro Plus 6.0. (B) Histogram represents the percent of β2 integrin-clustering cells (mean ± SEM; $n = 30$ cells from 3 independent experiments).

described in Section 2. The results showed that a burst of actin polymerization was increased to 14% above resting level in dHL-60 cells by fMLP stimulation, and the maximal change of fluorescence intensity was detected at 0.5 min after stimulation (Fig. 6). When dHL-60 cells were treated with BCPs, this treatment almost completely abolished actin polymerization compared with untreated control group. Those results indicated that BCPS inhibits fMLP-induced actin polymerization in neutrophils.

3.4. BCPs impaired the activation of Vav1 and Rac1

dHL-60 cells were stimulated with fMLP for 1 min at 37 °C. Vav1 was immunoprecipitated from cell lysates using anti-Vav1 Ab, further tyrosine phosphorylation level of Vav1 was detected by immunoblotting with PY20 Ab. The results showed that the tyrosine phosphorylation level of Vav1 was markedly increased in dHL-60 cells after fMLP stimulation (Fig. 7). When preincubated with BCPs, the tyrosine phosphorylation level of Vav1 was greatly reduced. Vav1 functions as guanine nucleotide exchange factors (GEFs) for Rac1, while, Rac1 is known to play a pivotal role in cell migration (Vedham, Phee, & Coggeshall, 2005). BCPs impaired

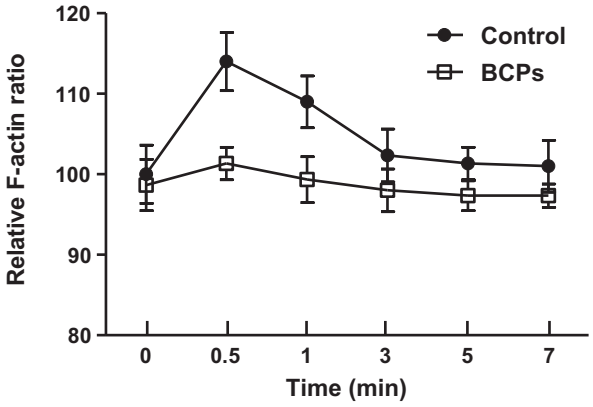

Fig. 6. Effects of BCPs on F-actin content in dHL-60 cells after fMLP stimulation. After treated with or without BCPs, dHL-60 cells were stimulated with fMLP (100 nM) for indicated times at 37 °C. F-actin was calculated by measuring fluorescence of extracted TIRTC-conjugated phalloidin in a Molecule Devicer CytoFluor II plate reader as described in Section 2. Bar graphs show the change in F-actin content expressed as a relative fold change in the mean fluorescence intensity of untreated control cells at time 0. Error bars represent the standard error of the mean (SEM) from 3 independent experiments per time point.

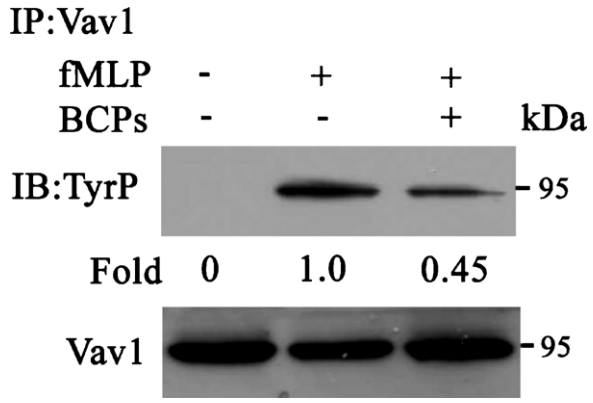

Fig. 7. BCPs inhibited fMLP-induced Vav1 activation. dHL-60 cells, treated with or without BCPs, were stimulated with fMLP (100 nM) for 1 min. Vav1 was immunoprecipitated from cell lysates, tyrosine phosphorylation level of Vav1 was detected by immunoblotting with anti-phosphotyrosine Ab. Then the blots were stripped and reprobed with anti-Vav1 Ab to demonstrate equal loading.

Vav1 activation, thus we further assayed Rac1-GTP level (the activated form of Rac1) by using a previously described GST pull-down method (Benard et al., 1999). As shown in Fig. 8, fMLP (100 nM) stimulation caused markedly increase of Rac1-GTP levels in dHL-60 cells. BCPs-treatment caused significant inhibition of activation of Rac1. Those results indicated that BCPs significantly impaired Vav1 and Rac1 activation induced by fMLP.

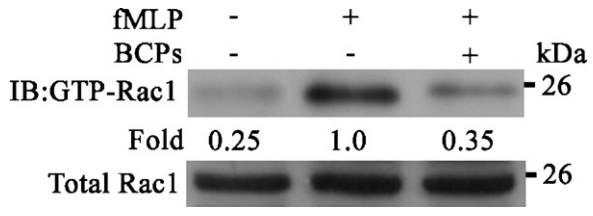

Fig. 8. BCPs inhibited fMLP-induced Rac1 activation. dHL-60 cells, treated with or without BCPs, were stimulated with 100 nM of fMLP for 1 min and lysed for the GST pull-down assay with the GST-PAK1-PBD (p21-binding domain), which specifically precipitates Rac1 in their GTP-bound form. Levels of total Rac1 were used to show cell lysate input. A representative experiment is shown from 3 independent experiments.

4. Discussion

Neutrophils are essential element of the innate immune response, and the rapid recruitment of neutrophils into inflammatory sites is crucial to defend against bacterial infection and remove damaged cells. However, neutrophils also contribute significantly to tissue damage in acute and chronic disease processes. Therefore, the destructive potential of neutrophils requires the tight control of their recruitment into tissue compartments, which has been considered as an effective strategy in amelioration of those disease processes.

Polysaccharides extracted from medicinal herbs have been proved as promising candidates for anti-inflammation. Natural polysaccharides affect multiple targets in the inflammatory progression. One of the interests in natural polysaccharides as anti-inflammatory agents is more and more evidence illustrating their ability to interfere with the migration of leukocytes to inflammatory sites. For example, in a rabbit model of bacterial meningitis, leukocyte rolling was markedly reduced by intravenous infusion of fucoidan, a sulfated polysaccharide from brown algae (Granert, Raud, Xie, Lindquist, & Lindbom, 1994). Similarly, polysaccharides from *Physalis alkekengi* and *Ginkgo biloba*, and fucoidan effectively reduced leukocyte recruitment to peritoneum in a model of peritoneal inflammation (Fei et al., 2008; Preobrazhenskaya et al., 1997; Tong et al., 2011). These anti-inflammatory effects were ascribed to the binding of polysaccharides to L- or P-selectins, which are essential cell adhesion molecules for the recruitment process of leukocytes. In addition, natural polysaccharides significantly decreased the expression of pro-inflammatory cytokines, such as IL-1β, IL-6 and INF-γ, whereas the anti-inflammatory cytokine, such as IL-10 and MIP-1β, was markedly increased, suggesting that the anti-inflammatory potential of polysaccharides might be *via* modulating pro-/anti-inflammatory cytokine secretion profiles (Liu & Lin, 2012; Ostergaard et al., 2000). Polysaccharides from *Padina tetrastromatica* significantly reduced the activity of inflammatory marker enzymes like lipoxygenases (LOX) and cyclooxygenase (COX) in peripheral blood mononuclear cells (PBMC) and increased the concentration of serum ceruloplasmin and myeloperoxidase (MPO), and also inhibited the nitric oxide synthase (iNOS) and COX-2 expression at mRNA level in carrageenan-induced inflammatory rat model (Mohsin & Kurup, 2011). PPS, a sulfated polysaccharide fraction from *Ecklonia cava*, significantly inhibited NO production, prostaglandin-E2 (PGE2) production and suppressed inducible iNOS and COX-2 expression in LPS-stimulated RAW 264.7 cells (Kang et al., 2011). Recently, Xie et al. (2012) found the polysaccharides from *B. chinense* (BCPs) attenuated LPS-induced acute lung injury (ALI) in mice, and the effect of BCPs against ALI might be related with its inhibitory effect on excessive activation of complement and on the production of pro-inflammatory mediators, such as MOP, TNF-α, and NO.

In the present study, we found a novel mechanism underlying the anti-inflammatory effects of polysaccharides from *B. chinense via* inhibiting the recruitment and migration of neutrophils by blocking chemoattractant receptor-mediated functions. We initially confirmed that BCPs, the polysaccharides from *B. chinense*, significantly inhibited neutrophil migration evaluated by *in vivo* and *in vitro* models. First, the effect of BCPs on neutrophil recruitment *in vivo* was investigated using a thioglycollate-induced acute peritonitis model. In order to convenient for tracking, the neutrophils were fluorescence-labeled with Calcein-AM, which is a bright green fluorescent compound and it is the most suitable indicator for staining viable cells due to its low cytotoxicity property, as well as it did not affect development of polarity and migration in neutrophil (Shin et al., 2010). The results showed BCPs severely impaired neutrophils infiltration *in vivo* and significantly fewer cells were recruited into the peritonea. Next, to confirm the effects of BCPs on neutrophil migration, two *in vitro* models, Transwell and cell-populated agarose drop assay, were applied to evaluate the inhibitory effects, and DMSO-induced differentiated HL-60 cells (dHL-60) were used. Neutrophil-like cells can be induced from human promyelocytic leukemia (HL-60) cells through differentiation with DMSO. The HL-60 cell line has been developed as a simple model system to study neutrophil cell migration without the need to derive cells from primary tissue (Millius & Weiner, 2010). The immortal cell line can be propagated for extended periods of time in culture and may be frozen for longer term storage. This is impossible with bone marrow or peripheral blood derived neutrophils. Both of the results from Transwell and cell-populated agarose drop assay confirmed that BCPs effectively inhibited neutrophil migration *in vitro*.

In circulating neutrophils, β2 integrin (CD11/CD18) are especially and highly expressed, and they are indispensable factors mediating the interactions of neutrophils with vascular endothelium and critically involved in firm adhesion and exsudation of neutrophils. A distinctive property of integrin is that their affinity and avidity are 'tunable', β2 integrin are in a low active state on resting neutrophils, the stimulation of chemoattractant fMLP modulates and switches the affinity from low to high, and avidity by clustering (He et al., 2011). Our results showed, under suspension state, addition of chemoattractant fMLP significantly caused β2 integrin activation detected by flow cytometry with the m24, an antibody identifying high-affinity configuration of β2 integrin, and pre-treatment with BCPs markedly reduced activation of β2 integrin and did not affect the total amount of β2 integrin on the cell surface. In addition, BCPs impacted fMLP-induced β2 integrin clustering. Those results indicated BCPs blocked active regulation of β2 integrin by chemoattractant fMLP, which may lead to significantly weaken the firm adhesion of neutrophils on the vascular endothelium and exsudation.

Localized actin filament formation induces membrane protrusion and pseudopod formation at the leading edge, which is required for neutrophil migration in response to chemoattractant signal (Van Haastert & Devreotes, 2004). The significant inhibition of fMLP-induced actin polymerization by BCPs allows us to reveal the signaling pathway, which BCPs might be involved in suppressing actin polymerization. fMLP stimulation causes the production and polymerization of actin filaments mainly *via* the activation of Rho family of small GTPases. As an important GEFs for their enzymatic activity of Rho family GTPases and a hematopoietic cell-specific signal transducer protein, Vav1 is dispensable for neutrophil migration and fMLP-induced chemotaxis (Gakidis et al., 2004). Vav1 activation is required for tyrosine phosphorylation by tyrosine kinase, such as Src- or Syk-family kinases, thus the levels of tyrosine phosphorylation reflect the activation degree of Vav1. Thus, we here confirmed, under suspension state, chemoattractant fMLP stimulation significantly induced Vav1 activation evaluated by immunoprecipitation and immunoblotting, while pre-treatment with BCPs markedly declined Vav1 activation. Rac1 activation, which further triggers Scar/WAVE- and WASP-mediated activation of the Arp2/3 complex, has been shown to be crucial for actin polymerization and cytoskeletal rearrangement dynamics, that will ultimately contribute to the regulation of neutrophil chemotaxis (Filić, Marinović, Faix, & Weber, 2012; Spiering & Hodgson, 2011). The present results showed that BCPs inhibited fMLP-induced Rac1 activation, which might be the main reason for inhibition of actin polymerization by BCPs. The *de novo* actin polymerization at the leading edge provides driving force for neutrophil migration, thus, that is one of the reasons for inhibiting neutrophil infiltration by BCPs.

In summary, BCPs impact the recruitment and migration of neutrophils by blocking fMLP chemoattractant receptor-mediated functions, including regulation of affinity and avidity of β2 integrin,

actin polymerization, and Vav1 and Rac1 activation. The polysaccharides from *B. chinense* could be considered as a promising candidate for amelioration of the inflammation-related disease process.

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