Peptidoglycan Suppresses Phagocytic Activities and Apoptosis of Macrophages in Colonic Mucosa Tissues of Crohn’s Disease Patients and In Vitro

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Background: Rac1 signaling plays a crucial role in controlling macrophage functions in CD. Peptidoglycan triggers several intracellular signaling pathways, including activation of Rac1, to regulate the function of macrophage. Suppressed Rac1 signaling in non-inflamed colonic mucosa of Crohn’s disease patients has been shown to correlate with increased innate immunity.

Material/Methods: We examined the effect of peptidoglycan on Rac1 signaling in macrophages and mucosal tissue samples collected from 10 patients with active Crohn’s disease and further investigated the effects of peptidoglycan on apoptosis and phagocytic activities of macrophages in vitro.

Results: Macrophage infiltration and Rac1 signaling was increased in inflamed mucosal tissues of Crohn’s disease patients. Immunoblotting assays revealed that peptidoglycan dose- and time-dependently increased the expression of Rac1-GTP, phosphorylated VAV1, and phosphorylated PAK1 in RAW264.7 macrophages, which, however, was attenuated by 6-thioguanine. Peptidoglycan also dose-dependently inhibited phagocytic activities of human peripheral blood mononuclear cells (PBMCs), which were partially abated by 6-thioguanine or NSC23766. Flow cytometry showed that peptidoglycan (3 μg/mL) decreased the proportion of apoptotic human PBMCs versus controls. The addition of 6-thioguanine or NSC3766 to peptidoglycan led to a sharper rise in the proportion of apoptotic human PBMCs than 6-thioguanine or NSC3766 alone.

Conclusions: Our findings suggest that Rac1 signaling is a common molecular target shared by peptidoglycan and immunosuppressive treatment in intestinal macrophages. Inhibiting Rac1 activation may be crucial for optimizing macrophage immunity for treatment of Crohn’s disease.

MeSH Keywords: Crohn Disease • Immunosuppression • Macrophages • Peptidoglycan • rac1 GTP-Binding Protein

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Background

Crohn’s disease, an inflammatory bowel disease, is characterized by the presence of granulomas and aggregates of macrophages (also known as giant cells), suggesting that macrophages play a pivotal role in the pathogenesis of Crohn’s disease [1,2]. Recent evidence shows that the number of CD68+ macrophages increased in inflamed intestinal biopsies from Crohn’s disease patients and CD14+ macrophages and the precursor monocytes express higher levels of proinflammatory cytokines in Crohn’s disease, supporting a central role of macrophages and monocytes in the pathogenesis of Crohn’s disease and macrophages and monocytes as a promising therapeutic target [3,4]. However, knowledge of the functions of macrophages and their regulatory signals in macrophages and monocytes is still limited.

Rac1, a small (~21 kDa) signaling G protein, is a member of the Rho family of GTPases and RAC1 is a susceptibility gene for inflammatory bowel disease [5]. Activated Rac1 is necessary to normal phagocytic activities of macrophages. However, our previous study showed that Rac1 activity and downstream signaling was strongly suppressed in non-inflamed colonic mucosa of Crohn’s disease compared to the inflamed tissue and significantly increased monocyte innate immune functions like bacterial phagocytosis [6]. It still remains unknown whether optimal function of macrophages requires balanced activation of Rac1 signaling.

Peptidoglycan is the major component of the cell wall of Gram-positive bacteria. The onset of Crohn’s disease often follows a bacterial infection and some Crohn’s disease patients have impaired innate immune responses and disequilibrium of bacterial flora in the intestine [7]. Recent studies have found that peptidoglycan binds to CD14 and Toll-like receptor 2 (TLR2) to trigger several intracellular signaling pathways, including activation of Rac1, to regulate the innate immune system of hosts [8,9].

In the present study, we examined the effect of peptidoglycan on Rac1 signaling in macrophages and mucosal tissue samples collected from 10 patients with active Crohn’s disease and further investigated the effects of peptidoglycan on apoptosis and phagocytic activities of macrophages in vitro.

Material and Methods

Tissues specimen acquisition

Tissue specimens were acquired from 10 patients (median age 33 years; range 22–40 years) with active CD with no prior therapy with immunosuppressive agents or 5-aminosalicylic acid. CD was diagnosed as previously described [3]. We also collected mucosa biopsy samples from 8 patients undergoing endoscopy for suspected irritable bowel syndrome (median age 35 years; range 25–42 years) as healthy controls. Two biopsy samples were collected from each patient from the ascending colon: one from the inflamed site and another from uninflamed site at least 20 cm away. The tissue specimens were snap-frozen in liquid nitrogen and stored at −80°C.

The study was approved by the Ethics Committee of Tianjin Medical University (Approval No. IRB2017-YX-045). All subjects gave written informed consent.

Cells

Blood samples were collected from 15 healthy volunteers (median age 31 years; range 24–41 years). Peripheral blood mononuclear cells (PBMCs) were prepared using Ficoll-Hypaque density-gradient centrifugation, followed by purification with CD14 microbeads (Miltenyi Biotec, Germany) [10]. PBMCs were cultured in RPMI 1640 (HyClone, China) supplemented with 100 U/mL penicillin/streptomycin (Sigma, USA) and 10% heat-inactivated fetal bovine serum (Gibco, USA). RAW264.7 cells were cultured in DMEM High Glucose (HyClone) supplemented with 10% heat-inactivated fetal calf serum at 37°C in 5% CO2, humidified air.

Immunofluorescent microscopy

Tissue sections were conventionally prepared and cryostat slides were fixed in cold acetone, washed extensively with phosphate-buffered saline (PBS), and blocked with 1% bovine serum albumin (BSA). Sections were then incubated in PBS containing a mouse monoclonal anti-human CD68 antibody (1: 200, Abcam; catalog No. ab955) and a rabbit monoclonal anti-human phosphorylated PAK1 antibody (1: 200, Abcam; catalog No. ab40852) overnight. Secondary antibodies included a donkey polyclonal secondary antibody to mouse IgG (1: 250, Alexa 488-green, Abcam; catalog No. ab150109) and a goat polyclonal secondary antibody to rabbit IgG (1: 200, Alexa 594-red, Abcam; catalog No. ab150080). Sections were examined under a confocal laser scanning microscope (Olympus, Japan).

Western blotting assays

Cell lysate was prepared as described previously [3] and samples were resolved in 10% SDS-PAGE. The following primary antibodies were used: rabbit monoclonal anti-mouse phosphorylated PAK1 antibody (1: 1000 dilution, Abcam, UK; catalog No. ab40852), rabbit polyclonal anti-mouse phosphorylated VAV1 antibody (1: 1000 dilution, CST, USA; catalog No. 2502), rabbit polyclonal anti-mouse Rac1 antibody (1: 2000 dilution, Abcam; catalog No. ab155938), rabbit polyclonal anti-mouse...
B-cell lymphoma extra-large (bcl-x) antibody (1: 1000 dilution, CST, 2 catalog No. 762), rabbit monoclonal anti-mouse signal transducer and activator of transcription-3 (STAT-3) antibody (1: 1000 dilution, CST; catalog No. 12640), rabbit polyclonal anti-mouse transcription factors of the nuclear factor κB (NF-κB) p65 antibody (1: 2000 dilution, CST, catalog No. 8242), and rabbit monoclonal anti-mouse β-actin antibody (1: 1000 dilution, CST; catalog No. 4970). The membrane was then incubated with a goat polyclonal anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (1: 1000 dilution, Boster, China; catalog No. BA1075) or goat polyclonal anti-mouse HRP-conjugated secondary antibody (1: 1000 dilution, Boster; catalog No. BA1054). Immunoreactivity was detected using an ECL method (Millipore, USA) and analyzed using Image-J software.

**Rac1 activity assay**

Rac1 activity was measured using active a Rac1 pull-down and detection kit as instructed by the manufacturer (Thermo, USA) [6]. Briefly, cell lysate was incubated with 20-μg GST-human PAK1-binding domain (PBD) at 4°C for 1 h in the spin cup containing glutathione resin. The resin was washed 3 times with lysis/binding/wash buffer, centrifuged at 6000×g for 30 s, and then solubilized in 20-μL 2×reducing sample buffer. Rac1-GTP in the solution was examined by Western blotting assays using an anti-mouse Rac1 antibody (1: 1000 dilution, Abcam, UK). Protein intensity was analyzed using Image-J software.

**Phagocytosis assay**

Phagocytosis assay was performed as previously described [6]. Briefly, human PBMCs were plated at 1×10⁵/mL and exposed to peptidoglycan (InvivoGen, from Escherichia coli) (1–20 μg/mL) dose- and time-dependently increased levels of phosphorylated VAV1, Rac1-GTP, and phospho-PAK2 (Figure 1C). Immunoblotting assays revealed that peptidoglycan caused a significant increase in the levels of phospho-PAK2 and Rac1-GTP (Figure 1A). Immunoblotting assays revealed that peptidoglycan (1 to 20 μg/mL) dose- and time-dependently increased the expression of Rac1-GTP (Figure 1B). Furthermore, peptidoglycan also caused a dose- and time-dependent elevation in the levels of phosphorylated VAV1, the major upstream activator of Rac1, and phosphorylated PAK1, an immediately downstream effector of Rac1 (Figure 1B). In addition, we treated CD14+ monocytes from the peripheral blood of healthy volunteers with LPS, peptidoglycan, and muramyl-dipeptide. Furthermore, 6-thioguanine noticeably attenuated peptidoglycan-induced elevation in phosphorylated VAV1, Rac1-GTP, and phospho-PAK2 (Figure 1C).

**Apoptotic assays**

Cells were stained with annexin V and propidium iodide using the Annexin V-PI FITC apoptosis detection kit as instructed by the manufacturer (BD, USA). Apoptotic cells were analyzed by FACS Calibur and FlowJo software. In situ apoptosis of CD68+ cells were examined by the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining (TMR red, Roche, Switzerland). Briefly, cryostat sections were fixed in cold acetone for 7 min at 4°C. After washing 3 times with PBS, sections were incubated with TUNEL reaction mixture for 60 min at 37°C in the dark. After rinsing slides 3 times with PBS, sections were incubated overnight at 4°C in mouse monoclonal anti-human CD68 antibody (1: 200, Abcam; catalog No. ab955). Sections were then incubated in donkey polyclonal secondary antibody to mouse IgG (1: 250, Alexa 488-green, Abcam; catalog No. ab150109), and counterstained with DAPI prior to analysis using a confocal laser scanning microscope. Quantification of apoptotic CD68+ cells was performed independently by 2 observers and 8 fields were selected randomly. Furthermore, caspase-3 activity was quantified using the caspase-3 colorimetric assay kit (USA, Biovision, K106) according to the manufacturers’ instructions. Caspase-3 activity was measured at 405 nm using a microtiter plate reader.

**Statistical analysis**

Data were presented as means ±SEM and analyzed using one-way ANOVA followed by Turkey’s test for multiple comparisons. Differences were considered significant when P<0.05.

**Results**

**Peptidoglycan activates Rac1 signaling in macrophages in vitro**

We examined the effect of peptidoglycan on Rac1 signaling in RAW264.7 macrophages. Immunoblotting assays revealed that compared with LPS and muramyl-dipeptide, peptidoglycan caused a significant increase in the levels of phospho-PAK2 and Rac1-GTP (Figure 1A). Immunoblotting assays revealed that peptidoglycan (1 to 20 μg/mL) dose- and time-dependently increased the expression of Rac1-GTP (Figure 1B). Furthermore, peptidoglycan also caused a dose- and time-dependent elevation in the levels of phosphorylated VAV1, the major upstream activator of Rac1, and phosphorylated PAK1, an immediately downstream effector of Rac1 (Figure 1B). In addition, we treated CD14+ monocytes from the peripheral blood of healthy volunteers with LPS, peptidoglycan, and muramyl-dipeptide. Furthermore, 6-thioguanine noticeably attenuated peptidoglycan-induced elevation in phosphorylated VAV1, Rac1-GTP, and phospho-PAK2 (Figure 1C).

**Rac1 signaling in colonic mucosal tissues of Crohn’s disease patients**

We examined Rac1 signaling in colonic mucosal tissues of Crohn’s disease patients. Immunofluorescence microscopy revealed that colonic mucosal tissues with inflammation in patients with Crohn’s disease exhibited markedly elevated levels of phospho-PAK1 in CD68+ macrophages versus paired uninflamed tissues or normal healthy colonic tissues (Figure 2). Peptidoglycan activates Rac1 signaling in macrophages in vitro, so this finding demonstrated that peptidoglycan activated Rac1 signaling in macrophages of colonic mucosal tissues of Crohn’s disease patients.
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A

Rac-GTP
Phospho-PAK2
GAPDH
Control LPS PGN MDP

B

Phospho-vav1
β-actin
GAPDH 0 1 3 10 20 (µg/ml)

Phospho-PAK1
β-actin
GAPDH 0 1 3 10 20 (µg/ml)

Rac1-GTP
β-actin
GAPDH 0 1 3 10 20 (µg/ml)
Peptidoglycan suppresses PBMC phagocytic activities via Rac1 signaling in vitro

We further investigated the effects of peptidoglycan on the phagocytic activities of macrophages. We found peptidoglycan dose-dependently inhibited phagocytic activities of human PBMCs (Figure 3A). Suppression of Rac1 signaling by 6-thioguanine, a competitive Rac1 antagonist, or NSC23766, a selective Rac1 inhibitor, significantly attenuated peptidoglycan-mediated suppression of phagocytic activities of human PBMCs (Figure 3B).

Figure 1. Activation of Rac1 signaling in macrophages was dose-dependently up-regulated by bacterial component peptidoglycan (PGN) and down-regulated by immunosuppressant 6-TG. (A) Peripheral blood CD14+ monocytes of healthy volunteers were treated with LPS, PGN and muramyl-dipeptide (MDP), levels of phospho-PAK2 and Rac1-GTP were significantly increased in PGN group compared to other groups (n=6, ** P<0.005). (B) RAW264.7 macrophages were treated with a given concentration of PGN for 24 h and levels of phospho-VAV1, phospho-PAK1, and Rac1-GTP were determined by Western blot or pull-down assay. (C) RAW264.7 macrophages were treated with 30 μg/ml PGN and various concentrations of 6-TG for a given time and levels of phospho-VAV1, phospho-PAK1, and Rac1-GTP of macrophages were determined by Western blot or pull-down assay.
Peptidoglycan inhibits apoptosis of macrophages

We examined the mucosal tissues of Crohn’s disease patients by immunofluorescent microscopy for CD68+ macrophages. We observed an apparently higher number of CD68+ cells in colonic mucosal tissues with inflammation versus paired uninfamed tissues or colonic mucosal tissues from healthy individuals, suggesting macrophage infiltration in inflamed colonic mucosa in Crohn’s disease patients (Figure 4). TUNEL assays further revealed presence of apoptotic CD68+ macrophages in paired uninfamed tissues while apoptosis was scant in colonic mucosal tissues with inflammation (Figure 5). Flow cytometry showed that peptidoglycan (3 μg/mL) decreased the proportion of apoptotic human PBMCs versus controls (Figure 6A). By contrast, the addition of 6-thioguanine or NSC3766 to peptidoglycan led to a sharper rise in the proportion of apoptotic human PBMCs than 6-thioguanine or NSC3766 alone (Figure 6B, 6C). Additionally, peptidoglycan (3 μg/mL) significantly elevated the levels of NF-κB, bcl-xL and STAT3 in RAW 264.7 macrophages, which, however, was partially aborted by NSC3766 and 6-thioguanine (Figure 6D). Peptidoglycan also markedly decreased caspase 3 activities while 6-thioguanine
or NSC3766 in combination with peptidoglycan enhanced caspase 3 activities to a greater extent than 6-thioguanine or NSC3766 alone (Figure 6E).

**Discussion**

We demonstrated that peptidoglycan dose-dependently activated Rac1 signaling in macrophages and blockade of peptidoglycan-induced Rac1 signaling led to enhanced phagocytic activities of human PBMCs.

Recent studies have shown that human immune system is more shaped by the environment than by heritable factors [11,12]. The inflamed mucosa of Crohn’s disease patients is characterized by a unique immune-microenvironment. On one hand, intestinal epithelial barrier dysfunction allows access of luminal bacteria and their antigens into the mucosal tissue, and on the other hand, immune-modulatory agents target intestinal immune cells during treatment [13]. It remains unanswered how these environmental factors influence innate immune cells, and especially macrophages. A recent report showed that CD68⁺ macrophages accumulated in inflamed biopsies of Crohn’s disease patients and produced more proinflammatory cytokines [3]. In this study, we showed that CD68⁺ macrophages accumulated in inflamed colonic mucosa of Crohn’s disease patients. Importantly, activities of Rac1 signaling were enhanced in accumulated macrophages of inflamed colonic mucosa. In peripheral blood monocytes and RAW264.7 macrophages, VAV1-Rac1-PAK1 signaling was dose-dependently stimulated by bacterial component peptidoglycan and inhibited by immunosuppressant 6-thioguanine. A recent study

![Figure 3. Overactivation of Rac1 signaling attenuated monocyte phagocytosis and suppression of Rac1 signaling ameliorated impaired phagocytosis induced by PGN. (A) Monocytes of healthy volunteers were treated with different concentrations of PGN (1 μg/ml, 3 μg/ml, 6 μg/ml, 10 μg/ml, and 20 μg/ml) (B) Monocytes of healthy volunteers were treated with different concentrations of PGN and/or 10 μM 6-TG/10 μM NSC23766 for 1 h and then challenged with FITC-labeled E. coli for 1 h (n=6, * P<0.05, ** P<0.005, *** P<0.0005).](image)
found that non-Rac-dependent TSC/Rheb-mediated PAK activation is characterized by a tuberous sclerosis complex; thus, it is interesting to measure the TSC/Rheb-PAK signaling in our patients [14]. These findings suggest that peptidoglycan and 6-thioguanine treatment have a common molecular target in innate immune cells and 6-thioguanine may be useful as a potent immunosuppressive agent in alleviating peptidoglycan-induced suppression of macrophage function in Crohn’s disease patients.

Rac1, as a member of the Rho family of GTPases, controls actin cytoskeletal movement and activation of the respiratory burst to combat bacterial infection. Rac1 is a susceptibility gene for inflammatory bowel disease [5]. Conditional disruption of Rac1 in macrophages protected mice against dextran sulfate sodium-induced colitis [4]. However, our previous study found that suppression of Rac1 signaling in non-inflamed colonic mucosa of Crohn’s disease patients was caused by GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs) rather than by differences in the mRNA levels of Rac1 [2]. The Rac1-GTPase switches between an active GTP-bound form and an inactive GDP-bound state. Whereas GEFs stimulate GTP loading, GAPs inactivate Rac1 by activating GTP hydrolysis to GDP [6]. Recent data have revealed that bacterial antigens preferentially target GTP-binding proteins, especially small GTPases like Rac. The inhibition of GTP hydrolysis is a common mechanism used by many bacterial antigens to activate GTPases [15–17]. This is in line with our findings that Rac1 signaling in macrophages was activated by bacterial component peptidoglycan in a dose-dependent manner. In addition, fecal supernatant from Crohn’s disease patients impairs intracellular bactericidal clearance in macrophages [18], suggesting a correlation between gut microbiota and inflammation of Crohn’s disease, especially impaired innate immunity. Our data provide important insights into the mechanism, implying that imbalanced Rac1 signaling under bacterial component stimulation and immunosuppressive inhibition may play a pivotal role in impaired innate immunity of Crohn’s disease patients.

We observed that peptidoglycan at low doses had no effect on phagocytic activities of monocytes, while at high doses it suppresses the phagocytic activities. Inhibition of Rac1 signaling ameliorated peptidoglycan-induced reduction in phagocytic activities. According to our previous finding of a bell-shaped dose response of Rac1 inhibitors in monocyte immunity [2], this finding poses an important hypothesis that the optimal function of

**Figure 4.** Over-accumulated CD68+ macrophages in inflamed CD biopsies. Inflamed and uninflamed colonic biopsies from CD patients (n=10) and biopsies of healthy controls from the same areas with the inflamed biopsies (n=8) were collected. Frozen sections were stained with anti-CD68 (Alexa 488-green, panels A–C). Merged images with DAPI (panels D–F) showed a higher amount of CD68+ macrophages in inflamed CD biopsies than in uninflamed CD biopsies and controls. Original magnification ×20, 10 fields were analyzed per patient.
Macrophages may require balanced activation of Rac1 signaling. Rac1 signaling regulates actin polymerization of cytoskeleton to form actin-enriched membrane cups during phagocytosis [19]. A recent study reported that activated Rac1 is necessary to assemble F-actin, but closing the phagocytic cup requires Rac1 to be deactivated [20], which supports our hypothesis.

Conclusions

One other important function of Rac1 signaling is to control apoptosis of cells. CD68 is a marker for various cells of the macrophage lineage, including monocytes and resident tissue macrophages [21]. Recent research shows that CD14⁺HLA-DR⁻macrophages derived from classical monocytes of peripheral blood account for the significant increase of macrophages in inflamed mucosa from active Crohn’s disease patients compared with controls and non-inflamed mucosa from active Crohn’s disease patients [22]. Reduction of CD68⁺ macrophages in the intestinal mucosa of patients with inflammatory bowel disease strongly correlate with endoscopic response and mucosal healing following infliximab therapy, highlighting the importance of macrophage apoptosis in Crohn’s disease treatment [3,22]. Our study confirms that peptidoglycan stimulated anti-apoptotic signaling NF-κB, STAT-3 and bcl-xL and protected monocytes from apoptosis, while inhibition of Rac1 by 6-thioguanine significantly increased peptidoglycan-associated monocyte apoptosis, indicating the importance of

Figure 5. Apoptosis of macrophages in inflamed CD mucosa. Biopsies from healthy controls, inflamed biopsies, and uninflamed biopsies from CD patients were stained with anti-CD68 (Alexa 488-green, panels A–C) and TUNEL (Alexa 590-red, panels D–F) for double-label immunofluorescence analysis. Merged images (panels G–I) show that apoptotic death rates of macrophages were comparable between groups. Original magnification ×40, 10 fields were analyzed per patient.
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Figure 6. Suppression of Rac1 induced PGN-associated apoptosis of monocytes. (A) Peripheral blood CD14+ monocytes of healthy
volunteers were cultured in the presence of PGN 3 μg/ml for 24 h; FACS analysis showed that the induction of apoptosis of
monocytes was decreased in 3-μg/ml PGN-treated cells (n=6, **P<0.005). (B) 6-TG slightly induced apoptosis of CD14+ macrophages (n=6, * P<0.05) and significantly induced PGN-associated apoptosis of monocytes (n=6, ** P<0.005). (C) NSC23766 slightly induced apoptosis of monocytes (n=4, * P<0.05) and significantly induced PGN-associated apoptosis of monocytes (n=4, ** P<0.005). (D) PGN significantly activated anti-apoptotic associated signals NF-κB, bcl-xL, STAT3 and β-actin (n=4, ** P<0.005, *** P<0.0005, respectively), compared with PGN groups. (E) Caspase-3 activities were reduced in 3-μg/ml PGN-treated monocytes (n=4, * P<0.05, ** P<0.005), compared with PGN group.

Rac1 in apotosis of monocytes. Azathioprine therapy offers a meaningful option in the management of steroid-dependent Crohn’s disease for up to 10 years [23]. Fine-tuning of Rac1 activation is a possible effect of azathioprine during Crohn’s disease treatment. Our study suggests peptidoglycan plays a pivotal role in modulating Rac1 activities of macrophages in the pathogenesis of Crohn’s disease, and finely-tuned Rac1 activation may be crucial for optimal macrophage immunity during Crohn’s disease treatment.

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