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EFhd2/swiprosin-1 regulates LPS-induced macrophage recruitment via enhancing actin polymerization and cell migration

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ABSTRACT

Macrophage motility is vital in innate immunity, which contributes strategically to the defensive inflammation process. During bacterial infection, lipopolysaccharide (LPS) potently activates the migration of macrophages via the NF-κB/iNOS/c-Src signaling pathway. However, the downstream region of c-Src that participates in macrophage migration is unclear. EFhd2, a novel actin bundling protein, was evaluated for its role in LPS-stimulated macrophage migration in this study. We found that LPS stimulated the up-regulation, tyrosine phosphorylation and membrane translocation of EFhd2 in macrophages. The absence of EFhd2 inhibited the recruitment of macrophages in the lungs of LPS-induced septic mice. LPS-induced macrophage migration was neutralized by the deletion of EFhd2. EFhd2-mediated up-regulation of NFPs (including Rac1/Cdc42, N-WASP/WAVE2 and Arp2/3 complex) induced by LPS could be used to explain the role of EFhd2 in promoting actin polymerization. Furthermore, the purified EFhd2 could directly promote actin polymerization in vitro. Dasatinib, a c-Src specific inhibitor, inhibited the up-regulation of EFhd2 stimulated by LPS. Therefore, our study demonstrated that EFhd2 might be involved in LPS-stimulated macrophage migration, which provides a potential target for LPS-activated c-Src during macrophage mobilization.

1. Introduction

The ability of monocytes/macrophages to migrate to the site of infection or inflammation is a crucial process in the developing innate immune response [1,2]. Classically, pro-inflammatory signals (including pro-inflammatory cytokines and microbial molecules) from inflamed foci induce monocyte emigration from the bone marrow, and circulating monocytes are targeted to inflammatory sites by rolling, adhesion and cytoskeleton-driven transmigration of local endothelial cells depending on adhesion molecules and chemokines [3,4].

Substantially, the initial response of a cell to migration is to extend protrusions driven by actin cytoskeleton reorganization [5]. Actin polymerization is mediated by the actin-related protein (Arp) 2/3 complex, which is activated by actin nucleation promoting factors (NPFs) such as Wiskott–Aldrich syndrome protein (WASP) and WASP-family verprolin-homologous protein (WAVE) family members at the plasma membrane [6,7]. For migration to occur, a protrusion must still be stabilized by attaching to surrounding matrix, forming a new point of cell adhesion, such as though the interaction of myosin II with actin filaments [5,8]. Rho proteins are believed to be important for regulating actomyosin contractility and actin polymerization by binding to Rho-associated coiled-coil forming protein kinase (ROCK) [5]. Myosin II activity is regulated by myosin light-chain (MLC) phosphorylation, which could be directly and positively regulated by ROCK [9].

Lipopolysaccharide (LPS), a morbigenous component of the outer membrane of Gram-negative bacteria, is also known to cause actin cytoskeleton reorganization in a variety of cells, including macrophages, which may directly contribute to the migration of monocytes/macrophages to the invaded region upon bacterial infection [10,11]. In vitro, LPS induces polarization, elongation and ruffling of macrophage-like J774 cells and RAW264.7 cells as well as accelerated cell movement, and LPS-induced activation of macrophage motility is linked to reorganization of the actin cytoskeleton [10,12]. Src family kinases (SFK), a family of non-receptor tyrosine kinases, phosphorylate and regulate the activity of NPFs that are required for actin polymerization [13]. For example, SFK activates N-WASP through tyrosine

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phosphorylation, which can induce Arp2/3 complex-mediated actin polymerization [14,15]. SFK comprises nine proteins that share similar structural and biochemical properties. Among these subtypes, c-Src is barely detectable in resting macrophages. However, an enhancement of c-Src expression upon LPS stimulation implies that it may have an unperceived role in transmitting LPS signaling [16]. In fact, LPS-mediated macrophage migration requires an enhancement of c-Src expression, which can be regulated by inducible nitric oxide synthase (iNOS) [17,18]. However, the molecular mechanism governing c-Src dependent migration of macrophage induced by LPS is unclear.

Recently, EFhd2 was identified as a novel actin bundling protein regulating cell spreading and migration in lymphocytes. EFhd2 was also found together with actin in the cytoskeleton fraction of human mast cells, NK-like cells and melanoma cells [19,20]. Morphologically, EFhd2 increased lamellipodia formation, whereas knock down of EFhd2 inhibited epidermal growth factor (EGF)-induced lamellipodia formation, and led to a loss of actin stress fibers at the leading edges [21]. One possible mechanism is that EFhd2 strongly facilitates the formation of entangled or clustered F-actin by directly binding to F-actin and dose-dependently inhibiting cofilin-mediated actin depolymerization by preventing the binding of cofilin to F-actin [21]. In the current study, the expression of EFhd2 was identified in murine peritoneal macrophages and RAW264.7 cells, and its expression was up-regulated under LPS exposure. Therefore, it is easy to speculate that EFhd2 may also regulate actin polymerization and participate in the migration of macrophages.

In this study, we observed the expression and distribution of EFhd2 in macrophage stimulated by LPS. Through a combination of loss-of-function and gain-of-function approaches, the effects of EFhd2 on cell migration, protrusion formation, cell spreading, actin polymerization, expression of Arp2/3 isoforms and NPFs were investigated. In addition, the regulation of c-Src on the expression of EFhd2 under LPS stimulation was studied.

2. Materials and methods

2.1. Materials

LPS (*E. coli*) was purchased from Sigma (St. Louis, Missouri, USA). A ProteoExtract Transmembrane Protein Extraction Kit (71772–3) was purchased from Merck Millipore (Massachusetts, USA). A G-Actin/F-Actin In Vivo Assay Kit (BK037), Actin Polymerization Biochem Kit (BK003), WASP-VCA Domain-GST Protein (VCG03), Arp2/3 Protein Complex (RP01P) and actin-stain 555 fluorescent phalloidin (PHDH1) were purchased from Cytoskeleton (Denver, Colorado, USA). Corning Costar Transwell inserts were purchased from Corning (New York, USA). Protein G Sepharose 4B (101241) was purchased from Invitrogen (Cedarhurst, NY, USA). F4/80 Antibody (12-4801-80) was obtained from eBioscience (San Diego, California, USA). Anti-EFhd2 (img-3387) was purchased from Novus (Littleton, Colorado, USA). DAPI (C1002), NP-40 (P0013F), SMT (iNOS inhibitor), BAY 11-7082 (NF-κB inhibitor) and dasatinib (c-Src specific inhibitor) were purchased from Beyotime (Shanghai, China). Anti-ARPC5 (sc-65165), anti-ARPC4 (sc-68394), anti-ARPC2 (sc-32195) and p-Tyr (sc-7020) antibody were purchased from Santa Cruz Biotechnology, Inc. (California, USA). Actin Nucleation and Polymerization Antibody Sampler Kit (8606), Myosin Light Chain 2 Antibody Sampler Kit (9776), anti-Rock-1 (4035), anti-Src (2109), anti-iNOS (13120), anti-phospho-c-Src (6943) and anti-IκBα (4814) were purchased from Cell Signaling Technology (Danvers, Massachusetts, USA). Cytochalasin D (BIA-C1170) was purchased from BioAustralis (Australia). Latrunculin A (L5163) was purchased from Sigma (St. Louis, Missouri, USA). PP2 (Src-family inhibitor) was purchased from Selleck Chemicals (Shanghai, China).

2.2. Mice

C57BL/6 mice were obtained from the Laboratory Animal Center of Second Military Medical University. Recombineering was used to generate a gene-targeting construct from a bacterial artificial chromosome (BAC) containing the *EFhd2* gene (C57BL/6 inbred strain). E14 ES cells were transfected with this construct, and successfully targeted ES cells were injected into C57BL/6 blastocysts. Mice bearing this targeted allele of exon 2–4 deletion in the germline were bred to generate *EFhd2* knockout mice [22]. All mice were housed and cared for in the Animal Care Facility at our Institution with free access to food and water. All protocols were approved by the Animal Care Committee at the Second Military Medical University.

2.3. Sepsis model

Sepsis was induced by single intraperitoneal (i.p.) injection of LPS (40 mg/kg). Clodronate liposome (CLOP) was used to deplete macrophages of C57BL/6J mice. Twenty-four hours after intravenous (i.v.) injection of CLOP (0.1 ml/10 g), 3×10^7 peritoneal macrophages isolated from *EFhd2* wild type (WT) of knock out (KO) mice were returned to macrophage-depleted mice before i.p. LPS.

2.4. Cell culture

Murine macrophage cell line RAW264.7 was obtained from the Type Culture Collection of the Chinese Academy of Sciences. Peritoneal macrophages were isolated from mice by peritoneal lavage 3 d after intraperitoneal injection with 3% (wt/vol) thioglycollate. Cells were washed with PBS and used for migration or cultured overnight in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin G, and streptomycin in a humidified 37 °C, 5% CO_2 incubator.

2.5. Transwell migration assay

As previously described before [23], cells were stimulated with or without LPS for 12 h. Cells of each group were then added to the upper wells (24-multiwell Corning Costar Transwell) at a density of 1×10^5 per well. Migrating cells could pass through the polycarbonate filter (8 μm). Cells were fixed in 4% paraformaldehyde for 10 min. The non-migratory cells were removed from the upper membrane surface carefully with cotton sticks. After cells were stained with crystal violet and imaged by a fluorescence microscope with a 40 × objective, the numbers of migratory cells were counted in five areas chosen randomly for each membrane. Ex vivo, the migration of peritoneal macrophages isolated from LPS-induced septic mice was observed 3 h or 6 h after being implanted.

2.6. G-actin/F-actin assay

G-actin and F-actin were measured using the in vivo assay kit according to the manufacturer's directions. Briefly [24], macrophages were lysed in a detergent-based buffer that stabilized and maintained the G and F forms of cellular actin. Only G-actin was solubilized by the buffer, and after a centrifugation step, F-actin was pelleted, whereas G-actin remained in the supernatant. The F-actin was depolymerizated and aliquots of supernatant and pellet were analyzed by Western blot.

2.7. F-actin polymerization assay

As previously described [25], we measured the change in fluorescence intensity to analyze actin polymerization by using the Actin Polymerization Biochem Kit. First, 1.5 × polymerization buffers were prepared for the assay, and WASP-VCA Domain-GST Protein and Arp2/3 Protein Complex were pre-incubated as the positive control. The final