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Photo-enhancement of macrophage phagocytic activity *via* Rac1-mediated signaling pathway: Implications for bacterial infection

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ABSTRACT

Phagocytosis and the subsequent destruction of invading pathogens by macrophages are indispensable steps in host immune responses to microbial infections. Low-power laser irradiation (LPLI) has been found to exert photobiological effects on immune responses, but the signaling mechanisms underlying this photobiomodulation of phagocytosis remains largely unknown. Here, we demonstrated for the first time that LPLI enhanced the phagocytic activity of macrophages by stimulating the activation of Rac1. The overexpression of constitutively activated Rac1 clearly enhanced LPLI-induced phagocytosis, whereas the overexpression of dominant negative Rac1 exerted the opposite effect. The phosphorylation of cofilin was involved in the effects of LPLI on phagocytosis, which was regulated by the membrane translocation and activation of Rac1. Furthermore, the photoactivation of Rac1 was dependent on the Src/PI3K/Vav1 pathway. The inhibition of the Src/PI3K pathway significantly suppressed LPLI-induced actin polymerization and phagocytosis enhancement. Additionally, LPLI-treated mice exhibited increased survival and a decreased organ bacterial load when challenged with *Listeria monocytogenes*, indicating that LPLI enhanced macrophage phagocytosis *in vivo*. These findings highlight the important roles of the Src/PI3K/Vav1/Rac1/cofilin pathway in regulating macrophage phagocytosis and provide a potential strategy for treating phagocytic deficiency *via* LPLI.

1. Introduction

The phagocytic engulfment of infectious pathogens by macrophages is an indispensable component of immunity (Wynn et al., 2013). This process is initiated *via* the interaction of opsonins on the surface of the particle to be internalized with phagocytic receptors on the surface of the macrophages. Then, a series of complex intracellular signaling event is triggered by ligated phagocytic receptors to perform efficient particle internalization (Aderem and Underhill, 1999). Although the details of the molecular mechanisms of phagocytosis have not been completely demonstrated, all phagocytic signaling pathways activate cytoskeleton-remodeling molecules to regulate cytoskeletal dynamics (Jaumouille and Grinstein, 2011; Masters et al., 2013; Tohyama, 2006).

Actin filaments, the major components of the cytoskeleton in most cells, function in particle internalization by forming pseudopod extensions (Rougerie et al., 2013). Previous studies have shown that Rac1 GTPase plays a pivotal role in regulating actin dynamics (Masters et al., 2013). Rac1 promotes actin filament formation by recruiting actin nucleation-promoting factors (NPFs) and the actin-nucleating complex Arp2/3 (DeMali et al., 2002; Isaac et al., 2010). Activated Rac1 can also suppress F-actin depolymerization *via* the p21-activated kinase (PAK)-mediated phosphorylation of cofilin (Petrilli et al., 2014). The function of Rac1 has been well characterized with respect to its effects on various actin-regulating proteins, but the upstream regulating pathway is highly complex and is not fully understood.

The activation of Rac1 GTPase involves the mechanistic transition from the inactive GDP-bound to the active GTP-bound state, which is catalyzed by guanine nucleotide-exchange factors (GEFs). Vav1 is a GEF that is primarily expressed in hematopoietic cells (Tong et al., 2013; Zenker et al., 2014). It has been reported that Vav1 can be phosphorylated *in vivo* by Src kinases (Fumagalli et al., 2013), suggesting that the guanine nucleotide-exchange activity

Abbreviations: CA, constitutively activated; CFP, cyan fluorescent protein; DN, dominant negative; FRET, fluorescence resonance energy transfer; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GEF, guanine nucleiotde-exchange factor; GST, glutathione-S-transferase; LPLI, low-power laser irradiation; NPF, nucleation-promoting factor; PAK, p21-activated kinases; PBD, the Rac/Cdc42 (p21) binding domain; PP1, 1-(1,1-dimethylethyl)-3-(4-methylphenyl)-1H-pyrazolo[3,4-d]pyrimidin; PI3K, phosphatidylinositol 3-kinase; Rac1, Ras-related C3 botulinum toxin substrate 1; ROS, reactive oxygen species.

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of Vav1 can be modulated by tyrosine phosphorylation. Vav1 may also be regulated *via* the interaction of its pH domain with the lipid products of PI3K (Gambardella and Vermeren, 2013; Navarro-Lerida et al., 2012). However, the exact regulatory mechanisms of Rac1 in phagocytosis need to be further elucidated.

Low-power laser irradiation (LPLI) is a non-damaging physical therapy that has been used clinically to accelerate wound healing (Conlan et al., 1996) and to reduce inflammation in various pathologies (Aimbire et al., 2008; Song et al., 2012). However, the biological mechanisms underlying the beneficial results observed in clinical trials are not fully understood. A growing body of experimental and clinical studies has demonstrated that LPLI may regulate cell survival, proliferation, and differentiation by triggering a series of complex intracellular signaling events (Feng et al., 2012; Huang et al., 2013; Liang et al., 2012; Ling et al., 2014; Meng et al., 2013; Zhang et al., 2012). This has attracted the interest of researchers to study the effect and molecular mechanism of LPLI on the broad spectrum of cellular processes.

In this study, we investigated the effects of LPLI on macrophage phagocytosis. Our results suggested that LPLI enhances macrophage phagocytic activity by activating the actin cytoskeleton regulatory signaling pathway. Moreover, LPLI decreased mortality and the organ bacteria load after Listeria monocytogenes infection, which correlated with increased phagocytosis of macrophages. In conclusion, our findings extend the understanding of the cellular signaling mechanisms underlying LPLI-induced immune responses and provide new insight into the therapeutic potential of LPLI for anti-infection applications.

2. Materials and methods

2.1. Chemical and plasmids

The following reagents were used: wortmannin, FITC-phalloidin and latex beads were obtained from Sigma (St. Louis, MO, USA); PP1 was obtained from BioVision (Milpitas, CA, USA). The following antibodies were used: Anti-Rac1, anti-Akt, and anti-β-actin antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies specific for phosphorylated Akt at Ser473 and cofilin at Ser3 were acquired from Cell Signaling Technology (Beverly, MA, USA). An anti-Vav1 antibody was acquired from Sangon Biotech (Shanghai, China). In addition, we used jetPEI-macrophage transfection reagent (Invitrogen, Carlsbad, CA, USA) to transfect plasmid DNA into cells, and the cells were examined 36–48 h after transfection.

The pRaichu-Rac1 plasmid was kindly supplied by Dr. Michiyuki Matsuda from Kyoto University (Yukinaga et al., 2014). The GST-PBD plasmid was kindly supplied by Dr. Weilin Jin from Shanghai Jiao Tong University (Guerrier et al., 2009). Rac1Q61L and Rac1T17N were obtained from Upstate Biotechnology (Lake Placid, NY, USA). The plasmids of S3A-cofilin (constitutively active cofilin), Vav1Y3F (active form of Vav1) and Vav1L213Q (GEF-deficient Vav1) were purchased from AddGene.

2.2. Mice

For the animal study, 6-week-old male mice (BALB/c) were used. The present study was performed in accordance with the guidelines in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council). This study was approved by the Institutional Animal Care and Use Committee of our university (South China Normal University, Guangzhou, China).

2.3. Cell culture and transfection

The mouse macrophage-like cell line RAW 264.7 cells were cultured in DMEM (GIBCO, Ltd., Grand Island, NY, USA). The human macrophage-like cell line U937 were cultured in RPMI 1640 medium (GIBCO). The mediums supplemented with 10% heat-inactivated fetal calf serum (FCS, GIBCO), 50 U/ml penicillin and 50 μg/ml streptomycin. Primary peritoneal macrophages were isolated from BALB/c mice using previously reported methods (Kim et al., 2010) and were cultured in RPMI-1640 medium (GIBCO) containing 10% FCS. The cells were maintained in a humidified 37 °C incubator in 5% CO_2. Transfection was performed using jetPEI-macrophage transfection reagent according to the manufacturer's instructions (Polyplus-Transfection, Strasbourg, France).

2.4. Laser treatment

The Joule calculation formula of the laser: $D = P/S \times T$. (D is energy density; P is power; S is area and T is time). For *in vitro* LPLI, the cells were serum-starved overnight and then irradiated using a fiber-optic light delivery system (632.8 nm, HN-1000; Laser Technology Application Research Institute Co., Ltd., Guangzhou, China) for 5 min at a fluence rate of 3.33 mW/cm^2, 6.67 mW/cm^2 or 13.33 mW/cm^2 (corresponding to doses of 1 J/cm^2, 2 J/cm^2, and 4 J/cm^2, respectively). Throughout each experiment, the cells were maintained in either a completely dark or a very dim environment, except when subjected to the light irradiation, to minimize ambient light interference.

For *in vivo* LPLI, the mice were directly irradiated using the 632.8 nm laser. Then, the abdomen was irradiated with a He-Ne laser for 5 min (corresponding to a dose of 2 J/cm^2). The rate loss of laser intensity through the mouse abdominal wall was measured before irradiation, and was about 5.823 times (the average value of five abdominal positions of 4 mice). The total laser fluence delivered to the abdominal surface was 11.646 J/cm^2, corresponding dose 2 J/cm^2 to reach the peritoneal cavity. The control groups were also maintained in a specially designed holder for the same amount of time used in the irradiated groups, but the laser source was not activated (sham irradiation).

2.5. Confocal laser scanning microscopy (LSM)

Cells were observed using a 40× oil objective lens. The stage of the LSM was equipped with a temperature- and CO_2-controlled small incubator (CTI-controller 3700 digital and Tempcontrol 37-2 digital; Zeiss, Jena, Germany), which maintained the cells at 37 °C, 5% CO_2 throughout the experiments. FITC or CFP fluorescence was excited using a 488 or 458 nm Ar ion laser, and the fluorescence emission was detected through a 500–530 nm, 470–500 or 535–545 nm band pass filter, respectively. During the experiments, the excitation power of the 458, 488, and 514 nm lasers was reduced to the level of 1%, 0.5%, and 0.5%, respectively, to reduce the possible effects of the exciting light.

2.6. Fluorescence resonance energy transfer (FRET) analysis

Raichu-Rac1 is a FRETprobe that consists of Rac1, the Rac-interactive binding (CRIB) domain of PAK, and the pair of YFP and CFP (Hirata et al., 2012). To monitor the Rac1 activation in the living cells, cells were transfected with Raichu-Rac1. When Raichu-Rac1 was activated by upstream regulators, the intramolecular binding of GTP-Rac1 to CRIB brought CFP close to YFP, increasing FRET from CFP to YFP.

FRET was performed on a commercial Laser Scanning Microscope (LSM510/ConfoCor2) combination system (Zeiss, Jena, Germany). For excitation, the 458 nm line of an Ar-Ion Laser CFP