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Regulation of phagocytosis and cytokine secretion by store-operated calcium entry in primary isolated murine microglia

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ABSTRACT

Microglia are immune effector cells in the central nervous system that participate in tissue repair, inflammatory responses, and neuronal degeneration. The most important signaling factor in the differentiation of immune-active cells after stimulation is the sustained high calcium concentration in the cytosol, which is called store-operated calcium entry (SOCE). Recently, the molecular identity of the store-operated channel (SOC) has revealed that Orai1, Orai2, Orai3, Stim1, and Stim2 constitute the most of SOC. In this study, we demonstrate that Orai1- and Stim1-mediated SOC regulated the phagocytic activity and cytokine release of primary isolated murine microglia. RT-PCR analysis revealed that primary cultured microglia from neonatal ICR mouse brains had Orai1, Orai2, Orai3, and Stim1. To elucidate the role of SOCE in the immune functions of microglia, pharmacological inhibitors or knockdown with Orai1 or Stim1 siRNA was applied, and UDP-induced phagocytic activity and LPS-induced cytokine secretion activity were compared. The pharmacological inhibition and siRNA effect was verified by measuring thapsigargin (TG)-, ATP-, or UDP-activated SOCE Ca^{2+} influx and proper siRNA-mediated knockdown was verified by western blot analysis. UDP-induced phagocytic activity was inhibited by pharmacological inhibitors of SOCE, such as SKF96365 or 2-APB, and knockdown of Orai1 and Stim1. Cytokine secretion of TNF-α and IL-6 by LPS treatment was also inhibited by SKF96365 and knockdown of Orai1 and Stim1. Meanwhile, LPS stimulation-induced NF-κB activation was not altered, but NFAT1 activity was attenuated with Stim1 knockdown. These results indicate that SOCE, which was composed of Orais and Stim1, regulates UDP-induced phagocytosis and LPS-stimulated cytokine secretion in microglia.

1. Introduction

The calcium ion (Ca^{2+}) functions as an important second messenger in most cells, including cells of the immune system [1,2]. Ca^{2+} signals are critical for properly activating the immune function of lymphocytes [1,2]. The major pathway that induces an increase in intracellular Ca^{2+} levels in lymphocytes is through store-operated calcium entry (SOCE) and calcium-release activated calcium channels (CRAC) [3,4]. An influx of Ca^{2+} into the cytosol occurs following the stimulation of immunoreceptors, such as the T cell receptor, B-cell receptor, and Fc receptors, as well as chemokine and co stimulatory receptors [5–7]. Immunodeficiency diseases in humans clearly indicated that the proliferation and activation of effector functions of lymphocytes are severely distorted in the absence of sustained Ca^{2+} increase via SOCE channels [5,8].

Recently, two molecules, stromal interaction molecule 1 (Stim1) and calcium release-activated calcium channel protein 1 (Orai1) (CRACM1 or TMEM142A), have been used to clarify our understanding of the regulation of Ca^{2+} signals in lymphocytes [8,9]. The Ca^{2+} pore of the store-operated channel (SOC) is composed of Orais, which are tetra-spanning plasma membrane (PM) proteins and function as highly Ca^{2+}-selective channels in the PM that are gated by Stim, which is the Ca^{2+} sensor in the endoplasmic reticulum (ER) [8]. Stim1 is a single-spanning membrane protein with the EF-hand Ca^{2+}-binding motif and appears to function as the sensor of ER luminal Ca^{2+} [10,11]. After store depletion, Stim1 changes its conformation in the ER membrane and then moves to the region of the PM to directly induce Ca^{2+} influx via Orais or transient receptor potential channels (TRPCs) to recharge the store[4,6].

Microglia are considered resident macrophages and play a pivotal role as immune cells in various types of injury and disease in the brain [12,13]. Their substantial activation in response to these pathological states results in the morphological change from the "ramified" to "amoeboid" cell shape, increase in phagocytic activity to remove cellular debris, and secretion of various chemical mediators, including pro-

Abbreviations: SOCE, store-operated calcium entry; SOC, store-operated channel; Stim1, stromal interaction molecule 1; UDP, uridine diphosphate; ATP, adenosine triphosphate; LPS, lipopolysaccharide; TG, thapsigargin; 2-APB, 2-aminoethoxyldiphenyl borate; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; ICR mice, imprinting control region mice; NFAT1, nuclear factor of activated T cells-1; i-κB, inhibitor of nuclear factor-κB; siRNA, small interfering RNA.

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inflammatory cytokines that propagate immunological actions [14]. The activation of microglia by stimuli, such as lipopolysaccharide (LPS), induces cytokine secretion or phagocytosis, which is dependent on the sustained increased of intracellular Ca^{2+} ($[Ca^{2+}]_i$) (referred to as SOCE [15,16]). Despite the crucial role of SOCE in microglia, the detailed molecular components of SOC in microglia have not been clearly identified in murine microglia yet, and their role in immune function has not been clearly reported. To address the molecular identity of SOC and its effect on immune functions of murine microglial cells, pharmacological inhibitors and the small interfering RNA (siRNA) knockdown system were applied to primary microglial cells. Our results demonstrate that microglial phagocytic activity and cytokine secretion were decreased by pharmacological inhibitors of SOC and Orai1 or Stim1 siRNA knockdown. Tumor necrosis factor-α (TNF-α) secretion was decreased and interleukin-6 (IL-6) secretion was considerably decreased by pharmacological inhibitors and knockdown of Orai1 or Stim1. These results indicate that microglial Orai1 and Stim1, which regulate the SOCE, influence phagocytosis and regulate cytokine secretion in murine microglia.

2. Materials and methods

2.1. Microglial primary culture

Primary cultures of mouse microglia were prepared from the cerebral cortices of 1-day-old ICR mice, according to the method of mild trypsinization [17]. Briefly, cortical cells were seeded in Dulbecco's modified Eagle's medium (DMEM)-F12 with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, and they were cultured at 37 °C in a humidified 5% CO_2 incubator after mechanical and chemical dissociation. The medium was replaced every 3–4 days, and confluency was achieved after 2 weeks. The upper layer of cells was removed in one piece by a 25-min treatment with trypsin solution (0.25% trypsin, 1 mM ethylenediaminetetraacetic acid [EDTA]) and diluted in DMEM-F12 (1:4). Floating non-microglial cells in media were removed, and microglial cells were grown in DMEM-F12 with 10% FBS.

2.2. Microglial staining for morphological analysis.

Primary cultured microglia treated with mock or LPS were fixed with 4% paraformaldehyde solution in PBS (19943 1 LT; Affymetrix) for 10 min at room temperature and then rinsed 3 times with PBS. For permeabilization, the cells were incubated in permeable sol (0.1% Triton X-100, 1% horse serum, 1% BSA in PBS (pH 7.4)) for 10 min at room temperature and then rinsed 3 times with PBS. To avoid non-specific binding of antibodies, the cell was blocking solution (5% Horse serum, 1% BSA, 0.1% Gelatin, 0.001% Sodium azide in PBS (pH 7.4)) for 30 min at room temperature. Tomato lectins (1:200, Dylight 594; Vector Labs, Burlingame, CA, USA) were used for cell body of microglia and nuclei were stained with 5 μg/ml DAPI (4, 6-diamidino-2-phenylindole) (D3571; Invitrogen). After rinsing, the coverslips were mounted with fluorescent mounting medium (S3023; Dako) and visualized on an inverted fluorescent microscope (IX73-F22PH; Olympus), and the morphological analysis of cells were quantified by Metamorph software (Molecular Devices).

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

To confirm the expression of Orai1-3, TRPC1-7, and Stim1 in microglial cells, the messenger RNA (mRNA) transcripts of Orais, TRPCs, and Stim1 were analyzed by RT-PCR. Total RNA was extracted by using Trizol solution (Invitrogen, Carlsbad, CA, USA), and an equal amount of RNA from each sample was reverse-transcribed by using an oligo (dT) primer and AccuScript high-fidelity reverse transcriptase (Agilent Technologies, Santa Clara, CA, USA). The complementary DNA (cDNA) was amplified with specific primers and a Taq polymerase (i-Taq, INTRON). The PCR primer sequences were as follows: TNF-α sense, GGG GCC ACC ACG CTC TTC TG; TNF-α antisense, TGG GCT ACG GGC TTG TCA CTC G; IL-6 sense, TCC GCA AGA GAC TTC CAG CCA G; IL-6 antisense, ATT AAG CCT CCG ACT TGT GAA GTG G; CD11b sense, TAT TTG TTC GGC TCC AAC C; CD11b antisense, TGG TTT GTT GAA GGC ATT TC; Orai1 sense, ATGC ATC GCC ACA TCG AGC; Orai1 antisense, AAA CTC GGC CAG CTC ATT GAG; Orai2 sense, TTG CGA TTT GCT ACC AAC CTG; Orai2 antisense, GCT GGC TTT GGG AAT TCA CC; Orai3 sense, TGC AAG CAT TGC CTC AGA AAC; Orai3 antisense, GGA TGC CAA CTG CAA AGC AG; Stim1 sense, GAT TTG ACC CAT TCC GAT TCG; Stim1 antisense, GCT CCT TAG AGT AAC GGT TCT G; TRPC1 sense, CCT TCT CAT ACT GTG GAT TAT TG; TRPC1 antisense CTG CTC TTT GGA AGT GTA CCC T; TRPC3 sense, GTG CAC GCA TCG AGA GGC; TRPC3 antisense GTT GGC AGT TGG GGT GAG; TRPC4 sense, ACG GTC CAG GCT CAA CAT C; TRPC4 antisense, ACA CGT CAC CAT CTT CAC CG; TRPC5 sense, ATC TAC TGC CTA GTA CTA CTG GCT; TRPC5 antisense, CAG CAT GAT CGG CAA TGA GCT; TRPC6 sense, TCT ATT GAG GAA GAA CGC TTT C; TRPC6 antisense, GAT TGC CTC CAC AAT CCG TAC; TRPC7 sense, CGT GCT GTA TGG GGT TTA TAA TG; TRPC7 antisense, GCT TTG GAA TGC TGT TAG AC; glial fibrillary acidic protein (GFAP) sense, TGA TGG AGC TCA ATG ACC G; GFAP antisense, CAT CTG CCT CCT GTC TAT ACG C; β-actin-sense, GAC CCA GAT CAT GTT TGA GAC C; and β-actin antisense, GGC CAT CTC CTG CTC GAA GTC.

2.4. siRNA transfection and western blot analysis

For siRNA transfections, plated microglia were transfected with RNAimax (Invitrogen, CA, USA) using 50 nM siRNA (Dharmacon, Lafayette, CO, USA). The expression levels of knocked down proteins were tested using western blot analysis at 48 to 72 h after transfections. For western blot analysis, seeded microglia were lysed in lysis buffer (150 mM NaCl, 5 mM Na-EDTA, 10% glycerol, 20 mM Tris–HCl [pH 8.0], 0.5% Triton X-100, and proteinase inhibitors [Complete, Roche Applied Science, IN, USA]). Antibodies directed against Orai1 (ACC-033; Cell Signaling, MA, USA), Stim1 (4917; BD Biosciences, CA, USA), phospho Ser32/36 IκBα (9246S; Cell Signaling), total IκBα (C-21) (sc-371; Santa Cruz, CA, USA), NFAT1 (pS54), phospho Ser54 NFAT1 (44944G, Invitrogen, CA, USA), total NFATc2 (sc-7296; Santa Cruz), aldolase A (N-15; sc-12059; Santa Cruz), and β-actin (sc-1616; Santa Cruz) as well as the appropriate secondary antibody (Thermo Scientific, MA, USA) were used. Protein lysates were separated on 4–12% pre-made gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels (Komabiotech, Seoul, Korea) and transferred to membranes. The quantification of protein expression was based on measurements of band intensity using the MultiGauge software (Fuji Film, Tokyo, Japan).

2.5. Enzyme-linked immunosorbent assay (ELISA)

The amounts of TNF-α and IL-6 that were released in the conditioned medium were measured using a sandwich ELISA kit that was specific for mouse TNF-α and IL-6 (Bio Legend, CA, USA). Cells were treated with 5 μg/ml LPS and incubated for 3–24 h. The supernatants were added to 96-well plates that were conjugated with the TNF-α- or IL-6-immobilized capture antibody. Next, a biotinylated anti-mouse detection antibody was added, followed by avidin-horseradish peroxidase. TMB substrate solution was subsequently added and final stop solution changed the reaction color from blue to yellow. The absorbance in each well was read at 450 nm. After cytokine production was measured, cells were lysed in 1 N NaOH. The amount of cytokines was calculated using the following formula: release = cytokine secretion from the supernatant (pg/ml)/cell lysate (μg).

2.6. Ca^{2+} measurements

Microglial cells were grown on glass coverslips and loaded for 30 min at room temperature with fura-2-acetoxymethylester (Fura-