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Research Paper

p32 is Required for Appropriate Interleukin-6 Production Upon LPS Stimulation and Protects Mice from Endotoxin Shock

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ABSTRACT

Sepsis is a major cause of morbidity and mortality in seriously ill patients and mitochondrial dysfunction is associated with poor outcomes in septic patients. Although interleukin-6 (IL-6) is a good prognostic marker for sepsis, the relationship between mitochondrial dysfunction and IL-6 remains poorly understood. We identified p32/C1QBP/HABP1 as a regulator of IL-6 production in response to lipopolysaccharide (LPS). LPS induced IL-6 overproduction in p32 deficient mouse embryonic fibroblasts (MEFs) through NF-κB independent but activating transcription factor (ATF) 4 dependent pathways. Short hairpin RNA-based knockdown of ATF4 in p32 deficient MEFs markedly inhibited LPS-induced IL-6 production. Furthermore, MEFs treated with chloramphenicol, an inhibitor of mitochondrial translation, produced excessive IL-6 via ATF4 pathways. Using a LPS-induced endotoxin shock model, mice with p32 ablation in myeloid cells showed increased lethality and overproduction of IL-6. Thus, this study provides a molecular link how mitochondrial dysfunction leads to IL-6 overproduction and poor prognosis of sepsis.

1. Introduction

Sepsis is a life-threatening condition that results from a harmful host response to infection. Sepsis-associated multiple organ dysfunction syndrome (MODS) is the predominant cause of mortality among seriously ill patients (Deitch, 1992). Despite improvements in laboratory testing and clinical therapy, the mortality rate of severe sepsis remains at approximately 30% and hospital admissions for severe sepsis have increased (Opal et al., 2013) (Caironi et al., 2014) (Lagu et al., 2012). Because around 750,000 patients are diagnosed with severe sepsis in the United States each year (Angus et al., 2001), improvements in care for severe sepsis remain a priority.

Mitochondria regulate many aspects of cellular signaling and metabolic pathways through the fatty acid oxidation (FAO), the tricarboxylic acid cycle and the electron transport chain. Several lines of evidence suggest that mitochondrial dysfunction is associated with poor outcomes in patients with sepsis and MODS (Brealey et al., 2002) (Brealey et al., 2004) (Duvigneau et al., 2008). While the causality is not yet confirmed, it does nevertheless suggest a new route for therapeutic intervention focused on either mitochondrial protection or acceleration of the recovery process during sepsis (Singer, 2014).

Endotoxin/lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, has been widely used in sepsis research. Expressed at the cell surface, toll like receptor (TLR) 4 detects LPS from Gram-negative bacteria. Interaction of TLR4 with LPS induces several intracellular signaling molecules, leading to the expression of nuclear factor-κB (NF-κB)-dependent pro-inflammatory cytokines or interferon regulatory factor-dependent type I interferons (IFNs) (Kawai and Akira, 2010). The inflammatory response is orchestrated by proinflammatory cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1, and IL-6.

IL-6 is a pleiotropic cytokine that plays a major role in host defense by regulating immune and inflammatory responses. During infection and chronic inflammation, IL-6 is produced by various types of cells, such as fibroblasts, macrophages, dendritic cells, T-cells, B-cells, keratinocytes, endothelial cells, mesangial cells, adipocytes and some tumor cells. Importantly, fibroblasts like other immune cells are a major source of IL-6. In chronic inflammation, fibroblasts are recognized as important regulators of inflammation through the production of IL-6 (Bernardo and Fibbe, 2013). In spite of the presence of several negative feedback mechanisms, constitutive overproduction of IL-6 is responsible for the pathogenesis of various inflammatory diseases, such as

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rheumatoid arthritis, systemic juvenile arthritis, and Crohn's disease (Kishimoto, 2010). Therefore, IL-6 signaling blockade with the humanized anti-IL-6 receptor antibody, tocilizumab, is a novel therapeutic strategy for various autoimmune and inflammatory diseases (Kishimoto, 2010).

Moreover, several studies have investigated the validity of using IL-6 for early sepsis diagnosis and predicting mortality (Viallon et al., 2000) (Gardlund et al., 1995) (Pettila et al., 2002). IL-6 has also been used as a prognostic marker for outcomes in septic patients, but it is still unknown whether sepsis-induced mitochondria dysfunction is associated with the production of IL-6.

Human p32/C1QBP/HABP has been cloned as a splicing factor 2-associated protein from human HeLa cells (Krainer et al., 1991). The p32 protein is a doughnut-shaped trimer, which primarily localizes to the mitochondrial matrix, but has also been reported to be present at the cell surface, in the nucleus and cytosol, as well as within secretory granules (Jiang et al., 1999) (Matthews and Russell, 1998) (van Leeuwen and O'Hare, 2001) (Soltys et al., 2000). We previously showed that p32 is synthesized as a pre-protein and is processed by proteolytic cleavage of the N-terminal amino acids containing the mitochondrial signal sequence (Muta et al., 1997). In addition, we reported that mitochondrial p32 is essential for mouse embryonic development dependent on mitochondrial translation (Yagi et al., 2012). Several previous siRNA studies of innate immunity suggest that p32 suppresses antiviral immune responses (Xu et al., 2009b) (Wang et al., 2014b) (Liu et al., 2015), but its molecular mechanisms are little elucidated. Much less is known about the role of p32 in antibacterial immunity.

Mitochondrial functions, such as mitochondrial membrane potential and mitochondrial reactive oxygen species (mROS), play important roles in innate antiviral immunity (Koshiba et al., 2011) (Tal et al., 2009) (Koshiba, 2013). On the other hand, many relationships between mitochondrial functions and sepsis remain to be revealed. In this paper, we present evidence that p32 is critically involved in TLR4-mediated IL-6 induction in vitro and in vivo. We have found that loss of mitochondrial translation due to a deficiency of p32 or the presence of inhibitors increases LPS-induced IL-6 overproduction via ATF4 activation in mouse embryonic fibroblasts (MEFs). Additionally, using a mice endotoxin shock model, we showed that p32 protects mice from endotoxin shock.

2. Material and Methods

2.1. Cell Preparation and Retrovirus–mediated Gene Transfer

MEFs were isolated from wild-type and p32 knockout mice as described previously (Yagi et al., 2012). MEFs were maintained at 37 °C and 5% CO2 in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, USA) supplemented with 10% FBS. To generate bone marrow-derived macrophages (BMMQs), bone marrow cells were cultured for 7 days with macrophage colony-stimulating factor (20 ng/mL; PeproTech, USA).

pSUPER retro puro GFP shRNA was a gift from John Gurdon (Addgene plasmid # 30519). The retroviral vector pSUPER retro puro were used to generate the plasmid encoding *GFP* shRNA or *Atf4* shRNA. The shRNA target sequences were: Mouse ATF4: 5′-AAGAGAAGGCAGATTCTCT-3′. The retroviral vector pMX was used to generate the plasmid encoding IRES–GFP (Empty), or *p32*–IRES–GFP. Retroviral transduction was done as previously described (Gotoh et al., 2010). These plasmids were transfected into Platinum-E packaging cells using FuGENE 6 transfection reagent (Roche Applied Science, Germany). The cell-culture supernatants were harvested 48 h after transfection, supplemented with polybrene (5 μg/mL; Sigma-Aldrich, USA), and used to infect MEFs and BMMQs. Before assay, two additional retroviral infections were performed at daily intervals.

2.2. Reagents

Actinomycin D, LPS, Rotenone, Antimycin, Oligomycin, Etomoxir and antibodies against β-actin were purchased from Sigma-Aldrich, USA, and Chloramphenicol were obtained from Wako Pure Chemicals, Japan. Actinonin was purchased from Enzo Life Sciences, Germany. Polyclonal antibodies against mouse p32 were raised in our laboratory. Antibodies against p38, phospho-p38, Erk1/2, phospho-Erk1/2, NFκB p65, phospho-NFκB p65, IκB, phospho-IκB, ATF4 were purchased from Cell Signaling Technology, USA. Anti-COX1 antibodies and anti-B23 antibodies were from Thermo Fisher scientific, USA. Total OHPHOS rodent antibodies cocktail were from Abcam, USA.

2.3. Real-time PCR

After treatment with RNase-free DNase I (QIAGEN, Germany), RNA samples were reverse transcribed PrimeScript™ RT Reagent Kit (TAKARA, Japan) according to the manufacturer's instructions. Quantitative real-time RT-PCR analysis was performed using specific primers (Supplementary Table S1). The expression of the genes was detected by qPCR with a thermal cycler (StepOne plus; Applied Biosystems).

MEFs were stimulated with LPS (100 ng/mL) for 3 h. Actinomycin D (10 μg/mL) was then added to cell culture medium to stop transcription. After incubated with actinomycin D, total RNAs were prepared at the indicated time periods.

2.4. Immunoblotting Analysis and Nuclear extraction

For direct immunoblotting, MEFs and macrophages were lysed with cell lysis buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl and 0.5% NP-40) and then subjected to immunoblotting using specific antibodies.

For nuclear extraction, MEFs were washed with cold Phosphate buffered saline (PBS). The cells were suspended in Hypotonic Buffer (10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 1 mM Dithiothreitol, and protease inhibitor cocktail). After incubation on ice for 15 min, the cells were added to the 0.6% IGEPAL CA-630 solution and vortexed vigorously for 10 s. Next, the cells were centrifuged at 10,000*g* for 30 s, and the supernatants were transferred to a fresh tube as cytosol fraction. The crude nuclei pellet was resuspended in extraction buffer (20 mM HEPES, pH 7.9, with 1.5 mM MgCl2, 0.42 M NaCl, 0.2 mM EDTA, 25% (v/v) Glycerol, 1 mM Dithiothreitol, and protease inhibitor cocktail), with intermittent vortexing for 15 min. Finally, cells were centrifuged for 5 min at 14,000*g* and the supernatant was transferred to a fresh tube as nuclear fraction.

2.5. Immunofluorescence Microscopy

After stimulation with LPS (100 ng/mL) at the indicated time periods, the cells were fixed with 4% paraformaldehyde/PBS for 10 min and permeabilized with 0.2% TritonX-100/PBS for 5 min. After being blocked with 1% bovine serum albumin (BSA)/PBS for 30 min, the cells were incubated with primary antibodies in 1% BSA/PBS for 1 h. Then, the cells were washed with PBS and incubated with Alexa 488 labeled anti-rabbit secondary antibody for 1 h. Cells were washed and Glass slides were mounted using Superfrost (Matsunami, Japan). Images were obtained using fluorescence microscope (BZ-9000, KEYENCE, Japan).

2.6. Mice

$p32^{flox/flox}$ mice have been described elsewhere (Yagi et al., 2012). *LysM-Cre* [B6.129P2-*Lyz2*$^{tm1(cre)Ifo}$/J] mice were purchased from The Jackson Laboratory, USA. Sex-matched $p32^{flox/flox}$/*LysM-Cre* + and littermate control $p32^{flox/flox}$/*LysM-Cre*- (wild type, WT) were used for all experiments in this study. Mice were kept under specific pathogen-free

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