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Suppression of TLR4-mediated inflammatory response by macrophage class A scavenger receptor (CD204)

Koji Ohnishi [a], Yoshihiro Komohara [a], Yukio Fujiwara [a], Kenichi Takemura [a], XiaoFeng Lei [a,b], Takenobu Nakagawa [a], Naomi Sakashita [a,c], Motohiro Takeya [a,*]

[a] Department of Cell Pathology, Graduate School of Medical Sciences, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan
[b] Department of Biochemistry, Showa University School of Medicine, Tokyo, Japan
[c] Department of Human Pathology, Institute of Health Biosciences, The University of Tokushima, Tokushima, Japan

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ABSTRACT

The class A scavenger receptor (SR-A, CD204), one of the principal receptors expressed on macrophages, has been found to regulate inflammatory response and attenuate septic endotoxemia. However, the detailed mechanism of this process has not yet been well characterized. To clarify the regulative mechanisms of lipopolysaccharide (LPS)-induced macrophage activation by SR-A, we evaluated the activation of Toll-like receptor 4 (TLR4)-mediated signaling molecules in SR-A-deficient (SR-A$^{-/-}$) macrophages. In a septic shock model, the blood levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6 and interferon (IFN)-β were significantly increased in SR-A$^{-/-}$ mice compared to wild-type mice, and elevated nuclear factor kappa B (NFκB) activation was detected in SR-A$^{-/-}$ macrophages. SR-A deletion increased the production of pro-inflammatory cytokines, and the phosphorylation of mitogen-activated protein kinase (MAPK) and NFκB *in vitro*. SR-A deletion also promoted the nuclear translocation of NFκB and IFN regulatory factor (IRF)-3. In addition, a competitive binding assay with acetylated low-density lipoprotein, an SR-A-specific ligand, and anti-SR-A antibody induced significant activation of TLR4-mediated signaling molecules in wild-type macrophages but not in SR-A$^{-/-}$ macrophages. These results suggest that SR-A suppresses the macrophage activation by inhibiting the binding of LPS to TLR4 in a competitive manner and it plays a pivotal role in the regulation of the LPS-induced inflammatory response.

1. Introduction

Macrophages are major innate immune cells that play an integral role in the immune responses of host defense against microbial infection. Macrophages are activated by recognition of microbial molecules by Toll-like receptors (TLRs), and up-regulates the host immune response. Bacterial lipopolysaccharide (LPS, endotoxin) is a strong stimulant for macrophage activation, and induces augmented pro-inflammatory cytokine production by macrophages via TLR4-responses, causing septic shock [1,2].

The class A scavenger receptor (SR-A, CD204) is one of the principal macrophage receptors which recognizes microbial molecules, including LPS, similar to TLRs [3–5]. SR-A is a type II trimeric transmembrane glycoprotein, and its extracellular collagenous domain can bind not only LPS but also various negatively-charged macromolecules such as modified low-density lipoprotein (mLDL), fucoidan, and fragmentary apoptotic cells [4–7]. We previously showed that SR-A attenuates myocardial injury after infarction through suppression of tumor necrosis factor (TNF)-α production from tissue macrophages [8], and SR-A inhibits nitric oxide production in macrophages to regulates hyperoxia-induced lung injury [9] and to promote EL4 lymphoma development [10]. Previous studies have demonstrated that SR-A-deficient (SR-A$^{-/-}$) mice are more susceptible to LPS-induced endotoxic mortality via overproduction of TNF-α and interleukin (IL)-6 [11,12]. Seimon et al. demonstrated that SR-A inhibits apoptosis in endoplasmic reticulum-stressed macrophages due to suppression of the TLR4-mediated interferon regulatory factor (IRF)-3/interferon (IFN)-β pathway [13]. These studies indicated that SR-A suppresses TLR4-mediated macrophage activation; however, the detailed mechanism is unclear. Many researchers inferred a possible mechanism, the binding of SR-A with LPS induces intracellular anti-inflammatory signals that regulate TLR4-mediated pro-inflammatory responses, although little evidence supports the notion of SR-A-mediated signals [14–19]. In this study, we investigated the differences of LPS/TLR4-induced signaling using SR-A$^{-/-}$ mice to elucidate the mechanisms involved in the SR-A regulation of TLR4-mediated macrophage activation.

* Corresponding author. Address: Department of Cell Pathology, Graduate School of Medical Sciences, Faculty of Life Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan. Fax: +81 96 373 5096.
E-mail address: takeya@kumamoto-u.ac.jp (M. Takeya).

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2. Materials and methods

2.1. Animals

SR-A$^{-/-}$ mice with C57BL/6J genetic background were kindly provided by Kodama and Suzuki [20]. Control wild-type (WT) mice (C57BL/6J background and Balb/c background) were obtained from Clea Japan (Tokyo, Japan). All mice were maintained under specific-pathogen-free conditions in Animal Resource Facility at the Kumamoto University. All animal procedures were approved by the Animal Research Committee at Kumamoto University.

2.2. LPS-injected septic shock model

8- to 10-week-old male WT (C57BL/6J) or SR-A$^{-/-}$ mice were treated with LPS from *Escherichia coli* O111:B4 (10 μg/g body weight) (Sigma–Aldrich, St. Louis, MO, USA) or an equal volume of sterile saline via intraperitoneal injection. The mice were monitored to assess survival for a period of up to 3 days. For cytokine measurement, blood samples were drawn at 8 h after injection.

2.3. Immunohistochemistry

The spleen tissue specimens were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Deparaffinized sections were immersed in 0.3% hydrogen peroxide solution and then were subjected to microwave pretreatment with pH 6.0 citrate buffer for anti-mouse macrophage antibody, F4/80 (Serotec, Oxford, UK) staining or with pH 8.0 ethylenediaminetetraacetic acid for phospho-nuclear factor kappa B (pNFκB) p65 staining. Isotype-matched rat or rabbit IgG were used as negative controls. After the reaction of each primary antibody, samples were incubated with horseradish peroxidase (HRP)-labeled goat anti-rat or goat anti-rabbit antibodies (Nichirei, Tokyo, Japan). The immunoreactions were visualized by using ImmPACT diaminobenzidine substrate kit (Vector Laboratory, Burlingame, CA, USA). For double immunostaining, an alkaline phosphatase-conjugated antibody (Nichirei) was used as the secondary antibody and the reaction was visualized by using Fast Red TR salt (Sigma).

2.4. Macrophage culture

For the bone marrow-derived macrophage (BMDM) cultures, femurs and tibias were excised from mice and cleaned of soft tissue. Briefly, marrow was flushed with sterilized PBS and erythrolysation was performed. Cells were seeded in polystyrene culture dishes with RPMI 1640 medium (Wako, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Wako). Cells were incubated in medium containing 20 ng/ml recombinant murine macrophage colony-stimulating factor (M-CSF; PeproTech, Rocky Hill, NJ, USA) at 37 °C/5% CO_2. Five days later, the dishes were rinsed carefully with sterilized PBS to remove non-adherent cells, and were incubated in serum-free medium for 24 h to prevent various macromolecules present in FBS that might bind SR-A [21]. For blocking the ligand-binding sites of SR-A, BMDMs were treated with 20 μg/ml acetylated LDL (AcLDL) in the presence of LPS. For the SR-A-blocking study, BMDMs from Balb/c mice were treated with 20 μg/ml rat anti-mouse SR-A antibody, 2F8 (Serotec) or control rat IgG2b (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min prior to administration of LPS (100 ng/ml).

2.5. Enzyme-linked immunosorbent assay (ELISA)

For cytokine measurement, a culture supernatant of macrophages and mouse serum were determined using mouse TNF-α, IL-6 (eBioscience, San Diego, CA, USA) or IFN-β ELISA kits (PBL, Piscataway, NJ, USA) according to the manufacturers' protocols.

2.6. Western blot analysis

Samples were run on 10% SDS–PAGE gels and then transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were incubated in 2% globulin-free bovine serum albumin (Wako) blocking buffer for 1 h, and then incubated with anti-phospho-extracellular signal regulated kinase (pERK) 1/2, anti-phospho-p38, anti-I-kappa B-alpha (IκBα) (all from Cell Signaling Technology, Danvers, MA, USA), with anti-phospho-c-Jun N-terminal kinase (pJNK) 1/2, anti-Lamin (both from Abcam, Cambridge, MA, USA), anti-TLR4, anti-phospho-NFκB p65, anti-β-actin, anti-NFκB, and anti-IRF-3 antibodies (Santa Cruz). After rinses with TBS containing 0.1% Tween 20, the membranes were incubated with the appropriate HRP-conjugated secondary antibody (GE Healthcare, Buckinghamshire, UK). The reaction was visualized with the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL, USA).

2.7. Nuclear extract preparation

Nuclear extracts were prepared using a NucBuster protein extraction kit (Novagen, Darmstadt, Germany) according to the manufacturer's protocol.

2.8. Statistical analysis

Data were expressed as the means ± SD. Mann–Whitney's *U*-test was used for two-group comparisons. The cumulative survival rate was compared between two groups using Kaplan–Meier survival analysis and generalized Wilcoxon test. A p-value of less than 0.05 was considered statistically significant. All data were representative of at least three independent experiments.

3. Results

3.1. SR-A deletion promotes pro-inflammatory cytokine production and results in high mortality against septic shock

To reconfirm previous septic shock model studies in SR-A$^{-/-}$ mice [11,12], a LPS-induced experimental septic shock was induced in WT and SR-A$^{-/-}$ mice. Consistent with previous reports, the results revealed a significantly higher mortality rate in SR-A$^{-/-}$ mice with augmented production of TNF-α, IL-6 and IFN-β (Fig. 1A and B). The LPS injection induced swelling of spleen and an immunohistochemical analysis revealed that the accumulation of macrophages in enlarged splenic red pulp was considerably increased after LPS-injection. Although their number was not different between WT mice and SR-A$^{-/-}$ mice, the intensity of nuclear staining of pNFκB p65 in macrophages significantly increased in the SR-A$^{-/-}$ mice after LPS-injection (Fig. 1C). Positive staining of pNFκB p65 was detected in the nuclei of most of F4/80-positive macrophages by double immunostaining (Fig. 1D).

3.2. SR-A deletion increases TNF-α, IL-6 and IFN-β production in vitro

We compared the LPS-induced production of TLR4-mediated cytokines such as TNF-α, IL-6 and IFN-β in cultured macrophages of WT and SR-A$^{-/-}$ mice. Although the expression of TLR4 was