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Macrophage C-type lectin is essential for phagosome maturation and acidification during *Escherichia coli*-induced peritonitis

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ABSTRACT

Sepsis is a life-threatening condition caused by an uncontrolled response to bacterial infection. Impaired bactericidal activity in the host is directly associated with severe sepsis; however, the underlying regulatory mechanism(s) is largely unknown. Here, we show that MCL (macrophage C-type lectin) plays a crucial role in killing bacteria during *Escherichia coli*-induced peritonitis. MCL-deficient mice with *E. coli*-induced sepsis showed lower survival rates and reduced bacterial clearance when compared with control mice, despite similar levels of proinflammatory cytokine production. Although the ability of macrophages from MCL-deficient mice to kill bacteria was impaired, they showed normal phagocytic activity and production of reactive oxygen species. In addition, MCL-deficient macrophages showed defective phagosome maturation and phagosomal acidification after *E. coli* infection. Taken together, these results indicate that MCL plays an important role in host defense against *E. coli* infection by promoting phagosome maturation and acidification, thereby providing new insight into the role of MCL during pathogenesis of sepsis and offering new therapeutic options.

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1. Introduction

Sepsis is a severe clinical condition caused by an overwhelming systemic inflammatory response to infection by bacteria or viruses. A high burden of infection, the presence of virulent factors, and resistance to opsonization and phagocytosis lead to progression of sepsis and triggers a ‘cytokine storm’: the secretion of large amounts of proinflammatory cytokines by immune cells [1]. Mortality rates associated with severe sepsis range from 20% to 50%, and currently there are no effective therapies [2]. However, some studies show that septic patients mount weak bactericidal immune responses, suggesting that boosting host immunity might increase survival [3,4].

The innate immune system is the first line of defense against bacterial infection. Early in the immune response, resident macrophages phagocytose pathogens [5]. Since nascent phagocytic vacuoles lack the ability to eliminate engulfed pathogens, the phagosomes subsequently acquire killing capability by fusing with endosomes, a process called “phagosome maturation” [4,6]. Phagosomes ultimately fuse with lysosomes to kill internalized microorganisms [6]. Because the process of phagosome maturation is connected to acquisition of an arsenal of oxidative, acidifying, and hydrolytic enzymes, it is important to ensure normal phagolysosome fusion to eradicate bacteria.

C-type lectin receptors (CLRs) are a large family of carbohydrate recognition domains (CRD) that act as pattern recognition receptors (PRRs) for pathogens; in this sense, they are similar to Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and NOD-like receptors (NLRs) [7]. Among the CLRs, MCL (macrophage C-type lectin, also called Clec4d, Clecsf8, and Dectin-3) is a type II transmembrane protein with a single extracellular CRD; MCL triggers intracellular signals by coupling with ITAM-bearing Fc receptor γ

Abbreviations: MCL, macrophage C-type lectin; CLRs, C-type lectin receptors; CRD, carbohydrate recognition domains; PRRs, pattern recognition receptors; TLRs, Toll-like receptors; FcRγ, Fc receptor γ; TDM, trehalose dimycolate; *E. coli*, *Escherichia coli*; BMDMs, bone marrow-derived macrophages; ROS, reactive oxygen species; LPS, lipopolysaccharide.

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(FcRγ) [8]. A recent study shows that MCL is an activating receptor that recognizes mycobacterial cord factor trehalose dimycolate (TDM) [9]. Initial recognition by MCL drives expression of Mincle, an inducible receptor that recognizes TDM. Moreover, MCL can form a receptor complex with Mincle to promote its surface expression, which boosts Mincle-mediated signaling [10]. Knockout mouse models of infection reveal that MCL plays a protective role against infection by *Klebsiella pneumoniae*, *Mycobacterium tuberculosis*, *Mycobacterium bovis,* and *Cryptococcus neoformans* [11–13]. Although MCL likely plays a role in regulating immune responses during pathogenic conditions, its direct role in bacterial sepsis is largely unknown.

Here, we examined whether deficiency of MCL impacts the outcome of *Escherichia coli* (*E. coli*)-induced peritoneal sepsis. MCL-deficient mice with a high bacterial burden in peritoneal lavage fluid exhibited increased susceptibility to *E. coli*-induced septic peritonitis. We then investigated the bactericidal activity of MCL and found defective bacterial killing by MCL$^{-/-}$ bone marrow-derived macrophages (BMDMs). In addition, MCL$^{-/-}$ macrophages showed defective phagosome maturation and phagolysosomal acidification after internalization of *E. coli*. Taken together, these data suggest that MCL plays a role in phagosome maturation and acidification during immune responses to *E. coli*-induced peritoneal sepsis.

2. Materials and methods

2.1. Mice

MCL$^{-/-}$ mice (Clec4dMCA) were kindly provided by the Consortium for Functional Glycomics (http://www.functionalglycomics.org) and were back-crossed for over ten generations to a C57BL/6 background. All mice were maintained in the specific pathogen-free facility at the Laboratory Animal Research Center at Yonsei University. Animals were maintained and all procedures performed with the approval of the relevant IACUCs of Yonsei University (permit number: IACUC-A-201509-463-02) in accordance to LABORATORY ANIMAL ACT of Korean Ministry of Food and Drug Safety for enhancing the ethics and reliability of animal testing through appropriate administration of laboratory animals and animal testing.

2.2. E. coli-induced peritonitis model

Wild-type (WT) and knockout mice received an intraperitoneal injection of a bacterial suspension containing 3×10^8 live *E. coli* cells (strain DH5-α, Invitrogen). Survival rate was monitored daily for up to 4 days after *E. coli* injection. To count bacteria, peritoneal lavage fluid was collected 24 h after initiation of *E. coli*-induced peritonitis [14]. Samples were serially diluted, plated on Luria-Bertani agar medium (Difco Laboratories), and incubated for 24 h at 37 °C. Bacteria were enumerated by counting the number of colony-forming units (CFU).

2.3. Enzyme-linked immunosorbent assay (ELISA)

Secretion of TNF-α, IL-6, MCP-1, IL-10, IL-12p40, and IFNγ secretion in culture supernatants of BMDMs was measured using Cytometric Bead Arrays (CBA; Mouse Inflammation Kit; BD Biosciences), according to the manufacturer's instructions.

2.4. Cell culture

BMDMs were prepared by isolating bone marrow cells from both femurs and tibiae of mice. Isolated cells were cultured for 7 days in 20% (v/v) L929 (ATCC) culture supernatant in basic Dulbecco's modified Eagle's medium (DMEM; ThermoFisher Scientific) supplemented with 20% fetal bovine serum (FBS; Gibco/ThermoFisher Scientific), 50 U/ml penicillin, and 50 mg/ml streptomycin (Invitrogen).

2.5. Immunoblotting

BMDMs derived from WT and knockout mice were lysed with 2 × Laemmli buffer containing a phosphatase inhibitor cocktail (Roche). Equal amounts of protein were analyzed by immunoblotting with anti-p38 (Cell signaling), anti-phospho-p38 (Cell signaling), anti-JNK (Cell signaling), anti-phospho-JNK (Cell signaling), anti-Erk1/2 (Sigma), anti-phospho-Erk1/2 (Sigma), anti-IκB (Sigma) using standard protocols. Signals were developed with Amersham ECL reagents and detected using the ImageQuant LAS 4000 system (GE Healthcare).

2.6. Measurement of E. coli phagocytosis and killing

To measure phagocytosis, fluorescein-labeled *E. coli* were opsonized with normal mouse IgG, mixed with BMDMs at a ratio of 1:100 (cells:*E. coli*), and rotated at 37 °C for the indicated times. Unbound *E. coli* were removed by washing three times with cold PBS. BMDMs were fixed with 3.7% paraformaldehyde and cell surface-bound bacteria quenched with trypan blue (2 mg/ml). Fluorescence was then measured in a flow cytometer (FACS Calibur; BD Biosciences). To measure intracellular bacterial killing, *E. coli* (DH5-α) were opsonized by incubation with commercial IgG for 0.5 h at 37 °C, followed by incubation with BMDMs (10^7 bacteria per 5×10^5 cells) at 37 °C for 30 min. Unbound bacteria were removed by differential centrifugation and the pellets resuspended in gentamicin solution (100 mg/ml) for 1 h at 37 °C to kill remaining extracellular bacteria. Next, cells were either lysed with 0.5% Triton X-100 by incubation on ice for 10 min or by incubation at 37 °C for 60 min before lysis under the same conditions. Surviving *E. coli* were cultured on selective agar plates and counted as described above. The experiment was performed more than three times.

2.7. Measurement of reactive oxygen species (ROS) production

BMDMs (1×10^5) were seeded into each well of a luminometer plate. Before the assay, cells were washed once with warm Hanks Balanced Salt Solution (HBSS). Next, 200 μl of HBSS containing 100 μM luminol and 5 U of horseradish peroxidase (Sigma) were added to each well for 10 min at 37 °C, followed by stimulation with *L. monocytogenes* (MOI, 10; ATCC), *E. coli* (MOI, 20; ATCC), or *C. albicans* (MOI, 2; ATCC). ROS production was monitored in a luminometer every 2–5 min for 2 h.

2.8. Analysis of phagosome maturation

BMDMs were plated on round 12-mm glass coverslips in 24-well tissue culture plates (2×10^5 cells/coverslip) and incubated overnight. Next, lysosomes were labeled for 30 min with 100 nM LysoTracker Red (Invitrogen). Cells were then washed three times with PBS. Next, opsonized *E. coli* labeled with fluorescein (Molecular Probes) were added at a cell:*E. coli* ratio of 1:50. At each time point, coverslips were washed vigorously five times with cold PBS and fixed in 3.7% paraformaldehyde (Sigma). After fixation, the coverslips were removed, mounted onto slides, and examined under a LSM 700 (Carl Zeiss) confocal microscope. Colocalization coefficients were calculated using Zen 2010 analysis software (Carl Zeiss) after randomly scanning ten cells in each test group (n = three independent experiments).

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