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Innate signaling mechanisms controlling *Mycobacterium chelonae*-mediated CCL2 and CCL5 expression in macrophages

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Mycobacterium chelonae **(Mch) is an atypical rapidly growing mycobacterium (RGM) that belongs to the** ***M. chelonae*** **complex, which can cause a variety of human infections. During this type of mycobacterial infection, macrophage-derived chemokines play an important role in the mediation of intracellular communication and immune surveillance by which they orchestrate cellular immunity. However, the intracellular signaling pathways involved in the macrophage-induced chemokine production during Mch infections remain unknown. Thus, the present study aimed to determine the molecular mechanisms by which Mch activates the gene expressions of chemokine (C-C motif) ligand 2 (CCL2) and CCL5 in murine bone marrow-derived macrophages (BMDMs) and** ***in vivo*** **mouse model. Toll-like receptor 2 (TLR2)-deficient mice showed increased bacterial burden in spleen and lung and decreased protein expression of CCL2 and CCL5 in serum. Additionally, Mch infection triggered the mRNA and protein expression of CCL2 and CCL5 in BMDMs via TLR2 and myeloid differentiation primary response gene 88 (MyD88) signaling and that it rapidly activated nuclear factor (NF)-κB signaling, which is required for the Mch-induced expressions of CCL2 and CCL5 in BMDMs. Moreover, while the innate receptor Dectin-1 was only partly involved in the Mch-induced expression of the CCL2 and CCL5 chemokines in BMDMs, the generation of intracellular reactive oxygen species (ROS) was an important contributor to these processes. Taken together, the present data indicate that the TLR2, MyD88, and NF-κB pathways, Dectin-1 signaling, and intracellular ROS generation contribute to the Mch-mediated expression of chemokine genes in BMDMs.**

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Introduction

Nontuberculous mycobacteria (NTM), which are also referred to as mycobacteria other than tuberculosis, are mainly inhabitants of natural environments (Falkinham, 2009) but are emerging as important human pathogens that are frequently identified as infectious agents in the lungs and skin/soft tissues (Cassidy *et al.*, 2009). NTM species are largely heterogeneous and the NTM pathogens related to pulmonary diseases are categorized into three main groups: the *Mycobacterium avium (M. avium)* complex, *M. kansasii*, and the *M. chelonae* (Mch) complex (Alvarez-Uria, 2010). Mch is an example of rapid growing mycobacteria (RGM) due to its ability to grow on Standard Methods agar in culture within 1 week (Ringuet *et al.*, 1999; Chan *et al.*, 2010). Of the various NTM, the Mch complex represent important human pathogens in immunocompetent and immunocompromised hosts due in part to the fact that they are closely related (Ringuet *et al.*, 1999; Alvarez-Uria, 2010; Chan *et al.*, 2010).

Innate immune responses are crucial components of early surveillance during anti-mycobacterial host defense following infection with NTM and mycobacteria (Chan *et al.*, 2010; Kim *et al.*, 2014a; Mortaz *et al.*, 2015). For example, chemokines participate in the migration of leukocytes and macrophages to the site of mycobacterial infection and, in turn, modulate various aspects of host defense and immunopathology (Dorhoi *et al.*, 2014; Torraca *et al.*, 2015). During *M. tuberculosis* infection, various chemokines including chemokine (C-C motif) ligand 2 (CCL2), CXCL3, CXCL5, and CXCL8, are produced by alveolar epithelial cells and human bronchial epithelial cells, which results in the formation of granulomas and activation of adaptive immunity (Monin and Khader, 2014). Polymorphisms of both the CCL2 and CCL5 genes are associated with susceptibility to human tuberculosis (Alqumber *et al.*, 2013; Gong *et al.*, 2013). Moreover, *M. tuberculosis*-induced CCL2 expression is highly enhanced in peripheral blood mononuclear cells isolated from patients with pulmonary tuberculosis (PTB) as opposed to patients with extrapulmonary TB (Hasan *et al.*, 2009). Additionally, expression of CCL5 in bronchoalveolar lavage fluid of patients with active PTB is increased relative to that in healthy control subjects (Sadek *et al.*, 1998). Although previous studies have shown that chemokines are a double-edged sword as they exert both protective effects in terms of bacterial elimination and lead to the progression of pulmonary dis-

eases, it remains unclear whether chemokines play a role in Mch-mediated innate immune responses, and the underlying molecular mechanisms are unknown.

Of the several classes of innate immune receptors, membrane-bound Toll-like receptor (TLR) 2 is one of the most characterized pattern-recognition receptors involved in NTM infections (Yim *et al.*, 2008). Early studies demonstrated that lipomannans from Mch robustly activate the mRNA expression and protein production of tumor necrosis factor (TNF)-α and interleukin (IL)-8 from human monocytic THP-1 cells (Vignal *et al.*, 2003). Previous studies have also shown that TLR2 and Dectin-1 are integrally involved in the *M. abscessus*-induced generation of proinflammatory cytokines and innate immune responses (Shin *et al.*, 2008) and that the induction of the nuclear factor (NF)-κB signaling pathway by TLR2 is required for the *M. abscessus*-mediated expression of CCL2, but not C-X-C motif ligand 2 (CXCL2), in macrophages (Kim *et al.*, 2012). Moreover, intracellular reactive oxygen species (ROS) play a crucial role as secondary messengers during the activation of innate immune responses following mycobacterial infection (Lee *et al.*, 2009a; Yang *et al.*, 2009). However, the intracellular and molecular mechanisms by which Mch modulates the innate immune responses of its host remain largely unknown compared to major human pathogens such as *M. tuberculosis* and other RGM species such as *M. abscessus*.

Thus, the present study examined the molecular signaling pathways that govern the gene expressions of CCL2 and CCL5, which are activated by the infection of murine bone marrow-derived macrophages (BMDMs) with Mch. The present results showed that Mch infection led to a robust increase in the mRNA levels of CCL2 and CCL5 and in TNF-α expression in BMDMs. The roles of TLR2, myeloid differentiation primary response gene 88 (MyD88), and Dectin-1 as well as the effects of NF-κB signaling and intracellular ROS generation during the Mch-mediated expressions of CCL2 and CCL5 in BMDMs were also examined.

Materials and Methods

Bacterial strains and culture conditions

Mch ATCC (35749) was obtained from The Korean Institute of Tuberculosis (Osong, Korea), maintained in Ogawa medium, and was grown in Middlebrook 7H9 broth (Difco Laboratories) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) (BD Pharmingen), 0.2% glycerol, and 0.05% Tween 80 (Sigma-Aldrich) at 32°C. The number of colony-forming units (CFU)/ml was determined on 7H10 agar supplemented with OADC at 32°C.

Mice and cells

Wild-type (WT) 6-to-8-week-old female C57BL/6 mice were purchased from SAMTAKO BIO KOREA. TLR2 and MyD88 knockout (KO) mice were kindly proved from Dr S. Akria (Osaka University, Japan). This study was approved by the Institutional Research and Ethics Committee at Chungnam National University (CNUH-014-A0008). All animal procedures were conducted in accordance with the guidelines of the Korean Food and Drug Administration (KFDA). BMDMs were isolated and then differentiated by growth for 5–7 days in medium containing M-CSF (25 μg/ml; R&D) as described previously (Yuk *et al.*, 2011).

Chemicals and reagents

Lipopolysaccharides (LPS; *Escherichia coli* 0111:B4) and synthetic bacterial lipopeptide (Pam3Cys-Ser-Lys4-OH) were from Invivogen. N-acetylcysteine (NAC), diphenyleneiodonium (DPI), BAY11-7082 (BAY), caffeic acid phenethyl ester (CAPE) were from Calbiochem. 4,5-Dihydroxy-1,3-benzene disulfonic acid disodium salt (Tiron) and dimethyl sulfoxide (DMSO; added to the cultures at 0.05% [v/v] as a solvent control) were from Sigma-Aldrich. Anti-β-actin (SC-1616) and anti-IκBα (SC-371) were purchased from Santa Cruz Biotechnology. All other reagents were obtained from Sigma-Aldrich, unless otherwise indicated

Cell counting kit (CCK)-8

The cytotoxic effects and cell viability of BMDMs were determined by using the CCK8 assay (Dojindo Molecular Technologies) according to the manufacture's protocol. Briefly, BMDMs were seeded on the 96-well culture plate and then infected with Mch at 37°C for indicated time. CCK8 solution (10 μl) was then added and incubated for 1 h at 37°C. Absorbance was measured at 450 nm. All experiments were performed in triplicate and cell viability was calculated as a percentage of control cells.

Mch infection and bacterial counts *in vivo*

Mice were inoculated with Mch (1×10^8 CFU in 0.1 ml PBS) by intravenous injection into lateral tail vein as described previously (Rottman *et al.*, 2007). Three mice per group were sacrificed at indicated periods and bacteria in lung, liver, and spleen homogenates were counted. For bacterial counting, numbers of viable bacteria in each organ were determined by plating serial dilutions of whole organ homogenates on Middlebrook 7H10 agar supplemented with ODAC (Difco). Colonies were counted after 4 days of incubation at 32°C and the results were calculated as the mean Log_{10} CFU/organ. Control mice were injected with sterile saline.

Reverse transcriptase (RT)-PCR

For semi-quantitative RT-PCR analysis, total RNA was extracted from cells using TRIzol (Invitrogen) as described previously (Yuk *et al.*, 2011). Primer sequences were as follows: *Tnf* (forward: 5′-CGGACTCCGCAAAGTCTAAG-3′, reverse: 5′-ACGGCATGGATCTCAAAGAC-3′), *Ccl2* (forward: 5′-ACTCAAGCCAGCTCTCTCTT-3′, reverse: 5′-TTCCTTCTTGGGGTCAGCAC-3′), *Ccl5* (forward: 5′-CCTCACCATCCTCACTG-3′, reverse: 5′-TCTTCTCTGGGTTGGCACAC-3′), *Actb* (forward: 5′-CCACCATGTACCCAGGCATT-3′, reverse: 5′-AGGGTGTAAAACGCAGCTCA-3′). The *Tnf*, *Ccl2*, *Ccl5*, and *Actb* annealing were performed 56°C for 40 sec. RT-PCR products were separated on 1.2% agarose gel and visualized by stained with ethidium bromide.

Enzyme-linked immunosorbent assay (ELISA) and Western blot analysis

For evaluating generation of mouse CCL2 and CCL5 protein, cell culture supernatants or mouse serum were collected and then analyzed using BD OptEIA ELISA Kit (BD Biosciences; mouse CCL2/MCP-1) and DuoSet ELISA kit (R&D System; mouse CCL5/RANTES). Sandwich ELISA assays were performed according to the manufacturer's protocol. Cytokine concentrations in the each sample were calculated using standard curves obtained from dilution of standard protein. For Western blot analysis, Cell lysis was performed with RIPA buffer (50 mM Tris-HCl; pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% deoxycholate, and protease inhibitors) at 4°C for 60 min. The protein extracts were boiled in SDS sample buffer and then subjected to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). There membranes were blocked with 5% non-fat milk. After incubation with specific primary antibody, horseradish peroxidase-conjugated secondary antibody was applied. The membranes were visualized by ECL solution (Millipore) and detected by UVitec Alliance mini chemiluminescence (UVitec).

Generation and transduction of small hairpin RNA (shRNA)

Gene silencing was achieved using shRNAs for the genes of interest, as described previously (Yuk *et al.*, 2009). The lentiviral construct vector of pLKO.1 and three packaging plasmids (pMDLg/pRRE, pRSV-Rev, and pMD2.VSV-G) were from Open Biosystems and sh*Clec7a*; *Dectin-1* (TRCN0000066928, TRCN0000066929, TRCN0000066930, TRCN0000066931, TRCN0000066932) were from Sigma-Aldrich as glycerol stocks. Lentiviruses were produced by transient transfection using packaging plasmids after Lipofectamine 2000-mediated transient transfection into HEK 293T cells After 72 h, the virus-containing HEK 293T cell culture supernatants were collected and filtered. Lentivirus determination was performed as described previously (Yuk *et al.*, 2009). The lentivirus particle mixed with 8 μg/ml polybrene (Sigma-Aldrich) and nonspecific shRNA (sh*NS*) or dectin-1 shRNA (sh*Dec-1*) into BMDMs, according to the manufacturer's instructions. After transduction, the BMDMs were harvested and the target gene-silencing efficiency was examined by RT-PCR analysis.

Measurement of intracellular ROS

Intracellular ROS levels were measured as described previously (Yang *et al.*, 2012). Intracellular ROS generation was measured with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA; Calbiochem) for 30 min and followed by analysis with a FACS Canto II flowcytometer (Becton-Dickinson). Analysis of 10,000 events/sample was quantified by FlowJo software (Tree Star).

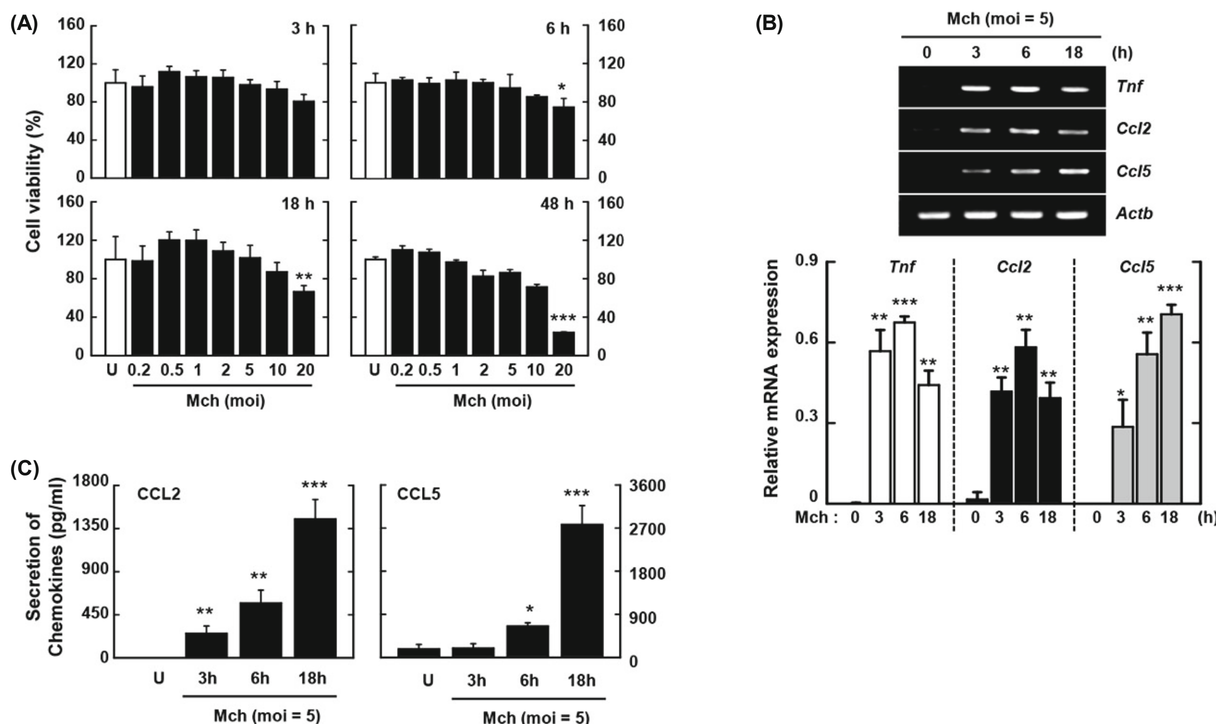

Fig. 1. Mch activates the mRNA and protein expression of chemokines such as CCL2 and CCL5 (A) BMDMs were infected with Mch (MOI from 0.2 to 20) for indicated times. Cell viability was assessed by CCK8 assay. (B and C) BMDMs were infected with Mch (MOI: 5) for the indicated times (0–18 h). (B) Cell lysates were collected and then subjected to semiquantitative RT-PCR (Top) for *Tnf*, *Ccl2*, and *Ccl5*. The densitometry values of the mRNA levels were normalized to those of β-actin (Bottom). A gel representative of three independent replicates with similar results is shown. (C) Culture supernatants were harvested to measure secretion of CCL2 and CCL5 protein using ELISA. The results are shown as the means ± SD of three independent experiments. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 (Student's *t*-test) compared to control cultures. U, uninfected.

Immunofluorescence microscopy of NF-κB p65 translocation

Translocation of NF-kB p65 into the nucleus was detected using immunofluorescence staining as previously described (Yuk *et al.*, 2011). Briefly, cells were infected with Mch for 30 min and then fixed with 4% paraformaldehyde in PBS for 10 min. Cells were permeabilized with 0.25% Triton X-100 in PBS for 10 min and stained with primary Ab (rabbit anti-mouse NF-κB p65, 1:400, for 18 h at 4℃; SantaCruz) and secondary Ab (anti-rabbit AlexaFluro 488, 1:400, for 2 h, Invitrogen) at RT. Nuclei were stained by incubated with DAPI (Sigma) for 3 min. After mounting, fluorescence images were acquired using a confocal laser-scanning microscope (LSM 710; Zeiss).

Luciferase reporter assays

NF-κB luciferase reporter assay was performed as described previously (Yuk *et al.*, 2011). Briefly, transduction with Adenovirus of NF-κB-Luciferase (Genetransfer Vector Core) for 36 h, and infected with Mch for 6 h. Infected cells were washed three times in PBS, and cell extracts were prepared by adding 100 μl of 1× Passive Reporter Lysis Buffer (Promega) Luciferase activity was measured using the Luciferase Assay System (Promega), according to the manufacturer's instructions.

Statistical analyses

All data were analyzed by Student's *t*-test with Bonferroni adjustment or ANOVA for multiple comparisons and are presented as the means ± SD. Differences were considered significant at $P < 0.05$.

Results

Mch infection up-regulates CCL2 and CCL5 production in macrophages

We first assessed the viability of BMDMs at the indicated time points after infection with Mch at various multiplicities of infection (MOI). As compared to uninfected controls, the viability of BMDMs infected with Mch (MOI: 20) was decreased significantly at 6–48 h (Fig. 1A). Using a MOI

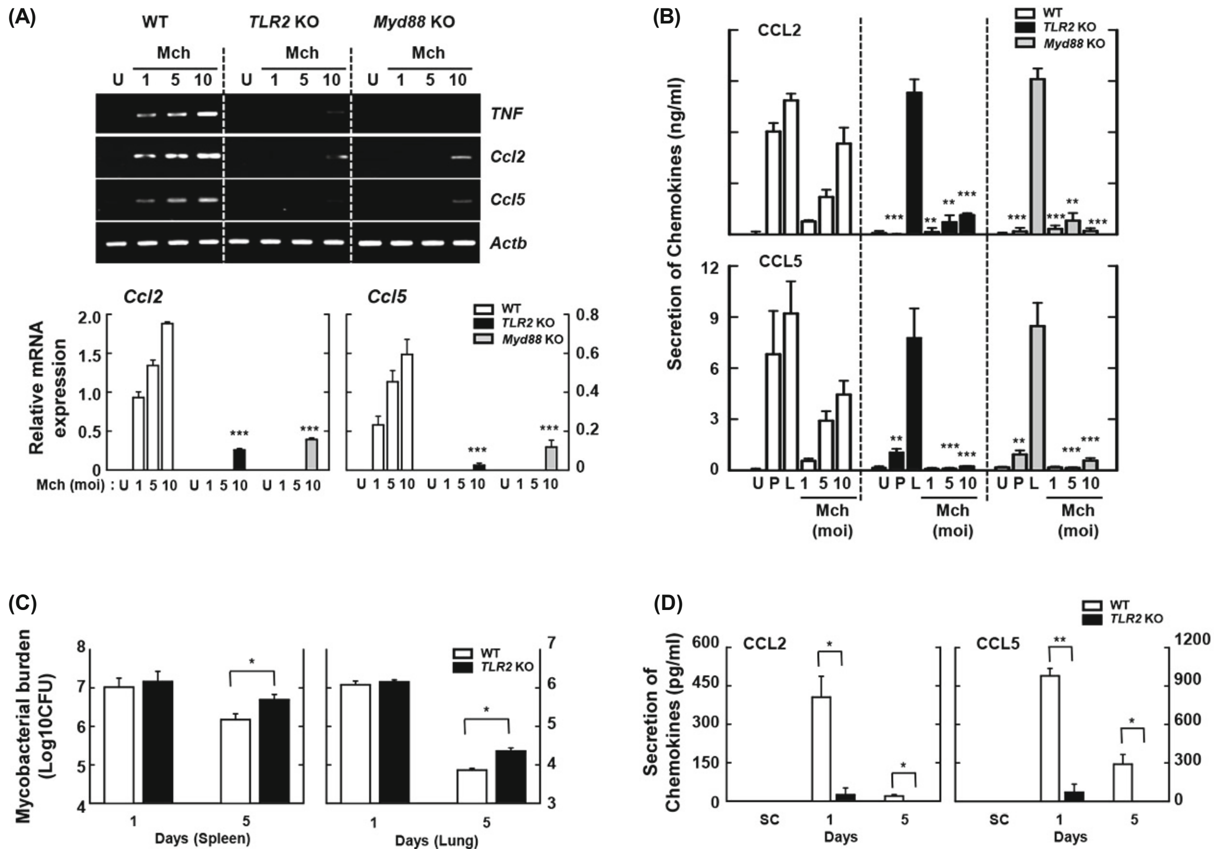

Fig. 2. TLR2 plays crucial roles in Mch-induced CCL2 and CCL5 production of macrophages and regulation of Mch growth *in vivo*. (A and B) BMDMs from WT, *Tlr2*-KO, and *Myd88*-KO mice infected with Mch (MOI: 5) for 6 h (for A) or 18 h (for B). (A) Cell lysates were collected and then subjected to semiquantitative RT-PCR (Top) for *Tnf*, *Ccl2*, and *Ccl5*. The densitometry values of the mRNA levels were normalized to those of β-actin (Bottom). A gel representative of three independent replicates with similar results is shown. (B) Culture supernatants were harvested and CCL2 and CCL5 expression level were measure by ELISA. (C and D) C57BL/6 WT or TLR2-KO mice (n = 3 per group) were intravenously infected with 1×10^8 CFU of Mch or treated with saline (SC) for the indicated time periods. (C) The mycobacterial loads of the lung and spleen tissues were determined at 1 and 5 days post-infection. (D) CCL2 and CCL5 production in serum were measured using ELISA assay. The results are shown as the means ± SD of three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (Student's *t*-test) compared to control cultures. U, uninfected. SC, distilled saline.

of 5, we next examined the expression pattern of CCL2 and CCL5 in macrophages. We found that Mch infection (MOI: 5) robustly activated the mRNA expressions of CCL2 and CCL5 as well as of TNF-α in BMDMs in a time-dependent manner (Fig. 1B). The Mch-mediated TNF-α and CCL2 expressions peaked at approximately 3–6 h after infection while CCL5 expression peaked at approximately 18 h after infection (Fig. 1B). Moreover, the secretion of CCL2 and CCL5 proteins into culture supernatants was increased by infection with Mch in a time–dependent manner (Fig. 1C).

TLR2 and MyD88 are required for CCL2 and CCL5-mediated host protection against Mch infection

TLR2 and MyD88 are key factors in the innate immune signaling and inflammatory responses that occur following infection with *M. tuberculosis* and atypical mycobacteria (Jo *et al.*, 2007; Shin *et al.*, 2008; Yuk and Jo, 2011; Kim *et al.*, 2014a). Additionally, Mch lipomannans activate the secretion of TNF-α and nitric oxide, which are dependent on TLR2 and the adaptor protein MyD88 (Quesniaux *et al.*, 2004a). However, the roles that TLR2 and MyD88 signaling play during the Mch-induced expression of chemokines in macrophages have yet to be fully characterized. A comparison of chemokine expressions in BMDMs from wild-type (WT), TLR2 knockout (TLR2-KO), and MyD88 knockout (MyD88-KO) mice revealed a marked attenuation of Mch-induced TNF-α, CCL2, and CCL5 mRNA (Fig. 2A) and protein (Fig. 2B) expressions in the TLR2-KO and MyD88-KO mice relative to WT mice. We next investigated whether TLR2 is required for the chemokine production and host defense during Mch infection *in vivo* in a mouse model. To examine the function of TLR2 in bacterial elimination, 3 mice/group were sacrificed 1 or 5 days post-infection and then bacteria were measured in lung, liver, and spleen homogenates. As shown in Fig. 2C, the bacterial burden was significantly higher 5 days, but not 1 day, post-infection in lung and spleen tissues from TLR2-KO mice than in those from WT mice. However, there was no significant difference in bacterial numbers in liver tissues (data not shown). Furthermore, serum CCL2 and CCL5 protein levels were significantly lower in TLR2-KO mice than in WT mice (Fig. 2D). These data indicate that TLR2 and MyD88 signaling are essential for the Mch-induced expression of CCL2 and CCL5 in BMDMs, and that TLR2-mediated CCL2 and CCL5 production is crucial for protective immunity against Mch *in vivo* in a mouse model.

Mch infection leads to activation of the NF-κB signaling pathway in macrophages

During mycobacterial infection, NF-κB signaling is crucial for the activation of inflammatory cytokine expression, innate effector molecules, and TLR-triggered innate immune responses in macrophages (Jo *et al.*, 2007; Yuk and Jo, 2011). Our research group has previously shown that *M.*

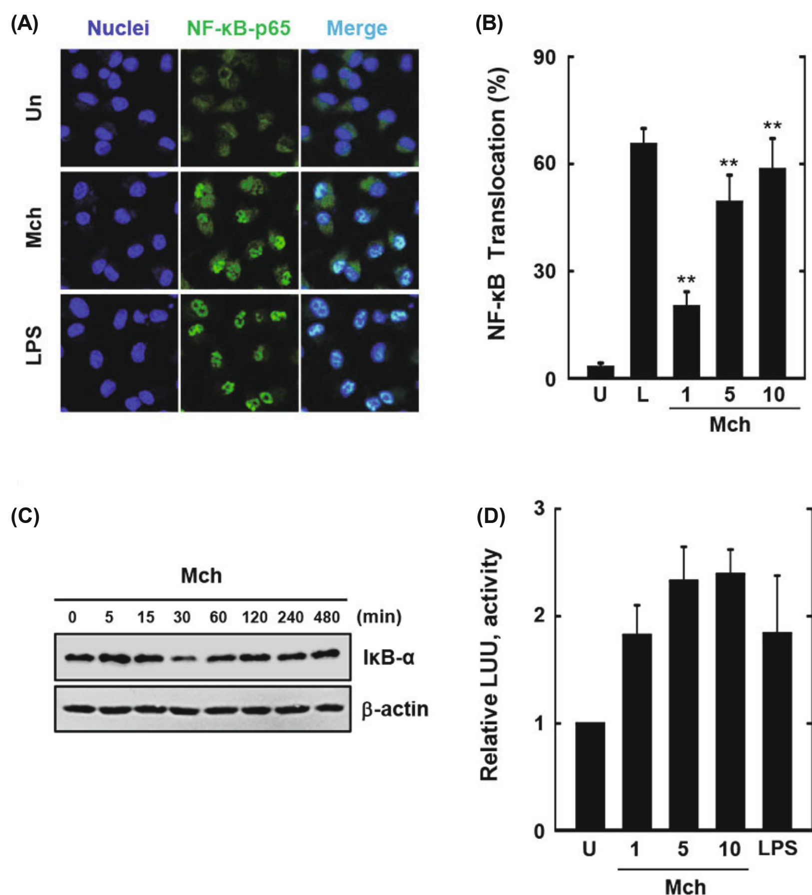

Fig. 3. **Mch results in the rapid activation of NF-κB signaling in macrophages.** (A and B) BMDMs were infected with Mch (MOI: 5) for 30 min and the cells were immunolabeled with anti-NF-κB p65 Ab and anti-rabbit-Alexa Fluor 488 (green) antibodies, and DAPI to visualize the nuclei (blue). Representative immunofluorescence images (for A) and the average mean fluorescence intensity of cells exhibiting NF-κB nuclear translocation (for B) are shown. (C) BMDMs were infected with Mch (MOI: 5) for the indicated times. The cell lysates were collected and then subjected to Western blot analyses of total IκB-α protein levels; β-actin was probed as a loading control. (D) BMDMs were transduced with NF-κB adenovirus luciferase construct and then infected with Mch (MOI: 1, 5, or 10) or LPS (100 ng/ml) for 6 h. The cell lysates were harvested and assayed for luciferase reporter activity. The results are shown as the means ± SD of three independent experiments. $^{**}P < 0.01$ (Student's *t*-test) compared with control cultures. U, uninfected.

abscessus-induced CCL2 expression is dependent on NF-κB signaling (Kim *et al.*, 2012). Thus, to examine the role of NF-κB signaling during Mch-induced chemokine expression, BMDMs were infected with Mch and it was determined whether Mch infection caused the nuclear translocation of NF-κB p65 from the cytoplasm. Mch infection markedly induced NF-κB p65 nuclear translocation within 30 min in BMDMs (Fig. 3A and B).

Following TLR stimulation, the initiation of NF-κB signaling is mediated via the rapid activation of the IκB kinase (IKK) complex, which phosphorylates IκB, resulting in its polyubiquitination and degradation (Karin and Delhase, 2000). Thus, the present study investigated the degradation of BMDMs by IκB-α following Mch stimulation. Mch infection led to marked IκBα degradation in BMDMs within 15–30 min; levels then recovered after 1 h (Fig. 3C). Next, a NF-κB luciferase assay was performed to evaluate the effects of NF-κB activity following Mch infection. To accomplish this, BMDMs were transduced with an adenovirus encoding a luciferase reporter plasmid containing response element for NF-κB (Ad-NF-κB-Luc) and the reporter gene activities were measured. Mch infection strongly activated NF-κB reporter gene activities in BMDMs transduced with Ad-NF-κB-Luc in a MOI-dependent manner (Fig. 3D). These data indicate that Mch induced NF-κB activation in macrophages.

Mch-induced TNF-α, CCL2, and CCL5 mRNA expression in macrophages was mediated via the NF-κB signaling pathway

Next, the effect of Mch-induced NF-κB signaling on the induction of TNF-α, CCL2, and CCL5 mRNA and protein expression in BMDMs was examined. The Mch-induced TNF-α, CCL2, and CCL5 mRNA expression in BMDMs was significantly attenuated in a dose-dependent manner by pretreatment with Bay 11-7085 (Bay) or caffeic acid phenethyl ester (CAPE), which are specific inhibitors of the NF-κB signaling pathway (Fig. 4A and B). Mch-induced production of CCL2 and CCL5 was also inhibited by pretreatment with Bay or CAPE (Fig. 4C). These data demonstrate that NF-κB signaling, as well as TNF, is required for Mch-induced CCL2 and CCL5 mRNA and protein expression.

Role of intracellular ROS during Mch-mediated production of CCL2 and CCL5 in BMDMs

Recently, intracellular ROS has been recognized as an important second messenger involved in various biological responses including TLR-triggered and mycobacteria-induced innate immune responses (Lee *et al.*, 2009a; Bae *et al.*, 2011; Bulua *et al.*, 2011). Thus, the present study aimed to determine whether Mch infections would result in the generation of

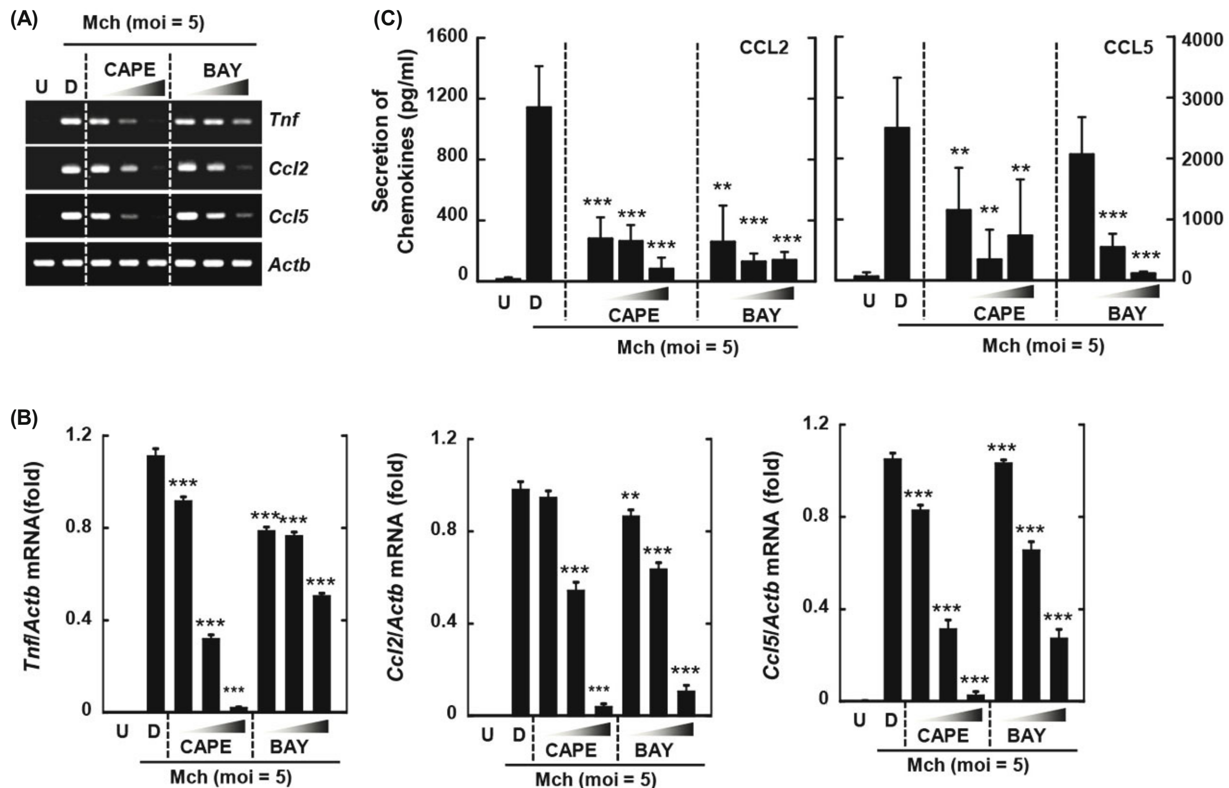

Fig. 4. NF-κB signaling is required for TNF-α, CCL2, and CCL5 expression in response to Mch. (A–C) BMDMs were infected with Mch in the presence or absence of BAY 11-7082 (BAY; 0.1, 1 or 3 μM, for 45 min) or CAPE (0.1, 1, 3 μM) for 6 h (for A and B) or for 18 h (for C). (A and B) The cell lysates were collected and subjected to semi-quantitative RT-PCR for *Tnf*, *Ccl2*, and *Ccl5*. A gel representative of three independent replicates with similar results is shown (for A). The densitometry values of each mRNA level were normalized relative to that of *Actb* (for B). (C) Culture supernatants were harvested to measure protein secretion of CCL2 and CCL5 using ELISA. The results are shown as the means ± SD of three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ (Student's *t*-test) compared with control cultures. U, uninfected; D, solvent control (0.05% DMSO).

ROS using dichlorodihydrofluorescein diacetate (DCFDA), which is a fluorescent dye that is induced by oxidation mediated by hydroxides, hydrogen peroxides, and hydroxyl radicals (Gomes *et al.*, 2005). The results showed a rapid increase in ROS within approximately 30 min of Mch infection in BMDMs (Fig. 5A). Additionally, the pretreatment of BMDMs with ROS scavengers such as the antioxidant N-acetylcysteine (NAC), the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor diphenyleneiodonium chloride (DPI), or the superoxide scavenger Tiron inhibited the Mch-induced CCL2 and CCL5 mRNA (Fig. 5B) and protein expression (Fig. 5C) in a dose-dependent manner, as well as TNF-α. These data suggest that ROS were involved in the Mch-mediated induction of inflammatory cytokines and chemokines in macrophages.

Dectin-1 signaling contributes to the Mch-induced TNF-α, CCL2, and CCL5 expression in BMDMs

Early studies demonstrated that Dectin-1 is integrally involved in the generation of proinflammatory cytokines in BMDMs after infection with *M. abscessus* (Shin *et al.*, 2008). To investigate the role of Dectin-1 in the Mch-induced TNF-α, CCL2, and CCL5 expression in macrophages, BMDMs were transduced with shRNA specific to Dectin-1 (shDectin-1), infected with Mch, and then subjected to RT-PCR analysis. The results showed that Dectin-1 knockdown significantly attenuated the Mch-induced TNF-α, CCL2, and CCL5 mRNA expression in BMDMs compared to BMDMs transduced with nonspecific shRNA (Fig. 6A and B). These data suggest that Dectin-1 was involved in the Mch-induced generation of TNF-α, CCL2, and CCL5 in macrophages.

Discussion

Currently, more than 150 NTM species are known and novel species are continuously being identified (Daley and Griffith, 2010). Of the various NTM, the incidence of infectious diseases associated with RGM is increasing worldwide, especially in Asia (Simons *et al.*, 2011). For example, the incidence of NTM diseases is increasing in a number of Asian countries, including South Korea, and this rate is negatively correlated with that of tuberculosis cases (Park *et al.*, 2010). However, the nature of and mechanisms underlying the immune responses of the host against NTM infections remain largely unknown. Mch is an important RGM, as is the Mch complex, as well as an emerging strain associated with various human pulmonary and soft tissue infections (Ringuet *et al.*, 1999; Alvarez-Uria, 2010; Chan *et al.*, 2010). The present study demonstrated that TLR2, MyD88, and NF-κB signaling, Dectin-1 signaling, and intracellular ROS contribute to the regulation of inflammatory responses and chemokine induction in macrophages during Mch infection.

In the present study, Mch robustly induced CCL2 and CCL5 gene expression through both the TLR2 and MyD88 sig-

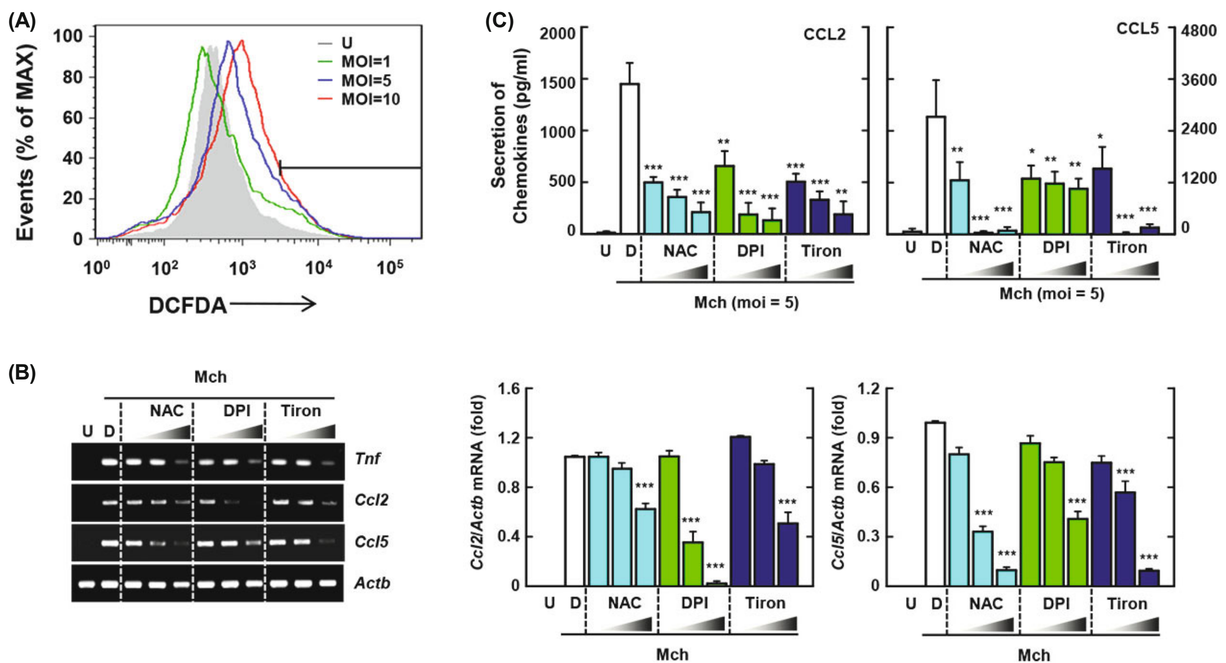

Fig. 5. Intracellular ROS is important for the Mch-mediated induction of CCL2 and CCL5 in macrophages. (A) BMDMs were infected with Mch (MOI: 1, 5, or 10) for 30 min and then stained with DCFDA. Intracellular ROS levels were analyzed using flow cytometry. (B and C) BMDMs were pretreated with an antioxidant (NAC; 5, 10, or 20 mM), NOX inhibitor (DPI; 1, 5, or 10 μM), or superoxide scavenger (Tiron; 5, 10, or 20 mM) for 45 min, and the cells were then infected with Mch (MOI: 5) for 6 h (for B) or 18 h (for C). (B) The cell lysates were collected and subjected to semi-quantitative RT-PCR for *Tnf*, *Ccl2*, and *Ccl5*. A gel representative of three independent replicates with similar results is shown (left). The densitometry values for each mRNA level were normalized to that of *Actb* (right). (C) The levels of CCL2 and CCL5 in the supernatants were measured by ELISA. The results are shown as the means ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ (Student's *t*-test) compared with control cultures. U, uninfected; D, solvent control (0.05% DMSO).

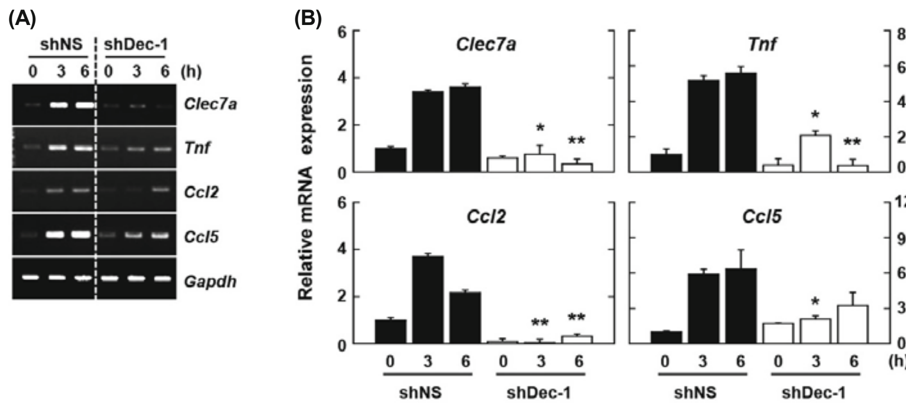

Fig. 6. Dectin-1 regulates chemokine production, including CCL2 and CCL5, in Mch-infected macrophages. (A and B) BMDMs were transduced with nonspecific shRNA or specific shRNAs against mouse *Clec7a/dectin-1* (sh*Dec-1*) following infection with Mch (MOI: 5) for the indicated times. The cell lysates were collected and subjected to semi-quantitative RT-PCR for *Tnf*, *Ccl2*, and *Ccl5*. A gel representative of three independent replicates with similar results is shown (for A). The densitometry values for each mRNA level were normalized to that of *Actb* (for B). The results are the means ± SD of three independent experiments. *$P < 0.05$ and **$P < 0.01$ (Student's *t*-test) compared with control cultures.

naling pathways in macrophages. Innate immune receptors-including TLRs, C-type lectin receptors, and Nod-like receptors-are involved in sensing and responding to *M. tuberculosis* and their ligands in various immune cells (Quesniaux *et al.*, 2004a; Basu *et al.*, 2012; Mortaz *et al.*, 2015), but the roles of pattern-recognition receptors in response to NTM infections remain largely unknown compared to that following infection with *M. tuberculosis*. Nevertheless, it is clear that TLR2 is crucial for the activation of inflammatory responses during NTM infections caused by *M. avium* (Pathak *et al.*, 2004), *M. abscessus* (Shin *et al.*, 2008), *M. massilience* (Kim *et al.*, 2014b), and *M. scrofulaceum* (Kim *et al.*, 2014a). Notably, lipomannans from Mch and *M. kansasii* activate the cell surface expression of CD40 and CD86 as well as the secretion of TNF-α and nitric oxide in a TLR2- and MyD88-dependent manner (Quesniaux *et al.*, 2004a, 2004b). Whether TLR2 plays a key role during Mch infection is unclear. In the present study, TLR2-KO mice showed an increased bacterial burden in lung and spleen tissues and decreased of CCL2 and CCL5 levels in serum. Our results suggest that TLR2 is essential for CCL2 and CCL5 production and activation of host protective immunity *in vivo*. Additionally, MyD88-deficient mice exhibit massive destruction of lung tissues and severe defects in the immune response to *M. avium* infection, which suggests a major role for MyD88 in determining the proinflammatory and innate host defenses following *M. avium* infection.

The C-C chemokines are potent chemoattractants and inflammatory mediators and also play essential roles in the recruitment and accumulation of monocytes and macrophages to sites of infection (Ono *et al.*, 2003; Moser *et al.*, 2004). CCL2 is also known as monocyte chemoattractant protein-1 (MCP-1) and initiates the recruitment of monocytes and leukocytes to inflammation sites (Madrigal and Caso, 2014). CCL2 plays an important role during the pathogenesis of tuberculosis, especially that related to human immunodeficiency virus type 1 (HIV-1) infection (Ansari *et al.*, 2013). Another chemokine, CCL5, is also referred to as Regulated on Activation Normal T cell Expressed and Secreted (RANTES) and acts as a crucial inflammatory mediator, which is a double-edged sword during viral infection (Glass *et al.*, 2003). For example, there is an increase in CCL2 levels in the bronchoalveolar lavage fluid of patients with PTB (Sadek *et al.*, 1998). In the present study, the NF-κB signaling pathway was required for the Mch-induced CCL2 and CCL5 mRNA expression in BMDMs. It is clear that the activation of the NF-κB-dependent pathway by TLR2 is required for CCL2 and CCL5 expression in various cell types. Early studies showed that the TLR4/lipopolysaccharide (LPS)-induced production of CCL2 and CCL5 in renal tubular epithelial cells is dependent on NF-κB signaling but not the extracellular signal-regulated kinase (ERK) pathway (Tsuboi *et al.*, 2002). The same study found that the LPS-induced production of CCL5 but not CCL2 is dependent on the c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) pathways. In peritoneal mesothelial cells, TLR4-induced CCL2 production is dependent on NF-κB signaling but not the activation of the MAPK pathway (Kato *et al.*, 2004). Pneumococcal proteins induce CCL2 and CCL5 mRNA expression via the activation of NF-κB and several MAPK signaling pathways in dendritic cells from CD14+ human monocytes (Bernatoniene *et al.*, 2008). Moreover, after infection with *Pseudomonas aeruginosa*, CCL5 production and NF-κB activation are dependent on the adaptor molecule Toll-IL-1R domain-containing adaptor-inducing IFN-β (TRIF) in murine alveolar and peritoneal macrophages (Power *et al.*, 2007), but the involvement of TRIF in the Mch-induced expression of chemokines was not detected in the present study (data not shown). Therefore, it would be interesting to investigate whether the MAPK subfamily members contribute to the Mch-mediated expression of CCL5 in BMDMs.

Numerous studies have shown that intracellular ROS, including superoxides, are important second messengers involved in a variety of biological signaling pathways (Bae *et al.*, 2011). Additionally, the TLR2 and MyD88 signaling pathways and the function of the phagocyte NADPH oxidase (NOX) are closely associated and act to enhance innate immune activation and bacterial killing (Laroux *et al.*, 2005). Previous studies have shown that ROS generated from NOX2 and p47phox are involved in innate and proinflammatory responses in macrophages and murine microglia following mycobacterial infection (Yang *et al.*, 2007, 2009). Furthermore, intracellular ROS generation is necessary for the expression of chemokines in response to the mycobacterial 30 kDa antigen in human monocytes (Lee *et al.*, 2009a) and TLR9-stimulated CCL2 expression in macrophages (Lee *et al.*, 2008). In vascular endothelial-like cells, the antioxidant

protein thioredoxin 1 suppresses oxidized low-density lipoprotein-stimulated CCL2 release via the prevention of the transcriptional activation of c-Jun/c-Fos (Chen *et al.*, 2010). Recent studies have revealed that microRNA-302, which is regulated by redox signaling, plays an essential role in the regulation of CCL5 mRNA levels in human fibroblasts (Kumar *et al.*, 2012). Although the present study did not involve investigation of the role of microRNAs, these findings demonstrate the important role of ROS in the activation of NF-κB, which regulates chemokine production in macrophages in response to Mch.

Dectin-1 is a C-type lectin-like receptor known for its ability to induce intracellular downstream signaling pathways that activate innate and adaptive immune responses after the recognition of its ligands from various species (e.g., fungi and mycobacteria) (Yadav and Schorey, 2006; Schorey and Lawrence, 2008; Plato *et al.*, 2013). Previous studies have shown that Dectin-1 is involved in *M. abscessus*-induced proinflammatory responses (Shin *et al.*, 2008). *M. tuberculosis* infection activates generation of inflammatory cytokines in type II airway epithelial A549 cells through Dectin-1 (Lee *et al.*, 2009a). In addition, Dectin-1 neutralizing antibody resulted in reduced generation of proinflammatory cytokines in macrophages infected with attenuated mycobacteria. However, these effects disappeared in macrophages infected with virulent *M. tuberculosis* H37RV or *M avium* 724 (Yadav and Schorey, 2006). *M. massiliense* activates TNF and IL-6 production through TLR2/Myd88, but not Dectin-1 (Kim *et al.*, 2014b). However, it remains unclear whether Dectin-1 plays a role in Mch-mediated innate immune activation. In the present study, the Mch-induced CCL2 and CCL5 expression was dependent on Dectin-1. In dendritic cells, the transcription factor NFATc2 is responsible for the regulation of the expression of numerous cytokines and chemokines, but future studies are needed to clarify the transcription factor or factors involved in the regulation of Mch-induced CCL5 expression (Yu *et al.*, 2015). Taken together, the findings of the present study suggest that Mch activated macrophage chemokine expression via TLR2- and MyD88-dependent NF-κB signaling and ROS generation. A better understanding of the mechanisms by which Mch regulates inflammatory signaling pathways will aid in the design of novel therapeutic against NTM infection.

Conflicts of interest

The authors have no financial conflict of interests.

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