Mol. Cells *29*, 35-39, January 31, 2010
DOI/10.1007/s10059-010-0003-5

Molecules and Cells

Mycobacterium tuberculosis-Induced Expression of Leukotactin-1 Is Mediated by the PI3-K/PDK1/Akt Signaling Pathway

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Chemokines function in the migration of circulating leukocytes to regions of inflammation, and have been implicated in chronic inflammatory conditions including mycobacterial infection. We investigated whether Leukotactin-1 (Lkn-1), a novel member of the CC-chemokines, is involved in the immune response of macrophages against *Mycobacterium tuberculosis* (MTB). In PMA-differentiated THP-1 cells, MTB infection increased mRNA expression of Lkn-1 in a dose-dependent manner. Lkn-1 induction peaked 12 h after infection, then declined gradually and returned to its basal level at 72 h. Secretion of Lkn-1 was elevated by MTB infection. The increase in expression and secretion of Lkn-1 caused by MTB was reduced in cells treated with inhibitors of phosphatidylinositol 3-kinase (PI3-K), 3-phosphoinositide-dependent kinase 1 (PDK1) and Akt. MTB-induced Akt phosphorylation was blocked by treatment with a PI3-K inhibitor or a PDK1 inhibitor, implying that PI3-K, PDK1, and Akt are associated with the signaling pathway that up-regulates Lkn-1 in response to MTB. These results suggest that Lkn-1 is novel member of the group of chemokines that is released by macrophages infected with MTB.

INTRODUCTION

Mycobacterium tuberculosis (MTB) is a major pathogen for tuberculosis, which causes 1.5-2 million deaths per year worldwide (World Health Organization, 2007). Infection with MTB affects the recruitment and activation of circulating effector leukocytes by influencing induction and secretion of cytokines from infected macrophages (Fenton, 1998). Infected macrophages release a variety of inflammatory cytokines as defense mechanisms against MTB (Ferrara et al., 2008; Law et al., 1996; Majumder et al., 2008).

Chemokines are cytokines that function as chemoattractants during pro-inflammatory responses (Chung et al., 2009; Yoshie et al., 2001). All chemokines are structurally related polypeptides that contain conserved cysteines that form internal disulfide loops (Fernandez and Lolis, 2002). Approximately 50 different chemokines have been identified to date, and are broadly classified into four distinct groups, CXC (α), CC (β), C (γ), and CX_3C (δ), based on the number and location of N-terminal cysteine residues (Zlotnik and Yoshie, 2000).

Recently, chemokines have also been implicated in recruiting immune cells and activating effector leukocytes in mycobacterial infection (Peters et al., 2003; Sadek et al., 1998). MTB is reported to induce the expression and secretion of CC-chemokines including monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α) and regulated upon activation, normal T-cell expressed and secreted (RANTES) (Algood et al., 2003; Lee et al., 2003; Riedel and Kaufmann, 1997) and these CC-chemokines inhibit intracellular growth of MTB (Saukkonen et al., 2002). Also, CC-chemokines such as RANTES are known to be potent leukocyte activators and chemoattractants in mycobacterial infection, providing a host defense mechanism against MTB (Stegelmann et al., 2005).

Leukotactin-1 (Lkn-1) is a recently characterized member of the CC chemokines (Youn et al., 1997). Although it is believed to play an important role in leukocyte trafficking and the development of inflammation, little information is available regarding its involvement and function in the chronic inflammation elicited by MTB. In this study, we aimed to elucidate the involvement of Lkn-1 in the host immune response against MTB infection. We observed mycobacterium-stimulated induction of Lkn-1 and evaluated the associated signal transduction pathway.

MATERIALS AND METHODS

Inhibitors

Specific inhibitors of p38 MAPK (SB202190), MEK1 (PD98059), classical PKC (Ro-31-8425), JNK (SP600125), PI3-K (Ly294002 and Wortmannin) and Akt (Akt inhibitor IV) were purchased from Calbiochem (USA). Specific inhibitors of phospholipase C (U73122) and PDK1 (OSU03012) were purchased from Cayman (USA). Dimetyl sulphoxide (DMSO) was obtained from Sigma-Aldrich (USA).

Preparation of mycobacteria

MTB H37Rv (ATCC 27294) used in this study was grown for

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Received June 8, 2009; revised September 18, 2009; accepted October 13, 2009; published online December 10, 2009

Keywords: 3-phosphoinositide-dependent kinase 1, Akt, Leukotactin-1, *Mycobacterium tuberculosis*, phosphatidylinositol 3-kinase

about four weeks at 37°C as a surface pellicle on Sauton medium enriched with 0.4% sodium glutamate and 3.0% glycerol. The surface pellicles were collected and disrupted by gentle vortexing with 6 mm glass beads. After clumps had settled, the upper suspension was collected and aliquots were stored at -80°C. Before infection, aliquots were thawed and quantitated for viable colony-forming units (CFU) on Middlebrook 7H10 agar (Difco, USA).

Cell culture and infection of MTB

The human monocytoid THP-1 leukemic cell line was maintained in RPMI 1640 medium with 2 mM glutamine, 10% heat inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco-BRL, USA) at 37°C under 5% CO_2. THP-1 cells were seeded in six-well plates and treated with 100 nM phorbol-12-myristate-13-acetate (PMA; Sigma) for 48 h to induce differentiation into macrophage-like cells, then washed three times with RPMI 1640 medium. Before infection, differentiated THP-1 cells were reconstituted in antibiotic free RPMI 1640 medium with 10% FBS. Cells were incubated with MTB H37Rv at multiplicity of infection (MOI) 0, 1, 2, 5, 10, 20 or 40 for 6 h. For time-dependent experiments, cells were infected with 10 MOI of MTB H37Rv for 0, 0.5, 1, 1.5, 2, 2.5, 3, 6, 9, 12, 24, 48 or 72 h. PMA-differentiated THP-1 cells were pretreated with inhibitors for 45 min before stimulation with MTB H37Rv for 4 h at MOI 10.

RNA extraction and semi-quantitative reverse transcriptase PCR (RT-PCR)

After removing nonphagocytosed bacilli, total RNA was extracted from cultured cells using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. cDNA was synthesized by reverse transcription with 2 μg total RNA, 0.25 μg of random hexamer (Invitrogen) and 200 unit of Murine Molony Leukemia Virus Reverse Transcriptase (MMLV-RT; Invitrogen) for 50 min at 37°C and 15 min at 70°C. Subsequent PCR amplification using 0.2 units of *Taq* polymerase (Cosmo Genetech, Korea) was performed in a thermocycler (Applied Biosystems, USA) for 40 cycles (94°C for 30s, 55°C for 30s, 72°C for 30s) using Lkn-1 primer (sense 5′-CCTCTCCTGCCTCATGCTTA-3′, antisense 5′-ACTGGGTTTGGCACAGACTT-3′). GAPDH was amplified as an internal control, with primers (sense 5′-CGGGAAGCTTGTGATCAATGG-3′, antisense 5′-GGCAGTGATGGCA TGGACTG-3′). PCR products were electrophoresed on 1.8% (w/v) agarose gels containing 0.5 μg/ml ethidium bromide, and the sizes of the products determined by comparison to 100 bp DNA ladder marker (Bioneer, Korea). The intensity of each band amplified by RT-PCR was analyzed using Gel Doc EQ Quantity One (version 4.5, Bio-Rad, Italy) and normalized to GAPDH mRNA in corresponding samples.

Enzyme-linked immunosorbent assay (ELISA)

Cell culture supernatants from MTB-infected THP-1 cells were collected 24 h after infection. Cell culture supernatants were analyzed using Duoset antibody pairs for detection of human Lkn-1 (R&D systems, USA), as recommended by the manufacturer. Lkn-1 concentrations in the samples were calculated using standard curves generated from recombinant Lkn-1, and the results expressed in picograms per milliliter.

Western blot analysis

PMA-differentiated THP-1 cells were grown in six-well plates and starved for 16 h, then infected with MTB for 0, 5, 15, 30, 60, 120 or 180 min. Cell lysates were prepared and western blot analysis performed with anti-phospho-Akt (Ser473) and anti-Akt antibodies (Cell Signaling Technology, USA). Immunoreactive proteins were detected by horseradish peroxidase-conjugated secondary antibody (Jackson Immunoresearch Laboratories, USA). The blot was developed with a chemiluminescent system (Pierce, USA).

Statistics

All values are given as mean ± standard deviation (SD). When a significant difference was detected, further analysis was performed using a Student's *t*-test. A p value of less than 0.05 was considered significant.

RESULTS

Infection of MTB increases mRNA expression of Lkn-1

Lkn-1, a novel member of the CC chemokines, is known to be involved in chronic inflammatory diseases such as atherosclerosis (Yu et al., 2004). To date, Lkn-1 involvement in tuberculosis has not been reported. We determined whether mycobacterial infection influences expression of Lkn-1 in differentiated THP-1 cells. THP-1 cells were treated with PMA for 48 h and incubated with MTB for 6 h, and mRNA level of Lkn-1 was detected by semi-quantitative RT-PCR. Expression of Lkn-1 was up-regulated by infection of MTB in a dose-dependent manner (Fig. 1), reaching a peak at 10 MOI of MTB. We also examined the effects of mycobacterial infection on the timing of Lkn-1 expression. As shown in Fig. 2, Lkn-1 expression was up-regulated in response to MTB in a time-dependent manner. The mRNA level of Lkn-1 peaked at 12 h after mycobacterial infection, and then declined gradually for 72 h. These results suggest that infection of MTB induces up-regulation of Lkn-1, implying that Lkn-1 may be involved in the immune response to MTB infection.

Mycobacterial infection enhances Lkn-1 secretion

MTB-stimulated macrophages release a variety of cytokines for recruiting and activating effector immune cells (Ferrara et al., 2008; Law et al., 1996; Majumder et al., 2008). To determine whether Lkn-1 secretion is also induced in response to MTB infection, PMA-differentiated THP-1 cells were incubated for 24 h, without or with MTB, and the culture media were collected and assayed by ELISA for secreted Lkn-1. Secretion of Lkn-1 could not be detected in uninfected control THP-1 cells (Fig. 3). However, significant amounts of Lkn-1 (approximately 2,000 pg/ml) were secreted from MTB-infected cells (Fig. 3). This demonstrated that macrophages release Lkn-1 in response to MTB infection and implied that secreted Lkn-1 may influence the immune response of macrophages infected with MTB.

Induction of Lkn-1 by MTB is mediated by the PI3-K/PDK1/Akt signaling pathway

To elucidate the mechanism by which mycobacterial infection affects expression of Lkn-1, we determined the signaling pathway associated with MTB-stimulated induction of Lkn-1. THP-1 cells were pre-incubated with inhibitors of specific signaling molecules for 45 min, then infected with MTB for 4 h. As shown in the Fig. 4A, treatment with PD98059 (an inhibitor of MEK1) or U73122 (an inhibitor of PLC) did not influence Lkn-1 induction by MTB. SB202190 (an inhibitor of p38 MAPK), Ro-31-8425 (an inhibitor of classic PKC), and SP600125 (an inhibitor of JNK) had slight negative effects on MTB-stimulated Lkn-1 expression. In contrast, pre-incubation with Ly294002 (an inhibitor of PI3-K) almost completely abolished MTB-induction of Lkn-1, demonstrating the involvement of PI3-K in this process (Fig. 4A). Pre-treatment with Wortmannin (an inhibitor of PI3-K),

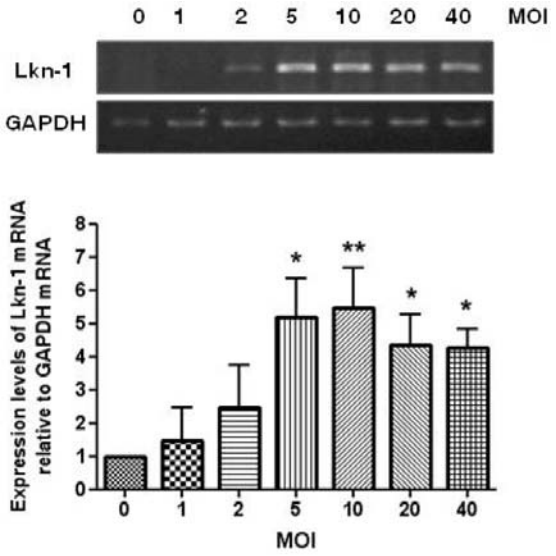

Fig. 1. Infection with MTB enhances expression of Lkn-1 in a dose-dependent manner. THP-1 cells were treated with 100 nM PMA for 48 h, and infected with the indicated concentrations (0, 1, 2, 5, 10, 20 or 40 MOI) of MTB for 6 h. Total RNA was extracted and cDNA was prepared. PCR analysis was performed using Lkn-1-specific primers. The PCR products were resolved by 1.8% agarose gel (upper panel), to detect Lkn-1. GAPDH was used as an internal control. Densitometric analysis was performed (lower panel). Data are expressed as the mean ± SD, and are presented as the expression levels of Lkn-1 mRNA relative to GAPDH mRNA (The expression level of Lkn-1 relative to GAPDH in the absence of mycobacterial infection was set to 1.0). The data represent results from three independent experiments. Statistical analysis was performed by Student's *t*-test. A p value of less than 0.05 was considered significant (* $p < 0.05$, ** $p < 0.01$ relative to uninfected control).

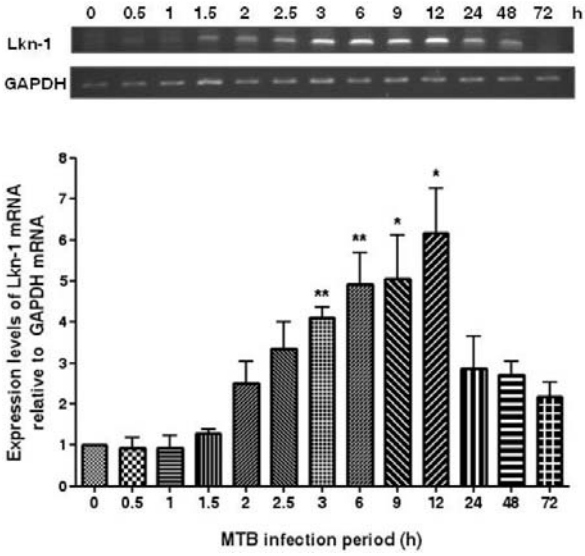

Fig. 2. MTB induces a time-dependent increase in Lkn-1 mRNA. THP-1 cells were differentiated for 48 h and incubated in the presence of MTB for the indicated times (0, 0.5, 1, 1.5, 2, 2.5, 3, 6, 9, 12, 24, 48 or 72 h). cDNA prepared from total RNA of infected cells was subjected to PCR to amplify Lkn-1. The PCR products were analyzed by 1.8% agarose gel (upper panel) to detect Lkn-1 expression. GAPDH was used as an internal control. Densitometric analysis was performed (lower panel). Data are expressed as the mean ± SD, and are presented as the expression levels of Lkn-1 mRNA relative to GAPDH mRNA (The level of Lkn-1 relative to GAPDH without infection by MTB was set to 1.0). The data represent results from five independent experiments. Statistical analysis was performed by Student's *t*-test. A p value of less than 0.05 was considered significant (* $p < 0.05$, ** $p < 0.01$ relative to uninfected control).

OSU03012 (an inhibitor of PDK1), or Akt inhibitor IV (an inhibitor of Akt), prevented induction of Lkn-1 mRNA in response to MTB infection (Fig. 4B). The secretion of Lkn-1 by MTB was also significantly blocked by pre-incubation with the PI3-K inhibitors Ly294002 and Wortmannin, the PDK1 inhibitor OSU03012, or the Akt inhibitor Akt inhibitor IV, in a dose-dependent manner (Fig. 4C). However, pre-treatment with PD98059, a specific inhibitor of MEK1, did not affect MTB-induced secretion of Lkn-1 (Fig. 4C).

In addition, MTB infection induced phosphorylation of Akt (Fig. 5A). When THP-1 cells were pre-treated with a PI3-K inhibitor (Ly294002) or a PDK1 inhibitor (OUS03012), Akt phosphorylation after MTB infection was severely blocked (Fig. 5B). These results suggested that MTB infection enhances expression of Lkn-1 via Akt activation, and that activation of Akt is linked to PI3-K and PDK1, which are upstream of Akt in the signal transduction pathway that regulates MTB-induced Lkn-1 expression.

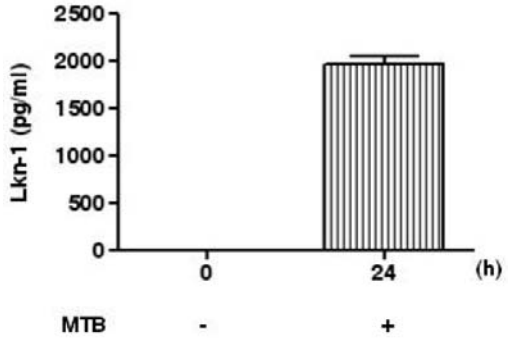

Fig. 3. Mycobacterial infection induces Lkn-1 secretion. Differentiated THP-1 cells were incubated in the absence or presence of MTB for 24 h, and secretion of Lkn-1 was measured by ELISA kit using cell culture supernatants. Data are expressed as the mean ± SD of three independent experiments and are presented as the concentration (pg/ml).

DISCUSSION

Mycobacterial infection induces both cellular and humoral immune responses. A variety of cytokines are proposed to recruit and activate immune cells in mycobacterial infection (Ferrara et al., 2008; Law et al., 1996; Majumder et al., 2008). However, until now, no whole expression profiles of cytokines in response to MTB infection have been reported, and the signaling pathways by which mycobacterial infection influences induction of cytokines have not been completely determined. In this work, we found that Lkn-1, a CC chemokine, is involved in the immune response to MTB, and demonstrated that infection of MTB induces expression and secretion of Lkn-1. We also found that up-regulation of Lkn-1 by MTB appears to be mediated by a signaling pathway that includes PI3-K, PDK1 and Akt.

Lkn-1 is involved in the recruitment of leukocytes, including monocytes, neutrophils, and lymphocytes, and is associated

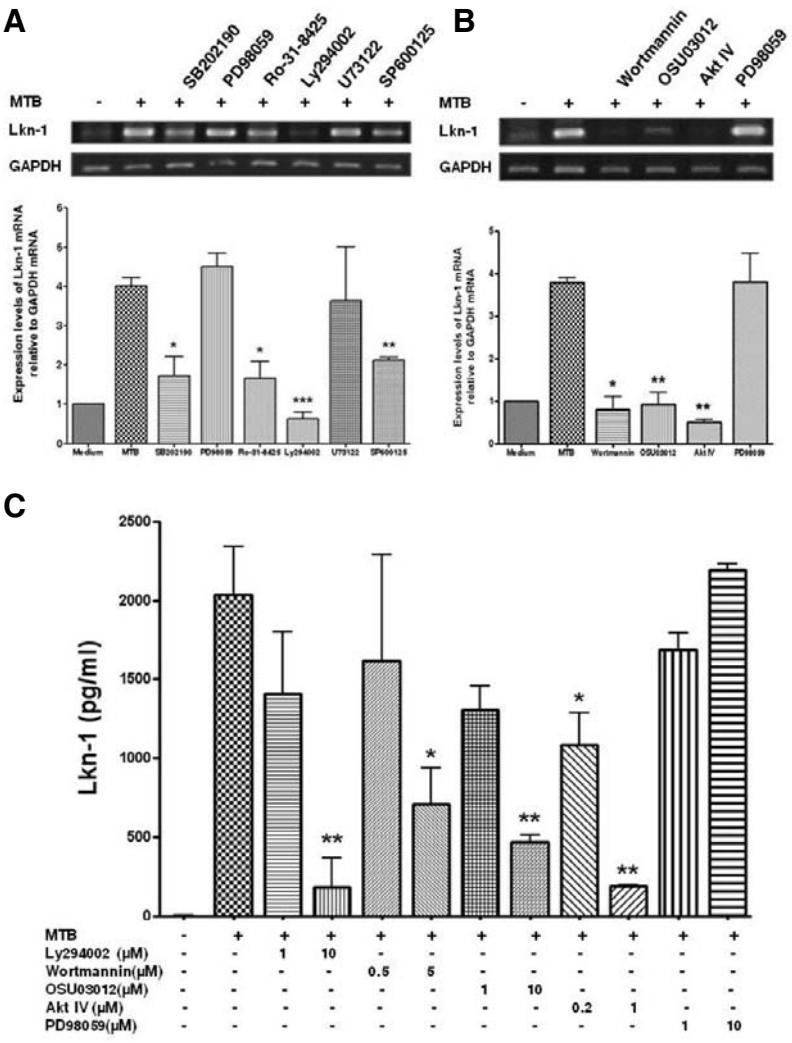

Fig. 4. MTB-induced expression and secretion of Lkn-1 is mediated by the PI3-K/PDK1/Akt signaling pathway. (A) PMA-treated THP-1 cells were pre-incubated with the inhibitors SB202190 (20 μM), PD98059 (50 μM), Ro-31-8425 (50 nM), Ly294002 (10 μM), U73122 (50 ng/ml), SP600125 (10 μM) for 45 min, followed by mycobacterial infection (10 MOI) for 4 h. (B) Differentiated THP-1 cells were pre-treated with PI3-K-, PDK1-, and Akt-specific inhibitors (100 nM Wortmannin, 1 μM OSU03012, 100 nM Akt IV inhibitor) for 45 min, followed by mycobacterial infection (10 MOI) for 4 h. cDNA was prepared from total RNA extracted from treated cells. PCR analysis was performed using Lkn-1-specific primers. PCR products were analyzed by 1.8% agarose gel (upper panel) to detect Lkn-1 expression. GAPDH was used as an internal control. Densitometeric analysis was performed (lower panel). Data are expressed as the mean ± SD, and are presented as the expression levels of Lkn-1 mRNA relative to GAPDH mRNA. (The level of Lkn-1 relative to GAPDH in the absence of mycobacterial infection was set to 1.0). The data are results from three independent experiments. Statistical analysis was performed by Student's *t*-test. A p value of less than 0.05 was considered significant. (* $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$ relative to MTB infection alone). (C) Differentiated THP-1 cells were pre-treated with indicated concentrations of Ly294002, Wortmannin, OSU03012, Akt IV or PD-98059 for 45 min, followed by MTB infection (10 MOI). Supernatants were harvested 24 h after infection and secretion of Lkn-1 was measured by ELISA. Data are the mean ± SD of three independent experiments. Statistical analysis was performed by Student's *t*-test. A p value of less than 0.05 was considered significant. (* $p < 0.05$ or ** $p < 0.01$ relative to MTB infection alone).

with the development of atherosclerosis (Youn et al., 1997; Yu et al., 2004). Its role in mycobacterial infection, however, was previously unknown. We show for the first time that expression and secretion of Lkn-1 is increased in macrophages infected with MTB. Induction of Lkn-1 was a relatively early response to mycobacterial infection, with Lkn-1 expression detected 2 h after infection and peaking 12 h after stimulation. At an early stage of infection, affected macrophages may release Lkn-1 for recruitment and/or activation of leukocytes in response to MTB. However, further studies are required to determine the exact function of Lkn-1 in the immune response against mycobacterial infection.

MTB escapes the mycobacterial killing activity of immune cells via various mechanisms. MTB inhibits apoptosis of host cells, allowing persistent survival, and the expression pattern of cytokines in infected macrophages is changed by mycobacterial infection, which affects leukocyte recruitment and activation (Cooper and Khader, 2008). As shown in Fig. 2, the expression level of Lkn-1 increased at a relatively earlier stage (up to 12 h), then began to decline at 24 h after infection, returning to basal levels by 72 h after infection. One hypothesis is that affected macrophages secrete Lkn-1 for recruiting or activating leukocytes to remove MTB, while MTB inhibits secretion of Lkn-1 by its host cells to cause chronic infection.

Expression of chemokines is regulated by various intracellular or extracellular stimuli that are delivered to the nucleus via signal transduction pathways, and result in the regulation of gene expression. Mycobacterial infection is reported to influence expression of a variety of chemokines via different signaling pathways (Mendez-Samperio et al., 2005; 2008; Roach and Schorey, 2002). Here, we found that MTB induces expression of Lkn-1 via the PI3-K/PDK1/Akt pathway. In addition, MTB infection induced phosphorylation of Akt at Ser473. Although we could not determine the phosphorylation site(s) of PDK1 and PI3-K that are affected by MTB infection, we determined that MTB-induced phosphorylation of Akt is prevented by the pre-treatment with Ly294002 (an inhibitor of PI3-K) or OSU0301 (an inhibitor of PDK1) (Fig. 5B). Therefore, Akt activation seems to be linked to PI3-K and PDK1. As shown in Fig. 4A, inhibition of p38, classic PKCs or JNK also caused diminished expression of Lkn-1 after MTB infection. Therefore, the other signaling path- way(s) associated with MTB-induced expression of Lkn-1 need to be elucidated by further studies.

The role of Lkn-1 in MTB infection remains to be elucidated.

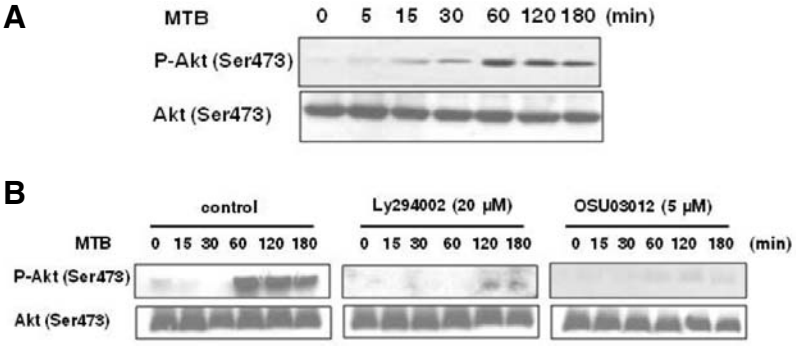

Fig. 5. PI3-K and PDK1 are involved in MTB-stimulated Akt phosphorylation. (A) Differentiated THP-1 cells were starved for 16 h and infected with MTB (10 MOI) for the indicated times before cell lysates were prepared, resolved on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Phosphorylation of Akt was detected by Western blotting using anti-phospho-Akt. (B) PMA-treated THP-1 cells were starved for 16 h, and treated with Ly294002 (20 μM) or OSU03012 (5 μM) for 45 min. Subsequently, cells were infected with MTB (10 MOI) for the indicated times before cell lysates were prepared. Akt phosphorylation was determined by Western blotting using anti-phopho-Akt. A representative of three independent replicates with similar results is shown.

One hypothesis is that the Lkn-1 that is released by infected macrophages is a leukocyte chemoattractant that provides a host defense mechanism against MTB. Recently, MTB was reported to use recruited, uninfected macrophages for its expansion (Davis and Ramakrishnan, 2009), so another possibility is that MTB induces expression of Lkn-1 from infected macrophages to recruit new and uninfected macrophages in which it can multiply and expand.

In this study, we demonstrated that Lkn-1 is induced by mycobacterial infection, and this induction is mediated by PI3-K/PDK1/Akt, implying that Lkn-1 may be involved in tuberculosis, a chronic inflammatory disease. While further studies are required to determine the exact role of Lkn-1 in mycobacterial infection, and to examine if MTB regulates Lkn-1 expression for its long-term survival, this is the first report implicating Lkn-1, a novel chemokine, in the immune response against MTB.

ACKNOWLEDGMENT

This work was supported by a grant of the Korean Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea (A010381).

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