Chapter

6

Leukocyte-specific protein 1 is essential for phagocytosis of *Candida albicans* by Dectin-1 on human macrophages

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Abstract

Dectin-1 is a pattern recognition receptor that is involved in both innate and adaptive immune responses to fungi. On macrophages, dectin-1 is one of the main receptors for phagocytosis and clearance of fungal pathogens. While several studies have identified the signaling pathways underlying dectin-1-mediated cytokine induction, the molecular mechanisms of dectin-1-mediated phagocytosis are still largely unknown. Here, we have identified the F-actin binding protein leukocyte specific protein 1 (LSP1) as a central mediator of dectin-1-induced phagocytosis. We show that LSP1 associates with dectin-1 on human macrophages. Silencing of LSP1 by RNA interference increased the expression of dectin-1 by impairing Dectin-1 internalization. Strikingly, LSP1 silencing abrogated *C. albicans* phagocytosis by dectin-1 and prevented clearance of *C. albicans* by primary macrophages. Thus, here we have identified a novel role for LSP1 in innate fungal defence by regulating dectin-1-mediated phagocytosis.

Introduction

Macrophages play an essential role in anti-fungal immunity by uptake and killing of fungal pathogens [1]. One of the main receptors involved in fungal recognition by macrophages is dectin-1, a C-type lectin that binds to β-glucans and is also expressed by other cells such as dendritic cells (DCs) and neutrophils [2]. Dectin-1 recognizes β-glucan carbohydrates on various fungi, including *Candida albicans*, and ligation mediates various cellular functions such as reactive oxygen production [3] and the induction of cytokines and chemokines [4, 5]. In addition, dectin-1 is one of the main receptors on macrophages that mediates fungi phagocytosis [2, 6].

Recently, several reports have characterized the molecular mechanisms of dectin-1-induced cytokine induction. Engagement of dectin-1 by fungal β-glucans leads to activation of the spleen tyrosine kinase Syk [7], which subsequently mediates the induction of cytokines and chemokines via caspase recruitment domain 9 (Card9)-dependent activation of nuclear factor (NF)-κB [8]. In addition, Syk is important for dectin-1-induced reactive oxygen production [3]. In contrast, the molecular mechanisms behind dectin-1-mediated phagocytosis are still largely undefined, but seem to be distinct from other phagocytic receptors, including the Fcγ receptors (FcγRs) and complement receptors (CRs) [9]. Although Syk is partially involved in dectin-1 mediated phagocytosis in DCs, phagocytosis is completely Syk-independent in macrophages [3, 9].

Here, we have identified a central mediator of dectin-1-mediated phagocytosis by human macrophages. We demonstrate that the F-actin binding protein leukocyte-specific protein 1 (LSP1) is associated with dectin-1. LSP1 regulates the cell-surface expression of dectin-1 by affecting the cellular distribution. Strikingly, we show that silencing of LSP1 in macrophages abrogates dectin-1-mediated phagocytosis of *C. albicans*, demonstrating that LSP1 is a crucial mediator of phagocytosis by dectin-1. In addition to heat-killed yeast, we show that LSP1 is also important for clearance of live *C. albicans* by dectin-1. Thus, here we have identified a novel role for LSP1 in fungal defence by regulating dectin-1-mediated phagocytosis. These findings provide new insight into the mechanisms of dectin-1-mediated immune responses.

Results and Discussion

Phagocytosis of *C. albicans* by human macrophages is mediated by dectin-1

Phagocytosis is crucial in the control of fungal pathogens such as *C. albicans* [12], with macrophages being the main phagocytic immune cells that combat fungal pathogens in infected tissues [6]. The most prominent membrane-bound receptors on macrophages that have been described to contribute to *C. albicans* phagocytosis are dectin-1, which recognizes β-glucans, and MR, which recognizes N-linked mannan [13]. To investigate the mechanisms of *C. albicans* phagocytosis by macrophages, we first examined the expression of these receptors on human primary monocyte-derived macrophages [6]. Human macrophages expressed dectin-1 and the MR, but not DC-SIGN (Figure 1a).

Phagocytosis of C. albicans was measured by incubating macrophages with FITC-labeled *C. albicans* for 60 min., after which macrophages were extensively washed. Extracellular fungi were quenched by trypan blue

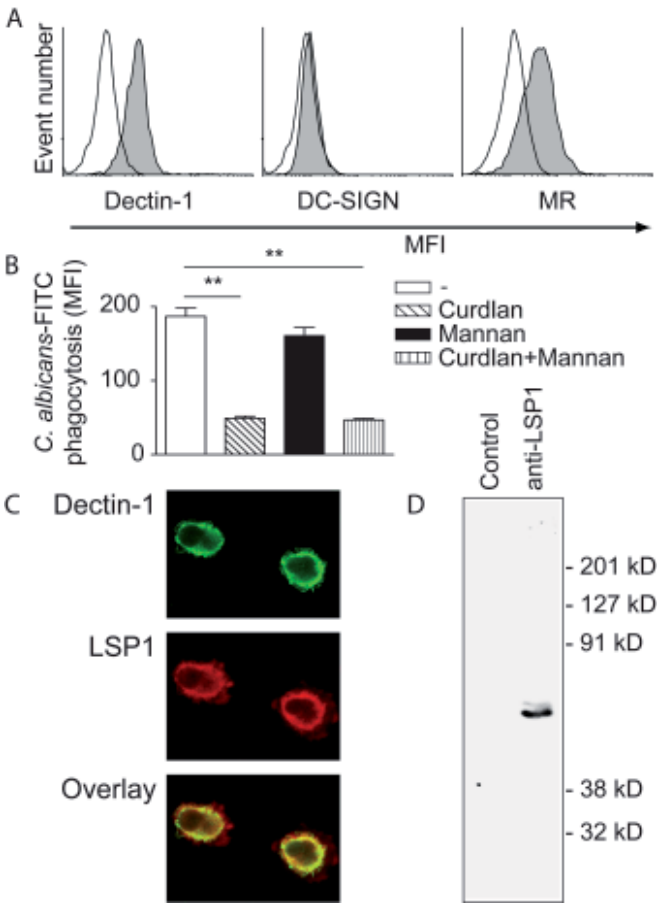

Figure 1. LSP1 is associated with Dectin-1 on human macrophages. a, Fluorescence intensity of dectin-1, DC-SIGN and MR expression by macrophages. Grey histograms represent staining with a specific antibody, solid lines represent staining with an isotype control. b, Uptake of FITC-labeled *C. albicans* by macrophages. Macrophages were pre-incubated with or without curdlan, mannan, or a combination to block dectin-1, MR, or both respectively. Subsequently, macrophages were incubated with FITC-labeled *C. albicans*. Extracellularly bound *C. albicans*-FITC was quenched with trypan blue and uptake of *C. albicans* was determined by analysing mean fluorescence intensity by FACS. **P < 0.01; *P < 0.05. c, Co-localization of dectin-1 with LSP1 on macrophages as determined by confocal imaging. Macrophages were stained for dectin-1 (green) and LSP1 (red). Images were overlayed to show co-localization. d, LSP1 associates with dectin-1 on macrophages. LSP1 was co-immunoprecipitated from macrophage lysate with antibodies against dectin-1. Immunoprecipitates were assayed for LSP1 by immunoblot.

and internalization was determined using flow cytometry. Macrophages strongly phagocytosed *C. albicans* (Figure 1b). To determine the contribution of dectin-1 or the MR, macrophages were pre-incubated with either β-glucan curdlan, which is a specific ligand for dectin-1 [14], or mannan, which specifically blocks mannose binding receptors such as the MR. Pre-incubation with curdlan strongly impaired phagocytosis of FITC-labeled *C. albicans*, whereas mannan did not affect the phagocytic capacity (Figure 1b). These data demonstrate that Dectin-1 is the major receptor for phagocytosis of *C. albicans* by human macrophages. However, since inhibition of Dectin-1 did not completely inhibit phagocytosis, other receptors beside Dectin-1 might also partially contribute to *C. albicans* phagocytosis.

LSP1 associates with dectin-1

Little is known about the mechanisms of dectin-1-mediated phagocytosis by human macrophages. Recent data demonstrate that the F-actin binding protein LSP1 associates with the C-type lectins DC-SIGN, Langerin and L-SIGN [15]. To investigate whether LSP1 associates with dectin-1, macrophages were grown on chamber slides, stained for dectin-1 and LSP1 and subsequently analyzed for co-localization by confocal imaging. LSP1 partially co-localized with dectin-1, suggesting that LSP1 is associated with dectin-1 (Figure 1c). Furthermore, LSP1 was co-immunoprecipitated from a macrophage lysate by an antibody against dectin-1 (Figure 1d), confirming that LSP1 associates with dectin-1.

Our finding that LSP1 associates with dectin-1 in addition to the previously reported binding to other C-type lectins [15] suggests that LSP1-association is a common feature of C-type lectins. A specific domain of LSP1, distinct from its F-actin-binding region, is required for binding to the C-type lectin DC-SIGN. Binding of LSP1 to DC-SIGN requires the YxxL motif present in the cytoplasmic tail of DC-SIGN [15]. Interestingly, dectin-1 bears two similar tyrosine motifs [16], which might account for LSP1 binding to dectin-1. The membrane proximal motif has been described to be essential for dectin-1-mediated phagocytosis [9], suggesting that LSP1 interacts with dectin-1 through this motif.

LSP1-silencing affects the expression and cellular distribution of dectin-1

To investigate the involvement of LSP1 in dectin-1-mediated phagocytosis, LSP1 was efficiently silenced by RNA interference in macrophages both at the mRNA and protein level in macrophages (Figure 2a and b). LSP1-silenced and control siRNA-treated macrophages

were stained for CD86, HLA-DR, CD11b, dectin-1 and MR. Remarkably, LSP1-silencing enhanced the expression of dectin-1, whereas it did not affect CD86, HLA-DR, CD11b and MR expression (Figure 2c). Next, we analysed dectin-1 expression on macrophages by confocal imaging. In control siRNA treated macrophages dectin-1 was expressed both extracellularly and intracellularly (Figure 2d). In contrast, dectin-1 in LSP-1-silenced macrophages was almost exclusively expressed on the cell surface (Figure 2d). These data indicate that LSP1 specifically affects the cellular distribution and thereby the cell-surface expression of dectin-1 in macrophages. This re-distribution of dectin-1 from internal vesicles to the plasma membrane most likely results from abrogation of dectin-1 internalization.

LSP1 is essential for phagocytosis and clearance of *C. albicans* by dectin-1

To determine whether LSP1 plays a role in dectin-1-mediated phagocytosis of fungi, the phagocytic capacity of LSP1-silenced macrophages was investigated. Strikingly, phagocytosis of *C. albicans* by LSP1-silenced macrophages was severely impaired compared to control cells after 60 minutes (Figure 3a). Pre-incubation with β-glucan curdlan did not further decrease phagocytosis (Figure 3a), strongly suggesting

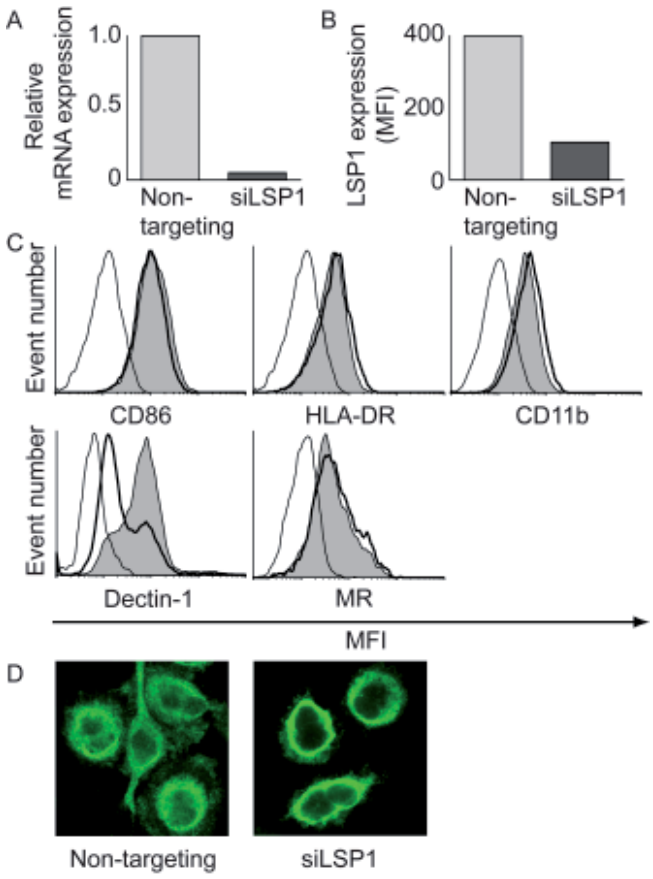

Figure 2. LSP1 silencing affects dectin-1 expression. a and b, mRNA expression determined by real-time PCR (a) and protein expression determined by FACS (b) of macrophages treated with siRNA against LSP1 or a non-targeting control. c, Dectin-1 expression by macrophages after LSP-1 silencing. LSP1-silenced or non-targeting-treated macrophages were stained for dectin-1, MR, CD86, HLA-DR or CD11b by FACS. Grey histograms represent LSP1-silenced cells, thick lines non-targeting siRNA treated cells and thin lines the isotype controls. d, Distribution of dectin-1 expression after LSP-1 silencing as determined by confocal imaging. LSP1-silenced or non-targeting-treated macrophages were stained for dectin-1 expression under equal conditions.

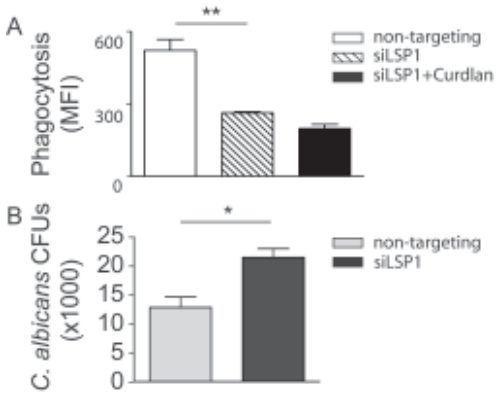

Figure 3. LSP1 is essential for phagocytosis and clearance of *C. albicans* by human macrophages. a, Phagocytosis of *C. albicans* by LSP-1 silenced macrophages. LSP1-silenced or non-targeting-treated macrophages were pre-incubated with or without curdlan to block dectin-1. Subsequently, macrophages were incubated with FITC-labeled *C. albicans*, extracellularly bound *C. albicans*-FITC was quenched with trypan blue and uptake was determined by FACS. b, Clearance of live *C. albicans* by LSP1 silenced macrophages. LSP1-silenced or non-targeting-treated macrophages were co-cultured with live *C. albicans* at 0.1 yeast particles per cell. After 24 h the amount of colony forming units in the supernatant was determined by counting serial step dilutions on agar plates. $^{**}P < 0.01$; $^{*}P < 0.05$.

that LSP1 silencing blocks dectin-1-mediated phagocytosis.

The main contribution of macrophages in anti-fungal immunity is their central role in clearance of fungal pathogens by uptake and killing [17]. However, thus far we only examined phagocytosis of heat-killed *C. albicans*, which has a more restricted expression of exposed β-glucans than live *C. albicans* [18, 19]. Therefore, we investigated whether LSP1 is also essential in clearance of live yeast. Macrophages were co-cultured with live *C. albicans* (0.1 yeast particles/cell) for 24 h, thereby allowing extensive *C. albicans* replication as well as uptake by macrophages. Subsequently, the amount of colony forming units (CFUs) in the supernatant was determined after 24 h by counting serial step dilutions of the supernatant on agar-plates. Strikingly, fungal outgrowth was significantly increased when *C. albicans* was cultured with LSP1-silenced macrophages compared to cells treated with non-targeting siRNA (Figure 3b), demonstrating that LSP1 is also important in clearance of live C. albicans by human macrophages.

Concluding Remarks

Dectin-1 is a PRR that plays a major role in anti-fungal immunity. Several reports have characterized the intracellular signaling pathways through which dectin-1 mediates the induction reactive oxygen production and inflammatory cytokines [3, 7, 8]. However, little is known about the molecular mechanisms of dectin-1-mediated phagocytosis. Here, we have identified LSP1 as an essential mediator of phagocytosis by dectin-1 and important for clearance of live C. albicans.

LSP1 is a 52-kD F-actin binding phosphoprotein expressed in all human leukocytes [20]. LSP1 has been described to play an important role

in leukocyte motility by rearrangement of actin filaments [21]. In addition, LSP1 interacts with the C-type lectin DC-SIGN and thereby facilitates the transport of HIV to the proteasome [15]. Here, we report yet another function: LSP1 is a crucial mediator of phagocytosis by associating with dectin-1. This finding correlates with its important role as regulator of microfilamentous cytoskeleton dynamics [22]. However, the exact mechanism through which LSP1 mediates the induction of phagocytosis requires further investigation. Interestingly, several reports have described that LSP1 can couple to downstream signaling, which might underlie dectin-1 mediated phagocytosis. LSP1 has been found to directly interact with PKC [23], which previously has been shown to be essential in dectin-1-mediated phagocytosis by macrophages [9]. However, future investigations are required to determine which downstream mediators are activated via LSP1 to induce phagocytosis.

In conclusion, we have identified LSP1 as an essential mediator of phagocytosis by dectin-1. Since dectin-1 interacts with both fungi and mycobacteria [24], LSP1 could play a critical role in innate immune responses against several pathogens.

Materials and Methods

Cells

Monocytes were isolated from buffy coats (Sanquin bloodbank, Amsterdam, The Netherlands) using a Ficoll and subsequent Percoll gradient, followed by culturing for 6 days in Teflon flasks (Nalgene) in RPMI with 5% human AB serum for 6 days, after which monocyte-derived macrophages were seeded in tissue culture plates.

Expression

Cells were stained with anti-dectin-1 (R&D Systems), anti-MR (BD Biosciences) and anti-DC-SIGN [10] followed by FITC-conjugated goat anti-mouse (Jackson Immunoresearch), or direct staining with anti-CD11b, anti-CD86-PE and anti-HLA-DR-PE (BD Pharmingen). LSP1 was detected by fixing cells in 3% para-formaldehyde, permeabilizing in 90% methanol and staining with rabbit anti-LSP1 (Cell Signaling) and PE-conjugated donkey anti-rabbit (Jackson Immunoreseach).

Phagocytosis

Macrophages were pre-incubated with 100 µg/mL curdlan (Sigma

C7821), 100 µg/mL mannan (Sigma M7504), a combination, or without block for 30 min. at 37°C in serum-free medium. Afterwards, cells were incubated for 60 min. with heat-killed *C. albicans*-FITC (moi 4), washed three times in PBS and detached from the culture plates using trypsin/EDTA. Extracellularly bound *C. albicans*-FITC was quenched with 0.008% trypan blue.

Immunofluorescence staining

Macrophages were cultured on Lab-Tek®II Chamber Slides (Nalge Nunc International Corp), fixed in 3% para-formaldehyde and permeabilized with PBS with 0.1% saponin. Cells were stained using mouse anti-dectin-1 (R&D Systems MAB1859) and rabbit anti-LSP1 (Cell Signaling) and anti-IgG2B-Alexa488 or anti-rabbit Alexa594 (Molecular Probes) respectively. Samples were analyzed by confocal microscopy (Leica AOBS SP2 confocal lasers scanning microscope (CLSM) system).

Immunoprecipitation and immunoblot

Dectin-1 was immunoprecipitated by incubating lysate overnight with anti-dectin-1 antibody (R&D) or an isotype control and protein A/G conjugated to agarose beads and loaded onto a 10% polyacrylamide gel for electrophoresis. Proteins were transferred to a nitrocellulose membrane and assayed for LSP1 by immunoblot.

RNA interference

Macrophages were transfected with 50 nM LSP1 SMARTpool (M-012640-00) and non-targeting siRNA pool (D-001206-13) as a control (Dharmacon) using transfection reagens DF4 (Dharmacon). At 72 hrs after transfection, cells were used for experiments.

Quantitative real-time PCR

PCR amplification was performed with the SYBR green method in an ABI 7900HT sequence detection system (Applied Biosystems) as described [11]. The normalized amount of target mRNA was calculated from the obtained Ct values for both target and GAPDH mRNA with $Nt = 2^{Ct(GAPDH)-Ct(target)}$. Nt in non-targeting samples was set at 1.

Clearance of live *C. albicans*

Macrophages per well were co-cultured with live *C. albicans* at 0.1 yeast particles per macrophage in serum-free medium. After 24 hours,

colony forming units (CFUs) were counted from serial step dilutions of the supernatant, which had been grown on agar plates.

Statistical analysis

Differences were assessed using a one-way analysis of variance (ANOVA). When the overall F-test was significant, differences were further investigated using the post-hoc Bonferroni test. Differences of yeast clearance were determined using the Student's t-test for paired observations. Significance was set at $p < 0.05$.

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