

Activated Dectin-1 Localizes to Lipid Raft Microdomains for Signaling and Activation of Phagocytosis and Cytokine Production in Dendritic Cells*

Received for publication, April 16, 2009, and in revised form, June 1, 2009. Published, JBC Papers in Press, June 12, 2009, DOI 10.1074/jbc.M109.009076

Shengli Xu^{†1}, Jianxin Huo[‡], Merry Gunawan[§], I-Hsin Su[§], and Kong-Peng Lam^{†1,2}

From the [†]Laboratory of Immunology, Bioprocessing Technology Institute, Agency for Science, Technology and Research, Singapore 138668, the [§]School of Biological Sciences, Nanyang Technological University, Singapore 637551, and the ¹Department of Pediatrics, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 119074

Lipid rafts are plasma membrane microdomains that are enriched in cholesterol, glycosphingolipids, and glycosylphosphatidylinositol-anchored proteins and play an important role in the signaling of ITAM-bearing lymphocyte antigen receptors. Dectin-1 is a C-type lectin receptor (CLR) that recognizes β -glucan in the cell walls of fungi and triggers signal transduction via its cytoplasmic hemi-ITAM. However, it is not known if similar to antigen receptors, Dectin-1 would also signal via lipid rafts and if the integrity of lipid raft microdomains is important for the physiological functions mediated by Dectin-1. We demonstrate here using sucrose gradient ultracentrifugation and confocal microscopy that Dectin-1 translocates to lipid rafts upon stimulation of dendritic cells (DCs) with the yeast derivative zymosan or β -glucan. In addition, two key signaling molecules, Syk and PLC γ 2 are also recruited to lipid rafts upon the activation of Dectin-1, suggesting that lipid raft microdomains facilitate Dectin-1 signaling. Disruption of lipid raft integrity with the synthetic drug, methyl- β -cyclodextrin (β mD) leads to reduced intracellular Ca²⁺ flux and defective Syk and ERK phosphorylation in Dectin-1-activated DCs. Furthermore, β mD-treated DCs have significantly attenuated production of IL-2, IL-10, and TNF α upon Dectin-1 engagement, and they also exhibit impaired phagocytosis of zymosan particles. Taken together, the data indicate that Dectin-1 and perhaps also other CLRs are recruited to lipid rafts upon activation and that the integrity of lipid rafts is important for the signaling and cellular functions initiated by this class of innate receptors.

Cell membranes are dynamic and laterally inhomogeneous bi-layered structures that are composed of multiple lipid and glycoprotein species, and located within cellular membranes are specialized detergent-resistant microdomains known as lipid rafts (1). Lipid rafts are envisaged as partially ordered membrane domains caused by the close packing of glyco-

phosphatidylinositol-anchored proteins with glycosphingolipids and cholesterol (2, 3). These lipid raft microdomains have been demonstrated to play an important role in facilitating the signaling of some transmembrane receptors, such as Fc receptors (4–6), cytokine receptors (7–9), and lymphocyte antigen receptors, namely the B-cell receptor (BCR)³ and T-cell receptor (TCR) (10–13). More recently, it was found that lipid rafts were also involved in the signal transduction of some innate pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs). For instance, TLR2 and TLR4 have been shown to translocate to lipid rafts upon their stimulation with specific agonists (14–16). However, it is currently not known if other classes of membrane-bound PRRs are also recruited to lipid rafts and if the integrity of lipid raft microdomains is important for the cellular functions initiated by these innate receptors.

Dectin-1 or Clec7a is a member of the C-type lectin receptor (CLR) family and functions as a pattern-recognition receptor by binding β -glucan found in the cell walls of pathogenic fungi such as *Candida albicans* (17–19). It is expressed mainly on innate cells such as macrophages, neutrophils, and dendritic cells (DCs) and plays an important role in anti-fungal immunity (17, 20, 21). Dectin-1 has been shown to mediate the phagocytosis of yeast and yeast-derived particles such as zymosan, and it could also activate the production of inflammatory cytokines in macrophages and DCs upon binding its ligands (18, 22–25).

Stimulation of Dectin-1 by zymosan or its specific ligand β -glucan activates Syk tyrosine kinase (23, 24) and leads to the subsequent activation of NF κ B via the Card9-Bcl10-Malt1 complex (24, 26). Recently, we demonstrated that stimulation of Dectin-1 also activated phospholipase C γ 2 (PLC γ 2) and led to the induction of Ca²⁺ flux and activation of NF κ B in DCs (27). Dectin-1 possesses an immunotyrosine-activated motif (ITAM) in its cytoplasmic tail (23, 24), suggesting that it is capable of mediating its own signal transduction, and more importantly, could signal in a manner analogous to the BCR and TCR. However, unlike the classical ITAM found in lymphocyte antigen receptors that comprises two tandem YXXL (where X is any

* This work was supported by the Biomedical Research Council of A*STAR.

¹ To whom correspondence may be addressed: Laboratory of Immunology, Bioprocessing Technology Inst., Agency for Science, Technology and Research, 20 Biopolis Way, 06-01 Centros, Singapore 138668, Singapore. Tel.: 65-64070026; Fax: 65-64789561; E-mail: xu_shengli@bti.a-star.edu.sg.

² To whom correspondence may be addressed: Laboratory of Immunology, Bioprocessing Technology Inst., Agency for Science, Technology and Research, 20 Biopolis Way, 06-01 Centros, Singapore 138668, Singapore. Tel.: 65-64070026; Fax: 65-64789561; E-mail: lam_kong_peng@bti.a-star.edu.sg.

³ The abbreviations used are: BCR, B-cell receptor; TCR, T-cell receptor; CLR, C-type lectin receptor; DC, dendritic cell; BMDC, bone marrow-derived DC; PRRs, pattern recognition receptors; TLR, Toll-like receptor; PLC, phospholipase C; Syk, spleen tyrosine kinase; NF κ B, nuclear factor κ B; IL, interleukin; ITAM, immunotyrosine-activated-motif; ERK, extracellular signal-regulated kinase; HRP, horseradish peroxidase; FITC, fluorescein isothiocyanate; CTB, cholera toxin B; DAPI, 4',6-diamidino-2-phenylindole; β mD, methyl- β -cyclodextrin; TNF, tumor necrosis factor.

Role of Lipid Rafts in Dectin-1 Signaling

amino acid) sequences, Dectin-1 has only a single YXXL and is frequently referred to as possessing a “hemi-ITAM.”

Given that Dectin-1 and BCR share some downstream signaling molecules such as Syk and PLC γ 2 but differ in the structure of their ITAMs, we wonder if Dectin-1 would signal via lipid rafts in a manner similar to BCR. In this report, we show that Dectin-1 as well as its downstream signaling molecules, Syk and PLC γ 2, translocate to the lipid rafts upon stimulation of DCs with zymosan or β -glucan. And more importantly, we demonstrate that the integrity of the lipid raft is important for Dectin-1 to induce Ca²⁺ flux and activate phagocytosis and cytokine production in DCs upon its activation.

EXPERIMENTAL PROCEDURES

Mice and Cells—C57BL/6 mice were bred and maintained in our animal facilities and used with approval from the Institutional Animal Care and Use Committee according to guidelines issued by the National Advisory Committee on Laboratory Animal Research. Bone marrow-derived dendritic cells (BMDCs) were differentiated as described (27). Briefly, bone marrow cells were cultured in Dulbecco's modified Eagle's medium supplemented with the necessary additives and 10% supernatant from GM-CSF-transduced X63 cells (a kind gift from Dr. C. Ruedl, Nanyang Technological University, Singapore). After 5–7 days of culture, BMDCs were purified with anti-CD11c mAb-coupled magnetic beads (Miltenyl Biotech), and cell purity was routinely verified by flow cytometry to be >90%.

Reagents—The following reagents were purchased: zymosan (Invivogen), zymosan-FITC (Invitrogen), curdlan (Wako), cytochalasin D (Calbiochem), methyl- β -cyclodextrin (β mD), and FITC-conjugated cholera toxin B (FITC-CTB) (Sigma). Antibodies used for flow cytometry were from BD Pharmingen and included those specific for CD11c (HL3), CD40 (3/23), and CD86 (GL1). Antibodies used for immunoblot analyses were as follows: from Santa Cruz Biotechnology: anti-ERK2 (C-14), anti-phospho-ERK (E-4), anti-PLC γ 2 (G-20), anti-caveolin-1 (N-20), anti-dectin-1 (N-16), goat anti-rabbit IgG-HRP, and goat anti-mouse IgG-HRP; from Cell Signaling: anti-Syk and anti-phospho-Syk (Tyr-525/526). Anti-mouse-Dectin-1-PE antibody was from R&D systems.

BMDC Stimulation Assay—For analysis of cytokine production and cell surface marker expression, 5×10^5 BMDCs were cultured in complete DMEM with or without $3 \mu\text{M}$ β mD and treated with $50 \mu\text{g/ml}$ zymosan or $100 \mu\text{g/ml}$ curdlan for 4 h in 12-well plates. Cytokines in the supernatants were measured using ELISA kits from BD Pharmingen. Cell surface markers expression was examined on a FACSCalibur (BD Biosciences) and analyzed with FlowJo software (TreeStar). For biochemical analyses, 5×10^6 BMDCs were stimulated for various times with $100 \mu\text{g/ml}$ zymosan or $500 \mu\text{g/ml}$ curdlan and processed for Western blotting.

Calcium Flux—For analysis of calcium flux, $5\text{--}10 \times 10^6$ ml of BMDCs were loaded with Indo-1 AM ($2 \mu\text{M}$, Molecular Probes) as described previously (27). After resting for 30 min, cells were stimulated with $100 \mu\text{g/ml}$ zymosan, and calcium flux was monitored on a LSR II or FACSAria flow cytometer in real time for 6–8 min. The kinetics studies of calcium flux were performed with FlowJo software.

Isolation of Lipid Raft Fractions—Lipid rafts were prepared as described previously (28). Briefly, BMDCs were lysed in 0.05% Triton X-100 in TNEV buffer (150 mM NaCl, 5 mM EDTA, and 25 mM Tris-HCl, pH 7.4), followed by addition of equal volume of 80% sucrose in lysis buffer and overlaid with 30 and 5% sucrose in the same buffer, respectively. Fractionation was performed in a SW60Ti rotor for 18 h at 4 °C and at $200,000 \times g$. Eleven fractions were collected and lipid raft fractions (corresponding to 3rd, 4th, and 5th fractions) were solubilized in 10 mM *n*-octyl β -D-glucopyranoside (Sigma).

Immunofluorescence and Confocal Microscopy—For immunofluorescence staining, BMDCs were allowed to adhere on fibronectin ($10 \mu\text{g/ml}$)-coated culture slides (BD Biosciences, Bedford, MA) for 2 h in 37 °C, 5% CO₂ incubator. DCs were stimulated with 50 ng/ml zymosan for 0, 10, and 20 min. The reaction was stopped by addition of ice-cold RPMI1640 medium (Invitrogen) containing $200 \mu\text{M}$ vanadate, and $500 \mu\text{M}$ sodium fluoride. After fixation in 1% paraformaldehyde, cells were stained with FITC-CTB and anti-mouse Dectin-1-PE, respectively. Slides were then mounted in Pro-Long Gold antifade mounting reagent with DAPI (Molecular Probes). Images were acquired with inverted laser-scanning confocal microscope (Carl Zeiss Inc.) and analyzed using Zen software (Carl Zeiss Inc.).

Western Blot Analyses—After stimulation, cells were collected and lysed with buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Igepal CA-630, 0.2 mM Na₃VO₄) and a protease inhibitor mixture (Roche Applied Science) to obtain whole cell extracts. The protein concentration was measured by a colorimetric assay (Bio-Rad). Subsequently, proteins were separated by SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and probed with various primary antibodies. Signals were visualized with SuperSignal West Pico/Dura chemiluminescent substrate (Pierce) after incubation with HRP-conjugated secondary antibodies.

Phagocytosis Assays—Phagocytosis of zymosan by BMDCs was quantified by flow cytometry as described before (23). Briefly, 1×10^6 BMDCs were nontreated or pretreated with 5 mM β mD for 30 min. When required, cells were further treated with $5 \mu\text{M}$ cytochalasin D for 40 min. Cells were stimulated with $50 \mu\text{g/ml}$ zymosan-FITC for 30 min, washed with phosphate-buffered saline (PBS), and resuspended in PBS containing 1 mM EDTA, 1 mM sodium azide, and 2.5 units/ml proteinase K (Sigma). The suspension was incubated at room temperature for 20 min to remove all noninternalized zymosan particles. Phagocytosis was examined on a FACSCalibur and analyzed with FlowJo software.

RESULTS

Dectin-1 Localizes to Lipid Rafts upon Stimulation of DCs with Zymosan or Curdlan—As Dectin-1 possesses a hemi-ITAM in its cytoplasmic tail and has been postulated to signal in a manner analogous to lymphocyte antigen receptors (23, 24), and it is known that BCR and TCR could translocate to cell membrane lipid raft microdomains upon antigen recognition (11, 13), we wondered whether Dectin-1 would also be recruited to lipid rafts upon its activation by ligands. To test this possibility, we stimulated BMDCs with zymosan, a yeast deriv-

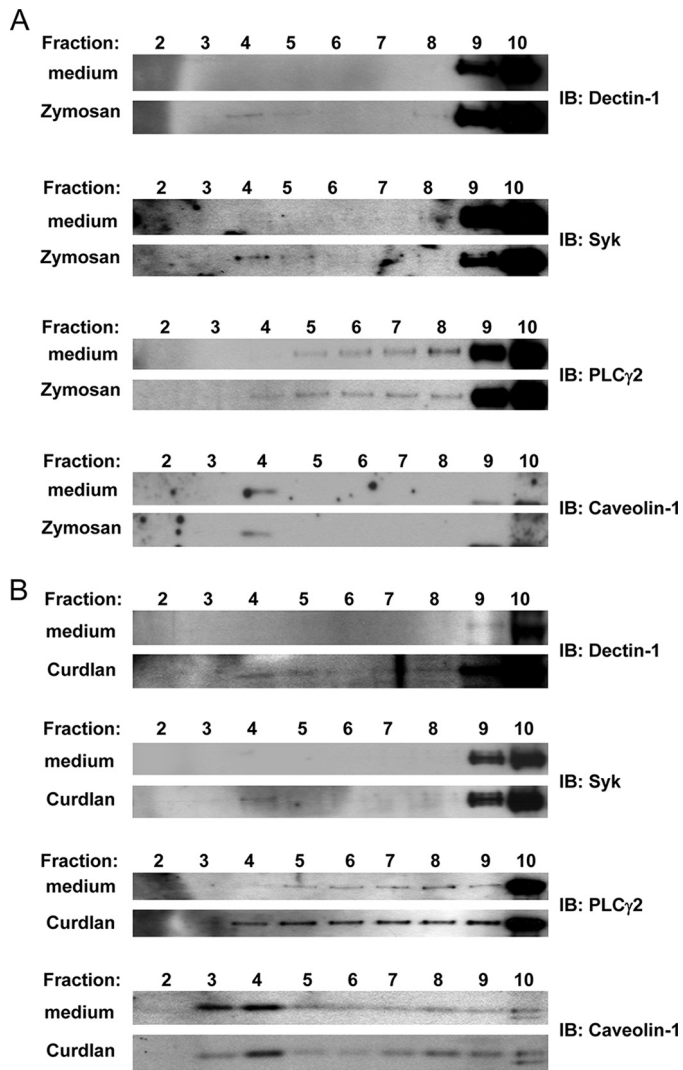


FIGURE 1. Stimulation of DCs with zymosan or curdlan leads to the localization of Dectin-1 and its associated signaling molecules Syk and PLC γ 2 to lipid rafts. Wild-type BMDCs were stimulated with zymosan (A) or curdlan (B), and whole cell lysates were separated into lipid raft and cytosolic fractions by sucrose gradient ultracentrifugation. The presence of Dectin-1, Syk, and PLC γ 2 in lipid rafts was determined by immunoblotting with specific antibodies. The anti-caveolin-1 blot was included to identify the lipid raft fractions. Data shown are representative of at least five independent experiments.

ative that could engage Dectin-1, and isolated lipid raft and cytosolic fractions from cell lysates to examine the localization of Dectin-1. As shown in Fig. 1A, there was no detectable Dectin-1 in the lipid rafts (fractions 3 and 4 as determined by the presence of the lipid raft-enriched caveolin-1) of BMDCs prior to any stimulation. However, treatment with zymosan readily resulted in the translocation of Dectin-1 to the lipid raft fractions of BMDCs, suggesting that the receptor sequesters to special cell membrane microdomains upon its activation.

Zymosan engages both Dectin-1 and TLR2 (18, 22) and it could be argued that Dectin-1 alone would not necessarily translocate to lipid raft upon activation. Thus we repeated the experiment using curdlan or β -glucan that specifically stimulated Dectin-1. Consistent with our finding in Fig. 1A, curdlan stimulation of BMDCs also resulted in the recruitment of Dectin-1 to the lipid rafts of BMDCs (Fig. 1B).

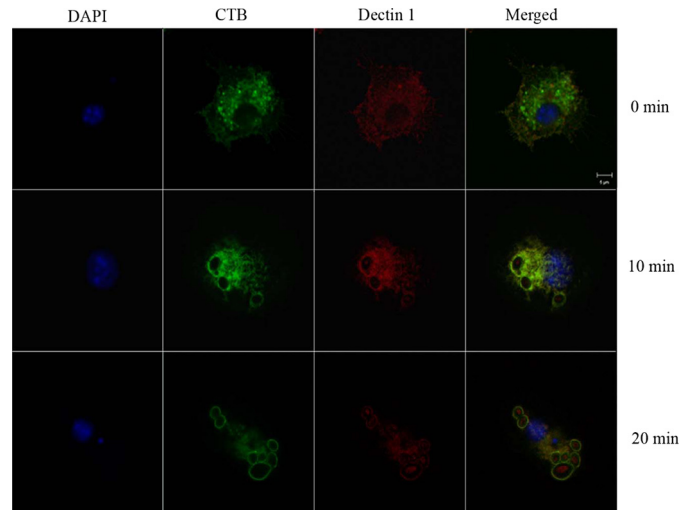


FIGURE 2. Visualization of Dectin-1 in lipid rafts of zymosan-stimulated DCs by confocal microscopy. Wild-type BMDCs were stimulated with zymosan and examined by confocal microscopy for the localization of Dectin-1 using PE-conjugated specific antibody (red). Lipid rafts were identified by staining with FITC-conjugated cholera toxin B (CTB) (green) while the nuclear compartment was visualized by DAPI-staining of DNA (blue). Data shown are representative of more than five independent experiments.

To independently verify that Dectin-1 could relocate to lipid raft upon its activation, we visualized the translocation by confocal microscopy using fluorochrome-conjugated anti-Dectin-1 antibody and cholera toxin B (CTB), which stains lipid rafts (Fig. 2). Prior to any stimulation, Dectin-1 was found to be diffusely expressed on the cell membrane of BMDCs. However, at 10 min after the addition of ligand, the CTB-stained lipid rafts were found to aggregate at the site of zymosan contact on the BMDCs. Dectin-1 receptors were also found to aggregate at the site of zymosan contact and more importantly, they co-localized with the CTB-stained lipid rafts. Interestingly, at 20 min after stimulation, both Dectin-1 and CTB-stained lipid rafts were found to be co-localized around cellular structures that appeared to be phagosomes. Thus, taken together, the data demonstrated unequivocally that Dectin-1 localizes to the lipid raft microdomains of BMDCs upon its activation.

Syk and PLC γ 2 Are Recruited Along with Activated Dectin-1 to Lipid Rafts of DCs—In lymphocytes, the activation and subsequent lipid raft localization of either BCR or TCR is accompanied by the recruitment of key proximal signaling molecules such as, respectively, the tyrosine kinase Syk or ZAP-70, the adaptor protein BLNK or SLP-76, and the phospholipases PLC γ 2 or PLC γ 1, to the lipid raft microdomains of B or T cells. Because Syk has been shown to be a proximal tyrosine kinase in Dectin-1 signaling (23, 24) and we have also demonstrated recently that PLC γ 2 is involved in Dectin-1 signal transduction (27), we proceeded to examine if these two molecules would also translocate to lipid rafts when we stimulated BMDCs with zymosan or curdlan. Indeed, as seen in Fig. 1A, both Syk and PLC γ 2 were recruited to the lipid rafts of BMDCs upon Dectin-1 activation by zymosan. Likewise, stimulation of BMDCs with curdlan also resulted in the localization of Syk and PLC γ 2 to the lipid rafts of these cells (Fig. 1B). Thus, the data so far were consistent with the recruitment of activated Dectin-1 and

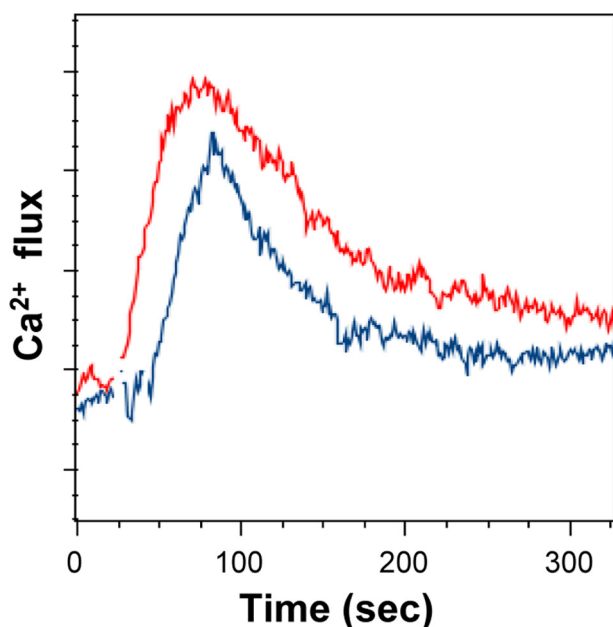


FIGURE 3. Disruption of lipid rafts with β mD attenuates Ca^{2+} signaling in zymosan-stimulated DCs. Indo-1 ($2 \mu\text{M}$) loaded wild-type BMDCs were either untreated (red histogram) or pretreated with 5 mM of β mD (blue histogram) for 30 min prior to being stimulated with $100 \mu\text{g/ml}$ zymosan and assayed for intracellular Ca^{2+} flux. Intracellular Ca^{2+} flux was depicted as a ratio of Indo-1 violet/blue fluorescence versus time. Data shown are representative of five independent experiments.

its associated receptor proximal signaling molecules to the lipid rafts of DCs to facilitate receptor signaling.

Disruption of Lipid Raft Microdomains Attenuates Dectin-1-induced Intracellular Ca^{2+} Flux and Syk, ERK Activation in DCs—To assess the significance of the localization of activated Dectin-1 to lipid rafts and especially with respect to the signaling functions of the receptor, we pretreated BMDCs with β mD, which is known to result in the dispersion of the cholesterol-rich lipid rafts by sequestering the cholesterol moieties (29), prior to stimulating the cells with zymosan or curdlan and examining the downstream signaling events triggered by the engagement of Dectin-1.

We had previously shown that the stimulation of Dectin-1 with zymosan led to the activation of intracellular Ca^{2+} flux in BMDCs (27). Hence, we examined the ability of Dectin-1 to trigger Ca^{2+} flux in BMDCs in the presence of β mD. As shown in Fig. 3, although zymosan could still induce a Ca^{2+} flux in BMDCs that had been pretreated with 5 mM of β mD, the pattern of Ca^{2+} signaling was, however, altered such that it was delayed and reduced in magnitude and became less sustained compared with the Ca^{2+} flux elicited in the control sample. These data suggested that the localization of activated Dectin-1 to lipid raft is important for its induction of maximal intracellular Ca^{2+} flux.

Next, we examined whether any other downstream signaling events would also be affected when the recruitment of Dectin-1 to lipid raft was disrupted. It is well documented that Dectin-1 signaling activates Syk and ERK (23, 24, 30), and we showed in Fig. 1 that Syk could also translocate to lipid rafts of BMDCs upon Dectin-1 engagement by zymosan or curdlan. Hence, we determined if the activation of either Syk or ERK or both via Dectin-1 would be compromised when the aggregation of lipid

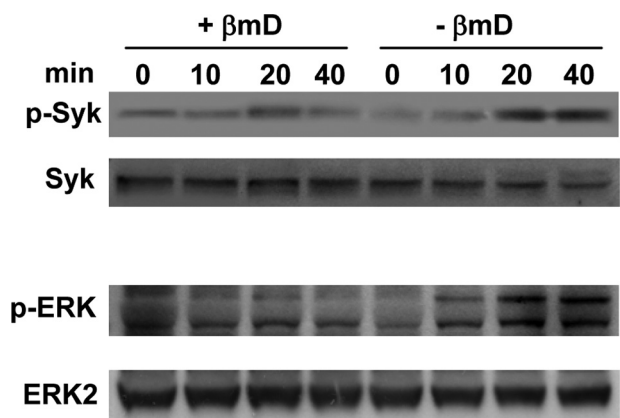


FIGURE 4. Compromised Syk, ERK activation in β mD-treated DCs upon curdlan stimulation. Wild-type BMDCs were non-treated (lanes 5–8) or pretreated with 5 mM β mD (lanes 1–4) and stimulated with $500 \mu\text{g/ml}$ curdlan for various times. Cell lysates were isolated and immunoblotted with anti-phospho-Syk and anti-phospho-ERK antibodies, and re-probed with anti-Syk, anti-ERK2 antibodies to control for the equal loading of cell lysates. Data shown are representative of 3–5 independent experiments.

rafts is prevented in BMDCs by treatment with β mD. Indeed, as shown in Fig. 4, curdlan-stimulated activation of Syk, as indicated by its phosphorylation, was delayed and appeared weaker and less sustained in the β mD-treated sample (lanes 1–4) compared with the control sample (lanes 5–8), suggesting that the activation of Syk was affected when lipid rafts were disrupted. More remarkably, the curdlan-induced activation of ERK, again as indicated by its phosphorylation status (Fig. 4), was drastically dampened and almost completely abrogated when the stimulated BMDCs were pretreated with β mD.

Lipid Raft Integrity Is Important for Dectin-1 Activation of DC Maturation and Secretion of Key Cytokines—We have so far demonstrated that Dectin-1 and its associated signaling molecules were recruited to lipid rafts upon the activation of BMDCs by zymosan or curdlan and that this translocation was critical for its downstream signal transduction process. Because the loss of lipid raft integrity leads to compromised Dectin-1 signaling, we next asked if it would result in any noticeable cellular defect in BMDCs.

Activation of DCs by surface innate receptors such as the TLRs or CLRs could trigger the cellular maturation of DCs and leads to their up-regulation of maturation or activation markers such as CD40 and co-stimulatory molecules CD80 and CD86 (30). Thus, we examined the expression of CD40 and CD86 as surrogate markers of DC maturation in experiments where DCs were activated by Dectin-1 in the presence of β mD. As shown in Fig. 5A, activation of DCs with zymosan or curdlan readily led to the up-regulation of CD40 on the cell surfaces of these cells. However, these ligands failed to induce the up-regulation of CD40 on BMDCs when the integrity of their lipid rafts was compromised by β mD treatment. A similar finding was obtained with CD86 (Fig. 5B) where prior β mD treatment compromised and affected the up-regulation of this co-stimulatory molecule on BMDCs upon their stimulation with zymosan or curdlan. Taken together, these data indicate that the integrity of the lipid raft microdomains is important for the maximal maturation of DCs upon Dectin-1 recognition of its ligands.

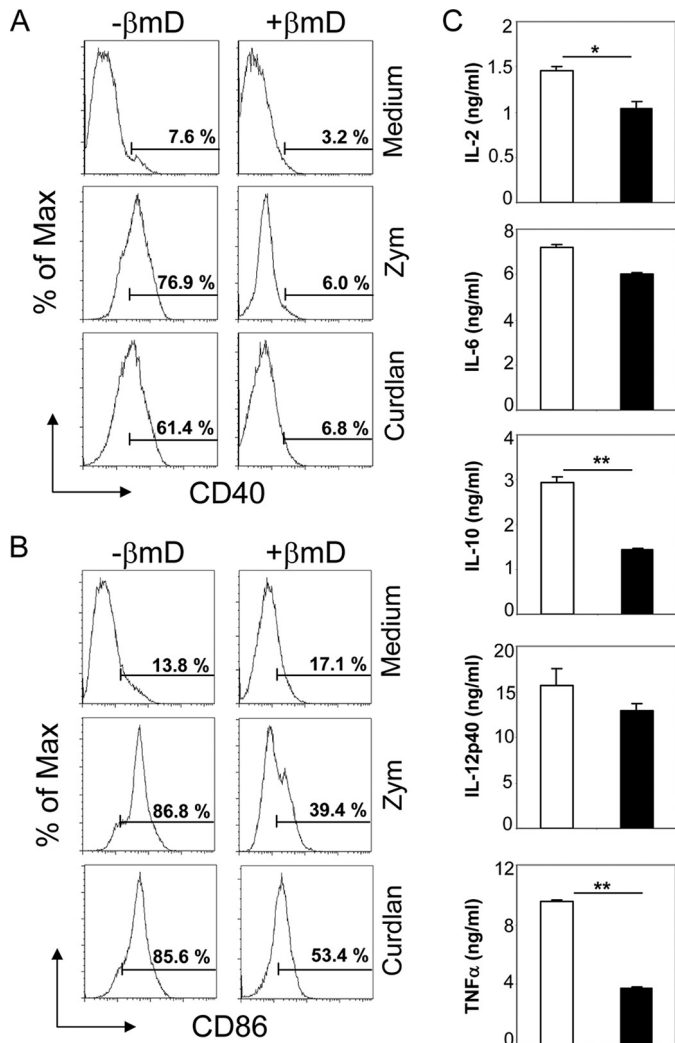


FIGURE 5. Disruption of lipid raft microdomains with β mD affects DC maturation and cytokine production in response to Dectin-1 stimulation. Wild-type BMDCs were stimulated with 50 μ g/ml zymosan (*Zym*) or 100 μ g/ml curdlan in the absence or presence of 3 mM β mD and their up-regulation of CD40 (A) and CD86 (B) monitored via flow cytometry. Cytokine production (C), measured via ELISA, by curdlan-stimulated BMDCs that were nontreated (white column) or pretreated with β mD (black columns). Results shown are mean values + S.D. of triplicates and are representative of five independent experiments. *, $p < 0.05$; **, $p < 0.01$.

Activated DCs are also known to secrete inflammatory cytokines upon the stimulation of their cell surface innate receptors. Dectin-1 is known to trigger the secretion of TNF α , IL-2, and IL-10 in BMDCs (23, 30). In particular, the Syk and ERK pathway downstream of Dectin-1 has been demonstrated to be intimately involved in the induction of IL-2 and IL-10 production in BMDCs upon Dectin-1 engagement (31). Hence, we examined the ability of zymosan- or curdlan-treated BMDCs to produce cytokines in the presence or absence of β mD treatment. As seen in Fig. 5C, the production of TNF α , IL-2, and IL-10 was significantly reduced when BMDCs were pretreated with β mD and subsequently stimulated with curdlan. On the other hand, the secretion of IL-6 and IL-12p40 was only marginally compromised by the β mD treatment. Nevertheless, our data collectively indicate that the recruitment of activated Dectin-1 to lipid raft is important for its induction of key cytokine production in DCs.

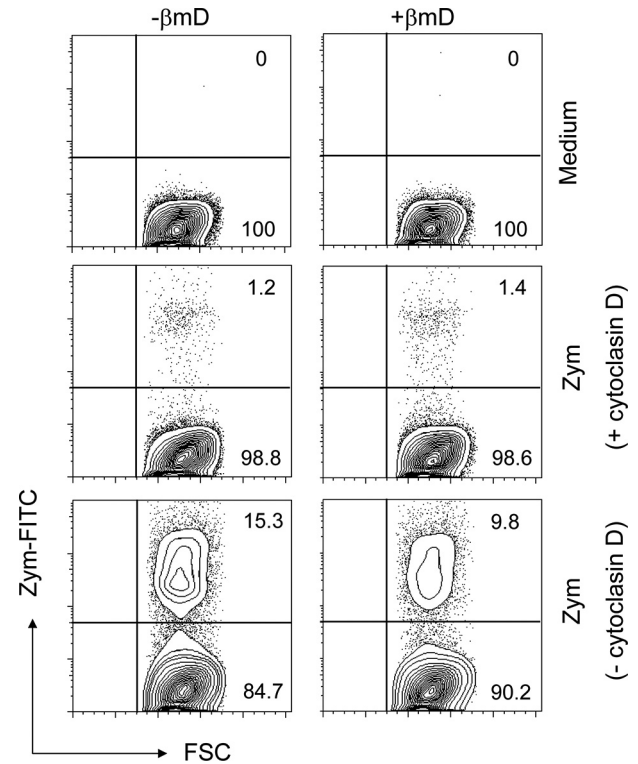


FIGURE 6. Phagocytosis of zymosan particles is impaired in β mD-treated DCs. Wild-type BMDCs were nontreated or pretreated with 5 mM of β mD for 30 min followed by incubation with 50 μ g/ml zymosan-FITC (*Zym-FITC*) for 30 min. Cytochalasin D was used to inhibit actin polymerization and served as a control. The extent of phagocytosis of zymosan by BMDCs was assessed by flow cytometry. Data shown are representative of four independent experiments.

The Integrity of Lipid Raft Microdomains Is Critical for Dectin-1-mediated Phagocytosis of Zymosan Particles in DCs—Our confocal microscopy analysis in Fig. 2 suggested that intact lipid rafts might also be important for the formation of phagosomes as both lipid rafts and Dectin-1 were shown to aggregate around the zymosan particles at 20 min after stimulation. Hence, we examined if the ability of Dectin-1-activated DCs to phagocytose fluorochrome-conjugated zymosan particles would be compromised when their lipid rafts were disrupted by β mD treatment.

We first treated BMDCs with the actin polymerization inhibiting reagent cytochalasin D to determine the background level of zymosan uptake by BMDCs in the presence or absence of β mD and found them to be equivalent and minimal (Fig. 6). Next, we showed that the pretreatment of BMDCs with β mD significantly reduced the level of phagocytosis as the fraction of cells that have ingested FITC-labeled zymosan (9.8%) was very much reduced when compared with the nontreated sample (15.3%). This suggests that the integrity of lipid rafts and the recruitment of Dectin-1 to lipid raft microdomains are important for Dectin-1-mediated phagocytosis of yeast derivatives.

DISCUSSION

The anti-fungal receptor Dectin-1 has previously been postulated to signal in a manner analogous to the lymphocyte antigen receptor (BCR and TCR), namely utilizing its hemi-ITAM containing cytoplasmic tail to activate downstream signaling

Role of Lipid Rafts in Dectin-1 Signaling

molecules upon receptor stimulation (23, 24). It has been established that following antigen stimulation, the BCR rapidly translocates into cholesterol- and sphingolipid-rich lipid rafts, which are specialized plasma membrane microdomains that serve as signaling platforms. We demonstrate here that apart from sharing some signaling components with the BCR signaling pathway, such as Syk and PLC γ 2, Dectin-1 also translocates to the lipid rafts of DCs upon its stimulation by zymosan or curdlan.

Although we can definitely detect the presence of Dectin-1 in the lipid raft fractions of zymosan- or curdlan-treated DCs but not in that of the unstimulated DCs using sucrose gradient ultracentrifugation, a considerably large amount of Dectin-1 is still in the soluble fractions. This could be due to the fact that both zymosan and curdlan are particulate agonists of Dectin-1, so at any one-time only a fraction of the DCs and hence Dectin-1 may have contact with the agonists. This is different from stimulations of BCR and TCR using soluble antibodies. Nevertheless, when we examined lipid rafts at a single DC level using confocal microscopy, we can clearly show the co-localization of Dectin-1 and lipid rafts marker CTB in zymosan-treated but not in resting DCs. Moreover, we found that both lipid rafts and Dectin-1 become more aggregated at the contact points of DCs with zymosan particles (Fig. 2). This could reflect the fact that prior to receptor stimulation, lipid rafts are small (<60 nm in diameter) and the random coalescence of small pieces of lipid rafts is not able to form stable platforms to trigger signaling. Upon stimulation, more stable and larger lipid rafts form and the engaged Dectin-1 receptors translocate to the same region of the membrane.

In BCR signaling, the translocation of the antigen receptor into lipid rafts leads to the recruitment of the adaptor protein BLNK, the tyrosine kinases Lyn and Syk as well as tyrosine phosphatase CD45 into the lipid rafts of B cells (32, 33). Similarly, we show here that two key immediate signaling molecules downstream of Dectin-1, Syk (23, 24), and PLC γ 2 (27), are also recruited to the lipid rafts of DCs upon Dectin-1 stimulation. These data would suggest that stimulation of Dectin-1 further results in the concentration of its key associated signaling transducers in lipid rafts for optimal signaling.

In support of this hypothesis, the extraction of cholesterol by the synthetic drug β mD that disrupts lipid rafts and its aggregation, results in a series of compromised signaling events downstream of Dectin-1, including lower and less sustained Ca²⁺ flux and defective Syk and ERK activation upon Dectin-1 stimulation in DCs. Consequently, the up-regulation of DC maturation markers CD40 and CD86 is dramatically impaired. Moreover, the production of cytokines such as IL-2, IL-10, and TNF α is also decreased. The impairment in IL-2 and IL-10 secretion could be directly attributed to the compromised ERK activation in the β mD-treated DCs as the ERK pathway has been shown to be critical in triggering the secretion of these two cytokines (26, 31).

Another interesting finding of our current work is that other than mediating the up-regulation of maturation markers and production of cytokines, lipid rafts may also be important for the formation of phagosomes in Dectin-1-activated DCs and concomitantly, the phagocytosis of yeast derivatives initiated

by the activation of Dectin-1. This is evident in our confocal microscopy study, in which we demonstrate a more obvious aggregation of lipid rafts and Dectin-1 around zymosan particles at the 20-min time point. It has been demonstrated previously that after 15 min of stimulation of macrophages with zymosan, hemi-ITAM-associated phosphorylated Syk and Src from Dectin-1 were found in phagosomes (23). Another previous study showed that Syk was required for the phagocytosis of zymosan by DCs (24). Our data further indicate that lipid rafts, in which the activated Dectin-1 localizes, are also associated with phagosomes. When lipid rafts are disrupted by β mD, the uptake of fluorochrome-labeled zymosan by DCs is drastically impaired (Fig. 5). Thus, the presence of intact and aggregated lipid rafts is important to initiate Dectin-1 proximal signaling cascades, leading to the phagocytosis of yeast derivatives and the activation of DCs in the production of cytokines.

It has been established that ITAM-containing receptors such as BCR, TCR, and FcR employ lipid rafts as a signaling platform to concentrate downstream signaling molecules and subsequently initiate signal transduction. Our study suggests that the hemi-ITAM bearing receptor Dectin-1, a member of the innate immune C-type lectin receptor family, also translocates into lipid rafts upon activation and utilizes lipid rafts for signal propagation and cell activation in DCs. It is likely that other members of the CLRs, which signal via ITAM or ITAM-like domains, also require lipid rafts microdomains for their signal transduction and the induction of their physiological functions.

Acknowledgments—We thank members of the LAM laboratory for insightful discussions.

REFERENCES

1. Simons, K., and Ikonen, E. (1997) *Nature* **387**, 569–572
2. Brown, D. A., and London, E. (1998) *Annu. Rev. Cell Dev. Biol.* **14**, 111–136
3. Kurzchalia, T. V., and Parton, R. G. (1999) *Curr. Opin. Cell Biol.* **11**, 424–431
4. Aman, M. J., Tosello-Trampont, A. C., and Ravichandran, K. (2001) *J. Biol. Chem.* **276**, 46371–46378
5. Locke, D., Chen, H., Liu, Y., Liu, C., and Kahn, M. L. (2002) *J. Biol. Chem.* **277**, 18801–18809
6. Lang, M. L., Chen, Y. W., Shen, L., Gao, H., Lang, G. A., Wade, T. K., and Wade, W. F. (2002) *Biochem. J.* **364**, 517–525
7. Vámosi, G., Bodnár, A., Vereb, G., Jenei, A., Goldman, C. K., Langowski, J., Tóth, K., Mátyus, L., Szöllösi, J., Waldmann, T. A., and Damjanovich, S. (2004) *Proc. Natl. Acad. Sci. U.S.A.* **101**, 11082–11087
8. Kondadasula, S. V., Roda, J. M., Parihar, R., Yu, J., Lehman, A., Caligiuri, M. A., Tridandapani, S., Burry, R. W., and Carson, W. E., 3rd (2008) *Blood* **111**, 4173–4183
9. Vereb, G., Matkó, J., Vámosi, G., Ibrahim, S. M., Magyar, E., Varga, S., Szöllösi, J., Jenei, A., Gáspár, R., Jr., Waldmann, T. A., and Damjanovich, S. (2000) *Proc. Natl. Acad. Sci. U.S.A.* **97**, 6013–6018
10. Seminario, M. C., and Wange, R. L. (2003) *Immunol. Rev.* **192**, 80–97
11. Pierce, S. K. (2002) *Nat. Rev. Immunol.* **2**, 96–105
12. Gupta, N., and DeFranco, A. L. (2007) *Semin. Cell Dev. Biol.* **18**, 616–626
13. He, H. T., Lellouch, A., and Marguet, D. (2005) *Semin. Immunol.* **17**, 23–33
14. Triantafílu, M., Gamper, F. G., Haston, R. M., Mouratis, M. A., Morath, S., Hartung, T., and Triantafílu, K. (2006) *J. Biol. Chem.* **281**, 31002–31011
15. Soong, G., Reddy, B., Sokol, S., Adamo, R., and Prince, A. (2004) *J. Clin. Invest.* **113**, 1482–1489

16. Nakahira, K., Kim, H. P., Geng, X. H., Nakao, A., Wang, X., Murase, N., Drain, P. F., Wang, X., Sasidhar, M., Nabel, E. G., Takahashi, T., Lukacs, N. W., Rytter, S. W., Morita, K., and Choi, A. M. (2006) *J. Exp. Med.* **203**, 2377–2389
17. Brown, G. D., and Gordon, S. (2001) *Nature* **413**, 36–37
18. Gantner, B. N., Simmons, R. M., Canavera, S. J., Akira, S., and Underhill, D. M. (2003) *J. Exp. Med.* **197**, 1107–1117
19. Taylor, P. R., Tsoni, S. V., Willment, J. A., Dennehy, K. M., Rosas, M., Findon, H., Haynes, K., Steele, C., Botto, M., Gordon, S., and Brown, G. D. (2007) *Nat. Immunol.* **8**, 31–38
20. Brown, G. D., Taylor, P. R., Reid, D. M., Willment, J. A., Williams, D. L., Martinez-Pomares, L., Wong, S. Y., and Gordon, S. (2002) *J. Exp. Med.* **196**, 407–412
21. Ariizumi, K., Shen, G. L., Shikano, S., Xu, S., Ritter, R., 3rd, Kumamoto, T., Edelbaum, D., Morita, A., Bergstresser, P. R., and Takashima, A. (2000) *J. Biol. Chem.* **275**, 20157–20167
22. Brown, G. D., Herre, J., Williams, D. L., Willment, J. A., Marshall, A. S., and Gordon, S. (2003) *J. Exp. Med.* **197**, 1119–1124
23. Underhill, D. M., Rossnagle, E., Lowell, C. A., and Simmons, R. M. (2005) *Blood* **106**, 2543–2550
24. Rogers, N. C., Slack, E. C., Edwards, A. D., Nolte, M. A., Schulz, O., Schweighoffer, E., Williams, D. L., Gordon, S., Tybulewicz, V. L., Brown, G. D., and Reis e Sousa, C. (2005) *Immunity* **22**, 507–517
25. Herre, J., Marshall, A. S., Caron, E., Edwards, A. D., Williams, D. L., Schweighoffer, E., Tybulewicz, V., Reis e Sousa, C., Gordon, S., and Brown, G. D. (2004) *Blood* **104**, 4038–4045
26. Gross, O., Gewies, A., Finger, K., Schäfer, M., Sparwasser, T., Peschel, C., Förster, I., and Ruland, J. (2006) *Nature* **442**, 651–656
27. Xu, S., Huo, J., Lee, K. G., Kurosaki, T., and Lam, K. P. (2009) *J. Biol. Chem.* **284**, 7038–7046
28. Xu, S., Huo, J., Tan, J. E., and Lam, K. P. (2005) *Mol. Cell. Biol.* **25**, 8486–8495
29. Harder, T., Kellner, R., Parton, R. G., and Gruenberg, J. (1997) *Mol. Biol. Cell* **8**, 533–545
30. LeibundGut-Landmann, S., Gross, O., Robinson, M. J., Osorio, F., Slack, E. C., Tsoni, S. V., Schweighoffer, E., Tybulewicz, V., Brown, G. D., Ruland, J., and Reis e Sousa, C. (2007) *Nat. Immunol.* **8**, 630–638
31. Slack, E. C., Robinson, M. J., Hernanz-Falcón, P., Brown, G. D., Williams, D. L., Schweighoffer, E., Tybulewicz, V. L., and Reis e Sousa, C. (2007) *Eur. J. Immunol.* **37**, 1600–1612
32. Guo, B., Kato, R. M., Garcia-Lloret, M., Wahl, M. I., and Rawlings, D. J. (2000) *Immunity* **13**, 243–253
33. Gupta, N., and DeFranco, A. L. (2003) *Mol. Biol. Cell* **14**, 432–444

Activated Dectin-1 Localizes to Lipid Raft Microdomains for Signaling and Activation of Phagocytosis and Cytokine Production in Dendritic Cells
Shengli Xu, Jianxin Huo, Merry Gunawan, I-Hsin Su and Kong-Peng Lam

J. Biol. Chem. 2009, 284:22005-22011.

doi: 10.1074/jbc.M109.009076 originally published online June 12, 2009

Access the most updated version of this article at doi: [10.1074/jbc.M109.009076](https://doi.org/10.1074/jbc.M109.009076)

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

This article cites 33 references, 17 of which can be accessed free at <http://www.jbc.org/content/284/33/22005.full.html#ref-list-1>