

Signal Transduction:
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Mast cells Dectin-1 in innate anti-fungal immunity

Dectin-1-mediated signaling leads to characteristic gene expressions and cytokine secretion via Syk in rat mast cells*

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Keywords

Dectin-1, Syk, mast cell, innate immunity, host defense, signal transduction, phosphotyrosine signaling, fungi

Capsule

Background: β -Glucan receptor Dectin-1 in dendritic cells and macrophages play important roles in anti-fungal immunity.

Results: Dectin-1 is expressed in rat mast cells and its tyrosine phosphorylation induces characteristic gene expression of transcription factors and cytokines through protein tyrosine kinase Syk.

Conclusion: Dectin-1 functions in rat mast cells.

Significance: Dectin-1-mediated signaling in mast cells may contribute to anti-fungal immunity.

ABSTRACT

Dectin-1 recognizes β -glucan and plays important roles for the anti-fungal immunity through the activation of Syk in dendritic cells (DCs) or macrophages. Recently, expression of Dectin-1 was also identified in human and mouse mast cells, although its physiological roles were largely unknown. In this report, rat mast cell line RBL-2H3 was analyzed to investigate the molecular mechanism of Dectin-1-mediated activation and responses of mast cells. Treatment of cells with Dectin-1-specific agonist Curdlan induced tyrosine phosphorylation of cellular proteins and the interaction of Dectin-1 with the Src homology 2 (SH2) domain of Syk. These responses depended on tyrosine phosphorylation of hemi-immunoreceptor tyrosine-based activation motif (hemITAM) in cytoplasmic tail of Dectin-1, whereas those were independent of γ -subunit of high-affinity IgE receptor (Fc ϵ RI γ). DNA microarray and real-time PCR analyses showed

that Dectin-1-mediated signaling stimulated gene expression of transcription factor Nfkbiz and inflammatory cytokines, such as monocyte chemoattractant protein-1 (MCP-1), IL-3, IL-4, IL-13 and tumor necrosis factor (TNF)- α . The response was abrogated by pretreatment with Syk inhibitor R406. These results suggest that Syk is critical for Dectin-1-mediated activation of mast cells, although the signaling differs from that triggered by Fc ϵ RI activation. In addition, these gene expressions induced by Curdlan stimulation were specifically observed in mast cells, suggesting that Dectin-1-mediated signaling of mast cells offers new insight into the anti-fungal immunity.

INTRODUCTION

Fungal infections are major health threat and clinical problems due to increasing numbers of immuno-compromised host because of the increase in immunosuppressive diseases such as AIDS and immunosuppressive therapies against chronic inflammatory diseases, autoimmune diseases, cancers and transplant rejections. Anti-fungal immunity, thus grows increasingly important, is initiated by recognition of fungal pathogens with innate immune receptors. Pattern recognition receptors, such as Toll-like receptors⁵ (TLRs) and C-type lectin receptors (CLRs), play important roles in the innate anti-microbial immunity by recognition of pathogen-associated molecular patterns (PAMPs), including carbohydrates, lipids, nucleic acids, and proteins. β -Glucan, a major carbohydrate component of fungal cell wall along with mannans and chitin, are known as a PAMP recognized by Dectin-1.

Dectin-1 is a type II transmembrane receptor which recognizes β -glucan. Dectin-1 was first identified by Ariizumi, *et al.* and originally thought to be a DC-specific receptor, from which its name ‘dendritic-cell-associated C-type lectin-1’ was derived (1). However the receptor is now known to be expressed by many other cell types, including macrophages, monocytes, neutrophils and T cells (2, 3). Especially in DCs and macrophages, Dectin-1-mediated mechanisms of anti-fungal immunity have been studied. The following shows the facts brought out by prior studies in DCs and macrophages.

Dectin-1 is composed of four domains, carbohydrate recognition domain (CRD), stalk domain, transmembrane domain, and cytoplasmic domain which possesses hemITAM. Alternative splicing produces two major isoforms, which vary by the inclusion, or exclusion, of the stalk region in rat (4), mouse (5) and human (6) (although Dectin-1 in human has eight splicing variants in total). The CRD of Dectin-1 specifically recognizes soluble and particle $\beta(1\rightarrow3)$ - and $\beta(1\rightarrow6)$ -linked glucan (2). In contrast to classic Ca^{2+} -dependent CLRs, CRD of Dectin-1 can recognize carbohydrate by Ca^{2+} -independent manner (2). Dectin-1 also recognizes impure particulate β -glucan zymosan, a stimulatory cell-wall extract of *Saccharomyces cerevisiae* that is composed mainly of β -glucan but also mannan, chitin, protein and lipid (7). A large number of receptors have been implicated in the recognition of zymosan, including mannose receptor (8), complement receptor 3 (9, 10), Dectin-1 (2) and TLR2 (11). Thereby, analysis using zymosan does not reflect the independent molecular mechanisms of Dectin-1, whereas zymosan acts as an ideal model of a complex microorganism displaying several PAMPs. Curdlan consists of purified $\beta(1\rightarrow3)$ -glucan polymer from *Alcaligenes faecalis*, therefore Curdlan was utilized as a specific agonist of Dectin-1 (12), in order to investigate independent molecular mechanism of Dectin-1 in this study.

Upon ligand binding, hemITAM of Dectin-1 is phosphorylated by Src family

protein-tyrosine kinases and recruits spleen tyrosine kinase (Syk) (10), which initiates a signaling cascade leading to nuclear factor- κ B (NF- κ B) (13, 14), nuclear factor of activated T-cells (NFAT) (15, 16), and MAPK activation (17-19). Traditional ITAM sequences, found in such as Fc receptors, consist of a tandem repeat of YXXI/L sequences (where X is any amino acid) which, on ligand binding and receptor clustering, become tyrosine-phosphorylated by Src kinases. In contrast to this, Dectin-1 has a single ITAM motif termed as hemITAM and phosphorylation of this hemITAM sequence is sufficient to mediate the interaction with Syk (which normally requires two phospho-tyrosines for binding) through an unknown mechanism (10, 20, 21). Syk kinase has two SH2 domains in tandem, which bind to specific phosphorylated tyrosine residues in protein and result in the assembly of signaling complexes (22). In the previous study used recombinant N-terminal (Syk-SH2(N)), C-terminal (Syk-SH2(C)), and tandem SH2 (Syk-SH2(NC)) domains of Syk to precipitate C-type lectin-like receptor 2 (CLEC2) both SH2 domains of Syk are required for binding and signaling downstream of CLEC2. This suggests that the mechanism of the binding of Syk to Dectin-1 is similar to CLEC2, because CLEC2 is a member of CLRs and has hemITAM as same as Dectin-1 (23). Through above, Dectin-1 activates a number of cellular responses, including phagocytosis (21) and reactive oxygen species (ROS) production (21, 24) and the production of various cytokines (IL-1, IL-2,

IL-6, IL-10, IL-12, IL-22, TNF- α) and chemokines (CCL17, CCL22) (25), leading to anti-fungal immunity.

Mast cells are now known to be critical effectors of not only allergic disease but also host defense (26, 27). Recently, it has been reported that Dectin-1 is expressed in mouse and human mast cells and its activation elicits leukotriene release, ROS production and Dectin-1 expression, indicating the relationship between mast cells and anti-fungal immunity (24, 28, 29). However, the signaling pathway and physiological roles of Dectin-1 in mast cells are still largely unknown. The purpose of this study is to investigate the molecular mechanism of Dectin-1-mediated activation and responses of mast cells in order to analyze how Dectin-1 in mast cells contributes to innate anti-fungal immunity.

EXPERIMENTAL PROCEDURES

Antibodies

Materials—Anti-phosphotyrosine (pY) (clone 4G10) and anti-GAPDH mAbs, and anti-phosphotyrosine (pY) (clone 4G10) agarose conjugate were purchased from Millipore (Bedford, MA, USA). Anti-dinitrophenyl (DNP) IgE mAb (clone SPE-7) was obtained from Sigma (St. Louis, MO, USA). Anti-mouse Dectin-1/CLEC7A polyclonal antibody was from R&D systems (Minneapolis, MN, USA). anti-phosphoERK, Anti-ERK polyclonal antibodies were from Cell signaling Technology (Danvers, MA, USA). Syk polyclonal antibody was raised against rat Syk-specific peptide (EPTGGAWGPDRGLC), as previously described (30). Anti-phospholipase C (PLC) γ 2 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protein A-agarose was from Sigma. Curdlan was from Wako (Osaka, Japan). Antigen DNP-BSA (30 mol of DNP/1 mol of BSA) was from LSL (Tokyo, Japan). Syk inhibitor R406 was from Selleck chemicals (Houston, TX, USA). BAY61-3606, PD98059, BAY11-7082, Cyclosporin A were from Wako. Curdlan was prepared as previously described (12). Curdlan is insoluble at neutral pH, thereby it was once solubilized with 0.15M NaOH solution at 10 mg/ml and then added to the culture medium to neutralize pH by diluting more than 100-fold.

cDNAs—The mouse Dectin-1A cDNA was obtained as follows. Total RNA from RAW 264.7 cells was isolated by using RNeasy mini kit

and

(Qiagen, Valencia, CA, USA) and the first-strand cDNA was generated by using superscript III (Life technologies, Carlsbad, CA, USA) with oligo(dT) primers, according to the manufacturer's instructions. The mouse Dectin-1A cDNA was subsequently amplified by PCR using following primers,

5'-CAAGTGCTCTGCCTACCTAGGGCCCTGT-3' (forward) and

5'-CACCATCTTTATATTCTCACATACATTTACAGTTCCTT-3' (reverse). The PCR product was subcloned into pGEM-T easy vector (Promega, Madison, WI, USA), and DNA sequence was verified. The cDNA fragment was inserted into pcDNA3.1(-) myc-His expression vector (Life technologies) to add epitope tag at the C-terminal site. The cDNA fragment encoding myc-His tagged Dectin-1A was transferred into pSVL expression vector (GE Healthcare, Buckinghamshire, UK). Substitution of Tyr¹⁵ to Phe (Y15F) by a point mutation of Dectin-1A cDNA was generated by QuickChange Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) using two primers, 5'-GAGAATCFTGGATGAAGATGGATTACTCAATTAGACTTCAGCAC-3' (forward) and 5'-GTGCTGAAGTCTAATTGAGTAAATCCATCTTCATCCAGATTCTC-3' (reverse). The resulted point mutation was confirmed by the DNA sequencing. The mutant cDNA (Dectin-1AY15F) was then transferred into the pSVL vector.

Cell culture and transfections—Rat basophilic leukemia RBL-2H3 cells were

maintained as monolayer cultures in DMEM with 100 U/ml of penicillin and 10% (v/v) heat-inactivated FCS. For stable transfection, 20 µg of linearized expression constructs and 2 µg of pSV2-neo vector were cotransfected into 5×10⁶ RBL-2H3 cells by electroporation (950 microfarads, 310 V) using GenePulserXcell (Bio-Rad, Hercules, CA, USA) as described (31). Stably transfected cell lines were selected with 0.4 mg/ml active G418 (Nacalai tesque, Kyoto, Japan). Cell lines were screened by the level of protein expression by the immunoblotting of detergent-soluble lysates with anti-Dectin-1 and anti-FcεRIβ antibodies (a gift from Dr. Reuben P. Siraganian (National Institutes of Health, MD, USA)).

Preparation of cell lysates, immunoprecipitation and immunoblotting—10⁶ cells were incubated without or with 100 µg/ml of Curdlan in the medium for the indicated periods of time, or cultured overnight with anti-DNP IgE mAb (1:5000) for sensitization and stimulated with 300 ng/ml of DNP-BSA for 10 min at 37°C. In some experiments, cells were pretreated with Syk inhibitor R406, prior to the stimulation. After the stimulation, cells were washed with ice-cold PBS twice and solubilized in the lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA, 100 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF and 2 µg/ml aprotinin) containing 1% Triton X-100. Preparation of detergent-soluble cell lysates, immunoprecipitation, and immunoblotting were performed as previously described (32-34).

FACS analysis—Parental or Dectin-1-expressing RBL-2H3 cells were reacted with anti-Dectin-1 antibody or goat anti-mouse IgG antibody (Jackson immunoresearch, West Grove, PA, USA) as a negative control for 30 min at 4°C. After washing with PBS, cells were stained with Alexa Flour 488 F(ab')₂ fragment of rabbit anti-goat IgG (Life technologies). Data from stained cells were acquired by FACScantII (Beckton Dickinson, Franklin Lakes, NJ, USA), and analyzed with FlowJo software (FlowJo, LLC, Ashland, OR, USA).

Pull-down assay—The GST-rat Syk-SH2 (both N- and C-terminal SH2 domains) expression construct was a gift from Dr. Reuben P. Siraganian. 5×10⁶ cells were incubated without or with 100 µg/ml Curdlan in medium, and were solubilized with the lysis buffer containing 1% Triton X-100. *In vitro* binding experiments were performed as previously described (32-34).

Microarray analysis—10⁶ cells were pretreated without or with R406 (2 µM) for 5min, and subsequently stimulated without or with 100 µg/ml of Curdlan for 2 h. Total RNAs were isolated and the qualities were evaluated by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The sense-strand cDNAs were generated using Ambion WT Expression kit (Life technologies), and the synthesized cDNAs were subjected to fragmentation and labeling by GeneChip WT Terminal labeling kit (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. Hybridization with

GeneChip Rat Gene 1.0 ST Array, scanning and generation of probe cell intensity data were carried out using an Affymetrix fluidics station 450 and GeneChip Scanner 3000 7G using Affymetrix GeneChip Command Console Software (Affymetrix). The data were imported into Subio platform version 1.16 (Subio, Kagoshima, Japan) for database management and quality control. All samples were assayed in two different biological replicates. Raw data were published in GEO database (GSE56246).

Quantitative Real-time PCR— 10^6 cells were seeded in 35-mm dishes and cultured overnight. Cells were pretreated without or with any of following, R406, PD98059, BAY11-7082, or Cyclosporin A and subsequently stimulated without or with 100 μ g/ml of Curdlan for 2 h at 37°C. Total RNA was extracted using High Pure RNA Isolation Kit (Roche, Mannheim, Germany) and the first-strand cDNAs were prepared using the superscript III with random primers. Real-time PCR was performed using KAPA SYBR FAST Universal qPCR Kit (KAPA Biosystems, Wilmington, MA, USA) according to the manufacturer's instructions. The primers used in this study were as follows: IL-3 (forward: 5'-ACAATGGTTCTTGCCAGCTCTAC-3'; reverse: 5'-AGGAGCGGGAGCAGCAT-3'), IL-4 (forward: 5'-CAGGGTGCTTCGCAAATTTTAC-3'; reverse: 5'-ACCGAGAACCCAGACTTGTT-3'), IL-13 (forward: 5'-AGGAGCTGAGCAACATCACAC-3';

reverse: 5'-CCATAGCGGAAAAGTTGCTT-3'), TNF- α (forward: 5'-GTAGCCCACGTCGTAGCAA-3'; reverse: AAATGGCAAATCGGCTGAC-3'), MCP-1 (forward: CGGCTGGAGAACTACAAGAGA-3'; reverse: 5'-CTCTTGAGCTTGGTGACAAATACT-3'), Nfkbiz (forward: 5'-TGCTCCAGGCAATCCAGAAG-3'; reverse: GTTGCTCCAGATCCACAAAC-3'), GAPDH (forward: 5'-TTCACCACCATGGAGAAGGC-3'; reverse: 5'-GGCATGGACTGTGGTCATGA-3'). The expression of the housekeeping gene GAPDH was used as a reference for normalization. The data were developed by using the StepOne Software version 2.1 (Life technologies) and the analyzed results were finally expressed as relative units.

Cytokine production— 10^6 cells were seeded in 35-mm dishes and cultured overnight without or with (for DNP-BSA) anti-DNP IgE mAb (1:5000) for sensitization. Cells were pretreated without or with R406, then stimulated with 100 μ g/ml of Curdlan or 30 ng/ml of DNP-BSA for 6 h at 37°C. Concentrations of MCP-1, IL-4, and TNF- α secreted into cell culture supernatants of untreated and stimulated cells were analyzed with plate-bound ELISA-kits (Quantikine ELISA, R&D systems) according to the manufacture's recommendations.

Statistical analysis—Significant differences were evaluated by the paired *t*-test; **P*<0.05, ***P*<0.01, ****P*<0.001 were considered

significant.

RESULTS

Dectin-1 stimulated with Curdlan triggers tyrosine phosphorylation of cellular proteins in RBL-2H3 cells—Fungal cell wall component β -glucan induces tyrosine phosphorylation of cellular proteins in macrophages (17) or in DCs (13). Therefore, first we analyzed whether Curdlan could induce tyrosine phosphorylation of cellular proteins in mast cell line RBL-2H3 cells (Fig. 1A). Tyrosine phosphorylation of cellular proteins gradually increased for 60 min after Curdlan stimulation, even though tyrosine phosphorylation was weaker and slower than that induced by Fc ϵ RI engagement. Dose-resonse experiments showed that the response to Curdlan reached a plateau at 1 μ g/ml (Fig. 1B), suggesting that a functional receptor for Curdlan is expressed in RBL-2H3 cells to trigger unidentified cellular responses.

Expression of Dectin-1 in RBL-2H3 cells was detected by RT-PCR. Two differential isoforms; Dectin-1A and 1B were confirmed by DNA sequencing (data not shown).

Generation of stable RBL-2H3 cell lines overexpressing Dectin-1 or its Y15F mutant—To analyze the function of Dectin-1 in mast cells, stable cell lines overexpressing Dectin-1 wild type (WT) or its inactive form were generated. Mouse Dectin-1 cDNA was transfected into RBL-2H3 cells (Fig. 2). Schematic diagram of Dectin-1 was shown in Figure 2A. Cytoplasmic tail of mouse Dectin-1 possesses two tyrosine residues (Y³ and Y¹⁵), however, only Y¹⁵ which is the component of

hemITAM is essential for the Dectin-1-mediated signaling in DCs (20). Furthermore, rat Dectin-1 does not possess a tyrosine residue corresponding to Y³ of the mouse Dectin-1 (Fig. 2A) (4). Therefore, Tyr¹⁵ was substituted with Phe (Y15F) to generate loss-of-function mutant of Dectin-1.

pSVL-myc-His-Dectin-1 or pSVL-myc-His-Dectin-1 Y15F mutant was cotransfected with pSV2-neo into RBL-2H3 cells. Each two clones in which the level of protein expression was highest were selected and utilized in the following study (Fig. 2B). Expression of wild type and mutant form of Dectin-1 on cell surface was confirmed by FACS analysis (Fig. 2C).

Tyr¹⁵ in hemITAM is required for Dectin-1-mediated cellular signaling in RBL-2H3 cells—Curdlan stimulation induces phosphorylation of ERK in macrophages (17) and DCs (18). Therefore, we examined whether Curdlan stimulation could induce tyrosine phosphorylation of cellular proteins and phosphorylation of ERK in the stable cell lines overexpressing Dectin-1 WT or Y15F mutant (Fig. 3A). As shown, treatment of cells overexpressing Dectin-1 WT with Curdlan increased inducible tyrosine phosphorylation of cellular proteins as well as phosphorylation of ERK rather than parental cells, and those were not observed in the cells overexpressing Dectin-1 Y15F mutant (Fig. 3A). In addition, inducible phosphorylation was detectable when the cells were stimulated with 1 μ g/ml of Curdlan, and was reached to the

maximum during 10 to 200 µg/ml of Curdlan (Fig. 3B). These results suggested that RBL-2H3 cells has Dectin-1-mediated signaling pathway and Tyr¹⁵ in hemITAM plays a critical role for Dectin-1-mediated cellular signaling pathway. It is important to note that pattern of protein-tyrosine phosphorylation by Curdlan stimulation was different from that induced by the engagement of FcεRI with DNP, although the receptor for Curdlan (Dectin-1) and FcεRI recruit and activate Syk, resulting in transmitting signal to downstream (35). Therefore Dectin-1-mediated signaling pathway though Syk might be different from that induced by the engagement of FcεRI in mast cells.

Curdlan induces FcεRIγ-independent interaction of Dectin-1 with the SH2 domain of Syk through tyrosine phosphorylation—Previous studies demonstrated that stimulation with β-glucan induces tyrosine phosphorylation of Dectin-1 and Syk in macrophages (14, 21), DCs (20), and tyrosine phosphorylation of these proteins is the key event for transmitting signal to downstream (18, 20, 21, 36). Therefore next we examined whether Curdlan could induce tyrosine phosphorylation of Dectin-1 and Syk in mast cells. Immunoprecipitation study demonstrated that Dectin-1 and Syk were tyrosine phosphorylated by Curdlan stimulation in RBL-2H3 cells overexpressing Dectin-1 WT (Fig. 4A, 4B). As expected, tyrosine phosphorylation of Dectin-1 Y15F mutant was not detected, suggesting that Y¹⁵ is required for Curdlan-induced tyrosine phosphorylation of Dectin-1 (Fig. 4A). In addition,

consistent with the previous report (20), Curdlan-mediated tyrosine phosphorylation of Syk was abrogated in Dectin-1 Y15F mutant cells (Fig. 4B).

Because tyrosine phosphorylation of hemITAM (Y¹⁵) is essential for the interaction between Dectin-1 and the SH2 domain of Syk in DCs (20), we examined the existence of this interaction in mast cells. Pull-down assay using GST-Syk-SH2 demonstrated that Dectin-1 WT, but not Y15F mutant, bound to the SH2 domain of Syk (Fig. 4C). The interaction between Dectin-1 and the SH2 domain of Syk was maximum at 40 min after Curdlan stimulation (Fig. 4C). Endogenous rat Dectin-1 was unable to be detected because anti-mouse Dectin-1/CLEC7A antibody could not react with rat species expressing in RBL-2H3 cells. In addition, engagement of FcεRI induces the interaction of FcεRIγ with the SH2 domains of Syk in mast cells (22), hence we examined whether the interaction between Dectin-1 and Syk was mediated by FcεRIγ. Patterns of protein-tyrosine phosphorylation shows that FcεRIγ was observed when the cells were stimulated with DNP through sensitized FcεRI, however, this band was not detected in the cells stimulated with Curdlan, suggesting that Dectin-1 associates with Syk in FcεRI-independent manner (Fig. 4C). These results demonstrated that signal transduction of Dectin-1 and that of FcεRI through Syk are independent each other, regardless of their similarity in the interaction between their hemITAM/ITAM and SH2 domains of Syk.

Syk plays a critical role in Dectin-1-mediated signal transduction in RBL-2H3 cells—By using the Syk inhibitor R406, which binds to the ATP binding pocket of Syk and inhibits its kinase activity as an ATP-competitive inhibitor (37), the importance of Syk in Dectin-1-mediated signal transduction in mast cells was evaluated. Preincubation of cells overexpressing Dectin-1 WT with R406 decreased Curdlan-induced tyrosine phosphorylation of cellular proteins and phosphorylation of ERK in concentration-dependent manner (Fig. 5A). Because almost all of protein-tyrosine phosphorylation induced by Curdlan stimulation were inhibited by R406, Syk plays a critical role in Dectin-1-mediated signal transduction in mast cells.

Curdlan stimulation induces tyrosine phosphorylation of PLC γ 2 which lies downstream of Syk, resulting in activation of ERK, JNK, and downstream transcription factors NFAT, and NF- κ B in DCs (15). As shown, tyrosine-phosphorylation of PLC γ 2 was induced by the stimulation with Curdlan, and was completely abrogated by the preincubation with R406 (Fig. 5B). These results demonstrated that Syk plays a critical role in Dectin-1-mediated protein-tyrosine phosphorylation including PLC γ 2 and phosphorylation of ERK in RBL-2H3 cells.

Treatment of cells with BAY61-3606 as well as R406 completely inhibits Curdlan-induced activation of ERK and PLC γ 2 (Fig. 5A and 5B). Therefore, we concluded that Syk is critical for

Curdlan-induced activation of ERK and PLC γ 2.

Curdlan stimulates the expression of characteristic genes in mast cells, different from those in macrophages or DCs—The microarray analysis was next performed to determine which transcription factors and cytokine genes were up-regulated by Curdlan in mast cells. The list of the significantly up-regulated genes in RBL-2H3 cells overexpressing Dectin-1 WT after 2 h of Curdlan stimulation was shown in Table 1. Consistent with previous studies in macrophages and DCs, gene expressions of TNF- α (12, 13, 15), IL-4/IL-13 (38, 39), early growth response transcription factor 3 (40, 41) and suppressor of cytokine signaling 1 (42) were also up-regulated in Curdlan-stimulated mast cells. Interestingly, Curdlan did not stimulate gene expressions which were previously reported in macrophages and DCs, such as IL-1 β (43, 44), IL-2 (18), IL-10 (16, 18-20), IL-12 (18, 20). Alternatively, gene expressions of IL-3, MCP-1, Nfkbiz were up-regulated by Curdlan stimulated mast cells, although those have not been reported in macrophages and DCs. These results demonstrated that Dectin-1 mediated signaling in mast cells plays a different role from those reported in macrophages and DCs. Therefore Dectin-1-mediated signaling in mast cells may play novel roles for anti-fungal immunity.

Dectin-1-mediated signal transduction in mast cells leads to characteristic gene expressions and cytokine secretion by Syk-dependent manner—Finally, quantitative real-time PCR was

performed to investigate Curdlan-mediated gene expressions. Gene expressions of IL-3, MCP-1, IL-13, IL-4, TNF- α , and Nfkbiz were significantly up-regulated by 2 h of Curdlan stimulation and potently inhibited by preincubation of cells with R406, suggesting that these gene expressions are regulated by Syk-dependent manner (Fig. 6A). Among the inducible genes, Curdlan-mediated secretion of MCP-1, IL-4 and TNF- α into the culture supernatants were examined using the cells overexpressing Dectin-1 WT (Fig. 6B). Consistent with the results of quantitative real-time PCR, secretion of MCP-1, IL-4, TNF- α induced by Curdlan was abrogated by pretreatment of Syk inhibitor R406 (Fig. 6B). These results demonstrated that Dectin-1-mediated signaling through Syk in mast cells stimulates characteristic gene expression of transcription factors and cytokines.

To further investigate the Syk-mediated cellular signaling, we next examined effects of inhibitor for MEK, NF- κ B and NFAT on Curdlan-induced mRNA expression of various cytokines. The treatment of Curdlan-stimulated cells with MEK inhibitor PD98059 dramatically suppressed mRNA expression of TNF- α mRNA (29% of control). Interestingly, the expressions of MCP-1 and IL-13 were up-regulated (142% and 176% of control, respectively) (Fig. 6C). Although we could not observe any significant effect of NF- κ B inhibitor BAY11-7082, the treatment of cells with Cyclosporin A, a Calcineurin/NFAT pathway inhibitor, caused dramatic suppression of

mRNA expression of IL-3, MCP-1, IL-4 and IL-13, but not TNF- α . These results suggest that Syk-dependent mRNA expression of MCP-1, IL-3, IL-4 and IL-13 in Curdlan-stimulated mast cells are dependent on the activation of NFAT, and that of TNF- α is dependent on the activation of ERK.

Finally, we examined whether treatment of parental RBL-2H3 cells with Curdlan stimulates secretion of MCP-1. As shown in Fig. 6D, Curdlan-mediated increase of secretion of MCP-1 from parental RBL-2H3 cells was observed. As expected, R406 strikingly suppressed the secretion of MCP-1.

DISCUSSION

Previously, Dectin-1 expression was reported in human cord blood-derived mast cells (28), human peripheral blood-derived mast cells (29) and mouse bone marrow-derived mast cells (24). Crucially, to begin with, Dectin-1 expressed in rat mast cell line RBL-2H3 cells was identified. With RT-PCR and DNA sequence, two functional isoforms expressing in RBL-2H3 cells were identified (data not shown), named as A and B (4). In addition, cellular proteins in RBL-2H3 cells are tyrosine-phosphorylated by Curdlan stimulation (Fig. 1). These results demonstrated that RBL-2H3 cells express Dectin-1, and have Dectin-1-mediated signal transduction pathway.

The importance of Syk in Dectin-1-mediated signal transduction have been demonstrated in macrophages and DCs. When hemITAM of Dectin-1 is tyrosine-phosphorylated, Syk is recruited and its SH2 domain binds to hemITAM of Dectin-1, resulting in tyrosine-phosphorylation of Syk and signal transduction to downstream (13, 20). Consistent with these facts, tyrosine phosphorylation of Dectin-1 and Syk, and the interaction between Dectin-1 and Syk, and the interaction between Dectin-1 and SH2 domain of Syk were detected after Curdlan stimulation of RBL-2H3 cells overexpressing Dectin-1 WT, whereas those in Y15F mutant is abrogated (Fig. 4). Interestingly, FcεRI does not have the interaction between its ITAM and SH2 domains of Syk after Dectin-1 stimulation with Curdlan (Fig. 4C). This indicates that the signal transduction of Dectin-1 and FcεRI

through Syk are completely independent each other, regardless of their similarity in the interaction between their hemITAM/ITAM and SH2 domains of Syk. Syk is a non-receptor type protein-tyrosine kinase and plays critical roles for B cell receptor, T cell receptor, FcεRI, Dectin-1, and CLEC2 to transmit those activation signal to downstream (45, 46). In this report, we found that Syk is important not only in macrophages and DCs but also in mast cells for Dectin-1-mediated signal transduction, by using R406 and BAY61-3606 (Fig. 5A, 5B). Furthermore, gene up-regulation of IL-3, MCP-1, IL-4, IL-13, Nfkbiz and TNF-α by Curdlan stimulation were also abrogated with R406 and BAY61-3606 (Fig. 6A), indicating that the Dectin-1-mediated regulations of these genes also depend on Syk.

As shown in figure 3A, we found that the pattern of protein-tyrosine phosphorylation is different between FcεRI and Dectin-1 stimulated cells. Although activation of Syk is essential in these signaling pathways, it is reported that FcεRI activates Fyn kinase independently activation of Syk (47). In addition, it is reported that protein tyrosine phosphatase SHP-1 could activate JNK in response to aggregation of FcεRI (48). These Syk-independent signaling pathways through FcεRI, but not Dectin-1, could account for the differences in gene expression triggered by these receptors.

With DNA microarray analysis, various up-regulated genes were detected in mast cells following Curdlan stimulation (Table 1). Six genes (IL-3, MCP-1, IL-4, IL-13, TNF-α, and Nfkbiz)

were focused on because they were well-analyzed in FcεRI signaling in mast cells and have a possibility corresponding to anti-fungal immunity. Quantitative real-time PCR analysis revealed that Curdlan could induce gene expression of IL-3, MCP-1, IL-4, IL-13, TNF-α and Nfkbiz and by Syk-dependent manner (Fig. 6A). MCP-1 is a CC chemokine and plays a critical role in the recruitment of monocytes into the site of inflammatory responses (49). Previous studies show that zymosan stimulates MCP-1 production, resulting in monocyte recruitment into inflammatory sites (50, 51). However, zymosan stimulates not only Dectin-1 but also TLR2 (14), thereby it has been still unknown whether MCP-1 is induced by only Dectin-1-mediated signaling pathway. In this study, it was first revealed that MCP-1 was produced through Dectin-1-mediated signal transduction by using Curdlan, a specific agonist of Dectin-1, in mast cells by TLR2-independent manner (Fig. 6B) (52, 53). IL-4 and IL-13, canonical type 2 cytokines, are closely related cytokines required for the generation of high-affinity IgE antibodies, mucus overproduction, and smooth muscle alterations (54). IL-4 and IL-13 highly up-regulate Dectin-1 expression on the cell surface through STAT6 activation in macrophages (54, 55). We demonstrated that mast cells stimulated through Dectin-1-mediated signal transduction produce IL-4 and IL-13 (Fig. 6). TNF-α, a well-known cytokine induced by Dectin-1 stimulation by Curdlan (12, 13, 15), is an essential cytokine

required for the successful control of many fungal pathogens (56-59). TNF-α production induced by Curdlan in mast cells indicates that mast cell contributes to innate anti-fungal immunity.

Nfkbiz, the IκBζ-coding gene, is rapidly induced by various inflammatory stimuli, however its physiological function remains largely unknown (60, 61). Recently, it has been reported in macrophages study that IκBζ is a transcriptional key regulator of MCP-1 following TLR stimulation (62). However, very importantly, it has never been reported whether Nfkbiz is up-regulated through Dectin-1-mediated signal transduction and exists in mast cells. In addition, although IκBζ is inducible in the MyD88-dependent part of the TLR/IL-1R signaling pathway (61), its regulatory mechanism remains unclear. Dectin-1-mediated signal transduction stimulates Nfkbiz transcription in mast cells, and was also pharmacologically identified that Syk is a key molecule for Dectin-1-mediated transcription of Nfkbiz, indicating the possibility for elucidation of novel mechanism of IκBζ induction (Fig. 6A). With immunoblotting using IκBα, IκB kinase and phospho-IκB kinase antibodies, NF-κB is not activated with Curdlan stimulation in RBL-2H3 cells (data not shown). Goodridge *et al.* demonstrated that although Dectin-1 signals directly activate NF-κB in mouse DCs, Dectin-1 signaling alone does not activate NF-κB in mouse macrophages (14, 42, 63). According to this, Dectin-1-mediated activation of NF-κB in mast

cells may not activate as same as that in macrophages. These results newly found out in this study are summarized in figure 7.

It is well known that the administration of zymosan into peritoneal cavity of mice induced massive infiltration of neutrophils with increased level of chemo attractants and inflammatory cytokine including MCP-1 and TNF- α . Given the fact that these effects of zymosan were significantly impaired in mast cell deficient W/W^V mice (64-66), mast cells might play important roles on the host defense against fungal infection. In addition to our present study, it was demonstrated that the treatment of bone marrow-derived mast cells with zymosan enhanced cell surface expression of Dectin-1 and stimulated ROS production, although these effects were significantly reduced in mast cells derived from TLR2-deficient mice (24). Further studies are required to understand how Dectin-1 and other innate immunoreceptors activated by fungal cell wall differently or cooperatively regulate mast cell activation.

In conclusion, these results potently suggest that mast cell contributes innate anti-fungal immunity. In addition, it has been reported that Dectin-1 has relationships with not only innate immunity but also autoimmune diseases (67-69), allergic diseases (29, 70) and metabolic diseases (71), indicating that mast cells may contribute to and offer new insights into these diseases.

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⁵**Abbreviations used are:** DCs, dendritic cells; SH2, Src homology 2; hemITAM, hemi-immunoreceptor tyrosine-based activation motif; FcεRIγ, γ-subunit of high-affinity IgE receptor; MCP-1, monocyte chemoattractant protein-1; TNF, tumor necrosis factor; TLR, Toll-like receptor; CLR, C-type lectin receptor, PAMP, pathogen-associated molecular pattern; CRD, carbohydrate recognition domain; Syk, spleen tyrosine kinase; NF-κB, nuclear factor-κB; NFAT, nuclear factor of activated T-cells; CLEC2, C-type lectin-like receptor 2; ROS, reactive oxygen species; DNP, dinitrophenyl; PLC, phospholipase C; WT, wild type

REFERENCES

1. Ariizumi, K., Shen, G-L., Shikano, S., Xu, S., Ritter III, R., Kumamoto, T., Edelbaum, D., and Morita, A. (2000) Identification of a novel, dendritic cell-associated molecule, dectin-1, by subtractive cDNA cloning. *J. Biol. Chem.* **275**, 20157-20167
2. Brown, G. D., and Gordon, S. (2001) Immune recognition: a new receptor for β -glucan. *Nature*. **413**, 36-37
3. Taylor, P. R., Brown, G. D., Reid, D. M., Willment, J. A., Martinez-Pomares, L., Gordon, S., and Wong, S. Y. (2002) The β -glucan receptor, dectin-1, is predominantly expressed on the surface of cells of the monocyte/macrophage and neutrophil lineages. *J. Immunol.* **169**, 3876-3882
4. Kato, Y., Adachi, Y., and Ohno, N. (2008) Characterization of rat β -glucan receptor dectin-1. *Microbiol. Immunol.* **52**, 418-428
5. Heinsbroek, S. E., Taylor, P. R., Rosas, M., Willment, J. A., Williams, D. L., Gordon, S., and Brown, G. D. (2006) Expression of functionally different dectin-1 isoforms by murine macrophages. *J. Immunol.* **176**, 5513-5518
6. Willment, J. A., Gordon, S., and Brown, G. D. (2001) Characterization of the human β -glucan receptor and its alternatively spliced isoforms. *J. Biol. Chem.* **276**, 43818-43823
7. Di Carlo, F. J., and Fiore, J. V. (1958) On the composition of zymosan. *Science*. **127**, 756-757
8. Taylor, P. R., Brown, G. D., Herre, J., Williams, D. L., Willment, J. A., and Gordon, S. (2004) The role of SIGNR1 and the β -glucan receptor (Dectin-1) in the nonopsonic recognition of yeast by specific macrophages. *J. Immunol.* **172**, 1157-1162
9. Thornton, B. P., Větrovická, V., Pitman, M., Goldman, R. C., and Ross, G. D. (1996) Analysis of the sugar specificity and molecular location of the β -glucan-binding lectin site of complement receptor type 3 (CD11b/CD18). *J. Immunol.* **156**, 1235-1246
10. Brown, G. D. (2006) Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nat. Rev. Immunol.* **6**, 33-43.
11. Brown, G. D., Herre, J., Williams, D. L., Willment, J. A., Marshall, A. S., and Gordon, S. (2003) Dectin-1 mediates the biological effects of β -glucans. *J. Exp. Med.* **197**, 1119-1124
12. Kataoka, K., Muta, T., Yamazaki, S., and Takeshige, K. (2002) Activation of macrophages by linear (1 $\rightarrow$ 3)- β -D-glucans. *J. Biol. Chem.* **207**, 36825-36831
13. Strasser, D., Neumann, K., Bergmann, H., Marakalala, M. J., Guler, R., Rojowska, A., Hopfner, K-P., Brombacher, F., Urlaub, H., Baier, G., Brown, G.D., Leitges, M., and Ruland, J. (2012) Syk kinase-coupled C-type lectin receptors engage protein kinase C- δ to elicit Card9

- adaptor-mediated innate immunity. *Immunity*. **36**, 32-42
14. Gantner, B. N., Simmons, R. M., Canavera, S. J., Akira, S., and Underhill, D. M. (2003) Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *J. Exp. Med.* **197**, 1107-1117
 15. Xu, S., Huo, J., Lee, K-G., Kurosaki, T., and Lam, K-P. (2009) Phospholipase C γ 2 is critical for Dectin-1-mediated Ca²⁺ flux and cytokine production in dendritic cells. *J. Biol. Chem.* **284**, 7038-7046
 16. Kelly, E. K., Wang, L., and Ivashkiv, L. B. (2010) Calcium-activated pathways and oxidative burst mediate zymosan-induced signaling and IL-10 production in human macrophages. *J. Immunol.* **184**, 5545-5552
 17. Olsson, S., and Sundler, R. (2007) The macrophage β -glucan receptor mediates arachidonate release induced by zymosan: essential role for Src family kinases. *Mol. Immunol.* **44**, 1509-1515
 18. Slack, E. C., Robinson, M. J., Hernanz-Falc3n, P., Brown, G. D., Williams, D. L., Schweighoffer, E., Tybulewicz, V. L., and Reis e Sousa, C. (2007) Syk-dependent ERK activation regulates IL-2 and IL-10 production by DC stimulated with zymosan. *Eur. J. Immunol.* **37**, 1600-1612
 19. Elcombe, S. E., Naqvi, S., van den Bosch, M. W., MacKenzie, K. F., Cianfanelli, F., Brown, G. D., and Arther, J. S. (2013) Dectin-1 regulates IL-10 production via a MSK1/2 and CREB dependent pathway and promotes the induction of regulatory macrophage markers. *PLoS One*. **8**, e60086
 20. 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) Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins. *Immunity*. **22**, 507-517
 21. Underhill, D. Rossnagle, M., E., Lowel, C. A., and Simmons, R. M. (2005) Dectin-1 activates Syk tyrosine kinase in a dynamic subset of macrophages for reactive oxygen production. *Blood*. **106**, 2543-2550
 22. Kihara, H., and Siraganian, R. P. (1994) Src homology 2 domains of Syk and Lyn bind to tyrosine-phosphorylated subunits of the high affinity IgE receptor. *J. Biol. Chem.* **269**, 22427-22432
 23. Fuller, G. L., Williams, J. A., Tomlinson, M. G., Eble, J. A., Hanna, S. L., P3hlmann, S., Suzuki-Inoue, K., Ozaki, Y., Watson, S. P., and Pearce, A. C. (2007) The C-type lectin

- receptors CLEC-2 and Dectin-1, but not DC-SIGN, signal via a novel YXXL-dependent signaling cascade. *J. Biol. Chem.* **282**, 12397-12409
24. Yang, Z., and Marshall, J. S. (2009) Zymosan treatment of mouse mast cells enhances dectin-1 expression and induces dectin-1-dependent reactive oxygen species (ROS) generation. *Immunobiology.* **214**, 321-330
 25. Vautier, S., MacCallum D. M., and Brown G. D. (2012) C-type lectin receptors and cytokines in fungal immunity. *Cytokine.* **58**, 89-99
 26. Galli, S. J., Nakae, S., and Tsai, M. (2005) Mast cells in the development of adaptive immune responses. *Nat. Immunol.* **6**, 135-142
 27. Voehringer, D. (2013) Protective and pathological roles of mast cells and basophils. *Nat. Rev. Immunol.* **13**, 362-375
 28. Olynych, T. J., Jakeman, D. L., and Marshall, J. S. (2006) Fungal zymosan induces leukotriene production by human mast cells through a dectin-1-dependent mechanism. *J. Allergy Clin. Immunol.* **118**, 837-843
 29. Ribbing, C., Engblom, C., Lappalainen, J., Lindstedt, K., Kovanen, P. T., Karlsson, M. A., Lundeberg, L., Johansson, C., Nilsson, G., Lunderius-Andersson, C., and Scheynius, A. (2011) Mast cells generated from patients with atopic eczema have enhanced levels of granule mediators and an impaired Dectin-1 expression. *Allergy.* **66**, 110-119
 30. Benhamou, M., Ryba, N. J., Kihara, H., Nishikawa, H., and Siraganian, R. P. (1993) Protein-tyrosine kinase p72^{syk} in high affinity IgE receptor signaling. *J. Biol. Chem.* **268**, 23318-23324
 31. Sada, K., Miah, S. M., Maeno, K., Kyo, S., Qu, X., and Yamamura, H. (2002) Regulation of FcεRI-mediated degranulation by an adaptor protein 3BP2 in rat basophilic leukemia RBL-2H3 cells. *Blood.* **100**, 2138-2144
 32. Shukla, U., Hatani, T., Nakashima, K., Ogi, K., and Sada, K. (2009) Tyrosine phosphorylation of 3BP2 regulates B cell receptor-mediated activation of NFAT. *J. Biol. Chem.* **284**, 33719-33728
 33. Ogi, K., Nakashima, K., Chihara, K., Takeuchi, K., Horiguchi, T., Fujieda, S., and Sada, K. (2011) Enhancement of B-cell receptor signaling by a point mutation of adaptor protein 3BP2 identified in human inherited disease cherubism. *Genes Cells.* **16**, 951-960
 34. Chihara, K., Kimura, Y., Honjoh, C., Sun, X., Takeuchi, K., and Sada, K. (2014) Tyrosine phosphorylation of 3BP2 is indispensable for the interaction with Vav3 in chicken DT40 cells. *Exp. Cell. Res.* **322**, 99-107

35. Sada, K., Zhang, J., and Siraganian, R. P. (2001) SH2 domain-mediated targeting, but not localization, of Syk in the plasma membrane is critical for FcεRI signaling. *Blood*. **97**, 1352-1359
36. LeibundGut-Landmann, S., Osorio, F., Brown, G. D., and Reis e Sousa, C. (2008) Stimulation of dendritic cells via the dectin-1/Syk pathway allows priming of cytotoxic T-cell responses. *Blood*. **112**, 4971-4980
37. Braselmann, S., Taylor, V., Zhao, H., Wang, S., Sylvain, C., Baluom, M., Qu, K., Herlaar, E., Lau, A., Young, C., Wong, B. R., Lovell, S., Sun, T., Park, G., Argade, A., Jurcevic, S., Pine, P., Singh, R., Grossbard, E. B., Payan, D. G., and Masuda, E. S. (2006) R406, an orally available spleen tyrosine kinase inhibitor blocks Fc receptor signaling and reduces immune complex-mediated inflammation. *J. Pharmacol. Exp. Ther.* **319**, 998-1008
38. Rand, T. G., Sun, M., Gilyan, A., Downey, J., and Miller, J. D. (2010) Dectin-1 and inflammation-associated gene transcription and expression in mouse lungs by a toxic (1,3)-β-D-glucan. *Arch. Toxicol.* **84**, 205-220
39. Rand, T. G., Robbins, C., Rajaraman, D., Sun, M., and Miller, J. D. (2013) Induction of Dectin-1 and asthma-associated signal transduction pathways in RAW 264.7 cells by a triple-helical (1,3)-β-D glucan, curdlan. *Arch. Toxicol.* **87**, 1841-1850
40. Goodridge, H. S., Simmons, R. M., and Underhill, D. M. (2007) Dectin-1 stimulation by *Candida albicans* yeast or zymosan triggers NFAT activation in macrophages and dendritic cells. *J. Immunol.* **178**, 3107-3115
41. Tassi, I., Cella, M., Castro, I., Gilfillan, S., Khan, W. N., and Colonna, M. (2009) Requirement of phospholipase C-γ2 (PLCγ2) for Dectin-1-induced antigen presentation and induction of TH1/TH17 polaryzation. *Eur. J. Immunol.* **39**, 1369-1378
42. Eberle, M. E., and Dalpke, A. H. (2012) Dectin-1 stimulation induces suppressor of cytokine signaling 1, thereby modulating TLR signaling and T cell responses. *J. Immunol.* **188**, 5644-5654
43. Kankkunen, P., Teirilä, L., Rintahaka, J., Alenius, H., Wolff, H., and Matikainen, S. (2010) (1,3)-β-Glucans activate both dectin-1 and NLRP3 inflammasome in human macrophages. *J. Immunol.* **184**, 6335-6342
44. Gringhuis, S. I., Kaptein, T. M., Wevers, B. A., Theelen, B., van der Vlist, M., Boekhout, T., and Geijtenbeek, T. B. (2012) Dectin-1 is an extracellular pathogen sensor for the induction and processing of IL-1β via a noncanonical caspase-8 inflammasome. *Nat. Immunol.* **13**, 246-255

45. Sada, K., Takano, T., Yanagi, S., and Yamamura, H. (2001) Structure and function of Syk protein-tyrosine kinase. *J. Biochem.* **130**, 177-186
46. Mócsai, A., Ruland, J., and Tybulewicz, V. L. (2010) The SYK tyrosine kinase: a crucial player in diverse biological functions. *Nat. Rev. Immunol.* **10**, 387-402
47. Parravicini, V., Gadina, M., Kovarova, M., Odom, S., Gonzalez-Espinosa, C., Furumoto, Y., Saitoh, S., Samelson, L. E., O'Shea, J. J., and Rivera, J. (2002) Fyn kinase initiates complementary signals required for IgE-dependent mast cell degranulation. *Nat. Immunol.* **3**, 741-748
48. Xie, Z. H., Zhang, J., and Siraganian, R. P. (2000) Positive regulation of c-Jun N-terminal kinase and TNF-alpha production but not histamine release by SHP-1 in RBL-2H3 mast cells. *J. Immunol.* **164**, 1521-1528
49. Lu, B., Rutledge, B. J., Gu, L., Fiorillo, J., Lukacs, N. W., Kunkel, S. L., North, R., Gerard, C., and Rollins, B. J. (1998) Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. *J. Exp. Med.* **187**, 601-608
50. Ajuebor, M. N., Flower, R. J., Hannon, R., Christie, M., Bowers, K., Verity, A., and Perretti, M. (1998) Endogenous monocyte chemoattractant protein-1 recruits monocytes in the zymosan peritonitis model. *J. Leukoc. Biol.* **63**, 108-116
51. Takahashi, M., Galligan, C., Tessarollo, L., and Yoshimura, T. (2009) Monocyte chemoattractant protein-1 (MCP-1), not MCP-3, is the primary chemokine required for monocyte recruitment in mouse peritonitis induced with thioglycollate or zymosan A. *J. Immunol.* **183**, 3463-3471
52. McIntosh, M., Stone, B. A., and Stanisich, V. A. (2005) Curdlan and other bacterial (1→3)-β-D-glucans. *Appl. Microbiol. Biotechnol.* **68**, 163-173
53. Ferwerda, G., Meyer-Wentrup, F., Kullberg, B.-J., Netea, M. G., and Adema, G. J. (2008) Dectin-1 synergizes with TLR2 and TLR4 for cytokine production in human primary monocytes and macrophages. *Cell. Microbiol.* **10**, 2058-2066
54. Van Dyken, S. J., and Locksley, R. M. (2013) Interleukin-4 and interleukin-13-mediated alternatively activated macrophages: roles in homeostasis and disease. *Annu. Rev. Immunol.* **31**, 317-343
55. Willment, J. A., Lin, H.-H., Reid, D. M., Taylor, P. R., Williams, D. L., Wong, S. Y., Gordon, S., and Brown, G. D. (2003) Dectin-1 expression and function are enhanced on alternatively activated and GM-CSF-treated macrophages and are negatively regulated by IL-10, dexamethasone, and lipopolysaccharide. *J. Immunol.* **171**, 4569-4573

56. Kawakami, K., Qifeng, X., Tohyama, M., Qureshi, M. H., and Saito, A. Contribution of tumour necrosis factor- α (TNF- α) in host defense mechanism against *Cryptococcus neoformans*. *Clin .Exp. Immunol.* **106**, 468-474
57. Netea, M. G., van Tits, L. J., Curfs, J. H., Amiot, F., Meis, J. F., van der Meer, J. W., and Kullberg, B. J. (1999) Increased susceptibility of TNF- α lymphotoxin- α double knockout mice to systemic candidiasis through impaired recruitment of neutrophils and phagocytosis of *Candida albicans*. *J. Immunol.* **163**, 1498-1505
58. Mehrad, B., R. Strieter, M., and Standiford, T. J. (1999) Role of TNF- α in pulmonary host defense in murine invasive aspergillosis. *J. Immunol.* **162**, 1633-1640
59. Allendoerfer, R., and Deepe, G. S. Jr. (1998) Blockade of endogenous TNF- α exacerbates primary and secondary pulmonary histoplasmosis by differential mechanisms. *J. Immunol.* **160**, 6072-6082
60. Yamazaki, S., Muta, T., and Takeshige, K. (2001) A novel I κ B protein, I κ B- ζ , induced by proinflammatory stimuli, negatively regulates nuclear factor- κ B in the nuclei. *J. Biol. Chem.* **276**, 27657-27662
61. Yamamoto, M., Yamazaki, S., Uematsu, S., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Kuwata, H., Takeuchi, O., Takeshige, K., Saitoh, T., Yamaoka, S., Yamamoto, N., Yamamoto, S., Muta, T., Takeda, K., and Akira, S. (2004) Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein I κ B ζ . *Nature.* **430**, 218-222
62. Hildebrand, D. G., Alexander, E., Hörber, S., Lehle, S., Obermayer, K., Münck, N-A., Rothfuss, O., Frick, J-S., Morimatsu, M., Schmitz, I., Roth, J., Ehrchen, J. M., Essmann, F., and Schulze-Osthoff, K. (2013) I κ B ζ is a transcriptional key regulator of CCL2/MCP-1. *J. Immunol.* **190**, 4812-4820
63. Goodridge, H. S., Shimada, T., Wolf, A. J., Hsu, Y. S., Becker, C. A., Lin, X., and Underhill, D. M. (2009) Differential use of CARD9 by Dectin-1 in macrophages and dendritic cells. *J. Immunol.* **182**, 1146-1154
64. Rao, T. S., Currie, J. L., Shaffer, A. F., and Isakson, P. C. (1994) In vivo characterization of zymosan-induced mouse peritoneal inflammation. *J. Pharmacol. Exp. Ther.* **269**, 917-925
65. Kolaczowska, E., Seljelid R., and Plytycz B. (2001) Role of mast cells in zymosan-induced peritoneal inflammation in Balb/c and mast cell-deficient WBB6F1 mice. *J. Leukoc. Biol.* **69** 33-42
66. Takeshita, K., Sakai, K., Bacon, K. B. and Gantner, F. (2003) Critical role of histamine H4 Receptor in Leukotriene B4 production and mast cell-dependent neutrophil recruitment

- induced by zymosan in vivo. *J. Pharmacol. Exp. Ther.* **307**, 1072-1078
67. Yoshitomi, H, Sakaguchi, N., Kobayashi, K., Brown, G. D., Tagami, T., Sakihama, T., Hirota, K, Tanaka, S., Nomura, T., Miki, I., Gordon, S., Akira, S., Nakamura, T., and Sakaguchi, S. (2005) A role for fungal β -glucans and their receptor Dectin-1 in the induction of autoimmune arthritis in genetically susceptible mice. *J. Exp. Med.* **201**, 949-960
 68. Plantinga, T. S., Fransen, J., Takahashi, N., Stienstra, R., van Riel, P. L., van den Berg, W. B., Netea, M. G., and Joosten, L. A. (2010) Functional consequences of *DECTIN-1* early stop codon polymorphism Y238X in rheumatoid arthritis. *Arthritis Res. Ther.* **12**, R26
 69. Salazar-Aldrete, C., Galán-Díez, M., Fernández-Ruiz, E., Niño-Moreno, P., Estrada-Capetillo, L., Abud-Mendoza, C., Layseca-Espinosa, E., Baranda, L., and González-Amaro, R. (2013) Expression and function of dectin-1 is defective in monocytes from patients with systemic lupus erythematosus and rheumatoid arthritis. *J. Clin. Immunol.* **33**, 368-377
 70. Lilly, L. M., Gessner, M. A., Dunaway, C. W., Metz, A. E., Schwiebert, L., Weaver, C. T., Brown, G. D., and Steele, C. (2012) The β -glucan receptor Dectin-1 promotes lung immunopathology during fungal allergy via IL-22. *J. Immunol.* **189**, 3653-3660
 71. Cortez-Espinosa, N., García-Hernández, M. H., Reynaga-Hernández, E., Cortéz-García, J. D., Corral-Fernández, N. E., Rodríguez-Rivera, J. G., Bravo-Ramírez, A., González-Amaro, R., and Portales-Pérez, D. P. (2012) Abnormal expression and function of Dectin-1 receptor in type 2 diabetes mellitus with poor glycemic control (HbA1c>8%). *Metabolism.* **61**, 1538-1546

FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. Curdlan induces tyrosine phosphorylation of cellular proteins in RBL-2H3 cells. (A) Analysis of time-course. RBL-2H3 cells were stimulated without or with 100 µg/ml of Curdlan for the indicated times (Curdlan), or preincubated overnight with anti-DNP IgE and then stimulated with 300 ng/ml of antigen DNP-BSA for 10 min (DNP). Cells were solubilized with the lysis buffer containing 1% Triton-X 100. Detergent-soluble lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-phosphotyrosine (pY) mAb, and anti-GAPDH mAb used as an internal control. Incubation of cells with solvent (NaOH) alone had no effect on pH changes of culture medium and protein tyrosine phosphorylation in RBL-2H3 cells (data not shown). (B) Analysis of dose dependency. RBL-2H3 cells were stimulated with indicated concentrations of Curdlan for 40 min. Cells were solubilized with the lysis buffer containing 1% Triton-X 100. Detergent-soluble lysates were separated by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies. Molecular size markers are indicated at the left in kDa. The results were representative of 3 independent experiments.

FIGURE 2. Generation of stable RBL-2H3 cell lines overexpressing Dectin-1 or its Y15F mutant. (A) Schematic diagram of Dectin1 wild type (WT) and Y15F mutant used in this study. Dectin-1 is composed of four domains, carbohydrate recognition domain (CRD), stalk domain (STALK), transmembrane domain (TMD), and cytoplasmic domain (CD) which possesses hemITAM. Underlines show amino acid sequence which constitutes hemITAM motif. Arrowed line shows an amino acid substitution of Tyr¹⁵ to Phe (Y15F mutant). (B and C) RBL-2H3 cells were stably transfected with pSVL-myc-His-Dectin-1 WT or pSVL-myc-His-Dectin-1 Y15F mutant, together with pSV2-neo, by electroporation. Clones resistant to G418 were selected and screened by the level of protein expression and surface expression. (B) Detergent-soluble lysates from the each cell line were separated by SDS-PAGE and analyzed by immunoblotting with anti-Dectin-1 and anti-FcεRIβ antibodies, respectively. Molecular size markers are indicated at the left in kDa. (C) Analysis of cell surface expression of Dectin-1 in established cell lines by flow cytometry. Cells were incubated with anti-Dectin-1 antibody (solid line) or control goat antibody (dashed line) followed by staining with Alexa 488-labeled secondary antibody. The results were representative of 3 independent experiments.

FIGURE 3. Overexpression of Dectin-1 increases Curdlan-mediated protein-tyrosine phosphorylation and ERK phosphorylation. Curdlan-induced tyrosine phosphorylation of cellular

proteins and ERK phosphorylation. (A) Analysis of time-course. Cell lines overexpressing Dectin-1 WT or Y15F mutant were stimulated without or with 100 $\mu\text{g/ml}$ of Curdlan for the indicated times (Curdlan), or preincubated overnight with anti-DNP IgE and then stimulated with 300 ng/ml of antigen DNP-BSA for 10 min (DNP). (B) Analysis of dose dependency. Cell lines overexpressing Dectin-1 WT and Y15F mutant were stimulated with indicated concentrations of Curdlan for 40 min. Cells were solubilized with the lysis buffer containing 1% Triton-X 100. Detergent-soluble lysates were separated by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies. Molecular size markers are indicated at the left in kDa. Similar results were obtained when the other cloned cell lines were examined. Data shown are obtained by using D12 (WT) and (YF22) Y15F.

FIGURE 4. Curdlan induces tyrosine phosphorylation of Syk through the interaction of Dectin-1 with the SH2 domain of Syk by Fc ϵ RI-independent manner. (A and B) Analysis of tyrosine phosphorylation of Dectin-1 and Syk in generated RBL-2H3 cell lines. RBL-2H3 cells (parental) and cells overexpressing Dectin-1 WT and Y15F mutant were stimulated without or with 100 $\mu\text{g/ml}$ of Curdlan for 40 min. Cells were solubilized with the denature lysis buffer (lysis buffer containing 1% Triton X-100, 0.1% SDS and 0.5% deoxycholic acid) and cell lysates were immunoprecipitated with anti-phosphotyrosine (pY) mAb conjugated agarose beads, and then immunoprecipitates (IP) and the source of precipitation (input) were separated by 10% SDS-PAGE and analyzed by immunoblotting with anti-Dectin-1 (A) and anti-Syk (B) antibodies. (C) Pull-down assay. RBL-2H3 cells (parental) and cells overexpressing Dectin-1 WT or Y15F mutant were stimulated without or with 100 $\mu\text{g/m}$ of Curdlan for the indicated times (Curdlan), or preincubated overnight with anti-DNP IgE and then stimulated with 300 ng/ml of antigen DNP-BSA for 10 min (DNP). Detergent-soluble lysates were reacted with GST-Syk-SH2 prebound to glutathione Sepharose 4B beads. The binding proteins (pull-down) and the source of precipitation (input) were separated by SDS-PAGE and analyzed by the immunoblotting with anti-phosphotyrosine (pY) and anti-Dectin-1 antibodies. Arrowhead shows the position of Fc ϵ RI γ . (A-C) Molecular size markers are indicated at the left in kDa. Similar results were obtained when the other cloned cell lines were examined. (A and C) Considering the difference in expression level of Dectin-1 between D12 and YF22 (shown in Fig. 2B), twice Y15F mutant cells (1×10^7 cells) more than Dectin-1 WT (5×10^6 cells) were used in this experiment.

FIGURE 5. The effect of Syk inhibitors on Dectin-1-mediated tyrosine phosphorylation of cellular proteins. (A) Cell lines overexpressing Dectin-1 WT were preincubated with the indicated

concentration of R406 or BAY61-3606 or equal amount of solvent DMSO for 5 min, and then stimulated without or with 100 µg/ml of Curdlan for 40 min. Detergent-soluble lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-phosphotyrosine (pY), anti-GAPDH, anti-phosphoERK (pERK) and anti-ERK antibodies. Arrowheads show phosphoERK1/2 or ERK1/2. (B) Cell lines overexpressing Dectin-1 WT were preincubated with the indicated concentrations of R406 or BAY61-3606 for 5 min, and then stimulated without or with 100 µg/ml of Curdlan for 40 min. Detergent-soluble lysates were immunoprecipitated with anti-PLCγ2 antibody, and then immunoprecipitates (IP) and the source of precipitation (input) were separated by 6% SDS-PAGE and analyzed by immunoblotting with anti-phosphotyrosine (pY) mAb and anti-PLCγ2 antibody. (A and B) Similar results were obtained when the other cell lines were examined. Molecular size markers are indicated at the left in kDa.

FIGURE 6. Dectin-1-mediated signaling activates various gene expression and cytokine secretion through Syk-dependent pathway in RBL-2H3 cells. (A) Quantitative analysis of Curdlan-induced gene expression by real-time PCR. Cells overexpressing Dectin-1 WT were preincubated without or with 2 µM of R406 for 5 min, and then stimulated without or with 100 µg/ml of Curdlan for 2 h. Total RNA was isolated, reverse transcribed, and cDNA of the indicated genes was analyzed by quantitative real-time PCR. The expression of the housekeeping gene GAPDH was used as a reference for normalization. All samples were assayed in three different biological replicates and are presented as mean ± SD. Significant differences were evaluated by the paired *t*-test; **P*<0.05, ***P*<0.01, ****P*<0.001 were considered significant. Similar results were obtained when the other cloned cell lines were examined. (B) Analysis of MCP-1, IL-4, TNF-α secretions induced by Curdlan. Cells overexpressing Dectin-1WT were preincubated without or with 2 µM of R406 for 5 min (R406), and then stimulated without or with 100 µg/ml of Curdlan (Curdlan), or preincubated overnight with anti-DNP IgE and then stimulated without or with 30 ng/ml of antigen DNP-BSA for 6 h (DNP-BSA). Cell culture supernatants were harvested and ELISA was performed. Significant differences were evaluated by the paired *t*-test; **P*<0.05, ***P*<0.01, ****P*<0.001 were considered significant. All samples were assayed in triplicates and are presented as mean ± SD. The results were representative 3 independent experiments. (C) Effects of inhibitor for MEK, NF-κB and NFAT on Curdlan-induced gene expression. Cells overexpressing Dectin-1 WT (D12) were stimulated with 100 µg/ml of Curdlan for 2 h in the presence of indicated concentrations of PD98059 (MEK inhibitor), BAY11-7082 (NF-κB inhibitor) or Cyclosporin A (inhibitor of Calcineurin/NFAT pathway). Gene expression was quantified by real-time PCR and is expressed relative to DMSO

control (100%). All samples were assayed in triplicates and are presented as mean $\pm$ SD. The results were representative of 3 independent experiments. (D) Analysis of MCP-1 secretion from parental RBL-2H3 cells. RBL-2H3 cells were preincubated without or with 2 μ M of R406 for 5 min (R406), and then stimulated without or with 100 μ g/ml of Curdlan (Curdlan). Cell culture supernatants were harvested and MCP-1 ELISA was performed. Significant differences were evaluated by the paired *t*-test (***P*<0.01). All samples were assayed in triplicates and are presented as mean $\pm$ SD. The results were representative 3 independent experiments.

FIGURE 7. Dectin-1-mediated signaling in mast cells may directly and indirectly contribute to anti-fungal immunity. This schema shows dectin-1-mediated signal transduction and subsequent increase of transcription factor and cytokines revealed in this study. Solid arrows show the results revealed in this study and dashed arrows show the results revealed in the past other reports (numbers in the parenthesis show the references). Dectin-1 is expressed in mast cells and recognizes β (1 $\rightarrow$ 3)-glucan. Tyr¹⁵ in hemITAM of Dectin-1 is required for subsequent recruitment of the SH2 domains of Syk, resulting in tyrosine phosphorylation of Syk. Dectin-1/Syk signaling activates intracellular signal transducers involving PLC γ 2, NFAT and ERK 1/2, resulting in stimulation of gene expression of cytokines and transcription factor, such as MCP-1, IL-3, IL-4, IL-13, TNF- α and Nfkbiz. MCP-1, IL-4/IL-13, Nfkbiz, TNF- α may assume induction of macrophage migration into the inflammation sites together with MCP-3, stimulation of Dectin-1 expression in macrophages, stimulation of MCP-1 gene expression, and inflammatory response against fungi, respectively. These events lead an individual to the successful control of fungal infections.

TABLE 1. Syk-regulated genes in mouse Dectin-1 transduced RBL-2H3 cells after 2 h of Curdlan stimulation

Gene	Description	Fold change	P value
IL3	Interleukin-3	17.37	<.01
Ccl2	CCl2 (MCP-1)	13.64	.01
Nr4a2	Transcription factor	11.37	.02
Nr4a3	Transcription factor	11.15	.02
Egr3	Transcription factor	9.38	<.01
IL13	Interleukin-13	8.89	<.01
Vom2r3	Vomer nasal 2 receptor, 3	5.06	.04
IL4	Interleukin-4	4.77	.01
Cish	Cytokine inducible SH2-containing protein	4.54	.01
Skil	Ski like oncogene	4.26	<.01
Nfkbiz	Nfkbiz (I κ B ζ)	3.67	.02
Osm	Oncostatin M	3.45	.01
Ccl7	CCl7 (MCP-3)	3.18	.02
Tnfrsf12a	TNF receptor	3.11	.01
Socs1	Socs1	3.04	.02
Gpr183	G protein-coupled receptor 183	2.75	.01
Csrnp1	Carbohydrate (karatan sulfate Gal-6) sulfotransferase 1	2.74	<.01
Ler3	Protocadherin 9	2.66	.02
Tnf	TNF- α	2.66	<.01

TABLE 1

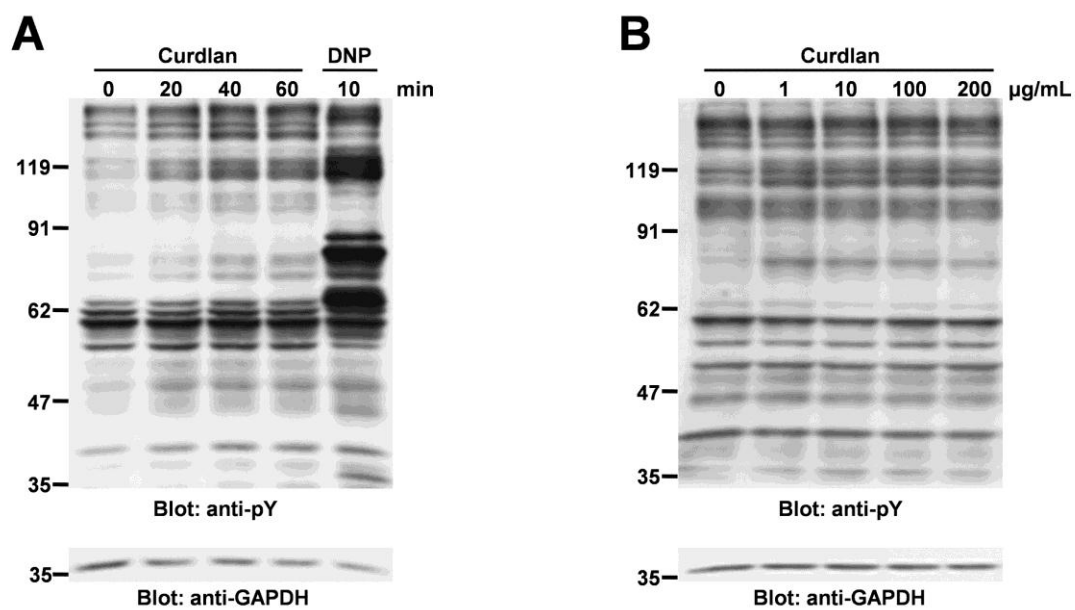


FIGURE 1

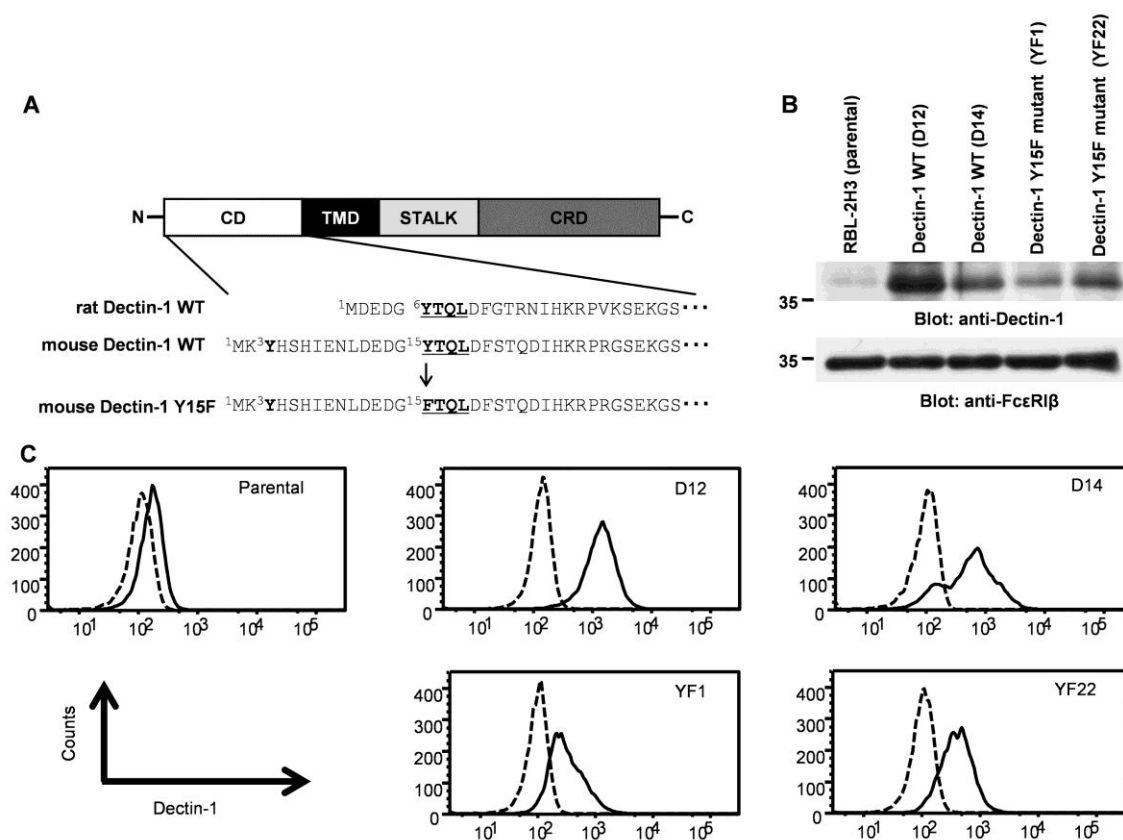


FIGURE 2

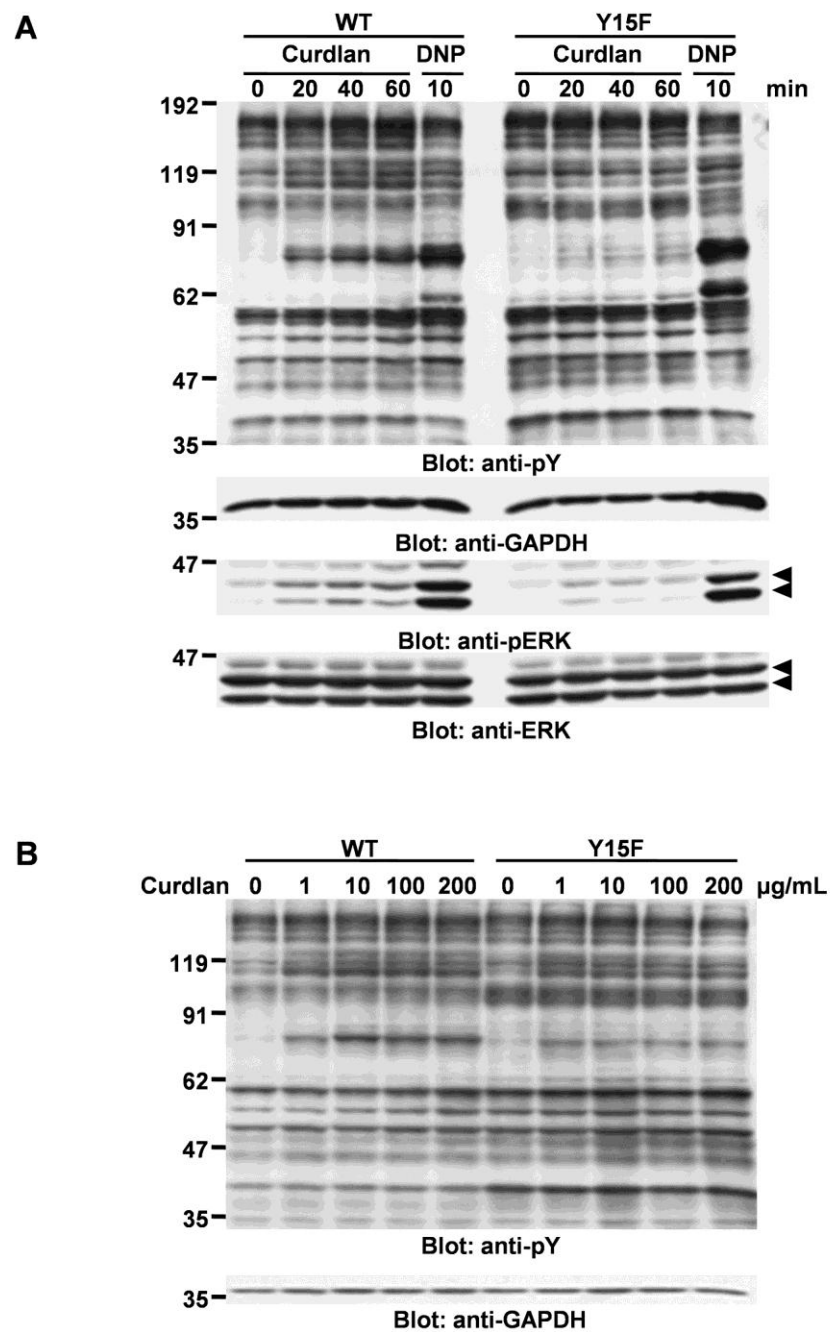


FIGURE 3

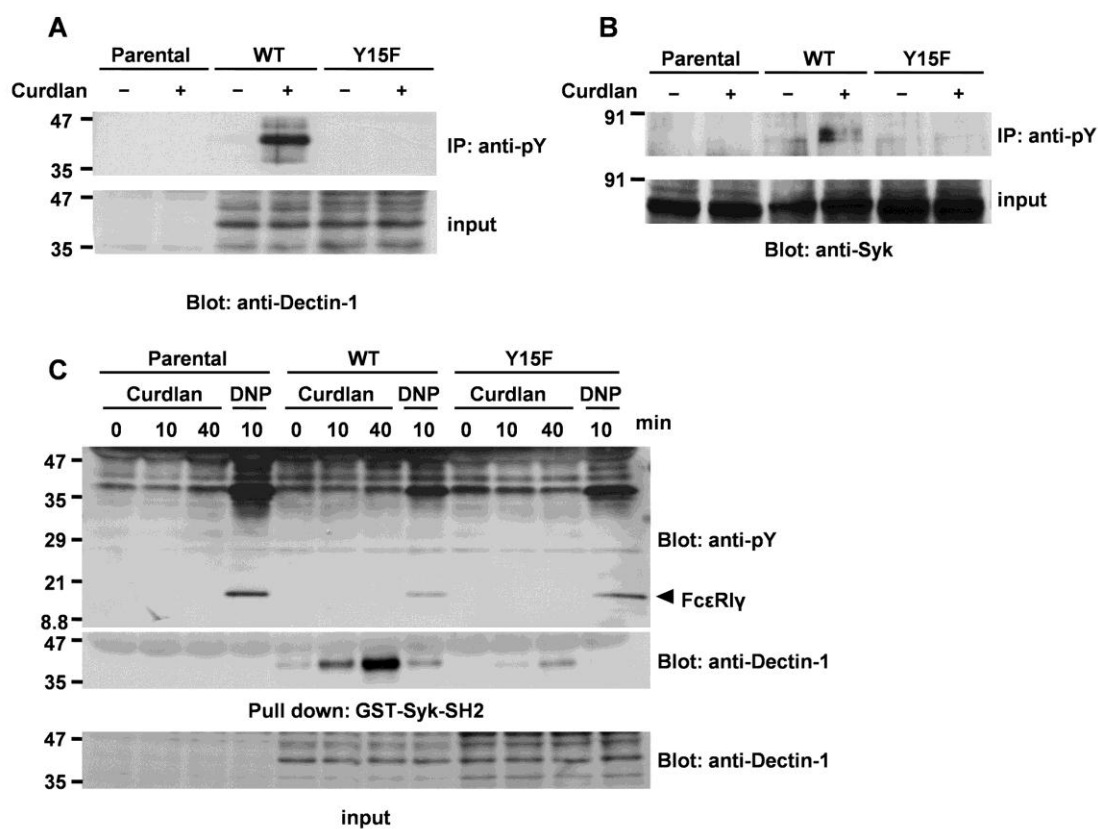


FIGURE 4

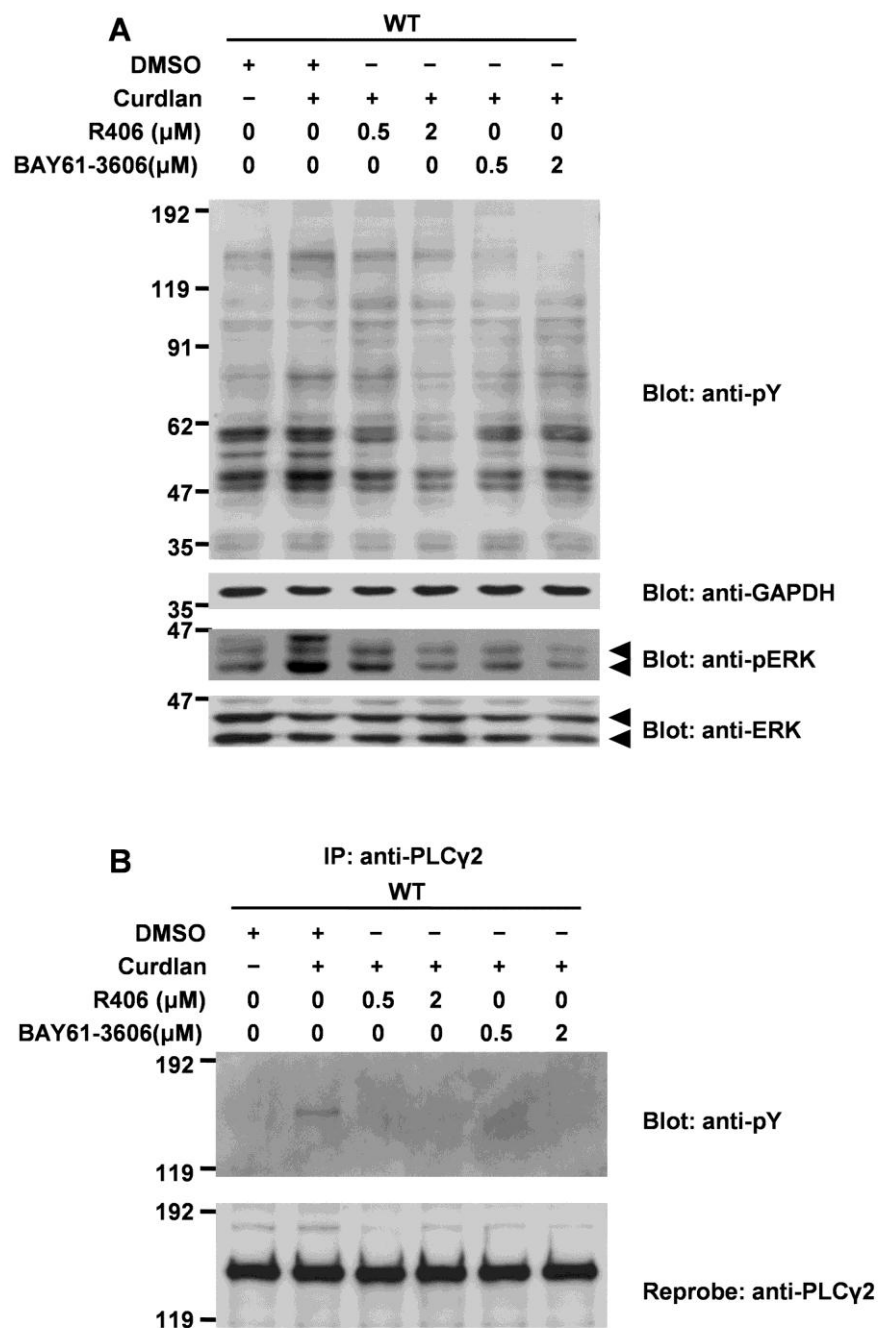


FIGURE 5

Mast cells Dectin-1 in innate anti-fungal immunity

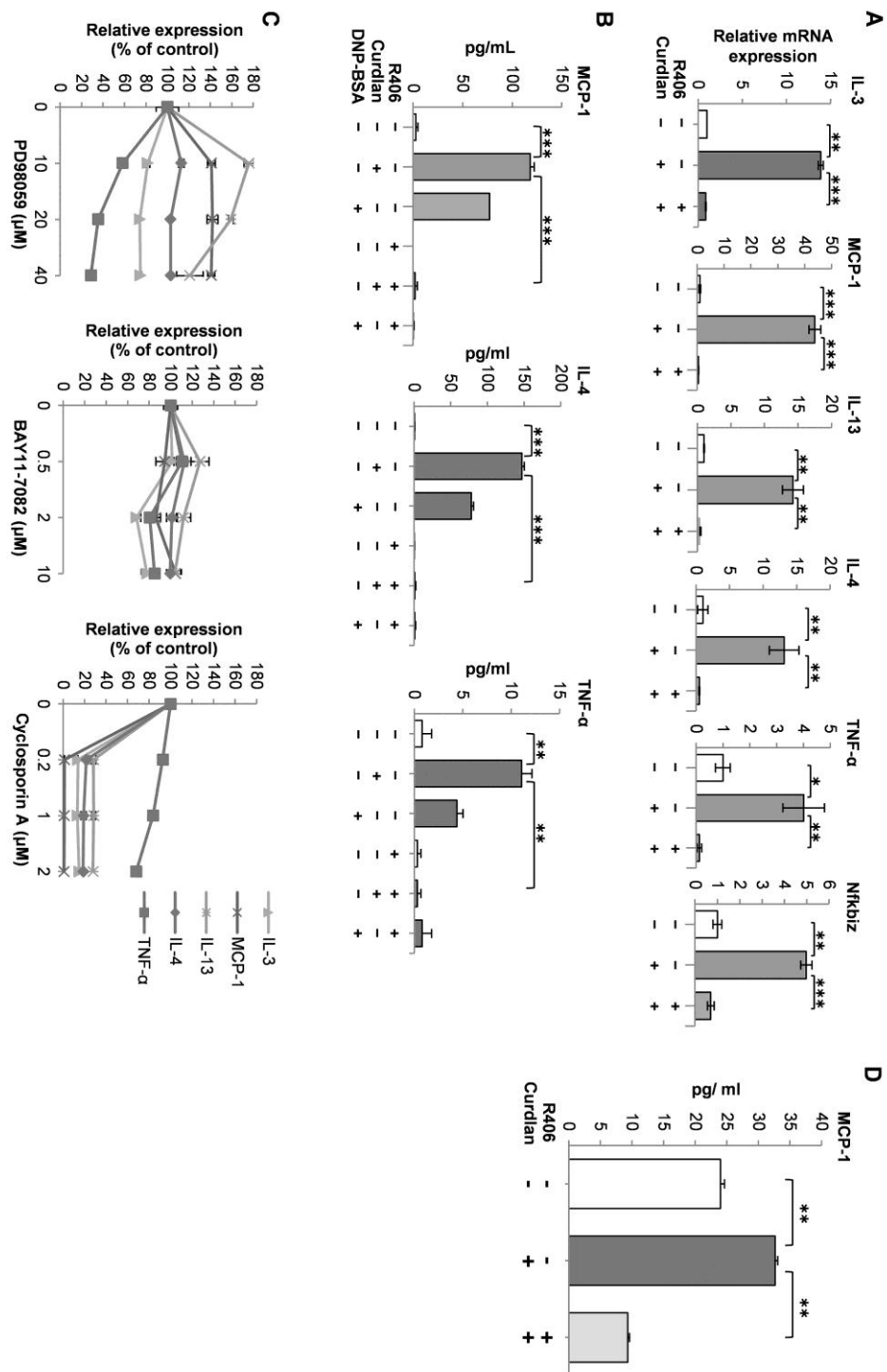


FIGURE 6

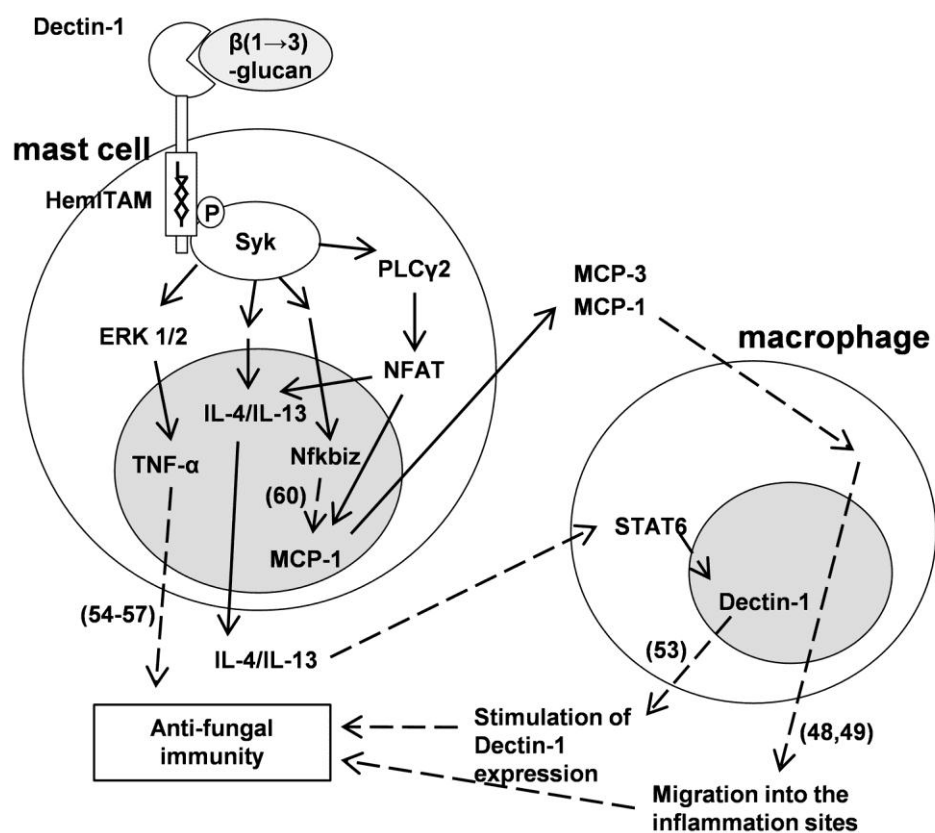


FIGURE 7