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Dectin-1 Pathway Activates Robust Autophagy-Dependent Unconventional Protein Secretion in Human Macrophages

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Dectin-1 is a membrane-bound pattern recognition receptor for β-glucans, which are the main constituents of fungal cell walls. Detection of β-glucans by dectin-1 triggers an effective innate immune response. In this study, we have used a systems biology approach to provide the first comprehensive characterization of the secretome and associated intracellular signaling pathways involved in activation of dectin-1/Syk in human macrophages. Transcriptome and secretome analysis revealed that the dectin-1 pathway induced significant gene expression changes and robust protein secretion in macrophages. The enhanced protein secretion correlated only partly with increased gene expression. Bioinformatics combined with functional studies revealed that the dectin-1/Syk pathway activates both conventional and unconventional, vesicle-mediated, protein secretion. The unconventional protein secretion triggered by the dectin-1 pathway is dependent on inflammasome activity and an active autophagic process. In conclusion, our results reveal that unconventional protein secretion has an important role in the innate immune response against fungal infections. The Journal of Immunology, 2014, 192: 5952–5962.

Fungi (e.g., yeast, filamentous forms) are heterotrophic eukaryotes that are associated with a wide spectrum of diseases in humans and animals, such as respiratory allergy and skin diseases. β-Glucans are naturally occurring carbohydrates that are the major structural components of the fungal cell wall and therefore represent potentially important immunostimulatory components of fungi. Particulate but not soluble β-glucans are potent activators of dendritic cells and macrophages (1). The major pattern recognition receptor (PRR) for β-glucans is dectin-1, a transmembrane C-type lectin receptor, which is predominantly expressed by the myeloid cells such as macrophages and dendritic cells (2–4). Receptor binding by β-glucans, including curdian and glucan from baker’s yeast (GBY), triggers a variety of protective cellular responses via the Syk kinase-signaling pathway. These responses include phagocytosis and killing, which is mediated through the respiratory burst, and the production of numerous cytokines and chemokines (5). In addition to dectin-1, the NLRP3 inflammasome is a PRR that participates in antifungal defense. The NLRP3 inflammasome is a caspase-1–activating molecular platform that regulates proteolytic processing of proinflammatory cytokines IL-1β and IL-18 (6, 7).

The “secretome” is generally referred to as the complex set of proteins secreted from living cells at a given time and under defined conditions. These proteins can be released through various mechanisms, including classical secretion and nonclassical, vesicle-mediated mechanisms such as exosome- and lysosome-mediated release of proteins (8). The secreted proteins can control and coordinate many biological activities in multicellular organism, such as growth, differentiation, apoptosis, and immune response, and they hold additional therapeutic importance as either targets for pharmacologic intervention in disease or cancer biomarkers. Studies using quantitative mass spectrometry (MS)-based proteome analysis combined with bioinformatics have highlighted the benefits of this method for elucidating which proteins are secreted in response to various stimuli (9–12). We have shown previously that β-glucans activate the membrane-associated dectin-1 and the cytoplasmic NLRP3 inflammasome in human macrophages, resulting in IL-1β gene transcription and IL-1β protein secretion, respectively (13), but the global protein secretion pattern and the associated intracellular signaling pathways after β-glucan stimulation have not been characterized. In this study, we have used MS-based quantitative proteomics—that is, the iTRAQ (isobaric tag for relative and absolute quantification) technique—to conduct a secretome analysis with the data being combined with transcriptionomics, bioinformatics, and functional studies.

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Abbreviations used in this article: ASC, apoptosis-associated speck-like protein, which contains a caspase recruitment domain; BMDC, bone marrow–derived dendritic cell; DAMP, danger-associated molecular pattern molecule; ER, endoplasmic reticulum; GBY, baker’s yeast; iTRAQ, isobaric tag for relative and absolute quantification; LDH, lactate dehydrogenase; 3-MA, 3-methyladenine; MS, mass spectrometry; PRR, pattern recognition receptor; siRNA, small-interfering RNA.
to characterize the global innate immune response of human primary macrophages stimulated through the dectin-1 pathway.

Materials and Methods

Cell culture and stimulations

PBMCs were isolated from healthy blood donors (Finnish Red Cross Blood Transfusion Service) and differentiated into macrophages in the presence of Macrophage-SFM medium (Life Technologies) supplemented with GM-CSF as described previously (14). The isolated cells were identified as macrophages by their typical morphology and CD14 expression pattern (14). Macrophages were untreated or stimulated with LPS (Escherichia coli O111:B4) or 1,3-β-glucans curdlan or GBY (all purchased from Sigma-Aldrich). The concentrations used in experiments were 1 μg for LPS, 10 μg for curdlan, 100 μg for GBY, or as indicated in the figures. Curdlan and GBY were suspended in PBS as a homogeneous dispersion. In the secretome analysis, the cells were stimulated for 18 h and subjected to gene expression microarray analysis for 6 h. Each macrophage sample represents a pool of separately stimulated cells from at least two different blood donors.

Caspase-1 inhibitor VI (Z-YVAD-FMK, 25 μM; Millipore), Syk tyrosine kinase inhibitor II (5 or 10 μM; Calbiochem), Src inhibitor PP2 (10 μM; Sigma), Src inhibitor-1 (20 μM; Sigma), and 3-methyladenine (3-MA; 10 mM; Sigma) were added to human macrophages 1 h before curdlan stimulation. Brefeldin A (Sigma) was used at 100 ng/ml and was added to cells 1 h after curdlan stimulation.

Mouse bone marrow-derived dendritic cells

Mouse bone marrow–derived dendritic cells (BMDCs) were derived from mouse bone marrow cells isolated from the femurs of wild type C57BL/6 (NOVA-SCB AB) and dectin-1 knockout mice (15). The animal study was approved by the Health Services of State Provincial Office of Southern Finland.

iTRAQ labeling and mass spectrometry

Before stimulation, the cells were washed and the culture media was changed to RPMI 1640. After stimulation, all cell culture media containing the secretome samples were collected and concentrated, and the proteins were precipitated with 2-D Clean-Up Kit (GE Healthcare), followed by protein alkylolation, trypsin digestion, and iTRAQ labeling (16) of the resulting peptides according to the manufacturer’s instructions (Applied Biosystems). The control sample was labeled with 114, LPS-stimulated was labeled with 115, Curdlan-stimulated was labeled with 116, and GBY-stimulated was labeled with 117 isobaric tag. After labeling, the samples were pooled and dried, and the peptides were fractionated by strong cation exchange chromatography using an Etan HPLC system (Akersham Biosciences) connected to a PolySULFOETHYL A column. Each SCX-fraction containing the labeled peptides was analyzed twice with nano-liquid chromatography-tandem mass spectrometry using Ultimate 3000 nano-nanomolar mass chromatograph (Dionex) and QSTAR Elite hybrid quadrupole time-of-flight mass spectrometer (Applied Biosystems) with nano-ESI ionization as described previously (11). MS data were acquired automatically using Analyst QS 2.0 software.

Gene expression microarray

Microarray experiments were performed at Biomedicum Genomics (Helsinki, Finland) using an Agilent Whole Human Genome 4 × 44K 1-Color Array (Agilent Technologies). Integrity and purity of the RNA were verified with Agilent 2100 Bioanalyzer (Agilent Technologies). Three independent experiments were performed.

Cell death assays

APOPercentage apoptosis assay was performed according to the manufacturer’s guidelines (Biocolor Life Science Assays). Photographs were taken with an Olympus DP70 Digital microscope camera, connected to an Olympus IX71 light microscope, and using software in DP Controller (version 2.2.1.227) and DP Manager (2.2.1.195; Center Valley, PA). The lactate dehydrogenase (LDH) assay was performed according to the manufacturer’s instructions (Roche Diagnostics). Cell viability was determined by measuring intracellular ATP using the CellTiter-Glo Lumi- nescence Cell Viability Assay (Promega).

Proteomics and transcriptomics data-analysis

Protein identification and relative quantitation were performed with the Paragon search algorithm (17) using ProteinPilot 2.0 interface (AB Scienx). Data files from both technical replicates of an iTRAQ sample set were processed together. Database searching was done against UniProt human database (version 2008-01-28 with 20330 human sequences) and ‘decoy’ database (the reverse amino acid sequence for false discovery rate estimation). The search criteria were: cysteine alkylation with MMTS, trypsin digestion, biological modifications allowed, thorough search, and detected protein threshold of 95% confidence (Unused ProtScore > 1.3). Importantly, no automatic bias correction was applied in the quantitation. The false discovery rates were calculated as described previously (18) and were 1.4% and 2% for the two biological replicates.

The secreted proteins were identified using their Gene Ontology annotations using GeneTrail (http://genetraill.bioinf.uni-bielefeld/ (19) and GoMiner (http://discover.nci.nih.gov/gominer/index.jsp) using UniProt as data source and Homo sapiens as the organism. Human diseases were left out of KEGG pathway results. SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/) was used to predict classically secreted proteins, and ExoCarta version 3.2 database (20) (http://www.exocarta.org/) was used to determine exosomal proteins.

The microarray expression values were preprocessed with LOWESS using the Agilent Feature Extractor and then median-centered, followed by reannotation of the probes using the Ensembl genomic database (version 60). Fold changes for genes were calculated for each stimulation versus the control, and the three replicates were combined using the median value. Gene Ontology enrichment was calculated with Fisher exact test using a genome-wide reference set for human genes as the reference. The pathway enrichment analysis was performed with SPIA (21), which uses the KEGG pathway database (22). All data analyses were performed with the freely available Anduril framework (23).

Exosome enrichment

Cells were stimulated in RPMI 1640 without FCS, after which the cell culture media were collected, and the cells and cell debris were removed (500 × g for 10 min and 3000 × g for 30 min). Exosomal fractions were enriched using centrifugal filter units (100 kDa cut-off), and the flow-through was further concentrated with 10-kDa centrifugal filter units. The enriched exosomal fractions were used directly for Western blotting, or the fractions were diluted with PBS and ultracentrifuged twice at 100,000 × g for 1 h. The purified vesicles were resuspended in PBS and used for electron microscopy or Western blotting.

Electron microscopy

The exosome suspension was fixed with 1% paraformaldehyde, transferred to Pioloform-carbon–coated copper grids, and allowed to absorb for 20 min. The grids were subsequently washed and contrasted with uranyl acetate to visualize proteins and viewed with a Jeol 1200 EX II transmission electron microscope.

Immunoblotting

To analyze the secreted proteins, cell culture media were concentrated with centrifugal filter units (cutoff, 10 kDa). The following Abs were used for immunoblotting: cleaved caspase-3 (Asp175) Ab (Cell Signaling Technology), anti–Alix (1A12, Santa Cruz), anti–tsg-101 (C-2; Santa Cruz), anti–annexin I (EH17a; Santa Cruz), anti–β-III-tubulin (Cell Signaling), anti–apoptosis-associated speck-like protein, which contains a caspase recruitment domain (ASC; TMS1; Millipore), anti–cathepsin B (Ab-3; Calbiochem), anti–cathepsin D (C-20; Santa Cruz), anti–Ag7 (D12B11; Cell Signaling), and anti–optineurin (C-2; Santa Cruz). The mAb against β2-integrin (R2E7B) was a gift from Prof. Gahmberg (Division of Biochemistry, University of Helsinki). The IL-1β Ab has been described previously (14).

To confirm equal loading and transfer of the protein, membranes were stripped and stained with ready-to-use SYPRO Ruby Protein Blot Stain (Sigma-Aldrich) or detected with anti–GAPDH ab (0411; Santa Cruz).

Luminex assay

The human cytokine Luminex Bio-Plex Pro immunoassay kit designed to detect chemokines TNF, CCL2 and CCL5 was from Bio-Rad Laboratories. The Luminex assay was performed according to the manufacturer’s instructions using Bio-Plex 200 system hardware and version 4.1.1 of Bio-Plex 200 software.

ELISA and RT-PCR

Human IL-1β and mouse IL-1β ELISAs were purchased from Diaclone and eBioscience, respectively. The experiments were performed three times with similar results.
Total cellular RNA was isolated using RNaseasy Plus Mini Kit (Qiagen) and were reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer’s instructions. Quantitative real-time PCR was performed with an ABI PRISM 7500 Sequence Detection System applying TaqMan chemistry and Predeveloped TaqMan qPCR FastMix (Quanta Biosciences). RT-PCR data were processed and quantified as described previously (24). The results are expressed as relative units. The experiments were performed three times with similar results.

Small interfering RNA approach

Macrophages were transfected twice with 200 nM non-targeting control small-interfering RNA (siRNA; AllStars Negative Control siRNA; Qiagen) and with 100 nM of each of two different beclin-1 siRNAs (Hs_BECN1_1, Hs_BECN1_3; Qiagen; final concentration being 200 nM) by using HiPerFect Transfection Reagent (Qiagen) according to the manufacturer’s instructions.

Accession codes

The Gene Expression Omnibus accession number for the microarray data reported in this article is GSE32282 (http://www.ncbi.nlm.nih.gov/geo/). The mass spectrometry data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (25) with the dataset identifier PXD000574 (http://proteomecentral.proteomexchange.org).

Results

β-Glucans induce significant gene expression changes and activate robust protein secretion from human primary macrophages

To characterize secretome upon dectin-1 activation in human macrophages, we stimulated the cells with either curdlan or GBY for 18 h, and subsequently the secreted proteins were analyzed by 4plex iTRAQ labeling combined with liquid chromatography-tandem MS analysis. In addition, bacterial LPS, a well-known inflammatory stimulus unrelated to the fungi activating TLR4 signaling, was used as a control. iTRAQ analysis was performed on two independent biological experiments resulting in the identification and quantification of 1597 distinct proteins with high-confidence (Supplemental Table I). Activation of the dectin-1 pathway effectively induced protein secretion, because the average ratio of total secreted protein compared with untreated cells was elevated by 2.7-fold when the cells were stimulated with curdlan compared with the 3.7-fold increase obtained with GBY, whereas the total protein secretion was only modestly (1.1 average ratio) increased by LPS (Supplemental Table I). Curdlan and GBY stimulation increased the secretion of 1258 and 1492 distinct proteins (fold change > 2), respectively (Fig. 1A). From these proteins, 1198 were common to both curdlan and GBY, evidence of a similar response to both β-glucans. Gene ontology analysis based on the known cellular locations for the identified proteins revealed that most of the secreted proteins detected were intracellular, including nuclear and cytosolic proteins and proteins from different cell organelles (Fig. 1A). Pathway analysis using the KEGG database of the identified proteins identified 14 pathways as being significantly (p < 0.05) overrepresented after β-glucan stimulation (Fig. 1A). Ten of these pathways are part of the immune system, including the phagosome, chemokine signaling pathway, leukocyte transendothelial migration, and the NOD-like receptor signaling pathway (marked as asterisk in Fig. 1A). To confirm that the robust secretion of intracellular proteins seen after dectin-1 activation was not a result of cell death, we first detected caspase-3 activation (Fig. 2B), which is a hallmark of apoptosis, in macrophages that had been activated through the dectin-1 or TLR4 pathways. Furthermore, both LDH release (Fig. 2C) and CellTiter-Glo Luminescent Cell Viability (Fig. 2D) assays demonstrated the lack of necrosis in these macrophages. We then performed gene expression microarray experiments to compare the global transcriptional response induced by β-glucans and LPS with their secretomes. We identified 767, 1447, and 1683 genes with >2-fold increase or decrease in curdlan-, GBY-, or LPS-stimulated macrophages, respectively (Supplemental Table II). The
the transcriptional profile of LPS-stimulated macrophages did not correlate with cytosolic DNA-sensing pathway whereas signaling pathways are being triggered by LPS and indicating that distinctly different downstream signaling pathways using the KEGG database (Fig. 1B). The main differentially expressed genes were analyzed further for their pathway associations using the KEGG database (Fig. 1B). Western blot analysis with caspase-3 Ab from total cell lysates. Human macrophages were stimulated with LPS, curdlan, or GBY or infected with influenza A virus for 18 h, after which the cell lysates were analyzed with anti–caspase-3 p19/17. Influenza A virus infection was used as a positive control. (C) LDH cytotoxicity assay was used to quantify lactose dehydrogenase (LDH) release into cell culture media. (D) Cell viability was determined by measuring intracellular ATR using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) after 18 h stimulation with LPS, curdlan, or GBY or influenza A virus infection.

high coefficient of determination ($r^2 = 0.5$) and scatter plot of curdlan- and GBY-stimulated genes demonstrated similar induction ratios after both β-glucan treatments (Fig. 1B). In contrast, the transcriptional profile of LPS-stimulated macrophages did not correlate with the transcriptional profile of β-glucan–stimulated macrophages ($r^2 = 0.28$; Fig. 1B), indicating that distinctly different downstream signaling pathways are being triggered by LPS and β-glucans. The differentially expressed genes were analyzed further for their pathway associations using the KEGG database (Fig. 1B). The main significantly ($p < 0.05$) overrepresented pathways identified from all three datasets were chemokine signaling pathway, cytokine–cytokine receptor interaction, and MAPK signaling pathways, whereas cytosolic DNA-sensing pathway, Jak–STAT signaling pathway, and NOD-like receptor signaling pathways, which were overrepresented after LPS but underrepresented after β-glucan stimulation.

Dectin-1 pathway activates both conventional and unconventional, vesicle-mediated protein secretion

To characterize the secretion mechanism activated by dectin-1 pathway in macrophages, the identified proteins were first classified using SignalP to determine the presence of signal peptide sequence needed for conventional protein secretion. Only 26% of curdlan-induced and 20% of GBY-induced secreted proteins were confirmed as secretory proteins, with the presence of signal sequences with a good prediction value and cleavage site position

FIGURE 2. Robust secretion of intracellular proteins after β-glucan stimulation is not a result of cell death. (A) Human macrophages were left untreated or stimulated with curdlan or cytosolic pI:C for 18 h, after which the possible ongoing apoptosis was studied with the APOPercentage apoptosis assay. Apoptotic cells are stained purple. pI:C, a cytotoxic viral dsRNA analog, was used as a positive control. Original magnification ×10, (B) Western blot analysis with caspase-3 Ab from total cell lysates. Human macrophages were stimulated with LPS, curdlan, or GBY or infected with influenza A virus for 18 h, after which the cell lysates were analyzed with anti–caspase-3 p19/17. Influenza A virus infection was used as a positive control. (C) LDH cytotoxicity assay was used to quantify lactose dehydrogenase (LDH) release into cell culture media. (D) Cell viability was determined by measuring intracellular ATR using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) after 18 h stimulation with LPS, curdlan, or GBY or influenza A virus infection.

The dectin-1 pathway activates both conventional and unconventional, vesicle-mediated protein secretion (Fig. 3A, Supplemental Table IIIA). This result led us to hypothesize that mechanisms other than conventional, signal peptide sequence–dependent secretion mechanisms were also being activated during β-glucan stimulation in human macrophages. The cells secrete the proteins into the extracellular space through membrane vesicles of endosomal and plasma membrane origin called “exosomes” and “microvesicles,” respectively (26). Based on the exosomal protein database ExoCarta, 49% of curdlan and GBY-induced secreted proteins were previously reported as being exosomal proteins (Fig. 3A, Supplemental Table IIIB). We then classified the secreted proteins based on their gene ontology annotations for “biological processes” (Fig. 3B). The exosomal proteins identified in our secretome data included typical exosomal proteins, such as proteins involved in protein metabolic processes (e.g., proteosome subunits and ribosomal proteins) and in vesicle-mediated trafficking (Rab protein family), signaling proteins (annexins and small GTPases), cell structure proteins (actin cytoskeleton proteins), and cell adhesion and migration proteins (integrins, ICAMs). The classically secreted proteins are mainly immune system proteins, such as chemokines and cytokines, and unclassified proteins include proteins involved in gene expression and RNA processing.

The exosomal nature of the protein secretion was confirmed in β-glucan–induced macrophages by conducting an experiment in which we enriched the fraction containing extracellular vesicles from the growth media by ultracentrifugation. Electron microscopy analysis revealed membrane-bound particles with a characteristic exosomal size and shape (Fig. 3C). Western blot analysis with Abs against known exosomal marker proteins including Alix, tsg 101, tubulin, and annexin I demonstrated their increased secretion after β-glucan stimulation (Fig. 3D). In addition, protein separation by SDS-PAGE and visualization with silver staining were used to estimate the total protein amount in exosomal fractions (Fig. 3E), further confirming that activation of the dectin-1 pathway notably increases the secretion of exosomal proteins.

Our secretome analyzes revealed that dectin-1 pathway also increased the secretion of several chemokines and cytokines, most of which were being secreted through conventional signal peptide–mediated pathway (yellow boxes in Fig. 4A, Supplemental Table IIIA). TNF and selected chemokines were quantified from the growth media using the Lumimex assay, with a high level secretion of TNF and chemokines after stimulation of macrophages with β-glucans and LPS (Fig. 4B). To characterize further the conventional protein secretion in macrophages, we used Brefeldin A, which inhibits transport of proteins from endoplasmic reticulum (ER) to Golgi, thereby blocking conventional protein secretion. Macrophages were stimulated with curdlan for 1 h, after which Brefeldin A was added. After 9 h of stimulation, the cell culture supernatants were collected. Lumimex assay revealed that Brefeldin A-treatment of macrophages completely inhibited conventional protein secretion including TNF and chemokines CCL-2 and CCL-5 in macrophages that had been activated through the dectin-1 pathway (Fig. 4C). However, Brefeldin A had no effect on dectin-1–induced unconventional IL-1β secretion (Fig. 4D).

Inflammasome activity is essential for dectin-1–induced unconventional protein secretion

The dectin-1 pathway activates NLRP3 inflammasome in human macrophages (13). After activation, the NLRP3 binds the adaptor protein ASC and procaspase-1 in the inflammasome multiprotein complex. The inflammasome assembly will spontaneously activate caspase-1, which cleaves pro–IL-1β and pro–IL-18 triggering a release of these cytokines. In line with previous study (13), our secretome data showed enhanced IL-1β and IL-18 secretion after
β-glucan stimulation, and this was confirmed by Western blot analysis (Supplemental Table I, Fig. 5A). Cathepsins are lysosomal proteases that are produced as inactive preproenzymes, which need to be cleaved into their active, mature forms (27). β-Glucan induced NLRP3 inflammasome activation has been shown to be dependent on cathepsin activity (13), and our current data indicate that there is a major increase in the secretion of mature cathepsins upon β-glucan stimulation (Fig. 5A). Interestingly, the mature form of cathepsin D was seen in macrophage secretome already at 3 h after dectin-1 activation. To characterize the secretion mechanism of inflammasome-components and regulators after curdlan stimulation in more detail, we enriched the extracellular vesicles from the cell culture media, and IL-1β, IL-18, ASC, and cathepsin secretion was analyzed (Fig. 5B).

Mature forms of IL-1β and IL-18 cytokines, as well as ASC isoforms p22 and p10, could be detected only in the flow-through fraction, which indicates that these molecules are not secreted by vesicles. Cathepsins are presumably released with secretory lysosomes, and as expected, we detected intact cathepsins in the flow-through fraction, which included also lysosomal proteins. Interestingly, the mature forms of cathepsins were detected only in the fraction containing vesicles. Caspase-1 has been implicated in the regulation of unconventional protein secretion in ultraviolet-activated human keratinocytes (28). We used a pharmacologic inhibitor of caspase-1 to examine the possible role of inflammasome activation in the dectin-1–induced protein secretion. Macrophages were stimulated through dectin-1 pathway in the presence or absence of the caspase-1 inhibitor, and cell culture supernatants were collected after 18 h of stimulation. Western blot analysis of cell culture supernatants indicated that in addition to total blockade of IL-1β secretion, the caspase-1 inhibitor also abolished the release of two unconventionally secreted proteins, tubulin and annexin I (Fig. 5C). However, the caspase-1 inhibitor had no effect on the secretion of the mature form of cathepsin D, indicating that cathepsins are activated upstream of the inflammasome.
analysis of cell culture supernatants revealed that caspase-1 inhibition has no effect on dectin-1–induced TNF and chemokine secretion (Fig. 5D). In conclusion, our results demonstrate that dectin-1–induced inflammasome activation is required to trigger unconventional protein secretion, but it is not essential for the release of conventionally secreted proteins.

**β-Glucan-activated protein secretion is dependent on dectin-1/Syk pathway**

Dectin-1 has an ITAM-motif within its cytoplasmic tail, and tyrosine phosphorylation of this motif by Src kinase followed by Syk recruitment is required for receptor activation and initiation of downstream signaling events (29). To confirm the role of dectin-1 in the β-glucan induced secretion of IL-1β and cathepsins, we used primary BMDCs from a mouse strain that is deficient in the dectin-1 receptor (15). Mouse bone marrow-derived macrophages are not responsive to β-glucans, and for this reason we used BMDCs (31). Dendritic cells from dectin-1<sup>−/−</sup> mice were unable to release the mature, biologically active form of IL-1β, and the secretion of mature forms of cathepsins were dectin-1 dependent (Fig. 6A, 6B). In contrast, expression of IL-1β mRNA in response to curdlan stimulation was only partially dependent on dectin-1 (Fig. 6C). These data indicate that other receptors, such as scavenger receptors CD36 and SCARF1 (30) or complement receptor CR3 (31) may be involved in the activation of IL-1β gene transcription in response to curdlan stimulation. Dendritic cells prepared from dectin-1<sup>−/−</sup> mice did not express IL-1β after stimulation with GBY, whereas dectin-1 deficiency had no effect on the LPS response (Fig. 6C).

The role of Syk and Src kinase in curdlan-induced secretion of inflammasome components and associated regulators was investigated using specific inhibitors for Syk (SykII) and Src (PP2 or Src inhibitor I). SykII clearly decreased the secretion of the mature forms of IL-1β, cathepsins B and D, and ASC p10 in macrophages.
In contrast, PP2 or Src inhibitor I treatment had no effect on the secretion of these proteins (Fig. 6D, Supplemental Fig. 1A). The effect of these inhibitors on the production of pro–IL-1β was also studied: the inhibitors alone had only a marginal effect on pro–IL-1β expression, but the combination of Syk and Src inhibitors completely abolished the production of pro–IL-1β (Fig. 6E). We also investigated the role of Syk and Src signaling in the total protein secretion: SykII alone clearly reduced curdlan-induced protein secretion, whereas PP2 or Src inhibitor I on their own had no effect on total protein secretion (Fig. 6F, Supplemental Fig. 1A). Western blot analysis of annexin I and tubulin demonstrated that secretion of these proteins was solely dependent on Syk, whereas the secretion of β2-integrin was inhibited only when both Syk and Src signaling were blocked (Fig. 6F).

**Inhibition of autophagy suppresses dectin-1–induced vesicle-mediated protein secretion**

Autophagy is a highly conserved biological process involved in the degradation of defective organelles and long-lived proteins. Previous studies have demonstrated that autophagy regulates inflammasome activation and IL-1β secretion (32–35); however, the results have been somewhat conflicting. Our secretome data revealed that several autophagy proteins were secreted during β-glucan stimulation (Fig. 7A). In addition, a recent report identified 94 autophagy-associated proteins (36), and 84 of them were detected in our secretome data (Supplemental Table IIIIC). This finding strongly suggests that autophagy participates in the protein secretion activated by dectin-1 pathway. To characterize the role of autophagy in protein secretion from human macrophages in response to β-glucan stimulation, we performed a Western blot analysis of LC3, a well-known marker of autophagy activation. This revealed that the conversion of the cytosolic LC3-I to the lipid-associated LC3-II, which is found on autophagy membranes, was increased in response to curdlan already at the 3-h time point (Fig. 7B). Next, the dectin-1–induced initiation of autophagy was inhibited using 3-MA (Fig. 7C), which inhibits the activity of class III phosphatidylinositol-3-OH kinase (PI(3)K) and the formation of autophagosomes. 3-MA treatment decreased significantly curdlan-induced IL-1β secretion (Fig. 7D), but this effect was not due to any reduction in IL-1β mRNA expression (Supplemental Fig. 1B). The silver-stained SDS-PAGE gel showed that the dectin-1–induced total protein secretion was clearly suppressed after 3-MA treatment (Fig. 7E). Western blot analysis of exosomal, inflammasome-associated and autophagy proteins indicated that the secretion of unconventionally secreted proteins was suppressed. To determine the involvement of beclin-1, a key component of the class III PI(3)K complex, in dectin-1–induced protein secretion we reduced beclin-1 protein expression with the siRNA approach. Transfection of macrophages with beclin-1–specific siRNA molecules reduced beclin-1 expression by 50% (Fig. 6D). In contrast, PP2 or Src inhibitor I treatment had no effect on the secretion of these proteins (Fig. 6D, Supplemental Fig. 1A). The effect of these inhibitors on the production of pro–IL-1β was also studied: the inhibitors alone had only a marginal effect on pro–IL-1β expression, but the combination of Syk and Src inhibitors completely abolished the production of pro–IL-1β (Fig. 6E). We also investigated the role of Syk and Src signaling in the total protein secretion: SykII alone clearly reduced curdlan-induced protein secretion, whereas PP2 or Src inhibitor I on their own had no effect on total protein secretion (Fig. 6F, Supplemental Fig. 1A). Western blot analysis of annexin I and tubulin demonstrated that secretion of these proteins was solely dependent on Syk, whereas the secretion of β2-integrin was inhibited only when both Syk and Src signaling were blocked (Fig. 6F).
Fungi are associated with a wide spectrum of diseases in humans and animals. These diseases include acute, self-limiting pulmonary manifestations and cutaneous lesions in immunologically competent individuals. In immunologically compromised patients, fungi can cause severe, life-threatening infections. An increased prevalence of cancer, chemotherapy, organ transplantation, and autoimmune disease has been reported to be associated with fungal infections in immunologically compromised patients. In this study, we have characterized the global innate immune response of human primary macrophages in response to dectin-1 activation using a systems biology approach. We demonstrate that the dectin-1 pathway induces significant gene expression changes and robust protein secretion in macrophages (summarized in Fig 8).

Many eukaryotic proteins are secreted through the conventional ER-Golgi secretory pathway, which is primarily regulated at the level of gene expression. These proteins contain N-terminal or internal signal peptides that direct them to the ER. In our quantitative secretome analysis, we identified many classically secreted proteins in cell culture supernatants of human macrophages in response to dectin-1 activation. The classically secreted proteins identified included many chemokines (e.g., CCL2-5, CXCL1-3,8) and cytokines (IL-6, IL-23, and TNF-α) that contribute to the innate immune response during microbial infections. In addition to classical protein secretion, there is extensive protein secretion through unconventional protein release was seen after dectin-1 activation. The unconventional protein secretion seen in macrophages activated through the dectin-1 pathway includes exosome-like vesicle-mediated release of proteins. This vesicle type of secretion delivers more efficiently signaling molecules to adjacent cells as compared with classically secreted proteins, which can diffuse throughout the extracellular environment (37).

One major group of inflammatory proteins identified in our secretome data were danger-associated molecular pattern molecules (DAMPs), which are preformed endogenous molecules, usually with a well-defined intracellular function, released or exposed following an injury or a stress. Our secretome and transcriptome data reveal that both curdlan and GBY stimulation clearly increased the secretion of several proteins that have been demonstrated, or at least proposed to have a role as endogenous danger signals (38, 39) (e.g., galectins, heat shock proteins, HMGB-proteins, S100-proteins; Supplemental Table I), but the gene expression levels of these molecules were not upregulated during cell stimulation (Supplemental Table II). Interestingly, all the identified DAMPs, except for HMGBs, are found in the ExoCarta (Supplemental Table IIIIB), evidence that DAMPs are secreted through exosomes. Galectin-3 was one of the DAMPs robustly secreted in response to dectin-1 activation; this is also
a PRR that recognizes the carbohydrates uniquely present in the cell walls of *Candida albicans*. Interestingly, it was recently shown that galectin-3 is directly associated with dectin-1 and is essential for pathogenic *C. albicans*-induced proinflammatory response (40). It is likely that the robust release of galectin-3 from human macrophages upon β-glucan stimulation results in the enhanced activation of dectin-1/Syk signaling pathway and more efficient antifungal defense.

One common feature of many types of vesicles is their expression of adhesion molecules. Our analysis demonstrated that β-glucan stimulation clearly induced the secretion of proteins involved in leukocyte migration like integrins, intracellular adhesion molecules and matrix metalloproteinases (Supplemental Table I). In addition, proteins that regulate actin cytoskeleton reorganization were highly upregulated in cell culture media. Leukocyte migration from the blood into tissues is vital for immune surveillance and inflammation.

During migration, the leukocytes bind to endothelial cell adhesion molecules and then migrate across the vascular endothelium. In this process, intracellular signals trigger an actin cytoskeleton reorganization and endothelial cell junction dissociation. These molecules are believed to mediate cell-to-cell communication by facilitating the interaction of secreted vesicles with recipient cells (41). After interacting with molecules on the recipient cell surface, the exosomes may fuse with the recipient plasma membrane, leading to the incorporation of proteins from the exosomal membrane into the membrane and the release of exosome contents into the cytoplasm of the recipient cell.

β-Glucans are potent activators of NLRP3 inflammasome in macrophages (13, 42); this is an essential part of the innate immune response to fungal infection also in vivo (43). Inflammasome activation is associated with the secretion of its central components, and active caspase-1 has been shown to be a regulator of unconventional

### Table

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### Figure 7

**Inhibition of autophagy suppresses unconventional protein secretion in macrophages that have been activated through the dectin-1 pathway.**

(A) List of autophagy proteins that were identified in the secretomes of β-glucan–stimulated macrophages. (B) Human macrophages were stimulated with curdlan for the times indicated. Next, cell lysates were prepared, and the expression LC3 was analyzed with immunoblotting. Numbers below lanes indicate the ratio of LC3-II/LC3-I. (C–E) Human macrophages were untreated or treated with 10 mM 3-MA for 1 h before curdlan stimulation (18). Next, the expression of LC3 was analyzed from cell lysates with Western blotting (C), and the expression of IL-1β was analyzed from cell culture supernatants by ELISA and Western blotting (D), respectively. The secreted proteins were separated by SDS-PAGE and visualized with silver-staining and Western blotting (E). (F) Human macrophages were transfected with control siRNA and beclin-1 specific siRNA molecules, after which they were left unstimulated or stimulated with curdlan for 18 h. Subsequently, cell culture supernatants were collected and concentrated, and the secreted proteins were separated by SDS-PAGE and visualized with silver-staining and Western blotting. (G) The IL-1β secretion was analyzed from cell culture supernatants with ELISA, and (H) TNF, CCL2, and CCL5 secretion was analyzed with Luminex assay.
However, our results show that Syk can act independently of Src in this process. In general, Src-family kinases and Syk tend to inhibit Syk kinase. In contrast, Src kinases had only a minor role on Syk kinase. The secretion of IL-1β-glucans upon dectin-1 activation was completely dependent on Syk kinase (50); this points to a role for LC3 in regulating phagocytosis, in addition to being a central player in autophagy.

In conclusion, we provide the first comprehensive characterization of the secretome and the associated intracellular signaling pathways involved in dectin-1/Syk signaling in human primary macrophages. In addition, we demonstrate an important role for unconventional, vesicle-mediated protein secretion in dectin-1-activated innate immune response.

Disclosures
The authors have no financial conflicts of interest.

References
9. Meissner, F., R. A. Scheltema, H. J. Mollenkopf, and M. Mann. 2013. Direct proteomic quantification of the secretome and the associated intracellular signaling pathways involved in dectin-1/Syk signaling in human primary macrophages. In conclusion, we provide the first comprehensive characterization of the secretome and the associated intracellular signaling pathways involved in dectin-1/Syk signaling in human primary macrophages. In addition, we demonstrate an important role for unconventional, vesicle-mediated protein secretion in dectin-1-activated innate immune response.

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