Spatiotemporal changes of the phagosomal proteome in dendritic cells in response to LPS stimulation

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**Abbreviations**

BMDC – bone marrow-derived dendritic cell

CATH – cathepsin

CANX – calnexin

CALR – calreticulin

DAVID – Database for Annotation, Visualization and Integrated Discovery

DC – dendritic cell

EEA1 – early endosome antigen 1

GO – gene ontology

IRG1 – immune-responsive gene 1

LAMP – lysosome-associated membrane protein

LBP – latex bead-containing phagosome

LPS – lipopolysaccharide

MΦ – macrophage

MHC – major histocompatibility complex

Mtb – *Mycobacterium tuberculosis*

NOS – nitric oxide synthase

NOX – NADPH oxidase

OVA – ovalbumin

PCV – pathogen-containing vacuole

ROS – reactive oxygen species

SLC – solute carrier protein

STX – syntaxin

STXBP – syntaxin-binding proteins

TFR – transferrin receptor

ZBP1 – Z-DNA-binding protein 1
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Abstract

Dendritic cells (DCs) are professional phagocytes that use innate sensing and phagocytosis to internalize and degrade self as well as foreign material, such as pathogenic bacteria, within phagosomes. These intracellular compartments are equipped to generate antigenic peptides that serve as source for antigen presentation to T cells initiating adaptive immune responses. The phagosomal proteome of DCs is only partially studied, in particular in response to inflammatory cues, and is highly dynamic as it changes during phagosome maturation, when phagosomes sequentially interact with endosomes and lysosomes. In addition, the activation status of the phagocyte can modulate the phagosomal composition and is able to shape phagosomal functions.

In this study, we determined spatiotemporal changes of the proteome of DC phagosomes during their maturation and compared resting and lipopolysaccharide (LPS)-stimulated bone marrow-derived DCs by label-free, quantitative mass spectrometry. Ovalbumin-coupled latex beads were used as phagocytosis model system and revealed that LPS-treated DCs show decreased recruitment of proteins involved in phagosome maturation, such as subunits of the vacuolar proton ATPase, cathepsin B, D, S and RAB7. In contrast, those phagosomes were characterized by an increased recruitment of proteins involved in antigen cross-presentation, e.g. different subunits of MHC I molecules, the proteasome and tapasin, confirming the observed increase in cross-presentation efficacy in those cells. Furthermore, several proteins were identified that were not previously associated with phagosomal functions. Hierarchical clustering of phagosomal proteins demonstrated that their acquisition to DC phagosomes is not only dependent on the duration of phagosome maturation but also on the activation state of DCs. Thus, our study provides a comprehensive overview of how DCs alter their phagosome composition in response to LPS, which has profound impact on the initiation of efficient immune responses.
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Introduction

Phagocytosis refers to the engulfment of large particles (≥0.5 μm) (1). In Protozoa, this process serves the uptake of nutrients (2), whereas in Metazoa it allows the clearance of dead cells and cell debris, as well as, the uptake and destruction of microbes during infection. The main populations of professional phagocytes that function in innate immunity are macrophages (MΦs), dendritic cells (DCs) and neutrophils (3).

After phagocytosis of a particle, the formed phagosome matures through interactions with endosomes and lysosomes and acquires a diversity of hydrolytic enzymes and antimicrobial peptides, leading to an increasingly degradative and antimicrobial phagosomal environment over time. In addition, the phagosome recruits the V-proton ATPase complex and the NADPH oxidase (NOX) complex, which promote acidification and production of reactive oxygen species (ROS), respectively, to enable optimal killing and destruction of pathogens (4) (5). Most MΦ and neutrophil populations completely degrade their phagocytic cargo, whereas DCs slow down phagosome maturation to preserve phagosomal antigen for presentation on MHC I and MHC II molecules to CD8+ and CD4+ T cells, respectively (6). Thus, the composition of the phagosome is dynamic and changes during phagosome maturation. Moreover, it varies between different types of phagocytes to support their specific function in immunity.

Various research groups have studied the phagosomal proteome of pathogen-containing vacuoles (PCVs) and latex bead-containing phagosomes (LBPs) in the past (7). Although LBPs are artificially induced phagosomes, they present key features of naturally occurring phagosomes and can be isolated at high purity on sucrose gradients, which is essential for further proteomic analysis (8) (9) (10) (11) (12) (13). Furthermore, inert latex beads can be coupled to specific ligands, such as lipopolysaccharide (LPS) or immunoglobulin G (IgG), to study the influence of a single ligand on the phagosomal proteome (14) (15) (16).

In early mass spectrometry studies, around 140 proteins could be identified in phagosome preparations (17). However, major advances in the field have made it possible to identify over 3500 phagosomal proteins (18) and to map almost 3000 phosphorylation sites on over 2400 phagosomal proteins in the last years (19). The phagosomal proteome is predominantly studied in MΦs, and so far only two studies tried to map the phagosomal proteome in DCs. Buschow et al. identified 328 proteins on LBPs of human monocyte-derived DCs, of which 90 were defined as phagosomal proteins by organelle enrichment ranking. They
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detected known as well as novel phagosomal proteins, such as galectin-9 (20). Li and colleagues studied *Mycobacterium tuberculosis*-containing phagosomes isolated from a bone marrow-derived MΦ-like cell line and BMDCs, and were able to map 1001 phagosomal proteins. In addition, they showed that antigen presentation pathways were suppressed to a greater extent in DCs compared to MΦs (21). To address this gap in knowledge on the phagosomal proteome in DCs, we aimed to analyze the phagosomal proteome in both resting and LPS-treated bone marrow-derived DCs (BMDCs) using label-free, quantitative proteomics. Stimuli in the DC environment and on phagocytosed particles can modulate phagosome maturation kinetics and antigen presentation efficacy, which is dependent on the type of stimuli and the duration of stimulation (16). LPS is the major cell wall component of Gram-negative bacteria, which consists of three different moieties, a core oligosaccharide, lipid A and the O-antigen (22). This bacterial product is known to induce the maturation of DCs during innate sensing, a crucial process that transforms immature DCs into full effectors of immunity by inducing antigen presentation capacities (23). While stimulation of DCs with LPS for long time periods (more than 20 h) decreases efficacy of cross-presentation of phagosomal antigens on MHC I molecules to CD8+ T cells (24), we showed previously that intermediate duration of LPS stimulation (7-20 h) restricts phago-lysosomal fusion and antigen degradation to transiently enhance cross-presentation capacity (25) (26). In this study, we focused on this duration of LPS stimulation and treated BMDCs for 16 h with LPS before phagocytosis was initiated in order to better understand the protein composition of mature DCs in comparison to their resting counterparts. As the phagosomal proteome is dynamic, we isolated LBPs at three different time points during phagosome maturation. Over 2800 proteins could be identified in the phagosomal proteome of DCs. Our results showed that the recruitment of proteins involved in phagosome maturation (e.g. subunits of the V-proton ATPase complex, cathepsin B, D, S and RAB7) was decreased in LBPs of LPS-treated DCs compared to resting DCs, whereas the recruitment of proteins involved in cross-presentation (e.g. MHC I subunits, TAP2 and proteasome subunits) was enhanced. Furthermore, several proteins with so far unknown phagosomal functions could be identified in the phagosomal proteome of LPS-stimulated DCs.
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Experimental Procedures

Animals
C57BL/6J female mice were purchased from Janvier and were used for the generation of BMDCs between 8 and 12 weeks of age. All mice were bred at the animal facility of the VIB-UGent Center for Inflammation Research (Ghent, Belgium) under specific pathogen-free conditions. All animal procedures were in accordance with the national guidelines and regulations of Ghent University, Belgium.

Cells
Bone marrow-derived dendritic cells (BMDCs) were generated from BM cells obtained from tibia and femur of mice by culturing them in IMDM/Glutamax medium (ThermoFisher Scientific) containing 10% supernatant from GM-CSF-producing J558 hybridoma cells (27), 10% heat-inactivated FBS (Biowest), 100 IU/ml penicillin, 100 μg/ml streptomycin and 50 μM β-mercaptoethanol (all from ThermoFisher Scientific). BMDCs were stimulated in vitro for 16 h with 100 ng/ml of ultrapure LPS from Escherichia coli 0111:B4 (Invivogen). BMDC generation and maturation was controlled by cell surface expression of CD11c, MHC I, MHC II and co-stimulatory molecules (CD80 and CD86) by flow cytometry.

Antibodies
The following antibodies were used for flow cytometry: rat anti-mouse CD32/CD16 (BD Pharmingen), CD11c APC (N418), CD80 PE (16-10A1), CD86 PE (GL1) MHC I PE (H-2Kb subunit, AF6-88.5.5.3), MHC II PE (I-A/I-E subunit, M5/114.15.2), fixable viability dye eFluor 780 and eFluor 506 (all from eBioscience), purified rabbit polyclonal anti-OVA (Sigma-Aldrich) and goat anti-rabbit Alexa Fluor 488 and 633 antibodies (ThermoFisher Scientific). Flow cytometry samples were measured on an LSR II cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

The following antibodies were used for confocal microscopy: PIP4K2B (sc-393246, Santa Cruz), Vinculin (V9131, Sigma-Aldrich), EEA1 (ab2900, Abcam), Ezrin (#3145, Cell Signaling), ArfGAP1 (sc-271303, Santa Cruz), Lysozyme (A0099, Dako), ATP6V1A (17115-1-AP, Proteintech) and LAMP-1 (13-1071, eBioscience). Goat anti-rabbit and anti-mouse Alexa Fluor 488 antibodies were purchased from ThermoFisher Scientific.
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The following antibodies were used for western blotting: EEA1 (ab2900), CATH D (ab6313), YKT6 (ab77150), IRG1 (ab122624, all from Abcam), RAB5 (C8B1), RAB7 (D95F2), NF-κB2 (#4882, all from Cell Signaling), Calnexin (H70), TGN38 (B-6), RAB6a (38-TB), RAB21 (B16K, all from Santa Cruz), TFR (H68.4, ThermoFisher Scientific), LAMP-1 (1D4B, eBioscience), GM130 (35/GM130, BD Pharmingen), ZBP1 (Zippy-1, Adipogen) and HOOK3 (15457-1-AP, Proteintech).

Isolation of latex bead-containing phagosomes (LBPs)

1 µm carboxylated latex beads (Merck Millipore) were activated with 250 mM N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride and 350 mM N-Hydroxysuccinimide in 100 mM 2-(N-Morpholino)ethanesulfonic acid buffer, pH 5, for 15 min at RT under tilt rotation (all Sigma-Aldrich). Next, beads were conjugated with 0.5 mg/ml low endotoxin ovalbumin (OVA, Worthington) in PBS for 2 h at RT. Resting and LPS-treated BMDCs were collected from culture dishes, resuspended at a cell density of 30 x 10^6 cells/ml in serum-free CO₂-independent medium (ThermoFisher Scientific) and incubated with OVA-coated beads at a bead:cell ratio of 125:1 for 30 min at 37°C. Non-phagocytosed beads were removed by three washes with ice-cold PBS (100 x g, 4 min, 4°C). Afterwards, BMDCs were incubated for different chase periods (15 min, 60 min and 120 min) at 37°C and 5% CO₂ to allow phagosome maturation to occur. The chase period was stopped by adding ice-cold PBS and LBPs were released from cells by mechanical lysis using a 1 ml syringe fitted to a 22G needle, as described previously (28). Afterwards, LBP purification was continued as described before (29).

Single-pot solid phase-enhanced sample preparation (SP3)

Phagosomes were lysed in 10% SDS, 50 mM triethylammonium bicarbonate, pH 8.5, sonicated and protein quantification was determined using the BCA protein assay kit (ThermoFisher Scientific). A volume corresponding to 20 µg for each sample was left to reduce for 30 min at 65°C and alkylated by 84 mM iodoacetamide (30 min, RT). Next, samples were processed using the SP3 method (30). Briefly, 50 µl of paramagnetic hydrophilic beads (Merck Millipore), 700 µl of acetonitrile and 50 µl of 10% formic acid were added to each sample. Samples were then vortexed and left to incubate at RT for 8 min and placed on a magnetic rack. Supernatants were discarded and beads were washed twice with 200 µl of 70% ethanol and once with 200 µl of acetonitrile. Proteins were eluted in 100 µl of 50 mM triethylammonium bicarbonate,
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10% trifluoroethanol, 0.05% SDS, pH 8.5, and digested with trypsin at a ratio of enzyme:substrate of 1:100 (w/w) at 37°C. After 3 h, a second aliquot of trypsin 1:100 (w/w) was added to the samples and incubated overnight at 37°C with agitation at 1300 rpm to avoid bead precipitation. After digestion, acetonitrile was added in excess and samples were incubated 8 min at room temperature to bind the peptides back to the beads. Samples were placed on the magnet rack, supernatants were discarded and beads were washed once with 200 µl of acetonitrile. Paramagnetic beads were dried for 10 min and peptides were eluted from the beads with 30 µl 2% DMSO, 50 mM triethylammonium bicarbonate, pH 8.5, in water. Samples were placed on the magnet rack and supernatants were collected in Protein LoBind tubes (Eppendorf) and acidified with 20 µl formic acid.

Mass spectrometry analysis

Samples were analyzed on an Ultimate 3000 rapid separation LC system (ThermoFisher Scientific) equipped with a C18 PepMap, serving as a trapping column (2 cm x 100 µm ID, PepMap C18, 5 µm particles, 100 Å pore size), followed by a 50 cm EASY-Spray column (50 cm x 75 µm ID, PepMap C18, 2 µm particles, 100 Å pore size; ThermoFisher Scientific) with a linear gradient of 2.4-20% (acetonitrile, 0.1% formic acid) over 175 min followed by a step from 20-28% acetonitrile, 0.1% formic acid over 30 min at 300 nl/min. Mass spectrometric identification was performed on an Orbitrap Fusion Tribrid mass spectrometer (ThermoFisher Scientific) operated in “Top Speed” data dependent and positive ion mode. FullScan spectra were acquired ranging from 400 m/z to 1600 m/z, at a resolution of 120 000 (at 200 m/z), with an automated gain control (AGC) of 300,000 and a maximum injection time of 50 ms. Charge state screening was enabled to exclude precursors with a charge state of 1. The intensity threshold for a MS/MS fragmentation was set to 104 counts. The most intense precursor ions are isolated with a quadrupole mass filter width of 1.6 m/z and HCD fragmentation was performed in one-step collision energy of 35% and activation Q of 0.25. MS/MS fragments ions were analyzed in the segmented linear ion trap with a normal scan range in rapid mode. The detection of MS/MS fragments was set up as the “Universal Method”, using a maximum injection time of 300 ms and a maximum AGC of 2,000 ions.

Data processing and quantitative analysis of label-free data
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Protein identification and label-free quantification were performed using MaxQuant Version 1.5.1.7 (31) with the following parameters: stable modification carbamidomethyl (C); variable modifications oxidation (M), acetylation (protein N terminus) and deamidation (NQ). Search was conducted using the Uniprot-Trembl Mouse database (54505 entries, downloaded 2015), using Trypsin/P as a specific protease, accepting a maximum of two missed cleavages and including common contaminants. Mass accuracy was set to 4.5 ppm for precursor ions and 0.5 Da for ion trap MS/MS data. The “match between runs” option enabled transfer of identifications across samples within a time window of 2 min of the aligned retention times. Identifications were filtered at a 1% false-discovery rate (FDR) at the protein level, accepting a minimum peptide length of 7 amino acids. Label-free quantification of identified proteins referred to razor and unique peptides and required a minimum ratio count of two and were calculated based on the raw spectral protein intensity of the MaxQuant software. For each condition, raw intensities were logarithmized and then normalized to the calculated average and used for downstream analyses in Perseus 1.5.3.1 (32). Student's t-test (two-tailed, homoscedastic) was performed on the normalized protein intensities, and proteins with \( P < 0.05 \) and a fold change > 1.5 were considered significantly altered in abundance between the samples. All raw datasets as well as the normalised dataset can be accessed in PRIDE (PXD012155) using the following login details: username: reviewer17591@ebi.ac.uk; password: 73m8TwPO.

Clustering of phagosomal proteins

Phagosomal proteins were clustered according to their log2 [LPS/resting] during the three different time points (15 min, 60 min and 120 min) in Perseus (33). The data were clustered hierarchically in 8 clusters, based on the k-means of cosine similarity.

LBP lysis and confirmation of phagosomal proteins by western blotting

Phagosome pellets were lysed in 2% (vol/vol) Triton X-100, 50 mM Tris, pH 8.0, 10 mM dithiothreitol, 2 x protease inhibitor cocktail (Roche) for 30 min on ice. Next, phagosomal proteins were recovered after spinning down the beads twice at 16.100 x g for 4 min at 4°C. Protein concentration was measured using the micro BCA protein assay kit (ThermoFisher Scientific), 5 x Laemmli sample buffer was added, and samples were boiled for 5 min at 95°C. Samples were loaded on 4-15% Criterion TGX protein gels (Bio-Rad) and run in 25 mM Tris, 192 mM glycine, 0.1% (m/vol) SDS, pH 8.3, at 100 V. Proteins were transferred
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by wet blotting in 25 mM Tris, 192 mM glycine, 20% (vol/vol) methanol overnight at 30 V. Equal loading of LBP samples was controlled by Ponceau S staining.

**Analysis of phagosome maturation by confocal microscopy**

Phagocytic uptake and phagosome maturation were analyzed as described previously (25) (34). Briefly, 3 μm amino beads (Polysciences) were pre-activated with 8% (vol/vol) glutaraldehyde in PBS for 4 h at RT under tilt rotation and protected from light. Next, beads were conjugated to 0.5 mg/ml low endotoxin OVA (Worthington) and 0.1 mg/ml Alexa Fluor 647 (ThermoFisher Scientific) overnight at 4°C. Reactive groups were quenched with 0.5 M glycine in PBS for 30 min at 4°C and beads were washed three times in PBS. BMDCs were seeded into poly-L-lysine pre-treated 96-well plates with #1.5 polymer coverslips (Ibidi) the day before the phagocytosis experiment and grown for 16 h in presence or absence of 100 ng/ml LPS. Cells were incubated with OVA/Alexa Fluor 647-coupled beads diluted in serum-free, CO₂-independent medium (ThermoFisher Scientific) at a bead:cell ratio of 15:1 for 30 min at 37°C and 5% CO₂. Cells were washed three times with PBS to remove unbound beads and incubated in complete BMDC medium for different chase periods (15 min, 60 min, 120 min) at 37°C and 5% CO₂. Samples were washed once with ice-cold PBS, fixed in PBS containing 4% (vol/vol) paraformaldehyde, pH 7.4, and 4% (vol/vol) sucrose for 20 min at RT, followed by quenching in PBS containing 50 mM ammonium chloride for 10 min at RT. Samples were blocked with normal goat serum for 60 min and incubated with primary antibodies overnight at 4°C. Subsequently, cells were washed three times in PBS and incubated with Alexa 488 secondary antibodies and Alexa Fluor Plus 405-labeled phalloidin (all from ThermoFisher Scientific) for 60 min at RT. After three washes in PBS, wells were sealed with Mowiol mounting medium and analyzed by confocal microscopy using an LSM 880 microscope (Zeiss) equipped with a 63x oil Plan-Apochromat objective (NA 1.4). Images of entire cells were acquired with a step width of 500 nm in z-direction using Zen software (Zeiss). In all samples, maximum projections of three focal planes were generated using Fiji software (ImageJ 1.51, NIH) to display the localization of labeled proteins of the phagosomes that are displayed in insets.

**Experimental Design and Statistical Rationale**
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For LC-MS/MS analysis, LBPs were isolated from BMDCs in independent experiments of four biological replicates (15 min time point of both, resting and LPS-treated cells) and three biological replicates (60 min and 120 min time point of both, resting and LPS-treated cells). Therefore, a total of 20 samples was analyzed by LC-MS/MS. Protein identification was performed using MaxQuant (v 1.5.1.7). Identifications were filtered at a 1% false-discovery rate (FDR) at the protein level, accepting a minimum peptide length of 7 amino acids. Label-free quantification of identified proteins referred to razor and unique peptides and required a minimum ratio count of two and were calculated based on the raw spectral protein intensity of the MaxQuant software. For each condition, raw intensities were logarithmized and then normalized to the calculated average and used for downstream analyses in Perseus 1.5.3.1 (32). Student’s t-test (two-tailed, homoscedastic) was performed on the normalized protein intensities, and proteins with P < 0.05 and a fold change > 1.5 were considered significantly altered in abundance between the samples. Further details can be found above in the paragraph ‘Data processing and quantitative analysis of label-free data’.

For WB validation, LBPs were isolated from BMDCs in two independent experiments comprising all time points (15 min, 60 min, 120 min) and both conditions (resting and LPS-treated). Therefore, a total of 12 samples was analyzed by western blotting.

For analysis by confocal microscopy, images of BMDCs of two independent experiments comprising all time points (15 min, 60 min, 120 min) and both conditions (resting and LPS-treated) were acquired.
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Results

Quantitative proteome analysis of DC phagosomes

In this study, we aimed to explore the effect of LPS stimulation for 16 h, applied at a concentration of 100 ng/ml, on the phagosomal proteome in DCs by label-free quantitative proteomics to follow spatiotemporal changes during phagosome maturation. To this end, we pulsed resting and LPS-treated BMDCs with 1 µm OVA-coated latex beads for 30 min. After the pulse of phagocytic particles, we removed non-internalized beads by several washes. Since it is known that the phagosomal proteome is very dynamic (35), we isolated LBPs after different chase periods (15 min, 60 min and 120 min) to analyze different time points of phagosome maturation. LBPs were released from cells by mechanical cell lysis and purified by ultracentrifugation on a discontinuous sucrose gradient. Replicates of phagosomal protein extracts were digested with trypsin and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) on an Orbitrap Fusion Tribrid mass spectrometer (Figure 1).

Data derived from three to four biological replicates were used to identify and quantify phagosomal proteins that were detected at least twice in each condition. 2843 proteins could be identified in the phagosomal proteome, which is in the same range as previously reported for the phagosome proteome of macrophages (18). In resting DCs, 80, 15 and 233 proteins were enriched at least 1.5-fold in 15 min LBPs, 60 min LBPs and 120 min LBPs (log2 [LPS/resting] ≤ -0.58, P-value < 0.05), respectively (Figure 1). Moreover, we found 113, 37 and 80 proteins to be significantly enriched in 15 min LBPs, 60 min LBPs and 120 min LBPs of LPS-treated BMDCs (log2 [LPS/resting] ≥ 0.58, P-value < 0.05) (Figure 1). Using the Database for Annotation, Visualization and Integrated Discovery (36) (37), we determined in a gene ontology (GO) analysis the major biological processes that are predominantly modulated in LBPs of resting and LPS-treated BMDCs during the different stages of phagosome maturation (shown in Figure 1). For this analysis, all proteins with a significant 1.5-fold change were included.

Early (15 min), intermediate (60 min) and late (120 min) phagosomes of resting BMDCs were enriched for proteins that function in different types of localization processes and membrane fusion. In 15 min LBPs, these proteins were associated with phagosomal acidification (ATP6v1b2, ATP6v1h), phagosomal antigen degradation (CATH S) and phagosomal fusion with endosomes and lysosomes (syntaxin (STX) 8, STX12, VTI11b). In 60 min LBPs of resting BMDCs, these proteins included VPS16, a subunit of the CORVET/HOPS
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complex, and SNAP29, which functions in the tethering of membranes to promote phagosomal fusion with endosomes and lysosomes (38) (39). In 120 min LBPs, we found a strong enrichment of various Rab proteins (RAB7, RAB8a, RAB11b, RAB18, RAB21), in addition to syntaxin-binding proteins (STXBP2, STXBP3) and SNAREs (SNAP23 and VAMP8) that function in the fusion of cellular membranes. Thus, LBPs of resting BMDCs were sequentially enriched for proteins that are directly involved in the progression of phagosome maturation, such as fusion with endocytic vesicles, phagosomal acidification and degradation of phagosomal cargo.

In contrast, LBPs of LPS-treated BMDCs were generally enriched with proteins associated with biological processes that are involved in eliciting an immune response, characterized by GO terms “immune system process” and “cell killing”. At the 15 min time point, we found enrichment of ATPase inhibitory factor 1 (ATPIF1), which was shown to modulate mitochondrial pH (40), and of different MHC I subunits (H2-K1, H2-T23) involved in cross-presentation of antigen to CD8+ T lymphocytes. The two main pathways for cross-presentation of phagosomal antigen are referred to as the “vacuolar” and “cytosolic” pathway (41). In the “vacuolar” pathway, antigen is processed by phagosomal proteases and the resulting peptides are loaded on MHC I molecules in the phagosome. In contrast, in the “cytosolic” pathway, antigen is exported out of the phagosome into the cytosol for degradation by the proteasome, after which the resulting peptides are imported back into the phagosome or into the ER for loading on MHC I molecules. Although 60 min LBPs and 120 min LBPs were also enriched in proteins associated with the GO term “immune system process”, these proteins are different from the ones of early phagosomes. In 60 min LBPs, we found among others SEC16a, a molecule involved in the export of proteins from the endoplasmic reticulum (ER) (42) and subunits of HSP90 (HSP90ab1 and HSP90aa1), which mediate correct protein folding in the ER (43). Late phagosomes (120 min LBPs) of LPS-treated DCs display an enrichment of interferon-induced proteins, such as BST2 (44) and interferon activated protein 204 (IFI204) (45). To conclude, LBPs in LPS-treated BMDCs were enriched in proteins involved in eliciting an immune response, such as MHC I molecules and proteins induced by interferons.

Proteins associated with similar phagosomal functions show similar kinetics of differential acquisition
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In order to identify phagosomal functions that are increased or decreased in phagosomes of DCs after LPS treatment, we grouped phagosomal proteins and the kinetics of their differential recruitment according to protein function (Figure 2). LBPs of resting BMDCs were more enriched in subunits of the V-proton ATPase complex and in many lysosomal and plasma membrane hydrolases, suggesting faster phagosomal acidification and antigen degradation kinetics in these cells. In contrast, proteins that function in cross-presentation of phagosomal antigen, such as TAP2 as well as different subunits of the proteasome (PSMA1, PSMD1, PSMD2, PSMD11 and PSMA12) and MHC I (H2-K1, H2-D1, H2-M2 and H2-M3), were mostly present in LBPs of LPS-treated BMDCs, suggesting more efficient cross-presentation activity to CD8+ T cells. Hence, these data support our previous findings showing that LPS-treated DCs delay phagosome maturation to transiently enhance cross-presentation efficiency, which is affected by the RAB34-dependent, peri-nuclear clustering of lysosomes (25).

We quantified different proteins involved in fusion and vesicular trafficking, such as syntaxins, sorting nexins, Rab proteins and vacuolar sorting proteins (Figure 2). Interestingly, some syntaxins (STX4, STX5), syntaxin-binding proteins (STXBP2 and STXBO3), sorting nexins (SNX1), Rab proteins (RAB6a, RAB11b, RAB21) and vacuolar sorting proteins (VPS4b) were more present in LBPs of LPS-treated BMDCs at an early time point (30 min/15 min), whereas they were more present in LBPs of resting BMDCs at a later time point (30 min/120 min). This suggests a faster recruitment of these proteins to LBPs of LPS-treated BMDCs. The same kinetics of differential acquisition were observed for HOOK3, a protein involved in trafficking of the phagosome along microtubules (46)(47). In contrast, SNX6 is more present in early LBPs of resting BMDCs and in late LBPs of LPS-treated BMDCs, suggesting faster recruitment to LBPs of resting cells. In addition, RAB7a, STX8, SNX27, VPS29 and VPS38 are enriched on LBPs of resting BMDCs, whereas CHMP4b, CHMP6, VPS33b and SNX12 are more present on LBPs of LPS-treated BMDCs.

Another protein group that was enriched in phagosomes of LPS-treated BMDCs included solute carrier (SLC) proteins. While the Toll-like receptors TLR7 and TLR13, involved in recognition of viral ssRNA (48)(49) and bacterial RNA (50), respectively, were enriched in early phagosomes of resting BMDCs, we found the cytosolic DNA receptor ZBP1 specifically enriched in LBPs of LPS-treated BMDCs. Similarly, our proteome data also showed an enrichment of the itaconate-generating enzyme IRG1 in LBPs after LPS stimulation.
Apart from its function in microbial killing and as a source of peptides for antigen presentation, the phagosome also functions as a signaling platform that integrates intra- and extracellular signals. We detected different signaling molecules in phagosomes, with some of them showing enrichment in LPS-treated BMDCs, such as NF-κB1 and NF-κB2. Similarly, nitric oxide synthase (NOS2) was enriched in those phagosomes, suggesting a higher production of nitrogen reactive species in phagosomes of LPS-stimulated DCs. In turn, nitrogen reactive species promote the nitrosylation of the NF-κB1 subunit p65, reducing its DNA-binding capacity and signaling properties (51). In this way, NOS2 might sequester NF-κB molecules to the phagosome. In addition, we detected several proteins on the phagosome that are involved in ubiquitination and SUMOylation, such as ubiquitin ligases (CBL, MARCH7, RNF13), as well as, the SUMO ligase RANBP2 and ubiquitin hydrolase FAM63 (Figure S1).

Clustering of phagosomal proteins reveals patterns of differential acquisition kinetics

In order to gain more insight into the spatiotemporal changes of phagosomal proteins, we clustered them according to the kinetics of their differential acquisition to LBPs of resting and LPS-treated BMDCs. Hierarchical clustering of phagosomal proteins based on the k-means of cosine similarity resulted in 2007 proteins in eight different categories covering 70% of the 2843 identified proteins in total (Figure 3, Supplementary Table 2). These clusters comprise the eight mostly observed patterns of differential protein appearance in LBPs when resting and LPS-treated BMDCs were compared during phagosome maturation. Proteins involved in cross-presentation were allocated to three different clusters. Cluster 5 contains TAP2, SEC61b and certain MHC I and proteasome subunits, which were enriched in LBPs of LPS-treated BMDCs during all three time points. However, TAP1, calreticulin (CALR) and ERAP1 showed transient enrichment in those phagosomes and were allocated to cluster 7 (named “alternately enriched in resting/LPS/resting”). Calnexin, SEC22b, SEC23b, the MHC I subunits H2-K1 and H2-D1, the proteasome subunits PSMD3 and PSMD11 and the MHC II subunits (H2-Eb1, H2-Ab1) were more present in LBPs of LPS-treated BMDCs during the early and intermediate time point (30 min/15 min and 30 min/60 min) and were attributed to cluster 8 (called “early/intermediate enriched in LPS”).

In addition to proteins with known phagosomal localization, cluster 5 also contains various proteins that were not detected in DC phagosomes before, such as ZBP1 and NF-κB2, which might have an unknown phagosomal function in addition to their known cellular functions as DNA/RNA sensor and transcription
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factor, respectively. In cluster 6 (named “enriched in resting at all time points”), we found proteins involved in fusion of phagosomes with early endosomes, such as Rab5a and subunits of the CORVET/HOPS complex (VPS16, VPS33a), and proteins involved in antigen processing, such as CATH S. Other proteins involved in antigen degradation (CATH D, B and Z), phagosomal acidification (ATP6v1c1, ATP6v1g1, ATP6v1e1, ATP6v1h, ATP6v1a, ATP6v1d) and phagosome maturation (LAMP-2) were attributed to cluster 7 (“alternately enriched in resting/LPS/resting”). In addition, this cluster contains many Rab proteins showing similar differential expression kinetics: RAB1b, RAB5c, RAB6a, RAB7a, RAB11b, RAB14, RAB18, RAB21. Other Rab proteins (RAB8a, RAB10, RAB44) as well as HOOK3 were allocated to cluster 8 (“early/intermediate enriched in LPS”). Hence, the kinetics of differential enrichment of proteins involved in phagosome maturation and antigen presentation in LBPs of resting and LPS-treated BMDCs follow different patterns.

To further validate our approach, we performed a similar phagocytosis experiment in BMDCs using fluorescently-labeled, OVA-coupled beads. After an initial pulse period of 30 min to induce uptake of OVA beads, resting and LPS-treated BMDCs were fixed after 15 min, 60 min and 120 min of phagosome maturation and labeled for selected proteins of the eight different clusters and analyzed by confocal microscopy. This method confirmed our previous observations and showed differential acquisition of proteins to LBPs depending on the duration of phagosome maturation and/or the analyzed cell type (resting and LPS-treated BMDCs). Representative examples of the different clusters were phosphatidylinositol 5-phosphate 4-kinase type-2 beta (cluster 1, Figure S2, upper panel), vinculin (cluster 2, Figure S2, lower panel), early endosome antigen 1 (cluster 3, Figure S3, upper panel), ezrin (cluster 4, Figure S3, lower panel), ADP-ribosylation factor GTPase-activating protein 1 (cluster 5, Figure S4, upper panel), lysozyme (cluster 6, Figure S4, lower panel), V-type proton ATPase catalytic subunit A (cluster 7, Figure S5, upper panel) and LAMP-1 (cluster 8, Figure S5, lower panel). In most of these images, the observed signal on LBPs represents a small proportion of the total protein distributed in the cell, which likely vary between proteins, even when they are located in the same cluster. This further supports our assumption that the bead-based purification approach used in our study is more suitable to characterize the phagosomal proteome unambiguously. Together, these findings demonstrate that the phagosomal acquisition of different proteins is not only dependent on the duration of phagosome maturation but also on the activation...
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state of DCs. Therefore, hierarchical clustering of phagosomal proteins might help to identify so far unknown molecular players involved in phagosome maturation, signaling and antigen presentation in DCs.

**Western blotting confirms presence of novel proteins on the phagosome**

After phagocytosis of particles, formed phagosomes mature over time through sequential fusion and fission events with endosomal and lysosomal compartments. During this process, the composition of early phagosomes changes into late phagosomal states and eventually into a phagolysosome, acquiring transiently different protein groups and changing their proteome over time. The three chase periods (15 min, 60 min and 120 min) when LBP-s were isolated for quantitative proteome analysis showed some differences between these three time points, but also between resting and LPS-stimulated DCs. In order to confirm the presence of identified proteins by a mass spectrometry-independent technique, we examined different phagosome maturation markers over time by western blotting (Figure 4A).

In early phagosomes (15 min LBPs), we detected higher amounts of transferrin receptor (TFR), early endosome antigen 1 (EEA1) as well as the small GTPase RAB5, as described previously in macrophages (15). These proteins are predominantly found in early and intermediate endosomes, suggesting fusion events and exchange of material between these compartments. The conversion between RAB5 and RAB7 on phagosomes correlates with the functional change of an organelle characterized by early endosomal properties into a compartment with more degradative functions, such as late endosomes and lysosomes (52). Although we were unable to see differences in the abundance of Rab7 between samples, we detected higher amounts of LAMP-1 and cathepsin D in 60 min LBPs and 120 min LBPs (Figure 4A), indicating that these fractions represent late phagosomes. Since we detected high amounts of cathepsin D in 120 min LBPs (Figure 4A), we consider these fractions to represent phagolysosomes, because this hydrolase is delivered by fusion events between phagosomes and lysosomes. Furthermore, we detected higher levels of cathepsin D in phagosomes of resting BMDCs compared to the ones of LPS-stimulated cells, again confirming that the latter are less progressed in phagosomal maturation.

Furthermore, we demonstrated the purity of our LBP preparations by verifying the absence of markers for other cell organelles. LBP fractions were free from contaminations of mitochondria and the Golgi, as demonstrated by the absence of cytochrome C (CYT C) as well as GM130 and TGN38, respectively (Figure 4A). In addition, we detected only very minimal amounts of YKT6 in our LBP preparations, an R-SNARE
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involved in homotypic vacuole fusion (53). Additionally, previous LBP preparations that were isolated in similar ways than our samples were shown by various techniques to be free of contaminants originated from other cellular organelles (19) (54) (55).

Nonetheless, despite our LBP preparations were free of contaminations of other cell organelle fractions, our quantitative mass spectrometry data of the phagosomal proteome showed the, sometimes unknown, presence of mitochondrial, cytosolic and nuclear proteins, mostly in phagosomes of LPS-stimulated BMDCs. Therefore, we analyzed the presence of some of these proteins also by western blotting (Figure 4B). Immune-responsive gene 1 (IRG1) is a mitochondrial enzyme that converts cis-aconitate to the antimicrobial metabolite itaconate (56). IRG1 expression is induced after LPS stimulation and results in the induction of A20 expression via ROS production (57). In return, A20 suppresses TLR signaling and IRG1 expression (58), resulting in a negative feedback loop that limits IRG1 activity. A cell-permeable itaconate derivate was recently shown to prolong survival against LPS-induced lethality in vivo (59) and to enhance IL-17 controlled skin pathology in a mouse model of psoriasis (60), highlighting the therapeutic potential of this regulatory pathway. In addition, we also found IRG1 in vacuoles containing Legionella pneumophila (61), and it was recently shown that IRG1 is important to regulate inflammatory responses during Mycobacterium tuberculosis (Mtb) infection as IRG1-deficient mice showed increased mortality after Mtb infection (62). We showed by western blotting that IRG1 was induced in both cellular and phagosomal lysates after LPS stimulation. In contrast to the MS data, we could detect IRG1 on LBPs of LPS-treated BMDCs at all three time points (Figure 4B), while we were unable to identify IRG1 in 60 min LBPs above threshold when we applied our quantitative MS approach.

Surprisingly, we detected NF-κB2, which is involved in non-canonical NF-κB signaling, in our LBP samples. This signaling molecule was not detected in previous studies investigating the phagosomal proteome of DCs and MΦ. Non-canonical NF-κB signaling is typically activated by members of the TNF family and involves the NF-κB-inducing kinase (NIK) (63). This kinase phosphorylates IKKα, resulting in ubiquitination and processing of the p100 NF-κB2 fragment to p52, which forms p52/RELB dimers that induce target gene expression (64) (65). The presence of the NF-κB2 p100 and p52 fragments in LBPs of LPS-treated BMDCs could also be confirmed by western blotting (Figure 4B).
ZBP1 is a cytosolic receptor that is upregulated by LPS and IFN-γ (66) and binds viral Z-DNA or Z-RNA (67). ZBP1 mediates virus-induced cell death during MCMV (68) and influenza infection (69). Similarly as IRG1 and NF-κB2, ZBP1 was enriched after LPS stimulation in both, cellular and phagosomal lysates at all three time points (Figure 4B).

Moreover, we investigated the presence of HOOK3 and two Rab proteins, RAB6 and RAB21, in the DC phagosome (Figure 4B). HOOK3 is important for assembly and activation of the dynein/dynactin complex (47) and binding of organelles, such as the phagosome to microtubules (46). It is targeted by the Salmonella SpiC protein to prevent phagolysosomal fusion and to promote Salmonella survival (70). RAB6a promotes the maturation of phagosomes containing Staphylococcus aureus (71), whereas RAB21 functions in the early endocytic pathway (72) and associates with macropinosomes during their maturation (73). Very recently, additional findings suggest that RAB21 regulates pro-inflammatory responses induced by LPS by the promotion of TLR4 endosomal trafficking and activation of downstream signaling (74). Our proteomic results showed a tendency of enrichment of these proteins in LBPs of LPS-treated BMDCs at an early time point (15 min LBPs) and a significant enrichment in LBPs of resting BMDCs at a late time point of phagosome maturation (120 min LBPs). For these proteins, we could not confirm the differential acquisition in response to LPS by western blotting. This might be due to differences in detection sensitivity between mass spectrometry and western blotting, making the latter technique less accurate in terms of quantitative levels in the analysis of complex protein mixtures.
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Discussion

In this study, we mapped the phagosomal proteome in DCs during different stages of phagosome maturation. In addition, we compared the phagosomal proteome between resting and LPS-stimulated DCs. The latter showed a reduced recruitment of lysosomal hydrolases and V-proton ATPase subunits, but higher levels of TAP2, MHC I and proteasome subunits. These changes in the phagosomal proteome of LPS-stimulated DCs support the delay in phagosome maturation and the transient increase in cross-presentation efficacy observed previously in these cells (25). Whereas certain proteins involved in fusion and vesicular trafficking were recruited faster or at higher levels to phagosomes of LPS-treated DCs, others were recruited faster or at higher levels to phagosomes of resting DCs. This indicates that phagosomes in resting and LPS-stimulated DCs may fuse with different types of vesicles of the endocytic pathway. In addition, most detected solute carriers were more abundant in phagosomes of resting BMDCs at the later phagosome maturation time point, suggesting a great need for export of building blocks formed after degradation of phagocytic cargo in these cells at later stages of phagosome maturation.

In our study, we uncovered the phagosomal presence of proteins that were so far not known to be recruited to or to reside in DC phagosomes and confirmed the presence of these proteins, such as IRG1 and ZBP1, by western blotting. Although the functional relevance of these findings awaits further exploration in DCs, studies performed in other cell types suggest an essential role of these molecules in the regulation of inflammation and infection. Itaconate generated by IRG1 is not only important to restrict intracellular growth of Legionella pneumophila (61), but also tempers inflammatory responses in myeloid cells during Mtb infection (62). Furthermore, this immunometabolite has also been shown to affect M2 macrophages, tumour-associated macrophages and immune-paralyzed monocytes and might have more consequences on host defense, immunity and tumorigenesis than previously thought (75). Recent data generated in neurons also show a functional link between IRG1 and ZBP1 by demonstrating the viral restriction in a neuroinvasive infection model helping to suppress the replication of viral genomes (76). Rsad2, also known as viperin, is another protein induced by pro-inflammatory cytokines and infection (77), which we identified in phagosomes of LPS-treated DCs. Recent work characterized an IRF7-mediated signaling pathway that is essential for DC maturation and function, for example to enable efficient antitumor activity (78).
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In our study, we demonstrated an increased recruitment of the p100 and p52 fragments of NF-κB2 in LBPs of LPS-treated BMDCs compared to resting BMDCs. Although NF-κB2 is mostly known for its function as transcription factor, it might have non-canonical functions in the phagosome. Previous work described NF-κB2 as a negative regulator of DC activation and presentation of soluble antigen to CD4+ T cells \textit{in vitro} and \textit{in vivo} (79) and to be important for efficient CD8+ T cell priming (80). Future work is needed to clarify whether NF-κB2 acts in the phagosome as a signaling molecule or whether it exerts a distinct phagosomal function.

So far the phagosomal proteome was mostly studied in MΦ cell lines. However, DCs show partially different functions compared to MΦs, as they display slower phagosome maturation kinetics and present phagosomal antigen to T cells, bridging innate and adaptive immunity (81). In this study, we determined the phagosomal proteome in LPS-stimulated DCs, previously shown to delay phagosome maturation and enhance cross-presentation (25). Therefore, the proteomic data presented here most likely contain novel regulators of phagosomal functions, such as phagosome maturation and antigen presentation. Future work using targeting MS-based techniques, such as SILAC, selected reaction monitoring and SWATH MS, will help to pinpoint and further characterize these novel molecules in DC phagosome biology.

Understanding how antigen presentation is regulated \textit{in vivo} in DCs is important to improve current DC-based vaccine strategies and immunotherapy that induce anti-cancer and anti-viral immunity. In addition, more knowledge on how phagosome maturation can be modulated may advance the development of therapeutics and host directed therapies against pathogens that block phagosome maturation to enhance their survival, such as \textit{Mycobacterium tuberculosis}. Although bead-containing phagosomes present many features of physiological phagosomes, their proteome is similar but not the same compared to the proteome of pathogen-containing vacuoles (PCVs) (82). The isolation of PCVs at high purity for analysis by MS remains challenging, however, recent progress in this field should enable the study of their phagosomal proteome in DCs (83). These studies combined with the findings presented here will hopefully enhance our understanding of how certain pathogens interfere with phagosome maturation and antigen presentation to increase their survival.
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Figure 1. Label-free quantitative proteomics of DC phagosomes. BMDCs were treated with 100 ng/ml LPS for 16 h, and resting and LPS-treated BMDCs were incubated with OVA-coupled beads for 30 min. Non-internalized beads were removed by several washes, and BMDCs were incubated for 15, 60, 120 min.
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to allow phagosome maturation to occur. After the different chase periods, cells were lysed mechanically and LBPs were isolated on a discontinuous sucrose gradient by ultracentrifugation. Phagosomal proteins were digested with trypsin and analyzed by LC-MS/MS on an Orbitrap Fusion spectrometer. Proteins were quantified using MaxQuant and were considered significantly altered in abundance between samples with a P-value < 0.05 and a fold change > 1.5. In the volcano plots, the number of significantly enriched proteins in LBPs of resting BMDCs (blue) and in LBPs of LPS-treated BMDCs (orange) is shown. Enrichment of biological processes was determined by gene ontology analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID). LPS: lipopolysaccharide; OVA: ovalbumin; LBP: latex bead-containing phagosome.
Figure 2. Kinetics of differential acquisition of phagosomal proteins on LBPs of resting and LPS-treated BMDCs. Differential abundance of phagosomal proteins, grouped according to their function, is shown for three time points (15, 60 and 120 min) during phagosome maturation. Differential acquisition of phagosomal proteins on LBPs isolated from resting and LPS-treated BMDCs is represented by their log2 ratio [LPS/resting]. Enrichment in LBPs of resting BMDCs is shown in blue, while increased abundance in LBPs of LPS-treated BMDCs is shown in orange.
Figure 3. Clustering of phagosomal proteins according to the kinetics of their differential acquisition. Phagosomal proteins were clustered based on their differential abundance (log2 ratio [LPS/resting]) at three time points (15, 60 and 120 min) during phagosome maturation. Proteins were grouped in 8 clusters by hierarchical clustering using Perseus, based on k-means of cosine similarity.
Figure 4. Confirmation of the presence of phagosomal proteins by western blotting. LBPs were isolated after a 30 min pulse with OVA-coupled beads, followed by a 15 min, 60 min or 120 min chase period. LBPs were purified by ultracentrifugation on a sucrose gradient, and LBP protein lysates were analyzed together with the total cell lysate (TCL) by SDS-PAGE and western blotting. (A) Analysis of phagosome maturation markers together with a validation of LBP purity demonstrated by the absence of a mitochondrial marker (CYT C), Golgi proteins (GM130 and TGN38) and an ER marker protein (YKT6). (B) The presence of novel phagosomal proteins (IRG1, NF-κB2, ZBP1, RAB6a, RAB21 and HOOK3) was also demonstrated by western blotting. TFR: transferrin receptor; EEA1: early endosomal antigen; RAB: Ras-related protein Rab; CANX: calnexin; LAMP-1: lysosome-associated membrane glycoprotein 1; CATH D:
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cathepsin D; CYT C: cytochrome C; GM130: golgi matrix protein 130; TGN38: trans-golgi network protein 38; YKT6: synaptobrevin homolog YKT6; IRG1: immune-responsive gene 1; NF-κB2: nuclear factor NF-κB p100 subunit; ZBP1: Z-DNA-binding protein 1; HOOK3: protein Hook homolog 3.