RESEARCH ARTICLE

# The endosomal proteome of macrophage and dendritic cells

*Sophie Duclos[1]*, Giovanna Clavarino[2,3,4]*, Gilles Rousserie[2,3,4], Guillaume Goyette[1], Jonathan Boulais[1], Voahirana Camossetto[2,3,4], Evelina Gatti[2,3,4], Sylvie LaBoissière[5], Philippe Pierre[2,3,4]** and Michel Desjardins[1,6]*

[1] Département de Pathologie et Biologie Cellulaire, Université de Montréal, Montréal, QC, Canada
[2] Centre d'Immunologie de Marseille-Luminy, Université de la Méditerranée, Marseille, France
[3] INSERM, U631, Marseille, France
[4] CNRS, UMR6102, Marseille, France
[5] Proteomics Unit, McGill University and Génome Québec Innovation Centre, Montreal, QC, Canada
[6] Département de microbiologie et immunologie, Université de Montréal, Montréal, QC, Canada

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The essential roles of the endovacuolar system in health and disease call for the development of new tools allowing a better understanding of the complex molecular machinery involved in endocytic processes. We took advantage of the floating properties of small latex beads (sLB) on a discontinuous sucrose gradient to isolate highly purified endosomes following internalization of small latex beads in J774 macrophages and bone marrow-derived dendritic cells (DC). We particularly focused on the isolation of macrophages early endosomes and late endosomes/lysosomes (LE/LYS) as well as the isolation of LE/LYS from immature and lipopolysaccharide-activated (mature) DC. We subsequently performed a comparative analysis of their respective protein contents by MS. As expected, proteins already known to localize to the early endosomes were enriched in the earliest fraction of J774 endosomes, while proteins known to accumulate later in the process, such as hydrolases, were significantly enriched in the LE/LYS preparations. We next compared the LE/LYS protein contents of immature DC and mature DC, which are known to undergo massive reorganization leading to potent immune activation. The differences between the protein contents of endocytic organelles from macrophages and DC were underlined by focusing on previously poorly characterized biochemical pathways, which could have an unexpected but important role in the endosomal functions of these highly relevant immune cell types.

**Keywords:**
Cell biology / Subcellular fractionation / MS / Vesicular trafficking

## 1 Introduction

Internalization of nutriments, recycling or degradation of cellular materials, downregulation of receptors and antigen presentation represent some of the numerous functions performed by the endocytic pathway. Receptor-mediated endocytosis through clathrin-coated vesicles has undoubtedly been the most investigated endocytic pathway [1, 2]. Other entry routes, such as caveolar uptake, have also been well characterized in recent years [2]. However, there is much less information about endosomes and lysosomes dynamics at different stages following internalization. The study of endosomes and lysosomes biogenesis is of particular interest with regards to a variety of diseases directly

**Correspondence:** Dr. Michel Desjardins, Département de pathologie et biologie cellulaire, Université de Montréal, C.P. 6128, Succ. Centre-ville, Montréal, QC,H3C 3J7, Canada
**E-mail:** michel.desjardins@umontreal.ca
**Fax:** +1-514-343-5755

**Abbreviations: DC,** dendritic cells; **EE,** early endosomes; **EM,** electron microscopy; **iDC,** immature dendritic cells; **LB,** latex beads; **LE/LYS,** late endosomes/lysosomes; **LPS,** lipopolysaccharide; **mDC,** mature dendritic cells; **PNS,** post-nuclear supernatant; **sLB,** small latex beads

*These authors contributed equally to this work.

**Additional corresponding author: Dr. Philippe Pierre
E-mail: pierre@ciml.univ-mrs.fr

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related to defects in the endocytic pathway. Lysosomal storage diseases including Chediak–Higashi syndrome or Hermansky–Pudlak syndrome type 2 [3–5] are caused by deficiencies in the process of lysosome-related organelles biogenesis. Cystic fibrosis, Niemann-Pick type C disease and a recently described immunodeficiency syndrome [6–8] constitute other endocytosis-related pathologies. Impaired endocytic downregulation of signaling receptors is frequently associated with cancer, since it can lead to increased and uncontrolled receptor signaling [9]. Moreover, many pathogens, including viruses (influenza virus, hepatitis C virus, vesicular stomatitis virus), parasites (*Leishmania*) and bacteria (*Brucella*), depend on endocytic trafficking pathways for their infectivity [10–14].

DC are hematopoietic cells specialized in antigen capture and presentation for initiation of primary and secondary immune responses upon infection. One of the most impressive features of DC activation by microbial products such as lipopolysaccharides (LPS) is the redistribution of MHC class II molecules from intracellular lysosomal compartments to the surface of activated cells [15]. The developmental activation of MHC class II transport in DC has been compared to a gearbox, through which immature ''idling'' DC ''gear up'' their antigen-presenting activity. The realization that DC lysosomes in activated cells can rescue selected molecules from degradation through proteins sorting, tubule formation and transport toward the cell surface [16, 17] has initiated a quest toward the understanding of the molecular machinery regulating this pathway and its ability to drive the immune system toward a productive and focalized antigenic response.

Most organelle proteomics studies rely on subcellular fractionation steps, which have been key in deciphering intracellular transport pathways. However, it is particularly difficult to reach in practice a sufficiently high degree of purification of endocytic organelles to perform reliable proteomics analyses, due to the complexity of vesicular structures of similar buoyant density and their intricate exchange dynamics. In this context, the use of latex beads (LB) has been an invaluable tool to isolate phagosomes and gain significant insights into the process of phagolysosomes biogenesis using MS [18]. We have developed a novel method to form and isolate endosomes based on the use of small latex beads (sLB) internalized by endocytosis, in order to compare and identify the proteome of endocytic organelles in macrophages, and late endocytic organelles isolated from immature DC (iDC) and mature DC (mDC).

## 2 Materials and methods

### 2.1 Cell culture

The murine macrophage-like cell line J774 was cultured in DMEM (Sigma) supplemented with 10% heat-inactivated FBS, 1% glutamine, 100 units/mL penicillin and 100 mg/mL streptomycin at 37°C in a 5% $CO_2$ atmosphere. Cells were grown to 80% confluency in Petri dishes prior to each experiment. Male C57BL/6 mice 7–8 weeks old were purchased from Charles River Laboratories (Cambridge, MA, USA). Bone marrow-derived DC were cultured as described previously [19]. Maturation was induced using 100 ng/mL LPS.

### 2.2 Organelle purification

In J774 macrophages, endosomes were formed by the internalization of red-dyed 100 nm LB (Merck-Chimie S.A.S./Estapor Microspheres, Fontenay Sous Bois, France) for either 15 min (early endosomes (EE)), or 15 min followed by a 60-min chase (late endosomes/lysosomes (LE/LYS)). In DC, 100 nm LB were internalized for 1 h, followed by 3 h chase. Purification of LE/LYS was either initiated or DC were incubated for 16 additional hours with LPS prior to purification. Endocytic organelles were then isolated on a sucrose gradient as already described [20], in three independent experiments for each sample. Approximately $20 \times 10^8$ J774 macrophages and $2 \times 10^8$ DC were needed to generate one sample. The pellets containing purified endosomes were resuspended in Laemmli buffer and quantified with the EZQ™ Protein Quantification Kit (Invitrogen). For each J774 sample, 40 μg was loaded on a 12% NuPAGE® Bis-Tris Gel from Invitrogen, while 20 μg of protein was loaded for each DC sample. Gel migration was stopped at 3 cm, and a silver staining was performed. Then, 1-$mm^3$ gel pieces were cut and placed in a 1% acetic acid solution in the wells of a digestion tray for proteomics analysis.

### 2.3 Proteomics analysis

Gel bands were subjected to reduction, alkylation and in-gel tryptic digestion in an automated MassPrep Workstation (Waters, Millford, MA, USA) as previously described [21]. All MS analyses were performed on a QTOF Micro (Waters) equipped with a Nanosource (New Objective, Woburn, MA, USA) modified to hold the PicoFrit column tip near the sampling cone. MS data were collected with the following data dependent acquisition settings: 1 s in MS mode, 1 precursor ion selected based on intensity (25 cps) and charge state (+2, +3 and +4), with a maximum collection time of 4 s in MS/MS acquisition mode.

MS/MS raw data were transferred from the QTOF Micro computer to a 50 terabytes server and automatically manipulated for generation of peak lists by employing Mascot Distiller version 2.3.1 (http://www.matrixscience.com/distiller.htmls) software with peak picking parameters set at 5 for signal/noise ratio and at 0.4 for correlation threshold. This reduced noise and produced a list of distinct peptide peaks in which all members of the isotopic clusters were collapsed into an equivalent monoisotopic peak. The

peak list data were then searched against the Universal Protein Resource (UniProt) (http://www.pir.uniprot.org/) database using Mascot (http://www.matrixscience.com) version 2.3, and restricting the search to a maximum of one missed (trypsin) cleavage, fixed carbamidomethyl alkylation of cysteines, variable oxidation of methionine, $\pm 0.5$ mass unit tolerance on parent and fragment ions. The search was limited to the *Mus musculus* taxonomy (November 24th, 2009, release; 55 729 sequences; 24 826 712 residues). Scaffold was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm [22]. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least two unique peptides. Protein probabilities were assigned by the Protein Prophet algorithm [23]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. The false discovery rate (FDR) was determined according to Choi and Nesvizhskii's [24] false-positive identification error method. With a protein probability of 99% and 1 peptide at 95%, the false discovery rate is 0.46% for the proteins and 0.012% for peptides. The identified proteins were further annotated using public databases that predict protein function (http://us.expasy.org, http://www.geneontology.org/, http://www.genecards.org/index.shtml). Data mining of the final list was conducted in a MySQL database using Navicat 7 (http://navicat.com/) as graphical user interface. The interactome was generated using a database dedicated to protein–protein interactions (http://string.embl.de/). Interaction networks were then visualized with the help of an open source bioinformatics software platform (Cytoscape: http://www.cytoscape.org/).

### 2.4 Immunoblotting and antibodies

For Western blot analysis, an identical amount of proteins from each sample was used, based on the protein concentration evaluated using the EZQ™ assay (Molecular Probes). The antibodies used were a mouse monoclonal anti-EEA1 (BD Biosciences); a rat monoclonal anti-LAMP1 (Developmental Studies Hybridoma Bank, Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, USA, and the Department of Biological Sciences, University of Iowa, Iowa City, IA, USA, under contract N01-HD-6-2915 from the NICHD); a mouse monoclonal anti-BiP (BD Biosciences); a mouse monoclonal anti-p62 (BD Biosciences); a mouse monoclonal anti-annexin A2 (BD Biosciences); a mouse monoclonal anti-prohibitin (Labvision corporation); a rabbit polyclonal anti-Rab5 (Santa Cruz Biotechnology); a rabbit polyclonal anti-cystatin C (Upstate Biotechnology); a rabbit polyclonal anti-MHC I (p8) (kind gift from J Neefjes, NKI, Amsterdam, The Netherlands); a rabbit polyclonal anti-H2-DM (kind gift from S. Amigorena, Institut Curie, Paris, France) and a rat monoclonal (clone IN1) anti-Ii (kind gift from Ira Mellman, Genentech).

### 2.5 Immunofluorescence and electron microscopy (EM)

Cells were harvested and let to adhere on 1% Alcian Blue-treated coverslips for 10 min at 37°C, fixed with 3% paraformaldehyde in PBS for 10 min at room temperature, permeabilized with 0.5% saponin in PBS/5% FCS/100 mM glycine for 15 min at room temperature and stained for 1 h with indicated primary antibodies. Alexa secondary antibodies (30-min staining) were from Molecular Probes (Invitrogen). Confocal microscopy (microscope model LSM 510, Carl Zeiss MicroImaging) was performed using a 63 × objective and accompanying imaging software. For EM, samples were fixed in 2% glutaraldehyde, post-fixed in 1% $OsO_4$, dehydrated in alcohol, processed for flat embedding in Epon 812 and observed at the Zeiss CEM 902 electron microscope as previously described [20].

## 3 Results

We developed a novel method to isolate endocytic organelles based on the use of sLB fed to cultured cells. We first tested the efficiency of the method on J774 macrophages, previously utilized to isolate and characterize phagosomes formed with larger beads ranging from 1 to 3 μm [25, 26]. Following the internalization of sLB for either 15 min or 15 min followed by a 60-min chase, we were able to fill early and late organelles of the endocytic pathway. Morphological analysis at the EM level indicated that sLB are initially internalized individually in small vesicles (Fig. 1A and B). However, within 15 min of internalization, J774 endosomes containing a large number of sLB are observed suggesting the coalescence of multiple small vesicles containing a single bead. These endocytic organelles are often observed at the cell periphery, and usually display a light electron density (Fig. 1A–C). After a 60-min chase, sLB are typically observed in more electron-dense organelles still displaying a large number of beads (Fig. 1D and E). After cell disruption, a few sLB-containing endosomes can be observed among the majority of other cellular membranes and organelles in the post-nuclear supernatant (PNS) (Fig. 1F, arrows). A simple centrifugation on a sucrose gradient followed by a pelleting step in PBS led to the isolation of highly enriched endosome preparations, apparently devoid of contamination by other organelles (Fig. 1G, arrows). Western blotting of EE or LE/LYS with several antibodies directed against protein markers of various cellular organelles confirmed the high enrichment of endosomes in our preparations and the absence of major

contamination by the Golgi apparatus, the nucleus and mitochondria (Fig. 1H).

With mouse bone marrow-derived DC, sLB were internalized for 1 h, followed by a 3 h chase. Purification of LE/LYS was either initiated or DC were incubated for 16 additional hours with LPS prior purification to induce DC activation and late endosomal reorganization. Efficient internalization and targeting of the beads to the LE/LYS compartments of DC were obvious from their colocalization with the late endosomal protein H2-DM and the lysosomal-associated membrane protein LAMP1 by confocal immunofluorescence (Fig. 2A). DC maturation was evaluated by the high level of lysosomal congregation induced by LPS in the vicinity of the MTOC [15]. Beads were almost exclusively found in H2-DM-expressing DC and not in other cells present at low levels in the culture (e.g. granulocytes) (not shown). Next, β-hexosaminidase activity was monitored using fluorescent substrate and found to be strongly associated with the fractions containing the colored beads (Fig. 2B), thus indicating the successful flotation of late endosomal and lysosomal organelles. Detection of H2-DM demonstrated a considerable enrichment in the beads-loaded endosomes fractions when compared to PNS. The purity of the isolated fractions was further confirmed by immunoblot, as shown by the absence of unprocessed Ii chain (ER-associated) and cystatin C (Golgi) (Fig. 2C).

The high enrichment of endocytic markers in our preparations, together with the minor contamination by other organelles, led us to pursue the analysis of endosomes of both J774 macrophages and DC by a quantitative proteomics approach. The proteins contained in the endosomes preparations were first separated by SDS-PAGE, and the tryptic digests were analyzed by LC MS/MS in a Q-TOF mass spectrometer. This approach led to the identification of 265 distinct proteins from J774 cells (Supporting Information Table 1) and 295 proteins from DC (Supporting Information Table 2). Manual annotation was then performed for each protein. A redundant peptide counting method recently used to compare the relative abundance of proteins in various organelles of the biosynthetic pathway [27] was then applied, allowing to sort proteins according to their relative abundance in early and late endocytic organelles (Supporting Information Tables 1 and 3) or according to the state of DC activation (Supporting Information Tables 2 and 3).

The efficiency of this methodology was confirmed by the correct assignment of several known markers of EE and LE/LYS to their respective organelle preparation in J774 macrophages. Hence, most of the hydrolases displayed a higher relative abundance in the late endocytic organelle preparation

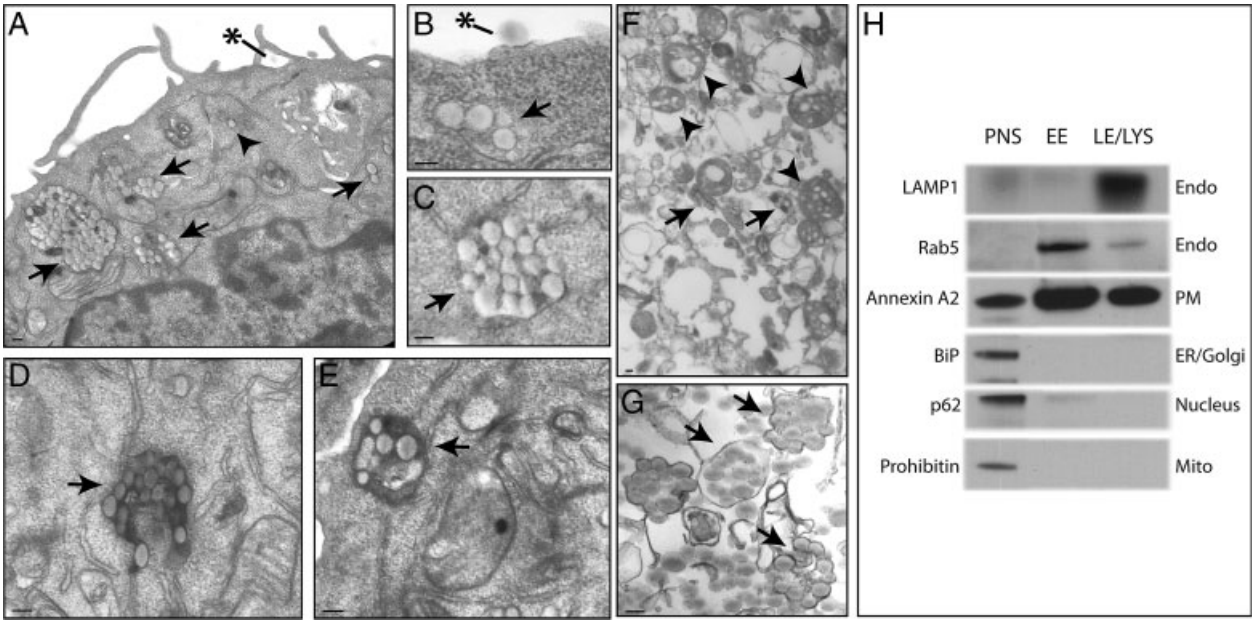



**Figure 1.** Analysis of J774 endosomes preparations. Morphology of sLB containing-endosomes in J774 cells at the EM. The sLB are internalized individually, as shown in panel A and B (asterisks). After 15 min of internalization, endosomes containing either 1 bead (A, arrowhead) or a larger amount of beads can be seen (A, B, C, arrows). Following a 60-min chase, endosomes still contain a large amount of beads, but they now have a typical electron-dense appearance (D, E). Endosomes preparations before and after centrifugation (F and G represent EE preparations, but the same results were obtained with LE/LYS). In the PNS (F), endosomes (arrows) can hardly be seen among a mixture of membranes, mitochondria (arrowheads) and other cell constituents. After centrifugation on the sucrose gradient, a highly enriched endosome preparation is obtained (G). Bars, 100 nm. (H) Western blot analysis of known markers of different organelles. Endocytic markers (LAMP1 and Rab5) are highly enriched in the endosomes preparations as compared to the PNS fraction. Annexin A2, a marker of the plasma membrane (PM) that has also been described on endosomes, is found in both EE and LE/LYS preparations. However, markers of the ER/Golgi, nucleus or mitochondria were not detected in the enriched endosomes preparations.

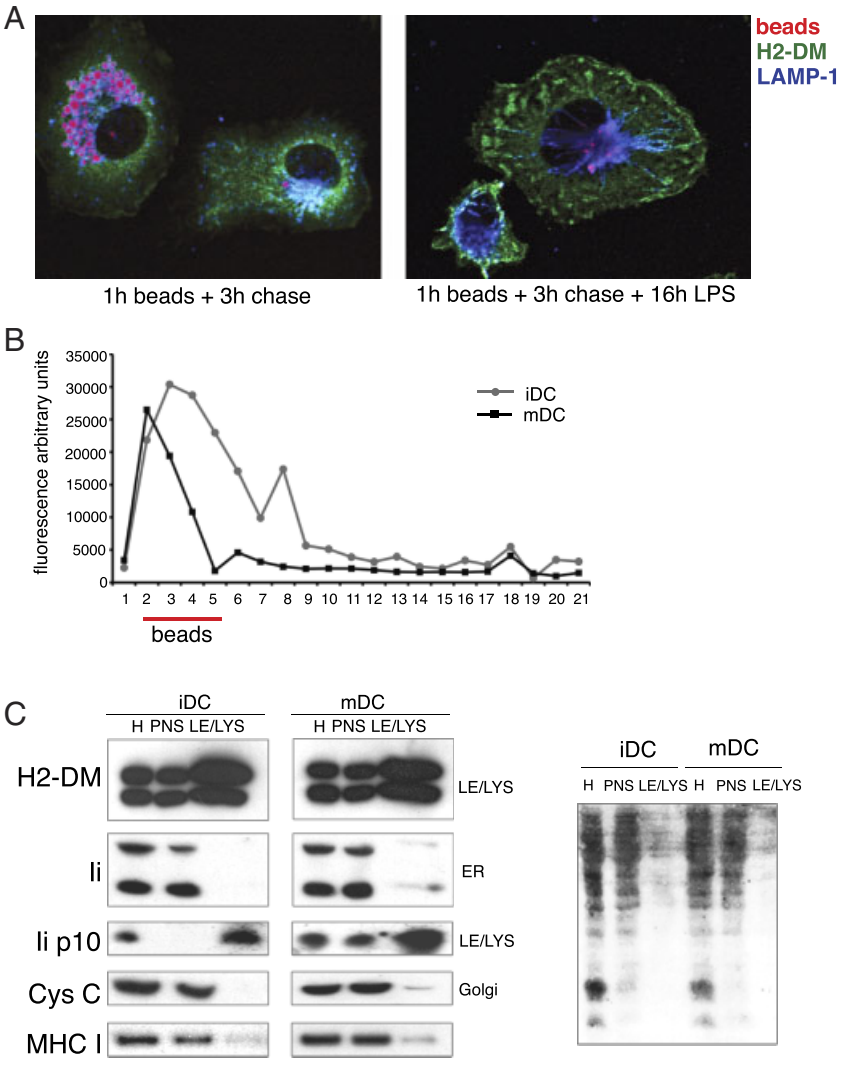
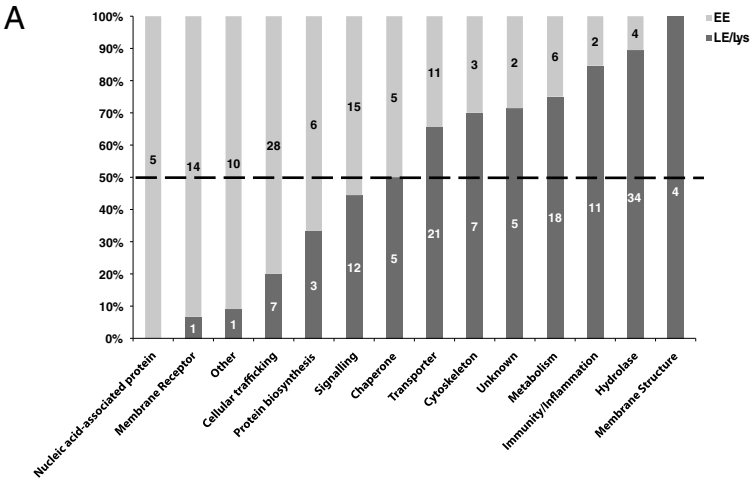



**Figure 2.** Analysis of DC LE/LYS preparations. (A) iDC were incubated for 1 h with sLB, and then chased for 3 h to allow beads internalization and access to the late endocytic compartments. Cells were either fixed (left panel) or stimulated with LPS for 16 h before fixation (right panel). Staining for H2-DM and LAMP-1 was performed before visualization at confocal microscopy. Scale bar, 10 μm. (B) The activity of lysosomal hydrolase β-hexosaminidase (β-hex) was measured by using a fluorescent substrate to evaluate the purity of the lysosomal fraction. β-hex activity was found mostly associated with fractions 2–5 containing the red beads. (C) Total cell homogenate (after mechanical lysis) (H), PNS and LE/LYS preparations from iDC and mDC were loaded on a SDS-PAGE gel and the purity of lysosomal fraction was evaluated by Western blot. H2-DM is enriched in the beads fraction. Invariant chain (Ii) p31 and p41 were not found in iDC and at the limit of detection in activated DC, whereas Ii proteolytic fragment p10 was enriched in the beads fraction. Cystatin C (Cys C) was absent from the beads fraction in iDC and the limit of detection in mDC, as well as MHC I. Coomassie staining shows the amount of proteins loaded for the different fractions (right panel).

of J774, while EE displayed higher levels of proteins like EEA1, Rab5 and the transferrin receptor, three well-characterized EE markers. To further validate our MS data, a literature search was undertaken. We found that 149 out of the total 265 proteins present in our list (56%) have previously been described in the endocytic pathway. Of these 149 proteins, 69 have been assigned endocytic localization by GO terms. In addition, 86 other proteins are plasma membrane proteins, likely to be found in endosomes following internalization. Furthermore, the relative abundance of proteins in early versus late organelles was confirmed by the literature for a large number of proteins. For example, Rab14, VAMP-3, Vps29, Snx17 and Rab21 localize to EE according to the literature [28–32], while Ifi30, Arl8b and Npc2 have all been described as LE/LYS proteins [33–35].

To further dissect the specific protein contents of early versus LE/LYS in macrophages, we compared the relative abundance of unique or enriched proteins as a function of their annotated functional group (Fig. 3A). As expected, hydrolases are highly enriched in LE/LYS, while early organelles contain a large number of proteins involved in cellular trafficking, as well as signaling proteins. Interestingly, although membrane receptors is not the most represented category in EE, 93% of all the membrane receptors found in the analysis are enriched in EE. Schematic representations of virtual endosomes were drawn according to the data obtained from the MS analysis (Fig. 3B). One of the observations from the macrophage virtual endosomes is that most of the Rab proteins are observed in EE. Since Rab proteins are involved in fusion events, this correlates with previous data showing that early endocytic compartments are more competent regarding to fusion [36].

After the sorting of proteins to their respective organelles in macrophages, networks of interactions were deduced based on interaction databases (Fig. 4). These networks highlight the close and complex interactions occurring between proteins preferentially associated to EE or LE/LYS. For example, the interactome showed interactions between Vps29 and Vps35 proteins. These proteins are part of the retromer complex, which is known to associate with EE [30].



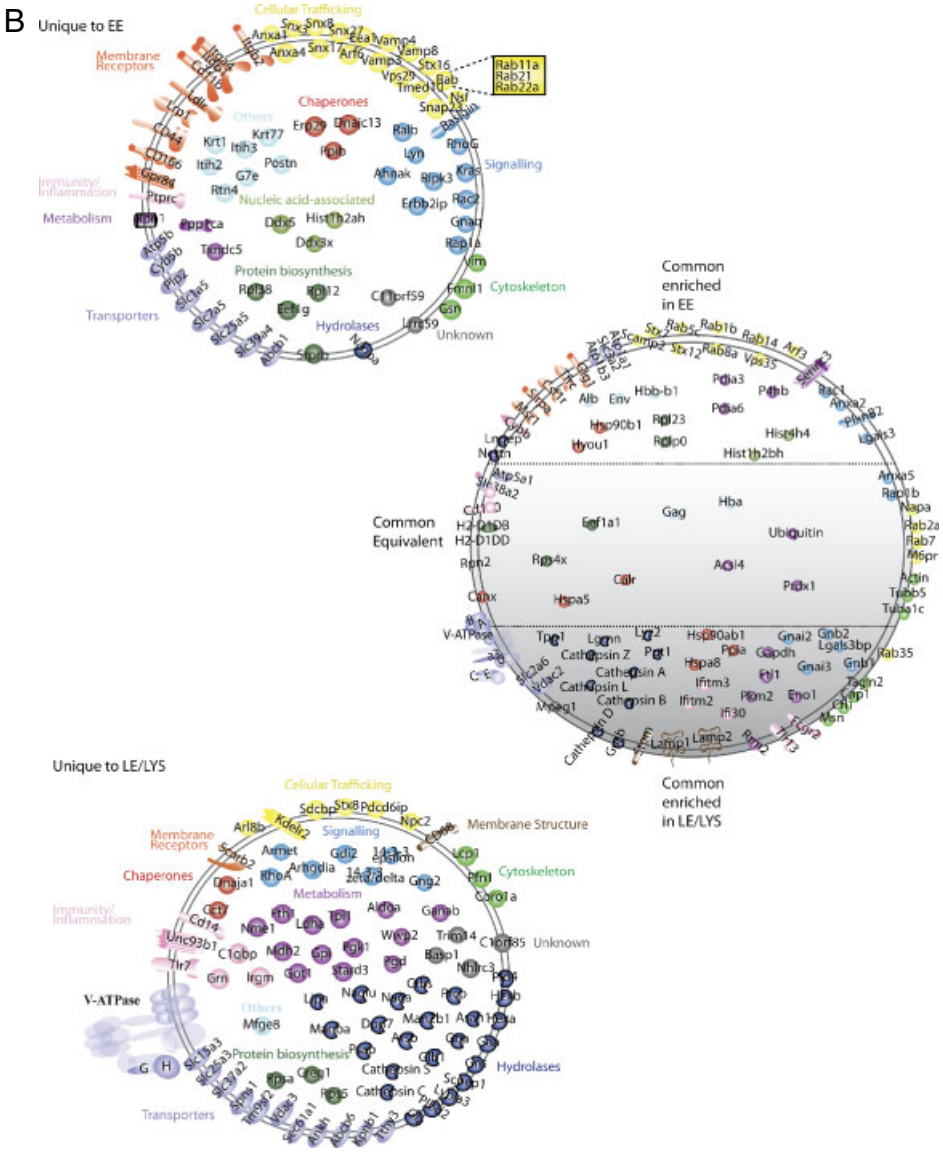




**Figure 3.** Distribution of proteins according to their functional category and virtual representation of J774 isolated EE and LE/LYS. (A) Distribution of proteins found in the MS analysis, enriched in or unique to J774 EE and LE/LYS, according to their functional category. (B) Schematic drawing of proteins unique (left) or common (right) to EE or LE/LYS, based on the RPC (Supporting Information Table 1). Common proteins include those that are either equivalent in both samples or enriched in one sample over the other.

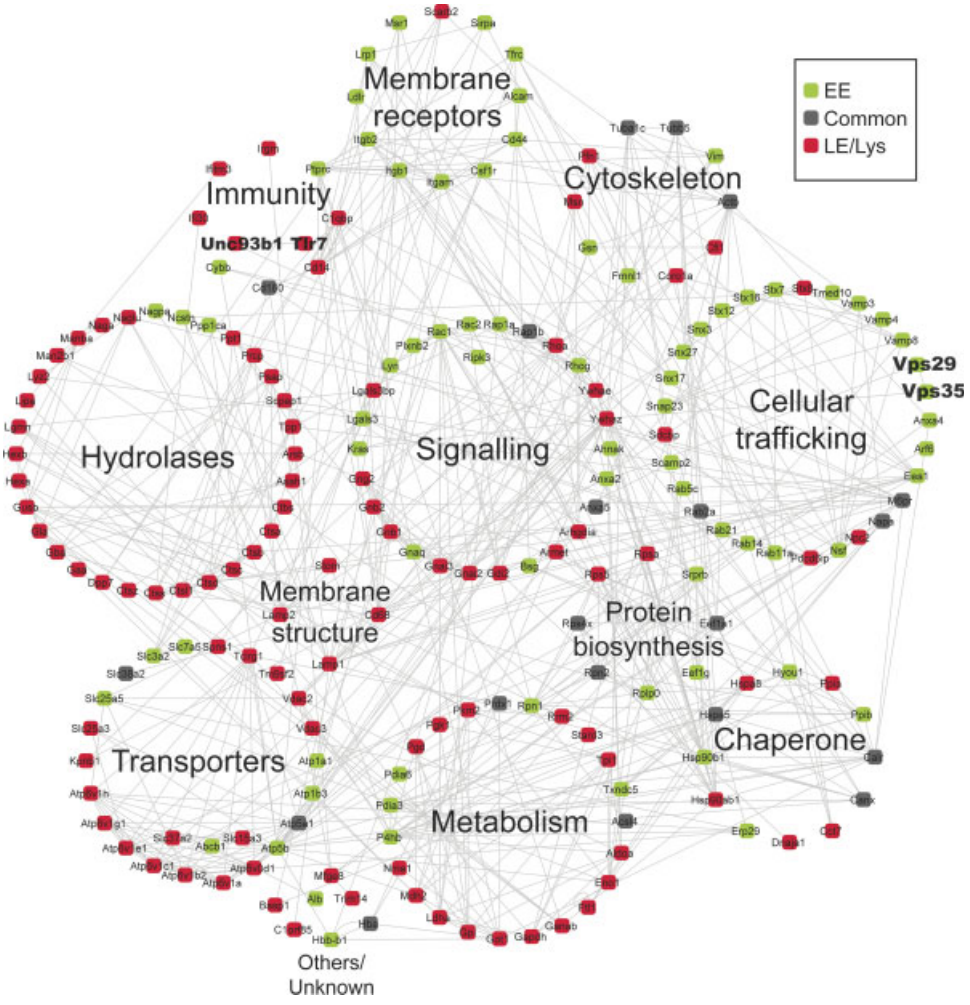



**Figure 4.** Interactome of J774 EE and LE/LYS proteins. Interaction network between proteins found in EE and/or LE/LYS, based on interactions reported in the STRING database. Specific interactions between Vps29 and Vps35 are highlighted, each being part of the retromer complex and either enriched in (Vps35) or unique to (Vps29) the EE sample. Interaction between TLR7 and UNC93B, found uniquely in the LE/LYS sample, is also highlighted.

In the case of LE/LYS, we observed a high level of interactions between hydrolases and proteins involved in metabolic processes. An interaction was also observed between UNC93B1 and TLR7. This is in agreement with the requirement for endocytic internalization during the activation of TLR7 by single-stranded RNA and the importance of UNC93B1 in endosomal TLRs (TLR3, 7, 9, 13) signaling [37]. Interestingly, TLR13 was also found enriched in LE/LYS (Fig. 3) although this interaction was not reported in the database. Moreover, the detection of UNC93B1 in the lysosomal purified samples suggests that this protein, which is normally retained in the ER and reaches the endosomal pathway after microbial stimulation [38] can be present on endosomes and act at this level.

For the DC, a total of 295 proteins were identified (Supporting Information Table 2). Among these, 62 were common (equivalent according to the RPC) to endosomes isolated in both iDC and mDC, 41 proteins were found unique to iDC, while 79 were unique to mDC. The functional categories mostly represented in the iDC are cellular trafficking, hydrolases and signaling. In mDC, metabolism-related proteins are by far the most abundant (Fig. 5A). Schematic representations of virtual DC endosomes were drawn according to the data obtained from the MS analyses (Fig. 5B). From these drawings, it can be observed that apart from the vacuolar ATPase (V-ATPase) complex, most transporters, and especially those from the solute carrier family (Slc), are highly enriched in mDC.

The lysosomal identity of the purified organelles is evident from the presence of multiple lysosomal-associated molecules (cathepsins, LAMP-1 and LAMP-2), and their DC origin demonstrated by the identification of most of the MHC II antigen presentation machinery, as well as DC-specific molecules such as DC-HIL (Gpnmb) and Dectin-2 (Clec6a). A majority of the molecules identified in DC LE/LYS have a metabolic, degradative (hydrolases), or transport function (cellular trafficking, transporter) or alternatively a role in immunity/inflammation processes (Fig. 5). A large collection of degrading enzymes was identified including cathepsins B, D, G, H and Z, along with the V-ATPase subunits responsible for the increase in endosomal acidification during DC activation. All these molecules have been shown to play a role in the processing of internalized antigens presented by MHC class II [39]. MHC class II as

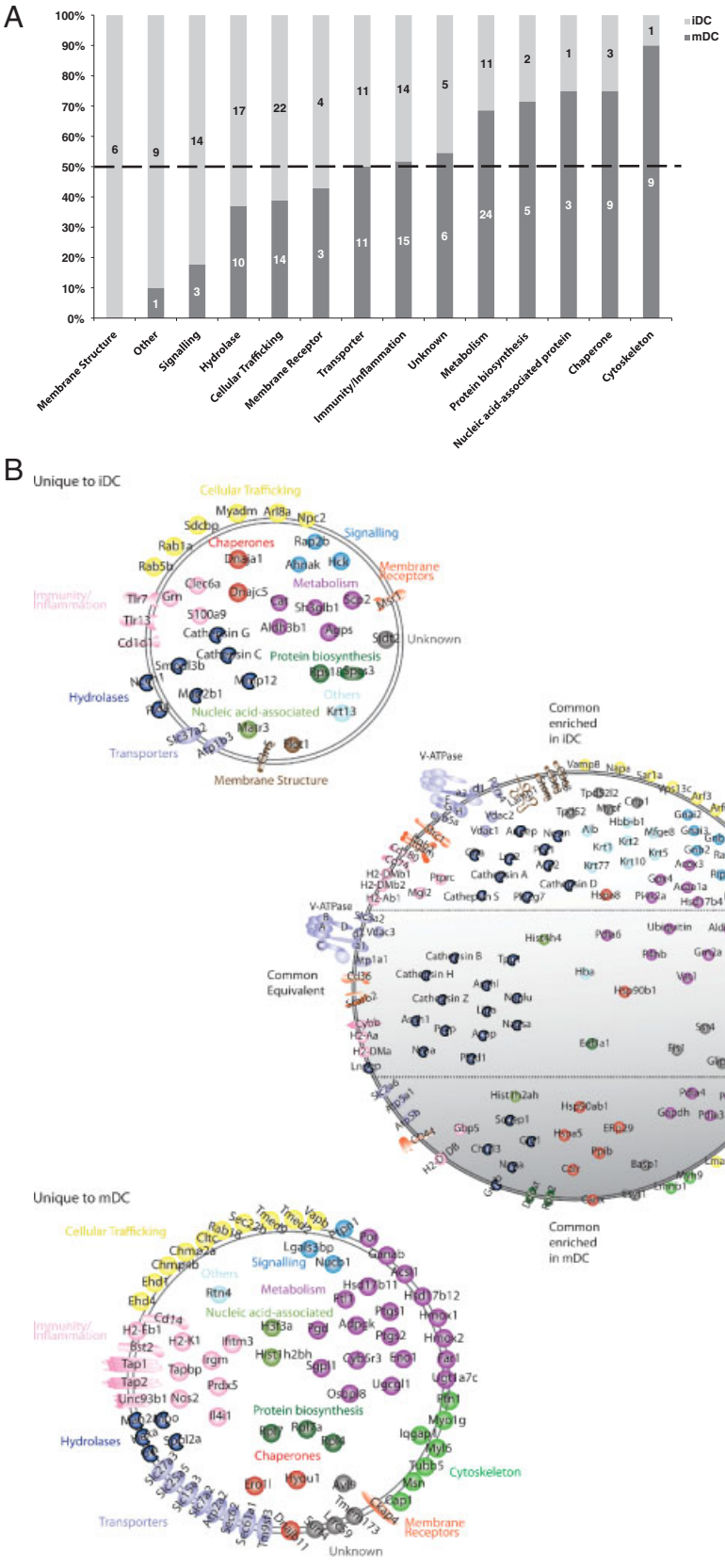



**Figure 5.** Distribution of proteins according to their functional category and virtual representation of mDC and iDC isolated LE/LYS. (A) Distribution of proteins found in the MS analysis, enriched in or unique to LE/LYS from iDC and mDC, according to their functional category. (B) Schematic drawing of proteins unique (left) or common (right) to LE/LYS from iDC or mDC, based on the RPC (Supporting Information Table 2). Common proteins include those that are either equivalent in both samples or enriched in one sample over the other.

well as H2-DM α β, Ii chain (CD74), Cathepsin S, CD1d and prosaposin were all identified, confirming the importance of lysosomal organelles in the processing of both proteins and lipids for antigen presentation. MHC class I molecules, along with the TAP transporters, were also detected in agreement with their capacity to access the endocytic pathway and participate in the cross-presentation of exogenous antigens [40]. Members of the ESCRT-III complex (Chmp2a and Chmp4b) have been found uniquely in mDC. The three ESCRT complexes and their associated proteins play critical roles in receptor downregulation, retroviral budding, and other normal and pathological cellular processes [41] and could be important for lysosomal dynamic upon DC maturation.

## 4 Discussion

The main approach used to isolate endocytic organelles involves isopycnic centrifugation, allowing the enrichment of EE and LE/LYS based on their intrinsic density. Endosomes isolated by this method have been shown to contain significant levels of contaminants from other organelles, including the Golgi apparatus [42, 43]. The approach developed here has the advantage of allowing the isolation of endocytic organelles by flotation on a sucrose gradient, in a region of the gradient where no other cell constituents are normally floating [20]. This enables the isolation of highly purified endosomes, as shown by EM and immunoblotting, providing evidence that sLB can be used to easily form and isolate endosomes. The flotation method has noticeable advantages over isopycnic centrifugation. For example, it allows the isolation of endosomes at various time points after their formation, potentially facilitating the fine characterization of the biogenesis of lysosomes. Another interesting aspect is the fact that sLB can be opsonized with ligands, enabling the study of the engagement of specific receptors during endocytosis. Hence, when the sLB were opsonized with insulin, we observed a significant enrichment of the insulin receptor by Western blotting in the isolated endosomes preparations compared to organelles isolated with unopsonized sLB (results not shown).

In the present study, sLB were used to isolate endocytic organelles from macrophages and DC in order to define their protein composition and highlight the potential functional properties of these compartments. Our data led to the first thorough characterization of endosomes purified from immune-competent cells. The spectral count method used to compare the relative abundance of proteins present in EE and LE/LYS preparations isolated from macrophages, and in LE/LYS isolated from iDC and mDC was validated by the striking observation that all the key markers of these organelles were effectively identified in higher abundance in their respective organelles (Rab5 and EEA1 in EE; v-ATPases and hydrolases in LE/LYS). These data support the notion that our quantitative analyses could effectively assign sets of proteins to their expected endosomal compartment.

This way, we were able to observe that various types of transporters are selectively distributed in the endocytic compartments of macrophages and DC. In macrophages, transporters of the V-ATPase family were exclusively enriched in the LE/LYS, while many subunits were common to iDC and mDC. On the other hand, the Slc transporters were differently distributed between EE and LE/LYS in macrophages, or between LE/LYS of iDC and mDC. For example, Slc7a2, Slc15a3 and Slc27a3, which are specifically expressed in DC and monocytes, were unique to the LE/LYS of mDC. Of the 10 Slc transporters found in macrophages (in both EE and LE/LYS), 5 were not detected in DC, while 2 out of 7 Slc transporters identified in DC were absent from the macrophages endosomes preparations. These data suggest that Slc transporters might participate in the distinct functions performed by macrophages and DC. Although some of the Slc proteins such as Slc15a3 and Slc39a4 have previously been found in endocytic organelles [44, 45], all the other Slc proteins in our analysis are described as endosomal components for the first time. Interestingly, most Slc15a transporters have been shown to mediate the internalization of muramyl peptides [46]. These are potent inducers of the nucleotide-binding oligomerization domain containing 2 (NOD2), an important cytosolic pattern recognition receptor that activates NF-κB and other immune effector pathways such as autophagy and antigen presentation. The presence of Slc15a3 in LE/LYS of macrophages and mDC could be important for the sensing of potential pathogens such as *Staphylococcus aureus* by transporting bacterial muramyl peptides in the cytosol. The coincident presence of Slc7a2, an arginine transporter, in mDC, [47], might be important for the generation of NO by Nitric oxide synthase 2 (NOS2), which metabolizes arginine into nitrites and is also abundant in mDC lysosomes. Thus, the presence of these transporters in the lysosomes might have crucial function for the antibacterial activity of macrophages and mDC.

Although a lot of information about the protein contents of EE and LE/LYS from macrophages has been gathered, only two time points have been analyzed so far. To have a more global picture of all the proteins involved in the endocytic pathway, more time points should be added. This would, for example, enable the identification of proteins that were not detected in our study because of their transient association with endosomes.

Another objective of this work was to compare the protein contents of LE/LYS from iDC and mDC, as well as to underline differences in endosomal proteins that could reflect the different functions of macrophages versus DC. The protein composition of iDC and mDC LE/LYS clearly reflects their antigen-presenting activity as well as their DC specificity. The major difference between LE/LYS from J774 and DC is in the level of antigen presentation molecules, significantly enriched in DC while almost absent in macrophages. However, the most striking feature of our

analysis was the abundant and differential expression of various Slc transporters, which defines these molecules as potential differentiation or activation markers for macrophages and DC, as well as suggests their involvement in the specific immune functions of these cells.

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