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Review

Phagosome proteomics to study *Leishmania*'s intracellular niche in macrophages

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ABSTRACT

Intracellular pathogens invade their host cells and replicate within specialized compartments. In turn, the host cell initiates a defensive response trying to kill the invasive agent. As a consequence, intracellular lifestyle implies morphological and physiological changes in both pathogen and host cell. *Leishmania* spp. are medically important intracellular protozoan parasites that are internalized by professional phagocytes such as macrophages, and reside within the parasitophorous vacuole inhibiting their microbicidal activity. Whereas the proteome of the extracellular promastigote form and the intracellular amastigote form have been extensively studied, the constituents of *Leishmania*'s intracellular niche, an endolysosomal compartment, are not fully deciphered. In this review we discuss protocols to purify such compartments by means of an illustrating example to highlight generally relevant considerations and innovative aspects that allow purification of not only the intracellular parasites but also the phagosomes that harbor them and analyze the latter by gel free proteomics.

1. Introduction

The phagocytosis of microorganisms by professional phagocytes constitutes a major pillar of the defense systems in metazoans (Ackerman et al., 2003; Brown, 1995; Stafford et al., 2002). In macrophages, the process involves formation of membrane delimited intracellular compartments referred to as phagosomes, which initiate the innate immune response and own a degradative capacity necessary to process antigens promoting the adaptive immune response (Pauwels et al., 2017; Russell et al., 2009). After sequestration of microorganisms phagosomes undergo remodeling *via* maturation and fusion with other endocytic organelles. Phagosome maturation may be controlled by Toll-like receptor signaling pathway upon stimulation with pathogens, even though it has been matter of debate for a long time and other factors could influence this process as well (Blander and Medzhitov, 2004; Tan and Kagan, 2017; Yates and Russell, 2005), recruits a large number of regulatory proteins and involves continuous membrane lipid modifications, which is ultimately responsible for the formation of the phagolysosome, a cellular compartment displaying digestive and microbicidal functions (Desjardins et al., 1994; Fairn and Grinstein, 2012; Lim et al., 2017). Many microbes, in particular pathogens such as *Mycobacterium*, *Legionella* and *Leishmania*, are known to interfere with this process but the molecular basis of this interference is only partly understood.

2. Proteomics of latex beads-containing phagosomes

Pioneering work on latex bead containing phagosomes has produced a blue print for using proteomics to gain insight into phagosomal functions and generated useful reference lists of the respective protein constituents. One of the first studies using gel-based proteomics identified a set of more than 140 proteins, which permitted the further characterization of phagosomes proposing novel components besides the proved phagolysosomal markers such as hydrolases, proton pump ATPase subunits and Rab proteins implicated in the fusion processes (Garin et al., 2001). Furthermore, comparative analyses of proteomes of macrophage early endosomes and late endosomes/lysosomes as well as proteomes of late endosomes of immature and lipopolysaccharide-activated (mature) dendritic cells highlighted functional properties of these compartments along phagosome maturation and phagocytes activation, respectively (Duclos et al., 2011). Goyette et al. (2012) demonstrated that proteomics and bioinformatics analyses represent a valid alternative approach to the classical imaging techniques and cell fractionation procedures to investigate spatio-temporal modifications of detergent-resistant membrane microdomains (DRMs) during phagolysosome biogenesis. This approach showed that only low proportions of phagosome proteins in early endosomes are distributed in DRMs and that the proportion increases during phagolysosome maturation, suggesting that DRMs actively modulate spatial reorganization of phagosomes (Goyette et al., 2012).

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With the advent of gel-free proteomics techniques based on liquid chromatography coupled to mass spectrometry (LC–MS), large scale studies have been conducted generating datasets of hundreds of proteins for each condition tested. Comparison of IFN-γ-activated to resting RAW264.7 macrophages showed that this cytokine affects phagosome maturation through the formation of a dense actin network. Moreover, these analyses demonstrated that IFN-γ delays fusion of phagosomes with lysosomes and, in addition to the strong induction of proteins involved in MHC class II antigen presentation, increases expression of molecules associated with MHC class I processing and presentation of exogenous antigens (Trost et al., 2009). Quantitative label-free mass spectrometry has been also used to quantify the abundance of proteins assigned to various organelles that contribute to the biogenesis of phagosomes in J774A.1 murine macrophages and it was found out that about one fifth of the early phagosome proteome originates from the endoplasmic reticulum (Campbell-Valois et al., 2012).

Comparative studies between phagosome of RAW264.7 and bone marrow derived macrophages (BMDMs) indicated that primary macrophages possess an increased phagocytosis rate, a faster phagosome acidification and a stronger proteolytic activity than secondary macrophages (Guo et al., 2015). In addition to the latex beads, proteomic analysis of isolated phagosomes from BMDMs incubated with particles conjugated with different ligands mimicking specific pathogen-associated molecules, opsonins and apoptotic markers have been conducted. This study identified 1891 proteins, with 1337 proteins that were quantified across all conditions, showing distinct effects on phagosome maturation and function (Dill et al., 2015).

3. Phagosome as intracellular niche for pathogens: learning from bacteria

In infection biology field studying the life cycle of intracellular pathogens, the molecular characterization of pathogens and their habitat represents one of the principal research topics in order to develop defined therapeutic strategies. Consequently, proteomics embodies an ideal approach to obtain large-scale information about processes associated with pathogen uptake, survival and replication, and immune response of the host.

In this context, according to the number of publications, the majority of the scientific articles reporting proteomics data related to infectious diseases deal with proteomes of pathogenic bacteria compared to eukaryotic parasites. Analogously, the most extensively studied intracellular niches are the vacuoles containing bacteria such as *Legionella*, *Mycobacterium*, *Salmonella* and *Chlamydia*. So far, protein composition of parasitophorous vacuoles using a proteomics approach has been rarely investigated.

One of the major hurdles working with bacteria is represented by their size. Since bacteria are small and their amount within the host cells is limited, extraction of sufficient bacterial material in the large excess of host protein is technically difficult (Bumann, 2010; Schmidt and Volker, 2011). Recently, many efforts have been done to enrich and isolate intracellular pathogenic bacteria and the compartments where they reside in order to perform proteomic analyses. As extensively summarized in the review of Herweg et al. (2015), three main strategies are followed to achieve this goal: (1) subcellular fractionation by density gradient centrifugation; (2) immune-affinity separation for example using magnetic beads-coupled antibodies; (3) sorting of internalized bacteria and/or host cell organelles by fluorescence activated cell sorting (FACS) (Herweg et al., 2015). Briefly, most of the bacteria-containing compartments are purified using a combination of the abovementioned methods. For instance, isolation using secondary antibodies coupled to magnetic beads followed by density gradient centrifugation represents now one standardize protocol to purify *Legionella*-containing vacuoles (Finsel et al., 2013; Hoffmann et al., 2012, 2013; Urwyler et al., 2010). Following the same concept, anti-epitope tag labeled magnetic beads are used to precipitate *Salmonella*-modified membranes after differential centrifugation (Vorwerk et al., 2015). An alternative to the magnetic beads coupled antibodies is represented by magnetic polystyrene beads coated with virulence factors. This strategy was employed to study *Mycobacterium tuberculosis* phagosome biogenesis, since beads-containing compartments can be isolated by magnetic purification, density gradient centrifugation and FACS (Axelrod et al., 2008; Geffken et al., 2015) and it differs from the classical centrifugation protocol (Rao et al., 2009; Shui et al., 2011). In addition, FACS was successfully used to isolate live *Salmonella* expressing green fluorescent protein (GFP) from infected mouse tissues (Becker et al., 2006). Due to technical and/or biological limitations, combination of different methods cannot be ubiquitously used. For example, *Chlamydia* inclusions are normally isolated and purified by filtration and centrifugation (Matsumoto, 1981), which could negatively affect their integrity. Nevertheless, a combined procedure using a ball homogenizer for cell lysis followed by Percoll gradient centrifugation and immune-magnetic purification was employed to improve isolation of chlamydial inclusions for SILAC-labeled proteomic analyses (Aeberhard et al., 2015).

4. *Leishmania* parasites and their intracellular habitat

Leishmania spp. are intracellular trypanosomatid parasites that cause a spectrum of diseases called leishmaniases that threaten more than 350 million people worldwide, with 12 million people that are currently infected, and about 2 million new cases occuring every year ((Herwaldt, 1999); http://www.who.int/gho/neglected_diseases/leishmaniasis/en/). Flagellated promastigotes are the extracellular form of *Leishmania* and replicate in the digestive tract of the insect host. After several morphological and metabolic differentiation steps, the highly virulent metacyclic promastigotes are transmitted into the skin of the mammalian host by the bite of female sandflies of the genus *Phlebotomus* or *Lutzomyia*. Once transmitted, the parasites are internalized by professional phagocytes such as macrophages where they lose their flagella, transforming into the amastigote form (Kaye and Scott, 2011). The ability to survive within macrophages is a long-known feature shared with other infectious organisms with a relevant impact on public health, including *Mycobacterium tuberculosis*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Toxoplasma gondii* and *Trypanosoma cruzi* (Alexander et al., 1999). Growth and replication of amastigotes takes place in a habitat designated as parasitophorous vacuole (PV), which resembles a late endosome, early lysosome according to the presence of late endosomal marker proteins Rab7 and the lysosomal-associated membrane proteins Lamp1 and Lamp2 (Antoine et al., 1990; Arango Duque and Descoteaux, 2015; Kima et al., 2010; Russell et al.,1992). In addition, real-time fluorescence imaging using primary macrophages generated from unique *gfp-rab5* transgenic mice showed that maturation of parasite-infested phagosome is uniform and residence of the parasites in the Rab5 compartment is shorter than in phagosomes containing latex bead (Lippuner et al., 2009).

Several investigations demonstrated at the molecular level that promastigotes interfere with the phagolysosome biogenesis in macrophages immediately after internalization. The main pathogenicity factor of promastigotes is the abundant surface glycolipid lipophosphoglycan (LPG) (Desjardins and Descoteaux, 1997; Moradin and Descoteaux, 2012). In the case of *L. donovani*, LPG modulates the remodeling of the vacuole by disrupting the phagosomal lipid microdomains, which control fusion of endosome with lysosomes, therefore contributing to the evasion of the microbicidal consequences of the phagosomal maturation process (Desjardins and Descoteaux, 1997; Tolson et al., 1990). In this context, LPG hinders the functional assembly of NADH oxidase complex and prevents the recruitment of vacuolar proton ATPase at the PV membrane, impairing the correct acidification of the vacuole necessary for antigen processing and initiation of the immune response (Lodge et al., 2006; Vinet et al., 2009). Moreover, LPG induces periphagosomal F-actin accumulation preventing the phagosome maturation (Holm et al., 2001). Taken together,