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Regulation of phagosome functions by post-translational modifications: a new paradigm

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Phagosomes are highly dynamic organelles formed by the uptake of particles through phagocytic innate immune cells such as macrophages. Their key roles in microbe elimination and antigen presentation make them essential for innate and adaptive immunity. However, phagosomes are also important for tissue homeostasis as even in healthy individuals billions of dead cells are phagocytosed each day. In this short review, we highlight how the use of latex beads as inert baits for phagocytosis and subsequent analysis by proteomics has changed our understanding of the phagosome. We further discuss recent data on post-translational modifications such as phosphorylation and ubiquitylation that regulate phagosome functions and demonstrate that the phagosome is not only a 'degradative organelle' but also serves as a subcellular signalling platform.

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Introduction

Phagocytosis is an evolutionary conserved process [1••,2] that enables cells to engulf and digest a variety of different particles. It serves as a vital source of nutrition in unicellular eukaryotes, but is also important for tissue remodelling [3] and innate immune defence in higher organisms [3]. Phagocytosis leads to the formation of a membranous vesicle called a phagosome that is formed within cells following engulfment of particles typically greater than 0.5 μm [4]. In animals, this process enables elimination of foreign bodies such as bacteria from infection sites. Phagocytosis also facilitates the removal and recycling of cellular debris, and clears the billions of apoptotic cells that are generated each day [4]. Phagosomes are key organelles for the presentation of antigens via MHC Class I (for antigen cross-presentation) and MHC Class II pathways, thereby linking innate and adaptive immunity (see Refs. [5,6] for review). Unsurprisingly, defects in phagocytosis and the phagosomal maturation pathway lead to several immune-related human diseases [4,7] highlighting its importance. Moreover, the phagosome or phagosome-related vacuoles are the intracellular niche for a number of important human pathogens such as *Mycobacterium tuberculosis*, Legionella, Brucella, Francisella and Leishmania [8]. Yet, relatively little is known about the molecular events that underlie the regulation of phagosome formation and maturation as recent data points to a highly sophisticated organelle, well beyond its traditional role in waste removal.

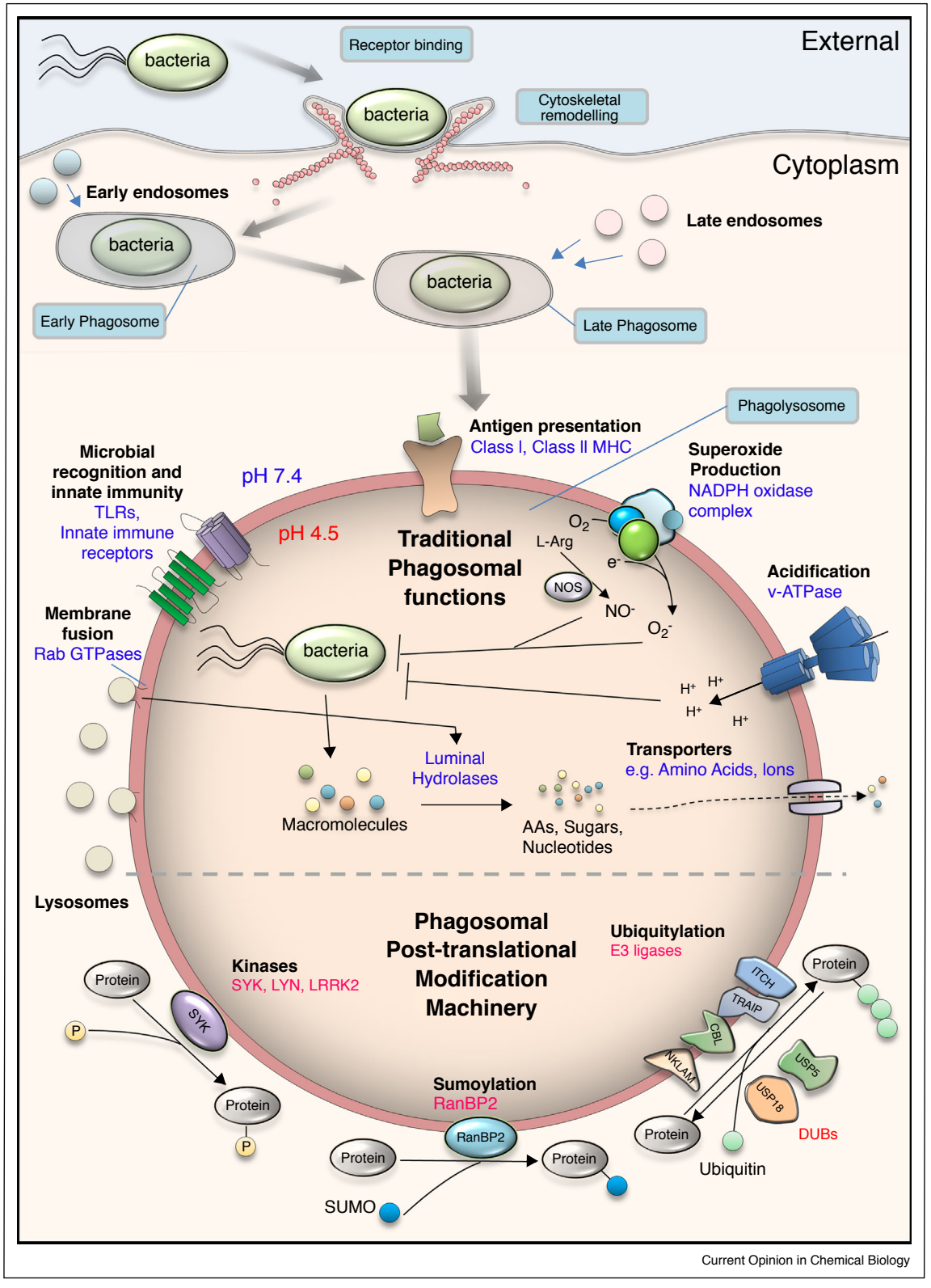
Phagosome formation begins with the recognition of a target ligand at the cell surface by a range of dedicated phagocytic receptors that include Scavenger and Fc-receptors and many others (for review see Refs. [4,9]). Receptor binding initiates signalling cascades that result in cytoskeletal remodelling and membrane protrusion around the particle [4], ultimately leading to membrane scission and the formation of an early phagosome [10,11]. The nascent phagosome then follows a choreographed pathway termed 'phagosome maturation' [9,12], whereby its proteome and physico-chemical properties dramatically change, driven by fission-fusion events with intracellular vesicles and organelles such as early and late endosomes, the ER and finally lysosomes [9,13,14••,15•]. Following the final fusion with lysosomes, the lumen of mature phagolysosome acquires a low pH, features a highly oxidative environment and contains many hydrolytic enzymes including proteases, DNAses, lipases and glycosidases, that all function at low pH and lead to the destruction of the internalised particle [12] (see Figure 1).

Phagosomes share most, if not all of the molecular machinery of the endocytic and autophagy pathways, such as the phosphatidylinositol-phosphate (PI3P) kinase complexes and Rab family GTPases that mediate vesicle trafficking to the lysosome [16]. As phagosomes can easily be isolated to high purity, unlike endosomes and autophagosomes, they represent an excellent model to study vesicular trafficking to the lysosome.

Phagosome proteomics and latex beads

Phagosomes can be isolated using several techniques including density gradient centrifugation [17] or affinity

Figure 1



The virtual phagosome. The phagosome has well established 'traditional' functions in acidification, degradation of exogenous particles, transport/recycling of building blocks and antigen presentation. Many of these functions and additional functions such as innate immune recognition and cell signalling are regulated through post-translational modifications of phagosomal proteins.

purification with magnetic beads [18–20] or biotin affinity purification [21,22]. However, the utilisation of latex or polystyrene beads, in conjunction with density gradient centrifugation, has revolutionised phagosome proteomics because of the exceptional high purity of phagosomes produced by this method (over 95% pure phagosomes [23••]). Phagocytic cells such as macrophages, efficiently phagocytose these beads, forming latex bead phagosomes (LBP) which can be separated from other cellular compartments by sucrose gradient centrifugation due to their low buoyant density (Figure 2a). Using cell cultured macrophages, relatively large amounts, that is hundreds of micrograms, of phagosome protein extracts can be obtained [23••,24]. Unfortunately, while bacteria-containing phagosomes have more *in vivo* relevance, they are much more difficult to purify as they have a density similar to that of other organelles such as mitochondria [25,26], and greater caution is needed when interpreting data using these phagosomes. While latex beads are non-biological, they can be coated with individual molecules to stimulate specific pathways, such as antibodies to trigger Fc-receptors, phosphatidylserine to trigger TAM receptors or bacterial lipopolysaccharide (LPS) to activate Toll-like Receptor 4 [27]. Importantly, LBP maturation dynamics are similar to that seen with bacteria, so they represent a good reductionist approach to study phagosome cell biology by biochemical methods and proteomics.

Advances in mass spectrometry (MS)-based proteomics over the past 25 years have seen a dramatic increase in the number of identified proteins on latex bead phagosomes [14••,17,23••,27,28•,29–31,32•,33••,34,35,36•] (Figure 2b). This sensitivity recently allowed for the identification of 2000–4000 proteins on phagosomes as well as post-translational modifications such as phosphorylation and ubiquitylation [23••,37]. The notion of the phagosome as a signalling hub, supported by recent data [9,23••,33••], implies that post-translational modifications play an important role in regulating phagosome functions. Here, we focus on enzymes in the protein phosphorylation and ubiquitylation space. These enzymes are considered to be well druggable [38–40] and good high-throughput screening and chemical biology tools exist [41–44]. This would allow for host-directed approaches for targeting diseases associated with dysregulated phagosome functions.

The phagosome and post-translational modifications

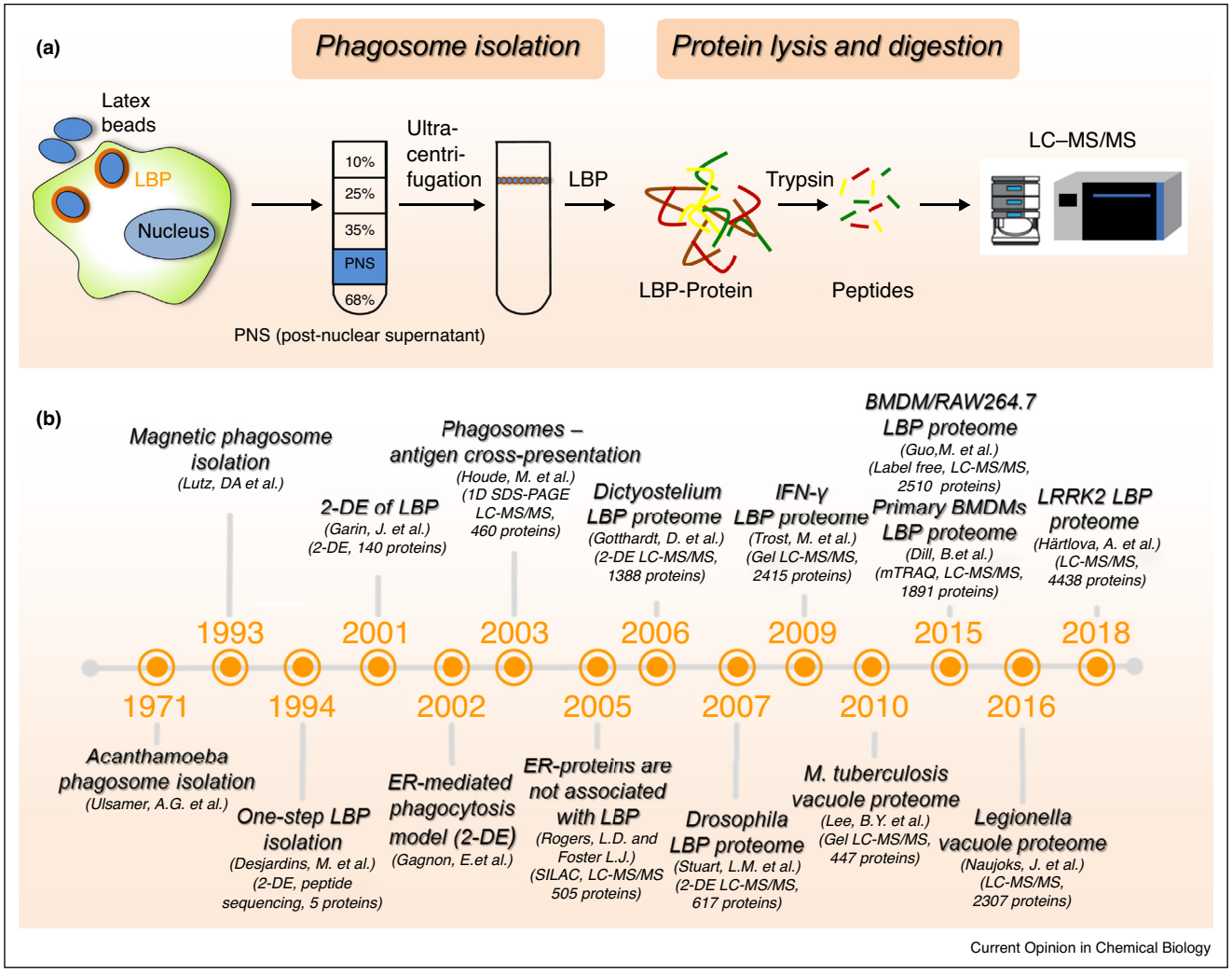
The idea of the phagosome as a molecular signalling platform is supported by proteomic data [9,23••,27,33••,34], which shows that the phagosomal proteome contains many enzymes that introduce post-translational modifications (PTMs) on other proteins (Figure 3). PTMs play important regulatory roles in biological processes, influencing a protein function's, stability, and localisation and increase the overall chemical diversity of the proteome. PTMs refer to the covalent attachment of chemical groups (phosphorylation, acetylation, methylation, nitrosylation, sulfation), more complex chemical structures (glycosylation, prenylation, AMPylation, ADP-ribosylation) and even small proteins (ubiquitylation and ubiquitin-like modifiers). Additionally, amino acid modification (deamidation, eliminylation) and proteolytic cleavage can influence protein activity. Given that PTMs play important roles in vesicle trafficking [45–47], it would not be surprising that they are important for phagosome biology.

Phosphorylation of phagosomal proteins

Phosphorylation is the reversible addition of a phosphate group on target proteins by protein kinases that generally act on the hydroxyl groups of serine, threonine, or tyrosine residues, although recently it was shown that other amino acids can also be phosphorylated [48]. Early pioneering work revealed the presence of tyrosine phosphorylation on phagosome-associated proteins, and variation in protein phosphorylation patterns has been observed during different stages of phagosome maturation [49]. Organelle-wide protein phosphorylation was highlighted by the first global analysis of phosphorylation on phagosome proteins using quantitative phosphoproteomics, which identified almost 3000 phosphorylation sites [23••]. This study revealed significant changes in the phosphorylation state of phagosomal proteins upon stimulation with the proinflammatory cytokine interferon-γ (IFN-γ) [23••] that increases the antibacterial activity of the cell. Many core phagosomal proteins were shown to be phosphorylated upon IFN-γ treatment including those involved in antibacterial activity such as NOS and v-ATPase, as well as membrane trafficking and cytoskeletal proteins [23••]. In the same study, over 100 kinases and phosphatases were also found on the phagosome, many of which were modulated by IFN-γ [23••]. A subsequent study [1••], comparing unicellular and multi-cellular organisms, also showed that while the phagosome has retained a core set of proteins during evolution, the level of phosphorylated proteins has dramatically increased, probably in line with increasing cellular complexity and the additional role of the phagosome in immunity [1••]. The functions of phosphorylation targets on the phagosome are widespread and cover most of its known functions including signalling, vesicle trafficking, cytoskeletal functions and transport across membranes [1••,23••].

Recent work by the Gutierrez and our own lab showed that the Parkinson's kinase LRRK2 was a negative regulator of phagosome maturation by regulating phosphatidylinositol 3-phosphate (PI3P) kinase complex around VPS34 [33••]. Pharmacological inhibition of LRRK2 kinase activity increased phagosome-lysosome fusion and thereby promoted killing of intracellular *M. tuberculosis*. Since highly selective LRRK2 inhibitors

Figure 2



Phagosome isolation and a short history of phagosome proteomics. (**a**) Phagocytosis is induced by presenting latex beads to macrophages which are internalised into phagosomes. Latex bead phagosomes (LBP) are isolated by placing the post-nuclear supernatant of the cell lysate onto the bottom of a sucrose gradient. LBPs float in this gradient upon ultracentrifugation and can be isolated to high purity. Phagosomal proteins are then extracted and digested by trypsin. Peptides are analysed by LC–MS/MS. (**b**) A short history of key phagosome proteomics papers [13,16,20–22,23••,24–27,28•,29,30].

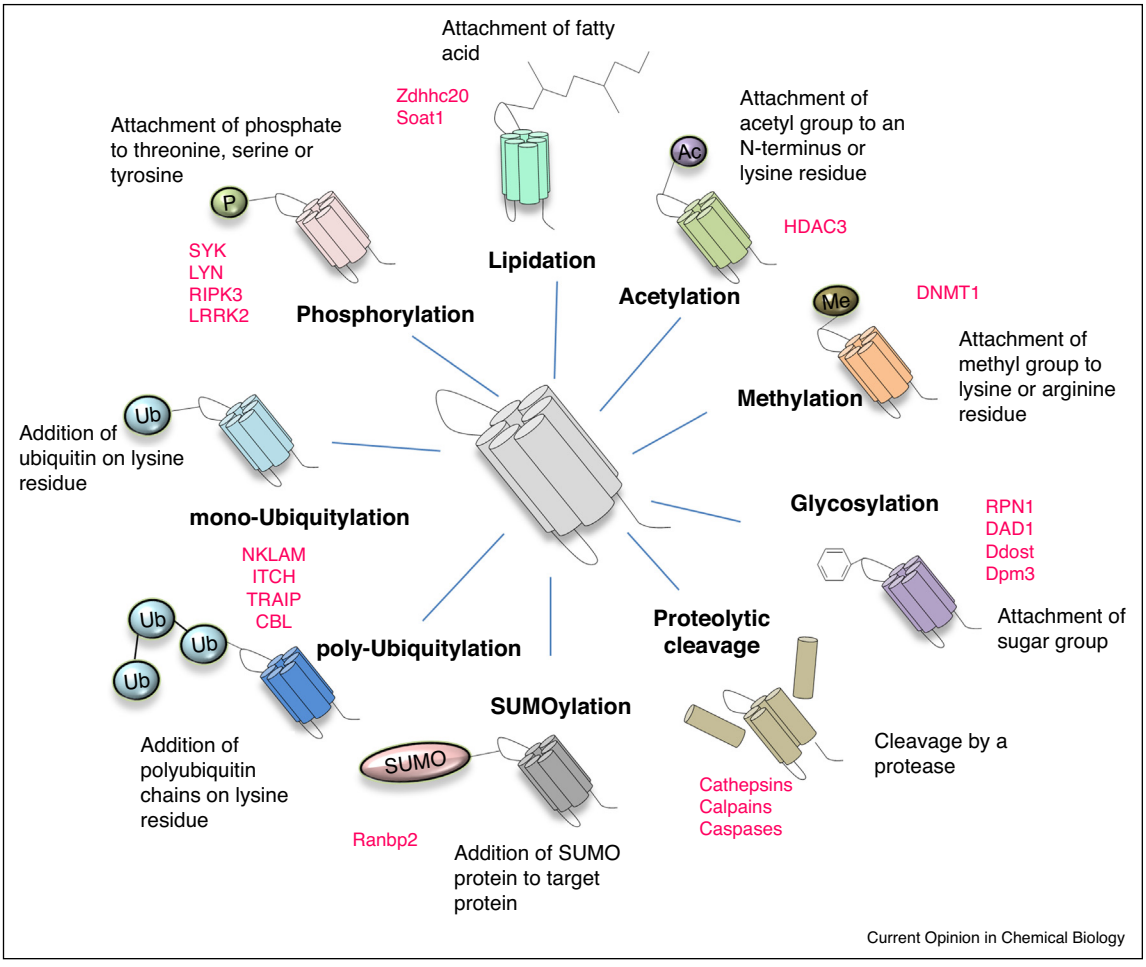
are available, these could serve as a host-directed strategy to help fighting intracellular pathogens. Moreover, in many neurodegenerative diseases, unfolded proteins are accumulating and are not properly degraded through the lysosomal pathway. LRRK2 inhibition might possibly enhance the capacity of cells to process cellular waste.

Considering the important role of phosphorylation in regulating cellular processes, it is not surprising that intracellular pathogens exploit this for their advantage. *M. tuberculosis* secretes a eukaryotic-like serine/threonine protein kinase G that has been shown to be important for blocking phagosome-lysosome fusion [50•]. Moreover, the *M. tuberculosis* secreted protein tyrosine phosphatase, PtpA, has been shown to dephosphorylate human vacuolar protein sorting 33B (VPS33B) to also inhibit phagosome-lysosome fusion [51] via exclusion of V-ATPase [52]. These examples clearly implicate an important role for protein phosphorylation in phagosome maturation, especially in mycobacteria-containing phagosomes.

Ubiquitylation of phagosomal proteins

Modification of cellular proteins by the covalent attachment of the 76-amino acid protein ubiquitin (Ub) is one of the most elaborate post-translational modifications in eukaryotic cells. It involves the concerted activities of three sequential enzymes (E): first, using ATP the Ub-activating E1 enzyme is 'charged' with Ub, which is then

Figure 3



Post-translational modification machinery present on the phagosome. Phagosomes are rich in enzymes regulating various cellular processes by post-translational modifications, including protein kinases, lipid transferases, acetyl transferases, methyl transferases, glycosyltransferases, proteases, ubiquitin and SUMO ligases. Specific examples are given in pink.

transferred to an Ub-conjugating E2 enzyme and finally the C-terminus of Ub is covalently attached to the ε-amino group of a lysine residue on target protein by an E3 Ub ligase, which determines the substrate specificity [44]. In humans, there are only two E1 enzymes, Uba1 and Uba6 while other members of the Uba family are involved in attachment of ubiquitin-like modifiers such as SUMO, NEDD8 and ISG15 [53]. There are tens of E2 and hundreds of E3 enzymes encoded in the human genome, which also contains a large number of deubiquitylases known as DUBs that reverse the ubiquitylation reaction [44,54–56]. As ubiquitin itself can be ubiquitylated on any of its seven lysines (K6, K11, K27, K29, K33, K48, and K63) and the N-terminal methionine (M1), a complex variety of ubiquitin chain types can be produced on target proteins, that can determine the fate of the protein. For example, K63-linked chain types are often involved in signalling whereas K48 chains target proteins for proteasomal destruction [54,55]. Many ubiquitin-like proteins, including SUMO, NEDD8 and ISG15 have been identified on phagosomes and it is likely that they will also play an important role in regulating phagosome functions.

Both mono-ubiquitylated and poly-ubiquitylated proteins have also been found on phagosomes [57]. Ubiquitylated proteins have been shown to be important in membrane trafficking [46,58], for example regulation of the PI3P kinase complex VPS34 [59]. Ubiquitylation was shown to affect Fc-receptor sorting on phagosomes [57], which may be linked to an overall role of ubiquitylation in receptor sorting [60]. A phagosome-associated E3 ubiquitin ligase, NKLAM, was shown to be enriched on phagosomes with elevated levels of ubiquitylated phagosome-associated proteins [61]. NKLAM was not essential for phagocytosis and knock-out of NKLAM led to reduced

inflammation and cytokine levels upon infection with *Streptococcus pneumoniae* [62]. Recent MS-based approaches have clearly shown the presence of a large contingent of ubiquitin-conjugation machinery on the phagosome [34] including E1, E2 and E3 enzymes and also deubiquitylases. While some of these proteins are relatively unknown, other phagosomal proteins such as MGRN1, CBL and ITCH are known to play important roles in endocytic trafficking [45].

The importance of ubiquitylation on the phagosome is demonstrated by bacterial pathogens specifically targeting this PTM during infection. *Legionella pneumophila* recruits polyubiquitin conjugates around the bacterial phagosome [63,64] that was shown to be dependent on the E3 ligase activity of a bacterial effector SidC [65•] that is translocated into host cells. The SidC ubiquitin ligase activity was also shown to be crucial for the recruitment of ER components to the bacterial phagosome [65•]. This highly significant study nicely demonstrates that ubiquitylation of phagosome proteins likely plays a crucial role in phagosome functions, but more work is required to decipher the exact role that ubiquitylation plays on the phagosome.

Conclusion

Post-translational modifications undoubtedly play a crucial role in phagosome biology. Yet, we have only just begun to scratch the surface of their importance along the phagosome maturation pathway. High-resolution mass spectrometry-based proteomics is integral to this discovery, and is uncovering the enormous complexity of the phagosomal proteome but also the diversity of phagosomal PTMs that allows the phagosome to react to ever changing stimuli and target cargo. The identification of specific enzymes on the phagosome that mediate protein phosphorylation and ubiquitylation indicate a proposed organelle-level control of signal transduction. It is thus tempting to speculate that the phagosome plays a role in initiating signal transduction, turning this 'degradative organelle' into a subcellular signalling platform.

Conflict of interest statement

Nothing declared.

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