A GUIDE TO...

A guide to measuring phagosomal dynamics

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Phagocytosis is an essential mechanism for immunity and homeostasis, performed by a subset of cells known as phagocytes. Upon target engulfment, *de novo* formation of specialized compartments termed phagosomes takes place. Phagosomes then undergo a series of fusion and fission events as they interact with the endolysosomal system and other organelles, in a dynamic process known as phagosome maturation. Because phagocytes play a key role in tissue patrolling and immune surveillance, phagosome maturation is associated with signaling pathways that link phagocytosis to antigen presentation and the development of adaptive immune responses. In addition, and depending on the nature of the cargo, phagosome integrity may be compromised, triggering additional cellular mechanisms including inflammation and autophagy. Upon completion of maturation, phagosomes enter a recently described phase: phagosome resolution, where catabolites from degraded cargo are metabolized, phagosomes are resorbed, and vesicles of phagosomal origin are recycled. Finally, phagocytes return to homeostasis and become ready for a new round of phagocytosis. Altogether, phagosome maturation and resolution encompass a series of dynamic events and organelle crosstalk that can be measured by biochemical, imaging, photoluminescence, cytometric, and immune-based assays that will be described in this guide.

Introduction

Initially described by Elie Metchnikoff more than 100 years ago [1,2], phagocytosis is conventionally defined as the regulated uptake of large particulate matter by specialized cells into membrane-bound vacuoles termed phagosomes. Indeed, phagocytic cells (phagocytes) recognize and internalize a wide array of targets for diverse purposes. Hence, 'phagocytosis'

serves as an umbrella term that encompasses all modalities of particle internalization, and while phagocytes spend a significant amount of energy and resources to engulf particles, the definition neglects the ultimate goal of the phenomenon: processing the captured prey. In addition, in immunity, phagocytosis is not a silent process; the prey is not kept secret. On the

contrary, in multicellular organisms, phagosomes become sensing and signaling hubs that communicate with other organelles and signaling pathways to optimize the phagocytic process and mount the right type of immune response [3–5]. Regardless of whether phagocytosis is used as a means for nutrient acquisition—as is the case with some unicellular organisms—to eliminate imminent threats or for homeostatic purposes in metazoans, internalized matter must be degraded, the resulting catabolites must be resolved, and the information gathered along the way must be conveyed throughout the cell.

Because of this, the phagocytic process has been broadly divided into major mechanistic stages: phagosome formation, phagosome maturation, and, more recently, phagosome resolution [6]. While phagosome formation is a remarkable process whereby phagocytes recognize prey and internalize it by remodeling their plasma membrane and rearranging their actin cytoskeleton, and has been extensively reviewed [7–9], this guide aims to discuss methods to study the subsequent stages, namely phagosome maturation and phagosome resolution (Fig. 1). Phagosome maturation was initially defined as the fusion of phagosomes with lysosomes—or granules in neutrophils—for cargo degradation [10,11]. Studies over the past 30 years have shown the complex molecular mechanisms that are necessary for a newly formed phagosome to be transformed into a degradative organelle—known as the phagolysosome—as it traffics and signals through the endocytic pathway (reviewed in Ref. [6,12–16]. While it is widely known that phagosomes undergo a sequential series of fusion and fission events with endosomes and lysosomes [17,18] (Fig. 1), it has become increasingly apparent that phagosomes also interact with several other organelles such as the endoplasmic reticulum (ER) [19], mitochondria [20,21], and the trans-Golgi network (TGN) [22] (Fig. 2). Moreover, only recently have several groups started investigating the consequences of breaching phagosome membrane integrity and the fate of the compartment and its catabolites following cargo degradation ([23–25]; Fig. 2). The importance of these phenomena is emphasized by fundamental processes such as inflammasome activation, induction of autophagy, antigen presentation, the recycling of organelles of the endocytic pathway, and the return to homeostasis so that the cells become readily available for additional rounds of phagocytosis. These late events—catabolite management and compartment resorption—encompass the recently described phase termed phagosome resolution [6]. The spatial and temporal sequence that orchestrates the mechanisms of phagosome maturation and resolution is tightly coordinated by a plethora of molecular events that can be studied by diverse methods. Here, we provide a guideline for diverse methods to study changes in phagosomal composition, signaling and integrity, cargo degradation, and phagosome resolution.

Chapter 1. Traditional methods to assess phagosome maturation

Changes in the biochemical composition and properties of the maturing phagosome

At a glance

As soon as a nascent phagosome is formed, it undergoes a drastic biochemical transformation, altering the composition of both its lumen and limiting membrane. The goal of these changes is to transform an 'innocuous' environment that resembles the extracellular milieu, into a hostile one that favors lytic reactions. This transition is driven by the trafficking and maturation of phagosomes through the endocytic pathway. Maturation is characterized by fusion and fission events with early and late endosomes, post-Golgi vesicles, and lysosomes [17,18], exquisitely coupled to the delivery and activation of a hydrolytic, oxidative, and acidifying machinery, as well as by centripetal movement of the vacuole along microtubules [26,27] and binding to F-actin [28,29]. Throughout maturation, the phagosome dynamically acquires stage-specific proteins and lipids (reviewed in Ref. [6,13–15,30,31]. These molecules can then be used as proxies to study the state of maturation of phagosomes by the methods described below. Perhaps, one of the most remarkable aspects of phagosome maturation is that the lumen of phagosomes—with some exceptions depending on the type of phagocyte [32,33]—undergoes acidification. This Potential for hydrogen (pH) decrease is in principle necessary for complete degradation of phagosomal cargo, since several hydrolases acquired by phagolysosomes have acidic pH optima. Additionally, reactive oxygen species (ROS) are generated in phagosomes to various degrees and with temporal variations depending on the nature of the phagocyte [32,34,35].

Biochemical methods

Phagosome isolation for biochemical studies

The principle of the method is the separation of phagosomes from cell homogenates based on their differential densities, as phagocytic cargoes are the predominant determinant of the density of the compartment. Because they have a density that is
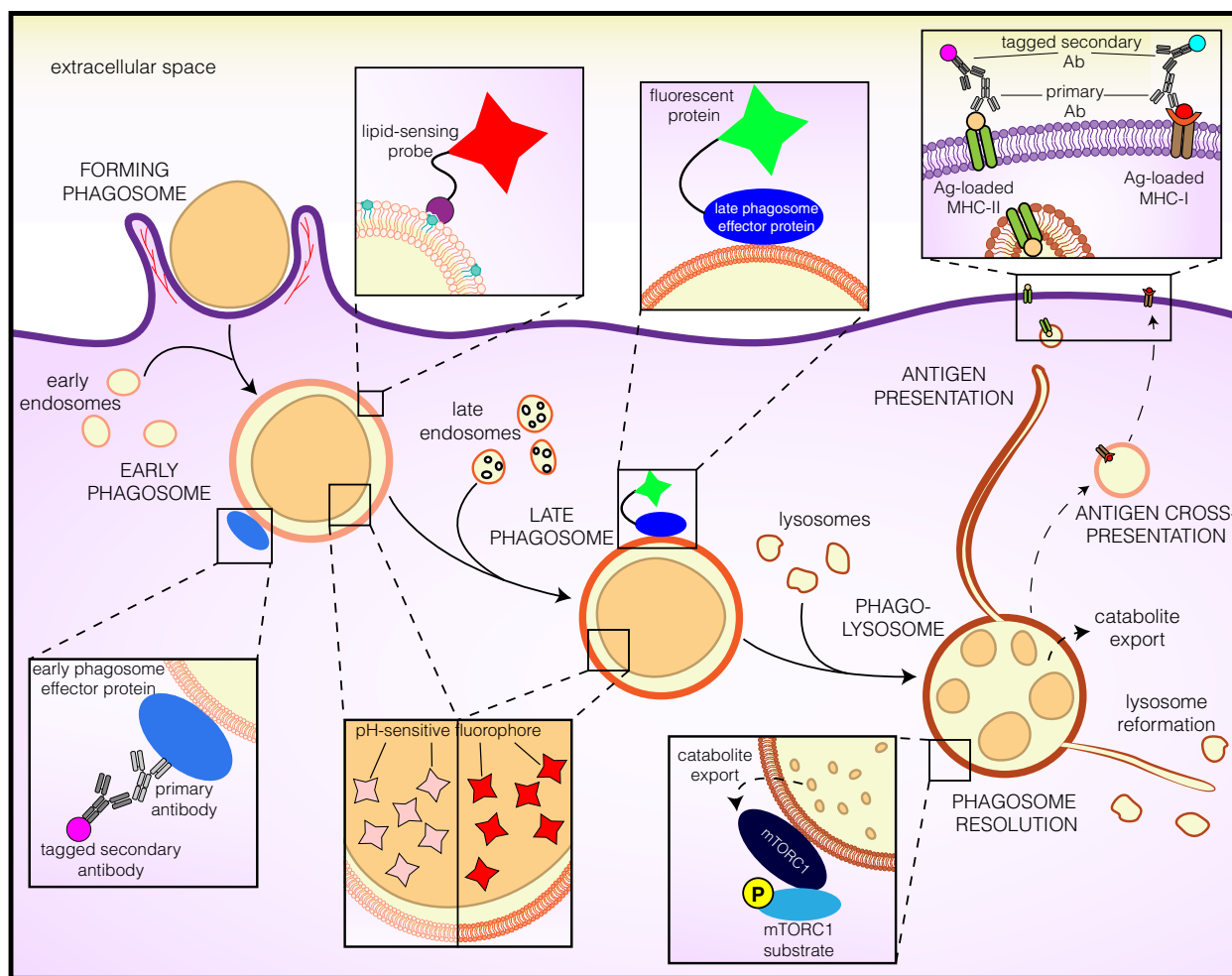
Fig. 1. The life cycle of phagosomes. Phagosomes are formed at the plasmalemmal level in an actin-dependent manner. Once inside cells, phagosomes mature through sequential fusion with early endosomes, late endosomes, and lysosomes. During maturation, phagosomes acquire effector proteins that can be probed in cells by immunolabeling and genetically encoded protein chimeras. Additionally, the lipid composition of the phagosomal membrane changes throughout maturation; such changes can be measured using genetically encoded lipid-sensing probes. In some phagocytes, as phagosomes mature, their lumen acidifies; this can be assessed through the use of pH-sensitive fluorophores. In some phagocytes, such as DCs, phagosomes are autonomous signaling organelles equipped for cargo degradation, peptide loading, and antigen presentation. The latter can be measured when peptides are loaded intracellularly or presented at the plasma membrane by immunolabeling methods or by measuring subsequent T-cell activation. Finally, after cargo degradation, phagosomes undergo resolution, which entails catabolite export, compartment resorption, and lysosome reformation. Export of catabolites (such as specific amino acids) can activate mTORC1 in the cytosolic leaflet of lysosomal membranes. mTORC1 activation can be measured by changes (e.g., phosphorylation) in its substrates.

markedly different from that of cellular organelles, latex beads have been widely used as model phagocytic targets. These methods, originally developed in the late 1960s and early 1970s [36,37], can be performed with a one-step density gradient [38]. Notably, phagosome isolation methodologies have been further optimized in order to increase homogeneity and purity [39–42]. Isolation techniques of physiologically relevant phagocytic prey such as bacteria, albeit significantly more challenging, have also been developed [43–48].

Many purification methods are based on Percoll or sucrose density gradients [46,49]. Phagocytes in culture are initially challenged with the phagocytic prey of choice and incubated for the desired time. Then, cells are scraped off or otherwise lifted and homogenized in the presence of protease inhibitors. The homogenate is then layered over a Percoll gradient or fractionated on a discontinuous sucrose gradient and centrifuged. Phagosomes are then collected from the appropriate interphase and their purity and properties assessed
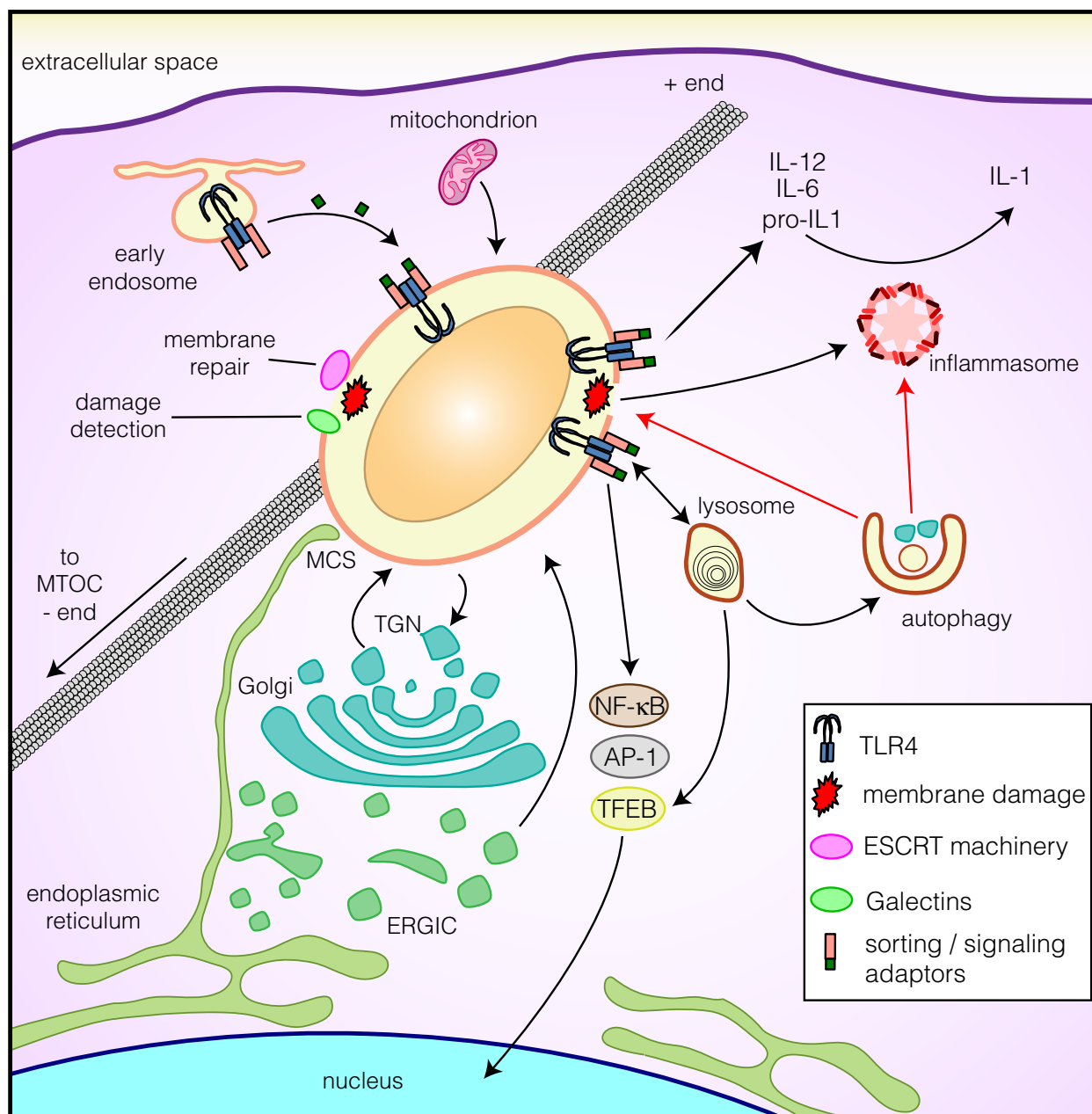
Fig. 2. Signaling and crosstalk of the maturing phagosome. During maturation, phagosomes are linked to microtubules through which they are transported centripetally toward the microtubule-organizing center. Additionally, phagosomes interact with the ER, the ERGIC, the TGN and mitochondria. As phagosomes mature, they become autonomous signaling entities by acquiring PRRs from endosomal compartments (e.g., TLR4). Receptor signaling promotes the production of proinflammatory cytokines—through NF-kB and AP-1 transcription factor activation—and enhances cargo degradation and antigen presentation depicted in Fig. 1. Some receptors also induce TFEB/TFE3 translocation to the nucleus and subsequent transcription of the coordinated lysosomal expression and regulation and proinflammatory gene networks. Additionally, some phagocytic cargoes can induce phagosomal membrane damage, which in turn results in phagosomal content leakage and inflammasome activation. These events can be targeted by autophagy. Additionally, mechanisms to detect and repair membrane damage include galectins and the ESCRT machinery, respectively, and can also be probed by immunoassays.

through downstream methods. Alternative strategies for phagosome isolation have emerged more recently, such as magnetic separation strategies, in which phagocytic targets can either be intrinsically magnetic (magnetic beads) or prelabeled with magnetic nanoparticles [44,47,50–52].

Downstream analysis methods

Proteomics

Some of the first systematic biochemical determinations of isolated phagosomes were done through two-dimensional gel electrophoresis using radiolabeled metabolites [38]. These studies rely on the identification of proteins by comigration with a limited number of standard proteins from a database. Phagosomes can also be analyzed by immunoblotting, whereby enrichment of specific proteins during the progression of phagocytosis can be assessed.

The development of advanced proteomic techniques, specifically high-performance liquid chromatography coupled to mass spectrometry, allowed large-scale, comprehensive analysis of the composition of phagosomes. Indeed, the evolving nature of phagosomes and the relative ease to isolate them from cell homogenates make phagocytosis an attractive process for proteomic studies. During the past ~ 20 years, diverse studies have systematically defined the phagosomal proteome at different stages of the process [39,53,54]. One of the major advantages of this technique is the possibility of performing unbiased experiments throughout the various stages of phagocytosis. Indeed, the development of quantitative proteomic techniques has shed light on the dynamics and composition of maturing phagosomes. In order for these approaches to represent individual stages of phagocytosis, it is highly recommended to synchronize the internalization through pulse-chase procedures (i.e., allow the phagocytes to engage prey for a limited period of time, before thoroughly washing unbound targets). However, as a bulk, 'end-point' method, proteomic analysis of a population does not provide information of individual phagosomes and it lacks spatial resolution. Perhaps, the major caveat of this approach is that complete purification of phagosomes is nearly impossible to achieve due to the complex interactions of the compartment with virtually every organelle in the cytoplasm. At the same time, this methodology allows to uncover possible membrane crosstalk between phagosomes and other organelles. Thus, criteria to discern between contaminants and actual organelle interactions are essential to control these approaches. This can be accomplished by incubating cargo with lysed cells, as one way to detect nonspecific interactions. Notably, over the years major strides have been made to maximize the purity of isolated phagosomes [39,40,42,55,56].

It is important to stress that the use of mass spectrometry as a platform to examine phagosomes carries the potential to fill important gaps in the field. While the phagosomal proteome has been widely investigated, other phagosomal 'omics' such as the lipidome and glycome have been studied to a significantly lesser extent [57,58]. Because of the increasingly recognized contribution of lipids, carbohydrates and metabolites to phagosomal dynamics, defining changes in their molecular landscapes in phagosomes, will likely render important advances in the field, especially in the understanding of host–pathogen interactions [59].

Cell-free fusion

Although exquisitely coordinated in time and space, each stage of phagocytosis is remarkably complex. Thus, accurately dissecting mechanisms at a molecular level can be challenging when studying phagocytosis in intact cells. To circumvent this, several groups have developed *in vitro* cell-free systems, which have been particularly useful to study phagosome maturation [17,60–63]. While these methods still rely on the use of phagosomes isolated from phagocytes, they are unique in that they utilize *in vitro* incubation of phagosomes with isolated cellular components. For these experiments, endocytic components of phagocytic cells are isolated and then incubated with purified phagosomes in the presence of a cytosolic extract from phagocytes with added adenosine triphosphate and protease inhibitors. This setup enables a stringent temporal control of phagocytic maturation events. After incubation, phagosomes can be analyzed by immunoblotting and electron and fluorescence microscopy, depending on the original experimental design. Cell-free systems were more commonly used with latex bead-containing phagosomes, though more recent efforts have allowed to study bacterium-containing vacuoles [62]. The versatility of these techniques was emphasized by studies showing binding of phagosomes to microtubules [64] and F-actin [28] as well as *de novo* F-actin assembly at phagosomal membranes [65]. However, caution should be used while designing this type of experiments, given that many phagocytic events are multifactorial and require several components and organelles.

Imaging and fluorescence-based methods

Because of the highly localized, dynamic and transient nature of phagosome maturation, microscopy techniques that provide high spatial and temporal resolution have enabled significant advances in the field. More recently, the continuous improvements in the resolution of imaging techniques have allowed for the identification of subphagosomal structures and regions

[66]. Additionally, specialized probes allow for qualitative and quantitative determination of phagosomal pH and ROS. Finally, fluorescence-based methods can also be used for bulk/ high-throughput measurements of phagosomes and phagocytes, and can also be adapted to flow cytometry. These methods will be described below.

Immunolabeling

Immunolabeling methods are useful techniques to probe protein recruitment to phagosomes. After challenging phagocytes with phagocytic targets, cells can be fixed at specific times during the process. Cells are then permeabilized and probed with antibodies raised against the proteins of interest. After washing, samples are probed with a tagged secondary antibody [e.g., with fluorophores for immunofluorescence (IF) microscopy or flow cytometry, with gold particles for electron microscopy (EM)]. As in most maturation assessments, it is critical to synchronize phagocytosis through pulse-chase protocols. Additionally, specific stage markers [i.e., early endosome antigen 1 (EEA1) for the early maturation stages; lysosome-associated membrane proteins (LAMP) for late maturation stages; or prelabeling lysosomes with fixable dextrans using pulse-chase protocols; Table 1] can be probed in parallel to confirm the maturation state of individual phagosomes. One of the main advantages of traditional imaging techniques is that they are compatible with primary cells and immortalized cell lines. Additionally, they represent the best option to study the localization of endogenous proteins within cells. However, it is worth noting that reliable antibodies are not always available for proteins of interest. Moreover, the number of proteins that can be probed per experiment is limited either by the species in which antibodies are raised (only one species per antibody), by the number of available fluorophores or number of fluorescent channels in the microscopy setup (for IF), or by the different sizes of gold particles (for EM). Thus, most experimental setups can probe 2–3 proteins. Additionally, the cell fixation and permeabilization methods that yield optimal results vary between antibodies and should be optimized.

The study of protein recruitment to individual phagosomes, along with their proteolytic activity, can also be achieved on isolated phagosomes by immunolabeling postnuclear homogenates of phagocytic cells after bead capture, followed by flow cytometry and gating on the bead population [67–69]. This approach allows the simultaneous detection of a wide array of proteins in a quantitative way. However, the number of parameters analyzed, albeit higher than in IF microscopy, is still limited by the fluorophore spectral overlap. In this regard, the recent development of mass cytometry has significantly increased the number of proteins that can be simultaneously quantified on a single-cell level by using probes coupled to heavy-metal isotopes instead of fluorophores, with little signal overlap between parameters [70].

Genetically encoded tools

While a carefully designed immunolabeling experiment can provide some temporal information on biological processes, live-cell imaging provides the best strategy to improve resolution. The most suitable approach to do this is the expression of fluorescently tagged genetically encoded tools. Through this approach, researchers can follow the fate of molecules during dynamic processes such as phagocytosis. An added advantage of using these methods is that in addition to proteins, several lipid species can be monitored by expression of lipid-sensing probes, which are designed from protein domains that bind a single lipid species with exquisite specificity and high sensitivity [71]. Several factors must be taken into consideration, most notably the fact that exogenous expression of proteins in most cases results in increased total expression of the protein of interest, which can result in diverse artifacts. Also, when expressing sensing probes, it is possible that they can outcompete binding of endogenous effectors and interfere with lipid metabolism and signaling. Thus, it is important to express these tools at levels as moderate as possible (i.e., close to those of endogenous proteins, as long as they are still detectable). This may be achieved by regulated viral vectors and/or conditional gene expression, such as the use of tetracycline-dependent transcriptional switches and more recently developed optogenetic tools [72,73]. Another limitation is that transfection/transduction of primary cells is significantly more challenging than that of transformed cell lines. However, several groups have optimized protocols (mainly using viral transduction) to study primary phagocytes [74,75]. Another consideration is that while traditional live-cell imaging techniques (i.e., confocal microscopy) provide suitable spatial and temporal resolution, techniques such as EM and super-resolution microscopy (in most cases exclusively applicable to fixed cells) are superior to spatial resolution [66]. Alternative approaches for live-cell imaging, albeit significantly more challenging, include endogenous tagging and the use of primary cells from transgenic mice [76].

Table 1. Selected reagents used in phagocytosis and phagosome maturation and crosstalk assays.

Reagent	Description	Applications	References
General phagocytosis assays			
Phagocytic targets			
Polystyrene beads	Inert particles	IF; immunoblot; proteomics; live-cell imaging	[42,96,176]
Magnetic particles	Inert particles	Magnetic isolation of phagosomes and phagosome-containing cells	[51,177]
RBC	Cell targets	Imaging-based methods; shear stress assessment	[166,178]
Apoptotic cells	Cell targets	Imaging	[179,180]
E. coli–OVA	Model Ag-expressing bacteria	Immunoassays	[126]
STm-OVA	Model Ag-expressing bacteria	Immunoassays	[128]
L. monocytogenes–OVA	Model Ag-expressing bacteria	Immunoassays	[181]
Zymosan	Yeast cell wall glycan component	Imaging-based methods	[177,182]
Immunoglobulin G (IgG)-opsonized particles	Polystyrene bead opsonization (for Fc receptor-mediated phagocytosis)	IF; live-cell imaging	[176]
IgG-opsonized RBC	RBC opsonization (for Fc receptor-mediated phagocytosis)	Imaging-based methods	[177,178]
Phagosome maturation assays			
Proteolytic activity			
OVA degradation	Quantitative assessment	Flow cytometry	[67]
DQ-BSA	Qualitative assessment	Fluorescence microscopy	[183–185]
pH measurement			
phRodo dyes	Qualitative assessment of acidification	Fluorescence microscopy	[186]
Cresyl violet	Qualitative assessment of acidification	Fluorescence microscopy	[187]
Fluorescein isothiocyanate (FITC)	Quantitative assessment	Ratiometric imaging	[33]
FITC/AF647	Quantitative assessment	Ratiometric flow cytometry	[67]
Oregon green 488 succinimidyl ester	Quantitative assessment	Ratiometric imaging	[88]
ROS measurement			
Luminol	Peroxidase-dependent detection of O_2^-	Luminometry	[80]
p-Hydroxyphenylacetate	Peroxidase-dependent detection of H_2O_2	Fluorometry	[80]
Dihydrorhodamine 123	General detection of ROS by oxidation of fluorophore	Fluorometry	[80]
p-Nitrotetrazolium blue	General detection of ROS by reduction of compound	Precipitation reaction (microscopy)	[188]
Oxyburst	General detection of ROS by oxidation of fluorophore	IF	[82,189]
Phagosome crosstalk and signaling assays			
Anti-EEA1	Early endosome marker (early maturation marker)	IF; EM	[126]
Anti-LAMP1	Lysosome marker (late maturation marker)	IF; EM; flow cytometry	[51,190]
Anti-LAMP2	Lysosome marker (late maturation marker)	IF; EM	[191,192]
Fixable fluorescent 10 kDa dextran	Prelabeling of endosomes/ lysosomes	Fluorescence-based imaging	[193]
Fluorescent wheat germ agglutinin	Prelabeling of endosomes/ lysosomes	Flow cytometry	[41]
BSA-gold conjugates	Prelabeling of endosomes/ lysosomes	EM	[194]
Tagged genetically encoded constructs	Fixed and live cells	Fluorescence-based imaging	[187]
Anti-ERGIC-53/p58	ERGIC protein	IF	[126,146]
Anti-TGN46	TGN protein	IF	[146]
Anti-TGN38	TGN protein	IF	[126]
Anticalreticulin	ER protein	IF	[146]
	ER enzyme	IF	[146]

Table 1. (Continued).

Reagent	Description	Applications	References
Antiprotein disulfide isomerase			
mTOR	mTOR localization	IF	[114,132]
Anti-S6K	mTORC1 activation	Immunoblot	[23,121]
TFEB/TF3	Translocation to nucleus	Immunoblot	[113]

ROS detection

During phagocytosis of microorganisms, phagocytes increase their oxygen consumption. In this process, the NADPH oxidase NOX2, transports electrons from cytosolic Nicotinamide adenine dinucleotide phosphate (NADPH) to the lumen of phagosomes where molecular oxygen is reduced to O_2^-, which is further converted into other highly microbicidal ROS [77–79]. Additionally, during inflammation, some phagocytes are capable of generating ROS extracellularly through activation of NADPH oxidase located at the plasma membrane [80]. ROS production plays additional roles in regulating phagosomal pH to preserve antigens for cross-presentation in dendritic cells (DCs) and may also impact phagosome membrane integrity [34,81–83], although pH-independent proteolytic functions of NADPH oxidase have also been demonstrated [84]. Diverse methods have been developed to measure ROS production inside and outside of cells. In general, these techniques—which are typically specific to a particular oxygen species—are primarily based on ROS-excitable dyes (e.g., luminol and isoluminol), substrate reduction (e.g., cytochrome c and *p*-nitroblue tetrazolium), or fluorophore oxidation (*p*-hydroxyphenylate, scopoletin, dichlorofluorescein, and dihydrorhodamine). These methods are detailed in various protocol guides adapted to the study of particular phagocytes [67,80,85].

pH measurement

Soon after the first description of phagocytosis, efforts were made to measure changes in phagosomal pH. Such methods that qualitatively determine pH changes have been commonly used in the field for the past ~ 60 years. The most common cellular and subcellular pH assessments rely on the use of indicator fluorophores such as the commercially available Lyso-Tracker, pHrodo, and cresyl violet. Technically, phagocytic targets can be prelabeled covalently with one of such dyes (e.g., pHrodo) and then fed to phagocytes, in which changes in fluorescence intensity can be tracked over time. Alternatively, acidic phagosomes can be labeled with targeted fluorophores, typically weak bases that partition into acidic compartments and accumulate therein. While these techniques are useful and informative for rapid (and relatively easy) determination, they are purely qualitative. Proper quantitative measurements of phagosomal pH require more specialized methods. Dual-excitation ratiometric imaging enables accurate and sensitive quantitative determinations at a subcellular level [86,87]. This technique relies on the use of a fluorophore that has a highly pH-sensitive excitation/emission wavelength peak and a second excitation/emission wavelength that is pH-independent. Even though changes in fluorescence intensity of the pH-sensitive wavelength reflect pH changes, they can also derive from photobleaching, dye leakage, or changes in focal plane. Hence, the second (pH-insensitive) wavelength is used to normalize for these potential artifacts. Thus, the ratio of fluorescence intensities at different wavelengths is used to monitor exclusively changes in pH. A fundamental aspect of this method is that such ratiometric values can be converted into pH values through the use of a calibration curve. This curve is developed *in situ* with the use of pH calibration solutions containing ionophores in order to adjust the pH of intracellular compartments or cells and measure the ratios of the fluorophore of interest at specific pH values. For phagocytosis assays, targets can be prelabeled with fluorophores such as fluorescein and Oregon green, and pH-insensitive fluorophores should also be included for ratiometric purposes [67,88].

Proteolytic and bactericidal activity

Along their maturation, phagosomes acquire degradative capacity, which in most phagocytes is supported by the increased acidity in their luminal environment [7]. Particularly in DCs, proteolytic activity is specifically regulated and geared toward the presentation of the resulting antigenic peptides to T cells [89]. Protein cargo degradation in phagosomes is generally assessed by the use of ovalbumin (OVA) or BSA-coated phagocytic targets, or cross-linked to red blood cells (RBC).

OVA degradation on phagosomes may be monitored over time after phagocytosis by flow cytometry, using anti-OVA antibodies or fluorophore-conjugated OVA. The evaluation of protein degradation on isolated phagosomes is performed by comparing the initial fluorescent peak corresponding to undegraded OVA (high mean fluorescence intensity, MFI) to the decrease in MFI observed over time as OVA is degraded during the phagosome maturation process [67,68]. Another frequently used reagent to evaluate phagosome proteolytic capacity is DQ-BSA, a self-quenched fluorophore—DQ™ Green or DQ Q™ Red —conjugated to BSA. This assay is based on the cleavage of DQ-BSA on the surface of phagocytic particles, by proteases present in the maturing phagosome, which leads to the generation of fluorescent products that can be analyzed by fluorescence microscopy [90–92]. More specific proteolytic activity measurements can be performed on isolated phagosomes at different maturation stages, by incubating phagosome extracts with fluorogenic cathepsin substrates and monitoring substrate degradation by fluorometric analyses [51,93].

Phagosomal bactericidal activity can be monitored over time after bacteria uptake, by sequentially allowing phagocytosis to occur, killing extracellular bacteria with gentamicin, lysing phagocytes, and plating cell lysates to count bacterial colonies as a measure of bacteria viability [94]. Replicating bacteria within phagocytes may also be detected by flow cytometry by colabeling bacteria with proliferation dyes (such as eFluor Q™) and regular fluorochromes. When bacteria replicates, the proliferation dye becomes increasingly diluted among daughter cells and is eventually undetected, while the regular dye remains constant. MFI ratio between both dyes are calculated over time to assess bacteria replication and may be compared between different phagocytes and/or cell treatments [95].

Chapter 2. Additional methods to assess phagosome maturation-associated pathways

Phagosome tubulation and crosstalk

At a glance

A striking transformation that some phagosomes undergo along their maturation pathway is the extension of phagosomal membrane tubules (phagotubules), which play different important roles according to the nature of the phagocyte. In DCs, phagotubules favor interphagosomal crosstalk and major histocompatibility complex (MHC)–class II antigen presentation [96]. Phagotubules may also favor the stimulation of pattern recognition receptors (PRR) in the cytosol by increasing the available surface for potential phagosomal leakage [97] (Fig. 2). In macrophages, the formation of distinct phagotubules serves different roles: Early on, it allows recycling of plasmalemmal components; at late stages, it promotes phagolysosomal formation and requires the association of phagosomes with microtubule-associated motor proteins [27]; and finally, as described in the third chapter of this guide, phagotubules are associated with phagosome resorption and resolution [23,24]. While the nature and role of phagotubules at different stages of the life cycle of phagosomes and also between phagocytes likely differ, the current lack of specific methodologies to study the diversity of phagotubules has limited their examination to general imaging techniques. Because of this, new methodological approaches are required for the unequivocal assessment of phagotubule identity and function, which will undoubtedly advance the understanding of these dynamic events.

Imaging and fluorescence-based methods

Given the dynamic nature of these events, the preferred method for the detection of phagosomal tubulation is live-cell imaging [24,98,99]. As a less efficient but in some cases more accessible alternative, phagotubules may also be visualized on fixed cells by IF or immunoelectron microscopy [27]. However, given their transient nature, it is recommended to use conditions that ensure phagotubule stability after fixation. These conditions are often analogous to the ones required to preserve the integrity of microtubules [100,101]. In our hands, the use of a periodate–lysine–paraformaldehyde fixative [102] followed by permeabilization with 0.1% saponin proved to be successful for the detection of OVA-containing phagotubules by fluorescence microscopy (manuscript in revision).

Phagosomal tubulation may also potentially favor the interaction between phagosomes and other organelles. These interactions can be detected by IF or immunoelectron microscopy, as discussed above. Flow cytometry also proved successful for the detection of phagosomal crosstalk among phagosomes carrying a Toll-like receptor (TLR) signature [96]. The same principle may be applied to the study of phagosomal recruitment of fluorescently tagged proteins present in different organelles. Of note, caution should be exerted when studying interorganelle membrane contact sites (MCS). Nonfunctional close proximity between organelles is common in the cytosol; thus, contaminants

are often found when performing biochemical methods, and imaging techniques can result in artifacts, both of which can be misinterpreted as functional crosstalk. Thus, the use of complementary approaches is highly recommended to study MCS, in addition to the design of critical functional assays (e.g., identification of membrane tethers and their manipulation) [19,24,103,104]

Phagosomal PRR signaling and MHC-II presentation

At a glance

Phagosomes are autonomous signaling organelles equipped with the necessary machinery for protein degradation, peptide loading, and subsequent antigen presentation on MHC-II molecules [68,105–108]. At the same time, signaling from PRR stimulated on phagosomes favors phagosome maturation and leads to the production of proinflammatory cytokines that shape the outcome of the immune response [5,109]. Furthermore, receptor-mediated phagocytosis or PRR signaling from phagosomes may also lead to the activation of the transcription factors TFEB and TFE3—master regulators of lysosomal biogenesis and function, and autophagy [110,111]—via mechanistic target of rapamycin (mTOR)-dependent and mTOR-independent mechanisms, enhancing phagosome degradative capacity and upregulating the transcription of proinflammatory and antimicrobial gene signatures (Fig. 2) [112–114] and thoroughly reviewed in Ref. [16].

The process of antigen MHC-II presentation has been comprehensively reviewed, and we direct readers to some examples of this excellent and extensive literature [115–120]. Given that we will refer to tools for the study of antigen presentation and recognition by T cells, we would like to point out that the standardized nomenclature for rat and mouse MHC can be found at the Jackson Laboratory homepage (http://www.informatics.jax.org/mgihome/nomen/). In particular, mouse MHC molecules are referred to as histocompatibility 2 (H2), and in the case of mouse MHC-II molecules H2-I-A or H2-I-E, the designation is frequently shortened to I-A or I-E followed by a superscript denoting the haplotype. In the case of human MHC, these molecules are designated as human leukocyte antigens (HLA; see below and Table 2). The standardized nomenclature is periodically revised by the World Health Organization and can be accessed via the international ImMunoGeneTips project/HLA database (https://www.ebi.ac.uk/ipd/imgt/hla/).

Biochemical assays

Phagocytosis and phagosome maturation-induced PRR signaling can be readily assessed by immunoblotting of whole-cell lysates at different time points after engulfment. Given that cell surface PRRs are initially triggered, a time course over phagocytosis together with a control with soluble PRR ligand is required to differentiate between plasmalemmal and phagosome-intrinsic PRR signaling. The detection of a second wave of phosphorylation of kinases present on the TLR pathway (such as p38) can be easily detected by immunoblotting [51]. This strategy can be applied to different PRR (or other phagosomal receptors of interest) signaling pathways.

With regard to the study of phagosome–lysosome crosstalk and signaling along the maturation process, lysosomal mTOR complex 1 (mTORC1) activation can be measured by immunoblotting for changes in mTORC1 substrates such as the phosphorylation of the ribosomal S6 kinase 1 [121]. Activation of TFEB and TFE3 can also be monitored by assessing their phosphorylation status and nuclear translocation by cellular fractionation and immunoblotting [113].

Fluorescence-based methods

MHC-II presentation from phagocytosed cargo can be assessed by IF microscopy and flow cytometry. Widely used procedures involve protein-coated polystyrene beads as phagocytic cargo and antibodies that recognize peptide:MHC-II complexes such as the pair $E\alpha_{52-68}$:I-A^b/ YAe antibody and others more recently developed [122]. Indirect readouts for MHC-II presentation include monitoring the cell surface expression of activation markers on T-cell clones specific to certain peptide:MHC-II complexes, such as murine OT-II (reactive to $OVA_{323-339}$:I-A^b) [123] or 1H3.1 (reactive to $E\alpha_{52-68}$:I-A^b) [124] T cells, or tetanus toxoid-specific human T cells [125]. The use of OVA or viral protein-expressing bacteria such as *Escherichia coli*, *Listeria monocytogenes,* or *Salmonella* typhimurium (STm) [126–128] as phagocytic cargo, followed by the coculture with antigen-specific T-cell clones, is also possible. However, the detection of MHC-II presentation of bacterial antigens would be desirable and more informative in terms of the evaluation of bacterial infections and host–pathogen interactions. In this regard, there are some available T-cell clones such as CN.B1 (reactive to STm flagellin $FliC_{427-441}$) [129] and T-cell receptor (TCR) transgenic mice such as CBir1Tg (specific to commensal intestinal bacteria flagellin) [130] and CN.B1 (specific to STm flagellin) [129].

Table 2. Selected reagents used in phagosome maturation-associated pathways and resolution.

Reagent	Description	Applications	References
Antigen class II presentation			
Eα_{52-68}:I-A^b YAe antibody	Antibody to peptide:MHC-II complex	IF; flow cytometry	[124,195]
1H3.1 mouse	TCR specific to Ealpha$_{52-68}$:I-A^b	Immunoassays	[124]
OT-II mouse	TCR specific to OVA$_{323-339}$:IA^b	Immunoassays	[123]
CN.B1 mouse and T-cell clone	TCR specific to STm flagellin FliC$_{427-441}$:I-A^b	Immunoassays	[129]
CBir1Tg mouse and T-cell clone	TCR specific to CBir flagellin$_{456-475}$: I-A^b	Immunoassays	[130]
Antigen cross-presentation			
OVA$_{257-264}$:25D1.16 antibody	Antibody to peptide:MHC-I complex	IF; flow cytometry	[126]
Influenza$_{365-380}$:H-2D^b antibody	Antibody to peptide:MHC-I complex	IF; flow cytometry	[154,155]
HSV$_{498-505}$:H-2K^b antibody	Antibody to peptide:MHC-I complex	IF; flow cytometry	[156]
OT-I mouse	TCR specific to OVA$_{257-264}$:H-2K^b	Immunoassays	[150]
B3Z hybridoma	TCR specific to OVA$_{257-264}$:H-2K^b	Immunoassays	[153]
OGDH hybridoma	TCR specific to OGDH:H-2K^b	Immunoassays	[157]
HSV hybridoma	TCR specific to HSV$_{498-505}$:H-2K^b	Immunoassays	[156]
gp100 human T-cell clone	TCR specific to peptide:HLA-A2	Immunoassays	[151]
MART1 human T-cell clone	TCR specific to peptide:HLA-A2	Immunoassays	[151]
Phagosome integrity			
FITC/TRITC dextran	Leakage to cytosol	IF	[164]
N-glycosylated Renilla luciferase	Enzymatic reaction in the cytosol	Luminescence	[158,159]
FRET probe CCF4 and β-lactamase	Enzymatic reaction in the cytosol	Fluorescence microscopy/flow cytometry	[83,146,170]
Anti-Galectin-3 antibodies	Membrane damage detection	IF	[167,169]
Anti-Galectin 8	Membrane damage detection	IF; immunoblot	[167,169]
Antibodies against ESCRT components	Membrane damage repair	IF	[167]
Phagosome resolution			
5- (and 6-) carboxytetramethylrhodamine succinimidyl ester	Phagolysosomal fission/ fragmentation	Lysosome reformation	[24]

Moreover, novel multiparametric flow cytometric-based approaches also allow the analysis of the expression on MHC-II molecules of different pathogen-specific antigens at the same time, by the use of various T-cell hybridomas expressing unique fluorescent reporters [131] (Table 2).

The study of the crosstalk between phagosomes and lysosomes can be assessed by IF microscopy to monitor mTORC1 localization to lysosomes or phagosomes and TFEB/TFE translocation to the nucleus [113,114,132].

Immunoassays

The production of cytokines after phagocyte stimulation by the phagocytic cargo (e.g., proinflammatory cytokines such as IL-6, IL-12, and Tumor necrosis factor alpha) [133] or by phagosomal damage [Interleukin-1 beta (IL-1β); see below] or after subsequent T-cell activation (such as IL-2) can be assessed by ELISA on fresh or frozen culture supernatant from stimulated cells. This method can be also applied to the activation of T-cell hybridomas, such as OT4H.1D5 and OT4H.2D5, specific to OVA$_{265-280}$:I-A^b [134]; 3A9, specific to hen egg lysozyme$_{48-62}$:I-A^k [135]; or various available T-cell hybridomas, specific to influenza hemagglutinin protein [136]. Most of these hybridomas contain the LacZ gene downstream the IL-2 promoter. Therefore, upon T-cell activation, β galactosidase can also be measured colorimetrically [137] in an easy and cost-effective—though less sensitive compared with other methods—way. Cytokine detection may also be accomplished by flow cytometry, which provides information about differences in the cell population. In the traditional setup, intracellular cytokines are detected after fixation and permeabilization. A variant of this method relies on their detection in live cells by using a matrix that retains cytokines on the cell surface. The advantage of this improved flow cytometric approach is that cells remain viable for further studies [138,139]. However, ELISA provides greater sensitivity, the possibility to quantify the

amount of the cytokines produced by the population and the option of assessing the samples at the most convenient time and on repeated occasions. Alternatively, the presence of cytokines or chemokines in culture supernatants can be detected by the use of commercially available cytokine dot-blot arrays, which confer the advantage of detecting multiple cytokines simultaneously albeit in a semiquantitative manner [113].

Antigen cross-presentation

At a glance

Antigen cross-presentation consists in the presentation of internalized exogenous antigens on MHC-I molecules and is particularly relevant for the activation of cytotoxic Cluster of differentiation 8 protein (CD8)[+] T cells in the development of an antiviral or antitumoral immune response [140–142]. In the case of phagocytosis, the process starts by the engulfment of targets and takes place more efficiently in a subset of DCs specialized in this mechanism [143,144]. Antigen cross-presentation is then mainly accomplished through the 'cytosolic' pathway. In this pathway, phagosomal antigens that gain access to the cytosol—by mechanisms currently not completely elucidated [145]—are degraded by the proteasome, and the resulting peptides are loaded onto MHC-I molecules within the ER.

Increasing evidence supports the notion that phagosomes become competent for antigen cross-presentation after delivery of MHC-I molecules and other ER-resident proteins from the ER, ER–Golgi intermediate compartment (ERGIC), or the endosomal recycling compartment (ERC) [105,126,146]. More recently, it has been proposed that phagosomes contain active proteasomes capable of generating intraphagosomal antigenic peptides and rendering phagosomes self-sufficient antigen cross-presentation organelles [147].

Fluorescence-based methods

Regardless of the relative contribution of the ER or the ERC to the phagosome maturation process, which remains controversial [104,148], these events are mostly being analyzed by IF and live-cell imaging. Antibodies to resident ER, ERGIC, or ERC compartments suitable for IF, as well as fluorescently tagged organelle markers, are readily available (Table 1).

Like MHC-II presentation, antigen cross-presentation from phagosomes can be assessed by IF microscopy and flow cytometry using protein-coated polystyrene beads as cargo and antibodies that recognize peptide:MHC-I complexes, such as anti-OVA$_{257-264}$:H-2K^b [149].

To evaluate T-cell activation after antigen cross-presentation, cell surface expression of activation markers is assessed on T-cell clones specific to certain peptide: MHC-I complexes, such as murine OT-I, reactive to OVA$_{257-264}$:H-2K^b [150]; or gp91, or melanoma-specific human T cells [151,152].

Immunoassays

The production of IL-2 from activated T cells can be measured by ELISA as described for MHC-II presentation assays. This also applies to the use of T-cell hybridomas B3Z, specific to OVA$_{257-264}$:H-2K^b [153]; hybridoma that recognizes influenza nucleoprotein$_{365-380}$:H-2D^b [154,155]; HSV-2.3.2E2 that recognizes herpes simplex virus glycoprotein B$_{498-505}$:H-2K [156]; or 2CZ that recognizes oxoglutarate dehydrogenase peptide:H-2K [157]. Despite being simple and rapid to test, the downside of using hybridomas and some Tcell clones resides mainly in the maintenance of cumbersome culture techniques and complex growing conditions.

Luminescence methods

A novel luciferase-based probe was designed to assess antigen translocation to the cytosol [158,159], in most cases required for proteasomal degradation and subsequent peptide loading in the cross-presentation process. The probe consists in an enzymatically inactive N-glycosylated variant of Renilla luciferase fused to the Fragment crystallizable (Fc) region of human IgG1. This variant becomes enzymatically active when deglycosylated by the cytosolic enzyme N-glycanase-1, generating cytosolic luminescence, which can be easily quantified. This strategy has the potential to be adapted to fusion proteins of interest and can be targeted to other phagocytic receptors as well.

Antigen translocation or exit to the cytosol can also be predicted by assessing phagosome integrity as will be discussed below.

Phagosome integrity

At a glance

Some phagocytic cargoes, such as cholesterol crystals, alum used in vaccine adjuvants, and bacterial pathogens may compromise phagosome integrity by causing membrane destabilization or active membrane damage [160]. Alternatively, lipid peroxidation mediated by the

NADPH oxidaseNOX2, may also lead to membrane integrity disruption [83]. When phagosome integrity is compromised, phagosomal content can gain access to the cytosol and trigger additional cellular processes and signaling cascades, such as inflammasome activation, autophagy induction, and/or antigen cross-presentation (Fig. 2). Moreover, compromising the integrity of the phagosomal membrane and the escape of its contents can ultimately result in cell death. Thus, the assessment of phagosome integrity is relevant to the study of the nature of the engulfed particle, the threat it potentially poses to the phagocyte, the cellular mechanisms activated by phagosome damage, and the type of immune response consequently triggered.

Biochemical methods

Phagosomal acquisition of proteins associated with membrane damage and/or repair (see below) may be assessed by immunoblotting [161] on isolated phagosomes. Controls for purity of phagosome preparations are required.

The production of active IL-1β—released if inflammasome activation is triggered after phagosome damage—compared to the pro-IL-1β form—triggered by inflammatory cargo in an intact phagosome—can be assessed by immunoblotting as an alternative indirect measure of phagosome integrity that also provides information about downstream inflammasome activity in the cytosol [162].

Fluorescence-based methods

Phagosome integrity can be measured by fluorescence microscopy approaches in live or fixed cells. One of these approaches is based on the dextran-release assay, which relies on the quantification of fluorescently labeled-dextran in the cytosol after phagolysosome formation and damage [163]. Phagocytic cells can also be preloaded with two discernible dextran–fluorophore conjugates as endolysosomal cargoes and then pulsed with a particulate phagosomal prey [164]. In this case, when lysosomes fuse with phagosomes in the process of phagosome maturation, dextran release to the cytosol (in the case of phagosomal damage) is quantified by ratiometric imaging between the two fluorophores. Alternatively, fluorescent dextran can be adsorbed to or loaded into some phagosomal cargoes (such as RBC) [165,166]. In this case, the assay can directly evaluate phagosomal leakage and becomes independent of lysosomal contribution. Another approach that is independent from phagosome maturation is the quantification of the recruitment of proteins that mark damaged membranes, such as galectins [167]. Galectins are cytosolic lectins that bind galactosides present on the luminal leaflet of organellar membranes and can therefore bind to phagosomal membranes when galactosides are exposed to the cytosol after membrane damage. Recruitment of galectins 3 and 8 to phagosomal membranes has been monitored by fluorescence microscopy [167–169]. Automated quantitative imaging of fluorescent puncta may be performed using high-content analysis platforms [167]. Binding of galectins to phagosomal membranes can also lead to the recruitment of proteins involved in membrane repair, such as Endosomal sorting complexes required for transport (ESCRT) complexes, or in damaged organelle removal, such as autophagy receptors or adaptors. Recruitment of ESCRT proteins and autophagy proteins, such as Microtubule-associated proteins 1A/1B light chain 3 and p62, can be assessed by fluorescence microscopy as an indirect measurement of phagosome damage [169].

Another possibility is based on a method used to measure escape to the cytosol of β-lactamase-expressing bacteria [170] and has also been applied to the study of antigen export to the cytosol in the antigen cross-presentation field [146,171]. The assay consists of preloading cells with the fluorescence resonance energy transfer (FRET) probe CCF4 prior to phagocytosis. When β-lactamase is present in the cytosol, it cleaves the probe, resulting in a loss of FRET signal at 535 nm and an increased emission at 450 nm, which can be quantified by fluorescence microscopy or flow cytometry.

Phagosome integrity after bacterial infection can also be assessed by flow cytometry, based on the assay described to quantify cytosolic versus vacuolar STm by differential permeabilization [172]. Based on the differences in abundance of cholesterol between the plasma membrane and intracellular organelles, treatment with digitonin under standardized conditions of time and concentration exclusively permeabilizes the plasma membrane, while saponin permeabilizes both plasma membrane and intracellular organelles. This assay can be extended to any phagosomal cargo, provided that the cargo (or a chemically modified version of it) can be detected by antibodies. If the phagosome is intact, the cargo will not be detected by antibodies in the presence of digitonin, but it will be detected in the presence of saponin. Conversely, if the phagosome is damaged, the antibodies will detect the cargo in the presence of either digitonin or saponin. Various degrees of detection (according to the level of availability of the cargo to the antibodies) over time after phagocytosis can be quantified by flow cytometry and

normalized to the total detection levels in the presence of saponin. Due to the requirement to maintain the conditions that prevent digitonin to permeabilize intracellular membranes, controls for the detection of intraorganellar and cytosolic epitopes such as ER proteins are essential to validate the assay.

Chapter 3. Methods to assess phagosome resolution

Phagosome resolution

At a glance

The biochemical composition of physiological phagocytic targets is usually complex. While phagolysosomes are equipped with a wide array of hydrolases to metabolize most macromolecules, the catabolites of this breakdown must be processed for the cell to resorb the compartment, recycle its components, and return to homeostasis in order to resume the immune response. Additionally, it is through processing of degradation products that antigen is presented on the surface of specialized phagocytes to lymphoid cells (as detailed in Chapter 2). Despite its obvious physiological significance, the resolution of phagosomes has rarely been studied and a comprehensive understanding of the molecular mechanisms that drive it is still lacking. Because interest in resolution emerged recently, the methods to study this stage are under development. Some of the techniques that have been used are adapted from studies of lysosomes and autophagy. Here, we discuss methodologies that have been used in a handful of studies on phagosome resolution (excluding antigen presentation, which is discussed above).

Biochemical methods

Detection of mTORC1

While phagosome resolution is by far the least understood stage of phagocytosis, some of its general aspects have been elucidated. Complete resorption of the phagosomal compartment is dependent on mTORC1. Indeed, the fission events that are necessary for membrane recycling and lysosome reformation are impaired upon mTORC1 inhibition [23]. Catabolite export from degraded cargo can promote mTORC1 activation, potentially promoting fission events. Thus, mTORC1 activation can be assessed as a proxy for the initiation of phagosome resolution. The caveat to this assessment is that mTORC1 should remain inactive during phagocytosis in order to detect significant changes in mTORC1 activation. To achieve this, cells can be amino acid-deprived before phagocytes are challenged with degradable phagocytic targets and for the duration of the experiment [23]. mTORC1 activation will occur when catabolites (such as specific free amino acids, e.g., leucine) resulting from robust target degradation are exported from the phagolysosome. These events can be measured by immunoblotting for changes in mTORC1 substrates as discussed in Chapter 2. However, while this method can be used as a readout of phagosome resolution, it does not represent a common physiological state of phagocytes. This emphasizes the need for the development of new methodologies that more closely represent phagocyte host environment.

Imaging and fluorescence-based methods

Phagotubule formation

During the very late stage of phagocytosis, the original phagosomal compartment undergoes a series of fission events mediated at least in part by robust membrane tubulation. Methods to study phagosomal tubulation have been described above (phagosome tubulation and crosstalk section). More recently, we have used lattice light-sheet microscopy (LLSM) to visualize dynamic phagosome tubulation [24]. Because frequent and continuous frame acquisition is critical to study these structures, LLSM minimizes photobleaching while enabling rapid acquisition of multiple focal planes. It is worth noting that this type of microscopy is highly specialized and not readily available yet to most researchers.

Cytosolic dispersion of phagosome-derived vesicles

One of the hypothesized consequences of phagosome resorption during the resolution stage is the reformation of terminal/ storage lysosomes, as in the case of autophagic lysosome reformation [173,174]. Thus, the above-mentioned fission events result in the dispersion of smaller compartments ('recovered' organelles) throughout the cytoplasm. This phenomenology has served as a proxy to assess resolution, as researchers can challenge phagocytes with prelabeled (degradable) phagocytic targets (e.g., RBC and apoptotic cells), with pH-insensitive dyes. After internalization, phagocytes are incubated for long time periods (at least 3–8 h) allowing phagocytosis to progress through resolution. Completion of the process can be measured through fluorescence microscopy by assessing the level of dispersion and size of vesicles of phagosomal origin

[23,24,175]. Recent unpublished studies suggest that lysosome and/or endolysosome regeneration indeed occurs at the phagosome resolution stage, by the use of assays to detect lysosome proteins, pH, and proteolytic activity on phagosome-derived vesicles. A more detailed description of these methods is accessible at: https://doi.org/10.1101/2020.05.14.094722.

Conclusions

The study of phagosome maturation and resolution interfaces with different fields within the biological sciences, including cell biology, immunology, and microbiology, and has recently returned to the spotlight with the increased interest in defining MCS and organelle crosstalk. The study of phagosome dynamics also bridges biological and physicochemical areas of expertise by the continuous development of new tools and technologies for visualization and quantification of phagosome-interrelated phenomena with increased precision, resolution, and sensitivity.

In this guide, we summarized some of the biochemical, imaging, fluorescence, luminescence, and immune-based methods currently available and widely used in the literature for the study of phagosomal dynamics, with a main focus on two subset of phagocytes: macrophages and DCs, an arbitrary decision motivated by our areas of expertise. We also aimed at describing methodologies to integrate the process of phagosome maturation with other downstream cellular signaling pathways, such as inflammasome activation, mTORC1 signaling, and autophagy induction. Additionally, we offered our point of view on the advantages and limitations of diverse methods in an effort to help researchers in their experimental design. Moreover, we attempted to point out some of the areas of study where new or improved methodology would be desired to advance current knowledge.

Ultimately, by providing information about methods applied in different fields to the study of phagosome maturation and resolution, we intended to highlight the crucial role played by phagosomes at the crossroads of fundamental cellular processes.

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Conflict of interest

The authors declare no conflicts of interest.

Author contributions

RL-K and ARM wrote and revised the manuscript and designed the figures.

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