UNVEIL THE COMPLEXITY OF THE MOLECULAR MACHINERY INVOLVED IN ERYTHROPHAGOCYTOSIS BY MACROPHAGES

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"O meu mundo não é como o dos outros, quero demais, exijo demais, há em mim uma sede de infinito, uma angústia constante que eu nem mesmo compreendo, pois estou longe de ser uma pessimista; sou antes uma exaltada, com uma alma intensa, violenta, atormentada, uma alma que se não sente bem onde está, que tem saudades... sei lá de quê!"

(Florbela Espanca)

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Amiga do coração

"Para ser grande, sê inteiro: nada
Teu exagera ou exclui.

Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.

Assim em cada lago a lua toda
Brilha, porque alta vive"

(Ricardo Reis)

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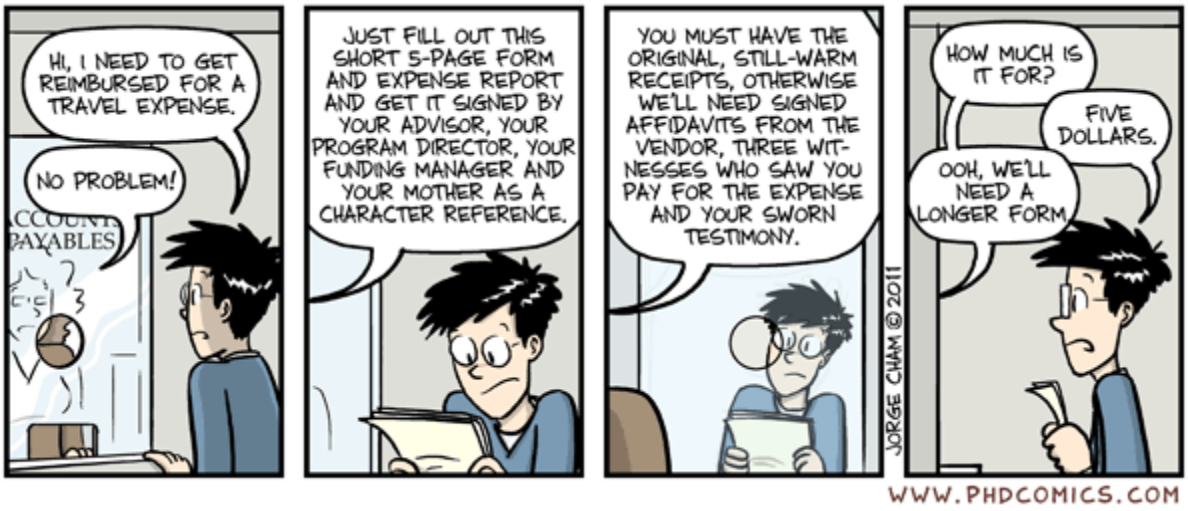

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Table of Contents

List of Figures

List of Tables

Abbreviation List

ABBREVIATION	FULL FORM
AE1	Anion exchanger
agRBC	Aged red blood cells
ARE	Antioxidant response element
ATG	Autophagy-related proteins
BFU-E	Burst-forming unit erythroid
bZIP	Basic leucine zipper
CFU-E	Colony-forming unit erythroid
CFU-GEMM	Colony-forming unit granulocyte erythroid monocyte and megakaryocyte
CNC	Cap´n collar motif
CR3	Complement receptor 3
dH_2O	Deionized water
EEA-1	Early endosome antigen-1
EP	Erythrophagocytosis
ESCRT	Endosomal sorting complex for transport
FBS Hi	Fetal bovine serum heat inactivated
FBS	Fetal bovine serum
$Fc_\gamma Rs$	Fc receptors
Fe	Iron
FLVRC1a	Feline leukemia virus subgroup C receptor 1a
Hb	Hemoglobin
HO-1	Heme oxygenase-1
IDR-1	Innate defense regulator-1
IgG	Immunoglobulin G
ILVs	Intraluminal vesicles
KEAP1	Kelch-like ECH-associated protein 1
LAMP	Lysosomal-associated membrane protein 1

LAP	LC3-associated phagocytosis
LC3	Microtubule-associated protein 1A/1B-light chain 3
LIR	LC3-interacting region
LRS	LC3 recognition sequence
MAF	Masculoaponeurotic fibrosarcoma
MHC	Major histocompatibility complex
MPR	Mannose-6-phosphate receptor
MPS	Mononuclear phagocyte system
MVB	Multivesicular body
NAbs	Naturally occurring antibodies
NBR1	Neighbour of BRCA1 1
NDP52	Nuclear dot protein 52
NLS	Nuclear localization signals
NOX2	NADPH oxidase 2
NRF2	Nuclear factor erythroid derived 2-like 2
p62/SQSTM1	Sequestosome
PB1	Phox and Bem1p
PE	Phosphatidylethanolamine
PI(3)P	Phosphatidylinositol-3-phosphate
PI3K	Phosphatidylinositol-3-kinase
PIB1	Receptor interacting protein-1
PMA	Phorbol-12-myristate-13-acetate
PS	Phosphatidylserine
RBC	Red blood cells/Erythrocytes
ROS	Reactive oxygen species
RPM	Red pulp macrophages
Rubicon	RUN domain protein as Beclin-1 interacting and cysteine-rich containing

SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SOD1	Superoxide dismutase 1
TFEB	Transcription factor EB
TIM4	Mucin domain protein 4
TNF-α	Tumour necrosis factor alpha
ULK1	Unc-51-like kinase 1
V-ATPase	Vacuolar proton ATPase
ZZ	Zinc finger region

Abstract

Erythrophagocytosis is a highly regulated process where sequential events ensure the proper internalization and clearance of red blood cells. However, details on these processes are scarce and not fully understood, making it difficult to modulate and possibly treat associated diseases, such as malaria and sickle-cell disease. After their development in the bone marrow, red blood cells have a life span of approximately 120 days. With time, the plasma membrane of red blood cells undergoes deleterious changes that make the cell susceptible to clearance by macrophages – a process known as senescence. Before this process, erythrocytes may undergo cell death – eryptosis - a similar process that occurs in nucleated cells. In both cases, clearance must occur efficiently in order to avoid the accumulation of free hemoglobin and heme and maintain the iron levels required for homeostasis. Processing of red blood cells by macrophages implies the interaction of nascent phagosomes with endocytic compartments until fusion with lysosomes where component degradation takes place –phagolysosome biogenesis. In the first part of this dissertation, this process was assessed in mouse macrophages from two different sources: bone marrow and the peritoneal cavity. As phagocytic particles involved in erythrophagocytosis, we generated three types of red blood cell models: 1) senescent/eryptotic red blood cells (aged red blood cells) from human blood, and opsonized-red blood cells from sheep blood, 2) IgG- and 3) complement-opsonized. After assessing the acquisition of early and late markers of phagolysosome biogenesis, we concluded that aged red blood cells containing phagosomes mature slower compared to opsonized targets and therefore reach the lysosomes with a delay. As aged red blood cells are the best suited model to mimic senescence/eryptosis due to the exposure of phosphatidylserine on the surface, another aim of this dissertation was to identify other molecular partners in the process of phagolysosome biogenesis. We have found, for the first time, that erythrophagocytosis is LAP-mediated, that is, it involves the recruitment of LC3. Additionally, we identified other proteins from the autophagy machinery such as p62, NBR1 and NDP52, and their association to phagosomes is dependent on ubiquitin. They exhibit different association hierarchies with phagosomal membranes and comparing differences between aged and IgG-Opsonized red blood cells, the most striking outcome was obtained for p62. This autophagy effector exhibited selectivity for aged RBC-containing phagosomal membranes and, surprisingly, it is crucial for their degradation. Interestingly, phosphorylation

of p62 on its Ser351 residue after erythrophagocytosis activates the non-canonical NRF2-signalling pathway, inducing the translocation of this transcription factor to the nucleus where p62, Hmox1 and Sod2 genes are up-regulated, contributing to the maintenance of cell homeostasis. In the absence of NRF2, degradation of red blood cells is also impaired. These outcomes imply a positive feedback loop between p62 and NRF2 in promoting efficient red blood cell clearance. Therefore, our findings contributed to unravelling the molecular mechanisms underlying phagolysosome biogenesis in erythrophagocytosis, opening new avenues to understand the severity of hemolytic diseases and possibly modulate the process.

Resumo

A eritrofagocitose é um processo altamente regulado onde eventos sequenciais asseguram a internalização e remoção de células vermelhas do sangue. No entanto, os detalhes acerca deste processo são escassos, dificultando a compreensão e a modelação de doenças associadas com os mesmos, como a malária ou anemia falciforme. Após o seu desenvolvimento na medula óssea, as células vermelhas têm um tempo de vida de aproximadamente 120 dias. Com o tempo, a membrana plasmática das células vermelhas passa por várias alterações nefastas, tornando-as susceptíveis a serem eliminadas pelos macrófagos - senescência. Antes deste processo, os eritrócitos podem sofrer morte celular – eriptose – processo semelhante que ocorre em células nucleadas. Em ambos os casos, o processo de remoção tem de ocorrer de forma eficiente, de modo a evitar a acumulação de hemoglobina e ferro livres e manter os níveis de ferro necessários à homeostase. O processamento das células vermelhas pelos macrófagos implica a interação entre os fagossomas que são formados após internalização e compartimentos da via endocítica, culminando na fusão com os lisossomas, onde ocorre a degradação dos seus constituintes – biogénese do fagolisossoma. Na primeira parte desta dissertação, este processo foi testado utilizando macrófagos de ratinho a partir de dois tecidos diferentes: medula óssea e cavidade peritoneal. Como partículas fagocíticas envolvidas na eritrofagocitose, geramos três distintos modelos de células vermelhas: 1) senescentes/eriptóticas (células envelhecidas) a partir de sangue humano, e células vermelhas opsonizadas com 2) IgG e 3) complemento, a partir de células vermelhas de ovelha. Após a avaliação da biogénese do fagolisossoma pela aquisição de marcadores iniciais e tardios da via endocítica, pudemos concluir que fagossomas contendo células vermelhas envelhecidas maturam mais lentamente quando comparadas com células vermelhas opsonizadas e, deste modo, fundem mais tardiamente com os lisosomas. Uma vez que células envelhecidas são o modelo que melhor mimetizam os processos de senescência/eriptose por externalizarem fosfatidilserina à superfície da membrana, outro dos objetivos desta dissertação foi tentar identificar proteínas adicionais envolvidas na biogénese do fagolisosoma. Verificámos, pela primeira vez, que a eritrofagocitose depende do processo LAP, isto é, envolve o recrutamento de LC3. Adicionalmente, identificámos outras proteínas da maquinaria da autofagia tais como o p62, NBR1 e o NDP52, cuja associação aos fagosomas depende da ubiquitina. Esta maquinaria apresenta diferentes hierarquias de associação aos

fagosomas e, comparando diferenças entre células vermelhas envelhecidas e opsonizadas com IgG, observamos que o p62 se associa preferencialmente aos fagosomas de células envelhecidas. Surpreendentemente, este efetor da autofagia seletiva é crucial para a degradação das mesmas. Curiosamente, a fosforilação do p62 no resíduo de serina 351 ocorre depois da eritrofagocitose ser estimulada, levando à activação da via de sinalização não canónica do NRF2, induzindo a translocação deste factor de transcrição para o núcleo e consequentemente a expressão de genes envolvidos na homeostase celular, como o *p62*, *Hmox1* e *Sod2*. Na ausência do NRF2, a degradação das células vermelhas envelhecidas é também afectada. Estes resultados levam a crer que existe um *feedback* positivo entre o p62 e o NRF2 na degradação eficiente das células envelhecidas. Desta forma, os nossos resultados contribuem para desvendar parte dos mecanismos moleculares envolvidos na biogénese do fagolisosoma após o processo de eritrofagocitose, abrindo novas portas para entender a severidade das doenças hemolíticas e possivelmente modular as mesmas.

CHAPTER I

Introduction and Aims

1. Why Do Red Blood Cells Matter?

1.1. Red Blood Cells – An Overview

Erythrocytes/Red blood cells (RBC) are the main components of human blood (about 5 x 10^6 erythrocytes per microliter) and have the crucial role as O_2 suppliers in tissues. They are produced by a controlled process known as erythropoiesis, which takes place in the bone marrow during the adult-age, and in the yolk sac in the early foetus. Blood accounts for 6-8% of body mass in adults and erythrocytes comprehends almost all the cellular elements in the blood: only 1 out of every 20 is a platelet and 1 out of 500 is a leukocyte [1]. The number of RBCs produced and released to the circulation is controlled by erythropoietin, a hormone secreted by the kidneys that drives the proliferation and differentiation of RBC precursors, upon a fall in O_2 pressure in respiring tissues.

Erythroid cells are derived from a pluripotent stem cell that, in addition of its self-replicative capacity, can give rise to separate cell lineages [2]. Therefore, erythropoiesis passes from the stem cell through to the multipotent progenitor colony-forming unit granulocyte erythroid monocyte and megakaryocyte (CFU-GEMM) to the burst-forming unit erythroid (BFU-E), so-called because its earliest progeny is motile, giving rise to a multi-subunit colony (or burst). Then, a more mature erythroid progenitor, colony-forming unit erythroid (CFU-E) is formed, being the first recognizable erythrocyte precursor in the bone marrow, named the pronormoblast. These precursors are five-fold to eight-fold more abundant than BFU-E in bone marrow and under normal circumstances do not appear in the circulation. As progenitors undergo the differentiation process, their numbers increase, with their proliferative potential simultaneously decreasing. These cells give rise to a series of progressively smaller normoblasts with increasing content of haemoglobin (Hb). The nucleus is finally extruded from the late normoblast progressing to mature red blood cells through to the reticulocyte stage. Erythropoiesis ends with the mature circulating red cell, which is a non-nucleated biconcave disc, without any organelles such as endoplasmic reticulum and mitochondria and, consequently, they are no longer able to proliferate but capable of sustaining their main function of oxygen delivery. Therefore, the concentration of erythrocytes depends on the O_2 supply to the tissues.

The final step of erythropoiesis in bone marrow has been reported to take place in a specialized microenvironment, consisting of clusters of erythroid cells at various stages of

differentiation surrounding a central macrophage – erythroblastic islands [3]. Marcel Bessis, who first described this niche in 1957, showed a structure containing a macrophage, known as nurse macrophage, surrounded by developing erythroblasts. He then oncluded that macrophages actively participate in erythroid development by providing iron for heme synthesis and by phagocytosing expelled nuclei during final erythroid differentiation (Figure 1). In humans, there are about 5-30 erythroblasts surrounding a central macrophage which have been proposed to secrete cytokines essential to erythroblast survival and maintenance, transport iron to erythroblasts, support the synthesis of large quantities of haemoglobin, and phagocytose and degrade erythroblast nuclei during nuclear extrusion, a step required to generate reticulocytes, as mentioned above [4].

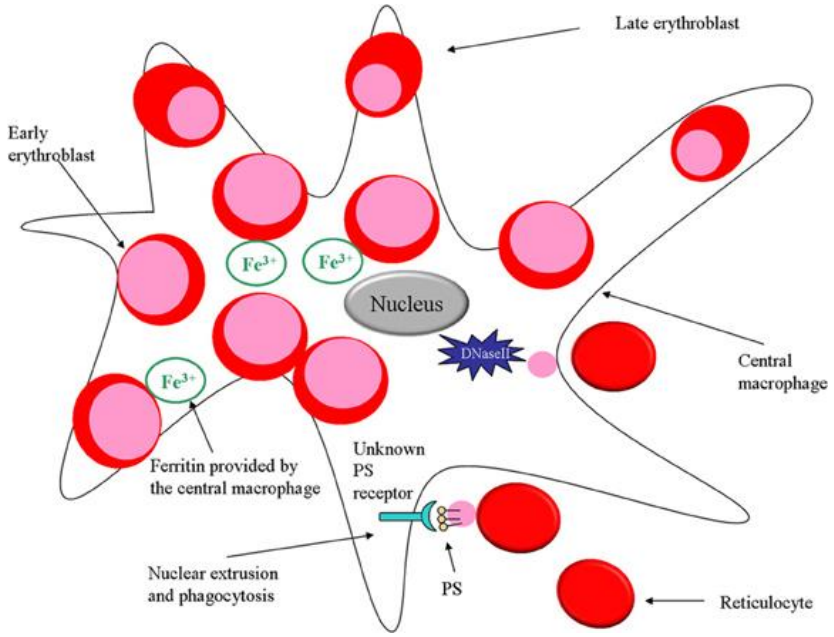

FIGURE 1: Erythroblastic island. A central macrophage is surrounded by erythrocytes at different erythropoiesis stages. Ferritin is endocytosed by erythroblasts with the consequent iron released becoming available for heme production. DNAse II inside the nurse macrophage is important to nuclei breakdown with subsequent expulsion and formation of reticulocyte. Figure reproduced from [3].

A single red blood cell is composed by 270 million molecules of haemoglobin and each haemoglobin has four heme *b* groups. Heme is a hydrophobic metallo-compound containing an iron (Fe) atom within its protoporphyrin IX ring and this structure is known as a “heme pocket”, characterized by a high frequency of aromatic amino acids conferring the hydrophobicity required to promote stable heme binding to hemoproteins [5]. In turn, iron is the most abundant element on earth and is indispensable for living organisms as it ensures the transport of oxygen and functionality of numbers of enzymes such as cytochromes

involved in the mitochondrial respiratory chain. The average human male adult contains about 4 g of iron, of which about 2.5 g is within the haemoglobin of erythrocytes and only 10% of dietary iron is absorbed in the gut. Therefore, the human body has evolved to create mechanisms towards iron recycling and the answer relies on red blood cells homeostasis. Human RBC have a limited lifespan of 120 days after which they become senescent and are engulfed and digested by macrophages through a process known as erythrophagocytosis (EP). Besides senescence, cell death-triggered damaged RBC also account for the majority of iron processed and recycled by the human body.

1.2. Red Blood Cell Removal from the Circulation

1.2.1. Senescence *versus* Eryptosis

The mature erythrocyte is unable to self-repair, synthesize lipids *de novo* and has no capacity to synthesize proteins. Therefore, its lifespan is finite and is further shortened when facing normoxic and hypoxic cycles and when the erythrocyte's ability to cope with damaging extracellular stimuli becomes impaired. Erythrocytes as oxygen transporters, must travel through the vascular circulation to supply the tissues and this function is assured by their highly deformable biconcave shape, allowing them to pass through narrow pores. During aging/senescence, membrane microvesiculation accompanied by haemoglobin loss, results in an increase in cell density and rigidity coupled with a decrease in deformability and flexibility, triggering red cell clearance [6]. Additionally, some metabolic alterations such as ATP depletion and decreases in glucose transport across the RBC are affected by senescence [7]. Haemoglobin, being the most abundant protein in RBC, is the main target of oxidative damage and at the same time, a scavenger of free radicals. Mature RBCs possess efficient enzymatic machinery to process and detoxify reactive oxygen species, including superoxide dismutase (SOD1), catalase, peroxidases and evidence demonstrates that the activities of some of these anti-oxidants decline during aging. Direct evidence of an *in vivo* SOD1 requirement was verified by Junichi Fujii and his collaborators [8], where SOD1 deficiency led to increased erythrocyte vulnerability by the oxidative stress. Consequently, the continuous destruction of oxidized erythrocytes appears to induce the formation of autoantibodies against certain erythrocyte components, resulting in anemia and compensatory activation of erythropoiesis. Auto-oxidation of haemoglobin is considered to be the major source of superoxide anion

production in senescent RBC. The reactive free radical species generated by Hb reactions and the interactions of Hb with membrane and cytoskeleton proteins, both induce oxidative stress and are involved in RBC aging. Band 3, also termed the anion exchanger (AE1), is an ion/trans-membrane transport protein channel, which enables anion transport via the RBC membrane and comprises 25% of the RBC total membrane protein. Similarly, it tethers the bilayer to the skeleton and therefore regulates the structure and function of the RBCs. RBC oxidative damage results in hemichrome (haemoglobin degradation products) generation and its binding to the cytoplasmic portion of band 3 protein, induces band 3 protein clusterization [9]. Several findings strongly suggested that senescent RBCs are recognized, *in vivo*, by low-affinity anti-band 3 naturally occurring IgG antibodies (NAbs) that bind to clustered band 3 proteins that have lost their anchorage to the cytoskeleton [10]. NAbs are antibodies present in the serum of healthy individuals that have been formed without apparent antigen exposure. However, the immunoglobulins formed are not efficient opsonins, due to their low affinity and low circulation numbers. Nevertheless, anti-band 3 NAbs appear to have higher affinity for complement C3 and by activating the classical complement pathway, stimulate complement amplification [11]. This results in a preferential $C3b_2$ -IgG complex formation, with RBC opsonization improvement [12].

Another mechanism that has been proposed to be important for senescent RBC removal is phosphatidylserine (PS) exposure at the cell surface of the RBC as demonstrated by *in vivo* experiments in some patients with hemolytic anemia [13]. In healthy RBCs, PS is usually located on the inner leaflet of the RBC membrane. For many years it was believed that exposure of phosphatidylserine on the outer leaflet of the membrane triggered a selective removal of senescent RBCs. In fact, aged RBCs present lower aminophospholipid translocase activity and higher levels of externalized PS, in comparison with younger ones [14] and the oldest erythrocytes are especially vulnerable to oxidation-triggered phosphatidylserine exposure and subsequent removal [15]. However, growing evidence has shown that clusterization of band 3 is the most prominent alteration in senescent RBCs triggering their removal by macrophages [16] and PS exposure occurs upon RBC vesiculation. This mechanism occurs as a self-protective mechanism while passing red pulp macrophages (RPMs) in the spleen for a “checkout” control and the effective disposal of such molecules would preserve the integrity of the erythrocyte and may expand its lifespan. Nevertheless, it is estimated that

human erythrocyte transit through the spleen every 20 min where they are subjected to several types of quality control [17].

Upon injury and prior to senescence, from < 1 h to 48 h, human mature erythrocytes may undergo suicidal death characterized by some features that are shared by but don´t resemble apoptosis of nucleated cells, because they lack nuclei and mitochondria. To distinguish the death of erythrocytes from apoptosis, some authors suggest the term **"eryptosis"** [18]. Additionally, RBC´s susceptibility to eryptosis increases with RBC age. One hallmark of eryptosis is: 1) cell shrinkage, resulting from an increase of intracellular Ca^{2+} leading to activation of Ca^{2+} -sensitive K^+ channels. Consequently, the subsequent efflux of K^+ hyperpolarizes the cell membrane, which drives Cl^- exit with osmotically obliged water, leading to cell shrinkage. [19]; the second hallmark is: 2) cell membrane scrambling, also due to an increase in intracellular Ca^{2+}. Ca^{2+} entry stimulates sphingomyelinase to form ceramide and both activate a scramblase [18], leading to the 3) exposure of phosphatidylserine (PS) and breakdown asymmetry of the cell membrane. Phosphatidylserine at the surface of eryptotic erythrocytes can bind to blood platelets and endothelial cells of the vascular wall and this interaction impedes microcirculation [20]. Moreover, phosphatidylserine-exposing erythrocytes may stimulate blood clotting and thus trigger thrombosis [21].

Eryptosis/senescence-mediated removal of damaged erythrocytes is likely to be involved in the pathophysiology of several clinical disorders. The percentage of phosphatidylserine-exposing erythrocytes in circulating blood is increased in diabetic patients [22]. Similarly, plasma from septic patients stimulates phosphatidylserine exposure, cell shrinkage, increases in cytosolic Ca^{2+} activity, and ceramide formation in erythrocytes compared to healthy individuals, implying severe eryptosis is observed in sepsis [23]. The malaria pathogen *Plasmodium falciparum* imposes oxidative stress on the host erythrocytes and thus activates several ion channels in the erythrocyte cell membrane [24], including the oxidant-sensitive Ca^{2+}-permeable erythrocyte cation channels. PS exposure is also enhanced in a substantial proportion of sickle red blood cells and erythrocytes of thalassemia patients and may contribute to the phagocytosis of these cells *in vitro* [25].

1.2.2. Erythrophagocytosis

Senescent and/or eryptotic RBCs (hereafter referred as damaged RBCs) are removed from the circulation through erythrophagocytosis, which takes place in macrophages from the mononuclear phagocyte system (MPS) - spleen, liver and bone-marrow [26]. In pathological conditions, such as infection and inflammation, circulating monocytes may enter the spleen and differentiate into RPMs [27]. It seems that in inflammatory situations, the resting macrophage populations are replaced with an inflammatory monocyte-derived pool of macrophages such as bone marrow-derived macrophages. Besides the spleen, the liver also has an important role in RBC clearance and iron recycling [28] and current research suggests that under pathological conditions the liver and not the spleen is the primary site of RBC clearance. The presence of stressed or damaged RBCs leads to a rapid recruitment of monocytes and these monocytes give rise to "transient macrophages" that are well equipped to phagocytose RBCs and recycle the iron. This specific macrophage population was only found in the liver. Besides macrophages, other phagocytes may contribute to RBC clearance in pathological situations as shown by Stijlemans *et al.* [29] where his team identified neutrophils and monocytes as phagocytes of the parasite *T. brucei.* This indicates that besides macrophages other phagocytes can play a role in RBC clearance during infection.

Although the relative contribution of this macrophage population to erythrophagocytosis is unknown, the processing and clearance mechanisms of RBC converge. Most of the research has focused on erythropoiesis while details on erythrophagocytosis are scarce. Indeed, free heme can act as a cytotoxic pro-oxidant since iron´s reactivity makes it quite toxic due to its ability to catalyze Fenton reactions and generate dangerous hydroxyl radicals (Figure 2). Cells can neutralize this cytotoxic effect by secreting Fe via Fe efflux pumps or by storing Fe inside ferritin.

$$Fe^{2+} + H_2O_2 \longrightarrow OH^- + OH^\bullet \quad (1)$$

$$Fe^{3+} + O_2^{\bullet-} \longrightarrow Fe^{2+} + O_2 \quad (2)$$

FIGURE 2: Fenton Reaction. *In vivo* Fenton chemistry (equation 1) in erythrocytes is initiated by H_2O_2, produced upon O_2 transport, inducing oxidation of Fe^{2+} with subsequent production of hydroxide (OH^-) and the highly reactive hydroxyl radical ($OH^\bullet$). Then, the superoxide anion $O_2^{\bullet-}$ can reduce the oxidized metal released by the Fenton reaction (equation 2).

Senescent/damaged RBCs are recognized by macrophages before internalization. There are a number of different receptors in the plasma membrane that recognize specific ligands. For example, red pulp macrophages express CR1 and CR3 that would enable them to recognize and phagocytose complement-opsonized RBC either alone or linked to Nabs on the RBC surface [30]. Initial studies showed that RBC-derived vesicles are rapidly removed from the circulation mainly by scavenger and PS receptors on liver Kupffer cells and to a lesser extent by other macrophages of the mononuclear phagocyte system [31]. Indeed, most of the knowledge regarding removal of PS-containing targets was based on previous studies on the clearance of apoptotic cells. These cells express a plethora of signals which some of them such as PS or complement opsonins are common with the signals expressed by RBC. Interestingly, researchers are currently attempting to understand the receptor dynamics and complexity of the phagocyte response to these different signals. In this matter, Flannagan *et al* [32] demonstrated that TIM4 and β1 integrins associate upon receptor clustering, suggesting a model in which TIM4 engages integrins as co-receptors to stimulate the signal transduction needed to internalize PS-bearing targets such as senescent/damaged RBCs.

Following recognition by macrophages, RBCs are internalized in an endocytic vacuole known as the erythrophagosome. Next, these erythrophagosomes interact with the endocytic pathway and are degraded in the erythrophagolysosome, where the breakdown of RBC contents occurs with subsequent storage and recycling. This is the major pathway of iron acquisition by tissue macrophages and the daily amount processed is estimated to be one erythrocyte per macrophage, thereby providing the iron required for erythropoiesis (Figure 3).

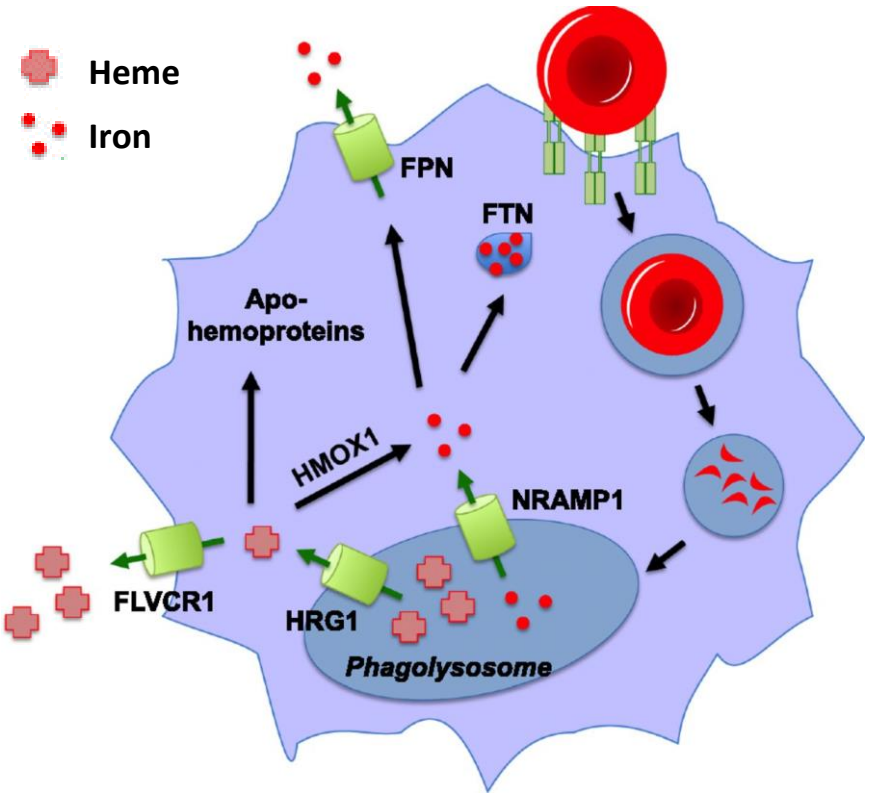

FIGURE 3: Heme and iron trafficking upon erythrophagocytosis. After internalization, senescent/damaged RBC reach the erythrophagolysosome on macrophages. There, heme is released from the RBC and transported out through the HRG1. In the cytosol, heme can leave the cell through FLVCR1 transporter or be degraded by heme oxygenase-1 (HMOX1), releasing iron, which can be stored by ferritin (FTN) or transported to the circulation through ferroportin (FPN). NRAMP1 is responsible for the import of iron to the cytosol. Figure adapted from [33].

In the phagocytic vacuole, the ingested erythrocyte is digested by exposure to reactive oxygen species (ROS) and to hydrolytic enzymes, leading to the degradation of haemoglobin and subsequent heme release. Heme is exported across the erythrophagolysosomal membrane to the cytosol through a conserved heme-transporting permease known as HRG1 [34]. Human HRG1 is expressed by macrophages of the MPS and is upregulated transcriptionally and at the protein level in response to treatment with heme or Fe during erythrophagocytosis. A recent study showed that HRG1 is localized to acidic endosomal and lysosomal organelles, colocalizing with lysosomal-associated membrane protein-1 (LAMP-1) and interacts with the c subunit of the vacuolar proton ATPase (V-ATPase) pump, enhancing endosomal acidification [35]. In the cytoplasm, heme can be transported out of the cell by the cell surface heme exporter, feline leukemia virus subgroup C receptor 1a (FLVCR1a), and bind to hemopexin which avoids free heme circulation. Interestingly, a different isoform, FLVCR1b, was identified in the membrane of mitochondria, where four steps of *de novo* heme synthesis

occur, aiming to export heme to the cytosol to complete the synthetic pathway [36]. Another fate of heme in the cytosol is to serve as heme oxygenase-1 (HO-1) substrate, which cleaves it into equimolar amounts of labile iron, carbon monoxide and biliverdin. Extensive research has been made throughout the years to demonstrate why heme is degraded in the cytosol, and the answer relies on the features of HO-1: 1) HO-1 is embedded in the endoplasmic reticulum (ER) membrane and the active site of this enzyme is oriented towards the cytosol [37]; 2) optimal pH activity of HO-1 is near 7.4; 3) maximal HO-1 activity is achieved only in the presence of biliverdin reductase which is responsible for biliverdin reduction and is located in the cytosol [38]. HO-1 is known to provide tissue damage control and host protection against infection as heme in the circulation is cytotoxic. The cytoprotective function of this enzyme also produces carbon monoxide as discussed above, as the binding of this gasotransmitter to Fe prevents the oxidation of hemoproteins as well as heme release from the haemoglobin [39].

After heme catabolism, iron can be stored inside the cell linked to ferritin or can be exported by ferroportin 1 (FPN1) cycling between endocytic vesicles and the macrophage plasma membrane, under erythrophagocytosis stimuli. The levels of iron in the plasma and extracellular fluids from dietary iron absorption and release from storage compartments, are negatively regulated by the hepatic hormone hepcidin which binds to ferroportin, inducing its endocytosis and proteolysis, and thereby causing iron sequestration in macrophages [40].

1.3. Phagocytosis and Phagosome Maturation

Phagocytosis is defined as the regulated uptake of large particles (>0.5 μm in diameter) into cytosolic, membrane-bound vacuoles called phagosomes. Phagocytosis is a complex, integrated biological process over space and time and determines host protective and/or deleterious outcomes. It shapes the nature and quality of dynamic innate and acquired immune responses to altered self- and foreign stimuli. The functionality of this process, however, has expanded to include a role in the clearance of debris in order to maintain homeostasis and tissue remodelling [41,42]. Notable examples include: the extrusion of erythroblast nuclei directly onto the surface of macrophages for immediate uptake, as mentioned; the clearance of outer segments shed by photoreceptor cells engulfed by nearby retinal pigment epithelial cells [43]; the removal of senescent red blood cells from circulation by macrophages in the spleen; and the clearance of millions of apoptotic germ cells that arise during spermatogenesis as well as those germ cells that display improper meiosis or other developmental abnormalities, by Sertoli cells [44]. These specialized phagocytes are hybrid, multi-functional phagocytes that are increasingly recognized for their importance in specific tissue contexts.

Among the plethora of cells that are able to phagocytose, they can be divided into **professional** or **non-professional** phagocytes [45]. **Professional phagocytes** include neutrophils, monocytes, macrophages, immature dendritic cells, osteoclasts, and eosinophils [46]. Bone marrow is the source of circulating neutrophils and monocytes that will replace selected tissue-resident macrophages and amplify tissue myeloid populations during inflammation and infection. During infection, neutrophils are most often the first cells on the scene, surveying for invading microorganisms, hence why they are relatively short lived [47]. Their capacity to generate and deliver microbicidal compounds into the phagosome underlies the elimination of potential pathogens. During development, macrophages emerge from precursors in the yolk sac, fetal liver, and spleen and persist as tissue-resident macrophages in many organs throughout adult life [48]. Macrophages can also clear pathogens at sites of infection, but serve the additional function of cleaning up the collateral damage caused by the exaggerated microbicidal response of neutrophils. Eosinophils, mainly involved in allergy and parasitic infection, can be longer lived and depend on granule release for their secretory effector functions. Immature dendritic cells derived from monocytic precursors lose their

phagocytic capacity upon maturation because they acquire a potent ability to present peptide antigens to naïve T-lymphocytes. Osteoclasts, multinucleated cells also derived from monocyte precursors, are able to take up bone and other particles, including bacteria and apoptotic cells. **Non-professional** phagocytes, such as epithelial cells, fibroblasts, smooth muscle cells and endothelial cells are unable to internalize microbes despite their ability to clear apoptotic cells under routine homeostatic conditions. Although they are called 'non-professional' due to the lack of efficient phagocytic receptor expression, in particular for opsonins, resulting in lower efficiency phagocytosis than that of professional phagocytes, non-professional phagocytes have a major role in tissues in which macrophages are scarce or where the access of macrophages to apoptotic cells is not readily achieved, such as in the alveoli of the lungs or in the intestinal epithelium, for example [46,49]. Owing to the number of different phagocyte types, the variety of their targets, and the complexity of their interactions, these engulfment processes are not always identical. Indeed, the process of phagocytosis (Figure 4) can be divided into different stages exploring the idea that at these steps phagocytes receive additional information about targets they engage and the nature of the threat [49].

Phagocytosis is initiated by engagement of surface receptors that recognize endogenous ligands exposed by the target particle (Figure 4): some phagocytic receptors recognize determinants inherent to the particle such as mannose receptors, which bind microbial polysaccharides and others interact with host serum factors, known as opsonins, that deposit on the surface of invading particles. Examples of opsonic receptors are complement receptor 3 (CR3) and Fc receptors ($Fc_{\gamma}Rs$) that associate with complement fragment iC3b and with immunoglobulin G (IgG), respectively [50], as already mentioned. Regarding Fc-mediated phagocytosis, besides the ligand-induced changes in the receptor conformation upon binding, it was also found that receptor clustering leads to the activation of signalling cascades [51].

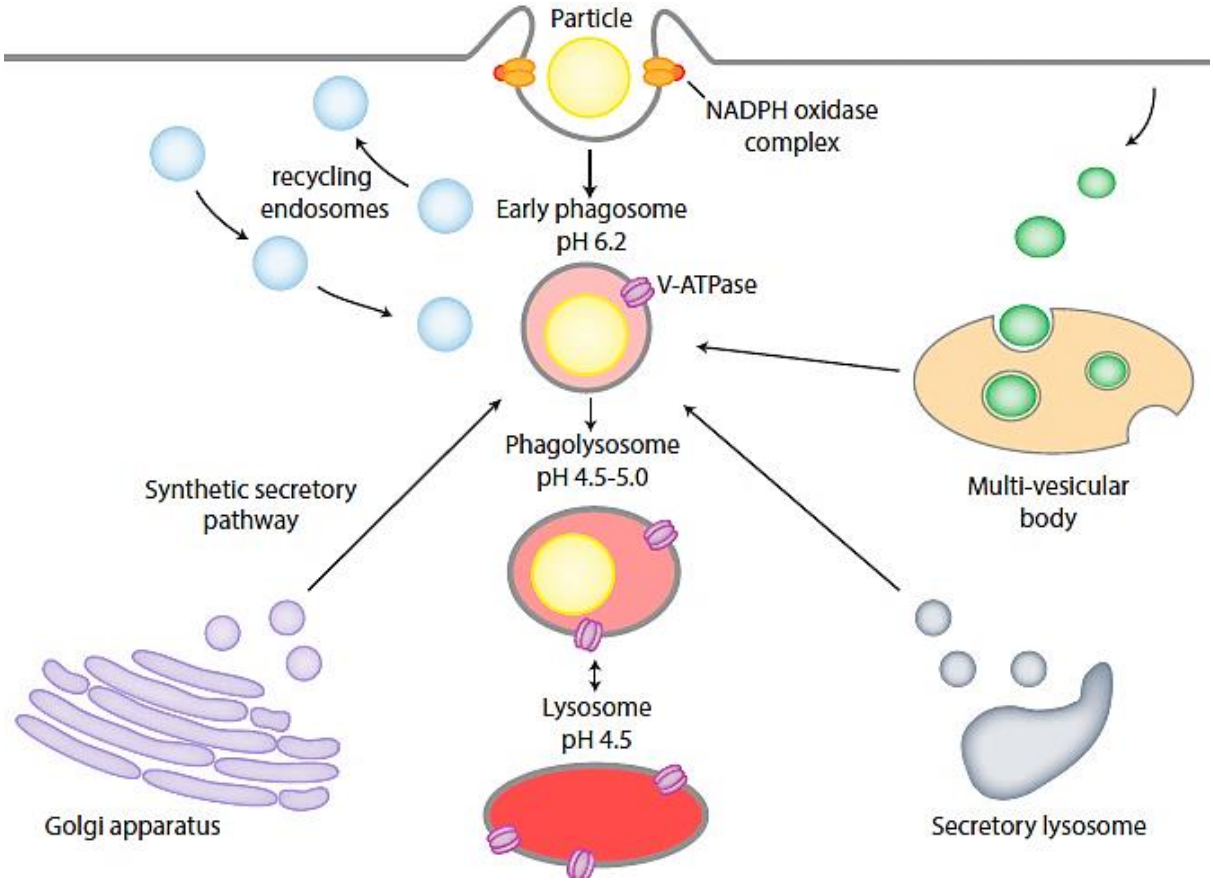

FIGURE 4: The Phagocytic Pathway. The phagocytic pathway starts with the interaction between the particle and the phagocyte. After recognition, the particle is internalized in a vacuole termed nascent phagosome. By interacting with components of the endocytic pathway, phagosomes mature until they fuse with lysosomes to form the phagolysosome, where particle degradation takes place. Figure reproduced from [46].

Receptor–ligand engagement triggers an intricate signalling network responsible for cytoskeletal and membrane remodelling that culminates in particle engulfment. Physiological targets often engage several of these receptors at the same time, simultaneously activating numerous signalling cascades that will act in parallel or in a cooperative fashion to induce particle uptake. Although being impossible to analyse this process as whole, studies on a single receptor make it possible to extract crucial information regarding the signalling pathways triggered by each receptor. The force that drives the extension of the membrane is generated by the polymerization of actin networks, cortical actin, directly under the plasma membrane [52]. In Fc-mediated phagocytosis, a phagocytic cup trapping the target is observed with pseudopod extension. CR3 is the best-studied phagocytic receptor after the FcγR and the signalling events elicited by CR3 and FcγR are markedly different. Early studies of CR3-

mediated phagocytosis by electron microscopy suggested that particles sink into the phagocyte without extending the pseudopods that are characteristic of FcγR phagocytosis, but accompanied by membrane ruffling [53]. In the case of apoptotic cells or senescent RBC, internalization is mediated by a process between micropinocytosis and complement-mediated phagocytosis with the target sinking [54].

Immediately after scission from the surface membrane, the newly formed phagosome, the nascent phagosome, resembles the plasma membrane in terms of lipid and protein composition and its fluid-phase contents are a reflection of the extracellular milieu. Next, there are a series of biochemical modifications that convert the nascent phagosome into a degradative organelle, through an ordered choreographed succession of events including membrane fission and fusion between the phagosomal membrane and the components of the endocytic pathway that radically alter the composition of the phagosome, while keeping its size nearly constant. This process is known as **phagosome maturation** (Figure 5). While the nascent phagosome in macrophages during ingestion of particles has a pH around 6.2, in neutrophils, Segal *et al.* demonstrated transient alkalinization of vacuolar pH before progressive acidification [55]. During maturation, the internalized plasma membrane-derived phagosomes move progressively towards the centrosome, receive further input from endocytic vesicles, and fuse selectively with lysosomes to form the phagolysosomes.

Phagosome maturation starts immediately after membrane scission [42,56] and F-actin dissociation. Changes both in the membrane and content, are brought by vesicular traffic to and from the phagosome. Early phagosomes are generated from the fusion of nascent phagosomes with early/sorting endosomes, acquiring RAB5. This small GTPase coordinates endocytic traffic and early phagosome biogenesis by recruiting and activating multiple effectors that facilitate the fusion events in early steps of phagosome maturation. The most studied effector is VPS34, a class III phosphatidylinositol-3-kinase (PI3K) transiently recruited by RAB5 that catalyzes the formation of the signalling lipid phosphatidylinositol-3-phosphate [PI(3)P]. This phosphatidylinositol allows the binding of proteins with PI(3)P binding domains to phagosomal membranes such as the early endosome antigen-1 (EEA-1). EEA-1 is a homodimer that besides a PI(3)P-binding domain (FYVE-domain) also has two RAB5 binding domains one in the C-terminus and the other in the N-terminus facilitating the docking between phagosomes and early endosomes. At the same time, this effector directly interacts with a soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE), which

is responsible for the fusion between donor and acceptor membranes. Under inflammatory conditions, NADPH oxidase complexes are recruited to the nascent phagosome membrane with a continuous production of ROS [57].

Despite vesicular fusion events, the surface area of the maturing phagosome remains constant due to fission events and recycling processes. As shown in Figure 5, the transferrin receptor is an example of a protein recycled back to the plasma membrane via the recycling pathway. This process is crucial as impairment of recycling inhibits the ability of phagocytes to ingest IgG-opsonized phagocytic targets or to process ingested apoptotic cells [58,59]. There is an additional process that enables the maintenance of phagosome size - the multivesicular body (MVB) pathway - which sorts integral membrane proteins via invagination and pinching of the limiting membrane of phagosomes into intraluminal vesicles (ILVs) [60] (Figure 5). MVB generation requires the endosomal sorting complex for transport (ESCRT) machinery [61]. Factors that initiate ILV formation are recruited to phagosomes at an intermediate stage, when the phagosome no longer resembles an early compartment but is not yet a late phagosome. Proteins targeted for degradation through MVB formation are usually mono- or polyubiquitinated.

The transition from early phagosome to late phagosome relies on the fusion with late endosomes/MVBs and this mature organelle is defined as a more acidic organelle (pH 5.5–6.0) due to the acquisition of V-ATPase proton pumps catalyzing the transport of H^+, across phagosomal membranes (see Figure 5). At this stage, distinct biochemical markers are acquired including the small GTPase Rab7, and loss of early markers, such as Rab5 [62]. Active Rab7 on the phagosomal membrane associates with the effector Rab7-interacting lysosomal protein (RILP), which coordinates microtubule-dependent vesicular traffic through direct association with the molecular motor dynein/dynactin. The motors not only displace phagosomes in the centripetal direction but, strikingly, promote the extension of phagosomal tubules toward late endocytic compartments, essential for efficient lysosomal fusion [63]. Additionally, the recruitment of Rab7 is facilitated by the presence of LAMP-1, which is a structural component of late phagosomes that simultaneously preserve membrane integrity and promotes microbicidal functions.

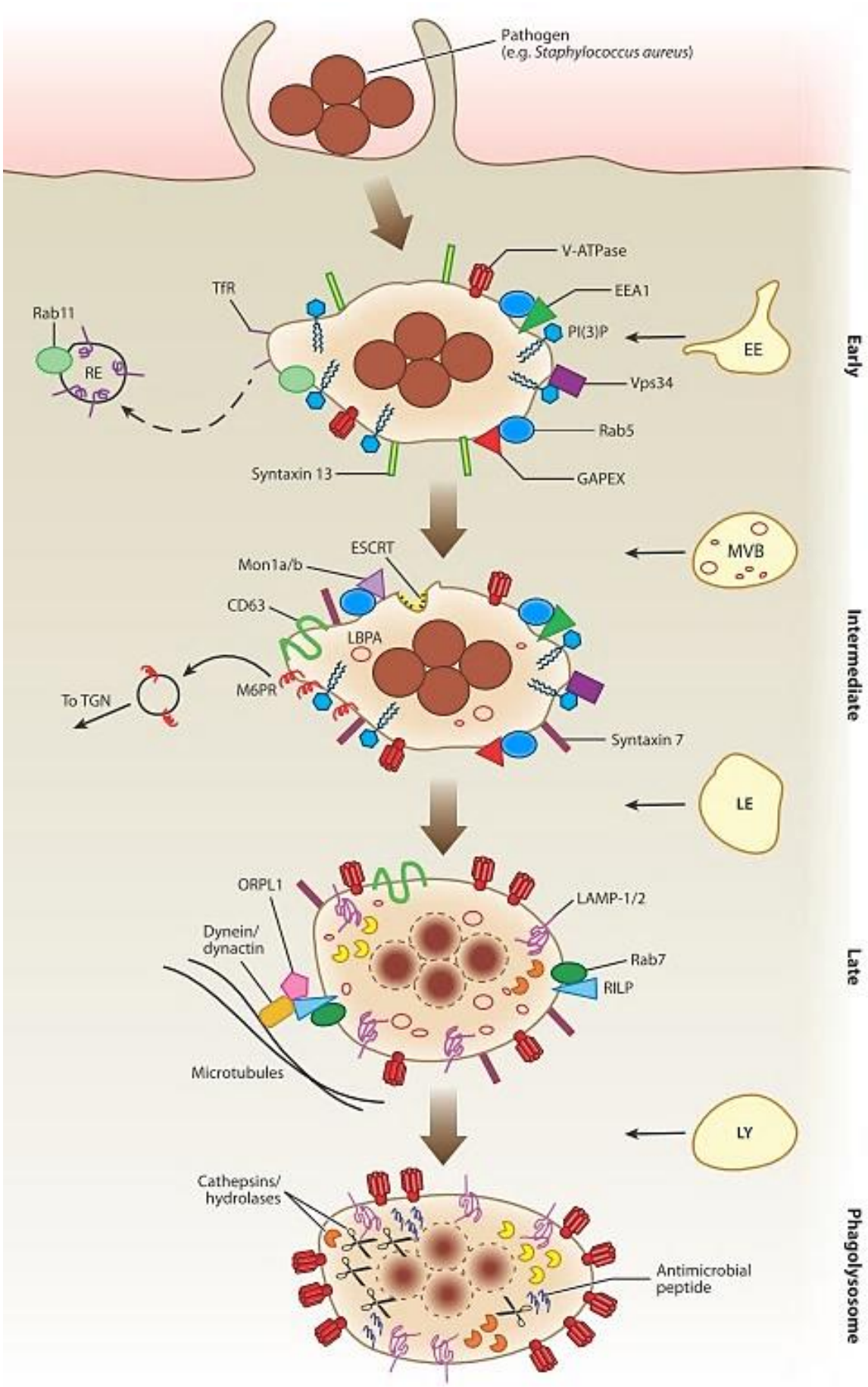
Pathogen
(e.g. Staphylococcus aureus)
V-ATPase
TfR
EEA1
Rab11
RE
PI(3)P
EE
Vps34
Rab5
Syntaxin 13
GAPEX
Early
MVB
ESCRT
Mon1a/b
CD63
LBPA
To TGN
M6PR
Syntaxin 7
Intermediate
LE
ORPL1
LAMP-1/2
Dynein/
dynactin
Rab7
RILP
Microtubules
Late
LY
Cathepsins/
hydrolases
Antimicrobial
peptide
Phagolysosome

FIGURE 5: Steps of Phagosome Maturation. The newly formed phagosome containing a pathogen interacts with early endosomes, yielding the early phagosome. During the maturation, phagosomes interact and acquire crucial machinery from the endocytic pathway allowing the process to proceed until fusion with lysosomes, forming the phagolysosome. Generally, the intravacuolar pH of phagosomes decreases during maturation, activating several proteolytic enzymes responsible for pathogen degradation. Figure adapted from [49].

The phagolysosome is the last organelle formed within this highly regulated process of maturation, resulting from the fusion between late phagosomes and lysosomes. These organelles are extremely acidic (pH 4.5–5.0) due to an enhancement in the V-ATPase proton pump number, leading to the activation of proteolytic enzymes responsible for target degradation and recycling of luminal contents. The magnitude of the H^+ gradient across the phagosomal membrane can vary greatly, depending on the type of phagocyte. For example, macrophages fully acidify their phagolysosomes and contain an arsenal of acid hydrolases that extensively degrade ingested macromolecules. On the other hand, dendritic cell proteolysis is incomplete (pH 6.0), and antigenic peptides become associated with major histocompatibility complex (MHC) polypeptides, which are displayed on their surface to activate of $CD4^+$ and $CD8^+$ T lymphocytes [64]. Neutrophils maintain a slightly alkaline phagolysosomal pH for extended periods, while phagosomes in M2 macrophages reach a very acidic pH minutes after sealing. In contrast, M1 macrophages maintain their pH similar to neutrophils as a consequence of the production of large quantities of ROS [65].

Lysosomes are well recognized as degradative organelles. In fact, materials that reach the lysosome are degraded by lysosomal hydrolases (there are 60 different hydrolases in animal cells, including proteases, peptidases, phosphatases, nucleases, glycosidases, sulfatases, and lipases) and the resulting breakdown products are used to generate new cellular components and energy in response to the nutritional needs of the cell. Most newly synthesized acid hydrolases are tagged with mannose-6-phosphate and traffic from the trans-Golgi network to lysosomes via attachment to a mannose-6-phosphate receptor (MPR). Then, they dissociate from the MPRs as a result of the acidic endosomal pH. The lysosomal membrane is composed by at least 25 proteins, of which the most abundant are LAMP-1 and LAMP-2. Glycosylations at their luminal domains form a glycocalyx, which is suggested to protect the membrane from the lytic enzymes within the lysosome. Most of these membrane proteins have sequence motifs in their cytosolic tails that target them to lysosomes [66]. Ongoing studies have shown that lysosomes are not simply “waste bags”, playing a central organelle to protect the cell

from different stimuli. These organelles may function as signalling hubs sensing the nutritional state of the cell and at the same time regulating the translocation of the transcription factor EB (TFEB), responsible for the expression of lysosomal genes and lysosome biogenesis, and further activation in the nucleus being crucial for the regulation of cellular energy metabolism [67]. In addition, lysosomes are also involved in plasma membrane repair, among others processes.

1.4. LC3-associated Phagocytosis

Autophagy (derived from the Greek words *auto* meaning "self" and *phagein* meaning "to eat") is an evolutionarily conserved and regulated self-digestion pathway, in eukaryotic cells which functions in response to various types of stress such as starvation, heat shock, oxidative stress, and the accumulation of damaged or superfluous organelles, comprising several steps, and at least 40 autophagy-related proteins (ATG). During macroautophagy (hereafter referred to as autophagy) [68], which is autophagy in its strictest form, the cellular components to be degraded are captured by a membrane structure that forms in response to signalling events (the phagophore) and then extended until the complete closure of the targeted material, in a double-membrane formed autophagosome. The formation of the phagophore (pre-initiation step) requires the activity of the unc-51-like kinase 1 (ULK1) complex (containing ULK1-ATG13-FIP200) which senses starvation and stressed signals, while its nucleation (initiation complex) requires a VPS34 lipid kinase complex (containing the PI3K/VPS34/Beclin1/ATG14). Then, a protein–lipid conjugation complex (containing ATG5, ATG12 and ATG16L1) attaches the ubiquitin-like protein microtubule-associated protein 1A/1B-light chain 3 (LC3), to the autophagosomal membranes contributing to membrane elongation and phagophore closure. Among the variety of LC3-like homologs, the isoform LC3B is by far the most studied [69]. The cytosolic form of LC3 (designated LC3-I) is conjugated to phosphatidylethanolamine (PE) and in this form (designated LC3-II) is recruited to the growing autophagosome membrane. Prior to the fusion of autophagosomes with lysosomes, the LC3-II located on the outer autophagosomal membrane is released by deconjugation of PE from LC3 by the proteolytic activity of ATG4. By contrast, LC3-II associated with the inner membrane is retained, although eventually degraded by acid hydrolases within the autophagolysosome. Autophagosome maturation is induced by the translocation of the SNARE protein syntaxin 17 [70] to the outer membrane of the completed autophagosome. This facilitates fusion of the autophagosome with lysosomes [70,71] and results in the acidification of the autophagolysosome lumen, the acquisition of lysosomal hydrolases and the degradation of the cargo as well as the inner of the two membranes.

Autophagy was initially characterized to be a nonspecific degradative process as an adaptive response to starvation. In this context, it mainly acts to replenish the nutritional state of the cell to feed essential metabolic pathways [72]. However, it is now accepted that

autophagy has the ability to selectively target structures, such as intracellular pathogens, damaged organelles and protein aggregates. This type of autophagy is called “selective autophagy” and is thought to play critical housekeeping roles to maintain intracellular homeostasis by eliminating unwanted structures. There are different modalities of selective autophagy according to the unwanted cellular structures: mitophagy (mitochondria), pexophagy (peroxisomes), lipophagy (lipid droplets), xenophagy (pathogens). This selectivity relies on the action of certain adaptor/receptor proteins, which bind to LC3 through the LC3-interacting region (LIR) [73] motif in humans or LC3 recognition sequence (LRS) motif in mouse in their structure, specifically sequestering and targeting the cargo to lysosomes for degradation. Among those studied, researchers focus on the adaptors that simultaneously bind ubiquitin and LC3, such as Sequestosome 1 (p62/SQSTM1), Neighbor of Braca 1 gene (NBR1) and Nuclear dot protein 52 (NDP52) [74–76] thereby linking ubiquitinated cargo with the autophagy machinery.

Contrary to other cytoplasmic digestive processes such as proteasomal degradation, autophagy offers the ability to capture and to eliminate large targets such as toxin protein aggregates, disused organelles and invading microorganisms. Thus, the elimination of potentially toxic components and the recycling of nutrients both support cellular survival. Basal levels of autophagy occur in most cells to maintain protein and organelle control by preventing the accumulation of protein aggregates and defective organelles. Additionally, autophagy is a key component of host innate immune defense against many pathogenic microorganisms, limiting bacterial escape from the phagosome, as well as targeting cytosolic bacteria to the lysosomes for clearance.

In 2007 [77], researchers observed that LC3 is not a unique marker of canonical-autophagy as the recruitment of this protein was induced after engulfment of *Escherichia coli* and yeast to phagosomes within 5 to 10 minutes after internalization, remaining associated while reaching the lysosome. Indeed, LC3 recruitment to the phagosomal membrane has also been shown to occur in the phagocytosis of different types of dying cells such as apoptotic bodies and necrotic cells [78] or entotic bodies [79], a process known as **LC3-associated phagocytosis** or **LAP** (Figure 6).

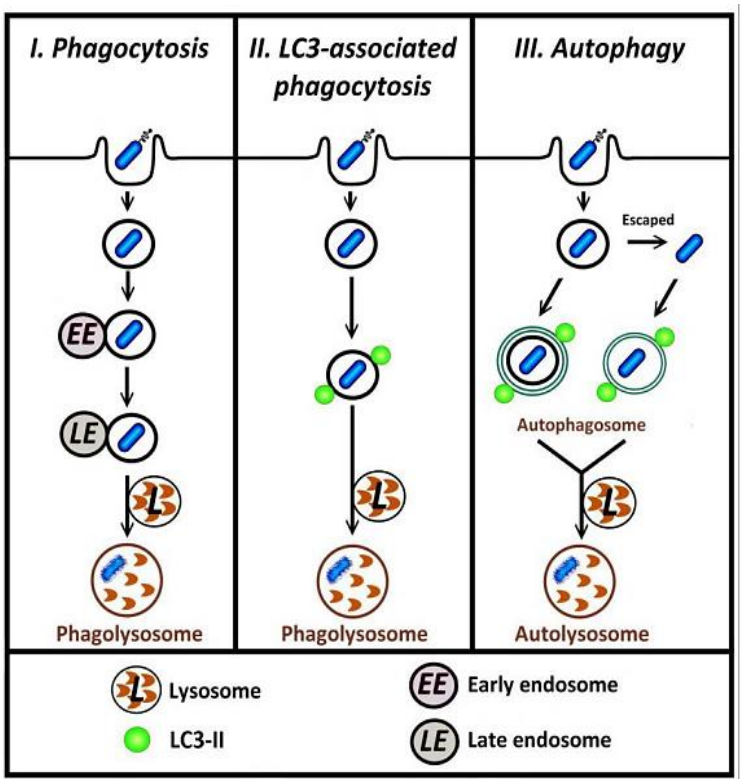

FIGURE 6: Different pathways targeting pathogens for degradation. I. Phagocytosis – The pathogen-containing phagosome interacts with the endocytic pathway until it fuses with lysosomes. **II.** LC3-associated phagocytosis (LAP) - entraps the pathogen into a single-membrane phagosome decorated with LC3-II. **III.** Autophagy – The pathogen is captured by a double-membrane phagosome (autophagosomes) decorated with LC3-II. The common feature in these 3 processes is that the pathogen is degraded by fusion with lysosomes. Figure reproduced from [80].

The exact mechanism responsible for triggering LAP is poorly understood, but there are some key features, which allow it to be distinguished LAP from canonical autophagy (Figure 6). Distinct from conventional autophagy where LC3 is incorporated into the double-membrane autophagosome, LC3 is recruited directly to the single-membrane phagosome (LAPosome), facilitating rapid phagosome maturation, degradation of engulfed targets, and modulation of the immune response. Another hallmark is that the pre-initiation complex (ULK1-ATG13-FIP200) crucial for autophagy is not required for LAP. As in autophagy, LAP recruits and activates Beclin1/VPS34 complex but in this case, it was demonstrated that Beclin1 is crucial for the recruitment of RAB5, a GTPase involved in the phagosome maturation [78]. The activity of the ATG5-ATG12-ATG16L conjugating system during LC3 recruitment is also required.

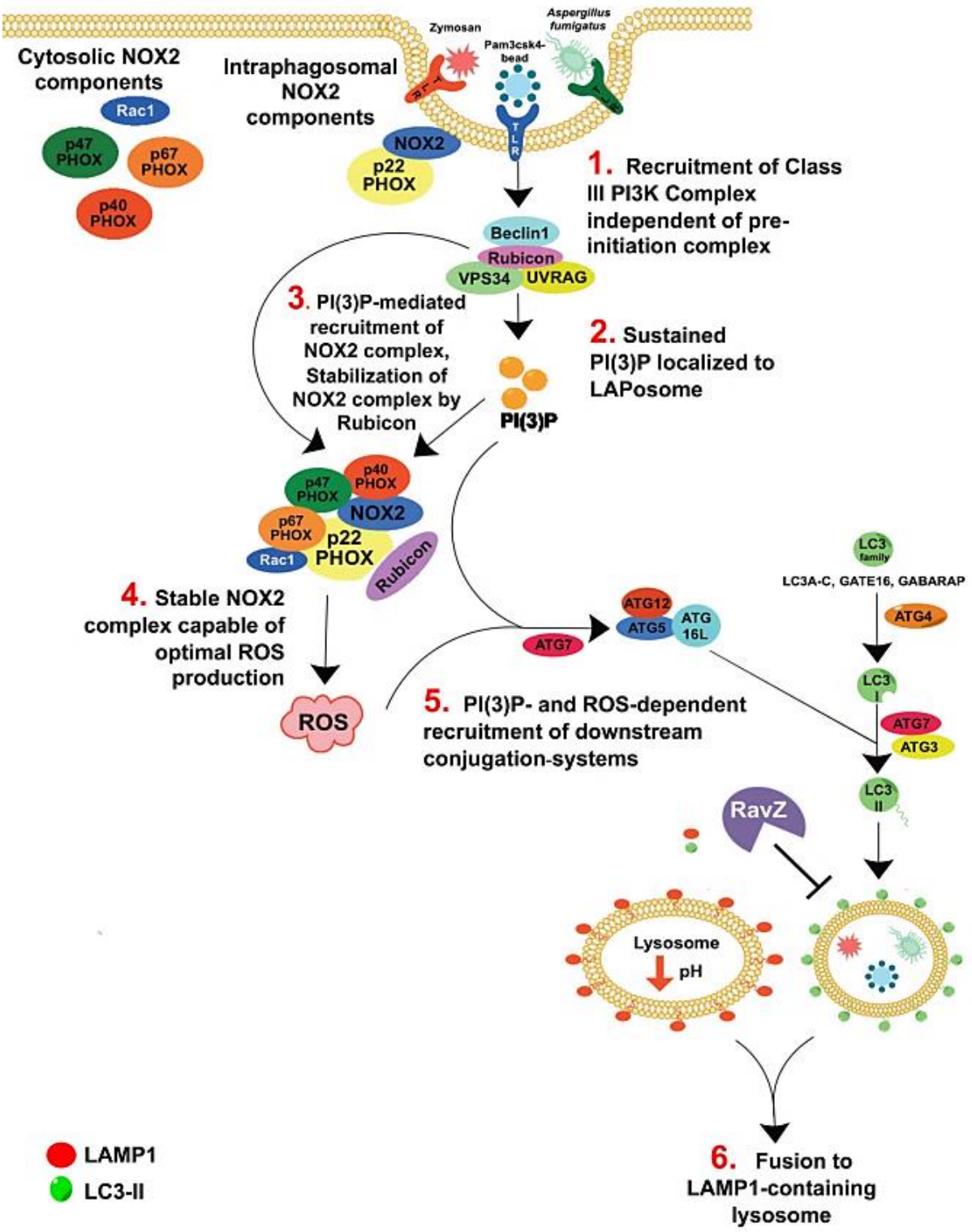

FIGURE 7: Proposed model for LAP. Recruitment of Rubicon at the LAPosome containing different targets, results in PI(3)P accumulation on the membrane, allowing for the recruitment of downstream signalling to the LAPosome. Rubicon also stabilizes the NOX2 complex promoting optimal ROS production for efficient killing. LC3-II is crucial for the progression of LAPosome until fusion with lysosomes. Figure reproduced from [81].

Numerous studies have focused on the role of LAP in the maturation and degradation of several pathogens-containing phagosomes [82–84]. Indeed, very recently [81], Martinez *et al* identified RUN domain protein as Beclin-1 interacting and cysteine-rich containing (Rubicon) (previously shown to be unnecessary for autophagy) as a molecular partner of Beclin1/VPS34, which is necessary for the production of PI(3)P in the LAPosome, crucial for LAPosome formation and progression. Upon microbial infection, pathogens are usually degraded by the action of NADPH oxidase 2 (NOX2) constitutively associated and located primarily in the membranes of phagosomes leading to the production of ROS in the form of superoxide within the phagosomal lumen [85]. Besides killing microbes, ROS also participate in host defense by inducing the activation of NF-κB and release of pro-inflammatory mediators, such as IL-1α, IL-6 and TNFα. Recently [86], it was reported that Rubicon is also an essential positive regulator of the NADPH oxidase complex by inducing ROS production upon microbial infection. Those two findings suggest that Rubicon targets the two innate immune machineries, autophagy and phagocytosis, to efficiently orchestrate intracellular antimicrobial responses (See Figure 7 for the sequential LAP steps suggested upon infection).

The clearance of dead cells by LAP requires the PS-receptor and mucin domain protein 4 (TIM4). Primary macrophages with reduced expression of TIM4 were deficient in dead cell-specific LAP as well as displaying lupus-like disease [87]. LAP-deficient macrophages produced IL-1β and IL-6 upon engulfment of dying cells. Strikingly, serum levels of these cytokines were acutely elevated in LAP-deficient animals, but not in LAP-sufficient animals. Also, LAP, but not canonical autophagy, is required for the production of IL-10 in response to apoptotic cell engulfment, and LAP suppresses production of inflammatory cytokines under these conditions [87]. Altogether, these data suggest that defective LAP results in failure of engulfed dying cells digestion, leading to elevated inflammatory cytokine production and a lupus-like syndrome. Interestingly, Rubicon$^{-/-}$ mice failed to degrade engulfed corpses in a LAP-dependent mechanism [87]. This identifies Rubicon as indispensable for LAP upon infection insult, and apoptotic cells or PS-exposed target clearance, working as a molecular switch between the repression of autophagy and the activation of LAP.

LAP has a number of different roles in the cell that include maintenance of cellular homeostasis and protection against invading pathogens, as discussed throughout this topic. It has become obvious that LC3 recruitment to the phagosomes shortly after engulfment aims to accelerate phagosome maturation and fusion with lysosomes, for a successful clearance

(Figure 8). In the absence of membrane-bound LC3, intraphagosomal pathogens obtain a survival advantage compared with control cells. In addition, apoptotic or entotic cell degradation is compromised upon LAP inhibition. On the contrary, antigen-presenting cells such as dendritic cells, which are more potent than macrophages, contain fewer proteolytic phagosome compartments. In this context, LAP can stabilize cargo for delayed processing and recognition, rather than immediate delivery for degradation [88]. LAP seems to contribute to antigen processing for MHC class II presentation, but only a subset of phagosomes within each cell is regulated by this pathway. In human phagocytes, LAP stabilizes substrates for delayed processing prior to MHC class II presentation to $CD4^+$ T cells.

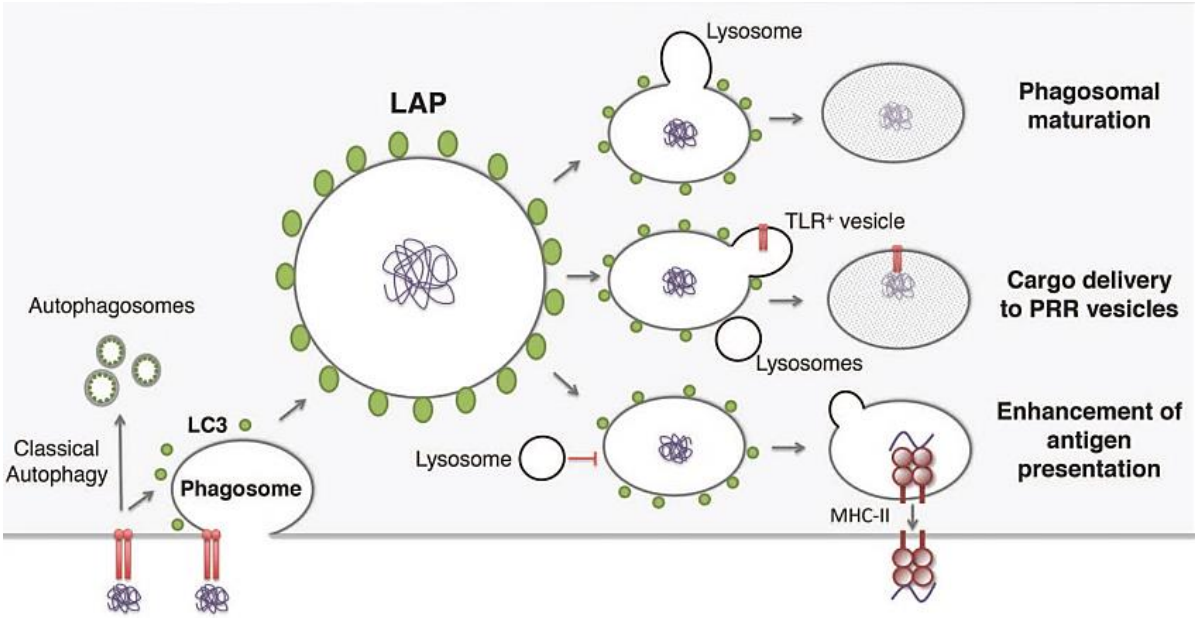

FIGURE 8: Different LAP roles. LC3 association to single-membrane phagosomes was suggested to promote phagosome maturation, pathogen delivery to vesicles containing specific receptors, and enhance antigen presentation. Figure reproduced from [89].

1.5. p62/SQSTM1: Structure and Biological Function

Since the identification of p62 [90], the number of reports concerning this protein has grown extensively. The same author recognized p62 as a noncovalent ubiquitin-binding protein that belongs to a new class of ubiquitin-binding proteins, affecting signal transduction at least partly through ubiquitination-mediated protein degradation [91]. He also found that p62 preferentially binds to ubiquitinated protein aggregates, which led him to coin the name "sequestosome" [92].

p62 is a 62 kDa ubiquitously expressed cellular protein, that is conserved in metazoa but not in plants and fungi. It is ubiquitously distributed in the cell: in the cytoplasm, localized in the nucleus, and within autophagosomes and lysosomes. Six distinct functional domains have been defined in its structure and include the following: N-terminal Phox and Bem1p (PB1) domain, a zinc finger (ZZ) region, a tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF6) binding (TB) domain, a LIR domain, Kelch-like ECH-associated protein 1 (KEAP1) interacting region (KIR) and a ubiquitin-associated (UBA) domain in the C-terminal (Figure 9).

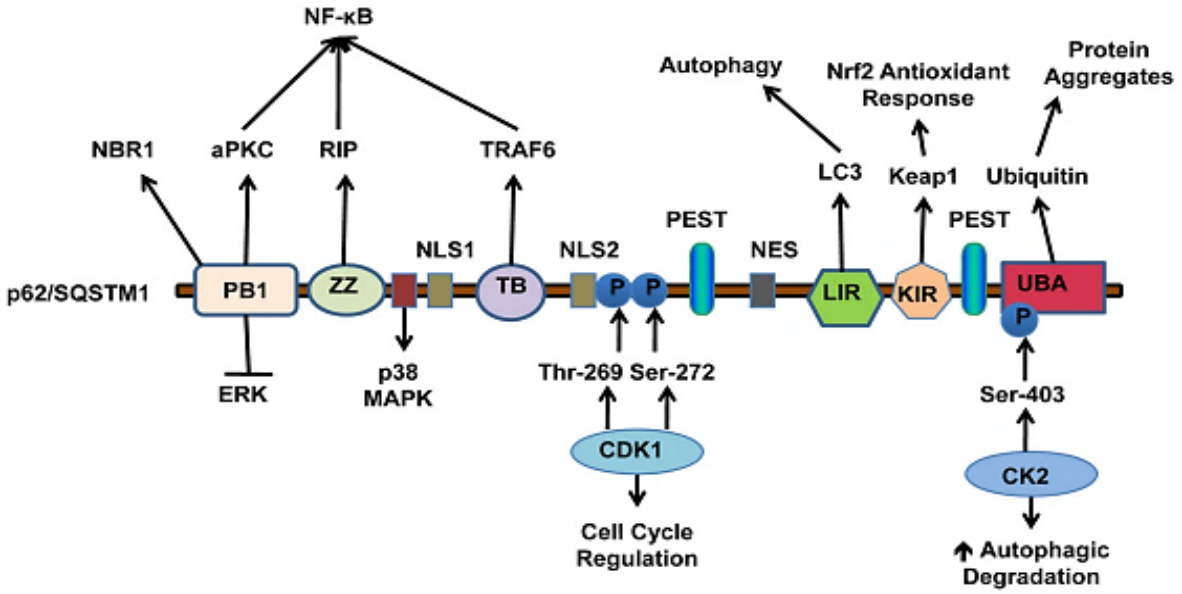

FIGURE 9: p62 domains. p62 is recognized as a signalling hub due to the diversity of functional domains responsible for different signalling pathways. The 6 domains are: PB1, ZZ, TB, LIR, KIR and UBA. Figure reproduced from [93].

As mentioned, p62 has generally been considered a cytosolic protein and little attention has been paid to its possible nuclear functions. Pankiv *et al* [94] found that p62 continuously shuttles between nuclear and cytosolic compartments using two nuclear localization signal

domains (NLS1 and NLS2) and one nuclear export motif (NES) in its amino acid sequence (Figure 9) and the cytosolic distribution of p62 represents the steady state of this dynamic shuttling process. The authors also demonstrated that this rapid nucleocytoplasmic shuttling is regulated by the phosphorylation of p62 in residues close to the NLS2 site, strengthening the notion that this adaptor is a phosphoprotein even in resting cells. Interestingly, p38 kinase has been described as a binding partner and co-localized in the nucleus with p62 [95]. The biological requirement of this process relies on the fact that p62 behaves as a sensor of nuclear and cytosolic proteotoxic stress.

p62 also contains two PEST sequences rich in proline (P), glutamic acid (E), serine (S) and threonine (T) thought to be proteolytic signals for rapid degradation, leading to short intracellular half-lives [93].

The ZZ domain is an atypical zinc finger domain that is not yet well characterized. It is localized next to the PB1 domain and is responsible for binding to the receptor interacting protein-1 (RIP1) controlling nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation through the TNF-α signalling pathway [96]. Through TB and PB1 domains, p62 is able to control NF-κB activation (Figure 9). In 2009 [97], p62 was identified as a molecular target of innate defense regulator-1 (IDR-1), a synthetic peptide with no antimicrobial activity that enhances microbial infection control while suppressing inflammation. The authors showed that IDR-1 specifically binds to the ZZ domain of p62, selectively stabilized TNF-α, inducing p62-RIP1 complex formation, and specifically modulated the downstream signalling pathways by activating p38, suppressing TNF-α. Hence, p62 allows for efficient communication between the sensing incoming pathogens and the initiation responses preventing infection.

Autophagy acts to regulate cellular homeostasis and signalling by altering the availability of key adaptor proteins, such as p62. The functional relevance of p62 did not emerge until an association between p62 and LC3 was reported, highlighting a role for p62 in autophagy [98]. In this study, p62 was found to mediate the formation of protein aggregates destined for autophagic turnover, which suggested that p62 facilitated selective degradation of protein cargo via autophagy. It is now understood, as already stated above, that p62 works as an adaptor, binding ubiquitinated protein aggregates and delivering them to the autophagosomes. This function is possible due to the LIR domain [73], in p62 structure which interacts with multiple sites on LC3. This interaction is indispensable for autophagic

degradation of p62, although not for translocation of p62 to autophagosome formation site [35]. Therefore, the impairment of p62-LC3 interaction leads to the formation of ubiquitin and p62-positive aggregates. These data suggest that the pathways controlling the activation of autophagy tightly control p62 levels to prevent homeostasis from being disrupted during inflammatory responses.

p62 self-oligomerizes in a PB1 domain-dependent manner to promote packaging of ubiquitinated cargos and delivery of packaged cargos to the autophagy pathway. Wurzer *et al* [99] show that the oligomers are able to bind to many ubiquitin tags on a single structure, enabling a p62 oligomer to form a stronger connection to the damaged protein than a single p62 unit can. At the same time, the oligomer can interact with many LC3 molecules and upon binding to damaged structures, drive the bending of the membrane around these structures (Figure 10). These findings reveal how the formation of oligomers allows p62 to specifically select and target damaged proteins that are covered with many ubiquitin tags for destruction. Additionally, an independent study [100] demonstrated that p62 forms flexible helical polymers from a PB1 domain scaffold, with the C-terminal part of p62 attached to ubiquitinated targets and LC3, forming the template for membrane elongation due to the many available LIR domains in the polymer. These outcomes corroborate the observations that mutations in the PB1 domain interfere with p62´s ability to oligomerize, inhibiting the recruitment of p62 to the autophagosome formation site.

Within its C-terminus, p62 binds non-covalently to both mono- and poly-ubiquitinated proteins via its UBA domain. The isolated UBA domain of p62 has relatively low affinity for ubiquitin compared with other ubiquitin receptor proteins like NBR1 [101]. It was recently shown that cells evolved to enhance this interaction [102]. The authors found that p62 is ubiquitinated at a specific lysine (K420) within its UBA domain by the KEAP1/Cul3 E3 ligase complex. Substitution of K420 to arginine, deletion of the UBA domain or mutation of the KIR domain in p62, decreases ubiquitinated inclusion formation, its LC3 interaction, and cell viability. Therefore, the ubiquitination status of p62's UBA domain may alter p62's oligomeric structure, promoting p62's sequestration activity, subsequent phagophore assembly, and autophagic degradation.

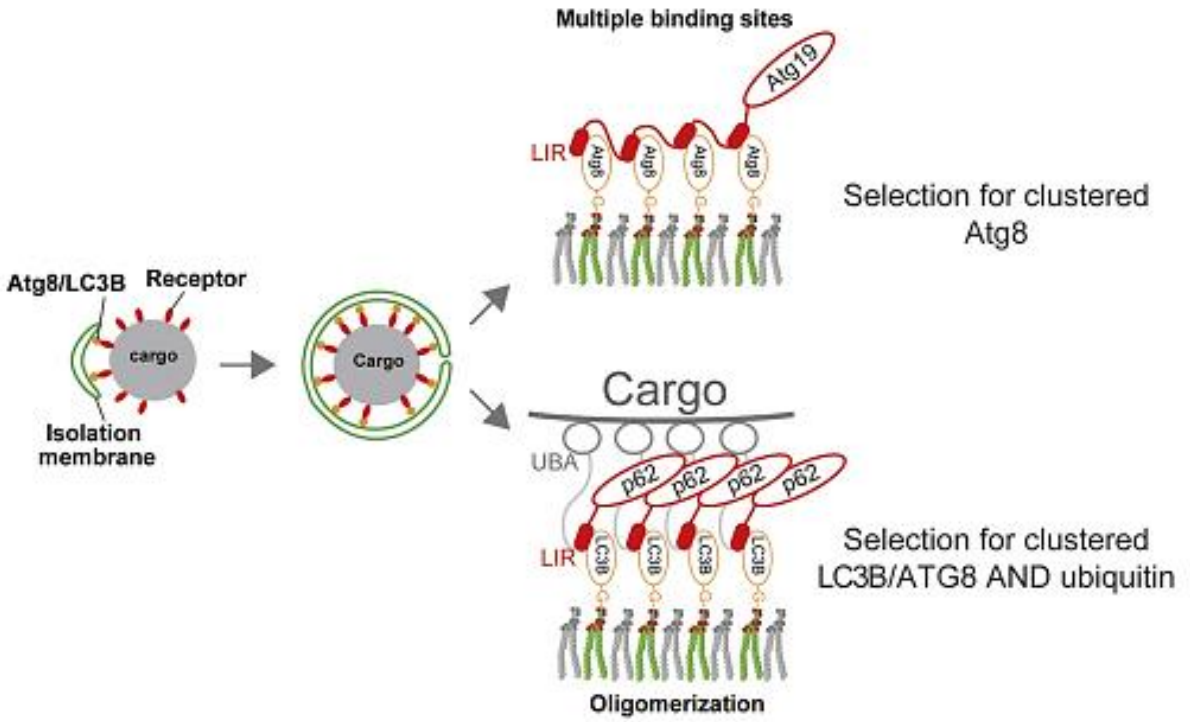

FIGURE 10: **p62 oligomerization to selectively target ubiquitinated cargo for degradation**. In this study, the authors have shown that the human cargo receptor p62 employs oligomerization to generate high-avidity interactions with ubiquitin and LC3B. Thus, oligomerization enables p62 to simultaneously select for concentrated ubiquitin and LC3B. The low affinity but high avidity interaction of the p62 oligomer with ubiquitin will select for these structures as ubiquitin is locally concentrated on them. Figure reproduced from [99].

Another well-known signalling pathway influenced by p62 is the oxidative stress response, which is regulated by the KEAP1-NRF2 system. Through its KIR motif, p62 is able to bind KEAP1, a Cullin3-ubiquitin E3 ligase complex adaptor protein. In turn, KEAP1-promoted polyubiquitination and subsequent proteasomal degradation of the transcription factor NRF2 are inhibited. Consequently, the expression of cytoprotective, antioxidant Nrf2 target genes is increased. Details on this non-canonical as well as canonical NRF2 signalling pathway are described in the next section.

p62 received more attention when mutations in its sequence were found in numerous patients with Paget's disease of bone (PDB) [103]. PDB is characterized by focal and disorganized increases in bone turnover, and primarily affects osteoclasts with an accumulation of inclusion p62-positive bodies in these cells. All of the mutations identified to date in PDB patients, are clustered either within or near the C-terminal region of the p62 protein that surrounds the UBA domain, and affect its interactions with multiubiquitin chains,

suggesting that an alteration of ubiquitin-chain binding by p62 promotes the development of PDB.

p62 is also a major component in Mallory-Denk bodies (MDBs), which are often associated with alcoholic and non-alcoholic steatohepatitis and p62-KO mice develop mature-onset obesity in addition to impaired glucose and insulin tolerance. As a scaffold multifunctional protein, p62 is required for the efficient and selective transmission of information during cell signal transduction, in particular, the inflammatory response. Hence, it is predicted that p62 may be a useful target for mechanisms impaired in different diseases. Based on this logic, a recent clinical trial [104] against solid tumours was conducted using a DNA vaccine encoding p62, called *Elenagen*. Interestingly, when patients with breast and ovarian cancer were pre-treated with *Elenagen*, they restored, at least partially, their sensitivity to conventional chemotherapy where it had previously failed. Remarkably, previous treatments in dogs markedly decreased the development of metastasis and suppressed chronic inflammation, which is a common feature of cancer, accelerating its progression and worsening its outcome. p62 is, therefore, a promising candidate for cell-specific targeting.

1.6. NRF2 Signalling Pathways

Human exposure to environmental toxins has been associated with the etiology of many diseases including cancer, cardiovascular and neurodegenerative disorders, sepsis and diabetes, all involving inflammation. Mammalian cells are frequently exposed to extrinsically and intrinsically generated toxic substances and to oxidative insults that disrupt their normal function by damaging nucleic acids, proteins, and membrane lipids. To overcome such insults, cells are equipped with elaborate defence systems. The basal level expression of detoxification enzymes and xenobiotic transporters appears to be sufficient to protect cells against low level stresses. The body also has the capacity to adjust to increasing levels of stress by upregulating the expression levels of a broad range of cytoprotective genes. The nuclear factor erythroid derived 2-like 2 (NRF2) is a potent transcription factor that plays a primary central role in the inducible cell defence system [105,106]. NRF2 is a member of the basic leucine zipper (bZIP) transcription factor subfamily featuring a cap´n collar motif (CNC) and contains six functional domains, known as Neh1–Neh6 (Figure 11), which are highly conserved across different species. The major regulatory domain is Neh2, which is located in the N-terminus of NRF2 and contains seven lysine residues used in ubiquitin conjugation as well as two binding sites, known as ETGE and DLG motifs as shown in Figure 11, that regulate NRF2 stability [107,108].

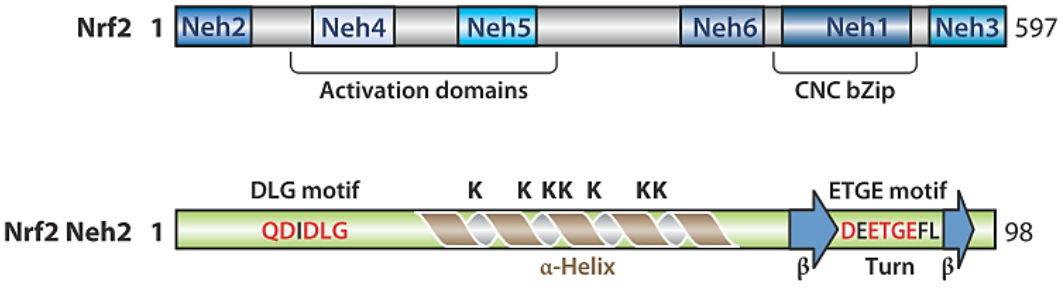

FIGURE 11: NRF2 structure. The transcription factor NRF2 is composed of 6 domains named Neh, and Neh2 is the major regulatory domain involved in the response against stress insults. Figure adapted from [108].

NRF2 target genes [109–111] include those involved in the regulation of the antioxidant response; of heme and iron metabolism; g for drug metabolizing enzymes; and metabolic genes. This so called “NRF2 battery”, which consists of over 100 genes, is continuously growing

as NRF2 activates a wide range of cellular defence processes, thereby enhancing the overall capacity of cells to detoxify and eliminate harmful molecules, maintaining cell homeostasis.

1.6.1. The Canonical NRF2-KEAP1 Signalling Pathway

NRF2 activation is regulated by the Kelch-like ECH-associated protein 1 (KEAP1) homodimer, an adaptor subunit of a Cullin 3 (CUL3)-based ubiquitin E3 ligase, which targets NRF2 constitutively for proteolytic degradation by the 26S proteasome, under basal conditions, in a stoichiometry of 2 KEAP1 :1 NRF2 molecule(s). KEAP1 possesses three domains (Figure 12): the BTB domain in the N-terminal which serves to dimerize KEAP1, enabling ubiquitin conjugation onto specific lysine residues located within the Neh2 domain of NRF2; the intervening region (IVR) rich in reactive cysteine residues, responsible for the interaction with Cullin 3 to promote NRF2 ubiquitination; and DC domain constituted by double glycine repeats (DGR or Kelch repeat) on the C-terminal region which binds to the Neh2 domain in NRF2. KEAP1 acts as a substrate adaptor to bring NRF2 into the E3 complex where the ubiquitin-conjugating enzyme (E2) binds to CUL3 C terminal region and catalyzes polyubiquitination of NRF2 protein on the lysine residues of Neh2 domain (see Figure 12).

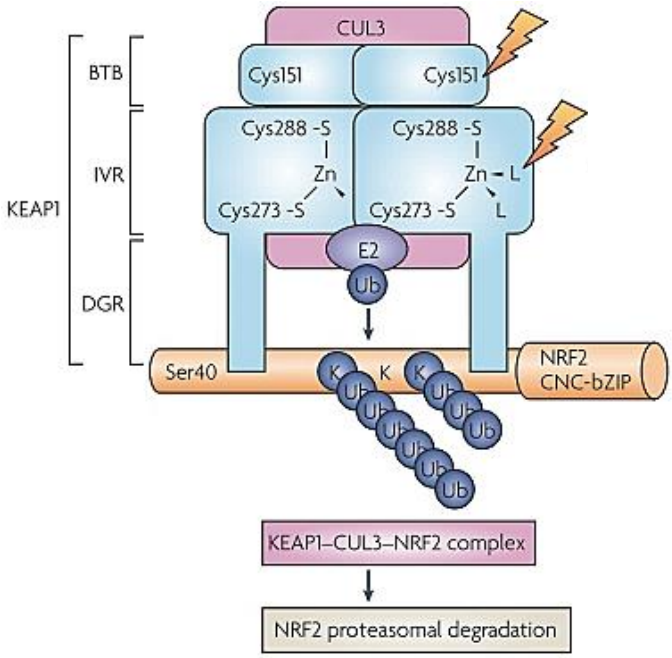

FIGURE 12: KEAP1 structure. KEAP1 is the negative regulator of NRF2 and its functional role relies on its peculiar structure. It contains different domains: Reactive cysteines in the BTB and IVR domains which are modified upon oxidative stress, releasing NRF2; DGR domain which binds to the Neh2 domain in NRF2; and interacts with CUL3, which is an E3 ligase targeting NRF2 to ubiquitination. Figure adapted from [112].

Although no crystal structure is available for NRF2, at basal conditions, it is known that the KEAP1 homodimer binds to the Neh2 domain of NRF2 in the cytoplasmic actin by means of two binding sites: the ETGE motif strongly binds to the Kelch KEAP1 domain and the DLG motif weakly binds to the other Kelch KEAP1 domain [113,114], as shown in Figure 13. Upon oxidative stress, a subset of cysteine residues in KEAP1 become oxidized and this modification changes the conformation of the complex, resulting in the inhibition of the ubiquitin E3 ligase activity of the KEAP1-CUL3 complex.

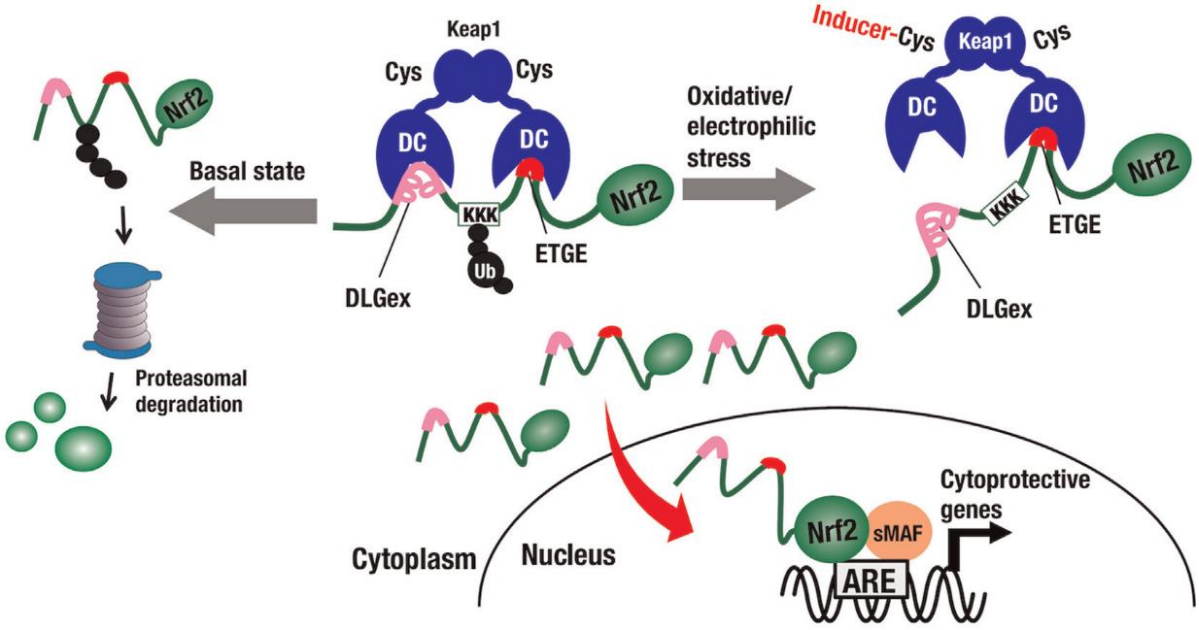

FIGURE 13: NRF2-KEAP1 signalling pathway. At steady state, KEAP1 binds NRF2 targeting this transcription factor for ubiquitination. Upon oxidative stress, KEAP1 is destabilized releasing NRF2 to the nucleus, inducing target gene transcription. Figure reproduced from[115].

Although NRF2 still binds one Kelch domain in KEAP1 through its ETGE motif, the disruption of the other interaction is sufficient to stop NRF2 ubiquitination in the lysine residues [113,116]. These findings led to a model named “cyclic sequential attachment and regeneration model of KEAP1-mediated degradation of NRF2” [117] where the authors found that the interaction between the NRF2 and KEAP1 relies on a unique close-open conformation, which is crucial for the signalling pathway activation. Basically, in the basal state, newly translated NRF2 binds sequentially to a free KEAP dimer, first through the ETGE motif to form the open conformation and then through the DLG motif, to form the closed conformation. Once in the closed conformation, NRF2 can be targeted for ubiquitination by the KEAP1-dependent E3-ubiquitin ligase. Ubiquitinated NRF2 is released from KEAP1 and degraded by

the proteasome. The free KEAP1 dimer is regenerated and able to bind to newly translated NRF2, and the cycle begins again. In the induced state, NRF2 is not released from KEAP1, free KEAP1 is not regenerated, and newly translated NRF2 accumulates and turns on the expression of cytoprotective genes. Inhibition of the proteasomal degradation of NRF2 by inducers blocks the cycle, leading to the accumulation of the KEAP1-NRF2 complex in the closed conformation. In the absence of new translation, NRF2 is not stabilized in the induced state, and NRF2-target genes are not upregulated, suggesting it is the *de novo* translated NRF2 which translocates to the nucleus, whereas the existing population of NRF2 remains bound to KEAP1 [117]. It is clear that within the numerous studies on NRF2-KEAP1 signalling pathway that KEAP1 is crucial for the proteasomal degradation of NRF2 and that its half-life is somehow dependent on the redox state of the cell, and is considered a negative regulator of this transcription factor.

When reaching the chromatin in the nucleus, NRF2 forms a heterodimer with small masculoaponeurotic fibrosarcoma (MAF) basic leucine zipper transcription factors and this complex is targeted specifically to the “NRF2 battery” of genes. These genes share a common DNA regulatory sequence in their promoters, named the antioxidant response element (ARE), where NRF2 binds to activate their expression [118,119]. As listed in Table I, these genes provide different functions in cellular regulation. Importantly, NRF2 has been shown to possess an ARE sequence within its promoter region providing a platform for NRF2 to initiate its own transcription, further enhancing the adaptive cell defence response [120]. Supplementary to its primary role in cytoprotection, NRF2 is also linked to differentiation, proliferation, growth, apoptosis and it is thought that NRF2 has evolved from an original role in hematopoiesis and regulation of cell differentiation from early lineages [121].

TABLE I: NRF2-target genes

General Classification	Genes	Symbol	Function	REF.
A. Antioxidants	*Peroxiredoxin 1*	*Prdx1*	Signal peroxidase to receive, transduce and transmit peroxide signals	[122]
	Thioredoxin 1	*Txn1*	Involved in the first unique step in DNA synthesis; oxidoreductase activity	[123]
	Hemoxigenase1	*Ho-1*	Breakdown of heme into biliverdin, iron and carbon monoxide	[124]
	Superoxide dismutase 2	*Sod2*	Mitochondria localization; catalysis of superoxide anion to hydrogen peroxide	[125]
	Superoxide dismutase 1	*Sod1*	Cytoplasm and intermembrane mitochondrial space localization; catalysis of superoxide anion to hydrogen peroxide; nuclear transcription factor to regulate oxidative stress resistance	[126]
	Gluthatione Peroxidase 1	*Gpx1*	Reduces lipid hydroperoxides to their corresponding alcohols; reduces free hydrogen peroxide to water	[127]
	Gluthatione Reductase	*Gsr*	Converts oxidized glutathione to two molecules of reduced gluthatione	[127]
	Catalase	*Cat*	Catalysis of hydrogen peroxide to water and oxygen	[126]
B. Xenometabolism (detoxification)	*Glutathione S-transferase*	*Gst*	Catalysis of the conjugation of electrophilic substrates to glutathione	[106]
	NAD(P)H: quinone oxidoreductase 1	*Nqo1*	Catalyze the two-electron reduction of quinones; their derivatives and direct scavenging of superoxide anion radicals	[106]

C. Autophagy	*p62*	*p62*	Adaptor protein in selective autophagy; participates in numerous signalling pathways	[128]
	Nuclear dot protein 52	*Ndp52*	Adaptor protein in selective autophagy	[129]
	UNC51-like kinase-1	*Ulk1*	Regulates the formation of the autophagophore	[130]
	Autophagy-related protein 7	*Atg7*	Autophagosome formation	[130]
	Autophagy-related protein 5	*Atg5*	Autophagosome elongation	[130]
D. Inflammation	*Interleukin 6*	*Il6*	Pro-inflammatory cytokine released by macrophages	[131]
	Interleukin 1b	*Il1b*	Considered the prototypic 'multifunctional' cytokine, affecting nearly all cell types, either alone or in combination with other cytokines; involved in the inflammasome protein complex	[131]
	Interleukin 12b	*IL12B*	Regulates inflammation by linking innate and adaptive immune responses; potent inducer of antitumor immunity in preclinical models.	[131]
	Cluster of differentiation	*Cd36*	Scavenger receptor required for oxidized LDL phagocytosis	[122]
	Macrophage receptor with collagenous structure	*Marco*	Scavenger receptor required for bacteria phagocytosis	[132]
E. Heme Metabolism	*Hemeoxigenase1*	*Ho-1*	Breakdown of heme into biliverdin, iron and carbon monoxide	[124]
	Ferritin	*Fth*	Stores Iron and has ferroxidase activity	[133]
	Ferroportin	*Fpn*	Non-heme iron exporter outside the cell	[134] [135]
	Heme responsive gene 1	*Hrg1*	Transports heme from the lysosome into the cytoplasm	[136]

NRF2 contains multiple nuclear localization signals (NLSs) which is known to facilitate NRF2 nuclear localization upon addition of ARE inducers [137]. Due to the larger size of the NRF2 protein compared to the size limit for diffusion through the nuclear pore complex, it is expected that during nuclear translocation, the nuclear localization signals (NLS1, NLS2, NLS3) of NRF2 molecules are recognized in the cytoplasm by soluble adaptor proteins termed importins, which upon binding, result in complexes that are transported through the nuclear pore to the nucleoplasm. These findings disagree with some authors who claim that NRF2 is primarily a nuclear protein and this nuclear localization is responsible for the basal expression of cell defence genes and that the degradation of NRF2 via KEAP1 is downstream of NRF2 transcriptional activity [138,139].

1.6.2. The Non-Canonical NRF2 Signalling Pathways

Although the most studied mechanism of NRF2 activation relies on KEAP1 cysteine oxidation where the oxidant sensor function of this protein is primarily to slow the ubiquitination and subsequent degradation of NRF2 at higher levels of oxidative stress, it has been demonstrated that alternative pathways are also involved. At a transcriptional level, it was found that there is a binding domain for NF-κB in the NRF2 promoter region, suggesting that the former could be involved in the regulation of the latter [140]. Interestingly, KEAP1 also negatively regulates the NF-κB signalling pathway [141]. NRF2 itself may contain an oxidant sensor that facilitates nuclear translocation, but that function remains poorly defined [142,143]. Furthermore, as NRF2 contains many serine, threonine and tyrosine residues providing sites for phosphorylation by different kinases, it was proposed that this modification might be involved in its nuclear exclusion and degradation. Protein kinase C has been shown to phosphorylate NRF2 at serine 40 in the Neh2 domain that binds to KEAP1, which appears to be an important event in the release of NRF2 from KEAP1 with further translocation to the nucleus [144] and it seems more likely that kinase signalling pathways are nearly always involved in the migration of NRF2 to the nucleus.

More recently, a new mechanism regulating NRF2 function independently of the redox state of cells has been studied although inducing the expression of antioxidant and detoxification genes. This promising non-canonical pathway of NRF2 regulation involves the action of autophagy mediators and KEAP1 – **p62-dependent NRF2 activation**. As mentioned

before, the p62 protein acts both as an adapter or scaffold protein in cellular signalling pathways and as a cargo receptor for degradation of ubiquitinated targets by autophagy. It has been shown that excessive accumulation of p62 due to a defect in autophagy causes an increase in oxidative stress, which can potentially lead to deregulation of several signal transduction pathways and altered gene expression [145]. Previous genetic studies on the autophagy-essential protein ATG7 also showed that loss of autophagy caused a marked accumulation of p62 along with robust induction of antioxidant genes, including *Nqo1* and *Gst* [98].

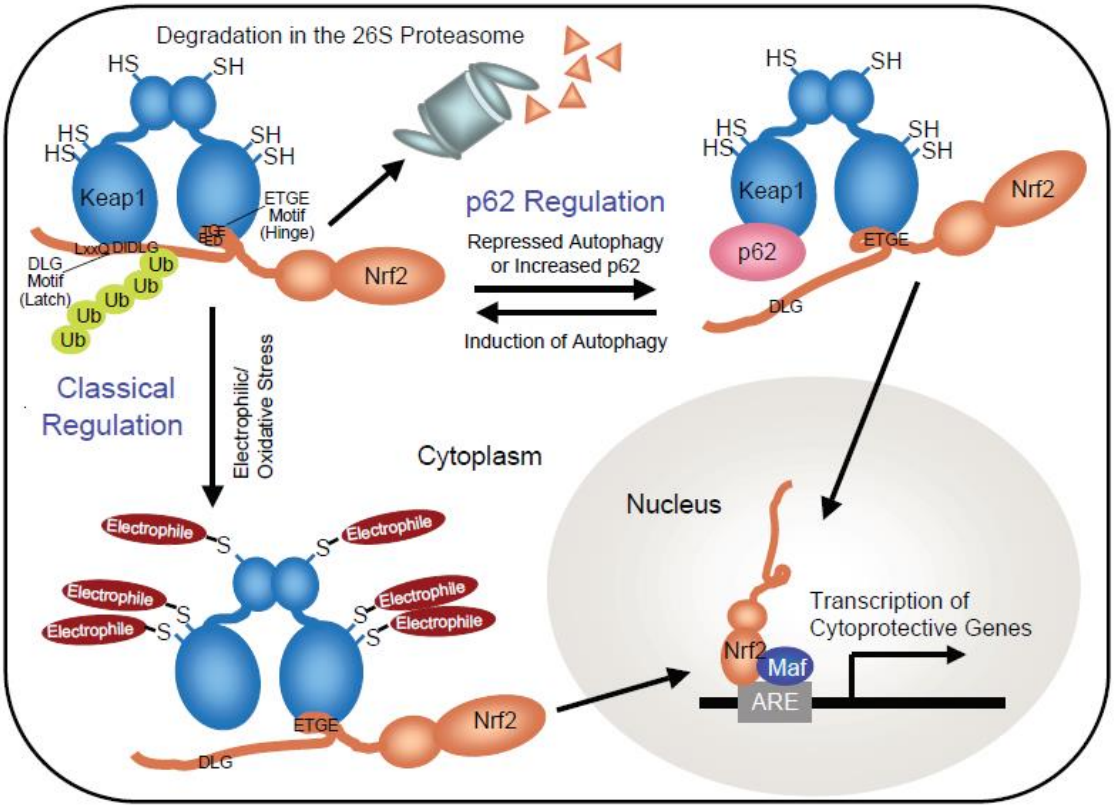

FIGURE 14: Comparison between the canonical and non-canonical NRF2-KEAP1 pathway. NRF2 translocation to the nucleus with subsequent transcription of cytoprotective genes can be achieved in two ways: upon oxidative stress, one KEAP1 domain detaches from NRF2 avoiding proteasomal degradation; and/or p62 can bind to KEAP1, releasing NRF2 with subsequent translocation to the nucleus. Figure reproduced from [146].

Komatsu *et al* [146] and Lau *et al* [147] reported the link between the KEAP1-NRF2 axis and autophagy by confirming the interaction between p62 and KEAP1. Through immunoprecipitation assays and the construction of several mutants, the authors found that the KEAP1-DC domain, but not the DGR domain, bound to both p62 and NRF2 suggesting that

the folded structure of KEAP1 is essential for its molecular recognition of p62, as is the case for NRF2. Additionally, the authors revealed that the interactions between the KEAP1-DC domain and p62-KIR heavily overlap with the interactions between the KEAP1-DC domain and NRF22-ETGE, strongly supporting the notion that p62-KIR binds to KEAP1 in a manner very similar to that of the NRF2-ETGE and NRF2-DLG motifs. Unlike NRF2, the binding stoichiometry between p62 and KEAP is 1:1, meaning that p62 binds to one KEAP1 molecule as shown in Figure 14. Interestingly and according to this result, p62 overproduction led to a marked decrease in NRF2 ubiquitination and consequent NRF2 stabilization, suggesting that p62 inhibits NRF2-DLG, but not NRF2-ETGE, from interacting with Keap1 (Figure 14). Consequently, it was shown an increase in the NRF2-target genes in the presence of a wild type-p62 compared to a mutant-p62, validating what was shown previously, that the presence of p62 by sequestering KEAP1 leads to the NRF2 stabilization and nuclear translocation.

In the same year, Jain *et al* [128] showed that NRF2 can induce *p62* expression by binding directly to a conserved ARE in its promoter/enhancer and that the p62 protein is able to augment its own expression via this element. Specifically, similarly to what Komatsu *et al* published, they found stimulation of NRF2 activity by binding to kelch domain on KEAP1 through the p62 KIR domain, sequestering it, and directing its degradation by autophagy. This p62 and KEAP1 association leads to stabilization of NRF2, enabling the transcription factor to induce gene expression from ARE-containing promoters, maintaining a positive feedback loop in the p62-NRF2 pathway. They also observed that KEAP1 is recruited to p62 inclusion bodies upon its overexpression and this sequestering of KEAP1 into p62 bodies may result in the displacement of KEAP1 from locations where it would otherwise interact with CUL3 and promote the ubiquitination and turnover of NRF2.

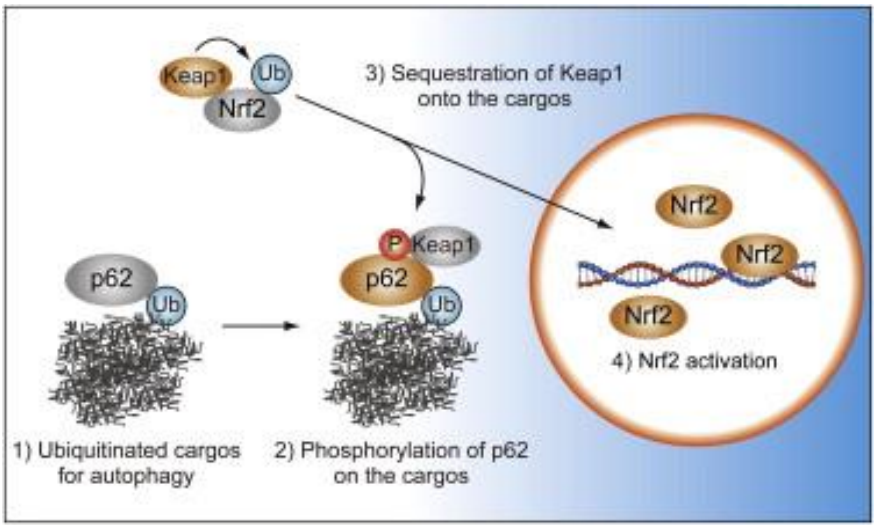

FIGURE 15: p62 phosphorylation. p62 phosphorylation enhances NRF2 translocation to the nucleus and therefore the induction of NRF2-target genes, independently of the canonical or non-canonical NRF2 signalling pathway. Figure reproduced from [148].

Interestingly, recent data [148,149] indicated that the KEAP1-NRF2 and selective autophagy pathways are connected to each other through the phosphorylation of p62 on two serine residues: S403 and S351. In normal cells, this functional interaction serves as a host defense mechanism (for a general view, see Figure 15), leading to the expression of antioxidant and anti-inflammatory enzymes and the subsequent selective clean up of cytotoxic structures concomitant with degradation of phosphorylated p62 and the KEAP1 complex. In the presence of ubiquitinated autophagic cargos (e.g., ubiquitinated protein aggregates, damaged mitochondria, and invasive bacteria – Figure 16), the S403 residue of the p62-ubiquitin-binding domain is initially phosphorylated by a specific kinase, which promotes the translocation of p62 to microbes, positive for ubiquitin. Then, p62 oligomerization occurs and this process is crucial for the phosphorylation of S351 in the p62-KIR domain to occur, in an mTORC1-dependent fashion. Furthermore, p62's affinity for KEAP1 increases significantly, followed by sequestration of KEAP1 on cargos. As a result, NRF2 is stabilized and translocates into the nucleus to induce cytoprotective NRF2 targets expression. The ubiquitinated cargos, together with phosphorylated p62 and the KEAP1 complex, are degraded by autophagy, leading to the elimination of cytotoxic components. However, there is a dual role of this phosphorylation process as this process also satisfies the metabolic demand of tumours to ensure tolerance of microenvironmental stress once tumours have attained a high level of proliferative activity, specifically in human hepatocellular carcinomas

(HCC), where S351 phosphorylation of p62 occurs constitutively. As a result, HCC acquires not only intact autophagic activity as a supplier of nutrients, but also constitutive activation of NRF2, thereby fulfilling the necessary conditions for tumour progression. In this case, the overexpression of p62 is sufficient for S351 phosphorylation, in an NRF2-dependent manner which is activated in malignant tumours [150]. Thus, activated Nrf2 induces p62 expression, leading to a vicious circle that promotes tumour progression. It is also known that autophagy has a significant impact on NRF2 activation, as p62 not only disrupts the KEAP1-NRF2 interaction but also removes KEAP1 from the cytosol via selective autophagy.

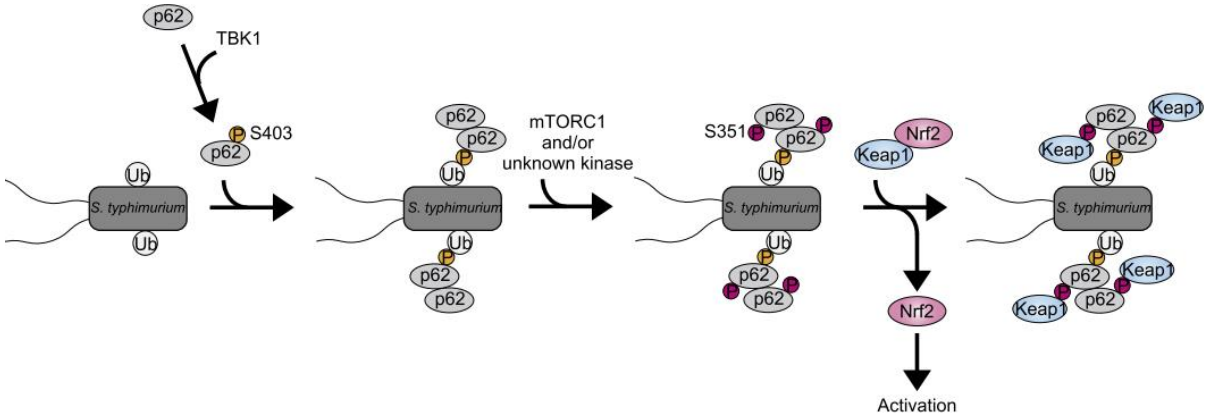

FIGURE 16: Sequential steps of NRF2 activation during xenophagy. After autophagic sequestration of pathogens and subsequent ubiquitination, p62 is phosphorylated on the S403 residue by the TANK-Binding Kinase 1 (TBK1) and binds to ubiquitin. Then p62 is again phosphorylated on the S351 residue enhancing the affinity for KEAP1, releasing NRF2 and its translocation to the nucleus. The pathogen is degraded by autophagy and NRF2-target genes are induced. Figure reproduced from [149].

Regarding this and previous observations, is intriguing that, whereas NRF2 is predominantly degraded through the proteasome pathway, KEAP1 is degraded through the autophagy pathway and this pathway maintains the integrity and homeostasis of the KEAP1–NRF2 system by governing KEAP1 turnover.

2. Aims of the Work

Senescence of red blood cells and subsequent clearance by erythrophagocytes are crucial biological processes that occur after travelling 120 days around the human body allowing for iron recycling and storage. Additionally, this premature internalization of erythrocytes avoids hemolysis and the release of free heme to the circulation where it could exert deleterious effects. Therefore, erythrophagocytosis must be successfully efficient and immunologically silent in order to maintain cellular homeostasis.

There is a lack of information regarding the molecular players involved in the removal of senescent and damaged RBC. Therefore, with this work we proposed to:

1. Address the maturation kinetics of phagosomes containing senescent/damaged RBC compared to IgG- and complement-opsonized RBC, the most known phagocytic models, in murine macrophages;

2. Identify the molecular machinery involved in erythrophagolysosome biogenesis;

3. Unveil the role of these molecular players involved in RBC degradation and how they are regulated.

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ez&rendertype=abstract

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CHAPTER II

Maturation of Phagosomes Containing Different Erythrophagocytic Particles by Primary Macrophages

This chapter is formatted based on the following manuscript submitted during the PhD experimental work:

- **Maturation of phagosomes containing different erythrophagocytic particles by primary macrophages**

In this work, Inês B. Santarino has carried out all the experiments and participate in the interpretation and discussion of the results, as well as the writing of the manuscript.

In the Material and Methods section of this manuscript, an extensive detailed description will be presented.

Maturation of phagosomes containing different erythrophagocytic particles by primary macrophages

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ABSTRACT

Erythrophagocytosis is a physiological process that aims to remove damaged red blood cells from the circulation in order to avoid hemolysis and restore the levels of iron inside the cells. Many efforts have been made to understand heme trafficking inside macrophages, but little is known about maturation of phagosomes containing different erythrophagocytic particles with different signals at their surfaces. Therefore, we performed a comparative study on the maturation of phagosomes containing three different models of red blood cells (RBC): aged/senescent, complement- or IgG-opsonized and using two types of professional phagocytes, bone marrow-derived and peritoneal macrophages. By comparing markers from different stages of phagosomal maturation, we have found that phagosomes carrying the aged RBC reach lysosomes with a delay compared to those containing IgG- or complement-opsonized RBC in both type of macrophages. These findings can contribute to understand the importance of the different signals at RBC surface in phagolysosome biogenesis as well as in dynamics of RBC removal.

INTRODUCTION

Red blood cells/erythrocytes (RBC) are the cellular components of blood which undergo an accurate maturation process losing their nucleus and organelles, although accumulating hemoglobin. After they are produced in the bone marrow, normal human RBC remain in the circulation for roughly 120 days before removal. The removal of aged/senescent RBC is conducted by macrophages of the reticuloendothelial system, found in the spleen, liver and bone marrow, through a process termed erythrophagocytosis [1]. This highly controlled and coordinated process is responsible for RBC degradation within the phagolysosome resulting in the breakdown of hemoglobin and the release of heme into the cytosol where its catabolism takes place leading to iron release [1]. The rapid removal of RBC from the circulation is extremely important for maintenance of iron/heme homeostasis, as the majority of iron required to sustain erythropoiesis is derived from senescent RBC and free iron is a strong pro-oxidant agent [2]

While aging, RBC undergo several metabolic and physical modifications such as: 1) membrane vesiculation disposing non-functional membrane patches [3] 2) oxidation-induced modifications of hemoglobin and membrane protein band 3 [4] energy depletion [5] 3)

progressive cell shape transformation and [6] 4) membrane remodelling such as the exposure of surface removal markers such as phosphatidylserine (PS) [7]. Directly or indirectly, these modifications trigger erythrophagocytosis.

It has been described that the central step in the clearance of RBC relies on the interaction between macrophage receptors and the protein band 3 that upon hemoglobin oxidation undergoes clustering promoting generation of epitopes on RBC cell surface. This provides a signal favouring immunologic recognition of redistributed band 3 by autologous IgG [8,9]. Nevertheless, the immunoglobulins formed are not efficient opsonins, due to their low affinity and low circulation numbers. For this reason, it has been hypothesized that phagocytosis of RBC can be enhanced by the activation of the classical pathway of the complement system after IgG binding [10]. These immunoglobulins preferentially generate C3b2–IgG complexes in the presence of active complement [11]. Furthermore, prior to senescence, RBC may enter eryptosis, a form of stress inducing programmed cell death resembling apoptosis in nuclear cells, characterized by RBC shrinkage and cell membrane scrambling with translocation PS from the inner leaflet to the outer leaflet of the membrane [12]. Despite some *in vivo* observations of PS exposure upon RBC ageing, there is no convincing evidences for the involvement of PS in physiological removal of aged RBC [13,14]. Thus, this topic is still under debate. However, very recently it was shown that senescent RBC are more prone to PS exposure upon oxidation than young RBC [15].

Since the bulk of iron required for the synthesis of new hemoglobin derives from RBC recycling, understanding phagocytosis of RBC is critical. Furthermore, in some pathologies such as chronic kidney disease and sepsis, erythrophagocytosis is altered due to RBC lifespan decrease and eryptosis increase, respectively [16][17]. Thus, it is fundamental to uncover details of the clearance of RBC as well as the role of different signals at their surfaces such as IgG, complement and PS in this process. Therefore, in the present study we aimed to address the interaction of different erythrophagosomes containing IgG- or complement- opsonised RBC or PS-enriched RBC (aged RBC) with components of the endocytic pathway namely early endosomes and late endocytic compartments. For that, we used primary mouse macrophages from bone marrow and peritoneal cavity. The taking home message is that phagosomes containing agRBC mature slower than the other models in primary mouse macrophages and this information can be important in pathologies with changes in RBC clearance.

MATERIAL AND METHODS

Cell Culture

Mouse Fibroblasts Clone 929 - L929 cell line - (kindly provided by Prof. Ira Tabas, Columbia University, NY, USA) were cultured in order to produce L-cell Conditioned Media (LCCM) enriched in mouse colony-stimulating factor (M-CSF) responsible for differentiating monocytes into macrophages [18]. Cells were maintained in DMEM high glucose supplemented with 10% of heat inactivated (HI)-FBS, 1% P/S, 1% sodium pyruvate and 1% HEPES. Cells were cultured for 7 days after which the culture media (LCCM) was harvested and filtered using a vacuum filter (Sarstedt, Nümbrecht, Germany) and stored at -80°C for long-term storage or -20°C for short-term storage.

Bone marrow-derived macrophages (BMDM) were obtained from 8-12 week old C57BL/6 wild-type mice and maintained for 7 days as described before [19]. Bone marrow was isolated from femurs and tibias, previously handled in aseptic conditions. Bones proximal to each joint were cut with a razor blade followed by flushing of the bone cavity with HBSS until it appeared white. After centrifugation, supernatant was discarded and total cells from the bone marrow were counted. $4x10^5$/mL cells were seeded in bacterial petri dishes in RPMI 1640 (with glutamine) media, 10% HI-FBS, 1% P/S and 30% LCCM. After 3 days, fresh media with P/S and 30% LCCM was added to the culture. At day 6, the cell suspension was completely removed and replaced by fresh media supplemented with 30% LCCM. On day 7, adherent macrophages were removed by gently scraping. After centrifugation, the supernatant was discarded and cells were counted and plated according to the experiment.

Peritoneal cells were collected from mice by flushing the peritoneal cavity with 30 mL ice cold Phosphate-Buffered Saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, 10 mM $NaHPO_4.2H_2O$, pH 7.4) [19]. Recovered cells were washed in DMEM (high glucose) supplemented with 20% HI-FBS and 1% P/S for 10 min, at 500 x *g*, 4°C and plated ($3x10^5$ cells/well) in coverslips in 24-well plates. After 2 h incubation, non-adherent cells were removed and the adherent cells were washed with warm sterile PBS followed by replacement with fresh DMEM. At 1st and 4th day, adherent peritoneal macrophages were washed with fresh DMEM and at day 5, the experiments were performed.

8-12 weeks old C57BL/6 mice (male and female) were purchased from Charles River Laboratories (Wilmington, MA, USA) and maintained under specific pathogen-free (SPF)

conditions according to protocols approved by the national (Portuguese Official Veterinary Department; *Direcção Geral de Veterinária*) ethics committees according to the Portuguese (Decreto-Lei 113/2013) and European (Directive 2010/63/EU) legislations.

Preparation of the different phagocytic particles

Human blood was collected into sodium heparin tubes (Greiner Bio-One, Kremsmünster, Austria) from healthy volunteers who signed consented forms approved by the Ethical Review Board of the Faculty of Medicine of the University of Coimbra and by the NOVA Medical School, New University of Lisbon. Sheep blood was collected from healthy sheep from Escola Superior Agrária de Coimbra and all protocols authorized by the national (Portuguese Official Veterinary Department; Direcção Geral de Veterinária) ethics committees according to the Portuguese (Decreto-Lei 113/2013) and European (Directive 2010/63/EU) legislations. Both human and sheep RBC (shRBC) were isolated [20] in aseptic conditions under a cell culture hood, using a Ficoll-Paque (GE Healthcare Life-Sciences, PA, USA) gradient. After centrifugation at 400 *g* for 30 min at 4ºC the RBC were located in the bottom of the centrifuge tube. Then RBC were washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, 10 mM $NaHPO_4.2H_2O$, pH 7.4) and finally resuspended in PBS supplemented with 0.1% glucose (20%, v:v). RBC in suspension were kept at 4ºC and used as native RBC.

Human RBC were aged (agRBC) in order to expose phosphatidylserine from the membrane to mimic what occurs physiologically in the human body during RBC turnover. After removing the glucose, RBC were incubated for 4 days at 37°C [20].

IgG-opsonized shRBC were opsonized as followed: fresh shRBC were fixed with 4% paraformaldehyde/sucrose (Sigma-Aldrich) and incubated at room temperature (RT) for 2h30 in the orbital rotator. A 10% shRBC suspension was mixed with phagocytosis media (CO_2-independent RPMI 1640 without serum) and a rabbit anti-sheep RBC at a ratio 1:50 (IgG:shRBC suspension). Opsonization was performed ON at 4°C in the orbital rotator.

C3bi-opsonized shRBC were prepared according to the protocol previously described [21]. 10% shRBC suspension was mixed with PBS and 180 ng/mL rabbit anti-sheep IgM (Cedarlane, Burlington, NC, USA) and incubated at RT for 1 h in the orbital rotator. Next, IgM-shRBC were washed with phagocytosis media to which C5-deficient human serum (Sigma-Aldrich) was added for a final concentration of 10% v/v. The mixture was incubated for 20 min at 37°C, in the thermomixer at 300 rpm. Under these conditions, the Fc region of the IgM pentamer

activates the classical pathway of the complement cascade and deposits C3b to the IgM-shRBC where it is rapidly converted to C3bi.

The day before phagocytosis assays, agRBC, IgG-opsonized RBC and C3bi-RBC were labeled with the vital dye CarboxyFluorescein Succinimidyl Ester (CFSE) according to the CellTracer™ CFSE Cell Proliferation Kit (Thermo Fisher Scientific Inc.) allow visualization under the microscope. CFSE was incubated with agRBC to a final concentration of 5 μM, for 15 min to allow crossing the membrane and extra 30 min to be metabolized. In the case of IgG-opsonized RBC these particles were incubated with CFSE prior to fixation with paraformaldehyde.

Phagocytosis and phagosomal maturation assays

Before the experiments, C3bi-shRBC, IgG-shRBC, IgG-opsonized beads and agRBC were washed twice with modified-RPMI 1640. All the phagocytic cells were also washed three times with modified-RPMI 1640 to remove cell debris and FBS to avoid the interference of other components.

Phagocytosis of agRBC and IgG-RBC was synchronized by keeping the macrophages together with the phagocytic particles on ice for 7 min before shifting the cells to 37°C for engulfment to occur.

For complement-mediated phagocytosis, C3bi-opsonized particles were incubated for 20 min at 37°C with macrophages to allow their specific bind to the surface receptors, CR3/CR1. Finally, Phorbol 12-myristate 13-acetate was added to a final concentration of 150 ng/mL for 15 min (BMDM pulse time) or 20 min (PM pulse time), which are the optimized time points for engulfment in these two different types of macrophages. Phagocytosis time in the two types of macrophages is different because the phagocytic capacity of the BMDM is higher compared with that of PM [22]. In all phagocytosis assays four RBC were added per macrophage. After phagocytosis, non-internalized RBC were removed by extensively washing or by hemolysis with deionized water and chased at 37°C for the time points referenced in the graphs abscissa, to follow phagosomal maturation.

Immunofluorescence and microscopy

After pulse-chase experiments, cells were fixed with 4% PFA for 30 min and washed with ice cold PBS before staining. For early endosome antigen 1 (EEA-1) staining, cells were permeabilized with 0.1% Triton X-100/ 200 mM glycine (Sigma-Aldrich) in PBS for 30 min and

treated with 50 mM ammonium chloride for 15 min to avoid autofluorescence. For LAMP-1 immunostaining, permeabilization was performed with cold methanol (previously place at -20°C for 10 min, instead of Triton X-100, as this agent destroys the antibody epitope). After washing with ice cold PBS, cells were incubated with blocking solution (Gelatin from cold water fish, Sigma-Aldrich) for 30 min. Primary and secondary antibodies used are listed in Tables I and II as well as their specificities. All primary antibodies were diluted in blocking solution and secondary antibodies in PBS.

TABLE II. List of primary antibodies used for immunostaining.

Antibody and host species	**Dilution**	**Incubation Time**	**Company; Reference**
Rat anti-LAMP-1	1:50	2 h at RT	Developmental Studies Hybridoma Bank; 1D4B
Mouse anti-EEA-1	1:50	1 h at RT	Sigma; E7659

Table III. List of secondary antibodies used for immunostaining.

Antibody and host species	**Dilution**	**Incubation Time**	**Company; Reference**
Cy3 Donkey anti-rat	1:500	1 h at RT	Jackson ImmunoResearch; 712-175-153
Cy3 Donkey anti-mouse	1:500	1 h at RT	Jackson ImmunoResearch; 715-165-150

Finally, stained coverslips were washed twice with ice cold PBS and once with dH_2O and mounted with Mowiol/DABCO (Merck Millipore, Oeiras, Portugal/Sigma-Aldrich). Images were acquired by a Carl Zeiss LSM 710 META laser scanning confocal microscope (ZEN software, Germany) using a 63x oil immersion objective N/A = 1.30, the argon laser (488 nm), DPSS 561-10 (561 nm) and spectral detection adjusted for the emission of the Cy3-fluorophores. Digital images were analyzed by using LSM Image Browser or Image-J software.

A phagosome was considered positive for a given marker when a fluorescent ring or a dotted patch was observed around the engulfed particle.

Statistical analysis

Statistical analysis (Two-way ANOVA followed by Bonferroni post-test) was performed using the GraphPad PRISM software version. 5.0. p <0.05 (*); p < 0.01 (**) and p <0.001 (***) were considered to be statistically significant. All results are presented as means ± standard error of the mean (SEM), from at least three independent experiments.

RESULTS AND DISCUSSION

Erythrophagocytosis is one of the main physiological processes responsible for the maintenance of tissue homeostasis. After recognition by specific receptors on macrophages membrane surface, targets are internalized culminating in the formation of a membrane-bound vacuole termed the phagosome, resembling the plasma membrane in terms of lipid and protein composition and its fluid-phase contents are a reflection of the extracellular milieu. Therefore, these nascent phagosomes are not capable to degrade cargo. However, soon after vacuole scission, there are a series of biochemical modifications that convert the nascent phagosome into a degradative organelle through an ordered choreographed succession of events including membrane fusion and fission between the phagosomal membrane and the components of the endocytic pathway – process known as phagosomal maturation [23–25].

In this work, we used human and sheep RBC to create different models of non-opsonized and opsonized RBC. agRBC were used as a model of senescent/eryptotic cell which the main signal for removal by primary phagocytes is the exposure of PS, as fully described before [20]. RBC were also opsonized with C3bi to mimic complement-mediated erythrophagocytosis and IgG to mimic Fc-mediated erythrophagocytosis, both opsonins being involved in the *in vivo* removal of senescent erythrocytes [26–28]. Because continuous subculture of cell lines may cause gene loss and impair macrophage immune functions, as phagocytic cells we decided to use two types of primary cells: bone marrow-derived macrophages and unstimulated peritoneal mouse macrophages. *In vivo*, the removal of damaged RBC is mainly carried out by the “mononuclear phagocyte system” cells of the bone marrow and under pathological conditions by tissue-resident macrophages in the spleen and liver [29–32]. However, the

majority of the *in vitro* studies of erythrophagocytosis have been performed in primary cultures of macrophages derived from bone marrow and peritoneal macrophages [33–38]. PM do not have a physiological role in the removal of damaged RBC however these phagocytic cells are among the best-studied macrophage populations in terms of cell biology and also in the identification of the molecular mechanisms involved in the removal of senescent RBC *in vitro* [33,35].

To assess the initial steps of phagosomal maturation, that is, the interaction between the nascent phagosome and the early components of the endocytic pathway, we followed the recruitment of the Rab5-effector EEA-1 (early endosome antigen 1), responsible for tethering early endosomes to nascent phagosomes, by immunofluorescence, for all the phagocytic particles, in BMDM and PM.

As observed in the Figure 1A, in BMDM there was a decrease with time of EEA-1 association with all the RBC-containing phagosomes, meaning that this effector interacts transiently with the phagosomal membranes. However, the loss of EEA1 in phagosomes with opsonised RBC was faster than in phagosomes with agRBC. At 15 min chase the higher percentage of EEA1-positive phagosomes was observed for phagosomes containing agRBC (50.07 ± 0.92 %) versus 14.67 ± 6.70 % and 11.47 ± 8.57 % for IgG-RBC and C3bi-RBC, respectively (Fig.1A and visualized in Fig. 1 B-D).

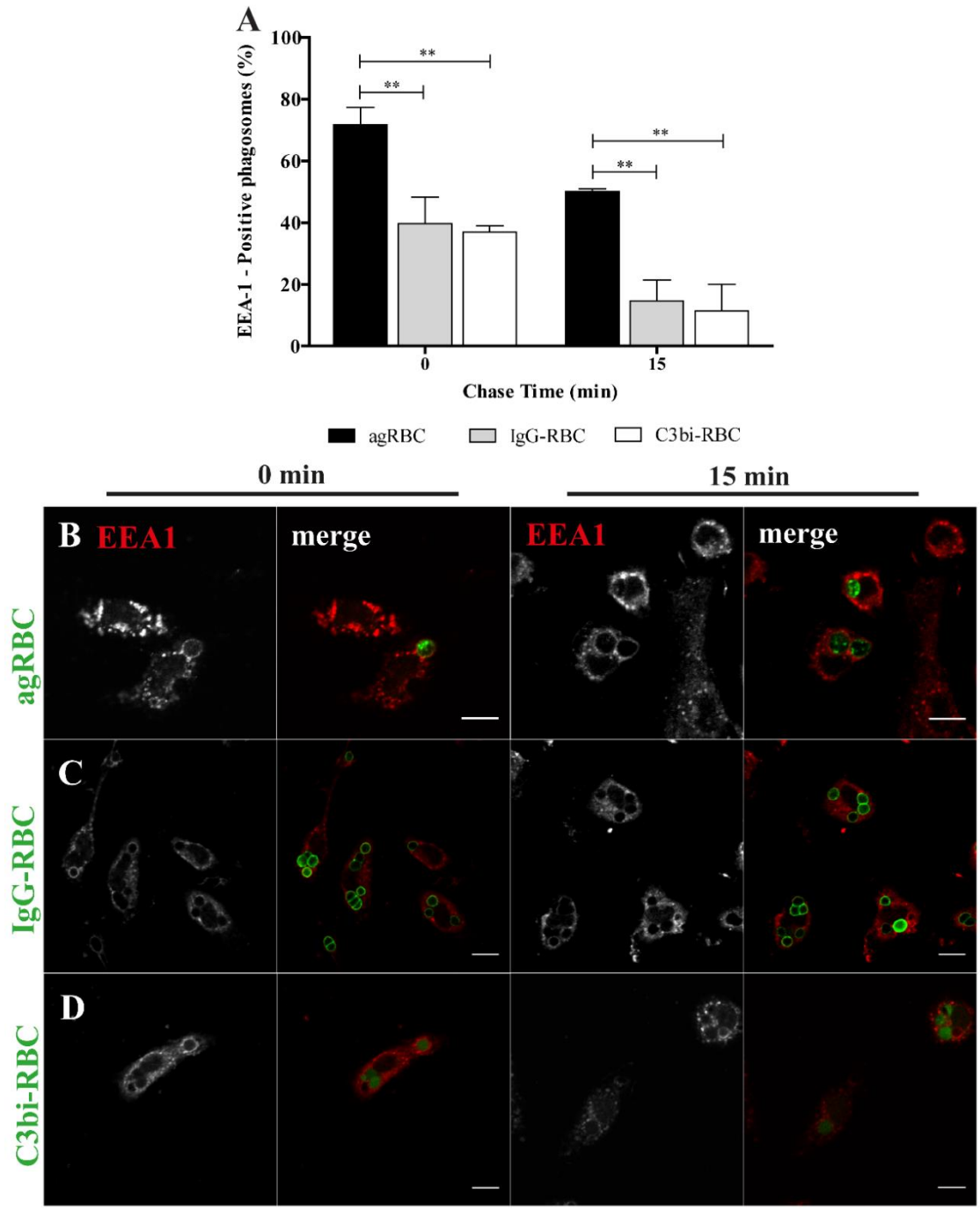

FIGURE 1. EEA-1 acquisition by phagosomal membranes containing different phagocytic particles in bone-marrow derived macrophages. The interaction of early endosomes with phagosomes containing different particles was assessed by the acquisition of EEA1 (A-D). BMDM were exposed to different phagocytic particles for 15 min and then chased for the times indicated in the graph abscissa. After pulse-chase experiments, cells were fixed, stained with EEA1 antibody and the positive phagosomes for the different chase time points were quantified in images acquired under a confocal microscope. A) Quantification of the EEA1-positive phagosomes. B) EEA1 staining of BMDM with agRBC-containing phagosomes. C) EEA-1 staining of BMDM with IgG-RBC-containing phagosomes. D) EEA1 staining of BMDM with C3bi-RBC-containing phagosomes. In B-D, for each time point, first column shows EEA1 distribution and the second column is a composite of EEA1 staining and the different RBC particles (green colour). In A, the values are means ± SEM of, at least, three independent experiments. At each time point 100 phagosomes were analysed. **, $p < 0.01$. Bars, 10 µm.

As the phagosomes mature, interaction with late components of the endocytic pathway such as late endosomes and lysosomes occurs culminating with phagolysosome formation. It is within this organelle that RBC degradation takes place. Interaction of phagosomes containing the different RBC with the late endocytic components was assessed by the acquisition of lysosomal-associated membrane protein (LAMP-1).

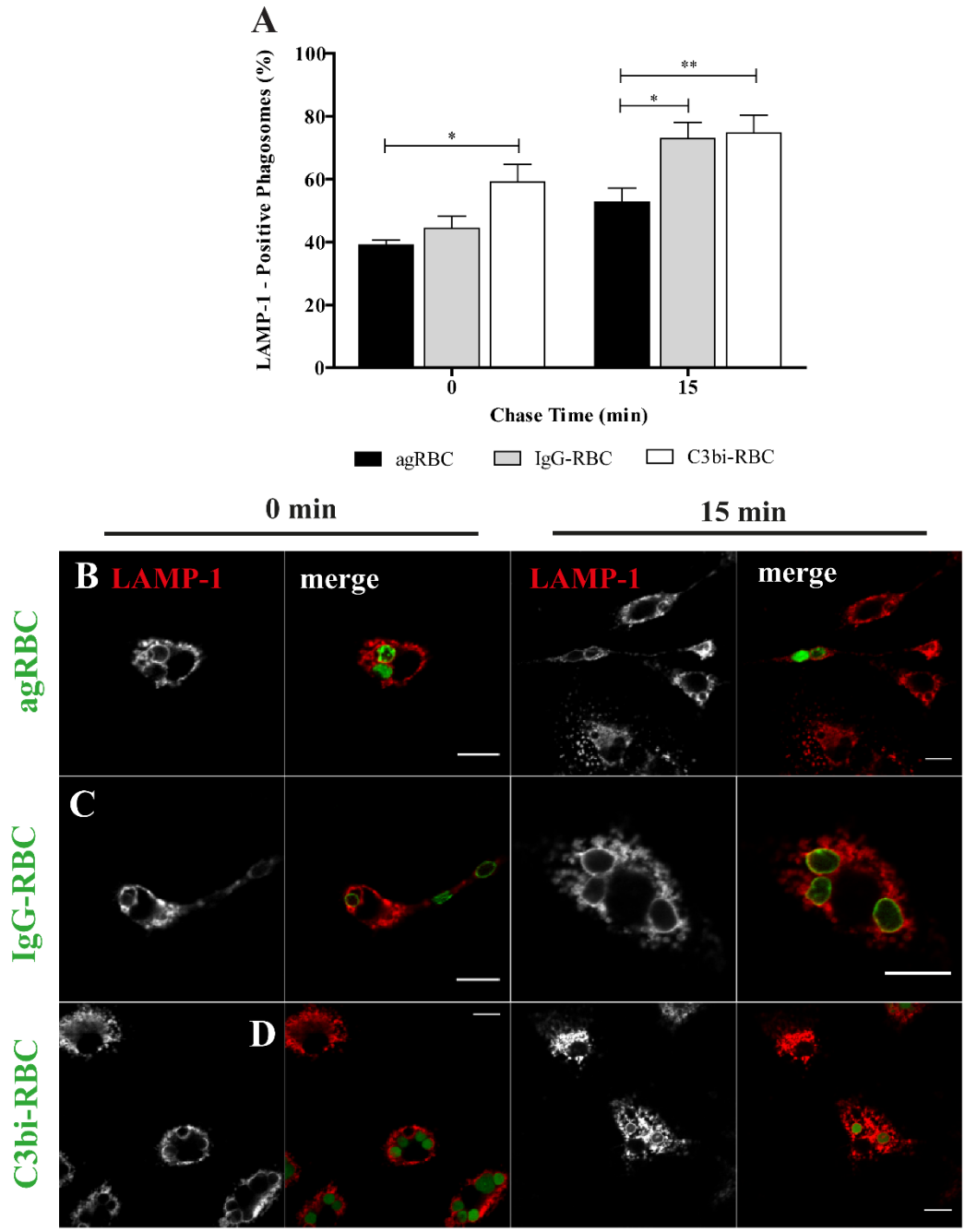

FIGURE 2. LAMP-1 acquisition by phagosomal membranes containing different phagocytic particles in bone-marrow derived macrophages. The interaction of the different phagosomes with late endocytic compartments (mainly late endosomes and lysosomes) was assessed by the acquisition of LAMP-1 (A-D). BMDM were exposed to different phagocytic particles for 15 min and then chased for the times indicated in the graph abscissa. After pulse-chase experiments, cells were fixed, stained with LAMP-1 antibody and the positive phagosomes for the different chase time points were quantified in images acquired under a confocal microscope. A) Quantification of the LAMP-1-positive phagosomes. B) LAMP-1 staining of BMDM with agRBC-containing phagosomes. C) LAMP-1staining of BMDM with IgG-RBC-containing phagosomes. D) LAMP-1 staining of BMDM with C3bi-RBC-containing phagosomes. In B-D, for each time point, first column shows LAMP-1 distribution and the second column is a composite of LAMP-1 staining and the different RBC particles (green color). In A, the values are means ± SEM of, at least, three independent experiments. At each time point 100 phagosomes were analysed. *, $p < 0.05$; **, $p < 0.01$. Bars, 10 μm.

At 15 min chase, LAMP-1 was present at higher levels in IgG-containing phagosomes (73.03 ± 5.08%) and in C3bi-containing phagosomes (74.60 ± 5.82%), compared to agRBC-containing phagosomes (52.70 ± 4.45%) in BMDM, as observed by the graph (Fig. 2A) and illustrated in Fig.2 B-D. Significant differences were also observed between agRBC- and C3bi-containing phagosomes (39.03 ± 1.69% and 59.20 ± 5.57%, respectively). These observations demonstrate that LAMP-1 acquisition is faster in IgG- and C3bi-containing phagosomes and is well correlated with the fast loss of EEA1 in these phagosomes (Fig.1A).

The maturation kinetic profile of phagosomes containing different RBC in PM was followed as described above for BMDM. Again, the association of EEA-1 with phagosomal membranes was transient for all three different phagocytic particles (Fig. 3A). However, EEA1 remained associated with phagosomes containing C3bi-opsonized RBC for longer periods of time when compared with the other phagosomes. Indeed, at 10 min chase time 63.10 ± 4.33 % of the phagosomes containing C3bi-opsonized RBC were still positive for EEA1 in contrast with 43.5 ± 1.03% and 40.33 ± 2.52 % for agRBC- and IgG-containing phagosomes, respectively (quantified in Fig.3A and visualized in Fig.3 B-D). Though, this delay in losing EEA1 by the phagosomes containing C3bi-opsonized RBC did not have any consequence in the interaction of these phagosomes with late endocytic components (Fig.4 A-D).

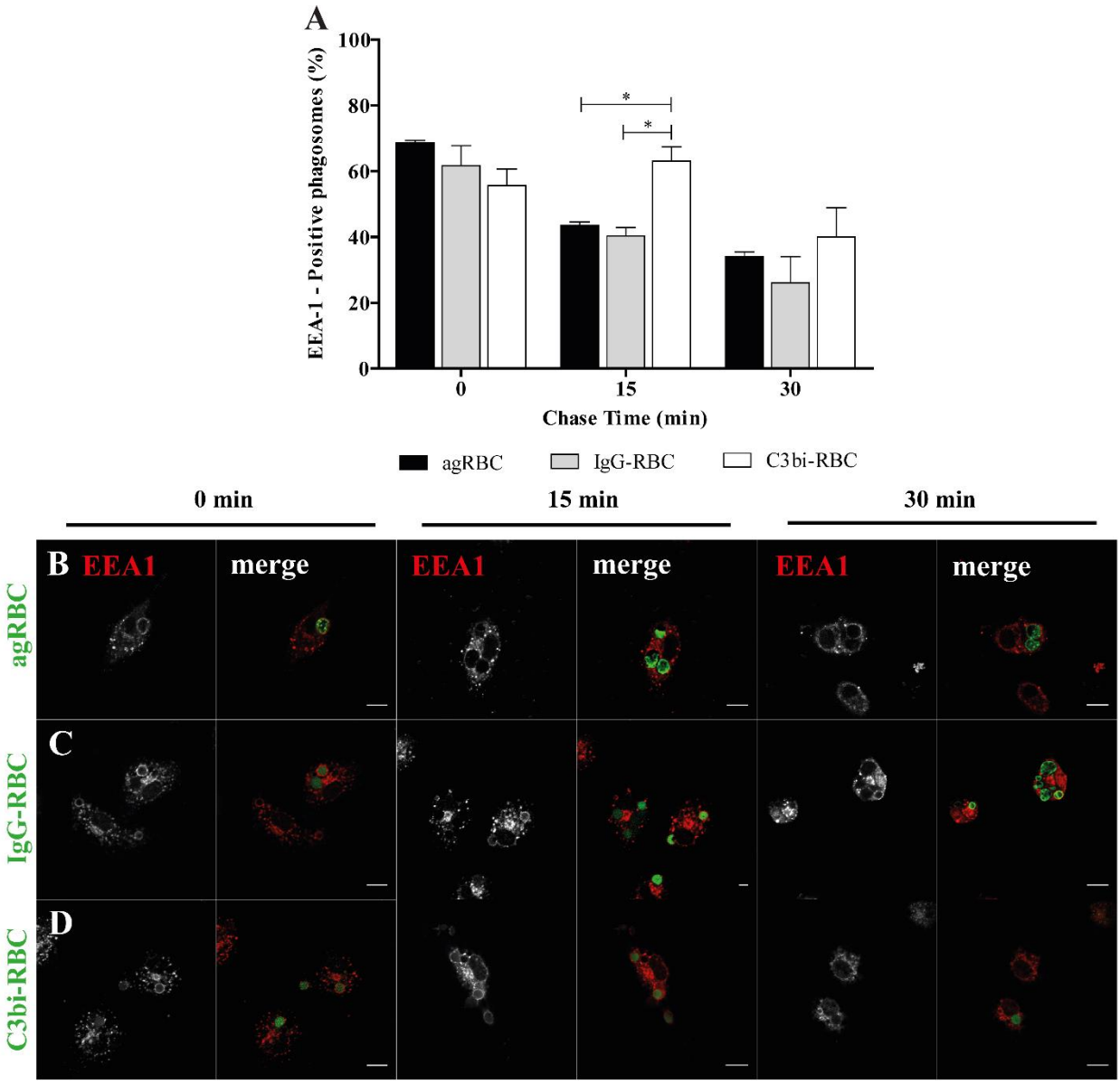

FIGURE 3. EEA1 acquisition by phagosomal membranes containing different phagocytic particles in peritoneal macrophages. The interaction of early endosomes with phagosomes containing different particles was assessed as described in the legend of Figure 1. PM were exposed to different phagocytic particles for 20 min and then chased for the times indicated in the graph abscissa. After pulse-chase experiments, cells were fixed, stained with EEA1 antibody and the positive phagosomes for the chase time points were quantified in images acquired under a confocal microscope. A) Quantification of the EEA1-positive phagosomes. B) EEA1 staining of PM with agRBC-containing phagosomes. C) EEA-1 staining of PM with IgG-RBC-containing phagosomes. D) EEA1 staining of PM with C3bi-RBC-containing phagosomes. In B-D, for each time point, the first column shows EEA1 distribution and the second column is a composite of EEA1 staining and the different RBC particles (green colour). In A, the values are means ± SEM of, at least, three independent experiments. At each time point 100 phagosomes were analysed. *, $p < 0.05$. Bars, 10 µm.

Comparing the results obtained in Fig.2 and Fig. 4, phagolysosome biogenesis is slower in PM than in BMDM for all RBC particles. Thus, besides the different phagocytic capacities previously reported [22], phagolysosome biogenesis in PM and BMDM is different. It is known that the peritoneal cavity has two PM subsets: large peritoneal macrophage and small peritoneal macrophage [39]. Under steady state conditions, large peritoneal macrophage appear to originate independently from hematopoietic precursors and retained the ability to proliferate in situ, maintaining physiological numbers [40]. The small PM are originated from circulating monocytes [39,40], and their numbers increase remarkably under inflammatory conditions. Therefore, small PM together with their precursor, the inflammatory monocyte population, are the major myeloid populations present in elicited peritoneal cavity. However, in our experimental conditions, we did not work with elicited-peritoneal macrophages and, in principle, our PM population is composed mainly by large PM that express high levels of Tim-4, a phosphatidylserine receptor, at the cell surface [39].

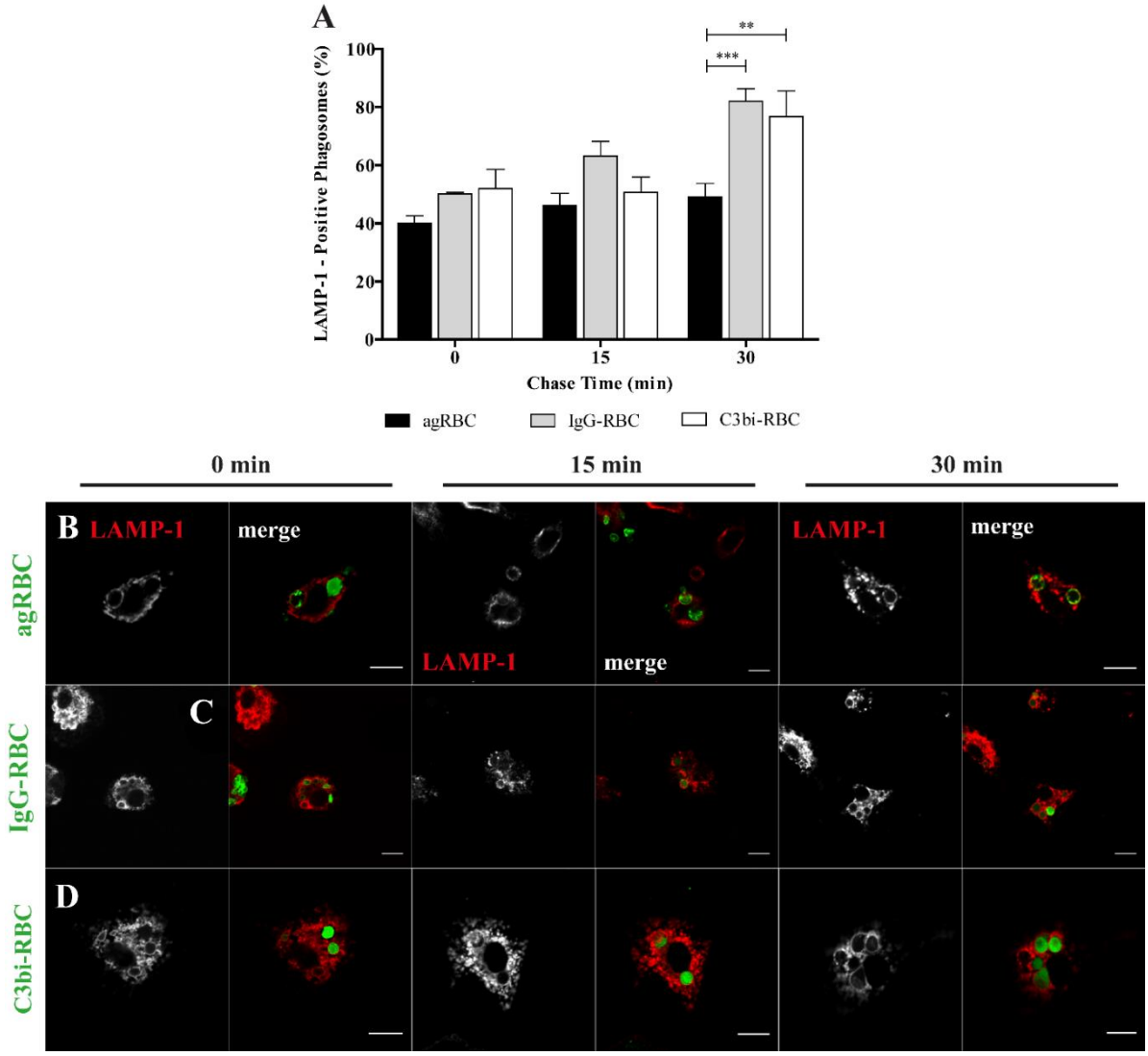

FIGURE 4. LAMP-1 acquisition by phagosomal membranes containing different phagocytic particles in peritoneal macrophages. The interaction of late endosomes/lysosomes with phagosomes containing different particles was assessed as described in the legend of Figure 2. PM were exposed to different phagocytic particles for 20 min and then chased for the times indicated in the graph abscissa. After pulse-chase experiments, cells were fixed, stained with LAMP-1 antibody and the positive phagosomes for the chase time points were quantified in images acquired under a confocal microscope. A) Quantification of the LAMP-1-positive phagosomes. B) LAMP-1 staining of PM with agRBC-containing phagosomes. C) EEA-1 staining of PM with IgG-RBC-containing phagosomes. D) LAMP-1 staining of PM with C3bi-RBC-containing phagosome. In B-D, for each time point, first column shows LAMP1 distribution and the second column is a composite of LAMP-1 staining and the different RBC particles (green color). In A, the values are means ± SEM of, at least, three independent experiments. At each time point 100 phagosomes were analysed. **, $p < 0.01$; ***, $p < 0.001$. Bars, 10 µm.

Similarly with what was observed for BMDM in PM phagolysosome formation is faster for opsonised RBC containing phagosomes than for those containing agRBC. These results are similar with those obtained by our group in smooth muscle cells [20] suggesting that processing of RBC is conserved among professional and non-professional phagocytes. Our data also demonstrate that different opsonins that interact with different phagocytic receptors leading to different signalling responses did not result in major differences in terms of phagolysosome biogenesis in both types if primary macrophages. The observation that phagosomes containing opsonised RBC mature more rapidly than those containing agRBC can be explained at least in part because Fc- and Complement- receptors are involved in the rapid removal from the circulation and killing of bacteria to avoid infection. Indeed, phagocytosis through either of these receptors usually results and reactive oxygen species formation and in the secretion of pro-inflammatory cytokines [41,42]. Interestingly and in contrast with Fc- and complement-mediated phagocytosis, PS-receptors mediated phagocytosis is immunologically silent and induces an anti-inflammatory response [43]. So, we can speculate that phagocytosis of RBC mediated by PS-receptors is crucial for keeping erythrophagocytosis immunological silent.

Finally, we want to state that the use of phagocytic cells and phagocytic particles of different origin could limit to some extent the conclusions at physiological level. However, in this work, different phagocytic particles were used as models to study different types of phagocytosis *in vitro* and, the information obtained is an important contribution in terms of cell biology of phagocytosis and membrane trafficking.

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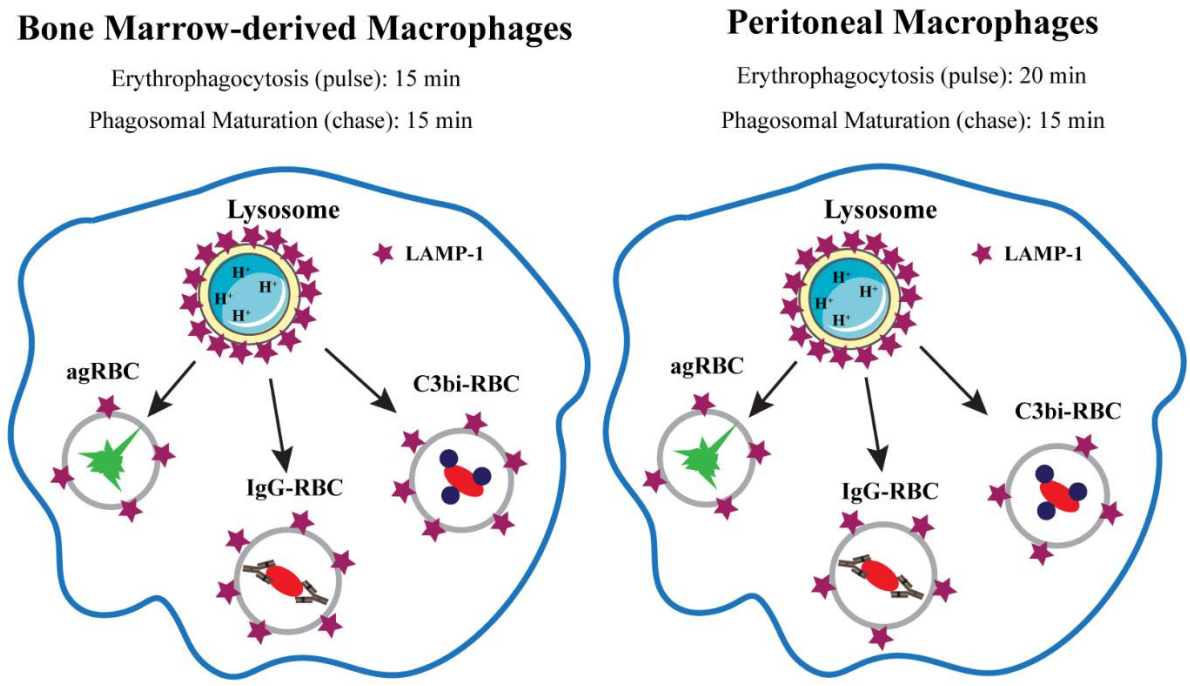
PhD fellowship reference: SFRH/BD/90258/2012.

GRAPHICAL ABSTRACT



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CHAPTER III

Involvement of the p62/NRF2 Signal Transduction Pathway on Erythrophagocytosis

This chapter is formatted based on the following manuscript submitted during the PhD experimental work:

- **Involvement of the p62/NRF2 signal transduction pathway on erythrophagocytosis**

In this work, Inês B. Santarino was involved in the experiments design and has carried out the majority of the work (from Figure 4 to Figure 8, and Suppl. Figure 2, including the western blot in the Figure 1 and the z-stack projections in Figure 3), as well as interpretation and discussion of the results. Inês also participated in the writing of the manuscript.

In the Material and Methods section of this manuscript, an extensive detailed description will be presented when new protocols are used.

Involvement of the p62/NRF2 signal transduction pathway on erythrophagocytosis

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ABSTRACT

Erythrophagocytosis, the phagocytic removal of damaged red blood cells (RBC), and subsequent phagolysosome biogenesis are important processes in iron/heme metabolism and homeostasis. Phagolysosome biogenesis implies the interaction of nascent phagosomes with endocytic compartments and also autophagy effectors. Here, we report that besides recruitment of microtubule-associated protein-1-light chain 3 (LC3), additional autophagy machinery such as sequestosome 1 (p62) is also acquired by single-membrane phagosomes at very early stages of the phagocytic process and that its acquisition is very important to the outcome of the process. In bone marrow-derived macrophages (BMDM) silenced for p62, RBC degradation is inhibited. P62, is also required for nuclear translocation and activation of the transcription factor Nuclear factor E2-related Factor 2 (NRF2) during erythrophagocytosis. Deletion of the *Nrf2* allele reduces p62 expression and compromises RBC degradation. In conclusion, we reveal that erythrophagocytosis relies on an interplay between p62 and NRF2, potentially acting as protective mechanism to maintain reactive oxygen species at basal levels and preserve macrophage homeostasis.

INTRODUCTION

Removal of damaged/aged red blood cells (RBC) from the circulation occurs through erythrophagocytosis, by tissue-resident macrophages in the spleen, liver and bone marrow[1–4]. Rapid removal of damaged RBC is important for maintenance of iron/heme homeostasis, as the majority of iron required to sustain erythropoiesis is derived from senescent RBC, and defects in erythrophagocytosis can lead to anemia and iron overload.[4]

Previous work identified receptor-ligand interactions and signaling pathways engaged during erythrophagocytosis. Namely, macrophages recognize damaged RBC by a range of senescence markers such as phosphatidylserine (PS), decreased levels of sialic acid, CD47 and binding of autologous immunoglobulins and opsonins [5]. Furthermore, some receptors involved in RBC clearance have also been established. Several *in vitro* studies have shown that PS recognition on the cell surface by stabilin-2 is important for RBC clearance, while others suggested that clearance of aged RBC by macrophages is likely dependent on scavenger receptors rather than specific PS receptors [2,6,7]. It is likely that under physiological conditions

the engulfment of RBC involves a myriad of receptors including the Fc- and complement-receptors.

Upon RBC recognition, macrophage actin cytoskeleton and cell surface remodeling takes place allowing for the formation of a specialized phagosome known as the erythrophagosome. Following scission from the plasma membrane, phagosomes undergo a maturation process involving a programmed change of their membrane and luminal composition resulting from a highly coordinated series of sequential membrane fusion and fission events with components of the endocytic pathway. Fusion with early-endosomes followed by interactions with late-endosomes and lysosomes culminates in the conversion of the phagosome into a lysosome-like organelle - the phagolysosome. It is within this organelle that RBC undergo degradation allowing for the reutilization of their components [4,8–10].

Beyond the involvement of vesicular traffic machinery, some components of the autophagy machinery are also involved in phagolysosome biogenesis, including the microtubule-associated protein 1 light chain 3 (LC3), an autophagy effector recruited to single-membrane phagosomes in a process termed LC3-Associated Phagocytosis (LAP). There is strong evidence to suggest that LAP facilitates rapid phagosome maturation while contributing to the degradation of engulfed phagocytic particles and modulation of immune responses [11–13]. In contrast to canonical autophagy, defined by the formation of a double-membrane autophagosome, LAP is associated with the recruitment of LC3 to single-membrane phagosomes carrying different types of cargo in an Atg5-, Atg7- and Beclin1-dependent manner, independently of the mammalian target of Rapamycin (mTor)-regulated ULK-ATG13-FIP200 complex [11,14]. Rubicon, an adaptor protein, was also identified as being required for LAP but not for autophagy [12]. NADPH oxidase-2 (NOX2) has also been identified as having a LAP-specific role [12,15]. It should be noted that this brief description of phagosomal maturation is a gross oversimplification of a highly complex and precisely choreographed process.

Although several studies have focused on intracellular mechanisms of heme trafficking during hemophagocytosis [16,17], few have addressed the molecular mechanisms underlying maturation and degradation of phagosomes containing RBC. We have recently shown that phagosomes containing RBC cells mature slower than phagosomes containing IgG-opsonized particles [18], in keeping with the notion that maturation of the phagosome in macrophages depends on the nature of the ingested cargo [19].

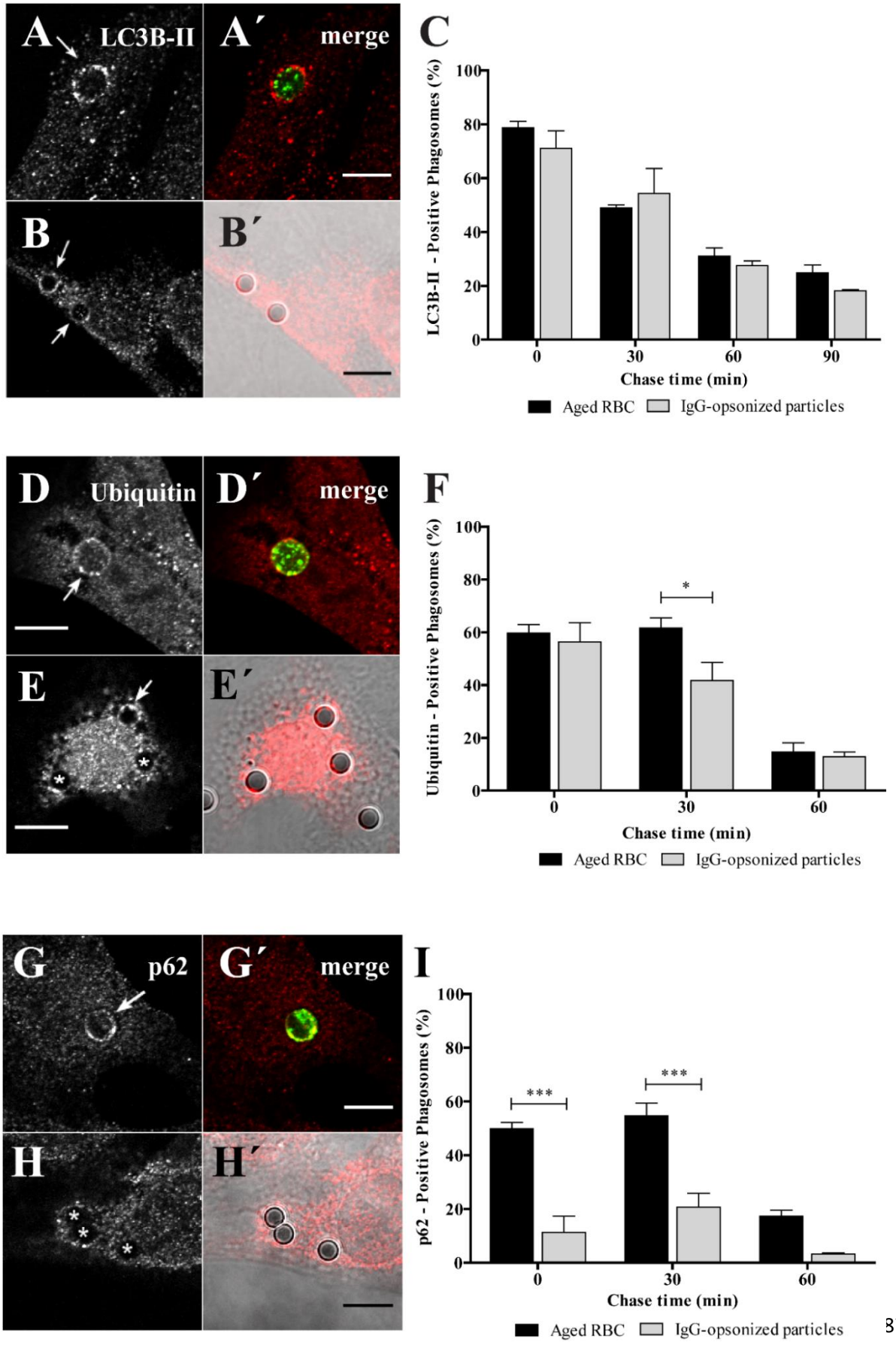
The present study was designed to identify the molecular machinery involved in maturation of phagosomes containing RBC. Of note, while erythrophagocytosis takes place mainly in erythrophagocytic macrophages such as bone marrow-derived macrophages (BMDM) it can also occur in non-professional phagocytes such as hepatic sinusoidal endothelial cells and vascular smooth muscle cells [20,2,21,22]. The process has some similarities with efferocytosis that occurs in pathological states like atherosclerosis and in which smooth muscle cells act as non-professional phagocytes in the arterial wall. In previous work we generated a smooth-muscle cell line that stably expressed Fcγ-RIIA receptors and described its use in studies of erythrophagocytosis [18]. Here we report mechanistic details of erythrophagocytosis by this non-professional phagocytic cell line as well as by primary BMDM. We show that beyond LC3, proteins associated with selective autophagy such as p62/SQSTM1 (Sequestosome 1), NBR1 (Neighbor of Braca 1 gene) and NDP52 (Nuclear dot protein 52)[23–25] are recruited to phagosomal membranes. The most striking phenotype was observed for p62 that associates preferentially with phagosomes containing RBC rather than to phagosomes containing IgG-opsonized particles. Moreover, we demonstrate that p62 is critical for RBC degradation. We also show that erythrophagocytosis triggers the nuclear accumulation of the transcription factor Nuclear factor E2-related factor 2 (NRF2) with subsequent up-regulation of *p62* expression s. In addition, NRF2 affects RBC degradation and p62 levels suggesting a link between these two molecular players in erythrophagocytosis.

RESULTS

The type of phagocytic particle determines the association of p62 with phagosomal membranes

We started by studying LAP in the non-professional phagocytes. Damaged/aged RBC were prepared by incubation in PBS (20% hematocrit) for 4 days at 37°C. This treatment triggers PS-exposure on the outer leaflet of the RBC membrane, resembling what happens to RBC during storage [26], or eryptosis – a form of programmed cell death similar to apoptosis in nucleated cells [27]. RBC phagocytosis was compared to phagocytosis of IgG-opsonized particles, the most studied phagocytic model. IgG-opsonized particles are known to be internalized via Fc-receptors. After exposing phagocytes to RBC or IgG-coated latex beads, LC3B association with phagosomal membranes was assessed by immunostaining of the endogenous protein by confocal microscopy., LC3B associated with phagosomes containing both particles,

immediately after phagocytosis, as evidenced by LC3B-II rims surrounding RBC and opsonized latex beads (Fig. 1A-B´).



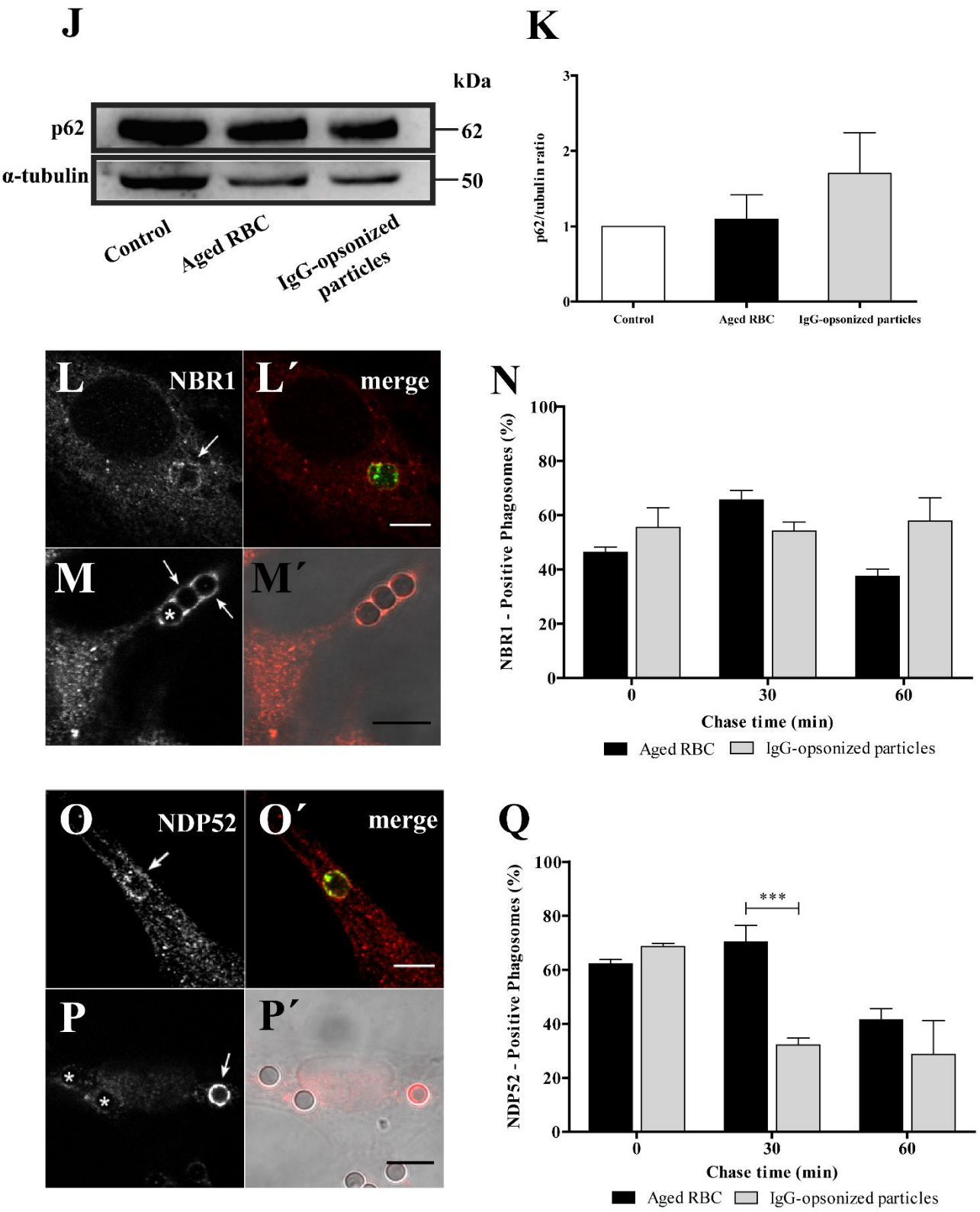

J
kDa
p62
α-tubulin
62
50
Control
Aged RBC
IgG-opsonized particles
K
p62/tubulin ratio
Control
Aged RBC
IgG-opsonized particles
L
NBR1
L´
merge
M
M´
N
NBR1 - Positive Phagosomes (%)
Chase time (min)
Aged RBC
IgG-opsonized particles
O
NDP52
O´
merge
P
P´
Q
NDP52 - Positive Phagosomes (%)
***
Chase time (min)
Aged RBC
IgG-opsonized particles

FIGURE 1. *Acquisition of LC3B-II and autophagy adaptor proteins by phagosomes in non-professional phagocytes.*
Phagocytes were challenged with RBC (A, A', D, D', G, G', L, L', O and O') or with IgG-opsonized particles (B, B', E, E', H, H', M, M', P and P') and then immunostained for the endogenous LC3B, ubiquitin, p62, NBR1 and NDP52 as indicated in the different figure panels. A, B, D, E, G, H, L, M, O and P are immunofluorescence images. A', B', D', E', G', H', L', M', O' and P' are the composite of the immunofluorescence image with the phagocytic particles visualized in green (RBC stained with CFSE) or by differential interference contrast (DIC, IgG-opsonized particles). In A-B' and G-H' images were acquired at 0 min chase time. In D-E', L-M' and O-P' the images were acquired at 30 min chase time. Arrows indicate positive- and asterisks (*) indicate negative-phagosomes for the indicated endogenous protein. Bars, 10 μm.
C, F, I, N and Q, Graphs showing the percentage of positive phagosomes for LC3B-II, ubiquitin and the different adaptor proteins. Quantifications were performed in non-professional phagocytes exposed to the different phagocytic particles for 30 min and chased for the times indicated in the graph abscissa. The values are means ± SEM of, at least, three independent experiments. At each time point, at least, 50 phagosomes were analyzed. *, $p<0.05$; ***, $p<0.001$ comparing differences between phagosomes with RBC and with IgG-opsonized particles.
(J) p62 levels in cell lysates of non-professional phagocytes exposed for 30 min to RBC or to IgG-opsonized particles. α-tubulin was used as loading control. (K) Ratio of p62/tubulin of quantified bands in cells exposed or not (control) to RBC or IgG-opsonized particles. Three independent experiments were performed.

Both types of phagosomes showed a rapid and transient LC3B membrane association,with peaks reaching a maximum of about 80% (78.7 ± 2.3%) for RBC and about 7 % (71.0 ± 6.5%) for opsonized beads, at 0 min chase. These results are in keeping with those reported by other groups showing that LC3B-II can be detected on phagosomes shortly after they are formed while LC3B-II-decorated autophagosomes can take hours to form [11,28]. LC3B-II gradually dissociated from both types of phagosomes, probably due to recycling from the phagosomal membranes (Fig. 1C). Since our phagocytic assays were performed in serum-free medium and canonical autophagy is activated under conditions of starvation [29], we tested whether nutrient deprivation was responsible for LC3B-II association with phagosomes containing RBC. As shown in Suppl. Fig. 1, no differences in the LC3B-II-phagosomal association pattern were observed when phagocytic cells were kept in the presence or absence of serum, suggesting that LAP machinery is independent of the nutritional status of the phagocytes, as previously described [11,13].

Beyond LC3B, phagosomal processing and autophagy share other players and mechanisms [12,30–33]. Therefore, we monitored phagosomal protein ubiquitination in phagosomes containing both types of phagocytic particles. Phagosomes containing either RBC or IgG-coated beads were associated with poly- and/or mono-ubiquitinated membrane proteins (Fig. 1D-E´). Namely, non-professional phagocytes presented, shortly after ingestion, a large

fraction (around 60% for both particles) of phagosomes positive for ubiquitinated components with some differences in the kinetics of signal loss over the maturation time (Fig. 1F), as assessed by the appearance of this tag in phagosomal membranes.

To acquire further insights into the autophagy-related molecular machinery involved in phagolysosome biogenesis, we looked at the phagosomal acquisition of p62, NBR1 and NDP52. These are receptors/adaptors which share the ability to simultaneously interact with the lipidated form of LC3B, LC3B-II and ubiquitinated substrates [32]. We started by testing intracellular distribution of p62 during both types of phagocytosis, a universal receptor for ubiquitinated cargo under physiological and pathological conditions [7,24,34–36]. Pulse-chase experiments revealed that RBC containing phagosomes displayed a different pattern of p62 association, as compared to IgG-opsonized beads (Fig. 1G-H). Namely, they showed similar kinetics for acquisition of p62 and ubiquitinated proteins while phagosomes containing IgG-opsonized particles showed only modest levels of p62 over time (compare Fig. 1I and Fig. 1F). That difference was not due to changes in expression levels of total p62 in phagocytic cells challenged with the two phagocytic particles, as confirmed by western blot (Fig. 1J and K).

Next, we analyzed the association of NBR1 with phagosomal membranes. As illustrated in Fig. 1L-M', NBR1 was recruited to both types of phagosomes. The time course of NBR1 dissociation from membranes of phagosomes that contained RBC was slightly different from the time course of NBR1 dissociation from phagosomes containing IgG-opsonized particles (Fig.1N). NBR1 dissociation from phagosomal membranes of IgG-coated beads was not observed even for the longest chase time tested. This may be due to a compensatory mechanism for the absence of p62 on the phagosomal membranes of IgG-coated beads.

Finally, we performed immunostaining for NDP52 to assess its acquisition by both types of phagocytic particles (Fig. 1O-P'). Phagosomes containing IgG-opsonized particles showed a transient NDP52 association with around 69% (68.64 ± 1.19%) of positive-phagosomes at 0 min chase and about 29% (28.69 ± 12.51%) at 60 min chase. Phagosomes containing RBC also showed a transient NDP52 association but this autophagy effector remained associated with these phagosomes for longer periods of time compared with those carrying IgG-opsonized beads (Fig. 1Q).

p62 and NBR1 are recruited to the phagocytic cups.

Since LAP and the autophagy receptors/adaptors tested in this work were acquired by the phagosomes at very early stages of phagocytosis, we enquired whether they were already present when the phagosomes were positive for F-actin. Phagosome formation is preceded by a dynamic set of events that induce actin cytoskeleton rearrangement in order to support pseudopod extension at sites of particle engulfment. This reorganization leads to a localized cup-shaped protrusion of the plasma membrane, the "phagocytic cup" (Figs. 2 and 3). This structure is enriched in actin filaments responsible for generation of the forces that alter the local shape of the cell surface. In the case of phagocytosis of RBC, membrane protrusions are formed upon actin polymerization, with particle sinking followed by the formation of the phagosome, through a process between complement-mediated phagocytosis and micropinocytosis[37] making the visualization of the phagocytic cups difficult, as can be observed in Fig. 2A (blue arrows in the XZ view point to F-actin). In contrast, in phagocytosis of IgG-opsonized particles, the actin cups are perfectly visualized (Fig. 3).

To assess how early the autophagy receptors/adaptors associate with the phagocyte particles, phagocytes were exposed to RBC and IgG-opsonized beads, fixed and co-stained for F-actin with Phalloidin and p62, LC3B, NBR1 or NDP52. Notably, p62 was found to be present in nascent RBC-containing phagosomes, co-localizing with F-actin as shown in Fig. 2A and absent in phagosomes containing IgG-opsonized particles, Fig. 3A, confirming the selectivity of this adaptor for RBC-containing phagosomes (Fig. 1I). As seen in Fig. 2B and D and Fig. 3B and D, LC3B-II and NDP52 were not co-localized with F-actin in any of the phagocytic particles incubated with phagocytes suggesting that they were acquired by the phagosomal membranes after actin dissociation and when phagosome maturation starts. NBR1 was present in the phagocytic cups of RBC and IgG-opsonized particles (Fig. 2C and Fig. 3C). Thus, during the phagocytic process, p62 and NBR1 were acquired earlier that NDP52 and LC3B-II, suggesting that the former proteins could be involved in the recruitment of the latter.

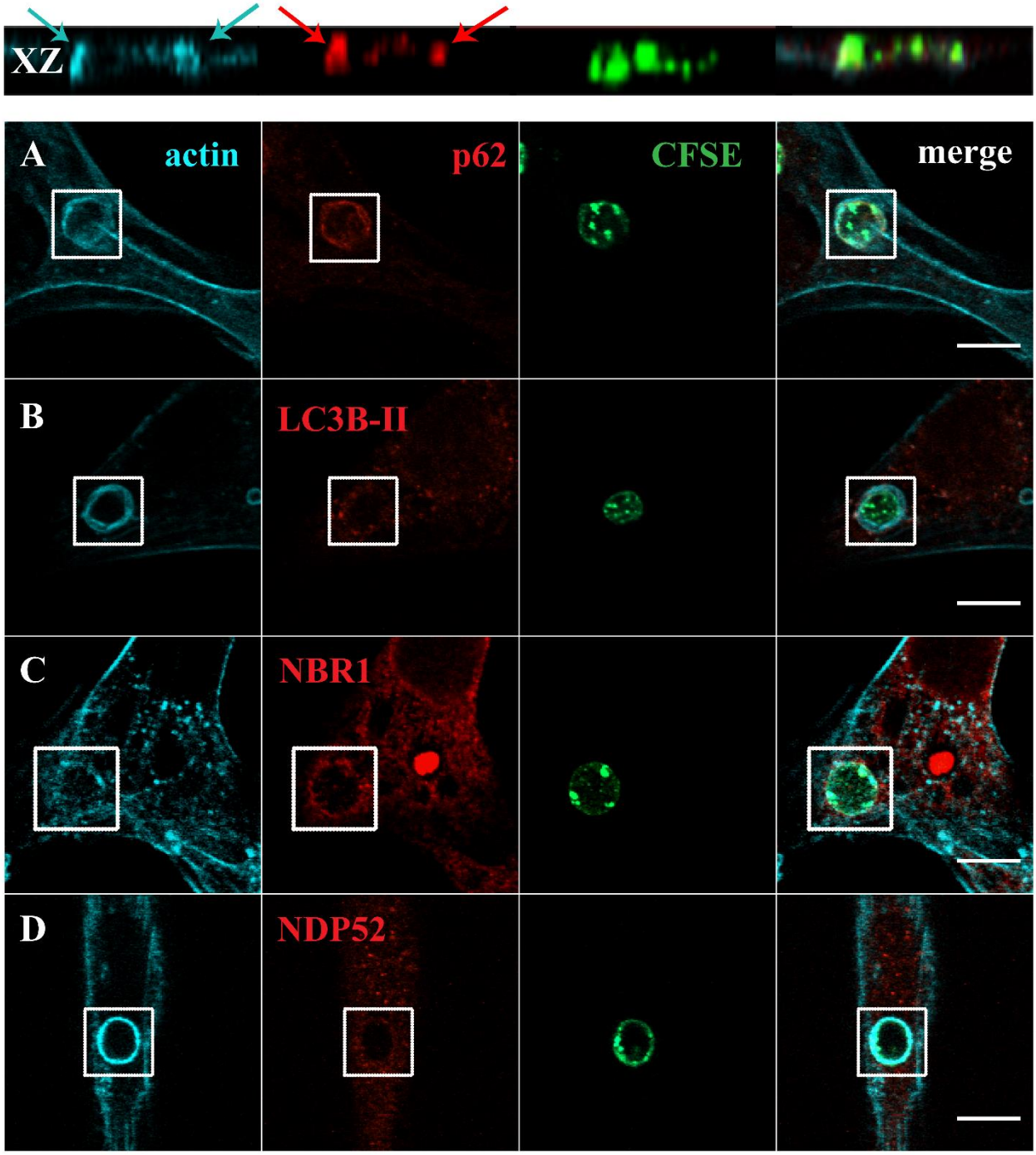


FIGURE 2. *Co-localization of LC3B-II and autophagy adaptor proteins with F-actin in phagosomes containing RBC.*

Non-professional phagocytes were fed with RBC for 30 min, fixed, and stained for F-actin with Phalloidin and for the endogenous LC3B or autophagy adaptors. A-D are representative images, obtained by confocal microscopy, of cells co-stained for F-actin and p62 (A), LC3B (B), NBR1 (C) or NDP52 (D). In A, side views (XZ) are merges of ten vertical sections of confocal stacks. Arrows indicate the nascent phagosome positive for F-actin (blue) and p62 (red). The first column represents cells stained for F-actin. The second column represents cells stained for the endogenous p62, LC3B-II, NBR1 or NDP52. The third column shows internalized RBC stained with CFSE. The fourth column represents merged images of F-actin with LC3B-II or autophagy adaptors and internalized RBC. The regions outlined by the boxes are nascent phagosomes. Bars, 10 µm.

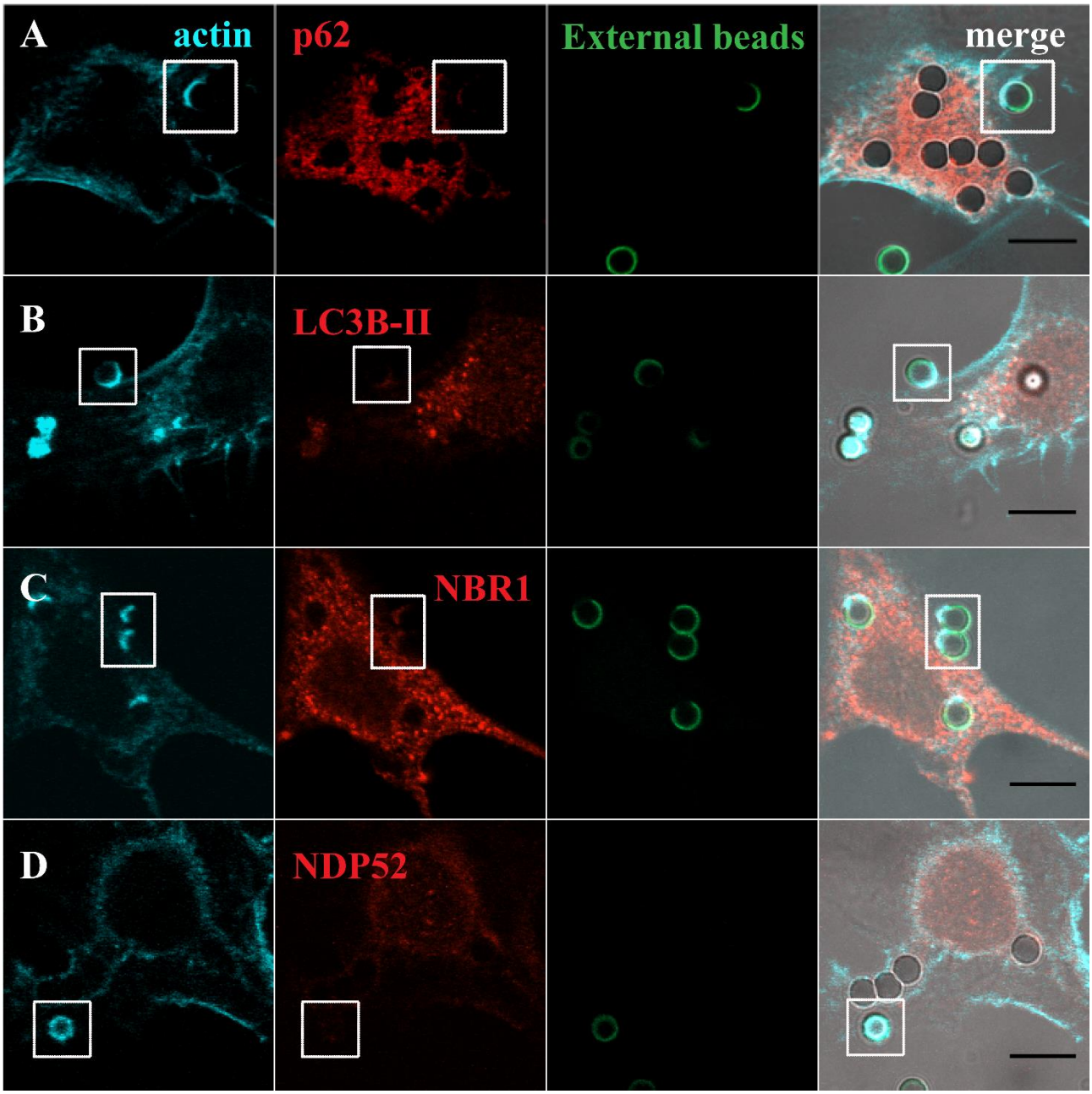
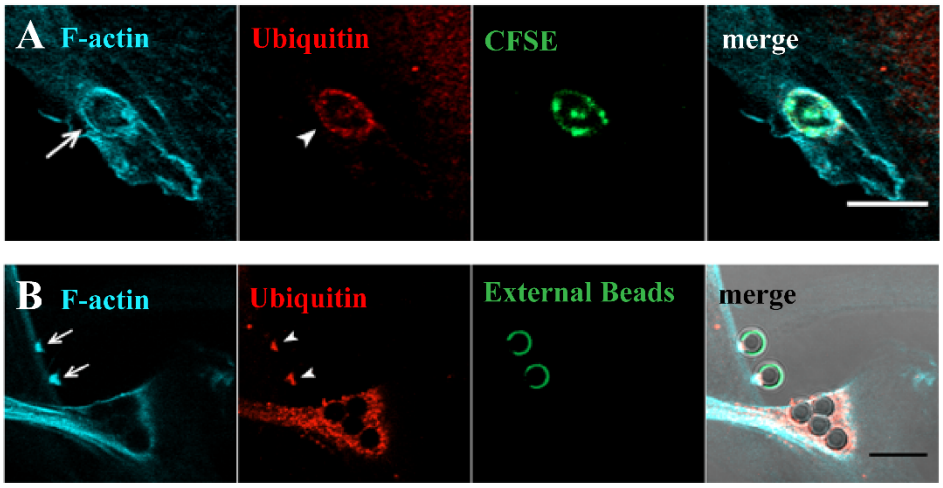


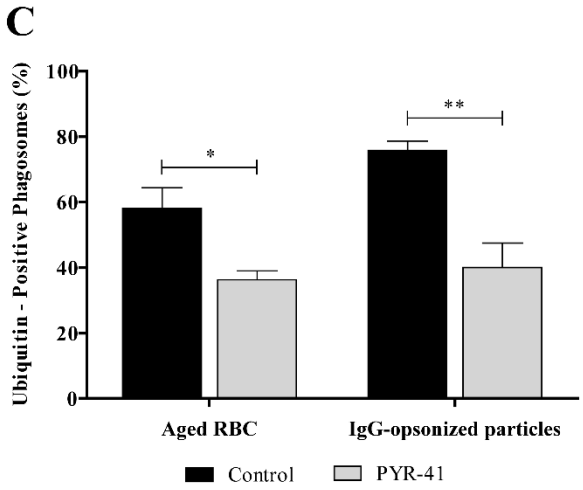
FIGURE 3. *Co-localization of LC3B-II and autophagy effectors in phagocytic cups of IgG-opsonized particles.*

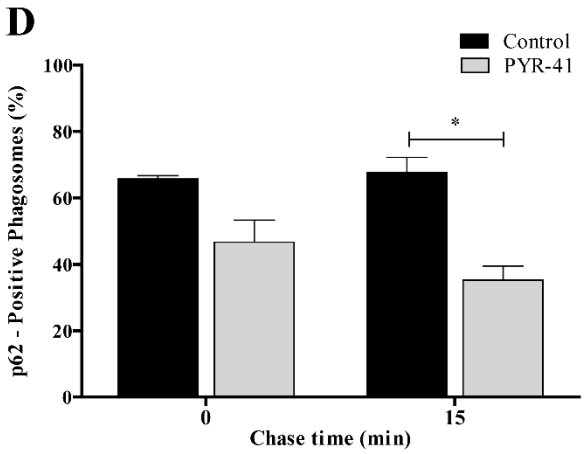
Non-professional phagocytes were fed with IgG-opsonized beads for 30 min, fixed, and co-stained for F-actin with Phalloidin and for the endogenous LC3B or autophagy adaptors. A-D are representative images of cells co-stained for F-actin and p62 (A), LC3B (B), NBR1 (C) or NDP52 (D). The first column represents cells stained for F-actin. The second column represents cells stained for the endogenous p62, LC3B-II, NBR1 or NDP52. The third column shows non-internalized beads stained with an anti-human IgG antibody conjugated with FITC. The fourth column represents merged images of F-actin with LC3B-II or autophagy adaptors and the opsonized latex beads (external and internal beads visualized by DIC). The regions outlined by the boxes are phagocytic cups formed upon the recognition of IgG-opsonized particles by the Fc-receptors. Bars, 10 µm.

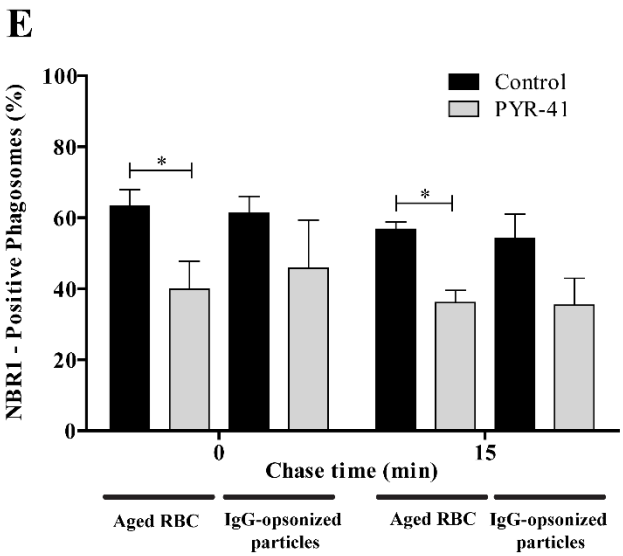
Ubiquitin is involved in the recruitment of the autophagy adaptors to the phagosomal membranes.

Since p62, NBR1 and NDP52 have ubiquitin binding domains and ubiquitination occurs during phagocytosis (Fig. 1D-F), we addressed the role of ubiquitin in the recruitment of these adaptors to phagosomes. First, we determined whether ubiquitin was associating with phagocytic cups. As observed in Fig. 4A and B, ubiquitin associated with phagocytic cups, visualized by F-actin staining, suggesting that phagosomal ubiquitination could be involved in the recruitment of p62, NBR1 and NDP52. An E1 ubiquitin-activating enzyme inhibitor, PYR-41, [38] reduced the percentage of ubiquitin-positive phagosomes containing RBC by 37.0% and by 47.0% for IgG-opsonized particles, when compared with control cells (Fig. 4C). Although inhibition of ubiquitination by PYR-41 of phagosomal membranes was not complete, the effect obtained was sufficient to affect the association of p62 (Fig. 4D), NBR1 (Fig. 4E) and NDP52 (Fig. 4F) with phagosomes containing RBC. The inhibitory effect of PYR-41 in the ubiquitination of phagosomal membranes containing IgG-opsonized particles was only observed for NDP52 recruitment to the phagosomal membranes (Fig. 4E and F). Together, these results suggest a new and differentiating role for ubiquitin in phagocytosis.


A
F-actin
Ubiquitin
CFSE
merge
B
F-actin
Ubiquitin
External Beads
merge


C
Ubiquitin - Positive Phagosomes (%)
100
80
60
40
20
0
*
**
Aged RBC
IgG-opsonized particles
Control
PYR-41


D
p62 - Positive Phagosomes (%)
100
80
60
40
20
0
Control
PYR-41
*
0
15
Chase time (min)


E
NBR1 - Positive Phagosomes (%)
100
80
60
40
20
0
Control
PYR-41
*
*
0
15
Chase time (min)
Aged RBC
IgG-opsonized particles
Aged RBC
IgG-opsonized particles

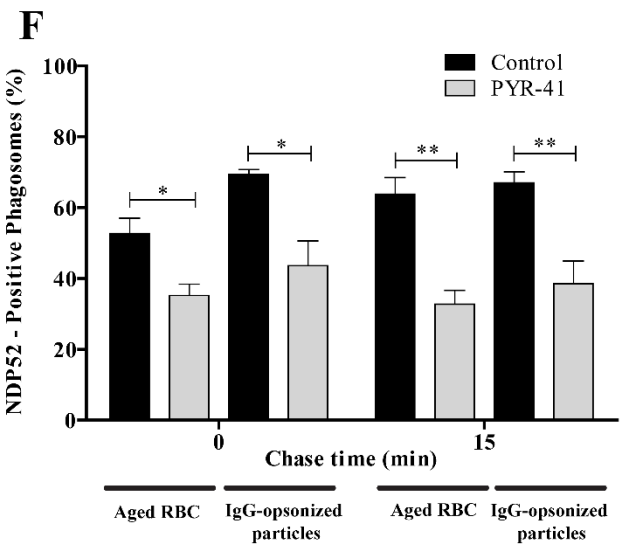

F
NDP52 - Positive Phagosomes (%)
100
80
60
40
20
0
Control
PYR-41
*
*
**
**
0
15
Chase time (min)
Aged RBC
IgG-opsonized particles
Aged RBC
IgG-opsonized particles

FIGURE 4. *Functional relevance of ubiquitin on the recruitment of autophagy effectors to phagosomes.* Non-professional phagocytes were fed with RBC or IgG-opsonized particles for 30 min, fixed, and co-stained for F-actin and ubiquitin. Representative image of a nascent phagosome (A) and a phagocytic cup (B) positive for actin (first panels) and ubiquitin (second panels). The third panels show internalized RBC labelled with CFSE and non-internalized beads stained with an anti-human IgG antibody conjugated with FITC. The fourth panels are composites of the 1st, 2nd and 3rd panels. Arrows and arrowheads indicate actin- and ubiquitin-positive phagosome, respectively. Bars, 10 μm. (C) Effect of PYR-41 in the ubiquitination of both RBC- and IgG-opsonized particles-containing phagosomes. Phagocytes were cultured and treated as described in Material and Methods section. (D) Quantification of PYR-41 effect on the acquisition of p62 by RBC-containing phagosomes. (E-F) Quantification of PYR-41 effect on the acquisition of the autophagy adaptor proteins, NBR1 and NDP52, respectively, by RBC- and IgG-opsonized particles-containing phagosomes. The values are means ± SEM of, at least, three independent experiments. At each time point, at least, 50 phagosomes were analyzed. *, $p<0.05$; **, $p<0.01$ comparing differences between adaptor-positive phagosomes containing RBC or IgG-opsonized particles in absence and in presence of the inhibitor PYR-41.

The role of p62 in phagocytosis of IgG-opsonized particles and RBC was compared in mouse BMDM. As shown in Fig. 5A-C the results for wild type BMDM exhibit a pattern that is very similar to the one described above for non-professional phagocytes with p62 associated mainly with RBC-containing phagosomes irrespective of the total p62 levels (Fig. 5D and E). Similarly, p62 associates with phagosomal membranes at very early stages of the phagocytic process in BMDM (Fig.5F). This suggests that the role of p62 is conserved in professional and non-professional phagocytes. Due to the residual levels of p62 detected in phagosomes containing IgG-opsonized particles we explored in further detail the role of p62, focusing only on RBC-containing phagosomes using mouse BMDM silenced for p62 or BMDM from p62-deficient mice ($p62^{-/-}$).

To ensure that RBC internalization and processing was a LAP-dependent process we assessed the recruitment of LC3B to phagosomal membranes in absence of Rubicon, an adaptor protein associated to the LAPosome and required for LAP.[12] As illustrated in Suppl. Fig. 2A-B, LC3B-II association with phagosomal membranes was inhibited in absence of Rubicon, when compared to control cells expressing Rubicon. This confirms that RBC internalization is mediated by LAP.

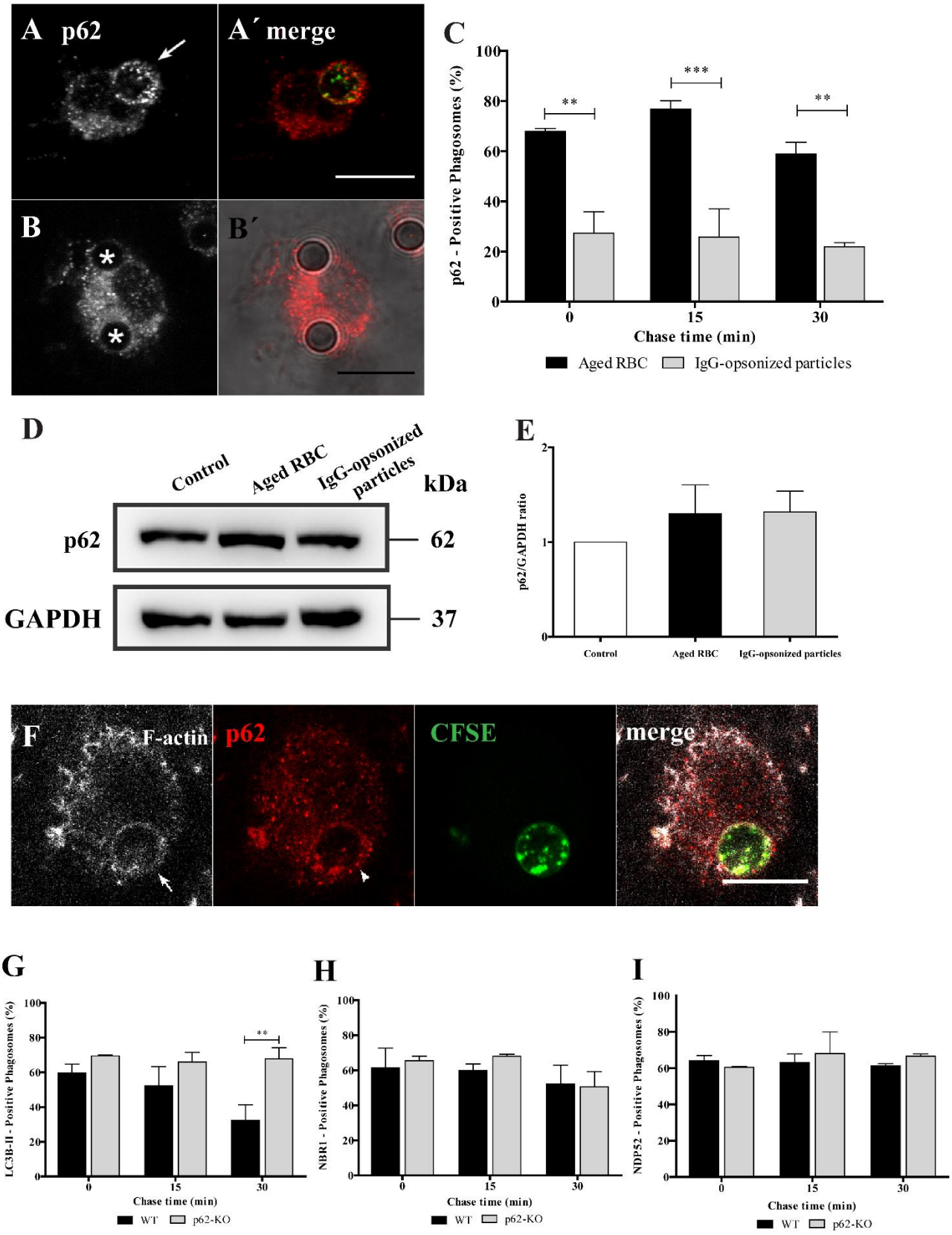

A p62
A´ merge
B
B´
C
p62 - Positive Phagosomes (%)
Chase time (min)
Aged RBC
IgG-opsonized particles
D
Control
Aged RBC
IgG-opsonized particles
kDa
p62
GAPDH
62
37
E
p62/GAPDH ratio
Control
Aged RBC
IgG-opsonized particles
F
F-actin
p62
CFSE
merge
G
LC3B-II - Positive Phagosomes (%)
Chase time (min)
WT
p62-KO
H
NBR1 - Positive Phagosomes (%)
Chase time (min)
WT
p62-KO
I
NDP52 - Positive Phagosomes (%)
Chase time (min)
WT
p62-KO

FIGURE 5. *Effect of p62 in the recruitment of LC3B-II, NBR1 and NDP52 to phagosomes containing RBC cells in BMDM.*
After pulse-chase experiments with RBC or IgG-opsonized particles, WT-BMDM cells were fixed and stained for p62. (A) WT-BMDM containing a p62-positive phagosome at 15 min chase time. (A') Corresponding merged image showing the internalized RBC stained with CFSE. (B) WT-BMDM containing p62-negative phagosomes at 15 min chase time. (B') Corresponding merged image showing the internalized IgG-opsonized particles in DIC. (C) Quantification of p62 positive-phagosomes. (D) p62 levels in total cell lysates of WT-BMDM exposed for 15 min to RBC or IgG-opsonized particles. GAPDH was used as loading control. (E) Ratio of p62/GAPDH of quantified bands in cells exposed or not to RBC, IgG-opsonized particles. Three independent experiments were performed. (F) Representative image of RBC-containing phagosome positive for actin (in white) and p62 (in red). Arrow and arrowhead indicate actin- and p62-positive phagosome, respectively. Quantification of LC3B-II- (G), NBR1- (H) and NDP52- (I) positive phagosomes in WT- BMDM (black bars) and p62-KO- BMDM (grey bars). The values are means ± SEM of, at least, three independent experiments. At each time point, at least, 50 phagosomes were analyzed. **, $p<0.01$; ***, $p<0.001$ comparing differences between p62-positive phagosomes containing RBC and IgG-opsonized particles or differences between of LC3B-II-positive phagosomes in WT- and p62-KO-BMDM.

Because p62 recruitment to the phagosomal membranes preceded that of LC3B-II (Fig. 2A and B), we assessed the requirement of the former in the phagosomal association of the latter. Figure 5G compares LC3B-II dissociation from phagosomal membranes of wild type ($p62^{+/+}$) and $p62^{-/-}$ BMDM and shows that in $p62^{-/-}$ BMDM LC3B-II did not dissociate from these membranes over the periods of time examined, as compared to $p62^{+/+}$ BMDM. As p62 can interact with NBR1 and NDP52 [39], we tested whether recruitment of these autophagy effectors to phagosomes was dependent on p62. As shown in Fig. 5H and I, neither NBR1 nor NDP52 association with phagosomal membranes required p62. Interestingly, the effect of p62 absence in LAP (Fig. 5G) delaying LC3B-II dissociation/degradation from the phagosomal membranes, seemed to have consequences in phagosome maturation and degradation. Phagolysosome biogenesis was assessed by the acquisition of the lysosomal membrane marker LAMP-1 in p62 silenced cells, in which p62 expression was reduced by 70.4 ± 0.08% assessed by qPCR and by Western blot (see Suppl. Fig. 2C,D). As illustrated in Fig. 6A-C, absence of p62 caused only a delay in LAMP-1 acquisition. Indeed, the percentage of LAMP-1-positive phagosomes in p62 silenced cells was only lower when compared to the phagosomes in control cells at 0 min chase time (23.8 ± 6.3% compared with 50.2 ± 9.1%, respectively).

This effect on phagolysosome biogenesis can have an impact in the RBC degradation which in turn can lead to a defective uptake and subsequent oxidative damage. Indeed, the absence of p62 impaired RBC degradation (Fig. 6D and E). Control and p62-silenced cells were

challenged with CFSE-labeled RBC for 15 min and then followed by live-cell confocal microscopy for 300 min. In control cells, the internalized RBC underwent efficient degradation after 60 min chase (assessed by the loss of fluorescence and disappearance of RBC by DIC, Fig.6 D and E upper panels, respectively). In contrast, the absence of p62 led to the failure of RBC degradation showed by the persistence of these RBC even after 300 min chase (Fig. 6D and E lower panels). This suggests that p62 is critical for phagosomal maturation and RBC degradation.

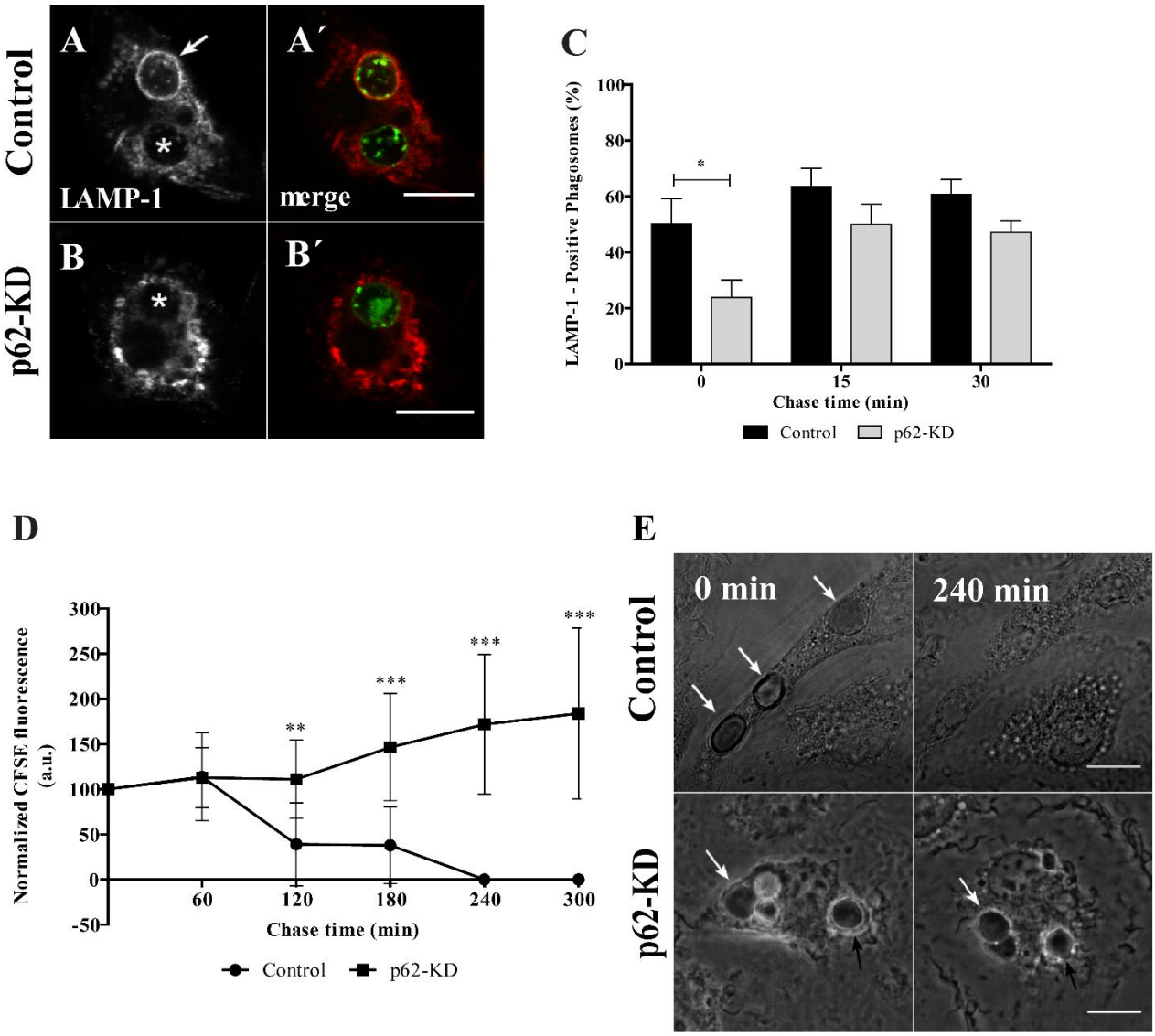
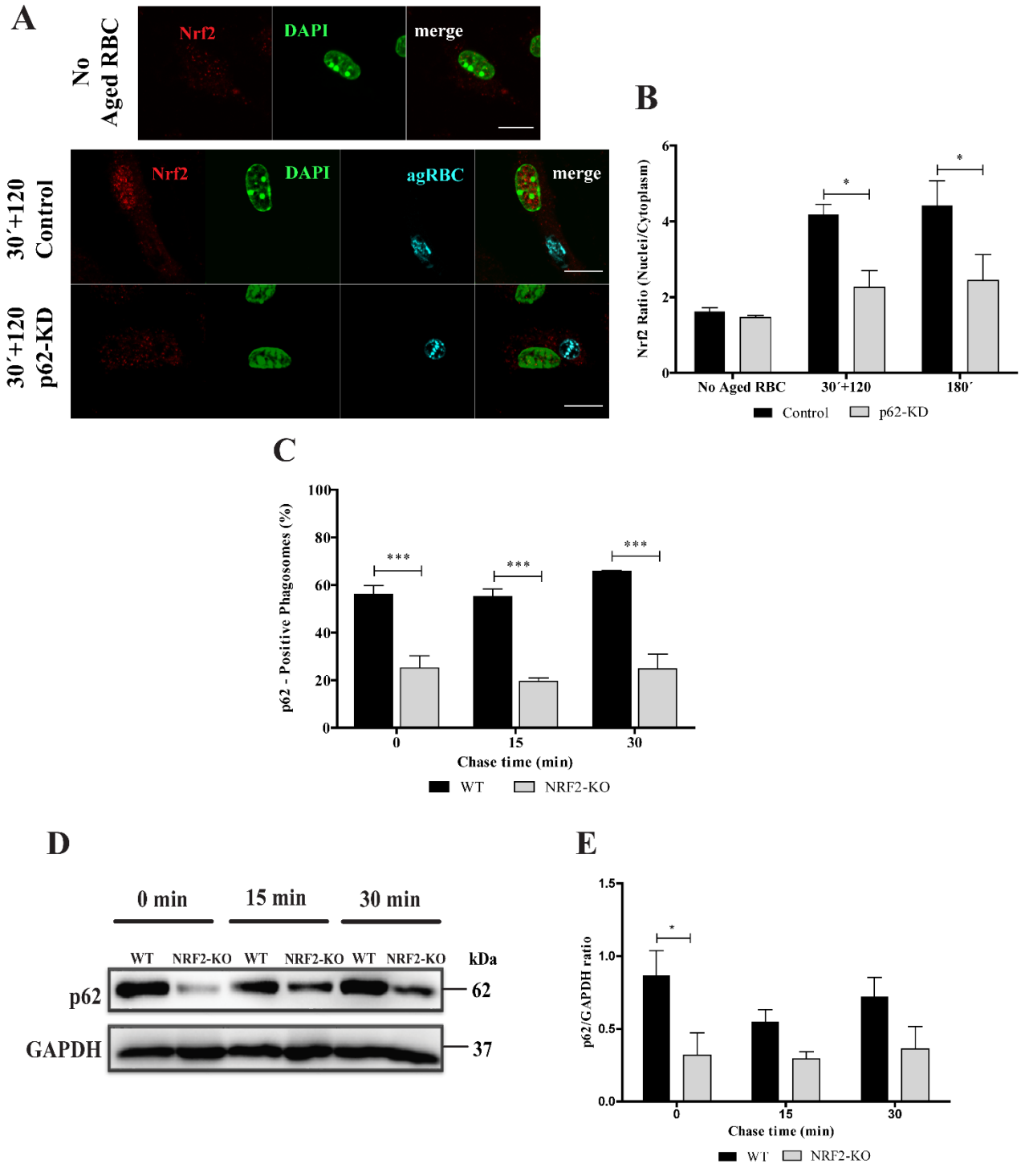


FIGURE 6. *Functional relevance of p62 in RBC degradation.*
After pulse-chase experiments with RBC, control- and p62-silenced BMDM were fixed and stained for LAMP-1. (A) Control-BMDM containing LAMP1-positive and LAMP1-negative phagosomes at 0 min chase time. (B) p62-KD-BMDM containing a LAMP-1-negative phagosome at 0 min chase time. (A´, B') Corresponding merged images showing the internalized RBC stained with CFSE. Arrow indicates a LAMP-1-positive phagosome and asterisks (*) indicate LAMP-1-negative phagosomes. (C) Quantification of LAMP-1-positive phagosomes in WT-BMDM (black bars) and p62-KD-BMDM (grey bars). The values are means ± SEM of, at least, three independent experiments. At each time point, at least, 50 phagosomes were analyzed. *, $p<0.05$, comparing differences between WT- and p62-KD-BMDM. (D, E) Time-lapse experiments of WT- and p62-KD-BMDM challenged with CFSE labeled-RBC for 15 min (0 min chase time) and followed for 300 min further to assess phagosome degradation. Phagosome degradation (D) was measured by the disappearance of fluorescence and the phagosome assessed by DIC (E). Bars, 10 μm. The values are means ± SD of 10 different phagosomes. **, $p<0.01$; ***, $p<0.001$ comparing differences between WT- BMDM and p62-KD-BMDM. (E) DIC images at 0 min and 240 min chase time of WT- BMDM and p62-KD-BMDM. Arrows point to RBC-containing phagosomes.

Erythrophagocytosis is associated with p62-dependent NRF2 activation.

Under basal conditions, NRF2 is ubiquitinated by Kelch-like ECH-associated protein 1 (KEAP1)-Cul3-E3 ubiquitin ligase complex and targeted to the 26S proteasome for degradation. Oxidative stress represses KEAP1 binding to the Cul3–Rbx1 complex, allowing newly transcribed NRF2 to undergo nuclear translocation[40–42]. NRF2 can also be activated via a non-canonical mechanism: phosphorylation of Ser351 on the KIR domain (Keap1-interacting region, aa 346–359) of p62 causing p62's affinity for KEAP1 to significantly increase [43]. Upon nuclear translocation NRF2 heterodimerizes with other basic leucine zipper transcription factors, such as small musculoaponeurotic fibrosarcoma (Maf) proteins and binds to antioxidant response element (ARE) in the promoter of NRF2-regulated genes to induce their transcription expression[44–46]. To acquire more mechanistic insights concerning the role of p62 in erythrophagocytosis we questioned whether erythrophagocytosis was associated with p62-dependent nuclear NRF2 accumulation. As illustrated in Fig. 7A,B, RBC internalization was associated with NRF2 nuclear accumulation, later in the phagocytic process, reaching maximum at 30 min pulse followed by 120 min chase or at 180 min. The fluorescence intensities ratio of NRF2 on nuclei and cytoplasm was roughly twofold in WT-BMDM exposed to RBC compared to unstimulated controls (Fig. 7A and B). This suggests that NRF2 nuclear translocation occurs only after fusion of phagosomes with lysosomes upon RBC degradation (Fig. 6C and D). In absence of p62, NRF2 nuclear translocation was reduced by 50%, when compared with control cells expressing p62 (Fig. 7A and grey columns in B). Next, we assessed the role of NRF2 on phagosomal maturation and RBC degradation, using BMDM

from *Nrf2*$^{-/-}$ mice. Through the entire maturation process the percentage of p62-positive phagosomes in *Nrf2*$^{-/-}$ BMDM was reduced (Fig. 7C) when compared with *Nrf2*$^{+/+}$ BMDM. This could be attributed to lower levels of p62 in *Nrf2*$^{-/-}$ BMDM when compared with *Nrf2*$^{+/+}$ BMDM as shown in the Western Blot (Fig. 7D) and quantified in Fig. 7E.



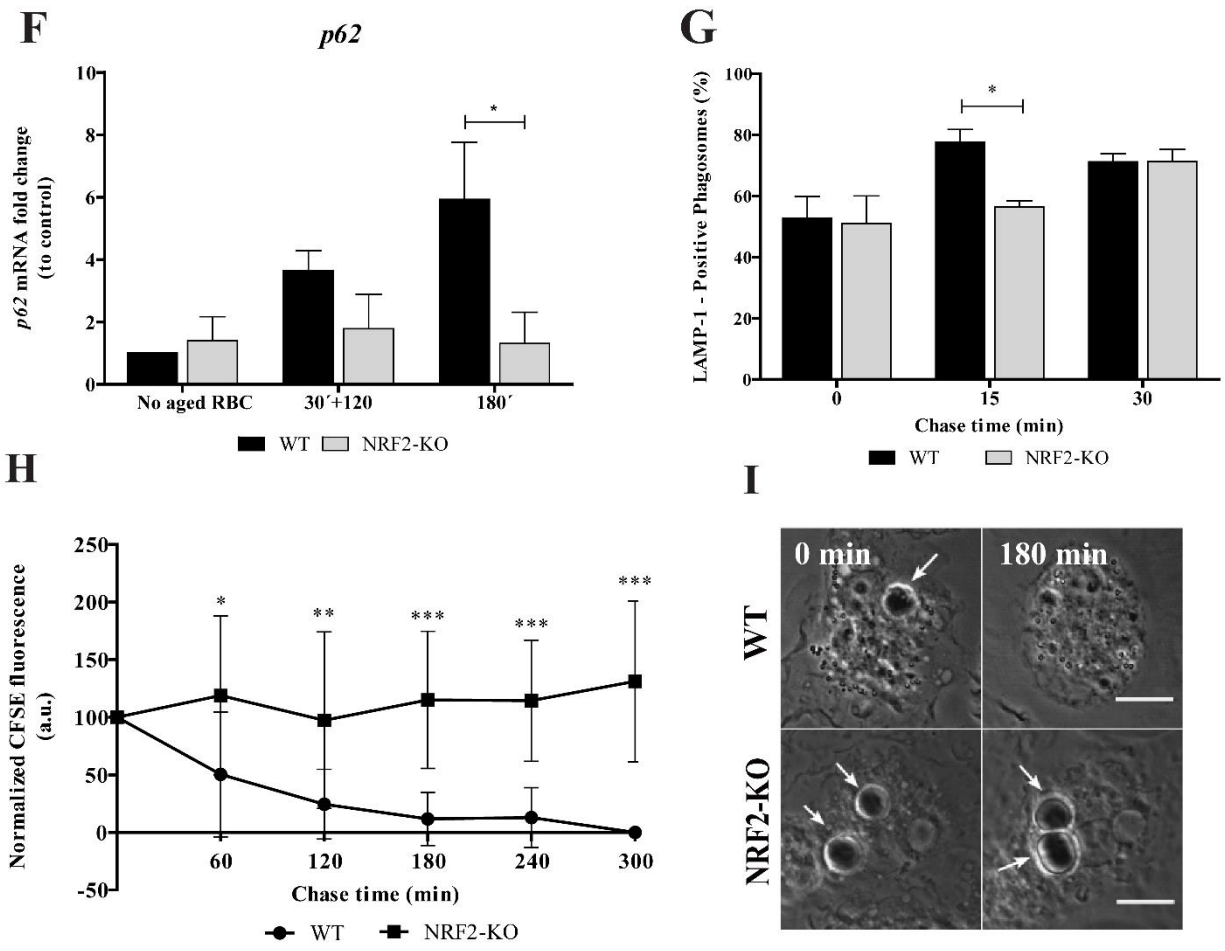


FIGURE 7. *NRF2 is critical for RBC degradation in BMDM.*
WT- and p62-KD-BMDM were fed with CFSE-stained RBC for 30 min and then chased for 120 min or fed for 180 min. (A) Translocation of NRF2 into the nucleus in WT- and in p62-KD-BMDM, assessed by immunostaining, in the absence (first row) or upon incubation with RBC (second and third rows). The second row represents NRF2-nuclear translocation in WT-BMDM. The third row represents NRF2-nuclear translocation in p62-KD-BMDM. NRF2 staining is represented in red, nucleus in green and internalized RBC in cyan. The last panels are merged images. Bars, 10 µm. (B) Quantification of NRF2 nuclear translocation expressed as a ratio of the fluorescence intensity between the nucleus and the cytoplasm. *, $p<0.05$ comparing differences between NRF2 fluorescence in WT-BMDM and p62-KD-BMDM challenged with RBC. (C-F) WT- and NRF2-KO-BMDM were challenged with RBC for 15 min (0 min chase) and then chased for the indicated times in the figures. (C) Quantification of p62-positive phagosomes after fixation and immunostaining for the endogenous protein. (D) p62 levels in total cell lysates of WT- and NRF2-KO-BMDM, for short time points. GAPDH was used as loading control. (E) Ratio of p62/GAPDH of quantified bands in cells exposed to RBC. Three independent experiments were performed. (F) WT- and NRF2-KO-BMDM were challenged with RBC for 30 min and then chased for 120 min or fed for 180 min. The expression of *p62* gene was assessed by RT-qPCR. Data were normalized to the endogenous *Hprt* and *Pgk1* genes. The values are means ± SEM expression levels of three independent experiments, each measured in two technical replicates. *, $p<0.05$.
(G) Quantification of LAMP-1-positive phagosomes after fixation and stained for LAMP-1. The values are means ± SEM expression levels of three independent experiments, each measured in two technical replicates. *, $p<0.05$.
(H, I) Time-lapse experiments of WT- and NRF2-KO-BMDM challenged with CFSE labeled-RBC for 15 min (0 min chase time) and followed for 300 min further to assess phagosome degradation.

Phagosome degradation was analyzed as described in the legend of Fig.6. The values are means ± SD of 10 different phagosomes. *, $p<0.05$; **, $p<0.01$; ***; $p<0.001$ comparing differences between WT- and NRF2-KO-BMDM. (H) DIC images at 0 min and 180 min chase time of WT- and NRF2-KO-BMDM. Arrows point to RBC-containing phagosomes.

Finally, we assessed whether NRF2 nuclear translocation during erythrophagocytosis is associated with p62 transcription (Fig. 7F). p62 expression increased after NRF2 translocation and absence of NRF2 showed a significant inhibition of p62 expression (5.93 ± 2.23 *versus* 1.31 ± 1.23) at 180 min of erythrophagocytosis. Altogether, these results strongly suggest the existence of a positive feedback between NRF2 and p62 in which p62 is required for NRF2 nuclear translocation during erythrophagocytosis, which induces the levels of P62 expression.

Transport of RBC-containing phagosomes to the lysosomes was only slightly affected in *Nrf2*$^{-/-}$ vs. *Nrf2*$^{+/+}$ BMDM (Fig. 7G), while the effect on RBC degradation was very striking (Fig. 7H and I). These two outcomes were similar to the results obtained in p62 silenced BMDM (Fig. 6D and E), suggesting that p62 and NRF2 are involved in the same pathway.

The non-canonical NRF2-signaling pathway activation[43] is associated with p62 phosphorylation at S351, by mTORC1, decreasing NRF2 binding affinity towards KEAP1 and therefore inducing the expression of NRF2-target genes. As illustarted in the Fig. 8A and quantified in the Fig. 8B, p62 S351 phosphorylation occurred in the phagosomal membranes containing RBC at early time points and tended to increase with time. In BMDM challenged with agRBC an increase in the total p62 S351 phosphorylation was observed when compared to BMDM not exposed to agRBC (Fig. 8C).

Since heme is a pro-oxidant molecule, we tested the involvement of NRF2 in the transcription of anti-oxidant genes, including thioredoxin-1, ferritin, glutathione S-transferase, peroxidoxin-1, heme oxygenase 1 (*Hmox1*), catalase, superoxide dismutase (*Sod*) 1 and 2. Among these, only the expression of *Hmox1* and *Sod2* were increased after NRF2 translocation in BMDM challenged with RBC (Fig. 8D and E, black bars). As observed in Fig. 8D, HO-1 expression was reduced in *Nrf2*$^{-/-}$ *vs.* *Nrf2*$^{-/-}$ BMDM, after erythrophagocytosis. Although, no statistical significances were observed for *Sod2* gene expression (Fig. 8E), it was possible to see a slight difference at 180 min pulse time in *Nrf2*$^{-/-}$ BMDM when compared with Nrf2$^{+/+}$ BMDM. Thus, the involvement of NRF2 on *Hmox1* and *Sod2* expression can be attributed to the intracellular release of potentially pro-oxidant labile heme, which occurs

after RBC degradation within the lysosomes. Accordingly, we conclude that NRF2 is both part of the machinery required for RBC degradation as well as for the anti-oxidative response.

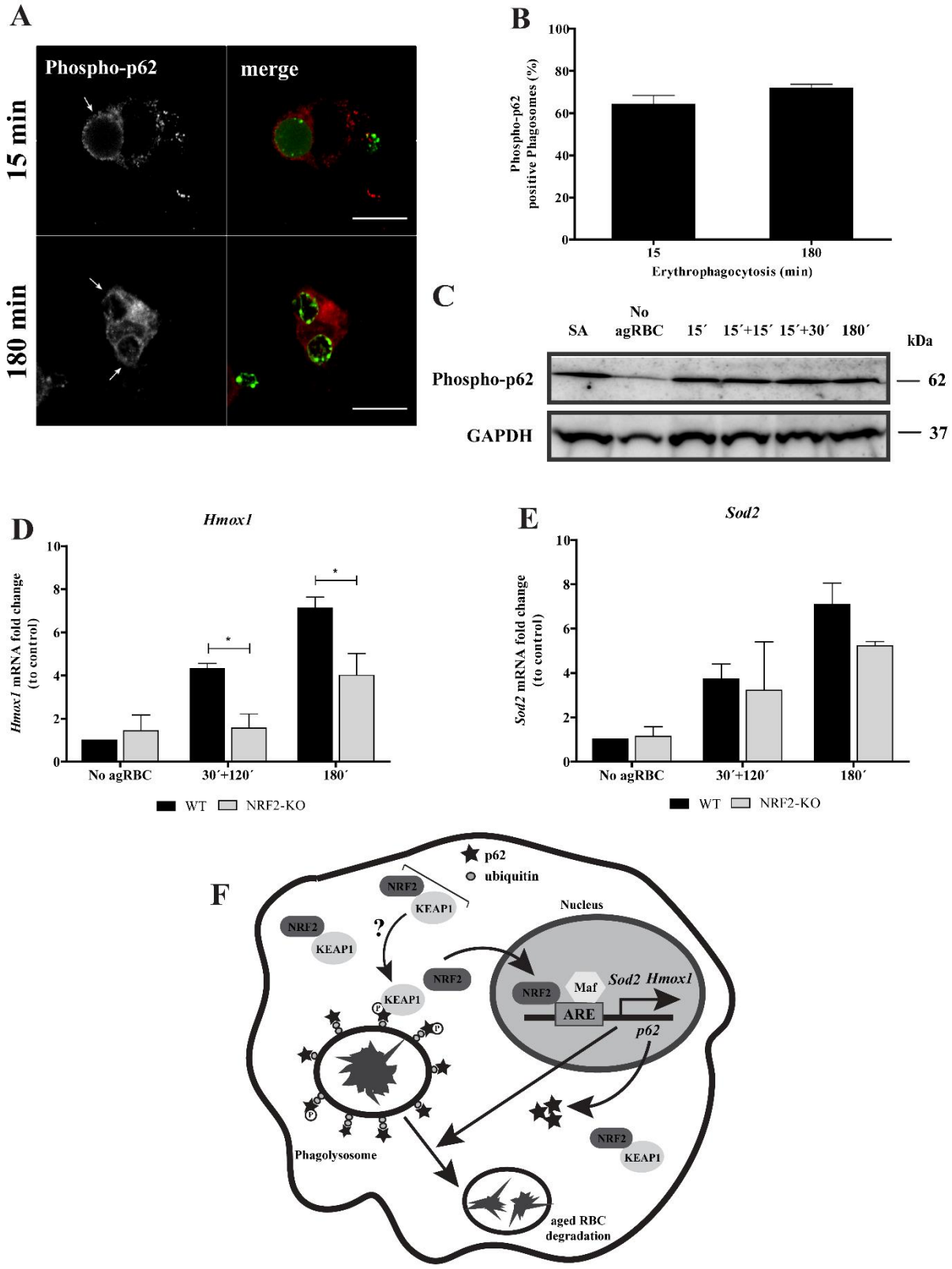


FIGURE 8. *p62 Phosphorylation and NRF2-target genes expression upon erythrophagocytosis.*
WT-BMDM were fed and chased with CFSE-labeled RBC for the times indicated and imunostained for phospho-p62 (A). (B) Quantification of phosphorylated p62-positive phagosomes. The values are means ± SEM expression levels of 2 independent experiments. (C) phosphorylated-p62 levels in cell lysates exposed to RBC. GAPDH was used as loading control. Two independent experiments were performed. As positive control, Sodium arsenite was added for 12 h to a final concentration of 10 µM. In D-E, WT- and NRF2-KO-BMDM were challenged with RBC for 30 min and then chased for 120 min or fed for 180 min. The expression of *Hmox1* (D), *Sod2* (E) genes was assessed by RT-qPCR. Data were normalized to the endogenous *Hprt* and *Pgk1* genes. The values are means ± SEM expression levels of three independent experiments, each measured in two technical replicates. *, $p<0.05$.
(F) Working Model: Our model suggests that degradation of RBC by macrophages is dependent on p62/NRF2 signaling pathway. p62 is recruited to RBC-containing phagosomes decorated with ubiquitin shortly after erythrophagocytosis, followed by phosphorylation in the S351 residue and KEAP1 acquisition, by an unknown mechanism. Then, after phagolysosome formation NRF2 is translocated to the nucleus where together with small Maf proteins, binds to ARE in promoter region of *Ho-1*, *p62* and *Sod2* genes, inducing their expression. This molecular machinery promotes aged RBC degradation and controls oxidative stress.
NRF2 - nuclear factor erythroid derived 2-like 2; KEAP1 - Kelch-like ECH-associated protein 1; ARE – antioxidant response element; Maf - small masculoaponeurotic fibrosarcoma; *Sod2* - superoxide dismutase 2; *Hmox1* – heme oxigenase 1.

DISCUSSION

While erythrophagocytosis is critical to the regulation of iron/heme metabolism and maintenance of homeostasis, our understanding of the molecular processes underlying the maturation of phagosomes containing RBC and their subsequent degradation by hemophagocytic macrophages is quite rudimentary. In this work we provide some new insights into the biogenesis of phagolysosomes containing RBC, their maturation, and the ordered degradation of RBC in both non-professional and professional phagocytes. We show that the process is complex and involves a convergence of endocytic and autophagic processes. When RBC are phagocytosed, p62 and NRF2 are critical for phagolysosome biogenesis and degradation. Our findings also show that beyond the involvement of LC3B-II, other components of the selective autophagy machinery such as NBR1, NDP52 and p62 are also recruited to the single membrane phagosomes.

Among the selective autophagy machinery tested, the most interesting outcome was observed for p62. This protein is recruited mostly to phagosomes carrying RBC but very weakly to phagosomes containing IgG-opsonized particles, suggesting not only that it has a negligible role in Fc-mediated phagocytosis but also the existence of different types of LAP. The fact that these results were observed in both non-professional and professional phagocytes suggests a

conserved role of p62 in RBC-containing phagosomes maturation and degradation. p62 associates with phagosomal membranes at very early stages of the RBC-phagocytic process, co-localizing with F-actin. We attempted to understand what signals the association of p62 with the phagocytic cups. Among other domains, p62 has a LC3-interacting motif (WXXL/I) called the LC3-recognition sequence (LRS) or the LC3-interacting region (LIR) as well as ubiquitin binding domains [23,47,48]. Our results indicate that p62 is not recruited to phagosomal membranes via interaction with LC3B-II since this protein associates with the phagosomal membranes after p62 and when F-actin is no longer detected on phagosomes. Using a pharmacological approach, we found that the recruitment of p62 might rely, at least in part, via the interaction of its ubiquitin-binding domain with ubiquitinated components of the phagosomal membranes.

The absence of p62 affects phagolysosome biogenesis with striking effects on RBC degradation. Though p62 has been shown to mediate intracellular xenophagic degradation of bacteria that undergo ubiquitination in response to their escape from phagosomes and subsequent formation of a double membrane organelle [34,36,49], to our knowledge this is the first time that the requirement of p62 for phagocytic cargo degradation within a single membrane organelle is reported. Interestingly, when RBC within the phagolysosome start to be degraded, NRF2 translocates to the nucleus. The translocation of this transcription factor is p62-dependent and in its absence, RBC degradation is blocked suggesting that p62 and NRF2 act together to degrade these phagocytic particles. We enquired how and why p62 and NRF2 interact upon erythrophagocytosis. In xenophagy, p62 is translocated to autophagosomes containing invasive *Salmonella* leading to Ser351 phosphorylation in the KIR motif, enhancing the interaction between p62-KEAP1 and consequently NRF2 translocation [50]. Furthermore, under amino acid rich conditions the mTORC1 has been identified as one of the kinases responsible for the phosphorylation of p62 [43]. Interestingly, NRF2-target genes are induced at the same time that autophagosomes entrap the microbes [50]. Our data, however, reveal a different sequential dynamic of p62 and NRF2 in response to RBC engulfment. In erythrophagocytosis, association of p62 with phagosomal membranes occurs at very early stages while NRF2 is activated later. Phosphorylation of p62 in its Ser351 residue occurs at early stages of phagocytosis and increases with time. We propose that once the RBC starts to degrade in the phagolysosome, and their contents are imported into the phagocyte cytosol for storage or recycling, phosphorylation of the p62 residue Ser351 increases, through an

unknown mechanism, culminating with NRF2 translocation to the nucleus and induction of some of its transcriptional targets (Fig. 8F). In erythrophagocytosis, we could detect the up-regulation of three well-known NRF2-target genes: *p62*, *Hmox1* and *Sod2*. The results obtained for p62 confirm the positive feedback that exists between NRF2 and p62 that has been already reported for other experimental settings [44–46]. The up-regulation of the other two genes could result from the products of RBC degradation and reactive oxygen species formation. In erythrophagocytic macrophages, RBC processing is followed by heme release from hemoglobin and its subsequent translocation from the phagolysosome lumen into the cytoplasm, via a mechanism assisted by the heme responsive gene 1 transporter (HRG1) [16,51,52]. Once in the cytosol, the heme catabolism enzyme HO-1 releases iron from the protoporphyrin ring for storage or reuse. Thus, the increase in concentration of cytosolic heme may explain the increase of HO-1 expression. Besides iron extraction from protoporphyrin, HO-1 generates equimolar amounts of biliverdin and carbon monoxide (CO), two anti-oxidant molecules[53,54]. *Sod2* up-regulation could be explained by an attempt of the macrophages to scavenge mitochondrial superoxide and thereby lower oxidative stress. Overproduction of reactive oxygen species has been described to be linked to impaired lysosomal function resulting from *changes in* cysteine residue of the Atp6v1a1 subunit of vATPase and its subsequent failure in acidifying the lysosomes [55–57]. Since lysosomal pH is a key determinant of lysosomal enzyme activity this could explain why in the absence of NRF2 or p62, RBC are not degraded. Finally, some autophagy genes (such as *Ndp52* and *Lc3b*) as well as *Lamp* were demonstrated to be upregulated by the NRF2 signaling pathway [58] which in turn can contribute to the rapid degradation of RBC reinforcing the critical role of NRF2 in this process.

Although the mechanism via which the p62/NRF2 pathway modulates the degradation of RBC is far from being elucidated, with this work we reinforce the view that this non-canonical signaling pathway is activated in the absence of oxidative stress or under autophagic conditions [44–46,59,60]. Furthermore, we were able to identify new molecular machinery involved in erythrophagocytosis of RBC, opening new avenues for specific targeting and modulation of this process. This new knowledge may have a critical role in a number of hemolytic disorders associated with defects in RBC function that can lead to premature RBC senescence, such as sickle cell disease.

MATERIAL AND METHODS

Cell culture

Rabbit vascular smooth muscle cells, used as a non-professional phagocytic cell line, were purchased from ATCC (Manassas, VA, USA) and maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% HI-FBS and 100 U/mL antibiotics. Cells were grown in a humidified incubator at 37ºC under 5% CO_2 atmosphere and passaged every 3 days with a 1/10 dilution.

Cells stably expressing Fcγ-RIIA were generated as described before [18,61]. Cells were plated in 24-multiwell plates at a density of 30×10^3 cells per well and grown on glass cover slips for 24 h. For experiments with the E1 inhibitor PYR-41 (Calbiochem, San Diego, CA, USA), 20×10^3 cells were plated per well and 24 h after, the drug was added.

L929 cell line (kindly provided by Prof. Ira Tabas, Columbia University, NY, USA) was cultured to produce L-cell conditioned media (LCCM) enriched in M-CSF to differentiate monocytes into macrophages, as previously described [62].

Bone marrow-derived macrophages (BMDM) were obtained from 8-12 week old C57BL/6J wild-type (WT), p62-knockout (kindly provided by Prof. Herbert Virgin, Washington University School of Medicine, St. Louis, MO, USA) and NRF2-KO mice. Primary macrophages were maintained as described [63], but in RPMI-1640 medium, containing 10% HI-FBS and 30 % LCCM. In the phospho-p62 experiments, BMDM were treated with 10 μM sodium arsenite (Sigma-Aldrich, St.Louis, MO, USA) for 12 h as positive control.

Mice were bred and maintained under specific pathogen-free (SPF) conditions, according to protocols approved by local (Instituto Gulbenkian de Ciência) and national (Portuguese Official Veterinary Department; Direcção Geral de Veterinária) ethics committees according to the Portuguese (Decreto-Lei 113/2013) and European (Directive 2010/63/EU) legislations. C57BL/6 $NRF2^{-/-}$ mice were provided by the RIKEN BioResource Center (Koyadai, Tsukuba, Ibaraki, Japan). C57BL/6J wild-type and $NRF2^{-/-}$ mice were co-housed from weaning (3-4 weeks old) to the date of the experiments.

Red blood cells were obtained from human blood collected from healthy volunteers at CNC and CEDOC. Written informed consent was obtained from all volunteers and approved by the Ethical Review Board of the Faculty of Medicine of the University of Coimbra and NOVA Medical School. RBC were isolated and aged as described before [18].

Phagocytosis and phagosomal maturation assays

Aged RBC were prepared as described before [18]. 3.87 µm Latex Beads (Bangs Laboratories Inc., IN, USA) were washed three times with PBS for 3 min at 13 200 rpm, resuspended with human serum IgG (Sigma-Aldrich) at a final concentration of 1.6 µg/µL in PBS and then incubated overnight (ON) at 4°C in an orbital rotator, at the maximum speed. After the pulse time, cells incubated with IgG-opsonized beads were incubated with Phalloidin 633 dye (1:800; ThermoFisher Scientific; A22284) for 5 min on ice, to label the non-internalized beads and then extensively washed with PBS.

Phagocytosis and phagosomal maturation assessment (pulse-chase) experiments were performed as explained in Material and Methods of the Manuscript 1. In pulse-chase experiments the pulse time was 30 min for non-professional phagocytes and 15 min for BMDM, followed by the chase times indicated in the graphs abscissa. To assess poly/mono-ubiquitylation of phagosomal membrane proteins, PYR-41 (given by Henrique Girão, CNC-IBILI, University of Coimbra), an E1 ubiquitin-activating enzyme inhibitor, was incubated ON at a final concentration of 5 µM for agRBC and 10 µM for IgG-opsonized beads, before the phagocytic assays. This inhibitor was present throughout the pulse-chase experiments.

When the purpose of the experiment was the visualization of phagocytic cups, the phagocytic cells were challenged with phagocytic particles without synchronization and without RBC lysis.

RNAi experiments

Seven days after BMDM differentiation, cells were plated in 48-(50 x 10^3 cells/well), 6-(44 x 10^4 cells/well) or 24-(88 x 10^3 cells/well) well plates. To knockdown (KD) p62 and Rubicon in BMDM, a siRNA smart pool (GE Healthcare Life-Sciences, Dharmacon) against p62 and a non-targeting sequence siRNA, scramble (control) were used for reverse transfection. siRNA´s were mixed with Lipofectamine® RNAiMAX Reagent (Thermo Fisher Scientific Inc.) in Opti-MEM® (Thermo Fisher Scientific Inc.). 72h after incubation and before the experiments knockdown of both genes was confirmed by Real-time quantitative RT-PCR (qPCR) and western blot (WB) (only for p62).

Immunofluorescence and microscopy

After phagocytic assays, cells were fixed with 4% PFA for 30 min, permeabilized using 0.1% Triton X-100 (with 200 nM glycine) for 30 min and blocked with 0.5% Gelatin from cold water fish skin in PBS for 30 min. The exceptions were: LAMP-1 staining in which cells were permeabilized using methanol for 10 min; phospho-p62 in which cells were permeabilized for 10 min and no blocking was performed; and for NRF2 in which permeabilization and blocking were performed with 0.25% Triton X-100 and 1% BSA/10% FBS in 1X PBS/0.1% Tween-20, respectively. Then, the cells were incubated with the appropriate primary antibody for 90 or 120 min at room temperature (RT) or ON at 4^0C, followed by incubation with secondary antibody, as listed in Tables III and IV.

Table IV. List of primary antibodies used for immunostaining.

	Antibody and host species	Dilution	Incubation Time	Company; Reference
Cell Lines	Rabbit anti-ubiquitin	1:50	1h 30 at RT	DAKO;20458
	Mouse anti-NBR1	1:80	ON at 4°C	Tebu-bio; H00004077-M01
	Rabbit anti-NDP52	1:80	ON at 4°C	Abcam; ab68588
	Rabbit-anti p62	1:80	2 h at RT	ABGENT; AP2183b
Primary Cells	Rat anti-LAMP-1	1:50	2 h at RT	Developmental Studies Hybridoma Bank; 1D4B
	Rabbit anti-p62	1:50	2 h at RT	ABGENT; AP2183b
	Rabbit anti-LC3B-II	1:50	1h 30 at RT	Cell Signaling; 2775
	Mouse anti-NBR1	1:50	ON at 4°C	Tebu-bio; H00004077-M01
	Rabbit anti-NDP52	1:50	ON at 4°C	Abcam; ab68588
	Rabbit anti-NRF2	1:50	2 h at RT	Cell Signaling
	Mouse anti-phospho(S351)-p62	1:50	1h 30 at RT	MBL Corporation; M217-3

Table V. List of secondary antibodies used for immunostaining.

Antibody and host species	Dilution	Incubation Time	Company; Reference
Cy3 Donkey anti-rat	1:500	1 h at RT	Jackson ImmunoResearch; 712-175-153
Cy3 Donkey anti-mouse	1:500	1 h at RT	Jackson ImmunoResearch; 715-165-150
Cy3 Donkey anti-rabbit	1:500	1 h at RT	Jackson ImmunoResearch; 711-165-152

For phagocytic cups visualization, phalloidin conjugated with Cy5 (1:100, Invitrogen), to stain F-actin, was added with the secondary antibodies. Stained samples were mounted with Mowiol/DABCO (Calbiochem) and analyzed by using a laser scanning confocal microscope (Carl Zeiss, Jena, Germany, LSM 510 software) or a Zeiss Cell Observer with a 63× oil immersion objective (NA = 1.30). The images were analyzed by using LSM Image Browser, Image-J software or Zen software.

For live-cell imaging, after differentiation, BMDM were plated on Glass Bottom Microwell Dishes of 35 mm (MatTek Corporation, Ashland, MA, USA) and silenced for p62. Before imaging, primary cells were preloaded with CFSE-labeled agRBC for 7 min on ice to synchronize and 15 min (0 min chase time) at 37°C for erythrophagocytosis. After this time point, agRBC were lysed in water and analyzed under the microscope. Confocal images were acquired on a Carl Zeiss LSM 710 META laser scanning confocal microscope (ZEN software, Germany), 63× oil immersion objective (NA = 1.30), the argon laser (488 nm), and spectral detection adjusted for the emission of the CFSE dye. Microscope temperature was turned on to 37°C and culture media was supplemented with HEPES. The time-course of degradation (chase time) of internalized agRBC was measured by the disappearance of CFSE fluorescence for 300 min and the phagosomes visualized by phase contrast**.** ImageJ 1.48v software was used to prepare the images and fluorescence intensity of RBC containing phagosomes at 15 min pulse was normalized to 100.

Quantitative PCR

Cells were seeded in 6-well plates when the aim of the experiment was to measure mRNA Nrf2-target genes expression. After the specific treatments, cells were washed three times with sterile ice cold PBS and total RNA extraction was followed using NZY Total RNA Isolation Kit (NZYTech, Lisboa, Portugal). Absorbance measurements at 260 nm in water were used to adjust the stock concentration of all RNA samples to 1 µg/µl, assuming an absorbance of 1 is equivalent to RNA at 40 µg/ml. cDNA synthesis, starting with 1 µg of total RNA, was performed using the iScript™ cDNA Synthesis Kit (Bio-Rad, Warszawa, Poland) in a PCR Bio-Rad T100 Thermal Cycler, following the manufacturer´s protocol. *p62* primers were designed using the Primer Blast from NCBI, *HPRT* primers were ordered from Bio-Rad and the remaining primers used in the experiments were provided by Miguel Soares (IGC, Oeiras, Portugal). Before proceeding for real-time quantitative PCR (qPCR) and after design to hybridize with murine-specific sequences exclusively, all pairs of primers (used at a 500 nM final concentration) were optimized in order to check the correct annealing temperature. Reactions were performed using SsoFast™EvaGreen®Supermix (Bio-Rad) and run in an Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific Inc.). Thermocycling conditions were: Enzyme activation, 95°C x 1 min, 1 cycle; Denaturation, 95°C x 15 sec, 40 cycles; Annealing, temperature variable x 15 sec, 40 cycles; Extension, 65°C x 30 sec, 40 cycles. The melting step was performed to confirm the presence of primer dimers: 95°C x 15 sec, 60°C x 30 sec, 95°C x 15 sec. The primers used in the experiments are listed on the Table V. The relative quantification of the target genes was calculated following the new mathematical model developed by Michael W.Pfaffl, taking into account the real-time PCR efficiencies of the target and reference gene transcripts, relative to control. Hypoxanthine phosphoribosyltransferase 1, *Hprt1*, (QIAGEN, Hilden, Germany) and *Pgk1* (Sigma-Aldrich, St.Louis, MO, USA) were used as housekeeping genes. At least three independent experiments were performed, each one measured in two technical replicates.

Table VI. Primers used to analyse NRF2-dependent genes expression and confirm *p62* and *Rubicon* Knockdown.

Target		Primer Sequence (5´to 3´)	Annealing Temperature(°C)
Sod2	Forward	TAAGGGTGGTGGAGAACCCAAAGGAG	59.0
	Reverse	TTATTGAAGCCAAGCCAGCCCCAG	
Hmox1	Forward	AAGGAGGTACACATCCAAGCCGAG	63.1
	Reverse	GATATGGTACAAGGAAGCCATCACCAG	
p62	Forward	GTCTTCTGTGCCTGTGCTGGAA	57.3
	Reverse	TCTGCTCCACCAGAAGATCCCA	
Pgk1	Forward	ATGGATGAGGTGGTGAAAGC	57.3
	Reverse	CAGTGCTCACATGGCTGACT	
Rubicon	Forward	GAGGCCCCAGGAATATCACC	55
	Reverse	GTGGGCGTTTTCCTTTTCCAG	

Western Blot Analysis

After experimental procedures, cells were lysed and the lysates blotted as described [64]. Cells were washed three times with sterile ice cold 1X PBS and lysed with 1X RIPA buffer (50 mM Tris-HCl pH 8.0, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS, 2 mM EDTA and 150 mM NaCl), supplemented with 25 X Protease Inhibitor Cocktail (Roche). Lysates were cleared by pipetting up and down for three times on ice. Then, a centrifugation at 4°C, 20 000 x *g*, for 30 min was performed and the supernatant was carefully removed and stored at -80°C. Protein was quantified using *DC*™Protein Assay (Bio-Rad) and 20 µg per sample were resolved on a 12% SDS polyacrylamide gel using the Mini-Protean®Tetra Cell (Bio-Rad). Separated proteins were transferred onto activated polyvinylidene difluoride (PVDF) membranes (Merck) in 1X transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) at 4°C, 300 mA, for 2 h. Membranes were blocked by incubation with blocking solution (5% non-dry fat milk in TBS-T (250 mM Tris-Base pH 7.6, 1.5 M NaCl, 0.1% Tween) for 1 h at RT in an orbital shaker. Antibodies used in WB are listed below in Table VI. Blots were developed with ECL (GE Healthcare Life-Sciences) using the Chemidoc XRS Imager (Bio-Rad). GAPDH and tubulin were used as loading controls. Primary antibodies were diluted in 5% blocking solution and

secondary antibodies in 1% blocking solution. Between each antibody incubation, membranes were washed three times in TBS-T for five minutes each.

Table VII. Primary and secondary antibodies used in Western Blot.

	Antibody and host species	Dilution	Incubation Time	Company; Reference
Primary Antibodies	Mouse anti-p62	1:500	ON at 4°C	Abnova; M01, Clone 2C11
	Mouse anti-tubulin	1:10 000	1h at RT	Sigma; T6199
	Goat anti-GAPDH	1:1000	1h at RT	SICGEN; Ab0049
	Mouse anti-phospho(S351)-p62	1:500	ON at 4°	MBL Corporation; M217-3
Secondary Antibodies	Sheep anti-mouse HRP (for p62)	1:10 000	1h at RT	GE Healthcare Life-Sciences; NA931V
	Goat anti-mouse HRP (for tubulin and phospho-p62)	1:5000	1h at RT	Bio-Rad; 1706516
	Rabbit anti-goat HRP (for GAPDH)	1:5000	1h at RT	Bio-Rad; 1721019

Statistical analysis

Statistical analysis (t-test or Two-way ANOVA followed by Bonferroni post-test) was applied using GraphPad PRISM software version 5. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$ were considered as statistically significant. Except for the live imaging experiments which standard deviation (SD) was applied, all results are presented as means ± standard error of the mean (SEM), from at least three independent experiments.

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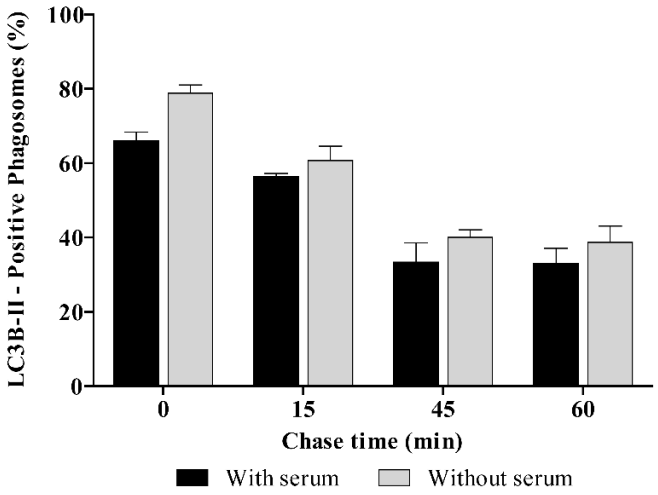
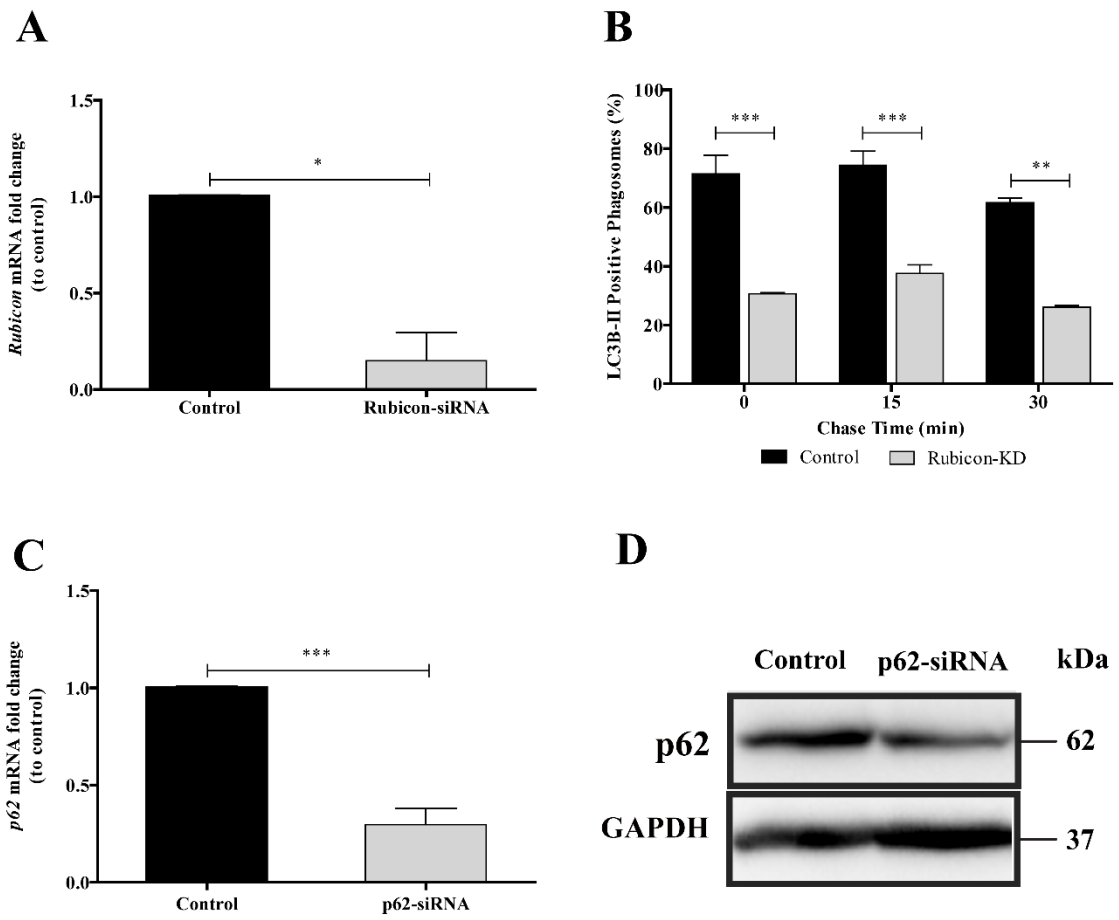
SUPPLEMENTARY MATERIAL



SUP FIGURE 1: *Association of LC3B-II with phagosomal membranes in the absence of serum.* Quantification of LC3B-II-positive phagosomes in non-professional phagocytes incubated in medium supplemented (black bars) or not/starved (grey bars) with serum, exposed to RBC and chased for the indicated times. The values are means ± SEM of, at least, three independent experiments. At each time point, at least, 100 phagosomes were analyzed.



SUP FIGURE 2: *Rubicon and p62 silencing and association of LC3B-II with phagosomal membranes in the absence of Rubicon, in BMDM.*
(A) *Rubicon* mRNA fold change in knockdown versus control cells (transfected with scramble RNAi) was determined by RT-qPCR. Data were normalized to the endogenous *Hprt* and *Pgk1* genes. The values are means ± SEM expression levels of two independent experiments, each measured in two technical replicates. *, $p<0.05$ comparing differences between scramble and knockdown cells. (B) Quantification of LC3B-II-positive phagosomes in BMDM compared control *versus Rubicon*-silenced cells, exposed to RBC and chased for the indicated times. The values are means ± SEM of two independent experiments. ***, $p<0.001$; **, $p<0.01$. (C) *p62* mRNA fold change in knockdown versus control cells (transfected with scramble RNAi) was determined by RT-qPCR. Data were normalized to the endogenous *Hprt* and *Pgk1* genes. The values are means ± SEM expression levels of four independent experiments, each measured in two technical replicates. ***, $p<0.001$ comparing differences between scramble and knockdown cells. (D) Western Blot of p62 expression levels in knockdown versus control cells (transfected with scramble RNAi). GAPDH was used as loading control.

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AUTHOR CONTRIBUTIONS:

Conceived and designed the experiments: IBS, MSV, AMR, MPS and OVV

Performed the experiments: IBS, MSV, NSD

Analyzed the data: IBS, MSV, MPS and OVV

Contributed reagents/materials/analysis tools: AMR, MPS and OVV

Wrote the paper: IBS, MSV, MPS and OVV

The authors declare no competing financial interest.

CHAPTER IV

General Discussion

4.1 Discussion

RBC due to their limited lifespan must be efficiently removed in order to avoid systemic hemolysis resulting in the accumulation of free heme, which is known as a pro-oxidant molecule [1]. Over the last few years, there has been a major effort to understand iron and heme metabolism following RBC internalization and the degradation of their contents. These findings contribute not only towards offering insights into the molecular mechanisms of iron handling by macrophages but more importantly, the physiological consequences when homeostasis is disrupted. However, less is known regarding the internalization and degradation of senescent/damaged RBC. Therefore, in the first part of this PhD project, we aimed to identify some of the players involved in the processing of senescent/damaged RBC models to understand if different targets-receptor engagement leads to differential responses in macrophages. For that, we developed an in vitro model as close as possible to the physiological erythrophagocytosis process, using primary macrophages and sheep or human RBC. The use of erythrocytes as phagocytic particles has several advantages: First, erythrocytes without surface alterations do not bind to phagocytes; Second, hypotonic lysis can be used to distinguish between bound particles and those that have been ingested by phagocytes; Third, most of the protocols used in the literature resemble RBC as models for phagocytic particles; Fourth, PS may be deliberately inserted into its membrane, allowing recreation of one of the hallmark characteristics of apoptotic cells. Although erythrocytes cannot undergo apoptosis due to the absence of nuclei, aged red blood cells are used as apoptotic models due to the exposure of PS that signals their removal from the circulation by macrophages (eryptosis as mentioned in chapter II).

The results from Chapter II showed that agRBC-containing phagosomes fuse with lysosomes with a delay compared to other phagocytic particles, and therefore the process of phagosomal maturation is retarded. This observation agrees with a publication that shows phagosomal maturation kinetics are dependent on the type of target [2]. Indeed, agRBCs stimulate macrophage membrane ruffling with the formation of a spacious phagosome implying a larger surface area and the involvement of a higher number of receptors. In contrast, Fc-mediated erythrophagocytosis leads to the formation of a tiny restricted phagosome. There is no consensus concerning the signals involved in actin remodeling and

how complement-opsonized targets are internalized [3,4]. However, our data shows that C3bi-mediated erythrophagocytosis triggers the formation of a phagosome similar to Fc-mediated erythrophagocytosis.

It has been shown [5,6] that upon RBC senescence, IgG NAbs bind to band 3 protein, mediating the internalization of agRBC. However, NAbs are not efficient opsonins in vivo, due to their low affinity and low circulation numbers. Additionally, it has been hypothesized that phagocytosis of senescent RBC can be enhanced by the activation of the classical pathway of the complement system after NAb binding. Once the classical pathway is activated, a significantly lower amount of NAb is needed for the induction of phagocytosis [7]. In our descriptive in vitro study, we found that there were no differences in the phagosomal maturation of IgG-opsonized RBC and C3bi-opsonized RBC and this outcome could be due to the fact that we induced the deposition of a single opsonin in the RBC membrane, producing different signalling responses in macrophages.

In the same study, we used BMDM and PM as the main phagocytes of RES and comparing the maturation kinetics of agRBC-containing phagosomes in these different subsets, it was possible to see a prominent reduction in agRBC maturation in the BMDM (black bars in Fig.1A, 2A), and therefore we may infer that PM (black bars in Fig.3A, 4A) have a higher capacity to handle the trafficking of these targets. Although there is a general assumption that PM are less phagocytic than BMDM [8], it is known that PM express high levels of TIM4, the receptor which recognizes and internalizes PS-enriched targets such as agRBC. As the internalization capacity affects phagosome maturation, the higher percentage of recognized and internalized agRBC, the probability of achieving degradation increases. The peritoneal cavity has two PM subsets, large and small peritoneal macrophages [9]. Under steady state conditions, large peritoneal macrophages appear to originate independently from hematopoietic precursors and retain the ability to proliferate in situ, maintaining physiological numbers [10]. The small PM originate from circulating monocytes [9,10], and their numbers increase markedly under inflammatory conditions. Therefore, small PM together with their precursor, the inflammatory monocyte population, are the major myeloid populations present in elicited peritoneal cavity. In this study, we used non-elicited PM so we assumed that the main population in the dishes were large peritoneal macrophages which have also been shown to express high levels of TIM4 [9], emphasizing the outcomes obtained. Finally, recent studies in the peritoneal cavity [11] demonstrate that even during an inflammatory response, tissue-

resident macrophages are maintained locally by proliferative self-renewal without substantial monocytic input, establishing peritoneal macrophages at the leading edge of tissue homeostasis.

Comparing the data between the 3 different types of RBC-containing phagosomes in both primary macrophages, we observed similar results between the two models of opsonized RBC. Both complement and IgG were opsonized with shRBC whereas agRBC were obtained from human blood. Additionally, macrophages were isolated from mice, which is a completely different source. Although this could introduce some redundancies and a misinterpretation of the results, the literature is clear about the use of sheep RBC from both in vitro and in vivo protocols. Accordingly, shRBC in vivo are non-replicating antigens that directly reach the spleen and are cleared within hours [12]. Nevertheless, we appreciate that if we wanted to adequately assess the impact of erythrophagocytosis on immune responses in vivo, we must use the same source.

Since the analysis of more than one receptor-target engagement and the respective signaling response is a challenging undertaking, researchers have focused on unveiling detailed mechanistic insights regarding each process, and Fc- and complement- mediated phagocytosis are the most well described processes in the literature. Interestingly, some authors are now focusing on deciphering the functional mechanism of PS-receptors. Very recently, Flannagan et al [13] demonstrated that TIM4 and β1 integrins associate upon receptor clustering, supporting a model in which TIM4 engages integrins as co-receptors to stimulate the signal transduction required to internalize PS-bearing targets such as senescent/damaged RBC.

Erythrophagocytosis implies the internalization and processing of RBC, but in the second part of this project, we focused on agRBC. Interestingly, stored agRBC is the best available model for studying erythrocyte ageing [14] and in addition to being used as a model of senescent RBC, agRBC may be also used to study eryptosis (damaged RBC) [15].

Understanding the molecular processes underlying the maturation and degradation of phagosomes containing agRBC is rudimentary and this knowledge becomes particularly important because malfunction in this finely tuned system can eventually lead to disease. Our findings contribute to the convergence of the phagocytic and autophagy pathways showing that beyond LC3B-II, components of selective autophagy machinery such as NBR1, NDP52 and p62 are also recruited to the single phagosomal bilayers containing IgG-opsonized particles

and agRBC. The association of LC3B-II to single phagosomal membranes containing several targets including apoptotic corpses was previously shown, and engagement PS receptors such as TIM4, seems to recruit LC3B-II to the membranes [16]. Conversely, in LAP-deficient mice, an increase in the serum levels of anti-dsDNA antibodies and anti-nuclear antibodies was observed. Also, LAP-deficient mice presented with IgG and complement C1q deposition in the glomeruli of kidneys, displaying evidence of kidney damage [17]. As agRBC expose PS on the outer leaflet membrane, it will engage with PS receptors, stimulating the association of LC3B-II upon phagocytosis, as we showed. Many studies monitored the recruitment of LAMP-1 to phagosomes, but did not distinguish LC3-positive versus negative phagosomes in these fusion events. However, in this work we observed that LC3B-II stays associated to the phagosomal membranes at the same time as LAMP-1.

Among the selective autophagy machinery tested, the most interesting finding was observed with p62. It has been reported that the presence of autophagic players facilitates the rapid processing of the cargo via fusion with the lysosomal pathway, having a critical role in their degradation [18]. Interestingly, the absence of p62 affects phagolysosome biogenesis with striking effects on agRBC degradation. Another plausible explanation may be related to lysosomal enzyme function. Phagolysosomal membranes contain several V-ATPase proton pumps, which acidify the lumen and activate lysosomal enzymes that degrade the cargo. It was demonstrated that NOX2 is acquired at the initial stages of autophagy before membrane sealing and is involved in the recruitment of LC3B-II to autophagosomes [19]. In fact, in our experimental settings, LC3B-II is also acquired at the early stages of phagosomal maturation after p62 association. Additionally, NOX2 is involved in the production of ROS inside the phagolysosome and when the rate of ROS production exceeds proton release, dismutation of superoxide occurs inside the phagolysosome, leading to lumen alkalinisation and therefore deactivation of proteolytic enzymes [20]. Thus, cargo cannot be degraded and accumulate inside the phagolysosome. As p62-silenced macrophages were not able to degrade agRBC it is worth speculating that this multifunctional protein somehow modulates ROS-driven biological responses. Indeed, the impairment of agRBC degradation could lead to the accumulation of heme inside the phagolysosome and ultimately propagate the pro-oxidant effect.

Upon erythrophagocytosis, we could observe the activation of the non-canonical NRF2 signalling pathway and the same response in p62- and NRF2-silenced cells in terms of agRBC degradation, which implies a mutual control of their function. Sod2 up-regulation could be

caused by macrophages attempting to scavenge reactive oxygen species formed in phagosomes through the activation of NOX2, as hypothesized before. Additionally, the detoxification of superoxide by SOD2 requires biliverdin and bilirubin to serve as scavengers for the mitochondrial superoxide. Bilirubin is a product of heme catalysis by HO-1, so both proteins work together as antioxidant enzymes. Although SOD2 is found solely in the mitochondria, some reactive species may diffuse from the phagolysosomal membrane [21]. Furthermore, it is possible that some mitochondria could be found juxtaposed to phagocytic vacuoles containing agRBC contributing for ROS production [22].

Erythrophagocytosis plays a protective role against oxidative stress and allows RBC regeneration but although the frequency of senescent RBCs is relatively low under steady-state conditions, there are some conditions that lead to disturbed homeostasis. For example, P. falciparum, the causative parasite of Malaria, invades human RBC and induces significant structural changes in the red cell membrane, including membrane invagination and phosphorylation of membrane skeletal components. After the asexual cycle, the red cell membrane is ruptured and new cells are infected by the parasite. Therefore, these infected RBCs become adhesive for a number of different cell types including new RBCs and vascular endothelial cells, causing obstruction of blood flow particularly in small diameter vessels of the microcirculation [23].

A human disease of this severity operating for thousands of years has evolved to offer some protection mechanisms against severe forms of the disease, such as developing of sickle-cell disease and β-thalassemia [24]. However, it is very important to note that none of these inherited red cell disorders offer complete protection against parasite infection; they only reduce the morbidity and mortality. The overall result is an increase in the rate of hemolysis, releasing a significant quantity of free Hb into the circulation. Hb exists in a dynamic equilibrium of tetramer and -subunit heterodimers, with a predominant dimer state at low plasma Hb concentrations. Dimers are of a relatively small molecular size allowing for protein translocation and access to vulnerable anatomic sites such as the vascular wall, possibly leading to vasoocclusion [25]. Also, in mouse models of sickle-cell disease, free heme is found at high levels and its hydrophobicity allows it to enter cell membranes and activate TLR4 signalling pathways that trigger pro-inflammatory cytokine production, thereby promoting blood cell adhesion and vasoocclusion [26]. Therefore, it is crucial to have effective

erythrophagocytosis and decipher the underlying molecular components of this mechanism in order to develop therapeutic treatments.

Finally, as PS-exposed RBC mimic both eryptosis and apoptosis, our findings contribute towards a greater understanding of the details of apoptotic cell clearance and why this mechanism fails in diseases such as Atherosclerosis.

4.2 Future Perspectives

In this project, we demonstrated that RBC bearing PS on the surface mature slower than opsonized-RBC, *in vitro*, in primary mouse macrophages. Although this experimental design allowed us to achieve the proposed outcomes, an *in vivo* protocol using peritoneal-elicited mouse macrophages would offer greater physiological insights on phagosomal maturation after injecting of the targets. Additionally, to enhance the impact of the work, it would be interesting to use inhibitors of phagolysosome formation such as chloroquine. This compound is currently used to treat Malaria as it avoids the excessive degradation of hemoglobin by enhancing lysosomal pH, and therefore preventing severe anaemia.

After assessing the association of LC3B-II to the phagosomes containing agRBC and IgG-opsonized particles, it was possible to observe that p62 is selectively recruited to the agRBC-phagosomal membranes. In addition, the degradation of these targets was also compromised. As this effect could contribute to a disruption in cell homeostasis, given infinite time and money, we must consider to look at the inflammatory response by macrophages. At the same time, it would be interesting to compare the process of erythrophagocytosis with heme stimulation, as this molecule was found to induce macrophage necroptosis through TLR-dependent signaling.

By using NRF2-KO mice and p62-silenced cells, we established a sequential mechanistic event after the engagement and internalization of agRBC, confirming that NRF2 is translocated to the nucleus upon erythrophagocytosis with consequent up-regulation of the genes involved in heme metabolism. However, there are some missing experiments that could be attempted, such as assessing the interaction between KEAP1-p62 on the agRBC-phagosomal membranes and therefore, gaining a better understanding of the p62 pool responsible for the agRBC degradation.

Finally, to show the impact of these *in vitro* results, a disease mouse model such as Sickle-cell disease could be crossed with p62-KO mice to yield a double KO in order to assess the impact on inflammation and therefore on mice survival.

4.3 Conclusions

In summary, agRBC-containing phagosomes mature slower compared to opsonized-shRBC, in primary mouse BMDM and PM. Internalization and processing of agRBC is mediated by LAP and several autophagy adaptors such as NDP52, NBR1 and p62 are also recruited to the phagosomes. The recruitment process of these adaptors depends on ubiquitin, and p62 and NBR1 are recruited before LC3B-II and NDP52.

We reported that once agRBC are phagocytosed, p62 and NRF2 are critical for phagolysosome biogenesis and degradation. p62 phosphorylation on its Ser351 residue is crucial to induce NRF2 translocation to the nucleus, inducing the up-regulation of *p62*, *Hmox1* and *Sod2* genes, upon erythrophagocytosis. These observations reveal the high complexity of these processes that goes beyond the interaction of phagosomes with components of the endocytic pathway and the recruitment of some autophagy machinery.

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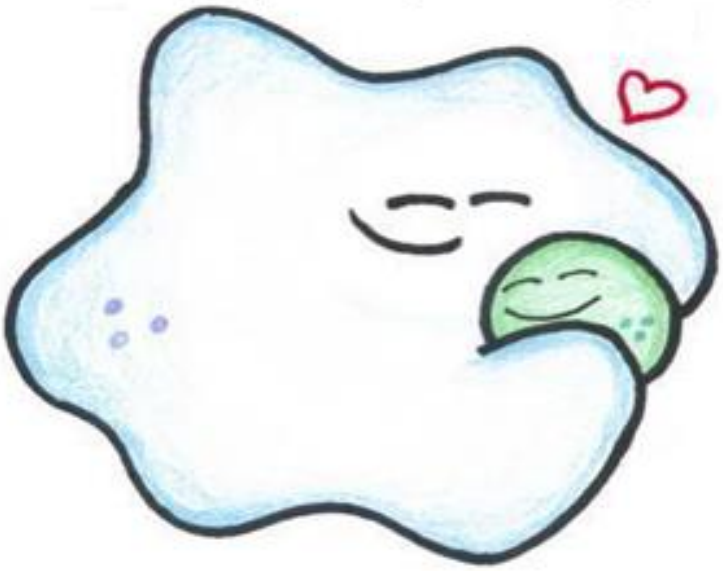
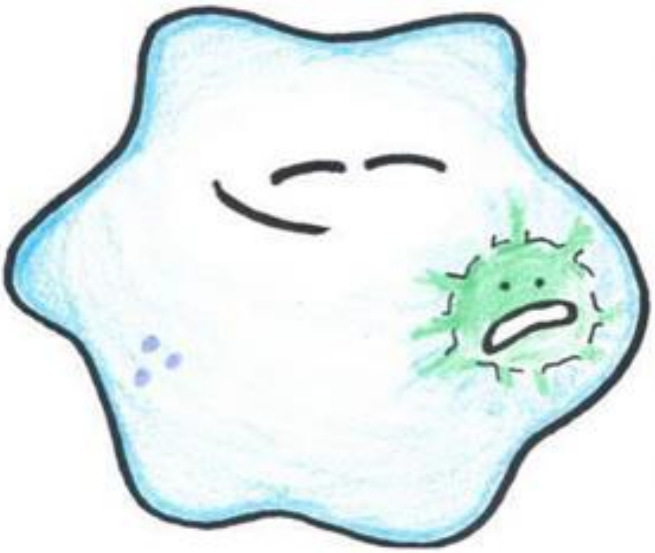
“Não há nada impossível. Uma frase para não esquecer naqueles dias fatídicos nos quais meterias a cabeça na sanita e puxarias o autoclismo sem piedade... Poupa o banho. Há sempre uma solução. Embora agora te pareça tudo muito negro, tu consegues. Fora, tudo é possível!”

Mr. Wonderful

Macrophage hugs





... are often fatal.