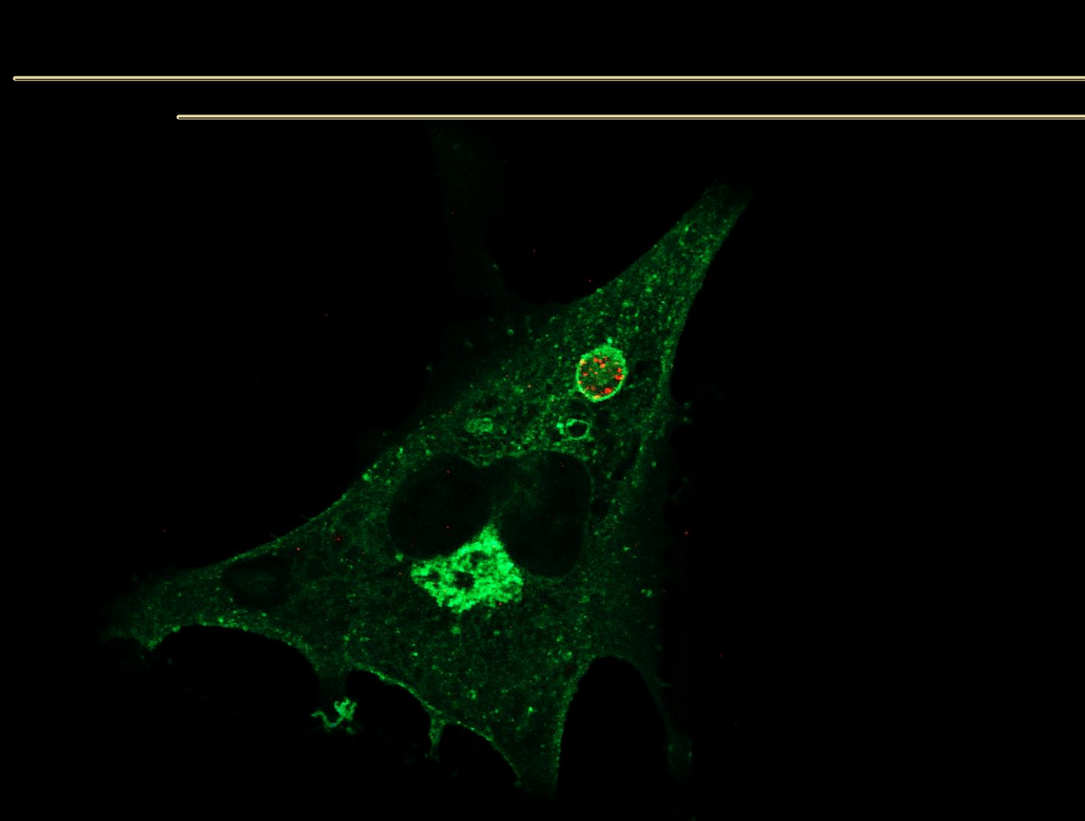
The role of the endocytic and autophagic molecular machineries in the removal of apoptotic cells

Michelle Stumpf Viegas

Universidade de Coimbra



Michelle Stumpf Viegas

The role of the endocytic and autophagic molecular machineries in the removal of apoptotic cells

Doctoral Thesis in Biosciences, speciality in Cell and Molecular Biology, under the supervion of Doctor Otília Vieira and co-supervision of Professor Doctor Emília Duarte, and presented to the Life Sciences Department of the Faculty of Sciences and Technology of the University of Coimbra.

2014

· U C ·

Universidade de Coimbra

Michelle Stumpf Viegas

The role of the endocytic and autophagic molecular machineries in the removal of apoptotic cells

2014

UNIVERSIDADE DE COIMBRA

Doctoral Thesis in Biosciences, speciality in Cell and Molecular Biology, under the supervision of Doctor Otília Vieira and co-supervision of Professor Doctor Emília Duarte, and presented to the Life Sciences Department of the Faculty of Sciences and Technology of the University of Coimbra.

Cover illustration: On the cover is a confocal image of a vascular smooth muscle cell transiently expressing the plasma membrane marker glycosyl-phosphatidyl-inositol-anchored yellow fluorescent protein (YFP-GL-GPI) visualized in green, which has internalized a human aged red blood cell stained with CMTMR and visualized in red.

Michelle Stumpf Viegas

O papel da maquinaria molecular endocítica e autofágica na remoção de células apoptóticas

2014

UNIVERSIDADE DE COIMBRA

Tese de Doutoramento em Biociências, especialização em Biologia Celular e Molecular, orientada pela Doutora Otília Vieira e co-orientada pela Professora Doutora Emília Duarte, e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

This work was performed at the Center for Neuroscience and Cell Biology (CNBC), University of Coimbra, Portugal and supported by Ph.D. grant SFRH/BD/62197/2009 awarded by the Portuguese Foundation for Science and Technology.

Este trabalho foi realizado no Centro de Neurociências e Biologia Celular (CNBC) da Universidade de Coimbra, Portugal, ao abrigo da bolsa de Doutoramento financiada pela Fundação para a Ciência e a Tecnologia (SFRH/BD/62197/2009).

Financiamento no âmbito do QREN - POPH, comparticipado pelo Fundo Social Europeu e por fundos nacionais do MCTES.

Agradecimentos

Ao longo de todo o percurso do meu doutoramento várias pessoas contribuíram não somente para o trabalho aqui apresentado, mas também para o meu desenvolvimento científico e pessoal. Assim, a todos que direta ou indiretamente me ajudaram a concluir esta etapa deixo aqui algumas palavras sinceras de agradecimento:

À minha orientadora, Doutora Otília Vieira, agradeço o acolhimento ao grupo, a oportunidade de poder ter trabalhado num projeto tão interessante, a confiança no meu trabalho e por todo apoio técnico e por vezes psicológico prestado nos momentos de maior dificuldade.

À Professora Doutora Emília Duarte agradeço o apoio e a disponibilidade em ter aceitado a co-orientação deste trabalho.

Aos meus colegas do grupo *Membrane Traffic and Disease*: Aos que ainda por aqui andam Ângela, Elda, Luís, Inês, Denisa, Marisa e Neuza; e também aos que já não fazem parte do grupo João, Kátia e Carla quero agradecer imenso o companheirismo, a animação diária, os "happy hours" e as palavras de compreensão e motivação quando as coisas não corriam tão bem. Queria ainda agradecer em particular ao Luís e à Inês por terem participado diretamente na conclusão de alguns resultados apresentados nesta tese. Ao Luís também pelas aulas de química e pela paciência. E em especial quero agradecer às minhas pequenas Ângela e Eldazita que muito mais do que colegas foram amigas em todas horas (boas e más); obrigada pelos conselhos, pelas trocas científicas, mas principalmente por sempre terem um sorriso para me dar, pelo carinho e por terem tornado os meus dias tão alegres no laboratório e fora dele. Ângela, tua boa disposição em ajudar-me foi determinante para o bom desfecho dessa história, serei sempre agradecida.

Ao Dani, que onde estiver eu sei que estará muito feliz por eu ter chegado aqui. Foste o maior responsável por eu ter vindo parar a Portugal e, portanto, ter realizado cá o doutoramento, assim vou agradecer eternamente a confiança e amizade.

Às amigas Daniela, Liane e Priscilla quero agradecer por indiretamente terem feito parte desta trajetória, pela amizade, por sempre torcerem por mim e terem uma palavra de incentivo.

A todo pessoal do CNC que de alguma forma contribuiu para a minha aprendizagem. Em especial quero agradecer à Dona Isabel e à Sílvia pelo carinho, por serem tão bem dispostas, solícitas e me terem ajudado quando ninguém mais podia.

À FCT pela credibilidade e pelo financiamento do meu doutoramento.

Aos meus familiares, minhas avózinhas, tios e primos pela grande torcida e motivação mesmo de tão longe.

Ao Ricardo, meu querido, melhor amigo e fiel companheiro por todos estes anos, quero agradecer pelo apoio emocional, pela compreensão, por me mostrar de maneira tão emocionante todas as qualidades deste país que tão bem me recebeu, por me ter ensinado os valores e a tradição de Coimbra e me ter feito sentir tanto orgulho em ser estudante nesta Universidade. Obrigada por ter feito esse caminho ser mais fácil de seguir e por me fazer sentir especial.

Ao Gabri, meu irmão, que me fez sentir tão honrada em ter seguido meus passos e ter também se aventurado pelo mundo das ciências biológicas, quero agradecer pelas conversas enriquecedoras, por se preocupar comigo, por me fazer rir e por sempre me motivar a seguir em frente.

Aos meus pais, meus maiores incentivadores, tenho muito a agradecer. Obrigada pelos valores que vocês tão bem me ensinaram, por terem formado o meu caráter, pelo esforço que fizeram ao longo de toda a vida pela minha educação e para que nada me faltasse, por me terem dado total liberdade para seguir meus sonhos, pela confiança, pelo amor incondicional, pela atenção e por todo o carinho que sempre tive de vocês. Se hoje cheguei aqui foi por vossa causa e quero que saibam que terão o meu eterno reconhecimento e gratidão. Mãe, és sem nenhuma sombra de dúvida a minha maior fonte de inspiração, força e perseverança. Divido esta alegria especialmente contigo, pois sem o teu fundamental apoio eu não teria chegado aqui.

"Alguns homens veem as coisas como são, e dizem 'Por quê?' Eu sonho com as coisas que nunca foram e digo 'Por que não?'"

(George Bernard Shaw, 1856 - 1950)

"Quando eliminamos o impossível, o que resta, ainda que improvável, deve ser a verdade."

(Arthur Conan Doyle, 1859 - 1930)

Table of Contents

Table of Figures

List of Abbreviations

agRBC	Aged red blood cells
Apo-B	Apolipoprotein B
ATG	Autophagy-related gene products
BAI-1	Brain angiogenesis inhibitor 1
BMDM	Bone marrow-derived macrophages
CFSE	Carboxyfluorescein-diacetate-succinimidyl ester
CMA	Chaperone-mediated autophagy
CMTMR	Chloromethyl-tetramethylrhodamine
CR3	Complement receptor 3
CRT	Calreticulin
CVD	Cardiovascular diseases
DIC	Differential interference contrast
DMSO	Dimethyl sulfoxide
DOCK180	Dedicator of cytokinesis
EEA-1	Early endosomal antigen-1
ELMO	Engulfment and cell motility protein 1
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complex for transport
FcR	Fc-receceptor
GABARAP	Gamma-aminobutyrate receptor-associated protein
Gas6	Growth arrest-specific 6
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
HOPS	Homotypic fusion and protein sorting
ICAM-3	Intercellular adhesion molecule 3
IgG	Immunoglobulin G
ILV	Intraluminal vesicles
Jak3	Janus kinase 3
LAMP	Lysosome-associated membrane proteins
LAP	LC3-associated phagocytosis
LBPA	Lysobisphosphatidic acid
LC3	Microtubule-associated protein 1 light chain 3
LCCM	L929-cells conditioned médium
LDL	Low density lipoprotein
LIR	LC3-interacting region
LOX-1	Oxidized low-density lipoprotein receptor 1
LPS	Lysophosphatidylcholine
M6PR	Mannose-6-phosphate receptor
MAP1LC3PE	Microtubule-associated protein 1 light chain 3

M-CSF	Macrophage-colony stimulating factor
MFG-E8	Milk-fat-globule-EGF-factor 8
MHC	Major histocompatibility complex
mTOR	Mammalian target of rapamycin
MVB	Multivesicular bodies
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NBR1	Neighbor of BRCA1 gene1
NDP52	Nuclear dot protein 52
Nix	Nip3-like protein X
NOX2	NADPH oxidase 2
OPTN	Optineurin
ox-LDL	Oxidazed-low density lipoprotein
PAMP	Pathogen-associated molecular patterns
PB1	Phox and Bem1p
PBS	Phosphate-buffered saline
PE	Phosphatidylethanolamide
PFA	Paraformaldehyde
PI	Phosphoinositides
PI3K	Phosphatidylinositol-3-kinase
PI3P	Phosphatidylinositol-3-phosphate
POPS	1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-l-serine
PtdSer	Phosphatidylserine
Rab	Ras-related proteins in brain
RBC	Red blood cells
ROS	Reactive oxygen species
RT	Room temperature
shRBC	Sheep red blood cells
SMC	Smooth muscle cells
SNARE	Soluble n-ethylmaleimide-sensitive factor attachment receptors
SQSTM1	Sequestosome-1/p62
TGF-β	Transforming growth factor-beta
TGN	Trans-Golgi-network
TIM	T cell immunoglobulin and mucin
TLR	Toll-like receptors
TNF-α	Tumor necrosis factor-alpha
UBD	Ubiquitin-binding domains
ULK	Unc-51 like kinase
UPS	Ubiquitin-proteasome system
UTP	Uridine-5'-triphosphate
Vps34	Vacuolar sorting protein 34
WHO	World health organization
YFP-GL-GPI	Glycosyl-phosphatidyl-inositol-anchored yellow fluorescent protein

Summary

Every day the human body turns over billions of cells ensuring the disposal of unwanted targets that die by apoptosis. The prompt and efficient removal of apoptotic cells by phagocytes, referred as to efferocytosis, plays an essential role during development, tissue repair and in the maintenance of homeostasis, triggering an immunological tolerance (Henson and Hume, 2006). On the other hand, defective clearance promote dying cell accumulation, converting harmless apoptotic cells into a risky secondary necrotic state that, eventually, expose self-antigens, which has been linked to the onset of several human disorders, including autoimmunity and chronic inflammatory diseases, such as atherosclerosis (Elliott and Ravichandran, 2010). Atherosclerosis remains the biggest cause of mortality and disabilities worldwide, especially in developing countries. The formation of the atheroma starts with the retention of low-density lipoproteins (LDL) inside the wall of blood vessels, where they become subjected to several chemical modifications. These modified-LDL induce the recruitment of monocyte-derived macrophages, which internalize the deposited fatty material. Over time, these lipid-loaded macrophages are no longer able to process the cholesterol, forming foam-cells that eventually undergo apoptosis. In early stages of atherogenesis, efferocytosis is very efficient; however in advanced lesions this process somehow fails, triggering an inflammatory response that, in turn, recruits more cells, including neighboring smooth muscle cells (SMC). Besides macrophages, SMC, the major cell type in the blood vessels wall, play an essential role by dealing with the dying cell accumulation, thus preventing atheroma progression (Moore and Tabas, 2011).

Although many efforts have been done to understand the machinery involved in the recognition of apoptotic cells by phagocytic cells (receptors and ligands), as well as the immune response elicited, very little is known about the intracellular transport of phagosomes containing apoptotic cells and its subsequent digestion into phagolysosomes, the final degradative compartment of the host cell (Hochreiter-Hufford and Ravichandran, 2013). Beyond that, *C. elegans* has been the model organism in studies of engulfment and degradation of apoptotic cells, which reinforce the need to have more information about the development of this process in mammalian systems. Thus, it is crucial to our understanding, to figure out the causes of the inefficient efferocytosis and how it contributes to the pathogenesis of certain diseases.

In this thesis, we have performed a detailed study on the maturation of phagosomes containing human aged red blood cells, our apoptotic cell model, using a mammalian phagocytic

cell line (vascular SMC). The maturation of phagosomes containing dying cells was compared with the processing of phagosomes loaded with IgG-opsonized particles, which are internalized via Fcγ-receptors and are the best characterized phagocytic model. At the present work, we provide evidence that the nature of the cargo modulates the phagocytic response, since phagosomes carrying apoptotic particles reach the lysosomes with a delay when compared to those containing IgG-opsonized particles. Furthermore, for the first time, we have identified some canonical autophagy effectors in phagolysosome formation, suggesting that LC3-Associated Phagocytosis (LAP), a process involved in phagosome maturation, implies more than the phagosomal recruitment of LC3 (Sanjuan et al., 2007). Indeed, experiments performed in bone marrow-derived macrophages from p62-KO mice clearly suggest that p62, despite not being required for LC3 recruitment, is important for phagolysosome biogenesis.

In summary, this data will improve our knowledge on the molecular machinery and mechanisms involved in efferocytosis. In the end, we hope to contribute to a better understanding of efferocytosis and the ways to modulate this process, which could culminate with the discovery of therapies that may benefit patients with atherosclerosis and other type of diseases in which efferocytosis is not efficient.

Resumo

Diariamente o corpo humano degrada bilhões de células garantindo a eliminação de alvos indesejados que morrem por apoptose. A remoção rápida e eficaz de células apoptóticas por células fagocíticas, processo conhecido como eferocitose, desempenha um papel essencial durante o desenvolvimento e na reparação e homeostase dos tecidos, sendo um fator crítico para tolerância imunológica (Henson and Hume, 2006). Por outro lado, uma remoção defeituosa promove a acumulação de células apoptóticas e subsequentemente necrose secundária, exposição de auto-antigénios (autoimunidade) e inflamação (Elliott and Ravichandran, 2010). Aterosclerose, uma doença inflamatória crónica, permanece a maior causa de mortes e incapacidades em todo mundo, especialmente em países em desenvolvimento. A formação do ateroma começa com a retenção de lipoproteínas de baixa densidade (LDL) dentro da parede dos vasos sanguíneos, onde estão sujeitas a várias modificações químicas. Estas LDL-modificadas induzem o recrutamento de macrófagos derivados de monócitos, os quais internalizam a matéria gorda depositada. Com o passar do tempo, estes macrófagos cheios de lípidos deixam de conseguir processar o colesterol, formando células espumosas que eventualmente sofrem apoptose. Nos estágios iniciais da aterogénese, a eferocitose é muito eficiente, entretanto em lesões avançadas este processo de alguma forma falha, provocando uma resposta inflamatória que, por sua vez, recruta mais células, inclusive células do músculo liso das artérias. As células musculares lisas, o tipo mais abundante de células na parede dos vasos sanguíneos, desempenham um papel essencial na eferocitose, contribuindo no atraso da progressão do ateroma (Moore and Tabas, 2011).

Embora muitos esforços tenham sido feitos para compreender a maquinaria envolvida no reconhecimento de células apoptóticas por células fagocíticas (recetores e ligandos), assim como a resposta imune suscitada, muito pouco é sabido sobre o transporte intracelular de fagossomas contendo células apoptóticas e sua subsequente digestão dentro de fagolisossomas, o compartimento degradativo final da célula hospedeira (Hochreiter-Hufford and Ravichandran, 2013). Além disso, *C. elegans* tem sido o organismo modelo em estudos de internalização e degradação de células apoptóticas, o que reforça a necessidade de mais informação sobre o desenvolvimento deste processo em sistemas mamíferos. Desta maneira, é crucial ao nosso entendimento, descobrir as causas de uma eferocitose ineficiente e como isto contribui para a patogenicidade de certas doenças.

Nesta tese, nós realizamos um estudo detalhado sobre a maturação de fagossomas contendo hemácias humanas envelhecidas, modelo apoptótico usado, utilizando uma linha de células do músculo liso como células fagocíticas. A maturação de fagossomas contendo células a morrer foi comparada com a maturação de fagossomas contendo partículas revestidas com IgG, as quais são internalizadas pelos recetores Fcγ, o mais bem caracterizado modelo fagocítico. No presente trabalho, nós fornecemos evidências de que a natureza da carga modula a resposta fagocítica, uma vez que fagossomas que carregavam partículas apoptóticas atingiram os lisosomas com um atraso em ralação àqueles contendo partículas opsonizadas por IgG. Além disso, pela primeira vez, nós identificamos elementos da autofagia canónica na formação dos fagolisossomas, o que sugere que a Fagocitose Associada à LC3 (LAP), um processo envolvido na maturação do fagossoma, implica mais do que o recrutamento de LC3 (Sanjuan et al., 2007). De facto, experiências realizadas em macrófagos provenientes de animais deficientes para a proteína autofágica p62 sugerem claramente que a p62, embora não necessária para o recrutamento do LC3, é importante para a biogénese do fagolisossoma.

Em síntese, estes dados vão contribuir para a nossa compreensão sobre a maquinaria molecular e mecanismos envolvidos na eferocitose. A longo prazo, esperamos que o conhecimento reportado nesta tese possa levar ao desenvolvimento de terapias para doenças em que a eferocitose não se processe normalmente.

Chapter I

Introduction

Summary

Introduction

1.1. Atherogenesis and apoptosis of pathological foam cells

Cardiovascular diseases (CVDs) include diseases of the heart, vascular brain accidents and blood vessels disorders, and remain the biggest cause of mortality and disabilities worldwide, especially in industrialized and developing societies (Roger et al., 2011; Rosamond et al., 2008). According to the World Health Organization (WHO), in 2008, more than 17 million people died from CVDs, representing 48% of the total number of deaths caused by non-communicable diseases, which are not transmissible among people. There are also new dimensions to this alarming situation, since over the past two decades, deaths from CVDs have been declining in high-income countries, but have increased at an astonishingly fast rate in low- and middle-income countries. Regarding to the different types of cardiovascular complications, atherosclerosis, an underlying disease process in blood vessels, was identified as the responsible for a large proportion of deaths: myocardial infarctions (heart attacks) were responsible for 7.3 million deaths (45%), while cerebrovascular diseases (strokes) were responsible for 6.2 million deaths (29%) (WHO, 2011) (see Figure 1.1).

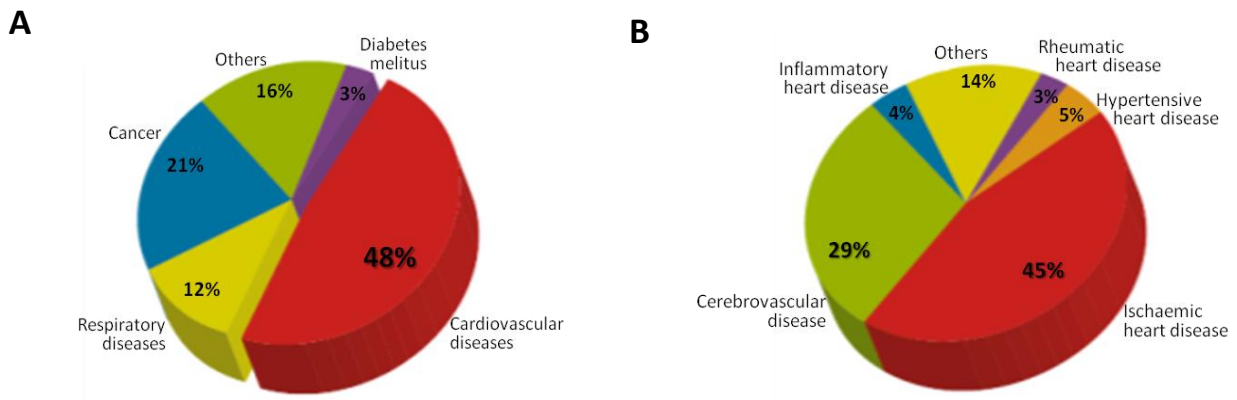


Figure 1.1. Cardiovascular disease mortality trends in 2008. General distribution of global non-communicable diseases by cause of death in both sexes (**A**); and distribution of global CVD burden due to heart attacks, strokes and other types of CVDs in males (**B**). [Adapted from (WHO, 2011)].

Yet, more than 3 million of these deaths occurred before the age of 60 and could have largely been prevented by reducing some of the risk factors associated to atherogenesis. This involve behavioral risks (e.g. tobacco, unhealthy diet, alcohol abuse and physical inactivity), metabolic risks (e.g. hypertension, cholesterol levels, diabetes and overweight), among others that are not modifiable, such as poverty, advancing age, male gender, genetic disposition and psychological factors. The prevalence of this disease continues to rise due to adoption of a "Western life-style" by an increasing fraction of the world population and is likely to reach epidemic proportions in the next few decades (Andreassi, 2009; Mehrabian et al., 1998).

In an overview, atherosclerosis is a chronic inflammatory disease of the arterial walls that develops over many years, starting in childhood and adolescence despite manifest mostly in advanced age. During atherogenesis, lipid material is deposited inside the wall of medium- and large-sized blood vessels. These fatty deposits together with some other elements (atheroma plaques) cause the inner surface of the blood vessels to become irregular and the lumen to become narrow, making it harder for blood to flow through (Ambrose et al., 1988; Ross, 1993). Blood vessels also become less pliable as a result. Eventually, the plaque can rupture, triggering the formation of a blood clot (thrombus). If the thrombus develops in a coronary artery, it can cause a heart attack; if it develops in the brain, it can cause a stroke. Moreover, sometimes, the thrombus can become loose and travel through the blood until to be trapped in small caliber vessels. The blocking of the blood flow cuts the supply of oxygen and nutrients, causing damage or death of the adjacent tissue, ultimately leading to thrombotic episodes. Another consequence can be seen when the thrombus clogs small vessels, leading to their complete disruption and bleeding, as aneurysms (Libby et al., 2011; Lusis, 2000).

In a molecular perspective, atherosclerosis is triggered by the subendothelial retention of apolipoprotein B-containing lipoproteins (Apo-B) in susceptible, but still pre-lesional areas of the arterial wall. These Apo-B lipoproteins, such as low-density lipoproteins (LDL), consist of a core of neutral lipids, markedly cholesteryl esters and triglycerides, surrounded by a monolayer of phospholipid, unesterified cholesterol and proteins. Their accumulation occurs predominantly at sites of turbulent laminar flow, notably, in arterial branch points and bifurcations, where endothelial cells have no particular orientation and polygonal shapes, thus increasing the permeability to certain macromolecules (Majesky, 2007; Williams and Tabas, 1995). After being transported across the intact endothelium, LDL become trapped in the extracellular matrix of the intima layer, space where they are subjected to several modifications to produce highly oxidized (ox-LDL) and aggregated LDL. Oxidation is believed to be the most atherogenic chemical

modification of LDL, highly contributing to trigger maladaptative responses to the retained material (Glass and Witztum, 2001; Navab et al., 1996; Steinberg and Witztum, 2010). Therefore, these ox-LDL are potent inflammatory inducers, stimulating the overlying endothelial cells to: 1) release chemotactic proteins to promote directional migration of monocytes (Dutta et al., 2012; Mestas and Ley, 2008); 2) express adhesion molecules to firmly adhere monocytes before their entry into the intima layer (Koenen et al., 2009); and 3) secrete growth factors, such as macrophage colony-stimulating factor (M-CSF), in order to differentiate monocytes into scavenging macrophages. Yet, recruited monocytes can be also differentiated into dendritic cells, which are more related to antigen-presentation (Paulson et al., 2010). Once embedded in the intima, the macrophages encounter native- and modified-LDL, which are endocytosed by different receptor-mediated processes or by fluid-phase: the native ones are internalized *via* the LDL-receptors, whereas the ox-LDLs are uptaken through scavenger receptors, such as CD36, or even *via* macropinocytosis (Febbraio et al., 2001; Johnson and Newby, 2009) (see Figure 1.2).

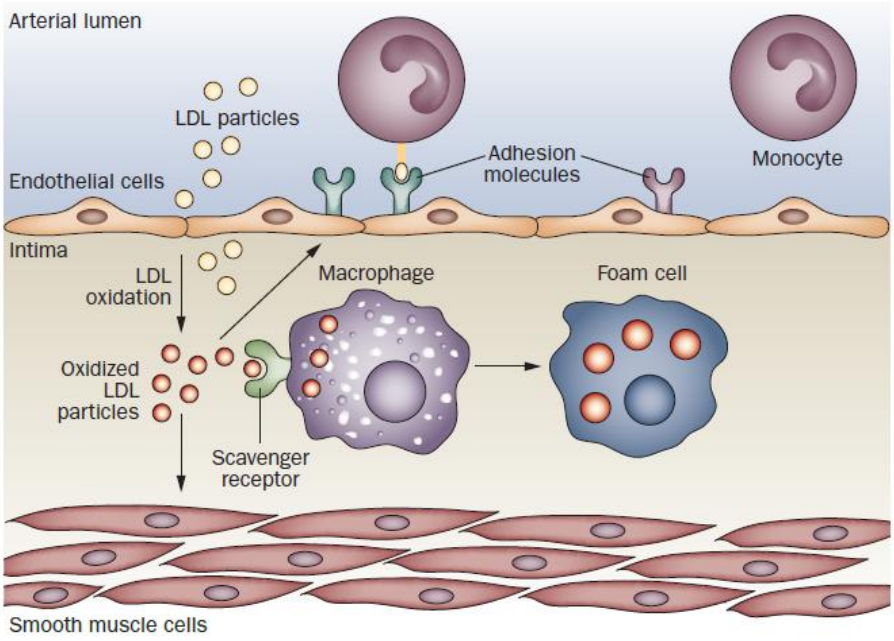


Figure 1.2. Effects of LDL particles on the vessel wall. Circulating LDL particles invade the arterial wall and accumulate in the intima, where they undergo chemical modifications, such as oxidation. Modified LDL can induce endothelial cell activation and expression of adhesion molecules. Furthermore, intimal macrophages can internalize modified LDL particles through scavenger receptors and become foam cells. Oxidized lipids probably modulate smooth muscle cell functions, for example increasing their adhesion to macrophages and foam cells in the plaque. [Adapted from (Rocha and Libby, 2009)].

In normal physiological conditions, the ingested LDL are efficiently delivered to lysosomes, where their cholesteryl esters portions are hydrolyzed to free cholesterol and the Apo-B

degraded to amino acids. This catabolic process is important for the normal turnover of LDL components, and lack of hydrolytic activity leads to an accumulation of undegraded substrates within the lysosomes. Then, from the lysosomes the free cholesterol can be exported from the cell by exocytosis (cholesterol efflux-reverse cholesterol transport) or transported towards the endoplasmic reticulum (ER) for re-esterification. Finally, the resulting cholesteryl ester molecules formed are storaged in the cytoplasm, inside a membrane-bound organelle called lipid droplets (Brown and Goldstein, 1983). The lipids stored in these cytosolic organelles can be later used by the cell for different processes, such as membranes and steroid hormone synthesis (Tall et al., 2008). However, with time the LDL in the arterial intima undergo modifications and these modified-LDL cannot be properly processed, resulting in intracellular irreversible accumulation of excess unesterified or free cholesterol into endolysosomal compartments (Maxfield and Tabas, 2005; Schmitz and Grandl, 2009). Eventually, the macrophages become enlarged and so full of lipoprotein-derived cholesterol that they assume a “foamy” microscopic appearance and thus are known as “foam cells” (de Duve, 1974) (see Figure 1.2). Foam cell formation in atherosclerotic plaques is initiated when the cholesterol removal becomes limited, either: 1) because the modified LDL in the arterial wall cannot be processed by the macrophages; or 2) because the ability of macrophages to efflux cholesterol becomes impaired. Notable, inefficient efflux of free cholesterol has toxic effects, inducing macrophage apoptosis likely by stress of the ER membrane bilayer (Feng et al., 2003).

As macrophages and apoptotic foam cells accumulate with time, they secrete additional adhesion molecules, growth factors and pro-inflammatory mediators that reinforce the lipoprotein retention and promote the recruitment of more monocytes, as well as T cells, mast cells, neutrophils and even smooth muscle cells (SMC) to handle apoptotic cell removal. Eventually, this extra assistance also fails to cope with the established disturbed scenario, contributing to foam cell formation and apoptotic cell death, thus triggering a chronic inflammatory response. All these process involving accumulation of cellular debris, extracellular lipids and inflammatory mediators, are predicted to form a lipid-rich pool called the necrotic core of the plaque (Tabas, 2010; Virmani et al., 2002). Once in the intima layer, the SMC recruited from the tunica media (the middle layer of the artery wall) start to proliferate in response to mediators (e.g. platelet-derived growth factor), and produce extracellular matrix molecules, including interstitial collagen, elastin and proteoglycans, in order to form a fibrous cap that

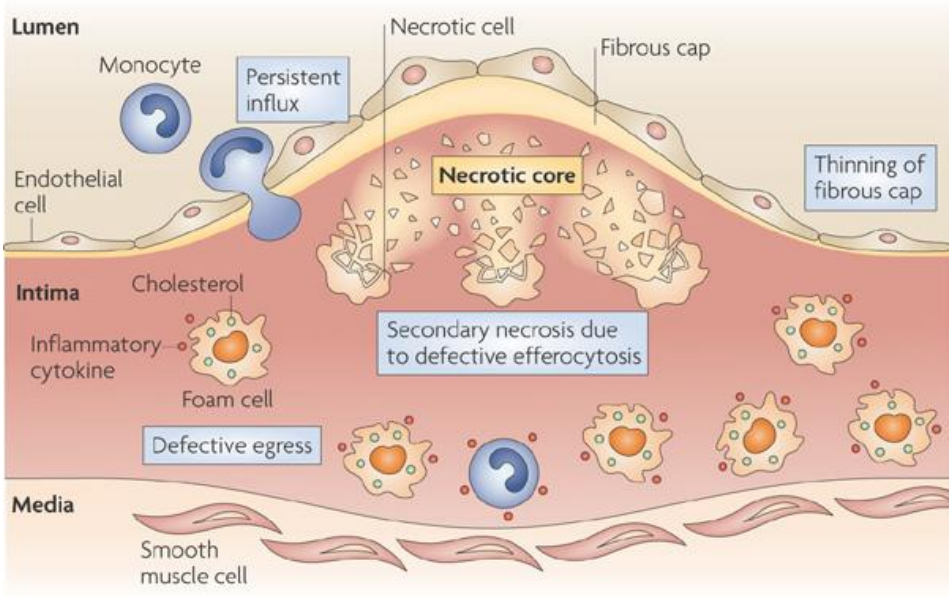


Figure 1.3. Hallmarks of defective resolution of inflammation in an atherosclerotic plaque. Inflammatory cells, including lipid-laden macrophage foam cells, accumulate in the intima owing to the persistent influx of new cells, particularly monocytes, and defective egress of the resident cells. Moreover, apoptotic macrophages are not efficiently cleared by efferocytosis and so they undergo secondary necrosis. This process contributes to the formation of the necrotic core, which promotes plaque disruption, particularly thinning of the fibrous cap. If the process continues, the fibrous cap breaches, leading to lumenal thrombosis and arterial occlusion. [Adapted from (Tabas, 2010)].

covers the necrotic core of the plaque (Fries et al., 2005; Negoro et al., 1995). Curiously, in contrast to many animal species used for atherosclerosis experiments, the intima layer of human arteries contains a resident population of SMC, which emphasize the role of this cell type in the progression of this disease. The fibrous cap, shaped by the SMC, protects the lesion by separating it from the arterial lumen; thus, the consequent death of SMC by apoptosis makes the plaque weaker, unstable and prone to rupture (Figure 1.3). Disruption of the fibrous cap exposes thrombogenic material, such as collagen to the circulation, which induces thrombus formation into the lumen of the vessel (Libby et al., 2011; Stone et al., 2011).

Early atherosclerotic lesions present only a few number of apoptotic cells, probably because they are efficiently cleared by phagocytic cells. In cooperation with neighboring phagocytes, apoptotic death is programmed to lead to compartmentalization and non- inflammatory metabolism of intracellular self-antigens. Indeed, the clearance of these dying cells renders this process not only harmless, but possibly beneficial, owing to the anti-inflammatory nature of apoptotic cell removal (Gregory, 2009; Henson, 2005). Nevertheless, in advanced lesions, for reasons that are still not completely understood, the clearance of apoptotic cells does not function properly, which is associated with secondary necrosis (Thorp, 2010). This

post-apoptotic state involves the loss of plasma membrane integrity, cell leakage, exposure of self-antigens and consequent activation of a pro-inflammatory response. Altogether, these events block the resolution phase, thus promoting persistent influx of monocytes coupled with defective egress of macrophages from the site of inflammation, as depicted in Figure 1.3 (Llodra et al., 2004). The intriguing point here may lie not only in the accelerated rates of apoptosis in advanced lesions, but perhaps even more so in the reasons for a defective phagocytic clearance of apoptotic cells (Kawane et al., 2006; Schrijvers et al., 2005; Tabas, 2005). Furthermore, besides macrophages, vascular SMC, that represent the major cell population in the vessel wall, may play an important role also by taking over the clearance of apoptotic cells when professional phagocytic cells are deficient, since the conditions of hypoxia generated inside the necrotic core may hinder local access to recruited monocytes. Therefore, because uncleared dead cells are a fundamental issue in the etiology of atherosclerosis, it would seem that the ability to modulate apoptotic cell clearance in this environment could serve as a useful and novel tool to prevent or even treat the disease.

1.2. Phagocytosis

1.2.1. Phagocytosis - an overview

Phagocytosis is defined as a specific form of endocytosis (process of moving cargo from outside towards the inside of a cell) involving the vesicular internalization of solid large particles (>0.5µm in diameter) and its subsequent elimination. Discovered in 1882 by Élie Metchnikoff, during his experiments observing the cellular movement within starfish larvae, this philogenetically conserved process is important throughout the animal kingdom, particularly within higher vertebrates. In unicellular eukaryotes, as certain protists, it is used as a means of feeding, providing their nourishment. Usually, in metazoans, phagocytosis is involved in the clearance of apoptotic cells during development and tissue remodeling, while in mammals it is also critical for the innate and adaptative immune response, contributing to our ability to fight pathogens. Owing to the number of different phagocytic cell types, the variety of targets and the extreme complexity of their interactions, the engulfment process is not always identical. In general, it involves ligand-receptor binding, intricate signaling networks, focal cytoskeletal rearrangement and a dynamic series of membrane fusion/fission events (Greenberg and Grinstein, 2002; Stuart and Ezekowitz, 2005; Vieira et al., 2002). Thus, the huge diversity

attributed to the phagocytic process might be related to both: the need to immune tolerate endogenous materials, coupled with the need to fight microorganisms that have developed, over thousands of years, various strategies to subvert the host-defense mechanisms, taking advantages of this process in order to survive and proliferate (Sarantis and Grinstein, 2012; van der Wel et al., 2007).

In this context, it is essential for the innate immune system, the first line of host defense, to be able to discriminate self from non-self-antigens. Therefore, phagocytic cells evolved several tactics based on the recognition of molecular patterns demarcating “non-self”, as well as “normal self” and “abnormal self”. These patterns are deciphered by a complex repertoire of receptors that either induce or inhibit an immune response, depending on the meaning of these signals (Medzhitov and Janeway, 2002). The first type of recognition, the risky “non-self”, depends on the ability of the host to recognize conserved products of microbial physiology that are unique to microorganisms and are not produced by the host, like lipopolyssacharides (LPS). Usually, after invasion, microbes encounter cells from the innate immune system, which identify them as a threat and immediately respond with inflammatory mediators (Underhill and Ozinsky, 2002). The second strategy of recognition, the “missing self”, depends on the detection of markers of normal self, which are products of metabolic pathways that are exclusive to the host and absent from microorganisms. The third strategy, the recognition of “abnormal self”, is based on the detection of altered self-markers that are induced upon infection or after cellular transformation, for instance tumor cells.

Different types of phagocytosis tend to be ligand specific; so distinct molecular patterns from bacteria (~0.5-3µm) or yeast (~3-4µm) are recognized by Toll-like receptors (TLR), for example (Akira and Takeda, 2004; Doyle et al., 2004). Microorganisms and endogenous particles can also be coated with complement proteins or antibodies (opsonins) and then taken up through complement receptors and Fc-Receptors (FcR), respectively (Anderson et al., 1990; Ross et al., 1992; van Lookeren Campagne et al., 2007). Still, in the case of apoptotic cells (~5-50µm) a multiple range of receptors are involved, suggesting a hierarchy of engulfment mechanisms and back-up systems, which include scavenger receptors and phosphatidylserine (PtdSer) receptors (Hoffmann et al., 2001). The array of receptors mediating these divergent responses is distinctly different and elicits dissimilar signaling and effector cascades. Despite the broad variety of receptors (over 20 different phagocytic receptors have been described to date) involved in the recognition of distinct targets is remarkable, biologically, the most important difference between the engulfment of apoptotic cells and the uptake of foreign agents is the immune response that

is elicited. The ingestion of pathogens or FcR-mediated phagocytosis often triggers an inflammatory response, followed by the release of pro-inflammatory cytokines (IL-1, IL-8, IL-12, TNF-α, etc.), whereas the ingestion of apoptotic cells is generally anti-inflammatory or immunologically silent, verified by the secretion of anti-inflammatory cytokines (IL-10 and TGF-β) (Devitt et al., 2004; Fadok et al., 1998a). That is such so important that, for instance, mice that are deficient in a specific receptor and fail to clear apoptotic cells, eventually generates auto-antibodies and develops autoimmune diseases (Hanayama et al., 2004).

Virtually, almost all cells are able to phagocytose particles; but still they are classified into professional or non-professional phagocytes according to their priority to execute phagocytosis among their other cellular functions (Parnaik et al., 2000; Rabinovitch, 1995). The professional phagocytes represented by macrophages, neutrophils and immature dendritic cells, are cells equipped with a complete arsenal of receptors that recognize all type of targets, even pathogens. They are experts in the art of entrap, kill and degrade microorganisms, although some phagocytic properties can vary according to their particular skills. In this regard, macrophages are considered the sentinels of the immune system, since they act as garbage collectors, antigen presenting cells and ferocious killers owing to their huge amount of endosomes and lysosomes (Miyake et al., 2007; Taylor et al., 2005). Meanwhile, neutrophils are highly microbicidal, in spite of play a negligible role in antigen presentation and in phagosome acidification, once they have few acidic organelles (Beertsen et al., 2008). Conversely, dendritic cells are less microbicidal and acidify their phagosomes more gradually in a manner conducive to controlled antigen degradation and presentation (Albert et al., 1998). On the other hand, non-professional “amateurs” phagocytes, represented by SMC, fibroblasts, epithelial cells, among others, are more limited than professional phagocytes in the nature of particles they can take up, since phagocytosis is not their principal function. Moreover, they lack efficient phagocytic receptors, in particular for opsonins attached to invaders by the immune system. In most sites within higher organisms, cell death will occur in the absence of a neighbor macrophage. In such situations, the usual phagocyte is likely to be a viable neighbor cell. Although the ability of amateur phagocytes to eat apoptotic cells has long been noted, its importance has been under-valued (Bursch et al., 1985; Cao et al., 2004; Dini, 1998; Dini et al., 1995; Monks et al., 2005). Given the strong conservation in clearance mechanisms (in terms of phagocyte receptors, bridging molecules and apoptotic cell ligands) between *C. elegans* and humans, the "traditional" phagocytes of dying cells are almost certainly the non-professional. They have important roles in particular tissues lacking a specific macrophage population (e.g. microglia in the brain, Kupffer cells in the liver and so on) or in

those that monocytes/macrophages cannot easily infiltrate (Henson and Hume, 2006; Hoopfer et al., 2006).

Conceptually, the engagement of phagocytic receptors initiates a complex signaling cascade that induces cytoskeleton and plasma membrane rearrangement, driven the extension of pseudopods and culminating with particle engulfment inside the phagosome, a membrane-bound vesicle containing the ingested particle (Greenberg, 1995; May and Machesky, 2001). The extension of pseudopods around the phagocytic targets requires either a continuous supply of membrane material and cytoskeleton reorganization, in order to form the phagocytic cup, a cup-shaped invagination or protrusion of the plasma membrane (Lee et al., 2007). Commonly, after formation, the phagocytic cup extends over the particle by sequential local responses to the ligand-coated surface, until closure at their distal margins, wrapping the target into the phagosome (Jaumouille and Grinstein, 2011). The localized supply of material to membrane extension mainly account with focal exocytosis, the accumulation and fusion of endosomes and other small vesicles with the plasma membrane in the phagocytic cup region (Cox et al., 1999). Moreover, the phagocytic cup organization also relies on the actin cytoskeleton, to both: shape the construction of the cup by active polymerization/depolymerization, and close the cup through contractile movements when associated with myosin. Actin is very concentrated in the advancing cups and persists until the closure of the phagosome (Swanson, 2008). Shortly after scission from the cell membrane, the nascent phagosome begins to disassembly the actin cytoskeleton surrounding it, this way enabling membrane interactions with distinct components of the endocytic pathway.

In conclusion, understanding phagocytosis is not purely an academic pursuit; derangement of the phagocytic process can have life-threatening consequences. Failure to ingest or kill pathogens can result in deadly infections, while inappropriate clearance of apoptotic bodies can give rise to inflammation and autoimmune disorders (Elliott and Ravichandran, 2010).

1.2.2. Phagosome Maturation

Immediately after sealing, the phagosome starts to evolve into a remodeling process termed phagosome maturation. Maturation is required because the nascent phagosome is not effectively degradative. Curiously, its soluble contents are a sampling of the innocuous extracellular environment and its membrane resembles the plasmalemma from which it was derived. Phagosome maturation proceeds through an ordered series of strictly choreographed

membrane fusion and fission events that radically change the lipid and protein composition of the phagosome membrane as well as the lumen properties. These continuous alterations are believed to enable phagosomes to preferentially interact with different endocytic vesicles, driving the stepwise progression of the process (Desjardins et al., 1994; Desjardins et al., 1997; Kinchen and Ravichandran, 2008; Vieira et al., 2002).

In many regards, phagosomal maturation reminds the progression of cargo along the endocytic pathway: from early endocytic vesicles to lysosomes for degradation, as schematically shown in Figure 1.4. Biochemical changes are mainly dictated by the vesicular traffic from and towards the phagosome membrane, which actively involve Rab GTPases proteins, phosphoinositide species, soluble N-ethylmaleimide-sensitive factor attachment receptors (SNAREs), motor proteins and microtubules [reviewed in (Huotari and Helenius, 2011)]. Rab proteins (Ras-related proteins in brain) are considering the master regulators of the intracellular membrane trafficking, defining the identity of the different compartments. Basically, they work as molecular switches by cycling between their inactive conformation (GDP-bound) and their active form (GTP-bound), whereby they associate with several effectors molecules and mediate a range of membrane trafficking events: tethering, budding, docking and fusion of vesicular intermediates (Rink et al., 2005; Schwartz et al., 2007; Smith et al., 2007; Stenmark, 2009). Besides that, are the phosphoinositides lipids (PI), which are produced by mono-, bis- and tris-phosphorylation of the inositol ring of phosphatidylinositol. These lipids also serve as markers of cell compartments by forming unique docking sites in part by conferring special curvature and charge to the membrane surface, improving electrostatic attraction and retention of effector proteins (Botelho et al., 2000b; Corrotte et al., 2006; Yeung et al., 2006). Regardless of function, Rab proteins and PI are also important targets for some pathogens that exploit the vesicular trafficking machinery of the host cell, thus impairing phagosome maturation in order to survive intracellularly. For instance, the *Mycobacterium tuberculosis* arrest the maturation process at early stages, generating a favorable niche to replicate, while avoiding progression to degradative stages (Russell et al., 2002; Vergne et al., 2004).

One of the earliest known maturation events in a number of systems is the recruitment of Rab5 to phagosomes, stimulating their fusion with early endosomes/sorting endosomes ((Bucci et al., 1992; Stenmark et al., 1994; Ullrich et al., 1994). The early endosomes are tubulovesicular compartments peripherally located, remaining transiently fusogenic with incoming plasma membrane cargoes before starting to mature further. Initially, Rabex-5, a guanine-exchange factor, is recruited to early endosomes, where it activates Rab5. Apart that, Rabex-5 also

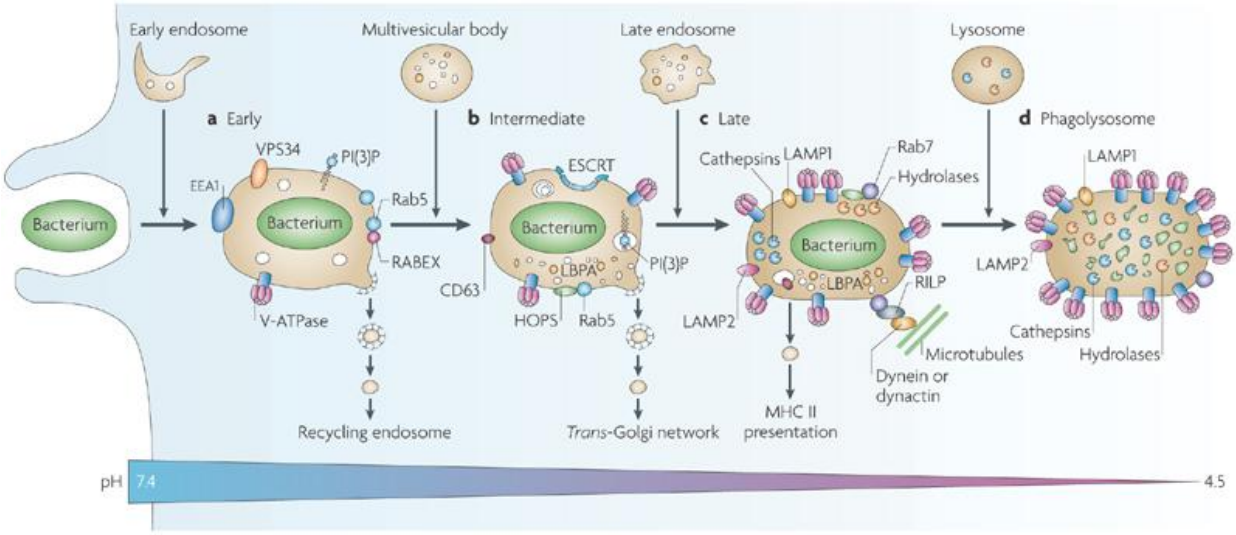


Figure 1.4. Schematic view of phagosome maturation. Shortly after target uptake, the phagosome undergoes a series of transformations that result from its sequential interaction with subcompartments of the endocytic pathway. Different stages of maturation are recognized: early (**a**), intermediate (**b**) and late (**c**) phagosomes - that culminate with the formation of phagolysosomes (**d**). During maturation, the phagosomes acquire various hydrolases and undergo a progressive acidification caused by proton pumping by the V-ATPase. [Adapted from (Flannagan et al., 2009)].

possesses an ubiquitin activity and can bind to ubiquitinated proteins, which is indeed required for its association with early endosomal membranes (Mattera et al., 2006). Activated Rab5 coordinates the endocytic traffic and early phagosome biogenesis by recruiting and activating effector proteins, such as the vacuolar sorting protein (Vps34) and the early endosomal antigen-1 (EEA-1) (Christoforidis et al., 1999; Simonsen et al., 1998). The Vps34, a class III PI3K, locally synthesizes the lipid phosphatidylinositol-3-phosphate [PI(3)P] on the cytosolic leaflet of early phagosomes, which facilitates the progression to further steps by creating docking sites to proteins containing PX and FYVE domains, like EEA-1 (Fratti et al., 2001). Although EEA-1 can directly binds Rab5, it can simultaneously engage PI(3)P, where it induces docking and fusion of early endosomes (Grosshans et al., 2006). The capacity of EEA-1 to promote fusion of membranes is due to its direct interaction with a specific SNARE protein (McBride et al., 1999). The SNARE proteins are universal mediators of membrane fusion by forming a hairpin-like complex responsible to bring donor and acceptor membrane compartments into direct opposition, so reducing the free-energy barrier for membrane fusion (Collins et al., 2002). The SNARE complexes are composed by v-SNARE proteins expressed on the membrane of the donor compartment (e.g. early endosome) and by t-SNARE proteins expressed on the acceptor membranes (e.g. nascent phagosome). After a transient association with the nascent phagosome, Rab5 rapidly dissociates

and Rab7 is acquired, allowing the phagosome interaction with late endocytic compartments (Vieira et al., 2003).

Indeed, complete bulk degradation requires that phagosomes keep following the degradative pathway, transiently moving from early to late stages of maturation, which is accompanied by loss of Rab5 and acquisition of Rab7, at this point forming hybrid organelles (Kinchen and Ravichandran, 2010). Rab7 plays an essential role in the recruitment and fusion of lysosomes with phagosomes (Yu et al., 2008). The conversion and activation (nucleotide exchange) of Rab7 on phagosomes is mediated for several players, including proteins that form the complex termed homotypic fusion and protein sorting (HOPS) (Rink et al., 2005; Wurmser et al., 2000). HOPS proteins are believed to be a tethering complex that binds Rab7 and promotes SNARE-mediated fusion of late endosomes with the phagosome (Brocker et al., 2012). Another effectors here are Rab7-interacting lysosomal protein (RILP) (Cantalupo et al., 2001) and the long splice-variant of oxysterol-binding protein related- protein 1 (ORP1L) that function together to link the phagosomes to microtubule motor proteins (dynein/dynactin), thereby coordinating the centripetal movement of Rab7-positive compartments. It is important because perinuclear localization of phagosomes is needed for efficient contact with lysosomes (Harrison et al., 2003; Johansson et al., 2007). Apart from Rab7, the phagosomes displaying a late endosome-like phenotype additionally expose the lysosome-associated membrane proteins (LAMPs) in their membranes. The LAMP proteins (LAMP-1, -2 and -3/CD63) are transmembrane proteins with a heavily glycosylated luminal domain, thus forming a continuous carbohydrate lining on the inner leaflet of late endosomes, lysosomes and mature phagosomes, sites where they are abundantly found (Eskelinen et al., 2003). They are thought to function in the maintenance of the structural integrity of membranes by protecting them from the hostile luminal environment. Moreover, LAMP proteins are in fact essential for Rab7 recruitment, lysosomal fusion and for acquisition of a microbicidal profile (Binker et al., 2007; Huynh et al., 2007).

Compared with early phagosomes (pH~6.0-6.8), the late ones are much more acidic (pH~5.0-6.0), a consequence of the action of additional proton pumps that accumulate on the phagosomal membrane along the maturation process. The activity of such pumps is catalyzed by the vacuolar-ATPase (V-ATPase), a multimeric complex able to translocate H^+ ions across endosomal/phagosomal membranes with expense of ATP. The V-ATPase complex consists in two functional subcomplexes: the cytosolic V_1 that mediates ATP hydrolysis and the integral membrane V_0 that constitutes the pore whereby the protons go through (Beyenbach and Wieczorek, 2006; Marshansky and Futai, 2008). Therefore, the progressive acidification of the

phagosomal lumen is a hallmark and critical step of phagosome maturation, as an acidic milieu is optimal for the activities of hydrolytic enzymes, the main responsibles for breaking down the phagosomal contents. These hydrolases include nucleases, glycosidases, lipases, phosphatases, sulfatases, phospholipases and even proteolytic enzymes, such as the cathepsins (Turk et al., 2000; Vieira et al., 2002). After being synthesized on the ER, the pre-enzymes (inactive hydrolases) bearing phosphomannosyl residues bind specifically to the mannose-6-phosphate receptor (M6PR) in the Golgi apparatus. There the resulting receptor-ligand complex is package into vesicles and transported through the Trans-Golgi-Network (TGN) until fusion with late endosomal compartments, wherein the low pH mediates the dissociation of the complex. Then, the M6PR is recycled back to the Golgi system, while the late endosome containing the pre-enzymes progresses on the endocytic pathway, until meeting with a lysosome (Griffiths et al., 1988).

Once the late endosome fuses with a lysosome, the key degradative compartment of the cell, the inactive hydrolases become activated owing to the marked acidity of this organelle (pH~4.5-5.0). Although at low pH levels the hydrolases function optimally, the impressive destructive capacity of the lysosomes is also collectively attributed to oxidant agents (reactive oxygen and nitrogen species), cationic peptides that permeabilize bacterial membranes and the NOX2, an NADPH oxidase enzyme complex (Savina et al., 2006). Furthermore, the drop in the pH levels is accompanied by an increase in Cl^- and remarkable changes in Ca^{+2}, Na^+ and K^+ concentrations (Hackam et al., 1997). Not surprisingly, if the late phagosomes ultimately fuse with lysosomes, forming the phagolysosomes, they acquire all these lytic mechanisms, which contribute to their microbiostatic, microbicidal and degradative features (Flannagan et al., 2009; Schwartz and Allen, 2006). Following the digestion of the cargo, the resulting building blocks leave the lysosomes to the cytosol either via diffusion or with the aid of specialized transporters, and there they can be further degraded to fuel energy metabolism or can re-enter biosynthetic pathways (Schulze et al., 2009).

Likewise, acidification of the phagosome is of such utmost importance that the process of antigen presentation relies on that. Molecules of the major histocompatibility complex class II (MHC-II) are yielded and assembled on the ER, where occlusion of the peptide-binding site prevents premature binding during their transport from the Golgi to late endocytic compartments. Within late endosomes the low pH degrades the protective portion of MHC-II leaving the peptide-binding region available to extracellular peptide antigens. Peptide loading onto MHC-II molecules is then delivered to the cell surface of antigen presenting cells (dendritic

cells, macrophages and B lymphocytes) by mechanisms that are still poorly understood, which further stimulates antigen-specific T cells for the appropriate immune response (Ramachandra et al., 2008). Phagosomal acquisition of MHC-II coincides with the gain of late endocytic markers, such as cathepsins and LAMP-2, suggesting that MHC-II is targeted to phagolysosomes, and to traditional late endosomes and lysosomes by the same intracellular transport pathways (Mantegazza et al., 2013).

Particularly curious, after the first rounds of fusion events the surface area of the maturing phagosomes remain nearly constant, although they become biochemically different from their predecessors. These observations imply that some phagosomal membranes are concomitantly removed, thus creating space for the next interaction to occur. Therefore, the constant fusion of vesicles with the phagosomes is balanced by the endosomal recycling pathway that return much of the endocytosed/phagocytosed proteins and lipids back to the plasma membrane (Maxfield and McGraw, 2004; van Ijzendoorn, 2006). The traffic machinery involved in components of phagosomal membrane back to the plasma membrane is mediated by Rab4 (fast recycling), Rab10 and also by Rab11, responsible for the slow recycling. These Rab proteins have been found on the limiting membrane of early phagosomes (Cardoso et al., 2010; Grant and Donaldson, 2009).

Another mechanism that contributes to the maintenance of the phagosome size is the direct delivery of the membrane-associated cargo for degradation into intraluminal vesicles (ILVs). This process is related to the assembly of multivesicular compartments inside the phagosome during intermediate stages of its maturation (Mobius et al., 2003). The sorting of the cargo to ILVs is thought to be facilitated by ubiquitination, that tag the target for subsequent degradation (Lee et al., 2005). Those ILVs are originated through the invagination and pinching of the phagosomal membrane, in a process akin to the formation of multivesicular bodies (MVB), similarly requiring the endosomal sorting complex for transport (ESCRT) machinery (Williams and Urbe, 2007). This complex is composed by ESCRT-0 that binds to ubiquitinated cargo, ESCRT-I and -II that catalyze the budding, while ESCRT-III mediates the scission of the ILVs (Gill et al., 2007). Additionally, distinct ILVs may form as a consequence of different lipid and/or protein cargo composition. For instance, the lipid *bis*monoacylphosphatidic or lysobisphosphatidic acid (LBPA) accumulates in internal membranes that are distinct from those membranes mediating the degradation of cell surface receptors. LBPA has a unique cone-shaped structure that may induce membrane curvature, facilitating its deformation when pH is acidic (Kobayashi and Hirabayashi,

2000; Piper and Katzmann, 2007). Moreover, there are some speculations about the role of LBPA in the cholesterol storage and efflux from late endosomes (Kobayashi et al., 1999).

1.2.3. Apoptotic cells and Efferocytosis

Based on the distinct features of the phagocytic process and immune consequences elicited, Peter Henson and colleagues have suggested the term “efferocytosis” (from the Latin “*effero*”, meaning “carrying the corpse to the grave”) to specifically refer to the engulfment of cells undergoing apoptosis (deCathelineau and Henson, 2003). This type of cell death occurs in all multicellular organisms, playing an essential role in development (particularly during organogenesis), tissue repair and maintenance of homeostasis (cell death is "balanced" against cell birth). Noteworthy, the adult human´s body turns over billions of cells every day (about 1 million cells per second) ensuring the removal of unwanted cells that die by apoptosis, while creating space for new living cells (Henson, 2005; Henson and Hume, 2006; Ravichandran and Lorenz, 2007; Vaux and Korsmeyer, 1999). These undesirable targets include superfluous cells, constantly arising from development, such on the maturation of T cells in the thymus or in specific niches of the brain throughout adult neurogenesis, where only a few number of these newly generated cells will survive and mature further. Aged cells also require constant corpse removal, for instance used leucocytes and effete red blood cells that are degraded in the spleen and liver; or the case of specialized retinal epithelial cells that enable normal vision by clearing senescent fragments shed by photoreceptor cells. Another condition that demand turnover are damage cells, which emerge from injury or infection, for example skin cells exposed to ultraviolet radiation, or macrophage invaded by bacteria. In fact, the efficiency of the efferocytic process appears to be huge when we consider that despite the massive daily loss of cells, the incidence of histologically detectable apoptotic cells is rarely observed in normal tissues (Gardai et al., 2006; Mochizuki et al., 1996; Scott et al., 2001; Vaux and Korsmeyer, 1999).

The term apoptosis (from the Greek: *apo*-off, *ptosis*-fallen) was first proposed in 1972 by the trio Kerr, Wyllie and Currie in their work describing a morphologically distinct form of cell death (Kerr et al., 1972). The induction of this genetic programmed type of cell death is the result of highly complex and sophisticated mechanisms, involving an energy-dependent cascade of molecular events, which implicate caspase activation. During the process of apoptosis the cell undergo some morphological changes starting with cell shrinkage and chromatin condensation. Then, extensive plasma membrane blebbing occurs, followed by nuclear and cell fragmentation

into apoptotic bodies (Coleman et al., 2001). At this point the organelle integrity is still maintained within an intact preserved plasma membrane (Bortner and Cidlowski, 2002). Regarding biochemical features, apoptosis induce modifications such as protein cleavage and cross-linking, DNA breakdown and the expression of several cell surface markers that contribute for the recognition of the dying cells by adjacent phagocytic cells, thus allowing their prompt removal (Elmore, 2007; Wyllie et al., 1980). All these series of controlled events ensure cellular remnants are contained and eliminated without initiating a reactive immune response against self-antigens. Tolerance is accomplished through several mechanisms, including suppression of pro-inflammatory cytokine production and release of anti-inflammatory cytokines, such as IL-10 and TGF-β (Fadok et al., 1998b). Defective removal lead to a persistent dying cell accumulation and activation of self-hydrolytic enzymes, responsible for causing swelling of the cell and irreparable damage to the plasma membrane, event known as secondary necrosis (Edinger and Thompson, 2004; Savill and Fadok, 2000; Silva et al., 2008). The loss of plasma membrane integrity compromises the surrounding tissue, since it is accompanied by leakage of the toxic intracellular contents, triggering an inflammatory response . Over the last years, many advances have been made to elucidate the mechanisms governing the removal of apoptotic cells, while the failure to properly clear them has been linked to non-resolving inflammation (atherosclerosis), many autoimmune conditions (systemic lupus erythematosus and rheumatoid arthritis) (Gaipl et al., 2005; Munoz et al., 2010a; Nagata et al., 2010; Nathan and Ding, 2010), respiratory diseases (chronic obstructive pulmonary disease and asthma) (Henson and Tuder, 2008; Hodge et al., 2005), neurodegenerative disorders (Parkinson´s, Alzhaimer´s and Hungtington´s diseases) (Mochizuki et al., 1996; Su et al., 1994; Thomas et al., 1995; Zhang et al., 1995) and tumorogenesis (Condeelis and Pollard, 2006; Solinas et al., 2009).

Based on work from many laboratories over the past decade, several broadly defined steps have been identified in the recognition and removal of apoptotic cells by phagocytes (Hochreiter-Hufford and Ravichandran, 2013). Current evidence suggests that these steps are very similar between professional and non-professional phagocytes, although the kinetics may differ, with professional phagocytes exhibiting higher rates and capacity for phagocytosis (Erwig et al., 2006; Parnaik et al., 2000). Each stage of the efferocytic process appears to be tightly regulated by signaling events involving both apoptotic cells and phagocytes, in a kind of teamwork, to ensure swift and efficient clearance. In general, phagocytes and dying cells have to be in proximity; the phagocytes then specifically engage the target through the recognition of signals exhibited by the dying cells; following, the phagocytes physically engulf the dying cell through cytoskeletal

reorganization of their plasma membrane; and lastly, the corpse have to be degraded through phagolysosomal processing as described above (Figure 1.5). Collectively, these events mediate the selective and immunologically silent versus immunogenic removal of apoptotic cells *in vivo* (Green et al., 2009; Griffith and Ferguson, 2011).

Early evidence from elegant genetic studies in *C. elegans* indicated that apoptotic cells may be recognized by phagocytes and cleared well before the apoptotic cells are fully dead, suggesting that might exist a mechanism by which the apoptotic cells advertise their imminent death (Reddien et al., 2001). In fact, now it is clear that prompt efferocytosis require that cells in very early stages of apoptosis announce their presence through the release of chemotactic factors, known as "find-me" signals, in order to recruit phagocytes to their proximity. These "find-me" signals released by different mechanisms, set up a concentration gradient within the tissue that allows phagocytes expressing their cognate receptors to migrate toward the site of death and locate the corpse (Peter et al., 2010). By assaying the ability of the culture supernatant from MCF-7 breast cancer cells to trigger chemotaxis of THP-1 monocytes, Lauber and coworkers (Lauber et al., 2003) first identified the lipid lysophosphatidylcholine (LPC) as a find-me signal. Since there, some other possible find-me signals have been reported: the sphingosine 1-phosphate (S1P), fractalkine CX3CL1, and even triphosphate nucleotides like ATP (in very low concentrations) and Uridine-5'-triphosphate (UTP). Among these, only fractalkine and the nucleotides have been shown to have relevance under *in vivo* conditions. Furthermore, UTP may also promote phagocytic activity of neighboring cells (not a recruited phagocyte), perhaps by upregulating phagocytic machinery (Elliott et al., 2009; Truman et al., 2008).

Although the find-me signals help bring phagocytes to the proximity of apoptotic cells, the phagocyte still has to specifically identify the dying cell among the neighboring of living ones. This requires that the apoptotic cells express specific markers on their surfaces, the called "eat-me" signals, for recognition by phagocytes via specific receptors (Paidassi et al., 2009; Ravichandran, 2011). At present many eat-me signals have been described, including the exposure of some normal intracellular components on the cell surface, and changes in the membrane charge and glycosylation patterns. Nuclear splitting and DNA fragmentation are well-established features of apoptosis. Meantime, it was demonstrated that nucleosome material associated with DNA is rapidly exposed at the surface of dying cells, probably by interactions between histones complexes and PtdSer, thus providing ligands for C1q binding (Furnrohr et al., 2007; Radic et al., 2004). Calreticulin (CRT), an endoplasmic reticulum-resident protein that takes part in calcium homeostasis, was also proposed as an "eat-me" signal, since apoptosis induce not only increased

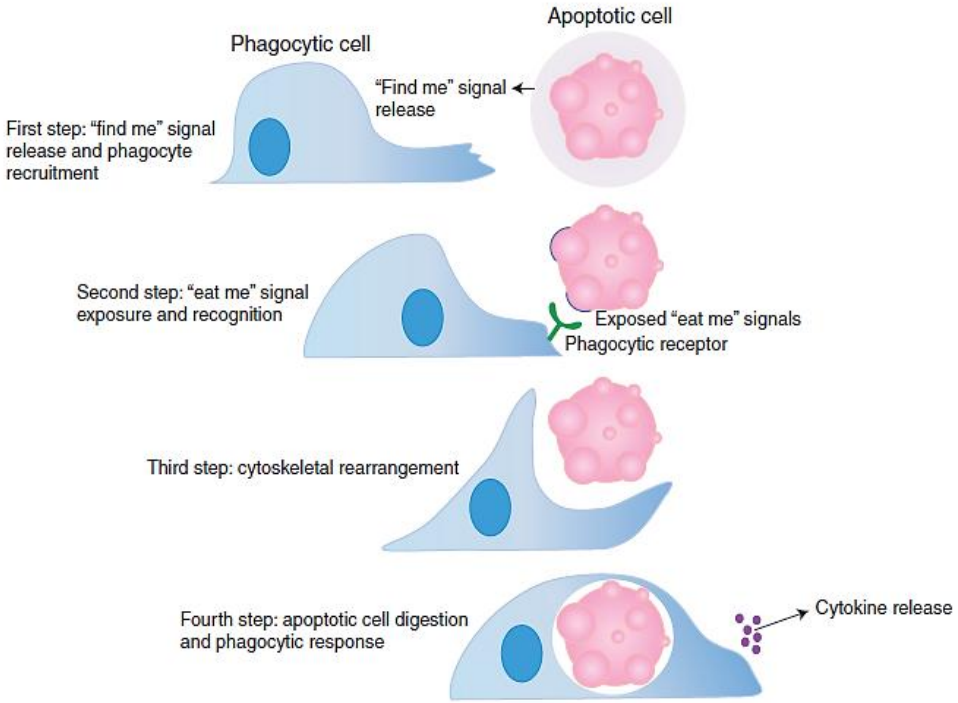


Figure 1.5. The steps of efficient apoptotic cell clearance. First, "find me" signals released by apoptotic cells are recognized via their cognate receptors on the surface of phagocytes. This is the sensing stage and stimulates phagocyte migration to the location of apoptotic cells. Second, phagocytes recognize exposed "eat me" signals on the surface of apoptotic cells via their phagocytic receptors, which leads to downstream signaling events culminating in cytoskeletal remodeling. Finally, further signaling events within the phagocyte regulate the digestion and processing of the apoptotic cell meal and the secretion of anti-inflammatory cytokines. [Adapted from (Hochreiter-Hufford and Ravichandran, 2013)].

cell-surface expression of CRT, but also its redistribution into membrane patches that colocalize with PtdSer, which facilitate the binding to the lipopolysaccharide receptor CD14 (Gardai et al., 2005; Williams, 2006). In addition to the appearance of newly exposed elements, the cell surface undergoes some changes during apoptosis, which applies to modifications of the cell glycosylation profile and oxidative status. Oligosaccharides attached to cell-surface glycoproteins become altered during apoptosis due to the removal of the terminal sialic acid from the carbohydrate chains. The loss of this terminal residue is associated with a decrease of the negative charges around the dying cell, allowing increased adhesion to phagocytes and binding to lectin proteins (Azuma et al., 2002). More, the oxidative stress induced by the apoptotic stimuli produce reactive oxygen species (ROS), ultimately, leading to membrane lipid peroxidation and oxidized-phospholipids that may be perceived as ligands to scavenger receptors (e.g. CD36, CD68 and LOX-1) (Oka et al., 1998; Savill et al., 1991). The expression of intercellular adhesion molecule 3 (ICAM-3), as well as the exposure of annexin-I and the binding of serum proteins such as

thrombospondin and complement C1q on the apoptotic cell surface are also signals for engulfment (Takizawa et al., 1996; Vandivier et al., 2002).

Although apoptosis induce many different modifications on cell surface, the most widely studied and universally seen alteration is the loss of phospholipid asymmetry and exposure of PtdSer on the outer leaflet of the plasma membrane. This evolutionarily conserved phenomenon is observed in many different cell types after multiple modalities of apoptosis induction, what is, perhaps, the reason why PtdSer externalization is a generally accepted definition for calling a cell apoptotic (Asano et al., 2004; van den Eijnde et al., 1998). PtdSer constitutes about 2-10% of the lipids in the plasma membrane in most eukaryotic cells. In healthy cells it is normally confined to the inner leaflet of the plasma membrane. However, when cells initiate the apoptotic program, there is a dramatic change in the amount of PtdSer exposed on the outer leaflet (more than 280-fold), which occur very early during apoptosis, when cells still retain their plasma membrane integrity (Borisenko et al., 2003). The exact mechanism by which PtdSer externalization occurs is just beginning to be better defined, but it appears to be a caspase-dependent event, in which ATP-dependent translocases that maintain PtdSer at the inner leaflet of the plasma membrane are inactivated, while Ca^{+2}-dependent phospholipid scramblases are activated, causing randomization ("flip-flop") of the membrane leaflet components (Sahu et al., 2007; Tang et al., 1996; Zullig et al., 2007).

The specific recognition of PtdSer appears to come in two kinds of receptors: those that are membrane proteins so can directly bind and recognize PtdSer, and those that indirectly recognize PtdSer through binding of soluble bridging molecule. The receptors capable of directly recognizing PtdSer include members of the T cell immunoglobulin and mucin (TIM) family (TIM-4, as well as TIM-1 and TIM-3) (Kobayashi et al., 2007; Miyanishi et al., 2007; Nakayama et al., 2009); the seven transmembrane brain angiogenesis inhibitor 1 (BAI1) (Park et al., 2007); and the atypical epidermal growth factor (EGF)-motif containing membrane protein Stabilin-2 (Park et al., 2008). On the other hand, the indirectly recognition is mediated by soluble proteins like the milk-fat-globule-EGF-factor 8 (MFG-E8) (Hanayama et al., 2002), the growth arrest-specific 6 (Gas6) and the serum Protein S. One region of MFG-E8 can bind PtdSer on apoptotic cells with high affinity, whereas a second region can simultaneously engage Integrin αvβ3 on phagocytes, thus mediating PtdSer-dependent uptake of apoptotic cells. Likewise, Gas6 and Protein S link up PtdSer exposed on apoptotic cells, being recognized in turn by Tyro-3-Axl-Mer family of receptors (denoted as TAM receptors) on phagocytes (Lemke and Rothlin, 2008; Rothlin et al., 2007; Scott et al., 2001). The most recent PtdSer receptor described is the receptor for advanced glycation

end products (RAGE), which curiously binds PtdSer in both its membrane-bound and soluble forms (He et al., 2011). Moreover, PtdSer oxidation itself was considered critical to induce efferocytosis, working as a ligand that binds to scavenger receptors on phagocytes (Figure 1.6).

Over the past decade, there has been a significant increase in our understanding in how PtdSer is recognized on apoptotic cells, although it is still a puzzle to understand why we need so many different receptors to do the same job, that is: grab the corpse. So far, the consensus in the field has been that not all receptors are expressed on a given phagocyte, and therefore multiple combinations of recognition are necessary depending on the tissue context (place, cell types involved, apoptotic stimuli, hierarchy affinity, etc.) (Bratton and Henson, 2008; Savill and Fadok, 2000). In agreement to this, several years ago, was hypothesized the model of "tethering and tickling", wherein some receptors may serve only with an adhesion purpose, whereas the others may mediate signaling (Henson et al., 2001). In fact, the interpretation of the current data suggest that PtdSer alone is not sufficient to mediate efferocytosis, which indicates that PtdSer on apoptotic cells is recognized by phagocytes in conjunction with one or more eat-me signals simultaneously, sequentially or even cooperatively to enhance engulfment (Hoffmann et al., 2001; Somersan and Bhardwaj, 2001).

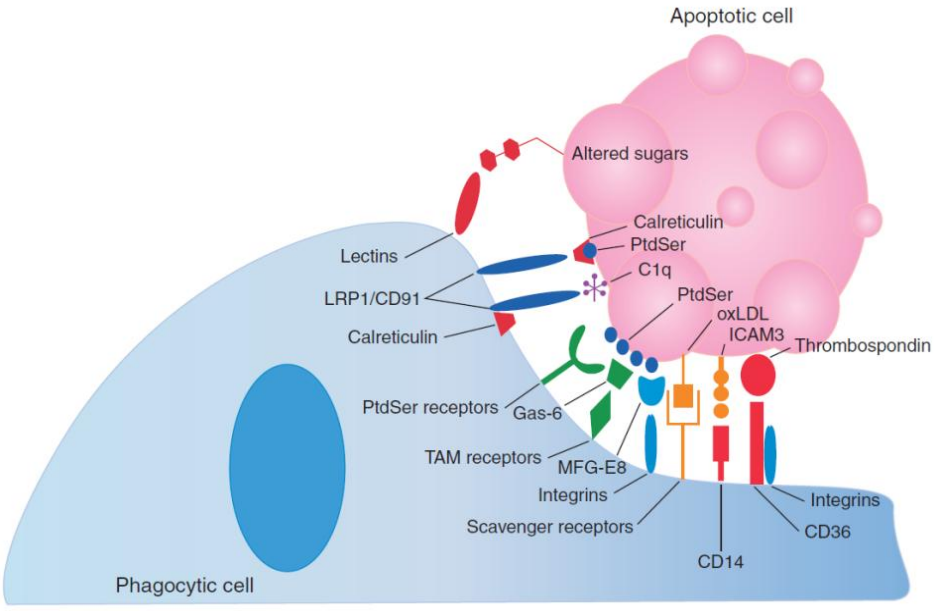


Figure 1.6. Apoptotic cell "eat me" signals and phagocytic receptors involved. As apoptotic cells undergo apoptosis, they expose "eat me" signals on their surfaces. PtdSer is the best studied "eat me" signal; however, several others are also pictured here. "Eat me" signals are recognized by phagocytic engulfment receptors either directly (as with PtdSer receptors including TIM-4, BAI1, and Stabilin-2) or indirectly via bridging molecules or accessory receptors (as with Gas-6/TAM receptors, MFG-E8/$\alpha_v\beta_{3/5}$, and $\alpha_v\beta_{3/5}$ in conjunction with CD36 in the recognition of thrombospondin). [Adapted from (Hochreiter-Hufford and Ravichandran, 2013)].

Receptor engagement to eat-me signals induces cytoskeletal reorganization in different types of cellular movements, which is regulated by members from the Rho-family GTPases, including RhoA and Rac1. Usually, apoptotic cell ingestion is described as a locally and temporarily controlled “zipperlike” mechanism, which implies that pseudopods follow the contour of the target particle and that both surfaces are tightly juxtaposed (as described before) (Krysko et al., 2006). Nevertheless, there are some evidence showing a different process, more akin to macropinocytosis (Hoffmann et al., 2001), which is associated with the uptake of surrounding fluid and the formation of spacious phagosomes (Erwig and Henson, 2008). Once again, both physical modes of uptake might be involved depending on the combination of “eat-me” signals exposed by the dying cell in a determined tissue situation. Activation of the protein RhoA seems to have a negative effect on engulfment, since it influences the phosphorylation status of the myosin light chain, promoting increased stress-fiber formation and cell contractility, which impair pseudopods extension (Tosello-Trampont et al., 2003). However, this inhibitory effect of RhoA contrasts with the positive effect of Rac1 that is activated by an upstream signaling pathway composed by the ELMO1-DOCK180 complex, which drives actin polymerization and the membrane ruffles formation, thus facilitating uptake at the phagocytic cup (Brugnera et al., 2002; Gumienny et al., 2001; Ravichandran and Lorenz, 2007).

After engulfment, the efferocytic process is still not complete, and must undergo phagosomal maturation to proper degradation and processing of the apoptotic material in order to maintain self-tolerance via cross-presentation of engulfed cell peptides through the MHC complex. The steps involved in apoptotic cell digestion are quite similar to the general process of phagosome maturation already discussed somewhere before (Bellone et al., 1997; Lu and Zhou, 2012; Zhou and Yu, 2008). However, this specific type of cargo initially recruits a different player to the phagocytic cup, the Dynamin, an evolutionarily conserved large GTPase that interacts with Vps34, leading to Rab5 recruitment and activation, which in turn promotes Vps34 activation and focal generation of PI(3)P on the phagosome surface (Praefcke and McMahon, 2004; Schmid and Frolov, 2011) . Data obtained from studies using the nematode *C. elegans* have shown that Dynamin controls both engulfment and phagosome maturation by stimulating the delivery of essential organelles to pseudopods and phagosomes (He et al., 2010; Yu et al., 2006). Furthermore, recent reports brought to light that components of autophagy machinery (but not intact autophagosomes) are associated with maturing phagosomes containing different types of cargo, including apoptotic cells. Apart from other common aspects shared by phagocytosis and autophagy, such as lysosome delivery of cargo, this additional crosstalk highlights the striking

conservation of regulatory factors between the two pathways (Lamb et al., 2013). So far, the appearance of autophagy effectors on phagosomal membranes is a quite unexplored field of research and, presumably, it works to enhance the degradative capacity of the phagosomes by promoting fusion with endosomes and lysosomes (Martinez et al., 2011; Sanjuan et al., 2007). Altogether, these new findings show us that the phagosome is not merely a large endosome and that phagosome maturation is a much more sophisticated process than previous thought.

1.3. Autophagy

1.3.1. Canonical Autophagy - general concepts

The term autophagy comes from the Greek words "*phagy*" meaning eat, and "*auto*" meaning self. By definition, this evolutionarily conserved self-eating mechanism is a stress-induced catabolic process that involves the sequestration and transport of cytosolic components to lysosomes for degradation. Notably, this is the only known mechanism that eukaryotic cells possess to dispose of intracellular organelles or protein aggregates that are too large to be degraded by the ubiquitin-proteasome system (UPS) (Ciechanover et al., 2000; Wong and Cuervo, 2010). Following lysosomal degradation, recycling occurs to replenish the cell with nutrients and building blocks for anabolic processes. The first descriptions of autophagy are from the early 1960s, when Christian de Duve described the presence of membrane vesicles containing parts of the own cytoplasm and organelles in various stages of disintegration inside normal cells. More, he pointed out that this process involving sequestering vesicles was related to lysosomes, stating that conditions such as starvation triggers its activation (De Duve and Wattiaux, 1966). Traditionally, autophagy was thought to be just a process of adaptation to nutrient deprivation, in which long-lived proteins and organelles were nonselective degraded to maintain cell homeostasis and survival (Mizushima, 2007). However, more recently, the scientific interests in the field remarkably increased and autophagy has been show to carry out a broad range of selective functions, including the turnover of superfluous organelles and misfolded proteins, as well as the clearance of defective organelles, protein aggregates and even intracellular pathogens, controlling aspects of immunity in multicellular organisms. Therefore, the autophagy machinery is thought to have evolved as a cellular response to allows unicellular eukaryotic organisms to survive during harsh conditions, probably by regulating energy homeostasis and/or protein and organelle quality control (Klionsky, 2007; Kubota, 2009).

Despite the most primordial function of autophagy is to protect cells by managing stressful conditions to maintain the cellular energetic balance, this degradative pathway is also involved in multiple biological processes including development (Levine and Klionsky, 2004), senescence (Young et al., 2009), lifespan extension (Vellai et al., 2009), tumor suppression immunity and defense against microbial invasion (Deretic and Levine, 2009). Usually, the cellular autophagic activity is very low under basal conditions, but can be markedly upregulated by numerous stimuli. The most well-known inducer of autophagy is nutrient deprivation (*in vitro* and *in vivo*, ranging from yeast to mammals), although it can also be activated by other physiological stress stimuli (e.g. hypoxia, energy depletion, ER-stress and high temperatures); hormonal stimulation; pharmacological agents (e.g. rapamycin that target mTOR); innate immune signals and diseases. Thus, an aberrant regulation and failure to properly degrade autophagic targets are related to many human pathologies, including cancer, myopathies, neurodegeneration, autoimmunity, heart and liver diseases, and gastrointestinal disorders (Cecconi and Levine, 2008; Levine and Kroemer, 2008; Mizushima et al., 2008). On the other hand, if autophagy is excessively induced, it can result in autophagic cell death, so-called type II programmed cell death, which would imply that autophagy is an upstream event of apoptosis or, alternatively, it could be independent of apoptosis, such as in situations in which autophagy-induced cell death does not exhibit any characteristic feature of apoptosis, like caspase activation and DNA fragmentation (Eisenberg-Lerner et al., 2009; Voss et al., 2010).

According to the different pathways by which cargo is delivered to lysosomes, autophagy can be divided into three main types: chaperone-mediated autophagy (CMA), microautophagy and macroautophagy. In CMA, a chaperone protein binds first to its cytosolic target and then to a LAMP-2 receptor on the lysosomal membrane, where the unfolding of the protein occurs. The unfolded cytosolic target protein is subsequentialy translocated into the lysosome for its degradation (Cuervo, 2010; Massey et al., 2004). CMA performs several general functions, such as the elimination of oxidazed proteins and misfolded proteins, and also provides amino acids during prolonged periods of starvation (Kaushik et al., 2008). Microautophagy directly engulfs soluble cytoplasmic materials into the lysosome for degradation either by invagination, protrusion or septation of the lysosomal membrane, in a process dependent on GTP hydrolysis and calcium. The maintenance of organellar size and cell survival under nitrogen restriction are the main functions of microautophagy (Li et al., 2012b; Uttenweiler and Mayer, 2008). Macroautophagy, in turn, is characterized by the formation of a cytosolic double-membrane vesicle called autophagosome that captures the intracellular target, undergoes maturation along

the endocytic pathway and reaches the lysosomes for content degradation (Yang and Klionsky, 2010a). Among the three main forms of autophagy, macroautophagy, hereafter referred as to autophagy, is the most widely studied and best characterized process.

The autophagic pathway consists in a very complex and dynamic series of events that depends on a set of well-conserved core of AuTophaGy-related gene products (ATG proteins), which are responsible for the regulation of the different steps of autophagosome formation: initiation/nucleation, elongation, binding of selective substrates and closure (Burman and Ktistakis, 2010; Rubinsztein et al., 2012; Yang and Klionsky, 2010b). In mammalian cells, the key upstream kinase that regulates the induction of most forms of autophagy is the UNC-51 like kinase1 (ULK1, the homolog yeast Atg1), which forms a complex with Atg13, Fip200 and Atg101. Upon induction signals the ULK1-complex is activated by the mammalian target of rapamycin (mTOR), the main regulator of the balance between cell growth and autophagy in response to cellular physiological conditions and environmental stress (Chan, 2009; Ganley et al., 2009; Jung et al., 2009). During the nucleation step, the active ULK1-complex is translocated from the cytosol to ER domains in order to recruit another kinase complex formed by Vps34, Beclin1/Atg6 and Vps15, which in turn generates PI3P on the outer leaflet of the ER (Funderburk et al., 2010; He and Levine, 2010; Levine and Deretic, 2007). Locally synthesized PI3P recruits effector proteins involved in the specific generation of ER-associated structures named omegasomes, which act as cradle for the formation of the phagophore, a precursor membrane that is able to expand to form the autophagosome (Matsunaga et al., 2010). Throughout this process, the concave side of a growing phagophore eventually becomes the luminal side of an autophagosomal inner vesicle, sequestering any cytoplasmic materials in that region. While converging evidence indicates that the phagophore originates from the ER, other sources of membranes such as the Golgi apparatus, mitochondria and the plasma and nuclear membranes have been implicated, leaving the question of its origin open (Hayashi-Nishino et al., 2009; Tooze and Yoshimori, 2010).

Following initiation, two different ubiquitin-like protein conjugation systems mediate the elongation of the autophagosome membrane. The Atg5-Atg12 conjugation system first conjugates Atg12 to Atg7 (E1-like activating enzyme), and this is followed by the transfer of Atg12 to Atg10 (E2-like conjugation enzyme). After that, Atg12 is transferred to Atg5 via a covalent bond. Then, Atg5-Atg12 conjugate forms a functional complex with Atg16, and this multimeric complex is crucial in autophagosome formation (Fujita et al., 2009; Itakura and Mizushima, 2010). The second conjugation system is initiated with the cleavage of the microtubule-associated

protein 1 light chain 3 (MAP1LC3 or just LC3) by the cysteine protease Atg4 to LC3-I, so exposing a glycine residue on the C-terminus end (Ichimura et al., 2000). Next, the cytosolic LC3-I is bound and activated by Atg7 (E1-like enzyme) and is transferred to Atg3 (E2-like enzyme). LC3-I is subsequently covalently linked to the lipid phosphatidylethanolamide (PE) (Geng and Klionsky, 2008). The previous formed Atg5-Atg12-Atg16 complex acts here as an E3-like ligase on the LC3 conjugation reaction to generate LC3-PE (LC3-II), which is incorporated into both the cytoplasmic and luminal faces of the elongating double-membrane, and facilitates closure of the autophagosome (Figure 1.7). In mammalian systems, most of the Atg proteins are only observed during phagophore formation and expansion, but never on the complete autophagosome (Longatti and Tooze, 2009). To date, only LC3, the mammalian homolog of yeast Atg8, is known to exist in autophagosomes, and therefore, this protein serves as a widely marker for autophagosomes (Kabeya et al., 2000; Mizushima, 2004).

While yeast has only a single copy of Atg8; and Drosophila and *C. elegans* have two copies; mammals have at least 6 homologues that can be grouped into two subfamilies: LC3 subfamily, which includes the LC3A (including 2 splicing variants), LC3B and LC3C isoforms; and the gamma-aminobutyrate receptor-associated protein (GABARAP) subfamily that comprises GABARAPL1, GABARAPL2 (GATE-16) and GABARAPL3 proteins (Geng and Klionsky, 2008; Weidberg et al., 2010b). All these members are involved in autophagy, but probably contribute for different aspects of autophagosome biogenesis, for instance LC3 isoforms are involved in phagophore membrane extension, whereas GABARAP family is required for later stages of autophagosome maturation. Despite extensive studies, the exact function of Atg8/LC3 is still unclear (Weidberg et al., 2010a). LC3 has been shown to be essential to drive autophagosome expansion possibly by mediating tethering and hemifusion of lipid membranes (Nakatogawa, 2007). Likewise, the amount of LC3 determines the size of the autophagosome (Xie et al., 2008). Furthermore, it is clear that LC3 associated with the inner membrane of the autophagosome is indispensable for the selective recognition and sequestration of specific cytosolic cargoes (Kanki et al., 2009; Noda et al., 2008).

The autophagosomes are ready to fuse with lysosomes once the vesicle membranes are sealed and the Atg machinery is disassembled and released back for reuse. They mature by either directly fusion with a lysosome to generate an autophagolysosome, or by fusing first with late endosomes to form an amphisome, which then fuses with the lysosome (Ganley, 2013). The interaction with compartments of the endocytic pathway could facilitate the autophagosome maturation, since several protein complex required for fusion with lysosomes are shared

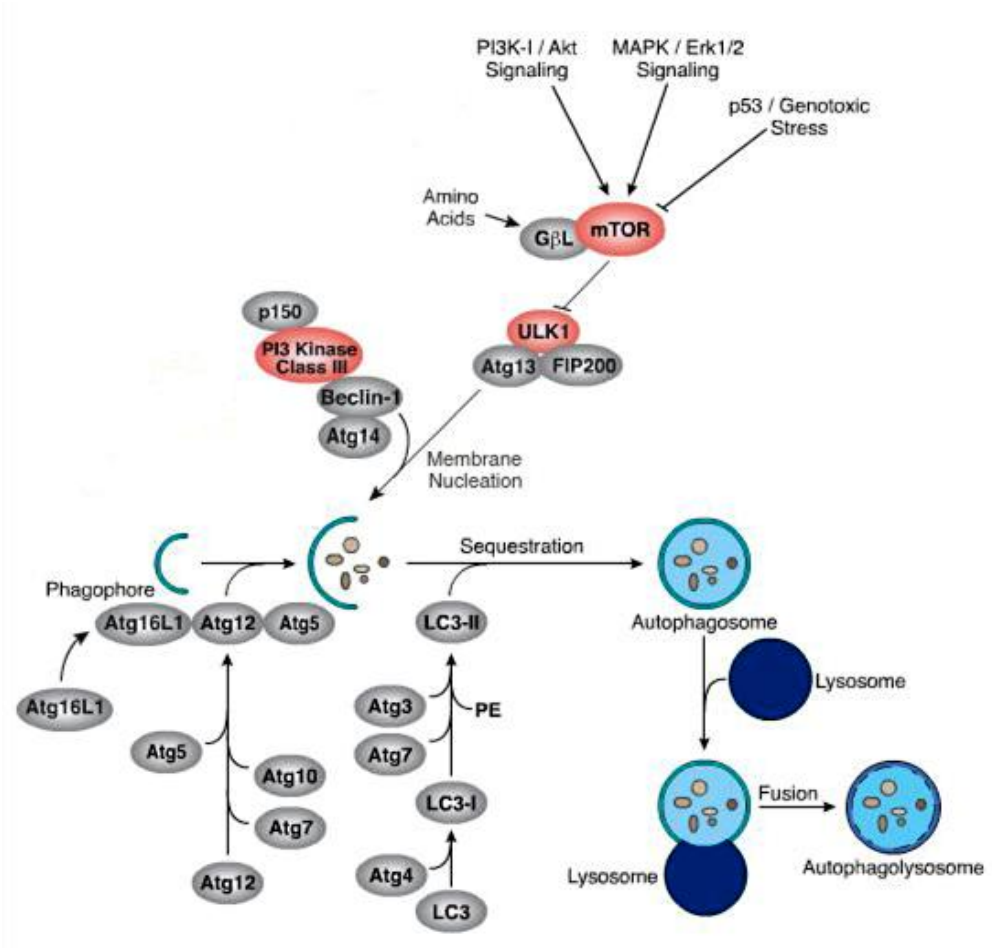


Figure 1.7. Diagram of the signaling pathway regulating autophagy. Under induction autophagy begins with the formation of the phagophore (membrane nucleation step). The concerted action of the autophagy core machinery proteins in the phagophore is thought to lead to its expansion around a cytosolic cargo (vesicle elongation), until its closure forming an autophagosome. When the outer membrane of the autophagosome fuses with a lysosome (docking and fusion steps) it forms an autophagolysosome. Finally, the sequestered material is degraded inside the autophagolysosome (vesicle breakdown and degradation) and recycled. (Source: http://www.cellsignal.com/).

between both pathways (e.g. ESCRT, HOPS and SNAREs complexes) (Kim et al., 2012; Lamb et al., 2013; Moreau et al., 2011; Nair et al., 2011). Moreover, this is involved in the transport of cytosolic antigens to MHC-loading endosomes to antigen presentation (Lee et al., 2010a; Schmid et al., 2007). Therefore, following the fusion with lysosomes, the lipidated LC3 on the outer membrane of the autophagolysosome is cleaved off by Atg4, which deconjugates LC3-PE, releasing LC3 back to the cytosol to be recycled (Yu et al., 2012). Meanwhile, the LC3-II on the inner membrane is degraded by lysosomal enzymes, together with the enclosed materials and the inner membrane itself. Amino acids and other building blocks that are generated by autophagic degradation are then returned to the cytoplasm for recycling or energy production.

1.3.2. Selectivity in autophagy

Sequestration of intracellular components inside an autophagosome was considered for a long time a non-selective bulk process by which cytosolic material was randomly captured and delivered to lysosomes. However, a number of recent reports present mounting evidence of selective autophagic degradation of protein inclusions caused by aggregate-prone or misfolded proteins (aggrephagy) (Filimonenko et al., 2010; Gamerdinger et al., 2009; Rubinsztein, 2006), of organelles such as mitochondria (mitophagy) (Okamoto et al., 2009; Tolkovsky, 2009), peroxisomes (pexophagy) (Kim et al., 2008), ribosomes (ribophagy) (Kraft et al., 2008), surplus ER (reticulophagy) (Bernales et al., 2006), lipids (lipophagy) (Singh and Cuervo, 2012), and even bacteria and virus (xenophagy) (Deretic, 2006; Dupont et al., 2009; Levine, 2005; Yordy et al., 2013). Mainly, these findings revealed the existence of a growing number of proteins dedicated to the tag and recognition of distinct cytosolic substrates. Moreover, the recruitment of these autophagy effectors toward the target is proposed to initiate the *in situ* formation of the autophagosome around the specific material in an LC3-dependent manner (Itakura and Mizushima, 2011).

Covalent attachment of ubiquitin to proteins has emerged as a highly versatile regulatory signal to several key cellular process including gene transcription, cell cycle progression, DNA repair, receptor-mediated endocytosis and different forms of selective autophagy (Haglund and Dikic, 2005). Yet, despite the binding of ubiquitin be the more often view post-translational modification to label selective targets to autophagy degradation, Galectin-8 bound to glycans on damage vesicles, phosphorylation and acetylation were also identified signals (Kirkin et al., 2009c; Thurston et al., 2012). The process of tagging a protein with ubiquitin is called ubiquitination and serves as the “kiss of death” signal for protein turnover. Ubiquitination occurs through an isopeptide bond formation between a specific amino group of a lysine residue in a target protein and the C-terminal carboxyl group of ubiquitin. Proteins can be modified by the conjugation of a single ubiquitin monomer (monoubiquitylation and multi-monoubiquitylation) or by a sequential conjugation of ubiquitin polymers (polyubiquitylation), in which ubiquitin moieties are most often connected via lysine-mediated isopeptide linkages (Ikeda and Dikic, 2008; Ye and Rape, 2009). Different chain linkage types arise from the fact that ubiquitin have seven different lysine residues, which serve as ubiquitin acceptor to another ubiquitin monomer (Behrends and Harper, 2011). This diversity in how ubiquitin can be attached to a certain substrate is decoded by distinct classes of ubiquitin-binding domains (UBDs). In selective

autophagy, at least 3 different ubiquitin-binding domains have been implicated in specific cargo receptors: ubiquitin-associated (UBA), ubiquitin binding in ABIN and NEMO (UBAN), and the ubiquitin-binding zinc finger (UBZ) domains (Dikic et al., 2009; Harper and Schulman, 2006). Thus, implementation of ubiquitin-binding domains in autophagy cargo receptors provides a flexible signal, which allows a much broader range of proteins to be targeted for autophagosome degradation. So far, a variety of cargos have been found to be depend on their ubiquitination to be efficiently incorporated into autophagosomes, it is the case of protein aggregates, mitochondria (via ubiquitination of outer mitochondrial membrane proteins), and microbes (via ubiquitination of bacterial membrane proteins or host binding proteins).

Autophagy receptors are defined as proteins being able to interact directly with both the intracellular structure that has to be specifically eliminated and the internal surface of growing phagophores, working as an adaptor to mediate cargo recognition, reason why they are also known as adaptor proteins (Johansen and Lamark, 2011; Rogov et al., 2014) (see Figure 1.8A). The binding of autophagy receptors to a determined cytosolic ubiquitin-tagged cargo is mediated by their UBDs, whereas the interaction with the expanding autophagosome is, usually, mediated by a specific hydrophobic sequence commonly referred to as the LC3-interacting region (LIR) motif, which in turn bind to the pool of Atg8/LC3 protein family members conjugated to the double-membranes (Noda et al., 2008; Shaid et al., 2013). For instance, an UBA domain is found in adaptor proteins like p62/Sequestosome-1 (SQSTM1) and Neighbor of BRCA1 gene1 (NBR1); while UBAN and UBZ domains are found in Optineurin (OPTN) and Nuclear dot protein 52 (NDP52), respectively (Rahighi et al., 2009). Beside these classical autophagy receptors characterized by their UBD and LIR motifs, some non-classical are appearing, targeting substrates selectively for autophagy independently of canonical UBD and LIR motifs. In mitophagy, for example, that mediates the elimination of damage or surplus mitochondria, UBDs are not always required. Despite the removal of mitochondria for organelle quality control is mostly mediated by the classical p62 receptor, the specific elimination of mitochondria from red blood cells, during reticulocyte maturation, critical involves the protein NIP3-like protein X (Nix) that has a LIR motif but is lacking a UBD (Novak et al., 2010; Sandoval et al., 2008). More, the promiscuous c-Casitas B-lineage lymphoma (c-CBL) shown to operates as an adaptor protein in selective autophagy independently of their catalytic activity, being also involved in proteasomal degradation (Sandilands et al., 2012) (Figure 1.8B).

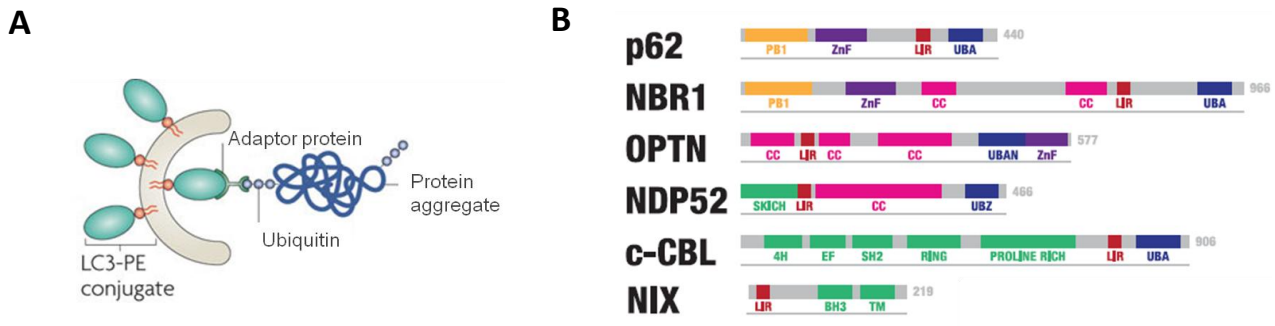


Figure 1.8. Machinery involved in selective autophagy. Ubiquitylation of cytosolic targets is a signal that triggers binding of the adaptor proteins, which also binds LC3 conjugated with phosphatidylethanolamine (PE) in the double membrane of the forming autophagosomes, allowing specific cargo sequestering (**A**) [Adapted from (Tyedmers et al., 2010)]. Some identified proteins involved in selective autophagy and their domain architecture. p62, NBR1, OPTN, NDP52 and c-CBL are autophagic adaptor proteins. Excluding c-CBL they all interact with both ubiquitin and LC3 to promote autophagic degradation. The UBD domain of c-CBL instead seems not to be involved in autophagic degradation. NIX is a mitochondrial membrane protein, which bind LC3/GABARP via their LIR motif (**B**). [Adapted from (Shaid et al., 2013)].

Regarding to the classical machinery involved in selective autophagy, p62 is the best-known mammalian adaptor protein and act as a universal receptor for ubiquitinated cargo (Bjorkoy et al., 2005; Pankiv et al., 2007). This protein is considered indispensable for basal levels of autophagy and has multifunctional roles in several biological processes such as cell signaling, differentiation and particularly in the removal of toxic misfolded proteins (Lee et al., 2010b; Moscat and Diaz-Meco, 2009; Moscat et al., 2007; Rodriguez et al., 2006). Mediated by its UBA domain, p62 is able to bind mono- or poly-ubiquitinated proteins, while its LIR motif allows the binding to LC3 in emerging autophagosomes. For instance, mutations in the UBA domain of p62 are related to chronic and metabolic disorders during bone remodeling (Goode and Layfield, 2010). Importantly, lack of autophagy leads to p62 accumulation into ubiquitin-positive inclusion bodies in neurodegenerative diseases and proteinopathies of the liver and muscles, in which p62 respond to stresses including amino acid starvation, ROS, accumulation of defective ribosomal products, so displaying a cytotoxic effect (Lamark and Johansen, 2012). Through its Phox and Bem1p (PB1) domain, p62 undergoes dimerisation, which allows interaction with protein kinases (e.g. MAP-kinases) and with the protein NBR1 (Lamark et al., 2003). The adaptor protein NBR1 also contain the N-terminal PB1 domain as well as the C-terminal UBA domain and the LIR sequence, but instead of p62, it undergoes dimerisation via the coiled coil (CC) domain. The shared common PB1 domain drives multimerasation of p62 and NBR1 in complex with ubiquitinated proteins, thereby amplifying the engagement of ubiquitinated substrates, what is

critical, for instance, in aggrephagy (Kirkin et al., 2009a; Lamark et al., 2009). Sequestration of misfolded proteins into protein aggregates likely shields aberrantly exposed hydrophobic surfaces from harmful interaction with essential cellular proteins, so it is regarded as a cellular defense mechanism (Kopito, 2000). Moreover, p62 and NBR1 were found to be recruited to the site of autophagosome formation, which is dependent on self-oligomerization but independent of most Atg proteins, including LC3; so suggesting that their localization may determine where autophagosomes are nucleated (Itakura and Mizushima, 2011). Although NBR1 can directly bind to p62 and cooperatively they act as a cargo receptor, it can also mediate autophagy independently, which is the case of the autophagic elimination of midbody derivates (Kuo et al., 2011). Once accumulation of midbody derivates is associated with cellular reprogramming of stem cells and enhanced tumorigenicity, NBR1 may have a role in cell differentiation and tumor suppression. Curiously, homologues of NBR1 are found throughout the eukaryotic domain, whereas the presence of p62 is unique for metazoans and likely the result of a duplication event early in the metazoan lineage (Svenning et al., 2011).

Together with NDP52 and OPTN, p62 also participates in the cellular defense mechanism against infection (Thurston et al., 2009; Wild et al., 2011; Zheng et al., 2009). Mammalian cells conjugate ubiquitin to pathogens that intrude the cytosol or reside into sequestered intact or damage phagosomes as part of their protective response thereby tagging microbes for destruction by xenophagy. For instance, the autophagic degradation of the bacteria *Salmonella Typhimurium* is suggested to be dependent in three autophagy receptors. Firstly, phagosomal membranes damaged by *Salmonella* entering the cytosol attract Galectin-8, which is specifically sensed by NDP52 Galectin-8, which is specifically sensed by NDP52 (Thurston et al., 2012), then once it escapes from the phagosome and becomes exposed to the cytosol, *Salmonella* is coat with host ubiquitin, which in turn bind to the UBA domain of p62, the UBZ domain of NDP52 and also the UBAN domain of OPTN (Rogov et al., 2013). All these adaptor proteins seem to be needed for restrict intracellular replication, although they are recruited independently to distinct microdomains surrounding the bacteria and cooperate for efficient degradation (Cemma et al., 2011). Yet, in spite of all three receptors act cooperatively in the same pathway, they are not redundant, since depletion of either protein induces bacterial proliferation, which suggest different roles, or even implicate a hierarchical and/or temporal recruitment. In addition, p62 and NDP52 were recently reported to target *Shigella* and *Listeria* to distinct autophagy pathways (Mostowy et al., 2011). Curiously, while p62, NBR1 and OPTN bind nonspecifically to multiple LC3/GABARAP proteins, NDP52 binds selectively to LC3C isoform through a non-canonical LIR

motif, termed CLIR that lack an aromatic residue, providing more specificity to handle different autophagic cargo and suggest a hierarchy among ATG8 orthologs (von Muhlinen et al., 2012). Another evidence of selectivity in xenophagy is the recruitment of p62 as an antiviral defense to clear surplus viral capsid proteins somehow protecting the host against cell death (Orvedahl et al., 2010).

In the past few years, research in the field uncovered new layers of complexity and functional diversity in terms of how this set of genes - originally characterized in the context of macroautophagy - may function to protect multicellular organisms against stressful conditions. However, many questions remain concerning identities of additional cargo and receptor pairs as well as signaling cascades leading to efficient cargo binding and recruitment to autophagic membranes under different physiological and pathophysiological conditions. Lastly, would be of great interest to elucidate the role of selective autophagy in different signalling pathways, especially to reveal how autophagy can control cellular homeostasis by modulating the capacity of intracellular signalling networks.

1.3.3. LC3-Associated Phagocytosis (LAP)

While endocytosis and autophagy were once considered largely separate modes of acquiring nutrients, recent evidence has shown extensive collaboration between them in mammalian cells, including the identification of an endocytic origin for vesicles utilized for autophagosome biogenesis (Ravikumar et al., 2010), fusion between autophagosomes and endosomes (Gordon and Seglen, 1988), lysosome delivery of cargo and co-regulation of endocytic trafficking and autophagy by Beclin1-Vps34 protein complexes (Funderburk et al., 2010; Lamb et al., 2013; Shui et al., 2008; Singh and Cuervo, 2011). However, one of the most surprising findings in this field is that autophagy proteins can control the degradation of several phagocytic targets, in an autophagosome-independent manner, what is called LC3-Associated Phagocytosis or simply LAP. This non-canonical recruitment of autophagy machinery to phagocytic compartments was first identified by the requirement of LC3, previously considered to be exclusively an autophagic marker of double-membrane vesicles, to single-membrane phagosomes harboring engulfed microorganisms in macrophages (Sanjuan et al., 2007). Thus, since this initial discovery of LAP occurring with agonists of TLR signalling, it has been proposed to occur in a variety of cellular contexts including in the clearance of apoptotic cells and other types of cell death removal, such as necrosis and RIPK3-dependent necrosis (Martinez et al., 2011), in FcγR-mediated engulfment

of IgG-opsonized substrates (Henault et al., 2012; Huang et al., 2009), zymosan internalization, macropinocytic uptake of fluid-filled vacuoles (Florey and Overholtzer, 2012) and in the ingestion and killing of live epithelial cells, a process named entosis (Florey et al., 2011). Similarly, in *C. elegans*, LGG-1 (homolog of LC3) is recruited to phagosomes taking apoptotic corpses, which reflects some evolutionary trend in this process (Li et al., 2012a). Furthermore, all described types of pathogen-related LAP seem to require bacterial viability and intact phagosome membrane (Lerena and Colombo, 2011). Hence, LAP has a number of different roles that include maintenance of cellular homeostasis and protection against invading pathogens (Martinez et al., 2013).

Although the participation of some phagocytic receptors are reported to induce LC3 translocation to single-membrane phagosomes, the precise mechanism responsible for triggering LAP remains to be discovered. In the case of pathogens, for instance, the recognition of bacterial LPS, by host trans-membrane TLR-2 and TLR-4 initiates the engulfing of the microbe into a newly formed phagosome that is rapidly decorated by LC3; whereas suppression of TLR signaling leads to failure of this process (Beutler, 2009; Sanjuan et al., 2009). Moreover, the generation of microbiocidal ROS through the activation of NOX2 NADPH oxidase by TLR or FcγR also resulted in LC3 recruitment to phagosomes (Huang et al., 2009). Still, the cytoplasm Nod-like Receptor-1 and -2 (NLRs), which are involved in the recognition of peptidoglycans on the surface of some bacteria, are predicted to recruit Atg16 to the site of invasion at the plasma membrane, potentially facilitating the request of LC3-II to the phagosome membrane (Travassos et al., 2010). On the other hand, the clearance of death cells by LAP seems to require the engagement of the phosphatidylserine receptor TIM-4. Macrophages with reduced expression of TIM-4 were deficient in death cell-specific LAP, so it is possible that this eat-me signal exposed in the surface of dying cells may regulate such process (Martinez et al., 2011).

So far, the easiest way to direct differentiate LAP from canonical autophagy is determine if the LC3 positive structure carrying the enclosed cargo is formed by a single or a double-membrane, although molecular differences also exist (Figure 1.9). The recruitment of LC3 to phagosomes seems to be independent of the ULK1-complex required for pre-initiation of canonical autophagy, since efficient knockdown of these proteins did not affect LAP for different phagocytic targets. The initiation of conventional autophagy also requires the activity of PI3K class-III, Vps34 in complex with Beclin1. In fact, both macrophage treatment with inhibitors of class-III PI3K and knockdown of Beclin1 displayed no LC3 translocation to apoptotic cell-containing phagosomes. Moreover, there is a general agreement that LAP requires Atg5-Atg12-

Atg16 conjugation system. Thus knockdown of either Atg5 or Atg7 significantly reduced the levels of LC3 surrounding phagosomes containing invading bacterial (Kageyama et al., 2011), apoptotic cells (Martinez et al., 2011) or live cells during entosis (Florey et al., 2011). Notably, these Atg7-deficient cells produced significantly less anti-inflammatory cytokines, such IL-10 and TGF-β, upon engulfment of dying cells (Martinez et al., 2011). Recent data also implicates this non-canonical form of autophagy in the normal visual cycle, where LC3 was found to associates with single membrane phagosomes containing engulfed photoreceptor outer segments in an Atg5 dependent manner that also requires Beclin1 (Kim et al., 2013).

Apart from the role of Atg5 and Beclin1 in LAP, these autophagy proteins are also involved in normal apoptotic cell removal by generate engulfment signals required for cell recognition. In a very elegant study about the role of autophagy in embryonic cavitation, Levine and co-workers have shown that mice lacking Atg5 and Beclin1 displayed defective apoptotic cell clearance during embryonic development owing a failure in expose PtdSer on their outer surface (Qu et al., 2007). Likewise, the inhibition of autophagy by silencing ATG5 and ATG7 genes enhances apoptosis simultaneously rendering the apoptotic cells less well recognized by efferocytes in mouse model for atherosclerosis, suggesting that LAP may be required in this conditions in order to maintain an anti-inflammatory environment (Liao et al., 2012).

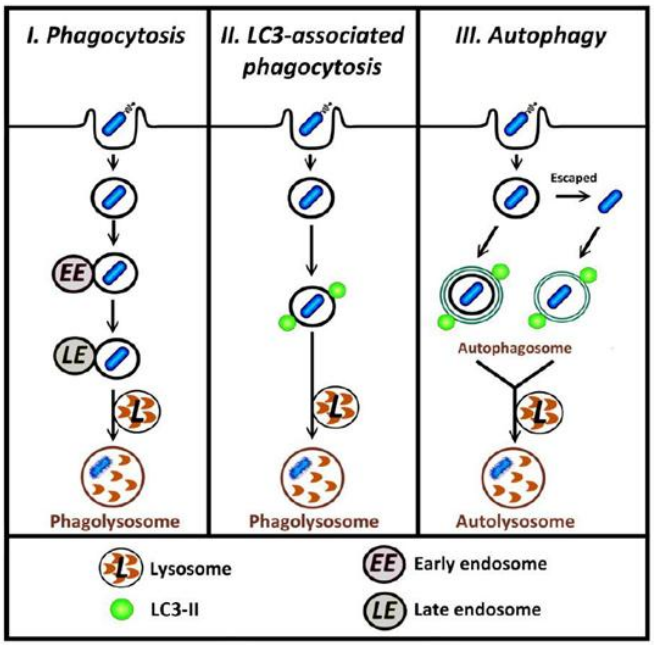


Figure 1.9. Different pathways by which bacteria can be degraded in phagosomes. I, in typical phagocytosis of bacteria, the phagosome may undergo fusion with endosomes and lysosomes (phagosome maturation) such that the bacterium is contained within a phagolysosome, the degradative compartment. **II**, in LC3-associated phagocytosis, autophagy proteins including LC3-II are recruited to the surface of phagosomal membranes; these vesicles subsequently fuse with lysosomes for degradation. **III**, bacteria that are retained in, or escape from, phagosome can be targeted by classical autophagy machinery [Adapted from (Lai and Devenish, 2012)].

The variety of vacuole types that are target by LAP activity suggest that this non-classical function of autophagy lipidation machinery may be a more general mechanism to facilitate lysosome fusion in cells than originally thought. Lipidated LC3 at single-membrane phagosomes may promote lysossomal fusion simply by facilitating membrane-membrane fusion directly or by recruiting other interacting proteins to the membranes. Altogether, these recent findings suggest that the participation of autophagy proteins in phagocytosis are related to accelerated levels in phagosome maturation and acidification, thus increasing the degradative capacity of phagolysosomes in digest apoptotic cells, simultaneously decreasing survival of internalized pathogens, contributing to the innate and adaptative immune responses, including in the maintenance of the anti-inflammatory response in tumor microenviroment (Kim and Overholtzer, 2013b). Therefore, the several potential implications of this recently identified cross-talk between phagocytosis and autophagy need to be better dissected, in order to create more therapeutic tools based on the modulation of phagosome maturation. This raises the possibility that patients with defective dying cell removal might benefit from treatments that target both: endocytic and LAP machineries, in order to accelerate apoptotic cell degradation.

1.4. Aims

Understanding the molecular mechanisms underlying efferocytosis is of the utmost importance for the successful implementation of therapeutic tools based on the modulation of apoptotic cell clearance. Since many efforts have been done to understand the first steps of recognition of apoptotic cells by phagocytes as well as the immune response elicited by efferocytosis, the main objective of this thesis is to examine the molecular machinery and mechanisms involved to the maturation process of phagosomes containing apoptotic cells in mammalian systems. This raises the possibility that patients who carry defective dying cell removal might benefit from treatments that target the machinery involved, in order to accelerate apoptotic cell degradation, ultimately preventing inflammation and autoimmunity, in diseases such as atherosclerosis that is one of the focuses of research in our laboratory. Thus, this work comprised the following objectives:

Study I:

- To characterize the kinetics of maturation of phagosomes containing apoptotic cells in comparison with phagosomes containing IgG-opsonized particles, the best-known phagocytic model.
- To address the role of PtdSer enrichment of apoptotic cell membranes on phagosome maturation.

Study II:

- To identify some novel components and regulators of the canonical autophagy pathway required to an effective efferocytosis.
- To evaluate the functional relevance of the recruitment of autophagy machinery to phagosome maturation of phagosomes containing apoptotic cells and IgG-opsonized particles.

Chapter II

Material and Methods

Summary

Material and Methods

2.1. Red Blood Cells isolation and aging

Human blood was collected from healthy volunteers at Center for Neuroscience and Cell Biology. Written informed consent was obtained from all volunteers, who signed informed consent forms for this purpose, approved by the Ethical Review Board of the Faculty of Medicine of the University of Coimbra. Red blood cells (RBC) from human blood were isolated using a Ficoll-Paque (GE Healthcare Life Sciences) gradient centrifuged for 30min at 400g at 4ºC; then RBC were washed twice with 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) and finally resuspended at 20% hematocrit in PBS supplemented with 0.1% glucose. These cells were kept at 4ºC and used as native RBCs. Aged RBC (agRBC) were prepared by incubating native RBCs in PBS (20% hematocrit) at 37ºC for 4 days. Sheep blood was obtained from Matadouro da Beira Serra and the sheep RBC (shRBC) were isolated and maintained as described for the human RBC.

Loss of phospholipid asymmetry of agRBC was assessed by flow cytometry (FACScalibur, Becton Dickinson) using Annexin V-FITC (BD Bioscience). Briefly, for these experiments, RBC (native or aged) were washed twice with cold PBS, and 10^6 cells were re-suspended in HEPES buffer pH 7.4 containing 2.5 mM calcium and incubated with 5 µL of Annexin V-FITC solution (BD Biosciences) for 15 min at room temperature (RT) protected from the light. Following washing, cells were gated for biparametric histograms FL1 versus FL2 (AnnexinV-FITC *versus* FSC-Height, respectively). For each condition at least 20.000 events were counted and conducted in duplicates.

The membrane integrity of the RBC and agRBC was confirmed by Trypan Blue assay. This dye exclusion method is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as Trypan blue, whereas dead cells do not. For that, a 1:1 dilution of the cell suspension was prepared using a 0.4% Trypan Blue solution (Gibco). Then, cells were counted under an inverted light microscope.

2.2. Preparation of Phosphatidylserine (PtdSer)-liposomes and their incorporation into agRBC membranes

Aqueous suspensions of 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), obtained from Avanti Polar Lipids Inc., were prepared by adding the hydration solution (0.11 M NaCl, pH 7.4) to the lipid powder in a water bath at 65ºC. The samples were submitted to several cycles of vortex/incubation at 65ºC for at least 1 h. The resulting multilamellar vesicle suspension was extruded through two stacked polycarbonate filters (Nucleopore) with a pore diameter of 0.1 µm using a minimum of 10 passages. During the extrusion the water-jacketed extruder (Lipex Biomembranes, Vancouver, British Columbia, Canada) was maintained at 65ºC. After extrusion the lipids were sonicated for 1 h and quantification of phospholipid concentration was done by a modified version of Bartlett's phosphate assay (Bartlett, 1959). The solution was kept in a nitrogen saturated atmosphere at 4ºC to avoid lipid oxidation. For enrichment of agRBC membranes in PtdSer, cells were incubated overnight (around 16 h) with phosphatidylserine (POPS)-liposomes at different RBC:PtdSer molecules (cell:lipid) ratios (1:10^3, 1:10^4 and 1:10^5). The incorporation of PtdSer into the outer leaflet of the plasma membrane of agRBC was also assessed by flow cytometry with Annexin V-FITC and membrane integrity by Trypan blue assay, as described above.

2.3. Opsonization of particles

Isolated shRBC were washed twice with PBS, resuspended with rabbit Immunoglobulin G (IgG) fraction against sheep red blood cells (MP Biomedicals) at a final concentration of 1.6 µg/µL in PBS, and then opsonized for 2 h at RT using an orbital rotator. Cells were then washed 3 times with PBS before use.

Latex beads (3.87µm diameter polystyrene microspheres from Bangs Laboratories) were washed 3 times with PBS, resuspended with IgG from human serum (Sigma) at a final concentration of 1.6 µg/µL in PBS, and then opsonized for 2 h at RT or overnight at 4ºC using an orbital rotator. Particles were then washed 3 times with PBS before use.

2.4. Cell culture and generation of a SMC stably expressing the Fcγ-RIIA

Rabbit vascular smooth muscle cells (SMC) were purchased from ATCC (Camden, NJ, USA) and maintained in RPMI-1640 medium (RPMI, Gibco) containing 10% fetal calf serum (heat inactivated) and 100 U/mL of antibiotics (penicillin and streptomycin). Cells were grown in a humidified incubator at 37ºC under 5% CO_2 atmosphere and used for assays during the exponential growth phase.

In order to generate SMC stably expressing Fcγ-RIIA, the human Fcγ-RIIA tagged with myc was subcloned into the retroviral vector pBABE-puro. The retrovirus production, cells infection and selection with puromycin were done as described before (Cardoso et al., 2010; Schuck et al., 2007).

Wild-type and engineered SMC expressing the Fcγ-RIIA were used between 1 and 8 passages. The splitting of cells was every three days using a Trypsin-EDTA solution (0.05% trypsin). For the assays wild-type or genetically modified SMC 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 at 37ºC under 5% CO_2 atmosphere.

2.5. Bone marrow-derived macrophage isolation and differentiation

Femurs from 8-10 week old C57BL/6 male mice (wild-type or p62 knockout animals) were provided by Dr. Herbert W. Virgin (Washington University in St. Louis, MO, USA). After dissection, the femurs were washed with ice-cold PBS and then both epiphyses were removed using sterile scalpel. The bones were flushed with a needle coupled to a syringe filled with cold Hank's Balanced Salt Solution (HBSS, Gibco) to extrude the whole bone marrow. Then, cell suspension was centrifuged at 1200 rpm at 4°C for 10 min and the pellet was gently resuspended in 4 mL of high glucose Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal calf serum and 100 U/mL of antibiotics (penicillin and streptomycin), and homogeneized in order to generate a fresh bone marrow cell suspension (a mixture of monocytes, platelets, RBC, fibroblasts, etc). After 24h of incubation at 37ºC under 5% CO_2 atmosphere, non-adherent bone marrow cells were collected, washed twice to remove cellular debris, counted and seeded in glass cover slips into 48-multiwell plates at a density of 7×10^5 cells per well, using DMEM supplemented with 10% L929-cells conditioned medium (LCCM) as a source of macrophage colony stimulating factor (M-CSF).

Cultures of mouse L929 fibrosarcoma cell line, kindly provided by Dr. Ira Tabas (Columbia University, NY, USA), were maintained in high glucose DMEM containing 10% fetal calf serum and 100 U/mL of antibiotics (penicillin and streptomycin) in a humidified incubator at 37ºC under 5% CO_2 atmosphere. The culture medium of these cells was replaced after 2 days and finally collected after two more days of incubation. Collected LCCM was centrifuged (850 rpm/5 min), and supernatant was filtered and stored at -80ºC to avoid protein degradation.

To completely differentiate monocytes into bone marrow-derived macrophages (BMDM), plated cells were cultivated for ten days in a humidified incubator at 37ºC under 5% CO_2 atmosphere. Every three days, cells were washed to eliminate cell contaminants (mostly platelets) and fresh medium (supplemented with 10% LCCM) was added in order to induce macrophage differentiation and cells reach the appropriated confluence (about 80%). The phagocytic assays were performed with BMDM cultures on the day eleven.

2.6. Binding, phagocytosis and phagosomal maturation assays

Before binding, phagocytosis or phagosomal maturation assays, native-, aged- and sheep-RBC were always labeled with Carboxyfluorescein-diacetate-Succinimidyl Ester (CFSE) or Orange-chloromethyl-tetramethylrhodamine (CMTMR) (both from Molecular Probes) at final concentration of 0.5 µM, for 15 min at 37°C. CFSE and CMTMR are vital dyes that passively diffuse into cells, where they are metabolized and cleaved by intracellular enzymes to yield highly fluorescent conjugates, which are well retained and fixed on the cytoplasm of living cells. In the case of agRBC enriched in PtdSer levels, this staining was performed right before incubation with POPS-liposomes. Then, RBC were washed with PBS and resuspended in CO_2-independent RPMI-1640 Modified medium (Sigma). Likewise, after IgG-opsonization, particles ware washed with PBS and resuspended in RPMI-Modified medium.

Before the experiments phagocytic cells (SMC or BMDM) were washed twice to discard cellular debris, and the culture medium was replaced with CO_2-independent RPMI. Finally, the different phagocytic particles were added. The onset of phagocytosis was synchronized for the different particles by plate centrifugation (800rpm/1min) and then cells were incubated at 37°C for different time points, according to the specific assay.

For binding experiments, phagocytic particles were added (3×10^5cell/well) to SMC cells, centrifuged and kept on ice at 4ºC for different time periods. Then, cells were shifted to 37ºC for only 35 s before 5 vigorous washes with ice-cold PBS to remove non-attached cells. Finally, cells

were fixed with 4% paraformaldehyde (PFA) for 30 min, permeabilized with 0.1% Triton X-100, stained for actin with Rhodamine-Phalloidin (1:500; Invitrogen) for 30 min at RT and analyzed under fluorescence-microscopy.

For phagocytic experiments, the cells were challenged for different time points with the different phagocytic particles and then non-internalized RBC were lysed using water for 5 seconds. Then, cells were fixed with 4% PFA for 30 min, permeabilized with 0.1% Triton X-100 and stained for actin with Rhodamine-Phalloidin (1:500) for 30 min at RT, while nuclei were stained with DAPI (Fluka) at final concentration of 30 nM for 20 min and analyzed under fluorescence-microscopy. Phagocytic phenotypes were quantified by averaging the percentage of SMC that have internalized at least one phagocytic particle, which are reported as the Phagocytic Index (PI).

Pulse-chase experiments were performed to assess phagosome maturation in SMC and BMDM. Briefly, after the pulse (period that phagocytic particles were allowed to be internalized by phagocytes) the non-internalized RBCs were lysed in water, while external IgG-opsonized latex beads were stained with an anti-human antibody conjugated with a fluorophore on ice. Then the cells were shifted again to 37ºC for different time points (chase) and fixed for further analysis.

2.7. Recycling of YFP-GL-GPI from the phagosomal membranes back to the plasma membrane

For the recycling of the plasma membrane marker glycosyl-phosphatidyl-inositol-anchored yellow fluorescent protein (YFP-GL-GPI), wild-type SMC and the variant SMC stably expressing Fcγ-RIIA were infected with adenoviruses expressing the YFP-GL-GPI for 1 h at 37ºC using Opti-MEM (Gibco), washed 3 times to complete removal of viral particles and incubated overnight in normal culture medium to cellular recover. Then, SMC transiently expressing the YFP-GL-GPI were used for phagocytic pulse-chase experiments as described above.

2.8. Assessment of phagosomal maturation and confocal microscopy

To estimate phagosomal pH over time, SMC were allowed to internalize phagocytic particles for 30 min, chased for different time points and then 50 nM LysoTracker Red DND-99 (Molecular Probes) was added to the cells for 5 min. After LysoTracker incubation, cells were

washed, fixed in 4% PFA and analyzed by fluorescence microscopy to determine the percentage of LysoTracker-positive phagosomes.

To stain lysosomal compartments, adherent SMC were incubated with 1mg/mL of Dextran Tetramethylrhodamine conjugate (10,000 MW, lysine fixable, fluoro-ruby, Molecular Probes) in a serum-free media for 24 h. Then the cells were washed and chased in normal culture medium for 2 h at 37ºC to ensure lysosome delivery of cargo. After lysosome staining, phagosomal maturation assays were performed as described above.

For immunocytochemistry experiments cells were, in general, fixed in 4% PFA for 30min, blocked in 0.5% Gelatin from cold water fish skin (Sigma) in PBS for 30min, permeabilized using 0.1% Triton X-100 (with 200nM glycine) for 30min and incubated with the appropriated primary antibody for 1h30min at RT, followed by incubation with secondary fluorescent antibody (1:800, from Jackson Immunoresearch) for 1h at RT. The exceptions were LBPA staining, where cells were permeabilized using a solution 0.05% Saponin (Sigma) for 15min; and LAMP-1 staining in BMDM, where cells were fixed and permeabilized using methanol for 10min. Details on primary antibodies used are described in the Table 2.1.

Primary Antibody	Host	Information	Company of Source	References	Dilution
EEA-1	goat	polyclonal (N-19)	Santa Cruz Biotecnology	SC-6415	1:30
LBPA	mouse	monoclonal (6C4)	Echelon Bioscience	117Z	1:80
LAMP-1	rat	mouse monoclonal	Developmental Studies Hybridoma Bank	1D4B	1:50
LC3-IIB	rabbit	polyclonal	Cell Signaling	2775S	1:100
SQSTM1 (p62)	rabbit	polyclonal (c-Term)	ABGENT	RB4614	1:80
NBR1	mouse	monoclonal (6B11)	Abnova	M01	1:80
NDP52	rabbit	polyclonal	Abcam	ab68588	1:80

Table 2.1. List of primary antibodies.

Stained samples were washed twice with PBS, mounted on glass slides using Mowiol with Dabco, and then analyzed by using a Carl Zeiss LSM 510 META laser scanning confocal microscope (LSM 510 software) or the Zeiss Cell Observer inverted widefield microscope (ZEN software), both with a 63x oil immersion objective. Digital images were analyzed by using LSM Image Browser or ImageJ software.

2.9. Pharmacological modulation of autophagy

JAK3 Inhibitor VI (Calbiochem) was added to the SMC at a final concentration of 5µM simultaneously with the phagocytic particles and was present throughout the pulse-chase experiments. Nortriptyline (Sigma) was added to SMC at a final concentration of 10µM (DMSO 0.01% was used as vehicle) 30 min before the addition of the phagocytic targets and was present throughout the pulse-chase experiment. Control cells were incubated only with the vehicle (DMSO 0.01%).

2.10. 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 as a statistically significant difference. Results are presented as means ± standard error of the mean (SEM) from at least three independent experiments.

Chapter III

Comparison of the kinetics of maturation of phagosomes containing apoptotic cells and IgG-opsonized particles

The work presented in this chapter was published in:
Viegas, M.S., Estronca, L.M., and Vieira, O.V. (2012). Comparison of the kinetics of maturation of phagosomes containing apoptotic cells and IgG-opsonized particles. PloS one *7*, e48391.

Summary

Comparison of the kinetics of maturation of phagosomes containing apoptotic cells and IgG-opsonized particles

3.1. Rationale

Vesicular trafficking plays a central role in the formation and maintenance of different intracellular compartments as well as in the communications between the cells and the environment. These distinct relationships are based on the endomembrane system, a set of interconnected sub-compartments comprising the ER, the Trans-Golgi Network (TGN), endocytic vesicles, lysosomes and the plasma membrane. In principle, cycles of membrane budding and fusion allow the lumen of any of those cellular structures to communicate with each other and with the cell exterior by means of transport vesicles (Bonifacino and Glick, 2004; Sanderfoot and Raikhel, 1999). According to the direction of the vesicles movement this system can be classified into exocytosis (materials are exported from the cell) or endocytosis (materials move inward the cell). The exocytic pathway is important in the expulsion of waste materials and secretion of cellular products, such as digestive enzymes, neurotransmitters or hormones *via* secretory vesicles (Sudhof and Rizo, 2011). On the other hand, the endocytic pathway involves the cellular internalization of extracellular fluids, molecules, foreign agents and apoptotic cells, likewise the sorting of plasma membrane proteins and lipids, thus being required for a vast number of functions: nutrient uptake, cell adhesion and migration, receptor signaling, pathogen entry and cell polarity. In this context, different types of endocytic processes can be distinguished by the size of the vesicle, the nature of the cargo and the machinery involved. In general, there are three types of endocytosis: phagocytosis (cell eating); pinocytosis (cell drinking); and receptor-mediated endocytosis, in which selective materials are uptaken (e.g. cholesterol, growth factors and antibodies) by binding to specific receptors on the cell surface, which folds inward to form coated pits (Botelho et al., 2000a; Grant and Donaldson, 2009; Sorkin and von Zastrow, 2009).

Phagocytosis, as previously discussed, represents an extremely complex cellular event by which large particles (>0.5μm in diameter) are actively recognized, engulfed into a phagosome and degraded through a finely tuned system known as phagosome maturation. Although the predominant interest in phagocytosis has been focused on its role in host defense against

infection, this process also plays a pivotal role in the removal of apoptotic cells, which is essential during development, tissue remodeling and for the maintenance of homeostasis in all multicellular organisms (Aderem and Underhill, 1999; Flannagan et al., 2012; Henson and Hume, 2006; Parnaik et al., 2000). Remarkably, even in tissues with high cellular turnover, apoptotic cells are rarely seen *in situ*, which is thought to be due to rapid and efficient disposal mechanisms evolved by phagocytic cells. Actually, this is particularly important to avoid non-resolving inflammation, many autoimmune conditions and developmental abnormalities, factors considered as triggers for numerous diseases, like atherosclerosis, lupus erythematosus and neurodegenerative disorders (Munoz et al., 2010a; Nathan and Ding, 2010). In these cases, professional phagocytes are not the only players involved in efferocytosis. For instance, the removal of apoptotic cell debris in atherosclerosis is known to specifically involve SMC that are not considered professional phagocytes to a very significant degree. In fact, in atherosclerosis, SMC represent the major phagocytic population in the vessel wall besides macrophages (Bennett et al., 1995; Kolb et al., 2007; Schrijvers et al., 2005).

The past decade has witnessed an impressive expansion on our knowledge regarding the fundamentals of the efferocytic process. So, based on the research from many groups, several distinguishable steps have been recognized in the engulfment and clearance of apoptotic cells. At very early stages of this type of programmed cell death, the cells release “find-me” signals establishing a chemotactic gradient of factors that advertise their status to local cells, while stimulate the migration of distant phagocytes (Elliott et al., 2009; Lauber et al., 2003; Munoz et al., 2010b; Truman et al., 2008). Then, the physical contact between the apoptotic cell and the phagocyte is mediated by ligands on the apoptotic cell (referred to as “eat-me” signals) and engulfment receptors displayed on the cell membrane of phagocytes. Among the vast array of identified “eat-me” molecules, the exposure of PtdSer on the outer leaflet of apoptotic cells appears to be a key marker (Fadok et al., 1992; Vandivier et al., 2006). This phospholipid, normally concentrated on the inner leaflet of the plasma membrane, loses its asymmetric distribution during apoptosis and is translocated to the outer leaflet of the plasma membrane (Fadok et al., 1992; Martin et al., 1995). Phagocyte recognition of PtdSer is mediated directly via one or more PtdSer receptors (e.g. Tim-4 and Stabilin-2); or indirectly through bridging molecules (e.g. MFG-E8 and Gas6) that simultaneously bind PtdSer on the apoptotic cell and a specific receptor expressed by phagocytes (Hanayama et al., 2004; Savill et al., 1990; Scott et al., 2001). For some of these receptors, direct or indirect binding to PtdSer, in combination with the recognition of other “eat-me” signals, results in Rac-dependent cytoskeletal reorganization,

which ultimately leads to the engulfment of the dying target (Ravichandran and Lorenz, 2007). Once inside the phagocyte, the ingested apoptotic cargo is processed via the phagolysosomal pathway, which shares features with the endocytic machinery, in spite of has some unique features of its own (Bohdanowicz and Grinstein, 2010; Erwig et al., 2006; Kinchen et al., 2008; Kinchen and Ravichandran, 2010; Yu et al., 2008). Specifically, the phagosomal membrane initially acquires markers of early endosomes that are subsequently lost and replaced by markers of late endosomes. Ultimately, lysosomal contents (e.g. cathepsins) and membrane constituents (e.g. LAMP-1, also present in late endosomes) are found in the terminal organelle, the phagolysosome, which represents the final degradative compartment of the host cell. All this coordinated sequence of events is accompanied by a progressive acidification of the phagosomal lumen, that correlates with the accumulation of V-ATPases (H^+ pumps) and the recycling of phagosomal membrane components back to the plasma membrane (Blander and Medzhitov, 2006; Erwig and Henson, 2008; Kinchen and Ravichandran, 2008; Zhou and Yu, 2008).

Genetically tractable organisms greatly contribute to our ability to make sense of this complexity of interactions and downstream signals resulting in the uptake of apoptotic cells. In this context, the nematode *C. elegans* has been a widely used model organism for studying the engulfment and degradation of apoptotic cells (often referred as cell corpses) owing its simple anatomy, well-established genetics, well-known cell lineage, rapid processing of dying cells and distinguishable apoptotic cell morphology (Kinchen et al., 2008; Lu and Zhou, 2012; Reddien and Horvitz, 2004). Meanwhile, in mammalian systems, reports exploring efferocytosis has shed light mainly on the immune response elicited by the removal of this nature of cargo and also on the early stages of engulfment (recognition and physical internalization); whereas the identification of the machinery required for subsequent steps remain unclear (Ravichandran and Lorenz, 2007). In other worlds, much attention has been focused on the participating ligands, receptors, and mechanisms of uptake, while still poorly is known about the disposition of the ingested cell within the phagosome. Furthermore, most part of the literature using mammalian tools has addressed uptake and processing of apoptotic cells by macrophages or immature dendritic cells (i.e. professional phagocytes), despite there are other cell types, such as fibroblasts, endothelial cells and SMC, that are also absolutely important to mediating efferocytosis in certain tissue contexts (Erwig et al., 2006; Parnaik et al., 2000). Concluding, our current understanding of the molecular processes that underlie maturation of phagosomes containing apoptotic cells in mammalian systems is rudimentary and so, deserves more attention.

Therefore, in this study, we investigated in detail the interaction of apoptotic cell-containing phagosomes with components from different stages of the endocytic pathway, recycling of phagosomal membrane components and lumenal acidification over time, likewise comparing those kinetics with the kinetics obtained from the maturation of phagosomes containing IgG-opsonized particles that are engulfed via Fc-Receptors, the best-studied phagocytic model. More, the experiments were performed in a non-professional phagocytic cell line: rabbit aortic smooth muscle cells, and thus to exclude the interference of the phagocytic cell type in our comparative study, we also generated SMC stably expressing the Fcγ-RIIA. We specifically chose these cells because one of our major research interests is to understand why the uptake or the processing of apoptotic cells is impaired in atherosclerosis and as stated above SMC represent the major phagocytic population in the vessel wall besides macrophages. As phagocytic particles, we used human aged red blood cells (agRBC) as our apoptotic cell model, and also IgG-opsonized sheep red blood cells (shRBC) or latex beads as control. Furthermore, since PtdSer exposure is the most important seen alteration on dying cells, we increase the normal levels of PtdSer in apoptotic cell membranes and observed its effect in efferocytosis and phagosomal maturation.

3.2. Phagocytic Particles

3.2.1. Human Aged Red Blood Cells

Mature red blood cells are highly specialized and terminally differentiated cells. Their main function is to transport oxygen (O_2) through the blood flow delivering it to the tissues. Very early in mammalian embryogenesis, nucleated RBCs are produced in the yolk sac, in a process called erythropoiesis. During later stages of embryogenesis and after birth it takes place in the liver and in the bone marrow, respectively. There they become enucleated in order to accommodate maximum space for haemoglobin and assume a biconcave shape to easily squeeze through thin capillaries. The cytoskeleton and the cell membrane of human RBC contain several different proteins, such as spectrin, ankyrin, actin and glycophorin, just to mention a few.

Healthy erythrocytes have a lifespan of about 120 days, after which they are cleared from the circulation mainly engulfed by Kupffer cells in the liver and by macrophages in the spleen (Clark, 1988; Piomelli and Seaman, 1993). The exact mechanism behind uptake and destruction of aged RBCs is still not completely understood, but it is clear that involves distinct morphological

changes including progressive shape changes, cell shrinkage, increased osmotic fragility due to micro-vesiculation of the plasma membrane bilayer and loss of lipid bilayer asymmetry. Klarl and co-workers (2006) demonstrated that the removal of extracellular glucose led to depletion of cellular ATP, stimulated PKC activity, enhanced serine phosphorylation of membrane proteins, decreased cell volume and increased PtdSer externalization (Klarl et al., 2006). Erythrocytes with PtdSer on their outer surface are then recognized and cleared by phagocytes (Schroit et al., 1985). Remarkable, these changes are very similar to the morphological hallmarks of classical apoptosis (Connor et al., 1994; Huang et al., 2011). However, because they lack some important apoptotic machinery, like nucleus and mitochondria, they are said to suffer an apoptotic-like process, called eryptosis (Lang et al., 2012). Moreover, mature RBCs demonstrated the presence of the pro-caspases-3 and -8, although treatment using ionophores (apoptotic inducer agents) lead to the activation of the cysteine protease calpain (Berg et al., 2001; Bratosin et al., 2001).

RBCs are considered very good phagocytic models since they offer many advantages compared to other cell types: 1) native RBCs cannot bind to phagocytes without previous modification on their surface; 2) they are easily induced to undergo an apoptotic-like process termed eryptosis, mimicking senescent cells; 3) the plasma membrane levels of PtdSer can be manipulated by incubating the cells with PtdSer-liposomes; 4) RBCs can also be opsonized with antibodies and then used to study receptor-mediated phagocytosis (e.g. Fc or complement - receptors); and 5) by applying a simple hypotonic shock the distinction between attached RBCs from those that were really internalized is easily done (Gigli and Nelson, 1968).

3.2.2. IgG-Opsonized Particles

Detection and clearance of foreign bodies, such as bacteria, fungi and parasites, was the first acknowledge function of phagocytosis, playing a crucial role in immune defense. This process involves a variety of receptors that are able to directly or indirectly identify particular microbial features. Direct recognition is often mediated by Toll-like receptors (TLR), which distinguish pathogen-associated molecular patterns (PAMPs) displayed on the surface of microorganisms, such as bacterial cell wall components or viral RNA. On the other hand, indirect recognition can be mediated by host serum factors circulating in the blood and in interstitial fluids, the called opsonins, which recognize extraneous antigens and components of the complement cascade deposited on foreign surfaces. Following their deposition on particles, these molecules are in turn engaged by specific opsonic receptors on the membrane of professional phagocytes, typified by

complement receptor 3 (CR3) and Fc-Receptors (FcR) that associate with complement fragments and with immunoglobulins, respectively (Aderem and Underhill, 1999).

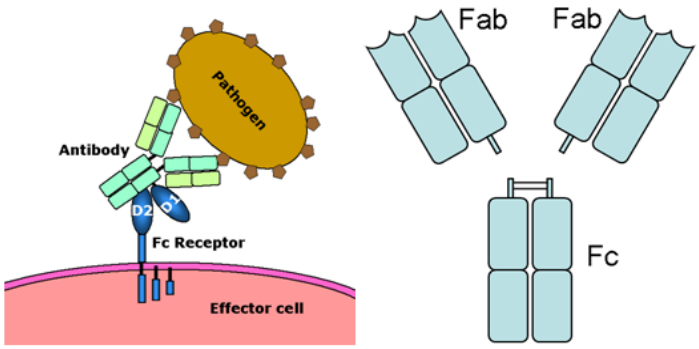


Figure 3.1. Fc-Receptor interaction with an antibody-coated pathogen and structure. (Source: http://www.sinobiological.com)

Our knowledge in phagosome maturation was almost entirely derived from studies using immunoglobulin G-opsonized particles, including IgG coated-red blood cells and -latex beads. Among the different Fc family of receptors, classified based on the type of antibody they recognize and affinity for the ligand, the Fc-gamma receptor (FcγR) is the one that specific binds to IgG (Ravetch and Bolland, 2001). Therefore, internalization of IgG-opsonized particles mediated by FcγR is by far the best-understood phagocytic model. The name of these receptors comes from their binding specificity for a part of an antibody known as the Fc (Fragment, crystallizable) region. The Fc region of the antibody points outwards, in direct reach of phagocytes, just after the IgG molecule binds to intruder agents through their Fab (Fragment, antigen binding) region (see Figure 3.1). Ultimately, the link between ligand-receptor, through the generic Fc-domain of FcR, elicits several biological responses beyond phagocytosis, like antibody-dependent cell cytotoxicity, release of inflammatory mediators, and regulation of lymphocyte proliferation and differentiation. Curiously, transfection of non-professional phagocytes (that lack opsonic receptors) with cDNAs encoding FcR dramatically increases the phagocytic rate (and, obviously, particle range) of these cells; thus this system has been widely used to dissect signaling pathways leading to particle internalization (Cai et al., 1994; Indik et al., 1995).

For simplicity, this receptor was mostly studied in isolation by use of model systems in which engagement of other receptors could be avoided or at least minimized. By contrast, in nature, multiple opsonic and non-opsonic receptors are engaged simultaneously, producing a complex and probably synergistic response, in which fast lateral diffusion and clustering occurs to

firmly attach the prey onto the phagocyte surface (Botelho and Grinstein, 2011). Actually, FcR clustering is required to elicit cellular responses, so bringing the cytosolic domain of multiple FcR into close proximity (Nimmerjahn and Ravetch, 2008). It has been proposed that grouping FcγR together enables them to associate with lipid microdomains (rafts) where downstream signaling elements (e.g. Src-family kinases) reside. So, activated Src-family kinases recruit a variety of adaptor and scaffolding proteins that link and expand the signaling network by providing additional docking nodes, which ultimately lead to particle engulfment by an actin-driven mechanism and cargo processing through phagosome maturation (Fitzer-Attas et al., 2000; Ghazizadeh et al., 1994; Hamada et al., 1993).

3.3. Results

3.3.1. Generation of agRBC and their enrichment in PtdSer as apoptotic cell models for phagocytosis and phagosomal maturation studies

We have generated agRBC and used them as our phagocytic apoptotic model. Indeed, although RBCs cannot undergo classical apoptosis because they lack a nucleus, mature RBC can undergo a rapid self-destruction process sharing several features with apoptosis, including PtdSer externalization, leading, in the presence of phagocytic cells, to their ingestion (Berg et al., 2001; Bratosin et al., 2001). To evaluate aging of RBC we used the FITC-labeled Annexin-V, which recognizes and binds PtdSer head group on the cell surface of dying cells, and then proceed to flow cytometry analysis. After being incubated in PBS at 37^{o}C during 4 days, agRBC efficiently translocated PtdSer from the inner to the outer leaflet of the plasma membrane (Figure 3.2), since 88.9 ± 3.5% of cells were positive for Annexin-V. In contrast, less than 1% of freshly isolated RBC (native RBC) were positive for Annexin-V and the fluorescence intensity was negligible (<3 %) (Table 3.1). The values obtained for aged- *versus* native-RBC were within the range reported previously by others (Kolb et al., 2007).

Data from many different laboratories have suggested that loss of phospholipid asymmetry and external expression of PtdSer are crucial for the recognition of apoptotic cells by macrophages and other phagocytes (Borisenko et al., 2003; Fadok et al., 2001). However, the literature is not clear on whether an extra amount of PtdSer on surface of apoptotic cells could somehow modulate the phagocytosis or phagosomal maturation. Therefore, we decided to load agRBC with more PtdSer by incubating them with PtdSer-liposomes (POPS) at different

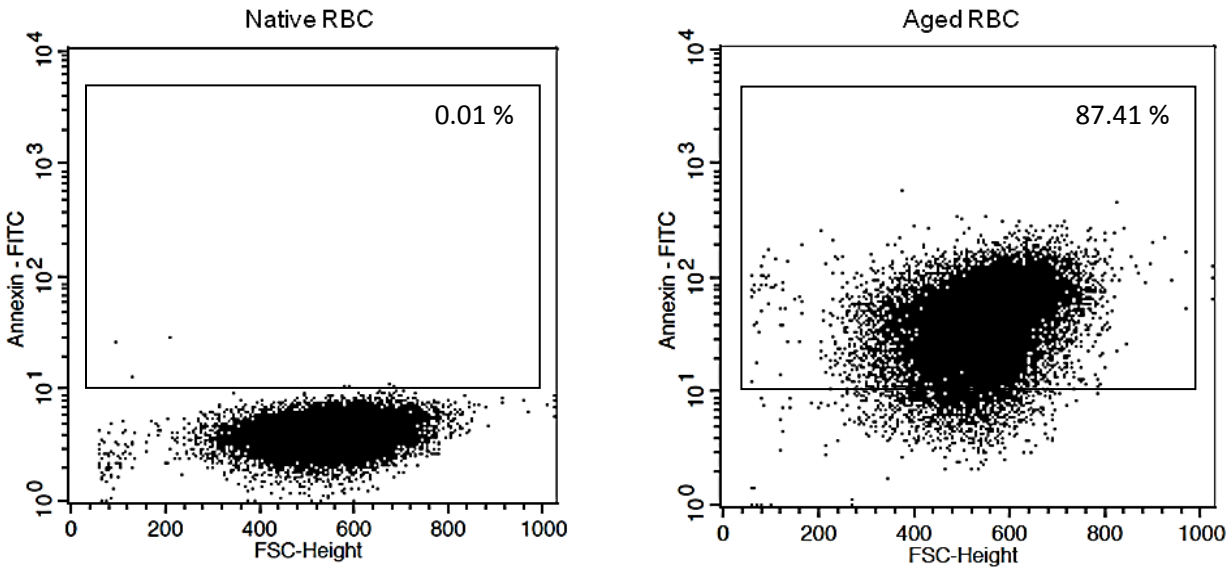


Figure 3.2. Detection of exposed PtdSer by flow cytometry. Native- and Aged- RBC were stained with Annexin-V conjugated with FITC. Aging process induced an increase in the population of Annexin-V positive cells (right panel). Data are expressed as a dot plot of one representative experiment conducted in duplicate.

agRBC:POPS (cell / PtdSer molecule) ratios (Figure 3.3). Although there are a wide range of individual variation in the incorporation of PtdSer, when agRBC were incubated for about 16h with different ratios of agRBC:POPS, the percentage of Annexin-V positive cells, in fact, showed a slight increase. However, comparing with the control (agRBC incubated only with the vehicle) we observed an increment of 5- and 8-fold in PtdSer levels for the ratios of $1{:}10^4$ and $1{:}10^5$ respectively, judged by the values of the mean fluorescence intensity (Table 3.1). Because no substantial enrichment was seen with the ratio $1{:}10^3$ this condition was ignored in further experiments.

Importantly, to exclude the possibility that during treatments the integrity of the plasma membrane of RBC was not affected, the cells were incubated with Trypan Blue (a vital dye that is negatively charged and does not interact with the cell unless the membrane is damaged). By light microscopy evaluation of agRBC or PtdSer-enriched agRBC after staining, no Trypan blue-positive cells were observed (results not shown), suggesting that the integrity of the plasma membrane was not affected by any of the treatments.

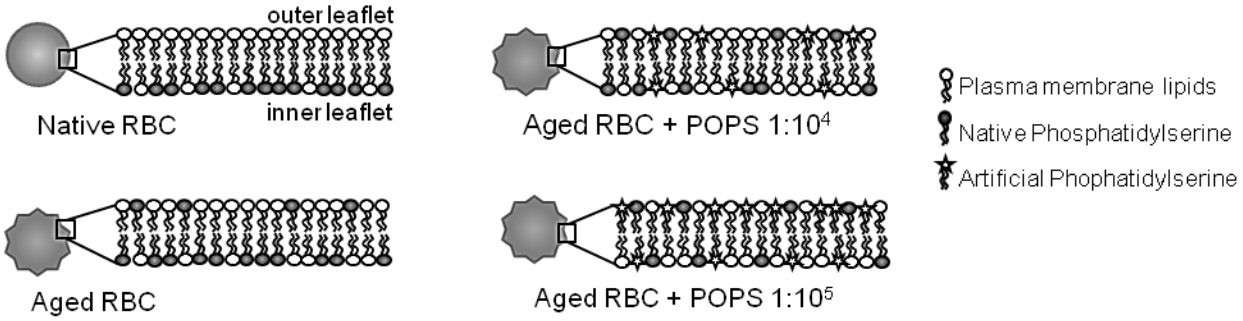


Figure 3.3. Schematic representation of different phagocytic models used in the experimental work.

Phagocytic Particle	Percentage of Annexin-V positive cells	Mean Fluorescence Intensity (%)
Native RBC	0.80 ± 0.23	3.19 ± 0.47
agRBC	88.9 ± 3.5	65.9 ± 8.6
agRBC+POPS 1:10^3	92.1 ± 2.9	56.8 ± 14.1
agRBC+POPS 1:10^4	98.8 ± 0.7	303.9 ± 72.9
agRBC+POPS 1:10^5	99.4 ± 0.2	520.8 ± 107.2

Table 3.1. Enrichment of agRBC membranes in phosphatidylserine. Results of flow cytometry analysis of Native RBC, agRBC and agRBC after overnight incubation with different cell:PtdSer liposome ratios labeled with Annexin V-FITC. The table shows the percentage of AnnexinV-positive cells and the Mean Fluorescence Intensity ± SEM of at least three independent experiments. A total of 20.000 events were analyzed in each condition.

3.3.2. Enrichment in PtdSer affected neither binding nor phagocytosis of agRBC

Apoptotic cell clearance by SMC has been demonstrated to occur *in vitro* and *in vivo*. Although they are classified as non-professional phagocytes owing to its non-myeloid origin, this cell type has the ability to bind and ingest cells undergoing apoptosis due to the exposure of PtdSer, thus playing an important role in the clearance of dying cells in disorders like atherogenesis. As previously mentioned, besides macrophages, SMC represent the major phagocytic population in the vessel wall (Bennett et al., 1995; Kolb et al., 2007; Schrijvers et al., 2005).

PtdSer exposure has traditionally been referred to as the key “eat-me” signal for receptor-mediated phagocytosis of apoptotic cells. However, the effect of PtdSer loading into apoptotic cells in binding (attachment of phagocytic particles to the cell surface of phagocytes),

phagocytosis (internalization of phagocytic particles by phagocytes) and phagolysosome biogenesis was never addressed. Thus, here we have analyzed the relationship between PtdSer levels in binding and phagocytosis of agRBC by SMC.

To study binding of agRBC and PtdSer-enriched agRBC, these particles were first labeled with the vital dye CFSE, fixed with 0.2% glutaraldehyde and then incubated with SMC, at 4°C, for different time points. Unbound cells were removed by extensive washing with cold medium, and samples were fixed with 4% PFA and stained for actin. The attached particles were then counted under fluorescence microscopy. In contrast to native RBC (result not shown), agRBC and PtdSer-loaded agRBC associated with SMC. Binding for all particles was time- and treatment-independent. The results obtained with agRBC and agRBC loaded with PtdSer were very similar, suggesting that increased PtdSer levels on the cell surface do not affect the rate of association between phagocytic particles and phagocytes. To validate our assay, we further incubated native RBCs enriched in PtdSer with SMC, such as described above. Indeed, in accordance to other groups (Borisenko et al., 2003), these cells were efficiently bound to the SMC. Our results suggest that PtdSer exposure is sufficient for binding, but enrichment of the plasma membrane with more PtdSer does not imply more binding (Table3.2).

Incubation Time	agRBC	agRBC+POPS 1:10^4	agRBC+POPS 1:10^5
30 min	6.43 ± 0.50	7.54 ± 0.50	8.30 ± 0.19
60 min	6.82 ± 1.10	7.35 ± 1.40	10 ± 0.5
90 min	7.35 ± 1.40	8.75 ± 0.10	9.90 ± 0.40

Table 3.2. Effect of PtdSer enrichment in binding of agRBC to SMC in function of time. After aging and incubation with different ratios of POPS cells were labeled and added to the SMC. Phagocytes were allowed to bind phagocytic particles on ice. Cells were then fixed and analyzed by light microscopy. The values show the number of particles bound per 100 phagocytic cells. Results are expressed as mean ± SD of at least three independent experiments. At each time point 100 phagosomes were analyzed.

Binding of apoptotic particles to phagocytic receptors eventually results in phagocytosis. To measure phagocytosis, human agRBC loaded, or not, with PtdSer were labeled with CFSE and incubated with SMC for different time points at 37°C. After phagocytosis cells were fixed, permeabilized, stained for nucleus and cortical actin with rhodamine-phalloidin, and analyzed by confocal microscopy (Figure 3.4). In these studies, the same number of phagocytic particles was added per different experimental conditions (i.e. 3×10^5 cells/well). Phagocytic phenotypes were quantified by averaging the percentage of SMC that have internalized at least one phagocytic

particle, which are reported as the Phagocytic Index. SMC engulfed all models of apoptotic particles tested. The phagocytic index ranged from 8.64 ± 1.31 to 10.25 ± 1.41 for agRCB and agRBC+POPS 1:10^5, respectively, at 30 min of phagocytosis (Table 3.3). Again, similar to what was described for binding experiments, the rate of phagocytosis was independent of incubation time (at least for the times we tested) and PtdSer concentration. The average number of particles engulfed by SMC was independent of the phagocytic particle analized, ranging between 1 and 2 particles (Table 3.4). The native RBC loaded in PtdSer, that were efficiently attached to SMC were not able to be engulfed (result not shown).

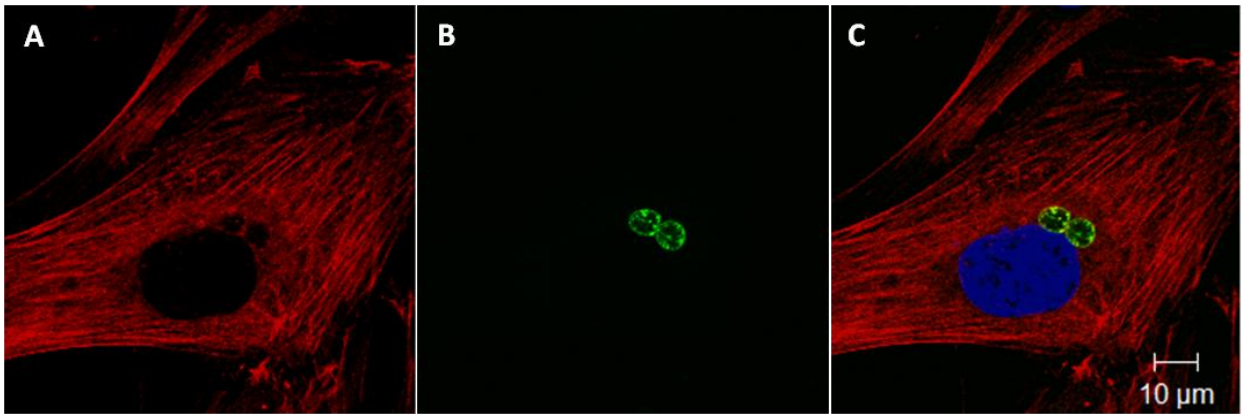


Figure 3.4. Representative image of two agRBC internalized by a SMC after 30 min of incubation at 37°C. A) Actin staining with rhodamine-phalloidin (in red). **B**) Engulfed agRBC stained with CFSE (in green). **C**) Corresponding merged image showing nucleus staining with DAPI (in blue). Bar, 10 µm.

Incubation Time	agRBC	agRBC+POPS 1:10^4	agRBC+POPS 1:10^5
30 min	8.64 ± 1.31	9.87 ± 0.64	10.25 ± 1.41
60 min	8.88 ± 3.46	10.76 ± 2.91	9.78 ± 1.31
120 min	10.65 ± 0.92	10.84 ± 1.46	9.88 ± 3.67

Table 3.3. Effect of PtdSer enrichment in phagocytosis of agRBC by SMC in function of time. After aging and incubation with different ratios of POPS cells were labeled and added to the SMC. Phagocytes were allowed to internalize particles at 37ºC. After lyses, cells were fixed and analyzed by light microscopy. The values show the percentage of SMC that have internalized at least one phagocytic particles (Phagocytic Index). Results are expressed as mean ± SD of at least three independent experiments. At each time point 100 phagosomes were analyzed.

Incubation Time	agRBC	agRBC+POPS 1:10^4	agRBC+POPS 1:10^5
30 min	1.37 ± 0.05	1.63 ± 0.22	1.51 ±0.37
60 min	1.41 ± 0.22	1.39 ± 0.14	1.34 ± 0.19
120 min	1.71 ± 0.27	1.66 ± 0.32	1.74 ± 0.62

Table 3.4. Effect of PtdSer enrichment in the number of particles ingested per phagocytic cell in function of time. After aging and incubation with different ratios of POPS cells were labeled and added to the SMC. Phagocytes were allowed to internalize particles at 37ºC. After lysis, cells were fixed and analyzed by light microscopy. The values show the average number of phagocytic particles ingested by SMC that have ingested at least one particle. Results are expressed as mean ± SD of at least three independent experiments. At each time point 100 phagosomes were analyzed.

There were some quantitative differences between binding and the Phagocytic index that were performed at different temperatures 4º and 37ºC, respectively. To explain this discrepancy we can envision the following scenarios: i) appearance of more phagocytic receptors at the plasma membrane, via exocytosis, at 37°C; ii) further modification of agRBC, with the appearance of more “eat me signals” also at 37°C.

In conclusion, our results showed that around 5- to 8- fold enrichment of agRBC in PtdSer is neither affecting binding nor phagocytosis, suggesting that probably the agRBC has enough of this phospholipid to saturate all the PtdSer-receptors at the plasma membrane of the SMC.

An interesting feature of phagosomes is that immediately after their formation at the plasma membrane they are unable to perform their main task: kill and degrade pathogens and apoptotic cells. Indeed, the acquisition of phagosome functional properties depends on a complex set of interactions with various cellular compartments, driving the biogenesis of phagolysosomes. Thus, we next assessed some features of the maturation of phagosomes containing apoptotic cells, comparing it with the maturation of phagosomes containing IgG-opsonized particles. Likewise we addressed the role of PtdSer loading in phagosomal maturation.

3.3.3. Phagosomes containing IgG-opsonized and agRBC particles mature at different rates

The efficient particle digestion and processing require dramatic remodeling of the phagosomal membrane and lumenal contents, the phagosomal maturation process. This process culminates with the fusion between phagosomes and lysosomes, followed by subsequent cargo

degradation (Kinchen and Ravichandran, 2010; Vieira et al., 2002). To better understand how phagosomes containing apoptotic cells mature, we examined their interactions with different endocytic compartments, as well as we tracked some characteristic changes along the time, like the recycling of phagosomal components back to the plasma membrane and the gradual dropping in pH levels. Thus, the maturation kinetics of phagosomes containing agRBC was compared with phagosomes carrying IgG-opsonized particles, which are recognized through the widely expressed and highly studied FcγR. Because SMC are non-professional phagocytes and so do not express this phagocytic receptor in normal conditions, we used a retroviral system to generate an engineered SMC line stably expressing the Fcγ-RIIA. As IgG-opsonized particles, our control, we used shRBC or inert latex beads cover with antibody. Furthermore, in order to check whether the amount of PtdSer exposed on cells undergoing apoptosis could affect phagosome maturation, we compared the maturation kinetics of phagosomes containing PtdSer-enriched agRBC with the ratios obtained for phagosomes containing agRBC.

To synchronize phagocytosis, the different targets were added to SMC or to the parental cell line (SMC stably expressing FcγR) and submitted to a short spin. Following this, cells were shifted to 37°C and particles were allowed to be phagocytosed for 30 min (pulse). This specific time of pulse was chosen according to the results obtained previously (see Table 3.3). After the pulse, cells were shifted to 4°C (to immediately stop phagocytosis) and the non-internalized agRBC were lysed in water, while the external latex beads were stained to permit further distinction. Then, cells were shifted again to 37°C to allow maturation of the new formed phagosomes for different time points (chase time). It is very important to keep in mind that all these procedures are crucial to study phagosome maturation, since an accurate differentiation is required to ensure that most phagosomes containing our targets of interest will mature with synchronized kinetics. Thus, we performed pulse-chase experiments to assess phagolysosome biogenesis. After each time-point of chase investigated, depending on the marker, cells were fixed, immunostained (or not, as required) and analyzed by confocal microscopy. A phagosome was considered positive for a given marker when a fluorescent ring was observed around the engulfed particle. In the case of the latex beads that were internalized after the pulse (made visible by labeling with secondary antibodies as described above) they were excluded to ensure that we were just following the phagosomes formed during the first 30 min of phagocytosis. By independently measuring, as a function of time, the acquisition of endocytic markers, recycling and phagosomal acidification under identical experimental conditions in pulse-chase experiments we can decouple internalization from maturation.

To track the association of the nascent phagosomes with early components of the endocytic pathway, we assessed, by immunofluorescence, the recruitment of the Rab5-effector EEA-1, responsible for tethering early endosomes to nascent phagosomes. For all phagocytic particles, EEA-1 association with phagosomal membranes was transient (Figure 3.5). So the phagosomes acquired the marker at early chase times and lost it in the course of time. As shown in Figure 3.5G, shortly after particle ingestion (0 min chase), a majority of phagosomes containing IgG-opsonized particles, agRBC and agRBC enriched in PtdSer ($1{:}10^5$ ratio) associated with EEA-1 (45 ± 1.53%, 53.7 ± 7.4% and 44 ± 4.5%; respectively). For the same time point the percentage of EEA-1 positive phagosomes carrying agRBC enriched in PtdSer ($1{:}10^4$ ratio) was lower when compared with the other phagosomes although not significantly so.

It is generally considered that phagolysosome biogenesis involves not only fusion with components of the endocytic pathway, but also fission events and recycling of plasma membrane components. Thus, we next examined the recycling of phagosomal components back to the plasma membrane, which is necessary for their constant remodeling and progressing of the maturation process. In order to follow trafficking of plasma membrane components, phagocytes were infected with adenovirus expressing the plasma membrane marker YFP-GL-GPI (a yellow fluorescent protein with glycosylation and glycosylphosphatidylinositol signals), and its recycling was assessed by looking at the elimination of the marker from phagosomal membranes in time. As shown in Figure 3.6 the plasma membrane protein YFP-GL-GPI was eliminated/recycled, with time, from all phagosomes. However, as shown in the graph (Fig. 15G) and illustrated in the Figure 3.6A-F, the recycling of YFP-GL-GPI from phagosomal membranes was faster in phagosomes containing IgG-opsonized particles compared with the other phagocytic particles. This effect was more pronounced at 15 min chase, where the percentage YFP-GPI-positive-phagosomes was significant higher for agRBC than for IgG-opsonized shRBC (61.0 ± 5.5% and 37.0 ± 6.2%; $p < 0.05$, respectively), suggesting that the recycling is delayed in phagosomes containing senescent particles. Since recycling of components of phagosomal membranes is crucial for normal progression of phagosome maturation we looked then at the acquisition of MVB/late endosomal markers to confirm that the kinetics of phagosomes containing senescent RBC and opsonized particles mature at different kinetics. The interaction of the different phagosomes with MVB/late endosomes was assessed by looking at the acquisition of LBPA. As shown in Figure 3.7G and illustrated in Figure 3.7A-F, phagosomes containing IgG-opsonized particles acquire LBPA much faster than phagosomes containing senescent particles with different levels of PtdSer. The difference was more notorious at 0 and 45 min chase time. Once

more, maturation kinetics of phagosomes sheltering PtdSer-enriched agRBC was not very affected compared to those containing control agRBC.

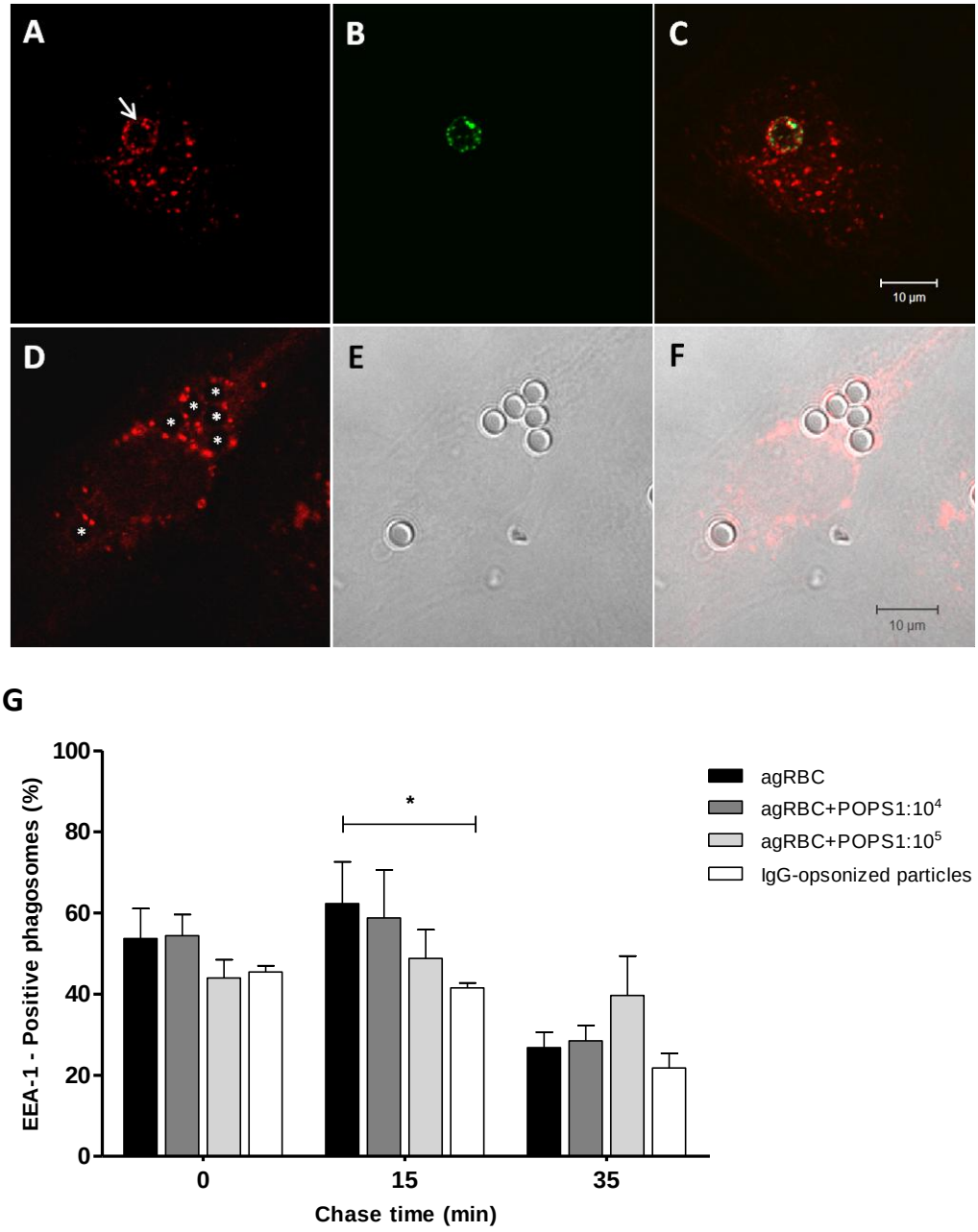


Figure 3.5. Interaction of phagosomes containing different phagocytic particles with early endosomes. The interaction of early endosomes with phagosomes containing different particles was assessed by the acquisition of EEA-1. After pulse-chase experiments, cells were fixed, stained with EEA-1 antibody and analyzed under confocal microscopy. **A)** EEA-1 staining of a cell containing an EEA-1-positive phagosome at 15 min chase time. **B)** Internalized agRBC stained with CFSE. **C)** Corresponding merged image. **D)** EEA-1 staining of a cell stably expressing the FcγR-IIA and containing six EEA-1-negative phagosomes after 15 min chase time. **E)** Corresponding differential interference contrast (DIC) image. **F)** Corresponding merged image. Arrow indicates a positive phagosome and asterisks indicate negative phagosomes. Bars, 10 µm. **G)** Quantification of the EEA-1-positive phagosomes. Wild type and engineered SMC were exposed to different phagocytic particles for 30 min and then chased for the time indicated in the graph abscissa. The values are means ± SEM of, at least, three independent experiments. At each time point 100 phagosomes were analyzed. *, $p < 0.05$ comparing differences between EEA-1 acquisition by phagosomes with agRBC and with IgG-opsonized latex beads.

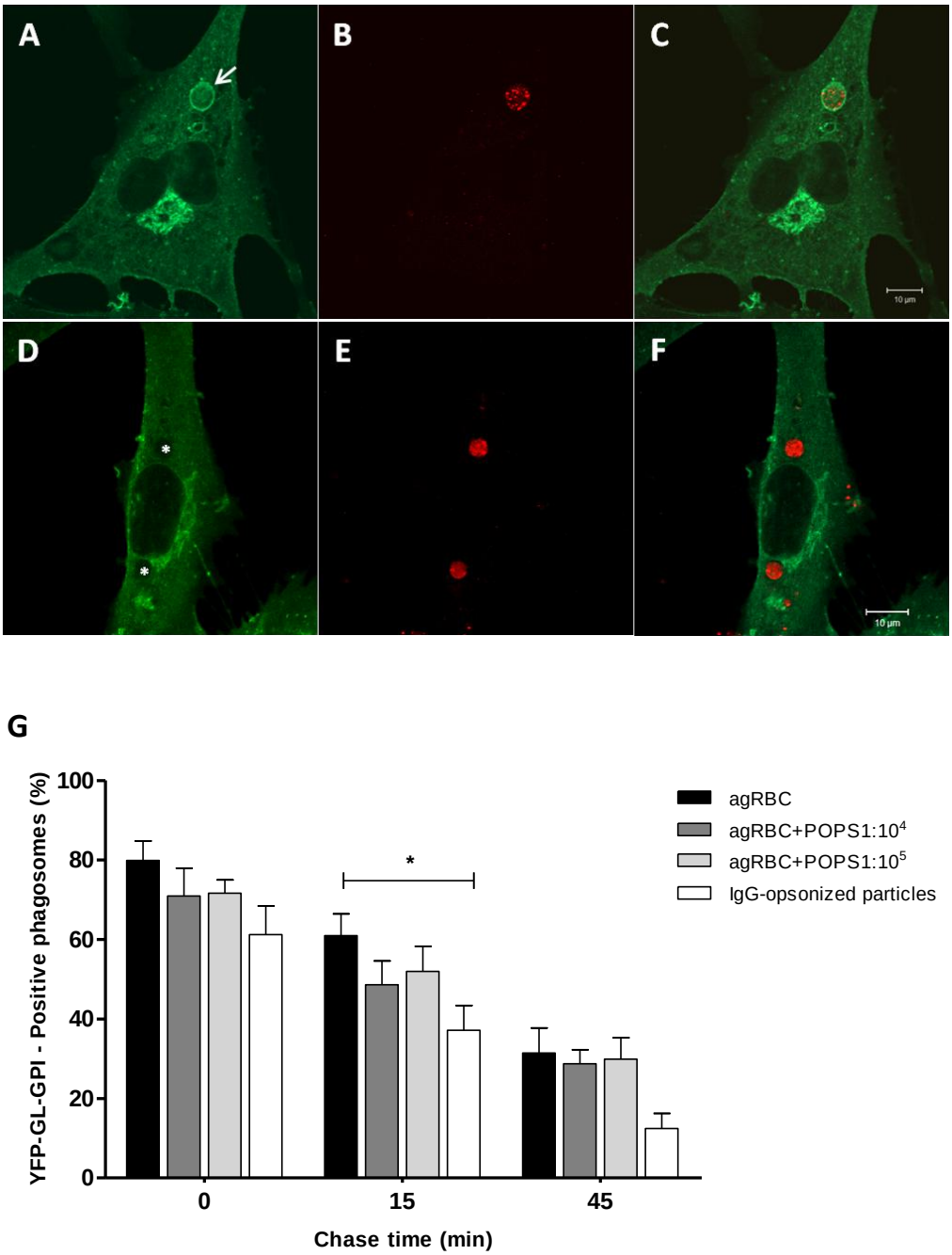


Figure 3.6. Recycling of YFP-GL-GPI from phagosomal membranes containing different phagocytic particles. The recycling of plasma membrane components from phagosomal membranes was assessed by the loss of the plasma membrane marker YFP-GL-GPI. Wild-type and engineered SMC were infected with adenoviruses expressing YFP-GL-GPI. **A**) Cell expressing YFP-GL-GPI and containing an YFP-GL-GPI-positive phagosome at 15 min chase time. **B**) Internalized agRBC stained with CMTMR. **C**) Corresponding merged image. **D**) SMC expressing the FcγR-IIA and YFP-GL-GPI showing two negative phagosomes for YFP-GL-GPI at 15 min chase time. **E**) Internalized IgG- opsonized shRBC stained with CMTMR. **F**) Corresponding merged image. Arrow indicates an YFP-GL-GPI-positive phagosome and asterisks indicate two YFP-GL-GPI-negative phagosomes. Bars, 10 µm. **G**) Quantification of the YFP-GL-GPI-positive phagosomes. Wild-type and engineered SMC expressing YFP-GL-GPI were exposed to different phagocytic particles for 30 min and then chased for the times indicated in the graph abscissa. The results are means ± SEM of, at least, three independent experiments. Samples were analyzed by fluorescence confocal microscopy. At each time point 100 phagosomes were analyzed. *, $p < 0.05$ comparing differences between loss of YFP-GL-GPI by phagosomes with agRBC and with IgG-opsonized shRBC.

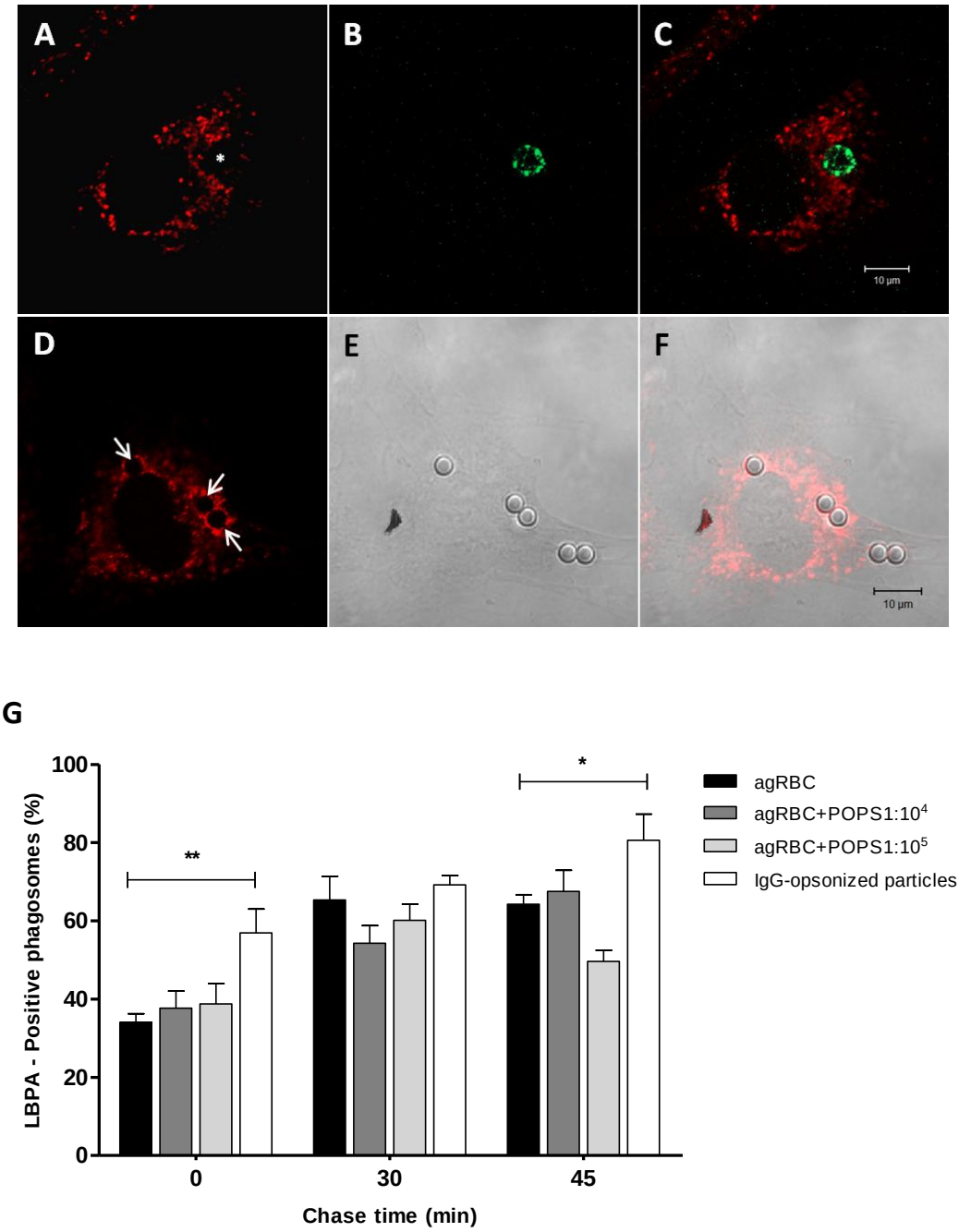


Figure 3.7. Interaction of phagosomes containing different phagocytic particles with MVB/Late endosomes. LBPA acquisition was used to assess the interaction of phagosomes containing different particles with MVB/late endosomes. **A**) LBPA staining of a cell containing a LBPA-negative phagosome at 45min chase time. **B**) Internalized agRBC stained with CFSE. **C**) Corresponding merged image. **D**) LBPA staining of a cell stably expressing the FcγR-IIA and containing three LBPA-positive phagosomes at 45min chase time. **E**) Corresponding differential interference contrast (DIC) image. **F**) Corresponding merged image. Arrows indicate three LBPA-positive phagosomes and asterisk indicates a negative phagosome. Bars, 10 µm. **G**) Quantification of the LBPA-positive phagosomes. Wild type and engineered SMC were exposed to different phagocytic particles for 30 min and then chased for the times indicated in the graph abscissa. After fixation the cells were stained with LBPA antibody and analyzed under confocal microscopy. Data shows the percentage of LBPA-positive phagosomes and are means ± SEM of, at least, three independent experiments. At each time point 100 phagosomes were analyzed. *, $p < 0.05$ and **, $p < 0.01$ comparing differences between LBPA-acquisition by phagosomes with agRBC and with IgG-opsonized beads.

Phagosomal maturation is accompanied by a gradual and profound decrease in pH levels, reported to be as low as 4.5 within late phagosomes. Lysotracker probes, fluorescent dyes that have been extensively shown be distributed into acidic compartments of the cell (e.g. late endosomes and lysosomes), were used to assess phagosomal lumen acidification in time (Borisenko et al., 2003). Thus, after performing the pulse-chase experiments for the different targets we monitored phagosomal acidification by incubating living SMC with Lysotracker for 5 min at 37ºC. Acidification rates in function of the time were quantitatively evaluated through confocal microscopy by determining the percentage of phagosomes that co-localized with the Lysotracker red fluorescence. Again, phagosomes containing IgG-opsonized particles acidified more rapidly than those carrying the apoptotic models, an effect that was more evident at the beginning (0 min) of the chase period (Figure 3.8). In the course of time the differences in acidification were attenuated.

The last event of phagosomal maturation includes fusion with lysosomes and consequent phagolysosome formation. In absence of good anti-LAMP antibodies (a classic lysosome-membrane marker) for immunofluorescence using rabbit cells, we decided to label the lysosomes with Dextran conjugated with Rhodamine. For this purpose wild-type and SMC expressing the Fcγ-RIIA were preloaded with fluorescent Dextran in serum-free media for 24 h followed by 2 h chase to ensure that all internalized Rhodamine-Dextran had reached the lysosomes. Then, the different phagocytic models were added to the phagocytes and normal pulse-chase experiments were performed. As shown in Figure 3.9, phagolysosome formation occurred faster for IgG-opsonized cargos. This effect is more notorious and significant at 0 min chase time. At later time points, phagosomes containing opsonized particles reach the same level of maturity as those containing apoptotic cells. Interestingly, for all cases phagosomal acidification occurs prior to lysosomal fusion.

Clearly, all the markers tested showed that different phagocytic particles, internalized by different receptors generated distinct phagosomes with different maturation kinetics. The less notorious results obtained for EEA-1 acquisition can be explained, at least in part, by the fact that the differences in phagosomal maturation kinetics were only delays and EEA-1 associates with phagosomal membranes only for few minutes. Thus, due to the short association of this endosomal marker with phagosomal membranes, delays in phagosomal maturation are very difficult to observe.

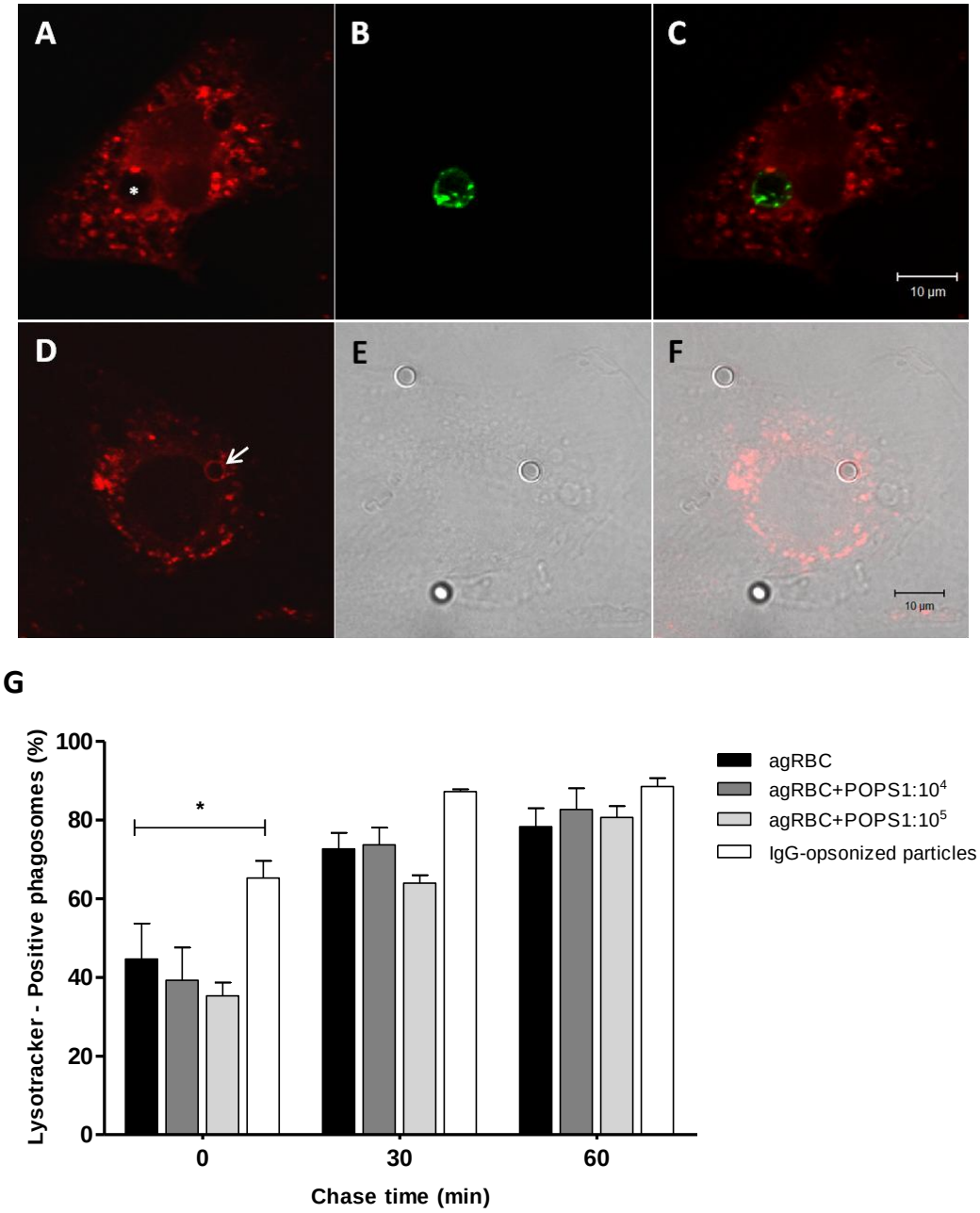


Figure 3.8. Acidification of phagosomes containing different phagocytic particles. Phagosome acidification was assessed with Lysotracker red. **A)** Lysotracker staining of a SMC containing a negative phagosome at 0min chase time. **B)** Engulfed agRBC stained with CFSE. **C)** Corresponding merged image. **D)** Lysotracker staining of SMC stably expressing FcγR-IIA with a Lysotracker-positive phagosome at 0min chase time. **E)** Corresponding DIC image. **F)** Corresponding merged image. Arrow indicates Lysotracker-positive phagosome and asterisk indicates a negative phagosome. Bars, 10 µm. **G)** Quantification of the Lysotracker-positive phagosomes. Wild type and engineered SMC were exposed to different phagocytic particles for 30 min and then chased for the times indicated in the graph abscissa. Before image acquisition, the cells were incubated with Lysotracker for 5 min. Data shows the percentage of Lysotracker-positive phagosomes and are means ± SEM of, at least, three independent experiments. At each time point 100 phagosomes were analyzed. *, $p < 0.05$ comparing differences between Lysotracker-acquisition by phagosomes with agRBC and with IgG-opsonized latex beads.

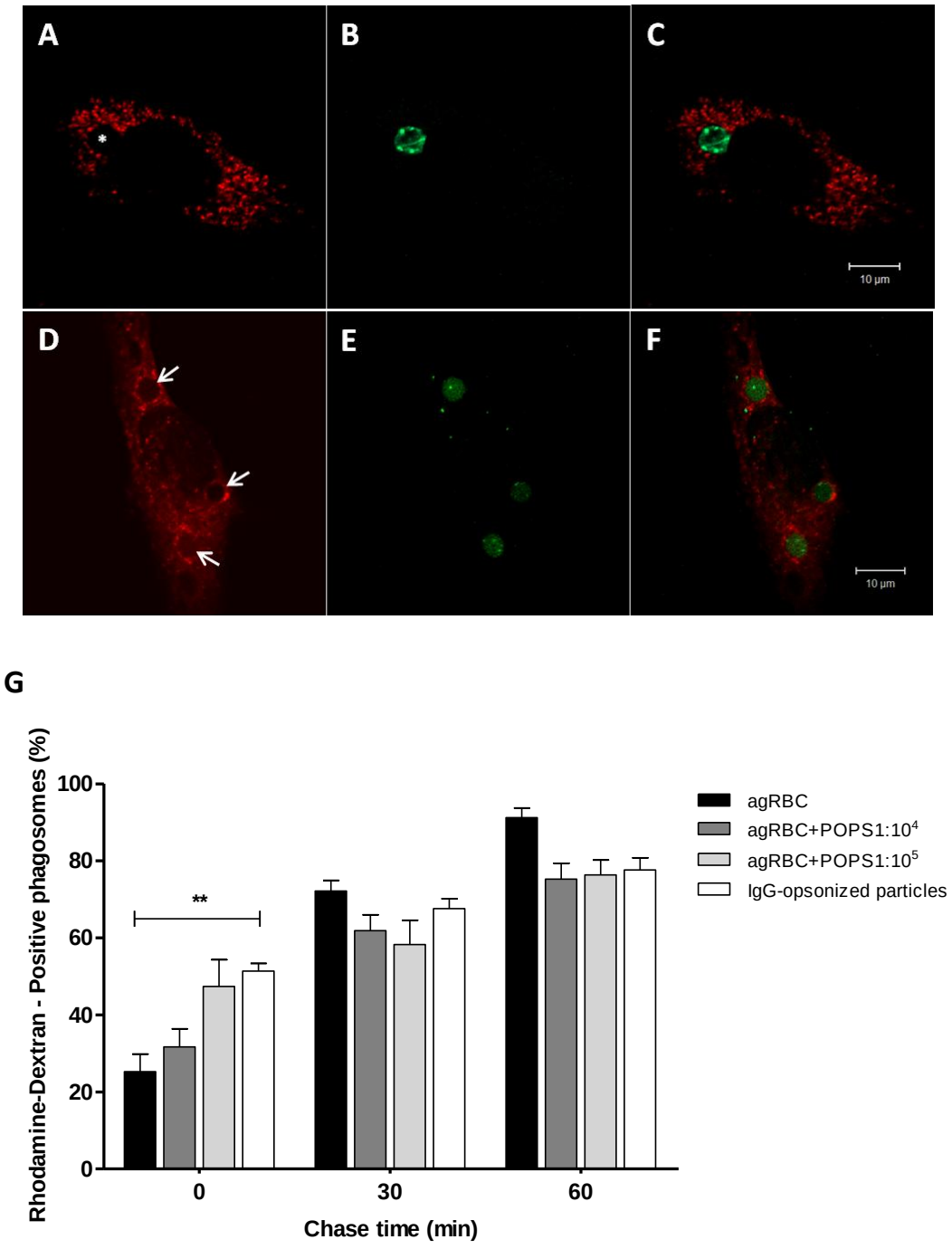


Figure 3.9. Interaction of phagosomes containing different phagocytic particles with lysosomes. Before challenging SMC with phagocytic particles, these cells were incubated with Rhodamine-Dextran and then chased to label lysosomes as described in the Material and Methods. Phagolysosome formation was then analyzed by looking at the phagosomal fluorescence acquisition. **A)** Representative image of lysosomal staining with Rhodamine-Dextran in a SMC containing a Rhodamine-Dextran-negative phagosome. **B)** Engulfed agRBC stained with CFSE. **C)** Corresponding merged image. **D)** Representative image of lysosomal staining with Rhodamine-Dextran in a SMC stably expressing the FcγR-IIA containing three Rhodamine-Dextran-positive phagosomes. **E)** Engulfed shRBCs stained with CFSE. **F)** Corresponding merged image. Arrows indicate three positive phagosomes and asterisk indicates a negative phagosome at 0min chase time (30 min pulse). Bars, 10 µm. **G)** Quantification of Rhodamine-Dextran-positive phagosomes. Wild type and engineered SMC were exposed to different phagocytic particles for 30 min and then chased for the times indicated in the graph abscissa. Data shows the percentage of Rhodamine-Dextran-positive phagosomes and are means ± SEM of at least three independent experiments. At each time point 100 phagosomes were analyzed. **, $p < 0.01$ comparing differences between Rhodamine-Dextran-acquisition by phagosomes with agRBC and with IgG-opsonized shRBC.

Finally, to exclude the possibility that the results obtained were not attributed to differences in cell type (SMC or SMC expressing FcγR) we further repeat some experiments only using SMC stably expressing FcγR as phagocytes. Thus, by comparing the maturation process of phagosomes containing agRBC and IgG-opsonized beads we found that, apparently, the accelerated ratios previously detected for phagosomes containing IgG-opsonized targets were in fact trustworthy, as presented in Figures 3.10A and 3.10B, showing the acquisition of LBPA and YFP-GL-GPI recycling, respectively, in time.

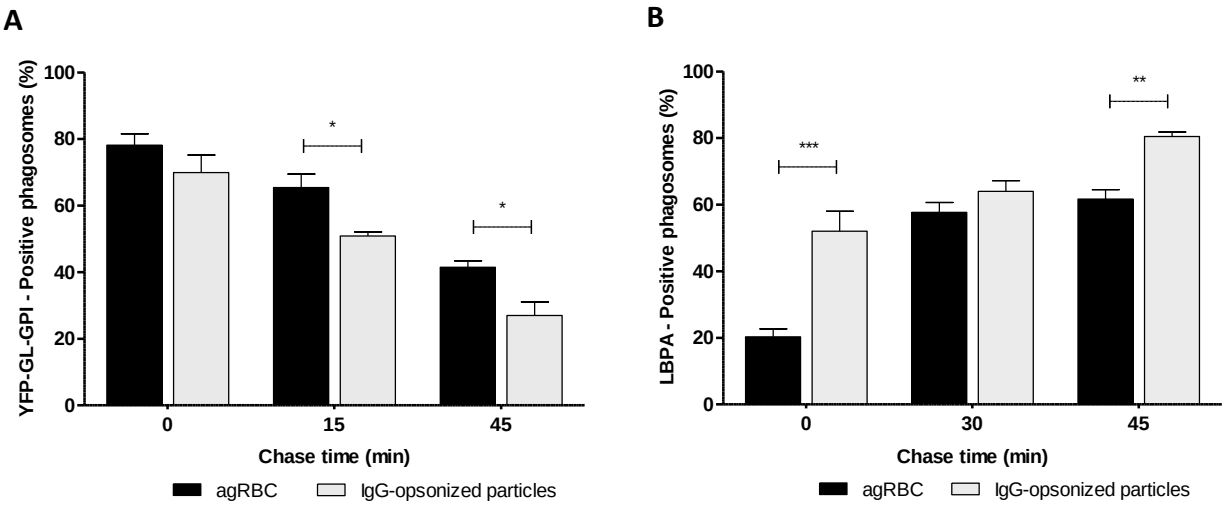


Figure 3.10. Phagosomal maturation kinetics of IgG-opsonized particles and agRBC in wild-type and SMC stably expressing the FcγR-IIA is similar. The expression of the FcγR-IIA in wild-type SMC does not change YFP-GL-GPI recycling and acquisition of LBPA of phagosomes containing IgG-opsonized particles and agRBC. **A)** Quantification of the YFP-GL-GPI-positive phagosomes. **B)** Quantification of the LBPA-positive phagosomes. The results are means ± SEM of, at least, three independent experiments. Samples were analyzed by fluorescence confocal microscopy. At each time point 100 phagosomes were analyzed. *, $p < 0.05$; **, $p<0.01$; ***; $p<0.001$ comparing differences between loss of YFP-GL-GPI or LBPA acquisition by phagosomes with agRBC and with IgG-opsonized particles. The experimental details have been described in the legends of the Figures 3.6 and 3.7.

3.4. Discussion

Little is known about how the final step of apoptotic cell clearance is regulated and more importantly how it differs from the processing of classically opsonized or microbial cells. Here, we tested the hypothesis that the particle itself can influence the intracellular trafficking of its phagosome inside a phagocyte. Especially, in this study, we characterized the maturation of phagosomes containing agRBC and the effect of PtdSer enrichment of the agRBC in phagosomal maturation. The maturation of phagosomes containing agRBC was compared with that of IgG-opsonized particles containing phagosomes in a mammalian non-professional phagocyte cell line.

The phagocytic particles used in this work are recognized by different receptors. The apoptotic cells are likely to be internalized by many different receptors that are believed to function cooperatively (Ravichandran and Lorenz, 2007). The IgG-opsonized particles are internalized via a single receptor, the FcγR, one of the best and most studied phagocytic receptor, also known to be tightly coupled to the production and secretion of pro-inflammatory molecules such as reactive oxygen intermediates and arachidonic acid metabolites (Aderem et al., 1985; Wright and Silverstein, 1983). More and more data support the notion that different phagocytic receptors send different signals to the actin cytoskeleton and initiate different mechanisms of internalization (Allen and Aderem, 1996; Swanson, 2008). Apoptotic signals stimulated membrane ruffling and formation of large and spacious fluid-filled vesicles; while Fc-Receptor stimulation drove pseudopod extension from the phagocytic cell, resulting in a smaller tight fitting phagosome without any extracellular fluid. Indeed, such differences reinforce the idea that phagocytic particle properties can determine the complexity of the actin arrangement that must be created to dictate membrane remodeling over the target, phagosome formation and, perhaps, further phagosome interactions (Aderem and Underhill, 1999; Champion and Mitragotri, 2006; Hoffmann et al., 2001).

All events associated with clearance of apoptotic cells are extremely organized and elaborated (Bratton and Henson, 2008). Any disturbance of this refined process can be translated into different disease states linked to inflammation and autoimmunity (Elliott and Ravichandran, 2010; Nagata et al., 2010). Although, a lot of efforts have been dedicated to the role of receptors and ligands involved in recognition and post-engulfment consequences, the maturation of apoptotic cells containing phagosomes in mammalian cells has remained elusive (Ravichandran and Lorenz, 2007). Phagosomes containing apoptotic cells undergo maturation to generate phagolysosomes, in which cell corpses are degraded but the regulation of the maturation process

is poorly understood. Several studies establish that the clearance of engulfed apoptotic bodies through phagosome maturation and fusion with lysosomes follows a path generally similar to that of other endosomes and phagosomes, but with some specific features. Recent studies in *C. elegans* have identified key factors involved in this maturation process, including Rab GTPases, PI3-kinases and components of the HOPS complex. It is also noteworthy that components required for phagosome maturation, such as Rab5, also contribute to engulfment itself and this outcome was never observed in Fc-mediated phagocytosis (Almendinger et al., 2011; Fullard et al., 2009; Guo et al., 2010; Kinchen et al., 2008; Kinchen and Ravichandran, 2008; Lu et al., 2008; Vieira et al., 2002; Yu et al., 2008; Zhou and Yu, 2008).

Here, the maturation of apoptotic cells containing-phagosomes was assessed by analyzing the time course of acquisition of early, late and lysosomal markers as well as by the loss of YFP-GL-GPI (a plasma membrane marker) and by acidification. To our knowledge this is the first time that such a systematic and comprehensive study has been done in mammalian cells. The rates of phagosome-lysosome fusion vary depending on the nature of the ingested particle. During Fc-mediated phagocytosis around 50% of the phagosomes have fused with lysosomes within 30 min (0 min chase). In contrast, for the same time point only 25% of the phagosomes containing agRBC have fused with lysosomes. Excluding the interaction with early endosomes, this trend was observed for all markers assessed, including recycling of plasma membrane components and acidification. This outcome, *i.e.*, phagosomes containing apoptotic cells maturing more slowly than those carrying opsonized particles, is not surprising, since intuitively, we may imagine that the fight against potentially harmful invaders is something that the body needs to face more aggressively, which is reflected in agility for a prompt destruction. On the other hand, apoptotic cells do not represent an eminent danger, since their effective removal is something that occurs almost constitutively in the body, illustrated by the negligible number of apoptotic bodies normally seen in damaged tissues (Blander and Medzhitov, 2004; Gardai et al., 2006). These observations indicate that phagocytic targets can differentially affect the maturation rate, perhaps, through their phagocytic receptors or other host-cell factors.

However, Erwig and colleagues (Erwig et al., 2006), in contrast with our results, showed that phagosomes containing apoptotic cells matured more rapidly than those containing opsonized cells in primary macrophages, macrophage cell lines and fibroblasts and this effect was independent of the phagocyte species or the ingested target cell. This discrepancy, in our opinion could have two possible explanations: i) differences between the phagocytes or the models of phagocytic particles used; and ii) experimental conditions used to assess phagosomal maturation.

We would like to emphasize that in our case the transport of phagosomes within the cells was followed by pulse-chase experiments. This experimental approach allows the decoupling of particle internalization from the kinetics of both phagosomal acidification and phagosomal-endosomal/lysosomal fusion. In contrast, Erwig and colleagues (Erwig et al., 2006) followed the acquisition of late endocytic markers and acidification at different times of internalization and thus, did not discriminate between the internalization and maturation of the phagosomes containing apoptotic cells. In our opinion this difference, in the experimental protocol, can explain, at least in part, the apparent discrepancy between their results and ours. In addition, our data was justified by a rigorous and meticulous process of marking and analysis of different stages of maturation, starting with sorting/early endosomes and culminating with lysosome interaction.

Finally, we addressed how the amount of PtdSer exposure on the surface of the cell corpse can affect its engulfment and maturation. For this purpose, we generated agRBC enriched in different amounts of PtdSer. The role played by PtdSer in phagocytosis of cells programmed to die is essentially linked to recognition and receptor engagement, in these circumstances, working as an “eat-me” signal. However, internalization by itself appears to involve and require additional ligands on apoptotic cells, PtdSer alone being insufficient to mediate phagocytic uptake (Borisenko et al., 2003). However, we did not observe any significant effect in engulfment of agRBC enriched in PtdSer or in posterior phagosome maturation suggesting that beyond a certain threshold the increase of negative charges at the surface of apoptotic cells by the incorporation of PtdSer, a critical eat-me signal, does not have any effect on the parameters measured in this work. While the literature suggests that the rate at which phagosomes mature may be related to the nature of the interaction between the particle surface and the phagosomal membrane, our results show that there may be a saturation threshold of PtdSer-receptors at the surface of the SMC. The types and abundance of receptors capable of binding PtdSer at the surface of SMC, to our knowledge, have still not been characterized. Another open question is whether the oxidized PtdSer (a process that seems to naturally occurs during apoptosis) is also critical to induce apoptotic cell phagocytosis (Kagan et al., 2002; Matsura et al., 2002).

Our data show that the interactions between the phagocytic models and the endocytic machinery of the SMC are significantly different in their kinetic characteristics. These findings can be relevant since the understanding of how defects in apoptotic corpse removal translate into disease states is not completely understood and enhanced phagocytosis of apoptotic cells may be exploited for therapeutic gain (Hart et al., 2008). Furthermore, studies using non-digestible

latex beads have shown that the inability to degrade a target can result in decreased uptake (Schrijvers et al., 2005). The challenges ahead include the identification of critical players as well as the signalling pathways that orchestrate the different stages of engulfment and maturation. It is already known, by studies of apoptotic cell removal in mammalian macrophages, that RhoA and ERM (ezrin-radixin-moesin) proteins have a role in the timely recruitment of Rab7 to the phagosome while no effect was observed on maturation of phagosomes containing IgG-opsonized particles (Erwig et al., 2006). These differences might be closely related to the different immunological responses induced by distinct phagocytic targets. Studies focusing on the degradation of apoptotic cells may provide new platforms for investigating the mechanisms underlying the differential processing of different phagocytic targets.

Chapter IV

Role of autophagy machinery on apoptotic cell clearance

Summary

Role of autophagy machinery on apoptotic cell clearance

4.1. Rationale

Efferocytosis is a process that must occur efficiently and accurately to discard unwanted cells that die every day in order to maintain tissue homeostasis. Wherever this process fails, dying cells accumulate; evolve into a critical secondary necrotic state and to the onset of several autoimmune and inflammatory diseases. The triumph of such a process depends on a series of synchronized events involving both apoptotic cells and phagocytic cells (Henson and Hume, 2006). Firstly, dying cells anticipate their own death and secret “find-me” signals to recruit not only professional phagocytes but also non-professional “neighboring” cells to their proximity (Truman et al., 2008). Non-professional SMC, for instance, are recruited to atherosclerotic lesions, where they play an important role by taking over local efferocytosis (Kolb et al., 2007; Schrijvers et al., 2005). After being located, apoptotic cells exhibit “eat-me” signals on their surface (e.g. PtdSer), which promote their recognition by phagocytes (Fadok et al., 1992; Paidassi et al., 2009). The engagement of phagocytic receptors and ligands eventually results in cytoskeletal reorganization, which drives the uptake of the cell corpse into the phagosome, likewise contributes to control inappropriate immune responses, so that apoptotic cells are removed in an anti-inflammatory manner (Griffith and Ferguson, 2011; Hochreiter-Hufford and Ravichandran, 2013). Once inside the phagosome, cargo will be processed through phagosome maturation, progressively acidifying, until be completely digested by lysosomal enzymes after fusion between the phagosome with lysosomes (Kinchen and Ravichandran, 2008; Vieira et al., 2002).

Another cellular process that also relies on lysosome delivery for cargo degradation is autophagy. According to the different ways that autophagic substrates reach the lysosomes, autophagy can be discriminated into three types: chaperone-mediated autophagy, microautophagy and the most extensively studied macroautophagy (referred to as autophagy hereafter) (Kubota, 2009; Levine and Klionsky, 2004; Yang and Klionsky, 2010a). This cellular “self-eat” process was originally considered a non-selective pathway activated under nutrient scarcity and metabolic stress, whereby cytosolic materials were enclosed into double-membrane

structures, called autophagosomes (Longatti and Tooze, 2009; Mizushima, 2007). However, recent studies have clearly demonstrated that autophagy has a greater variety of physiological and pathophysiological roles than expected, such as organelle quality control, development, anti-aging, elimination of microorganisms, cell death, tumor suppression and even antigen presentation (Deretic, 2006; Levine and Kroemer, 2008; Mizushima et al., 2008). In addition, refined mechanisms of selectivity start to emerge with the identification of autophagy receptors (Rogov et al., 2014). Also known as adaptor proteins, these mediators confer cargo specificity by simultaneously bind to ubiquitinated substrates in cytosolic materials and LC3 in autophagosomes (Johansen and Lamark, 2011; Shaid et al., 2013). In mammals, the role of LC3 (a protein hitherto considered as an exclusive autophagosome marker) is related to the expansion and closure of the phagophore (pre-autophagosomal membrane) to form the autophagosome, besides autophagosome maturation and fusion with lysosomes (Weidberg et al., 2010b). Nevertheless, over the past decade, LC3 has also been implicated to be recruited to single-membrane phagosomes, in a process termed LC3-Associated Phagocytosis or, for simplicity, LAP (Sanjuan et al., 2007). This crosstalk between autophagy and phagocytosis has been suggested to stimulate phagosome maturation by improving the degradative capacity of phagosomes containing distinct cargoes, including apoptotic cells, pathogens and entotic bodies (arising from live epithelial cells phagocytosis)(Florey et al., 2011; Florey and Overholtzer, 2012; Huang et al., 2009; Martinez et al., 2011; Sanjuan et al., 2009). Although this unusual translocation of LC3 to phagosomes has been shown to be dependent on several players of the regulatory machinery of autophagy, for example, Atg5, Atg7 and Beclin1; so far, there is no evidence about the phagosomal requirement of proteins associated with selective autophagy (Henault et al., 2012; Martinez et al., 2011). Thus, since the steps involving the processing of apoptotic cells into phagosomes are still full of open questions, the exploration of this newly discovered relationship between endocytic and autophagic machineries may provide new insights in the area.

Therefore, in this chapter we will show that LAP is involved in the maturation of phagosomes containing aged RBCs and IgG-opsonized particles in rabbit vascular SMC stably expressing the widely-studied Fcγ-RIIA. By using a pharmacological approach we evaluated the functional relevance of the recruitment of LC3 to the maturation of phagosomes containing the different phagocytic particles and have found that LAP manipulation may influence the normal rates of association between phagosomes and endocytic compartments. Furthermore, our results demonstrate, for the first time in the field, the involvement of adaptor proteins, such as p62 (also referred to as SQSTM1), NBR1 (Neighbor of Braca 1 gene) and NDP52 (Nuclear Dot

Protein 52) in phagosomal maturation (Bjorkoy et al., 2005; Kirkin et al., 2009b; Thurston et al., 2009; Waters et al., 2009). Since phagosomal membranes displayed ubiquitinated proteins, these effectors/adaptors from the selective machinery of autophagy can also be involved in selectivity of targets in LAP. Thus, unravel how the molecular mechanisms associated to apoptotic cell removal may have impact on tissue homeostasis and pathology (e.g. cardiovascular diseases) could be useful to make progress in our understanding about efferocytosis.

4.2. Results

4.2.1. Phagosomes containing agRBCs and IgG-opsonized particles acquired LC3-IIB at very early stages of phagocytosis

Recently, LAP was characterized in phagosomes carrying different types of dead cells (Martinez et al., 2011), so we asked if the engulfment of aged RBC was also able to recruit LC3 to their corresponding phagosomes. For that, eryptosis was triggered by incubating fresh RBCs at 37^{o} C during 4 days in a glucose-independent buffer. Senescence of RBC, translated by the loss of phospholipid asymmetry, were validated through flow cytometry analysis by using Annexin-V, as previously described (Viegas et al., 2012). SMC were plated, exposed to eryptotic targets for 30 min (pulse time) and LC3 association with phagosomes was confirmed by confocal microscopy of immunostained samples. Immediately after phagocytosis, the autophagic protein LC3-IIB (the isoform B of lipidated LC3) was translocated to nascent phagosomes containing agRBCs, as evidenced by phagosomes that appeared as an intense and continuous rim surrounding the apoptotic cell (see Figure 4.1A-B).

To determine the kinetics of LC3 association to phagosomes containing agRBC in time, we performed pulse-chase experiments and compared these results with the rates obtained for phagosomes containing IgG-coated latex beads. As previously stated, our group has shown that phagosomes containing these two different particles differ in the kinetic pattern of maturation, suggesting that more discrepancies may exist (Viegas et al., 2012). Additionally, IgG-opsonized particles were proposed to induce the recruitment of LC3 through the engagement of Fc-receptors (Henault et al., 2012; Huang et al., 2009), which was in fact confirmed to our own experimental conditions, as seen in Figure 4.1C-D. Because SMC are non-professional phagocytes, they do not naturally express the Fcγ-RIIA, so we used an engineered SMC line stably expressing the Fcγ-RIIA, a widely expressed and highly studied phagocytic receptor. We found that

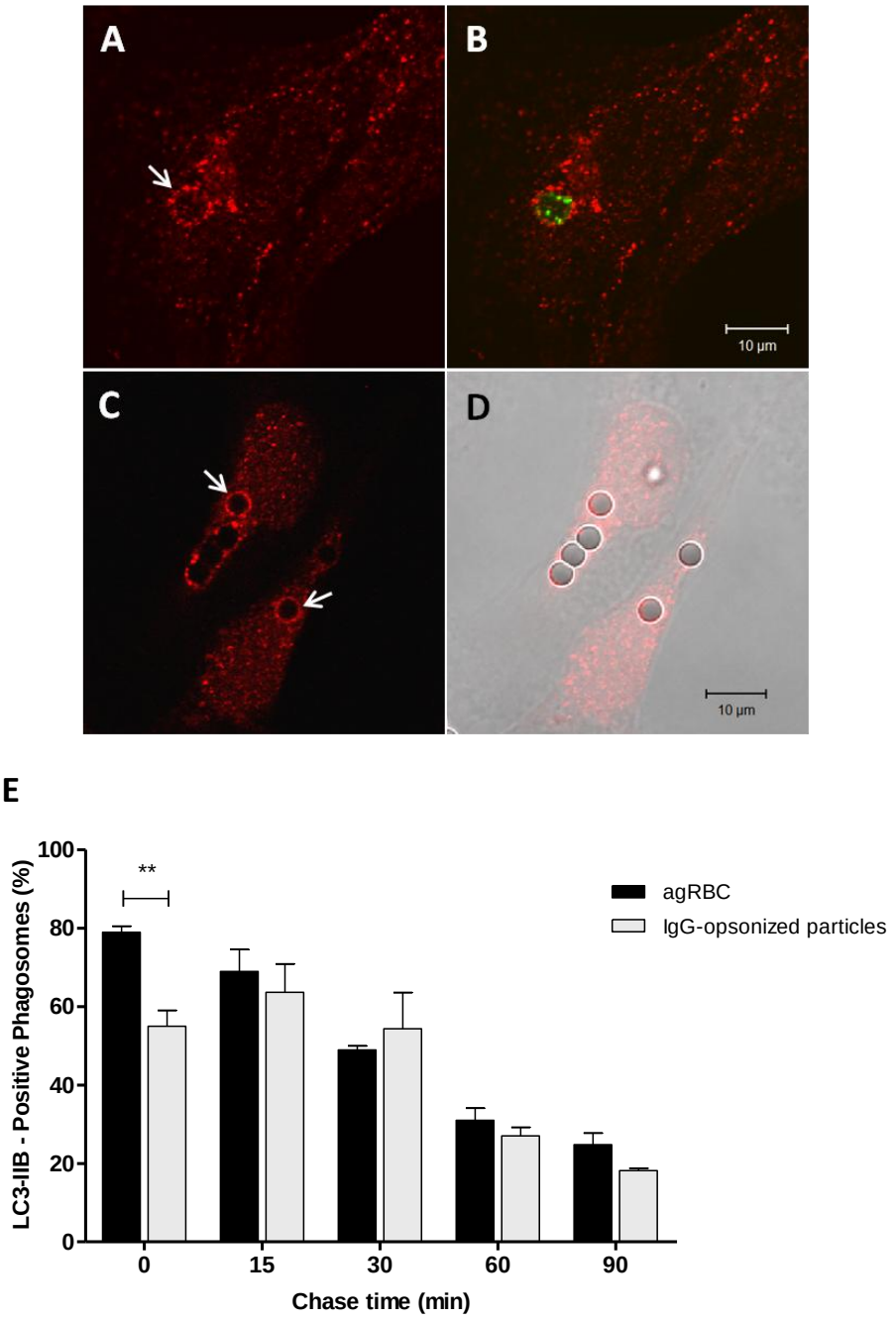


Figure 4.1. Association of LC3 to phagosomes containing different phagocytic particles. LAP was assessed by the acquisition of LC3-IIB by phagosomes. After pulse-chase experiments, cells were fixed, stained with LC3-IIB antibody and analyzed under confocal microscopy. **A)** LC3-IIB staining of a cell containing an LC3-positive phagosome at 15 min chase time. **B)** Corresponding merged image showing the internalized agRBC stained with CFSE. **C)** LC3-IIB staining of a cell containing two LC3-positive phagosomes at 15 min chase time. **D)** Corresponding merged image showing the internalized IgG-opsonized particles in DIC. Arrows indicate positive phagosomes. Bars,10 µm. **E**) Quantification of LC3-IIB-positive phagosomes. Engineered SMC were exposed to the different phagocytic particles for 30 min and then chased for the time indicated in the graph abscissa. The values are means ± SEM of, at least, three independent experiments. At each time point, at least, 100 phagosomes were analyzed. **, $p < 0.01$ comparing differences between LC3-acquisition by phagosomes with agRBC and with IgG-opsonized latex beads.

phagosomes containing the different targets (agRBC and IgG-opsonized beads) induced a rapid and transient association of LC3 onto their membranes, with peaks reaching a maximum of about 80% (79.0 ± 1.53%) after 0 min chase for agRBC and about 64% (63.67 ± 7.22%) after 15min chase for IgG-opsonized beads. Then, LC3 gradually dissociated from both types of phagosomes, probably due to hydrolytic lysosomal degradation and/or recycling of phagosomal components (Figure 4.1E).

Since autophagy, under normal physiological conditions, has a vital role in the maintenance of the amino acid pool during cellular starvation (Mizushima, 2007), and because our methodology for the phagocytic assays requires the use of serum-free medium, we further asked if the nutrient deprivation could affect the LC3 association to agRBC-containing phagosomes. Before that, we have determined the effect of the absence of serum in canonical autophagy and saw that after only 30 min of amino acid starvation the number of autophagosomes increases compared to fed SMC, judged by the higher number of punctuated LC3 structures (see Supplemental Figure 1 in the Appendix section). The autophagic activity in serum-deprived cells continued to increase until 60min of starvation; then the rates start to decline, likely because cells have reached the homeostatic balance. However, as shown in Figure 4.2, no changes were observed in the LC3 pattern distribution to phagosomes containing agRBC when we compared fed and starved SMCs stably expressing FcR, suggesting that LAP machinery is independent of the nutritional status of the phagocytes.

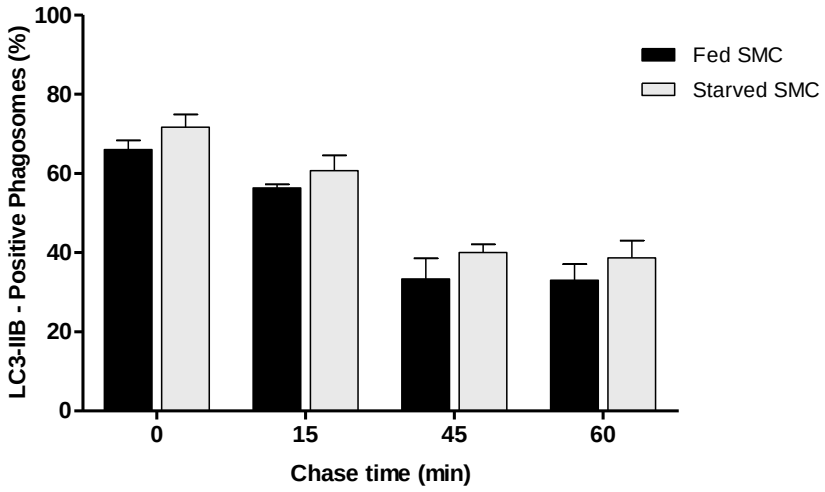


Figure 4.2. LC3 association to phagosomes containing agRBC in SMC subjected to different nutritional conditions. LAP was assessed by the quantification of LC3-positive phagosomes. Engineered SMCs were incubated in medium supplemented or not with serum, exposed to agRBC for 30 min, chased for the time indicated in the graph abscissa and then immunostained for LC3-IIB. The values are means ± SEM of, at least, three independent experiments. At each time point, at least, 100 phagosomes were analyzed.

4.2.2. Modulation of autophagic machinery affects LC3 association to phagosomal membranes as well as phagolysosome biogenesis

Since LAP represents a strait connection between phagocytosis and autophagy we decided to address the effect of two existing pharmacological drugs, Jak3 Inhibitor VI and Nortriptyline, in regulating LC3 translocation and LBPA acquisition during phagosome maturation. These compounds, whose actions are predicted to interfere with autophagy, were first evaluated by monitoring the "autophagic flux", a term used to denote the dynamic process of autophagosome synthesis, maturation and degradation inside the lysosome (autophagolysosome) together with the autophagic substrates (Mizushima et al., 2010).

In order to inhibit the autophagic flux we used Jak3 Inhibitor VI, a kinase target inhibitor characterized by suppress autophagosome formation without affecting the activity of PI3-kinases class-I or -III, involved in phagocytosis and phagosome maturation, respectively (Farkas et al., 2011; Leverrier et al., 2003; Vieira et al., 2001). To determine whether this compound was able to modulate the canonical autophagy in SMCs we first tested its effect on LC3 puncta formation by comparing fed cells (control) and cells starved with HBSS for 3h in the presence or absence of the Jak3 Inhibitor (5uM). In fact, after 30 min incubation the cells treated with the inhibitor almost entirely lost the punctuate distribution of LC3, confirming that the autophagic flux was being negatively affected (see Supplemental Figure 2 in the Appendix section). To demonstrate the effect of the autophagy machinery inhibition in LAP and phagosome maturation we have incubated SMC with the inhibitor for 30 min before the addition of the phagocytic particles, attempting to minimize its impact on engulfment. Shortly after internalization of agRBC, control cells reached their maximum of LC3-positive phagosomes (83.90 ± 3.46%), while the maximum for cells incubated with the drug was observed only after 60min of maturation (74.18 ± 3.24%), which clearly reveals a delay in the rates of association of LC3 with the phagosomal membrane. On the other hand, looking for phagosomes containing IgG-opsonized particles the effect of autophagy inhibition was different. In contrast with the results obtained for apoptotic particles, Jak3 Inhibitor VI was delaying the LC3 dissociation from the phagosomal membranes. Concerning that, we found that after 60 min of phagosome maturation control cells had less than 15% (14.98 ± 3.44) positive-phagosomes for LC3, while at the same time point treated cells still had almost 32% (31.60 ± 1.31) positive-phagosomes for LC3 (Figure 4.3).

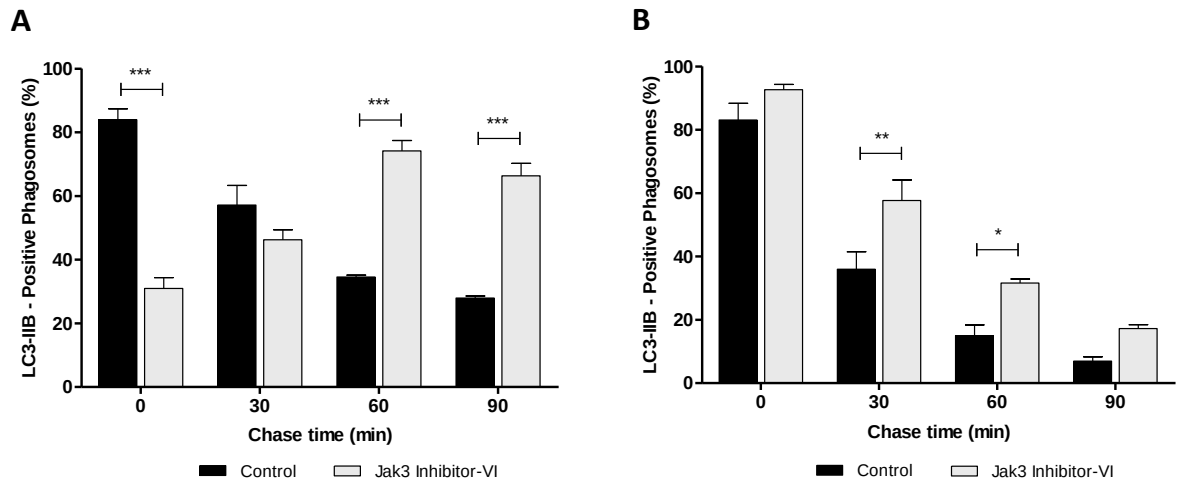


Figure 4.3. Effect of autophagy inhibition on LC3 acquisition. LAP was assessed by the quantification of LC3-positive phagosomes. Engineered SMC were incubated or not with Jak3 Inhibitor VI, exposed to the agRBC (**A**) and IgG-opsonized particles (**B**) for 30 min, chased for the time indicated in the graph abscissa and then immunostained for LC3-IIB. The values are means ± SEM of, at least, three independent experiments. At each time point, at least, 100 phagosomes were analyzed. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ comparing differences between LC3-acquisition by phagosomes with agRBC and with IgG-opsonized latex beads.

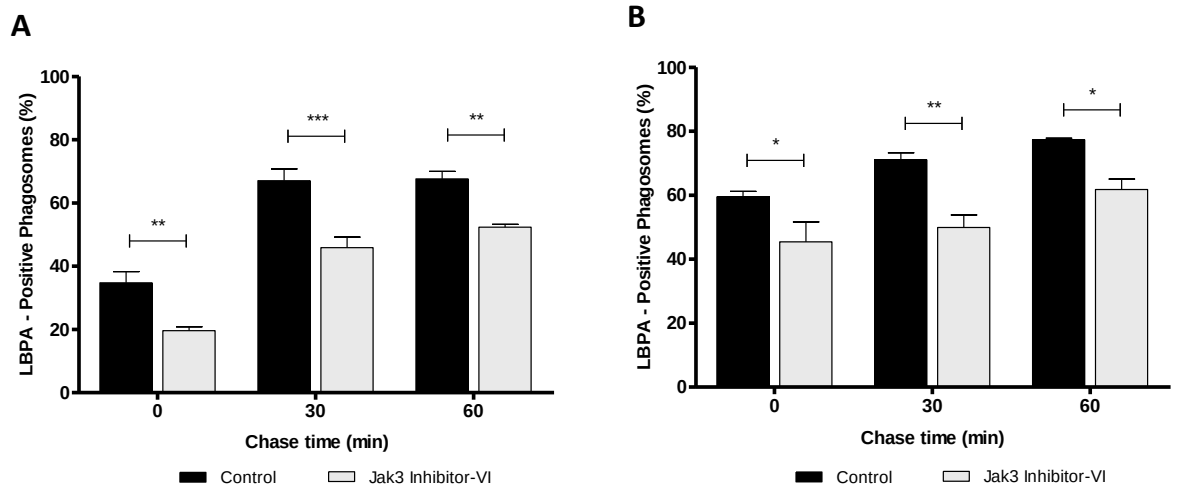


Figure 4.4. Effect of autophagy inhibition in phagosomal maturation. Phagosome maturation was assessed by the quantification of LBPA-positive phagosomes. Engineered SMC were incubated or not with Jak3 Inhibitor VI, exposed to agRBC (**A**) and IgG-opsonized particles (**B**) for 30 min, chased for the time indicated in the graph abscissa and then immunostained for LBPA. The values are means ± SEM of, at least, three independent experiments. At each time point, at least, 100 phagosomes were analyzed. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ comparing differences between LBPA-acquisition by phagosomes with agRBC and with IgG-opsonized latex beads.

Next, we addressed the role of Jak3 inhibitor in phagosome maturation by quantifying the acquisition of the lysobisphosphatidic (LBPA), a marker of MVB/late endosomes (Kobayashi et al., 1999).

For both types of phagocytic particles, the incubation with this drug was leading to a reduction of LBPA acquisition by the phagosomes, suggesting that LC3 acquisition is required for phagolysosome formation. Altogether, these results show that inhibition of the canonical autophagy machinery by Jak3 Inhibitor VI differently modulate LAP according to the phagocytic cargo, leading to a delay in the rates of LC3 association and/or dissociation, which in turn negatively influence phagosome maturation (Figure 4.4).

To check if SMCs treated with Nortriptyline, an autophagic inductor whose action is similar to starvation-induced autophagy, have opposite effects in LC3 associtions and phagsosomal maturation we have performed a set of experiments as described by Sundaramurthy and colleagues (Sundaramurthy et al., 2013). To validate the effect of the compound in our experimental conditions we examined LC3 puncta formation in SMCs not treated (control) or treated with Nortriptyline (10uM) for different incubation times. We found that after 30 min incubation was already possible to see an increased autophagic flux, indicated by the higher relative number and intensity of LC3 structures (autophagosomes/autophagolysosomes) in cells treated with the drug (see Supplemental Figure 3 in the Appendix section). The effect of the autophagic induction dictated by Nortriptyline in LAP and phagosome maturation was accessed, again, by looking at LC3- and LBPA- acquisition by phagosomal membranes. We saw that in treated cells, agRBC-containing phagosomes had an attenuated effect on the rates of LC3 dissociation, which became more visible after 90min of chase time, where our results indicated that around 50% of the phagosomes were still positives for LC3, while in control cells, this value as about 20% (Figure 4.5A). Additionally, for the same phagocytic particle (agRBC), we also found that Nortriptyline was able to accelerate phagosome maturation, confirmed by the fact that at 10 min of maturation, approximately 65% (64.90 ± 1.60%) of phagosomes had acquired LBPA in Nortriptyline-treated cells, while in control cells only 43% (43.67 ± 4.12%) of phagosomes were positives for this late endocytic marker (Figure 4.6A). Concerning the phagosomes containing IgG-opsonized cargo Nortriptyline do not appear to exert any significant effect neither in LAP nor in phagosome maturation kinetics (Figures 4.5B and 4.6B). Thus, these results emphasize that phagosomes obtained from different phagocytic particles and internalized by different receptors dissimilarly mobilize autophagic intermediates to phagosomal maturation.

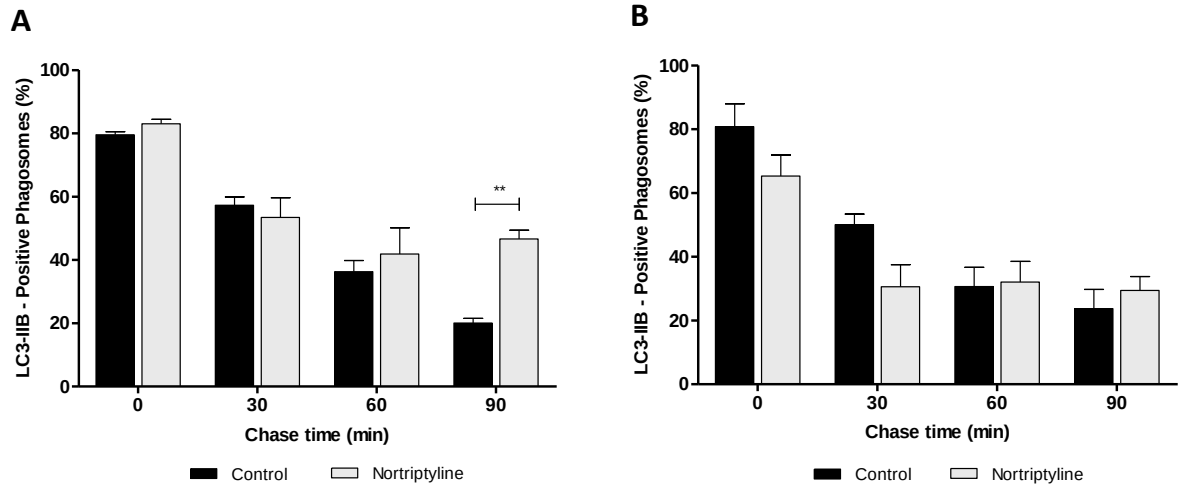


Figure 4.5. Effect of autophagy induction on phagosomal acquisition of LC3. LAP was assessed as described before. Engineered SMC were incubated or not with Nortriptyline, exposed to agRBC (**A**) and IgG-opsonized particles (**B**) for 30 min and then chased for the time indicated in the graph abscissa. The values are means ± SEM of, at least, three independent experiments. At each time point, at least, 100 phagosomes were analyzed. **, $p < 0.01$ comparing differences between LC3-acquisition by phagosomes with agRBC and with IgG-opsonized latex beads. **.**

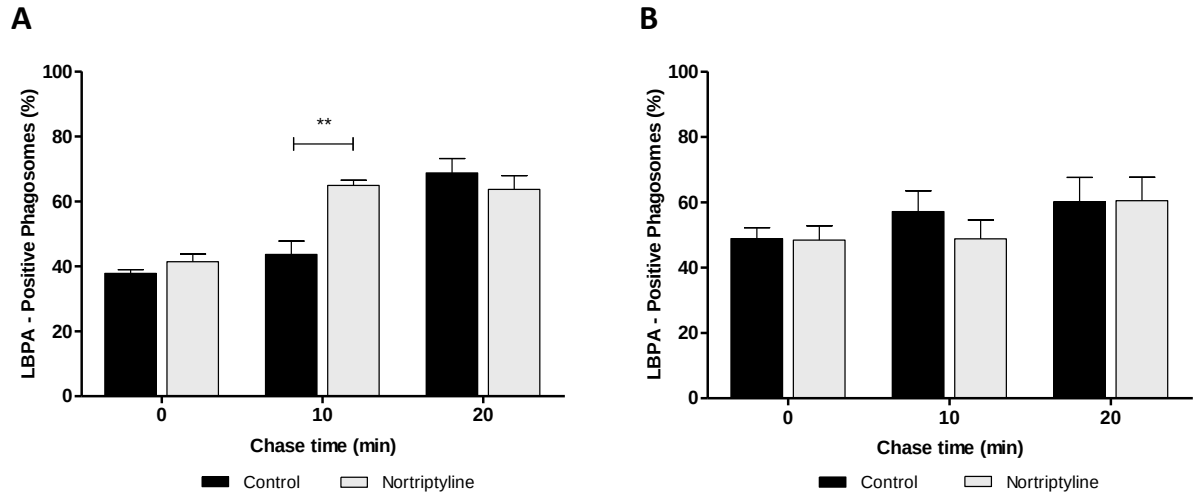


Figure 4.6. Effect of autophagy induction on phagosome maturation. Phagosome maturation was assessed by the quantification of LBPA-positive phagosomes. Engineered SMC were incubated or not with Nortriptyline, exposed to agRBC (**A**) and IgG-opsonized particles (**B**) for 30 min and then chased for the time indicated in the graph abscissa. The values are means ± SEM of, at least, three independent experiments. At each time point, at least, 100 phagosomes were analyzed. **, $p < 0.01$ comparing differences between LBPA-acquisition by phagosomes with agRBC and with IgG-opsonized latex beads.

4.2.3. Proteins on agRBC- or IgG-opsonized particles-containing phagosomes are ubiquitinated

Despite the major degradative pathway for autophagic substrates is considered non-specific, recent evidences demonstrated that some type of specific autophagic targeting mechanism also plays a role (Rogov et al., 2014). These selective mechanisms are based on proteins dedicated to tag and recognize distinct targets for degradation. Usually, the tag is mediated by ubiquitin, whereas recognition is conferred by autophagy receptors. To test whether ubiquitin can serve as a general signal to specifically target phagosomes for LAP, we performed pulse-chase experiments and immunostained the samples for the presence of poly-ubiquitinated proteins. Indeed, phagosomes containing agRBC and IgG-coated beads confirmed, by immunofluorescence followed by microscopy analysis, the presence of poly-ubiquitinated substrates on their membranes (Figure 4.7). By establishing a time course for the appearance of this tag in phagosomal membranes, we found that shortly after ingestion (0min chase) of both types of particles, SMC presented a high number of phagosomes positives for ubiquitinated components (around 60% for both particles). However, as the maturation progresses it is possible to distinguish two different patterns of dissociation, wherein phagosomes containing IgG-opsonized particles more quickly lose their link with ubiquitinated elements.

These results raised the question whether ubiquitin serves as a signal to promote LAP. Then, based on this outcome, we decided to address if LC3 adaptors that also bind ubiquitinated cargo via ubiquitin-binding domains were recruited to the phagosomal membranes.

4.2.4. The recruitment of LC3-adaptor proteins is cargo dependent

Because the phagosomes containing the different particles presented ubiquitinated proteins in their membranes, we further investigated the requirement of some described autophagy receptors/adaptors for this process, attempting to determine whether, like in canonical autophagy, these adaptor proteins could also have a role in provide LAP specificity according to the different phagosomal cargoes. The human genome encodes, at least, four ubiquitin-binding receptors involved in autophagy but here we have focused only in three: the signaling adaptor scaffold protein p62, NBR1 and NDP52 (Shaid et al., 2013). All of them share the ability to simultaneously interact with the lipidated form of LC3 in autophagosomes through a

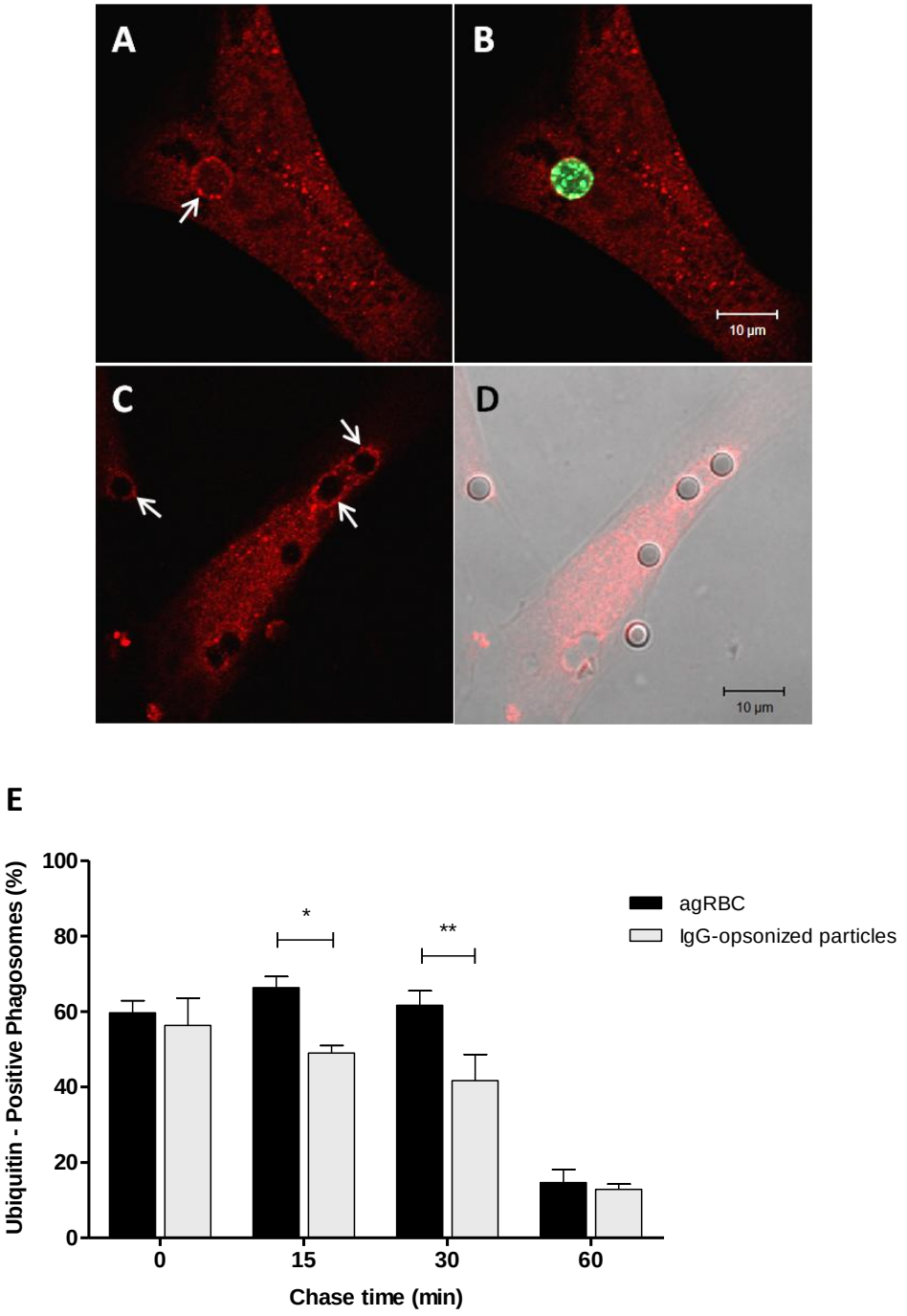
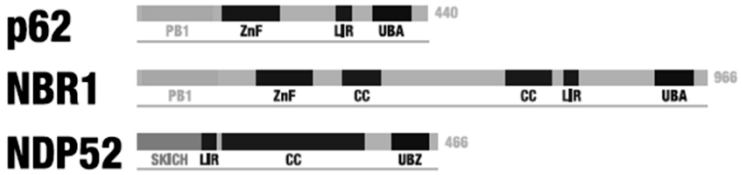


Figure 4.7. Distribution of ubiquitinated proteins on phagosomal membranes in function of time. After pulse-chase experiments, cells were fixed, immunostained for poly-ubiquitinated substrates and analyzed under confocal microscopy. **A)** Poly-ubiquitin staining of a cell containing an ubiquitin-positive phagosome at 15 min chase time. **B)** Corresponding merged image showing the internalized agRBC stained with CFSE. **C)** Poly-ubiquitin staining of a cell containing three ubiquitin-positive phagosomes at 15 min chase time. **D)** Corresponding merged image showing the internalized IgG-opsonized particles in DIC. Arrows indicate positive phagosomes. Bars,10 µm. **E**) Quantification of ubiquitin-positive phagosomes. Engineered SMC were exposed to the different phagocytic particles for 30 min and then chased for the time indicated in the graph abscissa. The values are means ± SEM of, at least, three independent experiments. At each time point, at least, 100 phagosomes were analyzed. *, $p < 0.05$; **, $p < 0.01$ comparing differences between ubiquitin-acquisition by phagosomes with agRBC and with IgG-opsonized latex beads.

LIR (LC3 Interaction Region) motif and with ubiquitinated substrates targeted to removal through an UBD (Ubiquitin Binding Domain), as illustrated in the scheme below:



Since p62 is the best-described adaptor protein and acts as a universal receptor for ubiquitinated cargo under physiological and pathological conditions, we have decided to initiate our investigation by testing its intracellular distribution during phagocytosis. As exhibited in Figure 4.8, after pulse-chase experiments, phagosomes containing agRBC or IgG-opsonized beads displayed completely different patterns of association for p62. Remarkably, phagosomes containing dying cells acquired p62 with a kinetic profile quite similar to that found for phagosomal protein ubiquitination. We observed the maximal percentage of p62 and ubiquitinated positive-phagosomes between 15 and 30 min chase, then proteins progressively dissociated from phagosomes. Meanwhile, phagosomes loaded with IgG-opsonized particles showed inexpressive levels of p62 along the time. Thus, from this set of results we can conclude that p62 is preferably acquired by agRBC-containing phagosomes than by IgG particles-containing phagosomes.

Once p62 can directly interact with NBR1 (through a PB1 domain), together acting as a cargo receptor for ubiquitinated substrates in classical autophagy, we have analyzed the association of this other autophagy receptor with phagosomal membranes (Lamark et al., 2003). As seen in Figure 4.9, NBR1 was recruited to both types of phagosomes, albeit with an unusual kinetic profile for phagosomes containing IgG-opsonized particles. For all the other markers analyzed so far we saw practically the same pattern of distribution, that is, a higher percentage of positive-phagosomes at early stages of maturation, followed by a progressive loss, reaching low percentage values at later stages, which is totally understandable by reasons already discussed (lysosome degradation *versus* recycling). However, for phagosomes containing IgG-coated beads the association of NBR1 did not appear to be attenuated over time, exhibiting quite constant levels along the maturation process. In contrast, agRBC-containing phagosomes did not demonstrate such surprising features in NBR1 recruitment, and even behaved similarly to the

pattern obtained for p62, with peak values of positive phagosomes obtained at around 30min chase (65.57 ± 3.58%).

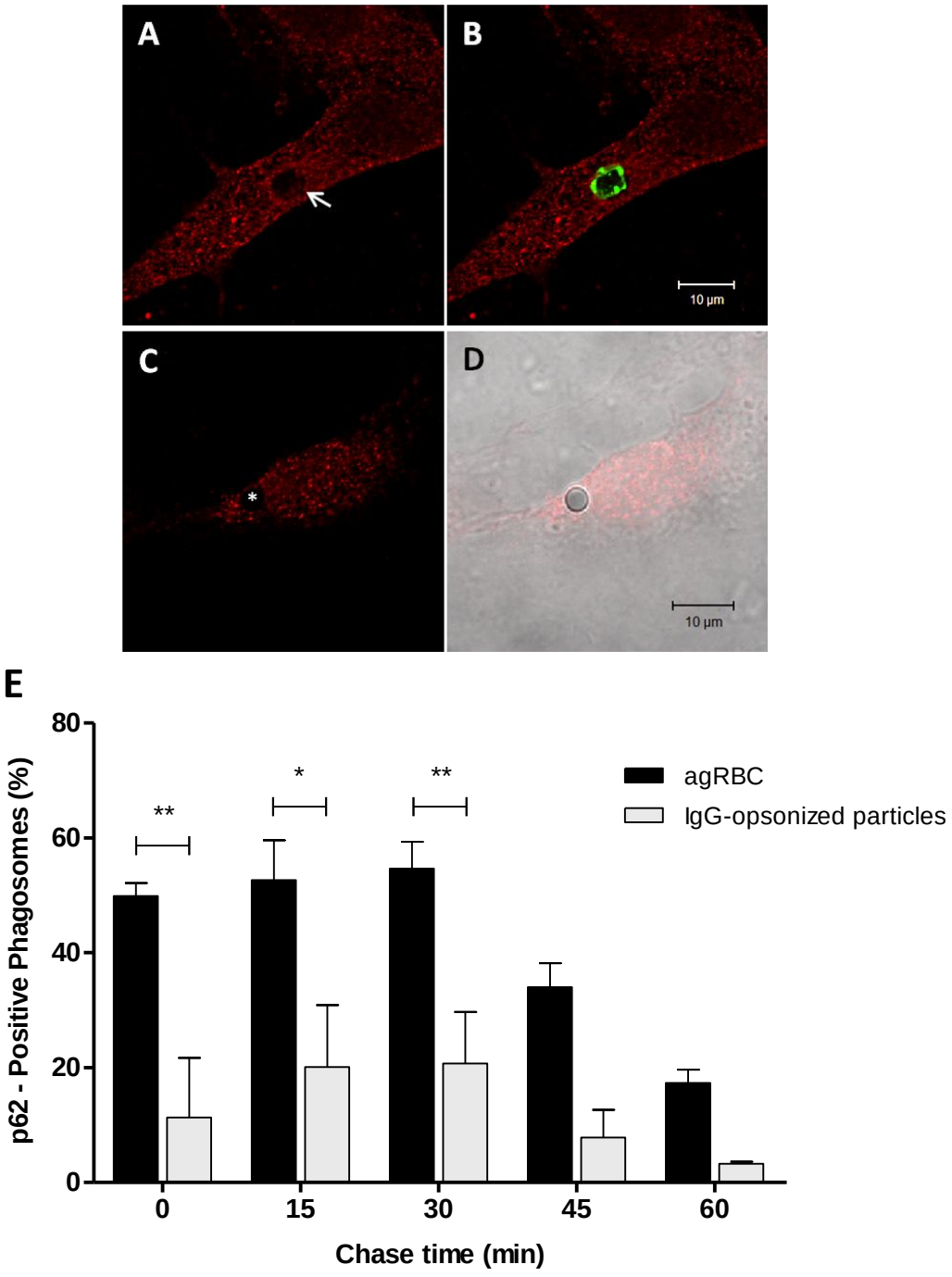
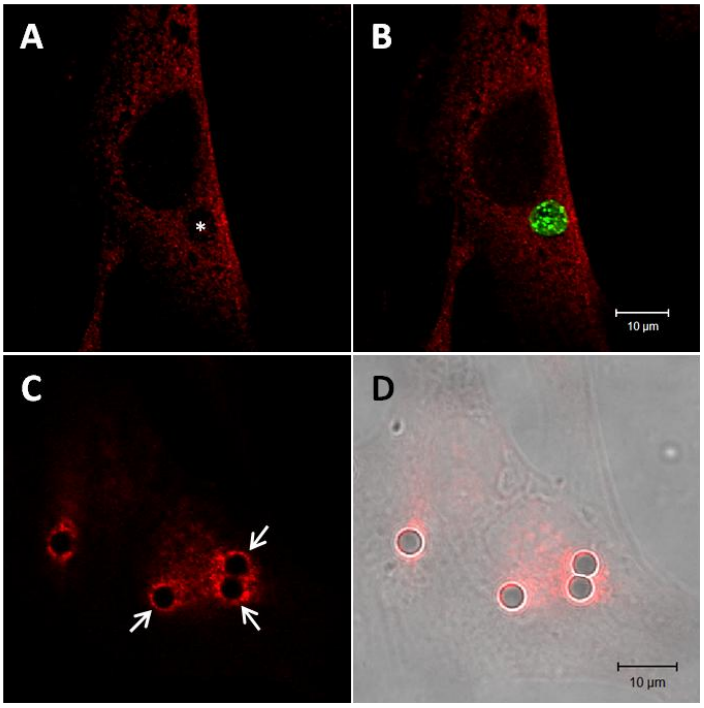


Figure 4.8. Acquisition of p62 by phagosomal membranes. After pulse-chase experiments, cells were fixed, stained for p62 and analyzed under confocal microscopy. **A)** p62 staining of a cell containing an LC3-positive phagosome at 30 min chase time. **B)** Corresponding merged image showing the internalized agRBC stained with CFSE. **C)** p62 staining of a cell containing one p62-negative phagosome at 0 min chase time. **D)** Corresponding merged image showing the internalized IgG-opsonized particle in DIC. Arrow indicates a positive phagosome and asterisk indicates a negative phagosome. Bars,10 µm. **E)** Quantification of p62 positive-phagosomes. Engineered SMC were exposed to the different phagocytic particles for 30 min and then chased for the time indicated in the graph abscissa. The values are means ± SEM of, at least, three independent experiments. At each time point, at least, 100 phagosomes were analyzed. *, $p < 0.05$; **, $p < 0.01$ comparing differences between p62-acquisition by phagosomes with agRBC and with IgG-opsonized latex beads.



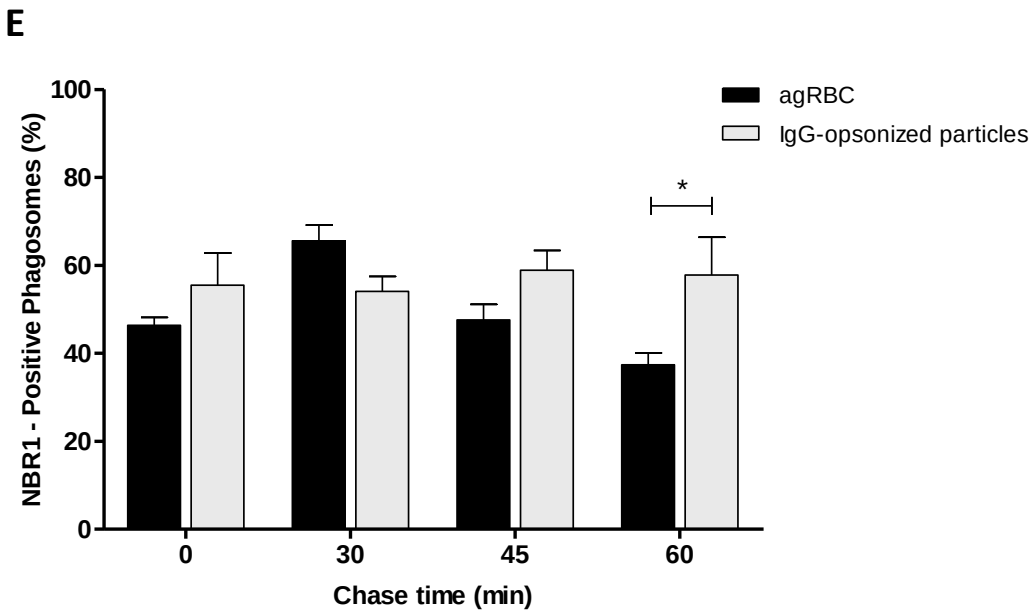


Figure 4.9. Acquisition of NBR1 by phagosomal membranes. After pulse-chase experiments, cells were fixed, stained for NBR1 and analyzed under confocal microscopy. **A)** NBR1 staining of a cell containing a NBR1-negative phagosome at 60 min chase time. **B)** Corresponding merged image showing the internalized agRBC stained with CFSE. **C)** NBR1 staining of a cell containing four NBR1-positive phagosomes at 60 min chase time. **D)** Corresponding merged image showing the internalized IgG-opsonized particles in DIC. Arrows indicate positive phagosomes and asterisk indicates a negative phagosome. Bars,10 µm. **E)** Quantification of NBR1 positive-phagosomes. Engineered SMC were exposed to the different phagocytic particles for 30 min and then chased for the time indicated in the graph abscissa. The values are means ± SEM of, at least, three independent experiments. At each time point, at least, 100 phagosomes were analyzed. *, $p < 0.05$ comparing differences between NBR1-acquisition by phagosomes with agRBC and with IgG-opsonized latex beads.

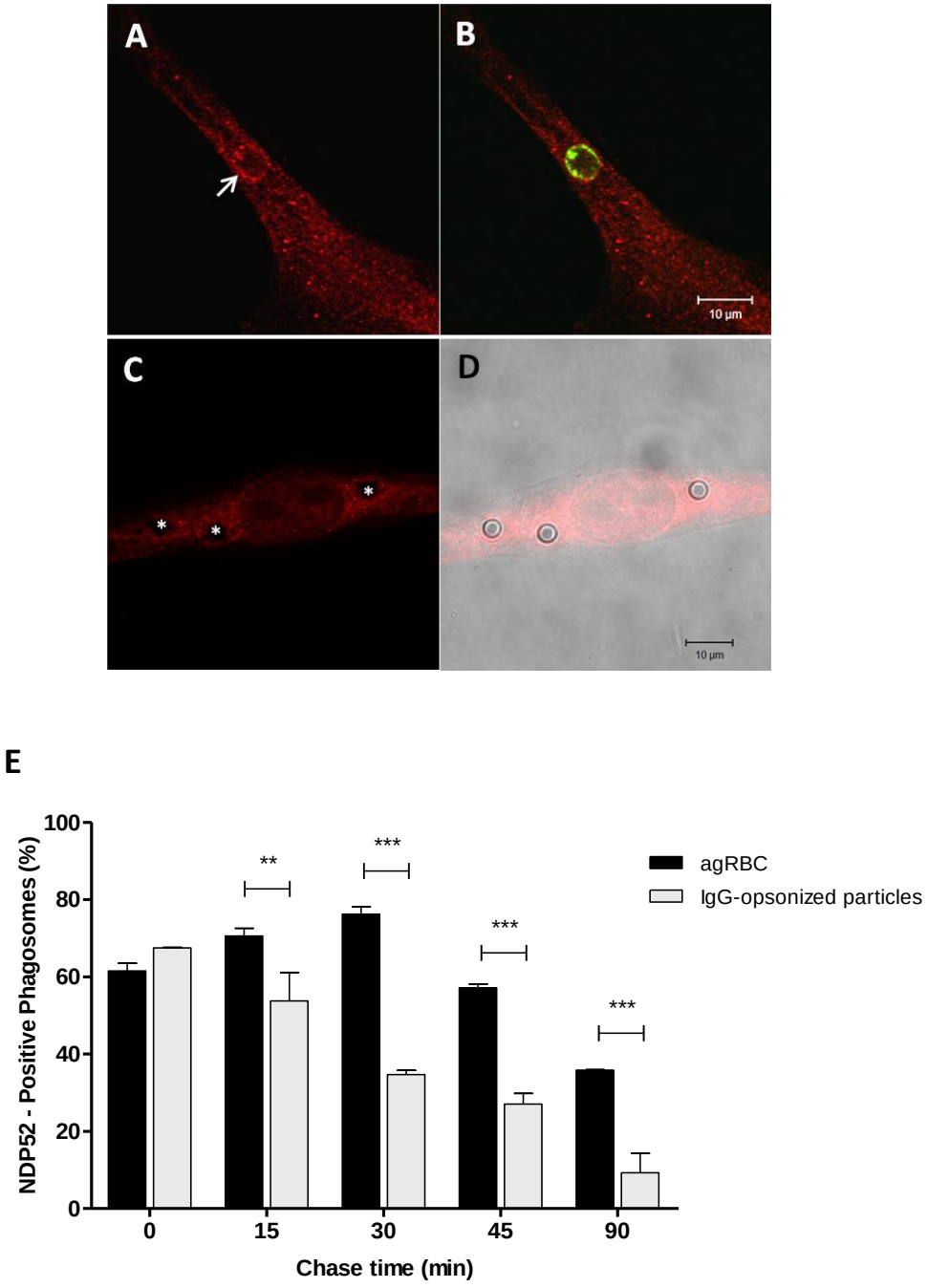


Figure 4.10. Association of NDP52 with phagosomal membranes. After pulse-chase experiments, cells were fixed, stained for NDP52 and analyzed under confocal microscopy. **A)** NDP52 staining of a cell containing a NDP52-positive phagosome at 30 min chase time. **B)** Corresponding merged image showing the internalized agRBC stained with CFSE. **C)** NDP52 staining of a cell containing three NDP52-negative phagosome at 30 min chase time. **D)** Corresponding merged image showing the internalized IgG-opsonized particles in DIC. Arrow indicates a positive phagosome and asterisk indicates a negative phagosome. Bars,10 µm. **E**) Quantification of NDP52 positive-phagosomes. Engineered SMC were exposed to the different phagocytic particles for 30 min and then chased for the time indicated in the graph abscissa. The values are means ± SEM of, at least, three independent experiments. At each time point, at least, 100 phagosomes were analyzed. **, $p < 0.01$; ***, $p < 0.001$ comparing differences between NDP52-acquisition by phagosomes with agRBC and with IgG-opsonized latex beads.

Likely NBR1, the cargo receptor NDP52 also interacts with p62; however, instead of directly bind to each other, they are independently recruited to autophagosomes, forming protein complexes (Cemma et al., 2011). To test whether NDP52 is also acquired by phagosomal membranes, we immunostained samples for this marker after pulse-chase experiments and confirmed that NDP52 was effectively acquired by phagosomes carrying both phagocytic particles, although with different kinetic patterns (Figure 4.10). Phagosomes containing IgG-opsonized particles showed a regular distribution of NDP52 over time, with almost 70% (67.46 ± 0.15%) of positive-phagosomes at 0min comparing to around 10% (9.30 ± 4.96%) at 90min chase. Beyond that, agRBC-containing phagosomes showed a delay on NDP52 association and dissociation compared with those carrying IgG-coated beads.

4.2.5. Some selective autophagy effectors are present in the phagocytic cups

Phagosome formation is preceded by a dynamic set of events that, ultimately, induce actin cytoskeleton rearrangement in order to support pseudopod extension at sites of particle engulfment. This reorganization yields a cup-shaped invagination or protrusion of the plasma membrane, named phagocytic cup (Lee et al., 2007). Such structure is enriched in actin filaments, which greatly contributes to generate the forces that alter the shape of the cell surface. Once all autophagy effectors tested in this work were acquired by phagosomes at very early stages of phagosome maturation, we investigated whether they were being acquired even before that, meaning by the phagocytic cups. For that, efferocytes were fed with agRBC or IgG-opsonized beads for 30min, fixed and stained for LC3-IIB, p62, NBR1, NDP52, as well as for cortical-actin (F-actin). Microscopic analysis revealed that LC3 and NDP52 were not exhibited in the phagocytic cup of any of the phagocytic particles offered to SMC; whereas NBR1 was present in the phagocytic cups of both particles. More selective, p62 was found only in the phagocytic cups of agRBC-fed SMC, suggesting that this protein may have a role in efferocytosis (Figure 4.11).

Altogether, our findings reinforce that the nature of the phagocytic targets dictates the differences in the interactions between phagosomes and autophagy effectors. Because p62 has shown the most exciting differences regarding the removal of dying cells in comparison with IgG-opsonized particles, a more detailed investigation about its functional relevance is mandatory to advance the characterization of the efferocytic process.

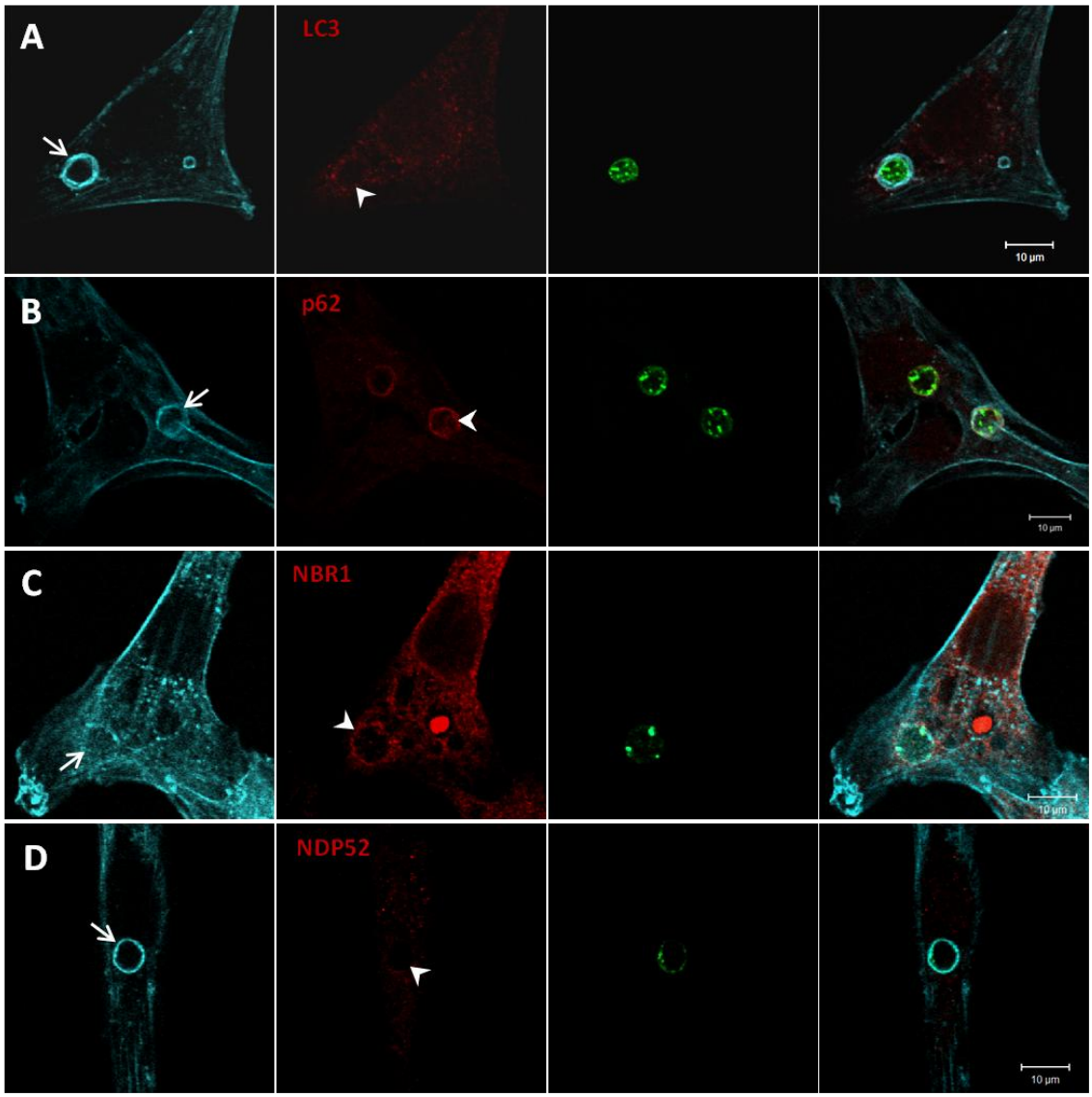


Figure 4.11. Association of autophagy effectors with the phagocytic cup of agRBC. SMC were fed with agRBC for 30min, fixed, and co-stained for actin filaments assembly using Cy5-phalloidin (in blue) and autophagy effectors (in red). **A**) Co-staining of actin-filament and LC3-IIB at the site of agRBC binding. **B**) Co-staining of actin-filament and p62 at the site of agRBC binding. **C**) Co-staining of actin-filament and NBR1 at the site of agRBC binding. **D**) Co-staining of actin-filament and NDP52 at the site of agRBC binding. Arrows and arrowheads indicate the phagocytic cups. Bars,10 µm.

4.2.6. p62 is not necessary for LC3 recruitment but its absence delays phagolysosome biogenesis

Regarding the residual levels of p62 detected in IgG-opsonized particles-containing phagosomes in contrast with the results obtained for apoptotic cells containing phagosomes, we decided to explore further the role of the signaling adapter p62 only in efferocytosis (Moscat et al., 2007). For this purpose we got bone marrow-derived macrophages (BMDM) from wild-type and p62 knockout (KO) mice and further analyzed LC3 acquisition and phagosomal maturation. According to our results, p62 is not important for the recruitment of LC3 to phagosomal

membranes. However, unlike phagosomes in wild-type macrophages, p62-deficient cells did not proper dissociate LC3 from phagosomes, showing quite constant levels of this marker over time (Figure 4.12A). Furthermore, BMDM lacking p62 affected phagolysosome biogenesis, judged by the acquisition of the lysosome membrane marker LAMP-1. As we can see in Figure 4.12B, cells from p62-KO animals demonstrated a delay in reaching the lysosomes compared to wild-type cells.

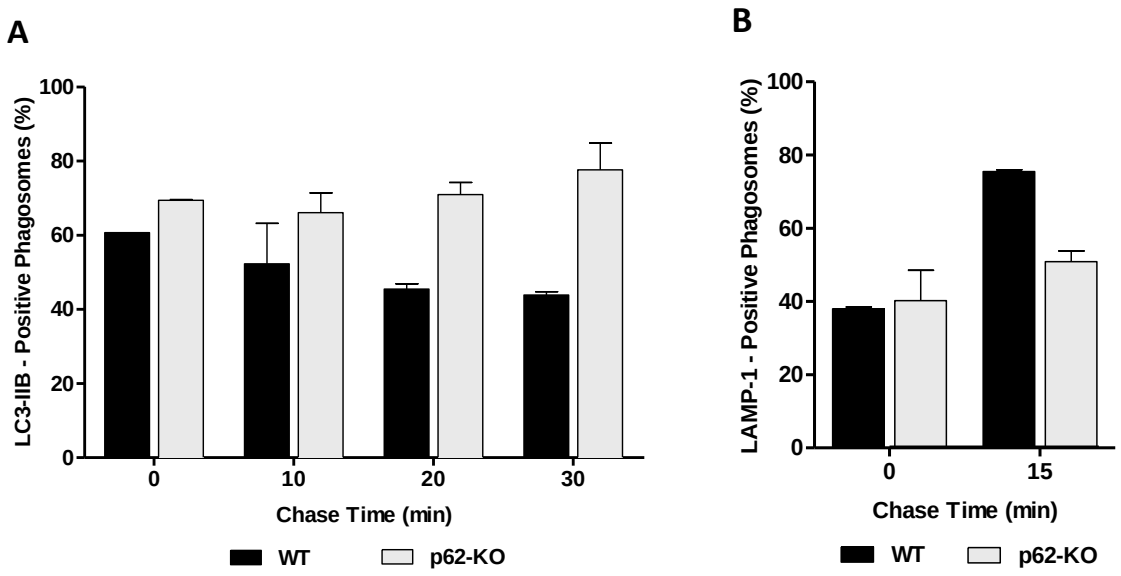


Figure 4.12. Requirement of p62 for LAP and phagolysosome formation in efferocytosis. BMDM from WT (black bars) or p62-KO (grey bars) mice were challenged with agRBC for 30 min and then chased for the time indicated in the graph abscissa. LAP was assessed by the quantification of LC3-positive phagosomes (**A**), while phagolysosome formation by the quantification of LAMP-1-positive phagosomes (**B**). The results are mean ± SD of two independent experiments.

4.3. Discussion

The quick removal of dying cells is a key final step, if not the ultimate goal of the apoptotic cell program. Over the last years, significant progress has been made in understanding the events involved in prompt efferocytosis, although our knowledge about the stages downstream from internalization remains unclear (Hochreiter-Hufford and Ravichandran, 2013). We have previous reported that the interactions between dying cells-containing phagosomes with components of the endocytic pathway are different from the behavior of phagosomes containing IgG-opsonized particles, the best understood phagocytic model (Viegas et al., 2012). Such observation stated that the nature of the phagocytic target controls the vesicular trafficking required for phagolysosome formation. In this study we aimed at clarifying some more discrepancies related to the maturation of phagosomes carrying different phagocytic particles, focusing on the role of autophagy effectors for the successful completion of the efferocytic process.

Sanjuan and collaborators were the first to identify LC3 as an important participant in the removal of cargo contained into single-membrane phagosomes (Sanjuan et al., 2007). From there, LAP has been suggested to contribute to the degradation of numerous phagocytic cargoes, including dying cells (Martinez et al., 2011). Our results showed that LC3 is not displayed on the phagocytic cups formed by agRBC and IgG-opsonized particles, but is quickly translocated to the nascent phagosomes containing the different internalized cargoes, demonstrating similar kinetics of association and dissociation throughout the degradative pathway. This outcome strengthens that LAP certainly impacts upon the immune system, by act as a defense mechanism against autoimmune and inflammatory responses generated by defective efferocytosis (Erwig and Henson, 2007; Kim and Overholtzer, 2013b; Martinez et al., 2013). Furthermore, the conjugation of LC3 to phagosomes containing agRBC proved to be totally independent of the nutritional status of the phagocytes, reinforcing that LAP does not relies on the inducer machinery of autophagy, composed by the ULK-complex and mTOR, upstream regulators that are sensitive to the environmental conditions of nutrients and metabolic stress.

The translocation of LC3 to dead cells-containing phagosomes was previously demonstrated to be dependent on the engagement of the PtdSer-receptor TIM4, and does not seems to occur in the absence of Atg5, Atg7 and Beclin1, regulators of the classical autophagic machinery (Martinez et al., 2011). Interestingly, Atg7-deficient macrophages produce increased levels of pro-inflammatory cytokines and significantly less anti-inflammatory mediators when fed with cells undergoing apoptosis (Martinez et al., 2011). Very elegant *in vivo* data using mouse model of advanced atherosclerosis, characterized by sustained inflammation, have shown that

macrophage lacking Atg5 enhances apoptosis and NADPH oxidase-mediated oxidative stress, simultaneously rendering the apoptotic cells less well recognized by efferocytes, which indicates that autophagy itself is occurring, but also LAP is an operational process in atherogenesis (Liao et al., 2012). On the other hand, the exposure of self-autoantigens resulting from the leakage of secondary necrotic cells was reported to form DNA-immune complexes that are targeted by anti-nuclear antibodies. The internalization of this immune reactive complex, in turn, requires the engagement of FcγR and also triggers the recruitment of autophagic elements in order to restrict autoimmunity (Henault et al., 2012). Thus, it becomes clear that Fc-mediated phagocytosis is also particular involved in LAP and subsequently in scenarios of non-resolving inflammation, such as seen in atherosclerosis (Tabas, 2010). Altogether, these recent findings justify, at least in part, our specific interest in these phagocytic models.

To further understand the functional relevance of the recruitment of LC3 to phagosomes containing our selected phagocytic models, pharmacological drugs were used in order to inhibit or induce the canonical autophagy machinery. Indeed, the effects of such modulation were reflected in LAP and phagosome maturation distinctively according to the type of phagosome. By inhibiting the autophagic flux using Jak3 inhibitor VI we found that phagosomes containing dying cells presented a delay in LC3 association, while IgG-opsonized particles induced a delay in LC3 dissociation from the membrane of their containing phagosomes. Since the inhibition of autophagy directly affected LAP we conclude that the unknown target of Jak3 Inhibitor VI is likely a protein shared by both machineries, otherwise such effect would not be so impressive. A good candidate could be the cysteine protease Atg4, since this protein plays a dual role in autophagy, catalyzing simultaneously the conjugation of LC3 to the lipid phosphatidylethanolamine (PE) during LC3 activation and in the deconjugation of LC3-PE after autophagolysosome formation (Yu et al., 2012). In addition, although Jak3 inhibitor VI has differently affected the recruitment of LC3 to phagosomes, the inhibition of autophagy was ultimately translated into delays in phagolysosome formation for both types of internalized particles, demonstrating the role of LAP in facilitates phagosome maturation and probably cargo degradation.

Curiously, when we increase the autophagic flux by inducing autophagosome formation using Nortriptyline we were not able to see such remarkable changes, only a slight delay in LC3 dissociation at later stages of phagosomal maturation. Most probably, the machinery shared between LAP and autophagy was sequestered by canonical autophagy, which was clearly favored under these circumstances. The elements involved in this competition could be the regulatory machinery of autophagy (e.g. Atg5, Atg7 or Beclin1), but also endocytic components (e.g. ESCRT,

HOPS and SNAREs complexes) and, of course, lysosomes (Funderburk et al., 2010; Kim et al., 2012; Martinez et al., 2011). If LC3 association with phagosomes is supposed to assist phagolysosome formation and Nortriptyline did not accelerated the process it is easy to understand why the maturation was not much affected as well, judged by the fact that LBPA was similarly acquired by phagosomes from treated and untreated cells.

The requirement of members from the selective machinery of autophagy was never addressed in the context of LC3 translocation to single-membrane vesicles (LAP). Ubiquitination is a classic signal for target cytosolic substrates for autophagic degradation; whereas in endocytosis this process was described to be required for formation of acidic multivesicular structures and sorting of endosomal proteins into intraluminal vesicles. The sorting of ubiquitinated proteins coordinated by the ESCRT complex are thought to be important in receptor down-regulation and antigen presentation, which is central to the function of phagosomes (Katzmann et al., 2002). Noteworthy, the activation of FcγR was reported to trigger protein ubiquitination, whereas normal FcR clearance from phagosomes also requires ubiquitination (Booth et al., 2002; Lee et al., 2005). In this scenario is not surprising that we have found the presence of ubiquitinated substrates on the membrane of both types of phagosomes. Actually, our data regarding the kinetic profile of this marker fits well with the literature, since the peak values found for poly-ubiquitinated substrates coincides with the time that is supposed the phagosomes interact with MVB. Additionally, phagosomes carrying IgG-opsonized particles have lost ubiquitinated components faster than agRBC-containing phagosomes, what could be explained by the fact that phagosomes loaded with IgG-coated particles mature faster compared with phagosomes loaded with dying cells (Viegas et al., 2012).

Since ubiquitination occurs as part of the host autophagic response to tag potential targets and because recent advances have indicated that adaptor proteins serve to connect ubiquitin with LC3 in order to promote autophagosome formation and selectivity of substrates in canonical autophagy, we further evaluated if these autophagy effectors are also involved in LAP (Raasi et al., 2005). For our entire surprise, the autophagy receptors p62, NBR1 and NDP52 were recruited to phagosomes containing agRBC and IgG-opsonized particles, although with some divergences in their pattern of acquisition over time. The differences found may be due to the signals triggered by the engagement of distinct phagocytic receptors, confirming again that the essence of the phagocytic target is important to outline the process of interaction with the cellular pathways that ultimately lead to the degradation of phagosomal contents. The residual levels of p62 recruited to phagosomes carrying IgG-opsonized cargo suggest that this protein apparently has a

negligible role in FcR-mediated phagocytosis. On the other hand, p62 rapidly associated with the membrane of nascent phagosomes containing dying cells and was even present on the phagocytic cup formed around the attached agRBC, implying that it could be specifically involved in efferocytosis. By using p62-deficient cells we proved that despite having a role in LC3 phagosomal dissociation and subsequently in phagolysosome biogenesis, this signaling adapter is not connected to LC3 recruitment at all. Based on the involvement of p62 in several cellular signaling process that seem unrelated to autophagy, it might be possible that p62 is implicated in the recognition of cells undergoing apoptosis, or simply works as a component that ensures LAP specificity (Bitto et al., 2014; Lee et al., 2010b; Pankiv et al., 2007). So, we can definitely exclude the possibility that p62 act as an adaptor protein promoting LC3 recruitment.

The adaptor protein NBR1 was recruited to the phagocytic cups of both particles; meanwhile NDP52 was not required at this early stage of phagosome formation in none of the particles we used. In contrast to others autophagy receptors that non-specifically bind to multiple Atg8/LC3 family members, NDP52 binds with more affinity to the LC3C isoform (von Muhlinen et al., 2012). Thus, in line with the involvement of NDP52 in LAP we may intuitively assume that the isoform LC3C must also perform a function in this non-canonical requirement of autophagy machinery. The results found for NBR1 and NDP52 also reinforce the principle that different cargoes distinctively modulate the phagocytic response, since the recruitment of each varied according to the target involved. Besides, although we were able to clarify some temporal issues about the recruitment of autophagy members to phagosomal membranes, their main function, the hierarchical requirement and the possibility of cooperativity remains to be unraveled.

In summary, this data show for the first time the recruitment of the selective machinery of autophagy in LAP, a non-canonical LC3 pathway, thus enhancing the number of players that can be target in therapies aimed at the resolution of inflammation and autoimmunity triggered by insufficient or impaired efferocytosis. Nevertheless, many questions still remain open, and elucidation of their answers is currently one of the most important tasks in the field.

Chapter V

General discussion and future perspectives

General discussion and future perspectives

Efferocytosis is one of the oldest fundamental cellular processes in multicellular organisms and, possibly, it represents the final aim of the apoptotic cell program. This very efficient mechanism of removal is controlled by the coordinated action of dying cells and phagocytes, whose interaction modulates signaling pathways underlying recognition, binding, engulfment, digestion and the responses that keep this process immunologically silent (Hochreiter-Hufford and Ravichandran, 2013; Poon et al., 2014). Indeed, even animals lacking adaptative immune responses do not show anything resembling inflammation or autoimmunity in the presence of uncleared dying cells (Gardai et al., 2006; Mochizuki et al., 1996). However, a defective clearance (failure or delay) of corpses brought severe complications when vertebrates increased their immune complexity to deal with pathogens that, in turn, have developed parallel ways to avoid detection, exploiting this new found niche in order to survive. Therefore, the evolution of the immune system is a direct consequence of the selection pressure exerted by microbes on multicellular organisms, increasing complexity in such way that expanded the chances of eliciting autoimmune reactions (Green et al., 2009; Gregory, 2009).

Unengulfed apoptotic cells have the propensity to leak their cellular contents over time, resulting in the exposure of self-antigens and a break in the immune tolerance (Savill and Fadok, 2000). Thus, deficient removal of apoptotic cells coupled with impaired phagosome maturation have been linked to the onset of several autoimmune and inflammatory human disorders, such as atherosclerosis, neurodegenerative diseases and lupus erythematosus (Elliott and Ravichandran, 2010; Moore and Tabas, 2011; Nagata et al., 2010). In this context, the search for therapies based on the modulation of the efferocytic response makes it imperative a detailed view about the key players, molecular mechanisms and pathways involved. Although much attention has been focused on the role of apoptotic cell-derived ligands and phagocyte receptors as well as to the post-engulfment signals mediating efferocytosis, very few is known about the processing of dying cells inside the phagosome, especially into mammalian systems (Erwig et al., 2006). Therefore, the challenge in this thesis was to advance our rudimentary knowledge about efferocytosis, focusing on steps downstream from internalization and in some unique aspects that differentiate the removal of apoptotic cells from the better understood phagocytic process,

the Fc-mediated phagocytosis. Moreover, by working with a cell type actively involved with the apoptotic cell clearance in blood vessels, i.e. smooth muscle cells, we could assess *in vitro* efferocytosis in cells that play a very important role in atherosclerosis, one of the diseases that kills more nowadays and a topic of great interest in our group (Kolb et al., 2007; Moore and Tabas, 2011; Schrijvers et al., 2005).

In this work we were able to systematically characterize the process of maturation of phagosomes containing agRBC and IgG-opsonized particles. By comparing the kinetic of interaction between the distinct types of phagosomes with components from different stages of the endocytic pathway, we found that dying cells induced a slower process of maturation compared with the process triggered by IgG-coated particles. For all markers analyzed: EEA-1, YFP-GL-GPI, LBPA, Lysotracker and Rhodamine-Dextran (used to identify lysosomes) the result was the same, stating that the nature of the phagocytic target modulates the vesicular trafficking involved in cargo degradation. If we assume that in mammals the accumulation of dying cells is very rare and that their immune responses do not evolved to cope with this specific problem, it is not surprising that phagosomes containing agRBC have shown the slowest process of maturation. Maybe our system is not fully prepared yet for such demand. The loss of membrane phospholipid asymmetry and early exposure of PtdSer have been documented on many different cell types undergoing apoptosis and the mechanisms mediating this membrane alteration are an area of active study (Fadok et al., 2001; Fadok et al., 1992; Fadok et al., 1998c; Ravichandran, 2010). The role of this phospholipid in promoting apoptotic cell internalization is still not completely clear, but seems that PtdSer alone is insufficient to mediate efferocytosis (Bratton and Henson, 2008; Hoffmann et al., 2001; Somersan and Bhardwaj, 2001). Moreover, according to our findings even if we increase the levels of PtdSer on the cell surface of apoptotic cells, it is still not enough to improve binding and engulfment, or influence phagosome maturation, suggesting that beyond a certain threshold more PtdSer does not affect efferocytosis.

In addition, we have shown that the endocytic machinery is not the only responsible for the processing of phagosomal contents, and that autophagic elements are more implicated in efferocytosis than previously thought. The link between phagocytosis and autophagy is recent and the non-canonical recruitment of the autophagic marker LC3 to phagosomes was suggested to promote phagosome maturation by accelerating lysosomal fusion, acidification and degradation of the cargo (Kim and Overholtzer, 2013a; Martinez et al., 2011; Sanjuan et al., 2007). We were able to go further and have identified effectors previously reported in the classic machinery of autophagy on the phagosomal membrane of our models of phagocytic particles.

Dying cells and IgG-opsonized particles rapidly induced the translocation of LC3 to their phagosomes, which was proved to be susceptible to the manipulation of the canonical autophagic machinery. In turn, LAP modulation has interfered in the communication between phagosomes and endocytic compartments, so disturbing phagosome maturation. Intriguingly, the ubiquitination of phagosomal proteins was observed as well as the recruitment of autophagy receptors, which should not be a mere coincidence, since this is exactly the same machinery used to confer autophagy selectivity of substrates (e.g. aggrephagy, mitophagy, xenophagy, etc) (Rogov et al., 2014; Shaid et al., 2013). Again, the different phagocytic targets have determined selectivity for the adaptors proteins recruited in each circumstance, and also induced differences regarding kinetics of association and dissociation with the effectors investigated. The protein p62 has shown the most striking results, being committed more to efferocytosis rather than to Fc-mediated phagocytosis. Although we have evidenced that p62 is not involved in LC3 recruitment, its role remains be further investigated, since BMDM lacking this signaling adapter have shown defective phagosome maturation.

Based on the work compiled in this thesis, the following conclusions have been drawn:

- Phagosomes containing IgG-opsonized particles mature faster than phagosomes containing dying cells;
- Aged RBC membrane enrichment in PtdSer does not affect phagocytosis or phagosomal maturation;
- LC3-IIB is translocated to phagosomal membranes and is required for phagosome maturation;
- Both phagocytic targets induce the ubiquitination of phagosomal components;
- The autophagy adaptors p62, NBR1 and NDP52 are recruited by the studied phagosomes with a specific demand and dissimilar kinetic of association and dissociation over time;
- The protein p62 is not involved in LC3 recruitment to phagosomal membranes in efferocytosis.

Finally, the data presented in this thesis has expanded our knowledge concerning the later stages of efferocytosis, showing that the molecular cascades controlling this process are much more sophisticated than previously thought. We hope this work will contribute to broaden the perspectives in the field, helping in the identification of host molecular targets for drug development, raising the possibility that patients might benefit from treatments that aim both endocytic and LAP machineries, in order to accelerate apoptotic cell degradation, as a strategy to fight human pathologies caused by defective efferocytosis.

Appendix

This section contains the supplemental information regarding the control experiments of some results presented in the Chapter IV of this thesis.

Supplemental Figure 1:

Autophagy, under normal physiological conditions, has a vital role in the maintenance of the amino acid pool during cellular starvation, thus protecting cells by managing stressful conditions to keep the energetic balance (Cecconi and Levine, 2008). Because our methodology for the phagocytic assays to further access LAP requires the use of serum-free medium, we first investigated the effect of the absence of serum in canonical autophagy. For that, adherent SMC were kept in the presence or absence of serum for different time points. We were able to observe that after 30 min of amino acid starvation the relative number of autophagosomes and/or autophagolysosomes increased comparing starved and fed SMC, judged by the higher number of punctuated LC3 structures (Figure S1). The autophagic activity in serum-deprived cells continued to increase until 60min of starvation; then the rates start to decline, likely because cells have reached the homeostatic balance (data not shown) (Mizushima and Yoshimori, 2007).

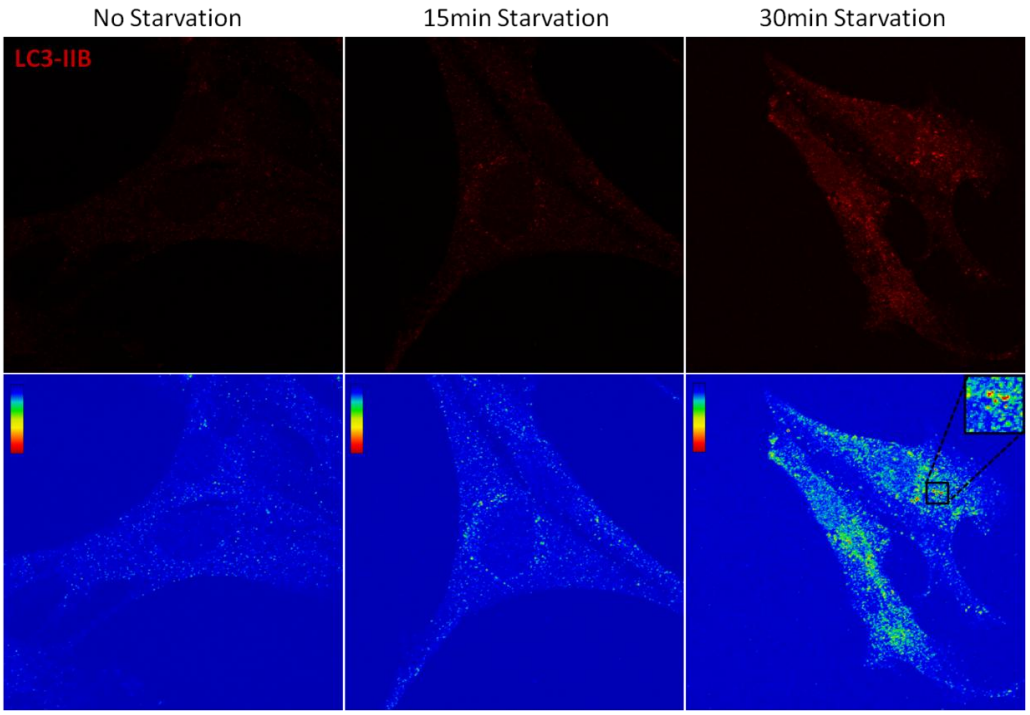


Figure S1. Canonical autophagy in SMC is affected by serum-starvation. SMC were untreated (no starvation) or treated (starvation) during 15 and 30min in a serum-free medium, followed by fixation and immunostaining for LC3-IIB. The images were converted into a color-coded based scale representing increasing fluorescence intensity using the software ImageJ. Hotter colors indicate the most intense fluorescence, while cooler colors represent the weakest fluorescence intensity.

Supplemental Figure 2:

To determine the effect of autophagy inhibition in LAP we first validated the role of Jak3 inhibitor VI in canonical autophagy itself. This compound is characterized by suppress autophagosome formation without affecting the PI3-kinases class-I or -III (Farkas et al., 2011). The activity of PI3K class-I is crucial to phagocytosis, while class-III is essential to phagosome maturation (Vieira et al., 2001), whose activities are necessary for LAP regulation. Curiously, the most commonly used pharmacological approaches to inhibit autophagy *in vitro* involves the use of PI3K inhibitors such as Wortmannin, LY294002 or 3-MA (Mizushima et al., 2010). To test Jak3 inhibitor VI activity, SMC were starved using HBSS for 3h to improve LC3 signal (which become easier to visualize the differences) and then treated with Jak3 inhibitor VI (5µM) for 30min. By comparing LC3 puncta formation in fed SMC (control) and SMC starved with HBSS in the presence or absence of Jak3 Inhibitor VI, we observed that after 30 min of incubation with the compound the HBSS starved cells almost entirely suppressed the punctuate distribution of LC3, confirming that the autophagic flux was being negatively affected (Figure S2).

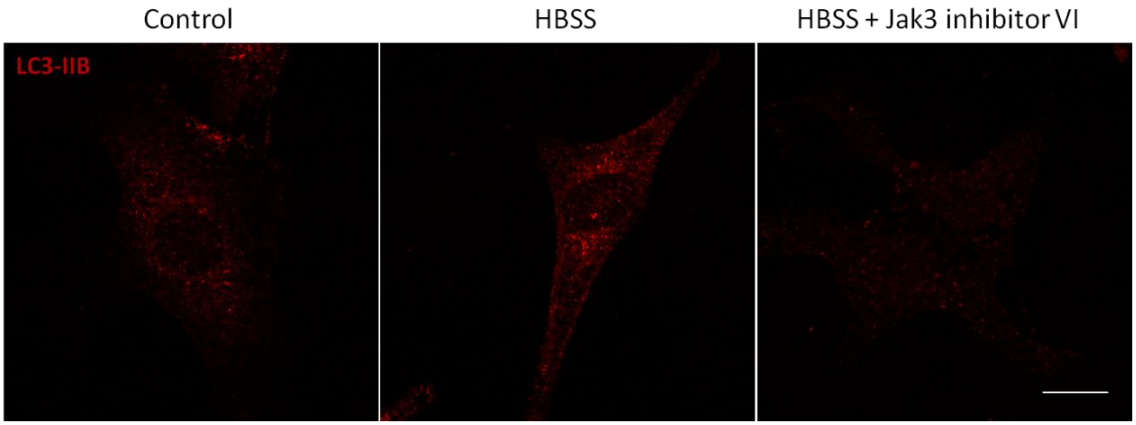


Figure S2. Effect of Jak3 inhibitor VI in canonical autophagy. SMC were untreated (control) or starved in HBSS for 3h in the presence or absence of Jak3 inhibitor VI (5µM) for 30min. Then cells were fixed and the autophagic flux was assessed by immunostaining cells for LC3-IIB. Bar, 10µm.

Supplemental Figure 3:

Autophagy is a dynamic process, so static measurements are not appropriate. Thus, the accumulation of autophagosomes is not always indicative of autophagy induction and may represent either the increased generation of autophagosomes and/or a block in autophagosome maturation and degradation (autophagic flux). In order to induce autophagy we have used Nortriptyline, an autophagic inductor whose action is similar to starvation-induced autophagy (Sundaramurthy et al., 2013). To evaluate the effect of autophagy induction in LAP we first validated the effect of Nortriptyline in canonical autophagy by examining LC3 puncta formation in SMC untreated or treated with Nortriptyline (10uM) for different time points in the presence or absence of the lysosomotropic reagent Chloroquine (Shiratsuchi et al., 2004). Cells cultured with choroquine, an agent that impairs lysosomal acidification, accumulate LC3-II even under normal (non starved) conditions because turnover of LC3-II by basal autophagy is blocked (Tanida et al., 2005). We found that after 30 min of incubation with the drug was already possible to see an increased autophagic flux, indicated by the higher relative number and intensity of LC3 structures (autophagosomes/ autophagolysosomes) in cells treated with Nortriptyline (Figure S3). The effect was more pronounced when cells were co-incubated with chloroquine (100µM) for 60min, visualized by the bigger size and fluorescent intensity of the vesicles, which confirm that canonical autophagy was induced by increase autophagosome formation.

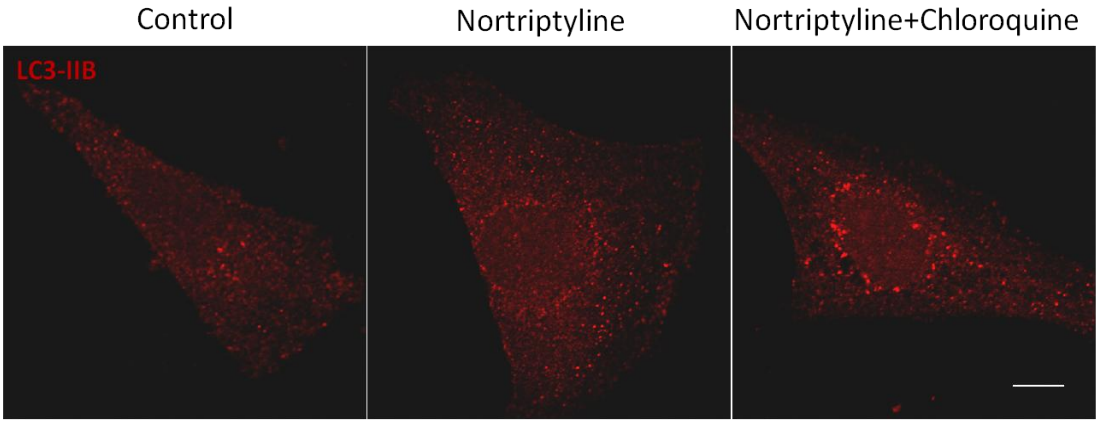


Figure S3. Effect of Nortriptyline in canonical autophagy. SMC were untreated (control) or treated with Nortriptyline (5µM) for 30min in the presence or absence of chloroquine (100µM) for 60min. Then cells were fixed and the autophagic flux was assessed by immunostaining cells for LC3-IIB. Bar, 10µm.

References

Aderem, A., and Underhill, D.M. (1999). Mechanisms of phagocytosis in macrophages. Annual review of immunology *17*, 593-623.

Aderem, A.A., Wright, S.D., Silverstein, S.C., and Cohn, Z.A. (1985). Ligated complement receptors do not activate the arachidonic acid cascade in resident peritoneal macrophages. The Journal of experimental medicine *161*, 617-622.

Akira, S., and Takeda, K. (2004). Toll-like receptor signalling. Nature reviews Immunology *4*, 499-511.

Albert, M.L., Pearce, S.F., Francisco, L.M., Sauter, B., Roy, P., Silverstein, R.L., and Bhardwaj, N. (1998). Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. The Journal of experimental medicine *188*, 1359-1368.

Allen, L.A., and Aderem, A. (1996). Molecular definition of distinct cytoskeletal structures involved in complement- and Fc receptor-mediated phagocytosis in macrophages. The Journal of experimental medicine *184*, 627-637.

Almendinger, J., Doukoumetzidis, K., Kinchen, J.M., Kaech, A., Ravichandran, K.S., and Hengartner, M.O. (2011). A conserved role for SNX9-family members in the regulation of phagosome maturation during engulfment of apoptotic cells. PloS one *6*, e18325.

Ambrose, J.A., Tannenbaum, M.A., Alexopoulos, D., Hjemdahl-Monsen, C.E., Leavy, J., Weiss, M., Borrico, S., Gorlin, R., and Fuster, V. (1988). Angiographic progression of coronary artery disease and the development of myocardial infarction. Journal of the American College of Cardiology *12*, 56-62.

Anderson, C.L., Shen, L., Eicher, D.M., Wewers, M.D., and Gill, J.K. (1990). Phagocytosis mediated by three distinct Fc gamma receptor classes on human leukocytes. The Journal of experimental medicine *171*, 1333-1345.

Andreassi, M.G. (2009). Metabolic syndrome, diabetes and atherosclerosis: influence of gene-environment interaction. Mutation research *667*, 35-43.

Asano, K., Miwa, M., Miwa, K., Hanayama, R., Nagase, H., Nagata, S., and Tanaka, M. (2004). Masking of phosphatidylserine inhibits apoptotic cell engulfment and induces autoantibody production in mice. The Journal of experimental medicine *200*, 459-467.

Azuma, Y., Inami, Y., and Matsumoto, K. (2002). Alterations in cell surface phosphatidylserine and sugar chains during apoptosis and their time-dependent role in phagocytosis by macrophages. Biological & pharmaceutical bulletin *25*, 1277-1281.

Bartlett, G.R. (1959). Phosphorus assay in column chromatography. J Biol Chem *234*, 466-468.

Beertsen, W., Willenborg, M., Everts, V., Zirogianni, A., Podschun, R., Schroder, B., Eskelinen, E.L., and Saftig, P. (2008). Impaired phagosomal maturation in neutrophils leads to periodontitis in lysosomal-associated membrane protein-2 knockout mice. J Immunol *180*, 475-482.

References

Behrends, C., and Harper, J.W. (2011). Constructing and decoding unconventional ubiquitin chains. Nature structural & molecular biology *18*, 520-528.

Bellone, M., Iezzi, G., Rovere, P., Galati, G., Ronchetti, A., Protti, M.P., Davoust, J., Rugarli, C., and Manfredi, A.A. (1997). Processing of engulfed apoptotic bodies yields T cell epitopes. J Immunol *159*, 5391-5399.

Bennett, M.R., Gibson, D.F., Schwartz, S.M., and Tait, J.F. (1995). Binding and phagocytosis of apoptotic vascular smooth muscle cells is mediated in part by exposure of phosphatidylserine. Circulation research *77*, 1136-1142.

Berg, C.P., Engels, I.H., Rothbart, A., Lauber, K., Renz, A., Schlosser, S.F., Schulze-Osthoff, K., and Wesselborg, S. (2001). Human mature red blood cells express caspase-3 and caspase-8, but are devoid of mitochondrial regulators of apoptosis. Cell death and differentiation *8*, 1197-1206.

Bernales, S., McDonald, K.L., and Walter, P. (2006). Autophagy counterbalances endoplasmic reticulum expansion during the unfolded protein response. PLoS biology *4*, e423.

Beutler, B.A. (2009). TLRs and innate immunity. Blood *113*, 1399-1407.

Beyenbach, K.W., and Wieczorek, H. (2006). The V-type H+ ATPase: molecular structure and function, physiological roles and regulation. The Journal of experimental biology *209*, 577-589.

Binker, M.G., Cosen-Binker, L.I., Terebiznik, M.R., Mallo, G.V., McCaw, S.E., Eskelinen, E.L., Willenborg, M., Brumell, J.H., Saftig, P., Grinstein, S*., et al.* (2007). Arrested maturation of Neisseria-containing phagosomes in the absence of the lysosome-associated membrane proteins, LAMP-1 and LAMP-2. Cellular microbiology *9*, 2153-2166.

Bitto, A., Lerner, C.A., Nacarelli, T., Crowe, E., Torres, C., and Sell, C. (2014). p62/SQSTM1 at the interface of aging, autophagy, and disease. Age (Dordr).

Bjorkoy, G., Lamark, T., Brech, A., Outzen, H., Perander, M., Overvatn, A., Stenmark, H., and Johansen, T. (2005). p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. The Journal of cell biology *171*, 603-614.

Blander, J.M., and Medzhitov, R. (2004). Regulation of phagosome maturation by signals from toll-like receptors. Science *304*, 1014-1018.

Blander, J.M., and Medzhitov, R. (2006). On regulation of phagosome maturation and antigen presentation. Nature immunology *7*, 1029-1035.

Bohdanowicz, M., and Grinstein, S. (2010). Vesicular traffic: a Rab SANDwich. Current biology : CB *20*, R311-314.

Bonifacino, J.S., and Glick, B.S. (2004). The mechanisms of vesicle budding and fusion. Cell *116*, 153-166.

Booth, J.W., Kim, M.K., Jankowski, A., Schreiber, A.D., and Grinstein, S. (2002). Contrasting requirements for ubiquitylation during Fc receptor-mediated endocytosis and phagocytosis. The EMBO journal *21*, 251-258.

Borisenko, G.G., Matsura, T., Liu, S.X., Tyurin, V.A., Jianfei, J., Serinkan, F.B., and Kagan, V.E. (2003). Macrophage recognition of externalized phosphatidylserine and phagocytosis of apoptotic Jurkat cells--existence of a threshold. Archives of biochemistry and biophysics *413*, 41-52.

Bortner, C.D., and Cidlowski, J.A. (2002). Apoptotic volume decrease and the incredible shrinking cell. Cell death and differentiation *9*, 1307-1310.

Botelho, R.J., and Grinstein, S. (2011). Phagocytosis. Current biology : CB *21*, R533-538.

Botelho, R.J., Hackam, D.J., Schreiber, A.D., and Grinstein, S. (2000a). Role of COPI in phagosome maturation. The Journal of biological chemistry *275*, 15717-15727.

Botelho, R.J., Teruel, M., Dierckman, R., Anderson, R., Wells, A., York, J.D., Meyer, T., and Grinstein, S. (2000b). Localized biphasic changes in phosphatidylinositol-4,5-bisphosphate at sites of phagocytosis. The Journal of cell biology *151*, 1353-1368.

Bratosin, D., Estaquier, J., Petit, F., Arnoult, D., Quatannens, B., Tissier, J.P., Slomianny, C., Sartiaux, C., Alonso, C., Huart, J.J*., et al.* (2001). Programmed cell death in mature erythrocytes: a model for investigating death effector pathways operating in the absence of mitochondria. Cell death and differentiation *8*, 1143-1156.

Bratton, D.L., and Henson, P.M. (2008). Apoptotic cell recognition: will the real phosphatidylserine receptor(s) please stand up? Current biology : CB *18*, R76-79.

Brocker, C., Kuhlee, A., Gatsogiannis, C., Balderhaar, H.J., Honscher, C., Engelbrecht-Vandre, S., Ungermann, C., and Raunser, S. (2012). Molecular architecture of the multisubunit homotypic fusion and vacuole protein sorting (HOPS) tethering complex. Proceedings of the National Academy of Sciences of the United States of America *109*, 1991-1996.

Brown, M.S., and Goldstein, J.L. (1983). Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. Annual review of biochemistry *52*, 223-261.

Brugnera, E., Haney, L., Grimsley, C., Lu, M., Walk, S.F., Tosello-Trampont, A.C., Macara, I.G., Madhani, H., Fink, G.R., and Ravichandran, K.S. (2002). Unconventional Rac-GEF activity is mediated through the Dock180-ELMO complex. Nature cell biology *4*, 574-582.

Bucci, C., Parton, R.G., Mather, I.H., Stunnenberg, H., Simons, K., Hoflack, B., and Zerial, M. (1992). The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway. Cell *70*, 715-728.

Burman, C., and Ktistakis, N.T. (2010). Autophagosome formation in mammalian cells. Seminars in immunopathology *32*, 397-413.

Bursch, W., Taper, H.S., Lauer, B., and Schulte-Hermann, R. (1985). Quantitative histological and histochemical studies on the occurrence and stages of controlled cell death (apoptosis) during regression of rat liver hyperplasia. Virchows Archiv B, Cell pathology including molecular pathology *50*, 153-166.

References

Cai, S.Y., Wang, Y.Y., and Yao, Z.J. (1994). Engineered bacterial Fc receptors. Science in China Series B, Chemistry, life sciences & earth sciences *37*, 454-461.

Cantalupo, G., Alifano, P., Roberti, V., Bruni, C.B., and Bucci, C. (2001). Rab-interacting lysosomal protein (RILP): the Rab7 effector required for transport to lysosomes. The EMBO journal *20*, 683-693.

Cao, W.M., Murao, K., Imachi, H., Hiramine, C., Abe, H., Yu, X., Dobashi, H., Wong, N.C., Takahara, J., and Ishida, T. (2004). Phosphatidylserine receptor cooperates with high-density lipoprotein receptor in recognition of apoptotic cells by thymic nurse cells. Journal of molecular endocrinology *32*, 497-505.

Cardoso, C.M., Jordao, L., and Vieira, O.V. (2010). Rab10 regulates phagosome maturation and its overexpression rescues Mycobacterium-containing phagosomes maturation. Traffic *11*, 221-235.

Cecconi, F., and Levine, B. (2008). The role of autophagy in mammalian development: cell makeover rather than cell death. Developmental cell *15*, 344-357.

Cemma, M., Kim, P.K., and Brumell, J.H. (2011). The ubiquitin-binding adaptor proteins p62/SQSTM1 and NDP52 are recruited independently to bacteria-associated microdomains to target Salmonella to the autophagy pathway. Autophagy *7*, 341-345.

Champion, J.A., and Mitragotri, S. (2006). Role of target geometry in phagocytosis. Proceedings of the National Academy of Sciences of the United States of America *103*, 4930-4934.

Chan, E.Y. (2009). mTORC1 phosphorylates the ULK1-mAtg13-FIP200 autophagy regulatory complex. Science signaling *2*, pe51.

Christoforidis, S., McBride, H.M., Burgoyne, R.D., and Zerial, M. (1999). The Rab5 effector EEA1 is a core component of endosome docking. Nature *397*, 621-625.

Ciechanover, A., Orian, A., and Schwartz, A.L. (2000). Ubiquitin-mediated proteolysis: biological regulation via destruction. BioEssays : news and reviews in molecular, cellular and developmental biology *22*, 442-451.

Clark, M.R. (1988). Senescence of red blood cells: progress and problems. Physiological reviews *68*, 503-554.

Coleman, M.L., Sahai, E.A., Yeo, M., Bosch, M., Dewar, A., and Olson, M.F. (2001). Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. Nature cell biology *3*, 339-345.

Collins, R.F., Schreiber, A.D., Grinstein, S., and Trimble, W.S. (2002). Syntaxins 13 and 7 function at distinct steps during phagocytosis. J Immunol *169*, 3250-3256.

Condeelis, J., and Pollard, J.W. (2006). Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. Cell *124*, 263-266.

Connor, J., Pak, C.C., and Schroit, A.J. (1994). Exposure of phosphatidylserine in the outer leaflet of human red blood cells. Relationship to cell density, cell age, and clearance by mononuclear cells. The Journal of biological chemistry *269*, 2399-2404.

Corrotte, M., Chasserot-Golaz, S., Huang, P., Du, G., Ktistakis, N.T., Frohman, M.A., Vitale, N., Bader, M.F., and Grant, N.J. (2006). Dynamics and function of phospholipase D and phosphatidic acid during phagocytosis. Traffic *7*, 365-377.

Cox, D., Tseng, C.C., Bjekic, G., and Greenberg, S. (1999). A requirement for phosphatidylinositol 3-kinase in pseudopod extension. The Journal of biological chemistry *274*, 1240-1247.

Cuervo, A.M. (2010). Chaperone-mediated autophagy: selectivity pays off. Trends in endocrinology and metabolism: TEM *21*, 142-150.

de Duve, C. (1974). The participation of lysosomes in the transformation of smooth muscle cells to foamy cells in the aorta of cholesterol-fed rabbits. Acta cardiologica *Suppl 20*, 9-25.

De Duve, C., and Wattiaux, R. (1966). Functions of lysosomes. Annual review of physiology *28*, 435-492.

deCathelineau, A.M., and Henson, P.M. (2003). The final step in programmed cell death: phagocytes carry apoptotic cells to the grave. Essays in biochemistry *39*, 105-117.

Deretic, V. (2006). Autophagy as an immune defense mechanism. Current opinion in immunology *18*, 375-382.

Deretic, V., and Levine, B. (2009). Autophagy, immunity, and microbial adaptations. Cell host & microbe *5*, 527-549.

Desjardins, M., Huber, L.A., Parton, R.G., and Griffiths, G. (1994). Biogenesis of phagolysosomes proceeds through a sequential series of interactions with the endocytic apparatus. The Journal of cell biology *124*, 677-688.

Desjardins, M., Nzala, N.N., Corsini, R., and Rondeau, C. (1997). Maturation of phagosomes is accompanied by changes in their fusion properties and size-selective acquisition of solute materials from endosomes. Journal of cell science *110 (Pt 18)*, 2303-2314.

Devitt, A., Parker, K.G., Ogden, C.A., Oldreive, C., Clay, M.F., Melville, L.A., Bellamy, C.O., Lacy-Hulbert, A., Gangloff, S.C., Goyert, S.M*., et al.* (2004). Persistence of apoptotic cells without autoimmune disease or inflammation in CD14-/- mice. The Journal of cell biology *167*, 1161-1170.

Dikic, I., Wakatsuki, S., and Walters, K.J. (2009). Ubiquitin-binding domains - from structures to functions. Nature reviews Molecular cell biology *10*, 659-671.

Dini, L. (1998). Endothelial liver cell recognition of apoptotic peripheral blood lymphocytes. Biochemical Society transactions *26*, 635-639.

Dini, L., Lentini, A., Diez, G.D., Rocha, M., Falasca, L., Serafino, L., and Vidal-Vanaclocha, F. (1995). Phagocytosis of apoptotic bodies by liver endothelial cells. Journal of cell science *108 (Pt 3)*, 967-973.

Doyle, S.E., O'Connell, R.M., Miranda, G.A., Vaidya, S.A., Chow, E.K., Liu, P.T., Suzuki, S., Suzuki, N., Modlin, R.L., Yeh, W.C.*, et al.* (2004). Toll-like receptors induce a phagocytic gene program through p38. The Journal of experimental medicine *199*, 81-90.

Dupont, N., Lacas-Gervais, S., Bertout, J., Paz, I., Freche, B., Van Nhieu, G.T., van der Goot, F.G., Sansonetti, P.J., and Lafont, F. (2009). Shigella phagocytic vacuolar membrane remnants participate in the cellular response to pathogen invasion and are regulated by autophagy. Cell host & microbe *6*, 137-149.

Dutta, P., Courties, G., Wei, Y., Leuschner, F., Gorbatov, R., Robbins, C.S., Iwamoto, Y., Thompson, B., Carlson, A.L., Heidt, T.*, et al.* (2012). Myocardial infarction accelerates atherosclerosis. Nature *487*, 325-329.

Edinger, A.L., and Thompson, C.B. (2004). Death by design: apoptosis, necrosis and autophagy. Current opinion in cell biology *16*, 663-669.

Eisenberg-Lerner, A., Bialik, S., Simon, H.U., and Kimchi, A. (2009). Life and death partners: apoptosis, autophagy and the cross-talk between them. Cell death and differentiation *16*, 966-975.

Elliott, M.R., Chekeni, F.B., Trampont, P.C., Lazarowski, E.R., Kadl, A., Walk, S.F., Park, D., Woodson, R.I., Ostankovich, M., Sharma, P.*, et al.* (2009). Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. Nature *461*, 282-286.

Elliott, M.R., and Ravichandran, K.S. (2010). Clearance of apoptotic cells: implications in health and disease. The Journal of cell biology *189*, 1059-1070.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. Toxicologic pathology *35*, 495-516.

Erwig, L.P., and Henson, P.M. (2007). Immunological consequences of apoptotic cell phagocytosis. The American journal of pathology *171*, 2-8.

Erwig, L.P., and Henson, P.M. (2008). Clearance of apoptotic cells by phagocytes. Cell death and differentiation *15*, 243-250.

Erwig, L.P., McPhilips, K.A., Wynes, M.W., Ivetic, A., Ridley, A.J., and Henson, P.M. (2006). Differential regulation of phagosome maturation in macrophages and dendritic cells mediated by Rho GTPases and ezrin-radixin-moesin (ERM) proteins. Proceedings of the National Academy of Sciences of the United States of America *103*, 12825-12830.

Eskelinen, E.L., Tanaka, Y., and Saftig, P. (2003). At the acidic edge: emerging functions for lysosomal membrane proteins. Trends in cell biology *13*, 137-145.

Fadok, V.A., Bratton, D.L., Konowal, A., Freed, P.W., Westcott, J.Y., and Henson, P.M. (1998a). Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. The Journal of clinical investigation *101*, 890-898.

Fadok, V.A., de Cathelineau, A., Daleke, D.L., Henson, P.M., and Bratton, D.L. (2001). Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts. The Journal of biological chemistry *276*, 1071-1077.

Fadok, V.A., McDonald, P.P., Bratton, D.L., and Henson, P.M. (1998b). Regulation of macrophage cytokine production by phagocytosis of apoptotic and post-apoptotic cells. Biochemical Society transactions *26*, 653-656.

Fadok, V.A., Voelker, D.R., Campbell, P.A., Cohen, J.J., Bratton, D.L., and Henson, P.M. (1992). Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immunol *148*, 2207-2216.

Fadok, V.A., Warner, M.L., Bratton, D.L., and Henson, P.M. (1998c). CD36 is required for phagocytosis of apoptotic cells by human macrophages that use either a phosphatidylserine receptor or the vitronectin receptor (alpha v beta 3). J Immunol *161*, 6250-6257.

Farkas, T., Daugaard, M., and Jaattela, M. (2011). Identification of small molecule inhibitors of phosphatidylinositol 3-kinase and autophagy. The Journal of biological chemistry *286*, 38904-38912.

Febbraio, M., Hajjar, D.P., and Silverstein, R.L. (2001). CD36: a class B scavenger receptor involved in angiogenesis, atherosclerosis, inflammation, and lipid metabolism. The Journal of clinical investigation *108*, 785-791.

Feng, B., Yao, P.M., Li, Y., Devlin, C.M., Zhang, D., Harding, H.P., Sweeney, M., Rong, J.X., Kuriakose, G., Fisher, E.A*., et al.* (2003). The endoplasmic reticulum is the site of cholesterol-induced cytotoxicity in macrophages. Nature cell biology *5*, 781-792.

Filimonenko, M., Isakson, P., Finley, K.D., Anderson, M., Jeong, H., Melia, T.J., Bartlett, B.J., Myers, K.M., Birkeland, H.C., Lamark, T*., et al.* (2010). The selective macroautophagic degradation of aggregated proteins requires the PI3P-binding protein Alfy. Molecular cell *38*, 265-279.

Fitzer-Attas, C.J., Lowry, M., Crowley, M.T., Finn, A.J., Meng, F., DeFranco, A.L., and Lowell, C.A. (2000). Fcgamma receptor-mediated phagocytosis in macrophages lacking the Src family tyrosine kinases Hck, Fgr, and Lyn. The Journal of experimental medicine *191*, 669-682.

Flannagan, R.S., Cosio, G., and Grinstein, S. (2009). Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. Nat Rev Microbiol *7*, 355-366.

Flannagan, R.S., Jaumouille, V., and Grinstein, S. (2012). The cell biology of phagocytosis. Annual review of pathology *7*, 61-98.

Florey, O., Kim, S.E., Sandoval, C.P., Haynes, C.M., and Overholtzer, M. (2011). Autophagy machinery mediates macroendocytic processing and entotic cell death by targeting single membranes. Nature cell biology *13*, 1335-1343.

Florey, O., and Overholtzer, M. (2012). Autophagy proteins in macroendocytic engulfment. Trends in cell biology *22*, 374-380.

References

Fratti, R.A., Backer, J.M., Gruenberg, J., Corvera, S., and Deretic, V. (2001). Role of phosphatidylinositol 3-kinase and Rab5 effectors in phagosomal biogenesis and mycobacterial phagosome maturation arrest. The Journal of cell biology *154*, 631-644.

Fries, D.M., Lightfoot, R., Koval, M., and Ischiropoulos, H. (2005). Autologous apoptotic cell engulfment stimulates chemokine secretion by vascular smooth muscle cells. The American journal of pathology *167*, 345-353.

Fujita, N., Saitoh, T., Kageyama, S., Akira, S., Noda, T., and Yoshimori, T. (2009). Differential involvement of Atg16L1 in Crohn disease and canonical autophagy: analysis of the organization of the Atg16L1 complex in fibroblasts. The Journal of biological chemistry *284*, 32602-32609.

Fullard, J.F., Kale, A., and Baker, N.E. (2009). Clearance of apoptotic corpses. Apoptosis : an international journal on programmed cell death *14*, 1029-1037.

Funderburk, S.F., Wang, Q.J., and Yue, Z. (2010). The Beclin 1-VPS34 complex--at the crossroads of autophagy and beyond. Trends in cell biology *20*, 355-362.

Furnrohr, B.G., Groer, G.J., Sehnert, B., Herrmann, M., and Voll, R.E. (2007). Interaction of histones with phospholipids--implications for the exposure of histones on apoptotic cells. Autoimmunity *40*, 322-326.

Gaipl, U.S., Voll, R.E., Sheriff, A., Franz, S., Kalden, J.R., and Herrmann, M. (2005). Impaired clearance of dying cells in systemic lupus erythematosus. Autoimmunity reviews *4*, 189-194.

Gamerdinger, M., Hajieva, P., Kaya, A.M., Wolfrum, U., Hartl, F.U., and Behl, C. (2009). Protein quality control during aging involves recruitment of the macroautophagy pathway by BAG3. The EMBO journal *28*, 889-901.

Ganley, I.G. (2013). Autophagosome maturation and lysosomal fusion. Essays in biochemistry *55*, 65-78.

Ganley, I.G., Lam du, H., Wang, J., Ding, X., Chen, S., and Jiang, X. (2009). ULK1.ATG13.FIP200 complex mediates mTOR signaling and is essential for autophagy. The Journal of biological chemistry *284*, 12297-12305.

Gardai, S.J., Bratton, D.L., Ogden, C.A., and Henson, P.M. (2006). Recognition ligands on apoptotic cells: a perspective. Journal of leukocyte biology *79*, 896-903.

Gardai, S.J., McPhillips, K.A., Frasch, S.C., Janssen, W.J., Starefeldt, A., Murphy-Ullrich, J.E., Bratton, D.L., Oldenborg, P.A., Michalak, M., and Henson, P.M. (2005). Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. Cell *123*, 321-334.

Geng, J., and Klionsky, D.J. (2008). The Atg8 and Atg12 ubiquitin-like conjugation systems in macroautophagy. 'Protein modifications: beyond the usual suspects' review series. EMBO reports *9*, 859-864.

Ghazizadeh, S., Bolen, J.B., and Fleit, H.B. (1994). Physical and functional association of Src-related protein tyrosine kinases with Fc gamma RII in monocytic THP-1 cells. The Journal of biological chemistry *269*, 8878-8884.

Gigli, I., and Nelson, R.A., Jr. (1968). Complement dependent immune phagocytosis. I. Requirements for C'1, C'4, C'2, C'3. Experimental cell research *51*, 45-67.

Gill, D.J., Teo, H., Sun, J., Perisic, O., Veprintsev, D.B., Emr, S.D., and Williams, R.L. (2007). Structural insight into the ESCRT-I/-II link and its role in MVB trafficking. The EMBO journal *26*, 600-612.

Glass, C.K., and Witztum, J.L. (2001). Atherosclerosis. the road ahead. Cell *104*, 503-516.

Goode, A., and Layfield, R. (2010). Recent advances in understanding the molecular basis of Paget disease of bone. Journal of clinical pathology *63*, 199-203.

Gordon, P.B., and Seglen, P.O. (1988). Prelysosomal convergence of autophagic and endocytic pathways. Biochemical and biophysical research communications *151*, 40-47.

Grant, B.D., and Donaldson, J.G. (2009). Pathways and mechanisms of endocytic recycling. Nature reviews Molecular cell biology *10*, 597-608.

Green, D.R., Ferguson, T., Zitvogel, L., and Kroemer, G. (2009). Immunogenic and tolerogenic cell death. Nature reviews Immunology *9*, 353-363.

Greenberg, S. (1995). Signal transduction of phagocytosis. Trends in cell biology *5*, 93-99.

Greenberg, S., and Grinstein, S. (2002). Phagocytosis and innate immunity. Current opinion in immunology *14*, 136-145.

Gregory, C. (2009). Cell biology: Sent by the scent of death. Nature *461*, 181-182.

Griffith, T.S., and Ferguson, T.A. (2011). Cell death in the maintenance and abrogation of tolerance: the five Ws of dying cells. Immunity *35*, 456-466.

Griffiths, G., Hoflack, B., Simons, K., Mellman, I., and Kornfeld, S. (1988). The mannose 6-phosphate receptor and the biogenesis of lysosomes. Cell *52*, 329-341.

Grosshans, B.L., Ortiz, D., and Novick, P. (2006). Rabs and their effectors: achieving specificity in membrane traffic. Proceedings of the National Academy of Sciences of the United States of America *103*, 11821-11827.

Gumienny, T.L., Brugnera, E., Tosello-Trampont, A.C., Kinchen, J.M., Haney, L.B., Nishiwaki, K., Walk, S.F., Nemergut, M.E., Macara, I.G., Francis, R.*, et al.* (2001). CED-12/ELMO, a novel member of the CrkII/Dock180/Rac pathway, is required for phagocytosis and cell migration. Cell *107*, 27-41.

Guo, P., Hu, T., Zhang, J., Jiang, S., and Wang, X. (2010). Sequential action of Caenorhabditis elegans Rab GTPases regulates phagolysosome formation during apoptotic cell degradation. Proceedings of the National Academy of Sciences of the United States of America *107*, 18016-18021.

Hackam, D.J., Rotstein, O.D., Zhang, W.J., Demaurex, N., Woodside, M., Tsai, O., and Grinstein, S. (1997). Regulation of phagosomal acidification. Differential targeting of Na+/H+ exchangers,

Na+/K+-ATPases, and vacuolar-type H+-atpases. The Journal of biological chemistry *272*, 29810-29820.

Haglund, K., and Dikic, I. (2005). Ubiquitylation and cell signaling. The EMBO journal *24*, 3353-3359.

Hamada, F., Aoki, M., Akiyama, T., and Toyoshima, K. (1993). Association of immunoglobulin G Fc receptor II with Src-like protein-tyrosine kinase Fgr in neutrophils. Proceedings of the National Academy of Sciences of the United States of America *90*, 6305-6309.

Hanayama, R., Tanaka, M., Miwa, K., Shinohara, A., Iwamatsu, A., and Nagata, S. (2002). Identification of a factor that links apoptotic cells to phagocytes. Nature *417*, 182-187.

Hanayama, R., Tanaka, M., Miyasaka, K., Aozasa, K., Koike, M., Uchiyama, Y., and Nagata, S. (2004). Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice. Science *304*, 1147-1150.

Harper, J.W., and Schulman, B.A. (2006). Structural complexity in ubiquitin recognition. Cell *124*, 1133-1136.

Harrison, R.E., Bucci, C., Vieira, O.V., Schroer, T.A., and Grinstein, S. (2003). Phagosomes fuse with late endosomes and/or lysosomes by extension of membrane protrusions along microtubules: role of Rab7 and RILP. Molecular and cellular biology *23*, 6494-6506.

Hart, S.P., Dransfield, I., and Rossi, A.G. (2008). Phagocytosis of apoptotic cells. Methods *44*, 280-285.

Hayashi-Nishino, M., Fujita, N., Noda, T., Yamaguchi, A., Yoshimori, T., and Yamamoto, A. (2009). A subdomain of the endoplasmic reticulum forms a cradle for autophagosome formation. Nature cell biology *11*, 1433-1437.

He, B., Yu, X., Margolis, M., Liu, X., Leng, X., Etzion, Y., Zheng, F., Lu, N., Quiocho, F.A., Danino, D.*, et al.* (2010). Live-cell imaging in Caenorhabditis elegans reveals the distinct roles of dynamin self-assembly and guanosine triphosphate hydrolysis in the removal of apoptotic cells. Molecular biology of the cell *21*, 610-629.

He, C., and Levine, B. (2010). The Beclin 1 interactome. Current opinion in cell biology *22*, 140-149.

He, M., Kubo, H., Morimoto, K., Fujino, N., Suzuki, T., Takahasi, T., Yamada, M., Yamaya, M., Maekawa, T., Yamamoto, Y.*, et al.* (2011). Receptor for advanced glycation end products binds to phosphatidylserine and assists in the clearance of apoptotic cells. EMBO reports *12*, 358-364.

Henault, J., Martinez, J., Riggs, J.M., Tian, J., Mehta, P., Clarke, L., Sasai, M., Latz, E., Brinkmann, M.M., Iwasaki, A.*, et al.* (2012). Noncanonical autophagy is required for type I interferon secretion in response to DNA-immune complexes. Immunity *37*, 986-997.

Henson, P.M. (2005). Dampening inflammation. Nature immunology *6*, 1179-1181.

Henson, P.M., Bratton, D.L., and Fadok, V.A. (2001). The phosphatidylserine receptor: a crucial molecular switch? Nature reviews Molecular cell biology *2*, 627-633.

Henson, P.M., and Hume, D.A. (2006). Apoptotic cell removal in development and tissue homeostasis. Trends in immunology *27*, 244-250.

Henson, P.M., and Tuder, R.M. (2008). Apoptosis in the lung: induction, clearance and detection. American journal of physiology Lung cellular and molecular physiology *294*, L601-611.

Hochreiter-Hufford, A., and Ravichandran, K.S. (2013). Clearing the dead: apoptotic cell sensing, recognition, engulfment, and digestion. Cold Spring Harb Perspect Biol *5*, a008748.

Hodge, S., Hodge, G., Holmes, M., and Reynolds, P.N. (2005). Increased airway epithelial and T-cell apoptosis in COPD remains despite smoking cessation. The European respiratory journal *25*, 447-454.

Hoffmann, P.R., deCathelineau, A.M., Ogden, C.A., Leverrier, Y., Bratton, D.L., Daleke, D.L., Ridley, A.J., Fadok, V.A., and Henson, P.M. (2001). Phosphatidylserine (PS) induces PS receptor-mediated macropinocytosis and promotes clearance of apoptotic cells. J Cell Biol *155*, 649-659.

Hoopfer, E.D., McLaughlin, T., Watts, R.J., Schuldiner, O., O'Leary, D.D., and Luo, L. (2006). Wlds protection distinguishes axon degeneration following injury from naturally occurring developmental pruning. Neuron *50*, 883-895.

Huang, J., Canadien, V., Lam, G.Y., Steinberg, B.E., Dinauer, M.C., Magalhaes, M.A., Glogauer, M., Grinstein, S., and Brumell, J.H. (2009). Activation of antibacterial autophagy by NADPH oxidases. Proceedings of the National Academy of Sciences of the United States of America *106*, 6226-6231.

Huang, Y.X., Wu, Z.J., Mehrishi, J., Huang, B.T., Chen, X.Y., Zheng, X.J., Liu, W.J., and Luo, M. (2011). Human red blood cell aging: correlative changes in surface charge and cell properties. Journal of cellular and molecular medicine *15*, 2634-2642.

Huotari, J., and Helenius, A. (2011). Endosome maturation. The EMBO journal *30*, 3481-3500.

Huynh, K.K., Eskelinen, E.L., Scott, C.C., Malevanets, A., Saftig, P., and Grinstein, S. (2007). LAMP proteins are required for fusion of lysosomes with phagosomes. The EMBO journal *26*, 313-324.

Ichimura, Y., Kirisako, T., Takao, T., Satomi, Y., Shimonishi, Y., Ishihara, N., Mizushima, N., Tanida, I., Kominami, E., Ohsumi, M.*, et al.* (2000). A ubiquitin-like system mediates protein lipidation. Nature *408*, 488-492.

Ikeda, F., and Dikic, I. (2008). Atypical ubiquitin chains: new molecular signals. 'Protein Modifications: Beyond the Usual Suspects' review series. EMBO reports *9*, 536-542.

Indik, Z.K., Park, J.G., Hunter, S., and Schreiber, A.D. (1995). The molecular dissection of Fc gamma receptor mediated phagocytosis. Blood *86*, 4389-4399.

Itakura, E., and Mizushima, N. (2010). Characterization of autophagosome formation site by a hierarchical analysis of mammalian Atg proteins. Autophagy *6*, 764-776.

Itakura, E., and Mizushima, N. (2011). p62 Targeting to the autophagosome formation site requires self-oligomerization but not LC3 binding. The Journal of cell biology *192*, 17-27.

Jaumouille, V., and Grinstein, S. (2011). Receptor mobility, the cytoskeleton, and particle binding during phagocytosis. Current opinion in cell biology *23*, 22-29.

Johansen, T., and Lamark, T. (2011). Selective autophagy mediated by autophagic adapter proteins. Autophagy *7*, 279-296.

Johansson, M., Rocha, N., Zwart, W., Jordens, I., Janssen, L., Kuijl, C., Olkkonen, V.M., and Neefjes, J. (2007). Activation of endosomal dynein motors by stepwise assembly of Rab7-RILP-p150Glued, ORP1L, and the receptor betalll spectrin. The Journal of cell biology *176*, 459-471.

Johnson, J.L., and Newby, A.C. (2009). Macrophage heterogeneity in atherosclerotic plaques. Current opinion in lipidology *20*, 370-378.

Jung, C.H., Jun, C.B., Ro, S.H., Kim, Y.M., Otto, N.M., Cao, J., Kundu, M., and Kim, D.H. (2009). ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. Molecular biology of the cell *20*, 1992-2003.

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y., and Yoshimori, T. (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. The EMBO journal *19*, 5720-5728.

Kagan, V.E., Gleiss, B., Tyurina, Y.Y., Tyurin, V.A., Elenstrom-Magnusson, C., Liu, S.X., Serinkan, F.B., Arroyo, A., Chandra, J., Orrenius, S.*, et al.* (2002). A role for oxidative stress in apoptosis: oxidation and externalization of phosphatidylserine is required for macrophage clearance of cells undergoing Fas-mediated apoptosis. J Immunol *169*, 487-499.

Kageyama, S., Omori, H., Saitoh, T., Sone, T., Guan, J.L., Akira, S., Imamoto, F., Noda, T., and Yoshimori, T. (2011). The LC3 recruitment mechanism is separate from Atg9L1-dependent membrane formation in the autophagic response against Salmonella. Molecular biology of the cell *22*, 2290-2300.

Kanki, T., Wang, K., Cao, Y., Baba, M., and Klionsky, D.J. (2009). Atg32 is a mitochondrial protein that confers selectivity during mitophagy. Developmental cell *17*, 98-109.

Katzmann, D.J., Odorizzi, G., and Emr, S.D. (2002). Receptor downregulation and multivesicular-body sorting. Nature reviews Molecular cell biology *3*, 893-905.

Kaushik, S., Massey, A.C., Mizushima, N., and Cuervo, A.M. (2008). Constitutive activation of chaperone-mediated autophagy in cells with impaired macroautophagy. Molecular biology of the cell *19*, 2179-2192.

Kawane, K., Ohtani, M., Miwa, K., Kizawa, T., Kanbara, Y., Yoshioka, Y., Yoshikawa, H., and Nagata, S. (2006). Chronic polyarthritis caused by mammalian DNA that escapes from degradation in macrophages. Nature *443*, 998-1002.

Kerr, J.F., Wyllie, A.H., and Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. British journal of cancer *26*, 239-257.

Kim, H.J., Zhong, Q., Sheng, Z.H., Yoshimori, T., Liang, C., and Jung, J.U. (2012). Beclin-1-interacting autophagy protein Atg14L targets the SNARE-associated protein Snapin to coordinate endocytic trafficking. Journal of cell science *125*, 4740-4750.

Kim, J.Y., Zhao, H., Martinez, J., Doggett, T.A., Kolesnikov, A.V., Tang, P.H., Ablonczy, Z., Chan, C.C., Zhou, Z., Green, D.R.*, et al.* (2013). Noncanonical autophagy promotes the visual cycle. Cell *154*, 365-376.

Kim, P.K., Hailey, D.W., Mullen, R.T., and Lippincott-Schwartz, J. (2008). Ubiquitin signals autophagic degradation of cytosolic proteins and peroxisomes. Proceedings of the National Academy of Sciences of the United States of America *105*, 20567-20574.

Kim, S.E., and Overholtzer, M. (2013a). Autophagy proteins regulate cell engulfment mechanisms that participate in cancer. Seminars in cancer biology *23*, 329-336.

Kim, S.E., and Overholtzer, M. (2013b). Autophagy proteins regulate cell engulfment mechanisms that participate in cancer. Seminars in cancer biology.

Kinchen, J.M., Doukoumetzidis, K., Almendinger, J., Stergiou, L., Tosello-Trampont, A., Sifri, C.D., Hengartner, M.O., and Ravichandran, K.S. (2008). A pathway for phagosome maturation during engulfment of apoptotic cells. Nature cell biology *10*, 556-566.

Kinchen, J.M., and Ravichandran, K.S. (2008). Phagosome maturation: going through the acid test. Nature reviews Molecular cell biology *9*, 781-795.

Kinchen, J.M., and Ravichandran, K.S. (2010). Identification of two evolutionarily conserved genes regulating processing of engulfed apoptotic cells. Nature *464*, 778-782.

Kirkin, V., Lamark, T., Johansen, T., and Dikic, I. (2009a). NBR1 cooperates with p62 in selective autophagy of ubiquitinated targets. Autophagy *5*, 732-733.

Kirkin, V., Lamark, T., Sou, Y.S., Bjorkoy, G., Nunn, J.L., Bruun, J.A., Shvets, E., McEwan, D.G., Clausen, T.H., Wild, P.*, et al.* (2009b). A role for NBR1 in autophagosomal degradation of ubiquitinated substrates. Molecular cell *33*, 505-516.

Kirkin, V., McEwan, D.G., Novak, I., and Dikic, I. (2009c). A role for ubiquitin in selective autophagy. Molecular cell *34*, 259-269.

Klarl, B.A., Lang, P.A., Kempe, D.S., Niemoeller, O.M., Akel, A., Sobiesiak, M., Eisele, K., Podolski, M., Huber, S.M., Wieder, T.*, et al.* (2006). Protein kinase C mediates erythrocyte "programmed cell death" following glucose depletion. American journal of physiology Cell physiology *290*, C244-253.

Klionsky, D.J. (2007). Autophagy: from phenomenology to molecular understanding in less than a decade. Nature reviews Molecular cell biology *8*, 931-937.

Kobayashi, N., Karisola, P., Pena-Cruz, V., Dorfman, D.M., Jinushi, M., Umetsu, S.E., Butte, M.J., Nagumo, H., Chernova, I., Zhu, B.*, et al.* (2007). TIM-1 and TIM-4 glycoproteins bind phosphatidylserine and mediate uptake of apoptotic cells. Immunity *27*, 927-940.

Kobayashi, T., Beuchat, M.H., Lindsay, M., Frias, S., Palmiter, R.D., Sakuraba, H., Parton, R.G., and Gruenberg, J. (1999). Late endosomal membranes rich in lysobisphosphatidic acid regulate cholesterol transport. Nature cell biology *1*, 113-118.

Kobayashi, T., and Hirabayashi, Y. (2000). Lipid membrane domains in cell surface and vacuolar systems. Glycoconjugate journal *17*, 163-171.

Koenen, R.R., von Hundelshausen, P., Nesmelova, I.V., Zernecke, A., Liehn, E.A., Sarabi, A., Kramp, B.K., Piccinini, A.M., Paludan, S.R., Kowalska, M.A*., et al.* (2009). Disrupting functional interactions between platelet chemokines inhibits atherosclerosis in hyperlipidemic mice. Nature medicine *15*, 97-103.

Kolb, S., Vranckx, R., Huisse, M.G., Michel, J.B., and Meilhac, O. (2007). The phosphatidylserine receptor mediates phagocytosis by vascular smooth muscle cells. J Pathol *212*, 249-259.

Kopito, R.R. (2000). Aggresomes, inclusion bodies and protein aggregation. Trends in cell biology *10*, 524-530.

Kraft, C., Deplazes, A., Sohrmann, M., and Peter, M. (2008). Mature ribosomes are selectively degraded upon starvation by an autophagy pathway requiring the Ubp3p/Bre5p ubiquitin protease. Nature cell biology *10*, 602-610.

Krysko, D.V., Denecker, G., Festjens, N., Gabriels, S., Parthoens, E., D'Herde, K., and Vandenabeele, P. (2006). Macrophages use different internalization mechanisms to clear apoptotic and necrotic cells. Cell death and differentiation *13*, 2011-2022.

Kubota, H. (2009). Quality control against misfolded proteins in the cytosol: a network for cell survival. Journal of biochemistry *146*, 609-616.

Kuo, T.C., Chen, C.T., Baron, D., Onder, T.T., Loewer, S., Almeida, S., Weismann, C.M., Xu, P., Houghton, J.M., Gao, F.B*., et al.* (2011). Midbody accumulation through evasion of autophagy contributes to cellular reprogramming and tumorigenicity. Nature cell biology *13*, 1214-1223.

Lai, S., and Devenish, R.J. (2012). LC3-Associated Phagocytosis (LAP): Connections with Host Autophagy. Cells 1(3), 396-408.

Lamark, T., and Johansen, T. (2012). Aggrephagy: selective disposal of protein aggregates by macroautophagy. International journal of cell biology *2012*, 736905.

Lamark, T., Kirkin, V., Dikic, I., and Johansen, T. (2009). NBR1 and p62 as cargo receptors for selective autophagy of ubiquitinated targets. Cell Cycle *8*, 1986-1990.

Lamark, T., Perander, M., Outzen, H., Kristiansen, K., Overvatn, A., Michaelsen, E., Bjorkoy, G., and Johansen, T. (2003). Interaction codes within the family of mammalian Phox and Bem1p domain-containing proteins. The Journal of biological chemistry *278*, 34568-34581.

Lamb, C.A., Dooley, H.C., and Tooze, S.A. (2013). Endocytosis and autophagy: Shared machinery for degradation. BioEssays : news and reviews in molecular, cellular and developmental biology *35*, 34-45.

Lang, F., Lang, E., and Foller, M. (2012). Physiology and pathophysiology of eryptosis. Transfusion medicine and hemotherapy : offizielles Organ der Deutschen Gesellschaft fur Transfusionsmedizin und Immunhamatologie *39*, 308-314.

Lauber, K., Bohn, E., Krober, S.M., Xiao, Y.J., Blumenthal, S.G., Lindemann, R.K., Marini, P., Wiedig, C., Zobywalski, A., Baksh, S.*, et al.* (2003). Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. Cell *113*, 717-730.

Lee, H.K., Mattei, L.M., Steinberg, B.E., Alberts, P., Lee, Y.H., Chervonsky, A., Mizushima, N., Grinstein, S., and Iwasaki, A. (2010a). In vivo requirement for Atg5 in antigen presentation by dendritic cells. Immunity *32*, 227-239.

Lee, S.J., Pfluger, P.T., Kim, J.Y., Nogueiras, R., Duran, A., Pages, G., Pouyssegur, J., Tschop, M.H., Diaz-Meco, M.T., and Moscat, J. (2010b). A functional role for the p62-ERK1 axis in the control of energy homeostasis and adipogenesis. EMBO reports *11*, 226-232.

Lee, W.L., Kim, M.K., Schreiber, A.D., and Grinstein, S. (2005). Role of ubiquitin and proteasomes in phagosome maturation. Molecular biology of the cell *16*, 2077-2090.

Lee, W.L., Mason, D., Schreiber, A.D., and Grinstein, S. (2007). Quantitative analysis of membrane remodeling at the phagocytic cup. Molecular biology of the cell *18*, 2883-2892.

Lemke, G., and Rothlin, C.V. (2008). Immunobiology of the TAM receptors. Nature reviews Immunology *8*, 327-336.

Lerena, M.C., and Colombo, M.I. (2011). Mycobacterium marinum induces a marked LC3 recruitment to its containing phagosome that depends on a functional ESX-1 secretion system. Cellular microbiology *13*, 814-835.

Leverrier, Y., Okkenhaug, K., Sawyer, C., Bilancio, A., Vanhaesebroeck, B., and Ridley, A.J. (2003). Class I phosphoinositide 3-kinase p110beta is required for apoptotic cell and Fcgamma receptor-mediated phagocytosis by macrophages. The Journal of biological chemistry *278*, 38437-38442.

Levine, B. (2005). Eating oneself and uninvited guests: autophagy-related pathways in cellular defense. Cell *120*, 159-162.

Levine, B., and Deretic, V. (2007). Unveiling the roles of autophagy in innate and adaptive immunity. Nature reviews Immunology *7*, 767-777.

Levine, B., and Klionsky, D.J. (2004). Development by self-digestion: molecular mechanisms and biological functions of autophagy. Developmental cell *6*, 463-477.

Levine, B., and Kroemer, G. (2008). Autophagy in the pathogenesis of disease. Cell *132*, 27-42.

Li, W., Zou, W., Yang, Y., Chai, Y., Chen, B., Cheng, S., Tian, D., Wang, X., Vale, R.D., and Ou, G. (2012a). Autophagy genes function sequentially to promote apoptotic cell corpse degradation in the engulfing cell. The Journal of cell biology *197*, 27-35.

Li, W.W., Li, J., and Bao, J.K. (2012b). Microautophagy: lesser-known self-eating. Cellular and molecular life sciences : CMLS *69*, 1125-1136.

Liao, X., Sluimer, J.C., Wang, Y., Subramanian, M., Brown, K., Pattison, J.S., Robbins, J., Martinez, J., and Tabas, I. (2012). Macrophage autophagy plays a protective role in advanced atherosclerosis. Cell metabolism *15*, 545-553.

Libby, P., Ridker, P.M., and Hansson, G.K. (2011). Progress and challenges in translating the biology of atherosclerosis. Nature *473*, 317-325.

Llodra, J., Angeli, V., Liu, J., Trogan, E., Fisher, E.A., and Randolph, G.J. (2004). Emigration of monocyte-derived cells from atherosclerotic lesions characterizes regressive, but not progressive, plaques. Proceedings of the National Academy of Sciences of the United States of America *101*, 11779-11784.

Longatti, A., and Tooze, S.A. (2009). Vesicular trafficking and autophagosome formation. Cell death and differentiation *16*, 956-965.

Lu, N., and Zhou, Z. (2012). Membrane trafficking and phagosome maturation during the clearance of apoptotic cells. International review of cell and molecular biology *293*, 269-309.

Lu, Q., Zhang, Y., Hu, T., Guo, P., Li, W., and Wang, X. (2008). C. elegans Rab GTPase 2 is required for the degradation of apoptotic cells. Development *135*, 1069-1080.

Lusis, A.J. (2000). Atherosclerosis. Nature *407*, 233-241.

Majesky, M.W. (2007). Developmental basis of vascular smooth muscle diversity. Arteriosclerosis, thrombosis, and vascular biology *27*, 1248-1258.

Mantegazza, A.R., Magalhaes, J.G., Amigorena, S., and Marks, M.S. (2013). Presentation of phagocytosed antigens by MHC class I and II. Traffic *14*, 135-152.

Marshansky, V., and Futai, M. (2008). The V-type H+-ATPase in vesicular trafficking: targeting, regulation and function. Current opinion in cell biology *20*, 415-426.

Martin, S.J., Reutelingsperger, C.P., McGahon, A.J., Rader, J.A., van Schie, R.C., LaFace, D.M., and Green, D.R. (1995). Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. The Journal of experimental medicine *182*, 1545-1556.

Martinez, J., Almendinger, J., Oberst, A., Ness, R., Dillon, C.P., Fitzgerald, P., Hengartner, M.O., and Green, D.R. (2011). Microtubule-associated protein 1 light chain 3 alpha (LC3)-associated phagocytosis is required for the efficient clearance of dead cells. Proceedings of the National Academy of Sciences of the United States of America *108*, 17396-17401.

Martinez, J., Verbist, K., Wang, R., and Green, D.R. (2013). The relationship between metabolism and the autophagy machinery during the innate immune response. Cell metabolism *17*, 895-900.

Massey, A., Kiffin, R., and Cuervo, A.M. (2004). Pathophysiology of chaperone-mediated autophagy. The international journal of biochemistry & cell biology *36*, 2420-2434.

Matsunaga, K., Morita, E., Saitoh, T., Akira, S., Ktistakis, N.T., Izumi, T., Noda, T., and Yoshimori, T. (2010). Autophagy requires endoplasmic reticulum targeting of the PI3-kinase complex via Atg14L. The Journal of cell biology *190*, 511-521.

Matsura, T., Serinkan, B.F., Jiang, J., and Kagan, V.E. (2002). Phosphatidylserine peroxidation/externalization during staurosporine-induced apoptosis in HL-60 cells. FEBS letters *524*, 25-30.

Mattera, R., Tsai, Y.C., Weissman, A.M., and Bonifacino, J.S. (2006). The Rab5 guanine nucleotide exchange factor Rabex-5 binds ubiquitin (Ub) and functions as a Ub ligase through an atypical Ub-interacting motif and a zinc finger domain. The Journal of biological chemistry *281*, 6874-6883.

Maxfield, F.R., and McGraw, T.E. (2004). Endocytic recycling. Nature reviews Molecular cell biology *5*, 121-132.

Maxfield, F.R., and Tabas, I. (2005). Role of cholesterol and lipid organization in disease. Nature *438*, 612-621.

May, R.C., and Machesky, L.M. (2001). Phagocytosis and the actin cytoskeleton. Journal of cell science *114*, 1061-1077.

McBride, H.M., Rybin, V., Murphy, C., Giner, A., Teasdale, R., and Zerial, M. (1999). Oligomeric complexes link Rab5 effectors with NSF and drive membrane fusion via interactions between EEA1 and syntaxin 13. Cell *98*, 377-386.

Medzhitov, R., and Janeway, C.A., Jr. (2002). Decoding the patterns of self and nonself by the innate immune system. Science *296*, 298-300.

Mehrabian, M., Wen, P.Z., Fisler, J., Davis, R.C., and Lusis, A.J. (1998). Genetic loci controlling body fat, lipoprotein metabolism, and insulin levels in a multifactorial mouse model. The Journal of clinical investigation *101*, 2485-2496.

Mestas, J., and Ley, K. (2008). Monocyte-endothelial cell interactions in the development of atherosclerosis. Trends in cardiovascular medicine *18*, 228-232.

Miyake, Y., Asano, K., Kaise, H., Uemura, M., Nakayama, M., and Tanaka, M. (2007). Critical role of macrophages in the marginal zone in the suppression of immune responses to apoptotic cell-associated antigens. The Journal of clinical investigation *117*, 2268-2278.

Miyanishi, M., Tada, K., Koike, M., Uchiyama, Y., Kitamura, T., and Nagata, S. (2007). Identification of Tim4 as a phosphatidylserine receptor. Nature *450*, 435-439.

Mizushima, N. (2004). Methods for monitoring autophagy. The international journal of biochemistry & cell biology *36*, 2491-2502.

Mizushima, N. (2007). Autophagy: process and function. Genes & development *21*, 2861-2873.

Mizushima, N., Levine, B., Cuervo, A.M., and Klionsky, D.J. (2008). Autophagy fights disease through cellular self-digestion. Nature *451*, 1069-1075.

Mizushima, N., and Yoshimori, T. (2007). How to interpret LC3 immunoblotting. Autophagy *3*, 542-545.

Mizushima, N., Yoshimori, T., and Levine, B. (2010). Methods in mammalian autophagy research. Cell *140*, 313-326.

Mobius, W., van Donselaar, E., Ohno-Iwashita, Y., Shimada, Y., Heijnen, H.F., Slot, J.W., and Geuze, H.J. (2003). Recycling compartments and the internal vesicles of multivesicular bodies harbor most of the cholesterol found in the endocytic pathway. Traffic *4*, 222-231.

Mochizuki, H., Goto, K., Mori, H., and Mizuno, Y. (1996). Histochemical detection of apoptosis in Parkinson's disease. Journal of the neurological sciences *137*, 120-123.

Monks, J., Rosner, D., Geske, F.J., Lehman, L., Hanson, L., Neville, M.C., and Fadok, V.A. (2005). Epithelial cells as phagocytes: apoptotic epithelial cells are engulfed by mammary alveolar epithelial cells and repress inflammatory mediator release. Cell death and differentiation *12*, 107-114.

Moore, K.J., and Tabas, I. (2011). Macrophages in the pathogenesis of atherosclerosis. Cell *145*, 341-355.

Moreau, K., Ravikumar, B., Renna, M., Puri, C., and Rubinsztein, D.C. (2011). Autophagosome precursor maturation requires homotypic fusion. Cell *146*, 303-317.

Moscat, J., and Diaz-Meco, M.T. (2009). p62 at the crossroads of autophagy, apoptosis, and cancer. Cell *137*, 1001-1004.

Moscat, J., Diaz-Meco, M.T., and Wooten, M.W. (2007). Signal integration and diversification through the p62 scaffold protein. Trends in biochemical sciences *32*, 95-100.

Mostowy, S., Sancho-Shimizu, V., Hamon, M.A., Simeone, R., Brosch, R., Johansen, T., and Cossart, P. (2011). p62 and NDP52 proteins target intracytosolic Shigella and Listeria to different autophagy pathways. The Journal of biological chemistry *286*, 26987-26995.

Munoz, L.E., Lauber, K., Schiller, M., Manfredi, A.A., and Herrmann, M. (2010a). The role of defective clearance of apoptotic cells in systemic autoimmunity. Nature reviews Rheumatology *6*, 280-289.

Munoz, L.E., Peter, C., Herrmann, M., Wesselborg, S., and Lauber, K. (2010b). Scent of dying cells: the role of attraction signals in the clearance of apoptotic cells and its immunological consequences. Autoimmunity reviews *9*, 425-430.

Nagata, S., Hanayama, R., and Kawane, K. (2010). Autoimmunity and the clearance of dead cells. Cell *140*, 619-630.

Nair, U., Jotwani, A., Geng, J., Gammoh, N., Richerson, D., Yen, W.L., Griffith, J., Nag, S., Wang, K., Moss, T.*, et al.* (2011). SNARE proteins are required for macroautophagy. Cell *146*, 290-302.

Nakatogawa, H. (2007). [Mechanisms of membrane biogenesis in autophagy]. Seikagaku The Journal of Japanese Biochemical Society *79*, 1065-1068.

Nakayama, M., Akiba, H., Takeda, K., Kojima, Y., Hashiguchi, M., Azuma, M., Yagita, H., and Okumura, K. (2009). Tim-3 mediates phagocytosis of apoptotic cells and cross-presentation. Blood *113*, 3821-3830.

Nathan, C., and Ding, A. (2010). Nonresolving inflammation. Cell *140*, 871-882.

Navab, M., Berliner, J.A., Watson, A.D., Hama, S.Y., Territo, M.C., Lusis, A.J., Shih, D.M., Van Lenten, B.J., Frank, J.S., Demer, L.L*., et al.* (1996). The Yin and Yang of oxidation in the development of the fatty streak. A review based on the 1994 George Lyman Duff Memorial Lecture. Arteriosclerosis, thrombosis, and vascular biology *16*, 831-842.

Negoro, N., Kanayama, Y., Haraguchi, M., Umetani, N., Nishimura, M., Konishi, Y., Iwai, J., Okamura, M., Inoue, T., and Takeda, T. (1995). Blood pressure regulates platelet-derived growth factor A-chain gene expression in vascular smooth muscle cells in vivo. An autocrine mechanism promoting hypertensive vascular hypertrophy. The Journal of clinical investigation *95*, 1140-1150.

Nimmerjahn, F., and Ravetch, J.V. (2008). Fcgamma receptors as regulators of immune responses. Nature reviews Immunology *8*, 34-47.

Noda, N.N., Kumeta, H., Nakatogawa, H., Satoo, K., Adachi, W., Ishii, J., Fujioka, Y., Ohsumi, Y., and Inagaki, F. (2008). Structural basis of target recognition by Atg8/LC3 during selective autophagy. Genes to cells : devoted to molecular & cellular mechanisms *13*, 1211-1218.

Novak, I., Kirkin, V., McEwan, D.G., Zhang, J., Wild, P., Rozenknop, A., Rogov, V., Lohr, F., Popovic, D., Occhipinti, A*., et al.* (2010). Nix is a selective autophagy receptor for mitochondrial clearance. EMBO reports *11*, 45-51.

Oka, K., Sawamura, T., Kikuta, K., Itokawa, S., Kume, N., Kita, T., and Masaki, T. (1998). Lectin-like oxidized low-density lipoprotein receptor 1 mediates phagocytosis of aged/apoptotic cells in endothelial cells. Proceedings of the National Academy of Sciences of the United States of America *95*, 9535-9540.

Okamoto, K., Kondo-Okamoto, N., and Ohsumi, Y. (2009). Mitochondria-anchored receptor Atg32 mediates degradation of mitochondria via selective autophagy. Developmental cell *17*, 87-97.

Orvedahl, A., MacPherson, S., Sumpter, R., Jr., Talloczy, Z., Zou, Z., and Levine, B. (2010). Autophagy protects against Sindbis virus infection of the central nervous system. Cell host & microbe *7*, 115-127.

Paidassi, H., Tacnet-Delorme, P., Arlaud, G.J., and Frachet, P. (2009). How phagocytes track down and respond to apoptotic cells. Critical reviews in immunology *29*, 111-130.

Pankiv, S., Clausen, T.H., Lamark, T., Brech, A., Bruun, J.A., Outzen, H., Overvatn, A., Bjorkoy, G., and Johansen, T. (2007). p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. The Journal of biological chemistry *282*, 24131-24145.

Park, D., Tosello-Trampont, A.C., Elliott, M.R., Lu, M., Haney, L.B., Ma, Z., Klibanov, A.L., Mandell, J.W., and Ravichandran, K.S. (2007). BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module. Nature *450*, 430-434.

Park, S.Y., Jung, M.Y., Kim, H.J., Lee, S.J., Kim, S.Y., Lee, B.H., Kwon, T.H., Park, R.W., and Kim, I.S. (2008). Rapid cell corpse clearance by stabilin-2, a membrane phosphatidylserine receptor. Cell death and differentiation *15*, 192-201.

Parnaik, R., Raff, M.C., and Scholes, J. (2000). Differences between the clearance of apoptotic cells by professional and non-professional phagocytes. Curr Biol *10*, 857-860.

Paulson, K.E., Zhu, S.N., Chen, M., Nurmohamed, S., Jongstra-Bilen, J., and Cybulsky, M.I. (2010). Resident intimal dendritic cells accumulate lipid and contribute to the initiation of atherosclerosis. Circulation research *106*, 383-390.

Peter, C., Wesselborg, S., Herrmann, M., and Lauber, K. (2010). Dangerous attraction: phagocyte recruitment and danger signals of apoptotic and necrotic cells. Apoptosis : an international journal on programmed cell death *15*, 1007-1028.

Piomelli, S., and Seaman, C. (1993). Mechanism of red blood cell aging: relationship of cell density and cell age. American journal of hematology *42*, 46-52.

Piper, R.C., and Katzmann, D.J. (2007). Biogenesis and function of multivesicular bodies. Annual review of cell and developmental biology *23*, 519-547.

Poon, I.K., Lucas, C.D., Rossi, A.G., and Ravichandran, K.S. (2014). Apoptotic cell clearance: basic biology and therapeutic potential. Nature reviews Immunology.

Praefcke, G.J., and McMahon, H.T. (2004). The dynamin superfamily: universal membrane tubulation and fission molecules? Nature reviews Molecular cell biology *5*, 133-147.

Qu, X., Zou, Z., Sun, Q., Luby-Phelps, K., Cheng, P., Hogan, R.N., Gilpin, C., and Levine, B. (2007). Autophagy gene-dependent clearance of apoptotic cells during embryonic development. Cell *128*, 931-946.

Raasi, S., Varadan, R., Fushman, D., and Pickart, C.M. (2005). Diverse polyubiquitin interaction properties of ubiquitin-associated domains. Nature structural & molecular biology *12*, 708-714.

Rabinovitch, M. (1995). Professional and non-professional phagocytes: an introduction. Trends in cell biology *5*, 85-87.

Radic, M., Marion, T., and Monestier, M. (2004). Nucleosomes are exposed at the cell surface in apoptosis. J Immunol *172*, 6692-6700.

Rahighi, S., Ikeda, F., Kawasaki, M., Akutsu, M., Suzuki, N., Kato, R., Kensche, T., Uejima, T., Bloor, S., Komander, D.*, et al.* (2009). Specific recognition of linear ubiquitin chains by NEMO is important for NF-kappaB activation. Cell *136*, 1098-1109.

Ramachandra, L., Boom, W.H., and Harding, C.V. (2008). Class II MHC antigen processing in phagosomes. Methods Mol Biol *445*, 353-377.

Ravetch, J.V., and Bolland, S. (2001). IgG Fc receptors. Annual review of immunology *19*, 275-290.

Ravichandran, K.S. (2010). Find-me and eat-me signals in apoptotic cell clearance: progress and conundrums. The Journal of experimental medicine *207*, 1807-1817.

Ravichandran, K.S. (2011). Beginnings of a good apoptotic meal: the find-me and eat-me signaling pathways. Immunity *35*, 445-455.

Ravichandran, K.S., and Lorenz, U. (2007). Engulfment of apoptotic cells: signals for a good meal. Nature reviews Immunology *7*, 964-974.

Ravikumar, B., Moreau, K., Jahreiss, L., Puri, C., and Rubinsztein, D.C. (2010). Plasma membrane contributes to the formation of pre-autophagosomal structures. Nature cell biology *12*, 747-757.

Reddien, P.W., Cameron, S., and Horvitz, H.R. (2001). Phagocytosis promotes programmed cell death in C. elegans. Nature *412*, 198-202.

Reddien, P.W., and Horvitz, H.R. (2004). The engulfment process of programmed cell death in caenorhabditis elegans. Annual review of cell and developmental biology *20*, 193-221.

Rink, J., Ghigo, E., Kalaidzidis, Y., and Zerial, M. (2005). Rab conversion as a mechanism of progression from early to late endosomes. Cell *122*, 735-749.

Rocha, V.Z., and Libby, P. (2009). Obesity, inflammation, and atherosclerosis. Nature reviews Cardiology *6*, 399-409.

Rodriguez, A., Duran, A., Selloum, M., Champy, M.F., Diez-Guerra, F.J., Flores, J.M., Serrano, M., Auwerx, J., Diaz-Meco, M.T., and Moscat, J. (2006). Mature-onset obesity and insulin resistance in mice deficient in the signaling adapter p62. Cell metabolism *3*, 211-222.

Roger, V.L., Go, A.S., Lloyd-Jones, D.M., Adams, R.J., Berry, J.D., Brown, T.M., Carnethon, M.R., Dai, S., de Simone, G., Ford, E.S*., et al.* (2011). Heart disease and stroke statistics--2011 update: a report from the American Heart Association. Circulation *123*, e18-e209.

Rogov, V., Dotsch, V., Johansen, T., and Kirkin, V. (2014). Interactions between Autophagy Receptors and Ubiquitin-like Proteins Form the Molecular Basis for Selective Autophagy. Molecular cell *53*, 167-178.

Rogov, V.V., Suzuki, H., Fiskin, E., Wild, P., Kniss, A., Rozenknop, A., Kato, R., Kawasaki, M., McEwan, D.G., Lohr, F*., et al.* (2013). Structural basis for phosphorylation-triggered autophagic clearance of Salmonella. The Biochemical journal *454*, 459-466.

Rosamond, W., Flegal, K., Furie, K., Go, A., Greenlund, K., Haase, N., Hailpern, S.M., Ho, M., Howard, V., Kissela, B*., et al.* (2008). Heart disease and stroke statistics--2008 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation *117*, e25-146.

Ross, G.D., Reed, W., Dalzell, J.G., Becker, S.E., and Hogg, N. (1992). Macrophage cytoskeleton association with CR3 and CR4 regulates receptor mobility and phagocytosis of iC3b-opsonized erythrocytes. Journal of leukocyte biology *51*, 109-117.

Ross, R. (1993). Rous-Whipple Award Lecture. Atherosclerosis: a defense mechanism gone awry. The American journal of pathology *143*, 987-1002.

Rothlin, C.V., Ghosh, S., Zuniga, E.I., Oldstone, M.B., and Lemke, G. (2007). TAM receptors are pleiotropic inhibitors of the innate immune response. Cell *131*, 1124-1136.

Rubinsztein, D.C. (2006). The roles of intracellular protein-degradation pathways in neurodegeneration. Nature *443*, 780-786.

Rubinsztein, D.C., Shpilka, T., and Elazar, Z. (2012). Mechanisms of autophagosome biogenesis. Current biology : CB *22*, R29-34.

Russell, D.G., Mwandumba, H.C., and Rhoades, E.E. (2002). Mycobacterium and the coat of many lipids. The Journal of cell biology *158*, 421-426.

Sahu, S.K., Gummadi, S.N., Manoj, N., and Aradhyam, G.K. (2007). Phospholipid scramblases: an overview. Archives of biochemistry and biophysics *462*, 103-114.

Sanderfoot, A.A., and Raikhel, N.V. (1999). The specificity of vesicle trafficking: coat proteins and SNAREs. The Plant cell *11*, 629-642.

Sandilands, E., Serrels, B., McEwan, D.G., Morton, J.P., Macagno, J.P., McLeod, K., Stevens, C., Brunton, V.G., Langdon, W.Y., Vidal, M.*, et al.* (2012). Autophagic targeting of Src promotes cancer cell survival following reduced FAK signalling. Nature cell biology *14*, 51-60.

Sandoval, H., Thiagarajan, P., Dasgupta, S.K., Schumacher, A., Prchal, J.T., Chen, M., and Wang, J. (2008). Essential role for Nix in autophagic maturation of erythroid cells. Nature *454*, 232-235.

Sanjuan, M.A., Dillon, C.P., Tait, S.W., Moshiach, S., Dorsey, F., Connell, S., Komatsu, M., Tanaka, K., Cleveland, J.L., Withoff, S.*, et al.* (2007). Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. Nature *450*, 1253-1257.

Sanjuan, M.A., Milasta, S., and Green, D.R. (2009). Toll-like receptor signaling in the lysosomal pathways. Immunological reviews *227*, 203-220.

Sarantis, H., and Grinstein, S. (2012). Subversion of phagocytosis for pathogen survival. Cell host & microbe *12*, 419-431.

Savill, J., Dransfield, I., Hogg, N., and Haslett, C. (1990). Vitronectin receptor-mediated phagocytosis of cells undergoing apoptosis. Nature *343*, 170-173.

Savill, J., and Fadok, V. (2000). Corpse clearance defines the meaning of cell death. Nature *407*, 784-788.

Savill, J., Hogg, N., and Haslett, C. (1991). Macrophage vitronectin receptor, CD36, and thrombospondin cooperate in recognition of neutrophils undergoing programmed cell death. Chest *99*, 6S-7S.

Savina, A., Jancic, C., Hugues, S., Guermonprez, P., Vargas, P., Moura, I.C., Lennon-Dumenil, A.M., Seabra, M.C., Raposo, G., and Amigorena, S. (2006). NOX2 controls phagosomal pH to regulate antigen processing during crosspresentation by dendritic cells. Cell *126*, 205-218.

Schmid, D., Pypaert, M., and Munz, C. (2007). Antigen-loading compartments for major histocompatibility complex class II molecules continuously receive input from autophagosomes. Immunity *26*, 79-92.

Schmid, S.L., and Frolov, V.A. (2011). Dynamin: functional design of a membrane fission catalyst. Annual review of cell and developmental biology *27*, 79-105.

Schmitz, G., and Grandl, M. (2009). Endolysosomal phospholipidosis and cytosolic lipid droplet storage and release in macrophages. Biochimica et biophysica acta *1791*, 524-539.

Schrijvers, D.M., De Meyer, G.R., Kockx, M.M., Herman, A.G., and Martinet, W. (2005). Phagocytosis of apoptotic cells by macrophages is impaired in atherosclerosis. Arteriosclerosis, thrombosis, and vascular biology *25*, 1256-1261.

Schroit, A.J., Madsen, J.W., and Tanaka, Y. (1985). In vivo recognition and clearance of red blood cells containing phosphatidylserine in their plasma membranes. The Journal of biological chemistry *260*, 5131-5138.

Schuck, S., Gerl, M.J., Ang, A., Manninen, A., Keller, P., Mellman, I., and Simons, K. (2007). Rab10 is involved in basolateral transport in polarized Madin-Darby canine kidney cells. Traffic *8*, 47-60.

Schulze, H., Kolter, T., and Sandhoff, K. (2009). Principles of lysosomal membrane degradation: Cellular topology and biochemistry of lysosomal lipid degradation. Biochimica et biophysica acta *1793*, 674-683.

Schwartz, J.T., and Allen, L.A. (2006). Role of urease in megasome formation and Helicobacter pylori survival in macrophages. Journal of leukocyte biology *79*, 1214-1225.

Schwartz, S.L., Cao, C., Pylypenko, O., Rak, A., and Wandinger-Ness, A. (2007). Rab GTPases at a glance. Journal of cell science *120*, 3905-3910.

Scott, R.S., McMahon, E.J., Pop, S.M., Reap, E.A., Caricchio, R., Cohen, P.L., Earp, H.S., and Matsushima, G.K. (2001). Phagocytosis and clearance of apoptotic cells is mediated by MER. Nature *411*, 207-211.

Shaid, S., Brandts, C.H., Serve, H., and Dikic, I. (2013). Ubiquitination and selective autophagy. Cell Death Differ *20*, 21-30.

Shiratsuchi, A., Watanabe, I., Takeuchi, O., Akira, S., and Nakanishi, Y. (2004). Inhibitory effect of Toll-like receptor 4 on fusion between phagosomes and endosomes/lysosomes in macrophages. J Immunol *172*, 2039-2047.

Shui, W., Sheu, L., Liu, J., Smart, B., Petzold, C.J., Hsieh, T.Y., Pitcher, A., Keasling, J.D., and Bertozzi, C.R. (2008). Membrane proteomics of phagosomes suggests a connection to autophagy. Proceedings of the National Academy of Sciences of the United States of America *105*, 16952-16957.

Silva, M.T., do Vale, A., and dos Santos, N.M. (2008). Secondary necrosis in multicellular animals: an outcome of apoptosis with pathogenic implications. Apoptosis : an international journal on programmed cell death *13*, 463-482.

Simonsen, A., Lippe, R., Christoforidis, S., Gaullier, J.M., Brech, A., Callaghan, J., Toh, B.H., Murphy, C., Zerial, M., and Stenmark, H. (1998). EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. Nature *394*, 494-498.

Singh, R., and Cuervo, A.M. (2011). Autophagy in the cellular energetic balance. Cell metabolism *13*, 495-504.

Singh, R., and Cuervo, A.M. (2012). Lipophagy: connecting autophagy and lipid metabolism. International journal of cell biology *2012*, 282041.

Smith, A.C., Heo, W.D., Braun, V., Jiang, X., Macrae, C., Casanova, J.E., Scidmore, M.A., Grinstein, S., Meyer, T., and Brumell, J.H. (2007). A network of Rab GTPases controls phagosome maturation and is modulated by Salmonella enterica serovar Typhimurium. The Journal of cell biology *176*, 263-268.

Solinas, G., Germano, G., Mantovani, A., and Allavena, P. (2009). Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. Journal of leukocyte biology *86*, 1065-1073.

Somersan, S., and Bhardwaj, N. (2001). Tethering and tickling: a new role for the phosphatidylserine receptor. The Journal of cell biology *155*, 501-504.

Sorkin, A., and von Zastrow, M. (2009). Endocytosis and signalling: intertwining molecular networks. Nature reviews Molecular cell biology *10*, 609-622.

Steinberg, D., and Witztum, J.L. (2010). Oxidized low-density lipoprotein and atherosclerosis. Arteriosclerosis, thrombosis, and vascular biology *30*, 2311-2316.

Stenmark, H. (2009). Rab GTPases as coordinators of vesicle traffic. Nature reviews Molecular cell biology *10*, 513-525.

Stenmark, H., Valencia, A., Martinez, O., Ullrich, O., Goud, B., and Zerial, M. (1994). Distinct structural elements of rab5 define its functional specificity. The EMBO journal *13*, 575-583.

Stone, G.W., Maehara, A., Lansky, A.J., de Bruyne, B., Cristea, E., Mintz, G.S., Mehran, R., McPherson, J., Farhat, N., Marso, S.P.*, et al.* (2011). A prospective natural-history study of coronary atherosclerosis. The New England journal of medicine *364*, 226-235.

Stuart, L.M., and Ezekowitz, R.A. (2005). Phagocytosis: elegant complexity. Immunity *22*, 539-550.

Su, J.H., Anderson, A.J., Cummings, B.J., and Cotman, C.W. (1994). Immunohistochemical evidence for apoptosis in Alzheimer's disease. Neuroreport *5*, 2529-2533.

Sudhof, T.C., and Rizo, J. (2011). Synaptic vesicle exocytosis. Cold Spring Harbor perspectives in biology *3*.

Sundaramurthy, V., Barsacchi, R., Samusik, N., Marsico, G., Gilleron, J., Kalaidzidis, I., Meyenhofer, F., Bickle, M., Kalaidzidis, Y., and Zerial, M. (2013). Integration of chemical and RNAi multiparametric profiles identifies triggers of intracellular mycobacterial killing. Cell host & microbe *13*, 129-142.

Svenning, S., Lamark, T., Krause, K., and Johansen, T. (2011). Plant NBR1 is a selective autophagy substrate and a functional hybrid of the mammalian autophagic adapters NBR1 and p62/SQSTM1. Autophagy *7*, 993-1010.

Swanson, J.A. (2008). Shaping cups into phagosomes and macropinosomes. Nature reviews Molecular cell biology *9*, 639-649.

Tabas, I. (2005). Consequences and therapeutic implications of macrophage apoptosis in atherosclerosis: the importance of lesion stage and phagocytic efficiency. Arteriosclerosis, thrombosis, and vascular biology *25*, 2255-2264.

Tabas, I. (2010). Macrophage death and defective inflammation resolution in atherosclerosis. Nature reviews Immunology *10*, 36-46.

Takizawa, F., Tsuji, S., and Nagasawa, S. (1996). Enhancement of macrophage phagocytosis upon iC3b deposition on apoptotic cells. FEBS letters *397*, 269-272.

Tall, A.R., Yvan-Charvet, L., Terasaka, N., Pagler, T., and Wang, N. (2008). HDL, ABC transporters, and cholesterol efflux: implications for the treatment of atherosclerosis. Cell metabolism *7*, 365-375.

Tang, X., Halleck, M.S., Schlegel, R.A., and Williamson, P. (1996). A subfamily of P-type ATPases with aminophospholipid transporting activity. Science *272*, 1495-1497.

Tanida, I., Minematsu-Ikeguchi, N., Ueno, T., and Kominami, E. (2005). Lysosomal turnover, but not a cellular level, of endogenous LC3 is a marker for autophagy. Autophagy *1*, 84-91.

Taylor, P.R., Martinez-Pomares, L., Stacey, M., Lin, H.H., Brown, G.D., and Gordon, S. (2005). Macrophage receptors and immune recognition. Annual review of immunology *23*, 901-944.

Thomas, L.B., Gates, D.J., Richfield, E.K., O'Brien, T.F., Schweitzer, J.B., and Steindler, D.A. (1995). DNA end labeling (TUNEL) in Huntington's disease and other neuropathological conditions. Experimental neurology *133*, 265-272.

Thorp, E.B. (2010). Mechanisms of failed apoptotic cell clearance by phagocyte subsets in cardiovascular disease. Apoptosis : an international journal on programmed cell death *15*, 1124-1136.

Thurston, T.L., Ryzhakov, G., Bloor, S., von Muhlinen, N., and Randow, F. (2009). The TBK1 adaptor and autophagy receptor NDP52 restricts the proliferation of ubiquitin-coated bacteria. Nature immunology *10*, 1215-1221.

Thurston, T.L., Wandel, M.P., von Muhlinen, N., Foeglein, A., and Randow, F. (2012). Galectin 8 targets damaged vesicles for autophagy to defend cells against bacterial invasion. Nature *482*, 414-418.

Tolkovsky, A.M. (2009). Mitophagy. Biochimica et biophysica acta *1793*, 1508-1515.

Tooze, S.A., and Yoshimori, T. (2010). The origin of the autophagosomal membrane. Nature cell biology *12*, 831-835.

Tosello-Trampont, A.C., Nakada-Tsukui, K., and Ravichandran, K.S. (2003). Engulfment of apoptotic cells is negatively regulated by Rho-mediated signaling. The Journal of biological chemistry *278*, 49911-49919.

Travassos, L.H., Carneiro, L.A., Ramjeet, M., Hussey, S., Kim, Y.G., Magalhaes, J.G., Yuan, L., Soares, F., Chea, E., Le Bourhis, L*., et al.* (2010). Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. Nature immunology *11*, 55-62.

Truman, L.A., Ford, C.A., Pasikowska, M., Pound, J.D., Wilkinson, S.J., Dumitriu, I.E., Melville, L., Melrose, L.A., Ogden, C.A., Nibbs, R*., et al.* (2008). CX3CL1/fractalkine is released from apoptotic lymphocytes to stimulate macrophage chemotaxis. Blood *112*, 5026-5036.

Turk, B., Turk, D., and Turk, V. (2000). Lysosomal cysteine proteases: more than scavengers. Biochimica et biophysica acta *1477*, 98-111.

Tyedmers, J., Mogk, A., and Bukau, B. (2010). Cellular strategies for controlling protein aggregation. Nature reviews Molecular cell biology *11*, 777-788.

Ullrich, O., Horiuchi, H., Bucci, C., and Zerial, M. (1994). Membrane association of Rab5 mediated by GDP-dissociation inhibitor and accompanied by GDP/GTP exchange. Nature *368*, 157-160.

Underhill, D.M., and Ozinsky, A. (2002). Phagocytosis of microbes: complexity in action. Annual review of immunology *20*, 825-852.

Uttenweiler, A., and Mayer, A. (2008). Microautophagy in the yeast Saccharomyces cerevisiae. Methods Mol Biol *445*, 245-259.

van den Eijnde, S.M., Boshart, L., Baehrecke, E.H., De Zeeuw, C.I., Reutelingsperger, C.P., and Vermeij-Keers, C. (1998). Cell surface exposure of phosphatidylserine during apoptosis is phylogenetically conserved. Apoptosis : an international journal on programmed cell death *3*, 9-16.

van der Wel, N., Hava, D., Houben, D., Fluitsma, D., van Zon, M., Pierson, J., Brenner, M., and Peters, P.J. (2007). M. tuberculosis and M. leprae translocate from the phagolysosome to the cytosol in myeloid cells. Cell *129*, 1287-1298.

van Ijzendoorn, S.C. (2006). Recycling endosomes. Journal of cell science *119*, 1679-1681.

van Lookeren Campagne, M., Wiesmann, C., and Brown, E.J. (2007). Macrophage complement receptors and pathogen clearance. Cellular microbiology *9*, 2095-2102.

Vandivier, R.W., Henson, P.M., and Douglas, I.S. (2006). Burying the dead: the impact of failed apoptotic cell removal (efferocytosis) on chronic inflammatory lung disease. Chest *129*, 1673-1682.

Vandivier, R.W., Ogden, C.A., Fadok, V.A., Hoffmann, P.R., Brown, K.K., Botto, M., Walport, M.J., Fisher, J.H., Henson, P.M., and Greene, K.E. (2002). Role of surfactant proteins A, D, and C1q in the clearance of apoptotic cells in vivo and in vitro: calreticulin and CD91 as a common collectin receptor complex. J Immunol *169*, 3978-3986.

Vaux, D.L., and Korsmeyer, S.J. (1999). Cell death in development. Cell *96*, 245-254.

Vellai, T., Takacs-Vellai, K., Sass, M., and Klionsky, D.J. (2009). The regulation of aging: does autophagy underlie longevity? Trends in cell biology *19*, 487-494.

Vergne, I., Chua, J., Singh, S.B., and Deretic, V. (2004). Cell biology of mycobacterium tuberculosis phagosome. Annual review of cell and developmental biology *20*, 367-394.

Viegas, M.S., Estronca, L.M., and Vieira, O.V. (2012). Comparison of the kinetics of maturation of phagosomes containing apoptotic cells and IgG-opsonized particles. PloS one *7*, e48391.

Vieira, O.V., Botelho, R.J., and Grinstein, S. (2002). Phagosome maturation: aging gracefully. The Biochemical journal *366*, 689-704.

Vieira, O.V., Botelho, R.J., Rameh, L., Brachmann, S.M., Matsuo, T., Davidson, H.W., Schreiber, A., Backer, J.M., Cantley, L.C., and Grinstein, S. (2001). Distinct roles of class I and class III phosphatidylinositol 3-kinases in phagosome formation and maturation. The Journal of cell biology *155*, 19-25.

Vieira, O.V., Bucci, C., Harrison, R.E., Trimble, W.S., Lanzetti, L., Gruenberg, J., Schreiber, A.D., Stahl, P.D., and Grinstein, S. (2003). Modulation of Rab5 and Rab7 recruitment to phagosomes by phosphatidylinositol 3-kinase. Molecular and cellular biology *23*, 2501-2514.

Virmani, R., Burke, A.P., Kolodgie, F.D., and Farb, A. (2002). Vulnerable plaque: the pathology of unstable coronary lesions. Journal of interventional cardiology *15*, 439-446.

von Muhlinen, N., Akutsu, M., Ravenhill, B.J., Foeglein, A., Bloor, S., Rutherford, T.J., Freund, S.M., Komander, D., and Randow, F. (2012). LC3C, bound selectively by a noncanonical LIR motif in NDP52, is required for antibacterial autophagy. Molecular cell *48*, 329-342.

Voss, V., Senft, C., Lang, V., Ronellenfitsch, M.W., Steinbach, J.P., Seifert, V., and Kogel, D. (2010). The pan-Bcl-2 inhibitor (-)-gossypol triggers autophagic cell death in malignant glioma. Molecular cancer research : MCR *8*, 1002-1016.

Waters, S., Marchbank, K., Solomon, E., Whitehouse, C., and Gautel, M. (2009). Interactions with LC3 and polyubiquitin chains link nbr1 to autophagic protein turnover. FEBS letters *583*, 1846-1852.

Weidberg, H., Shpilka, T., Shvets, E., and Elazar, Z. (2010a). Mammalian Atg8s: one is simply not enough. Autophagy *6*, 808-809.

Weidberg, H., Shvets, E., Shpilka, T., Shimron, F., Shinder, V., and Elazar, Z. (2010b). LC3 and GATE-16/GABARAP subfamilies are both essential yet act differently in autophagosome biogenesis. The EMBO journal *29*, 1792-1802.

References

World Health Organization (2011). Global Atlas on cardiovascular disease prevention and control, ISBN 978-92-4-156437-3. WHO Press, Geneva, Switzerland.

Wild, P., Farhan, H., McEwan, D.G., Wagner, S., Rogov, V.V., Brady, N.R., Richter, B., Korac, J., Waidmann, O., Choudhary, C*., et al.* (2011). Phosphorylation of the autophagy receptor optineurin restricts Salmonella growth. Science *333*, 228-233.

Williams, D.B. (2006). Beyond lectins: the calnexin/calreticulin chaperone system of the endoplasmic reticulum. Journal of cell science *119*, 615-623.

Williams, K.J., and Tabas, I. (1995). The response-to-retention hypothesis of early atherogenesis. Arteriosclerosis, thrombosis, and vascular biology *15*, 551-561.

Williams, R.L., and Urbe, S. (2007). The emerging shape of the ESCRT machinery. Nature reviews Molecular cell biology *8*, 355-368.

Wong, E., and Cuervo, A.M. (2010). Integration of clearance mechanisms: the proteasome and autophagy. Cold Spring Harbor perspectives in biology *2*, a006734.

Wright, S.D., and Silverstein, S.C. (1983). Receptors for C3b and C3bi promote phagocytosis but not the release of toxic oxygen from human phagocytes. The Journal of experimental medicine *158*, 2016-2023.

Wurmser, A.E., Sato, T.K., and Emr, S.D. (2000). New component of the vacuolar class C-Vps complex couples nucleotide exchange on the Ypt7 GTPase to SNARE-dependent docking and fusion. The Journal of cell biology *151*, 551-562.

Wyllie, A.H., Kerr, J.F., and Currie, A.R. (1980). Cell death: the significance of apoptosis. International review of cytology *68*, 251-306.

Xie, Z., Nair, U., and Klionsky, D.J. (2008). Atg8 controls phagophore expansion during autophagosome formation. Molecular biology of the cell *19*, 3290-3298.

Yang, Z., and Klionsky, D.J. (2010a). Eaten alive: a history of macroautophagy. Nature cell biology *12*, 814-822.

Yang, Z., and Klionsky, D.J. (2010b). Mammalian autophagy: core molecular machinery and signaling regulation. Current opinion in cell biology *22*, 124-131.

Ye, Y., and Rape, M. (2009). Building ubiquitin chains: E2 enzymes at work. Nature reviews Molecular cell biology *10*, 755-764.

Yeung, T., Ozdamar, B., Paroutis, P., and Grinstein, S. (2006). Lipid metabolism and dynamics during phagocytosis. Current opinion in cell biology *18*, 429-437.

Yordy, B., Tal, M.C., Hayashi, K., Arojo, O., and Iwasaki, A. (2013). Autophagy and selective deployment of Atg proteins in antiviral defense. International immunology *25*, 1-10.

Young, A.R., Narita, M., Ferreira, M., Kirschner, K., Sadaie, M., Darot, J.F., Tavare, S., Arakawa, S., Shimizu, S., and Watt, F.M. (2009). Autophagy mediates the mitotic senescence transition. Genes & development *23*, 798-803.

Yu, X., Lu, N., and Zhou, Z. (2008). Phagocytic receptor CED-1 initiates a signaling pathway for degrading engulfed apoptotic cells. PLoS biology *6*, e61.

Yu, X., Odera, S., Chuang, C.H., Lu, N., and Zhou, Z. (2006). C. elegans Dynamin mediates the signaling of phagocytic receptor CED-1 for the engulfment and degradation of apoptotic cells. Developmental cell *10*, 743-757.

Yu, Z.Q., Ni, T., Hong, B., Wang, H.Y., Jiang, F.J., Zou, S., Chen, Y., Zheng, X.L., Klionsky, D.J., Liang, Y.*, et al.* (2012). Dual roles of Atg8-PE deconjugation by Atg4 in autophagy. Autophagy *8*, 883-892.

Zhang, L., Kokkonen, G., and Roth, G.S. (1995). Identification of neuronal programmed cell death in situ in the striatum of normal adult rat brain and its relationship to neuronal death during aging. Brain research *677*, 177-179.

Zheng, Y.T., Shahnazari, S., Brech, A., Lamark, T., Johansen, T., and Brumell, J.H. (2009). The adaptor protein p62/SQSTM1 targets invading bacteria to the autophagy pathway. J Immunol *183*, 5909-5916.

Zhou, Z., and Yu, X. (2008). Phagosome maturation during the removal of apoptotic cells: receptors lead the way. Trends Cell Biol *18*, 474-485.

Zullig, S., Neukomm, L.J., Jovanovic, M., Charette, S.J., Lyssenko, N.N., Halleck, M.S., Reutelingsperger, C.P., Schlegel, R.A., and Hengartner, M.O. (2007). Aminophospholipid translocase TAT-1 promotes phosphatidylserine exposure during C. elegans apoptosis. Current biology : CB *17*, 994-999.