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# **(Auto)Phagocytosis in Atherosclerosis: Implications for Plaque Stability and Therapeutic Challenges**

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## **1. Introduction**

This chapter is intended to describe the interactions between cell death, phagocytosis and autophagic survival in atherosclerosis, and how these processes could be attractive therapeutic targets for atherosclerotic plaque stabilization.

Atherosclerosis is a long-term, progressive inflammatory disease characterized by the formation of atherosclerotic plaques in the intima of medium- and large-sized arteries. Progression of the disease is accelerated by well-known risk factors including gender, age, hypercholesterolemia, diabetes mellitus, hypertension, smoking, obesity and a sedentary life-style (Kannel et al., 2004). In the advanced stage, plaques can partially or totally occlude the blood vessel, known as arterial stenosis. However, not only the degree of stenosis, but also the composition and stability of the plaque determines the clinical outcome of the disease (Hansson, 2005). Indeed, plaques may become extremely unstable and prone to rupture through the presence of many inflammatory cells and mediators, a large necrotic core consisting of uncleared cell debris and lipids, a high degree of cell death leading to a scarce amount of smooth muscle cells, and the formation of leaky neovessels inside the plaque. Occlusive thrombi, as a result of plaque rupture, in turn cause acute (and often fatal) clinical manifestations, such as myocardial infarction and stroke. Current clinical therapy is focused on surgical interventions (stents, endarterectomy, bypass) and plasma cholesterol lowering drugs (e.g. statins). In addition, changes in diet and exercise have made significant inroads in preventing acute atherothrombotic events (Getz & Reardon, 2006). Although these aforementioned approaches have provided significant improvements, they are far from sufficient. Analysis of the cell death and phagocytosis machinery as well as survival strategies of cells in plaques could provide additional insights for development of new plaque stabilizing strategies.

## **2. Phagocytosis of apoptotic cells**

Billions of cells in the human body die each day by the process of apoptosis (Henson et al., 2001). A critical process, called phagocytosis, is coupled with each of these cell death events. Macrophages and nonprofessional phagocytes remove apoptotic cells (AC) via

phagocytosis in a rapid and efficient manner. This results in at least five critical protective effects: (1) it actively suppresses the secretion of inflammatory cytokines from the phagocyte, via autocrine/paracrine secretion of transforming growth factor beta (TGF $\beta$ ), prostaglandin E2 (PGE2), and platelet-activating factor (PAF) (Fadok et al., 1998), (2) it prevents leakage of proinflammatory intracellular material from the dying cell, before membrane damage occurs, (3) it triggers a potent survival pathway in the phagocyte, involving Akt, NF- $\kappa$ B and cholesterol efflux (Reddy et al., 2002; Kiss et al., 2006), (4) it can promote cell growth and wound healing through the release of vascular endothelial growth factor (VEGF) and TGF $\beta$ , respectively (Golpon et al., 2004; Sindrilaru et al., 2009), and (5) it has a vital role in maintaining immunological tolerance against cell-associated antigens, as the TGF $\beta$ , produced by the dying cells or the engulfing cells induce the differentiation of inducible regulatory T cells, which inhibit immune responses (Green et al., 2009). The entire process of phagocytosis (recognition, tethering, binding, internalization) is tightly regulated and involves a wide array of molecules, including receptors on the phagocyte, ligands on the AC and soluble bridging molecules (Figure 1). The multiplicity of ways in which phagocytes recognize and engulf AC suggests that a hierarchy of engulfment mechanisms and back-up mechanisms exists. Indeed, upon blocking essential receptors phagocytosis of AC is decreased but this inhibition is never complete, suggesting cooperation between different mechanisms of phagocytosis (Pradhan et al., 1997).

Changes in composition and molecular topology of the plasma membrane of the AC involve the loss of “don’t-eat-me” signals, also called self-associated molecular patterns (SAMPs), such as CD31 (Brown et al., 2002) and CD47 (Gardai et al., 2005) and the exposure of “eat-me” signals, also known as apoptotic cell-associated molecular patterns (ACAMPs). The most renowned ACAMP is the redistributed phosphatidylserine (PS) (Fadok et al., 1992, 1999; Callahan et al., 2000; Hoffmann et al., 2001), which on viable cells is actively confined to the inner leaflet of the plasma membrane. Annexin-1 is a caspase-dependent engulfment ligand that is recruited from the cytosol and exported to the outer plasma membrane leaflet, colocalizes with PS, and is required for efficient clearance of ACs (Arur et al., 2003). Similar to PS, calreticulin acts as a facultative recognition ligand on AC (Martins et al., 2010). Phagocyte pattern recognition receptors such as CD14 (Devitt et al., 1998; Schlegel et al., 1999), scavenger receptors such as CD36, and integrins such as  $\alpha$ v $\beta$ 3 (Savill et al., 1992), together with PS receptors such as BAI-1 (D. Park et al., 2007), TIM-4 (Miyanishi et al., 2007) and stabilin-2 (S.Y. Park et al., 2008), or PS bridging molecules such as MFG-E8 (lactadherin) and Gas-6 – which bind to PS on the AC and to integrins or MerTK on the phagocyte, respectively (Qingxian et al., 2010) – are well known phagocytic receptors. Upon engagement, strong tethering is achieved, the phagocytic synapse is set, and engulfment response pathways are activated. Other phagocyte receptors include an unidentified PS receptor (PSR), transglutaminase 2 (TG2) (Szondy et al., 2003), low density lipoprotein receptor-related protein 1 (LRP1) (Lillis et al., 2008), ATP binding cassette 7 (ABCA7), purinergic receptor P2Y2, CD91 (Martins et al., 2010) and fractalkine receptor 1 (CX3CR1).

Recognition signals and receptors are crucial for efficient phagocytosis. However, these signals can only be effective when the phagocyte and the AC are in close proximity. Soluble “come-get-me” signals are secreted by AC to attract phagocytes to sites of AC death (Grimsley & Ravichandran, 2003) (Table 1).

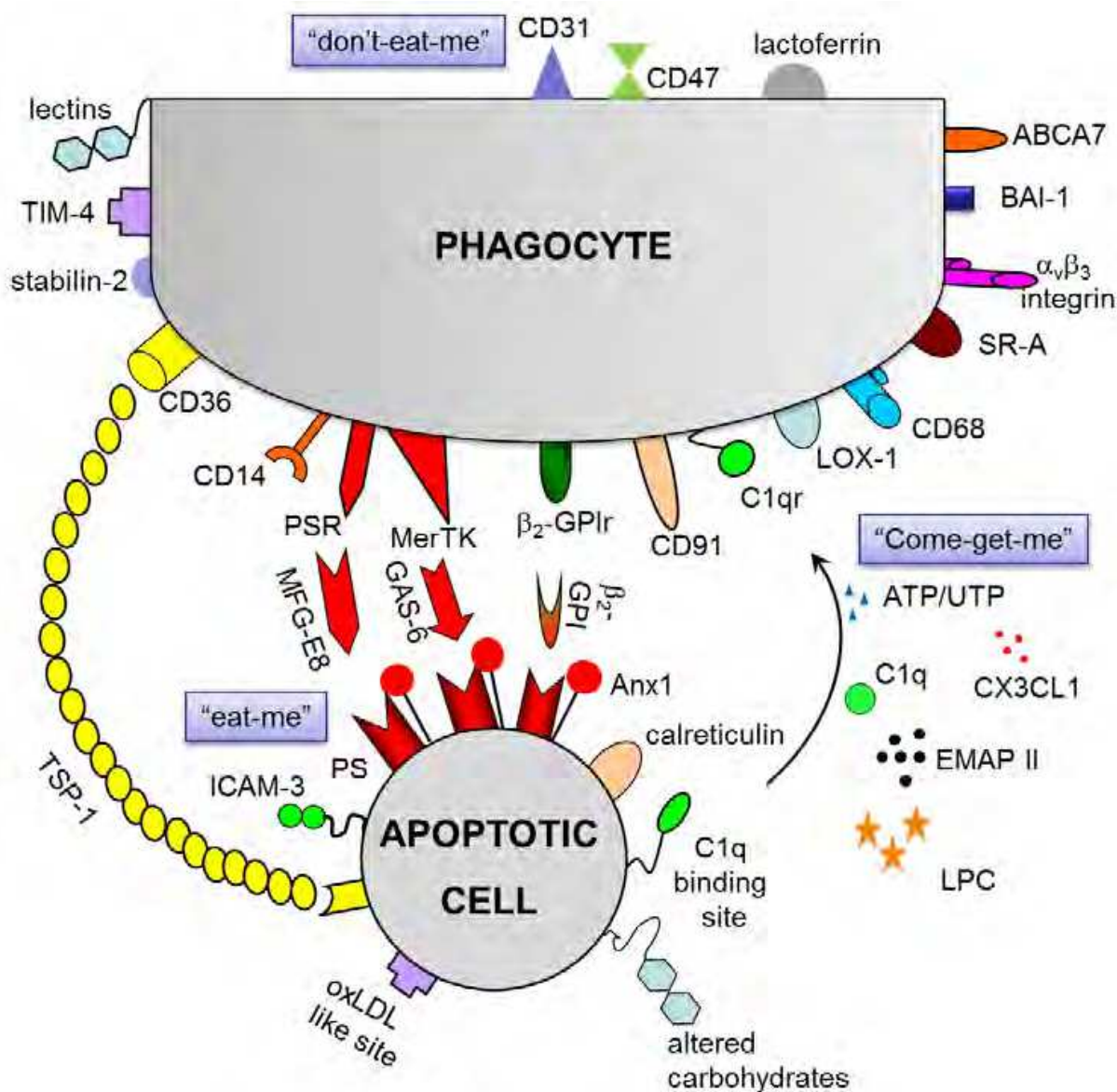


Fig. 1. Phagocyte recognition of apoptotic cells. Several receptors on the phagocyte membrane are involved and interact either directly with their ligands on the AC surface ("eat-me") or via bridging molecules. AC can send out chemoattractant signals to attract macrophages to the site of cell death ("come-get-me"). To prevent phagocytosis of healthy cells, the latter express molecules to identify themselves ("don't-eat-me"). ABCA7, ATP-binding cassette-7; CD14, lipopolysaccharide receptor; LOX-1, lectin-like oxidized low density lipoprotein receptor-1; CD36, thrombospondin receptor; CD68, macrophage scavenger receptor class A; FcR, Fc fragment of immunoglobulin G receptor; LRP, LDL receptor-related protein; CD91,  $\alpha_2$ -macroglobulin; GAS-6, growth arrest-specific gene 6;  $\beta_2$ -GPI, beta 2-glycoprotein 2; TSP, thrombospondin; PS, phosphatidylserine; PSR, PS receptor;  $\alpha_v\beta_3$ , vitronectin receptor; MFG-E8, milk-fat globule epidermal growth factor 8; ICAM-3, intracellular adhesion molecule-1; C1q, complement complex 1q; Anx1, annexin 1; BAI-1, brain angiogenesis inhibitor-1; TIM-4, T-cell immunoglobulin and mucin domain-containing protein 4; MerTK, c-met proto-oncogene tyrosine kinase; LPC, lysophosphatidylcholine; CX3CL1, fractalkine; EMAP II, endothelial monocyte-activating polypeptide II.



The first identified attraction signal of AC was the covalent dimer of ribosomal protein S19 (dRP S19) (Horino et al., 1998). It is an essential monocyte chemoattractant in the synovium of patients with rheumatoid arthritis (Nishiura et al., 1996). By using neutralizing antibodies, G-protein coupled receptor CD88 was identified as a crucial receptor for dRP S19. The most characterized AC-derived attractant is lysophosphatidylcholine (LPC), which is secreted in a caspase-3 dependent manner (Lauber et al., 2003). LPC exerts its chemotactic effect on monocytes via the G-protein coupled receptor G2A (Peter et al., 2008). Knockout mice that are deficient in G2A develop a typical autoimmune phenotype resembling systemic lupus erythematosus, which was already known to be caused by deficiencies in AC recognition (Le et al., 2001). Another AC-derived attraction signal is the endothelial monocyte-activating polypeptide II (EMAP II) that is proteolytically cleaved during apoptosis but not during necrosis to mature and release the active p23 protein (Knies et al., 1998). Processing of pro-EMAP II was observed to be a rather late event in the course of apoptosis, occurring several hours after poly (ADP-ribose) polymerase cleavage. Since AC are phagocytised at an early stage, it was suggested that mature EMAP II serves as a back-up signal at sites of excessive cell death (Behrendorf et al., 2000). Importantly, *in vitro* experiments showed that human pro-EMAP II is not cleaved by caspases and mature EMAP II could not be found in AC, as opposed to murine EMAP II. Thus, it is not entirely clear whether mature EMAP II or its precursor is the real cytokine (Martinet et al., 2010). The only receptor that has been reported in the context of EMAP II is CX3CR1, but it remains to be elucidated whether this is the crucial sensor for EMAP II p23 (Hou et al., 2006). C1q facilitates the ingestion of apoptotic cells by human macrophages via binding to calreticulin (Ogden et al., 2001). Thrombospondin-1 (TSP1) modulates a number of processes, including migration, proliferation and angiogenesis and was also found to be actively secreted during apoptosis (Savill et al., 1992). It serves as a bridging molecule to physically connect the phagocyte to the AC, though the phagocyte sensor involved remains obscure. Recently, nucleotides such as ATP and UTP, which can be recognized by purinergic receptor P2Y<sub>2</sub>, were found to be released from AC and incite migration of monocytes and macrophages (Elliott et al., 2009). Membrane-bound fractalkine (CX3CL1) can be released from AC by a so far unknown protease, to induce recruitment of macrophages (Truman et al., 2008). The CX3CR1-fractalkine system seems to be particularly important in germinal centers, and is limited to certain cell types, such as B cells and neurons. Two less characterized AC-derived chemokines are sphingosine-1-phosphate (S1P) and the N-terminal fragment of tyrosyl tRNA synthetases (mini TyrRS) (Gude et al., 2008; Wakasugi & Schimmel, 1999). Both were identified in *in vitro* settings and might contribute to the anti-inflammatory character of AC clearance. While it is clear that apoptosis can be executed in the absence of blebbing and that phagocytic engulfment is not restricted to 'bite-sized' apoptotic bodies, it has been suggested that fragmentation of the AC may be important for chemotactic signaling to mononuclear phagocytes (Segundo et al., 1999; Hugel et al., 2005; Simons & Raposo, 2009). Yet, the range of action and the phagocyte sensors remain to be resolved.

Note that plasma membrane-damaged cells (i.e. late apoptotic and necrotic cells) can also release or expose endogenous and exogenous signals, known as alarmins or damage-associated molecular patterns (DAMPs), to alert the organism to tissue injury (Patel et al., 2006; Peter et al., 2010) (Table 1). One such protein is the high mobility group box 1 protein (HMGB-1), which is released from necrotic cells, but not during apoptosis, and actively stimulates the secretion of proinflammatory cytokines (Scaffidi et al., 2002; Andersson et al.,

2000). Toll like receptors (TLR)-2 and 4 have been described to be engaged in HMGB-1 signaling (Yu et al., 2006). Another necrotic cell-derived danger signal, that is closely related to HMGB-1 is the hepatoma-derived growth factor (HDGF) (Nakamura et al., 1994). It is mainly located in the nucleus and remains trapped there during apoptosis, but is passively released during necrosis. The family of the calgranulin proteins, also referred to as the S100 proteins, are potent chemoattractant proteins and play a significant role in the propagation of inflammation (Donato, 2003; Foell et al., 2008). Specifically, the S100A8-S100A9 complex and S100A12 proved to be useful diagnostic markers of inflammatory diseases, like arthritis and COPD, where they are found in high concentrations in the blood, sputum, stool and synovial fluid (Foell et al., 2004). Future studies need to clarify whether these proteins also represent therapeutic targets. Heat shock proteins (HSP) 70, 90 and 98 are released during heat-induced necrosis, probably via exosomes (Basu et al., 2000). Yet, knowledge about the secretion and exact functions is confusing and needs clarification. For example, apart from their TLR-mediated pro-inflammatory signaling, HSP also display anti-inflammatory effects (Wieten et al., 2007; Tang et al., 2007). Finally, concentrations of uric acid are increased during cell injury. Uric acid has the ability to prime T cells in the generation of helper and cytotoxic T cells (Shi et al., 2002; Ghaemi-Oskouie & Shi, 2011).

“don’t-eat-me”	“eat-me”	“come-get-me”	“alarmins”
CD31	PS	LPC	HMGB-1
CD47	Anx1	EMAP-II (p23)	HDGF
lactoferrin	calreticulin	ATP/UTP	S100 proteins
		TSP1	HSPs
		CX3CL1	uric acid
		C1q	
		dRP S19	
		mini TyrRS	
		S1P	
		microblebs	

Table 1. Comprehensive list of signals that are presented and/or secreted by healthy cells (“don’t-eat-me”), apoptotic cells (cell surface “eat-me” signals and soluble “come-get-me” signals) or necrotic cells (“alarmins”). Abbreviations: PS, phosphatidylserine; Anx1, annexin 1; LPC, lysophosphatidylcholine, EMAP-II (p23), endothelial monocyte-activating polypeptide II active protein; TSP1, thrombospondin 1; CX3CL1, fractalkine; C1q, complement complex 1q; dRP S19, dimer of ribosomal protein S19; mini TyrRS, N-terminal fraction tyrosyl tRNA synthetase; S1P, sphingosine-1-phosphate; HMGB-1, high mobility group box 1 protein; HDGF, hepatoma-derived growth factor; S100 proteins, calgranulin family of proteins; HSP, heat-shock protein.

Recognition of necrotic cells by phagocytes is less well understood than recognition of ACs, but an increasing number of recent studies are highlighting its importance. (Krysko et al., 2006). C1q, mannose-binding lectin (MBL) and pentraxin-3 (PTX3) were demonstrated exclusively on late ACs and necrotic cells (Gaipf et al., 2001; Nauta et al., 2003; Rovere et al., 2000). C-reactive protein (CRP), an acute phase protein binds to secondary necrotic neutrophils (Hart et al., 2005). However, CRP does not uniquely bind to necrotic cells. The

same is true for TSP1. Future studies are needed to identify the differences and similarities between the macrophage interaction with AC and necrotic cells. A pitfall in this area is the lack of a good cell culture system to study this. Current cell culture models are still very variable, displaying a mixture of early and late ACs and necrosis. In addition, detailed information on mechanisms of binding and internalization of necrotic cells is sparse. Importantly, the trigger provoking necrotic death has an important impact on the kind of immunological response of phagocytes (Hirt & Leist, 2003). Phagocytic uptake of late apoptotic/necrotic cells by antigen-presenting cells, such as DCs, can lead to the presentation of autoantigens to autoreactive T cells in a proinflammatory context, thus facilitating the onset of an autoimmune response (Poon et al., 2010). However, there are several studies reporting non-inflammatory responses of phagocytes, including modulation of anti-tumor immunity and tissue repair via VEGF induction (M. Li et al., 2001).

For more comprehensive details of the molecular complexity of the clearance phases and engulfment signaling pathways, the reader is referred to recent reviews (Elliott & Ravichandran, 2010; Kinchen 2010; Poon et al., 2010; Devitt & Marshall, 2011).

### 2.1 Phagocytosis of apoptotic cells in atherosclerosis

In early human atherosclerotic plaques, phagocytic clearance of AC by macrophages is very efficient (Tabas, 2005) (Figure 2). Conversely, phagocytosis in advanced human atherosclerotic plaques is defective, resulting in secondary necrosis, expansion of the necrotic core and increased plaque vulnerability (Schrijvers et al., 2005; Thorp & Tabas, 2009; Thorp, 2010). Similar results have been found in mouse models of advanced atherosclerosis. The impact of defective clearance of AC on the plaque further exacerbated when essential effector molecules in phagocytosis were targeted. Examples of these effector molecules are MerTK (Thorp et al., 2008; Ait-Oufella et al., 2008), MFG-E8 (Ait-Oufella et al., 2007), C1q (Bhatia et al., 2007), LRP-1 (Yancey et al., 2010), TSP1 (Moura et al., 2008), TG2 (Boisvert et al., 2006) and ABCA7 (Jehle et al., 2006).

The mechanisms of defective phagocytosis in advanced atherosclerotic plaques are not entirely clear, although *in vitro* experiments have put forward several plausible explanations (Schrijvers et al., 2005). It is unlikely that overwhelming apoptosis is a major factor, since phagocytosis is very efficient in early plaques, where phagocytosis is not defective, when excessive apoptosis is induced through genetic manipulation (Tabas, 2010). In addition, AC in plaques are found in the vicinity of macrophages, which rules out a possible geographic problem. Moreover, atherosclerotic plaques are highly inflamed tissue and contain large areas of macrophage infiltration. Therefore, it is not the quantity of the phagocytes that is important for phagocytosis, but their functionality. Most likely, phagocytosis itself becomes ineffective in advanced plaques.

One of the first discovered mechanisms that contribute to defective clearance of AC in atherosclerosis is the competition of oxidized low density lipoproteins (oxLDL), but not acetylated LDL, with AC and oxidatively damaged cells for macrophage binding (Sambrano & Steinberg, 1995). AC, oxLDL as well as oxidized red blood cells share oxidatively modified moieties on their surfaces that serve as ligands for macrophage recognition, thereby profoundly inhibiting the phagocytosis of AC. Furthermore, secretion of MFG-E8 (lactadherin), a macrophage-derived molecule that facilitates phagocytosis of AC, is down-regulated upon free cholesterol-loading in macrophages (Su et al., 2005). Electron microscopy images from macrophage-derived foam cells in atherosclerotic plaques show

that these cells are often crammed with large lipid droplets. It is therefore tempting to speculate that these macrophages are no longer able to engulf AC ("full is full"). Uptake of AC in macrophages treated *in vitro* with an excess of platelets or aggregated LDL is not inhibited, most likely because foam cells in culture rarely reach the same degree of foam cell formation as seen in human plaques, but phagocytosis of beads results in a nearly complete inhibition of phagocytosis of AC (Schrijvers et al., 2005). These findings confirm a study by Moller et al. (2002) showing that uptake of indigestible, rigid particles by macrophages induces cellular 'stiffening' so that changes in cellular shape, which are needed to form pseudopodia for phagocytosis, are inhibited. In addition to foam cell formation, increased oxidative stress is a hallmark of advanced atherosclerotic plaques. Although several reactive oxygen species (ROS) can be found in human plaques, peroxynitrite (ONOO<sup>-</sup>) plays a central role in the pathophysiology of atherosclerosis as it induces protein nitrosylation and oxidative DNA damage. The peroxynitrite donor SIN1A concentration-dependently decreases phagocytosis of AC *in vitro* (Schrijvers et al., 2005), suggesting that PS or other factors present on macrophages or dying cells required for phagocytosis may be sensitive to oxidative conditions. Proteolytic cleavage of macrophage receptors exacerbates disease in cystic fibrosis and bronchiectasis (Vandivier et al., 2002). Potent proteases, such as matrix metalloproteinases and ADAM proteins (a disintegrin and metalloproteinase) are upregulated in advanced plaques. *In vitro* cleavage of MerTK leads to generation of soluble Mer, that binds to AC and prevents their uptake by macrophages (Sather et al., 2007). Interestingly, gene polymorphisms in CD31 ("don't eat me") and increasing levels of its soluble form have been associated with ischemic stroke, suggesting that extracellular CD31 could affect phagocyte interactions with AC (Wei et al., 2009). These, and other ideas, will require careful assessment in advanced atherosclerotic plaques.

The accumulation of AC has a number of consequences that promote plaque progression and destabilization (Figure 2). Firstly, impaired phagocytosis of AC results in enhanced secretion of proinflammatory cytokines including TNF $\alpha$  and IL-6 by the phagocyte, and a reduced release of anti-inflammatory proteins such as TGF $\beta$  and IL-10 (Fadok et al., 1998; Erwig & Henson, 2007). Deregulated expression of these cytokines may result in inflammatory autoimmune responses, as seen in systemic lupus erythematosus and rheumatoid arthritis, two non-atherosclerotic inflammatory disorders with a similar defect in phagocytosis of dying cells by macrophages. Secondly, tissue factor (TF) expression colocalizes with AC, especially around the lipid core of human atherosclerotic plaques, suggesting that uncleared AC are an important source of this molecule (Tedgui & Mallat, 2001). TF is a key element in the initiation of the coagulation cascade and mediates thrombus formation after rupture of an unstable plaque, when free AC are exposed to the blood stream. Thirdly, macrophages that have ingested cholesterol-loaded AC show a remarkable set of survival responses, some of which are not present during the loading of macrophages with lipoprotein-derived cholesterol (Cui et al., 2007). These responses include cholesterol esterification and massive cholesterol efflux, as well as the triggering of cell survival signal transduction pathways involving PI3-kinase/Akt and NF- $\kappa$ B. Consequently, impaired phagocytosis of AC renders the phagocyte more susceptible to cholesterol-induced death (Yvan-Charvet et al., 2010). Finally, expansion of the necrotic core is stimulated due to secondary necrosis of free AC. The interaction of macrophages with necrotic cells or compounds released from necrotic cells (alarmins) often results in an additional inflammatory response (Scaffidi et al., 2002). Necrotic cells also passively release matrix



degrading proteases. In this way, post-apoptotic necrosis may contribute to plaque instability through enlargement of the necrotic core, matrix breakdown and stimulation of inflammation and neovascularization (Figure 2).

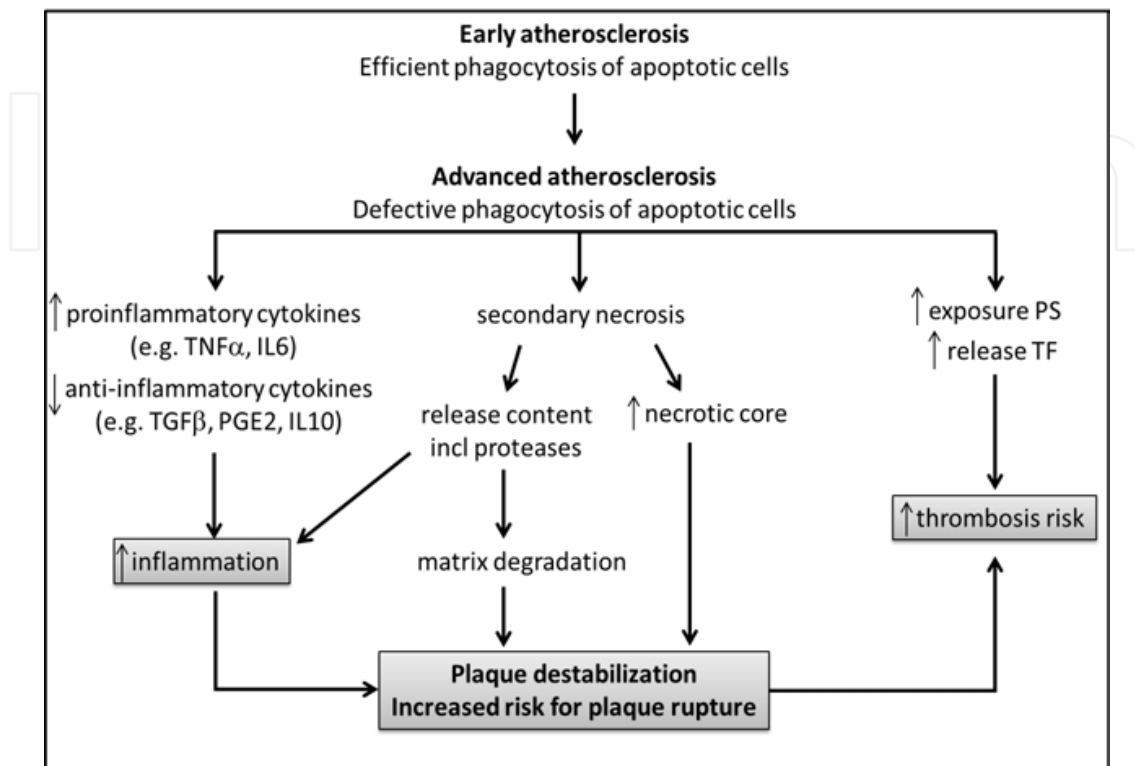


Fig. 2. Consequences of defective phagocytosis of apoptotic cells in atherosclerotic plaques. In early plaques, AC numbers are low and phagocytosis is very efficient, which stimulates active suppression of inflammatory pathways, prevents release of intracellular contents from the dying cells and promotes macrophage survival. In advanced plaques however, phagocytosis of ACs is severely impaired, leading to plaque progression and destabilization through various mechanisms. Unstable plaques are at risk for rupture, which results in the formation of an occlusive thrombus and subsequent, possible fatale, ischemic events. In addition, ACs expose phosphatidylserine (PS) and are a source of tissue factor (TF), both highly thrombogenic.

## 2.2 Therapeutic possibilities

Defective AC clearance can alter immune responses in ways that exacerbate human diseases, e.g. acceleration of SLE, COPD, cystic fibrosis and atherosclerosis. Therefore, a promising approach to stabilize atherosclerotic plaques would be to therapeutically enhance the phagocytic capacity of macrophages. Importantly, non-specific enhancement of phagocytosis is not desirable, because of the adverse effects associated with phagocytosis of lipoproteins, platelets or erythrocytes (Schrijvers et al., 2007).

Statins are an important class of drugs in the treatment of atherosclerosis. They were discovered as drugs that reduce cholesterol levels by inhibiting the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), but they also have an anti-inflammatory action by virtue of their ability to block prenylation of signaling molecules, i.e. Rho GTPases. Interestingly, it has been shown that lovastatin increases phagocytosis of ACs by

macrophages from COPD patients in an HMG-CoA reductase-dependent manner (Morimoto et al., 2006). In contrast, simvastatin impairs Fc-receptor mediated phagocytosis of bacteria (phagocytosis followed by oxidative burst and bacterial killing) and at the same time enhances the production of proinflammatory mediators (Benati et al., 2010). These data should be assessed to prevent potential side-effects in patients undergoing long-term treatment with this statin, who become exposed to an opportunistic pathogen such as *S. aureus*, that is commonly found on the skin and in the upper airways.

Because oxLDL or oxLDL antibodies interfere with phagocytosis of AC, it is tempting to speculate that antioxidants can improve clearance of dying cells. However, clinical trials in humans with antioxidants showed only limited success in preventing coronary artery disease. Possibly the antioxidants lose activity too quickly or do not penetrate the plaque deep enough to reach the phagocytes. This idea is supported by the fact that antioxidants do not prevent the oxidation of phagocytized lipoproteins in the lysosome of macrophages (Wen & Leake, 2007). It is possible that more encouraging results will be obtained in the future through the use of drugs that are targeted to specific oxidation reactions in atherosclerosis. It should also be noted that certain antioxidants may not promote but inhibit recognition of AC by phagocytes by inhibiting oxidation of externalized PS (Tyurina et al., 2004).

In obesity and type 2 diabetes, the defect in efferocytosis of macrophages seems to be related to increased concentrations of saturated fatty acids (FA) and/or decreased concentrations of the  $\omega$ -3 FA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (S. Li et al., 2009). This change in membrane lipid composition may lead to defective PI3K activation and failure to generate PIP3 in the macrophage phagocytic membrane. Interestingly, feeding ob/ob mice a fish oil diet increases  $\omega$ -3 FA without appreciable changes in the content of saturated FA and leads to a reversal of the defect in efferocytosis and a reduction in plaque size (S. Li et al., 2009). These findings suggest that the level of  $\omega$ -3 FA in the macrophage membrane is an important factor contributing to efficient efferocytosis.

Examples of other drugs that promote phagocytic clearance of AC are chemerin, an endogenous chemoattractant (Cash et al., 2010), the macrolide antibiotic azithromycin (Hodge et al., 2006), members of the lipoxin family (Godson et al., 2000), inflammation resolution mediators such as resolvins (Serhan et al., 2008), inhibitors of MerTK cleavage (Sather et al., 2007), PPAR $\delta$  activators (Mukundan et al., 2009), LXR activators which increase expression of MerTK, as well as compounds that induce secretion of the efferocytosis effector apolipoprotein E and ABCA1/G1-mediated efflux of toxic sterols (A-Gonzalez et al., 2009; Yancey et al., 2010; Yvan-Charvet et al., 2010).

Phagocyte populations in atherosclerosis are heterogeneous (Waldo et al., 2008; Shimada, 2009) and express different markers and phagocytic receptors, depending on their spatial location in the plaque and surrounding milieu. *In vitro*, alternatively activated macrophages (M2 phenotype) preferentially clear AC and secrete anti-inflammatory cytokines, including IL-10 and TGF $\beta$  (W. Xu et al., 2006). On the other hand, classically activated (M1) macrophages secrete proinflammatory cytokines, such as TNF $\alpha$  and IL-6, that can negatively affect phagocytosis. It would be interesting to elucidate whether communication between these subsets affects phagocytosis efficiency. Plaque phagocytes can polarize towards a spectrum of activation states, resulting in altered phagocytic capacities (Peng et al., 2009). Taking advantage of the fact that some subpopulations are more efficient phagocytes, might be a very attractive way to restore and enhance defective

AC clearance in atherosclerotic plaques. For example, activation of PPAR $\gamma$  stimulates macrophage polarization to the anti-inflammatory M2 phenotype (Bouhrel et al., 2007). Whether or not these aforementioned approaches will be effective at stabilizing atherosclerotic plaques through the amelioration of phagocytic clearance remains to be elucidated.

### 3. Autophagy

#### 3.1 General principles

While phagocytosis reflects clearance of non-self particles, autophagy represents a process for cells to dispose of their own unwanted or damaged proteins and organelles (Klionsky, 2007). Autophagy or "self eating" refers to a conserved cellular process for the turnover of organelles and proteins that occurs in all eukaryotic cells (Yoshimori, 2004). It is activated as an adaptive response to environmental stress (e.g. nutrient deprivation, hypoxia, oxidative stress, exposure to xenobiotics) to promote cell survival through the recycling of precursors (amino acids, free fatty acids, nucleotides) derived from the degradation of endogenous cellular components. Typical of autophagy is the formation of double-membrane structures, called phagophores, that engulf intracellular material such as protein aggregates, lipid droplets and complete organelles for degradation (Figure 3). The phagophore expands and, on completion, forms an autophagosome, which then fuses with lysosomes, thereby generating an autophagolysosome (Shintani & Klionsky, 2004). Incorporation of the outer autophagosomal membrane in the lysosomal membrane allows the degradation of the remaining inner single membrane and the cytoplasmic content of the autophagosome by lysosomal hydrolases.

Autophagy generally acts as a housekeeping mechanism, and is crucially involved in the maintenance of normal cellular homeostasis. Hereby, turnover of damaged or dysfunctional organelles, such as mitochondria, peroxisomes and endoplasmic reticulum, is facilitated to maintain a healthy population of these organelles. When stimulated by cellular stress conditions, autophagy functions as a self-cannibalization pathway that promotes cell survival in an unfavorable environment. This pro-survival function of autophagy has been demonstrated at the cellular and tissue level in different contexts, such as nutrient and growth factor deprivation, endoplasmic reticulum stress, development, microbial infection, and diseases characterized by the accumulation of protein aggregates. Preclinical studies have demonstrated that autophagy is associated with cancer, neurodegenerative disorders (e.g. Alzheimer's, Parkinson's and Huntington's disease), embryogenesis, aging and immunity, but also with cardiovascular disease including ischemia-reperfusion injury of the heart, cardiomyopathy and atherosclerosis (Mathew et al., 2007; Levine & Kroemer, 2008; Mizushima et al., 2008; Martinet & De Meyer, 2008,2009a). Despite tremendous recent advances in this field, the functional significance of autophagy in human disease remains incompletely understood, and potentially involves both adaptive and maladaptive outcomes.

There are three main types of autophagy, namely macroautophagy, microautophagy or chaperone-mediated autophagy (Klionsky et al., 2007). Macroautophagy is the most extensively analysed and quantitatively more important type of autophagy in which entire portions of the cytosol are sequestered by a de novo-formed phagophore that seals into an autophagosome (Figure 3). In addition to this 'in bulk' degradation, selective sequestration of cellular structures into autophagosomes is a common mechanism for removal of

organelles or particulate protein complexes (aggregates). This is then referred to as selective macroautophagy. The process of microautophagy is poorly characterized, at least in mammalian cells. Like in macroautophagy, entire cytosolic regions constitute the microautophagy cargo, but it is the lysosomal membrane itself that invaginates or protrudes to seclude the cytosolic components. Selective autophagy of soluble cytosolic proteins can occur via chaperone-mediated autophagy, the only type of autophagy that requires unfolding of the protein cargo before internalization into lysosomes (Dice, 2007). Selectivity is determined via the recognition of a pentapeptide in the amino acid sequence of the substrate proteins that are delivered to the surface of the lysosomes by a cytosolic chaperone (Hsc70). To reach the lysosomal lumen, these proteins interact with the lysosome-associated protein type 2A (LAMP-2A) receptor, and a complex of lysosomal chaperones at both sides of the lysosomal membrane.

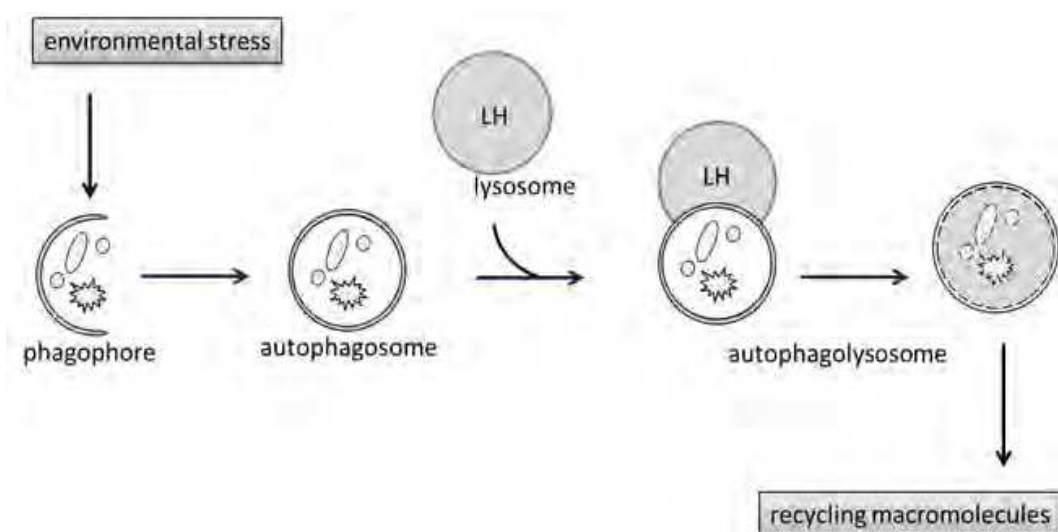


Fig. 3. Schematic depiction of the autophagy process. A phagophore is formed at the initiation of autophagy, in response to environmental stress (e.g. nutrient deprivation) to sequester cytoplasmic components for degradation. Upon completion, the phagophore forms a double-membraned vesicle called an autophagosome. After fusion with a lysosome, the content is degraded by lysosomal hydrolases (LH). Following breakdown, the resulting macromolecules (e.g. nucleic acids, amino acids, free fatty acids) are released back into the cytosol for reuse by the cell.

### 3.2 Molecular mechanisms of autophagy

The molecular machinery and signaling cascades that regulate autophagy are very complex (Ravikumar et al., 2010; Yang & Klionsky, 2010). Briefly, the key regulator of autophagy in human and murine cells is the mammalian target of rapamycin (mTOR) kinase, which suppresses autophagy in conditions of sufficient nutrients and growth factors, via class I phosphatidylinositol-3-kinases (PI3Ks) and Akt. Activation of the mTOR complex 1 (mTORC1) - and consequent repression of autophagy - can also be mediated by mitogen-activated protein kinases (MAPKs), by activation of the p90 ribosomal S6 kinase, as well as by the Wnt signaling pathway. Other prominent regulators of autophagy include (but are not limited to): AMP-activated protein kinase (AMPK), which inhibits mTOR in response to reduced ATP levels; eukaryotic translation initiation factor 2 $\alpha$  (eIF2 $\alpha$ ), which responds to



nutrient deprivation; c-Jun N-terminal kinase (JNK), which is involved in multiple signaling cascades activated by stressful conditions; members of the Bcl-2 protein family that contain a single Bcl-2 homology (BH) domain, the so-called BH3-only proteins, which displace (and hence derepress) the essential autophagy modulator Beclin 1 from inhibitory complexes with Bcl-2 or Bcl-XL; Sirtuin 1, which responds to high NAD<sup>+</sup> levels, de facto acting as a sensor of nutrient availability; the oncosuppressor protein p53, which inhibits autophagy when present in the cytoplasm; the I $\kappa$ B kinase (IKK) complex, which is also essential for the activation of NF- $\kappa$ B by stress; the inositol 1,4,5-trisphosphate (IP3) receptor (IP3R) at the level of the ER and transglutaminase 2. Finally, autophagy is positively regulated by the transcription factor activity of E2F1, FoxO3a, NF- $\kappa$ B and p53 among others.

### 3.3 Autophagy in atherosclerosis

Autophagy in atherosclerotic plaques has not yet been analysed in full detail due to technical limitations, in particular the lack of adequate marker proteins. As a consequence, the role of autophagy in atherosclerosis is unclear and currently under intense investigation. However, transmission electron microscopy of smooth muscle cells (SMCs) in the fibrous cap of advanced plaques revealed ultrastructural features of autophagy such as vacuolization and formation of myelin figures (Martinet & De Meyer, 2009b). The latter structures, composed of phospholipids and membrane fragments, refer to autophagic degradation of membranous cellular components. Moreover, Western blot analysis of human carotid plaques showed processing of microtubule-associated protein 1 light chain 3 (LC3) into the autophagosome-specific isoform LC3-II, indicating activation of autophagy. Importantly, these kind of data have to be interpreted with great caution. Autophagy is a dynamic process: formation of autophagosomes, followed by their degradation after lysosomal fusion (Figure 3). Many groups, including our own, have measured increased autophagosome formation or LC3 processing, and labelled this as increased autophagic activity. However, these parameters can also reflect impaired degradation. Therefore, it is essential to measure autophagic flux (Gottlieb & Mentzer, 2010). In tissue, this can be very challenging. A very elegant method now widely accepted to measure flux is using a tandem fusion protein of LC3 with the red acid-insensitive mCherry (or RFP) and the green acid-sensitive GFP proteins to measure formation of autolysosomes and their degradation (Kimura et al., 2007). GFP fluorescence is quenched in the acidic environment of the lysosome, while mCherry (or RFP) fluorescence is stable at low pH. As a consequence, detection of yellow fluorescence (overlap of GFP and mCherry) reflects impaired flux, and the presence of red fluorescence reflects quenching of GFP and thus ongoing degradation in the lysosome (Mizushima et al., 2010). Besides LC3, levels of other autophagy substrates can be used for measuring autophagic flux, of which the best studied is p62, an adaptor protein that links ubiquitinated proteins to LC3. Accumulation of p62 reflects impaired degradation in the autophagolysosome, whereas low p62 levels indicate active degradation. Unfortunately, no data is available on p62 levels during atherosclerotic plaque formation and destabilization.

*In vitro* studies identified several potential triggers for autophagy that are present in atherosclerotic plaques, such as inflammation (Heymann, 2006; Jia et al., 2006), ROS production (Scherz-Shouval & Elazar, 2011), accumulation of oxidized lipoproteins (Muller et al., 2011; Nowicki et al., 2007) and endoplasmic reticulum stress (Qin et al., 2010). Also hypoxia is a common feature of advanced human atherosclerotic plaques (Sluimer et al.,

2008), caused by inadequate vascularization, nutrient and growth factor deprivation, a well-known condition leading to induction of autophagy (Azad et al., 2008).

### 3.3.1 Dual role of autophagy in atherosclerosis

Autophagy can protect plaque cells against oxidative stress by degrading damaged intracellular material, in particular polarized mitochondria (Kiffin et al., 2006). In this way, successful autophagy of the damaged components promotes cell survival by limiting the release of pro-apoptotic proteins such as cytochrome c into the cytosol (Gozuacik & Kimchi, 2004). The protective role of autophagy in atherosclerosis was illustrated by *in vitro* findings showing that death of smooth muscle cells (SMC) induced by low concentrations of statins is attenuated by the autophagy inducer 7-ketocholesterol (Martinet et al., 2008). Also excess free cholesterol or exposure of SMCs to lipid peroxidation products such as 4-hydroxynonenal activates autophagy, thereby prolonging SMC survival (K. Xu et al., 2010). Also, autophagy safeguards efficient phagocytosis of AC (Qu et al., 2007), which is beneficial for plaque stability (Figure 2). Altogether, induction of autophagy represents a vital component of a general stress response in vascular cells and could therefore be an important determinant of the stability of atherosclerotic plaques (Figure 4).

In contrast, excessive autophagy in SMCs or endothelial cells may cause autophagic cell death (Levine & Yuan, 2005). SMC death in turn results in plaque destabilization owing to the reduced synthesis of collagen and thinning of the fibrous cap. Also endothelial cell death may be detrimental for the structure of the plaque as endothelial injury and/or death represents a primary mechanism for acute clinical events by promoting lesional thrombosis. In view of plaque stability and regression, these observations might advise against excessive autophagy induction in plaques. Conversely, autophagic death of macrophages is considered a promising approach to stabilize advanced plaques (Martinet et al., 2007).

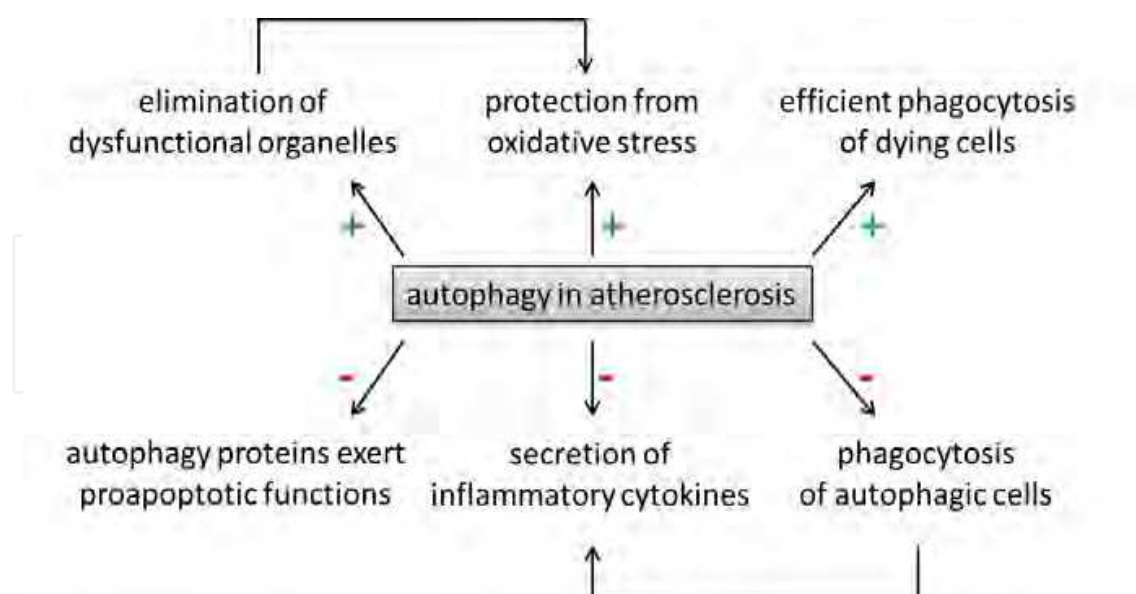


Fig. 4. Consequences of autophagy in atherosclerosis. While autophagy contributes beneficially to plaque stability by protecting cells from oxidative stress and ensuring efficient clearance of dead cells, it can also exert functions that are unfavorable in terms of plaque stability. Autophagy has a very potent inflammatory component and is very tightly linked to other forms of cell death, especially apoptosis.

However, recent evidence indicates that phagocytosis of cells dying through autophagy results in an inflammatory response through inflammasome activation, IL-1 $\beta$  release and subsequent IL-6 and TNF $\alpha$  induction in neighboring phagocytes (Petrovski et al., 2007) (Figure 4). Moreover, autophagy proteins themselves can exert proapoptotic functions. For example, Beclin-1 (Atg6), a key protein involved in autophagy, can interact with the anti-apoptotic proteins Bcl-2 or Bcl-xL. This interaction is pro-apoptotic and antiautophagic, at least under non-autophagic stimuli. However, during autophagic conditions, Beclin-1 binding to Bcl-xL is antiapoptotic (Pattingre et al., 2005; Wang, 2008). In addition, Atg5 (when cleaved by activated calpain) can bind to Bcl-xL and induce apoptosis (Yousefi et al., 2006; Yousefi & Simon, 2007). On the other hand, inactivation of autophagy genes (e.g. Atg5) also causes cell death (Komatsu et al., 2006), making matters even more complicated. These data demonstrate that the crosstalk between autophagy and apoptosis should not be neglected when designing interventions aimed at stabilizing atherosclerotic plaques via autophagy.

### 3.3.2 Is autophagy an interesting drug target for plaque stabilization?

Pharmacological modulation of autophagy can be achieved by targeting key players in the autophagy machinery (Rubinzstein et al., 2007). For example, rapamycin or its derivatives (e.g. everolimus) inhibit mTOR, thereby activating autophagy. Another way to modulate autophagy is to target the mTOR-independent route, mainly regulated by inositol-1,4,5-triphosphate (IP3) levels. This can be achieved with drugs such as lithium, sodium valproate and carbamazepine. The first line of evidence that demonstrates the beneficial effects of autophagy induction in atherosclerosis was obtained after stent-based delivery of the rapamycin derivative everolimus in plaques of cholesterol-fed rabbits (Verheye et al., 2007). This treatment resulted in a marked reduction of macrophages via autophagic cell death without affecting the SMCs. However, recent *in vitro* work in our laboratory has demonstrated that pharmacological induction of autophagy in macrophages triggers secretion of proinflammatory cytokines such as IL-6, MCP1 and TNF $\alpha$  (Martinet, De Meyer, unpublished data), suggesting that the autophagic process is not immunologically silent. This cocktail of cytokines has the potential to induce SMC death and exacerbate the inflammatory infiltration of leukocytes in the plaque. Combined treatment with anti-inflammatory agents such as glucocorticosteroids may help to prevent the adverse effects of drug-induced autophagy. On the other hand, induction of autophagy in free cholesterol-loaded SMCs by rapamycin inhibited AC death (K. Xu et al., 2010), possibly through the degradation of dysfunctional organelles such as mitochondria and endoplasmic reticulum. These findings provide a rationale for the use of rapamycin or analogs such as everolimus to stabilize advanced atherosclerotic plaques.

Several studies have shed light on the potential detrimental consequences of autophagy inhibition with regard to plaque stability. Firstly, autophagy is essential for efficient phagocytosis of dying cells (Qu et al., 2007). ACs derived from cells lacking essential autophagy genes (Atg5 or beclin-1) fail to present the “eat-me” signal PS and secrete lower levels of the “come-get-me” signal lysophosphatidylcholine (Qu et al., 2007). This will result in a marked decrease in the engulfment of apoptotic corpses, enhanced plaque necrosis and recruitment of inflammatory cells (Gautier et al., 2009). Thus, inhibition of autophagy may further destabilize advanced plaques, where phagocytosis of ACs is already compromised (Schrijvers et al., 2005). Additionally, inhibition of autophagy using 3-methyladenine in free

cholesterol-treated SMCs enhances apoptosis and necrosis (K. Xu et al., 2010), indicating that a strong interaction exists between autophagy and other forms of cell death (Maiuri et al., 2007). Of note, altering autophagy using pharmacological agents can be problematic, as they can interfere with general processes, such as glucose metabolism (e.g. metformin, deoxyglucose) or mitochondrial respiration (e.g. oligomycin). Broad-spectrum PI3 kinase inhibitors wortmannin and 3-methyladenine, widely used in autophagy research, inhibit Akt activation. Thus, results using these agents must always be interpreted carefully.

#### 4. Concluding remarks

(Auto)phagocytosis plays a key role in maintaining atherosclerotic plaque stability (Figure 5). Efficient phagocytosis of AC is required to prevent accumulation of dead cells, expansion of the necrotic core in plaques and inflammatory responses resulting from secondary necrosis of residual AC. These factors will undoubtedly promote plaque inflammation, destabilization and rupture, leading to possible fatal ischemic events (e.g. myocardial infarction and stroke). Autophagy may be viewed as a “cry for survival,” and such survival is the result of an adaptive response to fight against cellular stress. If this stress—such as oxidative damage in atherosclerotic plaques—is mild, autophagy is activated in order to

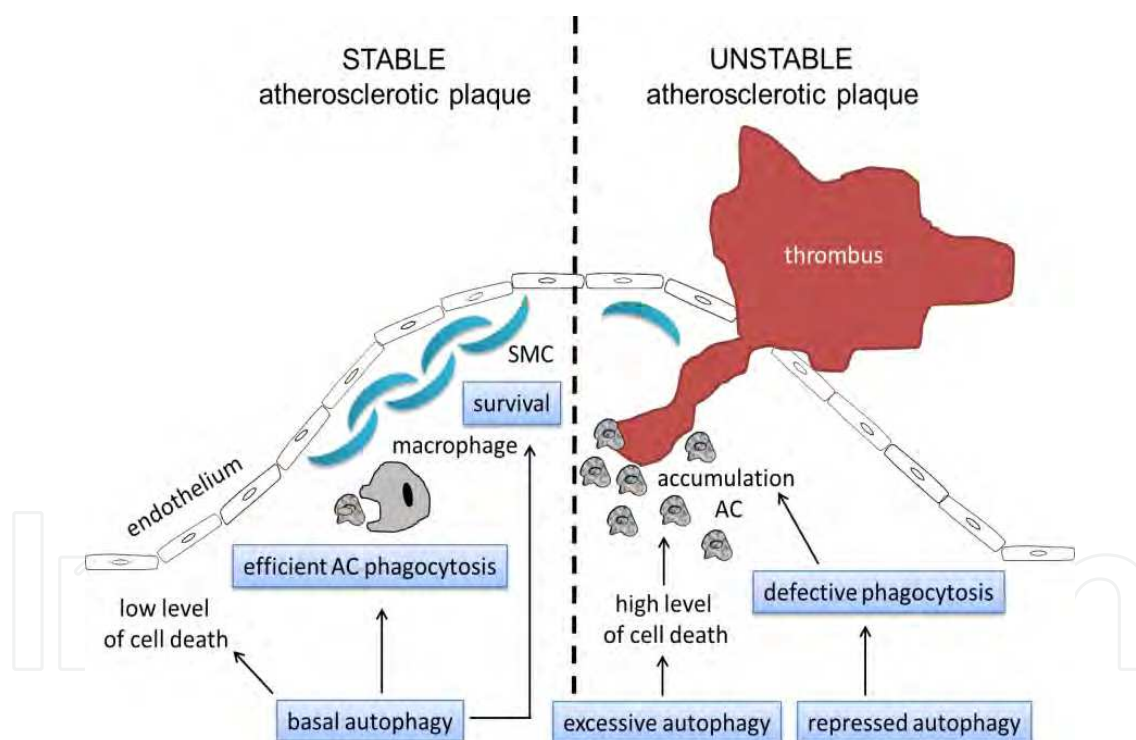


Fig. 5. Implications of dysfunctional phagocytosis and autophagy on the stability of atherosclerotic plaques. In stable plaques, AC numbers are low and phagocytosis is very efficient. Basal autophagy contributes to maintain efficient phagocytosis and promote cell survival. In unstable plaques, however, phagocytosis of AC is severely impaired, leading to accumulation of AC, expansion of the necrotic core, thereby increasing the risk for plaque rupture, which results in the formation of an occlusive thrombus. Decreased autophagy probably worsens phagocytosis efficiency, which is already compromised in advanced plaques, whereas excessive autophagy can result in cell death and inflammatory responses.



survive. On the other hand, if cellular stress is overwhelming or continuous, the adaptive response fails and cells undergo an autophagic type of death. Crossbreeding of mouse models for atherosclerosis (e.g. ApoE or LDL receptor knockout animals) with autophagy-deficient mice (e.g. conditional Atg5 or Atg7 knockout animals) will undoubtedly shed more light on the potential role of autophagy in atherosclerosis. Based on the literature and promising experiments in our lab and that of others, it is definite that cells need autophagy to be able to survive under stressful circumstances. Therefore, controlled (moderate) induction of autophagy, but not excessive induction or inhibition of autophagy, seems to be a promising strategy for stabilization of atherosclerotic plaques. The challenge for the future in the stabilization of atherosclerotic plaques will be to turn on the protective pro-survival effects of autophagy in a selective manner, without activating unwanted death pathways or proinflammatory signaling cascades. With progress being made in tissue-specific drug targeting using nanoparticles, one potential future approach to stabilize atherosclerotic plaques would be to develop macrophage-specific phagocytosis and/or autophagy inducers combined with anti-inflammatory drugs. However, stimulation of autophagy can only be beneficial if autophagic flux is not impaired, since this condition could lead to lysosomal leakage or ejection of autophagosomes as well as the induction of cell death. Altogether, (auto)phagocytosis is a crucial process involved in atherogenesis that significantly affects the stability of the atherosclerotic plaque.

## 5. Acknowledgements

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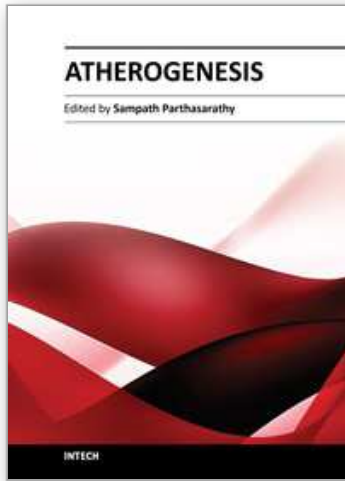


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