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"The Walking Dead: Macrophage inflammation and death in atherosclerosis"

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Abstract

Purpose of review—To highlight recent studies that describes novel inflammatory and signaling mechanisms that regulate macrophage death in atherosclerosis.

Recent Findings—Macrophages contribute to all stages of atherosclerosis. The traditional dogma states that in homeostatic conditions, macrophages undergo apoptosis and are efficiently phagocytosed to be cleared by a process called efferocytosis. In advanced atherosclerosis, however, defective efferocytosis results in secondary necrosis of these uncleared apoptotic cells, which ultimately contributes to the formation of the characteristic necrotic core and the vulnerable plaque. Here, we outline the different types of lesional macrophage death: apoptosis, autophagic and the newly defined necroptosis (i.e. a type of programmed necrosis). Recent discoveries demonstrate that macrophage necroptosis directly contributes to necrotic core formation and plaque instability. Further, promoting the resolution of inflammation using pre-resolving mediators have been shown to enhance efferocytosis and decrease plaque vulnerability. Lastly, the canonical “don't eat me” signal CD47 has recently been described as playing an important role in atherosclerotic lesion progression by impairing efficient efferocytosis. Although we have made significant strides in improving our understanding of cell death and clearance mechanisms in atherosclerosis, there still remains unanswered questions as to how these pathways can be harnessed using therapeutics to promote lesion regression and disease stability.

Summary—Improving our understanding of the mechanisms that regulate macrophage death in atherosclerosis, in particular apoptosis, necroptosis and efferocytosis, will provide novel therapeutic opportunities to resolve atherosclerosis and promote plaque stability.

Keywords

atherosclerosis; cell death; necroptosis; apoptosis; autophagy

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Introduction

Atherosclerosis is a chronic lipid-driven, maladaptive and non-resolving inflammatory disease of the vessel wall. Over several decades, atherosclerosis progresses in indolence and silence, eventually resulting in the formation of a life-threatening, rupture-prone atherosclerotic plaque. The mechanisms of atherosclerosis [1] and the pathophysiology of human atherosclerotic vulnerable plaques have been reviewed elsewhere [2,3]. In brief, the disease is triggered by sub-endothelial retention of infiltrated low-density lipoproteins (LDL) into the intimal space. The accumulated LDL undergoes oxidation and aggregation in the intima, presenting a source of chronic stimuli that instigates and propagates an innate immune reaction. This includes the recruitment, homing, migration and differentiation of monocytes into macrophages that avidly phagocytose modified cholesterol, secrete pro-inflammatory cytokines, enzymes and reactive oxygen species (ROS) and eventually undergo cell death. Together, these processes and the defective clearance of the dying cells (aka 'efferocytosis') propagate the inflammatory, forming a vulnerable atherosclerotic plaque. The key characteristics of a vulnerable plaque includes a very thin fibrous cap encapsulating acellular necrotic core region(s), lipid-laden macrophages and T lymphocytes and sometimes, intraplaque hemorrhage [4]. This review outlines recent insights into inflammatory and cell signaling mechanisms that regulate macrophage death in the formation of a vulnerable plaque.

The Fate of the Macrophage in Atherosclerotic Lesions

Atherosclerotic lesional macrophages have extraordinary plasticity and were historically thought to be derived entirely from circulating blood monocytes until recently, where macrophage-like cells have been shown to originate from smooth muscle cells [5,6]. Regardless of their lineage, these macrophages orchestrate a plethora of biological functions, including cholesterol efflux, phagocytosis, efferocytosis, proliferation, migration, secretion of pro-inflammatory and pro-resolving molecules and cell death, in response to various micro-environmental cues. Although *in vitro*, macrophages have been broadly classified into M1 pro-inflammatory (classical) and M2 anti-inflammatory (alternative), *in vivo* macrophages appear to be more transient and heterogeneous in their expression profiles. To date, based on their stimulus and functionality, *in vivo* macrophages have been classified as either pro-inflammatory and pro-atherogenic (i.e. M1, M4 or Mox etc) or anti-inflammatory and atheroprotective (i.e. Mhem, M(Hb) or M2)[7]. However, it's likely that the role of any single macrophage during its lifespan within the atherosclerotic lesion is more fluid and it could potentially transdifferentiate into another subset and/or express markers from different subsets simultaneously. Current and future studies identifying appropriate markers for macrophage type and lineage (i.e. SMC-derived versus monocyte-derived) will shed more light into macrophage classification and function.

Macrophage Death and the Vulnerable Plaque

The concept of cell death in atherosclerosis was suggested in the mid 1800's by Virchow, however it wasn't until Kerr *et al* in 1972, that the term 'apoptosis' or "falling off" was coined [8]. Although the precise mechanisms that regulate macrophage death remains to be

fully elucidated, the conventional dogma suggests that in early macrophage-rich lesions (i.e. fatty streaks), apoptotic macrophages are thought to be efficiently cleared by efferocytosis and thus, impede the accumulation of necrotic corpses and boost the resolution of inflammation [9–11]. However, as lesions progress and continue to accumulate free cholesterol, oxidized lipoproteins and fatty acids, macrophages undergo increased apoptotic cell death [12–14]. This combined with defective efferocytosis either due to an overwhelmed inflammatory response and/or impaired function of efferocytes (described in detail below), these apoptotic cells remain in the intima and ultimately undergo “post-apoptotic” or “secondary” necrosis. To date, secondary necrosis has been viewed as an unregulated process by which these apoptotic cells lose membrane integrity and release their entire cellular content, amplifying the inflammatory response and atherosclerotic lesion development [11,15]. Recent studies demonstrate that lesional macrophages can undergo several different types of death (Figure 1), including apoptosis, autophagic death and necroptosis and these pathways are outlined in this review.

Cell Death Pathways in Atherogenic Macrophages

Apoptosis

Apoptosis, also sometimes broadly referred to as programmed cell death (PCD), involves tightly controlled and regulated signaling pathways that induces cell membrane shrinkage, blebbing, nuclear and cytoplasmic condensation and cellular fragmentation, sending out a non-inflammatory and ‘tolerate me’ signal [8]. Apoptosis can be triggered extrinsically via the death receptor initiated pathway, or intrinsically via the mitochondria, with both pathways culminating in the activation of caspases and subsequent cell death (extensively reviewed in [16,17]). The extrinsic apoptotic pathway is initiated upon activation of death receptors from the tumour necrosis factor (TNF) receptor (TNFR) superfamily including TNFR1, Fas, death receptor-4 and -5 (DR4, DR5). These receptors are activated upon engagement of their cognate ligand on the cell membrane: TNF (for TNFR1), Fas ligand (for Fas) and TNF-related apoptosis-inducing ligand (TRAIL; for DR4/5) leading to recruitment of the death inducing signaling complex (DISC), consisting of Fas-associated death domain (FADD) and pro-caspase-8 (or -10). DISC formation and recruitment to the ligand-receptor complex then initiates the activation of caspase-3 resulting in apoptosis of cells (Figure 1). Unlike ligand-receptor mediated caspase activation, the intrinsic apoptotic pathway requires intracellular triggers to stimulate apoptosis. Here, signals including DNA damage, induce translocation of pro-apoptotic proteins BAX (BCL-2-associated X protein) and BAK (BCL-2 antagonist) to the mitochondrial outer membrane, promoting loss of mitochondrial transmembrane potential and the release of cytochrome *c* from the mitochondria to the cytosol. Cytochrome *c* binds Apaf-1 forming the “apoptosome”, resulting in caspase-9 and caspase-3 activation, fragmentation of DNA, membrane blebbing and, finally, cell death. Importantly, there is evidence of cross-talk between the extrinsic and intrinsic pathways, including Fas-mediated cell death can cleave Bid to activate the intrinsic pathway [18]. Similarly, TRAIL-DNA damage apoptotic synergy involves the activation of Bid by TRAIL [19].

To date, a number of atherogenic factors *in vitro* have been identified to induce macrophage apoptosis, including IL-23, TNF α , Fas, nitric oxide, hypoxia, high concentrations of oxidized LDL (oxLDL), mitochondrial dysfunction, insulin resistance, oxysterols, and intracellular accumulation of unesterified cholesterol [20–22]. However, *in vivo*, macrophage apoptosis is most likely multifactorial. In early- to mid-stage atherosclerosis, monocyte-derived macrophages engulf free cholesterol or modified-lipoproteins. This cholesterol is trafficked and esterified to cholesteryl esters (CE) to form lipid droplets [23]. However, should excess free cholesterol accumulate, this promotes ER stress, activates the unfolded protein response (UPR) and its effector C/EBP-homologous protein (CHOP) that consequently increases calmodulin kinase II (CaMKII) activity, death receptor signaling and mitochondrial dysfunction mediated apoptosis [13,24]. Deletion of various pro-apoptotic signaling targets, including Bax or the apoptotic inhibitor 6 (AIM/Spa/Ap16), in atherosclerotic apoE^{-/-} or Ldlr^{-/-} mice increases macrophage apoptosis and reduces early atherosclerosis [25,26]. Consistently, suppression of downstream apoptotic signaling (e.g. knocking out Jnk1) in the same model reduces macrophage apoptosis and increases lesion size [27]. Further, deleting TRAIL from, apoE^{-/-} mice, not only exacerbates atherosclerotic lesion progression but also its closely associated co-morbidity, diet-induced diabetes, implicating an important role for TRAIL-mediated apoptosis in reducing these cardiometabolic diseases [28]. Taken together, these studies suggest that specifically targeting molecules that promote macrophage apoptosis in nascent lesions could reduce early atherosclerotic disease progression.

Autophagy

Autophagy or “eating of self” was first described in the 1960’s as a protective mechanism by which cellular contents are recycled to promote survival in adverse environments [29]. To date, three forms of autophagy have been described: macro-, micro- and chaperone-mediated, all of which stimulate degradation of damaged organelles and unwanted proteins within lysosomes (reviewed in [30–32]). Macro-autophagy (herein referred to as autophagy) is the most common and widely studied form. In response to starvation and other stresses (e.g. hypoxia, infection), this catabolic process involves *de novo* formation of a limiting membrane (phagophore), which sequesters unwanted cytosolic components, protein aggregates and organelles into double membrane-bound vesicles or autophagosomes. A series of autophagy-related (Atg) proteins are recruited to the phagophore to tightly control the critical steps of autophagic process, including vesicle nucleation, elongation and completion (Figure 1). The mature autophagosome then fuses with the lysosome to create autolysosomes, where unwanted cytosolic components are degraded and recycled. In a fed-state, the well-known upstream regulators of autophagy, including insulin receptor, Phosphoinositide 3-kinase (PI3K) and the nutrient-sensing kinase, mechanistic target of rapamycin (mTOR), limit autophagy signaling. In contrast, during starvation, mTOR is inactivated to promote autophagy. In macrophage foam cells in early stages of atherosclerosis, lipophagy, a type of macroautophagy that targets cytosolic lipid droplets, plays an important role in hydrolyzing, recycling and processing triglycerides and cholesteryl esters for energy utilization (e.g. fatty acids for β -oxidation in the mitochondria) or removal of excess cholesterol (e.g. free cholesterol for macrophage cholesterol efflux) (reviewed in [33]). Damage-associated molecular patterns (DAMPs), toxins or cytokines

(e.g. $\text{INF}\gamma$, TNF, CD40L) can also induce inflammasome-dependent or -independent macroautophagy [34]. When autophagy is overstimulated, it is thought that cells eventually undergo autophagic cell death, possibly via Beclin-2 interaction with Bcl-2 in the mitochondria, followed by early degradation of organelles [35]. Unlike apoptosis, cytoskeletal elements remain intact until a later stage whereas autophagic cell death results in a 'tolerate-me' and non-inflammatory signal in a similar manner to apoptosis.

In advanced atherosclerosis, the various stimuli present in the atherosclerotic micro-environment suppresses autophagic mechanisms, ultimately resulting in defective autophagy in both pre-clinical experimental models [36–39] and in human carotid atherosclerotic tissue [40,41]. However, the precise mechanisms by which macrophages decide to undergo apoptotic or autophagic cell death remains to be fully elucidated as the inducers and the downstream signaling pathways overlap [42]. It is postulated that environments where nutrients are limited might activate autophagy as the default pathway to increase metabolism that is pro-survival. However, in an environment that has overwhelming stimuli (e.g. oxLDL, cholesterol crystals, death receptor activation), rapid apoptotic cell death pathways may be promoted instead of autophagy. For example, there is increased expression of the autophagic chaperone protein, p62 (SQSTM1), a marker of autophagic dysfunction, in mouse and human macrophages during atherosclerotic lesion progression [38]. In macrophage foam cells, the p62-enriched cytoplasmic inclusion bodies prevent cytotoxic protein aggregates in the absence of autophagy and thus, promote macrophage inflammasome activation and apoptosis [43]. Lastly, promising therapeutic interventions targeting the inflammasome by the NLRP3 inflammasome inhibitor, arglabin redirects macrophages to autophagic pathways, reducing inflammation and atherosclerosis [44]. Thus, promoting macrophage autophagy directly or indirectly by inhibiting inflammatory and cell death pathways may present novel therapeutic approaches to treat atherosclerotic disease.

Necroptosis

Until the last decade, necrosis was thought to be unregulated, accidental cell death that is the consequence of cell injury. However, the discovery of a class of receptor-interacting protein kinase (RIP) 1 and 3-targeting inhibitors, necrostatins, has led to the discovery of a process called necroptosis, which is a type of programmed necrosis [45]. Since then, necroptosis has been defined as a regulated cell death pathway that is caspase-independent and RIP1-, RIP3- and pseudokinase mixed lineage kinase domain-like protein (MLKL)-dependent lethal signaling pathway (Figure 1). Necroptosis activation results in cell rounding, cytoplasmic swelling, loss of membrane integrity and release of the entire intracellular contents or DAMPs, features commonly used to describe necrotic cells [46]. Necroptosis is initiated by the RIP1 phosphorylating RIP3 (necrosome complex I), which in turn, phosphorylates MLKL molecules that oligomerize into multimer complexes (necrosome complex II) that puncture the cell membrane [47,48]. RIP1 is a serine/threonine kinase that exerts strategic control over the cell's fate through tightly regulated ubiquitylation (pro-survival) and deubiquitylation (pro-death), resulting in the activation of (i) $\text{NF}\kappa\text{B}$ mediated inflammatory and survival pathways; (ii) caspase-dependent apoptosis (non-inflammatory); and/or (iii) RIP3-MLKL mediated necroptosis (inflammatory) [49–51]. Thus, RIP1 is recognized as a master regulator at the crossroads of inflammation and cell survival; however, dissecting the

different roles of RIP1 *in vivo* has been hampered by the perinatal lethality of RIP1 knockout mice [52–54]. Initially thought to entirely drive necroptosis, RIP3 now is known to also activate NLRP3 inflammasome activation and related inflammatory responses (e.g. IL-1 β secretion) [49–51] and re-direct cells to apoptotic pathways when necroptotic pathways are challenged [55]. Thus, phosphorylation of MLKL (pMLKL) and/or the MLKL complex are definitive indicators of the activation of necroptosis. The majority of the studies characterising necroptotic pathways in diseases have relied mostly or entirely on the non-physiological, pharmacological pan-caspase inhibitor, zVAD in combination with ligand of interest (e.g. TNF α) to block apoptosis to favor activation of necroptosis [56,57]. Although these studies have yielded tremendous insight into the players in these cell death pathways, the ‘decision’ of a cell to undergo necroptosis or apoptosis is tightly coordinated by overlapping cluster of regulatory molecules, including caspase 8, cellular inhibitors of apoptosis proteins (cIAPs), deubiquitinases A20 and cylindromatosis (CYLD) and cellular FLICE-like inhibitor protein (cFLIP) [58].

Given the presence of large necrotic core within atherosclerotic lesions, it was postulated that necroptosis also occurs in atherosclerosis. Indeed, a role for RIP3 in atherosclerosis was recently identified, where RIP3^{-/-} mice crossed to apoE^{-/-} mice develop less complex atherosclerotic lesions [59] and present with reduced inflammation, potentially through the dual decrease in necroptosis and activation of NLRP3 inflammasome, which is known to be activated in lesional macrophages [60]. However, the definitive activation of macrophage necroptosis (i.e. pMLKL or MLKL complexes) and the precise mechanisms by which physiological stimuli induce necroptosis in atherosclerosis was unclear. Our group recently demonstrated the presence of pMLKL within necrotic regions in both mouse and human advanced atherosclerotic lesions and the increased expression of both necroptotic genes, RIP3 and MLKL, in advanced human lesions [61]. Consistently, increased protein expression of RIP1 and RIP3 was detected in human atherosclerotic samples [62]. Mechanistically, sterile atherogenic ligand oxLDL or DAMPS released from necrotic cells can drive reactive oxygen species (ROS)-mediated increase in RIP3 and MLKL gene expression that ultimately drives macrophage necroptosis [61]. Consistent with the genetic studies, in apoE^{-/-} mice, we used a pharmacological inhibitor (Nec-1s) to block necroptosis activation and found that Nec-1s reduced lesion size and necrotic core formation, even when lesions were already established. However, the role of other functions of MLKL, independent of death (i.e. scaffold function in inflammasome activation [63] and the intersection of these complex pathways in atherosclerosis are yet to be deciphered.

Clearance and removal of dead cells: Efferocytosis

The prompt and efficient removal of dead cells by efferocytosis is essential to maintain tissue homeostasis. Phagocytosis of apoptotic cells through engulfment of condensed membrane-bound particles of various sizes is kinetically and quantitatively more efficient than that of necrotic cells, which are internalized as small cellular particles [64]. Efferocytosis is mediated through the interaction between phagocytic receptors (e.g. MerTK, SR-B1, LRP-1) and apoptotic cell ligands [(e.g. ‘eat-me’ signal, phosphatidylserine (PS), apoE] with the assistance of bridging molecules (e.g. Gas6) [65–69]. In advanced atherosclerosis, the efferocytotic process is defective, resulting in the accumulation of

apoptotic cells that are thought to undergo secondary necrosis [1,10]. Defective efferocytosis can result from phagocyte oxidative stress (due to reduced cholesterol efflux post-apoptotic cell engulfment [70]) and/or the cleavage of MerTK receptors by ADAM17, inhibiting the ability of efferocytes to engulf apoptotic cells [71]. A number of studies have identified other key players Cathepsin G and ERK5, in regulating efficient efferocytosis in atherosclerotic mouse models and necrotic core regions [72,73]. Consistent with previous observations [65], we observed that necroptotic macrophages are efferocytosed less effectively compared to apoptotic cells [61] which suggests that this may contribute directly to necrotic debris accumulation in the plaque. Of note, a recent study shows that phagocytosis of autophagic dead cells can activate the inflammasome and related inflammatory pathways that secrete IL-1 β , IL-6 and TNF α to neighbouring phagocytes [74], emphasizing the importance of the type of cell death in promoting clearance by efferocytosis. Nonetheless, whether primary necrotic or autophagic cells within the atherosclerotic lesions are directly contributing to the defective efferocytosis *in vivo* remains to be elucidated. Interestingly, administering specialized pro-resolving mediators such as Resolvin D1, Resolvin D2 or Maresin 1 to atherosclerotic Ldlr^{-/-} or apoE^{-/-} mice decreases oxidative stress and necrosis, promoting improved efferocytosis and stable atherosclerotic plaques, presenting novel therapeutic strategies to reduce the disease burden [75–77]. Recently, a study by Kojima et al [78] show that unengulfed macrophages and SMCs in advanced mouse and human atherosclerotic lesions express the “don't eat me” signal, CD47 in response to TNF α and NF κ B mediated activation. CD47 binds to its receptor signal regulatory protein (SIRP)- α on the efferocytes and prevents effective efferocytosis. Further and most significantly, delivery of anti-CD47 (or anti-TNF α) in atherosclerotic mouse models reversed the defective efferocytosis, increased apoptotic cell clearance and decreased overall atherosclerotic lesion size, identifying a novel therapeutic approach to treat atherosclerosis. Whether lesional macrophages undergoing necroptosis also express increased CD47 [79] that contributes to the decreased phagocytosis of these cells is yet to be investigated.

Concluding Remarks

Although we have made significant strides in the discovery of intricate mechanisms by which macrophage cell death and inflammatory pathways perpetuate atherosclerotic fibroatheroma progression, we have only hit the tip of the iceberg in our understanding of these mechanisms and many questions remain unanswered: What is the physiological ligand that blocks apoptosis and favors necroptosis *in vivo*? Which molecules connect the different cell death pathways? Can efferocytosis be restored therapeutically? Similarly, can necroptotic pathways be re-directed towards apoptosis to enable efficient clearance through efferocytosis? Overall, there are several potential opportunities to characterize the exact mechanisms that control macrophage cell death, which could potentially identify novel therapeutic targets to treat atherosclerosis.

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Abbreviations/Acronyms

EC	Endothelial cell
LDL	Low-density lipoprotein
SMC	Smooth muscle cell
CVD	Cardiovascular disease
RIP1/3	Receptor interacting protein kinase 1/3
MLKL	Mixed lineage kinase domain like protein
TNFα	Tumor necrosis factor α
zVAD	Benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (zVAD.fmk)
DAMP	Damage-associated molecular pattern
ROS	Reactive oxygen species
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
SRB-1	Scavenger receptor class B type I

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Article Summary

- Macrophages play an important role during all stages of atherosclerotic development, including recruitment and differentiation of immune cells and propagation of the inflammatory milieu through the secretion of pro-inflammatory cytokines.
- In early atherosclerotic lesions, lipid-loaded macrophages undergo apoptosis, a process by which the entire cellular contents are packaged into apoptotic bodies that can be effectively efferocytosed, resulting in a ‘tolerate me’ and non-inflammatory response.
- In response to excess lipid accumulation, macrophage foam cells in early lesions undergo autophagy, a regulated ‘recycling process’ that redirects metabolic pathways to promote fatty acid oxidation and cholesterol efflux - all of which are dysfunctional in advanced atherosclerotic lesions.
- In advanced atherosclerotic lesions, macrophage necroptosis, a type of programmed cell necrosis, induces cell rupture, releasing the entire cellular contents (DAMPs) that propagates inflammation, necroptotic death, and the resultant defective efferocytosis – all of which ultimately contribute to the formation of the necrotic core, a key characteristic feature of a vulnerable plaque.
- Effective efferocytosis in atherosclerotic lesions depends on the expression of functional phagocytic receptors (e.g. MerTK) and the decreased presence of “don’t-eat-me” signals (e.g. CD47).

Figure 1. Macrophage Apoptosis, Autophagy and Necroptosis in Atherosclerosis

In early lesions, modified lipoproteins (e.g. oxLDL) act as inflammatory stimuli within the vessel wall to recruit circulating monocytes, which avidly phagocytose these modified lipids to become macrophage foam cells. These cells then eventually undergo 3 main types of cell death within the vessel wall: apoptosis, autophagy-related cell death and necroptosis. Macrophages that undergo apoptosis can be efficiently efferocytosed to be cleared by other macrophages (purple), whereas necroptotic macrophages are ineffectively efferocytosed (grey), resulting in the accumulation of inflammatory stimuli within the intima that propagates atherosclerosis. Together, both increased cell death and defective efferocytosis ultimately results in the formation of the necrotic core, which is the hallmark of a rupture-prone, vulnerable plaque.