Programmed death of macrophages in atherosclerosis : mechanisms and therapeutic targets

Programmed cell death of macrophages in atherosclerosis: mechanisms and therapeutic targets

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Abstract

Atherosclerosis is a progressive inflammatory disorder of the arterial vessel wall characterized by substantial infiltration of macrophages. Macrophages in atherosclerosis exert both favorable and detrimental functions. Early in atherogenesis, macrophages can clear cytotoxic lipoproteins and dead cells, preventing cytotoxicity. Efferocytosis, the efficient clearance of dead cells by macrophages, is crucial for preventing secondary necrosis and stimulating the release of anti-inflammatory cytokines. In addition, macrophages can promote tissue repair and proliferation of vascular smooth muscle cell, thereby increasing plaque stability. However, advanced atherosclerotic plaques contain large numbers of pro-inflammatory macrophages that secrete matrix degrading enzymes, induce cell death in surrounding cells and contribute to plaque destabilization and rupture. Importantly, macrophages in the plaque can undergo apoptosis and several forms of regulated necrosis, including necroptosis, pyroptosis, and ferroptosis. Regulated necrosis plays an important role in the formation and expansion of the necrotic core during plaque progression, and several triggers for necrosis are present within atherosclerotic plaques. This review focusses on programmed cell death of macrophages in atherosclerosis and the pharmacological interventions targeting the several forms of macrophage cell death as potential means of stabilizing vulnerable plaques and improving the efficacy of currently available anti-atherosclerotic therapies.

Introduction

Atherosclerosis is a chronic, progressive inflammatory disorder of medium- and large-caliber arteries characterized by lipid accumulation in the intima, leading to complications such as myocardial infarction and stroke[1]. The pathogenesis involves low-density lipoprotein (LDL) cholesterol accumulation and endothelial cell activation, facilitating inflammatory cell recruitment. As monocytes/macrophages and other cells accumulate within the plaque and nutrient availability decreases, cell death increases, contributing to a large necrotic core in advanced atherosclerotic plaques[2] (FIG. 1). Intra-plaque neovascularization occurs due to hypoxia, with neovessel leakage causing intra-plaque hemorrhage and further recruitment of inflammatory cells, lipids, and erythrocytes. These processes contribute to plaque destabilization and rupture. Vulnerable plaques are characterized by a thin fibrous cap, extensive macrophage infiltration and inflammation, a large necrotic core, and intraplaque neovascularization and hemorrhage[3] (FIG. 1).

Macrophages exert both beneficial and harmful functions in atherosclerosis[4]. Macrophages can scavenge cytotoxic lipoproteins and dead cells, preventing cytotoxicity in early atherogenesis[5]. Efferocytosis, the efficient clearance of dead cells by macrophages, is essential to prevent secondary necrosis and triggers the release of anti-inflammatory cytokines. Macrophages can also enhance tissue repair and vascular smooth muscle cell (VSMC) proliferation, thereby increasing plaque stability. However, advanced plaques contain large numbers of pro-inflammatory macrophages that secrete matrix degrading enzymes, induce cell death in surrounding cells[6] and contribute to plaque destabilization and rupture.

In addition to inducing cell death, plaque macrophages themselves undergo several types of cell death of which apoptosis is the best-characterized. The large necrotic cores in advanced plaques result primarily from necrotic cell death. During necrosis, the intracellular contents of dying cells are released into the extracellular space, creating a highly inflammatory environment. Necrosis may occur accidentally or be induced through tightly regulated pathways such as necroptosis, pyroptosis or ferroptosis. Regulated necrosis plays an important role in necrotic core formation during plaque progression and several triggers for necrosis are present within atherosclerotic plaques[7] (FIG. 2). This review focuses on cell death of macrophages in atherosclerosis and pharmacological interventions targeting these forms of cell death, as they may help stabilize vulnerable plaques and improve the effectiveness of currently available anti-atherosclerotic therapies.

Apoptosis

Apoptosis is characterized by cellular shrinkage followed by chromatin condensation, membrane blebbing, nuclear fragmentation and ultimately separation of the cellular components into apoptotic bodies. Macrophage apoptosis is an important feature of atherosclerotic plaque development and can be initiated by multiple factors, such as oxidative stress, hypoxia, high cytokine concentrations (e.g. interferon-γ), and cholesterol overload[8] (FIG. 2). In early plaques, macrophage apoptosis is considered beneficial as it limits the cellularity of the lesions and suppresses plaque progression[8]. There is an inverse relationship between macrophage apoptosis and the early plaque area[9-12]. Thus, macrophage apoptosis in atherosclerotic plaques is necessary, at least initially, to reduce the macrophage content in the expanding plaques[13].

Apoptosis of macrophages in early plaques is not harmful because at that point in the time course of atherosclerosis the capacity of efferocytosis, the rapid engulfment of apoptotic bodies, is still sufficiently high. However, apoptosis of macrophages in plaques with reduced efferocytosis**,** which is the case in advanced plaques[8,14,15], carries risks as caspase-mediated cleavage of gasdermin E (GSDME),

a pore-forming protein, leads to membrane permeabilization and secondary necrosis (FIG. 3). Cleaved GSDME can also form mitochondrial membrane pores, releasing pro-apoptotic molecules such as cytochrome c and creating a positive feedback loop promoting caspase 3 activation and further GSDME cleavage. This ultimately augments apoptotic cell death and secondary necrosis.

Efferocytosis becomes impaired in advanced plaques[14,15] due to defects in the efferocytosis process itself or changes in the properties of apoptotic cells within the plaque making them poor efferocytosis substrates[16]. Apoptotic cells that are not cleared undergo secondary necrosis (FIG. 3), stimulating atherogenesis through induction of inflammation and enlargement of the necrotic core[15,17]. Non-cleared apoptotic cells are also an important tissue factor source, which increases plaque thrombogenicity[18]. Clearance of apoptotic cells is tightly regulated and phagocytes responsible for efferocytosis must discriminate between viable and dying cells. This is mediated by “don’t eat me” molecules such as CD47 on viable cells, interacting with the signal-regulatory protein α (SIRPα) on phagocytes and preventing their engulfment. In contrast, dying cells express “eat me” molecules such as calreticuline and phosphatidylserine on their surface, which interact with engulfment receptors on phagocytes such as Mer receptor tyrosine kinase (MerTK), transglutaminase 2, integrin αvβ5, low-density lipoprotein receptor-related protein 1, and scavenger receptor B through bridging molecules. In advanced atherosclerotic plaques, not only the phagocytic capacity of macrophages is impaired. Dying cells in advanced plaques also become poor substrates due to decreased expression of “eat me” and bridging molecules and increased expression of the “don’t eat me” molecule CD47 induced by TNFα. Targeting TNFα or CD47 with specific antibodies can inhibit this process. The abundance of oxLDL in the plaque can also mask “eat me” molecules and compete with dying cells for macrophage engulfment.

Necrosis

Necrotic cell death involves oncosis, organelle swelling, chromatin condensation, plasma membrane rupture, and release of intracellular material[19]. Macrophage necrosis contributes to atherosclerosis by forming and enlarging a central necrotic core in unstable plaques[20,21]. Plaque necrosis can be triggered by oxidative stress, Ca^{2+} overload, ATP depletion, and impaired efferocytosis[22]. Necrotic macrophages release pro-inflammatory cytokines and damage associated molecular patterns (DAMPs)[8], such as high mobility group box 1 protein (HMGB1)[23], which interacts with the receptor for advanced glycation end products (RAGE) and triggers NF-κB-dependent pro-inflammatory cytokine transcription, promoting further plaque development[24]. Neutralizing HMGB1 reduces the plaque area in $ApoE^{-/-}$ mice by inhibiting immune cell accumulation and macrophage migration[25]. Statins also attenuate plaque formation partly by reducing HMGB1 and RAGE expression[26-29].

Necrosis was long regarded as an unregulated process that occurs as a result of infection or injury, but now several forms of programmed necrosis, such as necroptosis, pyroptosis and ferroptosis are recognized. Upregulation of proteins related to necroptosis, ferroptosis and/or pyroptosis occurs simultaneously in atherosclerosis[30,31] through the coordinated action of multiple death-inducing stimuli (FIG. 2). Several stages of human atherosclerosis have been associated with both ferroptosis and pyroptosis, with significant interactions observed between these forms of cell death[30]. The expression of ferroptosis- and necroptosis-related proteins is also increased in atherosclerosis and is not altered by lipid lowering therapies[31]. These findings illustrate the contribution of different forms of regulated necrosis in macrophages to residual cardiovascular risk in atherosclerosis patients.

Necroptosis

Since the discovery of necrostatins, small molecules that inhibit receptor-interacting protein kinase (RIPK) 1-mediated cell death (subsequently called necroptosis), in TNFα-treated cells[32,33] necrosis is no longer considered unregulated. This discovery facilitated characterization of downstream necroptosis mediators such as RIPK3 and mixed lineage kinase domain-like pseudokinase (MLKL)[34-37] (FIG. 4). In atherosclerosis, triggers of necroptosis include TNFα under caspase inhibition, oxLDL and interferon-γ[38] (FIG. 2). Elevated expression of RIPK3 and MLKL is observed in human atherosclerotic plaques[39,40] and oxLDL increases ROS-mediated gene expression of RIPK3 and MLKL in macrophages, leading to necroptosis[39]. Advanced human atherosclerotic plaques also exhibit increased levels of RIPK1[40]. In human carotid plaques, RIPK1 colocalizes mainly with macrophages[15], supporting a role for macrophage necroptosis in atherosclerosis.

Pyroptosis

Pyroptosis is a pro-inflammatory regulated type of necrosis characterized by the formation of plasma membrane pores through the gasdermin (GSDM) protein family[41,42]. The process is initiated by formation of a large supramolecular complex called inflammasome (or pyroptosome) in response to intracellular danger signals[43]. Components of the NLRP3 inflammasome are present in human atherosclerotic plaques and are expressed in macrophages and foam cells surrounding the necrotic core[44,45]. In atherosclerotic plaques, the NLRP3 inflammasome is activated by ATP, derived from excessive cell death in advanced plaques, cholesterol and calcium phosphate crystals, and oxLDL[46-48] (FIG. 2). Subsequently, caspase 1- (an enzyme not involved in apoptosis) and caspase-11 mediated cleavage of gasdermin D (GSDMD) occurs, inducing pyroptosis and caspase 1-dependent release of the pro-inflammatory cytokines IL-1β and IL-18 (FIG. 4). Upregulation of GSDMD mRNA is observed in peripheral blood monocytes of patients with coronary artery disease and expression of GSDMD and NT-GSDMD (N-terminal active fragment)(FIG. 4) is increased in ApoE$^{-/-}$ and wild-type mice fed a high-fat diet[49], indicating that GSDMD is actively involved in pyroptosis during atherogenesis in both humans and mice. Triglyceride-induced macrophage cell death involves the activation of caspase-1 and triggers the pyroptosis pathway[50] (FIG. 2).

The expression of the NLRP3 inflammasome correlates with the severity of coronary atherosclerosis and cardiovascular risk factors[51], with the highest levels in unstable plaques[44], suggesting a potential role for the NLRP3 inflammasome in atherogenesis and plaque destabilization. Atherosclerotic plaques in LDLr$^{-/-}$ mice with NLRP3 or IL-1 deficient bone marrow are nearly 70% smaller compared to plaques of LDLr$^{-/-}$ controls[48]. Similar results were observed in ApoE$^{-/-}$ mice following NLRP3 knockdown or treatment with a selective NLRP3 inhibitor[52-55]. Atherogenesis is also decreased in ApoE$^{-/-}$ mice lacking functional caspase 1 due to genetic deletion or pharmacological inhibition[56,57]. Downstream of NLRP3 and caspase 1, administration of IL-18 increases plaque size in ApoE$^{-/-}$ mice while atherogenesis is reduced in IL-18 deficient ApoE$^{-/-}$ mice[58,59]. Likewise, IL-1β deficiency reduces atherosclerosis in ApoE$^{-/-}$ mice[60].

Importantly, other inflammasomes have been reported in atherosclerosis. NLRP1 and NLRC4 (NOD-like LRR -and CARD domain-containing protein 4) are upregulated in the peripheral blood of atherosclerotic patients[61]. Furthermore, the AIM2 (absent in melanoma 2) inflammasome, a cytosolic dsDNA sensor, is expressed near the necrotic core in human atherosclerotic lesions[62] (FIG. 4). Cytosolic dsDNA is released during sterile inflammation due to necrotic cell death or release of neutrophil extracellular traps (NETosis)[63]. In atherosclerosis, increased deposition of dsDNA occurs in advanced lesions along with increased expression of AIM2, primarily in macrophages[63]. AIM2 overexpression in

ApoE$^{-/-}$ mice exacerbates atherosclerosis while genetic ablation and pharmacological inhibition of AIM2 increases plaque stability[63,64].

Ferroptosis

Ferroptosis is a form of regulated necrosis hallmarked by iron-dependent accumulation of lipid hydroperoxides[65,66] through enzymatic peroxidation of poly-unsaturated fatty acids (PUFAs) in phospholipid bilayers or by non-enzymatic peroxidation via the Fenton reaction, which requires free ferrous iron (Fe^{2+}) from a cytosolic labile iron pool (FIG. 4). Glutathione peroxidase 4 (GPX4) mainly eliminates the lipid peroxides[65,67] (FIG. 4). However, when the lipid peroxides are not adequately eliminated or when the labile iron pool surpasses the buffering capacity of ferritin, excessive peroxidation of PUFAs occurs, altering the geometric properties of the lipid bilayer. This leads to the formation of membrane pores, culminating in cell lysis and death[68]. In addition, lipid peroxides are degraded to toxic lipid aldehydes such as malondialdehyde and 4-hydroxynonenal, contributing to cytotoxicity. Initiation of ferroptosis occurs by impairment of GPX4 activity through direct inhibition or by depletion of the substrate glutathione[65] (FIG. 4). Another initiator of ferroptosis includes accumulation of Fe^{2+} in the labile iron pool[69] due to decreased expression of ferroportin, increased expression of transferrin, or excessive activation of heme-oxygenase 1 (HMOX1). The latter process can occur in the context of intraplaque hemorrhage, when macrophages phagocytose red blood cells (erythrophagocytosis)[70-74] (FIG. 2), catalyzing the degradation of hemoglobin to Fe^{2+}, biliverdin and carbon monoxide. This iron loading of macrophages induces peroxidation of PUFAs (FIG. 4) and increases oxidation of LDL[75,76]. Indeed, in human atherosclerotic plaques, iron-positive foam cells are present and HMOX1 and ferritin accumulate[72,73,77], indicating ferroptosis in the plaques. This results in lytic cell death and the release of intracellular contents and DAMPs into the plaque, contributing to the growth of the necrotic core and plaque destabilization.

Parthanatos and autosis

Parthanatos, or poly(ADP-ribose) polymerase-1 (PARP-1) dependent cell death, is a form of programmed cell death resulting from poly(ADP ribose) (PAR) accumulation and the nuclear translocation of apoptosis-inducing factor (AIF) from mitochondria[78]. PARP-1 mediates parthanatos when it becomes over-activated in response to extreme genomic stress and synthesizes PAR, leading to the nuclear translocation of AIF[43]. While parthanatos has not been extensively studied in the context of atherosclerosis, evidence suggests that parthanatos in plaque macrophages may play a role in atherogenesis because advanced plaques contain high levels of oxidative stress and tissue damage due to peroxynitrite formation[6], which can oxidize DNA. Increasing DNA strand breaks are present in human and experimental plaques[79,80]. Oxidative DNA damage is associated with PARP-1 upregulation within macrophage-derived foam-cells. Inhibition of PARP-1 activity attenuates plaque development in ApoE$^{-/-}$ mice, enhances plaque stability and promotes regression of pre-established plaques[81,82].

Although autophagy is an important subcellular pathway that mediates macrophage survival, excessive autophagy can induce autosis, an autophagy-dependent, non-apoptotic, and non-necrotic form of cell death characterized by unique morphological features such as perinuclear ballooning[83,84]. Autosis may be triggered by conditions that induce autophagy, such as hypoxia-ischemia[85]. However, the role of autosis in atherosclerosis appears limited due to impaired autophagy in advanced atherosclerotic plaques[86], including within macrophages.

Biomarker-based patient selection for targeting macrophage cell death in atherosclerosis

Conventional parameters for risk assessment of atherosclerosis-related cardiovascular morbidity or mortality mellitus, such as plasma LDL and HDL levels, hypertension, smoking and diabetes, fail to provide insight into the presence and role of macrophage cell death in atherosclerotic patients. Given the close association between inflammation and necrosis, analysis of high sensitive C-reactive protein (hsCRP) indicative of chronic, low-level inflammation, could be interesting for its predictive value for future cardiovascular events[87] in individuals with or without a history of cardiovascular disease[88]. Elevated plasma levels of lipoprotein-associated phospholipase A_2 (Lp-PLA_2), an enzyme secreted by macrophages, have been associated with advanced atherosclerosis[89]. However, to accurately estimate necrosis involvement, hsCRP and Lp-PLA_2 plasma levels must be complemented by cell death biomarkers. Lactate dehydrogenase (LDH) in peripheral blood serves as an indicator of necrosis and tissue damage[90]. HMGB1, a DAMP released during necrosis, represents another peripheral blood indicator but may also be secreted by non-necrotic activated macrophages in inflammatory plaques[90]. HMGB1 is therefore not specific for necrosis but rather indicative of inflammation in general. While hsCRP, Lp-PLA_2, LDH and HMGB1 provide evidence for inflammation and necrosis, they do not offer sufficient insight into the specific cell death pathways involved.

Proteins involved in cell death execution are potential biomarkers to assess disease severity. Elevated serum levels of the necroptosis executor MLKL have been reported to predict mortality in critically ill patients and correlate with markers of organ failure[91]. Reduced levels of MLKL in plasma may serve as a potential prognostic biomarker[92]. RIPK1 and RIPK3 levels may also be of interest, although their phosphorylation, constituting the only evidence of necroptosis involvement, has rarely been reported[90]. To evaluate pyroptosis involvement, circulating levels of GSDMD, IL-1β and IL-18 can be analyzed[93,94]. In patients with atherosclerosis, elevated levels of GSDMD mRNA have been observed in peripheral blood monocytes, suggesting that GSDMD may represent a biomarker for pyroptosis in atherosclerotic plaques[49]. Identifying a biomarker for ferroptosis is challenging due to the lack of a specific executor and marker[95], although clinical lipidomics may aid in its detection[96]. Although circulating markers of specific cell death pathways are of interest, there is a need for large-scale clinical trials to evaluate their diagnostic value. Cell-free nucleic acids as biomarkers in cardiovascular and other diseases are also the subject of intense research[97,98]. Circulating levels of microRNAs involved in cell death pathway regulation may help determine the occurrence and types of cell death. Cell-free DNA released during cell death can be measured in blood and linked to specific tissues based on DNA methylation patterns[99]. Collectively, these techniques represent promising diagnostic developments with the potential to facilitate personalized medicine in cardiovascular patients.

Identifying plaques that may benefit from interventions that inhibit macrophage cell death

Imaging of plaques containing large necrotic cores and abundant macrophages and inflammation is important to identify plaques that may benefit from therapy targeting programmed cell death of macrophages. However, as discussed above, targeting of programmed cell death pathways in atherosclerosis is not always protective and stage-dependent involvement of cell death modalities must be taken into account. The stage of a plaque at a specific location can be determined using invasive imaging such as intravascular ultrasound, optical coherence tomography and near-infrared spectroscopy[100], or non-invasive techniques, such as positron emission tomography with radio-labelled tracers[101] taken up by metabolically active cells like macrophages, computed tomographic coronary angiography, and magnetic resonance imaging. Invasive and non-invasive imaging technique have

unique characteristics and limitations but can be used and combined to analyze morphological features of specific plaques[100], including macrophage infiltration, necrotic core area, and intra-plaque hemorrhage. Molecular imaging using probe-tagged nanoparticles that bind specific molecular targets[100] can also be used to evaluate plaque-specific involvement of cell death pathways by targeting programmed cell death markers. For example, pyroptosis-related proteins (NLRP3 and caspase 1) and ferroptosis-related proteins (e.g. GPX4) have been proposed as biomarkers for disease severity[30] but their diagnostic value in atherosclerosis patients remains to be evaluated. Nevertheless, several ferroptosis-related genes are upregulated in coronary artery samples from atherosclerosis patients compared to healthy vessel samples[102]. Collectively, these observations highlight the possibility of identifying programmed cell death pathways involved in specific plaques.

The complexity of targeting macrophage death pathways in atherosclerosis

Targeting macrophage apoptosis

With regard to targeting macrophage death pathways in atherosclerosis, several pertinent questions arise, including whether to induce or prevent macrophage cell death, the most effective type of cell death to target, and the stage of plaque development at which treatment should be administered. Early plaques may benefit from the selective removal of macrophages through macrophage-specific cell death, as this may have a plaque stabilizing effect. Therefore, a series of pharmacological strategies have been developed to selectively deplete plaque macrophages by inducing apoptosis[103]. Local administration of a protein synthesis inhibitor (e.g. cycloheximide or anisomycin) to atherosclerotic plaques removes macrophages via p38 MAPK-mediated apoptosis induction without affecting the viability of VSMCs or endothelial cells[104]. Indeed, plaque macrophages display high metabolic activity and are more sensitive to protein synthesis inhibitors than VSMCs and endothelial cells. Nonetheless, this approach presents challenges due to the reduced phagocytic capacity of plaques after macrophage depletion. Furthermore, the formulation of a therapy that selectively induces macrophage apoptosis in early plaques while preventing cell death in advanced lesions is a complex undertaking. In addition, the prevention of re-infiltration of circulating monocytes following macrophage depletion requires combined treatment with lipid-lowering and anti-inflammatory drugs.

Caution should be exercised in initiating apoptosis in macrophages in plaques that exhibit reduced efferocytosis, such as in advanced atherosclerosis. Indeed, the induction of macrophage depletion via apoptosis necessitates additional therapy to promote efferocytosis, alleviate inflammation, prevent the accumulation of free apoptotic cells and secondary necrosis[105,106] (FIG. 3). Overexpression of CD47 by TNFα in atherosclerotic plaques renders apoptotic cells impervious to phagocytic clearance. Therefore, antibodies that inhibit the anti-phagocytic signal CD47 can be used to stimulate efferocytosis. A synergistic effect on the clearance of apoptotic cells can be obtained by concurrent inhibition of CD47 and TNFα via anti-CD47 and anti-TNFα antibodies such as infliximab or etanercept. In addition, glucocorticoids can augment efferocytosis by several mechanisms, including increased expression of the MerTK receptor and bridging molecules such as annexin A1 and C1q[107-109]. However, long-term systemic administration of glucocorticosteroids can result in several adverse effects. Another approach involves increasing the production of specialized pro-resolving mediators (SPMs), including long-chain fatty acid-derived lipid mediators such as resolvin (Rv) D1, RvD2, RvE1, and RvE2. RvD1 levels are diminished in vulnerable regions of atherosclerotic plaques and administration of an SPM can impede the progression of atherosclerosis in mice[40,110-112]. This is due to stimulating efferocytosis through suppression of TNFα production[113], impeding the synthesis of pro-inflammatory mediators, and inhibiting leukocyte trafficking to inflammation foci[114].

Targeting macrophage necroptosis

The role of RIPK1 in atherogenesis is complex due to its dual function as an inactive scaffold versus kinase activity. Inhibition of RIPK1 reduces plaque size and promotes plaque stability in ApoE$^{-/-}$ mice[39,115]. However, the impact of myeloid RIPK1 gene deletion depends on the stage of atherogenesis. In early plaques, myeloid RIPK1 gene deletion results in increased apoptosis, slowing plaque progression. Despite the decreased macrophage content, the plaque and necrotic core are no longer reduced in more advanced plaques, probably due to the accumulation of free apoptotic and necroptotic cells[15]. Furthermore, inhibition of RIPK1 kinase activity by a RIPK1$^{S25D/S25D}$ mutation accelerated plaque progression in ApoE$^{-/-}$ mice and induced apoptosis in ApoE$^{-/-}$ Fbn1$^{C1039G+/-}$ mice with advanced atherosclerosis treated with the RIPK1 inhibitor GSK'547, which binds to RIPK1 with exquisite kinase specificity. Thus, it appears that targeting RIPK1 kinase activity does not limit atherogenesis[116]. Deletion of MLKL reduces the necrotic area in advanced but not early atherosclerotic plaques in ApoE$^{-/-}$ mice [117,118]. Furthermore, deficiency of RIPK3 reduces advanced atherosclerotic lesions in ApoE$^{-/-}$ and LDLr$^{-/-}$ mice without affecting earlier stages of plaque development[119], with loss of RIPK3 expression from bone-marrow derived cells primarily responsible for this atheroprotective effect[119].

Targeting macrophage pyroptosis

Targeting of IL-1β (FIG. 4) in atherosclerosis was evaluated in the CANTOS trial using canakinumab. This trial demonstrates that statin-treated patients with residual inflammatory risk benefit from additional anti-inflammatory therapy[120], consistent with previous findings in mice showing that targeting IL-1β inhibit plaque formation[121]. However, IL-1β neutralization in advanced plaques of ApoE$^{-/-}$ mice induces loss of VSMC and collagen within the fibrous cap while increasing macrophage content[122]. Moreover, targeting IL-1β with a specific antibody is associated with residual cardiovascular risk related to IL-6 and IL-18. Therefore, combined cytokine targeting[123] may be better but may include an increased infection risk[124]. Thus, combining cell death inhibitors with anti-inflammatory agents and lipid-lowering drugs may be beneficial in treating atherosclerosis. Furthermore, it is crucial to identify which patients benefit most from anti-IL-1β therapy and which might experience adverse effects[122]. Anakinra, an IL-1 antibody targeting both IL-1α and IL-1β (FIG. 4), is another option[125]. Colchicine, an inhibitor of NLRP3 inflammasome assembly, is a widely available alternative, and inhibits the release of both IL-1β and IL-18[126] (FIG. 4). In LoDoCo (Low-Dose Colchicine) and LoDoCo2 studies, colchicine reduced the risk of cardiovascular events in patients with coronary artery disease[127,128]. Collectively, these studies in mice and humans suggest that modulation of NLRP3-mediated pyroptosis may benefit patients with severe atherosclerosis. Furthermore, AIM2 overexpression in ApoE$^{-/-}$ mice exacerbates atherosclerosis while genetic ablation and pharmacological inhibition of AIM2 increases plaque stability[63,64,129] (FIG. 4).

GSDMD is the common executor of both inflammasome/caspase 1- and caspase 11-mediated pyroptosis and regulates IL-1 secretion by activated macrophages[130] (FIG. 4), rendering targeting of GSDMD an attractive approach for addressing pyroptosis in atherosclerosis. Although specific inhibitors of GSDMD are not available yet, disulfiram and dimethyl fumarate can inhibit GSDMD[131,132] and the latter reduces aortic plaque area in ApoE$^{-/-}$ mice[133].

Furthermore, macrophage polarization may play a role. Dipeptidyl peptidases 8 and 9 (DPP 8/9) are present in macrophage-rich plaque areas and play a role in macrophage death in vivo[134]. DPP9 is upregulated during differentiation from monocytes to macrophages, while inhibition of its activity reduces activation of pro-inflammatory M1-like but not of pro-resolution M2-like macrophages[134]. In atherosclerotic ApoE$^{-/-}$ mice, inhibition of DPP 8/9 with 1G244 results in a lytic form of cell death induction, probably pyroptosis, in activated macrophages[135]. The authors suggested that facilitating

the removal of M1 cells from the early stage of atherosclerosis with simultaneous preservation of M2 macrophages results in smaller plaques without increased signs of vulnerability.

Targeting macrophage ferroptosis

The first specific inhibitors of ferroptosis identified were ferrostatin-1 (Fer-1) and liproxstatin-1[65,136], with anti-ferroptotic activity attributed to their ability to act as potent radical trapping agents (RTAs) within lipid bilayers [137,138]. Pharmacological inhibition of ferroptosis with Fer-1 reduces plaque burden in ApoE$^{-/-}$ mice[77,139]. Reduced serum and aortic iron levels and increased expression of GPX4 and the SLC7A11 subunit of the glutamate-cysteine X_c-antiporter (FIG. 4) may explain the atheroprotective effects. Moreover, GPX4 overexpression in ApoE$^{-/-}$ mice inhibits plaque progression[140]. Fer-1 analogues with improved potency and ADME properties, including UAMC-3203[141,142], are currently available. Recently, we showed that erythrophagocytosis-induced ferroptosis during intra-plaque angiogenesis leads to larger atherosclerotic plaques, an effect that can be prevented by the ferroptosis inhibitor UAMC-3203[143]. In addition, IL-37, a newly identified anti-inflammatory factor, suppresses macrophage ferroptosis to attenuate atherosclerosis progression through activation of the nuclear factor erythroid 2-related factor 2 (NRF2) pathway[144].

The interplay between types of macrophage cell death

Apoptosis and necrosis have traditionally been considered mutually exclusive, but there is a balanced interplay between these types of cell death. Blocking one type of cell death can make cells susceptible to another death pathway, complicating therapeutic inhibition of cell death[145]. For example, inhibition of caspases by zVAD-fmk can prevent apoptosis but facilitate necroptosis (FIG. 4). Conversely, active caspase 8 promotes apoptosis while simultaneously cleaving RIPK1 and RIPK3, preventing necroptosis induction[146-148] (FIG. 4). Similarly, caspase 1 in pyroptosis is anti-apoptotic and its deletion is associated with non-pyroptotic cell death[149]. Moreover, during the execution phase of necroptosis, MLKL pores induce potassium efflux and activate the NLRP3 inflammasome[150-152], illustrating the interplay between apoptosis, necroptosis, and pyroptosis [153]. Ferroptosis and pyroptosis are also intertwined in atherosclerotic plaques. During erythrophagocytosis following intraplaque hemorrhage[72,154,155], iron from red blood cells can induce ferroptosis while heme can activate the NLRP3 inflammasome and induce pyroptosis[154,156]. Furthermore, oxLDL increases RIPK3 and MLKL gene expression leading to necroptosis while also inducing pyroptosis[39] (FIG. 2). RIPK1 kinase inhibition[116] or deletion of Gsdmd[157] can result in a switch to apoptosis, which may lead to secondary necrosis if efferocytosis is impaired. Therefore, the use of broad inhibitors that also block membrane permeabilization during secondary necrosis or compounds that activate plasma membrane repair mechanisms may be necessary. Thus, multiple cell death pathways may need to be targeted simultaneously. In addition, combining anti-inflammatory and lipid-lowering therapy with targeting upstream regulators and downstream executors of necrosis can minimize the presence of triggers of pro-inflammatory and necrotic pathways.

Pharmacological inhibition of macrophage necrosis through antioxidant therapy may represent an alternative approach for plaque stabilization. Although there is currently no convincing evidence that antioxidants such as vitamin C and E diminish atherosclerotic plaque progression[158], treatment with NecroX-7, a scavenger of mitochondrial ROS and peroxynitrite[159], reduces necrotic areas without affecting plaque size in ApoE$^{-/-}$ mice[160] and improves several features of plaque stability[160]. A phase I clinical trial showed that multiple intravenous administrations of NecroX-7 were well tolerated at

doses ranging from 3 to 30 mg[161]. Furthermore, local targeting of regulated necrosis of macrophages may limit plaque destabilization without affecting other tissues or cells. Drug-eluting stents can be used for this purpose[162]. Local treatment can also be achieved using nanoparticles containing therapeutic agents that bind specific cell-types such as macrophages or programmed cell death related proteins[163].

Targeting macrophage autophagy

In the context of pharmacological targeting of macrophage autophagy, the autophagy status of macrophages in atherosclerosis is crucial. Normal autophagy activity in macrophages is essential for cell viability and cellular homeostasis. Hypoactivity of autophagy in macrophages leads to decreased efferocytosis potential, lipid accumulation, and a pro-inflammatory status. Hyperactivity of autophagy in macrophages can result in autosis[85].

Autophagy can be pharmacologically induced by either an mTOR-dependent or an mTOR independent pathway (FIG. 5). mTOR is a serine/threonine protein kinase that regulates protein translation and cell proliferation. Inhibition of mTOR downregulates protein translation via dephosphorylation of downstream mTOR proteins, such as p70 S6 kinase and 4E-BP1, but also activates Atg genes, particularly the ULK1/Atg1 kinase complex (FIG. 5), which can selectively induce autosis in macrophages of atherosclerotic plaques if the level of autophagy induction is sufficiently high[164]. Because VSMCs are metabolically less active than plaque macrophages and thus less sensitive to inhibition of protein synthesis, they are resistant to cell death mediated by inhibition of protein translation. On the contrary, inhibition of protein translation in VSMCs induces a modulation toward a differentiated, quiescent, contractile phenotype[164]. Drug-eluting stents with an mTOR-inhibitor such as a rapamycin-derivative (rapalog, e.g., sirolimus or everolimus) are used clinically because they prevent in-stent restenosis. In atherosclerotic plaques, mTOR inhibition after stent-based administration of rapamycin-derivatives (rapalogs) such as everolimus induces selective induction of macrophage autosis and leads to clearance of macrophages in the plaque without altering the VSMC content[165] , promoting a stable plaque phenotype. Similarly, silencing of mTOR by mTOR-specific siRNA clears macrophages via induction of selective autophagy-mediated macrophage death and inhibits the progression and destabilization of atherosclerotic plaques[166,167].

Systemic administration of mTOR inhibitors in doses suitable for autophagy induction in macrophages leads not only to systemic immune suppression, but also to hyperlipidemia and hyperglycemia (FIG. 5), which is of obviously not beneficial, making only local treatment (as a drug-eluting stent) a justified approach for mTOR dependent clearance of plaque macrophages[86,168]. Moreover, one should be careful with this strategy, because prior to autosis everolimus-treated macrophages may secrete pro-inflammatory cytokines and chemokines through activation of p38 MAP kinase[169], thus providing a rationale for combining the local mTOR-inhibitor with an anti-inflammatory agent. To circumvent these problems, we therefore propose systemic application in patients with more advanced plaques of a selective mTOR-independent autophagy inducer, which induces low levels of autophagy without immunosuppression or other adverse effects, thereby inhibiting macrophage cell death and, importantly, increasing efferocytosis. Although mTOR-independent autophagy inducers already exist, they are low in specificity and selectivity and relatively low in potency. Therefore, much research is currently underway to develop selective (also for macrophages), specific mTOR-independent autophagy inducers.

Conclusion

Several forms of programmed cell death of macrophages play a role in atherogenesis and vulnerable plaque formation, including apoptosis, necroptosis, pyroptosis and ferroptosis. Targeting these forms of cell death may help stabilize vulnerable plaques and improve the effectiveness of currently available anti-atherosclerotic therapies. However, a balanced interplay exists between these cell death modalities. Consequently, it is important to consider that inhibiting one form of cell death may result in the activation of another. The stage of the atherosclerotic plaque is also a critical factor. In early plaques, the selective removal of macrophages via macrophage-specific cell death may have plaque-stabilizing effects. However, in more advanced plaques, sufficient efferocytosis capacity is essential to prevent secondary necrosis. Therefore, in addition to utilizing inhibitors of necroptosis, pyroptosis or ferroptosis, employing resolvins or autophagy-inducers represents a promising strategy for targeting macrophage programmed cell death as they can enhance efferocytosis capacity.

Figure legends

FIG. 1. Key steps in the progression of atherosclerosis. The formation of atherosclerotic plaques is initiated early in life with the development of intimal thickening in naïve arteries. This process evolves through the uptake of circulating lipoproteins, recruitment of inflammatory cells, and migration and trans-differentiation of vascular smooth muscle cells to form a stable plaque. Over time, macrophages and other inflammatory cells accumulate within the plaque and secrete matrix-degrading enzymes and factors that are toxic to vascular smooth muscle cells, leading to thinning of the fibrous cap and the formation of an early stage unstable plaque at risk of rupture or erosion. In the late stages of atherosclerosis, the plaque gradually expands and intra-plaque neovascularization occurs in response to the hypoxic environment. Rupture of the unstable plaque leads to thrombus formation, resulting in stroke or myocardial infarction.

FIG. 2. Main triggers of programmed cell death of macrophages in atherosclerosis. Apoptosis of macrophages can be triggered by oxidative stress, hypoxia, interferon-γ and cholesterol overload. Impaired efferocytosis can lead to secondary necrosis. Necroptosis of macrophages can be triggered by TNFα under caspase inhibition, oxLDL and interferon-γ. OxLDL, cholesterol and calcium phosphate crystals, ATP and triglycerides are triggers for macrophage pyroptosis. Ferroptosis of macrophages in atherosclerosis can be initiated by erythrophagocytosis and oxidative stress.

FIG. 3. Overview of key mechanisms in apoptosis and secondary necrosis. Apoptosis is a process of programmed cell death that can be initiated via two main pathways: the extrinsic and intrinsic pathways. The extrinsic pathway is activated upon binding of ligands, such as FAS ligand, to death receptors on the cell surface, including the FAS receptor. This results in the recruitment of the adaptor protein FADD and the initiator caspases, caspase-8 and/or caspase-10, to form the death-inducing signaling complex. Activation of these initiator caspases leads to the cleavage and subsequent activation of downstream effector caspases, including caspase-3 and caspase-7. The intrinsic pathway of apoptosis is facilitated by members of the Bcl-2 family, including activated BAX and BAK, leading to mitochondrial outer membrane permeabilization (MOMP). This results in the release of cytochrome c into the cytosol, which engages apoptotic protease activating factor-1 and forms the apoptosome, activating caspase-9 and subsequently effector caspases. The extrinsic pathway can interact with the intrinsic pathway through the cleavage of Bid (BH3 interacting domain death agonist) by caspase-8. This cleavage generates a truncated form of Bid (tBid) that translocates to the mitochondria and promotes the release of cytochrome c, triggering apoptosome formation and activating the intrinsic pathway. Secondary necrosis has long been viewed as an uncontrolled process that results in total lysis of the apoptotic cell. However, the progression from apoptosis to secondary necrosis is regulated by Gasdermin E (GSDME). GSDME is cleaved by caspase-3 to produce a N-GSDME fragment that targets the mitochondrial and plasma membrane for pore formation, facilitating the switch from apoptosis to secondary necrosis. Efferocytosis, the efficient clearance of dead cells by macrophages, is essential to prevent secondary necrosis and becomes impaired in advanced atherosclerosis. Treatment with resolvins, anti-CD47 or anti-TNFα antibodies can promote efferocytosis.

FIG. 4. Overview of the main mechanisms in regulated necrosis of macrophages (necroptosis, pyroptosis and ferroptosis) together with the most promising therapeutic targets.
Necroptosis is a form of regulated necrosis defined by TNF-induced necrotic cell death under caspase-deficient conditions. TNF binding to trimer TNFR1 leads to recruitment of TRADD and RIPK1. Subsequently, TRAF2/5 and cIAP1/2 are recruited to TRADD, allowing cIAP1/2 to conjugate RIPK1. The central event in necroptosis is the formation of a necrosome complex of receptor protein interacting kinase 1 (RIPK1) and receptor interacting protein kinase 3 (RIPK3), which promotes the phosphorylation of an important pro-death effector, mixed lineage kinase domain-like (MLKL). Finally, pore formation occurs in the macrophage. Although RIPK1 inhibitors can inhibit the necrosome, their use in the context of atherosclerosis is questionable because a switch to apoptosis may occur, which may lead to secondary necrosis if efferocytosis is impaired (see text for details).
Pyroptosis is a type of programmed cell death mediated by the formation of inflammasomes. Assembly of the NLRP3 inflammasome leads to caspase 1-dependent release of the pro-inflammatory cytokines IL-1β and IL-18, as well as to gasdermin D-mediated pore formation and pyroptotic cell death. The AIM2 inflammasome is another type of inflammasome that can mediate pyroptosis. The noncanonical inflammasome pathway leads to caspase-11 activation in response to lipopolysaccharides. Caspase-11 itself induces pyroptosis but requires NLRP3 and caspase-1 to promote cytokine secretion. Pyroptosis in macrophages can be inhibited in several ways, by NRLP3 inhibitors (e.g. by colchicine), AIM2 inhibitors, GSDMD inhibitors (e.g. disulfiram and dimethyl fumarate). The administration of antibodies specific to the cytokines IL-1β or IL-18 can neutralize their biological activity.
Ferroptosis is a form of regulated cell death characterized by intracellular iron-dependent peroxidation of polyunsaturated fatty acids (PUFA), leading to plasma membrane disruption. Glutathione peroxidase 4 (GPX4), a phospholipid hydroperoxidase, protects against lipid peroxidation by reducing lipid hydroperoxides to their respective alcohols using reduced glutathione (GSH) as a reducing agent. The oxidized form of glutathione (glutathione disulfide, GSSG), produced during the reduction of hydroperoxides by GPX4, is recycled. The cysteine/glutamate antiporter (x_c^-) takes cystine into the cell in exchange for glutamate. Radical-Trapping Antioxidants (RTAs), such as UAMC-3203 for example, suppress ferroptosis by interrupting the lipid peroxidation process.

FIG. 5. Autophagy induction in macrophage can lead to increased efferocytosis or autosis, depending of the level of autophagy induced. Autophagy is a degradation process that removes unnecessary or dysfunctional components of the cell through a lysosome-dependent regulated mechanism. The Unc-51 like autophagy activating kinase 1 (ULK1) complex plays a central role in the initiation stage of autophagy. AMP-activated protein kinase (AMPK) promotes autophagy by directly activating ULK1. Conversely, high mTOR activity prevents ULK1 activation by phosphorylating ULK1. The class III phosphatidylinositol 3-kinase (PI3K) complex produces phosphatidylinositol 3-phosphate in membranes related to autophagosome biogenesis. The ATG12-ATG5-ATG16L1 complex acts as a ubiquitin-like E3 enzyme, promoting the anchoring of LC3 proteins to the autophagosome membrane. It covalently links LC3 to phosphatidylethanolamine lipid groups in the phagophore membrane. This process converts LC3-I to LC3-II, which is associated with the autophagosome membrane. Subsequently, the autophagosome fuses with a lysosome, forming an autolysosome allowing the degradation of cellular substrates by hydrolytic enzymes. Only the main steps of the autophagic process are shown.
Autophagy in macrophages can be induced by inhibition of mTOR or by mTOR-independent pathways. Systemic administration of mTOR inhibitors at doses appropriate for autophagy induction in macrophages can result in systemic immunosuppression, hyperlipidemia, and hyperglycemia. These

adverse effects make local treatment, such as drug-eluting stents, a more justified approach for mTOR-dependent clearance of plaque macrophages in early atherosclerotic plaques. When an mTOR inhibitor, such as a rapalog, is administered via a drug-eluting stent, which is already used clinically to prevent in-stent restenosis, high concentrations can be achieved in the plaque, resulting in high levels of autophagy induction and accordingly macrophage autosis. Indeed, plaque macrophages are metabolically highly active and more sensitive to protein synthesis inhibition by mTOR inhibitors compared to other cell types in the vessel wall such as VSMCs. Another approach is the systemic application of a selective mTOR-independent autophagy inducer in patients with more advanced plaques. This would induce low levels of autophagy without immunosuppression or other adverse effects, inhibiting macrophage cell death and increasing efferocytosis. Novel mTOR-independent autophagy inducers are currently being developed.

Key points

- Programmed cell death of macrophages, including apoptosis, necroptosis, pyroptosis and ferroptosis, plays a role in atherogenesis and vulnerable plaque formation.
- A balanced interplay exists between these cell death modalities; inhibiting one form of cell death may result in the activation of another.
- In early plaques, the selective removal of macrophages via macrophage-specific cell death may have plaque-stabilizing effects.
- Sufficient efferocytosis capacity in advanced plaques is essential to prevent secondary necrosis.
- In addition to the use of inhibitors of necroptosis, pyroptosis or ferroptosis, employing resolvins or autophagy-inducers represents a promising strategy for targeting macrophage programmed cell death as they can enhance efferocytosis capacity.

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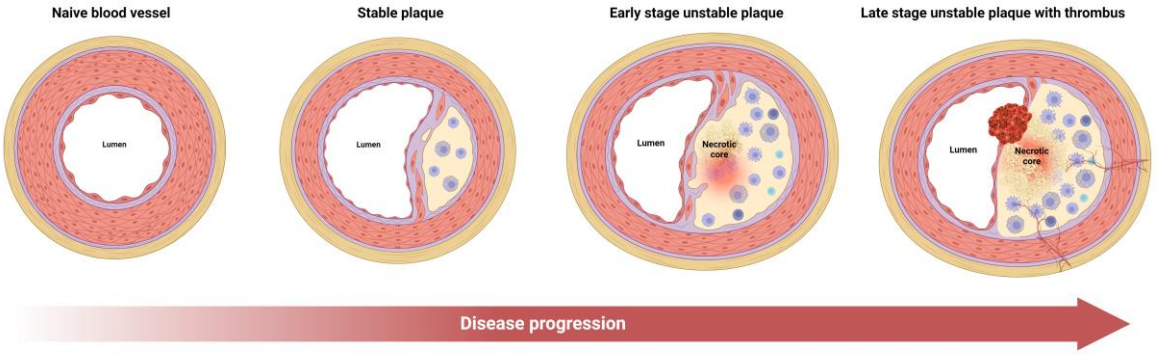
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Stable plaque
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Early stage unstable plaque
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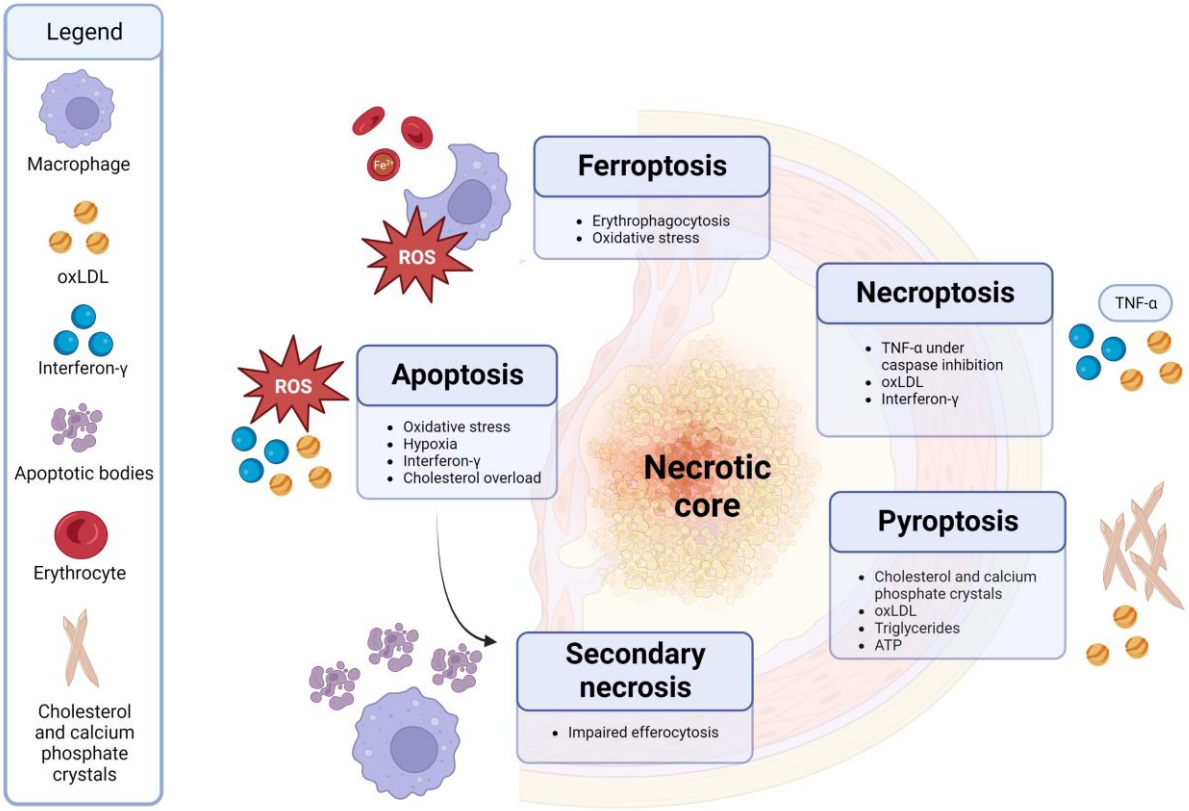
Necrotic core
Late stage unstable plaque with thrombus
Lumen
Necrotic core
Disease progression


Legend
Macrophage
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Apoptotic bodies
Erythrocyte
Cholesterol and calcium phosphate crystals
ROS
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ROS
Apoptosis
Oxidative stress
Hypoxia
Interferon-γ
Cholesterol overload
Necrotic core
Necroptosis
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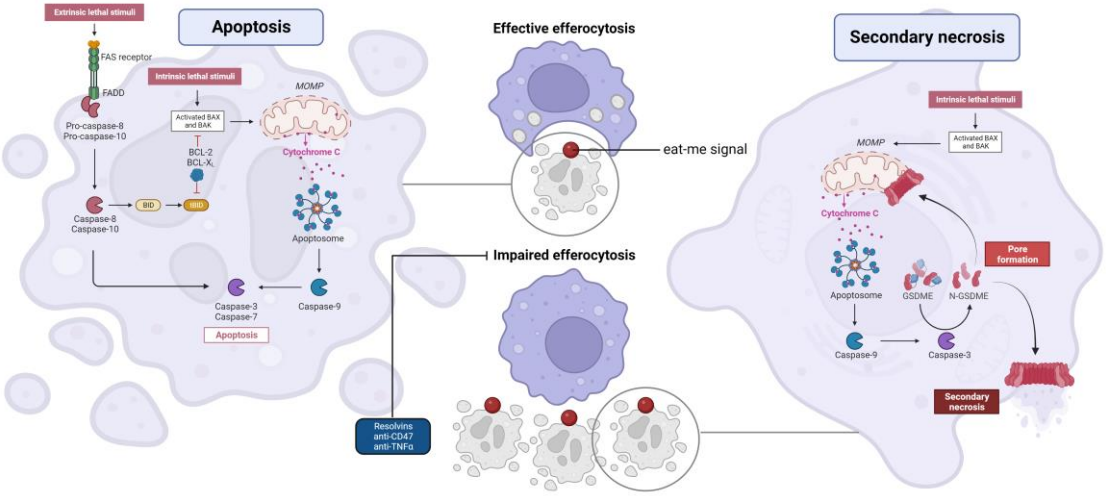
Extrinsic lethal stimuli
Apoptosis
FAS receptor
FADD
Pro-caspase-8
Pro-caspase-10
Caspase-8
Caspase-10
Intrinsic lethal stimuli
Activated BAX and BAK
BCL-2
BCL-X
BID
tBID
MOMP
Cytochrome C
Apoptosome
Caspase-9
Caspase-3
Caspase-7
Apoptosis
Effective efferocytosis
eat-me signal
Impaired efferocytosis
Resolvins
anti-CD47
anti-TNFα
Secondary necrosis
Intrinsic lethal stimuli
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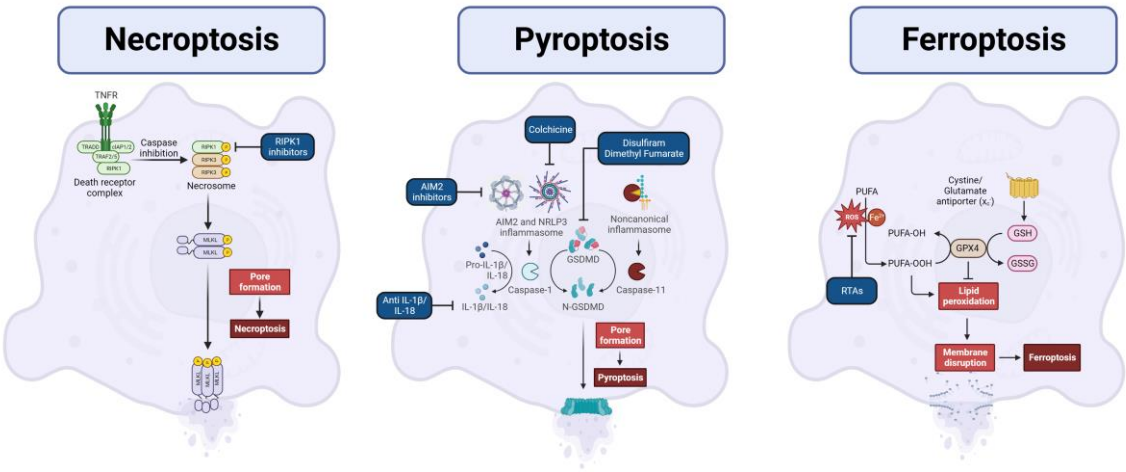
Secondary necrosis


Necroptosis
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Caspase inhibition
RIPK1 inhibitors
Death receptor complex
Necrosome
MLKL
Pore formation
Necroptosis
Pyroptosis
Colchicine
Disulfiram
Dimethyl Fumarate
AIM2 inhibitors
AIM2 and NRLP3 inflammasome
Noncanonical inflammasome
Pro-IL-1β/ IL-18
GSDMD
Caspase-1
Caspase-11
Anti IL-1β/ IL-18
IL-1β/IL-18
N-GSDMD
Pore formation
Pyroptosis
Ferroptosis
PUFA
ROS
Fe2+
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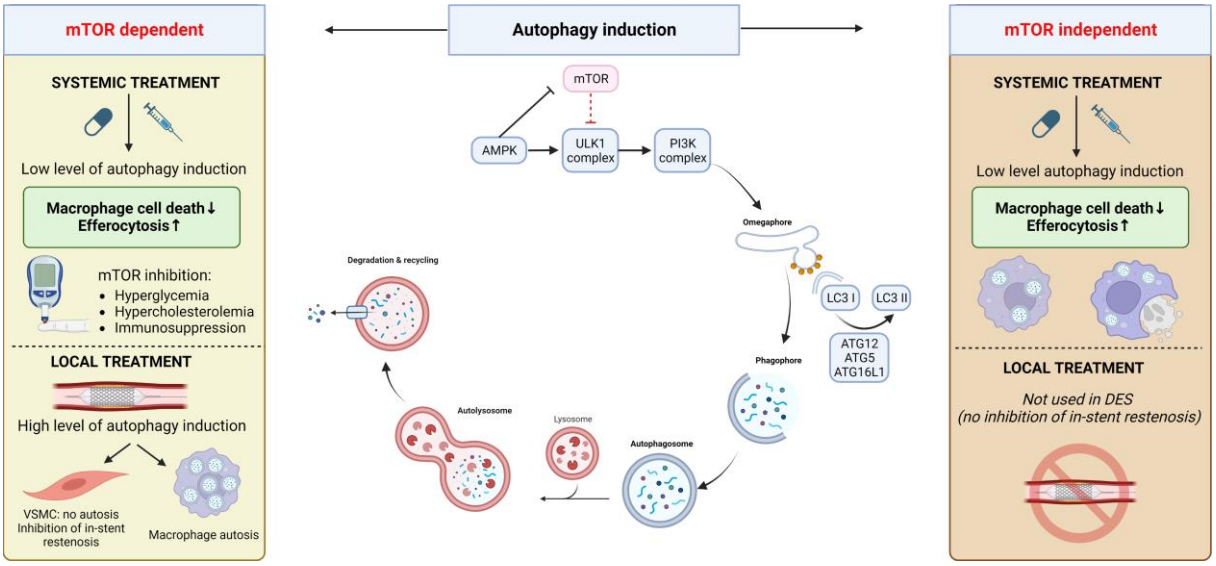
mTOR dependent
SYSTEMIC TREATMENT
Low level of autophagy induction
Macrophage cell death↓
Efferocytosis↑
mTOR inhibition:
Hyperglycemia
Hypercholesterolemia
Immunosuppression
LOCAL TREATMENT
High level of autophagy induction
VSMC: no autosis
Inhibition of in-stent restenosis
Macrophage autosis
Autophagy induction
mTOR
AMPK
ULK1 complex
PI3K complex
Omegaphore
LC3 I
LC3 II
ATG12
ATG5
ATG16L1
Phagophore
Autophagosome
Lysosome
Autolysosome
Degradation & recycling
mTOR independent
SYSTEMIC TREATMENT
Low level autophagy induction
Macrophage cell death↓
Efferocytosis↑
LOCAL TREATMENT
Not used in DES
(no inhibition of in-stent restenosis)