Arteriosclerosis, Thrombosis, and Vascular Biology

REVIEW

Regulated Necrosis in Atherosclerosis

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ABSTRACT: During atherosclerosis, lipid-rich plaques are formed in large- and medium-sized arteries, which can reduce blood flow to tissues. This situation becomes particularly precarious when a plaque develops an unstable phenotype and becomes prone to rupture. Despite advances in identifying and treating vulnerable plaques, the mortality rate and disability caused by such lesions remains the number one health threat in developed countries. Vulnerable, unstable plaques are characterized by a large necrotic core, implying a prominent role for necrotic cell death in atherosclerosis and plaque destabilization. Necrosis can occur accidentally or can be induced by tightly regulated pathways. Over the past decades, different forms of regulated necrosis, including necroptosis, ferroptosis, pyroptosis, and secondary necrosis, have been identified, and these may play an important role during atherogenesis. In this review, we describe several forms of necrosis that may occur in atherosclerosis and how pharmacological modulation of these pathways can stabilize vulnerable plaques. Moreover, some challenges of targeting necrosis in atherosclerosis such as the presence of multiple death-inducing stimuli in plaques and extensive cross-talk between necrosis pathways are discussed. A better understanding of the role of (regulated) necrosis in atherosclerosis and the mechanisms contributing to plaque destabilization may open doors to novel pharmacological strategies and will enable clinicians to tackle the residual cardiovascular risk that remains in many atherosclerosis patients.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: atherosclerosis ■ ferroptosis ■ necroptosis ■ necrosis ■ pyroptosis

Atherosclerosis is a progressive inflammatory disease of large- and medium-sized muscular arteries and typically leads to the formation of plaques, which can reduce blood flow to tissues. When a plaque develops an unstable phenotype, it is prone to rupture, which can lead to myocardial infarction, stroke, and sudden death. The global aim in the treatment of atherosclerosis is the prevention of cardiovascular complications.[1] Lifestyle changes, including dietary lipid lowering, regular physical activity, smoke cessation, and blood pressure control, are necessary measures in the prevention of the disease. If lifestyle changes are not sufficient, treatment with medications, such as those that lower circulating lipids, is recommended. However, despite tremendous advances in identifying and treating vulnerable plaques, the mortality rate and disability caused by such lesions still remains the number one health threat in developed countries. Statins and PCSK (proprotein convertase subtilisin/kexin type)-9 inhibitors reduce LDL (low-density lipoprotein)-cholesterol to low levels but do not eliminate residual cardiovascular risk as a result of other atherogenic lipoproteins or pathways for atherosclerotic cardiovascular disease, including inflammation, that are independent of LDL-cholesterol.[2] This highlights the need for additional therapeutic strategies to prevent atherosclerotic plaque formation and rupture. Because cell death is a prominent feature of advanced atherosclerotic plaques, with a major impact on atherogenesis and plaque destabilization,[3] pharmacological modulation of cell death in atherosclerosis represents a promising therapeutic approach. Indeed, plaque cells may undergo diverse types of death of which apoptosis is the best characterized. However, electron microscopic examination of human plaques showed that the vast majority of disintegrating macrophages and vascular smooth muscle cells have an ultrastructure typical of necrosis (30±18% necrotic versus 1±2% apoptotic cells).[4,5] This finding suggests that although cell death by apoptosis clearly occurs in advanced human plaques, cells that accumulate in vulnerable plaques die by necrosis.

Necrotic cell death is characterized by an increased cell volume (oncosis), organelle swelling and chromatin condensation, which eventually culminates in plasma membrane rupture and the release of intracellular compounds.[6]

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Nonstandard Abbreviations and Acronyms

ACSL-4	Acyl-CoA synthetase long chain family member 4
ADME	absorption, distribution, metabolism and excretion
AP	activator protein
ASC	apoptosis-associated speck-like protein containing a caspase recruitment domain
CANTOS	Canakinumab Anti-inflammatory Thrombosis Outcome Study
CARD	caspase recruitment domain
cIAP	cellular inhibitors of apoptosis
DAMP	damage-associated molecular pattern
DNL	Denali Therapeutics
ERK	extracellular signal-regulated kinase
FADD	fas-associated death domain
FasL	first apoptotic signal ligand
FSP	ferroptosis suppressor protein
GFH	Genfleet Therapeutics
GPX-4	glutathione peroxidase 4
GSDMD	gasdermin D
GSDME	gasdermin E
GSK	GlaxoSmithKline
GSR	glutathion-disulfide reductase
GSS	glutathion synthetase
GSSG	glutathion disulfide
HMGB-1	high-mobility group box 1
HMOX-1	heme oxygenase 1
IKK	IκB kinase
IL	interleukin
iNOS	inducible nitroc oxide synthase
JNK	c-Jun N-terminal kinase
LDL	low-density lipoprotein
LOX	lipoxygenase
LPCAT	lysophosphatidyl acyltransferase
MAPK	mitogen-activated protein kinase
MLKL	mixed lineage kinase domain-like protein
Nec-1s	necrostatin-1s
NEMO	NF-κB essential modulator
NF-κB	nuclear factor-κB
NLRP-3	nucleotide-binding oligomerization domain-like, leucine-rich repeat, and pyrin domain-containing receptor 3
Nrf-2	nuclear factor erythroid 2-related factor 2
NT-GSDMD	N-terminal cleavage product of gasdermin D
oxLDL	oxidized low-density lipoprotein
PAMP	pathogen-associated molecular pattern
PCSK-9	proprotein convertase subtilisin/kexin type 9
PRR	pattern-recognition receptor
PTGS2	prostaglandin-endoperoxide synthase 2
PYD	pyrin domain
RAGE	receptor for advanced glycation endproducts
RHIM	RIP homotypic interaction motif
RIPK	receptor-interacting protein kinase
ROS	reactive oxygen species
RSL3	Ras selective lethal 3
TAB	TGF-β activated kinase 1 (MAP3K7) binding protein
TAK	transforming growth factor β-activated kinase
TLR	Toll-like receptor
TNF-α	tumor necrosis factor α
TRADD	TNF receptor type 1-associated death domain protein
TRAIL	TNF-related apoptosis inducing ligand
TUNEL	terminal deoxynucleotidyl transferase dUTP nick-end labeling
zVAD-fmk	carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone

Highlights

- The majority of dying cells in advanced human atherosclerotic plaques undergo necrosis.
- Necrotic death stimulates atherogenesis and plaque instability through induction of inflammation and enlargement of a central necrotic core.
- Apart from accidental necrosis, regulated (programmed) necrosis is a major contributor to necrotic core formation and plaque destabilization.
- Cells in atherosclerotic plaques may undergo different types of regulated necrosis, including necroptosis, pyroptosis, and ferroptosis.
- Regulated necrosis can be efficiently blocked with potent and selective inhibitors targeting key regulators in the necrosis pathway.

Accumulation of necrotic cells and their contents triggers the formation and enlargement of a central necrotic core (Figure 1), which is a hypocellular region containing lipids and cellular debris.[7] The majority (80%) of necrotic cores in advanced human atherosclerotic plaques are larger than 1 mm^2, which compromises >10% of the lesion area.[8] However, in 65% of plaque ruptures, the necrotic core occupies >25% of the plaque area,[8] suggesting that it plays a pivotal role in unstable atherosclerotic plaques. Virtually all advanced human plaques have areas of necrosis.[9,10]

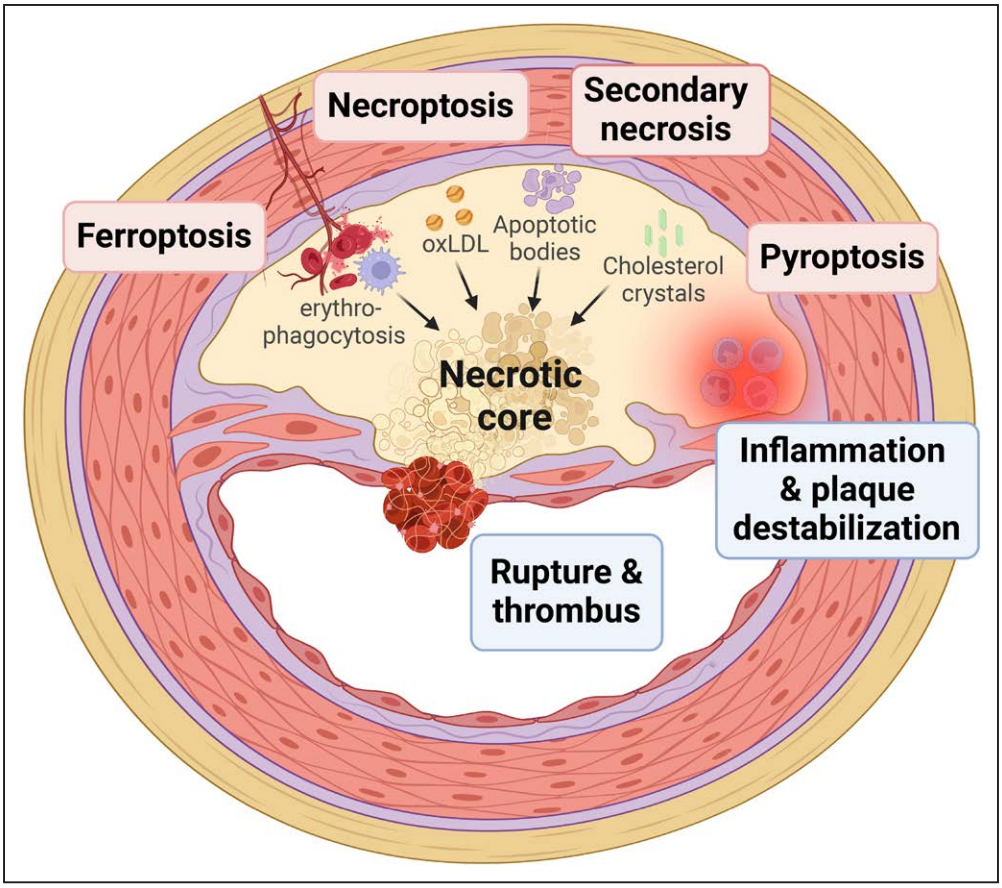

Figure 1. Overview of generally known regulated necrosis triggers in the atherosclerotic plaque and their impact on plaque progression and destabilization.

In atherosclerosis, necrosis can occur accidentally or be induced by tightly regulated pathways, eventually leading to the development of a large hypocellular region containing remnants of dead cells known as the necrotic core. During plaque progression, the hypoxic environment inside the plaque can induce neovascularization. Since intraplaque neovessels are commonly leaky, inflammatory blood cells are recruited, and intraplaque hemorrhage occurs. Macrophages surrounding intraplaque hemorrhage phagocytose erythrocytes (erythrophagocytosis), leading to HMOX-1 (heme oxygenase 1) activation, high intracellular levels of heme and iron, and consequently ferroptosis. Moreover, oxLDL (oxidized low-density lipoprotein) increases reactive oxygen species (ROS)-mediated RIPK (receptor-interacting protein kinase)-3 and MLKL (mixed lineage kinase domain-like protein) gene expression in macrophages, triggering necroptosis. As apoptotic bodies accumulate in advanced plaques, efferocytosis falls short and gets impaired, resulting in secondary necrosis. Cholesterol crystals in atherosclerotic plaques activate NLRP (nucleotide-binding oligomerization domain-like, leucine-rich repeat, and pyrin domain-containing receptor)-3 inflammasomes through lysosomal rupture leading to the induction of pyroptosis. Different forms of regulated necrosis contribute to expansion of the necrotic core which induces inflammation, plaque destabilization, fibrous cap rupture, and thrombus formation. Image created with Biorender.

Noteworthy is that both early and late stages of necrotic cores are recognized. Areas of early necrotic core formation typically show free cholesterol with mostly intact macrophages and extracellular matrix made up of proteoglycans. In contrast, late stage necrotic cores show numerous cholesterol clefts, cellular debris, and absence of extracellular matrix.[10] It should be noted that the core region is not only critical for plaque stability but also for thrombogenicity. The necrotic core contains high concentrations of tissue factor,[11] suggesting that plaque cells undergoing necrosis mediate thrombus formation after plaque rupture.

Apart from the formation and enlargement of a central necrotic core, necrotic macrophages in advanced plaques are a source of proinflammatory cytokines and damage-associated molecular patterns (DAMPs).[12] The release of DAMPs promotes inflammation in the plaque, thereby contributing to plaque instability. HMGB-1 (High-mobility group box 1) protein is one of the best studied DAMPs in atherosclerosis and is abundantly produced by plaque macrophages.[13] Once released in the extracellular space, HMGB-1 interacts with different receptors including RAGEs (receptor for advanced glycation end-products). Binding of HMGB-1 triggers the transcription of proinflammatory cytokines in an NF-κB (nuclear factor-κB) dependent manner, thereby promoting further plaque development.[14] Experimental evidence has shown that HMGB-1 expression increases during atherogenesis.[13] Neutralization of HMGB-1 in ApoE$^{-/-}$ mice reduces plaque area through inhibition of immune cell accumulation and macrophage migration.[15] Interestingly, increasing experimental evidence suggests that statins attenuate plaque formation partly by reducing the expression of

HMGB-1 and RAGE.[16–18] The release of HMGB-1 and other DAMPs or proinflammatory mediators is not only characteristic of necrosis but also occurs in senescent cells. Indeed, senescence is a dynamic process resulting in cell cycle arrest and accompanied by a proinflammatory senescence-associated secretory phenotype, which is proatherogenic.[19,20] Consequently, senescent cells are proinflammatory and undergo metabolic changes, but they remain viable. This contrasts with apoptotic and necrotic cells which lose viability irreversibly and are destined to disappear, either silently (apoptosis) or leaving a proinflammatory footprint behind (necrosis). Different intraplaque cell types, such as endothelial cells, vascular smooth muscle cells, macrophages, and T cells, can undergo senescence or cell death, but the total number of senescent versus dying cells in atherosclerotic plaques remains elusive and is complicated by their shared characteristics (eg, increased cell volume, DNA breaks, release of proinflammatory cytokines, and DAMPs) and interplay.[20,21]

Necrosis in atherosclerotic plaques can occur accidentally (eg, when cholesterol crystals puncture the plasma membrane) or can be induced following activation of tightly regulated pathways (Figure 1). In this review, we describe different types of regulated necrosis that may occur in atherosclerosis and how pharmacological targeting of these types of death can stabilize vulnerable plaques and contribute to the beneficial effects of currently applied plaque stabilizing therapies.

NECROPTOSIS

Research in the field of necrotic cell death was drastically changed by the discovery of small molecules, termed necrostatins, which inhibit RIPK (receptor-interacting protein kinase)-1-induced necroptosis in TNF (tumor necrosis factor)-α-treated cells.[22,23] This discovery led to the characterization of downstream necroptosis mediators, namely RIPK-3 and MLKL (mixed lineage kinase domain-like protein; Figure 2).[24–27] In addition to TNF-α, other necroptosis triggers have been identified, including TRAIL (TNF-related apoptosis inducing ligand), FasL (first apoptotic signal ligand), interferons, TLR (Toll-like receptor) ligands, and virus-activated pathways.[28]

Activation of the RIPK-1/RIPK-3/MLKL-Axis After TNF-α Stimulation

The response of cells to TNF-α is complex and primarily depends on the ubiquitination and phosphorylation profile of RIPK-1 (Figure 2).[29] Upon TNF-α binding, TRADD (TNF receptor type 1-associated death domain protein) and RIPK-1 are recruited to form a membrane-bound complex I. Subsequently, the default response to TNF-α signaling is ubiquitination of RIPK-1 by cIAP (cellular inhibitors of apoptosis)-1/2 and linear ubiquitin chain assembly complexes followed by the activation of prosurvival pathways including NF-κB and MAPK (mitogen-activated protein kinase) signaling. In this case, ubiquitinated RIPK-1 merely serves as a scaffold for binding and activation of mediators of NF-κB and MAPK pathways. In contrast, when protein synthesis of endogenous apoptosis inhibitors is blocked, for example by cycloheximide, or when ubiquitination and NF-κB activation are reduced, RIPK-1 switches from a prosurvival scaffold to a cytosolic prodeath protein.[30,31] In that case, necroptosis and apoptosis pathways compete and the presence and catalytic activity of caspase-8 plays a decisive role. Indeed, necroptosis proteins RIPK-1 and RIPK-3 are activated when caspase-8 is inhibited, combined with reduced RIPK-1 ubiquitination and NF-κB signaling.[30] Inhibition of caspase-8 can be obtained pharmacologically with the pan-caspase inhibitor zVAD-fmk (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone). However, the molecular mechanisms underlying caspase-8 inhibition and RIPK-1/RIPK-3 activation in atherosclerotic plaques are unclear. Most likely, oxidation or iNOS (inducible nitric oxide synthase)-driven S-nitrosylation (ie, inactivation) of a critical thiol-residue in the active site of caspase-8, in combination with proapoptotic signaling, triggers necroptosis induction. Macrophages overexpressing iNOS frequently surround the necrotic core of human plaques, which favors this hypothesis.[32] Interestingly, genetic deletion or deficiency of caspase-8 (or its adaptor TRADD) is associated with embryonic lethality in mice, which is attributed to necroptosis because deletion of RIPK-3 or MLKL rescues these mice. Lethality is mainly caused by defects in vascular development, underlining the importance of these pathways in cardiovascular disease.[33] Deletion of MLKL in caspase-8 deficient mice still causes perinatal lethality meaning that necroptosis-independent cell death is induced upon caspase-8 inhibition in later stages of embryonic development. Of note, this phenotype is rescued by deletion of ASC (apoptosis inhibitor speck-like protein) or caspase-1, both part of the pyroptosis machinery (vide infra), demonstrating that pyroptosis is induced when apoptosis and necroptosis are inhibited. Indeed, caspase-8 represents a molecular switch between apoptosis, necroptosis, and pyroptosis.[33]

Once activated, RIPK-1 undergoes autophosphorylation and interacts with RIPK-3 through a RHIM (RIP homotypic interaction motif) which results in RIPK-3 oligomerization and the formation of a necroptotic complex called the necrosome.[34,35] In the necrosome, RIPK-3 is activated resulting in a series of autophosphorylations and recruitment and phosphorylation of MLKL via its pseudokinase domain. As the name implies, the pseudokinase domain of MLKL topologically resembles a protein kinase domain but has no catalytic activity whatsoever and serves merely as an interaction domain with RIPK-3.[34] Finally, RIPK-3-induced phosphorylation of MLKL triggers its oligomerization. Phosphorylated MLKL oligomers associate with phospholipids in the plasma membrane, which

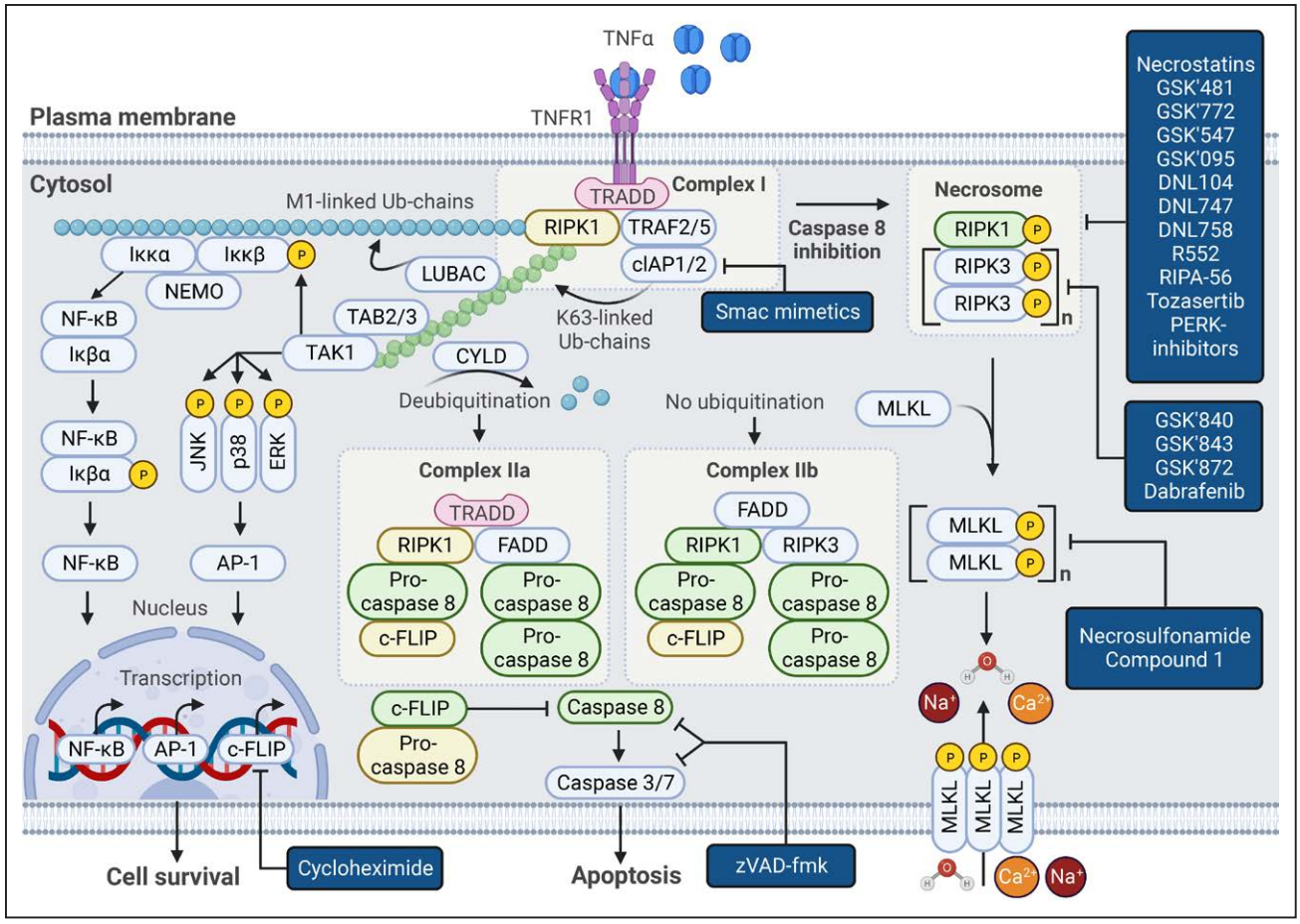

Figure 2. Overview of TNF (tumor necrosis factor)-α-induced apoptosis and necroptosis pathways and potential targets for pharmacological inhibition.

As TNF-α binds to trimeric TNFR (tumor necrosis factor receptor)-1, recruitment of tumor TRADD (TNFR1-associated death domain), and RIPK (receptor-interacting protein kinase)-1 is initiated. Consequently, TRAF (TNFR-associated factor)-2, TRAF-5, cIAP (cellular inhibitor of apoptosis protein)-1, and cIAP2 are recruited to TRADD, thereby forming complex I. cIAP-1/2 subsequently ubiquitinates RIPK-1 with K63-linked ubiquitin chains, allowing the recruitment of linear ubiquitin chain assembly complexes (LUBACs). Thereupon, LUBAC generates M1-linked ubiquitin chains, which then are added to RIPK-1. Subsequently, M1- and K63-ubiquitin chains act as a scaffold for the recruitment of IKK (IκB kinase)-α/IKK-β/NEMO (NF-κB essential modulator) and TAB (TGF-β-activated kinase 1-binding protein)-2/TAB-3/TAK (transforming growth factor β-activated kinase)-1, respectively. Next, TAK-1 phosphorylates IKK-β and the downstream MAPKs (mitogen-activated protein kinases) JNK (c-Jun N-terminal kinase), p38, and ERK (extracellular signal-regulated kinase), which activate transcription factor AP (activator protein)-1. Phosphorylated IKK-β activates IκBα resulting in the release of NF-κB (nuclear factor-κB). Subsequently, NF-κB translocates to the nucleus, resulting in the transcriptional upregulation of prosurvival genes. When cIAP-1/2 is depleted by smac mimetic-induced degradation or genetic ablation, RIPK-1 ubiquitination and NF-κB signaling are decreased. Simultaneous RIPK-1 deubiquitination by CYLD (cylindromatosis) protein will result in the release of RIPK-1 from complex I. Subsequently, RIPK-1 engages with FADD (fas-associated death domain), leading to the recruitment of procaspase-8 and c-FLIP (cellular FLICE-like protein long isoform) heterodimer and procaspase-8 homodimer, together forming complex IIa. Procaspase-8 and c-FLIP heterodimer inhibit the activation of caspase-8, thereby stimulating cell survival. In contrast, procaspase-8 homodimer generates active caspase-8, which in turn activates caspases-3 and -7 to induce apoptotic cell death. Whenever RIPK-1 is not ubiquitinated and transcription of apoptosis inhibitors is disrupted (eg, by cycloheximide), complex IIb, consisting of RIPK-1, RIPK-3, procaspase-8, and c-FLIP, will be formed. In contrast to complex IIa, the kinase activity of RIPK-1 is crucial for the induction of apoptosis via complex IIb. Pharmacological inhibition of apoptotic cell death can be achieved by pan-caspase inhibitors such, as zVAD-fmk. When caspase-8 is inhibited, activation of RIPK-1 will not result in RIPK-1-kinase dependent apoptosis but will initiate the necroptosis pathway. In the cytosol, RIPK-1 binds to RIPK-3, resulting in a series of autophosphorylation and transphosphorylations of RIPK-1 and RIPK-3. Phosphorylated RIPK-3 consequently recruits and phosphorylates MLKL (mixed lineage kinase domain-like protein) leading to MLKL oligomerization. MLKL oligomers migrate to the plasma membrane where they induce necroptotic cell death by membrane permeabilization and deregulation of calcium and sodium channels. The activation state (green = active and yellow = inactive) of RIPK-1, caspase-8, and c-FLIP plays a decisive role in the cell's fate. Pharmacological inhibition of necroptosis is achieved with inhibitors of RIPK-1 kinase activity, RIPK-3, or MLKL. DNL indicates Denali Therapeutics; GFH, Genfleet Therapeutics; GSK, GlaxoSmithKline; and zVAD-fmk, carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone. Image created with Biorender.

causes plasma membrane permeabilization, release of DAMPs and eventually necroptotic cell death.[27,36]

Necroptosis in Atherosclerosis

The expression of necroptosis mediators RIPK-3 and MLKL is elevated in human atherosclerotic plaques, both at the mRNA and protein level.[37,38] RIPK-3 and MLKL mRNA are specifically upregulated in subjects with unstable compared with stable atherosclerotic plaques.[37] RIPK-3 expression is also elevated in advanced plaques of LDLr$^{-/-}$ mice, predominantly in macrophages. Loss of RIPK-3 reduces advanced atherosclerotic lesions in ApoE$^{-/-}$ or LDLr$^{-/-}$ mice but has no effect on earlier stages of plaque development,[39] suggesting that macrophage necroptosis plays a major role in advanced plaques. Bone marrow transplantation showed that loss of RIPK-3 expression from bone marrow-derived cells is responsible for this atheroprotective effect.[39] Likewise, deletion of MLKL with antisense oligonucleotides or genetic deletion of MLKL reduces the necrotic area in advanced plaques but not in early atherosclerotic plaques of ApoE$^{-/-}$ mice.[40,41] These findings clearly illustrate a role for RIPK-3- and MLKL-mediated macrophage necroptosis in atherosclerosis.

Analogous with RIPK-3, RIPK-1 is mainly found in macrophages of human carotid lesions.[42] However, the role of RIPK-1 in atherogenesis is not straightforward and is complicated by 2 intrinsic, albeit conflicting activities, namely a scaffolding function that promotes cell survival versus a kinase activity that triggers cell death. A full RIPK-1 knockout affects both kinase-independent scaffolding functions and kinase-dependent cell death and inflammation.[42] It is worth mentioning that RIPK1$^{-/-}$ mice die perinatally due to the loss of prosurvival, RIPK-1-kinase independent signaling, such as the NF-κB pathway.[43] In contrast, mice with a RIPK-1 kinase dead (eg, K45A) or inactivating (eg, S25D) mutation are viable and display no obvious abnormalities. Similarly, mice with a deficiency of RIPK-3 and MLKL, which are key executioners of necroptosis and regulated by RIPK-1 kinase activity, are also viable and healthy. Therefore, inhibition of pathology-associated necroptosis may serve as a therapeutic target. However, in a comparative study using mice with inactive RIPK-1 kinase (RIPK1D138N/D138N), RIPK-3 deficiency (RIPK3$^{-/-}$), and MLKL deficiency (MLKL$^{-/-}$) in several models of necroptosis-related inflammatory diseases, MLKL deficiency offered little protection in a kidney ischemia-reperfusion model and no protection at all in a model of systemic inflammation, as opposed to RIPK1D138N/D138N and RIPK3$^{-/-}$ mice.[44] In general, this favors targeting upstream RIPK-1 and RIPK-3 over MLKL. RIPK-3 inhibitors have been developed and have been shown to block necroptosis in vitro and in vivo. However, they induce concentration-dependent apoptosis and, therefore, none of them moved into clinical trials.[45] In contrast, several specific RIPK-1 kinase inhibitors were safe and well tolerated in phase I (URL: https://www.clinicaltrials.gov; Unique identifiers: NCT02302404,[46] NCT03590613 and NCT03305419,[47] and NCT03757325) and phase II clinical trials (URL: https://www.clinicaltrials.gov; Unique identifiers: NCT02776033,[48] NCT02903966,[49] and NCT02858492[53]). Because the classical RIPK-1 kinase inhibitor Nec-1s (necrostatin-1s) suffers from selectivity and potency problems in vivo, several research groups have focused on the development of alternative RIPK-1 inhibitors over the past decade. Consequently, a new generation of alternative RIPK-1 kinase inhibitors have recently been developed with increased potency and improved pharmacokinetic profiles as compared to Nec-1s, such as GSK (GlaxoSmithKline)'547, GSK'772, DNL (Denali Therapeutics)747, DNL758, and GFH (GenFleet Therapeutics)312, which are currently being tested in animal models and humans.[50–52] After passing phase I safety and tolerability trials, phase II clinical studies were completed to evaluate the effects of GSK'772 on psoriasis, ulcerative colitis, and rheumatoid arthritis (URL: https://www.clinicaltrials.gov; Unique identifiers: NCT02776033,[48] NCT02903966,[49] and NCT02858492[53]). Importantly, promising results were reported for GSK'772 in patients with active plaque psoriasis.[48] DNL758/SAR443122 also passed phase I and is currently included in a proof-of-concept study in patients with cutaneous lupus erythematosus (URL: https://www.clinicaltrials.gov; Unique identifier: NCT04781816). Furthermore, GFH312 is currently included in a first-in-human trial (URL: https://www.clinicaltrials.gov; Unique identifier: NCT04676711). Altogether, RIPK-1 kinase inhibitors are readily moving into clinical stages and are reported to be safe and well tolerated so far, making the kinase activity of RIPK-1 an attractive therapeutic target for necroptosis in atherosclerosis.

Pharmacological inhibition of RIPK-1 by Nec-1s reduces plaque size and promotes plaque stability in ApoE$^{-/-}$ mice.[37] Similar observations apply after administration of RIPK-1 antisense oligonucleotides that reduce but do not completely abrogate Ripk-1 expression.[54] Additional in vitro experiments have unraveled the underlying mechanism by which necroptosis is induced in atherosclerosis. During plaque development, oxLDL (oxidized LDL) increases reactive oxygen species (ROS)-mediated RIPK-3 and MLKL gene expression in macrophages, which leads to necroptosis.[37] These findings demonstrate that inhibition of macrophage necroptosis could be a promising therapeutic strategy to prevent the development of a vulnerable plaque. However, ApoE$^{-/-}$ RIPK1S25D/S25D mice lacking active RIPK-1 kinase develop larger plaques compared with ApoE$^{-/-}$ RIPK1$^{+/+}$ controls.[55] Moreover, pharmacological inhibition of RIPK-1 with GSK'547 does not limit atherogenesis in ApoE$^{-/-}$ Fbn1$^{C1039G+/-}$ mice, a model of advanced

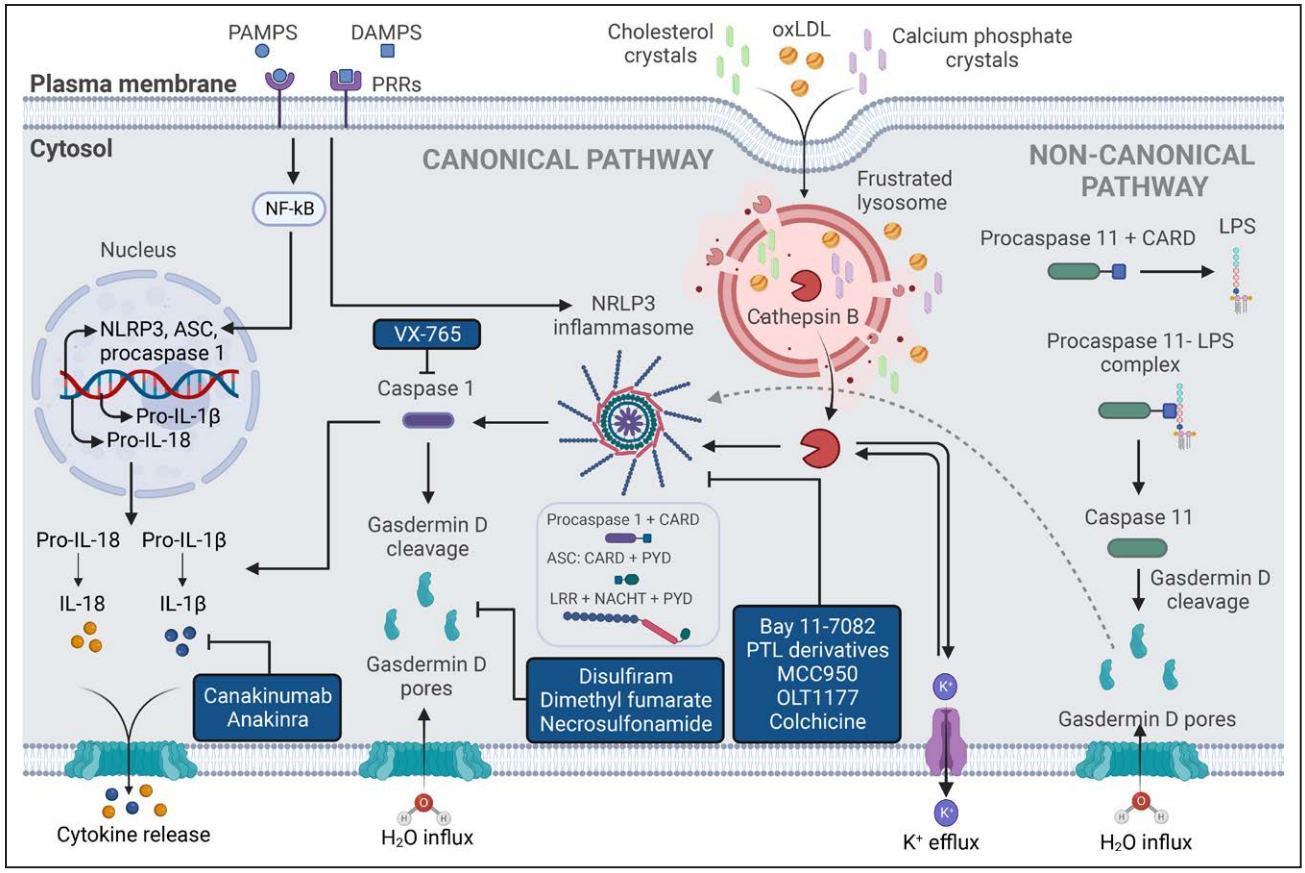

Figure 3. Overview of NLRP (nucleotide-binding oligomerization domain-like, leucine-rich repeat, and pyrin domain-containing receptor)-3-linked pyroptosis pathways and targets for pharmacological modulation.
Canonical pyroptosis is characterized by assembly and activation of inflammasomes, which are large supramolecular complexes required for caspase-1 activation. The NLRP-3 inflammasome consists of NLRP3, ASC (apoptosis inhibitor speck-like protein), and procaspase-1. NLRP-3 contains a C-terminal LRR, a central nucleotide domain called NACHT and an N-terminal PYD (pyrin domain). A priming step is required to increase the transcription of pro-IL (interleukin)-1β, NLRP-3, and ASC and is induced by recognition of extracellular molecules, such as lipopolysaccharide (LPS), TNF (tumor necrosis factor)-α, or IL-1β by PRRs (pattern-recognition receptors). Assembly of the inflammasome occurs through ASC which contains a PYD that interacts with the N-terminal PYD in NLRP3, and a CARD (caspase recruitment domain) for binding of procaspase-1. To allow proximity, and hence NLRP-3 activation, between NLRP-3 (mitochondria) and ASC (endoplasmic reticulum), activation of α-tubulin is required. The latter process can be pharmacologically inhibited by tubulin polymerization inhibitor colchicine. Activation of NLRP3 is induced by low intracellular potassium concentrations, for example, by potassium efflux through ionophores or cation channels. Cathepsins are also required for NLRP-3 activation and are released after lysosomal rupture induced by oxLDL (oxidized low-density lipoprotein), cholesterol, and calcium crystals. Activation of NLRP-3 results in cleavage of procaspase-1 to active caspase-1, which converts pro-IL-1β and pro-IL-18 to their bioactive forms and cleaves GSDMD (gasdermin D) N-terminally (NT). NT-cleaved GSDMDs oligomerize and form NT-GSDMD pores, which allow the secretion of cytokines and eventually results in membrane lysis and cell death. Caspase-1 can be pharmacologically inhibited with VX-765. Pharmacological inhibition of GSDMD can be obtained with disulfiram, dimethyl fumarate or necrosulfonamide. Neutralization of IL-1β with canakinumab or anakinra provides another strategy to block pyroptosis-induced inflammation. Alternatively, pyoptosis can be induced through caspase-11, which can directly sense cytoplasmic LPS without the need for an inflammasome, termed noncanonical pyroptosis. Caspase-11 contains an LPS-binding site located on its CARD domain through which a procaspase-11-LPS complex is formed. Subsequently, caspase-11 is activated which results in NT-cleavage of GSDMD and pyroptotic death. Caspase-11-induced NT-GSDMDs also serves as activator of the NLRP-3 inflammasome thus, caspase-11 indirectly triggers canonical pyroptosis. Image created with Biorender. DAMPs indicates damage-associated molecular patterns; and PAMPs, pathogen-associated molecular patterns.

atherosclerosis.[55] Accordingly, GSK'547 does not limit plaque formation in more advanced stages of atherogenesis while it decreases the plaque area in earlier stages.[56] Additionally, when RIPK-1 expression is reduced by antisense oligonucleotides,[54] there is a reduction in the macrophage ability to activate proinflammatory NF-κB while necroptotic cell death remains functional, suggesting RIPK-1 expression may be tightly regulated to balance its proinflammatory versus prodeath functions. Together, these results stress the complex and stage-dependent involvement of RIPK-1 kinase activity in atherosclerosis.

PYROPTOSIS

Besides necroptosis, other types of regulated necrosis have been observed in atherosclerotic plaques, although their significance is not always clear-cut. Among the most well-defined is pyroptosis, a proinflammatory form

of regulated cell death that is characterized by the formation of plasma membrane pores via caspase-1-dependent cleavage of GSDMD (gasdermin D), which is highly expressed in different tissues and cell types and well conserved in mammals.[57,58]

Canonical Inflammasome-Mediated Pyroptosis

Canonical induction of pyroptosis involves cleavage (activation) of caspase-1 through a large supramolecular complex, known as an inflammasome (Figure 3). The NLRP-3 (NOD [nucleotide-binding oligomerization domain]-like, LRR [leucine-rich repeat], and pyrin domain-containing receptor 3) inflammasome is currently the best characterized inflammasome and consists of NLRP-3, ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), and procaspase-1. First, a priming step, is required to increase the transcription of pro-IL (interleukin)-1β, NLRP-3, and ASC. The priming step is induced by recognition of extracellular molecules, such as lipopolysaccharide, TNF-α, or IL-1β by PRRs (pattern-recognition receptors). Subsequently, NF-κB will be activated and the C-terminal LRR on NLRP3 will be deubiquitinated, allowing NLRP3 activation. Assembly of the inflammasome occurs through ASC which contains a PYD (pyrin domain), that interacts with the N-terminal (NT) PYD in NLRP3, and a CARD (caspase recruitment domain) for binding of procaspase-1.[59]

Once assembled, the NLRP-3 inflammasome can be activated by low intracellular potassium concentrations. Indeed, potassium efflux induced by ionophores such as nigericin or by cation channels such as the P2X7 receptor and TWIK2 channel induced by ATP, are known inducers of NLRP3-dependent pyroptosis.[60,61] Furthermore, cathepsin B is required for NLRP3 activation.[62] Mounting evidence suggests that oxLDL, crystals of cholesterol and calcium phosphate, and fibrillar ligands in atherosclerotic plaques activate NLRP-3 inflammasomes through lysosomal rupture and subsequent cathepsin release, which in turn leads to cleavage and activation of procaspase-1.[63–66] Active caspase-1 exerts proinflammatory effects by converting pro-IL-1β and pro-IL-18 into their bioactive forms. Furthermore, GSDMD is N-terminally cleaved by caspase-1. Subsequently, NT-GSDMDs oligomerize and bind to phospholipids in the cell membrane where they induce pore formation. NT-GSDMD-induced pores are ≈10 to 15 nm in diameter, in contrast to the much smaller MLKL-induced channels, and a large number of these NT-GSDMD pores disrupt the plasma membrane and physiological ionic gradients.[67,68] The damaged membrane starts blebbing, a phenomenon that is also observed in apoptotic cells although in a much slower fashion.[67,69] Pyroptotic cells flatten while releasing cellular content through the NT-GSDMD pores, such as IL-1β, IL-18, ATP, HMGB-1, and cleaved GSDMs, which amplify inflammation and pyroptosis induction.[68,69] Interestingly, secretion of IL-1β and IL-18 does not require cell lysis and is temporally associated with GSDMD-dependent pore formation, suggesting that these pores are sufficient to mediate cytokine release.[70]

Noncanonical Pyroptosis Induction

Caspase-11 can directly sense cytoplasmic lipopolysaccharide without the need for TLR-4 or a canonical inflammasome, because it contains an lipopolysaccharide-binding site located on its CARD domain, forming a procaspase-11-lipopolysaccharide complex, also called the noncanonical inflammasome (Figure 3).[71] Active caspase-11 cleaves GSDMD which induces pore formation and pyroptosis, similar to caspase-1. Next to direct induction of pyroptosis, caspase-11-induced formation of NT-GSDMD also serves as an activator of the NLRP-3 inflammasome. In this way, caspase-11 indirectly triggers canonical caspase-1-dependent pyroptosis and, subsequently, the maturation and release of IL-1β and IL-18.[72]

Pyroptosis in Atherosclerosis

Recent studies have shown that components of the NLRP-3 inflammasome are present in human atherosclerotic plaques and are expressed in macrophages and foam cells around the necrotic core.[73,74] Both mRNA and protein levels of NLRP-3, caspase-1, ASC, IL-1β, and IL-18 are increased in human plaques compared with normal arteries.[74–77] In patients with coronary atherosclerosis, the aortic expression of the NLRP-3 inflammasome is correlated with disease severity and clinical risk factors for cardiovascular disease (eg, hypertension, diabetes, smoking, and LDL-cholesterol).[75] Moreover, the highest NLRP3 expression levels were observed in unstable lesions as compared to stable lesions and nonatherosclerotic arteries.[73] All these epidemiological studies highlight a possible role of the NLRP3 inflammasome in atherogenesis and plaque destabilization. Interestingly, it has been demonstrated that ATP and cholesterol crystals induce pyroptosis both in the presence and absence of lipopolysaccharide priming in cultured plaques.[76] Both inducers are relevant in the context of atherosclerosis as cholesterol is involved in every stage of plaque development, while extracellular ATP is expected to be more abundant in advanced stages of plaque development and destabilization due to excessive cell death.[66,76,78] Another possible activator of the NLRP-3 inflammasome in atherosclerosis is nicotine. Indeed, smoking is a major risk factor for atherosclerosis which is at least partly attributable to activation of the ROS/NLRP-3-axis and pyroptosis induction.[79,80]

Many studies have targeted proteins involved in canonical (NLRP3, ASC, caspase-1, IL-1β, and IL-18) and noncanonical (caspase-11) pyroptosis signaling in atherosclerotic models and reported beneficial effects

on atherogenesis (reviewed elsewhere: Xu et al[57] and Qian et al[81]). However, often these targets are not limited to pyroptosis signaling but are also involved in other proinflammatory and cell death pathways. For example, caspase-1 is reported to be proapoptotic, besides its pyroptotic properties, and deletion of caspase-1 activity is linked to lytic, nonpyroptotic cell death.[82] NLRP-3 is also linked to RIPK-1 and RIPK-3 signaling and subsequently, apoptosis and necroptosis.[83–85] In addition, targeting one single player might not suffice as both canonical and noncanonical pathways play a role in atherosclerosis. This is supported by the considerable risk for recurrent cardiovascular events in participants of the CANTOS trial (Canakinumab Anti-inflammatory Thrombosis Outcome Study) treated with the IL-1β antibody canakinumab.[86]

GSDMD is the common executor of both canonical and noncanonical pyroptosis. Interestingly, GSDMD mRNA is upregulated in peripheral blood monocytes of patients with coronary artery disease. Moreover, expression of GSDMD and NT-GSDMD is increased in ApoE$^{-/-}$ and wild-type mice fed a high-fat diet as compared to chow-fed controls.[87] Expression of NT-GSDMD was also observed in human endarterectomy specimens.[88] Thus, GSDMD is actively involved in pyroptosis during atherogenesis in both humans and mice, making GSDMD an attractive pharmacological target for pyroptosis in atherosclerosis. Disulfiram is a Food and Drug Administration-approved drug used for the treatment of alcohol abuse and was recently identified as a potent GSDMD inhibitor. It covalently modifies cys191 (in human, cys192 in mouse) in GSDMD to block pore formation at nanomolar concentrations[89] but is metabolized to carbon disulphide, which promotes atherosclerosis, thus not recommended for long-term antiatherosclerosis therapy. Dimethyl fumarate, used as an immunomodulator in multiple sclerosis, has been identified as an alternative GSDMD inhibitor,[90] and reduces aortic plaque area in hyperglycemic ApoE$^{-/-}$ mice.[91] More recently, genetic deletion of GSDMD as well as pharmacological inhibition with necrosulfonamide reduce infarct size and heart failure in a mouse model of acute myocardial infarction,[92,93] underlining the involvement of GSDMD in cardiovascular disease and the possibility to use it as a pharmacological target in atherosclerosis. This is supported by the observation that GSDMD deficiency decreases the formation of inflammatory plaques in ApoE$^{-/-}$ mice.[88] However, no effect on the initiation and formation of stable aortic plaques was observed. Thus, GSDMD is mainly involved in the transition to an inflammatory, vulnerable plaque phenotype in advanced stages of atherosclerosis and appears to be a promising target for limiting plaque destabilization.

Gasdermin E-Mediated Secondary Necrosis in Atherosclerosis

Besides the canonical caspase-1/inflammasome pathway that activates GSDMD, GSDME (gasdermin E) was recently identified as an alternative effector of programmed necrosis under proapoptotic conditions.[94] GSDME leads to membrane pores and programmed necrosis of apoptotic cells, known as secondary necrosis, after cleavage by caspase-3. Efficient clearance of apoptotic cells—a process called efferocytosis—is essential for preventing secondary necrosis. Unfortunately, efferocytosis is strongly impaired in advanced atherosclerosis,[95] making secondary necrosis a main feature of advanced plaques. Stimulation of efferocytosis is challenging as most phagocytes are lipid-filled foam cells with limited phagocytosis potential. Nevertheless, ongoing therapeutic efforts aimed at boosting efferocytosis have shown promising results.[96] It should be noted that GSDME, after cleavage by caspase-3, can also form pores in the mitochondrial membrane resulting in the release of proapoptotic molecules, such as cytochrome c. This event creates a positive feedback loop that promotes caspase-3 activation and further GSDME cleavage, ultimately augmenting apoptotic cell death and secondary necrosis. Surprisingly, there are no studies describing (cleaved) GSDME expression in atherosclerosis. It should be noted that GSDME is transcriptionally controlled by p53 and is essential in the p53-mediated response to DNA damage. Because DNA damage, phosphorylated (active) p53 and cleaved caspase-3 are abundantly present in advanced plaques,[97] this alternative, GSDME-mediated pathway of programmed necrosis, besides GSDMD, definitely needs further attention in the context of atherosclerotic plaque destabilization.

FERROPTOSIS

Ferroptosis was discovered when the small molecules erastin and RSL (Ras selective lethal)-3 were designed to induce cytotoxicity in cells expressing oncogenic mutant RAS proteins.[98–100] When characterizing the cytotoxicity induced by erastin and (1S,3R)-RSL3, a unique necrotic morphology featuring smaller mitochondria and increased membrane density was observed. Moreover, ferroptosis is characterized by excessive iron-dependent lipid peroxidation. This can occur by enzymatic peroxidation of polyunsaturated fatty acids in phospholipid bilayers through the ACSL (Acyl-CoA synthetase long chain family member)-4/LPCAT (lysophosphatidyl acyltransferase)-3/15-LOX (lipoxygenase) axis (Figure 4). Lipid peroxidation can also be induced nonenzymatically through Fenton chemistry, which forms hydroxyl radicals, and free radical chain reactions. Both enzymatic and nonenzymatic lipid peroxidation require free ferrous iron (Fe^{2+}), which resides in a cytosolic labile iron pool. Under physiological conditions, accumulation of ferrous iron in the cytosol and growth of the labile iron pool is limited by ferritin as it can store up to 4500 iron molecules.[101] Another

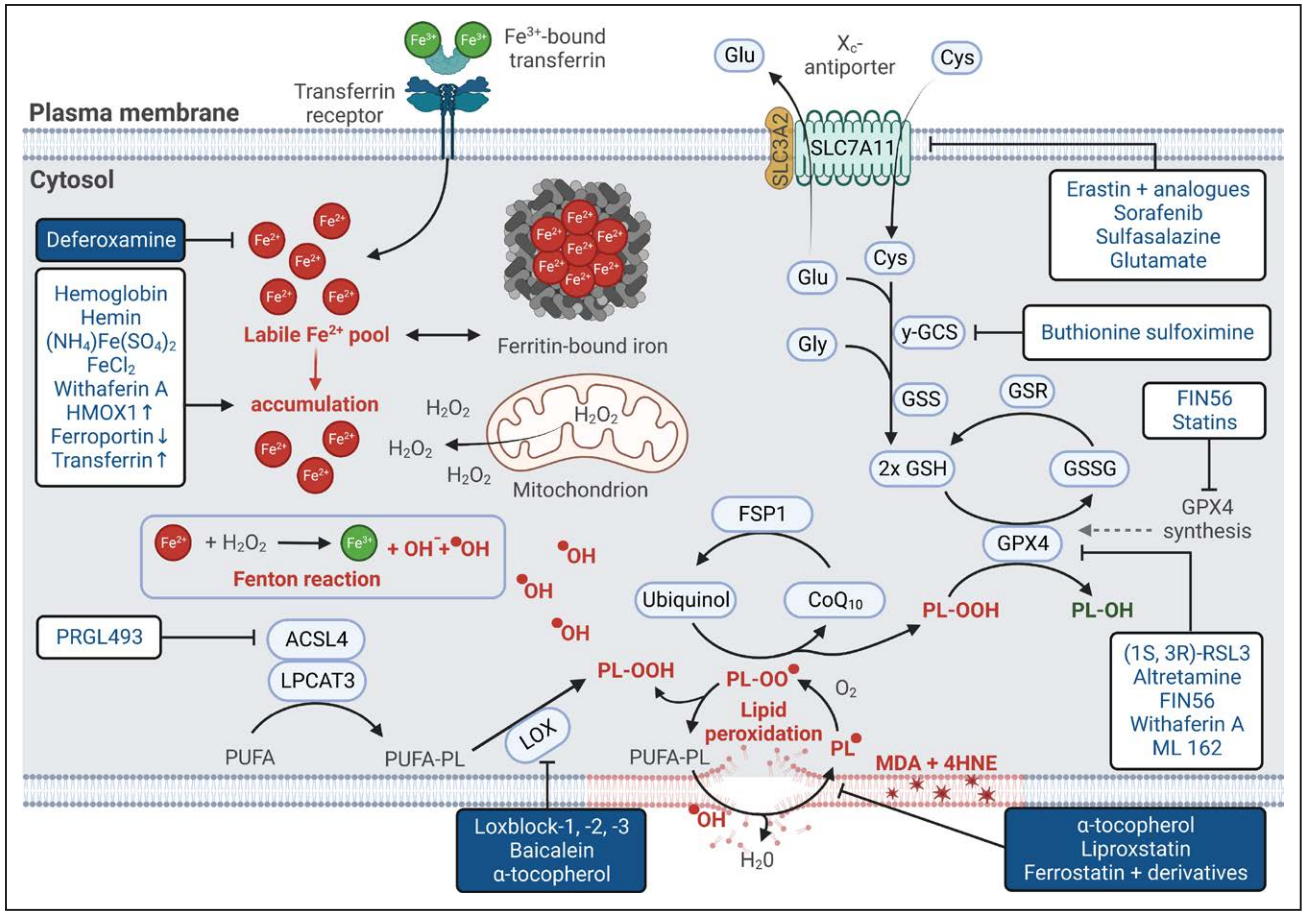

Figure 4. Overview of ferroptosis-inducing pathways and potential pharmacological targets.
Ferroptosis is characterized by excessive iron-dependent lipid peroxidation of poly unsaturated fatty acids (PUFAs) in phospholipid (PL) bilayers, which can be induced enzymatically or nonenzymatically and requires free ferrous iron (Fe^{2+}). Enzymatic lipid peroxidation occurs through the ACSL (Acyl-CoA synthetase long chain family member)-4/LPCAT (lysophosphatidyl acyltransferase)-3/15-LOX (lipoxygenase) axis. Nonenzymatic lipid peroxidation includes Fenton chemistry, which forms hydroxyl radicals, and free radical chain reactions. Free ferrous iron resides in a cytosolic labile iron pool. Growth of the labile iron pool is limited by ferritin, which stores Fe^{2+}. When the finely regulated iron balances are disturbed due to overloading of cells with iron, for example, with hemoglobin, hemin, ferrous ammonium sulfate or iron chloride, or due to excessive activation of HMOX (heme oxygenase)-1, decreased ferroportin (iron transporter) expression or enhanced transferrin (iron receptor) expression, the labile iron pool grows beyond the buffering capacities of ferritin, which suffices to induce ferroptosis. Under physiological conditions, lipid peroxides formed in cellular membrane environments are reduced by GPX (glutathione peroxidase)-4, thereby oxidizing glutathione (GSH). For the synthesis of GSH entry of cysteine (Cys) in exchange for glutamate (Glu) through the Xc-antiporter system is required. Inhibition of the Xc-antiporter with erastin, inhibition of glutathione synthesis or direct inhibition of GPX-4 with 1S,3R-RSL3 induces canonical ferroptosis through accumulation of lipid peroxides. Excessive lipid peroxidation of PUFAs in PL bilayers affects chemical and geometric properties of the lipid bilayer which destroys the barrier function of the plasma membrane and leads to cell lysis and eventually cell death. Physiologically ferroptosis is limited by ferritin and by FSP (ferroptosis suppressor protein)-1. FSP-1 reduces coenzyme Q10 (CoQ_{10}) to ubiquinol, which traps lipid peroxyl radicals. Pharmacological ferroptosis inhibition is obtained with radical trapping agents, such as α-tocopherol, liproxstatin, ferrostatin-1, and derivates, with iron chelators (eg, deferoxamine) or with blockers of the ACSL-4/LPCAT-3/15-LOX axis (eg, PRGL493, baicalcein, α-tocopherol, and LOX block-1). GSR indicates glutathion-disulfide reductase; GSS, glutathion synthetase; and GSSG, glutathion disulfide. Image created with Biorender.

ferroptosis limiting factor is FSP (ferroptosis suppressor protein)-1, which reduces coenzyme Q10 to ubuiqinol. The latter traps lipid peroxyl radicals, accompanied by the formation of coenzyme Q10, thereby preventing the formation of lipid peroxides.[102,103]

When growth of the labile iron pool exceeds the buffering capacity of ferritin (noncanonical pathway) or when formed lipid peroxides are not cleared properly (canonical pathway), excessive lipid peroxidation of polyunsaturated fatty acids in phospholipid bilayers occurs. This affects the chemical and geometric properties of the lipid bilayer, leads to membrane pore formation, and destroys the barrier function of the plasma membrane. Together, these events lead to cell lysis and eventually cell death.[104] Additionally, lipid peroxides are degraded to toxic lipid aldehydes, such as malondialdehyde and 4-hydroxynonenal, which adds an extra layer of cytotoxicity. Indeed, malondialdehyde and 4-hydroxynonenal easily bind covalently to proteins and DNA, thereby impairing several signaling processes. Furthermore, malondialdehyde binding on

epitopes generates oxidative self-epitopes which can be recognized by scavenger receptors on phagocytes or as DAMPs by PRRs and initiate innate immune responses. Malondialdehyde-modified proteins and lipoproteins also trigger adaptive immune responses.[105] Once lipid peroxides are formed, malondialdehyde and 4-hydroxynonenal may further amplify ROS signaling.

Canonical Induction of Ferroptosis

Canonical ferroptosis induction involves impaired GPX (glutathione peroxidase)-4, either through direct inhibition of GPX-4 or through depletion of its substrate glutathione (Figure 4).[106] GPX-4 catalyzes the reduction of lipid peroxides in cellular membrane environments and simultaneously oxidizes and consumes glutathione. Full-body depletion of GPX-4 in mice results in embryonic lethality and shRNA mediated GPX-4 knockdown sensitizes cells to undergo ferroptosis, underlining the importance of GPX-4 in normal physiology by preventing excessive lipid peroxidation.[107] (1S,3R)-RSL3 and erastin are both inducers of canonical ferroptosis. (1S,3R)-RSL3 (and not the other diastereomers of RSL3) directly targets and inhibits GPX-4.[107] Erastin inhibits the X_c^--antiporter system which transports cystine, a key precursor in the synthesis of glutathione, into the cell in exchange for glutamate. By inhibiting this system, erastin decreases the entry of cystine into the cell and induces downstream depletion of the cellular antioxidant glutathione, the substrate of GPX-4, thereby impairing GPX-4 activity and triggering accumulation of ROS.[107,108]

Noncanonical Induction of Ferroptosis

Noncanonical ferroptosis is induced when ferrous iron accumulates in the labile iron pool (Figure 4).[109] This occurs when the finely regulated iron balances are disturbed due to overloading of cells with iron (eg, overload with hemoglobin, hemin, ferrous ammonium sulfate, or iron chloride) or due to excessive activation of HMOX (heme oxygenase)-1, decreased ferroportin expression or enhanced transferrin expression.[109–111] Ferroportin and transferrin regulate iron export to and transport in the circulation, respectively. HMOX-1 is responsible for the catabolism of hemoglobin to heme and Fe^{2+}. This is highly relevant in the context of phagocytosis of erythrocytes, a process called erythrophagocytosis, which is responsible for the clearance of aged or damaged erythrocytes by macrophages.[112]

Ferroptosis in Atherosclerosis

Several epidemiological studies have reported a relationship between iron levels and atherogenesis.[113,114] Indeed, restriction of dietary iron intake or iron chelation with deferoxamine leads to a significant decrease in experimental plaque formation.[115–117] Moreover, iron depletion through frequent blood donation is associated with a decreased cardiovascular risk.[118–120] Given that lipid peroxidation, intraplaque hemorrhage and iron deposition are key features of advanced human plaques, ferroptosis is suggested to play a role in plaque destabilization, however, to date no direct evidence exists. Erythrocytes are released in the plaque during intraplaque hemorrhages, thereby increasing the cholesterol and iron content of the plaque. Macrophages surrounding intraplaque hemorrhages phagocytose erythrocytes leading to HMOX-1 activation and high intracellular levels of heme and iron.[32,121,122] In vitro experiments demonstrated that erythrophagocytosis by macrophages induces ferroptosis (Puylaert P, unpublished data). The latter results in the release of intracellular content and DAMPs into the plaque, which may contribute to exponential growth of the necrotic core, amplification of the inflammatory cycle, and eventually plaque destabilization. This hypothesis is supported by the observation that atherosclerotic lesions contain malondialdehyde, a toxic lipid peroxidation product, and adaptive IgG antibodies with specificity for malondialdehyde.[105] Malondialdehyde can modify epitopes and is, therefore, capable of inducing undesired proinflammatory responses in atherosclerosis. Malondialdehyde can also bind to LDL, leading to the formation of proatherogenic and immunogenic modified LDL. Interestingly, immunization studies have shown atheroprotective effects of neutralizing endogenous malondialdehyde.[105] Similarly, 4-hydroxynonenal can bind to apolipoprotein B on LDL, which leads to uptake of LDL by macrophages and contributes to foam cell formation. Next to the presence of lipid peroxidation products in plaques, iron-positive foam cells are present in human plaques, HMOX-1 expression is increased in human aortic endothelial cells and hemoglobin, HMOX-1 and ferritin accumulate in advanced human plaques.[32,122,123] These observations are suggestive of growth of the labile iron pool in plaque cells, and thus, combined with lipid peroxidation, of the occurrence of ferroptosis in plaques.

The first specific ferroptosis inhibitors that were identified by high-throughput screening of small molecule libraries are ferrostatin-1 and liproxstatin-1.[106,124] These are potent inhibitors of (1S,3R)-RSL3- and erastin-induced ferroptosis showing EC_{50} values in the nanomolar range. The antiferroptotic activity of both ferrostatin-1 and liproxstatin-1 can be attributed to their potent radical trapping effects, especially in lipid bilayers.[125,126] Another radical trapping agent that inhibits ferroptosis is α-tocopherol, albeit with a lower potency in lipid bilayers as compared to ferrostatin-1 and liproxstatin-1. Pharmacological inhibition of ferroptosis with ferrostatin-1 was recently reported to reduce plaque burden in $ApoE^{-/-}$ mice as well as in diabetic $ApoE^{-/-}$ mice.[123,127] Both studies also observed decreased iron levels in serum and in the aorta and increased GPX-4 and SLC7A11 (subunit of the X_c^--antiporter) expression which, at least partly, explains the observed atheroprotective effects. Accordingly, GPX-4 overexpression in $ApoE^{-/-}$ mice inhibited plaque progression.[128] Furthermore,

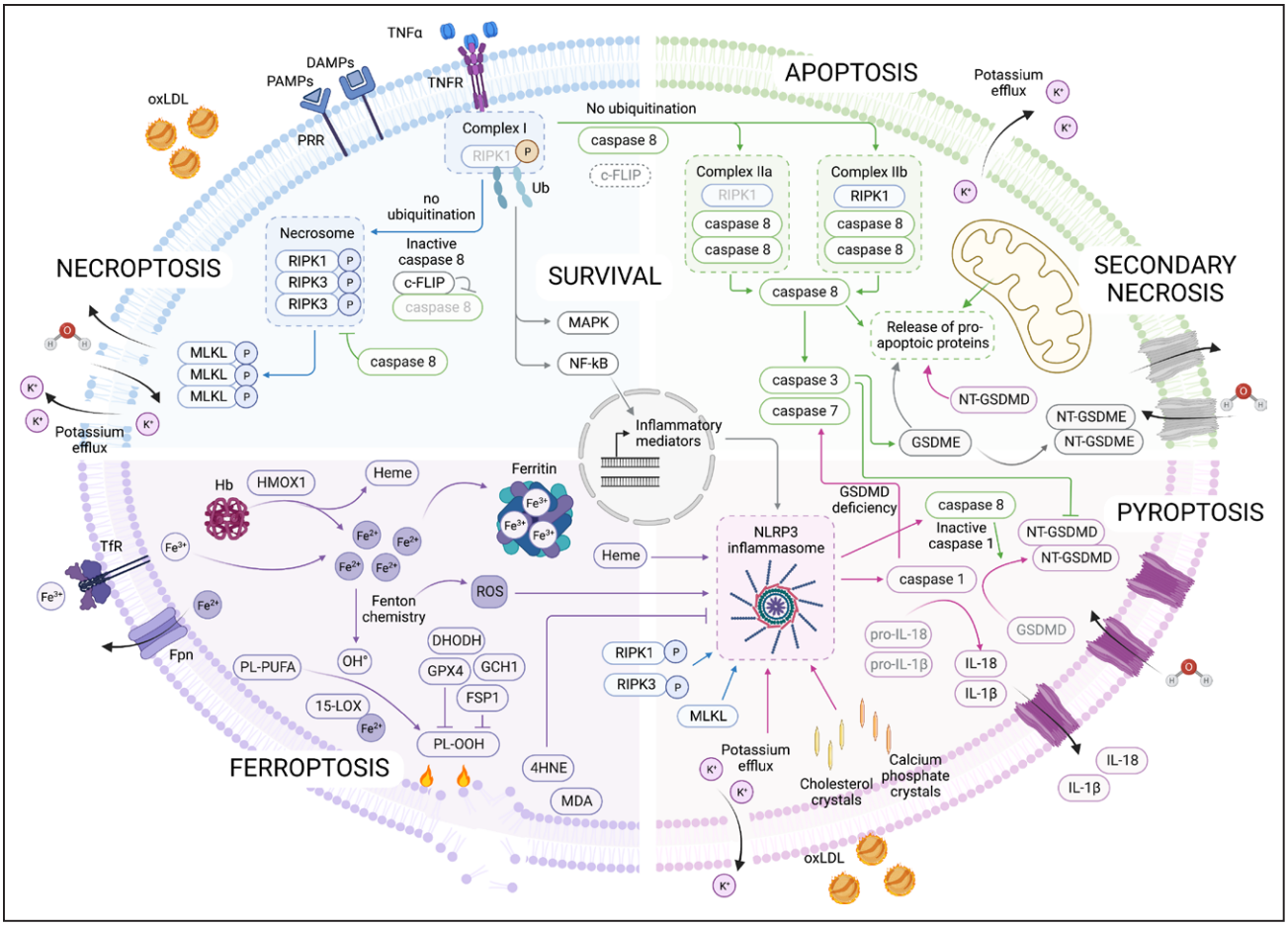

Figure 5. Cross-talk between necroptosis, apoptosis, secondary necrosis, pyroptosis, and ferroptosis.
When TNF (tumor necrosis factor)-α binds to its receptor (TNFR) membrane-bound complex I is formed which contains RIPK (receptor-interacting protein kinase)-1. In complex I RIPK-1 is ubiquitinated (Ub) and phosphorylated (P) which keeps it in an inactive conformation. RIPK-1 serves as a scaffold for prosurvival signaling, such as MAPK (mitogen-activated protein kinase) and NF-κB (nuclear factor-κB) pathways. Caspase 8 is inactivated by c-FLIP (cellular FLICE-like protein). When c-FLIP is absent, caspase-8 is released and engages with RIPK-1 to form complex IIa. If RIPK-1 is not ubiquitinated or de-ubiquinated it is released from complex I and engages with caspase 8 to form complex IIb. Both complex IIa and complex IIb activate caspase 8, which in turn activates apoptosis effector caspases 3 and 7. Caspase-8 also induces the mitochondrial release of proapoptotic proteins. Caspase 3 can cleave GSDME (gasdermin E), thus excessive caspase 3 activation leads to GSDME-mediated secondary necrosis. If RIPK-1 is not ubiquitinated and caspase 8 is inactivated (eg, by c-FLIP), RIPK-1 is released from complex I, undergoes activating autophosphorylations and phosphorylates RIPK-3. Phosphorylated RIPK-1 and RIPK-3 form a necrosome which recruits and phosphorylates MLKL (mixed lineage kinase domain-like protein). Subsequently, oligomerization of MLKL induces membrane permeabilization and eventually necroptosis. During necroptosis, apoptosis and secondary necrosis, potassium efflux occurs which is an activator of the NLRP-3 (NOD [nucleotide-binding oligomerization] domain-like, LRR [leucine-rich repeat]- and pyrin domain-containing receptor 3) inflammasome. Cholesterol crystals indirectly also induce potassium efflux. Caspase 1 is activated on the NLRP-3 inflammasome and in turn cleaves GSDMD (gasdermin D) N-terminally (NT) and activates IL (interleukin)-1β and IL-18. NT-GSDMDs oligomerize and form GSDMD pores leading to osmotic lysis and cytokine release. In the absence of GSDMD, caspase 1 induces apoptosis through activation of caspase 3. Reciprocally, caspase 3 limits pyroptosis through inhibition of GSDMD. In the absence of caspase 1, caspase 8 can also become activated on the NLRP-3 inflammasome and can cleave GSDMD. GSDMD and GSDME can also promote mitochondrial release of proapoptotic proteins, demonstrating intense cross-talk between pyroptosis, apoptosis, and secondary necrosis. Moreover, the necroptosis effector MLKL induces assembly of the NLRP-3 inflammasome, which can be activated by RIPK-1 and RIPK-3. Transcription of NLRP-3 inflammasome components can be induced through NF-κB pathways, linking TNF-α signaling and pyroptosis. Similarly, heme and oxidative stress can also activate the NLRP-3 inflammasome, providing a link between ferroptosis and pyroptosis. Indeed, heme is formed together with ferrous iron (Fe^{2+}) when hemoglobin (Hb) is catabolized by HMOX (heme oxygenase)-1. This contributes to growth of the labile iron pool, which is limited by ferritin. Growth of the labile iron pool beyond the buffering capacity of ferritin, for example, by excessive HMOX-1 activation, increased expression of TfR (transferrin receptor) or decreased expression of Fpn (ferroportin), can induce ferroptosis through Fenton chemistry and nonenzymatic lipid peroxidation. Alternatively, enzymatic lipid peroxidation by 15-LOX (lipoxygenase) can also induce ferroptosis. Excessive lipid peroxidation can be limited by GPX (glutathione peroxidase)-4 and FSP (ferroptosis suppressor protein)-1. During lipid peroxidation toxic lipid aldehydes are formed, such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). The latter was recently reported to inhibit NLRP-3 inflammasome activation. These examples illustrate that the fate of a cell depends on many factors (eg, cell death stimulus, cellular environment, cell type, and expression pattern of cell death executors) and is subjected to intense cross-talk between cell death pathways. Image created with Biorender.

Table 1. Necroptosis-, Pyroptosis-, and Ferroptosis-Related Proteins in Human Atherosclerotic Plaques

Cell death-related genes and proteins	Expression in human atherosclerotic plaques	Methods	Reference
RIPK-3	Gene expression upregulated in unstable carotid plaques	Gene expression analysis on carotid endarterectomy specimens (n=127 plaques from BiKE) vs disease-free artery (n=10 organ donors without cardiovascular history). Gene expression analysis on unstable (n=87 symptomatic patients) vs stable (n=40 asymptomatic patients) carotid plaques from BiKE.	Karunakaran et al[37] and Perisic et al[134]
	Protein levels increased in carotid lesions with necrotic core	Western blot analysis of plaques with vs without necrotic core (n≥6 per group from 12 autopsy samples and 6 carotid endarterectomies)	Tian et al[38]
RIPK-1	Protein level increased in carotid lesions with necrotic core	Western blot analysis of plaques with vs without necrotic core (n≥6 per group from 12 autopsy samples and 6 carotid endarterectomies)	Tian et al[38]
	Expressed in macrophages (but not smooth muscle cells) in carotid lesions	Double immunofluorescence staining of advanced carotid plaque selected from a library of endarterectomy specimens from patients with carotid stenosis >70%	Coornaert et al[42]
	Expressed in macrophages and endothelial cells in coronary plaques	Immunohistochemical analysis of early plaques vs arteries with pathological intimal thickening obtained from CVPath Institute Sudden Cardiac Death registry	Karunakaran et al[54] and Arking et al[135]
	Gene expression upregulated in early carotid plaques	Gene expression analysis on carotid endarterectomy specimens from 34 patients: plaque tissue (mostly stage IV and V lesions) vs adjacent macroscopically intact tissue (almost exclusively stage I and II lesions)	Karunakaran et al[54] and Ayari et al[136]
MLKL	Gene expression upregulated in unstable carotid plaques	Gene expression analysis on carotid endarterectomy specimens (n=127 plaques from BiKE) vs disease-free artery (n=10 organ donors without cardiovascular history). Gene expression analysis on unstable (n=87 symptomatic patients) vs stable (n=40 asymptomatic patients) carotid plaques from BiKE.	Karunakaran et al[37] and Perisic et al[134]
	P-MLKL levels increased in advanced coronary plaques	Immunohistochemical analysis of advanced lesions (n=11) vs arteries with pathological intimal thickening (n=5) obtained from CVPath Institute Sudden Cardiac Death registry	Karunakaran et al[37] and Arking et al[135]
NLRP-3	Gene and protein levels upregulated in unstable carotid plaques	Gene expression analysis on carotid endarterectomy specimens from 30 patients with >70% stenosis: unstable vs stable plaques (n=15 per group) based on plaque morphology. Immunohistochemical analysis of carotid endarterectomy specimens from 30 patients with >70% stenosis vs mesenteric arteries from 10 patients with early intestinal tumors. Western blot and immunohistochemical analysis of carotid endarterectomy specimens from 30 patients with >70% stenosis: unstable vs stable plaques (n=15 per group) based on plaque morphology.	Shi et al[73]
	Gene and protein levels upregulated in advanced coronary plaques	Gene expression analysis and immunohistochemistry of coronary artery specimens from 10 explanted hearts (4 patients undergoing heart transplantation, 6 donor hearts not fulfilling transplantation criteria, all male): advanced (stage IV–VI) vs early (stage I–III) lesions from the same coronary tree (n=10 per group)	Rajamaki et al[74]
	Expressed in aortic plaques and expression level correlated with coronary atherosclerosis severity and risk factors	Immunohistochemical analysis of ascending aorta specimens from 36 patients undergoing coronary artery bypass graft surgery (severity determined using Gensini scoring) vs 10 healthy renal arteries from kidney donors	Zheng et al[75]
	Gene levels upregulated in carotid plaques and expressed in intraplaque macrophages and smooth muscle cells	Gene expression analysis on carotid endarterectomy specimens (n=106 plaques from BiKE) vs disease-free arteries (n=9 iliac arteries, n=1 aorta intima from organ donors without cardiovascular history). Immunohistochemical analysis of 3 carotid endarterectomy specimens from BiKE.	Paramel Varghese et al[76]
	Expression positively correlated with degree of stenosis and plaque severity stage	Immunohistochemical analysis of 40 coronary artery samples obtained from 4 autopsy cases (causes of death: occupying lesions, cerebral hemorrhage, myocardial infarction, and diabetes)	Zhou et al[137]
ASC	Gene and protein levels upregulated in unstable carotid plaques	Gene expression analysis on carotid endarterectomy specimens from 30 patients with >70% stenosis: unstable vs stable plaques (n=15 per group) based on plaque morphology. Immunohistochemical analysis of carotid endarterectomy specimens from 30 patients with >70% stenosis vs mesenteric arteries from 10 patients with early intestinal tumors. Western blot and immunohistochemical analysis of carotid endarterectomy specimens from 30 patients with >70% stenosis: unstable vs stable plaques (n=15 per group) based on plaque morphology.	Shi et al[73]
	Gene and protein levels upregulated in advanced coronary plaques	Gene expression analysis and immunohistochemistry of coronary artery specimens from 10 explanted hearts (4 patients undergoing heart transplantation, 6 donor hearts not fulfilling transplantation criteria, all male): advanced (stage IV–VI) vs early (stage I–III) lesions from the same coronary tree (n=10 per group)	Rajamaki et al[74]

(*Continued*)

Table 1. Continued

Cell death-related genes and proteins	Expression in human atherosclerotic plaques	Methods	Reference
	Gene levels upregulated in carotid plaques and expressed in intraplaque macrophages and smooth muscle cells	Gene expression analysis on carotid endarterectomy specimens (n=106 plaques from BiKE) vs disease-free arteries (n=9 iliac arteries, n=1 aorta intima from organ donors without cardiovascular history). Immunohistochemical analysis of 3 carotid endarterectomy specimens from BiKE.	Paramel Varghese et al[76]
Caspase-1	Gene and protein levels upregulated in unstable carotid plaques	Gene expression analysis on carotid endarterectomy specimens from 30 patients with >70% stenosis: unstable vs stable plaques (n=15 per group) based on plaque morphology. Immunohistochemical analysis of carotid endarterectomy specimens from 30 patients with >70% stenosis vs mesenteric arteries from 10 patients with early intestinal tumors. Western blot and immunohistochemical analysis of carotid endarterectomy specimens from 30 patients with >70% stenosis: unstable vs stable plaques (n=15 per group) based on plaque morphology.	Shi et al[73]
	Gene and protein levels upregulated in advanced coronary plaques	Gene expression analysis and immunohistochemistry of coronary artery specimens from 10 explanted hearts (4 patients undergoing heart transplantation, 6 donor hearts not fulfilling transplantation criteria, all male): advanced (stage IV–VI) vs early (stage I–III) lesions from the same coronary tree (n=10 per group)	Rajamaki et al[74]
	Gene levels upregulated in carotid plaques	Gene expression analysis on carotid endarterectomy specimens (n=106 plaques from BiKE) vs disease-free arteries (n=9 iliac arteries, n=1 aorta intima from organ donors without cardiovascular history). Immunohistochemical analysis of 3 carotid endarterectomy specimens from BiKE.	Paramel Varghese et al[76]
	Cleaved caspase-1 upregulated in symptomatic plaques in vascular smooth muscle cells that are transdifferentiating to macrophages	Double immunofluorescence staining of carotid endarterectomy specimens: symptomatic vs asymptomatic patients (n=12 per group) with >70% stenosis	Burger et al[138] and Montecucco et al[139]
	Expression positively correlated with degree of stenosis and plaque severity stage	Immunohistochemical analysis of 40 coronary artery samples obtained from 4 autopsy cases (causes of death: occupying lesions, cerebral hemorrhage, myocardial infarction, and diabetes)	Zhou et al[137]
IL-1β	Gene and protein levels upregulated in unstable carotid plaques	Gene expression analysis on carotid endarterectomy specimens from 30 patients with >70% stenosis: unstable vs stable plaques (n=15 per group) based on plaque morphology. Immunohistochemical analysis of carotid endarterectomy specimens from 30 patients with >70% stenosis vs mesenteric arteries from 10 patients with early intestinal tumors. Western blot and immunohistochemical analysis of carotid endarterectomy specimens from 30 patients with >70% stenosis: unstable vs stable plaques (n=15 per group) based on plaque morphology.	Shi et al[73]
	Gene levels upregulated in carotid plaques	Gene expression analysis on carotid endarterectomy specimens (n=106 plaques from BiKE) vs disease-free arteries (n=9 iliac arteries, n=1 aorta intima from organ donors without cardiovascular history). Immunohistochemical analysis of 3 carotid endarterectomy specimens from BiKE.	Paramel Varghese et al[76]
	Expression increased in symptomatic plaques in vascular smooth muscle cells that are transdifferentiating to macrophages	Double immunofluorescence staining of carotid endarterectomy specimens: symptomatic vs asymptomatic patients (n=12 per group) with >70% stenosis	Burger et al[138] and Montecucco et al[139]
IL-18	Gene and protein levels upregulated in unstable carotid plaques	Gene expression analysis on carotid endarterectomy specimens from 30 patients with >70% stenosis: unstable vs stable plaques (n=15 per group) based on plaque morphology. Immunohistochemical analysis of carotid endarterectomy specimens from 30 patients with >70% stenosis vs mesenteric arteries from 10 patients with early intestinal tumors. Western blot and immunohistochemical analysis of carotid endarterectomy specimens from 30 patients with >70% stenosis: unstable vs stable plaques (n=15 per group) based on plaque morphology.	Shi et al[73]
	Gene levels upregulated in carotid plaques	Gene expression analysis on carotid endarterectomy specimens (n=106 plaques from BiKE) vs disease-free arteries (n=9 iliac arteries, n=1 aorta intima from organ donors without cardiovascular history). Immunohistochemical analysis of 3 carotid endarterectomy specimens from BiKE.	Paramel Varghese et al[76]

(*Continued*)

Table 1. Continued

Cell death-related genes and proteins	Expression in human atherosclerotic plaques	Methods	Reference
	Gene and protein levels upregulated in carotid plaques and correlated with plaque instability	Western blot analysis of 12 endarterectomy specimens (from 40 carotids collected from 35 patients) vs 5 control arteries (2 carotids and 3 mammary arteries collected at autopsy or during coronary artery bypass graft surgery). Immunohistochemical analysis of 6 endarterectomy specimens (from 40 carotids collected from 35 patients). Gene expression analysis on 22 endarterectomy specimens (from 40 carotids collected from 35 patients): symptomatic (n=13) vs asymptomatic (n=9) patients; unstable (n=14) vs stable (n=8) plaques (based on ulceration).	Mallat et al[77]
GSDMD	Cleaved GSDMD expressed in macrophage- and smooth muscle cell-rich areas of advanced carotid plaque	Double immunohistochemical staining of advanced carotid plaque selected from a library of endarterectomy specimens from patients with carotid stenosis >70%	Puylaert et al[88]
PTGS2	Expression positively correlated with degree of stenosis and plaque severity stage	Immunohistochemical analysis of 40 coronary artery samples obtained from 4 autopsy cases (causes of death: occupying lesions, cerebral hemorrhage, myocardial infarction, and diabetes)	Zhou et al[137]
GPX-4	Expression negatively correlated with degree of stenosis and plaque severity stage	Immunohistochemical analysis of 40 coronary artery samples obtained from 4 autopsy cases (causes of death: occupying lesions, cerebral hemorrhage, myocardial infarction, and diabetes)	Zhou et al[137]
Ferritin	Expressed in carotid plaques and upregulated in unstable plaques and symptomatic patients	Immunohistochemical analysis of 52 endarterectomy specimens from patients with >50% stenosis (Linköping Carotid Study): ruptured (n=19) vs vulnerable (n=9) vs stable (n=8) plaques (based on morphology and collagen staining); symptomatic (n=44) vs asymptomatic (n=8) patients	Li et al[140]
	Expressed in early plaques and accumulation in advanced plaques	Immunohistochemical analysis of 35 carotid endarterectomy specimens from patients with varying degree of carotid atherosclerosis (n=18 advanced and ruptured plaques, n=8 early plaques) vs healthy arteries (non-diseased parts of carotid arteries and n=4 mammary artery specimens)	Yuan et al[122]
	Expression in the intima of fatty streaks in areas rich in macrophages and TUNEL positivity	Immunohistochemical analysis of coronary artery and thoracic aorta specimens: fatty streak (n=12 autopsy cases with general atherosclerosis) vs normal arteries (n=19: normal areas of arteries from 12 autopsy cases with general atherosclerosis and 7 young autopsy cases without atherosclerosis)	Yuan[141]
Transferrin receptor	Expressed in carotid plaques and upregulated in unstable plaques and symptomatic patients	Immunohistochemical analysis of 52 endarterectomy specimens from patients with >50% stenosis (Linköping Carotid Study): ruptured (n=19) vs vulnerable (n=9) vs stable (n=8) plaques (based on morphology and collagen staining); symptomatic (n=44) vs asymptomatic (n=8) patients	Li et al[140]
HMOX-1	Gene and protein levels upregulated in symptomatic carotid plaques and correlated with intraplaque iron deposits and hemorrhage	Gene expression analysis on carotid endarterectomy specimens from 4 HeCES patients with bilateral stenosis: symptomatic vs asymptomatic side/plaques Gene expression analysis on carotid endarterectomy specimens from 40 HeCES patients with unilateral stenosis: symptomatic (n=22 patients with ipsilateral stroke symptoms) vs asymptomatic (n=18 patients without cerebrovascular symptoms) Western blot and immunohistochemical analysis of carotid endarterectomy specimens from 22 patients: symptomatic (n=13 patients with confirmed ipsilateral stroke) vs asymptomatic (n=9 asymptomatic patients with normal brain imaging)	Ijäs et al[142]
	Gene expression upregulated in advanced carotid plaques	Gene expression analysis on carotid endarterectomy specimens from 34 patients: plaque tissue (mostly stage IV and V lesions) vs adjacent macroscopically intact tissue (almost exclusively stage I and II lesions)	Ayari et al[136]
	Gene expression upregulated in unstable carotid plaques	Gene expression analysis on unstable (n=40 asymptomatic patients) vs stable (n=87 symptomatic patients) carotid plaques from BiKE	Perisic et al[134]
	Expressed in intraplaque macrophages together with hemoglobin and iron deposits	Immunohistochemical analysis of carotid endarterectomy specimens from 15 patients with >70% stenosis	Kockx et al[32]
	Expressed in atherosclerotic tissue and accumulation in advanced carotid plaques	Combined analysis of: Gene expression in carotid endarterectomy specimens from 34 patients: plaque tissue (mostly stage IV and V lesions) vs adjacent macroscopically intact tissue (almost exclusively stage I and II lesions). Gene expression in carotid endarterectomy specimens from 30 patients with >70% stenosis: advanced (n=8) vs early (n=9) plaques.	Ayari et al,[136] Wu et al,[143] and Döring et al[144]
	Gene expression in coronary plaque areas with hemorrhage	Immunohistochemical analysis of autopsy cases from sudden coronary deaths (CVPath Institute Sudden Coronary Death registry): plaque areas with prior hemorrhage vs areas without hemorrhage	Finn et al[145]

ASC indicates apoptosis-associated speck-like protein; BiKE, Biobank of Karolinska Endarterectomies; GPX, glutathione peroxidase; GSDMD, gasdermin D; HeCES, Helsinki Carotid Endarterectomy Study; HMOX, heme oxygenase; IL, interleukin; MLKL, mixed lineage kinase domain-like protein; NLRP-3, nucleotide-binding oligomerization domain-like, leucine-rich repeat- and pyrin domain-containing receptor 3; PTGS2, prostaglandin-endoperoxide synthase 2; RIPK, receptor-interacting protein kinase; and TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

Table 2. Human Intervention Studies Targeting Necroptosis, Pyroptosis, or Ferroptosis

Target	Drug	Study	Main findings	Reference
RIPK-1	GSK'772	Phase I single-center, randomized, placebo-controlled, double-blind study in healthy, male, adult volunteers (URL: https://www.clinicaltrials.gov; Unique identifier: NCT02302404)	Single and repeated doses were safe and well tolerated. Pharmacokinetic profiles showed dose linearity over the range tested (up to 120 mg bid). High level of RIPK-1 target engagement.	Weisel et al[46]
		Phase IIa multicenter, randomized, placebo-controlled, double-blind, repeat-dose study in patients with mild-to-moderate active plaque-type psoriasis (URL: https://www.clinicaltrials.gov; Unique identifier: NCT02776033)	60 mg bid or tid for 84 d was generally safe and well tolerated. Improved clinical efficacy measures and biomarkers in patients with mild-to-moderated active plaque psoriasis.	Weisel et al[48]
		Phase IIa multicenter, randomized, placebo-controlled, double-blind study in patients with active ulcerative colitis (URL: https://www.clinicaltrials.gov; Unique identifier: NCT02903966)	60 mg tid for 84 d was generally safe and well tolerated. No clinical improvement in patients with active ulcerative colitis.	Weisel et al[49]
		Phase IIa, multicenter, randomized, placebo-controlled, double-blind study in patients with moderate to severe rheumatoid arthritis (URL: https://www.clinicaltrials.gov; Unique identifier: NCT02858492)	60 mg bid or tid for 84 d was generally safe and well tolerated. No clinical improvement in patients with moderate to severe rheumatoid arthritis.	Weisel et al[53]
		Phase I single-center, randomized, placebo-controlled, double-blind study in healthy volunteers in the United Kingdom (URL: https://www.clinicaltrials.gov; Unique identifier: NCT03305419) and in Japan (URL: https://www.clinicaltrials.gov; Unique identifier: NCT03590613)	Similar pharmacokinetics and tolerability between Western and Japanese subjects	Tompson et al[47]
		Nonrandomized, open-label study to assess the pharmacokinetic profile of a modified-release prototype coated tablet formulation (URL: https://www.clinicaltrials.gov; Unique identifier: NCT03649412)	The GSK DiffCORE technology overcame food effects and can be used in once daily dosing regimen	Tompson et al[146]
	GSK3145095	Phase I/II open-label study in patients with solid tumors (URL: https://www.clinicaltrials.gov; Unique identifier: NCT03681951)	Study terminated	Cohen et al[147]
	DNL747/ SAR443060	Randomized, double-blind, placebo-controlled first-in-human study	Single and multiple ascending dosing 100–400 mg bid for 14 d was generally well tolerated and safe High level of RIPK-1 target engagement. Simultaneous preclinical studies revealed long-term toxicity. Production terminated.	Vissers et al[148]
		Phase Ib multicenter, randomized, double-blind, placebo-controlled crossover study in patients with Alzheimer disease (URL: https://www.clinicaltrials.gov; Unique identifier: NCT03757325)	50 mg bid for 28 d was safe and well tolerated High level of RIPK-1 target engagement (but lower than 200 mg bid). Simultaneous preclinical studies revealed long-term toxicity. Production terminated.	Vissers et al[148]
		Phase Ib multicenter, randomized, double-blind, placebo-controlled crossover study in patients with amyotrophic lateral sclerosis (URL: https://www.clinicaltrials.gov; Unique identifier: NCT03757351)	50 mg bid for 28 d was safe and well tolerated High level of RIPK-1 target engagement (but lower than 200 mg bid). Simultaneous preclinical studies revealed long-term toxicity. Study terminated.	Vissers et al[148]
	DNL758/ SAR443122	Phase IB, randomized, double-blind, placebo-controlled study in hospitalized patients with severe COVID-19 (URL: https://www.clinicaltrials.gov; Unique identifier NCT04469621)	300 mg bid for 14 d was considered well tolerated and safe. Preliminary results showed trends towards clinical improvement but a larger confirmatory trial to assess clinically significant effects is required.	Clot et al[149]
		Phase II, multicenter, randomized, double-blind, placebo-controlled proof-of-concept study in patients with moderate to severe subacute or chronic cutaneous lupus erythematosus (URL: https://www.clinicaltrials.gov; Unique identifier: NCT04781816)	Ongoing (estimated study completion: March 2023)	
	DNL788/ SAR443820	Phase I, open-label, crossover study in healthy volunteers (URL: https://www.clinicaltrials.gov; Unique identifier: NCT04982991)	"Robust target engagement was demonstrated at doses that were generally well tolerated." Published on Denali Therapeutics Inc website on October 6, 2021	[150]

(*Continued*)

Table 2. Continued

Target	Drug	Study	Main findings	Reference
		Phase II multicenter, randomized, double-blind, placebo-controlled study in patients with amyotrophic lateral sclerosis (HIMALAYA study, URL: https://www.clinicaltrials.gov; Unique identifier: NCT05237284)	Ongoing (estimated study completion: September 2023)	
	GFH312	A first-in-human, randomized, double-blind, placebo-controlled study in healthy subject (URL: https://www.clinicaltrials.gov; Unique identifier: NCT04676711)	Ongoing (estimated study completion: August 2022)	
IL-1β	Canakinumab	Randomized, double-blind, placebo-controlled, event-driven trial in the prevention of recurrent cardiovascular events among stable post-myocardial infarction patients with hsCRP≥2 mg/L (Canakinumab Anti-inflammatory Thrombosis Outcome Study, URL: https://www.clinicaltrials.gov; Unique identifier: NCT01327846)	Quarterly subcutaneous administration of 150 mg canakinumab reduced the risk for recurrent cardiovascular events independent of lipid lowering. Quarterly subcutaneous administration of canakinumab decreased hsCRP levels after 48 mo. A residual cardiovascular risk remains in patients despite treatment with high-intensity statins and canakinumab which was associated to IL-18 and IL-6.	Ridker et al[86,151]
NLRP-3 inflammasome	Colchicine (237 clinical trials on clinicaltrials.gov)	Phase III colchicine cardiovascular outcomes trial (COLCOT, URL: https://www.clinicaltrials.gov; Unique identifier: NCT02551094)	Low-dose colchicine (0.5 mg once daily) reduced the risk of ischemic cardiovascular events in patients recruited within 30 d after myocardial infarction. Patients benefit from early initiation of colchicine treatment (time-to-treatment initiation effect).	Tardif et al[152] and Bouabdallaoui et al[153]
		Phase III open-label, randomized, controlled trial to study the effect of low-dose colchicine on the natural history of patients with stable coronary artery disease (LoDoCo, identifier: ACTRN12610000293066)	Addition of 0.5 mg/d colchicine to standard therapy in patients with stable coronary artery disease reduced risk of cardiovascular events	Nidorf et al[154]
		Phase III double-blind, randomized, controlled, investigator-initiated, event-driven trial to study the effect of low-dose colchicine for secondary prevention of cardiovascular disease in patients with established, stable coronary artery disease (LoDoCo2, identifier: ACTRN12614000093684)	Addition of 0.5 mg/d colchicine to standard therapy in patients with stable coronary artery disease decreased the occurrence of cardiovascular events	Nidorf et al[155]
		Phase IV trial of anti-inflammatory therapy during percutaneous coronary intervention (URL: https://www.clinicaltrials.gov; Unique identifiers: NCT02594111 and NCT01709981)	No effect on the risk for post-percutaneous coronary intervention-related myocardial infarction. Colchicine attenuated the increase in inflammatory-markers after percutaneous coronary intervention.	Shah et al[156]
		Phase IV efficacy and safety study of colchicine in improving the stability of coronary plaques in patients with acute coronary syndrome (COLOCT, URL: https://www.clinicaltrials.gov; Unique identifier: NCT04848857)	Ongoing (estimated study completion: July 2023)	
	OLT-1177 (dapansutrile)	Phase I single-center, randomized, dose escalation study in healthy volunteers (URL: https://www.clinicaltrials.gov; Unique NCT02134964)	Daily oral administration of up to 1000 mg for 8 d was generally safe and well tolerated	Marchetti et al[157]
		Phase IIa open-label, dose adaptive, proof-of-concept study in patients with monoarticular gout flare	Orally administered 100–2000 mg/d was generally safe and well tolerated. Reduced target joint pain and joint and systemic inflammation were reported after 7 d treatment.	Klück et al[158]
		Phase Ib single-center, randomized, double-blind study in patients with NYHA II–III systolic heart failure (URL: https://www.clinicaltrials.gov; Unique identifier: NCT03534297)	Orally administered 500–2000 mg/d was generally safe and well tolerated in patients with stable HFrEF	Wohlford et al[159]
		Phase 2 pilot, single-center, open-label, proof-of-concept study in patients with Schnitzler syndrome (URL: https://www.clinicaltrials.gov; Unique identifier: NCT03595371)	Ongoing (estimated study completion: February 2023)	
		Phase 2 multicenter, randomized, double-blind, placebo-controlled study in patients with moderate COVID-19 symptoms and evidence of early cytokine release syndrome (URL: https://www.clinicaltrials.gov; Unique identifier: NCT04540120)	Ongoing (estimated study completion: July 2023)	

(*Continued*)

Table 2. Continued

Target	Drug	Study	Main findings	Reference
Caspase-1	VX765 (belnacasan)	Phase II randomized, double-blind, placebo-controlled study in patients with treatment-resistant partial epilepsy (URL: https://www.clinicaltrials.gov; Unique identifier: NCT01048255)	"The primary endpoint of the study was safety and tolerability, and results from the study showed a similar safety profile for VX-765 as compared to placebo. Secondary endpoints and additional analyses evaluated the clinical activity of VX-765, and results support the initiation of a larger and longer-duration." Published on Vertex Pharmaceuticals Inc website on March 10, 2011	[160]
		Phase IIb randomized, double-blind, placebo-controlled, parallel-group, dose-ranging study in patients with treatment-resistant partial epilepsy (URL: https://www.clinicaltrials.gov; Unique identifier: NCT01501383)	Terminated	
		Phase II randomized, double-blind, placebo-controlled study in patients with chronic plaque psoriasis requiring systemic therapy (URL: https://www.clinicaltrials.gov; Unique identifier: NCT00205465)	Completed: results to be reported	

COLCOT indicates Colchicine Cardiovascular Outcomes Trial; HFrEF, heart failure with reduced ejection fraction; HIMALAYA, Phase 2 Study for SAR443820 in Participants With Amyotrophic Lateral Sclerosis; hsCRP, high-sensitivity C-reactive protein; IL, interleukin; LoDoCo, low dose colchicine; NLRP-3, nucleotide-binding oligomerization domain-like, leucine-rich repeat- and pyrin domain-containing receptor 3; NYHA, New York Heart Association; and RIPK, receptor-interacting protein kinase.

inhibition of enzymatic lipid peroxidation with a pharmacological 15-LOX blocker decreased plaque progression in atherosclerotic rabbits with preestablished plaques and genetic deletion of 15-LOX resulted in decreased plaque burden in ApoE$^{-/-}$ mice.[129,130] Together, these studies are highly suggestive of involvement of iron-dependent lipid peroxidation in atherogenesis. Combined with the availability of novel ferrostatin-1 analogs with improved potency and ADME (absorption, distribution, metabolism and excretion) properties, such as UAMC-3203 and UAMC-3206,[131–133] this makes ferroptosis a promising target to explore in atherosclerosis.

REGULATED NECROSIS IN HUMAN ATHEROSCLEROTIC PLAQUES

As described above, several studies have reported the expression of proteins related to necroptosis, pyroptosis, or ferroptosis in plaques from both humans and animals. Observational studies in human atherosclerotic plaques are summarized in Table 1. Moreover, many compounds targeting regulated necrosis have been developed and some are already moving into clinical trials (Table 2). This makes targeting necroptosis, pyroptosis, and ferroptosis an interesting approach to explore in atherosclerosis.

CONCLUDING REMARKS

Research over the past 2 decades has demonstrated that necrotic cell death is critically involved in the formation and destabilization of atherosclerotic plaques. Different forms of regulated necrosis can be distinguished by analyzing a combination of mechanistic characteristics (eg, presence of phosphorylated MLKL during necroptosis and GSDMD-pore formation during pyroptosis) and morphological features (eg, mitochondrial abnormalities during ferroptosis, cytoplasm flattening, and membrane blebbing during pyroptosis). The most prominent characteristics of necroptosis, pyroptosis and ferroptosis are described in Table 3 (and more extensively reviewed elsewhere).[161,162] Hitherto, many qualitative analyses of morphological and mechanistic markers of regulated necrosis in atherosclerotic plaques have been performed. However, a quantitative analysis to estimate the true percentage of intraplaque cells undergoing necroptosis, pyroptosis, and ferroptosis (and hence their contribution to atherogenesis) is currently lacking.

Table 3. Overview of the Most Prominent Morphological and Mechanistic Characteristics of Necroptosis, Pyroptosis, and Ferroptosis

	Necroptosis	Pyroptosis	Ferroptosis
Morphological characteristics	Cell swelling	Lack of cell swelling	Mitochondrial abnormalities
	Plasma membrane rupture	Plasma membrane rupture	
	Moderate chromatin condensation	Membrane blebbing and formation of pyroptotic bodies	
		Moderate chromatin condensation	
Mechanistic markers	P-MLKL-mediated membrane disruption	Cleaved GSDMD pores	Lipid peroxidation
			Iron accumulation

GSDMD indicates gasdermin D; and MLKL, mixed lineage kinase domain-like protein.

Pharmacological inhibition of regulated necrosis improves several features of plaque stability, such as lowering plaque inflammation, reducing oxidative stress, and increasing collagen content and fibrous cap thickness. However, different forms of regulated necrosis can occur simultaneously, particularly in advanced atherosclerotic plaques, because of the coordinated action of multiple death-inducing stimuli. Indeed, severity stages in human coronary atherosclerosis positively associate with the expression of both ACSL-4 (ferroptosis), caspase-1, and NLRP-3 (pyroptosis).[137] Furthermore, the expression of several ferroptosis- and necroptosis-related proteins was upregulated in atherosclerotic rabbits and did not respond to atorvastatin or PCSK-9 antibody.[163] This finding highlights the possible contribution of these different forms of regulated necrosis to the residual cardiovascular risk that remains in atherosclerosis patients despite efficient lipid-lowering therapy. Accordingly, it may be necessary to target multiple death pathways. Dimethyl fumarate is a GSDMD inhibitor (pyroptosis) that reduces atherogenesis in hyperglycemic ApoE$^{-/-}$ mice by increasing Nrf (nuclear factor erythroid 2-related factor)-2 expression and decreasing ROS production.[91] Nrf2, once activated, binds to the antioxidant responsive element in the nucleus, thereby inducing the expression of antioxidant enzymes, such as HMOX-1.[164] Overexpression of HMOX-1 can trigger noncanonical ferroptosis induction, thus dimethyl fumarate indirectly targets ferroptosis. Moreover, dimethyl fumarate also inhibits NF-κB and promotes apoptosis. Therefore, the atheroprotective effects of dimethyl fumarate treatment are possibly attributable to combined targeting of Nrf-2, ferroptosis, pyroptosis, and apoptosis. Similarly, tanshinone IIA, a flavonoid used in traditional Chinese medicine, has atheroprotective effects due to targeting of either apoptosis,[165] ferroptosis,[166] or NLRP3-mediated pyroptosis.[167]

Another aspect that complicates therapeutic inhibition of regulated necrosis is the cross-talk between cell death mechanisms (Figure 5).[168] Apoptosis, autophagy, and (regulated) necrosis were initially considered mutually exclusive states. However, recent findings reveal a balanced interplay between these types of death so that blocking one type of death may sensitize cells to initiate another death pathway. For example, inhibition of caspases by the pan-caspase-inhibitor zVAD-fmk is sufficient for preventing apoptosis in many experimental models, but it may facilitate the necroptosis program downstream of TNF receptor. Conversely, active caspase-8 promotes apoptosis and simultaneously cleaves RIPK-1 and RIPK-3, thereby preventing activation of the RIPK-1/RIPK-3/MLKL-axis and necroptosis induction.[169,170] These observations underline the fine interplay between apoptosis and necroptosis pathways, with caspase-8 representing the molecular switch.[33] Importantly, caspase-8 can also be activated on inflammasomes when propyroptotic caspase-1 is absent or inhibited.[82] Moreover, necroptosis induction via TLR activation (eg, by poly(I:C) on TLR-3 or by lipopolysaccharide on TLR-4) upregulates the transcription of NLRP-3 while RIPK-1/RIPK-3 signaling can induce NLRP-3 inflammasome activation. During the execution phase of necroptosis, MLKL pores are formed which induce potassium efflux, a known activator of the NLRP-3 inflammasome.[83] Similarly, pannexin-1 channels are formed during apoptosis, which also induce potassium efflux and NLRP-3 inflammasome activation.[171] Altogether, there is a clear overlap and cross-talk between pyroptosis, apoptosis, and necroptosis pathways, which was recently termed PANoptosis.[172] It should be noted, however, that cross-talk with ferroptosis is also described. For example, NLRP3-dependent pyroptosis regulates downstream ferroptosis in a mouse model of type 2 diabetes-induced cardiac remodeling and contractile dysfunction. Moreover, the pyroptosis inhibitors MCC950 and necrosulfonamide inhibit high glucose/high fat-induced ferroptosis in vitro.[173]

In conclusion, the simultaneous occurrence of different types of regulated necrosis and intense cross-talk between cell death pathways provide a strong scientific rationale for recommending combination therapy to prevent necrotic core formation in advanced plaques. Simultaneous inhibition of different types of cell death by combination therapy could be an important emerging concept in the field of atherosclerosis, but so far there is little experimental evidence to support this approach.

ARTICLE INFORMATION

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Disclosures

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