



Published in final edited form as:

Immunometabolism. 2021 ; 3(2): . doi:10.20900/immunometab20210017.

Metabolic Consequences of Efferocytosis and its Impact on Atherosclerosis

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Abstract

Billions of cells undergo apoptosis daily and are swiftly removed by macrophages through an evolutionarily conserved program termed “efferocytosis”. Consequently, macromolecules within an apoptotic cell significantly burden a phagocyte with nutrients, such as lipids, oligonucleotides, and amino acids. In response to this nutrient overload, metabolic reprogramming must occur for the process of efferocytosis to remain non-phlogistic and to execute successive rounds of efferocytosis. The inability to undergo metabolic reprogramming after efferocytosis drives inflammation and impairs its resolution, often promoting many chronic inflammatory diseases. This is particularly evident for atherosclerosis, as metabolic reprogramming alters macrophage function in every stage of atherosclerosis, from the early formation of benign lesions to the progression of clinically relevant atheromas and during atherosclerosis regression upon aggressive lipid-lowering. This Review focuses on the metabolic pathways utilized upon apoptotic cell ingestion, the consequences of these metabolic pathways in macrophage function thereafter, and the role of metabolic reprogramming during atherosclerosis. Due to the growing interest in this new field, I introduce a new term, “efferotabolism”, as a means to define the process by which macrophages break down, metabolize, and respond to AC-derived macromolecules. Understanding these aspects of efferotabolism will shed light on novel strategies to combat atherosclerosis and compromised inflammation resolution.

Keywords

macrophage; metabolism; efferotabolism; efferocytosis; atherosclerosis

BACKGROUND AND SIGNIFICANCE OF EFFEROCTOSIS IN ATHEROSCLEROSIS

Innate and adaptive immunity guides development and organogenesis, clears pathogens, and actively returns wounded tissues to homeostasis through an active process known as “inflammation resolution” [1,2]. However, when normal immune cell function becomes

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CONFLICTS OF INTEREST

The author declares that there are no conflicts of interest.

dysfunctional, chronic inflammatory diseases manifest and homeostasis is no longer maintained [3]. Central to immune cell function is the removal of apoptotic cells, termed “efferocytosis”. In non-resolving diseases, particularly atherosclerosis, efferocytosis declines, areas of necrosis expand, and robust inflammatory responses arise [4]. Despite the multiple methods that lower circulating levels of cholesterol, atherosclerotic cardiovascular disease remains the leading cause of death worldwide. The recent success of the CANTOS and COLCOT clinical trials indicate that lowering inflammation is a critical arm of the treatment strategy to decrease the burden of atherosclerosis [5,6]. However, anti-inflammatory therapies leave individuals at risk for acute infections due to a reduced ability to mount an immune response [7]. Because efferocytosis instead activates pro-resolving pathways while leaving acute inflammatory responses intact [8,9], enhancing the clearance of dead cells becomes an attractive therapeutic strategy to curb atherosclerosis [8,10]. However, several areas related to efferocytosis remain unsolved. These include how macrophages handle the overwhelming burden of nutrients after internalizing an AC, what are the processes continuing the successive clearance of apoptotic cells, and what pathways are in place that transition the termination of inflammation into resolution. These topics are the focus of this Review. A timeline of significant events in the efferocytosis field are shown in Figure 1.

MECHANISMS OF EFFEROCYTOSIS

In areas of rampant cell death, apoptotic cells release “find-me” signals that include CX₃CL1 (CX₃C-chemokine ligand 1) [11], the nucleotides ATP and UTP [12], and the lipids lysophosphatidylcholine [13] and sphingosine 1-phosphate [14]. Upon arrival, macrophages bind externalized phosphatidylserine on an AC using cell-surface proteins, such as stabilin 1 [15], stabilin 2 [16], TIM1 (T cell immunoglobulin mucin receptor 1), TIM3, TIM4 [17], or BAI1 (ADGRB1) [18]. Alternatively, macrophages bind to bridging molecules, such as Gas6 and protein S, that tether externalized phosphatidylserine on ACs to the tyrosine kinases Tyro3, Axl, and MerTK [19]. Another bridging molecule, MFG-E8, links phosphatidylserine on ACs to the integrin heterodimers α V β 3 and α V β 5 [20–23]. These interactions between ACs and macrophages, whether directly or through bridging molecules, stimulate small GTPase signaling cascades that elicit dynamic actin remodeling to mediate internalization (Figure 2). As one example, macrophage BAI1 binding to AC phosphatidylserine activates the small GTPase Rac1 and drives the assembly of ELMO1/DOCK180 complexes [18]. Another example involves interactions between macrophage stabilin 2 and AC phosphatidylserine that drives GULP/thymosin β 4 complexes [24]. Because Rac1 signaling occurs in spatial proximity near the site where an AC engages a macrophage [25], WAVE1 activation of ARP2/3 polymerizes G-actin to F-actin to form a phagocytic cup around the bound AC [4]. Notably, another small GTPase, RhoA, is activated and stimulates retraction of the phagocytic cup into the macrophage [26]. When operating normally, the spatiotemporal dynamics between Rac1 and RhoA activation are elegantly coordinated such that the whole efferocytosis process may occur within minutes upon AC binding [26].

While apoptotic cell internalization demands intensive cytoskeletal remodeling, macrophages are simultaneously presented with the challenge of supplying enough plasma

membrane to both fully seal the AC and maintain the cell-surface area [27]. This phenomenon suggests that internal membranes rapidly mobilize to the cell surface upon AC engagement. This is supported by experiments that demonstrate inhibiting endosomal membrane trafficking to the cell surface using botulinum B or tetanus prevents the completion of phagocytosis [28]. Phosphoinositide 3-kinases (PI3Ks) and inositol phospholipids drive this fusion of recycling endosomes to the plasma membrane in proximity to the forming phagosome. Vesicular trafficking after AC internalization requires Drp1-dependent mitochondrial fission that then causes the endoplasmic reticulum to release stored calcium into the cytosol [29]. Calcium-dependent membrane sealing likely acts through synaptotagmin VII, as synaptotagmin VII causes exocytosis of lysosomal membranes [30]. The RAB family of GTPases also contributes to vesicular trafficking, as RAB17 distributes phagolysosomal membranes into recycling endosomes, which then fuse to the cell membrane [31]. Interestingly, RAB17-dependent trafficking of vesicles containing degraded AC constituents inhibits mixing with MHC class II-loading components [32], preventing antigen presentation and autoimmunity. Interestingly, when macrophages are challenged with a large apoptotic cell, such as a dead adipocyte, a process termed “digestive exophagy” occurs, whereby macrophages create a tight seal in contact with the AC, acidify the sealed area, and secrete lysosomal materials into the contact area [33,34]. This results in hydrolysis of an AC in a compartment that is outside the phagocyte.

After phagosome sealing, LC3-family proteins are conjugated to lipids at the phagosomal membrane by particular members of the canonical autophagy machinery, a process known as LC3-associated phagocytosis (LAP), to form a phagolysosome or LAPosome (Figure 2) [35]. Despite appropriating some members from the autophagy program, LAP is molecularly and cellularly distinct from autophagy. As an example, autophagosomes have double-membranes, whereas electron microscopy revealed that LAPosomes are single-membraned. Also, LC3-II can be detected on the phagosome in as little as 10 minutes, whereas autophagosomes may take hours to form. Notably, many studies investigating autophagy in vivo often target Atg5 or Atg7, which are also critical components of LAP [36]. Therefore, targeting these proteins not only impairs autophagy but also destroys LAP formation. Accordingly, the role of LAP function must be taken into consideration when interpreting studies focused on conserved autophagy-related proteins. A unique regulator of LAPosome formation is the involvement of Rubicon (RUN domain protein as Beclin 1 interacting and cysteine-rich containing), which promotes Class III PI3K activity that is essential for LC3 conjugation [36]. Impairments in LC3 conjugation to the phagosomal membrane prevent its fusion to lysosomes, thereby inhibiting phagosome acidification and apoptotic cell degradation. pH-dependent proteases and nucleases in the phagolysosome degrade the corpses into basic cellular components. For instance, DNase II reduces apoptotic cell DNA into oligonucleotides within the phagolysosome, and loss of DNase II causes an accumulation of undigested DNA originating from apoptotic cells within phagocytes [37]. Importantly, clearance of AC-derived DNA is critical for efferocytosis to remain non-phlogistic. Phagocytes incapable of degrading DNA within phagolysosomes cause a type-1 interferon response by activating intracellular nucleic acid sensors, leading to autoimmunity [38,39]. Importantly, disruption in any step of the efferocytosis program causes many chronic inflammatory diseases [4].

METABOLIC REPROGRAMMING IN MACROPHAGES DURING EFFEROCYTOSIS

Glycolysis

After Otto Warburg discovered that cancer cells preferentially carry out glycolysis, even in the presence of oxygen, it was soon found that immune cells could also carry out this phenomenon [40]. Initial studies identified that endotoxin activated neutrophils and stimulated aerobic glycolysis while oxygen consumption remained unchanged [41]. Additionally, it was later found that the bacterial cell wall component lipopolysaccharide (LPS, widely known as endotoxin) was driving glycolysis in macrophages through the activation of Toll-like receptor 4 (TLR4). Since then, the metabolic state of pro-inflammatory macrophages has been well-characterized and mechanistic studies have identified multiple mechanisms for how glycolysis orchestrates inflammation [42]. One example is that accumulation of succinate, due to a break in the TCA cycle, leads to HIF1 α stabilization and IL-1 β secretion [43]. Uptake of glucose, mediated by SLC2A1 (encoding GLUT1), typically initiates glycolysis and pyruvate synthesis, leading to ATP production and reduction of NAD⁺ to NADH during the process [42]. Glycolysis is regulated by the glycolytic enzymes pyruvate kinase isoenzyme M2 (PKM2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hexokinase 1 (HK1), and hexokinase 2 (HK2) [44]. Lactate is then formed from the reduction of pyruvate through lactate dehydrogenase (LDH), which recycles NADH into NAD⁺, and is exported from cells by SLC16A1 [44]. Why macrophages choose to carry out glycolysis as opposed to the more highly efficient mode of OXPHOS remains a mystery. Long-held explanations postulate that glycolysis prevents the imbalance of reactive oxygen species during OXPHOS and that glycolysis results in much more rapid ATP production. However, it has yet to be revealed what the evolutionary role of glycolysis in inflammatory macrophages is definitively.

Elegant studies have recently identified a protective role for glycolysis in macrophage function. RNA sequencing in phagocytes actively engulfing ACs revealed a genetic signature involving 33 members of the solute carrier (SLC) family of membrane transporters, of which two played critical roles in glycolysis [45]. The binding of ACs to phagocytes upregulated the glucose transporter SLC2A1 [45]. Simultaneously, the release of secretory factors by ACs increased Sgk1, a kinase that translocates SLC2A1 to the cell surface [45]. Increased expression of SLC2A1 and Sgk1 led to enhanced glucose uptake and activation of aerobic glycolysis (Figure 3) [45]. Flux through the glycolytic pathway was critical for the successive clearance of ACs as actin polymerization could be inhibited by blocking glycolytic flux or glucose uptake. In a later stage of efferocytosis, internalization of an AC enhanced expression of SLC16A1 that mediated the release of lactate, the end-product of glycolysis [45]. Lactate release acts in a paracrine manner to cause nearby cells to produce IL-10 and TGF β [45]. Thus, glycolysis induced by efferocytosis critically regulates at least two different facets of macrophage function: (1) glycolysis caused by SLC2A1 leads to dynamic actin remodeling to maintain the successive clearance of apoptotic cells, and (2) lactate release mediated by SLC16A1 contributes to the resolution program that is observed after efferocytosis [45]. While connecting glycolysis to the resolution program is surprising, it is certainly not controversial. For instance, glycolysis may contribute to macrophage

polarization towards a wound-resolving phenotype [46,47] and also participate in the differentiation of regulatory T cells (Tregs), which are well-documented to drive efferocytosis and promote resolution [48–50]. Also, because macrophages are presented with large amounts of cargo after engulfing an AC, metabolic processes to rapidly raise ATP levels, such as through glycolysis, would be expected to occur.

Fatty Acid Oxidation and Oxidative Phosphorylation

Polarization of macrophages towards a wound-resolving phenotype in vitro using IL-4 leads to a metabolically distinct profile compared to macrophages polarized towards a pro-inflammatory phenotype using LPS [51]. Specifically, IL-4 induces fatty acid oxidation (FAO) and mitochondrial biogenesis [52]. After its synthesis, pyruvate enters the mitochondria and initiates the TCA cycle, generating NADH, FADH₂, and GTP. NADH from the TCA cycle is then used to feed into the electron transport chain where 26 of the 30 ATP molecules are formed when glucose is oxidized to carbon dioxide and water, a process known as oxidative phosphorylation (OXPHOS). This metabolic pathway appears to be critical in macrophages because promoting OXPHOS enhances the expression of wound-resolving signature genes, whereas inhibiting OXPHOS reduces the anti-inflammatory properties of macrophages [53]. Metabolic pathways have certain degrees of flexibility where specific metabolites of one pathway will enter the pathway of another. Studies using conventional doses of 2-DG (2-deoxyglucose) suggested that polarization of macrophages towards the pro-resolving phenotype required glycolysis [42]. However, using lower doses of 2-DG and performing advanced cellular metabolomics revealed that glycolytic stimulation is unnecessary for polarization as long as OXPHOS remains intact [54]. Recently, lipin-1, a phosphatidic acid phosphatase and regulator of lipid metabolism, was shown to increase OXPHOS after IL-4 stimulation and contributed to macrophage polarization towards a pro-resolving phenotype [55,56]. Consistently, mice lacking lipin-1 show a selective defect in continual efferocytosis and display impairments in wound healing [55]. It has recently been shown that loss of myeloid CPT2 augments atherosclerosis, demonstrating that macrophage FAO is atheroprotective. This suggests that tipping the balance of macrophage metabolism towards FAO and OXPHOS may be a suitable therapeutic strategy [57].

In addition to glycolysis, it was recently discovered that macrophages internalizing an AC carry out mitochondrial FAO [58]. NAD⁺ production by FAO and complex III subunit 5-dependent electron transport promotes sirtuin-dependent PBX1 activation, which serves as a transcription factor for the pro-resolving cytokine IL-10 (Figure 3) [58]. There appear to be exciting roles for the mitochondria in efferocytosis beyond metabolism. Both uncoupling proteins and components of the mitochondria fission machinery promote the successive clearance of apoptotic cells [29,59]. Interestingly, the mitochondrial membrane potential (MMP) rises after phagocytes ingest ACs, which appears to be specific to the cargo load [59]. Safeguard mechanisms in phagocytes are in place to protect against excessive levels of MMP because this rise in MMP eventually subsides [59]. This decrease in MMP after efferocytosis involves the upregulation of UCP2, as silencing UCP2 results in persistently high levels of MMP after corpse engulfment [59]. Along with changes in MMP, mitochondria also undergo a rapid fission response soon after engaging an AC [29]. This

causes calcium buffering in a manner whereby stored calcium is released into the cytosol, which stimulates vesicular trafficking and phagosome sealing [29]. Interestingly, successful breakdown and degradation of an AC often leads to the rapid ingestion of a subsequent AC, termed “continual efferocytosis”. Because ACs often outnumber macrophages in areas of necrosis, understanding the mechanisms that drive the successive clearance of dead cells is becoming an active area of research.

Amino Acid Metabolism

Amino acid metabolism plays a significant role throughout all stages of atherosclerosis, including its regression. Glutaminolysis, which fuels the TCA cycle when glutamine is converted to glutamate, then to α -ketoglutarate, is linked to IL-1 β production in macrophages. Macrophages treated with LPS and oxPAPC (oxidized phospholipids from 1-palmitoyl-2-arachidonylsn-glycero-3-phosphorylcholine) increased mitochondrial respiration, accumulated oxaloacetate, and elevated glutaminolysis, which increased IL-1 β in a HIF1 α -dependent manner [60]. Glutaminolysis was shown to play a critical role in atherosclerosis, as inhibiting this pathway reduced IL-1 β expression in plaque macrophages. Another amino acid that is intimately linked to IL-1 β is serine. Conversion of serine into *S*-adenosylmethionine drives IL-1 β expression through epigenetic modifications [61,62]. Notably, the CANTOS trial demonstrated that targeting IL-1 β signaling reduced cardiovascular events [5], suggesting that targeting glutaminolysis or preventing serine metabolism into *S*-adenosylmethionine may offer therapeutic benefits.

Macrophage phenotypes are primarily specified by an exclusive expression of a repertoire of genes, with pro-inflammatory macrophages identified by the expression of inducible nitric oxide synthase (iNOS) and wound-resolving macrophages identified by arginase 1 (Arg1) expression [63]. Interestingly, these two enzymes share arginine as a common substrate. For pro-inflammatory macrophages, iNOS converts arginine into citrulline and generates nitric oxide as a byproduct, which rapidly elevates reactive oxygen and nitrogen species to induce a pathogen-killing oxidative burst [64]. Conversely, wound-resolving macrophages use Arg1 to hydrolyze arginine into ornithine, which, via ornithine decarboxylase (ODC), generates polyamines and proline to enhance cell proliferation and increase extracellular matrix deposition [65]. Arginine metabolism by iNOS and Arg1 plays particularly fascinating roles in atherosclerosis. Whereas iNOS-mediated nitric oxide production in macrophages elevates ROS [66], increases serum lipid peroxides [67], and blocks leukocyte emigration by initiating migratory arrest [68], macrophage Arg1 has been shown to enhance the successive uptake of ACs and contribute to extracellular matrix production [69]. Interestingly, loss of Arg1 in myeloid cells does not affect atherosclerosis progression [70]. However, Arg1 deletion in myeloid cells inhibits a downregulation in the pro-inflammatory cytokines TNF α and IL-1 β , reduces efferocytosis, decreases fibrous cap thickening, and prevents atherosclerosis regression [69]. These effects on atherosclerosis regression are dependent on putrescine synthesis by macrophages and raising putrescine levels in mice with pre-established atherosclerosis can blunt further progression [69].

Constitutive putrescine synthesis plays a crucial role in MerTK signaling also, as deletion of ODC in macrophages lowers MerTK expression and prevents a MerTK-Erk1/2 pathway that

stimulates IL-10 production [9]. Silencing ODC in plaque macrophages using macrophage-targeting nanoparticles diminishes IL-10 expression and prevents a reduction in plaque size and necrotic core area during atherosclerosis regression. In addition, spermidine synthase converts putrescine into spermidine, which acts as a critical switch between glycolysis and OXPHOS in macrophages through a mechanism involving eIF5A (eukaryotic initiation factor 5A) hypusination. Hypusine, a unique amino acid only found in eIF5A, promotes OXPHOS in IL-4-treated macrophages, and preventing eIF5A hypusination inhibits OXPHOS-dependent macrophage polarization towards a pro-resolving phenotype [71]. Interestingly, arginine metabolism down one pathway can actively suppress the metabolism of the other. For instance, nitric oxide-dependent S-nitrosylation of a critical cysteine residue in ODC inhibits its activity and prevents putrescine synthesis [72]. Altogether, these studies demonstrate that amino acid metabolism through multiple circuits has distinct and disparate effects on macrophage function.

Efferotabolism

Upon internalization and successful formation of the phagolysosome, macrophages rapidly breakdown the AC and reduce the cargo into basic components. These components burden the macrophage with potentially toxic macromolecules that must either be metabolized or effluxed [73]. As an example, intracellular cholesterol significantly rises every time a macrophage degrades an AC, which can be worsened if the AC is cholesterol-loaded, as is the case when macrophages encounter apoptotic foam cells. Efficient trafficking of phagolysosomal cholesterol to acyl-CoA:cholesterol acyltransferase (ACAT) in the ER esterifies free cholesterol to cholesterol fatty acid esters that restrict membrane-damaging effects of exuberantly high levels of free cholesterol (Figure 3) [74]. Simultaneously, AC-derived sterols activate the liver X receptor (LXR) and peroxisome proliferator-activated receptor (PPAR) families of nuclear receptors, which promote ABCA1 and ABCG1 expression to drive cholesterol efflux from the macrophage (Figure 3) [75–77].

Macrophages are also equipped to handle the considerable burden of DNA originating from ACs, as their lysosomes contain DNase II, a pH-dependent nuclease that cleaves chromosomal DNA [4]. Unfragmented DNA accumulates in macrophages lacking DNase II, and deletion of DNase II in mice leads to polyarthritis, which mimics the autoimmune disorder rheumatoid arthritis in humans [37]. Interestingly, AC-derived lipids have recently been shown to promote FAO in macrophages through lipin-1's transcriptional coregulation function [55]. Loss of lipin-1 in myeloid cells causes a specific defect in continual efferocytosis and impairs wound healing [56]. Interestingly, of all the amino acids arginine shows the highest relative elevation after efferocytosis [69]. When macrophages are polarized towards a pro-resolving phenotype, arginine from an AC is metabolized into the non-protein-encoding amino acid ornithine by arginase 1, which then becomes decarboxylated by the enzyme ODC into putrescine (Figure 3) [69]. This burgeoning field of study into how macrophages metabolize and respond to AC-derived components, including the metabolic rearrangements thereafter, deserves a new term, which I introduce as "efferotabolism". As macrophages are challenged with multiple other cargoes from ACs, future work is needed to identify how all these metabolites are processed and what effect they have on macrophage function.

CONSEQUENCES OF EFFEROCYTOSIS

A key consequence of efferocytosis is the termination of inflammation and stimulation of the active process of resolution [3]. Clearance of dying cells prevents post-apoptotic cell death and restricts the expansion of necrotic areas [2]. Also, efferocytosis stimulates specific signaling pathways that promote anti-inflammatory and pro-resolving pathways. For instance, apoptotic cell binding to TIM1 and stabilin 2 blunts the activation of NF- κ B, thereby reducing TNF α , IL-6, and CCL5, while also driving the production of TGF β [3,78,79]. Activation of MerTK and Axl, which belong to the TAM family of receptor tyrosine kinases, by ACs causes the expression of the E3 ubiquitin ligases suppressor of cytokine signaling-1 and 3 (SOCS-1 and SOCS-3) and blunts toll-like receptor and cytokine-dependent inflammation [80]. The pro-resolving cytokines TGF β and IL-10 are upregulated after efferocytosis, whereas the pro-inflammatory cytokines TNF α , IL-1 β , and IL-8 are downregulated[3]. As one such example, activation of the transcription factor PBX1 by efferotabolites, metabolites from ingested ACs or byproducts from the metabolism of AC-derived nutrients, drives the expression of IL-10 [58], which also requires putrescine synthesis to maintain a basal level of MerTK expression [9]. Being an essential class of efferotabolites, lipids derived from ACs activate PPAR γ , PPAR δ , and LXR α that promote TGF β and IL-10 secretion while simultaneously eliciting T cell differentiation into regulatory T cells (Tregs) and T helper 2 cells (Th2) that support inflammation resolution [3,81]. Furthermore, PPAR γ and LXRs preserve corepressor binding on the promoters of NF- κ B target genes and suppress the upregulation of pro-inflammatory cytokines [82,83].

Interactions between macrophages and ACs also enhance the production of specialized pro-resolving lipid mediators (SPMs), such as LXA4, RvE1, and PD1, while curbing the production of pro-inflammatory leukotrienes [10]. For example, MerTK activation drives the translocation of lipoxygenase 5 (5-LOX) to the cytoplasm from the nucleus and promotes LXA4 synthesis [84]. This protective pathway is lost if MerTK is cleaved by ADAM17. A cleavage-resistant version of MerTK prevents nuclear localization of 5-LOX, increases SPMs (particularly RvD1), raises the rate of efferocytosis, and decreases necrotic core expansion in *Ldlr*^{-/-} mice fed a Western diet [8,85]. Mechanistically, RvD1 suppresses CaMKII γ activity, leading to a decrease in MK2-dependent phosphorylation of 5-LOX and suppression of LTB₄ synthesis [86]. This pathway activates a feed-forward loop of resolution, as suppression of CaMKII γ activity also increases MerTK expression and efferocytosis [87,88]. These studies demonstrate that the execution of resolution and efferocytosis generates a positive amplification loop that can restore homeostasis.

IMPAIRMENTS IN EFFEROCYTOSIS DURING ATHEROSCLEROSIS

Low-density lipoproteins (LDLs) accumulate within the intima of medium to large-sized arteries [89,90]. Away from the antioxidant environment afforded by the blood, LDLs become oxidized and glycated, whereby these modified LDLs now activate the endothelium. Endothelial cell activation stimulates monocyte recruitment into the vessel wall, and these infiltrating leukocytes now begin engulfing modified LDLs to remove them [81,91,92]. However, these lipid-laden leukocytes eventually undergo apoptosis, and while they are efficiently cleared by surrounding macrophages early in atherosclerosis, efferocytosis fails

as atherosclerosis progresses [81]. This failure in efferocytosis results in post-apoptotic cell death and expands the necrotic core. Atheromas with particularly large necrotic cores are a hallmark of vulnerable plaques that are prone to rupture [93]. Advancements in molecular, genetic, and imaging technologies have identified that repairing defects in efferocytosis serves as an attractive therapeutic strategy to combat atherosclerosis.

Efferocytosis operates at high efficiency, so it is unlikely that exuberant plaque apoptosis is the leading cause of plaque necrosis. It is now exceedingly clear that defects in efferocytosis by plaque macrophages cause necrotic core formation, transforming benign lesions into vulnerable atheromas. One mechanism of impaired efferocytosis in atherosclerosis is the downregulation and proteolytic cleavage of AC-binding receptors. For instance, MerTK inactivation in Ldlr knockout mice fed a Western diet showed larger plaque size, increased necrotic core area, and reduced lesional efferocytosis [94]. Also, macrophages in advanced plaques of both mice and humans show heightened CaMKII γ (calcium/calmodulin-dependent protein kinase II γ) activity, which prevents a protective ATF6-LXR α pathway that drives MerTK expression [87]. Atheroprone mice lacking CaMKII γ in their myeloid cells show increased MerTK expression, improvements in efferocytosis, and reductions in necrotic core areas [87]. This mechanism can be therapeutically targeted, as the administration of macrophage-targeting nanoparticles loaded with CaMKII γ siRNA mimics the atheroprotective phenotypes observed in myeloid-specific CaMKII γ knockout mice [88]. A recent study demonstrated that MerTK expression was also sensitive to putrescine-dependent H3K9 di/trimethylation, as loss of the putrescine-synthesizing enzyme ODC (ornithine decarboxylase) reduced MerTK expression and prevented atherosclerosis regression [9]. Also, multiple pro-inflammatory stimuli activate ADAM17, which cleaves MerTK and impairs efferocytosis [95]. Consistently, the soluble fragment of MerTK, solMer, accumulates in advanced lesions of both mice and humans [96]. Mechanistically, solMer scavenges the bridging molecule Gas6 from binding to MerTK and prevents efferocytosis. Mutating a region within MerTK that confers resistance from ADAM17-mediated cleavage sustains efferocytosis and reduces necrotic core formation in mice [85]. Consistently, loss of MerTK function, whether through direct deletion or by replacing endogenous MerTK with a form containing an inactive kinase domain, results in deleterious effects on atherosclerosis caused by defective efferocytosis. Another AC receptor, low-density lipoprotein receptor-related protein 1 (LRP1), is also a target for cleavage by ADAM17, and loss of LRP1 in myeloid cells increases lesion size and necrotic core area in atheroprone mice [97–99]. In addition to LRP1 cleavage, macrophages treated with oxidized LDL cause internalization and degradation of LRP1 by epsin 1 and 2, a family of ubiquitin-binding endocytic adaptors [100]. Myeloid deletion of epsin 1 and 2 enhances efferocytosis and reduces plaque size and necrotic cores [100]. The bridging molecule MFG-E8 anchors ACs to the integrin α V β 3 and transglutaminase 2 (TG2) on phagocytes [21,101,102]. MFG-E8 expression declines as atherosclerosis advances and Ldlr knockout mice lacking MFG-E8 in hematopoietic cells show larger plaques and expanded necrotic cores [4,103]. Similarly, loss of TG2 in bone marrow cells advances atherosclerosis [104]. Loss of another bridging molecule, complement component 1q (C1q), shows enhanced plaque size and an increase in the number of apoptotic cells, likely due to impairments in efferocytosis [105]. Interestingly, high-mobility group box 1 (HMGB1) binds both phosphatidylserine and integrin α V β 3 to

inhibit efferocytosis [106,107]. HMGB1 levels increase in advanced plaques of both mice and humans, and administration of anti-HMGB1 antibodies reduces atherosclerosis. It should be noted that vascular smooth muscle cells (vSMCs) begin expressing macrophage markers and acquire the ability to phagocytose small particles after cholesterol loading [108]. However, these lipid-laden, macrophage-like vSMCs are unable to execute efferocytosis near the level observed with *bona fide* macrophages [109]. It is therefore conceivable that the expansion of efferocytosis-poor macrophage-like vSMCs can partially explain the decreased efferocytosis observed during atherosclerosis progression [110]. Dendritic cells (DCs) also play functional roles in all stages of atherosclerosis, including changes in efferocytosis. Mature DCs are relatively poor efferocytes and show impaired migration, which explains their propensity to often be found proximal to necrotic cores in advanced atherosclerotic lesions. When DCs are unable to carry out efferocytosis they produce and secrete inflammatory cytokines in the tissue microenvironment and can activate or reactive antigen-specific T cells, which may contribute to expansion of the necrotic core as atherosclerosis progresses [111–113].

There is now mounting evidence that ACs themselves become poor substrates for uptake by macrophages. For instance, in a TNF α -dependent manner the “don’t-eat-me” signal CD47 becomes upregulated on dying cells as lesions progress [114]. Administration of CD47-blocking antibodies to mice prone to atherosclerosis improves lesional efferocytosis and reduces necrotic core areas [114]. Furthermore, necroptotic cells are observed in plaques and show a particular resistance to being entirely engulfed and instead are “nibbled on” in a process known as trogocytosis [115]. In this setting, anti-CD47 blocking antibodies restore whole-cell engulfment of necroptotic cells and raise the level of resolution mediators in plaques [115]. In addition to the heightened presentation of “don’t-eat-me” signals, dead cells in lesions express lower levels of the “eat-me” signal calreticulin on their surface [116]. Apoptotic cells lacking *Cdkn2b* show decreased calreticulin levels and are resistant to being internalized by macrophages [116]. Furthermore, mice lacking *Cdkn2b* have increased plaque size and necrotic core area [116]. Interestingly, human carriers of the cardiovascular risk allele at chromosome 9p21 show lower calreticulin expression, suggesting that impaired apoptotic cell clearance could heighten cardiovascular disease risk in this population [117].

METABOLIC REPROGRAMMING OF MACROPHAGES DURING ATHEROSCLEROSIS

Glucose uptake in plaque macrophages of advanced lesions via GLUT1 has been demonstrated using positron emission tomography with the glucose analog ¹⁸F-fluoro-2-deoxy-D-glucose (18F-FDG) [118]. Increased 18F-FDG signal in plaques of rabbits correlated with increased levels of glycolysis and TCA cycle metabolites, suggesting that lesional macrophages are metabolically active [119]. Targeted metabolomics demonstrated that advanced plaques from human carotid arteries showed elevated glycolysis levels and decreased FAO [120]. However, because the cellular composition of advanced plaques differs widely from early lesions, studies on glycolysis in macrophages from human atherosclerotic specimens have yet to be fully defined. Several studies have been carried out to dissect the role of glycolysis in atherosclerosis. Mice deficient for GLUT1 in the

hematopoietic population, therefore unable to import glucose, showed defects in monocyte recruitment to lesions and reduced atherosclerosis [45,121]. Surprisingly, overexpression of GLUT1 under control of the CD68 promoter did not change monocytosis nor advance atherosclerosis [122,123]. These results suggest that while GLUT1 is necessary for inflammatory responses in macrophages, it is insufficient to protect against metabolic derangement during atherosclerosis. Another study found that the glucose-sensitive transcription factor ChREBP (carbohydrate-responsive element binding protein) was downregulated in LPS-stimulated macrophages [124]. Interestingly, ChREBP deletion in LPS-stimulated macrophages shows enhanced lactate production, indicating that ChREBP operates to suppress glycolysis [124]. Furthermore, ChREBP-deficient macrophages showed elevated levels of ROS and apoptosis, and in vivo, loss of ChREBP in hematopoietic cells worsened atherosclerosis [124].

Pro-inflammatory stimuli and hypoxic conditions activate the transcription factor HIF1 α , which leads to increased expression of GLUT1, HK2, and PFKFB3 (6-phosphofructo-2-kinase/fructose-2,6-biphosphatase), as well as decreasing the utilization of OXPHOS [42]. HIF1 α expression is elevated in atherosclerosis, particularly in macrophage-rich regions [125]. Furthermore, deletion of HIF1 α in myeloid cells reduced polarization of macrophages towards a pro-inflammatory phenotype, lowered GLUT1 expression and glucose uptake, and decreased atherosclerosis in *Ldlr*^{-/-} mice on a Western diet [126]. Exposing mice to high oxygen air using carbogen improved hypoxia in plaques and lowered necrosis, owed to increased MerTK-dependent efferocytosis [127]. While these studies are consistent with the hypothesis that glycolysis fuels inflammatory responses that promote atherosclerosis, glycolysis itself is insufficient to drive atherosclerosis. As mentioned above, macrophages transduced to overexpress GLUT1 increased glycolytic flux, but no differences in inflammatory gene expression and atherosclerosis were observed [123]. The insufficiency of glycolysis driving atherosclerosis is likely due to its opposing effects on efferocytosis-dependent resolution pathways, where metabolic flux through the glycolytic pathway is protective when a macrophage encounters an AC [45].

Compared to glycolysis, there is a paucity of evidence signifying the role of OXPHOS in macrophages during atherosclerosis. This is likely because plaque macrophages preferentially carry out glycolysis. However, in the setting of atherosclerosis regression, plaque macrophages take on a more alternatively activated phenotype, and these macrophages in culture perform high rates of FAO and OXPHOS [50,128,129]. Intracellular fatty acids in cells are bound to proteins, and loss of FABP4 (fatty acid binding protein 4) decreases NF- κ B activation and inflammatory gene expression [130]. Consistently, loss of FABP4 in hematopoietic cells lowers atherosclerosis burden [131]. Fatty acids can be thioesterified by long-chain acyl-CoA synthetases (ACSLs) into their acyl-CoA derivatives, allowing fatty acids to be then used for oxidation. Pro-inflammatory stimuli induced ACSL1, but surprisingly, macrophages lacking ACSL1 do not show impaired OXPHOS after treatment with LPS in the presence of palmitate [132–134]. Loss of ACSL1 in myeloid cells reduces atherosclerosis in diabetic mice but did not affect atherosclerosis in normoglycemic mice [135]. Interestingly, resolvin D1 (RvD1), which protects against atherosclerosis progression, promotes FAO and OXPHOS in macrophages, and inhibiting FAO using etomoxir prevents RvD1-dependent necroptotic cell uptake [136,137]. Future studies will be

required to delineate the specific role of FAO and OXPHOS in macrophage function during atherosclerosis and how this might be tuned to initiate a resolution response.

MACROPHAGE METABOLISM OF DEAD CELLS FROM NON-APOPTOTIC MEANS

It is now appreciated that lesional cells die in multiple ways, and macrophage responses to each form of cell death vary. Like apoptosis, necroptosis is a form of programmed cell death [138,139]. However, unlike the non-phlogistic nature of apoptosis, the cell membrane of necroptotic cells (NCs) loses integrity and causes the release of ECM degrading enzymes and DAMPs that stimulate a robust pro-inflammatory response [140]. Treating mice with necrostatin 1 (an inhibitor of RIPK1 that prevents necroptosis), deleting RIP3, or silencing MLKL using antisense oligonucleotides (ASOs) reduces necrotic core expansion [141–143]. Macrophages inefficiently take up necroptotic cells through a process known as “troglodytosis” because they present higher amounts of the “don’t-eat-me signal” CD47 on their cell surface [115]. This can be restored with either anti-CD47 blocking antibodies or RvD1 [115]. Because the membranes are not fully intact, the composition and quantity of cargo from necroptotic cells delivered to macrophages would be expected to be distinct from ACs. However, the response to fully engulfed necroptotic cells mimicked responses classically observed with ACs, suggesting that efferotabolites influencing the resolution response to fully engulfed necroptotic cells may be comparable to ACs [115,136]. Similar to necroptotic cells, pyroptotic cells are also highly inflammatory [144]. Uniquely associated with NLRP3 inflammasome activation and caspase 1 activity, pyroptotic cells release IL-1 β and IL-18 into the local tissue microenvironment, contributing significantly to atherosclerosis progression in the JAK2^{V617F} mouse model of Clonal Hematopoiesis of Indeterminate Potential, an independent risk factor for coronary heart disease [145–147]. Pyroptosis also shares some features with apoptosis. For instance, phosphatidylserine transfers to the outer leaflet, and chromatin condense during pyroptosis. Furthermore, caspases are involved in apoptosis and pyroptosis, with caspase 3, 5, and 7 being critical for apoptosis and caspase-1 being essential for pyroptosis [145]. Because gasdermin-dependent pore formation causes cell swelling and lysis, little is known about phagocytosis of pyroptotic cells, much less what metabolites macrophages are burdened with upon their ingestion. While currently unknown, an interesting question that arises from these studies is how much of the inflammasome activation during atherosclerosis is attributable to the release of DNA derived from ACs due to inefficient corpse clearance? Addressing this would be of great significance because repairing efferocytosis results in decreased IL-1 β production, and lowering inflammasome activation increases efferocytosis, potentially establishing a therapeutic feed-forward loop of inflammation resolution.

Macrophages play a central role in iron recycling. For instance, hemoglobin in aged erythrocytes phagocytosed by red pulp macrophages is degraded in lysosomes, with a portion of the inorganic iron being transported out of the cell via ferroportin [148,149]. However, free iron can escape into the cytosol, which is highly reactive with peroxides to produce lipid alkoxy and hydroxyl radicals, thereby generating ROS and lipid peroxidation products. Intracellular safeguards exist to circumvent these toxic effects of free iron,

including cytosolic ferritin, which stores intracellular iron in the form of oxidized iron (Fe^{3+}) [150]. However, if macrophages are over-burdened with iron, a form of programmed cell death termed “ferroptosis” occurs. In this setting, glutathione-dependent antioxidant systems fail, and unmitigated lipid peroxidation arises. Experimentally eliciting ferroptosis, evoked by Gpx4 inhibition or deletion, revealed that the oxidized phospholipid, 1-stearoyl-2–15-HpETE-*sn*-glycero-3-phosphatidylethanolamine (SAPE-OOH) serves as a novel “eat-me” signal presented on the surface of ferroptotic cells [151]. TLR2 was identified as the membrane receptor on phagocytes that recognized SAPE-OOH and interfering with SAPE-OOH formation or deleting TLR2 impaired the clearance of ferroptotic cells in vivo [151]. Inhibiting ferroptosis using ferrostatin-1 reduced lipid peroxidation in lesions and decreased atherosclerosis in atheroprone mice fed a Western diet [152]. Macrophages surrounding areas of intraplaque hemorrhage show activation of Nrf2 in response to heme and hemoglobin and uniquely express HO-1 (heme oxygenase 1) [153]. These macrophages, termed “Mhem” macrophages, are resistant to foam cell formation and stimulate anti-inflammatory pathways, suggesting that they are well-equipped to handle iron burden [154]. This is evidenced by the fact that Mhem macrophages have low intracellular iron and reduced ROS, likely through enhanced iron export through ferroportin. However, macrophages incapable of reducing lipid peroxides and metabolizing or exporting iron succumb to ferroptosis. An interesting question that arises from these studies is what happens to a macrophage upon ingestion of a ferroptotic cell? Macrophages must be equipped to handle elevated levels of iron and lipid peroxides, otherwise the consequence of ferroptotic cell uptake itself may lead to ferroptosis of the phagocyte, perpetuating an unmitigated loop of ferroptosis. Pathways utilized after phagocytosis of a ferroptotic cell likely include metabolic flux through iron metabolizing pathways, increasing iron efflux, balancing redox status by tuning the GSH/GSSG ratio, and activating Gpx4 to rid the phagocyte of lipid peroxides.

CONCLUSIONS

Phagocytosis of a dead cell loads a macrophage with metabolites and macromolecules contained within the corpse. To lower the burden of nutrient overload, macrophages must either incorporate these substances into metabolic circuits, expel them into the surrounding microenvironment, or store them in specialized organelles. It should be noted that the interplay between many factors impacts efferotabolism, from the type of dead cell being ingested, the quantity and composition of the cargo load, the cytoskeletal proteins used during engulfment, the repertoire of cell-surface receptors used to secure the corpse, and the crosstalk between macrophages and the local tissue microenvironment. Future studies will be needed to understand the links between efferotabolism, inflammation, and resolution, and identify strategies to promote efferotabolism in settings where it fails.

ACKNOWLEDGEMENTS

The author is supported by the NIH grant R00 HL145131.

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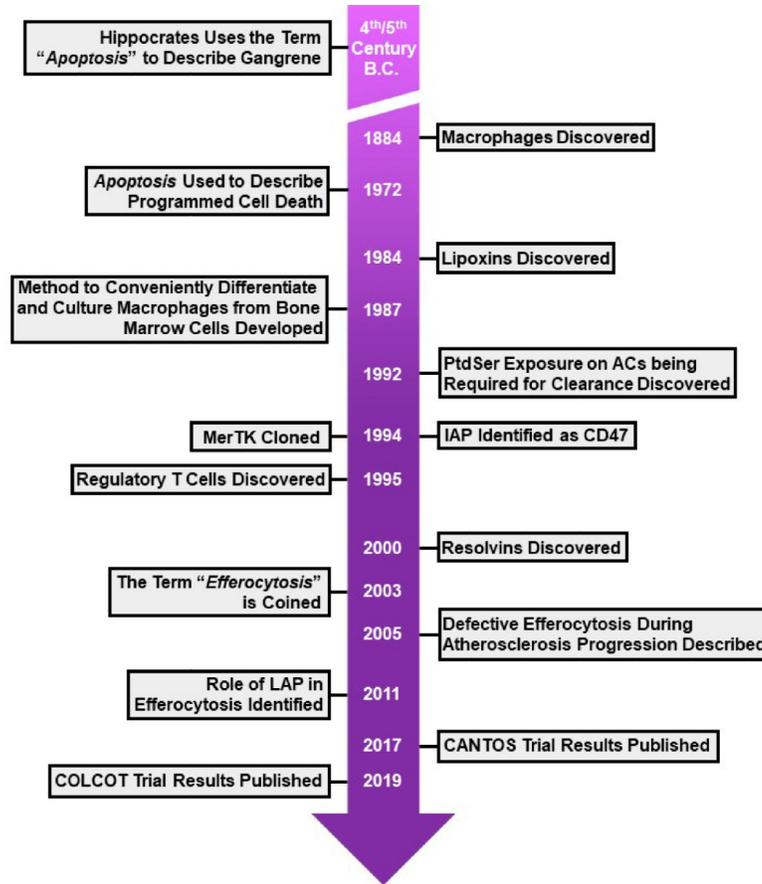


Figure 1.
Timeline of notable events in the history of efferocytosis.

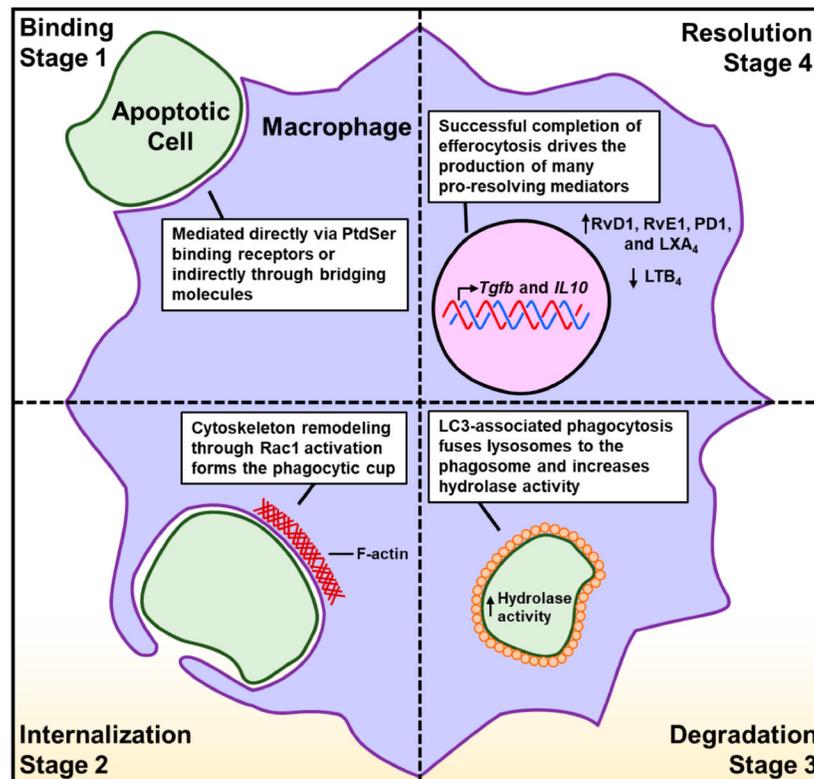


Figure 2. Mechanisms of Efferocytosis and its Consequences.

Macrophages bind externalized phosphatidylserine (PtdSer) on apoptotic cells using cell surface receptors, such as the TIM family of immunoglobulin receptors, Stabilin 1 or 2, or the GPCR BAI1. Alternatively, macrophages bind apoptotic cells indirectly through bridging molecules, such as Gas6 or proteins. Activation of cell-surface receptors stimulate Rac1 activation at the site of apoptotic cell adhesion to drive formation of the phagosome and mediate internalization. LC3-associated phagocytosis occurs through a Rubicon-dependent manner that facilitates lysosome fusion to promote apoptotic cell degradation. Completion of efferocytosis stimulates the production of anti-inflammatory cytokines and synthesis of SPMs to drive inflammation resolution.

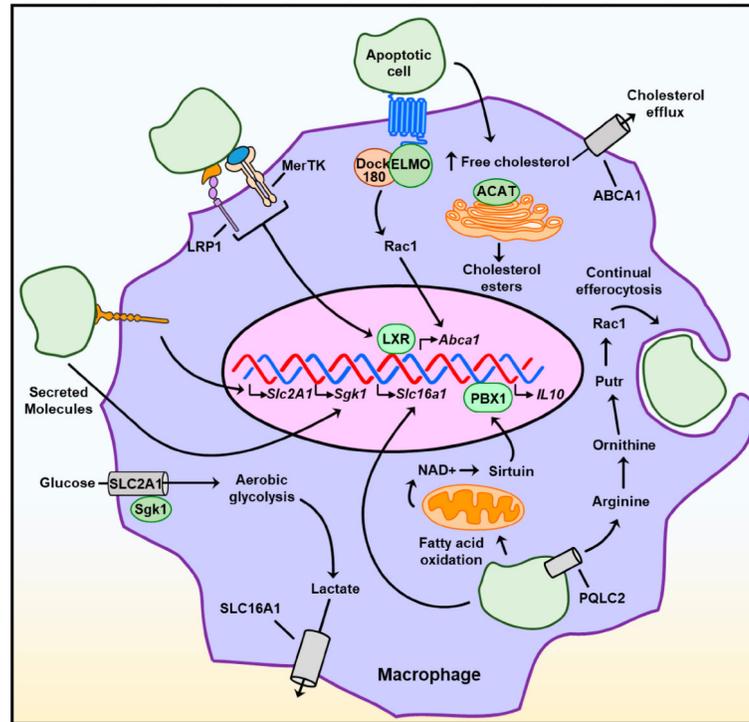


Figure 3. Current Understandings of Efferotabolism.

Macrophages are equipped with multiple mechanisms to process apoptotic cell-derived cargo, such as through metabolism or efflux. Acyl-CoA cholesterol acyltransferase (ACAT) esterifies free cholesterol from apoptotic cells into cholesterol esters that can either be stored or released through ATP-binding cassette transporter A1 (ABCA1). ABCA1 expression can be triggered through LXR-dependent or independent pathways that are both stimulated by apoptotic cell binding. Binding of apoptotic cells to cell-surface receptors upregulate SLC2A1 (encodes GLUT1), and secreted molecules from apoptotic cells stimulate Sgk1 expression, which stabilize SLC2A1 at the membrane. This localization of SLC2A1 allows glucose to be transported into the macrophage and undergo conversion to lactate via aerobic glycolysis. Internalization of an apoptotic cell stimulates the expression of another SLC family member, SLC16A1, which allows for lactate release. Secreted lactate acts in a paracrine manner to upregulate pro-resolving mediators in nearby macrophages. As another example, apoptotic cell-derived fatty acids undergo β -oxidation in mitochondria that stimulate PBX1-dependent IL-10 expression. Arginine from apoptotic cells is exported from the phagolysosome through PQLC2 and is then metabolized into putrescine, which stimulates continual efferocytosis in a Rac1-dependent manner.