Published in final edited form as:

Circulation. 2017 January 31; 135(5): 476–489. doi:10.1161/CIRCULATIONAHA.116.025684.

The Role of Efferocytosis in Atherosclerosis

Yoko Kojima, MD, PhD[1], **Irving L. Weissman, MD**[2], and **Nicholas J. Leeper, MD**[1,3]

[1]Department of Surgery, Division of Vascular Surgery, Stanford University School of Medicine, Stanford, CA

[2]Institute for Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, Stanford, CA

[3]Department of Medicine, Division of Cardiovascular Medicine, Stanford University School of Medicine, Stanford, CA

Abstract

The necrotic core has long been a hallmark of the vulnerable atherosclerotic plaque. While apoptotic cells are cleared very quickly in almost all other tissue beds, their removal appears to be significantly impaired in the diseased blood vessel. Emerging evidence indicates that this phenomenon occurs due to a defect in ‘efferocytosis’, the process by which apoptotic tissue is recognized for engulfment by phagocytic cells such as macrophages. Genetic and experimental data suggest that efferocytosis is impaired during atherogenesis due to dysregulation of so-called ‘eat me’ ligands which govern the ‘edibility’ of cells undergoing programmed cell death. Highlighted below is a summary of recent data indicating that efferocytosis is a major unappreciated driver of lesion expansion, but also a reversible defect that can potentially be targeted as a means to prevent plaque progression.

Keywords

Atherosclerosis; Vascular Biology; Efferocytosis; Macrophage; Necrotic Core

What is efferocytosis?

Even in health, the human body turns over more than one million cells per second through a process known as programmed cell death (PrCD), or apoptosis[1, 2]. This process occurs for a variety of physiological reasons (e.g. negative T cell selection), but is largely driven by the regular homeostatic turnover of aged and senescent cells[3]. To remove these superfluous cells, the body engages in an evolutionarily conserved process known as programmed cell removal (PrCR), or efferocytosis[4, 5]. Efferocytosis is a term derived from the Greek (meaning to carry the dead to the grave), and refers to the phagocytic engulfment of a

Correspondence to: Nicholas J. Leeper, MD, Divisions of Vascular Surgery and Cardiovascular Medicine, Stanford University, 300 Pasteur Drive, Room H3638, Stanford, California 94305, nleeper@stanford.edu, webpage: http://med.stanford.edu/leeperlab.html, Tel/Fax: 650 724 8475 / 650 498 6044.

Disclosures: NJL and ILW are cofounders and hold equity interest in 47 Incorporated.

cellular corpse[4, 6, 7]. Both professional phagocytes (e.g. macrophages) and non-professional (e.g. neighboring) cells participate in efferocytosis.

Because the body must carefully ensure that only apoptotic cells are removed, and that there is no off-target clearance of healthy tissue, efferocytosis is a highly regulated process. Dozens of molecules have now been implicated in the cross-talk which occurs between a dying cell and a potential phagocyte[4, 8, 9]. These include: 1. Chemoattractant 'find me' ligands which recruit phagocytes to the area of cell death; 2. 'Bridging molecules' that play an opsonin-like role while linking phagocytes to their target cells; and 3. Cell-surface 'eat me' ligands which physically engage and transactivate engulfment receptors on the phagocyte to initiate PrCR. Importantly, these molecules are counterbalanced by so-called 'don't eat me' ligands that are ubiquitously expressed on viable cells, but are rapidly downregulated during PrCD[10]. While these signaling pathways remain an area of intense research, it is clear that highly specific regulation of these apparently redundant 'eat me' and 'don't eat me' molecules ultimately determines whether a cell is viewed as 'inedible', and thus ignored by a phagocytic cell, or if it will be marked for engulfment and clearance from the body[1].

Evidence that efferocytosis is impaired in atherosclerosis

An interesting aspect of efferocytosis is the speed and efficiency with which the process occurs[9, 11]. It has been estimated that under almost all conditions, cells undergoing apoptosis are recognized and targeted for clearance in a matter of minutes. Indeed, these cells are cleared so efficiently that it is nearly impossible to identify TUNEL-positive apoptotic bodies (A.B.'s) in any healthy tissue bed[12], including organs with very high rates of cellular turnover such as the thymus, tonsil and bone marrow. In those rare instances when a pathologist is able to document an A.B. in healthy tissue, it is commonly in close proximity to a phagocytic cell. Such co-localization suggests that these apoptotic cells are in the process of being identified for clearance, and likely would not have been observed had the tissue been sampled a few minutes later.

One of the few exceptions to this rule is within the atherosclerotic plaque. Unlike the rest of the body, 'free' apoptotic bodies (which are not in close proximity to macrophages or other professional phagocytes) are commonly observed in the growing lesion. Careful quantification of the numbers of 'free' vs 'associated' A.B.'s has led Martinet and colleagues to conclude that the capacity for efferocytosis is reduced nearly 20-fold in plaque compared to elsewhere in the body[13]. Atheromas also contain far fewer phagocytes that have ingested multiple - as opposed to single - cellular corpses, again suggesting an impairment in efferocytosis[13]. While apoptosis is known to be increased during plaque formation, the capacity of the healthy phagocytes is normally so high that Tabas and colleagues have concluded in their review that the problem is not the consequence of too much cell death, but rather too little cell clearance[6].

How impaired efferocytosis might promote vascular disease

The accumulation of apoptotic and necrotic debris has long been associated with atherogenesis and plaque vulnerability[14–17]. At its most simplistic level, expansion of the necrotic core can be viewed as contributing to plaque expansion, which in turn impinges on luminal flow, thus reducing coronary perfusion. However, impaired efferocytosis likely induces a number of other maladaptive changes beyond this mechanical impediment to blood flow. Many of these may be directly causal for atherosclerosis and plaque vulnerability[13].

First, it is important to note that every time a phagocyte engulfs a dying cell it effectively doubles its intracellular content[18]. Because mammalian cells have difficulty metabolizing cholesterol, efferocytes must initiate reverse cholesterol transport machinery to avoid the intracellular accumulation of the membrane-derived lipids they have just ingested. Normally, externalized phosphatidylserine (PS) present on the surface of apoptotic cells upregulates ABCA1 in macrophages, which promotes the efflux of cholesterol to ApoA1[18, 19]. Interestingly, necrotic cells (which also express PS) do not elicit a similar response[18], and defective efferocytosis signaling has now been shown to suppress critical reverse cholesterol transport pathways in vascular cells[19]. The end result is the formation of foam cells, which are widely recognized as key drivers of atherosclerosis.

In addition to promoting salutary survival mechanisms that prevent the accumulation of potentially toxic cellular contents[20, 21], physiologic efferocytosis is also known to directly suppress inflammation. When a macrophage has successfully cleared a dying cell, beneficial factors such as IL-10 and TGF-β are released, presumably to signal that no additional inflammatory cells need to be recruited to the site of injury[22]. The opposite occurs when a phagocyte is unable to clear an A.B., however, and nonresolving inflammation or autoimmunity may ensue[19, 23, 24]. Thus, failed efferocytosis appears to trigger a molecular switch in phagocytic cells, converting them from champions of inflammation resolution to drivers of vascular inflammation.

A final point to consider is that the reason apoptosis is classically considered an ‘immunologically silent’ form of cell death may be because dying cells are normally cleared before they have had the opportunity to undergo secondary necrosis[4, 22]. When efferocytosis is impaired, however, apoptotic bodies very rapidly experience breakdown of their cell membranes, which leads to the release of previously-sequestered intracellular contents into the interstitium[16]. These factors - which include plaque destabilizing proteases[16], cytokines which promote plaque angiogenesis, and thrombogenic tissue factors[25] – each could accelerate atherosclerosis and promote lesion vulnerability.

Taken together, defects in efferocytosis could theoretically effect much more than the physical growth of the interlesional necrotic core. Rather, they may also stimulate plaque inflammation (via release of chemokinetic cytokines), foam cell accumulation (via impaired reverse cholesterol transport) and plaque vulnerability (via atherothrombotic changes within the ECM). What has classically been viewed as a simple ‘waste management’ issue may actually play a larger role in the vascular biology of atherosclerosis (Figure 1).

Why is efferocytosis impaired in atherosclerosis?

It is not yet understood why efferocytosis is dysfunctional within the atherosclerotic plaque, when it appears to be so highly preserved elsewhere throughout the healthy human body[13]. Conceptually, the accumulation of apoptotic debris within the plaque must be due to at least one of the following problems, as previously outlined by Thorp and Tabas[6]: 1. Overwhelming apoptosis within the plaque; 2. An impairment in the phagocytic capacity of lesional macrophages; and/or 3. Reduced 'edibility' of apoptotic vascular cells.

While programmed cell death is certainly known to be increased during atherosclerosis[26], experimental data suggest that the efferocytic system should have sufficient capacity to deal with this increase in apoptosis, at least initially[2, 8]. For example, studies in which macrophage apoptosis is induced in the beginning stages of lesion formation have found that triggered cell death actually prevents plaque expansion and promotes lesion stabilization[27–29]. Similarly, forced SMC apoptosis (accomplished by Bennett and colleagues via cell-specific expression of the human diphtheria toxin receptor), has no deleterious effect in normal vessels, and only becomes harmful when induced in the late stages of plaque development[30]. Thus, investigators have concluded that overwhelming apoptosis is not the reason uncleared cells accumulate in the diseased vessel wall, at least in the early stages of plaque development.

Conversely, several lines of evidence suggest that phagocytes may become less effective during atherogenesis. For example, macrophage 'skewing' toward the proinflammatory 'M1' phenotype in the plaque may reduce the number of beneficial 'M2' macrophages, thus inducing a relative deficit of those macrophages known to have a high phagocytic capacity[31, 32]. Additionally, reactive oxygen species such as peroxynitrite present in the plaque may impair the ability of macrophages to phagocytose apoptotic bodies[13] and dendritic cells are known to also have reduced efferocytic capacity as they mature[33, 34]. While lipid-laden foam cells have been shown to retain their phagocytic ability at least to some extent[13, 35, 36], vascular SMCs, which are known to possess potent 'non-professional' efferocytic capabilities, lose this functionality when exposed to oxidized lipids[37]. Thus, it is highly likely that the number or ratio of 'competent' efferocytes is reduced in or near the necrotic core, either through direct loss (e.g. apoptosis) or via the expansion of less effective phagocyte subpopulations (e.g. M1 macrophages and foamy SMCs).

Perhaps most importantly, diseased and dying vascular cells appear to become 'poor substrates' which are less 'appealing' to phagocytic cells than apoptotic tissue elsewhere in the body. This likely occurs for a number of reasons, including: 1. Genetic changes in subjects predisposed to atherosclerotic disease; 2. Inflammation-dependent modifications to efferocytosis signaling molecules within the plaque; and 3. The downstream sequelae of intralesional oxidized LDL.

First, studies investigating the heritable component of coronary artery disease (CAD) have found that carriers of the top genome-wide association study (GWAS) risk allele[38–40] have reduced intraplaque expression of a key 'eat me' ligand known as Calreticulin[19]. As a result, these subjects develop larger lesions replete with diseased vascular cells that are presumably

resistant to phagocytic clearance. The Calreticulin axis is discussed in further detail below, but these data suggest that impaired efferocytosis may be the mechanism underlying the most important commonly inherited locus for atherosclerosis (the 9p21 locus), and may explain how these variants promote risk independently of all known traditional risk factors (e.g. hypertension, dyslipidemia, diabetes and smoking)[41].

Second, emerging evidence has now linked inflammatory signaling directly to intra-arterial defects in efferocytosis-related ligand expression (Figure 2). For example, TNF-α, a cytokine known to be causal for- and upregulated in- atherosclerosis, triggers expression of a key 'don't eat me' molecule known as CD47 on the surface of apoptotic vascular SMCs[42]. Similarly, inflammation related to toll-like receptor signaling may suppress the expression of pro-efferocytic ligands such as MFGE8[43], and inactivating post-translational modifications to other key 'eat me' ligands such as MerTK and LRP1 are induced under inflammatory conditions[6, 8, 44, 45], as discussed below. Because failed efferocytosis induces the secretion of TNF-α[16], and TNF-α renders cells even more resistant to clearance, it is highly likely that an endless loop is active in the plaque, and that this may explain why efferocytosis is so impaired during atherogenesis. These data directly support the 'inflammatory hypothesis' of atherosclerosis[46].

Finally, the oxidized LDL present within the atherosclerotic plaque may also directly render apoptotic cells inedible[13]. OxLDL induces the generation of autoantibodies which decorate and presumably mask oxidized 'eat me' ligands on the surface of dying cells in the lesion[47, 48]. OxLDL is also known to directly compete with apoptotic bodies for scavenger receptors, making them less likely to be cleared through competitive inhibition[23].

Taken together, we are still learning why PrCR becomes defective during atherogenesis[13] and with ageing[49]. However, the problem appears to involve multiple pathways, including processes that impair the capacity of the phagocytes present in the plaque and factors that render diseased vascular cells less likely to be cleared. As with most other aspects of vascular biology, genetic and inflammatory factors appear to play central roles in the pathobiology of lesional efferocytosis, and will likely emerge as areas of focus for future translational studies.

Pathways already specifically linked to efferocytosis in vascular disease

Several factors involved in PrCR have now been experimentally linked to apoptotic debris retention or necrotic core expansion in the growing atherosclerotic plaque (Table). These include the bridging molecule, complement C1q[62], the phagocyte receptor, transglutaminase 2 (TG2)[63], and potentially other 'eat me' ligands such as lysoPC and Fas[65, 67]. However, it is likely that some phagocytosis-related molecules have greater relevance to vascular disease than others, and emerging studies have identified a handful of efferocytosis pathways that appear to have a disproportionate impact on atherogenesis.

Mfge8

First among these is the secreted glycoprotein, milk fat globule-EGF factor 8 (Mfge8, also known as lactadherin)[61]. Mfge8 functions as a bridging molecule that tethers the phagocyte

to its target, by simultaneously binding both αvβ3 integrin on the macrophage and externalized phosphatidylserine (PS) on the apoptotic body[68]. Mallat and colleagues reported that Mfge8 is expressed in human vascular tissue, but appears to be reduced in advanced plaque, particularly in lesions with a high burden of TUNEL-positive apoptotic cells. In animal models, atheroprone LDL receptor knockout mice (*ldlr*$^{-/-}$) transplanted with Mfge8$^{-/-}$ bone marrow developed systemic inflammation and advanced atherosclerotic plaques which had larger necrotic cores than control animals, even though loss of Mfge8 did not directly increase the susceptibility of macrophages to apoptosis in vitro. Of note, Mfge8 may also bind directly to TG2[69], another efferocytosis molecule previously linked to reverse cholesterol transport and plaque development[63]. Taken together, these data suggest that Mfge8 produced by bone marrow-derived macrophages is necessary to maintain efferocytosis and prevent inflammation, and that loss of this factor may promote atherosclerosis.

Mertk

The second efferocytic factor clearly linked to atherosclerosis is Mer receptor tyrosine kinase (mertk). This receptor is present on the surface of phagocytic cells and mediates engulfment of apoptotic bodies via the critical bridging molecule, Gas6[70]. Mallat and colleagues previously reported that MERTK is present on macrophages (but not SMCs) in human atherosclerotic plaque, and that atheroprone *ldlr*$^{-/-}$ mice transplanted with *mertk*$^{-/-}$ bone marrow develop significantly larger lesions that have larger necrotic cores, more uncleared apoptotic cells and increased inflammation, relative to control animals[51]. These findings were extended by Tabas and colleagues, who found that mice carrying a kinase-defective form of Mertk (Mertk^{KD}) generated lesions with more TUNEL-positive cells and more plaque necrosis than control animals on the apoe$^{-/-}$ background[50]. Mertk is also required for the clearance of apoptotic cardiomyoctyes after myocardial infarction, and its deficiency has been shown to promote cardiomyopathy in mouse models, extending its relevance to other cardiovascular disorders[71]. Of interest, the mertk receptor can be cleaved by metalloproteinases into an inactive soluble form (solMER), and this process leads to competitive inhibition of efferocytosis by providing a decoy receptor for Gas6[44]. Because solMer shedding is enhanced by pro-inflammatory stimuli commonly observed in vascular disease[44, 72], it is possible that this post-translational modification may play a role in suppressing efferocytosis during atherogenesis.

Calr/LRP1 and CD47

The final and perhaps most exciting PrCR pathway that has been linked to atherosclerosis is the one involving the pro-phagocytic Calr/LRP1 axis, and its counterbalancing ‘don’t eat me’ molecule, CD47. Calreticulin is a highly conserved chaperone protein that is now known to be upregulated and redistributed on the surface of cells undergoing programmed cell death[10]. After physically associating with phosphatidylserine (the other key ‘eat me’ ligand found on apoptotic cells), Calr transactivates LRP1 on the surface of adjacent phagocytic cells and induces engulfment. Emerging evidence suggests that Calr and LRP1 are critical mediators of efferocytosis, as supported by the fact that global knockout of either factor is embryonically lethal[73, 74].

Tissue-specific modulation of LRP1 has confirmed its central role in atherosclerosis. For example, *ldlr*$^{-/-}$ mice lacking LRP1 in the SMC (SM22Cre^{+}/LRP$^{flox/flox}$) develop dramatic, near-occlusive atherosclerotic lesions and aortic aneurysms[52]. Similarly, a series of studies by Fazio and colleagues have confirmed that loss of LRP1 in bone marrow derived macrophages impairs efferocytosis and promotes vascular inflammation, necrotic core accumulation and lesion growth, without having any impact on systemic lipid levels[53, 54]. The fact that loss of this efferocytosis receptor on *either* professional (e.g. macrophages) or non-professional (e.g. vascular SMCs) phagocytes was sufficient to significantly increase atherosclerosis highlights the importance of this pathway in vascular disease.

While no studies have specifically investigated conditional or cell-specific knockout of Calr in murine atherosclerosis models, other evidence has confirmed a role for LRP1's pro-efferocytic ligand in the prevention of atherosclerosis. For example, carriers of the risk allele at the chromosome 9p21 GWAS locus have now been shown to have reduced intraplaque expression of Calr due to an inherited defect in TGFB signaling[19, 39]. Mice deficient in one of the top 9p21 candidate genes (Cdkn2b) have reduced Calr expression and develop markedly larger atherosclerotic plaques that have several features of lesion instability including larger necrotic cores[19]. In vitro, apoptotic vascular SMCs deficient in Calr not only resist clearance by neighboring cells, but also promote juxtacrine changes in co-cultured macrophages, including a propensity to adopt a foam-cell phenotype, suppress reverse cholesterol transport, and secrete pro-atherosclerotic cytokines. Interestingly, these in vitro defects can be reversed with exogenous Calr peptide, suggesting that targeted reactivation of efferocytosis could prevent macrophage inflammation in atherosclerosis.

It is important to note, however, that Calr is also expressed on some non-apoptotic cells, suggesting the existence of a counterbalancing mechanism which prevents the off-target clearance of healthy tissue[10]. Oldenborg and colleagues have now shown that the key 'don't eat me' molecule, CD47, fulfills this role by triggering anti-efferocytic signaling cascades downstream of the SIRPα receptor on phagocytic cells[1, 75]. During PrCD, CD47 is rapidly downregulated and redistributed away from Calr, thus allowing unopposed LRP1 activation and successful engulfment[10].

Paradoxically, CD47 is upregulated in atherosclerosis[42]. This surprising observation results from a TNF-α-dependent signaling cascade through NFKB, which blunts the fall in CD47 expression normally expected to occur during apoptosis. As a result, these apoptotic vascular cells are rendered inedible and presumably contribute to the growth of the necrotic core. Confirming the critical role of this pathway in atherogenesis, CD47 blocking antibodies were found to have a profound antiatherosclerotic effect in several mouse models, including the ability to prevent progression of established lesions, protect against plaque rupture and induce regression of the necrotic core[42]. Taken together, the studies provide compelling evidence that perturbations in either pro-efferocytic Calr-LRP1 signaling or anti-phagocytic CD47-SIRP signaling is sufficient to cause atherosclerotic disease, and prioritize these axes for additional investigation.

Opportunities for translation

Efferocytosis has tremendous potential as both a diagnostic and therapeutic target. For example, CD47 is not only upregulated in patients with plaque compared to no plaque, but also in subjects with symptomatic carotid disease (e.g. those with TIA or stroke) compared to those with an asymptomatic stenosis[42]. Given the data that genetic variants may correlate with alterations in efferocytosis gene expression[19], it may be that a biomarker panel that combines genomic risk variants and expression profiling[76] could have utility in diagnosing individuals at risk for clinical events due to necrotic core expansion and plaque vulnerability.

Additionally, therapies that appear to specifically reactivate efferocytosis are already being tested in humans. In the oncology field, Weissman and colleagues have recently found that a major mechanism by which cancer cells evade the tumoricidal macrophage is by upregulating ‘don’t eat me’ molecules on their surface[77, 78]. Accordingly, humanized antibodies and decoy molecules have been developed that interrupt these pathways and restore the phagocytosis of malignant cells[79]. Preliminary studies suggest that these therapies may have an acceptable toxicity profile in non-human primates[80] and phase I cancer studies are already underway (ClinicalTrials.gov: NCT02678338). Assuming that these treatments are found to be safe and effective, there will be a very unusual opportunity to leverage the experience of the immuno-oncology field and rapidly translate these treatments into the cardiovascular realm.

Further, diseases associated with impaired efferocytosis seem particularly susceptible to combination therapies. In cancer models, anti-tumor antibodies such as rituximab synergize with pro-efferocytic therapies to dramatically accelerate tumor clearance[79, 81]. There is a clear mechanistic justification to consider a similar approach in cardiovascular disease, particularly given the link between vascular inflammation and the imbalance of efferocytosis ligand expression. As described above, levels of functional Mertk[72], LRP1[45] and Mfge8[43] all appear to be reduced under pro-inflammatory conditions. The hypothesis that anti-inflammatory therapies may restore pro-efferocytic signaling has specifically been tested in the case of CD47, which is now known to be directly downstream of TNF-α. Humans treated with the anti-TNF-α antibodies Infliximab or Etanercept have reduced in vivo expression of CD47, and mice treated with combination anti-TNF-α/anti-CD47 therapy display a modest incremental reduction in atherosclerosis over anti-CD47 alone[42]. Given that patients treated with anti-TNF-α therapy for rheumatological conditions appear to be protected from myocardial infarction and other adverse cardiovascular outcomes[82], there is a strong rationale for combining directed anti-inflammatory and pro-efferocytic therapies in the treatment of atherosclerosis.

A final consideration is that pro-efferocytic therapies may provide an actionable opportunity for the oft-mentioned concept of precision cardiovascular medicine[83]. Genetic studies pursuing the heritable component of cardiovascular disease have shown that the majority of genome-wide significant loci confer risk for myocardial infarction independently of all classical risk pathways, suggesting a novel mechanism of action which is not being addressed by lipid lowering or antihypertensive therapies[38, 84, 85]. Given that the top GWAS locus is now known to be associated with a reduction in intravascular ‘eat me’ ligand

expression[19], it is highly likely that these individuals will particularly benefit from genotype-driven, pro-efferocytic therapy, similar to oncology patients who receive tailored chemotherapy directed against their personal cancer mutation. While it is expected that all subjects with atherosclerosis will benefit from pro-efferocytic therapies, pharmacogenomic-based approaches could lead to outsized effects and even induce plaque regression.

Areas for future study

Although much has been learned about the role of efferocytosis in atherosclerosis over the last decade, several aspects of this process are yet to be explored. A better understanding of the nuances surrounding these pathways in health and disease should enhance our fundamental understanding of vascular biology and facilitate the translation of these findings from bench to bedside.

The first is the potential that pro-efferocytic therapies may have unanticipated drawbacks. For example, CD47 blocking antibodies are generally well tolerated, but do induce erythrophagocytosis and anemia under some conditions[42, 77]. While this toxicity can be ameliorated with dose escalation or reduced-dose combination therapy approaches[42, 80], other pro-efferocytic therapies may not be as specific for diseased tissue. Thus, the spatial and temporal changes in ‘eat me’ and ‘don’t eat me’ ligands that must occur in order to render a cell susceptible to phagocytic clearance need to be defined. Likely, the ratio and physical co-localization of these molecules provides an integrated ‘signature’ that determines a cell’s edibility[10]. Such information will explain how the body protects against off-target clearance of healthy tissue and should inform the development of pro-efferocytic therapies that do not induce removal of viable cells. Along these lines, the local delivery of pro-efferocytic therapies (e.g. with drug eluting stent[86] or targeted nanoparticle technology[87]) may prove highly effective, and could have a superior safety profile.

Second, the link between cancer and cardiovascular disease requires further investigation[19, 42]. The recent finding that a common pathway may underlie growth of both tumors and atheromas raises questions about whether the clonal hypothesis of atherosclerosis needs to be revisited[88]. Cancer stem cells are now known to express high levels of CD47 and they may use this factor to evade immune surveillance[89, 90]. Whether a similar process occurs on a pre-atherosclerotic clone could be investigated with advanced lineage tracing modalities. Recent studies have suggested there is far more plasticity of vessel wall cells during atherogenesis than previously appreciated[91, 92], and it may be that efferocytosis pathways are involved in such cell-fate decision making and expansion of a diseased subset of cells.

Third, the role of the vascular SMC in efferocytosis must be defined. While many of the studies described above focus on macrophage phagocytosis, it is clear that other vascular cells also participate in efferocytosis during plaque development. Elegant in vivo lineage tracing studies have now unequivocally confirmed that SMCs ‘de-differentiate’ and assume a ‘macrophage-like’ phenotype during atherogenesis, based on their ability to upregulate markers previously thought to be ‘macrophage-specific’, such as MAC-2[91, 92]. While the physiologic properties of these ‘phenotypically-modulated’ cells remains an area of intense

investigation, in vitro studies suggest that these cells have reduced phagocytic capacity compared to *bona fide* bone marrow-derived macrophages[93]. Because careful quantification of advanced lesions from lineage-traced mice has shown that a significant percentage of lesional 'macrophages' are actually of smooth muscle and not myeloid origin[92], we must determine if their efferocytic program is governed by pathways different from those reported in classical macrophages. If so, different therapies may be required to target specific components of the evolving lesion.

Finally, the intersection with related physiologic pathways and vascular disorders should be pursued. For example, several classical cardiovascular risk factors, including dyslipidemia, smoking and shear stress have been linked to efferocytosis[94–96]. Statins and PPAR-γ agonists have been shown to enhance efferocytosis, and experimental data supports the concept that enhancing blood flow or targeting of the angiotensin pathway could ameliorate defects in efferocytic signaling[31, 95–97]. Exciting new work exploring novel processes related to atherosclerosis including necroptosis (programmed cell necrosis)[98] and autophagy[99] has shown that these mechanisms may also regulate efferocytosis, and could become viable theranostic targets, as well. While efferocytosis has been studied in post-infarct cardiomyopathy[71], we do not yet know its role in other vascular diseases such as restenosis, pathologic angiogenesis or aneurysm disease. Given the simultaneous link to atherosclerosis, acute inflammation and clearance of apoptotic cardiomyocytes after myocardial infarction, pro-efferocytic therapies should be considered as a potential therapy for the early window after an acute coronary syndrome.

Conclusions

While the preceding 50 years have been marked by major improvements in survival from cardiovascular disease, many of these trends have begun to plateau or even worsen in recent years[100]. Some of these changes may be due to increases in obesity-related conditions such as diabetes, but others are likely due to the diminishing returns being achieved with the addition of new drugs that target risk factors for which effective therapies already exist. The recent discovery that efferocytosis is impaired in vascular disease represents a major advance in our understanding of the root cause of atherosclerosis and why necrotic debris accumulates over time. The advent of pro-efferocytic therapies that allow for the removal of diseased and apoptotic cells could allow for an entirely new platform of treatment that specifically targets the necrotic core, should these agents prove to be specific and safe in ongoing oncology trials.

Acknowledgments

Sources of Funding: This manuscript was supported by the National Institutes of Health (R01HL12522401 and R01HL12337001 to NJL).

References

1. Kinchen JM, Ravichandran KS. Phagocytic signaling: You can touch, but you can't eat. Curr Biol. 2008; 18:R521–524. [PubMed: 18579095]

2. Thorp EB. Mechanisms of failed apoptotic cell clearance by phagocyte subsets in cardiovascular disease. Apoptosis : an international journal on programmed cell death. 2010; 15:1124–1136. [PubMed: 20552278]

3. Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immunol. 1992; 148:2207–2216. [PubMed: 1545126]

4. Ravichandran KS, Lorenz U. Engulfment of apoptotic cells: Signals for a good meal. Nat Rev Immunol. 2007; 7:964–974. [PubMed: 18037898]

5. Hoffmann PR, deCathelineau AM, Ogden CA, Leverrier Y, Bratton DL, Daleke DL, Ridley AJ, Fadok VA, Henson PM. Phosphatidylserine (ps) induces ps receptor-mediated macropinocytosis and promotes clearance of apoptotic cells. The Journal of cell biology. 2001; 155:649–659. [PubMed: 11706053]

6. Thorp E, Tabas I. Mechanisms and consequences of efferocytosis in advanced atherosclerosis. J Leukoc Biol. 2009; 86:1089–1095. [PubMed: 19414539]

7. deCathelineau AM, Henson PM. The final step in programmed cell death: Phagocytes carry apoptotic cells to the grave. Essays in biochemistry. 2003; 39:105–117. [PubMed: 14585077]

8. Tabas I. Macrophage death and defective inflammation resolution in atherosclerosis. Nat Rev Immunol. 2010; 10:36–46. [PubMed: 19960040]

9. Savill J, Dransfield I, Gregory C, Haslett C. A blast from the past: Clearance of apoptotic cells regulates immune responses. Nat Rev Immunol. 2002; 2:965–975. [PubMed: 12461569]

10. Gardai SJ, McPhillips KA, Frasch SC, Janssen WJ, Starefeldt A, Murphy-Ullrich JE, Bratton DL, Oldenborg PA, Michalak M, Henson PM. Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of lrp on the phagocyte. Cell. 2005; 123:321–334. [PubMed: 16239148]

11. Henson PM, Bratton DL, Fadok VA. Apoptotic cell removal. Curr Biol. 2001; 11:R795–805. [PubMed: 11591341]

12. Ravichandran KS. Find-me and eat-me signals in apoptotic cell clearance: Progress and conundrums. J Exp Med. 2010; 207:1807–1817. [PubMed: 20805564]

13. Schrijvers DM, De Meyer GR, Kockx MM, Herman AG, Martinet W. Phagocytosis of apoptotic cells by macrophages is impaired in atherosclerosis. Arterioscler Thromb Vasc Biol. 2005; 25:1256–1261. [PubMed: 15831805]

14. Finn AV, Nakano M, Narula J, Kolodgie FD, Virmani R. Concept of vulnerable/unstable plaque. Arterioscler Thromb Vasc Biol. 2010; 30:1282–1292. [PubMed: 20554950]

15. Kolodgie FD, Narula J, Burke AP, Haider N, Farb A, Hui-Liang Y, Smialek J, Virmani R. Localization of apoptotic macrophages at the site of plaque rupture in sudden coronary death. Am J Pathol. 2000; 157:1259–1268. [PubMed: 11021830]

16. Martinet W, Schrijvers DM, De Meyer GR. Necrotic cell death in atherosclerosis. Basic Res Cardiol. 2011; 106:749–760. [PubMed: 21611880]

17. Tabas I. Pulling down the plug on atherosclerosis: Finding the culprit in your heart. Nat Med. 2011; 17:791–793. [PubMed: 21738159]

18. Kiss RS, Elliott MR, Ma Z, Marcel YL, Ravichandran KS. Apoptotic cells induce a phosphatidylserine-dependent homeostatic response from phagocytes. Curr Biol. 2006; 16:2252–2258. [PubMed: 17113390]

19. Kojima Y, Downing K, Kundu R, Miller C, Dewey F, Lancero H, Raaz U, Perisic L, Hedin U, Schadt E, Maegdefessel L, Quertermous T, Leeper NJ. Cyclin-dependent kinase inhibitor 2b regulates efferocytosis and atherosclerosis. J Clin Invest. 2014; 124:1083–1097. [PubMed: 24531546]

20. Moore KJ, Tabas I. Macrophages in the pathogenesis of atherosclerosis. Cell. 2011; 145:341–355. [PubMed: 21529710]

21. Cui D, Thorp E, Li Y, Wang N, Yvan-Charvet L, Tall AR, Tabas I. Pivotal advance: Macrophages become resistant to cholesterol-induced death after phagocytosis of apoptotic cells. J Leukoc Biol. 2007; 82:1040–1050. [PubMed: 17576822]

22. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/

paracrine mechanisms involving tgf-beta, pge2, and paf. J Clin Invest. 1998; 101:890–898. [PubMed: 9466984]

23. Schrijvers DM, De Meyer GR, Herman AG, Martinet W. Phagocytosis in atherosclerosis: Molecular mechanisms and implications for plaque progression and stability. Cardiovasc Res. 2007; 73:470–480. [PubMed: 17084825]
24. Ravichandran KS. Beginnings of a good apoptotic meal: The find-me and eat-me signaling pathways. Immunity. 2011; 35:445–455. [PubMed: 22035837]
25. Mallat Z, Hugel B, Ohan J, Leseche G, Freyssinet JM, Tedgui A. Shed membrane microparticles with procoagulant potential in human atherosclerotic plaques: A role for apoptosis in plaque thrombogenicity. Circulation. 1999; 99:348–353. [PubMed: 9918520]
26. Kockx MM, De Meyer GR, Muhring J, Jacob W, Bult H, Herman AG. Apoptosis and related proteins in different stages of human atherosclerotic plaques. Circulation. 1998; 97:2307–2315. [PubMed: 9639374]
27. Liu J, Thewke DP, Su YR, Linton MF, Fazio S, Sinensky MS. Reduced macrophage apoptosis is associated with accelerated atherosclerosis in low-density lipoprotein receptor-null mice. Arterioscler Thromb Vasc Biol. 2005; 25:174–179. [PubMed: 15499039]
28. Arai S, Shelton JM, Chen M, Bradley MN, Castrillo A, Bookout AL, Mak PA, Edwards PA, Mangelsdorf DJ, Tontonoz P, Miyazaki T. A role for the apoptosis inhibitory factor aim/spalpha/api6 in atherosclerosis development. Cell Metab. 2005; 1:201–213. [PubMed: 16054063]
29. Gautier EL, Huby T, Witztum JL, Ouzilleau B, Miller ER, Saint-Charles F, Aucouturier P, Chapman MJ, Lesnik P. Macrophage apoptosis exerts divergent effects on atherogenesis as a function of lesion stage. Circulation. 2009; 119:1795–1804. [PubMed: 19307478]
30. Clarke MC, Figg N, Maguire JJ, Davenport AP, Goddard M, Littlewood TD, Bennett MR. Apoptosis of vascular smooth muscle cells induces features of plaque vulnerability in atherosclerosis. Nat Med. 2006; 12:1075–1080. [PubMed: 16892061]
31. Yamamoto S, Yancey PG, Zuo Y, Ma LJ, Kaseda R, Fogo AB, Ichikawa I, Linton MF, Fazio S, Kon V. Macrophage polarization by angiotensin ii-type 1 receptor aggravates renal injury-acceleration of atherosclerosis. Arterioscler Thromb Vasc Biol. 2011; 31:2856–2864. [PubMed: 21979434]
32. Martinez FO, Helming L, Gordon S. Alternative activation of macrophages: An immunologic functional perspective. Annual review of immunology. 2009; 27:451–483.
33. Albert ML, Pearce SF, Francisco LM, Sauter B, Roy P, Silverstein RL, Bhardwaj N. Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and cd36, and cross-present antigens to cytotoxic t lymphocytes. J Exp Med. 1998; 188:1359–1368. [PubMed: 9763615]
34. Agrawal A, Agrawal S, Cao JN, Su H, Osann K, Gupta S. Altered innate immune functioning of dendritic cells in elderly humans: A role of phosphoinositide 3-kinase-signaling pathway. J Immunol. 2007; 178:6912–6922. [PubMed: 17513740]
35. Li Y, Gerbod-Giannone MC, Seitz H, Cui D, Thorp E, Tall AR, Matsushima GK, Tabas I. Cholesterol-induced apoptotic macrophages elicit an inflammatory response in phagocytes, which is partially attenuated by the mer receptor. J Biol Chem. 2006; 281:6707–6717. [PubMed: 16380374]
36. Van Vre EA, Ait-Oufella H, Tedgui A, Mallat Z. Apoptotic cell death and efferocytosis in atherosclerosis. Arterioscler Thromb Vasc Biol. 2012; 32:887–893. [PubMed: 22328779]
37. Clarke MC, Talib S, Figg NL, Bennett MR. Vascular smooth muscle cell apoptosis induces interleukin-1-directed inflammation: Effects of hyperlipidemia-mediated inhibition of phagocytosis. Circ Res. 2010; 106:363–372. [PubMed: 19926874]
38. Helgadottir A, Thorleifsson G, Magnusson KP, Gretarsdottir S, Steinthorsdottir V, Manolescu A, Jones GT, Rinkel GJ, Blankensteijn JD, Ronkainen A, Jaaskelainen JE, Kyo Y, Lenk GM, Sakalihasan N, Kostulas K, Gottsater A, Flex A, Stefansson H, Hansen T, Andersen G, Weinsheimer S, Borch-Johnsen K, Jorgensen T, Shah SH, Quyyumi AA, Granger CB, Reilly MP, Austin H, Levey AI, Vaccarino V, Palsdottir E, Walters GB, Jonsdottir T, Snorradottir S, Magnusdottir D, Gudmundsson G, Ferrell RE, Sveinbjornsdottir S, Hernesniemi J, Niemela M, Limet R, Andersen K, Sigurdsson G, Benediktsson R, Verhoeven EL, Teijink JA, Grobbee DE, Rader DJ, Collier DA, Pedersen O, Pola R, Hillert J, Lindblad B, Valdimarsson EM, Magnadottir

HB, Wijmenga C, Tromp G, Baas AF, Ruigrok YM, van Rij AM, Kuivaniemi H, Powell JT, Matthiasson SE, Gulcher JR, Thorgeirsson G, Kong A, Thorsteinsdottir U, Stefansson K. The same sequence variant on 9p21 associates with myocardial infarction, abdominal aortic aneurysm and intracranial aneurysm. Nat Genet. 2008; 40:217–224. [PubMed: 18176561]

39. Nanda V, Downing KP, Ye J, Xiao S, Kojima Y, Spin JM, DiRenzo D, Nead KT, Connolly AJ, Dandona S, Perisic L, Hedin U, Maegdefessel L, Dalman J, Guo L, Zhao X, Kolodgie FD, Virmani R, Davis HR Jr, Leeper NJ. Cdkn2b regulates tgfbeta signaling and smooth muscle cell investment of hypoxic neovessels. Circ Res. 2016; 118:230–240. [PubMed: 26596284]
40. Leeper NJ, Raiesdana A, Kojima Y, Kundu RK, Cheng H, Maegdefessel L, Toh R, Ahn GO, Ali ZA, Anderson DR, Miller CL, Roberts SC, Spin JM, de Almeida PE, Wu JC, Xu B, Cheng K, Quertermous M, Kundu S, Kortekaas KE, Berzin E, Downing KP, Dalman RL, Tsao PS, Schadt EE, Owens GK, Quertermous T. Loss of cdkn2b promotes p53-dependent smooth muscle cell apoptosis and aneurysm formation. Arterioscler Thromb Vasc Biol. 2013; 33:e1–e10. [PubMed: 23162013]
41. Cunnington MS, Keavney B. Genetic mechanisms mediating atherosclerosis susceptibility at the chromosome 9p21 locus. Curr Atheroscler Rep. 2011; 13:193–201. [PubMed: 21487702]
42. Kojima Y, Volkmer JP, McKenna K, Civelek M, Lusis AJ, Miller CL, Direnzo D, Nanda V, Ye J, Connolly AJ, Schadt EE, Quertermous T, Betancur P, Maegdefessel L, Matic LP, Hedin U, Weissman IL, Leeper NJ. Cd47-blocking antibodies restore phagocytosis and prevent atherosclerosis. Nature. 2016; 536:86–90. [PubMed: 27437576]
43. Komura H, Miksa M, Wu R, Goyert SM, Wang P. Milk fat globule epidermal growth factor-factor viii is down-regulated in sepsis via the lipopolysaccharide-cd14 pathway. J Immunol. 2009; 182:581–587. [PubMed: 19109191]
44. Sather S, Kenyon KD, Lefkowitz JB, Liang X, Varnum BC, Henson PM, Graham DK. A soluble form of the mer receptor tyrosine kinase inhibits macrophage clearance of apoptotic cells and platelet aggregation. Blood. 2007; 109:1026–1033. [PubMed: 17047157]
45. Thorp E, Subramanian M, Tabas I. The role of macrophages and dendritic cells in the clearance of apoptotic cells in advanced atherosclerosis. Eur J Immunol. 2011; 41:2515–2518. [PubMed: 21952808]
46. Libby P. Inflammation in atherosclerosis. Nature. 2002; 420:868–874. [PubMed: 12490960]
47. Chang MK, Bergmark C, Laurila A, Horkko S, Han KH, Friedman P, Dennis EA, Witztum JL. Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: Evidence that oxidation-specific epitopes mediate macrophage recognition. Proc Natl Acad Sci U S A. 1999; 96:6353–6358. [PubMed: 10339591]
48. Shaw PX, Horkko S, Tsimikas S, Chang MK, Palinski W, Silverman GJ, Chen PP, Witztum JL. Human-derived anti-oxidized ldl autoantibody blocks uptake of oxidized ldl by macrophages and localizes to atherosclerotic lesions in vivo. Arterioscler Thromb Vasc Biol. 2001; 21:1333–1339. [PubMed: 11498462]
49. Aprahamian T, Takemura Y, Goukassian D, Walsh K. Ageing is associated with diminished apoptotic cell clearance in vivo. Clinical and experimental immunology. 2008; 152:448–455. [PubMed: 18422728]
50. Thorp E, Cui D, Schrijvers DM, Kuriakose G, Tabas I. Mertk receptor mutation reduces efferocytosis efficiency and promotes apoptotic cell accumulation and plaque necrosis in atherosclerotic lesions of apoe−/− mice. Arterioscler Thromb Vasc Biol. 2008; 28:1421–1428. [PubMed: 18451332]
51. Ait-Oufella H, Pouresmail V, Simon T, Blanc-Brude O, Kinugawa K, Merval R, Offenstadt G, Leseche G, Cohen PL, Tedgui A, Mallat Z. Defective mer receptor tyrosine kinase signaling in bone marrow cells promotes apoptotic cell accumulation and accelerates atherosclerosis. Arterioscler Thromb Vasc Biol. 2008; 28:1429–1431. [PubMed: 18467644]
52. Boucher P, Gotthardt M, Li WP, Anderson RG, Herz J. Lrp: Role in vascular wall integrity and protection from atherosclerosis. Science. 2003; 300:329–332. [PubMed: 12690199]
53. Overton CD, Yancey PG, Major AS, Linton MF, Fazio S. Deletion of macrophage ldl receptor-related protein increases atherogenesis in the mouse. Circ Res. 2007; 100:670–677. [PubMed: 17303763]

54. Yancey PG, Blakemore J, Ding L, Fan D, Overton CD, Zhang Y, Linton MF, Fazio S. Macrophage lrp-1 controls plaque cellularity by regulating efferocytosis and akt activation. Arterioscler Thromb Vasc Biol. 2010; 30:787–795. [PubMed: 20150557]
55. Yancey PG, Ding Y, Fan D, Blakemore JL, Zhang Y, Ding L, Zhang J, Linton MF, Fazio S. Low-density lipoprotein receptor-related protein 1 prevents early atherosclerosis by limiting lesional apoptosis and inflammatory ly-6chigh monocytosis: Evidence that the effects are not apolipoprotein e dependent. Circulation. 2011; 124:454–464. [PubMed: 21730304]
56. Zhu L, Giunzioni I, Tavori H, Covarrubias R, Ding L, Zhang Y, Ormseth M, Major AS, Stafford JM, Linton MF, Fazio S. Loss of macrophage low-density lipoprotein receptor-related protein 1 confers resistance to the antiatherogenic effects of tumor necrosis factor-alpha inhibition. Arterioscler Thromb Vasc Biol. 2016; 36:1483–1495. [PubMed: 27365402]
57. Tao H, Yancey PG, Babaev VR, Blakemore JL, Zhang Y, Ding L, Fazio S, Linton MF. Macrophage sr-bi mediates efferocytosis via src/pi3k/rac1 signaling and reduces atherosclerotic lesion necrosis. Journal of lipid research. 2015; 56:1449–1460. [PubMed: 26059978]
58. Foks AC, Engelbertsen D, Kuperwaser F, Alberts-Grill N, Gonen A, Witztum JL, Lederer J, Jarolim P, DeKruyff RH, Freeman GJ, Lichtman AH. Blockade of tim-1 and tim-4 enhances atherosclerosis in low-density lipoprotein receptor-deficient mice. Arterioscler Thromb Vasc Biol. 2016; 36:456–465. [PubMed: 26821944]
59. Bolick DT, Skaflen MD, Johnson LE, Kwon SC, Howatt D, Daugherty A, Ravichandran KS, Hedrick CC. G2a deficiency in mice promotes macrophage activation and atherosclerosis. Circ Res. 2009; 104:318–327. [PubMed: 19106413]
60. Teupser D, Pavlides S, Tan M, Gutierrez-Ramos JC, Kolbeck R, Breslow JL. Major reduction of atherosclerosis in fractalkine (cx3cl1)-deficient mice is at the brachiocephalic artery, not the aortic root. Proc Natl Acad Sci U S A. 2004; 101:17795–17800. [PubMed: 15596719]
61. Ait-Oufella H, Kinugawa K, Zoll J, Simon T, Boddaert J, Heeneman S, Blanc-Brude O, Barateau V, Potteaux S, Merval R, Esposito B, Teissier E, Daemen MJ, Leseche G, Boulanger C, Tedgui A, Mallat Z. Lactadherin deficiency leads to apoptotic cell accumulation and accelerated atherosclerosis in mice. Circulation. 2007; 115:2168–2177. [PubMed: 17420351]
62. Bhatia VK, Yun S, Leung V, Grimsditch DC, Benson GM, Botto MB, Boyle JJ, Haskard DO. Complement c1q reduces early atherosclerosis in low-density lipoprotein receptor-deficient mice. Am J Pathol. 2007; 170:416–426. [PubMed: 17200212]
63. Boisvert WA, Rose DM, Boullier A, Quehenberger O, Sydlaske A, Johnson KA, Curtiss LK, Terkeltaub R. Leukocyte transglutaminase 2 expression limits atherosclerotic lesion size. Arterioscler Thromb Vasc Biol. 2006; 26:563–569. [PubMed: 16410462]
64. Lutgens E, Tjwa M, Garcia de Frutos P, Wijnands E, Beckers L, Dahlback B, Daemen MJ, Carmeliet P, Moons L. Genetic loss of gas6 induces plaque stability in experimental atherosclerosis. The Journal of pathology. 2008; 216:55–63. [PubMed: 18570189]
65. Feng X, Li H, Rumbin AA, Wang X, La Cava A, Brechtelsbauer K, Castellani LW, Witztum JL, Lusis AJ, Tsao BP. Apoe−/−fas−/− c57bl/6 mice: A novel murine model simultaneously exhibits lupus nephritis, atherosclerosis, and osteopenia. Journal of lipid research. 2007; 48:794–805. [PubMed: 17259598]
66. Aprahamian T, Rifkin I, Bonegio R, Hugel B, Freyssinet JM, Sato K, Castellot JJ Jr, Walsh K. Impaired clearance of apoptotic cells promotes synergy between atherogenesis and autoimmune disease. J Exp Med. 2004; 199:1121–1131. [PubMed: 15096538]
67. Thorp E, Tabas I. Differential effects of pioglitazone on advanced atherosclerotic lesions. Am J Pathol. 2009; 175:1348. [PubMed: 19661438]
68. Hanayama R, Tanaka M, Miwa K, Shinohara A, Iwamatsu A, Nagata S. Identification of a factor that links apoptotic cells to phagocytes. Nature. 2002; 417:182–187. [PubMed: 12000961]
69. Toth B, Garabuczi E, Sarang Z, Vereb G, Vamosi G, Aeschlimann D, Blasko B, Becsi B, Erdodi F, Lacy-Hulbert A, Zhang A, Falasca L, Birge RB, Balajthy Z, Melino G, Fesus L, Szondy Z. Transglutaminase 2 is needed for the formation of an efficient phagocyte portal in macrophages engulfing apoptotic cells. J Immunol. 2009; 182:2084–2092. [PubMed: 19201861]

70. Scott RS, McMahon EJ, Pop SM, Reap EA, Caricchio R, Cohen PL, Earp HS, Matsushima GK. Phagocytosis and clearance of apoptotic cells is mediated by mer. Nature. 2001; 411:207–211. [PubMed: 11346799]

71. Wan E, Yeap XY, Dehn S, Terry R, Novak M, Zhang S, Iwata S, Han X, Homma S, Drosatos K, Lomasney J, Engman DM, Miller SD, Vaughan DE, Morrow JP, Kishore R, Thorp EB. Enhanced efferocytosis of apoptotic cardiomyocytes through myeloid-epithelial-reproductive tyrosine kinase links acute inflammation resolution to cardiac repair after infarction. Circ Res. 2013; 113:1004–1012. [PubMed: 23836795]

72. Thorp E, Vaisar T, Subramanian M, Mautner L, Blobel C, Tabas I. Shedding of the mer tyrosine kinase receptor is mediated by adam17 protein through a pathway involving reactive oxygen species, protein kinase cdelta, and p38 mitogen-activated protein kinase (mapk). J Biol Chem. 2011; 286:33335–33344. [PubMed: 21828049]

73. Gold LI, Eggleton P, Sweetwyne MT, Van Duyn LB, Greives MR, Naylor SM, Michalak M, Murphy-Ullrich JE. Calreticulin: Non-endoplasmic reticulum functions in physiology and disease. FASEB J. 2010; 24:665–683. [PubMed: 19940256]

74. Boucher P, Herz J. Signaling through lrp1: Protection from atherosclerosis and beyond. Biochem Pharmacol. 2011; 81:1–5. [PubMed: 20920479]

75. Oldenborg PA, Zheleznyak A, Fang YF, Lagenaur CF, Gresham HD, Lindberg FP. Role of cd47 as a marker of self on red blood cells. Science. 2000; 288:2051–2054. [PubMed: 10856220]

76. Downing KP, Nead KT, Kojima Y, Assimes T, Maegdefessel L, Quertermous T, Cooke JP, Leeper NJ. The combination of 9p21.3 genotype and biomarker profile improves a peripheral artery disease risk prediction model. Vasc Med. 2014; 19:3–8. [PubMed: 24323119]

77. Willingham SB, Volkmer JP, Gentles AJ, Sahoo D, Dalerba P, Mitra SS, Wang J, Contreras-Trujillo H, Martin R, Cohen JD, Lovelace P, Scheeren FA, Chao MP, Weiskopf K, Tang C, Volkmer AK, Naik TJ, Storm TA, Mosley AR, Edris B, Schmid SM, Sun CK, Chua MS, Murillo O, Rajendran P, Cha AC, Chin RK, Kim D, Adorno M, Raveh T, Tseng D, Jaiswal S, Enger PO, Steinberg GK, Li G, So SK, Majeti R, Harsh GR, van de Rijn M, Teng NN, Sunwoo JB, Alizadeh AA, Clarke MF, Weissman IL. The cd47-signal regulatory protein alpha (sirpa) interaction is a therapeutic target for human solid tumors. Proc Natl Acad Sci U S A. 2012; 109:6662–6667. [PubMed: 22451913]

78. Chao MP, Majeti R, Weissman IL. Programmed cell removal: A new obstacle in the road to developing cancer. Nat Rev Cancer. 2012; 12:58–67.

79. Weiskopf K, Ring AM, Ho CC, Volkmer JP, Levin AM, Volkmer AK, Ozkan E, Fernhoff NB, van de Rijn M, Weissman IL, Garcia KC. Engineered sirpalpha variants as immunotherapeutic adjuvants to anticancer antibodies. Science. 2013; 341:88–91. [PubMed: 23722425]

80. Liu J, Wang L, Zhao F, Tseng S, Narayanan C, Shura L, Willingham S, Howard M, Prohaska S, Volkmer J, Chao M, Weissman IL, Majeti R. Pre-clinical development of a humanized anti-cd47 antibody with anti-cancer therapeutic potential. PLoS One. 2015; 10:e0137345. [PubMed: 26390038]

81. Chao MP, Alizadeh AA, Tang C, Myklebust JH, Varghese B, Gill S, Jan M, Cha AC, Chan CK, Tan BT, Park CY, Zhao F, Kohrt HE, Malumbres R, Briones J, Gascoyne RD, Lossos IS, Levy R, Weissman IL, Majeti R. Anti-cd47 antibody synergizes with rituximab to promote phagocytosis and eradicate non-hodgkin lymphoma. Cell. 2010; 142:699–713. [PubMed: 20813259]

82. Greenberg JD, Furer V, Farkouh ME. Cardiovascular safety of biologic therapies for the treatment of ra. Nature reviews Rheumatology. 2012; 8:13–21.

83. Antman EM, Loscalzo J. Precision medicine in cardiology. Nature reviews Cardiology. 2016; 13:591–602. [PubMed: 27356875]

84. Deloukas P, Kanoni S, Willenborg C, Farrall M, Assimes TL, Thompson JR, Ingelsson E, Saleheen D, Erdmann J, Goldstein BA, Stirrups K, Konig IR, Cazier JB, Johansson A, Hall AS, Lee JY, Willer CJ, Chambers JC, Esko T, Folkersen L, Goel A, Grundberg E, Havulinna AS, Ho WK, Hopewell JC, Eriksson N, Kleber ME, Kristiansson K, Lundmark P, Lyytikainen LP, Rafelt S, Shungin D, Strawbridge RJ, Thorleifsson G, Tikkanen E, Van Zuydam N, Voight BF, Waite LL, Zhang W, Ziegler A, Absher D, Altshuler D, Balmforth AJ, Barroso I, Braund PS, Burgdorf C, Claudi-Boehm S, Cox D, Dimitriou M, Do R, Doney AS, El Mokhtari N, Eriksson P, Fischer K, Fontanillas P, Franco-Cereceda A, Gigante B, Groop L, Gustafsson S, Hager J, Hallmans G, Han BG, Hunt SE, Kang HM, Illig T, Kessler T, Knowles JW, Kolovou G, Kuusisto J, Langenberg C,

Langford C, Leander K, Lokki ML, Lundmark A, McCarthy MI, Meisinger C, Melander O, Mihailov E, Maouche S, Morris AD, Muller-Nurasyid M, Nikus K, Peden JF, Rayner NW, Rasheed A, Rosinger S, Rubin D, Rumpf MP, Schafer A, Sivananthan M, Song C, Stewart AF, Tan ST, Thorgeirsson G, van der Schoot CE, Wagner PJ, Wells GA, Wild PS, Yang TP, Amouyel P, Arveiler D, Basart H, Boehnke M, Boerwinkle E, Brambilla P, Cambien F, Cupples AL, de Faire U, Dehghan A, Diemert P, Epstein SE, Evans A, Ferrario MM, Ferrieres J, Gauguier D, Go AS, Goodall AH, Gudnason V, Hazen SL, Holm H, Iribarren C, Jang Y, Kahonen M, Kee F, Kim HS, Klopp N, Koenig W, Kratzer W, Kuulasmaa K, Laakso M, Laaksonen R, Lind L, Ouwehand WH, Parish S, Park JE, Pedersen NL, Peters A, Quertermous T, Rader DJ, Salomaa V, Schadt E, Shah SH, Sinisalo J, Stark K, Stefansson K, Tregouet DA, Virtamo J, Wallentin L, Wareham N, Zimmermann ME, Nieminen MS, Hengstenberg C, Sandhu MS, Pastinen T, Syvanen AC, Hovingh GK, Dedoussis G, Franks PW, Lehtimaki T, Metspalu A, Zalloua PA, Siegbahn A, Schreiber S, Ripatti S, Blankenberg SS, Perola M, Clarke R, Boehm BO, O'Donnell C, Reilly MP, Marz W, Collins R, Kathiresan S, Hamsten A, Kooner JS, Thorsteinsdottir U, Danesh J, Palmer CN, Roberts R, Watkins H, Schunkert H, Samani NJ. Large-scale association analysis identifies new risk loci for coronary artery disease. Nat Genet. 2013; 45:25–33. [PubMed: 23202125]

85. Kullo IJ, Leeper NJ. The genetic basis of peripheral arterial disease: Current knowledge, challenges, and future directions. Circ Res. 2015; 116:1551–1560. [PubMed: 25908728]
86. Wang D, Deuse T, Stubbendorff M, Chernogubova E, Erben RG, Eken SM, Jin H, Li Y, Busch A, Heeger CH, Behnisch B, Reichenspurner H, Robbins RC, Spin JM, Tsao PS, Schrepfer S, Maegdefessel L. Local microrna modulation using a novel anti-mir-21-eluting stent effectively prevents experimental in-stent restenosis. Arterioscler Thromb Vasc Biol. 2015; 35:1945–1953. [PubMed: 26183619]
87. Fredman G, Kamaly N, Spolitu S, Milton J, Ghorpade D, Chiasson R, Kuriakose G, Perretti M, Farokhzad O, Tabas I. Targeted nanoparticles containing the proresolving peptide ac2-26 protect against advanced atherosclerosis in hypercholesterolemic mice. Sci Transl Med. 2015; 7:275ra220.
88. Benditt EP, Benditt JM. Evidence for a monoclonal origin of human atherosclerotic plaques. Proc Natl Acad Sci U S A. 1973; 70:1753–1756. [PubMed: 4515934]
89. Pang WW, Pluvinage JV, Price EA, Sridhar K, Arber DA, Greenberg PL, Schrier SL, Park CY, Weissman IL. Hematopoietic stem cell and progenitor cell mechanisms in myelodysplastic syndromes. Proc Natl Acad Sci U S A. 2013; 110:3011–3016. [PubMed: 23388639]
90. Jaiswal S, Jamieson CH, Pang WW, Park CY, Chao MP, Majeti R, Traver D, van Rooijen N, Weissman IL. Cd47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis. Cell. 2009; 138:271–285. [PubMed: 19632178]
91. Gomez D, Shankman LS, Nguyen AT, Owens GK. Detection of histone modifications at specific gene loci in single cells in histological sections. Nat Methods. 2013; 10:171–177. [PubMed: 23314172]
92. Shankman LS, Gomez D, Cherepanova OA, Salmon M, Alencar GF, Haskins RM, Swiatlowska P, Newman AA, Greene ES, Straub AC, Isakson B, Randolph GJ, Owens GK. Klf4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque pathogenesis. Nat Med. 2015; 21:628–637. [PubMed: 25985364]
93. Vengrenyuk Y, Nishi H, Long X, Ouimet M, Savji N, Martinez FO, Cassella CP, Moore KJ, Ramsey SA, Miano JM, Fisher EA. Cholesterol loading reprograms the microrna-143/145-myocardin axis to convert aortic smooth muscle cells to a dysfunctional macrophage-like phenotype. Arterioscler Thromb Vasc Biol. 2015; 35:535–546. [PubMed: 25573853]
94. Hodge S, Hodge G, Ahern J, Jersmann H, Holmes M, Reynolds PN. Smoking alters alveolar macrophage recognition and phagocytic ability: Implications in chronic obstructive pulmonary disease. American journal of respiratory cell and molecular biology. 2007; 37:748–755. [PubMed: 17630319]
95. Morimoto K, Janssen WJ, Fessler MB, McPhillips KA, Borges VM, Bowler RP, Xiao YQ, Kench JA, Henson PM, Vandivier RW. Lovastatin enhances clearance of apoptotic cells (efferocytosis) with implications for chronic obstructive pulmonary disease. J Immunol. 2006; 176:7657–7665. [PubMed: 16751413]

96. Isenberg JS, Hyodo F, Pappan LK, Abu-Asab M, Tsokos M, Krishna MC, Frazier WA, Roberts DD. Blocking thrombospondin-1/cd47 signaling alleviates deleterious effects of aging on tissue responses to ischemia. Arterioscler Thromb Vasc Biol. 2007; 27:2582–2588. [PubMed: 17916772]

97. Fernandez-Boyanapalli R, Frasch SC, Riches DW, Vandivier RW, Henson PM, Bratton DL. Ppargamma activation normalizes resolution of acute sterile inflammation in murine chronic granulomatous disease. Blood. 2010; 116:4512–4522. [PubMed: 20693431]

98. Karunakaran D, Geoffrion M, Wei L, Gan W, Richards L, Shangari P, DeKemp EM, Beanlands RA, Perisic L, Maegdefessel L, Hedin U, Sad S, Guo L, Kolodgie FD, Virmani R, Ruddy T, Rayner KJ. Targeting macrophage necroptosis for therapeutic and diagnostic interventions in atherosclerosis. Science Advances. 2016; 2:e1600224. [PubMed: 27532042]

99. Liao X, Sluimer JC, Wang Y, Subramanian M, Brown K, Pattison JS, Robbins J, Martinez J, Tabas I. Macrophage autophagy plays a protective role in advanced atherosclerosis. Cell Metab. 2012; 15:545–553. [PubMed: 22445600]

100. Sidney S, Quesenberry CP Jr, Jaffe MG, Sorel M, Nguyen-Huynh MN, Kushi LH, Go AS, Rana JS. Recent trends in cardiovascular mortality in the united states and public health goals. JAMA cardiology. 2016; 1:594–599. [PubMed: 27438477]

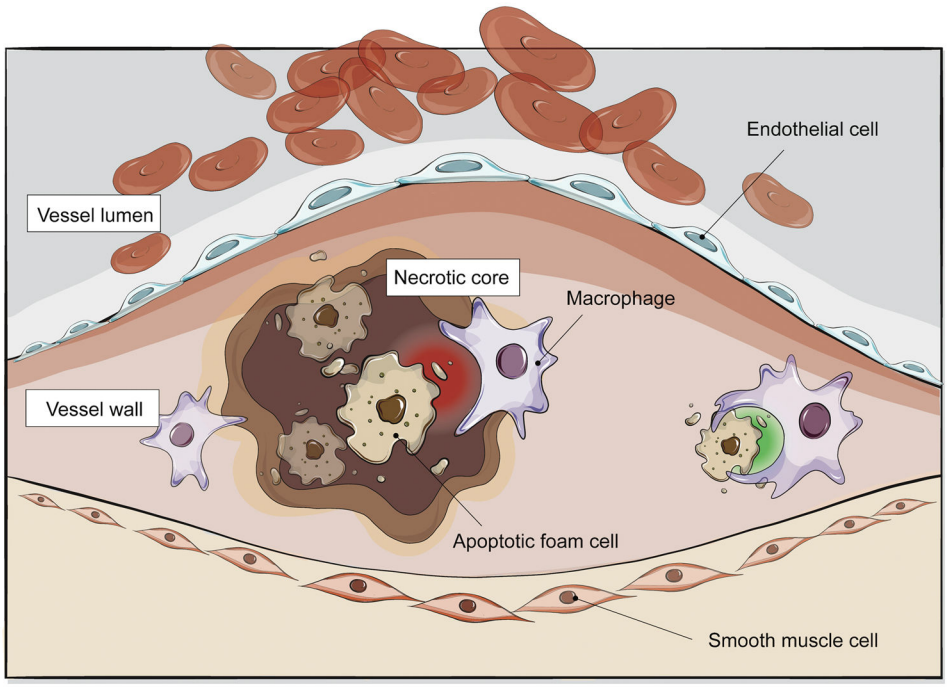

Figure 1. Impaired efferocytosis contributes to atherosclerosis

Diseased and apoptotic cells in the growing atherosclerotic plaque are not recognized for efficient phagocytic clearance by lesional macrophages. While the mechanisms which drive this pathology are still an area of active investigation, emerging data suggests that this defect may be due to impaired ‘eat me’ (green) and ‘don’t eat me’ (red) signaling that renders these cells ‘inedible’. As a result, foam cells accumulate to promote lesion expansion and apoptotic tissue undergoes secondary necrosis to accelerate vascular inflammation and lesion instability.

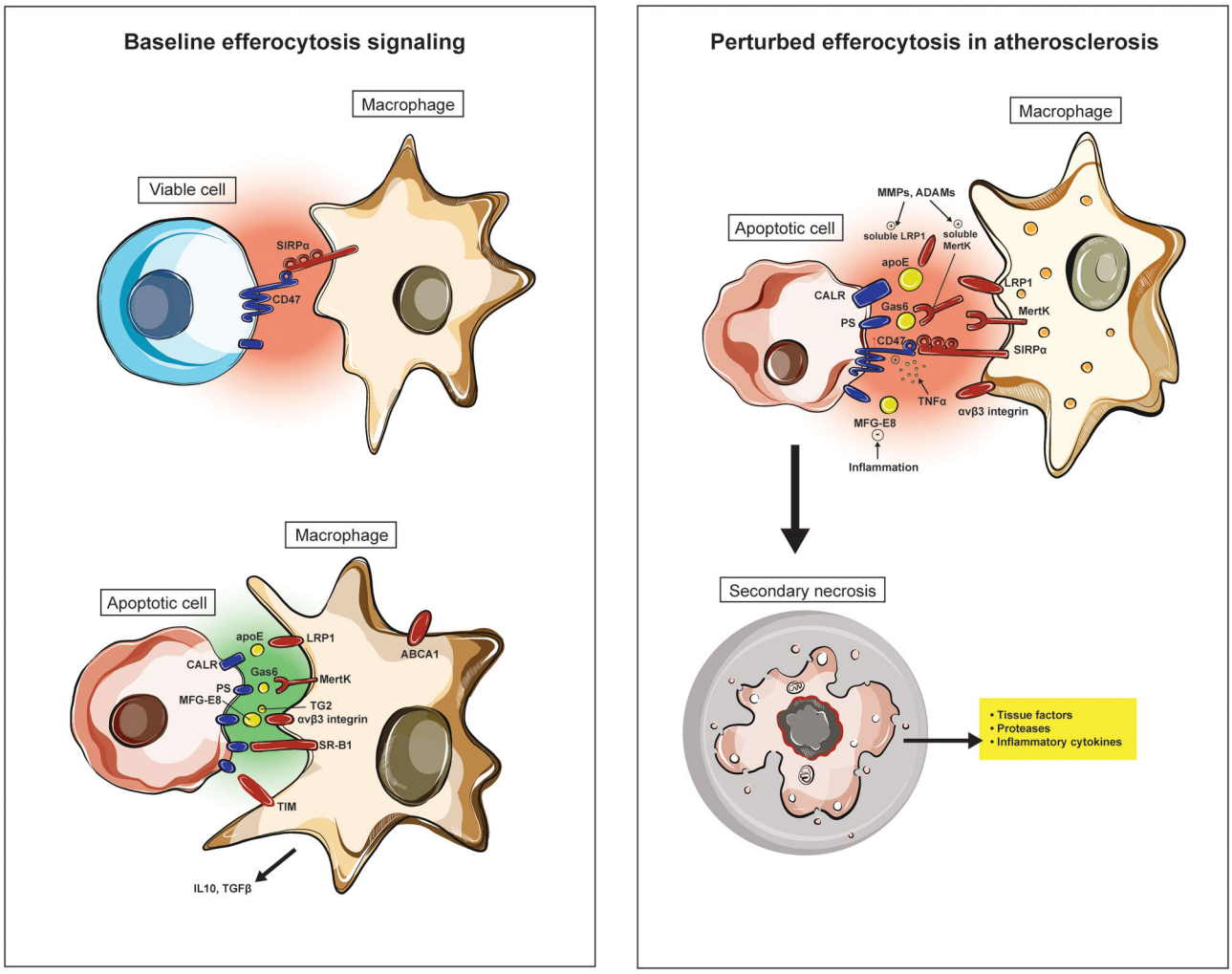

Figure 2. Impaired efferocytosis signaling in vascular disease

Experimental data suggests that pro-phagocytic signals (including calreticulin, Mfg-e8 and Mertk) are reduced in atherosclerosis due to inflammation, post-translational modifications and/or genetic variability. Exacerbating this loss of ‘eat me’ signaling is a concomitant upregulation of the CD47-Sirp-α ‘don’t eat me’ pathway, which furthers decreases the ‘edibility’ of cells within the necrotic core. The end result is that apoptotic cells in the growing plaque becomes poor substrates for phagocytic cells such as macrophages and dendritic cells. Such uncleared cells become secondarily necrotic and release additional proinflammatory stimuli, thus promoting a positive feedback loop.

Table

Efferocytosis-related molecules suggested to have a role in atherosclerosis

Table summary of putative ‘find me’, ‘eat me’ and ‘don’t eat me’ molecules that have been shown to alter atherosclerosis or features of plaque vulnerability, with notes about the animal models used and an interpretation of the authors’ findings.

Gene	Type of molecule	Mouse Model	In vivo findings	In vitro findings and additional notes	Expression	Ref
Mertk	Phagocytic receptor	$Mertk^{kd}$;$Apoe^{-/-}$	No change in plaque size (after 10 or 16 weeks of HFD) Increased: • TUNEL positive cells • Necrosis • Free apoptotic bodies	Reported to bind the bridging molecule, Gas6	Present on macrophages	50
Mertk	Phagocytic receptor	$Ldlr^{-/-}$ transplanted with $Mertk^{-/-}$ bone marrow	Increased: • Plaque size • TUNEL positive cells • Acellular core • Absolute macrophage content	• $Mertk^{-/-}$ MΦ did not have altered susceptibility to apoptotic death. Reduced IL10, • Increased TNF-α and IL12 observed (in splenocytes)	Present on MΦ. Reportedly not present on vascular SMCs	51
Lrp1	Phagocytic receptor	SMC $Lrp^{-/-}$;$Ldlr^{-/-}$	Increased: • Plaque size • Aneurysm formation • Elastic lamina disruption	Activation of PDGF-receptor signaling	Expressed on SMCs	52
Lrp1	Phagocytic receptor	$Ldlr^{-/-}$ transplanted with MΦ$Lrp1^{-/-}$ bone marrow	Increased: • Plaque size • Macrophage content • Elastic lamina breaks	• Increased MMP9, MCP1 in macrophages • $Lrp1^{-/-}$ macrophages secrete more TNF-α		53
Lrp1	Phagocytic receptor	$Ldlr^{-/-}$ transplanted with MΦ$Lrp1^{-/-}$ bone marrow	Increased: • TUNEL positive cells • Free apoptotic bodies	• Increased apoE, apoptosis, inflammatory cytokine production in $Lrp1^{-/-}$ MΦ • Decreased pAkt		54

Gene	Type of molecule	Mouse Model	In vivo findings	In vitro findings and additional notes	Expression	Ref
			• Necrotic core	• Reduced phagocytic capacity in Lrp1$^{-/-}$ MΦ		
Lrp1	Phagocytic receptor	MΦ Lrp1$^{-/-}$;Apoe$^{-/-}$ Ldlr$^{-/-}$ transplanted with MΦLrp1$^{-/-}$;Apoe$^{-/-}$ bone marrow	Increased: • Plaque size • Macrophage content • TUNEL positive cells • Necrotic core area • Free apoptotic bodies	• Increased macrophage apoptosis • Increased Ly6-C^{high} monocytes in spleen		55
Lrp1	Phagocytic receptor	Ldlr$^{-/-}$ transplanted with MΦLrp1$^{-/-}$, MΦLrp1$^{-/-}$;Apoe$^{-/-}$ bone marrow Treated with TNF-α inhibitor	Increased: • Plaque size • TUNEL positive cells • Necrotic core area • Free apoptotic bodies • Macrophage concent • M1 macrophage	• Increased TG • TNF-α blockade showed anti-atherosclerotic effect only when MΦ LRP1 present • TNF-α blockade had no effect on efferocytosis		56
SR-B1	Phagocytic receptor	Apoe$^{-/-}$transplanted with SR-B1$^{-/-}$; Apoe $^{-/-}$ bone marrow Ldlr$^{-/-}$ transplanted with SR-B1$^{-/-}$, SR-B1$^{-/-}$; Apoe $^{-/-}$ bone marrow	Increased: • Plaque size • Apoptotic cells • Necrotic core • Free apoptotic bodies Reduced collagen content, fibrous cap thickness	Reduced phagocytic capacity and anti-inflammatory cytokine production, more inflammatory cytokine production in SR-B1$^{-/-}$ macrophages		57
Tim-1/Tim-4	Phagocytic receptor	Ldlr$^{-/-}$ treated with Tim-1 or Tim-4 blocking antibodies	Increased: • Plaque size • Apoptotic cells • CD4^{+}T cells • Free apoptotic bodies	Reduced phagocytic capacity. Increased IL6 and MCP1 in Tim4 Ab treated macrophages	Present on T cells (Tim-1), macrophages and dendritic cells (Tim-4)	58

Gene	Type of molecule	Mouse Model	In vivo findings	In vitro findings and additional notes	Expression	Ref
G2A	Possible phagocytic receptor	$G2A^{-/-}$;$Apoe^{-/-}$ $Apoe^{-/-}$ transplanted with $G2A^{-/-}$; $Apoe^{-/-}$ bone marrow $Ldlr^{-/-}$ transplanted with $G2A^{-/-}$;$Ldlr^{-/-}$ bone marrow	Increased: • Plaque size • Collagen content • Necrotic core	• Increased pAkt, NFkB activation, inflammatory cytokine production • Reduced MΦ apoptosis • Reduced phagocytic capacity in $G2A^{-/-}$ MΦ • Increased plasma MCP-1, IL-6		59
CX3CL1	Find me ligand	$CX3CL1^{-/-}$;$Apoe^{-/-}$ $CX3CL1^{-/-}$;$Ldlr^{-/-}$	Decreased: • Plaque size and macrophage content in the brachiocephalic artery with inconsistent changes in the aortic sinus	Effects possibly due to reduced chemoattractant and adhesive properties		60
Mfge8	Bridging molecule	$Ldlr^{-/-}$ transplanted with $Mfge8^{-/-}$ bone marrow	Increased: • Plaque size • TUNEL positive cells • Acellular core • SMC and collagen content	• Reduced phagocytic capacity in $Mfge8^{-/-}$ macrophages • Reduced IL10; Increased IFN-γ (in splenocytes and spleen) • Altered T-reg function	Present on ECs, SMCs and MΦ. Downregualted on MΦs during atherogenesis	61
C1q	Bridging molecule	$C1q^{-/-}$;$Ldlr^{-/-}$ with standard diet	Increased: • Plaque size • Apoptotic cells	• Reported to bind calreticulin • Differences noted in this study depending on diet fed to mice		62
TG2	Bridging molecule	$Ldlr^{-/-}$ transplanted with $TG2^{-/-}$ bone marrow	Increased: • Plaque size • Necrotic core size • Macrophage content	• Reduced phagocytic capacity in $TG2^{-/-}$ MΦ • Decreased ABCA1 expression in $TG2^{-/-}$ macrophages • Triglycerides potentially reduced in vivo	Expressed in plaque intimal cells	63
Gas-6	Bridging molecule	$Gas6^{-/-}$;$Apoe^{-/-}$ with standard diet	No change in overall plaque size Increased:		Expressed by ECs, SMCs and MΦ in	64

Gene	Type of molecule	Mouse Model	In vivo findings	In vitro findings and additional notes	Expression	Ref
			• SMC, TGF-β and collagen content Decreased: • Necrotic core size, macrophage content		plaque. High in ruptured plaque, TCFA	
Fas	May stimulate Find me signaling	$Fas^{lpr/lpr}$;$Apoe^{-/-}$	Increased: • Plaque size • IgG deposition • TUNEL positive cells	Enlarged spleen, thymus, lymph node Renal damage Reduced circulating T cells, cholesterol, oxidized phosphor lipid on apoB-100 containing lipoprotein, bone mineral density		65
Fas ligand	May stimulate Find me signaling	Gld (fasL mutation);$Apoe^{-/-}$	Increased: • Plaque size • TUNEL positive cells • MΦ and T cell content • Free apoptotic cells in lymph node	• Splenomegaly, renal dysfunction • Reduced cholesterol • Infusion of lysoPC markedly increased apoptotic cells		66
Calreticulin	Eat me ligand	$Cdkn2b^{-/-}$;$Apoe^{-/-}$ (animals have reduced Calr, indirectly suggesting a role for this gene which was confirmed by in vitro assays)	Increased: • Plaque size • Necrotic core size Decreased: • Collagen content • Fibrous cap thickness	• $Cdkn2b^{-/-}$ apoptotic bodies showed reduced Calr expression, resistance to efferocytosis, reduced Abca1 expression and impaired cholesterol efflux		19
CD47	Don’t eat-me ligand	$Apoe^{-/-}$ treated with CD47 blocking antibody	Decreased: • Plaque size • Apoptotic cells • Necrotic core size • Free apoptotic bodies • Features of plaque vulnerability	• Splenomegaly and anemia observed due to transient erythrophagocytosis • Mechanism of upregulation shown to involve TNF-α	Upregulated in advanced atherosclerosis and in the necrotic core	42