Modulating Oxysterol Sensing to Control Macrophage Apoptosis and Atherosclerosis

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Atherosclerosis is the silent underlying cause of acute myocardial infarction and stroke, some of the most common causes of mortality worldwide. Aside from managing risk factors, such as hyperlipidemia, few therapies can be said to directly target atherosclerotic plaque progression partially because of remaining gaps in basic knowledge of the disease. Atherosclerosis is initiated by endothelial injury and deposition of lipids in the subendothelial layer called the intima. Circulating monocytes traffic to the site of injury, transmigrate through the endothelium, and differentiate to macrophages in an attempt to repair damage and remove lipids, much of which include deleterious oxidatively modified forms. Subsequently, plaque macrophages become central players in the progression of atherosclerosis because of a feedforward cycle of rampant lipid accumulation, proinflammatory cytokine release, cell death, and further monocyte recruitment.

A major area of focus in the field is in understanding why macrophages cannot efficiently clear lipid and cell debris deposits. Lipid species in the plaque, including oxidized low-density lipoprotein (oxLDL), are known to induce apoptotic cell death, though the precise lipid species and signal transduction mechanisms involved are unclear. This apoptotic response may actually be adaptive in the early stages of plaque development because secondary macrophages can adeptly clear apoptotic cells through efferocytosis before cell-intrinsic damage becomes overwhelming (Figure, top left). Indeed, many features of apoptotic cell death, including expression of eat-me surface signals, are purposed toward efferocytic clearance. In contrast, secondary necrosis occurs when apoptotic cells are left unengulfed, resulting in uncontrolled disintegration, release of cytosolic components, and damage to local tissue. Advanced plaques lack efficient efferocytosis such that many apoptotic cells progress to secondary necrosis, adding to plaque size and complexity (Figure, top right). Thus, understanding mechanisms of apoptotic cell death in atherosclerotic plaque macrophages and the impact on overall disease progression is of great interest. Oxysterols are a major constituent of oxLDL and are sensed by several signaling mediators, including the Insig, liver X receptor, and oxysterol-binding protein–related protein (ORP) families. These pathways coordinate both acute posttranslational and longer term transcriptional responses to tightly regulate cholesterol homeostasis and macrophage phenotype. However, it remains less clear how oxysterol sensing is mechanistically linked to regulation of apoptosis. Oxysterols derived from oxLDL have been proposed to promote caspase activation and apoptosis via direct activation of proapoptotic factors, such as Bax/Bad, lysosomal destabilization, and derangements in calcium homeostasis (Figure, bottom).

In the present issue of *Circulation Research*, Zhong et al describe a novel mechanism by which the oxysterol 25-hydroxycholesterol (25-OHC) binds an antiapoptotic member of the ORP family, ORP4L, to induce macrophage apoptosis in a manner that is atheroprotective. ORP4 was recently shown to have prosurvival and proliferative functions in immortalized cell lines and T cells, potentially through regulation of IP$_3$ and endoplasmic reticulum Ca$^{2+}$ release. Given its ability to bind oxysterols and the potential links to apoptosis, the authors first profiled ORP4 isoform expression across tissues and determined ORP4L to be the predominant macrophage variant. Primary macrophages from ORP4L knockout mice showed increased apoptosis at baseline and in response to lipopolysaccharide, tumor necrosis factor-α, an effect that was wholly rescued by reconstitutive ORP4L overexpression. Of the many influences ORP4L knockout could have on macrophage or physiological phenotypes related to atherosclerosis, apoptosis emerged as a strong candidate because of a lack of observed differences in the migratory capacity, oxLDL uptake, and phagocytic potential of ORP4L-deficient macrophages. Further, ORP4L overexpression inhibits apoptosis induced by a variety of atherosclerosis-relevant stimuli, including lipopolysaccharide, tumor necrosis factor-α, oxLDL, and 25-OHC.

The downstream mechanisms by which ORPs may regulate apoptosis are unknown. A communoprecipitation–proteomics approach identified G$_{q/11}$ and PLCβ3 (phospholipase C-β3) as ORP4L-binding partners that could mediate chemokine-induced G-protein–coupled receptor signal transduction. The chemokine C5a was used as a representative G-protein–coupled receptor–activating ligand, though many other atherosclerosis-relevant ligands, such as sphingosine-1-phosphate, lysophosphatidic acid, and monocyte chemotractant protein-1, could act similarly on this pathway. G$_{q/11}$, PLCβ3, and ORP4L are induced in concert during macrophage differentiation and colocalize at the plasma membrane, though the precise interaction of ORP4L with PLCβ3 was left
Figure. Model of oxysterol and oxysterol-binding protein–related protein 4L (ORP4L)–mediated apoptosis and impact on atherogenesis in early and late lesions. Top left, In early atherosclerotic lesions, macrophage foam cells undergoing apoptosis are efficiently cleared through efferocytosis, limiting plaque progression. Top right, In late atherosclerotic lesions, inefficient efferocytosis results in progression of apoptotic cells to secondary necrosis, contributing to necrotic core and eventually thrombus formation. Local proliferation of macrophages partially drives apoptotic cell accumulation. Bottom, ORP4L complexes with G\textsubscript{q/11} and PLC\textsubscript{B3} to facilitate constitutive IP\textsubscript{3}-Ca\textsuperscript{2+}-CREB signaling, resulting in Bcl-X\textsubscript{L} transcription and antiapoptotic activity. ORP4L signaling may also increase cellular proliferation. With oxysterol (mainly oxysterol 25-hydroxycholesterol [25-OHC]) binding, the ORP4L-G\textsubscript{q/11}-PLC\textsubscript{B3} complex is disrupted, resulting in (Continued)
unclear. Importantly, PLCβ3 deficiency has previously been linked to macrophage apoptosis and the reduction of atherosclerosis in mice7 through mechanisms highly concordant with those explored by Zhong et al, and G0 alpha, is a well-established transducer of chemokine inputs to PLCβ3 activity.8 To confirm this interaction, a yeast 2-hybrid screen identified the ORP4L amino acid residues 445 to 513 as containing the PLCβ3-binding domain, and absence of this domain reduces ORP4L-dependent apoptotic signaling on each of these intermediaries, though, of course, several components, such as Ca2+ and CREB, act broadly.

With a strong candidate mechanism in place for how ORP4L activates prosurvival BCL-XL signaling and prevents apoptosis, which are the relevant oxysterol interactions with ORP4L? Out of many potential oxLDL and plaque lipid constituents, 25-OHC was previously shown to bind ORP4L with high affinity and specificity,9 and the present study further confirmed this interaction using modeling and domain knockouts to identify the 25-OHC binding site. 25-OHC acted to disrupt ORP4L’s interactions with G0 alpha and PLCβ3 to prevent downstream signaling and induce apoptosis, whereas a mutant ORP4L lacking the putative 25-OHC binding site was insensitive to these effects.

Last, the impact of ORP4L signaling on atherosclerosis was assessed in vivo. ORP4L is most prominently expressed in macrophages in both murine and human lesions, and its impact on atherosclerotic progression was studied using several murine low-density lipoprotein receptor–deficient mouse models. Plaque burden was decreased in ORP4L knockout mice, which featured decreased aortic macrophage BCL-XL expression and apoptosis. These results were particularly consistent with the concept of macrophage apoptosis as a protective response: early-apoptotic (Annexin V+, PI−) macrophages were more prevalent, whereas late-apoptotic (Annexin V+, PI+) cells were less prevalent. This, along with reduced necrotic core size, suggests an efficient flux and effectoric clearance of these cells, although this was not directly assessed. Conversely, mice transplanted with bone marrow cells transduced to overexpress ORP4L showed reduced apoptosis and increased plaque burden. As the authors note, atherosclerotic phenotypes were assessed during a relatively advanced state of atherosclerosis, where increased macrophage apoptosis is generally thought to be maladaptive. We agree that this unexpected association may be because of a simple cumulative effect: induced apoptosis was protective enough in the early plaque to a degree that the later maladaptive effects of increased apoptosis were not realized. Longer term studies focused on truly advanced plaques, and the study of inducible Cre-Lox deletion or overexpression mouse models in the context of ORP4L, and other macrophage apoptotic factors will be needed to more definitively assess the impact of apoptosis in early (simple) versus late (complex) lesions.

Alternative aspects of ORP4L signaling may provide complementary explanations for its seemingly atherogenic role in macrophages. In advanced atherosclerosis, local macrophage proliferation is thought to supersede monocyte recruitment as the dominant contributor to macrophage abundance and plaque burden.10 Three studies have now identified pro-proliferative roles of ORP4L in several settings. Burgett et al11 showed that a class of antiproliferative natural compounds are potent and specific ligands for ORP4L and suggest that ORP4L activity is strongly proliferative. ORP4L is also crucial for proliferation in several cell lines, including Jurkat T cells.5,6 This phenotype may not have been obvious in nonproliferating peritoneal macrophages or other cells examined shortly after transient transfection in the present study and may be difficult to disentangle from antiapoptotic effects. Could the beneficial effects of ORP4L deficiency in more advanced plaques be partially explainable by reduced macrophage proliferation?

Given that 25-OHC is the predominant ligand for ORP4L,9 it is important to place the present study in the context of existing knowledge regarding macrophage 25-OHC and atherosclerotic macrophage biology. Compared with abundant oxysterols, such as 7-ketocholesterol and 7β-hydroxycholesterol, 25-OHC constitutes only a minor fraction of total oxysterol content in atherosclerotic plaques12 but has important and highly pleiotropic roles in cholesterol metabolism, inflammatory signaling, and atherogenesis.13 Unlike many other oxysterols formed through free radical oxidation, 25-OHC can be produced enzymatically by cholesterol 25-hydroxylase (CH25H) and is regarded as a signaling lipid (Figure, bottom). In conditions of high cholesterol bioavailability (such as the macrophage foam cell), 25-OHC serves as a feedback inhibition signal through Insig and liver X receptor signaling to potentially abrogate cholesterol biosynthesis and enhance reverse cholesterol transport. CH25H activity is also potently induced in response to inflammatory cues, including lipopolysaccharide and type I interferons, and limits macrophage inflammasome activation and interleukin-1β production.13 Thus, it is possible that CH25H-generated 25-OHC mediates the ORP4L-dependent apoptotic effects of lipopolysaccharide and tumor necrosis factor-α observed in the present study. To our knowledge, the impact of CH25H knockout on atherosclerosis has not been directly examined, but recent characterization of the CH25H repressor ATF3 provides preliminary insight. Knockout of ATF3 results in 25-OHC accumulation and atherogenesis attributed to enhanced foam cell formation,14 in contrast to what might be
expected if a proapoptotic ORP4L inhibitory role of 25-OHC was predominant.

Overall, it can be appreciated that inhibition of ORP4L/PLCβ3 antiapoptotic signaling is one of many complex influences of 25-OHC on macrophage fate and atherosclerosis. Of the possible oxysterol–ORP signaling cascades inducing apoptosis, the identified (25-OHC)–ORP4L interaction is perhaps the most significant uncovered to date. Additionally, Zhong et al’s identification of ORP4L as an essential component of the Gαq/11 and PLCβ3 prosurvival signaling cascade constitutes a substantial contribution to understanding apoptotic and oxysterol signaling in atherosclerosis. The persistent benefit of increased apoptosis in ORP4L knockout mice in a relatively advanced stage of atherosclerosis also further highlights the need to understand how apoptosis and other macrophage processes may impact atherosclerosis differentially depending on disease stage (Figure, top).

These findings place ORP4L among many ORP, liver X receptor, and Insig oxysterol signaling mechanisms regulating macrophage phenotype and atherosclerotic progression.

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References
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