Targeting macrophage necroptosis for therapeutic and diagnostic interventions in atherosclerosis

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Atherosclerosis results from maladaptive inflammation driven primarily by macrophages, whose recruitment and proliferation drive plaque progression. In advanced plaques, macrophage death contributes centrally to the formation of plaque necrosis, which underlies the instability that promotes plaque rupture and myocardial infarction. Hence, targeting macrophage cell death pathways may offer promise for the stabilization of vulnerable plaques. Necroptosis is a recently discovered pathway of programmed cell necrosis regulated by RIP3 and MLKL kinases that, in contrast to apoptosis, induces a proinflammatory state. We show herein that necrototic cell death is activated in human advanced atherosclerotic plaques and can be targeted in experimental atherosclerosis for both therapeutic and diagnostic interventions. In humans with unstable carotid atherosclerosis, expression of RIP3 and MLKL is increased, and MLKL phosphorylation, a key step in the commitment to necroptosis, is detected in advanced atheromas. Investigation of the molecular mechanisms underlying necroptosis showed that atherogenic forms of low-density lipoprotein increase RIP3 and MLKL transcription and phosphorylation—two critical steps in the execution of necroptosis. Using a radiotracer developed with the necroptosis inhibitor necrostatin-1 (Nec-1), we show that 123I-Nec-1 localizes specifically to atherosclerotic plaques in ApoE−/− mice, and its uptake is tightly correlated to lesion areas by ex vivo nuclear imaging. Furthermore, treatment of ApoE−/− mice with established atherosclerosis with Nec-1 reduced lesion size and markers of plaque instability, including necrotic core formation. Collectively, our findings offer molecular insight into the mechanisms of macrophage cell death that drive necrotic core formation in atherosclerosis and suggest that this pathway can be used as both a diagnostic and therapeutic tool for the treatment of unstable atherosclerosis.

INTRODUCTION

Atherosclerosis is characterized by the accumulation of lipid-rich plaques in medium to large arteries, which are replete with macrophages, T lymphocytes, lipids, and cholesterol crystals. Atherosclerosis is considered a benign disease until plaques weaken and rupture, leading to acute thrombus and subsequent myocardial infarction or stroke (1). A hallmark of such vulnerable lesions is the presence of a large necrotic core covered by a thin fibrous cap, which renders the plaque susceptible to rupture (2). Although the processes that underlie the initiation of inflammatory fatty lesions within the arterial wall are well understood, the mechanisms by which these benign lesions develop into rupture-prone culprit lesions are not. There is, thus, an urgent need to better understand the pathways that contribute to necrotic core formation and to develop strategies to target these processes therapeutically.

Atherosclerosis is initiated by the accumulation of excess low-density lipoprotein (LDL) cholesterol that becomes trapped in the subendothelial space, where it is modified in the oxidant-rich environment. According to the oxidation hypothesis, oxidized LDL (oxLDL) activates innate immune cells, particularly macrophages, to engulf the modified-self LDL via constitutively expressed scavenger receptors on their cell surface, triggering the activation of pattern recognition receptors, such as the Toll-like receptors and the inflammasome (3–6). OxLDL can be cytotoxic and induce apoptosis of macrophages and smooth muscle cells (SMCs), which in early plaques are effectively cleared by macrophages via efferocytosis (7). However, as macrophages accumulate lipids and undergo endoplasmic reticulum stress, efferocytosis has been shown to become defective, resulting in secondary necrosis and the uncontrolled release of inflammatory mediators, proteases, and coagulation factors—all factors that promote plaque vulnerability (8). Thus, macrophage cell death has been considered to be a major contributor to atherosclerosis and necrotic core formation in plaques.

The recent discovery and characterization of a pathway of programmed necrosis, or “necroptosis” (9), have expanded our understanding of the mechanisms leading to cell death. It is now understood that apoptosis and necroptosis have evolved as counterbalances in the first line of defense against inflammatory stimuli, either exogenous or self-derived. When proapoptotic caspase-8 is inhibited or overwhelmed, either through synthetic or naturally occurring inhibitors, the kinases RIP1 and RIP3 become phosphorylated, leading to recruitment and activation of MLKL by RIP3 and loss of plasma membrane integrity resulting in the release damage-associated molecular patterns (DAMPs) into the extracellular space (10–12). The upstream stimuli of necroptosis are beginning to be defined, and it is clear that both self- and non-self-ligands can contribute (13). Necroptosis and apoptosis share many overlapping factors; however, the small molecule necrostatin-1 (Nec-1)
uniquely inhibits the interaction of RIP1-RIP3 and subsequent downstream effectors of necroptosis (14); thus, Nec-1 can be considered a preferential inhibitor of necroptosis rather than apoptosis. Although the necrostatin class of small molecules has enabled the evaluation of RIP1-RIP3 in many pathologies and next-generation necrostatins have increased specificity for RIP1, the use of Nec-1 in long-term models of disease has not been explored (15). Furthermore, the recent development of Rip3 knockout mice has revealed a specific role for Rip3 in the development of a number of diseases (16), including atherosclerosis (17); however, the mechanistic insight into how necroptosis is driven in the plaque and whether this is relevant to human disease is still lacking.

Here, we set out to test whether necroptosis is activated in advanced atherosclerosis in humans and whether it could be targeted for diagnostic and therapeutic intervention in a model of established atherosclerosis. We find that the necroptotic pathway is triggered in human atherosclerotic plaques and is associated with markers of lesion vulnerability. Atherogenic ligands drive the expression of necroptotic genes, and this can be targeted as a therapeutic and diagnostic tool for advanced atherosclerosis in vivo. Together, our findings suggest that necroptosis underlies plaque vulnerability in humans and that dually targeting necroptosis for therapeutic and diagnostic interventions may benefit patients at high risk for vulnerable plaques and downstream adverse clinical events.

RESULTS
Necrotic pathway is activated in unstable atherosclerosis in humans

Although necroptosis has been shown to promote lesion progression in an experimental mouse model of atherosclerosis (17), to date there has not been compelling evidence that necroptosis is active in human plaques. Necrotic cells are frequently found within advanced human atheromas (18), and the presence of a necrotic core is a major predictor of rupture-prone lesions; therefore, we hypothesized that the necroptotic pathway is activated in advanced atherosclerosis in humans. We initially evaluated the gene expression of RIP3 and MLKL in carotid plaques from a large biobank of patients with atherosclerosis and in disease-free control arteries (19, 20). Gene expression analysis showed a significant increase in expression of both RIP3 and MLKL mRNA in atherosclerotic plaques compared to normal arteries (\( P \leq 0.0001 \); Fig. 1A). Because we hypothesized that necroptosis underlies lesion vulnerability, we examined whether expression of necroptotic genes may be further increased in unstable versus stable atherosclerotic plaques. Gene expression analysis of plaques from individuals with symptomatic carotid disease (that is, transient ischemic attack, minor stroke, and/or amaurosis fugax) revealed a significant elevation of both RIP3 and MLKL gene expression compared to plaques from asymptomatic individuals (\( P \leq 0.05 \) and \( P \leq 0.01 \), respectively; Fig. 1B). Traditional measures of cell death [for example, TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling) positivity] cannot distinguish between necroptotic and apoptotic cell death; however, the phosphorylation of MLKL—the last step in the execution of necroptosis—is considered to be the most definitive biomarker of necroptosis activity in vivo (21). To test whether necroptosis was indeed activated in vascular lesions in humans, we evaluated whether phosphorylated MLKL (pMLKL) could be detected in human coronary plaques with different stages of atherosclerotic lesions. Immuno-}

histrochemical analysis using an antibody that recognizes pMLKL showed that regions with advanced fibroatheroma lesions showed positive pMLKL staining in close proximity to the necrotic core, whereas within the same subject, early lesions (defined as pathologic intimal thickening) showed no positive pMLKL staining (Fig. 1C). Quantification of the pMLKL-positive area reveals that in subjects with advanced fibroatheromas, there is a significant elevation of pMLKL compared to subjects with intimal thickening (Fig. 1D). This is the first evidence that the necroptotic pathway is associated with human vascular disease and the first report that pMLKL is found within advanced atherosclerotic plaques, supporting the idea that this pathway could be contributing to lesion vulnerability.

OxLDL induces necroptosis in macrophages via RIP3

To further understand the mechanisms by which necroptosis is activated in plaques, we evaluated how atherogenic ligands trigger necroptosis by endogenous mechanisms in vitro. OxLDL is known to induce apoptosis; however, little is known whether oxLDL or other atherogenic ligands found within the plaque can endogenously promote necroptosis in the absence of nonphysiological apoptosis inhibitors (that is, zVAD.fmk) (17, 22, 23). Therefore, we tested the degree of necroptotic cell death in macrophages in vitro in bone marrow–derived macrophages (BMDMs) treated with oxLDL for 24 hours. Compared to unstimulated cells, oxLDL treatment resulted in a ~4-fold induction of cell death in macrophages, and this was significantly inhibited by cotreatment with the necroptosis inhibitor Nec-1 (3.9 ± 0.6–fold versus 2.3 ± 0.4–fold, respectively; \( P \leq 0.01 \); Fig. 2A and fig. S1A). Inhibition of apoptotic cell death with the pan–caspase inhibitor zVAD.fmk significantly enhanced cell death in response to oxLDL, similar to what had been reported previously (5.7 ± 0.7–fold; \( P \leq 0.001 \)), whereas Nec-1 treatment alone did not promote cell death (Fig. 2A and fig. S1B). To test whether the induction of cell death is dependent on RIP3 function, we measured the induction of cell death by oxLDL in both wild-type macrophages and macrophages deficient in RIP3 (\( \text{Rip}^3^-/- \)), and observed that \( \text{Rip}^3^-/- \)-macrophages are resistant to cell death in response to both oxLDL and oxLDL + zVAD.fmk (Fig. 2B). Because phospho-RIP3 and phospho-MLKL are required for the execution of necroptosis, we subsequently measured the degree of phosphorylation of RIP3 and MLKL in response to oxLDL, and we observed that oxLDL significantly induced both RIP3 and MLKL phosphorylation in macrophages, which was dampened by Nec-1 (Fig. 2, C and D). At the ultrastructural level, cells undergoing necroptosis have damaged plasma membrane integrity and translucent electron-light cytoplasm (24). Ultrastructural analysis of macrophages treated with oxLDL and oxLDL + zVAD.fmk using electron microscopy shows the typical electron-light zones within the cytoplasm that are not found in control-treated cells (Fig. 2E and fig. S1C). Because SMCs form the fibrous cap and play an important role in the rupture of atherosclerotic plaques, we were curious as to whether SMCs also undergo necroptosis in response to oxLDL but found that neither oxLDL nor oxLDL + zVAD.fmk significantly induced cell death in SMCs (fig. S2A). Together, these data demonstrate that oxLDL, a potent endogenous atherogenic ligand, can independently induce macrophages to undergo necroptosis in the absence of caspase inhibitors, suggesting that this pathway may be activated under physiologic conditions in the vessel wall.

Within the atherosclerotic milieu, various stimuli are present, in addition to oxLDL, that can promote the inflammatory response and propagate lesion formation. These include cytokines, chemokines,
reactive oxygen species (ROS), cholesterol crystals, and other modified self-ligands, and together are termed DAMPs. DAMPs are released from cells during the process of necroptosis, where uncontrolled leakage of intracellular components occurs after membrane integrity is lost (12). If these dying cells are not cleared away by efferocytosis, they can persist and allow for further DAMP release (25). We therefore hypothesized that efferocytosis of necrototic cells is impaired, which promotes DAMP release that exacerbates oxLDL-induced cell death. To test this, we treated BMDMs with staurosporine to induce apoptosis, or oxLDL + zVAD.fmk or LPS (lipopolysaccharide) + zVAD.fmk to induce necroptosis. Dead cells were collected and applied in equal number to naïve macrophages to allow for efferocytosis, after which unbound cells were washed extensively before uptake was quantified. As expected, cells treated with staurosporine (that is, apoptotic cells) were taken up by the naïve macrophages (Fig. 2F). In contrast, cells treated with either oxLDL + zVAD.fmk or LPS + zVAD.fmk (that is, necrototic cells) were not engulfed by the naïve macrophages to the same extent, thus confirming that necrototic cells are less efficiently efferocytosed than cells undergoing apoptosis. To test whether DAMP release might potentiate necrototic cell death induced by oxLDL, DAMPs generated by...
Fig. 2. OxLDL induces necroptotic cell death in macrophages. (A) BMDMs were treated with oxLDL (100 µg/ml) ± zVAD.fmk ± Nec-1 for 24 hours, and LDH (lactate dehydrogenase) release in the medium was measured. Data represent means ± SEM of five independent experiments. (B) Cell death in response to oxLDL ± zVAD.fmk in BMDMs from wild-type (WT) and Rip3−/− mice. (C) Western blot analysis of RIP3 after treatment with oxLDL ± zVAD.fmk ± Nec-1 for 8 hours. Band shift indicates phospho-RIP3 (pRIP3). (D) Western blot analysis of pMLKL after treatment with oxLDL for 12 hours or oxLDL ± zVAD.fmk for 8 hours. (E) Electron microscopy ultrastructural analysis of control and oxLDL-treated macrophages. Control macrophages had normal-looking cytoplasm, whereas oxLDL-treated macrophages had electron-light zones (arrows) that were not observed in control macrophages. Scale bar, 500 nm. (F) BMDMs were treated for 24 hours with medium alone (control) or medium containing 5 µM staurosporine (STS) (to induce apoptosis), oxLDL ± zVAD, or LPS ± zVAD (to induce necroptosis). Dead cells were collected, counted, resuspended in control medium, and applied to naive BMDMs at a ratio of 3:1. After 2 hours, cells were washed six times with cold phosphate-buffered saline (PBS), and fluorescence intensity was quantified using a plate reader. The graph depicts a representative experiment with a mean ± SD of n = 4 experimental replicates repeated at least three times. ****P < 0.0001, ***P < 0.001 versus nontreated (control). (G) BMDMs were subjected to freeze-thaw to generate necrotic DAMPs, which were added to cells with or without oxLDL ± zVAD.fmk ± Nec-1 for 24 hours, and cell death was measured. (H) Western blot analysis of RIP3 after treatment with oxLDL ± zVAD.fmk ± Nec-1 with or without DAMPs for 8 hours. Band shift indicates phospho-RIP3. (I) Cell death in response to oxLDL ± zVAD.fmk ± necrotic freeze-thaw DAMPs in BMDMs from WT and Rip3−/− mice. (J) Cell death in BMDMs from WT and Casp1−/− mice in response to oxLDL ± zVAD.fmk for 24 hours. *P ≤ 0.05, **P < 0.01, ***P < 0.001 by one-way or two-way analysis of variance (ANOVA). ns, not significant.
either mechanical (that is, freeze-thaw) or pharmacological (that is, LPS + zVAD.fmk) methods were coincubated together with oxLDL that significantly induced cell death in macrophages, which could be inhibited by Nec-1 (Fig. 2G and fig. S2B; P < 0.05). Similar to what is observed with oxLDL alone, cell death in response to DAMPs is dependent on RIP3, because Rip3−/− macrophages are resistant to cell death by oxLDL + DAMPs (Fig. 2H). Activation of RIP3 by phosphorylation is increased when macrophages are coincubated with oxLDL and DAMPs and can be inhibited by Nec-1 (Fig. 2I). These data indicate that in addition to oxLDL, DAMPs released from dying cells exacerbate necroptotic cell death and are dependent on RIP3 activation, suggesting that the multiple inflammatory ligands present in the atherosclerotic plaque may act concomitantly to promote necroptosis.

oxLDL is an inducer of the NLRP3 inflammasome and can induce the expression and secretion of interleukin-1β (IL-1β) (3, 4). We therefore asked whether the inflammasome was required for the induction of necroptosis by oxLDL. Macrophages from either wild-type mice or mice deficient in caspase-1 (Casp1−/−) had equivalent levels of cell death upon treatment with oxLDL (Fig. 2J). Similarly, cotreatment of macrophages with the caspase-1 inhibitor zVAD.fmk did not reduce or prevent cell death in response to oxLDL (fig. S2C). These data indicate that necrotic cell death in response to oxLDL does not depend on the induction of caspase-1, unlike what is observed for IL-1β secretion.

**OxLDL induces expression of necroptotic genes RIP3 and MLKL**

OxLDL is an inflammatory ligand that induces the activation of proinflammatory signaling events, eliciting cytokine and chemokine production (26). Because oxLDL induces necroptosis independently of caspase inhibition, we next sought to determine the mechanisms underlying the induction of necroptosis by oxLDL. We measured the expression of genes involved in the necrototic pathway in macrophages and found that the treatment with oxLDL induced the expression of RIP3 and MLKL at both the mRNA (Fig. 3, A and B) and protein levels (Fig. 3, C and D). OxLDL can induce the production of ROS (26). Therefore, we tested whether ROS production was critical for the induction of RIP3 and MLKL expression. We confirmed that treatment of macrophages with oxLDL induced the production of ROS, which was inhibited by the ROS scavenger diphenyleneiodonium (DPI) (Fig. 3E and fig. S3A). Pretreatment of macrophages with DPI inhibited the induction of both RIP3 and MLKL mRNA expression by oxLDL and oxLDL + zVAD.fmk (Fig. 3F). To assess whether this increase in gene expression was due to direct activation of the promoter by oxLDL, we used a synthetic construct with the promoter region of either RIP3 or MLKL upstream of a luciferase reporter. In RAW macrophages transfected with the promoter constructs, oxLDL treatment significantly induced the activation of the RIP3 reporter after 6 and 24 hours and the MLKL reporter after 24 hours (Fig. 3, G and H). Inhibition of ROS by treatment with DPI significantly blunted the promoter activity of RIP3 and MLKL, indicating that ROS-dependent activation of the promoter regions of RIP3 and MLKL by oxLDL (Fig. 3, G and H). In addition to MLKL, PGAM5 can play a role in necroptosis by inducing mitochondrial damage (27). We also found that PGAM5 was induced at the mRNA and protein levels by oxLDL, and its expression was similarly inhibited by DPI (fig. S3, B and C). These results indicate that oxLDL directly induces the expression of RIP3 and MLKL at the mRNA and protein levels, likely as a result of activation of the promoter regions of these genes by oxLDL signaling through ROS. Collectively, these data provide mechanistic insight into the observation in humans that necroptosis could be activated because of direct activation of the necroptotic program by atherogenic ligands in the plaque.

**Atherosclerosis can be visualized using a Nec-1 radiotracer**

Given the evidence that the necroptotic pathway is activated in human plaques and its expression is driven by atherogenic stimuli, we next test whether the necroptotic pathway could be targeted using molecular imaging to visualize its occurrence in atherosclerotic lesions in vivo. We developed an 125I-labeled Nec-1 compound and tested its localization to aortic lesions in ApoE−/− mice. Radiolabeled Nec-1 (125I-Nec-1) was injected into Apoe−/− mice with established lesions, and 2 hours later, aortas were harvested and analyzed for radiotracer uptake. Autoradiography imaging revealed that 125I-Nec-1 was primarily localized to areas of plaque accumulation in the en face aortic arch (Fig. 4A). Pretreatment of a cold nonradioactive Nec-1 compound significantly blocked aortic lesional uptake of 125I-Nec-1 (Fig. 4B), indicating that radiolabeled Nec-1 uptake is specific. When compared to Oil Red O (ORO) uptake within the aorta, which stains lipid-rich lesion areas, there was a significant correlation between the lesion area measured by 125I-Nec-1 and ORO (Fig. 4C), suggesting that this was localized to macrophage foam cells in plaques. These data demonstrate that necroptosis within atherosclerotic lesions can be targeted using a molecular imaging probe based on the small-molecule inhibitor Nec-1 to visualize atherosclerotic lesion areas.

**Blocking necroptosis in established lesions reduces plaque size and markers of vulnerability**

Active necroptosis was detected in advanced rupture-prone lesions in human subjects. Therefore, we next set out to test whether intervention using a small-molecule inhibitor of necroptosis could be used as a therapeutic strategy in mice with established atherosclerotic lesions (17). Necrostatin-1s (Nec-1s) was formulated into a time-release pellet and delivered to Apoe−/− mice, which had been fed a Western diet for 4 weeks to induce lesion development (Fig. 5A). After 6 weeks of treatment with either Nec-1 or placebo with Western diet feeding, quantification of en face lesion area in the ascending and ascending aorta revealed that Nec-1 treatment significantly reduced lesion burden by 68% compared to placebo-treated mice (9.9 ± 0.9% for placebo versus 3.1 ± 0.3% for Nec-1; P ≤ 0.0001; Fig. 5B). In the aortic root, lesion area was reduced by 27% compared to controls (0.50 ± 0.03 mm2 for placebo versus 0.37 ± 0.05 mm2 for Nec-1; P ≤ 0.05; Fig. 5C). Total plasma cholesterol and body weight were not affected by Nec-1 treatment (Table 1). These data demonstrate that intervention with an inhibitor of necroptosis can block the further progression of established lesions in mice with inflammatory atherosclerosis.

We next evaluated whether intervention with Nec-1 altered markers of lesion instability, namely, necrotic core area and SMC fibrous cap content. Evaluation of lesion characteristics demonstrated that Nec-1 reduced lesion necrotic core area by 62% compared to placebo (P ≤ 0.01; Fig. 5D) and increased smooth muscle actin area by 70% (P ≤ 0.05; Fig. 5E). Quantification of macrophage content within the lesions demonstrated that there was a slight but nonsignificant increase in CD68+ macrophage area with Nec-1 treatment, likely reflecting to the overall reduction in lesion size in the Nec-1–treated animals (34.4 ± 3.2% versus 43.5 ± 3.7% of lesion area, P = 0.18; Fig. 5F). We next assessed whether necrotic cell death was reduced in lesions from Nec-1–treated mice. We measured the presence of pMLKL as a measure of necrotic cell death and found that pMLKL expression was similarly inhibited in lesions from Nec-1–treated mice.
Fig. 3. OxLDL induces the expression of RIP3 and MLKL. (A and B) BMDMs treated with oxLDL (100 μg/ml) ± zVAD.fm for 3, 6, 12, or 24 hours were analyzed for gene expression of RIP3 (A) and MLKL (B) by qPCR (quantitative real-time polymerase chain reaction) and compared to control-treated cells. (C and D) Western blot analysis of RIP3 (C) and MLKL (D) expression in BMDMs treated with oxLDL. Quantification below of at least three independent experiments. (E) BMDMs were preincubated with 50 μM DPI and then treated with oxLDL or oxLDL + zVAD.fm for 3 hours before measuring ROS levels. The graph shows mean ± SD of technical triplicates and is representative of at least three experiments. Statistical analysis was performed using two-way ANOVA. **P < 0.01, ***P < 0.001. (F) RIP3 or MLKL mRNA expression measured by qPCR in BMDMs treated with oxLDL ± zVAD.fm, in the presence or absence of pretreatment with 50 μM DPI. (G and H) RAW macrophages were transfected with RIP3-promoter (G) or MLKL-promoter (H) luciferase constructs and treated for 6 or 24 hours with oxLDL in the presence or absence of pretreatment with 50 μM DPI before luciferase expression was examined and expressed as promoter-luciferase activation normalized to control (no treatment). *P ≤ 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 by one-way ANOVA.
death in situ (21) and found that Nec-1 treatment reduced the pMLKL-positive area by 63% compared to placebo controls ($P \leq 0.01$; Fig. 5G). Because RIP3 can also play a role in inflammasome activation and atherosclerosis progression (3, 4), we measured the levels of serum IL-1β as a readout of inflammasome activation in these mice and found that the serum levels of IL-1β were not different between the placebo- and Nec-1-treated groups ($775 \pm 70$ pg/ml versus $658 \pm 84$ pg/ml, $P = \text{ns}$; Fig. 5H). These results demonstrate that in mice with established atherosclerotic lesions, intervention with a pharmacological inhibitor of necroptosis can prevent further lesion progression and reduce markers of plaque instability (that is, necrotic core and necroptotic cell death), suggesting that necroptosis underlies both the development

Fig. 4. Radiolabeled Nec-1 can be used to visualize atherosclerotic plaques in Apoe$^{-/-}$ mice. (A) Left: Chemical structure of 7-$^{123}$I-Nec-1 tracer. Right: Representative images of aortic en face (no stain and ORO stain) and autoradiography from mice injected with $^{123}$I-labeled Nec-1 tracer. (B) Mice were injected with nonradioactive CI–Nec-1 compound 1.5 hours before being injected with radiolabeled $^{123}$I-Nec-1 tracer and subjected to autoradiography (images shown) and lesion uptake quantification ($n = 3$ mice per group). (C) Correlation of lesional uptake of ORO compared to $^{123}$I-Nec-1 ($n = 9$).
Fig. 5. Nec-1 therapy decreases atherosclerotic lesion progression and markers of instability in Apoe<sup>−/−</sup> mice. (A) Apoe<sup>−/−</sup> mice were fed a Western diet for 6 weeks before implantation of time-release pellets containing placebo or Nec-1s (2 mg/kg per day). After four additional weeks of Western diet feeding, the mice were harvested for morphometric analysis of atherosclerosis. (B) En face lesion area was measured in placebo- and Nec-1s–treated mice and is represented as lesion area as a percentage of total aorta area. (C) Lesion area in the aortic sinus in placebo- and Nec-1s–treated mice is represented as total area in μm<sup>2</sup>. (D) Necrotic core area within aortic sinus lesions. (E to G) Immunohistochemical staining of smooth muscle-α actin (SMC marker) (E), CD68 (macrophage marker) (F), and pMLKL (G) was performed on aortic sinus lesions and quantified with ImageJ. Representative images per group are shown. AU, arbitrary units. (H) Serum IL-1β in mice from placebo- or Nec-1–treated groups was measured at sacrifice by enzyme-linked immunosorbent assay (ELISA). *P ≤ 0.05, **P < 0.01 by Student’s t test.
and vulnerability of atherosclerotic plaques and can be targeted to reduce these markers of lesion instability.

**DISCUSSION**

Here, we show that necroptosis, an emerging pathway underlying inflammatory cell death in many pathologies, is active within the necrotic core in humans with advanced atherosclerosis and can be targeted for therapeutic and diagnostic interventions in mice. This study is the first to demonstrate that the necroptotic pathway is increased in vascular disease in humans, where MLKL is phosphorylated in advanced necrotic lesions. We report that necroptosis can be targeted with long-term therapeutic inhibition in a mouse model of established atherosclerosis to reduce lesion size and, importantly, markers of plaque vulnerability. Moreover, using ex vivo molecular imaging, we show that necroptosis can be used as a diagnostic tool to visualize this process in atherosclerotic lesions. Mechanistically, we demonstrate that the sterile ligand oxLDL, an inflammatory form of LDL that is abundant within developing atheroma, is sufficient to induce necrotic cell death in macrophages via direct activation of the RIP3 and MLKL promoter. Although oxLDL is known to induce apoptosis (22, 23), this is the first report that oxLDL can directly induce necrotic cell death in the absence of synthetic caspase inhibition. Overall, our study offers compelling evidence that necroptosis contributes to atherosclerosis lesion vulnerability in humans and offers the first mechanistic insight into how atherogenic ligands are driving this form of inflammatory cell death.

Mechanisms that govern necrotic core formation in vulnerable atherosclerotic lesions are poorly understood. Throughout virtually all stages of atherosclerosis, cell death is active and contributes to plaque progression, from early foam lesion formation to the advancement of unstable rupture-prone plaques (23). In the early stages of lesion initiation, apoptosis induced by oxidized lipids in the subendothelial space may serve as a protective mechanism by promoting rapid clearance of apoptotic cells by phagocytes via efferocytosis. In later stages, it is believed that efferocytosis is impaired and apoptotic cell debris accumulates and contributes to lesion destabilization (8). However, in advanced human atherosclerotic lesions, cells with necrotic morphology are more abundant than with apoptotic morphology, suggesting that other forms of cell death besides apoptosis endure in the advanced plaque and promote lesion destabilization (18). Our data suggest that so long as oxLDL persists within the plaque, macrophages undergo necrotic cell death, releasing DAMPs into the extracellular space to serve as a feed-forward loop to exacerbating the extensive necrotic core found in advanced lesions (10). The atheroma milieu is rich in other sources of DAMPs, such as other oxidized lipids (28), inflammatory cytokines, heat shock proteins, and mitochondrial DNA (29), and our data agree with the concept that the accumulation of DAMPs induced by atherogenic ligands further exacerbates necrotic cell death. Our data demonstrating that necrototic macrophages do not efficiently undergo efferocytosis further suggest that defective efferocytosis in the plaque may in part be driven by activation of the necroptotic pathway, potentiating the accumulation of inflammatory and necrotic debris and expansion of the necrotic core.

Apoptic cell death is triggered when caspase-8 is active, resulting in the cleavage of RIP1 and RIP3 and inactivation of the necroptotic program (12, 30–32). The expression of RIP3 is tightly correlated with the degree of necrosis (33), and induction of RIP3 expression is sufficient to overcome caspase-8 and trigger necroptotic cell death (34, 35). We show that oxLDL induces the expression of necroptotic genes RIP3 and MLKL and, importantly, promotes the up-regulation and phosphorylation of RIP3 protein—a requirement for the assembly of the RIP1-RIP3 complex and subsequent necroptosis (36). In addition, these data are the first to show that atherogenic ligands can induce the phosphorylation of MLKL, and this is active within the atherosclerotic plaque. Our data offer mechanistic insight into why Rip3<sup>−/−</sup> mice are protected from the transcriptional up-regulation of necroptotic cell genes RIP3 and MLKL. OxLDL is known to induce many downstream signaling events (26), including the generation of ROS, which can subsequently activate transcription factors to induce redox-sensitive gene expression (37). Similar to NLRP3, whose mRNA induction by oxLDL is considered a necessary priming step for inflammasome activation and is dependent on ROS (4, 38), we show that oxLDL induces mRNA and protein expression of RIP3 and MLKL and is sufficient to activate necroptosis, which is blocked upon scavenging cellular ROS. We find that the induction of necroptosis by oxLDL is independent of inflammasome activation, because cells deficient in caspase-1 or treated with caspase-1 inhibitors undergo necrotic cell death in response to oxLDL to the same degree as wild-type or untreated cells. This evidence could explain why gene expression of RIP3 and MLKL is positively associated with unstable atherosclerotic lesions compared to both stable lesions and healthy arteries. An improved understanding of how atherogenic ligands induce the expression of RIP3 and MLKL could assist in the rationale design of future therapeutics that block this mechanism to reduce atherosclerosis lesion vulnerability.

Our data establish the potential for the therapeutic treatment of established atherosclerosis with inhibitors of the necroptotic pathway. Within the atherosclerotic plaque, RIP3 contributes to inflammasome activation, which could be inhibited by blocking RIP1-RIP3 activation with Nec-1. However, we did not observe differences in serum IL-1β levels in mice treated with Nec-1, suggesting that at doses used in the current study, Nec-1 treatment did not impair inflammasome activation and thus likely did not contribute to the reduction in lesion size observed in the Nec-1–treated group. It has been suggested that some of the activities of Nec-1 render the RIP1-dependent apoptotic pathway inactive (39); thus, it is possible that in vivo, Nec-1 is serving to inhibit both apoptotic and necrotic cell death, where both pathways may ultimately contribute to lesion instability at later stages of atherosclerosis. Nevertheless, we observed a reduction in pMLKL in Nec-1–treated mice, specifically

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**Table 1. Body weight and serum cholesterol measurements in ApoE<sup>−/−</sup> mice.** TC, total cholesterol; HDL, high-density lipoprotein.

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<th>Placebo</th>
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<td><strong>Body weight (g)</strong></td>
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<tr>
<td><strong>TC (mg/dl)</strong></td>
<td>1109 ± 219</td>
<td>1046 ± 166</td>
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<td><strong>LDL (mg/dl)</strong></td>
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<td><strong>HDL (mg/dl)</strong></td>
<td>27 ± 11</td>
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indicating that necroptotic death is reduced in mice treated with Nec-1. We did not observe necroptosis of SMCs in response to oxLDL, possibly owing to the fact that SMCs take up modified lipids through distinct mechanisms compared to macrophages and may not trigger the same signaling pathways in response (40). However, we noted a thicker SMC cap in mice treated with Nec-1, which could be as a result of fewer pro- 

teases being released from macrophages upon necroptosis inhibition (that is, because intracellular contents are not released into the sur-

rounding area), which could result in the preservation of extracellular matrix and fibrous cap integrity. This is the first report of Nec-1 being used in a long-term therapeutic application and opens the door for next-generation necroptosis inhibitors with improved specificity and stability for the treatment of chronic inflammatory diseases, such as atherosclerosis.

Current clinical practice relies on invasive angiography to visualize plaques in patients with atherosclerosis, providing limited detail of plaque size but no insight into plaque inflammation or vulnerability. Imaging by positron emission tomography (PET) or single-photon emission computed tomography provides the advantage of being non-invasive, with the ability of detecting specific molecular processes that provide insight into the pathology of the plaque when specific molecular tracers are used (41). 18F-fluorodeoxyglucose (FDG) is a widely used nuclear tracer to detect inflammation tissues, because macrophages take up FDG at a higher rate than noninflammatory cells, making FDG a surrogate of plaque inflammation (42). FDG-PET has supported the concept that molecular imaging of dominant pathways within the atherosclerotic plaque correlates with lesion vulnerability and thus may be of added value when assessing overall cardiovascular risk (43). However, there remains a need to develop novel radiotracers that can detect cor-

nary artery inflammation without the complication of myocardial uptake and with less sensitivity to metabolic characteristics of the patient (that is, high fasting blood glucose). We found that 125I-Nec-1 colocalizes to the atherosclerotic plaques in Apoe−/− mice by autoradiographic imaging and strongly correlated with that of traditional ORO uptake. Although the uptake of the Nec-1 radiotracer was only measured ex vivo in the current study, similar radiotracers can be developed and labeled with PET radionucleides and allow Nec-1 to be used in the future for non-invasive PET imaging in animal models, with the ultimate goal of detect-

ing atherosclerotic lesions in patients with disease.

In summary, this study provides evidence that necroptosis activation is associated with necrotic core formation in advanced human atheromas and supports the therapeutic targeting of necroptosis to re-

ducing lesion progression and plaque vulnerability. Our study provides excitement for the targeting of necroptosis as a diagnostic tool using either nuclear imaging or/and biomarker expression to better identify patients at the highest risk for lesion rupture. Our findings offer mechanistic insight into how atherogenic ligands drive necrotic core develop-

ment to better devise therapies that specifically inactivate the necrotic pathway to directly treat the underlying causes of clinically vulnerable atherosclerosis.

MATERIALS AND METHODS

Reagents

Hi-TBAR (thiobarbituric acid reactive)–oxidized human LDL (BT-910X) and human LDL (BT-903) were purchased from Biomedical Technologies Inc. Macrophage colony-stimulating factor (M-CSF) and mouse IL-1β DuoSet ELISA kits were purchased from R&D Systems. zVAD.fmk and zVAD.fmk were purchased from BioVision Inc. and ApexBio, respectively. Nec-1 and DIP were obtained from Sigma-Aldrich. Cellular Reactive Oxygen Species Detection Kit was purchased from Abcam.

Human atherosclerotic lesion analysis

For gene expression studies, human arterial samples were obtained from BiKE at the Centre for Molecular Medicine, Karolinska Institute, as previously described (19, 20). In brief, control normal arteries (undi-

seased macroscopically atherosclerosis-free arteries, iliac, and one aorta) were obtained from organ donors without any current or history of cardiovascular disease. Atherosclerotic plaques were obtained from pa-

tients undergoing surgery for stable or unstable carotid stenosis. All samples were collected with informed consent, and the study was ap-

proved by the Ethical Committee of Northern Stockholm and met all international standards. Plaque tissues were ruptured with Tissue Rupturer/Homogenizer (Omni Inc.) and RNA-isolated. Gene ex-

pression profiles were obtained from n = 10 control arteries and n = 127 atherosclerotic plaque samples, and statistical analysis was per-

formed using Student’s t test with correction for multiple comparisons (P < 0.05). For details on patient characteristics, please see the study by Razuvaev et al. (19) and Perisic et al. (20). For immunohistochemical analysis, human coronary artery samples were obtained from the CVPath Institute Sudden Cardiac Death registry, as previously de-

scribed (44). Sudden death is defined as symptoms commencing within 6 hours of death (witnessed arrest) or death occurring within 24 hours after the victim was last seen alive in his or her normal state of health. Comprehensive analysis of coronary artery histology was performed for each subject. Formalin-fixed paraffin-embedded coronary artery blocks were further cross-sectioned at 5-μm thickness for analysis. After careful examination of hematoxylin and eosin and Movat pentachrome-stained sections, advanced fibroatheromas and control lesions with pathologic intimal thickening were selected. Hematoxylin and eosin images were obtained using Axios Scan.Z1 (Carl Zeiss). Immunohisto-

chemistry for pMLKL was performed using a primary antibody against human pMLKL (rabbit monoclonal antibody, Abcam) with a secondary antibody conjugated to biotin and visualized using diaminobenzidine (DAB) (Sigma). Quantification of pMLKL-positive areas was performed using ImageJ, and statistical significance between the two groups was analyzed using unpaired Student’s t test (P < 0.05).

Mice

C57BL6 wild-type and Apoe−/− and Casp1−/− mice were purchased from Jackson Laboratories. Rip3−/− mice were obtained from Genentech. All animal studies were approved by the University of Ottawa Animal Care and Use Committee in accordance with the international standards set forth by the Canadian Council on Animal Care.

Bone marrow–derived macrophages

BMDMs were isolated from femurs of adult wild-type or Rip3−/− mice and differentiated into macrophages using Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin plus either 20% L929 conditioned medium or mouse M-CSF (20 ng/ml) for 7 to 10 days. DAMPs were obtained by subjecting BMDMs to 3× 30-min freeze-thaw cycle or treatment with LPS (100 ng/ml) and 50 μM zVAD.fmk for 24 hours, after which the medium was collected and added to naive cells at indicated ratios.
Cell viability assays

Cell death was determined by measuring LDH release into the medium as previously described (45). Briefly, cells were treated with oxLDL (100 μg/ml) in the presence or absence of 50 μM MzVAD.fmk or 50 μM Nec-1 for 24 hours, and the medium was collected and centrifuged to pellet cell debris. The amount of LDH in the medium was measured in a kinetic assay by adding PBS containing 0.02% NADH (reduced form of NAD+) and 0.03% sodium pyruvate and measuring absorbance at 340 nm for 10 min at 1-min intervals. The slope of the curve provides a measure of cell death, which was expressed as fold change relative to control.

RNA isolation and qPCR

BMDMs were treated with control medium with or without oxLDL or oxLDL + zVAD.fmk for the indicated time points with or without 1-hour pretreatment with 50 μM DPI. Alternatively, BMDMs were polarized to M0, M1, or M2 before RNA isolation, as described above. Total RNA was isolated using TRizol reagent (Invitrogen) as per manufacturer’s instructions, and complementary DNA was synthesized using an iScript Reverse Transcription kit (Bio-Rad). qPCR was performed in triplicate using either SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) or TaqMan Gene Expression Assay, and the mRNA level of target genes was normalized to hypoxanthine-guanine phosphoribosyltransferase or β-actin housekeeping genes.

Western blot analysis

For Western blot analysis of RIP3, cells were lysed in 1.25× sample buffer [83 mM tris (pH 6.8), 6.7% SDS, 13.3% glycerol, 1.3% β-mercaptoethanol, and 0.03% bromophenol blue] and boiled at 100°C for 5 min before being subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis, as previously described (46). PVDF (polyvinylidene difluoride) membranes were blocked with 5% skim milk, followed by incubation with RIP3 (ProSci Inc., 1:500) or HSP90 (Santa Cruz Biotechnology, 1:1000) antibodies. For analysis of MLKL protein, cells were lysed in ice-cold M2 lysis buffer [50 mM NaF, 20 mM tris (pH 7.0), 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, and 3 mM EGTA, supplemented with Roche protease and phosphatase inhibitor cocktails] for 30 min before centrifugation to pellet insoluble component as described (47). Samples were mixed 3:1 with 4× sample loading buffer (Bio-Rad) before SDS-PAGE and Western blot analysis. PVDF membranes were blocked in 5% bovine serum albumin and probed with MLKL (Millipore, 1:500) or GAPDH (glyceraldehyde phosphate dehydrogenase) (Millipore, 1:1000) antibodies. Goat anti-mouse (1:2500), anti-rabbit (1:5000), or anti-rat (1:2500) IRDye secondary antibodies (Rockland) were used. The protein bands were visualized using Odyssey Infrared Imaging System (LI-COR Biotechnology).

Electron microscopy

Cultured cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.5) at 4°C. The fixed cells were washed in 0.1 M cacodylate buffer, postfixed in 1% OsO4, and rinsed with 0.1 M cacodylate buffer and distilled water. The fixed cells were lifted with 50 mM EDTA/Hanks’ balanced salt solution and collected by centrifugation. Cell sediments were washed in 1× PBS, dehydrated in an ethanol series, and embedded in Spurr resin. Resin blocks were sectioned by the ultramicrotome Leica EM UC6 using a diamond knife. Ultrathin sections were mounted on copper grids coated with formvar film. The sections were stained with 2% alcoholic uranyl acetate and Reynolds’ lead citrate. The stained sections were examined with a transmission electron microscope, JEOL 1230.

Efferocytosis assay

Efferocytosis assays were performed as previously described (48, 49). BMDMs were labeled with Vybrant Dye (5 μM, Invitrogen) for 25 min at 37°C, and unbound dye was removed with PBS washing. Labeled BMDMs were then treated for 24 hours with medium alone (control) or medium containing 5 μM staurosporine (to induce apoptosis), oxLDL (150 μg/ml + 100 μM zVAD), or LPS (200 ng/ml) + 100 μM zVAD (both to induce necroptosis). Dead cells were collected, counted, resuspended in control medium, and applied to naïve BMDMs at a ratio of 3:1. After 2 hours, cells were washed six times with cold PBS, and fluorescence intensity was quantified using a plate reader. Experiments were performed in quadruplicate technical replicates at least three independent times.

Measurement of ROS

BMDMs were preincubated with DPI for 30 min, followed by the cell permeant reagent 2′,7′-dichlorofluorescin diacetate fluorescent dye for 1 hour. Cells were then treated with oxLDL or oxLDL + zVAD.fmk, and fluorescence was measured at 3 and 24 hours as per manufacturer’s instructions (Abcam, ab113851).

Promoter luciferase assay

Luciferase reporter constructs containing the human MLKL and RIP3 promoters were purchased from SwitchGear Genomics. RAW264.7 macrophages (American Type Culture Collection) were plated in 24-well plates in 10% FBS DMEM and transfected using Lipofectamine 2000 (Life Technologies) with MLKL-promoter or RIP3-promoter plasmids (Renilla-based luciferase reporter) together with pGL3 basic (firefly-based reporter, Promega) to normalize for transfection efficiency. Twenty-four hours after transfection, the cells were treated with or without oxLDL (100 μg/ml) as above, for an additional 24 hours. Cells were harvested in 1× passive lysis buffer and luciferase measured using the Dual-Luciferase assay (Promega) according to the manufacturer’s instructions. All experiments were performed in quadruplicate technical replicates at least three times.

Atherosclerosis studies

All animal experiments were performed in accordance with the Animal Care and Use Committee, University of Ottawa, Canada. Eight-week-old apoE–/– mice (total n = 17 mice) were fed an adjusted-calories diet (21% fat, 0.2% cholesterol; Harlan Teklad) for 4 weeks to induce atherosclerotic progression. Mice were then injected with time-release placebo (n = 11) or stable Nec-1 (n = 8) (15) tablets (2 mg/kg per day), which were synthesized by Innovative Research of America. The mice were euthanized after 6 weeks of treatment and perfused with saline, and aortic roots were embedded in optimum cutting temperature compound medium and frozen. The aortic roots were sectioned (10 μm) and stained with hematoxylin and eosin for lesion area and necrotic core quantification, and a minimum of 10 sections per animal were measured across the length of the entire aortic root. For en face aortic lesion quantifications, aortas were dissected removing all branching vessels down to the femoral bifurcation and then sliced ventrally, and images of the aortas were digitally captured using Image-Pro. Lesion areas within the entire length of the aorta were quantified using ImageJ and expressed relative to the total aortic surface area. Immunohistochemistry to visualizing macrophages and SMCs was performed using primary
antibodies to CD68 (rat monoclonal antibody, Serotec) or phosho-MKL1 (rabbit polyclonal antibody, Abcam) with a secondary antibody conjugated to biotin and α-smooth muscle actin (monoclonal antibody, Sigma) conjugated to alkaline phosphatase and visualized using DAB or SIGMAFAST Red (Sigma), respectively. ImageJ was used to quantify the positive staining area, and the statistical significance between the two groups was analyzed using Student's t test (P < 0.05).

**Radiochemistry and ex vivo autoradiography study with ApoE−/− mice**

The tracer 123I-Nec-1 (7-123I-Nec-1) was synthesized by a Cu(I)-catalyzed direct halogen exchange reaction with the precursor 7-Br-Nec-1 and Na123I. Eight-week-old female apolipoprotein E knockout (ApoE−/−) mice were purchased from Charles River Laboratories and fed either a chow or Western diet (TD.10885, Harlan Laboratories) for 2 months (total n = 6 mice). 7-123I-O-Nec-1 (37.0 to 55.5 MBq) was administrated intravenously into the mice under anesthesia. After 2 hours, the mice were euthanized and perfused with PBS, followed by 10% formalin via left ventricle cannulation. Aorta was dissected from the heart at the base using a dissecting microscope following the removal of surrounding fat and connective tissue. En face specimens were immediately exposed to superresolution phosphor screens in an autoradiography cassette, as previously described (50). After overnight exposure at room temperature, the screens were scanned with Cyclone Phosphor Imager (PerkinElmer). Images were analyzed using OptiQuant 5.0 software. The regions of interest were drawn around the lesions on the aortic arch, and the counts in digital light unit (DLU) and surface areas in mm² were measured. The DLU was converted to activity in μCi using a set of calibration standards with known activities, which were exposed and scanned on the same screen used for the aorta samples, as previously described (50, 51). The percentage injected dose (%) ID was calculated from dividing the activity (μCi) of the lesion by the injected activity. Activity density in % ID/m² was calculated and normalized by animal body weight to get % ID × kg/m². The areas of the lesions and the whole aortic arch in mm² were also recorded using the OptiQuant 5.0 software, and the percentage of lesion within the area of the aorta vessel wall was calculated.

**Statistics**

Data shown are either means ± SD of a single representative experiment or means ± SEM of at least three independent experiments performed in triplicate and are indicated in the corresponding figure legends. Comparison between control and treatment was made using Student's t test (P ≤ 0.05) or comparison between groups by one-way ANOVA (P ≤ 0.05) or two-way ANOVA (P ≤ 0.05) using GraphPad Prism.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/2/7/e1600224/DC1

fig. S1. Percentage of macrophage cell death with oxLDL treatment and Nec-1.

fig. S2. Cell death in SMCs and macrophages treated with DAMPs.

fig. S3. Analysis of ROS production and PGAM expression in macrophages.

**REFERENCES AND NOTES**


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Targeting macrophage necroptosis for therapeutic and diagnostic interventions in atherosclerosis

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