**BASIC SCIENCES**

Resolvin D1 Enhances Necroptotic Cell Clearance Through Promoting Macrophage Fatty Acid Oxidation and Oxidative Phosphorylation

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**OBJECTIVE:** Plaque necrosis is a key feature of defective resolution in atherosclerosis. Recent evidence suggests that necroptosis promotes plaque necrosis; therefore, we sought to determine how necroptotic cells (NCs) impact resolution programs in plaques.

**APPROACH AND RESULTS:** To investigate the role(s) of necroptosis in advanced atherosclerosis, we used mice deficient of \textit{Mlkl}, an effector of necroptosis. \textit{Mlkl}^−/− mice that were injected with a gain-of-function mutant PCSK9 (AAV8-gof-PCSK9) and fed a Western diet for 16 weeks, showed significantly less plaque necrosis, increased fibrous caps and improved efferocytosis compared with AAV8-gof-PCSK9 injected wt controls. Additionally, hypercholesterolemic \textit{Mlkl}^−/− mice had a significant increase in proresolving mediators including resolvin D1 (RvD1) and a decrease in prostanoids including thromboxane in plaques and in vitro. We found that exuberant thromboxane released by NCs impaired the clearance of both apoptotic cells and NCs through disruption of oxidative phosphorylation in macrophages. Moreover, we found that NCs did not readily synthesize RvD1 and that exogenous administration of RvD1 to macrophages rescued NC-induced defective efferocytosis. RvD1 also enhanced the uptake of NCs via the activation of p-AMPK (AMP-activated protein kinase), increased fatty acid oxidation, and enhanced oxidative phosphorylation in macrophages.

**CONCLUSIONS:** These results suggest that NCs derange resolution by limiting key SPMs and impairing the efferocytic repertoire of macrophages. Moreover, these findings provide a molecular mechanism for RvD1 in directing proresolving metabolic programs in macrophages and further suggests RvD1 as a potential therapeutic strategy to limit NCs in tissues.

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

**Key Words:** atherosclerosis ▼ macrophage ▼ necroptosis ▼ resolvin ▼ thromboxane

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The resolution of inflammation is a protective process and is controlled by the balance between specialized proresolving mediators (SPMs) such as resolvins and proinflammatory lipid mediators (LMs) like leukotrienes and prostaglandins. In general, SPMs are protective in vivo, temper inflammation and promote tissue repair and regeneration in a manner that does not compromise host defense. A key cellular program of resolution is efferocytosis or the clearance of dead cells. Defective resolution underpins several diseases, including atherosclerosis and key features of impaired resolution in atherosclerosis are large necrotic cores, thin fibrous caps, defective synthesis of SPMs, and overproduction of proinflammatory LMs. Moreover, recent studies suggested that the SPM:proinflammatory LM ratios are associated with highly necrotic regions of human and murine plaques in advanced atherosclerosis, suggesting that plaque necrosis may be an important factor that impairs

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The Data Supplement is available with this article at https://www.ahajournals.org/doi/suppl/10.1161/ATVBAHA.120.315758.

For Sources of Funding and Disclosures, see page 1074.

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Arterioscler Thromb Vase Biol is available at www.ahajournals.org/journal/atvb
resolution. Indeed, major gaps remain in our understanding of how the drivers of plaque necrosis impinge on resolution programs.

In this regard, necroptosis is a form of necrotic cell death that has been shown to promote plaque necrosis.\(^5\)\(^-\)\(^7\) Mechanisms associated with how necroptotic cells (NCs) impact plaque necrosis are underexplored. Necroptosis is driven by specific signaling pathways that include the activation of RIP3 (receptor-interacting protein kinase 3) and MLKL (mixed lineage kinase domain) and unlike apoptosis is a highly proinflammatory mode of cell death.\(^8\) Moreover, while there is a plethora of knowledge regarding proinflammatory cytokines and chemokines that are released by NCs,\(^9\) there is little known about released LMs and how these factors impact resolution. Additionally, we previously demonstrated that NCs have high levels of CD47 and that removal of CD47\(^+\) cells reduced necrosis and promoted SPM synthesis in plaques.\(^10\) Direct causation that NCs impact SPM synthesis and efferocytosis in plaques is currently lacking.

Lastly, because several diseases (like atherosclerosis) are associated with an accumulation of NCs, understanding mechanisms associated with the clearance of NCs by macrophages is critically important. Recent evidence suggests that NC clearance is distinct from apoptotic cell removal by macrophages.\(^6\)\(^,\)\(^10\)\(^,\)\(^11\) Efferocytosis of apoptotic cells is achieved through modulating metabolic features of macrophages such as increasing fatty acid oxidation (FAO) and oxidative phosphorylation (OXPHOS).\(^12\) How NC clearance impacts these metabolic programs is not known. Moreover, how SPMs impact macrophage metabolism to promote efficient NC clearance is also not known.

Here, we provide causative evidence that the accumulation of NCs impairs resolution programs as well as mechanistic evidence as to how SPMs themselves can lead to efficient NC clearance. Briefly, we found that Mlkl\(^/-\) mice injected with AAV8 gof PCSK9 (adeno-associated viral vector serotype 8 expressing the gain-of-function mutation of mouse proprotein convertase subtilisin/kexin type 9) had significantly less plaque necrosis, increased fibrous caps, and improved efferocytosis compared with controls. Additionally, Mlkl\(^/-\) mice had a significant increase in SPMs including resolvin D1 (RvD1) and a decrease in prostanoids such as PGE\(_2\), PGD\(_2\), PGF\(_{2\alpha}\), and thromboxane. We found that NCs released a prostanoid storm in vitro that caused an impairment in efferocytosis of apoptotic cells and NCs through modulating OXPHOS in the ingesting macrophages. Treatment of macrophages with RvD1 rescued defective efferocytosis of apoptotic cells and NCs through an increase in p-AMPK (AMP-activated protein kinase) signaling, FAO, and OXPHOS. Together, these results suggest new mechanisms associated with clearance of NCs and offer a potential therapeutic strategy for the treatment of atherosclerosis.

### METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

### Murine Atherosclerosis

Mlkl\(^/-\) male and female mice on a C57BL/6J background were generously provided by Dr James Murphy at the Walter and Eliza Hall Institute of Medical Research\(^13\) and were housed at the Albany Medical College animal facility. We expanded our colony through simple breeding measures in which we crossed Mlkl\(^/-\) mice with C57BL/6J mice. We then crossed the heterozygotes to obtain homozygous Mlkl\(^/-\) mice or wt littermates for our studies. Wild-type (Wt) and Mlkl\(^/-\) mice were socially

| Nonstandard Abbreviations and Acronyms |
|---------------------------------------|
| ACC | acetyl-coa carboxylase |
| AMPK | AMP-activated protein kinase |
| BMDMs | bone marrow-derived macrophages |
| CM | conditioned media |
| FAO | fatty acid oxidation |
| FP | prostaglandin F\(_2\)\(_a\) receptor |
| LM | lipid mediator |
| MLKL | mixed lineage kinase domain |
| NC | necroptotic cell |
| Nec-1 | necrostatin 1 |
| OCR | oxygen consumption rate |
| OXPHOS | oxidative phosphorylation |
| OZ | oxidized LDL+ZVAD-FMK (general caspase inhibitor) |
| RvD1 | resolvin D1 |
| SPM | specialized proresolving mediator |
| TP | thromboxane receptor |
| Wt | wild type |

**Highlights**

- Mlkl\(^/-\) mice had significantly less plaque necrosis, increased fibrous caps, and improved efferocytosis.
- Mlkl\(^/-\) mice had an increase in specialized proresolving mediators and decrease in prostanoids.
- Exuberant prostanoids (eg, thromboxane) released from necroptotic cells impaired efficient clearance of both apoptotic and necroptotic cells.
- The specialized proresolving mediator resolvin D1 enhanced a proresolving phenotype by increasing p-AMPK (AMP-activated protein kinase) signaling, fatty acid oxidation, and oxidative phosphorylation in ingesting macrophages.
housed in standard cages at 22°C under a 12-hour light and 12-hour dark cycle. Wt and Mlk−/− mice (10–12 week old) were IV injected with 1x10^{12} gc/mL per mouse of gain-of-function mutant PCSK9.AAV8.TBG.Pim.PCSK9.D377Y;BG (or AAV8-gof-PCS9; Penn Vector Core) and fed the Western diet for 16 weeks to promote atherosclerotic plaques. Cholesterol levels were measured 2 weeks post injection and again at the time of euthanization. All procedures were performed according to the animal protocols approved by the Albany Medical College Institutional Animal Care and Use Committee.

Murine Atherosclerotic Lesion Analysis
Aortic roots from wt and Mlk−/− mice were harvested for histological analysis, and the aorta and BCA were subjected to liquid chromatography-tandem mass spectrometry (below). Lesion and necrotic area analysis were performed on hematoxylin and eosin-stained lesional cross-sections and were quantified using an Olympus camera and Olympus DP2-BSW software as previously described. Briefly, frozen specimens were immersed in OCT, cryosectioned, and 8 μm sections were placed on glass slides. Atherosclerotic lesion area, defined as the region from the internal elastic lamina to the lumen, was quantified by taking the average of 6 sections spaced =24 μm apart beginning at the base of the aortic root. To quantify fibrous cap thickness, lesional sections were stained for collagen using picrosirius red (Polysciences, Catalog No. 24901) as per manufacturer’s instructions and analyzed as previously described. Four sections per mouse were analyzed, and all images were taken with an Olympus microscope as above. In situ effectorcytosis was performed as previously described[14] and further details can be found in the Data Supplement.

Identification and Quantification of LMs by Liquid Chromatography-Tandem Mass Spectrometry
Aortas from wt and Mlk−/− mice were collected, immediately placed in 1 mL of ice-cold methanol and stored at ~80°C. Samples were then subjected to targeted metabololipidomics profiling via liquid chromatography-tandem mass spectrometry as described previously. Further details can be found in the Data Supplement.

NC Conditioned Media
Murine bone marrow-derived macrophages (BMDMs; 2x10^6 cells/well) were stimulated in a 6-well plate with 40 μmol/L Z-VAD-FMK (ApexBio, Catalog No. 1902) and 50 μg/mL of aggregated human oxidized LDL (low-density lipoprotein; Kalen BioMed, Catalog No. 770252-6) (OZ), or Nec-1 (necrostatin 1; 20 μmol/L) or OZ+Nec-1 in serum free DMEM. After 24 hours (37°C, 5% CO2) media was removed and replaced with fresh serum free DMEM for an additional 48 hours (37°C, 5% CO2). The media was then collected and centrifuged at room temperature for 5 minutes at 5000 rpm, and supernatant was used for functional assays. NC conditioned media (CM) was assayed for PGE_{2}, TXB_{2}, and RvD1 by ELISA (Cayman Chemical).

Clearance of NCs
To obtain NCs, murine BMDMs from C57BL/6J male mice were stimulated with OZ to obtain NCs as previously described. BMDMs were labeled with PKH67 (Sigma, Catalog No. PKH67GL-1KT) before O2 stimulation.

In parallel, murine BMDMs (1x10^6 cells/well) were labeled with PKH26 and plated in 8-well chamber slides in DMEM containing 10% FBS. The following day cells were treated with vehicle or 10 nmol/L RvD1 (Cayman Chemical, Catalog No. 10012554) for 20 minutes (37°C, 5% CO2). PKH67-labeled NCs were then added in a 1:2 ratio (BMDM:NC) for an additional 1 hour (37°C, 5% CO2). Nonengulfed dead cells were removed, and the BMDMs were then fixed with 4% PFA for 15 minutes before image acquisition and analysis. For some experiments, 1 μmol/L FCCP (carbonyl cyanide 4-[trifluoromethoxy] phenylhydrazone), 150 nmol/L SQ29548, 10 nmol/L RvD1, or a combination of FCCP and RvD1 or SQ29548 and RvD1 were incubated for 20 minutes (37°C, 5% CO2), and experiments were conducted as above.

In some experiments, BMDMs were incubated with vehicle, 3 μmol/L etomoxir (Sigma, Catalog No. E1905), 10 nmol/L RvD1, or 3 μmol/L etomoxir with 10 nmol/L RvD1 for 20 minutes (37°C, 5% CO2) after which green NCs were then added as above. In other experiments, 500 μmol/L AICAR (5-aminoimidazole-4-carboxamide ribonucleotide; Sigma, Catalog No. A9978) or 500 nmol/L compound C (Sigma, Catalog No. 171260) were incubated with BMDMs for 4 hours (37°C, 5% CO2), after which green NCs were then added as above. For all experiments, images were acquired on a Leica SPE confocal microscope at 40x magnification and analyzed with Image J software.

Seahorse Real-Time ATP Assay
BMDMs were seeded on a Seahorse XF96 cell culture plate (Agilent, No. 102601-100) overnight in DMEM containing 10% FBS. The following day BMDMs were treated with vehicle or 1 nmol/L RvD1 20 minutes before addition of NCs at a ratio of 1:2 as above for 2 hours (37°C, 5% CO2). To remove non-ingested dead cells, BMDMs were washed with the Seahorse XF DMEM media pH=7.4 (Agilent, No. 103575-100) supplemented with 1 mmol/L pyruvate (Sigma, Catalog No. S8638), 10 mmol/L glucose (Sigma, Catalog No. G8769), 2 mmol/L glutamine (Sigma, Catalog No. G8540), and then incubated at 37°C without CO2 for 1 hour. For measuring mitochondrial ATP production in live cells, oxygen consumption rate (OCR) was measured using the Seahorse XFe Real-Time ATP Rate assay kit (Agilent, No. 103592-100) and the Seahorse XF96 Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, CA). After 3 basal OCR measurements, the mitochondrial inhibitors oligomycin (1.5 μmol/L) and rotenone/antimycin A (0.5 μmol/L) were serially injected after every three measurements. The Mito ATP production rate was calculated according to equations described in the Agilent Seahorse XF Real-Time ATP Rate Assay user guide.

Immunofluorescence In Vitro
BMDMs (1x10^6 cells/well) were plated in 8-well chamber slides as above and were stimulated with vehicle or 10 nmol/L RvD1 for 1 hour (37°C, 5% CO2) after which media was removed and cells were immediately fixed with 4% PFA as above. Cells were permeabilized using perm wash buffer (BD Biosciences) for 20 minutes at room temperature. Cells were blocked in 2% BSA in PBS-T containing 10% goat serum for 30 minutes. Primary
antibodies for p-AMPK (Cell Signaling, Catalog No. 2535) or p-ACC (acetyl-coa carboxylase; Cell Signaling, Catalog No. 11818S) were prepared in 2% BSA in PBS-T at 1:100 and incubated with cells for 1 hour at room temperature. Primary antibodies were removed by washing (=2×). Alexa 647 anti-rabbit at 1:200 (2% BSA in PBS-T containing 5% goat serum) was then added for 1 hour at room temperature. Cells were counterstained with Hoechst for 10 minutes at room temperature and fixed again with 4% PFA for 10 minutes. Images were acquired on a Leica SPE confocal microscope at 63× magnification and analyzed with Image J software.

**Statistical Analysis**

Results are indicated as mean±SEM, and the significance of data was determined using Student 2-tailed t tests and 1-way ANOVA with Tukey multiple comparisons, or 2-way ANOVA with Tukey or Sidak multiple comparisons where appropriate. Prism (GraphPad Inc, La Jolla, CA) software was used for analysis, and \( P<0.05 \) was considered to be statistically significant. Details of the statistical analysis are present in the figure legends. The data from the hypercholesterolemic wt versus Mlkl−/− experiments were analyzed for normality using the Anderson-Darling test.

**RESULTS**

**Advanced Plaques From Mlkl−/− Mice Have Features of Improved Resolution**

Because MLKL is currently the most direct measurement of necroptosis, we questioned whether knockout of MLKL in mice would decrease necrosis, improve remodeling and enhance effecytosis in advanced atherosclerotic plaques. We injected wt and Mlkl−/− male mice with an AAV8-gof-PCSK9 that were immediately fed a Western diet for 16 weeks. Mice were then euthanized, and aortic roots were harvested for analysis. There was no significant difference in body weight, complete blood counts, and cholesterol levels between wt and Mlkl−/− mice (Table I in the Data Supplement). Representative hematoxylin and eosin images and quantification reveal significantly less necrosis in male Mlkl−/− mice compared with the wt controls (Figure 1A). However, no change was observed in the lesion area (Figure IA in the Data Supplement), which was consistent with the literature.9 Similar findings were observed in female mice (Figure II in the Data Supplement). One of the key features of advanced atherosclerotic plaques is the presence of a thin fibrous cap,10 and we recently found that improved fibrous caps thickness was associated with a resolving plaque.3 Indeed, the lesions of Mlkl−/− mice exhibited significantly thicker caps compared with wt mice (Figure 1B). Representative images are shown in Figure 1B and demonstrate increased red staining, which is indicative of more collagen. Lastly, another phenotypic feature of a resolving plaque is effecytosis. We stained aortic root lesions with F4/80 to label macrophages and TUNEL for apoptotic cells and found that Mlkl−/− mice had significantly more associated-to-free apoptotic cells, which suggests an improvement in effecytosis (Figure 1C). Total macrophage numbers were not significantly different between the groups (Figure IIIA in the Data Supplement). TUNEL+ cells were modestly decreased in the Mlkl−/− plaques but did not reach statistical significance (Figure IIB in the Data Supplement). Representative images are shown in Figure 1C and depict intraplaque TUNEL staining (red spots) and F4/80 (green) macrophages. Recent work suggests that Arg-1 (arginase 1) is a marker for efficient and continual effecytosis,19 and we found that Mlkl−/− plaques had a higher percentage of Arg-1+/F4/80+ cells compared with wt controls (Figure IB in the Data Supplement). Together, these results suggest that Mlkl−/− mice have an improved resolution phenotype because they have smaller necrotic cores, increased remodeling, and improved effecytosis during advanced atherosclerosis.

**Necroptosis Decreased the SPM:Prostanoid Balance in Atherosclerotic Plaques**

Imbalances between SPMs and proinflammatory mediators are associated with plaque necrosis in human atherosclerotic plaques and murine lesions.3,4 Because Mlkl−/− mice had decreased necrosis and other phenotypic features of resolution, we next questioned whether Mlkl−/− advanced plaques also had increased SPMs and an improved SPM:prostanoid LM ratio. Interaction network pathway analysis of docosahexaenoic acid and arachidonic acid LM metabolomes demonstrated that plaques from Mlkl−/− mice had increased SPMs and decreased prostanoids (Figure 2A and 2B, the Table). Specifically, 17-HDHA (ie, a biosynthetic pathway marker for RvD1) and RvD1 were significantly increased in Mlkl−/− plaques from male mice compared with controls (Figure 2C and 2D). This increase in RvD1 did not impact the levels of its receptor, called ALX/FPR2 (lipoxin A4 receptor) in plaques (Figure 2E). Conversely, the prostanoids thromboxane B2, (Figure 2F), PGE2 (Figure 2G), PGF2α (Figure 2H), and PGD2 (Figure 2I) were significantly lower in Mlkl−/− plaques compared with controls. The thromboxane synthase metabolite, 12-HHT was also significantly decreased in Mlkl−/− mice compared with controls (Table). Lastly, there was a significant increase in the SPM:prostanoid ratio (Figure 2J) in the Mlkl−/− mice compared with controls. Together, these results suggest that loss of necroptosis improves SPMs and decreases a prostanoid storm.
Figure 1. *Mlkl*−/− mice with advanced atherosclerotic plaques have reduced necrotic cores, increased cap thickness, and enhanced efferocytosis compared with wild-type (Wt) mice. 

**A**, Aortic root sections for AAV8-gof-PCSK9 injected Wt and *Mlkl*−/− male mice were stained with hematoxylin and eosin (H&E). Representative images are shown, and blazed hashed lines outline the lesion. Scale bar=50 µm. The right panel is the quantification of necrotic area. **B**, Representative images of picrosirius staining depict collagen in red. Black hashed lines demarcate the lesion. A and B, Images were acquired using an Olympus microscope, and analysis was done with Olympus DP2-BSW software. Scale bar=50 µm. **C**, Representative images of TUNEL (red) and F4/80 (green) stained lesions are shown. Efferocytosis was quantified by the ratio of associated TUNEL+ cells to free TUNEL+ cells in atherosclerotic plaques. Images were acquired with Leica SPE and quantified using Image J. Scale bar=10 µm. For all panels, results are mean±SEM, and each symbol represents an individual mouse. AC indicates apoptotic cell; and NC, necrotic core. *P<0.05 and ***P<0.001, Student t test.
NCs Release Prostanoids That Impair Efferocytosis of Apoptotic Cells

We next performed experiments to determine whether NCs themselves were a source of these LM imbalances. BMDMs were stimulated with OZ in the presence of vehicle or a necroptosis inhibitor called Nec-1, and necroptosis was evaluated based on an increase in a p-MLKL signal that was responsive to Nec-1 treatment (Figure V in the Data Supplement). To interrogate released factors from NCs, we generated NC-CM as described in the methods. We observed that NCs had significantly more PGE\textsubscript{2} (Figure 3A) and TXB\textsubscript{2} (Figure 3B), compared with cells in which necroptosis was blocked by Nec-1. We also obtained CM from apoptotic macrophages and found that there was significantly less PGE\textsubscript{2} (Figure 3A) and TXB\textsubscript{2} released by apoptotic cells (Figure 3B), compared with NCs. To corroborate these findings, we next generated NC-CM using OZ-stimulated BMDMs from either wt or Mlkl\textsuperscript{−/−} mice and found that there was significantly less PGE\textsubscript{2} (Figure 3C) and TXB\textsubscript{2} (Figure 3D) in Mlkl\textsuperscript{−/−} macrophages compared with wt controls. Importantly, we also observed a significant imbalance between the RvD1:PGE\textsubscript{2} (Figure 3E) and RvD1:TXB\textsubscript{2} (Figure 3F) ratios from NCs compared with NCs treated with Nec-1. The RvD1:PGE\textsubscript{2} (Figure 3G) and RvD1:TXB\textsubscript{2} (Figure 3H) ratios were also significantly higher in NC-CM from Mlkl\textsuperscript{−/−} macrophages compared with wt controls.

Figure 2. Specialized proresolving mediators (SPMs) are increased and prostanoids are decreased in plaques from Mlkl\textsuperscript{−/−} mice. Aortas from wild-type (WT) or Mlkl\textsuperscript{−/−} mice injected with AAV8-gof-PCSK9 virus and fed the Western diet (WD) were harvested and immediately frozen in ice-cold methanol. Tissues were analyzed by liquid chromatography-tandem mass spectrometry. Lipid mediators derived from (A) docosahexaenoic acid (DHA) and (B) arachidonic acid (AA) were identified, and quantified and changes between WT and Mlkl\textsuperscript{−/−} mice were visualized using interaction network pathway analysis. C, 17-HDHA, (D) resolin D1 (RvD1), (E) total SPMs, (F) thromboxane B\textsubscript{2} (TXB\textsubscript{2}), (G) PGE\textsubscript{2}, (H) PGF\textsubscript{2\alpha}, and (I) PGD\textsubscript{2} were quantified and are shown as pg/mg of protein. J, SPMs:prostanoids is shown as a ratio. Results are mean±SEM, n=6 mice for WT and n=8 mice for Mlkl\textsuperscript{−/−} mice. *P<0.05, **P<0.01, and ***P<0.001, Student t test.

Arterioscler Thromb Vasc Biol. 2021;41:1062–1075. DOI: 10.1161/ATVBAHA.120.315758
We next questioned whether another prevalent cell type in atherosclerotic lesions released similar LMs upon induction of necroptosis. For these studies, we induced necroptosis in human umbilical vein endothelial cells as described in the methods. We found that necroptotic human umbilical vein endothelial cells had significantly higher p-MLKL levels that were responsive to Nec-1 treatment (Figure VIA in the Data Supplement). We next generated NC-CM and found that necroptotic human umbilical vein endothelial cells significantly released PGE₂ (Figure VIB in the Data Supplement) and TXB₂ (Figure VIC in the Data Supplement), which was similar to necroptotic macrophages. Together, these results suggest that NCs (as opposed to apoptotic cells) release large amounts of prostanoids, such as PGE₂, and thromboxane.

To determine whether exuberant levels of PGE₂ or thromboxane released by NCs impacted resolution programs, we next performed efferocytosis experiments in the presence of control versus NC-CM as described in the methods. First, we found that NC-CM significantly impaired efferocytosis (Figure 3I). We next treated macrophages with AH6809 (200 μmol/L), which is an antagonist for PGE₂ and PGD₂ receptors and blockade of PGE₂ and PGD₂ signaling rescued NC-CM-induced defective efferocytosis (Figure 3I). Blockade of thromboxane signaling with SQ29548 (150 μmol/L), also significantly increased the clearance of apoptotic cells in this context. Because thromboxane is short lived, our data suggest that NC-CM stimulates macrophages to produce thromboxane in an amplification loop. Indeed, blockade of thromboxane signaling enhanced the clearance of NCs by macrophages (Figure VII in the Data Supplement). These results suggest that a prostanoid storm from NCs drives defective efferocytosis.

Because we observed that apoptotic cells release PGE₂ (Figure 3A), albeit to a much lesser degree as NCs, we next questioned whether the lower amounts of PGE₂ released by apoptotic cells impaired efferocytosis. For these experiments, we prepared apoptotic cell CM as described in the methods and performed efferocytosis as above. Interestingly, blockade of PGE₂ and PGD₂ signaling rescued apoptosis of macrophages (Figure VII in the Data Supplement). These results suggest that a prostanoid storm from NCs has a maladaptive impact on efferocytosis, that is, a prostanoid storm, and specifically thromboxane released by NCs has a maladaptive impact on efferocytosis, which is consistent with the literature, and a higher dose of PGE₂ impaired efferocytosis (Figure VIIIB in the Data Supplement). Therefore, our results suggest important context and that high levels of prostanoids released by NCs drives defective efferocytosis.

Importantly, because the anti-efferocytosis actions of thromboxane have not previously been reported, we next sought to determine whether thromboxane directly impairs efferocytosis. Indeed, we observed that the thromboxane receptor agonist, U46619, significantly impaired efferocytosis in a dose dependent manner (Figure 3J). Together, these results suggest that the prostanoid storm, and specifically thromboxane released by NCs has a maladaptive impact on efferocytosis, that is, a key cellular program of resolution.

Lastly, because we found that NCs do not readily biosynthesize RvD1, we next questioned whether this lack of RvD1 was a potential mechanism as to why prostanoids went unchecked to derange efferocytosis. We found that RvD1-stimulated macrophages have rescued efferocytosis in the presence of NC-CM (Figure 3I) or U46619 (Figure 3J). These results point to a crucial role for SPMs, like RvD1, to limit the anti-efferocytic actions of key released LMs from NCs.

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**Table. Lipid Mediators Identified in Advanced Plaques From Wt or Mlk⁻/⁻ Mice Injected With AAV8-gof-PCSK9**

| Mediator | Wt | Mlk⁻/⁻ |
|----------|----|--------|
| LXβ₁    | 177±17.3 | 404±188.9 |
| 15R-LXα₁ | 28.3±19.0 | 50.8±24.8 |
| 15R-LXβ₁ | 264±143.6 | 1801±2191.7 |
| LTB₄     | 273±86.1 | 334±169.8 |
| 6-trans,12epi-LTB₄ | 65±14.4 | 149±85.1 |
| PGE₂     | 3088±599.6 | 1610±430.2 |
| PGD₂     | 2332±502.2 | 1615±616.5 |
| PGF₂α    | 1159±265.0 | 850±225.4 |
| TXB₂     | 3769±875.6 | 2740±677 |
| 15-HETE  | 5620±1311.3 | 3582±820.7 |
| 12-HETE  | 68149±19833.3 | 94019±25291 |
| 5-HETE   | 573±158.8 | 845±265.2 |
| 12-HHT   | 11975±3113.3 | 8399±2814.4 |
| RvD1     | 2.7±8.6 | 14.7±11.4 |
| Mar1     | 0±0.0 | 13.6±15.4 |
| 12-trans Mar1 | 5.5±4.3 | 21.8±11.4 |
| Mar2     | 12.3±11.0 | 25.3±15.0 |
| 10S,17S-dHDA | 5.6±2.1 | 10.5±2.8 |
| 17-dHDA  | 1275±3180.0 | 2000±3533.2 |
| 14-dHDA  | 4848±1435.2 | 10762±3751.4 |
| 4-dHDA   | 65±17.1 | 170±35.7 |
| 18-HEPE  | 38±5.9 | 41.7±16.4 |
| 15-HEPE  | 222±26.2 | 219±60.7 |
| 12-HEPE  | 11360±3778.8 | 9625±6401.1 |
| 5-HEPE   | 54±14.6 | 54±12.9 |

Aortic lipid mediators were quantified as pg/mg of tissue. Results are mean±SD, n=6 mice for Wt and n=6 mice for Mlk⁻/⁻ mice. AAV8-gof-PCSK9 indicates adeno-associated viral vector serotype 8 expressing the gain-of-function mutation of mouse proprotein convertase subtilisin/kexin type 9; Mlk; mixed lineage kinase domain; and Wt, wild type.

*P<0.01, †P<0.05, and ‡P<0.001, Student t test.
Figure 3. Necroptotic macrophages release exuberant prostanoids to limit efferocytosis.

A, B, E, and F, Media from control (vehicle), necroptotic cell (NC)-conditioned media (CM), or apoptotic cells (AC-CM) were assayed for (A) PGE$_2$ and (B) thromboxane B$_2$ (TXB$_2$), by ELISA. Ratios of (E) resolvin D1 (RvD1):PGE$_2$ or (F) RvD1:TXB$_2$ were calculated. C, D, G, and H, Media from control (vehicle), NC-CM from wild-type (Wt) or Mlkl$^{-/-}$ macrophages were assayed by ELISA as above. Results for A–F are mean±SEM, n=3 separate experiments, *P<0.05, **P<0.01, and ***P<0.001. The statistical test for A and B was 1-way ANOVA with Tukey multiple comparisons; for C and D, 2-way ANOVA with Sidak and Tukey multiple comparisons, respectively; and E–H, a Student t test. I, Macrophages were treated with NC-CM, SQ29548 (SQ), or AH6809 (AH) before addition of apoptotic cells. Efferocytosis was performed for 30 min, and images were acquired with Bio-Rad Zoe fluorescence microscope and efferocytosis is represented as fold change. Results are mean±SEM, n=3 separate experiments, **P<0.01 and ****P<0.0001, 1-way ANOVA with Tukey multiple comparisons. J, Macrophages were stimulated with 100, 250, or 500 nmol/L of the thromboxane receptor agonist U46619 (U4) or U4 and RvD1 before addition of apoptotic cells. Experiments were carried out as in I. Results are mean±SEM, n=3 separate experiments. Nec-1 indicates necrostatin 1. *P<0.05, **P<0.01, and ****P<0.0001, 1-way ANOVA with Tukey multiple comparisons.
NC Prostanoid Storm and U46619 Derange Cellular Respiration in Macrophages to Limit Efferocytosis

Recent work suggests that FAO and OXPHOS are critical metabolic programs for efferocytosis. Therefore, we next questioned whether OXPHOS was disrupted by NC-CM or U46619. We quantified OXPHOS by measuring the OCR with a Seahorse XF96 Extracellular Flux Analyzer. We first confirmed previous findings in which macrophage uptake of apoptotic cells significantly increased OCR (Figure IXA in the Data Supplement). Efferocytic macrophages that were stimulated with NC-CM had significantly less basal and maximal OCR compared with control efferocytic macrophages (Figure IXA in the Data Supplement). Similar results were observed when efferocytic macrophages were stimulated with U46619 (Figure IXB in the Data Supplement). Representative Seahorse tracings clearly show decreased OCR when efferocytic macrophages were either stimulated with NC-CM or U46619 (Figure IXA and IXB in the Data Supplement). We next questioned whether boosting OXPHOS through mitochondrial uncoupling could rescue NC-CM- or U46619-impaired efferocytosis. Indeed, we observed that FCCP, (ie, an agent that uncouples mitochondria to promote cellular respiration), rescued defective efferocytosis induced by NC-CM or U46619 (Figure IXC and IXD in the Data Supplement). We also observed that macrophages ingesting NCs were rescued by FCCP (Figure VII in the Data Supplement). Lastly, we questioned whether macrophages from wt or Mlkl−/− mice exhibited differences in OCR values in response to efferocytosis and U46619. We did not observe any appreciable differences in macrophages from wt or Mlkl−/− mice (Figure IXE in the Data Supplement). Together, these results suggest that NCs or U46619 impinge on cellular respiration to clear apoptotic cells. Moreover, these findings suggest that disruption of cellular respiration in macrophages is a common mechanism as to why NCs limit the efferocytic repertoire of macrophages. How SPMs, like RvD1, impact OXPHOS during the clearance of dead cells is the next question.

RvD1 Enhances the Clearance of NCs Through Modulating FAO and OXPHOS

Uptake of apoptotic cells by macrophages is an efficient process that is accompanied with an increase in FAO and OXPHOS. The clearance of NCs is not an efficient process, and little is known about its impact on macrophage metabolism. Our results above suggest that improving OXPHOS (via FCCP uncoupling) enhances the clearance of NCs. We previously found that RvD1 promotes efficient clearance of NCs; therefore, we next questioned whether RvD1 promotes OXPHOS in macrophages during NC uptake. First, we observed that RvD1 enhanced the clearance of NCs in vitro, as we have previously shown (Figure 4A). We also observed that RvD1 increased the uptake of NCs in vivo (Figure VID in the Data Supplement). We found that RvD1-stimulated macrophages had a significantly higher basal OCR value compared with vehicle-treated macrophages during NC uptake (Figure 4B). The representative Seahorse tracing indicates higher OCR values with RvD1 treatment compared with the other conditions (Figure VA in the Data Supplement). Importantly, NCs alone had a significantly lower OCR compared with live cells (Figure VB in the Data Supplement), which suggests that NCs have minimal respiration and any observed changes in OCR are from macrophages ingesting NCs. In support of these findings, we show that RvD1-stimulated macrophages that were ingesting NCs promote a mitochondrial organization that is consistent with macrophages that have increased OXPHOS as their metabolic phenotype (Figure XC in the Data Supplement). Because the clearance of dead cells is a highly energetic process, we next calculated the rate of oxygen consumption that is coupled to mitochondrial ATP production during OXPHOS with the Seahorse Real-Time ATP Rate assay. We found that RvD1-treated macrophages had significantly increased mitochondrial ATP production compared with vehicle-treated macrophages that ingested NCs (Figure 4C). These results again suggest that RvD1 increased OXPHOS in macrophages during NC uptake.

We next questioned whether RvD1 enhanced mitochondrial membrane potential. For these experiments, macrophages were labeled with a MitoTracker dye in which fluorescence intensity is indicative of mitochondrial membrane potential changes. Representative images demonstrate a higher intensity in RvD1-treated macrophages that were ingesting NCs compared with controls (Figure 4D, left). We quantified the MitoTracker signal in macrophages and observed a significant increase in the MFI of MitoTracker in the RvD1-treated macrophages that were ingesting NCs, compared with vehicle-treated macrophages during NC uptake (Figure 4B). The representative Seahorse tracing indicates higher OCR values with RvD1 treatment compared with the other conditions (Figure VA in the Data Supplement), which suggests that NCs have minimal respiration and any observed changes in OCR are from macrophages ingesting NCs. In support of these findings, we show that RvD1-stimulated macrophages that were ingesting NCs promote a mitochondrial organization that is consistent with macrophages that have increased OXPHOS as their metabolic phenotype (Figure XC in the Data Supplement). Because the clearance of dead cells is a highly energetic process, we next calculated the rate of oxygen consumption that is coupled to mitochondrial ATP production during OXPHOS with the Seahorse Real-Time ATP Rate assay. We found that RvD1-treated macrophages had significantly increased mitochondrial ATP production compared with vehicle-treated macrophages that ingested NCs (Figure 4C). These results again suggest that RvD1 increased OXPHOS in macrophages during NC uptake.

As mentioned above, FAO has emerged as a critical metabolic program for efficient efferocytosis. To determine if RvD1 increases FAO during clearance of NCs, macrophages were incubated with vehicle or RvD1 before addition of NCs at a 1:2 ratio for 2 hours at 37°C and assayed for acylcarnitines by metabolomic analysis. We observed that RvD1 significantly increased several long chain acylcarnitines in macrophages ingesting NCs, compared with vehicle-treated macrophages ingesting NCs (Table II in the Data Supplement). Importantly, NCs alone or resting macrophages did not have increased acylcarnitines, which again suggests that the increases in acylcarnitines...
**Figure 4.** Resolvin D1 (RvD1) enhances fatty acid oxidation and oxidative phosphorylation during necroptotic cell (NC) clearance.

A. NC uptake was quantified by Leica SPE confocal microscope and analyzed with Image J. Results are mean±SEM, n=3 separate experiments, ****P<0.0001, Student t test. 

B. Seahorse assays were performed as described in the methods. Basal oxygen consumption rate (OCR) was quantified as fold change. Results are mean±SEM, n=4 separate experiments, *P<0.05, 1-way ANOVA with Tukey multiple comparisons. 

C. Mitochondrial ATP production was calculated using values obtained from the Seahorse assay in B. Results are mean±SEM, n=4 separate experiments, **P<0.01, 1-way ANOVA with Tukey multiple comparisons. 

D. Macrophages were incubated with MitoTracker before RvD1 stimulation, and experiments were performed as described in the methods section. Arrows denote mitochondrial structure and fluorescence intensity. Mitochondrial membrane potential was quantified as mean fluorescence intensity (MFI) by Image J analysis. Scale bar=5 µm. Results are mean±SEM, n=4 separate experiments, ***P<0.001, 1-way ANOVA with Tukey multiple comparisons. 

E. Macrophages were treated with 3 µmol/L etomoxir, RvD1, or in combination before the addition of NCs. Images were acquired with Leica SPE at 63x magnification and quantified as fold change. Results are mean±SEM, n=4 separate experiments, *P<0.05 and ***P<0.001, 1-way ANOVA with Tukey multiple comparisons. 

F. MitoTracker MFI was quantified with ImageJ analysis. Results are mean±SEM, of n=3 separate experiments. *P<0.05 and **P<0.01, 1-way ANOVA with Tukey multiple comparisons.
were a result of the macrophages ingesting the NCs (Table II in the Data Supplement). To prove causation for the importance of FAO oxidation and mitochondrial function, we inhibited the activity of the CPT1 enzyme (ie, a critical enzyme that initiates FAO) with etomoxir and found that etomoxir completely abrogated RvD1’s ability to efficiently engulf NCs (Figure 4E, Figure XI in the Data Supplement). We used 3 μmol/L etomoxir because this dose has been reported to block FAO in macrophages. Disruption of FAO with etomoxir significantly blocked RvD1’s ability to increase mitochondrial membrane potential (Figure 4F). Collectively, these results point to RvD1 controlling a FAO-OXPHOS program to generate ATP for efficient engulfment of NCs. We next questioned whether RvD1 directed signaling events that drive this metabolic program.

RvD1 Promotes AMPK Signaling to Enhance FAO and Engulfment of NCs

AMPK regulates cellular metabolism and can promote FAO and OXPHOS. We found that RvD1-treated macrophages had enhanced p-AMPK levels that were commensurate to AICAR, which is a known activator of AMPK (Figure 5A). A direct downstream target of AMPK signaling is ACC. AMPK activates ACC by phosphorylation, which directs the cell to shut down fatty acid synthesis to promote FAO. Therefore, we next questioned whether RvD1 increased p-ACC and indeed we observed that RvD1 increased p-ACC to similar levels as AICAR (5-amino-1-β-D-ribofuranosyl-1H-imidazole-4-carboxamide; Figure 5B). Importantly, RvD1 in the presence of compound C (ie, an inhibitor of AMPK) was unable to increase p-ACC levels (Figure XII in the Data Supplement). We next investigated whether RvD1’s ability to enhance the clearance of NCs was due to increases in p-AMPK. Indeed, compound C abolished RvD1’s ability to efficiently engulf NCs (Figure 5C). Moreover, activation of AMPK with AICAR mimicked RvD1’s ability to increase NC uptake and the combination of AICAR and RvD1 were not additive (Figure 5D), which again suggests that RvD1 acts through AMPK to efficiently engulf NCs. Compound C decreased mitochondrial membrane potential in RvD1-stimulated macrophages that were ingesting NCs. Representative images clearly depict MitoTracker intensity changes in which RvD1-treated macrophages have a brighter signal that is almost completely blocked by compound C (Figure 5E). Taken together, these data suggest that RvD1 initiates an AMPK signaling pathway to promote FAO and OXPHOS for efficient clearance of NCs.

DISCUSSION

We provide evidence that NCs impair key features of resolution such as the biosynthesis of SPMs like RvD1 and efferocytosis. Furthermore, our results suggest that NCs release a prostanoid storm in a manner that is imbalanced with RvD1 and that this imbalance drives defective efferocytosis of both apoptotic cells and NCs. Importantly, addition of RvD1 rescues defective clearance of NCs, through a mechanism that involves the activation of p-AMPK signaling, FAO, and OXPHOS in macrophages. Together, these results provide a new framework as to how NCs impact tissue repair programs in atherosclerosis.

NCs are widely known for their ability to exacerbate inflammation via the release of cytokines, chemokines, and DAMPs. However, before the studies herein, little was known about which LMs were released by NCs and how those LMs could impact the local environment. We present evidence that NCs produce a bolus of prostanoids such as PGE 2 α, PGF 2 α, and thromboxane in atherosclerotic plaques. Previous work revealed that TP (thromboxane receptor) knockout mice had improved atherosclerosis. Thromboxane receptors were also reported to be elevated in human atherosclerosis, which collectively suggests a maladaptive role for thromboxane in plaques. Moreover, other murine studies demonstrated that a knockout for FP (prostaglandin F 2α receptor) decreases atherosclerosis and protects against lipopolysaccharide-induced tachycardia. Thromboxane and PGF 2α activate GPCRs (G-protein-coupled receptors) that are coupled to Gq signaling and the release of calcium. PGF 2α via FP signaling was recently linked to CAMKII (Ca 2+/calmodulin-dependent protein kinase II) activation in other contexts. CAMKII has been shown to promote atheroprogresion through inhibiting efferocytosis. Therefore, in addition to our observations in which thromboxane limits OXPHOS, it is also possible that the prostanoid storm observed herein may limit efferocytosis through CAMKII related mechanisms as well.

Our work in context of the literature suggests that a source of thromboxane within plaques may be from NCs. Importantly, our findings suggest that released thromboxane may be an avoid me signal because of its ability to limit efferocytosis. Along these lines, our in vitro work proposes important context. We observed that necroptotic macrophages and endothelial cells release prostanoids, like thromboxane. Macrophages, platelets, and vascular smooth muscle cells of human arteries and veins are known to produce thromboxane. Undoubtedly, there are numerous cell types within the plaque and understanding (1) which cells types are most readily primed to undergo necroptosis in lesions and (2) whether LM signatures are distinct depending on the cell type are important next steps.

Additionally, we uncovered a mechanism in which RvD1 modulates the metabolism of the macrophage to promote efficient clearance that was not previously known. A recent study demonstrated that
Figure 5. Resolvin D1 (RvD1) promotes AMPK (AMP-activated protein kinase) signaling to enhance the uptake of necroptotic cells. 

A and B, BMDMs stimulated with vehicle, RvD1, or AICAR (5-amino-1-β-D-ribofuranosyl-1H-imidazole-4-carboxamide; an AMPK activator) as described in methods. Incubations were immediately stopped by fixation with 4% paraformaldehyde and immunostained with (A) p-AMPK or (B) p-ACC. Images were acquired on a Leica SPE confocal microscope, and mean fluorescence intensity was analyzed with Image J. Results are mean±SEM, n=3 experiments, **P<0.01 and ****P<0.0001, 1-way ANOVA with Tukey multiple comparisons.

C and D, For NC uptake assays, macrophages were stimulated with RvD1, compound C (ComC), AICAR or in combination and described in the methods. Images of NC uptake were taken on a Leica SPE confocal microscope and analyzed with Image J. Results are mean±SEM, n=4 separate experiments, *P<0.05, **P<0.01, ***P<0.001, 1-way ANOVA with Tukey multiple comparisons.

E, MitoTracker MFI was quantified as in Figure 4. Images were acquired using Leica SPE at 63× magnification. Scale bar=5 µm. Results are mean±SEM, n=3 separate experiments, *P<0.05, 1-way ANOVA with Tukey multiple comparisons.
efferocytosis of apoptotic cells by macrophages promotes OXPHOS. Moreover, FAO and OXPHOS are associated with tissue restorative macrophages. Essentially, mitochondria play a central role in cellular metabolism, providing a large source of cellular ATP through FAO and OXPHOS. Mitochondrial dysfunction has been associated with several chronic inflammatory diseases including atherosclerosis. Our work provides evidence that clearance of NCs by RvD1-treated macrophages promotes FAO and OXPHOS, which suggests that the presence of SPMs may augment these pathways in plaques to promote clearance of dead cells and limit necrosis. Moreover, we observed that RvD1 initiated AMPK signaling in macrophages, which is a known metabolic sensor that promotes FAO and OXPHOS. In agreement, RvD1 was also shown to increase p-AMPK in adipose tissue from db/db mice. The proresolving protein called annexin A1, that also binds the same receptor as RvD1, was recently shown to promote AMPK signaling in macrophages in a different context. Lastly, a previous study also found that AMPK played a crucial role in promoting efferocytosis. An interesting parallel to this work is with regard AMPK signaling to reduce atherosclerotic plaque burden and anti-miR-33-33 promoted FAO in obesity. How RvD1 or other SPMs may regulate miR-33 is of interest. Nevertheless, our results, in context with the literature, suggests that promoting macrophage FAO and OXPHOS are protective in atherosclerosis and possibly more globally in cardiometabolic diseases.

In conclusion, the findings of this study add new mechanistic insight into how necroptosis negatively impacts resolution. These results offer new insights to the LMs that are released by NCs and their function. Moreover, we present evidence that RvD1 directs metabolic programs in macrophages to enhance clearance of NCs. Because our results and others suggest that SPMs are defectively synthesized and are thus naturally lost during this atheroprogession, restoration of these key tissue reparative ligands may be a safer approach toward limiting atherosclerosis.
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