ERK5 Activation in Macrophages Promotes Efferocytosis and Inhibits Atherosclerosis

Running title: Heo et al.; ERK5 and efferocytosis

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Abstract

Background—Efferocytosis is a process by which dead and dying cells are removed by phagocytic cells. Efferocytosis by macrophages is thought to curb the progression of atherosclerosis, but the mechanistic insight of this process is lacking.

Methods and Results—When macrophages were fed apoptotic cells (ACs) or treated with pitavastatin in vitro, efferocytosis-related signaling and phagocytic capacity were upregulated in an ERK5 activity-dependent manner. Macrophages isolated from macrophage-specific ERK5 null mice exhibited reduced efferocytosis and levels of gene and protein expression of efferocytosis-related molecules. When these mice were crossed with LDLR<sup>−/−</sup> mice and fed a high-cholesterol diet, atherosclerotic plaque formation was accelerated, and the plaques had the more advanced and vulnerable morphology.

Conclusions Our results demonstrate that ERK5, which is robustly activated by statins, is a hub molecule that upregulates macrophage efferocytosis, thereby suppressing atherosclerotic plaque formation. Molecules that upregulate ERK5 and its signaling in macrophages may be good drug targets for suppressing cardiovascular diseases.

Key words: atherosclerosis, signal transduction, Efferocytosis, ERK5, Pitavastatin
**Introduction**

The necrotic core in advanced atherosclerotic lesions is a lipid-rich area containing dead and necrotic white blood cells, mainly macrophages, surrounded by inflammatory cells and contributes towards formation of vulnerable plaques and plaque disruption\(^1,2\). Rapid phagocytic clearance (efferocytosis) of the corpses by macrophages prevents necrotic core formation, but as the demand for efferocytosis increases, the system fails to meet this demand, hence the formation of necrotic cores\(^2-4\). In addition, recent evidence shows that efferocytosis becomes defective in advanced human lesions\(^2\). Preventive action of 3-hydroxy-3-methylglutaryl-coenzyme (HMG Co-A) reductase inhibitors (statins) on acute coronary syndrome independent of its effect of lowering plasma lipid levels is recognized\(^5\). In addition, lovastatin was implicated to regulate efferocytosis\(^6\).

“Find-me” and “eat-me” signals generated by apoptotic cells (ACs) and macrophages regulate efferocytosis\(^2-4\). When cells including foam cells undergo apoptosis, they release diffusible “find-me” molecules to which macrophages are attracted. ACs also express “eat-me” signals (such as phosphatidylserine) on their surface\(^7\), and via phosphatidylserine receptors such as mucin-domain-containing molecule (Tim-4), angiogenesis inhibitor 1 (Bai1), and Stabilin2, macrophages capture and engulf ACs. In addition to this direct binding of phosphatidylserine to its receptors, it also binds opsonins such as milk fat globule-EGF factor 8 (Mfg-e8) and thrombospondin (Thbs)1. Opsonins are bridging molecules between phosphatidylserine (on the surface of ACs) and various opsonin receptors (on the surface of macrophages), for example, transglutaminase (TG)2/Integrin for Mfg-e8, CD36 for Thbs1, and c-mer proto oncogene tyrosine kinase (MerTK) for growth arrest specific (Gas)6, which also promote macrophages’ engulfment activity\(^8\).
The involvement of efferocytosis-related molecules in atherosclerosis has been well established\textsuperscript{9,10}. Although ACs and statins are known to heighten the phagocytic activity of macrophages\textsuperscript{6,11}, the mechanism for this is not understood. Mukundan et al. recently reported that the expression of peroxisome proliferator-activated receptor \( \delta \) (PPAR\( \delta \)) and certain key opsonins including C1q, Mfg-e8, and Thbs1 was increased in macrophages that were incubated with ACs\textsuperscript{12}. Intriguingly, however, clearance of apoptotic debris by these macrophages was delayed despite increased opsonin production\textsuperscript{12}. Previously, we have demonstrated the involvement of ERK5 in transactivation of PPAR\( \gamma \) and PPAR\( \delta \) in endothelial and C2C12 cells\textsuperscript{13}. ERK5 is a member of the mitogen-activated protein kinase (MAPK) family activated by redox and hyperosmotic stresses, growth factors, and pathways activated by certain G-protein-coupled receptors\textsuperscript{14}. ERK5 is not only a kinase, but also has transcriptional activity, which is critical for activating transcription factors such as Kruppel-like factor 2 (KLF2) and PPARs\textsuperscript{13,15,16}. These studies suggest a possible role for ERK5 in phagocytosis by macrophages. Indeed, ERK5 is highly expressed in monocyte/macrophage, but its function, especially in the context of efferocytosis and cardiovascular diseases, is unknown.

In this study, we investigated whether ACs and statins could activate ERK5 in macrophages. We also examined whether this ERK5 activation has a role in phagocytosis of ACs. We found that in response to AC feeding and treatment with statins, macrophage ERK5 activity was significantly increased and also enhanced phagocytic clearance by upregulating expression of efferocytosis-related molecules. These in vitro results provide the mechanism for the effect of statins that, in our in vivo experiments, have inhibited apoptotic debris accumulation and formation of necrotic cores and atherosclerotic lesions. On the other hand, depletion of ERK5 in macrophages increased M1 (inflammatory)-related gene expression, atherosclerotic
plaque formation, and reduced the thickness of the fibrotic cap of advanced plaques. The molecules in the efferocytosis pathways should be attractive drug targets since they have unique features that distinguish them from other MAPK and signaling events.

Methods

Additional details of the experimental Procedures are included in the online supplemental information.

Mice

All animal procedures used in this study were approved by the Animal Care and Use Committee of the University of Rochester. All animals were housed in a temperature-controlled room under a light (12 hrs)/dark (12 hrs) cycle and under pathogen-free conditions. Macrophages deficient in ERK5 were obtained from ERK5fl/fl mice crossed with LysMCre+/− (C57BL/6 background) mice (ERK5-MKO). For atherosclerosis studies, these mice were crossed with LDLR−/− (C57BL/6J background) mice (ERK5-MKO/LDLR−/−). At 8 weeks of age, mice were placed on a high-cholesterol diet (D01061401C, Research Diets, Inc.) for 8 or 16 weeks. We prepared apoptotic cells by culturing thymocytes from 6- to 8-week old C57BL/6J WT mice in serum-free RPMI medium for 16-18 hrs or by treating them with dexamethasone (100 nM) for 3 hrs17. For FACS and microscopy analyses, we labeled apoptotic thymocytes by incubating them with 2 μM CMFDA (Molecular probes) for 30 min before feeding them to macrophages.

Statistical Analysis

Data are presented as mean ± S.D. Comparison between independent two groups was subjected to Wilcoxon rank-sum test. For multiple comparisons of >2 groups, Kruskal-Wallis test followed by Dunn’s post hoc analysis was performed using GraphPad Prism (GraphPad Software, Inc.,
CA). A $P$ value $<0.05$ was considered statistically significant.

**Results**

**ERK5 regulates macrophage efferocytosis.**

We have previously shown that ERK5 is involved in regulating PPAR$\delta$ transcriptional activity$^{13}$. Since PPAR$\delta$ plays a role in phagocytosis of ACs by macrophages$^{11}$, we first studied if ERK5 was activated in bone marrow derived macrophages (BMDMs) that were fed apoptotic thymocytes as ACs and found that ERK5 kinase activity was increased by $\sim 10 \pm 3.7$-fold. This ERK5 activation by ACs was specific, because necrotic thymocytes (NCs) failed to activate ERK5 (Fig. 1A). The ERK5 mRNA level (Fig. 1B) and protein expression (Supplementary Fig. 1) also increased in macrophages fed ACs, suggesting the importance of ERK5 in phagocytosis. Flow cytometry data on our thymocyte preparations indicated that practically all cells expressed a lymphocyte specific protein, CD3 (Supplementary Fig. 2A) and that cells with an NKT cell specific marker NK1.1 were negligible (0.496%) (Supplementary Fig. 2B). Therefore, the results shown in Fig. 1A are not due to apoptotic NKT cells. Since activated ERK5 detected in the experiment shown in Fig. 1A could come from engulfed CD3$^+$ ACs, we analyzed the presence of CD3 protein in macrophages fed ACs. This lymphocyte marker was not detected in AC fed macrophages, whereas it was readily detectible in ACs that was not phagocytized (Supplementary Fig. 2C). Thus, ERK5 detected in Fig. 1A comes from macrophages.

To determine the function of ERK5 in macrophages, we generated mice with macrophages deficient in ERK5 (ERK5$^{fl}$/LysMCre$^{+/-}$, ERK5-MKO). ERK5-MKO mice showed roughly 90% reduction in ERK5 protein expression in isolated peritoneal macrophages.
compared with those of non-transgenic littermate control (NLC) mice (Fig. 1C). Up to 6 months of age, we observed no difference in size or body weight between ERK5-MKO and NLC mice (Supplementary Fig. 3A), and no notable differences were found on their development and organ structures at the gross anatomical level (data not shown). Complete blood cell counts were performed on these animals, and we found no difference (Supplementary Fig. 4). Next, we examined cells isolated from bone marrow (BM), peripheral blood (PB), and peritoneal fluid (PE) of NLC and ERK5-MKO mice (Fig. 1D, E, and F). Compared with NLC mice, ERK5-MKO mice had similar numbers of monocytes (CD115+/Gr1−) in BM, but had more inflammatory (classical) monocytes (CD115+/Gr1+) in PB and PE and less resident (non-classical) monocytes (CD115−/Gr1+) in PB (Fig. 1D and E). There was no significant difference in the level of PE macrophages positive for F4/80, a maturation marker, between these mice (Fig. 1F).

To determine the role of ERK5 in phagocytosis by macrophages, BMDMs were fed CMFDA-FITC labeled ACs, and clearance of these fluorescent cells was quantified by flow cytometry. As shown in Fig. 1G, phagocytic activity of ERK5-MKO macrophages was lower by ~44% compared to that of NLC counterparts. Next, we examined AC clearance in vivo. We injected CMFDA-FITC labeled ACs to NLC and ERK5-MKO mice, and phagocytosis by CD11b+ splenic macrophages was measured. Although we injected the same number of fluorescent cells, phagocytosis by ERK5-depleted macrophages was severely compromised (Fig. 1H). Furthermore, resident peritoneal macrophages in ERK5-MKO mice showed a reduced capacity for clearance of fluorescent ACs after 6 hrs of injection (Fig. 1I, J, and K). ERK5 deficient macrophages also exhibited reduced ability to ingest ACs (Fig. 1I, J, and K). To test if ACs accelerate their own clearance by stimulating macrophages, BMDMs were primed by
feeding them with unlabeled ACs and then feeding them again, this time with fluorescent ACs, and measured uptake of fluorescent cells. Priming of macrophages from NLC mice increased their phagocytic capacity, whereas no significant priming was seen in ERK5-MKO macrophages (Fig. 1L). These results indicate ERK5’s critical role in the regulation of phagocytosis by the macrophages.

**ERK5 regulates efferocytosis-related gene expression and M1/M2-type polarization.**

To investigate the molecular mechanisms by which ERK5 regulates the capacity of phagocytosis and clearance of ACs, we investigated the expression of efferocytosis-related genes. We found a decreased level of PPARδ mRNA and a significant decrease in PPARδ protein expression in ERK5-MKO macrophages (Fig. 2A, C, and D). Next, we examined the expression of the following opsonins: Mer-tK, C1qa, C1qb, C1qc, Gas6, Mfg-e8, Thbs1, and Anxa1. Using quantitative RT-PCR (qPT-PCR), we found the expression of these opsonins, but not of CD36 and CD68, were reduced by ~40-80% in ERK5-MKO compared with NLC PE macrophages (Fig. 2B). Western-blot analyses revealed similar reduction in protein levels, although a less significant reduction in the Anxa1 protein level in ERK5-MKO cells was noted (Fig. 2C).

We then investigated the role of ERK5 in opsonin expression by gain-of-function studies. When primary BMDMs from wild-type (WT) mice were transduced by an adenovirus containing a constitutively active form of MEK5α (Ad-CA-MEK5α), opsonin mRNA levels, especially those of Mfg-e8 and Thbs1, were significantly increased compared with macrophages transduced with Ad-LacZ (Fig. 2E). In addition, CA-MEK5α increased Thbs1 and Mfg-e8 promoter activity in a PPARδ-dependent manner in macrophages, suggesting that ERK5 regulates transcription of certain opsonins at least in part via PPARδ (Fig. 2F-H). We found that mRNA expression of the “eat-me” signaling molecules including opsonins, Tim 4, Tyro3, Itgav, Bai1,
Stab2, and Rac1 (Fig. 3A,B), and of the “find-me” ligands such as S1P1 and CX3CL1, and of the “find-me” receptors, G2A and P2Y2 (Fig. 3C), were all increased after AC feeding to macrophages isolated from NLC, but not in those from ERK5-MKO, mice. This is in sharp contrast to the case of PPARδ whose depletion in macrophages did not affect the expression of “eat-me” signal receptors12.

Since a decrease in IL-10 and an increase in IFN-γ in Mfg-e8−/− macrophages have been reported9, we investigated if ERK5 could modulate the M1/M2 macrophage polarization status. At the basal level, we found that BMDMs isolated from ERK5-MKO animals had higher expression levels of M1-related genes such as iNOS, Tnf-α, and Mcp-1 and lower levels of M2-related genes such as arginase (arg) 1, resisin like molecule alpha 1 (Fizz1), and chitinase 3-like 3 (Chi3l3), demonstrating that ERK5 activation promotes M2 genes (Fig. 3D). In parallel, we found increased protein expression of iNOS and MCP-1 and decreased expression of Arg-1 and IL-10 in the peritoneal macrophages isolated from ERK5-KO compared with NCL mice (Fig. 3E).

Mfg-e8 secretion and phagocytic capacity are regulated by ERK5.
To determine whether opsonins secreted into the serum contribute to ERK5-mediated AC clearance by macrophages, we treated BMDMs with the serum obtained from ERK5-MKO or NLC mice. Incubation of BMDMs from NLC mice with the ERK5-MKO mouse serum reduced their phagocytic capacity while the serum from NLC mice rescued the phagocytic defect observed in ERK5-MKO BMDMs (Fig. 4A). Furthermore, addition of purified recombinant human Mfg-e8 to ERK5-MKO BMDMs improved their phagocytic capacity to ~80% of the normal level by NLC cells (Fig. 4B). Treatment of these cells with Thbs1 also improved their phagocytic ability (Supplementary Fig. 5). Consistent with these observations were reduced
levels of Mfg-e8 in the serum of ERK5-MKO mice detected by ELISA (Fig. 4C) and an
immunoprecipitation assay (Fig. 4D). Lastly, we determined opsonin levels in the liver of the
mice because Kupffer cells are the primary source of circulating opsonins. As expected, the
opsonin expression in the ERK5-MKO mouse liver was significantly lower than that in NLC
mice (Fig. 4E).

Statins induce opsonin expression via ERK5 activation.

Statins can increase efferocytosis, but the mechanism is unknown. We asked if macrophage
ERK5 had a role in statins-induced efferocytosis. We found that pitavastatin significantly
increased ERK5 phosphorylation and transcriptional activity in both mouse BMDMs (Fig. 5A,B)
and THP-1 cells (human monocyte/macrophage cell line; Supplementary Fig. 6A,B). The level
of pitavastatin-induced ERK5 transcriptional activation was comparable to that obtained by ACs
feeding (Fig. 5C). Furthermore, pitavastatin significantly increased opsonin expression
(Supplementary Fig. 6C) and the promoter activity of Thbs1 and Mfg-e8 in macrophages, all of
which were inhibited by the depletion of ERK5 (Fig. 5D,E). Interestingly, opsonin mRNA
expression levels in the liver were also significantly increased by pitavastatin injection in NLC
mice (Fig. 5F), but not in ERK5-MKO mice (Supplementary Fig. 7). These results show a
crucial role of macrophage ERK5 in statin-induced enhancement of efferocytosis.

Macrophage ERK5 deficiency accelerates formation of atherosclerotic plaques and
necrotic cores.

To determine the role of macrophage ERK5 in the development of atherosclerosis, NLC and
ERK5-MKO mice were bred into the LDLR<sup>−/−</sup> background and fed a high-cholesterol diet for 8
and 16 weeks. No differences were noted in their body weight, blood content of the total, LDL
and HDL cholesterol levels, and blood glucose tolerance test (Supplementary Fig. 3,8A).
However, the atherosclerotic lesion area observed in the en face sample of the aorta was significantly larger in ERK5-MKO/LDLR−/− than NLC/LDLR−/− mice (Fig. 6A, B and Supplementary Fig. 8B). Increased lesion size was also noted in histological cross-sections at the aortic valve area in ERK5-MKO/LDLR−/− animals compared with NLC/LDLR−/− mice (Fig. 6C, D, and Supplementary Fig. 8C). Plaque necrosis is a consequence of apoptotic cell accumulation in advanced lesions2,10. We, therefore, assessed the effect of depleted macrophage ERK5 on ACs accumulation identified by both apoptotic marker expression and loss of cellular integrity (Fig. 7A-C). We found that the number of TUNEL positive nuclei was significantly increased in ERK5-MKO/LDLR−/− lesions with larger necrotic cores compared with NLC/LDLR−/− mice (Fig. 7A-C, and E). Staining with anti-activated caspase 3 also gave the same results (Fig. 7D). We also detected an increased number of TUNEL-positive cells with cellular degradation and disintegration, which are known to be present in the advanced stage of apoptosis2,10, in ERK5-MKO/LDLR−/− compared with NLC/LDLR−/− mice (Fig. 7A-C). These data suggest that such advanced stage plaques develop as efferocytosis is severely compromised in ERK5-MKO macrophages. To examine if the ERK5 null condition makes macrophages more susceptible to apoptosis, we treated BMDMs with oxidized LDL (oxLDL). This treatment significantly increased TUNEL staining-positive cells and cleaved caspase 3 expressions in both ERK5-MKO and NLC BMDMs (Fig. 7F, G, and H), suggesting that apoptotic cell accumulation observed in the lesion of ERK5-MKO/LDLR−/− mice was not caused by increased apoptosis of ERK5 null cells but was due to defective clearance of ACs. Next, we examined composition of immune cells and SMCs in the lesion of ERK5-MKO/LDLR−/− compared to NLC/LDLR−/− mice by immunohistochemistry analysis using the following proteins—Macrophages (anti-Mac3), T cells (anti-CD3), and SMCs (anti-αSMA). As shown in
Supplementary Fig. 9, increased macrophages content in lesions of ERK5-MKO/LDLR^{−/−} compared with NCL/LDLR^{−/−} mice were detected. However, the total number of T lymphocytes was not different between the two groups. Lesions in ERK5-MKO/LDLR^{−/−} mice displayed increased cases of necrotic core formation. In contrast, the relative SMCs content was significantly less in the total lesions of the ERK5-MKO/LDLR^{−/−} mice, which represents the composition of vulnerable plaques. We also detected macrophage inflammatory mediators in the lesion by immunohistochemistry using anti-MCP-1 (M1 phenotype) and anti-IL-10 (M2 phenotype). We found increased MCP-1 expression, but decreased IL-10 expression in the intimal lesions of ERK5-MKO/LDLR^{−/−} compared with those in NCL/LDLR^{−/−} mice (Fig. 7I), which support in vitro data presented in Fig. 3D and E.

Advanced plaques lead to plaque rupture. We analyzed collagen contents and fibrous cap thickness of advanced lesions at the level of aortic valves. Mice were fed a high-cholesterol diet for 16 weeks and histological sections were examined after Masson’s Trichrome staining. We found a 30% decrease in collagen content (Fig. 8A,B) and a 45% decrease in fibrous cap thickness of lesions (Fig. 8C,D) in ERK5-MKO/LDLR^{−/−} compared with NCL/LDLR^{−/−} mice. Because the thickness of fibrous caps was not reduced in Mfg-e8 and Thbs1 knockout mice, we examined the expression and localization of Mfg-e8 in atherosclerotic lesions of mice fed a high-cholesterol diet for 8 and 16 weeks (Fig. 8E). Mfg-e8 was highly expressed in the plaque of the 8-week samples of NCL/ LDLR^{−/−} mice compared to ERK5-depleted mice and the 16-week samples of both genotypes. Mfg-e8 positive cells were also positive for Mac3 (a macrophage marker) but not for a smooth muscle cell marker (α-smooth muscle actin) in NCL/LDLR^{−/−} mice. Although Mac3 staining was increased in the 16-week samples of ERK5-MKO/LDLR^{−/−} mice, Mfg-e8 expression was reduced in the same area (Fig. 8E), suggesting that
ERK5 deficiency decreased Mfg-e8 expression in macrophages.

Discussion

During the past decade, evidence has accumulated supporting that atherosclerotic plaque formation is an inflammatory process. However, there is little evidence that anti-inflammatory agents are useful for the prevention of cardiovascular diseases including atherosclerosis. In contrast, beneficial effects of statins on preventing coronary events, which are independent of its effect on plasma lipid levels, have been recognized. Although statins have been suggested to inhibit inflammation and macrophage proliferation, the mechanism of such effects of statins is unknown. In this study we found that statins increased ERK5 kinase activity in macrophages and increased their phagocytic capacity. This effect was accompanied by increased expression of efferocytosis-related genes such as opsonins and molecules involved in the “eat-me” and “find-me” signaling. Thus, statins appear to activate these signaling cascades that are a part of macrophages’ normal defense mechanism, which is activated when macrophages come in contact with ACs, targets of macrophage efferocytosis. These “off-target” beneficial effects of statins are mediated by macrophage ERK5, as demonstrated by our studies using a macrophage specific ERK5 knockout mouse model.

It has been reported that a nuclear receptor, PPARδ works as a sensor of dying cells and orchestrates phagocytic responses. Since we have reported that ERK5 can regulate PPARs’ transcriptional activity, we have initially hypothesized that ERK5 may regulate phagocytosis through altering PPAR expression or activation. In this study, we found a significant decrease in PPARδ mRNA in ERK5-MKO macrophages, but a less robust reduction in its protein level was noted in these cells. A small but significant reduction in PPARδ expression in the intima was
also detected in vivo by immunocytochemistry (P<0.0377 compared to NLC/LDLR−/−).

**Supplementary Fig. 10.** It has been suggested that activation of PPARs by ligands can increase PPARs expression in a positive feedback manner\(^{26,27}\). It is possible that this small reduction in PPARδ expression is due to the inhibition of PPARs transcriptional activity in ERK5 deleted macrophages. Since depletion of PPARδ by siRNA inhibits CA-MEK5-mediated Thbs1 and Mfg-e8 promoter activity, ERK5-mediated PPARδ transcriptional activity may also be involved in some way in this regulation (Fig. 2E,F). In PPARδ−/− mice, the expression of only opsonins but not “eat-me” receptors was downregulated\(^{11}\). However, our study shows that ERK5 regulates the expression of not only opsonins but also other efferocytosis-related gene sets including “eat-me” and “find-me” molecules, indicating a more comprehensive role of ERK5 than PPARδ in regulating efferocytosis.

Deficiency in macrophage Mfg-e8 or Thbs1 has been shown to cause accumulation of ACs in the necrotic core, resulting in formation of large necrotic cores such as those found in advanced lesions in LDLR−/− and ApoE−/− mice\(^{2,10}\). In agreement with these reports, we found that macrophage ERK5 depletion in LDLR−/− mice resulted in accelerated formation of atherosclerotic lesions containing large necrotic cores with many more ACs compared with NCL/LDLR−/− mice (Fig. 5,6). Interestingly, however, we found less collagen content and a thinner fibrous cap in the atherosclerotic plaque of ERK5-MKO/LDLR−/− mice compared with NLC/LDLR−/− mice (Fig. 8A-D). These plaque phenotypes are different from the reported phenotypes of Mfg-e8 and Thbs1 KO mice\(^{9,28}\). Our study may suggest that depletion of macrophage ERK5 results in more advanced and vulnerable plaques than those in Mfg-e8 and Thbs1 KO mice. This effect, however, cannot be explained solely by ERK5’s role in regulating opsonin expression. As shown in Fig. 3, ERK5 regulates not only opsonin expression but also
“eat-me” and “find-me” signaling-related molecules and M1/M2 polarization. To put this in a different way, our study indicate that ERK5 is a more global regulator of macrophage efferocytosis, which may explain the superior effect of statins on inhibiting coronary events, compared with other anti-inflammatory reagents.

ERK5 can affect various macrophage functions that are dissimilar such as proliferation and inflammation. It is well known that a signaling molecule can cause two opposite effects based on its different compartmentalization, interacting partners, and post-translational modifications. For example, SUMOylated cytosolic p53 is pro-apoptotic while non-SUMOylated nuclear p53 is anti-apoptotic. As for ERK5, it is pro-proliferative under CSF-1 stimulation, but as we have shown in this study, activation of ERK5 by statin inhibits macrophage proliferation. Furthermore, ERK5 activation by Ad-CA-MEK5 could not upregulate macrophage proliferation (Supplementary Fig. 11), suggesting that ERK5 activation is necessary but not sufficient for increasing macrophage proliferation.

In summary, we have shown that macrophage ERK5 is important for not only AC-induced efferocytosis but also statin-induced clearance of ACs. Activation of ERK5 increases the expression of a broad range of anti-atherogenic molecules such as opsonins and “eat-me” and “find-me” signaling molecules in macrophages and also promotes their M2 polarization state. These multifaceted effects of ERK5 activation may explain the superior effect of statins, which robustly activates macrophage ERK5, on preventing coronary events compared with other anti-inflammatory agents. We suggest that our new findings on the role of macrophage ERK5 in efferocytosis will provide a new therapeutic strategy for reducing atherosclerosis and vulnerable plaque formation in advanced plaques.
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Conflict of Interest Disclosures: None.

References:


**Figure Legends:**

**Figure 1.** ERK5 kinase activation is involved in macrophage efferocytosis. **A, B,** ERK5 phosphorylation (A) and mRNA expression (B) in BMDMs fed apoptotic thymocytes (ACs) for 30 min (A) or 18 hrs (B), respectively. Feeding necrotic cells (NCs) did not increase ERK5 phosphorylation (A, left panel) and the graph represents densitometry data (A, right panel). -: no treatment. means ± S.D. (n=6), **P < 0.01.** C, ERK5 expression in peritoneal macrophages isolated from non-transgenic littermate control (NLC, LysCre<sup>+/−</sup>) and LysCre<sup>+/−</sup>ERK5<sup>fl/fl</sup> (ERK5-MKO) mice. Cell lysates were immunoblotted for total ERK5, ERK1/2, and tubulin. n=3 per genotype. **D-F,** Characterization of CD115<sup>+</sup> and Gr1<sup>+</sup> cells identified by specific immunolabeling. **D, E,** Representative flow cytometry results showing CD115<sup>+</sup>Gr1<sup>+/−</sup> cells in peripheral blood (D) and quantified data for bone marrow (BM), peripheral blood (PB), and peritoneal (PE) (E) cells from NLC or ERK5-MKO mice. **F,** Maturation levels of peritoneal macrophages from NLC and ERK5-MKO mice identified by the expression of CD115 and F4/80 markers. **G,** Representative flow cytometry data on AC phagocytosis by NLC and ERK-MKO macrophages *in vitro* after 60 min of incubation with CMFDA-labeled ACs at a 1:5 (BMDMs:ACs) ratio (n=5). **H-K,** *In vivo* clearance and phagocytosis assays. **H,** CMFDA-labeled ACs were injected intravenously and 12 hrs later, splenic macrophages (CD11b<sup>+</sup>) that have ingested labeled ACs were quantified by flow cytometry. **I,** CMFDA-labeled ACs were injected into the peritoneum of NLC and ERK5-MKO mice and 6 hrs later, free fluorescent cells
in the peritoneal cavity were quantified. J, Representative images showing phagocytized and free ACs. Anti-CD-11b staining (red) was used to identify macrophages. Delayed clearance of ACs by resident peritoneal macrophages in ERK5-MKO mice is illustrated. K, Phagocytic index 6 hrs after ACs injection. See Methods for detail. H, I, and K, means ± S.D. (n=4), *P < 0.05.

L, BMDMs isolated from NLC and ERK5-MKO mice were fed ACs 24 hrs prior to re-feeding them with CMFDA-labeled ACs. Macrophages with engulfed fluorescent ACs were quantified by flow cytometry 24hrs later. The phagocytic index was determined as described in Material and Methods. means ± S.D. (n=4). **P < 0.01. Comparison between independent two groups was subjected to Wilcoxon rank-sum test.

Figure 2. Macrophage ERK5 regulates expression of efferocytosis-related genes. A-C, Relative mRNA (A, B) and protein (C) expression of PPARs and opsonins in NLC and ERK5-MKO peritoneal macrophages were determined by quantitative RT-PCR (qPT-PCR) or Western blotting, respectively. means ± S.D. (n=4-5). CD36 and CD68 were used as control genes. *P < 0.05 D, Quantification of PPARδ expression is shown as fold change compared to the NLC BMDMs after normalization to the internal tubulin control. means ± S.D. (n=6). **P<0.01. E, Overexpression of CA-MEK5 in BMDMs increases mRNA expression of opsonins. Relative mRNA levels of indicated opsonins were assessed by qPT-PCR after Ad-CA-MEK5α or Ad-LacZ transduction. means ± S.D. (n=4). *P < 0.05. Hemagglutinin (HA) tagged CA-MEK5α expression was detected by immunoblotting with anti-HA (d, inset). F,G, Overexpression of CA-MEK5α increased Thbs1 (F) and Mfg-e8 (G) promoter activity via PPARδ. RAW264.7 cells were transfected with control siRNA (si-Control) or PPARδ siRNA (si-PPARδ) for 48 hrs followed by transfection of Thbs1-luc or Mfg-e8-luc promoter for further 18 hrs. The cells were
transfected with ERK5 or CA-MEK5α and then assayed for firefly and Renilla luciferase activities. means ± S.D. (n=6). *P < 0.05 and **P < 0.01. **H, Depletion of PPARδ protein by siRNA was confirmed by immunoblotting with anti-PPARδ and anti-tubulin (control). Comparison between independent two groups was subjected to Wilcoxon rank-sum test. Figs. 2F and G subjected to multiple group analysis using Kruskal-Wallis test followed by Dunn’s post hoc analysis.

Figure 3. ERK5 has a key role in induction by ACs feeding of efferocytosis-related genes in cultured BMDMs. A-C, Incubation of BMDMs with ACs for 16 hrs increased mRNA expression of opsonins (n=5) (A) and proteins involved in “eat-me” (n=4) (B) and “find-me” (n=4) (C) signals in an ERK5-dependent manner. Downregulation of CD47 (“don’t-eat-me” signal) expression by AC feeding did not occur in ERK5-MKO macrophages. means ± S.D. NLC vs. NLC+ACs; *P < 0.05 and **P < 0.01, and NLC+ACs vs. ERK5-MKO+ACs; #P < 0.05 and ##P < 0.01. D, Relative mRNA levels of inflammatory (left) and anti-inflammatory (right) genes in BMDMs isolated from NLC or ERK5-MKO mice were assessed by qPT-PCR. means ± S.D. (n=4). *P < 0.05 and **P < 0.01. E, Protein expressions of inflammatory (iNOS and MCP-1) and anti-inflammatory (Arg-1 and IL-10) proteins in BMDMs isolated from NLC or ERK5-MKO mice were assessed by Western blotting. Comparison between independent two groups was subjected to Wilcoxon rank-sum test. Figs. 3A, B, and C subjected to multiple group analysis using Kruskal-Wallis test followed by Dunn’s post hoc analysis.

Figure 4. ERK5 regulates phagocytic capacity of macrophages via Mfg-e8 secretion. A, B, Reduced phagocytic capacity of ERK5-MKO macrophages was restored by serum from NLC
mice (A) or purified recombinant Mfg-e8 (B). NLC and ERK5-MKO BMDMs were pre-treated with serum from NLC or ERK5-MKO mice (A) or purified Mfg-e8 (11 μg/ml) (B) for 24 hrs, and the phagocytic index was analyzed by incubation with CMFDA-labeled ACs at a 1:5 (BMDMs:ACs) ratio for 60 min as described in Fig. 1. means ± S.D. (n=5). *P < 0.05 and **P < 0.01. C, Decreased levels of Mfg-e8 in the serum of ERK5-MKO mice as quantified by ELASA. means ± S.D. (n=4). **P < 0.01. D, Circulating Mfg-e8 was determined by immunoprecipitating Mfg-e8 from serum followed by immunoblotting with anti-Mfg-e8 (Upper panel) and the graph represents densitometry data (Lower panel). means ± S.D. (n=4). **P < 0.01. E, ERK5 regulates opsonin expression in the liver. Relative mRNA levels of indicated opsonins in the liver of NLC and ERK5-MKO mice were assessed by qPT-PCR. means ± S.D. (n=5). *P < 0.05 and **P < 0.01. Comparison between independent two groups was subjected to Wilcoxon rank-sum test.

Figure 5. Pitavastatin upregulates opsonin expression via ERK5 activation. A, Pitavastatin increased ERK5 phosphorylation in BMDMs isolated from NLC mice after 3 hrs of treatment. B, RAW264.7 cells were transfected with pBind-ERK5 and pG5-luc for 18 hrs and then ERK5 transcriptional activity was assessed after 6 hrs of pitavastatin treatment or Ad-CA-MEK5 transduction. means ± S.D. (n=5). **P < 0.01. C, Incubation of RAW264.7 cells with 1 μM pitavastatin or ACs, but not NCs, for 6 hrs increased ERK5 transcriptional activity. Vehicle (veh); PBS, means ± S.D. (n=5). **P < 0.01. D, Relative mRNA levels of indicated opsonins and receptors in NLC and ERK5-MKO BMDMs treated for 18hrs with 1 μM pitavastatin were assessed by qPT-PCR. means ± S.D. (n=5). NLC vs. NLC BMDMs; *P < 0.05 and **P < 0.01, NLC BMDMs vs. NLC BMDMs + pitavastatin; #P < 0.05 and ##P < 0.01. E, Pitavastatin
increases Thbs1 and Mfg-e8 promoter activities. NLC and ERK5-MKO BMDMs were transfected with Thbs1-luc or Mfg-e8-luc for 18 hrs and stimulated with 1 μM pitavastatin for 8 hrs. The cells were assayed for firefly and Renilla luciferase activities. means ± S.D. (n=5). *P < 0.05 and **P < 0.01. F, Relative mRNA levels of indicated opsonins in the liver from NLC mice after 18 hrs of pitavastatin treatment (40 mg/kg) were assessed by qRT-PCR. means ± S.D. (n=5). *P < 0.05 and **P < 0.01. Comparison between independent two groups was subjected to Wilcoxon rank-sum test. Figs. 5B, C, D, and E subjected to multiple group analysis using Kruskal-Wallis test followed by Dunn’s post hoc analysis.

**Figure 6.** Accelerated atherosclerotic plaque formation in ERK-MKO/LDLR−/− mice. A, Whole aortas from each mouse group were stained by Oil-red O. C, Sections from the proximal aortas of each group were stained by H&E. Scale bars: 10 μm. B, D, The area covered by Oil-red O staining is expressed as percent of the total surface area of aorta from NLC/LDLR−/− and ERK5-MKO/LDLR−/− mice (B). The area of atherosclerotic plaques in sections of the proximal aorta was measured (D). means ± S.D. (B, n=20; D, n=15 per genotype). **P < 0.01. Comparison between independent two groups was subjected to Wilcoxon rank-sum test.

**Figure 7.** Increased apoptotic cell accumulation and necrotic core formation in ERK-MKO/LDLR−/− mice. A-C, Sections of proximal aortas from each group were labeled by TUNEL to detect apoptotic cells and counterstained with eosin to detect nuclei. Total TUNEL-positive (TUNEL+) cells were subdivided into intact (arrows) and disintegrated (sum of fragmented [open arrowhead] and degraded [closed arrowhead]) cells. Scale bars: 50 μm. B, The graph shows the percentage of TUNEL-positive cells (TUNEL+ cells/total cells counted) in the lesion area. Over
200 cells were counted in each section (n=5-6 per genotype). **P < 0.01. C, The percentage of disintegrated TUNEL-positive cells (disintegrated TUNEL\textsuperscript{+} cells/total TUNEL\textsuperscript{+} cells) in the lesion area (n=5-6 per genotype). **P < 0.01. D, Cleaved caspase 3 staining in the lesion area. Scale bars: 40 \( \mu m \). E, The area occupied by the necrotic core (acellular lipid core) is shown as percentage of total lesion area. Data are expressed as means ± S.D., n=7 per genotype **P < 0.01. F, Apoptosis was induced by incubation with oxLDL (100\( \mu g/ml \)) or PBS vehicle for 24 hrs in peritoneal macrophages isolated from ERK5-MKO or NLC mice and detected by TUNEL staining. G, The percentage of TUNEL-positive cells. means ± S.D., (n=5 per genotype). H, Cleaved caspase 3 expression induced by oxLDL in peritoneal macrophages isolated from ERK5-MKO or NLC mice. I, The area of atherosclerotic plaques in sections of the proximal aorta was stained with an antibody against MCP-1 (M1 phenotype) or IL-10 (M2 phenotype) and values represent the number of MCP-1 or IL-10 cells/% of total intimal lesion area. means ± S.D. (n=4 per genotype). **P < 0.01. Comparison between independent two groups was subjected to Wilcoxon rank-sum test.

**Figure 8.** Collagen content, fibrous cap thickness, and opsonin expression in atherosclerotic plaques in ERK-MKO mice. A, C, Masson’s trichrome staining for collagen (blue) in the lesions of NLC/LDLR\textsuperscript{−/−} and ERK5-MKO/LDLR\textsuperscript{−/−} mice. Scale bars: 60 \( \mu m \). B, Collagen in the lesion areas is expressed as the ratio of collagen (blue)/total intimal lesion area. means ± S.D., (n=9 per genotype). **P < 0.01. C, The area outlined by dotted lines is the fibrous cap and the distance between dotted lines was measured as the thickness of fibrous caps. The fibrous cap area (purple and blue) is composed of smooth muscle cells, macrophages, foam cells, and collagen. Scale bars: 100 \( \mu m \). D, Fibrous cap thickness is quantified in the advanced lesions. means ± S.D., (n=9 per genotype). **P < 0.01. Scale bars: 60 \( \mu m \). E, Expression and localization
of Mfg-e8 in advanced atherosclerotic lesions after feeding western diet for 8 and 16 weeks. Sections from the proximal aortas of each group were stained by control IgG or antibodies against SMA, Mac-3, or Mfg-e8. Scale bars: 50 μm. Comparison between independent two groups was subjected to Wilcoxon rank-sum test.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
ERK5 Activation in Macrophages Promotes Efferocytosis and Inhibits Atherosclerosis

Kyung-Sun Heo, Hannah J. Cushman, Masashi Akaike, Chang-Hoon Woo, Xin Wang, Xing Qiu, Keigi Fujiwara and Jun-ichi Abe

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Antibodies, siRNA, and reagents

Anti-phospho-ERK5 (T218/Y220, #3371), rabbit anti-ERK5 (#3372), Arg1 (#9819), and cleaved caspase 3 (#9664) were purchased from Cell Signaling. Antibodies made against Thbs1 (#SC-73158), C1qα (#SC-25856), PPARδ (#SC-74517), CD68 (#SC7084), MCP-1 (#SC-FL-148), and CD45 (#SC-70700) were purchased from Santa Cruz. Anti-CD3 (#D2712) and iNOS (#160862) were purchased from Thermo Scientific and Cayman Chemical, respectively. Human recombinant Thbs1 was purchased from Sigma-Aldrich (#SRP4805). Antibodies utilized for IHC were against smooth muscle actin (SMA; #M0851, DAKO), MAC3 (#550292, BD), Mfg-e8 (#D199-3, MBL), CD3 (#ab5690, Abcam), and IL-10 (#ABF13, Millipore). The ABC kit, secondary antibodies (biotinylated horseradish peroxidase conjugates), and diaminobenzidine (DAB) were purchased from Vector Laboratories (Burlingame, CA). CellTrcker™ Green CMFDA was from Molecular Probes (#C7025). The recombinant human Mfg-e8 (#2767-MF, R&D Systems), Thbs1 (#SRP4805, Sigma-Aldrich), and Mfg-e8 ELISA kit (DY2805, R&D Systems) were also purchased.

Plasmids

The Thbs1-*luc* reporter (Dr. Paul Bornstein, Addgene plasmid 12409)\(^\text{17}\) was obtained from the non-profit Addgene Plasmid Repository. The plasmid encoding Mfg-e8-2k-*luc* reporter was a kind gift from Dr. Iyoko Katoh (University of Yamanashi, Japan)\(^\text{18}\). Gal4-
ERK5 was created by inserting mouse ERK5 isolated from pcDNA3.1-ERK5 into BamH1 and Not1 sites of the pBIND vector. An adenovirus vector containing constitutively active (CA)-MEK5α was subcloned into the pENTR vector (Invitrogen) using specific enzyme sites, and then a recombinase reaction was performed to get a pDEST-based vector following manufacture’s instruction (#K4930-00, ViraPower Adenoviral Expression System, Promega). All constructs were verified by sequencing.

Mice

All animal procedures used in this study were approved by the Animal Care and Use Committee of the University of Rochester. All animals were housed in a temperature-controlled room under a light (12 hrs)/dark (12 hrs) cycle and under pathogen-free conditions. Macrophages deficient in ERK5 were obtained from ERK5fl/fl mice crossed with LysMCre−/+ (C57BL/6 background) mice (ERK5-MKO). For atherosclerosis studies, these mice were crossed with LDLR−/− (C57BL/6J background) mice (ERK5-MKO/LDLR−/−). At 8 weeks of age, mice were placed on a high-cholesterol diet (D01061401C, Research Diets, Inc.) for 8 or 16 weeks. We prepared apoptotic cells by culturing thymocytes from 6- to 8-week old C57BL/6J WT mice in serum-free RPMI medium for 16-18 hrs or by treating them with dexamethasone (100 nM) for 3 hrs. For FACS and microscopy analyses, we labeled apoptotic thymocytes by incubating them with 2 mM CMFDA (Molecular probes) for 30 min before feeding them to macrophages.

Mouse and human macrophage culture
Bone marrow-derived macrophages (BMDMs) were obtained from bone marrow cells isolated from femurs and tibias and cultured at the density of $1 \times 10^6$ cells/ml in DMEM (#11965118, Invitrogen) medium containing 10% FBS (v/v; Hyclone) and 10% (v/v) of the spent medium of L929 cells as the source of macrophage colony stimulating factor (M-CSF) for 5-8 days at 37˚C and 5% CO$_2$ in air. Peritoneal neutrophils and macrophages were obtained after i.p. injection of 2 ml autoclaved 3% (w/v) thioglycolate in H$_2$O (Sigma-Aldrich). After 3 days, peritoneal cells were harvested by injecting 10 ml DMEM containing 10% FBS into the peritoneal cavity, the abdomen gently massaged to dislodge cells, and cell suspension was collected. The mouse monocyte/macrophage cell line, RAW264.7 was obtained from ATCC (#TIB-71) and was propagated in DMEM containing 10% FBS at 37˚C and 5% CO$_2$ in air. The human monocyte/macrophage cell line, THP-1 was obtained from ATCC (#TIB-202) and was cultured in RPMI-1640 medium (#30-2001, Invitrogen) containing 10% FBS and 2-mercapto-ethanol at the final concentration of 0.05 mM at 37˚C and 5% CO$_2$ in air.

**Promoter activity and ERK5 transcriptional activity**

RAW264.7 and BMDMs were plated on 12-well plates at $5 \times 10^4$ cells/well. For a promoter assay, cells were transfected in Opti-MEM (#31985-088, Invitrogen) with Lipofectamine mixture containing the Mfg-e8-2k-luc or Thbs1-luc vector along with pRL-tk for 4 hrs. Cells were washed and fresh complete medium was added. Since pRL-tk contains the Renilla luciferase gene, the expression and transfection efficiencies were normalized with the Renilla luciferase activity. To detect ERK5 transcription activity, cells were transfected with Gal4-ERK5 and the Gal4-responsive luciferase reporter pG5-
Luc in Opti-MEM containing Lipofectamine 2000 (#11668-019, Invitrogen) per manufacture’s instruction. Opti-MEM was replaced by fresh complete culture medium 4 hrs post transfection. The cells were collected 36 hrs after transfection, and the luciferase activity was assayed with the dual luciferase kit (E1960, Promega) using a TD-20/20 luminometer (Turner Designs, CA). Transfections were performed in triplicate, and each experiment was repeated at least three times.

**Western blotting**

Expression levels of ERK5, C1qa, Gas6, Mfg-e8, Thbs1, Anxa1, and PPARδ in PE macrophages or BMDMs from NLC and ERK5-MKO mice were quantified by Western blotting. Macrophages were fed ACs for 8 hrs and whole cell lysates were prepared in RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 % Nonidet P-40, 0.1% SDS, 1 mM dithiothreitol, 1:200-diluted protease inhibitor cocktail (#P8340, Sigma-aldrich), and 1 mM PMSF). Total lysates were resolved by SDS-PAGE and electrotransferred onto a Hybond enhanced chemiluminescence nitrocellulose membrane, which was then incubated with antibodies against each of the proteins to be detected in the lysate. Bound antibodies were visualized by using the enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

**Real-time PCR assay**

Total RNA from mouse macrophages or tissue was extracted using the TRIzol reagent according to the manufacturer's instructions. Reverse transcriptase reactions (PCR) were
performed in 20 µl mixtures containing 1 mg of total RNA according to the manufacture’s protocol (Bio-Rad, #170-8890). For real-time PCR, mouse primers including Mertk (#MP202617), C1qa (#MP202861), C1qb (#MP202862), C1qc (#MP202863), gas6 (#MP205762), thbs1 (#MP216930), and Mfg-e8 (MP208190) were purchased from Origene with qSTAR qPCR primers. In addition, the following specific primers were designed using Primer Express 3.0 software (Tables 1 and 2).

Table 1. Specific primers for mouse ERK5, PPARs, Eat-me, Find-me, Don’t-eat-me and GAPDH

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Table 2. Specific primers for human opsonins, anti-inflammatory genes and GAPDH

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Real-time PCR was performed using the MyiQ™2 Two-Color Real Time PCR System (Bio-Rad) and SYBR Green (Bio-Rad) to measure gene expression. The cycling program was set as follows: thermal activation for 10 min at 95 °C and 40 cycles of PCR (melting for 15 s at 95 °C, followed by annealing/extension for 1 min at 60 °C). Specific gene expression data were normalized to GAPDH gene expression.

**In vitro apoptotic cell uptake and clearance**

To make apoptotic thymocytes (ACs), thymocytes were isolated from C57BL/6 WT mice and cultured overnight in RPMI 1640 containing 10% FBS. Apoptosis was triggered by...
incubating the primary murine thymocytes with 100 nM dexamethasone for 3 hrs. The cells were then labeled with CellTracker™ Green CMFDA-FITC (#C7025, Molecular Probe). For uptake assays, we added ACs to BMDMs at a 5:1 thymocyte to macrophage ratio for 60 min. Double discrimination flow cytometry was used to distinguish internalized from externally bound ACs. Phagocytic index was calculated from images taken by a fluorescence microscope as described in our previous report. Briefly, we counted over 200 cells manually per sample and applied the following formula: 

\[
\frac{(\text{number of macrophages containing one ACs}) + 2 \times (\text{number of macrophages containing two ACs}) + 3 \times (\text{number of macrophages containing three ACs}) + 4 \times (\text{number of macrophages containing more than four ACs})}{\text{total number of macrophages counted}}.
\]

Necrotic thymocytes (NCs) were made by incubating them at 56°C for 10 min. Cellular necrosis was confirmed by Trypan blue staining. For double feeding experiments, BMDMs plated on six-well plates were incubated with ACs (1:5) for 24 hrs and then rechallenged with CMFDA-labeled ACs. One day later, phagocytic index was determined as described above. For rescue experiments, BMDMs were cultured in DMEM containing M-CSF (10 ng/ml; Sigma-Aldrich, M9170) and 10% serum from NLC or ERK5-MKO mice. For rescue experiments with recombinant Mfg-e8 and Thbs1, BMDMs cultured in DMEM with 1% FBS and M-CSF (10 ng/ml) were treated with purified human Mfg-e8 (11 µg/ml; R&D, #2767) or Thbs1 (11 µg/ml; Sigma-Aldrich, #SRP4805) for 1 hr before feeding them ACs (1:5 ratio).

**In vivo apoptotic cell uptake and clearance**

For the splenic uptake and clearance assays, \(6 \times 10^7\) CMFDA-labeled ACs were injected
intravenously into 8-week-old NLC or ERK5-MKO mice. The mice were sacrificed 12 hrs after injection and splenocytes were isolated. Splenic macrophages were purified using magnetic beads coated with anti-CD11b, and flow cytometry was used to determine CMFDA-labeled ACs uptake by these cells. For the clearance assay, CMFDA-labeled-ACs were similarly injected into the peritoneal cavity, and 6 hrs later, cells were recovered by peritoneal lavage and counted under a fluorescence microscope.

**FACS analysis**

Cell proliferation was assessed by measuring new DNA synthesis by flow cytometry after EdU (5-ethyl-2'-deoxyuridine) staining per manufacture’s protocol (Click-iT® EdU Alexa Fluor® 488 Cell Proliferation Assay Kit, Molecular Probe; #C35002). To determine macrophage maturation, bone marrow cells (BM), blood monocytes (PB), and peritoneal macrophages (PE) were isolated from 7 or 8-week-old NLC or ERK5-MKO mice. The cells in suspension were washed in PBS and blocked by incubating them with 1 µg of FC blocks (CD16/32, eBioscience; #14-0161) for 10 min at RT. After washing them in PBS to remove free blocking antibodies, the cells were fixed in PBS containing 4% paraformaldehyde for 10 min and permeabilized by resuspending them in 1 ml of ice cold methanol for 20 min. Cells were suspended in FACS buffer and stained with PE anti-CD115 (Biolegend; #135506), APC-Cy7 anti-Gr1 (Ly6C/G, BD Biosciences; #557661), and Alexa Fluor® 647 anti-F4/80 (Biolegend; #123122), all at 1:1000 dilution, for 30 min and analyzed by flow cytometry.

**Analysis of opsonin (Mfg-e8) in serum**
Two mg of protein in the serum diluted in 1 ml of RIPA buffer was immunoprecipitated with 2 mg of anti-Mfg-e8 (SC-33546, Santa Cruz) for 18 hrs. Immunoprecipitates were captured by Protein A/G agarose beads (Invitrogen), washed to remove unbound protein, and bound Mfg-e8 determined by immunoblotting with anti-Mfg-e8 (D199-3, MBL). Circulating levels of Mfg-e8 in serum were also quantified using a commercially available ELISA kit per manufacturer’s protocol (R&D systems, DY2805).

**Metabolic parameters**

Mice were anesthetized and blood samples were collected from the abdominal artery. Plasma was prepared and total cholesterol, HDL, and non-HDL (LDL and VLDL) were measured enzymatically using commercially available kits (Abcam, #ab65390) according to the manufacturer’s instructions. For glucose tests, overnight-fasted mice were given an i.p. glucose (2 mg/g body weight) injection, and blood was collected from tail vein before (time 0) and at indicated times after injection for glucose measurement (Glucometer Elite; Bayer).

**Tissue preparation, histology, and quantification of the lesion size**

ERK5-MKO/LDLR<sup>+/−</sup> and NLC/LDLR<sup>+/−</sup> female mice were fed a high-cholesterol diet for 8 or 16 weeks and sacrificed by CO<sub>2</sub> inhalation. The arterial tree was perfused via the left ventricle with saline containing heparin (40 USPU/mL), followed by formaldehyde (4%, pH 7.3) in PBS for 10 min. The full-length of the aorta to the iliac bifurcation was dissected out and opened along the ventral midline. En face preparations were washed in distilled water, dipped in 60% isopropyl-alcohol, and stained for 40 min with 0.16% Oil-
Red-O dissolved in 60% isopropyl-alcohol/0.2 mol/L NaOH. Stained images were captured with a digital camera mounted on a Leica stereomicroscope and analyzed using Adobe Photoshop Extended software. The aortic sinus area attached to the heart was dissected after fixation as described earlier, embedded in paraffin and sections (5 µm) were cut. Serial sections were made through the entire aortic valve area and stained with hematoxylin and eosin (H&E) and Masson’s trichrome staining. The necrotic core was defined as an area free of H&E staining. The fibrosis cap was defined as the tissue between the vessel surface and the top edge of the necrotic core and the thickness of this tissue was measured at several places within each section of at least 3 consecutive serial sections. Plaque areas and necrotic core areas were also quantified. These studies were done blindly and the data was analyzed using ImageJ 1.64 (available as freeware from http://rsbweb.nih.gov/ij/).

**Immunohistochemistry**

To identify specific cells such as macrophages and smooth muscle cells (SMCs) and to detect specific protein expression within the plaque area, immunohistochemistry (IHC) was performed using the VECTASTAIN ABC system (Vector laboratories). Paraffin sections were blocked with 3% H$_2$O$_2$. Epitope retrieval (HIER) was performed by heating sections in the HIER buffer containing 10 mM sodium citrate and 0.05% Tween 20 (pH 6). The samples were blocked with 5% normal serum (Vector laboratories) matching the host of the secondary antibody for 30 min at room temperature (RT). Primary antibodies were against Mac3 (1:500, mouse monoclonal), alpha-actin (1:500, mouse monoclonal), Mfg-e8 (1:100, mouse monoclonal), and Thbs1 (1:100, mouse monoclonal).
monoclonal). Secondary antibodies (goat anti-mouse or anti-rabbit) were used at 1:500 dilutions. Sections were developed by DAB substrate (Vector laboratories) and counterstained with hematoxylin.

**In situ TUNEL and macrophage apoptosis assays**

To examine apoptosis, the aortic valve area was fixed and embedded in paraffin and sectioned (5 µm). TUNEL staining was performed using ApopTag® Peroxidase *In Situ* Apoptosis Detection Kit (#S7100, Millipore) following the manufacturer’s protocol. Briefly, paraffin sections were blocked for endogenous peroxidase activity (3% hydrogen peroxide in water) for 10 min at RT and treated with protein K (20 mg/ml) for 30 min. For a positive control, sections were treated with DNase I (1 mg/ml) for 10 min at RT. Terminal DNA labeling was performed at 37 °C for 1 hr with a reaction mixture containing fluorescein-conjugated nucleotides and terminal deoxynucleotidyltransferase enzyme. Slides were counterstained by eosin to identify nuclei. For each sample, a total of 200 nuclei were counted from 4 randomly selected fields and TUNEL positive cells were expressed as percent of total cells counted. Samples were examined using a 40X lens under an epi-fluorescence microscope (BX51, Olympus) equipped with a CCD camera (Spot, #22.1). To determine the active caspase 3 expressions in the section, samples were blocked with 5% Goat serum for 30 min at RT and treated with anti-caspase 3 overnight at 4 °C. Samples were then treated with biotinylated goat anti-rabbit IgG for 30 min at RT, followed by detection of biotin by DAB substrate (Vector laboratories) and counterstained with hematoxylin.
**Supplemental Expended Discussion**

ERK5 is not only a kinase, but also has transcriptional activity, which is critical for activating transcription factors such as Kruppel-like factor 2 (KLF2) and PPARs\(^5,6\). Previously we found the importance of ERK5 kinase activity in regulating ERK5 transcriptional activity\(^5\). In the study, we showed that dominant negative kinase form of ERK5 inhibited ERK5 transcriptional activity. Interestingly, we also found the kinase-independent regulation of ERK5 transcriptional activity\(^6\). We found that ERK5 transcriptional activity was inhibited by SUMOylation, which leads to the inhibition of KLF2 and eNOS expression\(^6\). We investigated whether SUMOylation of ERK5 could inhibit ERK5 phosphorylation and kinase activation. In here, we did not observe any difference in ERK5 phosphorylation and kinase activity between Ubc9 overexpressed and vector transfected cells. In addition, we did not find any difference in ERK5 phosphorylation between the ERK5 wild type and K6/22R SUMOylation sites mutant\(^6\). These data suggested that ERK5-SUMOylation did not change ERK5 phosphorylation and kinase activation, but inhibited its transcriptional activity and subsequent KLF2 and eNOS expression. Taken together, these data support the importance of both kinase and transcriptional activity of ERK5 in regulating its downstream events.

It is possible that ERK5 could phosphorylate or activate other molecules besides PPARs. The partial effect of PPAR\(\delta\) deletion on Thbs-1 or Mfg-e8 promoter activation as showed in Fig. 2F,G and 5E support this notion. Further investigation is necessary to clarify these issues.
Supplementary Figure 1. ERK5 expression in macrophages fed apoptotic T-cells.

(A) Peritoneal macrophages were fed apoptotic thymocytes for 0, 12, and 24 hrs and ERK5 expression was assayed by Western blotting using anti-ERK5. (B) Quantification of ERK5 expression is shown as fold changes compared to the control (first lane) after normalization to the internal tubulin control for each point. Shown is the mean ± S.D., (n =3). **P<0.01.
Supplementary Figure 2. Lack of a T cell antigen in macrophages fed apoptotic thymocytes.

(A) Apoptotic thymocytes were generated by serum starvation for 16-18 hrs as reported previously. Flow cytometric analysis of CD3 expression in apoptotic thymocytes. (B) Flow cytometric analysis of CD3 and NK1.1 expression in apoptotic thymocytes. The expression of NK1.1, which is a Natural killer T cell marker, is not detected. (C) BMDMs were either fed or not fed apoptotic thymocytes and macrophages or apoptotic thymocytes lysates immunoblotted for CD3, which is specifically expressed in lymphocytes. A representative experiment is shown from three independent experiments.
Supplementary Figure 3. Comparison of body weight, glucose tolerance, and plasma cholesterol levels between NLC/LDLR<sup>−/−</sup> and ERK5-MKO/LDLR<sup>−/−</sup> mice.

A, Body weight changes of NCL/LDLR<sup>−/−</sup> and ERK5-MKO/LDLR<sup>−/−</sup> mice fed with Western-type diet for 16 weeks show identical progression. Mean ± S.D. (n=8 per genotype).

B, After 16-week of Western-type diet, NCL/LDLR<sup>−/−</sup> and ERK5-MKO/LDLR<sup>−/−</sup> mice were fasted overnight (16 hours) before the glucose tolerance test. Glucose was injected intraperitoneally (2g/kg) and blood samples were taken before and at 0, 30, 60, 90, and 120 min after the injection for blood glucose measurements. means ± S.D., (n=6 per genotype). No difference was noted.

C, The same blood cholesterol profiles were observed in NCL/LDLR<sup>−/−</sup> and ERK5-MKO/LDLR<sup>−/−</sup> mice after 16 weeks on Western-type diet. means ± S.D., (n=6 per genotype).
Supplementary Figure 4. Blood cell counts in macrophage ERK5 deficient mice.

Blood cell counts in NLC and ERK5-MKO mice were made. ERK5 deficiency in macrophage did not affect the number of circulating red blood cells (RBC), monocytes (MCT), or lymphocytes (LYM). n=5 per genotype.
Supplementary Figure 5. Defective phagocytic capacity of ERK5-MKO macrophages was restored by purified Thbs1 protein.

NLC and ERK5-MKO BMDMs were treated with purified Thbs1 (11 ug/ml) for 24 hrs and fed CMFDA-labeled ACs at a 1:5 (BMDMs:ACs) ratio for 60 min. The phagocytic index was analyzed as described in Fig.1. means ± S.D. (n=5). *P < 0.05.
Supplementary Figure 6. Pitavastatin upregulates opsonin expression via ERK5 activation in human macrophages.

A, Pitavastatin increased ERK5 phosphorylation in THP-1 cells after 3 hrs of treatment. B, THP-1 cells were transfected with pBind-ERK5 and pG5-luc for 18 hrs and then ERK5 transcriptional activity was assessed after 6 hrs of pitavastatin treatment. means ± S.D. (n=3). **P < 0.01. C, Relative mRNA levels of indicated opsonins and a receptor in THP-1 cells after 18 hrs of 1 uM pitavastatin were assessed by qPT-PCR. means ± S.D. (n=3). *P < 0.05 and **P < 0.01. Vehicle (veh); PBS.
Supplementary Figure 7. Pitavastatin upregulates opsonin expression via ERK5 activation.

Relative mRNA levels of indicated opsonins and receptors in NLC and ERK5-MKO livers i.p. injected for 18hrs with 40mg/kg pitavastatin were assessed by qPT-PCR. *P < 0.05 and **P < 0.01 vs NLC livers, #P < 0.05 and ##P < 0.01 vs NLC livers + pitavastatin.
Supplementary Figure 8. Accelerated atherosclerotic plaque formation in ERK5-MKO/LDLR−/− mice.

A. Cholesterol profiles were determined in NCL/LDLR−/− or ERK5-MKO/LDLR−/− mice after feeding them a high cholesterol diet for 8 weeks. Mean ± S.D. (n=6 per genotype). There was no significant difference between these two groups. B,C. After 8 weeks of western-type diet, atherosclerotic lesions were quantified in NCL/LDLR−/− and ERK5-MKO/LDLR−/− mice. The quantified data are based on the whole aorta stained by Oil-red O (B) and sections from the proximal aorta stained by H&E (C) and expressed as mean percent lesion area relative to the total vessel surface area of the enface sample or the total valves area in the section. means ± S.D. (n=8 per genotype). **P < 0.01.
Supplementary Figure 9. Composition of immune cells in the atherosclerotic lesion area.

A,B, The area of atherosclerotic plaques in sections of the proximal aorta was stained with an antibody against CD3 (T lymphocytes) and Mac3 (macrophages) (A) values represent the number of CD3+ and Mac3+ cells/% of total intimal area (B), and the number of αSMA+ cells/% of total (intimal/medial) area. means ± S.D. (n=4 per genotype). **P < 0.01.
Supplementary Figure 10. Expression of PPARδ in the atherosclerotic lesion area.

A,B. The area of atherosclerotic plaques in sections of the proximal aorta was stained with an antibody against PPARδ (A) and values represent the number of PPARδ+ cells/mm² of total intimal area (B). means ± S.D. (P<0.0377 compared to NLC/LDLR−/−, n=5 per genotype).
Supplementary Figure 11. ERK5 activation and macrophage proliferation.

The BMDMs were transduced with Ad-LacZ (40 MOI) or Ad-CA-MEK5α (20 or 40 MOI) for 18 hrs. Cell proliferation was assayed by EdU (5-ethynyl-2’-deoxyuridine) incorporation for 30 min and labeled cells were quantified by flow cytometry. (n=3)
Supplemental Reference


