

Salicylate improves macrophage cholesterol homeostasis via activation of Ampk^S

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Abstract Atherosclerosis stems from imbalances in lipid metabolism and leads to maladaptive inflammatory responses. The AMP-activated protein kinase (Ampk) is a highly conserved serine/threonine kinase that regulates many aspects of lipid and energy metabolism, although its specific role in controlling macrophage cholesterol homeostasis remains unclear. We sought to address this question by testing the effects of direct Ampk activators in primary bone marrow-derived macrophages from Ampk $\beta 1$ -deficient ($\beta 1^{-/-}$) mice. Macrophages from Ampk $\beta 1^{-/-}$ mice had enhanced lipogenic capacity and diminished cholesterol efflux, although cholesterol uptake was unaffected. Direct activation of Ampk $\beta 1$ via salicylate (the unacetylated form of aspirin) or A-769662 (a small molecule activator), decreased the synthesis of FAs and sterols in WT but not Ampk $\beta 1^{-/-}$ macrophages. In lipid-laden macrophages, Ampk activation decreased cholesterol content (foam cell formation) and increased cholesterol efflux to HDL and apoA-I, effects that occurred in an Ampk $\beta 1$ -dependent manner. Increased cholesterol efflux was also associated with increased gene expression of the ATP binding cassette transporters, *Abcg1* and *Abca1*. Moreover, in vivo reverse cholesterol transport was suppressed in mice that received Ampk $\beta 1^{-/-}$ macrophages compared with the WT control. Our data highlight the therapeutic potential of targeting macrophage Ampk with new or existing drugs for the possible reduction in foam cell formation during the early stages of atherosclerosis.—Fullerton, M. D., R. J. Ford, C. P.

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Supplementary key words cholesterol efflux • adenosine 5'-monophosphate-activated protein kinase • lipid homeostasis • atherosclerosis • reverse cholesterol transport

Atherosclerosis is a chronic condition that stems from the delivery and unregulated uptake of circulating lipoproteins by macrophages in the vasculature. The retention and subsequent modification (oxidation, acetylation, or aggregation) of LDL particles results in the activation of endothelial cells at the branch points of arteries and increases the expression of various adhesion molecules, as well as chemoattractant proteins (1). This facilitates the recruitment and transmigration of circulating monocytes from the lumen to the subintimal space [for review see (2, 3)]. Infiltrating monocytes differentiate to macrophages (4) and upregulate scavenger receptors (5, 6) [such as scavenger receptor B1 (SR-B1), scavenger receptor-A (SR-A), and scavenger receptor CD36 (CD36)], which are

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Abbreviations: Acc, acetyl-CoA carboxylase; acLDL, acetylated LDL; Ampk, AMP-activated protein kinase; BMDM, bone marrow-derived macrophage; BTI, Biomedical Technologies Inc.; $\beta 1^{-/-}$, $\beta 1$ -deficient; CD36, scavenger receptor CD36; CIHR, Canadian Institutes of Health Research; Hmgcr, HMG-CoA reductase; LPDS, lipoprotein deficient serum; LSC, liquid scintillation counting; Lxr- α , liver X receptor α ; RCT, reverse cholesterol transport; SR-A, scavenger receptor-A; SR-B1, scavenger receptor B1; Srebp, sterol regulatory element binding protein.

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critical for the uptake of modified lipoproteins within the subendothelial space (7). The accumulation of lipid-laden macrophages (foam cells) results in the progression of atherosclerotic plaques. As atherosclerosis progresses, increased lipid content (mainly cholesteryl esters) of macrophages is intimately linked to the increased inflammatory tone in the plaque environment, and the infiltration and activation of numerous innate and adaptive immune cells (2, 3, 5, 8). Because the lipid burden of macrophage foam cells contributes directly to the inflammatory tone and risk of plaque rupture (3), strategies aimed at lowering the lipid content of foam cells may be useful therapeutically.

The physiological process known as reverse cholesterol transport (RCT), whereby cholesterol is removed from peripheral tissues and transported by HDLs to the liver for excretion through bile and ultimately the feces (9), has garnered therapeutic interest. In the macrophage, excess cholesterol is esterified to cholesteryl esters and stored in lipid droplets, leading to foam cell formation. However, cholesterol can be mobilized from lipid droplet stores (10, 11) and effluxed via active transport to extracellular acceptors, HDL and lipid-poor apoA-I, a process mediated by the ATP binding cassette transporters, *Abcg1* and *Abca1*, respectively (12–14). There is now strong evidence to suggest that increased cholesterol efflux from macrophage foam cells is a strong predictor of improved CVD risk profile (9, 15–17). Thus a mechanistic understanding of signaling pathways that promote cholesterol efflux and RCT is crucial for developing future therapeutic interventions.

AMP-activated protein kinase (Ampk) is a highly conserved serine/threonine kinase that activates catalytic processes to generate ATP (such as glucose uptake and FA oxidation), and inhibits anabolic pathways (such as lipid and protein synthesis) [for review see (18, 19)]. In mammals, Ampk exists as an $\alpha\beta\gamma$ heterotrimer (20), where the β subunit acts as a scaffold for the α -catalytic and γ -regulatory subunits (21). While there are many therapeutics [including the anti-diabetic drug metformin (22, 23)] that activate Ampk indirectly, the small molecule, A-769662 (24), and salicylate (25) increase Ampk activity directly through the $\beta 1$ subunit drug binding site (25–27). The activation of Ampk results in the inhibitory phosphorylation of acetyl-CoA carboxylase (*Acc*) (28, 29) and HMG-CoA reductase (*Hmgcr*) (30, 31), the rate-limiting enzymes controlling FA and cholesterol biosynthesis, respectively. Macrophages exclusively express an Ampk $\alpha 1\beta 1\gamma 1$ heterotrimer, which is essential for controlling rates of FA oxidation and reducing inflammation (32, 33); however, the importance of Ampk in controlling cholesterol metabolism has not been studied.

Aspirin (acetylsalicylate) is one of the most widely prescribed medications world-wide for the primary and secondary treatment of CVD. Aspirin irreversibly inhibits cyclo-oxygenases to disrupt prostaglandin synthesis (34) and reduces coagulation by inhibiting thromboxane A_2 production in platelets (35). It was initially thought that the benefits to aspirin therapy were entirely mediated by its anti-platelet effects; however, genetic disruption of

thromboxane A_2 synthesis and other anti-coagulant therapies do not display the same cardioprotective effect (36, 37). Upon ingestion, aspirin is rapidly deacetylated in the circulation to salicylate (38). Given the gastrointestinal and thrombotic side-effects of higher doses of aspirin and salicylate, a pro-drug of salicylate (salsalate), which is better tolerated, is now being used in clinical trials for CVD (TINSAL-CVD: NCT00624923). At clinical salsalate concentrations, salicylate directly activates Ampk and is required for increasing rates of FA oxidation in hepatocytes (25). The role of salicylates in regulating macrophage Ampk activity has not been studied.

Here we show that deletion of Ampk results in higher FA and cholesterol synthesis. Under lipid-laden conditions, Ampk $\beta 1$ -deficient ($\beta 1^{-/-}$) macrophages have more lipid accumulation and lower cholesterol efflux. In addition, direct activation of Ampk was able to restore cholesterol homeostasis in lipid-loaded macrophages through the suppression of lipid synthesis and foam cell formation, as well as stimulation of cholesterol efflux *in vitro* and *in vivo*.

MATERIALS AND METHODS

Mice

The generation and characterization of the Ampk $\beta 1^{-/-}$ mice has been previously described (39, 40). The Ampk $\beta 1^{-/-}$ and littermate WT control mice used in these studies were housed in specific pathogen-free micro-isolators, maintained on a 12 h light/12 h dark cycle with lights on at 0700, and had unlimited access to standard rodent chow and water. All animal experimental protocols used were approved by the McMaster University Animal Ethics Research Board and the University of Ottawa Animal Care Committee.

Cell culture

Bone marrow-derived macrophages (BMDMs) were generated as previously described (32). Briefly, mice were euthanized, tibias and femurs isolated, and the ends of each bone cut off. The tibia and femur from each leg were placed into a sterile 0.5 ml microfuge tube that had a hole punctured in the end with an 18 gauge needle, which was then placed inside a 1.5 ml microfuge tube before the addition of 100 μ l of DMEM (Invitrogen) to the 0.5 ml tube. Bone marrow cells were collected by centrifuging at 2,000 rpm for 4 min, resuspended and plated in 100 ml of DMEM supplemented with 10% FBS (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) in a T175 flask, and incubated at 37°C in a humidified atmosphere at 5% CO_2 . After 4 h, cells were plated into 10 cm tissue culture dishes in the presence of 20% L929 medium (as a source of macrophage colony stimulating factor) and left to differentiate for 7–8 days. One day prior to the experiment, cells were lifted into suspension in the existing L929-supplemented DMEM by gentle scraping and seeded into the appropriate plate for subsequent experiments.

Foam cell formation and lipid determinations

BMDMs were subjected to two distinct foam cell protocols. In foam cell protocol 1, BMDMs were incubated in the presence or absence of acetylated LDL (acLDL) (50 μ g/ml) [Biomedical Technologies Inc. (BTI)] for 30 h in the presence or absence of salicylate (3 mM) or A-769662 (100 μ M). In foam cell protocol 2,

cells were incubated with acLDL (50 $\mu\text{g}/\text{ml}$) for 30 h and then equilibrated for 12–16 h in 0.2% BSA DMEM stimulated in the presence or absence of salicylate (3 mM) and A-769662 (100 μM). At the completion of the incubation, cells were washed twice with PBS and cholesterol determined using the Amplex cholesterol kit (Invitrogen) as described (41).

Cholesterol efflux experiments

acLDL (50 $\mu\text{g}/\text{ml}$; BTI) was pre-equilibrated with 5 $\mu\text{Ci}/\text{ml}$ [^3H]cholesterol (Perkin Elmer) in DMEM supplemented with 5% lipoprotein deficient serum (LPDS) (BTI) for 12 h. BMDMs were then incubated with this radioactive mixture for a further 30 h. Radioactive medium was then removed and washed twice with PBS. Cells were then equilibrated in DMEM-LPDS supplemented with 0.2% BSA overnight (16 h) in the presence or absence of salicylate (3 mM), A-769662 (100 μM), or DMSO vehicle control. Fresh medium was then replenished in the presence or absence of 0.2% BSA, recombinant human apoA-I (5 $\mu\text{g}/\text{ml}$ -BTI), or human HDL (50 $\mu\text{g}/\text{ml}$; BTI), in the presence or absence of Ampk activators. After 24 h, medium was removed and radioactivity determined by liquid scintillation counting (LSC). Cells were lysed with 0.1 M NaOH and radioactivity determined by LSC. Efflux is expressed as a percentage of dpm for [^3H]cholesterol in medium/([^3H]cholesterol in medium + [^3H]cholesterol in cells) \times 100%. Efflux to either apoA-I or HDL was calculated by subtracting the effluxes of the wells containing only BSA without apoA-I or HDL from those containing it. The specific activity for each condition (genotype and treatment) was determined by assessing cholesterol mass and radioactivity prior to the addition of the apoA-I and HDL.

acLDL uptake

acLDL uptake was measured by determining the uptake of fluorescence-labeled acLDL (Dil-acLDL; BTI) as described previously (42). Briefly, BMDMs were plated into 24-well dishes and foam cells were induced by incubation with acLDL (50 $\mu\text{g}/\text{ml}$; BTI) for 30 h in the presence or absence of salicylate (3 mM), A-769662 (100 μM), or DMSO vehicle control. After foam cell formation, lipid-containing medium was removed and cells were incubated with Dil-acLDL (10 $\mu\text{g}/\text{ml}$) for 2 h. Dil-fluorescence was extracted using isopropanol and relative fluorescence intensity determined at 524 nm (excitation) and at 567 nm (emission) (43).

Lipogenesis and lipid extraction

BMDMs were labeled with [^3H]sodium acetate in the presence of 5 mM unlabeled sodium acetate, in the presence or absence of salicylate (3 mM), A-769662 (100 μM), or DMSO vehicle control for 16 h. Cells were then washed twice with ice-cold PBS and scraped in 200 μl PBS for lipid extraction. Lipids were extracted as described previously (44, 45). Radioactivity was determined by counting the chloroform phase containing total lipids or after saponification and extraction with petroleum ether, as previously described (45).

RNA isolation, cDNA synthesis, and quantitative PCR

RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was DNase-I treated (Invitrogen) and first strand synthesis was performed using SuperScript III reverse transcriptase (Invitrogen). cDNA was diluted 1:40 into ultrapure water, and mRNA expression determined using TaqMan assays (Invitrogen). For Sr-a determinations, primers were used as previously described (46). Relative expression was calculated using the $\Delta\Delta\text{Ct}$ method, as previously described (32, 47).

Western blotting

Cellular lysates were prepared, and Western blotting and quantification were performed as previously described (32). All antibodies were from Cell Signaling Technologies.

Flow cytometry

Cells were washed in PBS and removed from the wells via gentle pipetting into a 96-well 0.2 ml plate. Cells were then washed in PBS containing 1% BSA and pelleted at $500 \times g$. Cells were then incubated with 0.5 μg Fc block (CD16/32; BioLegend) for 30 min followed by incubation with the conjugated primary antibodies APC-CD36 (1:100; Miltenyl Biotec), FITC-SR-A (1:20; Miltenyl Biotec), and PE-SR-BI (1:20; Novus) for a further 30 min in a volume of 50 μl covered from light. Cells were then washed twice more with PBS containing 1% BSA and resuspended in a final volume of 200 μl . Cells were analyzed using a CyAnTM ADP analyzer and fluorescence intensity was calculated as fold increase over unstained genotype and treatment controls by FlowJo software (Tree Star).

In vivo RCT

In vivo RCT was performed as previously described (11, 48). In brief, BMDMs from Ampk $\beta 1^{-/-}$ and WT control mice were plated into 10 cm tissue culture plates. acLDL (50 $\mu\text{g}/\text{ml}$; BTI) pre-equilibrated with 5 $\mu\text{Ci}/\text{ml}$ [^3H]cholesterol (Perkin Elmer) in DMEM supplemented with 5% LPDS (BTI) for 12 h, was then added to the BMDMs for a further 30 h. Cells were washed and harvested by gentle scraping. Approximately 8×10^6 cells were injected into the intraperitoneal cavity of WT C57BL/6 mice. The radioactivity of each genotype preparation was determined prior to injection to account for changes in specific activity between isolations. Cellular cholesterol mass was also determined from each genotype to ensure equal lipid loading. Blood was collected at 48 h via cardiac puncture and livers were removed and weighed. Feces were collected over the 48 h period and total feces radioactivity was measured. Radioactivity was determined in serum, liver, and total feces (of equivalent wet weight) by LSC.

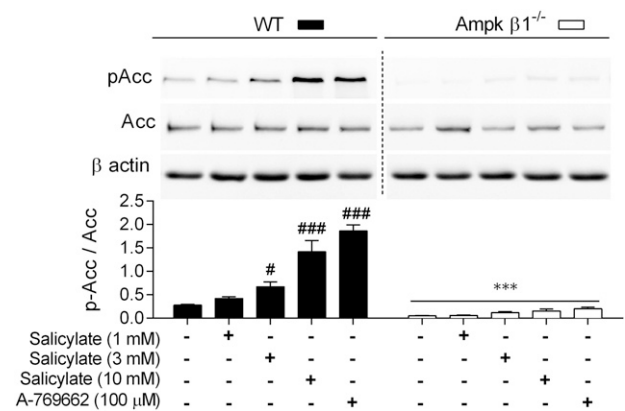


Fig. 1. Activation of Ampk signaling in primary BMDM. The phosphorylation of Acc at Ser 79 was assessed as a measure of Ampk activation in response to increasing concentrations of salicylate as well as A-769662 in BMDM from WT and Ampk $\beta 1^{-/-}$ mice. Duplicate gels were run for total Acc determination. WT and Ampk $\beta 1^{-/-}$ samples are loaded on the same gel, dotted line represents a cropped lane. Gels are representative of three separate bone marrow isolations per genotype, performed in duplicate. Data represent mean \pm SEM, where *** $P < 0.001$ compared within genotype and [#] $P < 0.05$ and ^{###} $P < 0.001$ are differences between treatment groups compared with vehicle control.

All [^3H]tracer measurements are expressed relative to the initial injected dose.

Statistics

All results are shown as mean \pm SEM. Results were analyzed using a two-tailed Student's *t*-test or two-way ANOVA where appropriate using GraphPad Prism software. A Bonferonni post hoc test was used to test for significant differences revealed by the ANOVA. Significance was accepted at $P \leq 0.05$.

RESULTS

We first sought to determine the efficacy of the direct Ampk $\beta 1$ activators in primary macrophages. Similar to hepatocytes (25), salicylate increased Ampk activity as indicated by increased phosphorylation of Acc1 (pSer 79) in a dose-dependent manner, as did A-769662, a result that we have shown previously (32) (Fig. 1). This effect was abolished in Ampk $\beta 1^{-/-}$ cells (Fig. 1). Neither treatment affected Ampk Thr172 phosphorylation (data not shown), which is in keeping with their allosteric mode of activation (24, 25, 27, 49). Phosphorylation of Acc1 by Ampk on Ser 79 inhibits its activity, resulting in reduced FA synthesis (22).

Similar to primary hepatocytes (22, 39), deletion of the Ampk $\beta 1$ subunit in BMDMs resulted in a higher lipogenic rate (incorporation of [^3H]acetate into lipids) compared with WT controls (Fig. 2A, B). The dysregulation of lipid synthesis resulted in a higher incorporation of labeled acetate into both FAs and sterols in Ampk $\beta 1^{-/-}$ BMDMs compared with WT cells (Fig. 2C, D). Upon Ampk activation with the direct $\beta 1$ activators salicylate and A-769662, both FA and sterol synthesis were inhibited in WT BMDMs, but were unaffected in the Ampk $\beta 1^{-/-}$ cells (Fig. 2A–D).

The progression of atherosclerosis stems from the unregulated uptake of modified lipoproteins by macrophages in the sub-endothelial space. We next treated WT and Ampk $\beta 1^{-/-}$ macrophages with acLDL to induce foam cell formation both in the presence and absence of Ampk activators. Exposure to acLDL caused a marked increase in cellular cholesterol content in cells from both genotypes (Fig. 3A), with no differences in triacylglyceride content (data not shown). When cells were lipid-loaded in the presence of salicylate or A-769662, there was a significant reduction in total cholesterol accumulation and foam cell formation in WT cells, but not Ampk $\beta 1^{-/-}$ cells. To further investigate how Ampk may regulate macrophage

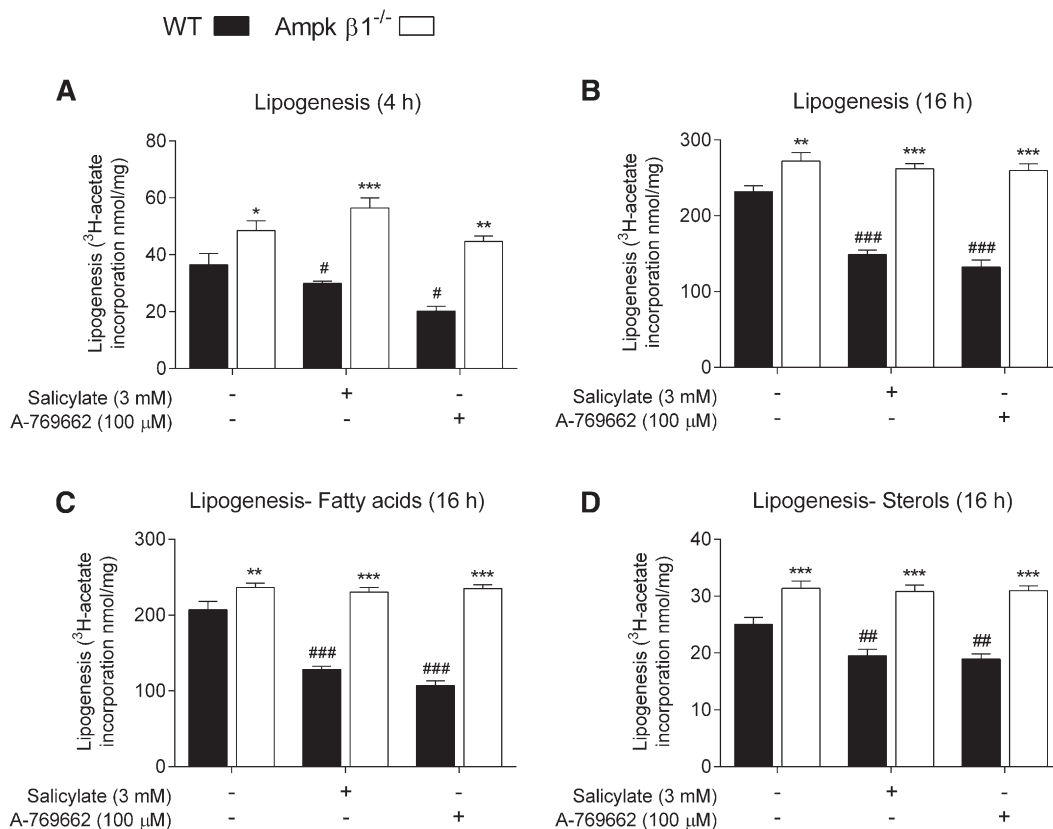


Fig. 2. Activation of Ampk alters basal lipid synthesis and content. The incorporation of [^3H]acetate into the total lipid fraction was measured as an indicator of lipogenesis after 4 h (A) and 16 h (B) treatments in the presence or absence of Ampk activators, A-769662 (100 μM), or salicylate (3 mM). Total lipids were then saponified and radioactivity determined from saponifiable (FAs) (C) and nonsaponifiable (sterols) (D) fractions, after 16 h. Data represent mean \pm SEM and are from three separate bone marrow isolations per genotype, performed in triplicate, where * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ are differences compared within genotype and # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ are differences between treatment groups compared with vehicle control.

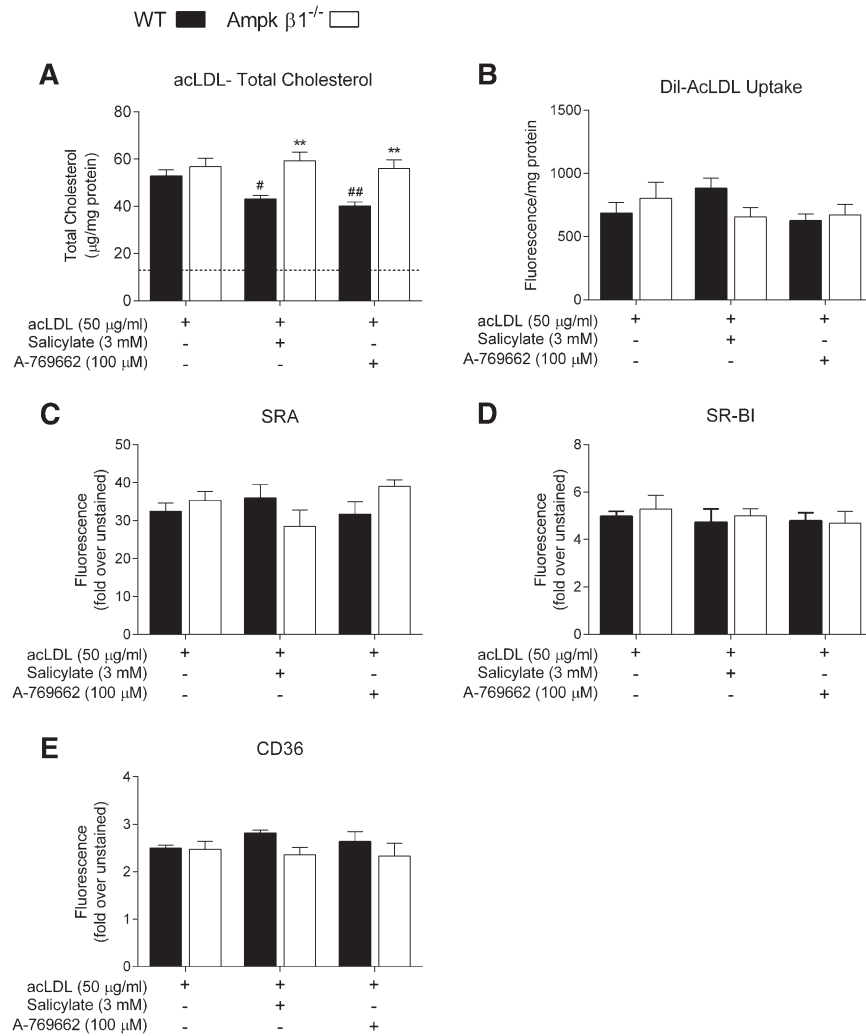


Fig. 3. Activation of Ampk alters cholesterol uptake and foam cell formation. WT and Ampk $\beta 1^{-/-}$ BM-DMs were lipid-loaded with acLDL (50 μ g/ml) for 30 h in the presence or absence of the Ampk activators, salicylate (3 mM), or A-769662 (100 μ M). After the loading period, total cholesterol (A) and the uptake of Dil-acLDL (B) (a further 2 h) were assessed. Surface expressions of scavenger receptors were determined by flow cytometry after lipid-loading in the presence or absence of Ampk activators. SR-A (C), SR-BI (D), and CD36 (E) were determined. Data represent mean \pm SEM and are from three separate bone marrow isolations per genotype, performed in triplicate, where ****** $P < 0.01$ are differences compared within genotype and **#** $P < 0.05$ and **##** $P < 0.01$ are differences between treatment groups compared with vehicle (acLDL) control.

cholesterol metabolism, we monitored the uptake of Dil-acLDL in BMDM cells that had been lipid-loaded in the presence or absence of the Ampk $\beta 1$ activators; however, there were no significant genotype or treatment differences (Fig. 3B). We next investigated the transcript and surface expression of the main scavenger receptor involved in acLDL uptake, SR-A, as well as SR-BI and CD36. There were no differences in transcript expression between genotype or treatment when cells were lipid-loaded in the presence of salicylate or A-769662 (supplementary Fig. 1A–C). To investigate further, we determined that the surface expression of SR-A, SR-BI, and CD36 were also unaltered (Fig. 3C–E). Because SR-A has been demonstrated to be the main effector by which acLDL is taken into the macrophage (50, 51), unaltered transcript and surface expression of SR-A (as well as other

scavenger receptors) is in keeping with the notion that Ampk activation has no effect on acLDL uptake.

The ability of macrophages to efflux cholesterol to extracellular acceptors is a critical component of RCT (9, 52). We treated BMDM cells with acLDL to induce foam cell formation, and then assessed cholesterol efflux to both HDL (mediated mainly by Abcg1) and lipid-poor apoA-I (mainly to Abca1) in the presence and absence of Ampk activators. Cholesterol efflux was lower in Ampk $\beta 1^{-/-}$ cells compared with WT control cells (Fig. 4A, B). Ampk activation via salicylate and A-769662 resulted in increased cholesterol efflux to both HDL and apoA-I (Fig. 4A, B), but only in WT macrophages, thus suggesting that Ampk-dependent processes contribute to macrophage RCT. Importantly, treatment with salicylate or A-769662 after the period of lipid loading

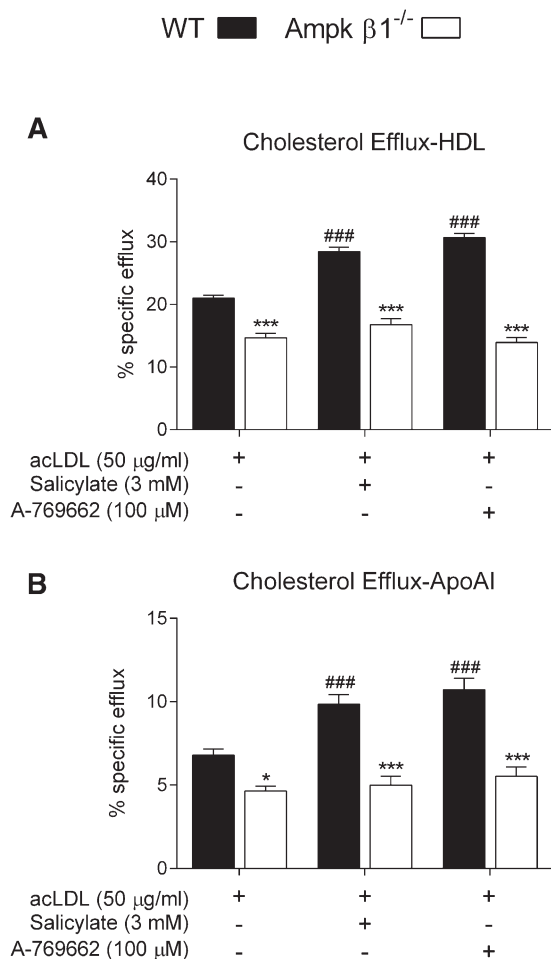


Fig. 4. Activation of Ampk increases cholesterol efflux. BMDMs were prepared from WT and Ampk $\beta 1^{-/-}$ mice and cells were labeled with acLDL (50 $\mu\text{g/ml}$) supplemented with [^3H]cholesterol (5 $\mu\text{Ci/ml}$). Percent specific efflux was measured to HDL (A) and apoA-I (B). Data represent mean \pm SEM and represent at least four separate bone marrow isolations per genotype, performed in triplicate, where * $P < 0.05$ and *** $P < 0.001$ are differences compared within genotype and ### $P < 0.001$ are differences between treatment groups compared with vehicle control.

did not alter cholesterol mass (supplementary Fig. 2), therefore specific activity was unaffected.

Given the significant effect of Ampk deletion on efflux to HDL and apoA-I, as well as the protective increase in efflux upon Ampk activation, we next assessed the transcript expression of the main cholesterol transporters in macrophages (48, 53). The expression of the Abcg1 and Abca1 transporters were reduced in Ampk $\beta 1^{-/-}$ BMDMs under vehicle-treated conditions (Fig. 5A, B), and both salicylate and A-769662 increased Abcg1 and Abca1 expression in WT but not Ampk $\beta 1^{-/-}$ cells (Fig. 5A, B). In addition to lipid transporter expression, we also monitored the expression of two important transcriptional regulators responsible for governing cholesterol homeostasis, liver X receptor α (Lxr- α) and sterol regulatory element binding protein (Srebp)2. Similar to the expression of Abca1 and Abcg1, Lxr- α expression was induced upon lipid loading, which occurred to a greater

extent in WT BMDMs (Fig. 5C). Exogenous lipid loading suppressed Srebp2 expression, although this effect was prevented in the Ampk $\beta 1^{-/-}$ macrophages (Fig. 5D). Ampk activation was associated with further increases in Lxr- α (Fig. 5C) and further suppression of Srebp2 (Fig. 5D).

We next investigated the role of macrophage Ampk in RCT in vivo. Radiolabeled, lipid-loaded macrophages from WT and Ampk $\beta 1^{-/-}$ mice (acLDL-[^3H]cholesterol) were injected into WT mice. We assessed radioactivity in the serum, liver, and feces after 48 h (Fig. 6A–C). Consistent with the observed genotype difference in cholesterol efflux, radioactivity in the serum ($P < 0.05$), liver ($P < 0.01$), and feces ($P < 0.001$) were all significantly lower in mice that received macrophages from Ampk $\beta 1^{-/-}$ mice, compared with WT (Fig. 6). Taken together, these data are entirely consistent with a regulatory role for Ampk in mediating the reverse transport of cholesterol.

DISCUSSION

Hyperlipidemia and other associated risk factors that predispose individuals to atherosclerosis lead to the overabundance of modified LDL-cholesterol and unregulated uptake via scavenger receptors, ultimately causing foam cell formation and atherogenesis (7). We show that Ampk plays an integral role in regulating macrophage cholesterol accumulation. The deletion of the Ampk $\beta 1$ subunit in macrophages results in a higher lipogenic rate and lower cholesterol efflux. Moreover, activation of Ampk using the direct Ampk $\beta 1$ activators salicylate and A-769662 resulted in a protective decrease in lipogenesis, cholesterol accumulation, and foam cell formation, and an increase in cholesterol efflux.

Ampk inhibits multiple facets of lipid metabolism. The synthesis of FAs is inhibited via phosphorylation of Acc1 at Ser 79, which reduces the production of the FA precursor malonyl-CoA (22, 29). Ampk also phosphorylates Hmgcr at Ser 871 (in mice), the rate-limiting enzyme in cholesterol synthesis (31); however, the physiological role of this regulation has yet to be examined in detail. In addition, Ampk directly phosphorylates Srebp1c (Ser 372) and Srebp2 (site unknown) (54), which are master transcriptional regulators that govern lipid levels via modulation of FA and cholesterol synthetic pathways, respectively. Upon activation of Ampk in macrophages, the synthesis of FAs and sterols was inhibited in WT cells, but not Ampk $\beta 1^{-/-}$ cells (Fig. 2). Ampk $\beta 1$ deficiency is associated with higher Srebp2 gene expression compared with WT control cells, and in response to Ampk activators, Srebp2 expression was significantly inhibited in WT cells, but not Ampk $\beta 1^{-/-}$ cells (Fig. 5D). The acute Ampk-specific inhibition of FA and sterol synthesis by salicylate and A-769662 after 4 h was likely mediated by the acute phosphorylation and inactivation of Acc1 and Hmgcr, respectively. However, during chronic experiments (24–30 h), the continued inhibition of lipogenesis and the modulation of lipid levels may reflect the net contribution of all affected pathways, including

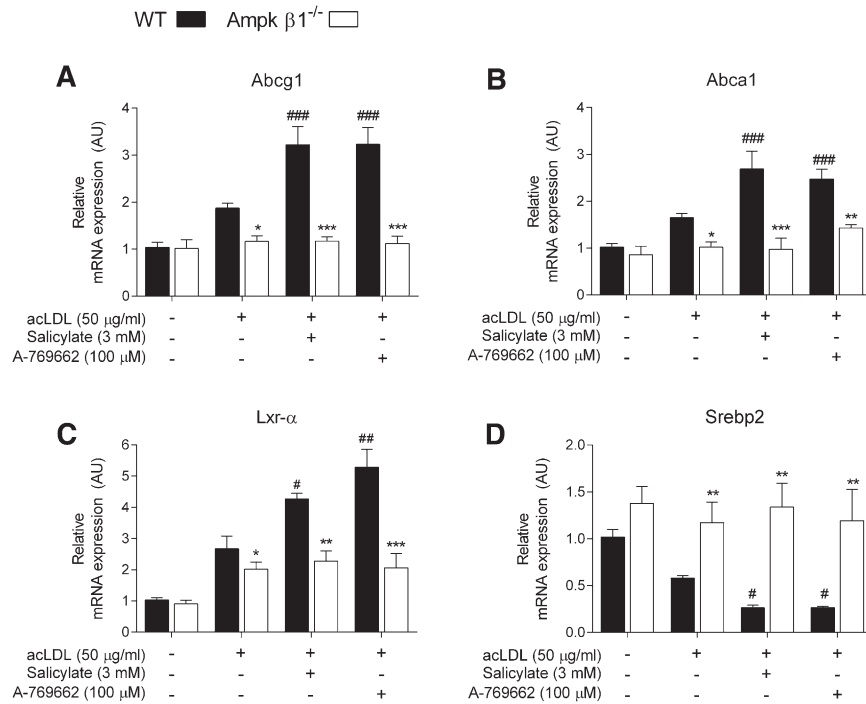


Fig. 5. Ampk activation is associated with increased transporter expression. BMDMs were prepared from WT and Ampk $\beta 1^{-/-}$ mice and treated in the presence or absence of acLDL (50 $\mu\text{g}/\text{ml}$) for 30 h and then in the presence or absence of A-769662 (100 μM) and salicylate (3 mM) for a further 24 h. Transcript expression of the transporters Abcg1 (A) and Abca1 (B), as well as the transcriptional regulators Lxr- α (C) and Srebp2 (D) were determined. Transcripts are shown relative to WT control in the absence of acLDL and expressed relative to β actin. Data represent mean \pm SEM and represent three separate bone marrow isolations per genotype, performed in quadruplicate, where * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ are differences compared within genotype and # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ are differences between treatment groups compared with vehicle (acLDL) control.

Srebp2. The relative contributions of the possible downstream regulators remain unclear.

In spite of differences in foam cell formation in the presence of Ampk activators (Fig. 3A), neither endogenous Ampk signaling, nor Ampk activation had any effect on the uptake of acLDL into lipid-loaded macrophages (Fig. 3B). This is consistent with previous studies demonstrating reductions in cholesterol accumulation with oxidized LDL and 5-aminoimidazole-4-carboxamide ribonucleoside treatment, although there were no measures of

uptake reported (55). In our experiments (supplementary Fig. 1) and those of Li et al. (55), this effect on foam cell formation was independent of alterations in scavenger receptor gene expression. Our investigation determined that the surface expression of key scavenger receptors (SR-BI and CD36), as well as the main receptor responsible for acLDL uptake, SR-A, were unaffected by Ampk signaling (Fig. 3C–E). This data suggests that Ampk activation alters cholesterol accumulations via uptake-independent mechanisms.

The ability of macrophages to efflux and the ability of HDL and other acceptors to transport cholesterol for the purpose of RCT have garnered therapeutic interest (15, 52, 56, 57). Many studies have used various cholesterol mobilization and transport protein knockout models to assess their role in macrophage RCT. Li et al. (55) demonstrated that 5-aminoimidazole-4-carboxamide ribonucleoside, an indirect Ampk activator, increased Abcg1-mediated efflux to HDL in oxidized LDL-loaded macrophages and endothelial cells (58), although the authors did not assess efflux to apoA-I, nor did they note a basal difference with transient transfection of a dominant negative Ampk. Human monocyte-derived macrophages incubated with the Ampk activators metformin and heme also demonstrate an increase in cholesterol efflux and stemming of foam cell formation (59). In the current study we found that exposure to direct Ampk activators

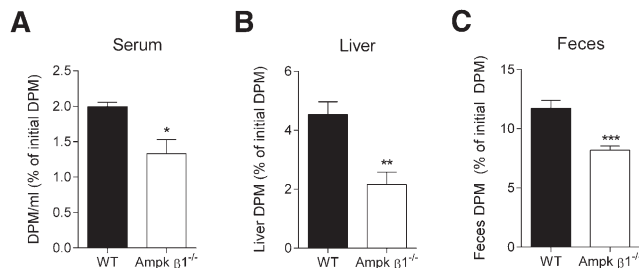


Fig. 6. Ampk facilitates RCT in vivo. Macrophages were prepared from WT and Ampk $\beta 1^{-/-}$ mice and lipid-loaded with acLDL (50 $\mu\text{g}/\text{ml}$ + [^3H]cholesterol). WT and Ampk $\beta 1^{-/-}$ cells were then injected into the peritoneal cavity of WT recipients and, after 48 h, radioactivity was determined in the serum (A), liver (B), and total feces (C). Data represent mean \pm SEM ($n = 6$ per group), where * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

increased Abcg1 and Abca1 expression and cholesterol efflux to both HDL and apoA-I (Figs. 4, 5). Basal levels of efflux and associated transporter expression were lower in Ampk $\beta 1^{-/-}$ macrophages, indicating that Ampk is important for controlling endogenous cholesterol efflux. In addition, we show for the first time that macrophage Ampk plays a critical role in mediating efficient *in vivo* RCT (Fig. 6). Interestingly, the process of *in vivo* RCT was diminished in Ampk $\beta 1^{-/-}$ macrophages (~30%) to a similar extent as cells that lack either Abca1 or Abcg1 (48, 53). This strongly suggests that Ampk is linked to important regulatory pathways that govern this process.

Our current study highlights a protective role for macrophage Ampk in regulating cholesterol metabolism. These effects are mediated through reductions in cholesterol synthesis under normal conditions and reductions in uptake and enhanced cholesterol efflux/RCT under lipid-loaded conditions. Importantly, we have tested A-769662 and salicylate and show that they reduce macrophage lipid synthesis and enhance cholesterol efflux via an Ampk $\beta 1$ -dependent pathway. While A-769662 has poor bioavailability (24), following salsalate and high-dose aspirin ingestion, salicylate is present in circulation at concentrations similar to those used in our studies (1–3 mM) (60, 61). Because Ampk $\beta 1$ is the predominant subunit in human macrophages (33), it is interesting to speculate that the activation of macrophage Ampk might mediate a portion of the beneficial effects of these drugs on CVD. Future studies testing this hypothesis in mouse models of atherosclerosis are warranted.

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