Macrophages represent a major immune cell population in atherosclerotic plaques and play central role in the progression of this lipid-driven chronic inflammatory disease. Targeting immunometabolism is proposed as a strategy to revert aberrant macrophage activation to improve disease outcome. Here, we show ATP citrate lyase (Acly) to be activated in inflammatory macrophages and human atherosclerotic plaques. We demonstrate that myeloid Acly deficiency induces a stable plaque phenotype characterized by increased collagen deposition and fibrous cap thickness, along with a smaller necrotic core. In-depth functional, lipidomic, and transcriptional characterization indicate deregulated fatty acid and cholesterol biosynthesis and reduced liver X receptor activation within the macrophages in vitro. This results in macrophages that are more prone to undergo apoptosis, whilst maintaining their capacity to phagocytose apoptotic cells. Together, our results indicate that targeting macrophage metabolism improves atherosclerosis outcome and we reveal Acly as a promising therapeutic target to stabilize atherosclerotic plaques.
Atherosclerosis is a lipid-driven chronic inflammatory disorder of the arteries in which macrophages are the most prevalent immune cells and define disease development\(^1\). One of the crucial initiating events in atherosclerosis is the infiltration and accumulation of cholesterol-rich lipoproteins within the intima of the arterial vessel wall. Subsequent modification of such lipoproteins induces an inflammatory response and as a result, monocytes are recruited to the vessel wall and differentiate into macrophages. Although macrophages can worsen disease progression by propagating inflammation, they can also stabilize atherosclerotic plaques by promoting the formation of a fibrous cap and by clearing apoptotic cells (ACs) to prevent necrotic core formation. Uptake of cholesterol gives macrophages their foamy appearance and this can lead to apoptosis\(^3\). Initially, ACs are efficiently cleared by neighboring macrophages in a protective process termed efferocytosis\(^8\). In advanced atherosclerosis, insufficient efferocytosis induces AC accumulation and necrotic core formation. In combination with thinning of the protective fibrous cap this can make plaques vulnerable to rupture and can cause cardiovascular events such as myocardial infarction and stroke\(^6\).

Given the prominent role of macrophages in the progression of atherosclerosis, modulating their responses is considered as a promising strategy to treat atherosclerosis\(^6\). The last decade, modulation of intracellular metabolic pathways has emerged as a new tool to reshape deranged macrophage functions and is considered as a new therapeutic opportunity\(^6\)–\(^9\). Indeed, recent immunometabolism research highlights that intracellular metabolic reprogramming is a key controller of macrophage activation\(^7\). High rates of glycolysis supports the inflammatory properties of macrophages that are activated with lipopolysaccharide (LPS), whereas increased mitochondrial oxidative phosphorylation supports interleukin (IL)-4 induced macrophage responses. Recent literature revealed an important role for ATP citrate lyase (Acly) in translating metabolic changes into altered macrophage phenotype and function\(^10\)–\(^12\).

Acly is a key metabolic enzyme that converts mitochondrial-derived citrate into acetyl-CoA and oxaloacetate within the cytosol. Acly-dependent acetyl-CoA incorporation into histone promotes chromosome accessibility and regulates both LPS- and IL-4-induced macrophage activation\(^10\)–\(^11\). In addition, acetyl-CoA fuels the synthesis of fatty acids, cholesterol, and the acetylation of non-histone proteins\(^13\)–\(^14\). Serving as a link between carbohydrate and lipid metabolism makes Acly a promising target to lower low-density lipoprotein (LDL) cholesterol levels and to reduce cardiovascular risk\(^15\)–\(^17\). Bempedoic acid is a competitive Acly inhibitor that is specifically activated in hepatocytes where it upregulates LDL receptor (LDLR) expression. Although promising data from clinical trials highlight that hepatic Acly inhibition reduces LDL cholesterol levels in hypercholesterolemic patients, the tools to study this key metabolic hub in macrophages in vivo remained absent.

Here, we first demonstrate that Acly is activated in inflammatory macrophages and in human atherosclerotic plaques. Next, we define its formerly unknown role in regulating both plaque and macrophage phenotype. Using a conditional genetic knockout mouse model, we find that Acly deficiency in myeloid cells induces a stable plaque phenotype as demonstrated by increased collagen content and fibrous cap thickness, along with a decreased necrotic core size. Further functional, lipidomic, and transcriptional characterization in vitro show that this in vivo plaque phenotype is linked to deregulated fatty acid and cholesterol biosynthesis and reduced liver X receptor (LXR) activation within the macrophages. This results in macrophage apoptosis, along with more efficient efferocytosis and increased clearance ACs. As such, we show that targeting macrophage Acly improve atherosclerosis outcome and can serve as a promising therapeutic target to stabilize atherosclerotic plaques.

### Results

**Acly is activated in inflammatory conditions.** Acly can be regulated at distinct levels, with phosphorylation at serine 455 serving as a key posttranslational modification to promote its enzymatic activity\(^18\). To study Acly expression and activity in inflammatory conditions, we first stimulated bone marrow-derived macrophages (BMDMs) with toll-like receptor 4 agonist (LPS) for 24 h as a prototypical model of inflammatory (also called classical or M1) activation. Although Acly gene expression and protein levels remained unaffected, we observed a marked increase of phosphorylated Acly (p-Acly) in inflammatory macrophages (Fig. 1a, b). As these data suggested that Acly activation might support inflammatory responses we next examined Acly phosphorylation within human atherosclerotic plaques. Whereas total Acly was present in most cells in and around the plaque, activated (p-)Acly predominantly colocalsed with CD45\(^+\)CD68\(^+\) macrophages in human atherosclerotic plaques (Fig. 1c, d, Supplementary Fig. 1a–c). In addition, unstable plaques showed increased abundance of activated Acly when compared to stable plaques (Fig. 1e). Rupture-prone plaque areas are known to be dominated by inflammatory macrophages\(^19\) and together with our observation that the levels of activated p-Acly were increased in inflammatory macrophages, this prompted us to study whether targeting macrophage Acly could improve atherosclerosis outcome.

**Macrophage Acly deletion stabilizes atherosclerotic plaques.** To examine the role of macrophage Acly in atherogenesis, we crossed Acly\(^\text{L/L}\) mice\(^20\) with mice expressing Cre recombinase under control of the Lyz2 promoter/enhancer regions (LysMccre) to generate a new genetic mouse model that specifically lacks Acly in the myeloid cell lineage (macrophages, monocytes, and neutrophils). After confirming the knockdown of ACLY in macrophages, myeloid dendritic cells, and neutrophils from those mice (hereafter referred to as Acly\(^\text{M-KO}\) (Supplementary Fig. 1d, e), we transplanted lethally irradiated atherosclerosis-susceptible Ldlr\(^-\) deficient mice with bone marrow from either Acly\(^\text{M-KO}\) mice or Cre-negative Acly\(^\text{L/L}\) littermate control mice. After validating efficient engraftment in both experimental groups (Supplementary Fig. 1f), the mice were fed a high-fat diet (HFD) (Fig. 2a). All mice exhibited a similar increase in body weight and plasma cholesterol, and similar end-state hepatic triglyceride levels, whereas the increase of plasma triglycerides was attenuated in Acly\(^\text{M-KO}\) mice (Supplementary Fig. 1g–i). Also, both groups showed a comparable blood leukocyte composition with only B-cell levels being slightly lower in Acly\(^\text{M-KO}\) mice (Supplementary Fig. 1k–o). As splenic B-cell levels remained unaltered, there is no apparent general defect in B-cell development or activation. It rather suggests an indirect, and yet unknown, effect of myeloid Acly knockdown on circulating B cells.

After 10 weeks of HFD, immunohistochemical and gene expression analysis on lesions confirmed Acly knockdown in myeloid cells within the atherosclerotic plaques of Acly\(^\text{M-KO}\) mice (Supplementary Fig. 2a, b). Although the atherosclerotic plaque size was similar, pathological scoring of plaques indicated an increase in thick fibrous cap atheromas in the Acly\(^\text{M-KO}\) group (Fig. 2b–d). The observation that the necrotic area was significantly smaller in Acly\(^\text{M-KO}\)-transplanted mice (Fig. 2e, f) indicated that the lesions of those mice are more stable as necrotic core formation is associated with plaque instability and rupture\(^3\). Moreover, analysis of Sirius red staining highlighted increased collagen content and fibrous cap thickness in plaques of
Acly\textsuperscript{M-KO}-transplanted mice (Fig. 2g–i), another sign of plaque stability. Immunohistochemical analysis revealed that the increase in collagen deposition was not accompanied by differences in macrophage and neutrophil content in the lesions (Fig. 2j, k, Supplementary Fig. 2c). Plasma triglyceride levels did not correlate with the plaque characteristics, indicating that lower triglyceride levels were not the cause of the more stable plaque phenotype (supplementary Fig. 2d). Transforming growth factor beta (TGF-\beta) is a key anti-atherogenic cytokine that can suppress immune effector functions and stabilizes atherosclerotic lesions by inducing fibrotic activities of smooth muscle cells and fibroblasts\textsuperscript{21}. We therefore assessed its expression in aortic arches and detected increased \textit{Tgfb1} expression in Acly\textsuperscript{M-KO}-transplanted mice (Fig. 2i). As labeling of TGF-\beta and the macrophage marker MOMA-2 suggested an increase of macrophage TGF-\beta but not total TGF-\beta (Fig. 2m–o), the increased \textit{Tgfb1} levels were probably caused by macrophages within the lesion. Collectively, these in vivo data demonstrate that Acly\textsuperscript{M-KO}-transplanted mice had a favorable plaque phenotype, reflected by decreased necrosis and increased collagen deposition and fibrous cap thickness.

**Acly regulates in vitro macrophage polarization.** A possible mechanism to explain the more fibrotic plaque phenotype in the Acly\textsuperscript{M-KO} mice is a change in macrophage polarization. Although inflammatory (M1) macrophages can promote atherogenesis, IL-4-induced macrophages (M2) are regarded as atheroprotective because of their anti-inflammatory and pro-fibrotic homeostatic properties\textsuperscript{1}. Previous in vitro studies with inhibitors suggested that Acly is involved in both inflammatory and IL-4-induced macrophage polarization\textsuperscript{10,22,23}. Therefore, we next sought to reassess these findings with the aim to study whether altered macrophage polarization could clarify the differences in plaque phenotypes (Fig. 3a).

Distinct macrophage subsets differentially affect plaque phenotype. As previous inhibitor studies reported an involvement of Acly in both M1 and M2 macrophage polarization\textsuperscript{10,11}, we next aimed to investigate whether altered macrophage polarization in the absence of Acly could clarify the differences in plaque phenotypes (Fig. 3a). Hereto, we first stimulated macrophages from control and Acly\textsuperscript{M-KO} mice with LPS to elicit an inflammatory macrophage response. Surprisingly, our genetic Acly\textsuperscript{M-KO} model indicated that Acly is not needed for inflammatory macrophage responses. The LPS-induced surface markers CD40, CD86, and major histocompatibility complex (MHC)-II were upregulated to the same extend in both groups and CD80 expression was higher in Acly\textsuperscript{M-KO} macrophages (Fig. 3b). Moreover, the LPS-induced levels of pro-inflammatory genes and factors such as IL-6, tumor necrosis factor (TNF), nitric oxide (NO), and reactive oxygen species (ROS) were even increased in the absence of Acly (Fig. 3c, d). Likewise, we detected increased inflammatory gene expression in vivo in the atherosclerotic lesions and peritoneal macrophages of the Acly\textsuperscript{M-KO} group (Fig. 3e, Supplementary Fig. 2e).

Next, we assessed the effect of Acly deficiency on IL-4-induced macrophage polarization (Fig. 4a). Hereto, we measured the IL-4-elicted expression of commonly used surface markers and detected reduced levels of CD206, CD273, and CD301 on the surface of Acly-deficient macrophages, along with blunted M2-associated gene expression (Fig. 4b, c). This decreased IL-4 response may be explained by lower histone 3 lysine 27 (H3K27) acetylation in the absence of Acly and confirms a previous study that applied Acly inhibitors during IL-4-responses in macrophages to link Acly-mediated production of acetyl-CoA to histone acetylation\textsuperscript{10}. Conversely, H3K27 levels were similar in naive and LPS-treated control and Acly\textsuperscript{M-KO} macrophages and thus histone acetylation is not the mechanism explaining the macrophage phenotype in this setting (Supplementary Fig. 2f). Together, the enhanced inflammatory activation and reduced IL-4 responses in the absence of Acly do not explain the advantageous plaque phenotype of Acly\textsuperscript{M-KO}-transplanted mice. Our data highlight that M1/M2 polarization in vitro not necessarily reflects the in vivo context and underscore that caution is necessary when interpreting inhibitor effects.
**Fig. 2** Acly deletion in macrophages elicits a favorable plaque phenotype. **a** In order to assess atherogenesis, bone marrow cells of either control of Acly^M-KO^ were transplanted in lethally irradiated Ldlr^−/−^ mice. Six weeks after transplantation, mice were put on a HFD for 10 weeks. **b** Representative toluidine blue staining of atherosclerotic plaques within the aortic roots of control and Acly^M-KO^ mice. Scale bar represents 500 μm. **c** Quantification of plaque size. **d** Plaque phenotypes as classified by an experimental pathologist. **e** Toluidine blue staining of atherosclerotic lesions with necrosis indicated in red. Scale bar represents 200 μm. **f** Quantification of the necrotic area. ^*^P = 0.0173. **g** Sirius red staining of plaques. Scale bar represents 200 μm. **h** Quantification of the collagen deposition. ^*^P = 0.0201. **i** Minimal cap thickness was measured at the thinnest region of the fibrotic cap surrounding the necrotic core as indicated by the arrows in **g**. ^*^P = 0.0292. **j** Quantification of MOMA-2^+^ area for macrophages. **k** Quantification of Ly6G^+^ area for neutrophils. **l** Normalized expression of Tgfb1 in aortic arches of control and Acly^M-KO^ mice. ^**^P = 0.0038. **m** Representative immunohistochemical analysis of macrophages (MOMA-2) and TGF-β in mouse plaques from control or Acly^M-KO^ mice (n = 6/6 ctrl/KO from one experiment). Scale bar represents 100 μm. **n** Quantification of colocalization, percentage of TGF-β^+^ area in MOMA-2^+^ area. **o** Quantification of TGF-β^+^ area in the lesion. Values represent mean ± SEM (n = 20/17 (ctrl/KO in b-l), n = 6/6 (ctrl/KO in n, o). ^*^P < 0.05; ^**^P < 0.01 by two-tailed Student’s t test (f, h, i, l). Source data are provided as a Source Data file (c, d, f, h-l, m, o).
Acly does not regulate core metabolic processes. To identify the mechanism by which myeloid deletion of Acly influences macrophage and plaque phenotype, we explored changes in core metabolic pathways as a potential explanation of the increased inflammatory response in Acly-deficient macrophages. Metabolomics revealed that the expected LPS-induced accumulation of citrate was more pronounced in the absence of Acly, signifying decreased Acly-mediated conversion of citrate into acetyl-CoA\textsuperscript{24,25}. Yet, loss of Acly led to no further changes in the abundance of other tricarboxylic acid cycle (TCA) cycle...
intermediates (Fig. 5b). Extracellular flux analysis, and glucose, lactate and ATP measurements showed no differences in mitochondrial function, glycolysis and ATP production, indicating that other mechanisms should explain the observed inflammatory phenotypes of Acly-deficient macrophages (Fig. 5c–g).

Deregulated cholesterol metabolism in the absence of Acly. To reveal such mechanism, we next performed RNA-sequencing on BMDMs from control and Acly<sup>M-KO</sup> mice. Plotting genes in a volcano plot and performing pathway analysis on the differentially expressed genes identified cholesterol biosynthesis as the most deregulated pathway, with most targets related to this pathway being upregulated in Acly<sup>M-KO</sup> macrophages (Fig. 6a, b). In addition to genes involved in cholesterol metabolism, fatty-acid metabolism, and cell cycle genes were differentially expressed. Distinct genes involved in cholesterol and fatty-acid import and transport (Ldlr, Cd36, Scarb1, Fabp3, Slc27a4) were increased in the absence of Acly. In contrast, Acly<sup>M-KO</sup> macrophages showed decreased expression of LXR-regulated genes that promote cholesterol efflux (Abca1, Abcg1, Apeo) or limit cholesterol uptake (Mlip) (Fig. 6c). The most significantly increased gene in Acly<sup>M-KO</sup> macrophages was Dhr24, encoding the enzyme that converts desmosterol into cholesterol as the terminal step in cholesterol synthesis (Fig. 6d). In line with increased Dhr24 expression, we measured lower levels of desmosterol and desmosterol-induced genes in Acly<sup>M-KO</sup> macrophages (Fig. 6e, f). By increasing cholesterol synthesis and import, and via limiting its export, Acly-deficient macrophages possibly try to cope with the reduced supply of Acly-mediated acetyl-CoA, and by doing so they manage to secure total cholesterol levels (Fig. 6g). Moreover, Acly-deficient macrophages might rescue acetyl-CoA production by upregulating Acss2 (Fig. 6h). This gene encodes acyl-coenzyme A synthetase short-chain family member 2, an enzyme that converts acetate into acetyl-CoA. This is in line with earlier reports that describe induction of Acss2 in adipocytes and mouse embryonic fibroblasts upon Acly deletion<sup>25,26</sup>.

The resulting deregulated cholesterol metabolism and suppressed desmosterol levels could explain the lower expression of LXR-target genes (Fig. 6f) and possibly also altered polarization (Figs. 3, 4) in Acly<sup>M-KO</sup> macrophages. Indeed, LXR activation is involved in reprogramming fatty-acid metabolism and inhibiting LPS-induced inflammatory-response genes in macrophages<sup>25</sup>. Supporting this hypothesis, exposing macrophages to LXR agonist GW3965 decreased inflammatory responses in both control and Acly<sup>M-KO</sup> macrophages (Supplementary Fig. 3a). As such, reduced LXR activation could provide one potential mechanistic explanation for the elevated inflammatory responses of Acly-deficient macrophages.

Fatty-acid synthesis is affected in the absence of Acly. Apart from cholesterol metabolism, reactome pathway analysis also indicated “fatty acyl-CoA biosynthesis” and “fatty-acid metabolism” as top deregulated pathways (Fig. 6b). As Acly provides acetyl-CoA as a precursor for fatty acid synthesis<sup>28,29</sup>, we
measured fatty acid levels in control and AclyM-KO macrophages by performing lipidomics measurements. Whereas RNA-sequencing analysis suggested a lipid-laden phenotype of AclyM-KO macrophages, total lipid pools were similar in control and AclyM-KO macrophages (Fig. 7a, b). Conversely, levels of omega 3 and 6 fatty acids, including arachidonic acid (AA) tended to be increased in AclyM-KO macrophages (Fig. 7c, d). Cyclooxygenase (COX)-1 and COX-2 produce prostaglandins...
**Fig. 6** Acly\(^{M-KO}\) macrophages exhibit deregulated cholesterol biosynthesis. **a** Volcano plot with up- or downregulated genes in Acly-deficient macrophages (BMDMs) highlighted in red or blue, respectively. **b** Ridgeline plot of significantly enriched Reactome pathways. Log\(_2\)FC distributions of member genes are plotted on the x axis. Color of the distributions represents enriched pathway P value. **c** Expression of genes involved in import/transport of cholesterol and fatty acids, and genes that promote cholesterol efflux. **d** Schematic view of cholesterol metabolism and the effect of Acly deficiency (red indicating upregulation; blue downregulation). **e** Desmosterol levels in control and Acly-deficient macrophages. **f** Genes previously found to be induced by desmosterol, plotted in a heatmap for control and Acly\(^{M-KO}\) macrophages. **g** Cholesterol levels in control and Acly-deficient macrophages. **h** Acss2 expression in control and Acly-deficient macrophages. Values represent mean from \(n = 2\) technical replicates from three pooled mice \(e, g\), or mean ± SEM \(n = 3\) technical replicates from three pooled mice (h). Source data are provided as a Source Data file \(e, g\) or deposited in GSE126690 (a-d, f, h).

**Acly\(^{M-KO}\) macrophages show increased apoptosis and efferocytosis.** In addition to cholesterol and fatty acid metabolism, pathway analysis identified “cell cycle checkpoints” and “TP53 regulates transcription of cell cycle genes” among the most-regulated pathways (Fig. 6b), with most genes related to these pathways being increased in Acly\(^{M-KO}\) macrophages (Fig. 8a). As the transcription factor p53 is a key modulator of both apoptosis and senescence, we assessed those cell cycle-related processes more detailed. We stained DNA of control and Acly\(^{M-KO}\) macrophages with propidium iodide and measured increased fractional DNA content (Sub-G1), a hallmark of ACs, in Acly\(^{M-KO}\) macrophages (Fig. 8c). Supporting those data, the expression of p53 from AA and these lipid mediators are known to affect inflammatory responses\(^{30}\). The genes encoding COX-1 and COX-2 (Ptgs1 and Ptgs2) and downstream prostaglandins (PGD\(_2\), PGE\(_2\), and in particular PGE\(_2\)) were deregulated in Acly\(^{M-KO}\) macrophages (Fig. 7d). Moreover, Acly\(^{M-KO}\) macrophages showed a decreased expression of prostaglandin E 4 receptor (EP4, Ptger4) (Fig. 7e). Loss of EP4 has previously been described to enhance inflammatory responses\(^{31,32}\). Subsequently, PGE\(_2\)- and LPS-treated Acly\(^{M-KO}\) macrophages display a reduced responsiveness to PGE\(_2\) when compared to control macrophages (Fig. 7f, g).

Together, these findings indicate that Acly\(^{M-KO}\) macrophages rewire cholesterol and fatty-acid metabolism to deal with the absence of Acly, and these metabolic changes are accompanied by altered inflammatory responses.
**Fig. 7** Deletion of Acly leads to deregulation of lipid mediators and decreased responsiveness to prostaglandin E2.

a. Total sum of measured lipids by metabololipidomics.

b. Neutral lipids analyzed by Bodipy 493/503.

c. Sum of AA, DHA, EPA, AdA, LA, ALA/GLA, DPAn-3, and DGLA omega 3 and 6 fatty acids (FA’s) measured by metabololipidomics.

d. Lipid abundances and genes involved in prostaglandin synthesis.

e. Ptger4 expression in control and Acly-deficient macrophages.

f. Effect of PGE2 treatment on cytokine secretion in control and AclyM-KO LPS-treated BMDMs.

g. Effect of PGE2 treatment on cytokine secretion in control and AclyM-KO LPS-treated BMDMs. Values represent mean ± SEM (n = 3 technical replicates from three pooled mice a-e), or mean (n = 2 technical replicates from three pooled mice f, g). Source data are provided as a Source Data file (a-d, f) or deposited in GSE126690 (d, e).
target genes related to cell cycle mostly increased in the absence of Acly (Fig. 8a). Noteworthy, the expression of the LXR-dependent anti-apoptosis genes CdS1 (AIM), Lbp, and Mafb (all known to promote macrophage survival33–35) was reduced in the absence of Acly, providing a potential link between reduced LXR activation (Fig. 6f) and increased apoptosis in these cells. Increased apoptosis within atherosclerotic lesions needs to be paralleled by increased clearance of those cells to prevent the growth of necrotic cores and plaque destabilization. Therefore, we questioned whether improved efferocytosis of apoptotic macrophages could explain the smaller necrotic cores in atherosclerotic lesions of AclyM-KO mice. Here, we co-cultured control or AclyM-KO BMDMs with apoptotic macrophages that were labeled with the pH-sensitive dye pHrodo and measured efferocytosis. Culturing the cells at 4 °C served as a negative control to set the pHrodo 

target for effective internalization of ACs was also validated by imaging flow cytometry (Fig. 8d). To validate these results in vivo, we analyzed apoptosis and efferocytosis in the atherosclerotic plaques from control and AclyM-KO mice by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) double staining with MAC3. In vivo apoptosis, but not efferocytosis, appeared to be increased in plaques from AclyM-KO mice (Fig. 8e, f). These assays highlighted that Acly-deficient macrophages demonstrate more-efficient efferocytosis in vitro, an atheroprotective macrophage function associated with stabilized atherosclerotic plaques in vivo (Fig. 8d).

Discussion

Targeting ACLY in liver cells has emerged as an attractive aid to lower serum LDL-C levels in hypercholesteremic patients16,17. In the present study, we demonstrate that myeloid deficiency for Acly deregulated fatty-acid and cholesterol metabolism and leads to enhanced apoptosis, combined with improved efferocytosis. In vivo, this results in more stable atherosclerotic plaques, reflected by smaller necrotic cores and more collagen deposition. As such we identify targeting macrophage Acly as a potential new therapeutic avenue in cardiovascular disease.

Noteworthy, Acly targeting with bempedoic acid (ETC-1002, a prodrug activated by acyl-CoA synthetase solute carrier family 27 member 2 (SLC27A2) in the liver) is already applied in the clinic as a new aid to lower serum LDL-C in hypercholesteremic patients36,37. Acly inhibition reduces cholesterol synthesis in hepatocytes, leading to the upregulation of LDLR expression and clearance of LDL-C particles from the blood38. Similar as in liver cells, Acly deficiency in macrophages leads to the upregulation of Ldlr, probably to compensate for deregulated cholesterol biosynthesis. In addition to elevated expression of the LDL receptor, loss of Acly enhances the expression of CD36 and SR-B1. Besides their role in cholesterol uptake, both class B scavenger receptors are key mediators of efferocytosis39,40. Impaired efferocytosis drives the accumulation of necrotic debris in atherosclerotic plaques and can lead to plaque rupture and cardiovascular events. In accordance with increased CD36 and SR-B1 expression in AclyM-KO macrophages, the capacity of these cells to clear ACs was improved. Silencing of ACLY was recently found to suppress DNA damage repair, resulting in genomic instability and cell death41. In agreement, Acly-deficient macrophages were more senescent and prone to apoptosis. Together, increased apoptosis and more effective clearance of such apoptotic macrophages may explain the reduced necrotic core formation in plaques of AclyM-KO mice. Along with reduced necrotic core formation, collagen deposition was increased in the plaques of AclyM-KO-transplanted mice. In line with the increase in collagen content and fibrospous cap thickness, the plaques of those mice showed increased mRNA levels of TGF-β1, a key pro-fibrotic cytokine which is secreted by macrophages upon efferocytosis42. Thus, efferocytosis might trigger the synthesis of TGF-β1 that drives the increased collagen deposition in plaques of myeloid Acly-deficient mice. Interestingly, although efferocytosis is generally believed to be an anti-inflammatory process, we observed augmented expression of inflammatory genes in plaques of AclyM-KO mice. Moreover, cultured Acly-deficient macrophages were found to be hyperinflammatory. We found that the expression of LXR-target genes and abundance of main LXR agonist desmosterol were diminished in both unstimulated and LPS-stimulated Acly-deficient macrophages. In addition to their role in cholesterol efflux, LXRs are established negative regulators of inflammatory macrophage responses43. Therefore, blunted LXR activation provides a potential mechanistic explanation for the elevated inflammatory responses of Acly-deficient macrophages. In addition, LXR activation is believed to promote macrophage survival33,35. LXR-target genes CdS1 (AIM), Lbp and Mafb have been found to inhibit apoptosis and promote macrophage survival and were all downregulated in Acly-deficient macrophages. Therefore, diminished LXR activation might contribute to the increase in apoptosis upon Acly deletion. Interestingly, Lauterbach et al.11 recently applied Acly inhibitors and siRNA-mediated knockdown to demonstrate the role of Acly in regulating acute LPS responses via histone acetylation. The difference between our observations and the ones of Lauterbach et al. might be explained by the knockdown method (i.e., short-term siRNA vs long-term LysMCre-driven) and other experimental variables. Clearly, the role of Acly in macrophages is stimulus- and time-dependent and also appears to depend on the experimental setting as Namgaladze et al. observed no effect of Acly silencing44. As such, this is an area that warrants future research.

Collectively, we demonstrate that macrophage-specific targeting of Acly elicits a favorable atherosclerotic plaque phenotype. With liver-specific inhibition of ACLY already being applied as a cholesterol-lowering aid, we anticipate our observations in macrophages to provide the development of new cell-specific ACLY inhibitors that target different cell types to tackle the disease from distinct angles.

Methods

Human tissue collection. Anonymous human plaque tissues for the determination of protein expression were obtained from the Maastricht Pathology Tissue Collection (MPTC) under approval of the Medical Ethical Committees AZmUM from the Maastricht University Medical Center. The formalin-fixed paraffin-embedded human carotid samples used for immunohistochemistry were collected at autopsy (n = 16, mean age 72.6 years, all men). Collection, storage in the MPTC and use of tissues were performed in agreement with the “Dutch Code for Proper Secondary use of Human Tissue”. None of the co-authors was involved in tissue collection during autopsy. No prior informed consent was directly obtained from the patients, but an opt-out arrangement was in place and tissues were not used in case of objection by relatives in line with the guidelines provided by the Dutch Code for Proper Secondary use of Human Tissue. Samples represented the following stages of atherosclerosis (n = 797 per, respectively, stable/unstable group): pathological intimal thickening and plaque with intraplaque hemorrhage45.

Mice experiments. C57Bl/6 mice with loxp sites flanking exon 9 of the Acly gene (Aclyflfl)20 were crossed with Lyz2-Cre transgenic mice to generate mice with a myeloid-specific deletion of Acly (Aclyfl/−/−). Ldlr−/− mice on a C57Bl/6 background were purchased from Jackson Laboratories. All mice were maintained in an animal facility at an ambient temperature of 20–24 °C with 40–70% relative humidity under a 12:12 h light–dark cycle. All mouse experiments were conducted after approval by the Committee for Animal Welfare (University of Amsterdam and VU university Amsterdam).

For bone marrow transplantation, female Ldlr−/− mice were lethally irradiated with six Gray on 2 successive days. After radiation randomization based on weight, mice were divided in two groups of 20 and injected intravenously with 5 × 106 bone marrow cells from either control or Aclyfl/−/− mice. From one week before until 5 weeks after transplantation, recipient Ldlr−/− mice received autoclaved tap water containing antibiotics (100 mg/L neomycin and 60,000 U/L polymyxin B sulfate). Bone marrow transplantation efficiency was evaluated by qPCR for Ldlr on DNA extracted from blood. Six weeks after the transplantation, mice received a HFD)}
Fig. 8 Deletion of Acly results in increased apoptosis, growth arrest, and more-efficient efferocytosis. 

a. Expression of genes associated with indicated reactome pathways. 

b. Flow cytometric analysis of control and AclyM-KO macrophages stained with propidium iodide to assess cell cycle distribution. *P = 0.0009, **P = 0.0385. 

c. Senescence in control and AclyM-KO BMDMs by β-galactosidase activity measurement. Scale bar represents 100 µm. **P = 0.0034. 

The in vitro phagocytosis of pHrodo-stained apoptotic cells by DiD-labeled control or AclyM-KO BMDMs measured by flow cytometry after incubation at 37 °C (or 4 °C as negative control) and validated by imaging flow cytometry (Imagestream, scale bar represents 7 µm). Quantification of efferocytosis at 37 °C as a percentage of DiD-positive cells that incorporated an apoptotic cell. ***P = 0.0002. 

e. Quantification of TUNEL+ cells in lesions of Ldlr−/− mice transplanted with control or AclyM-KO bone marrow. *P = 0.0203. 

f. Ratio of TUNEL+ cells bound to MAC3+ macrophages and free TUNEL+ cells. Values represent mean ± SEM (n = 3 technical replicates of one out of three representative independent experiments in b–d and n = 20/19 (ctrl/KO in e, f)). **P < 0.05; ***P < 0.01, ****P < 0.001 by two-way ANOVA with Bonferroni post hoc test for multiple comparisons b or two-tailed Student’s t test (c, d). Source data are provided as a Source Data file (b–f) or deposited in GSE126690 (a).
(15% fat and 0.15% cholesterol, Sniff Spezialdiëten). After 10 weeks HFD, mice were sacrificed by CO₂ asphyxiation. Hearts, livers, and spleens from killed mice were collected and washed. Bone marrow cells were isolated from femurs and tibias of control and AclyM-KO mice as previously described.[48]

**Histology.** Sections of 7 μm were cut on a Leica 3050 cryostat and stained with toluidine blue (0.2% in phosphate-buffered saline (PBS)) to quantify plaque area and collagen β content, respectively (antibodies detailed in the Supplementary Table 1). MOMA-2 (1:100, Abcam), Ly6G (1:200, Biolegend) or anti-ATP citrate lyase (Abcam, 1:100), anti-CD68 (Abcam, 1:75) or anti-ATP citrate lyase (Abcam, 1:100), and DAPI (Invitrogen, 1:1000) to visualize macrophages, neutrophil, total Acly and macrophage-related TGF-β content, respectively. (antibodies detailed in the Supplementary Table 1). MOMA-2, Ly6G, and Acly were captured using biotinylated rabbit anti-rat antibody (Vector Laboratories) as secondary antibody. Hereafter, the signal was amplified using the ABC kit (Vector Laboratories) and subsequently visualized using the ImmPACT AMEC Red substrate kit (Vector Laboratories). Combined MOMA-2 and TGF-β staining was visualized using Alexa-555-labeled goat anti-rabbit antibody (Molecular probes, 1:400) for MOMA-2 and Alexa-488-labeled goat anti-rat antibody (Molecular probes, 1:400) for TGF-β. Hereafter, the signal was measured by multispectral imaging using a Vectra Polaris slide imager (Perkin Elmer), and subsequently cleaned from autofluorescent spectra by measuring an unstained lesion. ImageJ was used for analysis of positive area and for colocalization with the ImageJ plugin JaCOP.

To analyze apoptosis and efferocytosis with TUNEL, sections were stained with MAC3 following paraformaldehyde fixation, and permeabilization with 0.1% triton X-100. MAC3 signal was detected using biotinylated rabbit anti-rat antibody, streptavidin-ABC-AP, and visualized using vector blue (all Vector Laboratories). TUNEL positive area was calculated using the ImmPACT AMEC Red substrate kit (Vector laboratories). X-100. MAC3 signal was detected using biotinylated rabbit anti-rat antibody, streptavidin-ABC-AP, and visualized using vector blue (all Vector Laboratories).

**Gene expression analysis.** Aortic roots were disrupted with glass beads in a Magna lyser system and total RNA was extracted from homogenates using TRIzol reagent (Life Technologies) and RNeasy Mini Kit (Qiagen). RNA from aortic arches and BMDMs was reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher) and gene expression analysis was performed with SYBR Green Fast mix (Applied Biosystems) on a ViiA7 system (Applied Biosystems). Levels of expression were normalized to housekeeping genes Rplp0 and Ppia. Primer sequences used for gene expression analysis are provided in Supplementary Table 2.

**Cytokine, NO, and ROS production.** Production of IL-6, TNF, and NO was quantified in supernatants using ELISA (Life Technologies) or the Griess test (Sigma) respectively, following the supplier’s protocols. In order to determine ROS production, macrophages were incubated with 20 μM CM-H2DCFDA (ThermoFisher) for 30 min at 37 °C (5% CO₂). Hereafter, cells were washed with PBS and fluorescence was measured on a Beckman Coulter CytoFLEX.

**Flow cytometry.** To examine surface marker expression, BMDMs were detached using citrate buffer (17 mM tri-sodium citrate dehydrate and 135 mM potassium chloride in water), transferred to V-bottom well plates, incubated with 1:50 anti-CD16/CD32 FC-block and stained for 20 min at room temperature in the dark with antibodies. Antibodies were directed to CD40-APC (Biologend, 1:100), HLC-II-PerCP/Cy5.5 (Biologend, 1:200), CD86-BV650 (Biologend, 1:100) and CD86-BV510 (Biologend, 1:100) for IL-4 stimulated macrophages or directed to CD71-PE (BD PharMingen, 1:250), CD206-APC (Biologend, 1:250), CD273-PE (BD PharMingen, 1:250), CD301-AF647 (Serotec, 1:250) or isotype controls (BioLegend) for IL-4 stimulated macrophages (IGG2A-PE, IGG2A-APC), diluted in staining buffer (PBS with 0.5% BSA and 2.5 mM ethylenediaminetetraacetic acid; EDTA). After staining, cells were washed and the supernatants were then transferred to a Beckman Coulter CytoFLEX or BD LSR Fortessa and analyzed using Flowjo (Tree Star). Surface expression was calculated as AMFI = (median fluorescence intensity/positive staining) - (median fluorescence intensity)isospecific or FMO control (MF1 controls (isotype or FMO) were measured on a pool of all samples.

**Metabolomics.** Macrophages were quenched by ice-cold methanol. D3-aspartic acid, D-serine, D-γ-glutamatic, γ-glutamatic, 13C2-pyruvate, 13C5-isoleucine, 13C6-glucose, 13C6-fructose-1,6-biphosphate, 13C5-glucose-6-phosphate, adenosine-15N1-monophosphate and guanosine-15N5-monophosphate (5 µM) served as internal standards. After addition of chloroform, samples were centrifuged and the supernatant was washed with acetone. Supernatants were lyophilized and resuspended in 12 N nature communications | https://doi.org/10.1038/s41467-020-20141-z | www.nature.com/naturecommunications
“polar” top layer was dried in a vacuum concentrator and dissolved in methanol/ water (6/4, v/v). Analysis was done using a Thermo Scientific (U)HPLC system coupled to a Thermo Q Exactive (Plus) Orbitrap mass spectrometer (Thallium) with a SeQuant ZIC-HILIC column. MS data were acquired in full scan, negative ionization mode with a mass resolution of 140,000.46 Interpretation of the data was performed on the Xcalibur software (Thermo Fisher). Further analyses were performed in R using ggplot2, vip, and mixOmics packages.

**Metabolic determinants and extracellular flux analysis.** Glucose (GOD-PAP method, Biobios) and ATP (Thermo Fisher) levels were enzymatically determined according to manufacturer’s protocol. Lactate was enzymatically determined on 3% metaphosphoric acid (MPA) deproteinized cell culture supernatants. After centrifugation (20,000 × g, 10 min) supernatants and t-lactic acid standards were measured for background fluorescence in a 0.5 M Glycine-0.4 M Hydrazine buffer, pH 9.0 with 3.6 mM NAD with a Mithras LB 940 reader at λex/λem = 340–410/450–105 nm every 2 min. Reactions were started by adding 0.5 M Glycine-0.4 M Hydrazine buffer, pH 9.0 with 50 μg/mL t-lactate dehydrogenase from rabbit muscle (Roche) and again measured at λex/λem = 340–410/450–105 nm every 2 min until a stable read was achieved.

For metabolic profiling, the XF-96 Flux Analyzer (Agilent) was used to assess OCR and ECR on macrophages from both phenotypes. BMDMs (8 × 10^4 cells/well) were plated on XF-96-cell culture plates (Agilent), treated with LPS or left untreated. In all, 1 hr before the assay, cells were washed and replaced with Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma-Aldrich) without glucose, phenol red, and sodium bicarbonate. The run consisted of 2 min run-in, 3 min measuring and subsequent four injections: glucose (final assay concentration 25 mM), oligomycin A (OM, final assay concentration 1.5 µM), FCCP (final assay concentration 1.5 µM) with sodium pyruvate (final assay concentration 1 mM), with last injection of antimycin A (AA, final assay concentration 2.5 µM) with rotenone (final assay concentration 1.25 µM). Data were analyzed using Wave software as detailed before.46

**Transcriptomics.** Total RNA was isolated from BMDMs using a RNeasy Mini Kit with DNase treatment (QIAGEN) and strand-specific libraries were constructed using the KAPA mRNA HyperPrep kit (KAPA Biosystems). Subsequently, samples were sequenced on an HiSeq 4000 instrument (Illumina)46. Afterwards, reads were aligned to the mouse genome mm10 by STAR 2.5.26. BAM files were indexed and filtered with SAMtools and raw tag counts and RPMK values were summed using HOMER2 package. Differential expression was assessed using the DESeq2 package in an R environment. Reactome pathway enrichment analysis was performed using the ReactomePA package for significantly differentially expressed genes (adjusted p value ≤ 0.05). Significantly enriched pathway list was filtered on redundant pathways that represent parent functionality. Summarized pathway list was visualized using ridge plots, by extracting gene ids and ploting fold change distributions using in-house scripts. Volcano plot and heatmaps were generated using the ggplot2, ggrepel, and pheatmap package.

**Analysis of sterols in macrophages.** Sterols were analyzed in 2 × 10^6 macrophages, homogenized in 100 μL PBS followed by sonication in a sonicator bath for 2 min. P-values < 0.05 were considered significant, with levels of significance being indicated as follows: *p < 0.05; **p < 0.01; ***p < 0.001.

**Metabololipidomics.** Lipids were extracted from macrophages (5 × 10^5 cells/concentration) in 24-well plates, cultured in serum-free phenol red-free DMEM medium supplemented with 25 mM HEPES, 2 mM l-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cell lysates were quantified by adding 1.5 mL methanol and 4 mL internal standard solution (0.05% propanol) was added and mixed followed by addition of 1.0 mL of saponification reagent (0.3 M KOH in ethanol). This was incubated for 2 h at 80 °C. After cooling, 2 mL of hexane and 0.5 mL of water was added and mixed. Three hundred microliters of the resulting trimethylsilyl (TMS)-ethers of the sterols was injected on an MSD9797A MS detector in the EI mode.

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