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ABCA1 and HDL_3 are required to modulate smooth muscle cells phenotypic switch after cholesterol loading

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ABSTRACT

Background and aims: Cholesterol-loaded smooth muscle cells (SMCs) modify their phenotypic behavior becoming foam cells. To characterize the role of ABCA1 and HDL_3 in this process, we evaluated HDL_3 effects on cholesterol-induced phenotypic changes in SMCs expressing or not ABCA1.
Methods: SMCs, isolated from the aortae of wild-type (WT) and Abca1 knock-out (KO) mice, were cholesterol-loaded using a "water-soluble cholesterol".
Results: Cholesterol loading downregulates the expression of *Acta2* and *calponin* (SMC markers), and increases the expression of *Mac*-2, CD11b and MHCII (inflammation-related genes and surface antigens) and *Abca1, Abcg*1. HDL_3 normalizes SMC marker expression and reduces the expression of inflammation-related genes/proteins in WT cells, an effect not observed with free apoA-I. The effect of HDL_3 is almost lost in Abca1 KO cells, as well as when Abca1 is silenced in WT SMC. HDL_3 does not differently affect cholesterol downloading in WT or KO cells and stimulates phospholipids removal in WT cells. Similarly, the expression of myocardin and its modulators, such as miR-143/145, is reduced by cholesterol loading in WT and Abca1 KO SMCs; HDL_3 normalizes their levels in WT cells but not in KO cells. On the contrary, cholesterol loading induces *Klf4* expression while HDL_3 restores *Klf4* to basal levels in WT cells, but again this effect is not observed in KO cells.
Conclusions: Our results indicate that HDL_3, by interacting with ABCA1, modulates the miR143/145-myocardin axis and prevents the cholesterol-induced gene expression modification in SMCs regardless of its cholesterol unloading capacity.

1. Introduction

Atherosclerosis is a complex disease initiated by the trapping of lipids in the subendothelial layer of the arterial wall, followed by the generation of biologically active species, which stimulate vascular cells to produce inflammatory molecules and recruit monocytes and T cells [1]. Smooth muscle cells (SMCs) are a major component of the media in human arteries, secrete extracellular matrix proteins and are considered protective against atherosclerotic plaque destabilization. However, like other cells such as monocyte/macrophages, SMCs can take up and store excess lipids and form foam cells [2–6]. Arterial SMC are not terminally differentiated and retain the ability to alter their phenotype in response to environmental cues [7] and, after exposure to atherogenic lipids, may undergo a phenotypic switch from a contractile to a dedifferentiated synthetic state to express inflammatory markers and a phagocytic activity. Upon cholesterol loading *in vitro*, mouse SMCs down-regulate the expression of typical smooth muscle markers, including *Acta2* (α-actin), *α-tropomyosin* and *myosin heavy chain,* and increase the expression of macrophage-related genes such as *CD68* and *Mac-2* [2,6]. These phenotypic changes result in a *trans*-differentiation to a macrophage-appearing state by down-regulating the miR-143/145-myocardin axis in a Kruppel-like factor 4 (Klf4)-dependent manner [6,8]. Another key driver toward the synthetic phenotype is platelet-derived growth factor (PDGF) expressed by many cell types in atherosclerotic lesions [9]. Klf4 has been shown to be necessary for SMC phenotypic transition in

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response to PDGF [10], regulating the transition toward a macrophage phenotype. This phenomenon is associated with increased proliferation, synthesis of extracellular matrix and proteases (such as matrix metalloproteinases) [11]. Thus, SMC phenotypic switching toward a synthetic phenotype may play a detrimental role in atherogenesis and gives a theoretical basis to the unexpectedly high percentage of foamy SMCs in the plaque, where they have been found to comprise more than 50% of total foam cells [2,3,6,8,12].

Human coronary SMCs express the ATP binding cassette (ABC) transporter ABCA1 and participate to the reverse cholesterol transport pathway, which allows lipid-laden cells to metabolize and export lipids to carriers that recycle them to the liver. ABCA1 mediates cholesterol and phospholipid efflux to small dense HDL_3 and to lipid-free apolipoprotein A-I (apoA-I) [13–15], thus modulating intracellular lipid levels, and interacts with apoA-I to form mature HDL [16,17]. It has been observed that foamy SMCs exhibit a selective loss of expression of ABCA1 in advancing lesion stage [3]. Moreover, the lack of ABCA1 causes Tangier disease, which is characterized in the homozygous state by the absence of HDL-cholesterol, hepatosplenomegaly, peripheral neuropathy [18,19]. Lipid vacuoles and/or other cytoplasmic lipid inclusions have been described in the aortic SMC of a Tangier disease patient [20].

To characterize the role of the ABCA1 transporter in SMC dedifferentiation, we evaluated the early phenotypic changes in wild-type (WT) and Abca1 knock-out (KO) mice-derived SMCs loaded with free cholesterol complexed to methyl-β-cyclodextrin [2], and how HDL_3 may affect these changes. Our results show that the addition of HDL_3, but not of apo A-I, to WT SMC can prevent the phenotypic changes induced by cholesterol accumulation. Interestingly, HDL_3 loses its preventive effects in cell not expressing Abca1 thus strengthening the fundamental role played by ABCA1 in protecting arterial SMCs from an excessive accumulation of cholesterol.

2. Materials and methods

2.1. Cell cultures

SMCs were isolated from the intimal-medial layer of aortae of littermate Abca1 WT and KO mice of both sexes (The Jackson Lab, Bar Harbor, ME, USA). The mice were originally on a DBA background and have been backcrossed into C57BL/6 mice for at least nine times. All mice were kept in accordance with guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes, and the Italian Ministry of Health and the local University of Milan ethics committee approved the protocol.

2.2. Cell treatment

Cholesterol was delivered to cells by using a Chol:MβCD complex as "water-soluble cholesterol" containing ≈50 mg of cholesterol/g solid (molar ratio, 1:6 cholesterol/MβCD) as shown before [2,6]. Subconfluent SMCs were incubated with DMEM supplemented with 0.2% Essential Fatty Acid Free albumin alone or containing Chol:MβCD (50 μg/mL) or HDL_3 (100 μg/mL) or apoAI (35 μg/mL) for 48 h or 7 days.

2.3. Flow cytometry

WT and KO SMCs were collected after 7 days of incubation and washed with PBS 1x. Cells were stained with mouse anti-CD11c AlexaFluor700 (eBioscience) and mouse anti-MHCII BV650 (BD bioscience) in MACS buffer (PBS 1x, 2% FBS, 2 mM EDTA) and incubated for 30 min at 4 °C in the dark. Cell were washed twice and resuspended in MACS buffer. 100 μL of cells suspension was acquired (Novocyte 3000) and analyzed with Novoexpress software (ACEA Biosciences). All flow cytometry antibodies were used at 1:200 dilutions; optimal antibody concentrations for staining were calculated based on manufacturer instructions.

A more detailed list of materials and methods is provided in the Data in brief article available online [21].

3. Results

3.1. Cholesterol loading induces mouse SMC phenotypic switch

In the first series of experiments, as shown also by Rong and Vengrenyuk [2,6], upon cholesterol loading of aortic SMCs isolated from C57BL/6 mice, we observed a significant and concentration-dependent decrease in *Acta2* mRNA levels (up to 50%, $p<0.05$ *vs.* control; see Fig.IA in Ref. [21]) and a parallel increase in the expression of *Mac-2, Abca1* and *Abcg1* mRNA (up to 10-fold, 16-fold and 160-fold, respectively). The effects on mRNAs were confirmed by Western blot analysis (see Fig. IB in Ref. [21]). As shown by Oil Red O staining (see Fig. IIA in Ref. [21]), Chol:MβCD incubation causes the transformation of SMCs into foam cells, with intracellular accumulation of lipid droplets. This is due to a three-fold increase in cellular total cholesterol content, consequent to an accumulation of both free and esterified cholesterol (see Fig. IIB in Ref. [21]). The increased cellular esterified cholesterol content is caused by a 2.5-fold stimulation of the activity of the esterifying enzyme ACAT that is completely blocked by the addition of Sandoz 58–035, a specific ACAT inhibitor (see Fig. IIIA in Ref. [21]). The way by which cholesterol is delivered to the cell, seems to play an important role. In fact, the addition of the same amount of cholesterol, either dissolved in ethanol or as lipoprotein cholesterol (AcLDL or native LDL) only slightly induced the activity of the ACAT enzyme (see Fig. IIIA in Ref. [21]), and did not cause the accumulation of lipid droplets into SMCs (data not shown), as recently shown also by others [22]. The cholesterol delivered as Chol:MβCD reaches the intracellular metabolic cholesterol pools, where it accumulates as free cholesterol, and is available for downloading in the presence of HDL_3, as it is suggested by the inhibition of cholesterol esterification rate after the addition of HDL_3 (see Fig. IIIB in Ref. [21]). Thus, the treatment with Chol:MβCD allows a fast delivery of cholesterol to metabolically active intracellular lipid pools, independently of the lipoprotein receptor(s) pathway.

3.2. Effects of Abca1 and HDL_3 or lipid free apoA-I on SMC or inflammation-related cells associated gene/protein expression in SMCs

To characterize the role of ABCA1 and HDL_3 in cholesterol-induced SMC dedifferentiation, we evaluated the phenotypic changes at the molecular level in cholesterol loaded SMCs isolated from *Abca*1 WT and KO mice. Cholesterol loading decreases *Acta2* and *calponin* (SMC markers) and increases *Mac-2* mRNA, or *CD11b and MHCII* surface antigens (inflammation associated markers) levels in both cell types (Fig. 1A and B; E-G). Interestingly, HDL_3 shows a different effect depending on the genotype. In fact, the phenotype observed is rescued in WT cells following the incubation with HDL_3 (Fig. 1A and B; E-G). On the contrary, the preventive effect of HDL_3 is completely lost in *Abca1* KO cells. In fact, the addition of HDL_3 does not significantly modify cholesterol effects on tested genes/surface antigens (Fig. 1A and B; E-G).

As ABCA1 mediates the cellular efflux of phospholipids and cholesterol to lipid poor HDL_3 and apoA-I [13,15,23], we evaluated if apoA-I addition is able to modify the cholesterol-induced

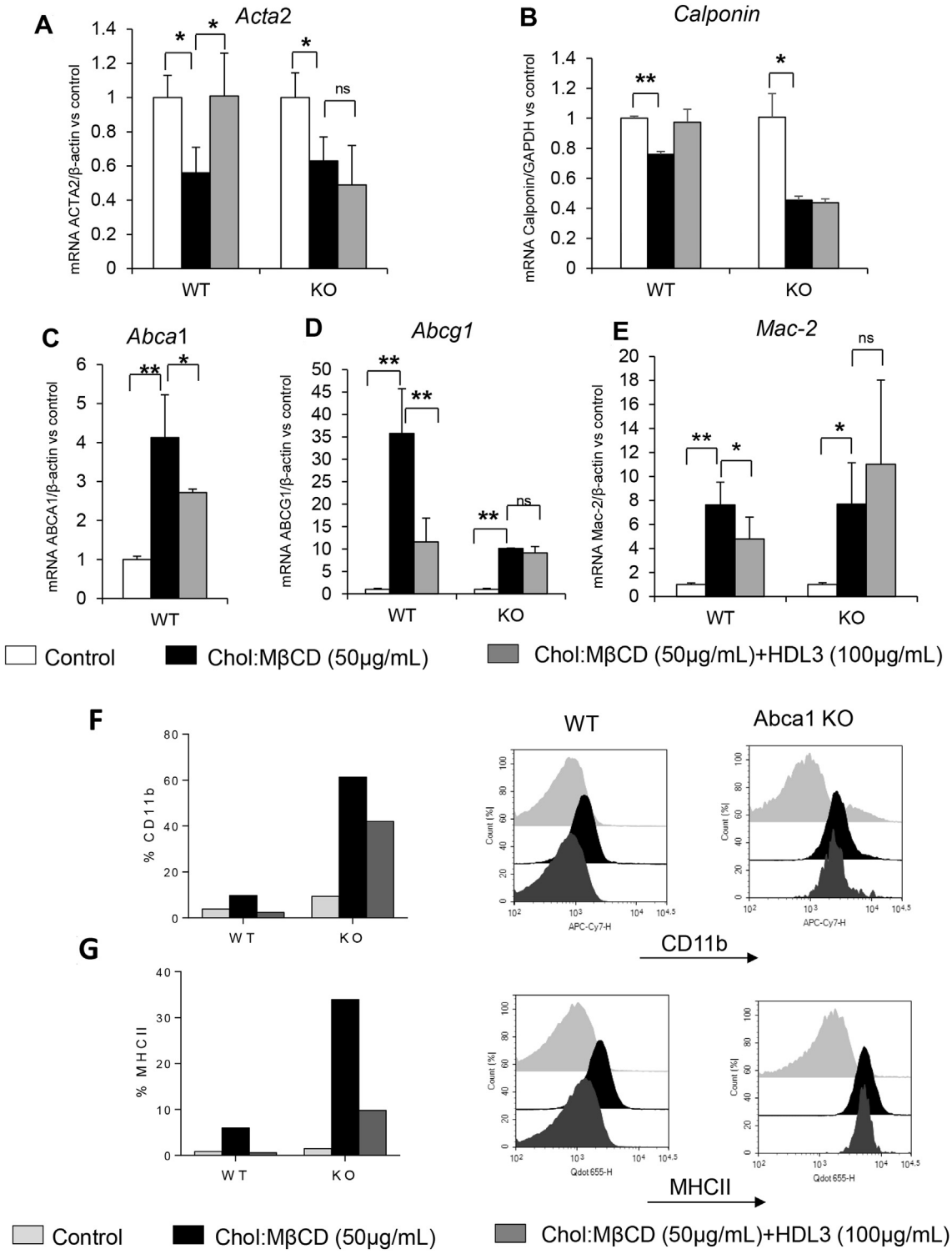


Fig. 1. HDL_3 differentially modulates the cholesterol-induced expression of genes/surface antigens in Abca1 WT and KO SMCs.
(A–E) Abca1 WT and KO mouse SMCs were treated with Chol:MβCD (50 μg/mL) in the presence or absence of HDL_3 (100 μg/mL). After 48 h, total mRNA was extracted and subjected to qRT-PCR analysis as described in Ref. [21]. Data are expressed as mean ± SD of three experiments performed in triplicates. (F–G) Cells were incubated as above, and after 7 days, surface antigens expression was analyzed by flow-cytometry as described in Materials and methods. ANOVA, Fisher's test: $^{*}p<0.05$; $^{**}p<0.01$.

phenotypic changes in Abca1 WT and KO SMCs. As shown in Table 1, cholesterol loading decreases *Acta2* and increases *Mac-2* mRNA levels, as shown above. However, apoA-I treatment has no effect on either *Acta2* or *Mac-2* mRNA levels in both cholesterol-loaded WT and KO cells.

3.3. *Abca1 knock-down blocks the preventive effects of HDL_3 on SMC phenotypic changes*

To confirm the involvement of ABCA1 in mediating the preventive effect of HDL_3 on SMC phenotypic modulation, we knocked

Table 1
Effect of lipid free apoA-I on *Acta2* and *Mac-2* mRNA levels in WT and Abca1 KO cells.

	WT			KO		
	Control	Chol:MBCD	Chol:MBCD + apoA-I	Control	Chol:MBCD	Chol:MBCD + apoA-I
	Fold of Control WT ± SD					
Acta2	1 ± 0.04	0.39 ± 0.05*	0.4 ± 0.1*	1 ± 0.1	0.6 ± 0.1*	0.65 ± 0.05*
Mac-2	1 ± 0.04	4.8 ± 0.4*	4.5 ± 1.1*	1 ± 0.1	10.1 ± 2.9*	9.25 ± 0.8*

Abca1 WT and KO mouse SMCs were treated with Chol:MβCD (50 μg/mL) in DMEM 0.2% EFAF, in the presence or absence of apoA-I (35 μg/mL). After 48 h, total mRNA was extracted and subjected to qRT-PCR analysis for *Acta2* and *Mac-2*, as described in Ref. [21]. Data are expressed as mean ± SD of three experiments performed in triplicates. ANOVA, Fisher's test: *$p < 0.05$ *vs*. Control.

down Abca1 expression in WT SMC by siRNA treatment. The incubation of WT SMC with *Abca1* siRNA reduced Abca1 expression to the levels observed in KO cells, as measured by either qRT-PCR or western blot analysis (see Fig. IV in Ref. [21]). As shown in Fig. 2, Abca1 knock-down completely abolishes the preventive effect of HDL_3 on cholesterol-induced phenotypic changes in WT SMC, evaluated by measuring *Acta2* and *Mac-2* expression.

3.4. Effects on Abca1 and Abcg1 mRNA expression in SMCs

Next, we analyzed the effect of cholesterol and HDL_3 on *Abca1* and *Abcg1* mRNA expression, membrane proteins involved in cholesterol efflux. QRT-PCR analysis shows that cholesterol-loading of aortic SMC increases mRNA expression of these transporters (Fig. 1C and D), as seen in C57BL/6 mice SMCs (Fig. I in Ref. [21]). As for *Acta2* and *Mac-2*, in WT cells HDL_3 significantly reduces the expression of *Abca1* and *Abcg1* induced by cholesterol treatment (Fig. 1C and D). However, in Abca1 KO cells again HDL_3 does not show any preventive effect on cholesterol-induced *Abcg1* expression (Fig. 1C and D).

3.5. Effects of HDL_3 on SMC phenotypic changes is independent from intracellular lipid removal

It has been shown that HDL_3 may reverse the cholesterol-induced phenotypic changes in SMCs [6]. Since we observed a different effect of HDL_3 on gene expression in Abca1 WT and KO cells, we tested whether this could be due to a different capacity of HDL_3 to remove cellular cholesterol from the two cell types. Abca1 WT and KO SMCs were incubated as before and intracellular lipids visualized by Oil Red O staining. As shown in Fig. 3 (upper panel), cholesterol loading of SMCs leads to foam cell formation, with an intracellular accumulation of neutral lipids (esterified cholesterol and triglycerides). The addition of HDL_3 does not differently affect lipid accumulation in both WT or Abca1 KO cells (Fig. 3 upper panel; C *vs*. B, and F *vs*. E). As expected, Abca1 KO SMC contain higher amount of both free and esterified cholesterol, as quantified by GLC (WT control: free cholesterol 31,1 μg/mg cell prot and esterified cholesterol 10,3 μg/mg cell prot; Abca1 KO control: free cholesterol 49,8 μg/mg cell prot, esterified cholesterol 63,5 μg/mg cell prot) and cholesterol loading causes a similar increase in intracellular free and esterified cholesterol in both WT and KO cells, (Fig. 3 lower panel). HDL_3 addition can lower free and esterified cholesterol levels in both cell types and at a similar level, regardless of the presence or absence of Abca1 (Fig. 3 lower panel, confirming the data already seen after Oil Red O staining). We tried to measure also intracellular triglycerides levels, but the amount was below the lower threshold levels of detection (data not shown). Since Abca1 modulates the removal of both cholesterol and phospholipids [15], we also measured the content of phospholipids in WT and KO cells. WT cells show a reduced phospholipid content both at basal and after HDL_3 treatment compared to KO cells (WT control: 30,1 μmol/mg cell prot; Fig. 3 lower panel). This could be due to the fact that the lack of functional ABCA1 causes phospholipid accumulation.

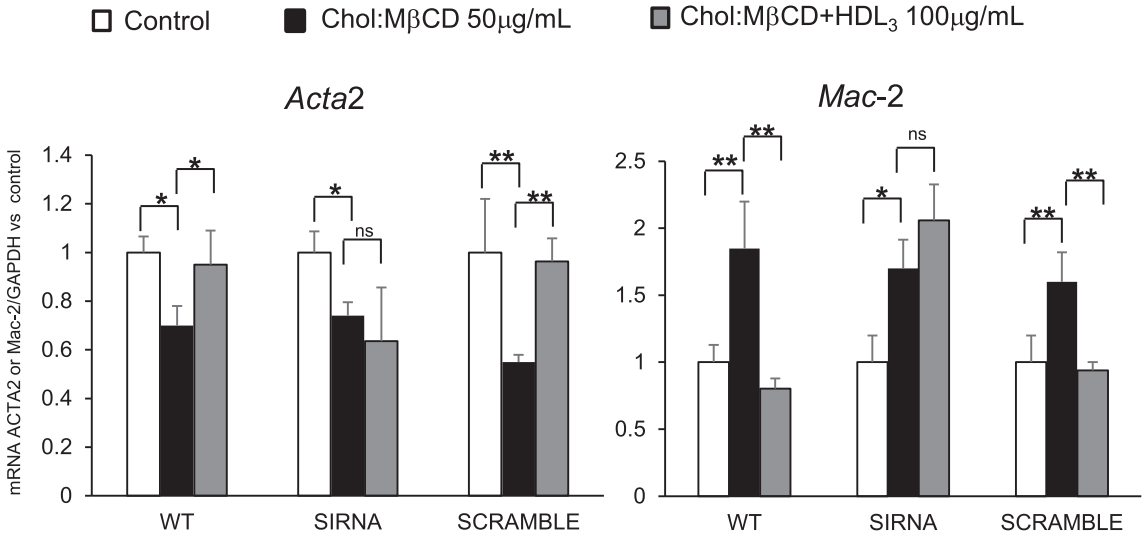
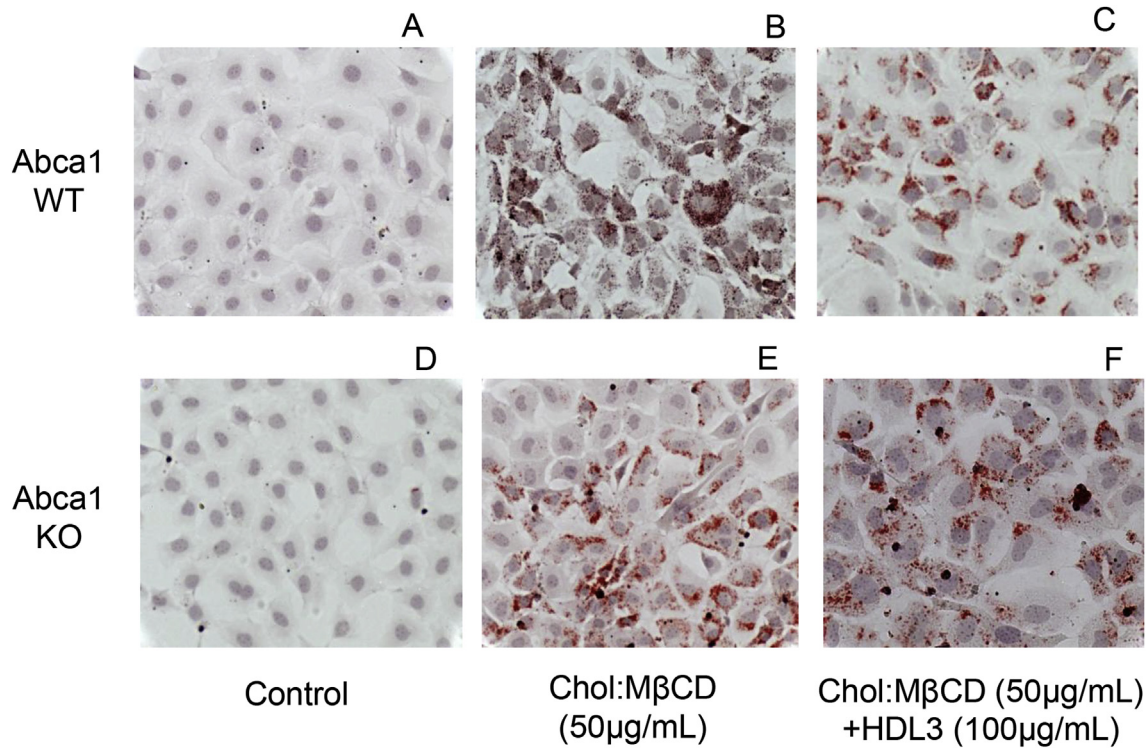


Fig. 2. Abca1 knock-down blocks the preventive effect of HDL_3 on cholesterol-induced phenotypic changes in WT SMC.
WT SMCs were treated for 24 h with *Abca1* siRNA or scramble siRNA. Then, cells were treated for 48 h with Chol:MβCD (50 μg/mL) in the presence or absence of HDL_3 (100 μg/mL). Total mRNA was extracted and subjected to qRT-PCR analysis for *Acta2* or *Mac-2* expression as described in Ref. [21]. Data are expressed as mean ± SD of five experiments performed in triplicates. ANOVA, Fisher's test: *$p<0.05$; **$p<0.01$.



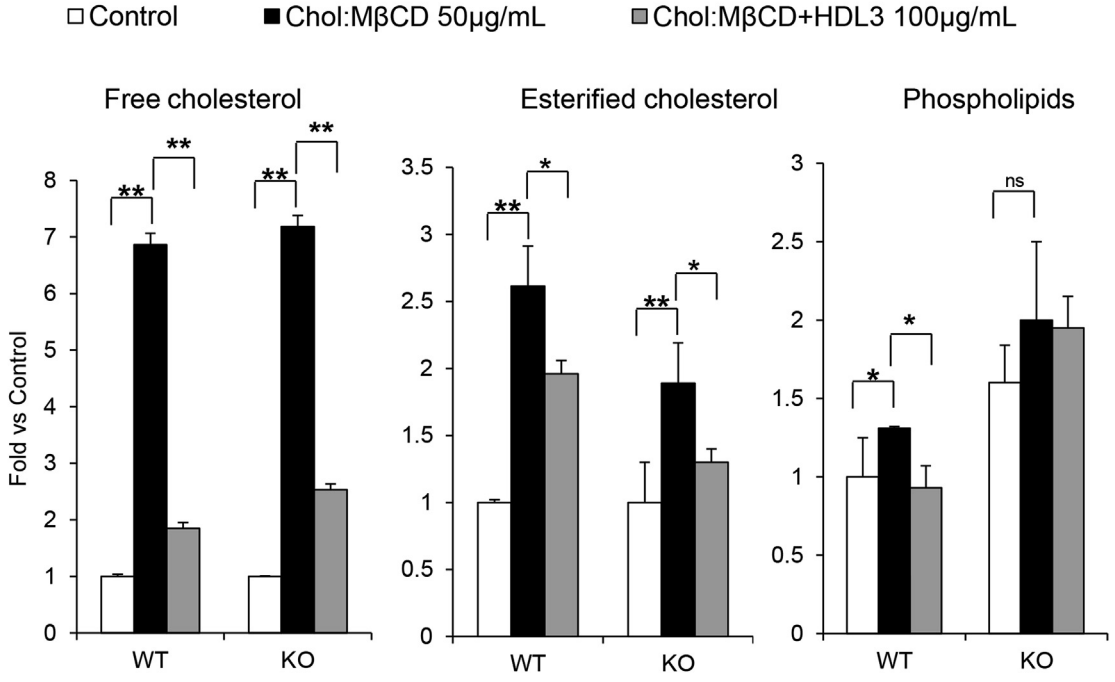


Fig. 3. HDL_3 does not differentially affect the accumulation of lipids in cholesterol-treated Abca1 WT and KO SMCs.
(Upper panel) Abca1 WT and KO mouse SMCs were treated with Chol:MβCD (50 μg/mL) in the presence or absence of HDL_3 (100 μg/mL). After 48 h, cells were fixed in 4% paraformaldehyde, stained with Oil Red O, and light microscopic images were acquired (original magnification 40X). (Lower panel) Abca1 WT and KO mouse SMCs were treated as described above. Then, cellular lipids (free and esterified cholesterol and phospholipids) were extracted and measured as described in Ref. [21]. Data are expressed as mean ± SD of four experiments performed in triplicates. ANOVA, Fisher's test: $^{*}p<0.05$; $^{**}p<0.01$.

3.6. Roles of miR-143/145, myocardin and Klf4 in modulating SMC phenotypic changes in response to cholesterol loading

Vengrenyuk, Fisher et al. [6] have very elegantly shown that cholesterol loading converts SMCs to a foam cell-appearing state by downregulating the miR-143/145-myocardin axis. We measured myocardin expression in our experimental conditions. The addition of cholesterol significantly reduces *myocardin* expression by up to 55% ($p<0.01$ *vs.* control) in both cell types. HDL_3 normalizes *myocardin* levels only in WT cells while it does not have any effect in Abca1 KO cells (Fig. 4A). Since miR-143 and miR-145 work together to regulate *myocardin* expression and SMC phenotypic switching [24], we measured the effect of cholesterol loading on miR-143/145 expression. As shown in Fig. 4B, cholesterol reduces the expression of miR-143 and of miR-145 in both cell types. HDL_3 normalizes miR-143/145 expression only in WT cells while it does not have any effect in KO cells.

To further characterize the effect on myocardin, we analyzed the involvement of KLF4, a repressor of myocardin [25], which is itself repressed by the miR-143/145-myocardin axis in a complicated feedback loop [6] and modulates SMC phenotypic transitions and functional properties [8]. In our experimental conditions, basal *Klf4* expression levels are increased in Abca1 KO cells compared to WT (Fig. 4A right panel), and this could explain the lower expression of *myocardin* observed in KO cells (left panel). After cholesterol loading, *Klf4* levels double in WT cells, while its expression is not affected in Abca1 KO cells. The addition of HDL_3 restores *Klf4* to basal levels in WT cells, while it does not have any effect in KO cells.

4. Discussion

Previous reports [2,6] have clearly shown that cholesterol-loading affects the plasticity of SMCs transforming them into foam cell-appearing cells, reducing the expression of *Acta2* and *calponin*, typical SMC markers, and increasing the expression of *Mac-2*, an inflammation-associated marker. In addition, cholesterol treatment increases also the expression of the transporters Abca1 and Abcg1, which participate in the reverse cholesterol transport pathway.

To analyze if the ABCA1 transporter has a role in SMC phenotypic switch, we evaluated *in vitro* the phenotypic changes induced by cholesterol loading both in WT and in Abca1 KO SMCs. Abca1 promotes the efflux of cholesterol and phospholipids especially onto lipid free and lipid-poor apoA-I [13,15]. Moreover, Abca1 modestly stimulates lipid efflux to smaller HDL_3 particles and does not promote cholesterol efflux to the larger HDL_2 fraction [13,26]. Interestingly, Haust [20] described lipid vacuoles and/or other cytoplasmic "inclusions" (ultrastructurally considered to present complex forms of lipids) in the aortic SMC in a Tangier disease patient, i.e. in the absence of a functional ABCA1. Concordantly, our results show that KO SMC have higher basal intracellular lipid levels compared to WT cells. The presence or absence of Abca1 does not influence, *per se*, the phenotypic changes in SMCs *in vitro*. In fact, after cholesterol loading, *Acta2* and *Mac-2* expression is modulated similarly in both WT and Abca1 KO SMCs (i.e., the expression of *Acta2* decreases and of *Mac-2* increases). Similarly, the expression of the transporters *Abca1* and *Abcg1*, and of inflammation-

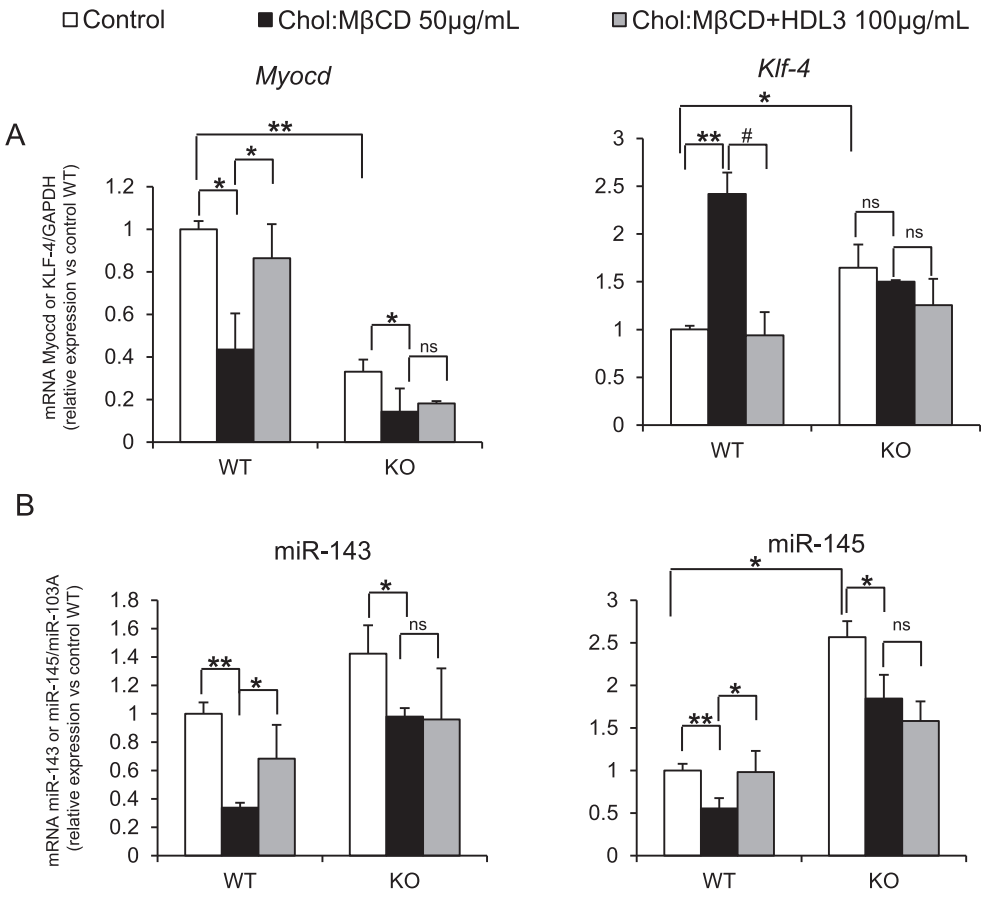


Fig. 4. HDL_3 prevents the cholesterol-induced modulation of *Myocd*, *Klf4*, and miR-143/145 mRNA in WT but not in Abca1 KO SMC.
Abca1 WT and KO mouse SMCs were treated with Chol:MβCD (50 µg/mL) in the presence or absence of HDL_3 (100 µg/mL). After 48 h, total mRNA was extracted and subjected to qRT-PCR analysis for *Myocd*, *Klf4*, and *miR143/145* expression as described in Ref. [21]. Data are expressed as mean ± SD of five experiments performed in triplicates. ANOVA, Fisher's test: $^{*}p<0.05$ *vs.* Control; $^{**}p<0.01$.

associated surface markers, such as CD11b and MHCII, also increases after cholesterol loading.

It has been previously reported that HDL may reverse the phenotypic changes induced by cholesterol loading of SMCs [6]. Interestingly, our data show that this is true only if a functional Abca1 is present. In fact, our results show that HDL_3 can effectively counteract the cholesterol-induced phenotypic changes only in WT cells, in which HDL_3 reverts SMC and inflammation-related gene mRNAs or surface antigens to the levels of control. On the contrary, HDL_3 is unable to counteract the phenotypic changes in Abca1 KO cells. The fundamental role of Abca1 in these processes is further demonstrated by the fact that knocking down *Abca1* expression in WT cells by siRNA prevents the rescue effect by HDL_3. Therefore, our results indicate that HDL_3 may play a protective role against SMC phenotypic switch but the presence of a functional Abca1 is mandatory.

Vengrenyuk et al. [6] previously showed a preventive effect of HDL_3 and apoA-I on SMC dedifferentiation postulating that this effect of HDL was because of the stimulation of cholesterol efflux in general. However, they did not actually measure the intracellular lipid content before and after cholesterol or HDL treatment. Our data show that the different behavior of WT and KO cells is not due to a different cholesterol efflux capacity in the presence of HDL_3. In fact, although KO SMC have a higher basal lipid content, HDL_3 can lower cholesterol levels in both cell types at a similar degree. Interestingly, HDL_3 stimulates intracellular phospholipids reduction in an Abca1-dependent manner. That is, HDL_3 induces the efflux of phospholipids only in the presence of Abca1. This is expected, since ABCA1 modulates phospholipids efflux [15] and the lack of a functional ABCA1 will not allow it.

Cholesterol loading modulates myocardin levels [6]. Myocardin is a powerful myogenic transcriptional coactivator which negatively regulates SMC inflammatory activation and vascular disease and its levels are reduced during atherosclerosis in association with SMC phenotypic switching [27–30]. We observed that *myocardin* basal expression in Abca1 KO SMCs is much lower compared to WT cells. This could be due to the increased expression of *Klf4* in SMC KO compared to what we observed in WT cells. Klf4 is a known repressor of myocardin expression [25], is required for phenotypic transition of cultured SMCs in response to PDGF [10] and regulates the transition toward an inflammatory phenotype [31]. The addition of cholesterol reduces *myocardin* expression in both WT and KO cells, reproducing the pathological situation observed during atherogenesis [27]. HDL_3 prevents this reduction only in WT cells. As expected, *Klf4* expression is increased by cholesterol treatment in WT cells and the addition of HDL_3 prevents this effect. In KO cells, we did not observe any effect of either cholesterol or HDL_3, suggesting that the absence of Abca1 affects Klf4 expression, but we do not have data yet to definitely prove this hypothesis. Studies are under way to unravel this matter.

MicroRNAs (miRNAs) are short non-coding RNAs that regulate gene expression by promoting degradation and/or repressing translation of specific target mRNAs. miR-143 and miR-145 may be co-transcribed in multipotent mouse cardiac progenitor cells before localizing into SMCs [32]. miR-143 and miR-145 are also involved in the modulation of SMC dedifferentiation upon cholesterol loading [6,33,34]. Therefore, we postulate an interaction between Abca1 and the miR-143/145-myocardin axis. In fact, if Abca1 is present, HDL_3 reverses the cholesterol-induced effects on both miR-143/145 and *myocardin* expression. On the contrary, in the absence of Abca1 HDL_3 preventive effect on SMC dedifferentiation is completely lost and this is paralleled by a lack of any effect(s) on myocardin, Klf4 and miRNAs.

Lipoproteins have been recently shown to carry miRNAs either through Abca1-mediated miRNA loading to HDL or through binding to circulating miRNAs [35]. miR-145 overexpression markedly reduces Abca1 levels in macrophages [36], while inhibiting miR-145 increases Abca1 protein levels and improves glucose-stimulated insulin secretion in hepatic cells [37]. In our experimental conditions, Abca1 KO SMCs express higher levels of miR-145 (Fig. 4B) compared to WT cells. Moreover, a reduced expression of miR-145 and miR-143 caused by cholesterol loading is paralleled by an increased expression of *Abca1* in SMCs, and HDL_3 may reverse these effects only if Abca1 is present. Thus, there is a clear link between miRNAs and Abca1, and the absence of Abca1 might affect the miRNA143/145-induced modulation of SMC genes. Several miRNAs (miR-128-1, miR-148a, miR-130b, and miR-301b) control the expression of key proteins involved in cholesterol-lipoprotein trafficking, such as the low-density lipoprotein receptor and ABCA1 [38,39]. It has been shown that *Abca1* is the target gene of miR-33 and the reduction of miR-33a might result in the increase of Abca1 protein by relieving translational repression at the 3′- UTRs of Abca1 [40]. Since Abca1 regulates cholesterol accumulation in SMCs, the increased Abca1 expression in these cells is a defense response leading to a reduction in SMC dedifferentiation and foam cells formation in the arterial wall. HDL_3 displays its beneficial properties by interacting with Abca1 and modulating these pathways. HDL_3, through its interaction with Abca1, may modulate miR-143/145 expression and SMC dedifferentiation in WT cells. The absence of Abca1 in the KO SMCs will block this regulatory pathway.

ABCA1 plays several fundamental roles in protecting from atherogenesis: it mediates cholesterol efflux to small dense HDL and to lipid-free apoA-I [13,14], thus modulating intracellular cholesterol levels, and participates to the reverse cholesterol transport pathway; it interacts with apoA-I to form mature HDL [16,17]; its deficiency, together with that of ABCG1, impairs macrophage migration [41] and accelerates atherosclerosis in mice [42]. The functions of ABCA1 are regulated both at the transcriptional and post-transcriptional level [43]. In addition, signaling cascades derived from the interaction of apoA-I with ABCA1 are involved in both ABCA1-mediated lipid efflux [14] and anti-inflammation [44]. Our results show that apoA-I/Abca1 interaction is not enough to counteract the dedifferentiation process, as apoA-I addition does not modify *Acta2* and *Mac-2* mRNA levels after cholesterol loading in both SMCs lines.

In conclusion, we demonstrate that HDL_3 prevents cholesterol-induced SMC phenotypic switching but only in the presence of a functional Abca1. Therefore, our studies add to the understanding of the atheroprotective effects of Abca1 and HDL_3. Abca1 and HDL_3, acting together, may modulate the metabolic pathways altered by cholesterol in SMCs reducing the formation of foam cells in the artery wall. Targeting this type of SMC phenotypic switching, might become a new strategy for developing new pharmacological intervention(s) to prevent or treat atherosclerosis.

Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Authors contributions

S.B. (corresponding author) conceived the entire study; S.B. and S.C. developed it; S.B., S.C., M.M., G.A.B., L.A., and M.C. performed the experiments, L.C. provided the apoAI; A.C. revised the manuscript, S.B. and S.C. analyzed and interpreted the data and wrote the manuscript. All authors have revised the manuscript critically for important intellectual content, and have given final approval of the version to be submitted.

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