■ A C C E L E R A T E D   P U B L I C A T I O N

Overexpression of STARD3 in human monocyte/macrophages induces an anti-atherogenic lipid phenotype

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A B S T R A C T

Dysregulated macrophage cholesterol homoeostasis lies at the heart of early and developing atheroma, and removal of excess cholesterol from macrophage foam cells, by efficient transport mechanisms, is central to stabilization and regression of atherosclerotic lesions. The present study demonstrates that transient overexpression of STARD3 {START [StAR (steroidogenic acute regulatory protein)-related lipid transfer] domain 3; also known as MLN64 (metastatic lymph node 64)}, an endosomal cholesterol transporter and member of the 'START' family of lipid trafficking proteins, induces significant increases in macrophage ABCA1 (ATP-binding cassette transporter A1) mRNA and protein, enhances [^{3}H]cholesterol efflux to apo (apolipoprotein) AI, and reduces biosynthesis of cholesterol, cholesteryl ester, fatty acids, triacylglycerol and phospholipids from [^{14}C]acetate, compared with controls. Notably, overexpression of STARD3 prevents increases in cholesterol esterification in response to acetylated LDL (low-density lipoprotein), blocking cholesteryl ester deposition. Thus enhanced endosomal trafficking via STARD3 induces an anti-atherogenic macrophage lipid phenotype, positing a potentially therapeutic strategy.

INTRODUCTION

Dysregulated macrophage cholesterol homoeostasis lies at the heart of early and developing atheroma, the principal cause of coronary heart disease. Macrophage 'foam cells', laden with cholesterol and cholesteryl ester, result from unregulated uptake of modified lipoproteins by macrophage scavenger receptors (CD36, CD68 and SR-AI/AII), and influence both plaque stability and progression [1]. Removal of excess cholesterol from macrophage 'foam' cells is central to lesion regression

and stabilization, and can be orchestrated, at least *in vitro*, by ABC (ATP-binding cassette) lipid transporters such as ABCA1, ABCG1 and ABCG4, and apo (apolipoprotein) acceptors, such as apoAI and apoE [2]. Efficient intracellular cholesterol transport is pivotal in marshalling appropriate cholesterol homoeostasis mechanisms, regulating sterol-responsive transcription factors, such as LXRα/β (liver X receptors α/β) and SREBPs (sterol-regulatory-element-binding proteins), and controlling cholesterol content of organelles, lipid rafts and membranes, and storage as cytosolic

droplets of cholesteryl ester [2,3]. Despite this, the proteins involved in non-vesicular cholesterol transport mechanisms remain poorly understood.

The START [StAR (steroidogenic acute regulatory protein)-related lipid transfer] domain is a 210 amino acid conserved 'helix-grip' fold, providing an adaptable binding site for lipids such as cholesterol, oxysterols, phospholipids and ceramides. In humans, START domains are found in 15 distinct proteins (STARD1–STARD15), implicated in non-vesicular lipid transport, cell signalling and lipid metabolism [4,5]. The prototypic member of this family, StAR (STARD1), delivers cholesterol from the outer to the inner mitochondrial membrane, to the CYP (cytochrome P450) side-chain-cleavage enzyme involved in steroidogenesis [6]. Overexpression of StAR can also decrease macrophage lipid content, inflammatory status and arterial cholesterol levels [7,8], and increase cholesterol efflux to apoAI via a mechanism dependent upon mitochondrial sterol 27-hydroxylase (CYP27A1), LXR activation and increases in ABCA1 mRNA and protein expression [9]. However, one 'downside' of StAR overexpression in macrophages is the induction of lipogenesis [9], possibly mediated by LXR-dependent increases in SREBP1c expression, a problem also associated with non-sterol LXR agonists.

The other member of the STARD1 subfamily STARD3 [also known as MLN64 (metastatic lymph node 64)] is a 54 kDa protein, co-amplified within chromosome band 17q12, a region containing the potent oncogene *ERBB2* in human breast carcinoma [10–12]. STARD3 is a late endosomal protein with two distinct conserved cholesterol-binding domains: a region of four transmembrane helices with three short intervening loops, called the MENTAL domain (MLN64 N-terminal domain), and the C-terminal 'START' domain [13]. The MENTAL domain may maintain cholesterol at the late endosomal membrane, prior to its shuttle to cytoplasmic acceptor(s) via the START domain.

Our previous studies have shown that cholesterol loading represses STARD3 expression, implicating this protein in dysfunctional cholesterol pathologies [14]. In the present study, we sought to establish a functional role for this endosomal cholesterol trafficking protein in macrophage lipid homoeostasis, testing the hypothesis that enhanced expression of STARD3 may be useful in the prevention of foam cell formation.

MATERIALS AND METHODS

Materials

Tissue culture reagents were purchased from Lonza; other reagents include pCMV.Script, and *STARD4* and *STARD*5 clones (Origene), *STARD3* clone (Stratagene), Amaxa monocyte/macrophage transfection reagent, NuPAGE gels and buffers (Invitrogen), antibodies

(AbCAM), primers and fluorescent probes (Eurogentec), and LDL (low-density lipoprotein) (Athens Research), acetylated as described previously [14]. Peripheral human monocyte/macrophages were purchased from Lonza, and human heart aorta RNA, derived from four to seven human heart aortae, was purchased from Clontech.

Cellular studies

Human (THP-1) monocytes (ECACC 880812101) were maintained in RPMI 1640 medium containing 10% (v/v) FBS (fetal bovine serum), as described previously [14,15]. STARD3, STARD4, STARD5 and empty vector control (pCMV; 0.5 μg of DNA) were delivered to THP-1 monocytes (10^7 cells) using an Amaxa Human Monocyte Nucleofector® kit (VPA-1007). Transfection efficiency using protocol Y001 (78.5%) was determined using the proprietary pmaxGFP® vector provided by Amaxa and flow cytometric analysis of 50000 cells. Transfected human THP-1 monocytes (1.5×10^6 cells/well) were differentiated into macrophages by addition of 100 nM PMA. Cellular lipids, RNA and cell protein lysates were collected 72 h post-transfection. Macrophage foam cell prevention experiments were initiated 48 h after transfection. Macrophages were radiolabelled with [^{3}H]oleic acid (1 μCi/ml; 10 μM) in the presence or absence of AcLDL (acetylated LDL; 50 μg/ml) and with or without an ACAT (acyl-CoA:cholesterol acyltransferase) inhibitor (447C88; 10 μM) for 24 h. Wild-type macrophages were incubated with progesterone (10 μM; 24 h) or U18666A (25 μM; 24 h) to inhibit endosomal trafficking, using ethanol vehicle (<0.1%). Cellular viability was assessed by conversion of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] into formazan. The caspase-Glo® 3/7 assay system (Promega) was used to detect apoptosis.

Lipid analyses

Flux of [^{3}H]oleic acid (1 μCi/ml; 10 μM) into the cholesteryl ester pool and of [^{14}C]acetate (1μCi/ml) into cholesterol, cholesteryl ester, fatty acid, triacylglycerol and phospholipid pools were assessed by TLC, as described previously [14,15]. Efflux of [^{3}H]cholesterol (0.5 μCi/ml) to apoA1 (10 μg/ml) and HDL (high-density lipoprotein; 10 μg/ml) were measured as described previously [13,15]. Mass of macrophage total cholesterol, triacylglycerol and choline-containing phospholipids were measured using Infinity™ and Phospholipids-B colorimetric assays (Alpha Labs) [14,15].

Gene and protein expression

Total RNA (Tri-Reagent; Sigma–Aldrich) was isolated from macrophages and reverse-transcribed to cDNA utilizing the MMLV (Moloney-murine-leukaemia virus) reverse transcriptase (Bioline). Expression of *STARD3*, *STARD4*, *STARD5*, *APOE*, *ABCA1*, *ABCG1*, *ABCG4*, *NR1H3* (encoding LXRα), *SREBP1* and *SREBP2*

Table 1   **Sequence of primers and probes used to quantify mRNA by PCR**

NR1H3 encodes LXRα.

Gene	Forward primer	Reverse primer	Probe sequence
STARD3	5′-CCTGCCCCGGTACCTCAT-3′	5′-GCGCTGTCGCAGGTGAA-3′	5′-AGAGCCTCGCGGCCACCATGT-3′
STARD4	5′-AACATCAGACAGGCCTTCCA-3′	5′-GAGAGCGGTGTGAGGTGCTT-3′	5′-TAGCGCGCCGTAGCTTCCA-3′
STARD5	5′-GTGGACTTGGTGCTAGTCAAGAGA-3′	5′-GACATAACGGATGCTCCACATG-3′	5′-ATGTGGACCATCAGTTCCAACGCCA-3′
ABCA1	5′TGTCCAGTCCAGTAATGGTTCTGT-3′	5′-AAGCGAGATATGGTCCGGATT-3′	5′-ACACCTGGAGAGAAGCTTTCAACGAGACTAACC-3′
ABCG1	5′-ACGTGCCCTTTCAGATCAATGT-3′	5′-GACGGCTGCGACGTCCATC-3′	5′-CCCAGTGGCCTACTGCAGCATCGT-3′
ABCG4	5′-GGTTCATGTCCCACGTGGTT-3′	5′-GCCGGTGTTGTTGAAGACCTT-3′	5′-TCTACCTGCATATTGGCGACGATGCC-3′
APOE	5′-TGGGTCGCTTTTGGGATTAC-3′	5′-CCATCAGCGCCCTCAGTT-3′	5′-CTGCTCAGCTCCCAGGTCACCCA-3′
NR1H3	5′-GAACAACTGGGCATGATCGA-3′	5′-AAGGAGCGCCGTTACACT-3′	5′-AAGCTCGTCGCTGCCCAGCAA-3′
SREBP1	5′-GCTCCTCCATCAATGACAAAATC-3′	5′-GCTTGAGTTTCTGGTTGCTGTGT-3′	5′-AAGGCCATCGACTACATTCGCTTTCTGC-3′
SREBP2	5′-CGGTAATGATCACGCCAACA-3′	5′-TGGTATATCAAAGGCTGCTGGAT-3′	5′-TCAGCACCACTCCGCAGACGAGG-3′
GAPDH	5′-CACATGGCCTCCAAGGAGTAA-3′	5′-TGAGGGTCTCTCTCTTCCTCTTGT-3′	5′-cTGGACCACCAGCCCCAGCAAG-3′

mRNA, relative to the housekeeping gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), was performed by Q-PCR (quantitative real-time PCR), using DNA engine Opticon 2 (MJ Research). PCRs contained cDNA template, Q-PCR mix, molecular biology grade water and 100 nM of each forward and reverse primer, and fluorescent probe [FAM (6-carboxyfluorescein)/TAMRA (6-carboxytetramethylrhodamine)]. Thermal cycling conditions were 15 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 20 s at 60 °C, with status at 50 °C. Primers and probes are given in Table 1. The comparative $2^{-\Delta C_t}$ method was utilized for quantification of each gene relative to *GAPDH* mRNA.

Macrophage lysates were collected in RIPA buffer plus Complete™ protease cocktail inhibitor (Roche). Protein lysates (15–30 μg/well) were separated by SDS/PAGE [NuPAGE; 10 % (w/v) gels], transferred on to a PDVF membrane and probed using rabbit anti-STARD3 (1:1000 dilution) or anti-GAPDH (1:1000 dilution) polyclonal antibodies, and a murine anti-ABCA1 monoclonal antibody (1:1000 dilution). Detection was achieved using appropriate secondary antibodies (1:1000 dilution) and the ECL (enhanced chemiluminescence) detection system [14]; densitometry was performed using Scion Image software.

Statistical analyses

Values are means ± S.E.M., and the number of independent experiments is indicated in the Figure legends. Significant differences ($P < 0.05$) were determined using a Student's *t* test or repeated measures ANOVA, followed by Dunnett's or Tukey–Kramer post-hoc tests, as appropriate. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ for comparisons are indicated.

RESULTS

Comparison of the endogenous expression of *STARD3* mRNA, relative to housekeeping gene *GAPDH*, in

Table 2   **Endogenous gene expression of *STARD3*, relative to *GAPDH*, in human (THP-1) monocytes and macrophages, human peripheral blood monocyte/macrophages and human heart aorta**

Results are from three individual cDNA preparations, derived from 4×10^7 human peripheral blood monocyte/macrophages (Lonza), the equivalent number of THP-1 monocytes and macrophages, and from a sample (50 μg) of four to seven pooled human heart aortae (Clontech).

Source of RNA	Expression of *STARD3* mRNA to *GAPDH* mRNA ($\times 1000$)
Human THP-1 monocytes	1.2 ± 0.2
Human THP-1 macrophages	0.8 ± 0.1
Human peripheral blood monocyte/macrophages	1.5 ± 0.4
Human heart aorta	0.4 ± 0.1

human THP-1 monocytes, THP-1 macrophages, human peripheral blood monocyte/macrophages and human heart aortae is shown in Table 2. Transient overexpression of *STARD3* in human THP-1 monocyte/macrophages increased mRNA levels by 87-fold (24 h; $P < 0.001$), 20-fold (48 h; $P < 0.001$) and 18-fold (72 h; $P < 0.05$) (Figure 1A), which translated into 2.2-fold ($P < 0.05$) increases in the levels of STARD3 protein after 72 h (Figure 2B) compared with the empty vector control; all values were normalized to the level of the housekeeping protein GAPDH. Cell viability was not altered by STARD3 overexpression after 72 h (25.4 ± 2.4 μM formazan in the empty vector control compared with 29.2 ± 6.4 μM formazan in the STARD3-overexpressing cells; $n = 6$ independent experiments; $P = 0.59$), and the levels of macrophage caspase 3/7 activity did not change when STARD3 overexpression was compared with the empty vector control (6971 ± 270 arbitrary fluorescent units in empty vector control compared with 7632 ± 1905 in STARD3-overexpressing cells; $n = 3$ independent experiments; $P = 0.509$). No
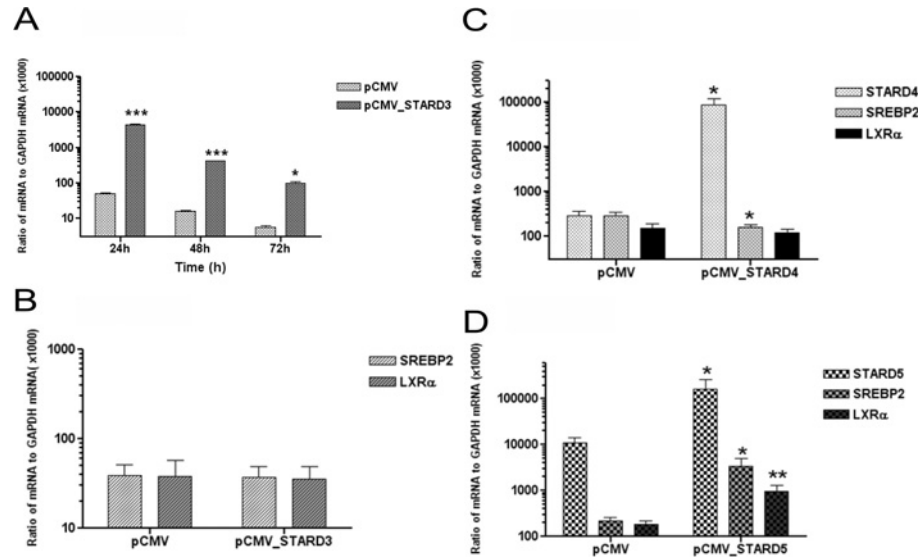
Figure 1   Overexpression studies: STARD3, STARD4 and STARD5

(A) Transient overexpression of *STARD3* compared with empty vector control (24–72 h) in human THP-1 monocyte/macrophages overexpressing STARD3. (B) Effect of STARD3 overexpression (72 h) on levels of *SREBP2* and *NR1H3* (LXRα) mRNA. Effect of overexpression of STARD4 (C) and STARD5 (D) on the gene expression of the same transcription factors in (B). Values are means ± S.E.M. of three independent experiments. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared with the empty vector control (pCMV).
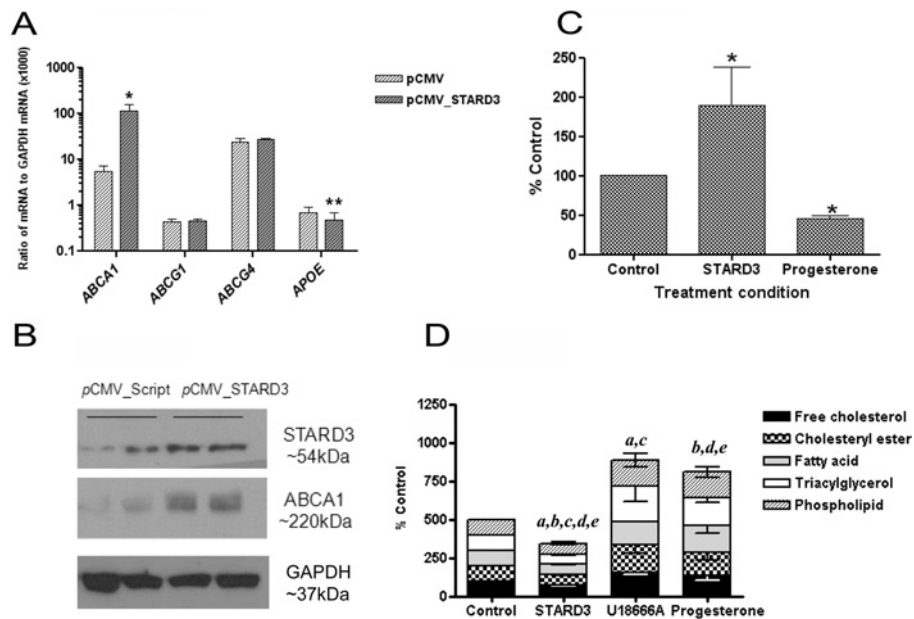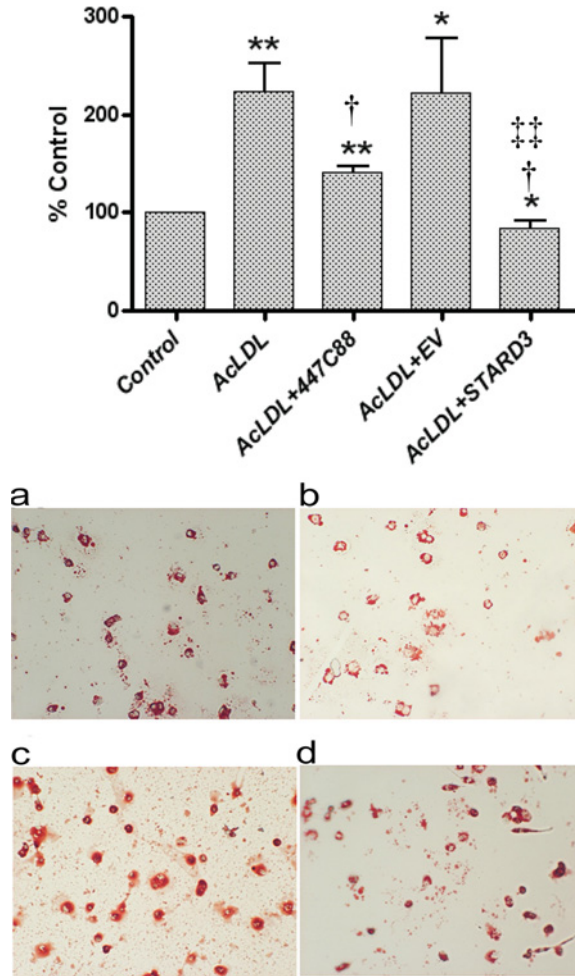

Figure 2   Effects of STARD3 overexpression on macrophage lipid homoeostasis

(A) Levels of *ABCA1*, *ABCG1*, *ABCG4* and *APOE* mRNA in monocyte/macrophages overexpressing STARD3 (72 h), compared with empty vector control, in three independent experiments. (B) Levels of STARD3 and ABCA1 protein, compared with the housekeeping protein GAPDH, in THP-1 monocyte/macrophages (72 h). Blots are representative of three independent experiments. The effect of STARD3 overexpression on (C) cholesterol efflux to apoAI (10 μg/ml) in five independent experiments, and (D) biosynthesis of lipids from [^{14}C]acetate (1 μCi/ml) in four independent experiments compared with empty vector controls,. Endosomal inhibitors U18666A (25 μM) and/or progesterone (10 μM) are included as positive controls. All values are means ± S.E.M. *$P < 0.05$ and **$P < 0.01$ compared with the empty vector control (pCMV). In (D), significant differences ($P < 0.05$) from the control incubation for each lipid are indicated as *a*, free cholesterol; *b*, cholesteryl esters; *c*, fatty acids; *d*, triacylglycerol; and *e*, phospholipids.

changes in steady-state levels of mRNA encoding sterol-responsive transcription factors *SREBP1* (not detectable), *SREBP2* or *NR1H3* were observed, as judged by Q-PCR (Figure 1B). In comparison, overexpression of genes encoding the cytosolic cholesterol transporters *STARD4* (Figure 1c) and *STARD5* (Figure 1D) exerted distinct influences on gene expression of these transcription factors: *STARD4* was associated with a reduced ($P < 0.05$) expression of *SREBP2*, whereas *STARD5* overexpression was associated with marked increases ($P < 0.05$) in both *SREBP2* and *NR1H3* mRNA levels.

Overexpression of STARD3 was associated with significant ($P < 0.05$) increases (20.7-fold) in ABCA1 mRNA (Figure 2A) and protein (2.6-fold) (Figure 2B); in contrast, no changes in *ABCG1* and *ABCG4* mRNA (Figure 2A) were observed between STARD3-overexpressing macrophages and control cells following normalization with GAPDH. These changes in gene expression strongly predicted functional increases in cholesterol efflux to apoAI, rather than to HDL, following STARD3 overexpression, and this proved to be correct, despite a small reduction in gene expression of the endogenous acceptor *APOE*. [^{3}H]Cholesterol efflux to apoAI (10 μg/ml; 24 h) was enhanced by 80% ($P < 0.05$) (Figure 2C), whereas efflux to HDL (10 μg/ml; 24 h) did not change significantly (5.27 ± 0.58% of HDL-specific efflux in empty vector control compared with 6.9 ± 2.5% of HDL-specific efflux in STARD3-overpexressing cells; n = 3; $P = 0.479$). In contrast, inhibition of endosomal trafficking using progesterone (10 μM), blocked apoAI-specific cholesterol efflux by 54% (n = 3; $P < 0.01$). Decreases in the biosynthesis of cholesterol (25.5%; $P < 0.05$), cholesteryl ester (34.1%; $P < 0.01$), fatty acid (27.3%; $P < 0.05$), triacylglycerol (41.9%; $P < 0.01$) and phospholipids (27.7%; $P < 0.05$) from [^{14}C]acetate were observed in STARD3-overexpressing macrophages (Figure 2D) compared with the empty vector control. No change in total cholesterol (26.1 ± 3.91 μg/mg of protein in empty vector control compared with 27.2 ± 3.15 μg/mg of protein in STARD3-overexpressing cells), triacylglycerol (83.7 ± 10.9 μg/mg of protein in empty vector control compared with 86.3 ± 21.3 μg/mg of protein in STARD3-overexpressing cells) or phospholipid mass (42.3 ± 3.96 μg/mg of protein in empty vector control compared with 47.1 ± 8.31 μg/mg of protein in STARD3-overexpressing cells) occurred over the time scale investigated. Again, inhibition of endosomal trafficking using U18666A (25 μM) or progesterone (10 μM) induced the opposite effect, increasing the incorporation of [^{14}C]acetate into each lipid pool ($P < 0.05$) (Figure 2D).

Importantly, when STARD3-overexpressing cells were challenged with AcLDL (50 μg/ml; 24 h), no significant increases in cholesterol esterification occurred, as judged by the incorporation of [^{3}H]oleate into the cholesteryl ester pool; in fact, a small but significant ($P < 0.05$)

Figure 3   Effect of STARD3 overexpression on macrophage foam cell formation

Upper panel, incorporation of [^{3}H]oleate (10 μM; 1 μCi/ml) into the cholesteryl ester pool following incubation with AcLDL (50 μg/ml; 24 h) in the presence or absence of the ACAT inhibitor 447C88 (10 μM) in wild-type cells, and in cells transfected with either empty vector (EV) or STARD3. Values are means $\pm$ S.E.M. of four independent experiments. *$P < 0.05$ and **$P < 0.01$ compared with controls; †$P < 0.05$ compared with AcLDL alone; and ‡‡$P < 0.01$ compared with empty vector control treated with AcLDL. Lower panel, Oil Red O staining in empty vector control (a and c) and STARD3-overexpressing cells (b and d) incubated in the absence (a and b) or presence (c and d) of AcLDL (50 μg/ml; 24 h).

decrease was observed. In contrast, in untreated and empty vector control cells, cholesterol esterification increased by approx. 2.2-fold ($P < 0.01$) when treated with AcLDL under the same conditions (Figure 3); a change blocked by the ACAT inhibitor 447C88 (10 μM). Equally, an apparent reduction in intensity of Oil Red O staining was observed in macrophages overexpressing STARD3, compared with empty vector control, after treatment with AcLDL (50 μg/ml; 24 h) (Figure 3).

DISCUSSION

The START family of lipid trafficking may be involved in non-vesicular cholesterol transport, regulating sterol-responsive transcription factors, controlling the cholesterol content of organelles, lipid rafts and membranes, and storage of cholesterol as cytosolic droplets of cholesteryl ester, although the mechanisms remain poorly understood at present.

Notably, our previous studies have demonstrated that overexpression of distinct cholesterol and oxysterol-binding proteins within the START family of lipid trafficking proteins, STARD1 (StAR) [9], STARD3, STARD4 and STARD5, exert different effects on gene expression of the sterol-responsive transcription factors SREBP2 and LXRα. Overexpression of the mitochondrial cholesterol transporter StAR is associated with the induction of lipogenesis [9], possibly via LXR-dependent induction of *SREBP1c*. Transient overexpression of cytosolic STARD4 decreased *SREBP2* mRNA levels by approximately half (Figure 1C), whereas overexpression of the gene encoding cytosolic STARD5 [16] protein resulted in marked increases in *SREBP2* and *NR1H3* mRNA levels compared with controls (Figure 1D). Overexpression of STARD4 and STARD5 have been shown previously to activate an LXR reporter plasmid in NIH-3T3 cells [17]. In contrast, STARD3 overexpression did not have an impact on either *SREBP2* or *NR1H3* mRNA levels (Figure 1B) and repressed lipogenesis (Figure 2D). Although some of the observed effects may be due to differences in gene expression and translation levels, despite the delivery of equivalent amounts of DNA to the same number of cells, they may also reflect the efficiency and/or directionality of the cellular cholesterol transport achieved [3,4].

Previously, we have speculated that STARD3 may traffic endosomally derived cholesterol to the ER (endoplasmic reticulum) and/or the plasma membrane [14]; even in the absence of lipoprotein-derived cholesterol, cholesterol cycles between the plasma membrane and endosomes [18]. Thus STARD3 could help to increase the cholesterol content of the ER membrane, and retention of SREBPs by the SCAP (SREBP2 cleavage-activating protein)/Insig (insulin-induced gene)-1(-2) complex, in agreement with the generalized repression of cholesterol, fatty acid and triacylglycerol biosynthesis observed in the present study. Cholesterol delivery to the ER might also be expected to expand the substrate pool available for esterification by ACAT1 (*SOAT1*). Instead, expansion of the cholesteryl [^{3}H]oleate pool following exposure to AcLDL was effectively blocked in cells overexpressing STARD3. One explanation for this finding is that delivery of sterol to the ER was just sufficient to sequester SREBPs, but does not reach the threshold required to activate ACAT. In turn, this must imply that the bulk of the endosomal cholesterol trafficked by STARD3 may

be efficiently directed elsewhere, perhaps to the plasma membrane to facilitate cholesterol efflux, possibly via vesicular transport facilitated by the Rab family of small GTPases. Dissociation between cholesterol transport to membrane-bound SREBP transcription factors and the substrate pool available for cholesterol esterification has been reported previously [19,20]. Kristiana et al. [20] observed clear differences in kinetics between endosomal delivery of LDL-derived cholesterol to SREBPs and ACAT in mutant CHO (Chinese-hamster ovary) cells with cholesterol trafficking defects [including NPC (Niemann–Pick type C)], contending that different cholesterol pools and/or transport pathways supply SREBPs and ACAT within the ER.

Our present findings also agree well with deletion studies of the START domain of STARD3 in mice *in vivo* [21]. Although relatively modest changes in lipid phenotype were observed, probably due to functional redundancies within the START family of cholesterol transfer proteins [21,22], significant increases in hepatic sterol ester were observed after feeding a high-fat diet, together with the reduced conversion of cholesterol into steroid hormones [21]. Use of a dominant-negative mutant of STARD3 (ΔSTART-STARD3) caused extensive cholesterol accumulation in CHO and COS-7 cells, accompanied by inhibition of late endosomal trafficking, similar to the phenotype caused by functional loss of NPC1/2 proteins [23]. Moreover, in cholesterol-laden cells, STARD3 becomes trapped at the periphery of cholesterol-laden lysosomes, reflecting a loss of dynamic cholesterol movement [23], and deletion of STARD3 is linked with disrupted actin-mediated dynamics of late endocytic organelles, suggesting that cholesterol binding or sensing by STARD3 in late endosomal membranes may govern actin-dependent fusion and degradative activity of that compartment [24]. Hepatic overexpression of STARD3 *in vivo* is associated with increased conversion into bile acids [25], although, in a separate study, apoptosis and hepatic toxicity were also reported, probably resulting from the grossly elevated and highly unphysiological levels of STARD3 utilized [26]; such changes were not observed in the present study.

Overexpression of STARD3 in the present study was also associated with increased expression of ABCA1 mRNA and protein. The latter suggests that STARD3, like NPC1, may facilitate trafficking of endosomal cholesterol and possibly ABCA1 protein through this compartment to the plasma membrane, increasing pools of membrane cholesterol and/or transporter available for efflux to apoAI [27]. The mechanism(s) by which STARD3 increases *ABCA1* mRNA levels are less obvious, as no induction of LXRα was observed in our present experiments, and *Npc1* inactivation reduces Abca1 protein levels, but does not alter *Abca1* mRNA levels, in murine macrophages [28]. However, SREBPs

(1 and 2) can exert repressive effects on ABCA1 expression, decreasing cholesterol efflux [29,30]. Thus it is possible that sequestration of SREBPs at the ER, as suggested by the co-ordinated loss of lipid biosynthesis observed in the present study, may relieve inhibition of *ABCA1* gene expression and increase cholesterol efflux to apoAI. Alternatively, STARD3 expression could alter levels of *ABCA1* mRNA by changing the ratios of saturated to unsaturated fatty acids [31], or perhaps via its involvement in actin-mediated dynamics of late endosomes [32] that trigger changes in actin-dependent gene expression [33].

In conclusion, STARD3 overexpression may be useful in limiting atherogenesis by up-regulating cholesterol efflux mechanisms, reducing cholesterol synthesis and inhibiting cholesterol esterification. *In vivo* studies are now needed to establish this contention, using murine models of atheroma.

AUTHOR CONTRIBUTION

Faye Borthwick initiated and performed the majority of the laboratory work, which was designed and supervised by Annette Graham; Anne-Marie Allen and Janice Taylor carried out additional experiments, including cell transfections, viability and caspase assays, and immunohistochemistry.

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