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ORIGINAL ARTICLE

Apolipoprotein A-I but not high-density lipoproteins are internalised by RAW macrophages: roles of ATP-binding cassette transporter A1 and scavenger receptor BI

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Abstract Accumulation of lipid-loaded macrophages (foam cells) within the vessel wall is an early hallmark of atherosclerosis. High-density lipoproteins (HDL) and apolipoprotein A-I (apoA-I) can efficiently promote cholesterol efflux from macrophages. Therefore, the interaction of HDL and apoA-I with macrophages appears to be important in the initial steps of reverse cholesterol transport, i.e. the transport of excess cholesterol from foam cells to the liver. However, although several cellular apoA-I and HDL receptors and transporters have been identified, it is as yet controversial how these interactions lead to cholesterol efflux from foam cells. In this study, we show that RAW264.7 macrophages bind HDL and apoA-I in a competable manner. Furthermore, cell surface biotinylation experiments revealed that apoA-I but not HDL is specifically internalised. Binding of HDL to macrophages is decreased by reducing the expression of scavenger receptor BI (SR-BI) with cyclic adenosine monophosphate (cAMP), acetylated low-density lipoprotein (acLDL) or RNA interference. In contrast, apoA-I cell association and internalisation is modulated in parallel with ATP-binding cassette transporter A1 (ABCA1) expression which is altered by stimulating cells with cAMP and acLDL or expressing short hairpin RNA (shRNA) against ABCA1. Consistent with this, cell surface trapping of ABCA1 with cyclosporin A (CsA) results in increased apoA-I binding but reduced internalisation. Furthermore, blocking apoA-I uptake inhibits its cholesterol efflux to apoA-I but not to HDL. Taken together, these data suggest that apoA-I- but not HDL-mediated cholesterol efflux may involve retroendocytosis.

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Abbreviations

HDL	High-density lipoprotein
apoA-I	apolipoprotein A-I

ABCA1	ATP-binding cassette transporter A1
SR-BI	scavenger receptor BI
LDL	low-density lipoprotein
acLDL	acetylated LDL
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CsA	cyclosporin A
siRNA	small interfering RNA
shRNA	short hairpin RNA

Introduction

Atherosclerosis is a progressive inflammatory disease that is characterised by the accumulation of lipids and fibrous elements within the wall of arteries [1]. Monocyte-derived macrophages are key players of both initiation and progression of atherosclerosis. Early in the development of atherosclerotic lesions, monocytes attach to endothelial cells and transmigrate into the subendothelial space where they can differentiate into macrophages [2]. The uncontrolled uptake of modified lipoproteins by macrophages via scavenger receptors leads to the accumulation of cholesteryl esters and the formation of macrophage-derived foam cells. This imbalance in cholesterol homeostasis is considered as an important step in the pathogenesis of atherosclerosis. Plasma levels of high-density lipoproteins (HDL) and their major protein constituent apolipoprotein A-I (apoA-I) are inversely correlated with the risk of atherosclerosis [3]. Among various atheroprotective properties of HDL and apoA-I, they serve as acceptor particles for macrophage cholesterol efflux, thereby initiating reverse cholesterol transport [4, 5]. For many years, the efflux of cholesterol was thought to occur primarily by passive aqueous diffusion, but recently it has become clear that this process is highly regulated and mediated by specific transporters and receptors including ATP-binding cassette transporter A1 (ABCA1) and scavenger receptor BI (SR-BI).

ABCA1 facilitates efflux of cellular phospholipids and cholesterol to lipid-poor apoA-I. Mutations in the ABCA1 gene are the underlying cause of Tangier disease [6–8], which is characterised by very low levels of plasma HDL, accumulation of cholesterol in tissue macrophages and moderately accelerated atherosclerosis. Mice with a targeted knock-out of ABCA1 in macrophages show enhanced atherosclerosis [9–11]. These data demonstrate the physiological importance of ABCA1 for cholesterol efflux. However, the molecular mechanism by which ABCA1 accomplishes cholesterol efflux is not yet resolved. Evidence is accumulating that lipid efflux requires physical interactions between apoA-I and ABCA1 [12–16], but it is not known whether this is a pure cell-surface event or involves the internalisation of apoA-I (reviewed in [17]).

In addition to ABCA1, SR-BI also promotes cholesterol efflux from macrophages, however to mature lipidated HDL rather than to lipid-poor apoA-I. SR-BI was originally characterised for its ability to mediate selective uptake of cholesteryl esters, phospholipids and triglycerides from HDL and other lipoproteins into liver and steroidogenic cells without whole-particle uptake [18]. However, SR-BI can also mediate bidirectional flux of cholesterol between macrophages and HDL [19]. Indeed, SR-BI-deficient mice as well as mice specifically lacking macrophage SR-BI show increased atherosclerosis [20–22]. However, although it is well accepted that the lipoprotein–macrophage interaction protects against foam cell formation and contributes to the prevention of atherosclerosis, the molecular mechanisms underlying this interaction are still not known.

In the present study, we investigated the interaction of HDL and apoA-I with macrophages to gain insight into the mechanism finally leading to cholesterol efflux. In contrast to other studies in this field, we directly compared the interaction of apoA-I and HDL within one well-defined cell system, namely, RAW264.7 cells. Thereby, we obtained evidence that ABCA1-dependent cholesterol efflux to apoA-I but not SR-BI-dependent cholesterol efflux to HDL requires internalisation of the agonist.

Materials and methods

Cell culture

The macrophage cell line RAW264.7 was obtained from the American Type Culture Collection and cultured in regular tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% fetal calf serum at 37°C in a humidified 5% CO_2, 95% air incubator.

Isolation and labelling of HDL and apoA-I

Plasma HDL ($1.063<d<1.21$ g/ml) was isolated from fresh normolipidemic human plasma of blood donors by sequential ultracentrifugation [23]. The purity of the lipoprotein preparation was verified by apolipoprotein composition analysis in a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to exclude contaminations with low-density lipoprotein (LDL) or albumin. HDL was iodinated with $Na^{125}I$ by the McFarlane monocholoride procedure as modified for lipoproteins [24]. Specific activities of approximately 300–900 cpm/ng of protein were obtained. Lipid-free human apoA-I was further extracted from HDL as described previously [25]. ApoA-I was labelled with ^{125}I using Iodo-Beads iodination reagent (Pierce) and $Na^{125}I$, according to the manufacturer's

instructions. Specific activities of 600–1,200 cpm/ng protein were obtained.

Binding and cell association

Cells were seeded in 12-well dishes at a concentration of 5×10^5 cells/well and grown until confluence (2 days). After washing the cells twice with DMEM, they were incubated for 2 h (or for the indicated time periods) at 4°C (binding) or 37°C (cell association) in DMEM/25 mM Hepes/1% bovine serum albumin (BSA) containing 5 μg/ml ^{125}I-apoA-I or 10 μg/ml ^{125}I-HDL, in the absence (total binding) or in the presence (unspecific binding) of a 40-fold excess of unlabeled apoA-I or HDL (or the indicated competitor). After incubation, cells were washed three times with cold phosphate-buffered saline (PBS) containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$. Cells were then lysed in 0.5 ml radioimmunoprecipitation assay (RIPA) buffer [10 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, complete protease inhibitor (Roche)]. The amount of bound radioactivity was determined using a γ-counter and normalised to the total protein content.

Cell surface biotinylation and internalisation

The assay was performed as described above for binding and cell association studies. After incubation with ^{125}I-apoA-I and ^{125}I-HDL, the cells were washed as described earlier and chilled on ice for 15 min. Cell surface proteins were biotinylated at 4°C for 1 h using 500 μg/ml EZ-link-sulfo-NHS-LC-biotin (Pierce) in PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$. After biotinylation, the PBS was saved, and radioactivity was measured and added to the amount of cell surface-bound protein measured later on. The reaction was stopped by 5 min incubation in DMEM. Cells were lysed in 0.5 ml RIPA buffer, and the lysates were incubated overnight at 4°C with 25 μl of 50% streptavidin-conjugated sepharose bead slurry (GE Healthcare) The biotinylated cell surface proteins were pulled down and thereby separated from not biotinylated internal proteins. The radioactive counts of the supernatant containing the internal proteins were measured and normalised to the protein contents.

Immunofluorescence microscopy

RAW264.7 cells were seeded in 24-well dishes and grown on coverslips until confluence. After washing the cells twice with DMEM, they were incubated for 2 h at 4 or 37°C in DMEM/25 mM Hepes/1% BSA containing 5 μg/ml apoA-I labelled with flourescein isothiocyanate (FITC) (Pierce). Thereafter, the cells were washed three times with PBS, fixed in 3% parformaldehyde and washed again with PBS. Cells were mounted in mounting media containing ProLong Antifade agents (Molecular Probes). Confocal microscopy and differential interference contrast microscopy was performed with a 63× oil-immersion lens.

SR-BI siRNA transfection

RAW264.7 cells were transfected with 67 nM BLOCK-iT™ fluorescent oligo only (noncoding) or in combination with 100 nM Stealth siRNA (both Invitrogen) against mSR-BI (5′-GGCTATGACGATCCCTTCGTGCATT-3′) with Lipofectamine 2000 in OPTIMEM (Invitrogen), according to the manufacturer's protocol. Six hours after transfection, the medium was replaced by DMEM 10% FCS without antibiotics. Binding, cell association and internalisation assays were performed 24 h after transfection. The efficiency of the silencing was evaluated by quantitative real-time polymerase chain reaction (RT-PCR) and Western blotting.

ABCA1 short hairpin RNA expressing plasmid and cell transfection

The plasmid expressing short hairpin RNA (shRNA) against mABCA1 was prepared using the GeneClip™ U1 Hairpin Cloning System (Promega). Two DNA oligonucleotides containing the hairpin siRNA target sequences mABCA1_sense 5′-TCTCGACCACCCTGGAAGAAA TAAAGTTCTCTTATTTCTTCCAGGGTGGTCCT-3′ and mABCA1_antisense 5′-CTGCAGGACCACCCTGGAA GAAATAAGAGAACTTTATTTCTTCCAGGGTGGTC-3′ (Microsynth) were cloned into the linearised pGeneClip™ neomycin vector. For stable transfection RAW264.7 cells were plated in a 6-well plate and transfected with the empty vector (mock) or with the vector containing the insert with Lipofectamine 2000. Two days after transfection, the cells were trypsinised and plated at low density in growth medium containing 1.0 mg/ml G418. Resistant colonies were picked and expanded. The efficiency of the silencing was evaluated by quantitative RT-PCR and Western blotting. Binding studies and cholesterol efflux experiments were performed in the absence of selective pressure.

Quantitative RT-PCR

Cells were harvested for total RNA using Tri Reagent according to the manufacturer's instructions (MRC). To remove contaminating genomic DNA from RNA, DNase I digestion (GE Healthcare) with subsequent DNase I inactivation was performed. Reverse transcription was performed using Superscript II RT (Invitrogen) following the standard procedure. Quantitative PCR was done with LightCycler FastStart DNA Master SYBR Green-I (Roche). Gene

specific primers were used as follows: mABCA1 5′-TGCC CTATGTGCTGTGCGTAG-3′ (forward), 5′-GGTGA GATTGAAGCCGTCCTC-3′ (reverse), annealing at 58°C, 3 mM $MgCl_2$; mSR-BI 5′-GGAATCCCCATGAACTG-3′ (forward), 5′-AGCCCTTTTTACTACCACTCC-3′ (reverse), annealing at 58°C, 3 mM $MgCl_2$. The transcription levels were normalised to mGAPDH (5′-CGACCCCTTCA TTGAC-3′ (forward), 5′-TCCACGACATACTCAGCAC-3′ (reverse), annealing at 58°C, 4 mM $MgCl_2$). Data analysis was performed using the ΔΔCt method.

Western blot

RAW264.7 cells were lysed in RIPA buffer. Equal amounts of total protein were separated by SDS-PAGE. Alternatively, cell surface proteins were determined by purifying biotinylated proteins by overnight incubation of 300 μg of cell proteins with 50 μg streptavidin-conjugated sepharose beads (GE Healthcare). The beads were washed three times with lysis buffer, and the pulled-down proteins were loaded on an SDS-PAGE. Proteins were transblotted onto a polyvinylidene fluoride membrane (GE Healthcare). The proteins were identified with the following antibodies: ABCA1 (ab18180, Abcam), SR-BI (ab3, Abcam). Expression levels were normalised to β-actin (A 5441, Sigma).

Lipid efflux

For cholesterol and phospholipid efflux experiments, RAW264.7 cells were labelled for 24 h with 2 μCi/ml [1,2-^{3}H]cholesterol and [methyl-^{3}H]choline chloride (Perkin Elmer), respectively. After the labelling period, cells were washed twice with DMEM and were allowed to equilibrate overnight in DMEM containing 0.2% BSA supplemented with cholesterol in the presence or absence of 0.3 mM 8-Br-cyclic adenosine monophosphate (cAMP; Sigma). After 24 h equilibration, the cells were washed and incubated for 6 h in DMEM containing 0.2% BSA in the presence or absence of 28 μg/ml lipid-free apoA-I or 100 μg/ml HDL. For some experiments, the cells were preincubated with 10 μM cyclosporin A (Sigma) 2 h before the assay and during the efflux period. Efflux media were collected and centrifuged to remove any detached cells and counted for ^{3}H. The cells were washed in PBS, lysed in 0.2 M NaOH/0.15 M NaCl and counted for radioactivity. Fractional cholesterol efflux was calculated as the percent of total [^{3}H]cholesterol or [^{3}H]choline released into the medium after subtraction of values obtained in the absence of apoA-I or HDL.

Statistical analysis

Each experiment shown is a representative of at least three similar experiments. Data are shown as means±SD. Statistical analyses were by analysis of variance (ANOVA) with Dunnett's multiple comparison post-test (for each group vs control) or Tukey's multiple comparison post-test (for all groups vs each other). All calculations were done using Prism software (GraphPad, San Diego, CA).

Results

ApoA-I and HDL specifically interact with macrophages

To determine the ability of apoA-I and HDL to function as ligands for macrophages, RAW264.7 cells were incubated with radiolabelled lipid-free apoA-I or HDL for 2 h at 4°C

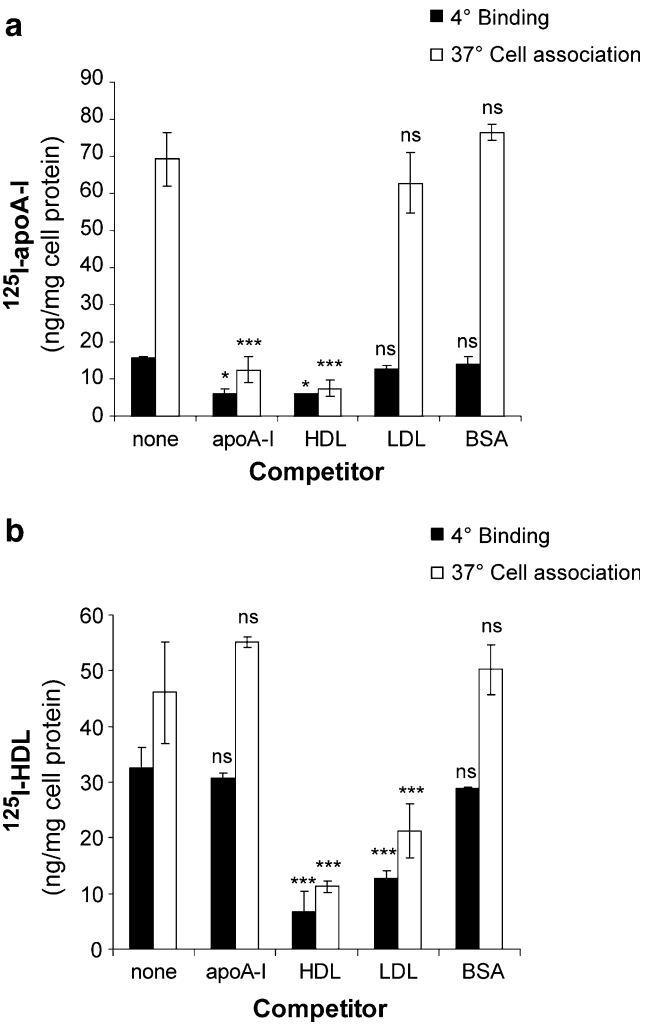

Fig. 1 ApoA-I and HDL are interacting in a specific manner with macrophages. **a** RAW264.7 cells were incubated at 4°C (*Binding*) or at 37°C (*Cell association*) for 2 h with 5 μg/ml ^{125}I-apoA-I in the presence or absence of a 40-fold excess of unlabelled competitor. **b** HDL binding and cell association were determined following the same method as for apoA-I, except that 10 μg/ml ^{125}I-HDL were used. *Single asterisk* $P<0.05$, *triple asterisk* $P<0.001$, *ns* not significantly different compared with binding/cell association in the absence of a competitor

(binding) or 37°C (cell association) in the presence or absence of a 40-fold excess of unlabelled competitors. ApoA-I binding and cell association were competed with an excess of apoA-I or HDL but not with an excess of LDL or BSA (Fig. 1a). HDL binding and cell association were extensively competed with an excess of unlabelled HDL, partially with LDL (~50%) but not at all with either BSA or, surprisingly, apoA-I (Fig. 1b). These results indicate that apoA-I and HDL can both interact specifically with macrophages although in a distinct manner.

ApoA-I but not HDL is internalised in macrophages

Next we analysed the cellular distribution of apoA-I and HDL in macrophages by using cell-surface biotinylation assays. RAW264.7 cells were incubated with ^{125}I-apoA-I or ^{125}I-HDL for 2 h at 4°C, a temperature where vesicular transport is blocked, or at 37°C. After this incubation period, cell surface-bound proteins were biotinylated at 4°C and separated from cellular lysate by immunoprecipitation with streptavidin beads. As shown in Fig. 2a, HDL was found on the cell surface, and no specific internalisation could be observed. In contrast to HDL, ~25% of the total cell associated apoA-I was found intracellularly (Fig. 2b). Similar results were also obtained by immunofluorescence microscopy (Fig. 2b). At 4°C, apoA-I was present at the cell surface only, whereas at 37°C, apoA-I clustered intracellularly, probably within vesicles in the cells. Furthermore, over time, apoA-I internalisation was increasing at 37°C, reaching saturation after 2 h, whereas at 4°C, as expected, no increase was observed (Fig. 2d). Upon analysis by SDS-PAGE, the internalised apoA-I was found intact as a single band of 28 kDA was detected (data not shown). Taken together, these results show that although both HDL and apoA-I can function as ligands for macrophages, only apoA-I is specifically internalised.

SR-BI is involved in the interaction of HDL with macrophages but does not affect the interaction of apoA-I

SR-BI not only promotes selective uptake of cholesterol from HDL but has also been shown to bind a variety of ligands including HDL, LDL and very low density lipoprotein (VLDL) [26–28]. Stimulation of RAW264.7 cells with 9-*cis*-retinoic acid (RA) and/or 22-hydroxycholesterol (22-HC), two pharmacological agents known to activate liver-X-receptors (LXRs), slightly reduced SR-BI expression on the mRNA level but did not affect its expression on the protein level (Fig. 3a,b). On the other hand, stimulation with acetylated LDL (acLDL), a modified form of LDL

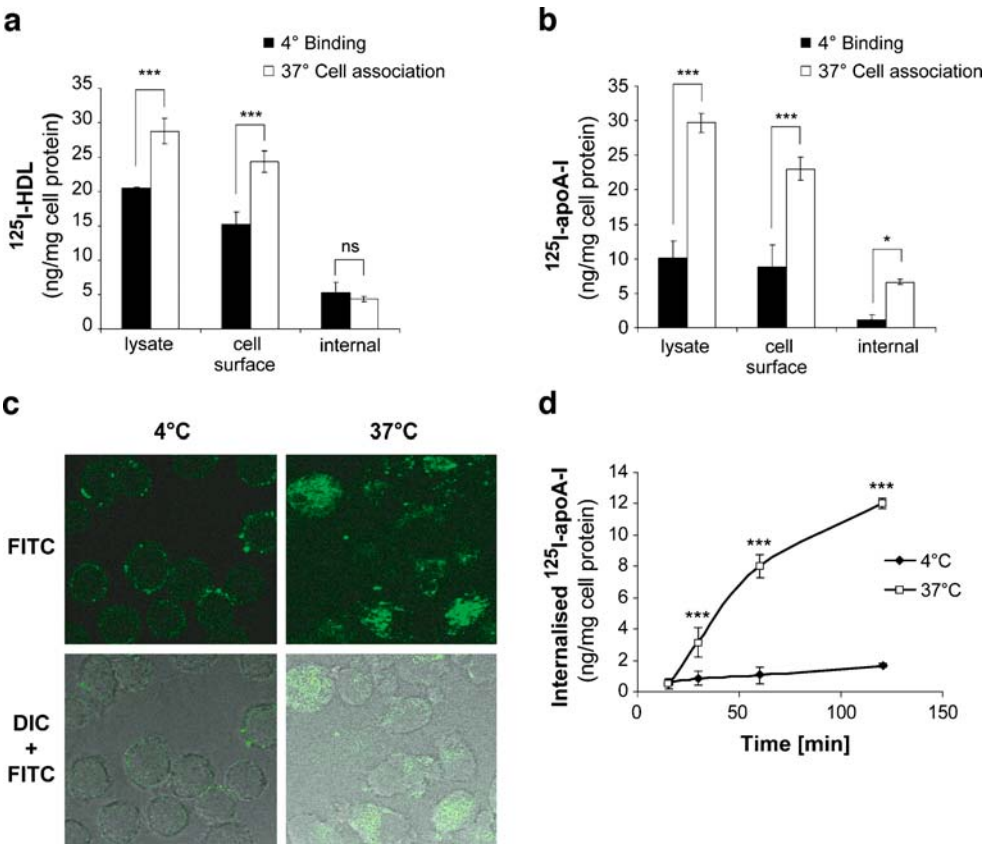

Fig. 2 ApoA-I but not HDL is specifically internalised by macrophages. RAW264.7 cells were incubated with ^{125}I-labelled protein at 4°C (control) or 37°C for 2 h. Cell-surface-bound proteins were biotinylated with 500 μg/ml biotin and captured with streptavidin beads, whereas not biotinylated proteins were counted as internal apoA-I or HDL. **a** Distribution of ^{125}I-HDL within the cell. **b** Distribution of ^{125}I-apoA-I with the cell after 2 h. **c** RAW264.7 cells incubated for 2 h at 4 or 37°C with 5 μg/ml FITC-labelled apoA-I. Images were analysed by confocal or by light differential interference contrast microscopy (×63). **d** Time course of ^{125}I-apoA-I internalisation. *Single asterisk* $P<0.05$, *triple asterisk* $P<0.001$, *ns* not significantly different compared with 4°C

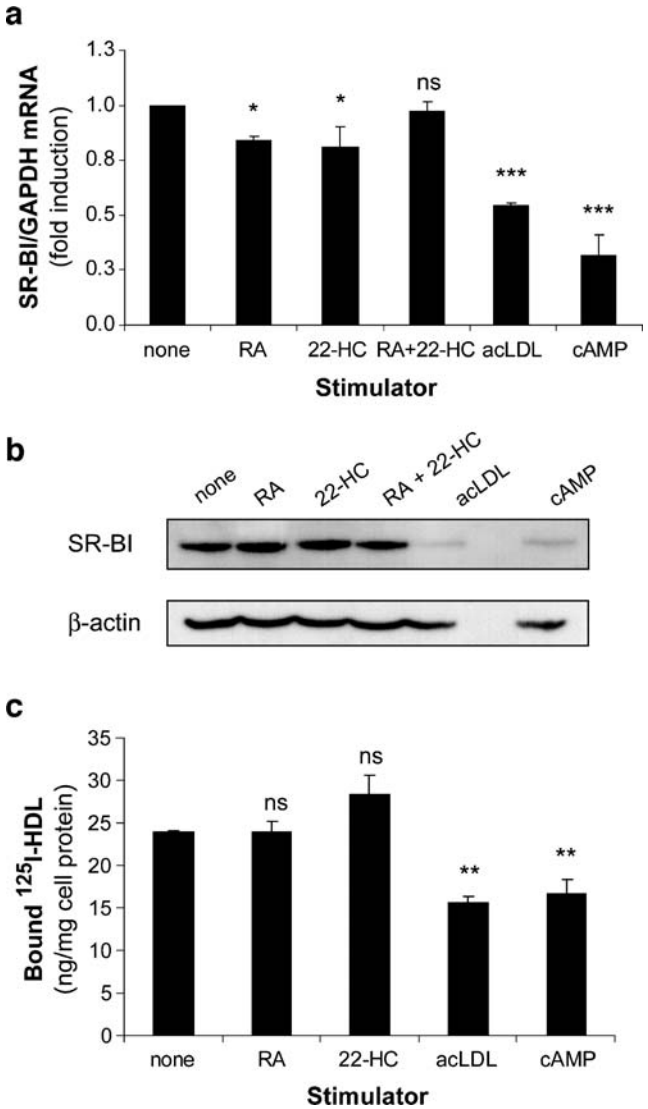

Fig. 3 Effect of SR-BI stimulation on HDL binding. **a** SR-BI mRNA and **b** protein levels were elucidated in cells treated with 10 μM 9-*cis*-retinoic acid (RA) or 10 μM 22-(*R*)-hydroxycholesterol (22-HC) for 24 h, 50 μg/ml acetylated LDL (acLDL) for 48 h, 0.3 mM 8-Br-cAMP for 16 h. **c** HDL binding in cells stimulated as in **a** and **b**. *Single asterisk* $P<0.05$, *double asterisk* $P<0.01$, *triple asterisk* $P<0.001$, *ns* not significantly different compared with not-stimulated cells

used to induce foam cell formation, and 8-Br-cAMP, an analogue of cyclic AMP that induces ABCA1 expression, reduced SR-BI expression (Fig. 3a,b) and diminished HDL binding by 40% (Fig. 3c). More direct evidence for the contribution of SR-BI to HDL binding was obtained by the use of RNA interference to reduce SR-BI expression. SR-BI transcription was reduced by 80%, and also the protein levels were significantly reduced in cells transfected with SR-BI-specific siRNA in comparison to controls (Fig. 4a,b). Furthermore, HDL binding was substantially lowered by more than 60% in siRNA transfected cells (Fig. 4c). We also investigated the involvement of SR-BI in apoA-I-binding and cell association. In contrast to HDL, none of the apoA-I interactions were affected in siRNA transfected cells compared to control cells (data not shown). The above experiments thus demonstrate that in RAW264.7 macrophages SR-BI modulates HDL binding but has no influence on apoA-I interactions.

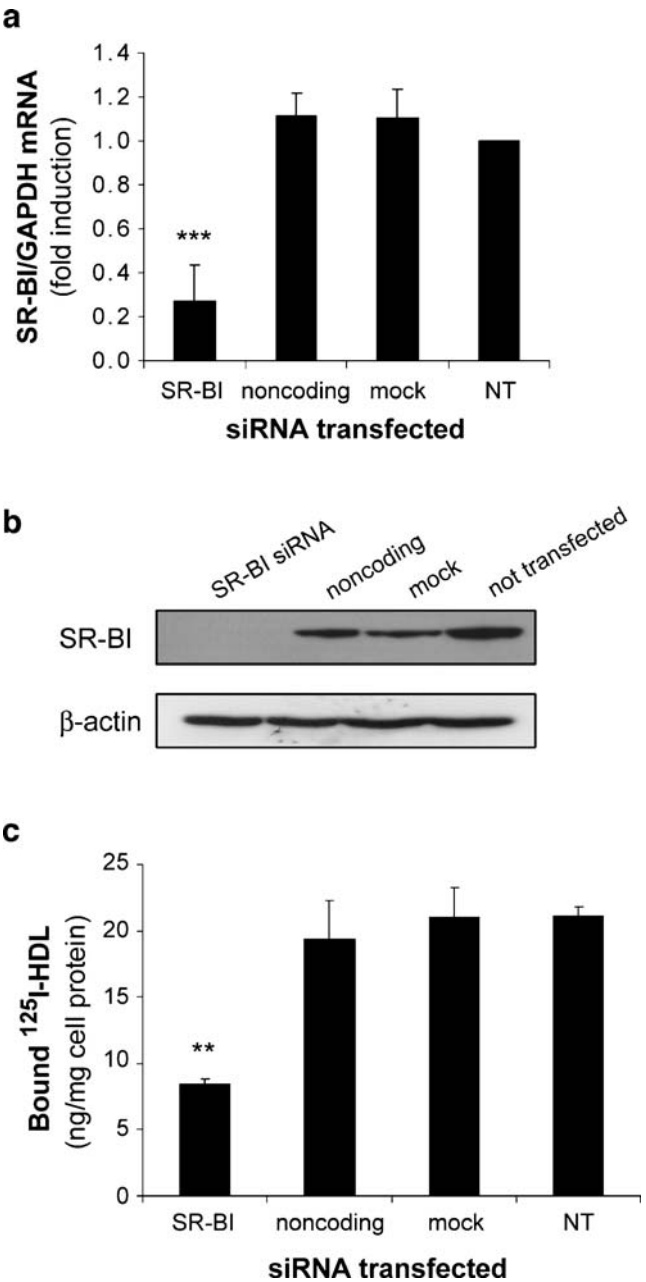

Fig. 4 Suppression of SR-BI expression decreases HDL binding. RAW264.7 cells were transfected with 100 nM siRNA specific for mSR-BI. SR-BI silencing was determined after 24 h **a** on the mRNA and **b** on the protein level. As control, noncoding siRNA, mock or not transfected (NT) cells were used. **c** HDL binding in siRNA transfected cells. *Double asterisk* $P<0.01$, *triple asterisk* $P<0.001$ compared with noncoding siRNA, mock or not transfected cells

ABCA1 modulates the binding, cell association and internalisation of apoA-I but has no effect on HDL binding

To examine the role of ABCA1 for binding apoA-I and HDL, RAW264.7 cells expressing shRNA against ABCA1 were used. In these cells, ABCA1 mRNA levels as well as protein levels were considerably reduced by 50% compared to control cells (Fig. 5a,b). HDL binding and cell association were identical in cells expressing shRNA against ABCA1 as well as in mock and not transfected control cells (data not shown). We then evaluated the interactions of apoA-I in cells with low ABCA1 expression. As shown in Fig. 5c and d, cell association and internalisation of apoA-I were significantly reduced by 40% in cells expressing shRNA against ABCA1. In addition, although stimulation with 8-Br-cAMP is known to induce ABCA1 expression in macrophages [29], apoA-I cell association and internalisation remained substantially reduced in these cells. Further evidence for the implication of ABCA1 in apoA-I cell association and internalisation was obtained by experiments done with cells stimulated with acLDL or 8-Br-cAMP, two treatments known to stimulate ABCA1 expression (Fig. 6a). In both cases, ABCA1 stimulation was accompanied by increased apoA-I cell association (Fig. 6b) and internalisation (Fig. 6c), indicating that modulation of ABCA1 expression not only affects apoA-I cell association but also internalisation.

The role of ABCA1 in apoA-I binding and internalisation was studied in more detail by treating RAW264.7 cells for 4 h with 10 μM cyclosporin A (CsA). As previously reported [30] and shown in Fig. 7a, the 4-h treatment led to a significant increase in cell surface ABCA1, which was further enhanced by stimulating the cells with 8-Br-cAMP. In parallel, binding of apoA-I was doubled after CsA treatment, and this increase was further enhanced by additional stimulation of the cells with cAMP (Fig. 7b). To exclude that the observed effect of CsA is ABCA1-independent, we repeated the same binding experiment in cells expressing the vector encoding for shRNA directed against ABCA1

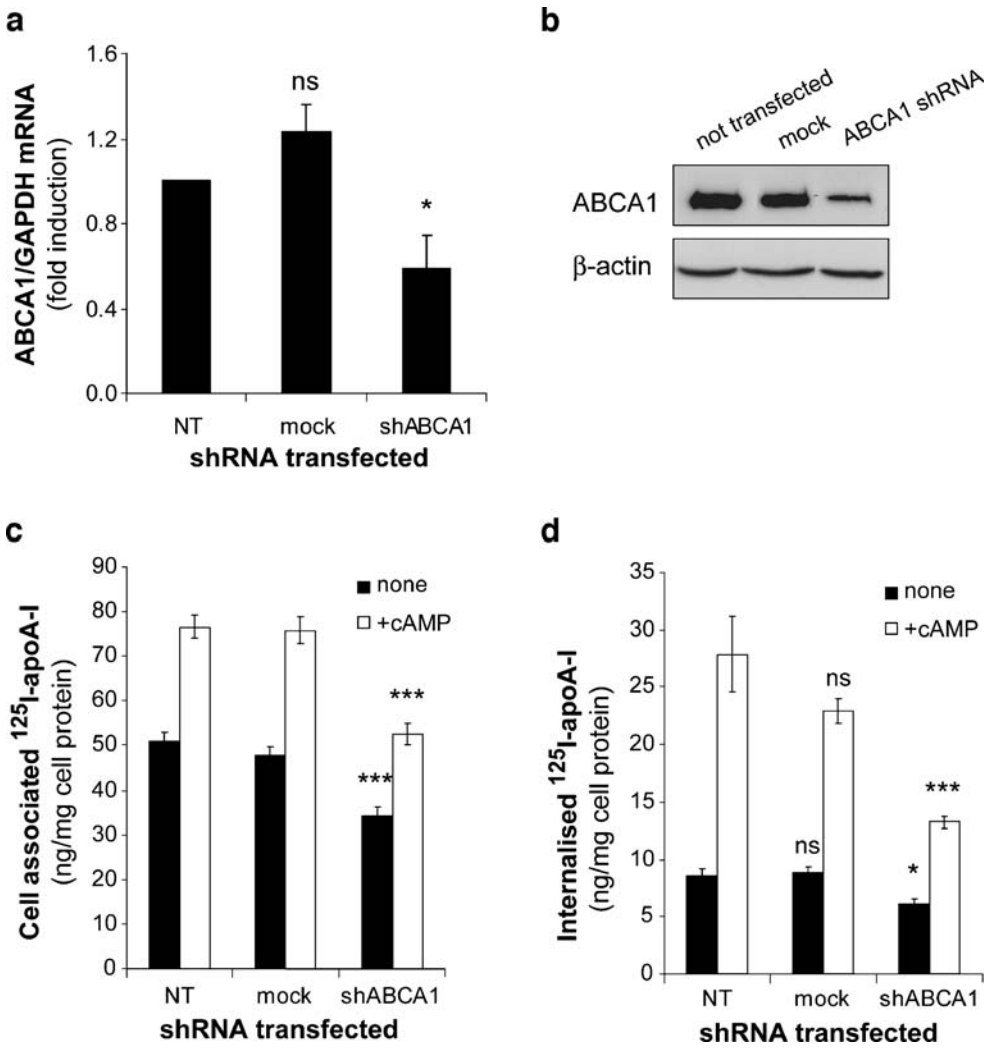

Fig. 5 ABCA1 plays a role in apoA-I cell association and internalisation. **a** Reduction in ABCA1 expression on the mRNA and **b** on the protein level was determined in cells stably transfected with a vector encoding for shRNA against mABCA1 (shABCA1). As control, cells transfected with the empty vector (mock) or not transfected cells (NT) were used. **c** ApoA-I cell association was measured in cells with low ABCA1 expression and in control cells in the presence or absence of 0.3 mM 8-Br-cAMP. **d** ApoA-I internalisation in cells treated as in **c**. *Single asterisk* $P<0.05$, *triple asterisk* $P<0.001$, *ns* not significantly different compared with mock or not transfected cells both in the presence or absence of 8-Br-cAMP

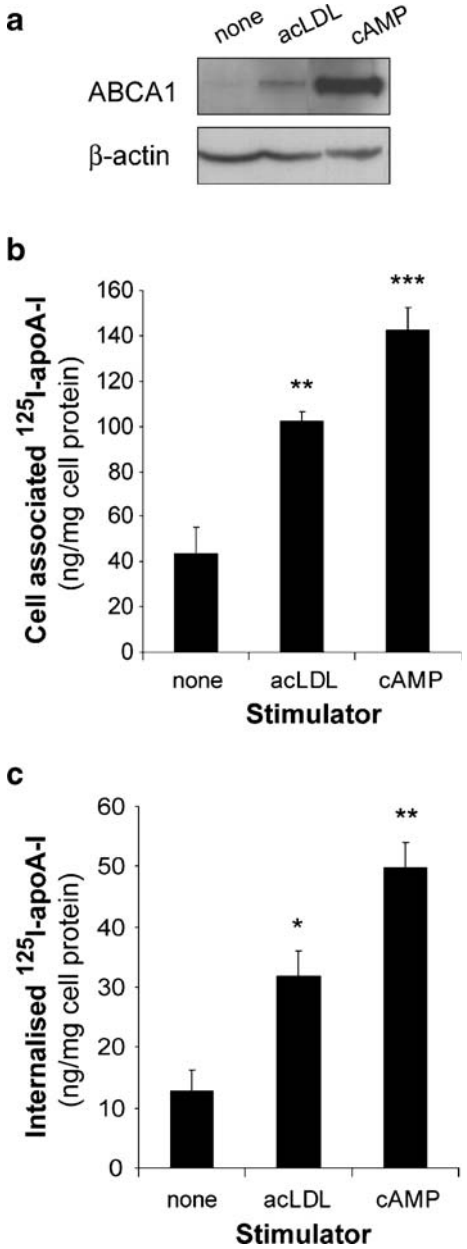

Fig. 6 ApoA-I cell association and internalisation correlate with ABCA1 expression. **a** ABCA1 expression on the protein level was determined in cells stimulated with 50 μg/ml acetylated LDL (acLDL) for 48 h or with 0.3 mM 8-Br-cAMP for 16 h. **b** ApoA-I cell association in stimulated cells. **c** apoA-I internalisation in stimulated cells. *Single asterisk* $P<0.05$, *double asterisk* $P<0.01$, *triple asterisk* $P<0.001$ compared with not-stimulated cells

(Fig. 7c). As expected, CsA treatment of these cells either in the absence or in the presence of 8-Br-cAMP did not affect apoA-I binding. It is interesting to note that apoA-I binding (Fig. 7b), in contrast to apoA-I cell association (Fig. 7d), was not significantly increased by stimulation with 8-Br-cAMP alone. Interestingly, apoA-I cell association (Fig. 7d) and internalisation (Fig. 7e) were strongly reduced by CsA treatment in 8-Br-cAMP-stimulated cells. Taken together, these results clearly demonstrate that ABCA1 plays a role in apoA-I binding, cell association and internalisation but is not involved in HDL binding.

Cholesterol efflux to apoA-I is inhibited when internalisation is reduced

Finally, cholesterol and phospholipid efflux to HDL and apoA-I were investigated under different conditions. Cholesterol efflux to HDL was doubled in cells stimulated with 8-Br-cAMP but was not altered in cells in which ABCA1 expression was reduced by transfection with shRNA (Fig. 8a). In contrast, after stimulation with 8-Br-cAMP, cholesterol efflux to lipid-free apoA-I was enhanced by a factor of 6 and was reduced in cells with knocked-down ABCA1 expression (Fig. 8b). This result provides additional evidence for the crucial role of ABCA1 in cholesterol efflux. To further elucidate if the reduction in cholesterol efflux was due to reduced apoA-I internalisation, we added CsA 2 h before the efflux period and again during the efflux period to inhibit apoA-I internalisation. Whereas cholesterol efflux from both unstimulated and 8-Br-cAMP stimulated cells to HDL remained unaffected by CsA (Fig. 8c), cholesterol efflux to apoA-I was almost completely inhibited by CsA in 8-Br-cAMP-stimulated cells (Fig. 8d). CsA also significantly reduced phospholipid efflux to apoA-I in 8-Br-cAMP stimulated cells (Fig. 8f), although to a lesser extent than cholesterol efflux. By contrast and like cholesterol efflux, phosholipid efflux to HDL remained unaffected by CsA treatment (Fig. 8e). Thus, it seems that ABCA1-dependent cholesterol and phsopholipid efflux is coupled to apoA-I internalisation.

Discussion

The role of HDL and its major apolipoprotein apoA-I in cholesterol efflux from macrophages has been studied extensively, but the molecular details underlying this interaction are still incompletely understood. In this paper, we directly compared the interactions of apoA-I and HDL with RAW264.7 macrophages, a cell system previously evolved as a well-accepted model for the study of cholesterol homeostasis in macrophages [31, 32]. Furthermore, we investigated the interactions of apoA-I and HDL with ABCA1 and SR-BI.

Consistent with previously published results, we showed that macrophages specifically bind HDL and apoA-I [12, 13, 33–35]. Based on competition assays illustrated in Fig. 1, we observed that while HDL competes for apoA-I binding, apoA-I is not a competitor for HDL binding. This observation suggests that HDL and apoA-I are binding to macrophages at least in part by distinct receptors. For

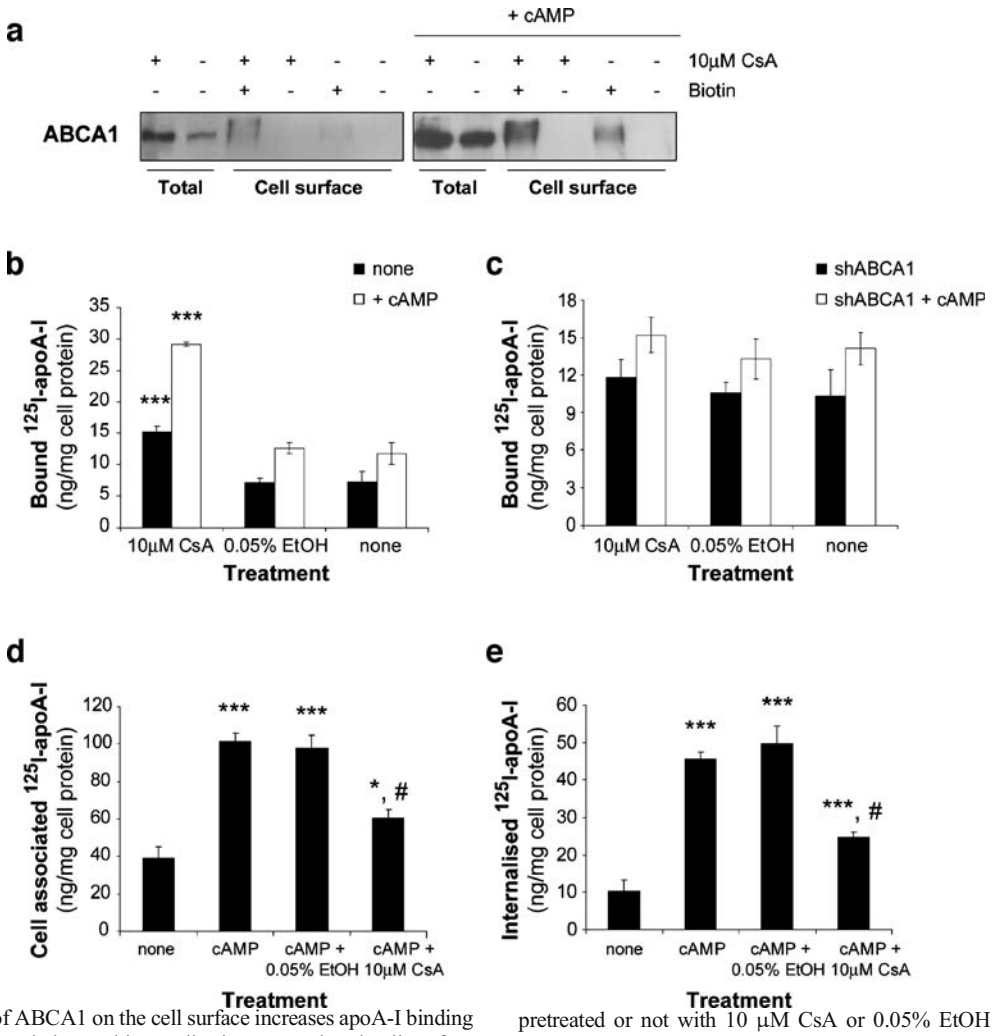

Fig. 7 Trapping of ABCA1 on the cell surface increases apoA-I binding but reduces cell association and internalisation. **a** Total and cell surface ABCA1 expression was measured in not-stimulated cells or in cells stimulated with 0.3 mM 8-Br-cAMP and incubated in the presence or absence of 10 μM CsA for 4 h. Cell surface proteins were biotinylated at 4°C and separated from cellular lysate by immunoprecipitation with streptavidin beads. Total and cell surface ABCA1 were detected by Western blot analysis. **b** ApoA-I binding was measured in not-stimulated cells or in cells stimulated with 0.3 mM 8-Br-cAMP pretreated or not with 10 μM CsA or 0.05% EtOH (control) for 4 h. *Triple asterisk* $P<0.001$ compared with control cells both in the presence or absence of 8-Br-cAMP. **c** ApoA-I binding in shABCA1 cells treated as in **b**. **d** ApoA-I cell association in 8-Br-cAMP stimulated cells in the presence or absence of 10 μM CsA. **e** ApoA-I internalisation in cells treated as in **d**. *Single asterisk* $P<0.05$, *triple asterisk* $P<0.001$ compared with cells treated in the absence of 8-Br-cAMP, *pound sign* $P<0.01$ compared with cells treated in the presence of 8-Br-cAMP

example, pre-β-HDL and lipid-free apoA-I are poor ligands for SR-BI [36], explaining the lack of competition of HDL binding by apoA-I. Conversely, it has been shown that apoA-I can dissociate from HDL, so that lipid-free apoA-I could be available for the competition of the apoA-I binding site by HDL [37].

While binding experiments performed at 4°C only monitor the amount of cell surface bound ligands, cell association at 37°C records both cell surface bound and internalised ligands. In agreement with this is our observation that cell association is always higher than binding (Fig. 1). Interestingly, this difference is higher for apoA-I than for HDL, providing initial evidence for distinct metabolic fates of apoA-I and HDL. In fact, using cell surface biotinylation assays and fluorescence microscopy, we were able to demonstrate that apoA-I but not HDL is internalised by macrophages in a specific and saturable manner (Fig. 2). Moreover, upon SDS-PAGE analysis, the internalised apoA-I was found to be intact rather than degraded (data not shown). Our finding of apoA-I internalisation is in line with findings of several other studies which also related this process to cholesterol efflux [38–41]. The lack of specific HDL internalisation is in agreement with some previous data [34, 42–45] but also in contrast to other data demonstrating HDL uptake [46–50]. This difference may reflect differences in cell types or in methods utilised to investigate uptake. Internalisation assays require methods that allow distinguishing intracellularly accumulated particles form particles bound at the cell sur-

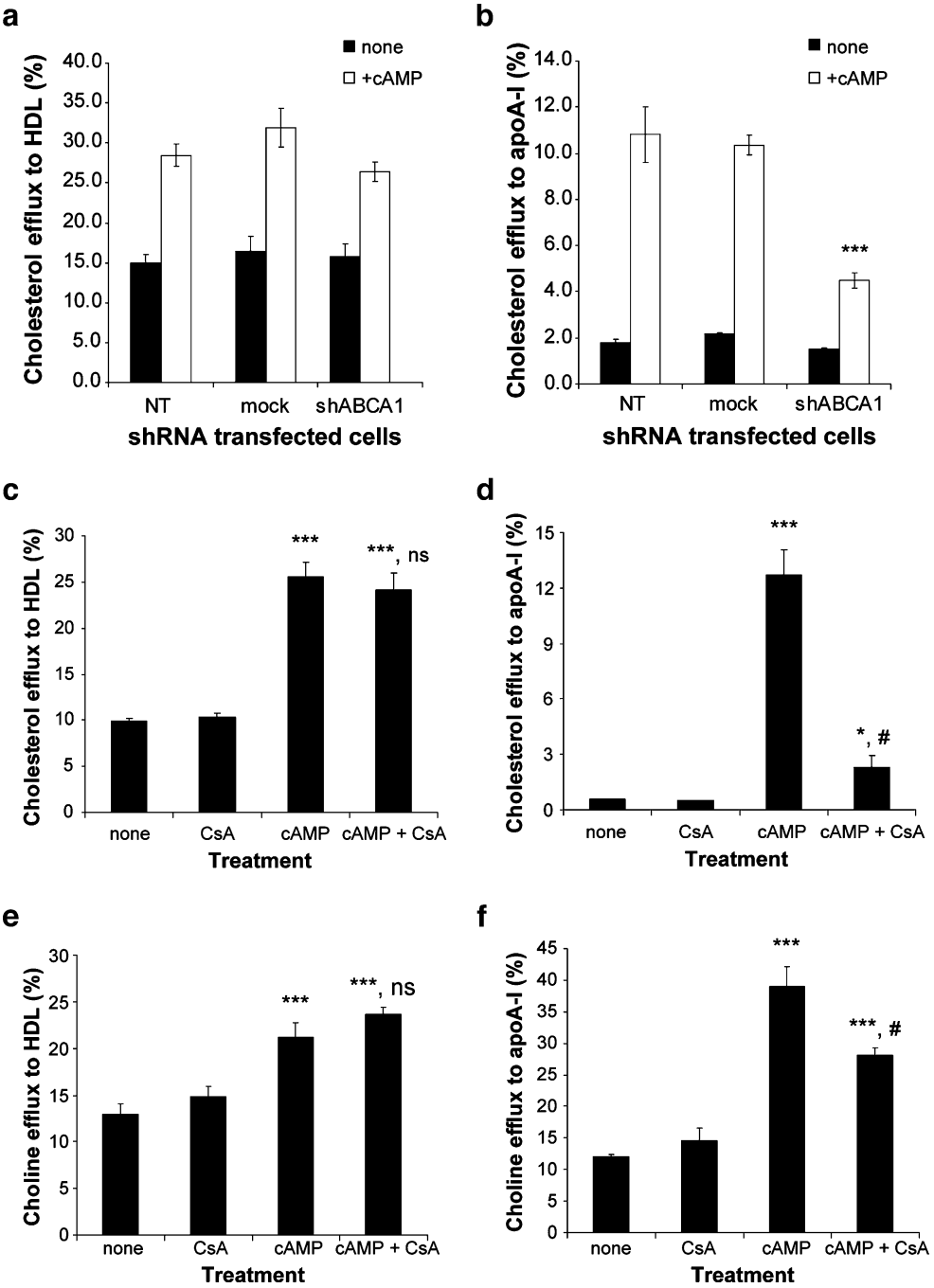

Fig. 8 Cholesterol efflux to apoA-I but not to HDL is inhibited when internalisation is blocked. **a** Cholesterol efflux to 100 μg/ml HDL for 6 h at 37°C from RAW264.7 cells stably expressing shRNA against ABCA1 or control cells pre-treated or not with 0.3 mM 8-Br-cAMP to induce ABCA1 expression. **b** Cholesterol efflux to 28 μg/ml apoA-I as described in a for HDL. *Triple asterisk* $P<0.001$ compared with mock or not transfected cells in the presence of 8-Br-cAMP. **c** Cholesterol efflux to 100 μg/ml HDL for 6 h at 37°C in the presence or absence of 10 μM CsA in cells stimulated with or without 0.3 mM 8-Br-cAMP. **d** Cholesterol efflux to 28 μg/ml apoA-I as described in c for HDL. **e** Choline efflux to HDL and **f** apoA-I as described above for cholesterol. *Single asterisk* $P<0.05$, *triple asterisk* $P<0.001$ compared with cells treated in the absence of 8-Br-cAMP, *pound sign* $P<0.01$, *ns* not significantly different compared with cells treated in the presence of 8-Br-cAMP

face. A commonly used approach is the displacement of extracellular bound particles with an excess of unlabelled particles at 4°C [51]. It should be noted that the efficiency to remove bound particles might be cell dependent and this could lead to the overestimation of internalised particles. The advantage of our protocol is that we extracted extracellular bound particles by exploiting the high affinity interaction between biotin and streptavidin. Moreover, it is important to emphasise that our experiments, by contrast to those reported by most other authors, were performed in parallel on apoA-I and HDL. Our discrepant findings on these two agonists strengthen the concept of distinct metabolic fates for apoA-I and HDL.

Next, we aimed to unravel the receptors modulating the interactions of HDL and apoA-I with macrophages. SR-BI is known as the receptor-mediating selective uptake of cholesterol esters from HDL by the liver and steroidogenic organs [27, 52]. Moreover, SR-BI is a multi-functional, multi-ligand receptor that facilitates the binding of a wide array of native and modified lipoproteins. With respect to

binding capacity, it is important to note that SR-BI has a known preference for lipidated apoA-I and HDL rather than lipid-free apoA-I [36, 53]. In agreement with this, reduction in SR-BI expression, either by lipid loading with acLDL or by stimulation with 8-Br-cAMP (Fig. 3) or by specific siRNA transfection (Fig. 4), significantly reduces HDL binding but has no effect on apoA-I binding, cell association and internalisation (data not shown). On the other hand, reduction in ABCA1 expression by the use of specific shRNA affects the interactions with apoA-I (Fig. 5) but does not modulate HDL interactions (data not shown). The effect of ABCA1 silencing on apoA-I cell association and internalisation became most prominently visible after stimulation with 8-Br-cAMP. In addition, while treatment of macrophages with acLDL or 8-Br-cAMP suppresses SR-BI expression, these stimulations increase ABCA1 expression as well as apoA-I cell association and internalisation (Fig. 6). Therefore, these findings not only suggest that HDL and apoA-I are interacting with distinct receptors, namely, SR-BI and ABCA1, but also that the interaction might be of specific importance under different conditions of altered cholesterol homeostasis. Moreover, it has been shown that also the relative proportions of lipid-free apolipoproteins and mature HDL in the plasma can affect the relative activities of ABCA1- and SR-BI-mediated cholesterol efflux [54, 55].

Whether ABCA1 mediates the binding of apoA-I directly and hence has to be considered as a receptor is still unclear. Overexpression of ABCA1 has been shown to increase apoA-I binding to the cell surface [13]. The direct interaction of apoA-I with ABCA1 is further supported by the study of both ABCA1 and apoA-I mutants. All ABCA1 mutants, except ABCA1(WS590S), which fail to promote cholesterol efflux also fail to cross-link apoA-I [15, 56]. Finally, apoA-I cross-linking to ABCA1 is possible only at room temperature [15]. We assessed the role of ABCA1 in apoA-I binding by using shRNA-mediated silencing. ABCA1 expression is reduced by about 50% on the mRNA level leading to a slightly lowered apoA-I binding (data not shown) compared to the more significant reduction in apoA-I cell association. This result clearly demonstrates the limitation of this approach especially for binding studies where the substantial residual ABCA1 activity still allows significant apoA-I binding. CsA has been shown to sequester ABCA1 on the cell surface [30]. In this paper, we observed that treating the cells with CsA indeed captures ABCA1 on the cell surface and in parallel increases apoA-I binding (Fig. 7a, b). Thus, these results provide additional evidence that ABCA1 can serve as a direct apoA-I binding protein. Moreover, apoA-I internalisation is reduced by CsA in 8-Br-cAMP stimulated cells. This observation well explains the observed reduction in apoA-I cell association in these cells and sustains the critical role of ABCA1 for apoA-I internalisation.

Our efflux studies finally reproduced previous findings that ABCA1 expression modulates cholesterol efflux to apoA-I but not to HDL (Fig. 8; reviewed in [57]). In addition, cholesterol and phospholipid efflux to apoA-I are inhibited whenever apoA-I internalisation is blocked, while lipid efflux to HDL is not affected. These data support the hypothesis of an ABCA1-dependent cholesterol efflux to apoA-I which involves internalisation of the ligand and probably resecretion of the lipidated particle, i.e. retroendocytosis. Indeed, several other studies are consistent with the need for endocytosis for lipid efflux to occur [38, 41, 58]. However, it is important to note that cholesterol efflux to apoA-I may also occur independently of apoA-I internalisation, suggesting that a more complex regulatory mechanism underlies this process. It might well be that apoA-I internalisation is needed to mediate cholesterol efflux from distinct intracellular cholesterol pools.

In summary, our study compares the interaction of HDL and apoA-I with macrophages. While HDL binds to the cells, for example via SR-BI, without being further internalised, apoA-I binding, cell association and internalisation correlate with ABCA1 expression. The expression and regulation of these two receptors ensures that both lipid-free apoA-I and HDL can remove excess cholesterol from macrophages under different circumstances. Future work will have to prove that our findings made in a transformed murine macrophage cell line are also valid for primary human monocyte-derived macrophages.

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