ABCA12 Regulates ABCA1-Dependent Cholesterol Efflux From Macrophages and the Development of Atherosclerosis

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Running title: ABCA12 – a New Regulator of Cholesterol Efflux

Summary

ABCA12 is involved in the transport of ceramides in skin, but may play a wider role in lipid metabolism. Here we show that in Abca12-deficient macrophages cholesterol efflux failed to respond to activation with LXR agonists. Abca12 deficiency caused a reduction in the abundance of Abca1, Abcg1 and Lxrβ; overexpression of Lxrβ reversed the effects. Mechanistically, Abca12 deficiency did not affect the expression of genes involved in cholesterol metabolism. Instead, a physical association between Abca1, Abca12 and Lxrβ proteins was established. Abca12 deficiency enhanced interaction between Abca1 and Lxrβ and degradation of Abca1. Overexpression of ABCA12 in HeLa/ABCA1 cells increased the abundance and stability of ABCA1. Abca12 deficiency caused accumulation of cholesterol in macrophages and formation of foam cells; it impaired reverse cholesterol transport *in vivo* and increased development of atherosclerosis in irradiated *Apoe*$^{-/-}$ mice reconstituted with *Apoe*$^{-/-}$*Abca12*$^{-/-}$ bone marrow. Thus, ABCA12 regulates cellular cholesterol metabolism via a LXRβ - dependent post-transcriptional mechanism.

Introduction:

Dysregulation of cholesterol homeostasis in macrophages is a pivotal event in the pathogenesis of atherosclerosis, the major cause of cardiovascular disease. Even small fluctuations of cellular cholesterol content cause apoptosis and necrosis. Macrophages, like all cells, possess a sophisticated regulatory network controlling cellular cholesterol homeostasis (Maxfield and Tabas, 2005). These pathways control, on the one hand, cholesterol biosynthesis and uptake of cholesterol in low density lipoproteins and on the other, the release of cellular cholesterol to the extracellular acceptors, such as apolipoprotein A-I (apoA-I) or high density lipoprotein (HDL). While mechanisms controlling cholesterol delivery have been investigated in great detail (Brown and Goldstein, 2009), the mechanisms responsible for regulation of cholesterol efflux are less clear. They are particularly relevant for macrophages, because cholesterol efflux in these cells is the major determinant of cholesterol homeostasis.

Three transporters responsible for regulated cholesterol efflux have been described: ABCA1, which is responsible for the efflux to lipid-free apoA-I, and ABCG1 and SR-B1, which regulate efflux to the mature HDL (Yvan-Charvet et al., 2010). However, numerous studies indicate that our understanding of the regulation of cholesterol homeostasis is far from complete (for review see (Fitzgerald et al., 2010)). We recently described a mouse carrying a mutation in another transporter, Abca12 (Smyth et al., 2008), a gene best known for being mutated in a skin disease, Harlequin Ichthyosis. In the skin, ABCA12 plays a critical role in the transport of ceramides in keratinocytes (Kelsell et al., 2005), however we found that in fibroblasts lacking Abca12 cholesterol homeostasis was also perturbed (Smyth et al., 2008). This prompted us to investigate the broader role of ABCA12 in cholesterol metabolism and the development of atherosclerosis.

Results:

Abca12 deficiency impairs cholesterol efflux from macrophages

To examine the role of Abca12 in cholesterol efflux we derived macrophages from the fetal livers of mouse embryos carrying the *El12* allele of *Abca12* (Smyth et al., 2008). ABCA1 is the sole determinant of specific cholesterol efflux to lipid-free apoA-I, therefore apoA-I was used as an acceptor to assess ABCA1-dependent efflux. In cells not activated with LXR agonist, cholesterol efflux to apoA-I was similar in cells of all genotypes (Fig. 1A). After activation of Abca1 expression with the LXR agonist TO-901317, cholesterol efflux was elevated in $Abca12^{+/+}$ cells, but not in mutant cells. Consequently, specific Abca1-dependent efflux was reduced in $Abca12^{+/El12}$ cells, and almost undetectable in $Abca12^{El12/El12}$ cells (Fig. 1A).

ABCG1 and SR-B1 are the principle mediators of specific cholesterol efflux to HDL; expression of ABCG1, but not SR-B1, is also activated by LXR agonists. We used HDL as an acceptor and cells activated with LXR agonist to test the effect of Abca12 deficiency on ABCG1-dependent efflux. In the absence of agonist, efflux was comparable in cells of all genotypes. Upon treatment with an LXR agonist, cholesterol efflux increased in $Abca12^{+/+}$, but not $Abca12^{El12/El12}$ cells (Fig. 1B). Similar results were obtained with macrophages derived from mice carrying the independent *Lx12* knockout allele of Abca12 (Yanagi et al., 2010). Fetal macrophages with this mutation exhibited similar defects in cholesterol efflux to apoA-I (Fig. 1C) and HDL (Fig. 1D) upon activation with LXR agonist. Non-specific efflux was unaffected by loss of Abca12 (Supplemental Table S1). These observations were also confirmed in the RAW 264.7 mouse macrophage cells upon siRNA mediated Abca12

knockdown (Fig. 1E, F). The effects of Abca12 deficiency on phospholipid efflux, a process also controlled by ABCA1, were similar to the effects on cholesterol efflux, except that phospholipid efflux was not impaired in heterozygous cells (Fig. 1G).

Since LXR activation triggers upregulation of Abca1 and Abcg1 expression, we measured the effect of Abca12 deficiency on levels of these proteins. Abca12 depletion by $siRNA^{Abca12}$ resulted in a substantial reduction in the levels of Abca1 and Abcg1, but not SR-B1, in cells activated with LXR agonist (Fig. 1H). The level of Abca1 after Abca12 silencing was 66.1 ± 4.4 % of control (mean ± SEM; n=7). Similar results were observed in $Abca12^{Lx12/Lx12}$ cells (Fig. 1I). Interestingly, in both siRNA treated RAW 264.7 cells and $Abca12^{Lx12/Lx12}$ -derived macrophages the abundance of Lxrβ, but not Lxrα was also reduced (Fig. 1J,K). The level of Lxrβ after Abca12 silencing was 58.5 ± 5.1 % of control (mean ± SEM; n=6). Collectively these data demonstrate that Abca12 deficiency impairs the ability of macrophages to up-regulate the levels of key ABC transporters and cholesterol efflux in response to LXR activation. Both ABCA1 and ABCG1 are known to play a significant role in cellular cholesterol homeostasis. While the precise role of ABCG1 is still contentious, the role of ABCA1 is well established; we therefore focused more detailed mechanistic studies on the relationship between ABCA12 and ABCA1.

The role of Lxrβ in impairment of cholesterol efflux in Abca12-deficient cells

Given that LXR primarily mediates its effects via the regulation of gene transcription, we examined the impact of Abca12 deficiency on the expression of genes involved in lipid metabolism. Using real time RT-PCR we assessed mRNA levels of *Lxrα*, *Lxrβ*, two LXR target genes (*Abca1* and *Abcg1*) and several other genes involved in cellular lipid metabolism (*Ldlr, Scarb1, Msr1, Abca3, Hmgcr*). As expected, *Abca12* mRNAwas undetectable in

Abca12$^{Lx12/Lx12}$ macrophages (Fig. 2A). Abca12 deficiency did not have a statistically significant effect on Abca1 and Abcg1 expression with or without stimulation by LXR agonists, nor on the expression of any other genes tested (Fig. 2A). Identical outcomes were observed when gene expression was analysed in RAW 264.7 cells with siRNA knockdown of Abca12 (Fig. 2B). Thus, loss of Abca12 does not impair cholesterol efflux via transcriptional regulation of the key mediators of cholesterol homeostasis. This is to be expected, given the functional redundancy between Lxrβ and Lxrα, and only partial decrease of Lxrβ levels in Abca12-deficient cells.

To control for agonist-specific effects, we treated cells with a chemically distinct LXR agonist, GW3965 (Joseph et al., 2002) and observed similar results (Fig. 2C,D). We also tested whether the effects of Abca12 deficiency are mediated through another regulator of ABC transporters, PPAR (Ogata et al., 2009). Treatment with the PPARα/γ agonist RO4509851 did not reverse the effect of Abca12 deficiency (Fig. 2C).

In addition to the LXR-dependent regulatory pathway, murine cells can activate *Abca1* expression and cholesterol efflux in response to treatment with cAMP (Haidar et al., 2002; Le Goff et al., 2006). When RAW 264.7 Abca12 knockdown cells were activated with cAMP, cholesterol efflux was elevated by about half the level observed in cells transfected with siRNA^{c} (Fig. 2E). cAMP modulates cholesterol efflux via several transcriptional (Le Goff et al., 2006) and post-transcriptional (Haidar et al., 2002) mechanisms. While the abundance of Abca1 protein was reduced in siRNA^{Abca12} transfected cells activated with LXR agonist, the levels of Abca1 mRNA (Supplemental Figure SI) and protein (Fig. 2F) were unaffected by Abca12 deficiency after activation with cAMP. We did not observe any effect of Abca12 deficiency on the cAMP induced changes in expression of 9 genes involved in the regulation of lipid metabolism (Supplemental Figure SI). These observations suggest that the effects of

Abca12 deficiency on Abca1 abundance are specific for the Lxr-dependent pathway. However, partial inhibition of cholesterol efflux indicates that Abca12 deficiency affected Abca1 functionality independently from its abundance.

To investigate the specific role of LXRβ in the impairment of cholesterol efflux in Abca12-deficient cells we conducted "rescue" experiments in which RAW 264.7 cells were transfected with $siRNA^{Abca12}$, *Lxrβ* cDNA or a combination of the two. Knockdown of Abca12 rendered cells unresponsive to treatment with LXR agonist, whereas transfection of Abca12-deficient cells with heterologous *Lxrβ* cDNA largely restored cholesterol efflux and Abca1 abundance (Fig. 2G,H). Transfection with *Lxrβ* cDNA alone had no effect (Fig. 2G). These findings confirmed that the effect of Abca12 deficiency on cholesterol efflux and Abca1 abundance is at least partially mediated by Lxrβ. Together these data demonstrate that Abca12 regulates Lxrβ abundance and Abca1 protein levels and functionality via a post-transcriptional mechanism.

Mechanisms of modulation of cholesterol efflux by Abca12

Previous studies have proposed that LXRβ can directly bind to ABCA1 reducing its activity and that LXR agonists interfere with this interaction reversing the inhibitory effect of LXRβ (Hozoji-Inada et al., 2011). A further post-translational mechanism that regulates ABCA1 activity and abundance is its re-localization: when ABCA1 is displaced from the cell surface it no longer supports cholesterol efflux and is rapidly degraded (Neufeld et al., 2001). We therefore investigated the effects of Abca12 deficiency on Abca1 localization and co-localization of Abca1 and Lxrβ. Abundance of Abca1 in non-activated RAW 264.7 cells was low and there was a degree of co-localization of Abca1 and Lxrβ (co-localization coefficient:

0.56±0.09) (Fig. 3A-C). In cells activated with LXR agonist the abundance of Abca1 increased considerably, but co-localization of Abca1 and Lxrβ did not change significantly compared to the non-activated cells (co-localization coefficient: 0.51±0.04) (Fig. 3D-F). Remarkably, the co-localization of Abca1 with Lxrβ dramatically increased in cells activated with LXR agonist and treated with $siRNA^{Abca12}$ (co-localization coefficient 0.81±0.04, $p<0.01$for cells treated with $siRNA^{Abca12}$ *versus* cells treated with $siRNA^{C}$) (Fig. 3D-I, lower magnification images are shown in Supplemental Figure SII A). The distribution of Abca1 was investigated using biotinylation of cell-surface Abca1. We observed a reduction in the abundance of cell-surface Abca1 in Abca12 deficient cells (Fig. 3J). This was also confirmed using cross-sections of individual cells (Fig 3K) and in confocal 3D reconstitutions which both show a reduction of plasma membrane Abca1 upon Abca12 knock-down (Control – supplemental movie S1, Abca12 knockdown – supplemental movie S2). The level of Abca12 in WT RAW 264.7 cells was insufficient for reliable detection, however, overexpression of heterologous Abca12 showed clear regions of overlap with Abca1 (Fig. 3L).

To investigate if Abca1stability was affected by Abca12 deficiency, we radioactively labelled newly synthesized Abca1 and performed pulse-chase experiments. Analysis demonstrated that the degradation rate of Abca1 was increased when Abca12 was knocked-down (Fig. 3M, N). The effect was further confirmed when cell surface Abca1 was labelled with biotin and the abundance of biotinylated Abca1 was followed; the rate of Abca1 degradation increased in Abca12-deficient cells (Supplemental Figure SII B).

Clearly, Abca12 is able to regulate the stability of Abca1. To investigate whether a physical interaction between Abca12, Abca1 and Lxrβ may form the basis for these effects, we employed a proximity ligation assay (PLA) (Baima et al., 2010). In this assay the number of fluorescent dots in a given cell is proportional to the number of interaction events between a

pair of heterotypic proteins. When the Abca12/Abca1 pair was studied, an abundant interaction was found and knockdown of Abca12 significantly reduced this interaction (Fig. 3O). When the Abca1/Lxrβ pair was analysed, activation of cells with an LXR agonist increased their interaction, and this was further increased after Abca12 silencing (Fig. 3P and quantitation in Supplemental Figure SII C). However, as expected, the abundances of both Abca1 and Lxrβ were affected by addition of LXR agonist and Abca12 silencing (Supplemental Figure SII D shows abundance of Abca12 and Lxrβ in this experiment) and when interaction between Abca1 and Lxrβ was normalized to the abundance of the interacting proteins, it was statistically significantly reduced after addition of LXR agonist and elevated after Abca12 silencing (Fig. 3P). We also observed an abundant interaction between Abca12 and Lxrβ, which was reduced when Abca12 was knocked-down (Fig. 3R).

We next examined HeLa cells stably transfected with GFP-ABCA1 expressed under the control of a CMV promoter (parental HeLa cells do not express homologous ABCA1) (Mukhamedova et al., 2007). When HeLa-ABCA1 cells were transfected with tGFP-ABCA12, the abundance of ABCA1 significantly increased (Fig. 4A). Overexpression of ABCA12 in HeLa cells caused modest elevation of cholesterol efflux, however in HeLa/ABCA1 cells this overexpression did not increase cholesterol efflux in addition to that caused by the presence of heterologous ABCA1 (Fig. 4B). We then investigated the distribution of ABCA1 and ABCA12 by confocal microscopy. Consistent with previous reports (Mujawar et al., 2006; Mukhamedova et al., 2007) most of the ABCA1 in HeLa/ABCA1 cells localized to the perinuclear area with a smaller proportion localized at the plasma membrane (Fig. 4C). Transfection with ABCA12 caused re-distribution of ABCA1 to the outer cytoplasm and plasma membrane (Fig. 4D), similar to the distribution of ABCA12 (Fig. 4E); there was a significant co-localization of ABCA1 and ABCA12 (Fig. 4F).

To investigate if overexpression of ABCA12 affected stability of ABCA1 we used biotin labelling of cell surface ABCA1 and found that while the abundance of total ABCA1 was considerably higher in cells transfected with ABCA12 (Fig. 4G, lane 1), levels of ABCA1 at the cell-surface were similar (Fig. 4G, lane 2). This observation explains the lack of effect of ABCA12 on cholesterol efflux in HeLa/ABCA1 cells. The rate of degradation of biotinylated ABCA1 was significantly reduced by ABCA12 (Fig. 4G,H).

In pulldown experiments, anti-ABCA1 antibody co-precipitated ABCA12 irrespective of whether or not proteins were cross-linked prior to immunoprecipitation (Fig. 4I). In the reciprocal experiment, when ABCA12 was immunoprecipitated, ABCA1 co-immunoprecipitated with ABCA12 (Fig. 4J). When LXRβ was immunoprecipitated from HeLa/ABCA1 cells transfected with ABCA12, both ABCA1 and ABCA12 co-immunoprecipitated with LXRβ (Fig. 4K). Experiments with the PLA assay confirmed interaction between ABCA1 and ABCA12 in this model (Fig. 4L). They also revealed that overexpression of ABCA12 increased interaction of ABCA1 and LXRβ and caused a re-localization of this complex to a region surrounding the nucleus (Fig. 4M). Note that cells in this experiment were not treated with LXR agonist and that the abundance of ABCA1 was elevated in cells overexpressing ABCA12.

Together these findings indicate that ABCA12 interacts with ABCA1 and that loss of ABCA12 reduces ABCA1 abundance on the cell surface and enhances its degradation. ABCA12 and ABCA1 are also likely to interact with LXRβ.

Abca12 deficient macrophages accumulate cholesterol

In macrophages, deficiencies in cholesterol efflux lead to accumulation of cholesterol and transformation of macrophages into foam cells. To investigate the potential contribution of Abca12 to this process, we assessed the rate of cholesteryl ester biosynthesis. In *Abca12*$^{El12/El12}$ cells the rate of cholesteryl ester biosynthesis was 3-fold higher compared to wild-type counterparts while under conditions of cholesterol loading this difference was 2-fold (Fig. 5A). Similar effects were observed in the Abca12-deficient RAW 264.7 cells (Fig. 5B). Significantly more cells were stained with Oil Red O in Abca12 knockdown cells compared to control cells (Fig. 5C,D).

We then performed a lipidomics analysis to investigate the effect of Abca12 knockdown on lipid accumulation in RAW 264.7 cells. Without cholesterol loading, Abca12 knockdown resulted in modest elevation of the abundance of cholesteryl esters, ceramides, and sphingomyelins (Fig. 5E). With cholesterol loading, Abca12 deficiency led to a significant accumulation of cholesterol, cholesteryl esters, ceramides, sphingomyelins, triglycerides and phosphatidylcholine (Fig. 5E). Full lipidomics data are presented in Supplemental Table S2.

Taken together these findings demonstrate that Abca12 deficiency results in the accumulation of lipids in macrophages.

Loss of Abca12 impairs reverse cholesterol transport and promotes atherosclerosis in vivo

We next assessed the effect of Abca12 deficiency on reverse cholesterol transport (RCT) *in vivo*. RAW 264.7 macrophages transfected with control or Abca12 siRNA were loaded with cholesterol, labelled and transplanted into the peritoneal cavity of mice. The amount of labelled cholesterol exported from macrophages with Abca12 knockdown to plasma and feces was reduced 2-fold compared with control macrophages (Fig. 6A,C). The amount of cholesterol in the liver was unaffected (Fig. 6 B), probably due to the transient nature of this

pool. The ratio of free cholesterol/cholesteryl esters in plasma was 0.56± 0.27 and 0.57± 0.49 (p=0.96) for control and Abca12 knockdown groups respectively.

We then investigated the effect of Abca12 deficiency on the development of atherosclerosis in the *Apoe*$^{-/-}$ mice. Since *Abca12*$^{El12/El12}$ mice are not viable (Smyth et al., 2008), we used a bone marrow chimera model, transplanting fetal liver cells (FLC) harvested from *Apoe*$^{-/-}$ *Abca12*$^{+/+}$ or *Apoe*$^{-/-}$ *Abca12*$^{El12/El12}$ embryos into *Apoe*$^{-/-}$ mice in which endogenous haematopoiesis had been ablated by irradiation. Eight weeks post-transplantation, bone marrow chimerism was confirmed by flow cytometric analysis of peripheral blood leukocytes, and mice exhibiting >90% donor engraftment commenced a high-fat diet. After 16 weeks of high-fat diet, mice transplanted with *Apoe*$^{-/-}$ *Abca12*$^{El12/El12}$ FLCs exhibited double the number of the plaques compared to *Abca12*$^{+/+}$ counterparts (Fig. 6D,H; $p<0.002$). The biggest effect was observed in the aortic arch, the area most susceptible to the development of atherosclerosis, (Fig. 6 E,H). There was a trend toward increased atherosclerosis in the thoracic aorta (Fig. 6 F) while no difference was found in the abdominal aorta (Fig. 6 G). Analysis of cross-sections of the aortic sinus revealed no difference between the two groups of mice in the abundance of macrophages in the plaque and expression of VCAM-1 on the surface of endothelium (Fig. 6 I,J). We found no difference in the abundances of collagen (Fig. 6 K), a marker of protein oxidation, nitrotyrosine (Fig. 6 L) or in the size of the necrotic core (Fig. 6 M).

Blood cell counts in the two groups of mice after 16 weeks on high-fat diet revealed two statistically significant differences: in *Apoe*$^{-/-}$ *Abca12*$^{El12/El12}$ mice % leukocytes were lower (6.3±0.5 *versus* 12.1±1.0; $p<0.002$) and % of mononuclear cells was higher (81.4±1.9 *versus* 69.9±1.4; $p<0.01$). Plasma triglycerides, total, non-HDL and HDL cholesterol levels in mice transplanted with *Apoe*$^{-/-}$ *Abca12*$^{+/+}$ or *Apoe*$^{-/-}$ *Abca12*$^{El12/El12}$ FLCs were similar

(Supplemental Table S3), indicating that the enhanced atherosclerosis was not the result of changes in plasma lipid profile.

These results indicate that Abca12 deficiency inhibits RCT *in vivo* and causes accumulation of cholesterol in macrophages promoting their transformation into foam cells and contributing to the development of atherosclerosis.

Discussion:

ABCA12 is a full-length ubiquitously-expressed ABC transporter. In skin, ABCA12 is responsible for transport of ceramides and its mutation leads to defects in skin barrier function, leading to dehydration (Kelsell et al., 2005). Recently we (Smyth et al., 2008) and others (Yanagi et al., 2008; Zuo et al., 2008) described mouse models of HI caused by mutations in *Abca12;* these mice are not viable and die shortly after birth. Using embryonic fibroblasts we have demonstrated that *Abca12*$^{-/-}$ cells have severe defects in cholesterol efflux and exceptional susceptibility to lipid overloading (Smyth et al., 2008). These findings suggested that the physiological role of ABCA12 may not be limited to skin and may be an element of a novel pathway regulating lipid metabolism in various tissues including macrophages, cells central to the development of atherosclerosis.

The main finding of this study is that ABCA12 is essential for the regulation of cellular cholesterol homeostasis. Loss of Abca12 renders macrophages unresponsive to stimulation by LXR agonists, reducing the abundance of cholesterol transporters and the rate of cholesterol efflux. These changes cause the accumulation of cholesterol in macrophages, their transformation into foam cells and development of atherosclerosis. In addition, Abca12

deficiency caused reduction in the abundance of LXRβ; reconstitution experiments employing heterologous Lxrβ suggest that LXRβ plays a key role in mediating the effects of loss of ABCA12. LXRs are nuclear receptors which control transcription of genes (Calkin and Tontonoz, 2010; Zelcer and Tontonoz, 2006); however, our findings support a post-transcriptional model of LXRβ action. It has been proposed that LXRβ binds to ABCA1 preventing ATP hydrolysis and interaction of ABCA1 with apoA-I; LXR agonists interfere with this interaction (Hozoji-Inada et al., 2011). Our studies have established an interaction between ABCA12, ABCA1 and LXRβ which is required for ABCA1 localization to the cell surface and is necessary for its normal activity and stability. We propose (Fig. 7) that in the presence of LXR agonist the ABCA1-ABCA12-LXRβ complex dissociates leading to a stimulation of cholesterol efflux as suggested by Hozoji-Inada (Hozoji-Inada et al., 2011). In the absence of ABCA12, LXRβ binds more firmly to ABCA1, and the complex may be mislocalized; as a result the LXR agonist is unable to induce dissociation. Consequently, ABCA1 remains dysfunctional and unable to bind apoA-I leading to its internalization and degradation along with LXRβ. Overexpression of LXRβ favours formation of LXRβ dimers which bind more weakly to ABCA1 allowing agonists to disrupt the complex. Indirect evidence in support of this hypothesis include: (i) binding of ABCA1 to ABCA12 and LXRβ; (ii) partial co-localization of ABCA12 and ABCA1; (iii) increased co-localization and interaction between LXRβ and ABCA1 after silencing of ABCA12; (iv) the ability of heterologous ABCA12 to cause a re-localization of ABCA1 and LXRβ similar to that was observed by Hozoji-Inada (Hozoji-Inada et al., 2011); (v) the complementary effects of ABCA12 deficiency and ABCA12 overexpression on ABCA1 stability and localization. On the other hand, when cells were activated with cAMP, ABCA12 deficiency did not affect ABCA1 abundance, but still partially reduced cholesterol efflux in the absence of LXR agonist. Further, ABCA12 deficiency only partially reduced ABCA1 abundance, but

completely blocked LXR-dependent elevation of cholesterol efflux. Together this suggests that there may be additional mechanisms underpinning the effects of ABCA12 deficiency on ABCA1 functionality. The only published mechanism that might explain the effects of cAMP on ABCA1 functionality is via phosphorylation of ABCA1 (Haidar et al., 2002), which may be affected by the disruption of ABCA1/ABCA12 complex. While the lack of direct evidence supporting this hypothesis means we cannot definitively exclude an unrelated mechanism, such as LXR-dependent ubiquitination (Zelcer et al., 2009), we consider this the most likely mode of action of ABCA12. It is important to recognize that the proposed mechanism of the effect of ABCA12 deficiency on ABCA1 cannot be *a priori* extended to ABCG1. Moreover, the effects of ABCA12 deficiency on ABCG1 may have contributed to the reduced reverse cholesterol transport and the development atherosclerosis in animal experiments.

Impairment of cholesterol homeostasis plays a central role in cardiovascular, neurodegenerative, skin and many other disorders. The current dogma suggests that the main players in regulating cholesterol homeostasis are the sterol regulatory element-binding protein (SREBP)-dependent pathways (which regulate the delivery of cholesterol to cells), and the LXR-dependent pathways (which regulate the removal of cholesterol from cells). In this study we have identified ABCA12 as a new player in the "removal" arm of cholesterol homeostasis. We show that ABCA12 plays a key role in posttranscriptional regulation of cholesterol transporter ABCA1 *in vitro* and demonstrate a pivotal role for ABCA12 deficiency in the development of atherosclerosis. Our findings also suggest that ABCA12 defines a part of a novel pathway involved in regulation of cholesterol homeostasis and a potential contributor to the development of atherosclerosis.

Experimental Procedures:

Cells

The *Abca12*^{El12} and *Abca12*^{Lx12} mouse strains were described previously (Smyth et al., 2008; Yanagi et al., 2010). *Abca12*^{El12} mice were backcrossed for 10 generations and the mutation was mapped to a narrow 4.7 Mb interval, only Abca12 gene in this interval was found to be altered. The uterus was removed on embryonic day (E)12.5 of pregnancy and embryos were isolated under aseptic condition. The embryonic liver was dissected; cells suspended and cultured in 6-well tissue culture plate in RPMI-1640 medium containing 10% FBS and 20% L929-cell conditioned medium as source of colony stimulating factors as described by Feng et al (Feng et al., 2005). After 24-hour depletion of adherent cells and debris, non-adherent macrophages are removed to fresh Petri dishes and grown to confluence. Embryos were genotyped and cultured macrophages were characterized morphologically as described by Suzuki et al (Suzuki et al., 2000); 95% of cells were deemed to be macrophages.

RAW 264.7 cells were maintained as described previously (Mukhamedova et al., 2008). HeLa-ABCA1 cells stably expressing GFP-tagged ABCA1 were a kind gift of Dr. A. Remaley and were described by us previously (Mukhamedova et al., 2007).

Transfections

RAW 264.7 mouse macrophages were transfected with Abca12-specific siRNA or scrambled siRNA (control) and treated with TO-901317 (4 μM) for 18 hours. Transfection was performed using Lipofectamine RNAi MAX (Invitrogen) following manufacturer's protocol. Abca12 siRNA and scrambled siRNA were from Santa Cruz Biotechnology.
Lxrβ and murine Abcva12 genes were inserted into pCMV6-AC-GFP plasmid (Origene). Human ABCA12 plasmid was a kind gift of Prof. D. Kelsell. Transfections with Lxrβ

plasmid were performed as described previously (Escher et al., 2005). Transfection of HeLa cells with ABCA12 plasmid was conducted as described previously (Mukhamedova et al., 2007).

HDL and apolipoprotein A-I

High density lipoprotein (HDL) (1.083<d<1.21 g/L) was isolated by sequential centrifugation in KBr solutions, delipidated and apoA-I was purified by gel filtration chromatography as previously described (Brace et al., 2010).

Cholesterol efflux

Cholesterol efflux was measured as described previously (Mukhamedova et al., 2008). Briefly, cells were incubated in serum-containing medium supplemented with [^{3}H]cholesterol (75 kBq/ml) for 48 h. Cells were then washed with PBS and incubated for 18 h in serum-free medium in the presence or absence of LXR agonist TO-901317 (final concentration 4 μM). ApoA-I or isolated HDL were then added to the final concentration of 30 μg/ml and cells were incubated for 2 h at 37^{o}C. The efflux was calculated as a proportion of radioactivity moved from cells to medium (minus efflux to medium without acceptors). ABC-dependent efflux was defined as a difference in the efflux to apoA-I or HDL from cells activated or not-activated with LXR agonist.

Western blot analysis

The antibodies against ABCA1, ABCG1, SR-B1, LXRα, LXRβ, and GAPDH were from Abcam and Aviva Systems Biology. Semi-quantitative analysis of Western blots was done by densitometry and presented as proportion of control after normalization to loading control.

For Abca12 analysis, cellular proteins were biotinylated with 2 mM EZ-Link NHS-LC-Biotin (Pierce) at room temperature for 1 h. Cells were then lysed with NP-40 lysis buffer and 200

µg each of the lysates were incubated with 2.5 µg goat anti-ABCA12 antibody (Novus). Complex of Abca12 protein and anti-Abca12 antibody was immunoprecipitated with Sepharose-protein G beads. Amount of protein equivalent to 100 µg of original protein lysate was run on the SDS-PAGE followed by Western blot and detected with HRP-conjugated streptavidin.

Immunoprecipitation

Equal amounts (150 µg) of total cell lysates were used to immunoprecipitate ABCA1, LXRβ and ABCA12-turboGFP proteins. Briefly, RIPA lysates were incubated with 2 µg of rabbit polyclonal ABCA1 (Novus Biologicals), turboGFP (Evrogen) or LXRβ (Abcam) antibody overnight at 4°C. Protein A-Sepharose beads (Pierce) were added to the antibody/protein mixture. Following incubation for 3 h with rotating at 4°C, beads were washed with PBS.

Pulse-chase experiments

RAW 264.7 cells were transfected with Abca12-siRNA or scrambled siRNA as described above. Forty-eight hours post transfection, the cells were activated with 4 µM TO-901317 for 3 h in culture medium, then incubated in Methionine/Cysteine-free RPMI (Sigma Aldrich) with TO-901317 for 1 h. The cells were pulsed with 100 µCi/ml of ^{35}S-labelled Methionine/Cysteine (PerkinElmer, EasyTag Express Protein Labeling Mix) for 2 h, washed in PBS, then chased in RPMI medium supplemented with 1.5 mg/ml L-methionine and 0.5 mg/ml L-cysteine (Sigma Aldrich) for the indicated times. ABCA1 protein was determined by immunoprecipitation with an affinity purified rabbit anti-ABCA1 antibody, separated by SDS-PAGE, transferred to PVDF membrane, and exposed to autoradiography film.

Biotinylation of cell-surface proteins

Cells were washed 3 times with ice-cold PBS and biotinylated with Sulfo-NHS-SS-biotin as described previously(Cui et al., 2012). After biotinylation, cells were washed twice with 50 mM Tris/150 mM NaCl to quench the reaction and once with PBS. Following incubation in serum-free RPMI medium at 37°C for 0 h, 4 h or 24 h, cells were lysed with RIPA buffer. Biotinylated proteins were purified using Ultralink Plus immobilized streptavidin gel (Pierce).

Cholesteryl ester biosynthesis

Cells were incubated for 18 h in serum-containing medium in the presence or absence of 50 μg/ml of acetylated LDL. Cholesteryl ester biosynthesis was then assessed by incorporation of [^{14}C] oleic acid into cholesteryl esters over 2 h as described previously (Mujawar et al., 2006). Staining of cells with Oil Red O was described previously (Smyth et al., 2008).

Real-time PCR

PCR primers were obtained from Taqman® Gene Expression Assays with follow gene assay IDs (Mm00442646_m1, Mm00550501_m1, Mm00613683_m1, Mm00437390_m1, Mm00443451_m1, Mm00437262_m1, Mm00450234_m1, Mm00446214_m1, Mm00440169_m1, Mm01282499_m1). Real-time PCR reactions were performed on 7500 Fast System from ABI Applied Biosystems. The relative amount of mRNA was calculated using the comparative C_T method. Gene expression was normalised to 18s rRNA.

Lipidomics

RAW264.7 cells were transfected with siRNA^{Abca12} or siRNA^{C}, activated with 4 μM TO-901317 for 24 hours, and incubated with or without 50 μg/ml acetylated LDL overnight. Cells were washed with PBS, resuspended into 20mM Tris/500mM NaCl solution containing

0.1 mM butylated hydroxytoluene and sonicated. Ten microlitres (20 µg of protein) each of the supernatant were subjected to lipids extraction and analysis using mass spectrometry.

Confocal microscopy

For imaging, RAW 264.7 or Hela-ABCA1 cells were grown in Lab-Tek Chamber Slide system (Nunc). In HeLa-ABCA1 cells ABCA1 was fused with GFP, in RAW 264.7 cells it was stained with monoclonal anti-ABCA1 antibody (Abcam) and AlexaFluor 488 anti mouse IgG secondary antibody (Invitrogen). Lxrβ was stained with polyclonal primary antibody (Abcam) and AlexaFluor 633 anti rabbit secondary antibody (Invitrogen). Abca12 staining in RAW 264.7 cells was performed using in house anti-Abca12 monoclonal antibodies and AlexaFluor 488 anti mouse IgG secondary antibody and in HeLa-ABCA1 cells using anti-ABCA12 antibody from Novus Biologicals and Texas Red 633 anti rabbit secondary antibody. Co-localization was quantitated using Zeiss LSM image analysis software as described by Zinchuk et al (Zinchuk and Grossenbacher-Zinchuk, 2011; Zinchuk et al., 2007) and expressed as unweighted co-localization coefficient of Lxrβ with Abca1on full images (10 images, 6-8 cells per image, 3 experiments).

In situ proximity ligation assay (Duolink)

The general principle of the Duolink *in situ* co-immunoprecipitation assay has been described previously (Baima et al., 2010). Cells were seeded at $1x10^4$ cells per well on 8-well Lab-Tek II Chamber Slides, incubated to 80% confluent. Forty-eight h post-transfection, cells were washed with PBS, fixed in 4% paraformaldehyde for 15 minutes, permeablized in 0.01% Triton X-100 for 10 minutes. Non-specific binding was blocked by incubating cells for 30 min at 37°C with a blocking solution provided by the manufacturer. The cells were incubated overnight at 4°C with two antibodies raised in different species. The proximity ligation assay was conducted using Duolink Orange Detection Kit. Images were quantified by counting

total number of PLA signals in each image and dividing by the number of nuclei in field of view. Counting was conducted in automatic mode using ImageJ software. A total of several hundred individual cells were counted for each group of samples.

In vivo reverse cholesterol transport assay

Cholesterol efflux *in vivo* was measured using a model described by Rader (Wang et al., 2007) with modifications described previously (Mukhamedova et al., 2008).

Bone marrow transplantation

After backcrossing 10 generations to C57BL/6, the *Abca12*^{El12} mutation was backcrossed onto a C57BL/6 Apoe-deficient background. C57BL/6 CD45.2 *Abca12*$^{-/+}$ *Apoe*$^{-/-}$ mice were then intercrossed and E13.5 livers harvested from embryos. Two million fetal liver cells were transplanted into adult C57BL/6 CD45.1 *Apoe*$^{-/-}$ recipients in whom endogenous hematopoiesis has been ablated by irradiation (11 Gy in 2 equal doses 2-3 hours apart). Eight weeks post-transplantation, donor engraftment was determined by flow cytometric analysis of peripheral blood leukocyte in conjunction with CD45-specific monoclonal antibody. Eight weeks post-reconstitution, bone marrow chimera mice were put on a high fat diet (21% fat, 0.15% cholesterol) for 16 weeks. Lipid accumulation and distribution and size (area) of atherosclerotic lesions in aorta were quantitatively assessed using en face analysis after staining with Sudan IV.

Heart tissue containing the aortic sinus was embedded in OCT compound (Tissue-Tek) and frozen for assessment of plaques within the aortic sinus region. Consecutive 10μm sections spanning 240μm of the aortic sinus were cut and collected. Sections were stained with Masson's trichrome stain to detect collagen. Sections were also examined for macrophage content, as well as inflammatory and oxidation markers by immunohistochemistry using the

following primary antibodies: anti-CD68 (AbD serotec), anti-VCAM-1 (BD Pharmingen), anti-Nitrotyrosine (Millipore). Images were quantified using ImagePro plus 6.0 software.

All animal experiments complied with the regulatory standards of, and were approved by, the Walter and Eliza Hall Institute Animal Ethics Committee and/or the AMREP Animal Ethics Committee.

Statistics

All *in vitro* experiments were performed in quadruplicates and repeated 2-3 times. Statistical analysis was made using Student's *t*-test and one-way ANOVA modified by the step down Bonferroni procedure; both tests produced identical results. Means ± SEM are shown.

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The authors declare no conflict of interests related to this paper.

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Figure Legends:

<u>Figure 1.</u> Cholesterol and phospholipid efflux from Abca12 deficient cells (see also Table S1).

A, B – cholesterol efflux to apoA-I (a) or HDL (b) from liver macrophages isolated from *Abca12*^{El12} strain. **C, D** – cholesterol efflux to apoA-I (c) or HDL (d) from liver macrophages isolated from *Abca12*^{Lx12} strain. **E –** abundance of Abca12 in RAW 264.7 cells after knockdown of Abca12 with siRNA. **F** – cholesterol efflux to apoA-I from RAW 264.7 cells after knockdown of Abca12 with siRNA. **G** – phospholipid efflux to apoA-I from liver macrophages isolated from *Abca12*^{El12} strain. **H –** abundance of Abca1, Abcg1 and SR-B1 in RAW 264.7 cells after knockdown of Abca12 with siRNA. **I** - Abca1 and Abcg1 abundance in liver macrophages isolated from *Abca12*^{Lx12} strain. **J** – Lxrα and Lxrβ abundance in RAW 264.7 cells after knockdown of Abca12 with siRNA. **K** - Lxrα and Lxrβ abundance in liver macrophages isolated from *Abca12*^{Lx12} strain. Activated cells refer to cells pre-incubated with LXR agonist TO-901317 (final concentration 4 μM). *p<0.01 (*versus* WT cells). Pooled data are represented as mean ± SEM.

<u>Fig. 2.</u> Involvement of Lxrβ in impairment of cholesterol efflux in Abca12-deficient cells (see also Figure SI).

A – expression of genes involved in lipid metabolism in *Abca12* $^{Lx12/Lx12}$ and *Abca12*$^{+/+}$ macrophages with or without activation with LXR agonist TO-901317 as assessed by real-time RT-PCR. *p<0.01 (*versus* non-activated cells). **B**– expression of genes involved in lipid metabolism in RAW 264.7 macrophages after knockdown of Abca12. Pooled data are represented as mean ± SEM; *p<0.01 (*versus* non-activated cells). **C** - cholesterol efflux to

apoA-I from RAW 264.7 cells after knockdown of Abca12 by siRNA and treatment with LXR agonist TO-901317 (4 μM) or GW3965 (1 μM) or a combination of TO-901317 (4 μM) and PPARα/γ agonist RO4509851 (5 μM). **D -** abundance of Abca1 in RAW 264.7 cells after knockdown of Abca12 by siRNA and activation with LXR ligands. **E -** cholesterol efflux to apoA-I from RAW 264.7 cells after knockdown of Abca12 and activation by LXR agonist TO-901317 (4 μM) or cAMP (0.3 mM). **F -** abundance of Abca1 in RAW 264.7 cells after knockdown of Abca12 and activation of Abca1 expression by TO-901317 or cAMP. **G -** cholesterol efflux to apoA-I from RAW 264.7 cells after knockdown of Abca12 and co-transfection with Lxrβ. Pooled data are represented as mean ± SEM; *p<0.01 (*versus* control cells), #p<0.001 (*versus* cells treated with siRNA). **H –** abundance of Abca1 in RAW 264.7 cells after knockdown of Abca12 and co-transfection with Lxrβ.

Fig. 3. Mechanism of modulation of cholesterol efflux by Abca12: studies on RAW 264.7 cells (see also Figure SII and Videos S1 and S2).

A – I – Confocal microscopy of Abca1 (A, D, G, green), Lxrβ (B,E,H, red) and merge between the two (C,F,I, yellow) in RAW 264.7 cells non-activated (A-C) or activated with LXR agonist TO-901317 (D-I) with (G-I) or without (A-F) Abca12 knockdown. Bar - 10 μm. **J** - abundance of total (upper panel) and cell-surface (bottom panel) Abca1 in RAW 264.7 cells with or without Abca12 knockdown. **K** - intracellular distribution of Abca1 in RAW 264.7 cells or RAW 264.7 cells after Abca12 knockdown. Top panels show cross-sectional images derived from stacking the images from sequential scanning of cells shown in the middle panels at different depth. The bottom panel shows florescence intensity measured along the arrow in the middle panel. **L** - intracellular distribution of Abca1 (green) and Abca12 (red) in RAW 264.7 cells overexpressing heterologous Abca12. Bar - 10 μm. **M, N** –

pulse-chase experiment of Abca1 degradation in RAW 264.7 cells with or without Abca12 knockdown. Proteins were pulse-labelled with [^{35}S]methionine/cysteine, Abca1 immunoprecipitated and isolated on a SDS-PAGE. Autoradiography of the Abca1 bands (G) and quantitation of the radioactivity in these bands (H) are shown. Means ± range of two experiments are shown. **O**– proximity ligation assay (PLA) of the Abca1/Abca12 pair in RAW 264.7 cells with or without Abca12 knockdown. **P** – PLA of the Abca1/Lxrβ pair in RAW 264.7 cells with or without activation with TO-901317 and with or without Abca12 knockdown. Graph shows PLA values normalized to the abundance of Abca1 and Lxrβ. **R** – PLA of the Abca12/ Lxrβ pair in RAW 264.7 cells with or without Abca12 knockdown. Quantitation of PLA assay (right panels in O-R) is described in Methods; Means ± SEM of PLA signal per cell are shown; *p<0.01(*versus* control); #p<0.01(*versus* non-activated cells).

Fig. 4. Mechanism of modulation of cholesterol efflux by ABCA12: studies on HeLa/ABCA1 cells.

A – abundance of ABCA12 and ABCA1 in HeLa/ABCA1 cells after transient transfection with ABCA12. **B** – cholesterol efflux to apoA-I after overexpression of ABCA12. *p<0.05 (*versus* un-transfected cells). **C, D** – intracellular distribution of ABCA1 in HeLa/ABCA1 cells transfected or not with ABCA12. **E** – intracellular distribution of ABCA12 in HeLa/ABCA1 cells transfected with ABCA12. **F** – merge of D and E. **G** – Time-course of degradation of biotinylated surface Abca1 in HeLa/ABCA1 cells with or without overexpression of ABCA12. **H** - quantitation of the bands in three experiments similar to that shown in G; Means ± SEM are shown, *p<0.01 (*versus* time zero). **I** – Western blot after immunoprecipitation of ABCA1 with anti-ABCA1 antibody and developing with anti-ABCA12 antibody. **J** – Western blot after immunoprecipitation of ABCA12 with anti-tGFP

antibody and developing with anti-ABCA1 antibody. **K -** Western blot after immunoprecipitation of LXRβ and developing with anti-ABCA1 antibody (top right quarter), anti tGFP (an ABCA12 tag) antibody (top left quarter) or anti LXRβ antibody (bottom half). No primary antibody was added to immunoprecipitation of the sample in the middle lane. HeLa/ABCA1 were transfected with ABCA12. **L** – PLA of the Abca1/Abca12 pair in HeLa/ABCA1 transfected or not transfected with ABCA12. **M** – PLA of the Abca1/Lxrβ pair in HeLa/ABCA1 transfected or not transfected with ABCA12. Means ± SEM of PLA signal per cell are shown in right panels; *p<0.01.

Fig. 5. Lipid accumulation in Abca12 deficient cells (see also Table S2).

A – cholesteryl ester biosynthesis in liver macrophages isolated from *Abca12*$^{El12/El12}$ strain with or without loading the cells with acLDL. **B** – cholesteryl ester biosynthesis in RAW 264.7 cells after knockdown of Abca12 with siRNA with or without loading the cells with acLDL. **C** – Oil Red O staining of RAW 264.7 cells after knockdown of Abca12 with siRNA. **D** – quantitation of Oil Red O staining of RAW 264.7 after knockdown of Abca12 with siRNA (percentage of stained cells, n=5); Pooled data are represented as mean ± SEM; *p<0.01 (*versus* WT or control cells). **E** – abundance of the individual lipid species in control *versus* Abca12 knockdown RAW 264.7 cells after activation with LXR agonist TO-901317, with or without loading with acLDL. *p<0.001 (*versus* acLDL loaded control cells); #p<0.01 *versus* non-acLDL loaded control cells.

Fig. 6. The effect of Abca12 K/O on reverse cholesterol transport and development of atherosclerosis *in vivo* (see also Table S3).

A-C - appearance of [^{3}H]cholesterol in plasma (A), liver (B) and feces (C) 24 h after transplanting cholesterol loaded and labelled RAW 264.7 macrophages transfected with either siRNA^{c} (n=6) or siRNA^{Abca12} (n=5). **D-G -** two groups of Apoe K/O mice were transplanted with either *Abca12* $^{El12/El12}$ (n=10) or WT (n=13) bone marrow. Animals were kept on a high fat diet for 16 weeks. Lipid accumulation and distribution and size (area) of atherosclerotic lesions in aorta were quantitatively assessed using *en face* analysis after staining with Sudan IV. Data are represented as individual values; horizontal dash represents mean ±SEM; *p<0.002. **H -** representative photographs of aortas after staining with Sudan IV. **I -** analysis of macrophage infiltration within the aortic sinus region after staining with anti CD68. **J -** analysis of the abundance of VCAM-1 in sections from the aortic sinus region. **K -** analysis of the abundance of collagen in sections from the aortic sinus region. **L -** analysis of the abundance of nitrotyrosine (NT) in sections from the aortic sinus region. **M -** analysis of the size of the necrotic core in sections from the aortic sinus region. **I-M -** % of staining in the plaque is shown; Mean ±SEM of n=6 in each group are shown.

Fig. 7. A proposed mechanism of regulation of ABCA1 by ABCA12 and LXRβ.

"Inactive" ABCA1 exists as a complex with ABCA12 and LXRβ. Addition of an LXR agonist leads to dissociation of LXR from ABCA1-ABCA12 and activation of ABCA1, enabling it to hydrolyse ATP, bind apoA-I and remain on the cell surface where it is stable. In the absence of ABCA12, LXRβ binds more firmly to ABCA1 and this association is not broken by LXR agonist; consequently ABCA1 remains inactive and unstable. Overexpression of LXRβ leads to the excess of LXRβ and formation of LXRβ dimers in the presence of an agonist; these dimers dissociate from ABCA1.