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Research Article

Low Doses of Innate Defence Regulator Peptide, IDR-1018, Enhances HDL-Mediated Cholesterol Efflux from Smooth Muscle Cells and Macrophages

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Abstract

Cardiovascular diseases due to atherosclerosis are the leading cause of death worldwide. In recent years, novel therapeutics designed to enhance high-density lipoprotein (HDL) activity as a treatment for atherosclerosis have been explored. Of particular interest is the use of amphipathic α-helical peptides to mimic the action of the main HDL protein, apolipoprotein (Apo)AI. Immunomodulatory peptides share many physical and functional properties with ApoAI and its mimetics; we therefore hypothesized that they too might be capable of enhancing cholesterol efflux. The aim of this study was to determine whether a potent immunomodulatory peptide, innate defence regulator (IDR)-1018, could promote cholesterol efflux from cells. Here, we report that IDR-1018 induced a dose-dependent increase in ApoAI on the cell surface and ATP-binding cassette transporter A1 (ABCA1) protein levels. Functional assays revealed that low doses of IDR-1018 improved HDL-mediated suppression of intracellular cholesteryl ester accumulation in macrophages and enhanced HDL-mediated cellular cholesterol efflux from smooth muscle cells and macrophages. Based on these results we propose that natural and synthetic immunomodulatory peptides like IDR-1018 represent a large new group of peptides that could be developed for the treatment of atherosclerosis.

Keywords: IDR-1018; Cholesterol efflux; Macrophage; Smooth muscle cells; High-density lipoprotein

Abbreviations: ABC: ATP-Binding Cassette; ACAT: Acyl-CoA Cholesterol Acyltransferase; Apo: Apolipoprotein; CE: Cholesteryl Ester; FAFA: Fatty Acid Free Albumin; FBS: Fetal Bovine Serum; FC: Free Cholesterol; HDL: High-Density Lipoprotein; HDP: Host Defence Peptide; HSF: Human Skin Fibroblast; IDR: Innate Defence Regulator;

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M-CSF: Macrophage-Colony Stimulating Factor; qRT-PCR: Quantitative Real Time-Polymerase Chain Reaction; SMC: Smooth Muscle Cell

Introduction

Atherosclerosis is characterized by aberrant accumulation of lipids, recruitment of immune cells and development of chronic inflammation in arteries [1]. Though numerous cell types are involved in the pathogenesis of this disease, macrophages and smooth muscle cells (SMCs) are thought to play a particularly significant role [1-5]. During the development of atherosclerosis, SMCs and macrophages internalize and store substantial amounts of cholesterol, developing into pro-inflammatory foam cells [2,3]. Reverse cholesterol transport by apolipoprotein AI (ApoAI) and high density lipoproteins (HDL) combats this process though the removal of excess cholesterol from peripheral tissues [6]. The initial stages of this process involve efflux of cholesterol from cells via the cholesterol transporters ATP-binding cassette (ABC)A1 and ABCG1 to lipid-poor ApoA1 and HDL particles, respectively [3,7-9]. ApoAI and HDL also exhibit extensive immunomodulatory activity [10-15]. Therefore, substantial efforts have focused on enhancing the cholesterol efflux and anti-inflammatory activities of ApoAI and HDL for atherosclerosis prevention [16]. This includes the use of ApoAI peptide mimetics [16], which have been designed largely on the structural and physical properties thought to confer the ability of ApoAI to induce cholesterol efflux [17,18]. The result has been a series of short amphipathic α-helical peptides with an ability to both induce cellular cholesterol efflux and modulate immune responses, both *in vitro* and *in vivo* [11,19-29].

Naturally occurring host defence peptides (HDPs) and their synthetic derivatives, termed innate defence regulators (IDRs), are powerful immunomodulatory peptides [30] that share several physical and biological characteristics with ApoAI and ApoAI peptide mimetics. They are short cationic amphipathic molecules that adopt a variety of conformations, including α-helices [30-32]. IDR-1018, a 12-amino acid peptide developed in our lab, adopts an amphipathic α-helical conformation in the presence of zwitterionic micelles [32]. It exhibits potent and pleiotropic immunomodulatory activities

including promotion of chemokine production, suppression of pro-inflammatory responses, enhancement of wound healing, and modulation of the differentiation state of macrophages [32-36]. IDR-1018 has also proven to be an effective treatment in animal models of *Mycobacterium tuberculosis* and *Staphylococcus aureus* infections and in preventing death due to severe (cerebral) malaria and perinatal brain injury [37-39].

The aim of this study was to determine if IDR-1018, previously developed for its immunomodulatory activities, was also capable of promoting cellular cholesterol efflux. Here we show that IDR-1018 bound serum ApoAI and facilitated its interaction with macrophages and, at low concentrations, enhanced HDL-mediated cholesterol efflux from macrophages and smooth muscle cells. Based on these results, we propose that IDR-1018 enhances cholesterol efflux from cells in a concentration dependent manner by binding to serum ApoAI, and improving its interaction with cells, thus promoting the transfer of cholesterol to ApoAI and HDL particles. This is a novel mechanism of action, unlike that of ApoAI peptide mimetics.

Materials and Methods

Blood collection and ethics statement

Blood and serum was collected from healthy volunteers according to the guidelines of the University of British Columbia clinical research ethics board (UBC-CREB# H04-70232).

Reagents

Solid phase F-moc chemistry followed by reverse phase HPLC was used to synthesize and purify the peptide, IDR-1018 (VRLIVAVRIWRR-NH$_2$) (CPC Scientific, Sunnyvale CA), to greater than 95% purity. Cholesterol and fatty acid free bovine serum albumin (FAFA) were purchased from Sigma Aldrich (St. Louis MO) and ApoAI was obtained from Academy Biomedical (Houston TX). [1,2-^{3}H]-cholesterol and

[1-^{14}C]-oleate were purchased from PerkinElmer (Waltham, MA). HDL$_3$ was isolated as described previously [40]. Delipidated plasma was generated from human plasma adjusted to a density of 1.21 using KBr, then spun at 400,000×g for 4 hours at 8°C in order to remove lipoproteins. Following lipoprotein removal, the plasma was dialyzed overnight (at 4°C) against a buffer containing 150 mM NaCl, 50 mM Tris-HCl and 0.3 mM EDTA, pH 7.4.

Cell culture

All cells used in this study were cultured in a humidified incubator at 37°C and 5% CO$_2$. Human monocyte-derived macrophages were generated as described previously [36]. For certain experiments, the differentiation process was carried out in medium containing penicillin (100 units/ml) and streptomycin (100 µg/ml) (Life technologies, Burlington ON) to prevent bacterial growth.

The human skin fibroblast (HSF) cell line was obtained from ATCC (Manassas, VA). The rat smooth muscle (SMC) cell line WKY3M-22 [41] was a generous gift from Dr. Joan Lemire (University of Washington, Seattle). Both cell lines were cultured in DMEM supplemented with 10% FBS (both from GE Healthcare Life Technologies), penicillin (100 units/ml) and streptomycin (100 µg/ml) (Life Technologies).

Western blot analysis

Following stimulation, cells were washed with PBS and lysed. Equal amounts of total protein were run on SDS-polyacrylamide (SDS-PAGE) gels. Membranes were probed with antisera to ApoAI (EMD Millipore), ApoE (Meridian Life Science, Memphis TN), ABCA1 (Santa Cruz Biotechnology Inc., Dallas TX), low-density lipoprotein receptor (Research Diagnostic Inc), and, for normalization, β-actin (Cell signaling Technology Inc., Danvers MA). ImageJ software was used for densitometric calculations.

RNA isolation and quantitative real-time PCR (qRT-PCR)

RNA isolation and qRT-PCR were carried out as described previously [36]. Primer sequences are presented in Table S1. Beta-2-microglobulin (B2M) was used as the reference housekeeping gene.

Cholesterol efflux assay

Cholesterol efflux experiments were carried out as described previously [42] with minor modifications. On day one of the macrophage differentiation process, media was removed and replaced with RPMI 1640 media containing 10% FBS, 10 ng/ml M-CSF, and 0.2 µCi/ml [1,2-^{3}H]cholesterol. Cells were incubated in this medium for five days. On day six, the medium was removed and cells were washed with PBS containing fatty acid-free albumin (FAFA) (1 mg/ml). The cells were then cholesterol loaded in RMPI 1640 media containing 2 mg/ml FAFA, 10 ng/ml M-CSF and 30 µg/ml free cholesterol for 24 hours. Subsequently, the medium was removed and cells were washed with PBS+FAFA (1 mg/ml) then equilibrated for 24 hours in RPMI 1640 medium supplemented with 1 mg/ml FAFA.

SMCs and fibroblasts were grown to approximately 60% confluence before being incubated with DMEM containing 10% FBS and 0.2 µCi/ml [1,2-^{3}H] cholesterol. Labeling continued until cells reached 100% confluence, at which point they were washed and cholesterol loaded.

Following labeling, cells were washed then incubated for 16 hours with ApoAI, HDL alone or HDL in combination with IDR-1018 in RPMI medium containing 1mg/ml FAFA. For the combination treatments, the HDL$_3$ concentration was kept constant at 0.12 µM (20 µg/ml) while IDR-1018 concentration was varied, generating a range of peptide:HDL molar ratios. Subsequently, radiolabelled extracellular cholesterol was collected from supernatant and intracellular free cholesterol and cholesteryl esters were extracted and separated by thin layer chromatography. Samples were then assayed for radioactivity. The amount of extracellular cholesterol and intracellular free cholesterol and cholesteryl esters was calculated as the percent of total radioactivity recovered.

Cholesterol esterification

On day 6 of the differentiation procedure,

macrophages were cholesterol loaded in RMPI 1640 media containing 2 mg/ml FAFA, 10 ng/ml M-CSF and 30 µg/ml free cholesterol for 24 hours. Cells were washed and treated as described for the cholesterol efflux experiments. After treatment, cholesterol esterification was assessed as described previously [42]. Data are presented as a percentage of the BSA control.

Statistical analysis

Data are expressed as mean ± SEM. Statistical differences were calculated with Graphpad Prism 6.0 (GraphPad Software, La Jolla CA) using a two-tailed Student's t-test. P values <0.05 were considered statistically significant.

Results

Stimulation with IDR-1018 increased the level of proteins associated with the cholesterol efflux pathway in macrophages

Since peptide IDR-1018 shares several physical and immunomodulatory properties with ApoAI and its peptide mimetics [10,11,18,29,32-34,36]. In addition, several natural immunomodulatory peptides have been previously shown to bind lipoproteins and, in some cases, facilitate the interaction of lipoproteins with cells [43-45]. Thus, we examined if IDR-1018 was able to modulate the mediators of cellular cholesterol efflux. Incubation of macrophages with 13 µM of IDR-1018 in medium containing human serum induced a significant increase in three major components of the cholesterol
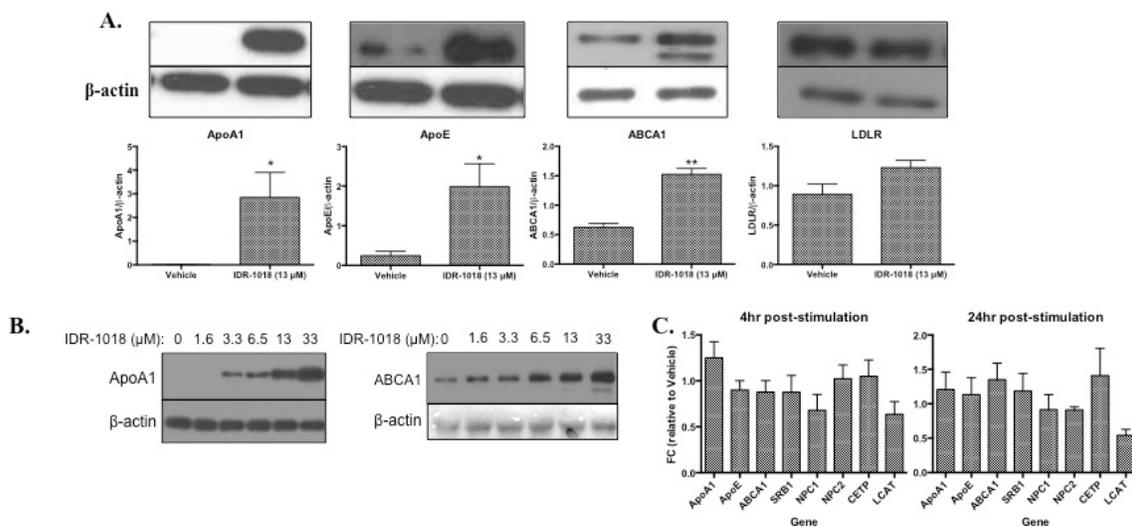

Figure 1: IDR-1018 increased the level of proteins associated with cellular cholesterol trafficking in macrophages.

A) Macrophages were stimulated with 13µM IDR-1018 for 24hr in RPMI media containing 2% human serum. Cell lysates were run on a 12% gel and probed for ApoAI, ApoE, ABCA1, LDLR and β-actin. Densitometry was completed using Image J software. Data is presented as the mean ± SEM. A minimum of three independent experiments were completed, data analyzed using Student's t-test. *p<0.05, **p<0.01 B) Macrophages were stimulated with increasing concentrations of IDR-1018 for 24hr in RPMI media containing 2% human serum. Cell lysates were run on a 12% gel and probed for ApoAI, ABCA1and β-actin. C) Fold change (FC) in gene expression determined by PCR in macrophages that were stimulated with 13µM IDR-1018 for 4 or 24hr in RPMI media containing 2% human serum. RNA was extracted and ApoAI, ApoE, ABCA1, SRB1, NCP1/2, CETP and LCAT expression were determined using qPCR. Data is presented as the mean ± SEM of at least three independent experiments.

efflux pathway, ApoAI, ApoE, and ABCA1 [7] (Figure 1A). Indeed, cellular ApoAI and ABCA1 levels were dose-dependently increased by IDR-1018 treatment (Figure 1B), consistent with the possibility that IDR-1018 might modulate cholesterol efflux by increasing the cell-associated levels of key proteins involved in the process.

Gene expression analysis revealed that the IDR-1018 induced a modest increase in ApoAI and no increase in ApoE and ABCA1 mRNA levels (Figure 1C). These results suggest IDR-1018 increased binding of serum or secreted ApoAI and ApoE to the cultured cells, and a reduction of ABCA1 protein degradation due to increased binding of apoAI to the cell surface [46,47]. IDR-1018 did not significantly alter expression of other genes associated with the cellular cholesterol efflux pathway.

IDR-1018 bound to HDL in serum as well as exogenous lipid-free ApoAI and promoted their interaction with macrophages

We then explored further the apparent increase in ApoAI binding to the cell surface in response to IDR-1018. Stimulation of macrophages with IDR-1018 for 15-60 minutes in medium containing human serum resulted in elevated cell-associated ApoAI, with the increase being observed as early as 15 minutes post-stimulation (Figure 2A). IDR-1018 has previously been shown to bind to and be internalized ([48], unpublished data) Thus, the rapid increase in ApoAI induced by IDR-1018 might have been a result of IDR-1018 binding to HDL or free ApoAI in the serum, which then associated with cells. To confirm whether IDR-1018 bound ApoAI in serum, macrophages were stimulated with IDR-1018 under a variety of serum conditions. Macrophages were also loaded with free cholesterol to mimic conditions under which macrophages would upregulate the cholesterol efflux pathway. As for previous experiments, a substantial (nearly 8-fold) increase in cell-associated ApoAI was observed for cholesterol-loaded macrophages stimulated with IDR-1018 in the presence of human serum (Figure 2B). This interaction was nearly abolished when delipidated serum was used. A similar result was observed for SMCs

(Figure S1). These data were consistent with those of Sorensen et al. [44], who showed that the binding of LL-37 to ApoAI and apoB did not occur in delipidated serum. Addition of exogenous lipid-free ApoAI and HDL both partially restored IDR-1018 induced association of ApoAI with macrophages and SMCs (Figure 2B and Figure S1). However, very little lipid-free ApoAI is found *in vivo* [49] therefore, the substantial increase in ApoAI observed when macrophages were stimulated with IDR-1018 in the presence of serum was likely the result of IDR-1018 binding to serum HDL, thus facilitating its interaction with cells.

Low molar concentrations of IDR-1018 enhanced HDL-mediated depletion of intracellular free cholesterol available for esterification

To determine if there were functional consequences of the binding of IDR-1018 to HDL, and the subsequent interaction of this complex with macrophages, we examined whether IDR-1018 influenced HDL-induced cholesterol efflux from macrophages. In these studies, the concentration of HDL was kept constant at 0.12 μM (20 μg/ml) while the concentration of IDR-1018 was varied, resulting in varying peptide:HDL molar ratios. Based on the dose-dependent increase in ApoAI observed when cells were stimulated with 3.3 to 33 μM of IDR-1018, high peptide:HDL ratios were initially utilized for the cholesterol efflux studies. Although, IDR-1018 alone had no effect on cholesterol efflux (data not shown), high concentrations inhibited HDL-mediated cholesterol efflux from cells (Figure S2). Therefore, the concentration of IDR-1018 was reduced substantially to generate peptide:HDL molar ratios ranging from 1:5 to 5:1.

To study the effect of these lower peptide:HDL ratios on cellular cholesterol levels, we initially utilized a cholesterol esterification assay. This assay accurately measures the degree to which cells are storing residual cholesterol, and thus also acts as an indicator of cholesterol efflux and has much lower backgrounds than other cholesterol assays. To store excess cholesterol in a non-toxic form, cells convert free cholesterol into cholesteryl esters, a reaction catalyzed by the endoplasmic reticulum enzyme Acyl-CoA cholesterol
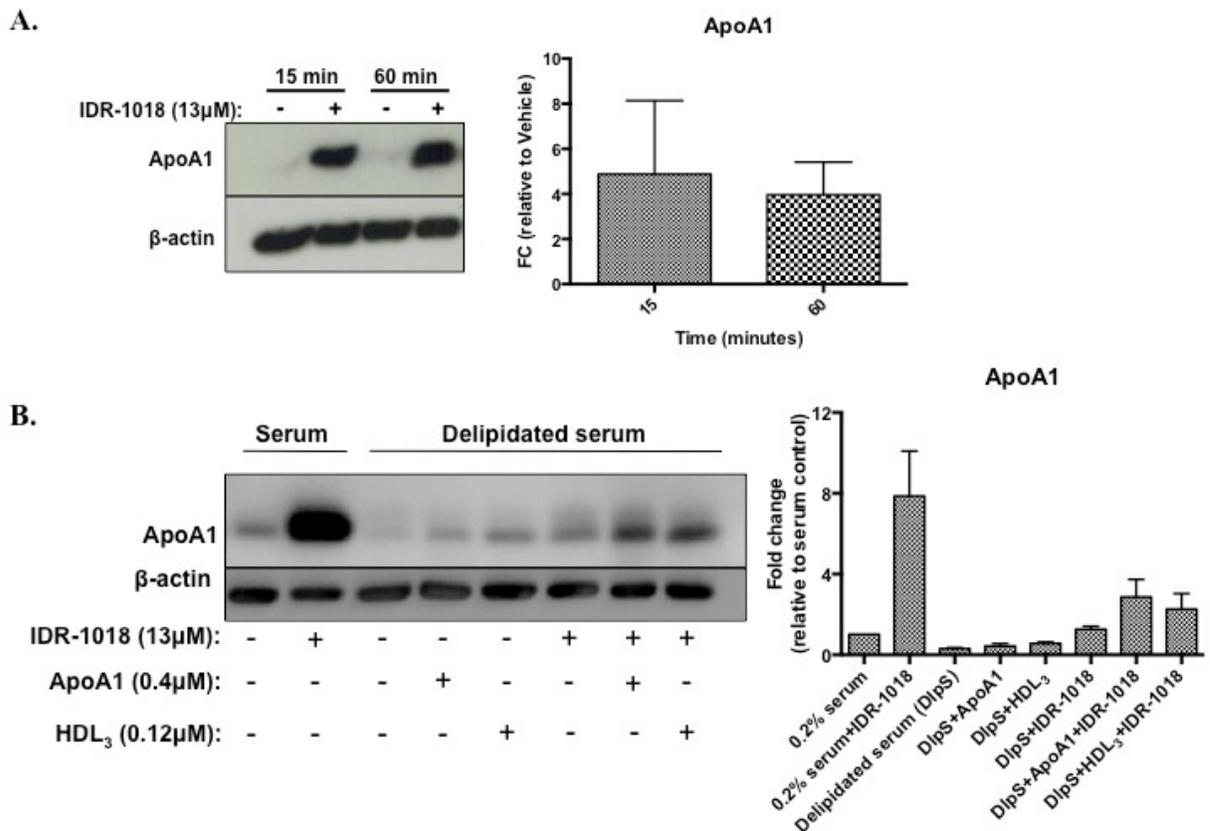
Figure 2: IDR-1018 rapidly increased ApoAI protein levels and interacted with HDL and exogenous lipid free ApoAI.

A) Protein levels in macrophages that were stimulated with 13µM IDR-1018 for 15 or 60 minutes in RPMI media containing 2% human serum. Cell lysates were run on a 12% gel and probed for ApoAI and β-actin (left). Densitometry (right) was calculated using Image J software and fold change (FC) is relative to no IDR-1018 control. Data are presented as the mean ± SEM. B) Macrophages were stimulated with 13 µM IDR-1018 (20 µg/ml) in the presence of media containing 0.2% human serum or de-lipidated human serum with or without lipid-poor ApoAI (10 µg/ml) or HDL (20 µg/ml) for 16 hours. Cell lysates were run on a 12% gel and probed for ApoAI and β-actin (left). Densitometry values (right) were calculated using Image J software. Data are presented as the mean of three independent experiments ± SEM.

acyltransferase (ACAT) [50]. In this assay, cells were stimulated with HDL alone or in combination with IDR-1018, followed by incubation with radiolabeled oleic acid. The oleic acid is used by ACAT as a substrate for the esterification of cholesterol [42]. The amount of radioisotope incorporated into cholesteryl esters estimates the level of cholesterol substrate available to ACAT. A decrease in cholesterol esterification is consistent with an increase in cholesterol efflux, which reduces the amount of intracellular free cholesterol available for esterification. A significant decrease in cholesterol esterification (55%) was observed in macrophages incubated with 20 µg/ml HDL for 16 hours (Figure 3). The 1:1 peptide:HDL molar ratio using 0.12 µM IDR-1018 enhanced the ability of HDL to deplete ACAT-accessible cholesterol a further 25%, suggesting that at low concentrations, IDR-1018 increased the HDL-mediated reduction in cholesterol storage. Thus, these results are consistent with the suggestion that, at low concentrations IDR-1018, promoted HDL-mediated efflux of free cholesterol from macrophages, leading to decreased cholesterol esterification. In contrast to
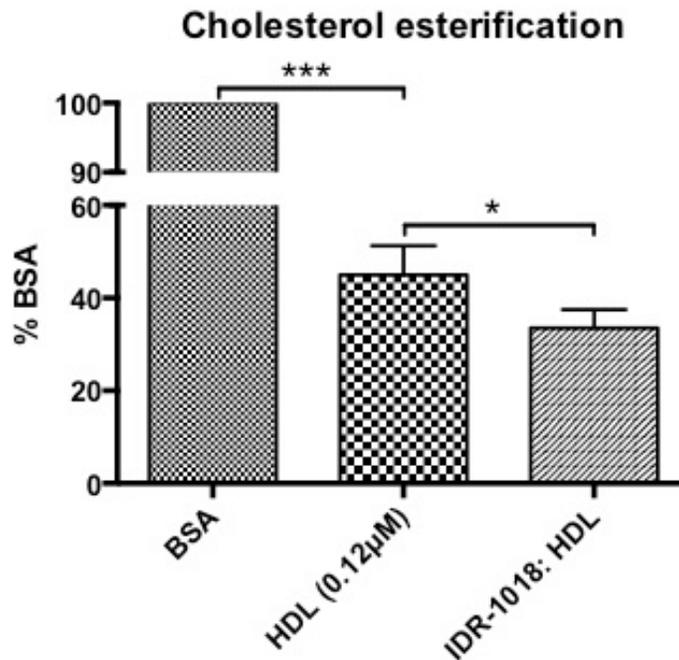
Cholesterol esterification

Figure 3: At low concentrations, IDR-1018 enhanced HDL-mediated inhibition of cholesterol esterification.

Macrophages were loaded with free cholesterol for 24 hour then stimulated with 0.12 µM HDL (20 µg/ml) alone or in combination with IDR-1018 for 16 hours. For the combination treatments (IDR1018:HDL), 0.12µM of IDR-1018 (0.18 µg/ml) and HDL (20 µg/ml) were added to the cells for a 1:1 molar ratio. Following the treatments, cells were incubated with [14C]oleate-alubumin for 1 hour. Media was removed and cholesteryl esters isolated from cell lysates. Data is presented as the mean percentage of BSA control ± SEM. Five independent experiments were completed, data analyzed using the paired Student's t-test ***p<0.001, *p<0.05.

the cholesterol efflux studies presented in Figure S2, higher concentrations of IDR-1018 appeared to have little effect on HDL-mediated depletion of excess intracellular cholesterol.

Low molar concentrations of IDR-1018 enhanced HDL-induced cholesterol efflux from macrophages and smooth muscle cells

To further characterize the effect of IDR-1018 on cholesterol efflux by HDL from macrophages and smooth muscle cells (SMC), a cholesterol efflux assay measuring extracellular and intracellular cholesterol was employed at the lower peptide:HDL molar ratios that proved effective in the ACAT assay. HDL induced a significant ~4-fold increase in extracellular cholesterol compared to the BSA control indicating that HDL was

potently inducing cholesterol efflux (Figure 4A). The 1:1 and 5:1 HDL:IDR-1018 ratios induced approximately 15% more cholesterol efflux from macrophages and SMCs, compared to HDL alone, suggesting that IDR-1018 increased HDL-mediated cholesterol efflux from SMCs (p<0.05) and possibly macrophages (Figure 4A and B). Consistent with the ACAT assay, HDL also induced a significant decrease in intracellular free cholesterol and cholesteryl esters compared to the BSA control (Figure 5). Addition of IDR-1018 significantly further decreased the intracellular free cholesterol content of macrophages compared to HDL alone but had no effect on cholesteryl esters (Figure 5A). This suggests that the increase in extracellular cholesterol observed with the addition of IDR-1018 might have resulted from IDR-1018 promoting the release of cholesterol that had yet to be esterified. Alternatively,
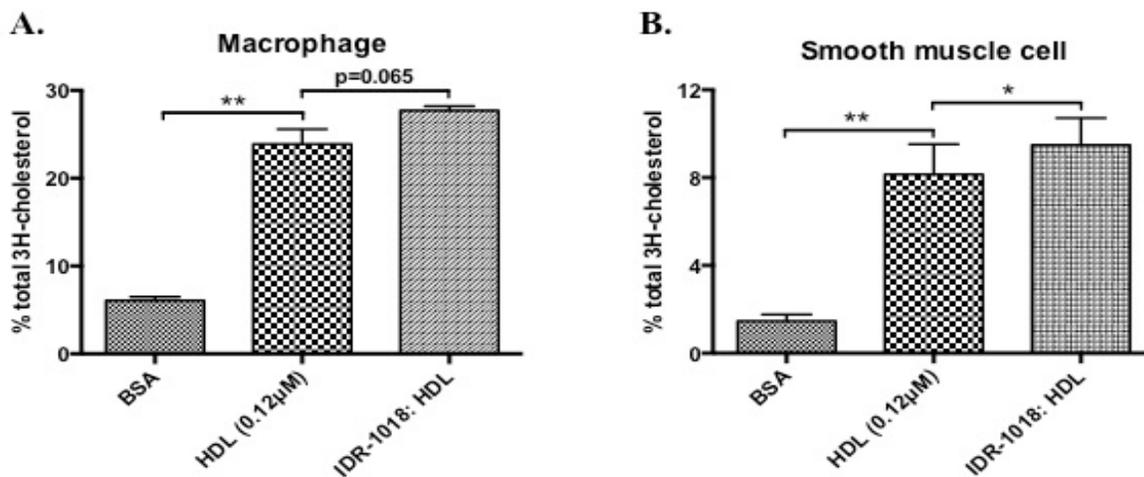
Figure 4: At low concentrations, IDR-1018 enhanced HDL-mediated cholesterol efflux from free cholesterol loaded cells.

Macrophages (A) and smooth muscle cells (B) were labeled with ^{3}H-cholesterol. Following the labeling period, cells were cholesterol loaded for 24 hours then equilibrated in media containing lipid free albumin for an additional 24 hours. Cells were then stimulated with 0.12 µM HDL (20 µg/ml) alone or in combination with IDR-1018 for 16 hours. For the combination treatment (peptide:HDL), 0.12 µM of HDL was combined with 0.12 µM (0.2 µg/ml) IDR-1018 for macrophages (1:1 ratio) or 0.6 µM (0.92 µg/ml) for smooth muscle cells (5:1 ratio). Following treatment, media was removed and used to measure extracellular cholesterol content while cell lysates were used to measure intracellular cholesterol content. Five independent experiments were completed for both macrophages and smooth muscle cells with one experiment on macrophages excluded as an outlier. Data is presented as the mean percentage of total cholesterol ± SEM, data analyzed using the paired Student t-test **$p < 0.01$, *$p < 0.05$.

IDR-1018 might have enhanced the transfer of radiolabeled cholesterol from the plasma membrane to HDL via the aqueous diffusion pathway [9].

As mentioned above, IDR-1018 significantly enhanced HDL-dependent cholesterol efflux from SMCs (Figure 4B). This increase correlated with a moderate but non-significant reduction in intracellular cholesteryl esters (Figure 5B). The results for the SMCs are of particular interest since these cells are now thought to represent more than 50% of the foam cells present in arteries [4].

At low molar concentrations, IDR-1018 did not alter ApoAI or ABCA1 protein expression

Immunoblot analyses of ApoAI and ABCA1 were completed for macrophages stimulated with the peptide:HDL ratios that enhanced HDL–mediated cholesterol efflux as well as the 114:1 ratio which is equivalent to 13 µM IDR-1018, a concentration that increased ApoAI and ABCA1 in macrophages stimulated in serum. HDL alone induced a modest increase in ApoAI. The 1:5, 1:1 and 5:1 ratios (0.023 µM, 0.12 µM and 0.58 µM, IDR-1018 respectively) induced a slight decrease in ApoAI compared to HDL alone (Figure 6A). Although 13 µM of IDR-1018 had no effect on ApoAI when used alone, combined with HDL (114:1 ratio) it increased ApoAI substantially, further supporting the hypothesis that IDR-1018 could interact with HDL in serum and promote its interaction with cells.

With respect to ABCA1, a different trend was observed compared to the results from macrophages stimulated with IDR-1018 in the presence of human serum. At time zero, the cholesterol-loaded macrophages exhibited elevated ABCA1 levels (Figure 6B), which was expected given that ABCA1 expression is known
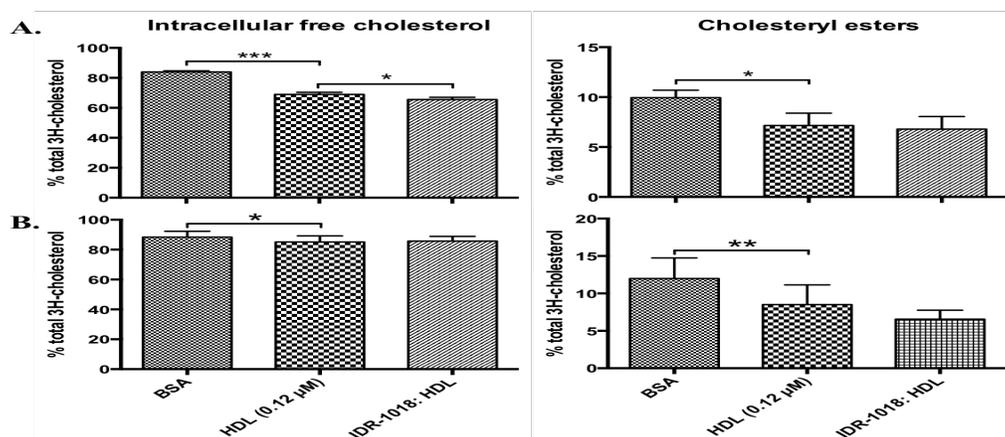
Figure 5: IDR-1018 further reduced the intracellular cholesterol content of HDL-treated cells.

Macrophages (A) and smooth muscle cells (B) were labeled with ³H-cholesterol followed by loading with cholesterol for 24 hours then equilibrated in media containing lipid free albumin for an additional 24 hours. . Cells were then stimulated with 0.12 µM HDL (20 µg/ml) alone or in combination with IDR-1018 for 16 hours. For the combination treatment (peptide:HDL), 0.12 µM of HDL was combined with 0.12 µM (0.18 µg/ml) IDR-1018 for macrophages (1:1 ratio) or 0.6 µM (0.92 µg/ml) for smooth muscle cells (5:1 ratio). Following treatment, cell lysates were used to measure intracellular free cholesterol (right graph) and cholesteryl esters (left graph). Results are average of 4 (macrophages) or 5 (SMC) experiments. Data is presented as the mean percentage of total cholesterol ± SEM, data analyzed using the paired Student t-test ***$p<0.001$, **$p<0.01$, *$p<0.05$.

to be upregulated in response to increased intracellular cholesterol levels [51-53]. After 16 hours the ABCA1 level declined slightly in the BSA control. This was attributed to passive aqueous diffusion of cholesterol from the cells [9]. Stimulation with HDL further decreased ABCA1, presumably as a result of HDL inducing efflux of cholesterol from the cells, reducing intracellular cholesterol content [52]. Interestingly, ABCA1 was nearly undetectable in cells stimulated with 13µM of IDR-1018 alone or in combination with HDL (114:1 peptide:HDL ratio). This contrasted with the results obtained when macrophages were stimulated with 13µM of IDR-1018 in the presence of human serum, but without additional HDL (Figure 1A and B). Much like the results for ApoAI, the low peptide:HDL ratios had little effect on ABCA1 levels compared to HDL alone.

Discussion

This study shows that IDR-1018 elevated the level of several proteins involved in cellular cholesterol efflux, aided HDL in depleting intracellular free cholesterol,

and enhanced HDL-mediated cholesterol efflux from SMCs and macrophages. Based on these data, we propose a model in which IDR-1018 enhanced HDL-mediated cholesterol efflux possibly by binding to HDL in serum and promoting its association with cells. It seems possible that this led to the improved transfer of cholesterol to HDL particles, while still allowing HDL to dissociate from the cell after efflux. At higher concentrations, IDR-1018 strongly enhanced the association of HDL with cells but efflux was inhibited, possibly because the dissociation of HDL from the cell was reduced (Figure 7).

Stimulation with IDR-1018 of macrophages that were not loaded with cholesterol, induced a significant increase in several components of the cholesterol efflux pathway including a dose-dependent increase in two of the most important components, ApoAI and ABCA1. The increase in ApoAI observed in response to IDR-1018 stimulation, was likely the result of IDR-1018 interacting with HDL in serum, thus facilitating its interaction with the plasma membranes of macrophages, since delipidation
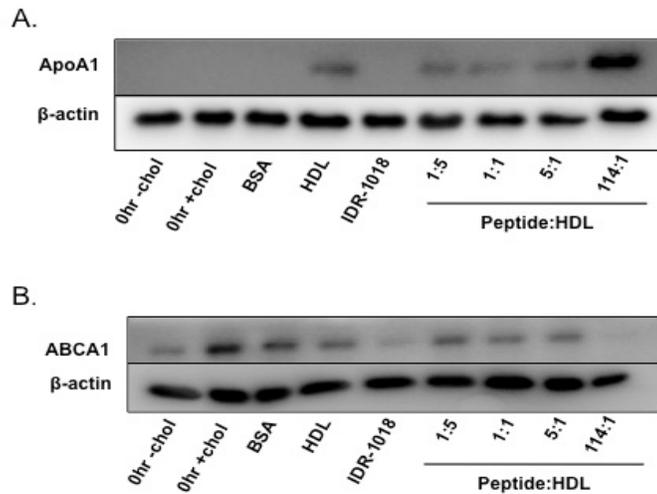
Figure 6: At low concentrations, IDR-1018 did not alter HDL-mediated changes in ApoAI or ABCA1 levels.

Macrophages were loaded with free cholesterol then incubated with 0.12 µM HDL (20 µg/ml) or 13 µM IDR-1018 (20 µg/ml) alone or in combination for 16 hours. For the 1:5, 1:1, 5:1, and 114:1 peptide:HDL molar ratios, the concentration of IDR-1018 was varied (0.023 µM, 0.12 µM, 0.58 µM, and 13 µM IDR-1018 respectively) while the HDL concentration was kept constant at 0.12 µM. Cell lysates were run on a 12% gel and probed for ApoAI (A), ABCA1 (B) and β-actin. Images are one experiment representative of three.

concentration of IDR-1018 used to generate the 1:1 peptide:HDL molar ratio (0.12 µM IDR-1018), appeared to enhance cholesterol efflux from macrophages and significantly reduced intracellular free cholesterol and the esterification of cholesterol for storage. IDR-1018 also significantly enhanced HDL-mediated cholesterol efflux from SMCs and reduced cholesteryl ester levels compared to HDL alone. Macrophages and SMCs play a significant role in the pathogenesis of atherosclerosis as the major sites of excess cholesterol sequestration and the resulting inflammation [1,4]. Therefore, the ability to enhance cholesterol efflux from these cell types is particularly relevant. The concentrations of IDR-1018 that enhanced HDL-mediated cholesterol efflux from macrophages did not visibly alter ApoAI protein levels. Based on these results, we propose that at low IDR-1018 concentrations, fewer peptide molecules were present to interact with each HDL particle. Thus, although IDR-1018 enhanced HDL association with cellular membranes, this interaction would have been relatively weak, allowing HDL particles to dissociate from the cell after taking up cholesterol. As the peptide:HDL ratio

increased, more peptide molecules would interact with each HDL particle, eventually coating HDL particles with peptide. While this likely enhanced the interaction of HDL with cellular membranes, it also potentially prevented the HDL particles from dissociating from the cells, thus inhibiting cholesterol efflux. A model of this hypothesis is presented in Figure 7.

Conclusions

The results presented in this study suggest that IDR-1018 enhanced HDL-mediated cholesterol efflux from multiple cell types, in part by facilitating the interaction of HDL with cells. These represent new biological properties for IDR-1018. These findings are especially important given that there has been little development for many of the ApoAI peptide mimetics that entered clinical trials for the treatment of atherosclerosis [16]. Although ApoAI mimetic peptides are able to directly induce cholesterol efflux, high concentrations (10-80 µg/ml) are required to achieve this effect [26,28,60]. In our system, low molar concentrations of IDR-1018
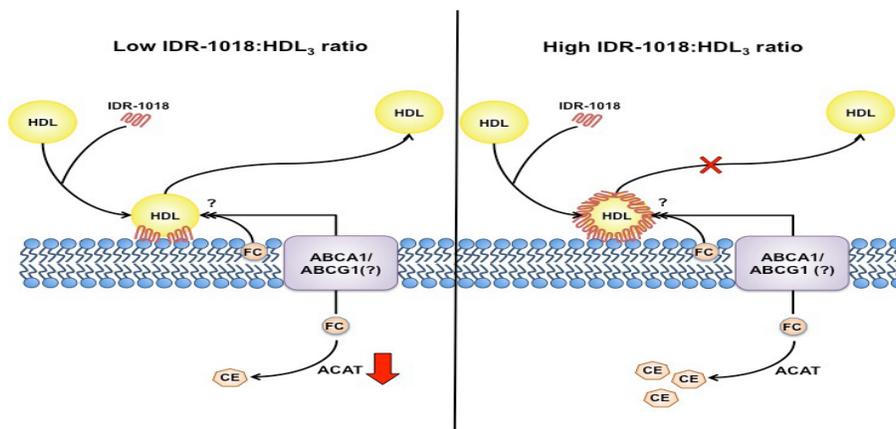
Figure 7: Proposed model for the enhancement of HDL-mediated cellular cholesterol efflux by IDR-1018.

At low concentrations, IDR-1018 enhanced HDL-mediated cholesterol efflux by binding to HDL particles in serum and facilitating their association with cells. This improved the transfer of free cholesterol (FC) to HDL particles via ABCA1, ABCG1 or passive aqueous diffusion, while still allowing its dissociation from cells. The addition of IDR-1018 also enhanced HDL-mediated suppression of cholesteryl ester (CE) synthesis in cells. At high concentrations, IDR-1018 became inhibitory possibly because the interaction between IDR-1018, HDL and the plasma membrane was too strong, preventing the dissociation of HDL from the cell. However, suppression of cholesterol esterification appeared unaffected by IDR-1018.

of plasma abolished the IDR-1018-mediated increases in ApoAI. A similar result was previously observed for another immunomodulatory peptide LL-37 that binds HDL particles through an interaction between its hydrophobic c-terminal domain and the lipids of HDL [44]. Several immunomodulatory peptides interact with both mammalian membranes and biomolecules such as lipoproteins and DNA [45,48,54-56]. This allows the peptides to facilitate interactions between the biomolecules and cells [45,54,57]. It seems likely that IDR-1018 interacted with HDL in a similar manner.

The increase in ABCA1 induced by IDR-1018 in macrophages stimulated in the presence of human serum, likely also occurred post-transcriptionally. ApoAI and several ApoAI peptide mimetics increase ABCA1 protein levels [58-60] through stabilization of the ABCA1 protein and prevention of its degradation, an important mode of post-translational regulation for this transporter [46,47,58,59,61,62]. The results suggest that IDR-1018 was able to increase ABCA1 levels by stabilizing it, although it is unclear if it did so directly or

through its interaction with lipid-poor ApoAI in serum. In cholesterol-loaded macrophages, IDR-1018 caused a substantial decrease in ABCA1 levels. Because the intracellular cholesterol level is also a major regulator of ABCA1, this result was initially thought to be due to IDR-1018 inducing cholesterol efflux from cells, which would result in a downregulation of ABCA1 [52]. However, since IDR-1018 alone had no effect on cholesterol efflux and at higher concentrations inhibited HDL-mediated cholesterol efflux, it seems unlikely that this was due to enhanced cholesterol efflux from cells and the consequent downregulation of ABCA1 [52].

Unlike ApoA1 and ApoA1 peptide mimetics, IDR-1018 was unable to directly induce cholesterol efflux from cells, however IDR-1018 did enhance HDL-mediated cholesterol efflux from SMCs and likely macrophages. This activity was dependent upon the concentration of IDR-1018 used. Concentrations of IDR-1018, similar to those that substantially increased ApoAI association with macrophages, actually inhibited HDL-mediated cholesterol efflux from cells. Conversely, the low

were needed, the equivalent of 0.12-0.58 µg/ml, to enhance cholesterol efflux normally induced by HDL. This is 10 times lower than the concentrations used in *in vitro* studies of the immunomodulatory properties of IDR-1018. Interestingly, the concentrations of IDR-1018 required to observe its immunomodulatory properties are similar to the concentrations of IDR-1018 that inhibited cholesterol efflux. Thus, testing in animal models of reverse cholesterol transport are needed to determine if the enhanced cellular cholesterol efflux induced by IDR-1018 is physiologically relevant. Regardless, the results presented here suggest that the immunomodulatory peptides currently in development as anti-infectives could also have potential use in the treatment of atherosclerosis.

Acknowledgements

References

1. Moore KJ, Tabas I (2011) Macrophages in the pathogenesis of atherosclerosis. Cell 145: 341-355.

2. Weber C, Noels H (2011) Atherosclerosis: current pathogenesis and therapeutic options. Nat Med 17: 1410-1422.

3. Moore KJ, Sheedy FJ, Fisher EA (2013) Macrophages in atherosclerosis: a dynamic balance. Nat Rev Immunol 13: 709-721.

4. Allahverdian S, Chehroudi AC, McManus BM, Abraham T, Francis GA (2014) Contribution of intimal smooth muscle cells to cholesterol accumulation and macrophage-like cells in human atherosclerosis. Circulation 129: 1551-1559.

5. Dubland JA, Francis GA (2016) So Much Cholesterol: the unrecognized importance of smooth muscle cells in atherosclerotic foam cell formation. Curr Opin Lipidol 27: 155-161.

6. Cuchel M, Rader DJ (2006) Macrophage reverse cholesterol transport: key to the regression of atherosclerosis? Circulation 113: 2548-2555.

7. Yvan-Charvet L, Wang N, Tall AR (2010) Role of HDL, ABCA1, and ABCG1 transporters in cholesterol efflux and immune responses. Arterioscler Thromb Vasc Biol 30: 139-143.

8. Fessler MB, Parks JS (2011) Intracellular lipid flux and membrane microdomains as organizing principles in inflammatory cell signaling. J Immunol 187: 1529-1535.

9. Rosenson RS, Brewer HB Jr., Davidson WS, Fayad ZA, Fuster V, et al. (2012) Cholesterol efflux and atheroprotection: advancing the concept of reverse cholesterol transport. Circulation 125: 1905-1919.

10. Smoak KA, Aloor JJ, Madenspacher J, Merrick BA, Collins JB, et al. (2010) Myeloid differentiation primary response protein 88 couples reverse cholesterol transport to inflammation. Cell Metab 11: 493-502.

11. Smythies LE, White CR, Maheshwari A, Palgunachari MN, Anantharamaiah GM, et al. (2010) Apolipoprotein A-I mimetic 4F alters the function of human monocyte-derived macrophages. Am J Physiol Cell Physiol 298: C1538-C1548.

12. Catapano AL, Pirillo A, Bonacina F, Norata GD (2014) HDL in innate and adaptive immunity. Cardiovasc Res 103: 372-383.

13. Feig JE, Rong JX, Shamir R, Sanson M, Vengrenyuk Y, et al. (2011) HDL promotes rapid atherosclerosis regression in mice and alters inflammatory properties of plaque monocyte-derived cells. Proc Natl Acad Sci U S A 108: 7166-7171.

14. Tang C, Liu Y, Kessler PS, Vaughan AM, Oram JF (2009) The macrophage cholesterol exporter ABCA1 functions as an anti-inflammatory receptor. J Biological Chem 284: 32336-32343.

15. Kim KD, Lim HY, Lee HG, Yoon DY, Choe YK, et al. (2005) Apolipoprotein A-I induces IL-10 and PGE2 production in human monocytes and inhibits dendritic cell differentiation and maturation. Biochem Biophys Res Commun 338: 1126-1136.

16. Kingwell BA, Chapman MJ, Kontush A, Miller NE (2014) HDL-targeted therapies: progress, failures and future. Nat Rev Drug Discov 13: 445-464.

17. Navab M, Anantharamaiah GM, Reddy ST, Hama S, Hough G, et al. (2005) Apolipoprotein A-I mimetic peptides. Arterioscler Thromb Vasc Biol 25: 1325-1331.

18. Getz GS, Wool GD, Reardon CA (2010) Biological properties of apolipoprotein a-I mimetic peptides. Curr Atherosclerosis Reports 12: 96.

19. Carballo-Jane E, Chen Z, O'Neill E, Wang J, Burton C, et al. (2010) ApoA-I mimetic peptides promote pre-beta HDL formation in vivo causing remodeling of HDL and triglyceride accumulation at higher dose. Bioorg Med Chem 18: 8669-8678.

20. Chen X, Burton C, Song X, McNamara L, Langella A, et al. (2009) An apoA-I mimetic peptide increases LCAT activity in mice through increasing HDL concentration. Int J Biol Sci 5: 489-499.

21. Navab M, Anantharamaiah GM, Fogelman AM (2008) The effect of apolipoprotein mimetic peptides in inflammatory disorders other than atherosclerosis. Trends in cardiovascular medicine 18: 61-66.

22. Ingenito R, Burton C, Langella A, Chen X, Zytko K, et al. (2010) Novel potent apoA-I peptide mimetics that stimulate cholesterol efflux and pre-beta particle formation *in vitro*. Bioorg Med Chem Lett 20: 236-239.

23. Tabet F, Remaley AT, Segaliny AI, Millet J, Yan L, et al. (2010) The 5A apolipoprotein A-I mimetic peptide displays antiinflammatory and antioxidant properties *in vivo* and *in vitro*. Arterioscler Thromb Vasc Biol 30: 246-252.

24. Yao X, Dai C, Fredriksson K, Dagur PK, McCoy JP, et al. (2011) 5A, an apolipoprotein A-I mimetic peptide, attenuates the induction of house dust mite-induced asthma. J Immunol 186: 576-583.

25. Qin S, Kamanna VS, Lai JH, Liu T, Ganji SH, et al. (2012) Reverse D4F, an apolipoprotein-AI mimetic peptide, inhibits atherosclerosis in ApoE-null mice. J Cardiovasc Pharmacol Ther 17: 334-343.

26. Sethi AA, Stonik JA, Thomas F, Demosky SJ, Amar M, et al. (2008) Asymmetry in the lipid affinity of bihelical amphipathic peptides. A structural determinant for the specificity of ABCA1-dependent cholesterol efflux by peptides. The J Biol Chem 283: 32273-32282.

27. Yancey PG, Bielicki JK, Johnson WJ, Lund-Katz S, Palgunachari MN, et al. (1995) Efflux of cellular cholesterol and phospholipid to lipid-free apolipoproteins and class A amphipathic peptides. Biochem 34: 7955-7965.

28. Remaley AT, Thomas F, Stonik JA, Demosky SJ, Bark SE, et al. (2003) Synthetic amphipathic helical peptides promote lipid efflux from cells by an ABCA1-dependent and an ABCA1-independent pathway. J Lipid Res 44: 828-836.

29. Gupta H, Dai L, Datta G, Garber DW, Grenett H, et al. (2005) Inhibition of lipopolysaccharide-induced inflammatory responses by an apolipoprotein AI mimetic peptide. Circ Res 97: 236-243.

30. Hilchie AL, Wuerth K, Hancock REW (2013) Immune modulation by multifaceted cationic host defense (antimicrobial) peptides. Nat Chem Biol

9: 761-768.

31. Hancock RE, Diamond G (2000) The role of cationic antimicrobial peptides in innate host defences. Trends Microbiol 8: 402-410.

32. Wieczorek M, Jenssen H, Kindrachuk J, Scott WR, Elliott M, et al. (2010) Structural studies of a peptide with immune modulating and direct antimicrobial activity. Chem Biol 17: 970-980.

33. Niyonsaba F, Madera L, Afacan N, Okumura K, Ogawa H, et al. (2013) The innate defense regulator peptides IDR-HH2, IDR-1002, and IDR-1018 modulate human neutrophil functions. J Leukocyte Biol 94: 159-170.

34. Mayer ML, Blohmke CJ, Falsafi R, Fjell CD, Madera L, et al. (2013) Rescue of dysfunctional autophagy attenuates hyperinflammatory responses from cystic fibrosis cells. J Immunol 190: 1227-1238.

35. Steinstraesser L, Hirsch T, Schulte M, Kueckelhaus M, Jacobsen F, et al. (2012) Innate defense regulator peptide 1018 in wound healing and wound infection. PloS one 7: e39373.

36. Pena OM, Afacan N, Pistolic J, Chen C, Madera L, et al. (2013) Synthetic cationic peptide IDR-1018 modulates human macrophage differentiation. PloS one 8: e52449.

37. Rivas-Santiago B, Castaneda-Delgado JE, Rivas Santiago CE, Waldbrook M, Gonzalez-Curiel I, et al. (2013) Ability of innate defence regulator peptides IDR-1002, IDR-HH2 and IDR-1018 to protect against Mycobacterium tuberculosis infections in animal models. PloS one 8: e59119.

38. Achtman AH, Pilat S, Law CW, Lynn DJ, Janot L, et al. (2012) Effective adjunctive therapy by an innate defense regulatory peptide in a preclinical model of severe malaria. Sci Transl Med 4: 135ra64.

39. Bolouri H, Savman K, Wang W, Thomas A, Maurer N, et al. (2014) Innate defense regulator peptide 1018 protects against perinatal brain injury. Ann Neurol 75: 395-410.

40. Francis GA, Oram JF, Heinecke JW, Bierman EL (1996) Oxidative tyrosylation of HDL enhances the depletion of cellular cholesteryl esters by a mechanism independent of passive sterol desorption. Biochem 35: 15188-15197.

41. Lemire JM, Potter-Perigo S, Hall KL, Wight TN, Schwartz SM (1996) Distinct rat aortic smooth muscle cells differ in versican/PG-M expression. Arteriosclerosis, thrombosis, and vascular biology 16: 821-829.

42. Waddington EI, Boadu E, Francis GA (2005) Cholesterol and phospholipid efflux from cultured cells. Methods 36: 196-206.

43. Wang Y, Agerberth B, Lothgren A, Almstedt A, Johansson J (1998) Apolipoprotein A-I binds and inhibits the human antibacterial/cytotoxic peptide LL-37. J Biol Chem 273: 33115-33118.

44. Sorensen O, Bratt T, Johnsen AH, Madsen MT, Borregaard N (1999) The human antibacterial cathelicidin, hCAP-18, is bound to lipoproteins in plasma. J Biol Chem 274: 22445-22451.

45. Higazi AA, Lavi E, Bdeir K, Ulrich AM, Jamieson DG, et al. (1997) Defensin stimulates the binding of lipoprotein (a) to human vascular endothelial and smooth muscle cells. Blood 89: 4290-4298.

46. Lu R, Arakawa R, Ito-Osumi C, Iwamoto N, Yokoyama S (2008) ApoA-I facilitates ABCA1 recycle/accumulation to cell surface by inhibiting its intracellular degradation and increases HDL generation. Arterioscler Thromb Vasc Biol 28: 1820-1824.

47. Wang N, Silver DL, Costet P, Tall AR (2000) Specific binding of ApoA-I, enhanced cholesterol efflux, and altered plasma membrane morphology in cells expressing ABC1. J Biol Chem 275: 33053-33058.

48. Mookherjee N, Lippert DN, Hamill P, Falsafi R,

Nijnik A, et al. (2009) Intracellular receptor for human host defense peptide LL-37 in monocytes. J Immunol 183: 2688-2696.

49. Hossain MA, Ngeth S, Chan T, Oda MN, Francis GA (2012) Lipid-bound apolipoproteins in tyrosyl radical-oxidized HDL stabilize ABCA1 like lipid-free apolipoprotein A-I. BMC Biochem 13: 1.

50. Chang TY, Li BL, Chang CC, Urano Y (2009) Acyl-coenzyme A:cholesterol acyltransferases. American J Physiol Endocrinol Metabol 297: E1-E9.

51. Hsieh V, Kim MJ, Gelissen IC, Brown AJ, Sandoval C, et al. (2014) Cellular cholesterol regulates ubiquitination and degradation of the cholesterol export proteins ABCA1 and ABCG1. J Biol Chem 289: 7524-7536.

52. Langmann T, Klucken J, Reil M, Liebisch G, Luciani MF, et al. (1999) Molecular cloning of the human ATP-binding cassette transporter 1 (hABC1): evidence for sterol-dependent regulation in macrophages. Biochem Biophys Res Commun 257: 29-33.

53. Lawn RM, Wade DP, Garvin MR, Wang X, Schwartz K, et al. (1999) The Tangier disease gene product ABC1 controls the cellular apolipoprotein-mediated lipid removal pathway. J Clin Invest 104: R25-R31.

54. Sandgren S, Wittrup A, Cheng F, Jonsson M, Eklund E, et al. (2004) The human antimicrobial peptide LL-37 transfers extracellular DNA plasmid to the nuclear compartment of mammalian cells via lipid rafts and proteoglycan-dependent endocytosis. J Biol Chem 279: 17951-17956.

55. Bandholtz L, Ekman GJ, Vilhelmsson M, Buentke E, Agerberth B, et al. (2006) Antimicrobial peptide LL-37 internalized by immature human dendritic cells alters their phenotype. Scand J Immunol 63: 410-419.

56. Yu HB, Kielczewska A, Rozek A, Takenaka S, Li Y, et al. (2009) Sequestosome-1/p62 is the key intracellular target of innate defense regulator peptide. J Biol Chem 284: 36007-36011.

57. Chamilos G, Gregorio J, Meller S, Lande R, Kontoyiannis DP, et al. (2012) Cytosolic sensing of extracellular self-DNA transported into monocytes by the antimicrobial peptide LL37. Blood 120: 3699-3707.

58. Arakawa R, Hayashi M, Remaley AT, Brewer BH, Yamauchi Y, et al. (2004) Phosphorylation and stabilization of ATP binding cassette transporter A1 by synthetic amphiphilic helical peptides. J Biol Chem 279: 6217-6220.

59. Arakawa R, Yokoyama S (2002) Helical apolipoproteins stabilize ATP-binding cassette transporter A1 by protecting it from thiol protease-mediated degradation. J Biol Chem 277: 22426-22429.

60. Tang C, Vaughan AM, Anantharamaiah GM, Oram JF (2006) Janus kinase 2 modulates the lipid-removing but not protein-stabilizing interactions of amphipathic helices with ABCA1. J Lipid Res 47: 107-114.

61. Martinez LO, Agerholm-Larsen B, Wang N, Chen W, Tall AR (2003) Phosphorylation of a pest sequence in ABCA1 promotes calpain degradation and is reversed by ApoA-I. J Biol Chem 278: 37368-37374.

62. Oram JF, Heinecke JW (2005) ATP-binding cassette transporter A1: a cell cholesterol exporter that protects against cardiovascular disease. Physiol Rev 85: 1343-1372.