

## RESEARCH PAPER

# High-density lipoprotein inhibits human M1 macrophage polarization through redistribution of caveolin-1

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**Received**

29 December 2014

**Revised**

19 August 2015

**Accepted**

25 August 2015

**BACKGROUND AND PURPOSE**

Monocyte-derived macrophages are critical in the development of atherosclerosis and can adopt a wide range of functional phenotypes depending on their surrounding milieu. High-density lipoproteins (HDLs) have many cardio-protective properties including potent anti-inflammatory effects. We investigated the effects of HDL on human macrophage phenotype and the mechanisms by which these occur.

**EXPERIMENTAL APPROACH**

Human blood monocytes were differentiated into macrophages in the presence or absence of HDL and were then induced to either an inflammatory macrophage (M1) or anti-inflammatory macrophage (M2) phenotype using LPS and IFN- $\gamma$  or IL-4, respectively.

**KEY RESULTS**

HDL inhibited the induction of macrophages to an M1-phenotype, as evidenced by a decrease in the expression of M1-specific cell surface markers CD192 and CD64, as well as M1-associated inflammatory genes TNF- $\alpha$ , IL-6 and MCP-1 (CCL2). HDL also inhibited M1 function by reducing the production of ROS. In contrast, HDL had no effect on macrophage induction to the M2-phenotype. Similarly, methyl- $\beta$ -cyclodextrin, a non-specific cholesterol acceptor also suppressed the induction of M1 suggesting that cholesterol efflux is important in this process. Furthermore, HDL decreased membrane caveolin-1 in M1 macrophages. We confirmed that caveolin-1 is required for HDL to inhibit M1 induction as bone marrow-derived macrophages from caveolin-1 knockout mice continued to polarize into M1-phenotype despite the presence of HDL. Moreover, HDL decreased ERK1/2 and STAT3 phosphorylation in M1 macrophages.

**CONCLUSIONS AND IMPLICATIONS**

We concluded that HDL reduces the induction of macrophages to the inflammatory M1-phenotype via redistribution of caveolin-1, preventing the activation of ERK1/2 and STAT3.

**LINKED ARTICLES**

This article is part of a themed section on Inflammation: maladies, models, mechanisms and molecules. To view the other articles in this section visit <http://dx.doi.org/10.1111/bph.2016.173.issue-4>

**Abbreviations**

BMDMs, bone marrow-derived macrophages; Cav-1<sup>-/-</sup>, caveolin-1 knockout; HDL, high-density lipoprotein; M $\beta$ CD, methyl-beta-cyclodextrin; M1, inflammatory macrophage; M2, anti-inflammatory macrophage; WT, wild-type

## Tables of Links

TARGETS	
<b>Transporters<sup>a</sup></b>	<b>Enzymes<sup>b</sup></b>
Na <sup>+</sup> /K <sup>+</sup> -ATPase	ERK1
	ERK2

LIGANDS	
Cholesterol	IL-6
H2O2	LPS
IFN- $\gamma$	MCP-1 (CCL2)
IL-4	TNF- $\alpha$

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (<sup>a,b</sup>Alexander *et al.*, 2013a,b).

## Introduction

Cardiovascular disease (CVD) remains the leading cause of morbidity and mortality globally (Greenland *et al.*, 2010). It is well accepted that plasma cholesterol levels are a major determinant in the risk and severity of atherosclerosis (Hoogeveen *et al.*, 2014) and that an inverse relationship exists between plasma high-density lipoproteins (HDL) and CVD risk (Gordon *et al.*, 1977), suggesting a protective role for HDL. One of the major functions of HDL is reverse cholesterol transport (RCT) from extra-hepatic organs including the vascular wall to the liver for excretion into bile (Zhu *et al.*, 2008; Hafiane and Genest, 2013; Baryliski *et al.*, 2014). However, HDL appears to exert a number of other anti-atherosclerotic effects including reducing endothelial inflammation (Barter *et al.*, 2004), reducing leukocyte activation (Rye and Barter, 2008; Murphy *et al.*, 2011) and decreasing the production of myeloid cells (Murphy *et al.*, 2013), which are intimately involved in the development and progression of the lesion. Increasing plasma levels of HDL also appears to directly impact macrophages in the atherosclerotic lesion and could contribute to atherosclerotic lesion regression (Feig *et al.*, 2011; Feig *et al.*, 2014).

Monocyte-derived macrophages serve as critical players in the initiation and progression of atherosclerosis. Tissue macrophages can adopt a wide range of functional phenotypes depending on their surrounding milieu. Monocytes/macrophages surrounded by inflammatory stimuli such as LPS and cytokines are able to derive macrophages to a 'classic' inflammatory M1 phenotype, associated with tissue destruction, the production of ROS and secretion of inflammatory cytokines. On the other hand, cytokines such as IL-4 and IL-10 promote macrophages to a 'non-classic' pro-resolving M2 phenotype, which supports tissue repair, clearance of cell debris, angiogenesis and secretion of anti-inflammatory cytokines (Tugal *et al.*, 2013). Through the use of mouse models, it is largely thought that atherosclerotic lesions have an imbalance between these macrophage subsets such that lesions are largely comprised of M1 macrophages (Swirski *et al.*, 2007; Tacke *et al.*, 2007), which impair the resolution of this chronic inflammatory disease (Tacke *et al.*, 2007). Dampening the M1 response or promoting M2 macrophages is a potential mechanism to promote the regression of atherosclerotic CVD.

Key findings from the Fisher laboratory have revealed that raising HDL levels regresses the atherosclerotic lesion in mouse models. Feig *et al.* (2011) discovered that increasing HDL levels via overexpression of human ApoA-I not only reduces the size of the lesion and the lipid content but also alters the genetic signature of lesional macrophages from an inflammatory (M1) to a pro-fibrotic (M2) phenotype. Increasing HDL levels may systemically have a number of effects that could result in lesional macrophages displaying an M2-like phenotype. As monocytes circulate in plasma containing increased HDL levels, the influence on the type of macrophage could begin at this point. In the current study, we determined whether HDL influences the maturation and phenotype of monocytes to macrophages and the mechanisms by which it does this.

## Methods

### Isolation of HDL

Human HDL was isolated and purified using sequential ultracentrifugation with potassium bromide for density adjustment as previously described (McPherson *et al.*, 2007). Plasma of healthy donors was obtained through the Red Cross Blood Bank of Australia. Plasma density was adjusted from 1.01 to 1.21 g·L<sup>-1</sup> to isolate pure HDL. SDS-PAGE gel was used to determine the purity of HDL using protein apoA-1 as a positive control.

### Isolation of human monocytes

Human monocytes were isolated from buffy coats (containing white blood cells, red blood cells, platelets and plasma) of healthy donors through the Red Cross Blood Bank of Australia. Peripheral blood mononuclear cells were isolated using Ficoll-paque density gradient centrifugation. Human primary monocytes were then purified using MACS Human Pan Monocyte Negative Selection Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The purity of isolated monocytes, assessed using CD14<sup>+</sup>/CD16<sup>+</sup> expression via flow cytometry, was greater than 85%.

### Monocyte to macrophage differentiation and polarization

Freshly isolated monocytes ( $1 \times 10^6$  cells·mL<sup>-1</sup>) were resuspended in culture medium (RPMI 1640 supplemented with 10% FBS, 1% Non-essential amino acids (NEAA), 100 IU·mL<sup>-1</sup> penicillin and 100 g·mL<sup>-1</sup> streptomycin). These cells were incubated for 7 days with or without 400 µg·mL<sup>-1</sup> human HDL, and refreshed every 2 days. To stimulate the differentiation of monocytes to macrophages, 100 ng·mL<sup>-1</sup> of human M-CSF were also added. On day 7, culture medium, without either HDL or M-CSF, was refreshed. LPS (100 ng·mL<sup>-1</sup>) plus IFN-γ (20 ng·mL<sup>-1</sup>) or IL-4 (20 ng·mL<sup>-1</sup>) were then added to induce differentiated primary macrophages (M0) into M1 or M2 phenotypes respectively.

### Bone marrow-derived macrophage isolation, differentiation and polarization

Murine bone marrow-derived macrophages (BMDMs) were obtained from both femur and tibia of WT (wild-type) C57Bl/6 and caveolin-1 knockout (*Cav-1*<sup>-/-</sup>) mice and cultured using a standard protocol similar to human monocytes to macrophage differentiation. Isolated cells were suspended with RPMI 1640 Medium (containing 20% L-cell-conditioned medium, 10% FBS, 50 IU·mL<sup>-1</sup> penicillin G and 50 µg·mL<sup>-1</sup> streptomycin). Cells were then seeded into cell culture plates for 7 days with or without human HDL (400 µg·mL<sup>-1</sup>), refreshed every 2 days. On day 7, medium (without HDL) was refreshed. LPS (100 ng·mL<sup>-1</sup>) plus IFN-γ (20 ng·mL<sup>-1</sup>) were then added to induce macrophages to the M1 phenotype.

### Surface marker expression

Collected macrophages were incubated in either APC-conjugated mouse anti-human CD192 or FITC-conjugated mouse anti-human CD64 to detect M1 macrophage surface markers, or Allophycocyanin (APC)-conjugated mouse anti-human CD206 and FITC-conjugated mouse anti-human CD200R to detect M2 macrophage surface markers for 30 min at 4°C. Cells were washed using centrifugation to get rid of any non-binding antibodies and were then analysed by flow cytometry using BD FACS Calibur (BD Biosciences, San Diego, CA, USA). Ten thousand cells were counted per sample, and the read-out was analysed using Cell Quest Pro software (BD Biosciences). All antibodies mentioned were purchased from BD Biosciences.

### ROS assay

Carboxy-H<sub>2</sub>DCFDA (Invitrogen, Carlsbad, USA) that detects intracellular hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was added 1 h before the cells were harvested to measure ROS production of macrophages. Fluorescent cells were determined by flow cytometry using BD Calibur flow cytometer (BD Biosciences). Again, 10 000 cells were counted per sample, and the read-out was analysed using Cell Quest Pro software (BD Biosciences).

### Real-time PCR

Total RNA was extracted from macrophages using TRIzol (Invitrogen, Carlsbad, CA, USA) and treated with RNase-Free DNase (Promega, Madison, WI, USA) before reverse-transcribing 2 µg of total RNA into cDNA using Moloney-

Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega). Then 100 ng cDNA was used to perform real-time PCR using FastStart Universal SYBR Green Master (Roche Applied Science, Indianapolis, IN, USA) on an ABI Prism 7500 Sequence Detection System (Applied Biosystems) to determine mRNA levels of target genes. Gene expressions were all normalized to 18S expression and assessed using the  $\Delta\Delta CT$  calculation method. Primers used were designed by Roche's ProbeFinder software and synthesized by GeneWorks. Primer sequences are available upon request.

### Cellular cholesterol labelling

BODIPY-Cholesterol (Avanti Polar Lipids, Alabaster, Alabama, USA) that detects cellular cholesterol as previously described (Murphy *et al.*, 2013), was added 1 h before the cells were harvested to measure cellular cholesterol content of macrophages. Flow cytometry was then used to analyse the MFI of fluorescent cells in the 10 000 cells per sample, with the read-out being analysed using Cell Quest Pro software (BD Biosciences).

### Extraction of protein from whole cell and membrane fraction

Whole cells were lysed using whole cell lysis buffer [10 mM of Tris (pH = 7.4), 0.1 M of NaCl, 1 mM of EDTA, 1 mM of EGTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X100, 10% glycerol and protease inhibitors (Roche Applied Science)]. Cell lysates were sonicated for 3 s at 14% intensity and incubated on ice for 30 min before centrifugation at 12 000 g in 4°C for 20 min to obtain supernatants of whole cell protein samples for further analysis. Membrane fractions were prepared via ultracentrifugation. Briefly, cells were first incubated with Buffer A [5 mM Tris-HCl (pH = 7.5) containing protease inhibitors (Roche Applied Science)] at 4°C for 30 min before being placed in -80°C for 15 min. Cells were then thawed on ice and passed through a 25 G needle before centrifugation at 250 g to get rid of the postnuclear supernatant fraction. Supernatants were collected and then spun at 150 000 g for 60 min in 4°C using Beckman Optima MAX-TL ultracentrifuge (Beckman Coulter, Brea, California, USA). Cell pellets were then resuspended using Buffer B [50 mM Tris-HCl (pH = 7.5) containing 2 mM mercaptoethanol, 1% triton X-100 and protease inhibitors (Roche Applied Science)]. All protein concentrations were then measured using Dc Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA).

### Western blot

Protein samples were separated by 10–12% SDS-PAGE gel and transferred onto a PVDF membrane. The membrane was then blocked using 5% fat-free milk and incubated with various primary antibodies (1:1000) overnight in 4°C including β-actin, Sodium Potassium ATPase (Abcam, Cambridge, UK), pSTAT1, pERK1/2, CAV-1, pSTAT3 and STAT3 (Cell Signalling Technology, Danvers, MA, USA). The membrane was then incubated with secondary antibodies (1:4000), including Anti-rabbit IgG (Cell Signalling Technology) or Goat anti-mouse IgG (H + L) – HRP Conjugate Antibody (BioRad Laboratories), and the protein bands were visualized using enhanced luminal reagents (PerkinElmer, Waltham, MA, USA) and quantified using NIH FIJI software.

## Animal experiments

Animals were bred and housed under standard conditions at the AMREP Animal Service Centre. Experimental procedures were approved by the Institutional Animal Ethics Committee in accordance with the Australian NHMRC guidelines. Male/female WT mice (on C57BL6 background) and *Cav-1*<sup>-/-</sup> mice of 10–12 weeks old were used. The *Cav-1*<sup>-/-</sup> mice were kindly provided by Robert Parton (University of Queensland) and were housed and bred at AMREP Animal Service Centre. A total of 39 mice were used in the current study. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

## Statistical analyses

Results are presented as mean ± SEM of *n* experiments. Each experiment was performed in triplicate to reduce error. If one of the triplicate values was anomalous, it was excluded. Triplicates were averaged to obtain one single value per experiment (*n*). Each value (*n* = 1) was then brought forward for statistical analysis. Statistical analyses used in these experiments were either one-way ANOVA followed by a Tukey *post hoc* test, or two-way ANOVA followed by a Tukey *post hoc* test. Values of *P* < 0.05 were deemed to be significant. Tukey *post hoc* tests were carried out only when *F* was significant (*P* < 0.05).

## Results

### HDL suppresses the induction of M1 pro-inflammatory phenotype

It is well established that human macrophages can be induced in an *in vitro* setting to express an M1 pro-inflammatory or M2 pro-resolving phenotype using LPS + IFN- $\gamma$  or IL-4 respectively (Murray *et al.*, 2014). To study the influence of HDL in this process, human monocytes were incubated with HDL during the differentiation and induction protocol. Incubating macrophages with LPS + IFN- $\gamma$  for 24 h significantly increased M1-related cell surface markers CD192 and CD64, while incubation with HDL significantly reduced both markers (Figure 1A,B). Consistent with this, LPS + IFN- $\gamma$ -induced macrophages demonstrated substantially increased gene expression of the M1-related cytokines, TNF- $\alpha$ , MCP-1(CCL2) and IL-6. Likewise, incubation with HDL markedly suppressed these M1-related genes (Figure 1C–E). When macrophages were induced with IL-4, the M2-related surface markers CD206 and CD200R significantly increased. However, incubation with HDL had no effect on either surface markers (Figure 1F,G) or the expression of M2-related genes CD206 and SR-B1 (Figure 1H,I).

### Preventing cell membrane lipid accumulation inhibits M1 polarization

Next, we explored the mechanism by which HDL suppresses the induction of macrophages to the M1 phenotype. Because the key function of HDL is its ability to remove lipids from cells (Tall *et al.*, 2012), we examined whether lipid removal may be important. We used methyl-beta-cyclodextrin (M $\beta$ CD) as a non-specific cholesterol acceptor to explore this pathway. Incubating macrophages with M $\beta$ CD significantly decreased

the expression levels of CD192, similar to levels seen with HDL (Figure 2A). To confirm that HDL and M $\beta$ CD were altering cholesterol content within these macrophages, we used a flow cytometry-based method to assess cellular cholesterol (Murphy *et al.*, 2013). We found that macrophages polarizing to an M1 phenotype had increased cellular cholesterol, while pre-treating cells with HDL and M $\beta$ CD could significantly prevent this increase to an equal degree, potentially through a decrease in cellular membrane cholesterol (Figure 2B). This suggests that preventing cell membrane lipid accumulation plays a significant role in the inhibitory action of HDL in M1 polarization.

### HDL prevents re-distribution of caveolin-1 to the membrane fractions of macrophages

Caveolae are specialized lipid raft domains that form flask-shaped invaginations of plasma membrane by coupling cholesterol with caveolin-1 in the membrane (Schlegel and Lisanti, 2001). Because HDL was demonstrated to decrease M1 induction via decrease in cellular cholesterol content, potentially from the membrane, we explored the role of caveolin-1 in this context. Membrane fractions of macrophages were separated, and abundance of caveolin-1 was assessed using western blot. Consistent with the notion that caveolae are formed when membrane cholesterol/lipid rafts are increased, we also saw an increased abundance of the major caveolae protein caveolin-1 in the membrane fraction of M1 macrophages compared with control (M0) (Figure 3A). Again, consistent with our cholesterol data (Figure 2B), HDL markedly reduced the abundance of membrane caveolin-1 in M1-induced macrophages (Figure 3A). To determine if the reduction in caveolin-1 in the membrane was due to an overall decrease in caveolin-1, we examined total whole cell lysates and found no change amongst the different treatments (Figure 3B), suggesting that caveolin-1 is retained within the cell and not shed. These data indicate that polarization of macrophages to an M1 phenotype favours the formation of caveolae by re-distributing caveolin-1 to the cell membrane and that incubation of these cells with HDL prevents this. Thus, it appears that reduced cellular cholesterol from macrophages inhibits cellular caveolin-1 from localizing to the membrane and in turn prevents the polarization of M1 macrophages.

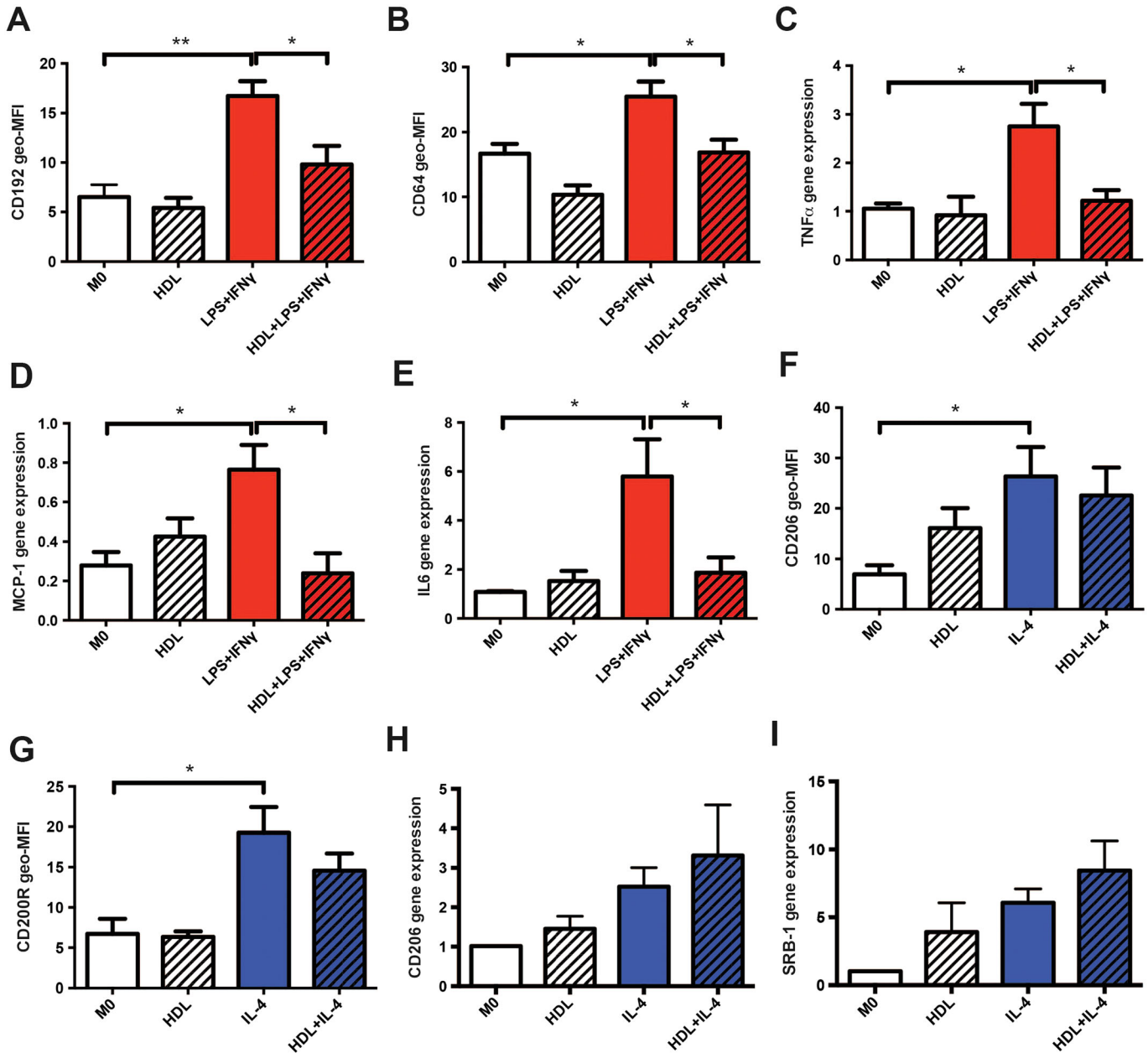
### Caveolin-1 is required for HDL to inhibit M1 macrophage polarization

To determine if HDL requires caveolin-1 to prevent the polarization of macrophages to an M1 phenotype, we cultured BMDMs from WT and *Caveolin-1*<sup>-/-</sup> (*Cav-1*<sup>-/-</sup>) mice. Consistent with our human macrophage data, WT macrophages incubated with HDL showed significant decrease in the expression of M1-related cell surface marker CD64 (Figure 4A) and M1-related genes, *Tnf- $\alpha$*  and *Il-6* (Figure 4B,C). Interestingly, BMDMs from *Cav-1*<sup>-/-</sup> mice failed to respond to HDL, differentiating into M1 macrophages. This along with the data in Figure 3 suggests that caveolin-1 is required for HDL to prevent the polarization of M1 macrophages and to inhibit the expression of inflammatory genes.

### HDL reduces phosphorylation of ERK1/2 via caveolin-1 in macrophages

Next, we examined how caveolin-1 could be involved in regulating the expression of inflammatory genes. There is an



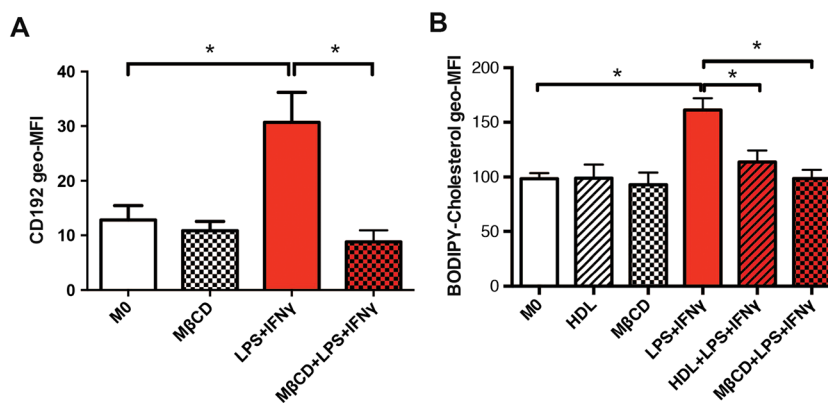


**Figure 1**

HDL reduces M1 macrophage polarization. Isolated monocytes were differentiated for 7 days to macrophages with or without HDL (400  $\mu\text{g}\cdot\text{mL}^{-1}$ ). They were then either polarized into an M1 or M2 phenotype using LPS + IFN- $\gamma$  or IL-4 respectively. Cell surface markers were determined using flow cytometry (A, B, F and G) and mRNA expressions were measured using RT-PCR (C, D, E, H and I). Data are presented as mean  $\pm$  SEM of 3–6 individual donors, each performed in triplicate and analysed using one-way ANOVA followed by Tukey's multiple comparisons test (\* $P < 0.05$ , \*\* $P < 0.01$ ).

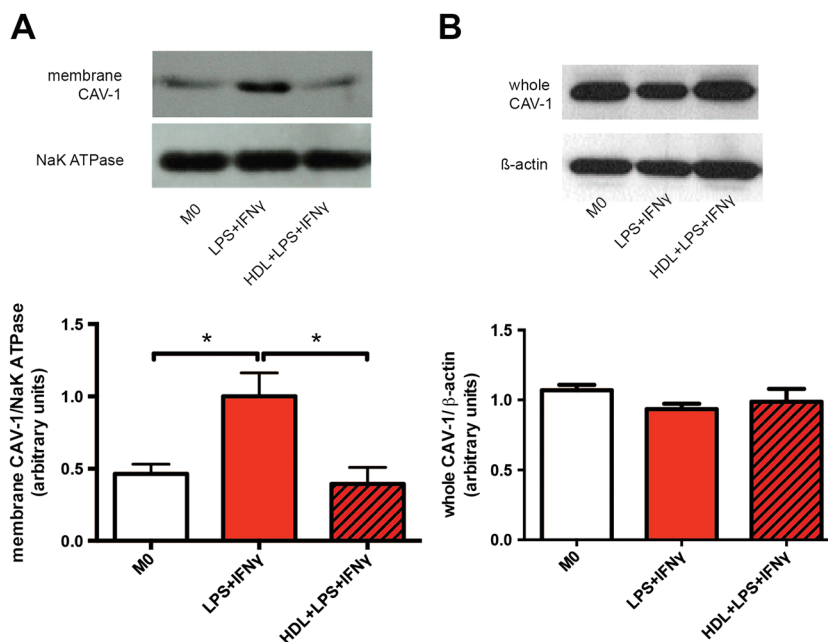
accumulation of evidence to suggest that caveolin-1 is important in mediating anti-inflammatory effects in murine macrophages through signalling pathways such as ERK1/2 (Medina *et al.*, 2006; Wang *et al.*, 2006). Therefore, to determine if HDL mediates its anti-inflammatory effects via ERK1/2 and if this requires caveolin-1, we examined ERK1/2 phosphorylation in WT and *Cav1*<sup>-/-</sup> BMDMs. Here, we discovered that HDL inhibited the phosphorylation of ERK1/2 in WT macrophages, consistent with inhibition of inflammatory

surface marker and gene expression. However, incubation of *Cav1*<sup>-/-</sup> macrophages with HDL failed to perturb the phosphorylation of ERK1/2 in response to LPS + IFN- $\gamma$  (Figure 5A). Evidence also indicates a role for caveolin-1 to interact or interfere with STAT3 to dampen the expression of key inflammatory genes (Shah *et al.*, 2002; Yuan *et al.*, 2011). However, we found that LPS + IFN- $\gamma$  did not influence the phosphorylation of STAT3 in WT or *Cav1*<sup>-/-</sup> BMDMs, nor did HDL affect STAT3 phosphorylation in WT BMDMs (Figure 5B).



**Figure 2**

Preventing cell membrane lipid accumulation inhibits M1 polarization. Following a 1 h pretreatment with MβCD (5 mM), 7 day-differentiated macrophages were then stimulated towards an M1 phenotype using LPS + IFN-γ for 24 h. The CD192 inflammatory cell surface marker (A) and BODIPY-cholesterol (B) were measured using flow cytometry. Data are presented as mean ± SEM of 5–9 individual donors, each performed in triplicate and analysed using one-way ANOVA followed by Tukey's multiple comparisons test (\**P* < 0.05).



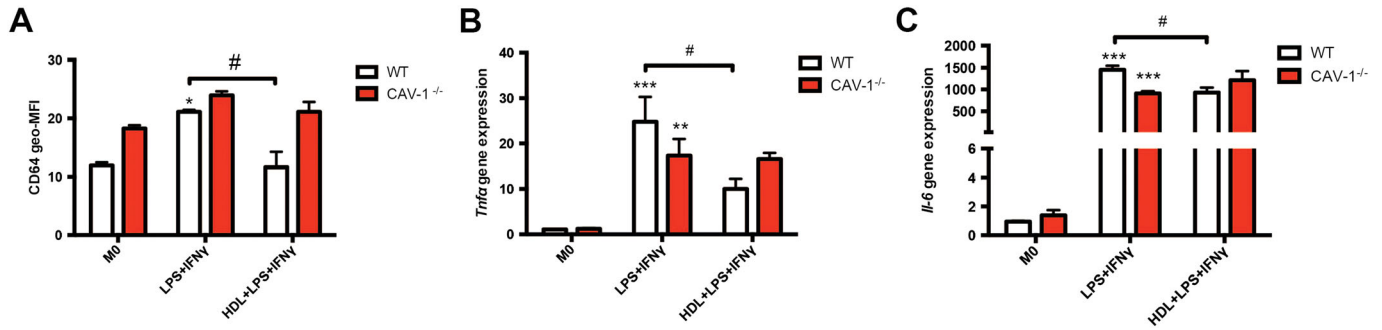
**Figure 3**

HDL prevents re-distribution of caveolin-1 to the membrane fractions of macrophages. Macrophages were pretreated with or without HDL (400 μg·mL<sup>-1</sup>) and induced into an M1 phenotype for 24 h. Protein of membrane (A) and whole cell (B) fractions were isolated from cells, run on a 12% SDS-PAGE gel and analysed using western blots. Na/K ATPase was used as a membrane loading control. Data are presented as a mean ± SEM of four individual donors, each performed in triplicate, and were analysed using one-way ANOVA followed by Tukey's multiple comparisons test (\**P* < 0.05).

### *HDL reduces phosphorylation of ERK1/2 and STAT3 in human macrophages*

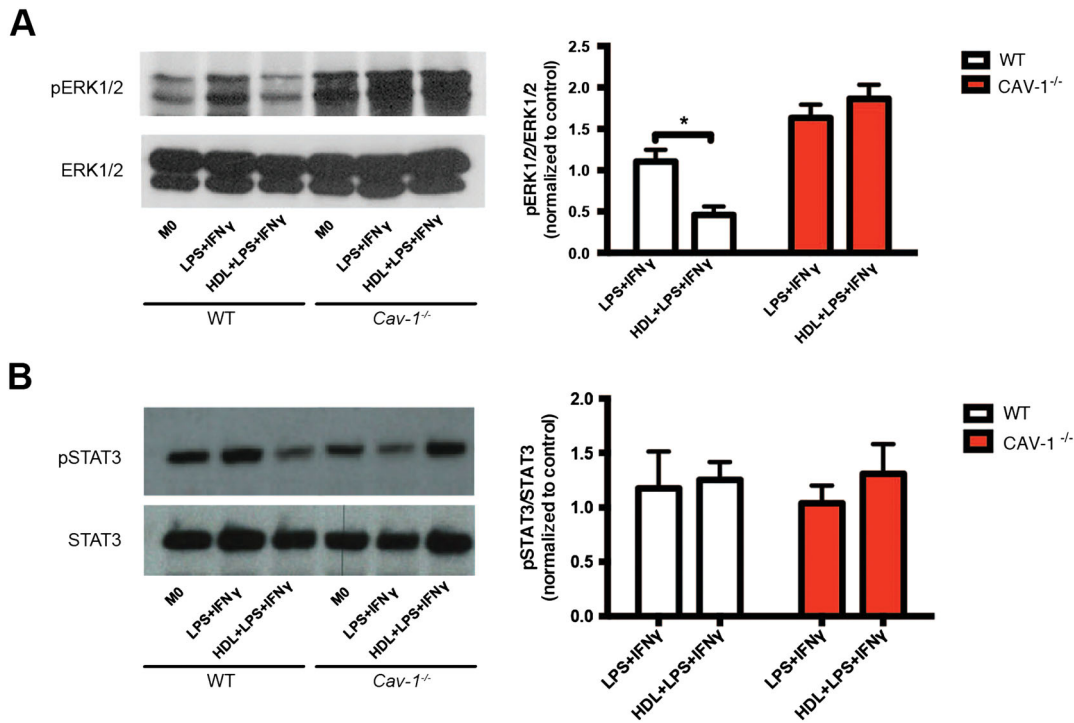
Our data in mice (Figure 5A) clearly shows a role for HDL in suppressing ERK1/2 signalling in macrophages. Thus, we aimed to determine if this was also true in human macrophages. As expected, when M0 human macrophages were polarized with LPS + IFN-γ, we observed a robust increase in ERK1/2 phosphorylation. HDL incubation significantly

decreased ERK1/2 phosphorylation recapitulating our results in mice (Figure 6A). We did also find a statistically significant increase in STAT3 phosphorylation upon LPS + IFN-γ induction, albeit to a lesser degree than pERK1/2, and this phosphorylation of STAT3 was prevented by HDL pre-treatment (Figure 6B). Thus, it is possible that STAT3 may be more important in human macrophage polarization and also suggests that pERK1/2 is likely the major pathway that HDL is



**Figure 4**

Caveolin-1 is required to reduce M1 macrophage polarization. WT and *Cav-1*<sup>-/-</sup> mice were killed when they were 10–12 weeks-old, and BMDMs were isolated. BMDMs were then cultured for 7 days with or without 400  $\mu\text{g}\cdot\text{mL}^{-1}$  of HDL before they were stimulated towards an M1 phenotype. The inflammatory M1-related cell surface marker CD64 (A) was measured using flow cytometry. Gene expressions of *Tnf- $\alpha$*  (B) and *Il-6* (C) were determined using RT-PCR. Data are presented as mean  $\pm$  SEM of 5–6 independent experiments, each performed in triplicate (two-way ANOVA followed by Tukey's multiple comparisons test, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared with M0 control group, #  $P < 0.05$ ).



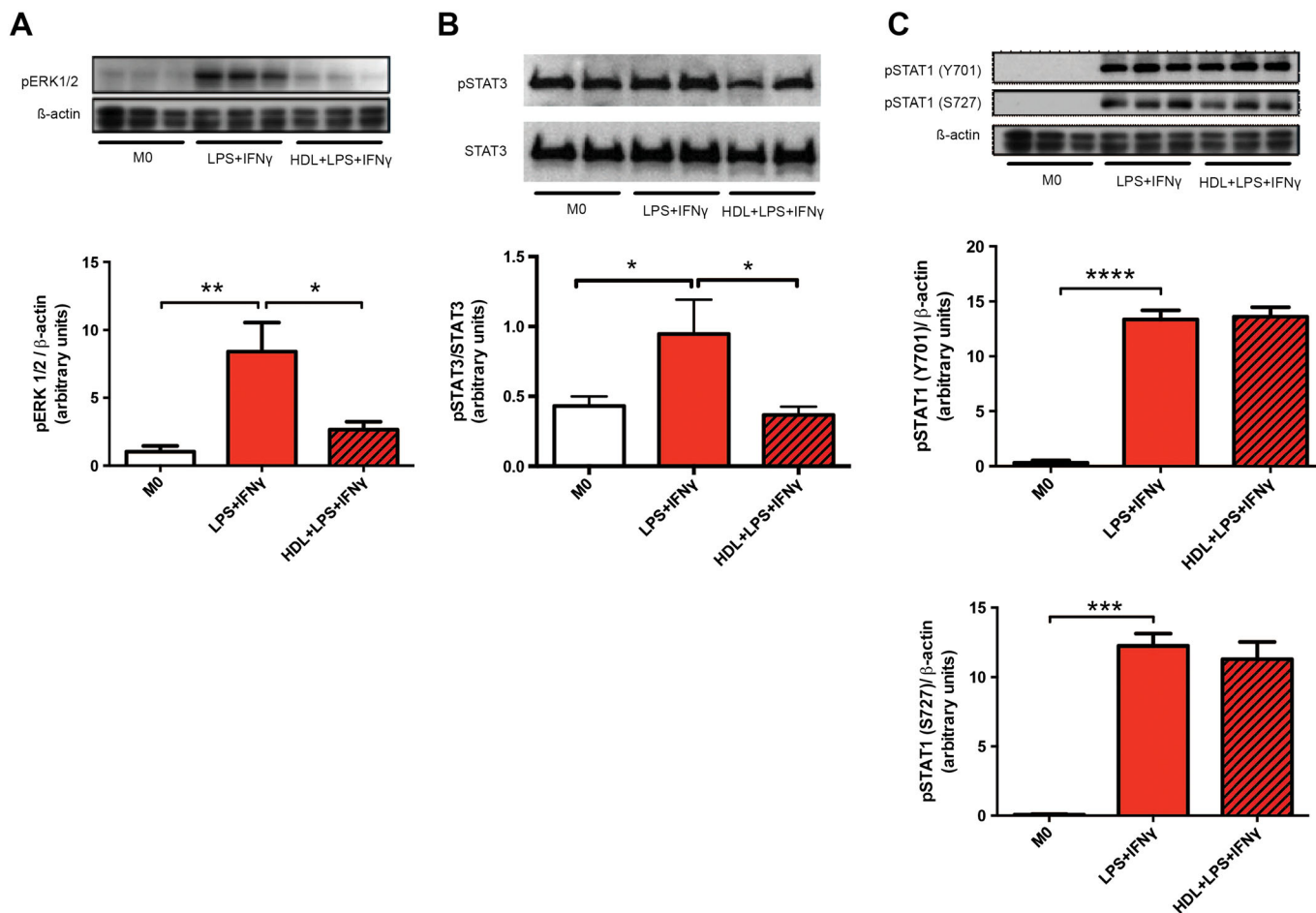
**Figure 5**

HDL mediated anti-inflammatory effects on ERK1/2, but not STAT3, via caveolin-1. WT and *Cav-1*<sup>-/-</sup> mice were killed when they were 10–12 weeks-old, and BMDMs were isolated. BMDMs were then cultured for 7 days with or without 400  $\mu\text{g}\cdot\text{mL}^{-1}$  of HDL before they were stimulated for 10 min with LPS + IFN- $\gamma$ ; they were then harvested for western blot analysis to detect the phosphorylation of ERK1/2 (A) and STAT3 (B). Data are presented as mean  $\pm$  SEM of 6–8 experiments, each performed in triplicate and normalized to M0 control values (one-way ANOVA followed by Tukey's multiple comparisons test, \*  $P < 0.05$ ).

inhibiting. We also examined the effect of STAT1, which can also play a role in the IFN- $\gamma$  induction of macrophages to an M1 phenotype (Ramana *et al.*, 2002). While we observed an increase in STAT1 phosphorylation at Y701 and S727, HDL failed to inhibit this event, suggesting that HDL does not inhibit M1 polarization via the STAT1 pathway (Figure 6C).

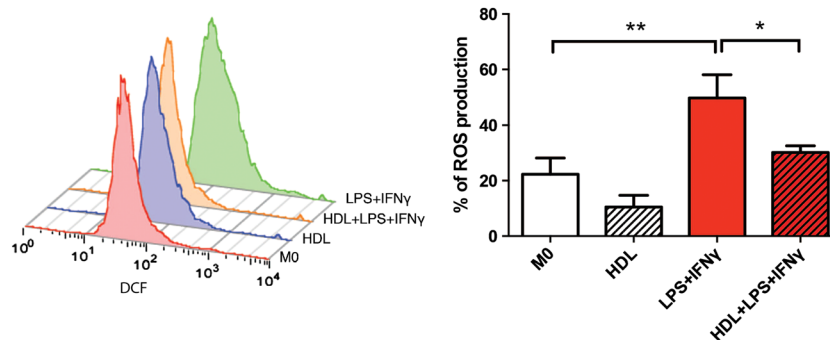
### HDL reduces ROS production of M1 macrophages

To determine if the decrease in M1 polarization as measured by cell surface and gene expression changes its function, we used carboxy-H2CDFDA to detect ROS production via flow cytometry as macrophages produce ROS in order to neutralize



**Figure 6**

HDL reduces the phosphorylation of ERK1/2 and STAT3, but not STAT1 of M1-induced macrophages. Seven day differentiated macrophages were pretreated with or without HDL before a 5 min (A) or 10 min (B and C) stimulation with LPS + IFN- $\gamma$ . pERK1/2, pSTAT3 and pSTAT1 were then measured using western blot analysis. Data are presented as mean  $\pm$  SEM of 3–4 individual donors, each performed in triplicate (one-way ANOVA followed by Tukey’s multiple comparisons test, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ ).



**Figure 7**

HDL reduces ROS production in M1 macrophages. After monocytes were differentiated to macrophages for 7 days in the presence or absence of HDL (400  $\mu\text{g}\cdot\text{mL}^{-1}$ ), macrophages were stimulated towards an M1 phenotype for 24 h. ROS production was measured using flow cytometry. Data presented as mean  $\pm$  SEM of seven individual donors, each performed in triplicate and analysed using one-way ANOVA followed by Tukey’s multiple comparisons test (\* $P < 0.05$ , \*\* $P < 0.01$ ).

pathogens (Mokgobu *et al.*, 2015). Stimulating macrophages towards an M1 phenotype with LPS + IFN- $\gamma$  demonstrated a greater capacity to produce ROS compared with basal

levels observed in M0 macrophages. When pre-treated with HDL, ROS production of M1 macrophages was significantly attenuated (Figure 7).



## Discussion and conclusions

In the present study, we demonstrated using flow cytometry, RT-PCR and functional assay of ROS production that HDL prevents human monocytes from differentiating into an M1 pro-inflammatory phenotype while having no effect on the M2 phenotype. We also show that caveolin-1 is critical for this effect of HDL and that this effect appears to be mediated through cell membrane lipid removal preventing the abundance of caveolin-1 at the cell membrane. This event is linked to a marked reduction in the phosphorylation of inflammatory ERK1/2 and STAT3 signalling pathways in M1-induced macrophages.

An inverse relationship between HDL-C and the risk of CVD events has long been well established (Castelli *et al.*, 1977; Gordon *et al.*, 1977). HDL exerts many atheroprotective effects ranging from inhibiting adhesion molecule expression on the endothelium to inhibiting the oxidation of LDL in the subendothelial space, as well as transporting cholesterol from cholesterol-laden peripheral tissues such as lipidated macrophages back into the liver for excretion (Tsompanidi *et al.*, 2010). Our findings identify yet another anti-inflammatory role of HDL, that is, that of suppressing inflammatory macrophage phenotypes. This is consistent with the recent publication by De Nardo *et al.* (2014) uncovering an anti-inflammatory role of HDL to down-regulate toll-like receptor-induced inflammatory cytokines in macrophages via activation of the ATF3 transcriptional regulator. In addition to the study, Feig *et al.* (2011) and Sanson *et al.* (2013) also demonstrate the ability of HDL to inhibit M1-related inflammatory genes in murine macrophages, however, both studies differ in that an up-regulation in M2-related genes observed in IL-4-induced murine macrophages was not observed in our human macrophages. As well, our data align with the recent study by Colin *et al.* (2014) where again, contrary to murine macrophages, HDL was not observed to influence human M2 macrophage polarization (Colin *et al.*, 2014).

The ability of HDL to prevent macrophages from being induced into an M1 phenotype provides further insight into the potential anti-atherosclerotic role of HDL in retarding plaque progression. Using immunological techniques, an abundance of M1 macrophages has been located in plaque lipid core, whereas M2 macrophages are predominantly situated in the stable cell-enriched regions of the atherosclerotic plaque away from the lipid core (Chinetti-Gbaguidi *et al.*, 2011). Furthermore, studies looking at plaque size and composition have found that administering native HDL significantly increases smooth muscle cell proliferation as well as decreasing MMP-9 expression, suggesting that the capacity of HDL to inhibit M1 macrophages from releasing inflammatory cytokines may result in a more stable plaque by attenuating fibrous cap thinning (Nicholls *et al.*, 2005).

Monocyte-derived macrophages to foam cell formation is a critical step in the formation of atherosclerotic lesions (Dushkin, 2012). Studies looking at cholesterol content within macrophages in lesions have noted a greater accumulation of lipids and bigger lipid droplets within M1 macrophages than M2 macrophages, further contributing to the burden of foam cells (Chinetti-Gbaguidi *et al.*, 2011) and highlighting the importance of reducing cholesterol level within M1 macrophages as well as macrophage number.

Interestingly, M1 macrophages appear to have better cholesterol transport handling than M2 macrophages due to an up-regulation of the cholesterol transporter genes ABCA1 and ABCG1 (Chinetti-Gbaguidi *et al.*, 2011). It is thus not unexpected that the removal of cellular cholesterol, potentially by modulating cell membrane levels using M $\beta$ CD or HDL in the current experiment effectively reduces the induction of macrophages to the M1 phenotype.

Interestingly, our data revealed an important and previously uncharacterized role for caveolin-1 in mediating the effects of HDL. Caveolin-1 is a structural protein that binds to membrane cholesterol, forming structural invaginations of the membrane known as caveolae (Simons and Toomre, 2000). Caveolin-1 is involved in many cellular processes such as lipid regulation, pathway signalling and also recently being reported to play a part in the development of atherosclerosis and inflammation (Gargalovic and Dory, 2003). Our data showed that membrane caveolin-1 was reduced by HDL but remained inside the cell, suggesting re-distribution within the cell. Many studies have suggested both pro-atherosclerotic and anti-atherosclerotic effects of caveolin-1, depending on their cell type and disease state (Pavlidis *et al.*, 2012). We have previously shown the critical role of caveolin-1 in the differentiation of monocytes to macrophages (Fu *et al.*, 2012). We now show that knocking out caveolin-1 diminished the effect of HDL on M1 induction, a finding consistent with reports by Wang *et al.* (2006) and Yuan *et al.* (2011) pointing towards an anti-inflammatory effect of caveolin-1 in macrophages (Wang *et al.*, 2006; Yuan *et al.*, 2011).

Further downstream, HDL was observed to prevent M1 macrophage polarization via ERK1/2 and STAT3, but not STAT1, signalling pathways. We found that ERK1/2 phosphorylation can be activated upon M1-induction and inhibited by HDL in both human and murine macrophages. Critically, ERK1/2 has been shown to be associated with caveolin-1 (Cohen *et al.*, 2003; Medina *et al.*, 2006). Therefore, our data suggest that HDL-induced, cholesterol efflux-associated, re-distribution of caveolin-1 prevents ERK1/2 phosphorylation and thus inhibits the expression of inflammatory M1-macrophage-associated genes. STAT3 has been previously shown to be induced upon inflammatory stimulation of macrophages with inflammatory mediators (Guo *et al.*, 2014; Vasamsetti *et al.*, 2015). Although in our hands, while this was true in human macrophages, we failed to see a robust increase of phosphorylation of STAT3 in murine BMDMs. This may reflect differences between human and mouse; however, this discrepancy between species also revealed a common pathway through ERK 1/2 that was suppressed in both mouse and human macrophages by HDL. Furthermore, previous studies show that IFN- $\gamma$  stimulation induces M1 polarization via STAT1 phosphorylation (Tugal *et al.*, 2013). Our data, while consistent with this, also clearly show that this is not the mechanism by which HDL acts.

Asides from examining M1 phenotype using cell surface markers and gene expression of M1-associated cytokines, the effect of HDL on the functional capacity of LPS + IFN- $\gamma$ -induced M1 macrophages was examined. Our data confirms an increase in ROS generation in M1 macrophages (Mokgobu *et al.*, 2015) where it is likely to trigger activation of the NF $\kappa$ B pathway, and in turn increase inflammatory cytokines expression (Fenyo and Gafencu, 2013). Furthermore, in the

setting of atherosclerosis, oxidative modification of LDLs induced by ROS results in an increase in foam cell formation (Yla-Herttuala, 1991). The anti-oxidative properties of HDL in the current study are thus consistent with its potential anti-atherosclerotic role.

In summary, we demonstrated that HDL prevents the induction of human macrophages into an M1 phenotype by preventing the accumulation of caveolin-1 to the cell membrane. These results provide further insights into the cardio-protective role of HDL, which may aid in atherosclerotic lesion regression/resolution and suggest that therapies targeted to redistribute membrane caveolin-1 in immune cells may be a novel therapeutic angle for dampening inflammation.

## Acknowledgements

M. K. S. L. and A. A-S. were supported by the Bright Sparks Program from Baker IDI Heart & Diabetes Institute Australia. A. J. M. was supported by a Career Development fellowship from the National Health and Medical Research Council (NHMRC), a future leader fellowship from the National Heart Foundation, and a Viertel award from Diabetes Australia Research Trust Australia. This work was also supported by an NHMRC programme grant (APP10363652) and by the Victorian Government's Operational Infrastructure Support (OIS) programme.

## Author contributions

M. K. S. L. designed and performed the experiments, analysed the data and wrote the manuscript. X. L. M. designed the experiments and analysed in part the data in the study. Y. F. designed and performed in part the experiments and analysed in part the data in the study. A. A-S. performed in part experiments in the study. D. D. helped perform in part the experiments in the study. M. A. F-R. created this line of C57/Black 6-backcrossed caveolin-1 knockout mice. R. P. provided intellectual input. D. S. provided intellectual input. A. J. M. helped design the experiments and wrote in part and finalized the manuscript. J. P. F. C-D. conceptualized the study and finalized the manuscript.

## Conflict of interest

Authors declare that they have no conflict of interest.

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