Accumulation of serum lipids by vascular smooth muscle cells involves a macropinocytosis-like uptake pathway and is associated with down-regulation of ATP-binding cassette transporter A1

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ABSTRACT

Purpose: Vascular smooth muscle cells (VSMC) are present in arterial intima before atherosclerotic plaques develop and are likely to be exposed to unmodified serum lipids as they enter the vessel wall. We examined the effects of sera from mice on morphology and function of mouse VSMC. **Methods & Results:** Incubation of a mouse VSMC line (MOVAS) with sera from normocholesterolemic (C57Bl6/J) or hypercholesterolemic (APOE$^{-/-}$) mice caused concentration-dependent increases in lipid accumulation as measured by AdipoRed, with the extent of lipid uptake significantly greater with the latter sera type. Inhibition of c-Jun N-terminal kinases (SP600125), Src kinases (AG1879) and clathrin-dependent endocytosis (monodansylcadaverine) to disrupt scavenger receptor-mediated uptake of lipids, had no effect on serum-induced lipid accumulation by VSMC. By contrast, inhibition of macropinocytosis with antagonists of PI-3 kinase (LY294002) and actin (cytochalasin D) markedly reduced lipid accumulation. Serum exposure reduced expression of ATP-binding cassette transporter A1 (ABCA1), consistent with impaired cholesterol efflux, but had no effect on expression of markers of VSMC differentiation. Moreover, expression of several inflammation and foam cell markers were unchanged (CCL2, CCL5, CD68) by mouse sera. **Conclusion:** Accumulation of unmodified serum lipids by VSMC involves a macropinocytosis-like uptake pathway and is associated with down-regulation of ATP-binding cassette transporter. We speculate that VSMC may play an atheroprotective role in arterial intima by acting as a 'sink' for unmodified lipids.

Key words: Serum lipids; vascular smooth muscle cells; foam cells; macropinocytosis; ATP-binding cassette transporter A1

INTRODUCTION

The 'response to retention' hypothesis posits that accumulation of unmodified serum lipoproteins in the sub-endothelial intima is an initiating event in atherosclerotic plaque formation (Williams and Tabas, 1995, Tabas et al., 2007). The lipoproteins are then thought to associate with extracellular matrix (ECM) proteoglycans via ionic interactions causing them to be retained within the intima, and also making them more susceptible to oxidation or enzymatic modification (Camejo et al., 1998, Chait and Wight, 2000). Such modifications convert lipoproteins from relatively innocuous species, into those that promote endothelial activation and chemoattraction of inflammatory cells such as macrophages and T cells (Tabas et al., 2007). Oxidized lipoproteins are also high affinity ligands for macrophage scavenger receptors such as CD36, type I (SR-A1) and type II (SR-A2) class A scavenger receptors, and lectin-like oxidized LDL receptor-1 (LOX-1), and are thus avidly taken up by these cells (Webb and Moore, 2007). Excessive uptake of lipids by macrophages causes their transformation into foam cells, the major constituents of fatty streaks (Webb and Moore, 2007). Furthermore, owing to their pro-inflammatory properties and propensity to undergo apoptosis, foam cells contribute to the ongoing development of more complex atherosclerotic lesions and their ultimate rupture (Bobryshev, 2006).

Although the majority of foam cells within atherosclerotic plaques are derived from macrophages, immunohistochemical studies on lesions from humans and non-human primates indicates that at least some of the lipid-laden cells are vascular smooth muscle cells (VSMC) (Takebayashi et al., 1972, Goldfischer et al., 1975, Stary et al., 1994). VSMC express several cholesterol uptake receptors including the low density lipoprotein (LDL) receptor, CD36, SR-A1, SR-A2, and CXCL16/SR-PSOX (Matsumoto et al., 2000, Wagsater et al., 2004, Lim et al., 2006, Ruan et al., 2006). Moreover, exposure of VSMC to inflammatory cytokines such as interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α)

and interferon gamma (IFN-γ), causes upregulation of these receptors and increased uptake of LDL that has been modified by acetylation, oxidation or enzymatically (Li et al., 1995, Wagsater et al., 2004, Ruan et al., 2006). This causes VSMC to assume a foam cell-like morphology characterised by the presence of lipid-filled vacuoles, down-regulation of VSMC differentiation markers, upregulation of macrophage markers, and increased production of pro-inflammatory cytokines (Rong et al., 2003, Barlic et al., 2007, Sima et al., 2010, Sun and Chen, 2010).

Diffuse intimal thickening (DIT) is evident in human arteries before the appearance of atherosclerotic plaques (Stary et al., 1992, Nakashima et al., 2002). Vascular smooth muscle cells (VSMC) are the major cellular constituents of DIT (Stary et al., 1992, Nakashima et al., 2002) and are thus likely to be exposed to serum lipoproteins that enter the vessel wall before they undergo modification. However, to our knowledge, no studies have examined the impact of exposure of VSMC to unmodified serum lipoproteins. Hence, we examined the effect of exposure of mouse VSMC to unmodified lipoproteins present in mouse serum in the belief that these studies may provide insights into the events that lead to the initiation of plaque formation. We demonstrate that VSMC in culture have a high capacity to take up and store the lipids that are present in sera from both normocholesterolemic (C57Bl6/J) and hypercholesterolemic (APOE$^{-/-}$) mice. However, unlike previous studies in which uptake of modified LDL was shown to be dependent on the activity of scavenger receptors and to cause induction of a pro-inflammatory, macrophage-like phenotype, we show that accumulation of unmodified serum lipids is the result of a macropinocytosis-like uptake pathway and is associated with the down-regulation of the cholesterol efflux transporter, ATP-binding cassette transporter A1 (ABCA1). Importantly, accumulation of serum lipids was not associated with a loss of VSMC differentiation markers or increased expression of pro-inflammatory cytokines. Based on these observations we propose that the VSMC may act as

a sink for unmodified serum lipoproteins. We further speculate that this action of VSMC within DIT and early atherosclerotic lesions may serve an atheroprotective function by preventing the interaction of lipoproteins with ECM proteoglycans and thereby limiting their oxidation.

MATERIALS AND METHODS

Cell culture

MOVAS (ATCC®^{CRL-2797})(Afroze et al., 2003) is a continuous mouse aortic vascular smooth muscle cell line that has been demonstrated to retain a VSMC-like phenotype, including a spindle cell morphology and the expression of VSMC-specific markers such as smooth-muscle α-actin and SM22-α. MOVAS were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated foetal calf serum (FCS) and 2 mM L-Glutamine. For experiments, cells were seeded into white 96-well Viewplates™ or clear 24-well plates as appropriate.

Sera collection

Mouse sera used in this study were obtained from male, C57BL6/J wild type (WT) or apolipoprotein E-deficient (APOE$^{-/-}$) mice (all aged 8-12 weeks) that had been fed a normal chow or Western-style diet (21% fat, 0.22% cholesterol), respectively, from 5 weeks-of-age (Specialty Feeds; Australia). FCS was obtained commercially (JRH Biosciences). For serum collection, mice were euthanized using isofluorane inhalation anaesthetic (Baxtor Healthcare, Australia). Whole blood was collected from the inferior vena cava using a sterile 26-gauge hypodermic needle (Terumo, Australia) and 1 mL Tuberculin syringe (Terumo, Australia) before being transferred into a sterile 1.5 mL microcentrifuge tube. Blood was left to clot for 45 min at room temperature before centrifugation at 16,100 x g for 8 min at 4°C. The serum was collected, transferred into a fresh sterile microcentrifuge tube and stored at -80°C until use. All sera used for experiments were non-heat inactivated.

Cell treatments

One day after seeding the VSMC, the conventional cell culture media (i.e. containing 10 heat-inactivated FCS) was replaced with an equivalent volume of media containing either non heat inactivated FCS, WT or APOE$^{-/-}$ mouse serum (10-50%). Cells were then incubated for up to 48 h. In some experiments, cells were exposed to drugs known to disrupt scavenger receptor- and macropinocytosis-dependent endocytotic pathways for the 3 h prior to, and the 48 h during exposure to serum. These included: amiloride (3,5-diamino-6-chloro-N-(diaminomethylene)pyrazine-2-carboxamide; 0.1–3.0 mmol/L) (Garcia-Perez et al., 2008); 5-(N-Ethyl-N-isopropyl) amiloride (10–300 µmol/L) (Martinez-Argudo and Jepson, 2008); LY294002 (2-morpholin-4-yl-8-phenylchromen-4-one; 100 µmol/L) (Kruth et al., 2005, Yao et al., 2009); cytochalasin D (1 µmol/L) (Kruth et al., 2005); monodansylcadaverine (10 µmol/L) (Jones and Willingham, 1999, Coller and Paulnock, 2001); SP600125 (1,9-Pyrazoloanthrone; 10 µmol/L) (Rahaman et al., 2006) and AG1879 (4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; 10 µmol/L) (Rahaman et al., 2006). Dimethyl sulfoxide (0.1%) was the vehicle in each case. To examine whether PDGF in mouse sera stimulated lipid uptake by VSMC, WT and APOE$^{-/-}$ mouse sera (20%) were incubated with a neutralizing anti-PDGF antibody (40µg/ml; Millipore) for 1 hour at 37°C, before treatment of cells for 48 h.

Oil red O staining

At time of assay, the cell culture medium was removed and VSMC were fixed with isopropyl alcohol. Cells were stained with oil red O (0.5% in 60% isopropyl alcohol) for 30 min and then de-stained with isopropyl alcohol. Cells were visualised at x20 magnification under bright field on an inverted microscope (PALM MicroBeam Carl Zeiss MicroImaging GmbH,

Germany). Images were captured by a digital camera under the control of PALM

RoboSoftware v3.1-0106.

Quantitation of VSMC lipid accumulation

Lipid accumulation was measured in VSMC plated in 96-well Viewplates^{TM} using AdipoRed™ assay reagent (Lonza, Basel, Switzerland), according to the manufacturer's instructions. After incubation of VSMC with FCS or mouse serum for 48 h, the cell culture medium was removed and the cells were rinsed with PBS. A volume of 200 μL of PBS was added to each well, followed by 5 μL of the AdipoRed™ assay reagent. Each plate was incubated at room temperature for 15 min and loaded into a Hidex Chameleon Plate Reader for detection of fluorescence at wavelengths 485 nm excitation and 570-90 nm emission. Background was subtracted from relative fluorescence units (RFUs) obtained and calculated as a % relative to a control.

Cell viability

Cell viability after serum treatment was assessed using CellTiter 96® AQueous One Solution Reagent (Promega, Madison, WI, USA). The reagent was diluted in PBS at a 1:5 ratio, added to each well and cells were incubated for 1 h in a humidified, 37°C, 5% CO_2 incubator. Formazan production by cells, which is directly proportional to the number of viable cells, was determined by measuring absorbance at 485 nm.

Serum lipoprotein sub-fractionation and analysis

FCS and mouse sera (stored at 4°C) were diluted (1:5) in running buffer (20mM sodium phosphate buffer, pH 7.8) and subjected to fast protein liquid chromatography (FPLC) (BioLogic DuoFlow, BioRad) using a Superdex 200, 10/300 GL column (GE Healthcare, UK). Plasma proteins were eluted by FPLC at a flow rate of 0.25 mL/min and monitored by measuring absorbance at 280 nm.

C. pneumoniae **infection of VSMC cells**

VSMC were infected with 10^4-10^6 inclusion forming units (IFU) of *C. pneumoniae* isolate A-03 (kindly provided by P. Timms; Queensland University of Technology, Australia) or a mock-inoculum, 1 h prior to the commencement of treatment with mouse serum.

Time-lapse video phase contrast microscopy

One day after seeding, cells in a 24-well plate (Nunc, Denmark) were treated with either non-heat inactivated FCS or mouse serum (10% and 50%) diluted in DMEM. After treatment, the plate was immediately placed on the stage of an inverted Leica AF6000 LX Live Cell Imaging Workstation (Leica Microsystems, Germany). Images were captured at 30 min intervals over a period of 48 h at 400x magnification, while the microscope stage was maintained under conventional cell culture conditions in a temperature- and CO_2- controlled chamber (37°C, 5% CO_2). Video images were collected and assembled using the Leica Application Suite AF Software (version 2.0).

Quantitative real-time PCR

Total RNA was harvested from cells using the RNeasy Mini Kit, and converted to cDNA with the Quantitect Reverse Transcription Kit (all from Qiagen, Germany), as per the manufacturer's instructions. Quantitative real-time PCR was performed using the following pre-designed TaqMan® gene expression assays (Applied Biosystems, USA): smooth muscle α-actin (ACTA2; Mm01546133_m1), ABCA1 (Mm00442663_m1), MCP-1 (CCL2; Mm00441242), RANTES (CCL5; Mm01302428_m1), CD36 (Mm01135198_m1), CD68 (Mm03047343_m1), Calponin H1 (CNN1; Mm00487032_m1), Mac-2 (Mm00802901_m1), SR-A1 (MSR1; Mm00446214_m1) and β-actin (4352341E). All real-time PCR reactions (20 µL) were prepared using diluted cDNA representative of RNA amounts (10-50 ng), 10 µL of

2x Taqman® Universal PCR master mix (Applied Biosystems) and 1 µL of the relevant 20x Taqman® Primer/Probe set. Detection was performed using the CFX 96™ real-time PCR machine (Bio-Rad, Hercules, Germany) using the following thermocycling program: 2 min at 50 °C for optimal Amp® Urase actvitiy, initial denaturation 10 min at 95 °C followed by 40 cycles of denaturation 15 s at 95°C and annealing and extension for 1 min at 60°C. All data were normalized to a house-keeping gene (β-actin) and expressed as fold-changes relative to a control, using the formula $2^{-\Delta\Delta CT}$.

Drugs and their sources

AG1879, amiloride, cytochalasin D, DMSO, EIPA, LY294002, monodansylcadaverine, SP600125 and oil Red O were all from Sigma Aldrich. The neutralizing anti-PDGF antibody was from Millipore.

Statistical analysis

All statistical analyses were conducted using GraphPad Prism software (version 5.03, GraphPad, USA). Where two experimental groups were compared, data were analysed by Student's unpaired t test. For comparisons across three or more treatment groups either one-way ANOVA followed by Dunnet's post-test, or two-way ANOVAs followed by Bonferroni post-test, was used. In all cases, $P<0.05$ was considered significant. N values represent numbers of replicates performed using different cell passages and sera from different mice.

RESULTS

Serum from mice induces lipid accumulation in mouse VSMC

When maintained under our conventional cell culture conditions (i.e. 10% heat-inactivated FCS in DMEM), murine VSMC displayed an epithelioid appearance, more reminiscent of intimal VSMC than the spindle-shaped VSMC of the medial layer of the vessel wall (Figure 1A). Incubation of VSMC with serum from either normocholesterolemic (WT) or hypercholesterolemic (APOE$^{-/-}$) mice for 48 h was associated with profound changes in morphology, most notably the appearance of intracellular vacuoles which stained positively for oil red O, indicating the presence of triglycerides and potentially other lipid species (Figure 1A; see also time-lapse phase contrast videos in Online Supplement Figure S1). Quantification of lipid accumulation with AdipoRed confirmed the above findings (Figure 1B). Cells exposed to increasing concentrations of FCS displayed minimal lipid uptake (Figure 1B). By contrast, exposure to serum from WT or APOE$^{-/-}$ mice caused a concentration-dependent increase in lipid accumulation in VSMC. Of note, the lipid content of VSMC exposed to serum from APOE$^{-/-}$ mice was greater than two-fold higher than that in cells exposed to WT serum at all concentrations tested (Figure 1B).

Reflecting the lipid uptake profiles described above, FPLC analysis demonstrated that whereas FCS was more or less devoid or serum lipids, serum from WT and APOE$^{-/-}$ mice contained appreciable amounts of LDL and HDL (Figure 1C). In addition, APOE$^{-/-}$ sera contained a high content of VLDL (Figure 1C). Finally, lipid accumulation and the change in cell morphology following exposure to the different sera did not have a significant impact on cell numbers/viability as revealed by MTS staining (Figure 1D).

Effect of pharmacological inhibitors of scavenger receptor- and macropinocytosis-dependent endocytosis on serum-induced lipid uptake in VSMC

Previously it has been shown that uptake of modified LDL by macrophage CD36 and SR-A scavenger receptors, and the subsequent transformation of macrophages into foam cells, is dependent on signalling pathways involving Src-family tyrosine kinases and/or the c-Jun N-terminal kinases, JNK1 and JNK2 (Ricci et al., 2004, Rahaman et al., 2006, Silverstein et al., 2010). However, in the present study, neither a Src kinase inhibitor, AG1879, nor a JNK inhibitor, SP600125, reduced lipid uptake by VSMC exposed to sera from WT or APOE^{-/-} mice (Figure 2A and B). Lipid uptake by VSMC was also unaffected by monodansylcadaverine (MDC; Figure 2A and B), an inhibitor of clathrin-dependent endocytosis, which is at least partially responsible for uptake of oxidised and acetylated LDL by macrophages (Jones and Willingham, 1999).

Macropinocytosis is an alternative pathway by which macrophages can accumulate lipids; especially lipids that have undergone minimal or no chemical modifications. Macropinocytotic uptake of lipids by macrophages has been shown to be sensitive to treatment with inhibitors of PI-3 kinase and actin polymerisation (Araki et al., 1996, Kruth et al., 2005). In the present study we showed that inhibition of PI-3 kinase with LY294002 markedly reduced lipid accumulation in VSMC exposed to serum from WT or APOE^{-/-} mice (Figures 2C and D). An actin polymerization inhibitor, cytochalasin D, also inhibited lipid uptake in VSMC exposed to WT serum (Figure 2C) but had little effect on cells exposed to APOE^{-/-} serum (Figure 2D). The effects of two additional macropinocytosis inhibitors, amiloride (0.1-3.0 mmol/L), and its analogue 5-(N-Ethyl-N-isopropyl) amiloride (10-300 µmol/L), were also examined. However, both of these inhibitors were found to be toxic to VSMC (data not shown) at concentrations previously demonstrated to inhibit macropinocytosis (Garcia-Perez et al., 2008, Martinez-Argudo and Jepson, 2008).

Platelet-derived Growth Factor (PDGF) and *Chlamydia pneumoniae* infection do not stimulate mouse sera-induced uptake of lipids by VSMC

Platelet-derived growth factor (PDGF) has been shown to induce cell membrane ruffling associated with macropinocytosis (Anton et al., 2003, Bryant et al., 2007). Thus, to determine if PDGF present in mouse sera was responsible for lipid accumulation by VSMC, WT and APOE$^{-/-}$ mouse sera were pre-treated with a neutralizing anti-PDGF antibody (40 µg/ml) prior to their addition to VSMC. Incubation of VSMC with untreated WT or APOE$^{-/-}$ serum was again associated with a marked increase in lipid uptake and these effects were not affected by blocking PDGF (Figure 3A and B).

Macropinocytotic lipid uptake in macrophages has previously been linked to activation of toll-like receptors (Choi et al., 2009b). Since VSMC are known to express TLR2 and TLR4 (de Graaf et al., 2006), we postulated that *C. pneumoniae*, a Gram-negative pro-atherogenic bacteria and agonist of toll-like receptors, would potentiate lipid accumulation in response to serum. Incubation of VSMC with *C. pneumoniae* was associated with the formation of inclusion bodies that were visible under a phase contrast microscope (x 400 magnification) after 48 h (data not shown)(Rivera et al., 2012). Nonetheless, infection with *C. pneumoniae* had no additional effects on lipid accumulation in VSMC either in the presence of FCS or mouse serum (Figure 3D).

Effect of mouse serum on expression of genes related to LDL uptake and cholesterol efflux in VSMC

The above findings indicated that there is likely to be little or no role for classical, receptor-mediated lipid uptake pathways in the effects of mouse serum on VSMC phenotype. Consistent with this idea, we found that the expression of the scavenger receptor, SR-A1, was

significantly downregulated in VSMC treated with 20% WT or APOE^{-/-} mouse sera (Figure 4A). Exposure of cells to 20% (non heat-inactivated) FCS also appeared to reduce SR-A1 expression compared to levels seen in cells maintained under conventional culture conditions, although this effect failed to reach statistical significance. Sera from WT and APOE^{-/-} mice also appeared to reduce expression of CD36, but again this effect was not statistically significant.

To provide an indication of cholesterol efflux capacity after serum treatment, we measured expression of ABCA1. Interestingly, mRNA expression of ABCA1 was rapidly downregulated (i.e. within 3 h) following exposure of VSMC to WT or APOE^{-/-} serum (Figure 4). Furthermore, expression of ABCA1 in mouse serum-treated cells remained suppressed for the remainder of the 48 h treatment period (P<0.001; Figure 5).

Effect of mouse sera on expression on markers of VSMC-differentiation and inflammation

A previous report demonstrated that, following treatment with cholesterol, mouse VSMC lose their VSMC properties and assume a macrophage-like phenotype (Rong et al., 2003). We therefore examined if lipid accumulation induced by mouse serum is associated with similar changes away from a VSMC phenotype. As reported previously (Afroze et al., 2003), MOVAS maintained under standard culture conditions expressed several markers of VSMC differentiation including smooth muscle α-actin, smooth muscle myosin heavy chain and calponin H1. The levels of each of these markers varied considerably over the course of the 48 h study period, even when the cells were maintained under standard culture conditions (i.e. 10% heat-inactivated FCS in DMEM). For example, expression of smooth muscle α-actin and calponin H1 appeared to initially increase from the 3 h to the 6 h time-points, and then steadily declined throughout the remainder of the experiment. By contrast, expression of

smooth muscle myosin heavy chain progressively increased over the 48 h incubation period (Figure 6A). Of note, treatment with a higher concentration (20%) of non-heat inactivated FCS, or with sera for either the WT or APOE$^{-/-}$ mouse strains had no significant effects on expression levels of these VSMC differentiation markers at any of the time points examined (Figures 6A-C).

To assess if VSMC gained a more macrophage-like phenotype in the presence of mouse serum, the expression of macrophage markers CD68 and Mac-2 were measured. In VSMC maintained under standard culture conditions, CD68 expression gradually increased during the incubation period such that it was 2.8-fold higher at the 48 h versus the 3 h time-point (Figure 6D). This temporal profile of CD68 expression was neither affected by increasing the FCS concentration to 20% nor by exposure of VSMC to WT or APOE$^{-/-}$ serum (Figure 6B). Contrasting the CD68 profile, expression of Mac-2 remained relatively constant over the 48 h incubation period in cells maintained under standard culture conditions (Figure 6E). Exposure to either WT or APOE$^{-/-}$ serum caused a 7-fold increase in Mac-2 expression at the 48 h time-point; however so too did exposure to the higher concentration of FCS (Figure 6B), suggesting that the change was not related to the uptake of lipids induced by mouse serum. Thus, to summarise, despite causing profound morphological changes, mouse serum does not appear to cause de-differentiation of VSMC away from a VSMC phenotype nor does it promote a more macrophage-like phenotype.

Mouse serum also appeared to have no major effects compared to FCS on expression of the pro-inflammatory chemokines/cytokines CCL2 and CCL5 (Figure 7). For example, CCL2 expression in VSMC maintained under standard culture conditions gradually declined over the 48 h incubation period, with similar profiles observed in the presence of the higher FCS concentration and following exposure to WT mouse serum (Figure 7A). Although there was a trend for the APOE$^{-/-}$ serum to cause an increase in MCP-1 expression at 3 h (by 3.5-

fold) and 6 h (by 2-fold), this was only transient, with expression levels declining thereafter such that by 24 h and 48 h they were no different to the levels seen in the other treatment groups (Figure 7A). CCL5 expression in the control group appeared to increase from the 3 h to the 6 h time-point, and then remained relatively stable for the remainder of the experiment (Figure 7B). By contrast, in cells treated with WT or APOE$^{-/-}$ serum there was no evidence of any early increases in expression. In fact, expression of CCL5 appeared to decline and, by 48 h, levels of CCL5 appeared to be lower in the mouse sera-treated groups than in the FCS-treated cells (Figure 7A). Thus, as for the macrophage differentiation markers, the expression of inflammatory markers was either unaffected or reduced by exposure to WT or APOE$^{-/-}$ mouse serum. Note, we also attempted to measure the effect of mouse serum on the expression of the pro-inflammatory cytokines TNF-α and IL-1β; however, these genes were below the detection limits of the assay in both the mouse sera- and FCS-exposed cell groups (data not shown).

DISCUSSION

Here we have shown that exposure of murine VSMC to serum from mice causes them to take up lipids and store them in intracellular vacuoles. Accumulation of serum lipids within VSMC was associated with down-regulation of the reverse cholesterol transporter ABCA1 and appeared to be dependent on the process of bulk fluid-phase endocytosis known as macropinocytosis.

As the major cellular component of DIT, VSMC are prevalent in arterial intima prior to the development of atherosclerotic plaques (Stary et al., 1992). Although the physiological function of DIT is largely unknown, its well-organized structure combined with its near universal presence in humans from early childhood irrespective of ethnicity, gender or family history of cardiovascular disease has led to the belief that it is a normal feature of healthy blood vessels and should not be considered as early evidence of atherosclerosis (Restrepo et al., 1979, Stary et al., 1992, Nakashima et al., 2008). Nevertheless, DIT occurs mainly at sites along the vascular tree that are predisposed to later development of atherosclerotic plaques (Stary et al., 1992, Nakashima et al., 2002). Hence, a better understanding of how the various cellular and non-cellular elements that make up DIT respond to potential pro-atherogenic insults may provide insight into the early events that either trigger or protect against atherosclerotic plaque formation.

According to the 'response to retention' hypothesis, atherosclerotic plaque formation begins with the entry of unmodified serum lipids into the subendothelial intima. These lipids then undergo ionic interactions with ECM proteoglycans rendering them susceptible to oxidation (Camejo et al., 1998, Chait and Wight, 2000). Following modification, serum lipids, especially LDL, act as inducers of endothelial activation, chemoattractants for inflammatory cells, and fodder for macrophage-derived foam cells (Tabas et al., 2007).

Hence, mechanisms that limit the interaction of LDL with proteoglycans and their subsequent modification in the vascular wall are likely to be atheroprotective.

VSMC present in DIT and early atherosclerotic plaques are likely to be exposed to serum lipids that enter the subendothelial intima even prior to them becoming proteoglycan-bound and modified. However, to our knowledge there is little information on the effects of unmodified serum lipids on VSMC morphology and function. Here we have shown that exposure of murine VSMC in culture to sera from either normocholesterolemic or hypercholesterolemic mice was associated with marked morphological changes, in particular the formation of lipid-filled vacuoles, which were apparent as early as 6 h after initiation of treatment. Yet, despite these changes, the VSMC retained their expression of several markers of VSMC differentiation, and did not appear to assume a pro-inflammatory phenotype.

Traditionally, foam cell formation involves the uptake of lipids, especially those that have been chemically modified through oxidation, acetylation, glycation or enzymatic processes, by scavenger receptors such as CD36, SR-A1 and SR-A2, and LOX-1 (Webb and Moore, 2007). Although macrophages are the major cell type that undergoes transformation into foam cells within the vessel wall, it is well established that VSMC also express several of these scavenger receptors and can become engorged with lipids when exposed to modified LDL, VLDL or cholymicrons (Floren et al., 1981, Klouche et al., 2000, Ishii et al., 2002). Uptake of lipids from modified lipoproteins by VSMC *in vitro* is associated with trans-differentiation into a more macrophage-like phenotype, characterized by reduced expression of VSMC-related genes and elevated expression of macrophage-related genes (Rong et al., 2003). In addition, expression of several pro-inflammatory chemokines (CX3CL1, CCL2), cytokines (TNF-α, macrophage migration inhibitory factor) and transcription factors (NF-κB, JNK) are also upregulated (Barlic et al., 2007, Chen et al., 2009, Sima et al., 2010, Sun and Chen, 2010). In the present study, we examined the effects of inhibitors (shown in

parentheses) of signalling enzymes that are essential for the uptake of modified lipids via scavenger receptor-dependent mechanisms including JNKs (SP600125), Src kinases (AG1879), and of clathrin-dependent endocytosis (monodansylcadaverine), which is partially responsible for uptake of oxidised and acetylated LDL by macrophages (Jones and Willingham, 1999, Coller and Paulnock, 2001, Rahaman et al., 2006). We found none of these compounds to have any inhibitory effects on VSMC lipid uptake induced by serum from WT or APOE$^{-/-}$ mice. As a further indicator that scavenger receptors were not involved, we found that exposure to mouse sera had either no effect or only transiently modulated expression levels of CD36 and SR-A1 in VSMC.

In contrast to its lack of effect on expression of scavenger receptors, mouse serum caused marked down-regulation of ABCA1 in VSMC. ABCA1 plays an important role in many cell types as the primary mediator of cholesterol efflux by facilitating its transfer across the plasma membrane and onto extracellular ApoA-1 (Yvan-Charvet et al., 2010). A previous report showed that ABCA1 expression was reduced in intimal versus medial VSMC from human atherosclerotic arteries, and that this was associated with an impaired ability to bind ApoA1 and transport lipids to the extracellular compartment (Choi et al., 2009a). Hence, down-regulation of ABCA1, leading to a reduction in reverse cholesterol transport, could provide at least some of the explanation for why, in the present study, VSMC accumulated lipids when exposed to mouse sera.

Macropinocytosis is a specialised form of endocytosis that allows the uptake of extracellular fluid in a bulk and non-selective manner (Kerr and Teasdale, 2009). Macropinocytosis is characterised by the formation of cup-shaped invaginations (or 'ruffles') of the plasma membrane, which eventually become closed off to form intracellular vacuoles known as macropinosomes (Kerr and Teasdale, 2009). Macropinocytosis is now recognised as an alternative pathway of lipid uptake in macrophages (Kruth et al., 2005, Yao et al.,

2009). Unlike traditional scavenger receptor-dependent mechanisms, which lead to preferential uptake of modified lipoproteins, macropinocytosis in macrophages is associated with accumulation of lipids from unmodified or minimally modified lipoproteins (Kruth et al., 2005, Choi et al., 2009b, Yao et al., 2009). Macropinocytosis is dependent on both actin polymerization and PI-3 kinase activity (Araki et al., 1996, Kerr and Teasdale, 2009). Hence, our observation that VSMC lipid uptake in response to WT mouse serum was sensitive to treatment with either cytochalasin D or LY294002, which inhibit actin polymerization and PI-3 kinase activity, respectively (Araki et al., 1996, Kruth et al., 2005), is consistent with a role for macropinocytosis in the process. While PI-3 kinase inhibition also partially prevented lipid uptake by VSMC following exposure to APOE$^{-/-}$ serum, cytochalasin D appeared to have no effect. The reason for the differential effect of cytochalasin D on lipid uptake induced by WT versus APOE$^{-/-}$ serum is currently unknown. It may simply relate to the different efficacies of the WT versus the APOE$^{-/-}$ serum in evoking lipid uptake, with the more powerful actions of the latter requiring a higher concentration of inhibitor to be surmounted. Unfortunately we were unable to test this as higher concentrations of cytochalasin D reduced cell viability (data not shown). Thus, we cannot exclude the possibility that alternative/additional mechanisms to macropinocytosis were at least partially responsible for lipid uptake following exposure to serum from APOE$^{-/-}$ mice.

Initiation of macropinocytosis occurs largely independently of the cargo that is endocytosed. Rather, it is thought to be a consequence of stimulation of growth factor or pattern recognition receptors (Kerr and Teasdale, 2009). Regarding the former, PDGF is one such growth factor that has been shown to stimulate membrane ruffling and macropinocytosis in other vascular (Zhao et al., 2011) and non-vascular (Anton et al., 2003) cell types. However, in the present study, depletion of PDGF from mouse sera with a neutralizing antibody, failed to prevent lipid accumulation by VSMC. Likewise, treatment of VSMC with

C. pneumoniae, a bacterial species that is known to activate toll-like receptors in VSMC, did not potentiate serum-induced lipid uptake by VSMC. Hence, at this point we can only speculate on which factor(s) present in mouse serum may have been responsible for triggering macropinocytosis.

One of the most striking observations from the present study was that despite the marked morphological changes in VSMC cells following macropinocytotic uptake of lipids, the cells retained expression levels of VSMC differentiation markers and did not appear to become pro-inflammatory. Were this to hold true *in vivo,* it might suggest that macropinocytotic lipid uptake by VSMC is a means of removing lipids from the extracellular compartment within the vascular wall thereby limiting their interaction with ECM proteoglycans and preventing them from undergoing oxidative modification. Hence, the presence of VSMC in DIT and early atherosclerotic lesions, and their ability to act as a 'sink' for unmodified serum lipoproteins that enter the vessel wall, might be important factors for limiting the initiation and/or progression of atherosclerotic plaques.

In conclusion, we have demonstrated for the first time that VSMC have a high capacity for uptake and intracellular storage of serum lipoproteins and that this process is likely the result of a macropinocytosis-like uptake pathway and possibly down-regulation of ABCA1-mediated cholesterol efflux. While insights into the (patho)physiological significance of these findings require further *in vivo* studies, we speculate that macropinocytotic uptake of serum lipid by VMSC may be an atheroprotective response to limit oxidative modification of lipids and the subsequent chemoattraction of inflammatory leukocytes.

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FIGURE LEGENDS

Figure 1. Serum from mice induces lipid accumulation in mouse VSMC. (A) VSMC were treated for 48 h with 10% (non-heat inactivated) FCS (control) or with serum from WT or APOE$^{-/-}$ mice before staining for intracellular lipids with oil red O. Images are representative of 3 separate experiments (scale bars = 150 μm). (B) Lipid accumulation after 48 h incubation with increasing concentrations of FCS, WT or APOE$^{-/-}$ sera was also quantified in VSMC using the AdipoRed fluorescence assay. Values (mean ± SEM; n=4-8) are expressed as relative fluorescence units (RFU). (C) FPLC fractionation of lipoproteins present in FCS, WT or APOE$^{-/-}$ sera. (D) Cell numbers/viability of VSMC following 48 h incubation under standard culture conditions (i.e. 10% heat-inactived FCS) or in the presence of 20% FCS, WT or APOE$^{-/-}$ serum. MTS conversion into it formazan derivative was assessed by measuring light absorbance at 482 nm (n=3). *$P<0.05$ vs control, #$P<0.05$ vs WT for 2-way ANOVA with Bonferroni post-test for 15 comparisons.

Figure 2. Inhibition of macropinocytosis- but not scavenger receptor-dependent endocytosis reduces serum lipid uptake in VSMC. VSMC were treated with pharmacological inhibitors of scavenger receptor- and clathrin-dependent pathways of lipid uptake (A & B) and of macropinocytosis-dependent endocytosis (C & D) for the 3 h prior to and 48 h during exposure to 20% WT or APOE$^{-/-}$ mouse serum. Lipid accumulation was monitored using the AdipoRed fluorescence assay. Values (mean ± SEM; n=4-8) are expressed as a percentage of the control group (i.e. cells maintained under normal culture conditions). *$P<0.05$ vs vehicle for 1-way repeated-measures ANOVA, with Dunnet's post-test.

Figure 3. PDGF and *C.pneumoniae* (Cpn) infection do not alter mouse-serum induced lipid uptake by VSMC. Pre-incubation of WT (A) or APOE$^{-/-}$ (B) mouse serum with a neutralizing anti-PDGF antibody (40 µg/ml) did not prevent their abilities to induce lipid accumulation by VSMC. (C) VSMC were incubated with either mock- or Cpn- (10^4-10^6 IFU) containing inoculum and subsequently treated with 20% WT serum for 48 h. Lipid accumulation was quantified using the AdipoRed fluorescence assay. Values (mean ± SEM) are expressed as a percentage of the FCS control group (n=3-6).

Figure 4. Effect of mouse sera on expression of scavenger receptors in VSMC. VSMC were either maintained under normal culture conditions (i.e. 10 % heat-inactivated FCS; control) or were treated with 20% FCS, WT or APOE$^{-/-}$ mouse serum and harvested after 3, 6, 24 or 48 h to measure mRNA expression of (A) SR-A1 or (B) CD36 by real-time PCR. Values (mean ± SEM; n=3-4) are expressed as fold-changes relative to the control 3 h time-point. *P<0.05 for 2-way ANOVA with Bonferroni post-test for 24 comparisons

Figure 5. Mouse serum downregulates expression of ATP-binding cassette transporter A1 (ABCA1). VSMC were either maintained under normal culture conditions (i.e. 10 % heat-inactivated FCS; control) or were treated with 20% FCS, WT or APOE$^{-/-}$ mouse serum and harvested after 3, 6, 24 or 48 h to measure mRNA expression of ABCA1 by real-time PCR. Values (mean ± SEM; n=4) are expressed as fold-changes relative to the control 3 h time-point. *P<0.05 vs control, #P<0.05 vs FCS for 2-way ANOVA with Bonferroni post-test for 24 comparisons.

Figure 6. Effect of mouse serum on markers of cell differentiation. VSMC were either maintained under normal culture conditions (i.e. 10 % heat-inactivated FCS; control) or were

treated with 20% FCS, WT or APOE$^{-/-}$ mouse serum and harvested after 3, 6, 24 or 48 h to measure mRNA expression of VSMC differentiation markers, smooth muscle α-actin (A), calponin HI (B) and smooth muscle myosin heavy chain (SM-MHC; C); and macrophage differentiation markers CD68 (D) and Mac-2 (E) by real-time PCR. Values (mean $\pm$ SEM; n=3-4) are expressed as fold-changes relative to the control 3 h time-point.

Figure 7. Exposure to mouse serum does not induce a pro-inflammatory state in VSMC. VSMC were either maintained under normal culture conditions (i.e. 10 % heat-inactivated FCS; control) or were treated with 20% FCS, WT or APOE$^{-/-}$ mouse serum and harvested after 3, 6, 24 or 48 h to measure mRNA expression of the chemokines CCL2 (A) and CCL5 (B) by real-time PCR. Values (mean $\pm$ SEM; n=3-4) are expressed as fold-changes relative to the control 3 h time-point.

A

control | 10 % WT | 50 % WT
10 % APOE$^{-/-}$ | 50 % APOE$^{-/-}$

B

Lipid accumulation (RFU x 10^6)

FCS, WT, APOE$^{-/-}$

% serum: 10, 20, 30, 40, 50

C

FCS
Absorbance (280 nm)
albumin
Elution time (s x 10^3)

WT
Absorbance (280 nm)
albumin
VLDL, LDL, HDL
Elution time (s x 10^3)

APOE$^{-/-}$
Absorbance (280 nm)
VLDL, albumin
LDL, HDL
Elution time (s x 10^3)

D

Cell number (MTS metabolism)

Control, FCS, WT, APOE$^{-/-}$

A

Lipid accumulation (% FCS)

Control, WT, WT + anti-PDGF

B

Lipid accumulation (% FCS)

Control, APOE-/-, APOE-/- + anti-PDGF

C

Lipid accumulation (% FCS)

Control, Cpn 10^6 IFU, WT, WT + mock, WT + Cpn 10^4 IFU, WT + Cpn 10^5 IFU, WT + Cpn 10^6 IFU

A

B