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Differential regulation of macropinocytosis in macrophages by cytokines: Implications for foam cell formation and atherosclerosis ☆

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ABSTRACT

A key event during the formation of lipid-rich foam cells during the progression of atherosclerosis is the uptake of modified low-density lipoproteins (LDL) by macrophages in response to atherogenic mediators in the arterial intima. In addition to scavenger receptor-dependent uptake of LDL, macropinocytosis is known to facilitate the uptake of LDL through the constitutive and passive internalization of large quantities of extracellular solute. In this study we confirm the ability of macropinocytosis to facilitate the uptake of modified LDL by human macrophages and show its modulation by TGF-β, IFN-γ, IL-17A and IL-33. Furthermore we show that the TGF-β-mediated inhibition of macropinocytosis is a Smad-2/-3-independent process.

1. Introduction

Atherosclerosis is a chronic inflammatory disease of the vasculature that is governed by many risk factors including genetic predisposition and diet. The disease itself is characterized by the accumulation and retention of apolipoprotein B (apoB)-containing lipoproteins, such as low-density lipoprotein (LDL), within the arterial wall and the subsequent formation of foam cell-rich fibrotic plaques that, upon rupture, result in myocardial infarction and stroke [1]. The formation of lipid laden foam cells in the arterial intima, as a result of modified LDL uptake by macrophages, represents a critical step during the progression of atherosclerosis. The uptake of modified forms of LDL is understood to take place through a receptor-dependent mechanism that is propagated by the increased expression of key genes, such as scavenger receptor-A (SR-A) and CD36, by invading macrophages [1,2]. Furthermore, this phenotypic change is known to be tightly regulated by a multitude of both novel and classical cytokines implicated in the control of atherogenesis, such as interleukin (IL)-33, interferon-γ (IFN-γ), and transforming growth factor-β (TGF-β) [3–5], and currently represents the most accepted and well documented paradigm of modified LDL uptake by macrophages.

In addition to this "classical" mode of lipid uptake, it has been shown that scavenger receptor-independent processes, such as macropinocytosis, may contribute significantly to the uptake of LDL by macrophages [6] and therefore drive the process of foam cell formation. Macropinocytosis is a form of fluid-phase endocytosis where solute uptake is directly proportional to the volume of liquid internalized and the solute concentration. The process itself involves the actin-dependent ruffling of the plasma membrane and the subsequent fusion of the membrane to itself in order to form intracellular fluid filled vacuoles 0.5–5 μM in diameter [7,8]. This process has been shown to contribute to the uptake of oxidized (Ox-) LDL by mouse RAW264.7 macrophages [9], an observation that may account for the appearance of LDL sized nano-particles within macrophages present in atherosclerotic lesions of apoE deficient mice [10]. Furthermore, macropinocytosis has also been shown to contribute to the uptake of native and modified forms of LDL by human peripheral blood-derived macrophages [11], phorbol 12-myristate 13-acetate (PMA)-derived macrophages [6] and macrophage colony-stimulating factor

Abbreviations: AcLDL, acetylated LDL; Apo, apolipoprotein; BMDM, bone marrow-derived macrophage; CD-36, cluster of differentiation 36; DiI, 1,1-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyane perchlorate; THP-1, human acute monocytic leukemia cells; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HMDM, human monocyte-derived macrophages; IL, interleukin; IFN-γ, interferon-γ; LDL, low-density lipoproteins; LY, lucifer yellow; OxLDL, oxidized LDL; SR-A, scavenger receptor A; shRNA, short hairpin RNA; TGF-β, transforming growth factor-β.

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(MCS-F)-derived macrophages *in vitro* [12,13]. In addition, it has also been shown that minimally oxidized LDL (mmLDL) can induce macropinocytosis in macrophages through a toll-like receptor (TLR) 4/spleen tyrosine kinase (SYK) dependent mechanism and therefore potentiate the uptake of both native and oxLDL thus contributing to the progression of atherosclerosis [14,15].

Despite its clear implication during the process of foam cell formation, the effect of novel and established foam cell regulators, such as IL-33, IL-17A, IFN-γ and TGF-β, on macropinocytosis remains unexamined. Therefore, the objective of this study was to confirm the role of macropinocytosis on modified LDL uptake in human macrophages and examine the effect of key cytokines known to regulate atherosclerotic progression on this process.

2. Materials and methods

2.1. Reagents and cell culture

All chemicals were purchased from Sigma–Aldrich (Poole, UK) unless otherwise stated. Recombinant human TGF-β, IL-33, IL-17A and IFN-γ were supplied by Peprotech (London, UK). Acetylated LDL (AcLDL) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethyllindocarbocyane perchlorate (Dil)-labeled AcLDL (DiI-AcLDL) were purchased from Intracel (Frederick, Maryland, USA).

Human monocyte-derived macrophages (HMDM) were differentiated from monocytes isolated from buffy coats supplied by the Welsh Blood service using Ficoll-Hypaque purification described elsewhere [3]. Ethical approval and informed consent for each donor was granted by the Welsh Blood Service for the use of human blood samples. Human acute monocytic leukemia cells (THP-1) and HMDM were maintained in complete RPMI-1640 supplemented with 10% (v/v) heat-inactivated FCS, penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (2 mmol/L) (all Invitrogen, Paisley, UK), at 37 °C in a humidified atmosphere containing 5% (v/v) CO_2. THP-1 monocytes were differentiated into macrophages using 160 nM PMA for 24 h and this ensured high expression levels of scavenger receptors and other genes implicated in the control of macrophage foam cell formation [16]. In all experiments cells were incubated for 24 h with either 30 ng/ml of TGF-β, 10 ng/ml of IL-33, 100 ng/ml of IL17A or 1000 U/ml of IFN-γ. THP-1 macrophages were pre-incubated with cytochalasin-D for 1 h prior to the addition of AcLDL, DiI-AcLDL or lucifer yellow (LY) dipotassium salt and cytokines. Cytokines and cytochalasin-D were reconstituted in PBS/0.1% bovine serum albumin (BSA) or DMSO respectively that were subsequently used as a vehicle controls.

2.2. DiI-AcLDL and lucifer yellow uptake assays

Cells were incubated for 24 h with DiI-AcLDL (10 μg/ml) or LY (100 μg/ml) in RPMI-1640 containing 0.2% (v/v) fatty-acid free BSA (Sigma–Aldrich) at 37 °C. The concentration of LY reflects that commonly used in the literature [17]. DiI-AcLDL and LY uptake were analyzed by flow cytometry on a FACS Canto (BD Biosciences, Oxford, UK) flow cytometer with at least 10,000 events acquired for each sample. DiI-AcLDL and LY uptake is represented as a percentage with the vehicle-treated control indicated as 100%.

2.3. Real-time quantitative PCR

RNA extraction, reverse transcription and real-time quantitative PCR analysis was performed as described elsewhere [3]. Oligonucleotides specific for 60S ribosomal protein L13a (RPL13A) (forward 5′-CCTGGAGGAGAAGAGGAAAGAGA-3′, reverse 5′-TTGAG GACCTCTGTGTATTTGTCAA-3′), CD36 (forward 5′-GAGAACTGTTA TGGGGCTAT-3′, reverse 5′-TTCAACTGGAGAGGCAAAGG-3′) [18] and SR-A (forward 5′-CCAGGGACATGGAATGCAA-3′, reverse 5′-CC AGTGGGACCTCGATCTCC-3′) [18] were purchased from Sigma Aldrich (Poole, UK). Fold changes in expression were calculated using $2^{-(\Delta Ct1\ -\ \Delta Ct2)}$, where ΔCt represents the difference between the threshold cycle (C_T) for each target gene and RPL13A mRNA transcript levels. Melting curve analysis was performed on each primer set to confirm amplification of a single product and all amplicons were sequenced to ensure reaction specificity (data not shown).

2.4. Adenoviral infection of THP-1

Adenovirus encoding shRNA against Smad-2, Smad-3 and GAPDH were prepared as previously described [19]. THP-1 monocytes were infected with RAd-GAPDH shRNA or RAd-Smad-2 shRNA or RAd-Smad-3 shRNA at a multiplicity of infection (MOI) of 100 for each virus in 0.5 ml RPMI-1640 medium for 2.5 h at 37 °C (rocking) prior to addition of 1 ml of RPMI-1640 medium (including 160 nM PMA to induce differentiation into macrophages) and incubation for a further 72 h. An MOI of 100 was sufficient to infect >98% THP-1 cells as measured by flow cytometry following infection with a GFP-expressing recombinant adenovirus (data not shown).

2.5. Statistical analysis

All data are presented as mean [±standard deviation (SD)] on the assigned number of independent experiments or, in experiments involving HMDM, experiments performed using samples from different donors. For single comparisons, values for p were calculated using the Student's t-test. For multiple comparisons, values of p were calculated using Welch's robust test of equality of means followed by Games–Howell post hoc analysis. Values of p were considered significant below 0.05.

3. Results

3.1. Cytochalasin-D inhibits the uptake of LY and AcLDL in human macrophages

The PMA differentiated THP-1 model is commonly used to delineate human macrophage cellular functions associated with atherosclerosis due to their conserved responses with primary HMDM and *in vivo* data [3,4,20]. This cellular system was therefore employed to examine the potential contribution of macropinocytosis during foam cell formation. In light of this we first examined the effect of the established macropinocytosis (not micropinocytosis [11]) inhibitor, cytochalasin-D, on the uptake of LY, a fluorescent dye commonly used to monitor macropinocytosis [7,21], by monocyte derived THP-1 macrophages. As shown in Fig. 1, cytochalasin-D, a cell permeable myotoxin that inhibits macropinocytosis through the de-polymerization of actin filaments and tubulin microtubules [11,12], attenuates the uptake of LY by approximately 70% (at 2.5 μg/ml) when compared to the vehicle control treated cells.

Next, in order to further substantiate the existing evidence suggesting a role for macropinocytosis in foam cell formation [7,12], we examined the effect of cytochalasin-D (at the concentration defined above) on the uptake of DiI-AcLDL, a form of LDL used extensively during *in vitro* foam cell formation assays due to its substantial intracellular accumulation [22]. As shown in Fig. 2A, cytochalasin-D significantly inhibited the uptake of AcLDL by THP-1 macrophages by approximately 37%, an observation that was also conserved in HMDM (Fig. 2B). This response was confirmed by an observed reduction in the intracellular cholesterol