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Lipin-1 contributes to modified low-density lipoprotein-elicited macrophage pro-inflammatory responses

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ABSTRACT

Atherosclerosis is a chronic inflammatory disease of large and medium-sized arteries and the underlying cause of cardiovascular disease, a major cause of mortality worldwide. The over-accumulation of modified cholesterol-containing low-density lipoproteins (e.g. oxLDL) in the artery wall and the subsequent recruitment and activation of macrophages contributes to the development of atherosclerosis. The excessive uptake of modified-LDL by macrophages leads to a lipid-laden "foamy" phenotype and pro-inflammatory cytokine production. Modified-LDLs promote foam cell formation in part by stimulating *de novo* lipid biosynthesis. However, it is unknown if lipid biosynthesis directly regulates foam cell pro-inflammatory mediator production. Lipin-1, a phosphatidate phosphohydrolase required for the generation of diacylglycerol during glycerolipid synthesis has recently been demonstrated to contribute to bacterial-induced pro-inflammatory responses by macrophages. In this study we present evidence demonstrating the presence of lipin-1 within macrophages in human atherosclerotic plaques. Additionally, reducing lipin-1 levels in macrophages significantly inhibits both modified-LDL-induced foam cell formation *in vitro*, as observed by smaller/fewer intracellular lipid inclusions, and ablates modified-LDL-elicited production of the pro-atherogenic mediators tumor necrosis factor-α, interleukin-6, and prostaglandin E2. These findings demonstrate a critical role for lipin-1 in the regulation of macrophage inflammatory responses to modified-LDL. These data begin to link the processes of foam cell formation and pro-inflammatory cytokine production within macrophages.

1. Introduction

Atherosclerosis, a chronic inflammatory condition of large and medium-sized arteries mediated by macrophages, is the most common cause of CVD [1]. Hypercholesterolemia can stimulate the deposition of cholesterol carrying low-density lipoproteins (LDL) in susceptible areas of the artery wall, initiating the development of atherosclerotic lesions [2]. The deposition of LDL in the arterial intima stimulates the recruitment of macrophages to remove cholesterol, thereby preventing cholesterol-induced necrosis [3]. If LDLs are not promptly removed from the intima, local reactive oxygen species can modify LDLs to oxidized-LDL (oxLDL) [4]. The oxidation of LDL causes it to be taken up by macrophages via scavenger receptors [5], resulting in a positive feed-back response and enhanced oxLDL uptake. Excessive oxLDL uptake initiates *de novo* lipid synthesis which is necessary to store incoming cholesterol and lipids. Free cholesterol-induced foam cell formation requires the activity of acyl-CoA synthetase, the enzyme responsible for the generation of fatty acid acyl-CoA precursors that are needed for glycerolipid synthesis and the esterification of cholesterol [6]. Additionally, fatty acid-induced intracellular lipid accumulation in the form of lipid droplets requires the generation of

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phosphatidylcholine [7]. Thus, the *de novo* production of lipids (both neutral and glycerophospho-lipids) is required for the intracellular storage and trafficking of lipids (i.e. endosomes and/or lipid droplets) [8]. In addition to enhanced uptake, macrophages loaded with oxLDL display a reduced ability to efflux cholesterol [9]. The net result of excessive LDL uptake and the inability to efflux cholesterol leads to lipid-laden macrophages called foam cells [10]. Eventually, foam cells begin to secrete pro-inflammatory mediators such as TNF-α, IL-6 and PGE_2. The combination of excessive uptake, reduced cholesterol efflux, and pro-inflammatory mediator production promotes atheroma formation, plaque progression, and ultimately plaque instability that culminate in the catastrophic events associated with CVD. It is currently unclear if lipid synthetic events elicited by oxLDL contribute to the foam cell pro-inflammatory state.

Lipin-1 belongs to the evolutionarily conserved three-member lipin family in mammals (reviewed in [11]). Lipin-1 converts phosphatidate into DAG through phosphohydrolase enzymatic activity and exhibits the highest phosphatidate-specific phosphohydrolase activity of the identified lipins [12]. Lipin-1 contributes to triglyceride and likely glycerophosholipid synthesis. The contribution of lipin-1 to glycerophospholipid synthesis is suggested to be necessary for new membrane generation during B cell differentiation [13]. In human and mouse macrophages, lipin-1 contributes to lipid droplet formation following fatty acid loading [14]. Additionally, we and others have demonstrated that lipin-1 regulates macrophage inflammatory activity in response to both the intracellular pathogen *Francisella tularensis* [15] and to lipopolysaccharide (LPS) [16]. These studies demonstrate that lipin-1 is involved in two cellular events that are known to exist in macrophage foam cells; storage of engulfed cholesterol/lipids and pro-inflammatory activity, suggesting to us that lipin-1 may link lipid synthesis pathways that contribute to foam cell formation to foam cell inflammatory mediator production. Therefore, we sought to determine if lipin-1 is present within atherosclerotic plaques and if it contributes to modified LDL-elicited foam cell formation and pro-inflammatory responses by macrophages.

2. Results

2.1. Macrophage-associated lipin-1 is present in atherosclerotic lesions

If lipin-1 contributes to atherosclerotic plaque progression, it should be present in atherosclerotic plaques. Staining of human atherosclerotic plaques revealed a strong localization of lipin-1 within the macrophage-rich region (Fig. 1A–B). Furthermore, lipin-1 in atherosclerotic plaques is located within $CD68^+$ cells, a surface marker for macrophages (Fig. 1C). Within $CD68^+$ cells lipin-1 was observed in both the cytosol and the nucleus (5 cells observed by confocal microscopy in various stages of plaque development). Furthermore, lipin-1 staining appears at a perinuclear position that could be the endoplasmic reticulum (ER). Due to the poor specificity of commercially available anti-mouse lipin-1 antibodies, a similar experiment demonstrating the presence of lipin-1 within murine plaques could not be performed. Therefore, we examined lipin-1 mRNA levels in a murine model of atherosclerosis. $ApoE^{-/-}$ mice were fed normal chow or a high-fat chow for 8 weeks, and lipin-1 mRNA expression in the aortic arch was measured by qRT-PCR. $ApoE^{-/-}$ mice fed a high-fat diet displayed a significant increase in lipin-1 expression compared to mice fed a normal diet (Fig. 1D). Taken together, these data suggest that macrophage-associated lipin-1 may contribute to atherosclerosis.

2.2. Lipin-1 contributes to modified-LDL induced foam cell formation

Lipin-1 regulates lipid droplet generation in hepatocytes and adipocytes, and recently it has been demonstrated that lipin-1 regulates saturated fatty acid-induced macrophage foam cell formation [14]. However, it is unknown if lipin-1 regulates modified LDL-elicited foam cell formation. Others have established that the treatment of macrophages *in vitro* with modified-LDL (oxidized or acetylated) generates lipid-laden macrophages that are equivalent to plaque macrophage foam cells [17]. OxLDL is inefficiently processed following uptake and a portion of the lipoprotein becomes trapped in the endocytic pathway [18,19] while the remaining can be broken down and the cholesterol transferred to lipid droplets. Conversely, acLDL is readily processed and leads to the accumulation of cholesterol ester-rich lipid droplets [20]. To investigate the contribution of lipin-1 to foam cell formation, we used the RAW264.7 macrophage cell line, in which we generated stable lipin-1 knockdown using a lentivirally delivered shRNA (80% knockdown). We have previously demonstrated both the successful and specific knockdown of lipin-1 in RAW264.7 macrophages [15]. Wild-type (WT), non-target (NT) or lipin-1 depleted RAW264.7 macrophages were mock treated or treated with oxLDL (10 μg/mL) or acLDL (50 μg/mL) for 24 h. To visualize intracellular neutral-lipid content we stained the macrophages with Nile Red, a stain that fluoresces intensely in neutral lipid-rich environments [21], which includes both lipid droplets and internalized lipoproteins [22]. We will refer to Nile Red positive structures as "lipid inclusions." To differentiate between internalized lipoproteins and lipid droplets we stained macrophages with anti-ADRP (adipose-related differentiation protein) antibody. ADRP is a protein known to be present on the outer surface of lipid droplets [23]. We acquired images of macrophages and quantified lipid inclusions and lipid droplets using the Cellomics (Thermo Scientific) high-content imaging system. Depletion of lipin-1 reduced both oxLDL-elicited lipid inclusions and acLDL-elicited lipid droplets in macrophages as observed by a reduction their number and intensity of staining when compared to WT macrophages (Fig. 2A–B). No differences were observed between WT and NT RAW264.7 macrophages in any analysis performed in this study, thus only WT data is shown in this manuscript. Together, these data suggest that lipin-1 contributes to foam cell formation regardless of the LDL species being metabolized.

2.3. Depletion of lipin-1 does not affect uptake of modified-LDL

The failure of lipin-1 depleted macrophages to become foam cells may be due to an inability to endocytose modified LDLs. The scavenger receptor CD36 is known to be the predominant oxLDL receptor present on the surface of macrophages [24]. Depletion of lipin-1 did not alter the amount of CD36 on the cellular membrane of macrophages either prior to, or after oxLDL treatment when compared to WT macrophages (Supp. Fig. 1). We next decided to measure uptake of labeled modified-LDLs. WT or lipin-1 deficient macrophages were treated with either with fluorescently labeled oxLDL (DiI-oxLDL), [^{3}H]-cholesterol loaded oxLDL, or [^{3}H]-cholesterol loaded acLDL. At 4 and 24 h after treatment, macrophages were collected and mod-LDL uptake was quantified by flow cytometry (DiI-oxLDL) or scintillation count ([^{3}H]-cholesterol mod-LDLs). Depletion of lipin-1 resulted in a partial reduction in the uptake of labeled cholesterol at both 4 and 24 h after treatment (Fig. 3A–C). However, this reduction is not sufficient to account for the nearly complete loss of mod-LDL-elicited lipid inclusions observed in lipin-1 depleted macrophages.

To address modified-LDL uptake by macrophages in a manner