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Macrophage molecular signaling and inflammatory responses during ingestion of atherogenic lipoproteins are modulated by complement protein C1q

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ABSTRACT

Background and aims: This study investigated the effect of innate immune protein C1q on macrophage programmed responses during the ingestion of atherogenic lipoproteins. C1q plays a dual role in atherosclerosis where activation of complement by C1q is known to drive inflammation and promote disease progression. However, C1q is atheroprotective in early disease using mouse models. Our previous studies have highlighted a non-complement associated role for C1q in polarizing macrophages towards an M2-like anti-inflammatory phenotype during ingestion of targets such as atherogenic lipoproteins. This study aims to investigate the molecular mechanisms involved.

Methods: We investigated the molecular signaling mechanisms involved in macrophage polarization using an unbiased examination of gene expression profiles in human monocyte derived macrophages ingesting oxidized or acetylated low density lipoproteins in the presence or absence of C1q.

Results: Expression of genes involved in Janus kinase and signal transducer and activator of transcription (JAK-STAT) signaling, peroxisome proliferator activating receptor (PPAR) signaling and toll-like receptor (TLR) signaling were modulated by C1q in this screen. C1q was also shown to significantly suppress JAK-STAT pathway activation (a maximum 55% ± 13% reduction, $p = 0.044$) and increase transcriptional activation of PPARs (a maximum 229% ± 54% increase, $p = 0.0002$), consistent with an M2-like polarized response. These pathways were regulated in macrophages by C1q bound to different types of modified atherogenic lipoprotein and led to a reduction in the release of pro-inflammatory cytokine IL-6.

Conclusions: This study identifies potential molecular mechanisms for the beneficial role C1q plays in early atherosclerosis.

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1. Introduction

Atherosclerosis is a chronic inflammatory disease. Increased accumulation of cholesterol-rich low-density lipoprotein (LDL) in the artery wall provides an initiating step. In this inflammatory environment, LDL can undergo modifications such as oxidation (oxLDL), or chemical modification (acLDL) in the intima which allows for pattern recognition by receptors of the immune system including scavenger receptors on macrophages, leading to uptake via receptor-mediated endocytosis [1]. There is high plasticity in macrophage phenotypes in vivo, with inflammatory (M1) polarized subsets likely to contribute towards disease progression in atherosclerosis [2,3]. In contrast, anti-inflammatory (M2) polarized macrophages likely play a role in the dampening or resolution of inflammation in this disease [4,5]. In addition to their role in modulating inflammation, macrophages also play an important role in removal of excess cholesterol through efflux via high density lipoprotein (HDL) loading [6]. In hypercholesterolemia, this pathway can become overwhelmed, leading to the accumulation of free cholesterol in the cell, and the formation of foam cells [7]. In late atherosclerosis a plaque forms. Inflammation contributes to damage in the plaque which can cause it to rupture and may lead to

Abbreviations: acLDL, acetylated low density lipoprotein; BMDM, bone marrow derived macrophages; GO, gene ontology; HDL, high density lipoprotein; HMDM, human monocyte derived macrophages; IFN, interferon; JAK, Janus kinase; LDL, low density lipoprotein; LDLR, low density lipoprotein receptor; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; oxLDL, oxidized low density lipoprotein; PPAR, peroxisome proliferator activating receptor; RLU, relative luciferase units; STAT, signal transducer and activator of transcription; TLR, Toll-like receptor.

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cardiovascular complications [8]. Thus macrophages play a key role in the progression of this disease, through their influence on cholesterol removal and inflammation [9].

C1q is a recognition component of the innate immune complement system, and plays a dual role in atherosclerosis. In the atherosclerotic plaque, C1q recognition of autoantibodies against oxLDL or direct binding to modified lipoproteins can activate the classical complement pathway [10–12]. This powerful innate immune response leads to production of inflammatory complement activation products such as C3 and C5a, and target deposition of the membrane attack complex (MAC), which contribute to disease progression in animal models [13,14] and are associated with risk of atherosclerosis and cardiovascular disease in humans [15]. However, macrophages can secrete C1q [16], and thus in early atherosclerosis, C1q may be present in the lesion in the absence of other complement components. We and others have shown that C1q bound to targets such as apoptotic cells or damaged lipoproteins can directly interact with macrophages, modulating macrophage responses [17]. The ability of C1q to enhance phagocytosis of numerous types of targets (bacteria, immune complexes, apoptotic cells, damaged lipoproteins) in various phagocytes is well described [18]. In addition to this, C1q has also been shown to modulate phagocyte inflammatory responses and signaling. C1q modulates phagocyte polarization towards an anti-inflammatory M2-like phenotype during apoptotic cell clearance, which may be important in prevention of autoimmunity and maintenance of normal tissue homeostasis [19–21]. C1q also promotes an M2-like anti-inflammatory macrophage phenotype during clearance of modified lipoproteins, and dampens inflammatory TLR signaling which suggests it can also reverse inflammatory macrophage polarization [22]. This may be important in resolving or restraining inflammatory diseases. A potential role for C1q in the modulation of NFκB transcription was also identified in these studies [19,22]. In addition to modulation of macrophage inflammatory polarization, ingestion of modified lipoproteins bound to C1q also triggers enhanced cholesterol efflux and decreases foam cell formation in human macrophages [23]. Importantly, we have previously shown that C1q does not bind unmodified LDL, and has no effect on cytokine or phagocytic responses when in solution, suggesting that multivalent presentation of the molecule is required for macrophage activation [19,23,24]. These non-complement associated roles of C1q may explain its protective role in mouse models of early atherosclerosis. In these studies, C1q deficient mice which were also low-density lipoprotein receptor deficient ($C1q^{-/-}LDLR^{-/-}$) exhibited a greater aortic root lesion size and accumulation of apoptotic cells compared to the C1q-sufficient $LDLR^{-/-}$animals [25].

In order to better understand the molecular signaling mechanisms by which C1q is reprogramming macrophage responses in atherosclerosis, these studies aim to investigate the effect of C1q-opsonization of modified lipoproteins on modulation of gene expression in macrophages during lipoprotein clearance and foam cell formation.

2. Materials and methods

2.1. Proteins and reagents

C1q is routinely isolated from plasma-derived normal human serum and validated for purity and activity as previously described [22]. Sterile filtered human lipoproteins (LDL, acLDL and medium oxLDL) were purchased from Kalen Biomedical (Montgomery Village, MD). Ultrapure LPS (E.coli 0111:B4) was obtained from Invivogen (San Diego, CA).

2.2. Cell isolation and culture

Human blood samples from healthy anonymized donors giving informed consent were collected into heparin by a certified phlebotomist according to the guidelines and approval of California University Long Beach Institutional Review Board. Human monocytes were isolated by counter current flow elutriation as described [26,27]. Cell purity was determined using the Scepter cell analyzer (EMD Millipore, Darmstadt, Germany) and monocyte populations used were greater than 90% pure. Isolated monocytes were cultured for 8 days in RPMI1640, 10% FCS, 2 mM L-Glutamine and 1% penicillin/streptomycin containing 25 ng/ml rhM-CSF (Peprotech, Rocky Hill, NJ) to stimulate differentiation into human monocyte derived macrophages (HMDM). Pooled HMDM from 3 to 10 donors were used to reduce donor variability. HMDM populations were 100% CD11b-positive and 0% CD3-positive by flow cytometry. Raw264.7 cells (ATCC), a murine macrophage cell line, were cultured as described [19].

2.3. Lipoprotein treatment, RNA and supernatant isolation

Macrophages (HMDM or Raw264.7) were harvested with Cellstripper (Corning, Tewksbury, MA) and added to 24- well tissue culture treated plates at 5×10^5 cells per well in X-Vivo15 (Lonza, Walkersville, MD) serum-free defined media. Cells were cultured for 3 h (for RNA isolation) or 18 h (for supernatant isolation) at 37 °C in 5% CO_2 with 10 μg protein/ml LDL, oxLDL or acLDL in the absence or presence of 75 μg/ml C1q. RNA from samples was isolated using Qiagen's RNeasy Mini Kit (Hilden, Germany). Supernatant was centrifuged at 300 × g, 10 min, and stored at −80 °C.

2.4. RNA-sequencing (RNA-seq)

Isolated RNA samples from triplicate experimental replicates were sent to the University of California Irvine Genomics High-Throughput Facility (Irvine, CA) for quantification and RNA integrity determination. All RNA samples used for cDNA library generation were assessed with RIN>9 on the Bioanalyzer RNA nanochip (Agilent Technologies, Santa Clara, CA). RNA-sequencing cDNA libraries were generated from 100 ng total RNA using the TruSeq Stranded mRNA Sample Preparation kit (Illumina, San Diego, CA). Libraries were validated on a Bioanalyzer High Sensitivity DNA chip (Agilent Technologies) and quantified using the KAPA Library qPCR kit at UCI Genomics High-Throughput Facility. Libraries were normalized, pooled, and were sequenced on two separate lanes (technical duplicates), 100bp, single-end on the HiSeq 2500 system (Illumina) at UCI Genomics High-Throughput Facility. Sequences were aligned to reference genome hg38 using CASAVA 1.8.2 software. Differential gene expression was determined using UCI's CyberT in-house software [28]. Statistically significant differences ($*p < 0.05$) were detected using an unpaired *t*-test. Gene ontology analysis was performed using DAVID software (https://david.ncifcrf.gov/) [29]. Select pathways of interest were identified by KEGG and GO pathway enrichment analysis [30]. Gene networks were generated with GeneMANIA (http://www.genemania.org/) [31]. Network interactions were limited to pathway and genetic interactions only. Cytoscape [32] was used to visualize genes within networks of interest that were significantly modulated by C1q in our RNA-seq assay ($p < 0.05$). Significantly up- or down-regulated genes were visualized by color-coding, with color intensity corresponding to extent of modulation.

2.5. Reverse transcription and quantitative real-time PCR (qRT-PCR)

100 ng of total RNA and Moloney murine leukemia virus reverse

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