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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 $55\% \pm 13\%$ reduction, $p = 0.044$) and increase transcriptional activation of PPARs (a maximum $229\% \pm 54\%$ 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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Conflict of interest disclosure

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Graphical abstract

Keywords

Inflammation; atherosclerosis; innate; LDL; JAK-STAT; PPAR

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 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.

Materials and methods

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).

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, 2mM L-Glutamine and 1% penicillin/streptomycin containing 25ng/ml rhM-CSF (Peprotech, Rocky Hill, NJ) to stimulate differentiation into human monocyte derived macrophages (HMDM). Pooled HMDM from 3-10 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].

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 hours (for RNA isolation) or 18 hours (for supernatant isolation) at 37°C in 5% CO₂ 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 xg, 10 minutes, and stored at -80°C.

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.

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

100 ng of total RNA and Moloney murine leukemia virus reverse transcriptase kit (Life Technologies) was used to synthesize cDNA according to manufacturer's instructions. TaqMan Gene Expression Assay probes (Life Technologies) were used to perform quantitative RT-PCR (StepOne, Applied Biosystems). Gene expression was normalized to endogenous control GAPDH, and mRNA levels were expressed as fold changes compared to untreated macrophages. The fold change was determined using the 2^{-C_t} method, as described [33].

STAT1 assays

Levels of total and phosphorylated STAT1 were measured in three populations of HMDM. In treatment group 1, unstimulated HMDM were fed 10 µg protein/ml oxLDL or 10 µg protein/ml acLDL in the absence or presence of 75 µg/ml C1q for 1 or 3 hours at 37°C, 5% CO₂. In group 2, HMDM were treated as in group 1, and stimulated with 20 ng/ml IFN γ 30 minutes prior to harvesting protein lysates. In treatment group 3, M1 -HMDM, cultured in the presence of 20 ng/ml IFN γ and 100ng/ml LPS for 24 hours, were treated as in group 1. The Milliplex MAP Cell Signaling Buffer and Magnetic Bead Detection Kit (EMD Millipore) was used to prepare lysates. Total STAT1 levels were measured on a Luminex Magpix System (EMD Millipore).

PPAR activation assay

Induction of PPAR transcriptional activity was evaluated using a luciferase reporter assay (Qiagen). RAW 264.7 cells were transfected with the provided mixture of the PPAR reporter construct and a constitutively active Renilla construct, using LipofectamineLTX and Plus reagent (Life Technologies) according to the manufacturer's instructions. After 24 hours, cells were incubated with 10-100 µg protein/ml LDL, oxLDL or acLDL in the presence or absence of 75 µg/ml C1q for a further 24 hours. In additional wells, cells were treated with 1 or 10 µM Rosiglitazone as a positive control. Cells lysates were then prepared and the relative firefly and Renilla luciferase activity were measured by the Dual Luciferase reporter assay system using a TD 20/20 luminometer (Promega, Madison, Wisconsin). The firefly luciferase activity was normalized to the Renilla luciferase activity to calculate the relative luciferase activity units (RLU).

IL-6 secretion assay

IL-6 was detected in HMDM supernatant by sandwich ELISA (Peprotech). ELISAs were performed according to manufacturer's instructions.

Statistical analysis

Numerical data represent the mean of at least three independent experiments (n) in which each condition was tested at least in duplicate, unless otherwise stated. Variance of means between two groups was analyzed by unpaired students *t*-test. **p*<0.05, ***p*<0.01. Statistical analyses were performed using GraphPad Prism 5.

Results

C1q modulates gene expression associated with signaling pathways involved in immune responses, transcription and metabolism in HMDM during atherogenic lipoprotein clearance

To perform an unbiased screen of C1q-modulated pathways in macrophages during ingestion of modified atherogenic lipoproteins, global transcriptional gene expression profiles of HMDMs incubated with oxLDL or acLDL in the presence or absence of C1q were analyzed using RNA sequencing (RNA-seq). C1q modulated numerous genes (Fig. 1A) in HMDM during ingestion of either oxLDL or acLDL. 720 genes modulated by C1q

were common to HMDM ingesting either oxLDL or acLDL. The biological processes significantly enriched by C1q as determined by Gene Ontology (GO) analysis, included regulation of intracellular signaling, transcription and immune responses (see Table 1 in [30]). KEGG pathway enrichment analysis and network pathway analysis demonstrated that signaling pathways modulated by C1q during ingestion of modified lipoproteins included downregulation of genes in the JAK-STAT pathway, upregulation of genes involved in nuclear receptor signaling and modulation of genes in TLR and cytokine signaling pathways (Figure 1B and [30]).

C1q downregulates genes involved in JAK-STAT signaling in HMDM during atherogenic lipoprotein clearance

C1q regulation of certain genes in the JAK-STAT signaling pathway identified by an unbiased RNA-seq screen was validated by quantitative RT-PCR (Fig. 2). Gene expression levels of *IFN γ* , *IRF9*, *JAK3*, *STAT1* and *STAT2* were significantly downregulated by C1q in HMDM during ingestion of oxLDL. A significant reduction in gene expression levels of *IRF9* and *JAK3*, and a trend towards a reduction in levels of *STAT2*, by C1q in HMDM during ingestion of acLDL was also observed, in accordance with the RNA-seq results. In contrast to RNA-seq results, C1q-mediated reductions in *STAT6* were not observed by QPCR.

C1q suppresses STAT1 levels and activity in HMDM during atherogenic lipoprotein clearance

To confirm if these observed C1q-mediated reductions in gene expression in the JAK-STAT pathway were seen at the protein level and conferred a functional reduction in activity of this pathway in HMDM, STAT1 assays were performed. Levels of total and phosphorylated STAT1(Tyr701) were measured by Milliplex MAPmate biomarker assay (Fig. 3A). oxLDL and acLDL significantly enhanced levels of total STAT1 (tSTAT1) as early as 1 hour post ingestion. Opsonization of the modified lipoproteins with C1q significantly reduced tSTAT1 to basal levels. IFN γ was added to some treatments for 30 minutes prior to the end of the incubation to induce pSTAT1 activation (Fig. 3B). As expected, IFN γ stimulated detectable levels of pSTAT1(Tyr701) in HMDM at 1 and 3 hours. This is in contrast to control macrophages which were not IFN γ treated, where all values for pSTAT1(Tyr701) were low (< 1 MFI normalized to β -tubulin) regardless of lipoprotein treatment. In IFN γ treated cells, both oxLDL and acLDL significantly enhanced levels of total STAT1 and pSTAT1(Tyr701) after 3 hours. Importantly, if C1q was bound to the modified lipoprotein, these observed increases were significantly reduced to levels similar to IFN γ treated cells which had not ingested lipoprotein. In HMDM polarized towards an M1-macrophage phenotype by chronic stimulation with IFN γ and LPS for 24 hours (Fig.3C), significantly higher levels of pSTAT1(Tyr701) were observed at all time points compared to unstimulated macrophages. Levels of tSTAT1 were similar at 1 and 3 hours, but as might be expected pSTAT1(Tyr701) activation increased over time. At 1 and 3 hours, significant decreases in tSTAT1 were observed in the presence of C1q. Levels of pSTAT1(Tyr701) were significantly increased by ingestion of oxLDL at 1 hour, and this increase was blocked by opsonization with C1q. acLDL had no significant effect on pSTAT1(Tyr701) levels. A similar trend was observed at 3 hours, but differences in pSTAT1(Tyr701) did not reach statistical significance.

C1q upregulates PPAR gene expression in human and mouse macrophages during atherogenic lipoprotein clearance

C1q modulation of *PPARα*, *PPARδ* and *PPARγ* gene expression was validated by quantitative RT-PCR (Fig. 4A). Modified lipoproteins alone downregulated levels of *PPARα* and upregulated levels of *PPARδ* compared to untreated human macrophages. However, levels of *PPARα*, *PPARδ* and *PPARγ* were significantly increased in HMDM when C1q was bound to either oxLDL or acLDL. No significant modulation of PPAR levels was observed in macrophages incubated with C1q alone, or in response to LDL±C1q. To determine if C1q triggered similar responses in mouse macrophages, gene expression of *PPARα*, *PPARδ* and *PPARγ* was also measured in Raw264.7 cells under similar conditions (Figure 4B). In Raw264.7, oxLDL reduced gene expression of all PPARs tested, suggesting that this mouse macrophage cell line does not regulate PPAR expression identically to human primary macrophages. However, similar to HMDM, C1q significantly increased expression levels of *PPARα*, *PPARδ* and *PPARγ* during oxLDL clearance in Raw264.7. This suggests that Raw264.7 macrophages provide an acceptable, if limited model to specifically investigate the role of C1q in PPAR activation.

C1q increases PPAR-mediated transcriptional activity in macrophages during atherogenic lipoprotein clearance

To determine if C1q upregulation of PPAR gene expression altered transcriptional activity of these nuclear receptors, a PPAR luciferase reporter assay was performed (Figure 4C). Incubation of transfected cells with known agonist rosiglitazone showed a dose-responsive increase in relative luciferase units (RLU), suggesting that the plasmid was successfully transfected and was responsive to activation. Incubation of transfected cells with unmodified LDL had no effect on transcription, regardless of the presence of C1q. Incubation of transfected cells with acLDL at 10 µg protein/ml or oxLDL at 100 µg protein/ml increased measured RLU. The presence of C1q significantly enhanced transactivation (RLU) in all conditions.

C1q downregulates IL-6 cytokine expression by HMDM during atherogenic lipoprotein clearance

Since JAK-STAT signaling and PPAR receptor activation have well documented roles in modulating immune responses, C1q modulation of inflammatory cytokine IL-6 was investigated in HMDM. We have previously shown that C1q downregulates IL-6 production during clearance of atherogenic lipoproteins in Raw264.7 mouse macrophages [22]. Gene expression in resting or LPS-activated (M1-like) HMDM was calculated relative to untreated macrophages (Fig. 5). Addition of LPS significantly increased levels of IL-6 at the gene and protein level in all treatment groups, as expected. Ingestion of oxLDL alone did not alter *IL-6* gene expression, but a significant reduction in *IL-6* gene expression was measured in LPS-activated HMDM when C1q was bound to the oxLDL compared to LPS-activated HMDM that had ingested oxLDL alone. To confirm the modulation of IL-6 at the protein level, the concentration of secreted IL-6 was measured by ELISA. Treatment with oxLDL enhanced IL-6 secretion from both resting and LPS-activated HMDM. Importantly, when

C1q was bound to oxLDL, the amount of secreted IL-6 decreased to below even basal levels. This is similar to our observations in mouse macrophages [22].

Discussion

We and others have previously shown that C1q can be a master regulator of macrophage function. Many studies focused on C1q modulation of macrophage responses during apoptotic cell clearance, and its role in the prevention of autoimmunity [20, 21, 33]. However, an additional and important role for C1q in reprogramming macrophage responses in inflammatory disease is also emerging. Data presented here show that C1q directly modulates JAK-STAT and PPAR signaling pathways in HMDM during ingestion of modified lipoproteins, influencing inflammatory responses such as IL-6 production, and potentially a number of other cellular responses such as cholesterol homeostasis and lipid metabolism.

C1q was previously shown to be protective in a murine model of early atherosclerosis [25], although the molecular mechanisms were not explored. Here we examined the responses of primary human monocyte-derived macrophages (HMDM) to interactions with C1q-opsonized lipoproteins, in order to identify potential signaling pathways that may be involved in reprogramming biological functions. Our previous study identified peak C1q transcriptional modulation at 3 hours [22], and therefore this current study was designed to focus on that early timeframe. An unbiased screen of genes that are regulated by physiological levels of C1q in HMDM during clearance of oxLDL or acLDL by RNA-seq determined that C1q significantly modulated 3198 genes (Fig.1A). While there were 720 common genes modulated by C1q bound to oxLDL or acLDL, there were also substantial numbers of genes uniquely modulated by C1q bound to either oxLDL (959 genes) or acLDL (1519 genes). This suggests that while some pathways are triggered by C1q irrespective of target, other pathways are target-specific and the direction or amplitude of modulation by C1q may vary accordingly. Significantly enriched biological processes highlighted a role for C1q in modulation of immune responses during lipoprotein clearance [30]. In addition, a role for C1q in modulation of defense/inflammatory responses and regulation of transcription was also suggested, corresponding to our previous findings in mouse macrophages [22]. Network pathway analysis demonstrated C1q-modulation of multiple genes involved in JAK-STAT pathway signaling. Several molecules of the JAK-STAT signaling pathway were also modulated by C1q in HMDM during apoptotic cell clearance [33] suggesting that this pathway is an important target of C1q-signaling in macrophages during clearance of multiple “damaged-self” targets. JAK-STAT signaling is an intracellular signal transduction cascade that is mediated by either class I or class II interferons binding to their respective receptors. Upon receptor engagement, JAKs phosphorylate and activate STATs, resulting in expression of genes involved in cell proliferation, and inflammation, leading to M1-polarization in macrophages [34, 35]. Type I interferons, IFN α and IFN β , have been shown to accelerate the development of atherosclerotic lesions [36, 37]. Similar studies have shown type II interferon, IFN γ , inhibits cholesterol efflux through STAT1 [38]. Thus, modulation of this pathway may be important in limiting disease progression. C1q-mediated downregulation of specific molecules involved in type I or type II IFN signaling was validated by qRT-PCR, including a reduction in expression of pro-inflammatory

cytokine IFN γ , signal transduction molecule JAK3 and heterotrimeric transcription factor complex components STAT1, STAT2 and IRF9 (Fig. 2). Consistent significant reductions of STAT1 protein levels were also measured in the presence of C1q, even as early as 1 hour after incubation with modified lipoprotein (Fig. 3). The functional relevance of these reductions in gene and protein expression of STAT1 was restricted to conditions where modified lipoproteins activated STAT1. Where oxLDL- and/or acLDL-mediated increases in pSTAT1(Tyr701) were observed in both an acute (30 minutes incubation with IFN γ) and chronic (24 hours incubation with LPS and IFN γ) inflammatory stimulation model, C1q was able to suppress STAT1 activity and restore it to levels similar to IFN γ stimulated macrophages that had not ingested lipoproteins. However, C1q did not reduce activation of STAT1 to basal, unstimulated levels. Macrophages are essentially biosensors, integrating signals from the environment and coordinating appropriate responses. These data suggest that C1q is able to blunt the specific pro-inflammatory signal by modified lipoproteins in macrophages, however activation of pSTAT1 in response to high levels of IFN γ $\pm$ LPS remain intact. Modulation of this pathway by C1q may be solely due to the reduction in tSTAT1 levels observed in the presence of C1q, but additional direct or indirect effects on the activation pathway by C1q signaling at the protein level cannot be ruled out. We have previously reported that C1q also modulates LPS signaling in monocytes/macrophages, including suppression of NF κ B activation [19, 21, 22]. RNA-seq data presented here also highlighted a role for C1q in modulation of TLR signaling pathways, including downregulation of MyD88, an important signal adaptor protein (Figure 1). Crosstalk between LPS and IFN γ signaling in M1 macrophages, is known to converge on STAT1 signaling [39], thus C1q may be regulating these pathways at multiple points. Interestingly, the RNA-seq data indicated that gene expression of anti-inflammatory cytokine IL-10 was downregulated by C1q at 3 hours (Fig. 1B). However, C1q increases secretion of IL-10 protein by HMDM during ingestion of oxLDL and acLDL at 18 hours (unpublished data). Thus, regulation of *IL-10* transcription by C1q is likely at a different, later, time point. This is similar to previous reports in which C1q upregulates IL-10 in HMDM during ingestion of apoptotic cells, where a significant modulation of gene expression of *IL-10* by C1q was not demonstrated until 18 hours [33]

Our RNA-seq screen also implicated C1q in modulation of nuclear receptor activation (Fig. 1B). Of particular interest in macrophages ingesting atherogenic lipoproteins was C1q-mediated upregulation of PPAR gene expression. PPARs are nuclear fatty acid-sensing receptors which play a key regulatory role in not only controlling glucose homeostasis and lipid metabolism, but also macrophage inflammatory responses, lipoprotein uptake and cholesterol efflux [40]. C1q increased gene expression of *PPAR α* , *δ* , and *γ* during ingestion of modified lipoproteins in both human (oxLDL and acLDL) and mouse macrophages (oxLDL) (Fig. 4AB). PPAR γ is of particular relevance to atherosclerosis, as activation of PPAR γ in models of atherosclerosis in vivo was shown to inhibit development of plaques and decrease inflammation in the atherosclerotic lesions [41-43]. Mechanisms of action have suggested that transcriptional activation of PPAR γ or PPAR α during oxLDL uptake improves cholesterol efflux by upregulating ABCA1 [44, 45]. C1q significantly increased PPAR transcriptional activity in macrophages in a luciferase reporter assay, during ingestion of different modified lipoproteins at a variety of doses (Fig. 4C). These increases may be due

to the increased uptake of modified lipoproteins in the presence of C1q (and thus, increased availability of ligand) and/or the increased receptor levels. Importantly, we have previously shown that C1q does not bind to, or increase ingestion of, unmodified LDL [46] and C1q only activates phagocyte responses (e.g. inflammation/phagocytosis) when presented in a multivalent manner, such as bound to a target surface [19, 24]. Therefore, as expected, treatment with soluble C1q or C1q + unmodified LDL had no effect on gene expression or transcriptional activity in our current study (Figure 4A, 4C).

Activation of PPAR γ and PPAR δ has been shown to inhibit inflammatory signaling, such as signaling via IFN γ or LPS, in macrophages, and promote M2-polarization [47, 48]. Thus, since C1q is reducing activation of JAK-STAT signaling, and increasing PPAR signaling, we investigated the outcome on the macrophage immune response through measuring expression of the inflammatory cytokine IL-6, a known target of the JAK-STAT pathway [39]. C1q significantly suppressed gene and protein levels of IL-6 in HMDM. This is similar to our previous data in mouse macrophages during ingestion of atherogenic lipoproteins [22], and in human phagocytes during ingestion of apoptotic cells [19, 20].

In summary, these data highlight an important role for C1q in modulating macrophage responses during clearance of atherogenic forms of lipoproteins. Our data corresponded with our previous finding that C1q modulates TLR signaling and suppresses NF κ B activation [19, 22], but also identified novel mechanisms by which C1q reprograms macrophage function. This includes a significant effect on JAK-STAT and PPAR signaling pathways at the gene and functional levels, leading to a measured reduction in inflammatory signaling in macrophages. Further studies are needed for the delineation of the crosstalk between these pathways and the specific contributions made to different types of inflammatory signals in macrophages. Activation of PPAR signaling may also yield additional potential atheroprotective effects on cholesterol metabolism and foam cell development which warrants further exploration. Improved understanding of mechanisms of macrophage programming is critical in the development and design of improved therapeutic strategies for atherosclerosis and other inflammatory diseases.

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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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Highlights

- Innate immune protein C1q modulates macrophage molecular signaling during atherogenic lipoprotein clearance
- C1q suppresses macrophage JAK-STAT signal transduction and activates PPAR-mediated transcription
- Modulation of these pathways by C1q leads to a reduction in inflammatory response in macrophages.

A.

B.

Fig. 1. RNA-seq analysis of biological processes modulated by C1q

HMDM pooled from 10 donors were incubated with oxLDL or acLDL $\pm$ C1q for 3 h in triplicate. Differentially expressed genes from RNA-sequencing were determined using Cyber-T software (n=3 experimental replicates analyzed in duplicate, $p < 0.05$, t -test).

(A) Libraries were compared to each other to show the intersection of all significant genes modulated by C1q between acLDL or oxLDL treatment. (B) Network diagrams of signaling pathways modulated by C1q in HMDM. Node colors correspond to changes in gene expression (blue=downregulated, white= not modulated, and yellow=up-regulated by C1q, $p < 0.05$).

Fig. 2. Validation of C1q modulation of genes involved in JAK-STAT signaling

Gene expression of *IFN γ* , *STAT1*, *IRF9*, *STAT2*, *JAK3*, and *STAT6* was measured by quantitative PCR from HMDM pooled from 10 donors incubated with oxLDL or acLDL $\pm$ C1q for 3 hours. Data are normalized to *GAPDH* and are fold differences compared to untreated HMDMs $\pm$ SEM ($n = 3$ independent experiments performed in duplicate, * $p < 0.05$, ** $p < 0.01$, t -test).

Fig. 3. STAT1 levels and activity in HMDM during clearance of modified lipoproteins
HMDM pooled from 3 donors were incubated with oxLDL or acLDL $\pm$ C1q for 1 h and 3 h. (A) Unstimulated HMDM, (B) HMDM stimulated with IFN γ 30 min before harvest, (C) M1 polarized HMDM. Total STAT 1 and pSTAT1 (Tyr701) levels in cell lysates were determined by Luminex immunoassay. Data are expressed as MFI $\pm$ SEM and are normalized to GAPDH (tSTAT1) or β -tubulin (pSTAT1) (n=3 independent experiments performed in duplicate, * p <0.05, ** p <0.01, t -test).

Fig. 4. C1q modulation of nuclear receptor signaling

HMDM pooled from 10 donors (A) or Raw264.7 mouse macrophages (B) were incubated with C1q only or LDL, oxLDL or acLDL $\pm$ C1q for 3 hours. Gene expression of *PPAR α* , *PPAR δ* , and *PPAR γ* was measured by quantitative PCR. Data are normalized to *GAPDH* and expressed as fold differences from untreated macrophages $\pm$ SEM (n= 3 independent experiments performed in duplicate, * p <0.05, ** p <0.01, t -test). (C) Raw 264.7 cells, transfected with a PPAR reporter construct and a constitutively active Renilla construct, were incubated with LDL, oxLDL or acLDL $\pm$ C1q, as indicated, or PPAR γ agonist

rosiglitazone for 24 hours. Data are expressed as average Relative Luciferase Units (RLU) $\pm$ SEM (n= 4 independent experiments performed in duplicate, ** $p<0.01$, t -test).

Fig. 5. Measuring cytokine gene expression in HMDM during clearance of oxLDL

HMDM pooled from 3 donors were incubated with oxLDL $\pm$ C1q for 3 h (RNA) or 18 hours (supernatant), in the presence or absence of LPS. (A) Gene expression of *IL-6* was measured in isolated mRNA by quantitative RT-PCR. Data are average fold change from untreated macrophages $\pm$ SEM (n=3 independent experiments performed in duplicate, * p <0.05, t -test). (B) Levels of IL-6 cytokine production were measured by ELISA from supernatants. Data are average concentration (pg/ml) $\pm$ SEM (n=3 independent experiments performed in duplicate, * p <0.05, t -test).