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Complement protein C1q promotes macrophage anti-inflammatory M2-like polarization during the clearance of atherogenic lipoproteins

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Abstract

Objective—Innate immune protein C1q plays a dual role in the chronic inflammatory disease of atherosclerosis. Complement activation via C1q exacerbates pathology in the atherosclerotic lesion in later stages of the disease. However, in early stages of disease C1q is protective. We hypothesize that complement-independent activities of C1q are involved in reprogramming macrophage inflammatory polarization.

Methods—The influence of C1q on macrophage inflammatory responses during clearance of oxLDL was examined. Changes in cytokines at the gene and protein level were measured by quantitative PCR and ELISA assay.

Results—C1q modulated cytokine expression in Raw264.7 macrophages during ingestion of oxLDL. Levels of proinflammatory cytokines IL-1β and IL-6 were downregulated by C1q, whereas levels of the anti-inflammatory cytokine IL-10 were increased. In addition, data from an NFκB-luciferase gene reporter assay suggest that C1q suppresses activation of NFκB during lipoprotein clearance in macrophages, providing one mechanism by which C1q downregulates pro-inflammatory cytokine production.

Conclusions—C1q-polarization of macrophages toward an anti-inflammatory (M2-like) phenotype may be important in dampening inflammation in the early atherosclerotic lesion. Further investigation of molecular pathways targeted by C1q may provide novel therapeutic targets for this disease.

Keywords

Complement; Macrophage; Cytokine; Innate immunity; Atherosclerosis; Oxidized LDL

Introduction

Innate immune protein C1q is a recognition component and initiator of the inflammatory complement cascade [1]. Complement activation by C1q exacerbates many chronic

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Conflict of interest

The authors declare no conflict of interest.

inflammatory diseases, including atherosclerosis [2], which is the predominant contributor to cardiovascular disease, the number one cause of death in the USA today [3]. Many complement components are associated with the atherosclerotic plaque [4], and studies suggest that complement activation products C3a and the membrane attack complex (MAC) promote atherosclerotic lesion development [5, 6]. However, in a mouse model of atherosclerosis ($LDLR^{-/-}$), C1q-deficient mice ($C1q^{-/-}LDLR^{-/-}$) showed an accumulation of apoptotic cells and a greater aortic root lesion size compared to the C1q-sufficient animals. This suggests C1q helps contain the size and complexity of early atherosclerotic lesions; however, the molecular mechanisms of its protective role have not been identified. Independent of its role in complement activation, C1q directly interacts with phagocytes and rapidly enhances phagocytosis of a range of targets, including apoptotic cells, immune complexes and damaged self-molecules [1]. The complement-independent role of C1q is well described in autoimmunity [7], but has not been fully explored in an inflammatory disease such as atherosclerosis.

Modified forms of cholesterol-rich low-density lipo-proteins (LDL), such as oxidized LDL (oxLDL), can accumulate when high concentrations of native LDL are in the blood (reviewed in [8]). Monocytes infiltrate the arterial intima differentiating into macrophages and target the ox-LDL. As a “damaged” molecule, damage-associated molecular patterns (DAMPs) on oxLDL are recognized by pattern recognition receptors on macrophages (reviewed in [9]), leading to the endocytosis of oxLDL via scavenger receptors such as CD36 and LOX-1 [10]. In this capacity macrophages are acting to clean up or “scavenge” these inflammatory molecules. In atherosclerosis, the balance between macrophage cholesterol uptake and efflux is disrupted leading to accumulation of free cholesterol in the cell, and the formation of macrophage foam cells, so-called for their ‘foamy’ lipid droplet-filled appearance [11]. In late stages of disease, inadequate/defective apoptotic foam cell removal by macrophages leads to secondary necrosis and plaque formation. Damage to this plaque by proinflammatory cytokine signaling, proteases and oxygen radicals can cause rupture and thrombus formation, and acute clinical complications such as myocardial infarction and ischemic stroke [12]. Furthermore, cholesterol crystals found inside lesions can activate the NLRP3 inflamma-some in macrophages to produce inflammatory cytokines, such as IL-1β that further push the environment toward inflammation and injury [13].

Macrophages exhibit high levels of plasticity in vivo, and, depending on the signals they receive, can be polarized toward inflammatory (M1) or anti-inflammatory (M2) subsets, with M1 macrophages likely contributing to the inflammatory environment in atherosclerosis [14–16]. The M1 phenotype is associated with production of IL-1β, IL-6, TNFα, and increased activity of iNOS (reviewed in [14]). This contrasts the expression profile of M2 macrophages, which is associated with IL-10 signaling and increased activity of arginase 1 [17, 18]. The proteins produced by macrophages in atherosclerosis have consequences important for the eventual outcome of the disease. Rupturing plaque lesions have been shown to contain predominantly M1 inflammatory cytokines, suggesting that the presence of M1 macrophages is detrimental in late-stage atherosclerosis. In addition, proteins associated with the M1 phenotype, such as IL-1β, are associated with unstable lesions [19]. Studies in $ApoE^{-/-}$ mice have shown that knockdown of IL-1β expression leads to a 30 % reduction in lesion size by the time they are 12–24 weeks old [20]. In contrast,

overexpression of anti-inflammatory M2-associated cytokine IL-10 was shown to suppress atherosclerosis in hyperlipidemic mice [21].

We have previously demonstrated that C1q modulates phagocyte cytokine responses toward a more M2-biased state and dampened M1 responses during the clearance of apoptotic cells [22–24]. This may be at least partially responsible for the autoimmune phenotypes of both human and mice genetically deficient in C1q [25]. In human monocytes we identified the C1q-generation of inhibitory NFκB complexes as one possible mechanism for the observed reduction in pro-inflammatory cytokines. C1q induced the translocation of NFκB p50p50 homodimers in contrast to the LPS-mediated induction of p50p65 heterodimers. NFκB p50p65 heterodimer complexes are known to bind κB promoter elements and induce expression of pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα, as well as other genes involved in initiation of atherosclerosis and foam cell formation (reviewed in [26]). NFκB p50p50 homodimer complexes bind to the κB elements but do not promote transcriptional activity of the promoters [27].

Recent reports have also begun to highlight crosstalk between pathways involved in cholesterol metabolism and in the immune response [13, 28, 29]. In recent studies, we have identified a novel role for C1q in modulation of macrophage lipid metabolism. These studies showed that C1q binds and enhances clearance of modified, atherogenic, lipoproteins, such as oxLDL and acetylated LDL (AcLDL), and increases cholesterol efflux, in cholesterol-loaded primary human monocytes and macrophages [30]. This may be important in preventing, slowing or even reversing foam cell development.

Most complement components are synthesized in the liver and are abundant in plasma. However, since macrophages can be a major source of C1q biosynthesis in vivo [31], C1q may be localized in macrophage-rich tissues, such as the early atherosclerotic lesion, in the absence of other complement components. Therefore, our central hypothesis is that complement-independent actions of C1q are polarizing macrophages toward a protective, anti-atherosclerotic phenotype during ingestion of modified lipoproteins.

Macrophage ingestion of cholesterol and inflammatory responses are key in progression of this disease. These studies aim to explore and define macrophage inflammatory polarization triggered by interaction with C1q during lipoprotein clearance in vitro. Elucidation of the molecules that regulate macrophage polarization and the signaling pathways involved should allow for the development of therapeutic strategies to reprogram inflammatory responses.

Materials and methods

Chemicals and reagents

1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) was obtained from Molecular Probes (Invitrogen, Eugene, OR). Sterile, filtered human LDL and the matched-lot modified form, oxLDL, were purchased from Intracel (Frederick, MD). Fluorescently labeled LDL was prepared using DiI (Molecular Probes) according to the manufacturer's instructions, and as previously described [30]. IL-4 and IFNγ were purchased from Peprotech (Rocky Hill, NJ). C1q was isolated from plasma-derived normal

human serum (NHS) by ion-exchange chromatography followed by size-exclusion chromatography according to the method of Tenner et al. [19] and modified as described [20]. The C1q preparations used were fully active, as determined by hemolytic titration, and homogeneous, as assessed by SDS-PAGE. Protein concentration was determined using an extinction coefficient ($E^{1\%}$) at 280 nm of 6.82 for C1q [21]. Ultrapure LPS was obtained from Invivogen (San Diego, CA).

Cells

Raw264.7 cells (ATCC), a murine macrophage cell line, were cultured in DMEM supplemented with 10 % Fetal Calf Serum (FCS), 100 U/ml Penicillin and 100 μg/ml Streptomycin (Invitrogen), as described [27]. C57bl6 control mice are commercially available (Jackson Laboratory). For obtaining bone marrow-derived macrophages (BMDM), murine femurs were dissected and flushed out with DMEM-2 % FCS. Non-adherent cells were cultured in DMEM supplemented with 10 % FCS, 15 % L929 conditioned medium, Pen/strep in 5 % CO_2 as described in detail in [32]. Expression of macrophage markers CD11b and F4/80 were assessed by flow cytometry using FITC-labeled antibodies (eBioscience, San Diego, CA, USA) to characterize and validate macrophage differentiation and only cells >90 % positive for those markers were used for each experiment.

Lipoprotein clearance assay

Cells harvested using Cellstripper (Corning), were resus-pended at 1×10^6 cells/ml in X-Vivo15 media supplemented with 1 % L-glutamine and 1 % penicillin/streptomycin, and added to 24-well tissue culture-treated plates. Cells were cultured for 30 min at 37 °C in 5 % CO_2 with 1–10 μg protein/ml DiI-labeled lipoproteins as described in the figure legends. Where indicated, DiI-labeled lipoproteins were preincubated with C1q (75 μg/ml) for 10 min at 37 °C prior to being added directly to the cells. After incubation, cells were harvested from wells using 0.25 % trypsin–EDTA (Invitrogen), and ingestion of DiI-labeled lipoproteins was analyzed in at least 10,000 cells by flow cytometry using the FACScan (BD Biosciences, San Jose, CA, USA).

Cytokine gene and protein expression measurements

Raw264.7 cells were added to 24-well tissue culture-treated plates, at 5×10^5 cells/well, in X-VIVO-15 media, which was supplemented with 1 % L-glutamine and 1 % penicillin/streptomycin. OxLDL at 10 μg protein/ml was added to wells in the presence or absence of 75 μg/ml C1q and in the presence or absence of 1–30 ng/ml LPS.

To measure gene expression: after 3 h, RNA was harvested from cells remaining in the wells using an Illustra RNA extraction kit (GE Healthcare), and cDNA was synthesized by RT-PCR using the Moloney murine leukemia virus reverse transcriptase kit (Invitrogen) according to the manufacturer's instructions. Levels of mRNA for GAPDH, IL-1β, IL-6, TNFα, and IL-10 were measured by quantitative RT-PCR (StepOne, Applied Biosystems) using TaqMan Gene Expression Assay probes (Invitrogen). The mRNA expression levels were normalized to GAPDH as an internal standard, and they were expressed as fold difference from Raw264.7-untreated macrophages.

To measure protein levels: after 18 h, supernatants were harvested and centrifuged to remove cellular debris. Secreted cytokine levels were quantified by ELISA using commercially available ELISA development kits from Peprotech (Rocky Hill, NJ; IL-6, TNFα, IL-10) or R&D Systems (Minneapolis, MN; IL-1β) according to the manufacturer's protocol. For IL-1β detection, to induce the production of mature IL-1β, 5 mM adenosine triphosphate (ATP) was added 3 h before harvest of supernatants. Supernatants were concentrated 20-fold via membrane ultrafiltration using a Vivaspin3 10 kDa MWCO (GE Healthcare, Uppsala, Sweden) before use in the IL-1β ELISA.

NFκB-luciferase assay

Induction of NFκB transcriptional activity was evaluated using a luciferase reporter gene driven by κ enhancer, pNFκB (Clontech, Palo Alto, CA). Raw 264.7 cells were transfected with the luciferase reporter construct together with pRL-TK Renilla luciferase internal control vector (Promega, Madison, WI) using Lipofectamine LTX and Plus reagent (Invitrogen) according to the manufacturer's instructions. After 24 h, cells were incubated with 10 μg protein/ml OxLDL in the presence or absence of 75 μg/ml C1q for a further 24 h. Cells were then harvested and the firefly luciferase activity and the Renilla luciferase activity were measured by the Dual Luciferase reporter assay system using a TD 20/20 luminometer (Promega). The firefly luciferase activity was normalized to the Renilla luciferase activity to control for variability in transfection efficiency and to calculate the relative luciferase activity units (RLU).

Results

C1q enhances ingestion of modified LDL by mouse macrophages

We and others have shown that C1q directly binds to apoptotic cells and enhances their clearance by human and mouse phagocytes [23, 33–35]. In addition, we have shown that C1q enhances human phagocyte clearance of oxLDL and AcLDL but not native LDL [30]. To assess C1q-modulation of clearance of atherogenic lipoproteins by murine macrophages, DiI-labeled LDL and oxLDL were incubated with primary murine bone marrow-derived macrophages (BMDM) isolated from control C57b6 mice, and Raw264.7, a mouse macrophage cell line in the presence or absence of purified C1q. Lipoprotein uptake was assessed by flow cytometry. A typical histogram is shown in Fig. 1a. C1q enhanced mouse macrophage clearance of modified LDL (OxLDL), but not unmodified LDL, similar to levels previously seen with human macrophages (Fig. 1b; [30]). Levels of uptake were dose responsive to concentration of oxLDL, and C1q-mediated enhancement of uptake was seen at concentrations of both 1 and 10 lg protein/ml oxLDL using multiple batches of lipo-protein (Fig. 1c). Ingestion of DiI-labeled modified LDL into vesicular compartments was confirmed by fluorescence microscopy (data not shown).

C1q modulates mouse macrophage cytokine expression

We have previously shown that C1q modulates cytokine production in human phagocytic cells during apoptotic cell clearance including upregulation of anti-inflammatory cytokine IL-10 in human monocyte-derived macrophages (HMDM) [23]. However, the degree and direction of modulation differed significantly depending on the differentiation state of the

phagocyte, and the target being ingested. Therefore, we examined the influence of C1q on macrophage cytokine production during ingestion of modified lipoproteins. Cytokine gene expression was measured in Raw264.7 cells incubated with oxLDL in the presence or absence of C1q for 3 h. Gene expression levels of pro-inflammatory cytokines IL-1β, IL-6 and TNFα and anti-inflammatory cytokine IL-10 were calculated relative to untreated macrophages (Fig. 2). Ingestion of oxLDL significantly enhanced macrophage gene expression levels of pro-inflammatory cytokines IL-6 and TNFα, and inhibited levels of IL-10 production in Raw264.7. However, when C1q was bound to oxLDL, gene expression levels of IL-1β and IL-6 were significantly downregulated and levels of anti-inflammatory IL-10 were significantly upregulated. Neither oxLDL nor C1q modulated levels of TNFα significantly. In LPS-activated (M1-like) Raw264.7, levels of all cytokines tested were significantly enhanced by addition of LPS, as expected. Interestingly, levels of LPS-induced IL-1β were downregulated by the presence of oxLDL. However, C1q bound to the oxLDL was able to further significantly downregulate pro-inflammatory cytokine gene expression, and enhance anti-inflammatory IL-10 expression.

To confirm these effects at the protein level, levels of cytokines released by Raw264.7 after ingestion of oxLDL in the presence or absence of C1q were measured by capture ELISA (Fig. 3). As expected, LPS significantly increased levels of IL-1 β produced by macrophages. Surprisingly, oxLDL significantly reduced IL-1β levels secreted by both untreated and LPS-treated macrophages. Levels of IL-1β in the absence of LPS were around the limits of detection for the ELISA (5 pg/ml) and no differences were observed in the presence or absence of C1q. However, C1q did significantly downregulate IL-1 β levels in LPS-activated macrophages. Similar to gene expression patterns, oxLDL enhanced levels of IL-6 protein production, which was downregulated by C1q to below basal levels. In agreement with the gene expression data, LPS treatment significantly enhanced levels of TNFα produced by macrophages but neither oxLDL nor C1q modulated levels of TNFα significantly. OxLDL alone did not modulate levels of anti-inflammatory IL-10 secreted by Raw264.7; however, levels were significantly enhanced when C1q was bound to oxLDL.

C1q inhibits NFκB-mediated transcription

oxLDL and LPS are known to stimulate pro-inflammatory cytokine gene expression through activation of transcription factor NFκB [36]. We have previously identified a role for C1q in the generation of inhibitory NFκB complexes in human monocytes, which correlates with the anti-inflammatory activity of C1q [27]. Therefore, to investigate the effect of C1q bound to oxLDL on transcriptional activity of NFκB, a luciferase reporter gene expressed under the control of κB elements was transfected into RAW 264.7 cells. Incubation of transfected cells with oxLDL alone significantly induced NFκB-driven luciferase activity, as did incubation with LPS (Fig. 4). However, cells which ingested oxLDL bound to C1q showed significant inhibition of oxLDL-induced transactivation in both resting and LPS-treated macrophages (Fig. 4). These data demonstrate that C1q mediates an inhibition of NFκB-stimulated promoter activity. Such inhibition may therefore play a significant role in the C1q modulation of pro-inflammatory cytokine expression in phagocytic cells.

Discussion

Complement protein C1q plays a dual role in atherosclerosis. Activation of complement via the C1q-mediated classical pathway can exacerbate inflammation and pathology. However, C1q was shown to have a protective role in early atherosclerosis in murine models of hyper-cholesterolemia [37]. Our central hypothesis is that complement-independent roles of C1q are responsible for the protective phenotype seen in early atherosclerosis. In support of this, data presented in this study show that opsonization of oxLDL by C1q polarizes macrophages toward an M2-like anti-inflammatory phenotype during the clearance of these atherogenic, damaged forms of lipo-proteins. The data presented here also suggest one molecular mechanism of action of C1q may be via inhibition of NFκB transcription factor activation in macrophages.

Here, we present data that C1q enhances uptake, or clearance, of oxidized LDL but not unmodified LDL (Fig. 1). Ingestion was enhanced in the presence of physiologically relevant amounts of C1q (serum concentration of approximately 75 μg/ml) by 1.59 or 1.55 fold in BMDM or Raw264.7 cells respectively. This is similar to the enhancement of oxLDL ingestion by C1q we have previously reported in human monocyte-derived macrophages (1.60 fold) [30] and thus, these data suggest that primary mouse macrophages (BMDM) and the Raw264.7 macrophage cell line are appropriate model systems for these studies. The role of C1q in activation of phagocytosis of a variety of targets including immune complexes and apoptotic cells is well reported [1]. OxLDL is taken up via scavenger receptors, however, the receptor(s) responsible for triggering the C1q modulation of phagocytosis are not definitively known [38, 39]. C1q-mediated clearance of apoptotic cells has recently been shown to act via upregulation of Mer tyrosine kinase (MerTK) [40]. However, the increased ingestion of C1q-opsonized oxLDL within 30 min is too rapid to require novel protein synthesis, and therefore unlikely to act via MerTK upregulation. It is important to note that although C1q increases ingestion of oxLDL, we have previously shown that the presence of C1q also leads to increased levels of cholesterol efflux and thus the enhanced ingestion of cholesterol does not lead to greater foam cell formation [30].

A role for C1q in prevention of autoimmunity is well described. We and others have previously shown that C1q enhances macrophage ingestion of apoptotic cells and modulates cytokine responses toward an anti-inflammatory M2-like response. This has been observed either in ‘resting’ otherwise unstimulated macrophages or LPS-activated inflammatory macrophages. However, since apoptotic cells themselves trigger anti-inflammatory macrophage responses, this study aimed to investigate if C1q could reprogram macrophage responses to oxLDL, which is not intrinsically anti-inflammatory, but provides a “damage-associated” signal to macrophages. Descriptions of the inflammatory nature of modified forms of LDL are variable in the literature. Cytokine profiles vary according to the extent of the damage (minimally oxidized versus oxidized), the dose (usually 10–100 μg protein/ml), and the type of cell investigated (monocytes, macrophages, endothelial cells, smooth muscle cells). Studies have reported that oxLDL activates the NLRP inflammasome and generates IL-1β in macrophages [41]. However, other studies suggest that observed increases in pro-inflammatory cytokine production may be due to LPS contamination of oxLDL preparations [42]. Using commercially available oxidized LDL (<0.2 endotoxin Units/ml) we did not see

an increase in IL-1β by oxLDL at gene or protein level. In addition, although we did detect significant upregulation of proinflammatory cytokines TNFα and IL-6 at the gene level, increases in secreted cytokine levels were not detected. We also observed a downregulation of gene expression of anti-inflammatory cytokine IL-10 in oxLDL-treated macrophages, but no significant differences in secreted IL-10 protein levels. Thus, ingestion of oxLDL via scavenger receptors in resting macrophages is not significantly modulating secretion of any of the cytokines investigated in this study.

As expected, LPS-activated macrophages expressed significantly enhanced levels of IL-1β, IL-6, TNFα and IL-10 at the gene and/or protein level. Levels of IL-6, TNFα and IL-10 were unchanged during ingestion of oxLDL. However, in contrast to resting macrophages, oxLDL significantly downregulated IL-1β levels in LPS-activated macrophages. This ability of oxLDL to modulate LPS (TLR-4) signaling in macrophages has been previously described [42, 43].

Importantly, when C1q was bound to the oxLDL during macrophage clearance, we observed significant downregulation of pro-inflammatory cytokine IL-6 at gene and protein levels. Pro-inflammatory IL-1β gene transcripts were also suppressed by C1q in resting and LPS-activated macrophages. Secreted IL-1β levels were very low and around the level of detection of the ELISA in the absence of LPS, but were significantly reduced by C1q in LPS-activated macrophages. Studies in human disease and mouse models of hyperlipidemia have shown that proinflammatory cytokines can play a central role in propagating disease progression and that reductions in proinflammatory cytokines like IL-1β and IL-6 could significantly contribute to a reduction in lesion size and abrogation of disease [20, 44].

C1q had no significant effect on TNFα gene or protein expression which is similar to our observations in human macrophages ingesting apoptotic cells. Interestingly, C1q increased levels of anti-inflammatory IL-10 expression at the gene and protein level in both resting and LPS-activated macrophages. Overexpression of IL-10 has previously been shown to suppress atherosclerosis in hyperlipidemic mice [21]. Thus, C1q appears to be able to re-polarize inflammatory M1-like (IL-1β and IL-6 producing) macrophages toward anti-inflammatory, resolving, M2-like (IL-10 producing) macrophages. Cytokine modulation by C1q may be an important mediator of resolution of inflammation in the atherosclerotic lesion, and one mechanism by which C1q is atheroprotective.

To begin to look at mechanisms by which C1q modulates cytokine expression, we investigated activation of transcription factor NFκB by oxLDL and the effect of C1q. NFκB transcription factor activation is important in inflammatory signaling [45]. In particular, Toll-like receptor activation of the canonical pathway, leading to nuclear translocation of NFκB family p65p50 heterodimers is known to be involved in pro-inflammatory cytokine production. We showed that oxLDL significantly increased NFκB transcriptional activity using an NFκB-luciferase reporter assay. OxLDL has previously been shown to activate NFκB in endothelial cells, via the lectin-like oxLDL receptor-1 (LOX-1) which is also expressed in macrophages [46]. In contrast, C1q down-regulated NFκB transcriptional activity in resting and LPS-treated macrophages during oxLDL ingestion. We have previously shown that C1q alters NFκB activation in LPS-treated human monocytes, and

directs nuclear translocation of NFκB family p50p50 homodimers instead [27]. Modulation of NFκB signaling may be one pathway by which C1q repolarizes macrophages toward an M2-like anti-inflammatory phenotype. Future studies will determine other molecular pathways and mechanisms that may be involved.

In summary we show for the first time that C1q down-regulates cytokine production in macrophages during clearance of oxidized LDL, a damaged self-molecule. While studies reporting associations between the complement cascade and atherosclerosis span over four decades, there is still little understanding of the non-complement cascade-related functions of complement proteins in this, or indeed any, inflammatory disease. C1q's protective role in the autoimmune disease is well described [25]. The studies here are the first to identify a similar protective role for C1q in regulating key steps in an inflammatory disease, via direct phagocyte interaction.

Elucidation of the molecular pathways regulating macrophage polarization should provide novel targets for therapeutic reprogramming of inflammatory responses in atherosclerosis. These may include strategies to enhance or mimic protective signaling mechanisms toward macrophage anti-inflammatory (M2) polarization, and/or inhibit detrimental terminal complement pathway activation. Overall, it is hoped that these studies will contribute to shifting the perception of complement as not just an ancient primary line of defense against pathogens, but also a significant regulator of normal phagocyte function.

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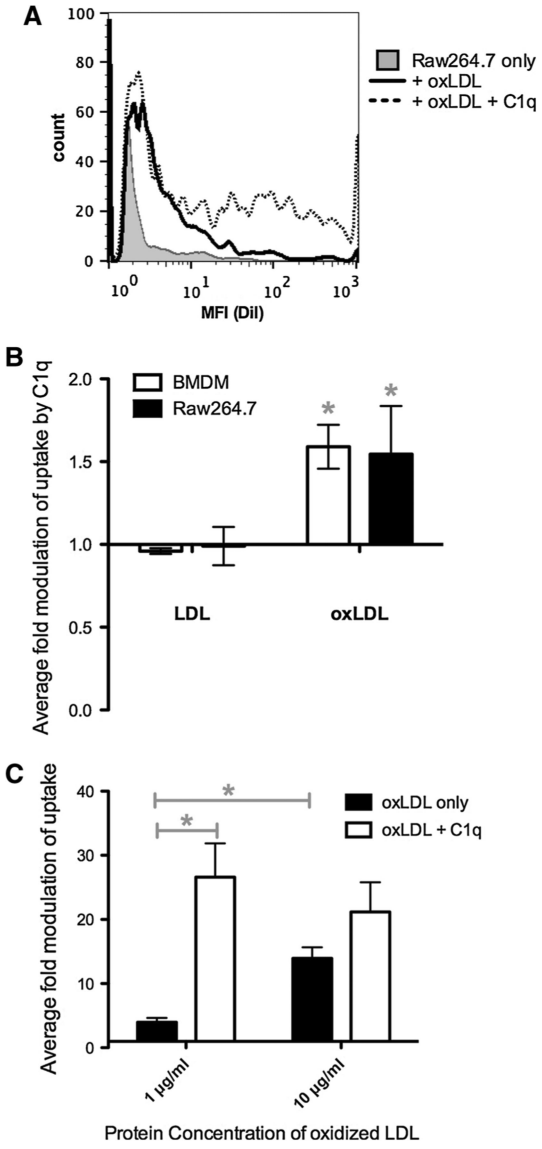

Fig. 1.

Measuring macrophage clearance of oxidized LDL (oxLDL). Raw 264.7 macrophages or murine bone marrow-derived macrophages (BMDM) were incubated with 10 µg protein/ml DiI-labeled LDL or oxLDL in the absence or presence of 75 µg/ml C1q for 30 min at 37 °C. Uptake of DiI-labeled lipoproteins was measured by flow cytometry, with a typical histogram shown (**a**). Levels of uptake of DiI-LDL or DiI-oxLDL were quantified by measuring mean fluorescence intensity (MFI) of macrophage populations. Data are expressed as average fold modulation of uptake (MFI) in the presence of C1q compared to levels of ingestion of DiI-oxLDL in the absence of C1q ($n = 3$, $^*p < 0.05$ paired Student's *t* test) (**b**). Raw 264.7 macrophages were incubated with 1–10 µg protein/ml DiI-labeled oxLDL and levels of uptake in the absence or presence of C1q were quantified by flow cytometry as described above. Data are expressed as average fold modulation of uptake (MFI) compared to untreated macrophages ($n = 3$, $^*p < 0.05$ paired Student's *t* test) (**c**)

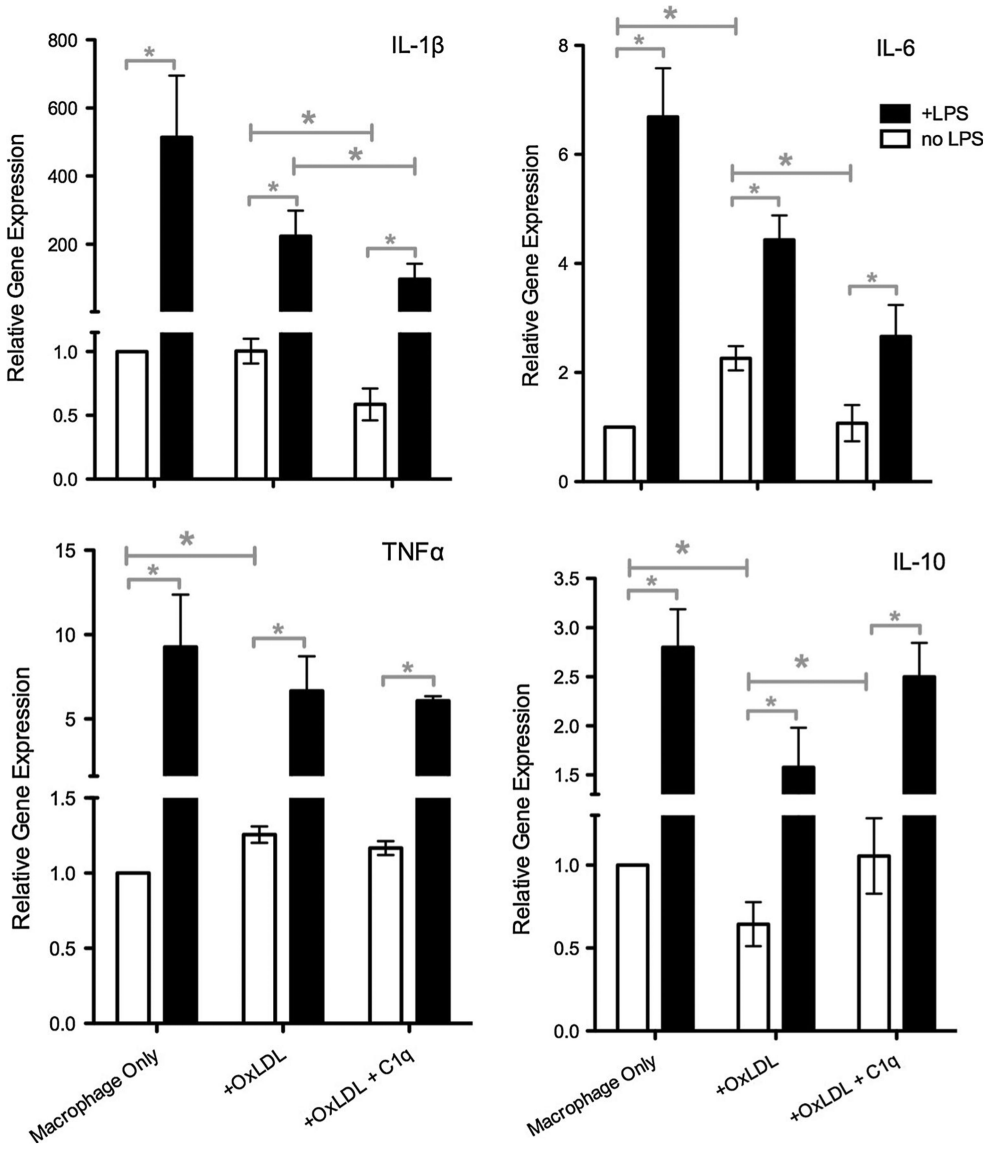

Fig. 2.

Measuring cytokine gene expression in macrophages during clearance of oxLDL. Raw 264.7 cells were incubated with 10 μg protein/ml oxLDL in the absence or presence of 75 μg/ml C1q for 3 h at 37 °C. In some samples, 1 ng/ml LPS was added to mimic infection/ inflammatory conditions. mRNA was isolated and cDNA was prepared. Gene expression of pro-inflammatory cytokines IL-1β, IL-6, TNFα and anti-inflammatory cytokine IL-10 was measured by quantitative PCR. Data are normalized to expression levels of housekeeping gene GAPDH, and are expressed as average relative gene expression compared to levels measured in untreated macrophages (macrophage only) ± SEM. Individual experiments were performed in duplicate, and data shown are averages from 3 to 6 individual experiments ($n = 3–6$, $*p < 0.05$ paired Student's t test)

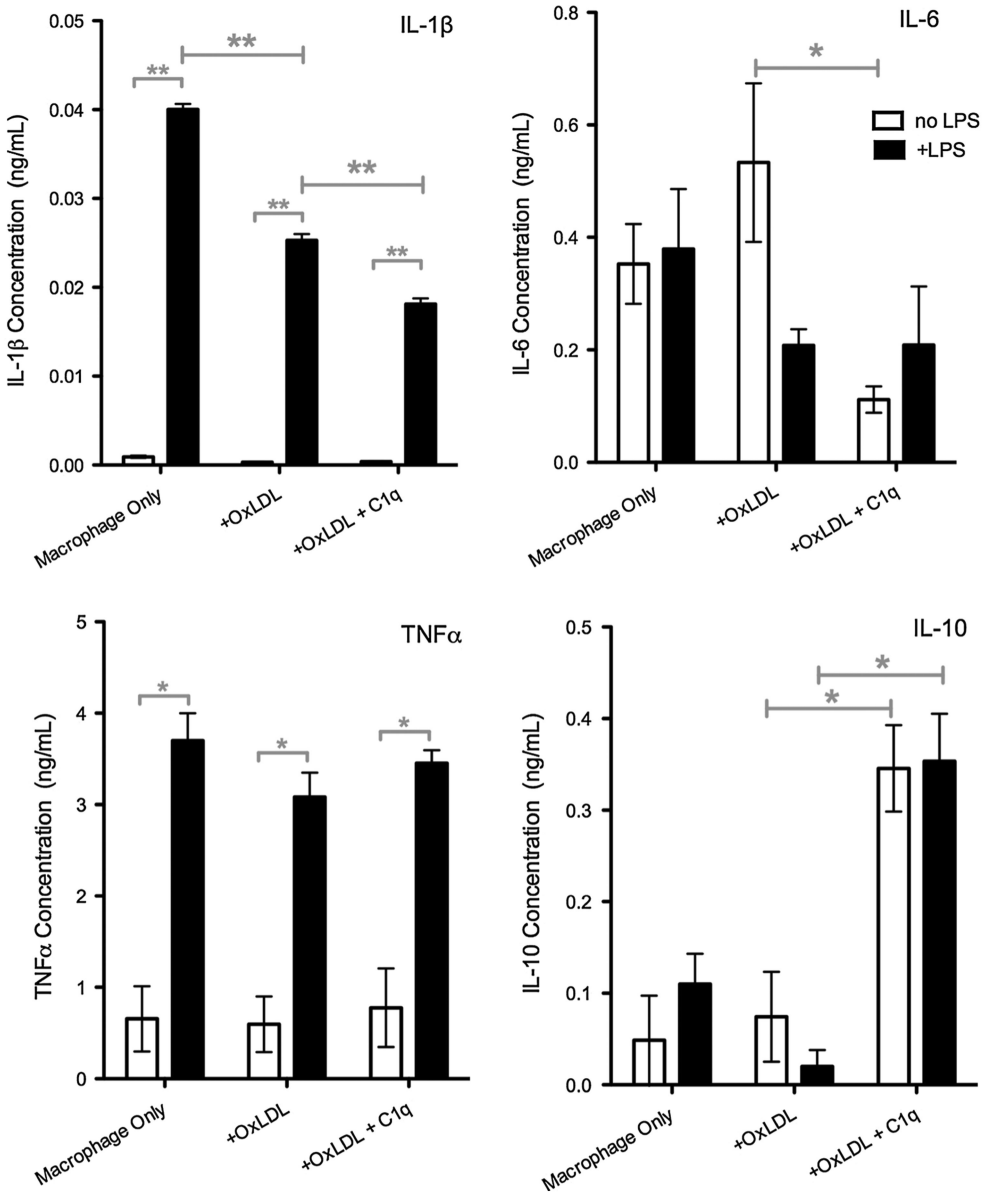

Fig. 3.

Measuring cytokine production by macrophages during clearance of oxLDL. Raw 264.7 cells were incubated with 10 μg protein/ml oxLDL in the absence or presence of 75 μg/ml C1q for 18 h at 37 °C. In some samples, 1 ng/ml LPS (IL-6, TNFα, IL-10) or 30 ng/ml LPS and 5 mM ATP (IL-1β) were added. Levels of cytokines produced were measured by ELISA. Data are expressed as average concentration (ng/ml) ± SEM ($n = 3–4$, *$p < 0.05$, **$p < 0.01$, paired Student's t test)

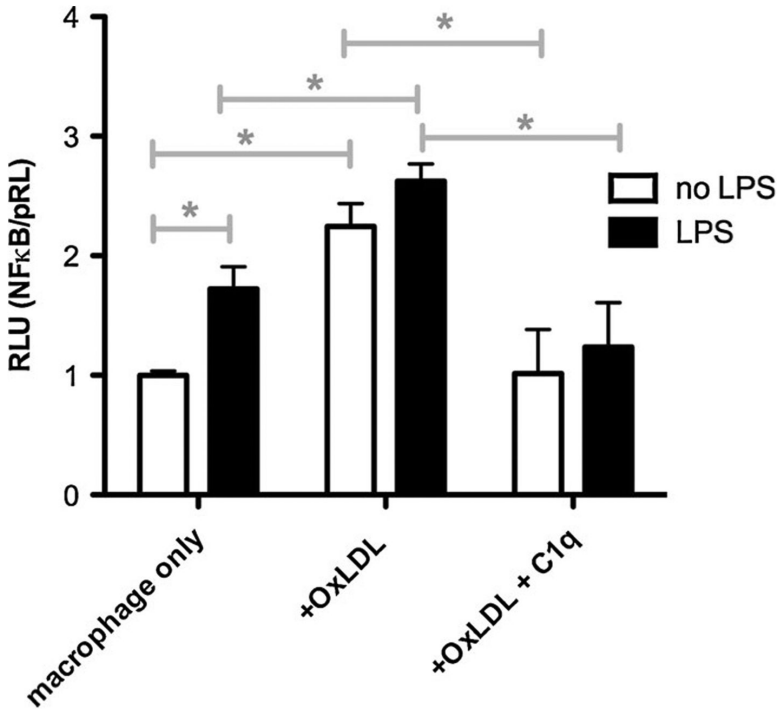

Fig. 4.

Measuring NFκB transcriptional activity by macrophages during clearance of oxLDL. Raw 264.7 cells co-transfected with an NFκB-luciferase reporter construct and pRL-TK Renilla transfection control plasmid, were incubated with 10 lg protein/ml oxLDL in the absence or presence of 75 μg/ml C1q for 24 h at 37 °C. In some samples, 100 ng/ml LPS was added to mimic infection/inflammatory conditions. Levels of luciferase produced were measured using a dual luciferase assay. Relative luciferase units (RLU) were calculated after normalizing to Renilla luciferase values. Data are expressed as average RLU ± SEM ($n = 3$, $^*p < 0.05$, paired Student's t test)