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Victoria Espericueta[1], Ayla O. Manughian-Peter[1], Isabelle Bally[2], Nicole M. Thielens[2] and Deborah A. Fraser[1]

[1.] Department of Biological Sciences, California State University Long Beach, CA, USA.

[2.] Univ. Grenoble Alpes, CEA, CNRS, IBS, F-38000 Grenoble, France.

Running Title

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Corresponding Author

Deborah A. Fraser
1250 Bellflower Blvd, CSULB, Long Beach CA 90840
Phone: 562-985 7597
Fax: 562-985 8878
Email: Deborah.fraser@csulb.edu

Key Words

Complement, C1q, phagocytosis, macrophage, inflammation

Abbreviations

CLR	collagen-like region
HMDM	human monocyte derived macrophages
HSA	human serum albumin
oxLDL	oxidized LDL
shEA	antibody-opsonized sheep erythrocytes

Abstract

Complement protein C1q plays a dual role in a number of inflammatory diseases such as atherosclerosis. While in later stages classical complement pathway activation by C1q exacerbates disease progression, C1q also plays a beneficial role in early disease. Independent of its role in complement activation, we and others have identified a number of potentially beneficial interactions of C1q with phagocytes *in vitro*, including triggering phagocytosis of cellular and molecular debris and polarizing macrophages toward an anti-inflammatory phenotype. These interactions may also be important in preventing autoimmunity. Here, we characterize variants of recombinant human C1q (rC1q) which no longer initiate complement activation, through mutation of the $C1r_2C1s_2$ interaction site. For insight into the structural location of the site of C1q that is important for interaction with phagocytes, we investigated the effect of these mutations on phagocytosis and macrophage inflammatory polarization, as compared to wild-type C1q. Phagocytosis of antibody coated sheep erythrocytes and oxidized LDL was measured in human monocytes and monocyte-derived macrophages (HMDM) respectively that had interacted with rC1q wild-type or variants. Secreted levels of cytokines were also measured in C1q stimulated HMDM. All variants of C1q increased phagocytosis in HMDM compared to controls, similar to native or wild-type rC1q. In addition, levels of certain pro-inflammatory cytokines and chemokines secreted by HMDM were modulated in cells that interacted with C1q variants, similar to wild-type rC1q and native C1q. This includes downregulation of IL-1α, IL-1β, TNFα, MIP-1α, and IL-12p40 by native and rC1q in both resting and M1-polarized HMDM. This suggests that the site responsible for C1q interaction with phagocytes is independent of the $C1r_2C1s_2$ interaction site. Further studies with these classical pathway-null variants of C1q should provide greater understanding of the complement-independent role of C1q, and allow for potential therapeutic exploitation.

1. Introduction

C1q is the recognition component of the classical complement pathway. In the blood C1q is predominantly found in complex with two copies each of proenzymes C1r and C1s ($C1r_2C1s_2$), termed the C1 complex (1). As a pattern recognition receptor (PRR) of the innate immune system, C1q is able to recognize a wide variety of targets including immune complexes, pathogen-associated molecular patterns (PAMPs), apoptotic cell-associated molecular patterns (ACAMPs) and damage-associated molecular patterns (DAMPs) such as oxidation neoepitopes on low density lipoproteins (oxLDL). Binding of C1q to a target leads to conformational changes within the C1 complex that allow for C1r to be cleaved autocatalytically, activating C1s, and resulting in downstream activation of the classical complement cascade. The three major outcomes of complement activation are opsonization of targets with complement fragment C3b to enhance phagocytosis, production of proinflammatory anaphylatoxins C3a and C5a leading to leukocyte recruitment to the area of activation, and lysis of targets via production of the membrane attack complex (MAC) (2). Complement activation by C1q is critical in controlling certain infections, but can also exacerbate many chronic inflammatory diseases, including atherosclerosis (3) and Alzheimer's disease (4, 5). However, C1q likely plays a dual role in inflammatory disease, and has been shown to have a protective role in mouse models of early atherosclerosis and Alzheimer's disease (6, 7). Many of the beneficial effects of C1q appear to be complement ($C1r_2C1s_2$) independent, and involve direct interactions of C1q with phagocytic cells. C1q modulates phagocyte responses including enhancement of phagocytosis/efferocytosis, and suppression of inflammatory responses (8, 9).

C1q is a complex molecule comprised of 18 polypeptide chains (6A, 6B and 6C). The A, B and C-chains are encoded by three individual genes (*C1QA, C1QB* and *C1QC*) (10). Each individual chain shares a similar structure, with a collagen-like region (CLR) comprising repeating Gly-X-Y triplets (where X is often proline and Y is often hydroxylysine or hydroxyproline) and a C-terminal globular head domain (gC1q). This structure is also shared by other members of the defense collagen family of proteins such as mannose binding lectin (MBL), surfactant proteins A and D and ficolins (8, 11). The CLR of C1q associate through disulfide bonds at the N-terminal ends to form A-B and C-C dimers. These dimers associate non-covalently (A-B-C) to form a triple helical structure in the CLR. Electron microscopy data show clearly that fully assembled C1q adopts a structure similar to a bouquet of flowers, with the 18 polypeptides forming the N-terminal

collagen-like tail diverging via a bend or hinge region to produce six individual globular head domains (12). The hinge is produced via a disruption in the collagen-like Gly-X-Y amino acid sequence and has been localized about half way through each of the A, B or C-chain CLRs. The binding site for C1r and C1s was also identified in the CLR, between the kink region and the globular domain (13). A previous study using recombinantly expressed C1q variants carrying mutations of LysA59, LysB61 and LysC58, identified specific lysine residues on the B- and C-chain (rC1qB, rC1qC) as critically important in binding and activation of the $C1r_2$-$C1s_2$ proenzymes (14).

A number of receptors have been identified to bind to C1q via its globular domains (gC1qR) or the collagen-like domain (cC1qR, CD91, CD93) (15, 16). However a single receptor through which C1q mediates its modulation of phagocyte functions has not been definitively identified. Studies using purified globular head domains of C1q (isolated by collagenase digestion of intact C1q), or purified collagen 'tails' of C1q (isolated by pepsin digestion of intact C1q) identified that modulation of phagocyte function is triggered via the collagen-like domain (17). The specific region within the C1q collagen-like domain that interacts with phagocytes has not yet been identified. However, previous studies identified a specific sequence in the collagen domain of MBL that is critical for the enhancement of phagocytosis (18). Since MBL and C1q share structural and functional similarities, it is likely that a similar domain exists in C1q. To gain further insight into the structural location of the site of C1q that is important for interaction with phagocytes, here we determined if the previously described C1q classical pathway-null variants containing lysine mutations in the C1q B-chain (rC1qB) or C-chain (rC1qC), retained their ability to modulate phagocytic cell functions (14).

2. Methods

2.1 Proteins and Reagents

Plasma 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). During the purification of C1q, serum depleted of C1q (C1qD) was collected after passage of plasma-derived serum in 25 mM EDTA (to dissociate C1q from C1r and C1s) over the ion-exchange resin and stored at -70˚C until use. Recombinant WT C1q (WT rC1q) and the variants of recombinant human C1q which contain a single amino acid mutation of the C1r/C1s binding site in either the B-chain (rC1qB) or C-chain (rC1qC) were expressed and purified from HEK293-F cells as described (14). Highly oxidized LDL (oxLDL) was purchased from Kalen Biomedical (Montgomery Village, MD). Fluorescently-labeled oxLDL was prepared using 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes) according to the manufacturer's instructions, and as previously described (21). Ultrapure LPS (E. coli 0111:B4) was obtained from Invivogen (San Diego, CA). Recombinant human macrophage-colony stimulating factor (rh-M-CSF) and IFNγ protein were purchased from Peprotech (Rocky Hill, NJ). All cell culture reagents were purchased from Life Technologies (Carlsbad, CA) unless otherwise stated.

2.2 Complement C1q Hemolytic Titer

Immune complexes of suboptimally opsonized sheep erythrocytes (shEA) were prepared as previously described (22) using a 1:3,200 dilution of hemolysin antibody (Complement Technology Inc., Tyler TX). Serial dilutions of C1q or HSA control were made into gelatin veronal buffer containing magnesium and calcium (GVB++) from a starting concentration of 300 μg/ml. 2 μl of each protein were added to 300 μl C1q-depleted serum supplemented with 3 mM Ca^{2+}, 100 mM Mg^{++} (C1qD), as a source of additional complement components and 80 ml shEA at 1 x 10^5 cells/ml. Tubes were incubated at 37ºC for 30 min before dilution with GVB++. Intact shEA were pelleted by centrifugation and absorbance of supernatant was read at 412 nm to determine relative amounts of hemoglobin released as a measure of lysis. A_{412} of shEA in water or GVB++ were used as positive (100% lysis) or negative (background, 0% lysis) controls respectively. Percent lysis relative to water for each dilution was calculated after removal of background. The measure of

complement hemolytic activity, $CH_{50,}$ is calculated as the concentration of protein able to induce 50% of maximal hemolysis.

2.3 Cell isolation and culture

Human blood samples from healthy anonymized donors giving informed consent were collected into EDTA by a certified phlebotomist according to the guidelines and approval of California State University Long Beach Institutional Review Board. Primary human monocytes were isolated using the Dynabeads Untouched Human Monocyte Kit from Invitrogen (Carlsbad, CA) according to the manufacturer's protocol. Cell purity was determined using the Scepter cell analyzer (EMD Millipore, Darmstadt, Germany). Isolated monocytes were used in phagocytosis assays (purity >93%) or cultured for 7-13 days in RPMI 1640, 10% FCS, 2-mM L-glutamine and 1% penicillin/streptomycin containing 25 ng/ml rhM-CSF (Peprotech, Rocky Hill, NJ) to stimulate differentiation in human monocyte derived macrophages (HMDM). Expression of macrophage markers CD11b and F4/80 were assessed by flow cytometry using FITC-labeled antibodies (eBioscience, San Diego, CA) to characterize and validate macrophage differentiation and were >94% population for each experiment.

2.4 Phagocytosis Assay

Phagocytosis assays were performed essentially as described previously (23). Briefly, LabTek chambers (Nunc, Rochester, NY) were coated with HSA, plasma C1q, recombinant WT C1q or recombinant C1q variants rC1qB or rC1qC at 5 μg/ml in coating buffer (0.1 M carbonate, pH 9.6), and incubated at 37ºC for 2 h. Chambers were washed twice with PBS and monocytes at 2.5 x 10^5/ ml in phagocytosis buffer (RPMI, 2 mM L-glutamine, 5 mM $MgCl_2$) were added to chambers. Slides were centrifuged at 70 g for 3 min and cultured for 30 min at 37ºC in 5% CO_2. Sheep erythrocytes suboptimally opsonized with IgG were used as phagocytic targets and prepared in gelatin veronal buffer (GVB++) as described above. 10^7 targets were added to each well and after centrifuging at 70 g for 3 min, incubated for an additional 30 min at 37ºC in 5% CO_2. Uningested targets were lysed with ACK, and cells were fixed in 1% glutaraldehyde in PBS. Cells were visualized using a modified Giemsa stain and at least 200 cells/well counted. Percent phagocytosis is the % cells scored that have ingested at least one target. Phagocytic index is the average number of ingested targets per 100 cells counted.

2.5 Lipoprotein Clearance Assay

Wells of a 96-well plate were coated with 5 μg/ml HSA or C1q (plasma derived, or recombinant variants) in coating buffer for 2 h at 37°C and washed 2x in sterile PBS. HMDM were harvested using Cellstripper (Corning), and resuspended at 1×10^6 cells/ml in phagocytosis buffer, before being added to the coated wells. Cells were cultured for 30 min at 37°C in 5% CO_2 before addition of 10 μg protein/ml DiI-oxLDL for an additional 30 min. After incubation, cells were harvested from wells using 0.25% trypsin-EDTA (Invitrogen), and ingestion of DiI-labeled lipoproteins was analyzed in at least 5,000 cells by flow cytometry using the Sony SH800 Cell Analyzer (Sony). Data analysis was performed using FlowJo software (Ashland, OR).

2.6 Lipoprotein Binding Assay

Wells of a 384-well plate (ThermoFisher) were coated with oxLDL at 50 μg protein/ml in PBS, and blocked with PBS/ 5% milk as described previously (21). Dilutions of control protein HSA or purified C1q in PBS/1% milk were incubated for 2 h at room temperature. After washing with PBS/0.05% Tween, monoclonal anti-C1q 1H11(24) (0.5 μg/ml in PBS/1% milk) was incubated in wells for 90 min at room temperature. Wells were washed prior to incubation with HRP-conjugated anti-mouse IgG secondary antibody (1:1,000 dilution; ThermoFisher Scientific) for 45 min at room temperature. The binding assay signal was developed by the addition of substrate TMB (ThermoFisher Scientific). Binding was assessed by measurement of the average absorbance of triplicate sample wells at 450 nm.

2.7 Cytokine Analysis

Wells of a 96-well plate were coated with 5 mg/ml HSA or C1q (plasma derived, or recombinant variants) in coating buffer as described above. HMDM were added at 1 x 10^6 cells/ml in HL-1 serum-free defined media (supplemented with 2 mM L-glutamine, 10 mM HEPES, 5 mM $MgC1_2$). Cells were cultured for 24 h at 37° C in 5% CO_2. In some wells, 20 ng/ml IFNγ and 100 ng/ml LPS were added for M1 macrophage polarization. ATP was added at 1 mM for the final 3 h of incubation (to provide a second signal for inflammasome activation, to measure IL-1β). Supernatants were harvested, and centrifuged to remove cellular debris and stored at -80° C until use. Secreted cytokine levels were quantified by Luminex multiplex analysis using the Milliplex Human Cytokine Panel (Millipore) according to the manufacturer's protocol.

211

212 *2.8 Statistical Analysis*

213 All individual experiments were performed using 2-3 technical replicates. Experiments using
214 cells (primary human monocytes or monocyte-derived macrophages) were repeated with cells
215 from 3-4 independent donors. Results were calculated as means ± SD. Treatment groups were
216 compared by one- or two-way ANOVA using GraphPad Prism as appropriate. Post-hoc multiple
217 comparisons tests were performed where indicated, as described in figure legends. Differences
218 were considered significant when p-value was <0.05.

219

3. Results

3.1 Recombinant C1q variants rC1qB and rC1qC do not activate complement.

Expression and functional characterization of recombinant C1q variants with mutations in B-chain Lys61 (rC1qB) or C-chain Lys58 (rC1qC) have previously been described (14). rC1qB and rC1qC were shown to have reduced interactions with C1r and C1s and a concomitant reduction in C1 activation. To validate these data, and to compare activity of recombinant WT C1q with native C1q purified from normal human plasma for these studies, a C1q hemolytic titer was performed (Figure 1). Concentrations of C1q used were from 0.1 – 300 μg/ml (physiological plasma levels of C1q are around 75 μg/ml). Concentrations of C1q proteins were normalized by A_{280nM} using an extinction coefficient ($E^{1\%}$) of 6.82 (25). As expected, plasma C1q exhibited a dose-dependent increase in hemolytic activity, (CH_{50} = 4.9 μg/ml). WT rC1q also activated complement to a similar extent (CH_{50} = 14.7 μg/ml). In this assay, importantly, the C1q B- and C-chain variants did not activate hemolysis above the background, HSA, control levels.

3.2 C1q variants rC1qB and rC1qC activate phagocytosis in human monocytes, similar to plasma C1q.

To determine if the variants rC1qB and rC1qC are able to enhance phagocytosis, a phagocytosis assay was performed in primary human monocytes using suboptimally opsonized sheep erythrocytes (shEA) as the immune complex-like target (Figure 2). HSA (control) or C1q proteins were immobilized on the surface of a chamber slide and allowed to interact with monocytes before addition of shEA. Both the % cells that ingested at least one target (% phagocytosis) and the average number of targets ingested per 100 cells (phagocytic index) were significantly increased in monocytes that interacted with any of the forms of C1q tested compared to the HSA control (one-way ANOVA with multiple comparisons). In addition, there were no significant differences observed among means of C1q samples for % phagocytosis ($p=0.4351$, one-way ANOVA) or phagocytic index ($p=0.0830$, one-way ANOVA).

3.3 C1q variants rC1qB and rC1qC bind and enhance HMDM clearance of oxLDL, similar to plasma C1q

We tested if C1q variants rC1qB and rC1qC could also promote clearance of a damaged-self target, oxidized LDL (oxLDL). C1q proteins or HSA control were immobilized on a plate (Figure 3), or added in solution at 75 μg/ml (Supplemental Figure 1A), along with addition of 10 μg protein/ml oxLDL, and interacted with HMDM. Ingestion of fluorescently-labeled oxLDL (DiI-oxLDL) by HMDM was measured by flow cytometry. Forward scatter (FSC) and side scatter (SSC) parameters were used to exclude dead cells/debris (Supplemental Figure 1B). Histograms of HMDM only (no oxLDL) were used to determine background fluorescence in each experiment (DiI-) and to set a gate to measure % cells that were DiI-positive (DiI+) (Supplemental Figure 1C). Similar to the phagocytosis assay using shEA targets, HMDM that interacted with any of the forms of C1q tested had significantly enhanced clearance of DiI-oxLDL compared to cells incubated with control protein HSA (Figure 3A). Both the % cells that were DiI+ and the median fluorescence intensity were significantly increased in the presence of C1q (one-way ANOVA with multiple comparisons). Again, there were no significant differences observed among means of C1q samples for % uptake ($P=0.0511$, one-way ANOVA) or MFI ($p=0.7773$, one-way ANOVA).

We have previously shown that C1q binds damaged-self molecules like modified (but not unmodified) forms of LDL, and enhances clearance by monocytes and macrophages (21). Here we investigated if C1q variants rC1qB and rC1qC retained this ability. A plate binding assay was performed, where oxLDL was coated on the surface of a well, and C1q binding was detected by immunoassay (Figure 3B). All forms of recombinant C1q (WT, rC1qB and rC1qC) showed dose-dependent increases in absorbance at 450 nm (A_{450}) as a measure of binding that was equivalent to the binding seen with plasma C1q. HSA was included as a background control.

3.4 C1q variants rC1qB and rC1qC modulate HMDM cytokine and chemokine responses, similar to plasma C1q

Interaction with C1q has been previously shown to modulate certain cytokines towards an anti-inflammatory response in numerous types of phagocytic cells (9). To determine if rC1qB and rC1qC variants modulate HMDM responses similar to plasma C1q and WT rC1q, Luminex assays were performed. Secreted levels of a panel of cytokines, chemokines and growth factors in supernatants from resting and M1-polarized HMDMs that had interacted with immobilized plasma C1q or recombinant forms of C1q were measured by multiplex analysis (Figures 4, 5 and

S2) and compared to HSA. As expected, levels of almost all secreted chemo/cytokines (14/15) were significantly higher in M1-polarized inflammatory macrophages compared to the resting HMDM. Interaction with plasma C1q and recombinant C1q (WT, rC1qB, rC1qC) significantly reduced the secreted levels of pro-inflammatory cytokines IL-1α, IL-1β, TNFα and IL-12p40 (Figure 4). Conversely, levels of anti-inflammatory cytokine IL-10 were increased in resting HMDM that interacted with C1q, compared to HSA, although this did not reach statistical significance. Interaction with all forms of C1q also significantly modulated the secreted levels of certain chemokines (Figure 5). Levels of CCL3/ MIP-1α and CXCL10/IP-10 were downregulated by C1q while CXCL1/GRO and CXCL8/IL-8 were upregulated by C1q. Expression levels of additional cytokines (IL-1Ra, IL-4, Il-6), chemokines (CCL2, CCL5, CCL11) and certain growth factors (EGF, FGF-2, VEGF) were not significantly affected by C1q (Supplemental Figure 2). Importantly, for all chemo/cytokines tested, no significant differences were observed between the levels secreted by HMDM that interacted with plasma or WT rC1q and the levels secreted after interaction with rC1qB and rC1qC variants.

4. Discussion

C1q plays an integral role in the defense against infection through complex with $C1r_2C1s_2$ and activation of the classical complement pathway. However, C1q also has an important non-complement associated role (in the absence of C1r or C1s) via direct opsonization of cellular debris or apoptotic cells. Here we present data showing that mutations of residues in the site of interaction with $C1r_2C1s_2$ do not affect the ability of these C1q variants to opsonize damaged-self targets, enhance phagocytosis and modulate macrophage inflammatory polarization. Understanding which regions of the C1q molecule are necessary for individual functions could assist in the design of therapeutic agents for inflammatory disease.

Expression of recombinant C1q, including variants containing B-chain Lys61-Ala mutations (rC1qB) or C-chain Lys58-Ala (rC1qC) mutations from wild-type were first described by Bally et al. (14). In this previous study, the C1q variants did not activate C1, unlike wild-type rC1q or serum derived C1q. This was tested using IgG-ovalbumin immune targets and assessed by C1s Western blot analysis. Here we performed an additional assay to test classical complement pathway activation by these variants using a C1q-specific hemolytic titer assay. Data in Figure 1 show that although dilutions of plasma purified C1q and wild-type rC1q were able to reconstitute complement activity (leading to hemolysis of immune complex targets) in C1q-depleted serum, the variants rC1qB and rC1qC could not. This is consistent with their reported defect in interaction with $C1r_2C1s_2$ and inability to form a stable C1 complex. The variation in CH_{50} activity between plasma derived C1q (CH_{50} = 4.9 μg/mL) and WT rC1q (CH_{50} = 14.7 μg/mL) is minor and may be due to the presence of a C-terminal FLAG tag in the C-chain of these recombinantly expressed proteins.

Many previous studies have shown that C1q enhances phagocytosis of a variety of targets in a wide range of phagocytic cells (9). We performed phagocytosis/clearance assays using primary human monocytes (Figure 2), and suboptimally opsonized sheep erythrocytes as our immune complex-like target and using human macrophages (Figure 3A) as our phagocytes and fluorescently labeled damaged-self molecule oxLDL as our target. For these studies C1q was immobilized on the surface of a plate, as a model system to mimic its multivalent presentation when attached to a target surface. In our phagocytosis assay, all forms of C1q tested significantly enhanced both the % cells undergoing phagocytosis and the average number of immune complex

targets each monocyte ingested above levels seen with HSA control protein. In addition, all forms of C1q modulated phagocytosis to a similar extent suggesting that the mutations in the B- and C-chains of C1q that abrogate complement activity do not affect phagocytic capabilities. This was supported in our clearance assay where HMDM were shown to ingest significantly higher amounts of DiI-labeled oxLDL when interacting with C1q compared to HSA, but no differences in levels between forms of C1q were identified (Figure 3A). Importantly, similar results were obtained when measuring HMDM phagocytosis of DiI-labeled oxLDL incubated with 75 μg/ml HSA, or C1q in a soluble system to more closely resemble physiologic conditions where C1q would be bound to a target (Supplemental Figure 1A). These data suggest that mutation of the region of interaction with $C1r_2C1s_2$ in the variants of C1q (rC1qB, rC1qC) does not negatively affect their ability to interact with, and activate, a variety of phagocytes. This is consistent with previous studies that identified a critically important region in the collagen-like domain of a similar molecule, MBL that is necessary for the enhancement of phagocytosis. MBL and C1q share structural similarities; MBL has an amino terminal collagen-like domain and a C-type lectin binding domain. It forms an oligomeric structure comprised of multimers of a 3 identical chain subunit. They also share functional similarities. For example, both are defense collagens, activating the classical (C1q) or lectin (MBL) pathways of complement, both are also able to directly interact with phagocytes and activate phagocytosis and modulate phagocyte inflammatory responses to certain targets (8, 21, 22). The specific sequence in MBL critical for phagocytosis was determined to be GEKGEP, found in each identical chain of the MBL oligomer, just below the hinge/kink region (18). It is likely that a similar domain exists in C1q, and the authors of this study hypothesize it may be the GEQGEP sequence in the human C1q A-chain, also located below the hinge region (see graphical abstract). Since C1q is a highly complex molecule, with a three-chain structure it has historically been problematic to express recombinantly. With these newly identified methods for producing active, intact rC1q, it may at last be possible for future studies to test the involvement of this GEQGEP sequence in C1q activation of phagocytosis, and to identify putative phagocytic receptors for C1q.

We and others have previously showed that C1q binds modified forms of LDL (21, 26). Our binding assay showed that C1q variants were also able to bind to oxLDL to a similar extent as plasma C1q or WT rC1q (Figure 3B). This is consistent with the idea that C1q likely binds to

356 modified forms of LDL via its globular head domain, thus leaving the collagen-like domain
357 available for phagocyte interactions.

358 C1q has a well-described role in the prevention of autoimmunity, and some beneficial roles
359 in inflammatory diseases like atherosclerosis and Alzheimer's disease (3, 27, 28). Most
360 complement components are synthesized in the liver and are abundant in plasma. However, since
361 macrophages can be a major source of C1q biosynthesis *in vivo* (29), C1q may be localized in
362 macrophage-rich tissue environments in the absence of other complement components such as C1r
363 and C1s. This includes production of C1q by infiltrating macrophages in the early atherosclerotic
364 lesion, or local synthesis by neurons in the brain after neuronal injury. Therefore, many of these
365 beneficial effects of C1q may be due to complement-independent actions of C1q. Beyond its ability
366 to enhance phagocytosis, we and others have shown that C1q dampens inflammatory responses in
367 phagocytes such as monocytes, macrophages, dendritic cells and microglia during ingestion of
368 damaged-self targets like apoptotic cells and oxLDL (21, 22, 30-36). To determine if the variants
369 of C1q retained this activity, secreted proteins from resting or inflammatory (M1) HMDM that had
370 interacted with C1q (or control protein HSA) were measured using Luminex multiplex analysis
371 (Figures 4, 5 and S2). While C1q differentially modulated certain levels of cytokines and
372 chemokines, importantly, the data show clearly that the variants of C1q (rC1qB and rC1qC) are
373 triggering the same macrophage responses as plasma C1q and WT rC1q. Data from 4 individual
374 donors were averaged, and there was some donor variability in the absolute amounts of each
375 protein measured from each donor (Figures 4A, 5A). However, when results were expressed as
376 fold differences from the HSA control levels within individual donors (Figures 4B, 5B), very clear
377 (and significant) patterns of modulation were evident. Similar to our previously reported data, C1q
378 suppressed secretion of proinflammatory cytokines IL-1α, IL-1β, TNFα and IL-12p40 in resting
379 and M1-polarized HMDM and showed a trend towards enhancing anti-inflammatory cytokine IL-
380 10 (Figure 4) (22, 33, 35). Macrophage secretion of inflammatory chemokine CCL3/ MIP-1α and
381 T-cell chemoattractant CXCL10/IP-10 were also suppressed by all forms of C1q. Interestingly,
382 neutrophil chemoattractants CXCL1 and CXCL8 were upregulated by C1q, which may suggest
383 that C1q differentially modulates the cellular composition within inflammatory sites. Since
384 macrophage responses to C1q differed in extent, direction (up, down or unchanged) and type of
385 macrophage (resting or M1), this supports the idea that C1q is able to actively reprogram

macrophage inflammatory responses, rather than having just a general inhibitory or activating effect.

This study clearly shows that the region on C1q that interacts with $C1r_2C1s_2$, and is important for classical complement pathway activation, is distinct from the region required for interaction with phagocytic cells. Narrowing down the functionally important regions of C1q is important for understanding the dual role of this molecule in inflammatory disease. Understanding the mechanism by which C1q exerts its effects on phagocytes, may help determine the region important for interacting with receptor(s). It is also a first step in the development of therapeutic agents for inflammatory diseases to exploit the beneficial non-complement actions of C1q. Importantly, therapeutic strategies should likely not focus on total complement inhibition. While complement fragments such as C3a/C5a are proinflammatory and may exacerbate disease, other complement protein fragments formed during activation of the complement cascade may also play beneficial roles in autoimmune/inflammatory disease. For example, a previous study showed that a patient homozygous for a similar mutation in C1q that abrogated C1r/C1s binding, but allowed C1q to bind to targets such as immunoglobulins and apoptotic cells, also developed lupus (along with multiple infections). This suggests that C1q alone may not be sufficient, and opsonins such as C3b and C4b, formed during complement activation, may also be required in protection against autoimmunity (37). Further studies with these and additional recombinant variants of C1q may provide a proof-of-concept for the long-term goal to develop therapeutic agents which enhance or mimic the demonstrated protective effects of C1q (and other opsonins), including enhancing removal of cellular debris/damaged-self molecules and reprogramming macrophages towards an anti-inflammatory polarized phenotype, without contributing to the inflammatory environment via complement activation.

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Figure Legends

Figure 1. Recombinant C1q variants rC1qB and rC1qC do not activate complement. A hemolytic assay was performed to compare classical complement pathway activation by recombinant variants of C1q to controls. HSA was used as a negative control and WT rC1q and plasma C1q were used as positive controls. Serial dilutions of proteins were made into GVB++ and incubated 30 min at 37ºC with hemolysin antibody suboptimally opsonized sheep erythrocytes (EA) in C1q-deficient serum. Lysis of sheep EA was determined by measuring A_{412} after centrifugation to pellet and remove intact cells. Maximum hemolysis was determined by incubation of EA with water and background was adjusted. Data are presented as % maximum hemolysis, average of experimental triplicates ±SD.

Figure 2. **C1q variants rC1qB and rC1qC activate phagocytosis in HMDM, similar to plasma C1q**. A) Photomicrographs of a typical experiment in which HMDM were adhered to plates coated with 5 μg/ml protein for 30 min prior to addition of sheep erythrocytes, sub-optimally opsonized with IgG, for an additional 30 min. B) Calculation of percent phagocytosis and phagocytic index. Percent phagocytosis is the number of cells ingesting at least one target/total number of cells scored x100. Phagocytic Index is the average number of ingested targets per 100 cells. Data are average ±SD from one experiment performed in duplicate, and are representative of data obtained from three individual donors. Statistics were calculated using a one-way ANOVA with multiple comparisons test. Differences from HSA control are shown. *$p<0.05$, **$p<0.01$.

Figure 3. C1q variants rC1qB and rC1qC bind and enhance HMDM clearance of oxLDL, similar to plasma C1q. A) HMDM were adhered to protein-coated slides and incubated with 10 μg protein/ml diI-labeled oxidized LDL (diI-oxLDL) for 30 min at 37ºC. Ingestion of oxLDL was measured by flow cytometry and average % cells that are DiI positive and average Median Fluorescence Intensity (MFI) +/-SD from 3 experimental replicates are shown. Statistics were calculated using a one-way ANOVA with multiple comparisons test. Differences from HSA control are shown. *$p<0.05$, **$p<0.01$. **B)** OxLDL was immobilized on a plate and incubated with 0 – 20 μg/mL C1q or HSA control. Binding was assessed by immunoassay and signal at 450nm detected. Data are average +/-SD from a single experiment performed in triplicate

Figure 4. C1q variants rC1qB and rC1qC modulate HMDM cytokine responses, similar to plasma C1q. Resting or M1-polarized HMDM were adhered to plates coated with 5 μg/ml protein for 24 h. Secreted cytokine levels were measured by Luminex multiplex analysis. Data are **(A.)** average concentration measured from 4 individual donors +/-SD and **(B.)** fold difference in cytokine level relative to HSA from 4 individual donors +/-SD. 2-way ANOVA statistical analysis with Tukey's post-hoc multiple comparisons test was performed using GraphPad Prism. Significant ($p<0.05$) differences between means of treatments are indicated with different letters (A,B,C) in (**A.**). Significant differences from HSA control (=1) are shown in (**B.**) *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

Figure 5. C1q variants rC1qB and rC1qC modulate HMDM chemokine responses, similar to plasma C1q. Resting or M1-polarized HMDM were adhered to plates coated with 5 μg/ml protein for 24 h. Secreted chemokine levels were measured by Luminex multiplex analysis. Data are **(A.)** Average concentration measured from 4 individual donors +/-SD and **(B.)** fold difference in chemokine level relative to HSA from 4 individual donors +/-SD. 2-way ANOVA statistical analysis with Tukey's post-hoc multiple comparisons test was performed using GraphPad Prism. Significant ($p<0.05$) differences between means of treatments are indicated with different letters (A,B,C) in (**A.**). Significant differences from HSA control (=1) are shown in (**B.**) *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

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Figure 1 C1q variants rC1qB and rC1qC do not activate the CCP

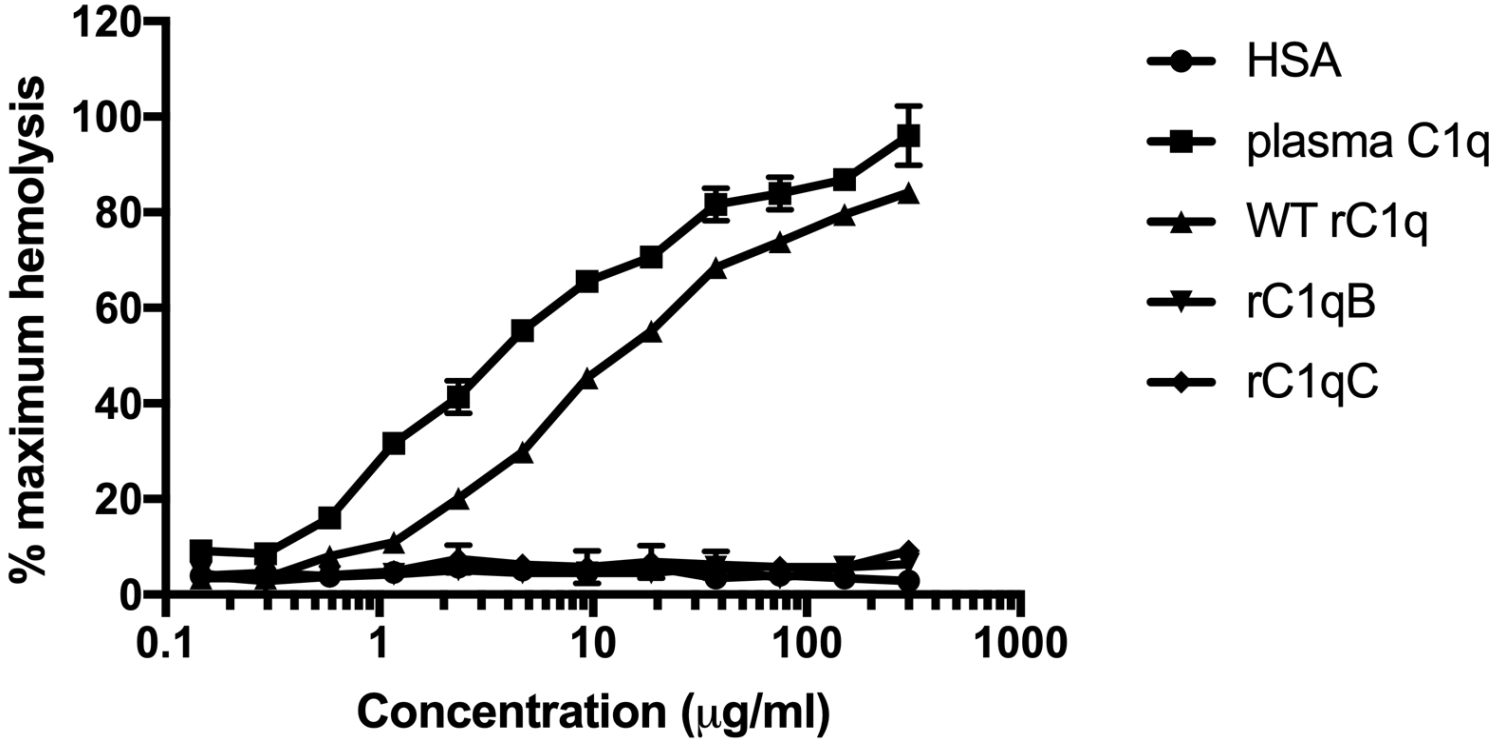


Figure 2: C1q variants rC1qB and rC1qC activate phagocytosis in HMDM, similar to plasma C1q

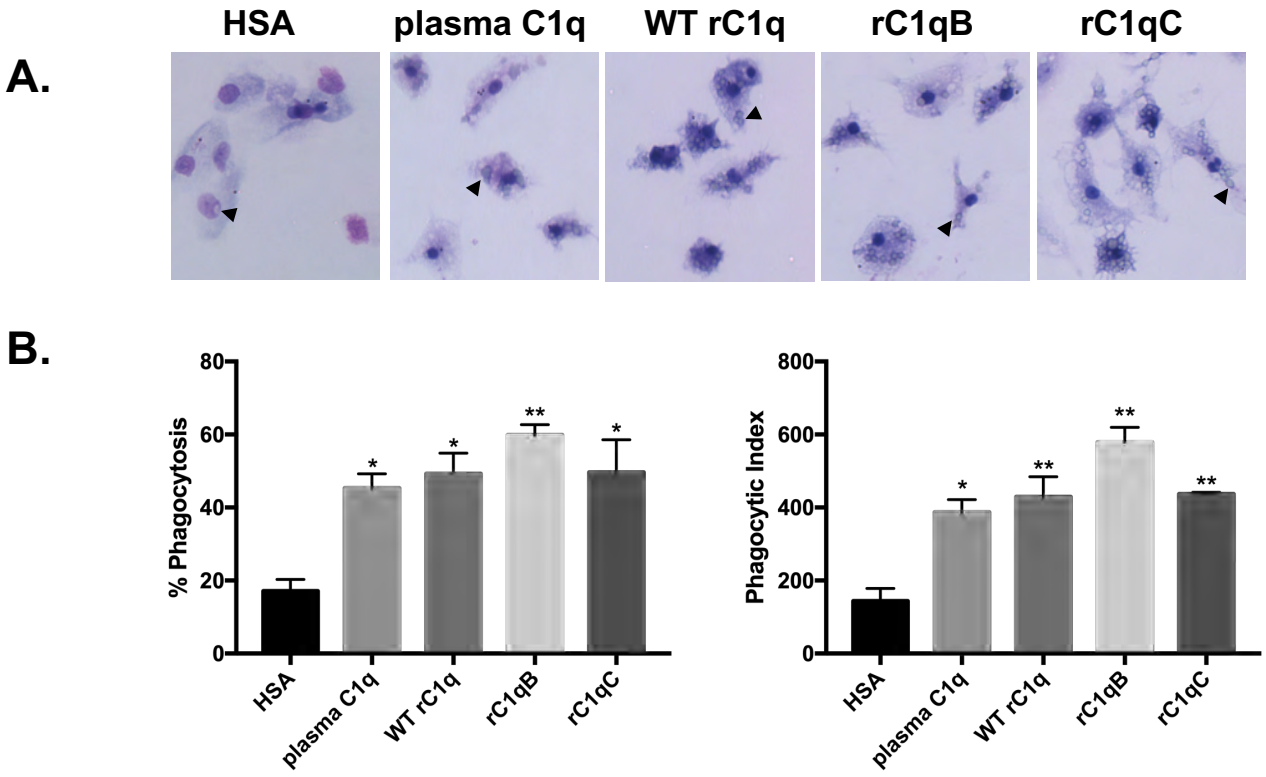


Figure 3: C1q variants rC1qB and rC1qC bind and enhance HMDM clearance of oxLDL, similar to plasma C1q

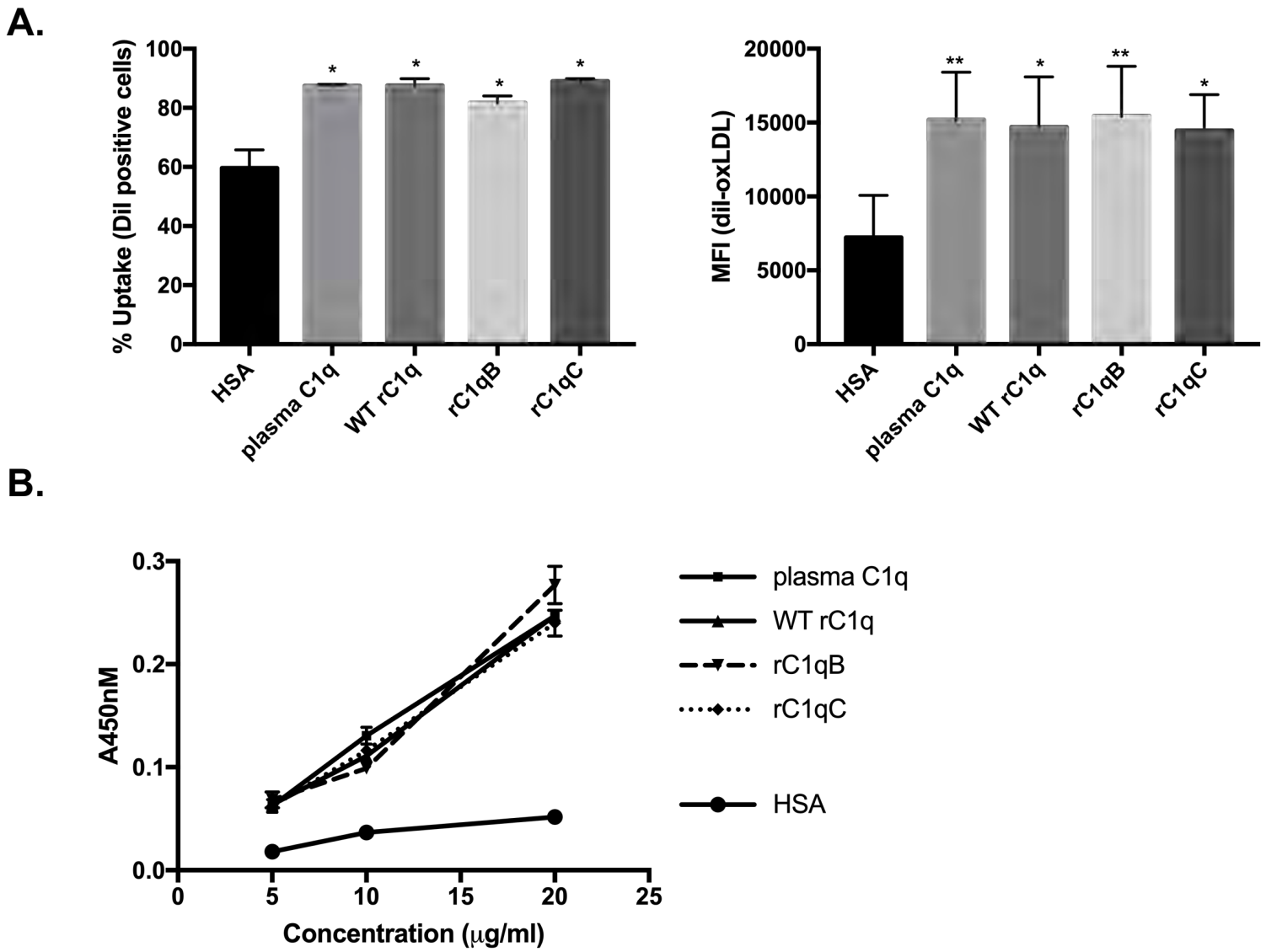


Figure 4 C1q variants rC1qB and rC1qC modulate HMDM cytokine responses, similar to plasma C1q

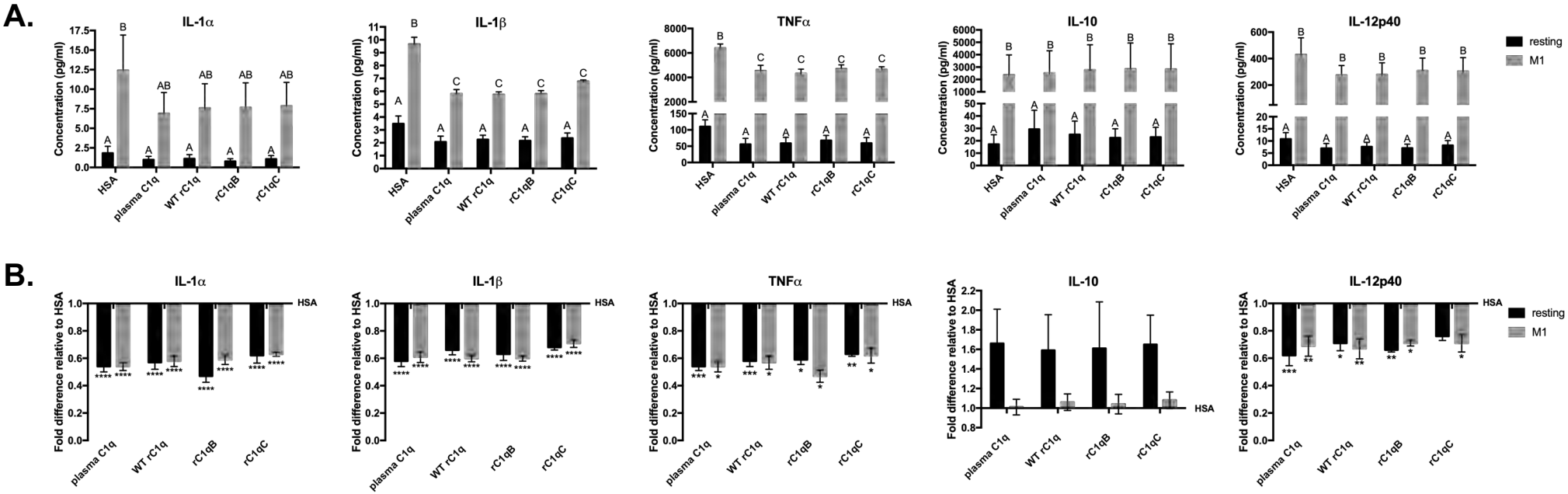
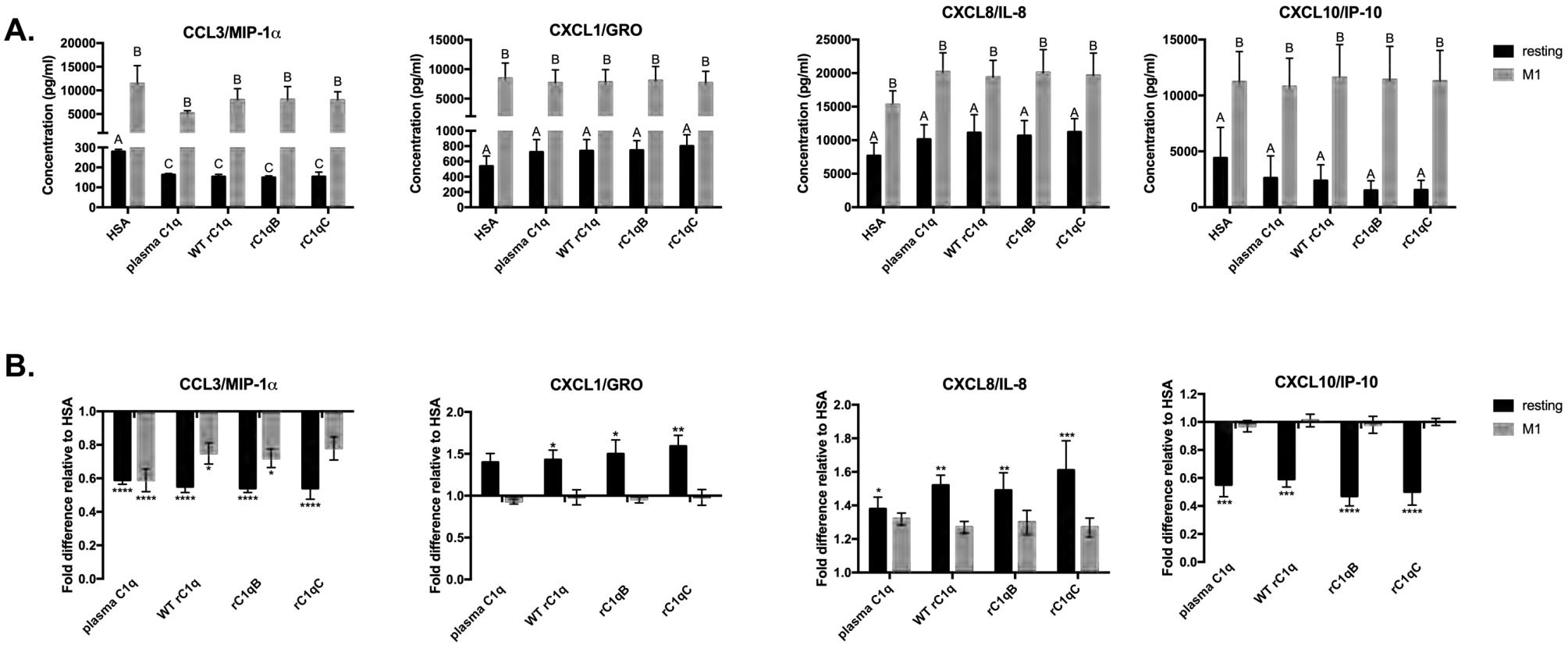
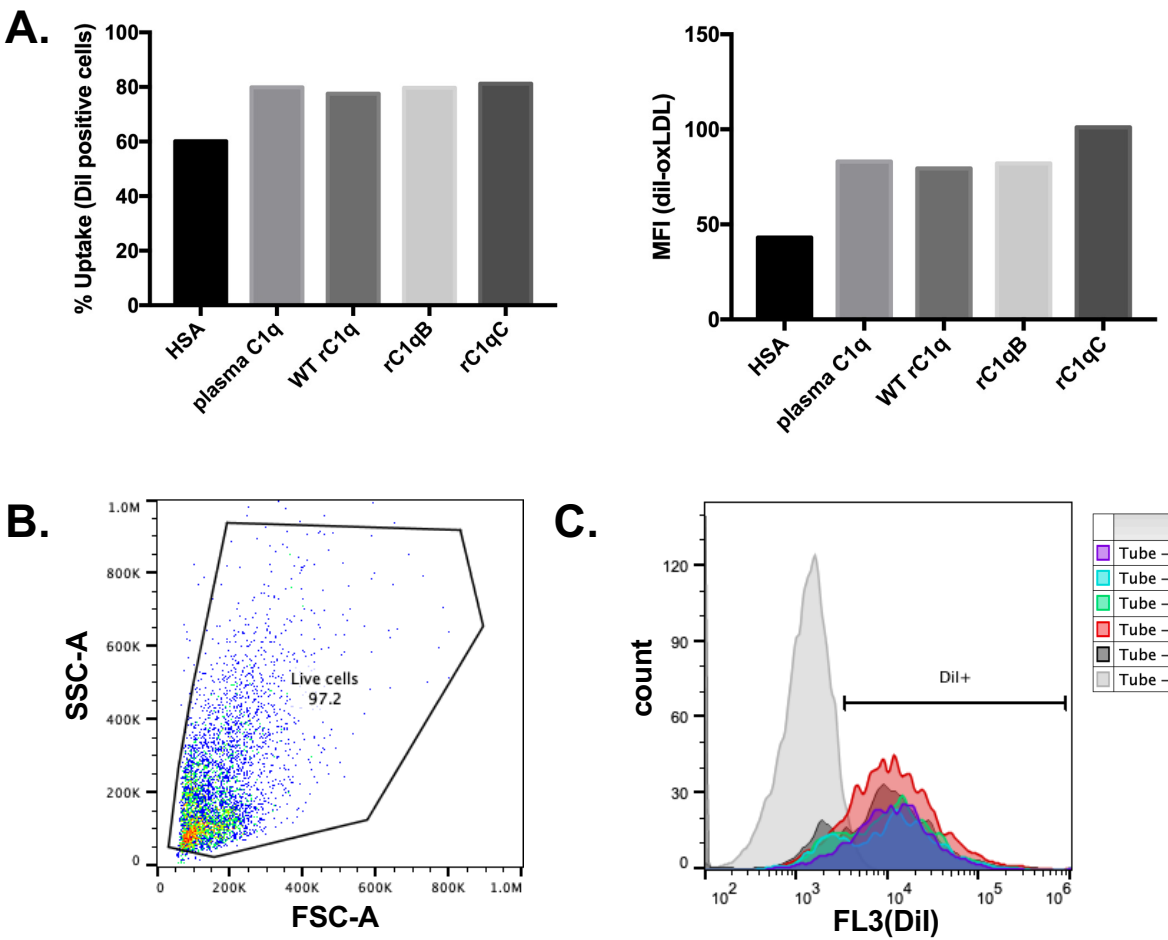


Figure 5 C1q variants rC1qB and rC1qC modulate HMDM chemokine responses, similar to plasma C1q



Supplemental Figure 1



	Sample Name	SampleID	Subset Name	Median : FL3-A
	Tube - 6_Data Source - 1.fcs	rC1qC	Live cells	11015
	Tube - 5_Data Source - 1.fcs	rC1qB	Live cells	10722
	Tube - 4_Data Source - 1.fcs	WT rC1q	Live cells	10941
	Tube - 3_Data Source - 1.fcs	Plasma C1q	Live cells	10650
	Tube - 2_Data Source - 1.fcs	HSA	Live cells	3760
	Tube - 1_Data Source - 1.fcs	Background	Live cells	1352

Figure S1. Measuring uptake by flow cytometry. (A.) HMDM were incubated with 10 μg protein/ml DiI-labeled oxidized LDL (DiI-oxLDL) with 75 μg/ml HSA or C1q for 30 min at 37°C. Ingestion of oxLDL was measured by flow cytometry and average % cells that are DiI positive and average Median Fluorescence Intensity (MFI) from a single experiment is shown. Representative dot plot (B.) and histogram (C.) from single experiment shown to demonstrate gating strategy. A. Flow cytometry data were gated first for live cells (using SSC and FSC). B. The gate for DiI-positive cells was set using histogram data for DiI fluorescence (FL3) on cells only (background, no DiI-oxLDL added, grey) ensuring that <5% DiI+ were present in this gate. This gate was applied to all additional samples, to determine % DiI positive (shift in FL3) for each treatment.

Supplemental Figure 2

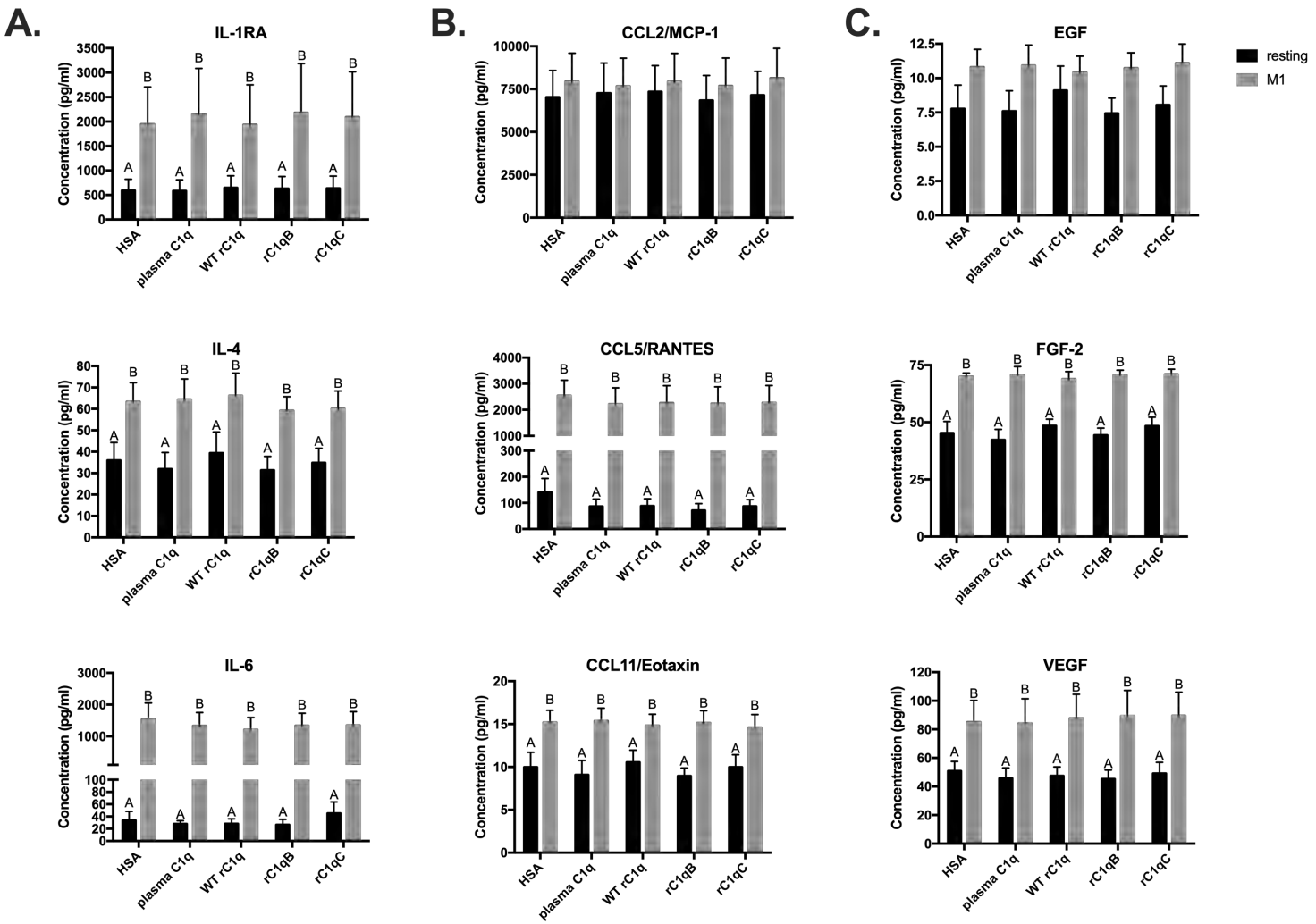


Figure S2. C1q variants rC1qB and rC1qC do not modulate certain HMDM secreted proteins, similar to plasma C1q. Resting or M1-polarized HMDM were adhered to plates coated with 5mg/ml protein for 24 hours. Secreted **(A.)** cytokine , **(B.)** chemokine, or **(C.)** growth factor levels were measured by Luminex multiplex analysis. Data are average concentration measured from 4 individual donors +/-SD . A 2-way ANOVA statistical analysis with Tukey's post-hoc multiple comparisons test was performed using GraphPad Prism. Significant ($p<0.05$) differences between means of treatments are indicated with different letters (A,B).