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Recombinant C1q variants modulate macrophage responses but do not activate the classical complement pathway

Victoria Espericueta¹, Ayla O. Manughian-Peter¹, Isabelle Bally², Nicole M. Thielens² and Deborah A. Fraser¹

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

C1q variants activate macrophage responses, not complement

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

40 **Abstract**

41 Complement protein C1q plays a dual role in a number of inflammatory diseases such as
42 atherosclerosis. While in later stages classical complement pathway activation by C1q exacerbates
43 disease progression, C1q also plays a beneficial role in early disease. Independent of its role in
44 complement activation, we and others have identified a number of potentially beneficial
45 interactions of C1q with phagocytes *in vitro*, including triggering phagocytosis of cellular and
46 molecular debris and polarizing macrophages toward an anti-inflammatory phenotype. These
47 interactions may also be important in preventing autoimmunity. Here, we characterize variants of
48 recombinant human C1q (rC1q) which no longer initiate complement activation, through mutation
49 of the C1r₂C1s₂ interaction site. For insight into the structural location of the site of C1q that is
50 important for interaction with phagocytes, we investigated the effect of these mutations on
51 phagocytosis and macrophage inflammatory polarization, as compared to wild-type C1q.
52 Phagocytosis of antibody coated sheep erythrocytes and oxidized LDL was measured in human
53 monocytes and monocyte-derived macrophages (HMDM) respectively that had interacted with
54 rC1q wild-type or variants. Secreted levels of cytokines were also measured in C1q stimulated
55 HMDM. All variants of C1q increased phagocytosis in HMDM compared to controls, similar to
56 native or wild-type rC1q. In addition, levels of certain pro-inflammatory cytokines and
57 chemokines secreted by HMDM were modulated in cells that interacted with C1q variants, similar
58 to wild-type rC1q and native C1q. This includes downregulation of IL-1 α , IL-1 β , TNF α , MIP-1 α ,
59 and IL-12p40 by native and rC1q in both resting and M1-polarized HMDM. This suggests that the
60 site responsible for C1q interaction with phagocytes is independent of the C1r₂C1s₂ interaction
61 site. Further studies with these classical pathway-null variants of C1q should provide greater
62 understanding of the complement-independent role of C1q, and allow for potential therapeutic
63 exploitation.

64 1. Introduction

65

66 C1q is the recognition component of the classical complement pathway. In the blood C1q
67 is predominantly found in complex with two copies each of proenzymes C1r and C1s (C1r₂C1s₂),
68 termed the C1 complex (1). As a pattern recognition receptor (PRR) of the innate immune system,
69 C1q is able to recognize a wide variety of targets including immune complexes, pathogen-
70 associated molecular patterns (PAMPs), apoptotic cell-associated molecular patterns (ACAMPs)
71 and damage-associated molecular patterns (DAMPs) such as oxidation neoepitopes on low density
72 lipoproteins (oxLDL). Binding of C1q to a target leads to conformational changes within the C1
73 complex that allow for C1r to be cleaved autocatalytically, activating C1s, and resulting in
74 downstream activation of the classical complement cascade. The three major outcomes of
75 complement activation are opsonization of targets with complement fragment C3b to enhance
76 phagocytosis, production of proinflammatory anaphylatoxins C3a and C5a leading to leukocyte
77 recruitment to the area of activation, and lysis of targets via production of the membrane attack
78 complex (MAC) (2). Complement activation by C1q is critical in controlling certain infections,
79 but can also exacerbate many chronic inflammatory diseases, including atherosclerosis (3) and
80 Alzheimer's disease (4, 5). However, C1q likely plays a dual role in inflammatory disease, and
81 has been shown to have a protective role in mouse models of early atherosclerosis and Alzheimer's
82 disease (6, 7). Many of the beneficial effects of C1q appear to be complement (C1r₂C1s₂)
83 independent, and involve direct interactions of C1q with phagocytic cells. C1q modulates
84 phagocyte responses including enhancement of phagocytosis/efferocytosis, and suppression of
85 inflammatory responses (8, 9).

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

96 collagen-like tail diverging via a bend or hinge region to produce six individual globular head
97 domains (12). The hinge is produced via a disruption in the collagen-like Gly-X-Y amino acid
98 sequence and has been localized about half way through each of the A, B or C-chain CLR. The
99 binding site for C1r and C1s was also identified in the CLR, between the kink region and the
100 globular domain (13). A previous study using recombinantly expressed C1q variants carrying
101 mutations of LysA59, LysB61 and LysC58, identified specific lysine residues on the B- and C-
102 chain (rC1qB, rC1qC) as critically important in binding and activation of the C1r₂-C1s₂
103 proenzymes (14).

104

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

119 **2. Methods**

120 *2.1 Proteins and Reagents*

121 Plasma C1q was isolated from plasma-derived normal human serum (NHS) by ion-
122 exchange chromatography followed by size-exclusion chromatography according to the method
123 of Tenner et al. (19) and modified as described (20). During the purification of C1q, serum depleted
124 of C1q (C1qD) was collected after passage of plasma-derived serum in 25 mM EDTA (to
125 dissociate C1q from C1r and C1s) over the ion-exchange resin and stored at -70°C until use.
126 Recombinant WT C1q (WT rC1q) and the variants of recombinant human C1q which contain a
127 single amino acid mutation of the C1r/C1s binding site in either the B-chain (rC1qB) or C-chain
128 (rC1qC) were expressed and purified from HEK293-F cells as described (14). Highly oxidized
129 LDL (oxLDL) was purchased from Kalen Biomedical (Montgomery Village, MD). Fluorescently-
130 labeled oxLDL was prepared using 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine
131 perchlorate (DiI, Molecular Probes) according to the manufacturer's instructions, and as
132 previously described (21). Ultrapure LPS (E. coli 0111:B4) was obtained from Invivogen (San
133 Diego, CA). Recombinant human macrophage-colony stimulating factor (rh-M-CSF) and IFN γ
134 protein were purchased from Peprotech (Rocky Hill, NJ). All cell culture reagents were purchased
135 from Life Technologies (Carlsbad, CA) unless otherwise stated.

136

137 *2.2 Complement C1q Hemolytic Titer*

138 Immune complexes of suboptimally opsonized sheep erythrocytes (shEA) were prepared
139 as previously described (22) using a 1:3,200 dilution of hemolysin antibody (Complement
140 Technology Inc., Tyler TX). Serial dilutions of C1q or HSA control were made into gelatin veronal
141 buffer containing magnesium and calcium (GVB++) from a starting concentration of 300 $\mu\text{g/ml}$.
142 2 μl of each protein were added to 300 μl C1q-depleted serum supplemented with 3 mM Ca^{2+} , 100
143 mM Mg^{++} (C1qD), as a source of additional complement components and 80 ml shEA at 1×10^5
144 cells/ml. Tubes were incubated at 37°C for 30 min before dilution with GVB++. Intact shEA were
145 pelleted by centrifugation and absorbance of supernatant was read at 412 nm to determine relative
146 amounts of hemoglobin released as a measure of lysis. A_{412} of shEA in water or GVB++ were used
147 as positive (100% lysis) or negative (background, 0% lysis) controls respectively. Percent lysis
148 relative to water for each dilution was calculated after removal of background. The measure of

149 complement hemolytic activity, CH_{50} , is calculated as the concentration of protein able to induce
150 50% of maximal hemolysis.

151

152 *2.3 Cell isolation and culture*

153 Human blood samples from healthy anonymized donors giving informed consent were
154 collected into EDTA by a certified phlebotomist according to the guidelines and approval of
155 California State University Long Beach Institutional Review Board. Primary human monocytes
156 were isolated using the Dynabeads Untouched Human Monocyte Kit from Invitrogen (Carlsbad,
157 CA) according to the manufacturer's protocol. Cell purity was determined using the Scepter cell
158 analyzer (EMD Millipore, Darmstadt, Germany). Isolated monocytes were used in phagocytosis
159 assays (purity >93%) or cultured for 7-13 days in RPMI 1640, 10% FCS, 2-mM L-glutamine and
160 1% penicillin/streptomycin containing 25 ng/ml rhM-CSF (Peprotech, Rocky Hill, NJ) to stimulate
161 differentiation in human monocyte derived macrophages (HMDM). Expression of macrophage
162 markers CD11b and F4/80 were assessed by flow cytometry using FITC-labeled antibodies
163 (eBioscience, San Diego, CA) to characterize and validate macrophage differentiation and were
164 >94% population for each experiment.

165

166 *2.4 Phagocytosis Assay*

167 Phagocytosis assays were performed essentially as described previously (23). Briefly, LabTek
168 chambers (Nunc, Rochester, NY) were coated with HSA, plasma C1q, recombinant WT C1q or
169 recombinant C1q variants rC1qB or rC1qC at 5 μ g/ml in coating buffer (0.1 M carbonate, pH 9.6),
170 and incubated at 37°C for 2 h. Chambers were washed twice with PBS and monocytes at $2.5 \times$
171 10^5 / ml in phagocytosis buffer (RPMI, 2 mM L-glutamine, 5 mM $MgCl_2$) were added to chambers.
172 Slides were centrifuged at 70 g for 3 min and cultured for 30 min at 37°C in 5% CO_2 . Sheep
173 erythrocytes suboptimally opsonized with IgG were used as phagocytic targets and prepared in
174 gelatin veronal buffer (GVB++) as described above. 10^7 targets were added to each well and after
175 centrifuging at 70 g for 3 min, incubated for an additional 30 min at 37°C in 5% CO_2 . Uningested
176 targets were lysed with ACK, and cells were fixed in 1% glutaraldehyde in PBS. Cells were
177 visualized using a modified Giemsa stain and at least 200 cells/well counted. Percent phagocytosis
178 is the % cells scored that have ingested at least one target. Phagocytic index is the average number
179 of ingested targets per 100 cells counted.

180 *2.5 Lipoprotein Clearance Assay*

181 Wells of a 96-well plate were coated with 5 µg/ml HSA or C1q (plasma derived, or recombinant
182 variants) in coating buffer for 2 h at 37°C and washed 2x in sterile PBS. HMDM were harvested
183 using Cellstripper (Corning), and resuspended at 1×10^6 cells/ml in phagocytosis buffer, before
184 being added to the coated wells. Cells were cultured for 30 min at 37°C in 5% CO₂ before addition
185 of 10 µg protein/ml DiI-oxLDL for an additional 30 min. After incubation, cells were harvested
186 from wells using 0.25% trypsin-EDTA (Invitrogen), and ingestion of DiI-labeled lipoproteins was
187 analyzed in at least 5,000 cells by flow cytometry using the Sony SH800 Cell Analyzer (Sony).
188 Data analysis was performed using FlowJo software (Ashland, OR).

189

190 *2.6 Lipoprotein Binding Assay*

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

200

201 *2.7 Cytokine Analysis*

202 Wells of a 96-well plate were coated with 5 mg/ml HSA or C1q (plasma derived, or
203 recombinant variants) in coating buffer as described above. HMDM were added at 1×10^6 cells/ml
204 in HL-1 serum-free defined media (supplemented with 2 mM L-glutamine, 10 mM HEPES, 5 mM
205 MgCl₂). Cells were cultured for 24 h at 37° C in 5% CO₂. In some wells, 20 ng/ml IFN γ and 100
206 ng/ml LPS were added for M1 macrophage polarization. ATP was added at 1 mM for the final 3
207 h of incubation (to provide a second signal for inflammasome activation, to measure IL-1 β).
208 Supernatants were harvested, and centrifuged to remove cellular debris and stored at -80° C until
209 use. Secreted cytokine levels were quantified by Luminex multiplex analysis using the Milliplex
210 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 \pm 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

220 **3. Results**

221

222 *3.1 Recombinant C1q variants rC1qB and rC1qC do not activate complement.*

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

234

235 *3.2 C1q variants rC1qB and rC1qC activate phagocytosis in human monocytes, similar to plasma*
236 *C1q.*

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

247

248 *3.3 C1q variants rC1qB and rC1qC bind and enhance HMDM clearance of oxLDL, similar to*
249 *plasma C1q*

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

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

272

273 *3.4 C1q variants rC1qB and rC1qC modulate HMDM cytokine and chemokine responses,* 274 *similar to plasma C1q*

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

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

295 4. Discussion

296

297 C1q plays an integral role in the defense against infection through complex with C1r₂C1s₂
298 and activation of the classical complement pathway. However, C1q also has an important non-
299 complement associated role (in the absence of C1r or C1s) via direct opsonization of cellular debris
300 or apoptotic cells. Here we present data showing that mutations of residues in the site of interaction
301 with C1r₂C1s₂ do not affect the ability of these C1q variants to opsonize damaged-self targets,
302 enhance phagocytosis and modulate macrophage inflammatory polarization. Understanding which
303 regions of the C1q molecule are necessary for individual functions could assist in the design of
304 therapeutic agents for inflammatory disease.

305 Expression of recombinant C1q, including variants containing B-chain Lys61-Ala
306 mutations (rC1qB) or C-chain Lys58-Ala (rC1qC) mutations from wild-type were first described
307 by Bally et al. (14). In this previous study, the C1q variants did not activate C1, unlike wild-type
308 rC1q or serum derived C1q. This was tested using IgG-ovalbumin immune targets and assessed
309 by C1s Western blot analysis. Here we performed an additional assay to test classical complement
310 pathway activation by these variants using a C1q-specific hemolytic titer assay. Data in Figure 1
311 show that although dilutions of plasma purified C1q and wild-type rC1q were able to reconstitute
312 complement activity (leading to hemolysis of immune complex targets) in C1q-depleted serum,
313 the variants rC1qB and rC1qC could not. This is consistent with their reported defect in interaction
314 with C1r₂C1s₂ and inability to form a stable C1 complex. The variation in CH₅₀ activity between
315 plasma derived C1q (CH₅₀ = 4.9 μg/mL) and WT rC1q (CH₅₀ = 14.7 μg/mL) is minor and may be
316 due to the presence of a C-terminal FLAG tag in the C-chain of these recombinantly expressed
317 proteins.

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

326 targets each monocyte ingested above levels seen with HSA control protein. In addition, all forms
327 of C1q modulated phagocytosis to a similar extent suggesting that the mutations in the B- and C-
328 chains of C1q that abrogate complement activity do not affect phagocytic capabilities. This was
329 supported in our clearance assay where HMDM were shown to ingest significantly higher amounts
330 of DiI-labeled oxLDL when interacting with C1q compared to HSA, but no differences in levels
331 between forms of C1q were identified (Figure 3A). Importantly, similar results were obtained
332 when measuring HMDM phagocytosis of DiI-labeled oxLDL incubated with 75 μ g/ml HSA, or
333 C1q in a soluble system to more closely resemble physiologic conditions where C1q would be
334 bound to a target (Supplemental Figure 1A). These data suggest that mutation of the region of
335 interaction with C1r₂C1s₂ in the variants of C1q (rC1qB, rC1qC) does not negatively affect their
336 ability to interact with, and activate, a variety of phagocytes. This is consistent with previous
337 studies that identified a critically important region in the collagen-like domain of a similar
338 molecule, MBL that is necessary for the enhancement of phagocytosis. MBL and C1q share
339 structural similarities; MBL has an amino terminal collagen-like domain and a C-type lectin
340 binding domain. It forms an oligomeric structure comprised of multimers of a 3 identical chain
341 subunit. They also share functional similarities. For example, both are defense collagens,
342 activating the classical (C1q) or lectin (MBL) pathways of complement, both are also able to
343 directly interact with phagocytes and activate phagocytosis and modulate phagocyte inflammatory
344 responses to certain targets (8, 21, 22). The specific sequence in MBL critical for phagocytosis
345 was determined to be GEKGEP, found in each identical chain of the MBL oligomer, just below
346 the hinge/kink region (18). It is likely that a similar domain exists in C1q, and the authors of this
347 study hypothesize it may be the GEQGEP sequence in the human C1q A-chain, also located below
348 the hinge region (see graphical abstract). Since C1q is a highly complex molecule, with a three-
349 chain structure it has historically been problematic to express recombinantly. With these newly
350 identified methods for producing active, intact rC1q, it may at last be possible for future studies to
351 test the involvement of this GEQGEP sequence in C1q activation of phagocytosis, and to identify
352 putative phagocytic receptors for C1q.

353 We and others have previously showed that C1q binds modified forms of LDL (21, 26).
354 Our binding assay showed that C1q variants were also able to bind to oxLDL to a similar extent
355 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-1 α , IL-1 β , TNF α 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-1 α 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

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

388

389 This study clearly shows that the region on C1q that interacts with C1r₂C1s₂, and is
390 important for classical complement pathway activation, is distinct from the region required for
391 interaction with phagocytic cells. Narrowing down the functionally important regions of C1q is
392 important for understanding the dual role of this molecule in inflammatory disease. Understanding
393 the mechanism by which C1q exerts its effects on phagocytes, may help determine the region
394 important for interacting with receptor(s). It is also a first step in the development of therapeutic
395 agents for inflammatory diseases to exploit the beneficial non-complement actions of C1q.
396 Importantly, therapeutic strategies should likely not focus on total complement inhibition. While
397 complement fragments such as C3a/C5a are proinflammatory and may exacerbate disease, other
398 complement protein fragments formed during activation of the complement cascade may also play
399 beneficial roles in autoimmune/inflammatory disease. For example, a previous study showed that
400 a patient homozygous for a similar mutation in C1q that abrogated C1r/C1s binding, but allowed
401 C1q to bind to targets such as immunoglobulins and apoptotic cells, also developed lupus (along
402 with multiple infections). This suggests that C1q alone may not be sufficient, and opsonins such
403 as C3b and C4b, formed during complement activation, may also be required in protection against
404 autoimmunity (37). Further studies with these and additional recombinant variants of C1q may
405 provide a proof-of-concept for the long-term goal to develop therapeutic agents which enhance or
406 mimic the demonstrated protective effects of C1q (and other opsonins), including enhancing
407 removal of cellular debris/damaged-self molecules and reprogramming macrophages towards an
408 anti-inflammatory polarized phenotype, without contributing to the inflammatory environment via
409 complement activation.

410

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419 **Figure Legends**

420 **Figure 1. Recombinant C1q variants rC1qB and rC1qC do not activate complement.** A
421 hemolytic assay was performed to compare classical complement pathway activation by
422 recombinant variants of C1q to controls. HSA was used as a negative control and WT rC1q and
423 plasma C1q were used as positive controls. Serial dilutions of proteins were made into GVB++
424 and incubated 30 min at 37°C with hemolysin antibody suboptimally opsonized sheep erythrocytes
425 (EA) in C1q-deficient serum. Lysis of sheep EA was determined by measuring A₄₁₂ after
426 centrifugation to pellet and remove intact cells. Maximum hemolysis was determined by
427 incubation of EA with water and background was adjusted. Data are presented as % maximum
428 hemolysis, average of experimental triplicates ±SD.

429
430 **Figure 2. C1q variants rC1qB and rC1qC activate phagocytosis in HMDM, similar to plasma**
431 **C1q.** A) Photomicrographs of a typical experiment in which HMDM were adhered to plates coated
432 with 5 µg/ml protein for 30 min prior to addition of sheep erythrocytes, sub-optimally opsonized
433 with IgG, for an additional 30 min. B) Calculation of percent phagocytosis and phagocytic index.
434 Percent phagocytosis is the number of cells ingesting at least one target/total number of cells scored
435 x100. Phagocytic Index is the average number of ingested targets per 100 cells. Data are average
436 ±SD from one experiment performed in duplicate, and are representative of data obtained from
437 three individual donors. Statistics were calculated using a one-way ANOVA with multiple
438 comparisons test. Differences from HSA control are shown. *p<0.05, **p<0.01.

439
440 **Figure 3. C1q variants rC1qB and rC1qC bind and enhance HMDM clearance of oxLDL,**
441 **similar to plasma C1q.** A) HMDM were adhered to protein-coated slides and incubated with 10
442 µg protein/ml diI-labeled oxidized LDL (diI-oxLDL) for 30 min at 37°C. Ingestion of oxLDL was
443 measured by flow cytometry and average % cells that are DiI positive and average Median
444 Fluorescence Intensity (MFI) +/-SD from 3 experimental replicates are shown. Statistics were
445 calculated using a one-way ANOVA with multiple comparisons test. Differences from HSA
446 control are shown. *p<0.05, **p<0.01. B) OxLDL was immobilized on a plate and incubated with
447 0 – 20 µg/mL C1q or HSA control. Binding was assessed by immunoassay and signal at 450nm
448 detected. Data are average +/-SD from a single experiment performed in triplicate

449

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

459

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

469

470

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- 570

Figure 1

C1q variants rC1qB and rC1qC do not activate the CCP

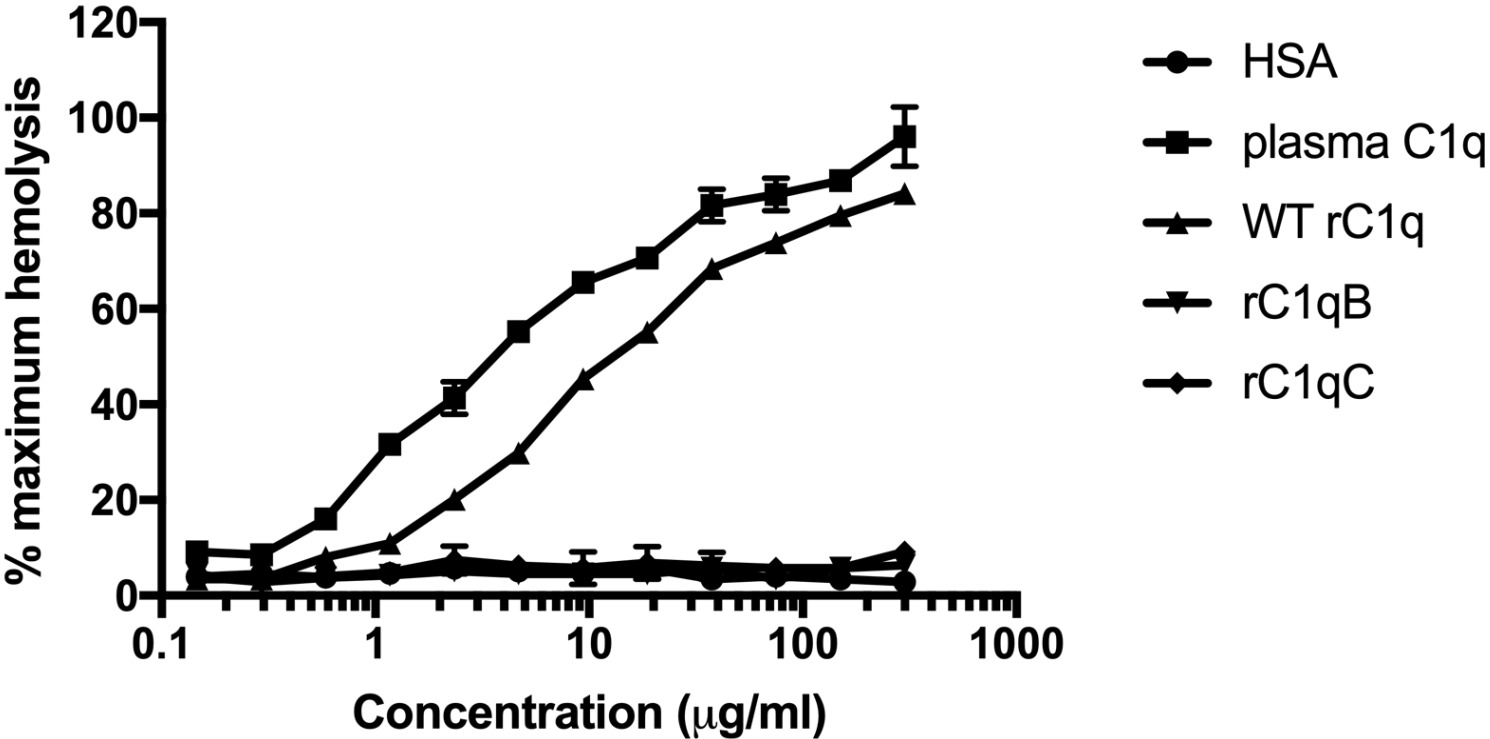


Figure 2: C1q variants rC1qB and rC1qC activate phagocytosis in HMDM, similar to plasma C1q

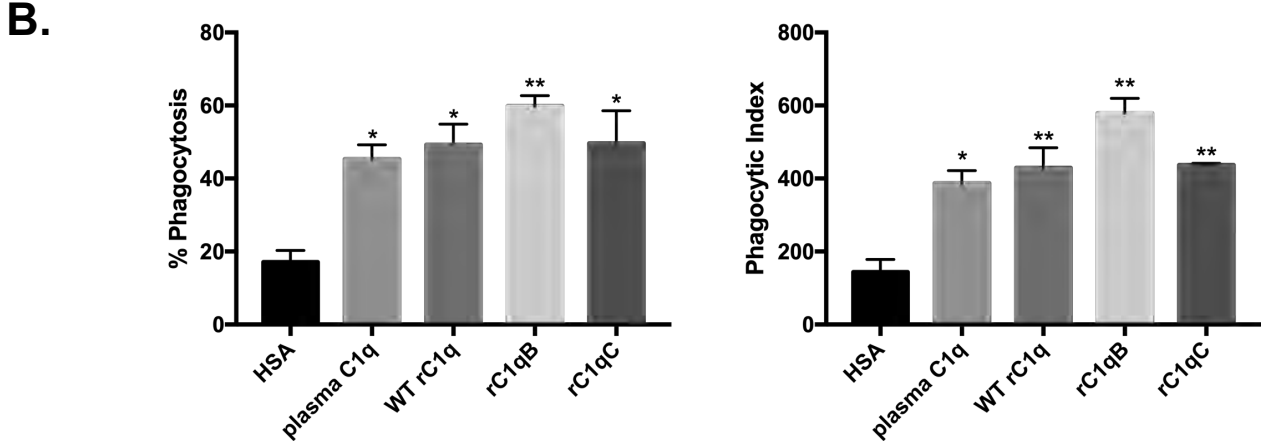
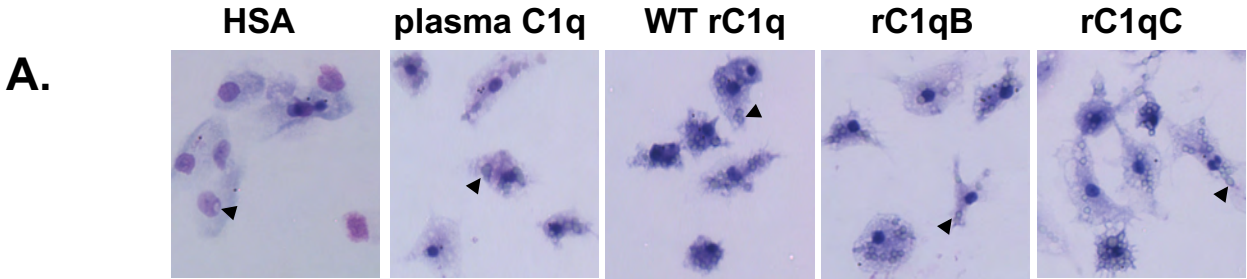
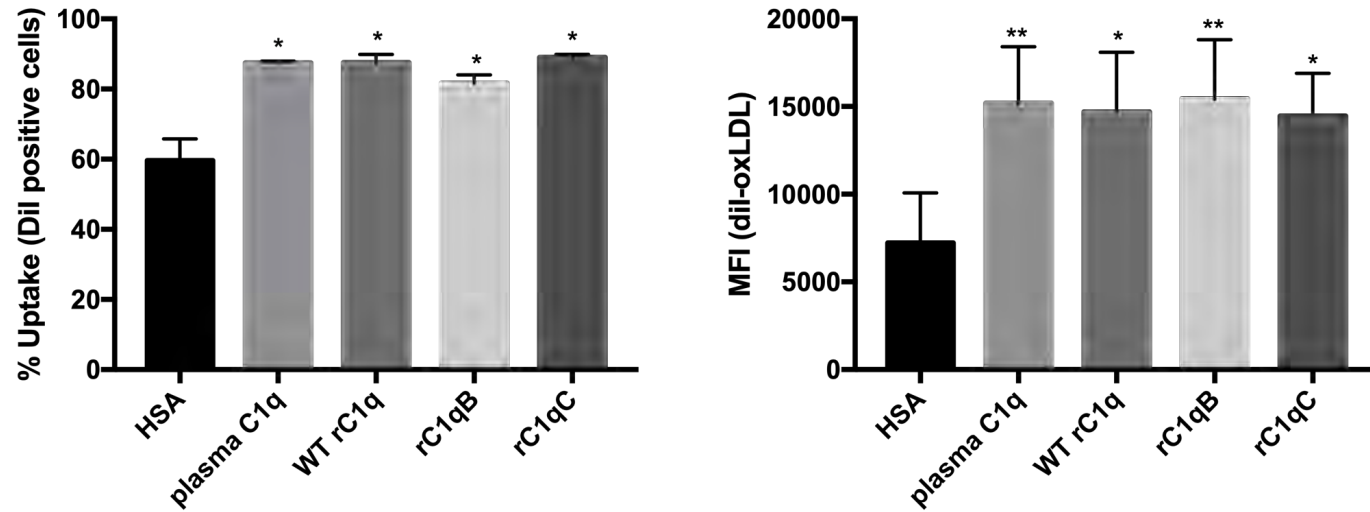


Figure 3: C1q variants rC1qB and rC1qC bind and enhance HMDM clearance of oxLDL, similar to plasma C1q

A.



B.

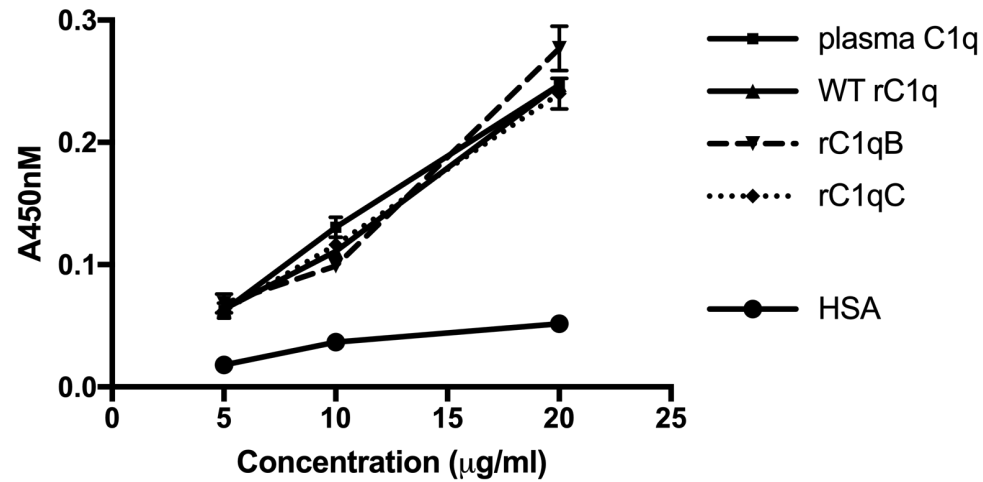


Figure 4 C1q variants rC1qB and rC1qC modulate HMDM cytokine responses, similar to plasma C1q

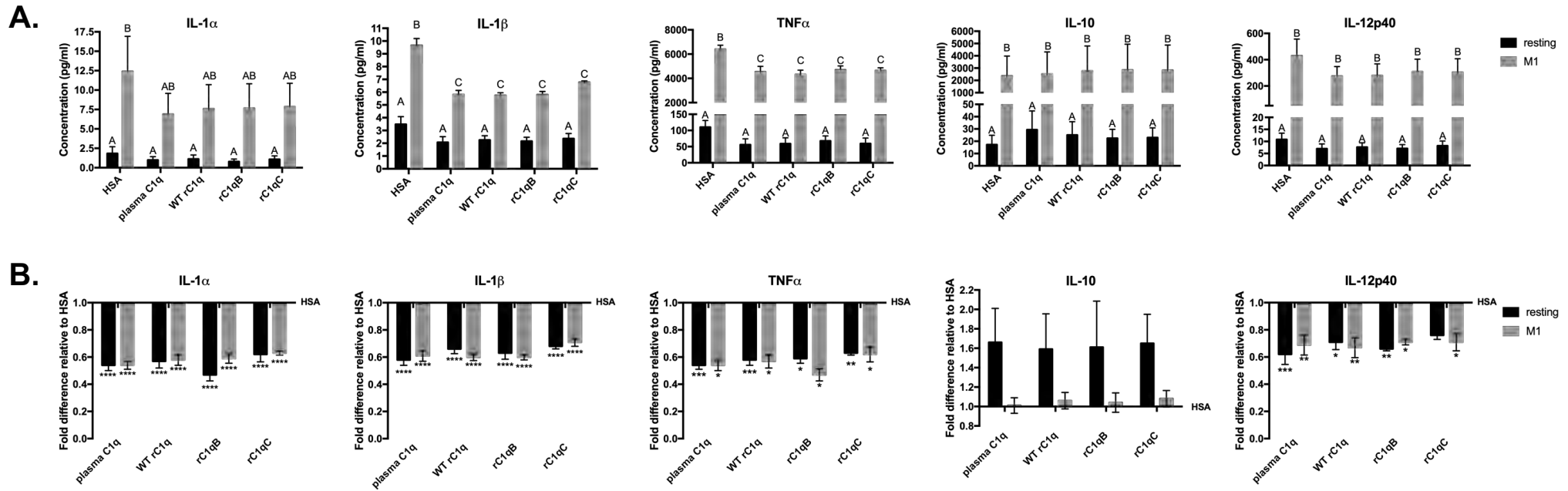
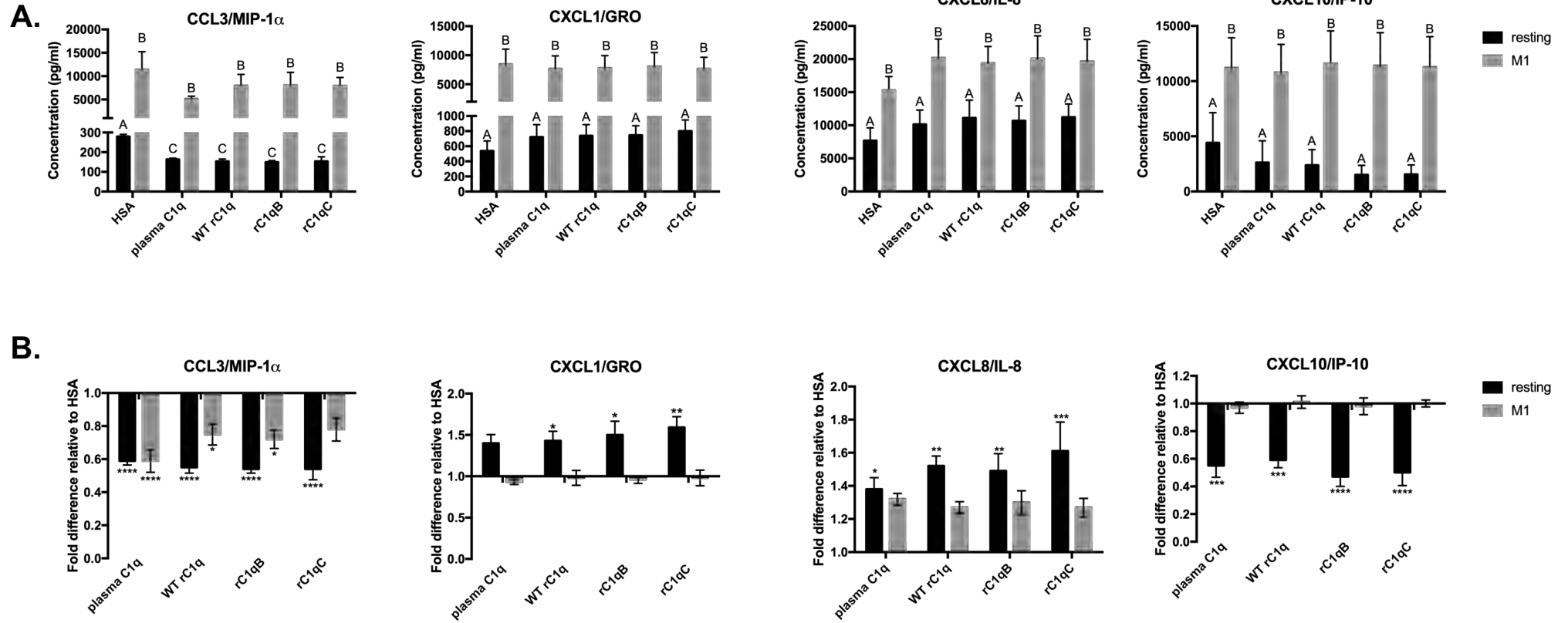


Figure 5 C1q variants rC1qB and rC1qC modulate HMDM chemokine responses, similar to plasma C1q



Supplemental Figure 1

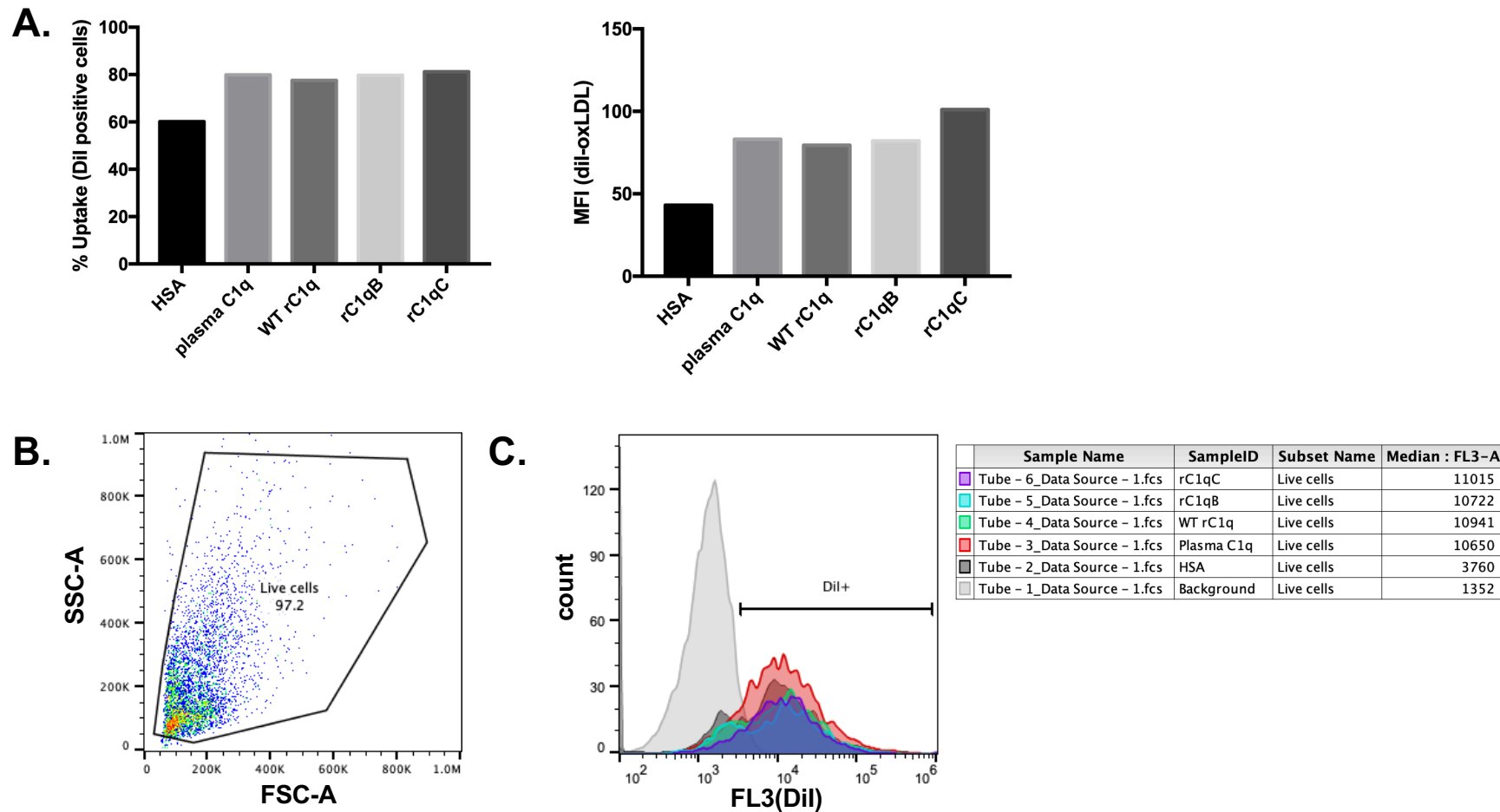


Figure S1. Measuring uptake by flow cytometry. (A.) HMDM were incubated with 10 μ g protein/ml dil-labeled oxidized LDL (dil-oxLDL) with 75 μ g/ml HSA or C1q for 30 min at 37°C. Ingestion of oxLDL was measured by flow cytometry and average % cells that are Dil positive and average Median Fluorescence 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 Dil-positive cells was set using histogram data for Dil fluorescence (FL3) on cells only (background, no Dil-oxLDL added, grey) ensuring that <5% Dil+ were present in this gate. This gate was applied to all additional samples, to determine % Dil positive (shift in FL3) for each treatment.

Supplemental Figure 2

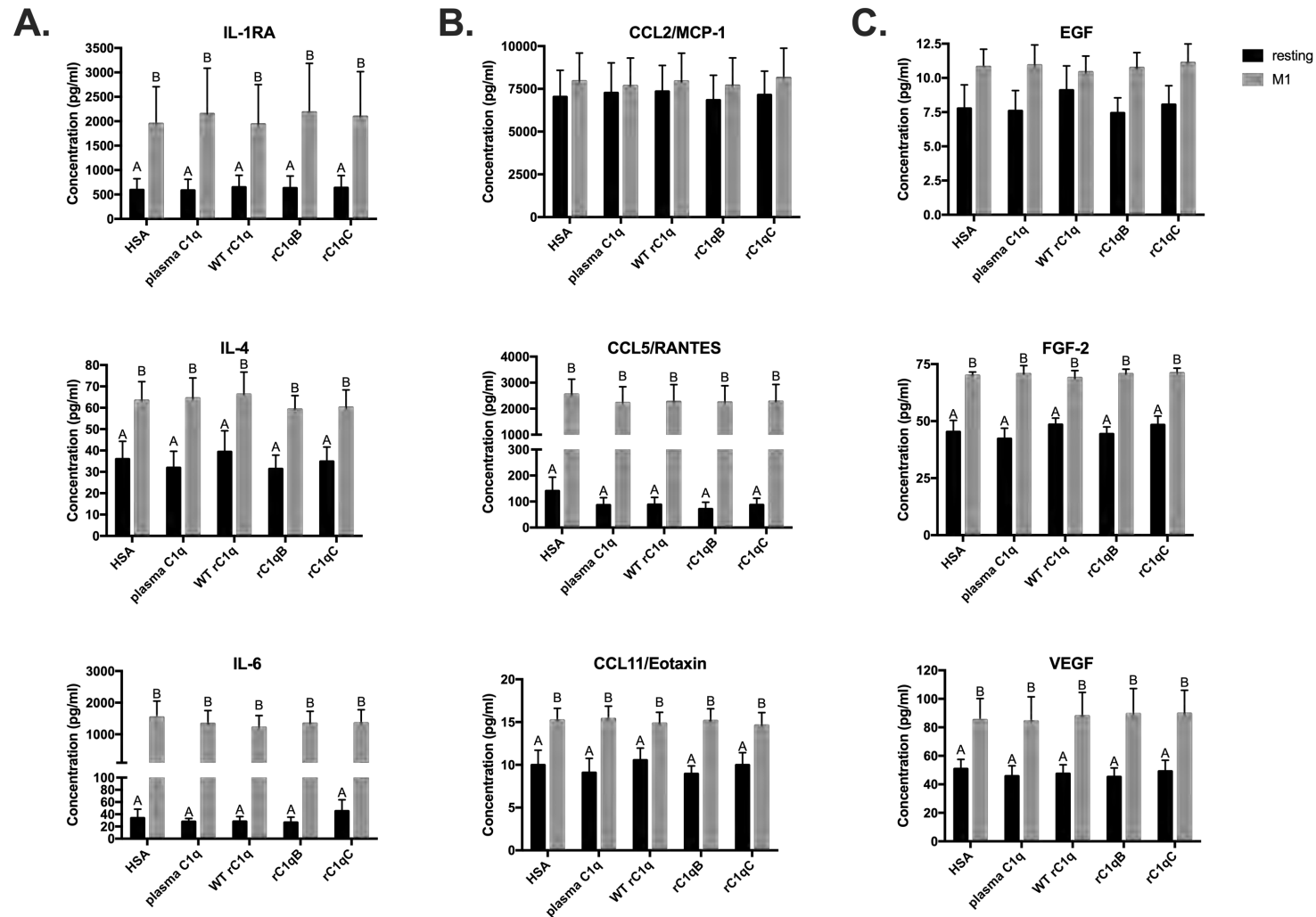


Figure S2. C1q variants rC1qB and rC1qC do not modulate certain HMDM secreted proteins, similar to plasma C1q. Resting or M1-polarized HMDM were adhered to plates coated with 5mg/ml protein for 24 hours. Secreted (A.) cytokine, (B.) chemokine, or (C.) growth factor levels were measured by Luminex multiplex 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).