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Insights into the structure-function relationships of dimeric C3d fragments

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Abstract

Cleavage of C3 to C3a and C3b plays a central role in the generation of complement-mediated defences. Although the thioester-mediated surface deposition of C3b has been well-studied, fluid-phase dimers of C3 fragments remain largely unexplored. Here we show C3 cleavage results in the spontaneous formation of C3b dimers and present the first X-ray crystal structure of a disulphide-linked human C3d dimer. Binding studies reveal these dimers are capable of crosslinking complement receptor 2 and preliminary cell-based analyses suggest they could modulate B cell activation to influence tolerogenic pathways. Altogether, insights into the physiologically-relevant functions of C3d(g) dimers gained from our findings will pave the way to enhancing our understanding surrounding the importance of complement in the fluid phase and could inform the design of novel therapies for immune system disorders in the future.

38 Introduction

39 Activation of the central complement component C3 (~1 mg mL⁻¹) to C3a and C3b by
40 classical/lectin (C4bC2a) or alternative (C3bBb) pathway C3 convertases plays an
41 essential role in the generation of complement-mediated defence mechanisms
42 against invading microbial pathogens. While the circulating C3a anaphylatoxin is
43 involved in inducing inflammatory immune responses, C3b (reported¹ normal plasma
44 concentration: ~210 ng mL⁻¹ but levels are markedly higher on infection and under
45 certain disease conditions although its short half-life (< 2 min) makes accurate
46 measurements difficult) facilitates opsonophagocytosis and clearance of immune
47 complexes through thioester-mediated opsonisation of primary amine- or hydroxyl-
48 containing antigenic and self surfaces. Attachment of multiple copies of C3b and its
49 breakdown products to antigenic surfaces in this way can result in C3d-complement
50 receptor 2 (CR2/CD21) and antigen-B cell receptor (BCR) co-ligation which
51 generates co-stimulatory signals for B cell activation in a C3d copy-dependent
52 manner^{2,3} and has been widely explored in vaccine design⁴⁻⁹.

53
54 Structure determination of native C3, C3b and C3c has provided crucial insights into
55 the mechanistic basis behind the activation of C3 to C3b^{10,11} while complexes of C3b
56 with factor I (FI) and the short consensus repeat (SCR) domains 1-4 of its cofactor
57 factor H (FH₁₋₄) have revealed the processes through which C3b is proteolytically
58 cleaved into its successive opsonin fragments iC3b and C3dg¹² (normal plasma
59 concentration: < 5.3 µg mL⁻¹, half-life: 4 hours¹³). Crystal structures have also shed
60 light upon the molecular basis underlying the thioester-mediated attachment of C3d
61 to antigenic surfaces¹⁴, provided explanations of how the interactions of C3d with its
62 receptors (CR2¹⁵ and CR3¹⁶) facilitate the recognition of opsonised antigens, and the
63 mechanisms by which pathogens such as *Staphylococcus aureus* utilise C3d-binding
64 proteins (e.g. Sbi¹⁷, Efb-C^{18,19} and Ecb/Ehp^{20,21}) to inhibit these interactions and
65 evade the immune system. Furthermore, complexes of C3d with FH SCR domains
66 19 and 20, which also bind host surface polyanionic markers such as
67 glycosaminoglycans and sialic acids, have been pivotal in understanding the
68 regulatory measures in place to protect host tissues against the indiscriminate
69 attachment of C3d to self versus non-self surfaces^{22,23}.

70

71 However, while these seminal structural studies alongside an abundance of
72 functional investigations have advanced our knowledge surrounding the interaction
73 of C3 fragments with self and non-self surfaces at a molecular level, our
74 understanding of the structural and functional aspects of fluid phase C3 activation
75 products remains incomplete. During activation in the fluid phase, the majority of C3
76 molecules do not covalently attach to surface-exposed hydroxyl- or amine-
77 nucleophiles but instead the highly reactive Cys–Gln thioester moiety within the
78 thioester-containing C3d domain (TED) of C3 undergoes hydrolysis resulting in the
79 generation of C3(H₂O) and formation of the C3(H₂O)Bb alternative pathway (AP) C3
80 convertase. Of the C3b generated by these fluid phase or surface-associated
81 convertases, only approximately 10% is deposited onto reactive surfaces²⁴, leaving
82 the remaining 90% to react with water wherein exposure of the cysteine free
83 sulfhydryl can lead to dimerisation of C3b and its subsequent breakdown products
84 iC3b and C3d(g) . Evidence of these dimers has been demonstrated via visualisation
85 of C3b generated from trypsin digestion of serum-derived C3 under non-reducing
86 conditions²⁵ and in C3dg preparations purified from human serum following ‘aging’ at
87 37 °C for 7 days²⁶.

88

89 C3b dimers, formed either by disulphide bonds or via other, undefined interactions,
90 have also been found to bind CR1 with 25-fold higher affinity than monomeric C3b²⁵,
91 induce histaminase release from human polymorphonuclear leukocytes²⁶, serve as
92 binding platforms for factor B fragment Bb during formation of AP C5
93 convertases^{27,28} and act as potent AP activators in complex with IgG²⁹. In addition,
94 dimers of C3dg have been isolated from C3-activated human serum following
95 omission of N-ethylmaleimide³⁰ and the propensity of recombinant C3d to dimerise
96 has been reported previously^{31,32}. A crystal structure of dimeric C3dg purified from
97 rat serum³³ provides further crucial evidence of the endogenous existence of these
98 dimers. However, aside from this severely truncated C3dg dimer which is believed to
99 have undergone proteolytic truncation during the crystallisation process³⁴, there is
100 currently a gap in knowledge surrounding the structural significance of disulphide-
101 linked dimers of C3 fragments as the thioester cysteine sulfhydryl is routinely
102 removed prior to structural analyses. For instance, the free cysteine of C3b has been
103 reacted with iodoacetamide prior to structural determination^{11,35,36} and the vast

104 majority of C3d constructs used for structural studies to date have harboured a
105 cysteine to alanine substitution in order to prevent dimerisation^{14,15,22,23}.

106

107 In this study we therefore aimed to delineate the molecular details and explore the
108 functional significance of dimeric human C3 fragments that can form following
109 activation of C3 in the fluid phase. We provide confirmatory evidence showing the
110 formation of disulphide-linked C3b dimers derived from serum-derived C3 and
111 subsequently present the first crystal structure of a human C3d dimer at 2.0 Å where
112 dimerisation is mediated by disulphide linkage of the thioester cysteine residues.
113 Through surface plasmon resonance (SPR) binding studies and preliminary cell
114 experiments using mouse splenocytes and human peripheral blood mononuclear
115 cells (PBMC) we show how dimeric C3d crosslinks surface-bound CR2 and could
116 modulate B cell activation to potentially influence tolerance mechanisms. In the
117 future a deeper understanding of these newly-discovered physiologically-relevant
118 roles of C3 fragment dimers could inform the design of autoimmune therapies and
119 help to further elucidate the significance of complement in the fluid phase as it
120 interacts with cells of the adaptive immune system and beyond.

121

122

123 **Results**

124 To elucidate the importance of dimeric human C3 breakdown fragments, we
125 investigated the formation of disulphide-linked dimers of C3b and C3d through
126 limited trypsin proteolysis of C3 and subsequently analysed the structural
127 characteristics of dimeric C3d using X-ray crystallography. SPR was used to
128 compare the binding kinetics and avidity of these dimers and monomeric C3d to CR2
129 and FH₁₉₋₂₀ and C3d-induced changes in the activation state of B cells were explored
130 using flow cytometric analyses.

131

132 **Cleavage of C3 results in the spontaneous formation of disulphide-linked C3b** 133 **dimers**

134 C3 purified from human serum was cleaved with trypsin under mild proteolysis
135 conditions and subsequently analysed using SDS-PAGE and anti-C3 α -chain
136 western blotting (**Figure 1**). Over 50% of C3 is cleaved to C3b following digestion
137 with trypsin after 2 minutes (**Figure 1a**) and a significant fraction of this C3b,
138 visualised under non-reducing conditions, spontaneously forms disulphide-bonded
139 dimers (**Figure 1b**). These dimers form instantly and remain stable for at least 2
140 hours (**Supplementary Figure S1**). In addition to the C3b dimers, a weak higher
141 molecular weight band suggestive of a dimeric form of C3 can also be observed at
142 $t=0$. The absence of these dimers in samples treated with reducing agent indicates
143 their formation is mediated by disulphide bonds. In a similar manner, recombinant
144 wild-type human C3d, with its native thioester cysteine intact (C3d^{17C}), also forms
145 disulphide-linked dimers under non-reducing conditions (**Supplementary Figure**
146 **S2**).

147

148 **Disulphide linkage of the thioester cysteine results in C3d dimerisation**

149 A crystal structure of wild-type human C3d, harbouring a cysteine at position
150 17/1010 (C3d numbering/intact pre-pro C3 numbering) (C3d^{17C}), was obtained at 2.0
151 Å resolution (**Figure 2, Supplementary Tables S1 and S2**). The structure clearly
152 shows the formation of a dimer mediated by partial disulphide linkage of the thioester

153 cysteine residues at position 17/1010 in both monomeric chains. This 17C-17C
154 disulphide creates a link between the two C3d monomers at the C-terminus of helix
155 α_1 , causing the convex molecular surfaces of the monomers to orient towards each
156 other whilst simultaneously exposing their concave binding faces (**Figure 2a**). Closer
157 examination of the C3d^{17C} dimer interaction surface (**Figure 2b**) confirms that the
158 overall (α - α)₆ barrel configuration of both monomers remains unchanged and
159 comparable to previously-published structures of monomeric C3d (0.61 Å (chain
160 A)/0.40 Å (chain B) main chain (M1-P294) RMSD relative to PDB: 1C3D). The 2Fo-
161 Fc electron density map at the C3d^{17C} dimer interface shows that chain B residue
162 17C (**Figure 2b** inset) adopts a dual conformation with one conformer existing in an
163 unpaired oxidized form, perhaps due to radiation damage. This indicates the
164 disulphide bond linking the two C3d monomers can occur in a partially disconnected
165 state which is consistent with results from size exclusion chromatography
166 experiments suggesting C3d^{17C} exists in a monomer-dimer equilibrium in solution
167 (**Supplementary Figure S2**).

168
169 Superimposition of the ligand-binding domains of CR2 (SCR1-2), the α_M integrin
170 domain of CR3 or SCRs 19-20 of FH on to the dimeric C3d^{17C} structure fails to
171 generate any molecular clashes (**Supplementary Figures S3a-d**). This important
172 observation suggests dimerisation does not cause any interference in the formation
173 of complexes between C3d or C3dg and their most physiologically-relevant binding
174 partners. *Staphylococcus aureus* immune evasion proteins such as Efb-C, Ecb/Ehp
175 and Sbi-IV are also predicted to bind the C3d^{17C} dimer without any hindrance as the
176 concave surfaces of both monomers are exposed and accessible. Significantly, as
177 CR2 and CR3 interact with the C3d^{17C} dimer via opposing surfaces, complement
178 receptor crosslinking could play an important role in the function of C3d dimers
179 (**Supplementary Figure S3c**). Moreover, the absence of steric hindrance following
180 superimposition of the C3d^{17C} dimer onto the C3b TED domain (**Supplementary**
181 **Figure S3e**), suggests dimerisation of C3b, as proposed previously^{37,38}, could occur
182 in a similar fashion to C3d without affecting the ability of C3b to interact with the
183 complement regulators FH and FI.

184

185 **C3d dimers can crosslink CR2 and FH₁₉₋₂₀**

186 As our structural analyses revealed the propensity of C3d^{17C} to dimerise, we next
187 analysed the binding interactions of C3d dimers in comparison to monomeric C3d^{17A}
188 using CR2 and FH₁₉₋₂₀ as two important known binding partners. Given that our size
189 exclusion chromatography experiments (**Supplementary Figure S2**) showed that
190 monomeric and dimeric C3d exist in equilibrium and therefore dimers can readily
191 dissociate into their monomeric constituents, we opted to create chemically stable
192 dimers of C3d^{17C} conjugated at the 17C position via a bromine-based linear linker
193 (N,N'-(propane-1,3-diyl) bis(2-bromoacetamide), see Materials and Methods;
194 **Supplementary Figures S4-S6b**). The N,N'-(propane-1,3-diyl) bis(2-
195 bromoacetamide) linker was used as this class of chemical compound has been
196 shown to selectively crosslink cysteine residues located within close spatial
197 proximity³⁹. Dimeric C3d^{17C} resulting from this chemical crosslinking reaction was
198 subsequently validated using particle analysis (**Supplementary Figure S6c**),
199 analytical ultracentrifugation (**Supplementary Figure S6d**) and mass spectrometry
200 (**Supplementary Tables S3 and S4, Supplementary Figure S6e**) and utilised in
201 SPR spectroscopy studies to gain insights into its binding patterns and kinetics.

202
203 In contrast to monomeric C3d^{17A} which displays a conventional association-steady
204 state-dissociation binding pattern when flowed over surface-immobilised CR2-Fc and
205 FH₁₉₋₂₀ (**Figure 3a** left), the binding of dimeric C3d^{17C} to the same ligands was found
206 to be noticeably distinct and suggestive of a two-state binding interaction (**Figure 3a**
207 right). At low concentrations up to the first replicate of 15.63 nM (dashed line), the
208 highly avid interactions with negligible dissociation indicate a bivalent binding mode
209 whereby the C3d^{17C} dimer crosslinks two CR2-Fc or two FH₁₉₋₂₀ molecules. During
210 the first injection of 15.63 nM C3d^{17C} dimer, 25 RU of material binds to the surface
211 and 10 RU remain avidly bound to the surface after regeneration. While at the
212 second 15.63 nM injection, 18 RU of material binds to the surface and only 2RU
213 remains avidly bound at the end (**Supplementary Figure S7**). In both cases an
214 equivalent amount of material is eluted during regeneration indicating the first
215 injection likely saturates the highly avid binding sites. As the surface cannot be fully
216 regenerated of these high avidity complexes, the subsequent cycle commences at a
217 higher baseline response. At this point and higher concentrations, the high avidity
218 binding sites for dimeric C3d^{17C} on CR2-Fc or FH₁₉₋₂₀ remain saturated causing the
219 binding mode to switch to less favourable/avid readily-disrupted interactions

220 suggestive of 1:1 binding between the C3d^{17C} dimer and CR2-Fc or FH₁₉₋₂₀ although
221 some cross-linked C3d^{17C} dimer-CR2-Fc and C3d^{17C} dimer- FH₁₉₋₂₀ complexes
222 persist (1-2 RU of material remaining bound to the surface after regeneration). At the
223 highest three concentrations of dimeric C3d^{17C} (62.5-250 nM), the less favourable
224 1:1 interactions (between 1 C3d^{17C} dimer: 1 CR2-Fc or FH₁₉₋₂₀ molecule) which are
225 readily eluted from the surface dominate (**Figure 3a** right inset). Consistent with
226 these results, the unusual binding patterns observed here were also evident in a
227 further two independent experiments (**Supplementary Figure S8**) and cannot be
228 attributed to higher order species of analyte or ligand as biophysical techniques
229 showed the dimeric C3d^{17C} and FH₁₉₋₂₀ preparations used did not contain aggregates
230 (**Supplementary Figures S6c, S6d and S9**). A model illustrating the binding events
231 described here is presented in **Figure 3b** (for CR2-Fc) and the superposition
232 displayed in **Figure 3c** illustrates the feasibility of CR2 crosslinking by dimeric
233 C3d^{17C} at a structural level.

234

235 **Dimeric C3d is a more potent modulator of B cell activation than monomeric** 236 **C3d**

237 Following on from our SPR studies which indicated dimeric C3d may have the
238 capacity to crosslink CR2, our next aim was to analyse the effects of dimeric
239 compared to monomeric C3d on B cell activation. Flow cytometry was employed to
240 examine changes in the expression of four surface-associated B cell activation
241 markers (CD40, CD69, CD71 and CD86) resulting from stimulation of isolated
242 human B cells with monomeric C3d^{17A} or chemically-linked dimeric C3d^{17C} alone or
243 in the presence of BCR-crosslinking anti-IgM F(ab')₂. As shown in **Supplementary**
244 **Figure S10**, although agonism of the BCR by anti-IgM significantly enhances
245 expression of all the activation markers (except CD40), neither monomeric C3d^{17A}
246 nor dimeric C3d^{17C} appears to influence the activation of isolated B cells in an
247 appreciable manner, as measured by the markers examined.

248

249 A more general approach, using Ca²⁺ influx as a measure of B cell activation was
250 therefore taken next. Here, incubation of B220⁺ mouse splenocytes with monomeric
251 C3d^{17A} or dimeric C3d^{17C} prior to stimulation with a suboptimal dose of a biotinylated-
252 anti-IgM/C3dg-biotin/streptavidin (a-IgM-b/C3dg-b/ST) BCR/CR2-crosslinking
253 complex was found to inhibit BCR/CR2-mediated Ca²⁺ influx in a concentration-

254 dependent manner (**Figure 4a, Supplementary Figure S11**). The observed blocking
255 effect was more pronounced following treatment with dimeric C3d^{17C}, particularly at
256 the lower concentration of 4 µg (**Figure 4a**), and for both constructs is only evident
257 when C3d is added ahead of the a-IgM-b/C3dg-b/ST complex and when a
258 suboptimal dose of anti-IgM (i.e. unable to trigger Ca²⁺ influx in the absence of CR2
259 engagement) within the crosslinking complex is used. Thus, the perceived inhibition
260 of Ca²⁺ influx and hence B cell activation is likely to result from sequestration of CR2
261 by monomeric C3d^{17A}, and to a greater extent, due to avidity and possibly via CR2-
262 CR2 crosslinking as suggested by our SPR experiments, dimeric C3d^{17C}, reducing
263 the proportion of CR2 available for crosslinking with the BCR.

264
265 In order to further investigate C3d-mediated changes in the activation state of B cells
266 within mixed populations of cells, as would occur *in vivo*, flow cytometry was utilised
267 to explore differences in the expression of CD40, CD69, CD71 and CD86 on CD19⁺
268 cells within donor human PBMC samples (see **Supplementary Figures S12 and**
269 **S13** for gating strategy applied). In contrast to the results gathered from isolated
270 human B cells (**Supplementary Figure S10**), a clear dose-dependent relationship
271 between C3d and B cell activation was observed in these experiments indicating in B
272 cell other mononuclear cell types may be involved responsiveness to free C3d, as
273 measured by expression of the markers analysed (**Figure 4b**). At concentrations ≥
274 10 nM, both monomeric C3d^{17A} and chemically-linked dimeric C3d^{17C} are able to
275 enhance expression of the early B cell activation markers CD69 and CD86 even in
276 the absence of BCR engagement by anti-IgM. In concert with anti-IgM although both
277 forms of C3d synergistically upregulate expression of these markers in a
278 concentration-dependent manner, dimeric C3d^{17C} is found to be approximately three-
279 fold more effective at enhancing activation than monomeric C3d^{17A} (47nM vs 139 nM
280 (CD69) and 18 nM vs 59 nM (CD86) geometric mean EC50s), perhaps through more
281 avid interactions with CR2.

282
283 Interestingly, in contrast to CD69 and CD86, both monomeric C3d^{17A} and dimeric
284 C3d^{17C} appear to downregulate CD40, particularly in the presence of anti-IgM, with a
285 more pronounced reduction in expression evident in the presence of dimeric C3d^{17C}.
286 Differently still, despite achieving a substantial increase in expression in the
287 presence of anti-IgM in the experimental time period, CD71 does not appear to be

288 influenced by either form of C3d. Importantly, the differential marker-specific trends
289 observed are consistent across cells gathered from all three donors analysed (data
290 from donors 2 and 3 can be found in **Supplementary Figure S14**) suggesting free
291 C3d (unattached to an antigen) may regulate B cell activation in a selective manner
292 and that dimeric C3d may have more potent modulatory roles than C3d monomers.

293 Discussion

294 Pre-treatment of C3b with sulfhydryl-alkylating agents and routine use of a
295 recombinant thioester cysteine deletion construct (C17A) in the past has prohibited
296 the structural and functional analysis of disulphide-linked dimers of C3 fragments
297 that can form following activation in the fluid phase. Concurrent with previous
298 findings²⁵, in this study we provide evidence showing trypsin-mediated cleavage of
299 C3 results in the spontaneous formation of a significant fraction of disulphide-linked
300 C3b dimers (**Figure 1**). Interestingly, our results additionally suggest the formation of
301 a dimeric form of C3. This dimeric fraction could conceivably involve the hydrolysed
302 form of C3 (C3(H₂O)) in which the exposed thioester cysteine sulphhydryl renders it
303 prone to the formation of disulphide-linked dimers as observed with C3b²⁵ as well as
304 the related thioester-containing complement protein fragments C4Ab and C4Bb⁴⁰.

305
306 Furthermore, here we verify that C3 breakdown product C3d, with its native thioester
307 cysteine intact (C3d^{17C}), forms disulphide-linked dimers in an analogous fashion to
308 C3b and in the first X-ray crystal structure of a human C3d dimer we confirm that
309 dimerisation is mediated by disulphide linkage of the thioester cysteine residues at
310 position 17/1010 (C3d numbering/intact pre-pro C3 numbering) (**Figure 2**) in a
311 manner that would also permit the analogous dimerisation of C3b (**Supplementary**
312 **Figure S3e**). Importantly, this dimer retains the ability of C3d to bind SCR domains
313 1-2 of CR2, the α M1 integrin domain of CR3 and SCR domains 19-20 of FH
314 (**Supplementary Figures S3a-d**).

315
316 In order to complement our structural studies, we next analysed the binding of a
317 stable chemically-linked C3d^{17C} dimer (**Supplementary Figures S5 and S6**) to CR2
318 (SCR1-4) and FH₁₉₋₂₀ using SPR. Here dimeric C3d^{17C} showed higher avidity binding
319 to both of the interacting partners examined, and in contrast to monomeric C3d, was
320 found to crosslink surface-associated CR2 as well as FH₁₉₋₂₀ (**Figure 3**,
321 **Supplementary Figures S7 and S8**). This crosslinking by disulphide-linked C3d^{17C}
322 dimers cannot be explained by the formation of higher order aggregates of dimeric
323 C3d^{17C} (**Supplementary Figures S6c and S6d**) or FH₁₉₋₂₀ (**Supplementary Figure**
324 **S9**) and is a finding that has not been observed previously but could indicate a
325 potential physiologically-relevant role of these dimers. Future investigations will

326 elucidate whether dimeric C3d^{17C} can crosslink its other receptor, CR3, or a
327 combination of CR2 and CR3, as suggested by our structural superpositions
328 (**Supplementary Figures S3b** and **S3c**).

329
330 Finally, we investigated the effects of dimeric compared to monomeric C3d on the
331 activation state of primary human and murine B cells using flow cytometry and Ca²⁺
332 influx experiments. In contrast to results from resting tonsillar B cells⁴¹, when
333 assayed in isolation, we found B cells purified from human PBMCs appeared to be
334 unresponsive to both forms of free C3d (**Supplementary Figure S10**). However,
335 both monomeric C3d^{17A}, and to a greater extent dimeric C3d^{17C}, inhibited BCR/CR2-
336 mediated Ca²⁺ influx in B220⁺ murine splenocytes when added prior to stimulation
337 with a BCR/CR2-crosslinking complex (**Figure 4a** and **Supplementary Figure S11**).
338 Further to previous reports using biotinylated C3dg (with a C17A mutation) in the
339 presence of streptavidin^{41,42}, these results suggest that pre-ligation of CR2 by
340 naturally-occurring fluid phase C3d(g) dimers could inhibit BCR/CR2 crosslinking-
341 mediated Ca²⁺ responses in B cells by sequestering the CR2/CD19/CD81 receptor
342 complex from the BCR with higher avidity than C3d monomers.

343
344 Both dimeric and monomeric C3d were also found to induce changes in the
345 expression of B cell activation markers on human CD19⁺ cells within PBMC samples
346 (**Figure 4b**, **Supplementary Figure S14**). Specifically, in the presence of anti-IgM,
347 both monomeric C3d^{17A} and to a three-fold greater extent dimeric C3d^{17C},
348 synergistically upregulated CD69 and CD86 which is consistent with previous reports
349 showing independent ligation of CR2 and the BCR (i.e. without crosslinking) by
350 simultaneous stimulation with biotinylated-C3dg/streptavidin complexes and anti-IgM
351 can augment B cell activation⁴³. Our results, however, additionally show that dimeric
352 C3d^{17C} is more efficient at augmenting CR2/BCR-dependent activation and that BCR
353 engagement may not be necessary for upregulation of certain activation makers as
354 at higher concentrations both forms of C3d were also capable of enhancing
355 expression of the early activation markers CD69 and CD86 in the absence of anti-
356 IgM. Although these findings in human PBMCs differ from a recent report suggesting
357 C3d inhibits the BCR-induced expression of CD69 on isolated tonsillar B cells⁴¹, they
358 are likely more representative of *in vivo* conditions, where interactions between
359 different cell types and associated factors occur continuously.

360
361 In contrast to CD69 and CD86, both monomeric and dimeric C3d appeared to
362 downregulate CD40, with a more pronounced reduction in expression in the
363 presence of dimeric C3d^{17C}. CD40 is involved in the regulation of several B cell
364 processes including germinal centre reactions⁴⁴, isotype switching⁴⁵ and somatic
365 hypermutation⁴⁶ and has also been shown to prevent B cells from becoming
366 anergic⁴⁷. Although further investigations are required to explain the C3d-mediated
367 downregulation of CD40 expression on B cells observed in our study, it is possible
368 that C3d stimulation of CR2 or CR3 expressed on other PBMC cell types (e.g. T
369 cells⁴⁸⁻⁵¹ and natural killer cells⁵²) induces the production of higher levels of soluble
370 CD40L that drive internalisation of CD40 or prevent efficient staining by occluding
371 the receptor. Alternatively, the known binding of CD40L to CR3⁵³ could be
372 outcompeted by CR3 interactions with C3d, particularly in its dimeric form, elevating
373 the levels of soluble CD40L available for binding CD40. Further experiments
374 investigating the effects of free monomeric and dimeric C3d on IgG titre and hence
375 B-cell differentiation or antibody class switching following activation of PBMCs with T
376 cell supernatants or co-culture with IL-2 or CD40L-producing feeder cells will help to
377 understand this process further.

378
379 Nonetheless, the preliminary data presented here suggest fluid-phase C3d(g),
380 particularly in its dimeric form, could alter the activation of B cells and may direct
381 them towards an anergic state. Although further verification is required, this
382 proposed role would be logical in terms of helping to limit the involvement of
383 complement in the generation of humoral immune responses in the absence of a
384 threat and is consistent with reports of CR2 ligation being involved in the anergy of
385 autoreactive B cells^{43,54,55}. Thus, in the future, C3d(g) dimers could have implications
386 for the development of novel therapies for autoimmune diseases, for example
387 through their effects on CD40/CD40L interactions, particularly as downregulation of
388 CD40 was shown to be a beneficial outcome of Rituximab treatment of systemic
389 lupus erythematosus (SLE) patients⁵⁶ and CD40/CD40L levels have been linked to
390 anti-DNA autoantibody titres in lupus patients⁵⁷ and mouse models⁵⁸. By extension,
391 these newly-uncovered functions of C3d could also offer a possible explanation as to
392 why humoral immune responses are inhibited, rather than enhanced, by certain

393 vaccine constructs composed of antigens linked to linear repeats of C3d placed in
394 close proximity to each other^{43,59}.

395

396 On the whole, our cell experiments not only suggest free fluid-phase C3d(g)
397 (unattached to a surface) may regulate B cell activation in a selective manner but
398 also that there are clear functional differences between monomeric and dimeric C3d
399 with the latter being a more potent modulator of the activation state of B cells as a
400 consequence of high avidity receptor interactions or through receptor crosslinking. In
401 addition, they indicate other PBMC cell types play an important role in the
402 responsiveness of B cells, in terms of the activation markers analysed, to C3d,
403 perhaps through the provision of sensitising or synergising co-stimulatory molecules
404 or via CR2-CR2 or CR2-CR3 crosslinking between cells. Although these preliminary
405 experiments have brought to light some of the potentially physiologically-relevant
406 functions of fluid-phase C3d(g) dimers, further investigations probing the molecular
407 mechanisms underlying these roles are warranted. In a wider context, it would also
408 be interesting to explore possible connections between the levels of C3d(g) dimers,
409 their distribution in the body and pathological conditions associated with uncontrolled
410 C3 activation, such as C3 glomerulopathy, as we surmise local upregulation of fluid
411 phase C3d(g) concentrations is likely to enhance C3d(g) dimerisation.

412

413 In summary, in this study we reaffirm the spontaneous formation of disulphide-linked
414 C3b dimers following cleavage of C3 and present the first structure of a fluid-phase
415 disulphide-linked human C3d dimer. Through accompanying functional analyses we
416 show that these dimers could have physiologically-relevant roles in crosslinking CR2
417 and selectively modulating B cell activation, possibly to trigger tolerogenic pathways.
418 Overall, our findings shed light on a fundamental aspect of complement biology that
419 is often overlooked and could have the potential to inform the design of novel
420 therapeutics for immune system disorders in the future.

421

422 **Materials and Methods**

423

424 **Purification and mild trypsin proteolysis of human C3**

425 C3 was purified from human serum by PEG precipitation⁶⁰, by slowly mixing the
426 serum with PEG 4000 (in precipitation buffer: 100 mM sodium phosphate, 150 mM
427 NaCl, 15 mM EDTA, 0.5 mM PMSF, pH 7.4) to a final concentration of 5% and then
428 incubating the mixture on ice for 30 mins. After centrifugation, the supernatant was
429 retained, and the process was repeated using PEG 4000 at a final concentration of
430 12%. The resulting pellet was resuspended in binding buffer and purified by weak
431 anion exchange chromatography (column: 1 mL HiTrap DEAE Sepharose FF
432 (Cytiva), binding buffer: 25 mM potassium phosphate, 5 mM EDTA, 50 mM EACA,
433 pH 7.0, elution buffer: 25 mM potassium phosphate, 5 mM EDTA, 50 mM EACA, 300
434 mM NaCl, pH 7.0).

435 The C3 containing fractions were subsequently pooled and 100 µg was digested with
436 Trypsin Gold protease (Promega) at 37°C for 2 mins before being quenched with 3%
437 (w/w) soybean trypsin inhibitor (Sigma Aldrich). An additional t=0 sample was
438 prepared by adding trypsin and trypsin inhibitor at the same time to a sample
439 containing 10 µg C3 before the incubation. The digested protein was then incubated
440 at 18°C for 2 hours, with timepoints taken every 15 mins and analysed using
441 reducing and non-reducing tris-acetate SDS-PAGE.

442 For western blot analyses, PVDF membrane was initially washed in methanol and
443 then soaked in western blot transfer buffer (methanol-free, Pierce) along with the gel
444 and filter pads. The proteins from the SDS-PAGE gel were then blotted onto the
445 PVDF membrane using a G2 semi-dry fast blotter (Pierce). After the membrane was
446 blocked and subsequently washed, and the immunodetection steps were completed
447 on a SNAP id 2.0 western blotting system (Merck Millipore) according to
448 manufacturer's instructions. The antibodies used include a polyclonal rabbit anti-C3d
449 (Dako) and a polyclonal goat anti-rabbit IgG (H+L) HRP conjugated (Invitrogen). To
450 detect the HRP conjugated antibody the membrane was incubated with ECL prime
451 western blotting substrate (Amersham) and then imaged on a Fusion SL (VILBER)
452 by chemiluminescence with molecular weight markers highlighted using a
453 WesternSure pen (LI-COR).

454 **Expression and purification of recombinant proteins**

455 The DNA sequence of human C3d (residues 1-310) comprised of C3 residues 996-
456 1303 (pre-pro C3 numbering) with a Cys to Ala mutation at position 17(C3d) /1010
457 (pre-pro C3) (C3d^{17A}) was previously cloned into the pET15b expression plasmid¹⁴.
458 To reproduce the wild-type sequence, the Ala at position 17 of the C3d^{17A} construct
459 was reverted back to a Cys (C3d^{17C}) using site-directed mutagenesis. Both C3d
460 constructs were expressed in the *Escherichia coli* BL21(DE3) (Sigma Aldrich) or
461 Shuffle T7 (NEB) cell lines and purified using cation exchange (column: HiTrap SP
462 HP [GE Healthcare], binding buffer: 50 mM MES pH 5.5, elution buffer: 50 mM MES,
463 500 mM NaCl pH 5.5) followed by size exclusion chromatography (column: HiLoad
464 16/600 Superdex 200 prep grade [GE Healthcare], buffer: 20 mM Tris, 150 mM NaCl
465 pH 7.4).

466 The human CR2(SCR1-4)-Fc and FH₁₉₋₂₀ constructs used in surface plasmon
467 resonance experiments were expressed in Chinese hamster ovary (CHO) cells or
468 *Pichia pastoris* respectively and purified as described previously (CR2(SCR1-4)-Fc:
469 ⁸; FH₁₉₋₂₀: ⁶¹). The monomeric state of FH₁₉₋₂₀ was confirmed using analytical
470 ultracentrifugation (**Supplementary Figure S9**).

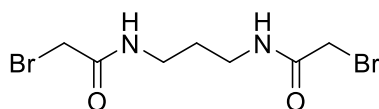
471

472 **Crystallisation, data collection and structure determination of dimeric C3d^{17C}**

473 Crystallisation was performed at 18°C using the hanging drop vapour diffusion
474 method. A 15 mg mL⁻¹ (432 µM) C3d^{17C} solution was subjected to a grid screen
475 containing 100 mM Tris pH 8, 50-300 mM NaCl and 16-26% PEG 4000. Crystals
476 appeared in the condition containing 100 mM Tris pH 8, 200 mM NaCl, 24% PEG
477 4000, were mounted on loops, flash-frozen in liquid nitrogen and X-ray diffraction
478 data collected on the IO4 beamline at the Diamond Light Source synchrotron
479 (Oxfordshire, UK) (See **Supplementary Table S1** for data collection statistics).
480 Integration of Dectris PILATUS 6M pixel detector diffraction images and data
481 reduction were performed using Xia2-DIALS and AIMLESS respectively. The
482 automated BALBES pipeline and COOT were used for molecular replacement and
483 model building. Refinement was carried out in REFMAC and Phenix (refinement
484 statistics can be found in **Supplementary Table S2**) and UCSF Chimera was used

485 for superpositioning and generation of images. The structure of the C3d¹⁷C dimer is
486 available on the PDB with the following accession code: 6RMT.

487 **Synthesis and characterisation of *N,N'*-(propane-1,3-diyl) bis(2-**
488 **bromoacetamide) linker**



489
490 A solution of K₂CO₃ (5.92 g, 42.8 mmol) in water (21 mL) was added to a solution of
491 1,3-diaminopropane (1.06 g, 14.3 mmol) in chloroform (35 mL) at 5°C with stirring. A
492 solution of bromoacetyl bromide (8.65 g, 42.8 mmol) in anhydrous chloroform (15
493 mL) was then added dropwise to the mixture and the reaction was left stirring at
494 room temperature for 18 hours. The resultant precipitate was filtered, washed with
495 water (6 x 10 mL), and dried under vacuum to yield *N,N'*-(propane-1,3-diyl)bis(2-
496 bromoacetamide) as a white solid (2.45 g, 55%). Subsequent characterisation of the
497 linker was performed using ¹H-NMR (**Supplementary Figure S4a**) and ¹³C-NMR
498 (**Supplementary Figure S4b**). High resolution electrospray ionisation time-of-flight
499 mass spectrometry m/z: [M + Na]⁺ calculated for C₇H₁₂Br₂N₂O₂Na = 338.9143 Da,
500 338.9143 Da was observed.

501

502 **Production, purification and characterisation of chemically-linked C3d dimers**

503 For the generation of chemically-linked C3d¹⁷C dimers, small-scale trials were
504 performed involving combination of C3d¹⁷C with the *N,N'*-(propane-1,3-diyl)bis(2-
505 bromoacetamide) linker in 0.1 M Tris, 0.15 M NaCl, 5 mM EDTA pH 7.5 at 0.55, 0.75
506 or 1.0 molar equivalences. Following overnight incubation at room temperature
507 (21°C), linker-mediated C3d¹⁷C dimerisation was confirmed using reducing SDS-
508 PAGE and electrospray time-of-flight mass spectrometry (**Supplementary Figure**
509 **S5**). A larger scale reaction at 0.75 molar equivalence (3.75 mg C3d¹⁷C, 0.026 mg
510 linker) was subsequently carried out as described above and subjected to size
511 exclusion chromatography to separate the chemically-linked dimeric C3d¹⁷C from
512 monomeric C3d¹⁷C (**Supplementary Figures S6a and S6b**). Particle size analysis
513 yielded a single species (**Supplementary Figure S6c**), analytical ultracentrifugation
514 confirmed the dimeric state of the chemically-linked C3d¹⁷C (**Supplementary Figure**
515 **S6d**) and both biophysical techniques showed a lack of aggregate formation.

516 Chemically-linked dimeric C3d^{17C} was subsequently digested with trypsin (Sigma
517 Aldrich) (1:50 ratio) at 37°C over a time course (**Supplementary Figure S6e**). The
518 digestion reaction was stopped by addition of a trypsin inhibitor (Sigma Aldrich) (1:2
519 ratio). Electrospray ionization time-of-flight mass spectrometry of the trypsin-digested
520 dimeric C3d^{17C} fragments followed by analysis using the Masshunter Qualitative
521 Analysis and BioConfirm (Agilent) software packages was used to confirm chemical
522 linkage at position 17C of C3d (**Supplementary Table S3**) and the presence of an
523 intact internal disulphide bond (**Supplementary Table S4**).

524

525 **Surface plasmon resonance**

526 All surface plasmon resonance experiments were performed on a Biacore S200
527 sensor (GE Healthcare) at 25°C with HBST (10 mM HEPES, 150 mM NaCl, 0.005%
528 Tween-20, pH 7.4) used as the running buffer. CR2-Fc and FH₁₉₋₂₀ were prepared in
529 10 mM sodium acetate pH 5 and immobilised at 300 RU (CR2-Fc: 20 µM, FH₁₉₋₂₀:
530 240 µM) to different flow cells of CM5 chips (GE Healthcare) using standard amine
531 coupling involving preparation of the dextran matrix with 100 mM N-
532 hydroxysuccinimide (NHS) and 40 mM 1-ethyl-3-(dimethylaminopropyl) carbodiimide
533 (EDC) followed by quenching with 1 M ethanolamine-HCl pH 8.5. Monomeric C3d^{17A}
534 and chemically-linked dimeric C3d^{17C} used as analytes were prepared to a fixed
535 concentration, serially diluted in HBST and injected in duplicate. 10 mM sodium
536 acetate, 1 M NaCl pH 4 was used as the regeneration buffer but could not
537 regenerate the chip surface of the highly avid interactions between dimeric C3d^{17C}
538 and CR2-Fc/FH₁₉₋₂₀. Data were analysed using the Biacore S200 Evaluation
539 Software 1.0. Responses from blank reference flow cells were subtracted from
540 ligand-immobilised flow cells and all data were double-referenced (buffer inject
541 subtracted).

542

543 **Flow cytometric analysis of B cell activation**

544 Human peripheral blood mononuclear cells (PBMC) were isolated from leukocyte
545 cone blood collected from healthy volunteers (NHS Blood and Transplant Service),
546 using density-gradient centrifugation in LeucosepTM tubes (Greiner-Bio-One).
547 PBMCs were frozen and stored in liquid nitrogen in accordance with UCB Biopharma
548 UK HTA License Number 12,504. Frozen PBMCs were thawed and diluted into cold

549 RPMI 1640 medium (Gibco) supplemented with 10% (v/v) foetal bovine serum, 1%
550 (v/v) penicillin-streptomycin (Sigma Aldrich) and 1% (v/v) GlutaMAX (Gibco). For
551 experiments on isolated B cells, B cells were purified from PBMCs by negative
552 selection using the Miltenyi Biotec Human B Cell Isolation Kit II as per the
553 manufacturer's instructions. Following centrifugation, cells were counted, assessed
554 for viability, which typically exceeded 90% for PBMCs and 80% for B cells, and re-
555 suspended to the desired density in ambient medium.

556 PBMCs or B cells were then seeded onto sterile V-bottom plates at a density of
557 150,000 or 40,000 cells/well respectively and allowed to recover at 37°C with 5%
558 CO₂ for 1h. Monomeric C3d^{17A} or chemically-linked dimeric C3d^{17C} were serially
559 diluted in media and added to the cells in duplicate to give final concentrations
560 ranging from 2 µM to 0.1 nM (based on the molecular weight of monomeric C3d^{17A}
561 for both constructs in order to control for the number of binding sites). Following a 30
562 min incubation period with the C3d constructs, additions of either goat F(ab')₂ anti-
563 human IgM LE/AF (Southern Biotech) at a final concentration of 10 µg mL⁻¹ or media
564 were made and the cells incubated for a further 18h at 37°C with 5% CO₂.

565 After a period of cooling on ice, the cells were stained for 1h with the LIVE/DEAD™
566 fixable near-infrared dead cell stain (1:1000 dilution, Invitrogen) along with the
567 following labelled antibodies diluted in an ice-cold staining buffer (PBS supplemented
568 with 1% BSA, 2mM EDTA and 0.05% NaN₃): PerCP-Cy™5.5 mouse anti-human
569 CD19 (1:40 dilution, Clone HIB19, BD Pharmingen) (for PBMC samples only), FITC
570 mouse anti-human CD40 (1:20 dilution, Clone 5C3, BD Pharmingen), Brilliant Violet
571 421™ mouse anti-human CD69 (1:40 dilution, Clone FN50, BioLegend), PE mouse
572 anti-human CD71 (1:20 dilution, Clone M-A712, BD Pharmingen) and APC mouse
573 anti-human CD86 (1:20 dilution, Clone 2331, BD Pharmingen). The cells were
574 subsequently washed and analysed on an Intellicyt® iQue Screener PLUS flow
575 cytometer. The gating strategy applied for live, singlet CD19⁺ B cells and activation
576 markers can be found in **Supplementary Figures S12** and **S13**, respectively.
577 Antibody capture beads were used for compensation. Data were expressed as mean
578 values from at least 2 replicates ± standard deviation from the mean and depicted as
579 scatter plots with curves fitted using a four-parameter variable slope non-linear
580 regression model in GraphPad Prism (version 8.4.1). The geometric mean

581 fluorescence intensity for the monomer and dimer were compared at both 0.1 nM
582 and 2000 nM C3d concentration using an analysis of variance on the log
583 transformed fluorescence fitting donor as a fixed effect. The downregulation of
584 CD40 expression is expressed as a percentage reduction in fluorescence of the
585 dimer compared to the monomer in the anti-IgM stimulated cells.

586

587 **Ca²⁺ influx experiments**

588 Intracellular Ca²⁺ measurements using flow cytometry were performed as described
589 previously^{42,62,63}. Briefly, isolated C57BL/6 mouse splenocytes maintained at 37°C
590 were Indo 1-AM loaded, stained with a rat anti-mouse CD45R/B220-APC antibody
591 (Clone RA3-6B2, BD Pharmingen) and analysed on a BD LSR II flow cytometer (BD
592 Biosciences) at RT. 4 or 10 µg of monomeric C3d^{17A} or chemically-linked dimeric
593 C3d^{17C} were added to the cells 30s after data acquisition. After 90s, cells were
594 stimulated with a suboptimal concentration of pre-mixed complexes composed of
595 0.056 µg mL⁻¹ biotinylated F(ab')₂ goat anti-mouse IgM (µ-chain specific) (Jackson
596 ImmunoResearch), ~3 µg mL⁻¹ C3dg-biotin (produced in house) and ~1.3 µg mL⁻¹
597 streptavidin (algM-b/C3dg-b/ST). Experiments were run for 10 min and intracellular
598 Ca²⁺ influx of gated B220⁺ cells was analysed using the FlowJo® software (FlowJo
599 LLC, BD).

600

601

602

603 **Data Availability**

604

605 The datasets generated and analysed during the current study are available from the
606 corresponding authors on request.

607

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830

831 **Author Contributions**

832 J.v.d.E, K.J.M., A.M., B.G.G. and A.A.W. designed the experiments. C.R.B.
833 performed preliminary structural studies. A.A.W. performed the crystallisation and
834 circular dichroism experiments. S.J.C. and A.A.W. reprocessed the crystallography
835 data and refined the structures. R.W.D. purified C3, guided by M.L., and carried out
836 the trypsin proteolysis, molecular modelling and structural analyses. A.G.W., B.A.

837 and R.L.M. synthesised and characterised the linker and conducted initial linkage
838 experiments. B.G.G. and A.A.W. performed the SPR experiments under the
839 guidance of K.J.M. and with helpful discussions from C.L.H. who also analysed the
840 data. L.K. completed the Ca²⁺ influx experiments. The B cell activation flow
841 cytometry experiments were performed by A.M. and K.W. with the assistance of
842 I.P.M. J.v.d.E. and A.A.W. wrote the manuscript with valuable contributions from all
843 the authors.

844

845 **Competing interests**

846 K.J.M. is a consultant for and receives funding or remuneration from Gemini
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856

857 **Additional information**

858 **Supplementary information** for this publication is available online

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861

862 **Figure Legends**

863

864 **Figure 1. Cleavage of C3 results in the spontaneous formation of disulphide-**
865 **linked C3b dimers.** (a) Reducing Tris-Acetate SDS-PAGE (left panel) and anti-C3
866 α -chain western blot (right panel) analyses of serum-derived human C3 subjected to
867 mild trypsin proteolysis at t=0 and t=2 minutes. Indicated are the intact and cleaved
868 C3 α -chains (120 and 111 kDa, respectively) and C3 β -chain (75 kDa). Anti-C3 α -
869 chain western blot analysis confirms the positions of the intact and cleaved C3 α
870 chains (right panel). (b) Non-reducing Tris-Acetate SDS-PAGE (left panel) and anti-
871 C3 α -chain western blot (right panel) analyses of human C3 subjected to mild trypsin
872 proteolysis at t=0 and t=2 minutes. Indicated are intact and cleaved C3 (195 and 186
873 kDa, respectively) as well as disulphide-linked C3b dimers (C3b², right panel) and a
874 faint band suggestive of a dimeric form of C3 (highlighted as C3² in grey font). Anti-
875 C3 α -chain western blot analysis confirms the positions of intact C3 and monomeric
876 and disulphide-linked dimeric C3b (right panel). Molecular weight markers shown are
877 HiMark (M1) and PageRuler Plus (M2). Raw SDS-PAGE gel and western blot
878 images can be found in Supplementary Figure S1.

879

880 **Figure 2. Structure of a disulphide-linked human C3d^{17C} dimer at 2.0 Å**
881 **resolution.** (a) The ribbon diagram shows disulphide linkage of the monomeric
882 subunits at position Cys17 results in the formation of a dimer 92.37 Å in length with a
883 0.61 Å (chain A)/0.40 Å (chain B) main chain (M1-P294) RMSD relative to the
884 structure of C3d^{17A} (PDB:1C3D). (b) Enlarged view of the C3d^{17C} dimer interface
885 showing the side chains of helix α 1 residues M1-C17. Inset: 2Fo-Fc electron density
886 contoured at 1.0 σ of the partially broken C17-C17 interchain disulphide bond (2.07
887 Å) resulting from oxidation of one conformer of Chain B Cys17. (c) Solid molecular
888 surface representation of the C3d^{17C} dimer in three different orientations rotated by
889 90° angles counter-clockwise. PDB accession code: 6RMT. See Supplementary
890 Tables S1 and S2 for data collection and refinement statistics.

891

892 **Figure 3. Dimeric C3d^{17C} cross-links CR2 and FH₁₉₋₂₀.** (a) SPR sensorgrams
893 showing serially-diluted concentrations of 250 nM monomeric C3d^{17A} (left) or dimeric
894 C3d^{17C} (right) flowed in duplicate over flow cells of a CM5 sensor chip immobilised

895 with CR2-Fc (top) or FH₁₉₋₂₀ (bottom). The binding of C3d^{17A} to CR2-Fc and FH₁₉₋₂₀
896 follows a conventional association-steady state-dissociation pattern while the binding
897 of dimeric C3d^{17C} to the same ligands generates an unusual two-state binding
898 interaction. At concentrations up to the first 15.63 nM replicate (dashed line) the
899 binding patterns depict highly avid interactions suggestive of the formation of dimeric
900 C3d^{17C}-CR2-Fc and dimeric C3d^{17C}-FH₁₉₋₂₀ crosslinked complexes which are not
901 fully eluted from the surface. Thus, the subsequent injection cycles commence at a
902 higher baseline response where the high avidity binding sites for dimeric C3d^{17C} on
903 CR2-Fc or FH₁₉₋₂₀ remain saturated. This causes the binding mode to switch to less
904 favourable, readily-disrupted interactions suggestive of the formation of 1:1
905 complexes, although some crosslinked complexes persist. Inset: baseline-adjusted
906 sensorgrams showing the less favourable 1:1 complexes (1 C3d^{17C} dimer: 1 CR2-Fc
907 or FH₁₉₋₂₀ molecule) which form at higher concentrations of dimeric C3d^{17C} (62.5-250
908 nM) and are readily eluted from the surface. Arrows depict the regeneration period.
909 See Supplementary Figure S7 for further details and Supplementary Figure S8 for
910 results from an additional two independent experiments. **(b)** Schematic model
911 depicting the proposed mechanistic basis behind dimeric C3d^{17C}-mediated
912 crosslinking of surface-associated CR2 (SCR 1-4). At low concentrations, C3d^{17C}
913 dimers crosslink two surface-associated CR2 (SCR 1-4) molecules via highly avid
914 interactions involving the acidic residue-lined concave face of C3d and SCRs 1 and
915 2 of CR2 (top). Once a critical threshold concentration has been surpassed, the
916 increase in dimeric C3d^{17C} molecules relative to available CR2 binding sites
917 outcompetes the second binding site on C3d^{17C} dimers and favours the formation of
918 1:1 complexes (middle). Unlike C3d^{17C} dimers, monomeric C3d^{17A} lacks the ability to
919 crosslink CR2 and is restricted to the formation of 1:1 complexes (bottom). **(c)**
920 Superposition of SCR1-2 of CR2 (PDB accession code: 3OED) onto its binding sites
921 on the C3d^{17C} dimer demonstrating how dimeric C3d^{17C} could crosslink CR2, as
922 indicated by the SPR data gathered, at a structural level.

923

924 **Figure 4. Monomeric C3d^{17A} and to a greater extent dimeric C3d^{17C} alter the**
925 **activation state of murine (a) and human (b) B cell populations. (a)** Ca²⁺ influx
926 experiment showing incubation with 4 µg C3d^{17A} monomer or C3d^{17C} dimer (30 s) 90
927 seconds prior to the addition of BCR/CR2-crosslinking complexes (a-IgM-b/C3dg-
928 b/ST) (120 s) significantly retards and reduces Ca²⁺ influx in CD45R/B220-gated Indo

929 1-AM-loaded C57BL/6 mouse splenocytes with a more pronounced blocking effect
930 apparent with dimeric C3d^{17C}. 10 µg of either form of C3d completely eliminates Ca²⁺
931 influx (Supplementary Figure S11) suggesting the observed blocking effect is
932 concentration dependent and likely a result of CR2 sequestration by monomeric
933 C3d^{17A}/dimeric C3d^{17C} reducing the proportion of CR2 available for crosslinking with
934 the BCR. BCR/CR2-crosslinking complexes were composed of a suboptimal dose
935 (0.056 µg mL⁻¹) of biotinylated F(ab')₂ goat anti-mouse IgM (a-IgM-b), C3dg-biotin
936 (C3dg-b) and streptavidin (ST). The C3d^{17A} monomer/C3d^{17C} dimer-mediated
937 blocking of Ca²⁺ influx was not evident when higher, more optimal concentrations of
938 a-IgM-b/ST were used or when all the reaction components were added
939 simultaneously. **(b)** Flow cytometric analysis of CD19⁺ B cells stimulated with
940 monomeric C3d^{17A} or dimeric C3d^{17C} in the presence or absence of BCR-
941 crosslinking anti-IgM F(ab')₂ (10 µg mL⁻¹) reveals C3d-induced changes in the
942 expression of surface-associated B cell activation markers. While no C3d-mediated
943 changes in CD71 expression are evident, at higher concentrations (≥ 3 nM) both
944 monomeric C3d^{17A} and dimeric C3d^{17C} appear to downregulate CD40, with a more
945 pronounced reduction in expression in the presence of dimeric C3d^{17C}. Conversely,
946 in the presence of anti-IgM, both monomeric C3d^{17A} and to a greater extent dimeric
947 C3d^{17C} synergistically upregulate CD69 and CD86 although at concentrations ≥ 10
948 nM both forms of C3d are also capable of enhancing expression of these activation
949 markers in the absence of anti-IgM. Data are of PBMC B cell populations from a
950 representative donor and displayed as mean values (n=2) ± standard deviation from
951 the mean with curves fitted using a non-linear regression model. Results from an
952 additional two donors can be found in Supplementary Figure S14.

953

954