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Clark, DJ; Catusse, J; Stacey, A; Borrow, P; Gompels, UA (2013)
Activation of CCR2+ human proinflammatory monocytes by hu-
man herpesvirus-6B chemokine N-terminal peptide. *The Journal*
of general virology, 94 (7). pp. 1624-35. ISSN 0022-1317 DOI:
<https://doi.org/10.1099/vir.0.050153-0>

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Activation of CCR2+ human proinflammatory monocytes by human herpesvirus-6B chemokine N-terminal peptide

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Running title: virus chemokine peptide activates CCR2+ monocytes

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Keywords: virus chemokine, CCR2, chemotaxis, macrophage, inflammation

Word and figure counts: Summary (Abstract): 206; Text: 4250; Figs and tables: 6

1 **ABSTRACT**

2 Human monocytes expressing CCR2 with CD14 or CD16 can mediate antigen presentation
3 and promote inflammation, brain infiltration and immunosenescence. Recently identified
4 roles are in HIV, parasitic and TB disease. Human herpesvirus 6B, HHV-6B, encodes a
5 chemokine, U83B, mono-specific for CCR2; distinct from related HHV-6A U83A, which
6 activates CCR1, CCR4, CCR5, CCR6 and CCR8 on immune effector cells and dendritic
7 cells. These differences could alter leukocyte-subset recruitment for latent/lytic replication
8 and associated neuroinflammatory pathology. Therefore, cellular interactions between U83A
9 and U83B could help dictate potential tropism differences between these viruses. U83A
10 specificity is maintained in the 38-residue N-terminal spliced-truncated form. Here we sought
11 to determine the basis for the chemokine receptor specificity differences and identify possible
12 applications. To do this we first analysed variation in a natural host population in sub-
13 Saharan Africa where both viruses are equally prevalent and compared these to global strains.
14 Analyses of U83 N-terminal variation in 112 HHV-6A and HHV-6B infections identified
15 6/38 U83A or U83B-specific residues. We also identified a unique single U83A-specific
16 substitution in one U83B sequence, 'U83BA'. Next, the variation effects were tested by
17 deriving N-terminal (NT) 17-mer peptides and assaying activation of *ex vivo* human
18 leukocytes, the natural host and cellular target. Chemotaxis of CCR2+ leukocytes was
19 potently induced by U83B-NT, but not U83BA-NT or U83A-NT. Analyses of the U83B-NT
20 activated population identified migrated CCR2+, but not CCR5+, leukocytes. The U83BA-
21 NT asparagine-lysine14 substitution disrupted activity, defining CCR2 specificity and a main
22 determinant for HHV-6A/B differences in cellular interactions. A flow cytometry-based
23 shape-change assay was designed, and used to provide further evidence that U83B-NT could
24 activate CCR2+CD14+CD16+ monocytes. This defines a potential anti-viral target for HHV-
25 6A/B disease and novel peptide immunomodulator for proinflammatory monocytes.

26 INTRODUCTION

27 Human monocytes can be classified into at least two distinct groups, classical and
28 non-classical based on CD14 and CD16 expression. These monocyte subsets express
29 chemokine receptors CCR2 and CX3CR1, respectively, which direct specific tissue migration
30 toward sites of selective chemokine secretion during infection (Ziegler-Heitbrock *et al.*,
31 2010). Recent studies have defined an intermediate monocyte group with intermediate CD14
32 and CD16 expression as well as intermediate levels of chemokine receptor expression
33 (Balboa *et al.*, 2011; Buckner *et al.*, 2011; Chimmá *et al.*, 2009; Lentz *et al.*, 2011; Williams
34 *et al.*, 2012; Ziegler-Heitbrock *et al.*, 2010). Transcriptome profiling of this group has
35 characterised these as antigen presenting cells with unique proinflammatory properties
36 (Merino *et al.*, 2011; Wong *et al.*, 2011; Zawada *et al.*, 2011; Ziegler-Heitbrock *et al.*, 2010).
37 Therefore the inflammatory response is fine-tuned depending on activation of specific
38 monocyte subsets. Recent evidence demonstrates monocytes in blood expressing CD14,
39 CD16 and CCR2, unlike classical or non-classical monocytes, have roles in mediating HIV
40 migration across the blood brain barrier and in increasing the severity of TB and
41 cardiovascular disease (Balboa *et al.*, 2011; Buckner *et al.*, 2011; Lentz *et al.*, 2011; Williams
42 *et al.*, 2012; Ziegler-Heitbrock *et al.*, 2010). These proinflammatory roles are combined with
43 increased antigen presentation in pleural effusions for TB, improved parasite inhibition, and
44 increased MHC class II and accessory molecules in donor gene expression studies (Balboa *et*
45 *al.*, 2011; Chimmá *et al.*, 2009; Wong *et al.*, 2011; Zawada *et al.*, 2011). Moreover, it has
46 been demonstrated that CD14, CD16, CCR2 expressing monocytes are senescent monocytes,
47 with shortened telomeres and increased chemokine receptor expression, which may
48 characterise increased inflammatory disease in the elderly including disposition to
49 cardiovascular disease (Merino *et al.*, 2011).

50 Human herpesvirus 6 is a ubiquitous pathogen in many populations, yet virus
51 reactivations from latent infection can be associated with severe inflammatory disease in
52 immunosuppressed patient populations, including post-transplant acute limbic encephalitis
53 (PALE), and cognitive impairment in hematopoietic stem cell transplantation as well as
54 myocarditis (Kuhl *et al.*, 2005a; Kuhl *et al.*, 2005b; Noutsias *et al.*, 2011; Schmidt-Hieber *et*
55 *al.*, 2011; Seeley *et al.*, 2007; Zerr *et al.*, 2011). By 2 years of age over 75% of infants have
56 acquired this pathogen and in adults HHV-6 seroprevalence is 83-100% worldwide (Hall *et*
57 *al.*, 2006). Primary infections of infants result in a usually self-limited fever and 10-24%
58 develop a skin rash; *exanthem subitum*, also called roseola (Hall *et al.*, 2006; Hall *et al.*,
59 1994; Zerr *et al.*, 2005). Recent evidence shows that approximately 0.1-1% of populations
60 examined have integrated HHV-6 genomes in the germline, giving inherited, chromosomally
61 integrated virus, ciHHV-6, with the potential to express virus genes as human alleles in every
62 cell (Arbuckle *et al.*, 2010; Arbuckle & Medveczky, 2011; Morissette & Flamand, 2010),
63 with implications for inflammatory disease.

64 HHV-6 is comprised of two variants, HHV-6A and HHV-6B, which have been
65 recently classified as distinct species (Adams & Carstens, 2012). They are highly similar in
66 terms of genome size, composition and structure, although there are differences in pathology,
67 cell tropism, and geographic distribution. Primary infant infections in Europe, USA and
68 Japan are predominantly infections with HHV-6B, 97-100%, while in Africa the reverse has
69 been found; 86-100% of healthy infants acquire HHV-6A as their primary HHV-6 infection
70 (Bates *et al.*, 2009; Hall *et al.*, 2006; Kasolo *et al.*, 1997; Sjahril *et al.*, 2009). HHV-6A and
71 HHV-6B lytically infect CD4⁺ T lymphocytes and undergo latency in monocytic, bone
72 marrow progenitor cells (Luppi *et al.*, 1999; Lusso *et al.*, 1988). In addition, it has been
73 shown *in vitro* that there are differences in cell tropism between HHV-6A and B. HHV-6A
74 has been reported to infect *in vitro* CD8⁺ T cells, NK cells, $\gamma\delta$ T cells, astrocytes and

75 oligodendrocytes (Ahlqvist *et al.*, 2005; Donati *et al.*, 2005; Lusso *et al.*, 1991; Lusso *et al.*,
76 1995).

77 HHV-6 encodes a specific chemokine, U83 which can mediate chemoattraction for
78 latent infection and dissemination in monocytes, but specificity and activity in subsets is not
79 defined. HHV-6B U83B, like the human chemokine CCL2 (formerly monocyte chemotactic
80 protein-1, MCP-1), is monospecific for CCR2, a chemokine receptor expressed on monocytes
81 (Luttichau *et al.*, 2003). Therefore U83B can both chemoattract cellular populations for
82 establishing latency yet also compete with CCL2 for chemokine receptor activation, thereby
83 diverting the host's cellular responses. This specificity is in contrast to properties of
84 homologous chemokine U83A, encoded by HHV-6A. The U83A chemokine has broad
85 chemokine receptor specificity: CCR1, CCR4, CCR5, CCR6 and CCR8, yet does not include
86 CCR2 (Catusse *et al.*, 2007; Dewin *et al.*, 2006). The properties of CCL2 have been shown to
87 be essential in a number of systems, including HIV, where it is critical for mediating
88 monocyte movement across the blood brain barrier and for subsequent correlates to
89 neuroinflammatory disease (Buckner *et al.*, 2011; Lentz *et al.*, 2011; Williams *et al.*, 2012).
90 During this infiltration, cells mature and become susceptible to HIV infection. HHV-6B is
91 linked with *status epilepticus* and subsequent temporal lobe epilepsy (Epstein *et al.*, 2012),
92 where there is evidence for a role for CCL2-CCR2 signaling (van Gassen *et al.*, 2008).
93 Therefore, we hypothesize that monocytes which express CCR2 together with CD14 and
94 possibly CD16 are targets for HHV-6B, with U83B a key candidate for this selectivity. In this
95 report the specificity of this chemokine is addressed and the effects on *ex vivo* human
96 leukocytes examined. In contrast, U83A is specific for chemokine receptors, CCR5 or CCR6,
97 which are present on dendritic cells and may affect antigen presentation by different
98 pathways. Experiments with a spliced version of U83A, which encodes a truncated version of
99 U83A, U83A-N, show the chemokine binding specificity is retained in the N-terminal half of

100 the molecule (Catusse *et al.*, 2007; Dewin *et al.*, 2006). Here this is further explored by
101 analysing strain variants in order to define U83B specificity for CCR2. Based on this
102 variation, synthesized peptides covering U83B-N are described to test specificity and
103 activities in mediating migration, using *ex vivo* human leukocytes to test possible effects on
104 inflammation. Specificity is defined as well as monocyte subset activation. Since
105 chemoattraction of cellular subsets can be a precursor to latent or lytic infection, this
106 specificity difference also defines a putative determinant of cellular tropism differences
107 between HHV-6A and B.

108

109 RESULTS AND DISCUSSION

110

111 U83 sequence variability and peptides

112 Prototype sequences for mature, spliced forms of U83A and U83B chemokines encoded by
113 laboratory reference strains of HHV-6A and HHV-6B were compared (termed U83A-N and
114 U83B-N) (Fig. 1). Additionally, comparisons of this region were made with sequences
115 derived from clinical samples. This included 38 sequences described here from clinical
116 strains in Zambia which were compared to 74 available on Genbank, from Japan, Germany,
117 USA, DR Congo and Uganda, total 112. This comparison showed U83 variation between
118 HHV-6A and B species, as previously identified (Bates *et al.*, 2009; Dewin *et al.*, 2006;
119 French *et al.*, 1999), typified by alignments from laboratory reference strains, HHV-6A strain
120 U1102 and HHV-6B strain Z29 (Fig. 1). This included 12 amino acid differences between
121 U83A and U83B in the mature full-length proteins. Seven differences were present in the
122 spliced N-terminal truncated form of the chemokine. Five of these were specific to either
123 HHV-6A or HHV-6B. However one HHV-6B clinical strain from those characterised here
124 from Zambia, N3, differed from the other 93 HHV-6B U83 sequences analysed. This
125 encoded a substitution of the U83A specific lysine (K) for the U83B specific asparagine (N)
126 at position 14 of the mature protein (Fig. 1). Such change in potential charge interactions
127 could alter ligand-receptor interactions affecting chemokine receptor specificity. Since the N-
128 terminal truncated spliced form still retained chemokine receptor binding specificity as
129 shown for U83A (Catusse *et al.*, 2007; Dewin *et al.*, 2006), peptides derived from this region
130 were examined in order to test specificity. To investigate this, peptides were synthesised
131 corresponding to the N termini of prototype U83A and U83B (laboratory strains U1102 and
132 Z29 respectively) and from clinical strain N3. These 17-mer peptides span 4 of the 6 differing

133 residues between spliced U83A and U83B and the corresponding peptides were termed NT
134 (N-terminal): U83A-NT, U83B-NT and U83BA-NT from the clinical strain N3 (Fig. 1).

135

136 **U83B-NT induces chemotaxis in *ex vivo* CCR2 positive human leukocytes**

137 U83B-NT, U83A-NT and U83BA-NT were measured for functional activity using a
138 chemotaxis assay in comparison to human chemokines, in physiological ranges between 0.1-
139 10nM. Chemotaxis was first tested in both a human monocyte cell line, THP-1, which
140 expresses CCR2 to establish the assay (Fig. 2A). Then chemotaxis assays were conducted on
141 *ex vivo* human leukocytes expressing CCR2, derived from multiple independent healthy
142 donors and the combined results analysed (Fig. 2B). CCR2 and CCR5 specific chemokines,
143 CCL2 and CCL4 respectively, were used as controls. Both CCL2, 10nM positive control, and
144 U83B-NT, 1-100nM, induced chemotaxis in the CCR2 expressing THP-1 cell line (Fig. 2A),
145 similar to that reported for a mature U83B-Fc fusion protein (Zou *et al.*, 1999). In the *ex vivo*
146 human PBMCs, maximal migration was similar for CCL2 and U83B-NT; significant
147 migration was induced by U83B-NT between 0.1-10nM, with a bell shaped response curve to
148 dilution gradients typical of chemokines. In contrast, stimulation with either U83A-NT or
149 U83BA-NT did not induce chemotaxis of *ex vivo* PBMC which had showed positive
150 migration to the CCL2 control (Fig. 2C). No chemotaxis was observed with U83A-NT,
151 U83BA-NT or U83B-NT when donor cells had levels of CCR2 or CCR5 surface expression
152 which were undetectable by flow cytometry and no response to positive control chemokines
153 CCL2 or CCL4, specific for CCR2 or CCR5, respectively (Fig. 2D). Efficient chemotaxis by
154 U83B-NT but not by U83A-NT or U83BA-NT, shows specificity for CCR2 by U83B resides
155 in this N-terminal region and defines Asn-14 as a key component. Full-length purified,
156 mature U83B effectively displaced radiolabeled CCL2 from binding to CCR2, with an EC50

157 of 79nM, compared to IC50 of 0.08nM for CCL2 (Luttichau *et al.*, 2003). In chemotaxis
158 assays, mature U83B, vCCL4, was similarly efficient as CCL2 in promoting migration of
159 CCR2 transfected murine L1.2 cell lines, albeit at higher concentrations of 100-1000nM,
160 compared to effective concentrations for CCL2 of 0.1-10nM (Luttichau *et al.*, 2003). U83B-
161 NT could not displace CCL2, up to 100nM (not shown) suggesting interactions at a distinct
162 site, while the chemotaxis mediated by U83B-NT was as potent as that induced by the human
163 chemokine CCL2, at 0.1nM-10nM. This suggests signalling is modulated by conformation of
164 the mature virus chemokine and exposure of the U83B N-terminal peptide is important for
165 potency. Human chemokines which bind CCR2 or CCR5 can induce rapid internalisation of
166 the receptor within minutes (Arai *et al.*, 1997; Catusse *et al.*, 2007; Signoret *et al.*, 2005). The
167 effects of CCL2 and U83B-NT were compared (Fig. 3). Although CCL2 induced some
168 reduction in surface CCR2 staining, indicative of internalisation of CCR2 as described (Arai
169 *et al.*, 1997) (Fig. 3A, B), there was no effect by U83B-NT by 10 minutes (Fig. 3C, D, in
170 duplicate at 50nM and in quadruplicate at 1, 5, 10 and 10nM). This resembles effects of
171 U83A on CCR5, which also induces efficient chemotaxis, but with a similar delayed receptor
172 internalisation different from the rapid internalisation/recycling induced by human
173 chemokines (Catusse *et al.*, 2009; Catusse *et al.*, 2007). Interestingly, CCL2 induced
174 chemotaxis has also been reported independent of CCR2 internalisation (Arai *et al.*, 1997).
175 The mature U83B, is monospecific for CCR2 and shows no interaction with CCR5 or other
176 human chemokine receptors including CX3CR1 (Luttichau *et al.*, 2003). This suggests
177 specificity for classical or intermediate monocytes.

178

179 **Migrated *ex vivo* PBMC induced by U83B peptide are enriched in CCR2**

180 In the chemotaxis assay donor PBMC bearing CCR2 could be specifically stimulated by
181 U83B-NT (and also CCL2) and positive chemotaxis could only be observed in donors where
182 there was a relatively high overall prevalence of CCR2 bearing cells in the PBMC
183 population. In order to further investigate this specificity, the phenotype of the actual
184 migrated population of *ex vivo* PBMC was characterised. Since this assay actually
185 phenotypes the migrated population, it could be performed on donor PBMC in which the
186 relative prevalence of CCR2+ leukocytes in the starting population from donor PBMC was
187 lower or even a minor group. The chemotaxed cells were collected and examined by flow
188 cytometry.

189 *Ex vivo* PBMC stimulated immediately after isolation with either U83B-NT or CCL2 in
190 transwell chemotaxis assays showed enrichment for CCR2+ cells post-migration relative to
191 the original PBMC population (stock cells) as well as the post-migration buffer control (Fig.
192 4, top panel). Similar CCR2 enrichment post-migration was shown in three further donors
193 tested after U83B-NT and CCL2 stimulation compared to buffer only treatment (Fig. 4A and
194 C, respectively). In these the starting population had low CCR2 expression as shown by
195 background migration to buffer only. Even with lower CCR2 expressing subsets, U83B-NT
196 could selectively enrich the CCR2 population. Full-length, mature U83A did not increase the
197 prevalence of CCR2 expressing cells (Fig. 4B), When cells were cultured to increase CCR5
198 expression, as shown previously (Catusse *et al.*, 2009; Catusse *et al.*, 2007), treatment with
199 U83B-NT did not increase migration of CCR5 bearing PBMCs. In contrast, there was
200 enrichment of CCR5, but not CCR2, bearing cells after migration towards full length mature
201 U83A (Fig. 4D), which has specificity for CCR5, but not CCR2 (Catusse *et al.*, 2007; Dewin
202 *et al.*, 2006) . Since the migration of CCR2 and not CCR5 bearing PBMCs was increased
203 with U83B-NT relative to the buffer-only treatment, this indicates CCR2 specificity rather
204 than general leukocyte activation.

205

206 **Shape change assay for cellular activation induced by U83B peptide**

207 Shape change is an indicator of cellular activation and can involve alterations in cell size,
208 granularity or overall morphology as shown for CCL4 and U83A treatment of CCR5
209 expressing human leukocytes by confocal microscopy and on eosinophils treated with CCL11
210 by flow cytometry (Catusse *et al.*, 2007; Sabroe *et al.*, 1999; Signoret *et al.*, 2005). CCR5
211 cells interacting with CCL4 show internalisation of CCR5 and a decreased cellular
212 morphology, whereas CCR3 expressing eosinophils treated with cognate ligand CCL11 react
213 with morphological changes leading to increased forward scatter in flow cytometry. A similar
214 flow cytometry based assay was established using *ex vivo* PBMCs to further investigate the
215 the CCR2 specificity of U83B-NT activation, and the affected monocyte subsets (Fig. 5).
216 Shape change in response to chemokine stimulation was first compared between CCR2 and
217 CCR5 expressing cells using gating strategy is shown in Fig. 5A-D. PBMCs were stimulated
218 with chemokine or buffer only treatment control then either CCR2 or CCR5 expressing cells
219 gated on (Fig. 5A, B) and CD3 expressing and/or dead cells gated out (lineage gating, Fig.
220 5C, D). Chemokine stimulated changes in forward scatter, either increased for CCR2 or
221 decreased for CCR5, were then analysed as compared to no treatment buffer only control
222 (Fig. 5E).

223 The effects of U83B-NT were compared with those induced by stimulation with human
224 chemokines specific for CCR2 or CCR5, CCL2 or CCL4 respectively, in relation to buffer
225 only treatment (Fig. 5E). Both CCL2 and U83B-NT showed similar shape change effects in
226 increasing forward scatter relative to the buffer control in the CCR2+/CD3- population (Fig.
227 5E). In contrast, CCL4 stimulation had little effect on these CCR2+/CD3- cells, while in the
228 CCR5+/CD3- cells, CCL4 stimulation resulted in a decrease in forward scatter, consistent

229 with alterations in cellular morphology previously observed. There was no effect on
230 CCR5+/CD3- PMBCs of either CCL2 or U83B-NT. This further shows the specificity of the
231 effect of U83B-NT in activating CCR2+ PBMCs. Monocytes are a major population
232 expressing CCR2. T-lymphocyte subsets may also express CCR2, but since CD3 expressing
233 cells were also gated out here, it is likely that monocytes are the main population responding.
234 Although NK or dendritic cells may also be present, these generally express CCR5 in
235 activated forms.

236

237 **U83B-NT stimulation of CCR2+CD14+CD16+ monocytic cells**

238 CCR2 expression has been identified in CD14+CD16- classical monocytes, and is decreased
239 or absent in non-classical CD14^{low}CD16+ monocytes. However, CCR2 is also expressed in
240 the recently defined intermediate monocyte subset which is CD14+CD16+. Therefore the
241 monocyte subsets activated by U83B-NT in the shape change assay were examined. In the
242 donor used for the experiment in Fig 5, there were sufficient CCR2+ cells to enable analysis
243 of the relative contribution of the different monocyte subsets to the response. Therefore, cells
244 activated after stimulation with chemokines as indicated by the shape change assay were
245 further analysed for monocytic markers, LPS receptor, CD14, and the FcγIII receptor, CD16.
246 CCR2+/CD3- cells and CCR5+/CD3- cells which changed shape (increase or decrease in
247 forward scatter profile, respectively) relative to the buffer median were assessed by flow
248 cytometry to enable the frequency of CD14+, CD16+ populations in the increased
249 (CCR2+CD3-) or decreased (CCR5+CD3-) forward scatter gates to be compared between
250 chemokine and buffer only treatments (Fig. 5E). Both the U83A-NT and negative control
251 human chemokine CCL4 (CCR5 specific) stimulation of CCR2+CD3- cells, showed no
252 difference to the buffer-only treatment (Fig. 5E and Table 1), while both U83B-NT and the

253 positive control human chemokine CCL2 (CCR2 specific) induced shape change (increased
254 forward scatter gate) and the responding population contained a higher frequency of both
255 CD14+ and CD16+ cells, as well as dually expressing cells showing the activated cells were
256 predominantly (>83%) CD14+ or CD16+ monocytic cells, with >77% of this population
257 showing dual staining. This was not seen in the CCR5+CD3- CCL4 activated population,
258 with only 0.14% CD14+CD16+ cells being present in the shape changed population (reduced
259 forward scatter gate) (Table 1). This indicates U83B-NT can activate specifically
260 CCR2+CD14+CD16+, intermediate monocytes.

261 Intermediate monocytes and non-classical monocytes, which express both CD14 and CD16,
262 have been increasingly defined as an intrinsic subset for virus interactions and some other
263 intracellular pathogens (Balboa *et al.*, 2011; Buckner *et al.*, 2011; Chikka *et al.*, 2009; Lentz
264 *et al.*, 2011; Williams *et al.*, 2012). With lower expression of CD14, human CD14dim
265 monocytes have roles in local tissue surveillance to detect nucleic acids and viruses via innate
266 TLR7 and TLR8 pathways and appear to correlate with motile monocytes which patrol the
267 vasculature (Cros *et al.*, 2010). In intermediate monocytes, CCR2 expression is also
268 intermediate, yet U83B-NT can activate this subset, despite the increased CCR2 expression in
269 classical CD14+CD16- subset, possibly indicating different CCR2 conformation or signalling
270 in this subset. The transition to CD16 expression from classical monocyte does appear to
271 coincide with expression of genes giving increased motility, so this could also explain this
272 finding. In the donor used for the experiments in Fig. 5/Table 1, CCL2 also activated this
273 subset, so this could be a donor specific finding, but still demonstrates that U83B peptide can
274 activate the intermediate subset. Monocytes generally comprise 10% of *ex vivo* human
275 PBMCs, and of these 85% are classical monocytes with CD14+CD16-, while the
276 intermediate monocyte subset CD14+CD16+ is approximately 5% (Ziegler-Heitbrock *et al.*,
277 2010), so 0.5% of starting input PBMCs collected could have properties of this subset

278 susceptible to U83B activation. This limited the number of cells available for analyses of
279 activation, particularly as CCR2 is induced in proinflammatory conditions, therefore lower or
280 not detected in healthy donors. The conditions were only available for *ex vivo* analyses of the
281 shape change phenotype assay in the donor indicated. It would be of interest to extend these
282 observations to patient cohorts with inflammatory disease. The other flow cytometry and
283 chemotaxis assays were all replicated in multiple healthy donors. A major strength of these
284 analyses is the use of *ex vivo* cells which have not been influenced by cytokine-activated
285 culture, therefore are most likely to represent physiologically active circulating subsets and
286 native interactions with the virus chemokine.

287 The CCR2+CD14+CD16+ phenotype has also been characterised as increased in senescent
288 cells, and can explain the increase in chronic inflammatory conditions in ageing populations
289 including those with cardiovascular disease (Merino *et al.*, 2011; Rogacev *et al.*, 2011;
290 Shantsila *et al.*, 2011). HHV-6B is associated with inflammatory conditions, including
291 encephalitis and myocarditis, where it is the most frequent virus identified together with
292 parvovirus 19 (Kuhl *et al.*, 2005a). Therefore, the U83B specificity further defined here,
293 provide a mechanism for modulation of the inflammatory response.

294 Since both HHV-6A and HHV-6B have also recently been identified as integrated genomes
295 ranging between 0.1 – 1.0% of global populations, up to 70 million people are potentially
296 exposed to effects of these virus genes (Arbuckle *et al.*, 2010; Arbuckle & Medveczky, 2011;
297 Morissette & Flamand, 2010). Evidence suggests the integrated HHV-6 is primarily in a
298 latent state, but there are reports of reactivation giving placental infection (Hall *et al.*, 2010).
299 Moreover, in the absence of other virus gene expression both HHV-6A and HHV-6B U83-N,
300 can be expressed, encoding the spliced truncated version which includes the U83B peptide
301 (French *et al.*, 1999). This immediate early profile suggests U83 is competent to be expressed
302 from the genome, could be expressed from every cell, thereby enhancing chemokine

303 activities in addition to CCL2 in inflammatory disorders. Notably, CCL2 has been described
304 in both neuroinflammatory and cardiovascular pathologies and U83B with similar properties,
305 but potentially wider cellular distribution as an integrated gene could contribute to this.

306 Therefore, properties of U83B shown here are relevant both to the virus and as a virus gene
307 expressed independently as a 'human' gene. Furthermore, to our knowledge, U83B-NT, as
308 characterised here, is the smallest CCR2 specific peptide which can function potently in
309 chemotaxis. Given its small size and efficacy, it could be used as a selective agent to
310 stimulate intermediate monocytes, as a novel adjuvant for increasing vaccine efficacy due to
311 the antigen presenting features, including MHC class II expression, of this cellular subset. It
312 may also have particular applicability to recently defined prime- chemokine 'pull'
313 vaccination strategies (Shin & Iwasaki, 2012).

314 **MATERIALS AND METHODS**

315 **Chemokine and peptide reagents.** Chemokines CCL2 (MCP-1), CCL3 (MIP-1 α), CCL4
316 (MIP-1 β) and CCL5 (RANTES) were purchased in lyophilized form from Peprotech (Rocky
317 Hill, NJ, USA) and reconstituted according to the manufacturer's instructions. 10mM aliquots
318 of the chemokines were prepared, diluting the reconstituted peptide in Phosphate Buffered
319 Saline (PBS), pH8. These aliquots were stored at -80°C. Working stocks (10 μ M) were
320 prepared when required from these aliquots in HEPES buffered saline solution (HBSS,
321 Sigma) with 0.1% bovine serum albumin (BSA, Sigma) and were stored at -20°C. Working
322 stocks were discarded after two freeze-thaw cycles. Viral chemokine peptides were
323 synthesised by Sigma-Genosys and reconstituted using the manufacturer's instructions.
324 Briefly, chemokine was reconstituted with DMSO (Sigma) to make a 5mM stock
325 concentration and then aliquots of 100 μ M and 10 μ M prepared using PBS/0.1% BSA and
326 stored at -20°C. After two freeze-thaw cycles aliquots were discarded.

327

328 **Polymerase chain reaction (PCR) amplification and nucleotide sequencing.** HHV-6 DNA
329 was isolated from infant sera samples collected in Zambia as part of analyses of infection
330 effects in a nutrition intervention study as described (CIGNISstudyteam, 2010). HHV-6 U83
331 was PCR amplified using Gotaq green mastermix (Promega) or Pfu polymerase (New
332 England Biosciences), as described (Bates *et al.*, 2009; French *et al.*, 1999). Outer primers
333 U83OF/OR were used followed by a nested set, U83IF/IR: U83OF
334 5'AGTTAACACGACGGGAACAAC3', U83OR 5'TTGGGATGATTATGGCAAAC3',
335 U83IF 5'GTAGGGAAAAAGACTTGTCGAA3', U83IR
336 5'AACCAGTATTAATGTCTTCGA3'. Gel purified DNA PCR products were sequenced
337 using Big dye terminator 3.1 (Applied Biosystems) and run on an ABI3730 (Applied

338 Biosystems). Sequences were analysed using Chromas pro (Technelysium) and compared to
339 chemokine sequences on Genbank using NCBI BLAST. Alignments were prepared using
340 ClustalW (Chenna *et al.*, 2003) and Jalview v2.4 (Waterhouse *et al.*, 2009).

341

342 **Human *ex vivo* peripheral blood mononuclear cells (PBMC) purification and culture.**

343 Whole blood was collected with 5mM EDTA (final concentration) from anonymously coded
344 healthy adult human blood donors (LSTHM, UK), with written consent, following local
345 phlebotomy guidelines. PBMCs were separated from whole blood using Histopaque-1077
346 (Sigma). PBMCs for culture were resuspended in RPMI-1640 with 10% autologous human
347 serum, 2mM Glutamax (Fisher), 50 U/mL penicillin (Sigma), 50 µg/mL streptomycin
348 (Sigma), plated in an ultra-low adherence flask (Corning, Corning, NY) and incubated at
349 37°C with 5% CO₂ for 72h as described (Catusse *et al.*, 2007).

350

351 **Chemotaxis assay.** PBMCs at a density of 2×10^6 /ml were incubated in HBSS with 0.1%
352 BSA and 1.7µM Calcein-AM (Invitrogen) for 30 minutes at 37°C, 5% CO₂. Cells were
353 washed in chemotaxis buffer (HBSS/0.1% BSA), then 1.5×10^5 cells, at a density of 3×10^6 /ml,
354 were plated out on a Neuroprobe ChemoTX™ microchemotaxis chamber (Receptor
355 Technologies, UK) on the filter above the lower chambers containing chemokine, peptide or
356 buffer. The assay was then run for 90 minutes by incubation of the cell and
357 chemokine/peptide filled microchemotaxis chamber at 37°C, 5% CO₂. Excess cells were
358 removed from the filter and cells migrated into the lower chamber were assayed using calcein
359 fluorescence measured with a Wallac Victor2 spectrometer (Perkin Elmer) with excitation
360 485nm and emission 535nm as described (Catusse *et al.*, 2007).

361

362 **Flow cytometry.** PBMC or THP-1 cells were stained with combinations of anti-human
363 receptor antibodies directly conjugated to fluorescent labels. These included CCR2-
364 phycoerythrin, PE, (FAB151P, R&D systems), CCR5-fluorescein isothiocyanate, FITC,
365 (FAB182F, R&D systems) CD3-pacific blue, PB, (BD Pharmagen #558124), CD14-
366 allophycocyanin, APC (BD 555399) or CD16-PE-Cyanin7, PE-Cy7, (BD 557744)
367 antibodies, as well as 'Dead' stain-violet with same spectrum and detected in same channel as
368 PB (Invitrogen, L34955). PBMCs were incubated with labelled antibodies, isotype or buffer
369 only controls for 30 minutes at 4°C, then washed with FACS buffer (PBS/0.1%BSA)
370 followed by fixing with 2% PFA for 15 minutes prior to analysis on a FACS Calibur (Becton
371 Dickinson). Data were analysed and compensation applied, where multicolour staining was
372 used, with FlowJo software (Treestar).

373

374 **Shape change assay.** *Ex vivo* PBMCs were incubated with or without positive control human
375 chemokines (CCL2 or CCL4), virus chemokine peptide (U83B-NT, U83A-NT, or U83BA-
376 NT) or buffer for 90 minutes at 37°C, same as for the chemotaxis assay. This was followed
377 by centrifugation, buffer wash, then staining for multi-colour flow cytometry, using
378 incubation with dead stain-PB and the following conjugated antibodies: CD3-PB, CCR2-PE,
379 and CCR5-FITC, CD14-APC and CD16-PE.Cy7. Samples were run on a FACS CyAn flow
380 cytometer (Beckman Coulter). For analyses, events were gated on flow cytometry markers
381 (dead stain-/CD3-/CCR2+ or dead stain-/CD3-/CCR5+) then forward and side scatter profiles
382 of these cells examined. CCR2+ or CCR5+ cells with changed forward scatter, relative to the
383 buffer, were gated after chemokine or peptide stimulation and then further analysed for CD14
384 and CD16 composition by gating for CD14-APC or CD16-PE.Cy7 staining.

385

386 **ACKNOWLEDGMENTS**

387 We thankCarolynn Stanley, LSHTM, for phlebotomy and all blood donors at LSHTM. We
388 also thank University of London for a postgraduate research award, LSHTM for a Graduate
389 Teaching Assistant PhD scholarship (DC) and early support from the Biotechnology and
390 Biological Sciences Research Council.

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FIGURE LEGENDS

554 **Fig. 1. U83 peptide sequences based on HHV-6A and HHV-6B clinical strains.**

555 Alignment of the amino acid sequences of the mature, spliced truncated form of U83, U83-N,
556 shown for HHV-6A, laboratory strain U1102, HHV-6B, laboratory strain Z29, and 38 clinical
557 strains analysed here in comparison to 74 strains available on NCBI. Those in bold and
558 prefaced by z are from Zambia and compared to representative clinical strains from previous
559 analyses (French *et al.*, 1999; Sjahril *et al.*, 2009). CD is a reference UK strain. The *
560 indicates amino acid differences. HHV-6A and HHV-6B strains are shown together, with
561 total numbers analysed indicated. The HHV-6B clinical strain N3 is shown separately as it
562 had a single substitution Asn-Lys14 only found in the HHV-6A strains. This substitution is
563 marked with an additional *. 17-mer peptides were derived from U83A and U83B N-terminal
564 regions and labelled U83A-NT and U83B-NT. To compare the effect of the Asn-Ly14
565 change between HHV-6A and HHV-6B U83, U83B-NT had a Gly-Cys3 substitution. The
566 HHV-6B clinical strain N3 peptide was labelled U83BA-NT.

567

568 **Fig. 2. Chemotaxis induced by U83B-NT.** Chemotactic index is calculated relative to buffer

569 only treatment which shows the background migration. This is given a value of 1, which
570 allows comparison between multiple assays; this cut-off is shown by the dotted line. (A.)

571 THP-1 human monocyte cell line expressing CCR2 (by flow cytometry), showing chemotaxis

572 to CCL2 and U83 peptide by flow cytometry assay using pooled cells (from 3 wells per

573 column), representative of two independent cultures. (B-D) Migration of *ex vivo* human

574 PBMC to stimulus in microchemotaxis chambers: (B.) CCR2 expressing *ex vivo* human

575 PBMC, show chemotaxis to CCL2 and U83B peptide; results for chemokine treatment were

576 combined from 4 donors using one way ANOVA with Dunnett's multiple comparison test for
577 statistical significance versus control buffer-only treatment, * $p < 0.05$, ** $p < 0.01$; (C.) U83A-
578 NT or U83BA-NT do not induce chemotaxis in *ex vivo* human PBMCs expressing CCR2
579 which respond to CCL2; results were combined from 3 donors as above. (D.) No chemotaxis
580 relative to buffer only treatment in *ex vivo* PBMC which had no response to CCL2 or CCL4
581 and no expression of CCR2 or CCR5 by flow cytometry, five donors.

582

583 **Fig. 3. CCL2 but not U83B-NT induces reduction in surface CCR2.** Internalisation assay
584 was conducted by measurement of any reductions in CCR2 surface staining after chemokine
585 stimulation. THP-1 cells were treated with chemokine or buffer only control for 10 min then
586 surface expression of CCR2 was assayed by flow cytometry. (A.) Control experiment without
587 any incubation. The dotted line shows the no staining control. The grey shading indicates
588 staining with the isotype control for the CCR2-PE antibody. The solid black line shows
589 staining with CCR2-PE antibody indicating CCR2 expression on almost all cells. (B.) Cells
590 were treated with CCL2, thick black solid and thick dotted lines, or buffer only control, thin
591 grey and thin dotted lines, for 10 min. CCL2 treatment resulted in lower levels of CCR2
592 staining. Results shown in duplicate of two independent experiments. (C.) Cells were treated
593 with buffer only control, thin grey line, or with U83B-NT 50nM, thick black line, which had
594 no effect on CCR2 surface staining. (D.) In a duplicate assay, cells were treated with different
595 dilutions of U83B-NT, 1, 5, 10, 20 and 50nM, and compared to buffer only control. There is
596 no evidence of a reduction in surface CCR2 at any concentration of U83B-NT.

597

598 **Fig. 4. Cells chemotaxed by U83B-NT are enriched for CCR2 bearing cells.** Human *ex*
599 *vivo* PBMC, stock cells expressing CCR2, which migrated through a 5 μ m filter during a 90

600 minute incubation with chemokine or buffer only treatment were collected post migration and
601 stained separately for CCR2 or CCR5 using antibodies conjugated with fluorescent tags (PE
602 and FITC respectively). The top panel demonstrates the assay set up with ex vivo PBMC
603 from a representative donor. The four graphs show the prevalence of the CCR2 staining of
604 the starting PBMC population compared to the cells that had undergone migration after
605 treatment with chemokines U83B-NT, CCL2 or buffer only. The light grey histograms show
606 the staining with an isotype control antibody and the solid line, clear histogram shows
607 staining with an antibody to CCR2. Y-axes show cell counts (expressed as % Max, the
608 percentage of the maximum number of cells) (A-D) Ex vivo PBMC from three further donors
609 were then tested and the prevalence of CCR2 or CCR5 bearing cells which have migrated
610 after chemokine stimulation (solid line, clear histogram) was compared to that of background
611 migration after buffer only treatment (dark grey histogram). (A.) U83B-NT 1nM, (B.) U83A
612 1nM, and (C.) CCL2 10nM incubation. In (D) PBMC were cultured 3 days in non-adherent
613 flasks to induce CCR5 expression, then treated with U83A as above.

614

615 **Fig. 5. Shape change assay further defines U83B-NT CCR2 specificity.**

616 Cells were stimulated with human chemokines, CCR2 specific CCL2 or CCR5 specific
617 CCL4, virus chemokine U83B-NT or buffer for 90 minutes and then stained for cell markers.
618 In this assay cells were stained for: CCR2 (PE, for gating CCR2 expressing cells), CCR5
619 (FITC, for gating in CCR5 expressing cells), CD3 (pacific blue, PB, for gating out T cells),
620 Dead cell stain (PB analogue, for gating out dead cells), CD14 (APC, For gating monocytic
621 cells), CD16 (PE-Cy7, for gating cells bearing this Fc receptor). In order to define
622 chemokine specific responses to chemokine receptors on the cell surface, cell populations
623 were first defined by chemokine receptor expression, either CCR2 or CCR5, which were

624 gated in separately to be able to compare their responses. Next the CD3+/dead cells were
625 gated out. Finally, the shape change effect on chemokine stimulated cells was evaluated by
626 changes in forward scatter relative to the no treatment buffer only control.

627 The flow cytometry gating strategy is summarised in (A-D). The grey histogram shows the
628 fluorescence of unstained cells, and the black line cells stained cells. (A.) Identification of
629 chemokine receptor positive cells, CCR2 or CCR5, within the total PBMC population (shown
630 here for CCR2-PE). The gate used to delineate CCR2+ cells is indicated. CCR5+ cells were
631 separately gated for comparison (CCR5-FITC). (B.) Dot plot showing the CCR2+ gated
632 population. (C.) Next, within either the CCR2 or CCR5 gated populations, cells were gated
633 out which stained for CD3 or were dead cells (CD3- PB). Similar gating was also performed
634 with the CCR5+ population. (D.) Dot plot showing the live CCR2+CD3- gated population.
635 (E.) Effects on forward scatter of chemokine treatment (black line) compared here to no treatment
636 buffer only control (grey tint) on cells expressing CCR2+CD3- or CCR5CD3- cells identified as
637 described above. Cells were stimulated with chemokines as indicated U83B-NT 1nM, CCL2
638 10nM, CCL4 10nM, or buffer only. In the left panels cells expressing CCR2 respond to specific
639 chemokine stimulation by increases in forward scatter, a larger, more granular morphology, as shown
640 for both CCL2 and U83B-NT stimulation relative to the buffer. In the right panels cells expressing
641 CCR5 respond only to specific chemokine CCL4 stimulation showed by decreases in forward scatter,
642 acquisition of a smaller, less granular morphology, relative to buffer. Cells were then gated (black
643 arrows) on the increased or decreased forward scatter profiles relative to the buffer control histogram
644 median and the relative prevalence of CD14 or CD16 cell surface marker in the shape-changed
645 population was further determined (Table 1).

Table 1.

Frequency of CD14⁺ CD16⁺ monocytes in chemokine stimulated CCR2⁺ CD3⁻ or CCR5⁺ CD3⁻ *ex vivo* human PBMCs relative to buffer in shape change assay

Stimulation	CCR2+CD3-			CCR5+CD3-		
	CD14 %	CD16 ⁺ %	CD14 ⁺ CD16 ⁺ %	CD14 ⁺ %	CD16 ⁺ %	CD14 ⁺ CD16 ⁺ %
Buffer	60.9	61.9	53.4	3.6	0.6	0.32
CCL4	65.3	65.8	57.1	1.4	0.4	0.14
CCL2	85.1	88.3	82.2	3.0	0.7	0.29
U83A-NT	67.5	68.4	62.9	4.1	0.7	0.34
U83B-NT	83.3	84.0	77.3	6.5	1.1	0.58

Fig. 1.

HHV-6A U83A-N (19)

U1102¹ FICSSPDAELFSEKSRMSSSVLLGCLLCMDWSAAVPV
AJ² FICSSPDAELFSEKSRISSSVLLGCLLCMDWSAAVPV

HHV-6B U83B-N (93)

Z29³ FIGSSPDAELSSSENSRISSSVLLGCLLCCTDWSAVVPV
BOU⁴ FIGSSPDAELSSSENSRISSSVLSGCLLCCTDWSAVVPV

N3 FIGSSPDAELSSEKSRISSSVLLGCLLCCTDWSAVVPV
* * * * *
*
*

U83A-NT FICSSPDAELFSEKSRM
U83B-NT FICSSPDAELSSENSRI
U83BA-NT FIGSSPDAELSSEKSRI

¹U1102 is same as Z27, Z5, GS, DA, CO7, **621, N25, C2, 35, 184, 538**

²AJ is the same as Z23, Z59, 5628-5-15

³z29 is same as HST, MBE, HT, ES16, BT499, 16-2-41, HST, ES17, BT552, **37, 39, N2, N3, N5, N8, N9, N11, N12, N13, N15, N17, N18, N19, N22, N23, N25B, N26, N28, N33**

⁴BOU is same as z3, z12, z13, z14, z25, Z317, AB69, St.W, BT348, BT344, ES11, **N35, CD**

Fig. 2.

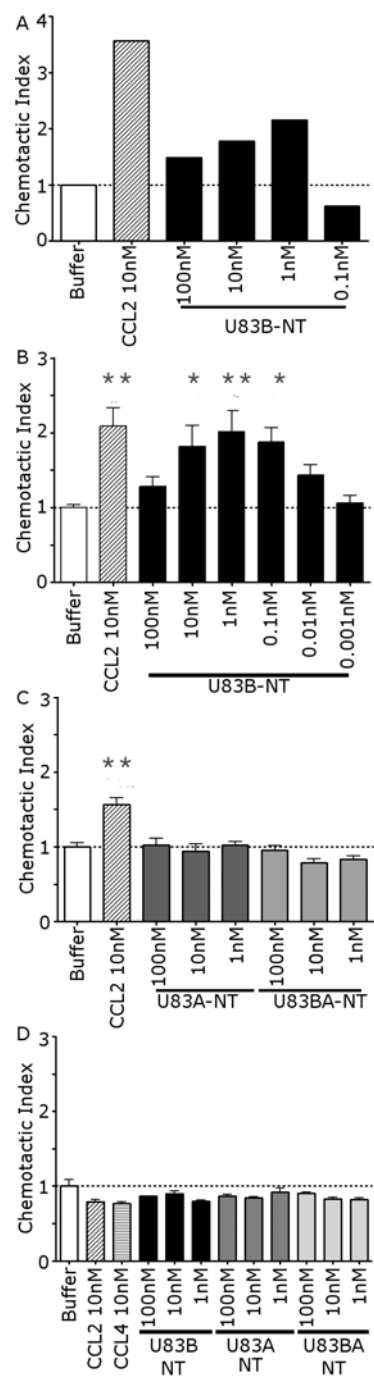


Fig. 3

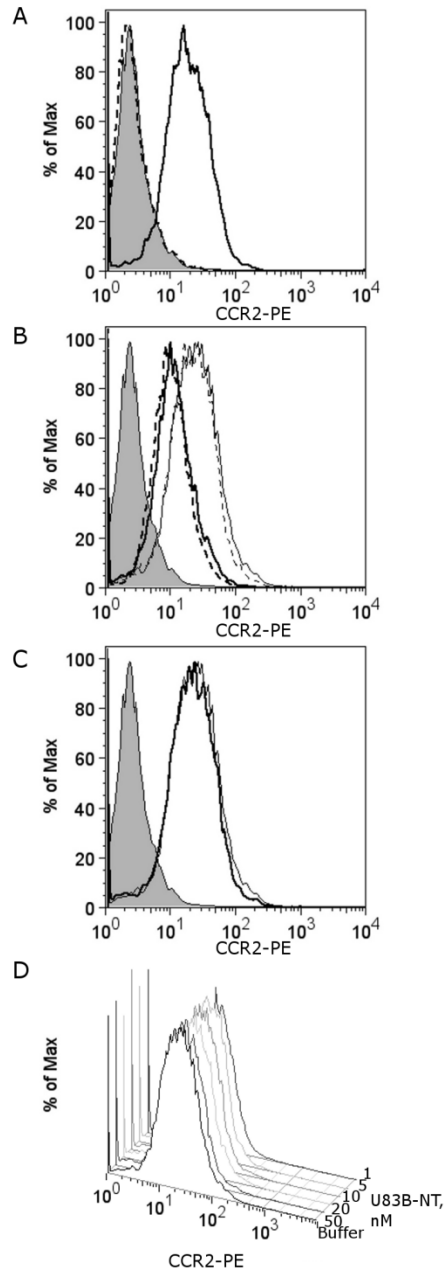


Fig. 4

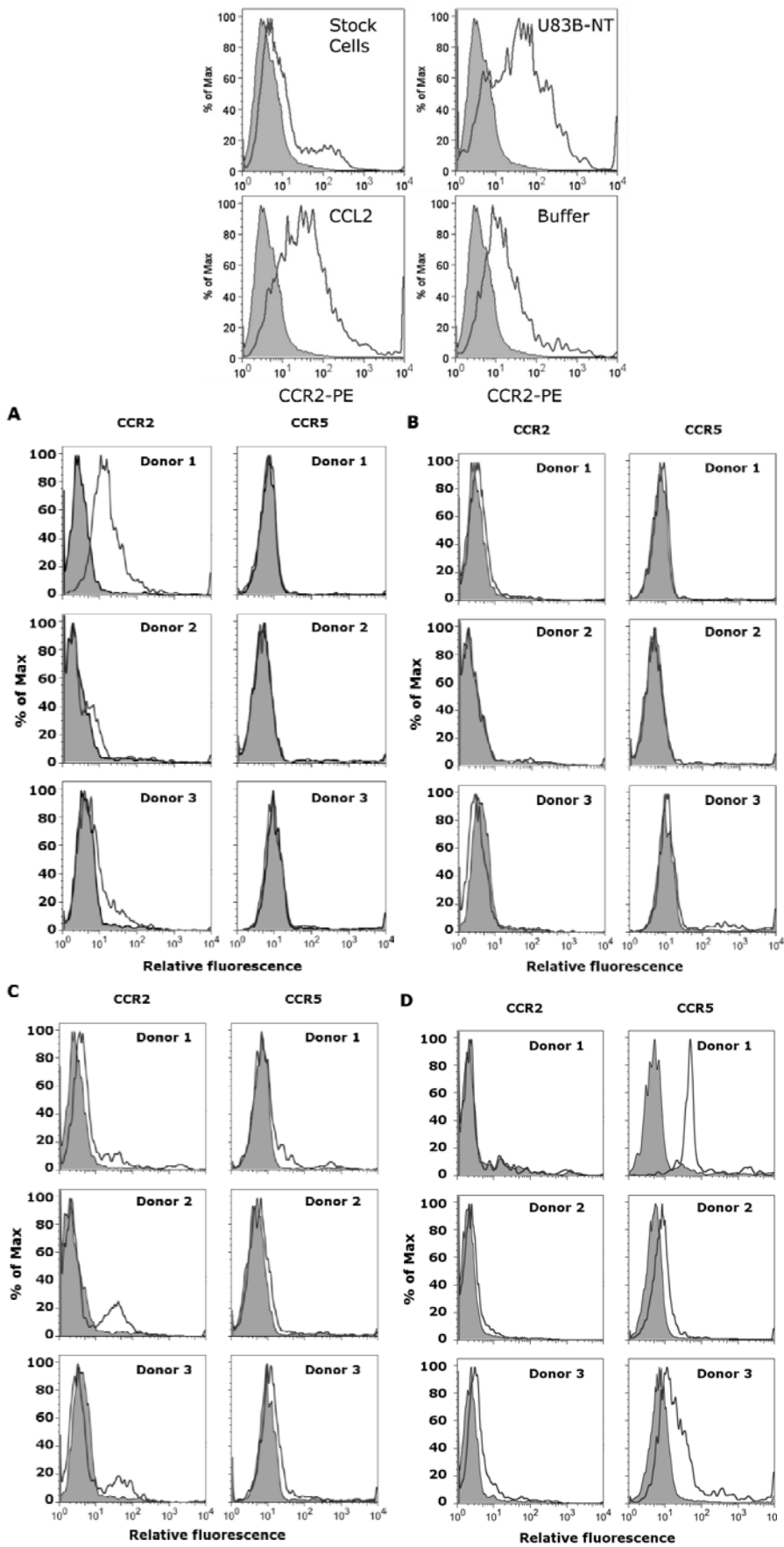
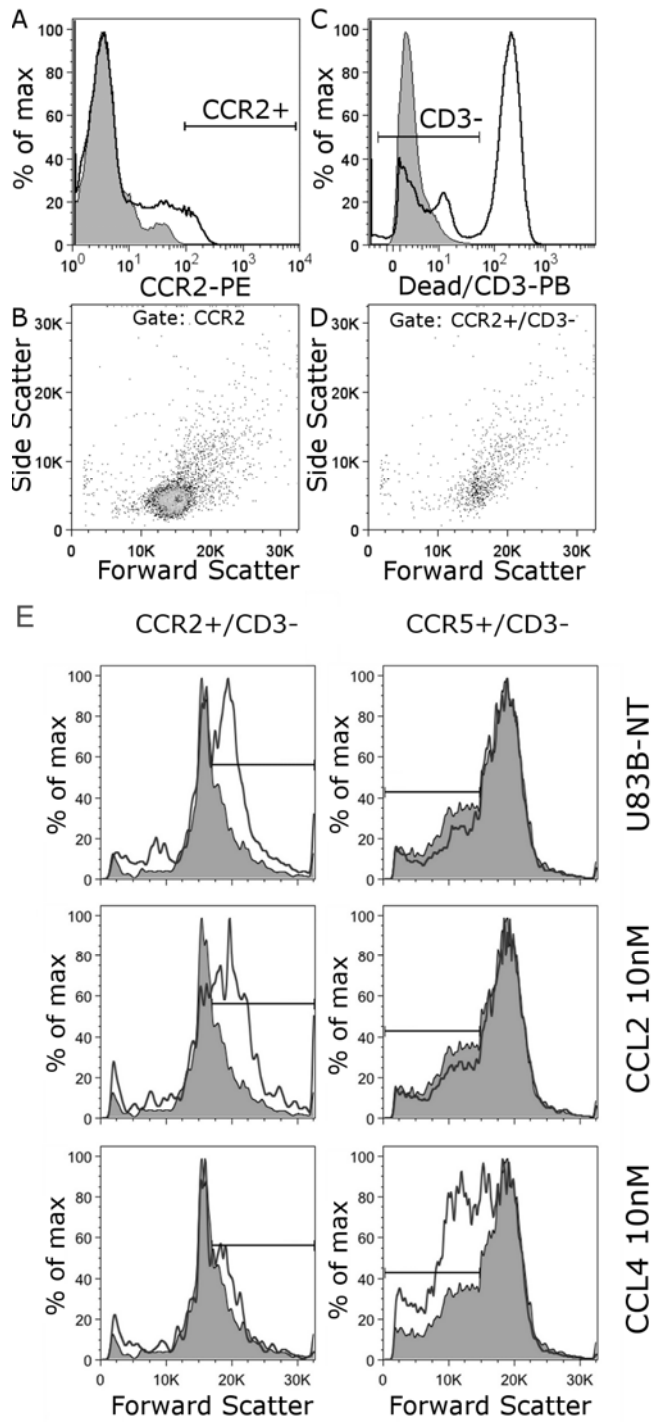


Fig. 5



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