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1 # Chemokine receptors ACKR2 and CCR1 coordinate macrophage dynamics and
2 mammary gland development

3

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13

14 ## Summary

15 In the mammary gland during puberty, availability of the chemokine CCL7 is controlled by a
16 scavenging receptor ACKR2 and provides a key signal to macrophages which have the
17 receptor CCR1. Together, this controls the timing of development.

18

19 ## Abstract

20 Macrophages are key regulators of developmental processes, including those involved in
21 mammary gland development. We previously demonstrated that the atypical chemokine
22 receptor, ACKR2, contributes to control of ductal epithelial branching in the developing
23 mammary gland by regulating macrophage dynamics. ACKR2 is a chemokine-scavenging
24 receptor, which mediates its effects through collaboration with inflammatory chemokine
25 receptors (iCCRs). Here we reveal that ACKR2, and the iCCR CCR1, reciprocally regulate
26 branching morphogenesis in the mammary gland, whereby stromal ACKR2 modulates levels

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27 of the shared ligand CCL7 to control the movement of a key population of CCR1-expressing
28 macrophages to the ductal epithelium. In addition estrogen, which is essential for ductal
29 elongation during puberty, upregulates CCR1 expression on macrophages. The age at
30 which girls develop breasts is decreasing, which raises the risk of diseases including breast
31 cancer. This study presents a previously unknown mechanism controlling the rate of
32 mammary gland development during puberty and highlights potential therapeutic targets.

33

34 **Keywords**

35 Chemokine, macrophage, mammary, branching, puberty, ACKR2, CCR1

36

37 **Running title**

38 Chemokine receptors regulate mammary gland development

39

40 **Introduction**

41 Breast development (thelarche) is the first visible sign of puberty in females, and typically
42 occurs between the ages of 8 and 13 (Merke and Cutler Jr, 1996). Globally, the age at
43 pubertal onset is falling (de Muinck Keizer-Schrama and Mul, 2001). Early puberty is
44 associated with an increased risk of disease in later life, including type II diabetes heart
45 disease, and cancer (Day *et al.*, 2015). Importantly, girls who develop breasts before the age
46 of 10 are 20% more likely to develop breast cancer (Bodicoat *et al.*, 2014). Therefore,
47 understanding the molecular and cellular mechanisms underlying breast development is of
48 key importance.

49 The mammary gland develops through branching morphogenesis, giving rise to ductal
50 epithelial networks. In the mouse this process begins at around 3 weeks (MM Richert, KL
51 Schwertfeger, JW Ryder, 2000), when highly proliferative structures known as terminal end
52 buds (TEBs) form at the end of epithelial ducts and drive network formation. Supporting this
53 process is a stromal population containing fibroblasts, extracellular matrix (ECM), adipocytes

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54 and immune cells (Wiseman and Werb, 2002). Prominent amongst the stromal immune cells
55 are macrophages which are found throughout the gland and surrounding TEBs.
56 Macrophages have been implicated in numerous developmental processes (Wynn, Chawla
57 and Pollard, 2013), and mammary gland development is severely impaired in macrophage-
58 deficient mice with altered TEB formation, ductal elongation during puberty and lobuloalveoli
59 development in pregnancy (Pollard and Hennighausen, 1994; Gouon-Evans, Rothenberg
60 and Pollard, 2000). Overall these studies indicate a key role for macrophages in the
61 regulation of ductal branching in the developing mammary gland.
62
63 Macrophages are recruited in a dynamic manner into the mammary gland throughout
64 development (Coussens and Pollard, 2011). The molecular mechanisms regulating the intra-
65 gland movement of macrophages as they migrate to terminal end buds to mediate their
66 developmental effects, are not currently understood and insights into these mechanisms will
67 enhance our overall understanding of how macrophages control mammary gland
68 development. Chemokines which comprise a family of proteins characterised by a conserved
69 cysteine motif, are important in vivo regulators of macrophage intra-tissue dynamics. The
70 chemokine family is subdivided into CC, CXC, XC and CX3C sub-families according to the
71 cysteine distribution, and chemokines act through G-protein coupled receptors to mediate
72 leukocyte migration (Nibbs and Graham, 2013). Within tissues chemokine distribution, and
73 gradients, can be regulated by members of the atypical chemokine receptor (ACKR) family,
74 which are 7-transmembrane spanning receptors that lack classical signalling responses to
75 ligands and which are typically stromally-expressed (Nibbs and Graham, 2013). Therefore,
76 together, signalling chemokine receptors and ACKRs regulate intra-tissue chemokine
77 function and coordinate leukocyte migration.
78
79 We have a long-standing interest in one of the atypical chemokine receptors, ACKR2.
80 ACKR2 scavenges and degrades inflammatory CC-chemokines thereby regulating their
81 intra-tissue concentration and spatial distribution (Nibbs and Graham, 2013). Accordingly it is

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82 a key player in the resolution of the inflammatory response with implications for
83 autoimmunity and cancer (Nibbs *et al.*, 2007; Di Liberto *et al.*, 2008; Shams *et al.*, 2017). We
84 previously demonstrated a role for ACKR2 in regulating branching morphogenesis in the
85 developing lymphatic system via control of macrophage dynamics around developing
86 vessels. More recently we have shown that ACKR2 also regulates branching morphogenesis
87 in the mammary gland and ACKR2-/- mice display precocious mammary gland development.
88 In essence, ACKR2 deficiency results in increased levels of monocyte and macrophage
89 attracting chemokines in the developing mammary gland and this is associated with
90 dysregulation of macrophage numbers and accelerated branching morphogenesis. The
91 chemokines scavenged by ACKR2 are ligands for the signalling chemokine receptors CCR1,
92 CCR2, CCR3, CCR4 and CCR5 (Fig. 1) (Nibbs and Graham, 2013; Bachelerie *et al.*, 2014).
93 It is likely therefore that the effects of ACKR2 on mammary gland development are indirect,
94 and a consequence of the regulation of levels of chemokines capable of modulating
95 macrophage function via one of these 5 receptors. Curiously, the dominant monocyte
96 recruitment receptor, CCR2, does not control the rate of branching morphogenesis in the
97 mammary gland (Wilson *et al.*, 2017; Jäppinen *et al.*, 2019), and mammary gland
98 macrophages do not express CCR4 (Wilson *et al.*, 2017). Together, this suggests that the
99 phenotype seen in ACKR2-/- mammary glands is a consequence of altered responses
100 through CCR1, CCR3 or CCR5. The purpose of this study was to determine which of these 3
101 receptors is the reciprocal partner of ACKR2, in the regulation of branching morphogenesis
102 in the developing mammary gland.
103
104 Here we identify CCR1, and its ligand CCL7, as key regulators working with ACKR2 in a
105 reciprocal manner to regulate macrophage numbers, and branching morphogenesis, in the
106 developing mammary gland. Collectively, this study sheds important light on the regulation of
107 macrophage dynamics during virgin mammary gland development.
108

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109 **Results**

110 **Ductal branching in the pubertal mammary gland is regulated by CCR1.**

111 To determine involvement of CCR1, CCR3 and CCR5 in the regulation of ductal branching
112 morphogenesis in the mammary gland we analysed carmine alum stained whole-mounts of
113 mammary glands from 7 week old WT and CCR1-/-, CCR3-/- and CCR5-/- mice (Fig. 2ai-iii).
114 The individual receptor deficient mice have different genetic backgrounds, therefore mice
115 from each strain were compared to their specific WT (Douglas P Dyer *et al.*, 2019).
116 Quantitative analysis of the whole-mounts indicated that branched area, ductal elongation,
117 TEB number and width were unaffected in CCR3-/- and CCR5-/- mice (Fig. 2aii-iii,
118 Supplementary Fig. 1). In contrast, CCR1-/- mice exhibited delayed mammary gland
119 development with decreased branched area at 7 and 8 weeks, reduced ductal elongation
120 and decreased number and width of TEBs at 7 weeks (Fig. 2ai and bi-iv). In addition, in
121 comparison to WT mice, CCR1-/- mice had thinner branches at 8 weeks (Fig. 2bv). This was
122 not seen for CCR3-/- or CCR5-/- mice (Supplementary Fig. 1e). As observed for ACKR2-/-
123 mice, by 12 weeks, when TEBs have regressed and ductal outgrowth is completed,
124 branched area and ductal elongation are equivalent between WT and CCR1-/- mice (Fig.
125 2bi-ii). Together these data show that CCR1 regulates mammary gland development at a
126 time point coincident with ACKR2 function in the same context.

127 Of note, in contrast to ACKR2-/- mice, no difference was observed in the distance between,
128 or density of, branches in WT and CCR1-/- mammary glands at any of the time points
129 investigated (Supplementary Fig. 2). This suggests that CCR1 does not regulate the density,
130 but the spread of the ductal network.

131 Importantly, previous publications have suggested potential redundancy in roles for CCR1, 3
132 and 5 in vivo (Mantovani, 1999; Schall and Proudfoot, 2011). Whilst we have shown this not
133 to be the case in acute inflammation (Douglas P. Dyer *et al.*, 2019), we have not examined
134 potential receptor redundancy in the context of mammary gland development. Therefore, to

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135 test for any potential redundancy between the CCRs, mammary gland whole-mounts were
136 obtained from iCCR-/- mice which have a compound deletion of CCR1, CCR2, CCR3 and
137 CCR5 (Douglas P Dyer *et al.*, 2019). As observed in the absence of CCR1, iCCR-/- mice
138 display similar delayed development at 7 weeks as demonstrated by reduced TEB number
139 (Supplementary Fig. 1c). No additional combinatorial effects of the receptors were observed
140 indicating that CCR1 is a non-redundant regulator of mammary gland development.

141

142 **CCR1 and ACKR2 are expressed surrounding epithelium in the mammary gland.**

143 We next examined the expression patterns of CCR1 and ACKR2 within the developing
144 mammary gland during late puberty. We used flow cytometry to identify the cell type(s)
145 expressing CCR1 within the mammary gland. As currently available antibodies to murine
146 CCR1 are of limited quality we included cells from CCR1-/- mice as a control. This analysis
147 demonstrated that CCR1 is only detectable on macrophages (CD45+SiglecF-
148 CD11b+F4/80+) within the mammary gland (Fig. 3a) and further in situ hybridisation showed
149 the CCR1+ cells to be intimately associated with the ductal epithelium (Fig. 3b). In contrast
150 to macrophages, eosinophils (CD45+ SiglecF+) and stromal and epithelial (CD45-) cells did
151 not express CCR1 (Fig. 3a). We next examined ACKR2 expression in the mammary gland.
152 Previously, we showed that ACKR2 is expressed by stromal fibroblasts in the developing
153 virgin mammary gland (Wilson *et al.*, 2017). Here we have used in situ hybridisation to locate
154 expression of ACKR2 to stromal cells in the vicinity of the ductal epithelium. Importantly no in
155 situ hybridisation signals were seen in the stroma of CCR1-/- or ACKR2-/- mammary glands
156 (Fig. 3b).

157 These data therefore demonstrate that CCR1 and ACKR2 are expressed in distinct cell
158 types surrounding TEBs in the developing mammary gland.

159

160 **Estrogen induces CCR1 expression on macrophages.**

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161 We next examined regulation of CCR1 expression on mammary gland macrophages.
162 Estrogen is essential for mammary gland development and ductal epithelial growth and
163 proliferation (Russell C. Hovey, Josephine F. Trott, 2002). ELISA-based analysis of estradiol
164 levels in the plasma of the developing mouse indicated that its production rises over the
165 same time-frame in which we observe altered ductal development in CCR1-/- mammary
166 glands (Fig. 4a). Notably, there was no difference in the levels of estradiol in WT and
167 ACKR2-/- mice, suggesting that the accelerated branching in ACKR2-/- mice is not caused
168 by increased levels of estrogen. To determine whether estrogen regulates CCR1 expression
169 on mammary gland macrophages we enzymatically digested mammary glands and exposed
170 the cells to DMSO (vehicle control) or 17β-estradiol for 1h at 37°C. CCR1 expression was
171 analysed by flow cytometry and shown to increase on CD45+ CD11b+F4/80+ macrophages
172 in response to 17β-estradiol (Fig. 4b). There was no significant difference between the level
173 of CCR1 expression on WT and ACKR2-/- macrophages after exposure, indicating that
174 ACKR2 does not regulate this process.

175 To determine whether this was a direct effect of estradiol on mammary gland macrophages,
176 CD11b+F4/80+ cells were isolated by FACS. In the absence of other cell types, CCR1
177 expression was increased following exposure to 17β-estradiol indicating that estrogen
178 induction of CCR1 results from a direct effect on mammary gland macrophages (Fig. 4c).

179 Notably, upregulation of CCR1 on macrophages in response to estradiol is age dependent,
180 as there is no difference in CCR1 expression in mice older than 8 weeks (Fig. 4d). In
181 addition, 17β-estradiol has no effect on macrophages isolated from the male fat pad or the
182 peritoneum of pubertal female mice (Fig. 4d-e). Taken together, this suggests that the effect
183 of estrogen on CCR1 expression is restricted to pubertal mammary gland macrophages and
184 limited to the key developmental time-frame we have identified.

185

186 **Chemokine levels are altered in the absence of CCR1 and ACKR2.**

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187 To identify the specific chemokines involved in regulating mammary gland development
188 through CCR1 and ACKR2, multiplex protein analysis of mammary gland lysates was carried
189 out. In keeping with our previous data we showed that, in the absence of scavenging by
190 ACKR2, the chemokines CCL7, CCL11 and CCL12 accumulate in the mammary gland at 6-
191 7 weeks (Fig. 5a) (Wilson *et al.*, 2017). The current analysis further revealed elevated CCL3,
192 CCL19, CCL22 and CXCL10 in the ACKR2 -/- mammary gland over this time-frame (Fig.
193 5a). Notably, other key chemokines associated with monocyte and macrophage migration
194 i.e. CCL2 and CCL5 are unchanged in the ACKR2-/- mammary gland (Fig. 5a). Importantly,
195 there were no significant differences in the levels of these chemokines in lysates obtained
196 from male WT and ACKR2-/- inguinal fat pads, indicating that the changes observed in
197 female lysates are specifically associated with the mammary gland (Supplementary Fig. 3).
198 In CCR1-/- mice, the levels of CCL7, CCL11 and CCL12 were unchanged, indicating that
199 ACKR2 is functional in these mice and able to scavenge chemokines normally. CCL3,
200 CCL19, CCL22, CXCL1 and CXCL12 are increased in ACKR2-/- mice and decreased in
201 CCR1-/- mice (Fig. 5). Given that CCL19, CXCL1 and CXCL12 are not ligands for either
202 ACKR2 or CCR1, it is likely that, along with CCL3 and CCL22, their altered levels reflect
203 variation in the numbers of chemokine-expressing immune or epithelial cells within the
204 mammary gland.

205

206 **CCR1 and ACKR2 reciprocally regulate CD206+ macrophages within the mammary**
207 **gland.**

208 Reciprocal regulation of leukocyte dynamics by CCR1 and ACKR2 in the developing
209 mammary gland should be reflected in complimentary changes in levels of key cellular
210 populations in CCR1-/- and ACKR2-/- mice. We detected no significant differences in the
211 lymphocyte populations or in non-macrophage myeloid cell populations investigated.
212 However, differences in a key macrophage population were identified. To investigate the
213 effects of CCR1 deficiency on macrophage levels in the mammary gland, flow cytometry of

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214 enzymatically digested 6.5 week old WT and CCR1-/- glands was carried out. The gating
215 strategy employed is described in Supplementary Fig. 4. CCR1-/- mice displayed no
216 significant differences in the bulk macrophage population (CD45+CD11b+F4/80+) (Fig. 6ai
217 and bi). However, we detected a significant decrease in the percentage of a small population
218 of macrophages expressing CD206 (mannose receptor) (CD45+SiglecF-F4/80+CD206+) in
219 CCR1-/- mice (Fig. 6aii, bii). Analysis of ACKR2-/- mice revealed a complimentary
220 phenotype to CCR1-/- mice in that they displayed an increase in the percentage of
221 macrophages in the mammary gland population and specifically of the CD206+ macrophage
222 subset (Fig. 6cii).

223 Finally, we examined the effects of estrogen on the CD206+ macrophage population. Our
224 data show that CCR1 expression was also increased on the surface of CD206+
225 macrophages in response to both 17β-estradiol and the estrogen mimic Bisphenol A (BPA)
226 (Fig. 6d). No effect of estrogen on CCR1 expression was observed in male macrophages
227 (Fig. 6d).

228 Thus, a key population of CD206+ macrophages are reciprocally regulated by ACKR2 and
229 CCR1. Importantly, CD206+ mammary gland macrophages have previously been implicated
230 in branching morphogenesis (Jäppinen *et al.*, 2019) and we propose that ACKR2 and CCR1
231 reciprocally control this population to coordinate branching morphogenesis in the pubertal
232 mammary gland.

233

234 **CCL7 regulates CD206+ macrophages and branching morphogenesis.**

235 Of the chemokines detected within the mammary gland, CCL7 is of particular interest as it is
236 shared between CCR1 and ACKR2 (Fig. 1), and is elevated in the pubertal mammary glands
237 of ACKR2-/- mice (Fig. 5aiv)(Wilson *et al.*, 2017). In addition, qRT-PCR analysis also
238 revealed that CCL7 is transcribed, by purified F4/80+ cells, at higher levels than other
239 ACKR2 ligands (Fig. 7ai). We therefore investigated its expression and function in the

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240 mammary gland. Using flow cytometry, intracellular staining revealed that CCL7 is produced
241 by immune cells, including SiglecF+ eosinophils, SiglecF- F4/80+ macrophages, and
242 SiglecF-Ly6C+ monocytes (Fig. 7aii). For each cell type, a markedly higher percentage of
243 cells obtained from the female mammary gland produced CCL7, than from male fat pad
244 cells. Notably, around 60% of female SiglecF+ cells produced CCL7 compared with 10% of
245 male cells (Fig. 7aii). The percentage of CCL7+ cells was unaffected in the absence of
246 ACKR2 (Fig. 7ai, ii). CCL7 is also produced by CD45- epithelial cells: mature (EpCAM+
247 CD49f-) and progenitor luminal (EpCAM+ CD49f+), and basal (EpCAM - CD49f+) cells (Fig.
248 7aiii, Supplementary Fig. 4b). Further, bioinformatic analysis confirmed that CCL7 is
249 produced by epithelial cells, including basal, luminal and myoepithelial cells (Supplementary
250 Fig. 5) (Bach *et al.*, 2017).

251 Given the notable CCL7 expression in the mammary gland, we next directly tested its
252 potential role in mammary gland development. PBS or 2 µg of CCL7 was administered
253 subcutaneously at the site of the mammary fat pad at the key time point of 6 weeks. After 3
254 days, mammary glands were harvested for cellular analysis by flow cytometry and carmine
255 alum whole-mount analysis. CCL7 administration alone was sufficient to increase the
256 percentage of CD206+ macrophages, and the area of branching within the mammary gland
257 (Fig. 7b). These data confirm that elevated levels of CCL7, as observed in ACKR2-/- mice,
258 leads to increased numbers of CD206+ macrophages in the mammary gland and
259 accelerated branching.

260 Overall these data demonstrate a role for CCL7, a ligand shared by CCR1 and ACKR2, in
261 branching morphogenesis. Lending further support to this conclusion is the fact that
262 bioinformatic interrogation of the precocious puberty (CTD Gene-Disease Associations)
263 dataset, using Harmonizome (Rouillard *et al.*, 2016), revealed that CCL7 and ACKR2 are
264 both associated with precocious puberty in children, with standardized values of 1.25588
265 (p=0.09) and 1.02634 (p=0.011) respectively.

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267 **Discussion**

268 The importance of macrophages in controlling developmental processes is well known
269 (Wynn, Chawla and Pollard, 2013). The role of chemokines and their receptors, which
270 provide molecular cues to guide and position macrophages during development, is an
271 emerging area of research (Lee *et al.*, 2014; Wilson *et al.*, 2017). Previously, we revealed
272 that the scavenging atypical chemokine receptor, ACKR2 controlled macrophages in the
273 mammary gland through a CCR2-independent pathway (Wilson *et al.*, 2017). Here we have
274 revealed a previously unknown immunological mechanism whereby, ACKR2 and the
275 inflammatory chemokine receptor CCR1, interact with their shared ligand CCL7 to
276 coordinate the levels of CD206+ macrophages, and thus, the extent of branching
277 morphogenesis in the pubertal mammary gland. Importantly, administration of CCL7 alone
278 was able to increase the percentage of CD206+ macrophages within the mammary gland
279 and drive accelerated branching morphogenesis. We propose that in CCR1-/- mice, although
280 CCL7 levels are unaltered, macrophages are unable to sense and respond to the ligand
281 without the cognate receptor, leading to delayed branching (Fig. 8).

282 Previously, it was thought that all mammary gland macrophages, at rest, and in
283 pathology, were derived from the bone marrow (Coussens and Pollard, 2011). In our
284 previous study, we showed that branching was unaltered in the absence of CCR2, indicating
285 that the macrophage population responsible for promoting branching morphogenesis was
286 unlikely to be bone marrow derived (Wilson *et al.*, 2017). Recently, a novel CD206+
287 macrophage population has been identified in the mammary gland, which is unaffected in
288 the absence of CCR2, but reduced in *plvap*-/- mice, which have reduced numbers of foetal-
289 derived macrophages (Jäppinen *et al.*, 2019). Branching is severely impaired in these mice
290 suggesting that foetal-derived macrophages play a key role in promoting branching
291 morphogenesis (Jäppinen *et al.*, 2019). We believe that the macrophage population
292 identified in our study may be derived from the same embryonic population (Jäppinen *et al.*,
293 2019).

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294 CCR1 is an inflammatory chemokine receptor which is expressed by immune cells,
295 and has been shown to be important in a number of pathologies, including: sepsis, viral
296 infections, cancer and autoimmune disease (Domachowske *et al.*, 2000; Katschke Jr. *et al.*,
297 2001; Ness *et al.*, 2004; Kitamura *et al.*, 2015). To our knowledge, this is the first description
298 of a key role for CCR1 in development. Of note, in the placenta, CCR1 has been shown to
299 be expressed by human trophoblasts as they switch to an invasive phenotype (Sato *et al.*,
300 2003). ACKR2 is highly expressed by placental trophoblasts, preventing excess levels of
301 inflammatory chemokines from entering the foetus, from the mother's circulation, by a
302 process of chemokine compartmentalisation (Teoh *et al.*, 2014; Lee *et al.*, 2019). As CCR1
303 expression has also been described in placental development, there could be wider
304 implications of the interaction described in this study.

305 In the mouse, sexual maturity occurs at around 6 weeks (Topper and Freeman,
306 1980). Here we report a marked increase in plasma estradiol levels between 6.5 and 7
307 weeks. This is the key time point in ACKR2/CCR1-dependent regulation of branching
308 morphogenesis. ACKR2 expression in the mammary gland specifically peaks at 6.5 weeks
309 and branching begins to accelerate at this time point (Wilson *et al.*, 2017). We show that
310 17β-estradiol increases CCR1 expression on macrophages. However, this is restricted to
311 pubertal mammary gland macrophages, as older female, male and peritoneal macrophages
312 do not respond. In addition to 17β-estradiol, the estrogen mimic, Bisphenol A also increased
313 CCR1 expression on CD206+ macrophages. This may be of concern as BPAs are widely
314 found in the environment and could potentially alter the immune response, and the extent of
315 branching in the mammary gland, in children during puberty. Previously CCR1 expression on
316 T cells was shown to be regulated by 17β-estradiol (Mo *et al.*, 2005). However this is the first
317 description of estrogen controlled CCR1 expression on macrophages. This observation
318 could have implications for our understanding of diseases where females exhibit increased
319 susceptibility. One example is rheumatoid arthritis, where CCR1 is also associated with
320 pathology (Katschke Jr. *et al.*, 2001; van Vollenhoven, 2009).

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321 Understanding the molecular signals which guide the rate of branching
322 morphogenesis in the mammary gland is highly important. Precocious puberty is a condition
323 where puberty begins before the age of 8, with some girls developing breasts as early as 4.
324 This results from early activation of the gonadotropic axis, leading to accelerated growth and
325 bone maturation, but ultimately reduced stature (Carel *et al.*, 2004). Potential risk factors
326 include exposure to endocrine disrupters, obesity, stress and ethnicity (Cesario and Hughes,
327 2007; Lee *et al.*, 2007; Meeker, 2012; Kelly *et al.*, 2017). As mammary gland development is
328 delayed in mice in the absence of CCR1, this could represent a novel therapeutic target to
329 treat aspects of precocious puberty. Several CCR1 antagonists are available and have been
330 used in a number of clinical trials (Lebre *et al.*, 2011). In addition, early breast development
331 leads to higher risks of breast cancer in later life (Bodicoat *et al.*, 2014), and women with
332 dense breasts are more likely to develop breast cancer (Nazari and Mukherjee, 2018). This
333 can be related to poor detection by mammography as the branches mask the cancer, but
334 may also be caused by genetic factors, parity and alterations in the breast stroma. Both
335 ACKR2 and CCR1 have been shown to be important in the progression of breast cancer,
336 therefore understanding early interactions between these receptors could reveal key
337 insights, which drive later pathology (Kitamura *et al.*, 2015; Shin *et al.*, 2017; Hansell *et al.*,
338 2018).

339 In this study, we have uncovered a novel mechanism by which estradiol upregulates CCR1
340 expression by pubertal mammary gland macrophages and stromal ACKR2 modulates levels
341 of CCL7, to control the movement of the CCR1+ macrophages to the ductal epithelium.
342 Overall therefore our data demonstrate that CCR1 and ACKR2 coordinately regulate
343 mammary gland branching morphogenesis.

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347 **Methods**

348 **Animals**

349 Animal experiments were carried out under the auspices of a UK Home Office Project
350 Licence and conformed to the animal care and welfare protocols approved by the University
351 of Glasgow. C57BL/6 mice, ACKR2-/- (Jamieson *et al.*, 2005), CCR1-/-, CCR3-/-, CCR5-/-
352 and iCCR-/- (Douglas P. Dyer *et al.*, 2019) mice were bred at the specific pathogen-free
353 facility of the Beatson Institute for Cancer Research.

354 **Carmine Alum Whole Mount**

355 Carmine alum whole mounts were carried out as described previously (Wilson *et al.*, 2017).
356 Briefly, fourth inguinal mammary glands were fixed overnight in 10% neutral buffered
357 formalin (NBF) (Leica) at 4°C. Glands were dehydrated for 1 h in distilled water, followed by
358 70% ethanol and 100% ethanol before overnight incubation in xylene (VWR international).
359 Tissue was rehydrated by 1 h incubation in 100% ethanol, 70% ethanol and distilled water,
360 before staining in Carmine Alum solution overnight at room temperature (0.2% (w/v) carmine
361 and 10 mM aluminium potassium sulphate (Sigma)). Tissue was dehydrated again before
362 overnight incubation in xylene. Finally, glands were mounted with DPX (Leica) and stitched
363 bright-field images at 10× magnification were taken using an EVOS FL auto2 microscope
364 (Thermofisher). Ductal elongation, and branched area from the lymph node, were measured
365 using ImageJ 1.52a (Schneider, Rasband and Eliceiri, 2012). 5 x brightfield images were
366 obtained using the Zeiss Axioimager M2 with Zen 2012 software. The numbers of branches
367 and branch thickness were counted as the average from 3 measurements from 6 individual
368 fields of view (F.O.V.) from each whole mount. TEBs were counted as the average from at
369 least 2 F.O.V. from each whole mount. All samples were blinded before measurements were
370 taken.

371 **RNAscope ® In situ hybridisation**

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372 Mammary glands were fixed in 10% neutral buffered formalin at room temperature for 24-36
373 hours before being dehydrated using rising concentrations of ethanol and xylene, and
374 paraffin embedded (Shandon citadel 1000 (Thermo Shandon). Tissue was sectioned onto
375 Superfrost plus slides (VWR) at 6 µm using a Microtome (Shandon Finesse 325 Microtome,
376 Thermo). Slides were baked at 60ºC for 1 h before pre-treatment. Slides were deparaffinised
377 with xylene (5 mins x 2) and dehydrated with ethanol (1 min x 2). Tissues were incubated
378 with Hydrogen peroxide for 10 mins at RT, then boiled in antigen retrieval buffer for 15 mins.
379 Slides were treated with protease plus for 30 mins at 40ºC. Slides were then hybridised
380 using the RNAScope® 2.5 Red Manual Assay (Advanced cell diagnostics) according to the
381 manufacturer's instructions using the Mm-Ccr1 and Mm-ACKR2 probes. Slides were
382 mounted in DPX (Sigma Aldrich) and imaged on an EVOS FL Auto2microscope.

383 **Mammary gland digestion**

384 The inguinal lymph node was removed from the fourth inguinal mammary gland, tissue was
385 chopped, and enzymatic digestion was carried out in a 37°C shaking incubator at 200 rpm
386 for 1 h, with 3 mg/ml collagenase type 1 (Sigma) and 1.5 mg/ml trypsin (Sigma) in 2 ml
387 Leibovitz L-15 medium (Sigma). The suspension was shaken for 10 s before addition of 5 ml
388 of L-15 medium supplemented with 10% foetal calf serum (Invitrogen) and centrifugation at
389 400 g for 5 min. Red blood cells were lysed using Red Blood Cell Lysing Buffer Hybri-Max
390 (Sigma) for 1 min and washed in PBS. Cells were washed in PBS with 5 mM EDTA,
391 resuspended in 2 ml 0.25% Trypsin-EDTA (Sigma) and incubated at 37°C for 2 min before
392 addition of 5 ml of serum-free L-15 containing 1 µg/ml DNase1 (Sigma) for 5 min at 37°C. L-
393 15 containing 10% FCS was added to stop the reaction and cells were filtered through a 40
394 µm cell strainer before a final wash in FACS buffer (PBS containing 1% FCS and 5 mM
395 EDTA).

396 **Flow cytometry**

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397 Antibodies were obtained from BioLegend and used at a dilution of 1:200: CD45 (30-F11),
398 CD11b (M1/70), F4/80 (BM8), SiglecF (S17007L), Ly6C (HK1.4), EpCAM (G8.8),
399 CD49f(GoH3), CCR1 (S10450E), and CD206 (C068C2) for 30 min at 4°C. Dead cells were
400 excluded using Fixable Viability Dye eFluor 506 (Thermo Fisher). Intracellular staining for
401 CCL7 was carried out using 1 in 100 biotinylated CCL7 antibody (R&D Systems) and
402 Strepdavidin BV605 (BioLegend) and eBioscience intracellular fixation and permeabilization
403 buffer. Flow cytometry was performed using an LSRII or Fortessa, (BDBiosciences) and
404 analysed using FlowJo V10.

405 **Proteomic analysis**

406 The inguinal lymph node was removed from the fourth inguinal mammary gland, tissue was
407 chopped, frozen in liquid nitrogen, crushed with a mortar and pestle, and resuspended in
408 dH_2O containing protease inhibitors (Pierce). Protein levels were determined using a custom
409 designed Magnetic Luminex Multiplex assay (R&D Systems), as described in the
410 manufacturer's instructions, and read with a Bio-Rad Luminex-100 machine. Data was
411 normalised to the protein concentration of tissue samples, determined by a BCA assay
412 (Pierce).

413 **Subcutaneous administration of CCL7**

414 2 μg of CCL7 in 200 μl PBS (R&D Systems) was injected subcutaneously into mice at 6
415 weeks of age. After 3 days, mice were culled and mammary glands were excised and
416 processed for whole mount and cellular analysis.

417 **17β-estradiol assays**

418 Fourth inguinal mammary glands were digested to obtain single cell suspensions. Cells were
419 plated at 0.5-1 x 10^5 cells in a 96 well plate in L-15 media containing 5% FCS and exposed
420 to DMSO (vehicle control) or 50 ug/ml 17-β estradiol or Bis-phenol A (Sigma) for 1 h at 37°C,
421 5% CO_2. The level of 17-β estradiol in plasma samples was determined using the Estradiol
422 parameter kit (R&D Systems) as described in the manufacturer's instructions.

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423 **Bioinformatic analysis**

424 Chemokine expression by epithelial cells was determined by searching the data repository
425 from Bach et al, 2017 (Bach *et al.*, 2017) at:
426 https://marionilab.cruk.cam.ac.uk/mammaryGland/.

427 **Statistical analysis**

428 Data were analysed using GraphPad Prism 8.1.2. Normality was assessed using Shapiro
429 Wilk and Kolmogorov–Smirnov tests. For data with normal distribution, two-tailed, unpaired t-
430 tests were used. Where data was not normally distributed, Mann–Whitney tests were used.
431 Significance was defined as $p<0.05$ *. Error bars indicate standard error of the mean
432 (S.E.M.).

433

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439 Wolfson Royal Society Merit award.

440 **Competing Interests**

441 The authors declare no competing interests

442 **Author Contributions**

443 GJW conceived the study, performed experiments, analysed data and wrote the paper. AF
444 performed experiments and analysed data. SRL, JK, MP and AJH performed experiments.
445 GJG conceived the study and wrote the paper.

446

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447 **Figures**

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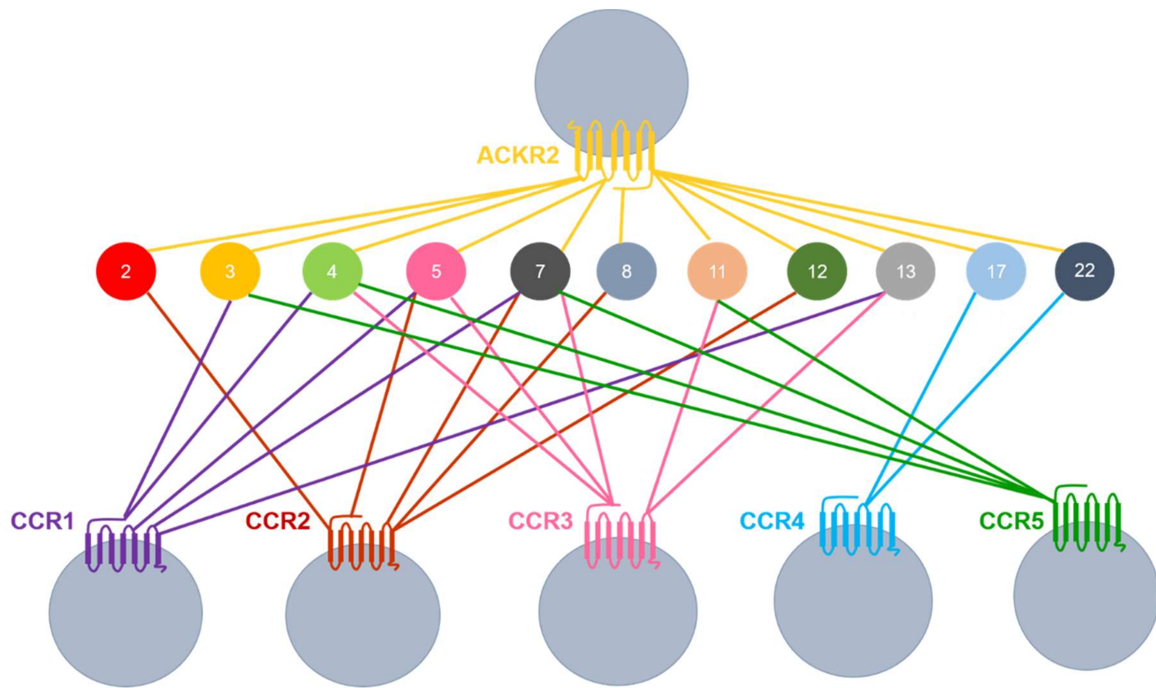

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450

451 **Figure 1: ACKR2 shares ligands with inflammatory chemokine receptors.** Coloured

452 lines indicate receptor ligand interactions. Data compiled from Bachelerie, F. *et al.,* 2014 and

453 Nibbs, R. J. B. & Graham, G. J., 2013.

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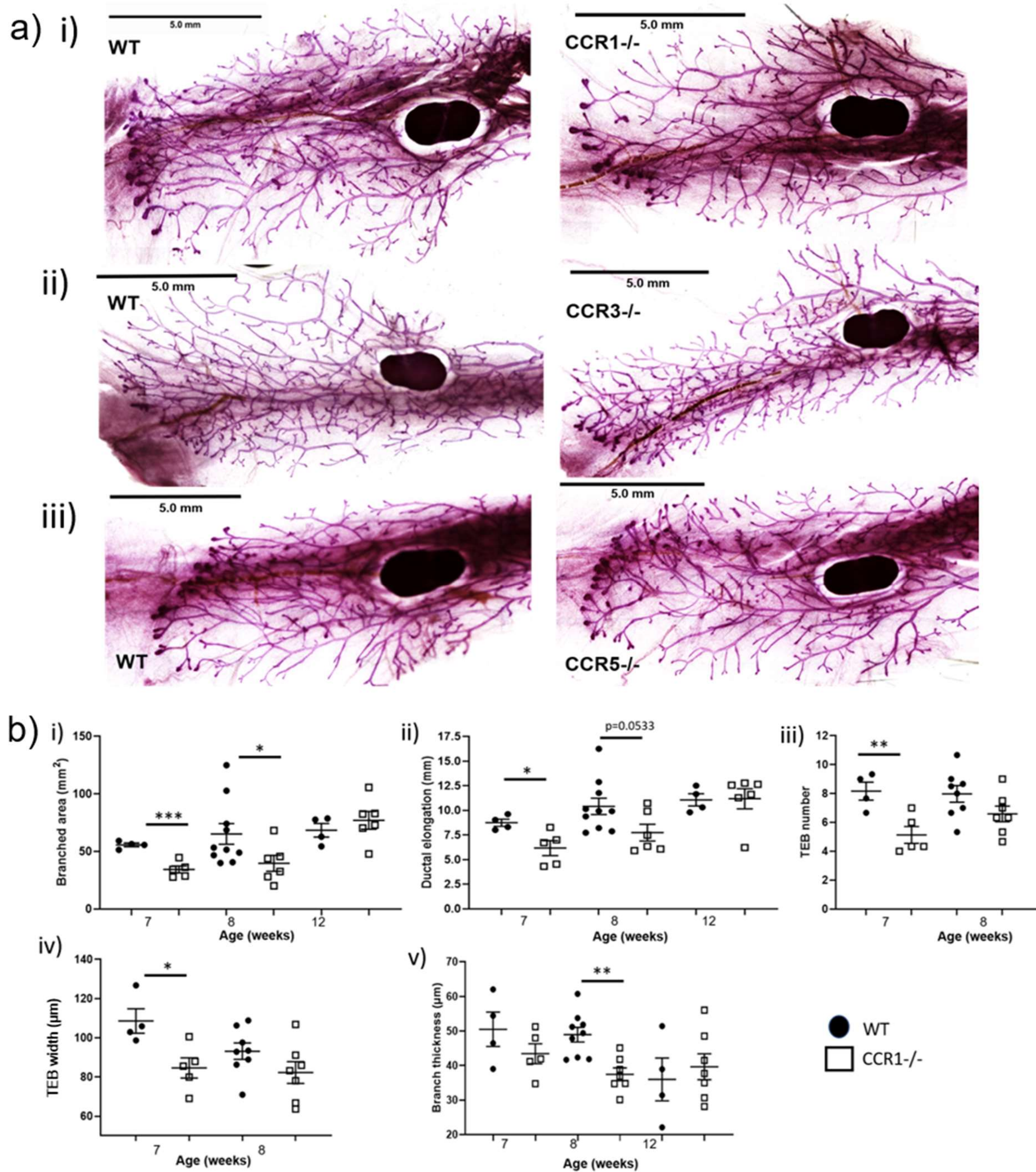

455

456 **Figure 2: Ductal branching in the pubertal mammary gland is regulated by CCR1. a)**
457 Representative carmine alum whole mount images of late pubertal (7 week old) virgin
458 mammary glands from **i)** wild-type and CCR1-/-, **ii)** CCR3-/- and **iii)** CCR5-/- mice. Scale
459 bars: 5 mm. **b)** Branching morphogenesis was quantified in 7 (WT n=4, CCR1-/- n= 5), 8
460 (WT n=10, CCR1-/- n= 7) and 12 (WT n=4, CCR1-/- n= 7) week mammary glands using
461 ImageJ, by measuring: **i)** the area of branching from the inguinal lymph node, and **ii)** ductal
462 elongation, measured from the middle of the inguinal lymph node to the furthest edge of

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463 ductal outgrowth. **iii)** The number of TEBs, was determined as the average number from at
464 least 2 individual fields of view (FOV) (5×) per gland. **iv)** The average width of all TEBs was
465 determined from at least 2 F.O.V (5x) per gland. **v)** Branch thickness was determined as the
466 average of 6 measurements from 3 F.O.V (5x) per gland. Significantly different results are
467 indicated. Error bars represent S.E.M.

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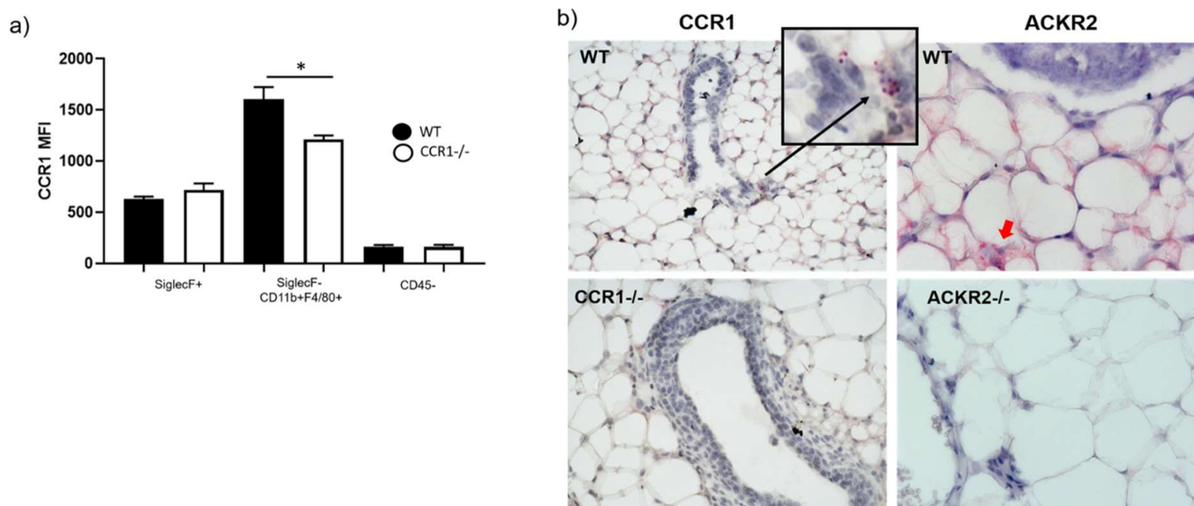

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471

472 **Figure 3: CCR1 and ACKR2 are expressed surrounding epithelium in the mammary**
473 **gland. a)** Flow cytometry analysis of CCR1 expression by enzymatically digested WT (black
474 bars, n=6) and CCR1-/- (white bars, n=4) mammary gland cells: CD45-, CD45+ SiglecF+,
475 and CD45+SiglecF-CD11b+F480+. **b)** RNAscope® in situ hybridization of CCR1 (highlighted
476 by black arrow) and ACKR2 (highlighted by red arrow), in the developing virgin mammary
477 gland of WT, CCR1-/- and ACKR2-/- mice. Significantly different results are indicated. Error
478 bars represent S.E.M.

479

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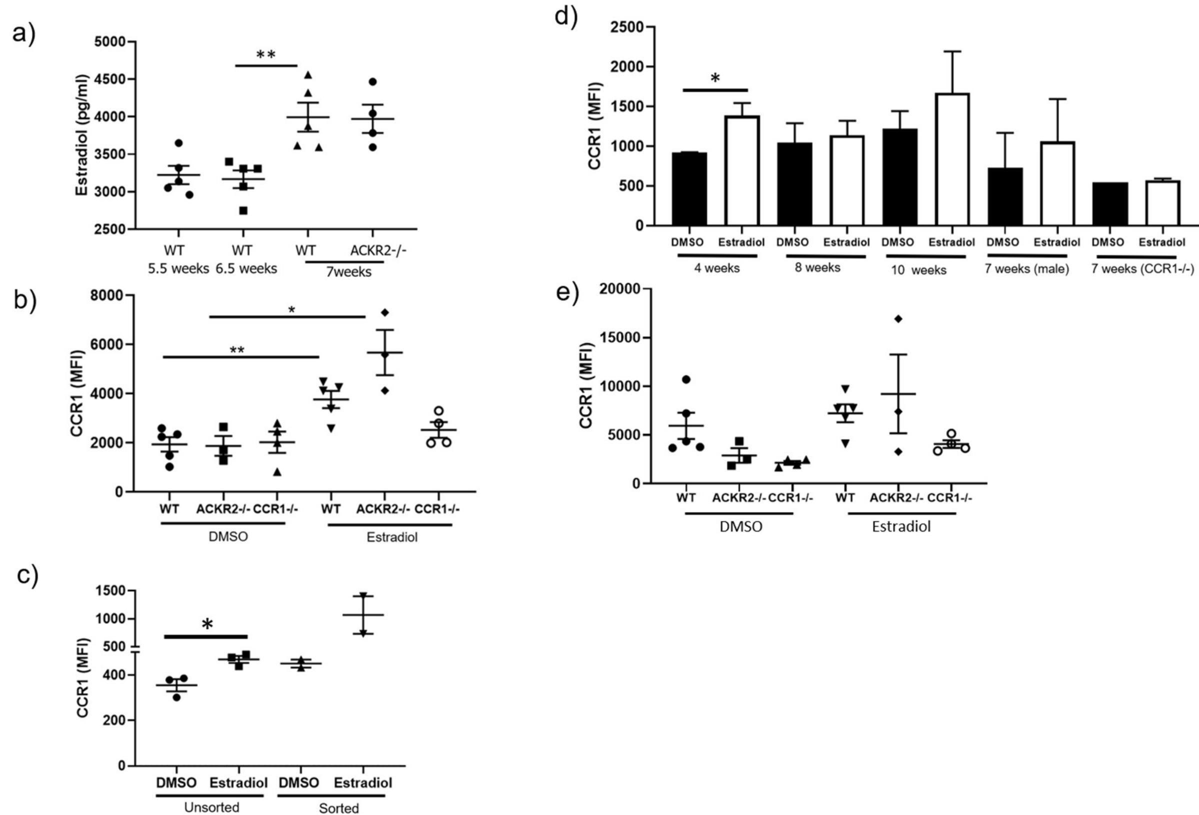

480

481 **Figure 4: Estrogen induces CCR1 expression on macrophages. a)** Estradiol levels in

482 plasma from 5.5 weeks WT (n=5), 6.5 weeks WT (n=5), 7 weeks WT (n=5), and 6.5 weeks

483 ACKR2-/- (n=4). **b)** CCR1 expression by CD11b+F4/80+ cells in response to DMSO and 50

484 µg/ml estradiol in WT (n=5), CCR1-/- (n=4), and ACKR2-/- (n=3). **c)** unsorted and

485 CD11b+F4/80+ FACS sorted cells from the mammary gland. **d)** CCR1 expression by

486 CD11b+F4/80+ cells from female WT mammary glands at 4, 8, 10 weeks old, female 7 week

487 old CCR1-/- mice and male WT inguinal fat pads from 7 week old mice, in response to

488 DMSO and 50 µg/ml 17β-estradiol (each group, n=3); and **e)** CD11b+F4/80+ cells from the

489 peritoneum. Significantly different results are indicated. Error bars represent S.E.M.

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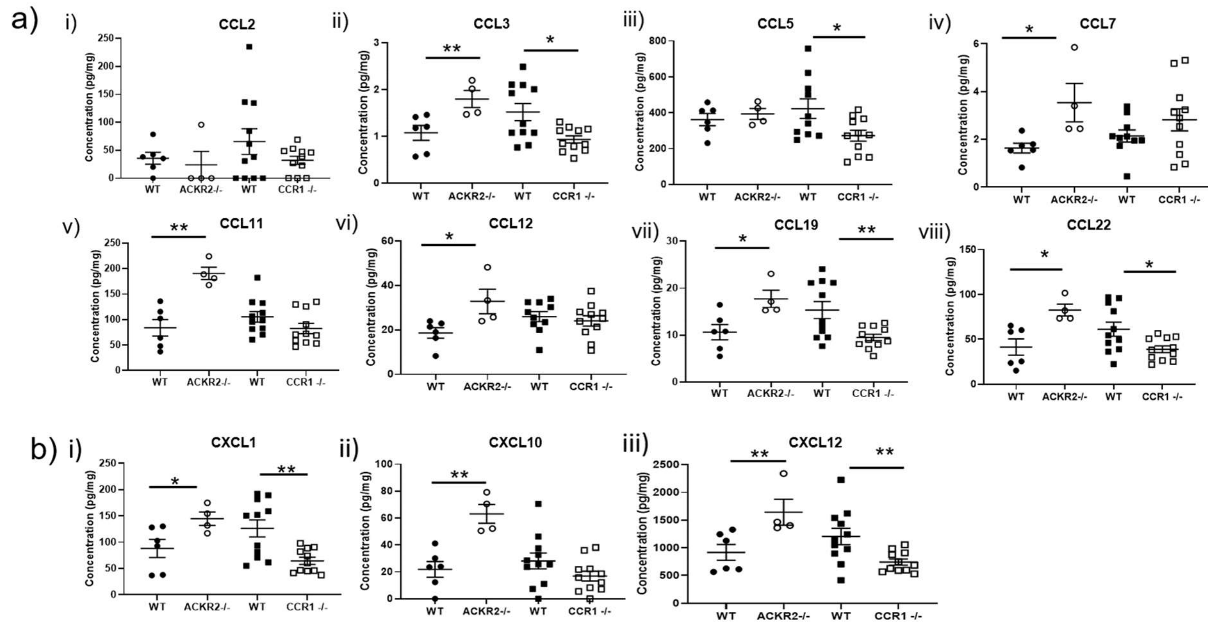

490

491 **Figure 5: Chemokine levels are altered in the absence of ACKR2 and CCR1.** Multiplex

492 measurement of protein concentration of **a)** inflammatory CC-chemokines; **i)** CCL2, **ii)**

493 CCL3, **iii)** CCL5, **iv)** CCL7, **v)** CCL11, **vi)** CCL12, **vii)** CCL19 and **viii)** CCL22, and; **b)**

494 inflammatory CXC chemokines **i)** CXCL1, **ii)** CXCL10 and **iii)** CXCL12 in whole mammary

495 gland homogenates. WT (CCR1) n=11, CCR1-/- n=11, WT (ACKR2) n=6, and ACKR2-/-

496 n=4. Significantly different results are indicated. Error bars represent S.E.M.

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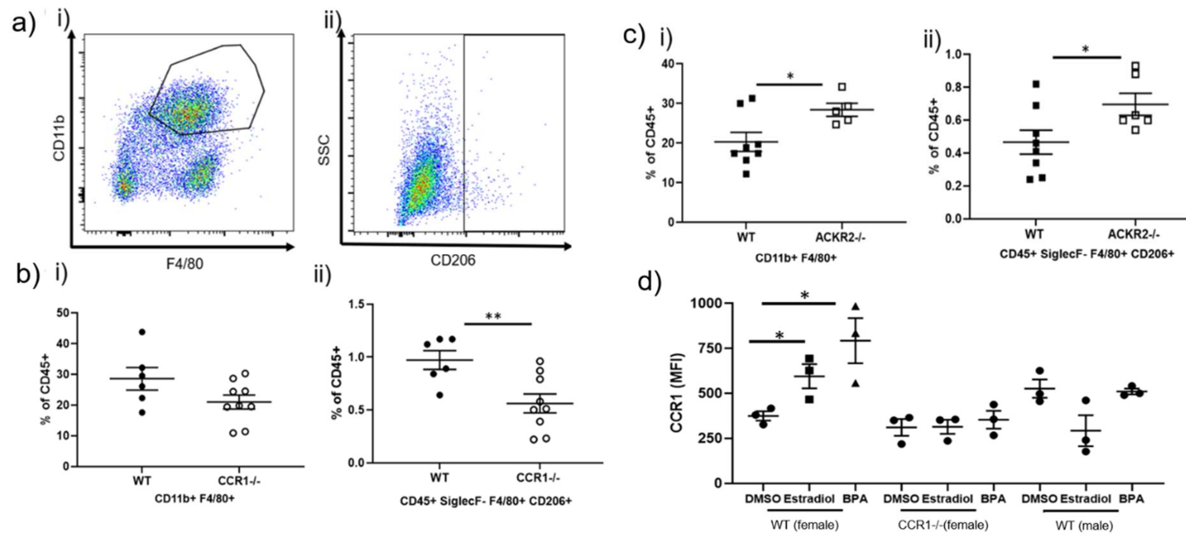

499

500 **Figure 6: CCR1 and ACKR2 reciprocally regulate CD206+ macrophages within the**
501 **mammary gland. a)** Flow cytometry was used to determine the percentage of i)
502 CD11b+F4/80+ and ii) SiglecF- F4/80+ CD206+ macrophages, within the CD45+
503 compartment of the 6.5 weeks old developing mammary gland. Flow cytometry of **b)** WT
504 (n=6) and CCR1-/- (n=9) and **c)** WT (n=8) and ACKR2-/- (n=5) mammary gland cells was
505 carried out. **d)** CCR1 expression by SiglecF- F4/80+ CD206+ cells in response to DMSO
506 and 50 µg/ml 17β-estradiol and Bisphenol A; female WT and CCR1-/- and male WT (n=3).
507 Significantly different results are indicated. Error bars represent S.E.M

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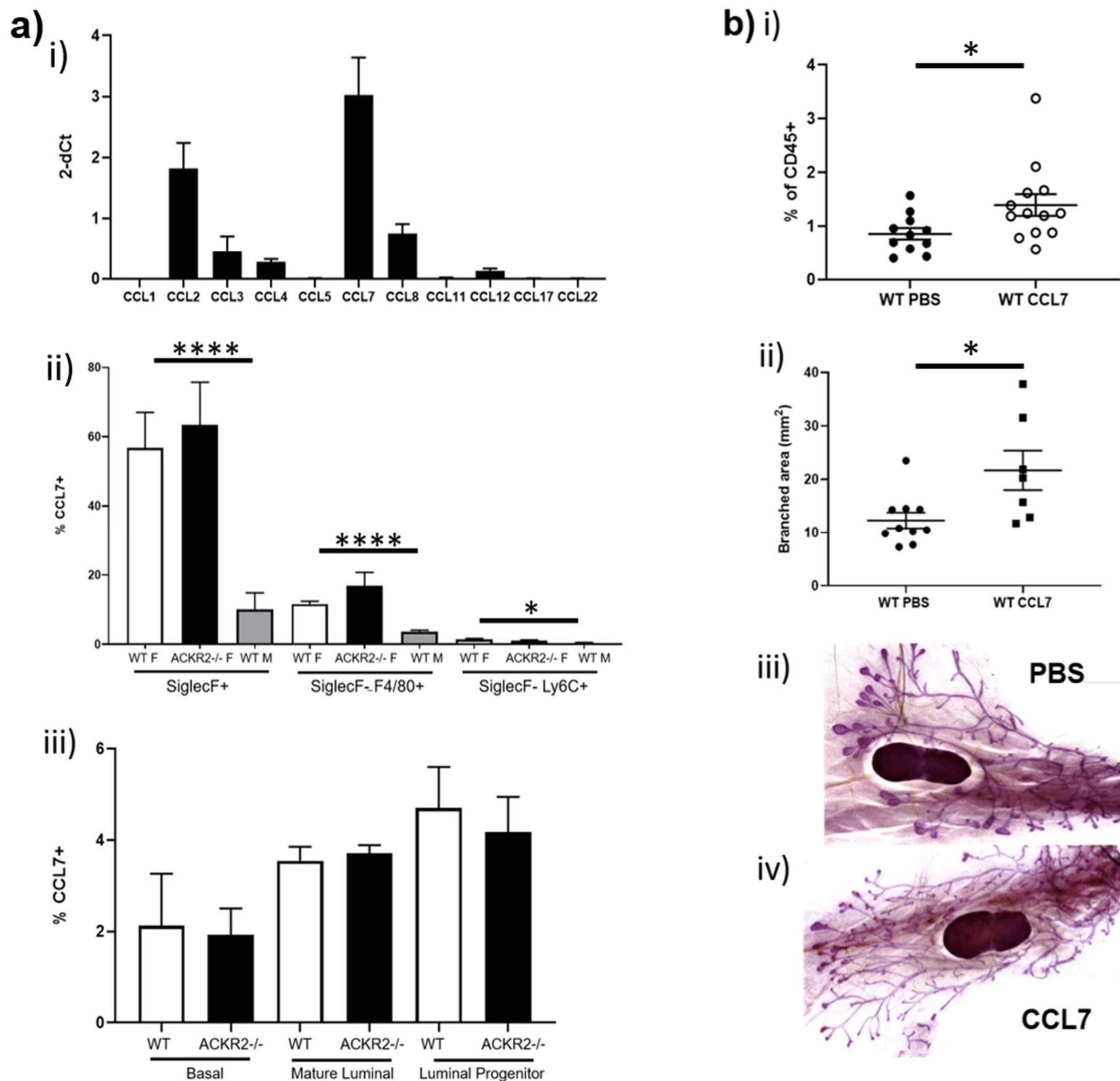

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509

510 **Figure 7: CCL7 controls CD206+ macrophages and branching morphogenesis. a)**

511 CCL7 is produced in the mammary gland by **i)** immune cells; including SiglecF+, SiglecF-

512 F4/80+ and SiglecF-Ly6C+, male WT (n=7) and female WT (n=6) and ACKR2-/- (n=4). **ii)**

513 Transcription of inflammatory chemokines by purified F4/80+ cells (n=3) and **iii)** CCL7

514 production by epithelial cells, Mature Luminal (EpCAM+ CD49f-), progenitor luminal

515 (EpCAM+ CD49f+), and basal (EpCAM- CD49f+), female WT (n=6) and ACKR2-/- (n=4). **b)**

516 3 days after subcutaneous administration of PBS or 2 μg CCL7 at 6 weeks, **i)** the

517 percentage of SiglecF-F4/80+CD206+ cells was measured by flow cytometry. (PBS, n=11,

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518 CCL7, n=13) and **ii)** the area of branching was measured using Image J (PBS, n=10, CCL7,
519 n=7). **iii)** and **iv)** Representative images of whole mounts from PBS and CCL7 injected mice.
520 Significantly different results are indicated. Error bars represent S.E.M.

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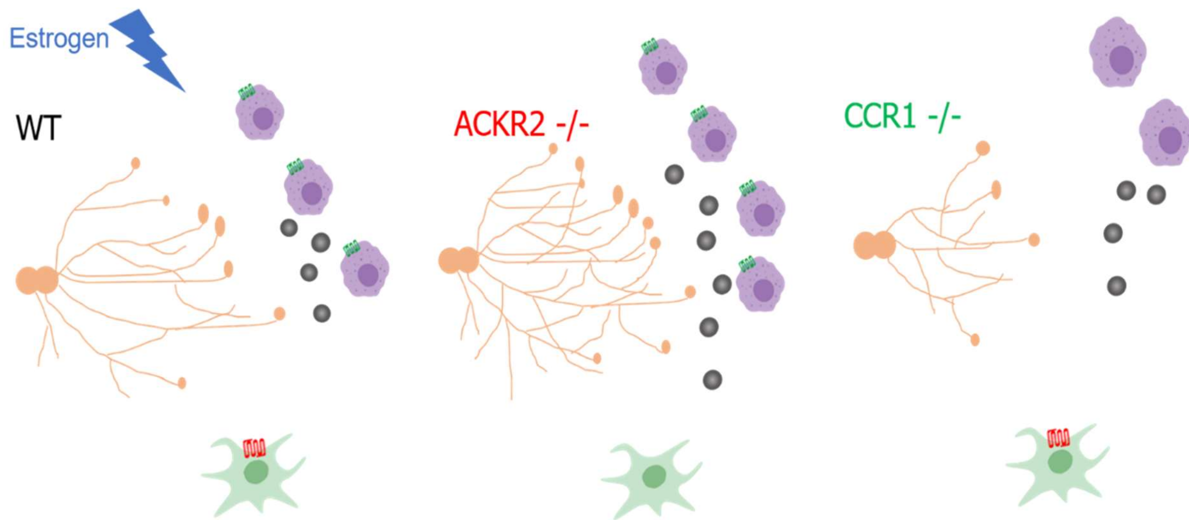

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525 **Figure 8: Proposed mechanism by which chemokine receptors CCR1 and ACKR2**
526 **coordinate mammary gland development.** Estrogen increases CCR1 expression on
527 macrophages (purple) during puberty, and stromal fibroblast (green) expressed ACKR2
528 modulates levels of CCL7 (grey circles) to control the movement of CCR1+ macrophages to
529 the ductal epithelium (orange). Schematic image was created with BioRender.

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