Chemokine receptors CCR1 and ACKR2 coordinate mammary gland development

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Abstract

Macrophages are key regulators of developmental processes. We previously demonstrated that the scavenging atypical chemokine receptor, ACKR2 has profound effects on branching morphogenesis, by regulating macrophage dynamics during pubertal mammary gland development. ACKR2 is a non-signalling receptor, which mediates effects through collaboration with inflammatory chemokine receptors. Here we reveal that the inflammatory chemokine receptor CCR1, is the reciprocal receptor controlling branching morphogenesis in the mammary gland. Delayed development, with decreased area of the ductal epithelial network and CD206+ macrophages, was observed in the glands of pubertal CCR1-/- mice. This is an inverse phenotype to that observed in ACKR2-/- mice. Subcutaneous administration of CCL7, a shared ligand between CCR1 and ACKR2, is sufficient to drive increased numbers of CD206+ macrophages and branching morphogenesis. In addition, estrogen, which is essential for ductal elongation during puberty, upregulates CCR1 expression on the surface of pubertal mammary gland macrophages. These data demonstrate that ACKR2 and CCR1 co-ordinately regulate the rate of branching

morphogenesis in the pubertal mammary gland, whereby stromal ACKR2 modulates levels of CCL7 to control the movement of macrophages expressing CCR1 to the ductal epithelium. The age at which girls develop breasts is steadily decreasing, which increases the risk of many diseases including breast cancer. This study presents a previously unknown immunological mechanism underpinning the rate of development during puberty which could reveal novel therapeutic targets.

Introduction

Breast development (thelarche) is the first visible sign of puberty in females, and typically occurs between the ages of 8 and 13[1]. Globally, the age at pubertal onset is falling[2]. Early puberty is associated with an increased risk of disease in later life, including type II diabetes heart disease, and cancer[3]. Importantly, girls who develop breasts before the age of 10 are 20% more likely to develop breast cancer[4]. Therefore, understanding the molecular mechanisms and immunological processes underlying this acceleration is of key importance.

Tissue remodelling by branching morphogenesis is a central developmental process. In most organs this occurs during embryogenesis or soon after birth. Uniquely, the majority of mammary gland development occurs postnatally, throughout the female reproductive lifetime. During murine embryonic development, an epithelial placode forms, and a rudimentary structure is present at birth. In the mouse, puberty begins at around 3 weeks[5], when ovarian hormones are released and terminal end buds (TEBs) form at the end of epithelial ducts. TEBs are highly proliferative structures which grow invasively through the fat pad, generating the ductal tree, until adulthood when they regress. By 8 weeks, the mouse is considered adult[5]. During pregnancy, proliferation of epithelial cells within ductal branches generates the lobuloalveoli (LAL) necessary to produce milk during lactation. Upon weaning, the epithelium undergoes involution, where an extensive programme of cell death remodels the gland to its pre-pregnancy condition[6].

55 The murine mammary gland consists of an epithelial network within the fat pad and a
56 stromal population containing fibroblasts, extracellular matrix (ECM), adipocytes and
57 immune cells[6]. Macrophages are found in all tissues within the body and are required for
58 immunological and developmental processes[7]. Mammary gland development is severely
59 impaired in mice which lack the main macrophage growth factor, colony stimulating factor 1
60 (CSF-1), with TEB formation, ductal elongation, branching during puberty and LAL
61 development in pregnancy all affected[8,9]. Macrophages are found surrounding TEBs,
62 produce proteinases and growth factors, and promote collagen fibrillogenesis[9,10].
63 Chemokines and their receptors play fundamental roles in controlling macrophage migration
64 and in regulating macrophage dynamics during tissue remodelling[11,12].

65 Atypical chemokine receptors (ACKRs) are a small family of 7-transmembrane-
66 spanning receptors that lack the DRYLAIV motif characteristic of conventional chemokine
67 receptors[13,14] and which do not directly support cellular migration. They are characteristically
68 expressed on stromal cells and, with the exception of ACKR1, internalise and degrade their
69 cognate ligands. They therefore function as chemokine scavenging receptors and thus
70 regulate chemokine availability within tissues. ACKR2 is a member of this family, which
71 internalises and degrades all 11 inflammatory CC-chemokines[14]. In so doing, ACKR2
72 mediates the resolution of inflammation in infection, autoimmunity and cancer [15–17]. In
73 development, ACKR2 is an essential regulator of macrophage dynamics controlling
74 branching morphogenesis in both the developing lymphatic system of embryonic skin, and
75 the pubertal mammary gland[11,12]. ACKR2-/- mice showed accelerated pubertal mammary
76 gland development as evidenced by increased branching and TEB number. Deficient
77 scavenging of macrophage attracting chemokines in ACKR2-/- mice, led to elevated
78 numbers of macrophages and a precocious developmental phenotype. This study provided
79 the first evidence of chemokine receptor involvement in postnatal development[11].

80 Through regulation of inflammatory CC chemokine availability, ACKR2 modulates
81 cell recruitment and migratory responses mediated by the conventional inflammatory

82 chemokine receptors with which it shares ligands[13,14]. Here we identify CCR1 as the
83 inflammatory chemokine receptor (iCCR) working in collaboration with ACKR2 to control
84 branching morphogenesis in the developing mammary gland. CCR1 is a conventional
85 chemokine receptor, which senses extracellular chemokines and signals to change the
86 behaviour of the cell[18]. CCR1-/- mice show delayed pubertal development, with decreased
87 TEBs, smaller area of the ductal epithelial network and a reduction in CD206+ (Mannose
88 Receptor) macrophages. This phenotype is opposite to that observed in ACKR2-/- mice.
89 Subcutaneous administration of CCL7, a ligand shared between CCR1 and ACKR2,
90 increased numbers of CD206+ macrophages and branching morphogenesis and in the
91 mammary gland. In addition, Estrogen, which is essential for ductal elongation during
92 puberty[19], upregulates CCR1 expression on the surface of pubertal mammary gland
93 macrophages. We also demonstrate that stromal ACKR2 modulates levels of CCL7, to
94 control the movement of macrophages expressing CCR1 to the ductal epithelium. This co-
95 ordination regulates the rate of branching morphogenesis in the mammary gland.
96 Collectively, this study reveals a previously unknown immunological mechanism, and sheds
97 important light on the regulation of macrophage dynamics during virgin mammary gland
98 development.
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100

101 **Results**

102 **Branching morphogenesis in the pubertal mammary gland is regulated by CCR1.**

103 We previously showed that CCR2-/- mice displayed only minor alterations in branch
104 morphology, with no effect on ductal elongation or branched area, suggesting the key
105 macrophage population in the mammary gland migrates independently of CCR2. ACKR2
106 also shares ligands with other iCCRs, including CCR1, CCR3, CCR4 and CCR5. CCR4 is
107 not expressed by macrophages and so our analysis has concentrated on CCR1, CCR3 and

108 CCR5[11]. These receptors have overlapping but distinct receptor ligand interactions with
109 ACKR2, summarised in Figure 1a[13,14]. To investigate if an iCCR collaborates with ACKR2 to
110 regulate branching morphogenesis during puberty, carmine alum whole mounts of mammary
111 glands were obtained from 7 weeks old WT and CCR1-/-, CCR3-/- and CCR5-/- deficient
112 mice (Fig. 1b i-iii). The individual receptor deficient mice are on different genetic
113 backgrounds, therefore each strain has been compared to their specific WT[20]. CCR1-/- mice
114 exhibit delayed mammary gland development with decreased area of branching at 7 and 8
115 weeks, decreased number and size of TEBs and reduced ductal elongation at 7 weeks (Fig.
116 1c i-iii). In addition, CCR1-/- mice have thinner branches at 8 weeks (Fig. 1c v). This
117 phenotype was previously observed in CCR2-/- but not ACKR2-/- mice[11]. Branched area,
118 TEB number and ductal elongation were unaffected in CCR3-/- and CCR5-/- mice (Fig. 1b ii-
119 iii, Supplementary Fig. 1). Previously we showed that ACKR2-/- mice had an increased area
120 of branching and numbers of TEBs at 6.5 and 8 weeks, and increased ductal elongation at 8
121 weeks[11]. This inverse phenotype in CCR1-/- mice suggests that CCR1 is the reciprocal
122 receptor for ACKR2, controlling the extent of branching, TEB number and ductal elongation.
123 As observed in ACKR2-/- mice, by 12 weeks, when TEBs have regressed and ductal
124 outgrowth is completed, branch area and ductal elongation is unaltered between WT and
125 CCR1 -/- mice (Fig. 1c).

126 Of note, in contrast to ACKR2-/- mice, no difference was observed in the density of,
127 or distance between, branches, measured within a 5 x field of view (F.O.V.) between WT
128 and CCR1-/- mice at any of the time points investigated (Supplementary Fig. 2). This reveals
129 that CCR1 does not regulate the density, but the spread of the network. CCR3-/- mice
130 display reduced distance between branches at 7 weeks, suggesting that ACKR2 and CCR3
131 may regulate the proximity of branches within mammary gland. Considerable data support
132 potential redundancy in roles for iCCRs in vivo. Therefore, to assess redundant and
133 combinatorial effects of multiple chemokine receptors, whole mounts were obtained from
134 iCCR-/- mice which have a compound deletion of the entire iCCR locus encoding CCR1,

135 CCR2, CCR3, CCR5 [20]. As observed in the absence of CCR1, iCCR-/- mice display delayed
136 development at 7 weeks with reduced TEB number (Supplementary Fig. 1). No additional
137 combinatorial effects were observed.

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

141 To investigate the involvement of chemokines in regulating mammary gland development
142 through ACKR2 and CCR1, multiplex protein analysis of mammary gland lysates was carried
143 out. In the absence of scavenging by ACKR2, inflammatory chemokines accumulate, and
144 can recruit immune cells. Previously we showed that ACKR2 limits the levels of inflammatory
145 chemokine ligands CCL7, CCL11 and CCL12 in the mammary gland at 6.5 weeks[11]. Here
146 we extend these observations and reveal that, in addition to CCL7, CCL11 and CCL12, the
147 ACKR2 ligands, CCL3, CCL19 and CCL22 are also elevated in the ACKR2 -/- mammary
148 gland at 7 weeks (Fig. 2a). In CCR1-/- mice, the levels of CCL7, CCL11 and CCL12 are
149 unchanged, indicating that ACKR2 is functional in these mice and able to scavenge
150 chemokines normally. As shown previously[11], CCL2, which is the key chemokine controlling
151 lymphatic development in embryonic skin, and CCL5 are unchanged in the ACKR2-/-
152 mammary gland (Fig. 2a). In ACKR2-/- and CCR1-/- mice, the levels of CCL3, CCL19 and
153 CCL22 are higher and lower respectively, which could reflect altered numbers of chemokine
154 expressing immune or epithelial cells. Similarly, the chemokines CXCL1 and CXCL12 are
155 increased in ACKR2-/- mice and decreased in CCR1-/- mice (Fig. 2b), and as these are not
156 ACKR2 ligands, this likely reflects the altered extent of branching morphogenesis.
157 Bioinformatic interrogation of publicly available mammary gland epithelial single cell RNAseq
158 data revealed that CXCL1 and CXCL12 are produced by basal and myoepithelial cells
159 (Supplementary Fig. 3)[21]. Importantly, there were no significant differences in the level of
160 chemokines in lysates obtained from male WT and ACKR2-/- fat pads, indicating that the

161 changes observed in the female lysates are specifically associated with the mammary gland
162 (Supplementary Fig. 4). Overall, chemokine levels are significantly altered in the absence of
163 both ACKR2 and CCR1 in the mammary gland.

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

165 Previously, we showed that ACKR2 is expressed by stromal fibroblasts in the developing
166 virgin mammary gland, and is markedly upregulated during late puberty at 6.5 weeks[11]. To
167 identify the cell type(s) expressing CCR1, mammary glands were obtained from 7 weeks old
168 WT and CCR1-/- mice, enzymatically digested and analysed by flow cytometry. Antibodies
169 against chemokine receptors often exhibit cross reactivity, therefore CCR1-/- cells were
170 included as a control. CCR1 is expressed by mammary gland macrophages, defined as
171 CD45+SiglecF-CD11b+F4/80+ cells, as the mean fluorescence intensity (MFI) of CCR1 was
172 significantly higher in WT than CCR1-/- cells (Fig. 3a). This is consistent with our previous
173 data which showed high levels of CCR1 transcription by isolated F4/80+ mammary gland
174 cells[11]. In addition, CCR1 expression by both human and mouse macrophages has been
175 well described[22–24]. Stromal and epithelial (CD45-) cells and eosinophils (CD45+ SiglecF+)
176 do not express CCR1 (Fig. 3a).

177 To further validate expression, and to identify the location of ACKR2+ and CCR1+ cells
178 within the mammary gland, RNAscope® in situ hybridisation was carried out, which detects
179 target RNA within intact cells. As shown in Fig. 3b, clear in situ hybridisation signals for both
180 CCR1 and ACKR2 were seen associated with ductal epithelium in WT but not CCR1-/- or
181 ACKR2-/- glands. Therefore, in situ hybridisation confirms stromal expression of ACKR2
182 surrounding the TEB, and CCR1 in close proximity to ductal epithelium, in the mammary
183 gland.

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186 **Estrogen levels increase in late puberty and induce CCR1 expression on**
187 **macrophages.**

188 ACKR2 expression in the mammary gland peaks at the defined time point of 6.5 weeks[11]. In
189 ACKR2-/- mice, branching begins to accelerate at that time point. One key change that
190 occurs around 6 weeks is the onset of sexual maturity[25]. To investigate whether there were
191 changes in the sex hormone estrogen at this time point, plasma levels of the main estrogen
192 estradiol were measured by ELISA. In WT mice, estradiol levels increase between 6.5 and 7
193 weeks (Fig. 4a). Notably, there is no difference between the levels of estradiol in WT and
194 ACKR2-/- mice, suggesting that the accelerated branching in ACKR2-/- is not directly caused
195 by increased levels of estrogen.

196 A previous study described upregulation of CCR1 on T cells in response to 17β-
197 estradiol[26]. To investigate if estrogen alters CCR1 expression on macrophages, cells
198 obtained from enzymatically digested mammary glands were exposed to DMSO (vehicle
199 control) or 17β-estradiol for 1h at 37°C and analyzed by flow cytometry. CCR1 expression
200 on CD45+ CD11b+F4/80+ macrophages increased in response to 17β-estradiol (Fig. 4b).
201 There was no significant difference between the level of CCR1 expression on WT and
202 ACKR2-/- after exposure, indicating that ACKR2 is not required for this process. To
203 determine whether another cell type present in the mammary gland was required for this
204 process, CD11b+F4/80+ cells were isolated by FACS. In the absence of other cell types,
205 CCR1 expression was increased after exposure to 17β-estradiol indicating that this is a
206 macrophage intrinsic mechanism (Supplementary Fig. 5c).

207 Notably, upregulation of CCR1 in response to estradiol is age dependent, as there is
208 no difference in CCR1 expression in mice older than 8 weeks (Supplementary Fig. 5a). In
209 addition, 17β-estradiol has no effect on macrophages isolated from the male fat pad or the
210 peritoneum of pubertal female mice (Supplementary Fig. 5a-b). Taken together, this
211 suggests that the effect of estrogen on CCR1 expression is restricted to pubertal mammary
212 gland macrophages.

213 **Macrophage dynamics are coordinated by CCR1 and ACKR2.**

214 ACKR2 is a key regulator of macrophage dynamics in both the developing lymphatic system
215 of embryonic skin, and the pubertal mammary gland[11,12]. To investigate the effect of CCR1
216 deficiency on macrophage levels in the mammary gland, flow cytometry of enzymatically
217 digested 6.5 weeks old WT and CCR1-/- glands was carried out. The gating strategy
218 employed is described in Supplementary Fig. 6. Unlike ACKR2, CCR1 deficiency had no
219 significant effect on the bulk macrophage population (CD45+CD11b+F4/80+) (Fig. 5a-b i),
220 however there was a significant decrease in the percentage of a small population of CD206+
221 macrophages (CD45+SiglecF-F4/80+CD206+) in CCR1-/- mice (Fig. 5a-c ii). The opposite is
222 observed in ACKR2-/- mice, with an increased percentage of CD206+ macrophages. Within
223 the CD11b+F4/80+ gate there is considerable heterogeneity, including a population of
224 CD11b+F4/80+SiglecF+ cells which are increased in ACKR2-/- glands but unaltered in the
225 absence of CCR1. The percentage of SiglecF+ eosinophils are unchanged between WT and
226 ACKR2-/- or CCR1-/- glands (Fig. 1a-c iii-iv). This indicates that the increase observed in the
227 bulk CD11b+ F4/80+ population previously reported in ACKR2-/- mice is unlikely to be key
228 for ACKR2 and CCR1 controlled mammary gland regulation. Instead, a small population of
229 CD206+ macrophages are co-ordinately regulated by ACKR2 and CCR1. Recently, CD206+
230 mammary gland macrophages which are derived in the foetus, have been identified and are
231 thought to promote branching morphogenesis[27]. In *plvap*-/- mice which have reduced
232 numbers of foetal liver-derived macrophages, including CD206+ macrophages, branching is
233 severely impaired[27]. We propose that ACKR2 and CCR1 reciprocally control this population
234 to coordinate branching morphogenesis in the pubertal mammary gland. CCR1 expression
235 was also increased on the surface of CD206+ macrophages in response to both 17β-
236 estradiol and the Estrogen mimic Bisphenol A (BPA) (Fig. 4b ii).

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239 **CCL7 controls CD206+ macrophages and branching morphogenesis.**

240 As CCL7 is a shared ligand between CCR1 and ACKR2 (Fig. 1a), which is elevated at both
241 6.5 and 7 weeks in the mammary gland (Fig. 2a)[11], we investigated its expression and
242 function. Using flow cytometry, intracellular staining revealed that CCL7 is produced by
243 immune cells, including SiglecF+ eosinophils, SiglecF- F4/80+ macrophages, and SiglecF-
244 Ly6C+ monocytes (Fig. 6a i). For each cell type, a markedly higher percentage of cells
245 produced CCL7 in female mammary gland, than male fat pad cells. In particular, around
246 60% of female SiglecF+ cells produced CCL7. In addition, qRT-PCR analysis of purified
247 F4/80 cells showed that CCL7 is transcribed at high levels (Fig. 6a ii). CCL7 is also
248 produced by CD45- epithelial cells: mature (EpCAM+ CD49f-) and progenitor luminal
249 (EpCAM+ CD49f+), and basal (EpCAM - CD49f+) cells (Fig. 6a iii). Further, bioinformatic
250 analysis revealed that CCL7 is produced by epithelial cells, including basal luminal and
251 myoepithelial cells (Supplementary Fig. 3). The percentage of CCL7+ immune and epithelial
252 cells was unaffected in the absence of ACKR2 (Fig. 6a i, iii).

253 To investigate the effect of elevated chemokine levels in vivo, either PBS or 2μg of
254 CCL7 was administered subcutaneously at the site of the mammary fat pad at the key time
255 point of 6 weeks. After 3 days, mammary glands were harvested for cellular analysis by flow
256 cytometry and carmine alum whole mount analysis. CCL7 administration alone was sufficient
257 to increase the percentage of CD206+ macrophages, and the area of branching within the
258 mammary gland (Fig. 6 b). This confirms that elevated levels of CCL7, as observed in
259 ACKR2-/- mice, leads to increased numbers of CD206+ macrophages in the mammary
260 gland and accelerated branching.

261 Thus, we propose that ACKR2 and CCR1 co-ordinately regulate the rate of branching
262 morphogenesis in the pubertal mammary gland, whereby estrogen increases CCR1
263 expression on macrophages, and stromal ACKR2 modulates levels of CCL7 to control the
264 movement of CCR1+ macrophages to the ductal epithelium. This proposed mechanism is
265 outlined as a schematic in Fig. 7.

266

267 **Discussion**

268 The importance of macrophages in controlling developmental processes is well known[7]. The
269 role of chemokines and their receptors, which provide molecular cues to guide and position
270 macrophages during development, is an emerging area of research[11,12]. Previously, we
271 revealed that the scavenging atypical chemokine receptor, ACKR2 controlled macrophages
272 in the mammary gland through a CCR2 independent mechanism[11]. This was unexpected, as
273 ACKR2 control of embryonic lymphatic development is dependent on CCR2+ macrophages
274 sensing levels of their shared ligand, CCL2[12]. Here we have revealed a previously unknown
275 immunological mechanism whereby, ACKR2 and the inflammatory chemokine receptor
276 CCR1, signal through their shared ligand CCL7 to coordinate the levels of a CD206+
277 macrophage population, and thus, the extent of branching morphogenesis in the pubertal
278 mammary gland. Importantly, administration of CCL7 alone was able to increase the
279 percentage of CD206+ macrophages within the pubertal mammary gland and drive
280 accelerated branching morphogenesis. This adds further evidence to support our hypothesis
281 that elevated levels of CCL7 in ACKR2-/- glands leads to increased macrophage
282 recruitment. We propose that in CCR1-/- mice, although CCL7 levels are unaltered,
283 macrophages are unable to sense and respond to the ligand without the cognate receptor,
284 leading to delayed branching (Fig. 7).

285 Previously, transcriptional analysis of mammary gland macrophages demonstrated
286 high expression of CCR1, CCR2 and CCR5, and low level expression of CCR3[11].
287 Interactions between inflammatory CC-chemokines and their receptors are known to be
288 highly complex, with the potential for redundancy[13,14] (Fig. 1a). This suggested that
289 macrophages could use a variety of chemokines and cognate receptors for movement into,
290 and within, the mammary gland. Instead, we describe here a highly specific interaction
291 between the scavenging atypical chemokine receptor ACKR2, inflammatory chemokine
292 receptor CCR1 and their shared ligand CCL7. These key chemokine receptors are located

293 on a tight chromosomal locus[28]. Compound chemokine receptor-null (iCCR-/-) mice have
294 recently been generated which target this locus[29]. Branching morphogenesis was analysed
295 in these mice and did not reveal any additional combinatorial effects, indicating that CCR1
296 deficiency, specifically, is responsible for delayed puberty.

297 Previously, it was thought that all mammary gland macrophages, at rest, and in
298 pathology, were derived from the bone marrow[30]. In our previous study, we showed that
299 branching was unaltered in the absence of CCR2, indicating that the macrophage population
300 responsible for promoting branching morphogenesis was unlikely to be bone marrow
301 derived[11]. Recently, a novel CD206+ macrophage population has been identified in the
302 mammary gland, which is unaffected in the absence of CCR2, but reduced in *plvap*-/- mice,
303 which have reduced numbers of foetal liver-derived macrophages[27]. Branching is severely
304 impaired in these mice suggesting that foetal derived macrophages play a key role in
305 promoting branching morphogenesis[27]. We believe that the macrophage population
306 identified in our study may be derived from the embryonic population described by Jappinen
307 et al, 2019[27].

308 CCR1 is an inflammatory chemokine receptor which is expressed by immune cells,
309 and has been shown to be important in a number of pathologies, including: sepsis, viral
310 infection, cancer and autoimmune disease[23,31–33]. To our knowledge, this is the first
311 description of a key role for CCR1 in normal development. Of note, in the placenta, CCR1
312 has been shown to be expressed by human trophoblasts as they switch to an invasive
313 phenotype[34]. ACKR2 is highly expressed by placental trophoblasts, preventing excess levels
314 of inflammatory chemokines from entering the foetus, from the mother's circulation, by a
315 process of chemokine compartmentalisation[35,36]. As CCR1 expression has also been
316 described in placental development, there could be wider applications of the ACKR2 and
317 CCR1 interaction described here.

318 In this study, we reveal the location of ACKR2+ cells beside the TEB. CCR1+
319 macrophages were identified by flow cytometry and the location of CCR1+ cells, tightly

320 associated with the ductal epithelium, was revealed. This suggests that CCR1+
321 macrophages directly interact with the ductal epithelium. Cross talk between immune cells,
322 the stroma and epithelium is highly important In the mammary gland, as disruption leads to
323 disordered development[37]. We reveal further information on detailed communication
324 between ACKR2+ stromal fibroblasts, CCR1+ macrophages and CCL7 producing epithelial
325 cells, through chemokine and receptor interactions.

326 ACKR2 expression in the mammary gland specifically peaks at 6.5 weeks[11]. In
327 ACKR2-/- mice, branching begins to accelerate at that time point. One significant event that
328 occurs around 6 weeks is the onset of sexual maturity[25]. We investigated whether there were
329 changes in the sex hormone estrogen at this key time point, and showed that estradiol levels
330 in mouse plasma are markedly increased between 6.5 and 7 weeks. Plasma estrogen levels
331 are not altered in WT or ACKR2-/- mice, indicating that increased estrogen does not explain
332 the acceleration of branching. However, estrogen does increase CCR1 expression on
333 macrophages. This upregulation in response to 17β-estradiol is restricted to pubertal
334 mammary gland macrophages, as older females, males and peritoneal macrophages do not
335 respond. In addition to 17β-estradiol, the estrogen mimic, Bisphenol A also increased CCR1
336 expression on CD206+ macrophages. This may be of concern as BPAs are widely found in
337 the environment and could potentially alter the immune response and the extent of
338 branching of the developing mammary gland, in children during puberty. Previously CCR1
339 expression on T cells was shown to be regulated by 17β-estradiol[26]. This is the first
340 description of estrogen controlled CCR1 expression on macrophages. This observation
341 could have implications for our understanding of diseases where females are more
342 susceptible than men. One such example is rheumatoid arthritis, where CCR1 is also
343 associated with pathology[8].

344 Understanding the molecular signals which guide the rate of branching
345 morphogenesis in the mammary gland is highly important. Precocious puberty is a condition
346 where puberty begins before the age of 8, with some girls developing breasts as early as

347 age 4. This results from early activation of the gonadotropic axis, leading to accelerated
348 growth and bone maturation, but ultimately reduced stature[38]. Potential causative factors
349 include exposure to endocrine disrupters, obesity, stress and ethnicity[39–42]. As puberty is
350 delayed in mice in the absence of CCR1, this could represent a novel therapeutic target to
351 treat precocious puberty. Several CCR1 antagonists are available and have been used in a
352 number of clinical trials[43]. Strikingly, bioinformatically interrogation of the precocious puberty
353 (CTD Gene-Disease Associations) dataset, using Harmonizome[44], revealed that CCL7 and
354 ACKR2 are both associated with precocious puberty in children, with standardized values of
355 1.25588 (p=0.09) and 1.02634 (p=0.011) respectively. In addition, early breast development
356 leads to higher risks of breast cancer in later life[4], and women with dense breasts are more
357 likely to develop breast cancer[45]. This can be related to poor detection by mammography as
358 the branches mask the cancer, but may also be caused by genetic factors, parity and
359 alterations in the breast stroma. Both ACKR2 and CCR1 have been shown to be important in
360 the progression of breast cancer, therefore understanding early interactions between these
361 receptors could reveal key insights, which drive later pathology[23,29,46].

362 In this study, we have uncovered a novel immunological mechanism by which estradiol
363 upregulates CCR1 expression by pubertal mammary gland macrophages, and stromal
364 ACKR2 modulates levels of CCL7, to control the movement of CCR1+ macrophages to the
365 ductal epithelium and drive branching morphogenesis.

366

367 **Methods**

368 **Animals**

369 Animal experiments were carried out under the auspices of a UK Home Office Project
370 Licence and conformed to the animal care and welfare protocols approved by the University
371 of Glasgow. C57BL/6 mice, ACKR2-/-[47], CCR1-/-, CCR3-/-, CCR5-/- and iCCR-/-[29] mice
372 were bred at the specific pathogen-free facility of the Beatson Institute for Cancer Research.

373 **Carmine Alum Whole Mount**

374 Carmine alum whole mounts were carried out as described previously[11]. Briefly, fourth
375 inguinal mammary glands were fixed overnight in 10% neutral buffered formalin (NBF)
376 (Leica) at 4°C. Glands were dehydrated for 1 h in distilled water, followed by 70% ethanol
377 and 100% ethanol before overnight incubation in xylene (VWR international). Tissue was
378 rehydrated by 1 h incubation in 100% ethanol, 70% ethanol and distilled water, before
379 staining in Carmine Alum solution overnight at room temperature (0.2% (w/v) carmine and
380 10 mM aluminium potassium sulphate (Sigma)). Tissue was dehydrated again before
381 overnight incubation in xylene. Finally, glands were mounted with DPX (Leica) and stitched
382 bright-field images at 10× magnification were taken using an EVOS FL auto2 microscope
383 (Thermofisher). Ductal elongation, and branched area from the lymph node, were measured
384 using ImageJ 1.52a[48]. 5 x brightfield images were obtained using the Zeiss Axioimager M2
385 with Zen 2012 software. The numbers of branches, average distance between branches and
386 branch thickness were counted as the average from 3 measurements from 6 individual fields
387 of view (F.O.V.) from each whole mount. TEBs were counted as the average from at least 2
388 F.O.V. from each whole mount. All samples were blinded before measurements were taken.

389 **RNAscope ® In situ hybridisation**

390 Mammary glands were fixed in 10% neutral buffered formalin at room temperature for 24-36
391 hours before being dehydrated using rising concentrations of ethanol and xylene, and
392 paraffin embedded (Shandon citadel 1000 (Thermo Shandon). Tissue was sectioned onto
393 Superfrost plus slides (VWR) at 6 µm using a Microtome (Shandon Finesse 325 Microtome,
394 Thermo). Slides were baked at 60°C for 1 h before pre-treatment. Slides were deparaffinised
395 with xylene (5 mins x 2) and dehydrated with ethanol (1 min x 2). Tissues were incubated
396 with Hydrogen peroxide for 10 mins at RT, then boiled in antigen retrieval buffer for 15 mins.
397 Slides were treated with protease plus for 30 mins at 40°C. Slides were then hybridised
398 using the RNAScope® 2.5 Red Manual Assay (Advanced cell diagnostics) according to the

399 manufacturer's instructions using the Mm-Ccr1 and Mm-ACKR2 probes. Slides were
400 mounted in DPX (Sigma Aldrich) and imaged on an EVOS FL Auto2microscope.

401 **Mammary gland digestion**

402 The inguinal lymph node was removed from the fourth inguinal mammary gland, tissue was
403 chopped, and enzymatic digestion was carried out in a 37°C shaking incubator at 200 rpm
404 for 1 h, with 3 mg/ml (w/v) collagenase type 1 (Sigma) and 1.5 mg/ml (w/v) trypsin (Sigma) in
405 2 ml Leibovitz L-15 medium (Sigma). The suspension was shaken for 10 s before addition of
406 5 ml of L-15 medium supplemented with 10% foetal calf serum (Invitrogen) and
407 centrifugation at 350g for 5 min. Red blood cells were lysed using Red Blood Cell Lysing
408 Buffer Hybri-Max (Sigma) for 1 min and washed in PBS. Cells were washed in PBS with 5
409 mM EDTA, resuspended in 2 ml 0.25% Trypsin-EDTA (Sigma) and incubated at 37°C for 2
410 min before addition of 5 ml of serum-free L-15 containing 1 µg/ml DNase1 (Sigma) for 5 min
411 at 37°C. L-15/10% FCS was added to stop the reaction and cells were filtered through a 40
412 µm cell strainer before a final wash in FACS buffer (PBS containing 1% FCS and 5 mM
413 EDTA).

414 **Flow cytometry**

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

423 **Proteomic analysis**

424 The inguinal lymph node was removed from the fourth inguinal mammary gland, tissue was
425 chopped, frozen in liquid nitrogen, crushed with a mortar and pestle, and resuspended in
426 dH2O containing protease inhibitors (Pierce). Protein levels were determined using a custom
427 designed Magnetic Luminex Multiplex assay (R&D Systems), as described in the
428 manufacturer's instructions, and read with a Bio-Rad Luminex-100 machine. Data was
429 normalised to the protein concentration of tissue samples, determined by a BCA assay
430 (Pierce).

431

432 **Subcutaneous administration of CCL7**

433 2µg of CCL7 in 200 µl PBS (R&D Systems) was injected subcutaneously into mice at 6
434 weeks of age. After 3 days, mice were culled and mammary glands were excised and
435 processed for whole mount and cellular analysis.

436

437 **17β-estradiol assays**

438 Fourth inguinal mammary glands were digested to obtain single cell suspensions. Cells were
439 plated at 0.5-1 x 10^5 cells in a 96 well plate in L-15 media containing 5% FCS, and exposed
440 to DMSO (vehicle control) or 50 ug/ml 17-β estradiol or Bis-phenol A (Sigma) for 1 h at 37°C,
441 5% CO_2. The level of 17-β estradiol in plasma samples was determined using the Estradiol
442 parameter kit (R&D Systems) as described in the manufacturer's instructions.

443

444 **Bioinformatic analysis**

445 Chemokine expression by epithelial cells was determined by searching the data repository
446 from Bach et al, 2017 [21]at: https://marionilab.cruk.cam.ac.uk/mammaryGland/.

447

448 **Statistical analysis**

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

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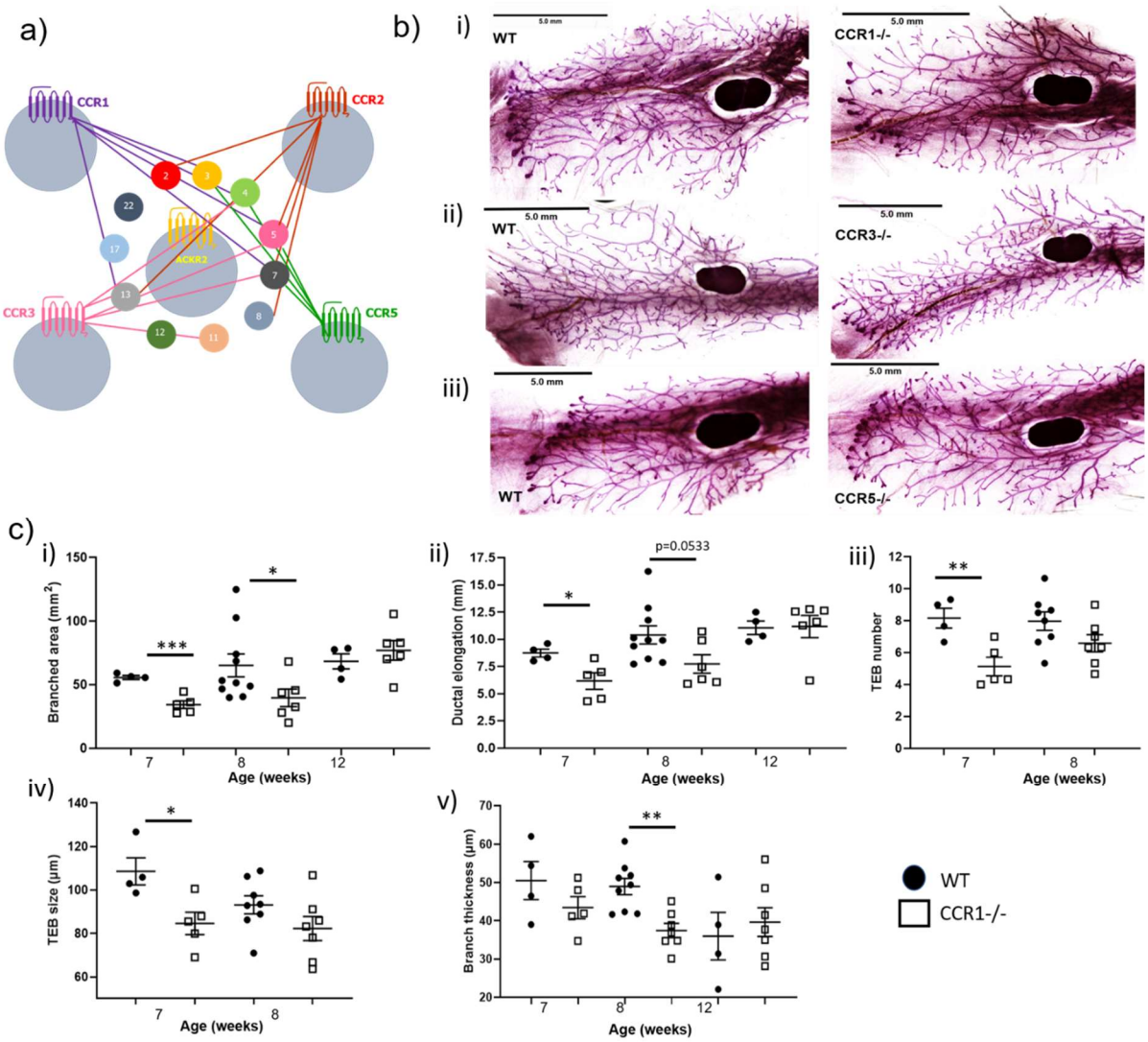
459 Society Merit award.

460 **Competing Interests**

461 The authors declare no competing interests

462 **Author Contributions**

463 GJW conceived the study, performed experiments, analysed data and wrote the paper. AF
464 performed experiments and analysed data. SRL, JK, MP and AJH performed experiments.
465 GJG conceived the study and wrote the paper.



466

467 **Figure 1: CCR1 controls mammary gland branching morphogenesis during puberty. a)**
468 ACKR2 shares ligands with inflammatory chemokine receptors. Data compiled from
469 Bachelerie, F. *et al.,* 2014 and Nibbs, R. J. B. & Graham, G. J., 2013. **b)** Representative
470 carmine alum whole mount images of late pubertal (7 week old) virgin mammary glands from
471 i) wild-type and CCR1-/-, ii) CCR3-/- and iii) CCR5-/- mice. Scale bars: 5 mm. **c)** Branching
472 morphogenesis was quantified in 7 (WT n=4, CCR1-/- n= 5), 8 (WT n=10, CCR1-/- n= 7)and
473 12 (WT n=4, CCR1-/- n= 7) week mammary glands using ImageJ, by measuring: **i)** the area
474 of branching from the inguinal lymph node, and **ii)** ductal elongation, measured from the
475 middle of the inguinal lymph node to the furthest edge of ductal outgrowth. **iii)** The number of
476 TEBs, was determined as the average number from at least 2 individual fields of view (FOV)
477 (5×) per gland. **iv)** The average width of all TEBs was determined from at least 2 F.O.V (5x)

478 per gland. **v)** Branch thickness was determined as the average of 6 measurements from 3

479 F.O.V (5x) per gland. Error bars represent S.E.M.

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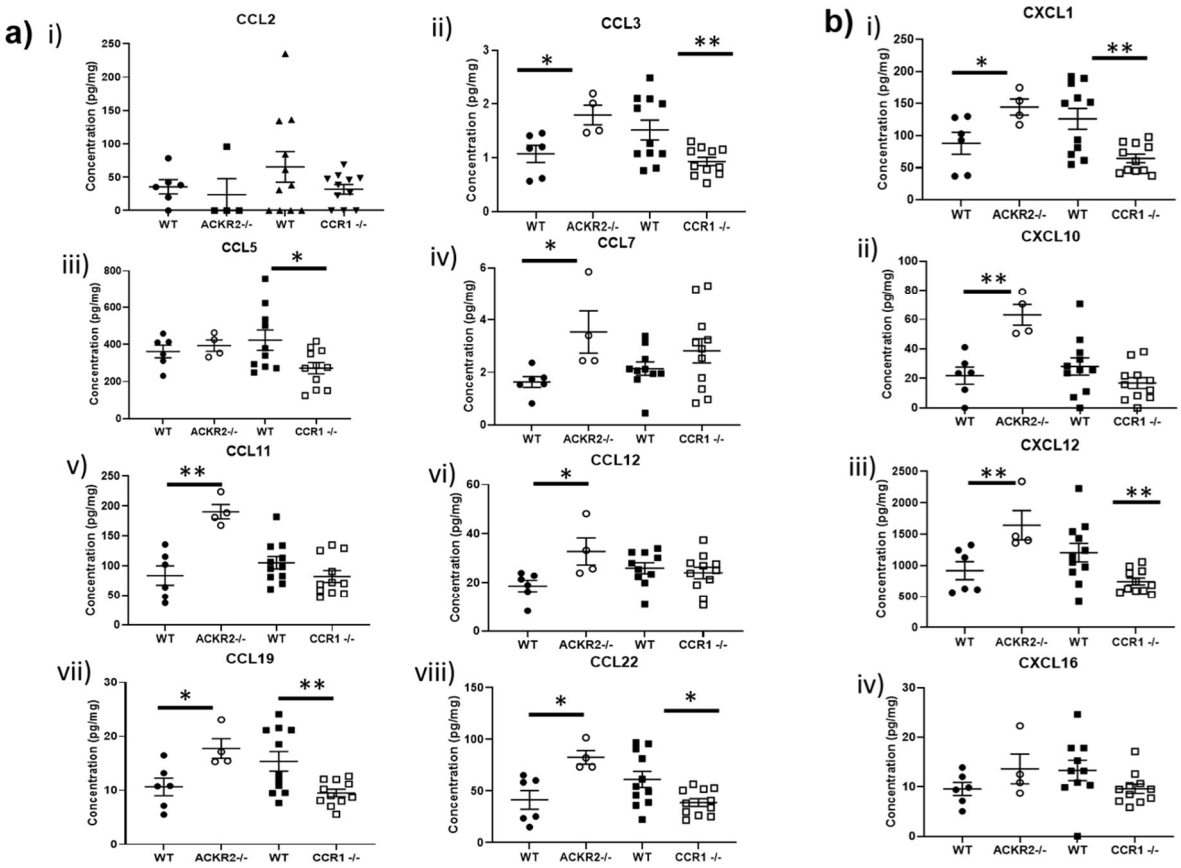


481

482 **Figure 2: Chemokine levels are altered in the absence of ACKR2 and CCR1** Multiplex

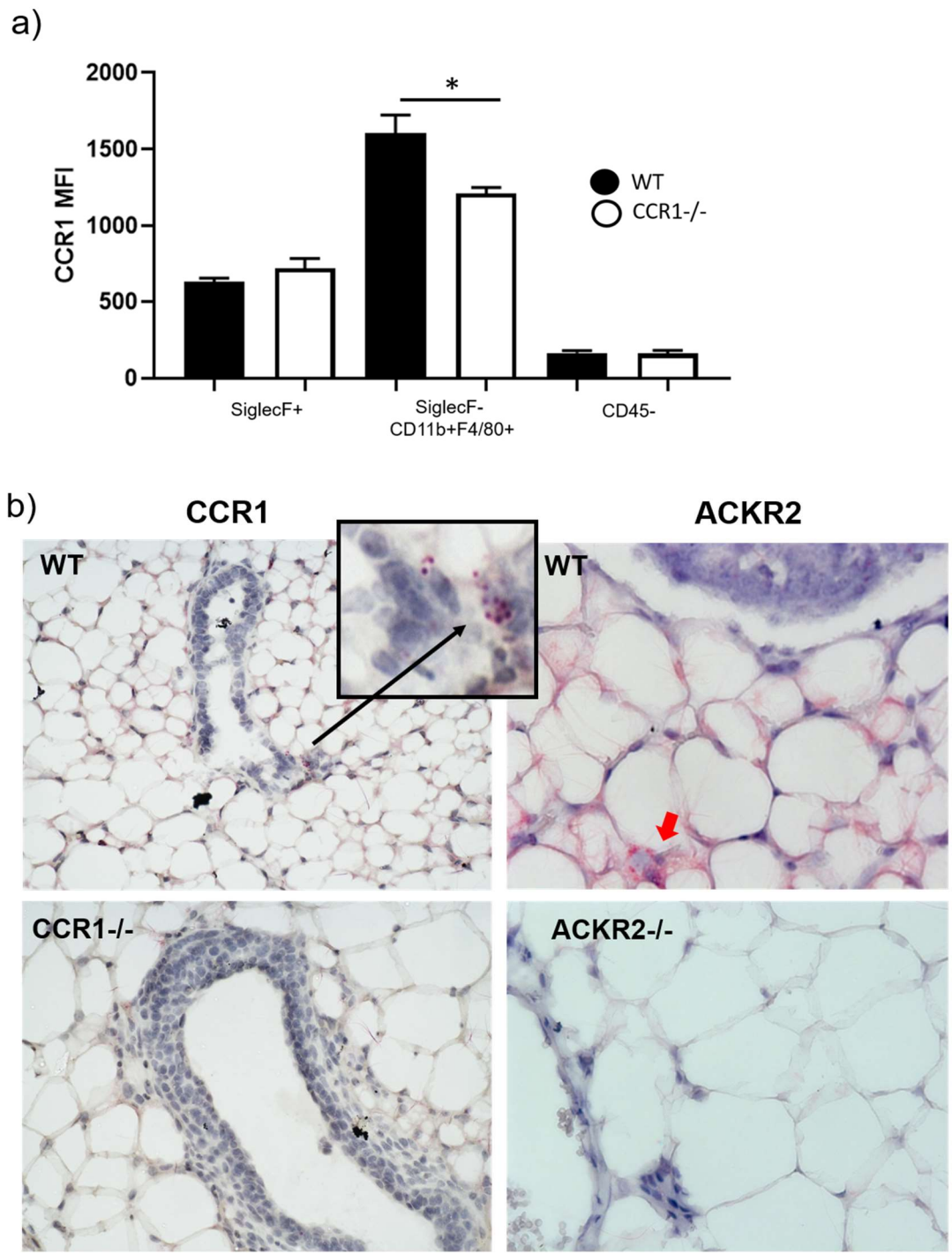
483 measurement of protein concentration of **a)** inflammatory chemokines; i) CCL2, ii) CCL3, iii)

484 CCL5, iv) CCL7, v) CCL11, vi) CCL12, vii) CCL19 and viii) CCL22, and; **b)** homeostatic

485 chemokines i) CXCL1, ii) CXCL10 iii) CXCL12 and iii) CXCL16 in whole mammary gland

486 homogenates. WT (CCR1) n=11, CCR1-/- n=11, WT (ACKR2) n=6, and ACKR2-/- n=4.

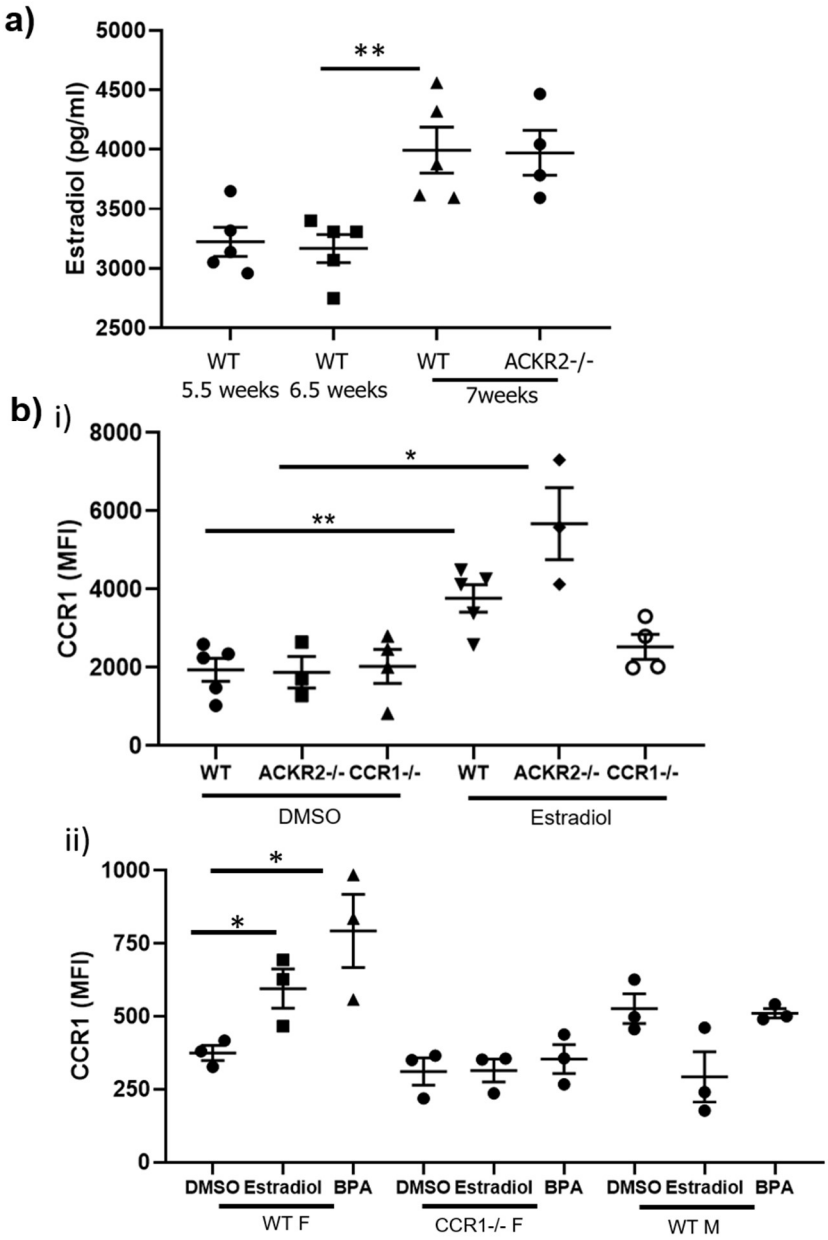
487 Significantly different results are indicated. Error bars represent S.E.M.



488

489 **Figure 3: CCR1 and ACKR2 are expressed in the mammary gland. a)** Flow cytometry
490 analysis of CCR1 expression by enzymatically digested WT (black bars, n=6) and CCR1-/-
491 (white bars, n=4) mammary gland cells: CD45-, CD45+ SiglecF+, and CD45+SiglecF-
492 CD11b+F480+. **b)** RNAscope® in situ hybridization of ACKR2 and CCR1 in the developing
493 virgin mammary gland of WT, CCR1-/- and ACKR2-/- mice. Significantly different results are
494 indicated. Error bars represent S.E.M.

495

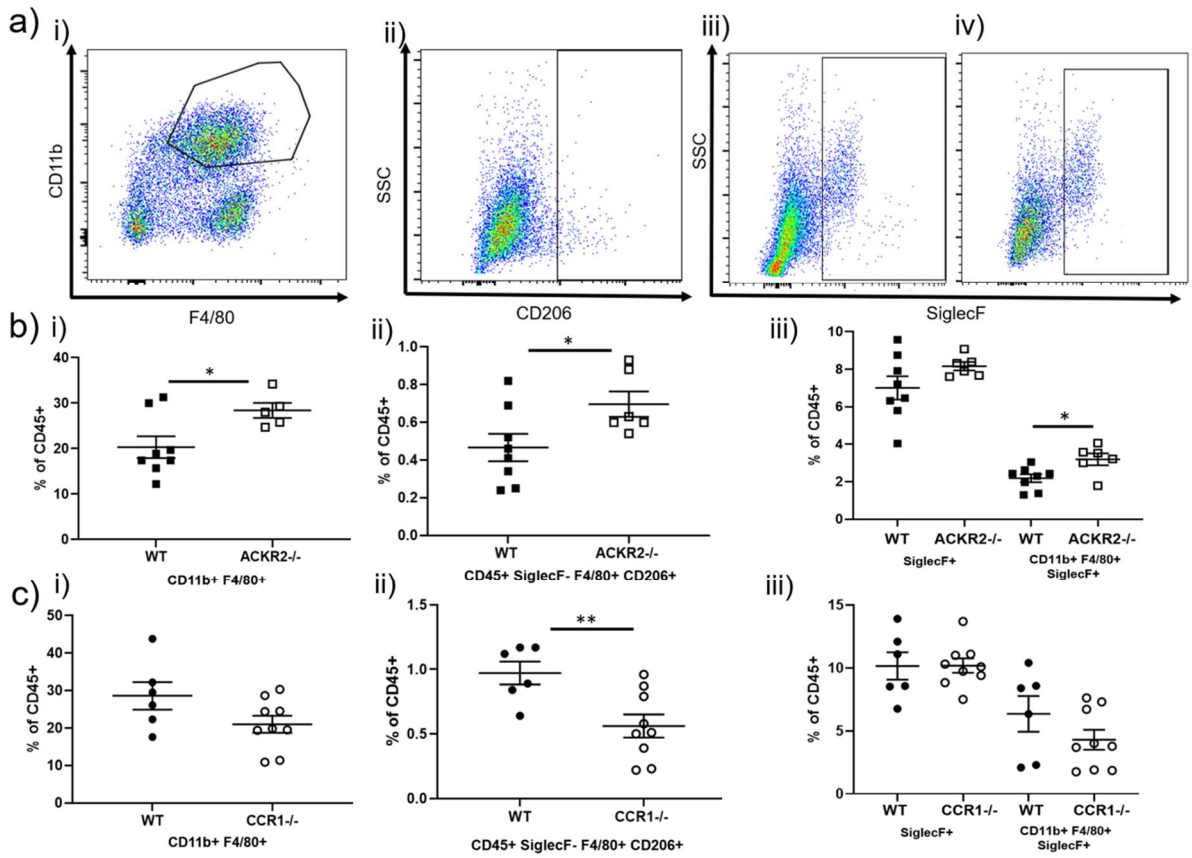


496

497 **Figure 4: Estrogen levels increase in late puberty and induce CCR1 expression on**
498 **macrophages. a)** Estradiol levels in plasma from 5.5 weeks WT (n=5), 6.5 weeks WT (n=5),
499 7 weeks WT (n=5), and 6.5 weeks ACKR2-/- (n=4). **b)** CCR1 expression by CD11b+F4/80+
500 cells in response to DMSO and 50 µg/ml estradiol in WT (n=5), CCR1-/- (n=4),and ACKR2-/-
501 (n=3), **c)** CCR1 expression by SiglecF- F4/80+ CD206+ cells in response to DMSO and 50

502 μg/ml 17β-estradiol and Bisphenol A; female WT and CCR1-/- and male WT (n=3).

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



504

505 **Figure 5: Macrophage dynamics are coordinated by CCR1 and ACKR2. a)** Flow

506 cytometry was used to determine the percentage of i) CD11b+F4/80+ and ii) SiglecF-

507 F4/80+ CD206+ macrophages, iii) SiglecF+ and CD11b+F4/80+ SiglecF+ cells within the

508 CD45+ compartment of the 6.5 weeks old developing mammary gland. Flow cytometry of **b)**

509 WT (n=8) and ACKR2-/- (n=5) and **c)** WT (n=6) and CCR1-/- (n=9) was carried out.

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

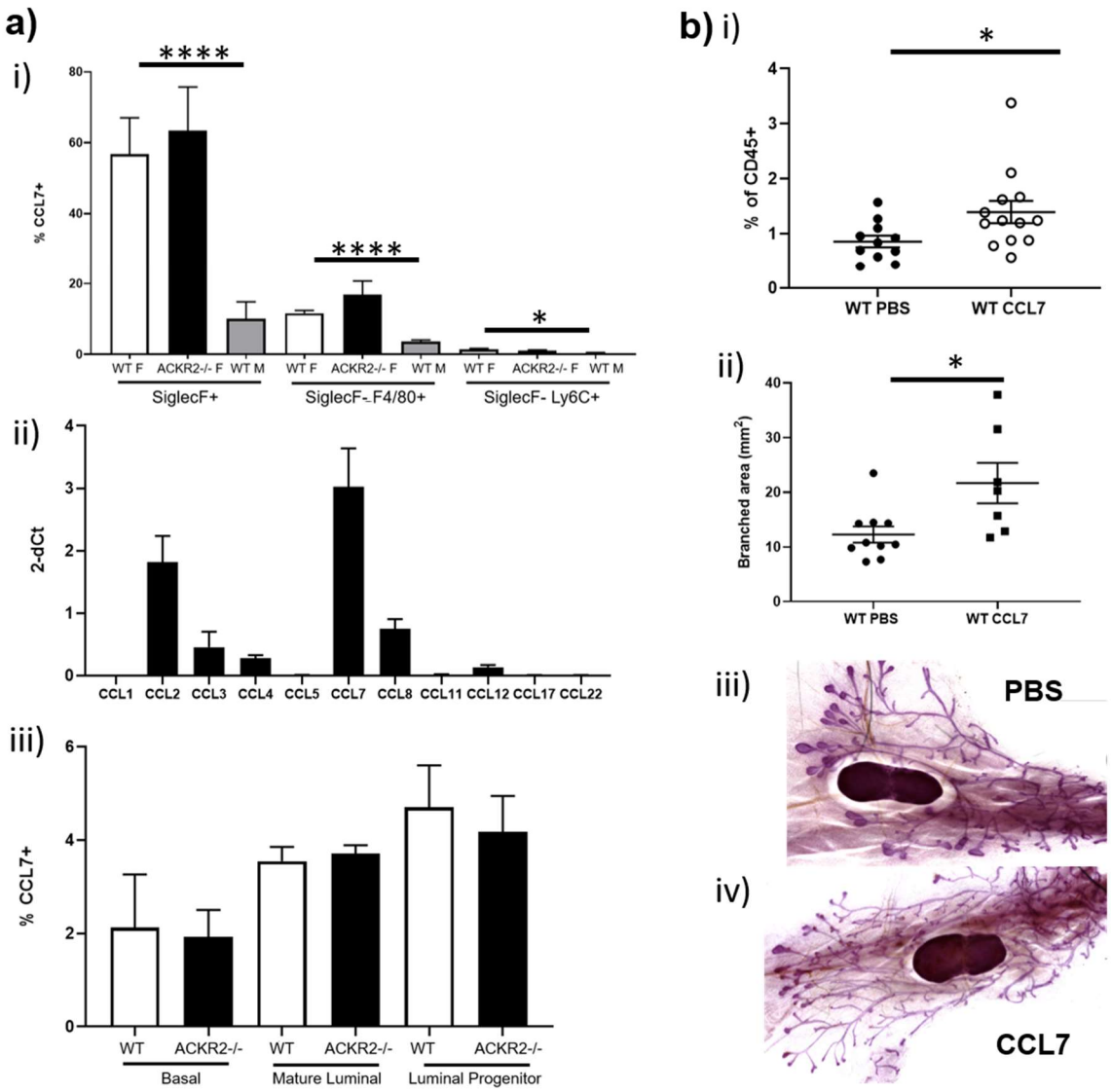
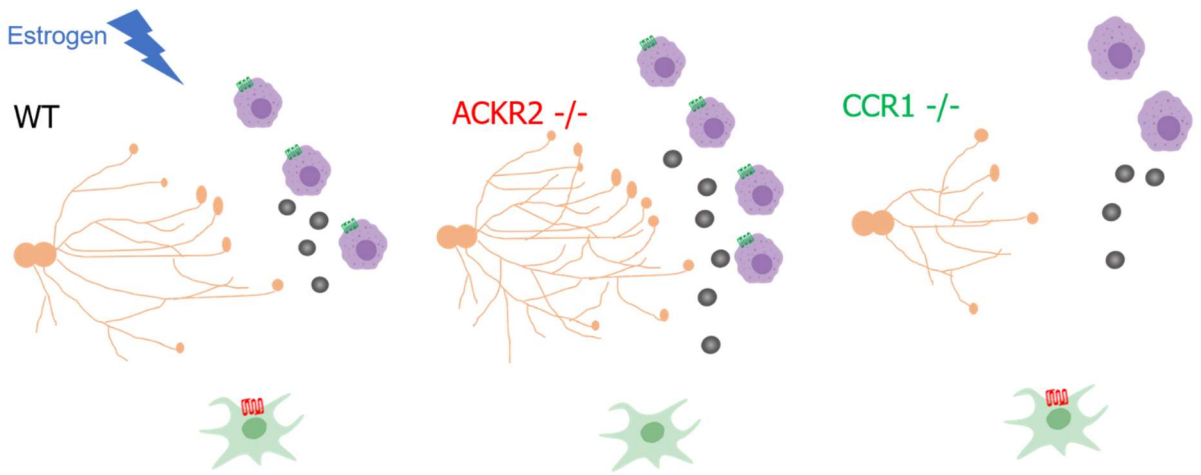


Figure 6: CCL7 controls CD206+ macrophages and branching morphogenesis. a) CCL7 is produced in the mammary gland by i) immune cells; including SiglecF+, SiglecF-F4/80+ and SiglecF-Ly6C+, male WT (n=7) and female WT (n=6) and ACKR2-/- (n=4). ii) Transcription of inflammatory chemokines by purified F4/80+ cells (n=3) and iii) CCL7 production by epithelial cells, Mature Luminal (ML, EpCAM+ CD49f-), progenitor luminal (EpCAM+ CD49f+), and basal (EpCAM- CD49f+), female WT (n=6) and ACKR2-/- (n=4). **b)** 3 days after subcutaneous administration of PBS or 2 µg CCL7 at 6 weeks, i) the percentage of SiglecF-F4/80+CD206+ cells was measured by flow cytometry. (PBS, n=11, CCL7, n=13) and ii) the area of branching was measured using Image J (PBS, n=10, CCL7, n=7). iii)

521 Representative images of whole mounts from PBS and CCL7 injected mice. Significantly
522 different results are indicated. Error bars represent S.E.M.

523



524

525

526 **Figure 7: Proposed mechanism by which chemokine receptors CCR1 and ACKR2**
527 **coordinate mammary gland development.** Estrogen increases CCR1 expression on
528 macrophages (purple) during puberty, and stromal fibroblast (green) expressed ACKR2
529 modulates levels of CCL7 (grey circles) to control the movement of CCR1+ macrophages to
530 the ductal epithelium (orange). Schematic image was created with BioRender.

531

532

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