

1 **ACKR2 IN HEMATOPOIETIC PRECURSORS AS A CHECKPOINT OF**
2 **NEUTROPHIL RELEASE AND ANTIMETASTATIC ACTIVITY**

3
4 Matteo Massara^{1,2,†}, Ornella Bonavita^{1,2,†}, Benedetta Savino^{1,2,†}, Nicoletta
5 Caronni^{1,2,†}, Valeria Mollica Poeta^{1,3}, Marina Sironi¹, Elisa Setten^{1,2}, Camilla
6 Recordati⁴, Laura Crisafulli^{1,5}, Francesca Ficara^{1,5}, Alberto Mantovani^{1,3,6}, Massimo
7 Locati^{1,2}, Raffaella Bonecchi^{1,3*}

8
9 ¹Humanitas Clinical and Research Center, via Manzoni 56, 20089, Rozzano (MI),
10 Italy; ²Department of Medical Biotechnologies and Translational Medicine, Università
11 degli Studi di Milano, Via Fratelli Cervi, 93, 20090 Segrate (MI), Italy; ³Department of
12 Biomedical Sciences, Humanitas University, Via Rita Levi Montalcini, 20090, Pieve
13 Emanuele (MI), Italy; ⁴Fondazione Filarete, viale Ortles 22/4, 20139, Milano, Italy;
14 ⁵Milan Unit, Istituto di Ricerca Genetica e Biomedica, CNR, via Manzoni 56, 20089
15 Rozzano (MI); ⁶ The William Harvey Research Institute, Queen Mary University of
16 London, Charterhouse Square, London EC1M 6BQ, UK.

17
18 † = these authors equally contributed

19
20 *** Corresponding author:**

21 Raffaella Bonecchi
22 Humanitas Clinical and Research Center
23 Humanitas University
24 Via Manzoni 113
25 20089 Rozzano, Italy
26 Tel +39.02.8224.5117
27 E-mail: raffaella.bonecchi@humanitasresearch.it

28
29 **Running title:** ACKR2 and metastasis

30
31 **Keywords:** chemokines; neutrophils; inflammation and cancer; metastasis.

32
33

34 **ABSTRACT**

35

36 Atypical chemokine receptors (ACKRs) are regulators of leukocyte traffic,
37 inflammation and immunity. ACKR2 is a scavenger for most inflammatory CC
38 chemokines and is a negative regulator of inflammation. Here we report that ACKR2
39 is expressed in hematopoietic precursors and downregulated during myeloid
40 differentiation. Genetic inactivation of ACKR2 results in increased levels of
41 inflammatory chemokine receptors and release from the bone marrow of neutrophils
42 with increased anti-metastatic activity. In a model of NeuT-driven primary mammary
43 carcinogenesis ACKR2 deficiency is associated with increased primary tumor growth
44 and protection against metastasis. ACKR2-deficiency results in neutrophil-mediated
45 protection against metastasis in mice orthotopically transplanted with 4T1 mammary
46 carcinoma and intravenously injected with B16F10 melanoma cell lines. Thus,
47 ACKR2 is a key regulator (checkpoint) of mouse myeloid differentiation and function
48 and its targeting unleashes the anti-metastatic activity of neutrophils in mice.

49

50 INTRODUCTION

51

52 Chemokines are key components of tumor microenvironment^{1,2}. Production of
53 chemokines sustains unresolving chronic inflammation which promotes
54 carcinogenesis. Moreover, oncogene activation drives chemokine production and
55 leukocyte recruitment in tumors epidemiologically unrelated to inflammatory
56 conditions³.

57 The repertoire of chemokines produced in the context of neoplastic tissues
58 shapes the leukocyte infiltrate^{1,2}. For instance, CCL2 and CXCL8 and related CC and
59 CXC chemokines, are major determinants of macrophage and neutrophil recruitment,
60 respectively. CCL17 and CCL22 have been associated with recruitment of Th2 and
61 Treg cells which tame effective antitumor immunity. Conversely CXCL10 driven
62 recruitment of CD8 T cells and Th1 cells is a driver of antitumor resistance^{1,2,4}.

63 Chemokine and chemokine receptors are important determinants of invasion
64 and metastasis⁵. For instance, the chemokine repertoire expressed by tumor cells
65 impacts on secondary seeding at distant sites such as the brain or lymph nodes⁶.
66 Moreover inflammatory chemokines precondition the metastatic niche and drive
67 tumor cell seeding at sites of secondary localization⁷.

68 Canonical chemokine receptors are seven transmembrane G protein coupled
69 receptors. Mature leukocyte subsets express distinct repertoires of signaling
70 chemokine receptors⁸. Moreover, the chemokine system includes a limited set (four)
71 of atypical chemokine receptors (ACKR). ACKRs bind a broad panel of inflammatory
72 (ACKR1 and ACKR2) or homeostatic (ACKR3 and ACKR4) chemokines and regulate
73 ligand availability by acting as decoys, scavengers, transporters or depot⁹⁻¹¹.

74 ACKR2 (previously known as D6) acts as a decoy and scavenger for most
75 inflammatory CC chemokines and is expressed by lymphatic endothelial cells,
76 trophoblasts in the placenta and some leukocytes such as innate B cells and alveolar
77 macrophages¹¹. In line with its scavenger function, ACKR2 is a negative regulator of
78 inflammation at different anatomical sites¹¹. Inflammation is a key component of the
79 tumor microenvironment¹². Accordingly, genetic inactivation of ACKR2 unleashes
80 tumor promoting inflammation in the skin and GI tract¹³⁻¹⁵. Moreover, downregulation
81 of ACKR2 in transformed cells has been associated with tumor progression and
82 oncogene activation in Kaposi sarcoma¹⁶⁻¹⁸.

83 The present study stems from an unexpected observation. In line with

84 previous carcinogenesis results^{13,14}, in an oncogene driven mammary carcinoma
85 model we found that ACKR2-deficient mice show enhanced tumor growth at the
86 primary tumor site. In contrast, ACKR2 gene-targeted mice are protected against
87 metastasis. This unexpected finding, extended to transplanted tumors, prompted a
88 dissection of underlying mechanisms. We found that ACKR2 is expressed in
89 hematopoietic progenitor cells (HPCs) and that it serves as a key regulator
90 (checkpoint) of myeloid differentiation and function. Targeting ACKR2 unleashed the
91 anti-metastatic potential of neutrophils.

92

93 **RESULTS**

94

95 *Ackr2^{-/-} mice are protected against tumor metastasis*

96 In order to extend previous studies on ACKR2 in carcinogenesis, we crossed
97 Balb/c *Ackr2^{-/-}* mice with Balb/c *NeuT* mice, which overexpress the rat HER2 (Neu)
98 oncogene under the mouse mammary tumor virus (MMTV) promoter and
99 spontaneously develop mammary carcinomas closely recapitulating human breast
100 carcinogenesis¹⁹. We followed primary tumor development measuring time of
101 appearance and volume, and found that in *NeuT/Ackr2^{-/-}* mice tumor masses in
102 mammary glands developed earlier (Supplementary Fig. 1a) and reached higher
103 volumes as compared to *NeuT/Ackr2^{+/+}* mice (Fig. 1a). This result is in accordance
104 with previous reports showing that ACKR2 genetic deficiency results in increased
105 growth of primary tumors^{13,14}. Unexpectedly, lung analysis revealed less metastatic
106 lesions in *NeuT/Ackr2^{-/-}* mice as compared to *NeuT/Ackr2^{+/+}* mice (Fig. 1b and 1c).

107 In an effort to strengthen and extend these findings, the transplanted tumor
108 lines 4T1 (mammary carcinoma) and B16F10 (melanoma), were used. 4T1 tumor
109 cells were transplanted orthotopically, whereas B16F10 melanoma cells were
110 injected i.v. in a classic “artificial” hematogenous metastasis model (see below).

111 When 4T1 cells were injected into the mammary glands of WT and *Ackr2^{-/-}*
112 mice, no difference in primary tumor growth was detected (Fig. 1d), but again the
113 number of spontaneous lung metastasis was significantly lower in *Ackr2^{-/-}* mice (Fig.
114 1e and 1f). Since 4T1 cells expressed little or no *Ackr2*, before and after in vivo
115 growth (Supplementary Fig. 1B), these results suggested that the regulatory function
116 of ACKR2 on metastasis is not cancer cell-intrinsic.

117 In order to understand which cells protect mice from metastasis, BM chimeric
118 mice were orthotopically injected with 4T1 cells. Mice were protected from metastasis
119 only when *Ackr2* was genetically inactivated in the hematopoietic compartment
120 (Supplementary Fig. 1c), demonstrating that protection phenotype was due to
121 hematopoietic expression of ACKR2.

122

123 *Increased myeloid cell mobilization in Ackr2^{-/-} mice*

124 The two breast cancer models used in our experiments are known to induce
125 expansion and mobilization of myeloid cells, which then promote tumor growth^{20,21}.
126 Interestingly, when animals were challenged with the 4T1 sibling cell line 66cl4,

127 which in contrast is unable to induce myeloid cell expansion and is less
128 metastatic^{22,23}, we did not find any difference in the number of metastatic lesions
129 between WT and *Ackr2*^{-/-} mice (Fig. 1f). We therefore focused on effects of *Ackr2*
130 genetic inactivation on the myeloid compartment of tumor-bearing mice as a potential
131 mechanism of protection from metastasis.

132 *NeuT/Ackr2*^{+/+} mice, at 25 weeks of age, have an increased number of circulating
133 Ly6C^{high} monocytes and Ly6G⁺ neutrophils (Fig. 2a and 2b, respectively; gating
134 strategy in Supplementary Fig. 2a) compared with Balb/c WT mice^{20,21}. As we
135 previously reported²⁴, *Ackr2*^{-/-} mice have increased number of circulating Ly6C^{high}
136 monocytes (Fig. 2a), while no significant difference was found in the number of
137 neutrophils (Fig. 2b) compared to WT mice. At 25 weeks of age, *NeuT/Ackr2*^{-/-} mice
138 presented a further significant increase of blood monocytes and neutrophils (Fig. 2a
139 and 2b) compared to *Ackr2*^{-/-} and *NeuT/Ackr2*^{+/+}, indicating that the lack of *Ackr2* was
140 a further driver of tumor-induced myelopoiesis and/or BM release of myeloid cells.

141 Increased number of inflammatory monocytes and neutrophils, but not alveolar
142 or interstitial macrophages, were also detected in the lungs of *NeuT/Ackr2*^{-/-} as
143 compared to *NeuT/Ackr2*^{+/+} mice (Fig. 2c and Supplementary Fig. 2b for the gating
144 strategy), while in basal conditions no difference was found between leukocyte
145 infiltrate in the lung of WT and *Ackr2*^{-/-} mice (Supplementary Fig. 2c). A higher
146 number of Ly6G⁺ neutrophils in the parenchyma of *NeuT/Ackr2*^{-/-} lungs compared to
147 *NeuT/Ackr2*^{+/+} mice was also found by immunohistochemistry, confirming the flow
148 cytometry data (Fig. 2d and 2e). Similar results were obtained analyzing blood and
149 lungs of WT and *Ackr2*^{-/-} mice orthotopically injected with 4T1 cells (Supplementary
150 Fig. 2d and 2e, respectively). In these mice, analysis of myeloid cells in the bone
151 marrow (BM) 14 days after tumor injection, showed a reduced number of monocytes
152 and neutrophils in *Ackr2*^{-/-} compared to WT mice (Fig. 2f). These results indicate that,
153 in tumor conditions, *Ackr2*^{-/-} mice show enhanced release of myeloid cells from BM,
154 which then accumulate in the blood and lungs.

155

156 *Increased chemokine-induced mobilization in Ackr2*^{-/-} mice

157 To investigate the role of ACKR2 in myeloid cells egress from the BM, we
158 performed leukocyte mobilization experiments. As previously reported and as shown
159 in Figure 3a, under homeostatic conditions, ACKR2-deficient mice have increased
160 frequency and absolute number of circulating Ly6C^{high} monocytes compared to WT

161 and a concomitant decrease in the frequency of the same cells in the BM (Fig. 3c),
162 whereas they showed similar number of circulating and BM neutrophils²⁴ (Fig 3b and
163 3d). After injection of CCL3L1, an ACKR2 ligand known to induce rapid mobilization
164 of both neutrophils and monocytes²⁵, *Ackr2*^{-/-} mice showed a significant higher
165 number of circulating monocytes and neutrophils compared to WT littermates (Fig. 3a
166 and 3b, respectively). Concomitantly, the decrease in monocytes and neutrophils in
167 the BM caused by CCL3L1 injection was more pronounced in *Ackr2*^{-/-} animals (Fig.
168 3c and 3d, respectively).

169 BM chimera experiments showed that both WT and *Ackr2*^{-/-} hosts when
170 transplanted with *Ackr2*^{-/-}, but not WT, hematopoietic cells had an increased number
171 of circulating monocytes and neutrophils (Fig. 3e and f; Supplementary Fig. 3a and
172 3b) and higher number of monocytes and neutrophils infiltrating the lung
173 (Supplementary Fig. 3c and 3d). These results demonstrated that the increased
174 mobilization of monocytes and neutrophils induced by CCL3L1 injection was caused
175 by the absence of ACKR2 in the hematopoietic compartment.

176 Finally, in order to understand whether there is a different localization of
177 leukocytes in the BM sinusoids of ACKR2 deficient mice, we performed in vivo
178 labelling experiments of monocytes with a 2-minute pulse of anti-Ly6C-PE antibody
179 as previously described²⁶. In ACKR2-deficient BM sinusoids there was a significant
180 higher percentage of monocytes located in this vascular compartment after
181 chemokine induced mobilization compared to WT controls (Supplementary Fig. 3e
182 and 3f).

183

184 *Neutrophils protect Ackr2*^{-/-} mice from metastasis

185 To investigate the relevance of the increased myeloid cell mobilization found in
186 *Ackr2*^{-/-} mice in the metastatic process, the B16F10 melanoma cell line was injected
187 i.v. in a classic “artificial” hematogenous metastasis model. In this experimental
188 setting, *Ackr2*^{-/-} mice showed increased number of circulating neutrophils while no
189 differences were found in the number of circulating monocytes, T and B lymphocytes
190 compared to WT mice (Supplementary Fig. 4a). Also in this model, there was a
191 significant reduction in the metastatic ratio in the lungs of ACKR2-deficient hosts
192 compared to WT animals (Fig. 4a). In order to understand which cells were
193 responsible for metastasis protection, we performed depletion experiments by using
194 monoclonal antibodies. Monocyte depletion by treatment with an α -CD115

195 monoclonal antibody significantly decreased the number of metastasis in WT mice,
196 but did not reverse the protection observed in *Ackr2*^{-/-} mice (Supplementary Fig. 4b).
197 We then performed neutrophil depletion with an α -Ly6G monoclonal antibody.
198 Neutrophil depletion caused a reduction in metastasis in WT mice while an increase
199 in metastatic ratio was observed in *Ackr2*^{-/-} mice (Fig. 4b). The protective role of
200 neutrophils in *Ackr2*^{-/-} mice was also demonstrated by performing depletion
201 experiments with the orthotopically transplanted 4T1 tumor line. Also in this model,
202 neutrophil depletion reduced the metastatic ratio in WT mice, while it increased
203 metastasis in *Ackr2*^{-/-} hosts (Fig. 4c). Finally, since ACKR2 was reported to be
204 expressed by B lymphocytes²⁷, we performed B lymphocyte depletion using an α -
205 CD20 monoclonal antibody with no rescue of the metastasis phenotype associated
206 with ACKR2 deficiency (Supplementary Fig. 4c).

207 The role of *Ackr2*^{-/-} neutrophils in protection against metastasis was further
208 investigated by adoptive cell transfer experiment. Transfer of *Ackr2*^{-/-}, but not WT,
209 neutrophils into WT tumor-bearing mice significantly reduced the metastatic ratio
210 (Fig. 4d) to values comparable to those observed in *Ackr2*^{-/-} tumor-bearing mice.

211

212 *Increased tumor-killing activity of Ackr2*^{-/-} *neutrophils*

213 As depletion and adoptive transfer experiments clearly pointed to neutrophils as
214 the key elements responsible for protection against metastasis observed in the
215 absence of ACKR2, we evaluated their reactive oxygen species (ROS) production,
216 one of the main mechanism of tumor cell killing. Neutrophils from B16F10-bearing
217 *Ackr2*^{-/-} animals produced significantly higher amounts of ROS compared to WT mice
218 (Fig. 5a). Similar results were obtained analyzing neutrophils in 4T1-bearing WT and
219 *Ackr2*^{-/-} mice (Supplementary Fig. 5a).

220 *Ackr2*^{-/-} neutrophils also showed a significant increase in transcript levels of the
221 chemokine receptors *Ccr1*, *Ccr2* and *Ccr5*, but not *Cxcr4* (Fig. 5b), while expression
222 levels of other genes associated with neutrophils activation, including *Tnf- α* , *Alox5*,
223 *Vegf-a*, and *Arg1*, were not different between WT and *Ackr2*^{-/-} cells (Fig. 5c).
224 Adoptive transfer experiments demonstrated that *Ackr2*^{-/-} neutrophils display
225 increased recruitment to the lung compared to WT neutrophils with a concomitant
226 reduction of their number in the blood (Fig. 5d and 5e, respectively).

227 Chemokines were reported to control not only neutrophil recruitment to
228 metastatic sites but also their potential anti-metastatic functions such as ROS

229 production²⁸. We therefore examined whether the increased levels of CC chemokine
230 receptors found on *Ackr2*^{-/-} neutrophils could result in increased ROS production.
231 Compared to WT cells, *Ackr2*^{-/-} neutrophils produced more ROS already under
232 resting conditions and even more so after stimulation with the CCR2 ligand CCL2
233 (Fig. 5f).

234 In order to assess the functional relevance of this observation, circulating
235 neutrophils were isolated from tumor-bearing mice and evaluated for their ability to
236 kill tumor cells in vitro. Neutrophils obtained from *Ackr2*^{-/-} mice displayed an
237 increased tumor killing activity (Fig. 5g) compared to cells isolated from WT mice.
238 The killing activity of both WT and *Ackr2*^{-/-} neutrophils was partially reversed by the
239 ROS inhibitor apocynin. Similar results were obtained with neutrophils isolated from
240 resting WT and *Ackr2*^{-/-} mice (Supplementary Fig. 5b).

241 Collectively, these results suggest that neutrophil activation during tumor
242 progression is constrained by ACKR2, which impinges on their expression of CC
243 chemokine receptors and inhibits their migration to the lung and their ability to
244 generate ROS, key mediators of neutrophil antitumoral potential^{29,30}.

245

246 *Ackr2* is expressed by HPCs and controls myelopoiesis

247 Results with BM chimeras demonstrated that ACKR2 expression by
248 hematopoietic cells was responsible for the more pronounced neutrophil mobilization
249 (Fig. 3e and 3f) and for protection against metastasis found in *Ackr2*^{-/-} mice
250 (Supplementary Fig. 1c). As *Ackr2* mRNA expression was very low in neutrophils
251 (Fig. 6b), we traced its expression on sorted myeloid lineage hematopoietic
252 precursors (gating strategy Supplementary Fig. 6a). *Ackr2* transcript level was
253 highest in Lin^{neg}/Sca-1⁺/cKit⁺ (LSK) cells, and was then downregulated in the more
254 mature common myeloid progenitors (CMP; Lin^{neg}/Sca-1⁻/cKit⁺/CD34⁺/FcγRII/III^{int})
255 and granulocytes-monocytes progenitors (GMP; Lin^{neg}/Sca-1⁻
256 /cKit⁺/CD34⁺/FcγRII/III^{high}) (Fig. 6a and b). Thus, the most immature progenitors have
257 the highest level of *Ackr2* expression, which then declines during the myeloid
258 differentiation, showing an opposite behavior as compared to CCR2, whose
259 expression is upregulated during maturation³¹ (Fig. 6c).

260 When we performed expression analysis of other chemokine receptors, we
261 found that, similarly to neutrophils, LSK, CMP and GMP isolated from *Ackr2*^{-/-} mice
262 expressed higher levels of *CCR1*, *CCR2* and *CCR5* compared to WT mice (Fig. 6c,

263 **Supplementary** Fig. 6b and 6c). FACS analysis confirmed an increased expression of
264 *CCR2* in *Ackr2*^{-/-} LSK, CMP and GMP cells and revealed that this was restricted to
265 the myeloid lineage, as no detectable levels of *CCR2* were observed in both WT and
266 *Ackr2*^{-/-} megakaryocyte-erythroid progenitors (MEP; Lin^{neg}/Sca-1⁻/cKit⁺/CD34⁻
267 /FcγRII/III⁻) (Fig. 6d). On the other hand, we did not find differences in *CXCR4* mRNA
268 and protein expression in WT and *Ackr2*^{-/-} HPCs (Fig. 6e and 6f, respectively). These
269 results indicate that ACKR2 is expressed by HPCs and controls the expression of
270 inflammatory CC chemokine receptors known to be involved in myeloid cell release
271 from BM.

272 WT and *Ackr2*^{-/-} BM did not differ in the absolute number of LSK and CMP,
273 while a trend of increase was observed in the number of GMP in the BM of *Ackr2*^{-/-}
274 compared to WT mice (Supplementary Fig. 6d). In addition, no difference in the
275 proliferation of hematopoietic progenitors (Supplementary Fig. 6e) and in the levels of
276 the hematopoietic growth factor G-CSF (Supplementary Fig. 6f) was found
277 comparing resting and tumor-bearing WT and *Ackr2*^{-/-} mice.

278 However, LSK cells sorted from BM *Ackr2*^{-/-} and cultured in vitro had increased
279 expression of the myeloid differentiation markers Ly6G, Ly6C, and CD11b, as
280 compared to their WT counterparts (Fig. 6g to 6i, respectively). Conversely, induction
281 of ACKR2 high expression levels by transfection in the human promyelocytic cell line
282 HL-60, which express low levels of *ACKR2* (Supplementary Fig. 6g and 6h), resulted
283 in a significant reduction of *CCR2* and *CD11b* expression, while no change in
284 *CXCR4* levels was detectable (Fig. 6j). The increased maturation of neutrophils was
285 also supported by in vivo experiments with a BrdU analog³². Indeed, in the blood of
286 *Ackr2*^{-/-} mice there were more mature neutrophils compared to WT mice
287 (Supplementary Fig. 6i).

288 Collectively, these data indicate that *Ackr2* genetic inactivation in early
289 hematopoietic precursors results in an accelerated maturation rate of neutrophils,
290 which are more efficiently mobilized by inflammatory chemokines and efficiently
291 recruited to metastatic lesions, where they perform enhanced anti-metastatic activity.
292 ACKR2 in hematopoietic precursors thus operates as a checkpoint for myeloid cell
293 mobilization and effector functions, and its targeting may pave the way to innovative
294 therapeutic strategies, unleashing myeloid cell-mediated protection against cancer.

295 **DISCUSSION**

296

297 Chemokines are essential mediators of cancer-related inflammation.
298 Chemokines and chemokine receptors are downstream of oncogene activation and
299 inactivation of oncosuppressor genes, as illustrated by CXCR4 and the VHL
300 pathway^{1,33}. Inflammatory chemokines contribute to shaping the landscape of
301 immunity in cancer by recruiting tumor promoting myeloid cells, polarized Th2 cells
302 and T reg cells and by promoting M2-like skewing of TAM¹.

303 Consistently with this general picture, inactivation of ACKR2, a decoy and
304 scavenger receptor for inflammatory CC chemokines, was associated with enhanced
305 carcinogenesis in the skin and GI tract^{13,14}. Conversely, downregulation of ACKR2
306 driven by Kras activation is associated with tumor progression in human Kaposi's
307 sarcoma and in a mouse vascular tumor model¹⁶. Tuning of monocyte recruitment
308 underlies the regulatory function of ACKR2 in tumor progression¹⁵.

309 In agreement with this set of previous data we found that in a model of primary
310 mammary carcinogenesis driven by Her2, an oncogene involved in human breast
311 cancer, ACKR2 deficiency was associated with accelerated appearance and growth
312 of primary lesions. Given the tumor promoting function of TAM in murine and human
313 breast cancer^{7,34,35}, it is reasonable to assume that enhanced tumor growth was
314 mediated by macrophages. Unexpectedly, in the same model, we found that ACKR2
315 deficiency was associated with protection against metastasis, an observation in
316 sharp contrast with the primary tumor phenotype.

317 Protection against lung metastasis in ACKR2 deficient hosts was also
318 observed in the 4T1 mammary carcinoma line transplanted orthotopically and in the
319 classic B16F10 melanoma cell line injected intravenously. The unexpected finding of
320 protection against metastasis prompted a dissection of underlying mechanisms
321 taking advantage of transplanted tumor models.

322 Several lines of evidence indicate that neutrophils mediate resistance to
323 metastasis in ACKR2 deficient hosts. ACKR2 deficiency was associated with
324 profound alterations in neutrophil phenotype and function. Indeed *Ackr2*^{-/-} neutrophils
325 had increased ROS production and cell-killing activity, a pattern that identifies mature
326 or activated neutrophils³². Moreover, neutrophils lacking ACKR2 had increased
327 expression of the chemokine receptors *Ccr1*, *Ccr2* and *Ccr5*, a chemokine receptor
328 profile again typical of activated neutrophils. Indeed, neutrophils express different

329 pattern of chemokine receptors depending on their activation state^{36,37}. IFN- γ the
330 prototypic Th1 cytokine, upregulates the expression of the CC chemokine receptors
331 CCR1 and CCR3³⁸ and inflammatory stimuli such as LPS induce CCR2 expression in
332 neutrophils³⁹. The expression of CC chemokine receptors by neutrophils is
333 functionally relevant not only in terms of recruitment to the inflamed site but also in
334 the activation of their effector functions such as respiratory burst, bacterial killing and
335 anti-metastatic activity^{28,36,40}. In line with these data *Ackr2*-deficient neutrophils
336 showed increased recruitment to the lungs (Supplementary Fig. 3d) and increased
337 tumor killing activity (Fig 5g) compared to WT neutrophils.

338 The role of neutrophils in protection against metastasis was also demonstrated
339 by depletion experiments. Neutrophil depletion rescued the phenotype of resistance
340 to metastasis observed in ACKR2 deficient hosts. Interestingly, in ACKR2-competent
341 mice neutrophil depletion resulted in reduced metastasis (Fig 4b and c), in line with
342 several observations of neutrophil-mediated tumor promotion (see below). Finally,
343 adoptive transfer of ACKR2-deficient neutrophils mediated resistance against
344 metastasis (Fig 4d). Thus, unleashing neutrophil mediated resistance underlies
345 protection against metastasis observed in ACKR2 deficient mice.

346 The finding that ACKR2 expression was vanishingly low in mature neutrophils,
347 raised the possibility of a regulatory function of this molecule upstream in
348 hematopoiesis. We found that ACKR2 is expressed by HPCs and that it is
349 downregulated during maturation in myeloid progenitors. Interestingly, ACKR2 was
350 cloned in 1997 from the BM⁴¹ but its role and expression in this compartment has not
351 been explored⁴². Here we found that *Ackr2*^{-/-} HPCs, like *Ackr2*^{-/-} neutrophils, express
352 extremely high levels of the chemokine receptors CCR1, CCR2 and CCR5 and, when
353 cultured with differentiating cytokines, acquire myeloid differentiation markers faster
354 compared to WT counterparts.

355 To directly assess the effect of ACKR2 on chemokine receptors in immature
356 hematopoietic elements, the HL-60 cell line was used. Here ACKR2 downregulated
357 the expression of *CCR2* and of the integrin *CD11b*. ACKR2 induces a signal
358 transduction cascade activating a β -arrestin dependent signaling that optimizes its
359 chemokine scavenging activity⁴³. It is likely that in HPCs β -arrestin signaling by
360 ACKR2 negatively controls the expression of CC chemokine receptors by interfering
361 with other signaling pathways or by activating mechanism of negative regulation as
362 previously described for other ACKRs. ACKR3, for example, negatively controls

363 signaling by CXCR4⁴⁴ and ACKR4 inhibits the expression of CCR7, CCR9, CXCR5,
364 and CXCR4⁴⁵. Our data are also consistent with previous published data reporting
365 that ACKR2 regulates neutrophil migration towards inflammatory CC chemokines⁴⁶.

366 Neutrophils have emerged as important players in cancer-related
367 inflammation. In general, several lines of evidence indicate that neutrophils are part
368 of the inflammatory network which promotes tumor progression and metastasis⁴⁷⁻⁴⁹.
369 In accordance with this line of evidence, we observed that neutrophil depletion using
370 an α -Ly6G mAb resulted in decreased lung metastasis in the B16F10 and in the 4T1
371 models (Fig. 4b and 4c). However, neutrophils can undergo functionally
372 reprogramming in response to tumor and host derived signals^{48,49} and accordingly
373 exert divergent influence on tumor growth⁵⁰, a dichotomy mirrored by prognostic
374 significance in different human cancers⁵¹.

375 Here we report that ACKR2 expressed by hematopoietic progenitor cells is a
376 key setpoint of neutrophil differentiation, mobilization and function (Supplementary
377 Fig. 7). Targeting hematopoietic ACKR2 may pave the way to innovative therapeutic
378 strategies unleashing myeloid cell-mediated protection against infection and cancer.

379

380 **MATERIALS AND METHODS**

381

382 *Cell lines*

383 4T1 and 4T1-66cl4 cells (kindly provided by Dr Claudia Chiodoni, Department of
384 Experimental Oncology and Molecular Medicine, Istituto Nazionale dei Tumori,
385 Milano, Italy) were grown in DMEM (Lonza) supplemented with 10% FBS (Sigma),
386 1% penicillin/streptomycin (Lonza), 1% L-glutamine (Lonza), 1% sodium pyruvate
387 (Lonza), 1% HEPES (Lonza). B16-F10, kindly provided by Prof Massimiliano Mazzone
388 (Vesalius Research Center, Leuven, Belgium), were grown in DMEM (Lonza)
389 supplemented with 10% FBS (Sigma), 1% penicillin/streptomycin (Lonza), 1% L-
390 glutamine (Lonza). 4T1-luc from PerkinElmer were grown in RPMI 1640 (Lonza),
391 10% FBS (Sigma), 1% penicillin/streptomycin (Lonza), 1% L-glutamine (Lonza), 1%
392 sodium pyruvate (Lonza), 5.4 g/l glucose (Sigma). HL-60 were purchased from ATCC
393 and grown in IMDM (Lonza), 20% FBS (Sigma), 1% Penicillin/Streptomycin (Lonza),
394 1% L-glutamine (Lonza), 1% Sodium Pyruvate (Lonza), and transfected with pEGFP-
395 N1 ACKR2 or mock vector⁵² by using the Nucleofector Kits for HL60 (Lonza)
396 according to the manufacturer's instructions. GFP positive cells were sorted for

397 mRNA analysis. Cells were tested for Mycoplasma and only Mycoplasma free cells
398 were used.

399

400 *Animals*

401 *Ackr2*^{-/-} mice were maintained on Balb/c and C57BL/6J genetic background. Balb/c
402 WT and *Ackr2*^{-/-} mice were crossed with NeuT mice (kindly donated by Professor
403 Federica Cavallo, University of Turin, Italy). WT and **WT CD45.1** mice were obtained
404 from Charles River Laboratories (Calco, Italy) or were cohoused littermates. All
405 colonies were housed and bred in the SPF animal facility at Humanitas Clinical and
406 Research Center in individually ventilated cages. Mice used for experiments were 8
407 to 12 weeks old. Procedures involving animals handling and care were conformed to
408 protocols approved by the Humanitas Clinical and Research Center (Rozzano, Milan,
409 Italy) in compliance with national (4D.L. N.116, G.U., suppl. 40, 18-2-1992) and
410 international law and policies (EEC Council Directive 2010/63/EU, OJ L 276/33, 22-
411 09-2010; National Institutes of Health Guide for the Care and Use of Laboratory
412 Animals, US National Research Council, 2011). The study was approved by the
413 Italian Ministry of Health (approval n. 88/2013-B, issued on the 08/04/2013). All
414 efforts were made to minimize the number of animals used and their suffering. Mice
415 were randomized based on sex, age and weight. The sample size was chosen on the
416 basis of past experience on tumor models in order to detect differences of at least
417 20% between the groups. In most in vivo experiments, the investigators were
418 unaware of the genotype of the experimental groups.

419

420

421 *Tissue collection*

422 Blood was collected from the retro-orbital plexus and by cardiac puncture as
423 described⁵³. Briefly, blood was collected in 2KD-EDTA spray coated tubes (BD
424 Bioscience), washed in FACS buffer (PBS^{-/-}, 1% BSA, 0.05 % sodium azide), red
425 blood cells were lysed, washed again and cells were stained as indicated. Lungs
426 were instilled with PBS for FACS analysis or 4% neutral buffer formalin for
427 histological analysis. For FACS analysis, lungs were minced, digested for 45 min in 1
428 mg/ml collagenase IV (Sigma) in PBS^{-/-}, filtered with 70 µm cell strainer. Red blood
429 cells were lysed and cells stained as indicated. BM was collected from femurs and

430 tibiae. Bones were harvested, cleaned, flushed and filtered with 70 μ m cell strainer.
431 Red blood cells were lysed and cells stained as indicated below.

432

433 *Tumor models*

434 Tumor volume was assessed with caliper using the formula: (Length x Width x
435 Width)/2. Tumor take in NeuT model was determinate by palpation as number of
436 mammary tumors per mouse. For 4T1 and 4T1-66cl4 models 5×10^5 cells were
437 injected in the mammary fat path of Balb/c mice. For lung metastasis evaluation in
438 NeuT, 4T1 and 4T1-66cl4 model, lungs were instilled and fixed for 24 h with 4%
439 neutral buffered formalin, routinely processed for paraffin embedding, sectioned at 4
440 μ m thickness, and stained with hematoxylin and eosin. Sections were evaluated in a
441 blinded fashion under a light microscope. Lung metastasis in NeuT, 4T1 and 4T1-
442 66cl4 models were classified according to their size into: small (<30 neoplastic cells),
443 medium (30–300 neoplastic cells), and large (>300 neoplastic cells). A total
444 metastatic score was then calculated for each lung as follows: number of small
445 metastases*1 + number of medium metastases*3 + number of large metastasis*5.
446 Representative images were acquired with Slide Scanner VS120 dotSlide (Olympus)
447 and analyzed with ImageJ. The melanoma cell line B16F10 (2×10^5 cells) was
448 injected i.v. in C57BL/6 mice and metastases were macroscopically counted as dark
449 nodules on the lung surface. For all the models, metastatic ratio was calculated as
450 ratio of metastasis in *Ackr2*^{-/-} or depleted mice compared to indicated control mice.
451 For monocyte depletion, mice were treated with 100 μ g of α -CD115 antibody (clone
452 AFS98, Bioxcell) the day before 2×10^5 B16-F10 injection and every two days for the
453 entire duration of the experiment. For neutrophils depletion, mice were treated with
454 200 μ g of α -Ly6G antibody (clone 1A8, Bioxcell) the day before 2×10^5 B16-F10
455 injection and with 100 μ g every three days for the entire duration of the experiment.
456 For B cell depletion, mice were treated with 250 μ g of α -CD20 (clone 5D2,
457 Genentech Inc.) three days before 2×10^5 B16-F10 injection. For adoptive transfer
458 experiments, neutrophils were isolated from WT and *Ackr2*^{-/-} BM using the Mouse
459 Neutrophil Isolation Kit (Miltenyi Biotec) and an autoMACS Pro separator (Miltenyi
460 Biotec). Cell purity was assessed by flow cytometry (CD45, CD11b, Ly6G) and used
461 only if neutrophils were $\geq 95\%$ on CD45⁺ cells. For B16F10 model, recipient WT mice
462 were injected i.v. with 5×10^6 WT or *Ackr2*^{-/-} neutrophils every 3 days for the entire
463 duration of the experiment. For adoptive transfer experiment, recipient CD45.1 mice

464 were injected i.v. with 2×10^6 CD45.2 WT or Ackr2^{-/-} neutrophils 15 minutes before
465 CCL3L1 (R&D) injection and after 1 hour lung and blood were collected and
466 leukocytes counted by flow cytometry.

467

468 *Immunohistochemistry*

469 Serial 4 μ m formalin-fixed and paraffin-embedded lung sections were deparaffinized
470 and underwent heat-induced epitope retrieval with pressure cooker. Endogenous
471 peroxidase activity was blocked by incubating sections in 3% H₂O₂ for 15 min. Slides
472 were rinsed and treated with Rodent Block M (Biocare Medical) for 30 min to reduce
473 nonspecific background staining and then incubated for 1 h at room temperature with
474 Ly6G antibody (1:200; clone 1A8; BD Bioscience), Sections were incubated for 30
475 min with Rat on Mouse HRP-Polymer kit (Biocare Medical). The immunoreaction was
476 visualized with 3,3'-diaminobenzidine (Peroxidase DAB Substrate Kit, Vector
477 Laboratories) substrate and sections were counterstained with Mayer's haematoxylin.
478 Negative immunohistochemical controls for each sample were prepared by replacing
479 the primary antibody with normal serum. Positive control sections were included in
480 each immunolabeling assay. Tissues were dehydrated with ethanol, mounted with
481 Eukitt and acquired with an Olympus BX61 virtual slide scanning system using Cell[^]F
482 software (Olympus). In each section 10 independent field of view were acquired. To
483 evaluate the extent of granulocytes infiltration in the lung parenchyma, the
484 percentage of Ly6G-positive area was analyzed with Image-Pro Analyzer 7.0 (Media
485 Cybernetics) software. Representative images were generated using the ImageJ
486 analysis program (<http://rsb.info.nih.gov/ij/>).

487

488 *Flow cytometry analysis*

489 Flow cytometry analysis were performed as previously described⁵³. To exclude death
490 cells from analysis, cells were stained with Violet dead cell stain kit (Thermo Fisher).
491 Single cell suspension was stained with antibodies listed in Supplementary Table 1
492 and related isotype. All antibodies were purchased from BD Bioscience, BioLegend,
493 eBioscience or AbD Serotec. Flow cytometry data were acquired using a FACSCanto
494 II (BD Bioscience) and LSR Fortessa (BD Bioscience) and data were analyzed with
495 FACS Diva (BD Bioscience) and representative images were generated with FlowJo
496 Software (Tree Star). To analyze ROS production, neutrophils were stained with 5
497 μ M CellROX Deep Red Reagent (Thermo Fisher) for 20 min at 37°C in RPMI 1%

498 FBS. Staining was blocked on ice, red blood cells were lysed, and neutrophils
499 analyzed by flow cytometry within 2 h from the staining. Where indicated, mice were
500 injected with 500 μg of Click-it EdU Plus (Thermo Fisher) resuspended in PBS.
501 Femurs were harvested two hours after injection for hematopoietic progenitor
502 analysis. For neutrophil analysis blood was collected 48 and 72 hours after injection.
503 Staining was performed according to manufacturer instruction. The absolute number
504 was determined by using TruCount beads (BD Biosciences) according to the
505 manufacturer instructions. Cell sorting was performed using a FACSAria III (BD
506 Bioscience).

507

508 *HPCs isolation and culture*

509 Lineage negative cells were isolated from WT and *Ackr2*^{-/-} BM using LS columns and
510 the Mouse Lineage Cell Depletion Kit (Miltenyi Biotec), according to manufacturer
511 instruction. Negative fraction was stained with Streptavidin-PB, Sca-1, c-kit, CD34
512 and Fc γ RII/III antibodies and sorted. LSK were seed (1×10^3 /well) in rounded bottom
513 96 wells plate in IMDM medium (Lonza) supplemented with 10% FCS (Sigma), 1% L-
514 glutamine (Lonza), 20 ng/ml SCF (Peprotech), 10 ng/ml IL-6 (Peprotech), 10 ng/ml
515 IL-3 (Peprotech), as previously described⁵⁴. Cells were harvested 3 and 6 days after
516 seeding, stained and analyzed by flow cytometry.

517

518 *Leukocyte mobilization*

519 Mice were injected i.p. with 3 μg CCL3L1 (R&D) and after 1 h blood was collected
520 and leukocytes counted by flow cytometry. To evaluate the percentage of monocytes
521 in BM sinusoids, mice were injected intravenously with 1 μg Ly6C-PE antibody 2 min
522 before the end of experiment.

523

524 *Generation of BM chimeras*

525 Recipient mice received gentamycin (0.8 mg/ml) in drinking water for 2 weeks
526 starting 10 days before irradiation. WT and *Ackr2*^{-/-} mice were lethally irradiated with
527 a total dose of 900 cGy. After 2 h, mice were injected in the retro-orbital plexus with
528 4×10^6 nucleated BM cells obtained by flushing of the cavity of a freshly dissected
529 femur from WT or *Ackr2*^{-/-} donors. Experiment were performed 16 weeks after
530 irradiation to allow complete myeloid repopulation.

531

532 *In vitro cell killing assay*

533 Neutrophils were isolated by magnetic separation as described above from blood of
534 14 days 4T1 tumor-bearing mice or from BM of untreated mice and seeded ($1 \times$
535 10^5 /well) in a 96 wells plate in which, 4 hours before, 5×10^3 4T1-luc cells were
536 plated in Optimem (Thermo Fisher) + 0.5% FBS. Cells were incubated overnight in
537 presence of Apocynin 100 μ M (Sigma) or DMSO control. Firefly luciferase activity
538 was detected with luciferase assay system (Promega) and Synergy H2 (Biotek). Cell
539 killing was calculated as percentage of tumor lysis by the following formula: % cell
540 killing = $(1 - [\text{luminescence of samples with neutrophils}] / [\text{luminescence of samples}$
541 $\text{in medium}]) \times 100$.

542

543 *Transcript analysis by quantitative PCR (qPCR)*

544 Total RNA was extracted from HPCs with miRNA easy Mini kit (Qiagen). Reverse
545 transcription was done using High Capacity cDNA Reverse Transcription Kit (Applied
546 Biosystems). Quantitative PCR was performed with TaqMan Gene Expression
547 Assays (Thermo Fisher) in a 7900 HT Fast Real-Time PCR System (Applied
548 Biosystems) with probes listed in Supplementary Table 2. Total RNA was extracted
549 from HL-60 cell and neutrophils using the TRIzol reagent (Thermo Fisher). Reverse
550 transcription was done using High Capacity cDNA Reverse Transcription Kit (Applied
551 Biosystems). Quantitative PCR was performed with TaqMan Gene Expression
552 Assays in a CFX Connect Real-Time PCR Detection System (BioRad) with probes
553 listed in Table S2. Relative mRNA expression was determined by using the $2^{-\Delta\text{Ct}}$
554 method, and normalized to the expression of the housekeeping gene *β -actin* or
555 *Gapdh*.

556

557 *Statistical analysis*

558 Data are represented as mean. In all figures sample variation is shown as SD. P
559 value was generated using the unpaired t test after normality test and F test to
560 exclude difference in the variance between groups (GraphPad Prism 5). * = $p < 0.05$,
561 ** = $p < 0.01$, *** = $p < 0.001$, ns = not statistically different.

562

563 *Data Availability statement*

564 The authors declare that all the other data supporting the findings of this study are
565 available within the article and its supplementary information files and from the
566 corresponding author upon reasonable request.

567

569 REFERENCES

- 571 1. Mantovani, A. et al. The chemokine system in cancer biology and therapy. *Cytokine*
- 572 *Growth Factor Rev* **21**, 27-39 (2010).
- 573 2. Chow, M.T. & Luster, A.D. Chemokines in cancer. *Cancer Immunol Res* **2**, 1125-1131
- 574 (2014).
- 575 3. Borrello, M.G. et al. Induction of a proinflammatory program in normal human
- 576 thymocytes by the RET/PTC1 oncogene. *Proc Natl Acad Sci U S A* **102**, 14825-30 (2005).
- 577 4. Lazenec, G. & Richmond, A. Chemokines and chemokine receptors: new insights
- 578 into cancer-related inflammation. *Trends Mol Med* **16**, 133-44 (2010).
- 579 5. Müller, A. et al. Involvement of chemokine receptors in breast cancer metastasis.
- 580 *Nature* **410**, 50-56 (2001).
- 581 6. Balkwill, F. Cancer and the chemokine network. *Nat Rev Cancer* **4**, 540-50 (2004).
- 582 7. Qian, B.-Z. et al. CCL2 recruits inflammatory monocytes to facilitate breast-tumour
- 583 metastasis. *Nature* **475**, 222-225 (2011).
- 584 8. Griffith, J.W., Sokol, C.L. & Luster, A.D. Chemokines and chemokine receptors:
- 585 positioning cells for host defense and immunity. *Annu Rev Immunol* **32**, 659-702 (2014).
- 586 9. Mantovani, A., Bonecchi, R. & Locati, M. Tuning inflammation and immunity by
- 587 chemokine sequestration: decoys and more. *Nat Rev Immunol* **6**, 907-18 (2006).
- 588 10. Bonecchi, R., Savino, B., Borroni, E.M., Mantovani, A. & Locati, M. Chemokine
- 589 decoy receptors: structure-function and biological properties. *Curr Top Microbiol Immunol*
- 590 **341**, 15-36 (2010).
- 591 11. Bonecchi, R. & Graham, G.J. Atypical Chemokine Receptors and Their Roles in the
- 592 Resolution of the Inflammatory Response. *Front Immunol* **7**, 224 (2016).
- 593 12. Mantovani, A., Allavena, P., Sica, A. & Balkwill, F. Cancer-related inflammation.
- 594 *Nature* **454**, 436-444 (2008).
- 595 13. Vetrano, S. et al. The lymphatic system controls intestinal inflammation and
- 596 inflammation-associated colon cancer through the chemokine decoy receptor D6. *Gut* **59**,
- 597 197-206 (2010).
- 598 14. Nibbs, R.J. et al. The atypical chemokine receptor D6 suppresses the development of
- 599 chemically induced skin tumors. *J Clin Invest* **117**, 1884-92 (2007).
- 600 15. Massara, M., Bonavita, O., Mantovani, A., Locati, M. & Bonecchi, R. Atypical
- 601 chemokine receptors in cancer: friends or foes? *J Leukoc Biol* **99**, 927-933 (2016).
- 602 16. Savino, B. et al. ERK-dependent downregulation of the atypical chemokine receptor
- 603 D6 drives tumor aggressiveness in Kaposi sarcoma. *Cancer Immunol Res* **2**, 679-689 (2014).
- 604 17. Wu, F.-Y. et al. Chemokine decoy receptor d6 plays a negative role in human breast
- 605 cancer. *Molecular Cancer Research* **6**, 1276-1288 (2008).
- 606 18. Bonecchi, R. et al. Atypical chemokine receptor 2: a brake against Kaposi's sarcoma
- 607 aggressiveness. *Oncoimmunology* **3**, e955337 (2014).
- 608 19. Rovero, S. et al. DNA vaccination against rat her-2/Neu p185 more effectively inhibits
- 609 carcinogenesis than transplantable carcinomas in transgenic BALB/c mice. *J Immunol* **165**,
- 610 5133-42 (2000).
- 611 20. Melani, C., Chiodoni, C., Forni, G. & Colombo, M.P. Myeloid cell expansion elicited
- 612 by the progression of spontaneous mammary carcinomas in c-erbB-2 transgenic BALB/c mice
- 613 suppresses immune reactivity. *Blood* **102**, 2138-45 (2003).
- 614 21. Casbon, A.J. et al. Invasive breast cancer reprograms early myeloid differentiation in
- 615 the bone marrow to generate immunosuppressive neutrophils. *Proc Natl Acad Sci U S A* **112**,
- 616 E566-75 (2015).

- 617 22. Pande, K. et al. Cancer-induced expansion and activation of CD11b⁺ Gr-1⁺ cells
618 predispose mice to adenoviral-triggered anaphylactoid-type reactions. *Mol Ther* **17**, 508-15
619 (2009).
- 620 23. Bidwell, B.N. et al. Silencing of Irf7 pathways in breast cancer cells promotes bone
621 metastasis through immune escape. *Nat Med* **18**, 1224-31 (2012).
- 622 24. Savino, B. et al. Control of murine Ly6C(high) monocyte traffic and
623 immunosuppressive activities by atypical chemokine receptor D6. *Blood* **119**, 5250-60
624 (2012).
- 625 25. Baba, T. & Mukaida, N. Role of macrophage inflammatory protein (MIP)-
626 1alpha/CCL3 in leukemogenesis. *Mol Cell Oncol* **1**, e29899 (2014).
- 627 26. Allende, M.L. et al. S1P1 receptor directs the release of immature B cells from bone
628 marrow into blood. *J Exp Med* **207**, 1113-24 (2010).
- 629 27. Hansell, C.A. et al. Universal expression and dual function of the atypical chemokine
630 receptor D6 on innate-like B cells in mice. *Blood* **117**, 5413-24 (2011).
- 631 28. Granot, Z. et al. Tumor entrained neutrophils inhibit seeding in the premetastatic lung.
632 *Cancer Cell* **20**, 300-14 (2011).
- 633 29. Zhang, D. et al. Neutrophil ageing is regulated by the microbiome. *Nature* **525**, 528-
634 532 (2015).
- 635 30. Coffelt, S.B., Wellenstein, M.D. & de Visser, K.E. Neutrophils in cancer: neutral no
636 more. *Nat Rev Cancer* **16**, 431-46 (2016).
- 637 31. Si, Y., Tsou, C.L., Croft, K. & Charo, I.F. CCR2 mediates hematopoietic stem and
638 progenitor cell trafficking to sites of inflammation in mice. *J Clin Invest* **120**, 1192-203
639 (2010).
- 640 32. Adrover, J.M., Nicolas-Avila, J.A. & Hidalgo, A. Aging: A Temporal Dimension for
641 Neutrophils. *Trends Immunol* **37**, 334-45 (2016).
- 642 33. Schioppa, T. et al. Regulation of the chemokine receptor CXCR4 by hypoxia. *J Exp*
643 *Med* **198**, 1391-402 (2003).
- 644 34. DeNardo, D.G. et al. CD4(+) T cells regulate pulmonary metastasis of mammary
645 carcinomas by enhancing protumor properties of macrophages. *Cancer Cell* **16**, 91-102
646 (2009).
- 647 35. Bottai, G. et al. AXL-associated tumor inflammation as a poor prognostic signature in
648 chemotherapy-treated triple-negative breast cancer patients. *NPJ Breast Cancer* **2**, 16033
649 (2016).
- 650 36. Bonavita, O., Massara, M. & Bonecchi, R. Chemokine regulation of neutrophil
651 function in tumors. *Cytokine Growth Factor Rev* **30**, 81-6 (2016).
- 652 37. Jablonska, J., Wu, C.F., Andzinski, L., Leschner, S. & Weiss, S. CXCR2-mediated
653 tumor-associated neutrophil recruitment is regulated by IFN-beta. *Int J Cancer* **134**, 1346-58
654 (2014).
- 655 38. Bonecchi, R. et al. Up-regulation of CCR1 and CCR3 and induction of chemotaxis to
656 CC chemokines by IFN-gamma in human neutrophils. *J Immunol* **162**, 474-9 (1999).
- 657 39. Souto, F.O. et al. Essential role of CCR2 in neutrophil tissue infiltration and multiple
658 organ dysfunction in sepsis. *Am J Respir Crit Care Med* **183**, 234-42 (2011).
- 659 40. Hartl, D. et al. Infiltrated neutrophils acquire novel chemokine receptor expression and
660 chemokine responsiveness in chronic inflammatory lung diseases. *J Immunol* **181**, 8053-67
661 (2008).
- 662 41. Nibbs, R.J., Wylie, S.M., Yang, J., Landau, N.R. & Graham, G.J. Cloning and
663 characterization of a novel promiscuous human beta-chemokine receptor D6. *J Biol Chem*
664 **272**, 32078-83 (1997).
- 665 42. Ottersbach, K. et al. Macrophage inflammatory protein-1alpha uses a novel receptor
666 for primitive hemopoietic cell inhibition. *Blood* **98**, 3476-8 (2001).

- 667 43. Borroni, E.M. et al. beta-arrestin-dependent activation of the cofilin pathway is
668 required for the scavenging activity of the atypical chemokine receptor D6. *Sci Signal* **6**, ra30
669 1-11, S1-3 (2013).
- 670 44. Levoye, A., Balabanian, K., Baleux, F., Bachelier, F. & Lagane, B. CXCR7
671 heterodimerizes with CXCR4 and regulates CXCL12-mediated G protein signaling. *Blood*
672 **113**, 6085-93 (2009).
- 673 45. Zhu, Y. et al. CCX-CKR expression in colorectal cancer and patient survival. *Int J*
674 *Biol Markers* **29**, e40-8 (2014).
- 675 46. Rot, A. et al. Cell-autonomous regulation of neutrophil migration by the D6
676 chemokine decoy receptor. *J Immunol* **190**, 6450-6 (2013).
- 677 47. Coffelt, S.B. et al. IL-17-producing gammadelta T cells and neutrophils conspire to
678 promote breast cancer metastasis. *Nature* **522**, 345-348 (2015).
- 679 48. Fridlender, Z.G. et al. Polarization of tumor-associated neutrophil phenotype by TGF-
680 beta: "N1" versus "N2" TAN. *Cancer Cell* **16**, 183-94 (2009).
- 681 49. Mantovani, A., Cassatella, M.A., Costantini, C. & Jaillon, S. Neutrophils in the
682 activation and regulation of innate and adaptive immunity. *Nat Rev Immunol* **11**, 519-31
683 (2011).
- 684 50. Eruslanov, E.B. et al. Tumor-associated neutrophils stimulate T cell responses in
685 early-stage human lung cancer. *J Clin Invest* **124**, 5466-80 (2014).
- 686 51. Shen, M. et al. Tumor-associated neutrophils as a new prognostic factor in cancer: a
687 systematic review and meta-analysis. *PLoS One* **9**, e98259 (2014).
- 688 52. Bonecchi, R. et al. Regulation of D6 chemokine scavenging activity by ligand- and
689 Rab11-dependent surface up-regulation. *Blood* **112**, 493-503 (2008).
- 690 53. Bonavita, O. et al. Chapter Twenty-Flow Cytometry Detection of Chemokine
691 Receptors for the Identification of Murine Monocyte and Neutrophil Subsets. *Methods in*
692 *enzymology* **570**, 441-456 (2016).
- 693 54. Ficara, F. et al. Pbx1 restrains myeloid maturation while preserving lymphoid potential
694 in hematopoietic progenitors. *J Cell Sci* **126**, 3181-91 (2013).

695

696 **Authors contribution**

697 RB, ML, AM, and FF conceived and designed the experiments.

698 MM, OB, BS, NC, MS, VMP, ES, CR, LC, and FF performed the experiments.

699 RB, MM, OB, BS, NC CR, and ML analyzed the data.

700 RB, ML and AM wrote the paper.

701

702 **Acknowledgments**

703 This study was supported by the Italian Association for Cancer Research (AIRC – IG
704 15438) and in part by a grant from the Italian Ministry of Health (GR-2010-2307975)
705 to FF. LC is recipient of a fellowship from Fondazione Nicola del Roscio. We
706 acknowledge the Humanitas Flow Cytometry and Imaging Core for the technical
707 assistance during the experiments.

708 **Competing financial interests**

709 The authors declare no competing financial interests.

710

711 **FIGURE LEGENDS**

712 **Figure 1: *Ackr2*^{-/-} mice are protected from lung metastasis**

713 a) *NeuT/Ackr2*^{+/+} (white squares) and *NeuT/Ackr2*^{-/-} (black squares) mice were
714 evaluated for tumor growth calculated as described in the Materials and Methods
715 section (n = 42 for *NeuT/Ackr2*^{+/+} and 23 for *NeuT/Ackr2*^{-/-} mice). b) Representative
716 images of hematoxylin and eosin staining of *NeuT/Ackr2*^{+/+} and *NeuT/Ackr2*^{-/-} lungs
717 at 25 weeks of age. Magnification: 10X. Scale bar: 3 mm. c) Metastatic ratio of
718 *NeuT/Ackr2*^{+/+} (white column) and *NeuT/Ackr2*^{-/-} (black column) mice, calculated as
719 described in the Materials and Methods section (n = 26 and *NeuT/Ackr2*^{+/+} and 16 for
720 *NeuT/Ackr2*^{-/-} mice, respectively). d) Tumor volume in WT (white symbols) and *Ackr2*^{-/-}
721 ^{-/-} (black symbols) mice injected orthotopically with 4T1 cells. (n = 14 for WT and 13
722 for *Ackr2*^{-/-} mice). e) Representative images of hematoxylin and eosin staining of WT
723 and *Ackr2*^{-/-} lungs at day 28 after 4T1 cell injection. Magnification: 10X. Scale bar: 3
724 mm. f) Metastatic ratio of WT (white columns) and *Ackr2*^{-/-} (black columns) mice at
725 day 28 after orthotopic injection of 4T1 or 4T1 66cl4 cells (n = 14 for WT and 13 for
726 *Ackr2*^{-/-} mice for 4T1, 4 for both WT and *Ackr2*^{-/-} mice for 4T1 66cl4). Data are
727 represented as mean (SD). p value was generated using the unpaired t test. * = p <
728 0.05, ** = p < 0.01, *** = p < 0.001, ns = not statistically different.

729 **Figure 2: Protection from metastasis in *Ackr2*^{-/-} mice is associated with**
730 **increased numbers of monocytes and neutrophils in blood and lungs**

731 a) Absolute number of circulating inflammatory monocytes (CD45⁺/CD11b⁺/Ly6C^{hi})
732 and b) neutrophils (CD45⁺/CD11b⁺/Ly6G⁺) in *NeuT/Ackr2*^{+/+} (white squares), and
733 *NeuT/Ackr2*^{-/-} (black squares), Balb/c WT (white triangles) and *Ackr2*^{-/-} (black
734 triangles) mice (n = 9 for *NeuT/Ackr2*^{+/+} and 7 for *NeuT/Ackr2*^{-/-}, 3 for both WT and
735 *Ackr2*^{-/-} at 10 and 15 weeks, 6 for *NeuT/Ackr2*^{+/+} and 5 for *NeuT/Ackr2*^{-/-}, 5 for both
736 WT and *Ackr2*^{-/-} at 25 weeks). c) Absolute number of neutrophils, inflammatory
737 monocytes, alveolar (CD11b^{low}/F4/80⁺/Ly6C^{int}/CD11c⁺/Ly6G⁻) and interstitial
738 macrophages (CD11b⁺/F4/80^{int}/Ly6C⁻/CD11c⁻/Ly6G⁻) in the lungs of *NeuT/Ackr2*^{+/+}
739 (white columns) and *NeuT/Ackr2*^{-/-} (black columns) mice at 15 weeks of age (n = 12
740 for *NeuT/Ackr2*^{+/+} and 6 for *NeuT/Ackr2*^{-/-} mice). d) Representative
741 immunohistochemical images of Ly6G staining in *NeuT/Ackr2*^{+/+} and *NeuT/Ackr2*^{-/-}
742 lungs at 25 weeks of age. Magnification: 20X. Scale bar: 100 μm. e) Quantification of
743 Ly6G immunohistochemical images as number of DAB positive cells on field of view

744 (n = 9 for *NeuT/Ackr2^{+/+}* and 8 for *NeuT/Ackr2^{-/-}* mice, respectively). f) Absolute
745 number of neutrophils and inflammatory monocytes in the BM of WT (white columns)
746 and *Ackr2^{-/-}* (black columns) mice on day 14 after orthotopic injection of 4T1 cells (n =
747 4 for both WT and *Ackr2^{-/-}* mice). Data are represented as mean (SD). p value was
748 generated using the unpaired t test. * = p < 0.05, ** = p < 0.01, *** = p < 0.001.

749

750 **Figure 3: Hematopoietic expression of ACKR2 increases monocyte and**
751 **neutrophil mobilization**

752 Absolute number of inflammatory monocytes (a) and neutrophils (b) in the blood of
753 C57BL/6J WT or *Ackr2^{-/-}* mice 1 h after i.p. injection of CCL3L1 or vehicle (n = 11 for
754 WT/PBS and 8 for *Ackr2^{-/-}*/PBS, 12 for WT/CCL3L1 and 10 for *Ackr2^{-/-}*/CCL3L1 for
755 monocytes; n = 8 for both WT/PBS and *Ackr2^{-/-}*/PBS, 7 for WT/CCL3L1 and 5 for
756 *Ackr2^{-/-}*/CCL3L1 for neutrophils). Percentage of monocytes (c) and neutrophils (d) in
757 the BM of the indicated mice 1 hour after CCL3L1 or vehicle i.p. injection (n = 19 for
758 WT/PBS and 15 for *Ackr2^{-/-}*/PBS, 4 for both WT/CCL3L1 and *Ackr2^{-/-}*/CCL3L1 for
759 monocytes; n = 12 for both WT/PBS and *Ackr2^{-/-}*/PBS, 7 for both WT/CCL3L1 and
760 *Ackr2^{-/-}*/CCL3L1 for neutrophils). Absolute number of inflammatory monocytes (e)
761 and neutrophils (f) in WT and *Ackr2^{-/-}* mice reconstituted with either WT (white
762 columns) or *Ackr2^{-/-}* BM (black columns) after i.p. injection of CCL3L1 (n = 3 for both
763 WT and *Ackr2^{-/-}* mice). Data are represented as mean (SD). p value was generated
764 using the unpaired t test. * = p < 0.05, ** = p < 0.01, *** = p < 0.001.

765

766 **Figure 4: *Ackr2^{-/-}* neutrophils are responsible of metastasis protection**

767 a) Metastatic ratio of C57BL/6J WT and *Ackr2^{-/-}* mice 10 days after i.v. injection of
768 B16-F10 cells (n = 14 for WT and 8 for *Ackr2^{-/-}*, sum of two independent
769 experiments). b) Metastatic ratio of C57BL/6J WT and *Ackr2^{-/-}* mice, treated with
770 isotype IgG or with α -Ly6G, 10 days after i.v. injection of B16-F10 cells (n = 6 for
771 WT/Isotype, 5 for WT/ α -Ly6G, *Ackr2^{-/-}*/Isotype and *Ackr2^{-/-}*/ α -Ly6G) c) Metastatic
772 ratio of Balb/c WT and *Ackr2^{-/-}* mice, treated with isotype IgG or α -Ly6G antibody, 28
773 days after orthotopic injection of 4T1 cells (n = 3 for WT/Isotype, WT/ α -Ly6G and
774 *Ackr2^{-/-}*/ α -Ly6G, 5 for *Ackr2^{-/-}*/Isotype). d) Metastatic ratio in WT mice 10 days after
775 i.v. injection of B16-F10 cells and adoptive transfer of WT (white dots) or *Ackr2^{-/-}*
776 neutrophils (black dots) or PBS (grey dots). Representative images of excised lungs
777 are shown on the left (n = 5 for PBS, 9 for WT neutrophils and 8 for *Ackr2^{-/-}*

778 neutrophils). Data are represented as mean (SD). p value was generated using the
779 unpaired t test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

780

781 **Figure 5: *Ackr2*^{-/-} neutrophils have increased tumor-killing activity and**
782 **expression of inflammatory CC chemokine receptors**

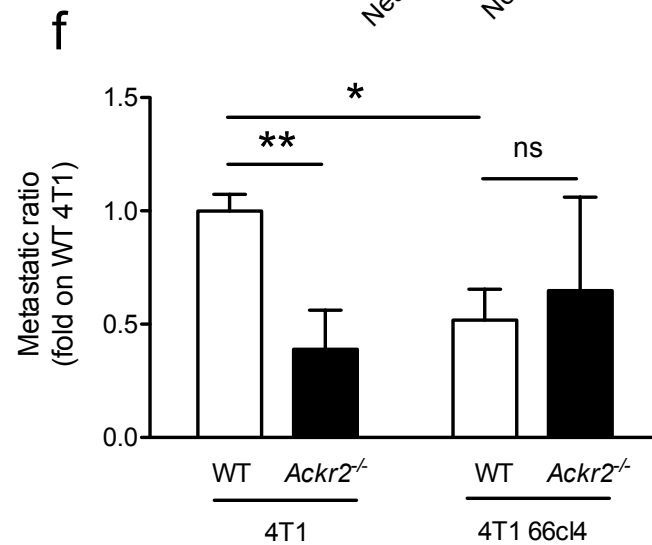
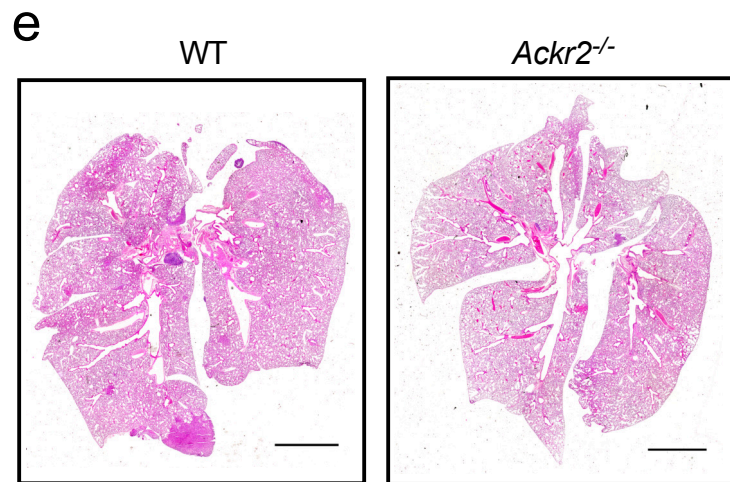
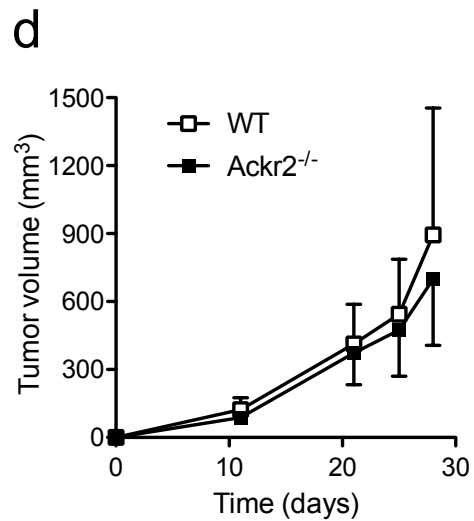
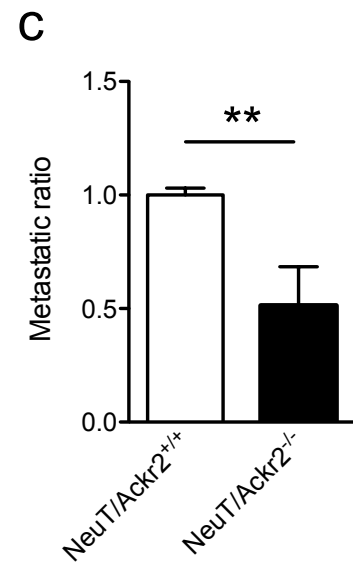
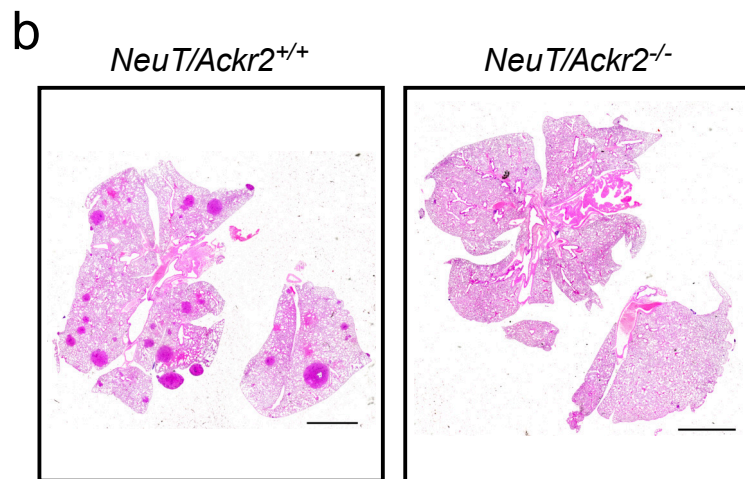
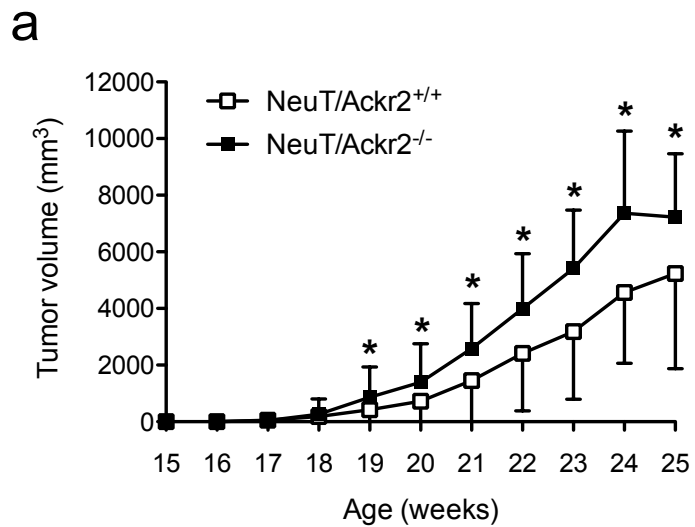
783 a) MFI of CellROX emission by WT and *Ackr2*^{-/-} neutrophils. Data are normalized on
784 MFI of WT neutrophils (n = 9 for WT and 5 for *Ackr2*^{-/-}). b) qPCR analysis of
785 chemokine receptors and c) activation markers in FACS sorted neutrophils taken
786 from unchallenged WT (white columns) and *Ackr2*^{-/-} (black columns) mice (n = 4 for
787 both WT and *Ackr2*^{-/-} mice, two independent experiments). Data are relative to *Gapdh*
788 expression. d) Percentage of WT and *Ackr2*^{-/-} CD45.2 neutrophils on total CD45.2
789 transferred neutrophils in the lungs of WT CD45.1 hosts and e) absolute number of
790 circulating WT and *Ackr2*^{-/-} CD45.2 neutrophils after one hour from adoptive transfer
791 in CD45.1 hosts and i.p. injection of CCL3L1 (n = 3 recipient for each group). f)
792 CellROX MFI in WT (white columns) and *Ackr2*^{-/-} (black columns) neutrophils
793 preincubated with PBS or LPS (100 ng/ml, 20 min) and stimulated with CCL2 (500
794 ng/ml, 30 min) or PMA (50 ng/ml, 30 min). CellROX MFI was normalized on basal
795 WT group (n = 4, two independent experiments for both WT and *Ackr2*^{-/-} mice). g)
796 4T1-luc cells killing by magnetically sorted circulating neutrophils taken from tumor-
797 bearing WT (white columns) and *Ackr2*^{-/-} (black columns) mice 21 days after 4T1
798 injection. Cells were treated with medium or LPS (100 ng/ml) + PMA (50 ng/ml).
799 Where indicated Apocynin (100 μ M) was added (n = 3, two independent experiments
800 for both WT and *Ackr2*^{-/-} mice). Data are represented as mean (SD). p value was
801 generated using the unpaired t test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

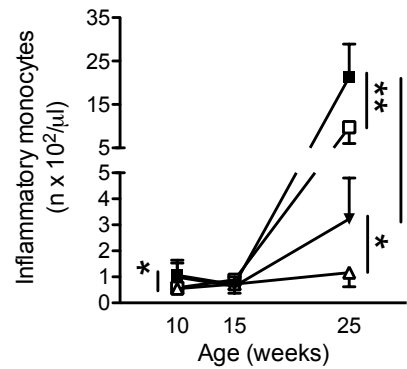
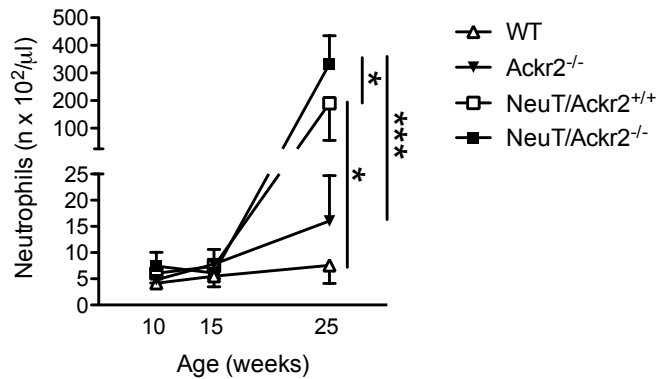
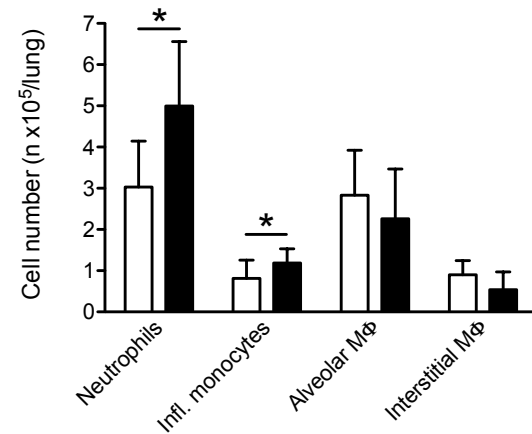
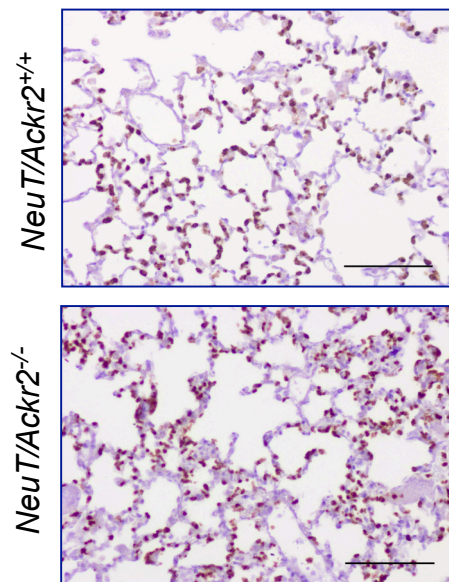
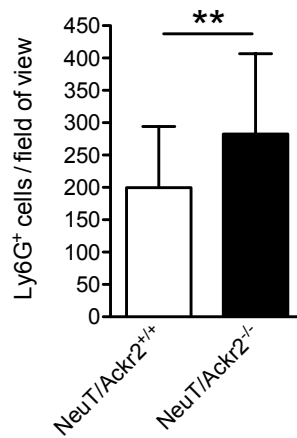
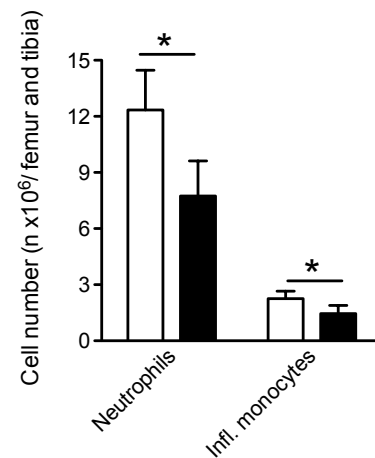
802

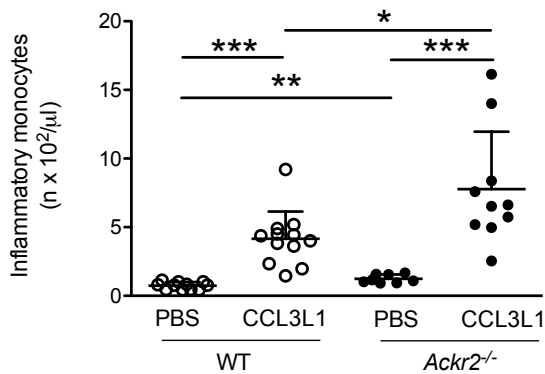
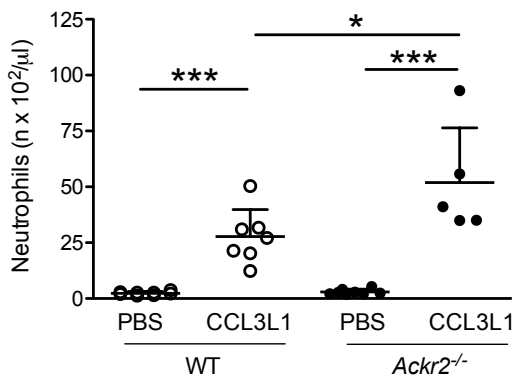
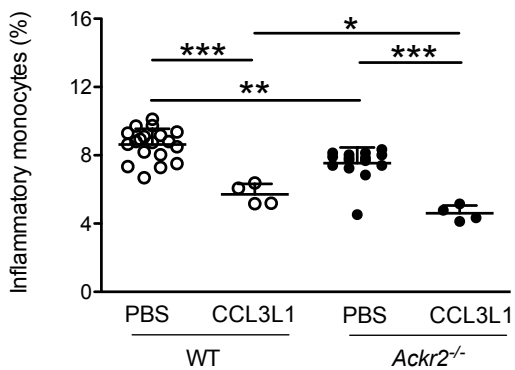
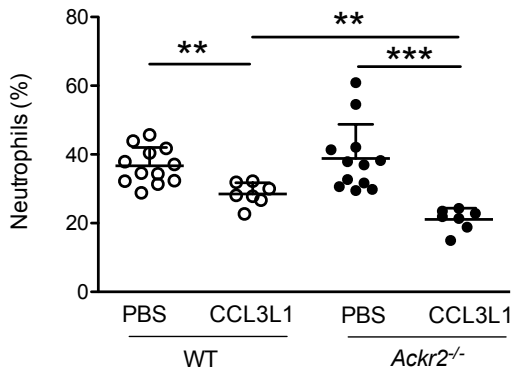
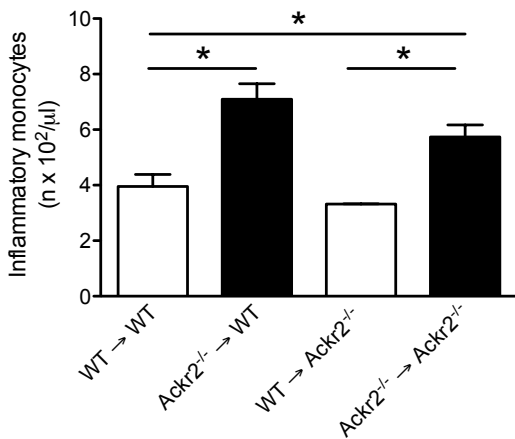
803 **Figure 6: *Ackr2* is expressed in HPCs and controls expression of CC**
804 **chemokine receptors**

805 a) Schematic representation of murine hematopoietic cell differentiation b) qPCR
806 analysis of *Ackr2* expression on sorted HPCs and neutrophils taken from BM of WT
807 mice (n = 7). c) qPCR analysis of *Ccr2* and e) *Cxcr4* expression on sorted HPCs
808 taken from WT (white columns) and *Ackr2*^{-/-} (black columns) mice. All qPCR data are
809 relative to β -actin expression (n = 7 for both WT and *Ackr2*^{-/-} mice). d) MFI of CCR2
810 and f) CXCR4 expression measured by FACS analysis in HPCs taken from WT
811 (white columns) and *Ackr2*^{-/-} (black columns) mice (n = 4 for both WT and *Ackr2*^{-/-}

812 mice). g) MFI of CD11b h) Ly6G i) and Ly6C expression on FACS sorted LSK cells
813 taken from WT (white columns) and *Ackr2*^{-/-} (black columns) cultured in vitro as
814 described in material and methods (n = 5 for WT and 7 for *Ackr2*^{-/-} at 3 days, 5 for
815 WT and 6 for *Ackr2*^{-/-} at 6 days). j) qPCR analysis of CCR2, CD11b, and CXCR4
816 expression in HL-60 cells transfected with ACKR2 (black columns) or empty vector
817 (white columns). qPCR data are relative to GAPDH expression and normalized on
818 mock transfected cells (n = 9 for mock and 13 for ACKR2 transfected cells, 3
819 independent experiments). Data are represented as mean (SD). p value was
820 generated using the unpaired t test. * = p < 0.05, ** = p < 0.01, *** = p < 0.001.
826



a**b****c****d****e****f**

a**b****c****d****e****f**