



HAL
open science

CXCR2 intrinsically drives the maturation and function of neutrophils in mice

Pauline Delobel, Benjamin Ginter, Eliane Rubio, Karl Balabanian, Gwendal Lazenec

► **To cite this version:**

Pauline Delobel, Benjamin Ginter, Eliane Rubio, Karl Balabanian, Gwendal Lazenec. CXCR2 intrinsically drives the maturation and function of neutrophils in mice. *Frontiers in Immunology*, 2022. hal-03798339

HAL Id: hal-03798339

<https://hal.science/hal-03798339>

Submitted on 5 Oct 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

CXCR2 intrinsically drives the maturation and function of neutrophils in mice

1

2 **Pauline Delobel¹, Benjamin Ginter¹, Eliane Rubio¹, Karl Balabanian^{2,3}, Gwendal Lazennec^{1,2*}**

3

4 ¹CNRS, SYS2DIAG-ALCEDIAG, Cap delta, 1682 rue de la Valsière, Montpellier, France.

5 ²CNRS, GDR 3697 "Microenvironment of tumor niches", Micronit, France.

6 ³Université Paris-Cité, Institut de Recherche Saint-Louis, INSERM U1160, Paris, France

7

8 *** Correspondence:**

9 Gwendal Lazennec

10 CNRS, SYS2DIAG, UMR9005, Cap delta, 1682 rue de la Valsière, Montpellier, F-34184, France.

11 Gwendal.lazennec@sys2diag.cnrs.fr

12

13

14 **Running Title:** CXCR2 function in Neutrophils

15

16 **Keywords:** chemokine receptors, Cxcr2, neutrophils, inflammation, tumor microenvironment

17

18 Abstract: 138 words / Text: 3860 words, 60 references / 8 Figures

19

20 **Abstract**

21 Neutrophils play a major role in the protection from infections but also in inflammation related to
22 tumor microenvironment. However, cell-extrinsic and -intrinsic cues driving their function at steady
23 state is still fragmentary. Using *Cxcr2* knock-out mice, we have evaluated the function of the
24 chemokine receptor *Cxcr2* in neutrophil physiology. We show here that *Cxcr2* deficiency decreases
25 the percentage of mature neutrophils in the spleen, but not in the bone marrow (BM). There is also an
26 increase of aged CD62L^{lo} CXCR4^{hi} neutrophils in the spleen of KO animals. Spleen *Cxcr2*^{-/-}
27 neutrophils display a reduced phagocytic ability, whereas BM neutrophils show an enhanced
28 phagocytic ability compared to WT neutrophils. Spleen *Cxcr2*^{-/-} neutrophils show reduced reactive
29 oxygen species production, F-actin and α -tubulin levels. Moreover, spleen *Cxcr2*^{-/-} neutrophils
30 display an altered signaling with reduced phosphorylation of ERK1/2 and p38 MAPK, impaired
31 PI3K-AKT, NF- κ B, TGF β and IFN γ pathways. Altogether, these results suggest that *Cxcr2* is
32 essential for neutrophil physiology.

33

34

35 Introduction

36 One of the first line of defense against pathogens such as bacteria, fungi, or parasites involves
37 neutrophils, which are key mediator of innate immunity and inflammation. Neutrophils use different
38 ways to clear the infection, including bacterial uptake (phagocytosis), phagolysosomal degradation of
39 bacteria with a cocktail of antimicrobial factors and reactive oxygen species (ROS) (oxidative burst)
40 or release of granules to neutralize extracellular pathogens (degranulation) (1). In addition, when
41 intruders are too large or have escaped the other microbial killing processes, neutrophils can extrude
42 a physical barrier to pathogen dissemination, called a neutrophil extracellular trap (NET), containing
43 DNA, histones and granule proteases (2).

44 Neutrophils are the most abundant circulating leukocytes, representing 50% to 70% of all circulating
45 leukocytes in humans and about 10 to 25% in mice (3). Neutrophils are relatively short lived cells
46 (4), even if recent studies have questioned this aspect and it is now believed that human and murine
47 neutrophils could have a half-life of 5 days or 18h, respectively (5).

48 Neutrophils arise from granulocyte–monocyte progenitors (GMPs), mostly within the bone marrow
49 (BM) during hematopoiesis in response to several cytokines, principally granulocyte colony–
50 stimulating factor (G-CSF) (6), but also from extramedullary tissues such as spleen (7-9). The first
51 progenitor that is ‘neutrophil committed’ is the neutrophil promyelocyte (10), which then matures
52 through granulocyte-committed precursors comprising myeloblasts, promyelocytes and myelocytes,
53 to a post-mitotic or transition pool of metamyelocytes, band cells and segmented neutrophils (11).

54 Mature post-mitotic neutrophils are released from the BM into the peripheral blood, extravasate from
55 circulation into the tissues under the coordinate regulation of various adhesion molecules and
56 chemokines (12). They are involved not only in the control of inflammation following infections or
57 injuries but have also pro or anti-tumoral actions as tumor associated neutrophils (TANs) (13-15). In
58 inflammatory sites, neutrophils fight the injury or infection and undergo apoptosis and are

59 phagocytosed by macrophages, once inflammation has been resolved (16). Senescent neutrophils can
60 also home back to the BM in a Cxcr4-dependent mechanism (17). The terminal differentiation of
61 neutrophils in the BM, before release into the bloodstream, is still a subject of debate, as some steps
62 of maturation could occur in other organs such as the spleen (9) and in the same line, neutrophil
63 progenitors have been found in the spleen (18). Maturation markers of neutrophils are also currently
64 discussed, but classically Ly6G, Cd101, Cxcr4 and Cxcr2 are used in mice (8, 12).

65 Cxcr2 appears as one of the key chemokine receptor expressed by neutrophils both in mice and
66 humans (19, 20). Cxcr2 binds the chemokines Cxcl1, 2, 3, 5, 6, 7 and 8 in human, which all have pro-
67 angiogenic properties and are located in a short cluster of chromosome 4 (21-23). Cxcr2 ligand action
68 is conditioned by its interaction with proteoglycans (24) and Cxcr2 signals through multiple
69 pathways including PI3K and Src (25). In addition, recent work has highlighted the role of Cxcr2 in
70 tumorigenesis, in particular through tumor-associated neutrophils (14, 23, 26, 27), but also in the
71 effects microbiota on pituitary function (28). *Cxcr2* knockout mice have been generated and are
72 characterized by a splenomegaly due to an increase of metamyelocytes, mature neutrophils and B
73 lymphocytes (29). These mice also display a defect in neutrophil recruitment after infection (30).

74 So far, the mechanism of Cxcr2 action in neutrophils remains poorly understood. In this study, we
75 have investigated the role of Cxcr2 in mouse neutrophils in the spleen and the BM, taking advantage
76 of *Cxcr2*^{-/-} mice. Our data show that Cxcr2 impairment in neutrophils affects differently spleen and
77 BM neutrophils in terms of maturation, phagocytosis and ROS production. Moreover, we analyzed at
78 the transcriptomic level the pathways that were altered by *Cxcr2* deletion in neutrophils. Altogether,
79 these results suggest that proper Cxcr2 expression and function is required for maturation and
80 effector functions of peripheral neutrophils.

81

82

83

84 Materials and methods**85 Animal Models and housing**

86 All animal experiments conformed to our animal protocols that were reviewed and approved by the
87 Institutional Animal Care and Use Committee. *Cxcr2*^{-/-} mice (29) were obtained from the Jackson
88 Laboratory. *Cxcr2*^{-/-} mice were backcrossed in FVB genetic background for more than 12 generations.
89 Control (WT) mice were also in a FVB background. All mice were housed in a SOPF (Specific and
90 Opportunistic Pathogen Free) animal facility.

91 Isolation of cells

92 Cells from the BM were isolated by centrifugation from the femurs and tibias of the animals, whereas
93 spleens were mashed on 100 µm nylon cell strainer. After centrifugation, red blood cells were
94 eliminated by treatment with ACK buffer (0.155 mM NH₄Cl, 1 mM KHCO₃, 0.1 mM EDTA) and
95 filtered on a 40 µm nylon cell strainer. After ACK treatment, cells were filtered on a 40 µm nylon cell
96 strainer. For neutrophil isolation, a first enrichment with EasySep™ Mouse CD11b Positive Selection
97 kit (StemCell technologies, Grenoble, France) was performed followed by cell sorting of CD45+
98 CD11b+ Ly6G+ F4/80- cells on an ARIA IIu FACS sorter (Becton Dickinson, Le Pont-de-Claix,
99 France).

100 Flow cytometry

101 Flow cytometry experiments were performed with the following conjugated antibodies from
102 Biolegend (London, United Kingdom): anti-mouse CD11b (clone M1/70), CD45 (clone 30-F11),
103 *Cxcr2* (clone SA044G4), Ly6G (clone 1A8), CD62L (clone MEL-14) or BD Biosciences (BD
104 Biosciences, Le Pont-de-Claix, France): *Cxcr4* (clone 2B11), or Ebiosciences (Fisher Scientific,
105 Illkirch, France): CD101 (clone Moushi101), Fixable viability dye (65-0866). Flow analysis was
106 performed on live singlets with a LSR II Fortessa flow cytometer (Becton Dickinson, Le Pont-de-
107 Claix, France). Data were analyzed using FlowJo (Tree Star).

108

109 Intracellular flow cytometry

110 Cells were first stained with antibodies directed against extracellular markers (CD45, CD11b, Ly6G)
111 and then permeabilized with Cytotfix/Cytoperm and Permash buffer (BD Biosciences). Intracellular
112 staining of F-actin was performed with Phalloidin, Fluorescein Isothiocyanate Labeled (Sigma-
113 Aldrich, ref P5282), whereas α -tubulin was labeled with α -Tubulin antibody (clone 11H10, Cell
114 Signaling, ref 2125) followed by secondary an anti-rabbit IgG antibody coupled to Alexa Fluor 555
115 (ThermoFisher, ref A27039). Phospho-p42/44 MAPK ERK1/2 (Thr 202/ Tyr 204) (Cell signaling, ref
116 4370) followed by secondary an anti-rabbit IgG antibody coupled to Alexa Fluor 555 (ThermoFisher,
117 ref A27039) was used to detect Phospho-p42/44 MAPK ERK1/2. p-p38 MAPK (Thr 180/ Tyr 182)
118 coupled to PE (cell Signaling 6908), was used to detect phospho-p38 MAPK.

119 Annexin V staining and measure of mortality

120 To determine the proportion of apoptotic and dead cells, fresh neutrophils were stained with Annexin-
121 FITC and propidium iodide (PI) according to manufacturer instruction (Invitrogen, ref V13242).

122 Phagocytosis assay

123 CD45⁺ CD11b⁺ Ly6G⁺ neutrophils were incubated for various times at 37°C with with E. coli Red
124 Phrodo bioparticles (ThermoFisher, Illkirch, France) at a concentration of 15 μ g/ml and analyzed in
125 a kinetic manner by flow cytometry with a LSR II Fortessa flow cytometer (Becton Dickinson, Le
126 Pont-de-Claix, France). Data were analyzed using FlowJo (Tree Star). When opsonized particles were
127 used, they were opsonized for 1h at 37°C with opsonizing reagent (E2870, ThermoFisher, Illkirch,
128 France), according to manufacturer instructions.

129 Reactive oxygen species and mitochondrial superoxide quantification

130 Reactive oxygen species and mitochondrial quantification was performed by labelling fresh CD45⁺
131 CD11b⁺ Ly6G⁺ neutrophils for 20 min at 37°C, using CellRox Orange reagent (ref. C10443) and
132 Mitosox reagent (ref. M36008) respectively, following the manufacturer's instructions (Molecular
133 Probes, ThermoFisher Scientific).

134

135

136 RNA extraction and RNA-seq data processing

137 Total RNA was isolated using TRIzol reagent (Fisher Scientific, Illkirch, France), as described by the
138 manufacturer. RNA integrity and quality were verified using RNA ScreenTape kit and Tapestation
139 2200 apparatus from AGILENT (Les Ulis, France). cDNA libraries were synthesized using
140 NEBNext® rRNA Depletion and Ultra™ II Directional RNA Library Prep Kit (New England Biolabs,
141 Evry-Courcouronnes, France). Library quality was checked on Tapestation 2200 apparatus from
142 AGILENT (Les Ulis, France) with DNA 1000 ScreenTape. Samples were sequenced on Novaseq
143 6000 (Illumina) with an average sequencing depth of 30 million of paired-end reads. Length of the
144 reads was 150 bp. Each 24 Plex Samples was sequenced on one Illumina SP FlowCell (2*800 million
145 of 150bases reads). Raw sequencing data was quality-controlled with the FastQC program. Low
146 quality reads were trimmed or removed using Trimmer (minimum length: 120 bp). Reads were aligned
147 to the mouse reference genome (mm10 build) with the Star tool. Gene counts were obtained by read
148 counting software Htseq. Normalization and differential analysis were performed with the DESeq2
149 package with Benjamini-Hochberg FDR multiple testing correction ($p < 0.05$; 1.5-fold or higher
150 change) comparing WT and KO animals. The data discussed in this publication have been deposited
151 in NCBI's Gene Expression Omnibus (31) and are accessible through GEO Series accession number
152 GSE209860 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE209860>).

153 Bioinformatic analysis

154 To assess biological interpretation of the most differentially expressed genes, we used Gene ontology
155 (GO) enrichment analysis. A gene set enrichment analysis (GSEA) was performed using signatures
156 from GSEA collections. A normalized enrichment score (NES) was calculated for each gene set and
157 only gene sets with an adjusted p value < 0.05 were selected.

158 Statistics

159 Statistical analyses were carried out using unpaired Mann-Whitney test.

160

161 **Results**162 ***Cxcr2* invalidation affects the maturation of neutrophils**

163 To evaluate the impact of *Cxcr2* knockout on neutrophil distribution and function, we first measured
164 the presence of CD45⁺ CD11b⁺ Ly6G⁺ neutrophils in the BM and the spleen by flow cytometry
165 (Fig.1A). Ly6G⁺ neutrophils were composed of two subpopulations with high (Ly6G^{hi}) or low
166 (Ly6G^{lo}) levels of Ly6 (Fig. 1A). In BM, the percentage of total Ly6G⁺ (Ly6G^{hi} + Ly6G^{lo})
167 neutrophils was increased by about 30% in *Cxcr2*^{-/-} compared to WT animals, and this was much
168 more pronounced in the spleen, with more than 8 - fold more neutrophils in *Cxcr2*^{-/-} animals (Fig.
169 1B). Of particular note, the percentage of CD45⁺ cells was not different in WT and KO spleens,
170 whereas it slightly increased (88.7% vs 95.2%) in KO BM compared to WT BM (Supplemental Fig.
171 1). No major change in FSC and SSC distribution was observed for Ly6G⁺ neutrophils
172 (Supplemental Fig. 2). In terms of absolute numbers, we observed a 5-fold increase of the total
173 number of CD45⁺ cells and CD45⁺ CD11b⁺ Ly6G⁺ neutrophils in the spleen of *Cxcr2*^{-/-} animals,
174 whereas minimal differences between *Cxcr2*^{-/-} and WT animals was seen in BM (no change of
175 the number of CD45⁺ cells and less than 30 % increase for CD45⁺ CD11b⁺ Ly6G⁺) (Supplemental
176 Fig. 3). This obviously creates a bias in the analysis of any type of subpopulation and for this reason,
177 we decided to continue to look at percentage of subpopulations of neutrophils among neutrophils, to
178 avoid this.

179 The level of Ly6G expression has been correlated with the degree of maturation of neutrophils with
180 Ly6G^{hi} neutrophils being the most mature (32). In BM, there was a slight reduction of the percentage
181 of immature Ly6G^{lo} neutrophils among total Ly6G⁺ in *Cxcr2*^{-/-} animals, whereas there was an
182 increase by 2-fold of immature Ly6G^{lo} neutrophils in *Cxcr2*^{-/-} spleen (Fig. 1C). To strengthen these
183 results, we used the CD101 marker, which characterizes mature neutrophils (8). Among neutrophils,
184 we observed a reduction of CD101⁺ mature neutrophils in the spleen, but an increase of mature
185 neutrophils in BM (Fig. 1D, F). Next, we also looked at *Cxcr4* expression in neutrophils, which is

186 correlated not only to immaturity of neutrophils (8), but also to aged neutrophils (33). The percentage
187 of Cxcr4⁺ neutrophils increased twice in the spleen, but was unaffected in the BM (Fig. 1E, G),
188 confirming the increase of the proportion of immature neutrophils in the neutrophil fraction of the
189 spleen. To assess more precisely whether these neutrophils corresponded to immature or aged
190 neutrophils in the spleen of KO animals, we used the marker CD62L (L-Selectin), in addition to
191 CXCR4 to identify "aged" CD62L^{lo}- CXCR4^{hi} neutrophils, as previously described (33). We
192 observed that the proportion of aged CD62L^{lo}- CXCR4^{hi} cells among neutrophils increased in KO
193 spleen compared to WT, but there was no significant change in BM (Supplemental Fig. 4).
194 To further assess the nature of neutrophils, we isolated by cell sorting CD45⁺ CD11b⁺ Ly6G⁺
195 neutrophils from WT and KO BM and spleen and colored them with Giemsa (Supplemental Fig. 9).
196 WT BM neutrophils displayed a condensed round nucleus, with a small cytoplasm, whereas KO BM
197 neutrophils seem to have a larger ring-shaped nucleus, which could suggest that they are more
198 mature. In the spleen, WT neutrophils had a strongly colored ring-shaped nucleus. Spleen KO
199 neutrophils exhibited a larger nucleus with a less pronounced staining and also a cytoplasmic center,
200 which could be reminiscent of band neutrophils (34). These morphological differences suggest that
201 WT and KO neutrophils might have distinct features.

202 ***Cxcr2*^{-/-} neutrophils display altered phagocytic ability**

203 Phagocytosis is one of the major function of neutrophils to eliminate infections. Thus, we analyzed
204 the phagocytic abilities of BM and spleen neutrophils. Kinetics of phagocytosis showed that BM
205 *Cxcr2*^{-/-} neutrophils had an enhanced phagocytic ability compared to WT neutrophils (Fig.2A),
206 whereas spleen *Cxcr2*^{-/-} neutrophils were less phagocytic than spleen WT neutrophils (Fig. 2B). We
207 have also performed an additional analysis, by separating Ly6G^{hi} and Ly6G^{lo} neutrophils
208 (Supplemental Fig. 5). Similar results were obtained for Ly6G^{hi} neutrophils as for Ly6G⁺
209 neutrophils, which is an increase of phagocytosis for BM KO neutrophils and a decrease of

210 phagocytosis for spleen KO neutrophils. Concerning Ly6G^{lo} neutrophils, they were less phagocytic
211 than Ly6G^{hi} neutrophils, both in the spleen and BM. Moreover, BM KO Ly6G^{lo} were also more
212 phagocytic than BM WT Ly6G^{lo} neutrophils. On the other hand, no difference was seen for spleen
213 Ly6G^{lo} neutrophils between WT and KO animals. In addition, when using opsonized bioparticles
214 (Supplemental Fig. 6), we obtained the same trend of difference between WT and KO neutrophils as
215 for non-opsonized particles.

216 To better understand the mechanisms underlying these changes in phagocytic ability, we looked at
217 ROS production and to actin-tubulin cytoskeleton organization of neutrophils, which are essential for
218 phagocytosis (35). We observed a strong reduction in the production of cytoplasmic ROS (Fig 3A, B)
219 and mitochondrial superoxide (Fig. 3C, D) of spleen *Cxcr2*^{-/-} neutrophils, whereas no difference
220 could be measured between BM WT and *Cxcr2*^{-/-} neutrophils. Spleen *Cxcr2*^{-/-} neutrophils had also
221 lower levels of F-Actin (Fig. 4A, B) as well as tubulin (Fig. 4C, D) compared to WT, but BM
222 neutrophils were unaffected by *Cxcr2* impairment. These results of decreased levels of F-actin and
223 tubulin were also confirmed, when comparing MFI values for F-actin and tubulin for the different
224 types of neutrophils (Supplemental Fig. 7). Altogether, these results could account for the lower
225 phagocytic ability of spleen *Cxcr2*^{-/-} neutrophils, which is in agreement with a lower maturity.

226

227 **Spleen *Cxcr2*^{-/-} neutrophils have a higher viability**

228 Neutrophil are short term living cells, so we wondered whether *Cxcr2* could modulate their survival
229 ability. We observed that the percentages of apoptotic or dead cells were reduced for spleen *Cxcr2*^{-/-}
230 neutrophils (Fig. 5B and 5C, respectively), whereas it was similar for WT and *Cxcr2*^{-/-} BM
231 neutrophils. So, this suggests that the reduced phagocytic ability of spleen *Cxcr2*^{-/-} neutrophils is not
232 a consequence of an impaired survival. We have also measured apoptosis and death after a 2h
233 incubation at 37°C, and obtained similar results (Supplemental Fig. 8), which suggests that an

234 increased apoptosis or death after 2h incubation does not affect the phagocytosis ability of
235 neutrophils.

236 **Transcriptomic analysis confirms the impaired maturation of spleen *Cxcr2*^{-/-} neutrophils**

237 We next focused on spleen neutrophils to decipher whether and how *Cxcr2* deficiency impacts their
238 molecular identity. RNAseq analyses of WT and *Cxcr2*^{-/-} spleen neutrophils showed that more than
239 2,500 transcripts were up-regulated and about the same number down-regulated in *Cxcr2*^{-/-} spleen
240 neutrophils (Fig. 6A, B). We then focused on their maturation using the neutrophil maturation
241 signature reported by Xie and collaborators (36). GSEA analysis confirmed that spleen *Cxcr2*^{-/-} were
242 less mature than their WT counterparts (Fig. 6D, E), with down regulation of genes encoding
243 Cathepsin D (*Ctsd*), JunB or IL-1 β involved in Netosis (2), Arginase-2 (*Arg2*) controlling extra-urea
244 cycle arginine metabolism and nitric oxide synthesis (12), C-type lectin receptor *Clec4d* crucial for
245 bacteria elimination (37) or *Selp1g* (CD162), important for the rolling of neutrophils (38) .
246 In addition, there was a cluster of GSEA Biological process (BP) signatures showing a down-
247 regulation of the chemotaxis and migration of spleen *Cxcr2*^{-/-} neutrophils (Fig. 6F), suggesting a
248 decrease in the migration ability of these neutrophils.

249 **ERK and p38 MAPK pathways are down-regulated in spleen *Cxcr2*^{-/-} neutrophils**

250 To look at the signaling that were affected in spleen *Cxcr2*^{-/-} neutrophils, we first focused on ERK
251 and p38 MAPK signaling, which are known to modulate the migration and adhesion of neutrophils
252 (39, 40). RNAseq data showed that both ERK1 and ERK2 cascade (Fig. 7A and C), as well as
253 p38MAPK cascade (Fig. 7B and D), were down regulated in spleen *Cxcr2*^{-/-} neutrophils. Concerning
254 ERK1/2 cascade, there is in particular a down-regulation of the AP-1 Transcription Factor Jun, of the
255 Cyclic AMP-Dependent Transcription Factor Atf-3, IL1 β , growth factors such as TGF β 1 and IGF1,
256 multiple chemokines such as Ccl3, 4, 15, 18, 23 and of a number of kinases such as Ptk2b (Protein
257 Tyrosine Kinase 2 Beta), the tyrosine-protein kinase Syk, Csk (C-Terminal Src Kinase) or the

258 serine/threonine protein kinase BRAF (Fig. 7C). For p38MAPK cascade, we observed in particular a
259 down regulation of Mitogen-Activated Protein Kinase 14 (Mapk14), Mitogen-Activated Protein
260 Kinase Kinase Kinase 3 (Map3k3), Map3k5, and cytokines such as IL1 β , HGF.

261 To confirm these results, we measured by flow cytometry the intracellular content of Phospho-p42/44
262 MAPK ERK1/2 and phospho-p38 MAPK (Thr 180/ Tyr 182). The mean of fluorescence (MFI) of p-
263 ERK1/2 and p-p38 were down-regulated by about 50% in spleen *Cxcr2*^{-/-} neutrophils compared to
264 WT neutrophils (Fig. 7E and 7F, respectively) confirming the alterations of these pathways.

265 **Several pathways are impaired in spleen *Cxcr2*^{-/-} neutrophils**

266 To further investigate the mechanisms involved in the changes observed in spleen *Cxcr2*^{-/-}
267 neutrophils, we explored the main pathways that were affected at the transcriptomic level. GSEA
268 analysis showed a reduction of PI3K-AKT signaling (Fig. 8A), TNF α signaling via NF κ B (Fig. 8B),
269 NIK-NF κ B signaling (Fig. 8C), as well of the cellular response to IL-1 (Fig. 8D) and TGF β signaling
270 (Fig. 8E) and signaling by IFN γ (Fig. 8F) signatures in spleen *Cxcr2*^{-/-} neutrophils compared to WT
271 neutrophils. Among the genes involved in PI3K-AKT pathway, we observed a down-regulation of
272 Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha, Delta and Gamma (Pik3ca,
273 Pik3cd and Pik3cg), and Phosphoinositide-3-Kinase Regulatory Subunit 1, 2 and 5 (Pik3r1, Pik3r2,
274 Pik3r5), Janus Kinase 1 (Jak1), Glycogen Synthase Kinase 3 Beta (GSK3b), and Kras, which are
275 crucial for this pathway.

276 NF- κ B pathway was also impaired with in particular down-regulation of genes such as NF- κ B
277 Subunits p52 (Nfkb2), Relb, C-Rel (Rel), TNF Alpha Induced Protein 2 and 3 (Tnfaip2 and Tnfaip3),
278 Tnfaip3 Interacting Protein 1 (Tnip1), TNF Receptor-Associated Factor 1, 3 and 5 (Traf1, Traf3,
279 Traf5) or TNF Receptor Superfamily Member 10b (Tnfrsf10b).

280 Upstream of NF- κ B activation, cellular response to IL-1 was also altered, with a down-regulation of
281 IL-1 itself, but also of Interleukin 1 Receptor Type 1 (Il1r1). Two other pathways were also linked to

282 PI3K/Akt, ERK, p38 and NF- κ B signaling, namely TGF β and IFN γ signaling. TGF β signaling was
283 impaired with a decrease in Transforming Growth Factor Beta 1 (Tgfb1), Transforming Growth
284 Factor Beta Receptor 1 and 2 (Tgfbr1 and Tgfbr2), and Smad2, 3, and 4. IFN γ signaling is also acting
285 through ERK and MAPK and PI3K signaling and exhibits in particular a down-regulation of Janus
286 kinase 2 (Jak2), suppressor of cytokine signaling protein 1n and 3 (Socs1 , Socs3), Signal transducer
287 and activator of transcription 4 (Stat4), Intercellular adhesion molecule-1 (ICAM-1) and Integrin
288 Subunit Beta 7 (ITGB7).

289

290 Discussion

291 Although the role of neutrophils in general inflammation but also tumor microenvironment
292 inflammation is growing, the factors controlling their function are still not completely identified. In
293 particular, the involvement of Cxcr2 in neutrophil physiology and features, as well as its impact on
294 signalization in neutrophils is still a matter of debate. In this study, we report that impairment of
295 *Cxcr2* leads to a modest increase of the percentage of neutrophils in the BM, but a strong one in the
296 spleen, in agreement with previous studies (29). We also show that Cxcr2 differentially affects the
297 maturation state of neutrophils in the spleen and the BM. More precisely, based on the maturation
298 markers Ly6G (32) and Cd101 (8) expression, *Cxcr2* deletion led an increase of Ly6G^{lo} or CD101-
299 immature neutrophils in the spleen, but to a decrease of immature neutrophils in the BM. The
300 presence of resident immature neutrophils in the spleen is thought to serve as a reservoir of
301 neutrophils, which will undergo a rapid proliferation and mobilization in case of infection to increase
302 the number of active mature neutrophils (18, 32). Spleen is also a site of accumulation and
303 destruction of neutrophils in human (41), so the presence of a high number of immature neutrophils
304 could correspond to both resident and recruited neutrophils. On the other hand, considering that the
305 BM is the main site of production of neutrophils, this would suggest that Cxcr2 impairment does not
306 alter the maturation process on neutrophils in the BM, or that there could be a retention of mature
307 neutrophils in BM. This latter hypothesis is unlikely, as we did not see any difference in the
308 percentage Cxcr4⁺ neutrophils in the BM. Cxcr4 is indeed crucial for the retention of neutrophils in
309 the BM (17), even though other studies have shown that the CXCR4 antagonist plerixafor did not
310 mobilize neutrophils from the BM, but rather enhanced the release of neutrophils in the circulation
311 from the marginated pool present in the lung (42). In contrast, the percentage of Cxcr4⁺ neutrophils
312 was increased 2-fold in the spleen, which could account for several features: this could be the sign of
313 more immature, since Cxcr4 expression is high in proliferating immature neutrophils (8). However,
314 high levels of Cxcr4 can also be seen in senescent or aging neutrophils (17, 43). To clarify this point,

315 we analyzed the presence of aged CD62L^{lo} – CXCR4^{hi} neutrophils as defined previously (33). This
316 shows that there is an increase of aged CD62L^{lo} – CXCR4^{hi} neutrophils in the spleen of KO animals,
317 but no change in BM. In addition, we also observed that the percentage of apoptotic or dead
318 neutrophils was reduced among spleen *Cxcr2*^{-/-} neutrophils compared to WT, whereas no difference
319 was seen in BM. Overall, our data suggest that spleen *Cxcr2*^{-/-} neutrophils are distinct from BM
320 neutrophils, as they are healthier and have more immature features (Ly6G^{lo} Cd101⁻ Cxcr4⁺) than
321 their WT counterparts.

322 To go further in the understanding of the effects of *Cxcr2* impairment, we have observed that spleen
323 *Cxcr2*^{-/-} neutrophils exhibited a reduced phagocytic ability than WT neutrophils. On the other hand,
324 BM *Cxcr2*^{-/-} neutrophils had a higher phagocytic ability than the WT. This difference between
325 spleen and BM neutrophils might be explained by their difference of maturation mentioned above.
326 This is also true for basal phagocytic ability of WT neutrophils, which is higher in the spleen than in
327 the BM. This might be due to a higher proportion of mature CD101⁺ neutrophils in WT spleen
328 compared to WT BM (Fig. 1F), as we have also shown that Ly6G^{hi} neutrophils had a better
329 phagocytic ability than Ly6G^{lo} neutrophils (Supplemental Fig. 5).

330 To explore the difference in phagocytic ability of WT and *Cxcr2*^{-/-} neutrophils more thoroughly, we
331 looked at ROS production and actin and tubulin cytoskeleton, which are key elements in
332 phagocytosis (35, 44). The percentage of cells with high cytoplasmic ROS, mitochondrial
333 superoxide, F-actin and α -tubulin was reduced among spleen *Cxcr2*^{-/-} neutrophils compared to WT,
334 but was not modified in BM neutrophils. This reduction could explain part of the reduced phagocytic
335 ability of spleen *Cxcr2*^{-/-} neutrophils, although one can also notice that decreased phagocytic ability
336 of WT BM neutrophils compared to WT spleen neutrophils is not correlated to their actin levels,
337 suggesting that other parameters might be involved. It is interesting to notice that aged neutrophils
338 are resistant to infections and display also a reduction of actin levels (43). Moreover, it has also been
339 shown that in some cases, immature neutrophils could produce less ROS (8).

340 It was essential to analyze the mechanisms underlying the differences between spleen *Cxcr2*^{-/-} and
341 WT neutrophils. RNAseq analysis of both types of isolated neutrophils confirmed the defect in
342 maturation of spleen *Cxcr2*^{-/-} neutrophils, with in particular a down regulation of genes involved in
343 Netosis (Cathepsin D, JunB or IL-1 β) (45), of Arginase-2 controlling extra-urea cycle arginine
344 metabolism and nitric oxide synthesis, of *Clec4d* implicated in bacteria elimination (46) or *Selp1g*
345 (CD162), essential for the rolling of neutrophils (47). Several GO pathways of migration and
346 chemotaxis constituted a down-regulated cluster, suggesting also an impairment of spleen *Cxcr2*^{-/-}
347 chemotaxis. This could explain an accumulation of neutrophils in the spleen, with a weak ability to
348 migrate to other tissues. Earlier studies have shown that in inflammatory conditions, *Cxcr2* was
349 essential for the recruitment of neutrophils (48).

350 In terms of signaling, we report a down regulation of ERK and p38 MAPK pathways, both at the
351 transcriptomic level, but also when assessing the phosphorylation of ERK and p38 at the protein
352 level. The ERK and p38 MAP kinases are strongly stimulated in neutrophils upon activation by G-
353 protein coupled receptors agonists. Moreover, p38 MAPK is critical for the release of primary and
354 secondary granules, but not that of secretory vesicles by neutrophils (49). p38 inhibition has been
355 shown to delay apoptosis of neutrophils (50), which is in agreement of the reduced rate of apoptosis
356 that we observed in spleen *Cxcr2*^{-/-} neutrophils. Moreover, p38 MAPK promotes chemotaxis
357 towards fMLP by interfering with GRK2-mediated desensitization, whereas ERK MAPK is
358 inhibiting it (51, 52). However, the role of ERK MAPK pathway in neutrophil functions remains
359 unclear, due to contradictory studies (37).

360 In addition, PI3K-Akt, TNF α signaling and NF- κ B signaling, were also altered. TNF α is essential to
361 trigger neutrophil activation and phagocytic activity (53). It is interesting to note that in neutrophils,
362 the production of ROS is dependent on PI3K and ERK (54). Moreover, PI3K, ERK and p38 are
363 necessary for efficient phagocytosis (55). It has also been reported that *PI3K γ* ^{-/-} neutrophils display a

364 defect in migration towards fMLP, C5a, Cxcl8 or Ccl3 and respiratory burst upon activation by C5a
365 or fMLP (56), but another study suggests that PI3K is not required for chemotaxis towards fMLP
366 (51).

367 Cellular response to IL-1 was also reduced. IL1 α and IL1 β are very potent mediators of
368 inflammation response, but their role in neutrophils remains poorly understood. Their main effect on
369 neutrophils could be to increase their survival (37).

370 We also observed a down-regulation of TGF β and IFN γ pathways. In the steady state situation, the
371 role of TGF β on neutrophil function is poorly understood. However, in cancer context, TGF β is
372 responsible for promoting the generation of pro-tumoral type N2 neutrophils (57), whereas type IFN β
373 and IFN γ might favor anti-tumoral type N2 neutrophils (58, 59). In non-cancerous situation, IFN γ has
374 also been shown to enhance, or prime, increased ROS production in combination with a secondary
375 stimulus and to promote phagocytosis (60), which could account for the decrease in ROS and
376 phagocytosis that we observed in spleen *Cxcr2*^{-/-} neutrophils. As treatment of PMNs with IFN γ
377 increases the production of TNF α and IL-1 β (61), this could also account for the down-regulation of
378 TNF α and IL1 β signaling that we report.

379 In conclusion, this work highlights the multiple roles played by the chemokine receptor Cxcr2 in
380 neutrophils and reinforces the importance of localization of neutrophils in terms of action and
381 features. The identification of the pathways that are dependent on Cxcr2 and their further
382 investigation will be essential to understand the roles of Cxcr2 in neutrophils in the steady state or
383 inflammatory situation but also in the tumoral context.

384

385

386 **1 Conflict of Interest**

387 *The authors declare that the research was conducted in the absence of any commercial or financial*
388 *relationships that could be construed as a potential conflict of interest.*

389

390 **2 Author Contributions**

391 P.B., B.G., E.R. and have contributed to the investigation. K.B. participated to writing review and
392 editing. GL was in charge of the conceptualization, investigation and supervision of the project,
393 funding acquisition and writing original draft preparation. All authors have read, revised and agreed
394 to the published version of the manuscript.

395

396 **3 Funding**

397 This work was supported by la Ligue contre le Cancer to GL.

398

399 **4 Acknowledgments**

400 We acknowledge the PCEA, RAM, MRI facilities in Montpellier. We are grateful to Institut du
401 cerveau et de la moëlle épinière in Paris for RNAseq experiments. We thank Vincent Rondeau and
402 Marie-Laure Aknin for useful advices in protocols.

403

404

405 **References**

406

- 407 1. S. Dohrmann, J. N. Cole and V. Nizet: Conquering Neutrophils. *PLoS Pathog*, 12(7),
408 e1005682 (2016) doi:10.1371/journal.ppat.1005682
- 409 2. S. S. Burgener and K. Schroder: Neutrophil Extracellular Traps in Host Defense. *Cold Spring*
410 *Harb Perspect Biol*, 12(7) (2020) doi:10.1101/cshperspect.a037028
- 411 3. L. G. Ng, R. Ostuni and A. Hidalgo: Heterogeneity of neutrophils. *Nat Rev Immunol*, 19(4),
412 255-265 (2019) doi:10.1038/s41577-019-0141-8
- 413 4. E. P. Cronkite, T. M. Fliedner, V. P. Bond and J. R. Rubini: Dynamics of hemopoietic
414 proliferation in man and mice studied by H3-thymidine incorporation into DNA. *Ann N Y Acad Sci*,
415 77, 803-20 (1959) doi:10.1111/j.1749-6632.1959.tb36943.x
- 416 5. J. Pillay, I. den Braber, N. Vrisekoop, L. M. Kwast, R. J. de Boer, J. A. Borghans, K.
417 Tesselaar and L. Koenderman: In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4
418 days. *Blood*, 116(4), 625-7 (2010) doi:10.1182/blood-2010-01-259028
- 419 6. H. K. Kim, M. De La Luz Sierra, C. K. Williams, A. V. Gulino and G. Tosato: G-CSF down-
420 regulation of CXCR4 expression identified as a mechanism for mobilization of myeloid cells. *Blood*,
421 108(3), 812-20 (2006) doi:10.1182/blood-2005-10-4162
- 422 7. N. Borregaard: Neutrophils, from marrow to microbes. *Immunity*, 33(5), 657-70 (2010)
423 doi:10.1016/j.immuni.2010.11.011
- 424 8. M. Evrard, I. W. H. Kwok, S. Z. Chong, K. W. W. Teng, E. Becht, J. Chen, J. L. Sieow, H. L.
425 Penny, G. C. Ching, S. Devi, J. M. Adrover, J. L. Y. Li, K. H. Liong, L. Tan, Z. Poon, S. Foo, J. W.
426 Chua, I. H. Su, K. Balabanian, F. Bachelierie, S. K. Biswas, A. Larbi, W. Y. K. Hwang, V. Madan, H.
427 P. Koefler, S. C. Wong, E. W. Newell, A. Hidalgo, F. Ginhoux and L. G. Ng: Developmental
428 Analysis of Bone Marrow Neutrophils Reveals Populations Specialized in Expansion, Trafficking,
429 and Effector Functions. *Immunity*, 48(2), 364-379 e8 (2018) doi:10.1016/j.immuni.2018.02.002
- 430 9. A. Hidalgo, E. R. Chilvers, C. Summers and L. Koenderman: The Neutrophil Life Cycle.
431 *Trends Immunol*, 40(7), 584-597 (2019) doi:10.1016/j.it.2019.04.013
- 432 10. J. B. Cowland and N. Borregaard: Isolation of neutrophil precursors from bone marrow for
433 biochemical and transcriptional analysis. *J Immunol Methods*, 232(1-2), 191-200 (1999)
434 doi:10.1016/s0022-1759(99)00176-3
- 435 11. L. W. Terstappen, M. Safford and M. R. Loken: Flow cytometric analysis of human bone
436 marrow. III. Neutrophil maturation. *Leukemia*, 4(9), 657-63 (1990)
- 437 12. A. Capucetti, F. Albano and R. Bonecchi: Multiple Roles for Chemokines in Neutrophil
438 Biology. *Front Immunol*, 11, 1259 (2020) doi:10.3389/fimmu.2020.01259
- 439 13. S. Jaillon, A. Ponzetta, D. Di Mitri, A. Santoni, R. Bonecchi and A. Mantovani: Neutrophil
440 diversity and plasticity in tumour progression and therapy. *Nat Rev Cancer*, 20(9), 485-503 (2020)
441 doi:10.1038/s41568-020-0281-y
- 442 14. C. Timaxian, C. F. A. Vogel, C. Orcel, D. Vetter, C. Durochat, C. Chinal, N. G. P, M. L.
443 Aknin, F. Mercier-Nome, M. Davy, I. Raymond-Letron, T. N. Van, S. D. Diermeier, A. Godefroy,
444 M. Gary-Bobo, F. Molina, K. Balabanian and G. Lazennec: Pivotal Role for Cxcr2 in Regulating
445 Tumor-Associated Neutrophil in Breast Cancer. *Cancers (Basel)*, 13(11) (2021)
446 doi:10.3390/cancers13112584

- 447 15. S. J. C. Mancini, K. Balabanian, I. Corre, J. Gavard, G. Lazenec, M. C. Le Bousse-Kerdiles,
448 F. Louache, V. Maguer-Satta, N. M. Mazure, F. Mechta-Grigoriou, J. F. Peyron, V. Trichet and O.
449 Herculat: Deciphering Tumor Niches: Lessons From Solid and Hematological Malignancies. *Front*
450 *Immunol*, 12, 766275 (2021) doi:10.3389/fimmu.2021.766275
- 451 16. C. Haslett: Granulocyte apoptosis and its role in the resolution and control of lung
452 inflammation. *Am J Respir Crit Care Med*, 160(5 Pt 2), S5-11 (1999)
453 doi:10.1164/ajrccm.160.supplement_1.4
- 454 17. C. Martin, P. C. Burdon, G. Bridger, J. C. Gutierrez-Ramos, T. J. Williams and S. M. Rankin:
455 Chemokines acting via CXCR2 and CXCR4 control the release of neutrophils from the bone marrow
456 and their return following senescence. *Immunity*, 19(4), 583-93 (2003) doi:10.1016/s1074-
457 7613(03)00263-2
- 458 18. S. Jhunjhunwala, D. Alvarez, S. Aresta-DaSilva, K. Tang, B. C. Tang, D. L. Greiner, P. E.
459 Newburger, U. H. von Andrian, R. Langer and D. G. Anderson: Frontline Science: Splenic
460 progenitors aid in maintaining high neutrophil numbers at sites of sterile chronic inflammation. *J*
461 *Leukoc Biol*, 100(2), 253-60 (2016) doi:10.1189/jlb.1HI0615-248RR
- 462 19. A. Chuntharapai, J. Lee, C. A. Hebert and K. J. Kim: Monoclonal antibodies detect different
463 distribution patterns of IL-8 receptor A and IL-8 receptor B on human peripheral blood leukocytes. *J*
464 *Immunol*, 153(12), 5682-8 (1994)
- 465 20. V. Marin-Esteban, J. Youn, B. Beaupain, A. Jaracz-Ros, V. Barlogis, O. Fenneteau, T.
466 Leblanc, F. Bellanger, P. Pellet, J. Buratti, H. Lapillonne, F. Bachelierie, J. Donadieu and C.
467 Bellanne-Chantelot: Biallelic CXCR2 loss-of-function mutations define a distinct congenital
468 neutropenia entity. *Haematologica*, 107(3), 765-769 (2022) doi:10.3324/haematol.2021.279254
- 469 21. C. L. Addison, T. O. Daniel, M. D. Burdick, H. Liu, J. E. Ehlert, Y. Y. Xue, L. Buechi, A.
470 Walz, A. Richmond and R. M. Strieter: The CXC chemokine receptor 2, CXCR2, is the putative
471 receptor for ELR+ CXC chemokine-induced angiogenic activity. *J Immunol*, 165(9), 5269-77 (2000)
- 472 22. I. Bieche, C. Chavey, C. Andrieu, M. Busson, S. Vacher, L. Le Corre, J. M. Guinebretiere, S.
473 Burlinchon, R. Lidereau and G. Lazenec: CXC chemokines located in the 4q21 region are up-
474 regulated in breast cancer. *Endocr Relat Cancer*, 14(4), 1039-1052 (2007)
- 475 23. G. Lazenec and A. Richmond: Chemokines and chemokine receptors: new insights into
476 cancer-related inflammation. *Trends Mol Med*, 16(3), 133-44 (2010)
- 477 24. K. Rajarathnam and U. R. Desai: Structural Insights Into How Proteoglycans Determine
478 Chemokine-CXCR1/CXCR2 Interactions: Progress and Challenges. *Front Immunol*, 11, 660 (2020)
479 doi:10.3389/fimmu.2020.00660
- 480 25. J. Sai, D. Raman, Y. Liu, J. Wikswo and A. Richmond: Parallel phosphatidylinositol 3-kinase
481 (PI3K)-dependent and Src-dependent pathways lead to CXCL8-mediated Rac2 activation and
482 chemotaxis. *J Biol Chem*, 283(39), 26538-47 (2008) doi:10.1074/jbc.M805611200
- 483 26. F. Boissiere-Michot, W. Jacot, O. Massol, C. Mollevi and G. Lazenec: CXCR2 Levels
484 Correlate with Immune Infiltration and a Better Prognosis of Triple-Negative Breast Cancers.
485 *Cancers (Basel)*, 13(10) (2021) doi:10.3390/cancers13102328
- 486 27. J. Yang, C. Yan, A. E. Vilgelm, S. C. Chen, G. D. Ayers, C. A. Johnson and A. Richmond:
487 Targeted Deletion of CXCR2 in Myeloid Cells Alters the Tumor Immune Environment to Improve
488 Antitumor Immunity. *Cancer Immunol Res* (2020) doi:10.1158/2326-6066.CIR-20-0312

- 489 28. C. Timaxian, I. Raymond-Letron, C. Bouclier, L. Gulliver, L. Le Corre, K. Chebli, A.
490 Guillou, P. Mollard, K. Balabanian and G. Lazennec: The health status alters the pituitary function
491 and reproduction of mice in a Cxcr2-dependent manner. *Life Sci Alliance*, 3(3) (2020)
492 doi:10.26508/lsa.201900599
- 493 29. G. Cacalano, J. Lee, K. Kikly, A. M. Ryan, S. Pitts-Meek, B. Hultgren, W. I. Wood and M.
494 W. Moore: Neutrophil and B cell expansion in mice that lack the murine IL-8 receptor homolog.
495 *Science*, 265(5172), 682-4 (1994)
- 496 30. L. Del Rio, S. Bennouna, J. Salinas and E. Y. Denkers: CXCR2 deficiency confers impaired
497 neutrophil recruitment and increased susceptibility during *Toxoplasma gondii* infection. *J Immunol*,
498 167(11), 6503-9 (2001) doi:10.4049/jimmunol.167.11.6503
- 499 31. R. Edgar, M. Domrachev and A. E. Lash: Gene Expression Omnibus: NCBI gene expression
500 and hybridization array data repository. *Nucleic Acids Res*, 30(1), 207-10 (2002)
501 doi:10.1093/nar/30.1.207
- 502 32. J. F. Deniset, B. G. Surewaard, W. Y. Lee and P. Kubers: Splenic Ly6G(high) mature and
503 Ly6G(int) immature neutrophils contribute to eradication of *S. pneumoniae*. *J Exp Med*, 214(5),
504 1333-1350 (2017) doi:10.1084/jem.20161621
- 505 33. M. Casanova-Acebes, C. Pitaval, L. A. Weiss, C. Nombela-Arrieta, R. Chevre, A. G. N, Y.
506 Kunisaki, D. Zhang, N. van Rooijen, L. E. Silberstein, C. Weber, T. Nagasawa, P. S. Frenette, A.
507 Castrillo and A. Hidalgo: Rhythmic modulation of the hematopoietic niche through neutrophil
508 clearance. *Cell*, 153(5), 1025-35 (2013) doi:10.1016/j.cell.2013.04.040
- 509 34. C. D. MacCalman, R. Farookhi and O. W. Blaschuk: Estradiol regulates N-cadherin mRNA
510 levels in the mouse ovary. *Dev Genet*, 16(1), 20-4 (1995)
- 511 35. N. Fine, S. Khaliq, S. Hassanpour and M. Glogauer: Role of the Cytoskeleton in Myeloid Cell
512 Function. *Microbiol Spectr*, 4(4) (2016) doi:10.1128/microbiolspec.MCHD-0029-2016
- 513 36. X. Xie, Q. Shi, P. Wu, X. Zhang, H. Kambara, J. Su, H. Yu, S. Y. Park, R. Guo, Q. Ren, S.
514 Zhang, Y. Xu, L. E. Silberstein, T. Cheng, F. Ma, C. Li and H. R. Luo: Single-cell transcriptome
515 profiling reveals neutrophil heterogeneity in homeostasis and infection. *Nat Immunol*, 21(9), 1119-
516 1133 (2020) doi:10.1038/s41590-020-0736-z
- 517 37. K. Futosi, S. Fodor and A. Mocsai: Reprint of Neutrophil cell surface receptors and their
518 intracellular signal transduction pathways. *Int Immunopharmacol*, 17(4), 1185-97 (2013)
519 doi:10.1016/j.intimp.2013.11.010
- 520 38. A. Zarbock, C. A. Lowell and K. Ley: Spleen tyrosine kinase Syk is necessary for E-selectin-
521 induced alpha(L)beta(2) integrin-mediated rolling on intercellular adhesion molecule-1. *Immunity*,
522 26(6), 773-83 (2007) doi:10.1016/j.immuni.2007.04.011
- 523 39. M. H. Pillinger, A. S. Feoktistov, C. Capodici, B. Solitar, J. Levy, T. T. Oei and M. R.
524 Philips: Mitogen-activated protein kinase in neutrophils and enucleate neutrophil cytoplasts: evidence
525 for regulation of cell-cell adhesion. *J Biol Chem*, 271(20), 12049-56 (1996)
526 doi:10.1074/jbc.271.20.12049
- 527 40. D. Kim and C. L. Haynes: The role of p38 MAPK in neutrophil functions: single cell
528 chemotaxis and surface marker expression. *Analyst*, 138(22), 6826-33 (2013)
529 doi:10.1039/c3an01076g

- 530 41. S. H. Saverymuttu, A. M. Peters, A. Keshavarzian, H. J. Reavy and J. P. Lavender: The
531 kinetics of ¹¹¹indium distribution following injection of ¹¹¹indium labelled autologous granulocytes
532 in man. *Br J Haematol*, 61(4), 675-85 (1985) doi:10.1111/j.1365-2141.1985.tb02882.x
- 533 42. S. Devi, Y. Wang, W. K. Chew, R. Lima, A. G. N, C. N. Mattar, S. Z. Chong, A. Schlitzer, N.
534 Bakocevic, S. Chew, J. L. Keeble, C. C. Goh, J. L. Li, M. Evrard, B. Malleret, A. Larbi, L. Renia, M.
535 Haniffa, S. M. Tan, J. K. Chan, K. Balabanian, T. Nagasawa, F. Bachelierie, A. Hidalgo, F. Ginhoux,
536 P. Kubes and L. G. Ng: Neutrophil mobilization via plerixafor-mediated CXCR4 inhibition arises
537 from lung demargination and blockade of neutrophil homing to the bone marrow. *J Exp Med*,
538 210(11), 2321-36 (2013) doi:10.1084/jem.20130056
- 539 43. J. M. Adrover, C. Del Fresno, G. Crainiciuc, M. I. Cuartero, M. Casanova-Acebes, L. A.
540 Weiss, H. Huerga-Encabo, C. Silvestre-Roig, J. Rossaint, I. Cossio, A. V. Lechuga-Vieco, J. Garcia-
541 Prieto, M. Gomez-Parrizas, J. A. Quintana, I. Ballesteros, S. Martin-Salamanca, A. Aroca-Crevillen,
542 S. Z. Chong, M. Evrard, K. Balabanian, J. Lopez, K. Bidzhekov, F. Bachelierie, F. Abad-Santos, C.
543 Munoz-Calleja, A. Zarbock, O. Soehnlein, C. Weber, L. G. Ng, C. Lopez-Rodriguez, D. Sancho, M.
544 A. Moro, B. Ibanez and A. Hidalgo: A Neutrophil Timer Coordinates Immune Defense and Vascular
545 Protection. *Immunity*, 50(2), 390-402 e10 (2019) doi:10.1016/j.immuni.2019.01.002
- 546 44. S. Dupre-Crochet, M. Erard and O. Nubetae: ROS production in phagocytes: why, when, and
547 where? *J Leukoc Biol*, 94(4), 657-70 (2013) doi:10.1189/jlb.1012544
- 548 45. L. W. Garratt: Current Understanding of the Neutrophil Transcriptome in Health and Disease.
549 *Cells*, 10(9) (2021) doi:10.3390/cells10092406
- 550 46. A. L. Steichen, B. J. Binstock, B. B. Mishra and J. Sharma: C-type lectin receptor Clec4d
551 plays a protective role in resolution of Gram-negative pneumonia. *J Leukoc Biol*, 94(3), 393-8 (2013)
552 doi:10.1189/jlb.1212622
- 553 47. Y. Q. Ma, E. F. Plow and J. G. Geng: P-selectin binding to P-selectin glycoprotein ligand-1
554 induces an intermediate state of alphaMbeta2 activation and acts cooperatively with extracellular
555 stimuli to support maximal adhesion of human neutrophils. *Blood*, 104(8), 2549-56 (2004)
556 doi:10.1182/blood-2004-03-1108
- 557 48. T. H. C. de Oliveira, P. E. Marques, F. Poosti, P. Ruytinx, F. A. Amaral, L. Brandolini, M.
558 Allegretti, P. Proost and M. M. Teixeira: Intravital Microscopic Evaluation of the Effects of a
559 CXCR2 Antagonist in a Model of Liver Ischemia Reperfusion Injury in Mice. *Front Immunol*, 8,
560 1917 (2017) doi:10.3389/fimmu.2017.01917
- 561 49. A. Mocsai, Z. Jakus, T. Vantus, G. Berton, C. A. Lowell and E. Ligeti: Kinase pathways in
562 chemoattractant-induced degranulation of neutrophils: the role of p38 mitogen-activated protein
563 kinase activated by Src family kinases. *J Immunol*, 164(8), 4321-31 (2000)
564 doi:10.4049/jimmunol.164.8.4321
- 565 50. K. Aoshiba, S. Yasui, M. Hayashi, J. Tamaoki and A. Nagai: Role of p38-mitogen-activated
566 protein kinase in spontaneous apoptosis of human neutrophils. *J Immunol*, 162(3), 1692-700 (1999)
- 567 51. B. Heit, L. Liu, P. Colarusso, K. D. Puri and P. Kubes: PI3K accelerates, but is not required
568 for, neutrophil chemotaxis to fMLP. *J Cell Sci*, 121(Pt 2), 205-14 (2008) doi:10.1242/jcs.020412
- 569 52. X. Liu, B. Ma, A. B. Malik, H. Tang, T. Yang, B. Sun, G. Wang, R. D. Minshall, Y. Li, Y.
570 Zhao, R. D. Ye and J. Xu: Bidirectional regulation of neutrophil migration by mitogen-activated
571 protein kinases. *Nat Immunol*, 13(5), 457-64 (2012) doi:10.1038/ni.2258

- 572 53. S. J. Klebanoff, M. A. Vadas, J. M. Harlan, L. H. Sparks, J. R. Gamble, J. M. Agosti and A.
573 M. Waltersdorff: Stimulation of neutrophils by tumor necrosis factor. *J Immunol*, 136(11), 4220-5
574 (1986)
- 575 54. L. Bei, T. Hu, Z. M. Qian and X. Shen: Extracellular Ca²⁺ regulates the respiratory burst of
576 human neutrophils. *Biochim Biophys Acta*, 1404(3), 475-83 (1998) doi:10.1016/s0167-
577 4889(98)00081-0
- 578 55. E. Uribe-Querol and C. Rosales: Phagocytosis: Our Current Understanding of a Universal
579 Biological Process. *Front Immunol*, 11, 1066 (2020) doi:10.3389/fimmu.2020.01066
- 580 56. Z. Li, H. Jiang, W. Xie, Z. Zhang, A. V. Smrcka and D. Wu: Roles of PLC-beta2 and -beta3
581 and PI3Kgamma in chemoattractant-mediated signal transduction. *Science*, 287(5455), 1046-9 (2000)
582 doi:10.1126/science.287.5455.1046
- 583 57. Z. G. Fridlender, J. Sun, S. Kim, V. Kapoor, G. Cheng, L. Ling, G. S. Worthen and S. M.
584 Albelda: Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2"
585 TAN. *Cancer Cell*, 16(3), 183-94 (2009)
- 586 58. L. Andzinski, N. Kasnitz, S. Stahnke, C. F. Wu, M. Gereke, M. von Kockritz-Blickwede, B.
587 Schilling, S. Brandau, S. Weiss and J. Jablonska: Type I IFNs induce anti-tumor polarization of
588 tumor associated neutrophils in mice and human. *Int J Cancer*, 138(8), 1982-93 (2016)
589 doi:10.1002/ijc.29945
- 590 59. A. Stoppacciaro, C. Melani, M. Parenza, A. Mastracchio, C. Bassi, C. Baroni, G. Parmiani
591 and M. P. Colombo: Regression of an established tumor genetically modified to release granulocyte
592 colony-stimulating factor requires granulocyte-T cell cooperation and T cell-produced interferon
593 gamma. *J Exp Med*, 178(1), 151-61 (1993) doi:10.1084/jem.178.1.151
- 594 60. T. N. Ellis and B. L. Beaman: Interferon-gamma activation of polymorphonuclear neutrophil
595 function. *Immunology*, 112(1), 2-12 (2004) doi:10.1111/j.1365-2567.2004.01849.x
- 596 61. L. Meda, S. Gasperini, M. Ceska and M. A. Cassatella: Modulation of proinflammatory
597 cytokine release from human polymorphonuclear leukocytes by gamma interferon. *Cell Immunol*,
598 157(2), 448-61 (1994) doi:10.1006/cimm.1994.1241
- 599

600 **Figure legends**601 **Fig.1: *Cxcr2* knock-out decreases the percentage of mature neutrophils in the spleen**

602 **A.** Representative dot plots of the gating strategy of CD45⁺ CD11b⁺ Ly6G^{hi} and CD45⁺ CD11b⁺
 603 Ly6G^{lo} neutrophils among CD45⁺ cells in WT and *Cxcr2*^{-/-} bone marrow (BM) and spleen (SP). **B.**
 604 Quantification of the percentage of the total neutrophils (CD11b⁺ Ly6G⁺: sum of Ly6G^{hi} and Ly6G^{lo})
 605 in the CD45⁺ fraction. **C.** Quantification of CD11b⁺ Ly6G^{hi} cells (left panel) and CD11b⁺ Ly6G^{lo}
 606 (right panel) in the CD11b⁺ Ly6G⁺ (Ly6G^{hi} and Ly6G^{lo} fractions) population. **D.** Gating strategy to
 607 identify CD101⁺ neutrophils in the CD45⁺ CD11b⁺ Ly6G⁺ fraction. **E.** Gating strategy to identify
 608 CXCR4⁺ neutrophils in the CD45⁺ CD11b⁺ Ly6G⁺ fraction. **F.** Percentage of mature CD101⁺
 609 neutrophils in the CD11b⁺ Ly6G⁺ fraction. **G.** Percentage of CXCR4⁺ neutrophils in the CD11b⁺
 610 Ly6G⁺ fraction. Data represent the mean \pm SEM of at least 6 animals (Mann-Whitney test, NS: non-
 611 significant, * p<0.05, ** p<0.01, *** p<0.001).

612

613 **Fig. 2: spleen *Cxcr2*^{-/-} neutrophils have an impaired phagocytosis ability**

614 **A.** To measure phagocytosis, BM CD11b⁺ Ly6G⁺ neutrophils were incubated with Red E. coli
 615 Phrodo bioparticles at 37°C for 0, 30 min, 1h or 2h and analyzed by flow cytometry. Upper panel:
 616 Gating strategy to identify phagocytic neutrophils (Phago⁺) in the CD11b⁺ Ly6G⁺ fraction at 2h time.
 617 Lower panel: Percentage of phagocytic Ly6G⁺ neutrophils. **B.** Same experiment with spleen
 618 neutrophils. Results are expressed as the percentage of phagocytic neutrophils in the CD11b⁺ Ly6G⁺
 619 population and represent the mean \pm SEM of at least 6 animals (Mann-Whitney test, NS: non-
 620 significant, * p<0.05, ** p<0.01, *** p<0.001).

621

622 **Fig. 3: spleen *Cxcr2*^{-/-} neutrophils display reduced ROS and superoxide levels**

623 **A.** Gating strategy to measure cytoplasmic Ros levels using CellRox Orange in CD11b⁺ Ly6G⁺
 624 neutrophils. **B.** ROS levels were quantified in spleen and BM CD11b⁺ Ly6G⁺ neutrophils using

625 CellRox Orange probe. Results are expressed as the percentage of ROS-positive neutrophils in the
626 CD11b+ Ly6G+ population and represent the mean \pm SEM of at least 6 animals (Mann-Whitney test,
627 NS: non-significant, ** $p < 0.01$). **C.** Gating strategy to measure Mitochondrial superoxide levels using
628 Mitosox in CD11b+ Ly6G+ neutrophils. **D.** Measure of the percentage of mitochondrial superoxide
629 -positive CD11b+ Ly6G+ neutrophils using Mitosox reagent. Data represent the mean \pm SEM of at
630 least 6 animals (Mann-Whitney test, NS: non-significant, ** $p < 0.01$).

631

632 **Fig. 4: Decrease of Actin⁺ and Tubulin⁺ levels in spleen *Cxcr2*^{-/-}**

633 **A.** Gating strategy to measure F-Actin levels (Actin⁺) in CD11b+ Ly6G+ neutrophils. **B.** Percentage
634 of CD11b+ Ly6G+ expressing Actin (mean \pm SEM of at least 6 animals; Mann-Whitney test, NS:
635 non-significant, ** $p < 0.01$). **C.** Gating strategy to measure α -Tubulin levels (Tubulin⁺) in CD11b+
636 Ly6G+ neutrophils. **D.** Percentage of CD11b+ Ly6G+ expressing Tubulin. (mean \pm SEM of at least
637 6 animals; Mann-Whitney test, NS: non-significant, ** $p < 0.01$).

638

639 **Fig. 5: spleen *Cxcr2*^{-/-} neutrophils exhibit a reduced apoptosis and mortality**

640 **A.** Gating strategy to identify alive, dead and apoptotic cells based on Annexin V and PI staining.
641 Control plot corresponds to cells labelled with cell surface markers, but not with Annexin V and PI
642 **B.** Measure of the percentage of apoptotic neutrophils in the spleen and BM of WT and *Cxcr2*^{-/-}
643 animals by annexin V staining. **C.** Same measure of dead cells by PI staining. Results are expressed
644 as the percentage of CD11b+ Ly6G+ neutrophils and represent the mean \pm SEM of 6 animals; Mann-
645 Whitney test, NS: non-significant, ** $p < 0.01$.

646

647 **Fig. 6: *Cxcr2*^{-/-} neutrophils display decreased maturity at the transcriptomic level**

648 **A.** Volcano plot showing the global changes in RNA expression patterns for spleen neutrophils
649 isolated from *Cxcr2*^{-/-} (SP KO) versus WT (SP WT) animals. Data represent analysis of cpm

650 estimates with a log of fold change of more than 1.5 fold and $p < 0.05$ of 4 animals per group. Grey
651 dots: NR: non-regulated genes; Green dots: genes with a log of fold change of more than 1.5 fold;
652 blue dots: genes with a p -value < 0.05 ; red dots: genes with a log of fold change of more than 1.5 fold
653 and $p < 0.05$. **B.** Number of differentially regulated genes for the spleen neutrophils. Up: genes up-
654 regulated in spleen isolated from *Cxcr2*^{-/-} versus WT animals. Down: down-regulated genes. NR: non
655 regulated genes. **C.** Simplified Heatmap of spleen *Cxcr2*^{-/-} versus WT neutrophils. **D.** Normalized
656 enrichment score (NES) after GSEA analysis of the transcriptome of spleen neutrophils isolated from
657 *Cxcr2*^{-/-} versus WT animals according to Neutrophil maturation of Xie et al. (36). **E.** Heatmap of the
658 significantly regulated genes ($p < 0.05$) of Neutrophil maturation signature. **F.** Cluster of chemotaxis
659 and migration GSEA analysis from *Cxcr2*^{-/-} versus WT animals according to Biologic process GO:
660 Cell-chemotaxis (NES= -1.64; $q = 0.0002$), Leukocyte-chemotaxis (NES= -1.62; $q = 0.001$),
661 Granulocyte chemotaxis (NES= -1.53; $q = 0.029$), Regulation of Chemotaxis (NES = -1.46; $q =$
662 0.013), Granulocyte migration (NES= -1.54; $q = 0.025$), Myeloid-leukocyte-migration (NES= -1.50;
663 $q = 0.012$), leukocyte-migration (NES = -1.48; $q = 0.003$).

664
665 **Fig. 7: ERK and p38 pathways are down-regulated in Spleen *Cxcr2*^{-/-} neutrophils.**

666 **A.** NES after GSEA analysis of the transcriptome of spleen neutrophils isolated from *Cxcr2*^{-/-} versus
667 WT animals according to ERK1 and ERK2 cascade signature. **B.** Same analysis for p38-MAPK
668 signature. **C.** Heatmap of the significantly regulated genes ($p < 0.05$) of GO-BP ERK1 and ER2 cascade
669 signature. **D.** Heatmap of the significantly regulated genes ($p < 0.05$) of GO-BP p38 MAPK cascade
670 signature. **E.** Phospho-p42/44 MAPK ERK1/2 (Thr 202/ Tyr 204) was analyzed by flow cytometry in
671 spleen CD11b⁺ Ly6G⁺ neutrophils. Left panel: representative Mean of Fluorescence (MFI) of WT
672 (red line) and KO (blue line) Ly6G⁺ neutrophils. Results are expressed as MFI of phospho-ERK
673 positive neutrophils in the CD11b⁺ Ly6G⁺ population and represent the mean \pm SEM of at least 6

674 animals (Mann-Whitney test, * $p < 0.05$). **F.** Phosphorylation of p38MAPK on Thr180/Tyr182 was
675 analyzed by flow cytometry in the same conditions (Mann-Whitney test, *** $p < 0.001$).

676

677 **Fig. 8: Multiple pathways are down-regulated in Spleen *Cxcr2*^{-/-} neutrophils**

678 GSEA analysis of the following signatures: **A.** WIKI-PI3K-AKT signaling. **B.** Hallmark-TNF α

679 signaling via NF κ B. **C.** GO-BP NIK-NF κ B Signaling **D.** GO-BP Cellular response to IL-1 **E.** WP-

680 TGF β signaling pathway **F.** Hallmark IFN γ -response

681