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1 **A Neutrophil-Centric View of Chemotaxis**

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26

27 **Abstract**

28

29 Neutrophils are key players of the innate immune system, that are involved in coordinating
30 the initiation, propagation and resolution of inflammation. Accurate neutrophil migration
31 (chemotaxis) to sites of inflammation in response to gradients of chemoattractants is pivotal
32 to these roles. Binding of chemoattractants to dedicated G protein coupled receptors initiates
33 downstream signalling events that promote neutrophil polarisation, a prerequisite for
34 directional migration. We provide a brief summary of some of the recent insights into
35 signalling events and feedback loops that serve to initiate and maintain neutrophil
36 polarisation. This is followed by a discussion of recent developments in the understanding of
37 *in vivo* neutrophil chemotaxis, a process that is frequently referred to as ‘recruitment’ or
38 ‘trafficking’. Here, we summarise neutrophil mobilisation from and homing to the bone
39 marrow, and briefly discuss the role of glucosaminoglycan-immobilised chemoattractants and
40 their corresponding receptors in the regulation of neutrophil extravasation and neutrophil
41 swarming. We furthermore touch on some of the most recent insights into the roles of
42 atypical chemokine receptors in neutrophil recruitment, and discuss neutrophil reverse
43 (transendothelial) migration together with potential function(s) in the dissemination and/or
44 resolution of inflammation.

45

- 46 **List of abbreviations**
- 47 ACKR – atypical chemokine receptors
- 48 COPD – chronic obstructive pulmonary disease
- 49 DAG – diacylglycerol
- 50 fMLF – N-formylmethionine-leucyl-phenylalanine
- 51 GAG – glycosaminoglycans
- 52 GAP – GTPase activating protein
- 53 G-CSF – Granulocyte colony stimulating factor
- 54 GEF – guanine nucleotides exchange factor
- 55 GPCR – G protein coupled receptor
- 56 GTPase – Guanosine triphosphatase
- 57 IP3 – inositol trisphosphate
- 58 LTB4 – leukotriene B4
- 59 MTOC – microtubular organising centre
- 60 PI3K – phosphoinositide 3-kinase
- 61 PIP3 – phosphatidylinositol-(3,4,5)-trisphosphate
- 62 PLC – phospholipase C
- 63 PTEN – phosphatase and tensin homologue
- 64 RasGRP – Ras guanyl releasing protein
- 65 SHIP – SH2-containing inositol phosphatase
- 66
- 67

68 **Introduction**

69 Chemotaxis is defined as directed cell migration in response to a gradient of a chemical
70 stimulus, with migration occurring towards a chemoattractant, or away from a
71 chemorepellent. Chemotaxis is critical during embryonic development, where it promotes
72 morphogenetic movements in response to growth factor receptor-mediated gradient sensing
73 by directional coordinated, collective cell migration. Examples of collective developmental
74 chemotaxis include the migration of neural crest cells and the angiogenic sprouting of blood
75 vessels towards growth factors [1]. In contrast, single cell chemotaxis provides a tightly
76 controlled mechanism throughout life by which immune cells are recruited, usually in
77 response to G protein coupled receptor (GPCR) stimulation by chemoattractants.

78

79 Chemotaxis has fascinated scientists for decades. Single cells are more amenable to *in vitro*
80 analysis than embryos. Neutrophils, the most abundant circulating leukocytes in man, are
81 short-lived immune cells of the granulocyte lineage. Neutrophils can produce reactive oxygen
82 species and degranulate, releasing cytotoxic products. Combined with their ability to
83 phagocytose and kill ingested microorganisms or to release chromatin-rich extracellular traps,
84 neutrophils provide a first line of defense against bacterial and fungal infections. They are
85 also key effectors in the inflammatory response (for general reviews [2, 3]). Neutrophils are
86 highly motile and migrate as single cells with exquisite speed and directionality in response
87 to chemotattractant stimulation. Chemotaxis *in vivo* is essential for many of the neutrophil's
88 functions throughout its lifetime.

89

90 **Experimental models of neutrophil chemotaxis**

91 The highly motile primary neutrophil is very short-lived and not amenable to culture,
92 transfection or transduction. Although they chemotax very well, freshly purified human

93 neutrophils are therefore not frequently used for chemotaxis experiments. Alternative models
94 that are not always representative of all facets of neutrophil functions are usually used
95 instead. Freshly isolated (often bone marrow-derived) neutrophils from mice that carry
96 genetic alterations of interest are frequently the model of choice. Mice, or indeed zebrafish,
97 offer in addition the opportunity to investigate neutrophil trafficking *in vivo*. Alternative more
98 tractable alternatives to primary neutrophils for the study of single cell chemotaxis *in vivo*
99 include cultured cells that can be differentiated to become neutrophil-like (e.g. HL-60), and
100 the social amoeba *Dictyostelium discoideum*. Over time, primary neutrophils obtained from
101 knock-out mouse models, transfected cell lines, and genetically modified *D. discoideum*
102 strains in combination with *in vitro* chemotaxis chambers have helped to decipher many
103 facets of the molecular regulation of chemotaxis [4]. Aided by increasingly powerful
104 intravital microscopy, in recent years such *in vitro* studies and relatively straightforward *in*
105 *vivo* recruitment assays have been supplemented with *in situ* observations of neutrophil
106 recruitment. Some of the recent advances in the understanding of the molecular regulation of
107 neutrophil chemotaxis *in vitro* and insights into the molecular control of neutrophil
108 trafficking *in vivo* are discussed here.

109

110 **Chemotaxis as a specialised form of cell migration**

111 In chemotaxis, receptor-mediated chemoattractant gradient sensing promotes cell polarisation
112 and thereby directional cell migration. General features of cell migration have been reviewed
113 in depth elsewhere [5, 6]. *In vitro* cell migration occurs by different modes, depending on
114 whether the cells are on a two-dimensional substrate or within a three-dimensional matrix. In
115 the first instance, cells adopt a flattened shape, form integrin-based adhesions to the
116 substratum, use actin-mediated propulsion led by a lamellipodium at their front, and are
117 characterised by a trailing end [7]. In the latter case, rather than relying on integrin-based

118 adhesions [8], neutrophils migrate in a frequently non-proteolytic, amoeboid fashion and
119 depend on actin-mediated protrusions and myosin II-mediated contractions to propel
120 themselves through a three-dimensional matrix [9]. *In vivo*, integrin-dependent steps involve
121 the breaching of barriers such as the vessel wall, whereas interstitial migration is thought to
122 be integrin-independent. ‘Amoeboid’ neutrophil migration in the interstitium involves the
123 selection of a path of least resistance, with neutrophils probing for gaps that permit passage
124 of their multilobular nuclei. Interestingly, leukocytes undergoing amoeboid chemotaxis
125 exhibit a typical microtubular organising centre (MTOC) position behind or, in the case of
126 the neutrophil, in between nuclear lobes. Amoeboid cell migration contrasts with the much
127 slower polarised ‘mesenchymal’ cell migration (that is exemplified by fibroblasts), which is
128 characterised by MTOC and Golgi apparatus polarisation in front of the nucleus [10-12]. An
129 elegant recent study that employed chemotactic mazes with channels of different sizes
130 demonstrated that the MTOC is a good indicator of the directional choice (or dominant pole)
131 of chemotaxing leukocytes [13]. Resting neutrophils are comparatively devoid of
132 microtubules, with chemoattractant stimulation causing microtubular polymerisation.
133 Interestingly, neutrophil chemotaxis on two dimensional matrices or elastase-dependent
134 invasion, but not transendothelial migration or crawling on immobilised chemoattractants
135 was shown to depend upon polymerisation of microtubules [14].

136

137 **Chemoattractant sensing by G protein coupled receptors**

138 Chemoattractants bind G protein coupled cell surface receptors (GPCRs) which usually
139 signal through $G\alpha_{i/o}$ containing heterotrimeric G proteins (reviewed in [15]). Although there
140 is some promiscuity, many chemoattractants have dedicated receptors. Several classes of
141 chemoattractants are known to act on neutrophils. They comprise lipids [e.g. leukotriene B4
142 (LTB₄)], formylated peptides of bacterial or mitochondrial origin [e.g. N-formylmethionine-

143 leucyl-phenylalanine (fMLF) which is frequently used *in vitro*], protein fragments (e.g. C5a
144 and C3a complement fragments) and classical chemokines, which are classed according to
145 their conserved cysteine residues into CC and CXC groupings. Table 1 provides a summary
146 of some major neutrophil chemoattractants together with their receptors. Many chemokines
147 can bind to extracellular glycosaminoglycans (GAGs) expressed by endothelial cells (or
148 outside of the vasculature to the extracellular matrix). This serves to essentially immobilise
149 the gradient, which is important, for example in the context of blood flow [16, 17]. The
150 directional cell movement on immobilised chemoattractants is sometimes referred to as
151 ‘haptotaxis’. Experiments involving the simultaneous application of several chemoattractants
152 *in vitro* established chemoattractants to exist in a hierarchy, with ‘end-target’ attractants (e.g.
153 fMLP or C5a) overruling intermediary chemoattractants (e.g. LTB₄). Unsurprisingly, the
154 signalling pathways employed by intermediary and end-target chemoattractants are non-
155 identical [18, 19].

156

157 **Molecular events regulating neutrophil polarisation.**

158 Chemoattractant-sensing GPCRs are distributed uniformly on the neutrophil’s surface. Both
159 directional and indeed uniform chemokine receptor stimulation of neutrophils in a dish
160 causes them to polarise, that is to say, adopt the morphology of the migrating cell described
161 above, prior to actually migrating in a directional, or random fashion by chemotaxis or
162 chemokinesis, respectively (Fig 1 for a simplified view of a polarised neutrophil).

163

164 On a molecular level, chemoattractant binding induces the G protein coupled chemoattractant
165 receptor to undergo a conformational change that allows it to activate heterotrimeric G
166 proteins, exchanging GDP for GTP on the G α subunit. This in turn induces the release of the
167 G $\beta\gamma$ subunits, so that both G α -GTP and G $\beta\gamma$ can activate downstream effectors, including

168 phospholipase C (PLC) β via $G\alpha$ and $G\beta\gamma$ as well as agonist-activated phosphoinositide 3-
169 kinase (PI3K) by $G\beta\gamma$ subunits [20, 21]. Four agonist-activated PI3Ks are expressed in the
170 neutrophil, PI3K α , PI3K β , PI3K δ and PI3K γ [22]. Of these, PI3K γ is activated directly by
171 $G\beta\gamma$ in concert with Ras-GTP [23], with Ras being activated downstream of PLC β by
172 RasGRP4 [24]. Both PLCs and agonist activated PI3Ks are well known regulators of
173 phosphoinositides, lipid components of cellular membranes, with PLCs catalysing plasma
174 membrane phosphatidylinositol(4,5)bisphosphate [PI(4,5)P₂] hydrolysis to generate inositol
175 trisphosphate (IP₃) and diacylglycerol (DAG), whereas PI3Ks phosphorylate PI(4,5)P₂ in the
176 3' position, generating the lipid second messenger phosphatidylinositol(3,4,5)trisphosphate
177 (PIP₃). Several PI3K isoforms are thought to be involved in chemotaxis, likely at least in part
178 because the individual PI3K isoforms cross-talk extensively [25]. The PI3K pathway
179 provides the mechanistic backdrop to the well-documented PIP₃ polarisation to the leading
180 edge of polarised neutrophils, neutrophil-like cells and dictyostelium [26-28]. Many PI3K
181 effectors are regulators of small GTPases, in particular guanine nucleotide exchange factors
182 (GEFs) and GTPase activating proteins (GAPs). These PIP₃-responsive regulators of small
183 GTPases in polarised neutrophils together promote actin-dependent protrusion, for example
184 by activating Rac1/2 and Arf6 and inactivating RhoA at the cell's pseudopod (reviewed in
185 [29, 30]). Research by many groups into the function of PI3K/PIP₃, and into individual PI3K
186 isoforms in chemotaxis resulted in somewhat contradictory reports. Taken together, this large
187 body of work suggests that individual PI3K isoforms, in particular PI3K γ/δ , regulate
188 chemokinesis and/or chemotaxis in an assay-, substratum- and in the case of human
189 neutrophils likely also priming-dependent fashion [31-38]. *D. discoideum* cells were shown
190 to be able to chemotax poorly even in the absence of any PI3K isoform [39]. Human
191 neutrophils from chronic obstructive pulmonary disease (COPD) patients and the elderly
192 were characterised by excessive PI3K activity and poor chemotactic directionality, and could

193 be rescued with low concentrations of inhibitors of the leukocyte-specific PI3K γ/δ that
194 partially inhibited these enzymes [40, 41]. This suggests that a ‘goldilocks principle’ applies
195 in chemotaxis, whereby too much PI3K activity may be just as disruptive as too little.

196

197 Neutrophil polarisation involves players including the above discussed PI3Ks and their
198 effectors, as well as PIP3 phosphatases. Amongst the numerous phosphoinositide
199 phosphatases that are expressed by leukocytes, the 3’ phosphatase PTEN and the 5’
200 phosphatase SHIP are best understood; both were shown to regulate chemotaxis [18, 26, 42].
201 PTEN-mediated regulation was found to be rather context-dependent, with it being suggested
202 to control chemotaxis in the presence of two opposing gradients, and in distinguishing
203 between end-point and intermediate point chemoattractants [18]. In contrast, SHIP-deficient
204 neutrophils were extremely spread and failed to polarise or chemotax effectively [26, 42].
205 Further important contributions are likely regulated by feed-back loops. For instance, Rho
206 GTPases, actin polymerisation and PIP3 polarisation at the cell’s front act in one such feed-
207 back loop [43-46]. Likely driven by PIP3-dependent Rac GEFs such as P-Rex1/2 and
208 DOCK2 [29], Rac activation has been shown to maintain neutrophil polarity through Hem-1,
209 which assists in polarising the neutrophil by facilitating actin polymerisation and excluding
210 myosin activity at the front of the cells, whilst also promoting Rac activity at the front in a
211 positive feedback loop [47]. The RhoA and Arf6 GAP ARAP3 is being recruited to the
212 plasma membrane in a PIP3 dependent fashion, regulating persistent PIP3 polarisation and
213 chemotactic directionality [48].

214

215 In the presence of uniform chemoattractant, neutrophils polarise randomly. Membrane
216 tension is one factor that has been shown to be involved in the regulation of such neutrophil
217 polarisation. Leading edge protrusions generate strong membrane tensions, thus inhibiting the

218 formation of secondary protrusions elsewhere in the neutrophil, and maintaining persistent
219 polarisation [49]. In neutrophils that make contact with the substratum, a further regulatory
220 input stems from altered membrane curvature. This is thought to be sufficient to break the
221 symmetry of the non-polarised neutrophil, establishing cytoskeletal back polarisation in the
222 adhering neutrophil in a PI4P, SRGAP2 and PIP5K1C90-dependent fashion [50].

223

224 ***In vivo* neutrophil chemotaxis (trafficking)**

225 Neutrophils migrate to new locations at least twice, and potentially more during their short
226 lives. All neutrophil trafficking events have their regulation by chemoattractant-mediated
227 chemotaxis in common. The remaining part of this minireview summarises some of the
228 recent insights into *in vivo* neutrophil chemotaxis in a range of situations (see Fig 2 for a
229 schematic diagram).

230

231 **Neutrophil chemotaxis during mobilisation and homing**

232 Neutrophil differentiation from progenitors occurs in the bone marrow, with 10^7 and 10^{11}
233 neutrophils released into the circulation each day in mouse and human, respectively. The
234 regulation of neutrophil release into the circulation has been elucidated with the help of
235 genetically modified mice. Immature and mature neutrophils are retained in the bone marrow
236 by CXCR4 chemokine receptor expression that is responsive to CXCL12 produced by bone
237 marrow stromal cells. The major mobilising cytokine G-CSF causes downregulation of
238 CXCR4 on neutrophils and of CXCL12 in the bone marrow [51], as well as upregulation of
239 CXCR2. With the CXCR2 agonists CXCL1 and CXCL2 constitutively expressed by bone
240 marrow endothelium, these changes drive neutrophil mobilisation from the bone marrow to
241 the circulation [52].

242

243 Circulating neutrophils under homeostatic conditions are short-lived, persisting in the
244 circulation for only one day before becoming senescent. Senescent neutrophils upregulate
245 CXCR4, which increases their sensitivity to CXCL12 that is expressed in the bone marrow.
246 In this way, senescent neutrophils are recruited or 'home' back to the bone marrow, where
247 they undergo apoptosis for clearance by stromal macrophages [53, 54]. Interestingly, both
248 neutrophil release into the circulation and clearance of senescent neutrophils occur in a
249 circadian rhythm, providing immunity while protecting the host [55, 56].

250

251 **Neutrophil recruitment to inflammatory sites – extravasation**

252 As the first circulating immune cells to be recruited to sites of inflammation, neutrophils
253 present a first line of cellular defense against infections. The initial step of neutrophil
254 recruitment from the circulation into the inflamed tissue is best understood in the inflamed
255 cremaster muscle, a site that is particularly amenable to intravital microscopy. Initially,
256 circulating, non-adhesive neutrophils form L-selectin and $\beta 2$ integrin-mediated interactions
257 on the luminal face of the wall of post-capillary venules [reviewed in [57]]. This is induced
258 by cytokine production (e.g. TNF) by resident macrophages, which in turn causes
259 upregulation of adhesion molecules (P- and E-selectins and integrin ligands including
260 ICAM1) as well as chemokines by the endothelium. Selectin-mediated interactions cause
261 neutrophil rolling along the endothelium, allowing neutrophil interactions with chemokines to
262 take place. Additional chemokine stimulation drives integrin activation and in turn integrin-
263 mediated neutrophil adhesion to the endothelial surface. Immobilisation of the chemokines to
264 the luminal face of the endothelium occurs due to CXCL1/CXCL2/CXCL8 binding to GAGs,
265 carbohydrate moieties that are expressed on the endothelial cell surface. In some
266 circumstances, GAGs not only bind, but transcytose chemokines [16, 17, 58]. GAG
267 chemokine immobilisation efficiency is chemokine-dependent, with chemokine

268 immobilisation avoiding chemokine diffusion despite the blood flow in the vessel. In this
269 way, GAG-dependent chemokine presentation ensures that rolling, but not circulating
270 neutrophils are activated while at the same time directing neutrophils to extravasate at
271 specific sites [59].

272

273 **Neutrophil swarming**

274 Neutrophil-mediated amplification of a chemotactic gradient by neutrophil-mediated release
275 of ‘intermediate-target’ chemoattractants (such as the lipid mediator LTB₄, which was to be
276 found stored in exosomes) can be induced by ‘end target’ chemoattractants such as C5a,
277 bacterial formylated peptides, and cell death, which leads to the release of formylated
278 mitochondrial proteins [60, 61]. This autocrine-paracrine chemoattractant signal
279 amplification loop causes directional collective neutrophil recruitment (‘swarming’) in
280 response to the activation of a leading neutrophil (Fig 2). By generating LTB₄, the leading
281 neutrophil instigates BLT1-mediated activation of the following neutrophils, which in turn
282 generate more LTB₄ [62, 63]. Interestingly, microlesions, such as those caused by the death
283 of individual parenchymal cells have recently been shown to be shielded by resident
284 macrophages. This neatly avoids a neutrophil swarming response to the released formylated
285 peptide and concomitant bystander host injury caused by neutrophil-derived inflammation
286 [64].

287

288 **Neutrophil recruitment to inflamed sites by series of chemoattractants**

289 In recent years it has come to be recognised that neutrophil recruitment to sites of
290 inflammation *in vivo* is regulated by a hierarchical series of chemoattractants. This principle
291 has been shown to hold true in several in models of sterile inflammation and injury. It is
292 illustrated for example by neutrophil recruitment to the inflamed joint of mice in the K/BxN

293 serum transfer model of rheumatoid arthritis. A series of elegant studies performed over a
294 number of years that combined mouse genetics and lately multiphoton intravital signalling
295 elucidated the sequential action of neutrophil chemoattractants in this disease model. Hence,
296 the deposition of immune complexes on the surface of the joint triggers the alternative
297 complement pathway, precipitating C5a generation and subsequent C5a deposition on the
298 luminal surface of the joint vasculature, where it is immobilised in a GAG-mediated fashion.
299 C5a binding to its receptor C5aR1 promotes β 2 integrin activation, causing neutrophils to
300 arrest, spread and crawl on the joint endothelium. C5a also causes neutrophil-driven
301 amplification of the chemotactic gradient by releasing LTB₄, and in turn promoting BLT1-
302 mediated extravasation into the joint tissue by autocrine-paracrine positive feedback loop.
303 Here, immune complex-mediated Fc γ R stimulation causes neutrophils to release IL-1 β . This
304 in turn induces the generation of endothelial cell- and synovial fibroblast-derived CCR1 and
305 CXCR2 chemokine receptors ligands. CCR1 promotes neutrophil crawling on the joint
306 endothelium with neutrophil-generated CXCL2 orchestrating CXCR2-dependent
307 amplification of neutrophil recruitment to the joint [65-68].

308

309 **Optimisation of neutrophil directional migration by atypical chemokine receptors**

310 In addition to G protein coupled chemokine receptors with signalling function, leukocytes
311 and stromal cells also express atypical chemokine receptors (ACKRs; table 1; Fig 2), which
312 do not signal through heterotrimeric G proteins. ACKRs are also known as scavenger or
313 decoy receptors, since some internalise and degrade chemokines, essentially functioning as
314 sinks to limit excessive inflammation [69, 70]. For example, ACKR2 was shown to limit
315 inflammation by reducing neutrophil directional migration to inflammatory chemokines by
316 competing for CCR1 ligands in a neutrophil autonomous fashion [71].

317

318 Neutrophil non-autonomous mechanisms also employ decoy receptors to finely tune
319 neutrophil migration. Unlike other atypical chemokine receptors, ACKR1 optimises
320 leukocyte extravasation by internalising and transcytosing chemokines [72, 73]. Some of the
321 latest studies in this area have coupled high resolution intravital imaging with genetics to
322 demonstrate how atypical chemokine receptors optimise neutrophil recruitment to inflamed
323 sites. Two atypical chemokine receptors were shown to jointly regulate neutrophil
324 recruitment to the inflamed joint in K/BxN serum transfer arthritis. Hence, C5aR2, an
325 atypical C5aR expressed by endothelial cells transports tissue-derived C5a across the
326 endothelium to be exposed on the luminal side, in this way aiding with arresting C5aR1-
327 expressing neutrophils. At the same time, endothelial ACKR1 was shown to transport
328 synovial tissue-derived CXCR2 ligands across the joint endothelium, facilitating neutrophil
329 adhesion and extravasation [74].

330

331 A separate study identified how the two CXCR2 ligands, CXCL1 and CXCL2 sequentially
332 direct neutrophil extravasation in the inflamed cremaster muscle. In this instance endothelial
333 and pericyte GAG-immobilised CXCL1 promoted neutrophil adhesion and crawling, whereas
334 CXCL2 controlled transendothelial migration. Fascinatingly, the source of CXCL1 was
335 endothelial cells and pericytes, whereas CXCL2 was generated and released by neutrophils in
336 another example of a paracrine amplification loop of directional neutrophil migration.
337 Neutrophil-derived CXCL2 was subsequently immobilised by ACKR1 expressed by
338 pericytes at venular cell-cell junctions, supporting the correct directionality of neutrophil
339 transendothelial migration [75].

340

341 **Reverse Migration**

342 To avoid excessive inflammation, neutrophils were long thought to undergo apoptosis,
343 followed by being cleared ('efferocytosed') by resident pro-resolution macrophages at sites
344 of inflammation [76]. Recent observations have, however, suggested that this may not be the
345 only possible fate of the neutrophil in sterile inflammation. Rather than undergoing apoptosis
346 and dying, neutrophils were found to migrate away from a sterile wound in zebrafish larvae,
347 including, on occasion, entering the vasculature [77]. Zebrafish neutrophils express two
348 chemokine receptors, CXCR1 and CXCR2, of which CXCR1 regulates recruitment to the
349 sterile wound, and CXCR2 promotes CXCL8-induced reverse migration, which interestingly
350 occurred by chemokinesis rather than chemotaxis [78], a view shared by a separate study
351 [79]. Interestingly, reverse migration may promote wound healing, since wounds in zebrafish
352 that are genetically deficient in CXCR2/CXCL8 displayed heightened inflammation [78].
353 This view is supported by other observations made in the zebrafish, where retaining zebrafish
354 neutrophils at the wound site and reducing neutrophil apoptosis by inducing HIF1 α was also
355 pro-inflammatory [80]. In a similar vein, tashinone IIA, an active compound from a Chinese
356 medicinal herb, that promoted neutrophil reverse migration was isolated in a zebrafish screen
357 aimed at identifying compounds that would aid the resolution of inflammation [81].
358 Interestingly, unlike their mammalian counterparts, neutrophils in zebrafish larvae are
359 generally tissue resident [82]. Therefore, the term reverse migration refers merely to the
360 direction of migration in the zebrafish, whilst it generally includes the breaching of the vessel
361 wall in the luminal direction (i.e. reverse transendothelial migration) in mammals. Reverse
362 migration has been suggested to occur, too, in humans. This view is controversial, however,
363 with circulating neutrophils that comprise a 'reverse migration signature' (CD54^{hi} CXCR1^{low})
364 being 4-8x more abundant in patients with systemic inflammation than in healthy individuals
365 [83]. Yet, there is evidence to support a potential role of neutrophil reverse migration in the
366 dissemination of inflammation from mouse models, where reverse migrated mouse

367 neutrophils observed after ischemia reperfusion injury augmented instances of inflammation
368 in the lung [84]. A subsequent study identified following ischemia reperfusion injury that
369 neutrophil-derived LTB4 and elastase were responsible for loss of junctional JAM-C,
370 permitting neutrophil reverse (transendothelial) migration, with reverse migrated neutrophils
371 again travelling to the lung to spread inflammation [85]. In a separate study the neutrophilic
372 response to a small localised burn in the liver was observed by intravital microscopy. This
373 induced neutrophil recruitment to the injury site, where neutrophils aided tissue repair,
374 phagocytosing dead tissue. Rather than undergoing apoptosis for phagocytosis at the injury
375 site, neutrophils once more left the wound, employing proteases to re-enter the vasculature by
376 reverse transendothelial migration. They entered the lung, and upregulated CXCR4 prior to
377 homing to the bone marrow for non-inflammatory clearance [86]. Clearly neutrophil reverse
378 migration is a very interesting area which remains to be further investigated and fully
379 understood. Does reverse migration only occur in response to sterile injury and, conversely,
380 is apoptosis at the site of inflammation followed by efferocytosis more typical of neutrophils
381 at sites of infection? Could reverse migration be involved in inducing lung injury under
382 certain but not all instances? It will be exciting to follow new developments in this area in the
383 future.

384

385 **Conclusion**

386 This minireview has highlighted key points of neutrophil chemotaxis, focussing on some
387 molecular events that were shown *in vitro* to regulate neutrophil polarisation and
388 summarising some exciting developments in neutrophil trafficking *in vivo*. The mind-
389 boggling complexity of the regulation of neutrophil chemotaxis is fascinating to the basic
390 scientist and provides evidence of the physiological importance of the process that is being
391 regulated. Meticulous regulation of neutrophil chemotaxis is required to balance neutrophilic

392 inflammation, to ensure adequate host defense while avoiding excessive host damage. As
393 evidenced by rare genetic diseases such as leukocyte adhesion deficiencies, in which $\beta 2$
394 integrins are absent or their signalling dysfunctional, interfering with leukocyte recruitment
395 leaves the body open to recurrent serious bacterial infections. Conversely, certain chronic
396 inflammatory diseases (e.g. rheumatoid arthritis or chronic obstructive pulmonary disease)
397 are characterised by excessive neutrophilic inflammation. Therapeutically targeting
398 neutrophil chemotaxis to alleviate such conditions may be feasible, but could result in
399 reduced host immunity as a trade-off.
400

401 **Summary Points**

- 402 • Chemotaxis is defined as directional cell migration towards a source of chemoattractant,
403 whilst chemokinesis is chemoattractant-induced cell migration in the absence of a gradient.
- 404 • Chemotaxis bears all the hallmarks of random migration, but in addition is characterised by
405 chemoattractant-induced polarisation, and directionality towards a source of chemoattractant
- 406 • Chemoattractants include lipids, peptides, protein fragments and chemotactic cytokines
407 (chemokines). They are classed into intermediary and end-point chemoattractants, and
408 operate in a hierarchical fashion. Chemoattractants signal through G protein coupled
409 receptors. Atypical chemoattractant receptors bind chemoattractant without inducing
410 intracellular signalling.
- 411 • Being amongst the fastest chemotaxing cells in the human body, neutrophils provide a first
412 line of defense against infections.
- 413 • Leukocyte recruitment to a site of inflammation is directed by chemoattractants and
414 therefore corresponds to chemotaxis *in vivo*. This area has been revolutionised by genetic
415 approaches in combination with intravital imaging. Many of the latest insights are concerned
416 with the integration of different chemoattractants by the migrating cell, paracrine
417 amplification loops ('swarming') and reverse migration (ie away from the source of
418 chemoattractant).

419

420

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	<i>Receptor</i>	<i>Chemoattractant</i>	<i>Alternative name</i>	<i>Function</i>
<i>Chemokine Receptors</i>	CXCR2	CXCL1	Gro- α (human) KC (mouse)*	Neutrophil recruitment & activation
	CXCR2	CXCL2	Gro- β (human) MIP2 (mouse)*	Neutrophil trafficking
	CXCR1	CXCL8	Interleukin 8 (IL-8)*	Neutrophil recruitment to sites of inflammation
	CXCR4	CXCL12	Stromal cell derived factor 1 (SDF1)	Bone marrow homing
<i>Chemoattractant Receptors</i>	BLT1	LTB4 (leukotriene B4)		Neutrophil recruitment and swarming
	FPR1 (also known as fMLPR) FPR2	Bacterial and mitochondrial formylated peptides, e.g. fMLF		Neutrophil recruitment
	C5aR	C5a		Neutrophil recruitment (eg in autoantibody induced disease)
	C3aR	C3a		Inhibitor of neutrophil mobilisation
<i>Atypical Chemokine receptors</i>	ACKR1 (formerly Duffy antigen receptor)			Chemokine transcytosis; Haematopoiesis and neutrophil blood counts [87]
	ACKR2 (formerly D6)	Inflammatory CC chemokines		Decoy / scavenger receptor

Michael and Vermeren, Table 1.

Figure and Table Legends.

Table 1. Common neutrophil chemoattractants and their receptors. In addition to chemotactic cytokines (chemokines), which bind to chemokine receptors that signal or atypical chemokine receptors that do not signal, neutrophils express a series of chemoattractant receptors, which bind to lipids, peptide, protein fragments or chemokines. In addition to atypical chemokine receptors (ACKRs), there are also atypical chemoattractant receptors, e.g. C5aR2, see main text. * Note, CXCL8/IL-8 and its receptor CXCR1 are lost in the mouse, where CXCL1/KC and CXCL2/MIP2 and their receptor CXCR2 appear to act as functional homologues.

Figure 1. Molecular signalling events in neutrophil polarisation allowing movement towards the chemotactic gradient. Binding of a chemoattractant to the G-protein coupled chemoattractant receptor induces intracellular signalling to regulate neutrophil polarisation. Polarised neutrophils are characterised by accumulation of PIP3 to the leading edge, where effectors such as Rho GEFs and GAPs promote actin polymerisation. Polarisation is maintained by feedback loops, for example inhibiting RhoA at the pseudopod. The bulky nucleus is used as a ‘mechanical gauge’ that together with the MTOC facilitates the cell’s progress through pores in the interstitium. Trailing end retraction is facilitated by microtubule depolymerisation, activating RhoA and triggering actomyosin contractility in addition to feedback loops involving RhoA, Rac and PTEN.

Figure 2. Neutrophils are controlled by chemotaxis. Clockwise, from top left: *Mobilisation and Homing.* CXCR2 signalling leads to the mobilisation of neutrophils from the bone marrow into the bloodstream, whereas upregulation of CXCR4 in senescent neutrophils promotes chemokine-driven homing back to the bone marrow. *Recruitment.*

Resident macrophages at inflammatory sites release pro-inflammatory mediators that promote selectin-mediated interactions between neutrophils and the endothelium. Neutrophils tether and roll along the endothelium, where GAG-immobilised chemokines guide the neutrophils through G protein coupled receptor signalling, regulating integrin-mediated neutrophil extravasation. *Atypical chemokine receptors* have been shown to aid neutrophil recruitment to sites of inflammation. *Swarming*. Certain end-target chemoattractants cause the release of LTB4 containing exosomes. An autocrine-paracrine feedback amplification loop promotes directional migration of many neutrophils in a 'swarm'. *Chemoattractant hierarchies*. *In vivo* the neutrophil encounters multiple chemoattractants, the response to which must be tightly regulated. For example, neutrophils choose 'end-target' chemoattractants over intermediate chemoattractants. *Reverse migration*. Neutrophil reverse (transendothelial) migration has been observed in many contexts and, perhaps depending on circumstances, may or may not have pro-inflammatory consequences. See text for further discussion.

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MM and SV wrote the paper and drafted the figures.

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Competing Interests

The authors declare that there are no competing interests associated with this manuscript.

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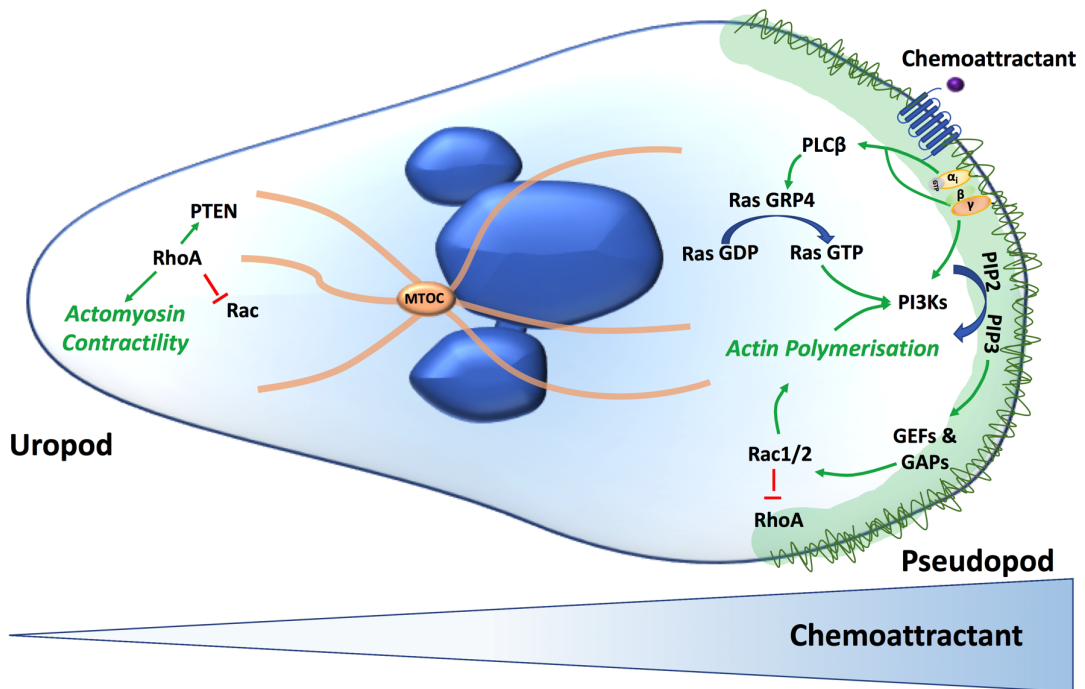
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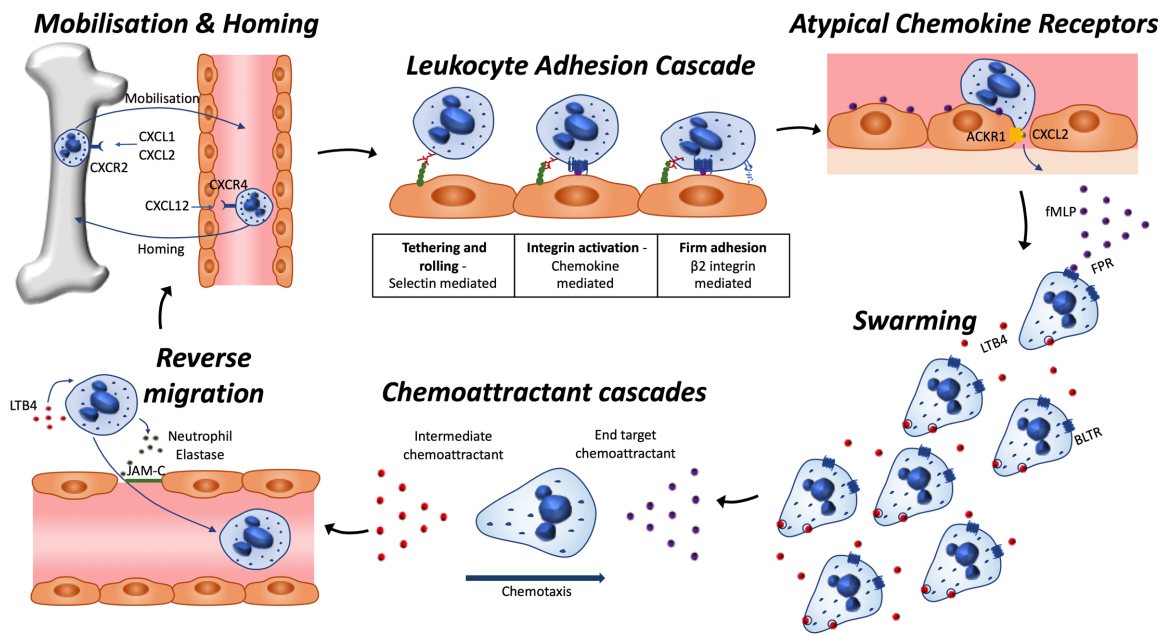
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Michael & Vermeren Fig 1



Michael and Vermeren Fig 2