bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

1 **Analysis of receptor-ligand pairings and distribution of myeloid subpopulations across**
2 **the animal kingdom reveals neutrophil evolution was facilitated by colony-stimulating**
3 **factors.**

4

5 Damilola Pinheiro[1], Marie-Anne Mahwin[1], Maria Prendecki[1] and Kevin J Woollard[1]

6

7 Affiliations

8 1. Centre for Inflammatory Disease, Department of Immunology and Inflammation, Imperial
9 College London. London. W12 0NN

10

11 Corresponding Authors

12

13 Damilola Pinheiro: d.pinheiro@imperial.ac.uk
14 Kevin J Woollard: k.woollard@imperial.ac.uk

15

16

17 **Short title: Analysis of receptor ligand pairing and myeloid cells across animal kingdom**
18 **reveal neutrophils evolved through colony stimulating factors.**

19 Impact statement: Colony-stimulating factors (CSFs) are important for myeloid phagocyte
20 development. The emergence of CSF3/CSF3R in tetrapod lineages has uniquely contributed
21 to physical, functional and structural adaptions observed in mammalian neutrophils.

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

22 Abstract

23 Neutrophils or heterophils constitute the largest population of phagocytic granulocytes in the
24 blood of mammals and birds. The development and function of neutrophils and monocytes is
25 primarily governed by the granulocyte colony-stimulating factor receptor family (CSF3R/CSF3)
26 and macrophage colony-stimulating factor receptor family (CSF1R/IL34/CSF1) respectively.
27 Using various techniques this study considered how the emergence of receptor:ligand pairings
28 shaped the distribution of blood myeloid cell populations. Comparative gene analysis
29 supported the ancestral pairings of CSF1R/IL34 and CSF3R/CSF3, and the emergence of
30 CSF1 later in tetrapod lineages after the advent of Jawed/Jawless fish. Further analysis
31 suggested that the emergence of CSF3 lead to reorganisation of granulocyte distribution
32 between amphibian and early reptiles. However, the advent of endothermy likely contributed
33 to the dominance of the neutrophil/heterophil in modern-day mammals and birds. In summary,
34 we show that the emergence of CSF3R/CSF3 was a key factor in the subsequent evolution of
35 the modern-day mammalian neutrophil.

36

37

38

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

39 ## Introduction

40

41 Phagocytes are key effector immune cells responsible for various biological processes; from
42 orchestrating responses against invading pathogens to maintaining tissue homeostasis and
43 neutrophils are the most abundant population of granulocytic phagocytes present in
44 mammalian blood [1-5]. Neutrophil and heterophils (their non-mammalian counterpart) arise
45 from a shared pool of haematopoietic stem cells and mitotic myeloid progenitor cells that can
46 also differentiate into monocytes, eosinophils, and basophils following exposure to the
47 relevant growth factor [1, 4, 6]. The development and life cycle of mammalian neutrophils
48 through a continuum of multipotent progenitors to a post-mitotic mature cell has been well
49 described and has been recently reviewed [2, 4].

50 The development and function of myeloid phagocytes is mediated through lineage-specific
51 transcription factors and pleiotropic glycoproteins - termed colony-stimulating factors (CSFs)-
52 acting in concert on myeloid progenitor cells. CSF1, CSF3 and their cognate receptors, are
53 lineage-specific and responsible for the differentiation and function of
54 monocytes/macrophages and neutrophils respectively [7-11]. There is a large body of
55 evidence demonstrating the requirement of CSFs for cell development as multiple studies in
56 knockout mice have shown that CSF1R/CSF1 and CSF3R/CSF3 are linked to the
57 development of monocytes and neutrophils *in vivo.* The loss of CSF3 or CSF3R directly affects
58 neutrophil populations resulting in a severe neutropenia, but not the complete loss of mature
59 neutrophils in the models studied [10-12]. The loss of CSF1 caused reduced cavity
60 development of the bone marrow, loss of some progenitor populations, monocytopenia and
61 reduced population of neutrophils in the bone marrow, although interestingly, elevated levels
62 of neutrophils were observed in the periphery [7, 8, 13].

63 Similarly, in humans, single gene mutations have been described in both CSF3 and CSF3R
64 resulting in severe congenital neutropenia (SCN). In contrast to some animal models,

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

65 individuals present as children with early onset life-threatening infections because they lack
66 mature neutrophils in the circulation as the neutrophil progenitors in the bone marrow do not
67 progress beyond the myelocyte/promyelocyte stage [14]. These studies demonstrate that CSF
68 receptor/ligand pairings are essential for homeostatic neutrophil development and are
69 intrinsically linked with neutrophil function, arguably making them ideal surrogates to study
70 neutrophil evolution. Through multiple methods we examined the emergence of the respective
71 CSF ligand and receptor genes and proteins across the Chordate phylum and demonstrated
72 how CSF1R/CSF1 and CSF3R/CSF3 pairings contributed to the evolutionary adaptions of the
73 mammalian neutrophil.

74

75 ## Results

76 **The neutrophilic/heterophilic granulocyte is the predominant granulocyte in the blood**
77 **of mammals and aves.**

78

79 The presence of analogous myeloid granulocytes and agranulocytes was examined by
80 comparing available complete blood count (CBC) data, where applicable, for various animal
81 orders and demonstrated the possible distribution of myeloid cells in blood across evolution.
82 The earliest chordates were represented by two groups, the first; jawed fish (4.10×10^8 MYA),
83 which included; coelacanths, elasmobranchs and whale sharks. Jawless fish containing
84 lampreys and hagfish (3.6×10^8 MYA) represented the second group. The next group
85 represented chronologically was the amphibians (3.5×10^8 MYA); containing anurans and
86 gymnophiona. The reptilian class was represented by three different orders, squamata ($3.3 \times$
87 10^8 MYA), crocodilia (2.4×10^8 MYA) and testudines (2.1×10^8 MYA). The following group
88 was the closely related avian class (7.0×10^7 MYA), which, contained birds from both
89 paleognaths and neognaths. The final class of chordates evaluated was Mammalia, where all
90 three orders; monotremata (1.1×10^7 MYA), marsupial (6.5×10^7 MYA) and placental ($6.25 \times$

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

91 10^7 MYA) were represented. All the species examined for cell distribution, and for the
92 subsequent gene sequence and/or protein homology studies were visualised in a species tree
93 (Figure 1a).

94 All classes within the phylum Chordata were evaluated for populations of neutrophils
95 /heterophils; eosinophils and basophils. Azurophils, a specialised population of granulocytes
96 – analogous to both the mammalian neutrophil and monocyte- but unique to reptiles were also
97 included. A meta-analysis was performed on aggregated data to show absolute counts per
98 cell type per order and finally, the proportional composition of myeloid cells only per cell type
99 (Figure 1b).

100 The presence of all myeloid cells was confirmed in at least one representative species from
101 each order examined. Elasmobranchs- rays and sharks- were unique in having both
102 heterophils (2.009 – 12.42 x10^9, n=3) and neutrophils (0.615 – 0.745 x10^9, n=3). All other
103 orders had either neutrophilic or heterophilic populations only. Heterophils were the
104 predominant granulocyte in Amphibians (the order of Anura; 1.10 – 4.60 x10^9, n=2), Reptiles
105 (the orders; squamata, 2.51 – 16.43 x10^9, n=6; testudines, 2.59 – 4.74 x10^9, n=2; and
106 crocodiles, 2.59 – 3.24 x10^9, n=3) and Aves (0.615 – 0.745 x10^9, n=5). In contrast, neutrophils
107 were the majority population in all three mammalian orders (monotremata, 5.97 – 8.01 x10^9,
108 n=2; marsupalia, 1.64 – 10.30 x10^9, n=4; and placental, 2.04 – 11.50 x10^9, n=5 [Figure 1bi]).
109 Eosinophils and basophils were over-represented in the orders of amphibians, reptiles and
110 aves, when compared to mammals, where they were either a minority population (eosinophils)
111 or absent (basophils) [Figure 1bi].

112 Analysis of the proportional composition of total myeloid white blood cells (WBC) showed
113 interesting changes in the distribution of granulocytes across the different species classes.
114 Again, elasmobranchs were unique amongst all classes as they retained both a majority
115 heterophil population (36.8% – 71.9%, n=3) and a minority neutrophil population (1.6% –
116 32.3%, n=3). The cold-blooded orders; rays (30.0% – 39.8%, n=3), anura (25.6% – 33.7%,
117 n=2), squamata (37.3% – 75.7%, n=6), testudines (23.9% – 54.7%, n=2) and crocodilia

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

118 (32.4% – 52.4%, n=3); retained a large population of neutrophils/heterophils; although
119 proportions of the population varied within related animal orders and between the different
120 species classes (Figure 1bii). In contrast, within the warm-blooded classes and orders; aves
121 (59.6% – 94.3%, n=5), monotremata (83.8% – 88.4%, n=2), marsupial (68.5% – 88.8%, n=4)
122 and placental (70.0% – 85.6%, n=5), the opposite distribution was observed and the
123 neutrophil/heterophil was the most abundant peripheral blood cell type (Figure 1bii).
124 Interestingly, basophils and eosinophils were well represented in the anuran order, 18.3% –
125 44.8%, n=2, and 8.6% – 37.7%, n=2, respectively. A similar pattern was observed in the
126 reptilian lineage; however, there were differences between orders as squamata (2.9% –
127 20.8%, n=6) and crocodilia (5.3% – 23.4%, n=3) had lower proportions of eosinophils
128 compared to testudines (27.7% – 47.6%, n=2). Conversely, basophils were elevated in
129 crocodilia (31.1% – 33.6%, n=3) compared to squamata and testudines (3.4% – 24.9%, n=6
130 and 1.2% – 2.19%, n=2 respectively) [Figure 1bii]. Suggesting that within closely related
131 orders that share similar habitats or environments, individual species favoured different
132 configurations of myeloid WBCs. Eosinophils were present as a small population in the
133 mammalian lineage, again variation between the respective classes was observed as
134 monotremata had a smaller proportion of cells (4.9% – 6.7%, n=2) compared to the marsupial
135 and placental orders, which had similar values (5.1% – 20.3%, n=4 and 3.8% – 20.8%, n=5
136 respectively). In contrast, compared to all the other classes, basophils seemed to have been
137 largely lost from some of the mammalian classes, as low proportions were observed in
138 monotremata (0.6% – 0.6%, n=2), marsupial ((0% – 1.85%, n=4) and placental classes (0% –
139 6.2%, n=5) [Figure 1bii].

140 Monocytes are blood agranulocytic cells that are closely related to neutrophils sharing both
141 growth and survival factors, early development pathways with common progenitors, and in
142 some instances, effector functions in the respective terminally differentiated cells [5, 15, 16].
143 Interestingly, the monocyte population peaked both in terms of number and proportion within
144 the reptilian class, in the orders; squamata (0.6 – 12.1 x10^9, n=6 and 15.4% – 38.5%, n=6

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

145 respectively) and testudines (2.9 – 15.5 x10^9, n=2 and 7.8% – 27.0%, n=2 respectively). The
146 azurophil – a specialised myeloid cell population- peaked in the crocodilian lineage (4.6 – 11.1
147 x10^9, n=3 and 0.2% – 0.9%, n=3 respectively), however; it remains unclear whether to classify
148 the azurophil as a distinct cell type, as we have done, or as a subset of monocyte or neutrophil.
149 Aves is the most closely related phylogenetic group to reptiles as they share a recent common
150 ancestor. Although Aves has lost the azurophil, they retain a small population of monocytes
151 (2.4% – 11.7%, n=5). Interestingly, a similar proportion is observed in the more distantly-
152 related mammalian class, across all three orders (monotremata (6.2% – 8.8%, n=2),
153 marsupial (5.1% – 10.2%, n=4) and placental (2.5% – 10.4%, n=5), suggesting that the advent
154 of endothermy may have played a role in the distribution of monocytes in warm-blooded
155 animals [Figure 1bii] In summary, we show that the majority of peripheral myeloid blood is
156 comprised of granulocytes in phylum Chordata, although the proportion of basophils,
157 eosinophils and neutrophils varies according to the respective orders and lineages. By the
158 advent of mammals and birds however, the neutrophil has become the predominant
159 granulocyte of the blood.

160

161

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

162 **Interrogation of CSF1/CSF1R and CSF3/CSF3R and C/EBP gene family reveals their**
163 **loss in the early lineages.**

164 The development and maturation of a neutrophil from a multipotent progenitor to a lineage
165 committed post-mitotic cell is driven by CSF3 working in concert with a number of transcription
166 factors, including Pu.1, GF1I and Runx, whose roles have been well established [4, 16]. One
167 of the most heavily involved family of transcription factors in neutrophil development is the
168 CCAT enhancer binding-protein family (C/EBP), comprising six members (C/EBPα, β, δ, γ, ε
169 and τ) [16-19]. Individual members are a necessary requirement for the different stages of
170 granulopoiesis under homeostatic and inflammatory conditions. Studies have shown that
171 *c/ebpα*$^{-/-}$ mice are neutropenic, whereas *c/ebpε*$^{-/-}$ mice lack functionally mature neutrophils,
172 thus demonstrating that *c/ebpα* and *c/ebpε* have distinct roles in the early (myeloid progenitor
173 progression) and later (maturation) stages of neutrophil development [16-19]. Having
174 established, the presence of the neutrophil/heterophils across the phylum Chordata, we then
175 evaluated the presence of CSFR/CSF and C/EBP genes in the different animal classes to
176 further understand the emergence of granulopoiesis in phylum Chordata.

177 Gene data for fifty-nine species was collected from the NCBI Gene and Ensembl databases
178 for the following genes; CSF1R, IL34, CSF1, CSF3R, CSF3, C/EBPα, C/EBPβ C/EBPδ and
179 C/EBPε and used to generate a heat map. Interestingly, CSF1R, CSF3R and CSF3 are absent
180 from the cartilaginous fishes; the Australian Ghost shark and the Whale shark (Figure 2a). In
181 contrast, the Coelacanth - another early lineage - retained CSF1R, IL34, CSF3R and CSF3
182 genes. CSF1 was absent from all the jawed fishes although IL34 was present. A similar pattern
183 was observed in the Jawless fish, CSF1R, IL34 and CSF3R were present in the Hagfish,
184 whereas CSF1 and CSF3 were absent. CSF1R was the only the receptor present in the
185 lamprey and all other CSF receptor and ligands were absent (Figure 2a). The Jawed/Jawless
186 fish accounted for the largest loss of genes within in an animal order as generally; CSF1R,
187 IL34, CSF1, CSF3R and CSF3 were largely all present in amphibia, reptilia, aves and
188 mammals. The exception being in the reptilian order of squamata, where IL34 had been lost.

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

189 There were also examples of gene loss at the species level, CSF1 was absent from the Gharial
190 and Ostrich; however, it had not been lost overall in the respective orders of crocodilia and
191 struthioniformes (Figure 2a).

192 The Jawed/Jawless fishes were again the most diverse in terms of coverage within the C/EBP
193 gene family. The Coelacanth had present all members examined (C/EBPα, C/EBPβ, C/EBPδ
194 and C/EBPε), the Hagfish had three members (C/EBPα, C/EBPβ, and C/EBPε) and the
195 Australian Ghost shark (C/EBPα and C/EBPε) and the Whale shark (C/EBPβ and C/EBPδ)
196 had two members each. Finally, the Lamprey had only one C/EBP gene present, C/EBPβ
197 (Figure 2a). Within the other animal orders, C/EBP gene loss was largely restricted to
198 individual species, such as the loss of C/EBPβ in the Eastern Brown snake, Central Bearded
199 dragon and Gecko; however, it was not lost across the whole order of Squamata. In the order
200 of testudines, both C/EBPδ and C/EBPε or C/EBPε only, had been lost from the Green Sea
201 Turtle and Chinese Softshell turtle respectively (Figure 2a). Similarly, in the Crocodilian order,
202 the Gharial (C/EBPδ and C/EBPε), Australian Saltwater Crocodile (C/EBPδ and C/EBPε), and
203 Chinese Alligator (C/EBPδ and C/EBPε) all had two different C/EBP genes present, in contrast
204 to the American alligator, which retained the complete set (C/EBPα, C/EBPβ, C/EBPδ and
205 C/EBPε). Interestingly, while the C/EBPε gene was missing from all birds examined, the
206 ostrich appeared to have no C/EBP gene family members present and the turkey only retained
207 C/EBPα (Figure 2a). Finally, there was some individual species-specific loss of C/EBP genes
208 in the mammalian class, with examples of C/EBPα and C/EBPβ loss being observed in some
209 members (Figure 2a). C/EBPε, which is required for the later stages of mammalian neutrophil
210 development was present across the order (Figure 2a). These results suggested that there
211 were local factors determining the retention and loss of genes as well as a strong level of
212 functional redundancy among the C/EBP gene family.

213 Interestingly, within the early lineages of the jawed fishes, IL34 was the only gene retained by
214 all representatives and the Coelacanth was the only species to have CSF1R, CSF3R and

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

215 CSF3 present. Given that these species all share a close common ancestor, this would
216 indicate that there had been local gene loss in both the Whale shark and Australian Ghost
217 shark. CSF1 was absent from all lineages and it was unclear as to whether this absence was
218 due to loss or that CSF1 had not yet evolved. To further address this, we used syntenic
219 methods to manually map out the orthologous gene locations in the Jawed fish. Syntenic maps
220 were generated for the CSF1R (CSF1R, IL34, CSF1) and CSF3R (CSF3R and CSF3) family
221 of genes using the human orthologue as a reference point (Figure 2b).

222 In humans, the CSF1R gene is located downstream of the HMGBX3 gene and upstream of
223 PGDFRB (a gene paralogue of CSF1R) and CDX1. A similar orientation is observed in the
224 Coelacanth, where CSF1R is downstream of HMGBX3 and adjacent to PDGFRB and CDX1.
225 The orientation is inversed in the Australian Ghost shark, where PDGFRB and CDX1 co-
226 localise together upstream but HMGBX3 and CSF1R have been lost (Figure 2b). Interestingly
227 in the Whale shark, CSF1R and all the flanking genes are absent, suggesting that this section
228 in its entirety may have been lost (Figure 2b). Human IL34 co-localises with FUK, COG4 and
229 SF3B3 upstream, and the MTSS1L and VAC14 genes immediately downstream. Both the
230 Coelacanth and the Whale shark have partially retained the syntenic combination but not in
231 the same location. IL34 is situated immediately downstream of COG4 and SF3B3, whereas
232 MTSS1L and VAC14 are located elsewhere on the Coelacanth chromosome (Figure 2b). In
233 the Whale shark, IL34 is immediately adjacent to MTSS1L and these genes are both
234 downstream of FUK and COG4, which have co-localised next to each other (Figure 2b). The
235 gene arrangement of the Australian ghost shark most closely resembles the human, as FUK,
236 COG4, SF3B3, MTSS1L and VAC14 all co-localise to the same region on the chromosome
237 and the absence of IL34, suggest it has been loss in the process of a local gene rearrangement
238 (Figure 2b). The CSF1 human orthologue has formed a contiguous block with AHCYL1 and
239 STRIP1 and is flanked upstream by AMPD2 and EPS8L3. Interestingly although CSF1 is
240 absent from all members, the flanking genes have largely been retained. Similar to the human
241 arrangement, in the Coelacanth; AMPD2 and EPS8L3 co-localised together, while AHCYL1

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

242 and STRIP1 are located in close proximity in a different location (Figure 2b). In both the
243 Australian Ghost and Whale Sharks, AHCYL1 and AMPD2 are located together, STRIP1 is
244 located elsewhere in the Australian Ghost shark and has been lost entirely from the Whale
245 shark (Figure 2b). These lineages arose early in evolution but have very similar synteny
246 structure to the later emerging human chromosome, suggesting that the CSF1 gene entered
247 this location in an ancestor that emerged after the jawed fish.

248 A similar pattern emerged in the CSF3 family of proteins when analysed. In humans, the
249 CSF3R gene is located downstream of STK40, LSM10, OSCP1 and MRPS15. The
250 Coelacanth is the only species to retain CSF3R and that is located immediately adjacent to
251 MRPS15, however STK40, LSM10 and OSCP1 have been lost. In contrast, although both the
252 Whale shark and Australian Ghost shark have presumably lost the CSF3R gene, they have
253 retained the four other flanking genes, either in one location as in the Australian Ghost shark,
254 or distributed along the chromosome, as in the Whale Shark (Figure 2b). Human CSF3 co-
255 localises downstream of GSDMA and PSMD3 and is adjacent to MED24. Again, a similar
256 arrangement is observed in the Coelacanth, with CSF3 flanked by PSMD3 and MED24
257 upstream and downstream respectively. As before, the Whale shark and Australian Ghost
258 shark are similar in their gene arrangements as GSDMA and PSMD3 have been lost, and the
259 only gene retained in the Australian Ghost shark is MED24 (Figure 2b). Intriguingly, in the
260 Whale shark, MED24 co-localises with an IL6-like gene, which could be a functional paralogue
261 of CSF3 (Figure 2b). These results suggest there is more than one receptor ligand family
262 involved in the development and maturation of heterophils and neutrophils in the early
263 lineages. Taken together, these data suggest that CSF3R/CSF3 and CSF1R were present in
264 a common ancestor to early lineages and there have been so local losses in selected
265 members. CSF1 appears to have evolved independently of its cognate receptor, after the
266 emergence of Jawed/ lineages and prior to the advent of the tetrapod lineages.

267

268

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

269 **Analysis of Chordate orthologous protein homology further supports the ancestral**
270 **pairing of CSF1R/IL34 and CSF3R/CSF3 in early lineages**

271

272 The syntenic analysis established the presence of the CSF1R and CSF3R gene families in
273 Chordates. Interestingly, CSF1R/IL34 and CSF3R/CSF3 had already evolved by the
274 emergence of Chordates, as evidenced by their existence in some of the early lineages of
275 Jawed/Jawless fish. However, the gene data in isolation did not provide a complete
276 understanding and additional analysis was needed. Orthologous proteins are considered to
277 have the same function in different species and therefore, it's broadly assumed that the
278 proteins will be largely conserved at the primary and structural levels [20]. To further elucidate
279 the evolutionary process, we compared the shared sequence similarity of the orthologous
280 CSF1R and CSF3R protein families, as this data is widely available. The protein sequences
281 for the following proteins; CSF1R, IL34, CSF1, CSF3R and CSF3 for fifty-nine species were
282 collated from the NCBI Protein database. The shared sequence similarity for each individual
283 sequence was generated using the NCBI BLAST engine tool and the relevant human
284 orthologue submitted as the query. The resulting data was then plotted in groups as identified
285 by animal order and visualised in a bar chart (Figure 3a).

286 The level of shared sequence similarity observed in the receptors, and therefore conservation
287 of the primary protein sequence, varied across the different animal orders. The highest levels
288 of shared sequence conservation for both CSF1R and CSF3R were observed in the placental
289 mammals (CSF1R; 86.5%, CSF3R; 81.6), which would be anticipated as the human is a
290 member of this group. The lowest level of shared sequence similarity was observed in the
291 Jawed/Jawless fish (CSF1R; 46.9%, CSF3R; 40.0%) (Figure 3a). Interestingly, the CSF1R
292 and CSF3R protein sequence values from early mammals, monotremata (CSF1R; 60.6%,
293 CSF3R; 53.8%) and marsupalia (CSF1R; 60.8%, CSF3R; 53.1%), ranged between the earlier
294 orders; Aves (CSF1R; 51.4%, CSF3R; 44.4%), Testudines (CSF1R; 53.3%, CSF3R; 46.4%),
295 Crocodilia (CSF1R; 52.8%, CSF3R; 45.2%), Squamata (CSF1R; 47.8%, CSF3R; 41.5%),

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

296 Amphibia (CSF1R; 47.2%, CSF3R; 39.4%) and placental mammals (Figure 3a). While there
297 is not a clear consensus as to what the minimum percentage of shared sequence similarity
298 needed to correlate with conservation of function is, it is notable that in this dataset for both
299 CSF1R and CSF3R - independently of each other- the baseline value of shared sequence
300 similarity was approximately 40% (Figure 3a).

301 The syntenic analysis also demonstrated that of the three ligands, IL34 emerged the earliest
302 and this was reflected in the protein data. As observed with the receptors, the highest level of
303 shared sequence similarity is in the placental mammal group (82.5%), and the lowest in the
304 Jawed/Jawless fish (38.8%) (Figure 3a). The IL34 protein sequence values from early
305 mammals; monotremata (50.0%) and marsupalia (50.2%), grouped very closely with the
306 earlier orders of; Aves (48.2%), Crocodilia (43.6%), Testudines (41.9%), Amphibia (38.0%)
307 and the Jawed/Jawless fishes (Figure 3a). IL34 was absent from the members of Squamata
308 examined. CSF3 is the next best conserved ligand, which again agreed with the synteny data.
309 The highest level of shared sequence similarity was in the placental mammal group (82.6%),
310 and the lowest in the Jawed/Jawless fish (30.2%) (Figure 3a). The CSF3 protein sequence
311 values were spread among the orders from monotremata (46.9%) and marsupalia (51.2%) to
312 Aves (41.0%), Crocodilia (35.8%), Testudines (38.2%), Squamata (30.5%) and Amphibia
313 (34.1%) (Figure 3a). Finally, CSF1, which is completely absent in Jawed/Jawless fish, had
314 similar values to CSF3, in terms of shared sequence similarity across the respective groups
315 of; placental mammals (80.0%), monotremata (46.5%), marsupalia (40.0), aves (32.2%),
316 Crocodilia (26.5%), Testudines (34.3%), Squamata (32.1%) and Amphibia (29.5%).
317 Intriguingly, the 40% baseline is applicable to the ligand data. In IL34, which is the oldest
318 ligand, the shared sequence similarity for all groups was above 40%. Whereas for both CSF3
319 and CSF1, which emerged later, in the majority of early groups the shared sequence similarity
320 was under 40% (Figure 3a).

321 The analysis of orthologous CSF1R and CSF3R protein families illustrated that the
322 mammalian proteins- largely within the placental mammals- had changed considerably

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

323 compared to the other lineages and therefore were excluded from subsequent analysis. To
324 further interrogate how the respective CSF1R and CSF3R families co-evolved in the early
325 lineages, the shared sequence similarities for each order were plotted against time (Figure
326 3b). Interestingly, the trajectories for CSF1R and IL34 and CSF3R and CSF3, largely tracked
327 to each other. This suggested that they evolved at a similar pace across the same period of
328 time and is indicative of evolutionary pressure restricting changes to cognate receptors and
329 ligands. As expected, CSF1 did not have the same restrictive pattern in the earlier lineages
330 as it emerged much later (Figure 3b). These results supported the early emergence of CSF3R,
331 CSF1R and IL34 of the respective ligands and receptors. Interestingly, although CSF3
332 developed later than CSF3R, the two have co-evolved in step together. In contrast, while
333 CSF1 would appear to be the principal ligand of CSF1R in mammals, the data supported the
334 ancestral pairing of IL34 and CSF1R in early lineages.

335

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

336 **The emergence of CSF3R/CSF3 and onset of endothermy likely influenced the**
337 **distribution of neutrophils in Chordates during evolution.**

338

339 The mammalian neutrophil shares many developmental and functional properties with
340 counterparts present in other animal orders such as phagocytosis, oxidative burst,
341 degranulation and cell motility [21, 22]. However; a notable species-specific difference is the
342 of distribution of the neutrophil/heterophil within peripheral blood, which suggests that
343 evolutionary pressures might also be involved. To further address this, we reconstructed a set
344 of related timescales plotting the percentage distribution of chordate myeloid WBC and the
345 percentage of protein shared sequence similarity of chordate CSFR/CSF *versus* time. To
346 simplify the model, the timescales were plotted chronologically, where applicable, and the
347 emergence point of the earliest member within an animal order was used. The timescales
348 were then divided into two distinct periods; the first focussed on the early lineages of Jawed/
349 Jawless fish, Amphibia and early reptilia and the second focussed on all reptilia, aves and
350 mammalia. We then considered the possible impact the emergence of CSF3 had on cell
351 distribution across the evolutionary timescale (Figure 4).

352

353 The synteny studies demonstrated that CSF1R and IL34 were already in existence prior to the
354 appearance of the Jawed/Jawless fish lineages. However, CSF3R and CSF3 were only
355 present in the Coelacanth and although an IL6-like paralogue was observed in the Whale
356 shark, a cognate receptor was not identified. These results suggest a greater level of diversity
357 in the growth factors responsible for WBCs in these lineages and is reflected in the distribution
358 of WBCs in Jawed/jawless fish, where heterophils, neutrophils, monocytes, eosinophils and
359 basophils are all present. While heterophils largely dominate, there was a more even
360 distribution of neutrophils and monocytes, with minority populations of basophils and
361 eosinophils (Figure 4ai). By the advent of amphibia, distinct neutrophil and heterophil

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

362 populations had seemingly been lost in favour of a single neutrophil or heterophil population.
363 The amphibian order was the only lineage to demonstrate a largely even distribution of
364 neutrophils, eosinophils, basophils and monocytes, although there was a slight decline
365 compared to the levels observed in Jawed/Jawless fish (Figure 4ab).

366 At some stage during tetrapod evolution both CSF3 and CSF1 emerged and haematopoiesis
367 largely transitioned to the tissue-specific compartment of bone-marrow, which likely had
368 implications for WBC distribution in subsequent orders. In early reptilia, represented by the
369 squamata order of lizards, the neutrophil becomes the dominant granulocyte and there is
370 concomitant reduction in basophils and eosinophils. Interestingly, there is an increase in
371 monocyte populations, which coincides with the emergence of the monocyte/macrophage
372 specific growth factor CSF1 (Figure 4ai). These changes occur in ectothermic species
373 suggesting that this is more in response to cell-intrinsic factors, rather than external factors
374 such as environment (Figure 4ai).

375 We hypothesised that the emergence of CSF1, CSF3 and CSF3R could be one of the cell
376 intrinsic factors as it facilitated the transition of haematopoiesis to the bone marrow through
377 modulating cell mobility. CSF3 and CSF3R in particular, are known to play prominent roles in
378 co-ordinating cell movement out of the bone marrow by disrupting the respective [C-X-C
379 chemokine receptor type 4 (CXCR4) / [C-X-C] motif ligand 12 (CXCL12) retention and
380 CXCR2/CXCL2 egress axes [1, 23, 24]. Although, CSF1R is mostly expressed by monocytes
381 and CSF3R by neutrophils [7, 10], multiple studies have shown that there is receptor cross
382 expression between them. Classical monocytes, the predominant monocyte population in
383 humans, express low levels of CSF3R protein and can be mobilised from the bone morrow in
384 response to exogenous CSF3 [25-27]. Similarly, CSF1R transgene expression has been
385 previously described both in *ex vivo* and cultured murine neutrophils [28]. We hypothesized
386 that the cross expression could be part of an evolutionary conserved mechanism that allowed
387 myeloid cells to modulate the speed of their movement and examined this further in preliminary
388 *in vitro* studies (supplementary Figure 1). Although there was low baseline CSF3R expression

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

389 (supplementary Figure 1a, monocytes that were cultured in CSF3 for 72 hours moved at
390 speeds comparable to that of freshly isolated neutrophils in *in vitro* mobility assays
391 (supplementary Figure 1b). Conversely, monocytes that were cultured in CSF1 moved the
392 slowest of the populations examined, suggesting that CSF1 and CSF3 signalling pathways
393 have contradictory effects on myeloid cell velocity (supplementary Figure 1b).

394 Intriguingly, we identified CSF3R expression in a minority population of $CXCR4^{hi}$ neutrophils
395 and negligible expression was observed on the $CXCR4^{int}$ and $CXCR4^{lo}$ populations ex *vivo*
396 (supplementary Figure 1c). CXCR4 expression is upregulated on neutrophils when they home
397 to the bone marrow as well as on populations that are retained in the bone marrow [23, 24]-
398 a microenvironment in which presumably minimal cell movement is required. Therefore, we
399 hypothesised that if this was an intrinsic property of the cell, CSF1R expression would be
400 upregulated and CSF3R downregulated on $CXCR4^{hi}$ neutrophils generated *in vitro*.
401 Neutrophils were cultured in media supplemented with 10% Foetal bovine serum (FBS) for 24
402 hours, which induced expression of CXCR4 and exogenous CSF3 (20ng/ml) was added to a
403 separate control stream (Supplementary Figure 1d). In all cultures, distinct $CXCR4^{lo}$ and
404 $CXCR4^{hi}$ populations were present after 24 hours and CSF1R was only observed in the
405 $CXCR4^{hi}$ populations with a concomitant reduction in CXCR2 and CSF3R (supplementary
406 Figure 1d). The reason for the induction of CSF1R by this subset of neutrophils remains
407 unclear, however, it appears to be cell intrinsic as the presence of exogenous CSF3 had no
408 effect on the induction of expression. While more experimental data is needed, our preliminary
409 in-vitro studies taken together suggest that the ability of neutrophils and monocytes to adjust
410 their velocity, potentially through switching of CSFR expression may have conferred a
411 functional adaption that then facilitated the increase of monocyte and neutrophil populations
412 during the transition from amphibia to early reptiles.

413 The reptilian lineage comprised three orders; squamata, crocodilia and testudines, who
414 emerged over a large timescale. Accordingly, there are many intra-order differences such as
415 the environments in which members live; i.e. in the water *versus* on land or the presence or

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

416 absence of limbs or exoskeletons. Interestingly, the neutrophils seemingly peaked in
417 Squamata as proportions steadily declined with the lowest levels observed in Testudines
418 (Figure 4bi). In contrast, Eosinophil levels, which remained low across the squamata and
419 crocodilia classes, peaked in testudines. However, across this period in time; CSF1R/CSF1
420 and CSF3R/CSF3 had become established as blood myeloid cell-specific factors and were
421 unlikely to be driving the changes in WBC distribution (Figure 4bi). Notably, as the neutrophil
422 populations declined, both the basophil and eosinophil populations increased, with basophils
423 peaking in the Crocodilia lineage before a dramatic decline in testudines. Suggesting,
424 environmental pressure on the immune response of certain orders favoured either the basophil
425 or the eosinophil at the expense of neutrophils and the other granulocyte (Figure 4bi). The
426 decline was not only restricted to neutrophil population, as the monocyte population had a
427 similar pattern. Monocytes were at their highest levels in squamata before a rapid decline in
428 crocodilia and becoming almost completely absent from Testudines. At the same time, the
429 azurophil emerged effectively taking over the role of the monocytes in crocodilia and
430 testudines (Figure 4bi).

431

432 Interestingly, by the advent of testudines, neutrophil/heterophils are no longer the dominant
433 granulocyte, although granulocytes overall still represent the largest proportion of myeloid
434 WBC (Figure 4bii). However, as the orders of aves, monotremata and marsupalia emerge
435 there is a noticeable change. A limitation of visualising data in this form is the implication of
436 the distinct sequential evolution of different animal orders. However; it's likely there would
437 have been a degree of overlap between the emergence of distinct orders. Therefore, it is
438 noticeable that there was a rapid increase in the both avian heterophil population and
439 monotremata neutrophil population compared to testudines (Figure 4bii). The increase in
440 neutrophils was mirrored by an equally rapid decrease in eosinophils in both Aves and the
441 mammalian orders (Figure 4bii). Neutrophils and eosinophils also share early common
442 progenitors as part of the development pathway in the bone marrow, therefore neutrophil

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

443 production appeared to be dominating the cell machinery. Interestingly, the switch to the
444 predominance of the neutrophil over the eosinophil, coincides with the emergence of
445 endothermy, strongly suggesting that external factors are behind these changes in myeloid
446 WBC distribution as there are no noticeable changes in the distribution of the CSF1R/CSF1
447 and CSF3R/CSF3 ligand/receptor pairings. However, the impact is filtering down to the
448 gene/protein level as there are changes in the shared sequence similarity of CSF3, as it
449 increases more rapidly between Testudines and Monotremata, than in the period between
450 Squamata and Testudines. Interestingly, these changes were not reflected in the CSF3R
451 trajectory (Figure 4bii) Presumably, the arrival of endothermy resulted in the appearance of
452 novel pathogens for which a neutrophil-mediated response was more appropriate than an
453 eosinophil-mediated one, leading to reductions in the eosinophil population and virtual
454 disappearance of basophils (Figure 4bii). The monocyte population had re-emerged as a
455 single population by the appearance of aves and the distribution remained consistent in both
456 the avian and mammalian orders (Figure 4bii). Intriguingly, the greatest period of change for
457 myeloid WBC distribution is between testudines and monotremata. However, the equivalent
458 period for protein homology happens much later between the mammalian orders of marsupalia
459 and placentalia (Figure 4bii) and is common across all CSFR/CSF pairings. Therefore, as
460 WBC function and distribution had previously been established in early lineages, this
461 suggested that another external factor was responsible for the rapid change, possibly the
462 emergence of internalized pregnancy (Figure 4bii).

463

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

464 **Discussion**

465

466 The mammalian neutrophil is a highly specialised cell that acts as a first responder to insults
467 against a host immune system as well as acting in an equally important sentinel role [2, 3].
468 They are functionally conserved across phylum Chordata and constitute the largest population
469 of myeloid cells in birds and mammals. In humans, for example, up to one billion neutrophils
470 per kilogram of body weight are produced in the bone marrow each day [4]. The immune
471 response has evolved in such a way as to be able to respond efficiently to a variety of threats,
472 and it is interesting that a resource such as the bone marrow should be expended on the
473 production and maintenance of the relatively short lived neutrophil at the expense of the
474 eosinophil and basophil. The timescale modelling demonstrates that prior to the advent of
475 tetrapoda lineages, the neutrophil was in a pool of different WBC populations at the disposal
476 of the jawed/jawless fish. However, the appearance of CSF3, altered the distribution and with
477 each emerging animal order, a different granulocyte was favoured, presumably, for cell-
478 specific adaptations in response to environmental challenges. Thus, suggesting that
479 CSFR1R/CSF1 and CSF3R/CSF3 signalling conferred adaptions on the neutrophil that
480 proved evolutionarily advantageous.

481

482 The bone marrow is an essential site for granulopoiesis and myelopoiesis. As multipotent
483 haematopoietic stem cells (HSCs) progress through different vascular niches within the bone
484 marrow they sequentially lose their potential to form other lineages in response to
485 environmental cues from bone marrow dwelling macrophages and stromal endothelial cells
486 [3, 4, 29]. The emergence of CSF1 in tetrapod lineages, that evolved after Jawed/Jawless fish,
487 lead to the appearance of bone and bone marrow. A consequence of this was the gradual re-
488 organisation of haematopoiesis away from existing haematopoietic tissues, such as the
489 eosinophil rich Leydig organ of sharks to tissue-specific compartments in the bone marrow.
490 CSF1, in conjunction with another factor, receptor activator of nuclear factor-κB ligand

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

491 (RANKL), co-ordinate the reabsorption of old bone through haematopoietically derived
492 osteoclasts to allow the generation of new bone. CSF1 and RANKL (which emerged at a
493 similar point in evolution) orchestrate bone-remodelling through their respective receptors,
494 CSF1R and RANK [30]. This suggests that by the time a bone structure had evolved in
495 amphibia, the bone marrow had become the principle site of haematopoiesis, although they
496 are some exceptions within various anurans [31].

497

498 In contrast to peripheral blood, CSF3 is expressed on a number of cells in the bone marrow
499 including; neutrophils, monocytes, B cells, myeloid progenitors and HSCs [29, 32-34].
500 Although CSF3 can likely act directly on HSCs through their receptor, it is believed to indirectly
501 mobilise HSCs through a monocytic intermediary that secretes CSF3, which leads to
502 suppression of the CXCR4:CXCL12 axis, alteration of the bone marrow niche, and the
503 subsequent release of HSCs [29, 32-34]. In a similar process, CSF3 can suppress B cell
504 lymphopoiesis by again targeting CXCL12 and suppressing other B cell tropic factors or
505 stromal cells that favour the lymphoid niche [35]. Thus, the emergence of CSF3R/CSF3
506 conferred the adaption, or advantage, of control of the biological machinery i.e. CSF3 provides
507 a mechanism through which haematopoiesis can be shaped and deployed in favour of
508 maximal neutrophil generation. This broadly aligns with the neutrophil starting to dominate
509 peripheral populations in the transition from Amphibia to the lizards of early Squamata
510 following the appearance of bone.

511

512 Mammalian neutrophil production occurs in the haematopoietic cords present within the
513 venous sinuses and the daily output is approximately $1.7x10^9$/kg [36]. As our data demonstrate
514 high levels of neutrophil output are common across warm-blooded chordates and
515 neutrophil/heterophils are the predominant granulocyte in birds and mammals, in what could
516 be considered an example of convergent evolution. One of the fundamental requirements of

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

517 the host innate immune response is to be able to respond rapidly to perceived threats that can
518 be present at any site in the body, which requires the cellular arm to be constantly present and
519 available. Theoretically this can be achieved in the steady state by having either low volumes
520 of long-lived cells or high volumes of short-lived cells circulating in the periphery. As a
521 consequence, there are potential biological trade-offs when considering each setting, firstly;
522 the longer a cell survives in a periphery, the more effort is required by the host to maintain its
523 survival in terms of providing appropriate cues and growth factors. Secondly, while a short-
524 lived cell does not need as much host input for survival, a high turnover is required in order to
525 ensure it doesn't compete for growth factors with other cell types or cause damage by being
526 retained beyond its usefulness. The latter setting fits with the observed neutrophil life cycle
527 and could be considered an evolutionary adaption. The CSF3R/CSF3 signalling pathway is
528 essential for generating high neutrophil numbers without adverse effects to the host.

529

530 The neutrophil has a short circulatory half-life in the steady state of approximately one day [3,
531 4], although this does remain controversial as some estimates of the circulatory lifespan are
532 as much as five days [37]. By contrast, a mature eosinophil has a short circulatory lifespan of
533 approximately 18 hours before relocating to the tissue, where it will survive for a further 2-5
534 days [38]. Similarly, intermediate and non-classical monocytes can survive in the periphery for
535 four and seven days respectively [39]. In human studies, CSF3 has been shown to "effectively"
536 shorten the lifespan of neutrophil myeloblasts and promyelocytes by decreasing their time
537 spent in cell cycle and accelerating their progress to maturation [40]. Although, it is unclear
538 what the exact effect is on the lifespan of post-mitotic neutrophils, studies have shown that the
539 addition of exogenous of CSF3 can delay apoptosis in mature neutrophils [41, 42]. This
540 suggests that under certain conditions; such as infection, CSF3 can extend the lifespan of a
541 mature neutrophil. Interestingly, classical monocytes also express CSF3R and have a short
542 circulating lifespan of approximately 24 hours [39] These studies suggest that under steady
543 state conditions and below a certain threshold, CSF3 has an as-yet-undocumented role either

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

544 directly or indirectly in maintaining a short neutrophil lifespan and thus allowing the efficient
545 turnover required to sustain high levels of granulopoiesis.

546

547 CSF3 orchestrates the life cycle of neutrophils in the bone marrow microenvironment by
548 marshalling neutrophil progenitors through different development stages through to maturity.
549 Accordingly, in response to this, CSF3R is expressed though every life stage, although at
550 differing levels across the neutrophil populations, with the highest levels observed on mature
551 cells where it is expressed at between two and three-fold more than on progenitors [43].
552 CSF3R/CSF3 signalling has a dual role in controlling the distribution of neutrophils by both
553 retaining a population of mature neutrophils in the bone marrow as a reservoir and facilitating
554 the egress of other neutrophils into the periphery. Thus, a major evolutionary adaptation that
555 CSF3R/CSF3 has conferred to the neutrophil is the ability to move, both on a population-wide
556 and individual cell level.

557

558 There is abundant production of neutrophils daily in the bone marrow; however, there are far
559 fewer neutrophils in circulation in the blood than are produced during granulopoiesis as the
560 total neutrophil population is effectively stored in the bone marrow or marginated in
561 intravascular pools within the spleen and liver [44]. Neutrophils can transit between sites in
562 response to CSF3 signalling and it is estimated that 49% of cells are present in the circulating
563 pool and the remaining 51% are marginated in discrete vascular pools [36, 45]. In the event
564 of an infection, neutrophils can be mobilised from the marginated pools and bone marrow in
565 response to GCSF-induced production of mobilising signals such as CXCL1 [46]. The co-
566 ordinated egress of neutrophils from the bone marrow is achieved by CSF3 interacting with
567 the CXCR4/CXCL12 and CXCR2/CXCL2 signalling pathways. CSF3 disrupts the CXCR4-
568 CXCL12 retention axis by reducing CXCL12 release from endothelial stromal cells and
569 reducing CXCR4 expression on neutrophils, thus allowing the movement of mature

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

570 neutrophils through the venous sinuses to the periphery. CSF3-induced expression of CXCR2
571 on neutrophils then causes their migration to the vasculature along a CXCL2 chemotactic
572 gradient [1, 23, 24]. The limited study data presented here demonstrated that the antagonism
573 between CXCR4 and CXCR2 is present *in vitro*, as in the absence of environmental cues from
574 endothelial cells, exogenous CSF3 induced CXCR2 expression and reduced CXCR4.
575 Interestingly, there was not a uniform impact as a minority of cells upregulated CXCR4 and
576 downregulated CXCR2 as well CSF3R. Furthermore, CSF1R expression was subsequently
577 induced on this minority population in a similar fashion observed to *ex vivo* cells. While this
578 study is too small to draw firm conclusions, it potentially suggests there is an antagonistic
579 interaction between CSF3R/CSF3 and CSF1R/CSF1 and work is ongoing to further define the
580 role this antagonism plays in neutrophil biology.

581

582 As neutrophils enters the bloodstream, they need to be able to migrate easily around the
583 circulatory system under high flow conditions. This requires the neutrophil's physical form to
584 have high deformability and flexibility as it encounters the different diameters of the
585 vasculature. The neutrophil nucleus is functionally adapted to this role because of its multi-
586 nucleated structure and the distinct protein composition of the nuclear envelope, features that
587 are widely conserved across mammalian species [47]. CSF3 in co-ordination with C/EBPε and
588 the ETS factors; Pu.1 and GA-binding protein (GABP) are responsible for the transcriptional
589 control of the essential neutrophil nuclear structural proteins Lamin A, Lamin C and Lamin B
590 receptor (LBR) [48, 49]. In comparison to other cell nuclear protein compositions, neutrophils
591 have a low proportion of Lamin A and Lamin C, which is believed to make the nucleus more
592 flexible for easier transit [47-49]. In contrast, the levels of LBR are increased, which is required
593 for nuclear lobulation and subsequent neutrophil maturation [47, 48]. Therefore, CSF3
594 signalling and C/EBPε are intrinsic to the evolution of neutrophil cell motility. Intriguingly, the
595 avian heterophil is not multi-lobulated, which aligns with our findings that C/EBPε had been

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

596 lost from the lineage. This suggests that a different C/EBP family member is involved in the
597 later stages of avian heterophil development.

598 The evolution of CSF3R/CSF3 has been essential to the development of the
599 neutrophil/heterophil in chordates and its own existence as the principle neutrophil growth
600 factor is evolutionarily advantageous. The Jawed/jawless lineages are unique in that they have
601 populations of both neutrophils and heterophils, whereas later linages favour either neutrophils
602 or heterophils. Our analysis shows that CSF3R/CSF3 emerged before the advent of
603 Jawed/jawless fish as both are present in the Coelacanth and absent from the other lineages.
604 Intriguingly, an IL6-like gene was present in the same syntenic location of the whale shark,
605 suggesting the possibility that heterophils and neutrophils were independently controlled by
606 an IL6-like protein and CSF3 in the early lineages. IL6 is an important pleiotropic pro-
607 inflammatory cytokine that plays key roles in infection, inflammation and haematopoiesis [50].
608 Although CSF3R/CSFR and IL6/IL6R, which are functional paralogues, diverged from each
609 other many millions of years ago, there is still functional redundancy at the cell level in modern-
610 day mammals, as neutrophils are present - though at vastly reduced numbers - in CSF3$^{-/-}$ and
611 CSF3R$^{-/-}$ mice [10, 11]. IL-6 can act on neutrophil progenitors and immature neutrophils thus
612 supporting neutrophil development in the bone marrow of CSF3 deficient mice [12]. However
613 mature neutrophils are refractory to IL-6 signalling as the expression of the IL-6R subunit
614 gp130 is lost during maturation [51]. From amphibia onwards, the protein analysis here
615 suggests that CSF3R and CSF3 co-evolved closely together and in contrast to
616 CSF1R/CSF1/IL34, CSF3 is likely the only ligand of CSF3R.

617

618 Our findings have demonstrated how essential CSF3R and CSF3 (and to a lesser extent
619 CSF1R and CSF1) are to the predominance of mammalian neutrophils in blood. Through the
620 course of evolution, CSF3R/CSF3 signalling has accrued many properties that are responsible
621 for the survival of the neutrophil and its ability to function, such as cell motility and mobility.
622 Interestingly, although CSF3 is present in jawed/jawless fish lineages, it's not until the

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

623 emergence of tetrapoda that CSF3R and CSF3 begin to dominate haematopoiesis. Our
624 current studies have been limited to chordates in which the data is publicly available. However,
625 we envisage exploring the phagocytic granulocyte of two model species. The amphioxus is
626 considered the basal chordate and a macrophage-like population has been identified,
627 although a neutrophil/heterophil has yet to be described within the rudimentary circulatory
628 system [52]. The lungfish, a primitive airbreathing fish, is unique among jawed/jawless
629 lineages as it lives in freshwater and can survive on the land for up to one year. Accordingly,
630 the lungfish has many adaptations and is considered to be a one of the closest living relatives
631 to tetrapods making it a good model species for further study [53]. Given how essential motility
632 and mobility are to neutrophil function and development, it would be useful to discern when it
633 emerged in evolution by identifying if equivalent cells and functional gene/protein orthologues
634 are present in either species. These comparative studies would answer fundamental
635 questions about the origin of the neutrophilic phagocyte.

636

637

638

639

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

640 ## Materials and Methods

641

642 ### Species selection

643

644 Ninety-seven animals from all the major classes were selected, thirty-eight of which, were
645 used for the calculation of haematological parameters and the remaining fifty-nine for the
646 bioinformatics-based studies. Analyses was performed on non- mammalian lineages
647 including; jawed/jawless fish, amphibia, non-avian reptiles – both non-crocodilian and
648 crocodilian-, aves, and the mammalian lineages; monotremata, marsupialia and placentalia.
649 Urochordates (tunicata), and cephalochordate (Amphoxi) were excluded from analysis as
650 there was insufficient coverage or insufficient annotation of sequence data in the Pubmed
651 Gene and Ensembl databases. Similarly, the lungfish (Dipnoi) was also excluded from this
652 analysis because of insufficient coverage of sequence data. Finally, teleost lineages were
653 excluded from the analysis owing to having gone through three rounds of genome wide
654 duplication, in contrast to all other chordates who have only undergone two rounds [54]. A
655 species tree was generated using the NCBI Common Taxonmy browser common tree tool
656 [55, 56] and visualised using the Interactive Tree of Life (iTOL) web browser tool [57].

657

658 ### Comparative analysis of haematological parameters and CSF/CSFR gene presence and
659 synteny in chordates

660

661 Complete blood count data for thirty-eight species representing each animal order or class
662 were collated from existing literature to perform a meta-analysis of myeloid WBC in phylum
663 Chordata. Only counts that were calculated to the standardised concentration (10^9/L) or
664 equivalent were used and all lymphoid data was excluded. The proportional composition for

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

665 each myeloid subset; neutrophil, eosinophil, basophil and monocytes as part of the overall
666 myeloid population was also calculated.

667 Gene sequence for fifty-nine species - representing different animal orders and classes - were
668 retrieved from the NCBI Gene databases [58] in most instances or from annotated entries in
669 Ensembl (v95) [59] for the following genes; CSF1R, IL34, CSF1, CSF3R, CSF3, C/EBPα,
670 C/EBPβ C/EBPδ and C/EBPε. A heatmap visualising gene presence, absence or presence of
671 orthologues was generated using maplotlib/seaborn library for Python 3.7.7.

672 Syntenic maps were generated manually for selected genes in the Jawed fish lineage. To
673 generate the maps, syntenic blocks were identified as a section of the human chromosome
674 containing the gene of interest flanked by *x* number of genes. The blocks were then manually
675 compared to similar regions in the chromosomes in Jawed fish to identify orthologous genes.
676 Between three and five genes were chosen per syntenic block to function as anchor points of
677 reference. Anchor points were selected based on their situational proximity, either upstream
678 or downstream, to gene of interest and were present in all species examined. Multiple genes
679 were identified as anchor points to mitigate for the random loss of genes during the process
680 of evolutionary gene rearrangement.

681

682 Generation of percentage shared sequence similarity plots and timescales

683

684 As with the gene sequences, protein sequences for the identified species were retrieved from
685 the NCBI Protein databases. Presumed orthologous sequences were screened using the
686 NCBI Basic Local Alignment Search tool (BLAST) to generate a percentage score of sequence
687 similarity. The parameters to determine shared sequence similarity were as follows; a
688 sequence was deemed to be homologous where coverage of the protein sequence was equal
689 to or greater than 40% of the total protein sequence examined and the e value was between
690 $0x10^{-20}$ - 0. Percentage scores were averaged per order. Shared sequence similarity was also

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

691 plotted against animal order or class. The same data was also used to generate two sets of
692 timescales for each order/ member for a given receptor/ligand family, where either the
693 calculated mean shared sequence similarity value or haematological parameters were plotted
694 on a timescale based on the emergence of the earliest known ancestor of that order *versus*
695 time (million years ago [mya]).

696

697 Isolation of peripheral blood myeloid cells and cell culture

698

699 Peripheral blood mononuclear cells (PBMCs) and Polymorphonuclear cells (PMNs) were
700 isolated from the blood of healthy donors using a triple density Percoll™ gradient. Both cell
701 fractions were washed and incubated in isotonic buffer solution for downstream applications.
702 Monocytes and neutrophils were harvested from the PBMC and PMN fractions using
703 immunomagnetic negative and positive selection respectively and the protocols were
704 performed according to the manufacturer's recommendation (Stemcell Technologies,
705 Cambridge, UK).

706 Mobility assays: Isolated monocytes were incubated in either CSF3 (20ng/ml) or CSF1
707 (20ng/ml) for 72 hours prior to the set-up of the assay. The CSF-treated cells were recovered
708 and washed in isotonic solution before being loaded into an Ibidi 2D chemotaxis chamber to
709 assess chemotaxis in response to a serum-based gradient. Migration velocity was tracked by
710 microscopy for one hour following seeding of cells. A parallel control stream using freshly
711 isolated neutrophils was also set up.

712 CXCR4 induction: Isolated neutrophils were cultured *in vitro* for 20 hours in 10% Foetal Bovine
713 Serum (FBS) supplemented RPMI 1640 culture medium to induce CXCR4 expression. A
714 separate control culture of neutrophils with the addition of exogenous CSF3 (20ng/ml) was
715 also established. At the conclusion of the incubation period, both cell cultures were recovered,
716 washed in isotonic solution and used for downstream analytical flow cytometry.

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

717 Flow cytometry

718

719 All antibodies used were purchased from Biolegend, London, UK. Freshly isolated PBMCs or
720 PMNs or cultured neutrophils were incubated with various antibodies as per manufacturer's
721 instructions for cell surface staining. For the identification of CSF3R and CSF1R expression,
722 cells were stained with fluorophore conjugated monoclonal anti-human antibodies against
723 CD3 (OKT3), CD19(HIB19), CD56(39D5), NKp46 (9E2) [lin^-], CD14 (63D3), CD16 (3G8),
724 CD66b (G10F5), CSF3R (LMM741), CSF1R (9-4D2-1E4) and HLA-DR (L2434). After
725 exclusion of cell debris and doublets based on side scatter characteristics (SSC), monocytes
726 were identified as SSC^{int} lin^- $CD14^+CD16^{+/-}HLA\text{-}DR^+$. The granulocyte population was
727 identified as SSC^{hi} $Lin^-CD66b^+CD16^{hi}HLA\text{-}DR^+$ and the minority eosinophil population was
728 excluded from neutrophils by differential CD16 expression. To further characterise CXCR4
729 induced neutrophils, the following monoclonal antibodies were also incorporated CXCR4
730 (12G5) and CXCR2 (5E8/CXCR2).

731

732

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

733 ## References

734

735 1. Adrover, J.M., et al., *A Neutrophil Timer Coordinates Immune Defense and Vascular Protection.*
736 Immunity, 2019. **51**(5): p. 966-967.
737 2. Hidalgo, A., et al., *The Neutrophil Life Cycle.* Trends Immunol, 2019. **40**(7): p. 584-597.
738 3. Ng, L.G., R. Ostuni, and A. Hidalgo, *Heterogeneity of neutrophils.* Nat Rev Immunol, 2019.
739 **19**(4): p. 255-265.
740 4. Yvan-Charvet, L. and L.G. Ng, *Granulopoiesis and Neutrophil Homeostasis: A Metabolic, Daily*
741 *Balancing Act.* Trends Immunol, 2019. **40**(7): p. 598-612.
742 5. Evrard, M., et al., *Developmental Analysis of Bone Marrow Neutrophils Reveals Populations*
743 *Specialized in Expansion, Trafficking, and Effector Functions.* Immunity, 2018. **48**(2): p. 364-
744 379.e8.
745 6. Mehta, H.M., T. Glaubach, and S.J. Corey, *Systems approach to phagocyte production and*
746 *activation: neutrophils and monocytes.* Adv Exp Med Biol, 2014. **844**: p. 99-113.
747 7. Dai, X.M., et al., *Targeted disruption of the mouse colony-stimulating factor 1 receptor gene*
748 *results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell*
749 *frequencies, and reproductive defects.* Blood, 2002. **99**(1): p. 111-20.
750 8. Yoshida, H., et al., *The murine mutation osteopetrosis is in the coding region of the*
751 *macrophage colony stimulating factor gene.* Nature, 1990. **345**(6274): p. 442-4.
752 9. Umeda, S., et al., *Effects of macrophage colony-stimulating factor on macrophages and their*
753 *related cell populations in the osteopetrosis mouse defective in production of functional*
754 *macrophage colony-stimulating factor protein.* Am J Pathol, 1996. **149**(2): p. 559-74.
755 10. Liu, F., et al., *Impaired production and increased apoptosis of neutrophils in granulocyte*
756 *colony-stimulating factor receptor-deficient mice.* Immunity, 1996. **5**(5): p. 491-501.
757 11. Lieschke, G.J., et al., *Mice lacking granulocyte colony-stimulating factor have chronic*
758 *neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil*
759 *mobilization.* Blood, 1994. **84**(6): p. 1737-46.
760 12. Basu, S., et al., *"Emergency" granulopoiesis in G-CSF-deficient mice in response to Candida*
761 *albicans infection.* Blood, 2000. **95**(12): p. 3725-33.
762 13. Hibbs, M.L., et al., *Mice lacking three myeloid colony-stimulating factors (G-CSF, GM-CSF, and*
763 *M-CSF) still produce macrophages and granulocytes and mount an inflammatory response in*
764 *a sterile model of peritonitis.* J Immunol, 2007. **178**(10): p. 6435-43.
765 14. Lakshman, R. and A. Finn, *Neutrophil disorders and their management.* J Clin Pathol, 2001.
766 **54**(1): p. 7-19.
767 15. Yáñez, A., et al., *Granulocyte-Monocyte Progenitors and Monocyte-Dendritic Cell Progenitors*
768 *Independently Produce Functionally Distinct Monocytes.* Immunity, 2017. **47**(5): p. 890-
769 902.e4.
770 16. Halene, S., et al., *C/EBPepsilon directs granulocytic-vs-monocytic lineage determination and*
771 *confers chemotactic function via Hlx.* Exp Hematol, 2010. **38**(2): p. 90-103.
772 17. Zhang, D.E., et al., *Absence of granulocyte colony-stimulating factor signaling and neutrophil*
773 *development in CCAAT enhancer binding protein alpha-deficient mice.* Proc Natl Acad Sci U S
774 A, 1997. **94**(2): p. 569-74.
775 18. Yamanaka, R., et al., *Impaired granulopoiesis, myelodysplasia, and early lethality in*
776 *CCAAT/enhancer binding protein epsilon-deficient mice.* Proc Natl Acad Sci U S A, 1997. **94**(24):
777 p. 13187-92.
778 19. Lekstrom-Himes, J. and K.G. Xanthopoulos, *CCAAT/enhancer binding protein epsilon is critical*
779 *for effective neutrophil-mediated response to inflammatory challenge.* Blood, 1999. **93**(9): p.
780 3096-105.

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

781 20. Forslund, K., I. Pekkari, and E.L. Sonnhammer, *Domain architecture conservation in orthologs.*
782 BMC Bioinformatics, 2011. **12**: p. 326.
783 21. Genovese, K.J., et al., *The avian heterophil.* Dev Comp Immunol, 2013. **41**(3): p. 334-40.
784 22. Styrt, B., *Species variation in neutrophil biochemistry and function.* J Leukoc Biol, 1989. **46**(1):
785 p. 63-74.
786 23. Eash, K.J., et al., *CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from*
787 *murine bone marrow.* J Clin Invest, 2010. **120**(7): p. 2423-31.
788 24. Eash, K.J., et al., *CXCR4 is a key regulator of neutrophil release from the bone marrow under*
789 *basal and stress granulopoiesis conditions.* Blood, 2009. **113**(19): p. 4711-9.
790 25. Hartung, T., et al., *Effect of granulocyte colony-stimulating factor treatment on ex vivo blood*
791 *cytokine response in human volunteers.* Blood, 1995. **85**(9): p. 2482-9.
792 26. Christopher, M.J., et al., *Expression of the G-CSF receptor in monocytic cells is sufficient to*
793 *mediate hematopoietic progenitor mobilization by G-CSF in mice.* J Exp Med, 2011. **208**(2): p.
794 251-60.
795 27. Boneberg, E.M., et al., *Human monocytes express functional receptors for granulocyte colony-*
796 *stimulating factor that mediate suppression of monokines and interferon-gamma.* Blood,
797 2000. **95**(1): p. 270-6.
798 28. Sasmono, R.T., et al., *Mouse neutrophilic granulocytes express mRNA encoding the*
799 *macrophage colony-stimulating factor receptor (CSF-1R) as well as many other macrophage-*
800 *specific transcripts and can transdifferentiate into macrophages in vitro in response to CSF-1.*
801 J Leukoc Biol, 2007. **82**(1): p. 111-23.
802 29. Greenbaum, A.M. and D.C. Link, *Mechanisms of G-CSF-mediated hematopoietic stem and*
803 *progenitor mobilization.* Leukemia, 2011. **25**(2): p. 211-7.
804 30. Kim, J.H. and N. Kim, *Signaling Pathways in Osteoclast Differentiation.* Chonnam Med J, 2016.
805 **52**(1): p. 12-7.
806 31. de Abreu Manso, P.P., L. de Brito-Gitirana, and M. Pelajo-Machado, *Localization of*
807 *hematopoietic cells in the bullfrog (Lithobates catesbeianus).* Cell Tissue Res, 2009. **337**(2): p.
808 301-12.
809 32. Petit, I., et al., *G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-*
810 *regulating CXCR4.* Nat Immunol, 2002. **3**(7): p. 687-94.
811 33. Semerad, C.L., et al., *G-CSF potently inhibits osteoblast activity and CXCL12 mRNA expression*
812 *in the bone marrow.* Blood, 2005. **106**(9): p. 3020-7.
813 34. Lévesque, J.P., et al., *Disruption of the CXCR4/CXCL12 chemotactic interaction during*
814 *hematopoietic stem cell mobilization induced by GCSF or cyclophosphamide.* J Clin Invest,
815 2003. **111**(2): p. 187-96.
816 35. Day, R.B., et al., *Granulocyte colony-stimulating factor reprograms bone marrow stromal cells*
817 *to actively suppress B lymphopoiesis in mice.* Blood, 2015. **125**(20): p. 3114-7.
818 36. Summers, C., et al., *Neutrophil kinetics in health and disease.* Trends Immunol, 2010. **31**(8): p.
819 318-24.
820 37. Pillay, J., et al., *In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days.*
821 Blood, 2010. **116**(4): p. 625-7.
822 38. Park, Y.M. and B.S. Bochner, *Eosinophil survival and apoptosis in health and disease.* Allergy
823 Asthma Immunol Res, 2010. **2**(2): p. 87-101.
824 39. Patel, A.A., et al., *The fate and lifespan of human monocyte subsets in steady state and*
825 *systemic inflammation.* J Exp Med, 2017. **214**(7): p. 1913-1923.
826 40. Lord, B.I., et al., *The kinetics of human granulopoiesis following treatment with granulocyte*
827 *colony-stimulating factor in vivo.* Proc Natl Acad Sci U S A, 1989. **86**(23): p. 9499-503.
828 41. van Raam, B.J., et al., *Granulocyte colony-stimulating factor delays neutrophil apoptosis by*
829 *inhibition of calpains upstream of caspase-3.* Blood, 2008. **112**(5): p. 2046-54.

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

830 42. Zimbelman, J., et al., *In vivo treatment with granulocyte colony-stimulating factor does not*
831 *delay apoptosis in human neutrophils by increasing the expression of the vacuolar proton*
832 *ATPase.* J Investig Med, 2002. **50**(1): p. 33-7.
833 43. Demetri, G.D. and J.D. Griffin, *Granulocyte colony-stimulating factor and its receptor.* Blood,
834 1991. **78**(11): p. 2791-808.
835 44. Ussov, W.Y., et al., *Granulocyte margination in bone marrow: comparison with margination in*
836 *the spleen and liver.* Scand J Clin Lab Invest, 1995. **55**(1): p. 87-96.
837 45. ATHENS, J.W., et al., *Leukokinetic studies. IV. The total blood, circulating and marginal*
838 *granulocyte pools and the granulocyte turnover rate in normal subjects.* J Clin Invest, 1961. **40**:
839 p. 989-95.
840 46. Köhler, A., et al., *G-CSF-mediated thrombopoietin release triggers neutrophil motility and*
841 *mobilization from bone marrow via induction of Cxcr2 ligands.* Blood, 2011. **117**(16): p. 4349-
842 57.
843 47. Manley, H.R., M.C. Keightley, and G.J. Lieschke, *The Neutrophil Nucleus: An Important*
844 *Influence on Neutrophil Migration and Function.* Front Immunol, 2018. **9**: p. 2867.
845 48. Malu, K., et al., *Cooperative Activity of GABP with PU.1 or C/EBPε Regulates Lamin B Receptor*
846 *Gene Expression, Implicating Their Roles in Granulocyte Nuclear Maturation.* J Immunol, 2016.
847 **197**(3): p. 910-22.
848 49. Cohen, T.V., et al., *The lamin B receptor under transcriptional control of C/EBPepsilon is*
849 *required for morphological but not functional maturation of neutrophils.* Hum Mol Genet,
850 2008. **17**(19): p. 2921-33.
851 50. Tanaka, T., M. Narazaki, and T. Kishimoto, *IL-6 in inflammation, immunity, and disease.* Cold
852 Spring Harb Perspect Biol, 2014. **6**(10): p. a016295.
853 51. Wilkinson, A.N., et al., *Granulocytes Are Unresponsive to IL-6 Due to an Absence of gp130.* J
854 Immunol, 2018. **200**(10): p. 3547-3555.
855 52. Han, Y., et al., *The primitive immune system of amphioxus provides insights into the ancestral*
856 *structure of the vertebrate immune system.* Dev Comp Immunol, 2010. **34**(8): p. 791-6.
857 53. Takezaki, N. and H. Nishihara, *Support for Lungfish as the Closest Relative of Tetrapods by*
858 *Using Slowly Evolving Ray-Finned Fish as the Outgroup.* Genome Biol Evol, 2017. **9**(1): p. 93-
859 101.
860 54. Glasauer, S.M. and S.C. Neuhauss, *Whole-genome duplication in teleost fishes and its*
861 *evolutionary consequences.* Mol Genet Genomics, 2014. **289**(6): p. 1045-60.
862 55. Sayers, E.W., et al., *Database resources of the National Center for Biotechnology Information.*
863 Nucleic Acids Res, 2009. **37**(Database issue): p. D5-15.
864 56. Benson, D.A., et al., *GenBank.* Nucleic Acids Res, 2009. **37**(Database issue): p. D26-31.
865 57. Letunic, I. and P. Bork, *Interactive Tree Of Life (iTOL) v4: recent updates and new*
866 *developments.* Nucleic Acids Res, 2019. **47**(W1): p. W256-W259.
867 58. Pruitt, K.D., T. Tatusova, and D.R. Maglott, *NCBI reference sequences (RefSeq): a curated non-*
868 *redundant sequence database of genomes, transcripts and proteins.* Nucleic Acids Res, 2007.
869 **35**(Database issue): p. D61-5.
870 59. Yates, A.D., et al., *Ensembl 2020.* Nucleic Acids Res, 2020. **48**(D1): p. D682-D688.

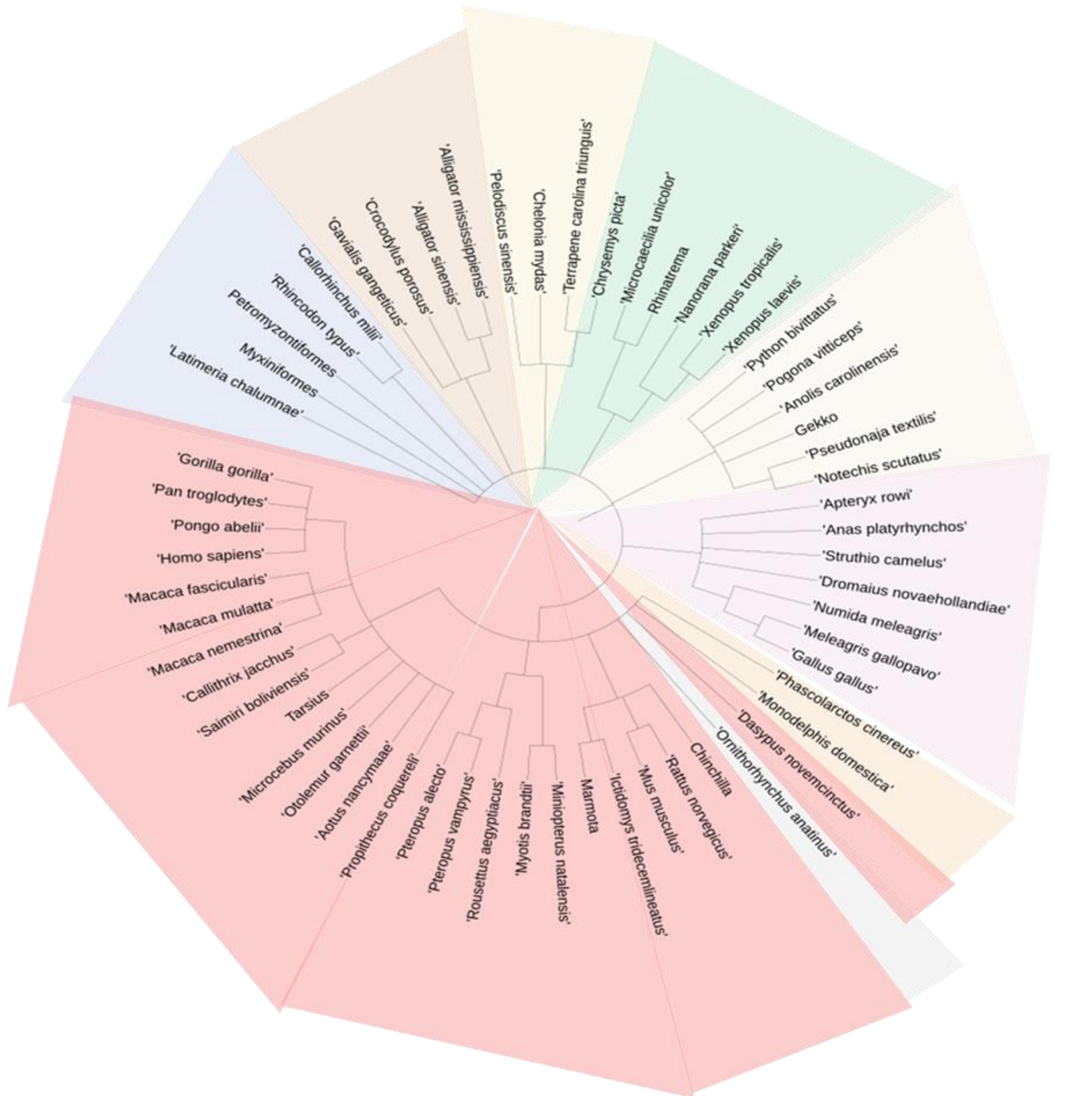
871

872

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

873

a)



Jawed/Jawless fish

- Jawed fish 4.10×10^{8} MYA
- Jawless fish 3.6×10^{8} MYA

Amphibia

- Amphibia 3.5×10^{8} MYA

Reptilia

- Squamata 3.3×10^{8} MYA
- Crocodilia 4.10×10^{8} MYA
- Testudines 2.1×10^{7} MYA

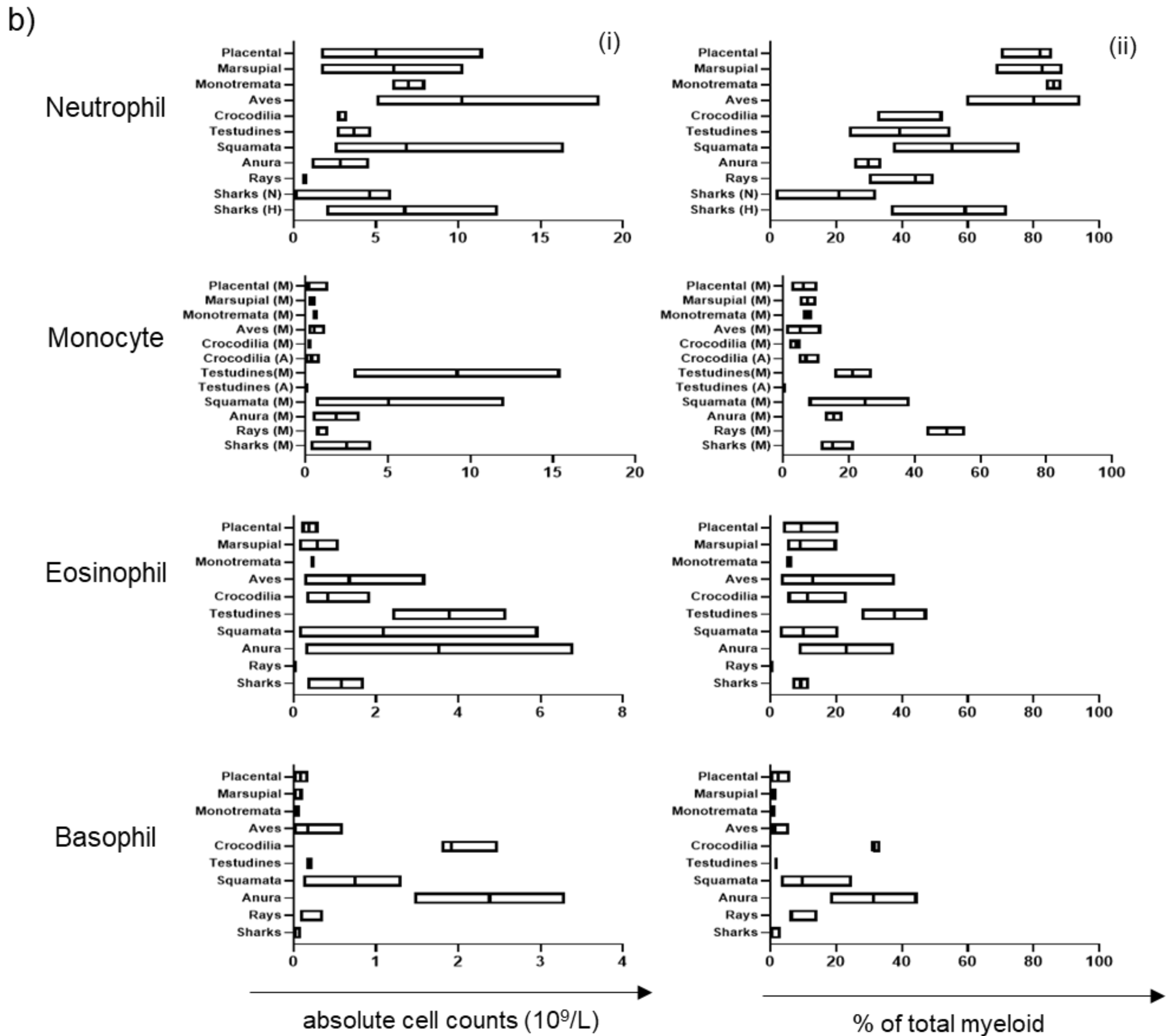
Aves

- Aves 7.0×10^{7} MYA

Mammalia

- Monotremata 1.1×10^{7} MYA
- Marsupalia 6.5×10^{7} MYA
- Placentalia 6.25×10^{7} MYA

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

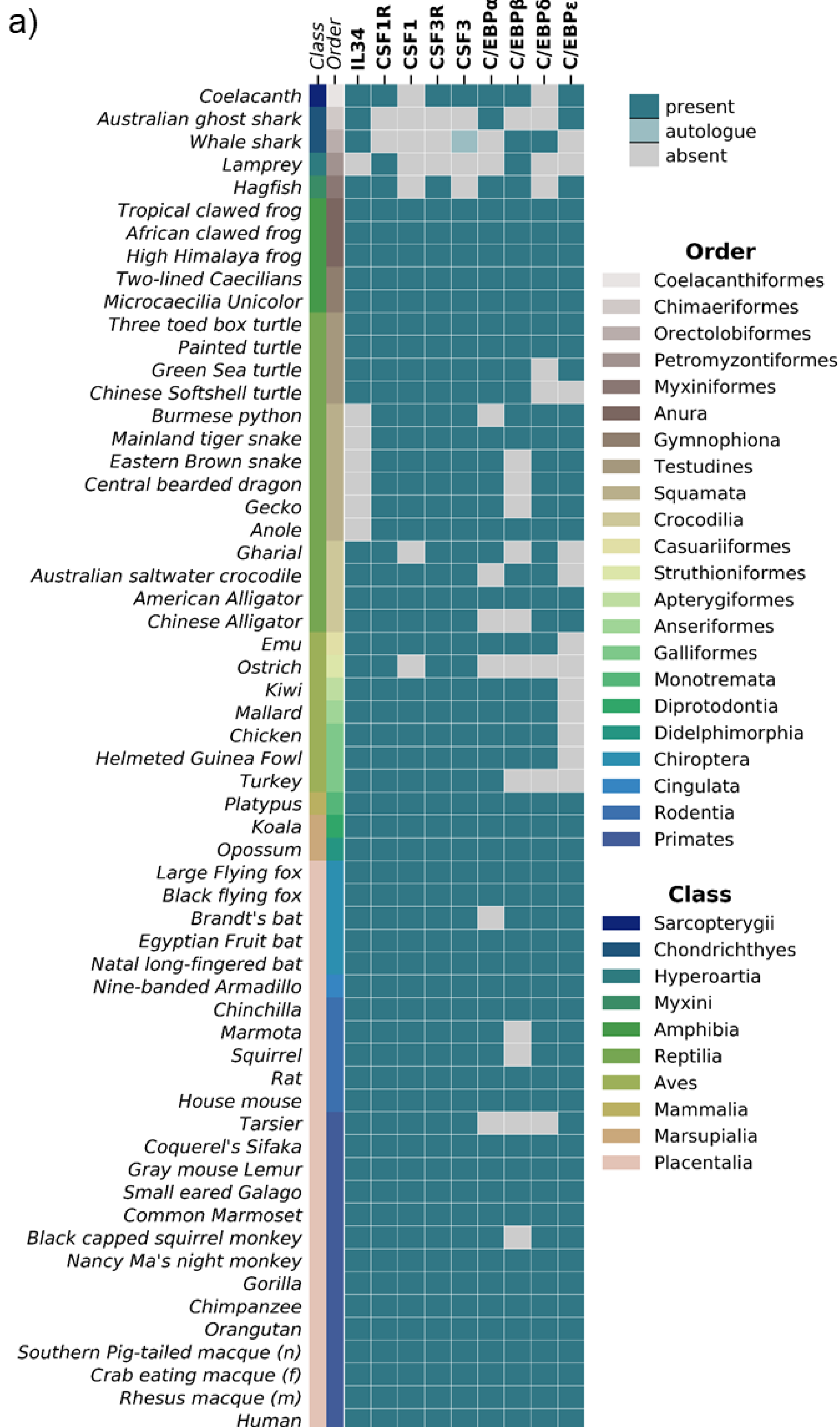


874

875

876 **Figure 1. Population comparison of blood myeloid cell subset distribution in**
877 **chordates demonstrates predominance of the neutrophilic granulocyte in birds**
878 **and mammals.**
879 A. Species tree of the animals within Phylum Chordata examined, sub-classified by animal
880 order or class. B. Meta-analysis of aggregated phylum Chordata complete blood cell counts
881 (excluding all lymphoid cells). Data is visualised as a floating bar and the line represents the
882 mean value and shows absolute counts per cell type per order (i) and composition of myeloid
883 cells per cell type (ii).
884

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.



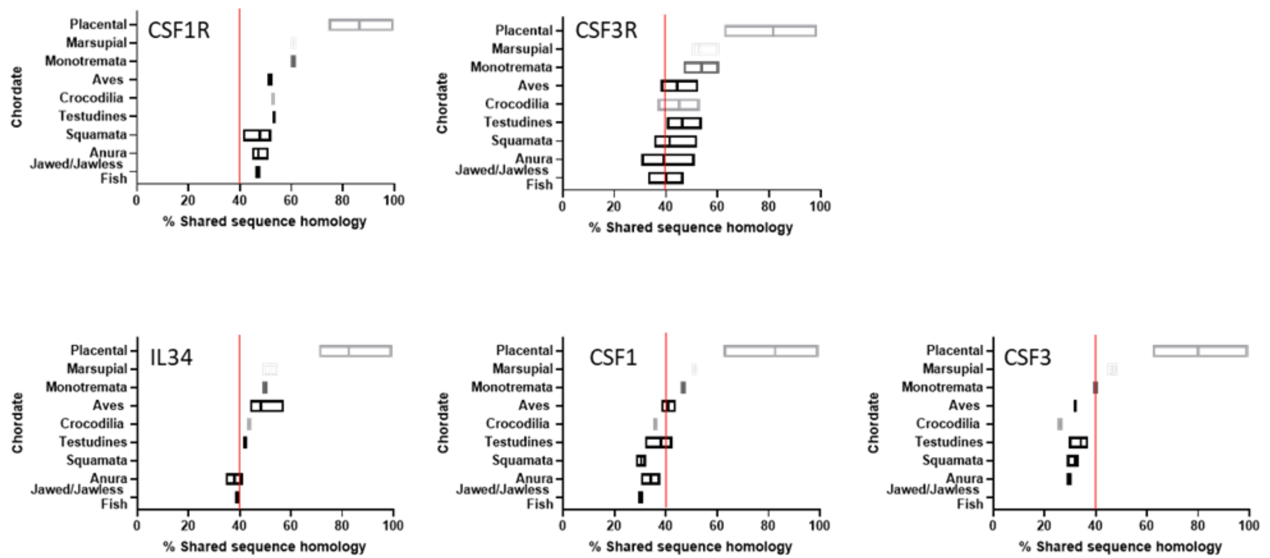
885

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

886
887 **Figure 2. Analysis of chordate CSF1/CSF1R, CSF3/CSF3R and neutrophil-related**
888 **transcription factors reveals their loss in Jawed/Jawless fish lineages.**
889 A. Heat map of CSF1R/IL34/CSF1, CSF3R/CSF3 and neutrophil-related transcription factors
890 in selected members of Phylum Chordata**.** B. Syntenic maps of CSF1R/IL34/CSF1 and
891 CSF3R/CSF3 in selected Jawed fish compared to human.
892

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

a)



b)

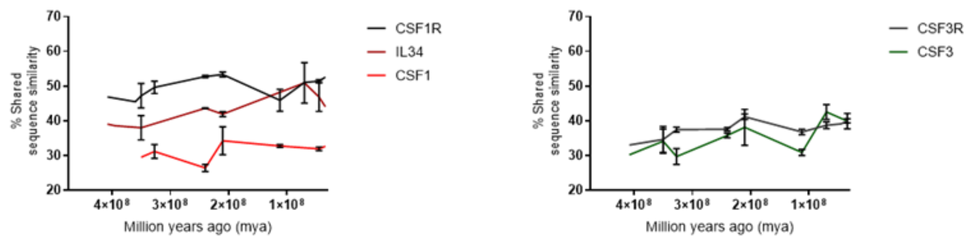
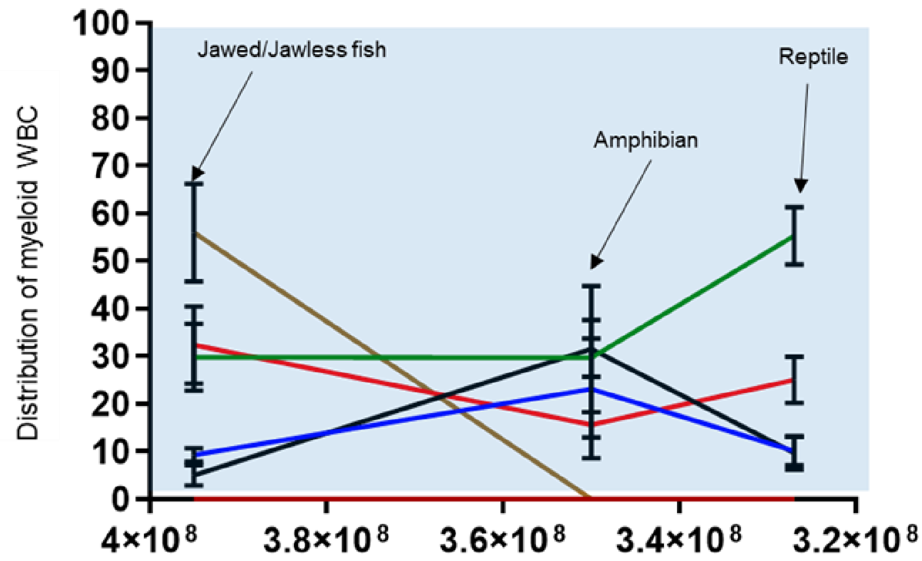
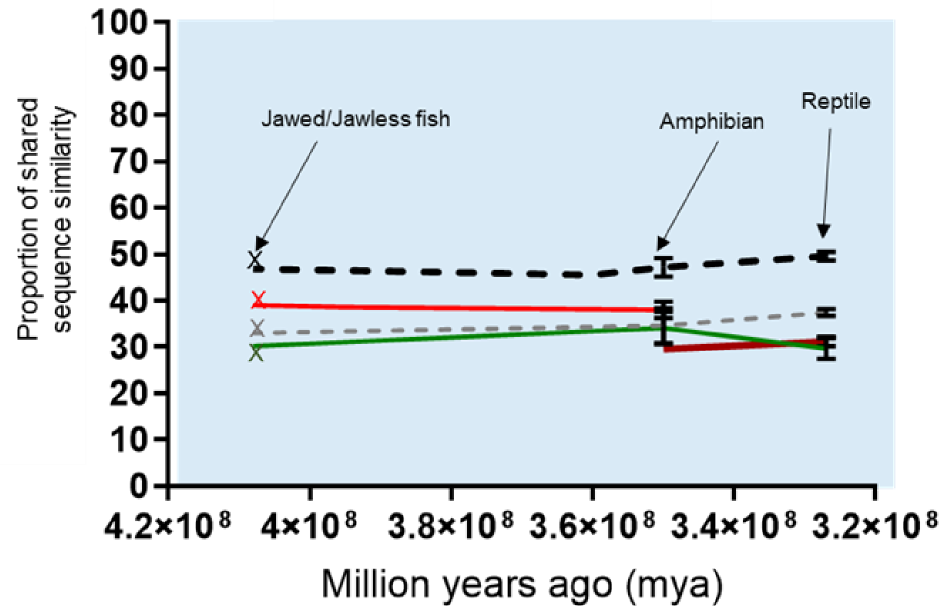


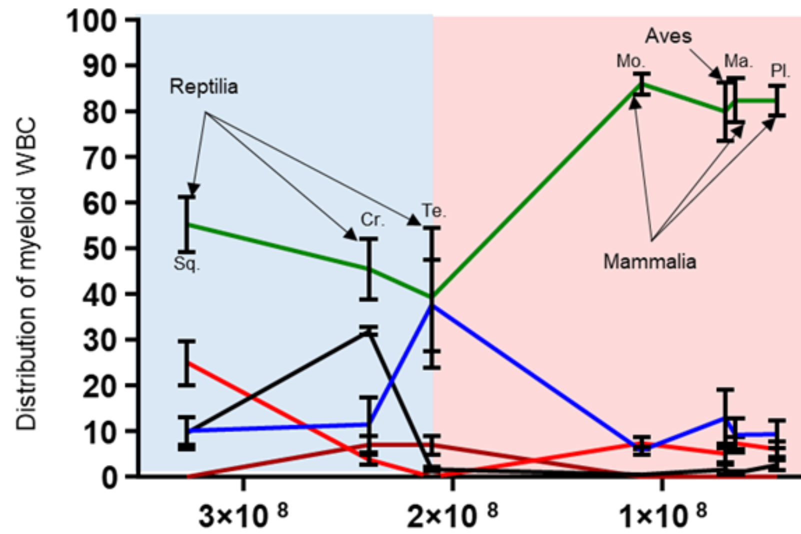
Figure 3. Shared sequence similarity analysis of Chordate CSFR/CSF protein homology further supports the ancestral pairing of CSF1R/IL34 and CSF3R/CSF3 in early lineages. A. Representative plots of % shared sequence similarity for CSF1R, CSF3R, IL34, CSF1 and CSF3 in the respective sub-groups of Phylum Chordata, data is visualised as a floating bar and the line represents the mean value. B. Graphical representation of % shared sequence similarity in sub-groups of Phylum Chordata *versus* time.

bi)





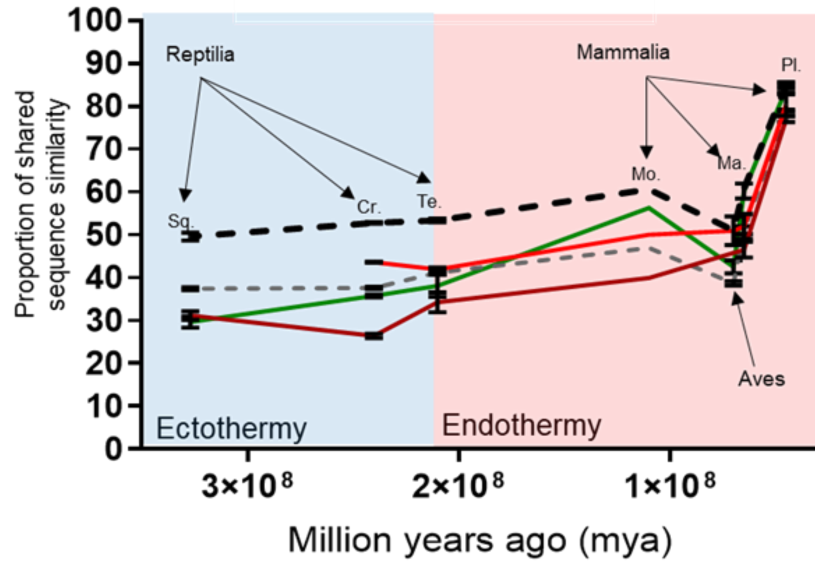
bii)



Keys

Neutrophils/Heterophil
Eosinophils
Basophils
Monocytes
Azurophil

Sq. = Squamata
Cr. = Crocodilia
Te. = Testudines
Mo. = Monotremata
Ma. = Marsupalia
Pl. = Placentalia



CSF1R
CSF3R
CSF3
IL34
CSF1

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.19.161059; this version posted July 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

Figure 4. Changes in Chordate blood granulocyte distribution from Jawed/Jawless lineages through to placental mammals are multi-factorial and likely driven by the emergence of CSF3 and onset of endothermy.

A. Graphical representation of % population distribution of myeloid white blood cells *versus* time (i) and % shared sequence similarity of CSF1R and CSF3R protein families *versus* time for Jawed/Jawless fish, Amphibia and the reptilian order of Squamata (ii). B. Graphical representation of % population distribution of myeloid white blood cells *versus* time (i) and % shared sequence similarity of CSF1R and CSF3R protein families *versus* time for the Reptilian orders of squamata, testudines and crocodilia, Aves, and the Mammalian orders of monotremata, marsupalia and Placental (ii).