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1 2	A layered erythromyeloid ontogeny ensures timely supply of erythrocytes and macrophages during development
3 4 5	Ramy Elsaid <sup>1,*</sup> , Aya Mikdache <sup>1</sup> , Patricia Diabangouaya <sup>1,#</sup> , Gwendoline Gros <sup>1,#</sup> and Pedro P. Hernández <sup>1,*</sup> .
6 7 8 9	<sup>1</sup> Institut Curie, PSL Research University CNRS UMR 3215, INSERM U934, 26 Rue d'Ulm, 75248 Paris Cedex 05, France.
10 11	# These authors contributed equally to this work.
12 13	*Corresponding author. Email: pedro.hernandez-cerda@curie.fr (P.P.H.) and ramy.elsaid@curie.fr (R.E.).
14 15	Short running title: Layered erythromyeloid ontogeny in zebrafish
15 16 17	Highlights
17 18	Primitive hematopoietic progenitors give rise and sustain embryonic erythromyeloid lineages.
19 20 21 22	Definitive hematopoietic progenitors show a late contribution to macrophages and erythrocytes during development.
23 24 25	Embryos show a layered ontogeny of macrophages that ensures their abundance for a timely contribution to tissue homeostasis and repair.
26 27	Selective ablation of definitive macrophages does not impair tail fin regeneration during early larval developmental stages.
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Abstract

In all organisms studied, from flies to humans, blood cells emerge in several sequential waves and from distinct hematopoietic origins. However, the relative contribution of these ontogenetically distinct hematopoietic waves to embryonic blood lineages and to tissue regeneration during development is yet elusive. Here, using a lineage-specific 'switch and trace' strategy in the zebrafish embryo, we report that the definitive hematopoietic progeny barely contributes to erythrocytes and macrophages during early development. Lineage tracing further show that ontogenetically distinct macrophages exhibit differential recruitment to the site of injury based on the developmental stage of the organism. We further demonstrate that primitive macrophages can solely maintain tissue regeneration during early larval developmental stages after selective ablation of definitive macrophages. Our findings highlight that the sequential emergence of hematopoietic waves in embryos ensures the abundance of blood cells required for tissue homeostasis and integrity during development Keywords Ontogeny; Hematopoiesis; Lineage tracing; Regeneration; Zebrafish 

#### 81 Introduction

82 Hematopoiesis is a complex biological process by which all mature blood lineages are 83 generated. In mammals, hematopoiesis originates from distinct blood progenitors that arise 84 during development resulting in a layered organization of the immune system (Elsaid et al., 85 2020). In mice, the first wave, also called primitive wave, starts in the extra-embryonic 86 microenvironment of the yolk sac (YS) around embryonic day (E) 7 (Palis et al., 1999; Kingsley 87 et al., 2004; Palis et al., 2001; Soares-da-Silva et al., 2021). Around E8.25, the YS also harbors 88 endothelial-derived multipotent erythro-myeloid progenitors (EMPs) which represent the 89 first definitive wave of hematopoiesis (Hoeffel et al., 2015; Gomez Perdiguero et al., 2015; 90 Bertrand et al., 2005; Gentek et al., 2018; Frame et al., 2016). At E9.5, other intra-embryonic 91 definitive hematopoietic progenitors emerge in the major arteries and they are the main 92 source of adult hematopoietic stem cells (HSCs) in the bone marrow (BM) (Cumano et al., 93 1996; Medvinsky and Dzierzak, 1996; Busch et al., 2015). These progenitors emerge through 94 an endothelial-to-hematopoietic transition mechanism (EHT) (Bertrand et al., 2010; Boisset 95 et al., 2010; Kissa and Herbomel, 2010b). At E10.5, the fetal liver (FL), the main hematopoietic 96 organ during embryonic development, harbors progenitors of dual origin derived from both 97 definitive and primitive hematopoietic progenitors (McGrath et al., 2015a). As in mammals, 98 zebrafish hematopoiesis also consists of multiple waves that emerge at distinct anatomical 99 locations (Elsaid et al., 2020). In the zebrafish embryo, primitive hematopoiesis emerges intra-100 embryonically around 11 hours post-fertilization (hpf) in the rostral blood island (RBI) and the 101 posterior lateral mesoderm (PLM) and generates primitive erythrocytes and myeloid cells 102 (Elsaid et al., 2020; Herbomel et al., 2001). Recently, the RBI was shown to be major source 103 of embryonic microglia (Ferrero et al., 2018; Xu et al., 2015). Other definitive or endothelial-104 derived hematopoietic waves are also produced in the developing organism (Elsaid et al., 105 2020). One of them is a transient wave that emerges from the posterior blood island (PBI) at 106 24-30 hpf via EHT and gives rise to EMPs like those in mammals (Bertrand et al., 2007; Ferrero 107 et al., 2018; Tian et al., 2017). At 36 hpf, other waves of HSCs and HSC-independent 108 progenitors emerge from the dorsal aorta (DA) via EHT (Bertrand et al., 2010; Kissa and 109 Herbornel, 2010b). These DA-derived hematopoietic progenitors then migrate to the caudal 110 hematopoietic tissue (CHT) (Murayama et al., 2006). By 4 days post-fertilization (dpf), 111 hematopoietic stem and progenitor cells migrate to the kidney marrow, the BM counterpart 112 in zebrafish, where they reside and differentiate to all blood cell lineages, establishing the 113 adult hematopoietic system (Murayama et al., 2006). Therefore, due to its similarity to 114 mammalian hematopoiesis, the zebrafish is an excellent model to study the ontogeny of 115 different hematopoietic waves during development.

116 Although it is well characterized that blood cells are produced in sequential and overlapping 117 waves (Elsaid et al., 2020), little is known about their contribution to different hematopoietic 118 lineages during development. However, recent studies in mice and in zebrafish suggest that 119 immune and blood cells might have distinct origins throughout life (Hoeffel et al., 2015; 120 Ferrero et al., 2018; Xu et al., 2015; Gentek et al., 2018; Gomez Perdiguero et al., 2015; Soares-121 da-Silva et al., 2021). Independent reports support the notion that definitive hematopoiesis 122 sustains embryonic blood lineages (Tian et al., 2017; Ulloa et al., 2021; Patel et al., 2022; 123 Stachura and Traver, 2011). However, primitive hematopoietic progenitors give rise also to 124 the erythromyeloid lineage and it is yet unknown to what extent these progenitors contribute 125 to embryonic blood lineages. This is mainly due to the lack of specific markers for primitive 126 hematopoietic progenitors in mammals (Elsaid et al., 2020; Ferrero et al., 2018; Xu et al., 127 2015; McGrath et al., 2015b).

128 The difficulty in accurately tracing the output of each wave in mammalian animal models has 129 hindered the full understanding of immune cell ontogeny. Recent studies in zebrafish showed 130 that embryonic and larval microglia originate from the RBI while adult microglia and other 131 adult macrophages arise from the ventral wall of dorsal aorta (VDA) (Xu et al., 2015; He et al., 132 2018; Ferrero et al., 2018). Despite their elegant experimental designs, only microglia and 133 adult macrophages origins were tested, without exploring the contribution of primitive 134 hematopoiesis to the embryonic/larval erythromyeloid lineage during tissue homeostasis and 135 regeneration.

136 In this study, we used the unique strengths of the zebrafish embryo to perform temporarily 137 resolved lineage tracing combined with live imaging to investigate the embryonic 138 erythromyeloid lineage ontogeny in vivo. We unveiled that embryonic erythrocyte and 139 macrophage lineages originate from primitive hematopoietic progenitors. In contrast, 140 definitive hematopoietic waves contribution to the erythromyeloid lineage occurred only at 141 the larval developmental stage. We further showed that upon tissue injury only primitive, but 142 not definitive, macrophages are recruited to the site of injury during embryonic development. 143 While both ontogenetically distinct macrophage populations were recruited to the site of 144 injury at early larval developmental stages, primitive macrophages were recruited to the 145 damage site earlier than their definitive counterparts. Our results also suggest that primitive 146 macrophages maintain tissue regeneration during early larval developmental stages after 147 selective ablation of definitive macrophages. Combined, our study reveals that the sequential 148 emergence of hematopoietic waves ensures the abundance of macrophages and erythrocytes 149 required for tissue homeostasis and integrity during development.

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#### 151 Results and discussion

# 152 Erythrocytes originate from primitive hematopoietic progenitors during embryonic153 development.

154 To set up a strategy to distinguish between primitive and definitive hematopoietic progenitors 155 based on their tissue of origin, we used an endothelial-specific tamoxifen-inducible transgenic 156 line Tq(fli1a:CreERT2) (Sánchez-Iranzo et al., 2018) in combination with different 157 hematopoietic lineage-specific switch and trace lines (Carney and Mosimann, 2018). All 158 definitive hematopoietic progenitors, including HSCs and EMPs, are generated as early as 24 159 hpf from endothelial progenitors (Bertrand et al., 2010; Kissa and Herbomel, 2010a; Tian et 160 al., 2017; Henninger et al., 2017; Ulloa et al., 2021; Bertrand et al., 2007). Thus, our inducible 161 labeling strategy at 24 hpf will label exclusively definitive hematopoietic progenitors as they 162 emerge without labeling primitive hematopoietic progenitors. To assess the efficiency of our 163 labeling strategy, we combined the Tq(fli1a:CreERT2) line with two different independent 164 transgenic lines, the lymphocyte-specific *Tq(lck:loxP-DsRedx-loxP-GFP)* (Tian et al., 2017; 165 Langenau et al., 2004) line (supplemental Figure 1A) and the Tq(coro1a:loxP-DsRedx-loxP-166 GFP) line which labels all leukocytes(Xu et al., 2015; Li et al., 2012) (supplemental Figure 1D). 167 We found that 80% of thymocytes in the thymus, which have only a definitive hematopoietic 168 origin (Tian et al., 2017; Elsaid et al., 2021), were labelled using either the Tq(lck:loxP-DsRedx-169 *loxP-GFP*) (supplemental Figure 1B-C) or the *Tg(coro1a:loxP-DsRedx-loxP-GFP*) lines 170 (supplemental Figure 1E-F), indicating the high labelling efficiency of our system.

To determine the origin of erythrocytes during embryonic development and early larval stages, we combined the Tg(fli1a:CreERT2) line with the erythrocyte-specific  $Tg(\alpha/\beta_{a2}$ *qlobin:loxP-DsRedx-loxP-GFP*) line (referred to hereafter as globin:switch) (Tian et al., 2017; 174 Ganis et al., 2012). In double transgenic zebrafish, 4-OH-tamoxifen (4-OHT) induces cre 175 recombination and removes the DsRed cassette, leading to permanent GFP expression in *fli1a*<sup>+</sup>-derived erythroid progeny (Figure 1A). To determine the contribution of definitive 176 177 hematopoietic progenitors to erythropoiesis during early stages of development, we exposed 178 embryos to either 4-OHT or ethanol (EtOH) starting at 24 hpf to label the aortic-endothelium-179 derived definitive hematopoietic progenitors (Bertrand et al., 2010; Kissa and Herbomel, 180 2010a; Tian et al., 2017), and then monitored GFP<sup>+</sup> definitive erythrocytes via live imaging 181 (Figure 1B and supplemental video 1). Primitive hematopoietic progenitors robustly 182 generated erythrocytes from early embryonic stages (Figure 1B). In contrast, GFP<sup>+</sup> definitive 183 erythrocytes started to emerge after 2 dpf, accumulated in the CHT region starting at 4 dpf (Figure 1C) and their contribution to the erythrocyte pool was not appreciable until later larval 184 185 developmental stages (~21 dpf, Figure 1C-F).

Using this labeling strategy, we demonstrated that under physiological conditions, definitive hematopoietic progenitors barely contribute to erythropoiesis during early stages of development. These results indicate that erythrocytes emerge in a layered organization during embryonic and early larval stages as in mammals (Soares-da-Silva et al., 2021), and that primitive hematopoietic progenitors generate a sufficient number of erythrocytes to sustain embryonic survival and tissue homeostasis (Palis, 2014).

#### 192 Embryonic macrophages originate exclusively from primitive hematopoietic progenitors

To assess the contribution of definitive hematopoietic progenitors to the embryonic macrophage pool, we marked the latter by combining the Tg(fli1a:CreERT2) line with the macrophage-specific Tg(mpeg1:LoxP-DsRedx-LoxP-GFP-NTR) line (referred to hereafter as 196 mpeg1:switch) (Lin et al., 2019; Ellett et al., 2011). As aforementioned, 4-OHT-induced cre 197 recombination leads to permanent GFP expression in  $fli1a^+$ -derived macrophages progeny (Figure 2A). Using this system, embryonic microglia cells, which are known to be of primitive 198 199 hematopoietic origin in zebrafish (Xu et al., 2015; Ferrero et al., 2018), were not labelled, 200 indicating the precision of this labelling system (supplemental figure 2A-B). While primitive 201 macrophages contributed robustly to the embryonic macrophage pool, we found that 202 definitive hematopoietic progenitors minimally contribute to macrophages during early 203 developmental stages, akin to erythrocytes ontogeny (Figure 2B). We showed further that 204 GFP<sup>+</sup> definitive macrophages started to emerge after 2 dpf and gradually increased in 205 numbers in the CHT region (Figure 2B and E) with a delayed modest contribution to the 206 macrophage pool in the periphery starting after 4 dpf (Figure 2B-D). GFP<sup>+</sup> definitive 207 macrophages contributed robustly to peripheral macrophages by 16 dpf, suggesting a distinct 208 differentiation kinetics of primitive and definitive macrophages throughout development (Figure 2C). 209

Altogether, our lineage tracing experiments show that aortic endothelium derived hematopoietic progenitors are barely contributing to embryonic erythroid and myeloid lineages (Figures 1 and 2). These data demonstrate that erythromyeloid lineages are mainly sustained by primitive hematopoietic progenitors during early developmental stages under physiological conditions.

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Ontogenically distinct macrophages are differentially recruited to the site of injury throughoutdevelopment.

218 It has been recently reported that distinct macrophage subpopulations play different roles to

219 promote tail regeneration after amputation (Wynn and Vannella, 2016; Morales and Allende, 220 2019). However, whether ontogenetically distinct macrophages display different functions 221 and recruitment behaviors to the site of injury remains poorly understood. Our tracing 222 experiments suggested that the definitive hematopoietic progenitors barely contribute to the 223 macrophage pool during early developmental stages under homeostasis. Therefore, we 224 sought to analyze if macrophages of distinct origins are recruited in a differential manner to 225 the damage site at different developmental stages. To track ontogenetically distinct 226 macrophages recruitment upon injury, we used the same strategy applied to trace them 227 under homeostasis (Figure 3A). We analyzed macrophages recruitment to the damaged site 228 in embryos (2 dpf) and larvae (5 dpf) since our lineage tracing experiments showed a different 229 abundance of ontogenetically distinct macrophages at these developmental stages (Figure 2). 230 We observed that only primitive macrophages were recruited to the site of injury in embryos 231 upon tail amputation (Figure 3B-C and supplemental Figure 3A). We then analyzed macrophage recruitment to the damage site in larvae, where both primitive and definitive 232 233 macrophage populations coexist in the periphery. We did not observe differences in the 234 recruitment of both primitive and definitive macrophages to the damage site 24 hours post 235 amputation (hpa) with a slight increase in the number of definitive macrophages 48 hpa 236 (Figure 3D-E and supplemental Figure 3B). Altogether, these results suggest that primitive 237 macrophages can alone maintain embryonic tissue homeostasis while both macrophage 238 populations are recruited to the damaged site and contribute to tail fin regeneration in the 239 larvae.

Previous reports showed that the first recruited macrophages play an important role in
proper tail fin regeneration (Morales and Allende, 2019; Nguyen-Chi et al., 2017; Petrie et al.,
2014), therefore, we further analyzed which macrophage population is recruited first to the

243 site of injury. We found that primitive macrophages are recruited earlier than their definitive 244 counterparts (Figure 3F-G). Since our observation of a delay in the recruitment of definitive 245 macrophages could be the result of differences in the number of primitive and definitive 246 macrophages in the periphery at the time of injury, we attempted to normalize their numbers. 247 Data was normalized by dividing the number of each recruited macrophage subpopulation by 248 their total number in the tail of the same larvae (Morales and Allende, 2019). After 249 normalization, we still observed that primitive macrophages are recruited earlier compared 250 to their definitive counterparts (Figure 3G). These data indicate that primitive macrophages, 251 as the first arrivals to the damage site, may have a specific role in the regeneration process 252 after amputation during development. Collectively, these results suggest that a layered 253 ontogeny of macrophages ensures their abundance for a timely contribution to tissue 254 homeostasis and regeneration.

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256 Primitive macrophages solely maintain tissue regeneration during early larval developmental

257 stages after selective ablation of definitive macrophages.

258 To evaluate the contribution of ontogenetically distinct macrophages to the regeneration of 259 the damaged tissue, we used the aforementioned strategy to trace and deplete macrophages 260 in a wave-specific manner during the regeneration process (Figure 4A). We used the mpeg1 261 switch line Tg(mpeg1:loxP-dsRed-loxP-eGFP-NTR) that allows selective ablation of 262 macrophages based on their origin. In this line, the expression of bacterial nitroreductase 263 (NTR) is under the control of the *mpeg1* promoter (Ellett et al., 2011). Thus, the NTR will be 264 expressed exclusively in the traced macrophages, thereby, traced macrophages could be 265 ablated by metronidazole (MTZ) treatment (Curado et al., 2008). Following this approach, we 266 evaluated the contribution of definitive macrophages selectively upon tissue injury. To assess

the efficiency of the NTR-MTZ system, we quantified the number of primitive and definitive
macrophages in the tail after treatment with either DMSO or MTZ for 48 hours from 4 to 6
dpf. We found that while primitive macrophages were not affected by the MTZ treatment,
definitive macrophages were significantly reduced after 48 hours of MTZ treatment (Figure
4B-C).

272 We next performed tail fin amputation in 5 dpf larvae that was treated with either DMSO or 273 MTZ from 4-6 dpf to ensure ablation of definitive macrophages through the regeneration 274 process. To assess if tail fin regeneration is affected when definitive macrophages are ablated, 275 we evaluated tail fin regeneration after amputation by measuring the tail fin area. While 276 primitive macrophage numbers and recruitment were not affected during the regeneration 277 process, definitive macrophages were significantly reduced during the first 48 hpa but 278 recovered by 72 hpa (Figure 4D-G and supplemental Figure 3C-D). We then performed tail fin 279 regeneration analysis and observed no differences in the regenerated tail fin area between larvae treated with either DMSO or MTZ (Figure 4H-I). Our results, thus, indicate that primitive 280 281 macrophages recruitment to the damage site is not affected and tail fin regeneration is not 282 impaired when definitive macrophages are depleted. Taken together, these data further 283 emphasize that primitive macrophages are sufficient to ensure a timely tissue regeneration 284 and to compensate the absence of other distinct macrophage populations during early 285 developmental stages.

Our study shows that the embryonic hematopoietic system follows a layered strategy to provide a timely supply of innate immune cells and erythrocytes. Under steady-state conditions, we found that the contribution of definitive hematopoietic progenitors to embryonic erythromyeloid lineage is limited. This is of particular interest as none of the 290 lineage tracing strategies performed in mice, despite their elegant designs, are specific to 291 definitive hematopoietic waves. Our results also complement the recently reported 292 observations that HSCs have a delayed contribution to the lymphomyeloid lineage during 293 development (Ulloa et al., 2021). Together, our observations indicate that the embryonic 294 erythromyeloid lineage is sustained by primitive hematopoietic progenitors under 295 homeostasis.

296 To investigate the temporal contribution of the macrophage lineage upon tissue injury, we 297 analyzed macrophage recruitment to the damaged tissue during embryonic and early larval 298 developmental stages. We found that primitive macrophages are the only ones recruited to 299 the site of injury during embryonic stages, however, both primitive and definitive macrophage 300 populations were recruited at early larval developmental stages. This difference in 301 recruitment behaviour between primitive and definitive macrophages could be due to that 302 the primitive macrophages are more sensitive to damage sensing and/or a consequence of 303 the immaturity of definitive macrophages at early developmental stages.

304 Furthermore, as timely recruitment of macrophages to the damage site is important to ensure 305 proper tail fin regeneration (Wynn and Vannella, 2016; Morales and Allende, 2019; Nguyen-306 Chi et al., 2017; Petrie et al., 2014), we found that the primitive macrophages are the first 307 responders after tail fin injury showed by their earlier recruitment than their definitive 308 counterparts. Thus, we propose that during development, primitive macrophages are more 309 prone to sense signals from the damage site and migrate toward it earlier than their definitive 310 counterparts. These intriguing results suggest that embryos and early larvae adopt a layered 311 ontogeny of macrophages to ensure their abundance for a timely contribution to tissue 312 regeneration.

313 A major finding of our study is the demonstration that primitive macrophages can solely 314 maintain tissue regeneration during development. This observation was supported by 315 selectively ablating the definitive macrophage pool and analyzing the tail fin area of 316 amputated larvae. We found that the number of recruited primitive macrophages to the site 317 of injury was not altered and tail fin regeneration was not impaired in definitive macrophage-318 ablated larvae. Together, these results suggest that primitive macrophages are sufficient to 319 ensure tissue regeneration and to compensate the absence of other distinct macrophage 320 populations during critical early developmental stages.

321 In conclusion, our study provides insights into the ontogeny of the erythromyeloid lineage 322 during embryonic/early larval developmental stages. Our findings support the notion that 323 embryos show a sequential emergence of hematopoietic waves to ensure the abundance of 324 macrophages required for tissue homeostasis and regeneration during a crucial 325 developmental time window. In line with our results, it has been recently reported in mice (Gomez Perdiguero et al., 2015; Patel et al., 2022; Yokomizo et al., 2022; Fanti et al., 2023) 326 327 and in zebrafish (Tian et al., 2017; Ulloa et al., 2021) that embryonic and adult HSCs do not 328 give rise to several blood subtypes at steady state, upon ablation of mature blood cells, and 329 in response to immune challenge. These observations indicate that ensuring a rapid response 330 to stress to maintain tissue homeostasis in an HSC-independent manner might be a conserved 331 mechanism between species throughout their life.

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#### 340 Materials and Data availability

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342 This study did not generate new unique reagents. The data sets generated during the

- 343 current study are available from the corresponding authors upon request.
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#### 345 Materials and methods

347 Zebrafish. Embryonic and adult zebrafish (Danio rerio) were maintained at 28°C on a 14-hour 348 light/10 hour dark cycle. The collected embryos were raised in fish water containing 349 0.01% methylene blue to prevent fungal growth. All fish are housed in the fish facility of our 350 laboratory, which was built according to the local animal welfare standards. All animal 351 procedures were performed in accordance with French and European Union animal welfare 352 guidelines and were evaluated by the ethic committee of the Curie Institute (Paris, France) 353 and approved.

354

**Transgenic lines.** The following lines of the AB stain were used:  $Tg(fli1a: CreER^{T2})$  (Sánchez-355 356 Iranzo et al., 2018), Tq(mpeq1:LoxP-DsRedx-LoxP-GFP-NTR)(Lin et al., 2019),Tq(coro1a:loxP-357 DsRedx-loxP-GFP) (Xu et al., 2015), Tg(globin:loxP-DsRedx-loxP-GFP) (Tian et al., 2017) and 358 *Tq*(*lck:loxP-DsRedx-loxP-GFP*) (Tian et al., 2017).

359

360 Fluorescence Microscopy. Zebrafish embryos, larvae and adults were anesthetized with 361 0.01% tricaine (A5040, Sigma) and mounted in 2.5% 2.5% methylcellulose in 35-mm imaging 362 dishes (MatTek) as described previously (Renaud et al., 2011). Fluorescent imaging was 363 performed with either Zeiss Axio Zoom V.16 upright microscope with an AxioCam HRm Zeiss 364 camera and Zeiss Zen 3.3 software or with Leica thunder imaging system with Leica LAS-X 365 software. Fluorescence was detected with dsRed and green fluorescent protein (GFP) filters. 366

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367 Image Analysis. All images were analyzed using FIJI software (Schindelin et al., 2012).

369 **CreER-loxP Cell Labelling (Lineage Tracing).** Zebrafish embryos at 24 hpf were treated with 370 5 μM 4-OHT (H7904, Sigma) for 24 hours. Controls were incubated in the equivalent amount 371 of Ethanol solution during the same period. Light exposure was avoided by using foil to cover 372 the plates as 4-OHT is light sensitive. After treatment, embryos were washed with fresh 373 embryo medium and placed back in incubator. From 5 dpf, larvae were transferred to the fish 374 facility nursery where they were kept and fed. Embryos were raised for further analysis at 375 different developmental stages.

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**Metronidazole treatment and cell ablation.** The *Tq(fli1CreER<sup>T2</sup>;mpeq1:loxP-DsRedx-loxP-*377 GFP-NTR) embryos were immersed in system water containing 10 mM metronidazole 378 379 (M1547, Sigma) for 48 hours, which caused an acute depletion of GFP-NTR<sup>+</sup> cells. The medium 380 containing the MTZ was changed every 24 hours. Controls were incubated in the equivalent 381 amount of DMSO solution during the same period. Light exposure was avoided by using foil 382 to cover the plates as MTZ is light sensitive. Zebrafish were further analyzed by fluorescence 383 imaging at different developmental time points.

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385 Tail Fin Amputation. 2 and 5 dpf larvae were anesthetized with 0.01% tricaine and amputated 386 with a sterile scalpel. The transection was performed by using the posterior section of the

ventral pigmentation gap in the tail fin as a reference, and immediately after amputation
 larvae were incubated in either E3 medium, or in MTZ in case of macrophage ablation at 28°C.

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**Quantification of Tail Fin Regeneration.** At 24, 48 and 72 hours post amputation or dpa (6,7and 8-days post fertilization, respectively), larvae were mounted in 2.5% methylcellulose, and regenerating tail fins were imaged in bright field. Tail fin area was measured from the anterior section of the ventral tail fin gap to the end of the regenerating fin, as previously described (Pei et al., 2016), using FiJi software. Tail fin areas were calculated and expressed in square micrometers (um<sup>2</sup>).

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**Quantification of Tail Macrophages.** Macrophages were quantified based on their location in the tail (periphery vs CHT) (Figure 2B) in non-amputated larvae. In amputated larvae, recruited macrophages to the site of injury were quantified. Normalization of recruited macrophage numbers was obtained by dividing the number of recruited macrophage subpopulation by their total number in the tail of the same larvae (periphery, CHT and recruited macrophages) (Morales and Allende, 2019).

403

404 Quantification and statistical analysis. Statistical analyses were performed by the GraphPad 405 Prism software (Prism 9). All experiments with only two groups and one dependent variable 406 were compared using an unpaired t-test with Welch's correction. Two-way ANOVA with 407 Tukey's multiple comparison was used for this analysis. Statistical data show mean ± s.e.m. 408 Each dot plot value represents an independent embryo, and every experiment was conducted 409 three times independently. The corrected total cell fluorescence (CTCF) was calculated using 410 the formula 'Integrated density whole – (area whole embryo x mean fluorescence 411 background)'. This formula is loosely based on a method described for calculating cell-412 fluorescence (Ulloa et al., 2021).

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#### 428 Authors contribution

429

430 Conceptualization: R.E. and P.P.H.; Methodology and data collection: R.E., A.M., P.D. and

431 G.G.; Writing - Original Draft: R.E.; Writing - Review & Editing: R.E. and P.P.H.; Funding

432 Acquisition: P.P.H.

433 434	Conflict of Interest
435 436	The authors declare no competing interests.
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606

#### **Steady state**



### **Caudal fin Injury**

During embryonic stages





Mo/ Mo Recruitment





Regeneration



During early larval developmental stages upon ablation of definitive macrophages







--- DsRed

--- GFP

\*\*\*\*

-<u>∓</u>

8 dpf

6 dpf

4 dpf









l 21 dpf

40-

20-0

2 dpf

40-

20-

0

2 dpf

4 dpf

6 dpf

8 dpf



0

2 dpf

6 dpf

8 dpf

4 dpf

16 dpf



Figure 2









### Supp Figure 1



100 80

> 60· 40· 20 0

÷

GFP+ DsRed+



Supp video



## CHT: caudal hematopoietic tissue

# Supp Figure 2.Related to Fig. 2









В



С



D



# Figure 1: Tracing the contribution of definitive hematopoietic waves to the erythroid lineage in the embryo and early larvae.

(A) Scheme of the 4-OHT-inducible transgenic lines used to assess the contribution of definitive hematopoietic waves to the erythroid lineage.

(B) Fluorescent images of EtOH non-switched controls (right) and 4-OHT-induced (left)

*Tg(fli1a:creERT2;globin:Switch)* embryos and larvae (2 dpf-8 dpf). Scale bars, 100 μm. Representative images are shown with quantification of erythrocytes in circulation in (E).

(C) Fluorescent images of 4-OHT-induced *Tg(fli1a:creERT2;globin:Switch)* embryos and larvae (2-8 dpf) in the CHT region. Non-switched primitive erythrocytes (right) and definitive erythrocytes (left). Scale bars, 100 μm. Representative images are shown with quantification of erythrocytes in the CHT in (F).

(D) Fluorescent images of 4-OHT-induced Tg(fli1a:creERT2;globin:Switch) (21 dpf) in the circulation. Scale bar, 100  $\mu$ m. Non-switched primitive erythrocytes (right) and definitive erythrocytes (left). Representative images are shown with quantification of erythrocytes in the circulation in (E).

(E) Quantification of DsRed and GFP fluorescence intensity percentage in the circulation was measured over a time course of 2-21 dpf. (2 dpf n=6; 4 dpf n=4; 6 dpf n=5; 8 dpf n=4; 21 dpf n=5). Mean  $\pm$  SEM of the

DsRed<sup>+</sup> and GFP<sup>+</sup> corrected total cell fluorescence (CTCF) percentage at each time point is shown. Two-way ANOVA with Sidak's multiple comparison was used for this analysis. \*\*\*\*p  $\leq$  0.0001.

(F) Quantification of DsRed and GFP fluorescence intensity percentage in the CHT was measured over a time course of 2-8 dpf. (2 dpf n=6; 4 dpf n=4; 6 dpf n=5; 8 dpf n=4). Mean  $\pm$  SEM of the DsRed<sup>+</sup> and GFP<sup>+</sup> corrected total cell fluorescence (CTCF) percentage at each time point is shown. Two-way ANOVA with Sidak's multiple comparison was used for this analysis. \*\*\*\*p  $\leq$  0.0001.

# Figure 2: Tracing the contribution of definitive hematopoietic waves to macrophages in the embryo and early larvae.

(A) Scheme of the 4-OHT-inducible transgenic lines used to assess the contribution of definitive hematopoietic waves to the macrophage lineage.

(B) Fluorescent images of EtOH non-switched controls (right) and 4-OHT-induced (left)

Tg(fli1a:creERT2;mpeg1:Switch) embryos and larvae (2 dpf-8 dpf). Macrophages were considered as peripheral macrophages (white area) or CHT-resident macrophages (blue area). Scale bars, 100 µm. Representative images are shown with quantification of macrophages in the periphery in (D) and the CHT in (E).

(C) Fluorescent images of 4-OHT-induced Tg(fli1a:creERT2;mpeg1:Switch) (16 dpf) in the periphery. Scale bar, 500  $\mu$ m. Non-switched primitive macrophages (right) and definitive macrophages (left). Representative images are shown with quantification of macrophages in the periphery in (D).

(D) Quantification of DsRed and GFP macrophage number in the periphery was measured over a time course of 2-16 dpf. (2 dpf n=6; 4 dpf n=11; 6 dpf n=7; 8 dpf n=4; 16 dpf n=3). Mean  $\pm$  SEM of the DsRed<sup>+</sup> and GFP<sup>+</sup> macrophage number at each time point is shown. Two-way ANOVA with Sidak's multiple comparison was used for this analysis. \*\*\*\*p  $\leq$  0.0001.

(E) Quantification of DsRed and GFP macrophage number in the CHT was measured over a time course of 2-8 dpf. (2 dpf n=6; 4 dpf n=11; 6 dpf n=7; 8 dpf n=4). Mean  $\pm$  SEM of the DsRed<sup>+</sup> and GFP<sup>+</sup> macrophage number at each time point is shown. Two-way ANOVA with Sidak's multiple comparison was used for this analysis. \*\*\*\*p  $\leq$  0.0001.

# Figure 3: Ontogenetically distinct macrophages exhibit different migration behavior and recruitment after tissue injury during early developmental stages.

(A) Scheme of the 4-OHT-inducible transgenic lines used to assess the contribution of ontogenetically distinct hematopoietic waves to the macrophage lineage upon tissue injury.

(B) Diagram showing the amputation plan, tail fin of Tg(fli1a:creERT2;mpeg1:Switch) were amputated at 2 dpf and macrophages recruitment to the site of injury was analyzed. Representative images are shown at 24 hpa. Scale bar:  $100\mu$ m.

(C) Diagram showing the counting region in the larvae. Quantification of DsRed and GFP macrophage number at the site of injury at 24 hpa (n=14) and 48 hpa (n=12). Mean  $\pm$  SEM of the DsRed<sup>+</sup> and GFP<sup>+</sup> macrophage number at each time point is shown. \*\*\*\*p  $\leq$  0.0001.

(D) Diagram showing the amputation plan, tail fin of *Tg(fli1a:creERT2;mpeg1:Switch)* were amputated at 5 dpf and macrophages recruitment to the site of injury was analyzed. Representative images are shown at 24 hpa. Scale bar: 100µm.

(E) Diagram showing the counting region in the larvae. Quantification of DsRed and GFP macrophage number at the site of injury at 24 hpa (n=10) and 48 hpa (n=6). Mean  $\pm$  SEM of the DsRed<sup>+</sup> and GFP<sup>+</sup> macrophage number at each time point is shown. \*\*\*\*p  $\leq$  0.0001.

(F) Tail fin of *Tg(fli1a:creERT2;mpeg1:Switch)* were amputated at 5 dpf and macrophages recruitment to the site of injury was analyzed. Representative images are shown at 3 hpA. Scale bar: 100µm.

(G) Quantification of DsRed and GFP macrophage number at the site of injury at 3 hpa (n=13). Mean  $\pm$  SEM of the DsRed<sup>+</sup> and GFP<sup>+</sup> macrophage number at each time point is shown (left). Quantification was normalized by the number of total macrophages in the tail of the respective larvae (the sum of peripheral, CHT and recruited macrophages of distinct origins).\*\*p  $\leq$  0.001;\*\*\*\*\*p  $\leq$  0.0001.

#### Figure 4: Selective ablation of definitive macrophages does not impair tail fin regeneration in early larvae.

(A) Diagram showing the macrophage ablation and tail fin amputation plan.

(B) Switched *Tg(fli1a:creERT2;mpeg1:Switch)* larvae were treated with DMSO as a control, or metronidazole (MTZ) to ablate definitive macrophages. Treatments were performed from 4 to 6 dpf. Representative images are shown 48 hours after treatments. Scale bars: 100 μm.

(C) Quantification of DsRed and GFP macrophage number in the tail region 48 hours after DMSO (n=9) or MTZ (n=9) treatments. Mean  $\pm$  SEM of the DsRed<sup>+</sup> and GFP<sup>+</sup> macrophage number is shown. \*\*\*\*p  $\leq$  0.0001.

(D) Switched *Tg(fli1a:creERT2;mpeg1:Switch)* larvae were treated with DMSO as a control, or metronidazole (MTZ) to ablate definitive macrophages. Treatments were performed from 4 to 6 dpf and tail fins were amputated at 5 dpf. Representative images are shown 24 hours after amputation. Scale bars: 100 µm.

(E) Quantification of DsRed and GFP recruited macrophage number in the tail region 24 hours after amputation. Larvae were treated at 4 dpf with either DMSO (n=11) or MTZ (n=20) for 48 hours. Mean  $\pm$  SEM of the DsRed<sup>+</sup> and GFP<sup>+</sup> macrophage number is shown. \*\*\*\*p  $\leq$  0.0001.

(F) Switched Tg(fli1a:creERT2;mpeg1:Switch) larvae were treated with DMSO as a control, or metronidazole (MTZ) to ablate definitive macrophages. Treatments were performed from 4 to 6 dpf and tail fins were amputated at 5 dpf. Representative images are shown 48 hours after amputation. Scale bars: 100 µm. (G) Quantification of DsRed and GFP recruited macrophage number in the tail region 48 hours after amputation. Larvae were treated at 4 dpf with either DMSO (n=17) or MTZ (n=9) for 48 hours. Mean ± SEM of the DsRed<sup>+</sup> and GFP<sup>+</sup> macrophage number is shown. \*\*\*\*p ≤ 0.0001.

(H) Representative images of regenerating tail fins of larvae at 72 hours after amputation. Larvae were treated with either DMSO or MTZ. Scale bars:  $100 \ \mu m$ .

(I) Tail fin area quantification of regenerating tail fins at 24 hpa, 48 hpa and 72 hpa in larvae treated with either DMSO or MTZ (24 hpa: DMSO n=21, MTZ n=20; 48 hpa: DMSO n=17, MTZ n=9; 72 hpa: DMSO n=14, MTZ n=9).

Sup. Figure 1: labeling strategy and the labeling efficiency of using thymocytes as a positive read out with *lck*:switch and *coro1a*:switch transgenic lines.

(A) Scheme of the 4-OHT-inducible transgenic lines used to assess the efficiency of the labelling strategy using the *Tg(fli1a:creERT2;lck:Switch)* double transgenic line.

(B) Fluorescent images of EtOH non-switched controls (right) and 4-OHT-induced (left)

Tg(fli1a:creERT2;lck:Switch) thymocytes at 5 dpf larvae. Representative images are shown. Scale bars, 100  $\mu$ m. (C) Quantification of DsRed and GFP fluorescence intensity percentage in the thymic region was measured at 5 dpf (n=14). Mean ± SEM of the DsRed<sup>+</sup> and GFP<sup>+</sup> corrected total cell fluorescence (CTCF) percentage is shown. \*\*\*\*\*p ≤ 0.0001.

(D) Scheme of the 4-OHT-inducible transgenic lines used to assess the efficiency of the labelling strategy using the *Tg(fli1a:creERT2;coro1a:Switch)* double transgenic line.

(E) Fluorescent images of EtOH non-switched controls (right) and 4-OHT-induced (left)

*Tg(fli1a:creERT2;coro1a:Switch)* thymocytes at 3 dpf embryos. Representative images are shown. Scale bars, 100 μm.

(F) Quantification of DsRed and GFP fluorescence intensity percentage in the thymic region was measured at 3 dpf (n=8). Mean  $\pm$  SEM of the DsRed<sup>+</sup> and GFP<sup>+</sup> corrected total cell fluorescence (CTCF) percentage is shown. \*\*\*\*\*p  $\leq$  0.0001. **Supp videos related to figure 1.** Fluorescence imaging of 4-OHT-induced *Tg(fli1a:creERT2;globin:Switch)* embryos and larvae over a time course of 2- 8 dpf. Videos are assembled from different Z-stacks projections at each defined developmental stage.

# Supp Fig.2. Quantification of GFP+ microglia in the head region at 2 dpf embryos. related to Fig.2 (A) Fluorescent images of EtOH non-switched controls (right) and 4-OHT-induced (left)

*Tg(fli1a:creERT2;mpeg1:Switch)* head regions in the developing embryos at 2 dpf. Representative images are shown Scale bar: 100μm.

(B) Quantification of GFP+ macrophage number in the head at 2 dpf (n=6). Mean  $\pm$  SEM of the and GFP<sup>+</sup> macrophage number is shown.

# Supp fig 3. Ontogenetically distinct macrophages recruitment after tail injury at different developmental stages. related to fig 3

(A) tail fin of Tg(fli1a:creERT2;mpeg1:Switch) were amputated at 2 dpf and macrophages recruitment to the site of injury was analyzed. Representative images are shown at 48 hpA. Scale bar:  $100\mu$ m.

(B) tail fin of *Tg(fli1a:creERT2;mpeg1:Switch)* were amputated at 5 dpf and macrophages recruitment to the site of injury was analyzed. Representative images are shown at 48 hpA. Scale bar: 100µm.

(C) Switched *Tg(fli1a:creERT2;mpeg1:Switch)* larvae were treated with DMSO as a control, or metronidazole (MTZ) to ablate definitive macrophages. Treatments were performed from 4 to 6 dpf and tail fins were amputated at 5 dpf. Representative images are shown 72 hours after amputation. Scale bars: 100 μm.

(D) Quantification of DsRed and GFP recruited macrophage number in the tail region 72 hours after amputation. Larvae were treated at 4 dpf with either DMSO (n=14) or MTZ (n=9) for 48 hours. Mean ± SEM of the DsRed<sup>+</sup> and GFP<sup>+</sup> macrophage number is shown.