Identification of polarized macrophage subsets in zebrafish

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Mai Nguyen-Chi1,2†, Béryl Laplace-Builhe1,2†, Jana Travnickova2,3, Patricia Luz-Crawford1,2, Gautier Tejedor1,2, Quang Tien Phan3, Isabelle Duroux-Richard1,2, Jean-Pierre Levraud4,5, Karima Kissa2,3, Georges Lutfalla2,3, Christian Jorgensen1,2,6‡, Farida Djouad1,2*‡

1Institut de Médecine Régénérative et Biothérapies, Institut national de la santé et de la recherche médicale, Montpellier, France; 2Université de Montpellier, Montpellier, France; 3Dynamique des Interactions Membranaires Normales et Pathologiques, Centre national de la recherche scientifique, Montpellier, France; 4Macrophages et Développement de l’Immunité, Institut Pasteur, Paris, France; 5Département de Biologie du Développement et Cellules Souches, Institut Pasteur, Paris, France; 6Clinical unit for osteoarticular diseases and Department for Biotherapy, Centre Hospitalier Universitaire, Montpellier, France

Abstract

While the mammalian macrophage phenotypes have been intensively studied in vitro, the dynamic of their phenotypic polarization has never been investigated in live vertebrates. We used the zebrafish as a live model to identify and trail macrophage subtypes. We generated a transgenic line whose macrophages expressing tumour necrosis factor alpha (tnfa), a key feature of classically activated (M1) macrophages, express fluorescent proteins Tg(mpeg1:mCherryF/tnfa:eGFP-F). Using 4D-confocal microscopy, we showed that both aseptic wounding and Escherichia coli inoculation triggered macrophage recruitment, some of which started to express tnfa. RT-qPCR on Fluorescence Activated Cell Sorting (FACS)-sorted tnfa+ and tnfa− macrophages showed that they, respectively, expressed M1 and alternatively activated (M2) mammalian markers. Fate tracing of tnfa+ macrophages during the time-course of inflammation demonstrated that pro-inflammatory macrophages converted into M2-like phenotype during the resolution step. Our results reveal the diversity and plasticity of zebrafish macrophage subsets and underline the similarities with mammalian macrophages proposing a new system to study macrophage functional dynamic.

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Introduction

Behind the generic name ‘macrophage’ hides various cell types with distinct phenotypes and functions. Currently, it is well established that macrophages are not just important immune effector cells but also cells with critical homeostatic roles, exerting a myriad of functions in development, homeostasis, and tissue repair and playing a pivotal role in disease progression (Wynn et al., 2013). Therefore, there is a high interest in a better characterization of these cells to establish an early and accurate diagnosis. The wide variety of macrophage functions might be explained by the outstanding plasticity and versatility of macrophages that efficiently respond to environmental challenges and changes in tissue physiology by modifying their phenotype (Mosser and Edwards, 2008). Although there is a consensus that macrophages are a diversified set of cells, macrophage subtypes are still poorly characterized. Indeed, although these cell populations have been extensively investigated in mouse and human, these studies were mostly performed in vitro using monocyte-derived macrophages induced under specific stimuli. A comprehensive characterization of macrophage
subsets that takes into account their specific behaviour, phenotypic diversity, functions, and modulation shall rely on a real-time tracking in the whole organism in response to environmental challenges.

Mouse and human macrophages have been classified according to their polarization state. In this classification, M1 macrophages, also referred as classically activated macrophages, are pro-inflammatory cells associated with the first phases of inflammation, while M2 macrophages, also known as alternatively activated macrophages, are involved in the resolution of inflammation and tissue remodelling (Gordon, 2003; Biswas and Mantovani, 2010; Sica and Mantovani, 2012). Differential cytokine and chemokine production and receptor expression define the polarization state of macrophages. However, it is worthwhile to note that such binary naming does not fully reflect the diversity of macrophage phenotypes in complex in vivo environments in which several cytokines and growth factors are released and adjust the final differentiated state (Chazaud, 2013; Thomas and Mattila, 2014). Macrophages might adopt intermediate activation phenotypes classified by the relative levels of macrophage subset-specific markers. Therefore, macrophage plasticity results in a full spectrum of macrophage subsets with a myriad of functions (Mosser and Edwards, 2008; Xue et al., 2014). Although the possible phenotype conversion of macrophages from M1 to M2 has been suggested in vitro studies, a recent study argues for the sequential homing of M1 and M2 macrophages to the site of injury (Stout et al., 2005; Sica and Mantovani, 2012; Shechter et al., 2013). Such controversies highlight the lack of accurate real-time tracing of macrophage subtypes in vivo in the entire animal.

Inflammation is a model of choice to study the wide range of macrophage subsets involved from its initiation to its resolution. Therefore, in the present study, we propose to decipher in vivo in real time the kinetic of macrophage subset recruitment, their behaviour and their phenotypic plasticity at the molecular level during a multiple-step inflammatory process. We used the zebrafish larvae model for its easy genetic manipulation, transparency, and availability of fluorescent reporter lines to track macrophages (Ellett et al., 2011). While the existence of macrophage subtypes in zebrafish embryos
Figure 1. The (tnfa:eGFP-F) reporter line recapitulates transcriptional activation of tnfα upon wound-induced inflammation and Escherichia coli infection. (A–E) Tumour necrosis factor alpha (tnfα) mRNA expression (blue, arrowhead) was detected by in situ hybridization using tnfα anti-sense probe: at 6 hpA in (A) intact (control) and (B) amputated fins from 3 dpf WT larvae, (C) in uninfected larvae (54 hpf, hours post-fertilization) and (D, E) E. coli infected larvae (24 hpi, 54 hpf). Arrows show melanocytes (black). (E) Imaging of tnfα mRNA expression in the muscle at higher magnification, asterisks show muscle fibres, scale bar in (B) = 100 μm and in (E) = 50 μm. (F, G) eGFP fluorescence (green) was analyzed by fluorescent microscopy in (F) intact (control) and (G) amputated Tg(tnfa:eGFP-F) fins at 6 hpA, dotted lines outline the caudal fin, scale bar = 100 μm and at 16 hpi in Tg(tnfa:eGFP-F) larvae injected with (H) PBS or (I, J) E. coli (red) in the muscle. Arrows show auto-fluorescent xanthophores. (J) Multi-scan confocal analysis of GFP expression in E. coli-infected Tg(tnfa:eGFP-F) larvae, scale bar = 20 μm. (K) tnfα mRNA and eGFP-F expressions were analyzed using microscopy at 6 hpA in amputated fins from 3 dpf Tg(tnfa:eGFP-F) larvae. Dotted lines delimit the caudal fin, arrowheads show overlapping signals, and arrows show the pigments. Scale bar = 100 μm. (L) Graphed data of representative fluorescence-activated flow cytometry analysis of eGFP+ cells in (M) Control or (M) Amputation. Green gates represent eGFP+ population and mean percentage of eGFP+ cells upon amputation. Tg(tnfa:eGFP-F) larvae were either kept intact (control) or amputated at 3 dpf, and cells were collected at 6 hr post-treatment. Green gates represent eGFP+ population and mean percentage of eGFP+ cells upon amputation.
has been suggested, they have not been fully characterized (Herbomel et al., 1999; Ellett et al., 2011; Cambier et al., 2013; Petrie et al., 2014). Here, we report a new reporter transgene for TNFa, a central inflammatory cytokine and well-established marker of M1 macrophages, instrumental to discriminate macrophage subsets during intravital imaging.

**Results and discussion**

**In vivo visualization of macrophage activation and polarization**

Fin wounding-induced inflammation and *Escherichia coli* inoculation in zebrafish larvae of 3 dpf are two well-established models triggering macrophage recruitment. Using in situ hybridization, we observed that the expression of the tumour necrosis factor alpha (*tnfa*), a consensus marker of M1 macrophages, was induced in cells accumulated in the caudal fin and the muscle following amputation (*nlarvae* = 29/33) and *E. coli* inoculation (*nlarvae* = 12/12), respectively (Figure 1A–E). To study the cells that express the *tnfa* transcripts, we established the *Tg(tnfa:eGFP-F*) transgenic zebrafish line expressing a farnesylated (membrane-bound) eGFP (eGFP-F) under the control of the *tnfa* promoter. While eGFP-F was undetectable in intact fins of *Tg(tnfa:eGFP-F*) fish to study macrophage activation by mating them with *tnfa* transgenic larvae, we performed a simultaneous detection of *tnfa* mRNA by in situ hybridization and GFP-F protein by immunofluorescence in amputated larvae 6 hpA. We observed a consistent overlap between *tnfa* and GFP-F signal in the fin (*nlarvae* = 11/11), showing the direct correlation of eGFP-F and *tnfa* transcriptional activation in the fin of the reporter line (Figure 1K). In addition, we FACS-sorted GFP+ cells from wounded *Tg(TNFα:eGFP-F*) larvae 6 hpA and performed RT-qPCR to analyze *tnfa* expression. We observed a significant increase of *tnfa* mRNA level in eGFP+ cells as compared to eGFP− cells (Figure 1L,M). Both together these results indicate that the *Tg(TNFα:eGFP-F*) reporter line recapitulates transcriptional activation of *tnfa*. Then, with the ability to specifically track *tnfa*-expressing cells, we used *Tg(tnfa:eGFP-F*) fish to study macrophage activation by mating them with *Tg(mpeg1:mCherryF*) fish in which macrophages express farnesylated mCherry (mCherryF) under the control of the macrophage-specific *mpeg1* promoter (Ellett et al., 2011; Nguyen-Chi et al., 2014). In intact *Tg(tnfa:eGFP-F/mpeg1:mCherryF*) larvae, no eGFP-F was observed in macrophages (Figure 2A). We imaged double transgenic larvae *Tg(tnfa:eGFP-F/mpeg1:mCherryF*) using 4D confocal microscopy from 45 min post-amputation and found that macrophages were recruited to the wound from 1 hpA, some starting to arrive at the wound (Video 1 and Figure 2C). Similarly, infection with a crimson-expressing *E. coli* in the muscle induced the expression of *tnfa* in phagocytes few hours following the infection (Figure 2—figure supplement 1, Video 2). Imaging of the double transgenic larvae *Tg(tnfa:eGFP-F/mpeg1:mCherryF*) showed that *tnfa*-expressing phagocytes were mainly macrophages (Figure 2—figure supplement 1, Video 2). These results show the dynamic macrophage activation in real-time in vivo including recruitment and rapid phenotypic change. During the revision of this paper, a similar result has been published (Sanderson et al., 2019).

**Morphology and behaviour of macrophage phenotypes**

To test whether *tnfa+* and *tnfa−* macrophages harboured different cellular characteristics, we first analyzed their morphology in fin-wounded *Tg(tnfa:eGFP-F/mpeg1:mCherryF*) larvae. *tnfa+mpeg1+* cells displayed flattened and lobulated morphology (Figure 2D), while *tnfa−mpeg1−* were elongated
Figure 2. Activation, morphology, and behaviour of TNF-α+ macrophages in (tnfa:eGFP-F/mpeg1:mCherry-F) transgenic larvae upon wound-induced inflammation. (A) eGFP-F (green) and mCherryF (red) fluorescence was analyzed by fluorescent microscopy in intact (control) and amputated Tg(mpeg1:mCherryF/tnfa:eGFP-F) fins at 6 hpA of 3 dpf larvae. Arrowheads show recruited macrophages that express tnfa, arrows show tnfa+ cells that are not macrophages, and asterisks show auto-fluorescent pigments. Dotted lines outline the caudal fin, scale bar = 100 μm. (B) Bright-field image of the wounded fin of a 3 dpf Tg(mpeg1:mCherryF/tnfa:eGFP-F) larva. Dotted red box shows the region imaged in C. (C) Representative time-lapse maximum projections show the activation of macrophages arriving at the wound in 3 dpf amputated Tg(mpeg1:mCherryF/tnfa:eGFP-F). The time pA is shown on top right Figure 2. continued on next page
Figure 2. Continued
corner and indicated in hours and minutes, white lines outline the caudal fin. The transcriptional activation of \textit{tnfa} (green) in recruited macrophage (red, arrowhead) was first observed from 3 hPA. Scale bar = 30 μm. White lines outline the caudal fin. (D, E) Maximum projections of confocal analysis of eGFP-F (green) and mCherryF (red) expressions in recruited macrophages at (D) 18 hPA and (E) 24 hPA in Tg(mpeg1:;mCherryF;tnfa:eGFP-F). tnfα+ mpeg1+ macrophages exhibit a round and protrusive morphology, while tnfα−mpeg1+ macrophages exhibit a dendritic morphology. (F) Velocity of tnfα+mpeg1+ and tnfα−mpeg1+ macrophages (N = 18). (G) Frequency of macrophage–macrophage contacts and (H) time length of the contacts of tnfα+mpeg1+ and tnfα−mpeg1+ cells. Measurements were extracted from three independent videos of amputated Tg(mpeg1:mCherryF/tnfa:eGFP-F), for contact frequency, N = 15 and for duration of the interaction, N = 11 macrophages. ****p < 0.0001.

(I) Representative time-lapse maximum projections show the behaviour of tnfα+mpeg1+ macrophages, starting at 19h20 pA during 42 min. Two macrophages (green + red) interact by cell–cell contact. These macrophages (eGFP in grey) remain attached up to 40 min. Scale bar 20 μm. (J) Representative time-lapse maximum projections show the behaviour of tnfα+mpeg1+ macrophages, starting at 25h12 pA during 52 min. Macrophages (red) barely establish cell–cell contact. Scale bar = 30 μm.

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The following figure supplement is available for figure 2:

Figure supplement 1. Activation of tnfα+ macrophages in (tnfa:eGFP-F/mpeg1:mCherry-F) transgenic larvae upon E. coli infection.

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and dendritic (Figure 2E). As we observed that tnfα+mpeg1+ cells were predominant at the wound at 18 hPA and tnfα−mpeg1+ cells at 24 hPA (data not shown), we imaged the behaviour of these macrophage populations in wounded fins from Tg(tnfa:eGFP-F/mpeg1:mCherryF) larvae at these time points. tnfα+mpeg1+ cells presented a lower velocity (0.32 μm/min) than tnfα−mpeg1+ macrophages (1.09 μm/min, Figure 2F) but a higher cell–cell contact frequency (0.036 VS 0.016 contacts/min) with other macrophages (Figure 2G,I,J and Videos 3, 4). Measurements of the duration of macrophage–macrophage contacts showed that these contacts lasted longer (48.6 min/contact) than that of tnfα−mpeg1+ macrophages (15.9 min/contact, Figure 2H–J and Videos 3, 4). All together these data highlight different morphology and behaviour of macrophage phenotypes in live zebrafish suggesting the existence of macrophage subsets exhibiting different functions.

Macrophages phenotypes are activated in a time-dependant manner

To quantify the respective frequency of tnfα+ macrophages (mCherry+eGFP− referred as dbl+) and tnfα− macrophages (mCherry+eGFP+ referred as mCh+), we performed flow cytometry analysis on cells isolated from Tg(tnfa:eGFP-F/mpeg1:mCherryF) larvae at different time points following caudal fin amputation or E. coli inoculation (Figure 3A,B). While only 5.6% ± 0.9 (s.e.m.) dbl+ cells were detected in the mpeg1+ population of the intact larvae, a steady increase of the dbl+ population from 6 to 20 hPA (up to 27.33 ± 0.2%) was observed. This percentage decreased dramatically at 26 hPA to 8.75 ± 1%. In E. coli inoculation experiments, the frequency of dbl+ cells increased as soon as 3 hpi (55.60 ± 0.6%) and remained stable until 26 hpi. These results demonstrate that wound-induced macrophage activation is transient compared to infection-induced macrophage activation.

Video 1. Transcriptional activation of tnfα in macrophages of (tnfa:eGFP-F/mpeg1:mCherry-F) transgenic larvae upon amputation. Representative time-lapse maximum projections show the transcriptional activation of tumour necrosis factor \textalpha (tnfα) in macrophages arriving at the wound in 3 dpf amputated Tg(mpeg1:;mCherryF/tnfa:eGFP-F). The time pA is shown on top right corner, white line outline the caudal fin. Scale bar = 30 μm. Image stacks were acquired every 3 min 30 s from 45 min pA to 7 hr 48 min pA at 2-μm intervals, 1024 × 512 pixel resolution using a confocal microscope TCS SP5 SPS inverted equipped with a HCX PL APO 40×/1.25-0.75 oil objective (Leica). Excitation wavelengths used were 488 nm for EGFP-F and 570 nm for mCherryF.

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To characterize at the molecular level $tnfa^-$ and $tnfa^+$ macrophage populations during early and late phases of inflammation, we FACS-sorted $dbl^+$ and $mCh^+$ cells from $Tg(tnfa:eGFP-F/mpeg1:mCherry-F)$ tail-amputated larvae (Figure 3C) and analyzed them by qRT-PCR. In mammals, M1 and M2 macrophages are reported to be involved, respectively, in the initial phase of inflammation and in the resolution phase. Cell sorting was thus performed at 6 hpA and 26 hpA following caudal fin amputation since the kinetic analysis of macrophage subset activation (Figure 3B) suggested that these two time points correspond to initiation and resolution of inflammation, respectively. As expected, high levels of $mpeg1$ expression was observed in the $mCh^+$ and $dbl^+$ sorted cells at 6 and 26 hpA (Figure 3D), and high levels of $tnfa$ expression was detected in double-positive populations at 6 hpA (Figure 3E). These observations demonstrated that fluorescence of these transgenes can be efficiently used to track and separate macrophage sub-populations. At 6 hpA, $dbl^+$ macrophages expressed high levels of $tnfb$, $il1b$, and $il6$ compared to $mCh^+$ macrophages (Figure 3F,G) that are well-known markers of M1 macrophages in mammals (Mantovani et al., 2002; Martinez et al., 2006). By contrast, $mCh^+$ macrophages expressed low levels of these pro-inflammatory cytokines at both 6 and 26 hpA (Figure 3F,G), but expressed high levels of $tgfb1$, $ccr2$, and $cxc4b$ (Figure 3H), that are specifically expressed in mammalian M2 macrophages (Mantovani et al., 2002; Martinez et al., 2006; Hao et al., 2012; Beider et al., 2014; Machado et al., 2014). Of note, neither Arginase 1 ($Arg1$), which is largely used as a M2 marker in mouse but not in human (Chinetti-Gbaguidi and Staels, 2011; Pourcet and Pineda-Torra, 2013), nor $il10$ (data not shown), a known M2 marker in mammals (Mantovani et al., 2002), was detected in zebrafish macrophages. Importantly, based on
the stability of the eGFP in Tg(tnfa:eGFP-F/mpeg1:mCherryF) larvae allowing us to specifically track the behaviour and fate of pro-inflammatory macrophages, we found that the dbl+ pro-inflammatory macrophages changed their phenotype at 26 hpA. Indeed, dbl+ macrophages negative for M2 markers at 6 hpA, displayed at 26 hpA, in parallel to a significant decrease of tnfα, il1b, and il6 expression level, a significant increased expression level of ccr2 and cxcr4b (Figure 3E,H). Of note, a tendency toward differential expression level was observed for tnfβ and tgfβ1 between 6 and 26 hpA. To go further and demonstrate that the same macrophages are present at the wound site during inflammation and its resolution, we generated the Tg(mpeg1:GAL4/UAS:Kaede) larvae to track macrophages exploiting the conversion of the native green fluorescence of Kaede into red fluorescence under UV light. Recruited macrophages were photoconverted 6 hpA and imaged at 26 hpA revealing that early recruited macrophages were still present at the wound area 20 hr later (Figure 4—figure supplement 1). Then, GFP+ macrophages were specifically tracked using time-lapse imaging of wounded Tg(mpeg1:mCherryF/TNFα:GFP-F) fins from 6 to 26 hpA. We show that initially recruited eGFP+ macrophages remain at the injury site and still express the GFP (Figure 4A–C and Video 5). The analysis of macrophage behaviour over time shows that among eGFP+ macrophages displaying an ameboid phenotype at the wound edge 6 hpA, 50% change toward a fibroblastic phenotype from 11 hpA when they moved distally (Video 5). All together these data show that pro-inflammatory macrophages underwent a phenotypic conversion toward an intermediate phenotype in which both M1 and M2 markers are expressed. In addition, this molecular characterization of macrophages in zebrafish reveals the conservation of macrophage subtypes between zebrafish and human.

In conclusion, we identified macrophage subsets in zebrafish and described their behaviour and fate during a process of inflammation (Figure 4D). Live imaging of transparent transgenic zebrafish larvae allowed the first real-time visualization of macrophage activation and polarization. In parallel, a molecular analysis of macrophage sub-populations highlights the evolutionary conservation of macrophages from fish to mammals. We propose that in response to wounding zebrafish, unpolarized macrophages are recruited to the inflammation site and adopt a M1-like phenotype. Subsequently, they progressively convert their functional phenotype from M1-like to M2-like in response to progressive inflammatory microenvironment changes within the tissue (Figure 4D). Live imaging of the new transgenic line we generated opens new avenues to study in real time in live vertebrates the full spectrum of macrophage activation, polarization, and functions.

Materials and methods

Ethics statement

All animal experiments described in the present study were conducted at the University of Montpellier according to European Union guidelines for handling of laboratory animals (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm) and were approved by the Direction Sanitaire et Vétérinaire de l’Hérault and Comité d’Éthique pour l’Expérimentation Animale under reference CEEA-LR-13007.

Nguyen Chi et al. eLife 2015;4:e07288. DOI: 10.7554/eLife.07288
Figure 3. Isolation and molecular characterization of macrophage phenotypes. (A) Graphed data of representative fluorescence-activated flow cytometry analysis of \textit{tnfa}^{+} \text{and} \textit{tnfa}^{-} macrophages upon inflammatory stimulations. \textit{Tg(mpeg1:mCherryF/tnfa:eGFP-F)} larvae were either kept intact (control), or amputated, or injected with PBS or with \textit{E. coli} at 3 dpf, and cells were collected at 6 hr post-treatment. Red, green, and yellow gates represent mCherry\textsuperscript{+}, eGFP\textsuperscript{+}, and mCherry\textsuperscript{+}eGFP\textsuperscript{+} populations, respectively. (B) Graph represents the kinetic of the frequency of \textit{mpeg1}^{+} \textit{tnfa}^{+} macrophages in macrophage population (\textit{mpeg1}^{+}) in three independent experiments following stimulation: amputation and \textit{E. coli} infection (\textit{E. coli}) at indicated time points. *p < 0.05.
Zebrafish line and maintenance

Fish and embryo maintenance, staging, and husbandry were as previously described (Nguyen-Chi et al., 2014). Experiments were performed using the AB zebrafish strain (ZIRC) and the transgenic line Tg(mpeg1:McHerryF) to visualize macrophages. For the photoconversion experiments, a cross of Tg(mpeg1:Gal4) produces Tg(UAS:kaede) lines was used, using breeders selected for progeny with negligible silencing of the UAS transgene.

Transgenic line construction

The TNFa promoter (Gene ID: 405785) was amplified from zebrafish genomic DNA using primers zTNFaP4 (CCCCCATGCTCAGCGTCCCTCC) and zTNFaE11N (TTATAGCGGCCGCCCTCAAGCTTCA). The resulting fragment was phosphorylated using T4PNK, digested by NotI and cloned in a farnesylated eGFP (eGFP-F) derivative of pBSKI2 (Thermes et al., 2002). The resulting plasmid (pZIPromTNFa-eGFP-F) harbours a 3.8-kb fragment of the zebrafish tnfa promoter, including part of the first coding exon. It uses the endogenous ATG codon of tnfa to drive the translation of eGFP-F. The expressed eGFP-F harbours the first 7 amino acids of zebrafish TNFa at its N-terminus (MKLESRA). The expression cassette is flanked by two I-SceI sites. pI2promTNFa-eGFP-F was co-injected in fertilized eggs with the enzyme I-SceI (New England Biolabs, France). Developing embryos were injected with non-pathogenic E. coli bacteria harboring either DsRed (van der Sar et al., 2012) or Crimson (Clontech, France) expression plasmid or no plasmid. Imaging was performed as previously described (Nguyen-Chi et al., 2014) using a confocal TCS SP5 inverted microscope with a HCXPL APO 40x/1.25–0.75 oil objective (Leica, France). Image stacks for time-lapse videos were acquired every 3–5 min, typically spanning 30–60 µm at 2-µm intervals, 1024 x 512 or 512 x 512 pixel resolution. The 4D files generated from time-lapse acquisitions were processed using Image J. They were compressed into maximum intensity projections and cropped. Brightness, contrast, and colour levels were adjusted for maximal visibility. Velocity of macrophages was measured using Manual Tracking Image J plugin. Frequency of macrophage–macrophage interaction and duration of interactions were measured manually on stack images. For tracking of macrophages, eGFP-F+ mCherryF+ cells from Tg(mpeg1:McHerryF/tnfa:eGFP-F) wounded fins were tracked using time-lapse imaging with FACS ARIA (BD Bioscience, France) and collected in 50% FCS/50% Leibovitz L-15 medium (21083-027, Gibco, France) on ice. To isolate total RNA, cells were...
Figure 4. M1-like macrophages convert their phenotype toward M2-like phenotype in the wounded fin. (A) Diagram showing the site where caudal fin was transected (dotted red line) in 3 dpf Tg(mpeg1:mCherryF/tnfa:eGFP-F) larvae. The black dotted box represents the region imaged by confocal microscopy. (B) Representative time-lapse maximum projections of 3 dpf Tg(mpeg1:mCherryF/tnfa:eGFP-F) amputated fins showing the fate of tnfa+ macrophages (magenta + green) at the indicated times pA (hours:minutes) from 6 hpA to 26 hpA. White lines delimit the caudal fin. Scale bar = 30 μm. (C) Tracking of tnfa+ macrophages from 6 to 26 hpA. The distinct colours of the lines correspond to the distinct macrophages that were indicated with an arrowhead in B. (D) Diagram representing macrophage activation and polarization in zebrafish. Unpolarized macrophages (mpeg1+) are mobilized and recruited to the wound following fin amputation. They are activated and polarized toward a M1-like phenotype (pro-inflammatory) few hours following fin amputation. After 24 hpA, in response to changes in environmental cues, the same macrophages progressively change their phenotype toward intermediate phenotypes and maybe fully polarized M2-like phenotype (non-inflammatory). Main markers of macrophage subtypes are indicated and resemble those found in human (tnfa/b indicates tumour necrosis factor alpha; il1b, interleukin 1-beta; il6, interleukin 6, tgfβ1, tumour growth factor beta 1; ccr2, c–c chemokine receptor type 2; cxcr4b, chemokine (C-X-C motif) receptor 4b).

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The following figure supplement is available for figure 4:

Figure supplement 1. Recruited macrophages remain in the region of tissue injury at 26 hpA.

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lysed in QIAzol Lysis Reagent (Qiagen, France) and RNA extracted using miRNeasy mini kit (Qiagen-21704, France). 20 ng of total RNA was reverse transcribed using High Capacity RNA Reverse Transcription kit (Applied Biosystems, France). qPCR were performed on a LightCycler 480 system (Roche, France), following manufacturer’s instructions (SYBR Green format, Roche Applied Science, Meylan, France) and using primers in Supplementary file 1: denaturation 15 s at 95°C, annealing 10 s at 64°C, and elongation 20 s at 72°C. Expression levels were determined with the LightCycler analysis software (version-3.5) from 5 independent experiments. The relative amount of a given mRNA was calculated by using the formulae \(2^{-\Delta Ct}\) with ef1a as reference.

**Statistical analysis**

Significance testing for Figures 1M, 3D–H was done using Mann–Whitney unpaired t-test, one-tail and Figure 2F–H using Mann–Whitney unpaired t-test, two-tails using GraphPad Prism 6 Software. *p < 0.05, **p < 0.01, ****p < 0.0001.

**In situ hybridization**

A tnfa probe was amplified from total cDNA by PCR using tnfa.55 and tnfa.58 primers (Supplementary file 1) and cloned in plasmid pCRII-TOPO. Digoxigenin (DIG)-labelled (Roche, France) sense and anti-sense RNA probes were in vitro transcribed (Biolabs, France). In situ hybridizations on whole-mount embryos were as previously described (Nguyen-Chi et al., 2012). For simultaneous detection of eGFP-F proteins and tnfa mRNA by immuno-detection and in situ hybridization, fixed and rehydrated Tg(tnfa:eGFP-F) larvae were permeabilised in ice in 100% ethanol for 5 min, then in a mixture of 50% Xylene-50% ethanol for 1 hr and in 80% acetone for 10 min at −20°C as described in Nagaso et al. (2001). After washes in PBS-0.1% Tween, larvae were post-fixed in 4% paraformaldehyde (PFA) for 20 min. Subsequent steps of hybridization, washes, and staining with NBT-BCIP (Roche, France) were as previously described in Nguyen-Chi et al. (2012). Next, unspecific-binding sites were saturated in PBS-1% bovin serum albumin (BSA)-1% lamb serum-10% Goat serum and larvae incubated 3 days with an anti-GFP antibody (MBL, 1/500). After extensive washes, larvae were incubated with a goat anti-rabbit antibody. Stained embryos were imaged using a MVX10 Olympus microscope with MVPLAPO 1× objective and XC50 camera and using a Zeiss Axioimager with a Zeiss 40× Plan-Apo 1.3 oil objective.

**Photoconversion of macrophage-specific Kaede protein**

Tg(mpeg1:GAL4/UAS:Kaede) embryos were raised to 3 dpf in the dark, and caudal fin was transected as described above. At 6 hpa, larvae were mounted in 1% low-melting point agarose. A 405-nm Laser Cube 405-50C on a confocal TCS SP5 inverted microscope with a HCXPL APO 40×/1.25–0.75 oil objective (Leica) was used to photoconvert the Kaede-labelled cells using 6% laser power scanning for 60 s (optimized before the experiments; data not shown). Fins were imaged before and after the photoconversion (at 6 and 26 hpa) in the green and red channels.

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Additional information

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Author contributions

MN-C, BL-B, FD, Conception and design, Acquisition of data, Analysis and interpretation of data, Drafting or revising the article; JT, PL-C, GT, QTP, ID-R, J-PL, GL, Acquisition of data, Analysis and interpretation of data; KK, CJ, Conception and design, Drafting or revising the article

Ethics

Animal experimentation: All animal experiments described in the present study were conducted at the University Montpellier 2 according to European Union guidelines for handling of laboratory animals (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm) and were approved by the Direction Sanitaire et Vétérinaire de l’Hérault and Comité d’Éthique pour l’Expérimentation Animale under reference CEEA-LR-13007.

Additional files

Supplementary file

- Supplementary file 1. Genes, accession numbers, and sequences of the primers.
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References


