Macrophages in Zebrafish Models of Liver Diseases

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Hepatic macrophages are key components of the liver immunity and consist of two main populations. Liver resident macrophages, known as Kupffer cells in mammals, are crucial for maintaining normal liver homeostasis. Upon injury, they become activated to release proinflammatory cytokines and chemokines and recruit a large population of inflammatory monocyte-derived macrophages to the liver. During the progression of liver diseases, macrophages are highly plastic and have opposing functions depending on the signaling cues that they receive from the microenvironment. A comprehensive understanding of liver macrophages is essential for developing therapeutic interventions that target these cells in acute and chronic liver diseases. Mouse studies have provided the bulk of our current knowledge of liver macrophages. The emergence of various liver disease models and availability of transgenic tools to visualize and manipulate macrophages have made the teleost zebrafish (Danio rerio) an attractive new vertebrate model to study liver macrophages. In this review, we summarize the origin and behaviors of macrophages in healthy and injured livers in zebrafish. We highlight the roles of macrophages in zebrafish models of alcoholic and non-alcoholic liver diseases, hepatocellular carcinoma, and liver regeneration, and how they compare with the roles that have been described in mammals. We also discuss the advantages and challenges of using zebrafish to study liver macrophages.

Keywords: Kupffer cells, monocytes, regeneration, NAFLD, ALD, hepatocellular carcinoma

INTRODUCTION

The liver is the largest internal organ in the body and exerts vital metabolic and immunological functions. Liver disease is a major health burden and accounts for ~2 million deaths per year worldwide (1). Liver transplantation is often the only curative option for patients with liver failure due to acute or chronic liver injury, and thus there is an urgent unmet need for alternative treatment.
The liver contains the largest number of tissue-resident macrophages that account for 80–90% of all macrophages in the body (2). These so-called Kupffer cells are considered to be self-renewing and non-migratory. During homeostasis, they exert phagocytic function to clear pathogens that reach the liver through the circulating blood. This macrophage population also maintains immunological tolerance in the liver to reduce accidental immune responses. During injury, Kupffer cells become activated and secrete pro-inflammatory cytokines and chemokines to recruit bone marrow-derived monocytes to the liver (3). Extensive research in samples from patients with liver diseases and rodent models of liver injury has revealed that both Kupffer cells and monocyte-derived liver macrophages play critical roles in hepatic steatosis, inflammation, fibrosis, and cancer, making them appealing therapeutic targets. Developing macrophage-based therapy, however, is challenging because it is a highly heterogeneous population. In fact, a recent study using single-cell RNAseq has identified 10 subpopulations of macrophages in human control and cirrhotic livers (4). Furthermore, macrophages are very plastic and often have multiple and sometime opposing functions in promoting liver disease progression vs. repairing injured liver (5).

The teleost zebrafish, an increasingly popular vertebrate model for studying development and genetics, has shown promise in bringing new insights into our understanding of the ontogeny of liver macrophages and their responses to injury. Zebrafish form a functional liver by just 4 days post fertilization (6). Despite some architectural differences, the zebrafish liver contains a highly similar parenchymal and non-parenchymal cell inventory as the mammalian liver. Taking advantages of the transparent larva and the accessibility to genetic manipulation, researchers have generated transgenic fluorescent reporter strains to mark individual liver cell types, enabling real-time tracking of their morphology and behaviors during development and injury (6). Zebrafish have been used in translational research modeling various liver diseases such as drug-induced acute liver failure, cholestasis, non-alcoholic liver disease, alcoholic liver disease, and cancer (6–8). These studies have demonstrated that the signaling pathways governing liver injury responses are highly conserved between zebrafish and mammals. Zebrafish are also an excellent in vivo model system for studying the innate immune system. The embryos have functional macrophages at 1 day post fertilization and neutrophils by 2 days (9). The zebrafish macrophages have conserved marker gene expression and functions as their mammalian counterparts. They can be easily visualized during homeostasis and inflammatory processes using the fluorescent reporter lines (9). Table 1 summarizes the tools for observing and manipulating macrophages in zebrafish.

Recent studies have confirmed the presence of macrophages in the livers of larval and adult zebrafish in physiological and pathological conditions. In this review, we provide an overview of the origin and development of hepatic macrophages in zebrafish. We highlight the recent advances where zebrafish transgenesis and imaging approaches reveal new aspects of macrophage functions in liver diseases. In particular, we focus on their roles in non-alcoholic and alcoholic liver disease, hepatocellular carcinoma, and liver regeneration. The capabilities and potential

| Table 1 | Tools to study macrophages in Zebrafish. |
|---|---|
| **Markers for macrophages** | |
| **Dye** | |
| Neutral Red | Marks live macrophages (10) |
| **Riboprobes for in situ hybridization** | |
| csf1ra | Also labels neural crests (11) |
| mpeg4 | (12) |
| cxcr3.2 | (12, 13) |
| mpeg1 | (12, 13) |
| mpeg1.2 | (14) |
| ptpn6 | (12) |
| **Antibody** | |
| L-plastin | Pan-leukocyte marker (15, 16) |
| Mpeg | (15) |
| WCL15 | Antigen unknown (11, 17, 18) |
| **Transgenic Reporter Line** | |
| Tg(mpeg1:GFP); Tg(mpeg1:mCherry); Tg(mpeg1:Gal4-VP16) | (13) |
| Tg(mpeg1:Dendra2) | Photoconvertible protein (19) |
| Tg(mpeg1:Kaede) | Photoconvertible protein (20) |
| Tg(mpeg1:Cre) | Applications include lineage tracing and tracking macrophage-dependent cytoplasmic transfer (21) |
| TgBAC(csfr1a:GFP) | Marks mononuclear phagocytes (22) |
| TgBAC(csfr1a:Gal4-VP16) | (23) |
| Tg(mpeg1:Cre); Tg(mpeg4:GFP) | (24, 25) |
| Tg(mpeg1:EGFP) | Marks activated macrophage (26) |
| Tg(mpeg4:EGFP) | Marks activated macrophage (27) |
| Tg(CORONIN1A:GFP) | Marks myeloid cells and lymphocytes (28) |
| **MACROPHAGE-SPECIFIC ABLATION MODELS** | |
| **Chemicals** | |
| Clodronate Lipoises | (29, 30) |
| Carrageenan | (31) |
| **Macrophage-deficient mutants and morphants (morpholino-injected animals)** | |
| Panther/csfr1a mutant | Reduced primitive macrophages (11, 20, 32) |
| irf8 mutant | Reduced macrophages and increased neutrophils (33, 34) |
| irf8 morphant | Reduced macrophages and increased neutrophils (33) |
| Pu.1 morphant | Lacks macrophage up to 3 days post fertilization; shows mortality after day 7. (35, 36) |
| **Nitroreductase-based macrophage ablation** | |
| Tg(mpeg1:NTR-eYFP) | (37) |
| Tg(mpeg1:Gal4-VP16; UAS:NTR-mCherry); | (38) |

(Continued)
of the zebrafish model in studying liver macrophages are also discussed (summarized in Figure 1).

THE ORIGIN OF HEPATIC MACROPHAGES IN ZEBRAFISH

Overview of Zebrafish Hematopoiesis

Similar to mammals, the development of the zebrafish hematopoietic system is characterized by several distinct waves (41, 42). The first wave, referred as primitive, occurs during early somitogenesis in the ventral lateral mesoderm and rostral blood island (RBI) at ~11 h post fertilization (hpf). The progenitors converge to the midline to form the intermediate cell mass, which is the primary site for primitive hematopoiesis and functionally equivalent to mammalian yolk sac blood islands. The process continues at ~24 hpf in the RBI during which the transient erythro-myeloid precursors (EMPs) are formed. The EMPs have limited lineage differentiation potential and lack the self-renewal capacity (43). The second or definitive wave of hematopoiesis starts at ~36 hpf when the first hematopoietic stem cells (HSCs) emerge from the ventral wall of the dorsal aorta (VDA) in the aorta-gonad mesonephros (AGM) region. This process is conserved among vertebrate species and gives rise to a multipotent cell type that can contribute to the entire hematopoietic lineage (44, 45). Another conserved feature between mammals and zebrafish is the migratory ability of the HSCs as they seed in different anatomical niches in order to differentiate and proliferate. Subsequently, hematopoiesis proceeds in the distal region of the tail, which is known as the caudal hematopoietic tissue (CHT) and represents the equivalent of the mammalian fetal liver (46). At about 96 hpf, the HSCs migrate either from the CHT or directly from the AGM to colonize the pronephros (47). There they will constitute the kidney, which corresponds to the mammalian bone marrow, to provide the adult zebrafish with hematopoiesis throughout their lifespan.

Tissue Resident Macrophages Arise From the HSC Origin, a Lesson From Fish

The zebrafish innate immune system is mainly composed of macrophages and neutrophils. Both are derived from the myeloid lineage that emerges during the primitive hematopoietic wave from the cells in the lateral plate mesoderm expressing Spi-1 proto-oncogene (spil) and lymphocyte cytosolic protein 1 (lcp1) also known as L-plastin (48, 49). Definitive hematopoiesis continues to contribute to the myeloid lineage and sustains its functionality throughout the lifespan. The innate immune system solely provides zebrafish with immune defense during the first month of life until the adaptive immune system fully develops (50).

The macrophage population consists of tissue-resident macrophages, bone marrow-derived recruited macrophages, and peritoneal macrophages. Resident macrophages are present in most tissues across the body and fulfill vital functions in homeostasis (51). It has been shown that the early EMPs populate different organs during development to form most of the resident macrophages in mice. This macrophage population acquires specialized, tissue-resident properties, and harbors self-renewing potential to maintain the adult population (52–55). One exception is the gut, where the macrophage population is continually replenished by circulating monocytes that differentiate into the mature resident macrophages (56).

Studies of resident macrophages in zebrafish have provided novel assessments of their origin. Recent work identified the age-dependent origin of microglia (57). While the primitive macrophages give rise to a transient population of microglia during the early larval stage, the adult microglia originate from the cmyb-dependent HSCs. Similar observations were made in adult zebrafish Langerhans cells and several other resident-macrophage populations (58), challenging the current model of the erythroid-myeloid origin of tissue macrophages. The zebrafish results are supported by a recent mammalian study (59), although there is still much controversy in the field (54).

Origin of Liver Resident Macrophages in Zebrafish

The liver is continuously exposed to antigens, microbial products, and xenobiotics. To adapt to such an environment, the liverharbors the largest population of macrophages among the solid organs and is constantly patrolled by circulating monocytes. Based on the ontogeny studies conducted mainly in mice, Kupffer cells originate from the yolk sac-derived erythro-myeloid progenitors that express macrophage colony stimulating factor 1 receptor (CSF1R) and are self-renewing (54). Recently, HSCs and some common circulating precursors have also been implicated in the development of Kupffer cells (53, 59, 60).

Resident macrophages have been observed in the adult zebrafish liver (58, 61–63). In elegant work, He and colleague utilized a laser-mediated temporal-spatially resolved cell labeling IR-LEGO-CreER-loxP system to mark cells within different hematopoietic compartments during distinctive waves of hematopoiesis and trace the destination of the labeled cells in adults (58). Followed by fine fate-mapping analysis, they showed that most of the primary tissue-resident macrophages in adult zebrafish, including those in the liver, are derived from the VDA, suggesting their HSC origin. This work illustrates how zebrafish can offer unique tools to elucidate the ontogeny of hepatic macrophages, which is a challenging topic in hepatology.
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Zebrafish Liver Macrophages

FIGURE 1 | Zebrafish, an emerging model for study hepatic macrophages. (A) Hepatic macrophages are present in the zebrafish liver at both larval and adult stages.  
(B) Increases in macrophage numbers have been observed in zebrafish models of liver pathology include non-alcoholic liver disease (NAFLD), alcoholic liver disease (ALD), and hepatocellular carcinoma (HCC), as well as in liver regeneration after partial hepatectomy and hepatocyte-specific ablation (left). Involvement of heterogeneous macrophage populations has been implicated in these models (right). (C) Current and potential applications available in zebrafish to study hepatic macrophages. Zebrafish larva is accessible for live imaging, allowing characterization of macrophage behaviors during early stages of immune responses. The live imaging platform in larva can also be utilized for laser-mediated localized manipulations of gene expression and cell ablation. Technologies such as GESTALT (genome editing of synthetic target arrays for lineage tracing) and single cell RNA-sequencing can be utilized to study the ontology and plasticity of macrophages in healthy and injured livers at a population level.

MACROPHAGES IN ZEBRAFISH MODELS OF LIVER DISEASES

Non-alcoholic and Alcoholic Liver Disease

Non-alcoholic fatty liver disease (NAFLD) and alcoholic liver disease (ALD) are among the leading causes of liver-related morbidity and mortality and primary indications for liver transplantation. In both diseases, extensive hepatic lipid accumulation caused by metabolic stress or alcohol consumption induces hepatocyte cell death (64). Damaged hepatocytes release danger-associated molecular patterns (DAMPS) to trigger activation of Kupffer cells and infiltration of circulating monocytes (3). Macrophages play divergent roles in NAFLD and ALD: they exhibit a pro-inflammatory phenotype during disease progression (65, 66) and become anti-inflammatory and tissue-protective during disease regression (66, 67). Feeding zebrafish
larvae with a high cholesterol diet (5% cholesterol w/w) for a week can cause elevated triglyceride and total cholesterol levels and lipid accumulation in the body. The animals develop macrovesicular steatosis in the liver by 1-week of feeding and display ballooning degeneration by 3 weeks (68). De Oliveira et al. showed that short-term feeding with a high fat diet (HFD) results in clustering of macrophages in the zebrafish larval liver (61). Whereas, the macrophages in the control livers constantly patrol the environment, the macrophages in the HFD-fed liver are more stationary and adopt a rounder morphology. They start to express TNFα, a consensus marker of M1 macrophages, consistent with activation and polarization of these cells (26).

It has been reported by multiple groups that acute and chronic ethanol treatment can induce hepatic steatosis in larval and adult zebrafish, respectively (69–73). In human and mouse, after alcohol consumption, ethanol enters the blood circulation through the gastrointestinal tract and reaches the liver via the portal vein (74). Ethanol is metabolized in the liver mainly by alcohol dehydrogenase ADH1 and cytochrome P450 2E1/CYP2E1 enzymes. Zebrafish have analogs of ADH1 and CYP2E1 that are capable of metabolizing ethanol (75). Treatment with pharmacological inhibitors of ADH1 and CYP2E1 blocks ethanol-induced hepatic steatosis in zebrafish larvae, indicating that steatosis is caused by ethanol metabolism (76). Zebrafish alcoholic injury models are achieved by aqueous exposure of the animals to ethanol. Thus, ethanol exposure can go through multiple routes, including the gastrointestinal tract, gill, and skin. Since the expression of ethanol-metabolizing enzymes has not been characterized at the tissue level in zebrafish, it is not clear whether tissues other than the liver participate in ethanol metabolism.

In the acute alcoholic liver injury model, exposing 4-day-old zebrafish larvae to 2% ethanol for 24 h causes hepatic steatosis (Figure 2) (71, 76). At this stage, the yolk provides the animal all the nutrients and is likely the source of fat in steatosis (76). Mammalian studies indicate that alcohol exposure increases the ratio of reduced nicotinamide adenine dinucleotide/oxidized nicotinamide adenine dinucleotide and subsequently impairs mitochondrial β-oxidation of fatty acids (77). Alcohol exposure also promotes lipogenesis and inhibits fatty acid oxidation by regulating the transcription factors of lipid metabolism. In the zebrafish acute alcoholic liver injury model, alcohol-induced lipogenesis requires activation of the sterol regulatory element binding protein (SREBP) transcription factors and involves the unfolded protein response pathway (71, 76, 78). In zebrafish larvae, acute ethanol exposure also prompts hepatic stellate cells to express extracellular matrix proteins and causes dilatation of the hepatic blood vessels (73). One day after ethanol is removed, there is an increase in the number of macrophages in the treated liver (Figure 2), accompanied with increased hepatic angiogenesis and hepatic stellate cell proliferation (73). In mammalian models of chronic liver injury, macrophages are the source of vascular endothelial growth factor that promotes angiogenesis (79). They also have dual function in fibrosis: both Kupffer cells and monocyte-derived macrophages are profibrogenic during fibrosis progression as they secrete TGFβ1 and PDGF to activate hepatic stellate cells and mediate the survival of myofibroblasts (80). When the insults are removed, monocyte-derived macrophages become antifibrotic to aid in the resolution of fibrosis. In our opinion, the zebrafish acute alcoholic liver injury model is useful for studying the initial responses of macrophages, endothelial cells, and hepatic stellate cells upon the addition and removal of ethanol. Such responses trigger the subsequent cascades of events underlying disease progression and regression.

It is important to note that NAFLD and ALD are chronic diseases progressing from hepatic steatosis to steatohepatitis, and further to fibrosis and cirrhosis, increasing the risk for hepatocellular carcinoma. The zebrafish NAFLD and ALD models described above are mainly based on short-term treatment at larval stages and do not recapitulate the full spectrum of the disorders in human. Fibrosis has not been observed in the larval NAFLD and ALD models, which could be due to the fact that the zebrafish liver does not have the portal-central arrangement as the mammalian liver. The duration of the experiments may not be long enough for fibrosis to develop. Therefore, it is important to validate the findings in adult chronic injury models and mammalian systems.

### Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is the most prevalent primary malignancy of the liver and results in ~800,000 deaths globally per year (81). It is the fastest growing cancer in the US. Accumulation of tumor-associated macrophages is commonly seen in the livers of patients with HCC and the number of macrophages correlates with HCC progression and poor prognosis (82, 83). As key components of the tumor microenvironment, macrophages are thought to be pro-inflammatory and pro-tumorigenic during HCC progression, but may switch to become anti-tumorigenic during HCC regression (79). In zebrafish, HCC can be induced by carcinogen and mutagen treatment, genetic mutations of tumor suppressor genes and oncogenes, and transgenic overexpression of oncogenes (7, 84). Zebrafish and human HCCs share similar histological features and gene signatures (85, 86). Increases in macrophage numbers have been observed in zebrafish HCC models with different tumorigenic triggers (35, 61, 87, 88).

By live imaging, De Oliverira et al. showed that HFD feeding induces changes in macrophage morphology and polarization in a transgenic zebrafish HCC model expressing activated β-catenin in the hepatocytes (61). Ablating macrophages prior to HFD feeding suppresses the exacerbated liver enlargement in HCC fish that is caused by HFD. Treatment with anti-diabetic agent metformin has similar inhibitory effects on HCC progression associated with HFD. Whereas, metformin has previously been proposed as a promising treatment for HCC, the zebrafish study provides direct in vivo evidence to show that it suppresses NAFLD-associated HCC progression by decreasing the number of pro-inflammatory macrophages and increasing T cell infiltration.

One challenge for investigating the roles of liver macrophages in HCC is that tumor formation often occurs in parallel with the progression of chronic liver disease. It is difficult to
Acute ethanol treatment causes hepatic steatosis and increases macrophage numbers in larval zebrafish. (A,B) Hematoxylin and eosin (H&E) staining of the paraffin sections showing the livers in a control larva (A) and a larva treated with 2% ethanol from 96 to 120 h post fertilization (B). The livers were harvested at 27 h post treatment (hpt). Scale bar, 20 \( \mu \)m. (C,D) Representative images of the whole-mount Oil Red O staining in the control (C) and ethanol-treated larvae (D). Dashed line outlines the liver. Lateral view, anterior is to the top. Oil Red O also stains the swim bladder (asterisk in C) and the residual yolk tissue (asterisk in D). Scale bar, 250 \( \mu \)m. (E,F) Confocal three-dimensional projections showing Tg(mpeg1:YFP)-expressing macrophages (green) in the whole liver at 27 hpt. Phalloidin staining (red) that labels cell cortex is used for recognizing various organs. Ventral views, anterior is to the top. Dashed line outlines the liver. Scale bar, 30 \( \mu \)m. (G) Numbers (mean±s.e.m.) of macrophages per liver at 0 hpt (left) and 27 hpt (right). Statistical significance was calculated by one-way ANOVA and Tukey’s post-hoc test. **p < 0.01, ns, not significant. This figure is reproduced with permission from Zhang et al. (73) and Disease Models & Mechanisms.
segregate the roles of macrophages in maintaining a prone-tumor inflammatory microenvironment vs. promoting HCC in response to tumor-derived signals (3). Multiple transgenic zebrafish lines utilizing chemically inducible expression systems (Tet-on, Tet-Off, and Mifepristone) have been generated to overexpress different oncogenes specifically in the hepatocytes (89). These models exclude the impact of chronic liver disease on HCC formation. Moreover, HCC can be induced in a temporally controlled manner and is regressed after removal of the chemicals, allowing investigation of macrophages at different stages of HCC progression and regression. In a transgenic zebrafish HCC model with inducible expression of oncogene Xmrk that encodes a hyperactive epidermal growth factor receptor (EGFR) homolog, the number of macrophages is increased during both HCC formation and regression (87). Interestingly, the macrophages are randomly distributed during HCC formation, and gradually show prominent blood vessel association as HCC regresses, implying that they have different functions at these two stages.

In vivo live imaging of the interactions between oncogenic hepatocytes and their microenvironment can be technically difficult in rodent models. Such analyses are readily feasible in zebrafish larvae due to their transparent body and availability of cell type-specific transgenic fluorescent reporter lines. Yang et al. investigated the responses of hepatocytes, innate immune cells and hepatic stellate cells during early stage of liver tumorigenesis in a kras12-induced HCC model (63). Upon hepatocyte-specific kras12 overexpression, there is sequential infiltration of neutrophils and macrophages, followed by proliferation and activation of hepatic stellate cells. Whereas, decreasing macrophage numbers by knocking down irf8 or pu.1 impairs both survival and activation of hepatic stellate cells, reducing neutrophils only affects their activation. The study further revealed reciprocal interactions between hepatic stellate cells and immune cells in HCC. Upon HCC induction, hepatocytes and macrophages increase expression levels of serotonin to regulate hepatic stellate cell survival and activation. In return, activated hepatic stellate cells secrete TGFβ1 to promote the pro-tumorigenesis function of neutrophils and macrophages. This work demonstrates the dynamic intercellular crosstalks within the tumor microenvironment that are crucial for liver tumorigenesis.

HCC is a male-biased disease with a male-to-female ratio of 2.4 worldwide (90). It is more aggressive and has worse prognosis in men than in women. The gender disparity also exists in rodent and zebrafish HCC models (91–93). In a series of reports from the Gong laboratory, the mechanisms of male-biased HCC carcinogenesis were explored in the transgenic zebrafish with inducible expression of oncogenes. In HCC models induced by kras12 and xmrk expression, there is an enhancement of hepatocarcinogenesis in male zebrafish compared to females (35, 94). Male HCC livers express higher levels of serotonin. It is accompanied with higher numbers of total hepatic stellate cells and activated hepatic stellate cells, as well as more severe infiltration of macrophages and neutrophils. The sex disproportion of HCC is thought to be not only due to varying risk factors in men and women, but also associated with the regulation of inflammatory responses in the tumor microenvironment by sex hormones (95, 96). Yet, the results of estrogen- and androgen-related clinical trials are inconclusive (97–99), suggesting the possible involvement of other hormones. One candidate is cortisol that is predominantly expressed in the male livers (35, 100). In the zebrafish kras12 and xmrk HCC models, cortisol induces expression of TGFβ1, which subsequently promotes infiltration of macrophages and neutrophils to accelerate hepatocarcinogenesis. The positive correlation between cortisol, TGFβ1, and macrophage/neutrophil infiltration has also been observed in patients with HCC (35).

MACROPHAGES IN ZEBRAFISH MODELS OF LIVER REGENERATION

Aligned with their involvement in liver diseases, macrophages are key participants in liver regeneration (101–103). Upon injury, liver macrophages infiltrate to the wound site to remove the dead hepatocytes. They also produce cytokines IL6 and TNFα that prompt hepatocytes to enter the mitotic cycle. Depletion of Kupffer cells in rodents by clodronate liposomes delays liver regeneration and exaggerates liver damage after partial hepatectomy (104, 105). Three liver regeneration models have been characterized in depth in zebrafish and the contribution of macrophages has been investigated. Following one-third partial hepatectomy, the adult zebrafish liver regains its original volume within 14 days via compensatory growth of the remnant hepatocytes (106–108). Macrophages accumulate at the amputation site within 48 h after the surgery to clear up neutrophils and resolve local inflammation (109). Digestive-organ-expansion-factor (Def) is a nuclear protein that mediates p53 degradation in the nucleus. In zebrafish with haploinsufficiency of Def, aberrant expression of cytokines halts the timely migration of macrophages to the amputation site. The resulting delay in neutrophil clearance and prolonged inflammation cause fibrotic scar formation.

Two hepatocyte-specific ablation models have been established in zebrafish. In one model, the transgenic zebrafish expressing the oxygen-insensitive NAD(P)H nitroreductase (NTR) in hepatocytes are treated with the antiprotozoal metronidazole. This prodrug is metabolized into a cytotoxin by NTR in hepatocytes (NTR) in hepatocytes are treated with the antiprotozoal metronidazole. This prodrug is metabolized into a cytotoxin by NTR in hepatocytes (110, 111). Treatment with metronidazole from 3.5 to 5 days post fertilization results in nearly complete hepatocyte ablation. The liver size fully recovers just 5 days after removal of the drug (112). In a second model, temporary knockdown of mitochondrial import gene tomm22 by morpholino oligonucleotide leads to hepatocyte degeneration. The liver in the morpholino-injected animal is smaller at 4 days post fertilization, but starts to regenerate as the morpholino effect expires and tomm22 expression is restored to the wild-type level (17). By 8 days post fertilization, the liver displays the size and structure that resemble the uninjected control. Unlike partial hepatectomy in which liver regeneration is driven by proliferation of existing hepatocytes, in both hepatocyte-NTR and tomm22-knockdown
models, extensive hepatocyte loss triggers dedifferentiation of biliary epithelial cells into liver progenitor cells to form new hepatocytes (112, 113). Robust recruitment of macrophages and engulfment of hepatocyte debris by macrophages are seen in both models (17, 114). In tomm22-knockdown model, the surviving hepatocytes turn on biliary markers to become hybrid cells that express both hepatocyte and biliary markers (38). Ablation of macrophages suppresses the formation of hydrid cells, which coincides with the reduction of Wnt/β-catenin signaling activity. This is consistent with the mammalian findings that macrophages produce Wnt3a to promote liver regeneration. This is consistent with the mammalian findings that macrophages produce Wnt3a to promote liver regeneration. It is possible to partially ablate the labeled macrophages by clodronate liposomes (120). Subsequently, the clonal composition can be assessed to identify the source of the recovering cells. Moreover, the labeled clones can be analyzed to determine how different subpopulations of macrophages react to various insults. The GESTALT system, which stands for genome editing of synthetic target arrays for lineage tracing, is another tool to add more depth to the understanding of liver macrophage ontogeny (121). It utilizes CRISPR genome editing to progressively introduce and accumulate distinct mutations in a DNA barcode over multiple rounds of cell division. The barcode can be used to dissect lineage relationship among liver macrophages via the mutation patterns shared between them. With the use of a heat shock inducible Cas9, it is also possible to laser-activate the GESTALT system in a spatio-temporally restricted manner to restrict the labeling to a specific site of interest 

By combining the liver regeneration models with the transgenic macrophage reporter lines, it is feasible to monitor macrophage recruitment, efferocytosis, and their interactions with other hepatic cells in vivo throughout the course of liver regeneration. The liver macrophages in tomm22-knockdown model exhibit a shift in morphology during the regeneration phase (38), suggesting that they undergo activation and polarization similar to their mammalian counterparts. In rodents, Kupffer cells and blood monocyte-derived macrophages play different roles in liver regeneration depending on the type of the original injury (2, 5). It will be interesting to utilize the zebrafish partial hepatocectomy and hepatocyte depletion models to compare the source of macrophages and their functions in hepatocyte- and biliary-driven liver regeneration, respectively.

### CONCLUSION AND FUTURE PERSPECTIVES

Several possible strategies can be used to design macrophage-based treatment for acute and chronic liver diseases: (1) Suppressing Kupffer cell activation; (2) blocking monocyte recruitment; (3) rendering macrophages toward a more restorative phenotype; and (4) macrophage cell therapy (3, 116). Not every aspect of liver macrophage biology can be easily investigated using in vitro systems and rodent models and including complementary animal models will be beneficial. Zebrafish has the complexity of a vertebrate system, established models of acute and chronic liver injury, conserved innate immune cells, and superior genetic and live-imaging capabilities, making it an attractive alternative animal model for studying macrophages in liver homeostasis and diseases. In this review, we have discussed the strengths of using zebrafish to visualize macrophages and monitor their interactions with other hepatic cells, and to manipulate these cells using genetic approaches.

The characterization of zebrafish liver macrophages is only at the beginning stage and much remains to be learned. Transcriptomic analysis of zebrafish macrophages has been performed in the context of Mycobacterial infection (117). However, the macrophage transcriptome has not been investigated in healthy and injured liver in zebrafish and to what degree this is comparable to humans is not clear. Few macrophage-specific antibodies are available in zebrafish. In particular, cell surface markers labeling macrophages at different polarization states have yet to be identified. Most of the zebrafish liver studies are conducted on larvae, as live imaging becomes less feasible beyond the larval stage when they are no longer transparent. Fibrosis and cirrhosis, however, are chronic processes and the duration of the larvae studies may not be long enough for fibrosis to develop. Another caveat of studying larvae is that the zebrafish immune system is primarily innate during the first month of life and the adaptive system only becomes fully functional afterwards (50). On one hand, the temporal separation of innate and adaptive immune systems permits exclusive interrogation of innate immune cell function without having significant influence from adaptive immunity. On the other hand, pathogenesis of human liver diseases does involve both innate and adaptive immunity and it is necessary to validate the larval findings in adult liver disease models. Comparative studies on human vs. zebrafish liver macrophages in physiological and disease conditions are very limited, and thus the human relevance of zebrafish findings should be evaluated.

Liver macrophages are highly polymorphic. The lack of tools to distinguish macrophages from different origins and at different activation states has prevented the assignment of specific functionalities to each subgroup, making it difficult to develop treatment that only targets the macrophage subgroups with detrimental effect. Some emerging technologies in zebrafish may open exciting revenues for interrogating the ontology, activation, heterogeneity, and plasticity of liver macrophages. The zebrafish model possesses an excellent toolbox for lineage-tracing and fate-mapping analyses to understand the ontology of different liver macrophage subgroups in normal and diseased livers. For instance, the Zebraway system allows tracing of the clonal origin of different liver macrophage subtypes (118, 119). Distinct clones can be sorted and sequenced separately to uncover the transcriptional states of different subpopulations. The multicolor labeling can also be utilized in adult zebrafish to assess the maintenance of liver macrophages population and distinguish between self-renewal and monocyte-based replenishment. It is possible to partially ablate the labeled macrophages by using clodronate liposomes (120). Subsequently, the clonal composition can be assessed to identify the source of the recovering cells. Moreover, the labeled clones can be analyzed to determine how different subpopulations of macrophages react to various insults. The GESTALT system, which stands for genome editing of synthetic target arrays for lineage tracing, is another tool to add more depth to the understanding of liver macrophage ontogeny (121). It utilizes CRISPR genome editing to progressively introduce and accumulate distinct mutations in a DNA barcode over multiple rounds of cell division. The barcode can be used to dissect lineage relationship among liver macrophages via the mutation patterns shared between them. With the use of a heat shock inducible Cas9, it is also possible to laser-activate the GESTALT system in a spatio-temporally restricted manner to restrict the labeling to a specific site of hematopoiesis and study the lineage relationship within this particular group. The GESTALT method can be combined with single-cell RNA sequencing to not only provide the identity of the subpopulations but also link each of them to a specific...
hematopoietic lineage and site (122). One may apply the GESTALT method in different liver pathologies to evaluate the liver macrophages plasticity at a population level. Lastly, a recent study from Paul and colleagues describes successful transplantation of primary human monocytes/macrophages into larval zebrafish, both directly into circulation and in an organ-specific manner (123). The human monocytes differentiate into functional macrophages at the physiological temperature of zebrafish, and survive for at least 2 weeks in the presence of zebrafish immunity. This methodology may permit in vivo characterization of human macrophages in zebrafish models of liver pathology at a cellular level. The new lines of experiments described above have the potential to advance our understanding of liver macrophage biology and contribute to the design of novel macrophage-targeted therapeutic strategies to treat liver diseases.

**AUTHOR CONTRIBUTIONS**

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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

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