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A fresh look at mycobacterial pathogenicity with the zebrafish host model

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Abstract

The zebrafish has earned its place among animal models to study tuberculosis and other infections caused by pathogenic mycobacteria. This model host is especially useful to study the role of granulomas, the inflammatory lesions characteristic of mycobacterial disease. The optically transparent zebrafish larvae provide a window on the initial stages of granuloma development in the context of innate immunity. Application of fluorescent dyes and transgenic markers enabled real-time visualization of how innate immune mechanisms, such as autophagy and inflammasomes, are activated in infected macrophages and how propagating calcium signals drive communication between macrophages during granuloma formation. A combination of imaging, genetic, and chemical approaches has revealed that the interplay between macrophages and mycobacteria is the main driver of tissue dissemination and granuloma development, while neutrophils have a protective function in early granulomas. Different chemokine signaling axes, conserved between humans and zebrafish, have been shown to recruit macrophages permissive to mycobacterial growth, control their microbicidal capacity, drive their spreading and aggregation, and mediate granuloma vascularization. Finally, zebrafish larvae are now exploited to explore cell death processes, emerging as crucial factors in granuloma expansion. In this review, we discuss recent advances in the understanding of mycobacterial pathogenesis contributed by zebrafish models.

KEYWORDS

autophagy, cell death, chemokine signalling, granulomas, inflammasomes, macrophages, Mycobacterium, tuberculosis, zebrafish model

1 | INTRODUCTION

Mycobacteria are the causative agents of some of the most serious human infectious diseases, including tuberculosis (TB), leprosy, and various other lung, skin, and disseminated infections (Kilinc et al., 2021). Pathogenic mycobacteria have developed highly effective virulence mechanisms to subvert the immune defenses of their host. In particular, mycobacterial pathogens are able to establish a replication niche inside macrophages and subsequently manipulate these innate immune cells to act as transport vehicles to invade host tissues (Houben et al., 2006). Following tissue invasion, inflammatory lesions, known as tuberculous granulomas, are

formed. These granulomas serve as a reservoir for the persistence of mycobacteria and are regarded as a defining feature of TB and related infectious pathologies (Pagan & Ramakrishnan, 2018). Studies in mammalian models, particularly mouse, guinea pig, rabbit, and non-human primates, contributed crucial insights into mycobacterial pathogenesis and are indispensable to advance TB research (Yang et al., 2021). However, no animal model system recapitulates all aspects of human pathogenesis faithfully, nor is equally suitable to different research questions. A notable example is the question of how granulomas are initiated, which requires access to early stages of infection that are difficult to access in mammalian models (Ramakrishnan, 2013). These considerations, together with an increasing desire for reducing the use of mammalian models in research, have created a favorable situation in which the zebrafish has been able to stand out. In this regard, the zebrafish model, especially at the early life stages, greatly complements the other existing models as it requires low maintenance, is easily genetically manipulable, ideally suited for in vivo imaging, and amenable to drug screening. For these and other reasons, zebrafish is widely appreciated for disease modeling, especially for the study of host-pathogen interaction (Meijer & Spaink, 2011; Varela et al., 2017; Yoshida et al., 2017).

TB is caused by the bacterium *Mycobacterium tuberculosis* (*Mtb*) but its close genetic relative *Mycobacterium marinum* (*Mmar*) in combination with its natural host, the zebrafish, have become an attractive tandem for deciphering host-pathogen interactions and advancing TB research (Cronan & Tobin, 2014; Meijer, 2016; Ramakrishnan, 2013). Both larvae and adult zebrafish can be infected with *Mmar* using different (micro) injection techniques (Benard et al., 2012; Saralahti et al., 2020). Adult zebrafish mount a full adaptive immune response and infection with *M. marinum* leads to the formation of tuberculous granulomas of similar structure as in human TB. Therefore, the adult zebrafish-*Mmar* model is well-appreciated for the study of latency and reactivation of the disease, a process leading to the active spreading of the bacteria and the appearance of symptoms of active TB in humans (Parikka et al., 2012). On the contrary, zebrafish larvae are suitable for the study of the early infection stages in the context of innate immunity, as the adaptive immune system only becomes fully functional 4 weeks post-fertilization (Masud et al., 2017). Moreover, the infection is tractable on a single cell level in the optically transparent larvae, allowing the in vivo application of advanced imaging techniques (Munoz-Sanchez et al., 2020; Rosowski, 2020b). Together, larval and adult zebrafish TB models have proven to be very useful for mechanistic molecular studies but also for drug research and vaccine development (Dalton et al., 2017; Saralahti et al., 2020).

The use of zebrafish has contributed not only to advance TB pathogenesis research but also to change long-standing dogmas within the TB field (Ramakrishnan, 2020). Recapitulating *Mtb* infection in humans, during the early phase of infection *Mmar* interacts with zebrafish innate immune cells (Davis et al., 2002). The role of phagocytes and their response to the invading pathogen is known to determine the fate of the infection. Zebrafish macrophages efficiently engulf the invading bacteria as do their human counterparts. Starting from a single infected cell, bacteria can be eliminated by host defenses or disseminated inside migrating phagocytes (Clay et al., 2007). The dissemination of the pathogen allows the formation of new infection foci that can lead to the persistence of the infection and the development of the typical granulomatous structures (Davis & Ramakrishnan, 2009). In the following sections, we will discuss how the zebrafish and its toolbox have contributed to the understanding of mycobacterial pathogenicity, and how there is still potential for the development of new tools that will expand the understanding of host-pathogen interactions.

2 | UNDERSTANDING THE INTRACELLULAR STAGES OF MYCOBACTERIAL INFECTION

A major advantage of using zebrafish larvae as a model system is that it enables subcellular imaging of infected phagocytes in a living host. In vivo imaging of how phagocytes respond to mycobacterial parasitism has been achieved label-free using differential interference contrast microscopy (e.g., Davis et al., 2002) and by fluorescent labeling using various dyes (as also commonly used in cell culture studies), and a growing collection of transgenic zebrafish lines that fluorescently label subcellular compartments or signaling proteins (e.g., Rosowski, 2020a, 2020b). Here, we illustrate these approaches with recent examples of *Mmar* infection studies.

Mycobacteria employ a mixture of two main virulence strategies to replicate inside their host cells. The first is that they are able to reside inside the phagosome by inhibiting its maturation and fusion with lysosomes, and can resist acidic pH even if the phagolysosomal pathway does progress (Gouzy et al., 2021; Levitte et al., 2016). The second is that they are able to gain access to the cytosol by permeabilizing the phagosomal membrane, which requires the mycobacterial type VII secretion system, ESX-1, encoded in the conserved RD1 virulence locus (Houben et al., 2012). The latter virulence strategy implies that mycobacteria have to be able to defend themselves at least to some extent against the primary defense mechanism in the cytosol, which is autophagy (Deretic et al., 2013). The capture of cytosolic *Mmar* by autophagic vesicles has been visualized by imaging of infection in GFP-Lc3 transgenic zebrafish (Hosseini et al., 2014). Like endogenous Lc3, the GFP-tagged protein is conjugated to the inner and outer membrane of nascent autophagosomes and this fluorescent signal remains detectable until the autophagosomal cargo is delivered to lysosomes (He et al., 2009). The analysis of GFP-Lc3 dynamics can be combined with LysoTracker dye to detect lysosomal acidification. This approach was used to study loss-of-function mutants of several autophagy-related genes, *sqstm1* (*p62*), *optn*, and *dram1*, demonstrating their requirement for the autophagic defense response (van der Vaart et al., 2014; Zhang et al., 2019, 2020). Notably, overexpressing these genes by mRNA injection enhanced the GFP-Lc3 response and the lysosomal delivery of *Mmar*, as well as the overall resistance of zebrafish larvae to the infection (van

der Vaart et al., 2014; Zhang et al., 2019). These studies suggest autophagy activation as a possible therapeutic strategy against mycobacterial infections (Munoz-Sanchez et al., 2020).

Breaching the boundary between the phagosome and cytosol not only triggers autophagy but also activates inflammasome signaling pathways that mediate interleukin 1 secretion and may culminate in pyroptotic cell death (Varela et al., 2019). Inflammasome pathway components are highly conserved in vertebrates, including zebrafish (reviewed in Forn-Cuni et al., 2019). The most extensively studied inflammasome in mammals, the NLR family pyrin domain containing 3 (NLRP3) inflammasome, has recently been found to be fully functional in zebrafish (Li et al., 2020). Interestingly, *Mtb* has evolved mechanisms to manipulate the activation of the NLRP3 inflammasome (Rastogi et al., 2021) but the conservation of this mechanism has yet to be studied in the *Mmar*-zebrafish infection model. Another constituent of a large subset of inflammasomes is an apoptosis-associated speck-like protein containing a CARD (Asc), which facilitates the formation of macromolecular complexes with pattern recognition receptors and inflammatory caspases (Forn-Cuni et al., 2019). Such macromolecular structures have been visualized in granulomas of *Mmar*-infected zebrafish larvae using an *asc*:*asc-gfp* transgenic zebrafish line (Forn-Cuni et al., 2019; Kuri et al., 2017). The Asc-dependent inflammasome pathway restricts intracellular mycobacterial growth and limits granuloma expansion in this model, while Asc-independent inflammasome signaling exacerbates inflammation and mycobacterial growth (Varela et al., 2019). Therefore, *Mmar* infection in zebrafish larvae provides a useful model system to unravel the triggers of different inflammasome pathways and to dissect their host-protective versus host-detrimental effects.

Proinflammatory macrophages release ATP into the extracellular space as a cell-to-cell communication mechanism, which is mediated by purinergic receptors like P2X7 and triggers transient intracellular calcium signals (Zumerle et al., 2019). Macrophage calcium dynamics was recently imaged during *Mmar* infection in vivo, using a zebrafish transgenic line with a genetically encoded calcium indicator, GCaMP6F, under control of the macrophage-specific *mfap4* promoter (Matty et al., 2019). This study identified a drug, clemastine, that potentiates the P2X7 receptor, thereby modulating the calcium signals and increasing host resistance to infection. This host-mediated anti-mycobacterial drug effect is independent of autophagy and requires the function of the inflammasome component, Asc. Calcium signals are also involved in oxidative stress and necrosis. These stress-related calcium signals were investigated in zebrafish larvae that are hypersusceptible to *Mmar* infection due to overexpression of *tnfa* (Roca et al., 2019). Two approaches were used to demonstrate mitochondrial Ca^{2+} overload in these animals: mitochondria-specific expression of a calcium indicator (GCaMP3) and administration of a fluorescent chemical probe for calcium (Rhod-2), accumulating in mitochondria. The in vivo application of these tools in zebrafish facilitated the identification of host-directed drugs limiting the mitochondrial calcium overload and associated necrosis that occurs under excess production of Tnfa (Roca et al., 2019).

3 | DECIPHERING THE RELATIVE CONTRIBUTION OF MACROPHAGES AND NEUTROPHILS TO PATHOGENESIS

When it comes to fighting infections, macrophages and neutrophils are the first responders of the innate immune system. Their main functions are to control the infection by taking up the pathogenic invaders and orchestrating the response of other immune cells. The application of fluorescent microscopy techniques and genetic cell ablation technologies has facilitated the dissection of the relative contribution of macrophages and neutrophils after mycobacterial systemic infection in zebrafish larvae. In the zebrafish embryo, macrophages and neutrophils are the first immune cell to develop, as early as 22 and 33 hr post-fertilization, respectively (Masud et al., 2017). This characteristic makes the zebrafish larvae model unique for intravital studies regarding host-pathogen interactions.

Early infection events have been analyzed using the zebrafish larvae-*Mmar* model in combination with transgenic zebrafish lines carrying fluorescent macrophages and neutrophils (Rosowski, 2020a). Several studies have shown that the initial interactions between *Mmar* and macrophages occur quickly at the site of infection (Davis et al., 2002; Davis & Ramakrishnan, 2009). Macrophages are the first immune cell to arrive at the site of infection and to phagocytose the invading pathogen (Clay et al., 2007). In contrast, the use of a transgenic line-carrying fluorescent neutrophils has shown that neutrophils are recruited to the initial infection sites but rarely interact with *Mmar* (Yang et al., 2012). As occurs during *Mtb* infection, neutrophils do not phagocytose extracellular bacteria even though they are attracted to the site of infection (Clay et al., 2007; Pedrosa et al., 2000; Yang et al., 2012). However, neutrophils appear to be important for cross-talk with macrophages and they exert protection during *Mmar* infection in zebrafish by reactive oxygen and nitrogen defense mechanisms (Elks et al., 2013; Yang et al., 2012). This protective role of neutrophils has been associated with the phagocytosis of dead infected macrophages (Yang et al., 2012).

Different signaling pathways seem to contribute to the pathogenesis of the disease but special attention has been put into unravel the role of macrophages and neutrophils in regulating the inflammatory response during *Mmar* infection. The use of fluorescent reporter lines has shed light on the in situ transcriptional activation of host inflammatory factors such as Il1b and Tnfa. In agreement with the well-known role of inflammatory mediators during mycobacterium infection, both *il1b*:GFP and *tnfa*:GFP are transcriptionally upregulated in infected macrophages early during *Mmar* infection in zebrafish larvae (Lewis & Elks, 2019; Ogryzko et al., 2019). On the contrary, in situ detection of the activity of reactive nitrogen species using whole-mount immunostaining, has stressed the importance

of surrounding neutrophils in the regulation of the inflammatory response through the hypoxia-inducible factor-1α (Hif-1a) signaling pathway and their implication in the oxidative killing of the pathogen (Elks et al., 2013; Ogryzko et al., 2019). Constitutive activation of Hif-1α has been found to enhance the protective abilities of neutrophils (Elks et al., 2013). Interestingly, this effect was retained in a comorbid setting that combined *Mmar* infection with injury-induced inflammation, supporting the potential of intervening with hypoxia signaling as a host-directed TB therapy (Oehlers et al., 2020; Schild et al., 2020).

In addition to fluorescent transgenic zebrafish lines, several cell ablation tools are available for specific cell depletion in zebrafish (Rosowski, 2020a). Ablation tools rely principally on genetic modifications or toxic effects on specific cells with different possibilities regarding spatial and/or temporal modulation of the ablation. Genetic ablation mediated by antisense oligonucleotide morpholinos targeting specific cell differentiation pathways, such as Pu.1 or Irf8, has been extensively used over the years in zebrafish (Rosowski, 2020a). Due to the lack of macrophages, *pu.1* morphants show increased *Mmar* growth but less tissue dissemination suggesting the importance of macrophages as propagators of the infection (Clay et al., 2007). In agreement, *irf8* morphants and adult *csf1r* mutant zebrafish, both presenting also a deficiency in macrophages, are hypersusceptible to *Mmar* as a result of the necrosis induced in granulomas due to the depletion of the macrophage supply (Pagan et al., 2015). The importance of neutrophils during *Mmar* infection has been shown with a neutropenic zebrafish line due to mutation of warts, hypo-gammaglobulinemia, infections, and myelokathexis (WHIM), which presented an increased susceptibility to the infection due to impaired neutrophil migration (Yang et al., 2012).

In summary, results in zebrafish have shown that the presence of both phagocytes is necessary for an efficient host response against *Mmar*, but the protective role of neutrophils seems not directly linked to the phagocytosis of the pathogen, in contrast to the role of the macrophages, which limit *Mmar* growth to some degree but are also exploited by the pathogen for replication and dissemination.

4 | NEW INSIGHTS INTO PHAGOCYTE MIGRATION AND DISSEMINATION OF MYCOBACTERIAL INFECTION

The migration of phagocytes is orchestrated by chemokine signaling axes, which are generally well conserved between humans and zebrafish (Sommer et al., 2020b). The zebrafish homologs of the human CCR2 chemokine receptor and its ligand CCL2 were shown to play an important role in establishing the initial infection. Injecting *Mmar* into the hindbrain ventricle revealed that tissue-resident macrophages are the first responders to the infection, followed by recruitment of monocytes from the circulation (Cambier et al., 2017). While the resident macrophages display anti-bacterial activity, the monocytes are permissive to *Mmar* growth, and their recruitment is mediated by the Ccr2 receptor (Cambier et al., 2014). Expression of the Ccl2 ligand is induced by a Sting-dependent signaling pathway in resident macrophages, in response to phenolic glycolipid found on the surface of *Mmar* and other pathogenic mycobacteria (Cambier et al., 2017). *Mmar* bacteria are then transferred by cell fusion events from the resident macrophages to the recruited population of Ccr2-expressing monocytes. These studies in zebrafish have highlighted a CCR2-CLL2-mediated interaction between phagocyte subsets proposed to be critical for establishing persistent infection also in human TB patients (Cambier et al., 2017).

The homologs of three members of the human CXCR family, CXCR2, CXCR3, and CXCR4, have also been studied in the context of *Mmar* infection in zebrafish larvae. Cxcr2 mediates the migration of neutrophils toward the chemokines Cxcl8a/Il8 and Cxcl18b. Imaging of *cxcl18b:gfp* transgenic zebrafish larvae revealed that *cxcl18b* expression is induced in uninfected stroma cells of granulomas and not detectable in the infected phagocytes (Torraca et al., 2017a). Consequently, Cxcr2-Cxcl18b signaling could aid host defense by mediating the recruitment of neutrophils, but in the same manner, it might also contribute to pathological inflammation in the granuloma.

The Cxcr3-Cxcl11 axis has been shown to control multiple aspects of macrophage function during infection. The expression of one of the *cxcl11* family genes, *cxcl11.1* (*cxcl11aa*) is strongly upregulated in *Mmar*-infected macrophages (Rougeot et al., 2019). Cxcl11aa signals through Cxcr3.2, the activity of which is antagonized by Cxcr3.3, an atypical receptor lacking the motif required for G-protein interaction (Sommer et al., 2020a). The opposite phenotypes of *cxcr3.2* and *cxcr3.3* loss-of-function mutants indicate that Cxcr3.2 signaling exacerbates infection by promoting the tissue dissemination of infected macrophages and granuloma formation, while Cxcr3.3 attenuates these effects (Sommer et al., 2020a; Torraca et al., 2015). Furthermore, live imaging of *cxcr3.2*-deficient macrophages showed that their aberrant migration is linked to an increase of the lysosomal compartment, which augments the antimycobacterial capacity (Sommer et al., 2021). The adverse effects of Cxcr3.2 signaling on host resistance to *Mmar* are in line with increased *Mtb* infection pathology observed in CXCR3-deficient mice (Chakravarty et al., 2007; Seiler et al., 2003).

Similar to Cxcr3.2, also the Cxcr4b receptor promotes *Mmar* pathogenesis, as it supports the vascularization of granulomas (Torraca et al., 2017b). Mechanistically, Cxcr4b-Cxcl12a signaling is hypothesized to facilitate vascularization through macrophage-mediated anastomosis downstream of Vegf signaling. This pathway could be a possible target for anti-angiogenic tuberculosis therapy, in line with the therapeutic effects of angiogenesis inhibitors that were previously demonstrated in zebrafish (Oehlers et al., 2015). However, it must be considered that the Cxcr4b-Cxcl12a axis has also been implicated in the recruitment of neutrophils that exert protection (Wright et al., 2021). This activity of the Cxcr4b-Cxcl12a axis is normally suppressed due to the expression of a microRNA, miR-206, in the host, which is induced by pathogenic mycobacteria

(Wright et al., 2021). Therefore, the therapeutic potential of modulating this chemokine axis requires further investigation.

All in all, owing to the powerful combination of genetics and in vivo imaging, studies in zebrafish have revealed that chemotactic processes mediated by several CCR and CXCR receptors on phagocytes frequently promote mycobacterial pathogenesis rather than contribute to the host defense response, which highlights how virulent mycobacteria have evolved effective mechanisms to exploit both pathways.

5 | LIFE AND DEATH INSIDE THE GRANULOMA

As pointed out in the previous sections, TB is characterized by the presence of granulomas. Granulomas are highly organized and compact aggregates of immune cells. The cell aggregates that form during *Mmar* infections of zebrafish larvae or adult fish recapitulate several aspects of granulomas in human TB, including the formation of a hypoxic and necrotic center (Figure 1) (Oehlers et al., 2015; Parikka et al., 2012; Ramakrishnan, 2013). Traditionally, granulomas have been associated with the presence of the adaptive immune system but research in zebrafish larvae showed that granulomas start to form in the context of only innate immunity (Davis et al., 2002). Furthermore, it can be observed in zebrafish larvae that infected macrophages egress from primary granulomas and seed secondary granulomas at distal sites (Figure 1) (Davis & Ramakrishnan, 2009). Importantly, opposite to the old vision of the granuloma as a host-protective structure, it has been revealed that pathogenic bacteria drive granuloma formation as a protective strategy against the host immune system (Cronan et al., 2016; Davis & Ramakrishnan, 2009; Ramakrishnan, 2013).

The zebrafish model has been widely used to study structural aspects of granuloma. Investigations in zebrafish suggest that the vascularization process of the granuloma is induced by mycobacterial factors in concert with host chemokine signaling (Oehlers et al., 2015; Torraca et al., 2017b). In addition to this pro-angiogenic signaling, mycobacteria induce vascular permeability through the induction of angiopoietin-2 in macrophages and stromal cells (Oehlers et al., 2017). This process is conserved between zebrafish and humans and therefore a promising therapeutic target (Hortle & Oehlers, 2020). Moreover, as in human TB, the structural maturation of the granuloma is tightly ligated to the reprogramming of macrophages into epithelioid cells (Cronan et al., 2016, 2021). Macrophage epithelization was found to rely on Stat6-mediated signaling, and disruption of this signaling axis in macrophages inhibited the progression of granulomas into necrotizing structures (Cronan et al., 2021). Another important aspect of human TB granuloma maturation that is recapitulated in zebrafish larvae and adult fish is the differentiation of macrophages into foam cells (Johansen et al., 2018). Foamy macrophages are sources of nutrients for the invading pathogen and their appearance has been also shown to be driven by the ESX-1 bacterial secretion system as well as by host thrombocytes (Hortle et al., 2019; Johansen et al., 2018). The appearance of foamy cells within the granuloma is a well-characterized effect of chronic TB suggesting that zebrafish is a suitable model for studying the reactivation of the disease that can occur spontaneously but also experimentally induced by irradiation (Parikka et al., 2012).

Contrary to the idea that arises when referring to a compact cell structure, the application of imaging techniques in vivo in the

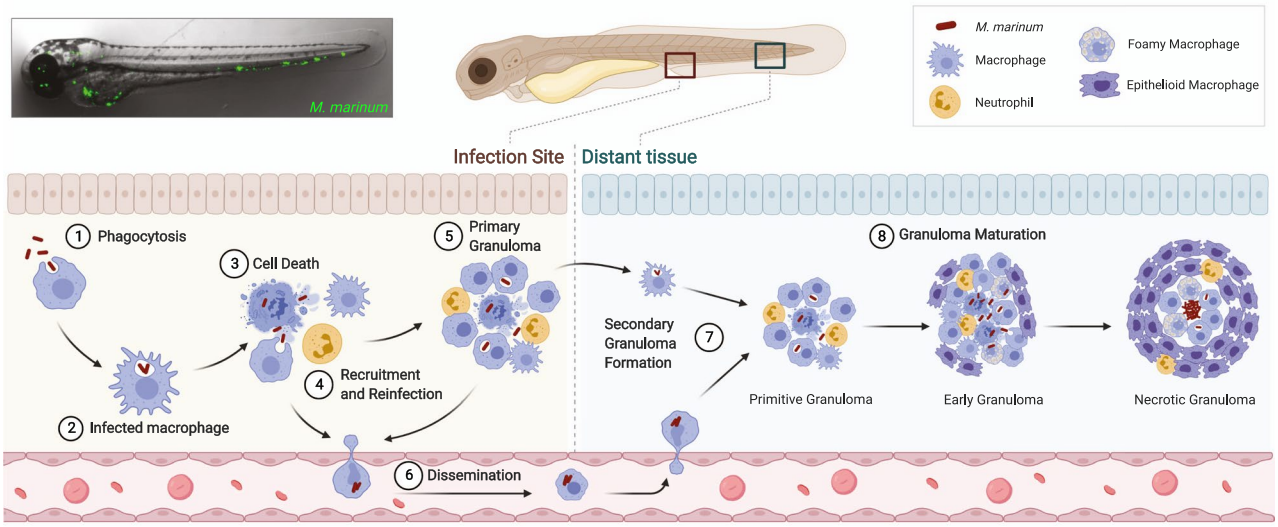

FIGURE 1 Mycobacterial infection and granuloma formation in zebrafish larvae. After infection, *Mycobacterium marinum* is primarily phagocytosed by macrophages (1). Infected macrophages (2) undergo cell death (3). The release of cellular contents favors the recruitment of new cells that become infected (4) initiating the formation of primary granulomas (5) at the infection site. Egression of infected cells initiates the dissemination (6) of the infection through the blood circulation or tissues leading to the formation of secondary granulomas (7) in distant tissues. Granulomas undergo a maturation process (8) in which size and cell composition vary from primitive granulomas to early granulomas and necrotic granulomas. In adult zebrafish, granulomas contain additional immune cells such as T lymphocytes. Created with Biorender.com

zebrafish model has proven that granulomas are highly dynamic structures (Davis et al., 2002). A considerable number of the cell movements and rearrangements observed in granulomas are driven by cell death processes. Signals from dead or dying granuloma macrophages recruit neutrophils, which then phagocytose corpses of infected macrophages (Yang et al., 2012). Moreover, as the infection progresses in larvae and adult zebrafish models, cell death processes inside de granuloma lead to the formation of a necrotic core in which the bacteria reside (Parikka et al., 2012; Ramakrishnan, 2013).

Cell death is also known to play a fundamental role during the formation of primitive granulomas and the dissemination of the disease. At early stages of the infection, the necrotic death of infected macrophages helps the multiplication and dissemination of the pathogen (Volkman et al., 2010). Cycles of death of infected cells and reinfection of naïve macrophages are behind the initiation of the pathogenesis as they favor bacterial proliferation but also granuloma formation and expansion (Davis & Ramakrishnan, 2009). Different cell death modalities have been implicated in the progression of the disease in zebrafish. Inflammasome activation and consequent pyroptotic cell death have been visualized in zebrafish larvae granulomas (Forn-Cuni et al., 2019; Varela et al., 2019). Moreover, Tnfa-mediated necrosis occurs in the presence of an excess of Tnfa in zebrafish larvae (Roca et al., 2019). Interestingly, although inflammasome-mediated and Tnfa-mediated cell death processes are molecularly regulated in a different way by the host, the pathogen is known to manipulate them in order to favor its release from infected cells, thereby increasing the virulence of the disease (Roca et al., 2019; Varela et al., 2019).

Finally, it is expected that with the development and application of new molecular and microscopy techniques in combined with the existing live models but also with new culture techniques, such as the explant of mature granulomas (Cronan et al., 2018), the zebrafish model will further contribute to the understanding of this fascinating cellular structure.

6 | CONCLUDING REMARKS

It has been 20 years since it was first demonstrated that *Mmar* can induce granuloma formation in the context of innate immunity in zebrafish larvae (Davis et al., 2002). This study kicked off a wide use of this model system and inspired research questions that could not have been addressed in other animal models, such as how granulomas are initiated by an interplay of host and bacterial factors (Ramakrishnan, 2020). Not only can hallmarks of human TB be recapitulated by *Mmar* infection of zebrafish, also the nerve damage characteristic for leprosy could be modeled in zebrafish larvae, using a *Mmar* strain with modified surface lipids to resemble *Mycobacterium leprae* (Madigan et al., 2017). Furthermore, the zebrafish host is permissive to non-tuberculous human mycobacterial pathogens, like *Mycobacterium abscessus*, *Mycobacterium kansassi* and *Mycobacterium fortuitum*, all inducing granuloma formation in immunocompetent larvae and showing extensive extracellular cord formation in immunocompromised larvae (Bernut et al., 2016; Johansen & Kremer, 2020a, 2020b).

Further use of the different mycobacterial infection models would benefit from expanding the toolbox for precision genetic engineering and the collection of fluorescent marker lines. Cell-specific gene inactivation has long been a bottleneck but has now been achieved for both neutrophils and macrophages (Isiaku et al., 2021; Wang et al., 2021), and this technology provides great opportunities to analyze gene functions in a lineage-specific manner. Proinflammatory responses in macrophages can be visualized using *tnfa:gfp* and *ilb:gfp* transgenic lines (Lewis & Elks, 2019; Ogryzko et al., 2019), but the tools are still lacking to detect other polarization states that make monocytes and macrophages permissive to mycobacterial growth. Additionally, there is a need for vesicle markers to trace the intracellular trafficking of mycobacteria and for fluorescently tagged proteins to study the activation of innate immunity signaling mechanisms in real time. Novel optogenetic tools are now being applied in zebrafish disease models and this technology opens new possibilities also for infection research, enabling local and timed activation of factors at the host-pathogen interface (Asakawa et al., 2021; Formella et al., 2018; Shkarina et al., 2021). These and other technological advances will maximize the use of zebrafish as a model host and will drive research into the mechanisms of innate host defense and pathogen evasion strategies, the necessary basis for therapeutic innovations.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

Data sharing not applicable—no new data generated.

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