Macrophages mediate flagellin induced inflammasome activation and host defense in zebrafish

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Summary

The inflammasome is an innate immune complex whose rapid inflammatory outputs play a critical role in controlling infection; however, the host cells that mediate inflammasome responses in vivo are not well defined. Using zebrafish larvae, we examined the cellular immune responses to inflammasome activation during infection. We compared the host responses with two Listeria monocytogenes strains: wild type and Lm-pyro, a strain engineered to activate the inflammasome via ectopic expression of flagellin. Infection with Lm-pyro led to activation of the inflammasome, macrophage pyroptosis and ultimately attenuation of virulence. Depletion of caspase A, the zebrafish caspase-1 homolog, restored Lm-pyro virulence. Inflammasome activation specifically recruited macrophages to infection sites, whereas neutrophils were equally recruited to wild type and Lm-pyro infections. Similar to caspase A depletion, macrophage deficiency rescued Lm-pyro virulence to wild-type levels, while defective neutrophils had no specific effect. Neutrophils were, however, important for general clearance of L. monocytogenes, as both wild type and Lm-pyro were more virulent in larvae with defective neutrophils. This study characterizes a novel model for inflammasome studies in an intact host, establishes the importance of macrophages during inflammasome responses and adds importance to the role of neutrophils in controlling L. monocytogenes infections.

Introduction

The innate immune response is critical for host defense against invading pathogens. Mechanisms have evolved to rapidly sense and protect the host from infection (Janeway and Medzhitov, 2002), including the activation of the multi-protein signalling complex known as the inflammasome (Martinon et al., 2009). The inflammasome is important for defense against many infections including intracellular bacterial pathogens (Sansone et al., 2000; Mariathasan et al., 2005; Lara-Tejero et al., 2006; Broz et al., 2010), as well as fungal and viral pathogens (Rathinam et al., 2010; van de Veerdonk and Joosten, 2015). To counter this host defense, many pathogens have evolved mechanisms to avoid or even actively inhibit inflammasome activation (Taxman et al., 2010; Lamkanfi and Dixit, 2011; Ulland et al., 2015). Despite substantial progress on the molecular mechanisms of inflammasome activation, little is known about how host protection is mediated and what role specific innate immune cell populations have during acute infection in vivo.

The inflammasome surveys the cytosol for signals of intracellular pathogens or host cell damage. Canonical inflammasome activation begins with the detection of a cytosolic ligand or danger signal, which induces the self-oligomerization of a corresponding inflammasome sensor (Martinon et al., 2002; Mariathasan et al., 2004; Faustin et al., 2007). Caspase-1 activates a number of inflammatory outputs including the activation of IL-1β, the production of eicosanoids and a pro-inflammatory form of programmed lytic host cell death termed pyroptosis (Chen et al., 1996; Martinon et al., 2002; Mariathasan et al., 2004; von Moltke et al., 2012). The molecular
mechanisms of inflammasome activation have been worked out in vitro largely using macrophages and dendritic cells, but it is not fully understood how these or other relevant cells of the immune system contribute to inflammasome-mediated host protection in vivo.

To address this gap, we have developed a model in zebrafish to study inflammasome activation and the contributions of innate immune phagocytes to host defense. The zebrafish is a powerful model system that is highly amenable to genetic manipulation and high-resolution imaging and has a conserved innate immune system (Meijer and Spaink, 2011; van der Vaart et al., 2012). Many of the components of the inflammasome are conserved in zebrafish. For example, a family of NLRs exist in the zebrafish genome (Laing et al., 2012). Many of the components of the inflammasome are conserved in zebrafish. For example, a family of NLRs exist in the zebrafish genome (Laing et al., 2012), although the ligands that they respond to have yet to be identified. The adaptor protein ASC is also conserved, and oligomerization of zebrafish ASC recruits and activates caspase A, the zebrafish functional homolog of caspase-1, into a characteristic speck structure (Masumoto et al., 2003). Furthermore, in ex vivo leukocytes from adult zebrafish, caspase A can cleave IL-1β upon stimulation with the fish-specific pathogen Francisella noatunensis (Vojtech et al., 2012). Live imaging of lytic macrophage death and hallmarks of pyroptosis have also been observed in a zebrafish larval model of Shigella flexneri infection (Mostowy et al., 2013). Infection with spring viremia of carp virus, a fish hemorrhagic virus, leads to a sharp decline in leukocyte number and induces membrane permeability. In this system, caspase A and IL-1β were co-localized by immunostaining with actively infected macrophages, showing IL-1β being released from macrophages (Varela et al., 2014). Collectively, these studies provide strong evidence that inflammasome signalling is conserved in zebrafish.

We developed a system to probe the inflammasome in zebrafish using a localized Listeria monocytogenes infection model. Listeria monocytogenes is a cytosolic pathogen that upon uptake into host cells produces a pore-forming cytolysin, listeriolysin O (encoded by the gene hly), to escape the phagosome (Gaillard et al., 1987). Once in the host cytosol, L. monocytogenes rapidly replicates and makes use of an actin nucleating factor ActA (actA) to hijack the host cytoskeleton and spread from cell to cell without leaving the intracellular niche (Kocks et al., 1992). These classic steps of L. monocytogenes virulence have been observed during systemic infection of zebrafish larvae (Levraud et al., 2009). To promote virulence and protect its intracellular niche, L. monocytogenes has evolved to largely evade inflammasome activation (Sauer et al., 2010; Sauer et al., 2011; Witte et al., 2012). However, it has previously been demonstrated that L. monocytogenes and other pathogens engineered to ectopically express flagellin in the host cytosol robustly activate the inflammasome and can be used to understand the role of the inflammasome in host defense and induction of adaptive immunity (Miao et al., 2010a; Sauer et al., 2011; Warren et al., 2011). Inflammasome activation leads to rapid and robust attenuation of L. monocytogenes virulence; however, as with Salmonella typhimurium, IL-1β and IL-18 are dispensable for this phenotype (Miao et al., 2010a; Sauer et al., 2011). Listeria monocytogenes that ectopically expresses flagellin (Lm-pyro) recruits LysM-positive cells to the spleen earlier than wild type (WT) infection; however, the inflammation also prematurely resolves (Williams et al., 2013). Listeria monocytogenes engineered to induce necrosis through misregulation of listeriolysin O activity is also highly attenuated, further demonstrating the importance of maintaining the intracellular replication niche (Glomski et al., 2003). Importantly, the virulence defects of necrotic L. monocytogenes strains can be rescued by neutrophil depletion (Glomski et al., 2003); however, neutrophil depletion does not rescue the virulence defect of Lm-pyro (Sauer et al., 2011), suggesting that the role of neutrophils following pyroptosis is complex. Taken together, these studies suggest that IL-1β/IL-18 production and neutrophil killing are not essential for bacterial clearance directly because of inflammasome activation. The roles of other innate immune cells during this process are not fully understood.

Here, we describe a model system to examine the role of the inflammasome in the cellular innate immune response to infection. As is observed in disseminated infection models in mice, bacteria that activate the inflammasome are attenuated in a localized zebrafish infection model. Inflammasome activation leads to a substantial increase in macrophage inflammation at the site of infection, and macrophages undergoing pyroptosis could be observed with high spatial and temporal resolution during infection. Leukocyte depletion supports a critical role for macrophages in inflammasome-mediated host defense. Conversely, neutrophils were important for general clearance of L. monocytogenes independent of inflammasome activation. This work establishes a tractable model for studying the innate immune response to inflammasome activation, making use of the unique features of both the zebrafish host and the intracellular pathogen L. monocytogenes, and highlights the novel finding that macrophages are essential for pathogen control in the context of inflammasome activation.

Results

Listeria monocytogenes hly and actA are required for localized zebrafish infection

Listeria monocytogenes can establish lethal infection in zebrafish larvae following intravenous inoculation
To allow for the tracking of cellular inflammatory responses to infection, we developed a hindbrain ventricle (HBV) localized \textit{L. monocytogenes} infection model, inoculating larvae at 48 h post fertilization (hpf). We chose this developmental stage as both neutrophils and macrophages have developed and are phagocytically active but are present in only small numbers at the HBV (Herbomel \textit{et al}., 1999; Herbomel \textit{et al}., 2001; Lieschke \textit{et al}., 2001; Le Guyader \textit{et al}., 2008). Following inoculation into the HBV, WT \textit{L. monocytogenes} was able to cause host death at each dose analysed, as low as 10 colony forming units (CFU) (Fig. 1A). Virulence increased in a dose-dependent manner, with an apparent LD$_{50}$ of 100 CFU. Mutant strains of \textit{L. monocytogenes} that are unable to escape the primary phagosomal compartment (Δhly) (Gaillard \textit{et al}., 1987) or maintain access to the cytosol via cell-to-cell spread (ΔactA) (Kocks \textit{et al}., 1992) were significantly attenuated at all doses examined (Fig. 1B–C).

To examine the kinetics of dissemination, we performed HBV infections using 100 CFU – the WT LD$_{50}$ – of WT and mutant strains constitutively expressing GFP and examined embryos for signs of disseminated infection. At 1 day post infection (dpi), no visible dissemination could be seen, likely because of low-resolution imaging. Between 2 and 3 dpi, patches of WT \textit{L. monocytogenes} emerged at distal points in the trunk and tail muscle along the neural tube (Fig. 1D), away from the inoculation site. From 3 to 4 dpi, these patches expanded as embryos succumbed to infection. Dissemination was not witnessed during infection with Δhly or ΔactA \textit{L. monocytogenes} (Fig. 1E–F) – which looked identical to phosphate-buffered saline (PBS)-inoculated larvae (Fig. S1) – nor were there signs of necrotic tissue damage as seen during WT infection. Taken together, these data suggest that localized HBV infection with WT \textit{L. monocytogenes} leads to disseminated disease and ultimately death across a wide range of doses dependent upon bacterial access to the host cytosol.

\textit{Lm-pyro} is attenuated in zebrafish larvae

Given that \textit{L. monocytogenes} zebrafish infection required access to the cytosol, we hypothesized that other adaptations that maintain the intracellular niche are similarly important for virulence. It has previously been demonstrated that \textit{L. monocytogenes} that activates host cell death is attenuated in vivo (Głomski \textit{et al}., 2003; Warren \textit{et al}., 2008; Sauer \textit{et al}., 2010; Sauer \textit{et al}., 2011; Warren \textit{et al}., 2011). \textit{Lm-pyro} is a strain genetically engineered to hyper-activate the mouse Nlrc4 inflammasome by secreting flagellin monomers exclusively when in the host cytosol (Sauer \textit{et al}., 2011). \textit{Lm-pyro} carries a genomically inserted construct that uses the actA promoter to drive secretion of the FlaA flagellin from \textit{Legionella pneumophila} specifically when the bacterium

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reaches the host cytosol and initiates actA expression. In agreement with previous studies (Sauer et al., 2011; Warren et al., 2011), Lm-pyro was attenuated following inoculation at a WT LD50 (Fig. 2A). In contrast to WT infection, the vast majority of larvae infected with Lm-pyro bacteria constitutively expressing GFP did not display signs of disseminated infection (Fig. 2B). Furthermore, Lm-pyro bacterial growth within larvae was decreased compared with WT infection (Fig. S2). Taken together, these data show that at the WT LD50, secretion of flagellin compared with WT infection (Fig. S2). Taken together, these data show that at the WT LD50, secretion of flagellin from the zebrafish host. Despite attenuation at lower doses, WT and Lm-pyro infection were equally virulent at 1000 CFU, and indeed, Lm-pyro was statistically more virulent at 10000 CFU (Fig. 2A). Thus, host responses downstream of sensing cytosolic flagellin can be overwhelmed with a high inoculum.

**Lm-pyro activates the inflammasome in zebrafish**

Lm-pyro is attenuated, both in macrophages and in vivo in mice, because of activation of the Nlr4 inflammasome (Sauer et al., 2011). We therefore examined if Lm-pyro activates the inflammasome in zebrafish, and if so, if this activation plays a role in controlling Lm-pyro infection. To determine if flagellin, and specifically Lm-pyro, activates a zebrafish inflammasome, we generated a GFP-tagged form of the zebrafish ASC, made mRNA by in vitro transcription and used this to assay for ASC speck formation. Zebrafish larvae overexpressing GFP-tagged ASC were inoculated with PBS, WT or Lm-pyro L. monocytogenes, and ASC specks at the inoculation site were quantified. Overexpression of GFP–ASC alone induced ASC specks, similar to cell culture systems (Masumoto et al., 2001a, b). Compared with mock inoculation with PBS, infection with WT bacteria did not lead to an increase in ASC specks, consistent with a lack of inflammasome activation by WT L. monocytogenes during in vivo infection (Sauer et al., 2010; Sauer et al., 2011; Witte et al., 2012). By contrast, infection with Lm-pyro led to an increase in the number of ASC specks compared with WT bacteria (Fig. 3A–B). Thus, the host response to cytosolic flagellin increased inflammasome activation as reported by ASC specks, whereas the presence of WT L. monocytogenes alone did not.

We next examined embryos actively infected with Lm-pyro for signs of pyroptosis, a programmed lytic host cell death downstream of inflammasome activation. To detect pyroptosis in vivo, we generated a zebrafish line with histone-2B tagged in macrophages (Tgmpeg1:mCherry-histone2B). We then crossed this line to zebrafish in which macrophages were tagged with cytosolic dendra2, allowing us to image both nuclear morphology and plasma membrane lysis. Larvae were infected with either WT or Lm-pyro bacteria expressing mCherry driven by the actA promoter, allowing us to focus on actively infected macrophages. At 24 hpi, we were able to image in vivo pyroptosis in detail during Lm-pyro infection (Fig. 3C and Video Clip S1). This process was characterized by rapid loss of cytosolic fluorescence immediately followed by nuclear condensation. The total process was complete within 10–20 min. Multiple macrophages could be seen undergoing pyroptosis, quickly followed by increased activity of both nearby macrophages and other unlabeled cells (Video Clip S1). Despite repeated attempts, we were unable to find any macrophages undergoing pyroptosis in WT-infected embryos. Thus, pyroptosis, one of the classic

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Fig. 2. Lm-pyro is attenuated in zebrafish larvae. A. Survival of larvae infected with WT and Lm-pyro at 10, 100, 1000 and 10 000 CFU. Larvae were infected as in Fig. 1. WT *Listeria monocytogenes* repeated with similar virulence. Lm-pyro displayed virulence defects at 10 and 100 CFU. At 1000 CFU, virulence was equivalent and slightly increased at 10 000 CFU. Each graph is the pooled data from three independent survival experiments. P-values and hazard ratios (HR) are relative to WT infection at the same dose of bacteria. B. Dissemination of GFP-expressing WT and Lm-pyro. Representative images of dissemination over the course of 100 CFU infection with WT and Lm-pyro. WT disseminated similar to Fig. 1. Lm-pyro did not disseminate. Scale bars are 500 μm.
outcomes of inflammasome activation, was witnessed during infection with Lm-pyro. Taken together, these data suggest that, consistent with previous observations in murine systems, wild-type \textit{L. monocytogenes} stimulates little to no inflammasome activation \textit{in vivo}, whereas Lm-pyro triggers classic hallmarks of inflammasome activation including ASC oligomerization and pyroptosis. Furthermore, these data suggest that zebrafish contain an intact inflammasome signalling cascade similar to the NAIP5/Nlrc4 inflammasome found in mice.

\textbf{Inflammasome activation attenuates Lm-pyro virulence}

The increased utilization of ASC combined with imaging pyroptosis strongly suggested that Lm-pyro was activating the inflammasome in our model system. Downstream events following inflammasome activation are ultimately executed by caspase-1. As virulence attenuation of Lm-pyro in other systems was dependent on inflammasome activation, we examined the ability of WT and Lm-pyro \textit{L. monocytogenes} to cause disease following administration of morpholino oligonucleotides to transiently knock down translation of caspase A (Masumoto \textit{et al.}, 2003), the zebrafish homolog of caspase-1. Knockdown efficiency was verified by western blot (Fig. S3). Caspase A knockdown had no effect on the virulence of WT \textit{L. monocytogenes} at the LD\textsubscript{50} (Fig. 4A), suggesting as previously reported (Sauer \textit{et al.}, 2011) that WT \textit{L. monocytogenes} minimally activates the inflammasome \textit{in vivo}. There was, however, a significant increase in Lm-pyro virulence following knockdown of caspase A (Fig. 4B), suggesting that the attenuated virulence of Lm-pyro is due to host signalling through the inflammasome. Of note, virulence during Lm-pyro infection was not fully rescued to WT levels upon caspase A knockdown (\(P < 0.0001\) comparing WT-infected vs. Lm-pyro-infected CaspA morphants). However, this is likely due to both the incomplete knockdown of caspase A as well as the return
of caspase A expression and hence inflammasome signalling at 4 dpf/2 dpi (Fig. S3). Together, these data suggest that, similar to mammalian systems, inflammasome activation in the zebrafish can be host protective in the context of intracellular bacterial infection.

**Inflammasome activation induces macrophage recruitment**

Having demonstrated that Lm-pyro activates the zebrafish inflammasome and that this response is host protective, we next began to assess the host leukocyte response to inflammasome activation. To determine the inflammatory response to inflammasome activation, we infected embryos with fluorescently labelled macrophages or neutrophils and quantified the number of cells recruited to the initial site of infection. WT infection did not induce macrophage inflammation compared with mock inoculation with PBS. In contrast, Lm-pyro infection elicited macrophage recruitment, resulting in an approximately twofold increase in the number of macrophages at the site of infection (Fig. 5A–B). Neutrophil recruitment, on the other hand, was increased during both WT and Lm-pyro infections, independent of inflammasome activation (Fig. 5C–D). Taken together, these data suggest that the acute inflammatory response to *L. monocytogenes* infection involves the recruitment of neutrophils whereas the macrophage compartment is specifically recruited during infection-induced inflammasome activation.

**Macrophages confer host protection to infection during inflammasome responses**

Given the specificity of the leukocyte response to bacterial infection with inflammasome activation, we next characterized the role of macrophages and neutrophils in controlling infection by depleting or severely impairing macrophage or neutrophil function respectively. Knockdown of cell populations was carried out in transgenic lines labelling the relevant cell type(s) and verified by fluorescence microscopy immediately prior to experimen-
Morpholino knockdown of the transcription factor Irf8 led to transient macrophage depletion until 2 days post infection in our model (Li et al., 2011). Control morphants were similar to WT larvae, as they were susceptible to WT infection and resistant to Lm-pyro (Figs 1A, 2A and 6A–B). Irf8 morphant fish, on the other hand, were highly susceptible to infection with both WT and Lm-pyro bacteria (Fig. 6A–B). Indeed, macrophage deficiency eliminated the difference in survival outcome following infection with WT and Lm-pyro (Figs 2A and 6A–B). This result was further verified using low-dose Pu.1 morpholino injection as an alternative method to deplete macrophages but not neutrophils (Rhodes et al., 2005) (Fig. S4). In contrast, Δhly bacteria remained avirulent in both control and Irf8 morphants (Fig. S5A), suggesting that macrophage deficiency does not simply lead to general susceptibility to infection.

To address the role of neutrophils downstream of inflammasome activation, we used a zebrafish model of the human disease leukocyte adhesion deficiency (Deng et al., 2011). Neutrophils expressing a dominant negative mutation in the small Rho family GTPase Rac2 have severely impaired neutrophil function (Deng et al., 2011). When expressed under a neutrophil-specific promoter (Tg: mpx-Rac2 D57N), neutrophils enter the circulation but are unable to exit the vasculature and cannot reach localized sites of infection. Tg:mpx-Rac2 D57N embryos were more susceptible to both WT and Lm-pyro (Fig. 6C–D). In contrast to macrophage depletion, however, Lm-pyro retained its virulence defect relative to WT bacteria when neutrophils were functionally defective (Fig. 6G). As with macrophage deficiency, the virulence of Δhly was unaffected in Tg:mpx-Rac2 D57N larvae (Fig. S5B). Depletion of both macrophages and neutrophils by knockdown of the transcription factor Pu.1 (Rhodes et al., 2005) made larvae highly susceptible to infection, causing rapid death during WT, Lm-pyro and even Δhly infection (Fig. 6E–G and Fig. S5C), suggesting a total loss of host defense and extracellular replication of L. monocytogenes. Taken together, these findings suggest that macrophage and neutrophil responses are crucial in the response to L. monocytogenes infection. Specifically, the loss of macrophages re-sensitized larvae to infections with inflammasome-activating bacteria, normalizing the virulence of WT vs. Lm-pyro infections, whereas neutrophil deficiency generally increased susceptibility to L. monocytogenes infection but did not specifically rescue Lm-pyro virulence.

**Fig. 6.** Macrophage depletion removes the virulence defect of Lm-pyro. (A, B) Survival of Irf8 morphant larvae lacking macrophages following infection with WT (A) and Lm-pyro (B), (A) and (B) at 100 CFU. (C, D) Survival of Tg:mpx-Rac2 D57N larvae with functionally defective neutrophils following infection with WT (C) and Lm-pyro (D). (C) and (D) at 100 CFU. (E, F) Survival of Pu.1 morphant larvae lacking both macrophage and neutrophil development following infection with WT (E) and Lm-pyro (F). (E) and (F) at 100 CFU. Each graph is the pooled data from three independent survival experiments. Statistical analyses compare phagocyte impaired with non-impaired condition. (G) Comparison table displaying Fisher’s exact test comparisons, assessing the significant difference of final survival outcomes.

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Discussion

In the present study, we generated a new localized infection model to probe the role of the inflammasome in host defense to bacterial infection. We established that localized L. monocytogenes infection model requires the classic virulence factors listerialysin O and ActA for pathogenesis. Taking advantage of the genetic tractability of L. monocytogenes, we also showed that canonical inflammasome signalling during the response to Lm-pyro, a strain of L. monocytogenes engineered to ectopically secrete flagellin into the cytosol of host cells, leads to attenuation of virulence. Macrophages were specifically recruited in response to inflammasome activation, and blocking macrophage development rescued the virulence of Lm-pyro infections to WT levels. Neutrophils, on the other hand, were recruited to both WT and Lm-pyro infections. Although neutrophils were required for controlling both infections, functionally defective neutrophils did not rescue the virulence defect of Lm-pyro relative to infection with WT L. monocytogenes.

Listeria monocytogenes has a wide natural host range in mammals but has also been used as a model pathogen in alternative hosts including Danio rerio (zebrafish) (Menudier et al., 1996; Levraud et al., 2009; Shan et al., 2015), Drosophila melanogaster (fruit fly) (Mansfield et al., 2003) and Galleria mellonella (greater wax moth) (Mukherjee et al., 2013). In our localized infections, the classic virulence factors listerialysin O and ActA were required for virulence, in agreement with systemic infection of zebrafish larvae with L. monocytogenes (Levraud et al., 2009), and other invertebrate models (Mansfield et al., 2003; Mukherjee et al., 2013). At high enough doses, ΔactA bacteria were virulent in our system, mirroring mouse models of listeriosis, where ΔactA mutants are lethal at an LD$_{50}$ roughly 1000-fold above WT (Goossens and Milon, 1992). This further supports the idea that even within non-natural hosts, L. monocytogenes’ ability to successfully complete the intracellular lifecycle within host cells is critical for virulence.

Having demonstrated that our localized infection model requires the L. monocytogenes intracellular life cycle for virulence, we sought to more fully establish the zebrafish larvae as a model system for examining inflammasome-mediated responses. Our studies show that zebrafish are able to sense cytosolic flagellin and activate an inflammasome complex. In mice, flagellin is sensed by the Nlrc4 inflammasome in conjunction with the adaptor proteins NAIP5/6 (Kofoed and Vance, 2011; Zhao et al., 2011). Both Lm-pyro and S. typhimurium that overexpresses flagellin are severely attenuated in mice dependent on this sensor (Miao et al., 2010a; Sauer et al., 2011). Humans have only one NAIP protein that does not recognize flagellin but rather senses type 3 secretion needle proteins (Yang et al., 2013). Mice have six NAIP proteins, some of which sense flagellin while others sense type 3 secretion inner rod proteins (Miao et al., 2010b; Kofoed and Vance, 2011; Zhao et al., 2011; Rayamajhi et al., 2013). A large family of NLR family genes has been identified in the zebrafish genome; however, the presence of NAIP in zebrafish was unclear (Laing et al., 2008). Still, a number of related baculoviral inhibitor of apoptosis containing family proteins are present. Although our work does not identify specific NAIP or Nlrc4 homologs, it does provide strong evidence that, as in humans and mice, this signalling axis is present in the zebrafish immune system and is important for host defense against infection. Future studies that are able to match inflammasome ligands to their cognate sensors within the zebrafish immune system will strengthen the power of this system.

Upon ligand sensing, the multi-protein inflammasome complex must form to achieve inflammatory outputs. Other studies in zebrafish larvae have also shown ASC-dependent effects and caspase A-dependent effects during the response to inflammasome-associated stimuli including cholesterol-induced gut inflammation (Progatzky et al., 2014), the response to viral infection (Varela et al., 2014) and tissue injury (Ogryzko et al., 2013). Using an ASC overexpression assay, we showed that ASC oligomerization increased only in response to Lm-pyro, but not to cytosolic bacteria alone. We also used a translation-blocking morpholino to demonstrate that the attenuated virulence of Lm-pyro was dependent on caspase A, the zebrafish caspase-1 homolog (Masumoto et al., 2003). We were also able to witness downstream outcomes of inflammasome activation, including the entire process of macrophage pyroptosis in vivo at high temporal resolution. Hallmarks of pyroptosis have been seen in the zebrafish during the response to S. flexneri and spring viremia of carp virus infection (Mostowy et al., 2013; Varela et al., 2014). Although these studies were the first to show pyroptosis occurring in vivo, our approach expands on these findings. Through the use of fluorescently labelled histones, we were able to track nuclear morphology through the complete process of pyroptosis. Furthermore, increased temporal and spatial resolution allowed us to track cells through the process of pyroptosis, observing intermediate stages of death. Finally, to our knowledge, our work represents the first real-time imaging of tissue-resident macrophages undergoing pyroptosis. Thus, our data demonstrate full conservation of the canonical inflammasome in zebrafish: from ligand sensing by a conserved sensor, oligomerization of the core inflammasome component ASC, subsequent caspase-1 activation and downstream outputs such as pyroptosis and control of bacterial infection.

Although it has been reported that wild-type L. monocytogenes activate the NLRP3, NLRC4 and AIM2 inflammasomes in cell culture (Wu et al., 2010), the in vivo
relevance of these findings has been controversial (Sauer et al., 2011). One early study suggested that caspase-1-deficient mice are more susceptible to L. monocytogenes infection (Tsuji et al., 2004); however, other studies have found only minimal inflammasome-dependent phenotypes, both in alternative cell culture methods and in animal models (Sauer et al., 2011). Indeed, other pathogens such as S. typhimurium can activate the inflammasome experimentally but have evolved elegant mechanisms to avoid doing so during in vivo infection (Miao et al., 2010b). We found no increase in macrophage recruitment or ASC speck formation over PBS inoculation following WT L. monocytogenes infection. Furthermore, there was no effect on the virulence of WT L. monocytogenes when caspase-1 was depleted, also consistent with some previous findings in mice (Sauer et al., 2011). Therefore, our findings support the idea that L. monocytogenes, as well as other exquisitely evolved intracellular pathogens (Miao et al., 2010b), largely avoids triggering the inflammasome to promote its virulence. However, a role for the inflammasome during WT L. monocytogenes infection cannot be entirely excluded.

Few studies have examined the role of innate immune cells during the response to inflammasome activation in vivo. Macrophages have been used for much of the in vitro characterization of inflammasome activation, although dendritic cells have also been examined (Storek and Monack, 2015). Lm-pyro elicits an early recruitment of LysM-positive cells – monocytes or granulocytes – to the spleen whose numbers quickly drop (Williams et al., 2013). Studies in the mouse have also suggested a role for macrophages in carrying out inflammasome responses in vivo as S. typhimurium engineered to overexpress flagellin is found within macrophages undergoing pyroptosis (Miao et al., 2010a). In support of these studies, we found that macrophages were specifically recruited to inflammasome activation induced by Lm-pyro, and we observed infected macrophages undergoing pyroptosis in real time. Surprisingly, WT infection did not induce macrophage recruitment above mock inoculation. However, this is potentially due to the wound incurred during inoculation, as macrophage number in the HBV following PBS inoculation was higher than reported in equivalent stage embryos during normal development (Herbmotel et al., 2001). Analysis of the kinetics of macrophage recruitment in this model will further clarify their role in the inflammatory response to L. monocytogenes infection; however, our findings suggest that inflammasome activation rapidly increases macrophage recruitment. Larvae lacking macrophages were equally susceptible to WT and Lm-pyro infection. We witnessed macrophage pyroptosis during Lm-pyro infection but were unable to witness pyroptosis during WT infection despite increased bacterial burden. Our findings, combined with previous reports (Miao et al., 2010a; Sauer et al., 2011), suggest that macrophage pyroptosis is likely to be a critical inflammasome output for bacterial clearance after inflammasome activation. Whether macrophages function as sentinel cells that undergo pyroptosis to recruit effector cells or as direct antimicrobial mediators remains unknown.

Following macrophage death in mice, S. typhimurium overexpressing flagellin are taken up and killed by neutrophils in an NADPH oxidase-dependent manner (Miao et al., 2010b), suggesting that neutrophils play an important role during the response to inflammasome activation. Neutrophil depletion did not, however, rescue the virulence defect of Lm-pyro in competitive index infections with WT L. monocytogenes (Sauer et al., 2011). In our experiments, neutrophils were recruited in equal number to WT and Lm-pyro infection. Furthermore, functionally defective neutrophils increased host susceptibility to both WT and Lm-pyro infection without removing the virulence defect of Lm-pyro. Therefore, while neutrophils were clearly important for controlling infection downstream of inflammasome activation, they were not required for inflammasome-dependent control of infection. Historically, the role of neutrophils in controlling L. monocytogenes has been unclear. Neutrophils have been deemed dispensable during L. monocytogenes infection because Ly6G antibody-mediated depletion of neutrophils did not increase susceptibility to infection (Shi et al., 2011), but antibodies were given to mice after inoculation with bacteria. When applied before infection, Ly6G antibody-mediated depletion does increase bacterial burden and host susceptibility as inoculating doses approached the LD$_{50}$ (Carr et al., 2011). Listeria monocytogenes strains that are cytotoxic independent of the inflammasome are killed by neutrophils and rescued by neutrophil depletion, suggesting that L. monocytogenes is highly susceptible to killing by neutrophils (Glomski et al., 2003). In a recent study, Sox2 was identified as a neutrophil-specific, cytosolic sensor of bacterial DNA that drives innate immune responses against L. monocytogenes (Xia et al., 2015). We found recruitment of neutrophils to the site of infection with L. monocytogenes, and a general increase in host susceptibility when neutrophil function was impaired. Our data therefore support an underappreciated role for neutrophils in the clearance of L. monocytogenes. Although this response was not specific to the inflammasome, understanding the role of neutrophils in response to L. monocytogenes infection is an ongoing focus of the lab.

Since its initial characterization approximately 13 years ago (Martinon et al., 2002), the study of the inflammasome has quickly become a burgeoning field (Kanneganti, 2015). During this time, impressive advances have been made in understanding the molecular mechanisms of how the inflammasome senses pathogens and danger signals.
to drive inflammatory outputs. The interaction between pathogens and host cells during infection is complex, and the study of inflammasome signalling in vivo has been difficult. Model systems such as ours that display conserved inflammasome signalling and outputs are genetically tractable and are amenable to techniques such as high-powered imaging will advance our understanding of how inflammasome activation and bacterial clearance are carried out by complex cell populations in vivo.

Experimental procedures

Zebrafish husbandry

All zebrafish used in this study were on the AB background (originally received from the Zebrafish International Research Center). Previously published transgenic zebrafish lines Tg(mpeg1:dendra2) (Harvie et al., 2013), Tg(lyz:eGFP) (Hall et al., 2007), Tg(actA:mpx:mCherry-2A-rac2-d57n) and Tg(mpx:mCherry-2A-rac2-d57n) (Deng et al., 2011) were used in this study. Adult zebrafish were maintained on a 14 h:10 h light/dark schedule. Upon fertilization, embryos were transferred into E3 buffer and maintained at 28.5°C. Before experimental manipulation, embryos were anaesthetised using E3 buffer containing a final concentration of 0.2 mg/ml Tricaine (ethyl 3-aminobenzoate; Sigma Aldrich). All adult and larval zebrafish handling was carried out in full compliance of National Institute of Health guidelines and approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee.

Generation of transgenic Tg(mpeg1:mCherry-histone2b) zebrafish

mCherry-histone 2B (von Dassow et al., 2009) flanked by XbaI and BamHI sites was cloned into the backbone vector with the mpeg1 promoter (Harvie et al., 2013) and minimal tol2 elements (Urasaki et al., 2006). Plasmid DNA and transposase mRNA were injected into early stage embryos, ∼30–60 mpf.

Generation of L. monocytogenes fluorescent reporter strains

To facilitate imaging ability, we constructed a set of fluorophore expressing strains of L. monocytogenes where fluorescence is driven either constitutively or downstream of the actA promoter. mCherry was codon optimized and synthesized as a gBlock gene fragment (Integrated DNA Technologies) flanked with EagI and Sall sites. The constitutive expression plasmid pPL2-GFP (Shen and Higgins, 2005) was digested with EagI and Sall, and EagI-mCherry-Sall was ligated in, generating a constitutive mCherry expression plasmid. From this plasmid, codon-optimized mCherry was PCR amplified with Clal and Sall flanking sites (forward primer TATTATcatgtATGTTAGTAAAGGTGAAGATAAT- ATG; reverse primer TTATAAgtcacTTATTTA- TAAATTCATCCACCTGACTATGA) and ligated into Clal-digested and Sall-digested actA promoter-driven RFP expression plasmid (Waite et al., 2011). GFP expression driven by the actA promoter was constructed in an identical manner (forward primer TATTATcatgtATGAGGAAAGGAGAACTTTCCACTGG; reverse primer TATTATgcacctATTTGTATAGTTCATTCA- TCCATGCCCATGTGAATC). In addition to these pPL2 constructs, constitutive expression vectors were shuttled into pPL1 and pPL2e; actA-driven expression vectors were shuttled into pPL2e. All primers were purchased from Integrated DNA Technologies.

Bacterial culture and preparation

All L. monocytogenes strains used were on the 10 403 s parental background. Listeria monocytogenes were grown in brain–heart infusion (BHI) media. For infection experiments, cultures were grown statically in BHI overnight at 30°C to reach stationary phase. Bacteria were subcultured for ∼1.5 h in fresh BHI (4:1, BHI:culture) to achieve growth to mid-logarithmic phase (OD600 ≈ 0.5). Bacteria were washed three times in PBS and resuspended to desired concentrations in PBS containing 10% glycerol and 2% PVP-40 (polyvinylpyrrolidine, Sigma Aldrich), to avoid bacterial clumping, and 0.3% phenol red for visualizing successful injections.

Injection of bacteria into zebrafish larvae

Manual dechorionation was performed using forceps at ∼36 hpf. E3 media containing 3% agarose was polymerized in a Petri dish on a shallow angle to make an injection ramp. At 48 hpf, anaesthetised larvae were placed in the deep end of the injection ramp. For injections, batches of larvae were pulled to the shallow end of the ramp with a glass rod, such that E3 media with Tricaine just covered the larvae. One nanoliter of bacterial solution was then directly injected into the HBV of embryos. Successful injections were monitored for the first minute post inoculation to verify that there was no leakage at the site of injection. Larvae were then transferred into 96-well flat bottom plates with fresh E3 lacking Tricaine to recover. For survival tracking, lack of a heartbeat was the readout for death.

CFU enumeration from zebrafish larvae

Larvae were injected as described previously. At the indicated time points, larvae were homogenized by shearing stress in a bead beater, without beads added, in 200 μl of 1% saponin (Sigma) in PBS, to lyse host cells but leave bacteria intact; 50 μl of homogenate was plated and
counted on LB agar with streptomycin (200 μg/ml; Sigma) using the Autoplate Spiral Plating System and QCount Colony Counter (Advanced Instruments).

**mRNA and morpholino injections**

GFP-ASC mRNA was cloned into the pCS2+ vector. pCS2+ was digested with BamHI and XhoI (both Promega). BamHI-GFP-EcoRI was ligated to EcoRI-ASC-XhoI, which was then ligated into the backbone. mRNA was synthesized in vitro using the mMessage M7 kit (Ambion) and cleaned up using the RNeasy mini kit (Qiagen). Morpholino oligonucleotides (Gene Tools) were stored in 1 mM stock concentrations at room temperature. All mRNA and morpholino oligonucleotides were injected into early stage embryos, ~30–60 mpf, in a volume of 3 nl [Pu.1, 500 μM for full knockdown of macrophages and neutrophils; 250 μM for macrophage only knockdown (Rhodes et al., 2005); Irf8, 400 μM (Li et al., 2011); CaspA, 250 μM (Masumoto et al., 2003); ASC–GFP mRNA, 100 ng/μl]. For experiments using morpholinos to target the knockdown of phagocyte populations, experiments were carried out in the transgenic lines listed previously, and phagocyte populations were verified immediately prior to the beginning of experiments by fluorescence microscopy. Phagocyte populations typically returned at 4 dpf. For caspase A knockdown, protein level was verified at 2 and 4 dpf by western blot (Fig. S3).

**Immunoblotting**

Whole protein lysates were taken from 50 larvae per sample for any given condition; 60 μg of lysate was loaded and run on an SDS-PAGE gel, transferred to nitrocellulose and immunoblotted for caspase A using a rabbit anti-zebrafish caspase A antibody (Anaspec). Actin was also immunoblotted as a loading control using a mouse anti-actin AC-15 antibody (Sigma). Blotting was imaged and quantified using an infrared Odyssey imaging system (LI-COR).

**Fluorescence microscopy**

Anaesthetised or 4% paraformaldehyde-fixed embryos were imaged on custom-made glass-bottom imaging dishes. For live time-lapse imaging, anaesthetised larvae were stabilized by mounting in 1% low-melt agarose with a final concentration of 0.2 mg/ml Tricaine as described in the preceding text. Still image and time-lapse microscopy was performed via zoomscope microscopy (EMSS/SyCoP3; Zeiss; 1X PlanNeoFluar Z objective), laser scanning confocal microscopy (Fluoview FV1000; Olympus; 0.75 NA/20X objective) or spinning disk confocal microscopy (AxioObserver.Z1; Zeiss; 0.80 NA/20X or 1.30 NA/63X objective). Confocal images and movies used in figures were run through de-speckling noise reduction in Fiji (Schindelin et al., 2012).

**Statistical analyses**

For survival curves, three independent experiments were performed. Results were pooled and analysed by Cox proportional hazard regression analysis, with experimental conditions included as group variables. For CFU enumeration, three independent replicate experiments were conducted. CFU enumeration levels were compared between experimental conditions using analysis of variance. The results were summarized in terms of least squares adjusted means and standard errors. Graphical representation shows each individual data point. For ASC speck and leukocyte quantification, three independent experiments were performed. Results were analysed by one-way ordinary analysis of variance with a Tukey’s multiple comparisons test. For comparing differences of final survival outcomes, results were analysed with Fisher’s exact test. For all tests, a P-value of < 0.05 was used to define statistical significance. Statistical analyses and graphical representations were all performed in R version 3 (R Development Core Team, 2013) or GraphPad Prism version 6.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Fig. S1. Mock inoculated larvae do not show signs of dissemination. Representative images of control larvae mock inoculated with PBS. Larvae were imaged daily via zoomscope microscopy.

Fig. S2. Lm-pyro growth in larvae is attenuated. Quantification of CFU per larvae. At 48 hpi fish were homogenized into 1% saponin, plated on LB-Strep and counted the following day. Statistics displayed represent pooled data from three independent experiments and was analyzed by a least squares means analysis. Individual larval burden from each experiment is shown, as indicated by symbols.

Fig. S3. Caspase A morpholino knockdown is transient. Following morpholino knockdown, 50 larvae were pooled and proteins extracted at 2dpf, the time of infection, and 4dpf, the time the morpholino has worn off.

Fig. S4. Low dose Pu.1 morpholino mediated macrophage depletion eliminates Lm-pyro’s virulence defect. Survival of low dose Pu.1 or control morphants following 100 CFU infection with WT (A) or Lm-pyro (B). Low dose Pu.1 morphants lack macrophages but retain neutrophils. Graph is the pooled data from two independent survival experiments. Statistical analyses compare phagocyte impaired to non-impaired condition. (C) Comparison table displaying Fisher’s exact test comparisons, assessing the significant difference of final survival outcomes.

Fig. S5. Δhly are only able to cause disease in the total absence of phagocytes. Survival of (A) Irf8 morphant larvae lacking macrophage development, (B) Tg(mpx-Rac2 D57N) larvae with functionally defective neutrophils, and (C) Pu.1 morphant larvae lacking both macrophage and neutrophil development following infection with Δhly with 100 CFU. Each graph is the pooled data from three independent survival experiments. Statistical analyses compare phagocyte impaired to non-impaired condition.