Zebrafish tissue injury causes upregulation of interleukin-1 and caspase-dependent amplification of the inflammatory response

Nikolay V. Ogryzko1,2, Emily E. Hoggett1, Sara Solaymani-Kohal1, Simon Tazzyman3, Timothy J. A. Chico1,2, Stephen A. Renshaw1,4,* and Heather L. Wilson2,*

ABSTRACT
Interleukin-1 (IL-1), the ‘gatekeeper’ of inflammation, is the apical cytokine in a signalling cascade that drives the early response to injury or infection. Expression, processing and secretion of IL-1 are tightly controlled, and dysregulated IL-1 signalling has been implicated in a number of pathologies ranging from atherosclerosis to complications of infection. Our understanding of these processes comes from in vitro monocytic cell culture models as lines or primary isolates, in which a range and spectra of IL-1 secretion mechanisms have been described. We therefore investigated whether zebrafish embryos provide a suitable in vivo model for studying IL-1-mediated inflammation. Structurally, zebrafish IL-1β shares a β-sheet-rich trefoil structure with its human counterpart. Functionally, leucocyte expression of IL-1β was detectable only following injury, which activated leukocytes throughout zebrafish embryos. Migration of macrophages and neutrophils was attenuated by inhibitors of either caspase-1 or P2X7, which similarly inhibited the activation of NF-κB at the site of injury. Zebrafish offer a new and versatile model to study the IL-1β pathway in inflammatory disease and should offer unique insights into IL-1 biology in vivo.

KEY WORDS: Inflammation, Interleukin-1, Zebrafish

INTRODUCTION
Interleukin-1 (IL-1) is an important activator of inflammation. Dysregulated IL-1β function has been described in the pathology of a number of auto- or chronic inflammatory diseases, leading to this cytokine being described as the ‘gatekeeper’ of inflammation (Dinarello, 2011c). Despite its key role in initiating inflammation, many aspects of IL-1β activity remain poorly understood, in part due to its unconventional secretion mechanism but also due to the complex array of proteins involved in its activation (Schroder and Tschopp, 2010). In macrophages, IL-1β transcription is induced and IL-1β mRNA stabilised following detection of pathogen-derived Toll-like receptor (TLR) ligands (Butler et al., 2004). Processing and secretion of inactive pro-IL-1β requires a secondary stimulus, the best-studied being ATP, a key damage-associated molecular pattern (DAMP) (Lister et al., 2007). ATP activates the P2X7 receptor, resulting in rapid assembly of the inflammasome, an IL-1β-activation and -processing platform. IL-1β is thereby processed into its active form, with concomitant secretion (Rathinam et al., 2012).

IL-1β secretion is proposed to occur via a number of different mechanisms, ranging from lysosomal and microvesicular to pyroptotic, dependent on the strength of the inflammatory stimulus and the cell type in question (López-Castejón and Brough, 2011). Our understanding of these mechanisms is built predominantly on cell-culture studies of various cell types, although, additionally, animal models have been used to evaluate the requirement of specific proteins in IL-1β-mediated inflammation (Horai et al., 2000; Kuida et al., 1995). However, it has not been possible to combine the key features of such models to determine, in an intact organism, the vesicular component of IL-1β secretion and how IL-1β is specifically targeted to effector cells.

The evolutionary origins of innate immunity predate the first vertebrates, with cytokine signalling pathways detected in simple organisms (Beck and Habicht, 1991). Much of the complexity of the human immune system is well established in ray-finned fish, making zebrafish a tractable model to study innate immunity and inflammation in vivo (Renshaw and Trede, 2012). Importantly, zebrafish possess orthologues of the known components of IL-1β signalling, including TLRs, NF-κB, IL-1 receptors I and II, and P2X7 (Huising et al., 2004; López-Castejón et al., 2007; Stein et al., 2007). Here, we use the zebrafish model, Danio rerio, to study IL-1β signalling, and demonstrate its value as a model of IL-1β biology through characterising the induction of IL-1β in response to injury and the attenuation of inflammatory signals through the use of inhibitors of the IL-1β pathway.

RESULTS
Zebrafish and human IL-1β are structurally conserved
Previous studies describing the use of the zebrafish as a tool for investigating IL-1β have highlighted the differences between zebrafish and human IL-1β; specifically, the lack of a conserved caspase-1 cleavage site on the zebrafish homologue (Bird et al., 2002). The two proteins share only 27% amino acid identity, but we observed greater identity (31%) in the C-terminal domain, representing the mature cytokine. We submitted the zebrafish IL-1β sequence to the Phyre structural prediction server (Kelley and Sternberg, 2009) generating a β-sheet-rich trefoil structure closely matching that of the mature human cytokine (supplementary material Fig. S1).

IL-1β message is induced in leukocytes throughout the zebrafish in response to injury
Having determined structural conservation of zebrafish IL-1β to its mammalian orthologues, we studied its expression and regulation in...
IL-1β pathway inhibitors reduce leukocyte recruitment to the site of injury

The early enhancement of IL-1β mRNA in inflammatory leukocytes suggests a role in initiating the inflammatory response in the zebrafish tailfin injury model. By analogy to mammalian IL-1β processing and release, we tested the involvement of the inflammasome in IL-1β processing by using inhibitors. The recruitment of innate immune cells is a key feature of the inflammatory response. We therefore developed an assay based on macrophage and neutrophil recruitment following tissue injury. Using an mpeg1:Gal4/UAS:Kaede reporter line we measured a reduction in the number of macrophages recruited to the site of injury after 6 hours when treated either with the caspase-1 inhibitor YVAD or with the P2X7 antagonists KN62 or BBG (Fig. 2A,B). The pan-caspase inhibitor qVD did not attenuate macrophage recruitment, suggesting perhaps that the effect on inflammasome inhibition might be balanced in vivo by suppression of normal apoptosis. Neutrophil recruitment was similarly attenuated by YVAD, Brilliant Blue G and KN62, whereas qVD had no significant effect upon this response (Fig. 2C,D). The effect of IL-1β and P2X7 on macrophage recruitment was confirmed by morpholino knockdown of IL-1β (Roca et al., 2008) and P2X7 (Chang et al., 2011) (supplementary material Fig. S2).

The caspase-1 inhibitor YVAD reduces the activity of NF-κB at the site of injury

Because IL-1β acts on target cells via the IL-1 receptor activating NF-κB, we tested whether NF-κB signalling was sensitive to treatment with inflammasome inhibitors. We generated a transgenic line using the NF-κB reporter constructs previously described (Kanter et al., 2011) and confirmed that, in this line, NF-κB was activated in response to injury (Fig. 3A,B). Using this line, we developed an inflammation assay based on quantification of the mean fluorescent intensity at the site of tailfin transection and observed that, when treated with the caspase-1 inhibitor YVAD, the activation of NF-κB at the site of injury was significantly reduced (Fig. 3C); this effect was not seen with the pan-caspase inhibitor qVD. P2X7 antagonists showed a trend towards reduced NF-κB activation, although this effect was not statistically significant (data not shown).

The expression of IL-1β in response to tailfin injury is reduced in response to treatment with IL-1β pathway inhibitors

Using the assay developed by de Oliveira et al. (de Oliveira et al., 2013), we investigated the expression of the same panel of inflammatory genes in response to tail injury and the effect of mammalian P2X7 and caspase-1 inhibitors BBG, KN62 and YVAD on expression of these genes. We tested whether zebrafish IL-1β induces its own downstream transcription, as described in numerous mammalian models (Dinarello, 2011a; Dinarello et al., 1987; Granowitz et al., 1992). We were able to detect the same induction of IL-1β, IL-8a and ptgs2b following injury as previously reported (de Oliveira et al., 2013) (supplementary material Fig. S3A-C). In addition, we measured a significant decrease in the expression of IL-1β but not IL-8 or ptgs2b in response to treatment with YVAD, BBG or KN62 at 2 hpi (Fig. 4A). Taken together, these results
demonstrate that the zebrafish inflammatory response is sensitive to inhibitors of inflammasome activity.

**DISCUSSION**

The investigation of IL-1β pathway biology has hitherto relied primarily on *in vitro* cell-culture techniques. Observing these processes in a living model could yield valuable insights into the release mechanisms of this cytokine as the IL-1β-producing cells interact with the cellular microenvironment in an inflammatory lesion. These complex interactions are not accessible to conventional monoculture techniques. Zebrafish are an ideal model for *in vivo* observation of the vertebrate immune system (Henry et al., 2013), although investigation of IL-1β in zebrafish has been confounded by concerns relating to the conservation of IL-1β processing in the zebrafish (Angosto et al., 2012). Recent publications have described the cleavage of zebrafish IL-1β by the caspase-1 homologues caspase-A and caspase-B (Vojtech et al., 2012), suggesting conservation of IL-1β processing. The functional significance of caspase activity in inflammation in zebrafish and the implications for IL-1β function have not previously been explored. Here, we describe the temporal induction of the IL-1β message in response to injury and characterise this response in terms of the innate immune cell lineages responsible. Comparable to mammalian data (Morris et al., 2005), the primary IL-1β-expressing cell in the inflammatory response following tail transection is the macrophage, although neutrophils were also implicated.

The zebrafish innate immune response was reduced in magnitude following treatment with inhibitors of P2X7, a key activator of IL-1β processing in mammalian systems (Ward et al., 2010). These data are supported by the reduction in both cellular and transcriptional
readouts of inflammation following treatment with the caspase-1 inhibitor YVAD, demonstrating a conserved role for this enzyme and for P2X7 in zebrafish immunity. Because apoptosis is widely regarded as an anti-inflammatory event (Fadok et al., 1992), it might be expected that inhibition of apoptosis with qVD leads to a reduction in apoptotic cells at the wound site, resulting in a net increase in the inflammatory burden at the site of injury, masking any underlying inhibition of IL-1β production. We also describe the reduction of IL-1β transcription following tissue injury by treatment with inhibitors of the IL-1 pathway, indicating that IL-1 amplifies the inflammatory response through further downstream IL-1 induction, similar to mammalian models. We were unable to detect the same reduction in expression of IL-8 and ptgs2b in response to injury, suggesting that both IL-1-dependent and IL-1-independent pathways regulate proinflammatory gene transcriptions in vivo.

The structural conservation of IL-1β between humans and zebrafish, alongside the induction of IL-1β in leukocytes following injury and the ability of inhibitors of mammalian caspase-1 and P2X7 to attenuate inflammation support the functional conservation of IL-1β signalling between mammalian and teleost lineages. Here, we demonstrate the versatility and unique advantages of the zebrafish model in studying the IL-1β pathway in vivo. The use of transgenic zebrafish lines to directly visualise inflammation offers exciting future opportunities to observe IL-1β function in vivo.

**MATERIALS AND METHODS**

**Transgenic line generation and maintenance**

Tg(mpeg1:Gal4.VP-16)s256 and Tg(pNF-κB:EGFP)s235 were generated as previously described (Ellett et al., 2011; Kanther et al., 2011). Tg(mpx:EGFP)i114 zebrafish were used to study neutrophils during inflammation (Renshaw et al., 2006). Zebrafish strains were maintained on a 14:10-hour light/dark cycle at 28°C as described (Nüsslein-Volhard and Dahm, 2002) in UK Home Office approved facilities in the MRC Centre for Developmental and Biomedical Genetics aquaria at the University of Sheffield. Tg(mpeg1:Gal4.VP-16)s256 was crossed to Tg(UAS:Kaede)s1999t to enable visualisation of macrophages.

**Expression analysis**

Zebrafish IL-1β (GenBank accession: AY340959.1) was cloned using 20 bp complementary primers (drIL-1βF: 5′-ATGGCATGCGGGCAATATGA-3′, drIL-1βR: 5′-CTAGATGCGCACTTTATCCT-3′) into dual promoter TOPO TA (Life Technologies, UK). Digoxigenin-labelled sense and antisense RNA probes were synthesised (Roche Diagnostics, UK) and WISH performed as previously described (Nüsslein-Volhard and Dahm, 2002). For antibody, TSA and FISH staining, the protocol was adapted from Prajsnar et al. (Prajsnar et al., 2012). Larvae were fixed in 4% w/v paraformaldehyde in PBS overnight at 4°C. Fixed larvae were briefly washed twice in PBS. Larvae were incubated in 1:50 TSA Cyanine5 (Perkin Elmer, UK) without PBS overnight at 4°C. Larvae were re-fixed for 20 minutes and endogenous peroxidase activity quenched with a 1-hour incubation in 0.3% H2O2. Larvae were probed for NF-κB B activation in response to injury. (A) Fluorescence photomicrograph of pNF-κB:EGFP embryos following tailfin transection at 1 hpi (i) and 8 hpi (ii) indicating the region quantified (square bracket). Scale bars: 100 μm. (B) There is a 2.4-fold increase in EGFP fluorescence in response to injury, quantifiable as average fluorescent intensity across the transection site (indicated in A). ****P<0.0001 by t-test. (C) Embryos treated with 50 μM YVAD show a reduction in EGFP fluorescence at 8 hpi compared with DMSO-treated controls and embryos treated with qVD. **P<0.01 by one-way ANOVA with Dunnett’s post-test. n=30 performed as three independent experiments.
**3D-structure prediction**

The 3D model of zebrafish IL-1β was generated by submitting the protein sequence (accession: NP_998009.1) to the Phyre Server (Kelley and Sternberg, 2009).

**Treatment with IL-1β-pathway inhibitors**

Embryos were pre-treated with compounds as described, for 2 hours before injury. Embryos were injured by tail transection at the distal-most point of the pigment gap at 3 dpf as described (Renshaw et al., 2006) and neutrophil or macrophage numbers at the site of injury counted at 6 hpi. Tg(pNF-kB:EGFP)sh235 embryos were injured as described, mounted in 0.75% LMP agarose at 8 hpi and imaged with a 10× NA 0.3 objective using a TE-2000U microscope (Nikon) and an Orca-AG camera (Hamamatsu, Japan) using Velocity™. The mean fluorescent intensity was measured between the line of injury and proximal end of the pigment gap, ignoring neutromasts, using Velocity™ for quantification.

**Analysis of gene expression**

Leukocyte-specific mRNA was obtained by dissociating mpeg:gal4;UAS:Kaede or mpx:GFP embryos in PBS with 0.4% trypsin on an orbital shaker for 1 hour with frequent trituration using a pipette. Cells were sorted by FACS analysis and RNA extracted using an RNA Isolate II micro kit (Bioline, UK). Tail-tissue RNA was obtained as described (de Oliveira et al., 2013) using Superscript 2 (Invitrogen). qRT-PCR was performed on a CFX96 detection system (Bio-Rad, UK) using Dyano Flash SYBR green mastermix (Thermo Scientific, UK) using primers described in de Oliveira et al. (2013).

**Statistical analysis**

Data were analysed using Prism 6 (Graphpad, USA). Data shown are mean ± s.e.m. using multiple t-tests with a Bonferroni correction or one-way ANOVA with Dunnett’s post-test.

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**Competing interests**

The authors declare no competing financial interests.

**Author contributions**

H.L.W., S.A.R. and N.V.O. conceived and designed the experiments. N.V.O. provided reagents. N.V.O., S.A.R. and H.L.W. wrote the paper.

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**Supplementary material**

Supplementary material available online at http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.013029/-/DC1

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