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The inflammatory bowel disease (IBD) susceptibility genes *NOD1* and *NOD2* have conserved anti-bacterial roles in zebrafish

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SUMMARY

Inflammatory bowel disease (IBD), in the form of Crohn's disease (CD) or ulcerative colitis (UC), is a debilitating chronic immune disorder of the intestine. A complex etiology resulting from dysfunctional interactions between the intestinal immune system and its microflora, influenced by host genetic susceptibility, makes disease modeling challenging. Mutations in *NOD2* have the highest disease-specific risk association for CD, and a related gene, *NOD1*, is associated with UC. *NOD1* and *NOD2* encode intracellular bacterial sensor proteins acting as innate immune triggers, and represent promising therapeutic targets. The zebrafish has the potential to aid in modeling genetic and environmental aspects of IBD pathogenesis. Here, we report the characterization of the Nod signaling components in the zebrafish larval intestine. The *nod1* and *nod2* genes are expressed in intestinal epithelial cells and neutrophils together with the Nod signaling pathway genes *ripk2*, *a20*, *aamp*, *cd147*, *centaurin b1*, *erbin* and *grim-19*. Using a zebrafish embryo *Salmonella* infection model, morpholino-mediated depletion of Nod1 or Nod2 reduced the ability of embryos to control systemic infection. Depletion of Nod1 or Nod2 decreased expression of dual oxidase in the intestinal epithelium and impaired the ability of larvae to reduce intracellular bacterial burden. This work highlights the potential use of zebrafish larvae in the study of components of IBD pathogenesis.

INTRODUCTION

The mammalian NOD genes encode proteins that have been implicated in the pathogenesis of immune-mediated diseases, including inflammatory bowel disease (IBD), graft-versus-host disease and uveitis (Hugot et al., 2001; Ogura et al., 2001; Rosenbaum et al., 2003; Holler et al., 2004; Brenmoehl et al., 2007). IBD is thought to arise through aberrant host-microbe interactions involving innate and adaptive immune signaling pathways. Genetic susceptibility loci for the major forms of IBD (Crohn's disease and ulcerative colitis) include components of the innate immune system (Mathew, 2008). The larval zebrafish (*Danio rerio*) is an established model for the investigation of in vivo innate immunity (Trede et al., 2004). *NOD2* (nucleotide oligomerization domain 2) was the first gene linked to IBD (Hugot et al., 2001; Ogura et al., 2001) and is the locus most strongly linked to the disorder. Our laboratory has analyzed the zebrafish NOD orthologs to explore the use of this model in understanding aspects of IBD genetics.

Mammalian NOD proteins share a common domain arrangement of a ligand-binding leucine-rich repeat domain, a central nucleotide oligomerization domain and an effector N-terminal caspase recruitment domain (CARD), with *NOD2* having

two CARDs. NOD proteins function as cytosolic microbial pattern recognition molecules and share many facets of form and function with the ancient family of plant disease resistance proteins (Litman et al., 2005; Ting et al., 2008; Sabbah et al., 2009). Zebrafish orthologs of *NOD1* and *NOD2* have been identified from the zebrafish genome, and it was found that these genes have conserved genetic synteny and the predicted proteins have identical domain structure to those of mammalian proteins (Laing et al., 2008; Chang et al., 2010). However, whether the biological roles of zebrafish and human NODs are similar has not been determined. Studies have explored the genomic evolution and tissue expression of the zebrafish Nod orthologs in adults, but have not investigated expression or function of zebrafish Nods in larvae (Stein et al., 2007; Laing et al., 2008; Chang et al., 2010).

To successfully apply the genetically amenable zebrafish platform to the study of complex immune-mediated disease, a broader understanding of zebrafish immunogenetics is required. Several studies have characterized zebrafish orthologs of many important mammalian cytokines, including members of the interleukin, interferon and tumor necrosis factor cytokine families (Pressley et al., 2005; Clay et al., 2008; Lu et al., 2008; Sieger et al., 2009; Oehlers et al., 2010). However, fewer in vivo studies have been carried out to examine the conservation of pattern recognition molecule function in zebrafish. Investigation into Toll-like receptor 4 (TLR4) signaling in zebrafish has revealed that zebrafish TLR4 functions as a non-lipopolysaccharide-sensing negative regulator of NFκB activation, whereas mammalian TLR4 functions as a lipopolysaccharide-sensing positive regulator of NFκB activation (Fan et al., 2008; Sepulcre et al., 2009; Sullivan et al., 2009). Conversely, undefined signaling through the TLR adaptor molecule MyD88 seems to have a conserved antimicrobial function in zebrafish (van der Sar et al., 2006; Hall et al., 2009). Furthermore, zebrafish MyD88 controls the evolutionarily conserved

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upregulation of intestinal alkaline phosphatase to detoxify lipopolysaccharide (Bates et al., 2007). Although these findings demonstrate some notable differences between mammalian and zebrafish immunity, they also illustrate the potential for in vivo zebrafish studies to produce novel insights into host-microbe interactions.

NOD1 and NOD2 are proposed to share a common signal transduction pathway towards NF κ B activation, by initially converging on a shared adaptor molecule, receptor-interacting protein serine/threonine kinase 2 (RIPK2) (Kobayashi et al., 2002; Kufer, 2008). In addition to effector signaling through RIPK2, mammalian NOD2 has been demonstrated to utilize the mitochondrial protein gene associated with retinoid- and interferon-induced mortality 19 (GRIM-19) for NF κ B activation and bactericidal activity (Barnich et al., 2005). Recent research has also demonstrated the production of reactive oxygen species (ROS), through dual oxidase (DUOX2), following NOD2 activation (Lipinski et al., 2009). Several negative regulators of NOD protein activation have been identified through their direct interaction with NOD proteins. These include angio-associated migratory cell protein (AAMP), CD147, centaurin β 1 and erbin (McDonald et al., 2005; Kufer et al., 2006; Yamamoto-Furusko et al., 2006; Till et al., 2008; Bielig et al., 2009). An indirect negative regulator of NOD and TLR signaling, the ubiquitin-editing enzyme A20, has been identified as an important regulator of intestinal immunity (Abbott et al., 2007; Hutti et al., 2007; Hitotsumatsu et al., 2008; Turer et al., 2008).

Here, to demonstrate the feasibility of using zebrafish to investigate IBD susceptibility gene function, we show that genes encoding multiple components of the Nod signaling pathway are expressed in the zebrafish intestine. We also demonstrate that, like their mammalian orthologs, zebrafish Nod proteins are important for antibacterial immunity.

RESULTS

Cloning of Nod signaling pathway genes and sequence comparison

To investigate the Nod pathway in zebrafish, we identified orthologs acting downstream of Nod1 and Nod2. We identified the predicted sequence *LOC564497* (http://www.ensembl.org/Danio_rerio/) as a putative zebrafish *a20* gene. BLAST searching predicted *zgc:153917*, *zgc:152984* and *zgc:73107* (ZFIN) to be putative zebrafish *centaurin b1*, *erbin* and *grim-19* transcripts, respectively. An ortholog of *AAMP* was identified as *zgc:85939*, herein referred to as *aamp* (Bielig et al., 2009). An ortholog of *CD147* was identified as *basigin*, herein referred to as *cd147*, consistent with current nomenclature (Ochrietor et al., 2003; Till et al., 2008).

Sequences encoding *a20*, *centaurin b1*, *erbin* and *grim-19* were isolated by reverse-transcriptase PCR (RT-PCR) from larval cDNA. Nucleotide sequences of recovered transcripts were matched to predicted open reading frames and analyzed using the online NCBI conserved domain search (supplementary material Fig. S1; <http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). The predicted A20 (*LOC564497*) protein contains homologous OTU-like cysteine protease and A20-like zinc finger domains. The predicted Centaurin b1 (*zgc:153917*) protein contains Pleckstrin homology, ArfGap and Ankyrin repeat domains. The predicted Erbin (*zgc:152984*) protein contains leucine-rich repeats and PDZ

domains. The predicted Grim-19 (*zgc:73107*) protein contains transmembrane helices and a coiled-coil domain.

Colocalized expression of Nod genes and genes encoding cognate signaling pathway molecules in the larval gut and neutrophils

Although the zebrafish Nod genes are expressed during development, the spatial expression patterns of the Nod genes have previously only been studied in adults (Laing et al., 2008; Chang et al., 2010; Oehlers et al., 2011b). We used whole-mount in situ hybridization to analyze the expression of genes involved in the Nod signaling pathway in 4- to 5-days post-fertilization (dpf) zebrafish larvae (Fig. 1A). Expression of *nod1* and *nod2* mRNAs was detected in the developing gut. Genes encoding the Nod signaling apparatus, including *ripk2*, *a20*, *aamp*, *cd147*, *centaurin b1*, *erbin* and *grim-19* were also expressed in the intestine. Additionally, expression of *cd147* was observed in the liver. Sense probes were used as controls for all genes and did not result in any staining (data not shown). Sections of these specimens confirmed expression in intestinal epithelial cells (supplementary material Fig. S2). Intestinal expression of the genes involved in the Nod signaling pathway is consistent with that previously described for the putative transporters of the Nod1 and Nod2 ligands *slc15a2* and *slc15a1*, respectively (Verri et al., 2003; Vavricka et al., 2004; Romano et al., 2006; Swaan et al., 2008). To analyze gene expression in zebrafish leukocytes, we performed fluorescence activated cell sorting (FACS) isolation of EGFP-positive neutrophils from Tg(*lyzC:EGFP*)¹¹⁷ larvae. Expression of *nod1*, *nod2* and genes involved in the Nod signaling pathway were detected in larval neutrophils by quantitative RT-PCR (qPCR) (Fig. 1B). With the exception of *grim-19*, an enrichment of transcripts for all genes investigated was observed in larval neutrophils when compared with other larval cells, with measurements for *centb1* and *ripk2* showing statistical significance ($P < 0.05$).

Because human peripheral blood neutrophils express *NOD2* but not *NOD1* (Ekman and Cardell, 2010), and zebrafish larval neutrophils seemed to express both *nod1* and *nod2*, we extended our investigation of Nod-related gene expression to adult zebrafish neutrophils. EGFP-positive neutrophils from Tg(*lyzC:EGFP*)¹¹⁷ adults were collected by FACS isolation from dissected whole kidney marrow, the site of adult hematopoiesis, and from gut, where mature neutrophils reside. The expression of our panel of nine genes in adult samples was compared with EGFP-negative larval cells by qPCR. We observed expression of all nine genes in neutrophils isolated from both the kidney and gut (Fig. 1C). Strikingly, we observed comparatively little expression of *nod2* in intestinal neutrophils when compared with kidney neutrophils.

Knockdown of zebrafish Nod1 or Nod2 does not affect embryonic development

Previous studies of Nod gene expression early in development (Chang et al., 2010) and our own observations of constitutive expression of the Nod signaling apparatus throughout zebrafish development (data not shown) suggested that Nod gene products might have important functions in developmental processes. To investigate a potential role for zebrafish Nod genes in development, we utilized splice-blocking morpholinos for gene-specific knockdown. RT-PCR across the target splice sites was used to examine morpholino efficacy. Morpholino knockdown of Nod1 was

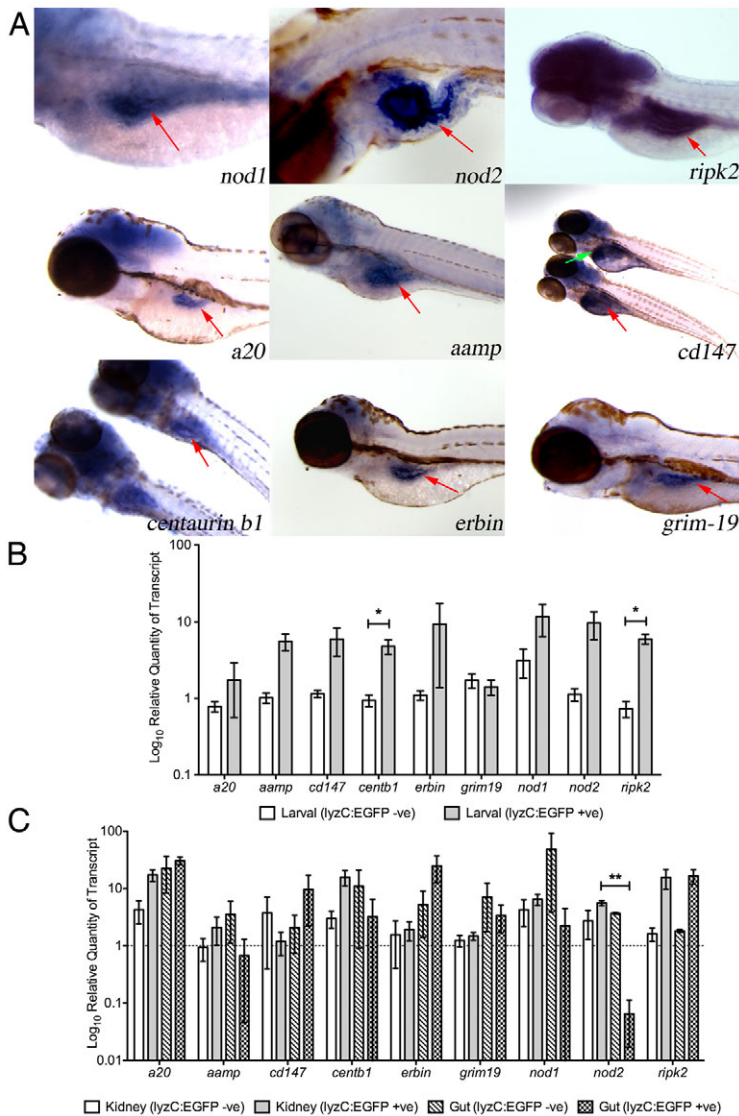


Fig. 1. Expression of genes encoding components of the zebrafish Nod signaling pathway. (A) Detection of gene expression, by whole-mount in situ hybridization, in 4 dpf (*nod1*, *ripk2*, *a20*, *erbin* and *grim-19*) and 5 dpf (*nod2*, *aamp*, *cd147* and *centaurin b1*) zebrafish larvae. All specimens are oriented anterior to the left, posterior to the right. Red arrows indicate the gut. Green arrow indicates liver. (B,C) Differential expression of Nod signaling pathway components in myelomonocytic cells from zebrafish larvae (B) and adults (C). Error bars represent s.e.m.; statistically significant differences of $P < 0.05$ (*) and $P < 0.01$ (**) are marked.

found to be effective until 3 dpf as evidenced by loss of the 647 bp PCR product (supplementary material Fig. S3A). Nod2 knockdown was effective until at least 7 dpf, because the major 598 bp PCR product was lost (supplementary material Fig. S3B). There were no obvious differences in development or survival, in the period up to 10 dpf, between control and morphant larvae. Because our gene expression analyses had demonstrated strong intestinal expression of genes potentially involved in the zebrafish Nod signaling pathway, we utilized a transgenic *ifabp:GFP* line that expresses GFP within intestinal epithelial cells to monitor gut development. Live imaging of transgenic morphant larvae did not reveal any macroscopic changes in intestinal development following Nod1 or Nod2 knockdown (supplementary material Fig. S4A–C).

The zebrafish *nod2* gene undergoes splice variation, similar to that of human *NOD2*

While validating the efficacy of Nod2 knockdown by RT-PCR, a minor splice variant of the *Nod2* transcript was detected in

control samples (supplementary material Fig. S3B). Short splice variants of human *NOD2* have been isolated from intestinal epithelial cells and leukocytes (Rosenstiel et al., 2006; Leung et al., 2007; Rosenstiel et al., 2007). Sequencing of the minor splice product revealed a cryptic splice site in exon 1 that, when translated in silico, resulted in a predicted Nod2 molecule with a single CARD but intact NOD and LRR domain [supplementary material Fig. S3C; NCBI conserved domain search (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>)]. This is similar to the documented mammalian splice variant (Rosenstiel et al., 2006).

Zebrafish Nod1 or Nod2 is required for embryonic resistance to infection by *Salmonella enterica*

To examine the role of zebrafish Nod1 and Nod2 in neutrophil-mediated systemic immunity, we utilized a *Salmonella enterica* yolk sac injection model (Fig. 2A; supplementary material Fig. S5). In embryos infected at 2 dpf, knockdown of Nod1 or Nod2 resulted in significantly decreased survival after infection with *S.*

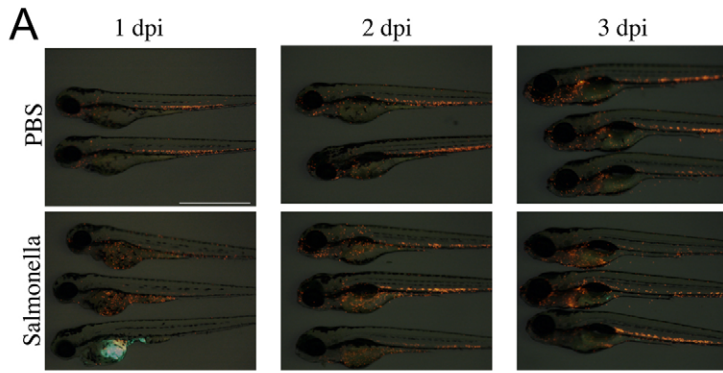
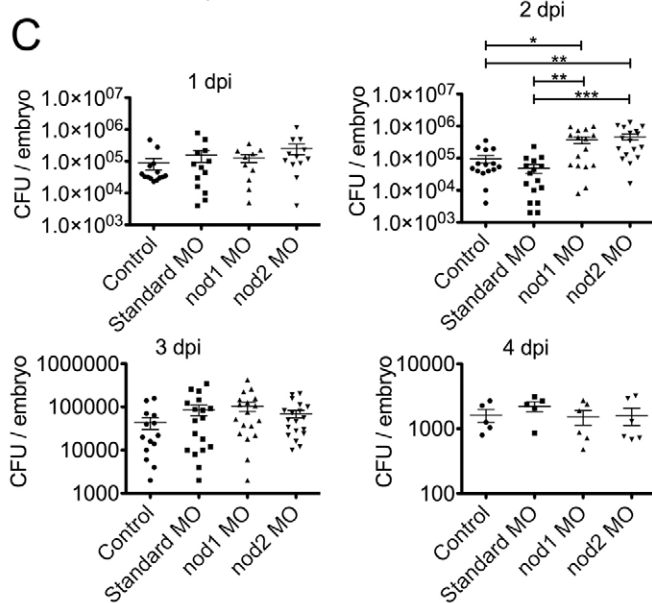
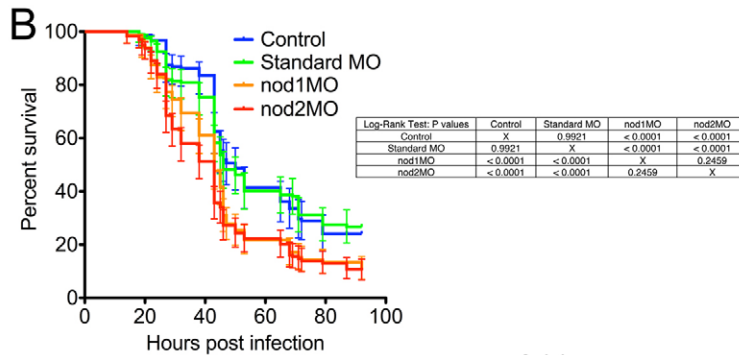


Fig. 2. Nod1 and Nod2 are required for systemic innate immunity in zebrafish embryos. (A) Time course of *Tg(lyzC:dsRed)⁵⁰* embryos injected with PBS or 200 CFU of GFP-tagged *S. enterica* into the yolk sac at 2 dpf. Scale bar: 1 mm. (B) Survival of embryos infected by yolk sac injection. Data represent means of triplicate replicates; 30 embryos per treatment group. All error bars represent a 95% confidence interval. (C) Scatter plots showing *S. enterica* CFU counts recovered from individual embryos infected by yolk sac injection with 200 CFU of GFP-tagged *S. enterica* at 2 dpf. Data represent three biological replicates. Error bars represent s.e.m.; statistically significant differences of $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) as determined by ANOVA are marked.



enterica ($P < 0.0001$; Fig. 2B). There was significant divergence of survival curves around 48 hours post-infection (hpi), and this was associated with an increase in *S. enterica* burden (Fig. 2C).

Zebrafish neutrophils are important mediators of systemic immunity in embryos and we hypothesized that depletion of Nod1 or Nod2 might affect the recruitment of neutrophils (Prajnsnar et al., 2008). Because yolk sac injection creates a focal point for infection, we were able to monitor, by fluorescence microscopy, the recruitment of neutrophils to the site of infection. However, neutrophils in Nod-depleted and control zebrafish were indistinguishable in their recruitment pattern (Fig. 3A).

Depletion of Nod1 or Nod2 did not significantly alter the number of neutrophils in unchallenged larvae (supplementary material Fig. S6A). However, we observed a clear difference between the proportions of embryos with a decreased number of neutrophils resident in the caudal hematopoietic tissue (CHT) after infection (see arrows in Fig. 3A). At 1 day post-infection (dpi), the CHT regions of control and morphant embryos were virtually devoid of neutrophils. From 2 dpi onwards, the CHTs of control and Nod2 morphant embryos were repopulated with neutrophils, whereas the CHT of Nod1 morphants remained depleted of neutrophils (Fig. 3B).

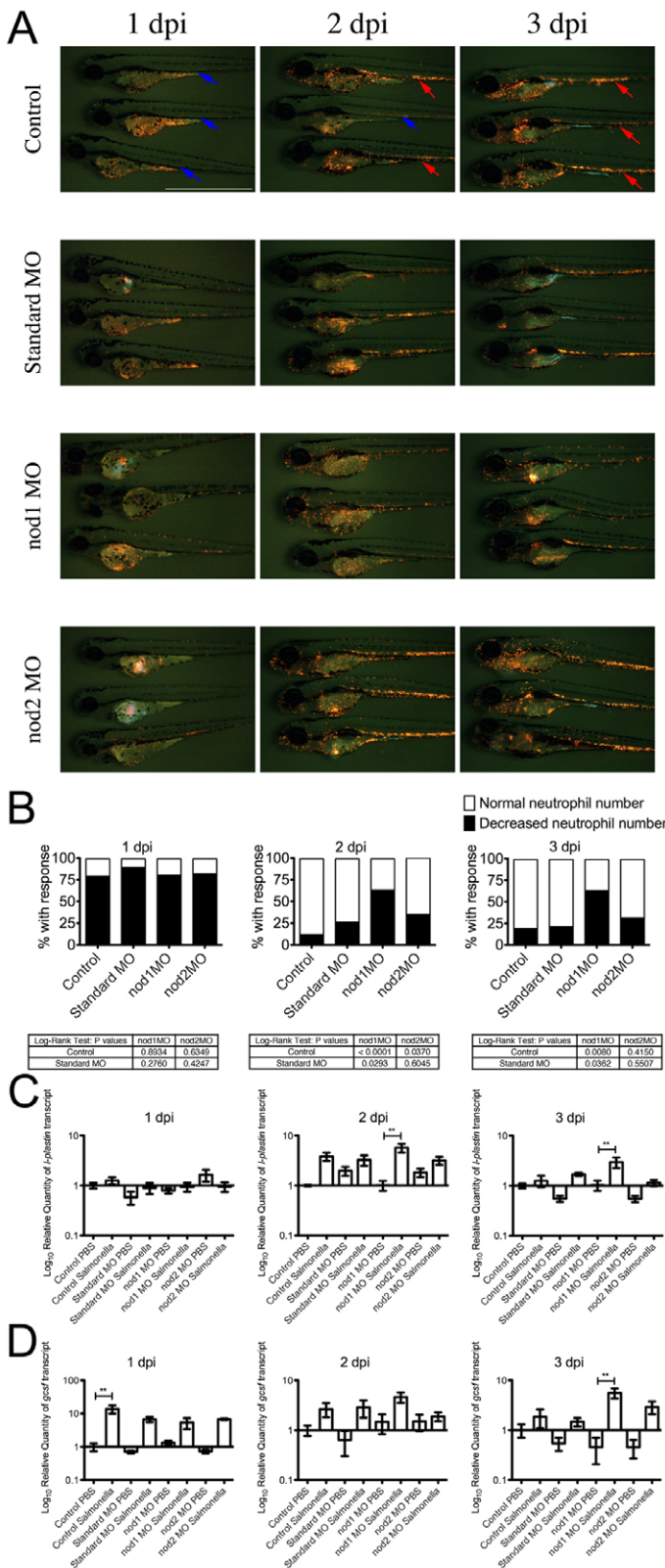


Fig. 3. Leukocytic response to yolk sac infection in control and morphant embryos. (A) Time course of Tg(*lyzC:dsRed*)⁵⁰ control and morphant embryos infected by yolk sac injection with *S. enterica* at 2 dpi. In control row: red arrows indicate embryos with a normal number of neutrophils in the CHT; blue arrows indicate embryos with a decreased number of neutrophils in the CHT. Scale bar: 1 mm. (B) Analysis of neutrophil number in the CHT of *S. enterica*-infected Tg(*lyzC:dsRed*)⁵⁰ embryos. Data represents three biological replicates; 50 embryos of each treatment were infected and followed for analysis. (C) Expression of *l-plastin* analyzed by qPCR. (D) Expression of *gcsfr* analyzed by qPCR. Data for qPCR experiments represents triplicate pools of five to ten embryos. Error bars represent s.e.m.; statistically significant differences of $P < 0.01$ (***) as determined by ANOVA are marked.

material Fig. S6B,C). Expression of the leukocytic markers *lymphocyte cytosolic plastin 1 (l-plastin)*, *lyzC* and *granulocyte colony stimulating factor receptor (gcsfr)*; also known as *csf3r*) were similar between morphant and control samples. Likewise, expression of the myelopoiesis-promoting cytokine *granulocyte colony stimulating factor (gcsf)*; also known as *csf3*) and the hematopoietic stem cell marker *runx1* were similar between controls and morphants at the early stage of infection. Surprisingly, an increase in *l-plastin* and *gcsfr* expression was noted in 2- and 3-dpi Nod1 morphants, demonstrating at least a normal, and potentially an increase, in the number of leukocytes in these surviving morphants. Alternatively, this observation might have been the result of elevated expression of these markers within a similar number of leukocytes. Nod1 morphants also had elevated *gcsf* and *runx1* expression at 3 and 2 dpi, respectively, consistent with the observation of elevated expression of leukocyte markers (Fig. 3D; supplementary material Fig. S6D).

Larval expression of dual oxidase is impaired following knockdown of zebrafish Nod1 or Nod2

Activation of mammalian NODs triggers an antimicrobial response in many cell types, including the expression of defensins and the production of ROS through DUOX2 (Kobayashi et al., 2005; Lipinski et al., 2009). To investigate the role of zebrafish Nod orthologs in mucosal immunity, we expanded our analysis of larval stage zebrafish and investigated whether Nod depletion affected the expression of zebrafish *dual oxidase (duox)* in response to immersion infection by *S. enterica*. Previous work by our laboratory has identified the intestinal epithelial cells as the main site of *S. enterica* adhesion during immersion infection (Hall et al., 2007). qPCR was used to examine the expression levels of *duox* in 6- and 24-hpi larvae. Expression of *duox* was decreased in uninfected Nod1 and Nod2 morphants at both 6 and 24 hpi compared with standard morpholino injected controls (Fig. 4A). Interestingly, we observed a trend for increased expression of *duox* in standard morpholino injected control groups compared with uninjected control groups (6 hpi, $P=0.1209$; 24 hpi, $P=0.3358$). Furthermore, Nod1 but not Nod2 morphants were found to have decreased *duox* expression at 6 hpi when infected with *S. enterica* when compared with the infected standard morpholino group.

Because the larval expression pattern of *duox* and *nod1/nod2* only overlap in the intestinal epithelium (Flores et al., 2010), we carried out whole-mount in situ hybridization to detect *duox*

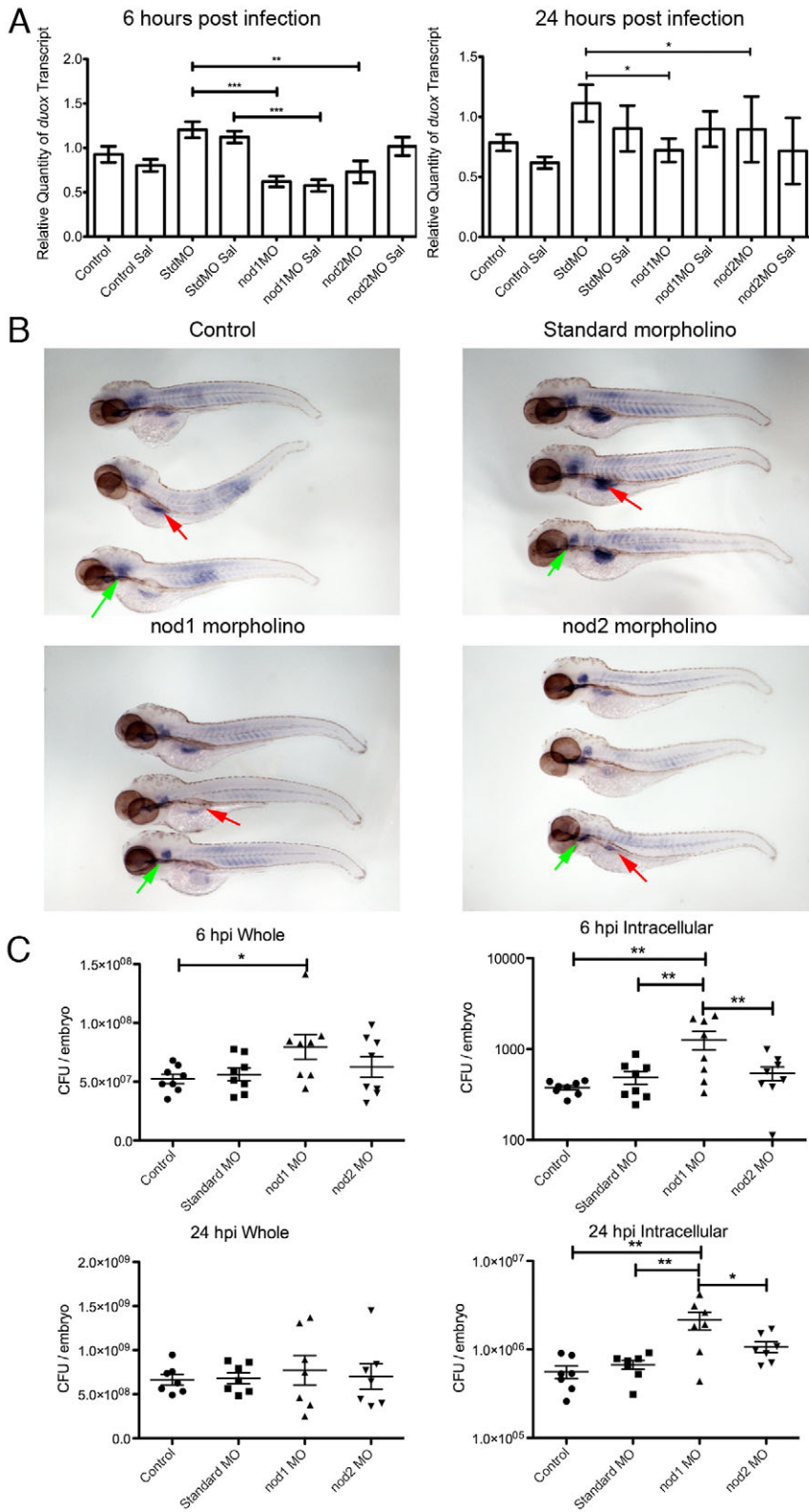


Fig. 4. Depletion of zebrafish Nod1 or Nod2 results in reduced expression of *duox* and increased *S. enterica* burden following infection in larvae. (A) Expression of *duox* examined by qPCR at 6 and 24 hpi. Data represents at least triplicate pools of 10 to 20 larvae. Error bars represent s.e.m.; statistically significant differences of $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.0001$ (***) as determined by ANOVA are marked. (B) Expression of *duox* in 4-dpf control and morphant larvae examined by in situ hybridization. Green arrows indicate thyroid expression; red arrows indicate intestinal epithelial cell expression. (C) Whole-larvae and intracellular bacterial recovery from 6 and 24 hpi larvae. Triplicate groups of five larvae were analyzed for each biological replicate represented by a single dot. Error bars represent s.e.m.; statistically significant differences of $P < 0.05$ (*) and $P < 0.01$ (**) as determined by ANOVA are marked.

transcripts in morpholino-injected 4-dpf larvae (Fig. 4B). Whereas epidermal and thyroid expression remained unchanged, gut expression was increased in standard morpholino injected larvae and decreased in *nod1* and *nod2* morpholino injected larvae.

Depletion of zebrafish Nod1 is associated with increased bacterial burden after immersion infection

Previous work in our laboratory has linked reduced *duox* expression to increased intracellular survival of *S. enterica* following immersion

infection (Flores et al., 2010). In mammals, production of ROS by DUOX2 is dependent on NOD2 activity (Lipinski et al., 2009). We therefore investigated the role of zebrafish Nod proteins in the control of *S. enterica* infection. Because survival rates of Nod-depleted larvae did not differ significantly from control groups during immersion infection (data not shown), total (whole fish) and intracellular bacterial burdens were enumerated at 6 and 24 hpi (Fig. 4C). Knockdown of zebrafish Nod1 resulted in an early increase of *S. enterica* burden at 6 hpi in both whole larvae and the intracellular compartment, whereas no change in *S. enterica* burden resulted from Nod2 depletion.

At 24 hpi, *S. enterica* burden was elevated in the Nod1 morphant intracellular compartment and a modest (1.9-fold and 1.6-fold increase compared with control and standard morpholino groups, respectively) but statistically insignificant increase in intracellular bacterial burden in the Nod2 morphants.

DISCUSSION

This study reports the first in situ analysis of Nod expression in larval zebrafish; this is the developmental stage that is the most relevant for investigation of zebrafish innate immunity (Trede et al., 2004). We have used a zebrafish reverse genetics approach to investigate the function of established IBD susceptibility genes (*NOD1* and *NOD2*) to demonstrate the feasibility of using zebrafish to investigate the function of other genes implicated in IBD etiology.

Knockdown of zebrafish Nod proteins did not seem to cause any major perturbation to embryonic development. This recapitulates observations in knockout mice and humans carrying variant alleles (Abraham and Cho, 2006). However, in the context of bacterial infection, this study shows that zebrafish Nod proteins do share an important antibacterial function with their mammalian counterparts (Kim et al., 2004; Kobayashi et al., 2005; Tattoli et al., 2007; Divangahi et al., 2008; Petnicki-Ocwieja et al., 2009).

Although the link between genetic variants of *NOD2* and Crohn's disease was the first genetic connection to IBD (Hugot et al., 2001; Ogura et al., 2001), the past decade has revealed several other major genetic risk factors for IBD. Recent work has shown the convergence of many molecular pathways containing IBD susceptibility genes on the Paneth cell and its antimicrobial functions (Wehkamp et al., 2004; Cadwell et al., 2008; Kaser et al., 2008; Bevins et al., 2009; Zhao et al., 2010). Because Paneth cell equivalents have yet to be identified in zebrafish, our study has focused on the expression of the zebrafish ortholog of DUOX2, an NAD(P)H oxidase family member responsible for the synthesis of ROS in response to inflammation and NOD2 (Lipinski et al., 2009; Niethammer et al., 2009; Flores et al., 2010). Whereas DUOX2 transcriptional regulation in humans seems to be independent of disease-associated NOD2 mutations (Hamm et al., 2010), there is little evidence regarding transcriptional regulation of DUOX2 by NOD2. Although ROS production is known to be increased in a NOD2-dependent manner, NOD2 knockout mice do not seem to have significantly lower basal ROS production (Lipinski et al., 2009). The measurement of ROS production does not directly address transcriptional regulation of DUOX2 in NOD2-deficient cells. In separate studies, our laboratory has correlated reduced *duox* expression in vivo with decreased bacterial control (Flores et al., 2010).

Studies identifying single CARD NOD2 splice variants in humans have concluded that normal NOD2 pro-inflammatory signaling is lost in non-canonical splice variants (Rosenstiel et al., 2006; Leung et al., 2007; Rosenstiel et al., 2007). The morpholino used in our study was very effective at preventing the formation of full-length *nod2* transcript, without affecting the formation of the shorter *nod2* splice variant. Thus, the larval zebrafish might prove to be a useful system to study the biological significance of such splicing events.

Our gene expression analysis of zebrafish neutrophils revealed expression of *nod1*, but very little *nod2*, in neutrophils from the gut (Fig. 1C). This is in contrast to human neutrophils, which strongly express NOD2 and not NOD1 (Ekman and Cardell, 2010). Furthermore, we observed a dependence on Nod1, but not on Nod2, for the replacement of neutrophils in the CHT of systemically infected embryos. Taken with our data showing an enhanced myelopoietic response to infection in surviving Nod1 morphants, it seems that myelopoiesis is normal in infected Nod1 morphants. However, depletion of Nod1 alters the response to infection, resulting in the more immediate recruitment of neutrophils away from the CHT. This subtle difference in the hematopoietic response to infection between Nod1 and Nod2 morphants might manifest owing to higher expression of Nod1 than Nod2 in neutrophils. The recent availability of macrophage-lineage transgenic lines will facilitate a more comprehensive analysis of zebrafish leukocyte expression of Nod1 and Nod2, and implications to immunity (Ellett et al., 2010; Gray et al., 2011).

Because most organ morphogenesis is completed by the larval stage of development, the zebrafish larva is an attractive target to undertake chemical-genetic studies of innate immune function. Key advantages of utilizing larval instead of adult zebrafish include the applicability of simple morpholino-mediated depletion of gene function, live imaging, amenability to chemical-genetic screening and the availability of techniques to raise germ-free animals (Zon and Peterson, 2005; Pham et al., 2008; Kaufman et al., 2009; Oehlers et al., 2011a). Specifically, once orthologs of IBD susceptibility genes are identified, morpholino-mediated gene knockdown could be used as a 'first pass' in vivo analysis to complement traditional investigation of loss-of-function phenotypes in knockout mice.

The splice-blocking efficiency of the *nod1* morpholino used in this study was not completely penetrant by the later stages analyzed. However, incomplete knockdown of MyD88 was sufficient to evoke a deficient immune response to systemic *Salmonella* infection in zebrafish larvae (van der Sar et al., 2006). In this present study, the partial knockdown of Nod1 resulted in decreased expression of *duox*, correlating with higher intracellular *S. enterica* burden when compared with the effects observed with the more efficient knockdown of Nod2 (Fig. 4C). This suggests that zebrafish Nod1 might be more important than Nod2 in mucosal immunity.

Interestingly, we observed increased intestinal epithelial cell expression of *duox* in standard morpholino injected larvae, a phenotype that did not seem to reflect better management of *S. enterica* infection in these larvae when compared with uninjected larvae (Fig. 4B). The selection of an appropriate control in morpholino-mediated innate immunity studies is an important consideration. A transcriptome study of *myd88* function in *Salmonella* infection demonstrated an effect of standard morpholino on cytokine induction without addressing baseline expression levels (Stockhammer et al., 2009). Because morpholino

injection results in the introduction of exogenous nucleic acid, we have also used the standard morpholino as a more appropriate mock-treatment control than using an uninjected larvae (Clay et al., 2007).

In summary, this study has demonstrated the functional conservation of the Nod signaling apparatus in zebrafish larvae. The work presented demonstrates the utility of the zebrafish model system for investigating the biological function of genes linked to human IBD.

METHODS

Cloning, probe synthesis and RT-PCR

Total RNA was isolated from zebrafish embryos using Trizol (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from 2 µg of total RNA with SuperScript III (Invitrogen) for RT-PCR or with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) for qPCR. Primers designed for this study are shown in supplementary material Table S1, all other primers have been previously described (Oehlers et al., 2011b; Oehlers et al., 2011a). qPCR and probe synthesis was carried out as previously described (Oehlers et al., 2011b).

Whole-mount in situ hybridization

Whole-mount in situ hybridization was carried out as previously described (Thisse and Thisse, 2008; Oehlers et al., 2010). Specimens were imaged and photographed with a Leica MZ16 FA stereomicroscope equipped with a Leica DFC490 camera. Stained specimens were mounted in paraffin, sectioned and counterstained with Nuclear Fast Red (Vector Labs) prior to imaging with a Leica DMR compound microscope equipped with a DFC420C camera.

Morpholino microinjection and analysis

Morpholinos (GeneTools, LLC) were designed to target the splice donor site after exon 1 of the *nod1* gene (5'-ACCAAATAAACATTACCTGGTCTGT-3') and the *nod2* gene (5'-GTTTAAAGGTGGTATTACCTGTTGTG-3'). Morpholinos were injected into one- to four-cell stage embryos at 0.75 pmol per embryo as described (Nasevicius and Ekker, 2000). Primers that spanned introns were used for RT-PCR of the targeted junctions.

For live imaging, larvae were anesthetized in tricaine and mounted in 3% methylcellulose for imaging on a Nikon SMZ1500 stereomicroscope equipped with a DS-U2/L2 camera.

FACS

Larval dissociation was performed as described (Covassin et al., 2006; Oehlers et al., 2010; Oehlers et al., 2011a). FACS sorting was carried out on a FACS Vantage flow cytometer (Becton Dickinson); cell count analysis experiments were carried out on a BD LSRII.

Yolk sac infection

Bacterial challenge was carried out with GFP-labeled *S. enterica* serovar Typhimurium (Hall et al., 2007), using a method adapted from Prajsnar et al. (Prajsnar et al., 2008). Bacteria were grown overnight in Luria broth at 37°C with shaking at 200 rpm. Bacteria were harvested by centrifugation and washed with sterile PBS prior to resuspension (1:1 ratio) in PBS. Injection doses were prepared by further diluting the resuspended bacteria 1:100 with PBS; 1 nl

TRANSLATIONAL IMPACT

Clinical issue

Crohn's disease and ulcerative colitis, collectively recognized as inflammatory bowel disease (IBD), are widely prevalent chronic immune disorders of the gastrointestinal tract. There has been little progress in developing effective long-term treatments for IBD, mainly because the disease is caused by a complex interplay between the intestinal immune system and its microbiota, and is therefore dependent on both host genetic and environmental factors. In terms of the genetic susceptibility to IBD, over 40 susceptibility genes have been identified for Crohn's disease. Functionally, many of the genes encode pivotal components of the innate immune system, including molecules involved in both the initial detection of invading bacteria and their subsequent autophagy. Two of the best-studied susceptibility genes, *NOD1* and *NOD2*, encode intracellular bacterial sensors that can trigger the innate immune system; these molecules are thus promising therapeutic targets. However, the mechanisms by which *NOD1* and *NOD2* influence the development and progression of intestinal inflammation are unclear, and addressing this issue in humans is extremely challenging. Therefore, studies of *NOD1* and *NOD2* function in a simple whole animal model are necessary to advance the understanding of IBD pathogenesis.

Results

This research shows that the role of *NOD1* and *NOD2* in humans is conserved and can be investigated in zebrafish larvae. The equivalent components of the human NOD signaling cascade are present in zebrafish, and are co-expressed with zebrafish Nod transcripts, suggesting conserved roles. Furthermore, zebrafish larvae lacking *nod1* or *nod2* are less likely than wild-type animals to survive following systemic infection with Salmonella. In mammals, *NOD2* cooperates with the enzyme dual oxidase (DUOX) to produce bactericidal reactive oxygen species in epithelial cells. Here, the authors show that depletion of zebrafish Nod1 or Nod2 significantly decreases zebrafish duox expression, which was associated with increased bacterial burden.

Implications and future directions

This work indicates that zebrafish Nod proteins, similar to their mammalian orthologs, are important for antibacterial immunity in the gut. Together, the data demonstrate the feasibility of using zebrafish to investigate the function of IBD susceptibility genes. This system will provide the international IBD research community with a 'fast-track' functional approach to confirm genotype-phenotype relationships relevant to IBD in order to prioritize translation of basic discoveries to the clinic.

of this solution (approximately 200 CFU) was microinjected into the yolk sac of anesthetized 2 dpf embryos mounted in methylcellulose. Injection doses were verified by plating onto LB agar. Embryos were recovered to E3, washed to remove excess methylcellulose and incubated for observations at 28°C.

Confocal microscopy

Infected larvae were briefly fixed in 4% paraformaldehyde, rinsed in PBS and mounted in 3% methylcellulose for imaging on a Nikon D-Eclipse C1 confocal microscope. Further image manipulation was carried out with ImageJ software version 1.43 (National Institutes of Health).

Bacterial immersion infection

S. enterica was grown overnight in Luria broth at 37°C with shaking at 200 rpm. The overnight culture was diluted 1 in 10 with Dulbecco's Modified Eagle's Medium and grown for a further 3 hours at 37°C with 200 rpm shaking. Bacteria were harvested by centrifugation and washed with sterile E3 prior to resuspension in

E3. Groups of 20 zebrafish larvae at 4 dpf were exposed to bacteria at a final concentration of 5×10^8 CFU/ml at 28.5°C. Bacterial concentration was checked by plating onto LB agar after resuspension.

Bacterial enumeration

The protocol for bacterial enumeration was adapted from previously described methods (Neely et al., 2002; van der Sar et al., 2003). Individual embryos or triplicate samples of ten larvae from each condition were collected into 1.5 ml microfuge tubes. Each sample was then washed three times in PBS to remove non-adherent bacteria. Samples were then homogenized in 100 μ l PBS in a fresh microfuge tube. A 50 μ l aliquot of homogenate was diluted for duplicate plating onto LB agar and grown overnight at 37°C. The intracellular bacterial load was determined by a modification of the gentamicin exclusion assay (Vaudaux and Waldvogel, 1979). The remaining 50 μ l of homogenate was incubated in 200 μ g/ml gentamicin at 28°C for 1 hour, washed and diluted for duplicate plating on LB agar and overnight growth allowed to occur at 37°C. *S. enterica* CFUs were enumerated by ImageJ (National Institutes of Health) analysis of GFP-expressing colonies from photographs taken with 460 nm epifluorescent illumination on a LAS-3000 (Fujifilm) equipped with a GFP510E filter.

Statistical analysis

All statistical analyses were performed with GraphPad Prism version 5.0a for Mac (GraphPad Software).

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COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

S.H.O.: prepared the manuscript. S.H.O. and M.V.F.: study design, data collection and analysis. C.J.H., K.E.C. and P.S.C.: study design and analysis of data. SS: study design. All authors edited the manuscript prior to submission.

SUPPLEMENTARY MATERIAL

Supplementary material for this article is available at <http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.006122/-/DC1>

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