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Article

Intestinal Alkaline Phosphatase Detoxifies Lipopolysaccharide and Prevents Inflammation in Zebrafish in Response to the Gut Microbiota

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SUMMARY

Vertebrates harbor abundant lipopolysaccharide (LPS) in their gut microbiota. Alkaline phosphatases can dephosphorylate and detoxify the endotoxin component of LPS. Here, we show that expression of the zebrafish intestinal alkaline phosphatase (Iap), localized to the intestinal lumen brush border, is induced during establishment of the gut microbiota. Iap-deficient zebrafish are hypersensitive to LPS toxicity and exhibit the excessive intestinal neutrophil influx characteristic of wild-type zebrafish exposed to LPS. Both of these Iap mutant phenotypes are dependent on Myd88 and Tumor Necrosis Factor Receptor (Tnfr), proteins also involved in LPS sensitivity in mammals. When reared germ-free, the intestines of Iap-deficient zebrafish are devoid of neutrophils. Together, these findings demonstrate that the endogenous microbiota establish the normal homeostatic level of neutrophils in the zebrafish intestine through a process involving Iap, Myd88, and Tnfr. Thus, by preventing inflammatory responses, Iap plays a crucial role in promoting mucosal tolerance to resident gut bacteria.

INTRODUCTION

LPS, the major constituent of the outer membrane of all Gram-negative bacteria, both pathogens and mutualists, was independently discovered as a bacterial-associated substance called endotoxin that elicits septic shock in animals (Beutler and Rietschel, 2003). We now know that LPS acts as a toxin by overstimulating Toll-like receptor (TLR) innate immune signaling, which induces pathogenic inflammatory responses. Mice deficient for TLR4, the TLR specific for LPS, or MyD88, a common adaptor downstream of TLRs, exhibit increased resistance to LPS toxicity (Kawai et al., 1999; Poltorak et al., 1998). TLR signaling through MyD88 promotes nuclear translocation of NF-κB and transcription of proinflammatory cytokines such as Tumor Necrosis Factor (TNF). TNF is an important mediator of septic shock, as demonstrated by the endotoxin resistance observed in mice treated with TNF blocking antibody (Beutler et al., 1985). However, TNF is not the only mediator of LPS toxicity, as indicated by the fact that mice deficient for TNFα (Pasparakis et al., 1996), its major receptor TNFRp55 (Pfeffer et al., 1993), or both its 75 and 55 kD receptors (Rothe et al., 1993) are as sensitive as wild-type mice to interperitoneal injection with high doses of LPS, although they are more resistant to low doses of LPS administered along with the hepatocyte toxin D-galactosamine. TNF and other proinflammatory cytokines induce vascular permeability, blood flow, and neutrophil recruitment to the LPS source as well as systemic responses such as fever, and in the extreme case of septic shock, disseminated intravascular coagulation, hypotension, and ultimately, organ dysfunction (Kasper and Harrison, 2005).

One of the most abundant sources of LPS encountered by vertebrates is their resident gut microbiota. Since the discovery of innate immune signaling pathways that recognize molecular signatures present on both pathogens and resident beneficial microbes, an unanswered question has been why gut microbial communities do not elicit pathological inflammation in their hosts. Indeed, inappropriate inflammatory responses to the microbiota manifest themselves in patients with inflammatory bowel diseases (Sartor, 2006). In healthy individuals, suggested protective mechanisms include sequestration of IgA coated gut microbes from the intestinal epithelium (Macpherson et al., 2005), barrier functions of the intestinal epithelium, and restricted expression of innate immune receptors in intestinal epithelial cells (Cario and Podolsky, 2005).

In addition to restricting innate immune signaling by sequestering proinflammatory ligands and their receptors, host cells can actively modulate the inflammatory response by modifying the proinflammatory microbial molecules themselves. Such a mechanism has been shown in the case of an acyloxyacyl hydrolase that cleaves acyl chains from the lipid A portion of LPS (Feulner et al., 2004). In mice lacking this enzyme, acylated LPS persists for longer periods of time after infection with Gram-negative pathogens and elicits increased B cell proliferation

and antibody production (Lu et al., 2005). Alkaline phosphatases (AP) have also been shown to modify LPS by dephosphorylating its lipid A moiety (Beumer et al., 2003; Koyama et al., 2002; Tuin et al., 2006; van Veen et al., 2005). Lipid A, which accounts for the toxicity of LPS, contains two phosphate groups coupled to glucosamines; removal of one of the phosphate groups generates a monophosphoryl lipid A that is a 100-fold less toxic than the unmodified lipid A (Schromm et al., 1998).

In vertebrates, APs are broadly distributed throughout different organs, but for the most part, their physiological substrates are unknown. Mice deficient for the ubiquitously expressed tissue-nonspecific AP (TNAP) die from seizures due to a defect in the metabolism of pyridoxal phosphate (Waymire et al., 1995), arguing that vitamin B6 is a natural substrate of TNAP in mice. The intestinal specific isozyme, intestinal alkaline phosphatase (IAP) has been used traditionally as a marker of enterocyte maturation, but its physiological function in the intestine is unclear. The protein is localized to the apical brush border and is enriched in surfactant-like particles that are secreted toward the intestinal lumen (Alpers et al., 1995). A sharp increase in activity of this enzyme occurs during the postembryonic development of mammals and fish, the period during which the gut microbiota is established (Bates et al., 2006; Henning, 1985; Zambonino Infante and Cahu, 2001). IAP has been thought to play a role in digestion and absorption of casein (Li-Chan and Nakai, 1989). However, IAP-deficient mice have no apparent digestion deficits and, in fact, exhibit accelerated transport of fat droplets through the intestinal wall, resulting in obesity when fed a high fat diet (Nakano et al., 2007; Narisawa et al., 2003).

We hypothesized that Iap functions to dephosophorylate LPS associated with gut bacteria, thereby modulating intestinal inflammation in response to the resident microbiota. We tested this idea using a zebrafish model because of the ease with which we could manipulate both bacterial associations, using gnotobiology, and host gene expression, using morpholino antisense oligonucleotides (MOs). The development and physiology of the teleost and mammalian digestive tracts are very similar (Wallace et al., 2005), and both share conserved responses to their resident microbiota (Cheesman and Guillemin, 2007). Here we show that zebrafish, like mammals, respond to LPS through a mechanism that involves Myd88 and Tumor Necrosis Factor Receptor (Tnfr). We report that upon colonization, gut microbes elicit low-level intestinal inflammation by a similar mechanism as exogenously administered LPS. Finally, we demonstrate that Iap function is required to detoxify LPS and to prevent intestinal inflammation in response to the resident microbiota.

RESULTS

Identification of the Zebrafish *intestinal alkaline phosphatase* Gene

In previous work we showed that brush border AP activity in the zebrafish intestine increases during the period when this organ is colonized by microorganisms, between 5 and 8 days postfertilization (dpf), but fails to increase in animals reared germ free (GF) (Bates et al., 2006). We found that by inoculating 5 dpf GF zebrafish with microbiota from conventionally reared (CV) controls, we could restore normal Iap levels by 8 dpf. Furthermore, we demonstrated that LPS exposure was sufficient to induce Iap activity in GF animals. To investigate the mechanism and functional significance of LPS regulation of Iap, we sought to identify zebrafish *iap* genes. APs in humans are encoded by four loci: *TNAP* on chromosome 1 and those corresponding to tissue-specific isozymes *IAP*, *placental AP*, and *germ-cell AP*, which are clustered at chromosomal position 2q37 and appear to have arisen from tandem duplications (Millán, 2006). To isolate *iap* genes in zebrafish, we compared the murine *Akp3* sequence that encodes IAP (accession number M61705.1) to the zebrafish genome, which yielded two *alkaline phosphatase* genes that mapped to chromosomes 11 and 22. Similar to humans, these *ap* genes were highly related but distinguished by their expression patterns (Le Du and Millan, 2002). In situ hybridization with the chromosome 11 *ap* transcript (accession number NM_201007), which was annotated as *alkaline phosphatase* (*alp*), exhibited low level, ubiquitous expression at 5 dpf (data not shown). In contrast, the unannotated chromosome 22 *ap* gene (accession number NM_001014353), which shared 75% sequence identity to murine *Akp3*, was expressed specifically in the intestinal epithelium of 5 dpf larvae (Figures 1A and 1B). RT-PCR analysis of dissected guts from 8 dpf larvae showed intestinal specific expression of the chromosome 22 *ap* gene, whereas the chromosome 11 *ap* transcript was present at low levels in the intestine as well as in the carcasses from which the intestines were removed (Figure 1C).

We next tested whether this gene encoded the AP activity in the zebrafish intestine by blocking expression of the gene with a splice-blocking MO specific to the gene (see Figure S1A in the Supplemental Data available with this article online). When reared to 8 dpf, the intestines of *iap*-MO injected animals displayed significantly reduced AP activity as compared to wild-type (WT) intestines (Figure 1D). The *iap*-MO specifically inhibited AP activity in the intestine and did not alter AP activity in the carcass lacking intestines (Figure 1D). We observed a similar intestinal-specific inhibition of AP activity in 8 dpf fish that had been immersed from 5 dpf in a solution of 10 mM L-phenylalanine (L-phen), a specific inhibitor of IAP isozymes (Fishman et al., 1963) (Figure 1D). Treatment with a control MO against an *α1,3galactosyl transferase* (*galT*) gene did not alter Iap activity (Figure 1E). Based on these results, we have designated the chromosome 22 *ap* gene the zebrafish *intestinal alkaline phosphatase* (*iap*). Intestines from *iap*-MO-injected animals retained some AP activity, likely due to incomplete inhibition by the *iap*-MO at 8 dpf, indicated by the presence of some WT *iap* transcript in these animals (Figure S1A). In addition, some AP activity in the intestine is likely to be encoded by the ubiquitously expressed *alp* gene because the *iap*-MO had no inhibitory

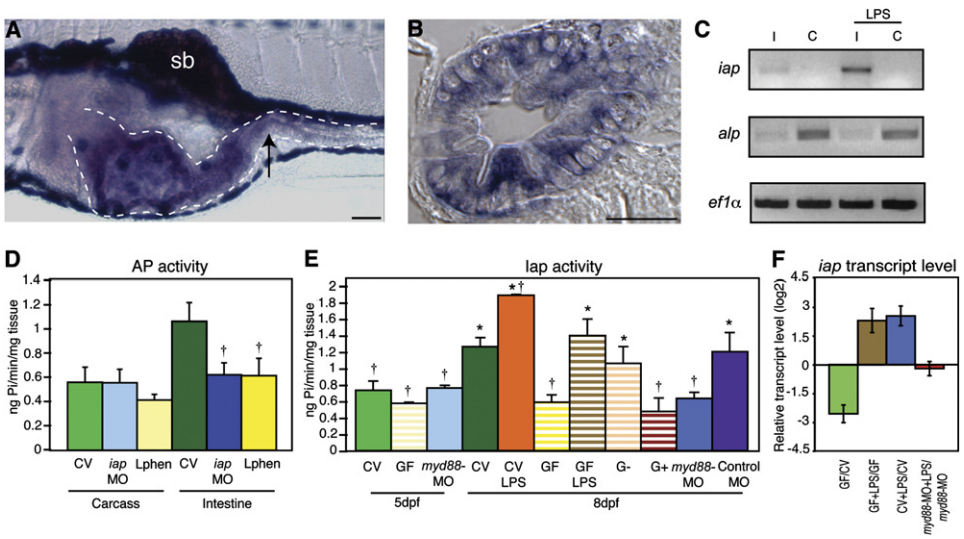

Figure 1. Iap Activity and *iap* Transcript Levels Are Regulated by LPS

(A and B) In situ hybridization of *iap* transcript at 5 days postfertilization (dpf) in (A) a whole mount larva and (B) a transverse section through the mid intestine (at the point indicated by the arrow in [A]). The *iap*-specific purple stain is present in the intestinal epithelium (outlined with the dotted line in [A]) and is distinct from the black pigment cells above and below the digestive tract and the swim bladder (sb) in (A). Scale bar in (A), 100 μm; scale bar in (B), 5 μm.

(C) Semiquantitative RT-PCR analysis showing *iap* and *alp* expression in dissected intestines (I) and carcasses from which intestines were removed (C) of 8 dpf larvae untreated or exposed to 50 μg/ml LPS for 24 hr. Levels of the housekeeping gene *ef1α* are shown as an amplification and loading control.

(D) AP activity in the carcasses and intestines of 8 dpf untreated CV WT larvae, *iap*-MO injected larvae, or larvae exposure to 10 mM L-phen from 5 dpf. †Indicates values that differ significantly as compared to the CV levels of each group (carcass or intestine), $p < 0.01$.

(E) AP activity in 5 and 8 dpf intestines from larvae reared CV (solid bars) left untreated, exposed at 5 dpf to 30 μg/ml LPS, or injected at the 1 cell stage with *myd88*-MO or the control *galT*-MO, or larvae reared GF (striped bars) left untreated, exposed at 5 dpf to 3 μg/ml LPS, or monoassociated at 5 dpf with a Gram-negative *Aeromonas* species (G−) or a Gram-positive *Streptococcus* species (G+). †Indicates values that differ significantly from CV at 8 dpf, *indicates values that differ significantly from GF at 8 dpf, $p < 0.01$. For (D) and (E), n = 10 dissected intestines/treatment for each trial, with at least 2 trials per treatment. Error bars represent standard deviation.

(F) *iap* transcript levels, measured by qRT-PCR, were reduced in 8 dpf GF versus CV animals, and elevated in 8 dpf CV and GF animals exposed for 24 hr to 30 μg/ml LPS, but not in 8 dpf *myd88*-MO injected animals-reared CV, and exposed for 24 hr to 50 μg/ml LPS. Data are representative of two repeated trials in which all samples were run in triplicate. Error bars indicate standard deviation. All animals were WT unless otherwise indicated.

effect on the total AP activity in 5 dpf intestine but reduced AP activity in 8 dpf intestines (Figure S1B), despite being more efficient at blocking *iap* transcript splicing at earlier time points (data not shown).

Bacterial LPS Regulates *iap* Transcription in Zebrafish

We previously reported that purified LPS at a concentration of 3 μg/ml was sufficient to induce Iap activity in GF larvae to levels of CV controls (Bates et al., 2006). Here, we tested whether Iap activity could be further induced by additional LPS. We observed that the Iap activity of CV larvae exposed to LPS at 30 μg/ml was significantly higher than untreated levels (Figure 1E). As we had shown previously, monoassociation of GF larvae with Gram-negative bacterial isolates from the zebrafish intestine (*Aeromonas* and *Pseudomonas* species) was sufficient to induce Iap activity to CV levels; however, when we monoassociated GF larvae with several Gram-positive isolates (*Streptococcus* and *Staphylococcus* species), which lack LPS in their cell walls, we observed no induction of Iap activity (Figure 1E and data not shown). These results indicated that LPS was sufficient and necessary to induce Iap activity in zebrafish.

In order to understand the mechanism of Iap induction, we examined *iap* transcript abundance in CV and GF larvae. Semiquantitative RT-PCR revealed that *iap* transcript levels increased in the intestine upon exposure to 50 μg/ml LPS for 24 hr, while transcription of *alp* remained invariant throughout the whole organism with this treatment (Figure 1C). Using quantitative reverse transcription PCR (qRT-PCR), we found that, similar to the regulation of Iap enzyme activity, *iap* transcript levels were significantly elevated in the presence of bacteria (Figure 1F). Furthermore, exposure to exogenous LPS (at 30 μg/ml for 24 hr) was sufficient to increase *iap* transcript levels in GF larvae and elevate *iap* above normal levels in CV larvae (Figure 1F).

Bacterial Regulation of Iap Activity Is Myd88 Dependent

The induction of *iap* in response to LPS suggested the possibility that this gene was regulated by Tlr signaling. Several orthologs of Tlr4 and a single gene encoding

Myd88 have been identified in the zebrafish genome (Jault et al., 2004; Meijer et al., 2004). To test whether Myd88 was important for LPS induction of *iap*, we used a splice-blocking MO to zebrafish *myd88* (Figure S1C). The *myd88*-MO-injected fish exhibited no gross morphological defects (data not shown) similar to reports of zebrafish treated with a different *myd88*-MO (van der Sar et al., 2006) and *Myd88*$^{-/-}$ mice (Iiyama et al., 2003). To test whether Myd88 was required for the larval upregulation of Iap activity, we dissected intestines from *myd88*-MO-injected larvae and assayed the tissue for AP activity. Whereas the AP activity from intestines of *myd88*-MO injected animals was indistinguishable between CV or GF WT animals at 5 dpf at the onset of bacterial colonization of the gut, intestinal AP activity in *myd88*-MO injected larvae failed to increase by 8 dpf, similar to GF WT animals (Figure 1E), suggesting that microbiota-dependent induction of Iap in CV animals was Myd88 dependent. Next we investigated whether *iap* transcript abundance was regulated by exogenous LPS in a Myd88-dependent manner. Using qRT-PCR, we found that *myd88*-MO injected larvae treated with 50 μg/ml purified LPS for 24 hr fail to increase *iap* (Figure 1F), indicating that Myd88 was required for LPS mediated induction of *iap*.

LPS Is Toxic to Zebrafish

Ingestion or intraperitoneal injection of high doses of LPS is toxic to mammals (Van Amersfoort et al., 2003). Vertebrate species exhibit a wide range of sensitivities to LPS toxicity: calves are extremely sensitive compared to rats and mice, whereas fish and frogs are more resistant (Berczi et al., 1966). We found that soaking 6 dpf zebrafish larvae in water containing high concentrations of LPS resulted in lethality, with percent survival and the kinetics of killing being proportional to the LPS dose. Larvae at 5 dpf were more resistant to LPS killing, possibly due to reduced expression of Tlr signaling pathway genes, which have been shown in some cases to have dynamic developmental expression profiles (Jault et al., 2004). At 6 dpf, a dose of 30 μg/ml LPS failed to cause death, a 50 μg/ml dose resulted in 100% mortality by 48 hr, a 150 μg/ml dose resulted in 100% mortality by 4.5 hr and a 250 μg/ml dose caused death in 100% of larvae in 2 hr (Figure 2A). Similar to mammals, zebrafish exposed to LPS exhibited organ failure, observed as decreased heart beating and lethargy, as well as edema and pooling of blood that was presumably a consequence of severe bradycardia (data not shown).

LPS toxicity has a characteristic histopathology in mammals including lesions in the liver, heart, and intestine, as well as edema and neutrophil infiltration in affected tissues. Histological analysis of livers from zebrafish larvae exposed to 100 μg/ml LPS for 24 hr and sacrificed prior to death revealed swollen, disorganized hepatocytes (Figure 2C) and infiltrates of myeloperoxidase (Mpo)-positive neutrophils (Figure 2F), similar to pathology reported in LPS-exposed mice (Inoue et al., 2005). As in LPS intoxicated mammals, we also observed intestinal infiltration of Mpo-positive neutrophils in LPS treated larvae, as discussed below.

A characteristic of LPS toxicity in mammals is the induction of high levels of transcription of the proinflammatory cytokine gene *Tnf*. qRT-PCR analysis of 7 dpf larvae treated with 50 μg/ml LPS exhibited a dramatic transient induction of both zebrafish *tnf* orthologs, *tnfa* and *tnfb*, with high levels of induction at 4 hr after exposure that declined significantly by 8 hr of treatment (Figure 2D). A similar transient *Tnf* induction was observed in a LPS induced rat model of bacteremia (Xuan et al., 2001) and in zebrafish embryos in a bacteremia model of infection with the Gram-negative fish pathogen *Edwardsiella tarda* (Pressley et al., 2005).

Another characteristic of LPS toxicity in mammals is the involvement of TLR and TNF signaling. We found that larvae injected with *myd88*-MO expressed lower levels of *tnf* transcripts upon LPS exposure (Figure 2D). In addition, *myd88*-MO injected larvae were significantly more resistant to LPS killing at two different LPS concentrations (Figure 2G, log rank test, $p < 0.0001$). To test for a possible role for Tnfr in responses to LPS, we used a splice-blocking MO to the zebrafish *tnfr1* (Figure S1D). *tnfr1*-MO-injected fish were also significantly more resistant to LPS than WT fish at two different LPS concentrations (Figure 2H, log rank test, $p < 0.0001$). Both the *myd88*- and *tnfr1*-MO-injected larvae did succumb to the LPS treatment, although with slower kinetics, probably due to Myd88- and Tnfr-independent mechanisms of LPS toxicity, respectively, as well as incomplete inhibition by the MOs (Figures S1C and S1D).

Iap Protects against LPS Toxicity

We hypothesized that a function of endogenous Iap was to detoxify LPS encountered by the intestinal epithelium. First, we tested whether dephosphorylated LPS was less proinflammatory and less toxic to zebrafish, as it is to mammalian cells (Koyama et al., 2002), by exposing 7 dpf larvae to 250 μg/ml LPS that had been pretreated with purified calf intestinal alkaline phosphatase (CIAP). Dephosphorylated LPS failed to induce *tnf* or *iap* expression (Figure 2D and data not shown) and was completely nontoxic to zebrafish, whereas mock-treated LPS at the same dose caused 100% lethality by 2 hr (Figure 3A).

Next we tested whether LPS sensitivity was increased in zebrafish in which Iap activity was depleted. Inhibition of Iap, both by immersion of 5 dpf larvae in a solution of 10 mM L-phen or injection with the *iap*-MO resulted in significantly increased sensitivity to LPS (Figures 3B and 3C). A dose of 30 μg/ml LPS administered to 6 dpf WT larvae caused insignificant mortality in a 48 hr period, whereas larvae pretreated with 10 mM L-phen at 4 dpf, followed by LPS exposure at 6 dpf, exhibited 100% mortality within the following 48 hr (Figure 3B, log rank test, $p < 0.0001$). Control larvae treated with L-phen alone showed no lethality over the same period (Figure 3B). L-phen-treated larvae were also markedly more sensitive than WT larvae when exposed to 150 μg/ml LPS at 7 dpf (Figure 3B, log rank, $p < 0.0001$). Similarly, *iap*-MO-injected larvae were more sensitive to LPS treatment with approximately 90% mortality by 2.5 hr when given a 150 μg/ml dose at 7 dpf,

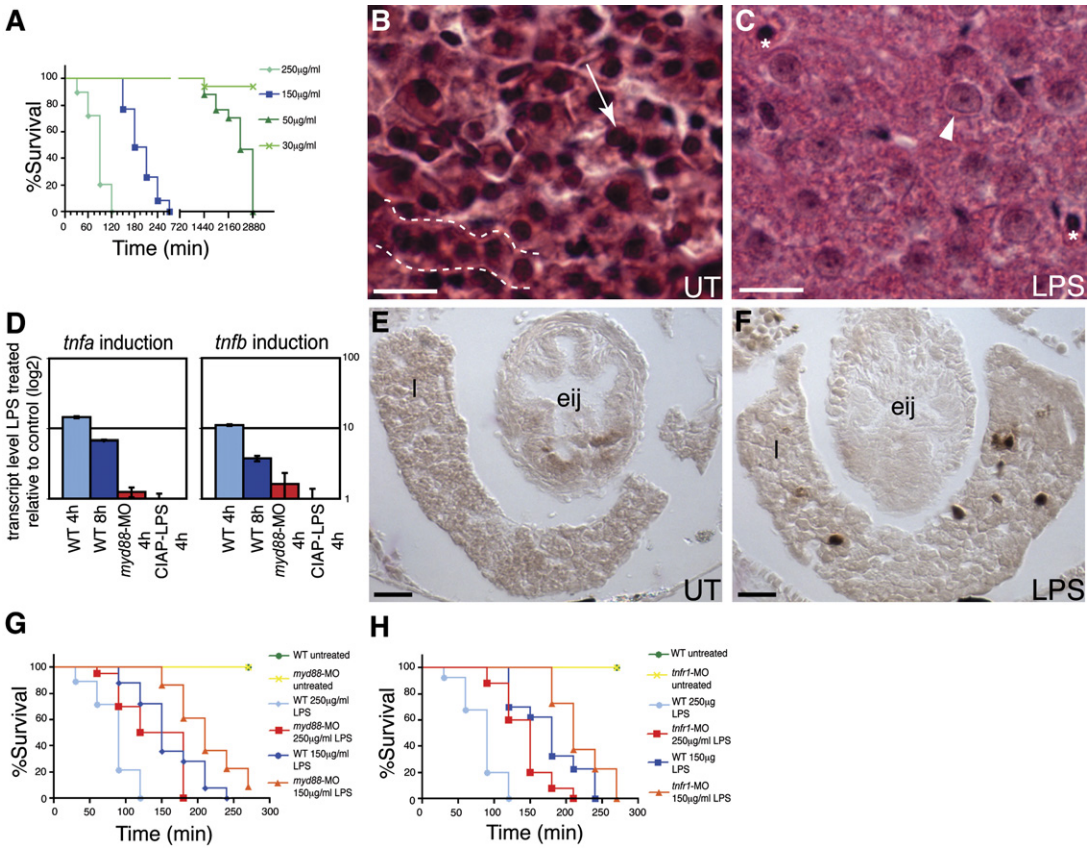

Figure 2. LPS Toxicity in Zebrafish

(A) Dose-dependent killing of wild-type animals exposed to LPS at 6 dpf. Analysis of survival curves show they are significantly different (log rank test, $p < 0.0001$).

(B and C) H&E stained liver sections of untreated (UT) 8 dpf larvae or exposed to 100 μg/ml LPS for 24 hr. (B) Hepatocytes show typical organization in cords (dashed line) with distinct nuclei (arrow). (C) LPS treatment resulted in disorganized tissue morphology with cell boundaries that are difficult to distinguish and swollen hepatocyte nuclei (arrowhead), in contrast to the normal-sized nuclei of red blood cells (asterisks). Scale bars in (B) and (C), 5 μm.

(D) *tnfa* and *tnfb* transcript levels, assayed by qRT-PCR in WT and *myd88*-MO-injected 7 dpf larvae exposed to 50 μg/ml LPS for 4 or 8 hr or WT exposed to 50 μg/ml CIAP treated LPS for 4 hr. Data are representative of two repeated trials in which all samples were run in triplicate. Error bars indicate standard deviation.

(E and F) Mpo-stained transverse sections of UT 8 dpf larvae or larvae exposed to 150 μg/ml LPS for 2 hr at the esophageal-intestinal junction (eij). Mpo-positive cells (dark brown) are present in the liver (l) of the LPS exposed animal in (F). Scale bars in (E) and (F), 10 μm.

(G and H) Survival curves of *myd88*-MO or *tnfr1*-MO injected 7 dpf larvae exposed to 150 or 250 μg/ml LPS. Survival curves are significantly different (log rank test, $p < 0.0001$). n = at least 30 total animals for each sample treatment in at least 2 independent trials. All animals were exposed to LPS at 7 dpf except those in (A), which all began treatment at 6 dpf to allow for a 48 hr time period to observe toxic effects of low doses of LPS (30–50 μg/ml) prior to termination of all experiments at 8 dpf.

whereas control animals experienced more prolonged survival (Figure 3C, log rank test, $p < 0.0001$). Finally, we observed that GF animals, which had reduced Iap activity (see Figure 1E), also exhibited increased sensitivity to 150 μg/ml LPS (Figure 3C, log rank test, $p < 0.0001$). Together, these data indicate that endogenous Iap conferred protection against a biologically relevant range of concentrations of exogenously administered LPS.

The Microbiota Regulate Homeostatic Numbers of Intestinal Neutrophils

We next asked whether Iap played a role in modulating the inflammatory response of the intestine to LPS associated with resident bacteria. We found no change in the level of *tnf* transcripts between GF and CV 8 dpf larvae, but this was not surprising given the transient nature of expression of these genes in response to proinflammatory stimuli (Figure 2D) or bacteremia challenge (Pressley et al., 2005). We characterized neutrophil cell homeostasis in the intestine in response to microbiota colonization. Mpo activity has been used as a marker of zebrafish granulocytes or neutrophils, which have been shown to infiltrate wound sites similar to mammalian neutrophils (Bennett et al., 2001; Lieschke et al., 2001; Mathias et al., 2006; Renshaw et al., 2006). Transcript levels of *mpo*, encoding a zebrafish Mpo homolog, are elevated in the intestines

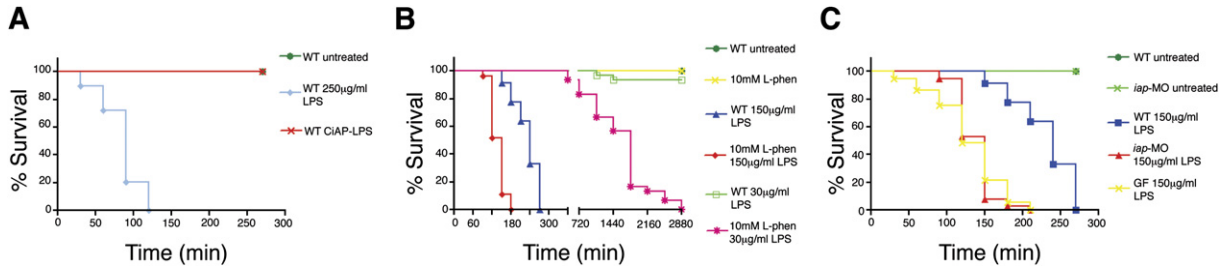

Figure 3. Iap Functions to Detoxify LPS

(A) LPS pretreated with CIAP was nontoxic to zebrafish at 250 μg/ml LPS in contrast to mock-treated LPS. Inhibition of IAP activity using (B) L-phen or (C) *iap*-MO, or by rearing larvae GF, significantly increased susceptibility of larvae to LPS killing. Survival curves are significantly different (except WT untreated and WT CIAP-LPS in [A]; WT untreated, 10 mM L-phen, and WT 30 μg/ml LPS in [B]; and *iap*-MO 150 μg/ml LPS and GF 150 μg/ml LPS in [C]) (log rank test, $p < 0.0001$). All animals were administered LPS at 7 dpf except in (B), where animals exposed to 30 μg/ml LPS began treatment at 6 dpf. All animals were reared CV, unless otherwise indicated. n = at least 30 total animals for each sample group in at least two independent trials.

of CV relative to GF zebrafish 6 dpf larvae (Rawls et al., 2004). We counted Mpo-positive neutrophils in the distal 140 μm of larval intestines, where we observed the preponderance of these cells to be distributed. We found that the average number of Mpo-positive neutrophils in the intestine increased during microbial colonization of this organ, with an average of 2 neutrophils in the distal intestine of 6 dpf CV larvae, increasing to an average of 7 neutrophils by 8 dpf (Figures 4Q and 4R). In contrast, GF intestines were entirely devoid of Mpo-positive neutrophils at 6 and 8 dpf, indicating that neutrophils were recruited into the intestine in response to the microbiota (Figures 4Q and 4R).

We next investigated the mechanism by which intestinal neutrophil recruitment occurs. We showed that the proinflammatory stimulus of 150 μg/ml LPS exposure for 2 hr resulted in a marked increased in Mpo positive intestinal neutophils (17 in 6 dpf and 22 in 8 dpf CV larvae, Figures 4D, 4E, 4Q, and 4R). In contrast, exposure to 150 μg/ml CIAP-treated LPS for 2 hr failed to elicit any increase in neutrophils in 8 dpf larvae (Figure 4R). Based on our results showing the importance of Myd88 and Tnfr1 in other responses to LPS, we tested whether the recruitment of intestinal neutrophils could be inhibited with the *myd88*-MO or the *tnfr1*-MO. We found that *myd88*-MO-injected larvae were devoid of Mpo-positive intestinal neutrophils when reared CV and had significantly reduced numbers of these cells upon exposure to LPS (Figures 4K, 4M, and 4R). Similarly, *tnfr1*-MO-injected larvae lacked Mpo-positive neutrophils in the gut epithelium when reared CV and upon exposure to LPS (Figures 4L, 4N, and 4R). Our results suggest that both Tlr and Tnf signaling play a role in neutrophil influx into the intestine in response to proinflammatory stimuli as well as for the homeostatic level of intestinal neutrophils established by the resident microbiota.

Iap Prevents Inflammatory Responses to the Resident Microbiota

We next examined the role of Iap in modulating the intestinal inflammatory response to the microbiota. In these experiments, we either inhibited Iap at 6 dpf with the *iap*-MO (when splice blocking was more effective, data not shown), or at 8 dpf with L-phen. We found that the number of Mpo-positive intestinal neutrophils in *iap*-MO injected larvae at 6 dpf or L-phen treated larvae at 8 dpf was significantly greater than in WT controls and was similar to the number seen in LPS exposed larvae (Figures 4C, 4F, 4O, 4Q, and 4R). We next tested whether this neutrophil influx was mediated by the same Myd88- and Tnfr1-based mechanisms as in WT animals exposed to LPS or microbiota. In larvae doubly injected with either a combination of *iap*- and *myd88*-MOs (Figures 4G and 4Q) or *iap*- and *tnfr1*-MOs (Figures 4H and 4Q), intestinal Mpo-positive neutrophil numbers were greatly reduced (Figure 4Q), indicating that Iap functions upstream of Myd88 and Tnfr1. Finally, to demonstrate that Iap function was required upstream of proinflammatory compounds associated with the resident microbiota, we examined neutrophil numbers in Iap-deficient larvae reared in the absence of microbes. When reared under GF conditions, both *iap*-MO-injected 6 dpf larvae and L-phen-treated 8 dpf larvae had very low numbers of Mpo-positive neutrophils in their gut epithelia (Figures 4H, 4P, 4Q, and 4R). Wild-type GF intestines were capable of neutrophil recruitment, as demonstrated by a robust neutrophil influx of these animals in response to LPS exposure (Figure 4R). These results argue that GF Iap-deficient larvae were devoid of intestinal neutrophils because they lacked the proinflammatory substrate of Iap, consistent with endogenous Iap functioning to detoxify LPS associated with resident gut bacteria.

DISCUSSION

Using a GF zebrafish model, we showed that LPS induces Iap activity during bacterial colonization of the gut (Bates et al., 2006). Although mammalian IAP is well characterized as a marker of enterocyte cytodifferentation, its endogenous substrates were unknown. IAP is capable of dephosphorylating a number of different LPS serotypes (Tuin et al., 2006), a reaction that produces a nontoxic form of the molecule. Here, we tested the hypothesis

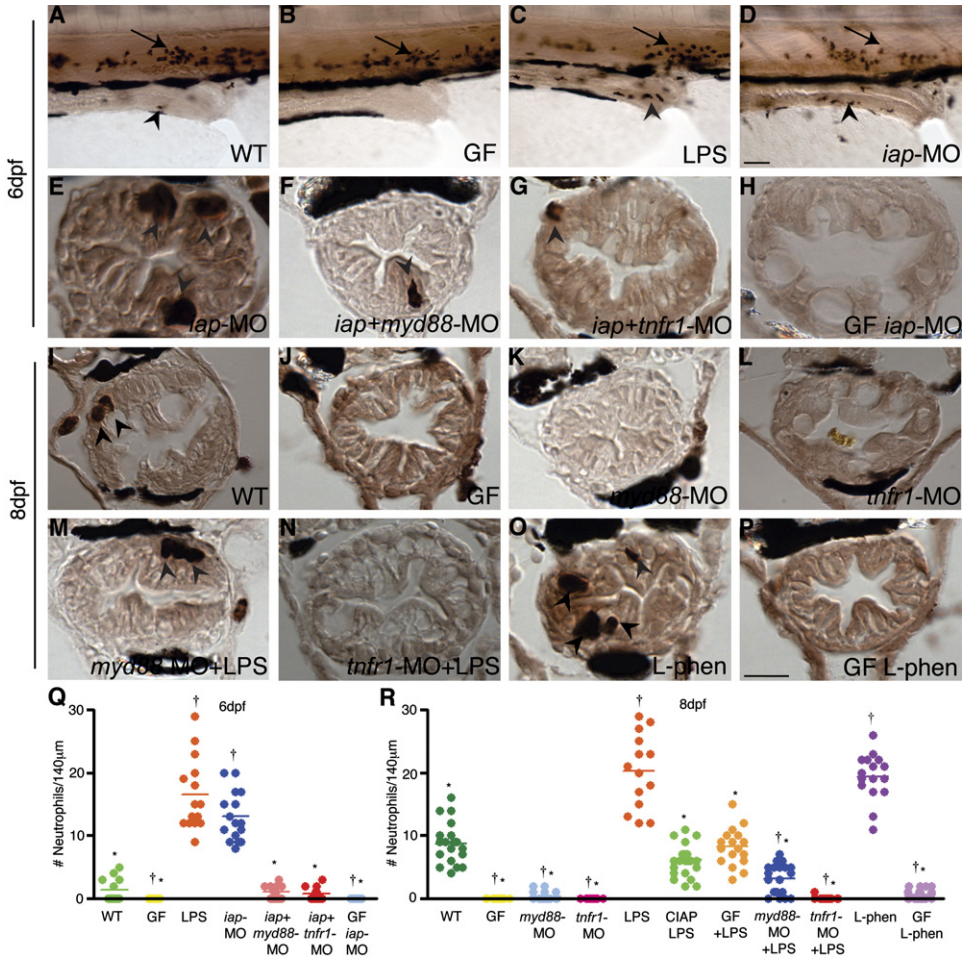

Figure 4. Iap Functions to Prevent Intestinal Neutrophil Infiltration in Response to the Microbiota

Whole-mount larvae at 6 dpf (A–D) and transverse sections through distal zebrafish intestines at 6 dpf (E–H) and 8 dpf (I–P) with Mpo-positive neutrophils visualized in dark brown in the intestinal epithelium (arrowheads) and posterior cardinal vein (arrows) (black cells surrounding the intestinal epithelium are pigment cells). The WT intestine contained low numbers of neutrophils at 6 (A) and 8 dpf (I), whereas GF intestines were devoid of all neutrophils (B and J). Neutrophil numbers increased significantly upon exposure to 150 μg/ml LPS for 2 hr (C) or with inhibition of endogenous Iap with *iap*-MO (D and E) or L-phen (O). Neutrophil infiltration was inhibited in *myd88*-MO (K) or *tnfr1*-MO-injected larvae (L), even upon exposure to 150 μg/ml LPS for 2 hr (M and N) or coinjection with *iap*-MO (F and G). In the absence of microbiota, Iap inhibition did not induce neutrophil influx (H and P). All animals were reared CV unless otherwise indicated. Scale bar in (D), 50 μm. Scale bar in (P), 5 μm. (Q–R) Quantification of neutrophils per 140 μm of distal intestine, n = at least 13 animals per treatment; bar indicates average value for each group. †indicates values that differ significantly from WT, *indicates values differ significantly from WT LPS treated, $p < 0.01$. One-way analysis of variance (ANOVA) show treatments differ significantly, (Q) $F = 99.24$, $p < 0.0001$ (R) $F = 142.7$, $p < 0.0001$.

that zebrafish Iap functions to detoxify LPS associated with resident gut bacteria.

LPS and the downstream products of its signaling are known to be toxic in mammals, but this response has not been previously described in zebrafish. We report that LPS treatment in zebrafish results in dose dependent death, organ failure, characteristic histopathology including swollen hepatocytes and neutrophil infiltration, and dynamic transcriptional induction of the inflammatory cytokine genes, *tnfa* and *tnfb*. It is difficult to compare the lethal dose of LPS between zebrafish and mice because we used a water-born route of administration that would lead to LPS ingestion as well as dermal and gill exposure. Within the 8 dpf larval zebrafish intestine, we estimate there to be approximately 4×10^5 bacteria, most of which are Gram-negative, within a volume of approximately 1 to 4 nl, resulting in a bacterial concentration of approximately 10^{11} cells/ml, similar to the bacterial concentration reported for the human colon (Savage, 1977). Based on measurements of total and shed LPS from *Salmonella* (Freudenberg et al., 1991), this bacterial density would put the concentration of total LPS in the zebrafish intestine on the order of mg/ml and shed LPS on the order of tens of μg/ml, within the range of concentrations used in this study. In mouse models of septic shock, LPS is typically administered intraperitoneally at doses of approximately 50 mg/kg body weight to achieve 100% lethality and is approximately 10-fold less toxic when administered orally

(Youngner, 1972). We showed that in zebrafish, as in mammals, the toxicity of LPS is ameliorated by inhibition of Myd88 and Tnfr. In addition, pretreatment of LPS with purified CIAP rendered it completely nontoxic to zebrafish. Finally, we demonstrated that inhibition of endogenous Iap, either pharmacologically or with a gene-specific MO, resulted in hypersensitivity to LPS toxicity.

To test the role of this enzyme in preventing inflammation in response to the microbiota, we investigated the regulation of neutrophil infiltration into the intestine. We showed that the homeostatic numbers of Mpo-positive neutrophils in the intestinal epithelium are established by the microbiota, with GF larvae lacking all such cells. We did not find evidence for macrophage involvement in the inflammatory response to gut microbes or exogenous LPS. Whereas RNA isolated from dissected guts of GF, CV, and LPS-exposed fish demonstrated significant differences in *mpo* levels correlated with intestinal neutrophil numbers, we observed no differences in transcript levels of the macrophage specific gene, *colony stimulating factor 1 receptor*, between these samples (J.M.B. and K.G., unpublished data). We showed that establishment of intestinal immune cell homeostasis upon microbiota colonization uses the same proteins, Myd88 and Tnfr1, that promote intestinal inflammation in response to exogenously administered LPS. These results suggest the importance of innate immune signaling in the maturation of mucosal immunity, similar to subtle developmental defects observed in the gut-associated lymphoid tissue of *Myd88*-deficient mice (Iiyama et al., 2003). In mice, MyD88 function has been shown to be required exclusively in myeloid cells and not the intestinal epithelium for microbiota-dependent responses to intestinal injury (Pull et al., 2005). It will be of interest to determine the cellular distribution and requirements for Myd88 function in the zebrafish intestine.

The microbiota's capacity to elicit intestinal inflammation through a similar mechanism as endotoxin highlights the importance of host strategies both to select for an appropriate microbial community (a process that may go awry in some inflammatory bowel diseases; Eckburg and Relman, 2007) and to limit the inflammatory impact of this community. Indeed, it is possible that the inhibition of innate immune responses, such as those mediated by Myd88, could alter the intestinal microbial community and, thus, indirectly result in a decrease in intestinal inflammation. However, when we cultured the microbiota of *myd88*-MO-injected and control larvae reared together in the same water, we observed no significant differences in bacterial load or colony morphology between the two groups (J.M.B., E.M., and K.G., unpublished data), arguing against a change in microbiota being responsible for the absence of Mpo-positive neutrophils in the Myd88-deficient intestines.

By characterizing neutrophil infiltration in Iap-deficient fish reared in the presence and absence of microbes, we show that Iap acts upstream of a proinflammatory signal associated with the microbiota. Together our results suggest the model that Iap, which is upregulated by the microbiota during colonization, functions to detoxify microbiota-associated LPS, thereby establishing a homeostatic negative feedback loop that prevents excessive Tlr and Tnf signaling and intestinal inflammation (Figure 5). Thus Iap plays an important role in limiting the proinflammatory potential of resident gut microbes and promoting mucosal tolerance to the Gram-negative bacteria that constitute a significant proportion of the zebrafish microbiota (Bates et al., 2006; Rawls et al., 2006).

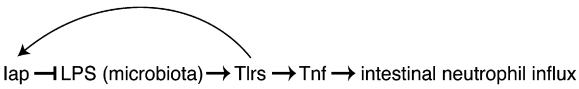

Figure 5. A Model of Iap Function in the Intestinal Epithelium
Iap is induced by microbiota-associated LPS and dephosphorylates this LPS, thereby establishing a homeostatic negative feedback loop that reduces signaling through Tlrs and Tnf and prevents excessive intestinal inflammation.

Importantly, Iap enzymatic activity is highly localized to the apical intestinal epithelium (Bates et al., 2006), and thus, its LPS detoxifying activity would not be expected to impair the host's detection of Gram-negative pathogens that have penetrated the intestinal epithelial barrier. In addition to its brush border localization, mammalian IAP has also been shown to be secreted by enterocytes (Alpers et al., 1995), raising the possibility that this enzyme may have extraintestinal activities, as has been suggested by studies in which rats injected intravenously with purified calf IAP followed by LPS exhibited reduced hepatocyte damage compared to control rats injected with LPS alone (Tuin et al., 2006). Just as the immune system is exquisitely specialized in different tissues, the localized modification of microbial products may represent an underappreciated aspect of microbial-host interactions. Localized expression of a host LPS-detoxifying acyloxacyl hydrolase (AOAH) to the renal cortex of the kidney and secretion of the enzyme into urine may help protect the urinary tract from excessive inflammatory responses to Gram-negative bacteria (Munford, 2005). Notably, no Aoah homologs have been found in fish genomes (Munford and Varley, 2006), suggesting that other LPS-detoxifying mechanisms, such as those involving APs, may play more important roles in these species. Another host enzyme expressed in lung epithelial cells, the mammalian lactonase, Paraoxonase-2 (PON2), has been shown to degrade the bacterial quorum sensing homoserine lactone autoinducer and interfere with signaling mechanisms necessary for *Pseudomonas aeruginosa* tracheal infection (Stoltz et al., 2007). Here we have demonstrated that Iap is another of what may prove to be a large number of host enzymes devoted to modifying bacterial signals encountered by specific tissues. Experiments to test whether IAP's function in preventing inflammatory responses to the microbiota is conserved in mammals are underway.

Humans exhibit a wide range of LPS responsiveness. Possible mechanisms for this phenotypic diversity include extensive polymorphism in the *TLR4* gene in human populations, with *TLR4* alleles that are less responsive to LPS

being correlated with greater incidence of Gram-negative bacterial infection but reduced risk for diseases associated with chronic inflammation (Miller et al., 2005). A considerable degree of genetic polymorphism also exists in mammalian *AP* genes (Millán, 2006) and allelic variants in *Akp2*, encoding TNAP, have been shown to regulate serum AP activity in mice (Foreman et al., 2005) suggesting the possibility of variation in *IAP* genes contributing to LPS responsiveness. Developmental variation in IAP activity may also have important implications for human health. For example, the premature intestine of infants afflicted with necrotizing enterocolitis may be prone to severe inflammatory responses due to a paucity of IAP activity.

Bacterial-animal mutualisms are characterized by careful negotiations between partners in order to optimize their shared environment. Reciprocal signaling exists in these mutualisms with microbial signals modulating host processes that then directly or indirectly influence the composition or activity of the microbiota. Microbial gut residents have been shown to actively suppress inflammation by inhibiting multiple steps in the NF-κB pathway (Ismail and Hooper, 2005). Here, we show that in zebrafish a signal common to many resident gut bacteria, LPS, upregulates Iap, which functions to prevent excessive intestinal inflammation, a response that would be detrimental to microbiota and host alike.

EXPERIMENTAL PROCEDURES

Gnotobiotic Zebrafish Husbandry

All experiments with zebrafish were performed using protocols approved by the University of Oregon Institutional Animal Care and Use Committee and following standard protocols (Westerfield, 1993). CV, GF, and mono-associated larvae were generated, and sterility of GF embryos was assessed as previously described (Bates et al., 2006). To generate GF *iap*-MO injected animals, embryos were produced by in vitro fertilization following the standard procedure (Westerfield, 1993), except for the use of antibiotic embryo medium as described previously (Bates et al., 2006) to fertilize the eggs, and then were injected with *iap*-MO as described below. The injected embryos were then derived GF as previously described (Bates et al., 2006), except the duration of soaking in 0.003% sodium hypochloride was reduced from 30 to 20 min and the embryos were additionally soaked in a solution of 0.1% poly(vinylpyrrolidone)-iodine for 2 min. Bacteria used for mono-associations were zebrafish isolates *Aeromonas veronii* biovar sobria, *Pseudomonas fluorescens* (Bates et al., 2006), *Streptococcus* and *Staphylococcus* strains (J.M.B., E.M., and K.G., unpublished data) and a *Streptococcus* isolate from mouse shown to colonize zebrafish (Rawls et al., 2006). Mono-associated animals were generated by injecting bacterial cultures into flasks of GF 5 dpf larvae to a final concentration of 10^6 colony forming units (CFU)/ml, which achieved a final bacterial load in 8 dpf larvae comparable to that of 8 dpf CV larvae (Bates et al., 2006; E.M. and K.G., unpublished data).

LPS and L-Phenylalanine Treatments

Filter-sterilized solutions of LPS (*E. coli* serotype 0111:B4, Fluka) were injected into flasks of GF and CV larvae to a final concentration of 3-250 μg/ml at 7 dpf unless otherwise indicated. Of note, LPS purchased from Sigma produced variable levels of killing, whereas four different lots of LPS purchased from Fluka gave very reproducible results. Commercial sources of LPS have been shown to contain trace contaminants of peptidoglycan (PGN) and lipoproteins (Hirschfeld et al., 2000). To test whether the LPS toxicity could be due to PGN contaminants, we exposed 7 dpf zebrafish larvae to solutions of purified PGN (*Staphylococcus aureus* 77140, Fluka) up to 1 mg/ml, but did not observe any killing over 24 hr with these treatments. To test whether the LPS toxicity could be due to lipoprotein contaminants, we exposed 7 dpf zebrafish to solutions of synthetic lipopeptide, $Pam_3Cys\text{-}Ser\text{-}(Lys)_4$, (ALX-165-066, Axxora), known to be an immune adjuvant (Erhard et al., 2000), up to 500 μg/ml, and also did not observe killing over 24 hr with these treatments. To generate dephosphorylated LPS, LPS was incubated with 0.148 U/μl CIAP purified from bovine intestinal mucosa (ALPI12G, Biozyme laboratories) for 4 hr at 37°C, then incubated at 80°C for 10 min to destroy CIAP activity. Mock-treated LPS was generated by incubating LPS under identical conditions without the addition of CIAP. To inhibit Iap, larvae were incubated in a solution of 10 mM L-phen for the duration of the experiment, starting at 5 dpf unless otherwise indicated. Some residual Iap activity was observed in the L-phen-treated larvae; however, higher doses of the compound were not readily soluble and appeared to be toxic to the fish.

Alkaline Phosphatase

AP activity was quantified as previously described (Bates et al., 2006). Briefly, dissected intestines from 10 larvae or the carcasses from which the intestines had been removed were pooled, weighed, homogenized, and incubated in p-nitrophenylphosphate liquid substrate system (Sigma) for 30 min, and absorbance was measured at 405 nm.

Morpholino Injections

Splice-blocking morpholinos (MOs) were obtained from Gene Tools (Corvalis, OR), except for MyD88e2i2, which was a generous gift from Dr. Lalita Ramakrishnan (University of Washington). All MOs were injected into embryos at the one cell stage at the indicated final amounts: IAPe2i2 (3 pmoles), TR1v1/TR1v2 (1.2 pmoles and 6 pmoles, respectively), MyD88e2i2 (25 pmoles), and galTe2i2 (5.9 pmoles). MO oligo sequences are as follows: IAPe2i2, 5′-TGTAAAGTCGTCTTCAT CACTCACC-3′; MyD88 e2i2, 5′-GTTAAACACTGACCCTGTGGAT CAT-3′; TR1v1, 5′-TACGTCCTTGTGCATTGCTGGCATC-3′; TR1v2, 5′-CTGCATTGTGACTTACTTATCGCAC-3′; and galTe2i2, 5′-AAATC ATTATGCACTCACCTGATGG-3′. Splice blocking was observed by RT-PCR analysis of cDNA from injected larvae using specific primers designed to span the splice site. Primer sequences are as follows: IAPe1F, 5′-TCAGAGGCTCGGGATGTGTTTG-3′; IAPe5R, 5′-GACTTT CCTTGTGCTTTGGCG-3′; MyD88e1F, 5′-TCTTGACGGACTGGGAAA CTCG -3′; MyD88e5R, 5′-GATTTGTAGACGACAGGGATTAGCC -3′; TR1F, 5′-GCATGGATCCATATCAGGACTTGGTGGA-3′; and TR1R, 5′-TCGAGAATTCTTACGAAACGCTTGTGTT-3′. The *myd88*-MO showed near-complete splice blocking, and the *tnfr1*-MO and *iap*-MO partially blocked splicing of the target transcripts at 8 dpf (Figure S1).

Semiquantitative RT-PCR Analysis

Dissected intestines or the remaining carcasses of 20 control animals or 20 animals treated with 150 μg/ml LPS for 2 hr were pooled, and RNA was harvested by homogenizing and extracting with Trizol reagent (Invitrogen). The RNA was further purified of contaminating genomic DNA using Turbo DNA-*free* kit (Ambion). The RNAs were used as templates for generating cDNAs with Superscript III reverse transcriptase and random primers (Invitrogen) following the manufacturer's instructions. cDNA amplification by PCR was subsequently carried out for 25 cycles, with each cycle consisting of denaturation at 94°C for 15 s, primer annealing at 59°C for 30 s, and extension at 72°C for 1 min. Assays were performed on 200 ng cDNA using Taq DNA polymerase (Roche) and 20 pmol of the gene-specific primers. Primer sequences are as follows: IAPF, 5′-ATGGGAGTGTCCACG GTTTCAG-3′; IAPR, 5′-CGATGCCAACAGACTTTCCTTG-3′; ALPF, 5′-GAAGGTCGTACAACTGCTTATCC-3′; ALPR, 5′-GATTCCCACT GATTTGCCTGC-3′; EF1AF, 5′- CGTCTGCCACTTCAGCATGTG-3′; EF1AR, 5′- ACTTGCAGGCGATGTGAGCAG-3′. Assays were performed using a TProfessional thermocycler (Biometra).

SYBR-Green Real-Time Quantitative (q)RT-PCR Analysis

RNA was harvested from GF, CV, or MO-injected whole larvae at 8 dpf, and cDNAs were generated as above. qRT-PCR assays were performed in 25 μl reactions with cDNA corresponding to 600 ng of total RNA, and 300 nM gene-specific or control primers. Gene-specific primers were designed using Primer Express 2.0 software (Applied Biosystems). Primer sequences are as follows; IAP151: 5′-GCCCTCA CACTGCCTCTCA-3′, IAP233: 5′-GAAACCGTGGACACTCCCATT-3′, TNF1F: 5′-TCCTCAGACCACGGAAAAGTG-3′, TNF1R: 5′-CAACCCA TTTCAGCGATTGTC-3′, TNF2F: 5′-GCTGGATCTTCAAAGTCGGGTG TA-3′, TNF2R: 5′-TGTGAGTCTCAGCACACTTCCATC-3′, EF1AF: 5′-CGTCTGCCACTTCAGCATGTG-3′, EF1AR: 5′-ACTTGCAGGCGATG TGAGCAG-3′. Assays were performed in triplicate using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Data were normalized to *ef1a* (ΔΔ Ct analysis, as described in the ABI User Bulletin No. 2, http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf).

Histology

Unless otherwise specified, zebrafish larvae were fixed in 4% paraformaldehyde (PFA) overnight, embedded in paraffin, and 7 μm thick sections were cut and mounted on glass slides. Samples were imaged on a Nikon TE2000 inverted microscope with Nomarski and fluorescence optics. Digital images were obtained with a CoolSNAP camera (Photometrics) or a QImage Micropublisher camera (QImaging Corp). Images were collected and analyzed using Metamorph and processed in Adobe Photoshop. Hematoxylin and eosin (H&E)-stained 7 μm transverse sections were analyzed for histopathology related to LPS treatment.

In Situ Hybridization

Larvae (5 dpf) were fixed in 4% PFA overnight and washed in 1XPBST. Whole-mount larvae were equilibrated into HYB^+ buffer (Westerfield, 1993) and prehybridized at 65°C for 4 hr. RNA probes were prepared according to Boehringer instructions (Cat. no. 1175025) and 100 ng of RNA probe in HYB^+ was heated at 65°C for 60 min. Larvae were hybridized with RNA probes for 36 hr. Probes were removed by soaking larvae for 30 min at 70°C in 50% formamide in 2× SSC, followed by rinsing for 30 min at 70°C in 2× SSC and rinsing 2 times for 30 min at 70°C in 0.2× SSC. Probes were detected according to the standard protocol (Westerfield, 1993), and larvae were sectioned as described previously for imaging.

Myeloid Peroxidase

Larvae were fixed in 4% PFA overnight and washed in 1× PBS. Whole-mount larvae were stained with Myeloperoxidase kit (Sigma) for histochemical identification of neutrophils. Mpo activity has been used as a marker of zebrafish neutrophils, although weak staining has also been observed in eosinophils and erythrocytes (Lieschke et al., 2001); therefore, care was taken not to overdevelop the Mpo stain. Following staining, larvae were washed in 1× PBS 0.1% Tween, embedded in paraffin, and 7 μm transverse sections were mounted on glass slides. Cells with strong Mpo staining in the intestinal epithelium were quantified in 20 serial sections rostral to the anus, corresponding to the distal 140 μm of the intestine.

Microbiota Enumeration and Analysis

To compare the microbiota of *myd88*-MO injected and WT siblings, these embryos were placed in polycarbonate cylinders with mesh bottoms and reared in the same tank in a shared microbiological aquatic environment. At 8 dpf, animals were euthanized with tricaine methane sulfonate (MS222, Sigma) and rinsed 3 times in sterile water. Ten wild-type or *myd88*-MO-injected larvae were each placed in 100 μl sterile water, homogenized, diluted, and cultured on tryptic soy agar to count CFU and examine colony morphologies. To estimate the concentration of bacteria in the larval zebrafish intestine from which we inferred LPS concentration, we measured the average number of 16S rRNA genes amplified from a known cell number of *Aeromonas veronii* biovar sobria, a dominant member of the zebrafish microbiota, to be approximately 4 copies/CFU. Based on our previous measurements of 16S rRNA copies amplified per zebrafish (Bates et al., 2006), we calculated the bacterial load per 8 dpf larva to be approximately 4×10^5. Based on measurements of histological sections and empirical observations from microinjecting known volumes of liquid into the larval gut, we estimate the volume of this organ to be approximately 1 to 4 nl.

Statistical Analysis

AP activity assays were repeated with at least two trials per treatment and data were analyzed using two-sample t tests assuming unequal variances in Excel (Microsoft Office). Survival curves were performed at least twice, with a combined total of at least 30 fish per treatment. Data for survival curves were graphed and analyzed using GraphPad Prism (Kaplan-Meier analysis, GraphPad Software, http://www.graphpad.com). Neutrophil cell counts were also graphed and analyzed using GraphPad Prism (Scatter plot using one grouping variable for one-way ANOVA) and were also analyzed using two-sample t tests assuming unequal variances in Excel (Microsoft Office).

Supplemental Data

The Supplemental Data include one supplemental figure and can be found with this article online at http://www.cellhostandmicrobe.com/cgi/content/full/2/6/371/DC1/.

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