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The pore-forming protein Aep1 is an innate immune molecule that prevents zebrafish from bacterial infection

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ABSTRACT

Following the *Aeromonas hydrophila* aerolysin, various aerolysin-like pore-forming proteins have been identified from bacteria to vertebrates. We have recently reported the mechanism of receptor recognition and *in vitro* pore-formation of a zebrafish aerolysin-like protein Dln1/Aep1. However, the physiological function of Aep1 remains unknown. Here we detected that *aep1* gene is constitutively expressed in various immune-related tissues of adult zebrafish; and moreover, its expression is significantly up-regulated upon bacterial challenge, indicating its involvement in antimicrobial infection. Pre-injection of recombinant Aep1 into the infected zebrafish greatly accelerated the clearance of bacteria, resulting in significantly increased survival rate. Meanwhile, the induced expression of cytokines such as interleukin IL-1β and tumor necrosis factor TNF-α in zebrafish upon injection of recombinant Aep1 suggested that Aep1 may be a pro-inflammatory protein that triggers the antimicrobial immune responses. However, compared to the overproduction of these cytokines in the infected zebrafish, pre-injection of Aep1 could significantly reduce the expression level of these cytokines, accompanying with a reduced bacterial load. Moreover, the expression profiles through the developmental stages of zebrafish demonstrated that *aep1* is activated at the very early stage prior to the maturation of adaptive immune system. Altogether, our findings proved that Aep1 is an innate immune molecule that prevents the bacterial infection.

1. Introduction

Pore-forming proteins (PFPs) have been found in all kingdoms of life (Bischofberger et al., 2009). They are featured with the capability to form a pore that penetrates the target membrane, usually upon drastic conformational changes from the water-soluble form to the transmembrane assembly (Dal Peraro and van der Goot, 2016; Iacovache et al., 2010). This unique capacity enables PFPs to execute various biological functions, including attack, defense and signaling (Bischofberger et al., 2009; Los et al., 2013). Based on the secondary structural elements that penetrate the membrane, PFPs could be classified into two classes: α-PFPs and β -PFPs (Gouaux, 1997; Iacovache et al., 2010). The β-PFPs, which possess a transmembrane β-barrel, have been the largest and most comprehensively studied class so far (Iacovache et al., 2010). As a popular family of β-PFPs, the aerolysin family was defined with a conserved core aerolysin motif of two β-strands separated by a pre-stem β-hairpin (Szczesny et al., 2011). The aerolysin family was named by the founding member, aerolysin from the Gram-negative bacteria *Aeromonas hydrophila* (Wilmsen et al., 1990); (Li et al., 2011). In fact, the aquatic bacteria *A. hydrophila* can attack fish mainly via aerolysin (Li et al., 2011; Rodriguez et al., 2008). Moreover, *A. hydrophila* aerolysin is also a major toxin contributing to the pathogenicity

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that causes gastroenteritis, deep wound, infections and sepsis in humans (Janda and Abbott, 1998). A previous phylogenetic analysis demonstrated that the aerolysin family is widely distributed from bacteria to vertebrate; and notably zebrafish encodes a dozen of putative aerolysin-like proteins (Aeps) (Szczesny et al., 2011).

Recently we reported the structure of the water-soluble dimeric form of zebrafish aerolysin-like protein Aep1 (previously termed Dln1), and revealed that Aep1 is composed of a C-terminal conserved aerolysin fold, fused with an N-terminal receptor-binding lectin domain (Jia et al., 2016). In combination with the cryo-electron microscopic structure of the octameric transmembrane-form structure, molecular dynamic simulations and biochemical analyses, we proposed a novel process of pore formation. Further biochemical analyses showed that Aep1 possesses a very high affinity toward yeast mannan and gp120 of human immunodeficiency virus (HIV) (Jia et al., 2016), which is similar to human immune protein ZG16p (Tateno et al., 2012) and cyanobacterial anti-HIV protein cyanovirin-N (Boyd et al., 1997). In addition, bioinformatic analysis revealed that Aep1 shares a sequence identity of ~60% to catfish and lamprey natterin-like proteins, which are hypothetical defense molecules against predators (Tamura et al., 2011; Xue et al., 2012). Thus, we hypothesize that zebrafish Aep1 might have a potential role of immune defense. It is known that the adult zebrafish have developed the complete innate (Herbomel et al., 1999, 2001) and adaptive (Davidson and Zon, 2004; Lieschke and Currie, 2007; Trede et al., 2004; Willett et al., 1999) immune systems. However, the cell-mediated and humoral immune responses of the adaptive immune system are not functional at the early developmental stages of larval zebrafish, until the maturation of lymphoid organs and cells (Lam et al., 2004). Therefore, the embryos/larvae of zebrafish in the first 4–6 weeks post-fertilization only possess the innate immunity in response to bacterial or viral challenge (Trede et al., 2004). This clearly defined temporal separation of innate immunity from adaptive immunity makes zebrafish an ideal model to study the innate immunity specific molecules (Novoa and Figueras, 2012; Stockhammer et al., 2009).

Innate immune responses play a key role in the eradication of invading microorganisms, therefore essential for the survival of vertebrates, including zebrafish (Renshaw and Trede, 2012). The innate immune cells, such as neutrophils, monocytes, and macrophages, can be activated by various cytokines to engulf pathogens and release toxic oxygen and nitrogen radicals (Medzhitov, 2010). Interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α) are key cytokines that initiate and amplify a wide variety of effects associated with the host response to microbial invasion (Brenner et al., 2015; Dinarello, 2009; Medzhitov, 2010; Sims and Smith, 2010). However, lowering down the bacterial load in infected host is often along with reduced level of cytokines (Agoro et al., 2017).

Here we investigated the profiles of constitutive expression of zebrafish *aep1*, as well as its induced expression in immune-related tissues upon intraperitoneal infection of bacteria. Pre-injection of the recombinant Aep1 can significantly accelerate the clearance of bacteria probably through inducing the expression of cytokines IL-1β and TNF-α, thus reducing the mortality rate of infected zebrafish. It is also in agreement with a relative low expression level of the cytokines after bacterial clearance assisted by Aep1. The *aep1* gene is activated at the dome stage, much earlier than the maturation of zebrafish adaptive immunity; and moreover, its expression in the larvae of 4 days post-fertilization could be significantly up-regulated upon bacterial infection, strongly suggesting Aep1 plays a role in innate immune response. These findings provide clues to the physiological function of this type of pore-forming protein that exclusively exists in bony fish and lamprey.

2. Materials and methods

2.1. Ethics statement

All animal procedures were conducted in strict accordance with the guidelines and regulations of the Animal Resources Center and University Animal Care and Use Committee of University of Science and Technology of China (USTC). The protocol was approved by the Committee on the Ethics of Animal Experiments of the USTC (Permit Number: USTCACUC1103013). All zebrafish surgeries were performed after anesthetization with tricaine methane sulfonate (MS-222, Sigma) treatment.

2.2. Preparation of biological materials

The wild-type embryos, larvae and adult zebrafish, and chloramphenicol-resistant *Staphylococcus aureus* (*S. aureus*) were used in this study. The embryos, larvae and adult (12–18 months old) zebrafish were reared at 28.5 °C under a 10/14 h light/dark rhythm condition according to the standard protocols. The 150 to 200 embryos were collected after spawning and allowed to develop in a petri dish. The embryos at one-cell, four-cell, dome, 50% epiboly, 80% epiboly, one-somite, five-somite, prim-5 and long-pec stage, and 5-day post-fertilization larvae were respectively selected and anaesthetized in 0.02% tricaine methane sulfonate, then quickly freezed in liquid nitrogen and stored at −80 °C for total RNA extraction. The 4-day post-fertilization larvae were chosen as the representative to construct the infection model. Stages of embryos were denoted by morphological features.

S. aureus of different doses were prepared for microinjection following a previous report (Li and Hu, 2012). The heat-killed *S. aureus* boiled at 95 °C for 1 h was used as control. The recombinant Aep1 protein used for intraperitoneal pre-injection was obtained as we reported before (Jia et al., 2016).

2.3. Microinjections of S. aureus into zebrafish

Adult zebrafish were anaesthetized by immersion in 0.02% tricaine methane sulfonate and intraperitoneal microinjected with *S. aureus* of various doses ranging from 10^5 to 10^9 colony forming units (CFU). The control group was microinjected with sterile phosphate buffered saline (PBS) buffer. Each group contained ten fish. The 4-day post-fertilization larvae were infected by microinjection of *S. aureus* suspension of 1700 CFU into the yolk, following a previous report (Li and Hu, 2012). Microinjection was performed with the following equipments: a pneumatic micro-pump (PICOSP RITZER III, USA), a micromanipulator (KANET EC, MB-K, Japan) and a stereoscopic dissecting microscope (SM20, TECH, China). The total RNA was extracted from the fish recovered in approximately 5–10 min following microinjection.

2.4. Pre-injection of recombinant Aep1 into zebrafish

To assess the effect of Aep1 on the survival rate of zebrafish, recombinant Aep1 protein of 0.0056–0.56 μg/g (protein/zebrafish) in sterile PBS buffer was intraperitoneally microinjected into the adult zebrafish 4 h before *S. aureus* infection following a previous report (Xiang et al., 2014). Sterile PBS buffer with the same volume was used as control. The percentages of survival zebrafish were determined at different time after infection. In addition, the zebrafish pre-injected with recombinant Aep1 at a dose of 0.056 μg/g were harvested at 4 and 8 h post-infection, and homogenated in sterile PBS buffer for the counting of rescued *S. aureus*. For bacterial counting, the homogenated fluids were serially diluted by the 10-fold dilution technique. Using the spread-plate

method, 100 μL of each dilution was plated out in triplicate sets of Luria-Bertani medium containing chloramphenicol. The plates were then incubated at 37 °C for 18 h, and the distinct colonies were counted. The bacterial numbers were expressed as log of CFU. As for the detection of cytokines IL-1β and TNF-α, Aep1 at a dose of 0.056 μg/g was pre-injected 4 h before injection by 10^8 CFU *S. aureus*, and then the spleens were extracted at 4 h post-infection.

2.5. Tissue samples, RNA extraction and real time PCR

The samples of skin, blood, gill, heart, spleen, liver, kidney, intestine and ovary were collected from adult zebrafish at 0, 4 and 8 h post-infected for the total RNA extraction. Various tissues of zebrafish in each group were dissected as previously described (Gupta and Mullins, 2010). Twenty embryos or larvae per developmental stage were collected together for the total RNA extraction. Similarly, each twenty 4-day post-fertilization larvae at 1.5 and 3 h post-infection were used to extract the total RNA, respectively. Each extraction was performed in triplicate.

We used Trizol Reagent solution (Takara, Japan) to extract the total RNA according to the manufacturer's protocol. The RNA quality was checked by agarose gel electrophoresis and a DU800 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). The cDNA was synthesized from DNA-free RNA by reverse transcription using a PrimeScript™ RT reagent Kit (Takara) with Oligo (dT) primers and random hexamer primers following manufacturers' instructions.

Real time PCR was performed on cDNA using FS universal SYBR Green Master (Roche) with the StepOne TM Real-Time PCR System (Applied Biosystems). Primers specific for *aep1* were designed using Primer Premier 5.0 software. The primers used are listed in Table 1. The transcription ratios of *aep1*, *IL-1β* and *TNF-α* to *β-actin* were calculated using the relative quantification analysis module of the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) based on Ct values. All real time PCR experiments were repeated three times.

2.6. Antibacterial experiments

Disk diffusion test was done to determine the antibacterial activity of Aep1 of different concentration against the chloramphenicol-resistant *S. aureus*, as marked by their zone of inhibition. Following a previous report (Graham et al., 1985), Aep1 impregnated on filter paper disks (~5 mm diameter) were placed on an agar plate having uniform *S. aureus* suspension (10^7 CFU/ml). The culture plates were then incubated at 37 °C for 24 h and the zone of inhibition was measured using photographic images of the agar plates.

2.7. Statistical analyses

Experiments were performed in triplicate. A log-rank test was used to compute *P* values in order to compare the Kaplan–Meier survival curves of zebrafish infected by *S. aureus*. Other data were analyzed using one-way ANOVA or *t*-test with GraphPad Prism version 5.0 (Prism, San Diego, CA, USA). The results are shown as mean ± S.E.M., and $P < .05$ is considered statistically significant. ** and *** represent $P < .01$ and $P < .001$, respectively.

Table 1
Sequences of primers used for detection of *aep1*, *IL-1β and TNF-α* in this study.

Primer	Sequence	Accession Number
β-actin-Forward	AAGCAGGAGTACGATGAGTCTG	NM_131031
β-actin-Reverse	GGTAAACGCTTCTGGAATGAC	
aep1-Forward	TCGGCTCTGGTTATTGTCTTGG	NM_001013304
aep1-Reverse	TCGTTTTTCTCATCCGTCTGCT	
IL-1β-Forward	TGGTGGATTCAGTGCCGTCTTAC	BC098597
IL-1β-Reverse	CCACCATCTGCGAATCTTCATAC	
TNF-α-Forward	CTTTACCGCTGGTGATGGTGTC	AY427649
TNF-α-Reverse	AAAGACACCTGGCTGTAGACAAA	

3. Results and discussion

3.1. The aep1 gene is constitutively expressed in immune-related tissues of adult zebrafish

To investigate the expression profile of *aep1* in various tissues, we dissected the immune-related tissues of adult zebrafish including skin, blood, gill, heart, spleen, liver, kidney, intestine (Trede et al., 2004) (Rauta et al., 2012; Zapata et al., 2006); and a non-immune-related tissue ovary, respectively for total RNA extraction. The *β-actin* gene was used as the internal reference, as it is one of the most stable housekeeping genes across different developmental stages and various different tissues (Chandrasekar et al., 2010). The real time PCR expression analysis showed that a given fragment of *aep1* could be detected from several immune-related tissues applied to the detection (Fig. 1), indicating that *aep1* is constitutively transcribed in these tissues. Compared to the expression level of *aep1* in the skin, which serves as the first barrier against bacterial invasion (Esteban, 2012), the heart, spleen, liver, gill and blood have a much higher expression level, and up to about 4 folds in the blood (Fig. 1). In contrast, the expression level of *aep1* in the kidney and intestine is much lower, and almost undetectable in the ovary.

3.2. Expression of aep1 is up-regulated upon bacterial infection

S. aureus has been widely used as an ideal zebrafish infection pathogen to study immunity, infection, and potential therapies (Li and Hu, 2012; Lin et al., 2007; Medina and Royo, 2013). To further explore whether the expression of *aep1* is inducible upon bacterial infection, we compared the expression level of *aep1* in adult zebrafish subject to *S. aureus* challenge. To minimize the interference of mechanical injury to the survival rate of zebrafish, we utilized intraperitoneal microinjection to infect zebrafish. We used a series of doses ranging from 10^5 to 10^9 CFU of *S. aureus* to infect zebrafish, and set the dose to 10^7 CFU for the following experiments

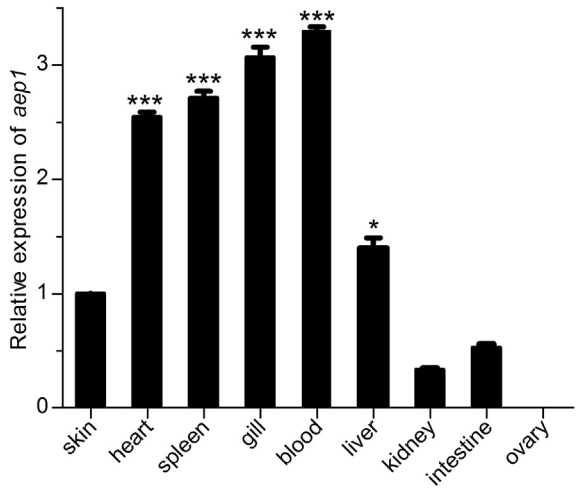

Fig. 1. Expression profiles of *aep1* gene in various zebrafish tissues by real time PCR. The *β-actin* gene was used as a control of housekeeping gene. The expression level of *aep1* gene in the skin was set to 1 unit. *** indicates significant difference, at $P < .001$.

of bacterial challenge (Supplementary Fig. 1). As expected, the infected zebrafish exhibited hemorrhage and swell in thoracic cavity, abdomen, and anal region, the same symptoms that have been previously reported (Li and Hu, 2012). Notably, the expression level of *aep1* in the spleen increased to about 80-fold both at 4 and 8 h post-infection (Fig. 2). It is in agreement with the fact that the spleen of zebrafish is a major lymphoid tissue (Zapata et al., 2006) and the center for antigen trapping, presentation, degradation and initiation of the adaptive immune (Rauta et al., 2012). Similarly in the liver where innate immune-related antimicrobial peptides and acute phase proteins are biosynthesized (Gao et al., 2008), the expression level of *aep1* gene increased to about 9-fold at 4 h and 7-fold at 8 h post-infection, respectively (Fig. 2). In addition, as a major immune organ responsible for phagocytosis, antigen processing through melanomacrophagic centers, the kidney also showed an approximately 5-fold up-regulation at 8 h post-infection (Fig. 2). Furthermore, the expression of *aep1* in the skin was up-regulated to about 3-fold at 4 h post-infection, whereas the expression in the intestine reached a much higher level, up to about 10-fold at 8 h post-infection (Fig. 2). The significantly up-regulated expression of *aep1* in immune-related tissues upon bacterial challenge indicated its involvement in antimicrobial infection.

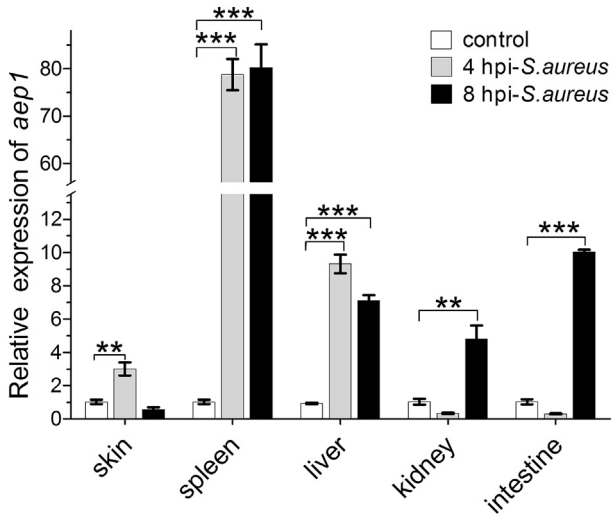

Fig. 2. The expression level of *aep1* in the adult zebrafish upon *S. aureus* challenge. The *aep1* transcripts were extracted from the corresponding tissues of infected zebrafish at 4 or 8 h post-injection (hpi), and quantified by real time PCR. Each histogram represents the average value of triplicate experiments (**$P < .01$ and ***$P < .001$).

3.3. Pre-injection of Aep1 protein can prevent zebrafish from bacterial infection

Because of the inducible expression of *aep1* upon bacterial challenge, we supposed that this protein might play a role in antimicrobial immune responses. Accordingly, we constructed a zebrafish peritonitis model to test this hypothesis. The pathogenic bacterium *S. aureus* was used to test the putative effect of recombinant Aep1 on the bacterial infection. Intraperitoneal injection of the bacteria resulted in the infection and consequent death of zebrafish. However, pre-injection of Aep1 4 h in advance significantly reduced the mortality of zebrafish upon bacterial infection (Fig. 3a). To explore the optimum dose of recombinant Aep1 protein that protects zebrafish from infection, we pre-injected 0.0056–0.56 μg/g (protein/zebrafish), respectively. As shown in Fig. 3a, intraperitoneal microinjection of Aep1 with 0.056 μg/g significantly improved the survival rate of zebrafish, whereas a dose of 0.0056 μg/g had no detectable effect. In contrast, pre-injection with a higher dose up to 0.56 μg/g seems to be toxic to the zebrafish, as it led to a lower survival rate compared to that of 0.056 μg/g (Fig. 3a). Therefore, we further semi-quantified the effect of pre-injection with a dose of 0.056 μg/g, and revealed a much more effective clearance of bacteria compared to the control group that pre-injected with PBS buffer, at both 4 and 8 h after infection (Fig. 3b). The results indicated that Aep1 prevent zebrafish from microbial infection in a dose-dependent manner.

3.4. Aep1 induces the production of cytokines in response to bacterial infection

As Aep1 had no direct killing or inhibitory effects on bacteria (Supplementary Fig. 2), we proposed that Aep1 might stimulate the antimicrobial immune responses of zebrafish. The induction of cytokines such as IL-1β and TNF-α is an important constituent of innate immune activation (van der Vaart et al., 2012). Thus we detected the *IL-1β* and *TNF-α* expression in the spleen of zebrafish only injected with recombinant Aep1. The results showed significantly increased expression levels of both *IL-1β* and *TNF-α* genes (Fig. 4), which indicated that Aep1 may be a pro-inflammatory protein involved in immune responses. In fact, bacterial infection also leads to the production of IL-1β and TNF-α, whereas, the overproduction of these cytokines can be deleterious, and cause morbidity and shorten lifespan (Rosas-Ballina et al., 2015; Tracey, 2002; van der Vaart et al., 2012). In agreement with these reports, microinjection of 10^8 CFU *S. aureus* brought a sharply increased expression of both *IL-1β* and *TNF-α* genes in the spleen (Fig. 4) and a higher mortality rate of zebrafish (Fig. 3a). However,

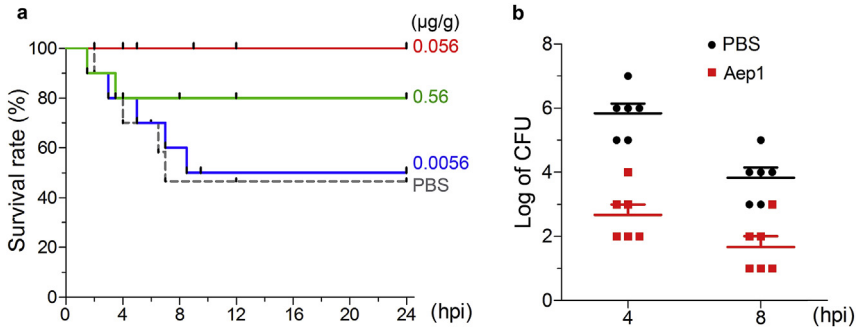

Fig. 3. (**a**) The survival rate of infected zebrafish pre-injected with Aep1 at various doses. The grey, blue, red and green lines refer to the zebrafish pre-injected with the PBS buffer and recombinant Aep1 of 0.0056, 0.056, 0.56 μg/g (Aep1/zebrafish), respectively. (**b**) Rescued *S. aureus* from the infected zebrafish. The red and black lines represent the number of rescued bacteria from the *S. aureus* infected zebrafish that were pre-injected with 0.056 μg/g (Aep1/zebrafish) or PBS buffer 4 h in advance, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

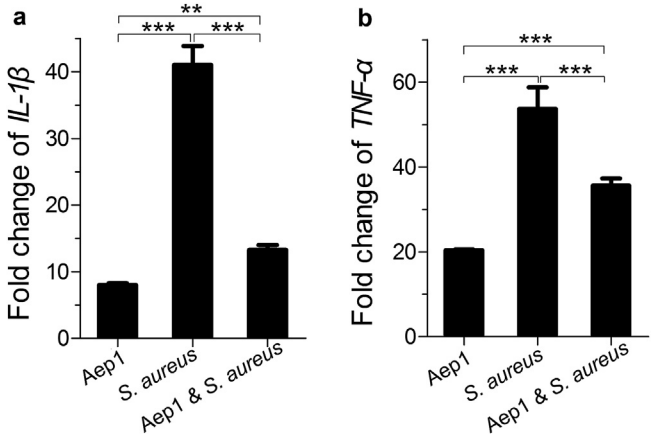

Fig. 4. The expression level of **(a)** *IL-1β* and **(b)** *TNF-α*. Zebrafish were injected with recombinant Aep1 protein only, and 10^8 CFU *S. aureus* with or without pre-injection of recombinant Aep1 protein 4 h in advance. ** stands for $P < .01$ and *** for $P < .001$.

these two cytokine genes showed remarkably decreased expression level in the infected zebrafish that were pre-injected with recombinant Aep1 4 h before infection (Fig. 4). We proposed that pre-injection of Aep1 induced the expression of cytokines such as IL-1β and TNF-α to activate the antimicrobial immune responses, and then significantly accelerated the clearance of bacteria. As a result, the reduced bacterial load in zebrafish (Fig. 3b) accompanied with a decreased expression level of related cytokines (Fig. 4), which resulted in an increased survival rate of zebrafish (Fig. 3a), as reported previously (Agoro et al., 2017). It is in agreement with a recent report that the frog aerolysin-like protein βγ-CAT is involved in the host innate immune responses against microbial infection via accelerated IL-1β release (Xiang et al., 2014).

3.5. The aep1 gene is active in the early developmental stages of zebrafish

The cytokines TNF-α and IL-1β are major innate immunity molecules in response to microbial challenges (Arango Duque and Descoteaux, 2014; Mizgerd et al., 2001). To characterize whether Aep1 is involved in innate immunity, we examined the expression of *aep1* gene in various developmental stages from the embryos to larvae, as zebrafish possess only the innate immune system, but not the adaptive immune system, in the first 4–6 weeks after fertilization (Davidson and Zon, 2004; Lieschke and Currie, 2007; Trede et al., 2004). Accordingly, we detected the temporal expression of *aep1* in zebrafish at ten defined developmental stages from the one-cell stage to the 5-day larvae (Fig. 5a). As shown in real time PCR results, the expression of *aep1* gene becomes detectable at the dome stage, and slowly increases along the following stages, eventually reaches a relatively high level at the long-pec and 5-day larvae stages (Fig. 5b). Therefore, the activation of *aep1* gene in zebrafish is much prior to the maturation of adaptive immunity. The high constitutive expression level of *aep1* at the long-pec stage of hatching period might be accompanied with the development of thymus, one of the major lymphoid tissues (Danilova et al., 2004; Zapata et al., 2006). Moreover, using the 4-day post-fertilized zebrafish as a representative, the expression level of *aep1* is sharply up-regulated 3 h after *S. aureus* infection (Fig. 5c). Altogether, it suggested that Aep1 plays a role in the innate immune response at the early developmental stages from embryos to larvae.

In summary, we systematically investigated the constitutive and induced expression profiles of zebrafish *aep1* gene, and antibacterial effects of recombinant Aep1 protein. Our findings demonstrated that Aep1 is an innate immune molecule that prevents zebrafish from bacterial infection.

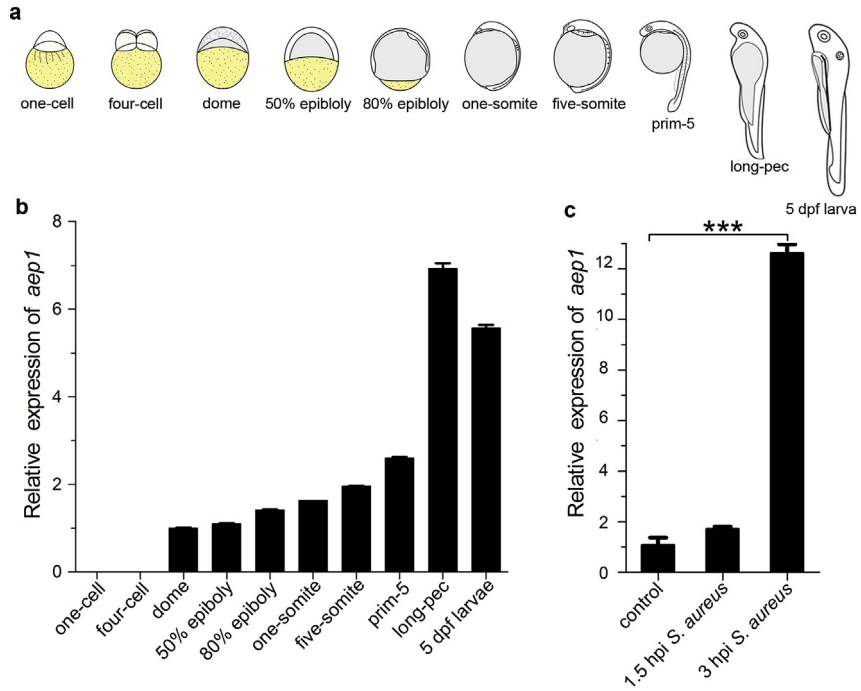

Fig. 5. The expression levels of *aep1* in various developmental stages from embryos to the 5-day larvae. (**a**) The ten stages include the nine stages during embryogenesis of embryos including one-cell, four-cell, dome, 50% epiboly, 80% epiboly, one-somite, five-somite, prim-5, long-pec, and the 5-day post-fertilization (dpf) larvae. (**b**) Relative expression levels of *aep1* in zebrafish at different developmental stages by real time PCR. Data are represented of three independent experiments. (**c**) Expression of *aep1* in 4-day larvae in response to *S. aureus* challenge. The *aep1* transcripts were extracted 1.5 or 3 h after *S. aureus* infection, and quantified by real time PCR. *** stands for $P < .001$.

Author contributions

L.L.C., N.J., J.X., B.H., Y.C., C.Z.Z. conceived and designed the experiments. L.L.C., N.J., Y.J.L., H.S. performed the experiments. L.L.C., N.J., J.X., W.F.L., B.H., Y.C., C.Z.Z. analyzed the data. J.X. drew the cartoon example of Fig. 5a. Y.J.L. contributed reagents/materials/analysis tools. L.L.C., J.X., D.D.C., Y.C., C.Z.Z. wrote the manuscript.

Statement of competing financial interests

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.dci.2018.01.003.

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