Published in final edited form as:

Fish Shellfish Immunol. 2009 February ; 26(2): 326–331. doi:10.1016/j.fsi.2008.12.004.

LPS response and tolerance in the zebrafish (*Danio rerio*)

B. Novoa¹, TV. Bowman², L. Zon², and A Figueras^{1,*}

¹ Instituto de Investigaciones Marinas, Consejo Superior de Investigaciones Científicas (CSIC), Eduardo Cabello, 6, 36208-Vigo, Spain

² Children's Hospital, Howard Hughes Medical Institute, Karp Family Research Laboratories, 7th Floor, Room 7211, One Blackfan Circle, Boston, MA 02115

Abstract

Zebrafish (*Danio rerio*) has been used in the present work to study the fish response to bacterial lipopolysaccharide (LPS) exposure and LPS tolerance. These mechanisms are not completely understood in mammals and, presently, are totally unknown in fish. Zebrafish larval survival was assessed following treatment with various types of LPS at a variety of concentrations to determine the sensitivity of zebrafish to LPS-induced immune activation. In addition, fish pre-treated with a sublethal concentration of LPS did not die after exposure to a lethal concentration of LPS demonstrating, for the first time, that LPS tolerance also happens in fish. The time interval between pretreatment and secondary exposure as well as the type of pretreatment dictated the strength of protection. Since zebrafish are in intimate contact with microorganisms, the high resistance of fish to LPS suggests that there must be a tight control of the LPS receptor cluster in order to avoid an excess of inflammation. One of these components is CXCR4, which has previously been shown to regulate the signal transduced by TLR4. Treating fish with AMD 3100, a specific inhibitor of CXCR4, increased LPS treatment associated mortality. Blocking CXCR4 via chemical or genetic inhibition resulted in a reversion of LPS tolerance, thus further supporting the negative regulatory role of CXCR4 in this inflammatory response. In support of an inhibitory role for CXCR4 in the inflammatory cascade, IL1 transcript levels were elevated in both unstimulated and LPS stimulated zebrafish *Odyseus* (CXCR4 deficient mutant) larvae.

Introduction

In mammals, microbial products such as lipopolysaccharide (LPS) or endotoxin, are potent inducers of inflammation that stimulates immune system cells after they are recognized mainly by Toll-like receptors (TLRs), a family of closely related transmembrane proteins that initiate signaling cascades. Gram-negative enterobacterial LPS signals through TLR4, which involves downstream molecules MyD88, TIRAP/Mal, IRAK and TRAF6, and leads to production of proinflammatory cytokines, proteases, eicosanoids, and reactive oxygen and nitrogen species [1]. Gram-positive bacteria usually activate cells in a TLR2-dependent fashion. In the case of *Pseudomonas aeruginosa* LPS, the involvement of both TLR2 and TLR4 has been reported [2]. If the inflammatory response to infection is not tightly controlled, several pathological processes may develop, including septic shock. Although LPS-induced proinflammatory molecules such as interleukin-1 (IL-1) or tumour necrosis factor (TNF α), are important for

*Corresponding Author. Contact: Antonio Figueras, Instituto de Investigaciones Marinas.CSIC. Eduardo Cabello, 6 36208 Vigo, Spain, Tel: 34 986 214462, Fax: 34 986 295762, E mail: antoniofigueras@iim.csic.es.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

avoiding the growth and dissemination of Gram-negative bacteria, their overproduction can lead to endotoxin shock which is a severe systemic inflammatory response triggered by the interaction of LPS with host cells, characterized by fever, myocardial dysfunction, acute respiratory failure, hypotension, multiple organ failure, and in a large number of cases, death [1,2]. It is well known in mammals, that a previous exposure to LPS induces “endotoxin tolerance” which is thought to protect the host from the endotoxic or septic shock, although the involved mechanisms are not fully understood. In fact contradictory results have been reported. Tolerance can limit neutrophil proinflammatory responses limiting neutrophil responses in vivo, potentially preventing excessive cell activation [3].

In recent years, zebrafish (*Danio rerio*) has been widely used in research areas such as cancer, stem cell research or development, however, due to its advantages it is starting to be used in other fields. It could also be appreciated in immunology and infectious diseases research: zebrafish larvae are transparent, easy to rear and only the innate immune system is present until several weeks postfertilization, thus simplifying analysis of immune responses [4]. In addition, physiological responses can be studied with the whole organism. Moreover, Purcell et al. [5] have characterized the key components of the TLR-signaling pathway including MYD88, TIRAP, TRIF, TRAF6, IRF3, IRF7 in zebrafish. It is also already reported that the main receptor for LPS is expressed in zebrafish at early times of infection [6,7], however, accessory molecules such as CD14, that are essential for the response to LPS in mammals, seem not to be present in the zebrafish genome [8].

The SDF1-CXCR4 system plays a role in hematopoietic cell migration during mammalian development [9] and recently has been implicated in germ cell and neuronal migration in developing zebrafish [10–13]. Cell migration is not only involved in aspects of development, organogenesis and organ function, but also plays a role in several pathological processes, such as the spread of tumour cells and formation of metastases [9] and the inflammatory responses. In fact, CXCR4 belongs to the cluster that participates in the LPS recognition after LPS binding protein and CD14 transfers the LPS from the extracellular space to the membrane, probably inhibiting the TLR4 in order to control an excess of inflammatory response [14].

In this work we have explored the use of zebrafish larvae as a new model for the study of LPS exposure associated mortality and LPS tolerance which is a hypo-responsive state to a second exposure to LPS.

Material and methods

Zebrafish and embryos were maintained according to standard protocols [15]. *Odysseus* mutants [11], which have a mutation in the CXCR4 gene, were also maintained according to standard protocols. All the mortality experiments were conducted using replicates of 10–15 fish each. Fish were maintained in 6 well plates at 28 °C during the treatments.

Zebrafish larvae were bathed in a range of concentrations of *Escherichia coli* 0111:B4, *P. aeruginosa* LPS (Sigma). Zebrafish larvae of 2, 5 and 10 days post fertilization (dpf) were treated with 0, 5, 25, 50, 150 and 200 µg/ml of 0111:B4 *E. coli* LPS. The same concentrations were used for *Pseudomonas aeruginosa* LPS (Sigma) Mortality was recorded regularly for 48 h.

Tolerance experiments were conducted using two days-old larvae that were exposed to sublethal LPS concentrations (50 µg/ml of *E. coli* 0111:B4 or 2.5; 5 and 10 µg/ml of *P. aeruginosa*). At different times post treatment, larvae were exposed to a lethal concentration of *E. coli* 0111:B4 or *P. aeruginosa* LPS.

Cross tolerance experiments were conducted using one or two pretreatments of 50 µg/ml of different PAMPs (*E. coli* 0111:B4 LPS, *E. coli* 055: B5 LPS (Sigma), *P. aeruginosa* LPS, β glucans (Macrogard), lipoteichoic acid from *Staphylococcus aureus* (Sigma), poly I:C (Sigma)) followed by a lethal concentration of *E. coli* LPS (150 µg/ml).

To investigate the involvement of CXCR4 on the larvae response to LPS, treatments with AMD3100 (Sigma) which is a pharmacological specific CXCR4 inhibitor, were studied. In order to find a nontoxic concentration for zebrafish larvae several concentrations were assayed (1 ng/ml, 10 ng/ml, 100 ng/ml, 1 µg/ml, 10 µg/ml, 100 µg/ml). Treatments with AMD 3100 alone or combined with a sublethal LPS pretreatment, previous to a lethal LPS concentration exposure, were used. Zebrafish *Odysseus* mutants, which have a mutation in the CXCR4 gene, were also used to clarify CXCR4 role in LPS tolerance.

Quantitative PCR assays were performed using the 7300 Real Time PCR System (Applied Biosystems) using pooled samples of 4–5 fish larvae. cDNA amplification was performed using specific primers designed by Primer 3 software [16]. 0.5 µl of each primer (10 µM) was mixed with 12.5 µl of SYBR green PCR master mix (Applied Biosystems) in a final volume of 25 µl. The standard cycling conditions were 95 ° for 10 min, followed by 40 cycles of 95 ° C 15 s and 60 ° C for 1 min. The comparative CT method (2-ΔΔCT method) was used to determine the expression level of analyzed genes [17]. The expression of the candidate genes was normalized using β-actin as a housekeeping gene. IL-1 was amplified with primers Forward ATC TCC ACC ATC TGC GAA TC and Reverse: AAC CTG TAC CTG GCC TGT TG and β-actin was amplified with primers: Forward: CAA CGG AAA CGC TCA TTG C and Reverse: CGA GCA GGA GAT GGG AAC C. Data were analyzed using a Student's *t*-test and differences were considered statistically significant at $p < 0.05$.

Results

1. Mortality caused by LPS exposure

Only the highest concentrations (150 and 200 µg/ml) of *E. coli* 0111:B4 were able to induce zebrafish larvae mortalities (Figure 1). However, LPS from *Pseudomonas aeruginosa*, was able to kill the fish at lower concentrations (50–100 µg/ml) than *E. coli* LPS (Figure 2).

The final *E. coli* LPS concentrations of 150 µg/ml or 50–100 µg/ml of *P. aeruginosa* were reproducibly lethal concentrations for wild type zebrafish embryos.

2. Induction of tolerance using LPS and pathogen associated molecular patterns (PAMPs)

In order to know if low LPS concentrations could produce tolerance in zebrafish, 2 dpf embryos were first treated with a sublethal concentration of 50 µg/ml of *E. coli* 0111:B4 LPS followed by an exposure to a lethal concentration of the same LPS serotype. The timing of administration of sublethal and lethal concentrations was critical (Figure 3). Tolerance was always observed when the time interval between pretreatment with the sublethal and exposure to the lethal concentration was at least 24 h. Exposure to a sublethal treatment 6 h before the exposure to the lethal concentration was not sufficient to confer protection. On the contrary, tolerance was observed when 4 days was the difference between treatments with the sublethal and lethal concentrations.

In all the conducted experiments, tolerance was always observed when LPS from *E. coli* 0:111 was used as sublethal concentration one day before of a lethal concentration of *E. coli* LPS (150 µg/ml) or *Pseudomonas* LPS (50–100 µg/ml). However, tolerance was not observed when larvae were pretreated with sublethal concentrations of *Pseudomonas* LPS (data not shown).

Other PAMPs were investigated to determine their ability to induce cross tolerance (protection) to LPS exposure. While some protection was observed with a single pretreatment, two treatments of 50 µg/ml of each PAMP were more effective in the induction of tolerance (Figure 4). In this case, LTA and the two *E. coli* LPS produced a complete protection of fish and poly I:C induced a delay in mortalities caused by a lethal concentration of LPS.

IL-1 transcript levels increased with time in larvae following a lethal exposure to LPS (Figure 5). A decrease in IL-1 transcript levels was observed in larvae treated with a sublethal concentration after 3 h.

3. CXCR4 involvement in the response to LPS treatment (AMD3100 treatment and *Odysseus* mutant fish)

With the aim to determine if CXCR4 has a role in the LPS tolerance of fish, we blocked its function via chemical and genetic approaches. First, after using a range of several concentrations of AMD3100, a pharmacological specific CXCR4 inhibitor [18,19], we found that the 10 µg/ml concentration was nontoxic for zebrafish larvae. Larvae exposed to AMD3100 were more sensitive to LPS treatment; lower concentrations of LPS led to lethality in the presence of AMD3100 (Figure 6A). Moreover, fish receiving AMD3100 treatment during the tolerization period were not able to survive after the exposure to a lethal concentration of LPS although they were treated previously with a protective sublethal concentration of LPS as in the experiments already described (Figure 6B).

Odysseus mutants, which are mutated in the CXCR4 gene, did not show higher sensitivity to LPS, but no LPS tolerance was observed (Figure 6B). To directly test the effect of CXCR4 loss-of-function on the downstream effects of the inflammatory cascade, IL-1 transcript levels were determined in *Odysseus* mutants before and after a sublethal exposure to LPS (Figure 6C). IL-1 levels were found to be high in *Odysseus* mutants without LPS exposure and additionally a significant increase in IL-1 level was seen in *Odysseus* mutants following treatment.

Discussion

The inflammatory cascade begins with the receptors involved in the binding and uptake of bacteria and their products by cells of the innate immune system. It continues with the production of proinflammatory cytokines, such as TNF- α , IL-1, and IL-8, lipid mediators, oxygen radicals, and tissue-damaging enzymes [20]. In this work we have shown that from an early age, zebrafish larvae (2dpf) are able to produce an inflammatory response when exposed to LPS.

The minimum lethal LPS concentration was much higher than in mammals and this led us to wonder why and how fish are so resistant to LPS and in general to other PAMPs as it has also been reported [8,21–23]. Although resistance to LPS has been observed in other non-mammalian vertebrates [8,24], fish live in water and therefore in intimate contact with a potentially high amount of microorganisms. If a high inflammatory reaction was triggered after each contact with external putative pathogens, fish simply could not survive. Thus, inflammation and sepsis should be tightly regulated in these animals due to their environment placement.

In agreement with what is reported for mammals [25] *P. aeruginosa* LPS was more lethal than *E. coli* LPS. Interestingly, the pretreatment of zebrafish larvae with different *P. aeruginosa* LPS concentrations did not protect to the subsequent exposure with a lethal concentration with the same LPS. On the contrary, the pretreatment with a non lethal LPS (*E. coli*) did protect when

fish were exposed to the *P. aeruginosa* LPS. This needs further research to clarify the involved molecular mechanisms.

As observed in mammalian macrophages, exposure of zebrafish larvae to high concentrations of LPS leads to death, probably related with a high production of proinflammatory cytokines and other molecules. However, the present work shows that if the fish are first treated with a sublethal concentration, this induces a hypo-responsive state to a second treatment of LPS that is known as LPS tolerance. In mammals, TLR homotolerance is consistently stronger than TLR heterotolerance (with other different PAMPs) [26]. This agrees with our experiments since although we could detect a complete protective role after two administrations of lipoteichoic acid (component of the surface of Gram-positive bacteria) to an exposure of a lethal concentration of LPS, this was not observed when only one pretreatment was given. However, no protection was achieved when β -glucan was used; this molecule interacts with a signalling non-TLR pattern-recognition receptor, dectin-1, but whether this is the cause of the lack of protection observed needs further research. Poly I:C, which mimics a viral infection, showed an intermediate effect. These different responses are incompletely understood mechanisms in mammals and, until now, totally unknown in fish.

IL-1 has been involved as a mediator of tolerance *in vivo* in mammals [27]. Our results show that the expression of IL-1 increases when fish are treated with high LPS concentrations, which would imitate the over-production of proinflammatory cytokines that is produced in the cases of sepsis. However, IL-1 decreases in the hypotolerized state induced by lower LPS concentrations which is in agreement with the reported inhibited expression of many cytokines, e.g., TNF α , IL-1 β , IL-6, and IL-12 in cases of LPS tolerance [28].

Several studies have pointed out that the chemokine receptor CXCR4 seems to be a functional part of the LPS-sensing apparatus [14,27], and could have a role inhibiting the signaling cascade initiated by TLR4. In the *Odyseus* fish, in which CXCR4 function is inhibited, the expression of IL-1 was higher than in wild types in the unstimulated state and was increased even more when fish were treated with LPS.

When wild type zebrafish larvae were treated with AMD3100, a pharmacological specific CXCR4 inhibitor [18,19] no tolerization was obtained. Similar results were obtained in *Odyseus* fish. These observations strongly suggest that CXCR4 could have a key role in modulating zebrafish immune responses to endotoxin LPS, since CXCR4 impairment (genetic or pharmacological) induces higher inflammatory responses and reversion of tolerance. These results suggest that zebrafish may have experienced an evolutionary selective pressure to avoid excessive inflammatory states that might be associated with an increased activity of the CXCR4 receptor. Our findings agree with previous studies, in which CXCR4 is described as involved in LPS binding but also responsible for triggering signalling. However, there is a controversy on this subject since some authors find that CXCR4 acts as an inhibitor of the LPS receptor TLR4 [29], but others state that CXCR4 interacts with TLR4 augmenting the LPS signalling [30].

Zebrafish might be a good model for studying infectious diseases, septic shock and tolerance to endotoxin. Zebrafish has several advantages compared with other models: a high number of individuals can be used, the availability of transgenic or mutant fish, and the ease of chemical manipulation, all of which facilitate observations of whole organism inflammatory reactions to external stimuli. This is a substantial benefit compared to other studies where only a cell line can be evaluated, and thus may not mirror the true *in vivo* response.

Acknowledgments

We want to thank all the people at the Dr. Zon Lab, Children's Hospital (Boston) for their hospitality and friendship. This work was partially supported by the project CSD2007-00002 Aquagenomics funded by the program Consolider-Ingenio 2010 from the Spanish Ministerio de Ciencia e Innovacion and 5R37DK55381-010 Characterizing the Zebrafish Genome funded by the National Institute of Diabetes and Digestive and Kidney Diseases at the National Institute of Health.

References

1. West MA, Heagy W. Endotoxin tolerance: a review. *Crit Care Med* 2002;30:S64–73.
2. Power MR, Peng Y, Maydanski E, Marshall JS, Lin TJ. The development of early host response to *Pseudomonas aeruginosa* lung infection is critically dependent on myeloid differentiation factor 88 in mice. *J Biol Chem* 2004;279:49315–49322. [PubMed: 15375173]
- 2 Sly LM, Rauh MJ, Kalesnikoff J, Song CH, Cristal G. LPS-induced upregulation of SHIP is essential for endotoxin tolerance. *Immunity* 2004;21:227–239. [PubMed: 15308103]
3. Parker LC, Jones EC, Prince LR, Dower SK, Whyte MK, Sabroe I. Endotoxin tolerance induces selective alterations in neutrophil function. *J Leuk Biol* 2005;78:1301–1305.
4. Lam SH, Chua HL, Gong Z, Lam TJ, Sin YM. Development and maturation of the immune system in zebrafish, *Danio rerio*: a gene expression profiling, in situ hybridization and immunological study. *Dev Comp Immunol* 2004;28(1):9–28. [PubMed: 12962979]
5. Purcell MK, Smith KD, Hood L, Winton JR, Roach JC. Conservation of Toll-Like Receptor Signaling Pathways in Teleost Fish. *Comp Biochem Physiol Part D Genomics Proteomics* 2006;1:77–88. [PubMed: 17330145]
6. Meijer AH, Krensa SFG, Medina Rodríguez IA, Hea S, Bitterb W, Snaar-Jagalsk BE, et al. Expression analysis of the Toll-like receptor and TIR domain adaptor families of zebrafish. *Mol Immunol* 2004;40:759–771. [PubMed: 14687933]
7. Jault C, Pichon L, Chluba J. Toll-like receptor gene family and TIR-domain adapters in *Danio rerio*. *Mol Immunol* 2004;40:759–771. [PubMed: 14687933]
8. Iliev DB, Roach JC, Mackenzie S, Planas JV, Goetz FW. Endotoxin recognition: in fish or not in fish? *FEBS Lett* 2005;579:6519–6528. [PubMed: 16297386]
9. Müller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, et al. Involvement of chemokine receptors in breast cancer metastasis. *Nature* 2001;410(6824):50–56. [PubMed: 11242036]
10. David NB, Sapède D, Saint-Etienne L, Thies C, Thies B, Dambly-Chaudière C, et al. Molecular basis of cell migration in the fish lateral line: Role of the chemokine receptor CXCR4 and of its ligand, SDF1. *Proc Natl Acad Sci USA* 2002;99:16297–16302. [PubMed: 12444253]
11. Knaut H, Werz C, Geisler R, Nüsslein-Volhard C. Tübingen 2000 Screen Consortium. A zebrafish homologue of the chemokine receptor Cxcr4 is a germ-cell guidance receptor. *Nature* 2003;421(6920):279–282. [PubMed: 12508118]
12. Knaut H, Blader P, Strähle U, Schier AF. Assembly of trigeminal sensory ganglia by chemokine signaling. *Neuron* 2005;47:653–666. [PubMed: 16129396]
13. Li Q, Shirabe K, Thies C, Thies B, Okamoto H, Masai I, et al. Chemokine signaling guides axons within the retina in zebrafish. *J Neurosci* 2005;25:1711–1717. [PubMed: 15716407]
14. Triantafilou M, Lepper PM, Briault CD, Ahmed MAE, Dmochowski JM. Chemokine receptor 4 (CXCR4) is part of the lipopolysaccharide sensing apparatus. *Eur J Immunol* 2008;38:192–203. [PubMed: 18081034]
15. Nusslein-Volhard, C.; Dahm, R. *Zebrafish, A Practical Approach*. Oxford University Press; New York, NY: 2002.
16. Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* 2000;132:365–386. [PubMed: 10547847]
17. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(-Delta Delta C(T)) method. *Methods* 2001;25:402–408. [PubMed: 11846609]
18. Fricker SP, Anastassov V, Cox J, Darkes MC, Grujic O, Idzan SR, et al. Characterization of the molecular pharmacology of AMD3100: A specific antagonist of the G-protein coupled chemokine receptor, CXCR4. *Biochem Pharmacol* 2006;72:588–596. [PubMed: 16815309]

19. Hatse S, Princen K, Bridger G, Clercq ED, Schols D. Chemokine receptor inhibition by AMD3100 is strictly confined to CXCR4. *FEBS Letters* 2002;527:255–262. [PubMed: 12220670]
20. Decker T. Sepsis: avoiding its deadly toll. *J Clin Invest* 2004;113:1387–1389. [PubMed: 15146235]
21. Renshaw SA, Loynes CA, Trushell DM, Elworthy S, Ingham PW, Whyte MK. A transgenic zebrafish model of neutrophilic inflammation. *Blood* 2006;108:3976–3978. [PubMed: 16926288]
22. Mathias JR, Perrin BJ, Liu TX, Kanki J, Look AT, Huttenlocher A. Resolution of inflammation by retrograde chemotaxis of neutrophils in transgenic zebrafish. *J Leukoc Biol* 2006;80:1281–1288. [PubMed: 16963624]
23. Yazawa R, Hirano I, Ohira T, Aoki T. Induction of Japanese flounder *tnf* promoter activity by lipopolysaccharide in zebrafish embryo. *Mar Biotechnol (NY)* 2005;7:231–235. [PubMed: 15864470]
24. Keestra AM, van Putten JP. Unique properties of the chicken TLR4/MD-2 complex: selective lipopolysaccharide activation of the MyD88-dependent pathway. *J Immunol* 2008;181:4354–4362. [PubMed: 18768894]
25. Koyama S, Sato E, Nomura H, Kubo K, Miura M, Yamashita T, et al. The potential of various lipopolysaccharides to release IL-8 and G-CSF. *Am J Physiol. Lung Cell Mol Physiol* 2000;278:658–666.
26. Dobrovolskaia MA, Medvedev AE, Thomas KE, Cuesta N, Toshchakov V, Ren T, et al. Induction of *in vitro* reprogramming by Toll-Like Receptor (TLR)2 and TLR4 agonists in murine macrophages: Effects of TLR “Homotolerance” Versus “Heterotolerance” on NF- κ B signaling pathway components. *J Immunol* 2003;170:508–519. [PubMed: 12496438]
27. Henricson BE, Neta R, Vogel SN. An interleukin-1 receptor antagonist blocks lipopolysaccharide-induced colony-stimulating factor production and early endotoxin tolerance. *Infect Immun* 1991;59:1188–1191. [PubMed: 1825485]
28. Medvedev AE, Kopydlowski KM, Vogel SN. Inhibition of lipopolysaccharide-induced signal transduction in endotoxin-tolerized mouse macrophages: dysregulation of cytokine, chemokine, and toll-like receptor 2 and 4 gene expression. *J Immunol* 2000;164:5564–5574. [PubMed: 10820230]
29. Kishore SP, Bungum MK, Platt JL, Brunn GJ. Selective suppression of Toll-like receptor 4 activation by chemokine receptor 4. *FEBS Letters* 2005;579:699–704.
30. Triantafilou M, Lepper PM, Briault CD, Ahmed MA, Dmochowski JM, Schumann C, Triantafilou K. Chemokine receptor 4 (CXCR4) is part of the lipopolysaccharide “sensing apparatus”. *Eur J Immunol* 2008;38:192–203. [PubMed: 18081034]

Figure 1.

Survival of zebrafish larvae exposed at different days post fertilization (dpf) to *E. coli* 0111:B4 LPS. Fish were bathed directly in water with the different LPS concentrations. Data correspond to a representative experiment conducted 4 times.

Survival of zebrafish treated with *P. aeruginosa* LPS

Figure 2.

Zebrafish larvae survival exposed at 2 dpf with *Pseudomonas aeruginosa* LPS. Results are $\pm$ standard deviations (n= 2 replicates with 15 fish each) of a representative experiment repeated three times.

Figure 3.

Summary of the different experiments conducted to demonstrate LPS tolerance in zebrafish. 2 dpf embryos were treated with a sublethal concentration of LPS (50 µg/ml of *E. coli* 0111:B4) and then at different times post-exposure to a lethal concentration (150 µg/ml) of the same LPS.

Figure 4.

Survival of zebrafish larvae incubated with other PAMPS to determine if they can induce tolerance to LPS. Treatments with the lethal concentration of *E. coli* LPS were conducted at 7 dpf. (A) one pretreatment at 3 dpf (B) two pretreatments at 3 and 6 dpf of PAMPS or two *E. coli* LPS serotypes. Results are $\pm$ standard deviations of $n=2$ replicates from a representative experiment out of three.

Figure 5.

Expression of IL-1 by qPCR showing its decrease after exposure to sublethal *E. coli* LPS concentration (50 µg/ml) compared with its increment when a lethal concentration was used (150 µg/ml). Results are $\pm$ standard deviations of $n=4$ pooled samples. *: indicates significant differences, $p<0.05$, with respect to control. #: indicates significant differences, $p<0.05$, with respect to the initial value after 30 min of treatment.

Figure 6. Involvement of CXCR4 in the sensitivity to LPS and tolerance

(A) Survival of wild type fish treated with AMD3100, a specific CXCR4 inhibitor, showing that AMD3100 treated fish do not survive exposure to LPS sublethal concentrations. Results are $\pm$ standard deviations (two replicates with 10 fish each). (B) Reversion of the tolerance to LPS in *Odysseus* fish and AMD3100 wild type treated fish compared with untreated wild type zebrafish. Fish were treated with a sublethal concentration of LPS and after one day they were exposed to a lethal concentration of *P. aeruginosa*. Results are $\pm$ standard deviations (two replicates with 10 fish each). (C) qPCR of IL-1 of *Odysseus* and Tubingen (wild type) fish 24 h after the treatment with 50 μ g/ml of LPS. Results are $\pm$ standard deviations of n= 4 pooled samples. *: indicates significant differences, $p < 0.05$, with respect to controls.