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Brain infection and activation of neuronal repair mechanisms by the human pathogen *Listeria monocytogenes* in the lepidopteran model host *Galleria mellonella*

Krishnendu Mukherjee,[1] Torsten Hain,[2] Rainer Fischer,[3] Trinad Chakraborty[2,*] and Andreas Vilcinskas[1,*]

[1]Institute of Phytopathology and Applied Zoology; Justus-Liebig-University of Giessen; Giessen, Germany; [2]Institute of Medical Microbiology; Justus-Liebig-University of Giessen; Giessen, Germany; [3]Fraunhofer Institute of Molecular Biology and Applied Ecology; Aachen, Germany

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Listeria monocytogenes the causative agent of the food-borne disease listeriosis in humans often involves fatal brainstem infections leading to meningitis and meningoencephalitis. We recently established the larvae of the greater wax moth (*Galleria mellonella*) as a model host for the investigation of *L. monocytogenes* pathogenesis and as a source of peptides exhibiting anti-*Listeria*-activity. Here we show that *G. mellonella* can be used to study brain infection and its impact on larval development as well as the activation of stress responses and neuronal repair mechanisms. The infection of *G. mellonella* larvae with *L. monocytogenes* elicits a cellular immune response involving the formation of melanized cellular aggregates (nodules) containing entrapped bacteria. These form under the integument and in the brain, resembling the symptoms found in human patients. We screened the *G. mellonella* transcriptome with marker genes representing stress responses and neuronal repair, and identified several modulated genes including those encoding heat shock proteins, growth factors, and regulators of neuronal stress. Remarkably, we discovered that *L. monocytogenes* infection leads to developmental shift in larvae and also modulates the expression of genes involved in the regulation of endocrine functions. We demonstrated that *L. monocytogenes* pathogenesis can be prevented by treating *G. mellonella* larvae with signaling inhibitors such as diclofenac, arachidonic acid, and rapamycin. Our data extend the utility of *G. mellonella* larvae as an ideal model for the high-throughput in vivo testing of potential compounds against listeriosis.

Introduction

The severe foodborne disease listeriosis is caused by the opportunistic gram-positive bacterial pathogen *Listeria monocytogenes*, and primarily affects pregnant women, newborns, the elderly, and immunocompromised adults. It is considered as the most severe bacterial foodborne infection with 1645 case in Europe (2009) and approximately 2500 cases per year in the US. Up to 30% of the cases are lethal and more than 50% correspond to septicemia.[1] *L. monocytogenes* is ubiquitous in the environment and can tolerate food preservation treatments based on extreme pH, salinity and temperature. The risk of listeriosis is increasing, reflecting the aging population and the growing number of immunocompromised individuals, creating a demand for novel therapeutic strategies. The complexity of the cell infection cycle of *L. monocytogenes* and its ability to target the central nervous system requires the development of adequate model hosts to discover novel anti-*Listeria* drugs.[1,2]

Ethical concerns and the high costs associated with the use of mammalian model hosts have encouraged researchers to explore alternative invertebrate models. The nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster* have been used to study *L. monocytogenes* pathogenesis.[3-7] However these models suffer from several disadvantages, including the inability to distinguish between *L. monocytogenes* strains that are pathogenic and non-pathogenic in humans.[8,9]

The larvae of the greater wax moth *Galleria mellonella* have been developed as model hosts for human pathogens. The larvae can be reared at 37 °C, the physiological temperature for human pathogens, which is often required for the synthesis and release of virulence factors.[10,11] In addition, they are particularly suited for high-throughput in vivo assays to test novel antibiotics and pathogen mutant libraries[12] and as a source of novel anti-infective drugs.[13] *G. mellonella* has also been established as a model to mimic oral infection with bacterial pathogens.[14]

*Correspondence to: Trinad Chakraborty; Email: Trinad.Chakraborty@mikrobio.med.uni-giessen.de;
Andreas Vilcinskas; Email: andreas.vilcinskas@agrar.uni-giessen.de
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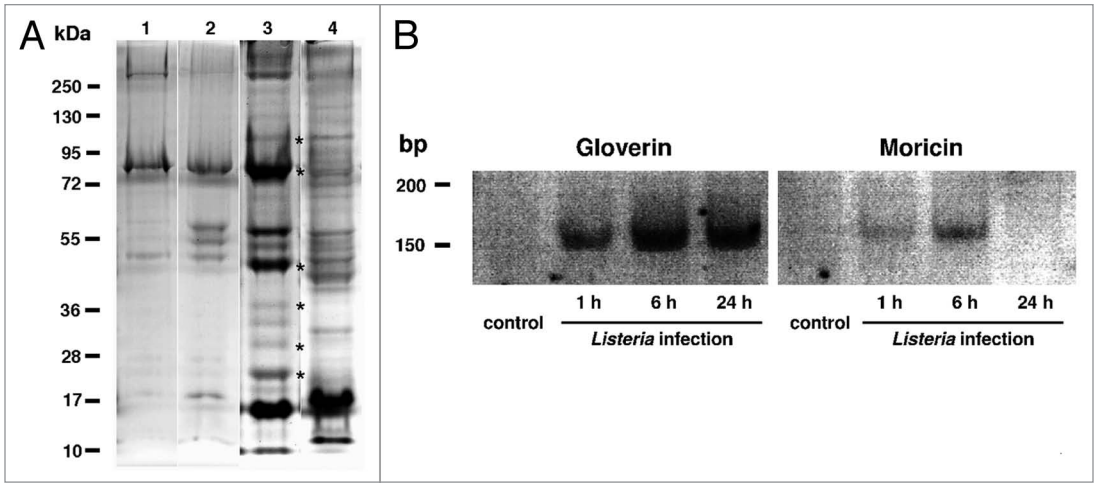

Figure 1. Induction of humoral immune responses in *G. mellonella* following *L. monocytogenes* challenge. (**A**) *G. mellonella* hemolymph proteins were isolated 24 h after infection with *L. monocytogenes* and were separated by Tris-Tricine-SDS-PAGE. Proteins were stained with Coomassie brilliant blue, completely destained with 70% (v/v) ethanol, and equilibrated in binding buffer. Lanes 1–4 represent protein samples from untreated, 0.9% NaCl injected, pathogenic *L. monocytogenes* injected and non-pathogenic *L. innocua* injected *G. mellonella* larvae, respectively. (**B**) The gloverin and moricin mRNA levels following injection with *L. monocytogenes* determined by RT-PCR and are shown relative to control larvae injected with 0.9% NaCl. The PCR products were separated by 9% Tris-Tricine-SDS PAGE at 20 °C. The experiments were repeated three times with similar results.

Independently, two groups have recently established *G. mellonella* as a suitable host for *L. monocytogenes*.[15,16] In addition, we have reported the presence of potent and inducible peptides in the hemolymph exhibiting inhibitory activity against *L. monocytogenes* such as Cecropin D.[17] We have also comprehensively documented the immune-gene repertoire in this lepidopteran model host by sequencing the transcriptome of different developmental stages and immune-activated larvae on the next-generation Roche 454-FLX platform combined with traditional Sanger sequencing.[18] Using these unique resources, here we have explored the suitability of *G. mellonella* as a model host to: (1) study brain infection in an insect model infected with human pathogenic *L. monocytogenes*, (2) address *L. monocytogenes* mediated developmental shifts in infected larvae, (3) document the pathogen-induced regulation of genes encoding growth hormones, (4) identify immunity and stress marker genes induced by *L. monocytogenes*, focusing on genes with known functions in cellular immunity and neuroendocrine/neuronal repair mechanisms and (5) develop a whole-animal, high-throughput platform suitable for the in vivo screening of drugs exhibiting activity against listeriosis.

Results

Infection with *L. monocytogenes* elicits immune responses in *G. mellonella*. To determine whether a virulent strain of *L. monocytogenes* can induce humoral immune responses in *G. mellonella* larvae, we compared the hemolymph proteins isolated from non-infected and infected larvae 24 h post-inoculation (hpi) with 10^6 cfu/larva as previously shown by Mukherjee et al. 2010.[16] We observed several new or more pronounced bands in the infected sample in comparison to 0.9% NaCl or non-pathogenic *L. innocua* injection, indicating the presence of proteins highly induced by pathogenic *L. monocytogenes* infection (**Fig. 1A**). We also extracted RNA from non-infected and infected larvae 1, 6 and 24 hpi to investigate the expression profiles of selected lepidopteran specific antimicrobial peptides.[19] *L. monocytogenes* infection in larvae resulted in the transient induction of gloverin and moricin in comparison to larvae injected with 0.9% NaCl (**Fig. 1B**).

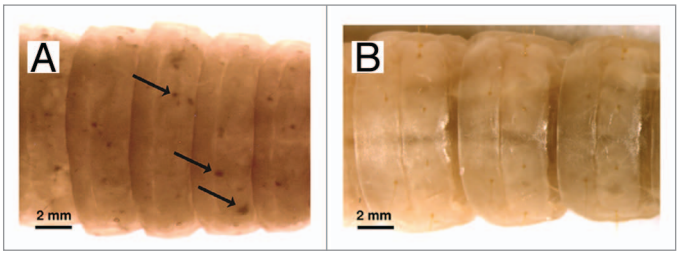

Figure 2. Ex vivo observation of *G. mellonella* larvae infected with *L. monocytogenes* Larvae were treated with (**A**) 1% DMSO and (**B**) diclofenac. Arrows show the melanized spots, indicating localized containment of *L. monocytogenes* in *G. mellonella* larvae after the administration of 1% DMSO as a control, whereas the aggregates do not form in larva treated with diclofenac.

Virulence factors required for the propagation of *L. monocytogenes* in humans are also essential for the infection of *G. mellonella*. Listeriosis in humans involves the formation of dark sub-epidermal spots (also on certain organs such as the liver) representing aggregates of macrophages, dendritic cells and entrapped bacteria.[20] We observed similar dark nodules in the integument of *G. mellonella* larvae infected with *L. monocytogenes* (**Fig. 2A**), but nodule development was inhibited by diclofenac, a non-steroidal, anti-inflammatory drug (NSAID) known for its listeriacidal activity (**Fig. 2B**). To confirm that the nodules observed in *G. mellonella* also represented cellular

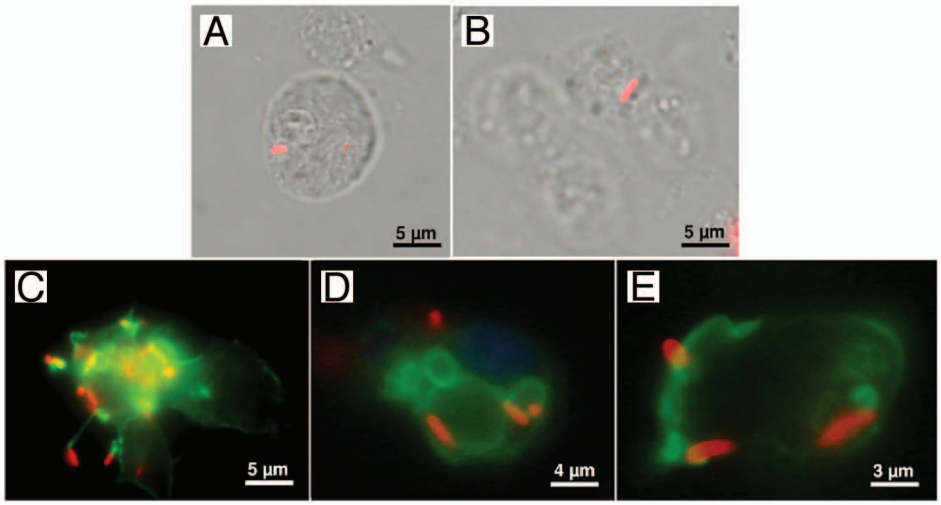

Figure 3. Cell-mediated immunity and infection in *G. mellonella* following *L. monocytogenes* challenge. (**A**) In vivo observation of *G. mellonella* hemocyte containing *L. monocytogenes* expressing red fluorescent protein marker 30 min after injection. (**B**) In vivo observation of *G. mellonella* larvae 48 h after injection showing hemocyte aggregates surrounding labeled *L. monocytogenes*, forming nodule like structures. (**C–E**) Immunofluorescence images of *G. mellonella* hemocytes 7 h after infection of 10^5 hemocytes per 24-well plate with *L. monocytogenes* EGD-e and isogenic deletion mutants EGD-eΔ*hly* and EGD-eΔ*act*A. Bacterial cells were stained with the M108 antibody (red fluorescence) and hemocyte actin was stained with Alexa Fluor 488 phalloidin (green fluorescence). *L. monocytogenes* spreads throughout the cytosol of the hemocyte and actin tails at the poles of some of the bacteria are visible (**C**) whereas EGD-eΔ*hly* (**D**) and EGD-eΔ*act*A (**E**) mutants fail to form actin tails.

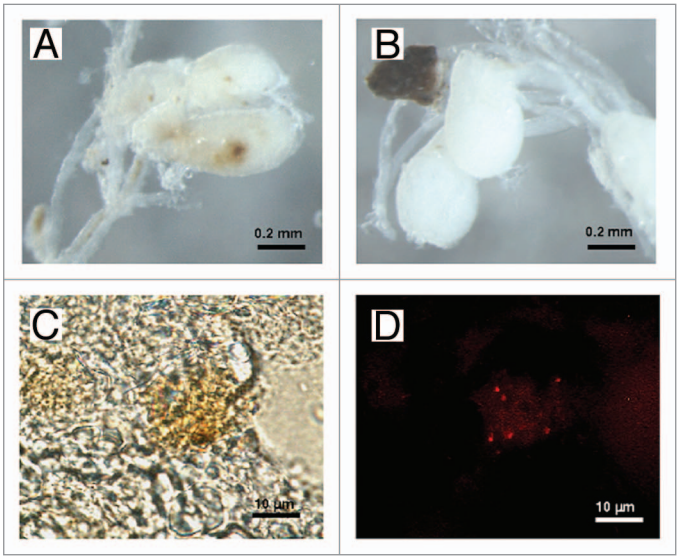

Figure 4. *G. mellonella* brain infected with *L. monocytogenes*. (**A**) Dissection of *G. mellonella* larvae infected with *L. monocytogenes* reveals the formation of dark spots in the brain. (**B**) Treatment with diclofenac prevents the formation of these spots. (**C**) Localized melanized regions are formed in the *G. mellonella* brain following infection with *L. monocytogenes*. (**D**) Persistence of labeled *L. monocytogenes* (red fluorescence) in melanized regions.

aggregates with entrapped or ingested bacteria, we inoculated the larvae with a recombinant *L. monocytogenes* strain expressing the marker protein DsRed-Express. Hemolymph samples collected after 30 min, 24 h and 48 h were analyzed by microscopy. Fluorescent bacteria were detected in hemocytes after 30 min, indicating phagocytic ingestion (**Fig. 3A**). The formation of hemocyte aggregates containing entrapped bacteria was observed 48 hpi (**Fig. 3B**). Bacteria in the cell-free hemolymph did not yield a fluorescent signal, indicating the hemolymph contained only dead bacteria (data not shown).

When *L. monocytogenes* infects humans, the virulence factor ActA is required for intracellular actin-based motility and cell-to-cell spreading of the bacteria, and the hemolysin listeriolysin O (encoded by the *hly* gene) is required for the bacteria to escape from the primary vacuole into the cytoplasm.[21] We tested the in vivo activity of ActA in *G. mellonella* larvae by isolating hemolymph samples and incubating the hemocytes with either a wild-type pathogenic *L. monocytogenes* strain or isogenic mutants lacking the virulence genes *act*A or *hly*. After 1 h we replaced the supernatant with fresh medium supplemented with 50 µg/ml gentamycin to kill extracellular bacteria. The intracellular bacteria were then stained with a monoclonal antibody (M108, red fluorescence) to distinguish them from actin filaments stained with Alexa Fluor 488 conjugated phalloidin (green fluorescence). No actin tail formation was observed in cells infected with EGD-eΔ*hly* (**Fig. 3D**) and EGD-eΔ*actA* (**Fig. 3E**) whereas intracellular bacteria either covered with actin "clouds" or undergoing rapid movement facilitated by actin "tails" were detected in hemocytes infected with wild-type EGD-e (**Fig. 3C**). These observations indicated that virulence factors required for the propagation of *L. monocytogenes* in humans are also essential for the successful infection of *G. mellonella*.

***L. monocytogenes* can infect the brains of *G. mellonella* larvae.** Listeriosis in mammals can develop into fatal brainstem infections,[1] therefore we investigated the possibility of using *G. mellonella* as a model host for human pathogens that infect the brain. We dissected the brains of larvae infected with 10^6 cfu/larva wild-type *L. monocytogenes* 6 d post injection and compared them to brains from non-infected larvae. The brains of the infected larvae displayed clear evidence of melanized nodules similar to those observed in the integument (**Fig. 4A**). These data confirm that *L. monocytogenes* induces an immune response in the brains of *G. mellonella* larvae, because the melanization of entrapped bacteria in multicellular aggregates indicates a locally-restricted cellular immune reaction.[22] Interestingly, larvae injected with 50 µM diclofenac before infection were devoid of brain nodules, showing that diclofenac prevents infection of the

Table 1. Selected *G. mellonella* stress markers used for real time RT-PCR analysis and their assignment to biological processes based on gene ontology categories

Biological processes	GenBank accession number of EST
Response to oxidative stress; glutathione metabolic process; peroxidase reaction	Contig17373_1.exp
Response to oxidative stress; phospholipid catabolic process	Contig03093_1.exp
Response to stress	Contig16593_1.f1.exp
Response to stress	GME-string_Contig_1489.0
Response to stress	Contig15219_1.exp
Response to stress	GME-string_Contig_2799.0
Response to stress	Contig01172_1.exp
Response to stress	Contig07949_1.exp
Neuromuscular junction development; regulation of cell shape; cortical actin cytoskeleton organization; phagocytosis, engulfment; axonogenesis; oocyte growth; cell projection assembly; myoblast fusion; bristle morphogenesis	Contig19101_1.f1.exp
G-protein coupled receptor protein signaling pathway; response to stress	Contig20595_1.exp
Response to stress	Contig15265_1.f1.exp
Defense response to Gram-positive bacterium; innate immune response; xenobiotic metabolic process; transport antifungal humoral response	Contig14128_1.exp
Signal transduction; cell proliferation; growth	GME-string_Contig_704.0
Hemocyte migration; signal transduction; cell proliferation; growth	GME-string_Contig_233.0
Axonogenesis	Contig17493_1.exp
Cell cycle, positive regulation of neuron apoptosis; cell division; embryonic development; neuron projection development; protein amino acid phosphorylation; serine family amino acid metabolic process	Contig02810_1.f1.exp

G. mellonella central nervous system by virulent *L. monocytogenes* (**Fig. 4B**).

The presence of entrapped bacteria within the melanized nodules (**Fig. 4C**) was validated by infecting larvae with the recombinant *L. monocytogenes* strain expressing the red fluorescent marker protein, confirming that the pathogen survives within the nodules of the insect brain (**Fig. 4D**).

Identification and transcriptional activation of stress and neuronal repair markers in *G. mellonella* following infection with *L. monocytogenes*. An analysis of the *G. mellonella* transcriptome revealed the presence of nearly 18 690 contigs with almost 40% of ESTs showing significant similarities to proteins in other insects.[18] We screened this comprehensive database for sequences matching known markers of stress responses and neuronal repair mechanisms (listed in **Table 1**) and then tested infected larvae by real-time RT-PCR to identify those induced by infection with virulent *L. monocytogenes*.

The resulting hits were queried against the NCBI database using BLASTx and BLASTn program and their functions were predicted by gene ontology (GO) analysis using Blast2GO. This revealed the induction of genes known to participate in oxidative stress responses, phagocytosis, cell proliferation and neuronal repair, as well as genes encoding heat shock proteins and antimicrobial peptides. The strongest induction was observed for genes encoding components of the cellular immune system, such as those involved in phagocytosis, hemocyte migration and the activities of G-protein-coupled receptors. The expression of some heat shock proteins peaked 1 h after infection, whereas others reached their peak after 4 d (**Fig. 5A**). Genes involved in neuronal repair mechanisms such as axonogenesis and neuron projection were also induced in response to infection (**Fig. 5B**).

***L. monocytogenes* infection induced developmental shifts in *G. mellonella*.** The infection of last-instar *G. mellonella* larvae with the human pathogenic *L. monocytogenes* strain EGD-e significantly delayed the formation of pupae compared with those injected with 0.9% NaCl or heat-killed EGD-e (**Fig. 6**). We found no significant difference in the rate of pupation between larvae injected with heat-killed EGD-e and 0.9% NaCl. The ability of the virulent *L. monocytogenes* strain to delay development of the infected larvae prompted us to study the impact of listeriosis on its endocrine system.

Metamorphosis in insects is controlled by the tightly regulated activity of hormones such as juvenile hormone and ecdysone, which are synthesized by the endocrine glands.[23,24] The opposite developmental effects of virulent and non-virulent microbes described above, suggested the possibility that human pathogenic *L. monocytogenes* can interfere with the regulation of the endocrine system in *G. mellonella*.

Infection with *L. monocytogenes* induces *G. mellonella* genes encoding growth hormones. To determine whether *L. monocytogenes* pathogenesis interferes with the endocrine regulation in *G. mellonella*, we monitored the expression of genes related to the synthesis and activity of juvenile hormone and ecdysone.

Four days post-injection with the virulent wild-type strain of *L. monocytogenes*, we found that several genes related to juvenile hormone were induced in infected *G. mellonella* relative to larvae

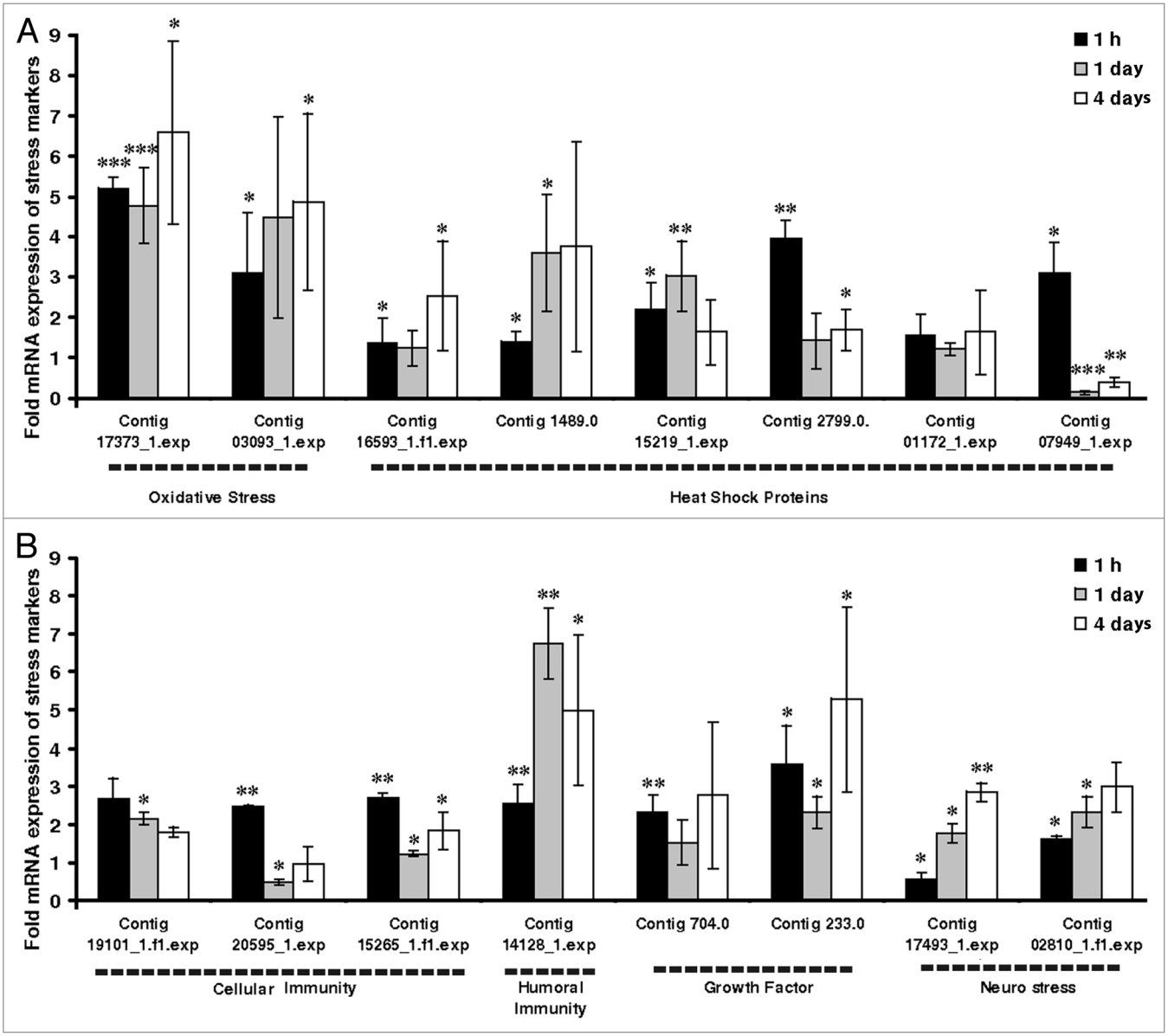

Figure 5. Transcriptional activation of potential stress markers in *G. mellonella* following *L. monocytogenes* infection. Transcriptional levels of putative markers of immune and stress responses and neuronal repair mechanisms following the infection of *G. mellonella* larvae with *L. monocytogenes*. Expression levels were determined by quantitative real-time RT-PCR and their significant induction are shown relative to control larvae injected with 0.9% NaCl. The selected ESTs from **Table 1** include (**A**) Contig 17373_1.exp, Contig 03093_1.exp, Contig 16593_1.f1.exp, Contig 1489.0, Contig 15219_1.exp, Contig 2799.0, Contig 01172_1.exp, Contig 07949_1.exp, and (**B**) Contig 19101_1.f1.exp, Contig 20595_1.exp, Contig 15265_1.f1.exp, Contig 14128_1.exp, Contig 704.0, Contig 233.0, Contig 17493_1.exp, Contig 02810_1.f1.exp. Values were normalized against expression levels of the housekeeping gene 18S r-RNA. The experiment was repeated three times with similar results. (*$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$).

injected with 0.9% NaCl (**Fig. 7A**) but only a transient impact was recorded for ecdysteriod gene expression (**Fig. 7B**).

Inhibitors of cell signaling enhance the survival of *G. mellonella* larvae infected with *L. monocytogenes*. The major virulence factors of *L. monocytogenes* modulate many cellular processes in vertebrate hosts, including autophagy and certain signaling cascades.[2] To explore the role of signaling cascades in *G. mellonella* we applied pharmacological agents associated with the induction of immune signaling pathways that modulate *L. monocytogenes* infections in vertebrate cells, i.e., diclofenac, arachidonic acid and rapamycin.

The NSAID diclofenac is an inhibitor of cyclo-oxygenases exhibiting activity against listeriosis,[25] arachidonic acid enhances phagocytosis and induces bacterial clearance from the *G. mellonella* hemolymph[26] and rapamycin is an mTOR inhibitor that induces autophagy, helping to eliminate *L. monocytogenes* from mammalian macrophages.[27-29] The separate administration of diclofenac (**Fig. 8A**), arachidonic acid (**Fig. 8B**) and rapamycin (**Fig. 8C**) to *G. mellonella* larvae at the onset of infection with *L. monocytogenes* significantly improved insect survival compared with controls treated only with the solvent. Therefore, the induction of cellular defense mechanisms can restrict *L. monocytogenes*

pathogenesis in *G. mellonella* as previously shown in mammalian systems.[30]

To determine whether these effects are manifested at the cellular level, we examined the effect of rapamycin on *G. mellonella* hemocytes prior to infection with *L. monocytogenes*. The pre-treatment of hemocytes with rapamycin significantly reduced the number of intracellular bacteria compared with hemocytes treated with DMSO alone (**Fig. 8D**). The induction of autophagy in hemocytes therefore restricts the intracellular growth of *L. monocytogenes*.

Discussion

Our study aimed to develop and extend *G. mellonella* as a surrogate model host for pathogens and parasites that can successfully cross the blood-brain barrier in humans[31] in order to further advance the use of this lepidopteran species in preclinical research. We selected *L. monocytogenes* because the pathophysiology of listeriosis lacks an easy-to-handle model host that reproduces all the hallmarks of the human disease.[1] Here we report for the first time that *L. monocytogenes* can target the central nervous system of *G. mellonella* larvae implicating the capacity of virulent strains to breach the blood-brain barrier of insects. Infection of the brain by *L. monocytogenes* was accompanied by the formation of melanized nodules similar to those observed beneath the integument of the larvae. In order to validate that these nodules contain entrapped *L. monocytogenes* we used a fluorescently labeled strain. We also examined genes whose expression are induced under these conditions including those encoding proteins related to neuronal repair mechanisms and stress responses, for example heat shock proteins, as well as genes contributing to cellular and humoral immunity. Our study shows that the ability of *L. monocytogenes* to induce immune responses in *G. mellonella* can be expanded beyond phagocytosis and the formation of cellular aggregates consisting of hemocytes and entrapped bacteria to include the induced syntheses of antibacterial proteins such as gloverin and moricin, which are specific for *Lepidoptera*.[13,19] However, neither cellular nor humoral immune responses prevented infected larvae from ultimate death upon establishment of *L. monocytogenes* infection. Therefore, we were interested in the identification of genes mediating resistance of *L. monocytogenes* against immune responses of *G. mellonella*. In our previous study we have elucidated the role of the virulence gene cluster (*vgc*) and particular virulence genes such as *hly*, *actA*, *mpl*, *plcB*, *plcA*, etc. in orchestrating mortal infections in this model host.[16] In this study we show that the expression of listeriolysin and the host actin polymerizing factor ActA is essential for acting tail formation in *G. mellonella* hemocytes. This is in contrast to the widely used *Drosophila* model, where deletion of *actA* or *hly* from *L. monocytogenes* only transiently alters the infective potential in comparison to the wild type pathogenic strain.[5,7]

A hallmark of our study is the observation that the virulent strain of *L. monocytogenes* was able to postpone the development of the infected insect host whereas heat-inactivated bacteria induced precocious formation of pupae. These developmental shifts can probably be attributed to the interference of

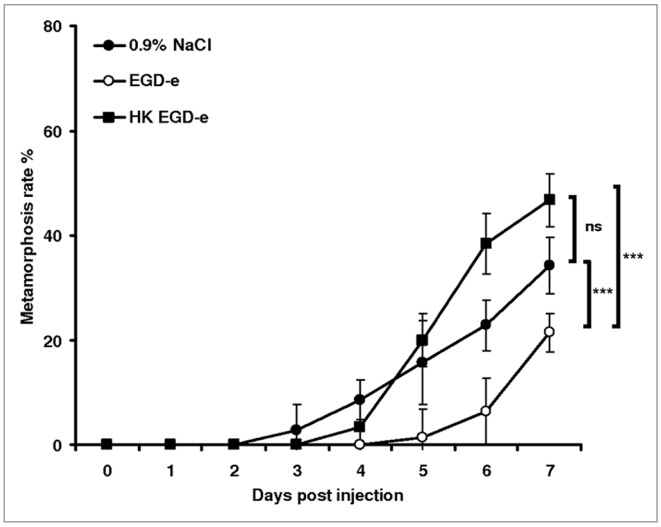

Figure 6. Metamorphosis of *G. mellonella* larvae following infection with pathogenic and non-pathogenic *L. monocytogenes*. Injection of pathogenic EGD-e or non-pathogenic heat killed (HK) EGD-e into last-instar larvae induced opposing developmental effects. Injection with HK EGD-e induced the precocious formation of pupae whereas infection with live wild type pathogenic EGD-e caused a significant delay in comparison to control larvae treated with 0.9% NaCl. The larvae were incubated at 37 °C and reared on an artificial diet. Results represent mean values of at least three independent measurements ± standard deviations from at least 20 larvae per treatment (***$P < 0.0005$; ns, not significant).

L. monocytogenes with the endocrine system of the infected host, because we determined induced transcription of genes related to processing or binding of the juvenile hormone. Metamorphosis in *Lepidoptera* and other holometabolous insects is initiated by decreasing juvenile hormon titers and increasing ecdyson concentrations.[23,24] Here we monitored that virulent *L. monocytogenes* enhanced expression of genes in *G. mellonella* which are related to binding and metabolism of juvenile hormone (**Fig. 7**) suggesting delayed metamorphosis as experimentally confirmed. Our observations are consistent with other studies showing that fungal pathogens can also postpone development of *G. mellonella* larvae[32] whereas stimulation of immune responses (e.g., by injection of heat-killed bacteria) has been reported to accelerate development of the model beetle *Tribolium castaneum*.[33] Further, bacterial toxins have been shown to imbalance the juvenile hormone titers in another lepidopteran species.[34] Thus the induction of expression of genes mediating processing or binding of either juvenile hormone or ecdysone imply that virulent *L. monocytogenes* can interfere with the endocrine system of the infected host ultimately resulting in delay of its development.

Interestingly, we were able to manipulate development of *L. monocytogenes* in *G. mellonella* by administration of pharmacological reagents that interfere either with the induction of autophagy (rapamycin) or inhibition of cyclo-oxygenase (diclofenac) in the host. Both compounds enhanced the survival of *G. mellonella* when administered at the onset of *L. monocytogenes* infection, replicating their reported anti-listeriosis activity in mammals.[25,27-29]

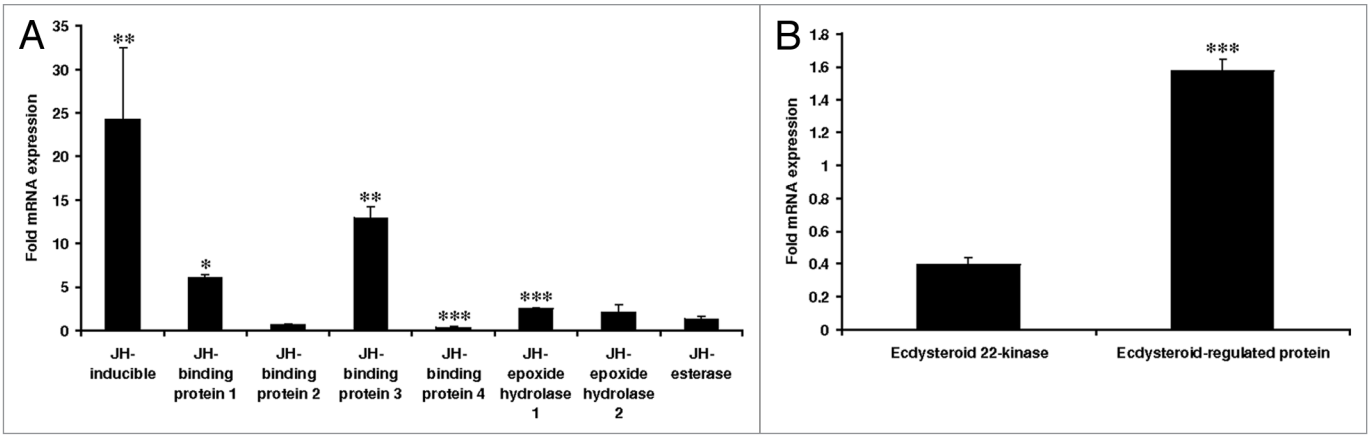

Figure 7. Transcriptional analysis of genes encoding growth hormone associated proteins in *G. mellonella* following *L. monocytogenes* infection The expression levels of genes encoding (**A**) JH-inducible, juvenile hormone binding proteins 1, 2, 3, and 4, JH-epoxide hydrolase 1 and 2, and JH-esterase and (**B**) ecdysteroid 22-kinase and ecdysteroid-regulated protein, in *G. mellonella* larvae following injection with virulent *L. monocytogenes* were determined 4 d after infection by quantitative real time RT-PCR. Significantly induced expression levels are calculated relative to control larvae injected with 0.9% NaCl. Values were normalized against the expression levels of the housekeeping gene 18S rRNA. The experiment was repeated three times with similar results. (JH, juvenile hormone; **P* < 0.05; ***P* < 0.005; ****P* < 0.0005).

To further explore the protective activity of rapamycin we treated *G. mellonella* hemocytes with this drug prior to infection with *L. monocytogenes*. Rapamycin reduced significantly the number of bacteria within the hemocytes, implicating that autophagy contributes to the mechanism which restrict intracellular growth of *L. monocytogenes* in *G. mellonella*. This conclusion is in accordance with the reported role of autophagy in eliminating *L. monocytogenes* from the fruit fly *Drosophila melanogaster* in which sensing of listerial cell wall components by the intracytosolically located peptidoglycan-receptor LE induces autophagy of infecting *L. monocytogenes*.[35]

Our results support the suitability of *G. mellonella* as a model host for human pathogens and as a valid whole animal-high-through-put system for testing of antimicrobial drugs.[12,36] In addition, we report for the first time its applicability for pathogens capable to penetrate the blood-brain-barrier and to reproduce in the brain.

Materials and Methods

Insects, bacteria, and media. *G. mellonella* larvae were maintained as described by Mukherjee et al.[16] We used *L. monocytogenes* strain EGD-e (serotype 1/2a)[37] the avirulent mutant strains EGD-eΔ*hly* and EGD-eΔ*actA*, and a recombinant strain transformed with the vector pJEBAN6 expressing the red fluorescent marker proteins HcRed and DsRed-Express.[38] Bacterial cultures were maintained aerobically in brain heart infusion (BHI) medium (Difco) at 37 °C and on BHI agar plates, supplemented with 5 μg/ml erythromycin in the case of the recombinant strain. For long-term storage, bacteria were frozen at −80 °C in BHI medium supplemented with 30% glycerol. For the injection experiments, we used logarithmic-phase bacterial cultures (10^9 cfu/ml) in 10 ml BHI broth. Bacterial inoculums were washed and serially diluted to 10^6 cfu/larva using 0.9% NaCl.

***G. mellonella* infections.** Bacterial inoculums were injected dorsolaterally into the hemocoel of last-instar larvae. After injection, larvae were incubated at 37 °C and reared on an artificial diet. Larvae were considered dead when they showed no movement in response to touch.

RT-PCR. RNA from untreated larvae and larvae injected with 0.9% NaCl and pathogenic *L. monocytogenes* was isolated at 1 h, 6 h, 24 h and 4 d after injection. A minimum of three larvae per treatment for each time point were homogenized in Trizol reagent (Sigma) and RNA was isolated according to the manufacturer's recommendations. Around 1 μg of RNA was used for cDNA (cDNA) synthesis. cDNA was prepared using the First Stand cDNA synthesis kit (Fermentas). Ethidium bromide gel staining was used to confirm RNA quality, and quantities were determined by spectrophotometry. RT-PCR was performed using the real-time PCR system Biorad (CFX 96) Mx3000P (Stratagene) and SsoFast EvaGreen Supermix (Biorad) according to the manufacturers' protocols, with gene-specific primers including the housekeeping gene 18S rRNA and actin, as listed in **Table S1**, and 50 ng template cDNA per reaction. PCR products were separated by 9% Tris-Tricine-SDS PAGE, and the gels were stained with ethidium bromide and visualized using a Umax PowerLook II scanner.

Analysis of hemolymph proteins by SDS-PAGE. *G. mellonella* hemolymph samples were collected in 98% acetone and centrifuged at 14000 rpm for 5 min. The pellet was dissolved in 8 M urea and centrifuged at 14000 rpm for 15 min. The supernatant containing proteins was fractionated by 9% Tris-Tricine-SDS PAGE at 20 °C. Gels were stained with colloidal Coomassie brilliant blue (Carl Roth) and visualized using a Umax PowerLook II scanner.

Ex vivo infection of *G. mellonella* hemocytes. *G. mellonella* hemocytes were maintained at 37 °C in Schneider medium (Bio Whittaker/Lonza) supplemented with 10% heat-inactivated fetal bovine serum (Biowest) and infections were initiated as

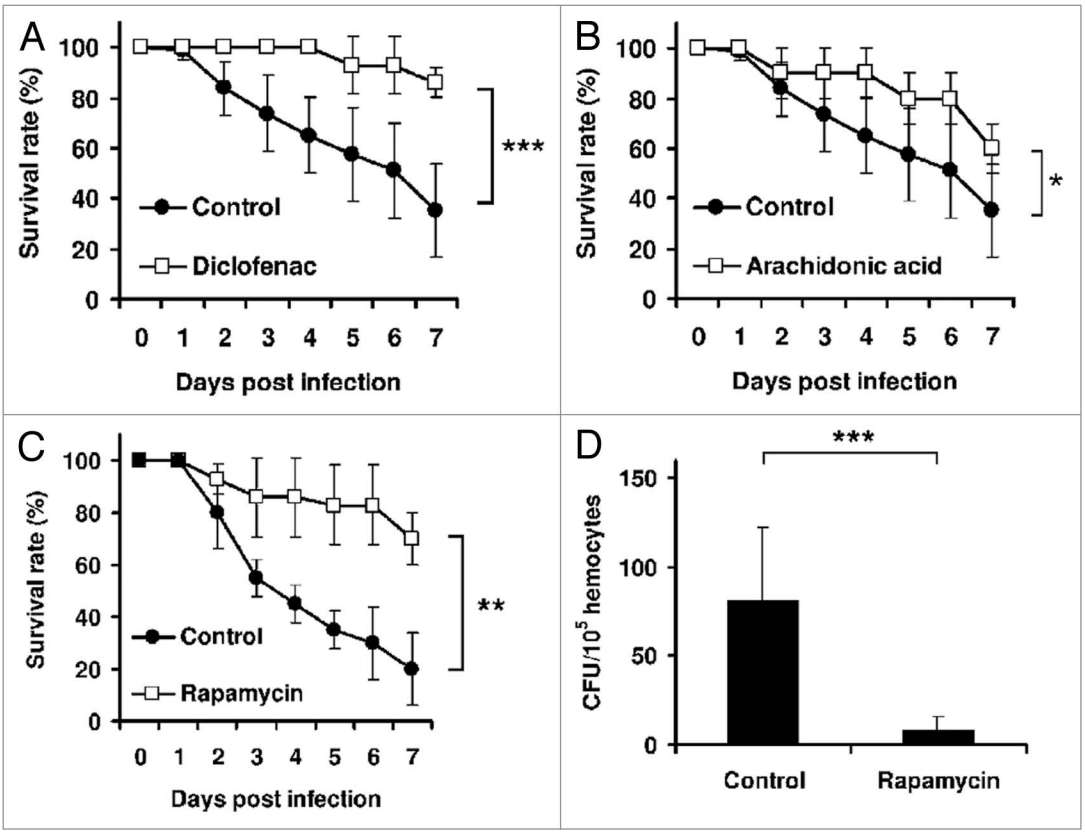

Figure 8. Pharmacological inhibitors of signaling pathways confer activity against *L. monocytogenes* infection in *G. mellonella*. Application of diclofenac (**A**), arachidonic acid (**B**) and the mTOR inhibitor rapamycin (**C**) prior to infection with *L. monocytogenes* resulted in significantly higher survival rates in *G. mellonella* larvae (□) compared with control solvents (●). (**D**) Ex vivo analysis shows that rapamycin reduced the replication of *L. monocytogenes* in *G. mellonella* hemocytes. Control hemocytes were treated with 1% DMSO. Results represent mean values of at least three independent determinations ± standard deviations. Each repetition comprises 30 larvae per treatment. Statistically significant differences were determined using Student t-test and are indicated as *$p < 0.05$; **$p < 0.005$ and ***$p < 0.0005$.

previously described.[16] In preparation for microscopy, cells were fixed in 3.7% paraformaldehyde on a coverslip and incubated at room temperature for 10 min, washed in PBS, permeabilized in 0.2% Triton X-100 in PBS for 1 min, and washed again in PBS. The coverslips were then incubated with monoclonal antibody M108 for 30 min at 33 °C, washed three times in PBS and incubated with a 1:100 diluted Cy3-labeled secondary anti-mouse antibody (Dianova) and Alexa Fluor 488 conjugated to phalloidin (diluted 1:100) (Molecular Probes, Invitrogen) in PBS containing 1% bovine serum albumin for 30 min at 33 °C. The coverslips were washed three times with PBS and mounted using Prolong Gold anti-fade reagent (Invitrogen).

The number of *L. monocytogenes* colony forming units were estimated as previously described[16] following the infection of *G. mellonella* hemocytes with wild-type bacteria (10^8 cfu/ml), incubating at 37 °C for 1 h, adding the appropriate compound and lysing the infected hemocytes after a further 3 h.

Microscopic analysis of infected brain tissue. *G. mellonella* larvae infected with *L. monocytogenes* were treated with 50 μM diclofenac and fixed in 5% formaldehyde. The brains of larvae infected with wild-type bacteria were dissected and photographed under a Leica binocular microscope, whereas those of larvae infected with the recombinant strain expressing red fluorescent protein were dissected and feathered over a glass slide, permeabilized with 0.2% Triton X-100 in PBS for 1 min, washed with PBS and photographed using a Zeiss axoplan microscope.

Anti-listeriosis-activity of signaling inhibitors. Signaling inhibitors such as 25 μM rapamycin in 1% DMSO (Calbiochem), 20 μM arachidonic acid in 1% ethanol (Sigma-Aldrich) and 50 μM diclofenac in 1% DMSO (Calbiochem) were injected into the hemocoel of last-instar *G. mellonella* larvae using 1-ml disposable syringes, resulting in approximate final in vivo concentrations of 1.25 μM rapamycin, 1 μM arachidonic acid and 2.5 μM diclofenac. The larvae were then inoculated with bacteria (10^6 cfu/larva) and controls were injected with 1% DMSO or 1% ethanol as appropriate. The injected larvae were incubated at 37 °C and reared on an artificial diet.

Data analysis. All experiments including the survival analysis of infected and therapeutically treated larvae were performed for a minimum of three times, and significant differences between the values were confirmed using the paired Student's t-test.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found at www.landesbioscience.com/journals/virulence/article/23629

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