Priming of innate antmycobacterial immunity by heat-killed Listeria monocytogenes induces sterilizing response in the adult zebrafish tuberculosis model

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Abstract

*Mycobacterium tuberculosis* remains one of the most problematic infectious agents due to its highly developed mechanisms to evade host immune responses combined with the increasing emergence of antibiotic resistance. Host-directed therapies aiming at optimal modulation of immune responses to improve bacterial eradication or to limit excessive inflammation are a new strategy for the treatment of tuberculosis. In this study, we have established a zebrafish-*Mycobacterium marinum* natural host-pathogen model system to study induced protective immune responses in mycobacterial infection. We show that priming adult zebrafish with heat-killed *Listeria monocytogenes* (HKLm) 1 day prior to *M. marinum* infection leads to significantly decreased mycobacterial loads in the infected zebrafish. Using rag1(-/-) fish we show that the protective immunity by HKLm priming can be induced through innate immunity alone. At 24 hours post infection, HKLm priming leads to a significant increase in the expression levels of macrophage-expressed gene 1 (mpeg1), tumor necrosis factor α (Tnfα) and nitric oxide synthase 2b (Nos2b) whereas superoxide dismutase 2 (Sod2) expression is downregulated implying that HKLm priming increases the number of macrophages boosts intracellular killing mechanisms. The protective effects of HKLm are abolished when the injected material is pretreated with nucleases or proteinase K. Importantly, HKLm priming significantly increases the frequency of clearance of *M. marinum* infection by evoking sterilizing immunity (25 % vs. 3.7 %, P=0.0021). In this study, immune priming is successfully used to induce sterilizing immunity against mycobacterial infection. This model provides a promising new platform for elucidating the mechanisms underlying sterilizing immunity and to develop host-directed treatment or prevention strategies against tuberculosis.
Introduction

Tuberculosis (TB) is an airborne respiratory disease caused by the intracellular bacterium *Mycobacterium tuberculosis* (Mtb). As few as one to five bacteria can lead to an infection (Rajaram, Ni et al. 2014, Cambier, Takaki et al. 2014). The outcome of tuberculosis is highly variable, ranging from rapid clearance by innate immune mechanisms, development of active disease, to the formation of a latent infection that can be actively contained inside granulomas but not eradicated. According to CDC estimates, even one third of the world population is infected with Mtb. However, only 5-10% of this population develops active, primary tuberculosis. Commonly, infection with Mtb leads to a latent, asymptomatic disease with the inherent ability to reactivate and disseminate into an active disease even decades after initial exposure, for example in the case of immunosuppression. In 2015, 1.4 million people died of tuberculosis and a total of 10.4 million new cases were reported along with an increasing number of multidrug resistant strains (World Health Organization 2016). Despite available multi-drug therapies and the Bacille Calmette-Guérin (BCG) vaccine, TB remains one of the leading infectious killers worldwide. According to a recent study, the standard 6-month antibiotic treatment against tuberculosis is ineffective in the eradication of *M. tuberculosis* even in patients with a successful follow-through of the antibiotic treatment (Malherbe, Shenai et al. 2016). As the current preventive and treatment strategies have proven insufficient, new approaches to control the global TB epidemic are urgently needed. Host-directed therapies offer a promising approach to improve the outcome of antituberculosis treatments. Host-directed therapies are a form of adjunctive therapy that aim to modulate the host immune responses to eradicate or limit mycobacterial infection (Tobin 2015).

Mycobacteria are especially successful in evading immune responses. Macrophages are known to limit mycobacterial growth in early infection to some extent (Clay, Davis et al. 2007). However, in many cases, the early events of mycobacterial infections are characterized by bacterial dominance. Pathogenic mycobacteria are able to avoid recognition by pattern recognition receptors and can lure mycobacterium permissive macrophages to the sites of infection (Cambier, Takaki et al. 2014). Upon phagocytosis, they block the fusion of phagosomes with lysosomes (Russell 2011), translocate to the cytoplasm (Simeone, Bobard et al. 2012, Houben, Demangel et al. 2012) and neutralize nitric oxide species (Flynn, Chan 2003) allowing them to survive within macrophages. Astonishingly, mycobacteria are even capable of exploiting macrophages for tissue dissemination (Clay, Davis et al. 2007). In addition to avoiding innate killing mechanisms, mycobacteria also inhibit transportation of mycobacterial antigens to lymph nodes (Wolf, Desvignes et al. 2008, Reiley, Calayag et al. 2008, ...
Gallegos, Pamer et al. 2008) thereby hampering the initiation of adaptive responses (Chackerian, Alt et al. 2002). Aggregates of innate and adaptive immune cells called granulomas are formed to contain the bacteria and to localize the infection to a limited area without eradicating the bacteria. Depending on the immune status of the host, either an active infection or a latent infection with a life-long risk of reactivation ensues (Barry, Boshoff et al. 2009).

Despite Mtb being good at evading host immune responses and having the ability to cause aggressive active or persistent latent infections, some people are known to be naturally protected against tuberculosis. There are significant differences in the ability of individuals to resist mycobacterial infection, reflecting the heterogenic nature of the human population. According to epidemiological data, a 7-43 % proportion of heavily exposed individuals are able to clear the infection before the onset of adaptive immunity resulting in negative tuberculin skin test and interferon gamma release assays (reviewed in (Verrall, G Netea et al. 2014). With this in mind, it should be possible to shift the balance of host-pathogen interactions in favour of the host by directing the immune response to the right immune activation at the early stages of infection, when the bacterial loads are rather small. Optimal immune activation could prevent mycobacterial evasion strategies, enhance killing of mycobacteria and ideally lead to sterilization of infection. However, to our knowledge, sterilizing antymycobacterial immunity has not been successfully induced in vivo.

In this study, we used the zebrafish model to study protective immune responses against mycobacteria at the early stages of infection. The zebrafish has recently become a well-accepted genetically tractable vertebrate model for human TB pathogenesis. Zebrafish are naturally infected by Mycobacterium marinum, a close genetic relative of M. tuberculosis. M. marinum causes a disease that shares the main pathological and histological features of human TB, including the formation of macrophage aggregates and granulomas (reviewed in (Meijer 2016, van Leeuwen, van der Sar et al. 2014). As the basic mechanisms of innate and adaptive immunity are conserved from zebrafish to man, the innate immune responses to M. marinum can be studied in zebrafish larvae (Ramakrishnan 2013), while the adult zebrafish has been proven an applicable model to study also the adaptive responses in TB (Hammarén, Oksanen et al. 2014, Parikka, Hammarén et al. 2012, Oksanen, Halfpenny et al. 2013). Even the transition of an acute primary infection to latency and its reactivation can be modeled in the adult zebrafish (Parikka, Hammarén et al. 2012), which has been difficult in other models. Our previous studies have shown that an infection with a low dose of M. marinum causes a latent mycobacterial disease with steady bacterial counts in the majority of the fish population, whereas in a small proportion of the fish (<1.5%) primary active disease leads to mortality
(Parikka, Hammarén et al. 2012). Around 10% of the fish are able to clear the mycobacterial infection (Hammarén, Oksanen et al. 2014). The spectrum of different disease outcomes in the *M. marinum*-zebrafish model thus resembles that of human tuberculosis.

Here, we have used the zebrafish model to test whether the number of individuals sterilizing the infection can be increased through injection with different priming agents to circumvent mycobacterial virulence strategies, which generally lead to persistent, latent infections (Parikka, Hammarén et al. 2012). Our study shows that the priming of zebrafish with heat-killed *Listeria monocytogenes* results in the sterilization of *M. marinum* infection in one fourth of individuals. The protective effect is caused by a protein and/or nucleic acid component of *L. monocytogenes* and is accompanied by the induction of *tumor necrosis factor α* (*tnfα*), *nitric oxide synthase 2b* (*nos2b*) and down regulation of *superoxide dismutase 2* (*sod2*). Hereby we show that the adult zebrafish is a feasible model for deciphering the mechanisms of sterilizing immunity, knowledge of which is crucial for the development of new preventive strategies and adjunctive therapies against tuberculosis.

**Results**

**Immune activation by heat-killed *Listeria monocytogenes* leads to lower mycobacterial burdens in adult zebrafish**

In this study, we set out to study protective immune responses at the early stages of an *M. marinum* infection. Our hypothesis is that the right immune activation at the early stages of an infection prevents mycobacterial evasion strategies and leads to protective immune responses, increased killing of mycobacteria, or even clearing of mycobacteria. To study our hypothesis, we wanted to direct the zebrafish immune response towards an optimal anti-TB response before the onset of an *M. marinum* infection. To find a factor that promotes a protective immune response in the early stages of an infection in adult zebrafish, fish were intraperitoneally injected with different priming agents one day before a low-dose intraperitoneal *M. marinum* infection (Fig 1A). Mycobacterial loads of the fish were determined with qPCR from internal organs 7 weeks post infection (wpi). We tested eight different agents including TLR ligands, vaccine adjuvants and heat-killed bacteria, namely, *M.*
marinum (HKMm), Listeria monocytogenes (HKLm), Streptococcus iniae (HKSi), Escherichia coli (HKEc), lipopolysaccharide (LPS), paclitaxel, muramyl dipeptide (MDP) and zymosan.

In this preliminary experiment, the tested priming agents had variable effects on mycobacterial loads. Priming of the adult fish with HKLm led to a clear decrease in median mycobacterial loads at 7 wpi compared to PBS controls. However, with most of the other tested priming agents, including HKMm, the reduction in mycobacterial loads was not as dramatic as with HKLm (Fig 1B). Importantly, HKLm priming was the most efficient way to induce bacterial clearance. The frequency of clearance increased by 4.8-fold compared to PBS-primed control. For paclitaxel, priming lead to an increase in mycobacterial loads. With the right dosing, paclitaxel can induce proinflammatory responses (Bracci, Schiavoni et al. 2014) but can also be toxic to immune cells (Tang, Hui Yuwen et al. 2017), which might explain the increase in mycobacterial load by paclitaxel. Our results indicate that early immune priming affects mycobacterial loads at 7 wpi and that heat-killed L. monocytogenes is the most promising priming agent with our experimental set up.

To ensure that the reduction in mycobacterial loads in adult fish was not due to direct bactericidal effects of HKLm priming, we incubated M. marinum in 7H9 medium together with different concentrations of HKLm (Fig S1). Based on the in vitro culturing, HKLm does not kill M. marinum indicating that the lowered mycobacterial burdens in zebrafish induced by HKLm priming is not due to direct killing of the mycobacteria by HKLm, but is mediated through effects on the host immune system.

To characterize the duration of the effect after HKLm priming, we injected fish either 1 or 7 days prior to infection with M. marinum. The mycobacterial loads were determined already at 4 wpi, because at this stage of the infection, bacterial counts have generally reached a steady state and they are easily measurable. Priming 1 day prior to infection was most efficient increasing the frequency of a clearing response by 3.7-fold compared control group (7d: 2.3-fold) in this preliminary experiment (Fig 1C). Based on these results, we continued with the -1d HKLm.
**Priming with heat-killed *L. monocytogenes* increases the frequency of clearance of mycobacterial infection in adult zebrafish**

To verify our finding from the preliminary priming experiments zebrafish were primed with an injection of HKLm (Fig 2A) one day prior to *M. marinum* infection, and PBS was used as a negative control. Compared to control treatments, priming with HKLm one day before infection consistently led to a significant decrease (14.1-fold decrease, on average) in mycobacterial loads in adult zebrafish at 4 wpi (Fig 2A shows a representative result of four separate experiments).

From our previous study, we know that without priming, approximately 10% of the *M. marinum* infected zebrafish are able to naturally clear the mycobacterial infection (Hammarén, Oksanen et al. 2014). Combining the results from four different experiments shows that priming with HKLm one day prior to *M. marinum* infection increases the frequency of sterilizing response by 6.8-fold in the wild-type fish at 4 wpi (PBS 3.7% (n=54) vs. HKLm 25 % (n=56), P=0.0021) (Fig 2B). However, also the individuals that have been unable to clear the infection, benefit from HKLm priming in terms of lowered bacterial loads (Fig S3). In a wild-type population with sustained infection, HKLm reduced the median bacterial load by 11.1-fold (P<0.0001).

**Innate immune mechanisms mediate the heat-killed *Listeria monocytogenes*-induced protection against mycobacterial infection**

Our results show a beneficial effect of HKLm priming on mycobacterial loads and clearance at 7 wpi (Fig 1B), 4 wpi (Fig 2A) as well as at 2 wpi (Fig 2C), all of which are late enough stages for both innate and adaptive responses to be active, although it is known that virulent mycobacteria cause a delay in the activation of T-cell responses (Gallegos, Pamer et al. 2008). To test, if HKLm priming has the same reducing effect on mycobacterial loads in the absence of an adaptive immune response, we used *rag1(-/-)* mutant fish. Fish lacking *rag1* do not undergo V(D)J recombination, which is essential for the production of the full variety of T cell and B cell receptors. The *rag1(-/-)* mutants therefore rely solely on their innate immune mechanisms in protection against infections (Wienholds, Schulte-Merker et al. 2002).

When *rag1(-/-)* mutants were primed with HKLm one day before *M. marinum* infection and bacterial loads were measured with qPCR at 4 wpi, HKLm priming caused a similar reduction in bacterial
loads in rag1(-/-) mutants (30-fold, P=0.0089) as in wild-type fish (9.7-fold, P=0.0013) (Fig 2D and Fig 2A). Also in rag1(-/-) mutants, priming with HKLm increased the frequency of sterilizing response from 0% (n=26) to 17% (n=23), P=0.0418 (Fig 5B). Looking at the fish with a sustained infection (unable to clear the infection), HKLm priming reduced the bacterial rag1 mutants by 7.2-fold (Fig S2B, not statistically significant). HKLm priming did not affect the cumulative mortality of low-dose infected wild-type adult fish (PBS: 11.7%; HKLm: 11.2%) but caused a trend of reduced mortality in rag1(-/-) mutant fish (PBS: 24.5%; HKLm: 11.1%) (Fig 2F-G). Together results imply an important role for innate immunity in the formation of protective HKLm-induced immune responses against mycobacterial infection. Importantly, they show that an optimal response induced by HKLm priming significantly increases the frequency of clearance of mycobacterial infection also in the absence of adaptive immunity.

Protective effects of heat-killed Listeria monocytogenes priming are not seen in the larval Mycobacterium marinum infection model

As our results showed that the protective effects of HKLm priming can be mediated through innate immune responses alone, we next used zebrafish larvae to test the effect of HKLm priming on M. marinum infection. During the first weeks of development, the zebrafish lack adaptive immune responses and rely solely on innate immune responses making it possible to study mechanisms of mycobacterial infection that arise from innate immunity. To this end, zebrafish larvae were primed with HKLm to induce a protective immune response against M. marinum infection. Zebrafish were primed by intravenous injection either at 1 dpf (day post fertilization) (Fig S2A) or at 2 dpf (Fig S2B). M. marinum infection was carried out intravenously at 2 dpf. Within this experimental setting, mycobacterial loads were not reduced in zebrafish larvae after priming with HKLm (Fig S2), possibly due to the immaturity of immune responses in the young larvae or due to the relatively lower HKLm dose that could be delivered into the larvae. As we were unable to see any protective effect in the zebrafish larvae, we continued using adult zebrafish for subsequent experiments.

Protective immunity against M. marinum infection is mediated by a protein and/or nucleic acid component of heat-killed L. monocytogenes

Based on the results of the preliminary experiment with different priming agents, HKLm seemed to contain a specific component responsible for the induction of sterilizing immunity. To characterize
the protective component(s), we carried out a set of experiments, in which zebrafish were primed with different preparations of HKLm 1 day prior to *M. marinum* infection and bacterial growth was analyzed 4 wpi. Priming with spent *L. monocytogenes* medium did not cause a significant reduction in the mycobacterial loads of the fish indicating that a secreted factor was not the causative agent behind the activation of antimycobacterial immune responses (Fig 3A). We also found that after autoclavage of *L. monocytogenes*, priming with the insoluble material provided a protective effect against *M. marinum* infection, whereas the soluble material was not protective (Fig 3B). However, we were able to show that treatment of the HKLm extract with either proteinase K or a cocktail of RNase and DNase abolished the protective effect (Fig 3C). These results indicate that the protective component is stable, resistant to high temperature and pressure, non-secreted, insoluble and likely consists of both a protein and nucleic acid component.

**Therapeutic potential of heat-killed Listeria monocytogenes treatment**

To further characterize the features of HKLm treatment, a 30-fold higher HKLm dose was injected 1 day prior to a low-dose *M. marinum* infection. With such a high dose, HKLm lost its beneficial effects (Fig 4A) and caused a trend of increased mortality of the fish by 4 wpi (PBS: 22.7%; HKLm: 41.7%) (Fig 4B). According to this results, it was concluded that by increasing the HKLm dose, it is not possible to further boost the protective effect but rather vice versa. The optimal dosage thus plays a crucial role in inducing a protective response.

We then tested, whether priming with HKLm 1 day prior to infection could protect fish against a high-dose infection challenge with 4883±919 cfu of *M. marinum*. In the context of a high-dose infection, HKLm did not induce significant difference in the mycobacterial loads, clearance or cumulative end-point mortality at 4 wpi (Fig 4C-D).

We next wanted to test whether HKLm can induce protective immune responses if the mycobacterial infection is already established. We have previously shown in the adult zebrafish that at 2 wpi, *M. marinum* infection is well-established and granulomas have already started to form (Parikka, Hammarén et al. 2012). We infected adult zebrafish with a low dose of *M. marinum*, injected HKLm two weeks after infection and measured mycobacterial loads as well as determined the cumulative mortality at 4 wpi. Based on our results, at a time-point in which the mycobacteria have already multiplied substantially and started forming granulomas, HKLm injection was not able to lower the bacterial loads, increase bacterial clearance or affect cumulative mortality (Fig 4E-F).
Primed adult zebrafish with heat-killed *Listeria monocytogenes* induces *mpeg1*, *tnfα* and *nos2* expression and downregulates *sod2* at the early phase of mycobacterial infection

We were interested in further characterizing the nature of the immune response induced by HKLm priming in adult zebrafish. We hypothesized that the protective effect could be mediated through enhanced killing of mycobacteria at the early phase of the infection due to changes in the numbers or activity of innate immune cells. To study the details of HKLm-induced immune activation by qPCR, the fish were primed with HKLm or PBS, infected with a low dose of *M. marinum* 1 day after priming and the total RNA was extracted from zebrafish organs collected 1 day post infection. First, the possible effects of HKLm treatment on the number of macrophages and neutrophils were by assessed by measuring the expression of *macrophage expressed gene 1* (*mpeg1*) (Ellett, Pase et al. 2011) and *myeloid-specific peroxidase* (*mpx*) (Lieschke, Oates et al. 2001), respectively. In the HKLm group, *mpeg1* expression was significantly higher than in the control group (Fig 5A HKLm 59.9-fold, PBS 39.3-fold, P=0.0352) suggesting that the number of macrophages was increased due to HKLm priming. The expression of *mpx* was not affected by HKLm (Fig 5B).

To assess the HKLm-induced changes at the level of immune cell activation, we measured the expression of a selection of markers related to the effective antimycobacterial functions of innate immune cells one day after *M. marinum* infection. Based on literature on the mechanisms limiting intracellular mycobacterial growth, the genes chosen for analysis were *tumor necrosis factor α* (*tnfα*) (Cobat, Poirier et al. 2015, Roca, Ramakrishnan 2013), *interferon γ* (*ifnγ*) (Flynn, Chan et al. 1993) and *nitric oxid synthase 2b* (*nos2b*) (Nicholson, Bonecini-Almeida Mda et al. 1996, Thoma-Uszynski, Stenger et al. 2001). *Ifnγ* was not differentially induced between PBS and HKLm treated groups (Fig 5C-D). The median expression levels of *tnfα* (Fig 5F, HKLm 10.3-fold vs. PBS 1.1-fold, P=0.0043) and *nos2b* (Fig 5G, HKLm 10.7-fold vs. 0.6 fold PBS, P=0.0001) were significantly increased in the HKLm primed group compared to the PBS control group. We also analyzed the expression levels of *sod2* (*superoxide dismutase 2*), which is a mitochondrial protein that converts the byproducts of oxidative phosphorylation to hydrogen peroxide and oxygen leading to neutralization of mitochondrial ROS (Pias, Ekshyyan et al. 2003) and *arg1* which is an alternative macrophage activation marker (Gordon, Martinez 2010). In the HKLm group, *sod2* expression was significantly more downregulated as compared to the PBS group indicating increased levels of ROS due to HKLm priming (PBS 0.59-fold vs. HKLm 0.27-fold, P=0.0022) (Fig 5H). *Arg1*, however, was not induced
in HKLm primed fish suggesting that alternative activation does not have an impact on the early mycobacteria elimination (Fig 5E). Together, these results suggest that HKLm induces M1-type classical macrophage activation leading to enhanced intracellular killing at the early stages of a mycobacterial infection.

**Priming of macrophages with heat-killed L. monocytogenes leads to decreased oxygen consumption in vitro**

Classically activated M1 macrophages have been shown to change their metabolism upon immune activation (Cheng, Quintin et al. 2014). To test whether this is also the case with HKLm priming, we set up RAW264.7 cell culture and primed them either with LPS (50 ng/µl) or HKLm (MOI: 530). 19 to 24h later oxygen consumed by these cells was measured using a Clark electrode. We used LPS as a positive control and showed that both treatments lower the oxygen consumption significantly (Fig 5I). Pooled result from three independent experiments concluded that HKLm or LPS primed mouse macrophages consume respectively 1.8-times (P=0.008) and 2.3-times (P=0.0042) less oxygen compared to untreated cells, *in vitro* (Fig 5I). This result imply that HKLm treated macrophages exhibit metabolic changes indicative of diminished oxygen similar to those observed during classical macrophage activation.
Discussion

Despite substantial progress in the field of medicine, tuberculosis still kills millions of people every year, and it has been declared a global public health crisis (World Health Organization 2016). The advances in the battle against tuberculosis have been hindered by the complex nature of the disease and the limitations of animal models to study tuberculosis. Tuberculosis manifests itself in a wide spectrum of disease, with most affected individuals being unable to eradicate the causative bacteria *M. tuberculosis* leading to the development of latent TB infection, which has a lifetime risk of reactivation in 5-10% of cases. The World Health Organization has estimated that 2-3 billion people are latently infected with *M. tuberculosis* (World Health Organization 2016) creating a huge pool of individuals with a potential to develop an active, transmissive disease. As current vaccination and antibiotic schemes have proven insufficient for controlling the global TB epidemic, host-directed therapies inducing protective immune responses are emerging as a novel strategy to treat TB (Tobin 2015). Efficient host-directed therapies used either alone or in combination with antibiotics are an approach potentially leading to sterilization of TB.

Although genome-wide association studies carried out in humans have given important clues on the nature of protective immune responses against TB (Azad, Sadee et al. 2012, Wilkinson, Llewelyn et al. 2000, Cobat, Gallant et al. 2009, Cobat, Poirier et al. 2015), animal models are essential for the execution of more mechanistic studies. The mouse, rabbit and macaque have been widely used to study TB (Myllymäki, Niskanen et al. 2015). To the best of our knowledge, spontaneous or induced sterilizing immunity has not been observed at the organismal level in mammalian animal models of TB. Clearance of cultivable mycobacteria occurs in the rabbit model (Subbian, Tsenova et al. 2012). However, standard bacterial culturing methods only detect the actively replicating mycobacterial populations, but not dormant bacteria (Chao, Rubin 2010), and in the rabbit, the clearance of cultivable mycobacteria indicates the establishment of a truly latent disease instead of sterilization (Subbian, Tsenova et al. 2012). The zebrafish has recently become a well-accepted genetically tractable vertebrate model for human TB pathogenesis to complement the more traditional mammalian models (Myllymäki, Niskanen et al. 2015). In our previous study, using a qPCR-based method, we were able to see spontaneous clearance of mycobacterial infection in the zebrafish-*M. marinum* infection model in approximately 10% of the fish at 4 wpi (Hammarén, Oksanen et al. 2014). As we observed no clearance at 2 wpi (Hammarén, Oksanen et al. 2014) spontaneous early clearance (likely induced by innate mechanisms) in our wild-type zebrafish population is a rare event. In this current study, we were able to increase the frequency of sterilizing mycobacterial infection by priming the innate immune response prior to *M. marinum* infection.
Our hypothesis was that priming or stimulation of the immune response in the adult zebrafish before *M. marinum* infection could lead to a sterilizing immune response instead of a lethal primary active disease or a latent infection that is prone to reactivation later in life (Parikka, Hammarén et al. 2012). By using an array of different priming agents, we wanted to study whether we could create a climate that would allow the immune response to circumvent their efficient virulence strategies and even eradicate the mycobacteria. Priming approaches have been successful in inducing protective responses against various other bacterial infections in the fruit fly, *Drosophila melanogaster* that relies solely on innate immunity (Pham, Dionne et al. 2007). Indeed, our results showed that sterilizing immunity can be induced in the *M. marinum* zebrafish model. In our hands, priming with heat-killed *L. monocytogenes* induces a sterilizing response in 25% of *M. marinum* infected fish at 4 wpi, whereas the percentage of spontaneous clearance was 3.7% in the PBS-primed control group.

The *M. marinum* -specific qPCR used to quantify bacterial loads is a sensitive method with a detection limit of approximately 100 bacterial genomes in the entire fish (Parikka, Hammarén et al. 2012, Hammarén, Oksanen et al. 2014). The major advantage of a qPCR-based method over culturing methods is that qPCR detects all bacterial genomes irrespective of metabolic state so that dormant bacteria are also detected. An advantage of the zebrafish is that, due to its small size, all infection target organs can be collected for determination of bacterial load, which is not practically feasible in larger animals. Based on the qPCR results, we are able to say that 25% of the wild type fish were able to clear the infection, indicating that HKLm priming indeed increases the frequency of sterilizing immune response. This model provides tools for elucidating the detailed mechanisms behind sterilizing immunity against tuberculosis.

To study whether the protective effects of HKLm priming require a functional adaptive immune system, we used *rag1* mutant zebrafish. Although *rag1* mutant zebrafish are known to be hypersusceptible to *M. marinum* infection due to a failure in the development of functional T and B cells (Swaim, Connolly et al. 2006, Parikka, Hammarén et al. 2012), their bacterial loads were significantly lowered and even cleared by HKLm priming suggesting that the protective response can be mediated by innate immunity alone. The role of innate immunity is also supported by the significant HKLm-induced reduction in bacterial loads as early as 2 wpi in wild-type fish, by which time adaptive responses are only starting to arise in mycobacterial infections (Andersen, Woodworth 2014). Despite the clear involvement of innate responses in HKLm-induced protective responses in adult fish, the results could not be reproduced in young zebrafish larvae that have only innate immunity, probably due to an immature immune response and technical difficulties to deliver high
enough doses of HKLm by microinjection methods. As adaptive responses are centrally involved in the pathogenesis of mycobacterial infections, using the adult zebrafish will likely better model the effects of immunomodulatory treatments on the immune response as a whole. On top of the protective effects of HKLm mediated through innate immunity, an additional level of protection was observed in the presence of a functional adaptive immune system in zebrafish. However, the observation that activation of innate immunity alone by immune priming with HKLm in some cases was sufficient for induction of a protective or sterilizing immunity opens new avenues for host-directed therapies or preventive strategies even in the absence of a fully functional adaptive immune system, such as in patients with a HIV co-infection.

At the moment, there is no immunotherapy that could cure an on-going mycobacterial infection by simply boosting the immune system. Therefore, we wanted to study whether HKLm could induce a protective response during an established mycobacterial infection. A single dose of HKLm was injected two weeks after *M. marinum* infection, when early granulomas have started to form and are visible in various organs (Parikka, Hammarén et al. 2012). However, this single injection of HKLm did not decrease mycobacterial loads at 4 wpi (Fig 4E-F). By this time-point, *M. marinum* has already had plenty of time to exert its early virulence strategies leading to effective avoidance of the pro-inflammatory host immune response (Elks, van der Vaart et al. 2014, Queval, Song et al. 2016, Bhat, Srivastava et al. 2017, Cambier, Takaki et al. 2014). Having gained a foothold within its host, mycobacteria are not as prone to the effects caused by a single therapeutic injection of HKLm as they are when entering a primed host. Adult zebrafish have also been used to model active fulminant tuberculosis by infecting individuals with a high *M. marinum* dose (Parikka, Hammarén et al. 2012). Individuals infected with a high *M. marinum* dose did not benefit from HKLm priming. The high infection dose of a few thousand mycobacteria leads to a disease state in which the capacity of the immune system rapidly becomes saturated allowing the bacteria to grow almost logarithmically (Parikka, Hammarén et al. 2012) possibly due to the limited number of macrophages (Pagán, Yang et al. 2015) compared to the high infection dose; a situation too challenging to overcome even in the presence of HKLm priming. However, in our preliminary experiments, we saw that in addition to priming 1 day prior to infection, protective effects were still visible when HKLm priming was delivered 1 week prior to *M. marinum* infection (Fig 1C), suggesting that this type of protective responses could be considered in designing new preventive strategies.

The protective effects of HKLm treatment delivered prior to infection could be mediated through the induction of trained immunity. It is known that innate immune cells can mediate an enhanced immune
response upon reinfection with same pathogen (Quintin, Saeed et al. 2012). The innate immune system can also cross-react with a new pathogen according to previous stimuli (Kleinnijenhuis, Quintin et al. 2014). It has been reported that some vaccines can produce durable cross-protection that cannot be explained by adaptive responses (Aaby, Kollmann et al. 2014). This type of nonspecific innate memory is referred to as trained immunity. Mechanisms of trained immunity have also been shown to be responsible for BCG induced by-stander protection against Candida albicans in the mouse (Van't Wout, Poell et al. 1992). Innate memory is mediated through reversible epigenetic changes rather than irreversible genetic recombination seen during the formation of classical immunological memory in adaptive immune cells and can last for weeks to months (reviewed by (Netea, Joosten et al. 2016). The effects of trained immunity in the context of susceptibility to tuberculosis is an interesting area of research and can yield new approaches in the development of preventive strategies based on innate immunity.

It has been reported that the number of macrophages is critical for the disease outcome and that macrophage deficiency is connected to accelerated progression of mycobacterial infection (Pagán, Yang et al. 2015). To assess the effect of HKLm priming on the number of macrophages and neutrophils, we measured the expression of the commonly used markers mpeg1 (macrophage expressed gene 1) (Ellett, Pase et al. 2011) and mpx (myeloid-specific peroxidase) (Lieschke, Oates et al. 2001), respectively. Based on the expression of these markers, the number of macrophages was significantly higher in HKLm primed fish (\(P=0.0352\)) whereas the amount of neutrophils remain unchanged (Fig 4A-B). This change seen in macrophages is potentially mediating the protective response against mycobacteria.

To decipher the type of protective immune response induced by HKLm priming, we measured an array of genes related to innate immune activation in the organs of M. marinum infected zebrafish 1 dpi. In line with in vitro studies on Listeria (Barbuddhe, Malik et al. 1998, Mirkovitch, König et al. 2006) in vivo priming with HKLm caused a significant increase in nos2b and tnfα with a simultaneous decrease in sod2 expression. Nos2b is one of the nitric oxide synthases in the zebrafish (Lepiller, Franche et al. 2009). NO is known to be mycobacteriocidal (Nicholson, Bonecini-Almeida Mda et al. 1996), but as pathogenic mycobacteria have developed evasion strategies to inhibit production of NO (Elks, van der Vaart et al. 2014, Queval, Song et al. 2016, Bhat, Srivastava et al. 2017), the NO levels naturally induced in Mtb-infected macrophages seem to be insufficient for lysing mycobacteria (Jung, Madan-Lala et al. 2013). Thus, the additional production of Nos caused by HKLm prior to infection likely potentiates the intracellular killing mechanisms. The beneficial effects of increased NO in
neutrophils (Elks, Brizee et al. 2013) as well as in macrophages (Cambier, Takaki et al. 2014) during early *M. marinum* infection have previously been demonstrated in zebrafish larvae. Cambier and colleagues showed that virulent mycobacteria avoid NO-mediated intracellular killing during the early phase of infection by hiding their TLR ligands under a phthiocerol dimycoceroserate coat (Cambier, Takaki et al. 2014). Co-infecting zebrafish larvae with *M. marinum* and *Staphylococcus aureus* or *Pseudomonas aeruginosa* lead to attenuation of mycobacterial infection (Cambier, Takaki et al. 2014). Also co-injection of live *M. marinum* with heat-killed *M. marinum* or with a mutant with exposed TLR ligands caused similar attenuation (Cambier, Takaki et al. 2014). It is likely that at least part of the protective effects caused by HKLm in the adult zebrafish are mediated through TLR ligands. The component analysis of HKLm suggested that nucleotides were important for HKLm-mediated protection against *M. marinum*. TLR9, which recognizes ddDNA, and leads induction of Nos2 (Ito, Wang et al. 2005) is a receptor potentially responsible for the protection. However, detailed analysis of the signaling pathways activated by HKLm treatment was beyond the scope of this study.

Tnfα is also known to mediate intracellular killing of mycobacteria by macrophages (Roca, Ramakrishnan 2013) and optimal levels of this cytokine have been proposed to lead to early clearance of TB in humans (Cobat, Poirier et al. 2015). Studies in zebrafish larvae have shown that high Tnfα levels alongside high ROS levels within macrophages, during the early days of mycobacterial infection, is bactericidal (Roca, Ramakrishnan 2013). Superoxide dismutase 2 is an enzyme that acts through neutralization of mitochondrial ROS (Pias, Ekshyyan et al. 2003). Its downregulation by HKLm should thus cause an increase in mitochondrial ROS, the high levels of which have been shown to enhance intracellular killing mechanisms within macrophages (reviewed in (Hall, Sanderson et al. 2014). Recently, in a human population study, a genetic variant leading to reduced activity of Sod2 was found to be associated with increased resistance to leprosy, a disease caused by *Mycobacterium leprae* (Ramos, Salomao et al. 2016). Therefore, a likely mechanism of clearing the mycobacterial infection by HKLm priming in the adult zebrafish model is mediated through increased Tnfα and decreased Sod2 production that together lead to higher, mycobacteriocidal levels of ROS within macrophages.

Roca et al. also showed that when the expression of Tnfα is endogenously high, continuously high ROS levels after the first days of infection leads to excessive inflammation, necrosis and exacerbation of the disease. This probably also explains the increased mortality with the higher HKLm dose. With a single small dose of HKLm used in our study, the effects of the treatment were undoubtedly positive, but it must be kept in mind that excessive or prolonged induction of Tnfα and ROS can also have
detrimental effects. The dosage of treatment as well as the genotype of the host affecting the baseline production of inflammatory cytokines will also need to be carefully considered in the development of host-directed immunomodulatory treatments.

Based on the gene expression data, the changes in the innate immunity induced by HKLm in the adult zebrafish seem to be mediated through an increased number and activation of M1 type macrophages. Recent research on the metabolism of different innate immune cells has shown that M1 macrophages have decreased oxygen consumption and increased glycolysis and lactate production (Cheng, Quintin et al. 2014). In our experiments, the oxygen consumption of mouse macrophages was significantly decreased by HKLm priming (Fig 5I) providing a further piece of evidence of M1 macrophages playing a central role in HKLm-mediated protection against mycobacterial infection. The result also implies that the type of activation caused by HKLm in the zebrafish are similar to those induced in mammalian macrophages.

Overall, we show that protective and even sterilizing immune responses can be induced in the zebrafish model for tuberculosis by priming with heat-killed \textit{Listeria monocytogenes}. The response is induced even in the absence of adaptive immunity and is accompanied by the increase in the number of macrophages, the induction of $\text{tnf}\alpha$, $\text{nos}\ 2b$ and the down-regulation of $\text{sod}\ 2$, likely leading to increased production of radical nitrogen and oxygen species and enhanced intracellular killing of mycobacteria. Based on our results, it seems that the type of activation induced by HKLm treatment is only effective when delivered at an early enough time-point prior to exposure to pathogenic mycobacteria. The model provides a platform in which both innate and adaptive mechanisms leading to sterilization of mycobacterial infection can be reliably studied. Such knowledge will contribute to the development of new vaccination strategies as well as host-directed therapies aiming at prevention of transmission and sterilizing treatment of TB disease.
Materials and methods

Zebrafish lines and housing
5-10 months old adult male and female AB wildtype zebrafish (*Danio rerio*) and *rag1(-/-) (hu1999)* mutant zebrafish (from ZIRC, Zebrafish International Resource Center, University of Oregon, OR, USA) were used in the experiments. The fish were housed in flow-through water circulation systems with a 14h/10h light-dark cycle.

Ethics Statement
All experiments were conducted according to the Finnish Act on Animal Experimentation (62/2006) and the Act on the Protection of Animals Used for Scientific or Educational Purposes (497/2013). ELLA (Eläinkoelautakunta, the National Animal Experiment Board in Finland under the Regional State Administrative Agency for Southern Finland) has approved the Tampere zebrafish facility and the animal experiments carried out in this project under the licenses ESAVI/6407/04.10.03/2012, ESAVI/8245/04.10.07/2015 and ESAVI/10079/04.10.06/2015.

Experimental *M. marinum* infections
*Mycobacterium marinum* (ATCC 927) was first pre-cultured on Middlebrook 7H10 plates with OADC enrichment (Fisher Scientific, NH, USA) at 29°C for one week. After plate culturing, *M. marinum* was transferred into Middlebrook 7H9 medium with ADC enrichment (Fisher Scientific, NH, USA) with 0.2% Tween 80 (Sigma-Aldrich, MO, USA), cultured for 3–4 days, diluted 1:10 and cultured for further 2 days until OD600nm reached 0.460–0.650. For adult zebrafish infections, *M. marinum* was first harvested by centrifuging for 3 min at 10,000 x g and was then resuspended and diluted in sterile 1x PBS with 0.3 mg/ml of phenol red (Sigma-Aldrich, MO, USA). 5 µl of the suspension (33±19 cfu/fish) was injected intraperitoneally (*i.p.*) with Omnican 100 30 G insulin needle (Braun, Melsungen, Germany) under 0.02% 3-aminobenzoic acid ethyl ester (pH 7.0) (Sigma-Aldrich, MO, USA) anesthesia. Infection doses were verified by plating 5 µl of the injection suspension on a 7H10 plate.

For larval infections *M. marinum* PTEC15 strain was used. This in-house made *M. marinum* wasabi-fluorescent strain was made by transforming a PTEC15 plasmid into *M. marinum* (ATCC 927) strain by electroporation. pTEC15 was a gift from Lalita Ramakrishnan (Addgene plasmid #30174). For larval infections, *M. marinum* PTEC15 strain was cultured for 4-5 days in supplemented 7H9 medium
with 75 µg/ml hygromycin (Merck, Darmstadt, Germany), diluted 1:10, cultured for 3 days until the OD600nm is 0.407-0.537 and harvested for infection by centrifugation.

Zebrafish larval infection experiments

To study the effect of heat-killed *L. monocytogenes* (HKLm) priming in zebrafish larvae, 1 nl of HKLm (240 cfu) or PBS control were injected into the caudal vein at 1 dpf (days post fertilization) under 0.0045% 1-phenyl-2-thiourea (Sigma-Aldrich, MO, USA) anesthesia. At 2 dpf the larvae were infected with 1 nl of *M. marinum* (39±13 cfu) into the blood circulation valley, transferred to fresh E3 medium and kept at 29°C. At 8 dpi, larvae were collected for DNA extraction with TRI Reagent (Fisher Scientific, NH, USA). DNA extraction was performed according to manufacturer’s instructions, after which *M. marinum* –specific qPCR was used to quantify mycobacteria.

For the quantification of PTEC15 fluorescence, 1 dpf (days post fertilization) wild type AB embryos were dechorionated and kept in E3 medium with 0.0045% 1-phenyl-2-thiourea (Sigma-Aldrich, MO, USA) at 29°C to prevent pigmentation. 1 nl of wasabi-fluorescent *M. marinum* suspension (39±16 cfu) with 0.6 mg/ml of phenol red (Sigma-Aldrich, MO, USA) and 490 cfu of HKLm were microinjected into the blood circulation valley at 2 dpf with a glass microcapillary. *M. marinum* infection doses were verified by plating the injection doses on a 7H10 agar plate. After infection, the larvae were kept in E3 medium with 0.0045% 1-phenyl-2-thiourea (Sigma-Aldrich, MO, USA) on 24-well plates at 29°C.

At 7 dpi (days post infection) larvae were anaesthetized with 0.02% 3-aminobenzoic acid ethyl ester (pH 7.0) (Sigma-Aldrich, MO, USA). The larvae were embedded on their side in 1% low melt agarose in E3 medium on black 96-proxiplates (Perkin-Elmer, MA, USA). Extra E3 medium with the anesthetic was added on top of the solidified low melt agarose to prevent the larvae from drying. The wasabi-fluorescent signal was measured three times using the EnVision plate reader (Perkin-Elmer, MA, USA) scanning program. The scan measurement was carried out on 5 horizontal and 5 vertical dots 0.5 mm apart from 6.5 mm height with 100% excitation at 493 nm, 509 nm emission and 500 flashes per point. The fluorescent signals of individual zebrafish larvae were normalized with the average signal from healthy non-infected larvae.
Preparation of heat-killed bacteria and priming injections

For the preparation of heat-killed bacteria, *Listeria monocytogenes* (10403S), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), *Salmonella typhimurium* (ATCC 14028) and *Streptococcus iniae* (ATCC 29178) were inoculated from glycerol stocks or blood agar plates and cultured in brain heart broth (BHB) (Sigma-Aldrich, MO, USA) at 37 °C until the OD600nm reached 0.9–1.0. Bacterial suspensions were plated on LB agar plates to verify bacterial concentrations. To heat-kill bacteria, the bacterial suspensions were autoclaved in BHB at 120 °C for 20 min and the sterility was confirmed by plating on LB plates after autoclaving. Injection doses of heat-killed bacteria for adult zebrafish were 0.5–1 x10^7 cfu (colony forming units). Injection doses for other priming agents were 13.5 µg/fish for lipopolysaccharide (Sigma-Aldrich, MO, USA), paclitaxel (Sigma-Aldrich, MO, USA), and Zymosan (Sigma-Aldrich, MO, USA) and 4.5 µg/fish for muramyl-dipeptide (Sigma-Aldrich, MO, USA). 5 µl priming i.p. injections were injected with Omnican 100 30 G insulin needle (Braun, Melsungen, Germany) under 0.02% 3-aminobenzoic acid ethyl ester (pH 7.0) anesthesia.

DNAse, RNAse and proteinase K treatment of heat-killed *Listeria monocytogenes*

DNase and RNase treatments were performed for autoclaved *L. monocytogenes* in BHB medium. Heat-killed bacterial suspension was incubated with 10 µg/ml of RNase A (Thermo Fisher Scientific, NH, USA) at 37°C for 18 h. After RNase treatment, the suspension was treated with 83 U/ml DNase I (Thermo Fisher Scientific, NH, USA) according to manufacturer’s instructions. Accordingly, HKLm was treated with 10 µg/ml of proteinase K (Thermo Scientific, NH, USA) at 37°C for 18 h and inactivated at 70°C for 15 min before injections.

RNA and DNA extractions from zebrafish samples

For RNA and DNA extractions, adult zebrafish were first euthanized with an overdose of 3-aminobenzoic acid ethyl ester anesthetic and then internal organs were collected from the body cavity. Organs were homogenized in TRI Reagent (Fisher Scientific, NH, USA) with ceramic beads using the PowerLyzer24 (Mobio, CA, USA) bead beater at 3200 rpm for 3×40 s. Samples were cooled on ice between the cycles. After homogenization, samples were sonicated for 9 min and the RNA and DNA were extracted according to the manufacturer’s instructions.
Gene expression studies and quantifying mycobacterial loads by quantitative PCR

Prior to quantitative PCR (qPCR) analysis, RNA was treated with DNase I (Thermo Fisher Scientific, NH, USA) to remove possible traces of genomic DNA according to the manufacturer’s instructions. After DNase treatment, RNA was reverse transcribed into cDNA with a Reverse Transcription kit (Fluidigm, CA, USA) according to the manufacturer’s instructions. Gene expression was measured by using SsoFast EvaGreen Supermix with Low ROX qPCR kit (Bio-Rad, CA, USA) with the CFX96 qPCR system (Bio-Rad, CA, USA). Zebrafish genes were normalized with expressed repetitive element loopern4 (Vanhauwaert, Van Peer et al. 2014) and compared to average induction of pooled baseline sample of healthy non-infected zebrafish. Results were analyzed using the ΔCt method and are shown as fold induction.

Mycobacterial loads were measured with the SensiFAST SYBR No-ROX qPCR kit (Bioline, London, UK) from genomic DNA according to manufacturer’s instructions. Each bacterial quantification qPCR run included standard curve of known amounts of M. marinum DNA. Primer sequences and gene association numbers are shown in table I.

Table I. Primer pairs used in qPCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene</th>
<th>Primer sequences 5’→3’</th>
</tr>
</thead>
</table>
| ifnγ1-2 | ZDB-GENE-040629-1 | F: GGGCGATCAAGGAAAACGACCC  
                      R: TAGCCTGCGGTCCTCTTTCGCT |
| ifnγ1-1 | ZDB-GENE-060210-1 | F: CCAGGATATTCACTCAGTCAAGGC  
                      R: TGTGGAGGCCCGATAATACACC |
| loopern4 | Expressed repetitive elements | F: TGAGCTGAAACTTTACAGACAT  
                      R: AGACTTTGGTGTCTCCAGAATG |
| nos2b  | ZDB-GENE-080916-1 | F: TCACCACAAAAGAGGCTGGAATTCG  
                      R: ACGCGCATCAAACAACCTGCAAA |
| sod2   | ZDB-GENE-030131-7742 | F: GGCCATAAAGGCGCTTGGACTTTG  
                      R: GCTGCAATCTCTCAATCTTCC |
| tnf    | ZDB-GENE-050317-1 | F: GGGCAATCAAAGAGGCTGGAAGAG  
                      R: GCAGCTGATGTGTCAAGACAC |
| arg1   | ZDB-GENE-040724-181 | F: TGGGAATAATAGGGCTCGGTTC  
                      R: TCTCTACCACACACACTTGC |
| mpeg1  | ZDB-GENE-081105-5 | F: CTTCTGTTTACAGCATCAGCGC  
                      R: ACGCGCATCAAACAACCTGCAAA |
**Measurement of oxygen consumption in HKLm-primed RAW264.7 cells**

To measure oxygen consumption, RAW264.7 cells (ATCC TIB-71) were cultured in Dulbecco’s modified eagle medium with 4.5 g/L D-glucose and L-glutamine (Gibco, Thermo Fisher Scientific, NH, USA) supplemented with 10% of heat-inactivated fetal bovine serum (Gibco, Thermo Fisher Scientific, NH, USA) and 100 U/ml penicillin-streptomycin (Thermo Fisher Scientific, NH, USA) at 37°C with 5% CO2. The cells were primed either with 50 ng/ml of LPS or heat-killed *L. monocytogenes* to correspond to MOI of 530. 19 to 24 hours later the media was changed to fresh media including priming agents. A minimum of 30 min later the oxygen consumption from 5 million cells was measured at 37°C in their culture media using a Clark electrode (Hansatech, UK). Mitochondrial respiration was measured as the total minus the background oxygen consumption; the latter being determined by exposing the cell suspension to 150-270 nM of antimycin A (Sigma-Aldrich, MO, USA), a potent inhibitor of the respiratory chain complex III.

**Statistical analyses**

GraphPad Prism software (5.02) was used to carry out statistical analysis. Non-parametric two-tailed Mann-Whitney test was used to compare differences between experimental groups. Bonferroni’s post-test was used to correct P-values for multiple comparisons. P-values smaller than 0.05 were considered as significant. The sample sizes for experimental fish groups were calculated with power and sample size program (version 3.1.2) by using data from our preliminary studies (Dupont, Plummer 1998).
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References


Fig 1. The effect of priming agents on *M. marinum* loads in adult zebrafish. A. Outline of the study. Adult wild type zebrafish were primed with 0.5-1x10^7 colony forming units (cfu) per fish of heat-killed bacteria or with other priming agents (13.5 µg per fish, except MDP 4.5 µg) 1 or 7 days prior to *M. marinum* infection (-1/7 day) with an intraperitoneal injection. The following day (0 day), a low dose of *M. marinum* was injected intraperitoneally into the zebrafish. Sterile 1x PBS was used as an injection control. Internal organs were collected either 2, 4 or 7 weeks post infection (wpi), DNA was extracted, and the bacterial counts were measured with *M. marinum*-specific quantitative PCR (qPCR). B. Priming with heat-killed *L. monocytogenes* (HKLm) reduces mycobacterial loads at 7 wpi. Zebrafish were primed with either PBS (n=8), heat-killed *M. marinum* (HKMm, n=9), HKLm (n=10), heat-killed *S. iniae* (HKSi, n=8), heat-killed *E.coli* (KHEc, n=9), lipopolysaccharide (LPS, n=8), paclitaxel (n=5), muramyl dipeptide (MDP, n=9) or zymosan (n=10) 1 day prior to *M. marinum* infection (16±4 cfu). Organs were collected 7 wpi. Priming with HKLm led to a bigger decrease in
the mycobacterial loads in adult zebrafish compared to other tested priming agents. Paclitaxel increased the mycobacterial loads in adult zebrafish. Medians are shown in the figure. C. Priming with HKLm 1 day prior to *M. marinum* infection increases the frequency of clearance. The fold change in the percentage of fish that were able to clear the *M. marinum* infection was higher in the group that was primed with HKLm 1 day prior (3.7 fold, PBS: n=12, HKLm=13) to infection compared to group that was primed 7 days (2.3-fold, PBS: n=12, HKLm=12) before the infection.
Fig 2. Priming with heat-killed *L. monocytogenes* significantly reduces mycobacterial loads in adult zebrafish via innate responses. A. Priming of adult zebrafish with 0.5x10⁷ cfu of heat-killed *L. monocytogenes* (HKLm) one day prior to *M. marinum* infection (27±2 cfu) led to a significant decrease in mycobacterial loads compared to control injection of sterile 1xPBS. The graph shows one representative experiment. Samples were collected at 4 wpi (PBS n=19, HKLm n=19). B. Priming with HKLm one day prior to *M. marinum* infection leads to sterilization of *M. marinum* in 25% of the WT zebrafish. Clearance percentage in the WT PBS control group was 3.7%. The data were collected from 4 independent experiments. *M. marinum* infection doses were 27±2 cfu, 26±13 cfu, 75±13 cfu and 26±8 cfu. (PBS n=54, HKLm n=56) C. Priming of adult zebrafish with HKLm leads to a significant decrease in mycobacterial loads compared to PBS controls already at 2 wpi. Infection dose 48±8 cfu, PBS n=11, HKLm n=12. D. HKLm priming one day prior to *M. marinum* infection significantly reduced mycobacterial loads in *rag1* (-/-) mutant fish compared to PBS control group at 4 wpi indicating a role for innate immune responses. The picture contains a combined result from two separate experiments. *M. marinum* infection doses were 48±8 cfu and 27±2 cfu. E. Priming with HKLm one day prior to *M. marinum* infection leads to sterilization of *M. marinum* in 17% of the *rag1* (-/-) mutant fish. Clearance percentage in the PBS control group was 0%. Data are pooled from two
independent experiments. PBS n=26 and HKLm n=23. **F-G.** HKLm priming did not affect cumulative mortality in WT adult fish (F) (PBS n=152, HKLm n=170), but caused a trend of reduced mortality in HKLm-injected \textit{rag1} (-/-) mutant fish (PBS n=53, HKLm n=55). P-values in A, C, and D were calculated with a two-tailed non-parametric Mann-Whitney test with GraphPad Prism. Medians for the individual experiments are shown in the figures. The P-values in B, E, F and G were calculated with Fisher’s test using GraphPad (QuickCales) online software.
Fig 3. Protective immunity against *M. marinum* is mediated by a protein and/or nucleic acid component of heat-killed *Listeria monocytogenes*. A-C) Zebrafish were primed with different components of HKLm and *L. monocytogenes* 1 day prior to *M. marinum* infection and mycobacterial loads were determined with *M. marinum*-specific qPCR 4 wpi. A) Protective immunity is not mediated by a secreted component in the *L. monocytogenes* growth medium. Infection doses 26±6 cfu, PBS n=10, medium n=16. B) Protective immunity is mediated by a component that was found in the insoluble phase. Infection dose 33±11 cfu, PBS n=10, insoluble n=12. C) Protective effect of HKLm priming was lost, when HKLm was treated with DNase and RNase or proteinase K. Infection dose 33±11 cfu, PBS n=10, DNase and RNase n=7, proteinase K n=8. P-values for all experiments were calculated with a two-tailed non-parametric Mann-Whitney test with GraphPad Prism and corrected with the Bonferroni’s method. Medians for each experiment are shown.
Fig 4. HKLm treatment does not protect against high dose or established *M. marinum* infection.  
A-B. Protective effect of HKLm priming is lost with high dose priming. Fish were injected with a high dose of HKLm (15.6x10⁷ cfu, 30-fold compared to previous dose) 1 day prior to *M. marinum* infection (34±11 cfu). Priming with a high dose of HKLm did not reduce mycobacterial numbers (A) and led to an increase in the mortality of the fish at 4wpi (B) (PBS n=10, HKLm n=10).  
C-D. HKLm priming does not protect from a high dose *M. marinum* infection. Fish were primed with HKLm 1 day prior to a high dose *M. marinum* infection (4883±919 cfu). No effect was observed on bacterial loads (C) or cumulative end-point mortality (D), PBS n=16, HKLm n=17.  
E-F. HKLm does not protect against an established *M. marinum* infection. Fish were injected with HKLm two weeks after an *M. marinum* infection (22±6 cfu). No effect on mycobacterial loads (E) or cumulative end-point mortality (F) was observed. PBS n=14, HKLm n=16. P-values for bacterial loads were calculated with a two-tailed non-parametric Mann-Whitney test with GraphPad Prism (A,C,E). Medians for the experiments are shown.
Fig 5. HKLm priming induces mpeg, tnfa and nos2b expression, downregulates sod2 expression in adult zebrafish and leads to decreased oxygen consumption in vitro. A-H. The expression levels of and mpeg (A) mpx (B). ifng1-1 (C) ifng1-2 (D) arg1 (E), tnfa (F), nos2b (G) and sod2 (H) were measured with qPCR from wild-type fish primed with HKLm or sterile PBS buffer as a control. One day after the priming the fish were infected with a low dose (67±16 cfu) of M. marinum. Samples for qPCR analysis were collected at 1 dpi. The results were normalized to uninfected wild-type baseline control. PBS n=10, HKLm n=11. HKLm priming leads to metabolic changes in vitro. RAW264.7 cells were primed with LPS or HKLm and oxygen consumption was measured 19-24h after priming. HKLm priming leads to a 1.8-fold decrease (P=0.008) in oxygen consumption compared to control. LPS was used as a positive control (2.3-fold decrease, P=0.0042) PBS n=9, LPS n=7, HKLm n=9. P-values for all experiments were calculated with a two-tailed non-parametric Mann-Whitney test with GraphPad Prism. Medians for each experiment are shown. Bonferroni correction was used in (C).
Figure S1. HKLm does not exhibit bactericidal effects on Mycobacterium marinum in vitro. M. marinum was incubated for 12 days in 7H9 medium together with different concentrations of HKLm. The bacterial cultures were plated on 7H10 plates and the colony forming units (cfu) of M. marinum were determined. 10^3, 10^4, and 10^5 denote the ratio of HKLm compared to the initial cfu in the M. marinum culture. Mm= Mycobacterium marinum, HKLm= Heat-killed Listeria monocytogenes. n=3 for each sample.
Figure S2

A

Larvae 1+2 dpf

\[ M. \text{marinum/fish} \cdot (\log_{10}) \]

PBS
HKLM

B

Larvae 2+2 dpf

\[ \text{RFU} (\log_{10}) \]

PBS
HKLM

Figure S2. HKLM priming does not protect zebrafish larvae from mycobacterial infection. Zebrafish larvae were primed with 240 cfu of HKLM 1 day post-fertilization (dpf) (A) or 490 cfu of HKLM 2 dpf (B). \textit{M. marinum} infections (39±13 cfu for A and 39±16 cfu for B) were carried out 2 dpf. Mycobacterial loads were determined with an \textit{M. marinum} -specific qPCR (A) or by measuring the fluorescent signal of a wasabi-\textit{M. marinum} strain (B) with a plate reader. \( n=10 \) for PBS and \( n=11 \) for HKLM in A and \( n=13 \) for PBS and \( n=17 \) for HKLM in B.
Figure S3. HKLm priming decreases the mycobacterial loads also in non-cleared populations. The data represented in A and B exclude those individuals that had bacterial counts below the detection limit of the M. marinum-specific qPCR method i.e. had cleared the infection. Also the wild-type individuals that were unable to clear the infection benefited from HKLm by showing significantly lowered mycobacterial loads (A). Rag1 (-/-) fish show a similar trend (B). P-values were calculated with a two-tailed non-parametric Mann-Whitney test with GraphPad Prism. Medians for the experiments are shown in the figures. Data are pooled from 4 individual experiments for A (representative experiment shown in Fig 2A, n=52 for PBS and n=42 for HKLm) and from 2 individual experiments in Fig. 2D for B (n=26 for PBS and n=19 for HKLm).