The molecular basis of metabolic syndrome: studies in zebrafish

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Chapter 3

The function of leptin in the defense against mycobacterial infection in zebrafish

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Abstract

Tuberculosis (TB) is a disease that has a high impact on metabolism and has been recently linked with activation of the leptin signal pathway. Leptin is a pleiotropic hormone that plays a dual role both in metabolism and as a cytokine, acting as a signaling molecule in the immune system. Mice that are deficient in leptin signaling show metabolic alterations, such as obesity and insulin resistance, and are also more susceptible to TB infection. In this study, we have established a zebrafish larval model system to study the effect of leptin deficiency on TB, using morpholino knockdown and a CRISPR/Cas9-generated mutant fish line. To model TB, we infected zebrafish embryos with *Mycobacterum marinum*, a close relative of *M. tuberculosis* which causes TB in humans. Morpholino knockdown of the genes encoding leptin b (*lepb*) and leptin receptor (*lepr*) show higher mortality and a more progressive course of infection. Using a *lepb* mutant line showed similar results. In contrast, morpholino knockdown of the *lepa* gene did not alter the rate of infection. In the *lepb* mutant, the infection-induced increase in the expression of two pro-inflammatory genes, *irg1l* and *il1b*, was abolished. Injection of a human recombinant leptin protein partially rescued the phenotype of the infected *lepb* mutant larvae: infection burdens in the infected leptin mutant were diminished and a higher expression of *irg1l* was found. Furthermore, the chemical inhibitor NSC-87877 also lowered bacterial burden in the infected larvae. Finally, glucose levels were elevated after mycobacterial infection in both mutant and wild-type larvae. Our findings underscore that the role of leptin in the immune defense against TB, and further exploration of this role may provide interesting novel possibilities for the development of novel host-directed treatments methods against TB.

Introduction

Tuberculosis (TB) remains a major public health problem as it is worldwide the most fatal disease caused by a single infectious agent and one of the top 10 leading causes of death overall. In 2019, an estimated number of 10 million people were infected and 1.4 million people died because of TB[1]. Although *Mycobacterium tuberculosis*, the causative agent of TB, has already been known for hundreds of years and antibiotics have been available for decades, its eradication is complicated. Besides the lack of possibilities for prompt and accurate diagnosis or effective treatment in many countries, this is also due to the increase of multi drug resistant (MDR) and extreme drug resistant (XDR) strains of the pathogen[1]. Therefore, alternative forms of therapies for TB are urgently needed to improve and support currently used antibiotics. The development of such novel therapies is very difficult because of the complexity of TB infection process in which many host-pathogen interaction mechanisms and metabolic adaptation play an important role in determining the course of the bacterial infection. An interesting approach towards a novel anti-TB therapy would be to improve host resistance, for instance by stimulation of proinflammatory cytokine responses.

An interesting member of the cytokine superfamily is leptin, a 16-kDa protein produced most notably by adipose tissue which for many years was thought to be mainly connected with the regulation of food intake and body weight[2]. However, it has now become clear that the functions of leptin are much broader. Leptin appears to be a key regulator of energy homeostasis, insulin secretion, angiogenesis, bone formation and reproduction[3,4,5,6,7]. The leptin protein is encoded by the *Lep* gene (also known as *Ob*) belongs to a cytokine family that also includes interleukin 6 (IL-6) and it can act by binding to the leptin receptor, which is a class I cytokine receptor encoded by the *LEPR* gene[8]. Mutations causing functional leptin deficiency in humans are rare, can be treated with leptin supplementation, but if left untreated result in severe obesity, insulin resistance and early death[9]. Polymorphisms in the *LEPR* gene are linked with the incidence of obesity[10]. In mice, mutations in the *Lepr* gene or the *Lep* gene result in severe obesity and a diabetic profile[11,12,13,14].

Interestingly, leptin also plays a role in the immune system. Leptin has been shown to modulate the response to inflammatory stimuli and its expression has been demonstrated to increase during infection and inflammation[15,16,17]. Mouse strains with mutations in the *Lep* and *Lepr* gene display immunosuppression reflected by thymic atrophy, a decreased number of leukocytes and a reduced cellular immune function[18,19,20,21]. Leptin stimulates the expression of several clusters of differentiation: CD39, CD69, CD25, CD71 and interleukin 1 receptor antagonist (IL1Rα) in human monocytes/macrophages and production of the proinflammatory cytokines IL-6 and TNF-α[22]. Furthermore, it plays a role in proliferation and phagocytosis of leukocytes and their production of eicosanoids, nitric oxide, leukotriene B4 (LTB4) and cyclooxygenase 2 (COX-2)[23]. Moreover, leptin promotes chemotaxis and the release of reactive oxygen species in neutrophils[24], and it protects these cells from apoptosis via PI3K- and MAPK-dependent pathways[25]. Fisher *et al.*[26] found that infection with *S. typhimurium* resulted in increased leptin receptor expression, both in mouse and human macrophages that inhibited bacterial clearance and promoted inflammation[26]. On the other hand, this study also showed that in mice, ablation of *Lepr* in macrophages and systemic treatment with a leptin receptor antagonist led to increased lysosomal activity, reduced inflammation and a reduced bacterial burden[26].

In addition, it was shown that during *M. tuberculosis* infection in mice leptin levels were shown to be elevated in the lungs, reaching their highest point at 2 weeks after infection[27]. Moreover, increased bacterial loads were found in the lungs of the infected mice with a mutation in the *Lep* gene (*ob*/*ob* mice), and this was accompanied with significantly higher mortality[27]. It was also shown in the *ob/ob* mice that the function of leukocytes in their lungs and activation of their T cells were altered, influencing lymphocyte function and granuloma formation[27]. This study thereby confirmed the results of earlier studies in human TB patients which showed that that leptin is a key factor in the early immune responses during *M. tuberculosis* infection[28]. Decreased leptin levels or leptin resistance contribute to immunosuppression and higher susceptibility to TB infection[28]. The leptin plasma concentrations in TB patients appeared to be affected by two opposing mechanisms that

depend on whether the infection is systemic or pulmonary. Acute immune responses at the systemic phase of the infection result in increased plasma leptin levels, leading to wasting syndrome and reduced appetite[28], whereas chronic infection with granuloma formation leads to a reduction in the production of leptin and general immunosuppression[28,29]. Therefore, leptin seems to be a factor that switches metabolism and immune responses during TB infection, depending on the course and phase of the disease. This could explain why leptin levels were significantly increased in patients who received anti-TB treatment[30].

Although *ob/ob* mice and rats are convenient animal models to study the function of leptin in mammals, they also have disadvantages, and using them for studying tuberculosis provides difficulties. The murine model differs from human metabolism in terms of energy partitioning and fat deposition[31]. Moreover, *M. tuberculosis* infection in mice progresses differently. For example, in mice the central necrosis in lung granulomata is absent upon infection via the respiratory tract and the distribution of macrophages and T cells in these tuberculous foci is different[32]. For our studies on the role of leptin during mycobacterial infection we have chosen to use a larval zebrafish model to overcome problems of the murine models. In the last two decades, zebrafish have widely been used as a model organism to study human diseases[33] including infectious diseases[34, 35, 36] such as mycobacterial infection[37]. *Mycobacterium marinum*, a close relative of *M. tuberculosis,* is able to systemically infect zebrafish larvae leading to an infection that is similar to the *M. tuberculosis* infection in humans, including granuloma formation[38]. The transparency of the larvae makes it is possible to track the infection *in vivo*, using fluorescent strains of the bacteria. It occurs often in zebrafish that there are paralogous copies of genes[39], and this is also the case for the leptin gene, whereas the gene for the leptin receptor (*lepr*) appears not to be duplicated. Zebrafish possesses two leptin genes leptin a (*lepa*) and leptin b (*lepb*). Although the two leptin proteins share only 24% amino acid identity and are 18% identical to human leptin, they share a characteristic gene structure and function with their mammalian orthologues. The two leptins are differentially expressed: in adults *lepa* is expressed mainly in the liver, whereas *lepb* is expressed in the ovaries. The latter was found to be downregulated during fasting[40].

In various studies, the expression of *lepa*, *lepb* and *lepr* has been knocked down using several approaches. Morpholino knockdown of *lepa* was found to lead to a reduction in the catabolism of lipids from the yolk, delayed development and reduced sensory structures, such as eyes and otic vesicles[41]. A homozygous ENU induced mutation of the *lepr* gene did not induce any effect of on body length and weight up to 100 days post fertilization[42]. However, this mutation did affect nutrient-induced β-cell compensation. Furthermore, using CRISPR/Cas9-mediated gene editing, mutations were made in the *lepr*, *lepa* and *lepb* genes. Mutation of *lepa,* but not *lepb*, led to a 17% increase in the number of β-cells, further confirming a role for leptin signaling in regulation of β-cell mass in larval zebrafish[42]. In contrast, a CRISPR/Cas-generated zebrafish line carrying a mutation in the *lepr* gene had a thinner body after 4 months post-fertilization (mpf) than the WT fish[41]. In addition, the

mutant fish showed a significant decrease of body weight and muscle fiber size, compared with heterozygous and WT fish[43].

In the present study, we have investigated the role of *lepb* as an immune modulator. Previously, we have observed a high induction of the expression of this gene in larval zebrafish during bacterial infection with *Staphylococcus epidermidis* and *M. marinum*[44], as well as a strong upregulation after *ptpn6* knockdown, which is known as a negative regulator of immune signaling pathways[45]. In particular, we have investigated how *lepb* deficiency and a chemical inhibitor NSC-87877 would influence mycobacterial infection. For this purpose, we have used morpholino knockdown of *lepb* and a *lepb* mutant strain that was generated using CRISPR/Cas9 gene editing[46]. Our results show that both *lepb* knockdown and knockout lead to increased bacterial burden and mortality, which can be partially restored to the wild type phenotype by exogenous leptin administration.

Materials and methods

Zebrafish husbandry

Zebrafish of the AB/TL (WT), *lepb*$^{+/+}$ (this thesis, Chapter 4), *lepb*$^{-/-}$ (this thesis, Chapter 4), *Tg(mpeg1:mCherry)*[47] and *Tg(mpx:GFP)*[48] lines were handled and maintained according to standard protocols (http://ZFIN.org), and in compliance with the directives of the local animal welfare body of Leiden University. Fertilization was performed by natural spawning at the beginning of the light period. Embryos were grown at 28.5 °C in egg water (60 µg/ml Instant ocean sea salt, Sera Marin). During injections and imaging, embryos were kept under anesthesia in egg water containing 0.02% buffered 3-aminobenzoic acid ethyl ester (tricaine, Sigma–Aldrich).

CRISPR/Cas9 mutagenesis

Site-specific CRISPR-Cas9 sgRNAs (actatagGGGGTCTCGGGATTGGGTAGgttttag) were generated using the online software CHOPCHOP according to Montague *et al.*[49]. *Lepb* mutant fish were generated using CRISPR-mediated gene knockout approach as described previously[50]. As described in Chapter 4 two different deletion mutants were generated. Homozygous F1 carriers were outcrossed once against WT, and were subsequently incrossed, resulting in *lepb*$^{-/-}$ and *lepb*$^{+/+}$ siblings that were used for experiments. For genotyping, genomic DNA was amplified using forward primer 5'-GAGACTCTCCTGAGGACACTGG-3' and reverse primer 5'-GCATGGCTTACACATTTCAGAG-3', amplifying a 201 base pair (bp) product containing the mutations, which can be detected using 2% agarose gel. However, in most experiments larvae with a heterozygous combination of the two mutations were used. This is because the fact that our procedure had generated two different mutations was discovered after the experiments described in this paper were performed.

Morpholino injections

For knockdown of the zebrafish *lepa*, *lepb and lepr* genes, morpholino oligomers (GeneTools, LLC, Philomath, OR, USA) were used that target the 5'UTR of the respective mRNAs (this thesis, Chapter 4). The morpholino was diluted to a concentration of 0.08 mM in 1× Danieau's buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, and 5.0 mM HEPES (pH 7.6)), and 1 nL of this solution was injected in the yolk sac of 1–2 cell stage embryos using an automated injection system (Life Science Methods, Leiden, The Netherlands). As a control, the standard control morpholino from Gene Tools LLC was used at the same concentrations as the other morpholinos (this thesis, Chapter 4).

Bacterial strains, growth conditions and injections

We compared two sites of micro-injection of bacteria into the embryo, which led to a systemic infection. *M. marinum* (Mma20 and E11 strain) containing a pMST3:mCherry expression vector, was grown as described in Carvalho *et al.*[51]. Two reaction vials with 1 ml of the culture were centrifuged for 1 min. The pellets were washed three times with 1 ml PBS. Suspensions were prepared based on the optical density at 600 nm, and by plating and subsequent CFU determination. The inoculates were suspended in 2% polyvinylpyrrolidone40 (PVP40, CalBiochem), 60 CFUs of E11 strain were injected either in the yolk sac at 4 hours post fertilization (hpf) or 150 – 200 CFUs of Mma20 were injected into the blood circulation of 28 hpf embryos via the caudal vein at the posterior blood island that caused a systemic infection. Blood island injection is a preferred injection site as described in Benard *et al*. [37], since embryos at this stage have phagocytically active macrophages but neutrophils have not yet matured. However, early injection of *M. marinum* into the yolk of embryos provides an alternative method to achieve a systemic infection. Control embryos were injected with PBS containing 2% PVP40.

NSC-87877 and human recombined leptin treatment

Treatment with NSC-87877 and human recombined leptin treatment was performed as described in this thesis, Chapter 4. The $lepb^{+/+}$ and $lepb^{-/-}$ embryos received 1nl of 10 µM NSC-87877 at 2 hpf into the yolk and were kept in 10 µM NSC-87877 after infection. 1nl of 25 µM of human recombinant leptin protein (HRL) was injected into the yolk sac at 2 hpf.

COPAS analysis

The COPAS™ XL (Complex Object Parametric Analyzer and Sorter, Union Biometrica, Holliston, MA, USA) large particle sorter has been designed for the analysis, sorting and dispensing of objects up to 1.5 mm in diameter based on size, optical density and fluorescence intensity. It is equipped with 488 nm and 561 nm Solid State lasers. Zebrafish embryos were measured alive at indicated time points, to determine their bacterial burden or the number of macrophages or neutrophils present in their bodies, with the COPAS XL using the following settings: photo multiplier tube voltage: 650 V for green/red and 0 V for yellow, optical density

threshold signal 975 mV (COPAS value: 50), and time of flight minimum 320 µs (COPAS value: 800)[44].

Microscopy

A fluorescence stereo microscope (MZ16FA, Leica Microsystems, Wetzlar, Germany), equipped with Leica DFC420C digital color camera, was used for imaging of the zebrafish embryos. Embryos were kept under tricaine anesthesia during imaging. The images were analyzed using custom-designed pixel quantification software as described by Stoop *et al.*[52]. Reference images were generated for estimating the average autofluorescence by calculating the sum of the pixels from the fluorescent red channel above background intensity.

Quantitative PCR

Total RNA was isolated using TRIzol (Life Technologies). RNA samples were treated with RQ1 DNAse (Promega) and reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories B.V.), according to the manufacturers' protocols. For the quantification of mRNA concentrations, qPCR was carried out using iQ SYBR Green Supermix (Bio-Rad Laboratories B.V.). The reactions were performed in an iCycler Thermal Cycler (Bio-Rad Laboratories B.V.) under the following conditions: 95 °C for 3 min, followed by 40 cycles of 15 s denaturation at 95 °C and 30 s at the corresponding annealing temperature and elongation at 72°C, and finally a melting curve was generated by 81 cycles from 55 to 95 °C (0.5 °C increments for every 10 s). Fluorescent signals were measured at the end of each cycle. Cycle threshold values (Ct values, i.e. the cycle numbers at which a threshold value of the fluorescence intensity was reached) were determined for each sample. To determine the gene regulation due to a treatment, in each experiment the average Ct value of the treated samples was subtracted from the average Ct value of the control samples, and the fold change of gene expression was calculated. Finally, this fold change was normalized against the expression level of the housekeeping gene *ppial* . The primer sequences were used as follows: *irg1l* forward 5'-GGTTAGAAGCAAGTCCTC and reverse 5'-TGTGTTCATCCTCCTCAG, *il1b* forward 5'-GAACAGAATGAAGCACATCAAACC and reverse 5'-ACGGCACTGAATCCACCAC.

Glucose measurements

Quantitative analysis of glucose levels was performed from whole body lysates using a glucose assay kit (Cayman Chemical, Ann Arbor, MI, USA). Ten zebrafish larvae in each experimental group per single experiment were sonicated in 30 µL Assay Buffer on ice. According to the instructions, standard curves were generated using glucose standard solution. A total of 25 µL assay Enzyme Mix (Cayman Chemical, Ann Arbor, MI, USA) was added and incubated for 10 min at 37°C. Fluorescence (514 nm) was measured using a BioTek plate reader equipped with GEN 5 software (v.2.04, BioTek, Winooski, VT, USA). The experiment was repeated three times.

Statistics

Statistical significance was analyzed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). The effect of morpholino knockdown on the bacterial burden was analyzed using either a 2-tailed t-test or a one-way ANOVA followed by Tukey's comparison test (multiple group comparisons). For qRT-PCR results, statistical significance was estimated by two-tailed t-tests on fold changes. Significance (P-value) is indicated with: ns, non-significant; *P < 0.05; **P < 0.01; ***P < 0.001, ****P < 0.0001. All data shown are means ± s.e.m.

Results

Knockdown of *lepb* and *lepr* increases bacterial burden in zebrafish larvae

Morpholino oligomers have already successfully been used to knock down *lepa* and *lepr* gene expression in a zebrafish study[54]. In the present study, we used a translation blocking morpholino to knock down the *lepb* gene, and a control morpholino was injected to embryos of the control group. The morpholinos were administered into the yolk sac of fertilized zebrafish embryos, within the first 30 minutes after fertilization (1-2 cell stage), using an automated injection system. Subsequently, the same embryos were infected into the yolk at approximately 4 hpf with the *Mycobacterium marinum* E11 mCherry-labeled fluorescent strain, using the same injection system.

The bacterial burden was monitored during the following 5 days, by measuring the fluorescent mCherry signal using COPAS flow cytometry. A significant difference between the control and *lepb* knockdown groups was observed at 2 day post infection (dpi), with average bacterial burdens of ~2000 AU in the control and ~2500 AU in the *lepb* morphants (Fig.1). Finally, at 5 dpi the burden increased to an average ~9000 AU in the control morpholino-injected larvae and ~13000 AU in the *lepb* morphants (Fig.1A). In addition, we determined the survival rate at 1-5 dpi. We found a significant difference between the infected control morpholino-treated and the infected *lepb* morpholino-treated groups, with ~60% survival in the control morpholino group, whereas the *lepb* morphant group showed mortality lower survival rate of ~45% (Fig.1B).

As a next step we used translation blocking morpholinos to knock down *lepa*, *lepb* and *lepr* expression and study the effect of these knockdowns on the course of systemic bacterial infection. The morpholinos against these genes, as well as the control morpholino, were ad ministered using the automated system within the first 30 minutes after fertilization. At 24 hpf, embryos were manually dechorionated, and at 28 hpf *Mycobacterium marinum* Mma20 strain labelled with the fluorescent mCherry protein was injected manually in the blood circulation at the posterior blood island. The bacterial burden progression was monitored between 1 and 4 dpi using COPAS flow cytometry.

At 1dpi, only the *lepr* group showed a significant difference compared to the group treated with the control morpholino. At 2 dpi we still found a significant difference between *lepr* knockdown group (~800 AU) and the control group (~900 AU), and also the bacterial burden

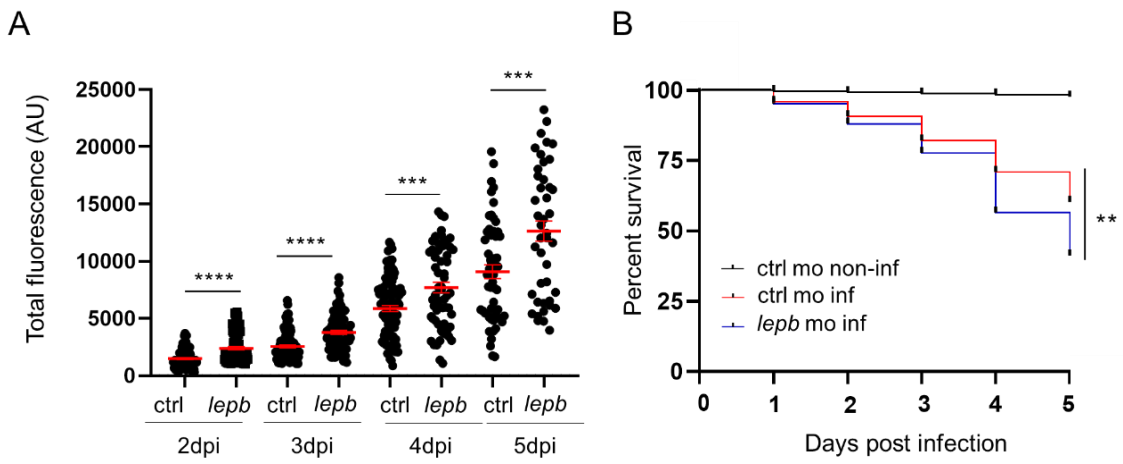


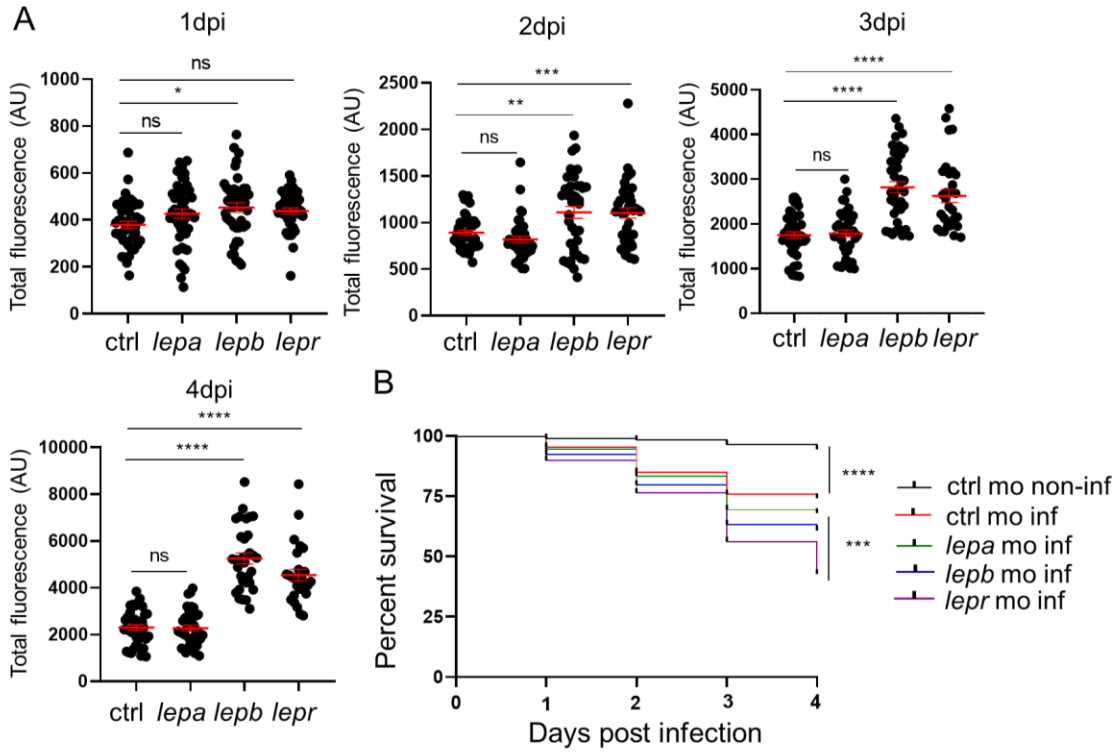
Figure 1. Morpholino studies: the effect of *lepb* knockdown on *M. marinum* yolk infection. (A) The bacterial burden in the control larvae and *lepb* morphants after *M. marinum* E11 infection in the yolk sac followed until 5 dpi. **(B)** Survival rates after *M. marinum* E11 yolk infection followed until 5 dpi. Data shown are pooled from three independent experiments and means ± s.e.m. are indicated. Statistical significance (determined using ANOVA with Tukey's post hoc test) is indicated by: **P<0.01; ***P<0.001; ****P<0.0001.

of the *lepb* morphants was significantly higher (~1200 AU) than that of the controls. At 3 and 4dpi the bacterial burden of the *lepa* group remained similar to that of the control group, whereas the *lepb* (~2700 AU and ~5000 AU) and *lepr* morphants (~2500 AU and ~4500 AU) still showed increased bacterial burden (Fig.2A). During this infection experiment we also monitored survival of the infected larvae. We only found a survival rate significantly different from that of the control infected group in the *lepr* deficient group, where the survival reached approximately 40%. Survival in the *lepb* and *lepa* deficient groups decreased to 60% and 70%, respectively, although the differences were not significant compared to the survival rate of the control group (Fig.2B).

Knockout of the *lepb* gene results in a more severe infection phenotype than observed after morpholino knockdown

Subsequently we used a *lepb* deficient mutant zebrafish line (*lepb*$^{-/-}$) that had previously been generated using CRISPR/Cas9-mediated gene editing (this thesis, Chapter 4). This mutant did not show any apparent developmental phenotype. Firstly, we infected embryos from this *lepb* mutant and wild type embryos at 4 hpf with *M. marinum* E11 strain , using the automated injection system. The progression of the infection was monitored by determining the bacterial burden using COPAS flow cytometry. We followed the bacterial burden up to 4 dpi. At 1dpi there was no difference in infection burden between the wild type (~1000 AU) and mutant group (~1200 AU). A significant difference in the bacterial burden was observed at 2 dpi, when it reached ~2500 AU in the wild type group and ~3000 AU in the mutants. The infection burden rose during the next two days of infection, reaching ~4000 AU and ~6500 AU in the wild types, as compared to ~8000 AU and ~9500 AU in the mutants (Fig.3A). In addition, in the mutant group the survival rate was significantly lower than in the wild type group. At 4 dpi survival

was 0% among mutants, whereas the survival in the wild type group was approximately 60%



(Fig.3B).

Figure 2. Morpholino studies: the effect of *lepa, lepb,* and *lepr* knockdown on *M. marinum* blood island infection. (A) The bacterial burden in the control larvae and morphant siblings after *M. marinum* Mma20 infection in the posterior blood island at 28 hpf, followed until 4 dpi. **(B)** Survival rates in the control larvae and morphant siblings after *M. marinum* Mma20 blood island infection, followed until 4 dpi. Data shown are pooled from three independent experiments and means ± s.e.m. are indicated. Statistical significance (determined using ANOVA with Tukey's post hoc test) is indicated by: *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; ns, non-significant.

As a next step we manually dechorionated embryos at 24 hpf and injected them at 28 hpf with an mCherry-labeled *M. marinum* Mma20 strain in the blood island. The bacterial burden was measured using COPAS flow cytometry during the following days. We observed that this systemic infection resulted in an increased bacterial burden in the mutants compared to the wild types from 1dpi up to 4dpi. Interestingly, this method of infection showed less variation in bacterial burden at 3 and 4 dpi than injection in the yolk sac at 4 hpf. The bacterial burden increased gradually, starting at ~900 AU at 1dpi in the wild type group and at ~1100 AU in the mutant group. Finally, at 4 dpi the bacterial burden reached ~3500 AU in the wild type larvae and ~7000 AU in the mutants (Fig.4A). Additionally, we studied survival rates which were relatively high compared to those observed in the yolk infection experiments and reached

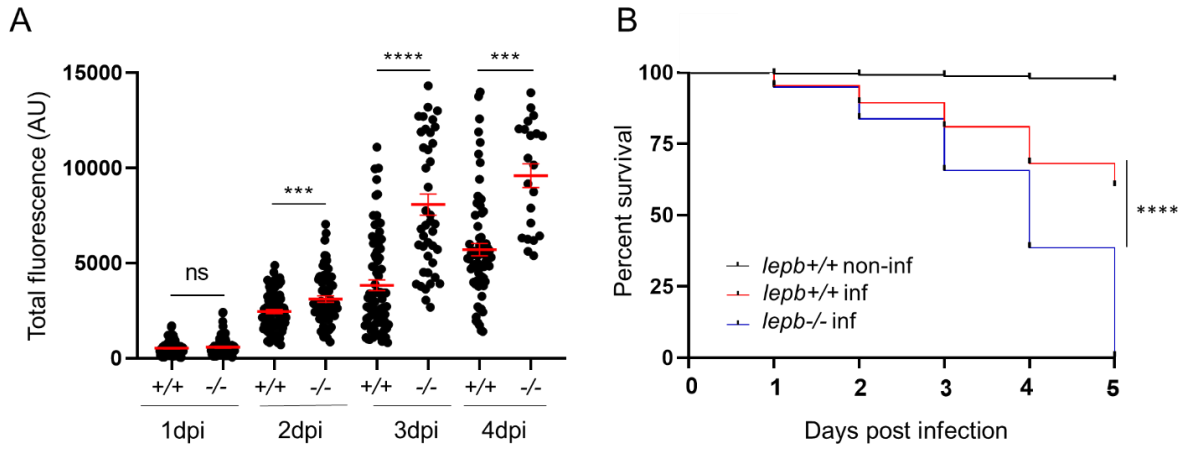


Figure 3. The lepb mutant zebrafish line: the effect of lepb knockdown on M. marinum yolk infection. (A) Bacterial burden in the wild type larvae (+/+) and *lepb*$^{-/-}$ mutants (-/-) after *M. marinum* E11 yolk infection, followed until 4dpi. **(B)** Survival rates in the wild type larvae and *lepb*$^{-/-}$ mutants after *M. marinum* E11 yolk infection, followed until 5dpi. Data shown are pooled from three independent experiments and means ± s.e.m. are indicated. Statistical significance (determined using ANOVA with Tukey's post hoc test) is indicated by: ***P<0.001; ****P<0.0001 ; ns, non-significant.

55% survival at 4 dpi in the mutant group, which was significantly increased compared to the wild types (Fig.4B). Furthermore, in an independent experiment, we compared the blood island infection in the *lepb* CRISPR/Cas mutant morpholino treatment (Fig. 4C). The results showed a significant difference in the infection rate between the *lepb* mutant and the morpholino-treated larvae. The infection in the mutant zebrafish larvae resulted in an increased bacterial burden that showed a total fluorescence of ~7500 AU at 4 dpi, as compared to ~5000 AU after morpholino knockdown (Fig.4C).

Leptin deficiency leads to a decreased number of macrophages but not neutrophils

To better understand why leptin deficiency influences bacterial infection rates, we measured the number of immune cells in the mutant and wild type groups. Zebrafish larvae from lines *Tg(mpeg1:mCherry);lepb*$^{+/+}$ and *Tg(mpx:GFP);lepb*$^{+/+}$, as well as *Tg(mpeg1:mCherry);lepb*$^{-/-}$ and *Tg(mpx:GFP);lepb*$^{-/-}$ were analyzed using COPAS flow cytometry at 5 days post fertilization (dpf).

The fluorescent signal in the *Tg(mpeg1:mCherry)* larvae, in which the macrophages are labeled with red fluorescence, was used to determine the relative number of macrophages, whereas the signal in the *Tg(mpx:GFP)* larvae, in which the neutrophils had a green fluorescent label, was used for assessment of the relative number of neutrophils. We observed that knockout of *lepb* results in a small decrease in the number of macrophages in the mutant (Fig.5A), with total fluorescence at approximately ~1700 AU in the *lepb*$^{+/+}$ group and ~1300 AU in the *lepb*$^{-/-}$ group. Next, we investigated the number of neutrophils in mutants

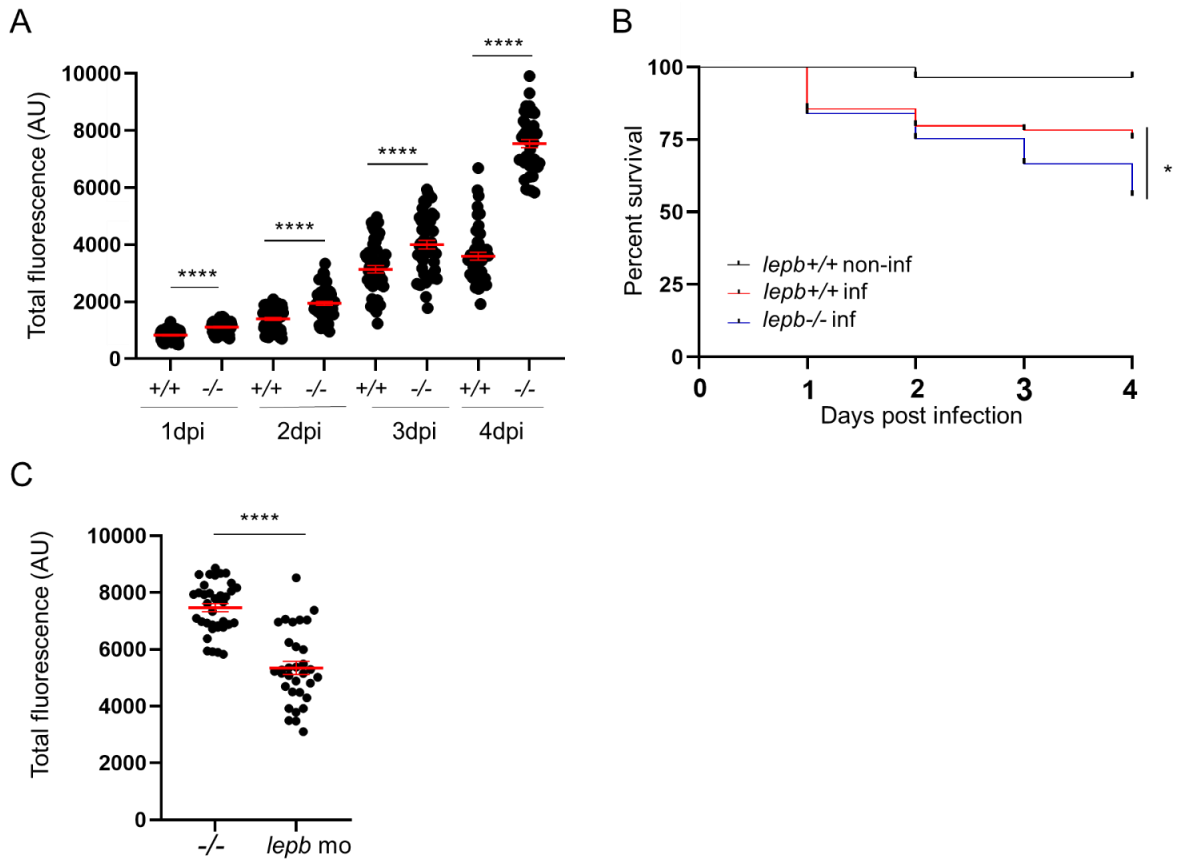


Figure 4. The *lepb* mutant zebrafish line: the effect of *lepb* knockdown on *M. marinum* blood island infection. **(A)** The bacterial burden in the wild type larvae (*+/+*) and *lepb* mutants (-/-) after *M. marinum* Mma20 blood island infection at 28 hpf, followed until 4dpi. **(B)** Survival rates in the wild type larvae and *lepb*$^{-/-}$ mutants after *M. marinum* Mma20 blood island infection, followed until 4dpi. **(C)** Bacterial burden in *lepb* morphants and *lepb*$^{-/-}$ mutants after *M. marinum* Mma20 blood island infection was followed until 4dpi. Data shown are pooled from three independent experiments and means ± s.e.m. are indicated. Statistical significance (determined using ANOVA with Tukey's post hoc test) is indicated by: *P<0.05; ****P<0.0001.

and wild types. The results (Fig.5B) showed no significant difference, with a total fluorescence of ~1550 AU in the wild type larvae, compared to ~1600 AU in the mutant larvae. Therefore, we can conclude that *lepb* deficiency decreases the number of macrophages, but does not affect the number of neutrophils.

Leptin b deficiency leads to downregulation of *irg1l* and *il1b* expression

The *irg1l* (a zebrafish ortholog of the mammalian immune responsive gene *irg1*)[55,56] and *il1b* gene have previously been established as transcriptional indicators for the response to bacterial infection of zebrafish larvae[57,58]. Quantitative real-time PCR (qPCR) was used to determine expression levels in *irg1l* and *il1b* expression in wild type and *lepb* mutant larvae between 1 and 4 days after *M. marinum* infection in the blood island at 28 hpf. The results

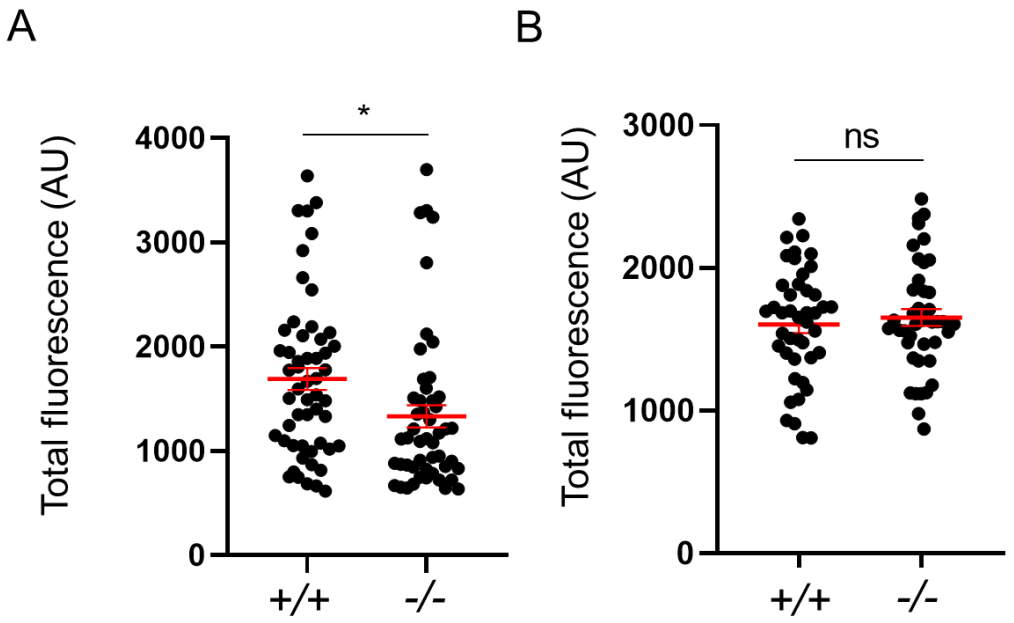


Figure 5. Determination of the number of macrophages and neutrophils in the lepb mutant line. (A) Fluorescence in *Tg(mpeg1:mCherry);lepb*$^{+/+}$ and *Tg(mpeg1:mCherry);lepb*$^{-/-}$ larvae at 5 dpf, measured by COPAS flow cytometry as a measure for the relative number of macrophages. **(B)** Fluorescence in *Tg(mpx:GFPgfp);lepb*$^{+/+}$, and *Tg(mpx:GFPgfp);lepb*$^{-/-}$ larvae at 5 dpf, as a measure for the relative number of neutrophils. Data shown are pooled from three independent experiments and means ± s.e.m. are indicated. Statistical significance (determined using t-test) is indicated by: *P<0.05; ****P<0.0001; ns, non-significant.

showed that in the wild type, as expected, the expression levels of *irg1l* and *il1b* are increased after infection, and this increase is already visible for both genes at 2 dpi. At all the time points after bacterial infection, we found that the levels of *irg1l* and *il1b* in the *lepb* mutant were significantly lower than in the wild types (Fig.6). The expression levels of *irg1l* and *il1b* after infection in the *lepb* mutant were even lower than in the uninfected wild type controls, and hardly changed over the course of the infection. Strikingly, at 4dpi the fold change of *irg1l* expression was five times higher in the infected *lepb*$^{+/+}$ group compared to the non-infected *lepb*$^{+/+}$ group, while in the infected *lepb*$^{-/-}$ group it was actually two times lower than in the infected *lepb*$^{+/+}$ group (Fig.6). Similarly, the fold change of *il1b* raised more than three times in the infected *lepb*$^{+/+}$ group compared to the non-infected *lepb*$^{+/+}$ group, whereas the expression in the infected *lepb*$^{-/-}$ group was one third lower (Fig.6). In conclusion, *lepb* deficiency in the zebrafish larvae leads to a decrease in the levels of *irg1l* and *il1b* after *M. marinum* infection.

Administration of human leptin decreases the infection rate and enhances the immune response in *lepb* mutants

Although the zebrafish leptin b protein shows only 18% similarity with its human orthologue, we tested whether we can rescue the phenotype of *lepb* morphants and mutants after infection using administration of human recombinant leptin (HRL). We injected zebrafish embryos at the 1-2 cell stage with the control, and the *lepb* and *lepr* targeting morpholinos

using the automated injection system. Subsequently, embryos were manually dechorionated at 24 hpf, and at 28 hpf injected with the Cherry-labeled *M. marinum* Mma20 strain into the blood island with or without HRL. The progression of the bacterial burden was measured using COPAS flow cytometry at 4 dpi. We found a significant difference in the bacterial burden between infected *lepb* morphants treated with HRL and infected morphants that had not received HRL. The HRL-treated larvae had an average total fluorescent of ~3500 AU, whereas the non-treated larvae showed a fluorescence of ~5500 AU, indicating that the HRL treatment partly rescues the phenotype of the *lepb* morphant. In contrast, the HRL administration did not change the progression of infection in the *lepr* morphants, in which the fluorescence remained at ~5000 AU (Fig.7A).

To study if we can observe a similar effect in *lepb* mutant larvae we injected embryos with HRL in the yolk within the first 30 min after fertilization. After manual dechorionation at 24 hpf, we infected the embryos into the blood island at 28 hpf with the mCherry-labeled fluorescent *M. marinum* Mma20 strain together with HRL. We determined the level of infection by measuring the fluorescence using the COPAS flow cytometry system at 4dpi. We found a significant effect of administration of HRL in that the total fluorescence reached approximately ~5000 AU in the mutant larvae, compared to ~3500 AU after HRL administration (Fig.7B). Thus, we again found a partial rescue of the phenotype induced by *lepb* deficiency upon HRL administration.

To further analyze the effect of the administration of HRL, we imaged the embryos using fluorescence microscopy, and assessed the bacterial burden by determining the fluorescent signal in the images. The results showed that administration of HRL decreased the bacterial burden in the leptin mutant zebrafish larvae to ~11500 AU, towards the level of the wild types ~6600 AU confirming the rescuing effect of the HRL treatment. In the mutant larvae fluorescent signal reached ~24900 AU (Fig. 7C,D).

Moreover, we wanted to test if administration of HRL would change the *irg1l* and *il1b* expression in the *lepb* mutant larvae. We infected embryos with bacteria into the blood island at 28 hpf. Analysis of the expression data at 4 dpi shows more than a two-fold increase in *irg1l* expression in the HRL-treated infected mutant larvae compared to infected mutants that had not received this treatment. However, the levels were still dramatically lower than those in the infected wild types where the fold change was six times higher than the non-infected control group (Fig.8A). The *il1b* expression was not significantly altered by the HRL treatment in the infected mutant larvae (Fig.8B). Non-infected mutant larvae showed decreased expression of *irg1l* and *il1b*, however the difference was not significant (Suppl.Fig.1).

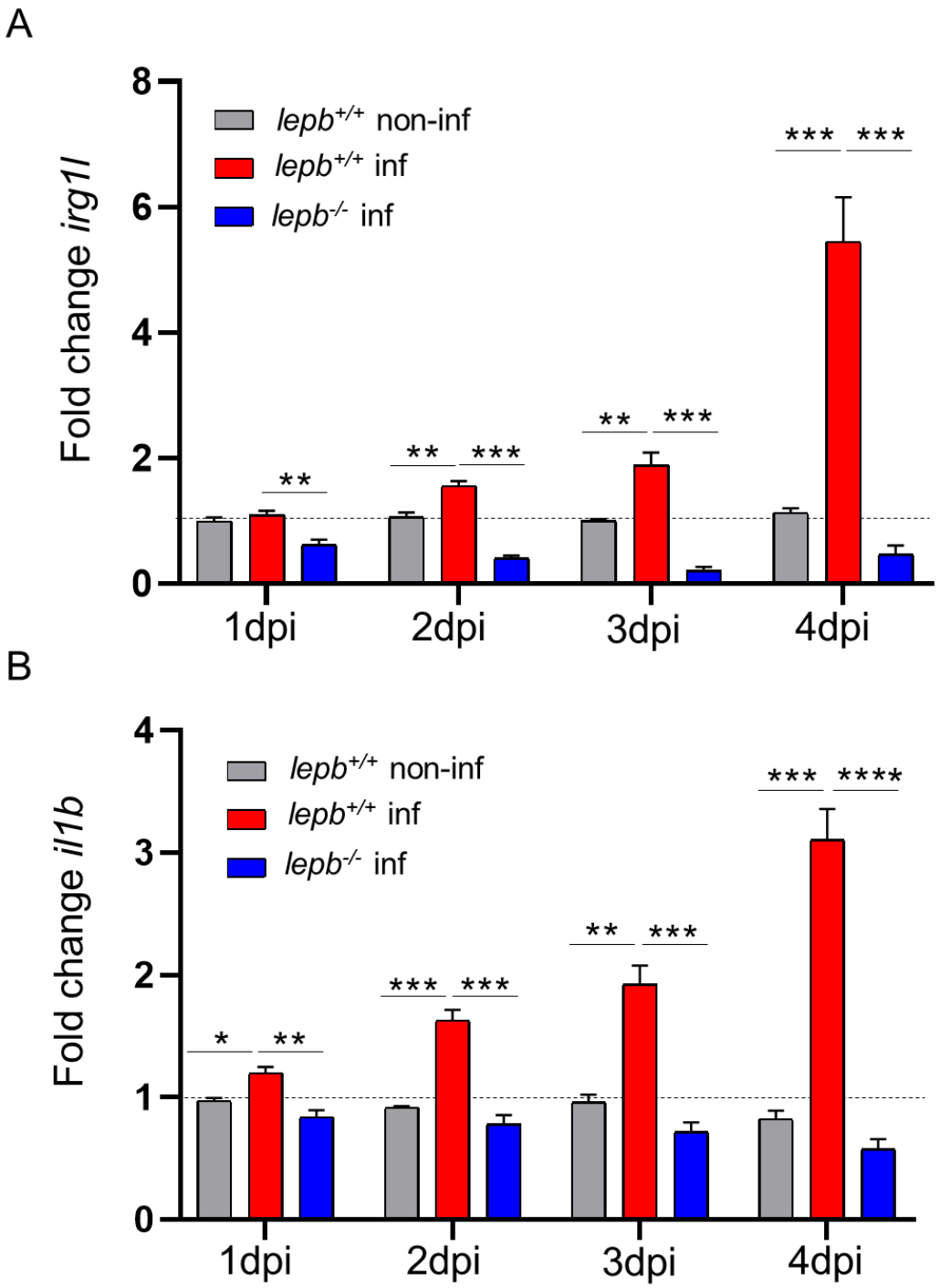


Figure 6. Expression of *irg1l* and *il1b* in the *lepb* mutant line after *M. marinum* blood island infection. (A) Expression *of irg1l* in the wild type larvae (+/+)and *lepb* mutants (-/-) after *M. marinum* Mma20 blood island infection at 28 hpf, starting from 1 dpi. **(B)** Expression of *il1b* in the wild type larvae (+/+) and *lepb* mutants (-/-) after infection, starting from 1 dpi. Data shown are means ± s.e.m. from three independent experiments. Statistical significance (determined using ANOVA with Tukey's post hoc test) is indicated by: *P<0.05; **P<0.01; ***P<0.001.

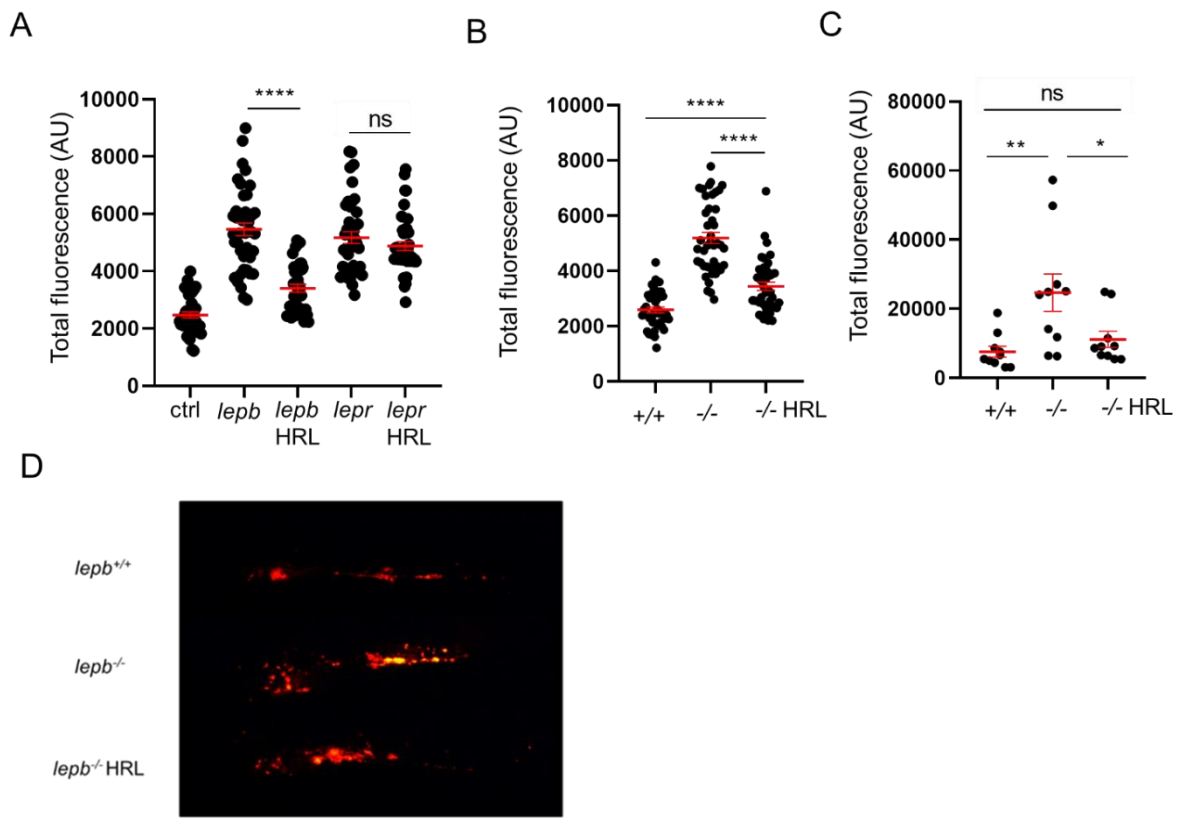


Figure 7. The effect of human recombinant leptin (HRL) administration on infection in *lepb* morphant and mutant larvae. (A) Bacterial burden in the control, *lepb* and *lepr* morpholino-treated groups, with and without HRL administration, measured at 4dpi. **(B)** Bacterial burden in the wild type (*+/+*) and *lepb* mutant (*-/-*) group, with and without HRL administration, measured at 4dpi. **(C, D)** Bacterial burden of same larvae used in B, based on fluorescent signals in microscopy images (shown in D). Data shown are pooled from three independent experiments and means ± s.e.m. are indicated. Statistical significance (determined using ANOVA with Tukey's post hoc test) is indicated by: *P<0.05; **P<0.01; ****P<0.0001; ns, non-significant.

NSC-87877 treatment decreases the infection rate in *lepb* mutants

In a previous study we have shown that treatment of *M. marinum* infected zebrafish larvae with NSC-87877, an inhibitor of Src homology region 2 domain-containing phosphatase-1 (SHP-1/2), led to a higher bacterial burden (this thesis, Chapter 2). Here we tested whether treatment with NSC-87877 influenced the bacterial burden in the *lepb* mutant. After yolk infection at 4 hpf with mCherry-fluorescent *M. marinum* Mma20, the embryos were kept in 10 µM NSC-87877 and the bacterial burden was measured by COPAS flow cytometry at 4 dpi. The results showed that at 4dpi NSC-87877 did not affect the bacterial burden in the *lepb* mutant (~8500 AU), compared to the non-treated *lepb*$^{-/-}$ group (~9600 AU), but still higher than infected control (~7500 AU) (Fig.9A). To study the effect of the NSC-87877 treatment after a blood island infection we manually removed the chorion of zebrafish embryos at 24 hpf, and at 28 hpf we injected the mCherry fluorescent *M. marinum* Mma20 strain into the blood island. After infection the embryos were kept in 10 µM NSC-87877 and the bacterial

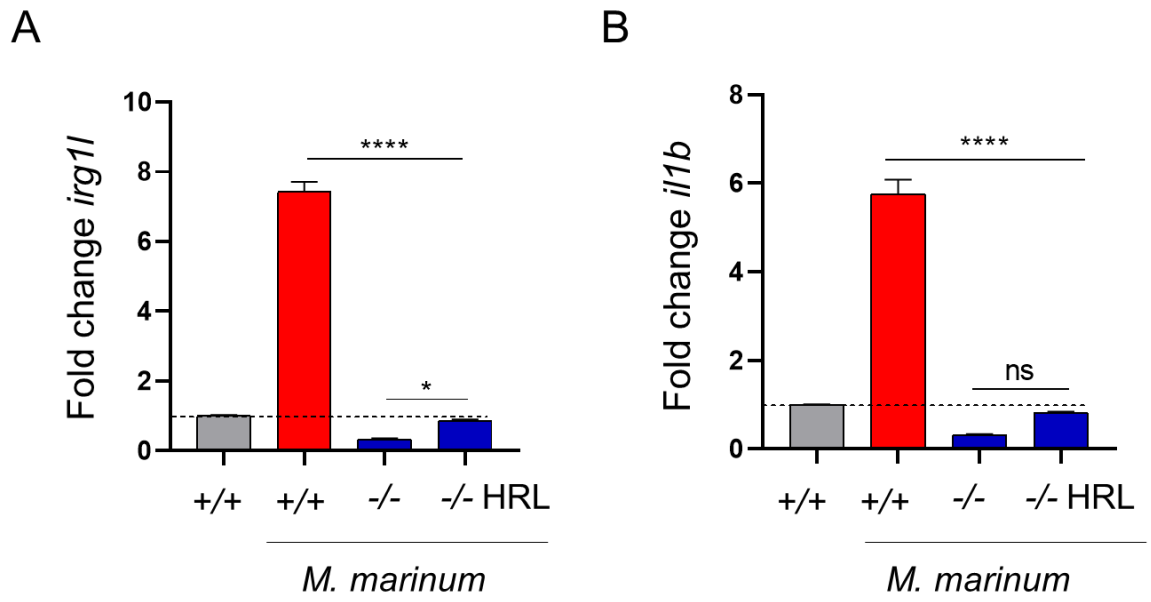


Figure 8. The expression of *irg1l* and *il1b* in *lepb* mutant after human recombinant leptin (HRL) administration. **A** qPCR analysis was performed at 4 days after *M. marinum* Mma20 blood island infection at 28 hpf. Expression levels are expressed as the fold change relative to the uninfected wild type control group. **(A)** *irg1l* and **(B)** *il1b* expression, measured in the *lepb* mutant (-/-) and wild type (+/+) larvae upon infection and/or treatment with HRL. Data shown are means ± s.e.m. from three independent experiments. Statistical significance (determined using wo-way ANOVA with Tukey's post hoc test) is indicated by: *P<0.05; ****P<0.0001; ns, non-significant.

burden was monitored by COPAS flow cytometry at 4 dpi. The results showed that NSC-87877 significantly lowered the bacterial burden in the infected *lepb* mutants (~2850 AU), compared to the untreated *lepb* mutants (~4300 AU). However the fluorescence level was still significantly higher compared to the control larvae (~2250 AU)(Fig.9B).

Leptin b deficiency increases basal and infection-associated glucose levels

Finally, we determined whether Leptin b deficiency affects the glucose metabolism at basal conditions and after infection with *M. marinum*. For this purpose, we used a colorimetric assay that measures free glucose. Since blood glucose after uptake is quickly converted to glucose-6- phosphate this assay gives a measure for glucose that is not taken up from the blood and glucose that is derived from gluconeogenesis[59]. Embryos were infected with the Mma20 strain in the blood island at 28 hpf. At 5 dpf, glucose levels were determined using samples from around ten pooled zebrafish larvae, infected and non-infected of both the *lepb*$^{-/-}$ and *lepb*$^{+/+}$. We observed that after infection, the glucose concentrations are significantly increased in the wild type (*lepb*$^{+/+}$) larvae (220 pmol/larva), compared to the non-infected control (80 pmol/larva) (Fig. 10). Interestingly, *lepb*$^{-/-}$ larvae showed this infection-induced increase as well, but had much higher glucose levels before (360 pmol/larva) and after infection (950 pmol/larva) compared to the wild types (Fig.10). The higher levels of glucose in the *lepb* mutant in the absence of infection is consistent with our previous results (this thesis, Chapter 4).

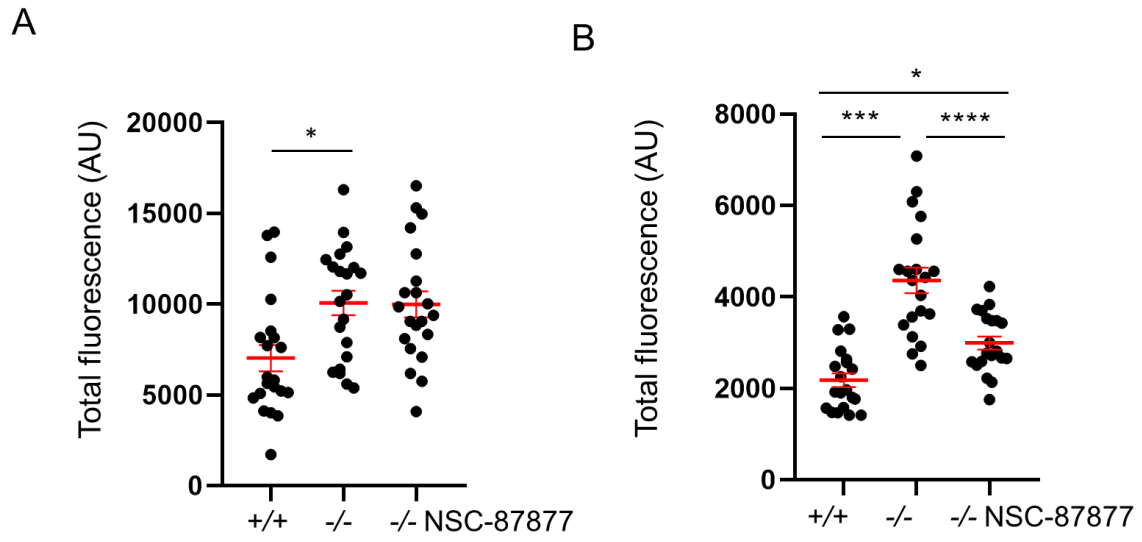


Figure 9. The effect of NSC-87877 treatment in *lepb* mutant fish after *M. marinum* infection. Bacterial burden in the control, *lepb*$^{-/-}$ larvae and *lepb*$^{-/-}$ larvae treated with NSC-87877, measured at 4dpi after yolk **(A)** and blood island **(B)** infection, at 4 and 28 hpf respectively. Data shown are pooled from three independent experiments and means ± s.e.m are indicated. Statistical significance (determined using two-way ANOVA with Tukey's post hoc test) is indicated by: *P<0.05; ***P<0.001; ****P<0.0001; ns, non-significant.

Discussion

In the present study, we have shown, using morpholino-induced knockdown of *lepb*, and *lepb* knockout using CRISPR/Cas9-mediated gene editing, that deficiency of *lepb* in zebrafish larvae results in an increased bacterial burden during *M. marinum* infection between 1 and 4 dpi. Knockout of *lepb* resulted in higher bacterial burden and mortality than after knockdown. Our results indicate that the leptin b protein plays an important role in the host defense during the early stages of a mycobacterial infection. In contrast, our morpholino studies showed that knockdown of *lepa* does not result in a significant change in the infection rate, whereas morpholino knockdown of *lepr* gives similar results as observed with the *lepb* morphants and mutants. We found that the effect of *lepb* deficiency was more severe after infection in the yolk at 4 hpf compared to infection in the blood island at 28 hpf. Finally, we observed a slight decrease in the number of macrophages in the *lepb* mutant larvae, whereas the number of neutrophils in leptin-deficient larvae was not changed.

In mammals, leptin is known to play a dual role both as a hormone and a cytokine. Its role as a hormone is connected with endocrine functions, bone metabolism and glucose homeostasis, whereas as a cytokine, leptin promotes inflammatory responses[60,62].

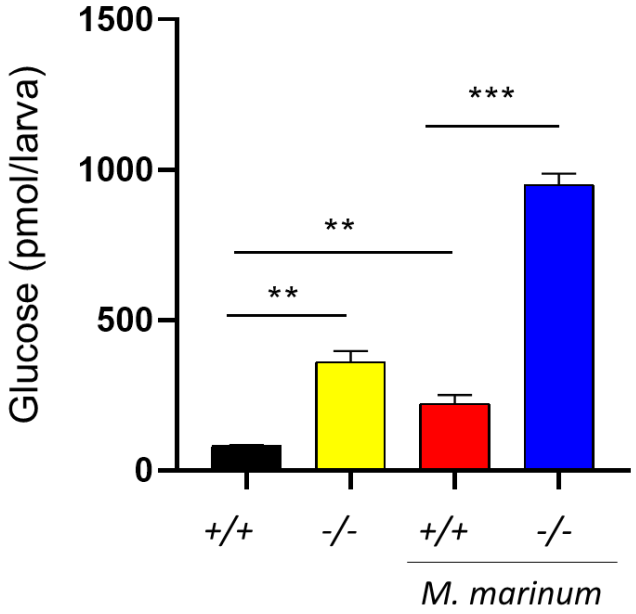


Figure 10. Glucose levels in *lepb* mutant and wild type larvae after *M. marinum* infection. Glucose levels (pmol/larva) in the wild type (*+/+*) and *lepb* mutant (*-/-*) larvae with and without *M. marinum* infection. Data are means ± s.e.m. from three independent experiments. Statistical significance (determined using two-way ANOVA with Tukey's post hoc test) is indicated by: **P<0.01; ***P<0.001.

In contrast, reduced levels of leptin are linked with an increased risk of infection and reduced cell-mediated immunity[15,19]. Leptin has been also found to play a significant role in the modulation of immune response upon TB infection[27,28,29,30].

This indicates that the observed effects of the *lepb* deficiency in our zebrafish model resembles phenotypes observed in leptin-deficient murine models. Our results are in line with findings in the *ob/ob* mice (which carry a mutation in the *Lep* gene) that show increased susceptibility to infection with *M. tuberculosis*[27]. In addition, previous research on the leptin receptor-deficient *db/db* mice showed that these mutants have downregulated recruitment of immune cells to the site of mycobacterial infection, delayed pulmonary expression of IFN-γ, (C-C Motif Chemokine Ligand 2) CCL2 and (inducible nitric oxide synthase) iNOS, as well as impaired granuloma formation[62]. Furthermore, research in murine models revealed that abnormalities in leptin or leptin receptor expression led to decreased macrophage numbers[63]. Thus, we have established a larval zebrafish model to study the role of leptin during TB infection that recapitulates the results of murine models.

In mice it has been shown that leptin regulates the expression of *il1b*[64], and that its deficiency leads to immunosuppression and downregulation of proinflammatory cytokines and markers[63]. Therefore, we tested whether *lepb* knockout would influence *irg1l* and *il1b* expression, which are zebrafish markers of the response to a mycobacterial infection[45,51,55-58]. Our results show that these genes are strongly induced upon *M. marinum* infection in the wild types, but are, in contrast, much lower expressed in the *lepb* mutant after infection. Furthermore, we found that co-injection of HRL with the mycobacteria was able to upregulate *irg1l* and *il1b* expression levels. We therefore conclude that *lepb* expression is crucial for a normal course of the immune response against this mycobacterial infection, as measured by

the transcription of *il1b* and *irg1l*. An effect of leptin on immune responses has also been shown by Loffreda *et al*.[63], who showed that exogenous leptin enhanced both macrophage function and expression of proinflammatory cytokines in mice *in vitro*.

Additionally, we have shown that NSC-87877 significantly reduced infection levels of *M. marinum* in the *lepb* mutant. This is surprising considering the observed effects of morpholino knockdown of *shp1* and NSC-87877 treatment in wild type larvae in other studies. A previous study from our group showed that morpholino knockdown of *shp1* expression increased the bacterial burden and caused hyper-induction of proinflammatory genes in zebrafish larvae upon infection with *Salmonella* Typhimurium or *M. marinum*[65]. In addition, we have recently shown that the SHP-1/2 inhibitor NSC-87877 similarly enhances the immune response and the induction of proinflammatory cytokines expression, and the mycobacterial infection burden was higher than in the wild type group (this thesis, Chapter 2). In contrast, NSC-87877 has recently been tested in mice infected with *B. pertussis*, where it resulted in lower bacterial survival and a decreased induction of the innate immune response[66]. We have recently generated a mutant of *shp1* (Bakker *et al.*, unpublished) that will be used to test the mechanism in which these two genes interact in further studies, for example by crossing with the *lepb* mutant.

Finally, because the function of leptin is associated with glucose metabolism (this thesis, Chapter 4), we have tested how glucose levels changed during mycobacterial infection in wild type and *lepb* mutant zebrafish larvae. TB is often associated with diabetes and impaired glucose metabolism that can be reversible if treated properly[67,68]. It has been found that during TB infection pro- and anti-inflammatory cytokines such as IL-1, IL-6, IL-10 and TNF-α that may have a direct effect on the glucose metabolism, and additionally induce the hypothalamic-pituitary-adrenal axis resulting in upregulated expression of stress hormones such as cortisol, which leads to stress hyperglycemia and a pre-diabetic state[69]. We found that after infection, the glucose concentration was significantly increased in wild type embryos. This effect of the mycobacterial infection has been observed before[31,32] and has been confirmed by NMR analyses and might be linked to a wasting syndrome that is caused by the infection[70]. Interestingly, *lepb*$^{-/-}$ larvae showed much higher glucose levels before and after infection than the wild types. Apparently, the increased glucose levels in the *lepb* mutants are anticorrelated the inflammatory response which were shown to be dramatically decreased in the mutants. We will further study changes in metabolism in the *lepb* mutant in the presence or absence of infection in future studies, which may show whether Leptin b is involved in modulating the metabolic syndrome observed in tuberculosis[70].

In conclusion, our results show how leptin can modulate the progress of infection and its role in immune responses. Furthermore, our data suggest that it could be possible to apply leptin as a supportive medication of current antibiotic therapy against infectious disease in patients who suffer from increased infection susceptibility due to a leptin deficiency.

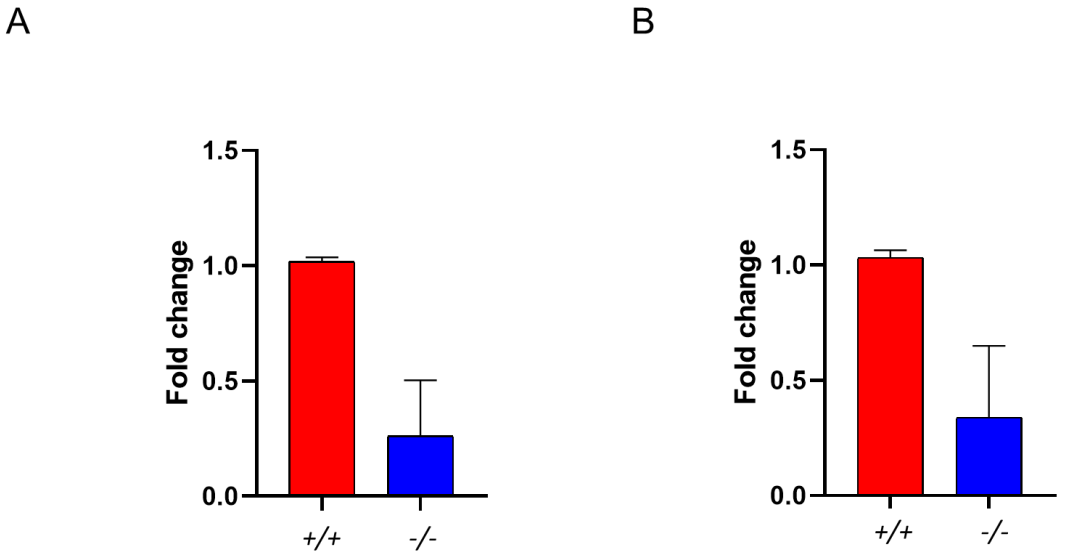
Acknowledgements

We thank Charlotte Welter for help with the qPCR experiments in the uninfected *lepb* mutant larvae. We thank Ulrike Nehrdich for help with the fish breeding.

Supplementary materials

Supplementary Table 1. List of morpholinos

Name	Sequence
lepa	5′-TTGAGCGGAGAGCTGGAAA-3′
lepb	5′-TTTTTTGCTTTGTTAATATCATCCCT-3′
lepr	5′-TCAAGACAGACATCATTTCACTTGC-3′
control moprholino	5′- CCTCTTACCTCAGTTACAATTTATA-3′



Supplementary Figure 1. *irg1l* and *il1b* expression in *lepb* mutant. qPCR was performed on 5 dpf larvae and compared with the uninfected sibling control. **(A)** *irg1l* and **(B)** *il1b* expression is measured in the non-infected *lepb* mutant. Data are mean±s.e.m. from two independent experiments.

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