The molecular basis of metabolic syndrome: studies in zebrafish

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Studies in zebrafish

Proefschrift

ter verkrijging van

de graad van doctor aan de Universiteit Leiden,

op gezag van rector magnificus prof.dr.ir. H. Bijl,

volgens besluit van het college voor promoties

te verdedigen op woensdag 30 maart 2022

klokke 13:45 uur

door

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geboren te Olsztyn, Polen

in 1987

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Chapter 1

Introduction

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I. Tuberculosis

Mycobacterium tuberculosis (Mtb) infection is currently one of the leading infectious diseases worldwide that has infected approximately one-third of the world population[1] and is a main cause of infection-related death [2]. Although the occurrence of tuberculosis (TB) in Europe is still at relatively stable levels, in some regions of Southeast Asia, Africa and the former Soviet Union, TB in combination with HIV infections contributes greatly to the TB epidemic[3]. What is making TB a global problem is the development of drug resistance by the bacterium. It is mainly caused by mutations that lead to alteration or overproduction of drug target proteins, which results in an altered drug target or titration of the drug respectively[4]. Moreover, it was shown that almost half of all TB-positive patients were infected by multiple-drug-resistant forms of the bacterium, whereas one quarter of them appeared to be resistant to all six drugs commonly used against the infection[5]. What is more, human immunodeficiency virus (HIV) infection has been associated with TB drug resistance, also resulting in inadequate responses to the available drug treatments[6].

TB results in primary immune responses of an organism[7]. During TB infection, bacteria invade and replicate in macrophages that results in cellular death by apoptosis or necrosis, and formation of granulomas[8]. Further bacterial replication is prevented by antigen specific T cells that promote an effective antimicrobial response via activation of cytokines and targeting the infected macrophages (Fig.1)[9]. In addition, secondary metabolic changes are observed during TB[8]. Prolonged infection leads to an alteration in carbohydrate metabolism and insulin activity that results in impaired glucose tolerance[10]. TB can also have an impact on pancreatic activity that results in pancreatitis and higher susceptibility to inflammation and amyloidosis[11]. Moreover, small numbers of bacteria can be disseminated to the visceral adipose tissue, which could be a cause for the development of a systemic metabolic syndrome in infected patients[12], because the pathogens can use locally stored fatty acids as a source of carbon. Interestingly, upregulation of some adipokines like MCP-1 (Monocyte Chemoattractant Protein-1), could be advantageous for the bacteria, since it contributes to recruitment of macrophages to the adipose tissue and to the development of insulin resistance[12]. Furthermore, patients with TB suffer from a lowered total cholesterol level and lowered albumin level, mainly during drug-resistant infection and HIV co-infection[13]. Eventually, the metabolic changes result in general undernutrition and lowered body mass index (BMI) of infected patients[14]. The mechanism of this process, which is also known as wasting syndrome, still remains unclear, but a low BMI has been linked to an increased risk of relapse of TB and strongly contributes to the mortality of the disease[15]. In most cases the patients gain weight during the treatment, which is taken as a positive marker of successful response to the medication[16].

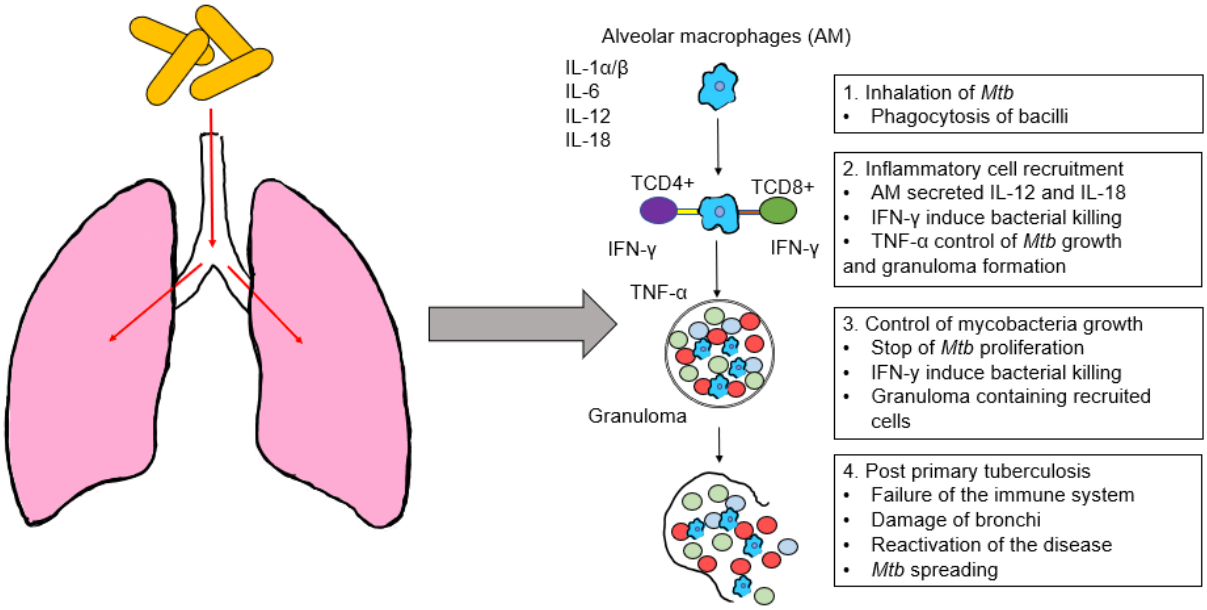

Figure 1. TB pathogenesis. TB pathogenesis can be divided in four stages. Macrophages interact through cellular receptors with inhaled mycobacteria which results in phagocytosis of the pathogens (stage 1). Mycobacteria survive and proliferate in the infected macrophages, which induces production of proinflammatory cytokines. The progression of inflammation induces the recruitment of monocytes, neutrophils and dendritic cells to the site of infection (stage 2). Expression of TNF-α leads to control of bacteria growth, followed by induction of T cells, which are organized in characteristic structures called granulomas that stop mycobacteria proliferation and spreading. A characteristic feature of granulomas is the presence of foam cells resulting from the differentiation of chronically activated macrophages (stage 3). At this stage infection can become latent and can become reactivated when immunosuppression occurs (stage 4)[17].

II. Tuberculosis and diabetes mellitus type 2

Diabetes mellitus (DM) has become a new worldwide epidemic, as a result of changes in diet, reduction in physical activity and increasing obesity[18]. Like many other factors, such as HIV, that lead to a higher susceptibility to TB, DM increases the risk of TB infection[19]. There are two types of DM which both increase the risk of TB infection about three-fold, as a result of chronic hyperglycemia, although the insulin-resistant DM type 2 (DM2) seems to have a larger contribution to the infection[20,21]. DM2 patients comprise around 90% of the global cases of DM and form a large proportion of the people afflicted with a dual TB/DM burden[22]. Autoimmune type 1 DM (DM1) is also associated with TB susceptibility, but to a smaller extent[23]. The exact mechanisms underlying this increased susceptibility is unclear, but in general it has been shown that patients that suffer from chronic inflammation show hyperglycemia and impaired glucose tolerance[24]. Moreover, an inflammatory state enhances secretion of pro-inflammatory cytokines, some of which have been shown to inhibit insulin signaling[25]. Studies performed in diabetic mice show that hyperglycemic animals have lower interferon-γ (IFN-γ) levels, impaired cellular immune responses to TB, higher bacterial burden and broadened infection[26,27]. In turn, TB leads to a higher prevalence of DM2 among infected

patients[28]. Infection affects the glucose metabolism, resulting in stress hyperglycemia and glucose intolerance in TB positive patients[29] and, as a result, a higher predisposition to DM2[30]. Stress hyperglycemia is a result of counter-regulation from multiple signaling pathways including hormones and cytokines. TB-induced hyperglycemia is possibly caused by changes in host immunity, metabolism and the endocrine system that occur during the disease, leading to an increased hepatic glucose production and peripheral insulin resistance[31]. During *Mtb* infection, pro- and anti-inflammatory cytokines, such as interleukin (IL)-1, IL-6, IL-10, interferon (IFN)-γ and tumor necrotic factor (TNF)-α, are produced. Furthermore, macrophages generate nitric oxide and reactive oxygen species, as well as activation of T-cells, and natural killer (NK) cells. Increased levels of pro-inflammatory cytokines, reactive oxygen species, and nitric oxide cause hyperglycemia that, through a cascade of inflammatory pathways, results in insulin resistance[32] and decreased glucose uptake[33]. In addition, metabolic and endocrine changes that contribute to the development of hyperglycemia include increased production of cortisol, adrenocorticotropic hormone (ACTH), prolactin and growth hormone that lead to higher glucose production and activated glycogenolysis in the liver and muscles. As a result of the described processes, chronic infection may lead to prolonged stress hyperglycemia (Fig.2) which may eventually develop into pre-diabetes or diabetes, that can persist even after a successful TB treatment[34].

III. Animal models in tuberculosis and diabetes research

The current challenges of TB research are connected with the complex pathology of TB infection and the difficulties in treatment. Therefore, there are various experimental animal models to study different aspects of TB, such as clinical signs, pathological changes, bacterial burden, progression of the disease and immunological parameters[35]. An ideal TB model would be able to mimic clinical signs, pathological lesions and metabolic changes that occur in humans. Mice, guinea pigs, rabbits, non-human primates and zebrafish are the most popular research models in TB research[35,36]. Each animal model is used for different research goals. Acute TB in mice models is suitable for evaluation of the efficacy of anti-TB drugs, owing to the possibility to use them to quantify pathological changes and bacterial burden[37]. Guinea pigs are characterized by a strong immune response, which is mostly used to evaluate anti-TB vaccines[38], whereas monkey TB models develop similar clinical signs and granuloma structures to human TB patients[39].

The zebrafish is a relatively new alternative animal model to study TB infection, but it is gaining popularity because of several important advantages compared to the other models. TB in zebrafish is caused by a natural pathogen *Mycobacterium marinum*, which causes a systemic disease that shows high similarity with human TB[41]. In humans, infection with *M. marinum* can cause a skin disease called 'tank granuloma'. By using zebrafish larvae, it is possible to track the mycobacterial infection *in vivo*, since larvae are small and transparent and have a fully functional innate immune system. Interestingly, larval zebrafish rely only on

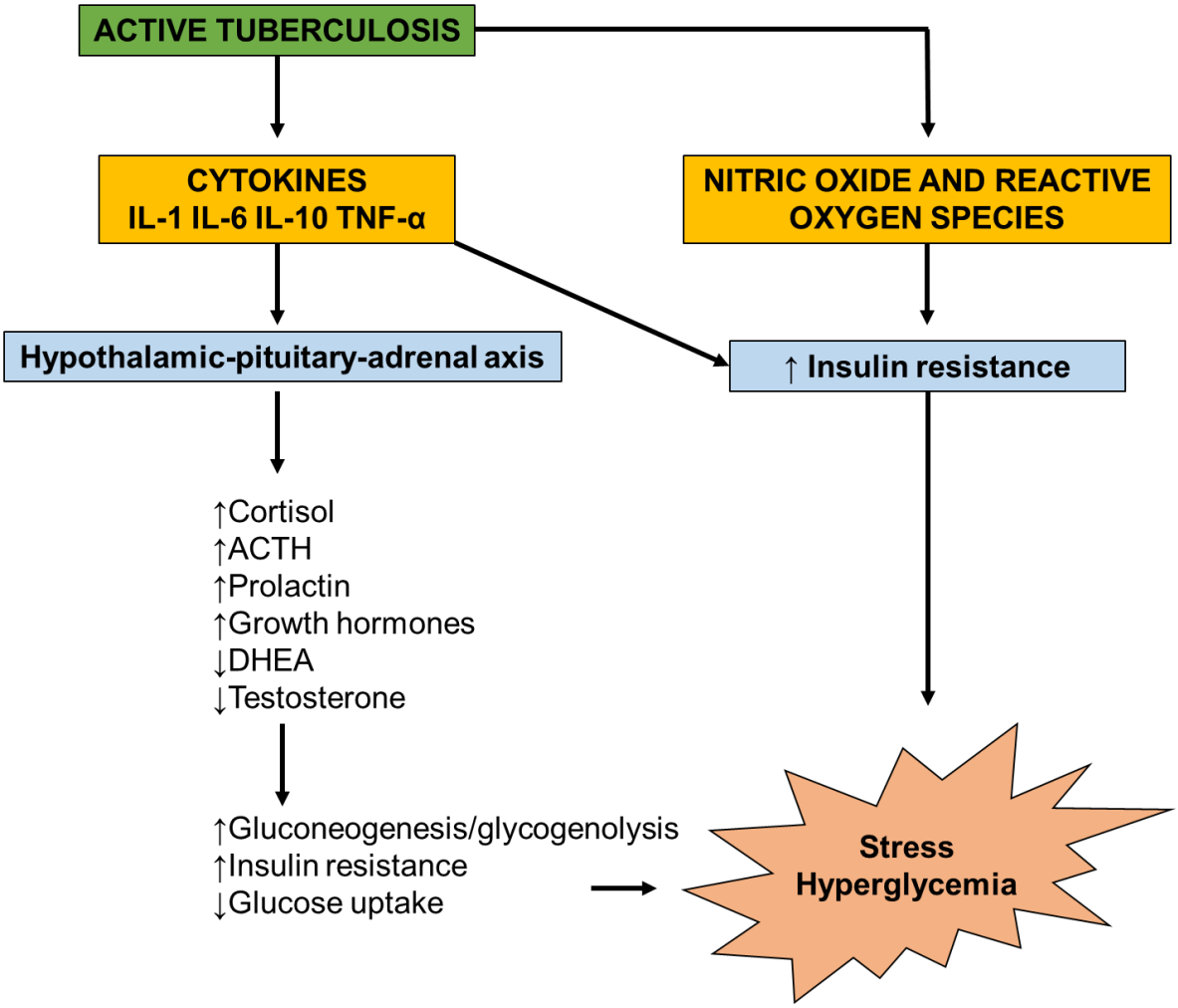

Figure 2. The role of tuberculosis in the pathophysiology of type 2 diabetes mellitus. Pro-inflammatory and anti-inflammatory cytokines released during active tuberculosis induce production of cortisol, ACTH, prolactin and growth hormone that leads to gluconeogenesis and glycogenolysis in liver and muscles, increase insulin resistance and decrease glucose uptake, which results in hyperglycemia. IL: Interleukin; TNF: tumor necrosis factor; ACTH: adrenocorticotropic hormone; T3 & T4: thyroid hormones; DHEA: dehydroepiandrosterone[40].

the innate immune system and have no developed adaptive immunity, which enables separate studies on innate immunity[42].

Animal models for DM include models with insulin resistance and models with dysfunction of insulin-producing pancreatic β-cells. Many DM2 animal models display obesity, similarly to humans, and suffer from glucose intolerance and insulin resistance[43]. One of the most popular animal models for diabetes is the Zucker Diabetic Fatty Rat (ZDF), which is characterized by a mutation in the leptin receptor that induces hyperphagia, obesity and glucose intolerance[44]. Another popular DM model is the *ob/ob* mouse, which is characterized by defective leptin signaling due to a mutation in the leptin (*Ob*) gene. The *ob/ob* mice suffer from severe obesity, hyperglycemia and hyperinsulinemia. Other metabolic dysfunctions include hyperlipidemia, lower physical activity and infertility[45]. In a similar model, the *db/db* mouse, alteration in the leptin receptor in also leads to alterations in glucose metabolism and insulin signaling. These

mice are hyperphagic, obese, hyperglycemic and diabetic, and they develop ketosis a few months after birth and have a shorter lifespan than wild types[46].

The zebrafish is a versatile animal model to study glucose metabolism and development of DM2. Zebrafish larvae become insulin resistant when injected with a high dose of human recombinant insulin, and this way they are used as a model to study insulin signaling and associated disorders[47]. The larvae provide an advantage over other models since they can be used in research on insulin resistance and immunity in a non-obese state. A mutation in the leptin receptor in larval zebrafish results in an increase in β-cell number and dysregulation in the expression of genes that are involved in glucose metabolism in the liver, whereas the adults show normal glucose levels and an increased β-cell mass[48]. Finally, zebrafish can become diabetic after diet-induced obesity (DIO) by overfeeding with artemia. The DIO zebrafish show higher blood glucose levels and insulin resistance after one week of overfeeding, which can be reversed by anti-diabetic treatment[49]. Recent research in adult and larval zebrafish show that it is a useful animal model to study DM2 and associated metabolic disorders[49,50].

IV. The role of leptin in tuberculosis and diabetes

As described above, the pathophysiology of TB and DM2 are strongly intertwined. During both conditions there are many organs and cell types that are involved in disease progression. One of the tissues being actively involved in both diseases is adipose tissue. Adipocytes secrete a number of pro-inflammatory cytokines, some of which, such as leptin, affect insulin signaling[51]. Leptin is a hormone, that belongs to the group of cytokines called adipocytokines, as it is mainly produced by adipocytes[52]. Leptin acts both as a hormone and as a cytokine. As a hormone, it regulates endocrine functions, bone metabolism, glucose and energy homeostasis, as well as food intake[53]. As a cytokine, leptin is involved in the inflammatory response and may cause autoimmune diseases[54].

Recently, it has been shown that leptin may have a significant role in the modulation of the immune responses during TB[55]. TB patients show lowered leptin levels, probably due to the loss of body weight[56]. Generally, during inflammation, infection or sepsis, leptin levels are elevated and correlate with the survival rate of patients[57]. Leptinemia, that occurs during bacterial infection, results from TNF-α and IL-6 activation and has been suggested to enhance pathogen clearance. In contrast, leptin-deficient *ob/ob* mice are more susceptible to infection than wildtype mice, and their increased susceptibility to infection can be reversed by leptin administration[58]. Neutrophils from leptin mutant mice were significantly attenuated in their response to infection, and alveolar macrophages are defective in defense against infection. Importantly, leptin administration restores the function of these cells[58]. Macrophages are especially important for leptin function since they express leptin receptor (LepRb), an isoform of the leptin receptor that can lead to activation of Signal Transducer And Activator Of

Transcription 3 (STAT3)[59]. Studies in *ob/ob* and *db/db* mice have shown that leptin expression is essential for a successful immune response against *Mtb*[60]. Leptin-deficient mice infected with *Mtb* suffer from dysregulated immune responses and impaired bacterial containment. Taken together, these studies indicate an important role of leptin in the response to infection, in particular in TB.

Additionally, leptin contributes to the development of DM2 by modulating glucose homeostasis and the inhibition of insulin synthesis and secretion[61]. Leptin-deficient *ob/ob* mice are characterized by insulin resistance and diabetes[62], whereas leptin injection lowers blood glucose and insulin levels[51]. Furthermore, leptin injection increases glucose uptake and oxidation in skeletal muscle and decreases the hepatic production of glucose[63]. The leptin signaling pathway works via JAK-STAT, affecting cAMP production and activation of phosphoinositide 3-kinase (PI3K)[64]. The mouse leptin receptor gene gives rise to six isoforms, from which only the long form of leptin receptor (LepRb) is capable to activate JAKs. Janus kinase 2 (JAK2) activation leads to autophosphorylation of multiple tyrosine kinase residues, that create a binding site for STAT molecules[65]. Phosphorylation of STAT3 leads to dimerization and nuclear translocation of this transcription factor, enabling it to regulate the transcription of various genes, such as suppressor of cytokine signaling 3 (SOCS3) that acts in a negative feedback loop to leptin signaling after prolonged stimulation[66]. Taken together, these data suggest that leptin regulates glycemic control in addition to energy homeostasis. Therefore, further studies on leptin signaling could contribute to the development of alternative therapies for restoration of glucose homeostasis.

V. The role of protein tyrosine phosphatases in immunity and glucose metabolism

Protein tyrosine phosphatases (PTPs) are key signaling regulators in many physiological processes. There are eight subtypes of PTPs, which are characterized by a single transmembrane spanning domain, variable N-terminal extracellular regions and a phosphatase domain[67,68]. Regulation of PTP activity occurs through dimerization of the phosphatase domain which results in an inhibition of the phosphatase activity[69]. Mutations in genes encoding PTPs are associated with many diseases, such as cancer[70], immune disorders[71] and diabetes[72].

The *motheaten* mouse carries a mutation in the Protein Tyrosine Phosphatase Non-Receptor Type 6 (*Ptpn6*) gene, which encodes the hematopoietic PTP SHP-1. This mouse develops autoimmune disease and inflammation[73] that are marked by alopecia, glomerulonephritis, dermatitis, inflammation of the paws, pneumonitis and high mortality[74]. Moreover, the mutant mice are characterized by overproduction and accumulation of macrophages and neutrophils in the lungs and skin, as well as a higher concentration of pro-inflammatory cytokines, serum immunoglobulins and auto-antibodies[74]. SHP-1 regulates macrophage

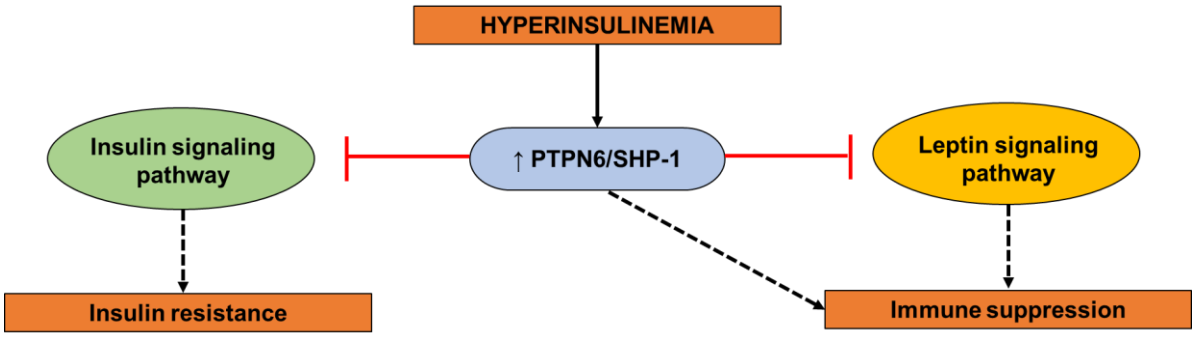

Figure 3. Model for hyperinsulinemia-induced immune suppression and insulin resistance via PTPN6/SHP-1. Hyperinsulinemia inhibits the insulin signaling pathway, leading to insulin resistance via induction of PTPN6, which encodes the phosphatase SHP-1. SHP-1 plays a role as a negative immune regulator by inhibiting the leptin signaling pathway that results in immune suppression[47].

function, downregulates IFN-γ-mediated macrophage nitric oxide production and IFN-γ-inducible gene expression[75]. SHP-1 phosphorylation is increased after TB infection and the protein co-localizes with TB phagosomes, indicating that bacterial phagocytosis by macrophages results in SHP-1 activation and its recruitment to the phagosome. Further phagosome maturation is regulated by the activity of (PI3K), which is inhibited by SHP-1 activation[76]. These findings are important for further TB research and imply the possible use of therapeutics that target SHP-1 for host-directed therapy against TB.

SHP-1-deficient mice show higher insulin sensitivity and glucose tolerance suggesting that this PTP is also involved in glucose metabolism and development of insulin resistance[77]. SHP-1 has also been studied in zebrafish larvae[47], and it was shown that zebrafish larvae are susceptible to injected human insulin, leading to the inhibition of gluconeogenesis and transient hypoglycemia.

Moreover, larvae treated with a high dose of human insulin develop insulin resistance and a loss of their primary insulin sensitivity. Interestingly, knockdown of *ptpn6* by morpholino oligonucleotides prevents the development of an insulin-resistant state as well as deregulation of other genes involved in the insulin signaling pathway such as genes encoding leptin, (phosphoenolpyruvate carboxykinase 1 (Pck1) and the insulin receptor[47]. Furthermore, *Ptpn6* activation leads to JAK2 dephosphorylation, that is involved in the leptin signaling pathway. These results support the hypothesis that hyperinsulinemia downregulates the insulin signaling pathway resulting in insulin resistance via activation of SHP-1. On the other hand, Shp-1 can play a role as a negative immune regulator by inhibiting the leptin signaling pathway[47]. In summary, SHP-1 regulates the transcription of metabolic and immune signaling pathways, acting as a mediator between insulin signaling regulation and the immune system (Fig.3).

VI. The effects of glucocorticoids on tuberculosis and diabetes

Glucocorticoids are a class of steroid hormones that regulate various processes in our body and are secreted in response to stress by the adrenal gland[78]. The main endogenous glucocorticoid in our body is cortisol, and its secretion is tightly regulated by the hypothalamic-pituitary-adrenal axis. During their general mode of action, glucocorticoids bind to the glucocorticoids receptor (GR)[79], which modulates the transcription of genes via two basic mechanisms: regulation of gene expression via interaction with glucocorticoid-responsive elements in the DNA, direct protein-protein interaction with transcription factors such as NF-κB and activator protein 1 (AP-1)[80].

Because of their immune-suppressive and anti-inflammatory effects, synthetic glucocorticoids such as dexamethasone and prednisolone are frequently prescribed drugs to control inflammation in diverse conditions such as infections and autoimmune diseases. They are widely used in many respiratory diseases like asthma, bronchiolitis, cystic fibrosis, COVID-19 and tuberculosis[81]. Currently, glucocorticoids, such as dexamethasone, are the only approved supporting chemotherapeutics for TB[82]. Glucocorticoid treatment has been proven to be beneficial for subsets of TB patients, by increasing their survival[83] and body mass[84]. This is most likely due to the suppression of the severe inflammatory response in these patients, but the exact mechanism of glucocorticoid action during the disease remains unclear.

Besides the immune-suppressive effects, the treatment with glucocorticoids can result in multiple adverse effects such as glaucoma, hypertension, skeletal muscle atrophy, osteoporosis, obesity and diabetes[85]. In people with TB, a high dosage of glucocorticoids results in hyperglycemia, fluid retention and hypertension[86,87]. It is well established that prolonged administration of glucocorticoids affects glucose metabolism and causes metabolic changes. It has been shown that glucocorticoid treatment is followed by elevated leptin concentrations via transforming growth factor beta (TGF-β) induction of the ALK5-Smad2/3 pathway[88], and if prolonged, can lead to leptin resistance and changes in fat metabolism[89]. Furthermore, glucocorticoids are well-known to interfere with the insulin pathway and glucose metabolism. Glucocorticoids affect cellular glucose uptake via glucose transporter type 4 (GLUT4)i glucose transporters in skeletal muscles, which are responsible for the majority of insulin-mediated glucose uptake, and induce protein degradation and a decrease in protein synthesis via the PKB/Akt and mTOR pathways. As a result, prolonged glucocorticoid administration is a risk factor for insulin resistance, DM2 and muscle wasting (Fig.4)[90,91].

Thus, glucocorticoids are on the one hand beneficial for TB patients since they suppress the inflammatory response, whereas on the other hand they may enhance the metabolic effects induced by TB and aggravate the TB-related insulin resistance and muscle wasting. A better understanding of the effects of glucocorticoid treatment on the progression of TB would not only improve TB treatment, but also could improve the quality of life of the patients after the infection has been cured.

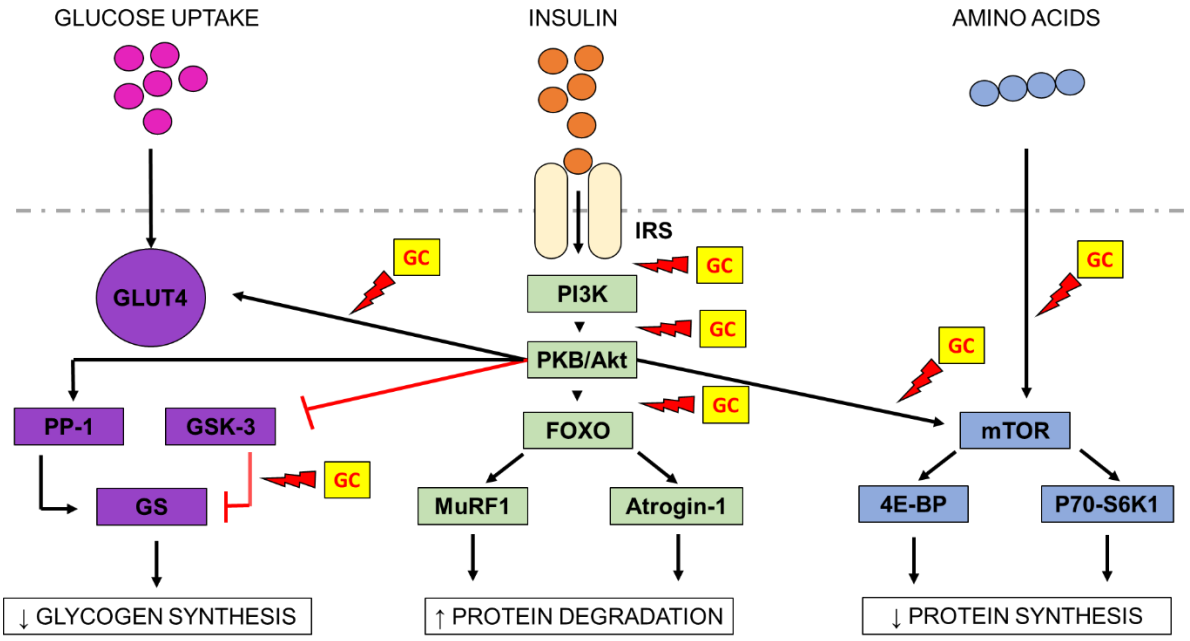

Figure 4. Molecular role of glucocorticoids in the insulin pathway and protein turnover. GLUT4 enables glucose uptake into cell. Glucocorticoids impair the insulin-mediated glucose uptake by directly interfering with the insulin signaling pathway leading to an increase in protein degradation and a decrease in protein synthesis[96].

VII. Conclusion

There is undoubtedly a strong association between TB and DM. A recent study shows that DM increases the risk of TB development three-fold. Moreover, DM increases TB severity and mortality in infected patients, as well as the risk of failure of the treatment[91]. In turn, TB patients develop DM more frequently than healthy individuals. TB patients are shown to suffer from stress hyperglycemia, subclinical diabetes, pre-diabetes and clinical diabetes. This process, from normoglycemia towards diabetes, may progress very rapidly in some patients [34]. Both diseases are emerging worldwide epidemics. DM is a major health problem based on lifestyle, diet and genetic predispositions, and TB is globally the infectious disease with the highest mortality, and Co-infection with both TB and HIV causes a serious health problem in developing countries. Moreover, the infection, in most of the cases, is complicated with rapid weight loss, called wasting syndrome that contributes largely to the mortality of the infected patients[92]. Interestingly, treatment with glucocorticoids improves body weight, plasma albumin level and increases appetite in subsets of treated TB patients[93].

Our immune system and metabolism are intricately intertwined. Production of pro-inflammatory cytokines such as leptin from adipose tissue contributes greatly to a cascade of metabolic changes such as hyperglycemia/insulin resistance, wasting syndrome, dyslipidemia, hypertension and microalbinuria[94]. Leptin is a protein that plays a dual role in an organism both as a hormone that modulates energy homeostasis and as a cytokine that

promotes the inflammatory response. In monocytes/macrophages, leptin promotes the production of pro-inflammatory cytokines such as IL-6 and TNF-α that induce proliferation and phagocytosis[95]. Furthermore, leptin protects neutrophils from apoptosis and activation of caspases, whereas it induces chemotaxis and expression of several adhesion molecules[40]. An acute inflammatory stimulus, such as TB, results in increased leptin levels[96], and this response has also been demonstrated in the zebrafish model[97].

In conclusion, the association between TB and DM is widely recognized, but little is known about the exact mechanisms of the interaction between these two pathologies, providing a strong motivation to understand the underlying mechanisms of TB and DM synergy. Understanding of the bilateral interactions is necessary to develop effective therapeutic strategies to minimize the dual aspects of the diseases.

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Chapter 2

A comparative transcriptome study of the effects of knock down of the Ptpn6 protein by treatment with morpholino or inhibitor NSC-87877 during mycobacterial infection in zebrafish larvae

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Abstract

Protein tyrosine phosphatases play an important role in many cellular processes such as cell survival, migration and immune responses. Protein tyrosine phosphatase nonreceptor type 6 (Shp-1/Ptpn6) knockdown in zebrafish larvae has previously been shown to result in immune deficiency and autoimmune disorders. In the present study, we compared the effects of *ptpn6* morpholino knockdown and the Ptpn6 inhibitor NSC-87877 on zebrafish embryos infected with *Mycobacterium marinum* and performed RNA sequencing (RNAseq) analysis on whole larvae. Infection data showed that mycobacterial infection progressed more rapidly after morpholino and NSC-87877 treatment compared to the control treatment, with the morpholino showing a larger effect than the inhibitor. The RNAseq analysis showed similar patterns in functional annotations and signaling pathways, such as synaptic transmission, ion transport, cell structure, proteolysis, immune response, apoptosis and gonadotropin secretion. Notably, inhibition caused by NSC-87877 and *ptpn6* morpholino knockdown resulted in upregulation of immune related genes. However, there are also some differences, such as, for instance, that *ptpn6* morpholino knockdown had more impact on the expression of metabolic genes. On the other hand, we found a number of genes of which the expression after bacterial infection was not affected by either treatment. The set of genes that show the same transcriptional response after *ptpn6* morpholino knockdown and NSC-87877 inhibitions are a useful reference set that can specifically linked to the function of Ptpn6. The obtained data stimulates further research on the NSC-87877 inhibitor in comparison with other inhibitors of Ptpn6.

Introduction

Protein dephosphorylation is a process that plays a role in all physiological processes inside the cells of living organisms. Dysregulation of this process leads to various diseases, such as cancer, diabetes, autoimmune disorders and neurological diseases[1,2,3,4,5]. The enzymes responsible for this process, protein tyrosine phosphatases (PTPs), form a large family of 107 enzymes divided into 4 groups depending on their protein structure and function[6,7,8,9]. Class I of PTPs is the biggest group and contains non-receptor PTPs. Two highly related proteins of this family are well conserved in vertebrates: Protein Tyrosine Phosphatase, Non-Receptor Type (PTPN)6, and PTPN11. They are also called Src homology region 2 domain-containing phosphatase (SHP)-1 and SHP-2, respectively, because they have two Src homology 2 domains N-terminal to the phosphatase catalytic domain[10] and are regulated by self-phosphorylation. PTPN6 is expressed mainly in the hematopoietic system, but expression has been observed in other cells as well, albeit at lower levels[11]. PTPN6 is also expressed by epithelial cells, and these cells express a different isoform type, which only differ in the first few amino acids that can result in differences in its function[12,]. PTPN6 plays a role in many different signaling pathways in cells. It is a key regulator of myeloid cell function and it impacts the function of diverse cytokine receptors, growth factor receptors and immunoreceptors[10]. PTPN11 is expressed more ubiquitously and is believed to be an essential component in several

oncogene signaling pathways[13]. The expression of PTPN11 is significantly lower in hematopoietic cells than that of PTPN6[14]

The function of the mouse *Ptpn6* gene has been intensively studied in various strains called *motheaten*, which carry a mutation in this gene. Besides a line with a genetically engineered mutation[15], there are also several *motheaten* mouse lines carrying spontaneous mutations. The first discovered spontaneous mutant, known as *me,* was a null mutation, where a frame shift mutation leads to a phosphatase-dead PTPN6[16]. The phenotype of this mutant is characterized by skin lesions and lethality within 6 weeks[17]. The *me* mutant also shows other severe disorders, such as autoimmunity and spontaneous inflammatory responses which are caused by a dysregulation of immune cell function [18]. A second spontaneous mutant strain, called *motheaten viable* (*mev*), survives for approximately 12 weeks and shows a similar phenotype as the *me* mutant, but less severe. In addition, two other *Ptpn6* mouse mutants, *spin* and *meB2*, display similar symptoms, which confirms an important role for PTPN6 in the function of myeloid cells and immune regulation[19].

The zebrafish larval model has several advantages for the modeling of disorders that requires studying the function of the innate immune system separate from the adaptive immune system, since the onset of the latter's activity happens later in zebrafish development[20]. In a previous study, we used a morpholino oligonucleotide knockdown approach in zebrafish larvae to study the function of the *ptpn6* gene. We showed that p*tpn6* deficiency in larval zebrafish results in a decreased ability to control a bacterial infection[21]. Our data showed that *ptpn6* acts as a negative regulator of the innate immune system, and thereby playing a crucial role during the response to a bacterial infection[21]. Moreover, knockdown of *ptpn6* in zebrafish leads to severe edema and skin lesions, resembling the murine *Ptpn6* knockout model.

Mycobacterium marinum, a close genomic relative of *M. tuberculosis*, is a natural pathogen of zebrafish which leads to systemic, chronic infection similar to human tuberculosis[22]. Injection of the pathogen in zebrafish larvae causes aggregation of infected macrophages into granuloma-like structures and the activation of granuloma-specific genes. Furthermore, advantages of zebrafish larvae such as their transparency, gives an opportunity to study natural host–pathogen interactions *in vivo* and to understand early host responses to tuberculous infection[23], in the absence of the adaptive immune system, which is not yet developed in the larvae[24].

In a different study, we demonstrated that morpholino knockdown of *ptpn6* prevents the downregulation of insulin- and immune-relevant genes in hyper-insulinemic zebrafish larvae, showing that Ptpn6 functions at the crossroads of immunity and insulin resistance[25]. In addition, Ptpn6 was shown to play a key role in insulin signaling pathways, acting as a mediator that regulates a switch between the insulin-sensitive and insulin-resistant states after hyperglycemia resistance[25].

In the present study, we have explored the effect of Ptpn6 deficiency in the absence and presence of a *Mycobacterium marinum* infection in zebrafish larvae using transcriptome analysis. Two approaches were used. First, we used the morpholino knockdown approach that we had previously used[21]. Second, looking for possible therapeutic opportunities, we tested a chemical inhibitor (NSC-87877) that has been shown to inhibit PTPN6, PTPN11, Dual specificity phosphatases and immune regulators such as cytokine signaling (SOCS) in human cells[26,27,28,29,30]. By comparing the effects of morpholino and inhibitor treatment we aim to establish a solid view of the role of Ptpn6 in the response to a mycobacterial infection.

Materials and methods

Fish maintenance

Wild type zebrafish of the AB/TL line 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. Eggs were collected and grown at 28.5 °C in egg water (60 µg/ml Instant ocean sea salt, Sera Marin).

Morpholino injections

For this study, a *ptpn6* morpholino oligonucleotide (GeneTools, LLC, Philomath, Oregon, USA) was used, which was previously described by Kanwal *et al.*[21] This splice-blocking morpholino induces the deletion of the phosphatase catalytic domain in the Ptpn6 protein. The morpholino (5'-ACTCATTCCTTACCCGATGCGGAGC-3') 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 the *ptpn6* morpholino solution or a standard control morpholino (5'- CCTCTTACCTCAGTTACAATTTATA-3') was injected into the yolk sac of approximately 60 embryos per experimental group at the 1-2 cell stage using an automated injection system[31] (Life Science Methods, Leiden, Netherlands).

Bacterial injections

The *M. marinum* E11 strain, containing an mCherry expression vector, was grown as described in Carvalho *et al.*[32]. Bacteria were injected as described in Benard *et al.*[33] Injections of *M. marinum* (20–40 CFUs, diluted in PBS containing 2% polyvinylpyrrolidone40 (PVP40)) were performed by injection (1 nl) into the yolk sac of approximately 50 zebrafish embryos per experimental group using an automated injection system (Life Science Methods), and took place around 4 hours post fertilization (hpf). Control embryos were injected with PBS. During injections fish were kept under anesthesia in egg water containing 0.02% buffered 3-aminobenzoic acid ethyl ester (tricaine, Sigma–Aldrich).

NSC-87877 treatment

The larvae were incubated from 4 hpf until 5 dpf in egg water that contained a solution of 10 µM NSC-87877 in egg water and DMSO, as the control group, larvae were incubated, during the same period of time, only in water with DMSO.

Microscopy

A Leica MZ16FA fluorescence stereomicroscope (Leica microsystems, Wetzlar, Germany) equipped with a 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 (previously described by Stoop *et al*.[34]), the results are written to a spread sheet so that further statistical analysis using GraphPad Prism 8 software (GraphPad Software, La Jolla, CA, USA) could be applied to the data.

COPAS analysis

Fluorescence intensities in zebrafish embryos were measured every 24 hours until 5 days post infection (dpi) using the COPAS XL (Union Biometrica, Holliston, Massachusetts, USA). The settings were as follows: photo multiplier tube (PMT) voltage was 650 V for green/red and 0 V for yellow, optical density threshold signal was 975 mV (COPAS value: 50), and the time of flight (TOF) minimum was 320 µs (COPAS value: 800).

RNA deep sequencing (RNAseq)

Thirty larvae at 5 dpi per sample, from three independent experiments, were homogenized in 1 ml of TRIzol reagent (Life Technologies), and total RNA was extracted according to the manufacturer's instructions. A total of 2 µg of RNA was used to make RNAseq libraries using the Illumina TruSeq RNA Sample Preparation Kit v2 (Illumina, Inc., San Diego, CA, USA). The manufacturer's instructions were followed with the exception of two modifications. First, in the adapter ligation step, 1 µl, instead of 2.5 µl, adaptor was used. Second, in the library size-selection step, the library fragments were isolated with a double Ampure XP purification with a 0.7× beads to library ratio (Beckman Coulter, Woerden, The Netherlands). The resulting mRNAseq library was sequenced using an Illumina HiSeq2500 Instrument (Illumina, Inc., San Diego, USA) according to the manufacturer's instructions with a read length of 2×50 nucleotides. Image analysis and base calling were done by the Illumina HCS version 2.0.12. Data analysis was performed using Genetiles software as previously described by Veneman et al.[35] and the Pathvisio software package (http://www.pathvisio.org)[36]. False discovery rate (FDR)-adjusted P values were calculated based on the algorithm of Benjamini & Hochberg (1990)[37]. Significance cutoffs at 1.5-fold change and a P value < 0.05 were used. Gene ontology was analyzed using the program DAVID[38].

Statistics

Statistical significance was analyzed using GraphPad Prism 8 software (GraphPad Software, La Jolla, CA, USA). Differences in total fluorescence intensities were statistically tested by unpaired t-test (comparison between 2 groups) or one-way ANOVA followed by Tukey's comparison test (multiple group comparisons).

Results

Transcriptomic profiling and pathways analysis in non-infected larvae

Knockdown studies of the *ptpn6* gene in zebrafish embryos were performed using a previously described splice-blocking morpholino[21]. The *ptpn6* (or standard control) morpholino was injected into the yolk sac of wild type embryos at the in 1-2 cell stadium (Fig.1). The morphants showed some developmental abnormalities such as smaller eyes and cardiac edema that have already been reported previously by Kanwal *et al.*[21]. The Ptpn6 inhibitor NSC-878777 or vehicle (DMSO) was added at 4 hpf. To characterize similarities between the effects of *ptpn6* morpholino and NSC-87877 treatment we performed an RNAseq based transcriptome analysis of zebrafish larvae at 5 dpf (Fig.1). We found 339 genes that were regulated by both the *ptpn6* morpholino and NSC-87877 treatment (Fig.2A). There were 182 upregulated genes shared between the two treatment groups, whereas 1123 genes were only upregulated in the morpholino group and 637 genes that were only upregulated after NSC-87877 treatment (Fig.2A). We found 157 genes that were downregulated by both treatments, 1704 genes that were only downregulated genes upon morpholino treatment and 325 genes that were only downregulated in the NSC-87877-treated group (Fig.2A). Moreover, from the volcano plots it is visible that more genes were downregulated after *ptpn6* knockdown, whereas upregulation is stronger in the NSC-87877-treated group, and that the morphants show a larger variation in the fold changes and p-values than the larvae treated with NSC-87877 (Fig.2B). In summary, these results show that the morpholino treatment induces a larger transcriptional effect than NSC-87877, regulating approximately 2.5 times more genes, and that approximately 10% of all genes regulated by the morpholino is also regulated by NSC-87877. Functional annotation clustering of the shared 339 genes significantly changed in both groups indicated that both methods of Ptpn6 inhibition affected regulation of genes associated with skin formation and regeneration (Table 1). Genes involved in processes involving intermediate filaments, keratin type I activity and fin regeneration were significantly enriched in the cluster of downregulated genes regulated by both morpholino- and NSC-87877-treatment (Table 1). Genes involved in neuroendocrine signaling and immunity were significantly enriched among the cluster of upregulated genes. Further analysis showed that the most enriched pathway among the upregulated genes was glyoxylate and dicarboxylate metabolism signaling, that is mainly responsible for biosynthesis of carbohydrates from fatty acids and is a part of the tricarboxylic acid cycle (TCA) cycle[32] (Table 1).

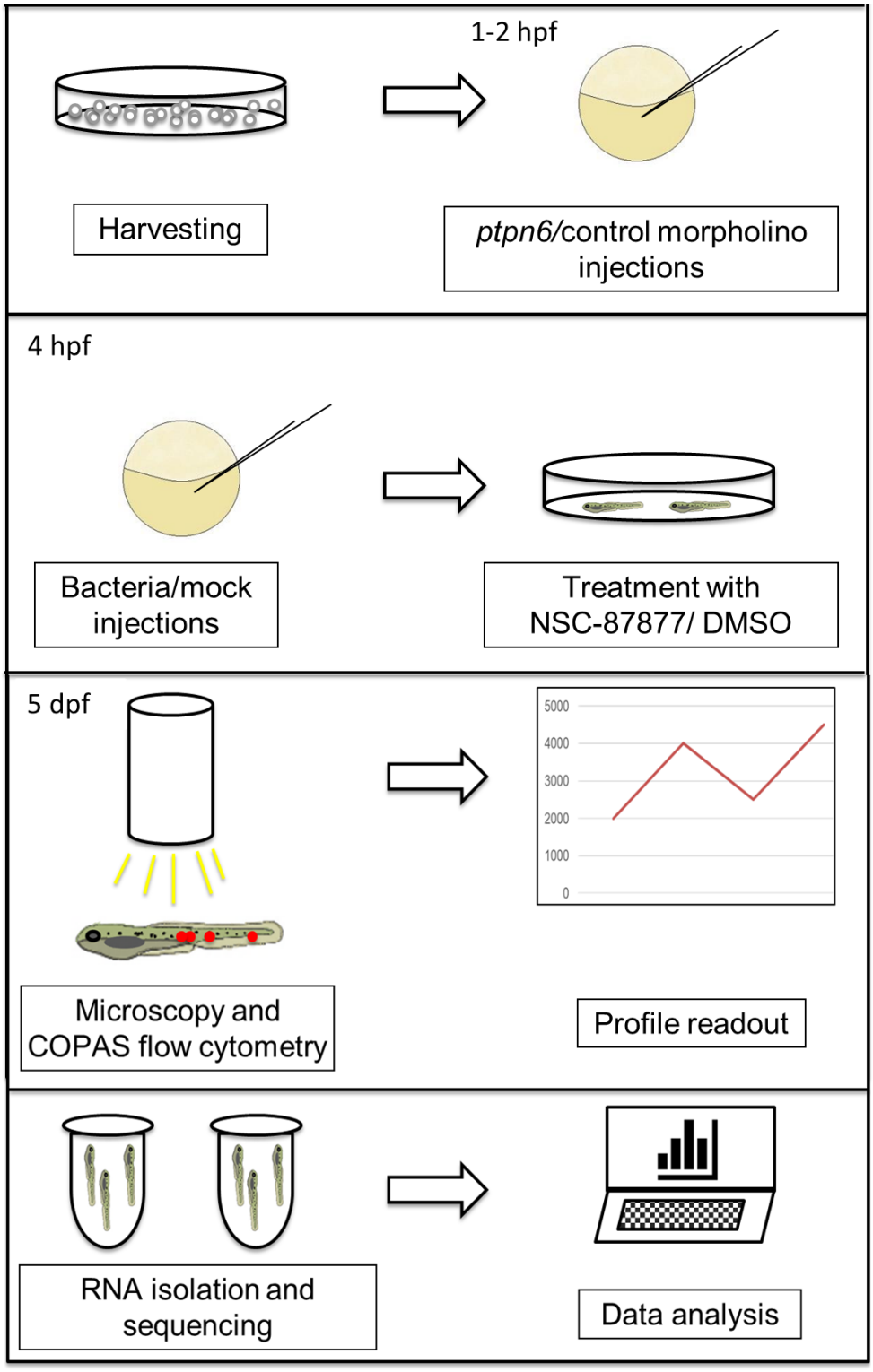


Figure 1. Experimental workflow. Zebrafish embryos were collected and immediately injected with morpholino. After bacterial injection at 4hpi, the embryos were kept in NSC-87877 or DMSO as a control. The bacterial burden was monitored using fluorescence microscopy and COPAS flow cytometry. At 5 dpi RNA of the larvae was extracted and analyzed by deep sequencing.

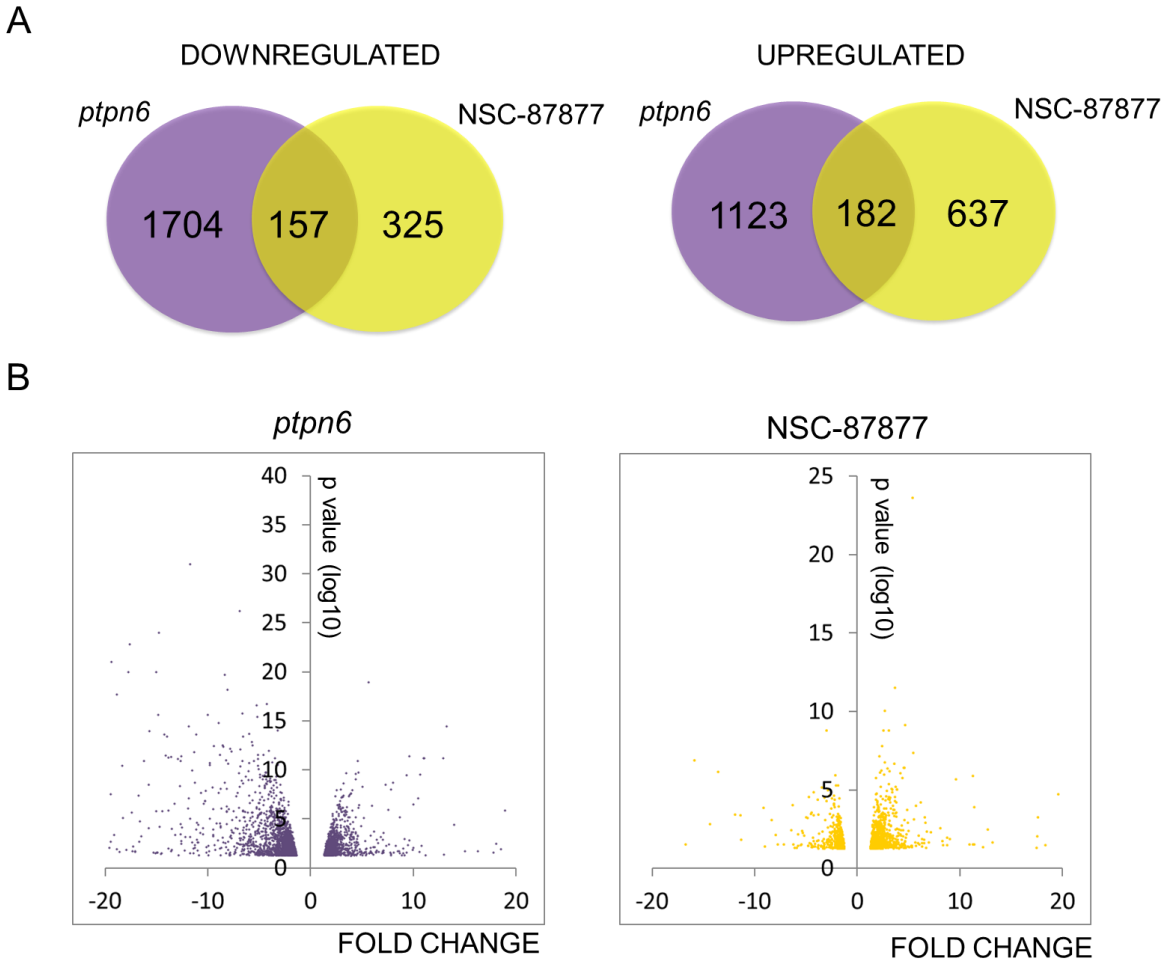


Figure 2. Transcriptome analysis by RNAseq showing modulation of gene regulation induced by *ptpn6* morpholino knockdown and NSC-87877 treatment. RNAseq analysis was performed on 5 dpf larvae treated with either *ptpn6* morpholino or NSC-87877 to inhibit Ptpn6 function. **(A)** Venn diagrams showing (overlaps between) clusters of all genes significantly upregulated or downregulated by *ptpn6* morpholino knockdown (compared to standard control morpholino, violet) or NSC-87877 treatment (compared to vehicle (DMSO) treatment, yellow). **(B)** Volcano plots indicating the fold change (x-axis) and P-value (y-axis) of the regulation for individual genes upon treatment with *ptpn6* morpholino (left panel) or NSC-87877 (right panel).

Morpholino knockdown of *ptpn6* leads to increased bacterial burden and mortality in zebrafish larvae

Zebrafish embryos were infected with *M. marinum* by injection into in the yolk sac at 4 hpf (Fig.1). We have previously shown that granuloma- like aggregates form within the body of the larvae several days post injection using this approach[28]. The infected embryos had been previously injected with the *ptpn6* morpholino or the standard control morpholino. During the infection we monitored the survival of the infected larvae. We found increased infection-induced mortality in the p*tpn6* morphants (~67% at 5 dpi) compared to the group treated with the control morpholino (~20%, Fig.3B).The bacterial burden was monitored for 5 days using COPAS flow cytometry. At 1 dpi there were no significant differences between the groups, with the fluorescence at approximately ~300 AU for all groups. The bacterial burden started

Table 1. Gene ontology analysis of the cluster of genes that are significantly regulated by both morpholino and NSC-87877 treatment using DAVID Functional Annotation Tool (Functional Annotation Clustering and KEGG pathways).

FUNCTIONAL ANNOTATION CLUSTERING		
Enrichment Score	UPREGULATED	DOWNREGULATED
3.15		Intermediate filament Keratin type I Fin regeneration Structural molecule activity
2.23	Neuropeptide hormone Extracellular space	
1.77	Arrestin - like Immunoglobulin E - set	
1.36		Extracellular region Transmembrane region
1.29		Secreted
1.27		Fibronectin, type III
PATHWAYS		
Enrichment Score	UPREGULATED	DOWNREGULATED
4.06	Protein processing in endoplasmic reticulum	
2.15		Tight junction Cell adhesion molecules (CAMs)
0.42		Fructose and mannose metabolism

to raise at 2 dpi up to ~700 AU in control morpholino-treated group, compared to ~1300 AU in the *ptpn6* morphants. At 3 dpi, the difference between the *ptpn6* knockdown group (~2500 AU) and the control group (~1600 AU) started to increase. This tendency continued at 4 dpi with an average ~2700 AU of total fluorescence in the control larvae, compared to ~4300 AU in the Ptpn6-deficient larvae, although large variation between single larvae in the *ptpn6* morpholino-treated group was observed. Finally, the infection results in a significant difference between the control group and the morphant group at 5 dpi (~ 7200 AU versus ~ 13560 AU) (Fig.3A). Similar results were obtained when the bacterial burden was monitored using fluorescence microscopy (Fig.4A). The microscopy data were quantified using pixel count software, showing a significant difference at 5 dpi between the control group (~80 AU) and the *ptpn6 morphants* (~490 AU)(Fig.4B).

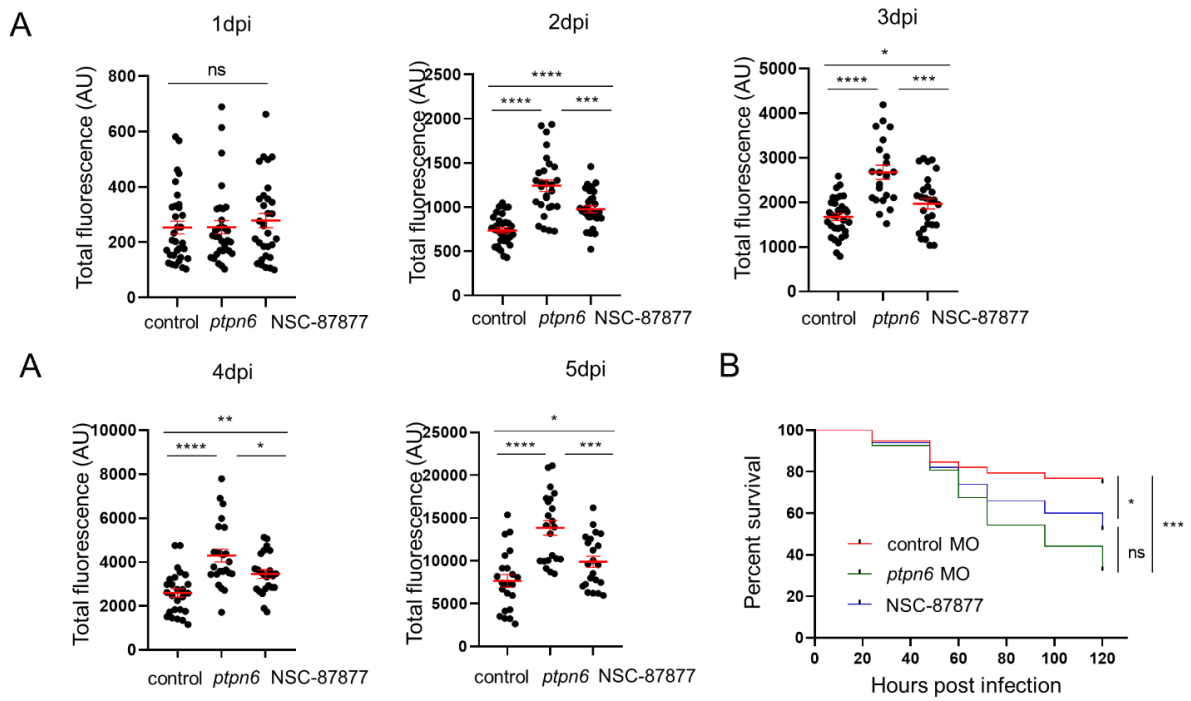


Figure 3. Inhibition of Ptpn6 function increases the bacterial burden and mortality rate after *M. marinum* infection. (A) Bacterial burden in the control, *ptpn6* morpholino- and NSC-87877-treated groups followed up to 5 dpi using COPAS flow cytometry. **(B)** Mortality rate in the control larvae, *ptpn6* morphants and after NSC-87877 treatment after *M. marinum* infection, followed until 5 dpi. Data shown are means ± s.e.m. from three independent experiments. Statistical significance is indicated as *P<0.05; **P<0.01; ***P<0.0 01; ****P<0.0001 (determined using ANOVA with Tukey's post hoc test).

Inhibition of Ptpn6 by NSC-87877 leads to a similar, but smaller, increase in infection rate as *ptpn6* morpholino knockdown

Next, we studied the effect of the Ptpn6 inhibitor NSC-87877 in four different concentrations of the drug (5, 10, 20 and 50 µM). The highest concentration resulted in rapid progression of the infection and enhanced mortality that reached 100% after 2 dpi, whereas 5 µM concentration showed no significant effect during infection (Suppl.fig.1). Treatment with 20 µM of the compound resulted in similar bacterial burdens and mortality rates and the concentration of 10 µM was chosen for further research to study if NSC-87877 has the same effect on the progression of the mycobacterial infection as *ptpn6* morpholino knockdown. Therefore, in the same experiment one group of *M. marinum* infected embryos was treated with 10 µM of NSC-87877 and one group with DMSO as a control (previously injected with control morpholino). Just like *ptpn6* morpholino treatment, NSC-87877 treatment resulted in increased mortality compared to the control-infected group, that reached (~40% at 5 dpi, compared to 65% for the control-treated group, Fig.3B). The progression of the infection was monitored during the following days using COPAS flow cytometry. We did not notice any significant effect of NSC-87877 treatment at 1dpi, but a significant difference was observed

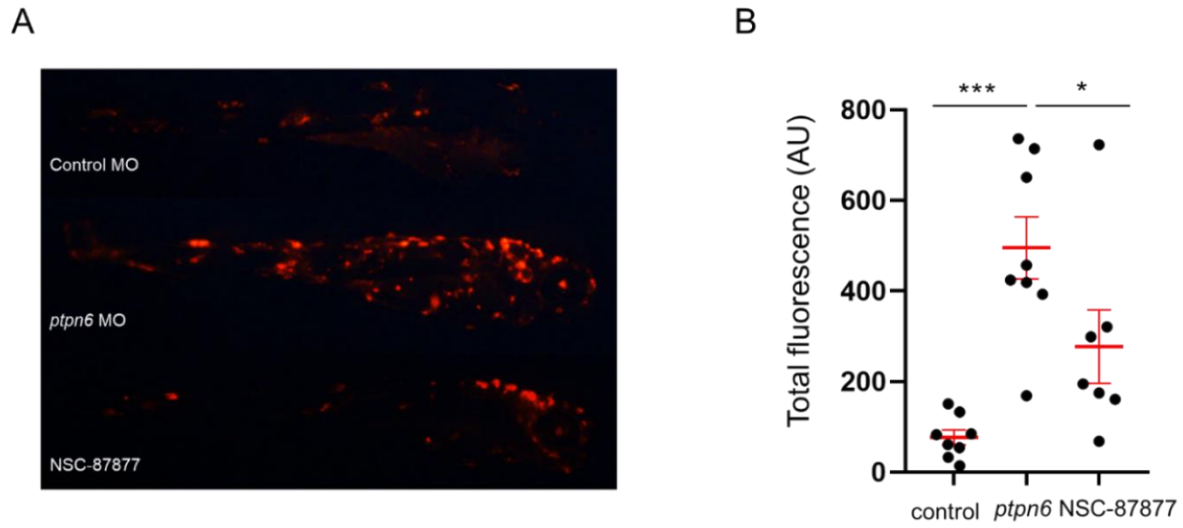


Figure 4. Bacterial burden after *M. marinum* infection. (A) Representative fluorescence microscopy images of bacterial infection in control, *ptpn6* MO- and NSC-87877-treated larvae at 5 dpi. Bacteria are shown in red. **(B)** Pixel counting of bacterial burden in the control/infection, *ptpn6* MO/infection and NSC-87877/infection groups at 5 dpi. Data shown are means± s.e.m. from three independent experiments. Statistical significance is indicated by: *P<0.05; ***P<0.001; (determined using ANOVA with Tukey's post hoc test).

at 2 dpi when a total fluorescence of ~1000 AU was observed in the NSC-87877-treated group (versus ~700 AU in the control group and ~1300 AU in the *ptpn6* morphants). At 3 dpi the difference between the NSC-87877 exposed group (~2000 AU), the control group (~1600 AU) and the *ptpn6* knockdown group (~2500 AU) was increased and this tendency continued at 4dpi with the average total fluorescence of and ~3400 AU in the inhibitor treated larvae (~2700 in the control group, ~4300 AU in the morphants,). At 5 dpi, the fluorescence of bacterial burden reached ~9800 AU in the larvae treated with inhibitor, ~7200 AU in the control group and ~ 13560 AU upon morpholino knockdown(Fig.3A).

Transcriptomic profiling and gene ontology analysis in *M. marinum* infected larvae

To study the effects of *ptpn6* morpholino and NSC-87877 treatment on the transcriptional response to infection, we used morpholino- and NSC-87877-treated embryos infected with *M. marinum* and monitored them up to 5 dpf, when they were collected and used for further analysis. First, the larvae were used to study the *ptpn6* mRNA levels after *M. marinum* infection, which was measured using qRT-PCR analysis. The *ptpn6* expression was shown to be upregulated at 4 dpi, showing >7-fold increased *ptpn6* mRNA levels in the infected larvae compared to non-infected larvae (Fig.5A). Second, we compared transcriptomic profiles to study the effect of *ptpn6* morpholino- and NSC-87877 treatment on the transcriptional response to infection. We found 234 upregulated and 214 downregulated genes in the control infected group (Fig.5B). Interestingly, these numbers were more than ten-fold higher upon *ptpn6* morpholino and infection treatment (2417 and 2447 respectively) and NSC-87877 and infection treatment (3336 and 2496 respectively, Fig.5B). Volcano plots conformed this effect,

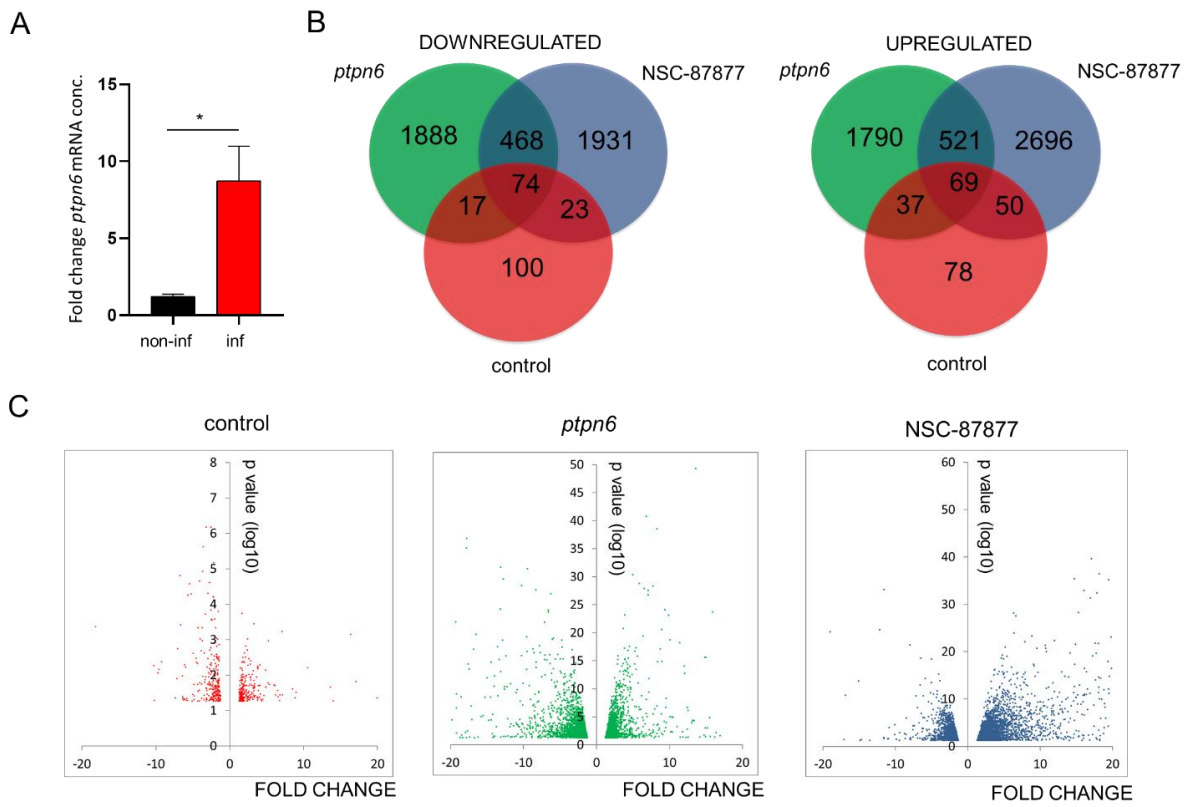


Figure 5. Transcriptome analysis by RNA-seq showing modulation of gene regulation induced by *ptpn6* MO knockdown and NSC-87877 treatment during *M. marinum* infection. **(A)** Expression of *ptpn6* at 5 dpi after *M. marinum* infection, determined using qRT-PCR. Data are mean ± s.e.m. from three independent experiments. Statistical significance is indicated by *P<0.05; (determined using student's t-test). **(B)** Venn diagrams showing significantly upregulated or downregulated genes during *M. marinum* infection in combination with control (red), *ptpn6* MO (green) and NSC-87877 (blue) treatment. **(C)** Volcano plots indicating the fold change (x-axis) and P-value (y-axis) of the regulation for individual genes.

showing dramatically larger variation in fold changes and p-values upon Ptpn6 inhibition (Fig.5C). The cluster of genes that was regulated by both morpholino/infection and NSC-87877/infection treatment, and not by the control/infection treatment included 989 genes (521 upregulated and 468 downregulated), which was only 11% of the total number of genes that are exclusively regulated upon Ptpn6 inhibition (Fig.5B).

We selected this cluster of shared 989 genes for further analysis. Gene ontology groups enriched in this cluster were mostly related to protein degradation for the upregulated genes, and synaptic transmission for the downregulated genes (Table 2). Analysis of enriched Kegg pathways revealed changes in MAPK,VEGF and p53 signaling pathways, which were found to be significantly upregulated in both *ptpn6* knockdown and NSC-87877 inhibited group, whereas some, such as purine metabolism were found to be downregulated (Table 3).

To further understand the effects of the *ptpn6* knockdown and NSC-87877 treatment on gene regulation, we manually analyzed the expression levels of the genes that are present in the signature sets of the *ptpn6* morpholino and NSC-87877 groups, but are not affected in the

control infection group. The analysis was performed using the Pathvisio software package (http://www.pathvisio.org). The most significant genes were grouped in immune response, matrix remodeling, prostaglandin, cytokine-cytokine receptor interaction and metabolic response (Fig.6).

Finally, analysis of several selected immune- and metabolism-related genes, described previously by Marín-Juez et al.[25], revealed that both Ptpn6 inhibition by NSC-87877 and *ptpn6* morpholino knockdown led to a stronger response from the immune system, with significant changes in the expression of *il1b, socs3a* and *socs3b*, and the largest increases in the expression of *irg1l, mmp9,* and *irak3* (Fig7A). The genes *tnfa* and *tnfb*, although highly induced after NSC-87877 inhibition, were not significantly changed by the *ptpn6* morpholino knockdown. Genes related to metabolism and protein degradation showed higher levels of regulation upon *ptpn6* morpholino treatment compared to NSC-87877 treatment. In the morpholino-treated group, an upregulation was observed for genes involved in insulin signaling pathway, such as *pck1*, *lepb* and *insb* as well as genes that play a role in protein degradation, such as *socs3a* and *fbxo32* (Fig.7B).

Discussion

Tyrosine (de)phosphorylation of proteins plays a critical role in the regulation of many immune-related processes. To study the role of the protein phosphatase PTPN6, we used zebrafish larvae as animal models and treated them with a small molecule inhibitor NSC-87877, that had previously been shown to inhibit human PTPN6 *in vitro*[26]. The effect of NSC-87877 treatment was compared with effects of morpholino knockdown of the *ptpn6* gene. Transcriptome analysis using RNA-seq of the non-infected *ptpn6* morpholino- and NSC-87877-treated groups identified enriched gene ontology groups of genes related to intermediate filaments and cell junctions, that are connected with skin structure and function. It has been previously been shown that *ptpn6* MO knockdown leads to inflammation, severe edema and skin lesions in zebrafish larvae[21], similar to the phenotypes that were found in murine Ptpn6 mutants *me*, *mev* and *spin*[39]. Moreover, Ptpn6^{spin} mice exhibit lesions that define neutrophilic dermatoses, including formation of intraepidermal pustules and cutaneous damage, associated with infiltrations with neutrophils[40]. However, the transcriptomes of non-infected *ptpn6* morphants and NSC-87877 treated larvae do not share many common genes that show regulation compared to the controls, and those that are shared are not obviously immune system- or neutrophil-related genes. Furthermore, within this cluster of these commonly regulated genes, enrichment of any specific pathway was not observed.

Table 2. Gene ontology analysis of the cluster of genes that are significantly regulated by ptpn6 MO/infection and NSC-87877/infection treatment and not by control/infection treatment using DAVID Functional Annotation Tool (Functional Annotation Clustering).

FUNCTIONAL ANNOTATION CLUSTERING		
Enrichment Score	UPREGULATED	DOWNREGULATED
7.24		Synapse Ion channel activity Cell junction GABA-A receptor complex Cell membrane
3.83	Protease Proteolysis Peptidase activity Hydrolase activity	
3.45	Protein catabolic process Proteasome ATPase TBP-class protein binding ER-associated ubiquitin Posttranslational modification	
3.29		Postsynaptic cell membrane NMDA receptor Glutamate receptor Extracellular ligand binding receptor
2.6	Endopeptidase activity Threonine protease	Nicotinic acetylcholine receptor Acetylcholine binding Cholinergic synaptic transmission
2.24	Hemoglobin complex Oxygen transport Globin Iron ion binding Heme	
2.19		Intermediate filament Structural molecule activity
2.06		Potassium transport Potassium channel BTB/POZ Voltage-gated channel

Table 3. Pathways analysis of the cluster of genes that are significantly regulated by ptpn6 MO/infection and NSC-87877/infection treatment and not by control/infection treatment using DAVID Functional Annotation Tool (Kegg pathways).

PATHWAYS	
UPREGULATED	DOWNREGULATED
Proteasome	Phototransduction
GnRH signaling pathway	Neuroactive ligand-receptor interaction
VEGF signaling pathway	Gap junction
p53 signaling pathway	Purine metabolism
Apoptosis	Calcium signaling pathway
Salmonella infection	
Regulation of actin cytoskeleton	
Glycine, serine and threonine metabolism	
MAPK signaling pathway	
Protein processing in endoplasmic reticulum	

Subsequently, we infected zebrafish embryos after both treatments with *M. marinum* mCherry fluorescent strain to explore the role of Ptpn6 in the response to a bacterial infection. Our results support previous results showing that reduced Ptpn6 activity impairs the ability of zebrafish embryos to control mycobacterial infection. Our data showed that the effect of NSC-87877 treatment on the bacterial burden was milder than the effect of *ptpn6* MO knockdown and resulted in lower mortality ratios. This difference in mortality might be connected with inhibition of the related protein phosphatase Ptpn11 by NSC-87877, as in murine cells PTPN11 has been shown to play a negative role in cell survival during inflammation and to accelerate apoptosis through the dephosphorylation of signal transducer and activator of transcription 5 (STAT5)[41]. At the transcriptional level, we found that the combination of either *ptpn6* MO or NSC-87877 treatment and infection regulated approximately ten-fold more genes than the infection in control larvae. Amon the genes that were regulated by both combination treatments and not in the control larvae we observed

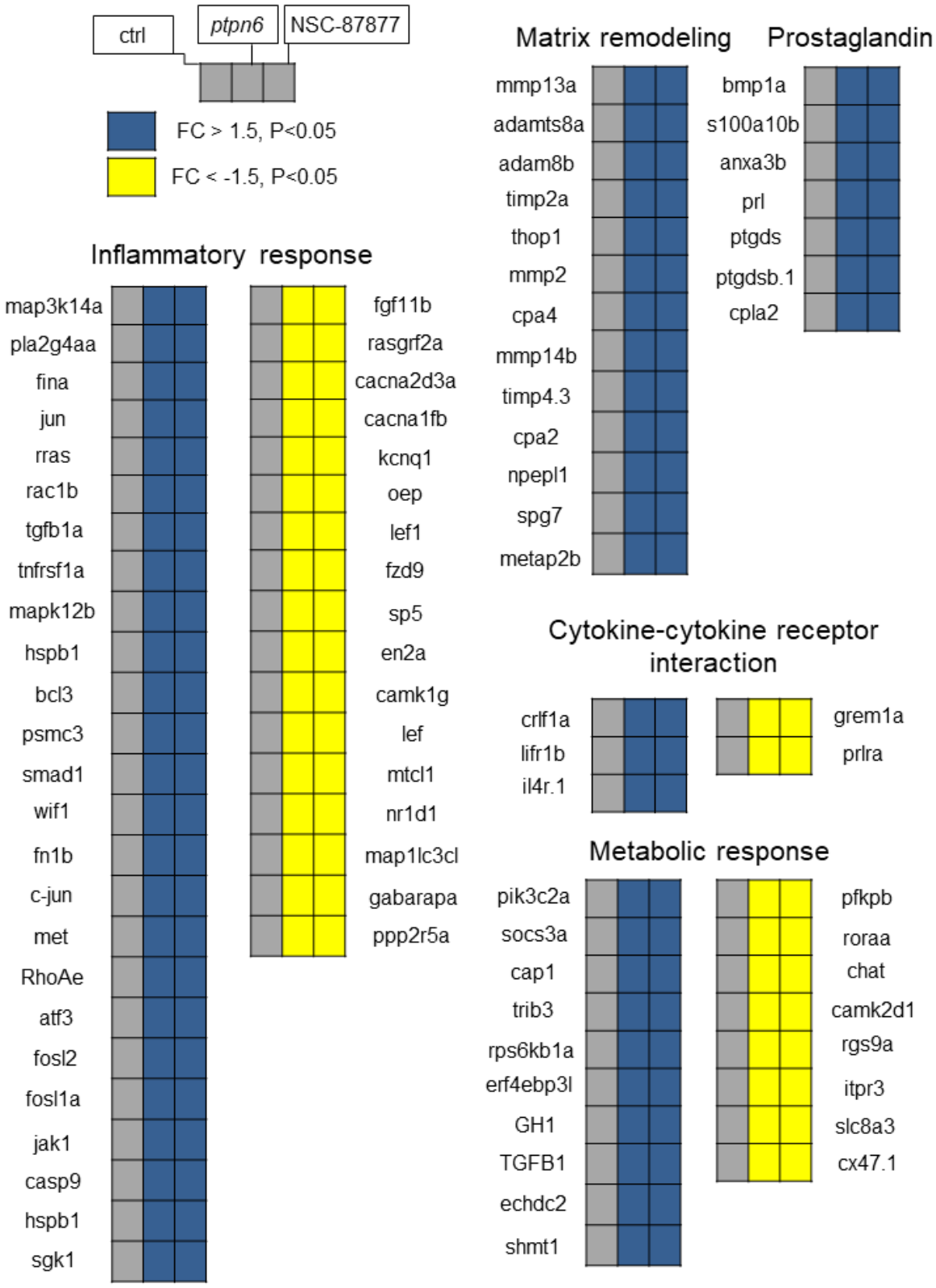


Figure 6. RNA-seq results for selected immune- and metabolism-related genes showing modulation of gene regulation by *ptpn6* MO knockdown and NSC-87877 treatment during *M. marinum* infection. Regulation is shown for the control infection experiment (left box), *ptpn6* MO infection experiment (middle box), and the NSC-87877 infection experiment (right box). Ratios are given for the infected versus the non-infection control group for each experiment. Blue boxes represent upregulation, yellow boxes represent downregulation, yellow boxes represent downregulation of gene expression.

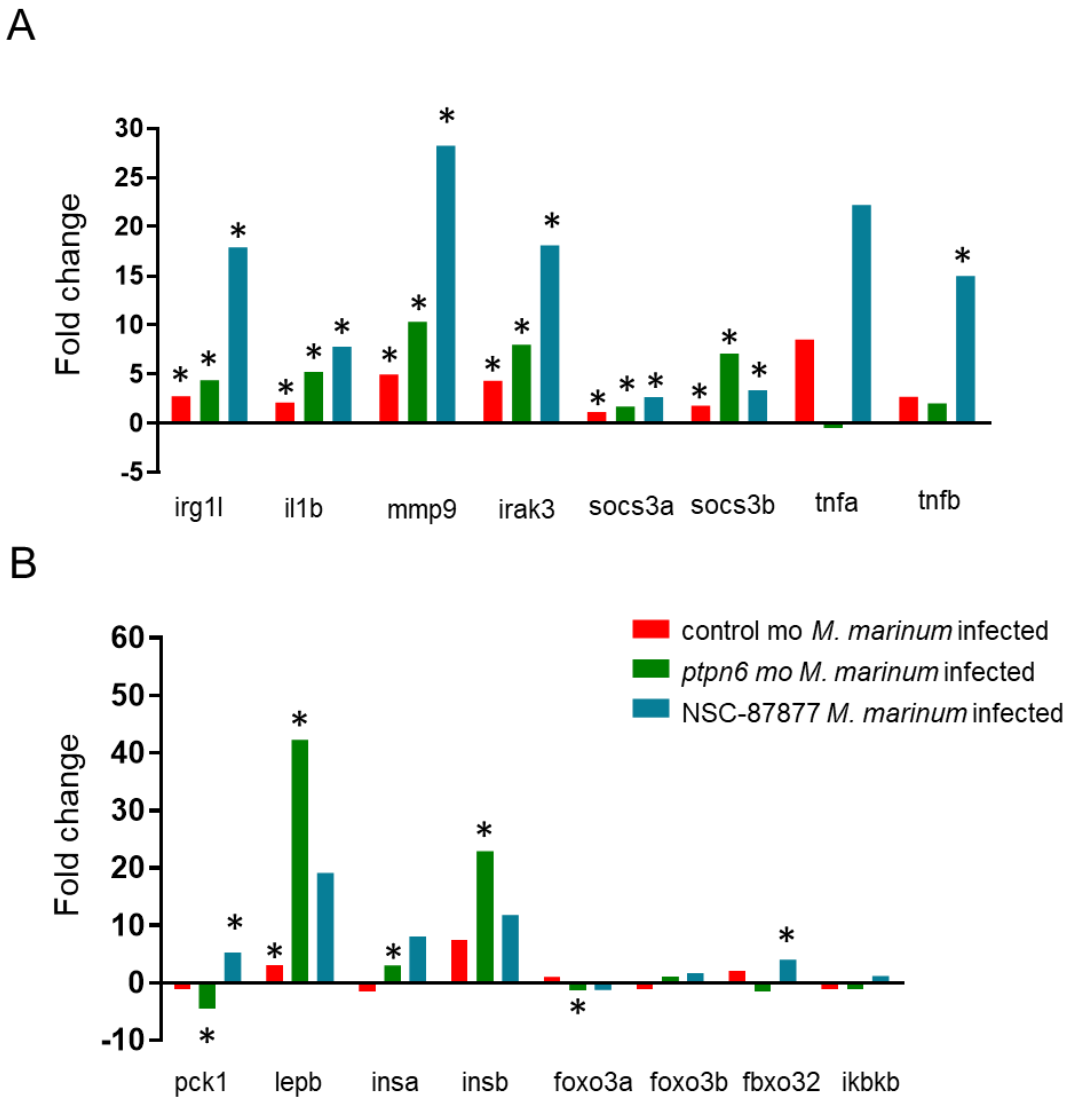


Figure 7. RNA-seq results for selected immune- and metabolism-related genes showing levels of gene regulation. The level of regulation (fold change) is shown in the control/infection (red bars), *ptpn6* MO/infection (green bars), and NSC-87877/infection (blue bars) groups compared to the control/non-infection group. Regulation of a selection of genes previously identified to be involved in innate immunity **(A)** and metabolism **(B)** is presented. Statistical significance (determined using analysis by Genetiles software) is indicated by *P<0.05

an enrichment of genes involved in synaptic transmission, ion transport, cell structure and proteolysis. Significantly changed pathways showed similar pattern with upregulation in some immune and inflammatory related pathways, cell structure and hormone release. Pathways related to synaptic transmission and transport were found to be downregulated. Dysregulation of MAPK signaling pathway and enhanced apoptosis were described previously in *ptpn6* morphants by Kanwal et al.[21], which corresponds with our findings.

Analysis of the regulation of various selected immune-related genes showed that both *ptpn6* MO knockdown and NSC-87877 treatment enhanced the expression of various genes involved in the innate immune response after *M. marinum* infection. We found that the most significantly upregulated immune-related gene after mycobacterial infection and NSC-87877

or *ptpn6* morpholino treatment was *mmp9*. Previous studies in zebrafish showed that *mmp9* is a crucial pro-inflammatory marker, which becomes highly induced by bacterial infection[42,43]. Other genes involved in immune processes that were also more highly induced after both *ptpn6* morpholino and NSC-87877 treatment were *irak3, irg1l, il1b, socs3a* and *socs3b*. These findings correspond with the data obtained by Kanwal et al.[21], who showed that *ptpn6* MO knockdown in zebrafish larvae infected with *S. typhimurium* resulted in hyperinduction of *mmp9* and *il1b*. These results suggest that *ptpn6* acts as a negative regulator of pro-inflammatory genes during the response to a bacterial infection. However, the results show some differences that are probably due to the fact that the microarray analysis performed by Kanwal et al.[21] were based on *Salmonella typhimurium* infection studies. Comparison of the results suggests that Ptpn6 has a stronger role in controlling responses to *S. typhimurium* infection than to *M. marinum* infection.

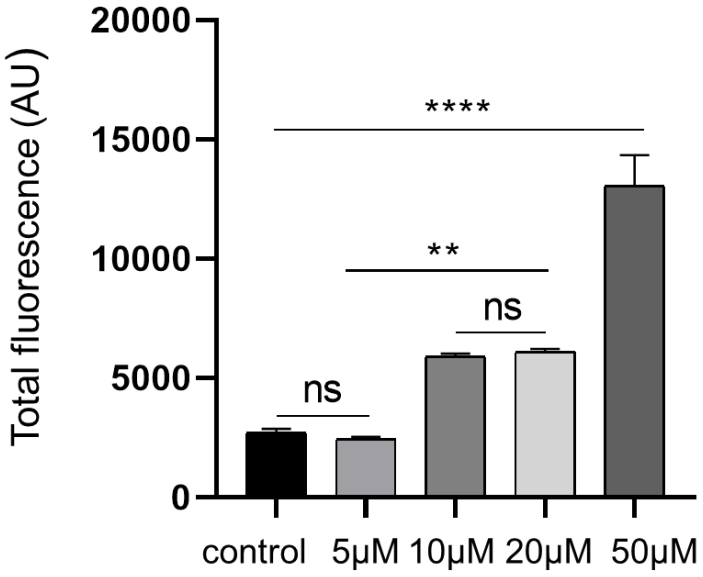
The most upregulated gene by the *ptpn6* MO/infection treatment that was also upregulated in the NSC-878777/infection group was *leptin b* that has previously been shown to be linked to *ptpn6* function (this thesis, Chapter 3) and to play both an immunological and metabolic role. This finding was not a surprise since previous studies in zebrafish larvae have already shown that *leptin b* becomes highly induced by mycobacterial infection[35]. Marín-Juez et al.[25] have suggested that Ptpn6 inhibits the leptin signaling pathway thereby suppressing the immune response. This conclusion was based on the studies where stimulation of PTPN6 by insulin led to JAK2 dephosphorylation, therefore interfering with the leptin signaling pathway[25]. Our finding that decreased Ptpn6 function caused hyperexpression of *leptin b* supports this hypothesis.

Most of the similarities between the *ptpn6* MO and the NSC-87877 treatments during infection were found in the group of genes involved in synaptic transmission, ion transport, cell structure, proteolysis, immune response, apoptosis and hormone release. However, there were differences in the regulation of the expression levels of many metabolic genes. In general, *ptpn6* MO/infection treatment had more impact on metabolic genes than NSC-87877/infection treatment. For instance, genes significantly changed by *ptpn6* MO knockdown were *insa*, *insb, pck1, fbxo32* and *foxo3a,* and these genes were not significantly regulated by NSC-87877. These genes are known to play a crucial role in the insulin signaling pathway and may play a role in the development of diabetes mellitus[44]. The mechanism behind the differences in the gene expression levels between the *ptpn6* MO- and NSC-87877-treated groups still remains unclear. NSC-87877 administration leads to stronger upregulation of expression of genes encoding cytokines and other immune-related proteins, its enhancement of the bacterial infection in zebrafish larvae is less severe than *ptpn6* MO knockdown. Administration of the *ptpn6* MO may resulted in several off-target effects and toxicity, which can result in an increased bacterial burden and mortality of the infected larvae. Relatively higher expression levels of immune-related genes and lower expression of some metabolic genes after treatment with NSC-87877 might be the result of a combination of both Ptpn6 and Ptpn11 inhibition. Knockout of *Ptpn11* in mice led to overproduction of the pro-

inflammatory cytokines IL-1β and IL-18 and increased sensitivity to peritonitis. In the mice with peritonitis, the infection showed a more severe course and was associated with excessive inflammasome activation[45]. Both PTPN6 and PTPN11 have been shown to modulate the insulin signaling pathway[25,46,47], which is in line with our observation of differences in the expression of metabolic genes, mainly involved in the insulin signaling pathway. Indeed, knockdown of *ptpn6* in zebrafish larvae has been shown to improve glucose uptake upon administration of a high dose insulin[25], whereas it was also shown that inhibition of PTPN11 in diabetic rats resulted in improved glucose uptake and reversed other diabetic alterations[46].

In summary, in the present study, we have compared *ptpn6* knockdown using morpholino oligonucleotides and Ptpn6 inhibition using NSC-87877, a non-specific PTPN6 inhibitor. Infection data show that both methods of decreasing Ptpn6 function compromises the defense against mycobacterial infection. Although we found some differences between the two treatments, we showed that the effects of these treatments show interesting similarities. The set of genes that show the same transcriptional response upon either treatment is a useful reference set that can specifically linked to the function of Ptpn6.

Supplementary materials



Supplementary Figure 1. Pixel counting of bacterial burden in the control/infection and NSC-87877/infection groups at 5 dpi. NSC-87877 was used in the concentrations of 5 μM, 10 μM, 20 μM and 50 μM. Data shown are means ± s.e.m. from three independent experiments. Statistical significance is indicated by: **P<0.01; ***P<0.0001; (determined using ANOVA with Tukey's post hoc test).

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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

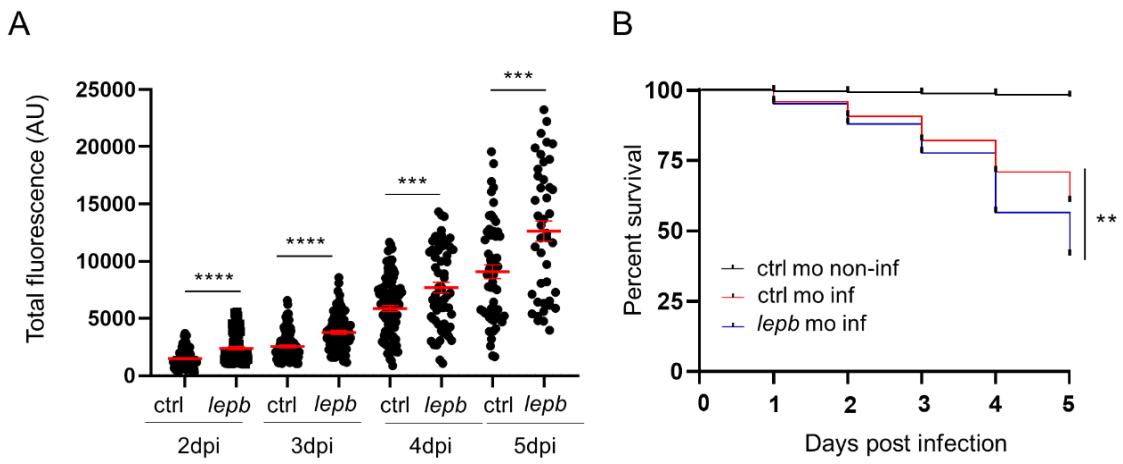


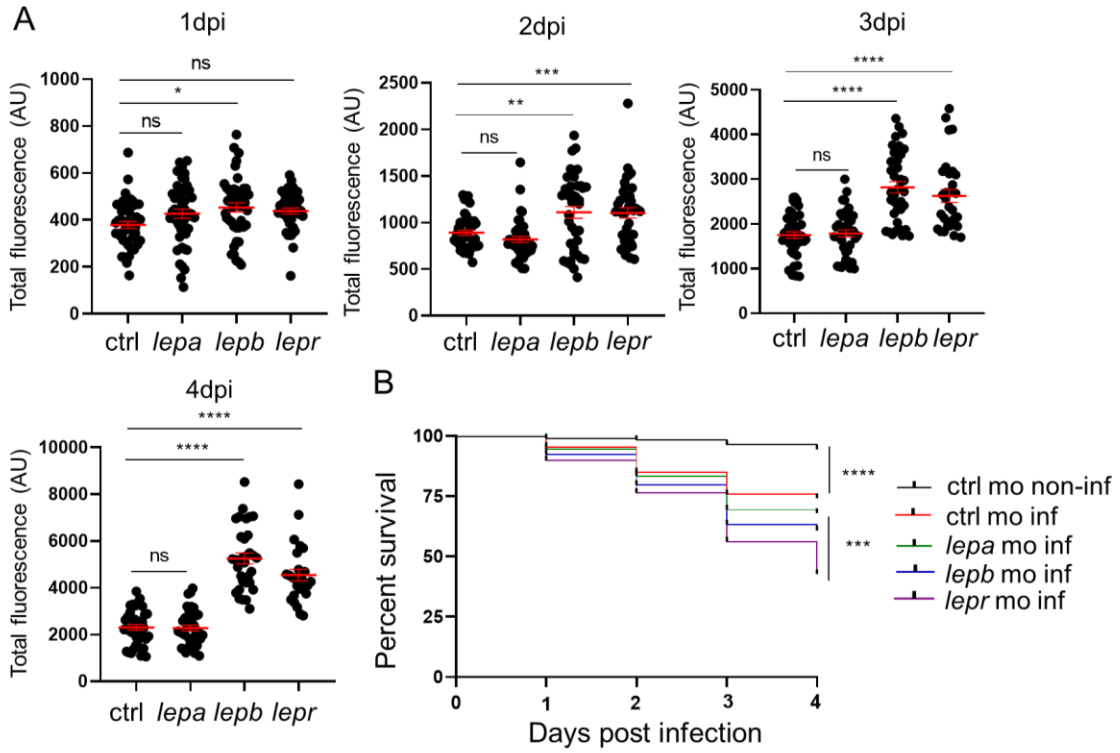
Figure 1. Morpholino studies: the effect of *lepb* knockdown on *M. marinum* yolk infection. (A) The bacterial burden in the control larvae and *lepb* morphants after *M. marinum* E11 infection in the yolk sac followed until 5 dpi. **(B)** Survival rates after *M. marinum* E11 yolk infection followed 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

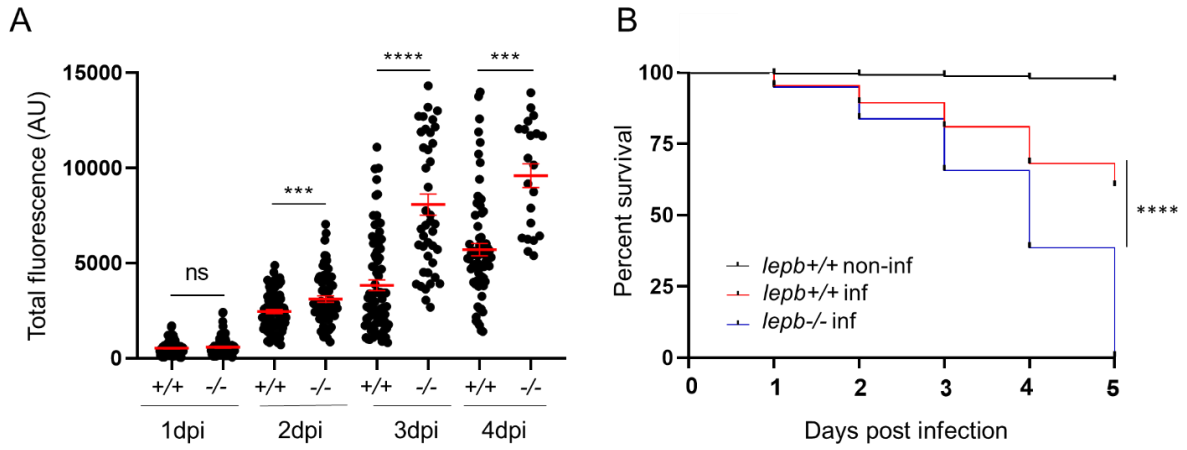


Figure 3. The lepb mutant zebrafish line: the effect of lepb knockdown on M. marinum yolk infection. (A) Bacterial burden in the wild type larvae (+/+) and *lepb*$^{-/-}$ mutants (-/-) after *M. marinum* E11 yolk infection, followed until 4dpi. **(B)** Survival rates in the wild type larvae and *lepb*$^{-/-}$ 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

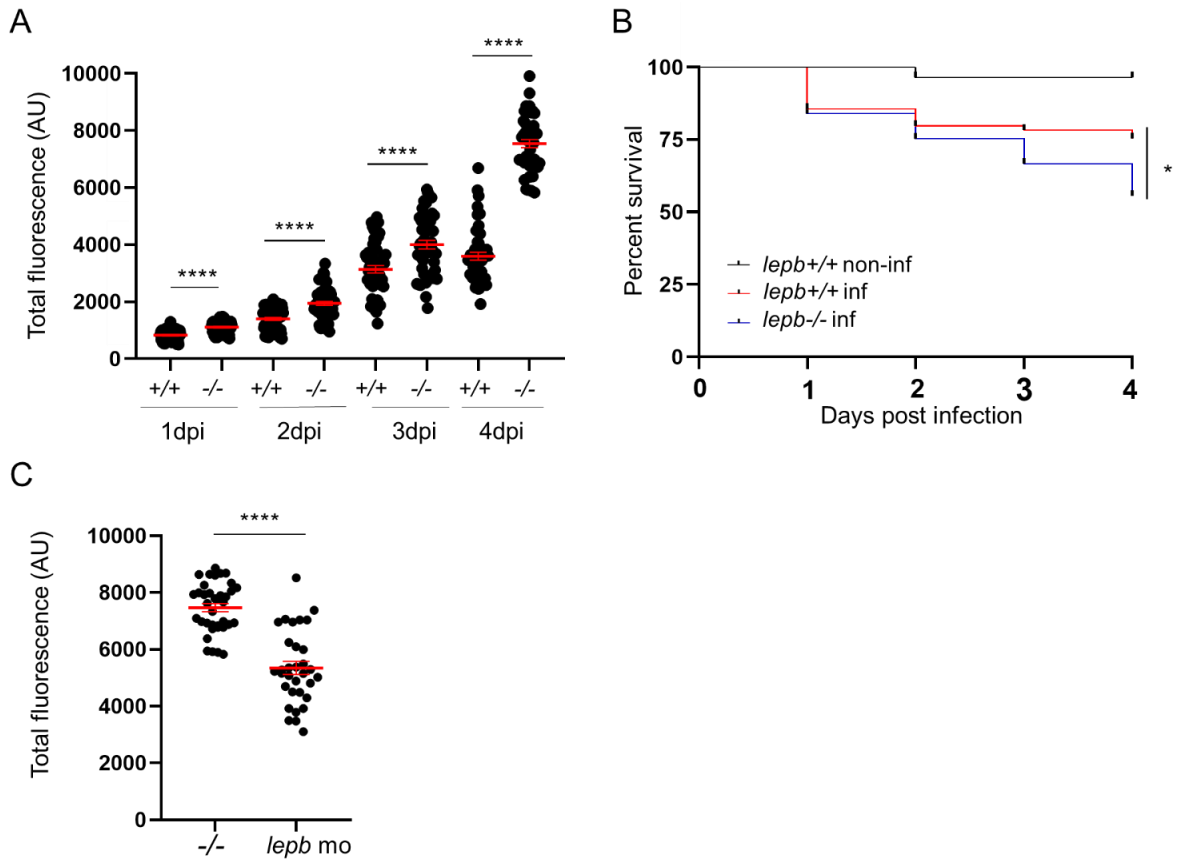


Figure 4. The *lepb* mutant zebrafish line: the effect of *lepb* knockdown on *M. marinum* blood island infection. **(A)** The bacterial burden in the wild type larvae (*+/+*) and *lepb* mutants (-/-) after *M. marinum* Mma20 blood island infection at 28 hpf, followed until 4dpi. **(B)** Survival rates 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

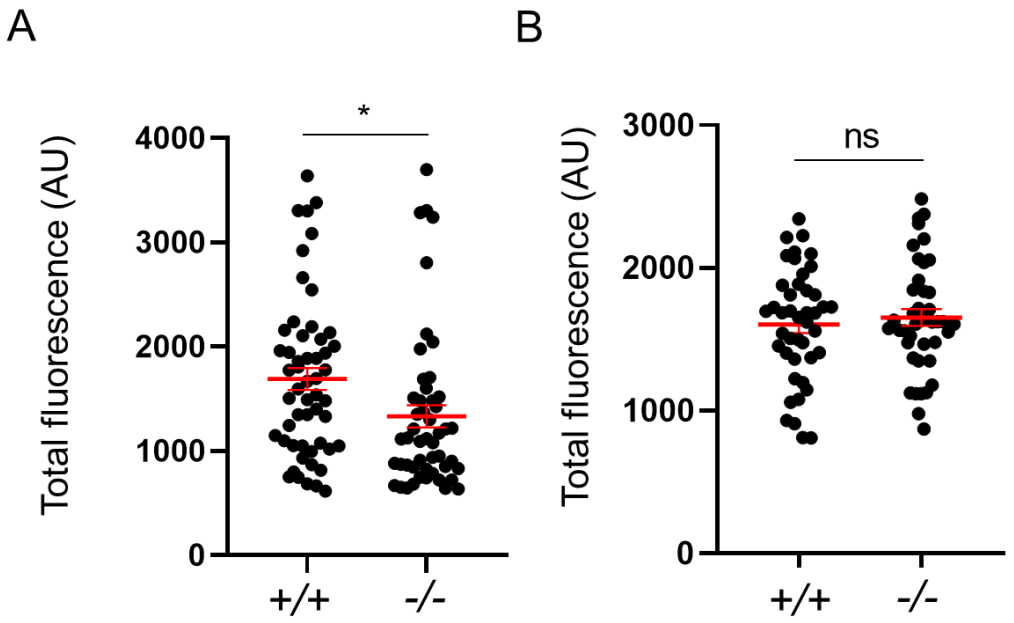


Figure 5. Determination of the number of macrophages and neutrophils in the lepb mutant line. (A) Fluorescence in *Tg(mpeg1:mCherry);lepb*$^{+/+}$ and *Tg(mpeg1:mCherry);lepb*$^{-/-}$ larvae at 5 dpf, measured by COPAS flow cytometry as a measure for the relative number of macrophages. **(B)** Fluorescence 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).

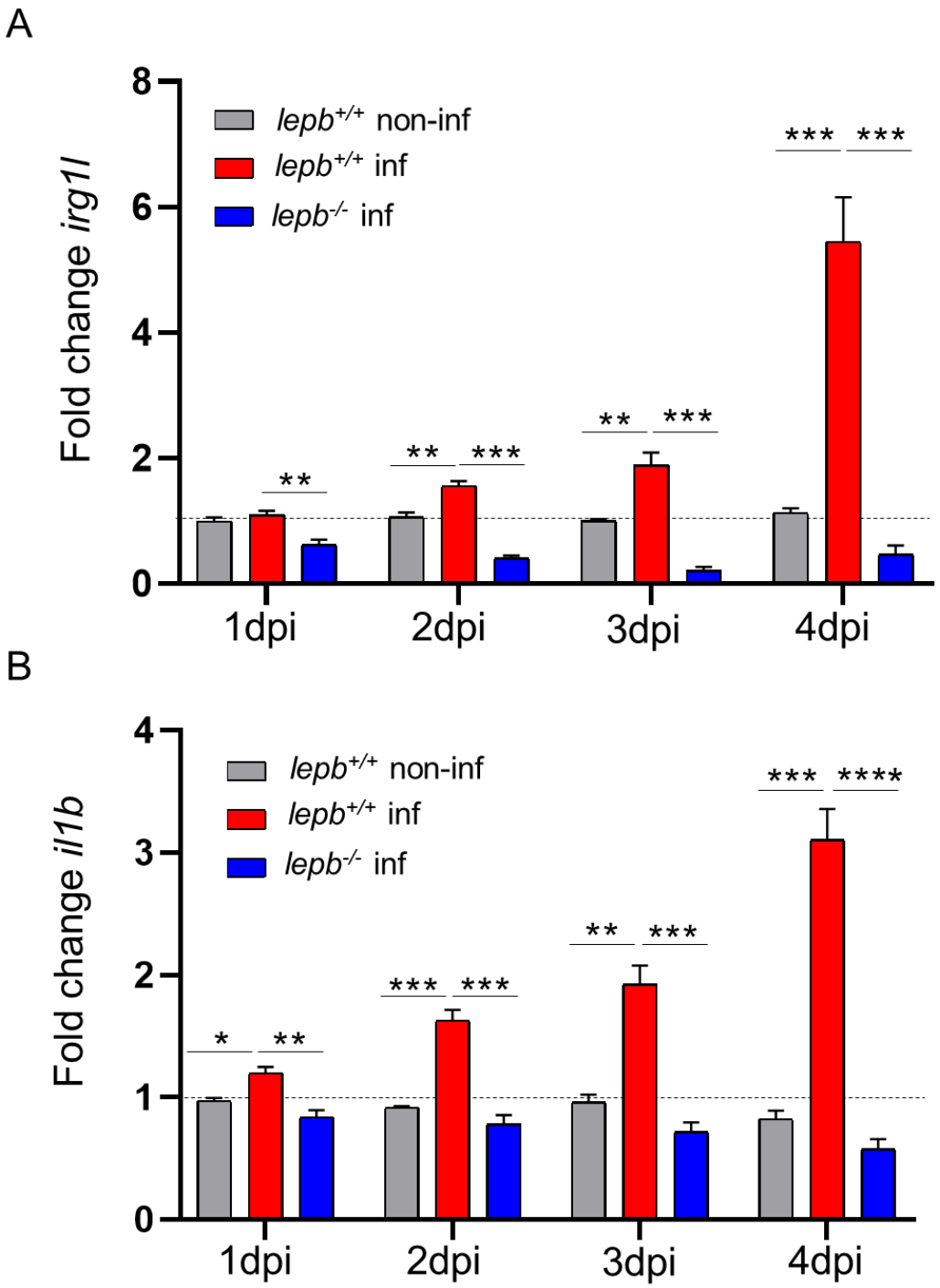


Figure 6. Expression of *irg1l* and *il1b* in the *lepb* mutant line after *M. marinum* blood island infection. (A) Expression *of irg1l* in the wild type larvae (+/+)and *lepb* mutants (-/-) after *M. marinum* Mma20 blood island infection at 28 hpf, starting from 1 dpi. **(B)** Expression of *il1b* 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.

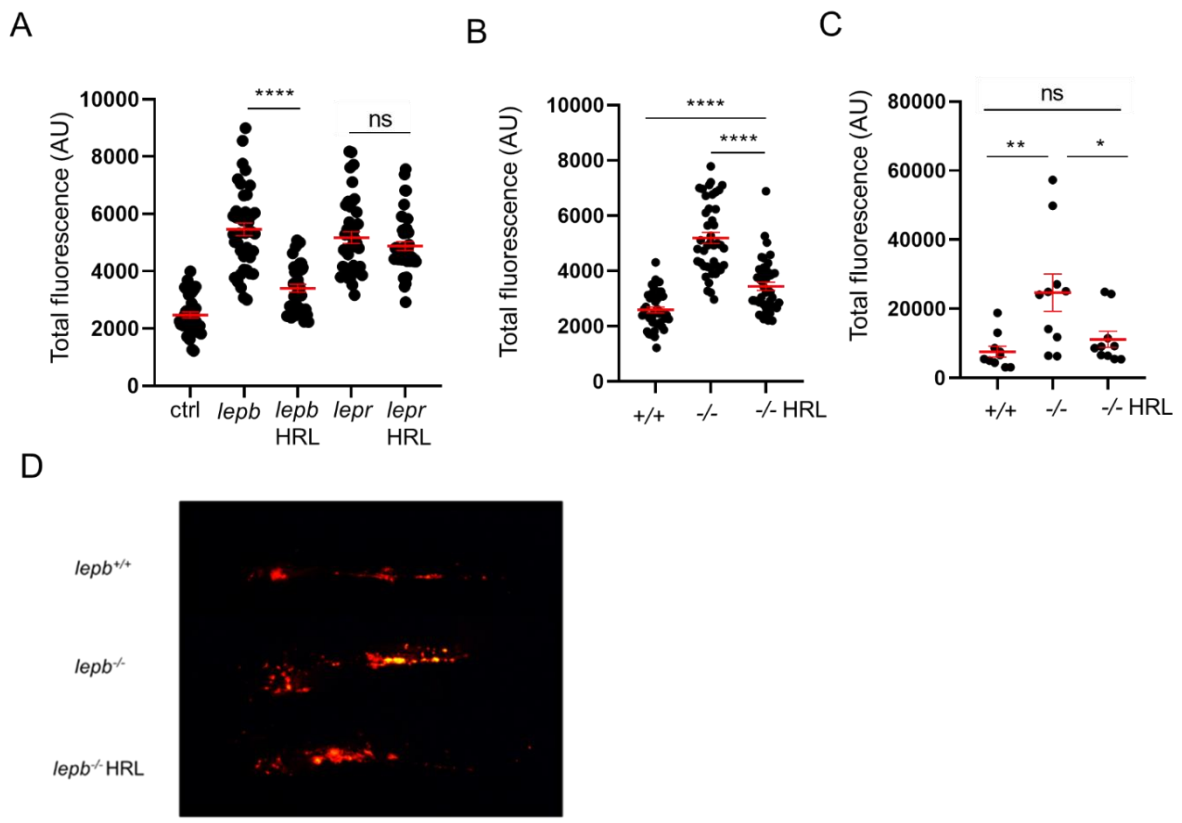


Figure 7. The effect of human recombinant leptin (HRL) administration on infection in *lepb* morphant and mutant larvae. (A) Bacterial burden in the control, *lepb* and *lepr* morpholino-treated groups, with and without HRL administration, measured at 4dpi. **(B)** Bacterial burden in the wild type (*+/+*) 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

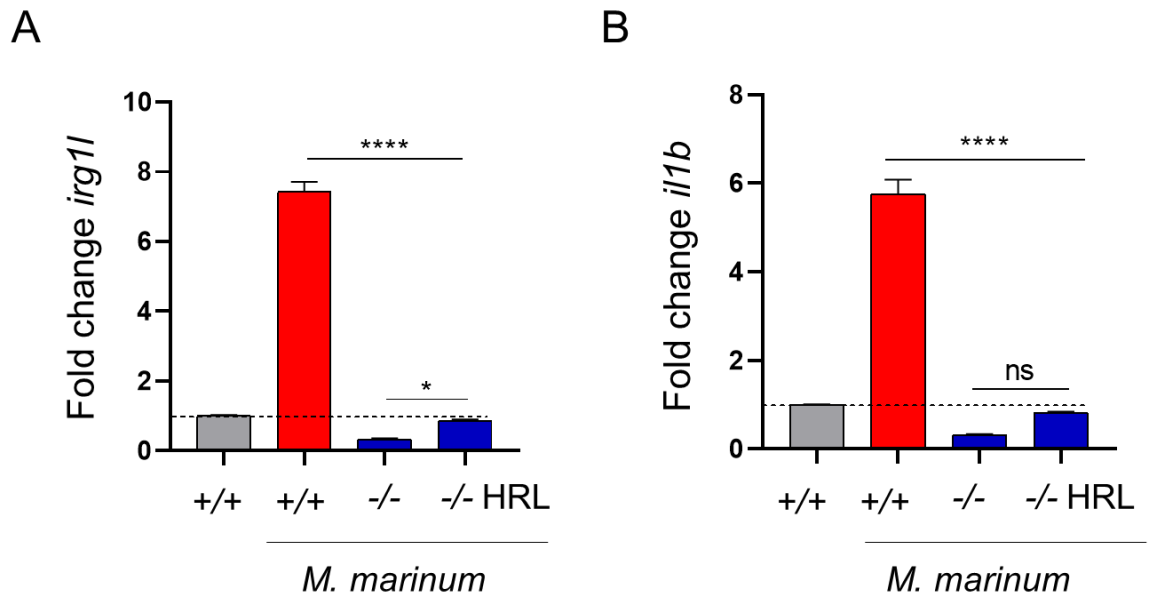


Figure 8. The expression of *irg1l* and *il1b* in *lepb* mutant after human recombinant leptin (HRL) administration. **A** qPCR analysis was performed at 4 days after *M. marinum* Mma20 blood island infection at 28 hpf. Expression levels are expressed as the fold change relative to the uninfected wild 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).

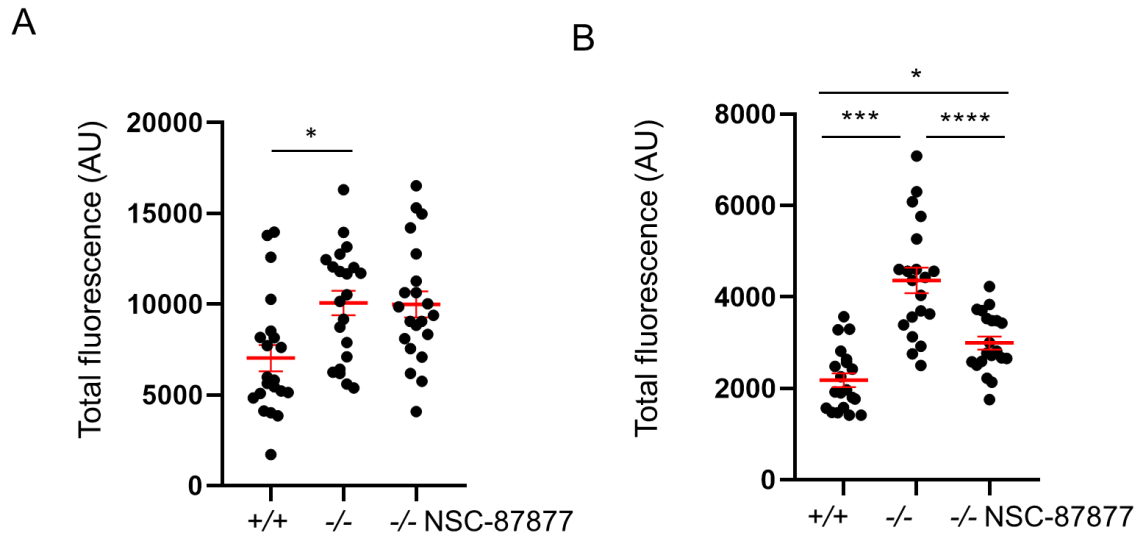


Figure 9. The effect of NSC-87877 treatment in *lepb* mutant fish after *M. marinum* infection. Bacterial burden in the control, *lepb*$^{-/-}$ larvae and *lepb*$^{-/-}$ larvae treated with NSC-87877, measured at 4dpi after yolk **(A)** and blood island **(B)** infection, at 4 and 28 hpf respectively. 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].

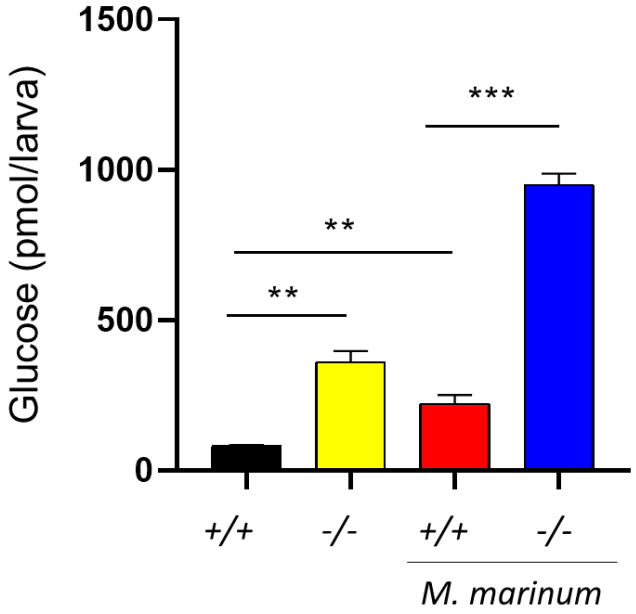


Figure 10. Glucose levels in *lepb* mutant and wild type larvae after *M. marinum* infection. Glucose levels (pmol/larva) in the wild type (*+/+*) and *lepb* mutant (*-/-*) larvae with and without *M. marinum* infection. Data are means ± s.e.m. from three independent experiments. Statistical significance (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.

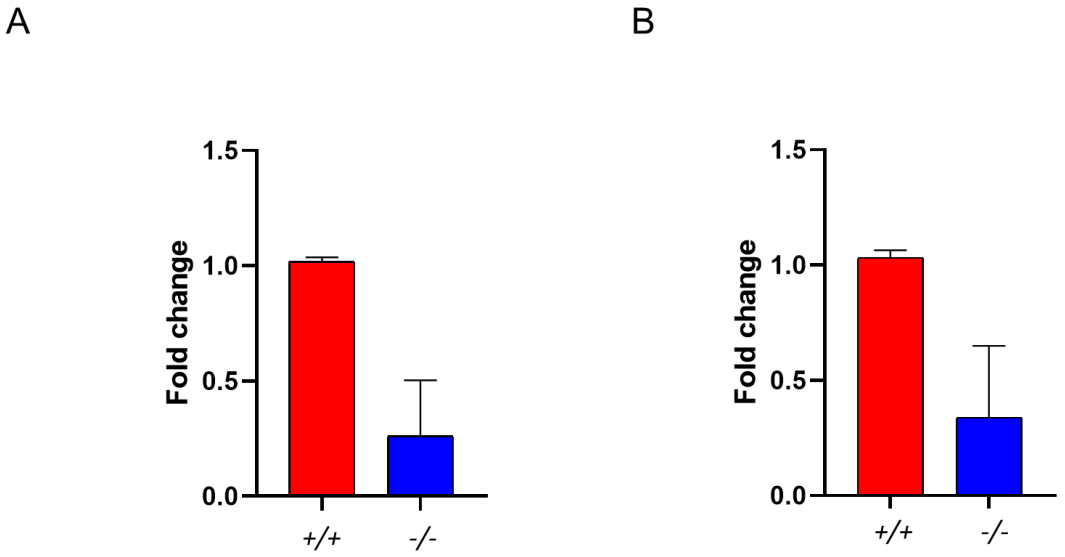
Acknowledgements

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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Chapter 4

Leptin is essential for insulin signaling during zebrafish embryogenesis

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Abstract

Leptin has been extensively studied in obesity and type 2 diabetes and has been shown to play a key role in whole-body energy homeostasis, however, its function in insulin signaling is still poorly understood. In this study, we show that leptin deficient zebrafish mutant embryos and larvae appear to be totally insulin resistant and show a diabetic phenotype at all stages of embryogenesis and larval development. This phenotype can be reversed by the injection of human leptin. Both metformin and the phosphatase inhibitor NSC-87877 are able to reverse this diabetic phenotype of the zebrafish larvae at the larval stage. In contrast to metformin, NSC-87877 was also active at early embryonic stages. Gene knockdown studies in the leptin mutant background indicate that the results are translatable to *Xenopus laevis* embryos and that the small non-receptor tyrosine phosphatase Ptpn6 is the most likely target responsible for the antidiabetic effect of NSC-87877.

Introduction

The metabolic syndrome is currently an increasing worldwide problem, affecting 425 million people of the world population (IDF Diabetes Atlas, 8th edn.). It includes type 2 diabetes mellitus (T2DM), which is increasing among the world population, reaching currently a pandemic form[1,2]. Current treatment methods of diabetes type 2 are still limited and fall in three categories, of which the insulin sensitizers such as metformin that remedy insulin resistance, the primary hallmark of diabetes 2, are the best studied and widely used[3]. Research on insulin resistance and type 2 diabetes has used human cell cultures and various animal models, mainly rodents. The mutant mice strains of the leptin and leptin receptor genes, the ob/ob, db/db strains, respectively have diabetic phenotypes and are therefore highly useful for diabetes research[4]. In addition, high fat diet mice, Zucker fatty rats and ZDF rats, have been widely used to study T2DM, obesity and the function of leptin signaling in metabolic syndrome[5,6].

Leptin is a cytokine produced mainly by mature adipocytes in white adipose tissue. In the brain it regulates food intake, appetite behavior and energy expenditure. Leptin mutations in rodents lead to hyper obesity and other abnormalities, which are described as main factors that influence development of diabetic symptoms[4]. The molecular mechanisms by which leptin controls insulin resistance in various target tissues are largely unknown[7]. Leptin's function is correlated with proteins tyrosine phosphatases that are key regulatory factors in many signal transduction pathways underlying vertebrate development[8]. Protein tyrosine phosphatase 1B (PTP1B) has emerged as a novel promising therapeutic target for the treatment of T2DM, as it plays an important role in the negative regulation of insulin signal transduction pathways[9]. Moreover, the expression of hypothalamic PTP1B is upregulated in leptin resistant animals[10]. It was recently shown that inhibition of low-molecular-weight

tyrosine phosphatase (LMPTP) in rodents results in attenuation of high-fat diet-induced diabetes[11].

In adult zebrafish leptin receptor and leptin genes have shown to have a conserved role in glucose homeostasis but, does not appear to play a role in adipose tissue homeostasis[12]. Zebrafish models have been proposed as alternative test systems for studying insulin resistance and T2DM that gives several opportunities to explore metabolic diseases, using numerous transgenic and knockout lines[13]. Although there are already established diabetic adult zebrafish models, which are based on a high fat feeding system[12,14], there is still lack of alternative early stages larval models, which provide the opportunity to perform fast and large scale screening assays, shortly after fertilization. As shown by Marin Juez *et al.* (2014)[15] zebrafish larvae are highly suited to study insulin resistance and are, therefore, a promising model system to study T2DM in a non-feeding situation. Marin Juez *et al.*, identified Shp-1 gene, also called *ptpn6*, in zebrafish larvae as a key factor in insulin resistance[15]. In this study we analyze the function of leptin and *ptpn6* in insulin resistance in zebrafish larvae. We have developed a novel high throughput method to test anti-diabetic drugs based on the fact that leptin deficient zebrafish larvae are totally insulin resistant and show as a result a diabetic phenotype already at very early stages of embryonic development. We show that metformin is highly effective for treating this diabetic phenotype in 4 days old zebrafish larvae and, using our high throughput test system, we have identified also the phosphatase inhibitor NSC-87877 as an alternative anti-diabetic drug that shows anti-diabetic effects at much earlier time points of development. Gene knockdown studies in the leptin mutant background indicate that Shp-1 is the most likely target responsible for the antidiabetic effect of NSC-8787.

Materials and methods

Zebrafish husbandry

Zebrafish lines were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (zfin.org). The breeding of adult fish was approved by the local animal welfare committee (DEC) of the University of Leiden (license number: 10612) and adhered to the international guidelines specified by the EU Animal Protection Directive 2010/63/EU. Adult zebrafish were not sacrificed for this study. All experiments in this study were performed on embryos/larvae before the free-feeding stage and did not fall under animal experimentation law according to the EU Animal Protection Directive 2010/63/EU.

Fish lines used in this work were the following: wild-type (WT) strain AB/TL, homozygous mutant (*lepb*$^{-/-}$) and WT siblings (*lepb*$^{+/+}$). Homozygous F1 carriers were outcrossed once against wild-type, 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 mutation, which can be detected using 2% agarose gel. Embryos were grown at 28.5 °C in egg water (60 µg/ml sea salt, Sera marin, Heinsberg, Germany). For live-imaging or injection assays, larvae were anesthetized in egg water medium containing 0.02% buffered Tricaine (3-aminobenzoic acid ethyl ester; Sigma-Aldrich, St Louis, MO, USA).

Insulin injection

1 nl of 100 nM human recombinant insulin (Sigma–Aldrich, the Netherlands) was injected into the caudal aorta of 4 days post fertilization (dpf) zebrafish larvae using a glass capillary as described in Juez *et al.*, 2014. 1 nl of PBS was used as a control injection.

Glucose treatment

Zebrafish larvae at 4 dpf were placed in 12 well plates (10 embryo per well) and immersed for two hours in 4 mL egg water, containing 250 mM of glucose (Sigma, USA, CAS. No. 50-99-7). After immersion first group was washed three times with egg water and collected for measurements, the rest were exposed to clean egg medium. Samples were taken after 120 min and after 240 min of washing period. As a control, larvae were exposed to mannitol (250 mM; Sigma, USA, CAS No. 69-65-8), instead of glucose, under the same conditions.

***In Vivo* Glucose Uptake Assay**

Lepb$^{+/+}$ and *lepb*$^{-/-}$ mutants were injected in the yolk with 2.5 mg/mL 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) at 4 hours post fertilization (hpf) and at 24 hpf, a fluorescent glucose analog (Life Technologies, Leiden, Netherlands), and incubated at 28.5 °C for 30-60 minutes. At the termination of the incubation period, seven embryos per condition from 1 day old group were anesthetized with 3-aminobenzoic acid ethyl ester methanesulfonate (Sigma-Aldrich), both groups were analyzed under a fluorescence stereomicroscope and a confocal microscope.

Metformin treatment

Lepb$^{+/+}$ and *lepb*$^{-/-}$ mutants, used for the ELISA assays, were treated with 10 µM metformin Cayman Chemicals, Ann Arbor, MI, USA) added to egg water containing DMSO from 3 dpf for 24 hours, as the control group, larvae were incubated only in water with DMSO. Embryos used for the fluorescent glucose assay received 10 µM metformin at 2 hpf under the egg chorion or into the yolk and the second dose together with fluorescent glucose injection at 24 hpf.

NSC-87877 treatment

Lepb$^{+/+}$ and *lepb*$^{-/-}$ mutants were incubated from 3dpf for 24 hours in egg water that contained NSC-87877 and DMSO, as the control group, larvae were incubated, during the same period of time, only in water with DMSO. Embryos used for the fluorescent glucose assay received 10 µM NSC-87877 at 2 hpf under the egg chorion or into the yolk and the second dose together with fluorescent glucose injection into the yolk at 24 hpf, 4 hpf and 6 hpf.

Glucose measurements

Quantitative analysis of glucose levels was performed from whole body lysates using a glucose assay kit (Cayman Chemical, Ann Arbor, MI, USA). Briefly, 7 zebrafish larvae in each experimental group 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).

Morpholino injections

For knockdown of particular genes, morpholino oligonucleotides (Gene Tools, LLC, Philomath, OR, USA) were injected into 1-cell zebrafish embryo. The morpholinos were diluted to a concentration of 0.5 mM in 1× Danieau's buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, and 5.0 mM HEPES (pH 7.6)) and 1 nL was injected using a Femtojet injector (Eppendorf, Hamburg, Germany). The *ptpn6* morpholino was used at a concentration of 0.08 mM as published previously by Juez *et al.*, 2014. Morpholino sequences are presented in Supplementary table 1.

CRISPR/Cas9 mutagenesis

Site-specific CRISPR-Cas9 sgRNAs (actatagGGGGTCTCGGGATTGGGTAGgttttag) were generated using the online software CHOPCHOP according to Montague *et al*., 2014[16]. *Lepb*$^{-/-}$ mutant fish were generated using CRISPR-mediated gene knockout approach and described previously[17]. As described in Suppl.fig.1 two different deletion mutants were generated. However, in most experiments, larvae with a heterozygote combination of the two mutations were used because the fact that there are two mutations was discovered later than the experiments were performed.

Frog husbandry and microinjection

All frog (*Xenopus laevis*) procedures and care were approved by the animal experiments committee (dierexperimentencommissie, DEC) of Leiden university. Frog embryos were collected by natural mating of wild-type females with males. For morpholino injection, morpholinos against the long-form and the short-form (Suppl.tabel 1) transcripts of leptin were mixed together. 30 ng of each was then injected into the blastomeres of the embryo at 2-cell or 4-cell stage. After injection, the embryos were cultured to stage 26 (staged according to Nieuwkoop and Faber) for glucose injection. For rescue experiments, 10 µM NSC-87877 was co-injected with the two morpholinos at 2-cell or 4-cell stage.

Images quantification

Bright-field images were obtained using Leica M165C stereomicroscope equipped with a DFC420C digital color camera (Leica Microsystems, Wetzlar, Germany). For fluorescent image acquisition, Leica MZ16FA stereo fluorescence microscope equipped with a DFC420C digital color camera (Leica Microsystems, Wetzlar, Germany) with GFP filter settings. For confocal laser scanning microscopy (CLSM) we used a Leica TCS SPE (Leica Microsystems, Wetzlar, Germany). For each larva a bright field image and a fluorescent channel image were obtained. In 24 hours post fertilization analyses of the glucose values first the overall shape of the larva was extracted from the bright field channel – this gives the total area. The yolk and yolk-extension were extracted from the fluorescent channel as these had the highest fluorescence, from this the surface areas for yolk and yolk-extension were established. The body of the larva could be found by excluding the area of yolk and yolk-extension and within the body the otic vesicle is taken as a boundary for the head. In this manner the body is divided in areas for each of which the surface area was computed, and which sum to the total surface. The area of the head was determined from the bright field image and was used as a mask in the fluorescent channel image to obtain the fluorescence for only the head area. This was expressed as a numerical density, that was the sum of the total fluorescence in the head area divided by the surface area of the head. The images and computations were corrected for a specific background fluorescence.

To estimate the fluorescence ratio between zygotic cell mass and yolk in early zebrafish embryos using wide field stereo microscopy, a square of 4 micrometer in center of these two parts of the embryos was quantified for fluorescence intensity. Ratio between the two blocks was measured using pixel counting software as described in Stoop *et al*. (2011)[18]. In *Xenopus laevis* embryos we compared the total fluorescence in the larvae to the fluorescence at the injection site that was arbitrarily defined as a square of 10 micrometer. To measure the fluorescence ratio between cell mass and yolk in early zebrafish embryos with CLSM we measured the total volume of these parts of the embryo and quantified the fluorescence in these parts using FIJI software.

Statistical analyses

Statistical differences were analyzed with Prism 6.0 (GraphPad Software, San Diego, CA, USA) using t-test for comparisons between two groups and one-way ANOVA (with Tukey's post hoc test correction) for multiple group comparisons and considered to be significant at $P<0.05$.

Results

General characterization of leptin b mutant zebrafish larvae

The zebrafish genome contains two leptin genes, *lepa* and *lepb*, and one leptin receptor gene[19] which have been previously studied by gene knock down and knock out studies[20,12]. Since pilot morpholino studies indicated a possible function of *lepb* in glucose transport of zebrafish larvae (Suppl.fig.2), the CRISPR/CAS9 gene editing tool was used to generate a *lepb* knock out zebrafish mutant line. The sgRNA was designed to target exon 2 of the *lepb* gene where the target site was located (Suppl.fig.1A). This resulted in two different mutations of 7 and b bp respectively, both predicted to lead to frameshift mutations (Suppl.fig.1A). Adult F0 fish from sgRNA injections were incrossed to obtain the F1 generation, where germline transmission of mutant alleles was confirmed by genotyping of its offspring. After outcrossing with the wild type line, and two incrosses we selected knock out mutant lines which were used for this research. Two groups of $lepb^{-/-}$ and $lepb^{+/+}$ larvae from the third generation were compared under normal embryo raising conditions to test for differences in unchallenged survival during development (data not shown). The $lepb^{-/-}$ mutants reached adulthood in a normal time span leading to adults with a normal fertility rate at 4 month post fertilization (data not shown).

Mutation of the *lepb* gene causes insulin resistance in the larval stage

To study how the $lepb^{-/-}$ zebrafish mutant responds to hyperinsulinemia, we injected insulin into the caudal aorta of a zebrafish larvae at 4 dpf. Glucose measurements were performed at 0, 30, and 240 minutes after the injection (Fig.1A). The results (Fig.1B) show a significant decrease in glucose level after insulin injection in wild type fish, whereas the glucose level rather increases after insulin administration in the mutant. Moreover, glucose basal levels at the first time point were much higher than in the wild type controls. These results indicate that the $lepb^{-/-}$ mutant is insulin resistant even prior to 4 dpf.

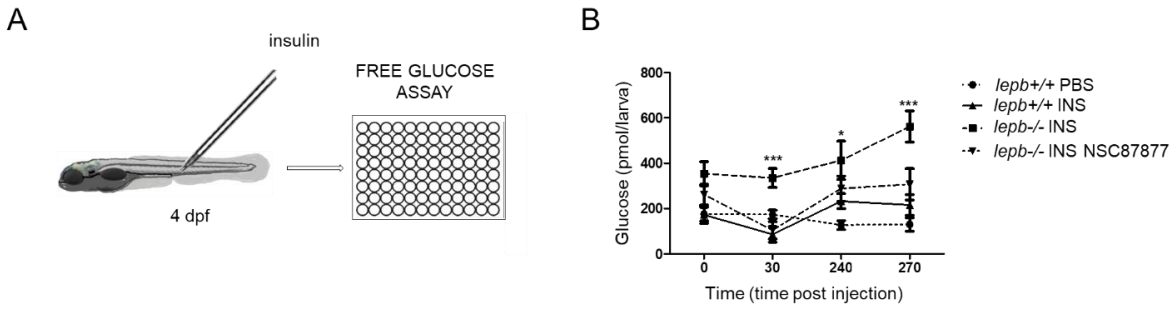


Figure 1. Insulin sensitivity and glucose levels of lepb mutant larvae at 4 dpf. (A) Insulin injection: Zebrafish larvae at 4 dpf receive 1nl of human recombinant insulin or PBS, into the caudal vein. The samples are collected at 0, 30 and 240 min post injections, to measure free insulin glucose level in the body, using ELISA Glucose Assay Kit. **(B)** Results of human recombinant insulin (INS) injection. Data, mean ± s.e.m, are combined from five biological replicates *P<0.05, ***P<0.001

We used the diabetic phenotype of our *lepb*$^{-/-}$ mutant to establish a method that corresponds to Oral Glucose Tolerance Test (OGTT) used in mice[21], to rapidly analyze glucose metabolism without injection procedures and stress-inducing anesthetic treatment. In this method, similar to the glucose tolerance test applied in mice, 4 dpf zebrafish larvae were immersed in egg water containing a 250 mM glucose concentration for two hours. Afterwards the larvae were incubated for 4 hours in glucose-free medium. The non-metabolizable compound mannitol was used as a control for osmotic effects. Samples were taken at 0, 120 and 240 minutes after washing by immediate homogenization of whole larvae in the buffer (Fig.2A). The results of the glucose measurement showed that in the control group, free glucose concentrations reached the basal level after 240 minutes post washing. In contrast, glucose levels remained at very high levels in the *lepb*$^{-/-}$ mutant after the washing step. These results show that the rapid glucose bathing method is highly efficient to demonstrate the diabetic characteristics of fish larvae at 4 days post fertilization (Fig.2B).

In order to further study glucose metabolism in the *lepb*$^{-/-}$ larvae, we used a previously published method based on the injection of 2-NBDG, a fluorescently labeled glucose analog in the yolk at 24 hours post fertilization embryos[22] (Fig. 3A). In agreement with this publication, we observed that in wild type larvae the fluorescent glucose is rapidly transported into the tissues of the embryo, with the brain as the most prominent destination (Fig. 3B). In contrast, in *lepb*$^{-/-}$ mutants there is no observable glucose uptake from injected yolk, where all the injected glucose remains.

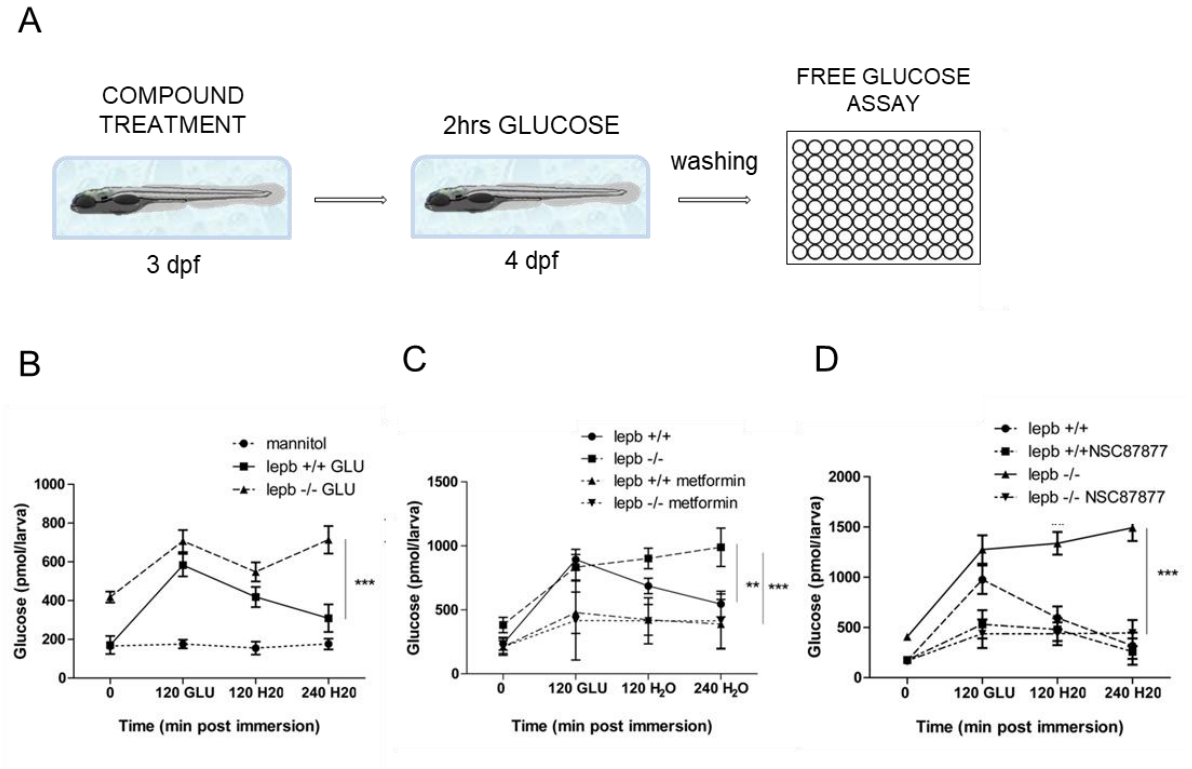
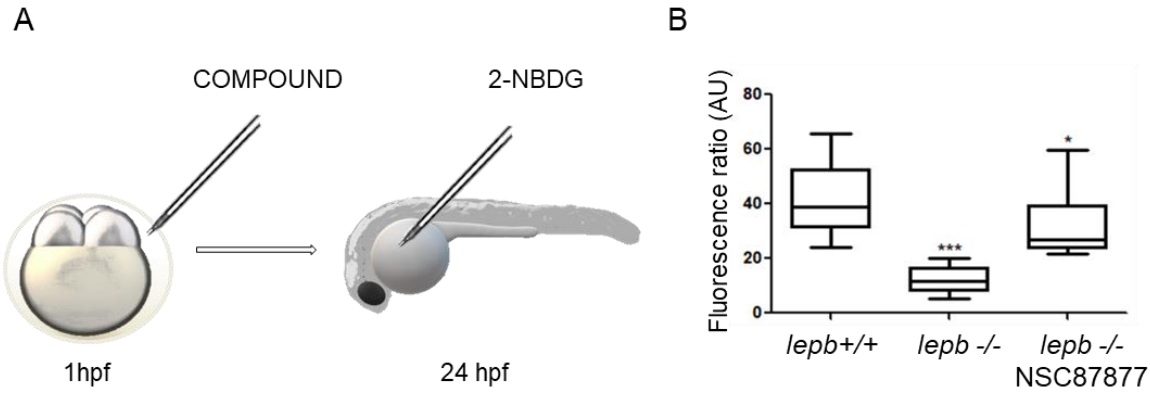


Figure 2. Glucose immersion. (A) Zebrafish larvae at 4 dpf are immersed in a medium containing 250 mM of glucose or mannitol. The samples are collected after 0 min,120 min of glucose immersion and 120 min and 240 min of washing in clean egg water, to measure free glucose level in the body, using ELISA Glucose Assay Kit. It is an alternative and less invasive method of testing insulin resistance in zebrafish larvae. **(B)** Glucose levels were determined after immersion in glucose using the ELISA method from Fig.7A **(C)** The effect of metformin on glucose levels **(D)** The effect of NSC-87877 on glucose levels. Data, mean ± s.e.m, are combined from five biological replicates (n=10 larvae/group), significance was measure between *lepb*$^{+/+}$ and *lepb*$^{-/-}$ groups unless indicated differently. **P<0.01, ***P<0.001

In conclusion, glucose transport in the *lepb*$^{-/-}$ mutant is completely blocked already at 24 hours post fertilization (Fig.3B).

Drug treatments of the *lepb*$^{-/-}$ mutant.

We have used our developed methods for measuring glucose uptake to test the effect of the antidiabetic drug metformin. Metformin was added to control and *lepb*$^{-/-}$ fish at 3 dpf and the glucose bathing assay was performed 24 hours later (Fig.2A). The results show that metformin at a concentration of 10 µM was highly effective in reverting the *lepb*$^{-/-}$ diabetic phenotype to the wild-type phenotype (Fig.2C). However, using the fluorescent glucose injection method we observed only a marginal effect of metformin at 24 hpf (Suppl.fig.3).



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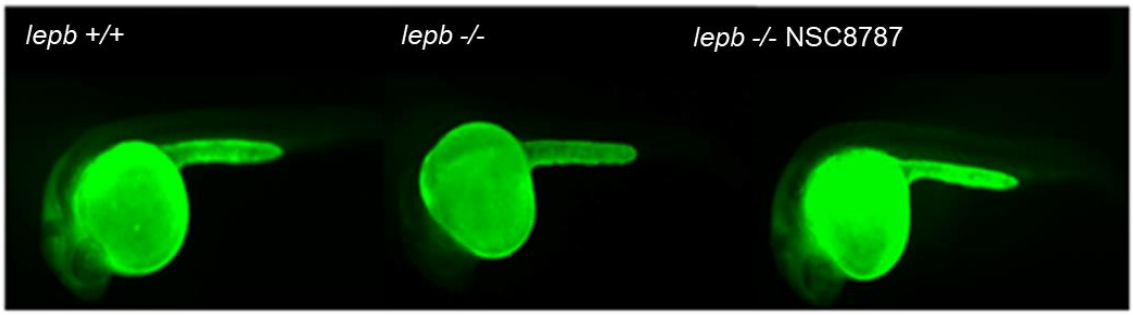


Figure 3. Quantification of glucose distribution in the brain after NSC-87877 treatment. (A) Injection of 2-NBDG in 24 hours embryos after NSC-87877 treatment **(B)** Glucose transport to the brain is decreased in *lepb*$^{-/-}$ mutant (**C)** Images were obtained by stereo fluorescence microscopy unless indicated otherwise. Data, mean ± s.e.m., are combined from three biological replicates (n=10 larvae/group). *P<0.05, ***P<0.001

We also tested other putative anti-diabetic drugs based on the published role of small non-receptor tyrosine phosphatases in insulin resistance[23]. One of the tested compounds, NSC-87877 has been published to inhibit Shp-2 and Shp-1[24,26] and also other targets such as the dual specificity phosphatase DUSP26[25,26]. NSC-87877 showed to be able to completely revert the diabetic phenotype of the *lepb*$^{-/-}$ at 4 dpf (Fig.2D). NSC-87877 was also able to significantly revert the glucose uptake deficiency at 24 hpf in the fluorescent glucose assay (Fig.3B,C). Moreover, we tested the effect of NSC-87877 on free glucose levels after insulin injection. The data show that NSC-87877 treatment of the *lepb*$^{-/-}$ mutant restores insulin sensitivity (Fig.1B).

***Lepb* controls glucose transport and insulin resistance during the early embryonic stages.**

Motivated by our results in the early larval stage we tested the function of the *lepb* gene during early embryonic stages. We found that knockout of *lepb* completely inhibited glucose transport between yolk and the developing zygotic cells even at very early stages, namely after 4 and 6 hpf. However, at earlier stages than the 64 cell stage, glucose transport was not influenced by the *lepb* mutation (data not shown). Moreover, we found that NSC-87877, injected under the chorion or in the yolk (Fig.4A), partially reversed glucose transport

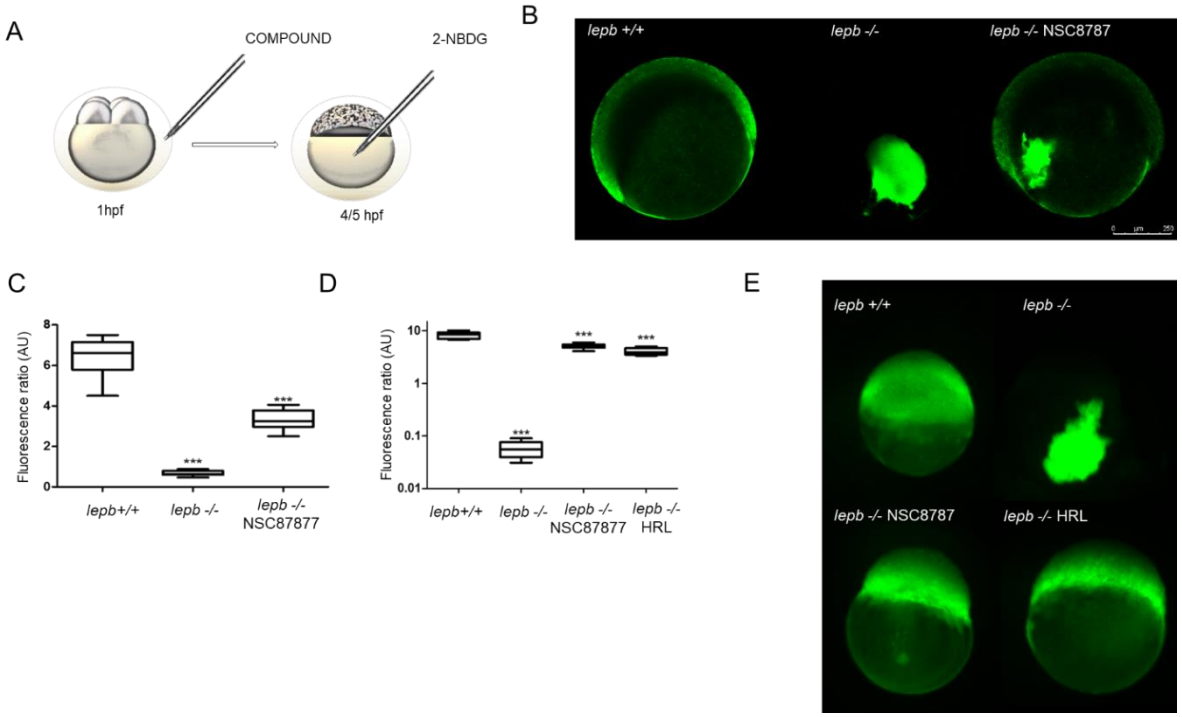


Figure 4. Early glucose injections. (A) Fluorescent glucose injection at 4-6 hpf: Drugs have been injected into the yolk of zebrafish embryos at early stages of embryogenesis (1 hpf). In the case of the 6 hpf analyses we made use of CLSM to distinguish the zygotic cells from the yolk. At 4 hpf stereo fluorescence microscopy was sufficient to discern the yolk from the zygotic cell mass. **(B, D)** Early stages (6 hpf) 2-NBDG injections according to method A. NSC-87877 was injected through the chorion or into the yolk sac at 1 hpf. Use was made of CLSM to discern the yolk form the zygotic cell mass. **(C, E)** Early stages (4 hpf) 2-NBDG injections according to method A. NSC-87877 or recombinant human leptin protein (HRL) was injected into the yolk sac at 2 hpf. Data, mean ± s.e.m., are combined from three biological replicates (n=10 larvae/group). ***P<0.001

inhibition at 4 and 8 hpf (Fig.4B,C). Interestingly, recombinant human leptin was also able to rescue glucose transport in the mutant (Fig.4D,E). In order to test whether the defect in glucose transport in early embryogenesis was related to insulin resistance we developed an assay for testing the effect of insulin at 4 hpf. This assay is based on the injection in the yolk of 1 nl glucose solution of 200 mg/ml in the yolk in the presence of the standard concentration of 2-NBDG (Fig.5A). In wild type embryos transport of the fluorescence glucose derivative is no longer observed due to competition with unlabeled glucose.

The apparent limitation of the glucose transport capacity at this glucose concentration could be overcome by the co-injection of human recombinant insulin showing the sensitivity of early embryos to insulin. In contrast glucose transport in the *lepb*$^{-/-}$ mutant was not significantly affected by injection of insulin, indicating insulin resistance (Fig.5B,C).

Gene knockdown studies for leptin signaling pathway analysis and translational studies in *Xenopus laevis*

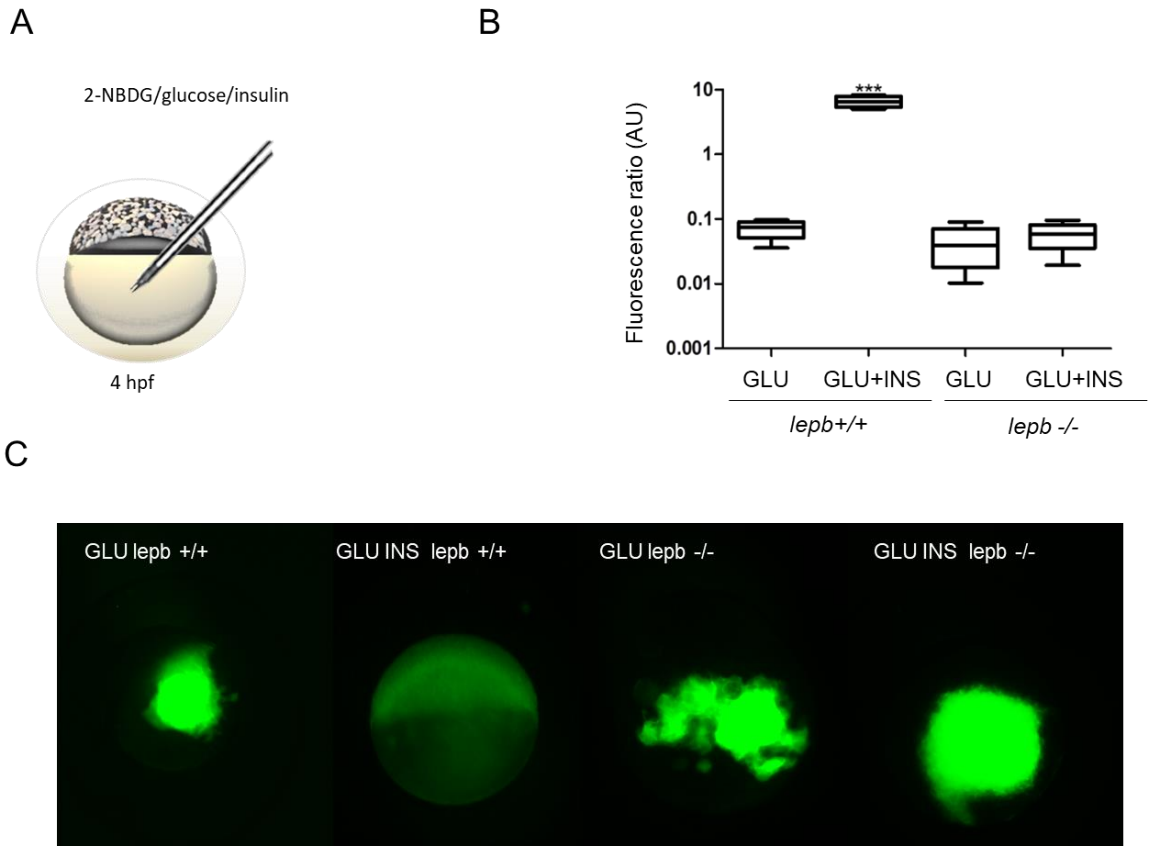


Figure 5. The effect of injection of human recombinant insulin into the yolk of 4 hpf zebrafish embryos. (A, B, C) Injections with 1 nl of glucose (200 mg/ml) in the presence of 2-NBDG results in accumulation of fluorescence in the yolk of 4 hpf embryos. 100 nM human recombinant insulin (INS) was co-injected with the 2-NBDG. Data, mean ± s.e.m) are combined from three biological replicates (n=10 larvae/group). ***P<0.001

We used morpholino anti sense technology to further study the signal transduction pathways underlying the identified function of leptin in early embryogenesis (Fig.6A). Firstly, we showed that the phenotype of knockdown of the *lepb* gene is glucose transport in early embryogenesis is also observed after injection of morpholino's against *lepb* (Fig.6B,C and Suppl.fig.2). Subsequently, we also tested morpholino's against the leptin receptor (*lepr*) and the second leptin gene (*lepa*). The results showed that *lepr* phenocopied accurately the effect of the *lepb* morpholino treatment. In contrast, *lepa* showed no significant effect on glucose transport in early embryogenesis (Fig.6B,C and Suppl.fig.2). These results indicated that a *lepb-lepr* signaling pathway was functionally similar to the function of leptin in humans. In order to get an indication on the target of NSC-87877, that was responsible for rescuing the *lepb* phenotype, we tested morpholino's against the most likely targets of this inhibitor[25,26]. Using the fluorescent glucose assay, we could demonstrate that knock down of the *ptpn6* gene completely reverted the *lepb*$^{-/-}$ mutant to the wild-type phenotype (Fig.6D,E). In contrast, knock down of other possible targets of NSC-87877, the closely related phosphatases *ptpn11a* (Shp-2a) and *ptpn11b* (Shp-2b), or the dual specificity phosphatase DUSP26 did not restore glucose transport (Suppl.fig.2B,2C). This indicates that *ptpn6* is the likely target of NSC-87877 responsible for the reversion of the *lepb*$^{-/-}$ phenotype. We used *Xenopus laevis* that has been used classically for embryogenic studies to show that the function of *lepb* in glucose transport is also relevant in embryos of other vertebrate organisms. Two morpholino's against the two leptin genes of *X. laevis* were designed and

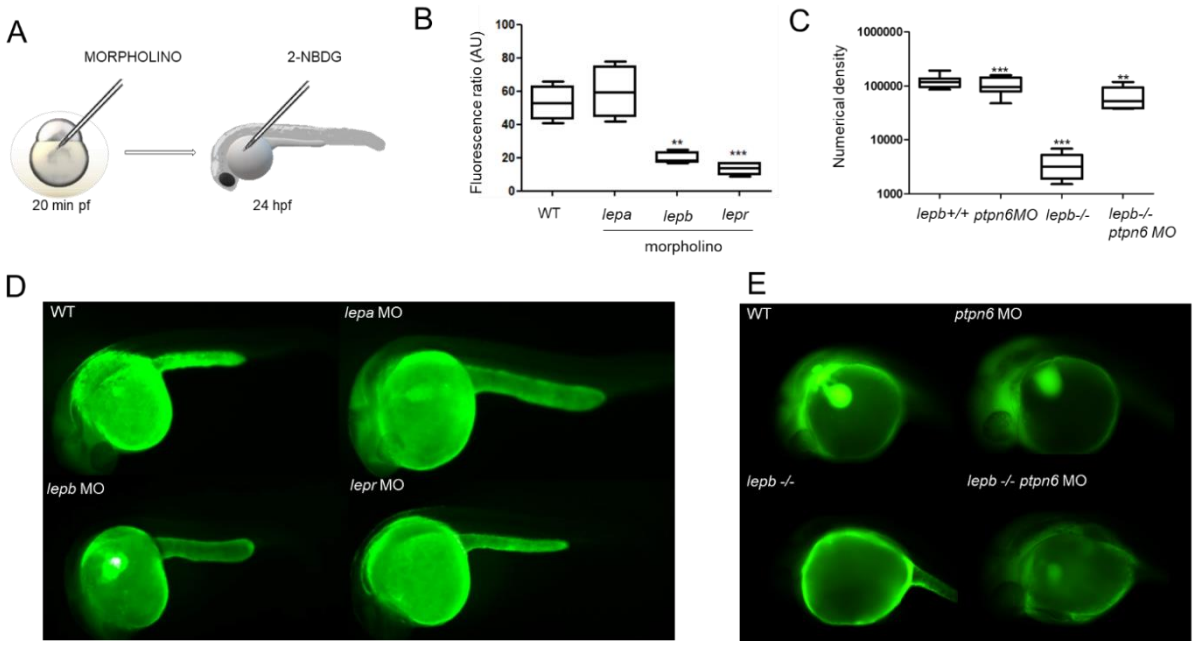


Figure 6. Late gene knockdown studies in zebrafish embryos. (**A**) Fluorescent glucose injection at 24 hpf: Gene expression was abolished in one cell stadium and at 24 hpf the embryos were injected into the yolk with fluorescent labelled glucose. One hour after injections, the accumulation of glucose in the brain of lepb$^{+/+}$ and lepb$^{-/-}$ zebrafish larvae can be observed with stereo fluorescence microscopy and has been quantified for the brain area using a custom written script **(B,D)** Knockdown of gene expression with morpholino's against *lepb, lepa* and *lepr* and imaging of glucose distribution at 24 hpf using method **(C,E**) Rescue of the *lepb* glucose transport deficiency by *ptpn6* morpholino at 24 hpf. Data, mean ± s.e.m., are combined from three biological replicates (n=10 larvae/group). **P<0.01, ***P<0.001

tested simultaneously as described in the material and methods (Fig.7A). Leptin knockdown results in inhibition of glucose transport after 24 hpf. Showing that lack of leptin expression in *X. laevis* leads to a similar glucose transport inhibition as in the zebrafish larvae. Moreover, morphant larvae show developmental abnormalities comparing to the control group. Importantly, injections with NSC-87877 together with morpholino not only rescue the developmental phenotype, but also glucose transport in the morphants (Fig.7B,C). These results clearly show that leptin plays a crucial role in glucose transport during early embryogenesis also in *X. laevis*.

Discussion

We show that a *lepb*$^{-/-}$ mutant zebrafish line is diabetic during larval development. This diabetic phenotype is characterized by insulin resistance and subsequent inhibition of glucose uptake at both systemic level and peripheral organs such as the brain. We also demonstrate that leptin b is essential for transport of glucose in the early stages of embryogenesis. Gene knockdown studies show that the leptin receptor is equally important to *lepb* but that *lepa* doesn't seem to play an important function. Knockdown of the *lepb* gene could be rescued by injection of human recombinant leptin even though this protein has only 18 percent of identity with the zebrafish leptin protein, showing the relevance of the results in our zebrafish test system for the function of mammalian leptin. In order to show that our findings are indeed relevant for the function of leptin in embryogenesis of other vertebrates models we tested the function of leptin in *Xenopus laevis* which is one of the few other vertebrate models

in which embryos can be easily handled. The results show that the two *X. laevis* leptins have a function in glucose transport during embryogenesis. Since the *X. laevis* leptins are distantly related in sequence to the zebrafish leptins, this indicates that the function of leptin in glucose transport is translatable to all vertebrates. The essential function of leptin in glucose transport during embryogenesis is surprising since in rodent models leptin is supposed to have a complex function in insulin resistance that involves systemic signaling via the blood stream[4], whereas at 4 hpf an organ system has not yet developed. This function of leptin b indicates a role of insulin receptors at the very early stages of embryogenesis that is confirmed by the effects of human recombinant insulin injected into the yolk sac. In previous work one of the zebrafish insulin receptors (*Insrb*) was reported to be expressed at 18 somite stage and both insulin receptors were maternally expressed in fertilized eggs[28]. In addition, two insulins have been described to be expressed during early zebrafish development. Of these two genes, *Insb*, was shown to be expressed at proliferating blastomeres at 3 and 4 hpf[29]. However, there is no knowledge which glucose transporters could be involved in glucose transport during embryogenesis. Considering the fact that glucose transport up to 64 cells stage was not dependent on leptin shows that such transporters and their control by leptin develops after the syncytial stage of embryogenesis. Our results suggested that also in adults, leptin was directly involved in glucose homeostasis, in line with the study of Michel *et al*, (2016)[12] who reported a diabetic phenotype in *lepr* knockdown zebrafish.

This supports the potential of the leptin signaling pathway for therapeutic purposes as reviewed by Coppari and Bjorbaek (2012)[30]. Although leptin has mainly been reported to be produced by adipocytes, and few other tissues such as the intestinal epithelium mainly during inflammatory conditions[31,32] there is also evidence that leptin is produced by human skeletal muscle in adults[33]. In a recent publication by Kang *et al.* (2016)[34] enhancer elements controlling *lepb* expression in zebrafish were shown to be triggered in injured tissues. Considering that these enhancer elements were also functional in mice tissue during wounding suggests a conserved function of leptin in wound repair. These and many other results indicate that in adults the function of leptin is much broader than the canonical adipocyte-brain axis[35, 36,37,38]. It has also been shown that in human placenta that there is an abundant production of the leptin protein[39]. Considering that leptin in mammalian cells can be transported by transcytosis to neighboring cells or tissues[40,41], it is an interesting idea that in mammalian embryogenesis placental leptin plays a role in glucose transport in the zygote.

In analogy with adult mammalian diabetes studies, the diabetic phenotype of zebrafish larvae can be reverted by external treatment with metformin at 3 days post fertilization. However, such treatment with metformin was not effective at earlier stages of development. The phosphatase inhibitor NSC-87877 can also revert the diabetic phenotype of the *lepb*$^{-/-}$ mutant at 3 dpf and even at much earlier stages of development. The fact that metformin was not active at earlier larval stages using fluorescent glucose uptake studies can be explained in several ways. A likely explanation is that there is no uptake of metformin through the skin and

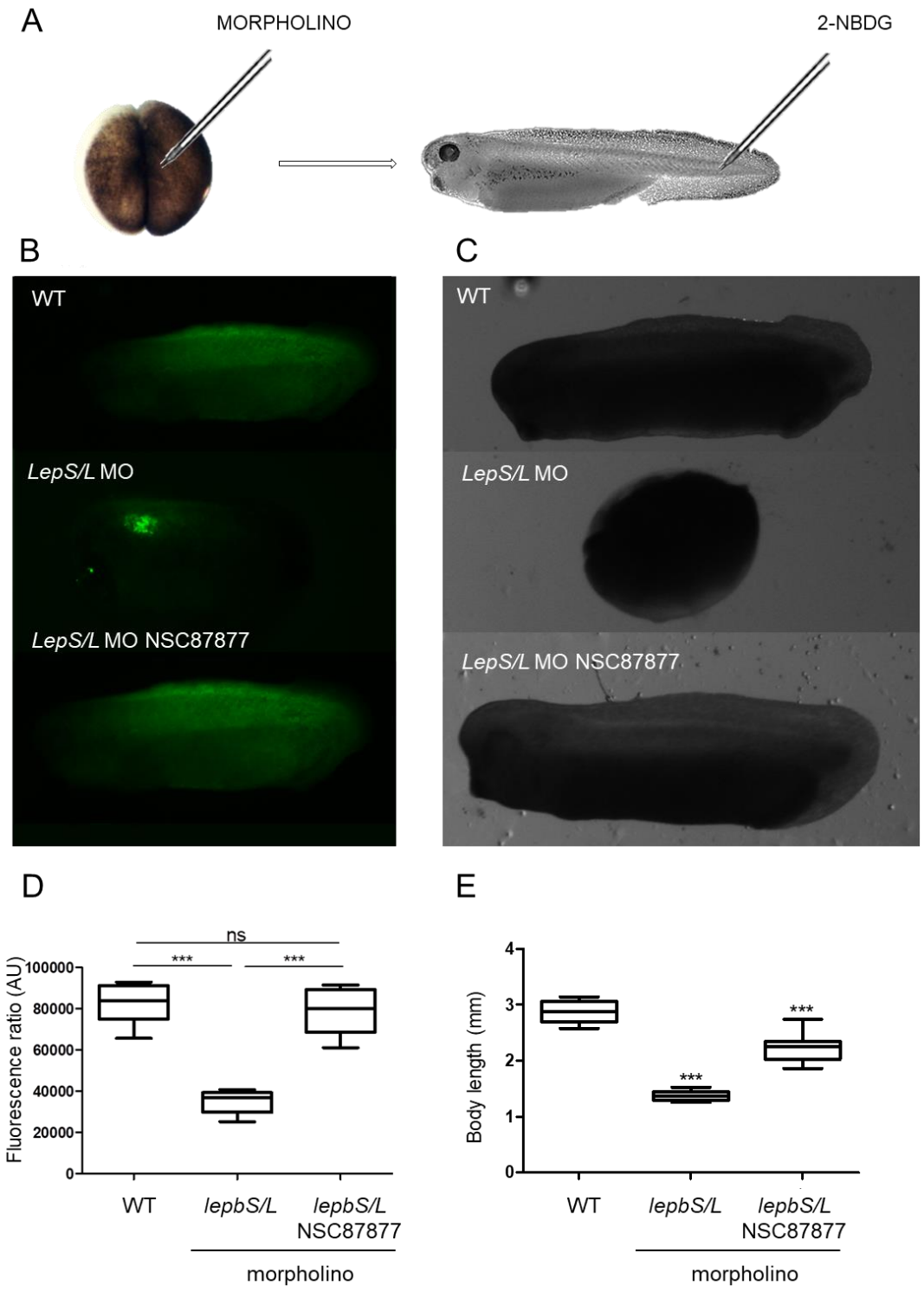


Figure 7. Gene knockdown studies in *Xenopus laevis* embryos. (**A**) Knock down of both the leptin S and leptin L genes of *Xenopus laevis* embryos. (**B, C**) 2-NBDG was injected at 48 hpf distribution was quantified using a custom script. Data, mean ± s.e.m., are combined from three biological replicates (n=10 larvae/group). ns = not significant, ***P<0.001

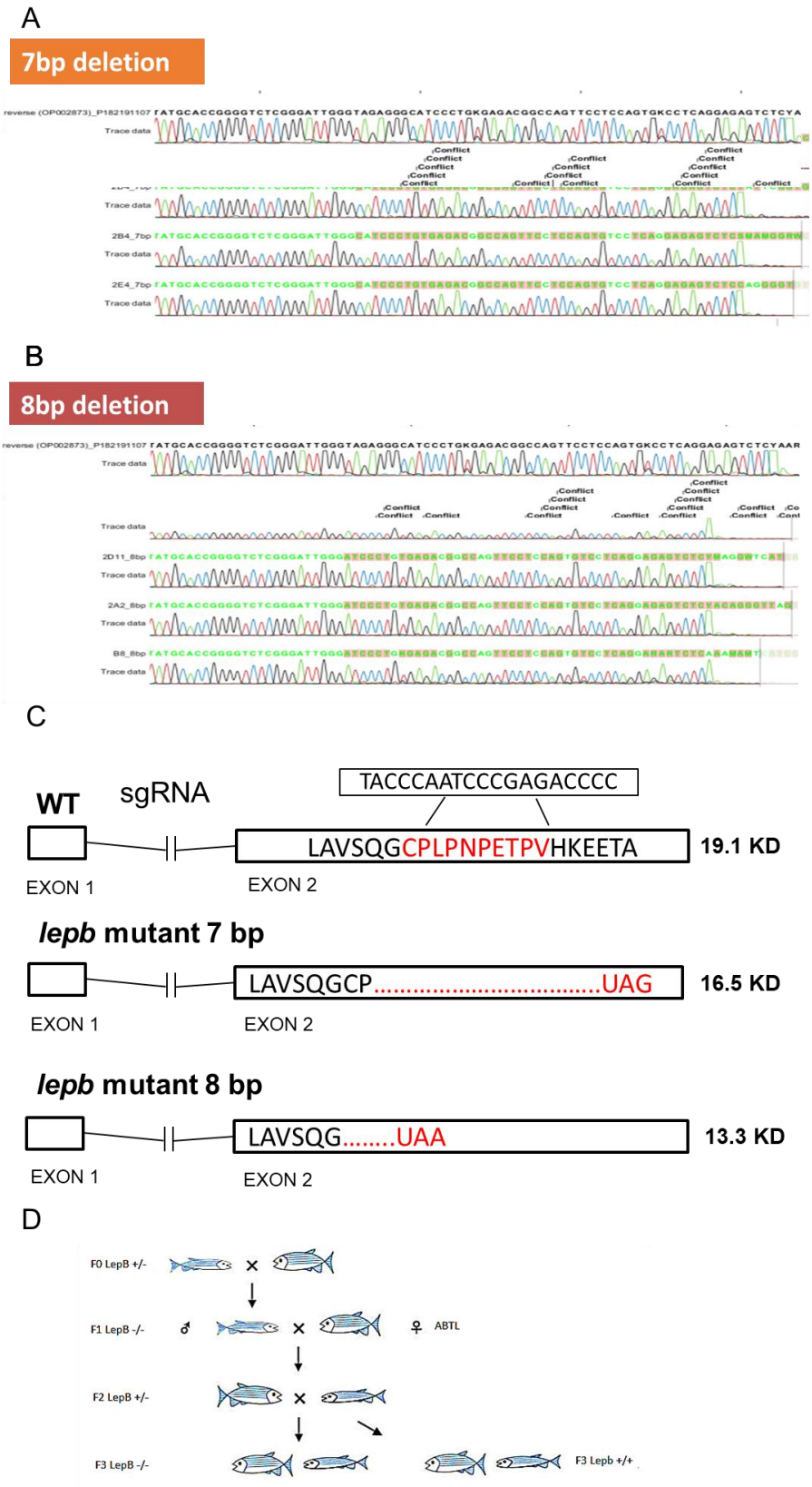
the observed effect a 4 dpf is through oral uptake when the mouth of the larvae has opened possibly because it might only function via ingestion in the intestinal track as observed in mammals[42]. In this respect NSC-87877 that is active after external treatment at 24 hpf, apparently is taken up through the skin and therefore could have applications in non-oral dosing systems.

Based on the reversal of the *lepb*$^{-/-}$ mutant by gene knockdown of *ptpn6*, the product of this gene is the most likely target of NSC-87877 underlying the anti-diabetic effect. Knockdown of other possible targets of NSC-87877, *ptpn11a* and *ptpn11b* and *dusp26* could not rescue the *lepb*$^{-/-}$ phenotype. *In vitro* inhibition studies showed that NSC-87877 has a similar inhibitory effect on truncated human PTPN6 and PTPN11 proteins at a five time higher IC50 than PTP1B[27]. It was later shown that NSC-87877 has a more potent inhibitory effect on the dual phosphatase DUSP26 than on full length human PTPN6 protein[26]. The expression pattern of *dusp26* that is restricted to neuroendocrine tissues in zebrafish larvae[43] supports our negative results in the rescue assay.

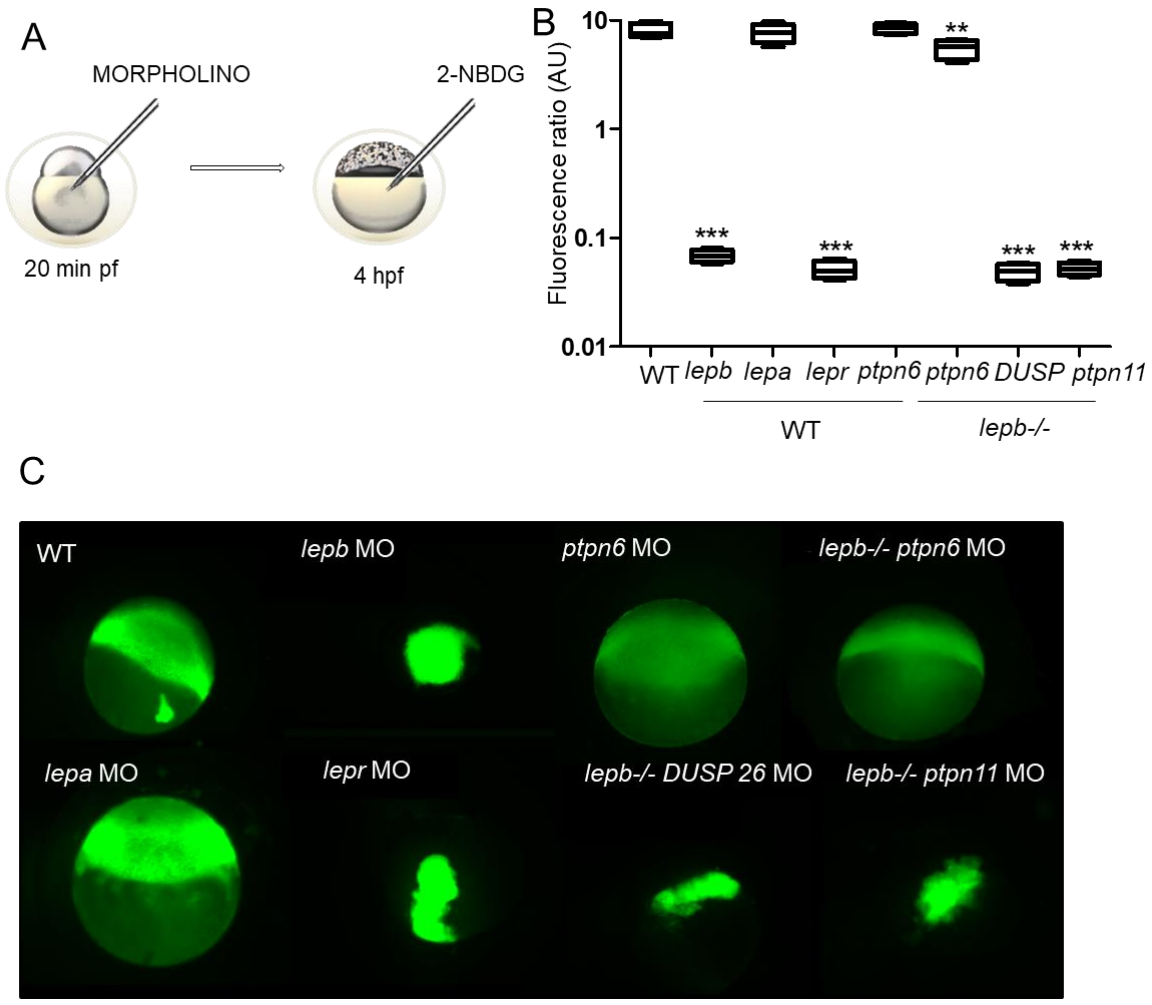
The direct effect of metformin and NSC-87877 in reversal of insulin resistance caused by a genetic defect shows the powerful action of these drugs at the level of insulin resistance. An effect of metformin on restoration of zebrafish larval beta cell development in a a *lepr* mutant was previously demonstrated by Michel *et al.* (2016)[12]. The drug metformin is already used for many years as first choice anti-diabetic drugs[44], however, its targets are multiple and therefore its function is still poorly understood[45,46]. In particular, the effect of metformin on insulin resistance, the hallmark of T2DM, needs further study[47,48].

The signaling pathway of insulin is highly conserved within all vertebrates. The positive effect of metformin in the zebrafish larval system shows that the results of mammalian diabetic test systems can be translated to lower vertebrate test systems. Considering that for treatment of T2DM it might be useful to overcome the very basic effect of insulin resistance indicates that our high throughput zebrafish model will be useful to identify new potentially antidiabetic drugs that can be further tested for their potential in mammalian studies.

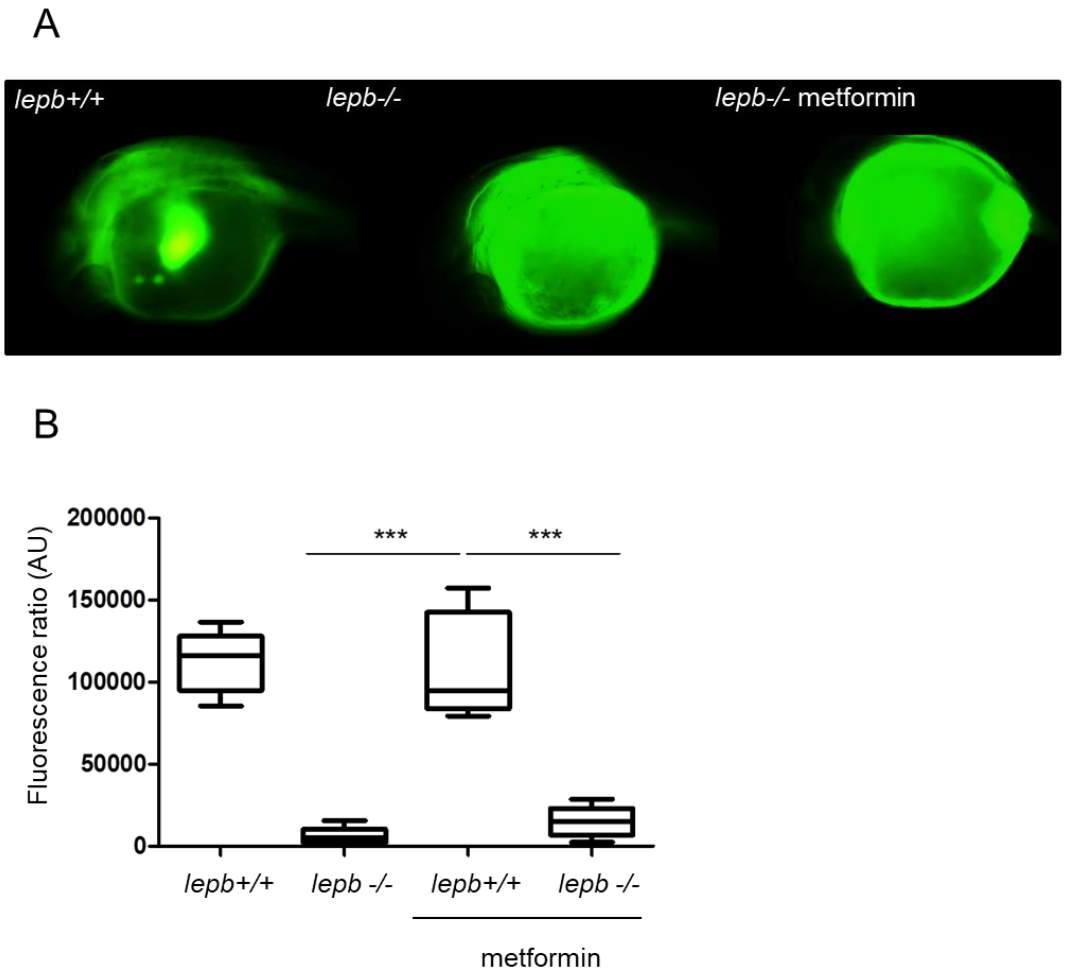
Supplementary materials



Supplementary Figure 1. An overview of the zebrafish leptin mutant. (A,B,C,D) We have found two leptin mutations 7bp and 8bp, which have the same phenotype and function.



Supplementary Figure. 2. Early gene knockdown studies in zebrafish embryos. (**A,B,C**) Knockdown of gene expression with morpholino's against *lepb, lepa*, *lepr, ptpn6* in lepb$^{+/+}$ embryos, as well as *ptpn6, DUSP 26* and *ptpn11*, in the lepb$^{-/-}$ embryos and imaging of glucose distribution at 4 hpf using method from Fig.2A. Data, mean ± s.e.m., are combined from three biological replicates (n=10 larvae/group). **P<0.01, ***P<0.001



Supplemental Figure 3. Effect of metformin. (**A**) We observe only a marginal effect of metformin in the *lepb*$^{-/-}$ larvae at 24 hpf. (**B**) After quantification of the fluorescent signal in the brain, there is no significant difference between *lepb* and metformin treated embryos. Cell to yolk fluorescent ratio, based on the confocal pictures, shows the difference in glucose uptake from the yolk between the three groups. Data, mean ± s.e.m., are combined from three biological replicates (n=10 larvae/group). ***P<0.001

Supplementary Table 1. List of morpholinos

Name	Sequence
lepb	5′-TTTTTTGCTTTGTTAATATCATCCCT-3′
lepa	5′-TTGAGCGGAGAGCTGGAAA-3′
lepr	5′-TCAAGACAGACATCATTTCACTTGC-3′
ptpn6	5′-ACTCATTCCTTACCCGATGCGGAGC-3′
DUSP26	5′-GAGACAATCTGGACATAAACGCCAT-3′
ptpn11 a and b	5′-GGTGGAACCACCTTCGGGATGTCAT-3′
control morpholino	5′- CCTCTTACCTCAGTTACAATTTATA-3′

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Chapter 5

Glucocorticoid modulation of metabolic changes induced by mycobacterial infection

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Herman P. Spaink, Marcel J.M. Schaaf

Abstract

People suffering from tuberculosis develop a state which is called wasting syndrome or cachexia and is characterized by dramatic weight loss and malnutrition. Glucocorticoid treatment may reduce the symptoms of wasting syndrome by inhibiting inflammatory signaling, but the metabolic effects of glucocorticoids in tuberculosis patients are still unclear. In the present study, we have investigated the effect of glucocorticoid treatment in a zebrafish model for tuberculosis, in which zebrafish larvae were infected with *Mycobacterium marinum*, a close relative of *M. tuberculosis*. Our results show that treatment with the glucocorticoid beclomethasone resulted in a higher bacterial burden and a slightly higher mortality, but that the infection-induced metabolic changes were attenuated. The increase in glucose levels was abolished and the decrease in muscle mass reduced by the beclomethasone treatment. Transcriptome analysis by RNA-sequencing was performed to study the molecular mechanisms underlying these changes, and the results of this analysis suggest that genes involved in glucose metabolism, insulin and leptin signaling play a crucial role in the observed metabolic changes resembling cachexia. Furthermore, these data demonstrated that during the mycobacterial infection the larvae are in a state of reduced sensitivity to glucocorticoids. Taken together, our data show that zebrafish larvae represent an interesting model system to investigate cachexia in human TB patients, which has provided novel insights into the molecular mechanisms underlying wasting syndrome and the possibilities for adjunctive glucocorticoid therapy to alleviate this metabolic state.

Introduction

Between a quarter and a third of the world's population, especially in low- and middle-income countries, is infected with the causative agent of tuberculosis (TB), *Mycobacterium tuberculosis*[1]. Most of these people develop a latent infection and do not show any symptoms[2,3], but approximately 5-10% of them develop clinically active TB[3,4]. Among those TB patients, the majority manifest a lung infection and around 20% show infection in other organs[3,5]. Currently, the standard treatment of TB consists of multiple antibiotic drugs, but for many years the bacterium has been developing resistance against mostly used antibiotics, and this resistance has made TB a major threat to global public health[6].

Patients with TB display a malnutrition state with significantly lower body weight, resulting from a depletion of both lean and fat tissue[7]. This process is called the wasting syndrome or cachexia and is a cardinal feature of TB. The dramatic weight loss and malnutrition have further consequences, including fatigue, delayed ambulation, higher risk for pulmonary embolism and secondary pulmonary infections, and represent the primary causes of death in TB patients[8,9]. A complex of different factors has been found to underlie this wasting syndrome. TB patients display a decreased appetite, increased glucose, fat and protein catabolism and a reduction in anabolic processes like protein synthesis[8]. Additionally, the disease has been associated with the development of impaired glucose tolerance and, as a

result, some patients can develop type 2 diabetes mellitus (T2DM) during the course of the infection[10,11]. Interestingly, both the weight loss and the altered glucose metabolism recover after successful TB treatment, although full recovery may not occur or take longer than the drug treatment[11,12,13]. The molecular mechanisms that cause these metabolic alterations have not been fully elucidated. In analogy with cachexia observed in other diseases, e.g. cancer, pro-inflammatory cytokines like TNF-α and IL-6 are often considered as important drivers of wasting in TB, possibly by increasing leptin levels[14]. However, in several studies the circulating TNF-α levels did not significantly correlate with body weight in TB patients[15,16,17], and leptin levels have been shown to be increased in TB patients in some studies[14,17,18], but decreased in others[19,20,21].

Glucocorticoids are a class of steroid hormones, which are mainly produced in response to stress. The main endogenous glucocorticoid in humans and fish is cortisol. After activation of the glucocorticoid receptor (GR), glucocorticoids suppress the immune response[22]. Synthetic glucocorticoids like prednisolone and beclomethasone are commonly used clinically as anti-inflammatory drugs in the treatment of many chronic immune-related diseases[23]. It has been shown that adjunctive glucocorticoid therapy improves the survival in subgroups of TB patients, suffering from tuberculous meningitis and pericarditis[24,25,26,27]. In pulmonary TB adjunctive glucocorticoid therapy is used to prevent complications, but the effect on survival is controversial[28]. The exact mechanism of action underlying these effects of glucocorticoids is unclear, which is particularly true for the effect of glucocorticoid therapy on cachexia. Since glucocorticoids have been shown to suppress TNF-α production in in hyper-inflammatory infection[29], it may be hypothesized that glucocorticoids reduce symptoms of wasting syndrome through inhibition of inflammatory signaling. However, in the absence of an infection, glucocorticoids are known to induce symptoms of the wasting syndrome rather than suppress them. They have been shown to induce an increase in blood glucose level, which can lead to diabetes mellitus[30], and are known to be important mediators of muscle wasting. Upregulation of forkhead box protein O1 (FOXO1) and deregulation of (myoblast determination protein 1) MyoD are the main mechanisms of glucocorticoid-induced muscle wasting[31]. Another process that is involved in the glucocorticoid-induced muscle atrophy is insulin resistance and increased calcium levels that enhance protein degradation[32]. Taken together, previous studies have shown that glucocorticoids regulate both the immune response and induce metabolic changes, but that little is known about their mechanism of action during TB and their impact on metabolism during the infection. In the present study, we have investigated the effect of glucocorticoids on the metabolic changes seen during TB infection.

As a model system to study effect of glucocorticoids, we have used the zebrafish (*Danio rerio*). The zebrafish is a powerful research model for studies on various metabolic diseases, such as diabetes and other metabolism-related diseases[33,34]. In addition, zebrafish larvae represent a versatile model to study mycobacterial infection[35]. In zebrafish, *Mycobacterium marinum,* a

close relative of *M. tuberculosis*, is able to cause a systemic and chronic infection, with containment of bacteria in granulomas that show strong structural similarity to those caused by *M. tuberculosis* in humans[29,36]. Since the progression of this infection shows strong similarities with human TB, it is an interesting model to study the metabolic changes associated with this mycobacterial infection. The zebrafish model is also used to study the molecular mechanisms of glucocorticoid action. Zebrafish express a Gr with high structural and functional similarity to its human equivalent[37], and this zebrafish Gr has also been shown to modulate metabolism and the immune system in a similar way to the human GR[38,39]. In recent studies, we have used the zebrafish model to investigate the inhibitory effect of glucocorticoids on the inflammatory response, in particular their effect on macrophages[39,40].

Our focus in the present study was on the effect of glucocorticoid treatment on the metabolic changes induced by *M. marinum* infection in zebrafish larvae. Our results show that treatment with the glucocorticoid beclomethasone attenuates the effect of the infection on glucose levels and muscle wasting. RNA-sequencing was performed to study possible molecular mechanisms underlying the effects of beclomethasone. Interestingly, the results of this analysis indicate that beclomethasone hardly changes gene transcription in infected larvae, suggesting that the infection induces a glucocorticoid-resistant state.

Materials and methods

Fish maintenance and handling

Zebrafish of the AB/TL line were maintained and handled according to the guidelines from the Zebrafish Model Organism Database (http://zfin.org), and in compliance with the directives of the local animal welfare committee of Leiden University. They were housed under a 14 hours light and 10 hours dark cycle at 28.5 °C. 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).

Bacterial preparation and zebrafish injections

M. marinum injections were performed as described by Benard et al.[35]. A *M. marinum* strain, containing an expression vector for the fluorescent protein Wasabi, was cultured as described in Benard et al.[35]. Two vials with 1 ml of the mycobacterial culture were centrifuged at 14680 rpm for 1 min. The pellets were washed three times with 1 ml phosphate-buffered saline (PBS). Suspensions were prepared based on the optical density at 600 nm, which corresponds to 1.0 x 10^8 colony forming units (cfu)/ml. The inoculums were suspended in 2% polyvinylpyrrolidone40 (PVP40, CalBiochem) to a concentration of 2.0 x 10^8 cfu/ml. Between 20 and 40 cfu were injected into the blood island of 28 hours post fertilization (hpf) embryos, which were kept under anesthesia in egg water containing 0.02% tricaine (3-aminobenzoic acid ethyl ester (Sigma). Non-infected (control) embryos were injected with a carrier solution

(PBS). The embryos were treated with beclomethasone (25 µM) or vehicle starting at 2 hours before the infection.

Measurement of mortality and bacterial burden

The infected larvae were monitored every 24 hours post infection to determine mortality rates. The larvae used for the survival assay were kept individually in 96 well plates. The larvae used for other assays were kept in petri dishes. We used the Complex Object Parametric Analyzer and Sorter (COPAS, Union Biometrica) system to determine the total level of green fluorescence, which was used as a measure for the bacterial burden. The following parameters were used: optical density threshold (extinction) = 390 mV (COPAS value: 20), minimum time of flight = 280 ms (COPAS value: 700), red photomultiplier tube (PMT) voltage = 500 V, green PMT voltage = 0 V, yellow PMT voltage = 0 V, fluorescent density threshold = 800. The fluorescence intensities of approximately 50 injected embryos were measured daily (every 24 hr) with the COPAS system until 4 days post infection (dpi). Embryos that were not successfully infected and did not show proper fluorescence were discarded from the experiment. Measurements were performed daily, and after each measurement the medium was refreshed. Data were pooled from three independent experiments. Data shown are means ± s.e.m.

Glucose measurement

Quantitative analysis of glucose levels was performed from whole body lysates using a glucose ELISA kit (Cayman Chemical, USA), according to the manufacturer's instructions. Fifteen zebrafish larvae per experimental group were sonicated in 30 µl Assay Buffer on ice. A total of 25 µl assay Enzyme Mix was added and the solution was incubated for 10 min at 37°C. Standard curves were generated using glucose standard solutions. Fluorescence was measured using a BioTek plate reader (excitation wavelength 514 nm) equipped with GEN 5 software (v.2.04, BioTek). Data shown are means ± s.e.m. of three independent experiments, which were performed in triplicate.

Measurement of muscle mass

A phalloidin staining was used to determine the muscle mass. Larvae were put into microcentrifuge tubes at 4 dpi, and fixed in 0.5 ml 4% paraformaldehyde. The tubes were kept at 4°C overnight. The next day, the fixative was removed and the larvae were rinsed three times for 5 min in 0.5 ml PBS/0.1%Tween20. After removing the PBS/ 0.1%Tween20, a solution of 0.5 ml PBS/2%TritonX-100 was added to each tube. The tubes were then gently rocked for 1.5 hour at room temperature. The liquid was removed and 19 µl of PBS/2%TritonX-100 was added to each tube, along with 1µl of AlexaFluor555Phalloidin. The tubes were gently rocked at 4°C overnight. The next day, the larvae were washed three times with 0.5 ml PBS. Fixed larvae were imaged using a Leica MZ16FA fluorescence stereomicroscope (Leica Microsystems). Quantification of the fluorescent signal was done

using dedicated bacterial pixel count software as previously described by Stoop et al.[41]. Three individual experiments were performed, which were done with at least ten replicates per experimental group. Data were pooled from three experiments, and data shown are means ± s.e.m.

RNA-sequencing (RNA-seq) analysis

In three independent experiments, pools of 20 embryos of four experimental groups (veh/non-inf, beclo/non-inf, veh/inf, beclo/inf) were collected at 4 dpi, snap-frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted according to the manufacturer's instructions, using TRIzol (Life Technologies). A total of 2 µg of RNA was used to make RNA-seq libraries using the Illumina TruSeq RNA Sample Preparation Kit v2 (Illumina, Inc., San Diego, CA, USA). The manufacturer's instructions were followed with the exception of two modifications. In the adapter ligation step, 1 µl adaptor was used (instead of 2.5 µl), and in the library size-selection step, the library fragments were isolated with a double Ampure XP purification with a 0.7× beads to library ratio (Beckman Coulter). The resulting mRNA-seq library was sequenced using an Illumina HiSeq2500 Instrument (Illumina Inc.), with a read length of 2×50 nucleotides. Image analysis and base-calling were done by the Illumina HCS version 2.0.12. Data analysis was performed using GeneTiles Software (www.genetiles.com, described in Veneman et al. [42]), and gene ontology and pathway analysis were performed using DAVID Functional Annotation Tool (https://david.ncifcrf.gov/). Finally a detailed gene ontology analysis was performed manually by gathering information from zfin.org, to select genes involved in metabolism.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA). The survival rates (Fig.1A) were analyzed using a Log-rank Mantel-Cox test. The bacterial burden (Fig.1B) was analyzed using two-way ANOVA followed by Sidak's multiple comparisons. The glucose concentrations (Fig.2A) were analyzed by using two-way ANOVA followed by Tukey's post hoc test for multiple group comparisons. Data from the phalloidin staining (Fig.2B) were also analyzed by two-way ANOVA followed by Tukey's post hoc test for multiple group comparisons. RNA-seq data (Figs.3-6) were analyzed using GeneTiles software and DESeq2. Statistical significance was accepted at $p<0.05$.

Results

Beclomethasone increases the severity of *M. marinum* infection in zebrafish larvae

To study the effect of glucocorticoids on the course of the mycobacterial infection, wild type zebrafish embryos were injected with fluorescently (Wasabi-)labeled *M. marinum* strain at 28 hpf. The embryos were treated with beclomethasone or vehicle starting at 2 hours before the infection, and the survival was monitored during the next 4 days. We found that the mortality

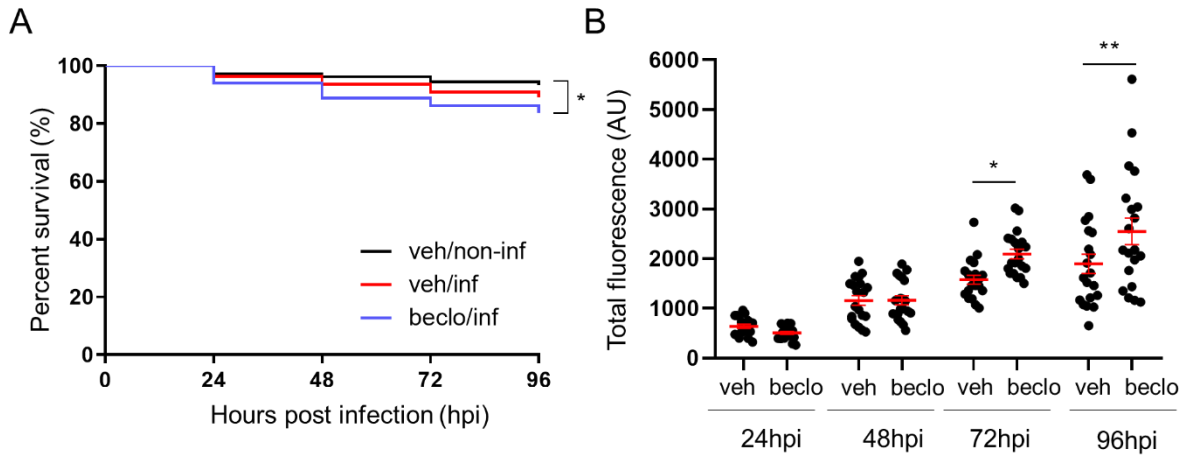
in the infected beclomethasone-treated (beclo/inf) group was significantly higher compared to survival in the non-infected control (veh/non-inf) group (reaching respectively ~17% and ~7% at 96 hpi). No difference was observed between the mortality in the infected vehicle-treated (veh/inf) group (which reached a mortality rate of ~11% at 96 hpi) and any of the other groups (Fig.1A).

In addition to their survival, the bacterial burden of the infected larvae was monitored during this period. To study the bacterial burden in the surviving larvae, we used COPAS flow cytometry to follow the progression of the infection. We found that at 1 and 2 dpi the average total fluorescence was not different between the vehicle- and beclomethasone-treated group, reaching levels of 614±42 Arbitrary Units (AU) and 525±31 AU at 1 dpi respectively, and 1257±97 AU and 1204±90 AU at 2 dpi (Fig.1B). A significant difference was noticeable at 3 dpi when the fluorescence was at 1630±89 AU in the vehicle-treated group and at 2070±97 AU in the beclomethasone-treated group. At 4 dpi the difference remained with a fluorescence level of 2086±194 AU in the vehicle-treated group and 2755±269 AU in the beclomethasone-treated group (Fig.1B).

Taken together, these data show that beclomethasone treatment significantly increases the severity of the *M. marinum* infection. It increases the mortality and the bacterial burden, although the effects are relatively small.

Beclomethasone abolishes infection-induced changes in glucose level and muscle mass

To study the effects of infection and beclomethasone treatment on the glucose metabolism during *M. marinum* infection, we measured the glucose levels in four different treatment groups: control-treated larvae (veh/non-inf), larvae that were treated with beclomethasone (beclo/non-inf), larvae that were infected (veh/inf), and larvae that received the combined treatment (beclo/inf). Embryos were infected at 28 hpf with the fluorescently labelled *M. marinum*. The glucose concentrations were measured at 1, 2, 3 and 4 dpi using an ELISA-based approach (Fig.2A). Both beclomethasone and infection enhanced glucose levels in the larvae at 2, 3 and 4 dpi, with glucose concentrations of 277±4 pmol/larva in the beclo/non-inf group and 291±37 pmol/larva in the veh/inf group at 4 dpi, compared to veh/non-inf control group. However, in the beclo/inf group, which received the combined treatment, the glucose level was significantly lower than the levels in both the beclo/non-inf and the veh/inf group at 2, 3 and 4 dpi, with a concentration of 213±22 pmol/larva at 4 dpi. Subsequently, to study muscle wasting, we used phalloidin staining, which labels actin fibers in the larvae, to visualize muscle tissue at 4 dpi[43,44] (Fig.2B). We found that beclomethasone treatment alone did not affect the muscle mass in the larvae, but that infection caused severe muscle loss, indicated by a decrease of ~55% in phalloidin staining in the veh/inf group (2548±532 AU), compared to the veh/non-inf group (6487±283 AU). Interestingly, the combined infection and beclomethasone treatment did not result in a change in muscle mass, indicated by a level of phalloidin staining in the beclo/inf group (4762±496 AU), which was not significantly different from the veh/non-



inf group. A representative images of the phalloidin staining of a larva from the veh/non-inf group is shown in Fig.2C.

Figure 1. The effect of beclomethasone treatment on *M. marinum* infection in zebrafish. (A) Survival curves of zebrafish upon *M. marinum* infection at 28 hpf. Survival was monitored between 24 and 120 dpi. Infected fish were either vehicle-treated (red line, veh/inf) or beclomethasone-treated (blue line, beclo/inf) and a vehicle-treated non-infected group was added as control (black line, veh/non-inf). At 120 hpi the percentage survival was approximately ~75% in the beclo/inf group and ~82% in the veh/inf group, compared to ~90% in the veh/non-inf group. A significant difference was observed between the beclo/inf and the veh/non-inf group. **(B)** The bacterial burden in larvae from the veh/inf and beclo/inf groups was determined between 1 and 4 dpi based on fluorescence intensities. In the beclo/inf larvae the bacterial burden at 3 and 4 dpi was significantly higher than in the veh/inf larvae. Data were pooled from three independent experiments. Data shown are means ± s.e.m. *P<0.05; **P<0.01

In summary, these results indicate that beclomethasone treatment abolishes both the infection-induced increase in glucose concentration and the loss in muscle mass due to the infection.

Transcriptome analysis by RNA-sequencing

To study the transcriptional changes underlying the observed effects of beclomethasone treatment during bacterial infection, we performed RNA-sequencing-based transcriptome analysis. RNA was isolated from whole larvae at 4 dpi, from the four experimental groups: veh/non-inf, beclo/non-inf, veh/inf and beclo/inf. In the analysis of the RNA-seq results, mRNA levels in the latter three groups were determined relative to the levels of the veh/non-inf group. Significance cutoffs of fold change<1.5 or >1.5, and p value<0.05 were used. Our data showed that 2542 genes were significantly regulated in the veh/inf group (Suppl.Table 1), 1407 in the beclo/ inf group (Suppl.Table 2), and 2319 in the beclo/ non-inf group (Suppl.Table 3) (see Venn diagrams in Fig.3).

About two times more genes were upregulated than downregulated in all studied groups (Figs.3B,C). Interestingly, the largest overlap (1284 genes) was observed between the clusters

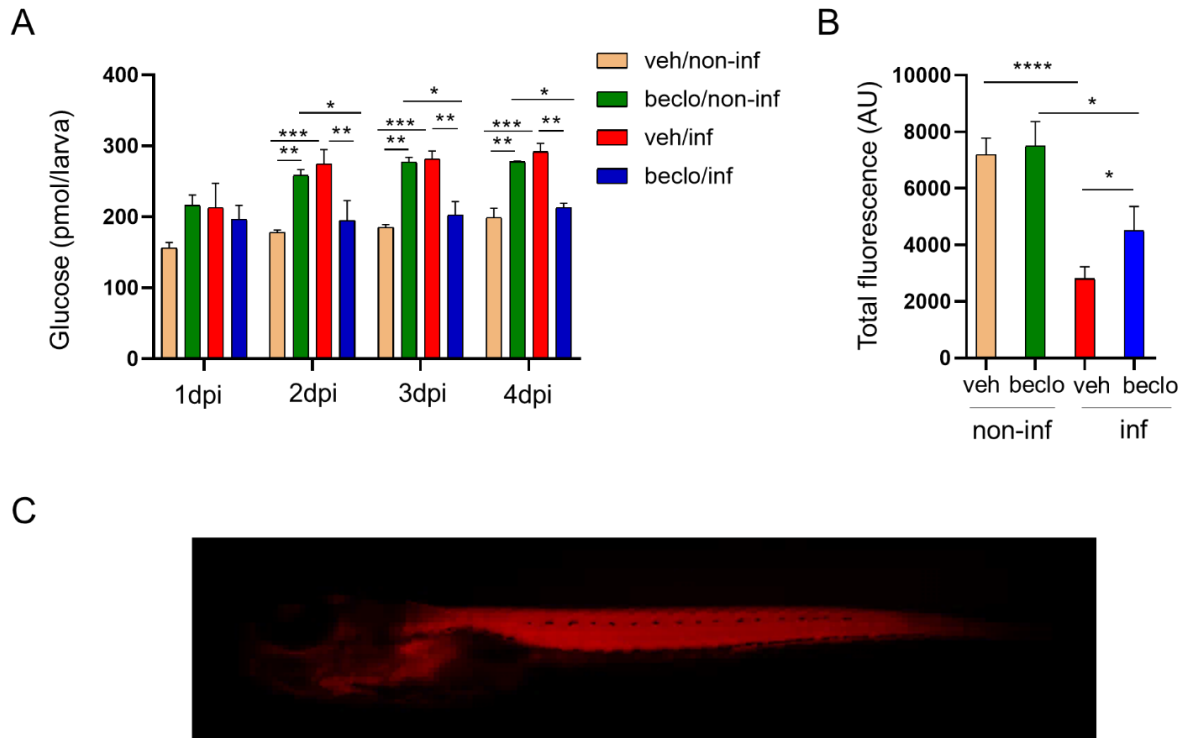


Figure 2. Effect of beclomethasone on the glucose level and muscle mass in *M. marinum* infected zebrafish. (A) Glucose levels in zebrafish larvae from the veh/non-inf, beclo/non-inf, veh/inf and beclo/inf groups at 4 dpi, determined by ELISA. Significant differences in glucose levels were observed between 2 and 4 dpi. The glucose level was increased in the beclo/non-inf and the veh/inf group compared to the veh/non-inf group. When beclomethasone treatment and infection were combined (beclo/inf treatment), the glucose levels were not different from those in the veh/non-inf group. **(B)** Muscle wasting in zebrafish larvae at 4 dpi, determined using phalloidin staining and quantitation of fluorescence in larvae. Larvae from the veh/inf group showed significantly lower muscle mass compared to the non-infected groups (veh/non-inf and beclo/non-inf). The combined beclo/inf treatment did not result in a significant change in muscle mass compared to the veh/non-inf group, and showed a significantly higher muscle mass compared to the veh/inf group. **(C)** Representative image of phalloidin staining of a larva at 4 dpi from the veh/non-inf group, captured using fluorescence microscopy. Data were pooled from three independent experiments, and data shown are means ± s.e.m. . *P<0.05; **P<0.01; *P<0.001; **P<0.0001.

of regulated genes in the veh/inf group and the beclo/inf group, which indicates that the effect of beclomethasone on transcription during an infection is limited. In addition, volcano plots of the gene regulation (p value plotted versus fold change for each individual gene) showed that the plots of the veh/inf group and the beclo/inf group were very similar (Figs.4A,B).

Furthermore, the plot for the beclo/non-inf group mainly showed lower fold changes for the upregulated genes (Fig.4C), and all plots showed more upregulated genes than downregulated ones (Figs.4A,B,C).

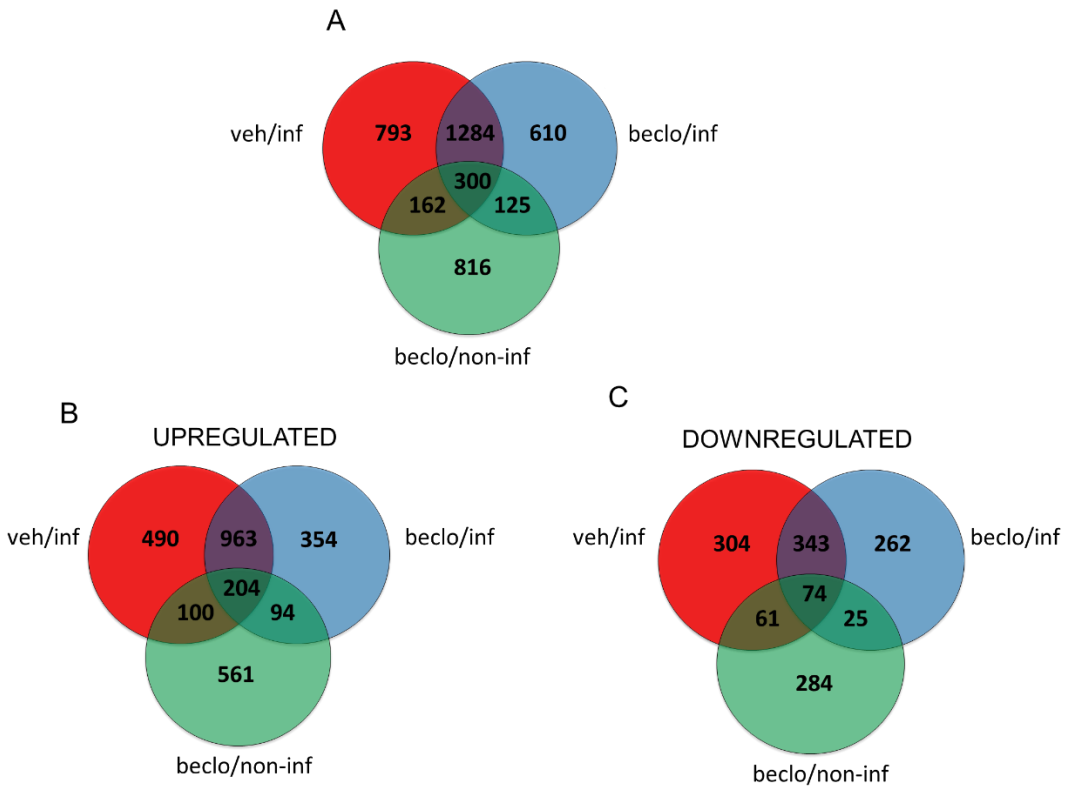


Figure 3. Venn diagrams showing numbers of regulated genes per treatment and overlaps between clusters of regulated genes. (A) Clusters of genes significantly regulated (compared to veh/non-inf treatment) by vehicle and infection (veh/inf), beclomethasone treatment (beclo/non-inf) and the combined infection and beclomethasone treatment (beclo/inf). The diagram shows that the largest number of genes was regulated by infection (veh/inf: 2542 genes). Combined treatment changed the expression of a relatively high number of genes as well (beclo/inf: 2319 genes), and the beclomethasone itself resulted in the lowest number of changes in gene expression (beclo/non-inf: 1407 genes). A large overlap (1284 genes) was observed between the veh/inf and the beclo/inf cluster. **(B)** Clusters of genes upregulated by the three different treatments (compared to veh/non-inf treatment). **(C)** Clusters of downregulated genes by the three different treatments (compared to veh/non-inf treatment).

To further investigate how beclomethasone treatment affects the transcriptome of *M. marinum* infected larvae, we compared the beclo/inf with the veh/inf group. A cluster of only 246 genes showed significantly different expression levels between these two groups (Suppl.Table 4), confirming the limited number of differently regulated genes between these two groups that we saw in the earlier analysis. This cluster should contain the genes that are responsible for the differences in glucose concentration and muscle mass that were observed between these two groups. Analysis of gene ontology (using the DAVID online functional annotation tool) showed enrichment of gene ontology groups related to processes involved in immune response ('defense response to virus', 'response to xenobiotic stimuli', 'humoral immune response' and 'chemotaxis'), as well as many general processes (such as, 'transport' and 'signal transduction' and 'potassium ion transmembrane transport') (Table 1).

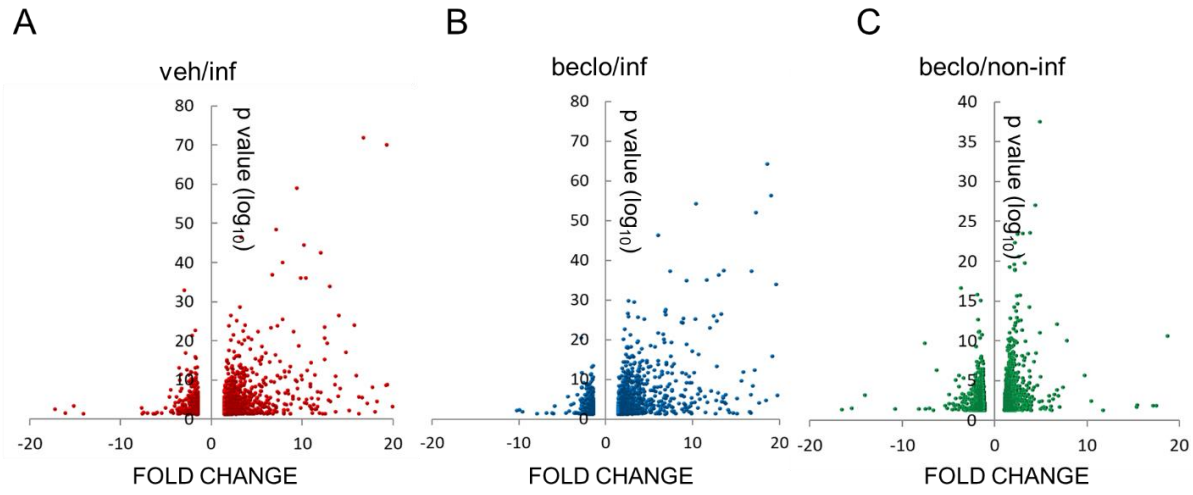


Figure 4. Volcano plots presenting the p-value as a function of the fold change for individual genes. (A) Gene regulation in the veh/inf group. **(B)** Gene regulation in the beclo/inf group. **(C)** Gene regulation in the beclo/non-inf group.

A look at significantly enriched KEGG pathways showed enrichment of four pathways, of which two pathways were connected to immunity: 'intestinal immune network' and 'cytokine-cytokine receptor interaction'. The other two significantly enriched pathways were 'retinol metabolism' and 'PPAR signaling pathway' (Table 2)(Suppl.Table 5).

A more detailed analysis of gene ontology showed that out of the 246 genes differentially expressed between the veh/inf and the beclo/inf group. Interestingly, 40 of these genes are directly or indirectly related to glucose metabolism and the insulin signaling pathway. Of these 40 genes, 25 were expressed at a lower level in the presence of beclomethasone (Suppl.Table 6). Some of these downregulated genes are known to be involved in glucose metabolism: *ptgs2a*[45,46], *slc27a1a*[47], *kcnj1a.4* and *kcnj1a.5*[48], *nfe2*[49,50] and *claudin*[51]. Downregulated genes that are known to be directly connected with insulin resistance and T2DM2 were *ghsrb* (which is also involved in leptin secretion and appetite stimulation[52]), *tent5c* (also connected with obesity[53]), *bcl6b*[54], *cxcr4a* (also an immune-related gene) and *serpine1*[55]. In this cluster of genes, we also distinguished two genes that are known to be involved in the PPAR signaling pathway, *slc27a1a* and *fabp7b*[56], one gene that regulates myoblast proliferation and differentiation, *odc1*, and *socs3a* which has been suggested to play a role in the development of leptin resistance[57] (Suppl.Table 6).

Only 15 genes were expressed at a higher level in the presence of beclomethasone (Suppl.Table 7), and some of these genes have been shown to be involved in the pathogenesis of T2DM: *arnt* and *ace2,* and *alpi.2* (known to act protectively against DM2)[58]. Other genes in

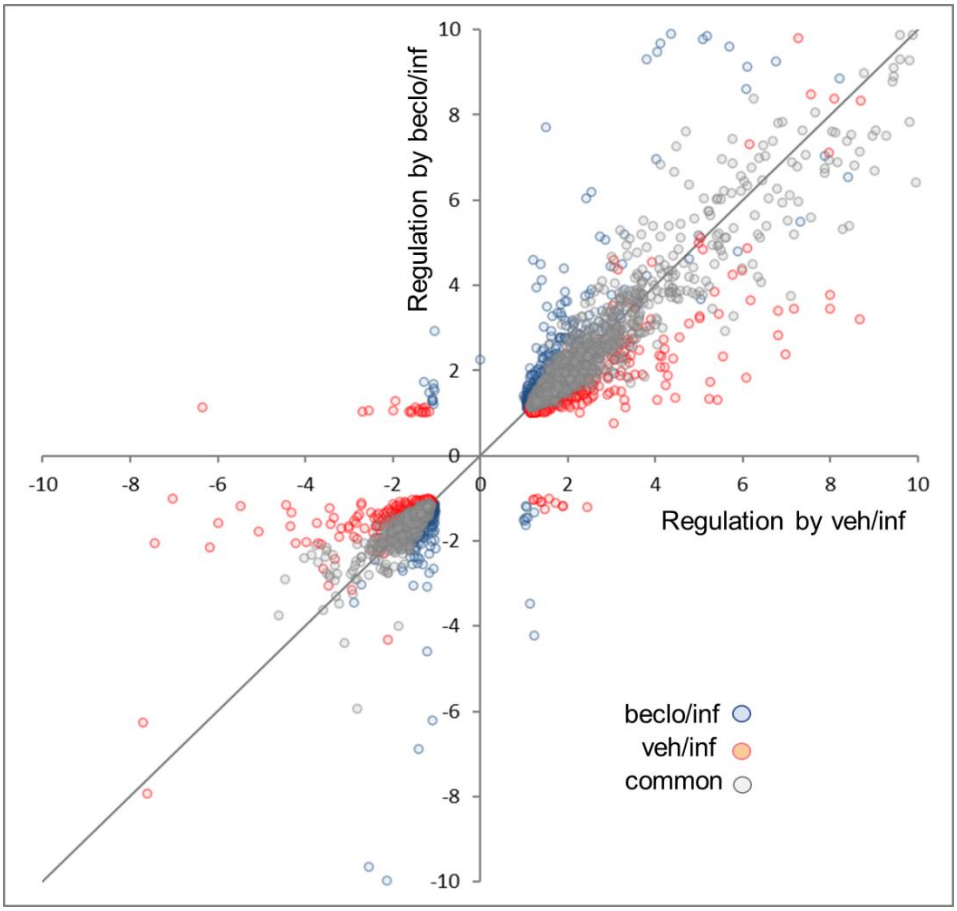


Figure 5. Scatter plot showing the effect of beclomethasone treatment on infection-regulated gene expression. For all genes showing significant regulation upon veh/inf treatment or the beclo/inf treatment, the fold change in the beclo/inf group was plotted as a function of the fold change due in the veh/inf group. Genes significantly regulated in the veh/inf group are indicated by red markers, genes significantly regulated in the beclo/inf group by blue markers, and genes significantly regulated by both treatments are indicated by gray markers. The diagonal line indicates the point at which beclomethasone treatment does not affect infection-induced changes. The plot shows that the vast majority of genes is similarly regulated in the absence and presence of beclomethasone, indicating that beclomethasone hardly affects the transcriptional response in the presence of an infection. Significantly regulated genes were selected by using a $p<0.05$ and $|FoldChange|>1.5$ cutoff.

this group were *grp,* that has been implicated in gastrointestinal and metabolic diseases[59], *serpinb1*, which is highly expressed in individuals with both diabetes and obesity[55], and the chitinase genes *chia.3* and *chia.1* which are known to be highly expressed in obesity and insulin resistance[60]. In addition, *ca4b* was found in this group, which lowers glucose concentration in the plasma[61], as well as *dgat1a* that plays a crucial role in the triglyceride and glucose metabolism[62] (Suppl.Table 7). Genes involved in lipid metabolism were *pdzk1*, involved in cholesterol metabolism[63], *faah2b* that participates in fat storage via the leptin signaling pathway[64], *asah2* which is essential for degradation of sphingolipids[65], and *igf1*[66] (Suppl.Table 7). Other upregulated genes were *rgs9b*, which is involved in *leptin a* transcription[67], and *abcc6a* of which insufficiency leads to mineralization of cardiovascular, ocular and dermal tissues[68] (Suppl.Table 7).

Discussion

In the present study, we have investigated whether glucocorticoid treatment alters the metabolic changes during *Mycobacterium marinum* infection in zebrafish. Our data show that the infection decreases the muscle mass and increases the glucose levels in the larvae, reflecting the metabolic changes related to infection-induced cachexia. Treatment with the glucocorticoid beclomethasone attenuated the decrease in muscle mass and abolished the increase in glucose concentration. RNA-sequencing analysis was performed to unravel the molecular mechanisms underlying these metabolic changes. Our data showed that beclomethasone had a relatively minor effect on transcription during *Mycobacterium marinum* infection, suggesting that the infection induces a glucocorticoid-resistant state. Still, a number of immunity- and metabolism- related genes were identified of which

Table 1. Gene ontology for genes showing significantly different expression levels between the veh/inf and beclo/inf groups. Performed using The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8.

TERM	GENES
Chemotaxis	4
G-protein coupled receptor signaling pathway	14
Defense response to virus	3
Transport	15
Intercellular signal transduction	7
Chitin metabolic process	2
Thrombin receptor signaling pathway	2
Potassium ion transport	4
Humoral immune response	2
Chitin catabolic process	2
Signal transduction	15
Potassium ion transmembrane transport	3
Response to xenobiotic stimulus	2
Metabolic process	7

Table 2. Pathway analysis for genes showing significantly different expression levels between the veh/inf and beclo/inf groups, performed using The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8.

PATHWAY	GENES
Retinol metabolism	3
Intestinal immune network for IgA production	3
Cytokine-cytokine receptor interaction	4
PPAR signaling pathway	3

beclomethasone altered the expression level during the infection. These genes may reveal the molecular pathways responsible for the infection-induced metabolic changes.

Although *Mycobacterium marinum* infection in zebrafish larvae is a well-established model for studying the pathogenesis of TB, the metabolic changes during this infection have hardly been studied. In the present study, we demonstrate a decrease in muscle mass and increased glucose levels in infected larvae. These changes are highly reminiscent of the metabolic alterations observed in human TB patients, which include increased protein degradation and reduced protein anabolism resulting in muscle atrophy and increased gluconeogenesis possibly resulting from insulin resistance, which may lead to T2DM[69]. Previously, the zebrafish *Mycobacterium marinum* infection was used to study changes in lipid metabolism, which also resembled alterations observed in TB patients[70]. Thus, the zebrafish *Mycobacterium marinum* infection model system shows translational value for studies on the metabolic changes that are observed during TB.

When the infected larvae were treated with the synthetic glucocorticoid beclomethasone, the infection-induced increase in glucose concentration was abolished and the reduction in muscle mass was attenuated. Interestingly, in the absence of an infection beclomethasone increased the glucose levels. This is a well-established glucocorticoid effect[71], that has previously been demonstrated in zebrafish embryos as well[38]. In contrast, beclomethasone did not affect the muscle mass in the uninfected larvae. Apparently, beclomethasone and infection do not simply have opposite effects on glucose levels and muscle mass, but beclomethasone modulates the metabolic response of the larvae to the infection, in line with conditional, context-specific action of the GR described in many other studies[72]. The observed attenuation of the metabolic effects was not due to diminished levels of infection, since the bacterial burden in the beclomethasone-treated larvae was actually increased.

In order to unravel the molecular mechanisms that lead to the metabolic changes during the *Mycobacterium marinum* infection, a transcriptome analysis was performed using RNA-

sequencing. Even though this analysis revealed that the infection induced a state of reduced glucocorticoid sensitivity, beclomethasone did modulate the transcription of a number of genes during infection, and these changes most likely underlie the altered glucose levels and muscle mass and studying this relatively small number of genes with altered expression could reveal crucial pathways contributing to the wasting syndrome in TB. Gene ontology analysis revealed that this cluster contains many-immune related genes, but also metabolism-related genes were enriched in this cluster. Out of 40 metabolism-regulated genes in this cluster, 25 genes showed a lower expression level in the presence of beclomethasone. Most of these genes were involved in glucose metabolism, leptin signaling pathway, insulin resistance and the pathogenesis of T2DM. In the cluster of 15 genes that showed a higher expression level in the presence of beclomethasone, most genes were associated with other metabolic processes, responsible for fat storage and cholesterol metabolism. However, some of them are also participating in the insulin signaling pathway.

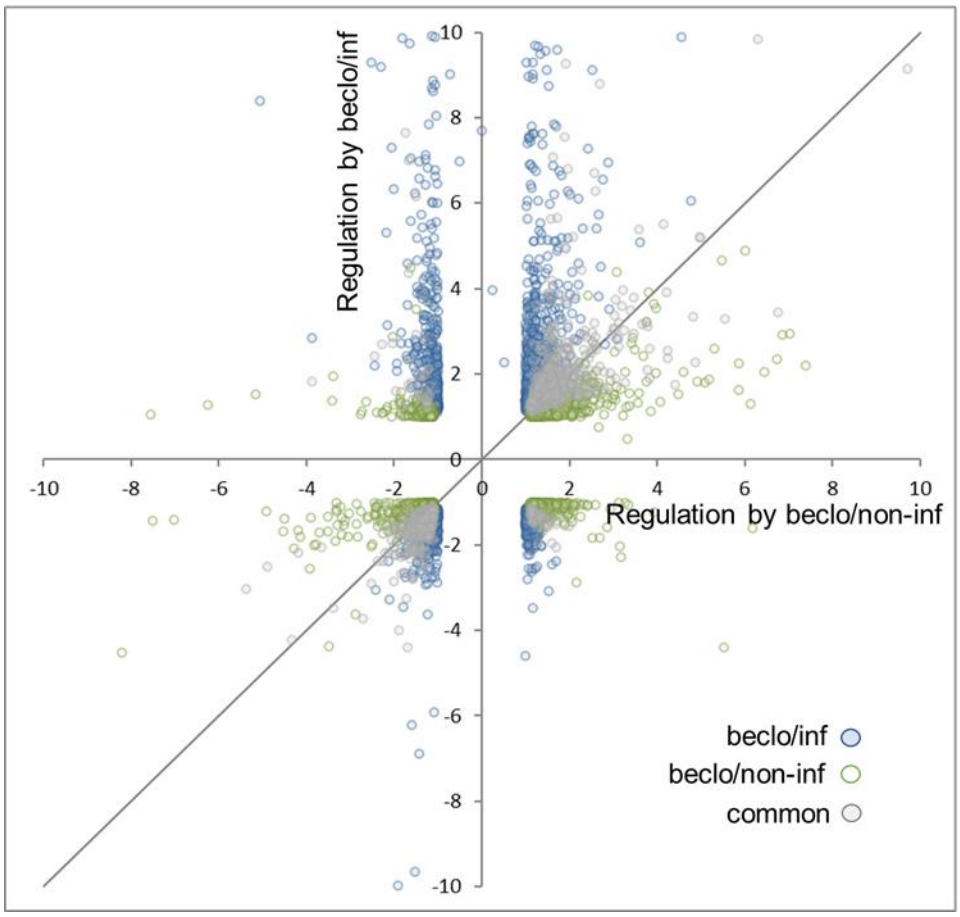
The glucocorticoid-resistant state induced by the *Mycobacterium marinum* infection was reflected by the RNA-sequencing results in two ways. First, the Venn diagrams (Fig.3) and scatter plot (Suppl.Fig.1) show that there was very little similarity between the gene regulation by beclomethasone in the absence of infection (beclo/non-inf) and the regulation by the combined beclomethasone/infection treatment (beclo/inf). These results indicate that most of the beclomethasone-induced gene regulation that is observed in the absence of infection does not occur in infected larvae. Second, when comparing the regulation in the beclo/inf and the veh/inf groups using the Venn diagrams (Fig.3) and scatter plot (Fig.5), we found that most of the gene regulation of highly similar between these groups, indicating that the regulation of the vast majority of genes is not affected by the beclomethasone administration. A direct comparison between these groups showed a cluster of only 246 genes regulated differently. These data show a striking contrast to previous work from our laboratory in which zebrafish larvae were subjected to tail fin amputation. In these experiments, beclomethasone attenuated almost the entire transcriptional response to the amputation, suggesting that the resistance to beclomethasone observed in the present study during *Mycobacterium marinum* infection, is specific to certain infectious and/or inflammatory conditions.

The observed glucocorticoid-resistant state has been associated with TB and other infectious and/or inflammatory conditions. Previously, it was shown in PBMCs from patients suffering from severe TB that the ratio between the expression level of the canonical GR and an alternative splice variant that may act as a dominant-negative inhibitor, GRβ, was reduced. This reduced ratio appeared to correlate with levels of pro-inflammatory cytokines, and could underlie a decreased sensitivity to glucocorticoids[732,74]. Bacterial lipopolysaccharide (LPS) has been shown to reduce glucocorticoid sensitivity in in various cell types[75,76,77], and a variety of bacterial toxins, like the anthrax lethal toxin have been shown to reduce GR function in a variety of cell types *in vitro* and *in vivo*[74]. Viral infections have been demonstrated to decrease

glucocorticoid sensitivity in lung epithelial cells[78,79,80], and the NF-κB, JNK[81] and TGF-β[82] pathways have been shown to be implicated, possibly resulting in posttranslational modification of the GR protein[79].

In summary, the results of this study show that *Mycobacterium marinum* infection in zebrafish larvae represents an interesting model system to investigate mechanisms underlying cachexia in human TB patients. We observed increased glucose levels and reduced muscle mass upon infection. These changes were abolished and attenuated respectively by treatment with the glucocorticoid beclomethasone, even though transcriptome analysis showed that the larvae were in a glucocorticoid-resistant state. The few transcriptional changes elicited by beclomethasone suggest that genes involved in glucose metabolism, insulin and leptin signaling play a crucial role in the observed metabolic changes resembling cachexia.

Supplementary materials



Supplemental Figure 1. Scatter plot showing the effect of infection on beclomethasone treatment. For all genes showing significant regulation upon beclo/non-inf treatment or the beclo/inf treatment, the fold change in the beclo/inf group was plotted as a function of the fold change due in the beclo/non-inf group. Genes significantly regulated beclo/non-inf group are indicated by green markers, genes significantly regulated in the beclo/inf group by blue markers, and genes significantly regulated in both conditions are indicated by gray markers. Significantly regulated genes were selected by using a $p<0.05$ and $|FoldChange|>1.5$ cutoff.

Supplemental Table 5. Enriched gene ontology groups in the cluster of significantly regulated genes between the veh/inf and beclo/inf groups, determined using The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8.

ENRICHMENT SCORE: 2.84	COUNT
Transmembrane helix	63
Transmembrane	63
Membrane	66
Integral component of membrane	65
Membrane	64
ENRICHMENT SCORE: 1.65	**COUNT**
Chemokine receptor family	4
Chemokine receptor activity	4
Cxc chemokine receptor 4	3
Chemotaxis	4
G-protein coupled receptor signaling pathway	14
G-protein coupled receptor , rhodopsin like	11
Intestinal immune network for Iga production	3
Signal transduction activity	15
Cytokine-cytokine receptor interaction	4
ENRICHMENT SCORE: 1.11	**COUNT**
Metal ion binding	21
Zinc ion binding	12
Zinc finger	8
B30.2/spry domain	7
SPRY	6
BBOX	5

Supplemental Table 6. List of significantly downregulated genes between the veh/inf and beclo/inf groups, and their possible role in the development of metabolic disorders. The analysis of their possible roles was performed using The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8, and manually by using zfin.org and pubmed.ncbi.nlm.nih.gov.

GENE ID	NAME	PATHWAYS AND PROCESSES
ENSDARG00000004539	ptgs2a prostaglandin-endoperoxide synthase 2a	Prostaglandins signaling pathway Wasting, glucose metabolism, diabetes
ENSDARG00000006240	slc27a1a solute carrier family 27 (fatty acid transporter)	PPAR Signaling Pathway lipid uptake, metabolism, and transport in the Larval Zebrafish
ENSDARG00000090635 *ENSDARG00000089060*	kcnj1a.4 kcnj1a.5 potassium inwardly-rectifying channel, subfamily J, member 1a,	change in fasting glucose, onset diabetes glucose metabolism
ENSDARG00000009544	claudin b	glucose metabolism, diabetes
ENSDARG00000035198	gcnt4a glucosaminyl (N-acetyl) transferase 4, core 2, a	carbohydrate metabolism, lipid metabolism
ENSDARG00000056561	asb11 ankyrin repeat and SOCS box containing 11	pancreatic β-cells function, diabetes
ENSDARG00000056021	sostdc1b sclerostin domain containing 1b	Bmp/Wnt inhibitor signaling pathway diabetes type 1, enhances pancreatic islet function
ENSDARG00000057117	ghsrb growth hormone secretagogue receptor b	appetite leptin secretion diabetes type II
ENSDARG00000020606	nfe2 nuclear factor, erythroid 2	promote gluconeogenesis, onset and progression of type 2 diabetes mellitus inflammatory cytokines
ENSDARG00000017653	rgs13 regulator of G protein signaling 13	Inflammation, diabetes type I
ENSDARG00000010437	tent5c terminal nucleotidyltransferase 5C	Diabetes, obesity
ENSDARG00000056784	aire autoimmune regulator	type I diabetes, insulin autoimmunity
ENSDARG00000034650	fabp7b fatty acid binding protein 7, brain, b	PPAR signaling pathway diabetes type II, lipid metabolism

ENSDARG00000029866	slc6a14 solute carrier family 6 member 14	obesity
ENSDARG00000069335	bcl6b transcription repressor	diabetes type II
ENSDARG00000007377	odc1 ornithine decarboxylase	regulates myoblast proliferation and differentiation
ENSDARG00000056795	serpine 1	diabetes type II, obesity
ENSDARG00000025428	socs3a suppressor of cytokine signaling 3a	insulin resistance, leptin signaling
ENSDARG00000100564	sil1 SIL1 nucleotide exchange factor	glucose-stimulated insulin secretion
ENSDARG00000077130	bcl10 BCL10 immune signaling adaptor	diabetes type II, metabolic syndrome
ENSDARG00000015902	stat6 signal transducer and activator of transcription 6, interleukin-4 induced	beta cells, diabetes type I
ENSDARG00000088048	fgf18a fibroblast growth factor 18a	Obesity, diabetes type II
ENSDARG00000010169	myd88 MYD88 innate immune signal transduction adaptor	increases risk of diabetes, obesity
ENSDARG00000013855	slc12a3 solute carrier family 12 member 3	Diabetes, diabetic nephropathy

Supplemental Table 7. List of significantly upregulated genes between the veh/inf and beclo/inf groups, and their possible roles in the development of metabolic disorders. The analysis of their possible roles was performed using The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8, and manually by using zfin.org and pubmed.ncbi.nlm.nih.gov.

GENE ID	NAME	PATHWAYS AND PROCESSES
ENSDARG00000021855	ARNT Aryl hydrocarbon receptor nuclear translocator	diabetes type II, ARNT expression is reduced in diabetic human islets and β cell
ENSDARG00000045156	rgs9b regulator of G protein signaling 9b	leptin a transcription
ENSDARG00000022261	pdzk1 PDZ domain containing 1	cholesterol metabolism
ENSDARG00000016918	ace2 angiotensin I converting enzyme 2	diabetes type II, diabetic nephropathy
ENSDARG00000053774	alpi.2 alkaline phosphatase, intestinal, tandem duplicate 2	is protective against type 2 diabetes mellitus
ENSDARG00000054786	faah2b fatty acid amide hydrolase 2b	fat storage
ENSDARG00000094132	IGF-1 insulin-like growth factor 1	Low- and high-normal IGF-I levels are both related to insulin resistance.
ENSDARG00000043074	grp gastrin-releasing peptide	both gastrointestinal inflammatory states and classical chronic metabolic diseases such as diabetes
ENSDARG00000042293	ca4b carbonic anhydrase IV b	type I diabetes
ENSDARG00000103503	dgat1a diacylglycerol O-acyltransferase 1a	metabolic processes, conversion of diacylglycerol and fatty acyl CoA to triacylglycerol
ENSDARG00000009612	chia.3 chia.1 chitinase, acidic.3, acidic.1	increased in CHI3L1 levels in obesity, insulin resistance
ENSDARG00000016750	abcc6a ATP-binding cassette, sub-family C	diabetic vascular calcification, elastic fibre mineralisation and fragmentation
ENSDARG00000012829	asah2 N-acylsphingosine amidohydrolase 2	impaired glucose tolerance, lipids and amino acids metabolism
ENSDARG00000055416	serpinb1	Promotes Pancreatic β Cell Proliferation

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SUMMARY AND DISSCUSSION

Tuberculosis (TB) is currently one of the most fatal infectious diseases in the world and, as one of the top ten leading causes of death, remains a big threat to public health worldwide. Although *Mycobacterium tuberculosis* (*Mtb*), the pathogen that causes TB, has been known for many years, its eradication has so far been unsuccessful. A surge of multi drug resistant (MDR) and extreme drug resistant (XDR) strains of the pathogen demands alternative forms of therapies against the disease to support current treatment[1]. Complexity of host-pathogen interaction mechanisms and metabolic adaptation during the course of the disease play an important role in determining the outcome of the bacterial infection. These complex mechanisms require a broader approach towards a novel anti-TB therapy, for example by increasing the host's natural resistance. Because of the complicated nature of TB infection, the research described in this thesis focused on different aspects of insulin resistance and glucose metabolism during the infection[2]. We used the zebrafish larval model which has several advantages for the modeling of disorders and enables studying the function of the innate immune system separate from the adaptive immune system. The transparency, rapid development and possibility of genetic manipulation make zebrafish larvae perfect model organisms for our research[3].

Protein tyrosine phosphatases (PTPs) are factors that play an important role in many cellular processes such as cell survival, migration and immune responses[4]. There are four classes of them of which class I is the biggest group that contains non-receptor PTPs. The protein tyrosine phosphatase non-receptor type 6 (*PTPN6*) gene is expressed mainly in the hematopoietic system, but expression has also been observed in epithelial cells[5]. The product of this gene, SHP-1, plays a role in many different signaling pathways. It is a key regulator of myeloid cells, cytokine receptors, growth factor receptors and immunoreceptors. *ptpn6* knockdown in zebrafish leads to immune suppression, severe edema and skin lesions, similar to the findings in murine *Ptpn6* mutants *me*, *mev* and *spin*[6].

In **Chapter 2** of this thesis, we compared the effects of *ptpn6* morpholino knockdown and the small molecule Shp-1 inhibitor NSC-87877 which had previously been shown to inhibit human SHP-1 *in vitro*[7]. Both methods were used on zebrafish embryos infected with *Mycobacterium marinum* (*Mm*), a close relative of *Mtb.* The results of this study showed that reduced SHP-1 activity impairs the ability of zebrafish embryos to control the mycobacterial infection. Moreover, the progression of infection was more rapid after both morpholino and NSC-87877 treatment compared to the control treatment, with this effect being more severe after the *ptpn6* morpholino knockdown than after the NSC-87877 inhibition. This difference might be caused by inhibition of the related protein phosphatase Ptpn11 by NSC-87877, as in murine cells this phosphatase plays a negative role in cell survival during inflammation and accelerates apoptosis through the dephosphorylation of STAT5[8]. In addition, RNA sequencing (RNAseq) analysis was performed using RNA isolated from whole infected larvae. This analysis

showed that reduced SHP-1 activity results in significant changes in the expression levels of genes involved in processes such as synaptic transmission, ion transport, cell structure, proteolysis, the immune response, apoptosis and gonadotropin secretion. It is worth mentioning that both the inhibition by NSC-87877 and *ptpn6* morpholino knockdown resulted in upregulation of immune-related genes. Still some differences between the two treatments were found. The *ptpn6* morpholino knockdown had more impact on the expression of metabolic genes than NSC-87877 inhibition. Furthermore, the expression of a large number of infection-regulated genes was not affected by either the morpholino or the inhibitor treatment. These data show that both methods of inhibiting SHP-1 function change the course of the mycobacterial infection. In addition, the set of genes that showed the same transcriptional response upon the morpholino and the inhibitor treatment makes a useful reference set that can specifically be linked to the function of SHP-1 and supports further research on the NSC-87877 in comparison with other SHP-1 inhibitors.

Leptin is a protein that acts both as a pleiotropic hormone[9] and as a cytokine[10]. As a hormone, it regulates energy homeostasis, food intake, insulin secretion, angiogenesis, bone formation and reproduction[9]. Mutations that cause functional leptin deficiency in humans are rare and can be treated with leptin supplementation, but if left untreated they may cause severe obesity, insulin resistance and death[11]. In mice, mutations in the gene encoding leptin or the leptin receptor (*Lep* or *Lepr* respectively) result in multiple metabolic alterations, resulting in severe obesity and insulin resistance[12]. Notably, these mice are also more susceptible to TB, which in turn leads to increased leptin levels in the lungs, reaching their highest point at two weeks post infection. Moreover, increased bacterial presence was found in the lungs of infected mice with a mutation in the *Lep* gene (*ob*/*ob* mice), which was associated with a significantly higher mortality[13].

In **Chapter 3**, we have investigated the role of *lepb* as an immune modulator by establishing a zebrafish larval model system to study the effect of leptin deficiency on TB infection. The zebrafish genome contains two leptin genes: leptin a (*lepa*) and leptin b (*lepb*). Although the two proteins have only 24% of their amino acids in common, and are only 18% identical to human leptin, they share a characteristic gene structure and function with their mammalian orthologues. The two leptin genes are differentially expressed: in adults *lepa* is expressed mainly in the liver, while *lepb* is expressed in the ovaries[14]. Previous studies showed a high induction of the expression of the *lepb* gene in larval zebrafish during bacterial infection with *Staphylococcus epidermidis* and *Mm*, as well as a strong upregulation after *ptpn6* knockdown[15]. Using morpholino knockdown and a CRISPR/Cas9-generated mutant fish line, we studied the effect of leptin signaling in *Mm*-infected zebrafish embryos. Morpholino studies showed that knockdown of both *lepb* and *lepr* led to a higher mortality and a more rapid course of infection. On the other hand, knockdown of *lepa* did not have any significant impact on the course of the infection. We found that the effect of the CRISPR/Cas9-generated *lepb* mutation was more severe than after the morpholino knockdown, especially after yolk infection at 4 hpf. Furthermore, we observed a slight decrease in the number of macrophages

in the *lepb* mutant larvae, while the neutrophil number was not changed. In the *lepb* mutant, the expression of two pro-inflammatory genes, *irg1l* and *il1b*, was downregulated after the infection. However, injection of a human recombinant leptin protein partially rescued the phenotype of the infected *lepb* mutant larvae: the bacterial burden in the infected leptin mutant dropped and *irg1l* expression increased. In mice, leptin has also been shown to regulate the expression of *il1b*, and its deficiency leads to immunosuppression and downregulation of pro-inflammatory cytokines and markers[16]. Additionally, we tested how glucose levels change during mycobacterial infection in wild type and *lepb* mutant zebrafish larvae. We found that after *Mm* infection, the glucose concentration was significantly increased in wild type embryos, whereas *lepb* mutant larvae showed much higher glucose levels both before and after the infection. Taken together, our results indicate that the leptin b protein plays an important role in the host defense during the early stages of a mycobacterial infection underlining the role of leptin in the immune defense against TB. These results also support further exploration of its possibilities for the development of novel host-directed treatments methods against TB.

As mentioned above, leptin has also been studied extensively in obesity and type 2 diabetes mellitus (DM2), as it has been shown to play a leading role in whole-body energy homeostasis[17]. However, the molecular mechanisms by which leptin controls insulin resistance are largely unknown. Research on insulin resistance and DM2 has been conducted on human cell cultures and in various animal models, mainly rodents[18]. The mutant mouse strains of the leptin and leptin receptor genes, the *ob/ob* and *db/db* strains respectively, show diabetic phenotypes and are therefore highly useful for diabetes research. Additionally, high fat diet mice, Zucker fatty rats and Zucker diabetic fatty rats have widely been used to study DM2, obesity and the function of leptin signaling in the metabolic syndrome[19]. Zebrafish models have been used as alternative test systems for studying insulin resistance and DM2 as they provide multiple opportunities to explore metabolic diseases, using numerous transgenic and knockout lines[20]. In adult zebrafish the leptins and the leptin receptor have a conserved role in glucose homeostasis, but they do not have any impact on adipose tissue[17]. Although there are already established diabetes models in adult zebrafish, there is a demand for an alternative early stage larval model, which would allow to perform fast and large-scale screening assays. Previous research has shown that zebrafish larvae form a promising model system to study DM2 under non-feeding conditions[17]. Shp-1 acts, as mentioned previously, as a negative immune modulator, but it is also as a key factor in insulin resistance[21].

We have analyzed the role of leptin b and SHP-1 on insulin resistance in early zebrafish larvae in **Chapter 4**, by developing a novel high-throughput method to test anti-diabetic drugs. The screening was possible due to the fact that leptin-deficient zebrafish larvae were totally insulin-resistant and showed a diabetic phenotype already at very early stages of their development. We also showed that metformin, although highly effective for treating this diabetic phenotype in 4-day-old zebrafish larvae, did not have anti-diabetic effects at earlier stages of development. This finding indicated that insulin receptors are functional at the very

early stages of embryogenesis which was also confirmed when we studied the effect of injecting human recombinant insulin into the yolk sac. One of the zebrafish genes encoding an insulin receptor (*Insrb*) was reported to be expressed at the 18-somite stage and both insulin receptor genes were maternally expressed in fertilized eggs[22]. In addition, two insulin genes have been described to be expressed during early zebrafish development[23]. The *Insb* gene has been was shown to be expressed at proliferating blastomeres at 3 and 4 hours post fertilization[23]. Unfortunately, there are no data available about which glucose transporters could be involved in glucose transport during embryogenesis. Using our high-throughput test system we identified the chemical inhibitor NSC-87877 as a potential alternative anti-diabetic drug that was able to revert the diabetic phenotype of the *lepb* mutant at 3 days post fertilization and at earlier stages of development. We established that Shp-1 is the most likely target responsible for the antidiabetic effect of NSC-8787, since knockdown of genes encoding other possible targets of NSC-87877, *ptpn11a*, *ptpn11b* and *dusp26,* could not rescue the *lepb* mutant phenotype. It has been shown that NSC-87877 has a stronger inhibitory effect on the dual phosphatase DUSP26 than on the human SHP-1 protein[24]. The expression pattern of *dusp26* that is restricted to neuroendocrine tissues in zebrafish larvae is in line with the results of our rescue experiments[25]. Finally, we tested the function of leptin in *Xenopus laevis* which is one of the few other vertebrate models in which embryos can easily be manipulated. We showed that in *X. laevis* embryos two leptin proteins play a role in glucose transport during embryogenesis, which indicated that the function of leptin in glucose transport is likely to be translatable to all vertebrates. In conclusion, our high-throughput zebrafish model has potential to identify new anti-diabetic drugs that can be further tested in mammalian studies.

Patients that suffer from TB develop a state which is called wasting syndrome or cachexia that is characterized by severe malnutrition and significant weight loss[26]. This state can lead to secondary health issues, such as fatigue, delayed ambulation, a higher risk of pulmonary embolism and secondary pulmonary infections, which are primary causes of death in TB patients[27]. Glucocorticoids are a class of steroid drugs, which are analogues of the endogenous hormone cortisol that are clinically used as immune-suppressive and anti-inflammatory drugs[28]. It has been shown that glucocorticoids, most likely as a result of their ability to attenuate inflammatory responses, improve the survival of a subset of TB patients, suffering from tuberculous meningitis and pericarditis[29]. In pulmonary TB, adjunctive glucocorticoid therapy is used to prevent complications, but the effect on mortality is not clear[30]. Interestingly, glucocorticoids may also reduce the symptoms of wasting syndrome in TB, but the metabolic effects of glucocorticoids in TB patients have not been elucidated yet.

In **Chapter 5**, we have investigated the effect of glucocorticoid treatment in a zebrafish model for TB after *Mm* infection. Our results showed that treatment with the glucocorticoid beclomethasone results in a higher bacterial burden and a slightly higher mortality. Interestingly, beclomethasone treatment inhibited the infection-induced metabolic changes. The *Mm*-induced increase in glucose levels was abolished and the infection-related decrease

in muscle mass was reduced by the beclomethasone treatment. In contrast, beclomethasone did not have any effect on the muscle mass of the uninfected larvae. Beclomethasone and infection most probably do not have opposite effects on glucose levels and muscle mass, but beclomethasone modulates the metabolic response of the larvae during the infection. The observed inhibition of the metabolic effects was not due to the level of infection itself, since the bacterial burden in the beclomethasone-treated larvae was actually increased. Transcriptome analysis by RNA-seq was performed to study the molecular mechanisms underlying these changes and the results of this analysis suggest that genes involved in glucose metabolism, insulin and leptin signaling play a role in the observed metabolic changes. Gene ontology analysis revealed that many immune- and metabolism-related genes are significantly changed after beclomethasone treatment. Most of the downregulated genes were involved in glucose metabolism, leptin signaling, insulin resistance and the pathogenesis of DM2. Upregulated genes were more associated with other metabolic processes, mainly fat storage and cholesterol metabolism, although some of them are involved in insulin signaling. Furthermore, these data demonstrated that during the mycobacterial infection the larvae are in a state of reduced sensitivity to glucocorticoids, which has previously been associated with TB and other infectious or inflammatory conditions. Taken together, our data show that zebrafish larvae represent an interesting model system to investigate the possibilities for adjunctive glucocorticoid therapy to improve patients' metabolic status. In addition, they form a novel model for studies on the molecular mechanisms underlying glucocorticoid resistance, which is a phenomenon that severely limits the clinical use of these anti-inflammatory drugs.

In conclusion, the research described in this thesis has, using the zebrafish as a model system, shed new light on the intricate relationship between TB and DM2, in particular on the role of leptin, SHP-1 and glucocorticoids. Leptin plays an important role during TB infection and has a huge impact on insulin sensitivity in zebrafish larvae. Similarly to what has been observed in the murine model, leptin deficiency in zebrafish increased the bacterial burden and mortality during the infection, leading to hyperglycemia and the development of insulin resistance. In addition, a novel SHP-1/SHP-2 inhibitor, NSC-87877, was shown to represent a promising anti-diabetic drug that can be used for further DM2 research, as it is able to rescue the phenotype of the leptin-deficient zebrafish and to restore glucose transport to the tissues. In contrast to metformin, NSC-87877 can act at very early developmental stages and inhibits the function of SHP-1 and factors that underly impaired glucose metabolism, whereas metformin is mostly known to improve insulin sensitivity. Additionally, treatment with the glucocorticoid beclomethasone attenuates the metabolic changes associated with the infection, and transcriptional alterations induced by beclomethasone treatment suggest that genes involved in glucose metabolism, insulin and leptin signaling all play an important role in the modulation of the metabolism. Our data show that zebrafish larvae represent an interesting model system to investigate the complex pathology of TB, and the studies described in this thesis in which this model has been used have provided novel insights into

the molecular mechanisms underlying wasting syndrome and the possibilities for adjunctive glucocorticoid therapy to alleviate this metabolic state.

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SAMENVATTING

Tuberculose (tbc) is momenteel één van de meest dodelijke infectieziektes ter wereld en blijft, als een van de tien belangrijkste doodsoorzaken, een grote bedreiging voor de volksgezondheid wereldwijd. Hoewel *Mycobacterium tuberculosis* (Mtb), de ziekteverwekker die tuberculose veroorzaakt, al vele jaren bekend is, is de uitroeiing ervan tot dusver niet gelukt. Een golf van multi drug resistente (MDR) en extreme drug resistente (XDR) stammen van de ziekteverwekker vereist alternatieve vormen van therapieën tegen de ziekte ter ondersteuning van de huidige behandeling. Complexiteit van interactiemechanismen tussen gastheer en ziekteverwekker en metabole aanpassing tijdens het ziekteverloop spelen een belangrijke rol bij het bepalen van de uitkomst van de bacteriële infectie. Deze complexe mechanismen vereisen een bredere benadering van een nieuwe anti-tbc-therapie, bijvoorbeeld door de natuurlijke weerstand van de gastheer te verhogen. Vanwege de gecompliceerde aard van tbc-infectie, richtte het onderzoek dat in dit proefschrift wordt beschreven zich op verschillende aspecten van insulineresistentie en glucosemetabolisme tijdens de infectie. We gebruikten het zebravislarvenmodel dat verschillende voordelen heeft voor het modelleren van aandoeningen en het mogelijk maakt om de functie van het aangeboren immuunsysteem los van het adaptieve immuunsysteem te bestuderen. De transparantie, snelle ontwikkeling en mogelijkheid tot genetische manipulatie maken zebravislarven perfecte modelorganismen voor ons onderzoek.

Eiwittyrosinefosfatasen (PTP's) zijn factoren die een belangrijke rol spelen bij veel cellulaire processen zoals celoverleving, migratie en immuunresponsen. Er zijn vier klassen waarvan klasse I de grootste groep is die niet-receptor-PTP's bevat. Het eiwit tyrosinefosfatase non-receptor type 6 (*PTPN6*) gen komt voornamelijk tot expressie in het hematopoëtische systeem, maar expressie is ook waargenomen in epitheelcellen. Het product van dit gen, SHP-1, speelt een rol in veel verschillende signaalroutes. Het is een belangrijke regulator van myeloïde cellen, cytokinereceptoren, groeifactorreceptoren en immunoreceptoren. ptpn6 knockdown bij zebravissen leidt tot immuunsuppressie, ernstig oedeem en huidlaesies, vergelijkbaar met de bevindingen in muizen Ptpn6-mutanten me, mev en spin.

In **Hoofdstuk 2** van dit proefschrift vergeleken we de effecten van ptpn6 morfolino knockdown en de kleine molecule Shp-1 remmer NSC-87877 waarvan eerder was aangetoond dat het humaan SHP-1 in vitro remt. Beide methoden werden gebruikt op zebravisembryo's die waren geïnfecteerd met *Mycobacterium marinum* (Mm), een naaste verwant van Mtb. De resultaten van deze studie toonden aan dat verminderde SHP-1-activiteit het vermogen van zebravisembryo's om de mycobacteriële infectie onder controle te houden, schaadt. Bovendien was de progressie van infectie sneller na zowel morfolino- als NSC-87877-behandeling in vergelijking met de controlebehandeling, waarbij dit effect ernstiger was na de knockdown van *ptpn6* morfolino dan na de remming van NSC-87877. Dit verschil kan worden veroorzaakt door remming van het verwante eiwitfosfatase Ptpn11 door NSC-87877,

aangezien dit fosfatase in muizencellen een negatieve rol speelt bij celoverleving tijdens ontsteking en apoptose versnelt door defosforylering van STAT5. Bovendien werd RNA-sequencing (RNAseq)-analyse uitgevoerd met behulp van RNA geïsoleerd uit hele geïnfecteerde larven. Deze analyse toonde aan dat verminderde SHP-1-activiteit resulteert in significante veranderingen in de expressieniveaus van genen die betrokken zijn bij processen zoals synaptische transmissie, ionentransport, celstructuur, proteolyse, de immuunrespons, apoptose en gonadotropinesecretie. Het is vermeldenswaard dat zowel de remming door NSC-87877 als de knockdown van *ptpn6* morfolino resulteerde in opregulatie van immuungerelateerde genen. Toch werden er enkele verschillen tussen de twee behandelingen gevonden. De knockdown van *ptpn6* morfolino had meer invloed op de expressie van metabole genen dan remming door NSC-87877. Bovendien werd de expressie van een groot aantal infectie-gereguleerde genen niet beïnvloed door de morfolino- of de remmerbehandeling. Deze gegevens laten zien dat beide methoden voor het remmen van de SHP-1 functie het verloop van de mycobacteriële infectie veranderen. Bovendien vormt de set genen die dezelfde transcriptionele respons vertoonden op de morfolino- en de remmerbehandeling een bruikbare referentieset die specifiek kan worden gekoppeld aan de functie van SHP-1 en ondersteunt verder onderzoek naar de NSC-87877 in vergelijking met andere SHP-1 remmers.

Leptine is een eiwit dat zowel als een pleiotroop hormoon als een cytokine werkt. Als hormoon reguleert het de energiehomeostase, voedselinname, insulinesecretie, angiogenese, botvorming en reproductie. Mutaties die functionele leptinedeficiëntie bij mensen veroorzaken, zijn zeldzaam en kunnen worden behandeld met leptinesuppletie, maar als ze niet worden behandeld, kunnen ze ernstige obesitas, insulineresistentie en overlijden veroorzaken. Bij muizen leiden mutaties in het gen dat codeert voor leptine of de leptinereceptor (*Lep* of *Lepr*) tot meerdere metabole veranderingen, resulterend in ernstige obesitas en insulineresistentie. Deze muizen zijn met name ook vatbaarder voor tuberculose, wat op zijn beurt leidt tot verhoogde leptinespiegels in de longen, die hun hoogste punt twee weken na infectie bereiken. Bovendien werd een verhoogde bacteriële aanwezigheid gevonden in de longen van geïnfecteerde muizen met een mutatie in het *Lep*-gen (*ob/ob* muizen), wat gepaard ging met een significant hogere mortaliteit.

In **Hoofdstuk 3** hebben we de rol van het *lepb* gen als immuunmodulator onderzocht door het opzetten van een zebravislarvaal modelsysteem om het effect van leptinedeficiëntie op tbc-infectie te bestuderen. Het genoom van de zebravis bevat twee leptine-genen: leptine a (*lepa*) en leptine b (*lepb*). Hoewel de twee eiwitten slechts 24% van hun aminozuren gemeen hebben en slechts 18% identiek zijn aan humaan leptine, delen ze een karakteristieke genstructuur en functie met hun zoogdierorthologen. De twee leptine genen komen differentieel tot expressie: bij volwassenen komt lepa voornamelijk tot expressie in de lever, terwijl lepb tot expressie wordt gebracht in de eierstokken. Eerdere studies toonden een hoge inductie van de expressie van het *lepb*-gen in larvale zebravissen tijdens bacteriële infectie

met *Staphylococcus epidermidis* en *Mm*, evenals een sterke opregulatie na *ptpn6* knockdown. Met behulp van morfolino knockdown en een CRISPR/Cas9-gegenereerde mutante vislijn, bestudeerden we het effect van leptine-signalering in *Mm* geïnfecteerde zebravisembryo's. Morfolino onderzoeken toonden aan dat knockdown van zowel lepb als lepr leidde tot een hogere mortaliteit en een sneller verloop van infectie. Aan de andere kant had knockdown van *lepa* geen significante invloed op het verloop van de infectie. We ontdekten dat het effect van de door CRISPR/Cas9 gegenereerde lepb-mutatie ernstiger was dan na de morfolino-knockdown, vooral na een dooierinfectie bij 4 hpf. Verder zagen we een lichte afname van het aantal macrofagen in de *lepb* mutant larven, terwijl het aantal neutrofielen niet was veranderd. In de *lepb* mutant werd de expressie van twee pro-inflammatoire genen, *irg1l* en *il1b*, na de infectie gedownreguleerd. Injectie van een humaan recombinant leptine eiwit redde echter gedeeltelijk het fenotype van de geïnfecteerde lepb-mutante larven: de bacteriële belasting in de geïnfecteerde leptine-mutant nam af en de *irg1l* expressie nam toe. Bij muizen is ook aangetoond dat leptine de expressie van *il1b* reguleert, en de deficiëntie ervan leidt tot immunosuppressie en downregulatie van pro-inflammatoire cytokines en markers. Daarnaast hebben we getest hoe glucosespiegels veranderen tijdens mycobacteriële infectie in wildtype en lepb mutant zebravislarven. We ontdekten dat na *Mm* infectie de glucoseconcentratie significant was verhoogd in wildtype embryo's, terwijl lepb-mutante larven zowel voor als na de infectie veel hogere glucosespiegels vertoonden. Alles bij elkaar genomen geven onze resultaten aan dat het leptine b eiwit een belangrijke rol speelt in de afweer van de gastheer tijdens de vroege stadia van een mycobacteriële infectie, wat de rol van leptine in de immuunafweer tegen tbc onderstreept. Deze resultaten ondersteunen ook een verdere verkenning van de mogelijkheden voor de ontwikkeling van nieuwe gastheergerichte behandelmethoden tegen tbc.

Zoals hierboven vermeld, is leptine ook uitgebreid bestudeerd bij obesitas en diabetes mellitus type 2 (DM2), omdat het aangetoond is dat het een leidende rol speelt in de energiehomeostase van het hele lichaam . De moleculaire mechanismen waarmee leptine de insulineresistentie regelt, zijn echter grotendeels onbekend. Er is onderzoek gedaan naar insulineresistentie en DM2 op menselijke celculturen en in verschillende diermodellen, voornamelijk knaagdieren. De mutante muizenstammen van de leptine- en leptinereceptorgenen, respectievelijk de *ob/ob*- en *db/db*-stammen, vertonen diabetische fenotypes en zijn daarom zeer nuttig voor diabetesonderzoek. Bovendien zijn vetrijke dieetmuizen, Zucker-vette ratten en Zucker-diabetische vette ratten op grote schaal gebruikt om DM2, obesitas en de functie van leptine signalering bij het metabool syndroom te bestuderen. Zebravismodellen zijn gebruikt als alternatieve testsystemen voor het bestuderen van insulineresistentie en DM2 omdat ze meerdere mogelijkheden bieden om metabole ziekten te onderzoeken, met behulp van talrijke transgene en knockoutlijnen . Bij volwassen zebravissen spelen de leptine en de leptinereceptor een behouden rol in de glucosehomeostase, maar ze hebben geen invloed op het vetweefsel. Hoewel er al diabetesmodellen bestaan bij volwassen zebravissen, is er vraag naar een alternatief

larvalemodel in een vroeg stadium, dat het mogelijk zou maken om snelle en grootschalige screeningsassays uit te voeren. Eerder onderzoek heeft aangetoond dat zebravislarven een veelbelovend modelsysteem vormen om DM2 te bestuderen onder niet-voedende omstandigheden. Shp-1 werkt, zoals eerder vermeld, als een negatieve immuunmodulator, maar het is ook een sleutelfactor in insulineresistentie.

We hebben de rol van leptine b en SHP-1 op insulineresistentie bij vroege zebravislarven geanalyseerd in **Hoofdstuk 4**, door een nieuwe high-throughput methode te ontwikkelen om antidiabetica te testen. De screening was mogelijk vanwege het feit dat leptine deficiënte zebravislarven volledig insulineresistent waren en al in een zeer vroeg stadium van hun ontwikkeling een diabetisch fenotype vertoonden. We toonden ook aan dat metformine, hoewel zeer effectief voor de behandeling van dit diabetische fenotype bij 4 dagen oude zebravislarven, geen antidiabetische effecten had in eerdere stadia van ontwikkeling. Deze bevinding gaf aan dat insulinereceptoren functioneel zijn in de zeer vroege stadia van embryogenese, wat ook werd bevestigd toen we het effect bestudeerden van het injecteren van humane recombinante insuline in de dooierzak. Een van de zebravisgenen die coderen voor een insulinereceptor (*Insrb*) werd naar verluidt tot expressie gebracht in het 18-somietenstadium en beide insulinereceptorgenen werden door de moeder tot expressie gebracht in bevruchte eieren. Bovendien is beschreven dat twee insulinegenen tot expressie worden gebracht tijdens de vroege ontwikkeling van de zebravis . Het is aangetoond dat het Insb-gen tot expressie wordt gebracht bij prolifererende blastomeren 3 en 4 uur na de bevruchting. Helaas zijn er geen gegevens beschikbaar over welke glucosetransporters betrokken zouden kunnen zijn bij het transport van glucose tijdens de embryogenese. Met behulp van ons high-throughput testsysteem identificeerden we de chemische remmer NSC-87877 als een potentieel alternatief antidiabetisch medicijn dat in staat was om het diabetische fenotype van de *lepb*-mutant 3 dagen na de bevruchting en in eerdere stadia van ontwikkeling om te keren. We hebben vastgesteld dat Shp-1 het meest waarschijnlijke doelwit is dat verantwoordelijk is voor het antidiabetische effect van NSC-8787, aangezien het uitschakelen van genen die coderen voor andere mogelijke doelwitten van NSC-87877, *ptpn11a*, *ptpn11b* en *dusp26*, het *lepb*-mutante fenotype niet kon redden. Er is aangetoond dat NSC-87877 een sterker remmend effect heeft op het dubbele fosfatase DUSP26 dan op het menselijke SHP-1 eiwit. Het expressiepatroon van *dusp26* dat beperkt is tot neuro-endocriene weefsels in zebravislarven is in overeenstemming met de resultaten van onze reddingsexperimenten . Ten slotte hebben we de functie van leptine getest in *Xenopus laevis*, een van de weinige andere gewervelde modellen waarin embryo's gemakkelijk kunnen worden gemanipuleerd. We toonden aan dat in *X. laevis* embryo's twee leptine eiwitten een rol spelen bij het transport van glucose tijdens de embryogenese, wat erop wijst dat de functie van leptine bij het transport van glucose waarschijnlijk vertaalbaar is naar alle gewervelde dieren. Concluderend kan worden gesteld dat ons zebravismodel met hoge doorvoer het potentieel heeft om nieuwe antidiabetica te identificeren die verder kunnen worden getest in zoogdierstudies.

Patiënten die aan tuberculose lijden, ontwikkelen een toestand die verspillend syndroom of cachexie wordt genoemd en die wordt gekenmerkt door ernstige ondervoeding en aanzienlijk gewichtsverlies. Deze toestand kan leiden tot secundaire gezondheidsproblemen, zoals vermoeidheid, vertraagd lopen, een hoger risico op longembolie en secundaire longinfecties, die primaire doodsoorzaken zijn bij tbc patiënten. Glucocorticoïden zijn een klasse van steroïde geneesmiddelen, die analogen zijn van het endogene hormoon cortisol en die klinisch worden gebruikt als immuunonderdrukkende en ontstekingsremmende geneesmiddelen. Het is aangetoond dat glucocorticoïden, hoogstwaarschijnlijk als gevolg van hun vermogen om ontstekingsreacties te verzwakken, de overleving verbeteren van een subgroep van tbc-patiënten die lijden aan tuberculeuze meningitis en pericarditis. Bij longtuberculose wordt adjuvante behandeling met glucocorticoïden gebruikt om complicaties te voorkomen, maar het effect op de mortaliteit is niet duidelijk. Interessant is dat glucocorticoïden ook de symptomen van het wasting-syndroom bij tbc kunnen verminderen, maar de metabole effecten van glucocorticoïden bij tbc-patiënten zijn nog niet opgehelderd.

In **Hoofdstuk 5** hebben we het effect onderzocht van behandeling met glucocorticoïden in een zebravismodel voor tuberculose na *Mm* infectie. Onze resultaten toonden aan dat behandeling met het glucocorticoïde beclomethason resulteert in een hogere bacteriële belasting en een iets hogere mortaliteit. Interessant is dat behandeling met beclomethason de door infectie veroorzaakte metabole veranderingen remde. De *Mm* geïnduceerde stijging van de glucosespiegels werd teniet gedaan en de infectiegerelateerde afname van de spiermassa werd verminderd door de behandeling met beclomethason. Daarentegen had beclomethason geen effect op de spiermassa van de niet-geïnfecteerde larven. Beclomethason en infectie hebben hoogstwaarschijnlijk geen tegengestelde effecten op glucosespiegels en spiermassa, maar beclomethason moduleert de metabole respons van de larven tijdens de infectie. De waargenomen remming van de metabole effecten was niet te wijten aan het infectieniveau zelf, aangezien de bacteriële belasting in de met beclomethason behandelde larven juist was verhoogd. Transcriptoomanalyse door RNA-seq werd uitgevoerd om de moleculaire mechanismen te bestuderen die ten grondslag liggen aan deze veranderingen en de resultaten van deze analyse suggereren dat genen die betrokken zijn bij glucosemetabolisme, insuline en leptinesignalering een rol spelen in de waargenomen metabole veranderingen. Genontologie analyse onthulde dat veel immuun- en metabolisme-gerelateerde genen significant veranderd zijn na behandeling met beclomethason. De meeste van de gedownreguleerde genen waren betrokken bij het glucosemetabolisme, leptinesignalering, insulineresistentie en de pathogenese van DM2. Opgereguleerde genen waren meer geassocieerd met andere metabolische processen, voornamelijk vetopslag en cholesterolmetabolisme, hoewel sommige van hen betrokken zijn bij insulinesignalering. Bovendien toonden deze gegevens aan dat tijdens de mycobacteriële infectie de larven in een staat van verminderde gevoeligheid voor glucocorticoïden verkeren, wat eerder in verband werd gebracht met TB en andere infectieuze of inflammatoire aandoeningen. Samengevat

laten onze gegevens zien dat zebravislarven een interessant modelsysteem vormen om de mogelijkheden voor aanvullende glucocorticoïde therapie te onderzoeken om de metabole status van patiënten te verbeteren. Bovendien vormen ze een nieuw model voor studies naar de moleculaire mechanismen die ten grondslag liggen aan resistentie tegen glucocorticoïden, een fenomeen dat het klinische gebruik van deze ontstekingsremmende geneesmiddelen ernstig beperkt.

Concluderend heeft het onderzoek beschreven in dit proefschrift, waarbij de zebravis als modelsysteem is gebruikt, nieuw licht geworpen op de ingewikkelde relatie tussen TB en DM2, in het bijzonder op de rol van leptine, SHP-1 en glucocorticoïden. Leptine speelt een belangrijke rol bij tbc infectie en heeft een enorme invloed op de insulinegevoeligheid van zebravislarven. Net als wat is waargenomen in het muizenmodel, verhoogde leptinedeficiëntie bij zebravissen de bacteriële belasting en mortaliteit tijdens de infectie, wat leidde tot hyperglykemie en de ontwikkeling van insulineresistentie. Bovendien werd aangetoond dat een nieuwe SHP-1/SHP-2-remmer, NSC-87877, een veelbelovend geneesmiddel tegen diabetes is dat kan worden gebruikt voor verder DM2-onderzoek, omdat het in staat is het fenotype van de leptine-deficiënte te redden. zebravis en om het transport van glucose naar de weefsels te herstellen. In tegenstelling tot metformine kan NSC-87877 in zeer vroege ontwikkelingsstadia werken en remt het de functie van SHP-1 en factoren die het glucosemetabolisme ondermijnen, terwijl het vooral bekend is dat metformine de insulinegevoeligheid verbetert. Bovendien vermindert de behandeling met het glucocorticoïde beclomethason de metabole veranderingen die gepaard gaan met de infectie, en transcriptieveranderingen die worden veroorzaakt door de behandeling met beclomethason suggereren dat genen die betrokken zijn bij het glucosemetabolisme, insuline en leptinesignalering allemaal een belangrijke rol spelen bij de modulatie van het metabolisme. Onze gegevens laten zien dat zebravislarven een interessant modelsysteem vormen om de complexe pathologie van TB te onderzoeken, en de studies beschreven in dit proefschrift waarin dit model is gebruikt, hebben nieuwe inzichten opgeleverd in de moleculaire mechanismen die ten grondslag liggen aan het cachexie metabool syndroom en de mogelijkheden voor adjuvante glucocorticoïde therapie om deze metabolische toestand te verlichten.

List of Abbreviations

AB/TL: AB/Tupfel long fin
BMI: body mass index
Cas9: CRISPR associated protein 9
CFU: colony forming units
COPAS: Complex Object Parametric Analyzer and Sorter
CRISPR: Clustered regularly interspaced short palindromic repeats
DAVID: The Database for Annotation, Visualization and Integrated Discovery
DM: diabetes mellitus
DMSO: Dimethyl sulfoxide
dpf: days post fertilization
dpi: days post infection
hpf: hours post fertilization
HRL: human recombinant leptin
lep: leptin
Mm/tb: Mycobacterium marinum/ tuberculosis
MO: morpholino
qPCR: quantitative PCR
PTPN: Tyrosine Phosphatase, Non-Receptor Type
PTPs: protein tyrosine phosphatases
T2DM: type 2 diabetes mellitus
TB: tuberculosis
Tg: transgenic
WT: wild-type

Curriculum vitae

Natalia Nowik was born in Olsztyn, Poland on August 31, 1987. After graduating from No. III High school in Olsztyn in 2006, she started a 6-years program in veterinary medicine at the Faculty of Veterinary Medicine, University of Warmia and Mazury (UWM) in Olsztyn.

In 2009, during the second year of the studies, she started a one year internship at the Laboratory of Genomics and Transcriptomics under the supervision of dr. Piotr Podlasz, where she conducted research on the role of the neuropeptide galanin using zebrafish as the model organism. She was awarded a prize for her scientific work as a young researcher in a poster session of Veterinary Medicine and Animal Based Products Section for young scientists.

After graduation in 2012, she moved to the Netherlands, where she worked as a veterinarian and a food production specialist. In 2014 she joined dr. Podlasz's group again and after a few months, in 2015, she started her scientific project, as a guest researcher, at dr. Spaink's lab. One year later she was admitted as a Ph.D. candidate at the Institute of Biology, Leiden University. In 2017 she won a grant, PRELUDIUM 11, funded by the Polish National Science Centre that is a prestigious funding opportunity intended for pre-doctoral researchers about to embark on their scientific career. The work done during her time at Leiden University is presented in this thesis.

List of Publications

Nowik N, Prajsnar TK, Przyborowska A, Rakus K, Sienkiewicz W, Spaink HP, Podlasz P. The Role of Galanin during Bacterial Infection in Larval Zebrafish. *Cells*. 2021 Aug 6;10(8):2011. doi: 10.3390/cells10082011.

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Acknowledgments

It would be impossible to write this thesis without help of many people that supported me in many different ways during my PhD project.

Foremost, I would like to thank my promotors prof. dr. Herman Spaink and dr. Marcel Schaaf for supporting and helping me during all this time. Thank you, Herman, for giving me this chance and supervising me through all these years. As your PhD student, I really appreciate your positive attitude and professionalism. You are not only a prominent researcher, but also a great person. Marcel, you are a really dedicated supervisor that takes all the responsibilities very serious. Thank you for all your help and mentoring. Your professionalism and friendly attitude makes coo peration with you a pleasure.

I would like to thank prof. dr. Annemarie Meijer and prof. dr. Ewa Snaar-Jagalska for their support and scientific discussions during our weekly meetings. Your suggestions and ideas had a substantial contribution to my scientific work.

When I have started my work at IBL, the first person that I met was Wouter whom I want to thank for being my mentor during the first months of my stay. Thanks to you, I have learnt all the necessary techniques that helped me to build up my scientific work presented in this thesis. Arwin, Claudia and Lanpeng, thanks to you my first days in Leiden went smoothy, and I immediately felt welcomed. I would also like to thank all the people in the lab: Shuxin, Yasuhito, Quanchi, Kelly, Hurzos, Elena, Samrah, Vincenzo, Rui, Tomek, Bjørn, Natalia, Ralf, Monica, Yufei, Radek, Salome, Gabriel, Mahmoud, Marlon, Francisco (Panchito) and Michiel. Min and Hilda thank you for our scientific and non-scientific chats when we were sitting in the same office. I still miss those days and your companionship. I cannot skip Saskia who has always been kind and helpful. I am still missing her stories about life in Uganda; I wish these memories were published one day. Thank you Retno and Cikra for our time together. It was a pleasure working with you. And finally, thank you Wanbin and Yi for being in one team with me. You are very talented young researchers, do not miss your opportunities.

I am deeply grateful for my students, I mentored during my PhD. This experience ta ught me a lot, not only about science, but also about myself. It was not an easy task as we had many ups and downs, but I hope that you all remember it as a good time. Many thanks Charlotte, Joyce, Monisha and Roxan. I wish you all the best in the future.

My scientific work could not be finished without the zebrafish team. Karin, Guus, Urlike, Michelle, Corrina you are a fantastic people. Thank you for all the fun that we had together.

My sincere thanks go to dr. Piotr Podlasz who showed me the zebrafish w orld and, without whom, I would not be where I am now.

PhD is not only about science, but also friendship. Thank you, Frida, for being there with me through all these years. You were (and still are) my mental support during hard times. I wish we could work together in the future again.

I am grateful to all members of my Promotion Committee for reviewing my manuscript. Piotr thank you very much for the extensive feedback that you provided.

Last but not least, I would like to thank those closest to me. Mom and Dad, thank you for your love and for believing in me. Pawel you are not only my husband, but also my best friend. I am grateful for your support and love, I would not achieve it without you. I would like to thank my sister Paulina for always being there for me and my daughter Lena for showing me different aspects of life.