CHARACTERIZATION OF MYCOBACTERIUM MARINUM-INDUCED GRANULOMAS IN ZEBRAFISH AND THE ROLE OF FATTY ACID ACCUMULATION AND HYPOXIA IN ESX-5-MEDIATED HYPERVIRULENCE

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Human granulomas induced by *M. tuberculosis* are highly organized structures that can contain dormant mycobacteria for decades. Several animal models have been employed to study tuberculosis (TB) and granulomas. In this study, we characterized granulomas formed in adult zebrafish upon infection with the fish pathogen *M. marinum*, and found a high degree of similarity with human TB lesions. The macrophage core of the *M. marinum*-induced granulomas was found to contain epithelioid and multinucleated giant cells and we could show a ring of CD4+ T cells, including FoxP3+ regulatory cells, surrounding the structure. In addition, we established that *M. marinum*-induced granulomas in zebrafish can be hypoxic, a prerequisite for mycobacterial dormancy. Dormancy is associated with an accumulation of TAG, composed of (host-derived) fatty acids. Interestingly, we found that a hypervirulent strain of *M. marinum* that is impaired in ESX-5-mediated protein secretion is significantly reduced in its ability to import extracellular fatty acids. Fatty acid uptake could partially be restored by rerouting the ESX-5-dependent lipase LipY to the ESX-1 secretion system, leading to expression of this protein on the bacterial cell surface of ESX-5-deficient *M. marinum*. However, rerouting LipY did not revert the hypervirulent phenotype in adult zebrafish, indicating that other ESX-5-dependent factors are involved in this process.
INTRODUCTION

The causative agent of tuberculosis, *Mycobacterium tuberculosis*, is able to persist in the human body for decades, without being eradicated or causing disease. During this latent infection, bacteria are maintained within granulomas that are mainly present in the lungs. A fine balance between bacterial growth and antibacterial mechanisms of the host exists in these organized immune structures. Granulomas consist of a core of macrophages, some of which are harboring bacilli. Among these macrophages are epithelioid cells, lipid-rich foamy macrophages and multinucleated giant cells [1].

The macrophage core is surrounded by a layer of fibroblasts that can form a fibrous cuff, and finally a region enriched for CD4+ and CD8+ T lymphocytes. Particularly the CD4+ T cells in this layer are important for limiting bacterial growth and maintaining the granuloma structure [2]. Among the CD4+ T cells, a subpopulation of regulatory CD25+FoxP3+ T cells has been described to accumulate in lung granulomas [3]. There, these cells are thought to limit pathology-causing inflammatory processes. Within one infected individual, several granuloma types can be found at the same time. Solid granulomas, which mainly consist of macrophages and a limited number of lymphocytes, coexist with necrotic granulomas. Mycobacteria often reside extracellularly in the hypoxic center of this second lesion type, which consists of necrotic, sometimes caseous material, derived from dead macrophages. When latent TB progresses into active disease, the granuloma becomes increasingly necrotic. Due to increased oxygen levels that result from cavity formation, bacterial numbers rise.

The granuloma eventually ruptures, leading to the release of bacteria into the lung airways and transmission of *M. tuberculosis* to a new host.

TB pathogenesis and granuloma formation have been well described in human patients. To faithfully reproduce and study the specific characteristics of these processes, several different animal models have been developed. However, their resemblance to human TB varies substantially. Mice, which have been used for most studies and for which many tools are available, normally do not develop chronic disease and lack well-organized, hypoxic granulomas [4]. Recently, mouse strains that develop granulomas with more resemblance to those formed in humans have been described [5]. Although disease progression in guinea pigs and rabbits is more similar to human TB, molecular tools and reagents are missing for these models [6]. Experiments in non-human primates best reproduce human granulomas, but are expensive and difficult to perform [7]. A relatively new model organism that has gained popularity over the last years is the zebrafish. Infection with *Mycobacterium marinum*, a close relative of *M. tuberculosis* and a natural pathogen of ectothermic animals such as fish, leads to a course of disease that is comparable to human TB [8,9]. Infected zebrafish have been shown to develop latent mycobacterial disease with necrotizing granulomas in their organs [10,11]. In addition, transparent zebrafish embryos are increasingly employed to study the process of early granuloma formation [12,13]. The rapid development of new tools, ease of genetic manipulation and low costs make the zebrafish model an attractive alternative to the current ones for studying mycobacterial disease.
During the initiation of latent disease, *M. tuberculosis* undergoes a number of transcriptional changes that allows it to persist in the hypoxic and nutrient-limited environment of the granuloma. These dormant bacteria have become metabolically inactive, do not, or minimally, replicate and accumulate lipids as a storage of energy [14]. In addition, they develop tolerance to many antibiotics, making it extremely difficult to reach complete clearance during latent infection. One of the key regulators that initiates these structural changes is DosR. In response to hypoxia, this transcription factor regulates the induction of a set of 48 genes, also known as the DosR regulon [15]. Many of the DosR-regulated genes encode factors involved in metabolic adaptation to anaerobic conditions. Several of these proteins are involved in lipid metabolism. One of them is Tgs1, which is essential for the accumulation of triacylglycerol (TAG), a critical event during dormancy [16]. Another hypoxia-induced, although DosR independent protein that is involved in lipid metabolism is the PE family protein LipY [17-19]. LipY, which contains a lipase domain, is the most active mycobacterial enzyme involved in the hydrolysis of long-chain TAG into single fatty acids [18]. As the lipY gene is up-regulated under hypoxic conditions, hydrolysis of stored TAG by LipY has been proposed to play a role in energy utilization during dormancy [17]. In support of this theory, an *M. tuberculosis* lipY mutant is attenuated in its capacity to hydrolyze stored TAG during dormancy-mimicking conditions [18]. Interestingly, LipY is the only of 24 putative lipases encoded in the *M. tuberculosis* genome that contains a PE domain. Many PE and PPE family proteins are secreted or transported to the cell surface via the type VII secretion (T7S) system ESX-5 [20]. Also for LipY of *M. tuberculosis* and *M. marinum*, which in the latter contains a PPE instead of PE domain, ESX-5-dependent cell surface localization has been shown [21]. Because of its presence on the bacterial cell surface, LipY may not only be involved in the hydrolysis of bacterial TAG stored in the cytoplasm, but also in the hydrolysis of TAG from host macrophages during infection. In line with this, it was recently shown that the accumulation of TAG in *M. tuberculosis* during a macrophage infection was mainly due to *de novo* synthesis of TAG from imported fatty acids derived from the host [17]. LipY may therefore play a dual role in energy-related processes, by facilitating uptake of fatty acids from the host and by liberating fatty acids from bacterial storages.

In absence of ESX-5, LipY and many other PE and PPE proteins cannot be transported to the cell surface [20-22]. Recently, we found that blocking ESX-5 secretion leads to hypervirulence of *M. marinum* in adult zebrafish [11]. Bacterial numbers and granuloma formation increase rapidly in fish infected with an ESX-5-deficient strain, leading to a considerable increase in disease progression and early death of the host. This mutant displays normal virulence in macrophages and zebrafish embryos, demonstrating that the observed effects are specific for adult fish. As we could rule out a role for the adaptive immune system by using rag1 mutant zebrafish, we hypothesized that ESX-5-deficient mycobacteria may not be able to go into dormancy during infection of adult zebrafish. In this study, we followed up on this hypothesis. First we characterized the granulomas that are formed in adult zebrafish upon *M. marinum* infection. We found a high degree of similarity with human TB granulomas, including their hypoxic nature,
which would enable *M. marinum* to become dormant. By studying the characteristics of ESX-5-deficient *M. marinum* under dormancy-inducing conditions, we found that this strain is indeed impaired in its ability to import fatty acids. Moreover, we established a role for the C-terminal domain of LipY in this process by redirecting LipY to the ESX-1 secretion system. These experiments simultaneously show that ESX-substrates can be rerouted for secretion via a different T7S system, by replacing the N-terminal domain. However, redirection of LipY did not reverse the hypervirulent phenotype of ESX-5-deficient *M. marinum*, indicating that factors other than LipY-mediated fatty acid import are involved in ESX-5-associated hypervirulence.

**RESULTS**

*M. marinum*-infected zebrafish develop well-organized granulomas that are surrounded by a ring of T cells

Previously, we have shown that an *M. marinum* mutant impaired in ESX-5-mediated protein secretion is hypervirulent and induces early onset of granuloma formation in adult zebrafish [11]. The organization of these granulomas does not seem to differ from those induced by wild-type bacteria at a later stage. In order to analyze whether the granulomas induced in zebrafish are structurally similar to those formed in the human body in response to *M. tuberculosis* infection, we examined histological sections of zebrafish infected for 4 weeks with the ESX-5-deficient hyper-granulomatous *M. marinum* espG₅ transposon mutant strain. We found that granulomas were mainly present in the pancreas, liver and spleen of infected fish (Fig. 1A). Antibody staining demonstrated the presence of a population of CD4+ T cells restricted to the outer layer of the granuloma (Fig. 1A and B). FoxP3+ cells were also found in this area, indicating the presence of regulatory cells among the T helper cells (Fig. 1B). A similar staining pattern was observed for granulomas in zebrafish that developed during a prolonged time period of infection with the *M. marinum* wild-type strain (Fig. S1). A close examination of the inner layer of macrophages surrounding the necrotic center revealed the presence of multinucleated giant cells and epithelioid macrophages (Fig. 1C). In addition, we observed that granulomas with necrotic centers as well as solid granulomas were present at the same time, indicating heterogeneity in developmental stages (Fig. 2). Since fish were infected with *M. marinum* strains expressing the red fluorescent protein mCherry, localization of mycobacteria within tissues could be determined by performing fluorescent microscopy on zebrafish sections. This revealed the presence of a high amount of bacteria in the caseous necrotic center of the granuloma (Fig. 2B), which is also observed in progressive *M. tuberculosis* infection in human tissues [28]. In solid granulomas bacteria could not be detected with fluorescence microscopy (Fig. 2A), indicating that in these lesions the number of bacteria is relatively small. Together, our data shows that granulomas formed upon *M. marinum* infection in zebrafish have a similar stratified composition as those induced by *M. tuberculosis* in human patients, with a central (necrotic) core surrounded by a layer of macrophages containing epithelioid and multinucleated giant cells, and finally a ring of T lymphocytes. The structure of these granulomas in combination with the chronic nature of the infection and accompanying
pathology indicates that infection with *M. marinum* in zebrafish resembles the human situation during infection with *M. tuberculosis*.

*M. marinum* granulomas in zebrafish are hypoxic
Within granulomas, mycobacteria often reside in a dormant state, which is characterized by low metabolic activity and low or non-replicating persistence. An important inducer
of dormancy is hypoxia. The granuloma provides such a hypoxic environment, as has been shown for several animal models upon *M. tuberculosis* infection [27]. Although we established several structural similarities between human and zebrafish granulomas, it is unknown whether the granulomas in chronically infected fish are also hypoxic, allowing dormancy. Therefore, we employed the hypoxia marker pimonidazole to visualize oxygen content *in vivo*. This probe becomes active in hypoxic cells, leading to adduct formation. Pimonidazole was injected in chronically infected zebrafish 24 hours prior to sacrifice. By antibody staining of adducts in sectioned zebrafish, we found that the *M. marinum*-induced granulomas in adult zebrafish are hypoxic (Fig. 3A and B). Adduct staining was only observed in granulomatous lesions that contained mycobacteria (Fig 3C), indicating specific localization of hypoxic areas. Therefore, we can conclude that granulomas induced by *M. marinum* can be hypoxic and create an environment that enables bacterial dormancy.

**ESX-5-deficient *M. marinum* is impaired in fatty acid uptake**

Previously, we found that during infection of zebrafish with an *M. marinum* espG5 mutant strain, bacterial numbers increase significantly over a relatively short time period [11]. Possibly, these bacteria are unable to enter the dormant state but continue to multiply and induce acute disease. Dormant, hypoxic mycobacteria are known to accumulate TAG, composed of fatty acids imported from the host cell [17,29]. We hypothesised that hypoxia and lipid accumulation might be required for mycobacteria to go into dormancy and that the ESX-5-deficient *M. marinum* espG5 mutant strain is unable to accumulate
TAG. To test this hypothesis, we incubated *M. marinum* strains with fluorescently-labelled fatty acids under hypoxic conditions and determined mycobacterial accumulation of these lipids by fluorescence microscopy. We found that the fluorescence intensity was highly decreased in the espG₅ mutant, which indicates that these bacteria are impaired in fatty acid import from the extracellular environment (Fig. 4A and B). Complementation of ESX-5 function by introduction of the interrupted espG₅ gene restored fatty acid uptake, indicating that this process is mediated by ESX-5-dependent proteins.

**ESX-5-deficiency does not lead to an altered intracellular TAG content**

Inside mycobacteria, fatty acids are converted into bacterial TAG, which is the main source of energy during dormancy. We questioned whether the impaired ability of ESX-5-deficient *M. marinum* to incorporate external fatty acids, affects intracellular TAG levels. To study this, we grew bacterial strains under normoxic and hypoxic conditions and performed TLC analysis. We observed a clear increase in TAG content during growth under low oxygen levels as compared to normoxic conditions (Fig. 5), which is characteristic for dormant bacteria. However, our results did not show major

**Figure 3.** *M. marinum*-induced granulomas in zebrafish are hypoxic. A. Hydroxyprobe-1-stained section of *M. marinum*-infected zebrafish injected with PIMO. Hypoxic areas within zebrafish tissue appear in brown color. Inset of granuloma is enlarged in (B), and (C) Ziehl-Neelsen staining shows the presence of mycobacteria within the same granuloma. Black arrows point to mycobacteria (in purple color), white arrows indicate border of the granuloma D. HE-staining of the granuloma.
differences in intracellular TAG content between wild-type and espG5 mutant strains. These data show that ESX-5 has no major effect on intracellular TAG levels, suggesting that ESX-5-dependent import of extracellular fatty acids does not contribute to the accumulation of TAG under the conditions tested.

**Bacterial growth and gene expression of the dormancy regulon are not affected by ESX-5-deficiency under hypoxic conditions**

Under hypoxic conditions, macrophages accumulate TAG that can be taken up by mycobacteria as fatty acids during infection [17]. This coincides with an up-regulation of dormancy gene transcription by mycobacteria. As we found that ESX-5-deficient *M. marinum* is impaired in the uptake of fatty acids, we hypothesized that the lipid-rich environment within hypoxic macrophages might induce dormancy in wild-type but not in the hypervirulent espG5 mutant bacteria. To investigate this possibility, we infected macrophages with wild-type or espG5 mutant *M. marinum* strains, and incubated
these cells under hypoxic conditions. CFU analysis showed that hypoxia indeed limits intracellular bacterial growth, but only during the first 3 days of infection (Fig. 6A). However, there is no difference in growth between wild-type and espG5 mutant bacteria, suggesting that hypoxia by itself is not responsible for the different course of infection of these two strains in adult zebrafish. Previously, we had already established that there is also no difference in growth rate when the two strains are grown in 7H9 culture medium under hypoxic conditions [11]. In order to investigate whether there is an altered response to hypoxia on the gene expression level, we measured mRNA levels of genes from the dormancy regulon during growth in hypoxic or normoxic culture medium. We observed an increase in transcription of dosR, hpX_1 (encoding the DosR-dependent alpha-crystallin antigen Acr) and tgs1 (encoding the DosR-dependent TAG synthase Tgs1), as can be expected under hypoxic conditions (Fig. 6B). Although up-regulation of the lipY gene has been described to occur in hypoxic macrophages [17], it was not induced in the oxygen-deprived culture medium used in our experiments. Remarkably, expression levels of the unrelated esx-1 genes eccCa1 and esxA that we used as a control, were down-regulated under these hypoxic conditions. As we could not detect any difference in expression levels for the genes analysed between the M. marinum wild-type and espG5 mutant strains, our data indicates that ESX-5 does not affect the DosR transcriptional response at low oxygen levels.

Figure 5. Intracellular TAG levels are not affected by ESX-5-deficiency. TLC of apolar lipids extracted from M. marinum strains grown under aerobic (left) or anaerobic (right) conditions. Tri- (TAG), di- (DAG) and monoacylglycerol (MAG) are indicated.
LipY can be rerouted for secretion via ESX-1, which restores the uptake of fatty acids by ESX-5-deficient *M. marinum*

We have established that the uptake of fatty acids is a process that can be mediated by ESX-5-dependent proteins. The ESX-5-secretion system of *M. marinum* is responsible for the surface localisation of numerous PE and PPE proteins [20]. One of these PPE proteins is LipY, a lipase with high hydrolytic activity towards long-chain TAG [18,21]. Since LipY is the only known ESX-5-dependent protein with such a clear role in lipid metabolism, we hypothesized that it may be involved in the release and possibly even uptake of extracellular fatty acids. In order to investigate this, we explored the possibility to complement the *M. marinum* espG₅ mutant strain with LipY. LipY should be relocated to another secretion pathway to reach this goal. Therefore, we attempted to reroute LipY to the ESX-1 secretion system by constructing an HA-tagged fusion protein consisting of the lipase domain of
LipY and the PPE-domain of PPE68. This specific PPE protein is dependent on ESX-1 for its export to the bacterial cell surface [21,30]. In order to determine whether the newly constructed LipY fusion protein was indeed redirected to ESX-1 for secretion, we expressed it in *M. marinum* wild-type, ESX-1-deficient *eccCb1* and ESX-5-deficient *espG* transposon mutant strains. Western blot analysis of bacterial fractions showed that the PPE68-LipY fusion protein was indeed located on the cell surface and in the secreted fractions of both wild-type and *espG* mutant bacteria (Fig. 7A). Despite its efficient expression, the LipY fusion protein was absent from the cell surface and supernatant of *eccCb1* mutant bacteria (Fig. 7B). Interestingly, expression of the ESX-1-dependent protein EspE was highly increased in this strain, indicating that the fusion protein influences expression of ESX-1 substrates that cannot be transported to the cell surface. Furthermore, the PPE68-LipY chimera was present in the uncleaved form on the cell surface and in the secreted fraction of wild-type and *espG* mutant bacteria (Fig 7A), indicating that secretion through ESX-1 affects the normal processing of LipY [21]. Together, these data show that the newly created LipY fusion protein is transported to the cell surface via ESX-1. Moreover, our results indicate that by exchanging PPE domains, proteins can be redirected for secretion via a different type VII secretion system.

We next used the *espG* mutant strain expressing PPE68-LipY to investigate whether cell surface exposure of the LipY fusion protein could complement the inability of ESX-
5-deficient *M. marinum* to import extracellular fluorescently labeled fatty acids. Our results show that this is partly the case, as the fluorescence intensity of these bacteria increases to ~60% of wild-type levels (Fig. 4A and B). This partial complementation may be due to the fact that, in contrast to wild-type LipY, a portion of the PPE68-LipY protein is secreted instead of bound to the cell surface (Fig 7A) [21]. Nevertheless, our finding indicates that LipY is involved in the uptake of fatty acids. In order to investigate whether the lipase domain of LipY should be functional for this effect, we mutated the active site by replacing the serine residue on position 402 of the protein for an alanine residue. Analysis of confocal fluorescence microscopy images showed that this inactive enzyme could also partially complement fatty acid uptake by ESX-5-deficient *M. marinum*, indicating that the active site of LipY is not required for this process (Fig 4A and B).

**LipY does not revert hypervirulence of ESX-5-deficient *M. marinum***

As the introduction of ESX-1-transported LipY in the espG5 mutant of *M. marinum* can partly restore the import of extracellular fatty acids, we questioned whether this could be the missing signal for these bacteria to go into dormancy in vivo. To study whether LipY could revert the hypervirulent phenotype of ESX-5-deficient *M. marinum*, we infected adult zebrafish with the wild-type, espG5 mutant, and espG5 mutant strain expressing PPE68-LipY. Analysis of CFU counts within zebrafish organs after two weeks of infection revealed that introduction of PPE68-LipY in ESX-5-deficient *M. marinum* could not reduce the large increase in bacterial loads (Fig 8). Introduction of two different constructs, inducing either high or low expression of the fusion protein (PPE68-LipY_H and PPE68-LipY_L, respectively), did not lead to a decrease in hypervirulence of the espG5 mutant. Therefore we can conclude that cell surface localization of the C-terminal domain of LipY does not affect virulence in vivo, despite its role in the import of fatty acids.

![Figure 8](image-url). PPE68-LipY does not revert hypervirulence of ESX-5-deficient *M. marinum* in zebrafish. Adult zebrafish were infected with the *M. marinum* wild-type, espG5 mutant, or espG5 mutant strain expressing PPE68-LipY. This fusion protein was either highly expressed (PPE68-LipY_H), by the hsp60 promotor, or expressed in lower amounts (PPE68-LipY_L), under control of the pe35 promotor. After 14 days of zebrafish infection, CFU numbers in spleens and livers were determined by organ plating on 7H10 plates.
DISCUSSION

In this study we have characterized *M. marinum*-induced granulomas in zebrafish, a natural host for this pathogen. Using zebrafish-specific CD4 and FoxP3 antibody staining, we showed that granulomas in zebrafish are surrounded by a dense layer of helper and regulatory T cells. Among the population of macrophages, we could detect epithelioid and multinucleated giant cells. In addition, we observed heterogeneity in lesion types within animals, with the presence of necrotic and solid granulomas at the same time. Necrotic centers of the granuloma were found to contain high amounts of bacteria. Furthermore, we showed that granulomas in *M. marinum*-infected zebrafish can be hypoxic. All of these features are also characteristic for the human TB granuloma [1]. Zebrafish granulomas have previously been suggested to contain few lymphocytes [8]. Our findings suggest that experimental setup and choice of strains may make a large difference in phenotype and course of infection within the same model organism. Taken together, our results demonstrate that granulomatous lesions in zebrafish induced by *M. marinum* are highly similar to those that develop in human TB, and underline the applicability of the zebrafish infection model in studying mycobacterial disease.

The hypoxic environment within the zebrafish granuloma allows *M. marinum* to induce the DosR regulon, and probably as a result of that to reach a dormant state. Recently, the presence of a dormant population of *M. marinum* bacteria has been demonstrated in zebrafish with latent mycobacterial disease [10]. Dormant bacteria do not replicate or do so minimally. They accumulate lipids, which may serve as energy storages. Our study indicates that hypoxia indeed limits intracellular growth of *M. marinum*, but only during the first days of macrophage infection. Previously, we found that inactivation of the ESX-5 protein secretion system leads to increased bacterial growth and virulence in vivo. We hypothesized that possibly, these bacteria are not able to reach a dormant state and studied the specific characteristics of these espG₅ mutant bacteria under dormancy-mimicking conditions. We found that ESX-5 is involved in the uptake of lipids, as the ESX-5-deficient espG₅ mutant was severely attenuated in its ability to import fluorescently labeled fatty acids. By introducing the C-terminal domain of the ESX-5-dependent lipase LipY on the cell surface of these bacteria via ESX-1, the uptake of fatty acids could be partly restored. LipY has been implicated in energy release during dormancy by hydrolysis of intracellular TAG storages [18]. As this protein is also cell surface localized and has a binding site for TAG and/or fatty acids, it is conceivable that it may facilitate import of host derived free fatty acids as well, by guiding them to a -yet unidentified- import system. Our experiments indicate that an active lipase domain is not required for the uptake of fatty acids, strengthening this hypothesis. In addition, recent data from our lab indicates that possibly, PPE or PE proteins form small specific channels in the outer membrane that could help to facilitate nutrient transport (Houben and Bitter, manuscript in preparation). If LipY-mediated fatty acid import would be involved in the induction of dormancy, we hypothesized that the introduction of an ESX-1-dependent LipY protein would reduce hypervirulence of espG₅ mutant *M. marinum* in zebrafish. Although we were able to
achieve heterologous secretion, our infection data showed that LipY-mediated uptake of lipids is not essential for dormancy and suggests that LipY does not play a major role in virulence in vivo. Therefore our data indicates that one or more other ESX-5 substrates must be involved in the hypervirulence phenotype.

In this study, we show that ESX-substrates can be rerouted for secretion via a different T7S system, by replacing their N-terminal domain. Using this approach we could redirect the ESX-5-dependent LipY to the ESX-1 system by replacing the entire PPE domain of LipY for that of the ESX-1-dependent PPE68. Our data indicates that the factor that determines system specificity is located within the N-terminal PPE domain. This finding may be exploited for applications that require transport of specific proteins to the cell envelope. For example, the current BCG vaccine may be improved by rerouting dominant ESX-1-dependent T-cell epitopes to one of the other ESX systems, in order to express them on the bacterial cell surface. Replacement of the N-terminal domain for an ESX-5-specific PE/PPE domain in combination with small modifications may be sufficient to reroute these proteins to ESX-5.

Taken together, we established that *M. marinum*-induced granulomas in zebrafish are highly similar to those formed in human TB. The hypoxic nature of these zebrafish granulomas allows bacteria to become dormant, which is characterized by the accumulation of host-derived fatty acids. We found that the uptake of extracellular fatty acids is reduced in the hypervirulent ESX-5-deficient *M. marinum* strain. Rerouting the ESX-5-dependent lipase LipY to the ESX-1 secretion system could partially complement fatty acid uptake. Hypervirulence however, could not be reverted by this LipY fusion protein, indicating that LipY-mediated lipid uptake does not play a major role in this process.

**EXPERIMENTAL PROCEDURES**

**Strains and growth conditions**

The *M. marinum* wild-type strain E11, its isogenic eccCb1 transposon mutant (eccCb1::Tn), espG5 transposon mutant (espG5::Tn) and complemented espG5 mutant (espG5::Tn-c) used in this study have been described previously [9,13,20]. *M. marinum* strains were grown at 30˚C in Middlebrook 7H9 medium enriched with ADC supplement. To create anaerobic conditions, bacteria were grown according to the Wayne model [23]. For protein analysis, *M. marinum* bacteria grown to mid-logarithmic phase were washed with PBS and grown for 24 hours in 7H9 culture medium supplemented with 0.2% glycerol and 0.2% dextrose.

**Infection of adult zebrafish**

One-year old male *Danio rerio* zebrafish were anaesthetized in 0.02% MS-222 (Sigma) and injected intraperitoneally with 2*10⁴ *M. marinum* Wt, espG5::Tn, espG5::Tn-c, espG5::Tn+PPE68-LipY_H or espG5::Tn+PPE68-LipY_L bacteria. After 14 days, zebrafish were euthanized by MS-222 overdose (A-5040; Sigma) and livers and spleens were isolated. Organs were homogenized in BBL MycoPrep and plated in serial dilutions on 7H10 agar plates to determine bacterial CFU, as described previously [13].
Infection of human macrophages

The human monocytic cell line THP-1 was cultured in RPMI1640 + glutamax (Gibco) supplemented with FCS (Gibco) at 37°C and 5% CO₂. 1*10⁶ Cells per well were differentiated into macrophages in 12-wells plates by 24 hours of stimulation with 25 ng/ml PMA. Bacteria were washed with PBS and subsequently used to infect THP-1 macrophages for 2 hours at a multiplicity of infection (MOI) of 0.5 at 33°C and 5% CO₂. Subsequently, infected cells were washed with RPMI and incubated at 33°C, in 1 or 21% O₂ and 5% CO₂. After 72 and 120 hours, cells were lysed with 1% Triton X-100 in PBS and intracellular bacteria were plated on 7H10 agar plates in serial dilutions.

Lipid uptake assay

*M. marinum* strains were incubated with 4 μg/ml of the fluorescently labeled fatty acid Bodipy 558/568 C₁₂ (Life Technologies) under hypoxic conditions. After 72 hours, bacterial cells were washed with PBS and fixed in 4% paraformaldehyde. Bacteria were heat-fixed on cover slips and viewed with confocal microscopy (Leica TCS SP2). Imaging was performed using Leica confocal software with identical settings for each bacterial strain. Quantification of fluorescence intensity was performed on confocal images with ImageJ software. For each biological replicate, quantification was performed on a minimum of 1,000 bacteria per strain and mean fluorescence intensity of the *M. marinum* wild-type strain was set to 100%.

RNA isolation and qRT-PCR

Pelleted bacteria grown to mid-logarithmic phase under hypoxic or normoxic conditions were homogenized in Trizol by bead-beating with 0.2 mm silica beads. Disrupted bacteria were centrifuged at 16.000g and supernatant was extracted with chloroform for 5 minutes. After centrifugation at 16.000g, RNA was precipitated from the supernatant with isopropanol. By centrifugation at 16.000g, RNA was pelleted and subsequently washed with 70% ethanol. Air-dried RNA pellets were dissolved in RNAse-free water and treated with DNAsel for 20 minutes at 37°C. cDNA was generated with a SuperScript VILO cDNA synthesis kit (Life technologies). qRT-PCR was performed on an equivalent of 10 ng RNA with SuperScript III Reverse Transcriptase (Life Technologies) on a LC480 (Roche). Ct values of target genes were normalized to sigA levels.

Construction of ESX-1_LipY

In order to redirect LipY to the ESX-1 secretion system, a fusion protein consisting of the PPE domain of PPE68 and the C-terminal domain of LipY was constructed using a nested PCR approach. To this end, pe35 and the PPE domain of ppe68 (Mmar_0185-0186) were amplified from the *M. marinum* genome by PCR, using a reverse primer containing an overhang complementary to the DNA region directly following the PPE domain of *lipY* (0186LipY_Rv, primers sequences in Table S2). In addition, the C-terminus of the *lipY* gene (Mmar_1547) was amplified from the *M. marinum* genome by PCR, using a forward primer containing an overhang complementary to the end of the PPE domain of *ppe68* (0186LipY_F). Then, the two PCR products from the reactions described above were
used as input DNA for a third PCR reaction, where a pe35 forward primer with Eco21I restriction site and a lipY reverse primer with 3’ HA epitope and HindIII restriction site were used (0185_Fw and LipY_Rv_HA). The resulting PCR product and an empty pSMT3 cloning vector were digested with Eco21I and HindIII (Fermentas), followed by ligation of the digested PCR product into the vector with T4 ligase (Fermentas) to generate pSMT3::PPE68-LipY. An active site mutant of the LipY lipase domain was generated according to the strategy described above, where the primers 0186LipY_Rv and 0186LipY_Fw were replaced by LipY_S402A_Fw and LipY_S402A_Rv, respectively, and pSMT3::PPE68-LipY was used as template DNA. In this pSMT3::PPE68-LipY_S402A construct, the serine on position 402 of LipY was replaced by an alanine. In order to remove the constitutively active hps60 promoter from the plasmid and regulate transcription of the fusion gene only by the promoter located upstream of pe35, the pSMT3::PPE68-LipY was digested with XbaI and BamHI. Restriction sites were blunted with T4 polymerase (Fermentas) after which the plasmid was religated with T4 ligase. All constructs were introduced in M. marinum wild-type and ESX-1 mutant strains by electroporation. All primer sequences are listed in Table S1.

**Analysis of protein expression and secretion**

Secreted proteins were precipitated from the culture supernatant with 10% TCA (Sigma-Aldrich). Pelleted M. marinum bacteria were washed with PBS and cell wall proteins were extracted by incubation for 30 minutes in 0.5% Genapol X-080 (Sigma Aldrich). Genapol-treated M. marinum cells were disrupted by sonication. Proteins were separated according to their molecular weight on 12-15% SDS-PAGE gels and subsequently transferred to nitrocellulose membranes. Immunostaining was performed with mouse monoclonal antibodies directed against the HA-epitope (HA.11, Covance) or EsxA (Mab Hyb76-8) [24], or with rabbit polyclonal sera recognizing EspE [25].

**TAG extraction of M. marinum and TLC**

M. marinum bacteria were grown aerobic or anaerobic in Middlebrook 7H9 medium for 24 hours. 50 OD units of bacterial culture was pelleted and washed with PBS. Apolar mycobacterial lipids were extracted with petroleum ether as described previously [26]. Thin layer chromatography was performed on Silica gel 60 TLC plates (Merck Millipore) using heptane:diethylether:formic acid (40:10:1) as a solvent system.

**Histopathological analysis and immunohistochemistry**

One-year old male Danio rerio zebrafish were infected for four weeks with M. marinum espG::Tn, or eight weeks with the wild-type strain, and fixed in 4% paraformaldehyde after terminal anesthesia by incubation in an overdose of MS-222 (A-5040; Sigma). Fixed animals were embedded in paraffin and cut in coronal serial sections from ventral to dorsal. Tissue sections were deparaffinized in xylene and rehydrated up to 70% alcohol. Endogenous peroxidase was inactivated by incubation in 0.3% H₂O₂ in methanol for 30 minutes. After 10 minutes of pre-incubation in 1% BSA, sections were incubated with a rabbit polyclonal CD4 antibody (1:1000) or FoxP3 antibody
in 0.1% BSA at 4% paraformaldehyde. All incubations were performed at room temperature. A Zeiss Axioskop light microscope equipped with a Leica DC500 camera was used for imaging. ImageJ software was used to adjust brightness and contrast of images.

Staining of PIMO adducts
In order to visualize hypoxia within tissues, pimonidazole hydrochloride (PIMO) was injected intraperitoneally at a dose of 30 μg/g body weight in one adult zebrafish that was infected for 8 weeks with the M. marinum wild-type strain. After 24 hours, the fish was euthanized by terminal anesthesia and fixed in 4% paraformaldehyde. Detection of PIMO adducts was performed on zebrafish sections with a monoclonal Hypoxyprobe-1 antibody (1:50) as described previously [27].

REFERENCES
9. van der Sar AM et al. Mycobacterium marinum strains can be divided into two distinct types based on genetic diversity and virulence. Infect Immun (2004); 72(11): 6306-12.
10. Parikka M et al. Mycobacterium marinum causes a latent infection that can be reactivated by gamma irradiation in adult zebrafish. PLoS Pathog (2012); 8(9): e1002944.


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Table S1. Nucleotide sequences of primers used in this study.
Figure S1. Histological section of CD4- and FoxP3-antibody stained granulomatous zebrafish tissue, derived from a fish infected for 8 weeks with an M. marinum wild-type strain. Stained sections appear in brown colour.