Tampere University

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A Zebrafish Model for Developing Vaccines against Tuberculosis

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ACADEMIC DISSERTATION
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ACADEMIC DISSERTATION
Tampere University, Faculty of Medicine and Health Technology,
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To my Family

"We gain strength, courage and confidence every time we look fear in the face.
We must do that which we think we cannot do."
-Eleanor Roosevelt

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The journey to become a Doctor of Philosophy, has offered both challenges and successful moments, which I have luckily been able to experience with my lovely colleagues at work, as well as with family and friends out of the office. I have learnt that despite the competitive nature of science, it is nearly impossible to succeed without a good team. Therefore, at first, I would like to thank Professor Mika Rämet for allowing me to work in such an inspiring research team. I appreciate your expertise and leadership skills, and I feel that I have received support whenever I needed it, despite of the long distance between Oulu and Tampere. I would like to especially thank you for the support during the final steps of my writing, which was crucial in pushing me forward within a relatively strict deadline.

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Tampere, January 2020

Mirja Niskanen

ABSTRACT

Tuberculosis (TB) is one of the most serious diseases worldwide, leading to the death of 1.5 million people annually. According to the World Health Organization, every year, 10 million people are infected by *Mycobacterium tuberculosis*, the causative agent of tuberculosis. Depending on the strength of the immune system, the infection develops into a granulomatous pulmonary disease, which can be progressive or is controlled by the immune system to maintain latency. An estimated 1.7 billion people have an asymptomatic latent TB, having a 5-10% risk of developing the active disease in their lifetime. The only licensed tuberculosis vaccine, the Bacillus Calmette Guérin, which was developed already in the 1920's, protects only against severe forms of tuberculosis in infants. Currently, there is no vaccine available that effectively prevent tuberculosis in adults or the reactivation of a latent disease. The latest vaccine research focuses on safer immunization approaches, such as subunit vaccines, which do not contain live particles of the pathogen.

Despite the long history of tuberculosis research, our understanding of the exact mechanisms of the diseases at the cellular level and the complex interactions between the bacilli and the immune system remain incomplete. One of the challenges in tuberculosis research has been a lack of appropriate animal models. A zebrafish (*Danio rerio*) model, based on an infection with *Mycobacterium marinum* leading to a disease, which resembles the main features of human tuberculosis, was utilized in this thesis. It is known that in an experimental set up, a high dose infection with *M. marinum* causes a progressive disease, whereas a low dose infection leads to the formation of a latent infection in adult zebrafish. In this study, it was shown that by suppressing the fish immune system with dexamethasone, the latent infection can be reactivated.

The main goal of this thesis was to identify novel vaccine antigens to prevent tuberculosis. To this end, potential antigens were selected based on the literature and on a transcriptomic level analysis performed with *in vitro* reactivated *M. marinum* samples. In total, 22 different genes were tested as DNA vaccines. Seven of these

were derived from reactivation-associated genes identified using the transcriptomic analysis and 15 genes were selected from the literature. 16 antigens were tested against the primary infection, and four (cdh, PE5, PE31 and RpfE) showed protection in a bacterial count analysis after 4 weeks of infection. Furthermore, the RpfE-derived antigen enhanced survival in a 12-weeks follow-up. Correspondingly, the preventive effects of 15 antigens against reactivation were tested with the dexamethasone-based reactivation method. With this model, three antigens, namely RpfB, MMAR_4207 and MMAR_4110, inhibited reactivation.

This thesis showed that an adult zebrafish- *M. marinum* infection model, and the developed dexamethasone-induced reactivation model are feasible for testing the effectiveness of novel DNA vaccines. As the genomes of *M. tuberculosis* and *M. marinum* are highly similar, the *M. tuberculosis* homologues of the identified promising antigens are potential targets for developing novel vaccines and drugs against tuberculosis.

TIIVISTELMÄ

Maailmanlaajuisesti tuberkuloosi on yksi merkittävimmistä kuolemansyistä aiheuttaen vuosittain 1.5 miljoonan ihmisen kuoleman. Maailman terveysjärjestön mukaan, tuberkuloosia aiheuttava patogeeni, *Mycobacterium tuberculosis*, tartuttaa joka vuosi 10 miljoonaa ihmistä. Sairaus voi olla etenevä tai latentti. Latentissa infektiossa elimistön immuunipuolustus pystyy rajaamaan infektion granuloomarakenteiden sisään. Arviolta 1.7 miljardilla ihmisellä on tälläinen oireeton, latentti tuberkuloosi, ja siten 5-10% elinikäinen riski aktiiviselle sairaudelle. 1920-luvulla kehitetty, ainoa saatavilla oleva tuberkuloosirokote, Bacillus Calmette-Guérin, suojaa ainostaan imeväisikäisiä tuberkuloosin vakavampia tautimuotoja vastaan. Nykyisin ei ole saatavilla rokotetta, mikä antaisi tehokkaan suojan aikuisille tai estäisi latentin infektion uudelleenaktivaation. Viimeaikoina rokotetutkimus on keskittynyt turvallisempiin menetelmiin, kuten alayksikkörokotteisiin, jotka eivät sisällä elävää patogeenia.

Huolimatta tuberkuloositutkimuksen pitkästä historiasta, tuberkuloosin taudinaiheuttamismekanismeja sekä mykobakteerien ja immuunipuolustuksen välistä vuorovaikutusta ei edelleenkään täysin tunneta. Yksi tuberkuloositutkimuksen haasteista on ollut käyttökelpoisten mallieläinten puute. Tässä tutkimuksessa hyödynnettiin seeprakalamallia (*Danio Rerio*), jonka *Mycobacterium marinum*-infektio muistuttaa monilta piirteiltään ihmisen tuberkuloosia. Tiedetään, että laboratorio-olosuhteissa, korkea infektioannos aiheuttaa etenevän sairauden ja matala-annoksinen infektio puolestaan latentin infektion. Tässä tutkimuksessa osoitettiin, että heikentämällä kalan immuunipuolustusta deksametasonilla, latentin infektiot uudelleenaktivoituvat.

Tämän väitöskirjatutkimuksen päätavoitteena oli löytää uusia rokoteantigeeneja tuberkuloosia vastaan. Tavoitteen saavuttamiseksi valitsimme lupaavia *M. marinumin* geenejä sekä kirjallisuuden että lähetti-RNA-sevensointituloksen perusteella. *In vivo*-tutkimuksessa testattiin DNA-rokoteantigeeneinä yhteensä 22 eri geeniä. Näistä seitsemän on erityisesti reaktivaatiossa ilmentyvää ja 15 kirjallisuuden perusteella valittua geeniä. 16 eri antigeenin teho testattiin primaari-infektiota vastaan. Testatuista antigeeneistä neljä (cdh, PE5, PE31 ja RpfE) antoivat suojan neljän

viikon kuluttua infektiosta bakteerimääritykseen perustuvassa tutkimuksessa. Näistä yksi (RpfE) paransi myös kalojen selviytymistä 12 viikon infektionjälkeisessä serannassa. Lisäksi testattiin 15 eri antigeenin mahdollinen suojavaikutus latentin infetion uudelleen aktivaatiota vastaan deksametasoni-hoitoon perustuvan koemallin avulla. Tutkimuksessa löydettiin kolme antigeenia, RpfB, MMAR_4207 ja MMAR_4110, jotka estivät uudelleenaktivaatiota.

Tässä väitöskirjatutkimukssa osoitetaan, että seeprakalan *M. marinum*-infektiomalli sekä deksametasoniin perustuva latentin infektion uudelleenaktivaatiomalli ovat käyttökelpoisia testattaessa uusien DNA-rokotteiden tehoa. Johtuen *M. tuberculosiksen* ja *M. marinumin* genomien samankaltaisuudesta, löydettyjen lupaavien antigeenien *M. tuberculosis*-homologit ovat mahdollisilta kohdegeenejä uusien tuberkuloosirokotteiden kehittämiselle.

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ABBREVIATIONS

Ad5	Adenovirus 5
Ad35	Adenovirus 35
Ag85	*Mycobacterium tuberculosis* antigen 85
APC	Antigen presenting cell
BCG	Bacillus Calmette-Guérin vaccine
BCR	B cell receptor
rBCG	Recombinant Bacillus Calmette-Guérin vaccine
CFP-10	10-kDa culture filtrate antigen
CFU	Colony forming unit
ChAd	Chimpanzee adenovirus
CMV	Cytomegalovirus
CpG	Deoxycytidylate-phosphate-deoxyguanylate motif
CT	Computed tomography
DBD	Dextran binding domain
DC	Dendritic cell
EFa1	*Elongation factor 1-alpha*
EP	Electroporation
ESAT-6	6-kDa early secretory antigen target
ESX-1	ESAT-6 secretion system 1
FACS	Fluorescence associated cell sorting
FLU	Replicon-deficient influenza virus
GFP	Green fluorescence protein
GLA-SE	Glycopyranosyl lipid adjuvant in a stable oil-water
GM-CFS	Granulocyte macrophage colony stimulating factor
Hly	Listeriolysin
HIV	Human immunodeficient virus
Hsp	Heat shock protein
i.m.	Intramuscular
i.p.	Intraperitoneal
IFNγ	Interferon γ

Ig	Immunoglobulin
IGRA	Interferon-gamma release assay
IL	Interleukin
LTBI	Latent tuberculosis infection
MCS	Multiple cloning site
MDR-TB	Multidrug resistant *Mycobacterium tuberculosis* strain
MHC	Major histocompatibility complex
MMITS	*M. marinum* internal transcribed spacer
Mtb	*Mycobacterium tuberculosis*
MVA	Modified Vaccinia Ankara virus
rMtb	Recombinant *Mycobacterium tuberculosis* strain
NK cell	Natural killer cell
p.o.	Per oral
PC	Principal component
PCR	Polymer chain reaction
PE	Proline-glutamic acid family protein
PLGA	Poly(lactic-co-glycolic acid)
PPE	Proline-proline-glutamic acid family protein
PBS	Phosphate-buffered saline
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
Rpf	Resuscitation promoting factor
RSV	Rous sarcoma virus
RR-TB	Rifampicin resistant *Mycobacterium tuberculosis* strain
SV	Simian virus
TB	Tuberculosis
Th	T helper cell
TNF	Tumor necrosis factor
TST	Tuberculin skin test
XDR-TB	Extensively drug-resistant *Mycobacterium tuberculosis* strain
WHO	The World Health Organization

ORIGINAL PUBLICATIONS

The study presented in this thesis is based on original publications, which are listed below. The publications are reproduced with the permission of the copyright holders.

Publication I Myllymäki H, **Niskanen M**, Oksanen KE, Sherwood E, Ahava M, Parikka M, Rämet M. Identification of novel antigen candidates for a tuberculosis vaccine in the adult zebrafish (*Danio rerio*). *PLoS One*. 2017 Jul 25; 12(7):e0181942.

Publication II Myllymäki H*, **Niskanen M***, Luukinen H, Parikka M, Rämet M. Identification of protective postexposure mycobacterial vaccine antigens using an immunosuppression-based reactivation model in the zebrafish. *Dis Model Mech*. 2018 Mar 13; 11(3):10.1242/dmm.033175.

Publication III Myllymäki H*, **Niskanen M***, Oksanen K, Rämet M. Immunization of adult zebrafish for the preclinical screening of DNA-based vaccines. *J Vis Exp*. 2018 Oct 30; (140).

Publication IV **Niskanen M**, Myllymäki H, Rämet M. DNA vaccination with the *Mycobacterium marinum* MMAR_4110 antigen inhibits reactivation of a latent mycobacterial infection in the adult zebrafish. *Vaccine*. 2020;38(35):5685-5694.

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1 INTRODUCTION

Over the centuries tuberculosis has plagued mankind. Tuberculosis is a pulmonary disease, caused by the intracellular pathogen *Mycobacterium tuberculosis* (Furin et al, 2019). Tuberculous bacilli were identified for the first time in 1882 by Robert Koch and since then tuberculosis has been actively studied (Luca & Mihaescu, 2013). Although researchers have made many life-changing medical breakthroughs, such as developed antibiotics and vaccines, *M. tuberculosis* continues its victorious journey. According to the World Health Organization, WHO, tuberculosis remains the most lethal bacterial disease in the world. In 2018, 10 million people contracted tuberculosis and the infection led to the death of 1.5 million people. Besides active tuberculosis, it has been estimated that roughly 1/5th of the human population have an asymptomatic, latent form of the disease (WHO, 2019).

The most prominent feature of tuberculosis are granulomas found in the lung tissue both in the active and latent phases of the disease. Granulomas are organized immune cell clusters that isolate the invading mycobacteria and thus prevent dissemination of the infection (Guirado & Schesinger, 2013). However, mycobacteria have developed many coping mechanisms due to which they can survive inside the hostile environment of granulomas and macrophages (Lin & Flynn, 2015). In the latent infection phase, bacteria fall into so-called dormancy, where their metabolic activity and replication slow down. The latent infection can remain dormant for years or even decades, before reactivation, which occurs typically as a result of immunosuppression of the host (Esmail et al, 2014). Therefore, people with a latent infection are an enormous reservoir of *M. tuberculosis* and have a 5-10% lifetime risk of developing an active, and contagious, tuberculosis and thus further spreading the disease (WHO, 2019).

Because of the various phases of the disease, the prevention and treatment of tuberculosis has turned out to be challenging. As an intracellular pathogen with effective coping mechanisms, like dormancy, the clearance of a *M. tuberculosis* infection requires long-term treatment with a combination of several different antibiotics (WHO, 2019). This has led to the formation of multidrug resistant *M. tuberculosis* strains, which complicates the treatment further. Similarly, the methods

for preventing tuberculosis are limited. Until now, immunization against *M. tuberculosis* relies on the Bacillus Calmette-Guérin vaccine (BCG), which is a live attenuated vaccine, derived from *Mycobacterium bovis*. Unfortunately, the protective effect of BCG is insufficient (Luca & Mihaescu, 2013). While it protects infants from a systemic infection, it does not provide protection to adults or against reactivation of the latent infection. Besides the poor efficiency, many side effects and safety issues have questioned its use further (Lahey & von Reyn, 2016). An effective vaccine would be the most efficient way to control or even eradicate tuberculosis. Therefore, more and more interest and resources are focused on research into tuberculosis vaccines.

Although, tuberculosis has been studied for years, the cellular and molecular mechanisms of the infection are poorly understood. Similarly, complex immunological responses to a mycobacterial infection remain partly elusive. One of the reasons why the research on ways of combating tuberculosis has progressed slowly is the lack of appropriate animal models. Conventional animal models, such as mice, have been widely utilized due their practicality and the availability of immunological and genetic tools (Singh & Gupta, 2018). However, the disease outcome in mice variates in several aspects, including differences in pathology of granulomas and T cell responses, as compared to human tuberculosis. During the past two decades, the zebrafish (*Danio rerio*) has emerged as an applicable model for studying different human diseases (Myllymäki et al, 2015). In addition, it turned out that a *Mycobacterium marinum* infection in zebrafish resembles the features of human tuberculosis, including the active and latent phases of the infection with granulomas and reactivation of the latent infection (Swaim et al, 2006; Parikka et al, 2012; Myllymäki et al, 2016).

The WHO has set a goal to end the global tuberculosis epidemic by 2050. To meet this ambiguous goal, novel approaches to develop safe and effective vaccines both against a primary infection and against the reactivation of a latent infection are urgently needed. As a response to this challenge, this doctoral thesis utilizes the DNA vaccination technique and the ethically sound zebrafish-*M. marinum* infection model for studying novel vaccine antigens against tuberculosis.

2 REVIEW OF THE LITERATURE

2.1 Tuberculosis

2.1.1 Epidemiology and common features of tuberculosis

Tuberculosis (TB) is an ancient disease caused by *Mycobacterium tuberculosis* (Mtb), an intracellular pathogen discovered by Robert Koch in 1882 (Sakula, 1982; Luca & Mihaescu, 2013). As an airborne disease, the most common clinical manifestation of an Mtb infection is pulmonary TB, although it can also affect other sites of the body causing versatile extrapulmonary forms of TB (WHO, 2019). According to the latest Tuberculosis report by the WHO (2019), globally 1.7 billion people are infected with Mtb and 5-10% of these individuals will develop the disease during their lifetime. Therefore, TB is diagnosed in 10 million people and it is the cause of death of approximately 1.5 million people every year (WHO, 2019). Diagnosis of TB relies mainly on medical history of a patient, microbiological examination and X-ray imaging. Immunocompromised individuals are more prone to the infection, consequently TB incidence rates are higher in areas where HIV is endemic, such as in South-East areas of Africa, the Eastern Mediterranean and the Western Pacific (Zhang et al, 2019; WHO, 2019). TB incidence rates are also high in India, China and the Russian Federation, where drug resistant strains of Mtb have become a problem. Thus far, an estimated 484 000 of all diagnosed TB cases are caused by either a rifampin or a multidrug resistant Mtb (RR/MDR-TB) strain (WHO, 2019). The conventional treatment of TB consists of four chemotherapeutic agents (isoniazid, rifampin, pyrazinamide, and either ethambutol or streptomycin) with six to nine months of treatment (WHO, 2019). Compliance to the extensive, long-term treatment has turned out to be challenging, which further increases the number of MDR-TB (resistance to at least isoniazid and rifampicin) and extensively drug-resistant (XDR-TB, resistant to at least four of the regular anti-TB drugs) cases.

Besides the diagnosed active TB cases, the latent TB challenges the current approaches for treating, diagnosing and preventing TB. A roughly estimated 1/5th of the human population carry a latent, asymptomatic form of TB, and therefore

have a 5-10% lifetime risk of reactivation and developing an active disease (WHO, 2019). With the available diagnostic tests, such as the tuberculin skin test (TST) and interferon-gamma release assays (IGRAs), it is not possible to predict which individuals will develop the active disease (Esmail et al, 2014), which in turn complicates the identification and targeting of individuals who would need preventive treatment.

2.1.2 Active *M. tuberculosis* infection and transmission

Typical symptoms of an active Mtb infection are a persistent cough with increased sputum formation, haemoptysis, weight loss, weakness, night sweats and fever (Sia & Rengarajan, 2017). Besides the clinical symptoms, the diagnosis of TB is based on a radiography examination and the detection of Mtb bacilli in the sputum of the patient (Sia & Rengarajan, 2017). By X-ray imaging the chest, it is possible to detect granulomas in the patient's lungs, and computed tomography (CT) can be used to obtain more detailed information, for instance about the manifestation of the disease in the lymph nodes (Kienzl-Palma and Prosch, 2016). As acid-fast bacilli, mycobacteria van be detected by microscopy from sputum, however microbiological culturing and pcr-based methods are required to identify the strain (Sia & Rengarajan, 2017). In addition, an Mtb infection can be diagnosed with the TST by exposing the patients' skin to Mtb antigens and monitoring the skin response for 24 to 48 hours (Ahmad, 2011). However, the test is not specific, and it might give a false positive result in case of individuals who have been vaccinated with BCG or have been exposed to some other mycobacteria (Singla et al, 2005; Ahmad, 2011; Piñeiro et al, 2012). In addition, false negative results are possible due to incorrect administration of the antigens or interpretation of the results. The other more sensitive test is IGRA, which detects the release of IFN-γ after stimulation with Mtb specific antigens (ESAT-6 and CFP-10) (Madariaga et al, 2007). BCG vaccination and environmental mycobacteria do not interfere these tests, nevertheless patients with both an active or a latent infection can test positive (Madariaga et al, 2007; Ahmad, 2011).

Persons with active TB can infect healthy individuals. Transmission occurs through so called droplet nuclei, tiny water droplets (1-5μm in diameter) containing viable bacteria, which are released when a person with TB cough or sneezes (Figure 1.) (Nardell, 2015). Typically, transmission requires indoor environments and relatively long exposure times, thus any environments where people spend longer periods,

such as households, hospitals, schools, workplaces and prisons are potential places for transmission (Churchyard et al, 2017). When a susceptible individual inhale a droplet nucleus, (s)he may become infected and develop an active disease. The outcome after exposure to bacteria depends on the person's immune system, the number of inhaled bacteria as well as on the virulence of the bacteria (Nardell, 2015). If the immune system succeeds in controlling the bacteria, it can result in the elimination of bacteria and clearance of the disease, or the formation of an inactive latent phase of the TB (Figure 1.). Instead, if the immune system fails to control the growth of the mycobacteria (about 5% of infected people), liquefied caseous foci form inside mature lesions, which enable bacteria to disseminate into other sites of the lung tissue and infect healthy persons (van Crevel et al, 2002). (Figure 1.) Majority of infected individuals are able to contain the bacterium and develop LTBI, whereas a small fraction of populations are either completely resistant or especially susceptible (native populations of America and sub-Saharan Africa) to the infection (Möller et al, 2018).

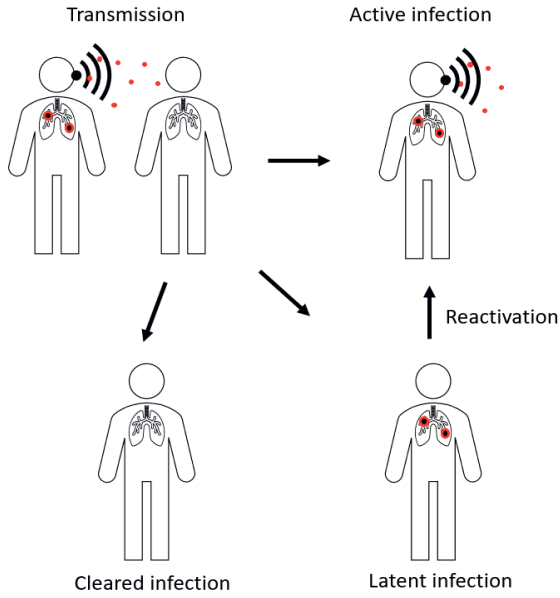

Figure 1. Transmission and infection phases of tuberculosis. Typically, the transmission of tuberculosis takes a place via the airways when an individual with active tuberculosis coughs or sneezes. Depending on the immune status of the exposed healthy individual, the infection may cause an active disease, it can be cleared completely or controlled in the form of latent infection. Further, the latent infection can be reactivated, and it can progress into an active, contaminating disease.

2.1.2.1 Immunopathogenesis of *M. tuberculosis* infection

When a tuberculous bacillus enters the lung tissue, it activates the innate immune responses of the host. As a first line defense mechanism, alveolar macrophages recognize Mtb through pattern recognition receptors, such as C-type lectins, toll-like receptors and Nod-like receptors, and phagocytose the bacteria in attempting to destroy them (Ferwerda et al, 2005; Mishra et al, 2017; Lugo-Villarino et al, 2018). Recognition results in the activation of several signaling pathways, and the production of inflammatory cytokines and chemokines (TNF, IL-1, IL-12), and the further recruitment of macrophages, dendritic cells, monocytes and NK cells to the site of infection (Allen et al, 2015; Sia & Rengarajan 2019). However, Mtb has several mechanisms to prevent recognition by its host, interrupt phagosomal maturation and change its metabolism with the intention to survive and replicate inside macrophages (Hart et al, 1987; van Crevel et al, 2002; Ehrt & Schnappinger 2009). As the infection proceeds, the number of Mtb inside macrophages increases, which finally leads to necrosis and disruption of the macrophages and dissemination of the bacteria (Ahmad, 2011). Consequently, further inflammatory cells filtrate into the lung tissue, forming early tuberculous lesions. At this stage, bacteria start growing exponentially inside macrophages. However, tissue damages and observed symptoms remain modest, while even more macrophages accumulate to the infection site (van Crevel et al, 2002).

Within two to three weeks after an Mtb infection, T cell mediated immunity (adaptive response) against the mycobacteria develops (van Crevel et al, 2002). During this time, lung dendritic cells migrate to local lymph nodes and prime T cells to become antigen-specific CD4+ and CD8+ T cells (Ahmad, 2011). After this, mature T cells migrate via lymphatic vessels and blood circulation from the lymph nodes to the infection site and activate macrophages and other antigen presenting cells to kill the intracellular bacteria by secreting interferon gamma (IFN-γ) and interleukins, which in turn restrain the growth of the bacteria and facilitates the maturation of granulomas (van Crevel et al, 2002). CD4+ T cells and especially the IFN-γ they produce have been shown to have an essential role in controlling an Mtb infection in both animal and human studies (Green et al, 2013; Usman et al, 2017). Therefore, the depletion of CD4+ T cells, as in HIV-infected individuals, is clearly connected to impaired immune responses against Mtb (Theuer et al, 1990). The immunological memory against Mtb relies mainly on the formation of specific memory T cells, which are detected in both active and chronic infections in human patients (Tonaco et al, 2017). Recent studies suggest that patients with active TB

have increased numbers of CD4+ central memory cells (a subset of memory cells, which are resided in lymphoid organs), whereas patients with a latent infection have more CD8+ effector cells (Tonaco et al, 2017).

The role of the humoral immunity in the defense of intracellular pathogens is assumed to be secondary, and it is mainly associated with opsonization and complement activation (Achkar et al, 2015). With opsonization, the immune system induces the elimination of intracellular Mtb by enhancing phagolysosomal fusion and altering the environment inside macrophages so that it becomes advantageous for mycobacterial killing (Armstrong et al, 1975; Malik et al, 2000; Arckar et al, 2015). Conversely, several studies show that the role of the humoral immunity in an Mtb infection is more complicated. For instance, mice treated with human polyclonal IgG survive better against an Mtb infection than do untreated mice (Olivares et al, 2006), whereas both B cell and IgG deficient mice seem to be more susceptible to mycobacterial infections than wild-type mice (Vordermeier et al, 1996; Rodriguez et al, 2005). Moreover, it is known that the BCG vaccination induces Th1 responses (Kozakiewicz et al, 2013), and some RNA/DNA vaccine candidates induce both cellular and humoral responses (Chang-hong et al, 2008). In addition, high numbers of B cells are present in granulomas and thus they may have a significant role in organizing granulomas and controlling the dissemination of bacteria (Achkar et al, 2015).

2.1.3 Latent *M. tuberculosis* infection

Most individuals infected with Mtb can control the growth and dissemination of the bacteria but do not manage to clear the infection (Lin & Flynn, 2015). Clinically this chronic phase of an Mtb infection is called a "latent tuberculosis infection" (LTBI), where patients are asymptomatic, non-contagious and have a delayed response to the TST (WHO, 2018). Since the available LTBI tests cannot distinguish between individuals who have cleared the infection and individuals who have the latent infection, the exact global burden of LTBIs is not known (Esmail et al, 2014; WHO, 2019). However, it has been estimated that more than roughly 2 billion people are infected with Mtb serving as a remarkable reservoir of potential disease (WHO 2018; WHO, 2019).

While CD4+ T cells are mainly responsible for immune responses against Mtb both in the acute and chronic phases of the infection, studies with mice and macaques highlight the role of cytotoxic CD8+ T cells in controlling the growth of

Mtb in the granulomas and thus containing the infection in the latent stage (van Pinxteren et al, 2000; Chen et al, 2009; reviewed in Lin & Flynn, 2015). In a LTBI, CD8+ T cells seem to produce mainly IFN-γ, TNF and IL-2, whereas in active TB the effect is based on IFN-γ/TNF in combination or on IFN-γ alone (Rozot et al, 2013). Besides cytokine production, mature CD8+ T cells can destroy bacteria via the formation of toxic granules, by inducing the apoptosis of infected macrophages or by directly killing Mtb with granulysin (Lin & Flynn, 2015). However, the CD8 T cell-mediated protection is not enough alone, and CD4 T cells are needed to stimulate CD8 T cells and as a primary source of IFN-γ.

2.1.4 Granuloma structures in a *M. tuberculosis* infection

Pathologically the granuloma structures, which are observed in both active and latent Mtb infections, are the hallmark of TB (Guirado & Schesinger, 2013). Granulomas are well-organized immune cell clusters, consisting of different subtypes of macrophages, monocytes, T and B cells, NK cells, dendritic cells and fibroblasts, which enclose the infected macrophages and therefore constrain the dissemination of the mycobacteria (Guirado & Schesinger, 2013; Cadena et al, 2017) (Figure 2.). The granuloma structure is dynamic and changes in the course of the infection depending on the environment, state and number of bacteria, and the host's immune capacity (Cadena et al, 2017). It has also been shown that granuloma populations are heterogeneous in different phases of the infection (Sia & Rengarajan, 2019). For instance, the numbers and phenotypes of T cells, the degree of macrophage polarization, and the extent of fibrosis can vary between granulomas (Marino et al, 2015; Sia & Rengarajan 2019).

The formation of a granuloma starts shortly after Mtb enters the lung tissue where macrophages phagocytose the invading pathogen and transport it to adjacent lymph nodes (Guirado & Schesinger, 2013). This initiates a cascade where infected macrophages recruit inflammatory cells by secreting chemokines to migrate to the original infection site and form the early granulomatous lesions (four weeks post infection), which are also called nascent granulomas (Cadena et al, 2017). As the infection proceeds, a necrotic core, containing mostly dead macrophages, forms inside the granuloma (Ahmad, 2011). At this point, these so called caseous granulomas are encapsulated by fibroblasts, they can become calcified and the microenvironment inside the granuloma changes. Typically, the pH decreases, the amount of nitric oxide and carbon monoxide increases, and some areas may become

hypoxic (Ahmad, 2011). These changes induce the expression of dormancy-associated mycobacterial genes, which in turn enables the survival of Mtb inside the granulomas in dormancy. The hypoxic environment also induces angiogenesis in the granulomas, which in turn improves bacterial growth, since oxygen and nutrients become available (Walton et al, 2018). On the other hand, vessels provide access for immune cells to the center of the granuloma, and thus facilitates the work of the immune system (Matty et al, 2015). The overall function of the granulomas is two-sided, one the one hand they provide a mechanism for the immune system to constrain dissemination of the mycobacteria, but on the other hand granulomas provide an environment for Mtb to survive inside the host.

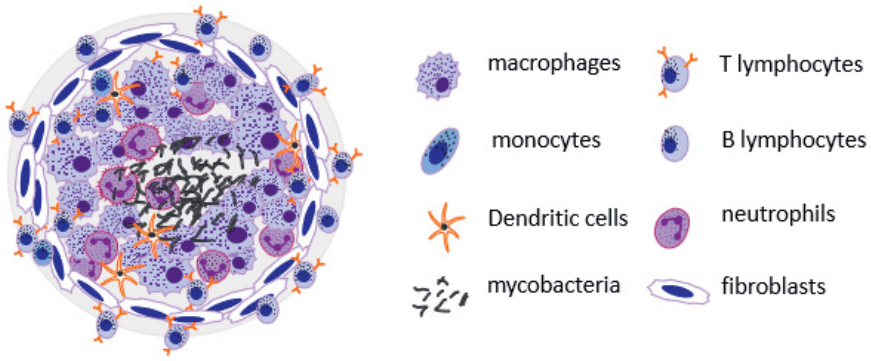

Figure 2. Granuloma structure. Modified from Cadena et al, 2017.

Beside the classic, caseous granulomas, histologically granulomas can be divided into different subtypes according to their typical characteristics. Classification is often based on the research interest and there are no standardized rules for categorization. For instance, the terms necrotic, neutrophil-rich, mineralized, fibrotic, fibrocaseous, caseous, vascularized, hypoxic, capsulated, calcified and cavitary granulomas are used (Cadena et al, 2017). In addition, the maturity of the granuloma can be used for classification. In this case nascent granulomas are often called early granulomas, whereas caseous granulomas are called mature granulomas and disrupted granulomas either resolved or disseminated granulomas.

2.1.5 Reactivation of a latent *M. tuberculosis* infection

Individuals with a LTBI have a 5-10% lifetime risk of reactivation and progression into an active disease (WHO, 2019). Reactivation can occur years after the original exposure to bacteria, however the risk for reactivation is not constant over time (Esmail et al, 2014). Most of the reactivation cases are diagnosed within 18 months of infection, and the risk decreases gradually over time, to only 0.5% 10 years after infection (Esmail et al, 2014). Several factors and medical conditions, all associated with immunosuppression, are connected to the increased risk of reactivation of a LTBI. An HIV-infection is the best characterized risk factor, increasing the risk for reactivation 10-110-fold compared to individuals without an HIV-infection (Ai et al, 2016). Accordingly, individuals whose immune system is chemically weakened, for instance by immunosuppressive medication after transplantation, due to the use of high-doses of corticosteroids or anti-tumor necrosis factor (TNF) therapy, have a higher risk of reactivation (Ai et al, 2016). In addition, it has been reported that silicosis, diabetes, smoking, cancer, vitamin D depletion and being underweight are risk factors for reactivation (Ai et al, 2016). Moreover, studies show an association between age and reactivation, and it seems that both infants and elderly persons are more prone to reactivation (Negin et al, 2015).

Granuloma structures have a significant role in restraining the dissemination of bacteria, and dysregulation of the immune system can promote granuloma progression and reactivation (Guirado & Schlesinger, 2013). Even though the reactivation mechanisms are inadequately known, it is known that the balance between pro-inflammatory and anti-inflammatory responses is important (Guirado & Schlesinger, 2013). So far, there is no single factor that triggers the reactivation, instead imbalance in any of the controlling mechanisms may trigger disruption of the granuloma and thus the dissemination of bacteria and the development of an active disease.

2.2 Tuberculosis vaccines

Regardless of the long history of Mtb research, there is only one available vaccine, Bacillus Calmette-Guérin (BCG), against TB. The current vaccine development is mainly focused on finding novel vaccines to replace (preventive vaccines) or boost BCG (booster vaccines) or on developing vaccines that can be used for immunotherapy (therapeutic vaccines). The 13 vaccine candidates that are currently

in clinical studies are divided into four groups; recombinant mycobacteria vaccines, inactivated whole-cell or fragmented vaccines, viral vector vaccines and protein/adjuvant vaccines (Summarized in Table 1.). The following two chapters introduce the different types of vaccines studied to prevent TB in recent history, providing a detailed summary of DNA vaccines and their potential in preventing TB.

2.2.1 Bacillus Calmette-Guérin

The history of the BCG vaccine begins in 1908, when Robert Koch and Camille Guérin managed to attenuate a virulent *M. bovis* stain and started to test it as a vaccine against TB (Calmette et al, 1927 reviewed in Luca & Mihaescu, 2013). The first clinical tests with healthy infants started already in 1921 (Pottenger, 1929), and the vaccine was considered safe, until the Lübeck disaster in 1930, where 72 children died when they received a vaccine that was contaminated with a virulent strain of Mtb (Fox et al, 2016). This event was an onset for critical research to study both the safety and the efficacy of the BCG. Today several different BCG vaccine strains are approved for clinical use (e.g. BCG-Pasteur, BCG-Glaxo and BCG-Phipps and BCG-Tokyo), which are genetically different and provide a broad range of efficiency, ranging from 0 to 80 percent (Luca & Mihaescu, 2013). Despite the uncertain efficiency of BCG, it is still used in high-risk areas, where even a minor increment in protective immunity can improve protection against TB at the population level when large groups of children are vaccinated. In addition, it has been observed that immunization with BCG protects against TB meningitis and miliary disease among children, and it has also been shown to provide protection against some non-tuberculous mycobacteria, such as *M. leprosy* and *M. buruli ulcer* (Smith et al, 1976; Rodrigues et al, 1993; Lahey et al, 2016). There is also evidence showing that vaccination with BCG significantly decreases neonatal mortality by reducing neonatal sepsis, respiratory infections and fever (Elguero et al, 2005; Shann, 2010; Shann, 2013). The mechanisms behind the phenomenon are presumably related to epigenetic effects on innate immune cells (Kleinnijenhuis et al, 2012). Moreover, BCG has been found to act as an immunostimulant by inducing strong (Th1) cytokine responses, which can enhance the effects of other vaccines administered to newborns (Ota et al, 2002). Similarly, the immunostimulant effect is utilized as an adjuvant in the treatment of bladder cancers and metastatic melanomas in adults (DeGeorge et al, 2017; Benitez et al, 2019). Mice studies have also suggested that the

Table 1. Summary of the tuberculosis vaccines in clinical trials.

Vaccine	Description	Effect	Phase
Recombinant mycobacterial vaccines			
VMP1002 (SII, Max Planck, VPM, TBVI)	rBCG vaccine	therapeutic	III
MTBVAC (Biofabri, TBVI, Zaragoza)	rMtb vaccine	preventive	IIa
Inactivated whole-cell or fragmented vaccines			
RUTI (Archivel Pharma)	fragmented Mtb in liposomes	therapeutic	IIa
Vaccae (Anhui Zhifei Longcom)	inactivated *M. vaccae*	therapeutic	III
DAR-901 (Dartmouth Uni., Aeras)	inactivated *M. obuense*	preventive	IIb
Immuvac (MIP) (Cadila Pharma)	heat-killed *M. indicus pranii*	therapeutic	III
Viral vector vaccines			
ChadOx.1 MVA85A (Oxford Uni, TBVI)	antigen 85A	preventive, booster	I
Ad5 85A (McMaster, CanSino)	antigen 85A	booster	I
TB/FLU-04L (RIBSP)	antigen 85A	therapeutic	IIa
Protein/adjuvant vaccines			
M72/AS01E (GSK, IAVI)	Rv1196, Rv0125 + adjuvant	therapeutic	IIb
ID93 + GLA-SE (IDRI/WT)	Rv2608, Rv3619, Rv3620, Rv1813 + adjuvant	therapeutic	I, IIa
H56:IC31 (SSI, Valnevam Aeras)	ESAT-6, Ag85B, Rv2660c + adjuvant	therapeutic	I, IIb
GamTBvac (MoH Russia)	Ag85A, ESAT-6-CFP10 + DBD + adjuvant	preventive, booster	I

strong Th1 response induced by BCG could provide protection against inflammatory and especially Th2-driven autoimmune diseases, such as allergic asthma (Kowalewicz-Kulbat & Locht, 2017). Exact protective effects of BCG in autoimmune diseases remain elusive, however it seems that Th1/Th2 balance and increased levels of TNF may have a significant role. For instance, in case of type 1 diabetes, it has been shown that TNF selectively eliminates autoreactive Treg cells and increases beneficial Treg cells (Faustman, 2018).

Although the BCG vaccine is considered safe, there are several possible BCG-related complications. The most common mild adverse effects after BCG vaccination are induration, pain, erythema and ulceration of the injection site, and more than 70-95% of the patients suffer from some of these symptoms (Lahey et al, 2016). Severe adverse effects are rare and can be divided in localized or systemic reactions. The most typical severe local effects are lymphadenitis and abscess (Lotte et al, 1984). Osteomyelitis is a rare, serious reaction to BCG (incidence 0.01-300 per million) and the incidence varies with the BCG strain (Lahey & von Reyn, 2016). BCG vaccination is contraindicated for immunocompromised children, since it can cause disseminated infections (Lotte et al, 1984; Al-Hammadi et al, 2017). In addition, it has been observed that in individuals with a genetic defect in the IFN-γ receptor, IFN-γ production is impaired and these persons are thus highly susceptible to TB (Casanova, 1997). Due to this genetic difference, such infants may develop a severe, systematic infection even after exposure to low-virulent mycobacteria, such as the attenuated *M. bovis* in the BCG vaccine. Because of its limited efficiency and the many problems connected to BCG, most of the low-risk countries have removed BCG from their vaccination programs for infants.

2.2.2 Recombinant mycobacterial vaccines

The first attempts to improve the BCG vaccine were focused on producing genetically modified recombinant BCG strains (rBCG). Currently, the most advanced recombinant BCG vaccine candidate is **VPM1002** (SII, Max Planck), where the urease C gene of *M. bovis* is replaced by Listeriolysin (Hly) of *Listeria monocytogenes* (Grode et al, 2005). With these modifications, the produced BCG ⊿*ureC*::*hly* secretes listeriolysin, which improves perforation of the phagosomal membrane, which in turn improves access to the antigens and thus enhances the CD8+ T cell immune response (Grode et al, 2005; Nieuwenhuizen et al, 2017). Besides the leakage of antigens, also bacterial DNA is released into the cytosol, which promotes autophagy, inflammasome activation and apoptosis (Nieuwenhuizen et al,

2017). Preclinical studies with VPM1002 have demonstrated its safety and enhanced protective efficacy when compared to the BCG vaccine (Grode et al, 2005; Desel et al, 2011; Vogelzang et al, 2014; Gengenbacher et al, 2016a; Gengenbacher et al, 2016b). Currently, VPM1002 is in phaseIIb clinical trials in Africa, where its efficacy in HIV infected infants is tested. Further, the phaseII/III trial to test post-exposure efficacy has received regulatory approval (Nieuwenhuizen et al, 2017).

The **MTBVAC** (Biofabri and University of Zaragosa) vaccine was the first live attenuated Mtb (from the Mt103 strain) containing vaccine that entered into clinical trials (Aguilo et al, 2016). In MTBVAC, the attenuation of Mtb is based on the deletion of the *phoP* and the *fadD26* genes, which are two major virulence factors of Mtb (Marinova et al, 2017). PhoP is a transcription factor, which regulates more than 2% of the Mtb genes, including the production of cell-wall lipids and secretion of ESAT-6 (Gonzalo-Asensio et al, 2008; Frigui et al, 2008). The deletion of FadD26 prevents the synthesis of phtiocerolmycocerosates (PIDM), which is a virulence lipid of the mycobacterial envelope (Cox et al, 1999). The vaccine has shown similar safety and an improved protective compared to BCG in preclinical studies (Arbues et al, 2013; Aguilo et al, 2016). Phase I clinical studies demonstrated the safety and immunogenicity of MTBVAC in BCG naïve adults (Spertini et al, 2015). The vaccine is currently in phase IIa studies, where its safety and immunogenicity are tested in South African newborns (Biofabri, 2018). The study is estimated to end in February 2020.

2.2.3 Subunit vaccines

According to the definition by the WHO, "Subunit vaccines, like inactivated whole-cell vaccines, do not contain live components of the pathogen. They differ from inactivated whole-cell vaccines, by containing only the antigenic parts of the pathogen." As subunit vaccines do not contain live particles, they are considered safe. In addition to inactivated vaccines, viral vector and protein-based vaccines, DNA vaccines are typical examples of subunit vaccines.

A great advance of subunit vaccines is the possibility to select the used antigen or antigen combinations, which enables tailor-made vaccine design and targeted immune responses. The most utilized Mtb antigens in the current TB subunit vaccines are listed in the Table 2. While the attenuation of the *M. bovis* strain used in the BCG vaccine is based on a genomic deletion in the RD-1 locus, containing virulence genes such as 6 kDa early secretory antigen target (ESAT-6) and 10 kDa

culture filtrate antigen (CFP-10), these Mtb genes are widely utilized in subunit vaccines. Besides the RD-1 locus related antigens, many metabolism related genes, PE/PPE family members and dormancy associated genes are tested as vaccine antigens in subunit vaccines (Table 2.). Often the desired immunological responses are elicited with subunit vaccines; however, the challenge seems to be in forming the correct immunological memory.

Table 2. Mtb antigens used in subunit vaccines.

Antigen	Description	References
RD-1 locus related antigens		
ESAT-6	Rv3875, 6 kDa early secretory antigen target (EsxA)	Lin et al, 2012
Rv3619	putative ESAT-6 like protein (EsxV)	Bertholet et al, 2010
Rv3620	putative ESAT-6 like protein (EsxW)	Bertholet et al, 2010
CFP-10	Rv3874, 10 kDa culture filtrate antigen (EsxB)	Tkachuk et al, 2017
CFP-7	Rv0288, low molecular weight protein antigen 7 (EsxH), TB10.4	Havenga et al, 2006
Metabolism related antigens		
Ag85A	Rv3804c, Fibronectin protein A (FbpA), mycolyl transferase	Verreck et al, 2009
Ag85B	Rv1886c, Fibronectin protein B (FbpB), mycolyl transferase	Cervantes-Villgrana et al, 2013
Rv0125	mtb32a, Serine protease (PepA)	Skeiky et al, 2004
PE/PPE family members		
Rv1196	Mtb39a, PPE18	Skeiky et al, 2004
Rv2608	PPE42	Bertholet et al, 2010
Dormancy antigens		
Rv2660c	Encodes a hypothetical protein	Yihao et al, 2015; Lin et al, 2012
Rv1813c	Encodes a hypothetical protein, coregulated by MprA and DosR	Bretl et al, 2012; Bertholet et al, 2010

2.2.3.1 Inactivated whole-cell or fragmented vaccines

Before the era of the BCG vaccinae, inactivated mycobacterial vaccines were broadly tested both in experimental models and human studies to prevent TB (Opie & Freund, 1939). Many of the tested vaccines showed efficacy, however further development stopped when BCG became the golden standard to prevent TB (Opie & Freund,1939; Kaufmann et al, 2017). Nowadays, inactivated mycobacteria vaccines, such as RUTI, Vaccae and Immuvac, are mainly being tested as immunotherapeutic vaccines to treat TB or as a booster for the BCG vaccine (DAR-901).

RUTI (Archievel Farma SL) contains detoxified, fragmented Mtb packed in liposomes and it has been shown to stimulate a cellular response against persistent mycobacteria (Cardona, 2006). RUTI is administrated as a combination with standard chemotherapy and in preclinical studies in mice and guinea pigs, it has demonstrated its safety and protective efficacy (Cardona, 2006). Similarly, phase I and II clinical trials have shown the safety, immunogenicity and tolerability of the vaccine (Guirado et al, 2008; Nell et al, 2014; Usman et al, 2017). In a phase IIa clinical trial, where its efficacy and immunogenicity were tested in HIV-positive individuals with a LTBI, the vaccine was showed to be well-tolerated and immunogenic (Nell et al, 2014). Presently, a phase IIa clinical trial, where the therapeutic effect of RUTI is tested in patients with MDR-TB after a successful first phase chemotherapy treatment, is ongoing (ClinicalTrials.gov; NCT02711735).

Another therapeutic vaccine, ***Vaccae*** (ICMR, Cadila Pharmaceuticals), has been studied for years. The vaccine contains an inactivated, whole-cell extract of a *Mycobacterium vaccae* strain (Zhu et al, 2018). The safety and efficacy of the vaccine have been proven in preclinical studies. Besides its therapeutic potential, Vaccae's ability to prevent TB has also been tested in a phase III clinical trial in China. This study was completed at the end of 2017; however, the results are not publicly available (ClinicalTrials.gov, NCT01979900).

SRL172 (Dartmouth University) is a heat-inactivated, therapeutic vaccine derived from an environmental mycobacterium, *Mycobacterium obuense*. To scale-up the vaccine production, the manufacturing method for SRL172 was changed, and therefore it is nowadays called **DAR-901** (von Reyn et al, 2017; Kaufmann et al, 2017). The results of studies on SRL172 and DAR-901 have been variable. Phase I and II clinical studies confirmed the safety, tolerability and immunogenicity of the vaccine (Waddell et al, 2000; Vuola et al, 2003; Lahey et al, 2010)., whereas SRL172 did not improve antituberculosis treatment in HIV-infected adults with TB (Mwinga

et al, 2002). Recently, DAR-901 has also been tested as a prophylactic vaccine. In a study in mice, where DAR-901 was used to boost the efficacy of BCG, immunization elicited cellular and humoral responses and boosted protection against Mtb (Lahey & von Reyn, 2016). A phase II study to prevent Mtb infections in healthy adults, previously immunized with BCG, is ongoing in Tanzania (ClinicalTrials.gov, NCT02712424).

Immuvac (ICMR, Cadila Pharmaceuticals) consists of a heat-killed *Mycobacterium indicus pranii* (MIP) and, was originally developed against leprosy (Andersen & Kaufmann, 2014). However, it was found to have a therapeutic effect against TB (Gupta et al, 2012). Currently Immuvac is in phase III clinical trials, and it is licensed for clinical use as a therapeutic vaccine in India. Coadministration of BCG and MIP encapsulated in alginate is one of the latest improvements to the BCG vaccine, and preclinical studies have shown an enhanced protective effect in mice (Nagpal et al, 2019).

2.2.3.2 Viral vector vaccines

Viral vectors are convenient for immunization purposes as viruses can efficiently transfer genes to target cells and induce immune responses and cellular immunity without additional adjuvants (Ura et al, 2014; Zhu et al, 2018). Several different viral vectors have been utilized in TB research (Table 3.) (Kaufmann et al, 2017). Some of the viral vector vaccines can be administered by the mucosal (intranasal) route and they can thus induce antigen specific T cell responses directly in the lung tissue (Zhu et al, 2018). However, it seems that viral vector vaccines induce less central memory T cells than adjuvanted protein vaccines (Billeskov et al, 2013; Zhu et al, 2018).

Table 3. Viral vectors used for tuberculosis vaccines (Kaufmann et al, 2017).

Name	Vector
MVA	Modified vaccinia Ankara virus
Ad5	Adenovirus 5
Ad35	Adenovirus 35
ChAd	Chimpanzee adenovirus
FLU	Replicon-deficient influenza virus (H1N1)

One of the most common approaches in TB research has been the utilization of the Mtb antigen 85A (Satti et al, 2014; Geldenhuys e al, 2015; Luabeya et al, 2015;). High hopes were placed on a viral vector vaccine candidate, **MVA85A**, constructed of recombinant, modified vaccinia virus Ankara (MVA) that expresses the Ag85A antigen from Mtb. MVA85A was tested as a booster vaccine to BCG. Most of the preclinical studies showed improved protection when compared to BCG alone (Goonetilleke et al, 2003; Williams et al, 2005; Hatherill et al, 2016). Even one study with non-human primates (NHP) demonstrated the boosting effect of MVA85A after an intratracheal challenge with Mtb (Verreck et al, 2009). However, in the similar study setup, where the Mtb challenge was conducted using aerosol administration, the improvement in the efficiency was not seen (White et al, 2013). Both preclinical and clinical studies demonstrated the safety and immunogenicity of the MVA85A in versatile experimental setups; with different doses and routes of administration and with different populations, for instance among healthy persons, latently infected individuals, adults, children, HIV-infected and BCG-vaccinated individuals (Hatherill et al, 2016). Because of these promising results, MVA85A was tested in phase II clinical trials, which confirmed that it was safe, tolerated and immunogenic but failed to show any improved efficacy (Tameris et al, 2013; Ndiaye et al, 2015; Kashangura et al, 2019). However, recently MVA85A was tested as a booster for **ChAdOx185A**, a vaccine candidate composed of a replicon-deficient chimpanzee adenovirus (ChAd) expressing Ag85 (Stylianou et al, 2015). It was found that vaccination with MVA85A followed by ChAdOx185A showed better protection than BCG alone in mice (Stylianou et al, 2015). The combination was tested in a phase I clinical trial with healthy adults in the UK, which demonstrated that the vaccination method was immunogenic and well-tolerated (Wilkie et al, 2019).

Two different replication-deficient adenoviral vectors, **Ad35** (serotype 35 vector, Crucell) and **Ad5** (serotype 5 vector, McMaster) have been utilized in TB vaccines (Kaufmann et al, 2017). The vaccine candidate **AERAS-402** comprised Ad35 vector with the Mtb antigens 85A, 85B and 10.4 (Havenga et al, 2006). The vaccine was considered safe and it induced immunological responses both in preclinical and clinical studies but failed to protect against the actual disease (Abel et al, 2014; Darrah et al, 2014; Tameris et al, 2015; Nyendak et al, 2016). Studies with the **Ad5Ag85A**, a serotype 5 vector expressing Ag85A (McMaster), have been more promising, however. It has been tested as a booster for BCG, and intranasal administration seems to enhance the protection of the vaccine (Wang et al, 2004; Santosuosso et al, 2006; Xing et al, 2009). Ad5Ag85A has been shown to be safe and well-tolerated both in preclinical and clinical studies and it seems to elicit both CD4+ and CD8+

T cell immune responses (Smaill et al, 2013). A phase I clinical trial, where the safety and immunogenicity of the vaccine, after intramuscular and intranasal administration, are tested in healthy adults with a history of BCG immunization, is ongoing in Canada and is estimated to be completed in 2021 (ClinicalTrials.gov, NCT02337270).

TB/FLU-04L (Research Institute of Influenza, Saint Petersburg, Russia) is a mucosal-vectored vaccine (administrated via the intra-nasal route) based on an attenuated replication-deficient influenza A virus vector expressing the antigens Ag85A and ESAT-6 (Stukova et al, 2006; Hatherill et al, 2016). Like Ad5Ag85A, the intranasal administration of the TB/FLU-04L vaccine is safe and induces T cell responses (Stukova et al, 2006). In preclinical studies, immunization with the vaccine has shown improved efficacy, however the phase I clinical trial did not show a protective effect in boosting BCG. Currently, TB/FLU-04L is under evaluation for a phase IIb trial to study its protective effect in individuals with LTBI.

2.2.3.3 Protein/adjuvant vaccines

Protein vaccines contain purified or recombinant antigens of pathogens. Typically, they manage to elicit immune responses, however the enhancement in the protective effect *in vivo* requires coadministration of immunostimulatory adjuvants.

M72/AS01 (adjuvant system 01, GlaxoSmithKline) is a vaccine combined of a 72 kDa recombinant fusion protein and the AS01 adjuvant. The M72 fragment contains three subcomponents, a 14-kDa C-terminal fragment of **Mtb 32A** (Rv0125 encoding PepA), the full length **Mtb 39A** (Rv1196 encoding PPE 18) and a 20-kDa N-terminal fragment of Mtb32A, in tandem (Skeiky et al, 2004). The vaccine antigens were selected based on their ability to stimulate T cells in TST-positive individuals (Skeiky et al, 1999; Al-Attiyah et al, 2004). The AS01 adjuvant system of the M72/AS01 vaccine contains two immunostimulants, MPL (3-O-desacyl-4'-monophosphoryl lipid A) and the saponin QS-21 in liposomes (Didierlaurent et al, 2016). MPL activates innate immune responses via TLR4 and QS-21 seems to stimulate antigen-specific antibodies and cytotoxic T cells (Casella et al, 2008; Newman et al, 1997). The known hemolytic activity of QS-21 in the AS01 adjuvant system is eliminated with the coadministration of cholesterol derived from liposomes (Beck et al, 2015). In preclinical studies, immunization with the M72/AS01 generated a strong immunological response, especially against the C-terminal Mtb 32A fragment (Skeiky et al, 2004). The vaccine protected mice against an aerosol challenge with Mtb, and the immunization of guinea pigs resulted in prolonged

survival as compared to BCG (Skeiky et al, 2004). Consequently, M72/AS01 was the first recombinant TB vaccine to be tested in clinical trials. The safety and immunogenicity (increased number of CD4+ T cells) of the vaccine has been demonstrated in several human studies (Day et al, 2013; Idoko et al, 2014; Penn-Nicholson et al, 2015; van der Meeren et al, 2018; Tait et al, 2019). A clinical study by Van der Meeren et al. (2018) showed, that immunization with the M72/AS01 vaccine provides 54% protection against the progression of active TB among IGRA-positive individuals, giving high expectations for further studies.

Another recombinant protein vaccine, called **ID93-GLA-SE** (IDRI/WT), combines fragments of three Mtb virulence-associated (Rv2608, Rv3619, Rv3620) genes and one latency-associated (Rv1813) gene, and a synthetic **g**lycopyranosyl **l**ipid **a**djuvant in a **s**table oil-water **e**mulsion (GLA-SE) (Bertholet et al, 2010). Studies with mice have shown that immunization with the ID93-GLA-SE vaccine elicits protection against Mtb (Baldwin et al, 2015), and boosts the protective effect of BCG (Kwon et al, 2019). The phase I clinical trial proved the appropriate safety profile and a phase II trial to study the preventive efficacy of the vaccine was completed (March 2019), but the results of the study have not been published (Coler et al, 2018; NCT02465216).

Several different multistage, subunit vaccines (e.g. H1:IC31, H4:IC31 and H56:CAF01) have been tested for their ability to prevent TB. Nowadays only one, namely **H56:IC31** (SSI, Valnevam Aeras), is in clinical trials. H56:IC31 fuses the commonly known Mtb antigens 85B and ESAT-6 with Rv2660c, which is a latency-associated gene of Mtb, and the IC31 adjuvant (Lin et al, 2012). The adjuvant is a combination of an antibacterial peptide and a synthetic oligodeoxynucleotide (TLR9 agonist) (Aagaard et al, 2011). Mouse studies demonstrate both the protective effect of the H56:IC31 vaccination in containing a latent infection and in preventing a primary infection (Aagaard et al, 2011). Studies with non-human primates support the data from mice studies (Lin et al, 2012).

GamTBvac (MoH Russia) is the latest multistage subunit vaccine to have entered into clinical studies. The vaccine is a combination of two different recombinant proteins composed of a dextran binding domain (DBD) and a Gly-Ser spacer fused either with the Mtb Ag85A or Mtb ESAT-6 and CFP-10 antigens (Tkachuk et al, 2017). Dextran core nanoparticles are used as an adjuvant. The protective effect of the GamTBvac vaccine against a primary infection and in boosting the effect of BCG, as well as its safety, were shown in preclinical studies with mice and guinea pigs (Tkachuk et al, 2017). The phase I clinical trial with healthy adults demonstrated acceptable safety and tolerability of the vaccine (Vasina et al, 2019). A phase II study

testing the vaccines safety, reactogenicity (the degree to which the vaccine can provoke adverse side effects through excessive immune responses, such as a fever, or swelling at the injection site) and immunogenicity is ongoing in Russia (ClinicalTrials.gov, NCT03878004).

2.3 DNA vaccines

DNA vaccination was introduced for the first time in the early 1990s and it is said to be the third-generation immunization method (Tang et al, 1992; Tregoning et al, 2014). The technique enables tailor-made immunization with a minimal risk of virulence reversion. The method is utilized in developing vaccines against several medical conditions, such as cancer, allergy, autoimmunity and infections (Khan et al, 2013). Until now, three DNA vaccines have been licensed for use in veterinary medicine, but none have been approved for clinical use in humans (Garver et al, 2005; Grosenbaugh et al, 2011; Myhr, 2017). In preclinical studies with non-human primates, DNA vaccines have shown good protection against an Mtb infection, which emphasizes the potential of DNA vaccines for controlling TB (Kita et al. 2005; Okada et al, 2007; Cervantes-Villagrana et al, 2013).

2.3.1 Principle of DNA vaccination

In DNA vaccination, antigens are introduced to the cells within expression plasmids. Typically, DNA vaccines are injected into muscle tissue, where the injected plasmid-DNA enters myocytes (Khan et al, 2013). In the nucleus, RNA-polymerase copies the DNA sequence of the plasmid and forms mRNA, which is transferred from the nucleus to ribosomes in the cytosol or in the endoplasmic reticulum (Khan et al, 2013) (Figure 3.). Ribosomes translate the genetic code of the mRNA into peptides, which after folding, have the immunogenic characteristics of the antigen (Rivas-Santiago & Cervantes-Villagrana, 2014). The produced antigens are secreted into the extracellular space, where they induce immunological responses (Figure3) (Rivas-Santiago & Cervantes-Villagrana, 2014). Typically, antigens are expressed several weeks after injection.

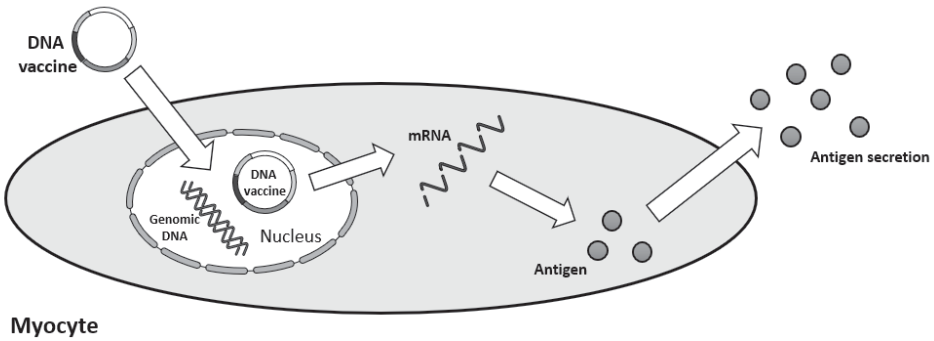

Figure 3. The basic principle of the DNA vaccination. The DNA vaccine plasmid is introduced into a myocyte, where it enters the nucleus. The plasmid is translated into a messenger RNA, which is translated into a protein and secreted. Modified from Rivas-Santiago & Cervantes-Villagrana (2014).

2.3.2 DNA vaccine plasmid design and construction

Construction of a DNA vaccine is based on conventional cloning methods. The basic construct includes an expression vector, which is safe to humans (e.g. pVAX1, and pcDNA3.1 vectors) (Williams, 2013; Gómez et al, 2018). The vector contains a **promoter** or enhancer sequences of viral origin, for instance the cytomegalovirus (CMV), Rous sarcoma virus (RSV) and simian virus (SV) 40 promoters, which can promote antigen expression in various tissues (Figure 4.) (Hasson et al, 2015). The sequence of the antigen is inserted into the **multiple cloning site** (MCS), which is located next to the promoter (Hasson et al, 2015). The MCS contains a selection of different restriction sites and thus enables the cloning of several antigens or adjuvants into the same expression vector (Gómez et al, 2018). Typically, the **Kozak consensus sequence** (A/GCCAUGG), which enhances ribosomal binding in eukaryotic cells, is added before the start codon (AUG) of the antigen (Gómez et al, 2018). To facilitate the correct folding of each immunogen, **cleavage sites** (e.g. RGRKRSS) can be added between the antigens (Yan et al, 2009). Alternatively, immunogens can be cloned without cleavage sites forming a fusion protein with several epitopes (Chu et al, 2016). The plasmid has a stop codon (TAG, TAA or TGA), after the antigen sequence(s) for translation, followed by a **poly-A-tail** to improve the translation of the mRNA (Gómez et al, 2018). In addition, the expression vector includes a bacterial **replication origin** and **antibiotic resistance gene**, which facilitate the microbiological production of the recombinant plasmid (Hasson et al, 2015). At the structural level, the efficacy of the DNA vaccines can be

improved by codon optimization, selection of alternative promoters, or by removing bacterial elements (Li et al, 2012).

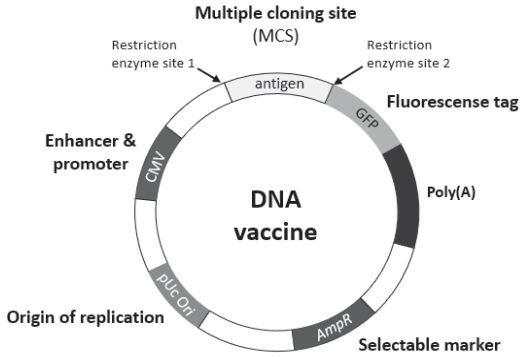

Figure 4. Common structure of a plasmid-based DNA vaccine encoding a fusion protein. Modified from Rivas-Santiago & Cervantes-Villagrana (2014).

2.3.3 Immunological responses to DNA vaccines

DNA vaccination elicits many immunological responses. Plasmid DNA that enters the target cells (e.g. myocytes and keratocytes) can induce both humoral and cellular immune responses (Figure 5.). Transfected myocytes translate and secrete antigens into the extracellular space, where dendritic cells (DCs) recognize antigens and engulf them (Gómez et al, 2018). Further DCs process the engulfed antigens and present them either on MHC class I or II molecules on their surface (Tregoning & Kinnear, 2014). Antigens which have a naïve protein conformation are presented via class II molecules, and vaccine-derived peptides on class I molecules (Rivas-Santiago & Cervantes-Villagrana, 2014). Antigen-loaded dendritic cells migrate to draining lymph nodes, where they stimulate T lymphocytes (Suschak et al, 2017). An antigen presented on a MHC class I molecules stimulate CD8+ T cells and antigens presented on MHC II molecules stimulate CD4+ T cells (Suschak et al, 2017). In addition, it is possible that the plasmid DNA directly transfects antigen presenting cells. In this case, it is likely that APCs present the antigens on MHC class I molecules, and therefore activate CD8+ T cell responses (Khan, 2013).

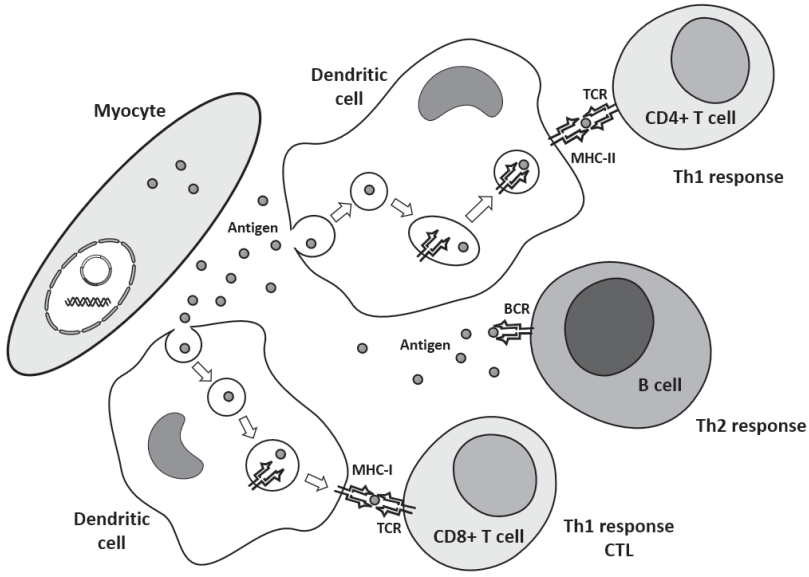

Figure 5. Induction of the cellular and humoral immunity by DNA vaccines. The myocyte transfected with the DNA vaccine secretes antigens, which are recognized by dendritic cells and B cells. Dendritic cells present antigens via MHCI and MHCII receptors to CD8+ and CD4+ T cells, inducing a Th1 immune response. B cells recognize antigens directly with the B cell receptors (BCRs) and maturein to effector B cells. Modified from Rivas-Santiago & Cervantes-Villagrana (2014).

After vaccination, only part of the injected plasmid DNA enters the target cells. The naked DNA outside the nucleus is recognized by pattern recognition receptors of innate immune cells, which start to produce inflammatory cytokines, which in turn stimulate antigen presentation of the APCs to T cells (Tregoning & Kinnear, 2014). It is also possible, that transfected cells are phagocytosed by APCs, which present the antigens to T cells (cross-priming) (Khan et al, 2013).

B cells can directly recognize antigens that are secreted by myocytes, and thus induce humoral responses (Figure 5.) (Khan, 2013). An antigen interaction with a B cell receptor (BCR) provokes the production of antigen specific antibodies and the formation of a B-cell memory against the antigen (Khan, 2013).

2.3.4 Delivery of DNA vaccines

The transfer of the plasmid DNA into target cells is a critical step in DNA vaccination. Despite the plasmid do not integrates to genome of the host, the

plasmid needs to enter the nucleus to utilize the translation machinery of the cell. To get to the nucleus, the DNA needs to pass two barriers, the cell membrane and the nuclear membrane, and it is estimated that only 0.1% of the injected plasmid DNA is translated (Capecchi et al, 1980). To improve the efficacy of DNA vaccine antigens, many administration routes and delivery techniques are being tested. An ideal delivery method should result in optimal expression, immunogenicity and practicability with minimal costs (Tregoning & Kinnear, 2014).

Intramuscular injection is the most common method for administration, however several different **delivery routes** including subcutaneous, intradermal, intranasal, intravenous, vaginal, rectal, sublingual and oral are being investigated (McCluskie et al, 1999, Hamajima et al, 2002; Bivas-Benita et al, 2005; Schautteet et al, 2011; Mann et al, 2013). Mucosal administration would be an attractive delivery route for TB vaccines, as it has the potential to elicit local immune responses in lung tissue (Tregoning & Kinnear, 2014). The challenge, especially in mucosal administration, is degradation of the plasmid, as mucus contains many degrading chemicals and enzymes (Tregoning & Kinnear 2014). To reduce degradation, different **DNA packaging** methods are being studied, and there are currently three main approaches for packaging; cationic polymers, liposomes and virosomes (Greenland & Letvin, 2007; Kheiri et al, 2012). Since the backbone of DNA is negatively charged, it can interact with **cationic polymers**, such as polyethyleneimine, chitosan and poly(lactic-co-glycolic acid) (PLGA), or it can be packed into **liposomes** or **virosomes** (Greenland & Letvin, 2007). The formed carrier vesicles can fuse with the membrane of the target cell and thus transfer plasmid DNA into the cytosol.

To improve the expression of the DNA vaccine, several different device-mediated techniques are being applied. In animal studies, **electroporation** (EP) has proved to be the most efficient method to date (McCluskie et al, 1999; Chu et al, 2016). During EP, a short electric pulse(s) is targeted to the injection site, which temporarily changes the permeability of the cell membrane (Rosazza et al, 2011). Both in clinical and preclinical studies, EP has been shown to improve antigen expression 100- to 1000-fold compared to naked injection (Low et al, 2009; Vasan et al, 2011; Villarreal et al, 2013; Grant-Klein et al, 2015). Other device-mediated techniques use physical force for delivery. In the **gene gun** method the DNA is coated with (gold) particles and fired at the skin (Yang et al, 1990). In **Jet-injectors** liquid containing the plasmid DNA is compressed with gas or springs through a filter, which leads to the formation of tiny droplets that can be forced into cells (Shergold et al, 2006). Additional delivery techniques include **tattooing** and

microneedle patches. Although tattooing improves expression of the antigens, it requires relatively large skin areas, which limits its use (Grunwald et al, 2014; Tregoning & Kinnear, 2014). **Microneedle patches** are one of the most promising methods for the delivery of DNA vaccines. In this technique microneedles are coated with plasmid DNA and applied in as a patch to the patient's skin (DeMuth et al, 2013). Within the method, it is possible to adjust the delivery depth and administer several antigens and adjuvants simultaneously (Tregoning & Kinnear, 2014).

2.3.5 Molecular adjuvants of DNA vaccines

Numerous molecular adjuvants, including cytokines, chemokines and co-stimulatory molecules, are being tested to improve the immunological responses of DNA vaccination (Williams, 2013). The most utilized molecular adjuvants tested in DNA vaccines for TB are presented in Figure 6. Molecular adjuvants are can be co-administered with the antigenic vaccine plasmid, as "back-bone adjuvants", or they can be introduced to the cells within a separate adjuvant plasmid (Suschak et al, 2017). The cloning of molecular adjuvants into the expression vector is preferred, as thereafter they are expressed as long as the vaccine antigens. Transfected cells produce and secrete adjuvants, similarly to antigens, to the surrounding tissue, which stimulate local APCs and cells in lymph nodes (Suschak et al, 2017).

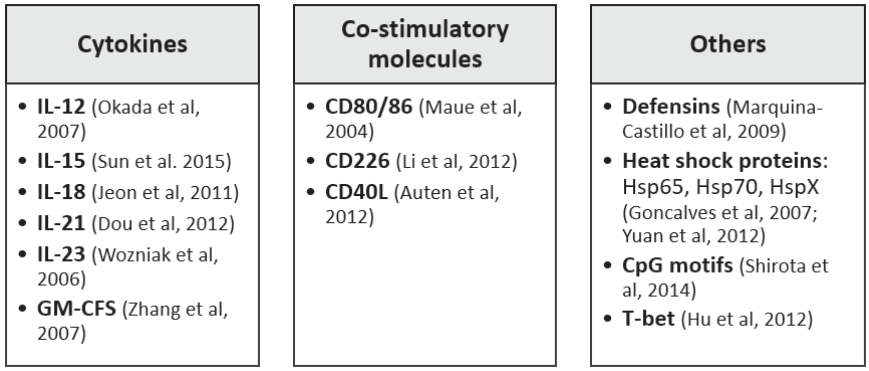

Figure 6. Examples of molecular adjuvants tested with DNA vaccine antigens against mycobacterial diseases. Abbreviations: IL, interleukin; GM-CFS, granulocyte macrophage colony stimulating factor; CD, cluster of differentiation; CpG motif, deoxycytidylate-phosphate-deoxyguanylate motif; T-bet, a T-box transcription factor

Cytokines have a critical role in cell signaling, and they have been utilized as molecular adjuvants in DNA vaccines. In TB vaccines, the most tested molecules are interleukin 12 (IL-12), interleukin 21 (IL-21) and granulocyte macrophage colony stimulating factor (GM-CFS) (Rivas-Santiago & Cervantes-Villagrana, 2014). These cytokines are being tested as adjuvants in coadministration with both BCG and with DNA vaccines expressing Mtb antigens (e.g. Ag85, ESAT-6) with variable results (Okada et al, 2007; Zhang et al, 2007; Jeon et al, 2011). Typically, adjuvants elicit immunological responses, although the improvement in the protection is not clear. For instance, an IL-12 adjuvant increases the production of IFN-γ and therefore enhances Th1 responses. However, it has failed to improve the efficiency of the vaccine *in vivo* (Jeon et al, 2011).

Immunoglobulin superfamily proteins, such as CD226, are expressed on majority of human immune cells. They can be adhesive molecules or receptors that transmit immunological responses (e.g. NKs and CD8+ T cells), and they have been tested as **co-stimulatory molecules** in DNA vaccines (Figure 6.). In animal studies with mice and cattle, the administration of co-stimulatory molecules has been shown to enhance immunogenicity and improve protection against mycobacterial infection (Maue et al, 2004; Auten et al, 2012; Li et al, 2015).

In addition, antimicrobial peptides (e.g. β-defensin 2), heat shock proteins, and transcription factors are being tested as molecular adjuvants (Figure 6.). Antimicrobial peptides are the first line immune response against microbes, and they stimulate various immune responses, including phagocytosis, chemotaxis and cytokine production (Mohan et al, 2013). In mouse studies, the co-administration of **defensins** with ESAT-6 and Ag85B, improved the survival of the animals after a mycobacterial infection (Rivas-Santiago & Cervantes-Villagrana, 2014). **Heat shock proteins** (Hsp) are intracellular molecules which function as chaperons in numerous cellular processes and they have the potential to induce many immune responses. In TB vaccines, for instance Hsp65, Hsp70 and HspX, are exploited, and they seem to enhance the effect of both the BCG and DNA vaccines (Gonvalves et al, 2007; Yuan et al, 2012). T-bet (TBX21) is a member of the T-box family of **transcription factors**, which induces Th1 responses especially in NK cells (Hu et al, 2012). T-bet has been tested as a back-bone adjuvant in combination with Ag85B, and the adjuvant seems to enhance the efficiency of the vaccine (Hu et al, 2012).

Furthermore, the plasmid DNA itself contains endosomal unmethylated deoxycytidylate-phosphate-deoxyguanylate (**CpG**) motifs, which work as in-built adjuvants (Shirota et al, 2014). Additional CpG motifs can easily be inserted to the

DNA vaccine construct, or the existing motifs can be modified or deleted. The produced CpG oligos activate human DCs and B cells, and thus induce the production of IgG (Bode et al, 2011). They also stimulate monocytes and macrophages to produce cytokines and enhance both CD4+ T cells and CD8+ cytotoxic T cells (Bode et al, 2011). When coadministrated with antigens, CpG motifs promote CD8 T cell responses (Suschak et al, 2017).

2.3.6 Advantages and disadvantages of DNA vaccines

DNA vaccines have many advantages as compared to conventional vaccines (Figure 7.). DNA vaccines elicit long lasting immune responses, both humoral and cellular, against specific antigens (Khan, 2013). Since they do not contain any live particles of the pathogen, there is no risk of infection (Gómez et al, 2018). In many studies they have been proven to be well tolerated and safe. The construction of DNA vaccines is relatively easy and enables the selection of tailormade antigens (Khan et al, 2013). In addition, production is inexpensive and the vaccine construct is resilient to temperature, which facilitates storage and shipping (Gómez et al, 2018).

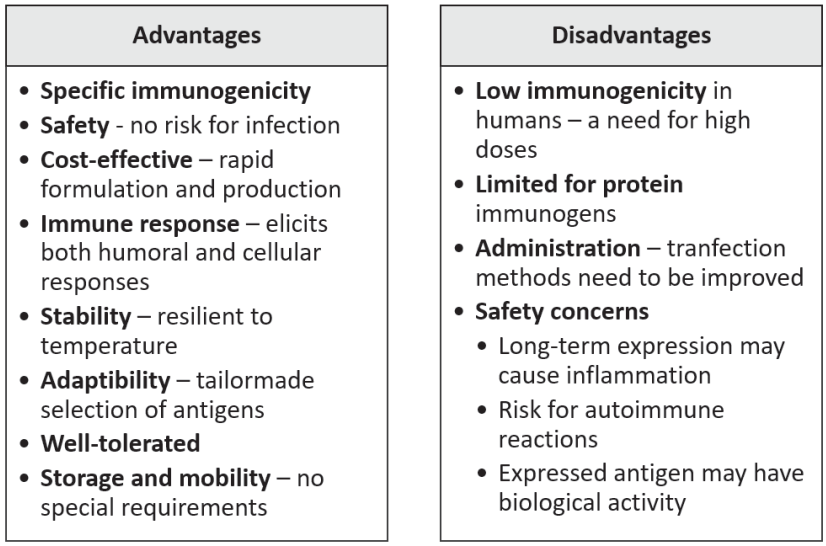

Figure 7. Advantages and disadvantages of DNA vaccines. (Modified from Khan, 2013 and Gómez et al, 2018).

The greatest disadvantage of DNA vaccines is their relatively poor immunogenicity in humans (Li et al, 2012). Despite the fact that many animal models show improved efficiency, similar results have not been achieved in humans (Khan, 2013). The required high doses and physiological differences between humans and animal models can explain poorer protection (Gómez et al, 2018). The other challenge of DNA vaccines is their administration. Intramuscular injections alone are inefficient, and more convenient methods to introduce vaccine plasmids into the target cells are needed (Gómez et al, 2018). Due to the expression technique, DNA vaccines are limited for use as protein immunogens, and therefore they are not applicable for all types of vaccines, for instance for vaccines that utilize polysaccharide antigens (Li et al, 2012). The method has also raised some safety concerns; the long-term expression of the antigens may cause inflammation or induce autoimmune reactions (Gómez et al, 2018. It is also possible that the expressed antigens have unpredicted biological activity (Li et al, 2012).

2.3.7 Experimental DNA vaccines against tuberculosis

Currently there are no DNA vaccines in clinical trials against TB. However, many research groups are studying the immunogenicity, protective and therapeutic effects of various antigens as DNA vaccines mostly in murine models. The study approaches include testing of single antigens, such as Rv2628, Rv3407 and Rv2190c, or a combination of several antigens (Liang et al, 2017; Liang et al, 2018; Liang et al, 2019). Antigens are often commonly known virulence factors or latency-associated proteins. Table 4. summarizes the most promising DNA vaccines that have been tested in the past two years in animal models.

2.4 Conventional animal models for tuberculosis research

Throughout the history of TB research, several different animal models have been utilized for evaluating drug and vaccine efficiency and the molecular mechanism of the infection. In 2018, more than 60% of all preclinical studies on TB were performed with **mice** (Singh & Gupta, 2018). The small size, relatively low housing costs, easy handling and availability of immunological tools explains the popularity of the model (Myllymäki et al, 2015). The Mtb infection in mice stimulates similar immunological responses as in humans, although the pathology of the disease is

Table 4. Summary of experimental DNA vaccines against tuberculosis in preclinical studies during the past two years.

Antigen	Antigen characterization	Protocol	Response to vaccine	Reference
Rv2628	Latency-associated, induced in hypoxia	3 doses, mice Reactivation model	Decreased lung CFU counts	Liang et al, 2019
Rv3407	Detoxification (antitoxin VapB47)	3 doses, mice	Induced IFNγ and Th1	Liang et al, 2018
Ag85B-ESAT-6-Rv2660c	Virulence factors and latency-associated	1 dose with EP, mice	Increased number of CD8+ T cells	Tang et al, 2018
Ag85A-Rv3425-Rv2029c	Early and latency-associated	3 doses, mice	Macrophage activation, Induced Th1	Su et al, 2017
Mtb32C-HBHA	Induce aggregation of mycobacteria	Boosting BCG, mice	Induced IFNγ	Teimourpour et al, 2017
Bfrb and Mpt32	Metabolism, cell wall and cell processes	mice	Strong immune response	Shahzad et al, 2017
PE35, PPE68, EsxA, EsxB, EsxV	PPE family proteins, virulence factors	1 dose, mice	*In vivo* expression and immunogenicity	Hanif et al, 2017
Rv2190c	Virulence, detoxification, adaptation	3 doses, mice Therapeutic	IFNγ, Th1	Liang et al, 2017
Hsp65 and IL-12	Heat shock protein and interleukin	3 doses, monkeys, therapeutic vaccine	Therapeutic efficacy against MDR-TB and XDR-TB	Okada et al, 2017
Ag85B-ESAT6-RpfE	Virulence factors and resuscitation promoting factor	Boosting BCG, zebrafish	Boosts BCG	Oksanen et al, 2016
Ag85-Tb10.4	Culture filter proteins, virulence	3 doses, mice	IFNγ, IL-12	Meshkat et al, 2016
Rv0577	Culture filtrate protein	Boosting BCG	Immunogenic, boosts BCG	Gu et al, 2016

different, and genetic susceptibility seems to determine the lung pathology (Rhoades et al, 1997). Overall, an infection leads to progressive disease with the formation of inflammatory lesions lacking the typical features of human TB granulomas (including necrosis, caseation and cavitation) (Myllymäki et al, 2015). In addition, there are differences in bacterial control and T cell responses as compared to humans (Singh & Gupta, 2018).

Guinea pigs were the first animals used for TB research by Robert Koch (Koch, 1891; Kaufmann, 2001). In general, guinea pigs are sensitive to Mtb infection and the disease outcome recapitulates the features of human TB in many aspects (Myllymäki et al, 2015). The granulomas are necrotic and caseating, although they do not form liquified cavities. In comparison to mice, laboratory space requirements and costs are higher for guinea pigs. An Mtb infection in guinea pigs cause a progressive disease, which can be protected against with BCG, and therefore the model is useful in vaccine safety and efficacy studies, as well as for evaluating drugs (Myllymäki et al, 2015; Singh & Gupta, 2018).

A mycobacterial infection in **rabbits** captures many of the disease pathologies of human TB. The granulomas can be necrotic, caseating, hypoxic, and they form cavities and induce the formation of vessels (Mendez et al, 2008). The susceptibility to an Mtb infection varies with the rabbit strain (Manabe et al, 2003). In general, rabbits are less susceptible than guinea pigs to an Mtb infection, and they are highly susceptible to a *M. bovis* infection (Singh & Gupta, 2018). The rabbit model is useful for transmission research and for studying rarer forms of TB, such as bone, meningeal and cutaneous TB (Singh & Gupta, 2018). The relatively large size and lack of immunological reagents limits the use of rabbits in TB research (Myllymäki et al, 2015).

Due to the close evolutionary relationship between **non-human primates** (NHPs) and humans, an Mtb infection in NHPs recapitulates the human TB best (Myllymäki et al, 2015). The phases of the infection, immune responses and disease pathology are identical to those in humans. However, the susceptibility to an Mtb infection can vary between species; cynomolgus macaques are relatively resistant, and thus a convenient model for LTBI, whereas rhesus macaques are more susceptible (Singh & Gupta, 2018). Regardless of poor availability of animals, high housing and experimentation costs and ethical concerns, NPHs are often used to evaluate efficacy and safety of promising drug and vaccine candidates prior to clinical studies (Singh & Gupta, 2018).

2.5 The adult zebrafish as a model for tuberculosis

Zebrafish has turned out as a useful animal model for studying tuberculosis. The immune system of the adult zebrafish recapitulates strikingly well the human immune system; it possesses both the innate and adaptive arms and includes most of the same immune cell types, such as macrophages, neutrophils, DCs and lymphocytes with conserved functions (Myllymäki et al, 2016). The natural fish pathogen, *Mycobacterium marinum*, causes a similar granulomatous infection in fish as Mtb does in humans (Table 5.) (Swaim et al, 2006; Parikka et al, 2012). Studies with zebrafish larvae have shown that the infection results in the phagocytosis of mycobacteria by macrophages, and the formation of granulomas (Davis et al, 2002;

Table 5. Comparison of characteristics between *M. tuberculosis* and *M. marinum*. Data are collected from Aubry et al, 2017 and Gordon&Parish, 2018.

	M. tuberculosis	***M. marinum***
Typical features and size	Aerobic, acid-fast bacilli 2-4 μm by 0.3-0.5μm rods	Aerobic, acid-fast bacilli 1-4 μm by 0.3-0.5μm rods
Prevalence	Worldwide, human lung tissue	Environmental, waterborne
Optimal growth temperature	25-37 °C	25-35°C
Replication rate	12-24 hours	3-4 hours
Natural infection route	Through air droplets	Via water
Natural hosts	Human	Fish and amphibians, (opportunistic infections in humans)
Possible outcomes of infection	Spontaneous healing, active infection or latent infection	Spontaneous healing, active infection or latent infection
Symptoms of infection	Coughing, chest pain or pain while breathing, unintentional weight loss, fatigue, fever, night sweets, chills and loss of appetite	Uncoordinated swimming, abdominal swelling, loss of weight, skin ulceration, granuloma formation
Susceptibility to antimicrobial agents	Isoniazid, rifampicin, pyrazinamide, ethambutol, streptomycin	Most of antibiotics, including rifampin and doxycycline

Davis &Ramakrishnan, 2009). In a systemic infection in adult zebrafish, the granulomas become caseating and share structural features with the granulomas in human TB (Swaim et al, 2006; Parikka et al, 2012; Myllymäki et al, 2018; Cheng et al, 2020). An *M. marinum* infection in adult zebrafish can cause either an active or a latent disease in a dose dependent fashion (Swaim et al, 2006; Parikka et al, 2012). In experimental setups, latent infection has been difficult to replicate, thus the naturally persistent infection in fish provides a feasible model to study a LTBI. Furthermore, the latent infection can be reactivated by immunocompromising the fish, providing a model for studying reactivation from the side of both the host and the pathogens (Parikka et al, 2012; Myllymäki et al, 2018).

On the bacterial side, the *M. marinum* and Mtb genomes are 85% conserved (Stinear et al, 2008). Due to relatively high homology, many virulence genes of Mtb are conserved in *M. marinum*. For instance, it has been shown that the RD1 virulence locus and the ESX-1 secretion system, are similarly critical for bacterial virulence in fish as in humans (Volkman et al, 2004; Swaim et al, 2006; Stoop et al, 2011). Because of the similar infection phases and homology of the pathogens, the zebrafish is a useful model for studying the effects of novel vaccine and drug candidates against human TB (Oksanen et al, 2013; Oksanen et al, 2016; Myllymäki et al, 2016). Indeed, for example immunization with DNA vaccines, expressing the commonly used antigens (Ag85, ESAT-6 and CFP-10), protects zebrafish against a *M. marinum* infection (Oksanen et al, 2013).

On a practical level, zebrafish are small in size, reproduce high number of offspring and are thus relatively cost-effective animals to maintain (Myllymäki et al, 2015). The availability of different transgenic and reporter lines, as well as molecular biological tools, such as antibodies, has increased as the model has become more popular. The obvious limitations of use of zebrafish as model for TB are related to differences in anatomy between humans and fish; the lack of lungs and lymph nodes, differences in T cell subpopulations and different infection routes of Mtb and *M. marinum* (Myllymäki et al, 2015; Myllymäki et al, 2016). Moreover, as a teleost fish, zebrafish have a diploid genome, which may complicate studying of gene expressions and production of targeted knockout strains (Saralahti et al, 2020). However, mention the fact that the zebrafish is the lowest developed vertebrate animal model, providing a more ethical model for research purposes than conventional animal models (Myllymäki et al, 2015; Myllymäki et al, 2016).

2.5.1 DNA vaccines used to prevent fish diseases

DNA vaccines have been proven to be effective in immunizing fish against viral infections (Evensen & Leong., 2013). Two DNA vaccines, pCMV4-G against viral hemorrhagic septicemia and Apex-IHN against infectious hematopoietic necrosis, are used to immunize farmed fish (Lorenzen et al., 1997; Evensen & Leong, 2013). Besides the variety of viral infections, several DNA vaccines against e.g. pathogens of the Streptococcal, Vibrio and Enterococcus species and parasites are under study (Hølvold et al., 2014). Immunization with a DNA vaccine carrying Ag85A, which is a typical component in DNA vaccines candidates for preventing a TB infection in humans, has correspondingly been shown to increase the survival of vaccinated fish over control fish after challenging them with *M. marinum* (Pasnik &Smith, 2005). However, the Ag85A DNA vaccine does not provide long-term protection (Hashish et al, 2018).

3 AIMS OF THE STUDY

Even though tuberculosis is an ancient disease and novel preventative and different treatment options are being studied intensely, tuberculosis remains the deadliest bacterial disease in the world. The latent form of the Mtb infection, which is carried by an estimated 1/5th of the human population, further increases the global threat of tuberculosis. The available tuberculosis vaccine, BCG, provides only limited protection to infants and it is unable to protect adults or against reactivation of the latent infection, highlighting the evident need for effective immunization methods. Even now the protective mechanisms of the BCG vaccine and the mechanisms of a mycobacterial infection at the cellular level are poorly known, which has hampered the development of improved vaccines against tuberculosis. Recent research has aimed tailormade and safe immunization, making the DNA vaccination method an appealing approach. The traditional animal models used in tuberculosis research are infected with mycobacterial species which are not natural pathogens of the host. These models therefore inadequately resemble human tuberculosis infection, so the interest towards to the zebrafish (*Danio rerio*) – *M. marinum* infection model has increased. In this respect, the specific aims of this thesis were:

1. To test, whether the adult zebrafish – *M. marinum* infection model can be utilized for screening the effects of DNA vaccine antigens against primary mycobacterial infections.

2. To develop a model for reactivating latent mycobacterial infections in the adult zebrafish by suppressing the fish immune system with dexamethasone and using the model to test the efficiency of novel DNA vaccine antigens against reactivation of the latent infection.

3. To find novel reactivation-associated mycobacterial genes and test their protective effect as DNA vaccines against reactivation with the dexamethasone-induced reactivation model.

4 MATERIALS AND METHODS

4.1 *M. marinum* strains and culturing of bacteria

The *Mycobacterium marinum* strain ATCC 927 (isolated from fish) was used in all *in vivo* infection experiments and in most of the *in vitro* bacterial studies. The only exception was the *in vitro* antibiotic susceptibility test, where a bioluminescent *M. marinum* strain, ATCC BAA535, with the pMV306 plasmid (Addgene plasmid # 26161) was utilized. Mycobacteria were cultured with standard procedures either on 7H10 Middlebrook OACD plates or in 7H9 Middlebrook medium (BD Biosciences, Franklin Lakes, NJ) supplemented with either OADC or ADC Middlebrook growth supplements, 4 mL/L glycerol and 1g/L Tween®80 (only 7H9 medium). Both liquid cultures and bacterial plates were incubated in the dark at 29°C. Bacterial cultures were maintained by plating onto a new 7H10 plate weekly; every two weeks bacteria were inoculated from a bacterial glycerol stock, stored in a -80°C freezer.

4.1.1 Producing of hypoxic *M. marinum* cultures

The Wayne's low oxygen model for *M. tuberculosis* was used for producing hypoxic *M. marinum* cultures. In the protocol, a clump of bacteria was cultured in 10 mL of 7H9 medium (BD Biosciences, Franklin Lakes, NJ) for two days at 29°C where after the bacteria were diluted to an optical density (OD) value between 0.07 to 0.1 (measured at 600 nm) and incubated for three more days. After incubation, the cultures were diluted to 0.07 and divided into 14-mL tubes. Each tube contained 6.7 mL of bacterial suspension and as a redox control, 160 μL of methylene blue solution (100mg/mL) was added to the control tubes before sealing the tubes airtightly with laboratory film. The tubes were incubated at 29°C on a shaker (125 rpm) and OD_{600} values were measured daily for 18 days (Figure 8). The change in the redox status was followed by observing the color changes in the control tubes.

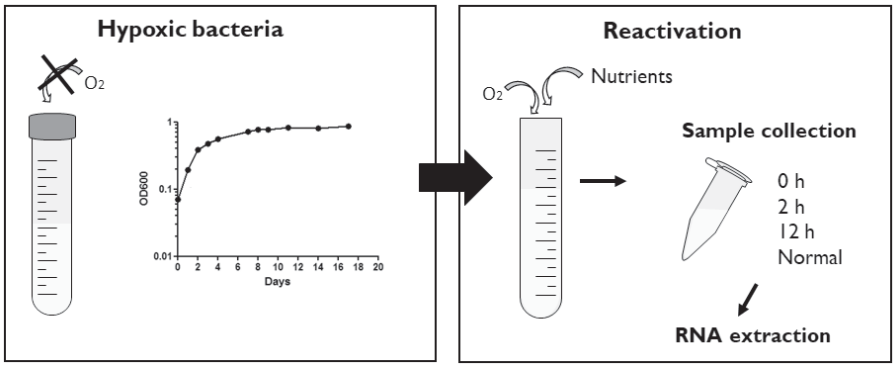

Figure 8. Production and reactivation of hypoxic *M. marinum* cultures.

4.1.2 *In vitro* reactivation of the dormant cultures

To study reactivation of the dormant *M. marinum*, hypoxic cultures were diluted in a 1:1 ratio in 7H9 medium (BD Biosciences, Franklin Lakes, NJ) and incubated at 29°C on a shaker (125 rpm) until the indicated time points (2 and 12 hours) (Figure 8). Hypoxic cultures and exponentially growing bacteria were used as controls. After reaeration, the growth of the bacteria was monitored by both measuring OD_{600} values and by plating bacteria dilutions onto 7H10 plates (BD Biosciences, Franklin Lakes, NJ), whose colonies were counted after 5 days of incubation at 29°C. The rest of the bacteria were collected by centrifugation, 6000 x g at 4°C for 10 minutes, and the pellets were suspended in 700 µL of RTL buffer (RNAeasy Mini Kit, Qiagen) supplemented with β-mercaptoethanol (10 µL/mL) (Sigma-Aldrich). Bacteria suspensions were homogenized in 2-mL cap tubes containing 6 ceramic beads (2.8 mm) with a PowerLyzer24 bead beater (Mobio). The mechanical homogenization step included three 40 s cycles at 3200 rpm and samples were cooled on ice between the cycles. Homogenization was completed by ultrasound sonication (10 minutes) in a water bath. RNA extractions were further performed according to the manufacturer's manual (Qiagen), except 30 µL of sterile water was used in the final elution step instead of the elution buffer. Isolated RNAs were purified from DNases with the RapidOut DNase removal kit (Thermo Fischer Scientific) and used either for a transcriptomic analysis or for quantitative reverse transcriptase PCR (qRT-PCR).

4.1.3 Transcriptomic analysis of reactivated *M. marinum* samples

Before the transcriptomic analysis, the concentration and quality of extracted RNAs were confirmed with the Fragment Analyzer (Agilent) using the Fragment Analyzer RNA Kit (Agilent). The Finnish Functional Genomics Centre (University of Turku and Åbo Akademi University) performed the sequencing with the HiSeq2500 instrument using single-end sequencing chemistry, and the service included also the analysis of the data. Briefly, 1 μg of each sample (four biological samples at each time point) were sequenced using a 50 bp read length. The data were analyzed with the R (version 3.2.2.) and the Bioconductor (version 3.2.) programs. The data were normalized with the TMM (Trimmed Means of M values) normalization method (edgeR R/Bioconductor package), in which highly expressed genes and genes that have a large variation are excluded, and the clustering of samples was performed using Euclidean metrics. Differentially expressed genes were identified from the data using a 2-fold change and a p-value below 0.01 as thresholds.

A new set of reaerated samples was prepared (as described above) to verify the results of the mRNA sequencing with qRT-PCR. For the qPCR, the iTaq universal SYBR® Green One-step Kit (Bio-Rad) kit was used with gene specific primers presented in the Original publication II. The qPCR runs were performed with the CFX96™ Real-Time PCR Detection System (Bio-Rad) using the following program: 1. initial denaturation 95°C 5 min., 2. denaturation 95°C 5 s, 3. annealing 65°C 10 s and 4. elongation 72°C 10 s. Steps from 2. to 4. were repeated 39 times, followed by a melting curve between 65 to 95°C with 0.5°C increments.

4.1.4 Testing the *in vitro* susceptibility of *M. marinum* to antimicrobial agents

To test the susceptibility of mycobacteria to different antimicrobial agents, a bioluminescence *M. marinum* ATCC BAA535 strain, containing the LuxABDE cassette was used. The study was performed in 96-well plates, where each well contained 200 μL of the bacterial suspension (500 CFU/μL) and different concentrations of the chemicals (ethambutol E4630, metronidazole M3761, isoniazid I3377 and amikacin A0368000; Sigma-Aldrich). For each of the tested antibiotic concentrations, six replicate samples were prepared, and water was used as a negative control. Plates were incubated at 29°C for seven days and the bioluminescence was measured on days 0 to 4 and 7 with a 2014 EnVision Multilabel

Reader (PerkinElmer, Waltham, MA, USA). The EnVision Workstation 1.12 (PerkinElmer) was utilized for the data analysis.

4.2 Zebrafish and ethical statements

Adult wild type AB fish from the Tampere zebrafish Core Facility were used in all of the *in vivo* experiments, with the exception of the reactivation-related FACS analysis, where Tg:lck(*lck-EGFP*) fish (obtained from the Zebrafish International Resource Center (ZIRC), University of Oregon, USA) were utilized. At the beginning of the experiments, the fish were 5 to 9 months old and for the infection experiments only male fish were used.

During the experiments, fish were maintained in a flow-through salt water system (AquaSchwarz GmbH, Germany). The quality of the water (temperature 28°C, pH 7.6 and conductivity 800 mS) was monitored and maintained daily. The circadian rhythm of the fish followed 10/14h light/dark cycle. Fish were fed either once a day with the GemmaMicro 500 (Skretting, USA) or twice a day with the SDS400 (Special Diet Services, UK) dry foods. To avoid stress, fish were maintained in groups, with a maximum of 7 fish per L of water. The well-being of the fish was monitored daily, and the fish showing signs of discomfort or sickness, or fulfilling any criteria for humane endpoints, were euthanized with 0.04% 3-aminobenzoic acid ethyl ester pH 7.0 (A5040, Sigma-Aldrich).

The fish experiments were conducted with good ethical practice and with trained personnel. The Animal Experiment Board of Finland has approved all of the fish experiments included in the study (ESAVI/8125/04.10.07/2013; ESAVI/10823/04.10.07/2016; ESAVI/12135/04.10.07/2017). In addition, experiments were performed according to the EU-directive 2010/63/EU and the Finnish legislations on animal experiments.

4.3 Development and production of DNA vaccines

4.3.1 Selection of vaccine antigens and design of the vaccine construct

The detailed vaccine design is described in the III publication of this thesis. The selection of the genes utilized as DNA vaccine antigens was based either on the

literature (I-III Publication) or on RNA sequencing results of the *in vitro* reactivation of hypoxic *M. marinum* (IV Publication). The immunogenic parts of the genes were predicted with the Predicted Antigen Peptides tool (http://imed.med.ucm.es/Tools/antigenic.pl). In addition, by using the same tool, predicted transmembrane and extracellular parts of the protein were preferred.

In all of the constructs, the pCMV-EGFP (Addgene plasmid # 11153) was used as an expression plasmid. The vaccine antigens were cloned into the multiple cloning site (MCS) next to the GFP tag and under a CMV promoter. The start codon and the Kozak consensus sequence were added to the forward primers. An additional nucleotide(s) was added to the reverse primer to keep the GFP tag in frame.

4.3.2 Cloning of vaccine antigens into the expression vector

The standard Phusion protocol (Phusion Hot Start II DNA polymerase, Thermo Scientific) was used to amplify antigen sequences from the *M. marinum* genomic DNA with the cloning primers presented in the original publications (I, II and IV). PCR products were purified with the GeneJet PCR purification kit (Thermo Scientific), and both the insert and the plasmid were digested with the selected restriction enzymes (Fast Digest, Thermo Scientific) with standard procedures. The digested vector was treated with alkaline phosphatase (FastAP, Thermo Scientific) and ligated with the insert using the T4 ligase protocol (Thermo Scientific). 4 μL of ligation mix was transformed into a suspension of chemically competent One Shot TOP10 cell suspension (Invitrogen) according to the manual and plated onto LB agar plates (Miller, Neogen) supplemented with ampicillin (20 μg/mL, Sigma-Aldrich). The bacterial plates were incubated at 37°C over night. The success of the cloning was confirmed by colony-PCR using the iTaq™ DNA polymerase (Bio-Rad) and the plasmid specific primers; CMV-F forward: 5'-CGCAAATGGGCGGTAGGCGTG-3' and EGFP-N reverse: 5'-CGTCGCCGTCCAGCTCGACCAG-3'. The PCR products were run on an agarose gel and DNA extractions were performed from colonies with the right sized insert using the Miniprep DNA extraction kit (Qiagen). The successful cloning was further confirmed by sequencing the cloning site with the same primers used for the colony-PCR. The sequencing core facility of Tampere University performed the sequencing and the results were analyzed with the ClustalOmega multiple sequence alignment tool (EMBL-EBI).

For the immunization experiments, larger quantities of vaccine plasmid DNAs were produced with the Plasmid Plus Maxi Kit (Qiagen) according to the manual; plasmid DNA was extracted from 120 mL of bacterial culture and 120 mL of sterile water was used in the final elution step.

4.4 Analysis of vaccine antigen production in zebrafish

The detailed DNA vaccination technique is presented in the III publication, including both the written protocol and video shots of the most critical steps. Briefly, fish were anesthetized one by one with 0.02% 3-aminobenzoic acid ethyl ester pH 7.0 (A5040, Sigma-Aldrich). A 6 to 8-µL vaccine dose, containing 12 µg of plasmid in 1xPBS supplemented with Phenol red, was injected into the dorsal muscle. The operation was performed under a light microscope with a microinjector. Immediately after vaccination, the injection site was electroporated with six 40 V, 50 ms pulses. After the procedure, the recovery of the fish was carefully monitored.

4.4.1 Fluorescence microscopy

5-7 days after the immunization, the *in vivo* expression of the experimental vaccine antigens was verified with fluorescence microscopy. Before imaging with a fluorescence microscope (Nikon AZ100), the fish were anesthetized with 0.02% 3-aminobenzoic acid ethyl ester pH 7.0 (A5040, Sigma-Aldrich) and set on a Petri dish. Both light field and fluorescence images were taken from the same view and images were combined with the ImageJ software (https://imagej.net). In a large-scale experiment, the *in vivo* expression of fusion proteins was confirmed under UV light, where anesthesia was not needed.

4.4.2 Western blotting and GFP ELISA

To verify that the *in vivo* expressed fusion proteins (antigen-GFPs) were correct, the Western blot analysis was utilized. In addition, GFP-ELISA was used to quantify the *in vivo* expression of the fusion proteins. For both analyses, 7 days post vaccination three fish were euthanized with 0.04% 3-aminobenzoic acid ethyl ester pH 7.0 (A5040, Sigma-Aldrich), and the fluorescence part of the dorsal muscle was dissected and collected into a homogenization tube. Samples were homogenized mechanically

in TRI Reagent (Molecular Research Centre, Inc) as described in paragraph 4.3.1., without the ultrasound sonication step. Proteins were extracted according to the TRI Reagent protocol. Protein concentrations were defined with the Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific) using a BSA (1mg/ml) serial dilution as a standard.

For the Western blot, 15 µL of each protein sample was denaturized with 4x Laemmli buffer, supplemented with β-mercaptoethanol (1:10 volume ratio), and heated for 5 minutes at 95°C. 7.5 – 15 µg of protein was loaded onto a 4–20% Mini-PROTEAN® TGX™ Gel (BioRad). A sample extracted from a fish immunized with an empty pCMV-EGFP plasmid was used as a positive control and a sample collected from a non-immunized fish as a negative control. After the run, proteins were transferred onto a nitrocellulose membrane with the Trans-Blot® Turbo™ Mini Nitrocellulose Transfer Packs (BioRad). The membrane was blocked with milk and the GFP of the fusion protein was detected with a horse radish peroxidase conjugated GFP Tag Monoclonal Antibody (GF28R, Thermo Fisher).

To detect the relative expression levels of the fusion proteins, the GFP ELISA Kit (Cell Biolabs) was used according to the standard protocol. Three biological samples of each vaccine group were measured, and samples from non-immunized fish were used as negative controls. After absorbance measurements, a GFP standard was used to calculate the protein concentrations in each sample.

4.5 DNA vaccination experiments

4.5.1 Experimental infection in the adult zebrafish

Experimental infections of fish were conducted by intraperitoneal injections under anesthesia with 0.02% 3-aminobenzoic acid ethyl ester pH 7.0 (A5040, Sigma-Aldrich). Depending on the experimental setup, either a low dose (30 - 50 CFU) or a high dose (10 000 – 20 000 CFU) of bacteria was used. For the infections, *M. marinum* ATCC 927 was cultured to the exponential growth phase in two steps. First, a clump of bacteria was cultured in 10 mL of 7H9 medium (BD Biosciences) and incubated for two days at 29°C. After incubation, the bacteria were diluted to the OD_{600} value of 0.07 and incubated for three more days. On the day of the infections, 1 mL of bacterial suspension (OD_{600} 0.5 – 0.6) was pelleted by centrifugation, 10 000 x g for 3 min., and the pellet was diluted with sterile 0.9 x phosphate buffered saline

(PBS). The dilutions for the injection mixes were calculated so that one 5 µL-infection dose contained the desired number of bacteria in PBS and 0.5 µL Phenol red (0.03 mg/mL). To ensure homogeneity of the dilution, the mix was mixed by pulling it six times through a 27 G needle with a syringe. The actual infection doses were confirmed by plating onto 7H10 plates (BD Biosciences), and counting colonies after 5 five days of incubation at 29°C.

4.5.1.1 DNA extraction from fish samples

To collect the samples for DNA extractions, one fish at a time was euthanized with 0.04% 3-aminobenzoic acid ethyl ester pH 7.0 (A5040, Sigma-Aldrich). The internal organs of the fish were dissected and collected into a homogenization tube containing six ceramic beads (2.8 mm, OMNI International). Samples were kept on ice or stored in a -80°C freezer. DNA was extracted from the samples according to the extraction protocol of the TRI Reagent (MRC). Briefly; TRI Reagent was added to the tissue sample tubes to a total volume of 1.5 mL, and the samples were mechanically homogenized as described in paragraph 4.1.2. Homogenized samples were centrifuged at 4°C for 10 minutes at 12 000 x g, and 1 mL of cleared homogenate was mixed vigorously with 200 µL of chloroform (Sigma-Aldrich). After 3 minutes of incubation, the samples were centrifuged at 12 000 x g for 15 minutes at 4°C, and the upper, RNA-containing phase, was discarded. The DNA in the lower phases was precipitated by mixing with 400 µL of isopropanol (Sigma-Aldrich) and incubating for 10 minutes at room temperature. Precipitated DNAs were collected by centrifugation (12 000 x g, 15 min., 4°C) and the pellet was washed three times with 950 µL of 70% ethanol. Finally, the washed and air-dried pellets were diluted in 200 µL of sterile water and their concentrations were measured with a NanoDrop instrument (Thermo Scientific).

4.5.1.2 Determination of bacterial loads with quantitative PCR

For qPCR, DNA samples were diluted in sterile water to a maximum concentration of 333 mg/µL. The SensiFAST™ SYBR® No-ROX kit (Biolane Reagents Ltd, UK) with the *M. marinum* internal transcribed spacer (MMITS) primers (forward: 5'-CACCACGAGAAACACTCCAA-3' and reverse:5'-ACATCCCGAAACCAACAGAG-3') was used to measure the bacterial counts. A DNA sample, whose bacterial count was previously confirmed, was used for

preparing a standard (a 1:5 dilution series). The qPCR was performed according to the manual (Biolane) with the CFX96™ Real-Time PCR Detection System (Bio-Rad). The used PCR program was the following: 1. initial denaturation 95°C 5 min., 2. denaturation 95°C 5 s, 3. annealing 65°C 10s and 4. elongation 72°C 10s. Steps from 2. to 4. were repeated 39 times, and thereafter a melting curve was detected between 65 to 95°C with 0.5°C increments.

4.5.2 DNA vaccination against a primary *M. marinum* infection

The protective effect of experimental DNA vaccines was tested against both primary infection and reactivation. In the primary infection experiments, adult zebrafish were at first immunized with experimental DNA vaccine antigens as described in 4.4. (N= 15-19 fish/group) (Figure 9). Five weeks post vaccinations, fish were infected with a low dose of *M. marinum* (30-50 CFU/fish), and four weeks later the fish were euthanized with 0.04% 3-aminobenzoic acid ethyl ester pH 7.0 (A5040, Sigma-Aldrich) and bacterial counts in each fish were quantified as described in the previous paragraphs 4.6 and 4.6.1. In the survival experiments, the fish were infected with a high dose of mycobacteria (10 000 – 20 000 CFU/fish, N=20-25 fish/group) and the survival of the fish was followed for 12 weeks after infections. Fish immunized with an empty expression plasmid were used as negative controls.

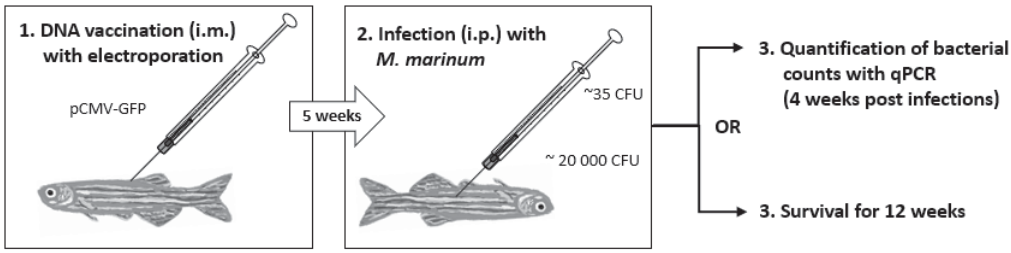

Figure 9. A schematic presentation of testing the protective effect of experimental DNA vaccines against primary *M. marinum* infection. **1.** Adult zebrafish are immunized with experimental DNA vaccines. **2.** Five weeks post vaccination the fish are infected with either a low dose or a high dose of mycobacteria. **3.** Four weeks later, bacterial counts are quantified with qPCR from fish infected with a low dose of *M. marinum*, or after a high dose infection the survival of the fish is followed for 12 weeks.

4.5.3 DNA vaccination against reactivation of the latent infection

The outline for testing the protective effect of experimental DNA vaccines against reactivation of latent infection is presented in Figure 10. At first, fish were infected with a low dose of *M. marinum* (30 – 50 CFU/fish). 5 weeks post infection, the fish were immunized intramuscularly with experimental DNA vaccines as described above (4.4.). 5 weeks later, the latent infections were reactivated with an immunosuppressive treatment as described in the following paragraph (4.6.3.1.). Bacterial loads in each fish after reactivation were quantified with qPCR (4.6.1.). Fish immunized with an empty pCMV-EGFP plasmid and treated with an immunosuppressant were used as negative controls.

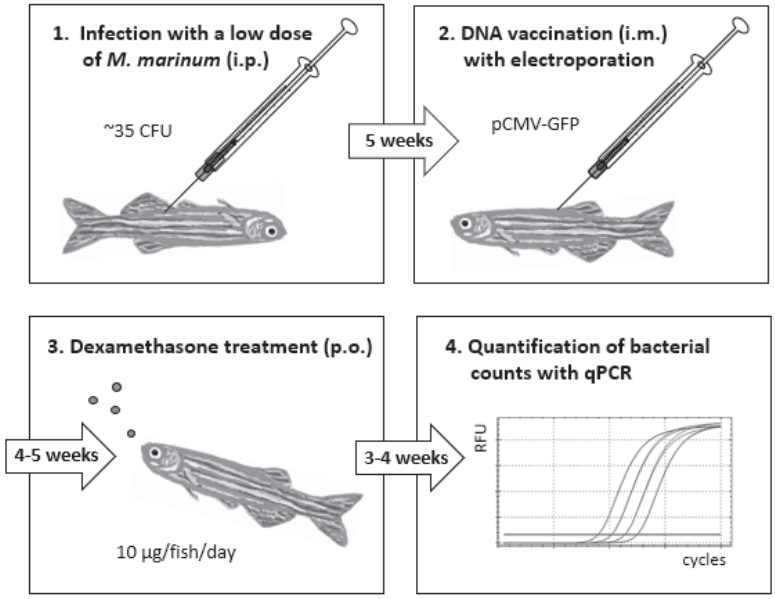

Figure 10. The dexamethasone-based protocol for the reactivation of a latent *M. marinum* infection in an adult zebrafish. **1**. Fish are infected with a low dose of *M. marinum* (~35 CFU/fish). **2.** Five weeks after infections, fish are immunized with an experimental DNA vaccine. **3.** 4 to 5 weeks post immunization, latent infections are reactivated with a dexamethasone treatment for 3 to 4 weeks. **4.** Bacterial counts in each fish were quantified with qPCR.

4.5.3.1 Reactivation of latent infections with immunosuppressants

The immunosuppressive effect of four different drugs (azathioprine A4638, dexamethasone D4902, prednisolone P6004 and methylprednisolone M1755200, Sigma-Aldrich) was tested with fish with a latent *M. marinum* infection. First fish were infected with a low dose of *M. marinum* (30-50 CFU/fish) as described in 4.3., and five weeks later the fish were exposed for 3 to 4 weeks to different immunosuppressive agents. The chemicals were administrated orally within gelatin coated fish feed (2.5mg/g). The coated feed was prepared by mixing 25 mg of each immunosuppressants with 2 mL of 70% ethanol, which was further mixed with a melted bovine gelatin (G9391, Sigma-Aldrich) water solution (400 mg/5mL). The warm gelatin mixture was spread evenly on 10 g of dry SDS400 fish feed (Special Diet Services) and left to air dry. The dry, coated feed was homogenized in a mortal with a pestle and stored at 4°C, covered from light. The daily doses per tank of fish were calculated and weighed. During the feeding, the water circulation in the tanks was stopped, which prevented the spreading of the chemicals into other tanks in the same unit.

The testing of the effect of different antibiotics to treat reactivated infections was carried out with a similar feeding method. The immune system of fish with a latent infection were weakened with a four-week treatment with dexamethasone. After reactivation, antimicrobial drugs were administered within the gelatin coated fish feed. To prepare coated feeds, 125 mg of each antibiotic (ethambutol E4630, metronidazole M3761, isoniazid I3377 and amikacin A0368000, Sigma-Aldrich) was mixed with ethanol, and the feed was further prepared as described above.

4.5.3.2 Histological visualization of granulomas

Histological samples were prepared to visualize granuloma structures at different phases of the reactivation. At the designated timepoints, fish were euthanized with 0.04% 3-aminobenzoic acid ethyl ester pH 7.0 (A5040, Sigma-Aldrich). Heads and tails were removed, and the bodies of the fish were fixed in fixed in 10% phosphate buffered formalin (Oy FF-Chemicals Ab) for 7 days. After fixation, samples were decalcified by incubating in 0.5M EDTA solution (pH 8.0) for 5 days, washed with rinsing water for 2 hours and dehydrated in increasing alcohol series (70%, 96% ethanol; 2 hours in each). Finally, samples were cast in paraffin blocks. Each block was cut through and 5-µm sections were collected every 200 µm on SuperFrost®Plus glasses (Thermo Fisher Scientific). The sections were fixed to the glasses by

incubating for 2 hours at 60°C, and the slides were deparaffinized in xylene (3 x 4 min.) and rehydrated with a series of decreasing alcohol concentrations (absolute ethanol 2 x 2 min., 96% ethanol 2 x 2min., 70% ethanol 1 x 1min.) to distilled water.

Mycobacteria inside granulomas and in tissues were visualized with Ziehl-Neelsen staining and fibrous capsules of granulomas with Mallory's Trichrome staining (Figure 11.). Hypoxic lesions inside the granulomas were stained with the HypoxyProbe-1 kit (HP1-100Kit, Hypoxyprobe). In this protocol, fish were treated with pimonidazole hydrochlorid (60 μg/fish) 5 minutes before euthanasia. The detailed protocol is presented in Original publication II. After staining, the glasses were dehydrated with a series of increasing alcohol concentrations to xylene and mounted with the Eukitt® (Sigma-Aldrich) or Coverquick 2000 (VWR Chemicals). Slides were scanned with an Olympus BX43 microscope and analyzed with the JPEG2000 virtual slide and the ImageJ software. The counting of the number of granulomas and classification of each granuloma was performed manually from each slide.

Ziehl-Neelsen	Mallory's Trichrome
• Deparaffinization • **Carbolfuchsin** 4-5 hours • Running tap water 5 min. • Acid-alcohol, rinse • Running tap water 5 min. • Distilled water, rinse • **Methylene blue**, 30 s • Running tap water 5 min. • Distilled water, rinse • 70% ethanol 1 x 1 min. • 96% ethanol 2 x 1min. • Abs. Ethanol 2 x 1min. • Xylene 3 x 4 min. • Embedding with Eukitt®	• Deparaffinization • Bouin's fluid, over night • Distilled water, rinse • **Acid fuchsin** 3 min. • Distilled water, rinse • **Phosphomolybdic acid solution** 2 min. • Distilled water, rinse • **Aniline blue / Orange G** solution 15 min. • Distilled water, rinse • 96% ethanol 2 min. • Abs. Ethanol 3 x 2 min. • Xylene 3 x 4 min. • Embedding with Eukitt®

Figure 11. The used Ziehl-Neelsen and Mallory's Trichrome staining protocols.

4.5.3.3 Analyzing the effects of dexamethasone on the fish immune system with FACS

Fluorescence associated cell sorting (FACS) was performed to analyze the effect of the dexamethasone treatment on the fish immune system. Uninfected fish (AB and Tg:lck(*lck-EGFP),* 12 fish in each group, were fed for one week with the feed containing dexamethasone (10μg/fish/day). The control fish were fed with the normal fish food. After the treatment, the fish were euthanized with 0.04% 3-aminobenzoic acid ethyl ester (A5040, Sigma-Aldrich), and the kidneys were collected and homogenized in 500 μL of fetal bovine serum (Sigma-Aldrich) in PBS by pipetting. The lysate was filtered through a 35 μl filter by centrifugation. In all of the steps, samples were kept on ice. A FACSCanto II device (Becton Dickinson Biosciences) was used to sort the different cell populations (lymphocytes, blood cell precursors, granulocytes and monocytes) and the data were analyzed with the FACSDiva software (Becton Dickinson Biosciences). The gating of different cell populations was based on GFP expression, the granularity and size of the cell. Each sample was run with medium speed (1000 events/s) and 20 000 events were recorded.

4.5.3.4 Analyzing the effects of dexamethasone on the immune system with qRT-PCR

qRT-PCR was used to analyze the expression levels of different cytokines and T cell markers from kidney samples after the dexamethasone treatment. At first, the immune system of wild type AB zebrafish was weakened with dexamethasone and the kidneys were collected as described in the previous paragraph 4.5.4.3. Kidney samples were homogenized in 700 μL of the RLT buffer and the lysates were used for RNA extraction with the RNeasy Mini Kit (Qiagen). The extracted RNAs were purified with the RapidOut DNase Removal kit (Thermo Scientific) and used for qRT-PCR with the iTaq universal SYBR® Green One-step Kit (Bio-Rad) as described in 4.1.3. The expression levels of each marker gene were normalized to the expression level of *elongation factor 1-alpha* (*EFa1*; *eef1a1/1)*. All of the used primer sequences are presented in Original publication II.

4.6 Group size calculations and statistical analysis

A sample size calculator (https://clincalc.com/stats/samplesize.aspx) was used to determine the group sizes in the fish experiments. The parameters inserted into the calculator were based on our previous pilot studies. The calculator compares two independent study groups with a continuous primary end point. Within the type I/II error rates, alpha 0.05 and power 0.8, the minimum groups size was 16 fish. In practice, 17 to 20 fish per group were used, as some fish may not survive all the steps of the experiment.

Most of the statistical analysis were performed with the GraphPad Prism software (version 5.02, GraphPad Software Inc.). The differences in bacterial loads in infected fish were compared with the Mann-Whitney test. The statistical significance in the survival experiments was analyzed with the log rank Mantel–Cox test. In the FACS study, differences between different groups were analyzed with two-way ANOVA with the Bonferroni posttest. The unpaired Student's *t*-test and Fisher's exact test of independence were used to analyze differences in granuloma counts after reactivation. One-way ANOVA with the Friedman's test were used to analyze the efficacy of different antibiotics in the *in vitro* experiment. P values <0.05 were considered significant.

In the mRNA sequencing data analysis, the R Package Limma was utilized to analyze differences between the test groups. A 2-fold change and p values <0.01 were set as the threshold.

5 SUMMARY OF THE RESULTS

5.1 Generation of a pCMV-GFP plasmid to express DNA vaccine antigens in the adult zebrafish

It has been shown that DNA immunization can elicit effective protection against viral infections in farmed fish. In this thesis, we utilized the zebrafish- *M. marinum* infection model to test novel DNA vaccine candidates against mycobacterial infections. Antigens were cloned into the pCMV-GFP expression vector next to the GFP site. The expression of the produced fusion protein was confirmed *in vivo* in adult zebrafish (Figure 12). Typically, five to seven days post vaccination, the green fluorescence protein at the injection site was detected with a fluorescence microscope (Figure 12 B). There was some variation in the intensity and duration of the antigen expression between different antigens, for instance the expression of the MMAR_1093 antigen was detected for only a short time three to four days post immunization, and MMAR_0444 was produced at high levels for more than four weeks (Original publication IV, Fig. S3). The expression levels of vaccine candidates were quantified with GFP-ELISA from protein samples extracted from the dorsal muscle of the fish (Figure 12 C). There was a similar variation between different antigens as detected by fluorescence microscopy. While most of the antigens were expressed at similar levels as the empty expression plasmid (expressing only GFP), RpfA, RpfB and MMAR_3501 showed relatively low expression levels and the RpfE fusion protein showed higher expression levels than the GFP controls (Figure 12 C. and Original publication I, Fig.3.). Further, a Western blot analysis was performed using similar protein samples to confirm the correct size of the produced fusion proteins (Original publication I, Fig.4. and Figure 12 D).

5.1.1 Selection of antigens for DNA vaccines

Depending on the experimental set up, the choice of vaccine antigens was based on different criteria. In most of the experiments, where we tested the protective effect against a primary *M. marinum* infection, the selection was based on previous literature.

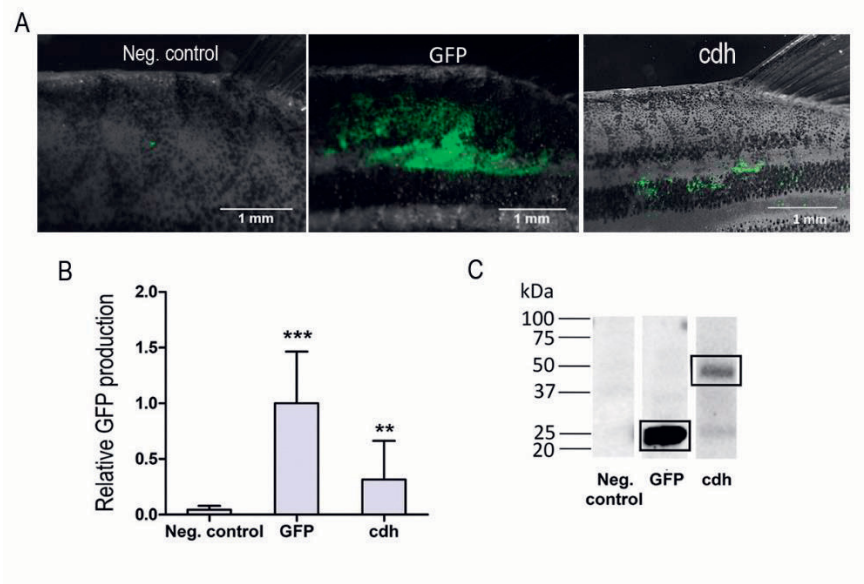

Figure 12. Validation of *in vivo* expression of the pCMV-GFP vaccine constructs. Adult zebrafish were immunized with an experimental vaccine (cdh as an example) or with an empty expression plasmid (GFP) as a positive control and non-immunized fish were used as a negative control.12µg of the plasmid was injected into the dorsal muscle and the injection site was electroporated with six 50 ms pulses of 40V. (**A**) Five to seven days post immunization, the *in vivo* expression of the produced fusion proteins was imaged with a fluorescence microscope. (**B**) The fluorescence part of the dorsal muscle was used for protein extraction and the expression levels of the produced fusion proteins were quantified with GFP-ELISA. The measured absorbance values were normalized with the average of the control values. The bars show the mean with SD. N≥4 per group, **p<0.01 and ***p<0.001, two-tailed Mann-Whitney test. (**C**) The correct sizes of the proteins were confirmed with a Western blot analysis. The correct sizes of the blotted proteins; GFP 27 kDa and cdh 52 kDa. Figures B and C are modified from Original publication I, Fig 3. and 4.

In Original publication I, we selected antigens which are expressed in different phases of bacterial growth and are known to contribute to the virulence of mycobacteria. The genes were classified into four groups; resuscitation promoting factors (Rpfs), PE/PPE family proteins, transmembrane proteins and secreted factors and metabolic enzymes (listed in the Table 6). Only parts of the sequences of the selected genes were used as vaccine antigens, where extracellular or transmembrane-associated parts were favored. In addition, *M. marinum* genes with a known *M. tuberculosis* homologue were preferred.

Table 6. Summary of the tested DNA vaccine antigens. Minus (-) means no difference and plus (+) means a statistically significant difference ($p<0.05$) in the bacterial loads compared to the bacterial counts measured in the control group fish (Mann-Whitney test).

M. marinum gene identifier	Gene name	The effect against primary infection	The effect against reactivation
Resuscitation promoting factors			
MMAR_2772	Rpf-like protein	-	-
MMAR_3776	RpfE	+	-
MMAR_4479	RpfB	-	+
MMAR_4665	RpfA	-	-
PE/PPE proteins			
MMAR_0641*		not tested	-
MMAR_2670	PE19_1	-	-
MMAR_4241	PE_31	+	-
MMAR_5258	PE5_1	+	-
Transmembrane proteins and secreted factors			
MMAR_0552*		not tested	-
MMAR_0444*		not tested	-
MMAR_2220	lprG	-	-
MMAR_2674	esxM	-	-
MMAR_3501		-	-
MMAR_4207		-	+
MMAR_4524*		not tested	-
MMAR_4637	ompA	-	-
Metabolic enzymes			
MMAR_0514*		not tested	-
MMAR_1093*	CycA	not tested	-
MMAR_3112	cysQ	-	-
MMAR_3445	cdh	+	-
MMAR_4110*		-	+
MMAR_4629	cysM	-	-

*Antigens selected based on the mRNA sequencing results of the *in vitro* reactivation

In order to find novel vaccine antigens to prevent reactivation of the latent mycobacterial infection, we set up an *in vitro* reactivation model for *M. marinum*. Hypoxic mycobacteria cultures were reactivated by reaeration and the samples, collected at the designated timepoints (0, 2 and 12 hours), were used for a transcriptomic analysis (N=4/group). Exponentially growing bacteria were used as a control. The results of the mRNA sequencing were visualized by the Principal Component Analysis (PCA) (Figure 13 A). The 2-hour samples and control samples were well clustered, whereas the hypoxic and 12-hour samples were more dispersed (Figure 13 A). By comparing the groups, we identified 379 differentially expressed genes (Figure 13 B). In the 2-hour reactivation group, the expressions levels of 165 genes were upregulated compared to exponentially growing bacteria and 27 genes were upregulated compared to hypoxic bacteria, and the expression levels of seven genes were upregulated in both comparisons (Figure 13 C). These genes, MMAR_0444, MMAR_0552, MMAR_4524, MMAR_0514, MMAR_4110, MMAR_1093 and MMAR_0641, were membrane proteins, transporters, PE/PPE family proteins and metabolism related proteins (Figure 13 C).

In the reactivation experiments (Original publications II and IV), the selection of antigens was based both on the previous literature and on the mRNA sequencing data obtained from the *in vitro* reactivation experiment. All of the selected antigens and their protective effect against a primary infection and against reactivation of the latent infection are listed in Table 6.

5.1.2 Immunization with the *M. marinum* antigens, RpfE, PE5_1, PE_31 and cdh, protects against a primary mycobacterial infection

An effective vaccine, which would control the infection and prevent the progression into an active disease, could be one of the key elements in controlling the dissemination of TB. With the zebrafish- *M. marinum* infection model, it is possible to mimic the human primary *M. tuberculosis* infection (Swaim et al, 2006; Parikka et al, 2012). The effects of the selected antigens (listed in the Table 6.) were tested with this primary infection model, where fish were first immunized with experimental DNA vaccines and five weeks later infected with a low dose of *M. marinum*. Four weeks post infections the fish were sacrificed, and the bacterial counts in each fish were quantified with qPCR. Of the tested antigens, immunization with four different antigens, namely RpfE, PE5-1, PE_31 and cdh, showed protection against a primary

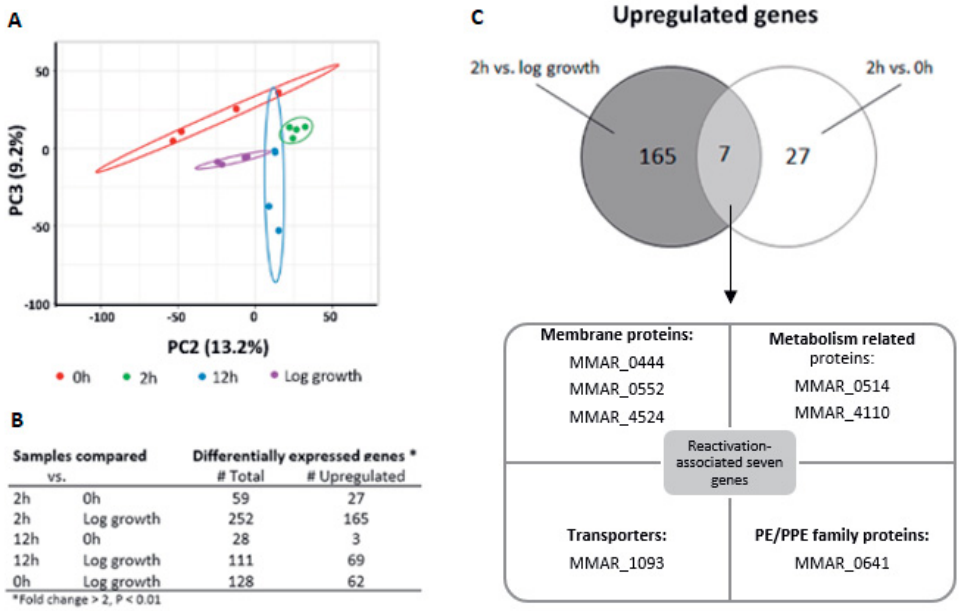

Figure 13. The expression levels of seven *M. marinum* genes were upregulated during reactivation. mRNA sequencing was performed on RNA samples extracted from hypoxic, reaerated and exponentially growing bacteria. (**A**) Principal Component Analysis shows clustering of the samples. The X and Y axes show the principal components 2 and 3, and ellipses show the area inside which a new observation falls with a 0.95. (**B**) Fold-change >2 and p-value<0.01 were used as the criteria for filtering upregulated genes between the groups. (**C**) Seven of the genes were upregulated during reactivation in both hypoxic and exponentially growing bacteria. Figures are modified from Original publication IV, Fig.1.

infection (two-tailed Mann-Whitney test) (Figure 14 A, Table 6 and Original publication I Fig 5.). An 88% reduction in median bacterial burdens was quantified in fish immunized with the RpfE antigen and immunization with PE5_1, PE_31 and cdh led to 56%, 50% and 62% reductions in bacterial loads, respectively.

The protective effect of these four antigens was further tested against a high dose *M. marinum* infection (Figure 14 B and original publication I Fig 6.). In these experiments, the survival of the fish was followed for 12 weeks after infection. The survival curves of each immunization group were compared to the survival of the control group fish, which were immunized with the empty expression plasmid (Figure 14 B). Immunization with the RpfE antigen led to an improved survival of the fish; after a high dose infection, 40% of the fish were alive after the 12 weeks

follow-up, whereas in the control group only 16% of the fish survived (**p<0.01, Log-rank Mantel-cox test).

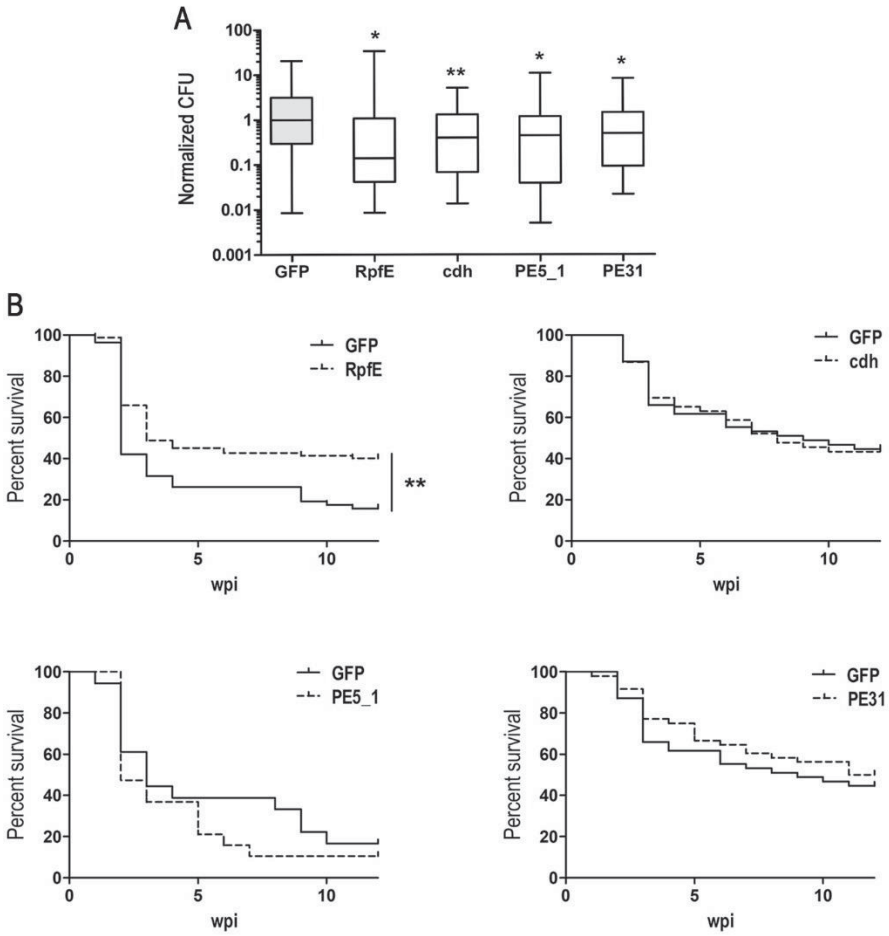

Figure 14. Immunization with *M. marinum* antigens, RpfE, PE5_1, PE31 and cdh, provides protection against a primary mycobacterial infection. Adult zebrafish were immunized with the experimental DNA vaccines or with the empty expression plasmid (control group). Five weeks post immunization, fish were infected with either a low or a high dose of *M. marinum*. (**A**) Four weeks after a low dose infection, bacterial loads in each fish were quantified with qPCR. Immunization with RpfE, PE5_1, PE31 and cdh led to reduced median bacterial counts as compared to the control group. The CFU values were normalized with the median CFU of the GFP controls of the same experiment. N=10-29 fish/group. *p<0.05, **p<0.01, two-tailed Mann-Whitney test. (**B**). Survival of the immunized fish was followed for 12 weeks after a high dose infection. Immunization with the RpfE led to a significantly improved survival of the fish. N≥19 fish/group. **p<0.01, Log-rank Mantel-cox test. Figures modified from Original publication I, Fig 3. and 4.

5.2 Dexamethasone treatment reactivates a latent mycobacterial infection in the adult zebrafish

Immunosuppression often triggers the reactivation of a latent TB infection in humans. Similarly, in zebrafish, gamma irradiation leads to immunosuppression and therefore to the reactivation of the latent disease (Parikka et al, 2012). To further study the reactivation mechanisms and to find a more feasible method to reactivate latent infections, we tested the ability of four different immunosuppressants (azathioprine, dexamethasone, methylprednisolone and prednisolone) to reactivate latent *M. marinum* infections in the adult zebrafish. Fish with a latent *M. marinum* infection were treated with immunosuppressive medications (10μg/fish/day, N=17-19 fish/group). One of the tested glucocorticoids, namely dexamethasone, led to significantly higher median bacterial burdens as compared to the bacterial burdens in the control group fish ($p=0.04$, one-tailed Mann-Whitney test) (Figure 14 A and Original publication II, Fig 1A. and 1B.). A similar trend was seen with the azathioprine treatment; however, the tested doses of methylprednisolone and prednisolone did not trigger the reactivation (Original publication II, Fig 1A.), and based on these results, dexamethasone was selected for further reactivation studies.

5.2.1 Dexamethasone treatment disrupts granuloma structures, increases the number of granulomas and decreases the hypoxic areas in the granulomas

Granulomas are the most common feature of human TB. A *M. marinum* infection in zebrafish leads to the formation of similar granuloma structures. To study the effect of feeding dexamethasone on the granulomas in fish with a latent mycobacterial infection, we collected histological samples at different timepoints during the dexamethasone treatment. Ziehl-Neelsen staining was used to visualize the mycobacteria and Trichrome staining to show the fibrous tissue around the granulomas (Figure 15 B). In addition, Hypoxyprobe staining was utilized to detect hypoxia inside the granulomas. The dexamethasone treatment weakened the granuloma structures; after two weeks of treatment, granulomas appeared larger and disrupted, and a week later bacteria started to escape from existing granulomas (Figure 15 B). Different types of granulomas were counted to quantify the changes in granulomas during the immunosuppression. Granuloma numbers in fish with a latent infection (control group) remained relatively constant at all the timepoints (an

average 9 to 17 granulomas per fish) (Figure 15 C), whereas the dexamethasone treatment increased the total number of granulomas from an average of 17 granulomas to an average of 57 granulomas in three weeks (Figure 15 C). It was also observed that dexamethasone increased the numbers of necrotic and multi-centric

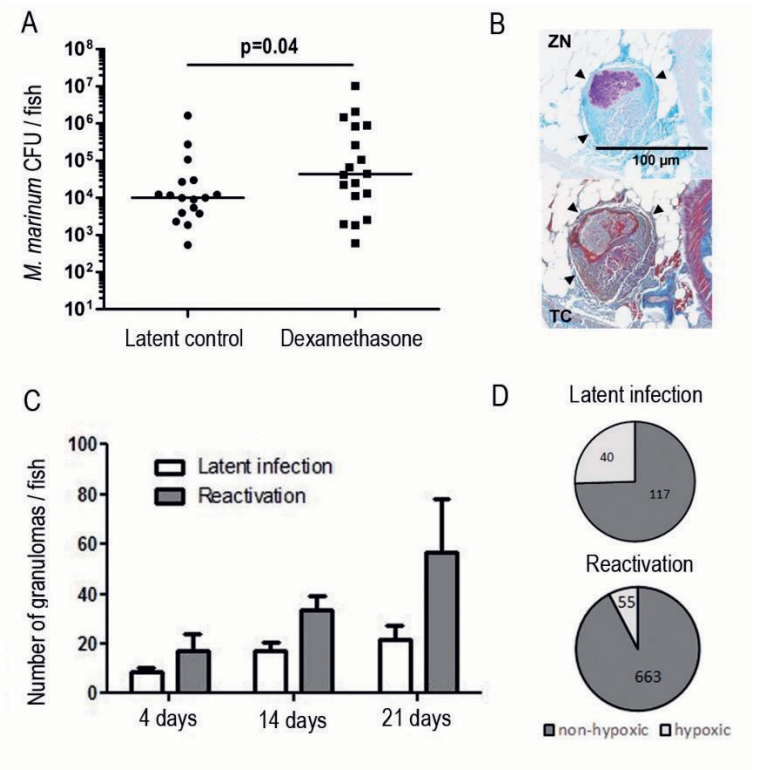

Figure 15. Dexamethasone treatment reactivates latent *M. marinum* infections in adult zebrafish, which is seen as an increased number of granulomas, decreased hypoxia in the granulomas and increased bacterial counts in fish with latent infections. (**A**) Adult zebrafish with a latent *M. marinum* infection were treated for four weeks with dexamethasone (p.o., 10µg/fish/day). Bacterial loads in each fish were determined with qPCR. A dexamethasone treatment led to increased bacterial burdens as compared to CFUs in latent control group fish (N=17-19 fish/group, one-tailed Mann-Whitney test). (**B**) The Ziehl-Neelsen and the Trichrome staining show the purple bacteria and fibrous tissue in granuloma structures. Arrow heads point to fibrous tissue. (**C**) The total number of granulomas at different time points of the dexamethasone treatment (4, 14 and 21 days) compared to latently infected fish (N=3-6 fish/group, means with SD, unpaired Student's t test). (**D**) The proportion of hypoxic and non-hypoxic granulomas in the latent phase of an infection and after a two-week treatment with dexamethasone. Figures are modified from Original publication II, Fig. 2.

granulomas (Figure 15 C and Original publication II, Fig S2B), as well as the number of nascent granulomas (Original publication II, Fig 2B).

During the latent phase of the infection, the hypoxic environment inside the granulomas induce the metabolic adaptation of mycobacteria towards the dormant state. Hypoxyprobe staining was utilized to visualize the effects of the dexamethasone treatment on hypoxia inside the granulomas. In the latent infection phase, 25% of the fish granulomas contained hypoxic areas (Figure 15 D). After a two-week dexamethasone treatment, only 8% of the granulomas were hypoxic (Figure 15 D).

The data suggest, that the dexamethasone-induced reactivation affects to the granuloma structures by decreasing hypoxia and weakening the integrity of the granulomas, which eventually leads to the escape of bacteria from the granulomas and the formation of an active disease.

5.2.2 Dexamethasone treatment decreases the number of lymphocytes in both infected and un-infected adult zebrafish

To study the influence of dexamethasone on immune cell populations in the fish, we utilized a flow cytometric analysis. As the kidney is the main hematopoietic organ of fish, kidney samples were used for the analysis. Different cell populations; lymphocytes, blood cell precursors and granulocytes and monocytes, were separated based on size (defined by the forward scatter, FSC) and granularity (defined by the side scatter, SSC) (Figure 16 A). In the first experiment, samples were collected after one, two and four weeks of immunosuppression. The one-week dexamethasone treatment decreased the proportion of lymphocytes (from 19.3±3.5% to 12.4±1.6%, $p<0.01$, two-way ANOVA), while it did not have an influence on the proportions of other cell populations (Original publications II, Fig S3). The results were similar after the longer treatment periods (two and four weeks).

Comparable results were obtained when the experiment was repeated with the transgenic fish line, Tg:lck(*lck-EGFP*), in which mature T cells are labeled with GFP. The analysis was performed at one, two and four weeks after the dexamethasone treatment. After one week of immunosuppression, the proportion of GFP+ lymphocytes decreased significantly (from 12.5±3.4% to 6.3±2.4%, $p>0.001$, two-way ANOVA) and remained at the same level throughout the dexamethasone treatment (Original publications II, Fig. 3D). Moreover, the proportion of the GFP- cell population, containing B cells and immature T cells, decreased (from 18.5±2.8%

to 10.3±1.7%, *P*<0.001, two-way ANOVA) after four weeks of immunosuppression (Original publications II, Fig 3D).

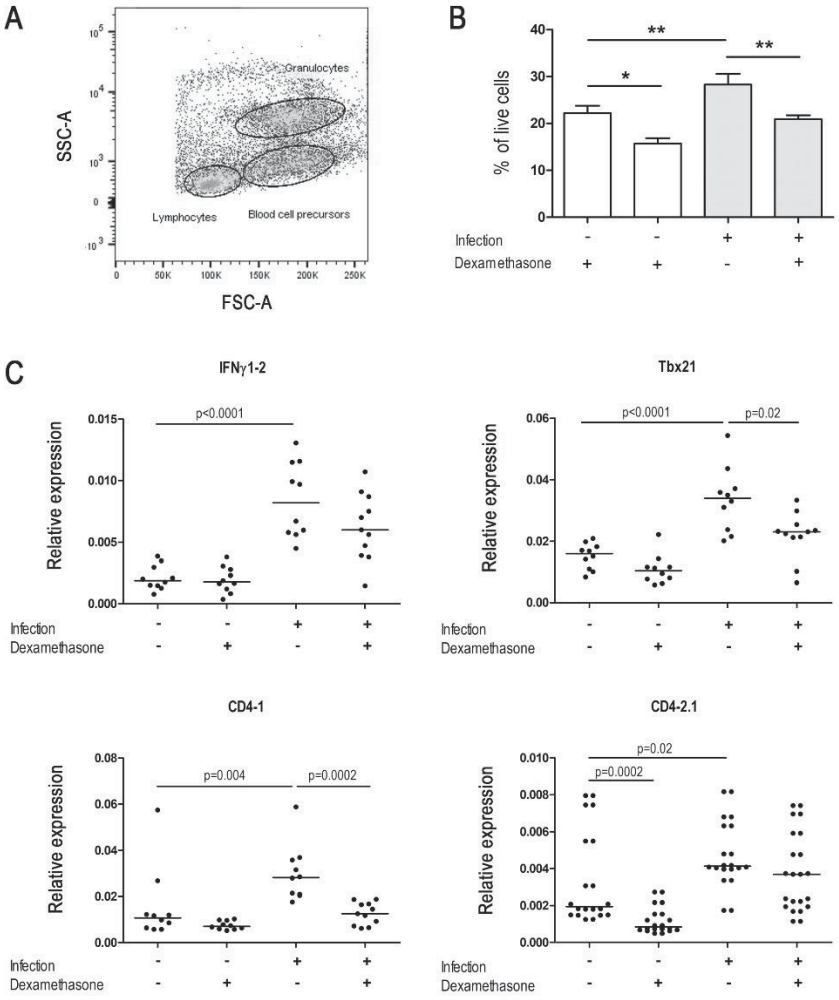

Figure 16. Dexamethasone treatment decrease the number of lymphocytes in both infected and uninfected fish. (**A**) Kidney cell populations of adult zebrafish were sorted with FACS based on size (side scatter, SSC-A) and granularity (forward scatter, FSC-A). (**B**) The number of lymphocytes is elevated in infected fish and reduced during a one-week dexamethasone treatment (two-way ANOVA with Bonferroni posttest, *p<0.05 and **p<0.01. Panels **C-F** show the relative expression levels of the inflammatory marker *ifng* (**C**) and immune cell markers, *cd4-1* (**D**), *cd4-2.1* (**E**) and *IgM* (**F**). Expression levels were normalized to the expression level of the housekeeping gene *EF1a*. Horizontal lines represents the median value of each group. N=10-11 fish/group. The two-tailed Mann-Whitney test was used for the statistical analysis. Figures are modified from Original publication II, Fig. 3 and Fig. 4.

The differences in the proportions of lymphocytes between infected and uninfected wild type fish were examined further after a one-week dexamethasone treatment. The latent infection status increased the number of lymphocytes ($p<0.01$, two-way ANOVA with the Bonferroni posttest) and the dexamethasone treatment decreased the proportion of lymphocytes from 24.6±3.2% to 21.7±4.5% ($p<0.01$, two-way ANOVA with the Bonferroni posttest), to the same level seen in uninfected fish (Figure 16 B).

To study the effect of the dexamethasone treatment on lymphocyte functions, the expression levels of different T and B cells were studied with qRT-PCR. As expected, the expression levels of inflammatory markers *tnf* and *ifng1-2* were elevated in fish with latent infections compared to uninfected fish (Figure 16 C and Original publications II Fig 4B and Fig S5B).

CD4+ and CD8+ lymphocytes seem to have a critical role in controlling a mycobacterial infection. To this end, we measured the expression levels of the CD4+ markers (*cd4-1* and *cd4-2.1*) and the CD8+ marker *cd8a*. Bothe *cd4-1* and *cd4-2.1* were induced by the infection and reduced by the following dexamethasone treatment (Figure 16 D and E). Similarly, the infection induced the expression of *cd8a*, however the dexamethasone treatment did not have an effect on its expression levels (Original publications II, Fig. 4E). In addition, the expression levels of the B cell marker *IgM* remained constant in all of the study groups (Figure 16 F).

Overall, the data indicate that the immunosuppressive effect of dexamethasone in fish is based on a general depletion of lymphocytes, especially CD4+ T cells.

5.2.3 An experimental DNA vaccine antigen, MMAR_4110, protects against reactivation of a latent mycobacterial infection

The seven genes, whose expression was upregulated upon reactivation, were selected for a further experiment to study their preventive effect as DNA vaccines against the reactivation of a latent mycobacterial infection. Predicted immunological parts of the genes were cloned to the pCMV-GFP expression plasmid. Fish with a latent infection were immunized with these experimental DNA vaccines and their protective effect against reactivation was tested with the dexamethasone treatment. One of the tested antigens, namely MMAR_4110, inhibited reactivation significantly ($p<0.05$, one-tailed Mann-Whitney test) (Figure 17A and Table 6). A similar effect was seen with three other MMAR_4110 antigens, designed from three different

immunological parts of the same gene (N=14-17 fish/group, $p<0.05$, one-tailed Mann-Whitney test) (Figure 17 B). The original MMAR_4110 vaccine was also tested in the primary infection set up. Results showed that immunization with the MMAR_4110 antigen does not protecting against a primary infection, which was confirmed with both bacterial quantification 4 weeks after a low dose infection (Figure 17 C) and with a 12-weeks survival follow up after a high dose infection (Figure 17 D).

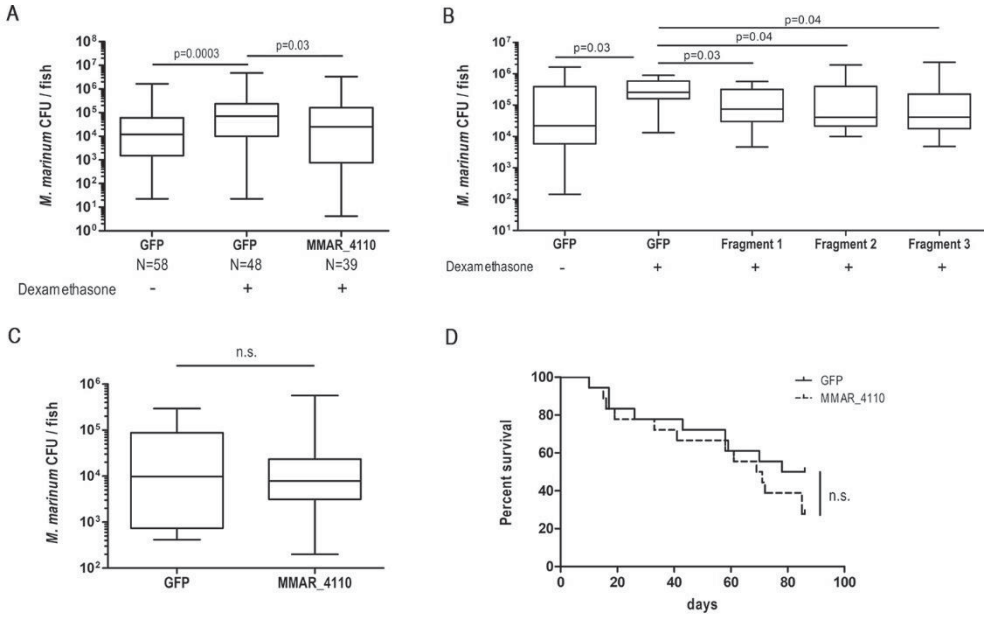

Figure 17. DNA vaccine antigen MMAR_4110 provides a protection against reactivation of a latent *M. marinum* infection. (**A**) Fish with a latent *M. marinum* infection were immunized with the experimental DNA vaccines. An empty expression plasmid (GFP) was used as a control. Five weeks post vaccinations, latent infections were reactivated with dexamethasone. The MMAR_4110 vaccine inhibited the reactivation of the latent infection ($P<0.05$, one-tailed Mann-Whitney test). Lines show the median bacterial loads per group and the whiskers the min and max values of a group (**B**). The three additional antigens derived from the same *M. marinum* gene were tested with the same protocol. All of the tested antigens showed protection against reactivation (N=15-19 fish/group, one-tailed Mann-Whitney test). (**C** and **D**) The effect of the MMAR_4110 vaccine was tested against a primary infection. (C) Five weeks after immunization, fish were infected with a low (**C**) or a high (**D**) dose of *M. marinum*. Four weeks after a low dose infection, bacterial counts in each fish were quantified with qPCR, or the survival of the fish was followed for 12 weeks after a high dose infection. The vaccine did not provide protection against a primary infection. The Mann-Whitney and the log-rank Mantel-cox tests were used for statistical analyses.

5.2.4 *M. marinum* derived DNA vaccine antigens RpfB and MMAR_4207 inhibit the reactivation of a latent mycobacterial infection

The effect of the vaccine antigens, which were tested against a primary *M. marinum* infection, were also tested against reactivation with the same dexamethasone-based reactivation model (Table 6). Two of the tested DNA vaccine antigens, namely RpfB and MMAR_4207 significantly prevented reactivation (p-values 0.01 and 0.003, two-tailed Mann-Whitney test).

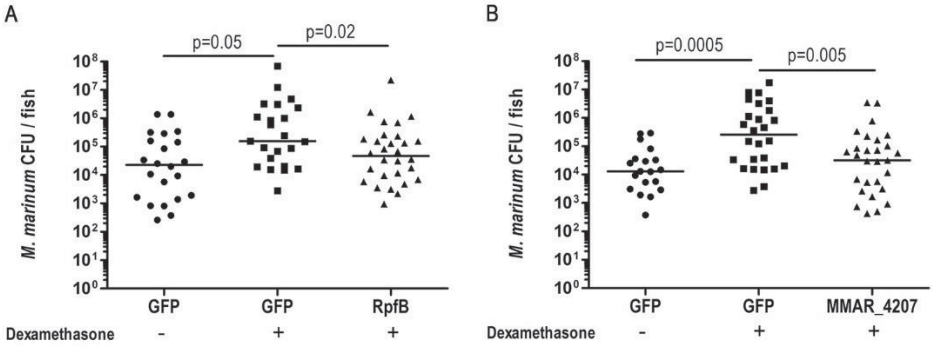

Figure 18. *M. marinum* antigens RpfB and MMAR_4207 inhibit the reactivation of a latent mycobacterial infection in the adult zebrafish. Adult zebrafish were infected with a low dose of M. marinum (~35 CFU/fish). Five weeks later, fish were immunized with the experimental DNA vaccines or with the control vaccine (empty vector). Five weeks after the vaccinations, latent infections were reactivated with the dexamethasone treatment (10µg/fish/day) for three to four weeks. Bacterial counts in each fish were determined with qPCR. Immunization with the RpfB and MMAR_4207 led to inhibition of reactivation (p=0.01 and p=0.03, two-tailed Mann-Whitney test). Horizontal lines show the median bacterial loads in each group.

Overall, with the dexamethasone-induced reactivation model, we found three antigens, which we consider potential candidates for further studies for vaccine development against tuberculosis.

6 DISCUSSION

6.1 Reactivation of latent mycobacteria

6.1.1 Lessons learnt from *in vitro* experiments

Mycobacteria have an ability to survive in hostile environments by falling into dormancy, where they can persist even for years before they reactivate in more favorable conditions (Batyrshina & Schwarz, 2019). This phenomenon is typical in a LTBI, where Mtb is arrested inside macrophages and granulomas (Esmail et al, 2014). However, the mechanisms by which mycobacteria survive in a dormant state and resuscitate to the actively replicating state, are poorly understood. Studying dormancy and reactivation *in vivo* has turned out to be challenging due to the complex interactions between the bacteria and the immune system, and therefore several different *in vitro* models have been developed. The most used model for Mtb is the hypoxia-induced dormancy model of Wayne (Wayne & Hayes, 1996). A similar, slowly-replicating persistent stage can be achieved, for instance, by limiting the availability of nutrients and metal ions or by lowering the pH (Batyrshina & Schwarz, 2019). In this study (Original publication IV), we selected a modified Wayne's method to produce dormant *M. marinum* cultures, where the low oxygen level is considered the main factor involved in the adaptation of Mtb in the latent infection phase (Wayne and Hayes, 1996). The obvious limitation of the model is that it only takes into account hypoxia without any other aspects of dormancy, such as adaptation of the bacilli to the nutrition status, low pH or oxidative and nitrosative stresses (Braz et al, 2015; Del Portillo et al, 2018). The model has also been criticized for the heterogeneity of Mtb in hypoxic cultures, where a relatively high portion of the bacilli seem to retain their viability (Alnimr, 2015). In addition, the lack of standardization makes the comparison between studies performed in different environments difficult. Nevertheless, all *in vitro* models have their limitations; they are valuable for obtaining preliminary data, which needs to be later validated *in vivo*.

The aim of our *in vitro* reactivation study was to identify *M. marinum* genes, whose expression is induced during reactivation. We hypothesized that these genes could

be potential targets for developing novel DNA vaccine antigens against the reactivation of a latent mycobacteria infection. With a transcriptomic analysis, we identified nearly 400 differentially expressed genes, between different conditions (i.e. logarithmic growth, dormancy and reactivation), which included genes from various gene families (e.g. PE/PPE proteins, membrane components and metabolism-associated proteins), (Original publication IV). The Wayne's model has been utilized in hundreds of studies to investigate the dormancy of Mtb (Batyrshina & Schwarz, 2019). However, the reactivation of other mycobacteria is less studied. According to my knowledge, this was the first transcriptomic level *in vitro* reactivation study with *M. marinum*, providing novel information about the genes involved in the early phases of reactivation (Original publication IV). Du et al. (2016) published a somewhat similar study with Mtb, where they studied gene expression in dormancy and in the reaeration lag phase. Despite the differences in the study set up, certain types of genes, such as the lipid metabolism related *fadD* genes and some *PPE family* genes were upregulated in both studies. This was expected, as lipid metabolism is required for cell division and 10% of the mycobacterial genome encodes PPE/PE family proteins (Cole et al, 1998; Sarmiento et al, 2019). There were also clear differences in the identified genes, which can be explained by the different time points; the Mtb reactivation study focused on the lag phase after reaeration, whereas we were interested in the early phases of the reactivation.

In the future, it would be appealing to repeat the transcriptomic level reactivation analysis using *in vivo* samples. Thus far, we have not succeeded in quantifying bacterial gene expression from whole fish or kidney samples. The challenge has been relatively low quantities of mycobacteria in samples and thereby difficulties in gene expression analysis. However, the latest reports show that zebrafish granulomas can be dissected and maintained in an *ex vivo* culture for up to three days (Cronan et al, 2018). This micro-dissecting technique could be an option for studying the *in vivo* effect of dexamethasone on both mycobacteria and on immune cells in granulomas in the zebrafish-*M. marinum* infection model.

6.1.2 Lessons learnt from *in vivo* experiments

The commonly used animal models, with the exception of non-human primates, poorly resemble human LTBI, especially the reactivation of the latent infection (Myllymäki et al, 2015). Mtb is not a natural pathogen of these animals, and therefore the immune responses differ from human TB. As described above in chapter 2.4, *M.*

marinum is a natural pathogen to fish, and is suited for studying both the latent and active phases of the mycobacterial infection in zebrafish (Swaim et al, 2016; Parikka et al, 2012). In addition, it has been shown that by suppressing the fish immune system with gamma irradiation, latent infections reactivate (Parikka et al, 2012). Further, in Original publication II, we showed that reactivation can be caused by dexamethasone-induced immunosuppression. The result was not surprising, as it is known that immunosuppressive conditions in humans can cause the reactivation of a LTBI (Ai et al, 2016). In our study, the dexamethasone-induced reactivation was most effective in the bacterial count analysis compared to other tested chemicals. Exposure to two other glucocorticoids (prednisolone and methylprednisolone) were not able to trigger the reactivation. However, this might be related to the tested dose; higher doses might cause a similar response as dexamethasone, but further studies are needed to both confirm this assumption and the safety of higher doses to fish.

The exact mechanisms of action of azathioprine are not well understood. After absorption, azathioprine is metabolized to 6-mercaptopurine, which inhibits purine synthesis resulting in acute cytotoxic effects especially against cytotoxic T lymphocytes, NKs and B cells, whereas the function of Th cells seems to remain intact (Winkelstein, 1979). Long-term treatment seems to lead to leucopenia (Fraser et al, 2002). In our reactivation study with azathioprine, we saw a similar increasing trend in bacterial counts as with the dexamethasone treatment, suggesting that with an extended exposure period, azathioprine could reactivate latent mycobacterial infections. However, for the screening to be efficient, and for ethical reasons, prolonged treatment periods are not desired, and therefore this option was not studied further in this thesis.

As for azathioprine, the cellular and sub-cellular mechanisms of glucocorticoids are not completely understood. It is known that in humans their effect is mainly based on the suppression of most of the immune cells, leading to a decrease in the number of monocytes, macrophages, T cells, eosinophils and basophils (Strehl et al, 2019). Only the number of circulating neutrophils increases during the treatment (Strehl et al, 2019). Our FACS analysis showed that the dexamethasone treatment reduced the number of lymphocytes in zebrafish kidneys, and a subanalysis of different lymphocytes using qPCR showed that the effect was particularly strong for CD4+ T cells (Original publication II). The number of granulocytes and monocytes increased after two weeks of dexamethasone treatment but remained relatively stable at other time points (Original publication II).

Granuloma structures are similar in an Mtb infection in humans and in a *M. marinum* infection in zebrafish (Swaim et al, 2006; Parikka et al, 2012; Myllymäki et

al, 2016). We visualized the effect of a dexamethasone treatment on granulomas using a histological staining and found out that the immunosuppression increases bacterial loads, weakens the integrity of the granulomas and decreases the hypoxia inside them (Original publication II). In addition, the number of disseminating bacteria increased during reactivation. Generally, the variation between fish and even between granulomas in the same fish was relatively high. This is in line with a macaque study, where macaques with a latent infection were treated with TNF neutralization (Gideon et al, 2015). The study showed that during a latent infection, granulomas are heterogeneous and individuals with a higher number of granulomas were not more prone for reactivation. Instead, they found that local inflammation, often around a single granuloma, predicted against reactivation.

Overall the data suggest that in the adult zebrafish, dexamethasone treatment suppresses the fish immune system, especially T lymphocytes leading to increasing number of bacteria, weakening of granuloma structures and finally, dissemination of the disease. Especially the influence of dexamethasone on bacterial counts was evident, indicating the usefulness of the model for studying the efficiency of novel vaccine and drug candidates against reactivation.

6.2 An adult zebrafish as a model for DNA vaccine research against tuberculosis

The BCG vaccine, which is derived from a live attenuated strain of *M. bovis*, is the only available vaccine and therefore the golden standard for preventing TB. However, the protective effect of BCG is poor, providing protection only against severe forms of TB, whereas protection against pulmonary TB is inadequate (Luca & Mihaescu, 2013). The lack of both in depth knowledge of the disease mechanisms and appropriate animal models, have held back the development of effective TB vaccines. An *M. marinum* infection in zebrafish recapitulates the features of human TB better than any of the conventional animal models. Besides the similarities in the course of the infection and disease pathology between humans and fish, 84% of disease related zebrafish genes have human orthologues (Howe et al, 2006). Similarly, the genomes of *M. tuberculosis* and *M. marinum* are highly conserved (Swaim et al, 2006; Stinear et al, 2008). Because of these facts, the zebrafish- *M. marinum* infection model is a useful tool for TB research.

The applicability of the zebrafish model to TB vaccine research was validated in a previous study, where adult zebrafish were immunized with the BCG vaccine and

4 weeks later infected with *M. marinum* (Oksanen et al, 2013). As in humans, the BGC vaccine did not result in a sterilizing immunity against a mycobacterial infection in fish, but it improved the overall survival of the infected individuals (Oksanen et al, 2013). The zebrafish model was further tested for the DNA vaccination approach. Oksanen et al. (2013) showed that immunization with the commonly tested *M. tuberculosis* virulence factors, Ag85B, ESAT-6 and CFP-10, resulted in the production of IFNγ in individuals infected with a high dose of *M. marinum*. Moreover, immunization with these antigens led to improved survival rates (Oksanen et al, 2013). Furthermore, a DNA vaccine combining Ag85B, ESAT6 and a resuscitation-related gene RpfE was able to boost the partial protection gained by BCG in this model (Oksanen et al., 2016).

As a consequence of these promising results, the zebrafish model was utilized in this thesis to screen the effect of novel DNA vaccines (Original publications I, II and IV). In general, immunization with the pCMV-GFP plasmid, encoding mycobacterial antigens, was well tolerated by the fish. We did not observe any adverse effects connected to the antigens or to the expression plasmid, which is in line with the previous DNA vaccination studies performed with other animal models and in clinical studies, highlighting the safety of DNA immunization methods (Khan et al, 2013; Gómez et al, 2018). In addition, the vaccination technique, including an intramuscular injection followed by electroporation, was relatively well tolerated in our studies. The rare complications were connected to a failed injection technique or an unexpected error in the electroporation equipment. However, less than 1% of the vaccinated fish were euthanized because of these complications (Original publication III). In studies with mammals, electroporation is rarely used to improve DNA vaccination. Similarly, in zebrafish, Oksanen et al. (2013) showed that injection without electroporation leads to expression of the antigen-GFP fusion protein, at a level which was measurable with GFP-ELISA. However, the challenge is that the optimal dose of antigen needed to elicit adequate immune responses is currently not known. In this respect, for screening purposes, the use of electroporation is justified. By using the electroporation and relatively high doses of vaccines, we can minimize the possibility of false negative results of our screening studies.

Including appropriate controls for a vaccine screen is critical. It is known that both a mock injection with a saline solution and the GFP protein itself can induce immunological responses (Ansari et al, 2016). And we have noted a similar trend in our vaccination studies with zebrafish (data not shown). In most of our experiments, vaccine antigens were expressed as a fusion protein with the GFP. Only in Original publication IV, the MMAR_4110 fragments 1 to 3 were not fused with the GFP. To

exclude the effect caused by immune responses of the GFP, fish in a control group were immunized with an empty expression plasmid, expressing only the GFP.

In general, due to its low developmental stage, the zebrafish is considered a more ethical vertebrate model than mammals. This aspect advocates the use of zebrafish over other models for preliminary preclinical studies. However, it is also important to recognize the limitations of the model and accept that no model is capable of recapitulating human tuberculosis completely. For instance, the anatomical differences between fish and humans is an obvious limitation of the zebrafish model, and further studies with mammals, and primates are needed before human trials. However, by using zebrafish as a screening model for novel vaccine candidates, it is possible to reduce the number of animals with a more developed nervous system, which supports the 3R goal set for animal experiments. The additional ethical aspect related to animal experiments is the determination of the correct group sizes. Both too large and too small numbers of used animals may cause an ethical dilemma. The small group sizes may skew the real variation seen in a population, and therefore give false positive or false negative results. On the other hand, by increasing the group sizes it is possible to increase the differences between the groups. In this thesis, all the group sizes were considered before performing any experiments. For the evaluation of the correct group sizes, pilot studies and group size calculators were utilized, and experiments were approved by the Animal Experiment Board of Finland.

6.2.1 Screening of DNA vaccines against a primary and a latent mycobacterial infection

Due to the above discussed shortcomings of the BCG vaccine, there is an obvious need for novel, effective TB vaccines. In this thesis, the zebrafish model was utilized to test the effectiveness of DNA vaccine candidates against both a primary infection and the reactivation of a latent infection. Two different approaches to test the effect of a primary infection were utilized. The first line screening method was based on the quantification of bacterial loads of immunized fish four weeks after a low dose *M. marinum* infection. During this thesis project, in total the effects of 16 antigens were screened with this method (Original publications I, II and IV). Immunization with four of the tested antigens (cdh, RpfE, PE5_1 and PE31) led to lower bacterial burdens compared to GFP-immunized fish. Furthermore, immunization with RpfE improved the survival of the fish (Original publication I). All of the positive results

were obtained with the antigens that were selected based on previous literature. Two of them were PE family proteins, which have been shown to elicit immune responses and some of them have been studied as DNA vaccine candidates (Hanif & Mustafa, 2017). As assumed, one of the Rpfs, which are highly connected to the resuscitation and virulence of mycobacteria, protected the fish against a primary infection. In addition, one of them (cdh) is a membrane protein with metabolic activity in the biosynthesis of phospholipids. All of these *M. marinum* genes have Mtb orthologues, and thus it will be interesting to test the effect of the corresponding Mtb homologs in mammalian models.

When we set out to identify novel vaccine antigens against the reactivation of a latent infection, our hypothesis was that genes expressed particularly in the early phase of the reactivation are potential targets for vaccine development. We tested the protective effect of 24 different antigens with the dexamethasone-based reactivation model (Original publications II and IV). Seven of these genes were selected based on the transcriptomic analysis of the *in vitro* reactivation experiment and the rest of the antigens were selected from previous literature. We found three antigens (RpfB, MMAR_4207 and MMAR_4110), which inhibited reactivation. It turned out that another Rpf protein, as a DNA vaccine antigen, was able to inhibit the dexamethasone-induced reactivation (Original publication I). While it is known that the expression profiles of *Rpf*s differ depending on the infection phase (Gupta et al, 2010), it is plausible that one of them provides protection against a primary infection and another against reactivation. In addition, our finding that one of the Rpfs is protecting against primary infections, corroborates the notion that latency is dynamic phenomenon with no clear-cut border to active infection (Barry et al, 2009).

Not much is known about MMAR_4207. Based on the Mycobrowser database, its Mtb orthologue Rv1234 is a transmembrane protein, which is expressed in starvation. Similarly, the information on MMAR_4110 is lacking. It is classified as an aldehyde dehydrogenase, with no clear Mtb orthologue. To gain more information about the biological significance of these genes on the mycobacterial side, it would be worth producing knock-out strains and studying their virulence and pathogenicity both *in vitro* and *in vivo*. This would increase our knowledge of the mechanisms underlying reactivation. Of the tested 24 antigens, 29% (7/24) gave a positive result either against a primary infection or the reactivation of a latent infection. If we only look at the seven antigens selected based on the transcriptomic analysis, we find that 14% (1/7) of the tested antigens worked as assumed. As far as I can see, the success rate is relatively high.

Overall, these zebrafish vaccine studies together with the previous studies (Parikka et al, 2012; Oksanen et al, 2013; Oksanen et al, 2016) show the applicability of the zebrafish model for the preliminary screening of novel vaccine antigens. In the future, it would be interesting to study the immunological responses behind the protective effect of these novel vaccine antigens, and certainly test if the Mtb homologues of these antigens are able to protect mammals against mycobacterial infections.

6.3 DNA vaccines against tuberculosis– challenges and future perspectives

As a conventional vaccine, protective effect of the BCG is based on antigen recognition and induction of adaptive immune responses. However, contrary to other infections that result in lifelong protection, individuals with a history of TB have a higher risk of developing a severe disease after second exposure to mycobacteria. Moreover, there is evidence that most of Mtb's T cell epitopes are conserved, suggesting that inducing of adaptive immunity is beneficial to Mtb (Comas et al, 2010; Coscolla et al, 2015). Furthermore, large-scale cohort studies have failed to show that protective TB vaccines are based on T cell responses (Kagina et al, 2010), implicating that novel strategies to prevent primary TB infection are needed. Currently, trained immunity (including innate immunity) is discussed as a possible mechanism of protection against primary infection. However, duration of the elicited trained immunity responses is uncertain. Thus, there are still a lot of uncharted territories for future TB vaccine development both related to preventive and therapeutic vaccines.

The WHO has set an ambitious goal to eradicate TB by 2050. Despite global programs (StopTB, End TB Strategy of WHO, Sustainable Developmental Goals) the incidence rates of TB have decreased very slowly, and TB remains one of the deadliest diseases worldwide (WHO, 2019). An efficient vaccination would be the most effective way to prevent and control TB. During the history of TB, researchers have developed methods to improve the BCG vaccine and to design novel vaccines. So far, none of the tested TB vaccines, including BCG, have achieved sterilizing immunity against Mtb (Darrah et al, 2019). The key issue in TB vaccine development is the lack of knowledge of the immune mechanisms against an Mtb infection at the cellular and molecular level. The fact that 90% of the people with a LTBI are asymptomatic, indicates that an Mtb infection can be controlled by the immune

system. In this respect, an in-depth understanding of the Mtb infection and its immunology is the key element when inventing effective vaccines.

There are potential serious adverse effects related to use of BCG and thus for example in Finland only newborns who are considered to have a high risk of infection are vaccinated. The safety concern can be minimized with DNA vaccines, which are generally safe and well-tolerated over the other types of vaccines. Despite of this clear advantage, DNA vaccination is less studied in controlling TB, mainly due to its poor efficiency. Thus far, there have been many DNA vaccine candidates that have shown promising results in preclinical studies, nevertheless clinical trials have failed. Based on the years of testing the immunogenicity, protective and immunotherapeutic efficiencies of DNA vaccines, it seems that there are some common features among promising candidates. First, the protective effect of these antigens is based mainly on the induction of Th1 responses (Du et al, 2017; Liang et al, 2017; Liang et al, 2018). This implicates that the antigen needs to be translated, APCs needs to recognize the translated peptides and present them to Th cells; and problems in any of these steps may lead to impaired protection. Besides these steps, the expression of the vaccine antigen needs to be high enough and long-lasting to elicit a proper immune response. Second, the number of produced specific antibodies increases after the second (and third) immunization, implicating that boosting is an efficient method to increase efficiency of DNA immunization (Meshkat et al, 2016; Du et al, 2017; Liang et al, 2017; Okada et al, 2017; Liang et al, 2018; Liang et al, 2019). In this respect, it would be intriguing to test if the vaccine antigens, which showed a protective effect in this study, could achieve better protection with the prime-boost method. Similarly, the effect of our promising antigens in boosting BCG, could be one of the future options to test. However, since the goal of our zebrafish studies is to identify potential antigens, rather than develop the most effective fish vaccine against a *M. marinum* infection, it is logical and ethical to save these tests for mammalian studies focusing to the most promising vaccine candidates. Third, it seems that the codelivery of IL-2 and IFNγ seems to increase the effectiveness of DNA vaccines (Meshkat et al, 2016; Okada et al, 2017). The role of IFNγ is not surprising, since the assumption is that DNA immunization leads to the maturation of memory T cells, which produce IFNγ (Zhu et al, 2018).

One of the challenges in developing DNA vaccines, is the selection of antigens. The choice is difficult due to the different phases of Mtb infection. In the same manner, the metabolic activity of mycobacteria is different in replicating and dormant bacteria, leading to different gene expression profiles. There are genes, such as ESAT-6, which are continuously expressed, but for instance Ag85B is expressed

in the early phases of the infection (Moguche et al, 2017). Furthermore, there is heterogeneity in the metabolic status of the bacteria even in the same phase of the infection depending on the microenvironment around the bacilli. Therefore, it seems plausible that combining antigens that are expressed at different stages of the infection in the same vaccine would induce a better a protective effect than vaccines that contains only one antigen (Luabeya et al, 2015). One of the advantages of the DNA vaccination is, that it allows combining multiple different antigens into the same expression plasmid. In this thesis, each antigen was tested individually, which is rational when novel protective antigens are sought after. However, in the following experiments, it would be interesting to combine the effective antigens in the same vaccine and see if the effect is improved. For instance, we could combine the MMAR_4207 antigen with a dormancy-associated gene of mycobacteria. Similarly, it would be possible to combine antigens with previously tested antigens, such as Ag85 and ESAT-6, which have been used in many vaccine studies (Aagaard et al, 2011; Luabeya et al, 2015; Liu et al, 2016). The combination of Ag85 and ESAT-6 has also been tested in zebrafish, where it elicited a protective effect (Oksanen et al, 2016). This combination was also utilized in Original publication II, where it was successfully used as a positive control in the vaccination study. However, there is also conflicting data about the ESAT-6 antigen from mice studies. Even though, the ESAT-6 improved the protective effect of a DNA vaccine, it seemed to induce a hypersensitivity response and cause the death of infected mice (Liang et al, 2016). Therefore, some researchers caution the use of *esat6* or the EAST-6 protein as a vaccine antigen (Gong et al, 2018). In our experiments, no increase in the mortality of fish immunized with the Ag85 and ESAT-6 antigens and infected with *M. marinum* was observed (Original publication II).

By changing the administration method or by adding adjuvants, the poor immunogenicity of the DNA antigens can be improved. In mice studies, it has been observed that using electroporation decreased the amount of needed DNA by roughly 50%, which decreases the needed amounts of thevaccine, which in turn decreases the expenses. In our zebrafish studies, electroporation was used routinely. However, a more convenient and practical method would be optimal for large scale immunization. In the future, improvements to the administration technique might have a critical role in achieving proper immune responses in humans after DNA vaccination. In the same way, adjuvants may have a pivotal role in TB vaccine development. Already now, most of the protein vaccines are combined with some adjuvant. So far, based on previous studies, the optimal administration methods and adjuvants remain elusive.

For now, DNA vaccines are not utilized to prevent TB, and in fact there are very few viable candidates for future studies. However, the promising results of the M72 vaccine give hope that a subunit vaccine could elicit protective immune responses against TB also in humans. This thesis introduces five novel antigen candidates; however, the real potential of these antigens needs to be evaluated in the future in studies with mammals.

7 SUMMARY AND CONCLUSIONS

Despite the rapid progression in medical research, tuberculosis remains as a major global health burden. *Mycobacterium tuberculosis* infects 10 million people and causes the death of 1.5 million annually. Moreover, roughly 1/5th of the human population have the latent infection, and a 5-10% lifetime risk of reactivation. The current methods to prevent and treat tuberculosis, including antibiotics and vaccines, are limited. The limited knowledge of the complex and dynamic interplay between the bacteria and the host's immunological responses, together with a lack of appropriate animal models have hampered tuberculosis research. The only available vaccine, the Bacillus Calmette-Guérin (BCG), has turned out to be inefficient in preventing the spread of the disease. Furthermore, the BCG vaccine has many serious adverse effects such as osteomyelitis, it even carries the risk of causing an active disease in immune-compromised children. Although researchers have tried to both improve the BCG vaccine and develop novel approaches for immunization, there are no other vaccines, whose safety and efficiency have been proven in clinical trials.

In this doctoral thesis, I utilized the previously established adult zebrafish-*M. marinum* infection model, which recapitulates key features of the human tuberculosis infection. The model was used to screen for the protective effects of novel vaccine antigens introduced as an expression plasmid to the dorsal muscle of the fish. The study shows that the zebrafish model is applicable for screening for the effects of novel DNA vaccines against a primary infection, which was validated by both a bacterial count analysis and by survival experiments. Further, to study the protective effect of vaccine antigens against reactivation, a novel, dexamethasone-based, reactivation method in adult zebrafish was developed. An orally administered glucocorticoid treatment led to increased bacterial counts in fish infected with a low dose of mycobacteria. The glucocorticoid treatment significantly decreased the number of T lymphocytes in zebrafish kidneys, which was associated with changes in the structure of granulomas and in an increased number of bacteria inside the granulomas, and finally dissemination of the bacteria outside of the granulomas. The developed reactivation model was effective and reducible making it a convenient model for the (preliminary) screening of the effects of novel vaccine antigens before mammalian studies.

To identify vaccine antigens to prevent the reactivation of a latent mycobacterial infection, the *in vitro* reactivation of *M. marinum* was performed by utilizing the Wayne's low-oxygen model for *M. tuberculosis*. With a transcriptomic level analysis, we identified genes, whose gene expression is up-regulated in reactivation. Of the nearly 400 differentially expressed genes, the expression of seven genes was induced during reactivation in comparison with both logarithmically growing and hypoxic cultures. The genes were members of PE/PPE proteins, metabolic enzymes or they were associated with the mycobacterial membrane. It must be noted, that no such experiment had been previously performed with *M. marinum*. Therefore, this study provides novel information of the *M. marinum* genes in reactivation, but on the other hand it is impossible to evaluate the results against the data from previous studies, which have been performed with other mycobacterial species, mostly with *M. tuberculosis*. Further, antigens from these seven genes, together with 16 other antigens which were selected based on the literature, were tested with the dexamethasone-induced reactivation model in the adult zebrafish, where four of them inhibited the reactivation.

In conclusion, this doctoral thesis shows the applicability of the adult zebrafish as a model for the preclinical screening of novel DNA vaccines against tuberculosis. By using the model, we found novel antigens, which are potential candidates for further mammalian studies, and perhaps, later on can be tested in clinical trials. In addition, both *in vitro* and *in vivo* reactivation models provide novel tools for studying the mechanisms of reactivation from the perspective of both the host and pathogen, allowing us to increase our knowledge of the reactivation of a mycobacterial infection.

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PUBLICATIONS

PUBLICATION I

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RESEARCH ARTICLE

Identification of novel antigen candidates for a tuberculosis vaccine in the adult zebrafish (*Danio rerio*)

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Abstract

Tuberculosis (TB) remains a major global health challenge and the development of a better vaccine takes center stage in fighting the disease. For this purpose, animal models that are capable of replicating the course of the disease and are suitable for the early-stage screening of vaccine candidates are needed. A *Mycobacterium marinum* infection in adult zebrafish resembles human TB. Here, we present a pre-clinical screen for a DNA-based tuberculosis vaccine in the adult zebrafish using an *M. marinum* infection model. We tested 15 antigens representing different types of mycobacterial proteins, including the Resuscitation Promoting factors (Rpf), PE/PPE protein family members, other membrane proteins and metabolic enzymes. The antigens were expressed as GFP fusion proteins, facilitating the validation of their expression *in vivo*. The efficiency of the antigens was tested against a low-dose intraperitoneal *M. marinum* infection (≈ 40 colony forming units), which mimics a primary *M. tuberculosis* infection. While none of the antigens was able to completely prevent a mycobacterial infection, four of them, namely RpfE, PE5_1, PE31 and cdh, led to significantly reduced bacterial burdens at four weeks post infection. Immunization with RpfE also improved the survival of the fish against a high-dose intraperitoneal injection with *M. marinum* (≈ 10.000 colony forming units), resembling the disseminated form of the disease. This study shows that the *M. marinum* infection model in adult zebrafish is suitable for the pre-clinical screening of tuberculosis vaccines and presents RpfE as a potential antigen candidate for further studies.

Introduction

Tuberculosis (TB) remains a major health problem that has been extensively studied in recent years. *Mycobacterium tuberculosis*, the causative agent of TB, caused 1.4 million deaths and 10.4 million new infections in 2015 [1]. The prevalence of TB is highest in Africa and Asia,

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where 75% of all new cases are diagnosed [1]. The World Health Organization (WHO) estimates that one third of the human population have a latent TB infection and carry up to a 10% lifetime risk of reactivation into an active disease [1]. In addition, the multi-drug resistant *M. tuberculosis* strains and HIV co-infections hamper the treatment of TB [1,2].The WHO has set an ambitious goal to eliminate TB as a global health problem by the year 2050 [1]. To reach the goal, new innovative approaches are needed.

Interest in developing novel tuberculosis vaccines has grown over the years. The only available TB vaccine, Bacillus Calmette Guérin (BCG), protects young children, but its ability to induce long-term cell mediated immune responses varies and the protection it provides against pulmonary TB or against the reactivation of latent TB is limited [3–5]. Therefore, new vaccines that protect from the primary infection, boost BCG induced immunity or prevent the reactivation of a latent infection, are needed to overcome TB.

A central issue in TB research has been the paucity of good animal models [6]. *M. tuberculosis* is not a natural pathogen of traditional animal models such as mice, rabbits and guinea pigs, and natural hosts, non-human primates, can be used only very selectively for experiments [6]. In the past ten years, the zebrafish (*Danio rerio*) has emerged as an advantageous animal to model a TB infection. An infection with *Mycobacterium marinum*—a close relative to *M. tuberculosis*—in zebrafish leads to a disease that resembles human TB in many aspects [7]. *M. marinum* is a natural pathogen of fish and an infection can lead to either an active or a naturally latent form of the disease [8–10]; reviewed in [11]. As a vertebrate, the zebrafish has both an innate and an adaptive immunity with essentially the same immune cell populations as are present in humans, including neutrophils, macrophages and both T and B cells. Also, zebrafish CD4+ and CD8+ lymphocytes perform similar functions as in humans [12–17]. Although there are physiological differences between humans and zebrafish, most importantly fish lack lungs and are smaller than humans, there is accumulating evidence for the similarities in immune responses involved in mycobacterial infections in zebrafish and humans, and factors increasing susceptibility to infections [17–26]. In addition, similar virulence factors and immune evasion strategies are used by both *M. marinum* and *M. tuberculosis* [19,27–31]. The data obtained from the zebrafish studies has already proven useful in the design of novel drugs and therapies against TB [21,25,30,32].

Despite the increasing knowledge on mycobacterial pathogenesis, the development of new TB vaccines has turned out to be challenging. Currently there are 14 vaccine candidates in the clinical trial pipeline, including inactivated or attenuated whole-cell vaccines, and subunit vaccines containing mycobacterial antigens. [4,33]. Expression of a bacterial antigen leads to the production of cytokines, including Interferon gamma (IFN-γ), and antigen presentation via the major histocompatibility complex of dendritic cells and the development of antigen specific memory cells [34]. An important advantage of DNA vaccines over BCG and other live attenuated vaccines is that they can be safely administered to immunocompromised people. [1,4,35].

A key step in the design of DNA vaccines is the choice of the antigen(s), especially since DNA vaccines tend to have a relatively weak immunogenicity in humans [34]. Even though there are methods for predicting the immunogenicity of selected antigens, *in vivo* infection models are required to assess the efficacy of the novel vaccine candidates as there are currently no reliable biomarkers for predicting the efficacy of protection against TB [4,36,37]. We have previously shown that adult zebrafish can be partially protected against mycobacteriosis with the BCG vaccine or with a DNA vaccine expressing a combination of antigens [38,39]. The current study was designed to test the applicability of the adult zebrafish-*M. marinum* infection model in the pre-clinical screening of DNA-based tuberculosis vaccines. Based on literature and online databases, MarinoList and TubercuList [40,41], we selected 15 *M. marinum*

antigens that have a homologue in *M. tuberculosis* and predicted or experimentally shown immunogenicity. We selected molecules that belong to different functional categories and are expressed during different stages of mycobacterial growth, including four Resuscitation promoting factors [42], three PE/PPE family members [43] and five other membrane associated proteins together with three proteins involved in metabolism. The selected antigens were tested as prophylactic DNA vaccines using two variations of the zebrafish mycobacterium infection model: a low-dose infection that mimics a primary TB infection leading to latency; and a high-dose infection that replicates miliary tuberculosis.

Materials and methods

Fish

Adult (5–7 month-old) wild type AB zebrafish were used for all experiments and maintained as in (Parikka et al, 2012). Animal studies were approved by the National Animal Experiment Board in Finland (Approval number ESAVI/8125/04.10.07/2013) and conducted in accordance with the EU-directive 2010/63/EU on the protection of animals used for scientific purposes.

Culture of *M. marinum* and qRT-PCR

The *Mycobacterium marinum* strain ATCC 927 was cultured on 7H10 Middlebrook OACD plates (BD Biosciences, Franklin Lakes, NJ) at +29˚C, inoculated to a fresh plate every 7 days, and a fresh stock was thawed after every two passages. Liquid cultures for RNA isolation and infections were grown in 7H9 Middlebrook medium (BD Biosciences, Franklin Lakes, NJ), see below for details.

Expression of the *M. marinum* genes corresponding to the selected antigens was confirmed by qRT-PCR from *M. marinum* RNA. The *M. marinum* ATCC 927 strain was cultured in 7H9 (BD Biosciences) medium to the log phase (OD600 of 0.6). Bacteria from six separate liquid cultures were collected by centrifuging for 5 minutes at 800 x g. The pellets were used for RNA extractions with the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Before qRT-PCR, the RNA samples were treated with DNase (RapidOut DNase Removal kit, Thermo Fischer Scientific, Waltham, MA USA). The expression of mycobacterial genes was verified with the iScript™ One-Step RT-PCR Kit with SYBR® Green (Bio-Rad, California, USA) according to the manufacturer's instructions. The *M. marinum internal transcribed spacer* (*MMITS*) [8] was used as a reference gene, and the qRT-PCR results were analyzed by the ΔCt-method [44]. The primers used for qRT-PCR were designed using the Primer3Plus software [45] and are listed in S1 Table.

Construction of DNA vaccines and immunizations

Homology between the *M. tuberculosis* and *M. marinum* genes was analyzed with the Clustal Omega sequence alignment tool [46]. The cellular location of the chosen *M. marinum* proteins was determined based on experimental evidence available in the literature or by prediction of transmembrane protein topology with a hidden Markov model [47]. Antigen sequences of different lengths were selected for expression in the candidate vaccine, however, when possible, all or part of the extracellular region of the *M. marinum* protein was included in the vaccine antigen. The Expasy Compute pI/Mw tool [48] was used to calculate the expected molecular weight of the antigen-GFP fusion proteins. The primers used for cloning the antigens are listed in S2 Table.

DNA vaccine constructs were prepared and the DNA vaccinations performed as described in [38]. In brief, the chosen antigen regions were amplified from *Mycobacterium marinum* ATCC grown on 7H10 Middlebrook OACD plates (BD Biosciences, Franklin Lakes, NJ) by colony PCR. Purified PCR products were restriction cloned into the pCMV-*eGFP* expression vector to be expressed with a C-terminal GFP tag (Addgene plasmid 11153), transformed into *E. coli* One Shot TOP10 cells (Invitrogen) and confirmed by sequencing. For DNA immunizations, plasmid DNA was purified using the QIAGEN Plasmid Plus Maxi Kit (Qiagen, Venlo, The Netherlands). The pCMV-EGFP plasmid without mycobacterial inserts was used for control vaccinations.

For vaccine immunizations, the fish were briefly anaesthetized in 0.02% 3-aminobenzoic acid ethyl ester (pH 7.0) (Sigma–Aldrich) and injected in the dorsal muscle with 12 μg of the vaccine or the *pCMV*-eGFP plasmid using aluminosilicate capillary needles and a PV830 Pneumatic PicoPump microinjector (World Precision Instruments, Sarasota, FL). The injection was followed by electroporation (6 pulses, 40 V, 50 m s each) using the GenePulser-electroporator (Bio-Rad, Hercules, CA) with tweezer-type electrodes (BTX/Harvard Apparatus, Holliston, MA) [39].

Fluorescence microscopy, Western blotting and GFP ELISA

In vivo expression of the plasmid DNA-derived protein products (GFP and its antigen recombinants) was verified by fluorescence microscopy, Western blotting and ELISA using naïve fish as a negative control. Nikon AZ100 fluorescent microscope was used for the microscopy. For Western blotting and ELISA, the fish were dissected under UV light and their dorsal muscles that showed the fluorescence indicative of vaccine antigen expression were collected for analysis. The samples were homogenized in TriReagent (Molecular Research Centre, Inc., Cincinnati OH, USA) with ceramic beads (MO BIO Laboratories, Carlsbad CA, USA) using a PowerLyzer™ 24 Bench Top Bead-Based Homogenizer (MO BIO Laboratories), followed by a protein extraction protocol according to the manufacturer's instructions.

The Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) was used to define the total protein concentration of each lysate. For Western blotting, a volume corresponding to a total protein amount of 7.5–15 μg of each fish homogenate was resolved on a 4–20% Mini-PROTEAN® TGX™ Gel (BioRad) and blotted onto a nitrocellulose membrane using Trans-Blot® Turbo™ Mini Nitrocellulose Transfer Packs (BioRad). The horse radish peroxidase conjugated GFP Tag Monoclonal Antibody (GF28R) (Thermo Fisher) was used for detection of the target protein. The GFP ELISA Kit (Cell Biolabs, San Diego, CA) was used for determining the relative levels of GFP according to the manufacturer's instructions. The absorbance values were transformed into GFP concentrations using a GFP standard, and the amount of GFP in each sample was normalized with the total protein concentration of the sample and with the average of the GFP controls in the experiment. Non-immunized AB fish were used as a negative control in both Western blots and ELISA.

M. marinum infections

Fish were infected either with a low (~40 cfu) or high (~10,000 cfu) dose of *M. marinum* four weeks after immunization. *M. marinum* ATCC 927 was cultured at 29˚C in standard mycobacterium medium, 7H9 (BD Biosciences), and prepared for infections as described in [8]. For infections, fish were anesthetized with 0.02% 3-aminobenzoic acid ethyl ester. The desired dose of *M. marinum* diluted in 0.2 M sterile KCl was injected intraperitoneally (i.p.). Thereafter, the fish were immediately released into a recovery tank. Infection doses were verified by plating the bacteria onto 7H10 plates (BD Biosciences). Following the infections, the well-

being of the fish was monitored daily, and fish showing signs of stress or mycobacterial disease during the experiment follow-up period were euthanized with 0.04% 3-aminobenzoic acid ethyl ester.

Nucleic acid extraction and quantification of bacterial burdens

To assess vaccine efficacy on a primary infection, AB fish immunized with experimental or control (GFP) antigens (~15 fish/group) were infected with a low dose (~40 cfu) of *M. marinum* four weeks post-immunization. Five weeks after infection, the fish were subjected to DNA extraction. Fish showing signs of disease during the five-week follow-up interval were euthanized immediately and included in the cfu analysis. The contents of the peritoneal cavity, including the visceral organs, of euthanized fish was collected into homogenization tubes (Mobio, California, USA) and homogenized in 1.5 ml of TRI reagent (MRC, OH, USA) using the PowerLyzer24 bead beater (Mobio). Homogenized samples were sonicated using an m08 water bath sonicator (Finnsonic, Lahti, Finland) and DNA extractions were then carried out as in [38]. The bacterial burden per fish was measured from the DNA samples by qPCR with *M. marinum*-specific primers using a standard curve with previously determined bacterial loads as described in [8]. Antigen immunizations that showed a protective effect (or tendency) were repeated one or two more times with similarly sized groups.

Survival follow-up

For survival experiments, the control and experimental fish (19–34 fish/group) were infected with a high dose (~10.000 cfu) of *M. marinum* and followed for twelve weeks. Fish included in the survival experiments were monitored daily for their well-being and humane end point criteria ratified by the national ethical board were followed. Fish showing signs of discomfort or disease were euthanized using 0.04% 3-aminobenzoic acid ethyl ester. Antigen immunizations that showed a protective effect (or tendency) (RpfE, PE31, MMAR_3501, esxM, cdh) were repeated one or two more times with similarly sized groups.

Power calculations and statistical analyses

The required sample size (n) for each experiment was calculated using the following formula: $n = 2(Z_a + Z_{1-\beta})^{2\sigma^2,}/\Delta^2$, in which Z_α (1.96) is a constant set based on the accepted error α (0.05), $Z_{1-}\beta$ (0.8416) is a constant set according to the power of the study (0.8), σ is the estimated standard deviation (0.5). Δ is the difference in the effects of the two treatments compared (estimated effect size), and was set to 0.5 (50%) relating to a reduction in the bacterial burden or improvement in the survival percentage. This is approximately the same as the effect that is achieved by the BCG vaccination [38,39]. Based on these calculations, the minimum group size was set to 14 fish [49].

Statistical analyses were done using the GraphPad Prism 5.02 software (GraphPad Software Inc., California, USA). The statistical tests used were the log rank Mantel–Cox test for the survival experiments, and the Mann–Whitney test for bacterial counts and ELISA results. Values of $p \leq 0.05$ were considered significant.

Results

Choice of antigens and antigen construction

For the vaccine antigen screen, we selected genes that belong to diverse functional categories and are expressed at different stages of the mycobacterial life cycle. In addition, we chose antigens with different (observed or predicted) cellular locations, although we focused on secreted

and membrane-associated proteins, as these presumably are more likely to elicit responses by the host immune system [50]. We chose the antigens based on literature (see below) and homology data in online databases Tuberculist [40] and Marinolist [41].

Resuscitation promoting factors (Rpf) are proteins with peptidoglycan-hydrolysing activity and are thought to be important for mycobacterial virulence and especially for resuscitation from dormancy. Mutant bacterial strains with *Rpf* deficiencies display defects in replication, reactivation and in persistence to stress, presumably due to alterations in the structure of their cell wall [10,51–53]. There are five *rpf* genes in the *M. tuberculosis* genome (*rpfA-E*) [42] and four in *M. marinum* (*rpfA*, *-B* and *-E*, and *resuscitation-promoting factor-like protein* (*mmar_2772*) homologous to *M. tuberculosis rpfC*, which is hereafter referred to as *rpfC*). Despite the name, the expression profiles of the *M. tuberculosis rpf* genes differ according to the infection phase, suggesting that they have distinct functions [54]. As Rpfs have also been reported to have immunogenic properties in mice [55] and in humans [56]; for a review see [57], we included all four *M. marinum* antigens in our screen.

Another relatively well-studied group of potential mycobacterial antigens are the PE/PPE proteins, which are named after the proline-glutamic acid (PE) and proline-proline-glutamic acid (PPE) motifs near their N-termini. The *pe*/*ppe* genes constitute ∼10% of the genome of pathogenic mycobacteria, and their expression is differentially regulated by stress and other environmental conditions, including inside granulomas. PE/PPE proteins are commonly localized to the bacterial cell surface or are secreted, enabling them to elicit and modulate host immune responses. Many PE/PPE proteins have been shown to be highly antigenic [58] and several have been studied as vaccine candidates, of which a candidate comprising a polyprotein of Mtb32 (PepA) and Mtb39 (PPE18) has progressed to clinical studies [59]. For our screen, we chose three members of this protein family that had not yet been tested as vaccine candidates, namely PE5_1, PE19_1 and PE31.

We also included proteins with a signature expression profile in certain phases of the infection. For this, we chose the outer membrane protein A (ompA), whose homolog in *M. tuberculosis* induces strong IFN-γ responses in cattle [60], and the predicted transmembrane protein MMAR_3501 encoded by the Dormancy survival regulon (DosR), that is highly immunogenic, especially in patients with LTBI [61]. In addition, we selected the Early secreted antigenic target (ESAT)-6/10-kDa culture filtrate protein (CFP-10) family member esxM, whose homolog in *M. tuberculosis*, Rv3620c, is a secreted, antigenic protein [62]; the lipoprotein lprG, whose homolog in *M. tuberculosis* has been shown to induce the activation of memory T cells in humans [63]; and MMAR_4207, a predicted transmembrane protein of unknown function with a highly conserved homolog in *M. tuberculosis* [40,41].

Knowing that mycobacteria undergo extensive metabolic changes during the different stages of their lifecycle [64], we chose components of metabolic pathways as vaccine antigens. The biosynthesis of cysteine is needed in the oxidative defense and for dormant mycobacteria to persist inside infected macrophages. Therefore, we selected cysQ and cysM, two critical enzymes of this pathway [65,66]. In addition, we chose cdh, a predicted membrane protein and CDP-diacylglycerol pyrophosphatase, which is involved in the biosynthesis of phospholipids, and whose *M. tuberculosis* homolog Rv2289 shows high abundance in the virulent H37Rv strain, but is nearly absent from the avirulent H37Ra strain [67].

The selected antigens together with their *M. tuberculosis* homologs are listed in Table 1. For clarity, the same grouping according to the (predicted) function of the antigen proteins will be used throughout the paper.

Table 1. The selected antigens and their *M. tuberculosis* homologs with predicted functions.

Accessionnumber	Protein name	*M.*tuberculosis protein (% homology)	Protein Size (aa)	Predicted protein function	Reference
Resuscitation Promoting Factors					
MMAR_4665	RpfA	Rv0867c (84%)	386	Peptidoclycan hydrolase. May promote the resuscitation of dormant cells.	[42,51,53]
MMAR_4479	RpfB	Rv1009 (85%)	363	Peptidoclycan hydrolase. May promote the resuscitation of dormant cells.	[8,42,52]
MMAR_2772	resuscitation-promoting factor-like protein	Rv1884c/RpfC (66%)	138	Peptidoclycan hydrolase. May promote the resuscitation of dormant cells.	[54–56]
MMAR_3776	RpfE	Rv2450c (74%)	244	Peptidoclycan hydrolase. May promote the resuscitation of dormant cells.	[40,41,57]
PE/PPE proteins					
MMAR_5258	PE5_1	Rv1386/PE15 (70%)	103	Membrane protein of unknown function.	[43,58]
MMAR_2670	PE19_1	Rv1788/PE18 (89%)	99	Membrane protein of unknown function.	[43,58]
MMAR_4241	PE31	Rv1195/PE13 (70%)	99	Membrane protein of unknown function.	[43]
Transmembrane proteins and secreted factors					
MMAR_4207		Rv1234 (95%)	175	Conserved hypothetical membrane protein of unknown function.	[40,41]
MMAR_3501		Rv1733c (38%)	193	Conserved hypothetical membrane protein of unknown function.	[61]
MMAR_4637	ompA	Rv0899/ompA (67%)	332	Structural outer membrane protein that may protect the integrity of the bacterium.	[60]
MMAR_2674	esxM	Rv3620c/esxW (87%)	98	Secreted, ESAT-6/CFP-10 family protein, function unknown.	[62]
MMAR_2220	lprG	Rv1411c/lprG (78%)	233	Conserved lipoprotein of unknown function.	[63]
Metabolic enzymes					
MMAR_3112	cysQ	Rv2131/cysQ (78%)	263	Monophosphatase involved in sulphur metabolism.	[65,66]
MMAR_4629	cysM	Rv1336/cysM (76%)	314	Cysteine synthase.	[65,66]
MMAR_3445	cdh	Rv2289 (68%)	264	Secreted CDP-diacylglyserol pyrophosphatase involved in phospholipid biosynthesis.	[67]

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Expression of selected antigens in *M. marinum*

The expression of the selected *M. marinum* genes in the ATCC 927 strain was verified by qRT-PCR (Fig 1). For this purpose, we used a log phase bacterial culture (OD600 of ~0.6), which represents actively growing, infectious mycobacteria. Although the relative expression levels of the candidate genes observed in our bacterial culture varied in a range of a ten times higher expression (*RpfA* and *esxM*) to 10^{-5} (*pe31*) compared to the reference gene *MMITS* expression, each of the selected genes was verified to be expressed. Based on this, all 15 antigens were selected for further studies and cloned into an expression vector as GFP–tagged fusion proteins.

Verification of antigen expression by the vaccines

One of the key issues in DNA vaccination is achieving adequate antigen expression in the target tissue. To assess this, the in vivo expression of the vaccine constructs was analyzed with three different methods, each utilizing the GFP tag fused with the antigen. First, antigen expression was visualized in situ, in the dorsal muscles of the fish, with a fluorescent microscope. Although not quantitative, visual inspection provides a quick and easy way to evaluate successful vaccinations and antigen expression in each individual fish without harming them.

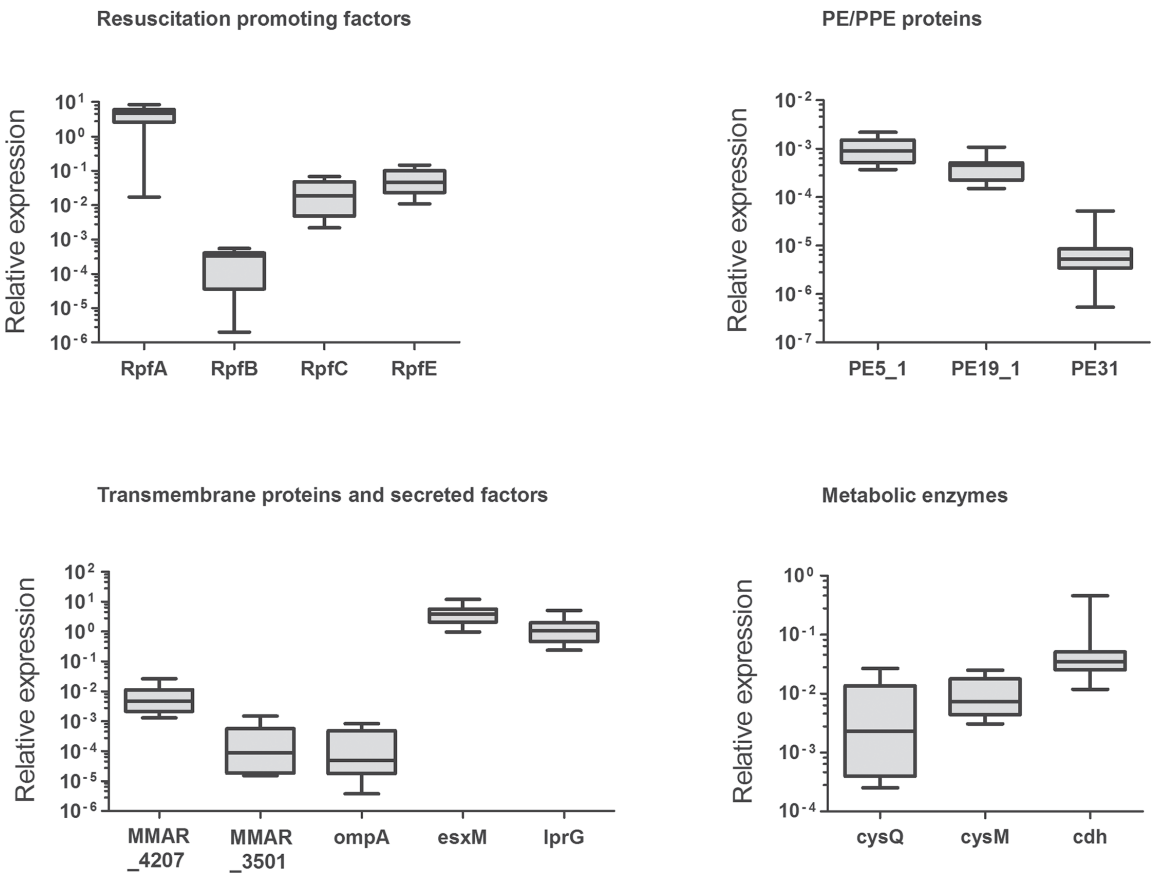

Fig 1. Expression of the antigens in the *M. marinum* ATCC 927 strain. A liquid culture of *M. marinum* was grown to a log phase, bacteria were harvested by centrifugation and subjected to RNA extraction and DNase treatment. Antigen expression was confirmed by qRT-PCR using primers specific for each antigen (S1 Table). The *M. marinum* transcribed internal spacer (*MMITS*) was used as a reference gene. The horizontal lines represent medians and the bars and whiskers represent minimum and maximum values. N = 6.

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As shown in Fig 2, all of the tested antigens showed detectable GFP expression seven days after vaccination. Fluorescence above the background level of non-immunized fish was observed for all of the candidate antigens, and this was always located near the injection site in the dorsal muscle.

To quantify antigen expression, a GFP enzyme-linked immunosorbent assay (ELISA) was used for proteins extracted from the dorsal muscles of the vaccinated fish. The quantitated expression of the recombinant constructs relative to the GFP control (samples from fish injected with an empty plasmid) is shown in Fig 3. All immunizations led to quantifiable GFP expression. Most antigens had expression levels comparable to the GFP control, while RpfA, RpfB and MMAR_3501 antigens had a rather low expression (10–16% of the GFP control), and the RpfE fusion protein showed expression levels exceeding that of the GFP control.

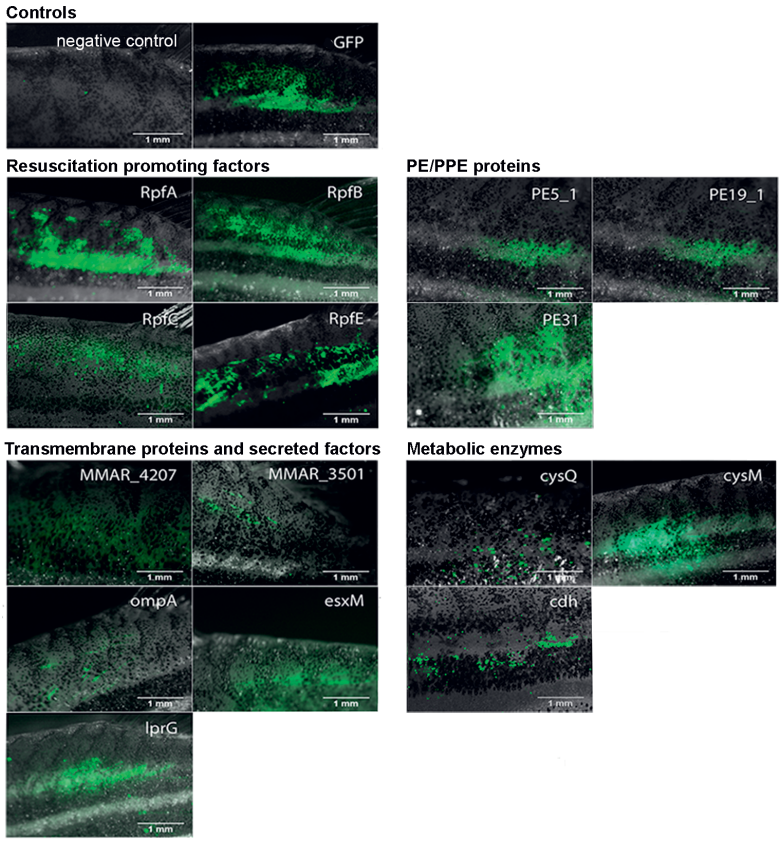

Fig 2. In situ GFP expression in immunized zebrafish. AB fish were immunized with 12 μg of experimental or control vaccine plasmids, followed by electroporation. Seven days post-injection, the successful vaccinations and expression of the antigen-GFP fusion proteins were verified by fluorescence microscopy. The fluorescence resulting from the expression of the antigen-GFP fusion protein is seen in the dorsal muscle near the injection site. For each antigen, a representative example is shown. Non-immunized AB fish were used as a negative control.

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To validate the correct size of the recombinant antigen fusion proteins, they were visualized with Western blotting, using a HRP-conjugated GFP antibody (Fig 4). The GFP control (fish immunized with an empty plasmid) produced a strong band of the expected size (GFP protein, 27 kDa). Importantly, expression of all of the antigens resulted in a detectable band corresponding to the calculated molecular weight of the GFP fusion protein (Fig 4).

Vaccine efficiency against a low-dose *M. marinum* infection

In most humans, a *M. tuberculosis* infection most often leads to a sub-clinical, latent infection, where the infection retains the potential to reactivate [68,69]. Ideally, a TB vaccine would prevent new infections; however, a more realistic goal would be a vaccine that helps the host to limit and control the infection and to prevent the dissemination into a fulminant disease [33].

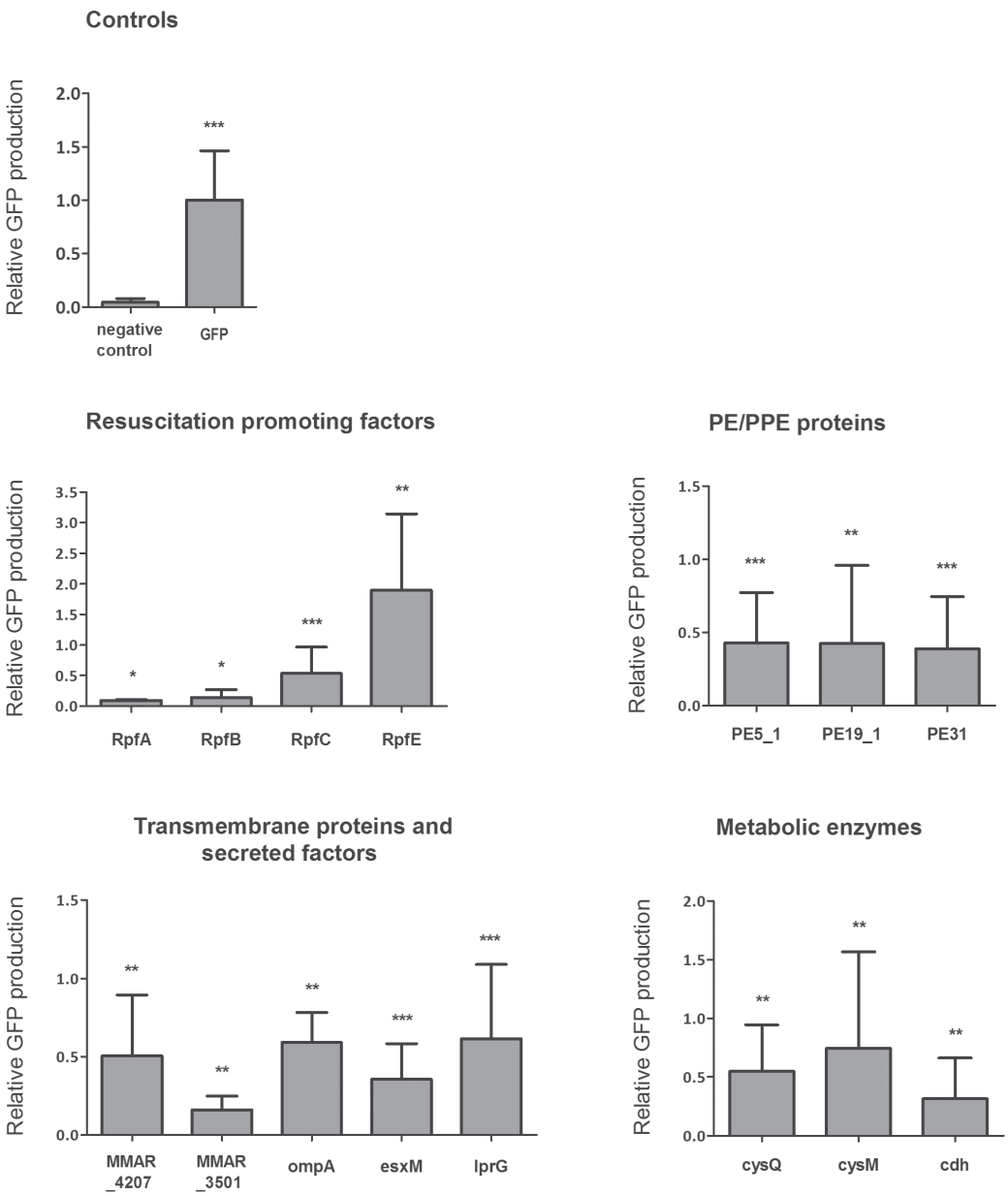

Fig 3. Quantification of mycobacterial antigen expression with GFP ELISA. AB fish were immunized with 12 μg of experimental or control vaccine plasmids, followed by electroporation. Seven days post-injection, fish were dissected under a UV light and the dorsal muscles were collected and homogenized with ceramic beads, followed by protein extraction. 7.5–15 μg of each protein lysate in a 1% SDS buffer was used for a GFP ELISA analysis. A standard curve was used to quantify the absorbance values, which were then normalized with the average of the control values of each experiment before the values were pooled. Non-immunized AB fish were used as the negative control. Mean ±SD is shown. N≥4 per group. * p<0.05, ** p<0.01, *** p<0.001 (Two-tailed Mann-Whitney test).

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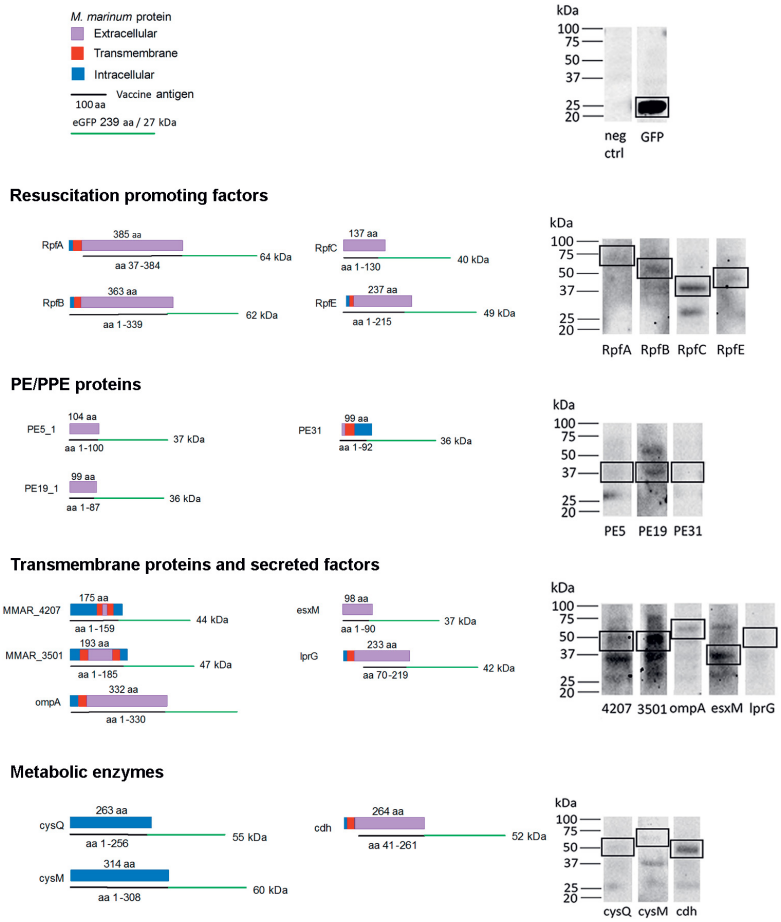

Fig 4. Schematic representation of the vaccine antigens. The *M. marinum* proteins are represented by bars, different colors indicate cellular location based on the literature and/or Trans Membrane prediction using Hidden Markov Models (TMHMM). The vaccine antigen-GFP fusion proteins are represented by lines, together with their expected molecular weights (See legend for more details). On the right, an immunoblot analysis of antigen-GFP fusion proteins. For the analysis, AB fish were immunized with 12 μg of experimental or control (empty plasmid with GFP only) vaccines, followed by electroporation. Seven days post-injection, fish were dissected under UV light and the dorsal muscles were collected and homogenized, followed by protein extraction. 7.5–15 μg of each protein lysate was run on an SDS-PAGE gel, blotted onto a nitrocellulose membrane followed by immunodetection with a horse radish peroxidase (HRP) conjugated anti-GFP antibody. Non-immunized AB fish were used as the negative control.

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In the adult zebrafish, a primary infection can be modelled by a low-dose *M. marinum* infection, which in most fish leads to a latent disease with stable bacterial counts [8]. To assess the efficacy of the selected antigens against a primary infection, the fish were first immunized with the experimental and control vaccine plasmids, and four weeks later i.p. infected with ~40 cfu of *M. marinum*. Five weeks post-infection, the fish were sacrificed and the bacterial burden of each fish was quantified by qPCR (Fig 5). To enable the comparison of data from multiple

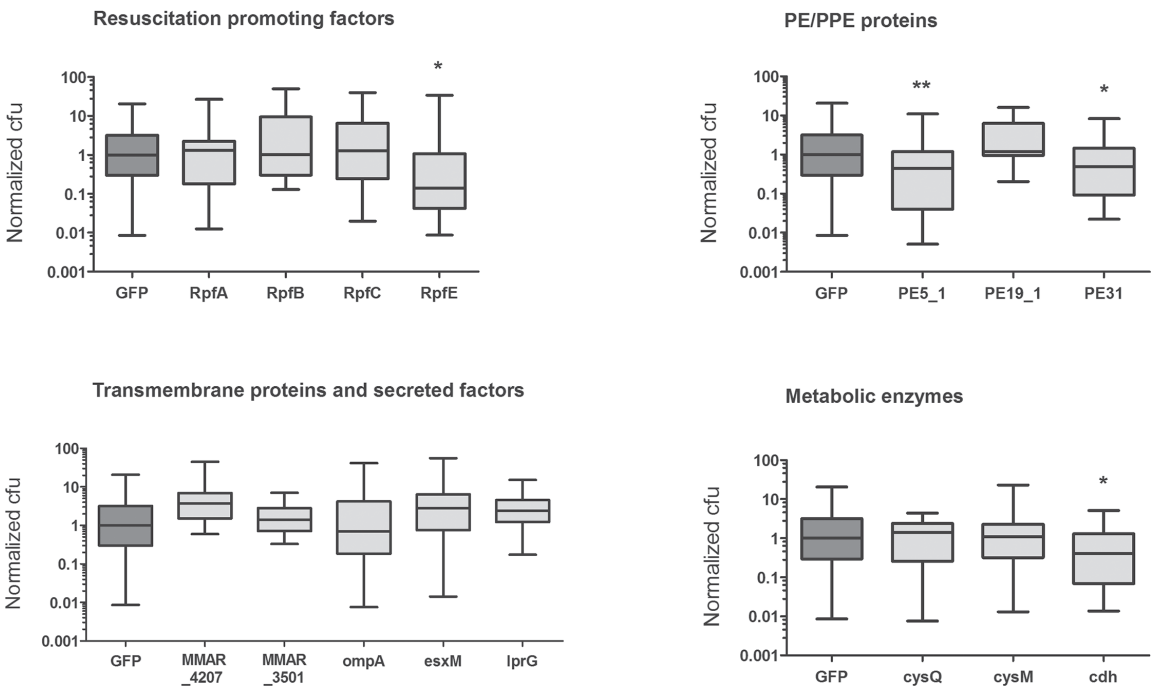

Fig 5. RpfE, PE5_1, PE31 and cdh antigens reduce bacterial burdens in adult zebrafish infected with a low-dose of *M. marinum*. AB fish were immunized intramuscularly with the experimental and control (GFP) antigens, followed by an intraperitoneal infection with ~40 cfu of *M. marinum*. Five weeks post-infection, the fish were euthanized, and their internal organs were dissected, homogenized and subjected to DNA extraction. The bacterial burden in each fish was determined by qPCR with *M. marinum* specific primers. The experimental cfu values in each experiment are normalized with the median cfu of the GFP controls of the same experiment. The lines represent median values, and the bars and whiskers the minimum and maximum values for each group, respectively. N = 10–29 per group. * p<0.05, ** p<0.01 (two-tailed Mann-Whitney test).

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experiments without bias from variations in the basal levels, the bacterial count of each sample was normalized with the median cfu value of the GFP control group of the same experiment. The raw values of the bacterial counts in each sample compared to the control group(s) are shown in S1 Fig. While most of the 15 antigens tested did not affect the progression of the infection in terms of bacterial numbers and none of them was able to clear the infection completely, four of the candidate vaccines reduced the bacterial burden significantly (two-tailed Mann-Whitney test). These included RpfE, which led to an 88% reduction in median bacterial counts; together with two PE protein family members, PE5_1 and PE31, and the metabolic protein cdh, which reduced the bacterial burden by 56%, 50% and 62%, respectively.

Vaccine efficiency against a high-dose *M. marinum* infection

In young children, a *M. tuberculosis* infection may lead to an acute, fulminant infection. The BCG vaccine protects children against this miliary TB, but due to safety issues, the use of BCG is limited in low-risk areas and excluded from HIV co-infected patients [1,5,35,69]. Therefore, a safer vaccine for preventing the dissemination of TB is required. To model a miliary TB infection in the adult zebrafish, we used a high-dose *M. marinum* infection that leads to an acute disease and relatively high mortality [8]. As a proof-of-concept, we have shown that

zebrafish can be partially protected against a high-dose *M. marinum* infection by BCG vaccination, indicated by improved survival [38,39]. We used a similar approach to test the effect of the candidate DNA vaccines. Of the original 15 antigens, we chose 10 for assessment in a high-dose infection assay, including the four that significantly reduced the bacterial burden in the low-dose infection assay. As previously, the fish were immunized with the experimental and control antigens and infected with *M. marinum* five weeks later, this time with ~10.000 cfu. Survival of the fish was monitored for 12 weeks, during which all fish showing signs of disease were euthanized. The survival curves of each immunization compared with the control group of the same experiment are shown in Fig 6. One of the tested antigens, RpfE, led to a significantly improved survival (40% compared to the 16% of the control group). In addition, immunization with RpfA slightly enhanced fish survival from week 10 post infection onwards, although the effect was not statistically significant.

Discussion

TB has a long history with mankind and it still remains a global challenge [70]. The bacterium has had time to evolve and adapt to its human host, and to develop means to avoid host immune responses or to use them for its own benefit [64,71]. Due to the complicated interactions between the bacterium and its host, proper *in vivo* models are needed for studying TB. The zebrafish, together with its natural pathogen *M. marinum*, have emerged as a feasible system to model TB [8–11]. Studies in zebrafish larvae and adults have shown several similarities in immune responses against mycobacterial infections in zebrafish and humans. These include the Toll-like receptor (TLR) signaling [18,19,72], leukotriene A_4 hydrolase and the Tumor necrosis factor signaling [22,73,74], Th2 type cells [23,24] and lysosomal trafficking [25] and furin [26]. In addition, the zebrafish model has been used to study mycobacterial virulence factors and immune evasion strategies, revealing that many of them are used by both *M. marinum* and *M. tuberculosis*. Examples of this include the genes in the *RD1* locus [19, 27,29]; the chemokine CXC-motive containing receptor 3 (CXCR3) signaling [20]; efflux pumps to achieve antibiotic tolerance [28], or the use of surface-associated membrane lipids to prevent the induction of TLR signaling [31]. Mycobacteria are also able to exploit the host's resources for their own benefit, for example by inducing the expression of matrix metalloproteinase-9 (MMP9) in the host for the recruitment of macrophages [29] or by initiating granuloma-associated angiogenesis [30]. Consequently, the zebrafish model has already been used for designing novel drugs and therapies against TB [21,25,30,32]. Moreover, owing to its small size, fast production of offspring and relatively low housing costs, the zebrafish is also a suitable model for large scale biomedical screening studies [75]. Considering the scale of the global TB problem, the emergence of multi-drug resistant *M. tuberculosis* strains and the difficulty of predicting protective immune responses, the discovery of new drugs and vaccines likely will require such screening models [6].

Although attenuated, the BCG vaccine is a live pathogen, and thereby imposes a risk of a disseminated disease in immunocompromised individuals. This has limited its use in low-risk countries [35]. Tragically, the people in high-risk areas, who could benefit from the BCG vaccination, also have high a incidence of a co-infection with HIV, which prevents the use of BCG in these individuals [1,3]. Therefore, safer vaccine alternatives are being actively investigated and 14 candidates are currently in different phases of clinical trials. Subunit vaccines are generally considered safer than whole-cell vaccines, and several candidates are being studied at the moment [33]. The antigens chosen for a subunit vaccine depend on the intended protective category: a pre-exposure vaccine would contain antigens expressed in metabolically active and replicating *M. tuberculosis*, while a post-exposure vaccine would consist of antigens expressed

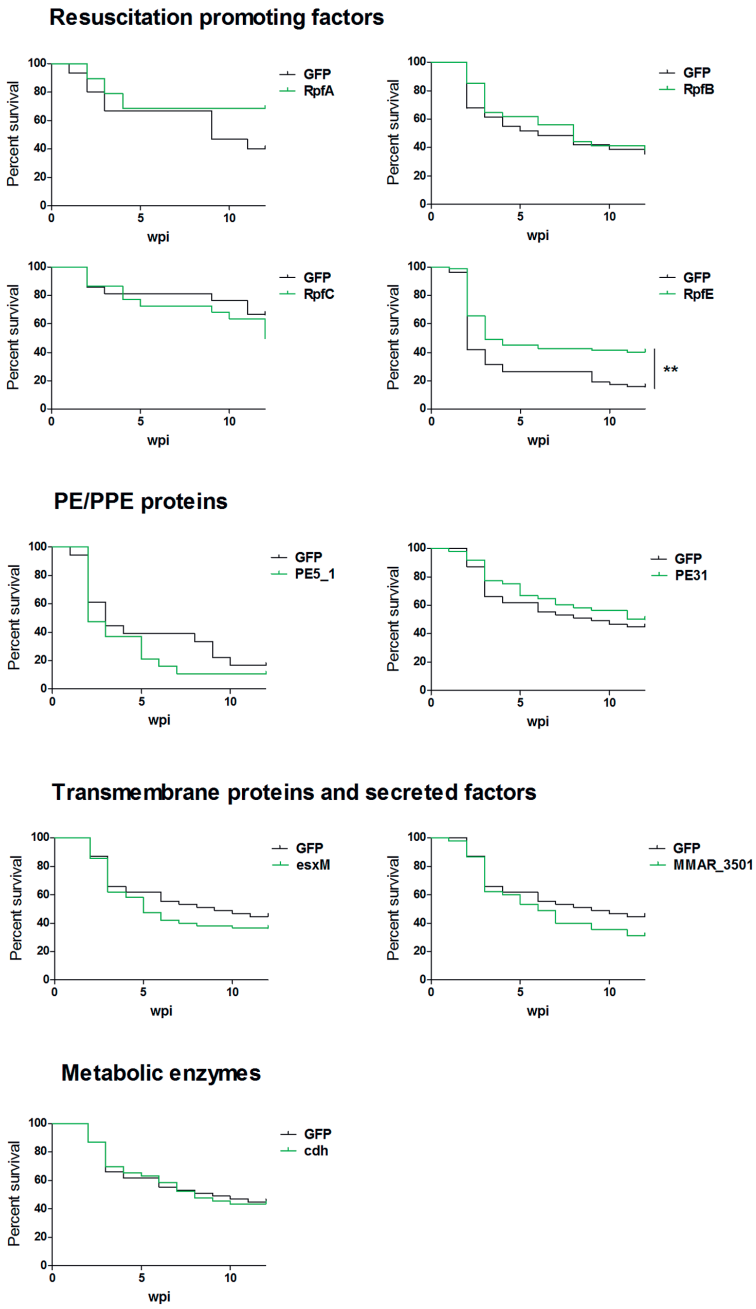

Fig 6. RpfE antigen improves survival of the fish infected with a high dose of *M. marinum*. AB fish were immunized intramuscularly with the experimental and control (GFP) antigens, followed by an intraperitoneal

infection with ~10.000 cfu of *M. marinum*. Fish were then followed for 12 weeks for survival. The survival curve for each antigen immunization is shown separately with the GFP control group of the same infection experiment(s). ** p<0.01 (Log-rank (Mantel-Cox) test). N≥19 in each group.

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during dormancy. As the subunit vaccine technology facilitates the use of several antigens, a combination of them would ideally give protection against both the active and latent stages of TB [33,76]. In our study, we tested 15 antigens that are expressed at different stages of the mycobacterium lifecycle and belong to different functional categories. We chose not to use BCG as a positive control because the most effective administration route for BCG in the zebrafish is an intraperitoneal injection, while DNA vaccines are injected intramuscularly. In addition, BCG is unable to replicate or form granulomas in the zebrafish and thus its protection is rather modest and variable [38, 39].

Prior to the screening in the infection assays, we verified the expression of the corresponding mycobacterial genes in the ATCC 927 strain by qRT-PCR. In the vaccine plasmid, the antigens were expressed as GFP fusion proteins, which facilitated the verification of their expression in vivo. For this, we used fluorescence microscopy, ELISA and Western blotting to allow the analysis of the expression of the antigens in situ, quantitatively and qualitatively, respectively. All of the fusion proteins were detected in each of the assays. As fluorescent microscopy allows the detection of antigen expression easily and without harming the fish, we used it to assess the success of each vaccination during the screening.

We used two assay settings to study the efficiency of the antigens against a mycobacterial infection: a low-dose infection followed by the quantification of the bacterial burden five weeks after infection, and a high-dose infection followed by the monitoring of survival for 12 weeks. The former is set to simulate a primary infection, and the latter a fulminant disease. As the stress caused to the fish by a high-dose infection and a survival assay is higher than that caused by a low-dose infection, for ethical reasons, we decided to exclude some of the antigens that did not show any protective effect against the low-dose infection from the survival study, even though the infection phases studied by the assays are different.

Four antigens were found to have protective effects against a low-dose mycobacterial infection. These include the probable CDP-diacylglycerol pyrophosphatase cdh, and two antigens belonging to the PE/PPE family, namely PE5_1 and PE31, and RpfE. Of these, cdh remains rather poorly characterized. Both the PE5_1 and PE31 antigens led to an approximately 50% reduction in the median cfu counts compared to the control group in the low-dose *M. marinum* infection assay. Their *M. tuberculosis* homologs, PE15 (Rv1386) and PE13 (Rv1195), have been studied using a recombinant *M. smegmatis* strain. Both recombinants led to the enhanced survival of bacteria within macrophages, presumably due to interference with host (innate) immune signaling pathways [77,78]. The expression of *pe13* was upregulated by diverse types of stress, and led to the increased production of interlukin-6 (IL-6) and IL-1β in macrophages [77], while PE15 upregulated anti-inflammatory cytokines and down-regulated proinflammatory cytokines and nitric oxide [78]. Thus, it is possible that both of these proteins are involved in evading the host immune response thereby promoting the survival of the mycobacteria. Further studies are required to determine the usefulness of these antigens as vaccine candidates. For example, they could be studied as a combination of two or more antigens, or if they are able to boost the protection offered by the BCG vaccination.

Of the mycobacterial antigens included in our screen, the Rpf proteins are probably the best studied, both considering their role in mycobacterial pathogenicity and their potential medical use. The latter is supported also by the results of this study, where RpfE was the only antigen that provided protection against both a primary (low dose) and a fulminant (high dose)

infection. This is in line with previous results from mouse studies. In a mouse *ex vivo* model, RpfE induced the maturation of dendritic cells via the TLR4 leading to the generation of Th1 and Th17 cell mediated immunity, without stimulating the suppressive regulatory T cells [79]. RpfE has been also studied to some extent as a DNA vaccine candidate in the mouse model, where it has shown high immunogenicity and variable protection against *M. tuberculosis* both in terms of cfu burdens and survival times [76,80]. Considering that Rpf proteins are variably expressed during reactivation from dormancy, and that the *M. marinum* infection in adult zebrafish displays a natural latency that can be reactivated experimentally or spontaneously [8,23], the zebrafish model provides a promising platform to study Rpfs as vaccine candidates against the reactivation of latent TB. This is an important aspect in the TB research, as immunization of the latent *M. tuberculosis* carriers, especially adolescents and young adults, who are the main source of TB transmission, would effectively limit new infections [81]. We have previously shown that the adult zebrafish is partially protected against a *M. marinum* infection by the BCG vaccine [38,39], and that this protection can be boosted by immunization with a DNA vaccine consisting of RpfE combined with two other well-studied antigens ESAT-6 and Ag85 [39]. This makes the zebrafish a promising model for developing booster vaccines for BCG.

In conclusion, this study indicates that the *M. marinum* infection model in the adult zebrafish is suitable for early-stage pre-clinical TB vaccine screening and that the PE/PPE proteins and Resuscitation promoting factors, especially RpfE, are interesting candidates for further studies as antigens for DNA vaccines against TB.

Supporting information

S1 Fig. Bacterial counts in adult zebrafish after immunization and a low dose M. marinum infection. AB zebrafish were vaccinated intramuscularly with experimental antigens and a control (GFP), followed by an intraperitoneal *M. marinum* infection (~40 cfu). Five weeks post infections, fish were euthanized and their internal organs were collected for DNA extractions. Bacterial burdens were determined from the extracted DNAs by qPCR with *M. marinum* specific primers. Figures show the pooled results of different experiments, which are indicated with different colors. Each dot represents the bacterial count in one fish, and the horizontal lines represent median values. N = 10–29. * $p<0.05$ (two-tailed Mann-Whitney test). Abbreviations: PE5, PE5_1; PE_19, PE19_1; 4207, MMAR_4207; 3501, MMAR_3501.
(TIF)

S1 Table. The primers used for qRT-PCR analysis.
(DOCX)

S2 Table. The primers used for cloning the antigens.
(DOCX)

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S1 Fig.

Resuscitation promoting factors
PE/PPE proteins
Transmembrane proteins and secreted factors
Metabolic enzymes
M. marinum cfu / fish
GFP
RpfA
RpfB
RpfC
RpfE
PE5_1
PE19_1
PE31
MMAR_4207
MMAR_3501
ompA
esxM
IprG
cysQ
cysM
cdh
*

S1 Fig. Bacterial counts in adult zebrafish after immunization and a low dose M. marinum infection.

AB zebrafish were vaccinated intramuscularly with experimental antigens and a control (GFP), followed by an intraperitoneal *M. marinum* infection (~40 cfu). Five weeks post infections, fish were euthanized and their internal organs were collected for DNA extractions. Bacterial burdens were determined from the extracted DNAs by qPCR with *M. marinum* specific primers. Figures show the pooled results of different experiments, which are indicated with different colors. Each dot represents the bacterial count in one fish, and the horizontal lines represent median values. N = 10–29. * $p<0.05$ (two-tailed Mann-Whitney test). Abbreviations: PE5, PE5_1; PE_19, PE19_1; 4207, MMAR_4207; 3501, MMAR_3501.

https://doi.org/10.1371/journal.pone.0181942.s001

S1 Table. The primers used for qRT-PCR analysis.

Gene	Forward primer	Reverse primer
RpfA	ATGTCCACGAGGCTTCGACC	TCGTTGCCGCTGATGTTCTG
RpfB	AGTTTCTGCGTCCAAGACCG	CTGGACGATGTCGATCACCC
RpfC	CACCTGGGAGGAATACGGTG	TTGGCGATCGCGATTTGTTG
RpfE	CCAAGGTCTACACCGTGAACT	GTGTTGATGCCCCAGTTACC
PE5_1	GAGTTATTCCCGAGGGTTTG	GGATCACCGCCGTAATGG
PE19_1	AGATGTATCAAGCCGTGAGCG	GTACGACCCTGAGCTGATGC
PE31	GTCTATCGGAGCGGCATTGA	GCAAGCAGTTCGGAAACCTC
MMAR_4207	TTCGGCCTGATTACCTCAGC	ATCGCAGAGCACGTCATAGC
MMAR_3501	GTTGTGGTGCAAGTGCGATG	ATACTTGGATACGCTGGCCC
ompA	ATTTCTCCAACGCCGAACCT	AAGGTGATGGTGTCCCGTTC
esxM	CATGGGCCAGATGAACACCG	TGCTCTTGCTGCTCGTAGTTG
lprG	GATCACCGGAAAGGTCACTG	GTTCTCCTGAATCCACACGG
cysQ	CGGCAATCCTCTATCACGTC	AGATAGGCATCGGCTTTGC
cysM	TCTACGGGACCGAGATCATA	GGTAGAGCAGGACCCATTC
cdh	GGGTGGCCATAGTTATCACG	GCGTTTTCCATCTTCTTGCC
MMITS	CACCACGAGAAACACTCCAA	ACATCCCGAAACCAACAGAG

S2 Table. The primers used for cloning the antigens.

Gene	Forward primer	Reverse primer
RpfA	CACACAGCTAGCCACCATGGTCAG CGTCGCCAAGATC	CACACAGAATTCCGATCTTGGCGA CGCTGAC
RpfB	CACACAGCTAGCCACCATGTTGCG CGCTGTGG	CAGCCAGAATTCCCACCGCGATCT GTTCTTC
RpfC	CACACAGCTAGCCACCATGACACA CATCGCGAAAC	CACACAGAATTCGGGGCGTGAAT ATCGAGATG
RpfE	CACACAGCTAGCCACCATGAAGAA CGTCCGCAACAG	CACACAGAATTCCTCGGATCTGCT GCTCTTCAC
PE5_1	ACACAGCTAGCCACCGTGATGTTG CGAGTTATTCCC	CACACAGAATTCCAAAAGCGCCGT AGCC
PE19_1	ACACAGCTAGCCACCATGCATACG TGACCACAC	ACACGAATTCCGTACGACCCTGAG CTG
PE31	ACATAGCTAGCCACCATGTCTTCT GTTACGGCTCGAC	TCAGAATTCCGGTTTCGGTGCTCA GG
MMAR_4207	ACATAGCTAGCCACCATGACTAGC CCCTTCCAG	ACGAATTCGATCGCAGAGCACGTC ATAGC
MMAR_3501	ATATAGCTAGCCACCATGGAGACG TTCAAACTGGAC	ACGAATTCCGTCTTGCAGACAACT GAGC
ompA	CACACAGCTAGCCACCATGGTGG GTACCGACGCGG	CACACAGAATTCCCACAATTTCGA CACGGC
esxM	ACATAGCTAGCCACCATGACTGCA CGCTTTATGAC	ACGAATTCCCTGCTCTTGCTGCTCG
lprG	ATATAGCTAGCCACCATGAAGACG CTCTCCGGC	ACGAATTCTGGACAAGGTCATCTC GACG
cysQ	ATATAGCTAGCCACCATGCGGGAA GAGGTTGG	ACGAATTCCATCGAGCAGCACAG GC
cysM	ATATAGCTAGCCACCATGACCCGC TACGACTCAC	ACGAATTCTAGGTGCCGGGTGTTC ATA
cdh	CACACAGCTAGCCCACCATGGCCG ACCGGGATCAAC	CACACAGAATTCCTTTGGCGACCG AGCAG

PUBLICATION II

Identification of protective postexposure mycobacterial vaccine antigens using an immunosuppression-based reactivation model in the zebrafish

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RESEARCH ARTICLE

Identification of protective postexposure mycobacterial vaccine antigens using an immunosuppression-based reactivation model in the zebrafish

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ABSTRACT

Roughly one third of the human population carries a latent *Mycobacterium tuberculosis* infection, with a 5-10% lifetime risk of reactivation to active tuberculosis and further spreading the disease. The mechanisms leading to the reactivation of a latent *Mycobacterium tuberculosis* infection are insufficiently understood. Here, we used a natural fish pathogen, *Mycobacterium marinum*, to model the reactivation of a mycobacterial infection in the adult zebrafish (*Danio rerio*). A low-dose intraperitoneal injection (~40 colony-forming units) led to a latent infection, with mycobacteria found in well-organized granulomas surrounded by a thick layer of fibrous tissue. A latent infection could be reactivated by oral dexamethasone treatment, which led to disruption of the granuloma structures and dissemination of bacteria. This was associated with the depletion of lymphocytes, especially $CD4^+$ T cells. Using this model, we verified that ethambutol is effective against an active disease but not a latent infection. In addition, we screened 15 mycobacterial antigens as postexposure DNA vaccines, of which RpfB and MMAR_4207 reduced bacterial burdens upon reactivation, as did the Ag85-ESAT-6 combination. In conclusion, the adult zebrafish-*M. marinum* infection model provides a feasible tool for examining the mechanisms of reactivation in mycobacterial infections, and for screening vaccine and drug candidates.

This article has an associated First Person interview with the first author of the paper.

KEY WORDS: Tuberculosis, Reactivation, Zebrafish, Vaccine antigens

INTRODUCTION

Tuberculosis (TB) remains one of the major global health problems. *Mycobacterium tuberculosis* (Mtb), the causative agent of TB, led to 1.4 million deaths and 10.4 million new infections in 2015 (WHO, 2017). The World Health Organization (WHO) estimates that one third of the human population carries a latent TB infection, and therefore has up to a 10% lifetime risk of it reactivating into an active disease. Both vaccines and antibiotics have their limitations in combating TB. Curative antibiotic treatments are lengthy and further complicated by the emergence of multidrug-resistant Mtb strains (WHO, 2017). The only available TB vaccine, Bacillus Calmette Guérin (BCG), is still widely used. Although the BCG vaccine can protect infants from disseminated TB, its ability to induce long-term cell-mediated immune responses is limited, and therefore it does not properly prevent the reactivation of a latent TB infection (Tang et al., 2016). To reach the ambitious goal of eliminating TB by the year 2050, innovative approaches are needed.

As nonhuman primates are the only animal models that fully replicate all phases seen in human TB, it has been challenging to study this area, especially the latency and reactivation of mycobacterial infections (Capuano et al., 2003; Myllymäki et al., 2015). It is known that reactivation of a latent TB infection is often associated with immunosuppression, such as human immunodeficiency virus (HIV), chemotherapy or immunosuppressive drugs (Ai et al., 2016), but the more detailed mechanisms of reactivation remain largely elusive on both the bacterial and host side (Dutta and Karakousis, 2014; Peddireddy et al., 2017). Considering the number of latent TB carriers, preventing the reactivation of latent TB would be a key step in the battle against TB (Matteelli et al., 2017).

During the past couple of decades, the zebrafish (*Danio rerio*) has proven an applicable alternative for modeling TB (Myllymäki et al., 2016). For this purpose, a natural fish pathogen and a close relative of Mtb, *Mycobacterium marinum*, is used (Stinear et al., 2008). Depending on the infectious dose, a *M. marinum* infection in adult zebrafish can lead either to an active or to a naturally latent form of the disease (Parikka et al., 2012; Swaim et al., 2006). In the latent form of human TB, the bacteria are contained in structures termed granulomas, which are surrounded by immune cells and a fibrotic capsule. Although granulomas were long thought to be a protection method elicited solely by the host, it has been found that they also promote persistence of the bacteria (O'Garra et al., 2013; Davis and Ramakrishnan, 2009). Upon a *M. marinum* infection, both adult zebrafish and larvae form granulomas that are highly similar in structure to those in humans (Parikka et al., 2012; Davis et al., 2002), and the immune responses they elicit against mycobacteria share similarities to those in humans (Parikka et al., 2012; Swaim et al., 2006; Hammarén et al., 2014; Clay et al., 2008; Torraca et al., 2015; Pagán et al., 2015; Yang et al., 2012). This has facilitated the translation of some of the results from fish studies to humans (Tobin et al., 2010; Adams et al., 2014; Berg et al., 2016; Thuong et al., 2017).

In the zebrafish, a latent mycobacterial infection can spontaneously reactivate into an active disease (Hammarén et al.,

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2014). In the current study, we have developed an experimental method to reactivate a latent mycobacterial infection in the adult zebrafish by feeding them the glucocorticoid dexamethasone. We use this model for the characterization of the cellular and molecular mechanisms associated with the reactivation of a mycobacterial infection. In addition, we show that the model can be used in screening for antibiotics and novel vaccine candidates against the reactivation of a latent mycobacterial infection.

RESULTS

Dexamethasone treatment leads to an elevated bacterial burden in zebrafish with a latent *M. marinum* infection

In humans, the reactivation of a latent TB infection often follows immunosuppression. Similarly, immunosuppression by gamma irradiation leads to the reactivation of a mycobacterial infection in zebrafish (Parikka et al., 2012). To further study the mechanisms of the reactivation of a mycobacterial infection in zebrafish, we tested the effects of various immunosuppressive medicines on a latent *M. marinum* infection (Fig. 1A). The fish were first infected with a low dose of *M. marinum*, which leads to a latent infection in most fish, and the immunosuppressive treatments were started 5 weeks later. For this, we selected chemicals that are commonly used in humans to alleviate excessive immune reactions associated with various medical situations, such as organ transplantation, arthritis and inflammatory bowel disease, and have been reported to increase risk of the reactivation of TB in individuals with a latent infection (Jick et al., 2006; Maltzman and Koretzky, 2003). The drugs included azathioprine, dexamethasone, methylprednisolone and prednisolone. To minimize additional stress, the drugs were administered orally, as a gelatin mix that was used to coat the fish food pellets. The experimental fish were treated with the drugs, 10 µg/fish/day, for 4 weeks, whereas the control fish received gelatin-coated food with no chemicals. After the treatment, the fish were euthanized and the DNA extracted from the intraperitoneal cavity of each fish was used to determine the number of mycobacteria.

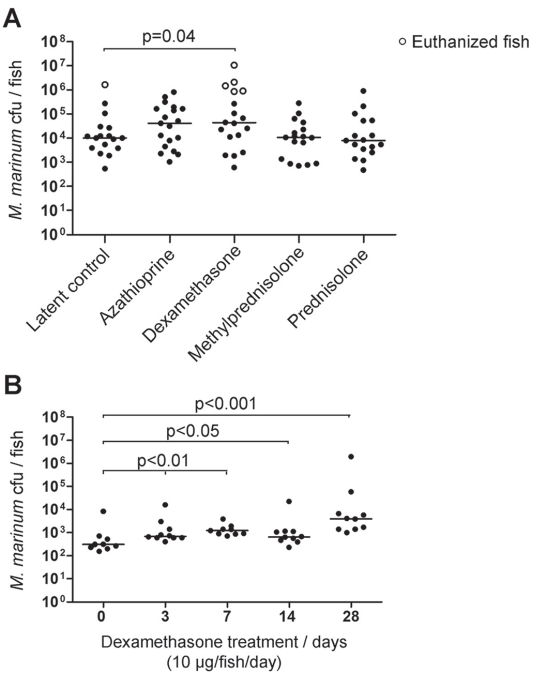

Fig. 1. Dexamethasone treatment increases bacterial loads in adult zebrafish with a latent *M. marinum* infection. (A) Zebrafish with a latent mycobacterial infection were treated with immunosuppressants. Each dot shows the bacterial count in one fish after 4 weeks of exposure to immunosuppressive medication (10 µg/fish/day), (n=17-19 fish/group). (B) Dots represent bacterial loads after 3, 7, 4 and 28 days of dexamethasone treatment of fish with a latent mycobacterial infection (n=9-10 fish/group). Horizontal lines represent the median bacterial count of each group. Statistical significance was analyzed with the one-tailed Mann–Whitney test. See also Fig. S1.

We found that feeding with dexamethasone led to an increase in bacterial burdens (P=0.04, one-tailed Mann–Whitney test); azathioprine produced a similar trend, though the increase was not statistically significant (Fig. 1A). Within the anticipated dose of 10 µg/day, prednisolone and methylprednisolone did not lead to elevated bacterial burdens. One fish from the control group and five fish from the group fed with dexamethasone were euthanized according to the humane endpoint criteria during the treatment – these fish were also included in the analysis. Consistently, a high number of mycobacteria [average of $2.9\times10^6\pm4.8\times10^5$ colony-forming units (cfu)] was detected in the euthanized fish (Fig. 1A), suggesting that the signs of discomfort were due to the progression of the *M. marinum* infection rather than the immunosuppression itself. In line with this, the feeding of dexamethasone did not cause signs of discomfort in uninfected fish. Therefore, the increase in the bacterial counts following a dexamethasone treatment is likely to be caused by the progression of a latent mycobacterial infection into an active phase.

The kinetics of this progression were analyzed by measuring the bacterial numbers at several time points following a dexamethasone treatment in two independent experiments (Fig. 1B; Fig. S1A). An increase in the bacterial burden was observed 3 days after the onset of the feeding of dexamethasone (P=0.007, one-tailed Mann–Whitney test), eventually leading to an approximately 1000-fold increment at 4 weeks. A higher dose of dexamethasone (20 µg/day/fish) had an essentially identical effect on bacterial numbers (Fig. S1B). Therefore, the lower dose of dexamethasone (10 µg/day) was selected for subsequent experiments. A pretreatment with dexamethasone prior to infection did not affect the outcome of a low-dose infection (Fig. S1C), suggesting that dexamethasone is more likely to affect the host's ability to control a latent infection than to suppress the responses against mycobacteria during an early infection. To obtain a more mechanistic insight into this, we next investigated the cellular and molecular events associated with the reactivation of a latent mycobacterial infection in the adult zebrafish.

Reactivation of a latent mycobacterial infection alters the quality and quantity of granulomas

The formation of granulomas is defined as the hallmark of tuberculosis, and granulomas also form in the adult zebrafish during the course of an *M. marinum* infection. To demonstrate the presence of granulomas during reactivation, we used Ziehl–Neelsen staining to visualize mycobacteria in histological sections, and trichrome staining to visualize the fibrous capsule typically found around mature granulomas (Parikka et al., 2012). In fish with a latent infection, the granulomas were generally well organized and surrounded by a thick layer of fibrous tissue confining the mycobacteria inside the granulomas (Fig. 2A). After 2 weeks of dexamethasone treatment, the granulomas appeared larger and looser in structure, showing thinning and disruption of the

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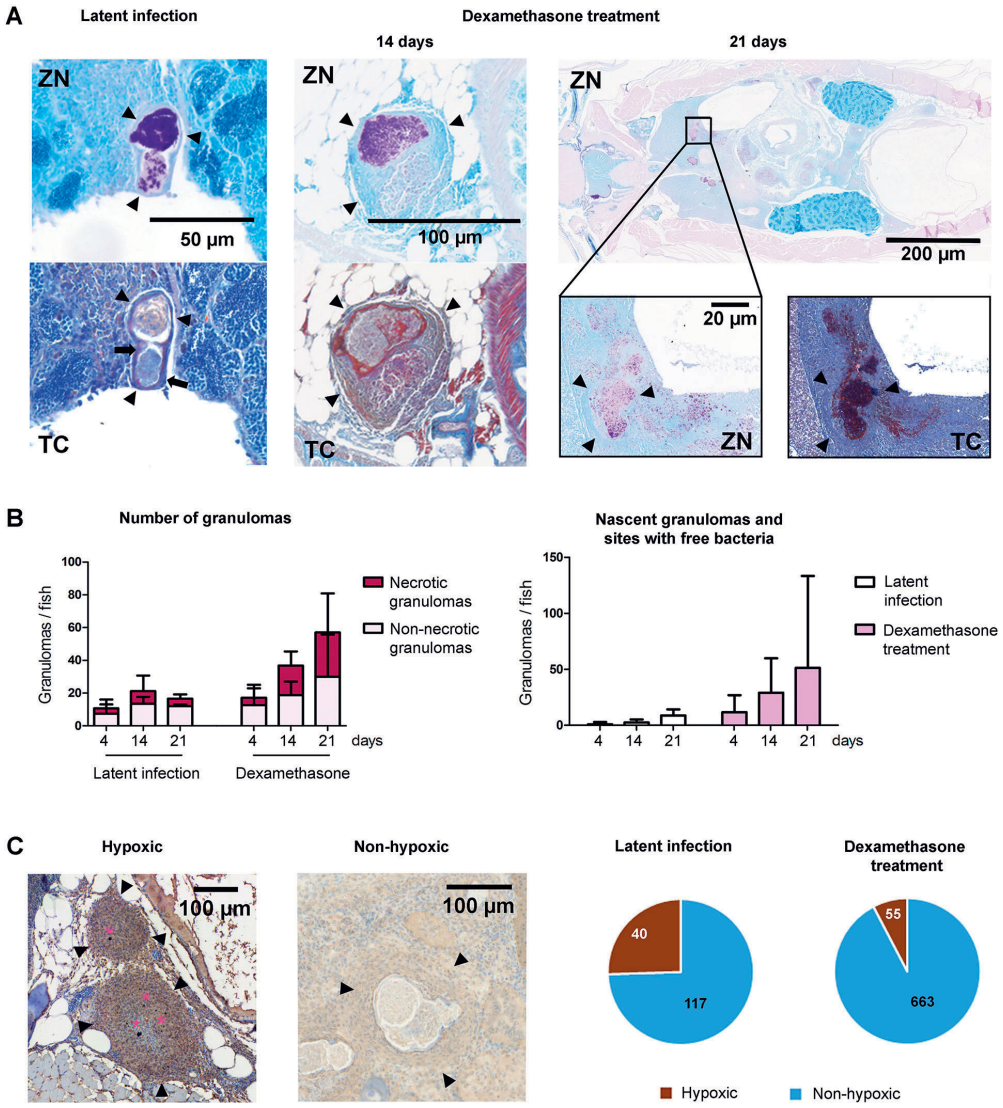

Fig. 2. Dexamethasone treatment leads to disruption of the granuloma structure, increase in the number of granulomas and loss of hypoxia in zebrafish with a latent mycobacterial infection. (A) Ziehl–Neelsen staining (ZN) of mycobacteria (purple). Black arrowheads indicate the outline of a granuloma. Trichrome staining (TC) of fibrous tissue around granulomas (blue) (black arrows). (B) Quantification of the total number of granulomas and the proportion of necrotic and non-necrotic granulomas (left), and the number of nascent granulomas and sites with non-capsulated mycobacteria (right) per fish after 4, 14 and 21 days of dexamethasone treatment or normal feeding (n=3-6 fish/group). Data are presented as mean±s.d. Statistical significance was assessed by unpaired Student's t-test. (C) Hypoxic staining shows hypoxic lesions inside granulomas in dark brown (pink stars). The proportions of hypoxic and non-hypoxic granulomas during a latent infection, and after 2 weeks of dexamethasone treatment, are quantified in the pie chart. See also Fig. S2.

surrounding fibrous layer. After 3 weeks of dexamethasone treatment, further loss of granuloma integrity was observed, together with bacteria escaping from the granulomas and spreading into tissues, indicating progression of the infection into an active state.

To quantify the dexamethasone-induced changes in the number and types of granulomas, we counted the total number of granulomas per fish at different time points during the treatment (4, 14 and 21 days, n=3-6) (Fig. 2B). In the control group, the number of granulomas remained relatively constant (average of 9-17 granulomas/fish), while in the dexamethasone-treated group, the number of granulomas increased from an average of 17 granulomas at day 4 to an average of 57 granulomas per fish at 21 days. To further characterize the process, we calculated the number of

necrotic (Fig. 2B) and multicentric granulomas separately (Fig. S2A-C). Both are subtypes of mature granulomas that contain a high number of mycobacteria, indicating an advanced stage of a mycobacterial disease (Parikka et al., 2012). We found that after 3 weeks of dexamethasone treatment, the number of necrotic granulomas had increased from an average of 4.5 at day 4 to 27 at day 21 (P=0.02, one-tailed Mann–Whitney test) (Fig. 2B), while there was also an increase in the number of multicentric granulomas (Fig. S2B) and the proportion of necrotic versus non-necrotic granulomas (Fig. S2C). Consistent with the qualitative analysis, there was also a clear increase in the number of nascent granulomas and sites with noncapsulated mycobacteria, from a mean of 11 granulomas or sites at day 4 to 51 at day 21 (Fig. 2B). This suggests that the treatment leads to the escape of mycobacteria from existing granulomas and the seeding of new ones.

Hypoxia has been shown to have an important role in mycobacterial pathogenesis. A low-oxygen environment induces metabolic adaptation in bacteria, which allows the bacteria to persist inside the host during a latent infection. To visualize hypoxic areas in granulomas, pimonidazole treatment and Hypoxyprobe staining were used (Fig. 2C) (Matty et al., 2015). Small hypoxic areas were found adjacent to 25% of the granulomas in the fish with a latent infection. Following a 21-day dexamethasone treatment, hypoxic lesions were observed in only 8% of all granulomas (P<0.0001, Fisher's exact test) (Fig. 2C).

Altogether, these data suggest that during the reactivation of a latent disease, mycobacteria first replicate within existing granulomas. This leads to bacteria escaping and forming new granulomas, as well as the appearance of free bacteria, which is associated with the disruption of the granuloma structure and the loss of hypoxia inside the granulomas.

Dexamethasone treatment leads to a decrease in the amount of lymphocytes

Next, we investigated the alterations caused by dexamethasone in immune cell populations in the kidney, which is the site of hematopoiesis in the adult zebrafish. For this, we first treated wild-type fish with dexamethasone for 1, 2 and 6 weeks and dissected their kidneys for a flow cytometric [fluorescence-activated cell sorting (FACS)] analysis. For the analysis, the cells in the live gate were separated into populations based on size [defined by the forward scatter (FSC)] and granularity [side scatter (SSC)]. These included lymphocytes, granulocytes and monocytes, and blood cell precursors. Upon dexamethasone treatment, the proportion of lymphocytes decreased from 19.3±3.5% to 12.4±1.6% (P<0.01, two-way ANOVA), while the relative amount of granulocytes and monocytes and blood cell precursors remained unchanged (Fig. S3). To study the effect of dexamethasone treatment on lymphocytes in more detail, we used the Tg:lck(*lck-EGFP*) fish line, which expresses *GFP* under the tyrosine kinase promoter specific for mature T cells, NK-like cells and myeloid-like cells in the kidney of adult zebrafish (Langenau et al., 2004; Carmona et al., 2017). *lck-GFP* fish were treated with dexamethasone for 1, 2 and 4 weeks, followed by FACS analysis of the kidney cell populations as above (Fig. 3; Fig. S4B). As expected, the GFP^+ cell population mainly fell into the lymphocyte gate (Fig. 3A; Fig. S4B,C) (Langenau et al., 2004). Again, upon dexamethasone treatment, the proportion of lymphocytes decreased from 30.1±4.0% to 18.2±3.1% (P<0.001, two-way ANOVA), while there was a slightly increasing trend in the relative amount of granulocytes and monocytes and blood cell precursors (Fig. 3B,C; Fig. S4B,C). More specifically, the proportion of GFP^+ lymphocytes decreased during the first week of dexamethasone treatment, from the original 12.5±3.4% of live cells in untreated fish to 6.3±2.4% (P<0.001, two-way ANOVA), after which it remained the same throughout the treatment (Fig. 3D). In addition, the GFP^- lymphocyte population, which contains B cells and immature T cells (Langenau et al., 2004), decreased from 18.5±2.8% to 10.3±1.7% of live cells (P<0.001, two-way ANOVA) after 4 weeks of treatment (Fig. 3D). The proportion of GFP^+ lymphocytes compared to GFP^- ones decreased slightly at the 1- and 2-week time points, but by 4 weeks, the GFP^+: GFP^- ratio had returned to the same as that in the control group (40.2% and 43.0% of GFP^+ lymphocytes) (Fig. S4D).

Dexamethasone treatment alters the expression of T cell markers upon infection

To further characterize the effects of dexamethasone treatment on the immune response against mycobacteria, we compared the kidney lymphocyte population of uninfected and infected wild-type zebrafish after 1 week of treatment with dexamethasone (Fig. 4; Fig. S5). A latent mycobacterial infection led to an increase in the lymphocyte population (from 21.7±4.5% to 28.3±7.3%; P<0.01, two-way ANOVA with Bonferroni posttest). Treating fish with a latent infection with dexamethasone led to a decrease in the proportion of lymphocytes, to the level seen in uninfected fish (24.6±3.2%, P<0.05, two-way ANOVA with Bonferroni posttest) (Fig. 4A).

To investigate the effect of dexamethasone on lymphocyte function, we studied the expression levels of marker genes of different types of T and B cells from the isolated kidney cells using quantitative reverse transcription PCR (qRT-PCR) (Fig. 4B-G; Fig. S5B-G). Of the studied inflammation markers, both *tnfa* and *interferon gamma 1-2* (*ifng1-2*; *ifng-1*) were induced in the fish carrying a latent infection, with the former being slightly upregulated (Fig. S5B), and the latter slightly downregulated (Fig. 4B), by dexamethasone. The expression of *tnfa* correlated with the bacterial burden (Spearman r 0.8180, P<0.0001), which likely explains its relative elevation in the dexamethasone-treated individuals, whereas no such correlation was observed for *ifng1-2* expression (r –0.1740).

Zebrafish have $CD4^+$ and $CD8^+$ lymphocyte populations with functions similar to those in humans (Langenau et al., 2004; Yoon et al., 2015; Renshaw and Trede, 2012). Consistently, the expression of both *cd4-1* and *cd4-2.1* was induced upon infection (P=0.004 and P=0.0002, respectively), whereas their expression was reduced following dexamethasone treatment: *cd4-1* was suppressed following reactivation (P=0.0002) (Fig. 4C), and *cd4-2.1* was reduced in both uninfected (P=0.02) and infected (P=0.088) fish (Fig. 4D). Expression of *cd8a*, a marker gene for cytotoxic T cells, was induced in infected fish (P<0.0001), but its expression was not altered by the dexamethasone treatment (Fig. 4E).

The balance between the $CD4^+$ Th1 and Th2 cells has been shown to play a role in the control of a mycobacterial infection (Hammarén et al., 2014). To study the $CD4^+$ subpopulation more specifically, we used the expression of the *tbx21* transcription factor as a marker for the Th1 population, and the transcription factor *gata3* and *il4*, *il10* and *il13* as markers for Th2 cells. *tbx21* expression was induced upon infection (P<0.0001) (Fig. 4F), as was the expression of *il10* (P=0.004) (Fig. S5C), while the expression of the other Th2 markers tested remained relatively constant (Fig. S5D,E). Dexamethasone treatment suppressed the expression of *tbx21* in uninfected fish (P=0.052) and in fish with a latent infection (P=0.02) (Fig. 4F). Of the Th2 markers, a decreasing expression for *gata3* was seen after dexamethasone treatment in

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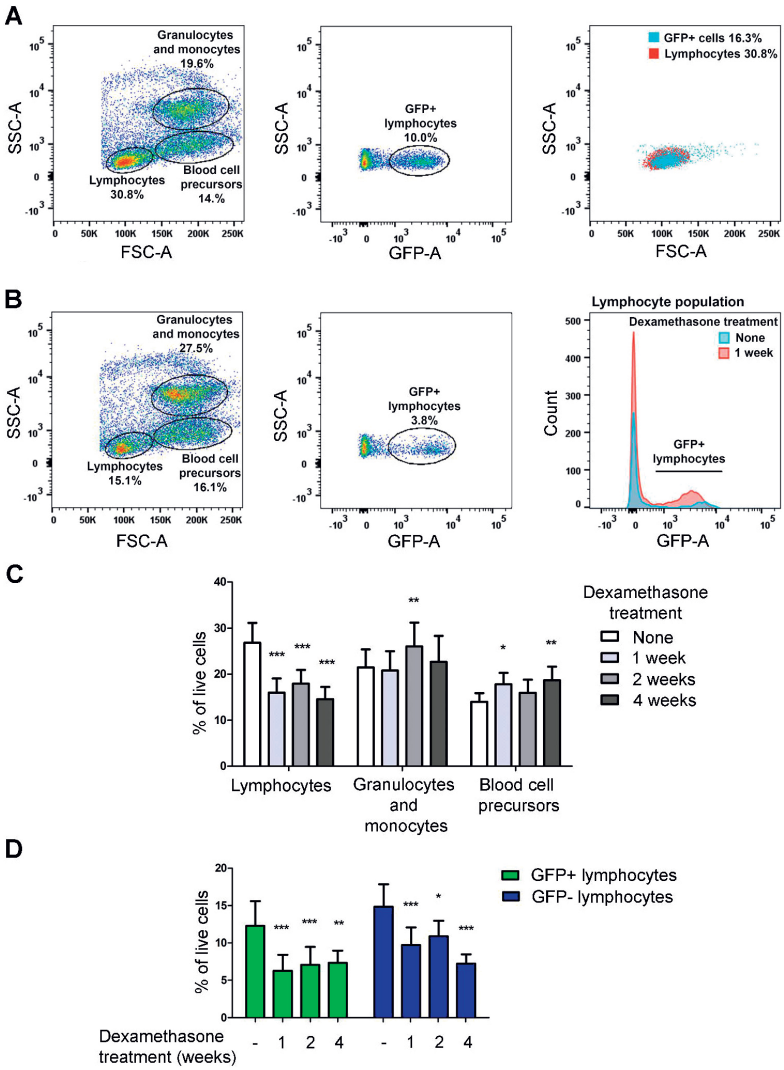

Fig. 3. Dexamethasone treatment leads to a depletion of lymphocytes in the adult zebrafish. (A) Sorting of the kidney cell populations of *lck-GFP* fish based on size (side scatter, SSC-A) and granularity (forward scatter, FSC-A) (left). The lymphocyte population is further separated into GFP$^+$ and GFP$^-$ subpopulations (middle), and the majority of the GFP$^-$ cells fall into the lymphocyte gate by the SSC-A and FSC-A. (B) After a 1-week dexamethasone treatment, the kidney cells fall into the same gates, but the number of both GFP$^+$ and GFP$^-$ lymphocytes is depleted (right), as quantified in C and D. n=12. Data are presented as mean±s.d. Statistical significance is analyzed by two-way ANOVA with Bonferroni posttest, *P<0.05, **P<0.01, ***P<0.001. Also see Figs S3 and S4.

uninfected fish (P=0.03) and a corresponding trend was observed in infected fish (Fig. 4G). The expression of *IgM* (*ighm*), which was used as a marker for B cells, remained relatively constant in both infected and dexamethasone-treated fish (Fig. S5F), whereas *tgfb1b*, a marker for regulatory T cells (Tregs), was suppressed upon infection in the immunocompetent fish (P=0.0007), and upregulated in the dexamethasone-treated group (P=0.03) (Fig. S5G). However, the expression of *foxp3a*, a transcription factor characteristic for Tregs was not affected by dexamethasone treatment (Fig. S4H).

Overall, these data indicate that a dexamethasone treatment leads to the general depletion of lymphocytes. The most prominent effect is seen in the expression of *cd4-1*, *cd4-2.1* and the Th1 marker *tbx21*. Although dexamethasone-treated fish are able to induce an IFN-γ response against mycobacteria, the suppression of specific T cell populations, together with the induction of the inhibitory cytokine *tgfb1b*, are associated with compromised ability to control infection.

The zebrafish model can be utilized to assess the efficacy of drugs at different phases of a mycobacterial infection

Owing to their small size and fast reproduction rate, zebrafish are well suited for large-scale pharmaceutical screening studies (Lohi et al., 2013). On this premise, we investigated whether the dexamethasone-based reactivation model can be used for testing the effectiveness of antimicrobial medicines against reactivated mycobacteria.

We chose four different antimicrobial drugs that have been used for treating mycobacterial diseases, including ethambutol, isoniazid, amikacin and metronidazole. Each of the antibiotics

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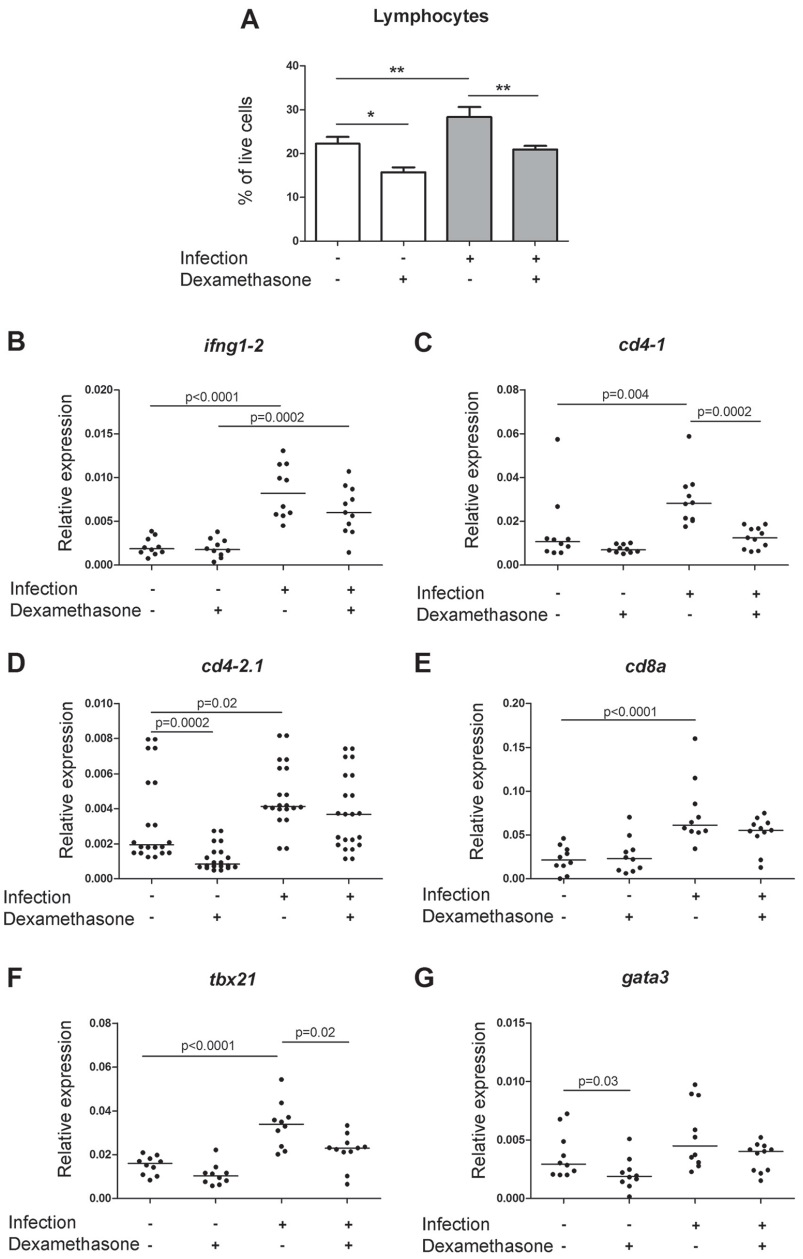

Fig. 4. Dexamethasone treatment decreases the expression of CD4$^+$ T lymphocyte markers in uninfected and infected zebrafish. (A) Lymphocytes are expanded in the zebrafish kidney upon a latent *M. marinum* infection and depleted by dexamethasone treatment. The kidney cell populations of AB fish were analyzed with FACS. Bars show the mean percentage of lymphocytes±s.d. *P*-values were calculated using two-way ANOVA with Bonferroni posttest, **P*<0.05, ***P*<0.01, ****P*<0.001. (B-G) Relative expression levels of inflammatory genes and T cell markers. Each dot represents the expression level of the marker gene relative to the housekeeping gene (*EF1a*). Horizontal lines show the median value in each group. *n*=10-11 fish/group. *P*-values were calculated using the two-tailed Mann–Whitney test. (B) *ifng1-2*, (C) *cd4-1*, (D) *cd4-2.1*, (E) *cd8a*, (F) *tbx21*, (G) *gata3*. See also Fig. S5.

was first tested for their effect against *M. marinum in vitro* using a bioluminescent strain, which allowed the quantification of bacterial growth at different time points with a luminometer. Two different doses were tested for each antimicrobial drug. During the 7-day follow-up period, all antibiotics, excluding isoniazid, inhibited the growth of *M. marinum* in a dose-dependent manner, compared to bacteria cultured in medium without antibiotics (Fig. 5A). Higher doses of ethambutol (2.4 µg/ml), and amikacin (45.5 µg/ml), completely prevented bacterial growth (*P*<0.05, respectively, one-way ANOVA with Friedman's test).

Next, zebrafish with a latent *M. marinum* infection were treated for 3 weeks with dexamethasone, followed by 4 weeks of treatment with antibiotics (50 µg/fish/day each). Metronidazole (160 µg/ml),

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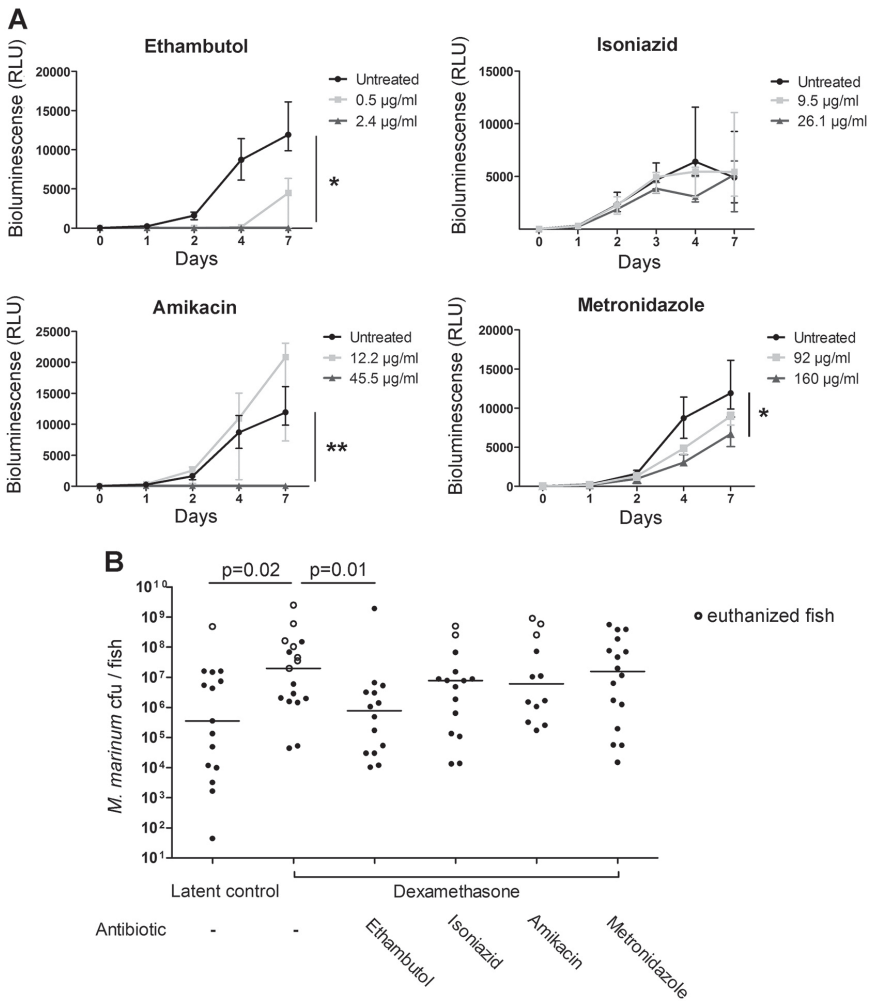

Fig. 5. Ethambutol inhibits the growth of *M. marinum* both *in vitro* and upon dexamethasone treatment *in vivo*. (A) The effects of selected antibiotics on the growth of a bioluminescent strain of *M. marinum in vitro*. The graphs represent the detected relative bioluminescence (RLU) of bacteria at the indicated time points after exposing them to an antibiotic. n=5-6. One-way ANOVA with Friedman's test and Dunn's multiple comparison test was used for statistical analysis, *P<0.05, **P<0.01. (B) Adult zebrafish with a latent mycobacterial infection were treated for 3 weeks with dexamethasone (10 µg/fish/day) followed by 4 weeks of treatment with selected antibiotics. Each dot represents the bacterial burden per fish. Horizontal lines show the median value of each group. The statistical analysis was performed with the two-tailed Mann–Whitney test.

which is designed to target anaerobic microbes, inhibited the growth of *M. marinum in vitro* by 48% compared to controls, but did not limit the bacterial burden *in vivo* in a latent (Fig. S6A) or reactivated infection (Fig. 5B).

However, ethambutol decreased the median bacterial counts in dexamethasone-treated zebrafish by 96% compared to untreated controls (P=0.01, Mann–Whitney test), which is close to the level seen in a latent infection (Fig. 5B). Of note, a 2-week treatment with ethambutol did not affect bacterial counts in zebrafish with a latent *M. marinum* infection (Fig. S6B), consistent with the knowledge that ethambutol acts by preventing the replication of mycobacteria (Forbes et al., 1962). This further demonstrates that dexamethasone treatment causes the reactivation of a latent mycobacterial infection in zebrafish, leading to the active replication of the bacteria and susceptibility to ethambutol.

Identification of novel protective postexposure vaccine antigens against the reactivation of TB in the zebrafish model

We have previously shown that the zebrafish-*M. marinum* infection model is suitable for the preclinical screening of novel vaccine candidates against a mycobacterial infection (Oksanen et al., 2013, 2016). The zebrafish can be partially

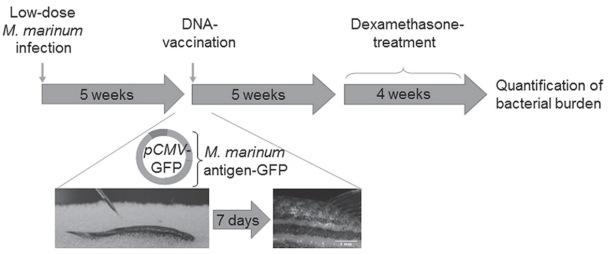

Fig. 6. Schematic representation of the experimental protocol for testing vaccine candidates against the reactivation of a latent mycobacterial infection. Fish with a latent *M. marinum* infection are immunized with a DNA plasmid carrying a GFP-tagged mycobacterial antigen, which allows detection of successful immunizations by fluorescent microscopy. After 5 weeks, the fish are subjected to dexamethasone treatment (10 µg/fish/day) for 4 weeks, followed by determination of bacterial burdens by qPCR.

protected against a primary *M. marinum* infection by BCG, or by prophylactic DNA-based vaccines consisting of a combination of previously studied mycobacterial antigens (Ag85 and ESAT-6), as well as with some novel antigens (Myllymäki et al., 2017).

Based on this, we studied the applicability of the dexamethasone-based reactivation model for the preclinical screening of new DNA-based vaccines by examining the therapeutic effect of the selected mycobacterial antigens against the reactivation of a latent *M. marinum* infection. The zebrafish were first infected with a low dose of *M. marinum*, and vaccinated 5 weeks postinfection. Four weeks after the vaccinations, the fish were subjected to dexamethasone treatment (10 µg/fish/day) for 4 weeks, after which their bacterial burdens were quantified by qPCR (the protocol is outlined in Fig. 6). The antigens showing a significant reduction in bacterial counts in the initial screen were subjected to a replicate experiment to confirm the effect. We first tested the effect of the Ag85-ESAT-6 antigen combination against reactivation, and found that this immunization decreased the bacterial counts by 56% or 0.35 $\log_{10}$ (P=0.02, two-tailed Mann–Whitney test) compared to the control group immunized with an empty *GFP* plasmid (Fig. 7).

The other antigens tested involved genes from different functional categories, including the four *M. marinum* Resuscitation promoting factors (Rpf proteins), three members of the PE/PPE protein family, four conserved membrane proteins, one secreted factor and three metabolic enzymes. Compared to the GFP-immunized control group, most of these did not affect the bacterial burden of the fish upon reactivation. Noteworthy, immunization with the antigens consisting of RpfB and MMAR_4207, led to a significant reduction in bacterial counts (by 63% or 0.53 $\log_{10}$ and 85% or 0.81 $\log_{10}$, respectively) (Fig. 7).

Altogether, these data show that the dexamethasone-based reactivation model provides an amenable tool for the preclinical screening of both novel antimicrobials and therapeutic vaccines against the reactivation of TB. For the latter, we identified two promising candidates, RpfB and the conserved membrane protein MMAR_4207.

DISCUSSION

TB remains a major health problem, and new antimicrobial medicines and vaccines are needed to prevent new infections and to prevent the reactivation of a latent TB infection (WHO, 2017). During the past decade, the zebrafish, with its natural pathogen *M. marinum*, has become a powerful model for studying the mechanisms associated with mycobacterial defense as well as new therapeutic strategies to fight mycobacterial infections. Some of the results have been already translated into combatting human TB infections (Adams et al., 2014; Tobin et al., 2013, 2012; Oehlers et al., 2017). In the current study, we utilized the adult zebrafish-*M. marinum* model to establish a tool for studying the control and reactivation of latent mycobacterial infections. This was achieved by the administration of the glucocorticoid dexamethasone in the fish food. In humans, glucocorticoids are used as immunosuppressants to treat various diseases, such as arthritis, and long-term use at a high dose has been associated with the reactivation of a latent TB infection, but the exact mechanisms underlying the reactivation are poorly understood (Jick et al., 2006).

TB remains one of the major killers of HIV-coinfected individuals: people with HIV are 20-30 times more likely to develop active TB; and there were 0.4 million of such deaths in 2015 (WHO, 2017). In humans and macaques, an HIV or a Simian immunodeficiency virus (SIV) infection is associated with the loss of $CD4^+$ T cells, which has long been thought to be the main factor promoting the reactivation of a latent TB infection (Barnes et al., 1991; Diedrich et al., 2010). This is also supported by data from the mouse model (Kupz et al., 2016a,b). Using the adult zebrafish, we observed a depletion of the lymphocyte population by dexamethasone treatment in uninfected fish, and a failure to induce lymphocyte expansion in infected fish. This is consistent with the depletion of T cells by dexamethasone seen in zebrafish larvae (Langenau et al., 2004). Moreover, our results suggest that dexamethasone, in particular, decreases the amount of conventional $CD4^+$ lymphocytes, including both Th1 and Th2 cells, whereas the expression of *tgf-b1b*, induced by the immunosuppressive Treg cell population, is upregulated. Dexamethasone has been shown to alleviate allergic symptoms in mice by upregulation of Treg cells (Zhang et al., 2016), but the exact role of these cells and the associated cytokines in the control of mycobacterial infections remains to be studied.

Considering the functional similarities between human and zebrafish $CD4^+$ and $CD8^+$ T cells (reviewed in Renshaw and Trede, 2012), $CD4^+$ cells could be a key player in controlling a latent mycobacterial infection also in the zebrafish. However, the susceptibility to the reactivation of a latent TB varies even among HIV-coinfected individuals, and in a macaque model, $CD8^+$ and B cells have been shown to elicit a protective effect in some of the coinfected individuals (Foreman et al., 2016). In the zebrafish, the expression levels of *cd8a* and the B cell marker *IgM* remained constant during the dexamethasone treatment, suggesting that these cells are left unaffected by the treatment. Furthermore, the individual zebrafish within a single line have been shown to differ in their ability to control a mycobacterial infection, probably due to genetic heterogeneity, leading to differences in the immune response (Hammarén et al., 2014). We also observed that some fish managed to maintain a relatively low mycobacterial count despite the dexamethasone treatment, which could be explained by the protective effect of CD8 and/or B cells, mimicking the situation in humans and macaques (Foreman et al., 2016; van Meijgaarden et al., 2015). The number of granulocytes and monocytes was not decreased by dexamethasone. Macrophages and neutrophils are needed for the formation of granulomas and

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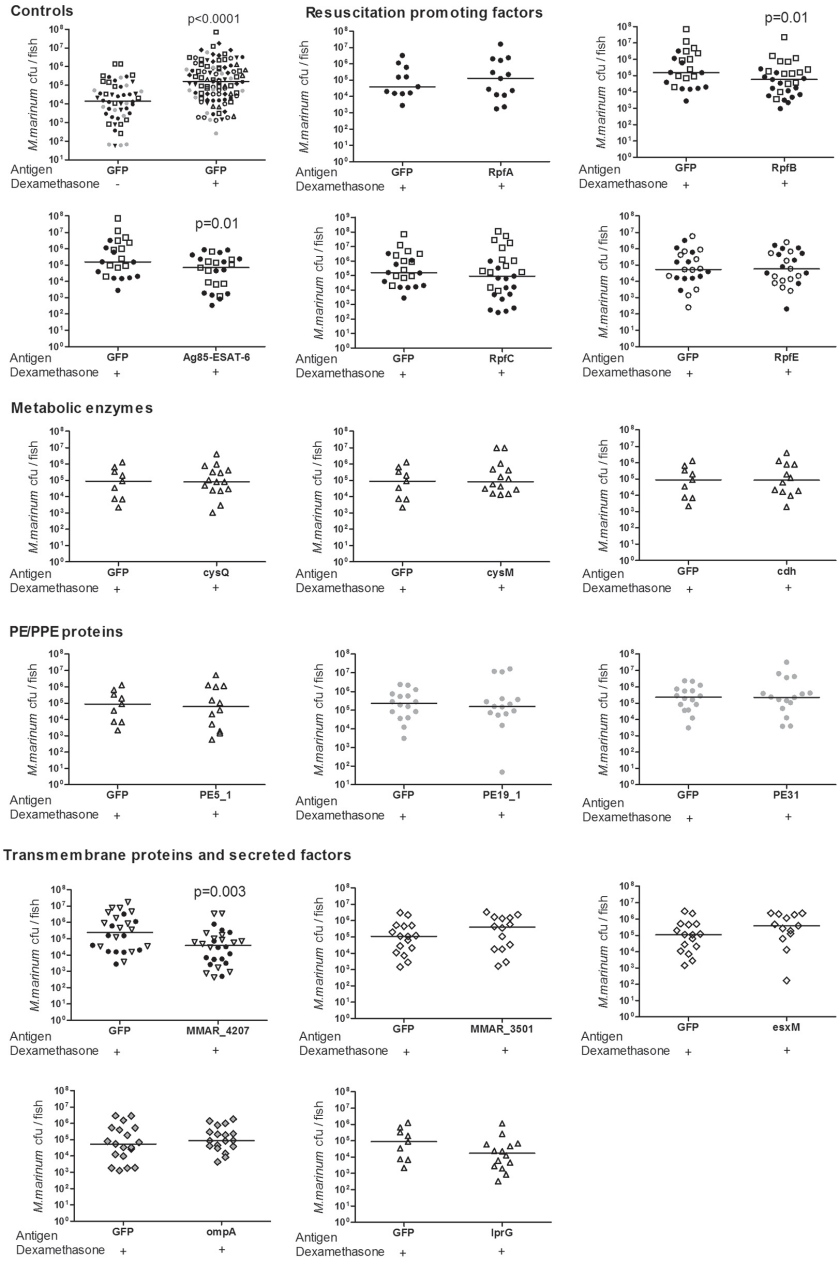

Fig. 7. Ag85-ESAT-6, RpfB and MMAR_4207 antigens decrease bacterial burdens upon reactivation of a latent *M. marinum* infection in the adult zebrafish. Fish with a latent *M. marinum* infection were immunized with the GFP control or experimental vaccines and 5 weeks later treated for 4 weeks with dexamethasone. Each dot represents the bacterial load in one fish. Horizontal lines show the median values of pooled experiments (n=11-29 fish/group). Results from different experiments are marked with different symbols. A two-tailed Mann–Whitney test was used to calculate P-values.

for the early control of a mycobacterial infection (Davis et al., 2002; Yang et al., 2012; Davis and Ramakrishnan, 2009). Although treatment with dexamethasone prior to an infection does not increase susceptibility to mycobacteriosis, but rather compromises the control of a chronic infection, confirming that dexamethasone does not affect the properties of macrophages or

neutrophils would require functional assays. In the infected, dexamethasone-treated fish, we observed a strong negative correlation (Spearman r –0.88, P=0.0007) between the bacterial burden and the relative amount of macrophages and neutrophils in the kidney, but it remains elusive whether there is any causal relationship in either direction.

In adult zebrafish, granuloma structures reflect the stage of the disease (Parikka et al., 2012), and, similar to observations in macaques, rabbits and guinea pig, hypoxia is observed in granulomas in adult zebrafish with a latent *M. marinum* infection, mainly in the cells adjacent to necrotic areas (Via et al., 2008; Oehlers et al., 2015). Upon reactivation, we observed loss of hypoxia, which was associated with increasing granuloma counts, their necrosis and multicentricity, and disruption of the surrounding fibrous capsule, together with the emergence of disseminated bacteria at several sites within tissues and the formation of nascent granulomas. The latter are normally associated with a recent infection that has not yet reached a latent stage (Parikka et al., 2012), but, in the context of dexamethasone treatment, their appearance likely indicates progression of the infection from a latent state into an active one. As the mechanisms that trigger the exit of mycobacteria from the granuloma remain largely elusive (Boggiano et al., 2017), this model provides a feasible tool for examining the role of different immune cells in the control of mycobacterial infections.

Owing to their cell wall properties and ability to persist inside granuloma structures, mycobacteria have been a difficult pathogen to target by current antibiotics (Peddireddy et al., 2017). Multidrug-resistant strains present a further challenge for treatments, and new antimicrobial drugs are therefore urgently needed (WHO, 2017). We observed that ethambutol effectively prevented the growth of *M. marinum in vitro*, and after dexamethasone treatment, mycobacteria could be successfully targeted *in vivo* with ethambutol, whereas a 2-week treatment had no effect on a latent infection. Of the other antibiotics tested in this study, a larger dose of amikacin could inhibit mycobacterial growth *in vitro*, thus a larger dose could improve the result *in vivo* as well. Metronidazole is designed to exhibit its antibacterial activity under anaerobic conditions. It has been shown to prevent the reactivation of latent TB in the macaque model (Lin et al., 2012), but not in the guinea pig (Hoff et al., 2008), and the human trial was discontinued owing to neurotoxicity (Carroll et al., 2013). In our hands, metronidazole inhibited the growth of *M. marinum in vitro* by ∼50%, but the tested dose was ineffective against both a latent and a reactivated infection in zebrafish. This could be due to a lack of anaerobiosis (despite the presence of hypoxic lesions) in the zebrafish granulomas.

In previous studies, we showed that the zebrafish-*M. marinum* infection model is feasible for use in the preclinical screening of vaccines against a mycobacterial infection (Oksanen et al., 2013; Myllymäki et al., 2017). This was also assessed in the current study in the context of preventing reactivation of a latent mycobacterial disease. We screened the effectiveness of an antigen combination Ag85B-ESAT-6, which has been studied in HIV-positive Mtb carriers (Reither et al., 2014), together with 15 novel conserved mycobacterial antigens. We found that the Ag85B-ESAT-6 combination was able to improve the control of dexamethasone-activated infection, reducing the median bacterial burden by 85%. Importantly, two of the tested novel mycobacterial antigens, namely RpfB and MMAR_4207, significantly decreased the bacterial burden in the dexamethasone-treated fish. Their protective effect is comparable to the effect of the BCG vaccination in a primary *M. marinum* infection in the zebrafish (82% or 0.76 $\log_{10}$ reduction in bacterial burden) (Oksanen et al., 2016), or an *M. tuberculosis* infection in mice (0.35-1.2 $\log_{10}$ reduction in bacterial counts) (Jeon et al., 2008). As its name implies, RpfB is thought to promote the resuscitation and growth of dormant, nongrowing cells, and the potential of the Rpf proteins as vaccine antigens has been studied to some extent (Romano et al., 2012); thus, the promising result is not completely unexpected. MMAR_4207 is a conserved membrane protein with an unknown function, which makes this antigen an interesting novel candidate for further studies. In our previous study, these antigens did not affect the bacterial burden as prophylactic vaccines in a primary infection assay (Myllymäki et al., 2017), suggesting that they would more effectively target mycobacteria upon reactivation. However, it is also possible that these vaccines reduce the bacterial load during the latent phase of infection before the immunosuppression takes place. Therefore, determining the exact mechanism of their action requires further studies. Vaccine candidates that elicit a strong CD4-based response generally provide a rather low level of protection (Tameris et al., 2013; Sakai et al., 2016), and, recently, other protection mechanisms, such as the $CD8^+$ and B cells and the humoral responses, have also received attention (Achkar et al., 2015; van Meijgaarden et al., 2015; Kamath et al., 2006). A model system, where the CD4 arm of immunity is hampered, could provide an interesting means to explore these. Moreover, a therapeutic vaccine that would improve the control of a latent TB, also in the immunocompromised, would be a great asset in the battle against TB. Based on the results of this study, the zebrafish model can provide a time- and cost-effective tool for the early screening of such vaccine candidates, followed by studies in larger animal models, such as macaque SIV-TB coinfection (Diedrich et al., 2010; Foreman et al., 2016; Capuano et al., 2003).

In conclusion, the results of this study show that a latent *M. marinum* infection can be reactivated with dexamethasone treatment in the adult zebrafish. We used this method to characterize the mechanisms associated with the process and found that in zebrafish, like in humans, the depletion of lymphocytes, especially the $CD4^+$ T cell population, is associated with the impaired control of a latent mycobacterial infection. This method also provides a tool for screening the effectiveness of novel antimicrobial drugs and vaccines against the reactivation of a latent mycobacterial infection. Importantly, we identified two novel mycobacterial antigens – RpfB and MMAR_4207 – that show protection against the reactivation of a latent mycobacterial infection as postexposure DNA vaccines.

MATERIALS AND METHODS

Zebrafish

In the experiments, 5- to 9-month-old, wild-type AB zebrafish (*Danio rerio*) from the Tampere Zebrafish Core Facility were used. In addition, for the flow cytometry, adult Tg:lck(*lck-EGFP*) fish [obtained from the Zebrafish International Resource Center (ZIRC), University of Oregon, USA] were used. Fish were maintained in a 10 h/14 h light/dark cycle, in a flow-through salt water system at 28°C and were fed twice daily. The Animal Experiment Board in Finland approved all animal experiments (ESAVI/8125/04.10.07/2013) and studies were carried out in accordance with the EU directive 2010/63/EU on the protection of animals used for scientific purposes. Humane endpoint criteria were applied throughout all experiments, the wellbeing of the fish was monitored carefully, and any fish showing signs of illness or discomfort were immediately euthanized with 0.04% 3-aminobenzoic acid ethyl ester pH 7.0 (A5040, Sigma-Aldrich, St Louis, MO, USA).

Experimental infections

The *M. marinum* strain ATCC 927 was used for the infections. The culturing of bacteria and infections were carried out as described earlier (Parikka et al., 2012), except that bacteria were diluted with sterile 0.9× phosphate buffered

saline (PBS) and 0.03 mg/ml Phenol Red before the infections. The infection dose was 33±21 cfu/fish in all experiments, and was confirmed by plating on 7H10 Middlebrook OACD agar plates (BD Biosciences, Franklin Lakes, NJ, USA).

Preparation and administration of medicine food

Immunosuppressive agents and antibiotics were administrated per os in the fish food. The gelatin-coated fish food contained 2.5 mg/g of the immunosuppressant or 12.5 mg/g of the antibiotics. Bovine gelatin (G9391, Sigma-Aldrich; 400 mg) was diluted in 5 ml distilled water by heating (45°C). Then, 25 mg of each immunosuppressant (azathioprine A4638, dexamethasone D4902, prednisolone P6004 and methylprednisolone M1755200, Sigma-Aldrich) or 125 mg of antibiotic (ethambutol E4630, metronidazole M3761, isoniazid I3377 and amikacin A0368000, Sigma-Aldrich) was mixed with 2 ml of 70% EtOH and the suspension was combined with the gelatin solution. The gelatin mixture was spread evenly on 10 g of dry fish food pellets (SDS400, Special Diet Services, Essex, UK) and air dried overnight. The food was weighed and homogenized using a mortar and a pestle. The increase in the weight of the food, from the added gelatin and medicines, was taken in account when the daily doses of prepared food were calculated and weighed, so that each fish received 4 mg of food containing the desired dose of the drug. During the reactivation treatments, the fish were fed once daily. The water circulation was stopped during the feeding to avoid the spreading of chemical-containing food into other tanks of the unit.

Histology

For histological staining, fish with a latent infection were treated with dexamethasone (10 µg/fish/day) and samples were collected after 4, 14 and 21 days of treatment (n=3-6 fish/group). Untreated groups of fish were collected as controls at each time point. The fish were euthanized with 0.04% 3-aminobenzoic acid ethyl ester (A5040, Sigma-Aldrich) and fixed in 10% phosphate buffered formalin (Oy FF-Chemicals Ab, Haukipudas, Finland) for 7 days. Decalcification, embedding into paraffin and the cutting of sections were performed according to Parikka et al. (2012). Two parallel 5-µm sections were collected every 200 µm on two SuperFrost®Plus glasses (Thermo Fisher Scientific), deparaffinized and used for either Ziehl–Neelsen or trichrome staining. Ziehl–Neelsen staining was performed as described in Parikka et al. (2012). The trichrome staining protocol was a modified version from Mallory's trichrome staining; before treatment with acid fuchsin, the sections were incubated in Bouin's solution (HT10132, Sigma-Aldrich) overnight at room temperature. All slides were scanned with an Olympus BX43 microscope and analyzed with the JPEG2000 virtual slide and ImageJ software. Different types of granulomas (necrotic, multicentric or nascent) and disseminated bacteria were classified according to examples shown in Fig. S2A. Granulomas were counted manually from each section to determine the total number of granulomas in each fish (Oksanen et al., 2013).

Hypoxic lesions in granulomas were visualized with the HypoxyProbe-1 kit (HP1-100Kit, Hypoxyprobe, Burlington, MA, USA) in fish with a latent infection and after 3 weeks of reactivation with dexamethasone. Fish were anesthetized with 0.02% 3-aminobenzoic acid ethyl ester and intraperitoneally injected with pimonidazole hydrochloride (60 µg/fish), which forms covalent adducts with sulphydryl groups within protein structures under hypoxic conditions. The fish were transferred to fresh water for 5-10 min before euthanasia and histological sections were prepared as described above. The staining followed the manufacturer's instructions with some modifications. Glasses were first deparaffinized and boiled in a 10 mM sodium citrate buffer with 0.05% Tween (pH 9) at 98°C for 15 min to retrieve antigens. To block endogenous peroxidase activity, glasses were treated for 5 min with 3% hydrogen peroxidase (88597, Sigma-Aldrich) before a 1-h incubation with the primary antibody, a 1:600 dilution of Hyproxyprobe-1 Mab1 (Batch No. 5.23.15). The secondary antibody, universal immuno-enzyme polymer-peroxidase conjugate (mouse) (414131F, Nichirei Biosciences Inc., Tokyo, Japan), was incubated for 30 min. For the staining reaction, 15 µl/ml of the chromogen (ImmPACT DAP chromogen, Vector Laboratories, Burlingame, CA, USA) was mixed with ImmDACT™ DAB (Vector Laboratories), and incubated for 5 min. Mayer's Hematoxylin solution (Reagena, Toivala, Finland) was used as a counterstain for 2 min. TBS with 0.05% Tween buffer was used for all washing steps and the staining was performed with a Autostainer 480 machine (Lab Vision, Thermo Fisher Scientific). Finally, glasses were dehydrated within increasing alcohol series to xylene and embedded with Coverquick 2000 (VWR Chemicals, Leuven, Belgium), and scanned as described above. The total number of hypoxic and nonhypoxic granulomas was counted from both groups (n=2 fish/group).

FACS

For the FACS analysis, wild-type AB fish and Tg:lck(*lck-EGFP*) fish were fed with dexamethasone-containing food for the indicated times, together with an untreated control group (12 fish/group); and latently infected and uninfected AB fish were treated with dexamethasone or normal fish food for 1 week. Fish were euthanized with 0.04% 3-aminobenzoic acid ethyl and their kidneys were dissected in 500 µl of 0.5% fetal bovine serum (Sigma-Aldrich) in PBS on ice. Tissues were homogenized and filtered through a 35 µm filter cap by centrifugation at 200 ***g*** for 1 min. Cell sorting was performed with a FACSCanto II device (Becton Dickinson Biosciences, San Jose, CA, USA) and with the FACSDiva software (Becton Dickinson Biosciences). The samples were run at medium speed (1000 events/s) and 20,000 events were recorded for each sample, and used for the analysis of different cell populations (lymphocytes, blood cell precursors, granulocytes and monocytes) based on GFP expression, granularity and size with FlowJo software.

qPCR and qRT-PCR

Bacterial counts for each fish were obtained with qPCR (SensiFAST™ SYBR® No-ROX kit, Biolane Reagents Ltd, UK). The contents of the intraperitoneal cavity from each fish was used for DNA extractions and the Tri-reagent was used for the isolations as described previously (Parikka et al., 2012). In qPCR, primers specific for the *M. marinum* internal transcribed spacer forward (5′-CACCACGAGAAACACTCCAA-3′) and reverse (5′-ACATCCCGAAACCAACAGAG-3′) were used. To exclude the possibility of a background *M. marinum* infection, a group of uninfected fish treated with dexamethasone was also subjected to the qPRC analysis.

For the expression analysis of cytokines and T cell markers, whole kidneys were dissected and used for RNA isolation with the RNeasy Mini Kit (Qiagen). RNA samples were purified with the RapidOut DNase Removal kit (Thermo Fischer Scientific) and then used for qRT-PCR (SensiFAST™ SYBR No-ROX Kit, Biolane Reagents Ltd), according to the manufacturer's instructions. Expression levels of cytokines were normalized to the expression level of *elongation factor 1-alpha* (*EFa1*; *eef1a1/1*) in each sample. The primers used are shown in Table 1. The qPCR was run with a Bio-Rad CFX96™ thermal cycler, and the data were analyzed with the Bio-Rad CFX96™ Real Time System software. Excluding the kidney, the rest of the internal organs were used for DNA extraction and determining the bacterial counts as above.

In vitro antibiotic testing with a bioluminescent *M. marinum*

To test the effectiveness of antimicrobial drugs *in vitro*, we exposed a bioluminescent *M. marinum* strain, ATCC BAA535, with the pMV306 plasmid (Addgene plasmid 26161), containing the LuxABDE cassette and kanamycin resistance (Andreu et al., 2010), to different concentrations of antibiotics (ethambutol E4630, metronidazole M3761, isoniazid I3377 and amikacin A0368000, Sigma-Aldrich) and measured the light emitted by the bacteria with a 2014 EnVision Multilabel Reader (PerkinElmer, Waltham, MA, USA). Antibiotic concentrations were selected based on the literature (de Steenwinkel et al., 2010). For each tested concentration, six replicate samples were prepared in a white 96-well plate; in each well the desired antibiotic dilution was added to 200 µl of a bacterial dilution (500 cfu/µl). Water was used as a negative control. Mycobacteria were incubated at 29°C for 7 days and the bioluminescence was measured on days 0, 1, 2, 3, 4 and 7. The results were analyzed with EnVision Workstation 1.12 (PerkinElmer).

DNA vaccinations

DNA vaccine constructs were prepared as in former studies (Oksanen et al., 2013; Myllymäki et al., 2017). Briefly, antigens were cloned into plasmids (pCMV-*EGFP*, Addgene plasmid 11153) and transformed into chemically

Table 1. Primers used in qRT-PCR

Marker	Forward primer	Reverse primer
cd4-1	5′-CTGTTTCTGTTATAGACCTTGCC-3′	5′-CTGGTCGGTCTTAAATGAAACT-3′
cd4-2.1	5′-CTTCAATGTCACTCTGATAAATGTGC-3′	5′-TTACGTGCCGTGTAGATCTG-3′
cd8a	5′-GGAGTACCAGGTGGGCTTTT-3′	5′-GAGGAAAAGTCCACAACCTC-3′
efa1	5′-CTGGAGGCCAGCTCAAACAT-3′	5′-ATCAAGAAGAGTAGTACCGCTAGCATTAC-3′
foxp3a	5′-CAAAAGCAGAGTGCCAGTGG-3′	5′-CGCATAAGCACCGATTCTGC-3′
gata3	5′-GGATGGCACCGGTCACTATT-3′	5′-CAGCAGACAGCCTCCGTTT-3′
ifng1-2	5′-CTTTCCAGGCAAGAGTGCAGA-3′	5′-TCAGCTCAAACAAAGCCTTTCG-3′
IgM	5′-AGATCCAATACAAAGATACTATGC-3′	5′-TGGTGAAATGGAATTGTGG-3′
il10	5′-ATTTGTGGAGGGCTTTCCTT-3′	5′-AGAGCTGTTGGCAGAATGGT-3′
il13	5′-GGAAGCTGTGTTAGTCAATCC-3′	5′-GCCTGACAGAAATAATCATGC-3′
il4	5′-TGGTCCCCGTTTCTGAGTC-3′	5′-ACCCTTCAAAGCCATTCCTG-3′
tbx21	5′-GGCCTACCAGAATGCAGACA-3′	5′-GGTGCGTACAGCGTGTCATA-3′
tgfb1b	5′-CTGTTTGTACTGAAAAGTCTGAGG-3′	5′-TCCAGACATAGGAGCAAGAGC-3′
tnfa	5′-GGGCAATCAACAAGATGGAAG-3′	5′-GCAGGTGATGTGCAAAGACAC-3′

competent *E. coli* (One Shot TOP10 cells, Invitrogen). Plasmid DNA was isolated with a Plasmid Plus Maxi Kit (Qiagen, Venlo, The Netherlands). Fish were infected with a low dose of mycobacteria, and 5 weeks postinfection vaccinated with 12 µg plasmid or a mixture of plasmids into the dorsal muscle, followed by electroporation to the injection site (six pulses of 40 V, 50 ms each) (Oksanen et al., 2013). The *EGFP* expression of vaccine constructs was detected in each fish under UV light 5-7 days postvaccination (Myllymäki et al., 2017). Five weeks postvaccination, dexamethasone feeding (10 µg/fish/day) was started lasting for 4 weeks, followed by determination of bacterial burden in each fish as described above. In the primary screen, 11-18 fish per group were immunized with the antigen candidates. Of these, the antigens leading to a significant reduction in bacterial counts (two-tailed Mann–Whitney test) were chosen for a replicate experiment with similar group sizes to confirm the original result.

Statistical analyses and power calculations

Statistical analyses were carried out with GraphPad Prism 5 version 5.2 (Software Inc.). Bacterial burdens per fish were analyzed with the one- or two-tailed Mann–Whitney test, and the granuloma counts were analyzed with unpaired Student's *t*-test and Fisher's exact test of independence for hypoxic and nonhypoxic granulomas. Two-way ANOVA with a Bonferroni posttest was used for the statistical analysis of the cell populations in the FACS analysis, and the qRT-PCR results were analyzed with a two-tailed Mann–Whitney test. The effectiveness of the antibiotics *in vitro* was assessed with one-way ANOVA with Friedman's test. In each case, $P<0.05$ was considered significant. In the antibiotic and vaccine screening experiments, the sample size of each group was determined based on power calculations as in Myllymäki et al. (2017).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: H.M., M.P., M.R.; Methodology: M.N., H.M., H.L.; Validation: M.N., H.M.; Formal analysis: H.M., M.N., H.L.; Investigation: M.N., H.M., H.L.; Resources: M.R.; Writing - original draft: H.M., M.N., M.P., M.R.; Writing – review & editing: H.M., M.N., M.R.; Visualization: M.N., H.M.; Supervision: H.M., M.P., M.R.; Project administration: H.M., M.R.; Funding acquisition: M.R., H.M., M.N., H.L., M.P.

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Supplementary information

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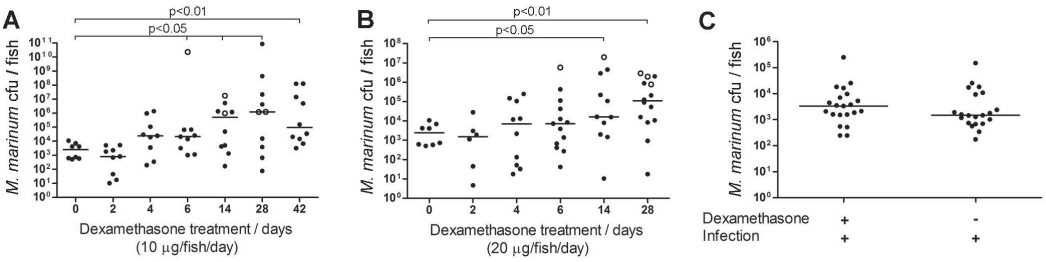
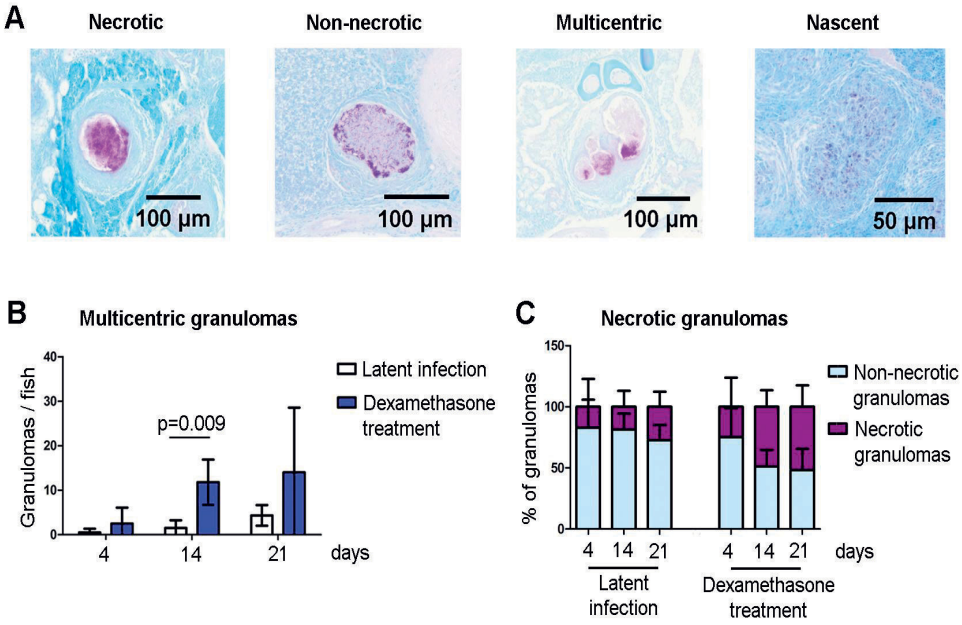


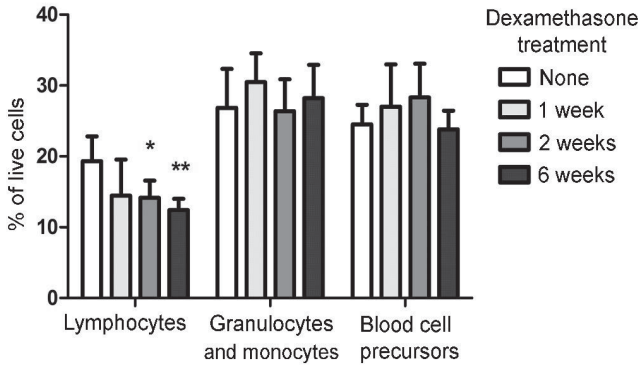
Supplementary Figure 1. Dexamethasone treatment increases bacterial loads in zebrafish with a latent mycobacterial infection.

(A) Zebrafish with a latent *M. marinum* infection were treated with dexamethasone 10 µg/fish/day (A) or 20 µg/fish/day (B) for indicated times. Each dot shows the bacterial count in one fish (n=8-14 fish/group). (C) The fish were treated with dexamethasone (10 µg/fish/day) for four weeks prior to a low dose *M. marinum* infection. The bacterial burden was quantified 4 weeks post infection. Horizontal lines represent the median bacterial count of each group. p values were calculated using the one-tailed Mann-Whitney test.



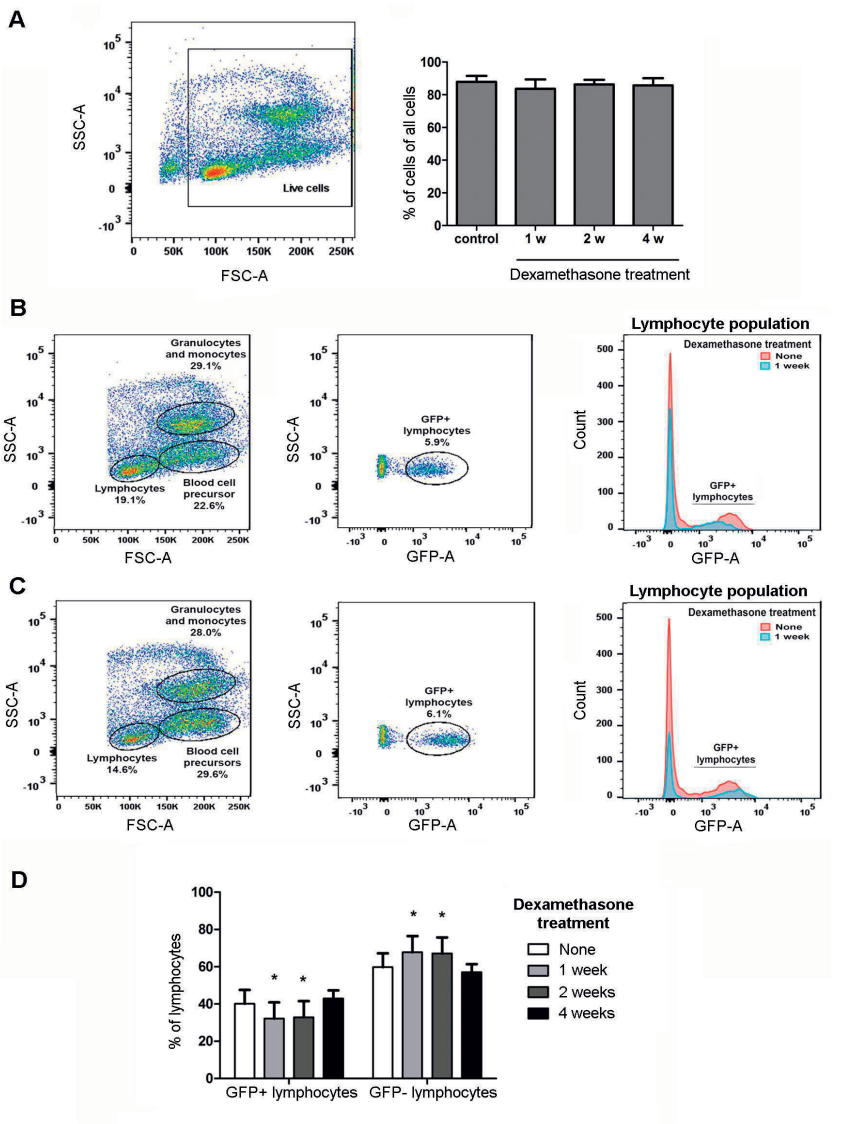
Supplementary Figure 2. Dexamethasone treatment increases the number of multicentric granulomas and the relative proportion of necrotic granulomas.

(A) Classification of different types of granulomas with Ziehl-Neelsen staining used for the quantification in Figure 2 and in this figure. (B) Quantification of multicentric granulomas per fish in a latent infection compared to a dexamethasone treatment. The bars shows mean±s.d. (n=3-6 fish/group). (C) The proportion of necrotic and non-necrotic granulomas in a latent infection and after dexamethasone treatment (n=3-6 fish/group). Bars show the mean percentage±s.d. p values are calculated with two-way ANOVA.



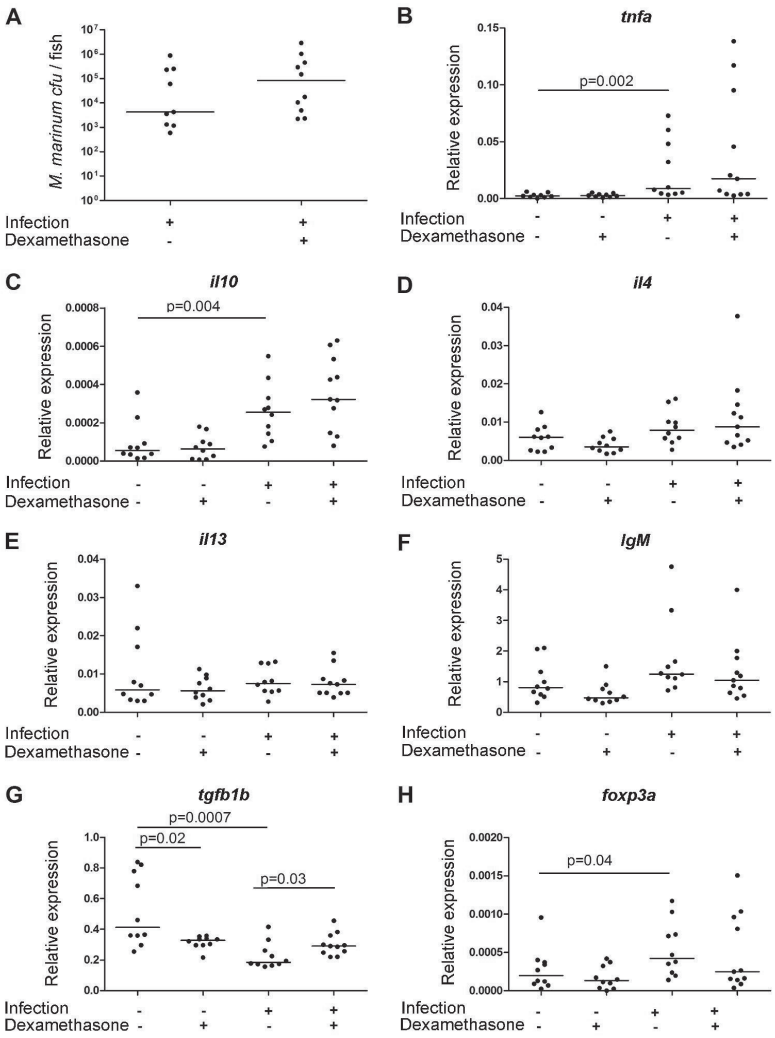
Supplementary Figure 3. Dexamethasone treatment decreases the relative amount of lymphocytes in the zebrafish kidney, but does not affect the granulocyte and monocyte or blood cell precursor populations.

Quantification of cell populations in the zebrafish kidney by FACS analysis following a dexamethasone treatment for 1, 2 and 6 weeks. n=7-9 fish/group. Data is presented as mean ±s.d. The data was analyzed with two-way ANOVA with Bonferroni's posttest, * $p<0.05$, ** $p<0.01$.



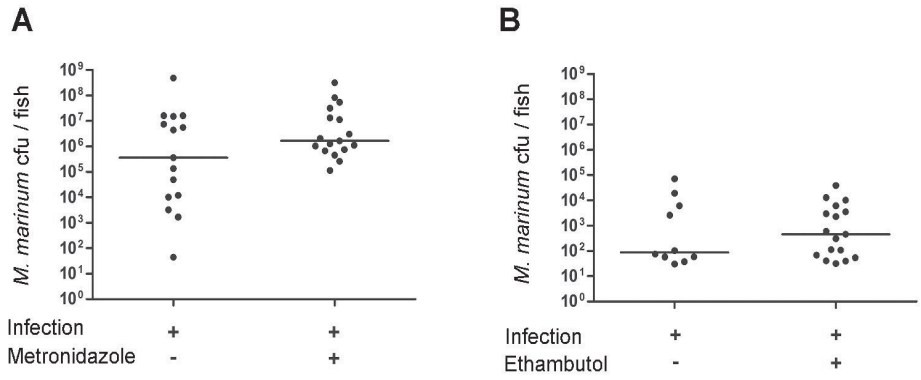
Supplementary Figure 4. Dexamethasone treatment does not affect the number of live cells in the zebrafish kidney or their gating, but has a mild effect on the proportion of GFP+ and GFP- lymphocytes.

A) The cells in the live gate were included in the FACS analysis and their number remained constant during a dexamethasone treatment. B and C) Gating of the kidney cells based on size (SSC-A) and granularity (FSC-A) and GFP expression after two (B) and four (C) weeks of a dexamethasone treatment. C) The relative proportion of GFP+ lymphocytes decreases during the first two weeks of a dexamethasone treatment, but is restored to the level of untreated fish by four weeks. n=12. Data is presented as mean±s.d. The data was analyzed with two-way ANOVA with Bonferroni's posttest, * p<0.05, ** p<0.01, *** p<0.001.



Supplementary figure 5. Mycobacterial infection increases the expression level of *tnfa* and *il10* and a dexamethasone treatment increases the expression of *tgfb1b* upon infection.

(A) The bacterial burden per fish in a latent infection and after a one-week dexamethasone treatment. Horizontal lines are median values of each group (n=9-10 fish/group). (B –H) Relative expression levels of cytokines and lymphocyte markers in zebrafish kidneys. Expression levels were normalized with the expression level of *ef1a*. Horizontal lines show median values of each group (n=9-10 fish/group). p values were calculated using the two-tailed Mann-Whitney test. (B) *tnfa*, (C) *il10*, (D) *il4*, (E) *il13*, (F) *IgM*, (G) *tgfb1b*, (H) *foxp3a*.



Supplementary Figure 6. Metronidazole and ethambutol treatment against a latent mycobacterial infection in adult zebrafish.

(A) Fish with a latent mycobacterial infection were treated three weeks with metronidazole (50 µg/fish/day), and (B) two weeks with ethambutol (50 µg/fish/day). Each dot represents the bacterial count in one fish. Horizontal lines show the median value of each group. n=10-15 fish/group. The two-tailed Mann-Whitney test was used for the statistical analysis.

PUBLICATION III

Immunization of adult zebrafish for the preclinical screening of DNA-based vaccines

Myllymäki H, Niskanen M, Oksanen K. and Rämet M.

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Video Article

Immunization of Adult Zebrafish for the Preclinical Screening of DNA-based Vaccines

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Abstract

The interest in DNA-based vaccination has increased during the past two decades. DNA vaccination is based on the cloning of a sequence of a selected antigen or a combination of antigens into a plasmid, which enables a tailor-made and safe design. The administration of DNA vaccines into host cells leads to the expression of antigens that stimulate both humoral and cell-mediated immune responses. This report describes a protocol for the cloning of antigen sequences into the pCMV-EGFP plasmid, the immunization of adult zebrafish with the vaccine candidates by intramuscular microinjection, and the subsequent electroporation to improve intake. The vaccine antigens are expressed as green fluorescent protein (GFP)-fusion proteins, which allows the confirmation of the antigen expression under UV light from live fish and the quantification of expression levels of the fusion protein with ELISA, as well as their detection with a western blot analysis. The protective effect of the vaccine candidates is tested by infecting the fish with *Mycobacterium marinum* five weeks postvaccination, followed by the quantification of the bacteria with qPCR four weeks later. Compared to mammalian preclinical screening models, this method provides a cost-effective method for the preliminary screening of novel DNA-based vaccine candidates against a mycobacterial infection. The method can be further applied to screening DNA-based vaccines against various bacterial and viral diseases.

Video Link

The video component of this article can be found at https://www.jove.com/video/58453/

Introduction

The first DNA vaccine studies were performed in the 1990s[1], and since then, DNA vaccines have been tested against various infectious diseases, cancer, autoimmunity, and allergy[2]. In mammals, a DNA vaccine against West Nile virus in horses and a therapeutic cancer vaccine for canine oral melanoma have been licensed, but these are not currently in clinical use[2]. In addition to the interest evoked by mammalian studies, DNA vaccination has turned out to be a convenient way to immunize farmed fish against viral diseases. A vaccine against fish infectious hematopoietic necrosis virus (IHNV) has been in commercial use since 2005, and a vaccine against infectious pancreatic necrosis virus (IPNV) was recently licensed[3]. In addition, several DNA vaccines against fish pathogens are being developed.

As traditional vaccines often contain inactivated or live attenuated pathogens, they pose a potential risk of transmitting the disease[2]. DNA vaccines, in turn, avert this risk, as they are based on the administration of plasmid encoding bacterial or viral antigens, rather than the whole pathogen itself[2,4]. DNA vaccines are produced with DNA recombination techniques, which allows the precise design of vaccine antigens and the flexible formulation of antigen combinations and adjuvants in a single vaccine construct[5]. Furthermore, the production of DNA vaccines is faster, easier and more cost-efficient than that of protein-based recombinant vaccines, which is a major advantage for vaccine candidate screening purposes, but also, for example, in the case of pandemic outbreaks[2].

In fish, the most common administration routes for DNA vaccines are intraperitoneal, intramuscular, and oral[3,6,7], while in mammals, subcutaneous and intradermal routes are additional options[2]. After an intramuscular injection, the administrated DNA plasmids enter the cells at the administration site (*e.g.*, mostly myocytes, but also resident antigen-presenting cells [APCs]). The proportion of transfected cells can be significantly increased by electroporation[2,8]. After entering the cell, some plasmid DNA enters the nucleus, where the genes encoded by the plasmid are transcribed[2]. In this protocol, we utilize the pCMV-EGFP plasmid that has a strong ubiquitous promoter optimized for eukaryotic

expression[9]. In this construct, the antigens are translated as a fusion protein with a GFP. The GFP enables the confirmation of a successful vaccination and the correct antigen product by the simple visualization of antigen expression with a fluorescence microscope in live fish.

In mammals, DNA vaccines have been shown to stimulate different types of immune responses depending on the transfected cell types[2,5]. Transfected myocytes secrete antigens into the extracellular space or release them upon cell death, and the antigens engulfed by APCs are, subsequently, presented on major histocompatibility complex II molecules[2]. This triggers CD4 and CD8 T cell responses, especially, in addition to B cell responses[2,5,10]. In fish, T and B lymphocytes, as well as dendritic cells (DCs), have been identified, yet their division of labor in antigen presentation is less well understood[11]. Zebrafish DCs, however, have been shown to share conserved phenotypic and functional characteristics with their mammalian counterparts[12]. Furthermore, DNA vaccination has been shown to elicit similar immune responses in fish and in mammals, including T and B cell responses[6,13,14,15,16].

Both larvae and adult zebrafish are widely used to model different infectious diseases, such as the fish *M. marinum* infection model of tuberculosis used in this protocol[17,18,19,20,21,22]. In comparison with mammalian model organisms, the advantages of zebrafish include their small size, fast reproducibility, and low housing expenses[23]. These aspects make the zebrafish an ideal animal model for large-scale preclinical screening studies for novel vaccines and pharmaceutical compounds[23,24,25].

In this protocol, we describe how novel vaccine antigen candidates against mycobacteriosis can be evaluated by the DNA-based vaccination of adult zebrafish. First, we describe how antigens are cloned into the pCMV-EGFP expression plasmid, followed by a detailed protocol for the intramuscular injection of vaccine plasmids and the subsequent electroporation into muscle. The expression of each antigen is confirmed by fluorescence microscopy one-week postimmunization. The efficacy of the antigen candidates is then tested by experimentally infecting vaccinated fish with *M. marinum*.

Protocol

Experiments including adult zebrafish require a permission for animal experimentation for both the vaccination and the subsequent studies with the infection model. All methods and experiments described here are approved by the Animal Experiment Board of Finland (ESAVI) and the studies are carried out in accordance with EU directive 2010/63/EU.

1. Cloning of DNA Vaccine Antigens

1. Select an expression plasmid optimized for the eukaryotic expression of the antigen(s) of interest under a strong, constitutive promoter, such as the cytomegalovirus (CMV) immediate early promoter. To enable the *in vivo* verification of the antigen expression, select a vaccine plasmid that encodes a fluorescent tag, such as the pCMV-EGFP plasmid[9] used in this protocol.
2. Use appropriate web-based and bioinformatic tools to select a potentially immune-protective antigen sequence (or several sequences) of approximately 90–600 nt (30–200 aa)[26] from the gene(s)-of-interest of the pathogen that will be used in the subsequent infection model.
3. Use available software, or manually design primers to amplify the antigen genes from the pathogen genome and to clone the antigen sequence(s) into the multiple-cloning site of the expression plasmid. Make sure to preserve the correct reading frame when selecting the antigen.
4. Include both a Kozak sequence (CCACC)[27] and a start codon (ATG) in the 5' primer. To preserve the C-terminal EGFP tag, avoid intervening stop codons (TAG, TAA, TGA) in the antigen sequence and the 3' primer. Also, ensure that the GFP tag remains in the same reading frame with the antigen of interest.
5. Use RNA or DNA extracted from the pathogen as a template to generate an adequate amount of PCR product with the cloning primers. Preferably, use a proofreading DNA polymerase to preserve the correct antigen sequence.
6. Purify the PCR product and check the correct size of the product by gel electrophoresis. Digest and ligate the PCR product with the digested vaccine plasmid.
7. Transform the ligation mix into competent bacterial cells according to a suitable protocol. Use an appropriate antibiotic selection for positive colonies and plasmid production in *E. coli*; the pCMV-EGFP plasmid, for example, contains an ampicillin resistance gene for this.
8. Use Sanger sequencing to confirm the insertion of the correct antigen sequence. The following primers can be used for sequencing antigens in the pCMV-EGFP plasmid: CMV forward 5'- CGCAAATGGGCGGTAGGCGTG-3' and EGFP-N reverse 5' CGTCGCCGTCCAGCTCGACCAG-3'.
9. Produce and purify a sufficient amount of the vaccine construct. Dissolve or elute the plasmid in sterile water. Make sure that the produced plasmid DNA is of high quality and the concentration is at least 0.72 µM or 2,000 ng/µL.

2. Pulling the Microinjection Needles

1. Prepare microinjection needles in advance. Use 10-cm aluminosilicate glass capillaries. Note that borosilicate glass capillaries are too brittle for injecting adult fish.
2. Pull the needles with a micropipette-needle-fabricating device.
   NOTE: The needles should look similar to the one presented in **Figure 1**. With the device used in this protocol (**Table of Materials**), the following settings result in the desired kind of needles:
   1. Set the glass capillary in the V-groove in the puller bar and tighten the clamping knob lightly.
   2. Move the holder next to the filament and gently push the capillary through the filament into the puller bar on the other side of the filament. Avoid touching the filament with the capillary.
   3. Tighten the clamping knobs, set down the safety glass, and press the pull button.
      CAUTION: The filament is hot.

3. Place the needles on a piece of reusable adhesive inside a 15-cm Petri dish plate to protect the needle tips. Keep the dish covered to keep the needles clean.

3. Filling the Micropipette Needles

1. Prepare the vaccine mix. Use 0.5–12 µg of plasmid per dose. If combining several different plasmids in one vaccination mix, use a maximum total DNA concentration of 12 µg per fish.
2. Calculate the volume of the vaccine "master mix" according to the number of fish in each group (see below). Add 1 µl of sterile-filtered phenol red to each injection dose to ease both the filling of the capillary needles and the observation of the injection. Add sterile 1x PBS up to a maximum total volume of 5–7 µl in one injection dose[8].
   NOTE: Injection volumes higher than 7 µL can result in the occasional leakage of the injection solution and should, therefore, be avoided.
3. Place a piece of tape, glue side up, on an appropriate holder, for example, the side of an empty tip box. Gently attach the capillary needles to the tape.
4. Pipet a maximum of 7 µL of the vaccine mix onto a piece of laboratory film. Using a loading tip, transfer the vaccine from the film into the needle. Pipet slowly and carefully, avoid pipetting air bubbles into the needle.
5. Let the needles settle for 15–30 min to remove possible remaining small air bubbles.

4. Setting the Microinjector and Electroporator for Immunization

1. Set the micromanipulator and a light source into the right position. Switch the air pressure tap to the open position.
2. Adjust the parameters for the pneumatic pump (see also **Figure 2**) as follows.
   1. Set the **vent** knob on the **Eject port** to **hold** to prevent backfilling of the pipette by capillary action. Set the **tubing** from the eject port to the micropipette. Do not use the Vacuum port in this protocol.
   2. Adjust the **pulse length**: use the **timed mode**, where an electronic timer controls the duration of the time the pressure solenoid stays open. Check that the green lamp next to the Eject pressure gauge illuminates when the pressure solenoid is open (energized).
   3. Set the pulse **range** to 10 s; with this setting, the pulses may be further set from 100 ms to 10.1 s. Use the **10-turn period dial** for fine-tuning the pulse length—every turn of the dial is 1.0 s. If needed, this can also be adjusted during an injection.
   4. Use the pulse initiator ("start button") on the front panel of the pneumatic pump, or a remote **foot switch** (recommended). This is connected to the front panel of the pneumatic pump.
3. Set the needle onto the micropipette holder of the micromanipulator. Cut the tip of the needle with tweezers so that liquid can be pushed out, and use the microscope to view the correct position. Press the foot switch once to see that a 1-s pulse pushes a small droplet out of the needle.
4. Use the following settings for the **electroporator**: voltage = 40 V; pulse length = 50 ms, number of pulses = 6. Connect the tweezers to the electroporator. See that the actual voltage and pulse length shown on the monitor do not differ significantly from the settings.

5. Injection of the DNA Vaccine and Electroporation

1. For immunizations according to this protocol, use healthy, 6 to 12 month-old adult zebrafish. Keep the fish in a flow-through system with a 14/10 h light/dark cycle, with a maximum of seven fish per 1 L of water.
2. Prepare a 0.02% 3-aminobenzoic acid ethyl ester (tricaine) solution (pH 7) in tank water for anesthetizing the fish[28]. Use a 10 cm Petri dish or something similar.
3. Prepare a recovery tank by filling a 5 L beaker with 3 L of clean system water.
4. Prepare a vaccination padding to keep the fish in a fixed position during the vaccination. Take a 5 x 7 cm^2 piece of a 2 to 3 cm-thick sponge. Cut a groove into the sponge with a scalpel blade or sharp scissors.
   NOTE: The same vaccination padding can be used in multiple experiments. Disinfect the sponge between the experiments by soaking it in 70% ethyl alcohol and allow to dry.
5. Thoroughly soak the sponge in the system water and set the sponge on a Petri dish.
6. Fast the fish 24 h before the vaccinations.
7. Anesthetize one zebrafish by placing it on a Petri dish containing 0.02% tricaine. Wait until the fish does not respond to touch stimulation and until there is no movement of the gills. Anesthetize a single fish at a time.
8. Using a plastic spoon, transfer the anesthetized zebrafish onto the wet sponge and set the fish's ventral side down into the groove. In the correct position, ensure that the head and most of the body of the fish are inside the groove and the dorsal fin and the tail are protruding out from the groove.
9. Under the microscope, carefully place the needle in an approximately 45° angle close to the zebrafish's dorsal muscle, using the x- and y-axis fine-tuning wheels on the micromanipulator.
10. Find the small spot without scales in front of the dorsal fin, where pushing the needle does not demand force. If resistance is felt, try an adjacent spot. Avoid injuring the spine, the dorsal fin, or the scales.
    NOTE: If the needle bends while pushing, shorten the needle slightly by cutting it.
11. Use the foot switch to gradually inject the vaccine solution into the muscle. Observe the injection through the microscope: phenol red is visible as it enters the muscle tissue. Adjust the duration of the pulse if needed.
    NOTE: Alternatively, use the pulse initiator button on the front panel of the pneumatic pump for the injection. However, the use of a remote foot switch allows using the other hand for adjusting the pulse length by the 10-turn dial wheel. Avoid injecting the solution too fast, since this may cause excessive tissue damage. Make sure not to inject any air.
12. Electroporate the fish immediately after the injection. Make sure that the fish is still under anesthesia. Keep the fish on the sponge and set the fish between tweezer-type electrodes, so that the electrodes are located on each side of the injection site. Do not press the electrode tweezers too tight but keep both electrodes in contact with the fish.
    1. Press the start button on the electroporator to give six 40 V, 50 ms pulses.

2. Gently transfer the fish to the recovery tank.
3. Clean the electrodes after each electroporation by swiping them with 70% ethanol.
   NOTE: Carefully monitor the well-being of the fish after the vaccination. Euthanize any fish showing signs of discomfort (a slow recovery from anesthesia, aberrant swimming, gasping) in 0.04% tricaine. After recovery, transfer the fish to the flow-through unit and feed it normally.

6. Visualization and Imaging of Antigen Expression

1. Anesthetize the fish in 0.02 % tricaine 2–7 days after immunization, and use a UV-light to see EGFP expression near the injection site.
   NOTE: Visual inspection under a UV light is an easy and non-invasive operation that is suitable for the routine verification of successful vaccinations, also in large-scale experiments. If no images of the fish are required, this step can also be performed in regular fish tanks without the need to anesthetize or move the fish.
2. To capture images, use a fluorescence microscope to visualize EGFP expression at the injection site[8]. Use a 2X objective lens and select the correct filter to visualize fluorescence or visible light views.
3. Keep the anesthetized fish still by pressing the ventral fin gently with tweezers toward a Petri dish bottom. Take both light-microscope and fluorescence images of the same area.
4. Merge the light-microscope and fluorescence images using the appropriate software[29]. Add a scale bar.

7. Quantification of the Expression Level and Size of the Antigens

1. Euthanize the fish in 0.04% tricaine. Dissect the fluorescent part of the dorsal muscle with a scalpel and tweezers under UV-light. Extract proteins from the samples[8].
2. Verify the correct size of *in vivo*-produced proteins with a western blot analysis, using a horseradish peroxidase (HRP)-conjugated GFP antibody (or similar)[8]. Include a negative control (unimmunized fish) to exclude any background signals and unspecific binding, together with a control expressing EGFP without a fused antigen.
   NOTE: For the western blot analysis, the expected size (in kDa) of the antigen-fusion proteins can be calculated, for example, by the equation:
   Molecular Weight (MW) of dsDNA = (number of nucleotides x 607.4) + 157.9; or by using web-based tools.
3. Quantify the expression of each antigen using a GFP-ELISA[8] (optional).

8. Combining the Vaccination Protocol with an *M. marinum* Infection Model

1. Determine the group size required for the reliable determination of the effectiveness of a novel vaccine candidate in the infection model and the assay used (see, for example, Myllymaki *et al.*[24] and Charan and Kantharia[30]). Carry out the appropriate power calculations while planning the experiments.
2. To evaluate the effectiveness of vaccine candidates, infect the fish 5 weeks postimmunization. Use an intraperitoneal infection with approximately 30 colony-forming units (cfu) of *M. marinum*, which leads to a latent infection in most fish[8,20,31].
   NOTE: When using *M. marinum*, follow a BSL2 safety protocol. The preparation of the bacterium or virus depends on the pathogen.
3. Quantify the number of pathogens in each fish. Euthanize the fish 4 weeks postinfection and determine the bacterial burden in each fish from the extracted DNA with qPCR using primers specific for *M. marinum*[20,24].
   NOTE: Be sure to include an appropriate control group and use the correct statistical methods for analyzing the results. Generally, a group of fish immunized with the empty pCMV-EGFP plasmid is a suitable negative control.
4. Confirm positive results with antigens without the GFP tag. Clone the antigens as described in step 1 and repeat the vaccination experiment.

Representative Results

The steps involved in the DNA vaccination protocol of adult zebrafish are illustrated in **Figure 3**. At first, the selected antigen sequences are cloned into a pCMV-EGFP plasmid and plasmid DNA is produced and purified[24] (**Figure 3**).Vaccine candidates are then injected intramuscularly with a microinjector and the injection site is electroporated to improve the intake of the plasmid into cells (**Figure 3**). The used vaccination dose was optimized by injecting different amounts of the pCMV-EGFP plasmid and measuring the GFP expression with ELISA (**Supplementary Figure 1**). Two to seven days postvaccination, the expression of the fusion protein is detected under UV light and visualized with fluorescence microscopy (**Figure 3** and **Figure 4**). The expression levels of different antigens may vary from very intensive (antigen 1) to a faint expression (**Figure 4**). In addition, GFP expression can be observed across the dorsal muscle (antigen 1), or in a more limited area (antigen 2) (**Figure 4**). However, if no fluorescence is detected within 10 days, it is recommended to make sure that there are no mistakes in antigen cloning or primer design. To confirm that the expressed fusion protein is of the correct size, proteins can be extracted from the muscle tissue around the injection site and used for a western blot analysis.

The effect of the vaccine candidates is evaluated by challenging the fish with a low dose of *M. marinum* by an intraperitoneal injection (**Figure 5**). Four to five weeks postinfection, the bacterial counts are determined with qPCR and compared to bacterial loads in the control group (**Figure 5**). Furthermore, the effectiveness of the most promising vaccine candidates can be tested by monitoring the survival after a high dose *M. marinum* infection(**Figure 5**). However, in addition to giving a quantitative result on the progression of the infection, instead of merely a status of alive or dead, the qPCR-based cfu quantification requires less time and smaller group sizes and is, therefore, a more ethical approach for a primary screen. Overall, this protocol facilitates the screening of the effectiveness of novel vaccine antigens within 12 weeks (**Figure 5**).

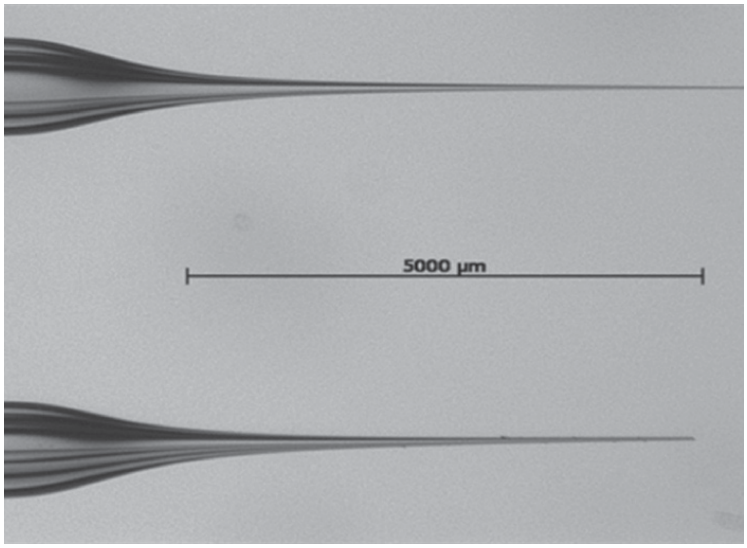


Figure 1: Close-up (12X) of aluminosilicate needles used in the adult zebrafish *intra muscular* injections. The tip below has been cut with tweezers and is ready to be used for microinjections. This figure has been adapted from Oksanen[35]. Please click here to view a larger version of this figure.

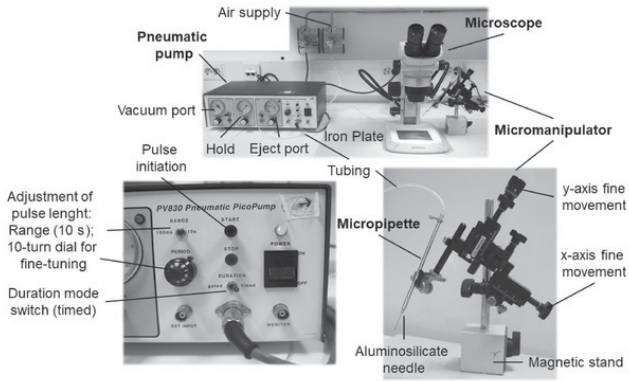


Figure 2: Microinjection equipment and set-up. The main components of the equipment needed for the DNA vaccination of adult zebrafish are highlighted in bold. The critical adjustments are indicated. Please click here to view a larger version of this figure.

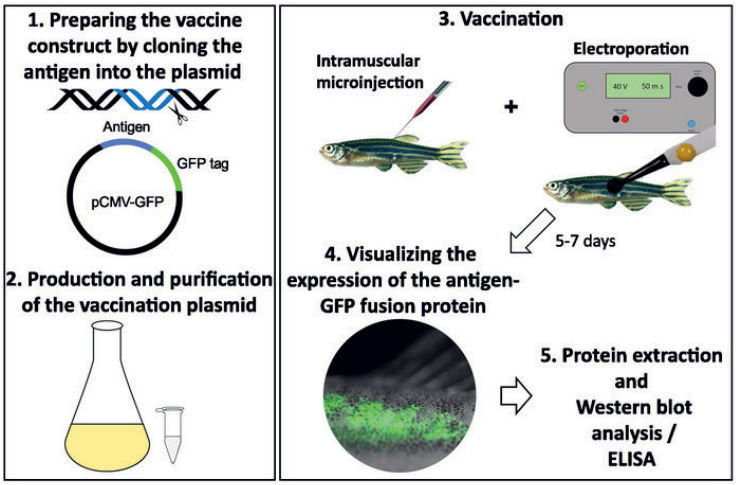


Figure 3: Preparing the DNA vaccine plasmids and the immunization procedure. (**1**) Selected antigens are cloned adjacent to the GFP tag in the pCMV-EGFP plasmid. (**2**) The vaccine construct is produced microbiologically, concentrated, and purified. (**3**) 12 µg of plasmid is injected into the dorsal muscle of an anesthetized adult zebrafish with a microinjector, and the injection site is subsequently electroporated with six 40-V, 50-ms pulses. (**4**) Two to seven days postvaccination, the GFP expression of the antigen-GFP fusion protein is visualized with a fluorescence microscope. (**5**) The fluorescent part of the dorsal muscle can be dissected and used for protein extraction. The size of the fusion protein is confirmed with a western blot analysis and the expression level with GFP-ELISA. Please click here to view a larger version of this figure.

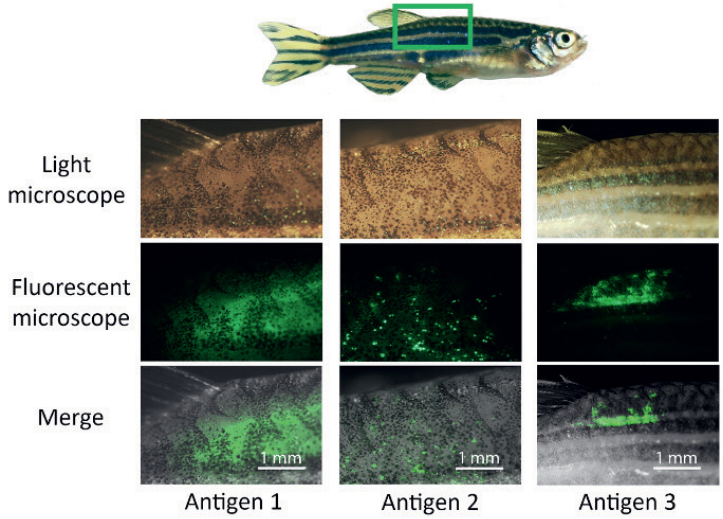


Figure 4: Visualizing the expression of the antigen-EGFP fusion protein. Anesthetized adult zebrafish are vaccinated with 12 µg of experimental vaccine antigens (antigen 1–3) and the injection site is electroporated, subsequently, with six 40 V, 50 ms pulses. Two to seven days postvaccination, the injection site is imaged with a microscope. First, the expression of GFP is detected under a fluorescence microscope. The area is inspected using a 2X magnitude objective and imaged and saved in .tiff form. The light microscope image of the same area is merged with the fluorescence image using the ImageJ software. The quantity and position of the antigen expression may vary between antigens and individual fish. For example, the expression of antigen 1 is observed across the dorsal muscle and the expression of antigen 2 is seen as small spots, whereas antigen 3 is strongly expressed in a more limited area. Please click here to view a larger version of this figure.

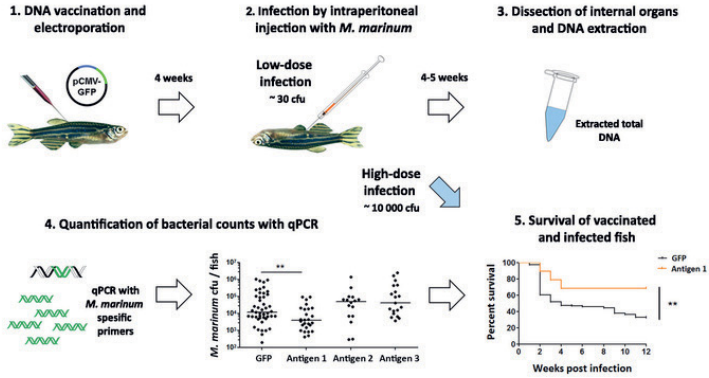
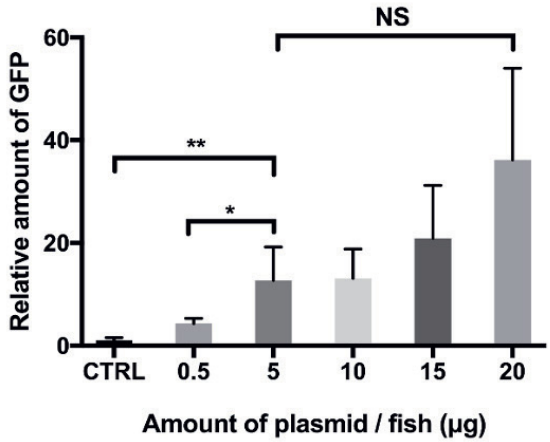


Figure 5: Testing the effectiveness of vaccine candidates against a mycobacterial infection. (**1**) Adult zebrafish are vaccinated with experimental DNA vaccines against mycobacteriosis. (**2**) Five weeks postvaccination, the fish are infected with a low dose of *Mycobacterium marinum* (~30 cfu). (**3**) Four weeks later, the internal organs are dissected and used for DNA extraction. (**4**) The bacterial count in each fish is quantified with qPCR using *M. marinum*-specific primers. Immunization with antigen 1 led to a significant decrease in the bacterial counts (p < 0.01, two-way ANOVA), while antigens 2 and 3 had no effect. (**5**) The protective effect of the most promising vaccine candidate (antigen 1) is further evaluated in a survival experiment, where fish are infected with a high dose (~10,000 cfu) of bacteria and their survival is monitored for 12 weeks. Consistent with the decrease in the bacterial burden observed in panel **4**, vaccination with antigen 1 also improved the survival of the fish upon an *M. marinum* infection (p <0.01), suggesting this antigen could be a promising candidate for a novel vaccine against tuberculosis. Please click here to view a larger version of this figure.



Supplementary Figure 1: Amount of plasmid DNA affects plasmid-derived EGFP expression in adult zebrafish. Groups of fish (n = 5 in each group) were injected with 0.5–20 µg of pCMV-EGFP, and electroporation (six pulses of 50 V) was used to enhance the transfection. Control fish (CTRL) were injected with 2 µg of the empty pCMV plasmid not containing the EGFP gene. GFP-ELISA was performed 3 days postinjection to define the relative EGFP expression in fish homogenates. P-values: *p <0.03, **p <0.004. The error-bars represent standard deviations. NS = not significant. This figure has been adapted from Oksanen[35]. Please click here to view a larger version of this figure.

Discussion

The procedure of immunizing adult zebrafish with DNA-based vaccines requires some technical expertise. Even for an experienced researcher, vaccinating a single fish takes approximately 3 min, excluding preparations. Thus, a maximum of roughly 100 fish can be immunized within a day. If more than 100 fish are required for the experiment, the immunizations can be divided between up to 3 days. In addition to the quality of the experiment, sufficient training of the researcher(s) for handling the fish and performing the immunization is essential for the well-being of the fish. Make sure to follow local legal and animal welfare rules and guidelines when it comes to housing the fish, planning the experiments, and the qualifications required for the personnel carrying out the experiments.

In summary, there are several critical steps to avoid complications in the immunization protocol. For the successful immunization, ensure that **1)** the fish to be immunized are healthy and sufficient in age and size (the immunization of more juvenile fish can require down-scaling the vaccine volume and the electroporation settings); **2)** the fish are properly anesthetized with no stronger than 0.02% 3-aminobenzoic acid ethyl ester, and they remain anesthetized throughout the entire procedure (anesthesia should be kept as short as possible to ensure the recovery of the

fish); **3)** the sponge pad is properly soaked; **4)** liquid is injected in each pulse from the pneumatic pump and, if not, the pulse length is adjusted (pulling the needle slightly backwards along the y-axis can help); **5)** there are no air bubbles with the vaccine solution; **6)** the electroporation settings and the actual pulse voltage and length are correct; **7)** the electrodes do not cause skin damage on the electroporation site (during the electroporation, keep the electrodes in gentle contact with the fish, and release the fish immediately into the recovery tank after electroporation).

It is important to monitor the fish after the electroporation in the recovery tank and to euthanize any fish showing signs of discomfort. Furthermore, it is necessary to practice the procedure before starting a large-scale experiment, to ensure a fluent workflow. If possible, ask a sufficiently trained colleague for assistance with filling the needles and the electroporation.

The DNA vaccination method enables the tailor-made design of vaccine antigens. It is possible to clone the whole antigen or, preferably, select parts of the antigen based on cellular localization and immunogenicity[24]. In addition, the method enables combining several antigens or adjuvants into one vaccine construct or injecting several separate plasmids at the same time[2]. By including a stop codon after the antigen sequence or by excising the EGFP gene from the plasmid, it is possible to utilize the same plasmid vector also to express the antigen without the subsequent N-terminal GFP tag. This may be reasonable in confirming the positive screening results, as the relatively large size of GFP can affect the folding of the antigen and, thus, restrict humoral responses potentially evoked by the vaccination.

A higher antigen expression has been linked to DNA vaccine immunogenicity[2]. Electroporation after injection has, thus, been included in this protocol, as it has been shown to increase the expression of antigens or reporter genes from fourfold to tenfold in zebrafish[32]. Furthermore, electroporation as a technique causes moderate tissue injury, thus inducing local inflammation that further promotes the vaccine-induced immune responses[2]. On the other hand, electroporation is generally well-tolerated. With the equipment used here, practically 100% of adult zebrafish will recover well from the six pulses of 40 V used in this protocol[35].

In addition to using electroporation to enhance the entry of the vaccine plasmid into the cells, we use a strong ubiquitous promoter in the vaccine plasmid and a polyA tail at the 3' end of the antigen to improve antigen expression in the transfected fish cells. In some cases, if the codon usage of the target pathogen significantly differs from the vaccinated species, codon optimization has been found useful in further increasing target gene expression[2]. In this zebrafish-*M. marinum* model, however, codon optimization had no significant effect on the expression levels of two mycobacterial model genes, *ESAT-6* and *CFP-10,* and has, thus, been deemed unnecessary in this model[35].

Target gene expression profiles have some temporal variation between the antigens, depending, for instance, on the size and the structure of the antigens in question. However, antigen expression is usually similar within a group of fish immunized with the same vaccine. Typically, the brightest EGFP expression is observed four days to one-week postvaccination, but a scale of 2–10 days is possible. It is recommended to validate the expression of each antigen-EGFP fusion protein in a small group of fish (2–3) before including the antigen in a large-scale experiment. If no GFP expression is observed at any point 2–10 days after immunization, make sure that **1)** the immunization protocol was carefully followed. Always have a group of fish immunized with the empty pCMV-EGFP plasmid as a positive control and make sure that **2)** the antigen design and molecular cloning was carried out correctly (adequate primer design; the antigen and the EGFP tag are both in the same reading frame and no intervening stop codons are included). In some cases, despite the correct antigen design, GFP cannot be detected. This may be due to the incorrect folding or rapid breakdown of the fusion protein. In these circumstances, it may be necessary to redesign the antigen.

In vaccines that are used to immunize farmed fish, the plasmid dose used is typically 1 µg or less[7,33,34]. In zebrafish, reporter gene expression can also be detected after at least a 0.5 µg plasmid injection following electroporation; however, the relative target gene expression significantly increases with a higher amount of plasmid per fish (**Supplementary Figure 1**). In fish injected with the pCMV-EGFP reporter plasmid, an injection with 5–20 µg of plasmid resulted in four to eight times higher EGFP levels in comparison with fish injected with 0.5 µg. Therefore, to ensure a high enough target gene expression, yet have injection volumes that are small enough (≤7 µL) to prevent any excess tissue damage or vaccine leakage, we chose to use 5 to 12 µg per fish for the preliminary screening purposes. In addition to vaccine immunogenicity, a high enough target gene expression is required to detect reporter gene expression with a fluorescence microscope and with western blot, which is necessary for screening purposes to confirm the correct in *vivo* translation of the target antigen. However, lower plasmid doses (0.5–1 µg) can be useful for other types of experimental uses.

In conclusion, this protocol for the immunization of adult zebrafish with a DNA plasmid can be used in the preclinical testing of novel vaccine candidates against various bacterial or viral infections. The expression of the vaccine antigen as a GFP-fusion protein allows the visualization of a successful immunization event and antigen expression. We apply this method for the preclinical screening of novel vaccine antigen candidates against tuberculosis. For this, we infect the zebrafish five weeks postvaccination and determine the bacterial counts in each fish with qPCR[20,24].

Disclosures

The authors have nothing to disclose.

Acknowledgements

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PUBLICATION IV

DNA vaccination with the *Mycobacterium marinum* MMAR_4110 antigen inhibits reactivation of a latent mycobacterial infection in the adult zebrafish

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DNA vaccination with the *Mycobacterium marinum MMAR_4110* antigen inhibits reactivation of a latent mycobacterial infection in the adult Zebrafish

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ABSTRACT

Background: Tuberculosis is a major challenge for health care, as options for its treatment and prevention are limited. Therefore, novel approaches, such as DNA vaccination, to both prevent primary infections and the reactivation of latent infections need to be developed. A *Mycobacterium marinum* infection in adult zebrafish (*Danio rerio*) recapitulates features of the human *Mycobacterium tuberculosis* infection, providing a convenient preclinical animal model for studying tuberculosis.
Methods: Hypoxic *M. marinum* cultures were produced with the Wayne model, and further reaerated to replicate the *in vivo* reactivation *in vitro*. Expression levels of *M. marinum* genes were studied with mRNA sequencing from exponentially growing bacteria, anaerobic cultures and at 2 and 12 h after reaeration. Seven reactivation-associated genes were selected for further studies, where their antigen potentiality as DNA-vaccines to prevent reactivation of a latent mycobacterial infection was investigated in the adult zebrafish model. The Mann-Whitney test was used to evaluate differences in bacterial counts between the groups.
Results: The mRNA sequencing data showed that, seven *M. marinum* genes, MMAR_0444, MMAR_0514, MMAR_0552, MMAR_0641, MMAR_1093, MMAR_4110 and MMAR_4524, were upregulated during reactivation when compared to both dormant and logarithmic growing bacteria. Four different MMAR_4110 antigens prevented the reactivation of a latent mycobacterial infection in the adult zebrafish.
Conclusion: This study provides novel information about reactivation-related *M. marinum* genes. One of the antigens, MMAR_4110, inhibited the reactivation of a latent *M. marinum* infection in zebrafish, implicating that the characterized genes could be potential targets for further vaccine and drug development against mycobacterial diseases.

1. Background

Mycobacterium tuberculosis, the causative agent of human tuberculosis (TB), infects 10 million people annually. TB is difficult to treat and thus it causes the death of 1.5 million people annually [1]. In addition, approximately 1/5 of the human population carry a latent infection with a 5–10% life time risk for reactivation [1,2]. The only available vaccine, Bacillus Calmette-Guérin (BCG), protects effectively against severe forms of TB in infants, while it provides poor protection for adults or against reactivation of a latent disease [1,3,4]. An effective vaccine against human TB is required to control the spread of the disease. Several different approaches to develop novel TB vaccines, including conventional, therapeutic, prophylactic, booster and re-infection preventing vaccines, are under clinical studies [5]. Considering safety aspects, a DNA vaccine, which does not contain live or attenuated parts of bacteria, is an appealing alternative.

During the past two decades, DNA vaccines have been gaining importance as a promising therapeutic approach to prevent infectious diseases, cancer, autoimmune disorders and allergy [6–9]. A DNA vaccine is composed of an expression vector with a gene, which encodes an antigen under a eukaryotic promoter[10]. The technique enables the *in vivo* expression of tailormade antigens and immunization without a live or attenuated microbe [10,11]. For example, the DNA vaccination technique has shown its

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applicability in immunizing fish against a Novirhabdovirus infection [12]. Two DNA vaccines, pCMV4-G against viral hemorrhagic septicemia and Apex-IHN against infectious hematopoietic necrosis, are commercially available and used in immunizing farmed fish [12–15]. Despite these advances, the development of DNA vaccines against bacterial diseases has turned out to be more challenging both in fish and in mammals [6]. However, recent results with the M72 vaccine candidate against TB are promising and show that tailor-made protein vaccines can elicit adequate immune responses also in mammals [16–18].

The high genetic similarity between the fish natural pathogen *M. marinum* and the human *M. tuberculosis* allows utilizing the zebrafish-*M. marinum* infection model in TB research [19–24]. A *M. marinum* infection in adult zebrafish (*Danio rerio*) recapitulates several features of human tuberculosis, and an infection can cause a progressive or an asymptomatic latent disease in a dose-dependent fashion [14,20,21,25]. In a latent infection, the fish immune system is capable of controlling the invading bacilli in granuloma structures, whereas in the acute disease bacteria can disseminate without restraint [25,26]. Both in humans and in fish, during the latent infection phase, the metabolic environment inside granulomas changes, and consequently mycobacteria adapt by adopting a dormant state [26,27]. Even years later, as a result of the weakened immune system of the host, dormant bacteria can reactivate and cause an active TB [1,28]. Similarly, by suppressing the fish immune system with either gamma irradiation or dexamethasone treatment, it is possible to induce reactivation of a latent infection [21,27].

One of the challenges in developing effective vaccines against TB is that the mechanisms of host-pathogen interactions during a mycobacterial infection are inadequately understood [29]. Knowledge of the expression levels of different mycobacterial genes in different phases of the mycobacterial life cycle could provide new insights into mycobacteriosis. Here, we focused on identifying novel DNA vaccine antigens against reactivation of the latent mycobacterial infection. Unlike BCG or other live vaccine strategies, this kind of a vaccine would be safer method to immunize immunocompromised individuals, for instance HIV positive populations, against reactivation of the latent TB. To select potential mycobacterial antigens, we set up an *in vitro* reactivation model with *M. marinum* and identified genes, whose expression was induced during reactivation. Selected genes were then further tested as DNA vaccine antigens to prevent reactivation of a latent infection in the adult zebrafish.

2. Methods

2.1. Culture of M. Marinum

All experiments were conducted with the *Mycobacterium marinum* ATCC 927 strain from the culture maintained in this laboratory as described in [30]. All liquid culture experiments were performed in 7H9 Middlebrook medium supplemented with ADC Middlebrook growth supplement, glycerol (4 mL/L) and Tween®80 (1 g/L). The medium was aseptically dispensed into sterile tubes or culturing flasks. Bacteria was plated on Middlebrook 7H10 agar, prepared according to the manufacturer's instructions from Becton Dickinson. All bacteria cultures were incubated at 29 °C.

2.2. Hypoxic M. Marinum cultures

The Wayne low oxygen model for *M. tuberculosis* was utilized to produce hypoxic *M. marinum* cultures. The liquid bacteria culture was diluted (OD_{600} 0.07) and divided into 14 mL VWR Culture Tubes with Dual-Position Caps (6.7 mL in each). Tubes were sealed air tightly with laboratory film and incubated on a shaker (125 rpm) at 29 °C. To detect the redox stage in the sealed tubes, 160 μL of methylene blue solution (100 mg/mL) was added in a control tube before sealing. The color change in the control tube was checked daily. The growth of the bacteria was followed by measuring OD_{600} values in each tube for 18 days.

2.3. Reaeration of hypoxic M. Marinum and RNA extractions

To reactivate hypoxic *M. marinum* cultures, the sealed tubes were opened and inoculated into 7H9 medium (1:1 ratio), followed by incubation at 29 °C on a shaker (125 rpm) for indicated times. Logarithmically growing cultures were used as control samples. Growth of the bacteria was followed by measuring OD_{600} values and by plating dilutions on the 7H10 agar plates before harvesting. At the selected time points (0 h, 2 h and 12 h) cells were harvested by centrifugation (6000 × g at 4 °C for 10 min). Pellets were suspended to 700 μL of RTL buffer (RNAeasy Mini Kit, Qiagen) and samples were homogenized with 6 ceramic beads (2.8 mm, OMNI International) 3 times 3200 rpm 40 s with a homogenizer (PowerLyzer24 bead beater, Mobio). RNAs from the homogenized samples were extracted with the RNAeasy Mini Kit (Qiagen) with the standard procedure, and RNAs were eluted in 30 μL of sterile water. Extracted samples were purified with a dnase removal kit (RapidOut DNA Removal Kit, Thermo Fisher Scientific). The quality of the extracted RNA was confirmed with Fragment Analyzer (Agilent) using the Fragment Analyzer RNA Kit (Agilent).

2.4. mRNA sequencing

Four *M. marinum* RNA samples (1 μg/sample) at each time points were sequenced in the Finnish Functional Genomics Centre (University of Turku and Åbo Akademi University). The HiSeq2500 instrument was used for sequencing by single-end sequencing chemistry with a 50 bp read length. The RNA-seq data analysis was carried out in the Medical Bioinformatics Centre of the Turku Bioscience Centre using R (version 3.2.2) and Bioconductor (version 3.2.) In short, the reads were aligned against the *Mycobacterium marinum* M reference genome (AssemblyGCA_000018345.1) downloaded from Ensembl using Bowtie version 2–2.2.6, and only uniquely aligned reads were used for further analyses. The data were normalized and converted into RPKM values (Reads Per Kilobase of exon per Million reads mapped) using the TMM normalization method of the edgeR R/Bioconductor package. Clustering of the samples was done using Euclidean metrics. R Package Limma was used for performing the statistical testing between the groups. A 2-fold change and a p-value below 0.01 were set as the thresholds used for filtering differentially expressed genes. Principal component analysis (PCA) was carried out with the ClustVis web tool [31].

2.5. qRT-PCR

Expression profiles of selected *M. marinum* genes were verified by qRT-PCR from a new set of samples with gene specific primers (Table 1). qRT-PCR was performed with the iTaq universal SYBR® Green One-step Kit (Bio-Rad) and the CFX96™ Real-Time PCR Detection System (Bio-rad).

2.6. Zebrafish maintenance and infection with M. Marinum

For all the fish experiments, adult wild type AB zebrafish (6–8-month-old) were used and maintained as described in [27]. During the experiments, the wellbeing of the fish was monitored twice a day and fish showing any signs of disease or discomfort were euthanized with 0.04% 3-aminobenzoic acid ethyl ester.

Table 1
M. marinum gene specific primers for qRT-PCR.

Gene specific primer	Sequence 5′ -> 3′
MMAR_0444 forward	CAGCTGGTTGTTCATCGC
MMAR_0444 reverse	GGATGGGGATGACGATCG
MMAR_0514 forward	CCTGACAACGAGGGGATG
MMAR_0514 reverse	CAGCCCATACCCGGATTC
MMAR_0552 forward	CTGTGCGTCATTCATGGC
MMAR_0552 reverse	GGCAACGTGTACAACAGC
MMAR_0641 forward	CAGCACCTTGGACATCGA
MMAR_0641 reverse	CGTTGTAGAAGCCGGACA
MMAR_1093 forward	CTGGCGAATCTCTGGAACGA
MMAR_1093 reverse	CCACCAGTTCCACTCCGATA
MMAR_4110 forward	CGAGAACGTCAACTATCGCC
MMAR_4110 reverse	AGATTGTCCAGTGCCCCTTC
MMAR_4524 forward	CCCCGACACCAACCTCTC
MMAR_4524 reverse	CACATCGGTTTGGGATCCGT

For the infection experiments, *M. marinum* was cultured and prepared as described in [21]. Fish were anesthetized in 0.02% 3-aminobenzoic acid ethyl ester and injected into the intraperitoneal cavity either with a low (35 CFU) or with a high (20 000 CFU) dose of bacteria following the protocol shown in [32]. Infection doses were confirmed by plating on 7H10 plates (BD Biosciences).

2.7. RNA extraction and quantification of M. Marinum gene expression in the adult zebrafish

Infected fish were euthanized with 0.04% 3-aminobenzoic acid ethyl ester, and their kidney and rest of the internal organ block were dissected into two separate 2.0 mL reinforced tubes with screw caps (OMNI International) containing 6 ceramic beads (2.8 mm, OMNI International). Kidney samples were homogenized with 700 μL of RTL buffer (RNAeasy Mini Plus kit, Qiagen) as *M. marinum* pellets described above. RNA from the homogenized samples was extracted with the RNAeasy Mini Plus kit (Qiagen) according to the manufacturer's instructions. DNA from the organ blocks was extracted with the TRI Reagent (MRC) as described in [30,33].

M. marinum gene expression levels were quantified from the kidney-RNA samples by qRT-PCR with the gene specific primers listed in Table 1. iTaq universal SYBR® Green One-step Kit (Bio-Rad) was used for qRT-PCR and the expression level in each fish was normalized with the *M. marinum* bacterial load in the same fish. The latter was quantified by qPCR from the DNA samples, extracted from internal organs of each fish, with *M. marinum internal transcribed spacer* (MMITS) primers (F: 5′-CACCACGAGAAACACTCCAA-3′, R: 5′-ACATCCCGAAACCAACAGAG-3′) as described in [33].

2.8. DNA vaccine constructs

The hidden Markov model was used to predict the cellular location of the selected *M. marinum* proteins [34], and transmembrane or extra-cellular regions were favored as antigens in the DNA vaccine constructs. The selected antigen sequences were cloned between the NheI and EcoRI sites in the pCMV-GFP vector (Addgene plasmid # 11153; http://n2t.net/addgene:11153; RRID: Addgene_11153), which was a gift from Connie Cepko. A cacaca-overhang and a Kozak sequence with the start codon (caccatg) were added to the forward primers, and the overhang plus one extra nucleotide to keep the GFP tag in the correct reading frame were added to the reverse primers (Table 2). While cloning MMAR_4110 fragments 1–3, a stop codon (tta) was included in the reverse primers to block translation of the GFP tag. The regions for the antigen fragments were chosen based on an immunogenicity prediction by the Predicted Antigen Peptides tool (http://imed.med.ucm.es/Tools/antigenic.pl). All the cloning and plasmid production steps were performed as described in [33]. The vaccine constructs were sequenced with the CMV-F (*5′-CGCAAATGGGCGG TAGGCGTG-3′*) and EGFP-N (*5′-CGTCGCCGTCCAGCTCGACCAG-3′*) primers.

Table 2
The primers used to clone *M. marinum* antigens into the pCMV-GFP expression vector.

Cloning primer	Sequence 5′ -> 3′
MMAR_0444 forward	CACACA-GCTAGC-CACCATG-AACTCGCTGAGCACTACC
MMAR_0444 reverse	CACACA-GAATTC-A-GATCCAGTTGAAAAGCGA
MMAR_0514 forward	CACACA-GCTAGC-CACCATG-GATTCGATCGGAAAACCTG
MMAR_0514 reverse	CACACA-GAATTC-A-GAATGAGGCTGGATCGTC
MMAR_0552 forward	CACACA-GCTAGC-CACCATG-GCACTGACTTCCCAACAAC
MMAR_0552 reverse	CACACA-GAATTC-A-GTCCAACTGACTCACGTGT
MMAR_0641 forward	CACACA-GCTAGC-CACCATG-ATGAGTTTTGTGTTGTTG
MMAR_0641 reverse	CACACA-GAATTC-A-GTGGTAACCCAGCATGGC
MMAR_1093 forward	CACACA-GCTAGC-CACCATG-CCTGGCGAATCTCTGGAAC
MMAR_1093 reverse	CACACA-GAATTC-A-CGACCGAAGATTCCCGG
MMAR_4110 original forward	CACACA-GCTAGC-CACCATG-TATGTGGCCGAACCGATC
MMAR_4110 original reverse	CACACA-GAATTC-A-GTCAAACGTTTTCGAGATG
MMAR_4524 forward	CACACA-GCTAGC-CACCATG-CACCAACCTCTCCGAGGTG
MMAR_4524 reverse	CACACA-GAATTC-A-CAGCGTGCGTTTGTAGCTC
MMAR_4110 fragment 1 forward	CACACA-GCTAGC-CACCATG-ATGATCGCCGAGTTCGA
MMAR_4110 fargment 1 reverse	CACACA-GAATTC-TTA-CAGCTTCTCCTGCACCAG
MMAR_4110 fragment 2 forward	CACACA-GCTAGC-CACCATG-CCGTCCAACACCTACTTCAAC
MMAR_4110 fragment 2 reverse	CACACA-GAATTC-TTA-CGGGTGCTCGTAGAACAG
MMAR_4110 fragment 3 forward	CACACA-GCTAGC-CACCATG-GTGTTGACCTCGTCAATGC
MMAR_4110 fragment 3 reverse	CACACA-GAATTC-TTA-ATTGGACATGCTCGTTCG

2.9. Immunization with the DNA antigen candidates

In each of the vaccination experiments, zebrafish were immunized with the experimental DNA vaccine constructs as described in [35]. Briefly, fish were anesthetized with 0.02% 3-aminobenzoic acid ethyl ester and 12 μg of plasmid DNA was injected into the dorsal muscle, and the injection site was electroporated (6 pulses, 40 V, 50 m s each). *In vivo* expression of the fusion proteins was confirmed by checking the fish under UV light 5–7 days after injections, and by fluorescence microscopy with a Nikon AZ100 microscope. A plasmid expressing GFP without any mycobacterial antigens was used as a negative control in every experiment.

In the reactivation experiments, a dexamethasone-induced reactivation model was utilized [27]. Fish were infected with a low dose of *M. marinum* and 4 weeks later the fish with latent infections were immunized with the DNA vaccine constructs. 5 weeks after immunization, the fish were treated with a dexamethasone-containing feed (10 μg/fish/day) for 3–4 weeks. The wellbeing of the fish was carefully monitored, and the experiment was terminated if several fish (>30%) showed symptoms of mycobacteriosis. The *M. marinum* load in each fish was quantified with qPCR and the one-tailed Mann-Whitney test was used to assess statistical differences between the groups.

In the primary infection experiment, fish were first immunized with the original MMAR_4110 antigen or the control plasmid. 5 weeks post immunizations, the fish were infected with a low

dose of *M. marinum*. 4 weeks post infection, the fish were euthanized and the bacterial load in each fish was determined from DNA samples with qPCR as described above. In the survival experiment, the fish were injected with a high dose of *M. marinum* (22 000 CFU/fish) and the survival of the fish was followed 12 weeks post infection (as described in [35]).

2.10. Statistical analysis and power calculations

The two-tailed Mann-Whitney test was used to analyze differences in gene expression profiles from the *M. marinum in vitro* reactivation samples.

In all of the fish experiments, the required group sizes were calculated with a sample size calculator based on the results or our previous studies [36]. The following parameters were inserted into the calculator; two independent study groups and a continuous primary endpoint, type I/II error rates alpha 0.05 and power 0.8. Within these parameters the minimum group size was set to 16 fish. The one-tailed Mann-Whitney test was used to analyze bacterial counts, and the log rank Mantel-Cox test for the survival. Statistical details (significance and n value) can be found in the figure legends.

A statistical analysis was performed and a graphical representation of the data were created using the GrapPad Prism 5.02 software. P values < 0.05 were considered significant.

3. Results

3.1. Transcriptomic analysis of hypoxic M. Marinum after reaeration

To study the reactivation-related genes of *M. marinum*, we first produced dormant bacteria cultures by modifying the Wayne low oxygen model for *M. tuberculosis* [37]. Four days after sealing the cultures, the bacilli transformed into a non-replicating (anaerobic) stage, which was evident by methylene blue decolorization and stabilized growth (**Figure S1**). Next, we resuscitated the non-replicating persistent state cultures by reaeration and harvested the cells after 2 and 12 h. Samples from hypoxic cultures and logarithmic growing bacteria were used as controls. The viability of the hypoxic cultures and the growth of reaerated cultures were confirmed by spectrophotometric measurements at 600 nm and by plating.

At the defined time points (0 h, 2 h, 12 h and log phase) harvested cells were used for RNA extraction and mRNA sequencing (n = 4 /each time point) (**Tables S1 and S2**). A total of 5133 genes showed expression in one or more samples. The groups were visualized by Principal Component Analysis (PCA, Fig. 1**A**), which indicated that samples form hypoxic cultures and the samples aerated for 12 h were most dispersed, indicating diverse gene expression profiles among these cultures.

In a further comparison between the groups, we identified a total of 379 differentially expressed *M. marinum* genes from the RNA-seq datasets (Fig. 1**B** and **Table S3**). To classify these, we searched for genes that were upregulated following culture aeration compared to normally grown and hypoxic cultures. As shown in Fig. 1**B**, altogether 27 genes were upregulated compared to the hypoxic culture already at 2 h after reaeration indicating a rapid change in gene expression during reactivation. We therefore focused on the 2-hour reaeration group in the further analysis. As shown in Fig. 1**C, 165** genes are upregulated in this group compared to logarithmic growing bacteria and 27 compared to the hypoxic culture (Fig. 1**C**).

To gain information about the nature of the cellular events induced during the early reactivation process, we ran a pathway enrichment analysis on these gene lists. Among the significantly enriched GO terms ($p < 0.05$, Classic fisher test) in the 2 h vs. hypoxic culture upregulated gene list, many (9/18) were involved in transmembrane transportation (**Table S4**). Likewise, in the 2 h vs. logarithmic growth group, 6/27 of the significantly enriched GO terms were involved in transport (of inorganic ions, namely Potassium), while the majority of the GO terms were associated with various metabolic and biosynthetic processes (18/27; **Table S5**). In addition to ion transmembrane transport (GO:0034220), the GO terms enriched in the list of upregulated genes from both comparisons (2 h vs. hypoxic culture and 2 h vs. logarithmic growth) included Alcohol metabolic process (GO:0006066) and Organic hydroxy compound metabolic process (GO:1901615).

In terms of individual genes, there were only seven, namely MMAR_0444, MMAR_0514, MMAR_0552, MMAR_0641, MMAR_1093, MMAR_4110 and MMAR_4524, genes that were upregulated in both comparisons (2 h vs. hypoxic culture and 2 h vs. logarithmic growth) (Fig. 1**D** and Table 3). We focused on these genes in more detail. The mRNA sequencing results for the seven genes were verified from a new set of samples by qPCR (Fig. 1**E**) with gene specific primers (Table 1). The enhanced expression of *MMAR_0444, MMAR_0514, MMAR_0552, MMAR_1093* and *MMAR_4524* were confirmed by qPCR.

Since the *in vitro* expression profiles of *M. marinum* genes can differ from those seen in *in vivo* environment, we studied the expression levels of these seven *M. marinum* genes from infected (~35 CFU/fish) adult zebrafish after dexamethasone induced reactivation (n = 6–8 fish/group). RNAs extracted from the kidneys were used for qRT-PCR to analyze the gene expression profiles with gene specific primers and DNAs extracted from the organ block were utilized to analyze the bacteria counts in each fish. Expression of all the genes studied (except for MMAR_4524) was detected in the *in vivo* samples (**Figure S2**).

3.2. Immunization with the MMAR_4110 antigen protects against reactivation of a latent M. Marinum infection in the adult zebrafish

We hypothesized that genes expressed specifically in the early stage after reaerating of the dormant bacteria could be potential targets for vaccine development against reactivation. To assess this, we cloned the corresponding sequences into the pCMV-GFP expression vector (Fig. 2). To enable visualization of the *in vivo* expression of the antigens, they were cloned under a strong CMV promoter next to the GFP site. The expression of the fusion proteins was verified at the injection site 3–7 days post immunization. GFP expression was detected in all vaccine constructs; however, the expression of the MMAR_1093-GFP protein was faint and was detected only at 3 days after injection whereas the others were expressed at high level for at least two weeks (**Figure S3** and data not shown).

To assess the ability of the antigens to protect against the reactivation of a latent mycobacterial infection, we utilized a model where adult zebrafish with a latent mycobacterial infection are immunized intramuscularly with the experimental vaccine antigens or with the GFP control, and five weeks later treated with dexamethasone for 3–4 weeks to cause reactivation of the infection [27]. In each of the experiments, increased bacterial counts were measured in GFP-vaccinated and dexamethasone-treated fish when compared to bacterial burdens in latent control group fish, indicating reactivation of the infection as expected ($p < 0.05$, n = 17–20 fish/group) (Fig. 3). Each antigen immunization was repeated 2–3 times. While most antigens had no or little effect on the bacterial counts, the expression of antigen MMAR_4110 protected against the dexamethasone-induced reactivation of a mycobacterial infection ($p = 0.03$, one-tailed Mann Whitney test) (Fig. 3**F and Table S6**).

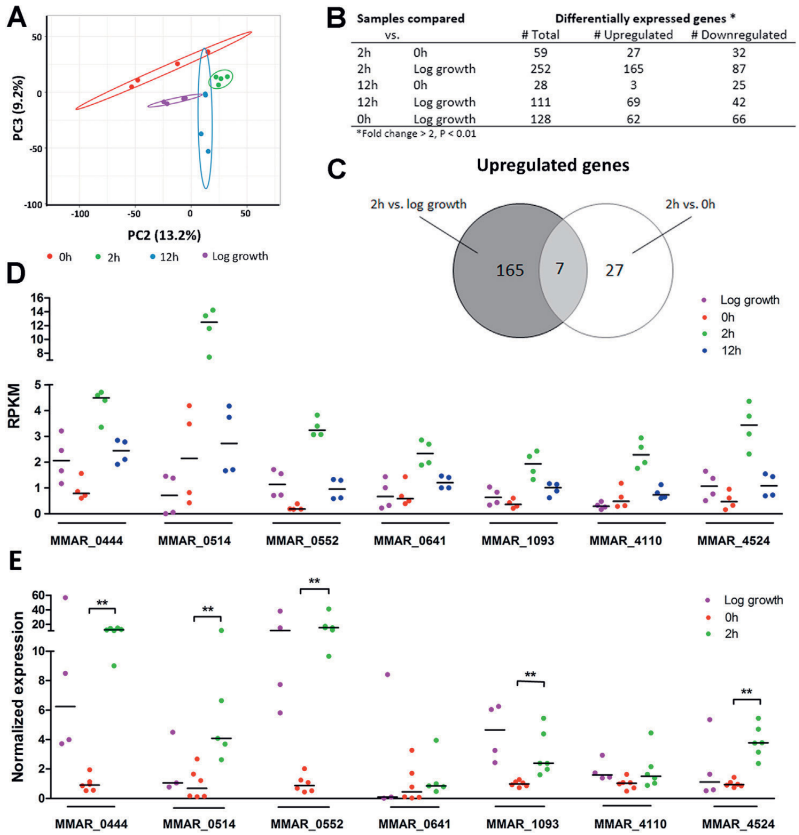

Fig. 1. The expression of seven *M. marinum* genes were induced in reactivation compared to both hypoxic and logarithmic growing bacteria. RNA from reaerated and logarithmic growing *M. marinum* were extracted and used for mRNA sequencing. **A**) PCA plot. Unit variance scaling is applied to rows; SVD with imputation is used to calculate principal components. The X and Y axes show principal component 2 and principal component 3 that explain 16.5% and 9.2% of the total variance, respectively. Prediction ellipses mean that with a probability 0.95 a new observation from the same group will fall inside the ellipse. N = 16 data points. **B**) Summary of differentially expressed genes between groups. Fold change > 2 and p-value < 0.01 were used as criteria for filtering the differentially expressed genes. **C**) 165 genes were upregulated during reactivation at 2 h as compared to logarithmic growing bacteria. The expression of seven *M. marinum* genes was induced compared to both logarithmic growth and dormancy. **D**) RPKM values (Reads Per Kilobase of exon per Million reads mapped, mean values with SD) of seven differentially expressed *M. marinum* genes in different conditions (0 h, 2 h, 12 h and log growth). N = 4 samples per group. **E)** New *M. marinum* RNA samples were produced to validate the mRNA sequencing data *in vitro*. The expression levels of each *M. marinum* genes were analyzed from samples collected from hypoxic (0 h), reaerated (2 h) and logarithmic growing bacteria, n = 4–6 samples / group. The values were normalized to the average SQ-value of hypoxic samples. All the genes showed trends towards higher expression after two hours of reaeration when compared to hypoxic bacteria (MMAR_0444, *MMAR_0514*, *MMAR_0552*, *MMAR_1093*and *MMAR_4524* being statistically significant). P-values (**<0.01, *<0.05) were calculated with the two-tailed Mann-Whitney test.

We next examined whether the MMAR_4110 antigen is effective against a primary infection with *M. marinum* in adult zebrafish. First, we immunized fish with the MMAR_4110 antigen, and 5 weeks later exposed them to a low-dose *M. marinum* infection (~35 CFU/fish), and then followed the progression of the disease by measuring bacterial counts 4 weeks after infection (Fig. 4**A**) and by studying survival of the fish 12 weeks post exposure to 20 000 CFU/fish of *M. marinum* (Fig. 4**B**). Both experiments showed that the MMAR_4110 antigen does not protect against a primary infection (Fig. 4), suggesting that the vaccine antigen provides protection specifically against the reactivation of a latent infection.

3.3. Three additional antigen fragments of MMAR_4110 derived antigens protect adult zebrafish against the reactivation of a latent M. Marinum infection

To confirm that the protective effect of the MMAR_4110 antigen is caused by an immune response targeting the MMAR_4110 gene, we selected three additional regions of the gene (Fig. 5**A**). This time, the selected antigen regions (that were predicted to be immunogenic) were relatively short (89 bp, 75 bp and 38 bp, respectively), and they were expressed without the GFP fusion. All the tested antigen fragments of the MMAR_4110 gene protected against the reactivation of a mycobacterial infection, which was seen as lower bacterial burdens when compared to the GFP-immunized control group ($P < 0.05$, one-tailed Mann Whitney test, N = 11–17 fish / group) (Fig. 5**B**).

4. Discussion

The latest Tuberculosis report by the WHO estimates that one fifth of human population are asymptomatic carriers of *M. tuberculosis* and therefore at a risk of reactivation and progression into an active disease [1]. Among immunocompromised individuals, the risk for reactivation is high and the need for a preventive is evident. Recent advances in vaccine development point towards

Table 3
Detailed information of the seven *M. marinum* genes upregulated in reactivation as compared to both dormant and logarithmic growing bacteria. Information collected from the Mycobrowser database [38].

Gene	Product	Gene ontology terms / Functional category
MMAR_0444	Conserved transmembrane protein	Integral to membrane
MMAR_0514	PEP phosphomutase	Catalytic activity
MMAR_0552	Conserved hypothetical membrane protein	Cell wall and cell processes
MMAR_0641	PPE family protein	PE/PPE
MMAR_1093 (***CycA***)	D-serine/D-alanine/ glycine transporter	Amino acid transmembrane transport activity, Amino acid transport, Integral to membrane
MMAR_4110	Aldehyde dehydrogenase	Acetaldehyde dehydrogenase (acetylating activity), Alcohol dehydrogenase (nad) activity, Alcoholic metabolic process, Carbon utilization, Metal ion binding, Oxidation-reduction process
MMAR_4524	Conserved hypothetical membrane protein	Cell wall and cell processes

PEP, phosphoenolpyruvate mutase; **PE/PPE**, conserved Pro-Glu / Pro-Glu-Glu motifs; **nad**, nicotinamide adenine dinucleotide.

tailor-made and safe immunization methods that could provide long-lasting protection against tuberculosis and the reactivation of a latent disease.

The challenge in developing novel vaccines has been the lack of in-depth knowledge about the protective immunity during different phases of mycobacterial infection. Due to active and latent disease phases of TB, different approaches to select antigens against primary infection or reactivation of latent disease should be considered. Genes related to cell growth or membrane structure are likely chosen in vaccines preventing primary infection, whereas genes expressed in resuscitation might be better suited in vaccines against reactivation. However, so far there is no golden standards available how to select an optimal antigen, and often the same antigens are tested in both experimental setups. In this study, we utilized an *M. marinum* species that is a close relative to *M. tuberculosis*, to search for mycobacterial genes that are induced during the early phases of reactivation after hypoxia. Using mRNA sequencing, we identified nearly 400 genes that were differentially expressed between our experimental conditions. Since the *M. marinum* genome is 85% homologous to the *M. tuberculosis* genome [19], it is possible to use this information to identify *M. tuberculosis* genes that could be potential targets for drug and vaccine development against human TB.

Out of the 379 differentially expressed genes identified in our mRNA sequencing, we found several groups of genes that might have potential for vaccine development. For instance, genes upregulated at 2 h compared separately to genes upregulated at 0 h or exponentially growing bacteria, would be interesting to study further. This study was focused on seven genes, which were induced in reactivation when compared to both hypoxic cultures and logarithmic growing bacteria. The functions for most of these genes remain poorly defined, although there are data suggesting that some of these genes could have a role in dormancy or reactivation of *M. tuberculosis*. Three of the seven genes, MMAR_0444, MMAR_0552 and MMAR_4524, are conserved transmembrane proteins with unknown functions [38]. MMAR_0444 Mtb homologue Rv0204c and MMAR_4524 Mtb homologue Rv0990c are identified as components in a virulence gene cluster [39,40]. BALB/c mice infected with both Rv0204c and Rv0990c transposon mutated bacteria had reduced bacteria counts. In addition, infection with a Rv0990c mutant strain increased the survival of the animals when compared to mice infected with the wild type bacteria [39,41]. The function of the MMAR_0552 and its Mtb homologue Rv0293c remain poorly known. Even though membrane proteins are assumed to be potential targets for vaccine antigens [42], in our experimental setup, these proteins did not provide protection against reactivation.

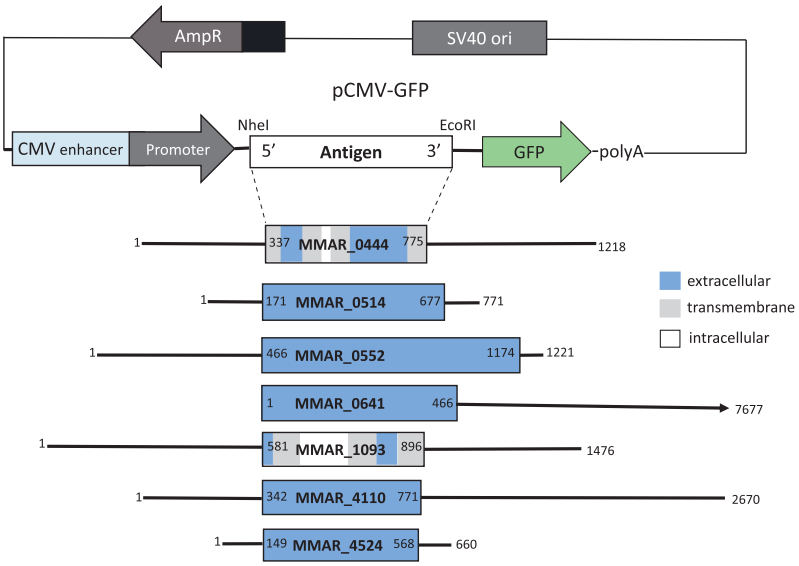

Fig. 2. DNA vaccine constructs and *in vivo* expression of antigen-GFP fusion proteins. Seven different antigen candidates were cloned into the pCMV-GFP expression vector between the NheI and EcoRI restriction sites next to a GFP tag, under a strong CMV promoter. According to the TMHMM topology prediction, most of the protein regions selected for the antigens were located outside of the cell or were transmembrane.

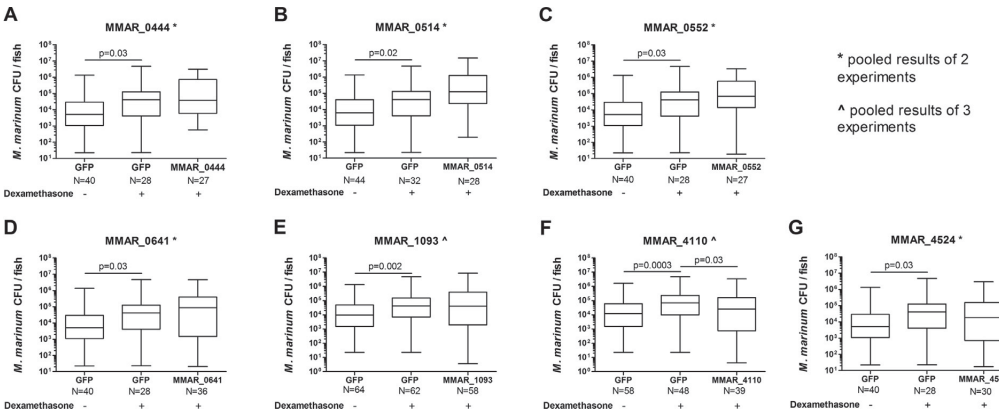

Fig. 3. Immunization with the MMAR_4110 antigen prevents reactivation of a latent *M. marinum* infection in the adult zebrafish. AB fish were infected with a low dose of *M. marinum* (~35 CFU). 5 weeks post-infection, the fish were immunized with the GFP control or an experimental vaccine candidate; **A**) MMAR_0444, **B**) MMAR_0514, **C**) MMAR_0552, **D**) MMAR_0641, **E**) MMAR_1093, **F**) MMAR_4110 or **G**) MMAR_4524. 5 weeks later, the fish were treated for 3–4 weeks with p.o. dexamethasone (10 μg/fish/day). The bacterial burden in each fish was determined with qPCR with *M. marinum* specific primers. The lines represent median values, and the bars and whiskers the minimum and maximum values for each group, respectively. The figure shows the results of two or three pooled experiments, N = 27–64 fish/group. The one-tailed Mann-Whitney test was used to calculate P-values.

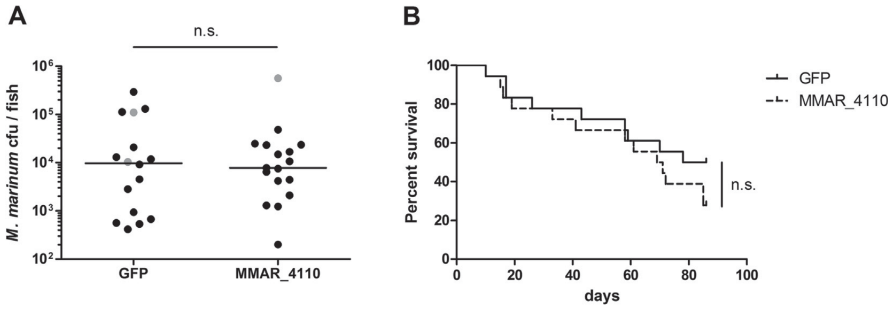

Fig. 4. The MMAR_4110 vaccine antigen does not protect against a primary *M. marinum* infection in adult zebrafish. AB fish were immunized with the experimental vaccine candidate, MMAR_4110, or the GFP expression vector and five weeks later infected intraperitoneally with mycobacteria, 35 CFU (A) or 10 000 CFU (B). **A**) Four weeks later bacterial counts in each fish were determined with qPCR. Each black dot represents bacterial counts in one fish and grey dots bacterial counts in fish euthanized before the end of the experiment. Horizontal lines represent the median value of the group, N = 16–17 fish/group, the One-tailed Mann-Whitney test. **B**) Survival of immunized and infected fish 12 weeks post infection. N = 18 fish/group, log-rank (Mantel-Cox) test.

Interestingly, it is known that the *M. tuberculosis gene Rv3347c (PPE55), which is* homolog to the PPE family protein of *M. marinum*, MMAR_0641, is differentially expressed in pulmonary TB granulomas [43] and it has been suggested that it has a role in the survival of the bacteria under stress [44]. Until this study, the antigen potential of MMAR_0641 had not been studied. However, in our hands, the MMAR_0641 antigen did not protect against the reactivation of a latent infection.

MMAR_0514 is, according to the Mycobrowser database, a conserved hypothetical PEP phosphonomutase [38]. It shares 32% identity with the Mtb gene Rv1998c, which has been shown to be induced by hypoxia in a DNA microarray analysis [45]. Lately, the immunogenicity of the Rv1998, among 68 other antigens, was tested in an *ex vivo* experiment, where blood samples, collected from human TB patients, were stimulated with Mtb antigens, and the expression levels of the cytokines were measured 7 days post stimulation [46]. In this setting, the Rv1998 antigen showed immunogenicity, which was seen in increased IL-8, IL-6 and IL-17 expression levels [46]. In our hands, immunization with the MMAR_0514 antigen did not protect against reactivation.

The MMAR_1093 (cycA) and its *M. tuberculosis* homologue (Rv1704c) are permeases, which transport D-alanine, D-serine and glycine across the cytoplasmic membrane [38]. Until now, their antigenic potential was not studied. Our experiments showed insufficient protection by the MMAR_1093 antigen against the reactivation of a latent mycobacterial infection. However, it is worth noting that this antigen was poorly expressed as a GFP fusion protein *in vivo* compared to the other tested antigens. While it is known that vaccines need to induce a strong enough immune response with the right timing [47], the low expression might explain the lack of protection.

In this study, all of the four antigens directed against the aldehyde hydrogenase MMAR_4110 protected against the reactivation of a latent mycobacterial infection in the adult zebrafish, while the antigen had no effect against a primary infection. Of note, one of the GO terms specifically enriched among the upregulated genes in the 2 h reaeration samples was Alcohol metabolic process (GO:0006066), and MMR_4110 is associated with this. Thus, it is plausible that this process could be therapeutically targeted when aiming at hindering mycobacterial reactivation after hypoxia. Unfortunately, there is no clear *M. tuberculosis* homologue for MMAR_4110. However, a BLAST analysis shows that MMAR_4110 shares 26% similarity with the *M. tuberculosis* bifunctional acetaldehyde (adhE1, Rv0162), suggesting that *M. tuberculosis*

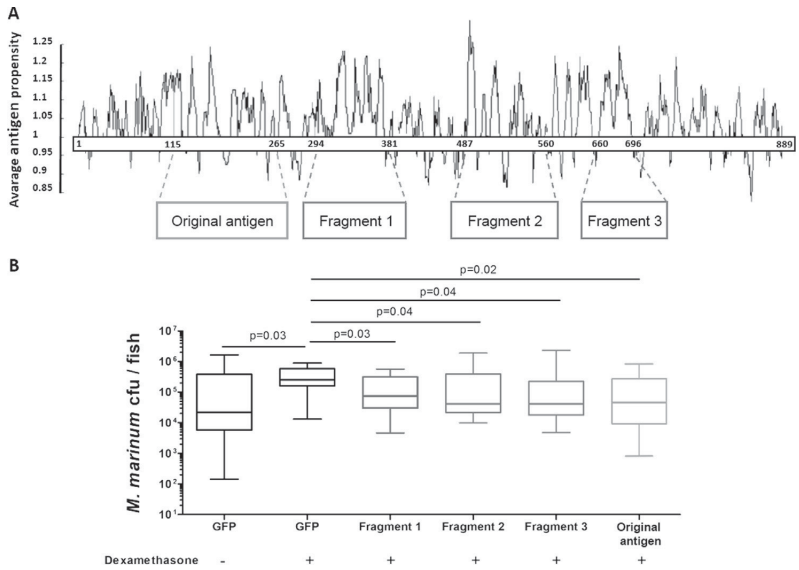

Fig. 5. Three different MMAR_4110 vaccine antigens inhibit reactivation of a latent mycobacterial infection in the adult zebrafish. Different parts of the MMAR_4110 gene for vaccine antigens were selected using a immunogenicity prediction tool, Predicted Antigen Peptides. Areas that showed a higher antigen propensity (>1) were selected (blue areas) and cloned into the pCMV-GFP expression vector (A). The original MMAR_4110 antigen, which data is represented also in the Fig. 3, was a fusion protein with GFP, whereas Fragments 1, 2 and 3 were expressed without the GFP tag. Adult zebrafish having a latent infection (original infection dose 35 CFU) were immunized with the empty pCMV-GFP vector or with the experimental DNA vaccines and treated 5 weeks post immunization for 4 weeks with dexamethasone (10 μg/fish/day) (B). Horizontal lines show the median and the bars and whiskers the minimum and maximum values of each group, N = 15–19 fish/group. A one-tailed Mann-Whitney test was used to calculate P-values. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

alcohol hydrogenases could be potential targets for vaccine and drug development. Immunization with the MMAR_4110 antigen led to a median decrease of 65% in bacterial counts compared to the control group immunized with an empty *GFP* plasmid. In a previous study using a similar model, a combination of the previously studied Ag85-ESAT-6 antigens led to a decrease of bacterial counts by 56% [35].

There are studies indicating that a large tag, such as the GFP fusion protein used in this study could form a physical barrier and thereby hinder antigen recognition [48]. In addition, it has been shown that GFP expression and an injection itself can trigger immunological responses, which may lead to improved protection against mycobacteriosis [48]. Therefore, we confirmed that both the original MMAR_4110 antigen with the GFP tag and the three Fragments that lacked the tag provided comparable protection against reactivation. Moreover, in this study, we used GFP-immunized fish as a control group, thereby avoiding the bias caused by the expression of the GFP tag.

Since *M. marinum* is a natural fish pathogen and DNA vaccines are shown to induce good immune responses in fish [12,15], the MMAR_4110 antigens could also be useful in the prevention of mycobacteriosis in fish farms or aquariums. In addition, based on the BLAST analysis, the MMAR_4110 gene has conserved homologues among many other mycobacterial species, and therefore this gene could be a potential target when novel therapeutic agents are developed against other mycobacterial diseases.

5. Conclusions

Tuberculosis remains a global health burden affecting millions of people worldwide. Besides the high number of active tuberculosis cases, 20% of the human population carry the latent form of the infection and are therefore at risk of reactivation. The current prevention method of tuberculosis is based on the BCG vaccine, which provides only partial protection for infants. Overall, novel immunization methods have a central role in attempts to eradicate tuberculosis. DNA vaccination has become as a feasible method for immunization against infectious diseases. However, incomplete knowledge about the cellular and molecular level mechanisms of a *M. tuberculosis* infection has prevented the discovery of effective vaccine antigens against tuberculosis. In our current study, mycobacterial genes, whose expression is upregulated in reactivation were studied as DNA-based vaccine antigens for their ability to inhibit the reactivation of a *M. marinum* infection in the adult zebrafish. One of the tested seven *M. marinum* antigens, MMAR_4110, provided protection against reactivation of the latent infection. Although there is no direct *M. tuberculosis* homologue for the MMAR_4110 gene, *M. tuberculosis bifunctional acetaldehyde dehydrogenase* could be a potential target for developing novel vaccines and therapeutics against human tuberculosis.

6. Ethical approval and consent to participate

All of the fish experiments were approved by the National Animal Experiment Board in Finland (Approval number ESAVI/10823/04.10.07/2016 and ESAVI/12135/04.10.07/2017) and performed in line with the EU-directive 2010/63/EU on the protection of animals used for scientific purposes

7. Consent for publication

Not applicable.

8. Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author at a reasonable request.

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10. Authors' contributions

All authors contributed to the conception and design of the experiments. MN and HM performed all mycobacteria and fish experiments and laboratory analyses. HM was a major contributor in analyzing the mRNA sequencing data. All authors contributed to interpreting the data. MN wrote the original draft of the manuscript, and MR and HM revised the content. All authors read and approved the final manuscript.

CRediT authorship contribution statement

Mirja Niskanen: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Visualization. **Henna Myllymäki:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - review & editing. **Mika Rämet:** Conceptualization, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2020.06.053.

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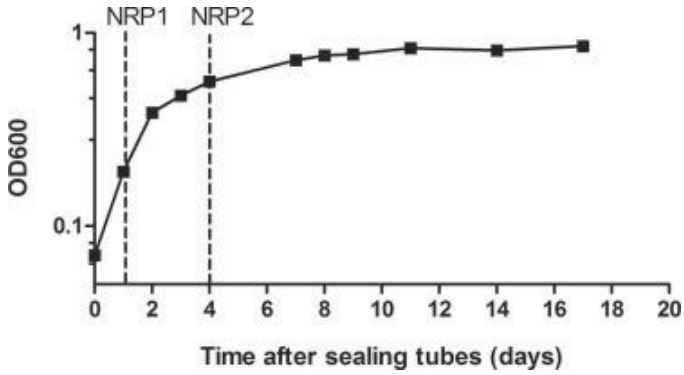

Figure S1. The adjusted Wayne low oxygen model for producing hypoxic *M. marinum* cultures. Bacterial cultures were grown in sealed tubes to produce a gradual reduction of oxygen. After one day of incubation, the bacteria reached microaerobic conditions (non-replicating stage 1, NRP1). Three days later the bacteria reached the anaerobic, non-replicating stage 2 (NRP2), where they had consumed the available oxygen, which was detected by decolorization of the methylene blue in the control tubes and a slow replication rate measured by optical density at 600 nm. The growth curve shows the mean OD value with SEM measured from 10 cultures.

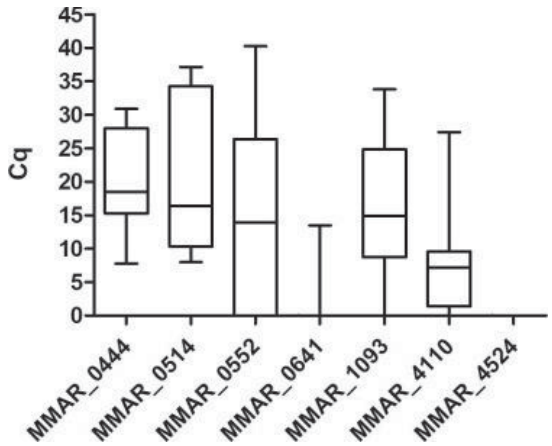

Figure S2. Cycle quantification (Cq) values of the seven studied *M. marinum* genes *in vivo*. Adult zebrafish were infected with a low dose of *M. marinum* (34 CFU/fish) and five weeks later, latent infections were reactivated with a one-week treatment of dexamethasone feeding. Gene expression levels were measured from kidney RNA samples. The box and whisker plot showing Cq values of each studied gene. Black lines and boxes represent the median and 25/75 percentiles. Whisker caps represent the minimum and maximum values. N = 6-8 samples / group.

Table S6. Statistical summary of the tested DNA vaccine antigens. The same data are visualized in the Figure3.

Group	N	Median CFU	10% Percentile	90% Percentile	% reduction compared to reactivated GFP	P
GFP latent	40	$5{,}15 \times 10^3$	$3{,}09 \times 10^2$	$1{,}09 \times 10^5$	87,6	0.03
GFP reactivation	28	$4{,}14 \times 10^4$	$4{,}08 \times 10^1$	$7{,}67 \times 10^5$	N/A	
MMAR_0444	27	$3{,}80 \times 10^4$	$2{,}02 \times 10^3$	$1{,}36 \times 10^6$	8,2	NS
MMAR_0552	27	$7{,}04 \times 10^4$	$1{,}81 \times 10^2$	$1{,}40 \times 10^6$	N/A	
MMAR_4524	30	$1{,}87 \times 10^4$	$4{,}01 \times 10^1$	$9{,}54 \times 10^5$	54,8	
MMAR_0641	36	$8{,}55 \times 10^4$	$1{,}11 \times 10^2$	$1{,}56 \times 10^6$	N/A	
GFP latent	44	$6{,}16 \times 10^3$	$3{,}27 \times 10^2$	$1{,}03 \times 10^5$	85,1	0.02
GFP reactivation	32	$4{,}14 \times 10^4$	$7{,}45 \times 10^1$	$7{,}15 \times 10^5$	N/A	
MMAR_0514	28	$1{,}25 \times 10^5$	$2{,}16 \times 10^3$	$2{,}25 \times 10^6$	N/A	NS
GFP latent	64	$1{,}01 \times 10^4$	$3{,}53 \times 10^2$	$1{,}62 \times 10^5$	76,2	0.002
GFP reactivation	62	$4{,}25 \times 10^4$	$8{,}54 \times 10^2$	$6{,}31 \times 10^5$	N/A	
MMAR_1093	58	$4{,}07 \times 10^4$	$2{,}21 \times 10^1$	$1{,}64 \times 10^6$	4,2	NS
GFP latent*	54	$1{,}21 \times 10^4$	$3{,}45 \times 10^2$	$1{,}60 \times 10^5$	83,0	0.0003
GFP reactivation*	48	$7{,}10 \times 10^4$	$2{,}55 \times 10^2$	$7{,}67 \times 10^5$	N/A	
MMAR_4110*	39	$2{,}52 \times 10^4$	$7{,}66 \times 10^1$	$6{,}11 \times 10^5$	64,5	0.03

*The data are also shown in Fig.5.