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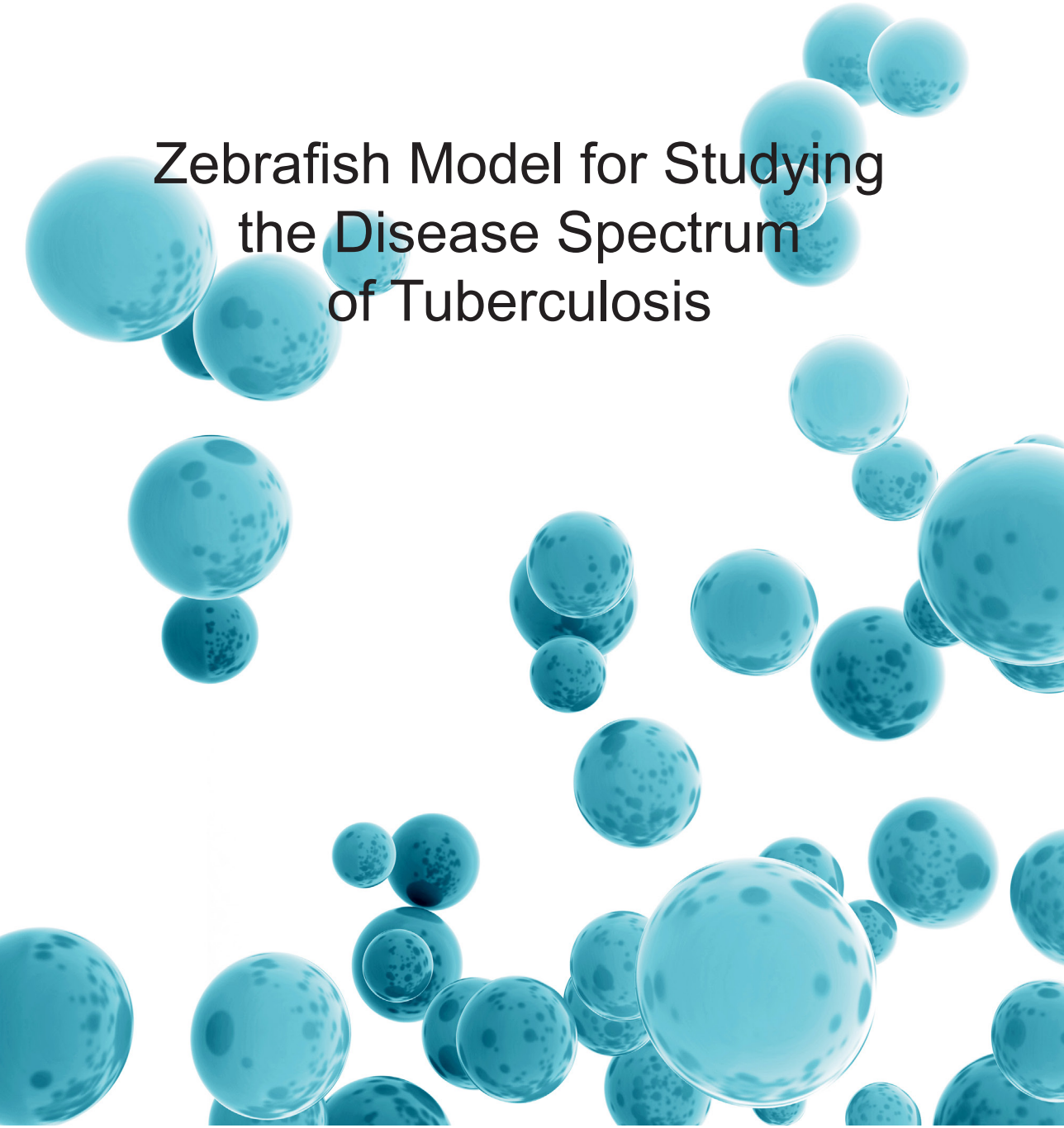
Zebrafish Model for Studying the Disease Spectrum of Tuberculosis



MILKA HAMMARÉN

Zebrafish Model for Studying the Disease Spectrum of Tuberculosis

ACADEMIC DISSERTATION
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UNIVERSITY OF TAMPERE

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ABSTRACT

Tuberculosis (TB) remains one of the deadliest infections worldwide killing 1.8 million people every year. Infection with *Mycobacterium tuberculosis* can lead to a spectrum of different outcomes including primary active and latent infection, the latter of which usually remains asymptomatic but has the potential to cause a severe, reactivated infection after decades of quiescence. The successful pathogenic strategies of *M. tuberculosis* allow the bacteria to circumvent and direct host immune responses to promote their own survival. The lack of an efficient and safe vaccine and the decreasing efficacy of antibiotic treatments against TB substantiate the urgent need for more powerful weaponry to control this devastating disease. Knowledge of the nature of protective immune responses furthers the development of prevention and treatment strategies against TB.

A myriad of studies elucidating the details of mycobacterial pathogenesis has been carried out in *in vitro* models. However, the outcome of an infection is a product of the complex interplay between the bacteria and a multitude of host cells necessitating the use of *in vivo* models. In this thesis, I have developed new methodology to utilize the adult zebrafish (*Danio rerio*) and its natural pathogen *Mycobacterium marinum* to model the pathogenesis of human tuberculosis. *M. marinum* is a close relative of *M. tuberculosis* and has been successfully used in zebrafish larvae to elucidate the interactions between the bacterium and innate immune cells. As the adaptive arm of immunity is known to centrally affect the pathogenesis of human TB, the adult zebrafish with a fully developed adaptive immune system was exploited in this study to establish an even more comprehensive model of mycobacterial disease.

In this study, injecting a large dose (~2000 bacteria) of *M. marinum* caused a progressive infection with high mortality. In contrast, injection of a small dose (<100 bacteria) created a vast disease spectrum closely resembling that seen in human TB with bacterial clearance (~10 %), primary active (~10 %), stable latent (~65 %) and reactivated infection (~15 %). The majority of the low-dose-infected fish remained asymptomatic during the 8-month follow-up, although the presence of mycobacteria could generally be verified by qPCR. Dormant bacteria were detected in the latently infected individuals with qPCR as well as by a novel plating method. The latent

infection could be experimentally reactivated by depletion of lymphocytes by gammairradiation.

The efficacy of lymphocyte depletion in causing reactivation of infection demonstrated the central role of these cells in controlling *M. marinum* infection in the fish. The importance of adaptive immunity was further verified by showing that r*ag1* (-/-) mutants lacking functional lymphocytes had higher mortality and developed higher mycobacterial loads with less dormant bacteria.

With the qPCR methodology developed for this study, it was possible to measure mycobacterial loads, host immune responses and mycobacterial dormancy from each individual to find associations between the host immune response and the control of mycobacterial growth. Spontaneous reactivation of *M. marinum* infection was accompanied by an increase in total T cell number. *Foxp3* expression was specifically increased compared to latent infection reflecting an increase in regulatory T helper responses in a reactivated infection. The role of regulatory T cell responses in the context of the reactivation of human tuberculosis should be assessed in future investigations. Among the individuals with an asymptomatic infection, high T cell numbers were found to be associated with limited mycobacterial growth. In the individuals with the most progressed mycobacterial infection, T helper 2 (Th2) responses were specifically lacking. The low Th2 response was also seen in spontaneously reactivated infection. So far, TB diagnostics and vaccination strategies have heavily relied on the measurement and induction of Th1 responses. The biomarker potential of Th2 responses to estimate the risk of reactivation should be studied in human blood samples. Moreover, based on these results, the possible protective effects Th2 responses in the context of vaccine induced immunity against tuberculosis should be reassessed.

The adult zebrafish-*M. marinum* model was also used to test immunoactivators to study, whether the host immune response could be directed to more efficiently kill mycobacteria. Injection with heat-killed *Listeria monocytogenes* 1 day prior to low-dose infection with *M. marinum* led to significantly lower mycobacterial loads. Importantly, the proportion of individuals able to clear the infection was significantly increased by priming. The bacterial clearance occurred also in the absence of adaptive immunity as shown by using *rag1 (-/-)* fish. The protective effect was accompanied by the up-regulation of the genes encoding tumor necrosis factor α (Tnfα), nitric oxide synthase 2b (Nos2b) and the down-regulation of the gene encoding superoxide dismutase 2 (Sod2), suggesting that intracellular killing mechanisms were more activated in the primed group. These findings pave the way for the development of

host-directed therapies based on the direct induction of optimal innate immune responses against pathogenic mycobacteria.

The adult zebrafish-*M. marinum* system is a convenient non-mammalian model of the entire spectrum of mycobacterial disease, including spontaneous latency with dormant mycobacteria. It could serve as an *in vivo* platform to screen experimental vaccination strategies against latent and reactivated infection or treatments targeted at dormant mycobacteria. Due to the small size of the zebrafish and the sensitive qPCR-based method developed for detecting both actively dividing and dormant mycobacteria, bacterial clearance can be assessed in the zebrafish more reliably than any other animal. Thus, the zebrafish is an indispensable model in the quest for sterilizing treatments against tuberculosis.

TIIVISTELMÄ

Tuberkuloosi on yksi maailman tappavimmista infektioista: vuosittain 1,8 miljoonaa ihmistä kuolee *Mycobacterium tuberculosis* -bakteerin aiheuttamaan tautiin. Tuberkuloosibakteerin aiheuttama tautikirjo on laaja, ja bakteerille altistuminen voi johtaa akuuttiin infektioon tai latenttiin eli oireettomaan tautiin, joka saattaa aiheuttaa vakavan taudin jopa vuosikymmenien hiljaiselon jälkeen. Mykobakteerien pitkälle kehittyneet virulenssimekanismit estävät ja muovaavat isäntäeliön puolustusjärjestelmän toimintaa, mikä mahdollistaa mykobakteerien selviytymisen elimistössä. Nykyisen tuberkuloosirokotteen heikon tehon ja turvallisuusongelmien vuoksi rokote on poistettu suomalaisesta rokoteohjelmasta, ja lisääntyvä antibioottiresistenssi hankaloittaa taudin hoitoa yhä enenevissä määrin. Uusien, tehokkaampien rokotteiden ja hoitomuotojen kehittämiseen tarvitaan yksityiskohtaista tietoa taudilta suojaavien immuunivasteiden luonteesta.

Suuri osa mykobakteeri-infektioiden patogeneesin yksityiskohdista on selvitetty soluviljelymalleja käyttäen. Koska bakteerin ja eri tyyppisten isäntäsolujen yhteispeli vaikuttaa infektion kulkuun monimutkaisella tavalla, laajempien tutkimuskysymysten selvittelyyn tarvitaan eläinmalleja. Tätä väitöstutkimusta varten olen kehittänyt metodiikkaa, joka hyödyntää aikuisen seeprakalan (*Danio rerio*) ja *Mycobacterium marinum* -infektion uudenlaisena ihmisen tuberkuloosimallina. *M. marinum* on luontainen kalapatogeeni, joka on läheistä sukua *M. tuberculosis* -bakteerille. *M. marinum*ia on käytetty menestyksekkäästi seeprakalan poikasmallissa mykobakteerien ja synnynnäisen immuniteetin solujen välisen vuorovaikutuksen tutkimiseen. Koska hankinnaisen immuniteetin tiedetään vaikuttavan keskeisesti tuberkuloosin patogeneesiin, tässä väitöstutkimuksessa käytettiin aikuisia kaloja, joilla nämäkin mekanismit ovat pitkälle kehittyneet.

Tässä väitöskirjatutkimuksessa havaittiin, että suuren *M. marinum* -annoksen (n. 2000 bakteeria) injektointi aiheutti vakavan taudin suurelle osalle kaloista ensimmäisten viikkojen aikana. Pienen annoksen infektio (<100 bakteeria) sen sijaan aiheutti ihmisen luonnolliselle tuberkuloosi-infektiolle tyypillisen laajan tautikirjon, jossa n. 10 % kykeni välttämään infektion, n. 10 % sai aktiivisen primääri-infektion, n. 65 % kehitti latentin infektion ja n.15 % itsekseen uudelleen aktivoituneen taudin latentin vaiheen jälkeen. Matalan annoksen infektio oli suurimmalla osalla kaloista

oireeton koko 8 kuukauden seurantajakson ajan. Latenttia infektiota kantavista kaloista pystyttiin todentamaan uinuvia mykobakteereja sekä qPCR:n että tässä tutkimuksessa kehitetyn maljausmenetelmän avulla. Latentin infektion aktivaatio voitiin aiheuttaa kokeellisesti poistamalla lymfosyyttejä gammasäteilytysmenetelmällä.

Lymfosyyttien poistamisen aiheuttama latentin taudin aktivaatio oli yksi osoitus näiden solujen tärkeydestä kalan *M. marinum* -infektiossa. Hankinnaisen immuniteetin tärkeys varmistui kokeissa, joissa matalan annoksen *M. marinum* -infektio aiheutti lymfosyyttipuutteisille *rag1* (-/-) -kaloille vakavan taudin. Verrattuna villityypin kaloihin, *rag1*-mutanttien infektiossa bakteerimäärät kasvoivat korkeammiksi, uinuvia bakteereja oli vähemmän ja kalojen kuolleisuus oli korkeampi.

Tarkoituksiimme sopiviksi kehittämieni qPCR:ään perustuvien menetelmien avulla oli mahdollista mitata mykobakteerien määrää, kalan immuunivastetta ja mykobakteerin uinumista samasta yksilöstä ja etsiä yhteyksiä immuunivasteen ja mykobakteerien kasvun välillä. Itsestään uudelleen aktivoituneeseen infektioon liittyi T-solujen kokonaismäärän kasvu. Erityisesti säätelevään auttaja-T-soluvasteeseen liittyvän *foxp3*:n tuotto lisääntyi. Säätelevien T-soluvasteiden merkitys ihmisen latentin tuberkuloosin aktivaatiolle on mielenkiintoinen tulevaisuuden tutkimusaihe. Oireettomassa populaatiossa korkea T-solujen kokonaismäärä oli yhteydessä isännän kykyyn rajoittaa mykobakteerien kasvua. Korkea mykobakteerimäärä oli yhteydessä matalaan tyypin 2 auttaja-T-soluvasteeseen (Th2). Th2-vaste oli matala myös uudelleenaktivoituneessa taudissa. Ihmisen tuberkuloosidiagnostiikassa ja rokotteiden kehityksessä on tähän asti korostettu Th1-vasteita. Olisi syytä tutkia, voisiko Th2-vasteita mitata ihmisen verinäytteistä latentin taudin aktivoitumisriskin arvioimiseksi. Th2-vasteiden mahdollista hyödyllisyyttä tuberkuloosirokotteissa olisi myös hyvä arvioida uudelleen.

Seeprakalamallissa testattiin myös immuunivastetta muokkaavia aineita, joita injektoitiin kaloihin ennen infektointia. Tarkoituksena oli löytää käsittely, joka aktivoisi immuunivastetta tuhoamaan mykobakteereja tehokkaammin. Lämpötapetun *Listeria monocytogenes* -bakteerin injektointi päivää ennen matalan annoksen *M. marinum* -infektiota laski mykobakteerien määrää sekä lisäsi infektoitumiselta suojautuneiden kalojen osuutta. Käsittely suojasi myös lymfosyyttipuutteisia *rag1* (-/-) -kaloja. Käsittely aiheutti tuumorinnekroositekijä alfaa (Tnfα) sekä typpioksidisyntaasi 2b:ä (Nos2b) koodaavien geenien ilmentymisen lisääntymistä ja superoksididismutaasi 2:a (Sod2) koodaavan geenin ilmentymisen vähenemistä, mikä viittaa solunsisäisten tappomekanismien tehostumiseen.

Löydökset ovat tärkeitä uudentyyppisten, isännän immuunivasteita ohjailevien hoitomuotojen kehittämisen kannalta.

Aikuisen seeprakalan *M. marinum* infektio on yksinkertainen ja käyttökelpoinen malli, jossa voidaan tutkia tuberkuloosin tautikirjoa. Seeprakalassa kehittyy latentti infektio, jossa nähdään myös uinuvia mykobakteereja. Se on lupaava *in vivo* -malli, jossa voitaisiin tutkia aivan uudenlaisia, latenssia ja uudelleenaktivaatiota estäviä rokotteita sekä etsiä uinuviin bakteereihin tehoavia hoitomuotoja. Tässä työssä kehitetyllä herkällä qPCR-menetelmällä voidaan havaita sekä aktiiviset että uinuvat mykobakteerit seeprakalan kaikista kudoksista. Seeprakalan *M. marinum* -infektio on näin ollen verraton työkalu tuberkuloosi-infektion sterilointiin tähtäävien hoitomuotojen kehittämiseen.

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LIST OF ORIGINAL COMMUNICATIONS

The thesis is based on the following original publications, which are referred to by their Roman numerals.

I Parikka M*, **Hammarén MM***, Harjula SK, Halfpenny NJ, Oksanen KE, Lahtinen MJ, Pajula ET, Iivanainen A, Pesu M, Rämet M. *Mycobacterium marinum* causes a latent infection that can be reactivated by gamma irradiation in adult zebrafish. PLoS Pathog. 2012 Sep;8(9):e1002944.

II **Hammarén MM**, Oksanen KE, Nisula HM, Luukinen BV, Pesu M, Rämet M, Parikka M. Adequate Th2-type response associates with restricted bacterial growth in latent mycobacterial infection of zebrafish. PLoS Pathog. 2014 Jun 26;10(6):e1004190.

III Luukinen HM, **Hammarén MM***, Vanha-aho LM*, Svorjova A, Kantanen L, Luukinen BV, Hytönen V, Parikka M. Priming of innate antimycobacterial immunity by heat-killed *Listeria monocytogenes* induces sterilizing response in the adult zebrafish tuberculosis model.*(Submitted manuscript)*

*Equal contribution

ABBREVIATIONS

BCG	Bacillus Calmette-Guérin
Cfu	colony-forming unit
CT	computer tomography
DC	dendritic cell
Dpf	days post fertilization
Ef1α	elongation factor 1 alpha
FDG PET/CT	[18F]-fluoro-2-deoxy-D-glucose (FDG) positron emission tomography combined with computed tomography
Foxp3	forkhead box P3
Gapdh	glyceraldehyde 3-phosphate dehydrogenase
GltA1	citrate synthase
Gy	Gray
HIV	human immunodeficiency virus
HKEc	heat-killed *Escherichia coli*
HKLm	heat-killed *Listeria monocytogenes*
HKMm	heat-killed *Mycobacterium marinum*
HKSi	heat-killed *Streptococcus iniae*
Ifnγ	interferon gamma
Ig	immunoglobulin
IGRA	interferon gamma release assay
IL	intereukin
i.p.	*intraperitoneal*
LDLN	lung-draining lymph node
LPS	lipopolysaccharide
Lta4h	leukotriene-A4 hydrolase
LTBI	latent tuberculosis infection
MDP	muramyl-dipeptide
MDR-TB	multi-drug resistant tuberculosis
MHC	major histocompatibility complex

MMITS	*Mycobacterium marinum* internal transcribed spacer
Mpi	months post infection
Mtb	*Mycobacterium tuberculosis*
NCC	non-specific cytotoxic cells
NK-cells	natural killer cells
NO	nitric oxide
Nos2	nitric oxide synthase 2
PET	positron emission tomography
QPCR	quantitative polymerase chain reaction
Q-RT-PCR	quantitative reverse transcription polymerase chain reaction
Rag	recombination-activating gene
ROC	receiver operating characteristic
ROS	reactive oxygen species
Rpf	resuscitation-promoting factor
TB	tuberculosis
Tbx21	t-box transcription factor 21
TDR-TB	totally drug-resistant tuberculosis
Th	T helper
Tnf	tumor necrosis factor
TLR	toll-like receptors
Treg	regulatory T cell
TST	tuberculin skin test
WHO	World's Health Organization
Wpf	weeks post fertilization
Wpi	weeks post infection
wt	wild-type
XDR-TB	extremely drug-resistant tuberculosis
ZIRC	Zebrafish International Resource Center

1 INTRODUCTION

Between the seventeenth and nineteenth centuries, *Mycobacterium tuberculosis* (*Mtb*) was the pathogen responsible for 20 % of deaths in the Western world (Comas, Coscolla et al. 2013). Based on whole-genome sequence analysis, *Mtb* has plagued the human kind for at least 70,000 years (Comas, Coscolla et al. 2013). During this time, *Mtb* has refined its virulence strategies to perfection. Despite the advances of modern medicine, antibiotics and the Bacillus Calmette-Guérin (BCG) vaccine, tuberculosis (TB) remains one of the deadliest infections killing 1.8 million people every year (World Health Organization 2016).

Mtb is enters the body through the inhalation of droplets containing aerosolized mycobacteria released from the airways of a person with active TB. In 85 % of the cases, the lungs are the main site of infection, although almost any other tissue can get colonized by this pathogen. (Dheda, Barry 3rd et al. 2016). In pulmonary TB, the most apparent symptom is the prolonged cough lasting more than three weeks. General symptoms also include fever, weight loss and night sweats. (Sia, Wieland 2011). In an immune competent host, instead of a primary active infection, more commonly, *Mtb* causes a latent, subclinical infection, during which the majority of the bacteria are thought to remain in a dormant state (Lin, Flynn 2010). Should the host immune system become weakened, the latent infection can reactivate causing severe, contagious disease even after decades after the initial infection (Lin, Flynn 2010).

The efficacy of the BCG vaccine is highly variable ranging between 0 and 80 % (Fine 1995) and although it protects children against active disease, it is inadequate in preventing pulmonary TB in adults (Andersen, Doherty 2005) or latent and reactivated infection (Andersen, Woodworth 2014). As BCG contains live, weakened bacteria, there are also some safety concerns, which have limited its use (Andersen, Woodworth 2014). Despite enormous efforts, to date, the scientific community has not been able to develop a vaccine superior to BCG (Andersen, Woodworth 2014). A vaccination strategy leading to sterilizing immunity against TB is yet to be developed.

Antibiotic treatment of TB is also problematic. A person with active T disease is treated for 6 months with a cocktail of four different antibiotics. According to recent,

rather unsettling findings, even when successfully completed, in at least 35 % of the cases, this regimen does not lead to eradication of the pathogen, but rather drives the bacteria to a quiescent mode with the potential to cause relapse of the disease (Malherbe, Shenai et al. 2016). Antimicrobial treatments that efficiently kill such quiescent, dormant mycobacteria are required for the total eradication of *Mtb*. In addition, the emergence and spread of *Mtb* strains with a genetic resistance to all currently used antibiotics poses a massive challenge for controlling TB. Therefore, alternative treatment approaches, such as host-directed therapies based on optimal modulation of the immune response (Tobin 2015) should be developed.

There are some antibiotic regimens that are useful in preventing reactivation of latent infection (Vernon 2013, Ai, Ruan et al. 2016). However, as latent TB (LTBI) is so common, prophylactic antibiosis against reactivation should be limited only to high-risk populations. Allocation of the prophylactic treatment is difficult due to the lack of reliable diagnostics to assess the risk of reactivation. (Schnappinger, Ehrt 2016)

Unravelling the mysteries of mycobacterial infections that result from the complex interplay between the host and pathogen, require the use of appropriate animal models. Mice, rabbits and monkeys have been widely used for modelling human TB (Myllymäki, Niskanen et al. 2015). In this Doctoral Thesis, I have set up, characterized and put to use a system, in which adult zebrafish (*Danio rerio*) are infected with *Mycobacterium marinum* to elucidate the pathogenesis of TB in a simple vertebrate model.

2 REVIEW OF THE LITERATURE

2.1 Epidemiology of TB

According to World's Health Organization (WHO), in 2015, 10.4 million people developed active TB, and 1.8 million people died of the infection. These numbers make TB one of the deadliest infections on the globe. (World Health Organization 2016). Although compared to any other pathogen, *Mtb* has a rather low infection rate (a person with active TB disease infects 3-10 people per year (Van Leth, Van der Werf et al. 2008), one third of the human population are estimated to be infected with *Mtb* (World Health Organization 2016). However, only around 5-15 % of these individuals develop an active disease, whereas others remain asymptomatic (Vynnycky, Fine 1997).

One most prominent host-related, environmental risk factors for developing active TB is a co-infection with human immunodeficiency virus (HIV) (Havlir, Getahun et al. 2008). Other significant risk factors (generally increasing the risk more than 10-fold, listed starting from the highest risk) include chronic renal failure requiring dialysis, use of transplantation-related immunosuppressives and undernourishment (Dheda, Barry 3rd et al. 2016). In addition, cancer, silicosis, alcohol abuse, diabetes, smoking as well as the use of gluococorticoids or tumor necrosis factor alpha (Tnfα) inhibitors are associated with heightened TB-risk. (Dheda, Barry 3rd et al. 2016) Although 12 % of the new active TB cases are diagnosed in HIV positive individuals (Havlir, Getahun et al. 2008), it has to be noted that undernutrition and indoor air pollution are estimated to contribute even more to prevalence of active TB (27 % and 22 %, respectively) (Lönnroth, Castro et al. 2010). The radical >100-fold reduction in TB-related deaths in the US and Europe from 1900 to 1980 is thought to mainly result from the improved hygiene and socioeconomic conditions (Lienhardt, Glaziou et al. 2012).

The disease-burden is geographically unevenly distributed, so that African countries are most heavily affected (Pai, Behr et al. 2016), primarily due to high prevalence of HIV (World Health Organization 2016). Based on the data presented in the previous paragraph, also the poor living conditions in developing countries contribute to the high disease load. It can be speculated that in addition to co-

morbidity and environmental factors, genetic traits of the host affect the geographical distribution as many genetic polymorphisms within the human population have been reported to affect susceptibility to TB (Hawn, Dunstan et al. 2006, Thuong, Dunstan et al. 2008, Ma, Liu et al. 2007).

The recent emergence of antibiotic resistant strains of *Mtb* pose a tremendous challenge to controlling the TB epidemic. Multi-drug resistant TB (MDR-TB, resistant to both rifampicin and isoniazid) has a global prevalence of ~5%. The prevalence is highest in the area of the former Soviet Union (>20 %)(World Health Organization 2014). Lately, resistance genes have also started emerging in TB strains in China, where ~25 % of cases are caused by *Mtb* resistant to either rifampicin or isoniazid (Zhao, Xu et al. 2012). In India, even totally drug resistant strains have developed (Udwadia, Amale et al. 2012). The situation with antibiotic resistance is grim and calls for immediate action both in national governments and re-direction of the efforts and funding of medical industry.

2.2 Diagnostics of TB

The tuberculin skin test (TST), a.k.a. the Mantoux test, is carried out by injecting purified protein derivative intradermally and measuring the transverse diameter of the localized reaction around the injection site (Pai, Denkinger et al. 2014). This delayed-type hypersensitivity reaction is indicative of T-cell-mediated immune responses to tuberculous antigens (Pai, Denkinger et al. 2014). The reaction, however, can also develop due to previous BCG vaccination or exposure to non-tuberculous mycobacteria leading to false positive results (Farhat, Greenaway et al. 2006). False negative results can arise due to T cell anergy caused by active TB infection or in immunocompromised patients, who actually often are at greatest risk of developing active TB once infected (Menzies 2000).

IGRA (interferon gamma release assay) is a more modern method to assess previous or current exposure to *Mtb*. IGRA is more *Mtb*-specific than TST as it does not respond to T cells induced by BCG vaccination (Pai, Denkinger et al. 2014). IGRA is based on measuring the Ifnγ (interferon gamma) release from T cells stimulated with early secreted antigenic target 6 and culture filtrate protein 10 (Pai, Denkinger et al. 2014). As these proteins are also present in other virulent mycobacteria, such as *M. marinum* and *M. kansasii* (Arend, van Meijgaarden et al. 2002) IGRA is not entirely *Mtb*-specific.

Both TST and IGRA generally remain positive during a latent infection or even after successful clearance of the infection in case T cell responses have been activated (Barry, Boshoff et al. 2009, Esmail, Barry et al. 2014). Therefore, these methods alone are not sufficient in deciphering the status of the infection. Reliable diagnosis of active tuberculosis is traditionally based on an X-ray or more recently on a computer tomography (CT) scan, in which abnormal findings will lead to further microbiological testing, such as microscopy and the culturing of sputum samples. A later addition to TB diagnostics is the Xpert MTB/RIF based on the amplification of mycobacterial DNA and is now recommended as the first-line diagnostic method by WHO (Pai, Behr et al. 2016).

Despite the vast variety of methods currently available, the diagnostic tool pack is incomplete as diagnostic markers for the risk of reactivation of LTBI are still lacking (Escalante, Peikert et al. 2015). Such markers would allow the development of novel diagnostics to avoid unnecessary prophylactic antibiosis during LTBI.

2.3 Spectrum of TB

The outcome of infection with *Mtb* are various and can be anything between full clearance and a lethal, generalized infection. Both bacterial and host factors as well as environmental conditions affect the outcome of disease as described in the section "Epidemiology of TB". An over-simplified dichotomy divides the outcomes of primary infection to either active or latent TB according to whether symptoms develop or not: Active TB with overt signs of disease is induced in 5-15% of *Mtb*-exposed individuals, whereas the rest remain asymptomatic (Delogu, Goletti 2014). However, there is a spectrum of different disease states within this asymptomatic population. Some have been able to clear the infection by innate immune responses while others have mounted suboptimal adaptive responses leading to a latent infection with a 10 % life-time risk of reactivation (Delogu, Goletti 2014).

2.3.1 Primary active TB

Although pulmonary TB is the most common form of TB, *Mtb* can colonize essentially any organ. General symptoms of TB include fever, weight loss and night sweats. In pulmonary TB, the most apparent symptom is the prolonged cough lasting more than three weeks. The diagnosis of active TB is based on the clinical,

epidemiological, radiographic, microbiological as well as histopathological features. Controlling active pulmonary TB is of high epidemiological importance, as coughing patients with active TB spread infectious aerosols leading to transmission of the disease. (Sia, Wieland 2011)

2.3.2 Latent and reactivated TB

In LTBI, the immune system is able to limit and wall off the primary infection, but the disease has the potential to reactivate later in life, should the immune system of the host become weakened (Barry, Boshoff et al. 2009, Esmail, Barry et al. 2014). Necropsies collected from individuals with LTBI reveal fibrotic and necrotic granulomas with calcification (Dannenberg Jr 2006). According to a commonly supported hypothesis, these hypoxic lesions contain dormant, non-replicating mycobacteria, which, when resuscitated from dormancy, cause the reactivated infection (Barry, Boshoff et al. 2009).

LTBI is diagnosed when a person without typical symptoms of TB has a positive reaction in TST marking previous or on-going T-cell exposure to mycobacterial antigens (Barry, Boshoff et al. 2009). However, according to the current understanding, LTBI has a wide clinical spectrum within this asymptomatic group: Some TST-positive individuals may have actually cleared the infection completely with the help of adaptive responses, whereas others have lesions with actively replicating bacteria (Barry, Boshoff et al. 2009). The hypothesis has initially arisen from imaging studies utilizing PET (positron emission tomography) and CT combined with ^{18}F-fluorodeoxyglucose, which assess the metabolic activity of lung lesions in individuals with latent TB. A range of lesion types was found, some of which were indistinguishable from lesions in patients with active TB (Goo, Im et al. 2000). Results gained using the non-human primate model for TB have supported the idea of disease spectrum within the latent population (Lin, Rodgers et al. 2009).

The research efforts to understand, diagnose and prevent latent mycobacterial infection are necessary for controlling the TB epidemic, as the huge burden of latent TB poses a severe threat through the constant risk of reactivation. The fact that the bacteria can persist within the host for decades also makes the evolution of antimicrobial resistance within the mycobacterial genome more likely. The factors leading to the establishment and control of latent TB are poorly characterized. Neither is there a diagnostic method for estimating the risk of reactivation currently available (Schnappinger, Ehrt 2016). As the current vaccine does not prevent latent

or reactivated infection (Andersen, Woodworth 2014), more knowledge on these forms of TB are needed for the development of comprehensive treatment and prevention modalities.

2.3.3 Early and late clearance of TB

There is both epidemiological as well as genetic data supporting the idea of the early clearance of TB (Verrall, G Netea et al. 2014). As reviewed by Verrall et al., in different human populations, a proportion between 7 and 43 % of individuals remains TST-negative despite severe exposure to *Mtb* suggesting early (innate) resistance to the pathogen (Verrall, G Netea et al. 2014). Some genetic loci are associated with lack of TST response despite continuous exposure to *Mtb* (Cobat, Gallant et al. 2009). Genetic variability in the area encoding TNFα is one of the factors linked to innate resistance to TB in humans (Cobat, Poirier et al. 2015). This finding is in line with the notion that anti- TNFα therapies used for treating inflammatory disorders cause increased susceptibility to active TB (Dobler 2016).

However, based on studies assessing TST reversion (spontaneous change of a TST-response of a non-treated, latently infected individual from positive to negative between two different measurements) it has been hypothesized that ~10 % of individuals in whom mycobacterium specific adaptive response becomes activated are able to clear the infection (Jasenosky, Scriba et al. 2015). This population could be named as "late clearers" as adaptive immunity is thought to be involved in the process.

Better understanding of the mechanisms behind innate and adaptive resistance to mycobacterial infection provide the basis for the development of adjunctive therapies aiming at the optimal modulation of host immune responses and novel vaccine strategies, respectively. The methodology available in human studies to gain knowledge on the mechanisms required for mycobacterial clearance are rather limited. The results presented above rely on the (faulty) assumption of negative TST and/or IGRA test (*i.e.* lack of adaptive immune response toward mycobacterial antigens) reflecting the clearance of tuberculous bacteria, the direct and thorough verification of which is not feasible in humans (Verrall, G Netea et al. 2014). Therefore, studying the mechanisms behind mycobacterial clearance necessitate the use of animal models, in which the direct assessment of the mycobacterial loads is possible.

2.4 Infection biology of TB

Considering the vast disease spectrum caused by *Mtb* as well as the fact that humans (and other primates (Robertson, Altmann et al. 2012)) are the only reservoir(s) for *Mtb*, the bacterium can be regarded as both a symbiont and a pathogen of humans (Comas, Coscolla et al. 2013). Based on whole-genome sequence analysis, the two species have lived together and co-evolved for at least 70,000 years (Comas, Coscolla et al. 2013). *Mtb* has been successful in developing mechanisms to maintain itself within the host for a prolonged period of time and to transmit itself to a new host at a sufficient rate ensuring its survival. Studying the infection biology of such closely evolved host-bacterium pair has intrigued researchers around the world, and so far, many details about the course of infection have been elucidated, mainly using various cellular and animal models. However, crucial bits of information, namely those required for the development of sterilizing treatments, are still lacking.

2.4.1 The cornerstones of innate immune responses against TB

Mtb enters the body through inhaled droplets and enters alveolar macrophages (Guirado, Schlesinger 2013). A single bacterium is thought to be sufficient for the establishment of infection (Rajaram, Ni et al. 2014). Alveolar macrophages play a dual role in the pathogenesis as on one hand, they form the first line of defense but on the other hand, also serve as host cells for the mycobacteria often allowing their initial growth and establishment of infection (Leemans, Juffermans et al. 2001, Leemans, Thepen et al. 2005, Samstein, Schreiber et al. 2013). The mycobacteria enter macrophages via receptor-mediated phagocytosis (Berrington, Hawn 2007, Jo 2008). The pathogen recognition receptors, such as toll-like receptors (TLRs) on macrophages contribute to the activation of intracellular killing mechanisms (Jo 2008, Jo, Yang et al. 2007, Reiling, Ehlers et al. 2008, Kleinnijenhuis, Oosting et al. 2011) which has been thought to mainly rely on the production of nitric oxide (NO) (Nicholson, Bonecini-Almeida Mda et al. 1996) and reactive oxygen species (ROS)(Roca, Ramakrishnan 2013). However, although NO is efficient in killing mycobacteria within murine macrophages in a TLR2-dependent manner (Thoma-Uszynski, Stenger et al. 2001, Chan, Xing et al. 1992), the efficacy of this mechanism in human macrophages has been questioned (Jung, Madan-Lala et al. 2013). At the level of cytokines, Ifnγ (Flynn, Chan et al. 1993) and Tnfα (Roca, Ramakrishnan 2013) have been regarded as crucial for limiting intracellular growth. However, in

many cases, innate responses are insufficient for the efficient control of TB, as virulent mycobacteria hamper intracellular killing mechanisms and modify the host immune response for their advantage to spread to new locations in the body (See section 2.4.3). More knowledge on how to optimally boost innate immune responses in TB is needed for the development of host-directed immunomodulatory treatments.

2.4.2 Adaptive immune responses in TB

T cells can be roughly divided in two main groups: CD8+ cytotoxic T cells and CD4+ T helper (Th) cells. Cytotoxic CD8+ cells recognize foreign antigens presented on MHCI (major histocompatibility complex) proteins on the surface of infected host cells and exert their cell lysing functions on such cells. CD4+ T helper cells recognize antigens presented on MHCII molecules that sample the contents of phagocytic vesicles. T helpers do not attack cells directly but rather provide assistive functions through cytokine secretion and the licensing of antibody-producing B cells through providing the required co-stimulatory signals. (Murphy, Weaver 2016)

Th cells can differentiate into various subtypes with characteristic cytokine profiles leading to appropriate antigen-specific effector functions. The best characterized T helper cell subgroups are Th1, Th2, Th17 and Treg (regulatory) cells. Th1 responses are important in the eradication of intracellular pathogens, whereas the Th2 subtype is induced as a response to extracellular pathogens or phagocytosed antigens. Th1 cytokines enhance the lysis of intracellular pathogens and aid the production of opsonizing antibodies from B cells. Th2 cytokines, at the other end, promote humoral responses from B cells and the production of antibodies to contain extracellular infections, especially helminth and parasite infections. Th17 responses activate neutrophils to combat extracellular bacteria, whereas Tregs serve an entirely different role though their distinct cytokine profile that suppresses all other T cell responses. (Murphy, Weaver 2016)

Th cells are known to play a critical role in inhibiting active TB (Boom, Canaday et al. 2003, Lin, Rutledge et al. 2012). Mice lacking Th cells are hypersusceptible to TB (Mogues, Goodrich et al. 2001) and reduced Th levels in HIV positive people increases the risk of infection and the development of reactivated TB (Pawlowski, Jansson et al. 2012). The classical paradigm underlines the critical contribution of Th1 CD4+ helper cells to controlling TB (reviewed in (Mayer-Barber, Barber 2015)), as low levels of Ifnγ, an important Th1 cytokine associate with increased

susceptibility to active TB (Rossouw, Nel et al. 2003), (Altare, Lammas et al. 1998, Altare, Durandy et al. 1998, Fieschi, Dupuis et al. 2003, Picard, Fieschi et al. 2002). Th1 and Th2 are generally thought to counteract each other, and therefore Th2 cells have at some point been claimed to even be detrimental in TB (Orme, Roberts et al. 1993, Hernandez-Pando, Orozcoe et al. 1996). Later, evidence supporting the opposite view have also emerged suggesting a possible role for Th2, B cells and antibodies in controlling TB (Chan, Mehta et al. 2014, Jung, LaCourse et al. 2002, Hammarén, Oksanen et al. 2014). In addition, Th17 may exert protective effects in TB, especially in the early stage of the disease (reviewed in (Jasenosky, Scriba et al. 2015)).

Polyfunctional T helper cells producing more than one cytokine are generally thought to provide more extensive control over infection compared to those with a narrower cytokine profile. Such cells have been under intense research during the last years. In the context of TB vaccination, polyfunctional T cells producing Ifnγ, TNFα and IL-2 are considered to be the most effective, as they promote T cell proliferation alongside with effector functions (Seder, Darrah et al. 2008). However, in a large number of studies assessing the frequency of these trifuncional T cells in patients with active TB versus latent TB have produced controversial results (reviewed in (Jasenosky, Scriba et al. 2015)), suggesting that there are many other factors besides trifunctional helper T cells that determine the outcome of infection.

There are less studies assessing the role of CD8+ cells in TB. Mouse studies on the role of CD8+ killer T cells in TB are controversial, but in the non-human primate model also these cells seem to have the ability to limit TB (Chen, Huang et al. 2009). In human patients with active TB, the cytotoxic activity of CD8+ cells is impaired (Smith, Klein et al. 2000, Andersson, Samarina et al. 2007). Terminally differentiated CD8+are reduced in patients with active TB (Caccamo, Guggino et al. 2009). Overall, it seems that in patients with active TB, CD8+ cells are arrested to an intermediate differentiation stage in which their proliferative as well as cytotoxic effector functions are impaired (Jasenosky, Scriba et al. 2015). It therefore seems plausible that even though the (terminally differentiated) CD8 cells would have the capacity to limit mycobacterial growth, in active TB cases, CD8 cells are "paralyzed" and are likely not the factor that has initially determined the outcome of infection.

Although adaptive immune responses against TB have been well characterized in various models, the attempts to induce a sterilizing adaptive immune response by vaccination in humans so far have been unsuccessful (Andersen, Woodworth 2014). The knowledge on some key components of the induction of an optimal adaptive immune response against pathogenic mycobacteria is still missing.

2.4.3 Bacterial manipulation of host responses

Detailed comparisons of the avirulent BCG vaccine strain of *M. bovis* with virulent mycobacteria led to the identification of the mycobacterial RD1 locus as a centrally important virulence locus (Mahairas, Sabo et al. 1996, Lewis, Liao et al. 2003). The locus encodes a secretory system called ESX-1 (Abdallah, Van Pittius, Nicolaas C Gey et al. 2007) and has been extensively studied. It is the most important locus linked to the intracellular survival of mycobacteria in alveolar macrophages. Firstly, ESX-1 has been found to block the fusion of phagosomes with lysosomes allowing the bacteria to survive within phagocytic vesicles (Russell 2011). It has also been shown to be responsible for the translocation of virulent mycobacteria to the cytoplasm of the host cell to escape killing within phagosomes (Simeone, Bobard et al. 2012, Houben, Demangel et al. 2012). Virulent mycobacteria differ from avirulent ones also in that they produce enzymes with the ability to neutralize NO species providing an additional mechanism to escape intracellular killing (Flynn, Chan 2003).

In addition to its ability to avoid intracellular killing by phagocytic cells, *Mtb* has been found to delay the onset of adaptive immune response in many different ways. This delay provides a prolonged time for the bacteria to replicate prior to initiation of effective adaptive responses (Chackerian, Alt et al. 2002). Effector T cells peak only at around 28 days of initial *Mtb* exposure (Gallegos, Pamer et al. 2008). During the first week of infection, it seems that antigens cannot be efficiently presented (Gallegos, Pamer et al. 2008). Dendritic cells (DC) are a central target for pathogenic manipulation by mycobacteria, as these cells play a central role in the activation of adaptive responses. Mannosylated lipoarabinomannan on the surface of pathogenic mycobacteria induces the production of IL10 from DCs leading to impairment of DC maturation and co-stimulatory molecules required for efficient effector T cell activation (Geijtenbeek, Van Vliet et al. 2003). Mycobacteria are also known to inhibit antigen presentation by down-regulating MHCII expression (Wolf, Linas et al. 2007) Another mechanism to delay the functions of adaptive immunity is through the modulation of the type of cell-death of phagocytic cells (Behar, Divangahi et al. 2010). Virulent *Mtb* strains promote necrosis and inhibit apoptosis (Behar, Divangahi et al. 2010). A pro-apoptotic strain of *Mtb* was found to be more efficient in inducing CD8+ T cell responses compared to wild-type strains (Hinchey, Lee et al. 2007). In another study, virulent *Mtb* was found to modify the eicosanoid metabolism of the host to inhibit apoptosis of phagocytic cells and to delay the onset of protective T cell responses (Chen, Divangahi et al. 2008). Modification of the immune response in favor of apoptosis could therefore be a useful strategy to induce

protective adaptive responses. Various studies have shown that transportation of the mycobacteria to the lung-draining lymph nodes (LDLN) for antigen presentation and T cell activation is slower than in other infections (Wolf, Desvignes et al. 2008, Reiley, Calayag et al. 2008, Gallegos, Pamer et al. 2008), providing one mechanistic explanation to the kinetics of mycobacterial infections.

During the last decade, data gained from mouse models (Scott-Browne, Shafiani et al. 2007, Kursar, Koch et al. 2007, Shafiani, Tucker-Heard et al. 2010)as well as from human patients (Guyot-Revol, Innes et al. 2006, Ribeiro-Rodrigues, Resende Co et al. 2006) suggest that virulent mycobacteria could induce regulatory T cell responses to promote their own survival within the host. Regulatory T cells undergo rapid proliferation in parallel to effector T cell population after antigen-presenting DCs reach the LDLNs (Shafiani, Tucker-Heard et al. 2010). T cell transfer experiments carried out in mice have revealed that mycobacterial infection can induce expansion of Treg population specific to a mycobacterial antigen Ag85A. In addition, the implantation of a relatively small number of such cells caused further delay in the arrival of effector T cells to the site of infection and lead to increased bacterial burdens in the lungs of *Mtb*-infected mice (Shafiani, Tucker-Heard et al. 2010). Induction of mycobacterium-specific regulatory T cells is an interesting mechanism that could, in part, account for the success of this "symbiotic pathogen". This theory is supported by the findings stating that immunodominant mycobacterial antigens recognized by CD4+ T cells are hyperconserved across strains and lineages so that it might even be beneficial for mycobacteria to be recognized by (regulatory) T cells (Comas, Chakravartti et al. 2010, Russell 2013). Recently, it was also reported that virulent *Mtb* induces tolerogenic DCs by an IL-10 dependent mechanism (Kim, Kwon et al. 2017), which might be how Tregs are induced. The actual antigens recognized by Tregs during infection and their role for pathogenesis are still under investigation.

2.4.4 Granulomas and other mycobacterial niches within the host

As macrophage are often unable to kill intracellular mycobacteria right after initial infection, the bacteria spread to lung interstitial tissue by directly infecting the epithelial cells or within the macrophages. The activation of phagocytic cells leads to cytokine and chemokine production, which lures more immune cells to the site of infection and subsequent formation of a tight cell aggregate known as the granuloma – the hallmark of mycobacterial infections. Traditionally, they are thought to be host-

protective structures that wall off the infection, although the bacteria cannot be eradicated. These structures are initially tight aggregates of innate immune cells, but once the adaptive immune response is active, also T and B cells gather around the granuloma and keep the bacteria at bay. A tight, fibrotic cuff often forms to surround the structure. The core of the granuloma may compose of living macrophages with mycobacteria within them or in other cases it may become necrotic, caseate and/or calcify. (Guirado, Schlesinger 2013) Despite the fact that granulomas are often located in the lungs, they have been shown to contain hypoxic areas, which are thought to promote mycobacterial dormancy (Via, Lin et al. 2008). Granulomas can be thought of as a sort of a compromise between the host and mycobacteria: when the host is unable to eradicate the bacteria, they are encapsulated within a granuloma and some bacteria persist within these structures as commensalistic tenants as long as the immune status of the host is stable and able to maintain latency.

In addition to granulomas, mycobacteria have been shown to exist in other compartments. During a latent infection, *Mtb* has been suggested to reside outside granulomas within endothelium, fibroblasts, macrophages, type II pneumocytes (Hernandez-Pando, Jeyanathan et al. 2000), as well as in fat cells around the heart, lymph nodes, stomach and kidneys (Neyrolles, Hernández-Pando et al. 2006). In active TB in HIV positive individuals, *Mtb* exists in non-organized lesions, not in confined granulomas (de Noronha, Bafica et al. 2008). There are thus a spectrum of different niches for the bacteria to reside in and it is likely that these various microenvironments can induce specialization of different mycobacterial subpopulations, such as actively dividing intracellular, extracellular as well as slowly diving or non-dividing dormant mycobacteria (De Chastellier 2009).

2.4.5 Mycobacterial dormancy

Dormancy in mycobacteria is induced as a stress response in the hostile environment created by the immune response mounted by the infected host cells (Chao, Rubin 2010). Such conditions are present within macrophages or within granulomas and include nutrient starvation, nitric oxide, carbon monoxide and low oxygen concentration (Kumar, Toledo et al. 2007). Exposure to such conditions activates the transcription of mycobacterial genes within the dormancy regulon (Voskuil, Visconti et al. 2004). So-called toxin-antitoxin loci that produce RNA-binding proteins also contribute to dormancy during hypoxia and starvation (Korch, Contreras et al. 2009).

Dormant bacteria are viable but non-culturable, which is why their detection has been difficult by traditional diagnostic methods. Dormant bacteria greatly differ from their actively dividing counterparts in terms of shape and energy metabolism. They utilize anaerobic metabolism as demonstrated by the up-regulation of the glyoxylate cycle and triacylglycerol biosynthesis and the down-regulation of the central metabolism as well as sensitivity to metronidazole, an antibiotic that requires anaerobic metabolism for its activity. An important aspect of mycobacterial dormancy is its reversible nature. Dormant mycobacteria can be resuscitated from dormancy should the environment become favorable for its growth. (Chao, Rubin 2010)

Dormant mycobacteria are hypothesized to be important for establishment of persistent latent infections (Chao, Rubin 2010). The first pieces of evidence to support their existence in human patients came from a pioneering study by (Hernandez-Pando, Jeyanathan et al. 2000), in which *Mtb* DNA was discovered from lung necropsies of asymptomatic LTBI patients without histological evidence of TB (Hernandez-Pando, Jeyanathan et al. 2000). Subsequently, *Mtb* DNA has also been discovered in adipose cells of latently infected individuals (Neyrolles, Hernández-Pando et al. 2006).

The presence of a small dormant mycobacterial population also during active TB has been proposed to be at least partly responsible for the delayed efficacy of antibiotic treatments used against TB (Andersen, Woodworth 2014). In addition, if such bacterial populations reside within the human body for decades in latent infection (Lillebaek, Dirksen et al. 2002), during this time, they can, in theory, accumulate genetic resistance to various antibiotics. For thorough control of TB through novel vaccination and antimicrobial strategies, understanding the biology and regulation of mycobacterial dormancy is essential.

2.5 Treatment and prevention of TB

2.5.1 Vaccination against TB

Currently, the only available vaccine against TB is the intradermally injected BCG, which was released more than 80 years ago. It contains live, attenuated *Mycobacterium bovis* and it is administered widely despite its variable efficacy and potential to cause disease in immunocompromised individuals (Andersen, Woodworth 2014). The

efficacy of BCG ranges between 0 and 80 % (Fine 1995). Although it protects children against active disease, it is inadequate in preventing pulmonary TB in adults living in TB-endemic areas (Andersen, Doherty 2005) and does not protect against latent or reactivated infection (Andersen, Woodworth 2014).

BCG induces a strong Th1 type response with Ifnγ, Tnfα, and IL2 secretion (Soares, Scriba et al. 2008, Soares, Kwong Chung et al. 2013). Also some proliferation of CD8+ cells(Soares, Scriba et al. 2008, Soares, Kwong Chung et al. 2013), as well as IL-17 production from CD4+ cells (Kagina, Abel et al. 2010, Gopal, Lin et al. 2012) is induced by BCG. Th1-type responses have been considered protective and the assessment of the efficacy of novel vaccines has been mainly relying on the measurement of these immune responses (Andersen, Woodworth 2014). However, in a prospective study among BCG-vaccinated infants, there was no correlation between the magnitude of Th1-type responses and the protection against TB-disease (Kagina, Abel et al. 2010). Also, although one of the most promising subunit vaccines to boost BCG, MVA85A, efficiently induced Ifnγ responses from Th1 cells, it failed to increase protection against TB in a human efficacy trial (Tameris, Hatherill et al. 2013). These results indicate that the promotion of Th1 responses as such may not be the appropriate vaccination strategy to combat TB clearly demonstrating a need for the development of entirely new approaches.

2.5.2 Antibiotic treatment of active TB

Where vaccines as well as natural immune responses of the host fail, active TB disease ensues requiring immediate medical attention and antibiotic treatment. Untreated TB is detrimental and lethal in 53-86 % of cases (Tiemersma, van der Werf, Marieke J et al. 2011). Antibiotic treatment of TB can thus significantly improve disease control, but in many respects, it is far from optimal.

The current antibiotic regimen against active TB consists of isoniazid and rifampicin taken for 6 months combined with pyrazinamide and ethambutol for the first two months. Although called the short regimen, compared to many other antibiotic treatments, it is rather long. Many factors can be speculated to account for the requirement of such a long treatment. The slow replication rate or even non-replication of a part of the mycobacterial population (dormant bacteria) (Vernon 2013) blunts the efficacy of bacteriostatic antibiotics. The thick mycobacterial cell

wall as well as compartmentalization of the bacteria within granulomas complicate the penetration of drugs into mycobacteria.

According to a recent report in Nature Medicine by Malherbe and colleagues, it seems that the standard course of antibiotics in treating genetically drug-sensitive TB may in many cases end up being insufficient and result in relapse. Using a FDG PET/CT scan ([18F]-fluoro-2-deoxy-D-glucose (FDG) positron emission tomography combined with computed tomography), an increase in pulmonary lesions was found in one third of the patients during the treatment. One year after treatment, as many as 70% had residual pulmonary lesions. Alarmingly, *Mtb* mRNA could be detected in 35% of the culture-negative sputum samples after 6 months of treatment, suggesting that a separate (unculturable) mycobacterial population dodges chemotherapy (Malherbe, Shenai et al. 2016). This population is likely to account for the relapse cases after a seemingly successful treatment.

Evidence from animal models (Andersen 2007, Shi, North et al. 2004, Barry, Boshoff et al. 2009), as well as from necropsies from humans (Hernandez-Pando, Jeyanathan et al. 2000) suggest that in LTBI, compared to active TB, an even greater proportion of bacteria enter a dormant, non-replicative state. A person diagnosed with LTBI may benefit from a 3-month course of isoniazide-rifapentine (Vernon 2013) or isoniazid monotherapy (Ai, Ruan et al. 2016). However, as the risk of reactivation of TB in general population is rather low (5-10% during life-time with most of the risk concentrated within 2 years of infection) (Jasenosky, Scriba et al. 2015), the side-effects, such as hepatotoxicity outweigh the benefits of prophylactic treatment in the majority (Vernon 2013). Currently, prophylaxis is limited to groups defined by external, well-known risk factors (such as HIV co-infection, silicosis or organ transplantation)(Ai, Ruan et al. 2016), as there are no diagnostic tools to quantify the actual risk of reactivation within a single LTBI patient (Schnappinger, Ehrt 2016). New markers assessing the risk would allow better allocation of prophylactic treatment and limit the unnecessary use of antibiotics (Esmail, Barry et al. 2014).

The long duration as well as the side effect related to treatment come with problems in treatment compliance (Dheda, Barry 3rd et al. 2016). Although it seems that the majority of patients adhere to the intensive phase of therapy for the first two months, there is a steady increase in the proportion of patients that leave the therapy prior to completion of the 6-month regimen (Kruk, Schwalbe et al. 2008). Likely for the same reasons, with the added effect of the asymptomatic nature of LTBI, the adherence to LTBI therapy is unsatisfactory (Norton, Holland 2012). Poor treatment compliance is likely one of the main accelerators of the development of resistant *Mtb*

strains. According to WHO description, MDR-TB is resistant to isoniazid and rifampicin whereas extremely drug-resistant TB has additional resistance to fluoroquinolones and to at least one of the injectable second-line antibiotics (amikacin, capreomycin or kanamycin). Alarmingly, even totally drug-resistant, incurable forms of TB have emerged (Dheda, Barry 3rd et al. 2016). There are various promising drug candidates as well as repurposed molecules in clinical trials that offer hope for patients infected with *Mtb* (Dheda, Barry 3rd et al. 2016).

As stated previously in this paragraph, there are also persistent mycobacterial populations that seem to resist chemotherapy even in the absence of genetic resistance against the antibiotics used. A new arsenal of weapons specifically targeted at these phenotypically resistant mycobacterial populations is urgently needed for comprehensive TB control. The development of models to understand the functions of these subpopulations that can be speculated to consist of dormant mycobacteria or mycobacteria residing in biofilm matrix can help in designing and testing novel treatment strategies leading to total recovery from TB infection.

2.5.3 Adjunctive therapy modulating host immune responses

In the hopes of avoiding the caveats of antibiotic treatment, most importantly the frontier of antibiotic resistant strains, researchers have started looking for ways to harness the immune system to more efficiently target mycobacteria or, alternatively, to limit the excessive inflammation that accounts for the massive disease pathology in some patients. As in many other diseases, the result of a balancing act between sufficient and excessive inflammatory responses determines whether an infection can be successfully controlled (Casadevall, Pirofski 2003). As such adjunctive drugs target and bind to host molecules, it is less likely for the bacteria to gain mutations that would render a treatment inefficient.

Glucocorticoids with general immunosuppressive effects, such as dexamethasone and prednisolone, are widely used to limit excessive inflammation that is characteristic in the pathology of TB meningitis and pericarditis (Hakim, Ternouth et al. 2000, Mayosi 2002, Thwaites, Fisher et al. 2009). According to a vast meta-analysis, using glucocorticoids combined with antibiotic treatment led to a 17 % reduction in TB mortality (Critchley, Young et al. 2013). In the development of host directed therapies, customization of treatment according to the patient's individual inflammatory status is required. For example, in a retrospective analysis on the efficacy of adjunctive glucocorticoids for TB meningitis, it was noted that only

individuals with two copies of the high-activity promoter of the gene *lta4h* encoding leukotriene-A4 hydrolase leading to high Tnfα levels, benefitted from glucocorticoid treatment (Tobin, Roca et al. 2012)

Immunosuppressives with better defined targets (such as phosphodiesterases regulating Tnfα levels) are being tested in laboratory settings with the aim of restricting pathological inflammation (reviewed in (Tobin 2015)). Host-targeted treatments open entirely new avenues in the TB field and the detailed analysis of the characteristics of an optimal response against mycobacterial infection will form the foundation of novel immunomodulatory therapies.

2.6 Mammalian animal models for tuberculosis

A lot of work to elucidate the detailed mechanisms of TB pathogenesis has been done *in vitro* using macrophages as host cells (Koul, Herget et al. 2004). However, such simple, yet ethical, models cannot replicate the complex interplay between the host and the pathogen or reveal the efficacy of novel vaccination or chemotherapeutic interventions. Data collected from human TB patients would of course be most relevant, but experimental work in animals is in many cases required prior to testing the applicability of new treatment strategies in humans.

As the clinical outcome of TB is both dose and host dependent (Lin, Flynn 2010), choosing a relevant model is of tremendous importance. Mice have been successfully used for TB-drug discovery (Barry, Boshoff et al. 2009), but unfortunately the highly organized, necrotic, fibrotic caseating granulomas, the central features in human tuberculosis, are absent in the mouse (Alexander, Liu 2006, Russell, Barry 3rd et al. 2010, Flynn 2006, Barry, Boshoff et al. 2009). The TB disease in mice does not replicate the disease spectrum seen in humans, as they generally do not develop latent infection, but rather a chronic, progressive disease with a high bacterial load (McCune Jr, Tompsett et al. 1956). Guinea pigs develop tissue patterns that are more granuloma-like (Russell, Barry 3rd et al. 2010, Flynn 2006), but they are hypersusceptible to TB and develop a fatal infection even with a small infection dose (Wiegeshaus, McMurray et al. 1970, McMurray 2001). The guinea pig model is thus inappropriate for assessing the mechanisms behind latency and reactivation. TB in the rabbit seems to nicely recapitulate the human disease with pathology distinguished by caseous necrotic granulomas (Manabe, Dannenberg et al. 2003) and latency as the usual outcome of the infection (Manabe, Kesavan et al. 2008). So far, the model best reproducing the pathology and the full spectrum of human TB is

undoubtedly the non-human primate model utilizing macaques (Flynn 2006). However, due to ethical issues and the high expenses of the rabbit and macaque models, their use should be carefully considered and limited to studies where other models are not applicable.

2.7 The zebrafish model for tuberculosis

During the last fifteen years, the zebrafish (*Danio rerio*) infected with *M. marinum* has emerged as a convenient model for mycobacterial disease with the typical granulomatous pathology (Berg, Ramakrishnan 2012, Ramakrishnan 2013, Meijer 2016). The high level of conservation of the immune mechanisms from fish to humans as well as from *M. tuberculosis* to *M. marinum* set the stage for using a simple non-vertebrate model for mechanistic large-scale experiments on host-pathogen interactions in mycobacterial disease.

2.7.1 Comparison of *Mycobacterium marinum* with *Mycobacterium tuberculosis*

The genus *Mycobacterium* comprises more than 70 species (Harmsen, Dostal et al. 2003). Most of them are harmless environmental organisms, while others such as *M. tuberculosis, M. leprae* and *M. ulcerans* cause severe infections in humans. *M. fortuitum, M. abscessus*, *M. chelonae* and *M. marinum* infect mainly aquatic species (van der Sar, Appelmelk et al. 2004). *M. marinum,* previously known as *M. balnei* (Stinear, Seemann et al. 2008)*, M. platypoecilus* and *M. anabanti* (Decostere, Hermans et al. 2004) is a natural pathogen of fish and amphibians and the infectious agent behind fish tuberculosis (van der Sar, Appelmelk et al. 2004, Decostere, Hermans et al. 2004, Tobin, Ramakrishnan 2008). *M. marinum* is transmitted through cannibalism, injuries, skin abrasions, external parasites or consumption of contaminated feed (Decostere, Hermans et al. 2004, Stamm, Brown 2004). The symptoms include uncoordinated swimming, abdominal swelling, weight loss and the development of skin ulcers (Stamm, Brown 2004). Fish tuberculosis is a systemic disease (Watral, Kent 2007, Swaim, Connolly et al. 2006, Davis, Clay et al. 2002) that causes granuloma formation in various organs (Stamm, Brown 2004). In humans, *M. marinum* causes "swimming pool granulomas" and "fish-fanciers fingers" manifested as skin ulcerations (Decostere, Hermans et al. 2004, Stinear, Seemann et al. 2008). The dermal

granulomas caused by *M. marinum* and *M. tuberculosis* are histologically indistinguishable (Tobin, Ramakrishnan 2008).

The histologically observed similarities reflect the genetic relatedness between the two mycobacterial species. Sequencing and annotation of the entire *M. marinum* genome 2008 confirmed the close genetic relationship with *M. tuberculosis*. The two species seem to have developed from a common ancestor (Stamm, Brown 2004, Stinear, Seemann et al. 2008, Tobin, Ramakrishnan 2008) and they share 3000 orthologous proteins with an average amino acid identity of 85 % (Stinear, Seemann et al. 2008). Importantly, the central mycobacterial virulence determinants are present in both species (Gao, Guo et al. 2004, Cosma, Klein et al. 2006, Tobin, Ramakrishnan 2008, Stinear, Seemann et al. 2008). Cross-species complementation experiments have demonstrated the functional conservation of these factors: avirulent *M. marinum* has been shown to retrieve its virulence when transformed with the orthologous virulence genes of *Mtb* (Tobin, Ramakrishnan 2008, Stamm, Brown 2004). Not surprisingly, immunization of mice with *M. marinum* can provide protection against *Mtb* (Collins, Montalbine et al. 1975).

Compared to *M. tuberculosis, M. marinum* has many practical assets making it an attractive model to study mycobacterial pathogenesis. Firstly, *M. marinum* grows more rapidly in laboratory settings: the generation time of *M. marinum* is between 4 and 8 hours (Clark, Shepard 1963, Gao, Manoranjan 2005), whereas the duplication of *M. tuberculosis* takes close to 24 hours (Cole, Brosch et al. 1998). Under optimal growth conditions, *M. marinum* forms visible colonies on agar plates in 5–7 days whereas *M. tuberculosis* requires 3 weeks (Stamm, Brown 2004, Cosma, Swaim et al. 2005, Gao, Manoranjan 2005). As a human pathogen with the ability to cause a potentially lethal disease, *M. tuberculosis* has been classified as a Biosafety Level 3 (BSL-3) pathogen, with which the safety considerations and regulations in a laboratory setting are much heavier (Alderton, Smith 2001) than with *M. marinum,* classified as BSL-2 (Gao, Manoranjan 2005).

2.7.2 The immune system of the zebrafish

Zebrafish is a rather new model in the field of immunology, but despite the evolutionary distance between fish and mammals, the innate and adaptive immune systems are astonishingly similar (Trede, Langenau et al. 2004). In evolution, rag (recombination-activating gene)-dependent adaptive immunity first developed in jawed fish (Lieschke, Currie 2007) and the zebrafish is developmentally one of the

simplest vertebrates with both innate and rag-dependent adaptive immune systems. Zebrafish is thus an extremely convenient two-in-one model of immunity. During the first 4 weeks of life, zebrafish mainly rely on the innate arm of immunity (Trede, Langenau et al. 2004, Lieschke, Trede 2009, Sullivan, Kim 2008), which allows the separate assessment innate functions (Sullivan, Kim 2008).

2.7.2.1 The innate immune system of the zebrafish

Apart from basophilic neutrophils, counterparts for most innate immune cells of humans have been identified in the zebrafish (Meeker, Trede 2008). Zebrafish operate with a phagocytic machinery soon after fertilization: a population of phagocytosing primitive macrophage cells (Lieschke, Trede 2009) can be detected as early as 25 hours post fertilization (hpf) (Herbomel, Thisse et al. 1999). Mature macrophages are found in great numbers in adult zebrafish, both in the blood circulation and as tissue resident cells (Sullivan, Kim 2008, Lieschke, Currie 2007). Similarly to mammalian macrophages, lysosomes in zebrafish macrophages contain superoxide and reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radicals, and hypochlorites, which are highly efficient against most pathogens (Sullivan, Kim 2008).Zebrafish harbor granulocytes starting from ~48 hpf (Lieschke, Oates et al. 2001, Willett, Cortes et al. 1999). Neutrophilic granulocytes (Bennett, Kanki et al. 2001, Renshaw, Loynes et al. 2006) as well as eosinophilic granulocytes (Balla, Lugo-Villarino et al. 2010) in the zebrafish bear great resemblance to their mammalian counterparts. Adult zebrafish also harbor an additional class of granulocytes with shuffled characteristics of mammalian eosinophils and basophils (Bennett, Kanki et al. 2001). Neutrophils eliminate microbes by phagocytosis, secretion of antimicrobial substances, production of ROS and release of neutrophil extracellular traps. Furthermore, they serve an important role as inflammatory mediators through release of cytokines and chemokines (Henry, Loynes et al. 2013, Harvie, Huttenlocher 2015).

DCs have been identified in the zebrafish. DCs are phagocytic cells with professional antigen-presenting properties. DCs engulf microbes trafficking their antigens to secondary lymphoid tissues to be presented to antigen-specific T cells. These antigen-presenting cells have an important role as bridging innate immune responses with adaptive ones for example through co-stimulation of T cells. (Lugo-Villarino, Balla et al. 2010).

Most apparent differences between the human and zebrafish immune systems are found in cytotoxic innate cells. Cytotoxic cells called non-specific cytotoxic cells

(NCC) are found in bony fish but are lacking in humans. These recognize and destroy foreign objects such as transformed or virally infected cells. NCCs bear resemblance to mammalian natural killer cells (NK-cells) or monocytes (Yoder 2004). Another class similar to mammalian NK-cells has been isolated from zebrafish kidneys. Instead of NK-receptors these cells express novel immune-type receptors that are not present in mammals. However, these receptors activate the same signaling pathways as human NK-receptors (Yoder 2004).

Looking at the central innate immune functions in more detail, more similarities can be found. Zebrafish express an arsenal of Toll-like receptors (TLRs) important for the first-line defense against microbes. *In silico* analysis has identified the orthologues of mammalian TLRs in the zebrafish. Similarly, most adaptor proteins acting down-stream of TLRs exist in the fish. Compared to mammals, the TLRs in zebrafish show 50 % higher diversity, possibly allowing the recognition of a wider repertoire of microbial patterns (Lieschke, Trede 2009). The activation of TLR signaling causes alterations in gene expression, more specifically in the production of different cytokines with paracrine and autocrine functions in modulation of inflammatory responses (Sullivan, Kim 2008). Importantly for a TB model, the cytokines corresponding to mammalian Tnfα (Praveen, Evans et al. 2006) as well as type II Ifns (including Ifnγ) (Igawa, Sakai et al. 2006), have been identified in the zebrafish. It needs to be noted, however, that substantial expansion in the number of cytokines has occurred in teleost fish (Lieschke, Trede 2009) possibly leading to some level of functional differences between the immune systems of fish and other vertebrates. Besides cytokines, chemokines are another group of secreted inflammatory signaling molecules that are important in guiding inflammatory processes. More specifically, they lure leukocytes to the site of injury acting as initiators in inflammation. Members of both two classes of chemokines (CC (Peatman, Liu 2006) and CXC (Long, Quint et al. 2000)) have been identified in zebrafish.

Finally, zebrafish also have an active complement system (Trede, Langenau et al. 2004, van der Sar, Appelmelk et al. 2004, Lieschke, Trede 2009) that can be activated via three different pathways: the mannose binding lectin, the classical anti-body dependent or the alternative and pathway (Holland, Lambris 2002). The activation of the complement releases proteases that activate target proteins by cleaving. Activated complement enhances phagocytosis or forms so-called membrane attack complexes on pathogens. (Lieschke, Trede 2009)

2.7.2.2 The adaptive immune system of zebrafish

Besides the highly efficient innate immune system, zebrafish also operate with a fully functional adaptive immune system, which provides an additional level of malleability and is a prerequisite for developing immunological memory protecting an animal against recurrent infection. In the zebrafish, the thymus and the kidney are the primary lymphoid organs responsible for lymphocyte development and maturation (Al-Adhami, KUNZ 1977, Murayama, Kissa et al. 2006), whereas the kidney, the spleen and the gut are the secondary lymphoid organs with important functions in lymphocyte activation (Traver, Paw et al. 2003). Thus, one of the most apparent differences in the anatomy of the mammalian and fish immune systems is that in the fish, the kidney serves as the main lymphatic organ instead of the red bone marrow (Al-Adhami, KUNZ 1977). Despite having a vast network of lymphatic vessels (Yaniv, Isogai et al. 2006), zebrafish seem to lack distinct lymph nodes (Trede, Langenau et al. 2004, Meeker, Trede 2008, Lieschke, Trede 2009) and the intestinal lymphoid tissue in the fish is less organized compared to the Peyer's patches of mammals (Langenau, Ferrando et al. 2004, Danilova, Steiner 2002). The zebrafish spleen does not have germinal centers (Meeker, Trede 2008), and the anatomical location of B cell activation in the zebrafish is still under investigation (Page, Wittamer et al. 2013, Lewis, Del Cid et al. 2014).

T cell responses responsible for cell-mediated immunity (Lam, Chua et al. 2002) along with B cells, responsible for adaptive humoral responses through production of antibodies (Lam, Chua et al. 2004), are active in the zebrafish starting from 4 weeks post fertilization (wpf). The central lymphoid organs the thymus, the kidney and the spleen as well as peripheral tissues harbor large numbers of T and B cells, whose development relies on the same V(D)J recombination mechanisms that operate in mammalian lymphocytes (Wienholds, Schulte-Merker et al. 2002). The production of the B cell and T cell receptor repertoire through V(D)J recombination requires the function of Rag1 and Rag2 (Willett, Cherry et al. 1997).

As opposed to the five antibody isotypes expressed by mammalian B cells, zebrafish only produce three immunoglobulin isotypes (IgM, IgD and IgZ), of which IgZ is not found in mammals (Willett, Cherry et al. 1997, van der Sar, Appelmelk et al. 2004, Danilova, Bussmann et al. 2005). IgM seems to be the predominant antibody in zebrafish (van der Sar, Appelmelk et al. 2004) and it has been suggested that the various IgM species in zebrafish (monomers, dimers, trimers and tetramers) likely provide IgM molecules with an array of effector functions that in mammals are mediated through different isotypes (Costa, Danz et al. 2012). Affinity

maturation of antibodies has not been detected in zebrafish (Trede, Langenau et al. 2004, Meeker, Trede 2008, Lieschke, Trede 2009, Wakae, Magor et al. 2006).

Overall, there are some differences in the anatomy of the adaptive immune systems as well as the antibody repertoire between fish and mammals, yet, the basic principles of adaptive immunity are astonishingly similar. The importance of functional lymphocytes and adaptive immunity in the adult zebrafish has been demonstrated in a study in which the lack of *rag1* caused hypersusceptibility to mycobacterial infection (Swaim, Connolly et al. 2006), although it has to be noted that this is not the case in all infections (Lewis, Del Cid et al. 2014).

2.7.3 Lessons learnt from the zebrafish-*M.marinum* model

Important discoveries made using *M. marinum* –infected zebrafish larvae have changed some of the central dogmas in the TB field. Traditionally, granulomas have been regarded as host-protective impenetrable structures. The larval *M. marinum* model has provided new insights according to which mycobacteria could actually benefit from granulomas by recruiting macrophages to the site to use them for propagation and spreading. (Davis, Ramakrishnan 2009, Volkman, Clay et al. 2004)

Another finding stemming from a forward genetic screen carried out in zebrafish larvae provided important insight on the importance of optimal inflammatory balance in controlling mycobacterial infections by Lta4h-mediated regulation of Tnfα (Tobin, Vary Jr et al. 2010). Importantly, the finding was verified in human genome wide association studies, in which a single nucleotide polymorphism in the promoter region of *lta4h* was found to be associated with the severity of TB meningitis as well as leprosy, another problematic mycobacterial infection caused by *M. leprae* (Tobin, Vary Jr et al. 2010). These parallels attest the applicability of this non-vertebrate model in solving the mystery of TB.

The larval model is highly useful for studying the interactions between mycobacteria and innate immune cells *in vivo* (Meijer 2016, Berg, Ramakrishnan 2012). However, as the adaptive immune system is known to affect the pathogenesis of human TB (North, Jung 2004, Cosma, Sherman et al. 2003), the adult zebrafish is likely a more comprehensive model. Some pioneering studies assessing the *M. marinum* infection in the zebrafish in terms of histological features, kinetics, immune responses and pathogenicity between different strains were published between 2003 and 2009 paving the way for the adult zebrafish as a TB infection model (Prouty, Correa et al. 2003, Meijer, Verbeek et al. 2005, Swaim, Connolly et al. 2006, Harriff,

Bermudez et al. 2007, Harriff, Bermudez et al. 2007, van der Sar, Abdallah et al. 2004, van der Sar, Spaink et al. 2009, Hegedus, Zakrzewska et al. 2009, Watral, Kent 2007). Transcriptomic studies measuring the gene expression induced by *M. marinum* infection have verified important immunological similarities in human and fish TB (van der Sar, Spaink et al. 2009, Hegedus, Zakrzewska et al. 2009).

The first comprehensive study describing the central aspects of the adult zebrafish-*M. marinum* showed that the kinetics and severity of the infection with the M strain of *M. marinum* are affected by the initial dose and that a dose as small as 5 colony-forming units (cfu) is sufficient to cause infection (Swaim, Connolly et al. 2006). The infection was progressive even with a small dose causing close to 50 % mortality in 16 weeks. The granulomas formed were necrotic closely resembling those seen in active human TB (Ulrichs, Kaufmann 2006). Using *rag1* -/- fish lacking adaptive immunity (Wienholds, Schulte-Merker et al. 2002) in a survival experiment, the authors concluded that adaptive immunity is required for limiting mycobacterial growth in the zebrafish (Swaim, Connolly et al. 2006). Overall, the model seemed to reflect the pathogenesis of progressive human TB.

3 AIMS OF THE STUDY

The life cycle of pathogenic mycobacteria within the host is complex and the factors affecting the outcome and pathogenesis of mycobacterial infections are poorly understood. Importantly, the nature of a protective response against tuberculosis is still unknown (Weiner, Kaufmann 2014) and the efficacy of vaccination against TB is suboptimal (Andersen, Woodworth 2014).

The antibiotic treatment of TB is complicated and requires a prolonged regimen consisting of a cocktail of multiple drugs to reach sputum negativity, and still, recent research suggests that unculturable, dormant populations of mycobacteria can persist even after a seemingly successful treatment (Malherbe, Shenai et al. 2016). As dormant bacteria are thought to dominate in latent infections, i*n vivo* models with spontaneous latency are needed to study these phenotypically resistant bacterial populations and to test new compounds directed against dormant mycobacteria.

In addition to phenotypic resistance to antibiotics, acquired genetic antibiotic resistance is spreading and causing infections that are currently not treatable. To avoid selection in favor of such resistant strains, the use of antibiotics among latently infected individuals should be limited. New diagnostics are needed for addressing the disease status among the asymptomatic patients to facilitate the allocation of prophylactic treatment only to the patient groups with the highest probability of reactivation.

To circumvent the dilemma of antibiotic resistance in *Mtb* strains, future therapies should be developed upon alternative approaches such as host-directed therapies that boost the host immune response to more efficiently lyse bacteria. Boosting innate responses is an applicable approach also in patient groups with deficiencies in adaptive immunity, most importantly patients with a HIV co-infection. Detailed understanding of the optimal innate responses at different stages of mycobacterial disease can only be acquired by using apt *in vivo* models.

The specific aims of this Doctoral Thesis were:

- To establish an adult zebrafish model with the required methodology to study the entire spectrum of tuberculosis including primary active, latent, reactivated or cleared infection.
- To determine, whether adaptive immunity modulates the outcome of infection in the adult zebrafish-*M. marinum* system to allow it to be used for modelling adaptive immune responses in the pathogenesis of TB.
- To find out, whether the natural disease spectrum seen in the zebrafish is associated with differences in T helper polarization as knowledge on these associations enables the characterization of the optimal adaptive responses as well as the development of biomarkers for TB diagnostics.
- To establish a zebrafish model for studying ways to induce immune responses leading to improved control or sterilization of mycobacterial infection.

4 MATERIALS AND METHODS

4.1 Zebrafish maintenance

AB wild-type fish aged 5-10 months were used for most experiments. For assessing the role of adaptive responses, adult *rag1 (−/−)* hu1999 mutant fish (Wienholds, Schulte-Merker et al. 2002)(from ZIRC, Zebrafish International Resource Center, University of Oregon, OR, USA) were used. Fish were housed in a flow-through system with a 14/10 h light/dark cycle and fed with dry fish food SDS-400 (Special Diets Services, UK) twice a day.

4.2 Ethics statement

All experiments have been accepted by the Animal Experiment Board in Finland (under the Regional State Administrative Agency for Southern Finland) and were carried out in accordance with the EU-directive 2010/63/EU on the protection of animals used for scientific purposes and with the Finnish Act on Animal Experimentation (62/2006). Permit numbers for the facility and the experiments are the following: LSLH-2007-7254/Ym-23, ESLH-2008-07610/Ym-23, 20.10.2010, ESAVI-2010-08379/Ym-23, ESAVI/6407/04.10.03/2012, PH1267A, ESAVI/733/04.10.07/2013, ESAVI/8245/04.10.07/2015 and ESAVI/10079/04.10.06/2015.

4.3 Immunomodulation

For the preparation of heat-killed bacteria, *Listeria monocytogenes* (10403S), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), *Salmonella typhimurium* (ATCC 14028) and *Streptococcus iniae* (ATCC 29178) were inoculated from glycerol stocks or blood agar plates and cultured in brain heart broth (Sigma-Aldrich, MO, USA) at 37 °C to an OD_{600} of 0.9–1.0. The bacterial concentration was determined by plating dilutions on LB agar and incubating at 37°C for 24 h. Bacteria were autoclaved in

brain heart broth at 120°C for 20 min and samples were plated to confirm the efficacy of the heat-killing treatment. The immunomodulatory solutions were diluted appropriately to inject an amount originating from 0.5–1 x10^7 bacteria. The doses of commercially available immunomodulators (Sigma-Aldrich, MO, USA) were 13.5 μg/fish for lipopolysaccharide (LPS), paclitaxel and zymosan; and 4.5 μg/fish for muramyl dipeptide (MDP). Fish were anesthetized with 0.02% 3-aminobenzoic acid ethyl ester (pH 7.0) (Sigma-Aldrich, MO, USA) and immunomodulators were delivered by *i.p.* injection in a volume of 5 μl using Omnican 100 30 G insulin needles (Braun, Melsungen, Germany) one day prior to experimental low-dose infection with *M. marinum*. Sterile phosphate buffered saline (PBS) composed of 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, was injected as a control.

4.4 Experimental infections

M. marinum (ATCC 927) was cultured in 7H9 medium (Becton, Dickinson and Company (BD), NJ, USA) as described in (Swaim, Connolly et al. 2006) with slight modifications: culture at 29°C, concentration of Tween 80 0.2%. After a one-week culture on 7H10 plates (BD), 1 μl of bacteria were transferred into liquid medium and cultured for 4 d, diluted ~ 1:10 and cultured to an OD_{600} of 0.495-0.680, diluted with sterile 0.2 M KCl + 0.3 mg/ml phenol red (Sigma-Aldrich, MO, USA) or PBS. The fish were anesthetized in 0.02% 3-aminobenzoic acid ethyl ester (pH 7.0) (Sigma-Aldrich, MO, USA) and *i.p.* injected with 5 μl using an Omnican 100 30 G insulin needle (Braun, Melsungen, Germany). Replicate bacterial samples were plated onto 7H10 plates to verify the infection dose. In article I, the low dose was 34 ± 15 cfu and the high dose 2029 ± 709 cfu. In article II, the low dose was 21±7 cfu and the high dose 1783±364 cfu. In article III, the low dose was 33±19 cfu.

4.5 Survival experiments

Survival data was collected during infection experiments by marking the date by which the humane end point criteria were met. Animals showing any of the following were euthanized: lack of response to touch, abnormal swimming, gasping, observable swelling, observable wasting or loss of scales.

4.6 Nucleic acid extraction

4.6.1 DNA extraction

The organs of euthanized zebrafish were collected from the peritoneal cavity and frozen at -80 °C. The organs were weighed and a modified enzymatic lysis buffer (20 mM Tris-HCl pH 8.0, 20 mM sodium EDTA pH 8.0, 1.2% Triton X 100) was added. Samples were homogenized using the PowerLyzer24 (Mobio) at speed 3,200 for 3 x 20 second cycles with 30 second pauses. A portion of the homogenate containing <25 mg was used for further DNA extraction. The samples were sonicated a water bath sonicator (m08, Finnsonic, Lahti, Finland) for 9 min. To improve lysis of mycobacteria, 20 mg/ml of lysozyme (Sigma-Aldrich, MO USA) was used and the samples were incubated at 37°C for 2 h. Modified enzymatic lysis buffer was added to the samples to equalize the volumes. QIAGEN DNeasy Blood & Tissue Kit manufactures protocol for DNA extraction from gram-positive bacteria was followed from this point on.

4.6.2 DNA-RNA co-extraction

To extract samples for mycobacterial load measurements (DNA) or gene expression analysis (RNA). TRI reagent for DNA-RNA co-extraction (MRC, OH, USA) as previously described in (Stamm, Morisaki et al. 2003). Briefly, the peritoneal cavity of the euthanized zebrafish was emptied and put in tube on dry ice and stored at -80°C until extraction. The sample was homogenized in TRI reagent using PowerLyzer24 (Mobio) at 3,200 rpm for 3 x 40 second cycles in with ceramic beads. RNA extraction was carried out according to the manufacturer's protocol. To extract DNA an equal volume of back extraction buffer (4 M guanidine thiocyanate (Sigma-Aldrich, MO, USA), 50 mM sodium citrate, 1 M Tris) was added on top of the lower phase after phenol-chloroform phase separation. DNA was precipitated with isopropanol, washed with ethanol twice and dissolved in sterile ddH_2O.

4.7 Quantitative polymerase chain reaction (qPCR)

4.7.1 Quantification of *M. marinum*

Bacterial loads were measured by qPCR from DNA samples using SENSIFAST NO-ROX SYBR kit (Bioline) with *M. marinum* specific primers as described in (Stamm, Morisaki et al. 2003). The specific primers for *M. marinum* 16S–23S ITS sequence were F: 5'-caccacgagaaacactccaa-3' R: 5'-acatcccgaaaccaacagag-3'. The final composition of the reaction was as follows: 1x SENSIFAST NO-ROX SYBR GreenPCR Master Mix (stock 2x), 0.4 μM MMITS1 (*M. marinum* internal transcribed spacer) forward primer, 0.4 μM MMITS1 reverse primer, 3 μl of template (conc. <300 ng/μl). A dilution series of DNA extracted from mycobacterial culture was used as a standard and DNA extracted from three healthy fish was used as a negative control. CFX96 cycler (Bio-Rad, CA, USA) was used with the following settings: 1. 3 min 95°C , 2. 5 s 95°C, 3. 10 s 65°C, 4. 5 s 72 °C, 5. 39 cycles from 2. to 4. 6. Melting curve 55-95°C at 0.5 intervals. The limit of detection was ~100 bacteria/fish.

4.7.2 Quantification of gene expression

To remove remnants of genomic DNA, prior to RT(reverse transcription)-qPCR RNA was treated with DNAse (Fermentas) according to the manufacturer's protocol. In part I and II, Bio-Rad iScript One-Step RT-PCR Kit with SYBR Green was used according to manufacturer's protocol. In part III, RNA was used as the template in a separate reaction to produce cDNA with a Reverse Transcription kit (Fluidigm, CA, USA) according to the manufacturer's instructions. Gene expression was thereafter measured with SsoFast EvaGreen Supermix with Low ROX qPCR kit (Bio-Rad, CA, USA). CFX96 cycler (Bio-Rad, CA, USA) was used in all measurements. Host gene expression was normalized to the expression of genes encoding glyceraldehyde 3-phosphate dehydrogenase (*gapdh*)(I, II), elongation factor 1 alpha *(ef1a)*(II) or to expressed repetitive element called *loopern* (III). The expression levels of the mycobacterial dormancy gene encoding citrate synthase (*GltA1)* was normalized to the total mycobacterial load (I&II). The results were analyzed using the ΔCt method comparing infected samples to an RNA sample extracted from a pool of healthy, non-infected zebrafish and shown as fold induction compared to

baseline levels. *GltA1* levels are in arbitrary units/mycobacterial genome. Gene accession numbers and primer sequences can be found separately in Table 1.

Table 1. Primer sequences

ZEBRAFISH

TARGET	ID	Forward	Reverse
TBX21	ZDB-GENE-080104-3	GGCCTACCAGAATGCAGACA	GGTGCGTACAGCGTGTCATA
GATA3	ZDB-GENE-990415-82	GGATGGCACCGGTCACTATT	CAGCAGACAGCCTCCGTTT
IL4	ZDB-GENE-100204-1	GCAGGAATGGCTTTGAAGGG	GCAGTTTCCAGTCCCGGTAT
IL13	ZDB-GENE-100727-2	GGAAGCTGTGTTAGTCAATCC	GCCTGACAGAAATAATCATGC
FOXP3A	ZDB-GENE-061116-2	CAAAAGCAGAGTGCCAGTGG	CGCATAAGCACCGATTCTGC
IL12	ZDB-GENE-060724-1	AGCATGGCTCTGGCTCTGGC	TGCTCCTTCATCTTTCCCTCCTTCT
IFNΓ1-2	ZDB-GENE-040629-1	GGGCGATCAAGGAAAACGACCC	TAGCCTGCCGTCTCTTGCGT
NOS2B	ZDB-GENE-080916-1	TCACCACAAAAGAGCTGGAATTCGG	ACGCGCATCAAACAACTGCAAA
TNFA	ZDB-GENE-050317-1	GGGCAATCAACAAGATGGAAG	GCAGCTGATGTGCAAAGACAC
GAPDH	ZDB-GENE-030115-1	AGTGTCAGGACGAACAGAGGCT	GCCAATGCGACCGAATCCGTTA
EF1A	ZDB-GENE-990415-52	CTGGAGGCCAGCTCAAACAT	ATCAAGAAGAGTAGTACCGCTAGCATTAC
LOOPERN4	expressed repetitive element	TGAGCTGAAACTTTACAGACACAT	AGACTTTGGTGTCTCCAGAATG
ST2	ZDB-GENE-060621-4	CGCTTTACTGTGGAGAGATGG	TGCTGCTGTGTTTGATGCTC
STAT6	ZDB-GENE-030131-9359	GGTAGTCAGGAAATCAATGC	ACCTCAGACATGAACTTACTGC
SOD2	ZDB-GENE-030131-7742	GGCCATAAAGCGTGACTTTG	CTGCAATCCTCAATCTTCC
M. MARINUM			
TARGET	**ID**	**Forward**	**Reverse**
16S–23S ITS	locus AB548718	CACCACGAGAAACACTCCAA	ACATCCCGAAACCAACAGAG
GLTA1	MMAR_1381	CCACAGCCACATGAGTTACG	GCTCGAAGGTATCCACAACC

4.8 Dormancy plating

Micrococcus luteus is known to secrete resuscitation-promoting factor (Rpf) into its growth medium under appropriate culturing conditions (Mukamolova, Kaprelyants et al. 1998). An inoculate from a glycerol stock of *M. luteus* was cultured in 10 ml of LB liquid medium at 37°C o/n in constant agitation to on OD605 of 0.100). The culture was diluted 1:25 using lactate minimal medium (prepared as in (Mukamolova, Kaprelyants et al. 1998) except for the concentration of lithium L-lactate (0.5% w/v) and rotated (150 rpm) at 30°C for 4 days until OD605 was 0.705. The culture was centrifuged (10,000 g, 3 min) and the supernatant was sterile filtered using 0.2 μm filters (Whatman, GE), aliquoted and stored at -80°C. 7H9 (BD) antibiotic plates were prepared as described (Cosma, Swaim et al. 2005) with 10 μg/ml amphotericin B, 25 μg/ml polymyxin B, 20 μg/ml trimethoprim, 50 μg/ml carbenecillin (Sigma-

Aldrich, MO, USA). In addition, 20 μg/ml of azithromycin was added (Sigma-Aldrich, MO, USA). To make plates with Rpf, 500 μl of the prepared solution was absorbed on each 7H9 antibiotic plates. On –Rpf control plates, an equal volume of fresh, sterile medium was absorbed. Fish were homogenized with PowerLyzer24 (Mobio) 3x30s 3200 rpm in sterile PBS 0.5% Tween 80 (v/v). Dilutions were plated +/- Rpf and incubated in the dark at 25°C for 15-17 days. The average load of culturable *M. marinum* was determined and number of colonies plated +/- Rpf were compared pair-wise to assess the presence of dormant mycobacteria. The efficacy of Rpf in revealing dormant populations was confirmed by plating active logarithmic *M. marinum* broth cultures and old stationary *M. marinum* broth cultures that had been kept in closed bottles at +29°C for 5-8 months were plated +/- Rpf.

4.9 Immunosuppression for induction of reactivation

Fish were put in 100 ml glass flasks (5 fish/80 ml of water/flask) and irradiated with 25 Gray (Gy) using Gammacell 1000 irradiator. Low-dose infected fish were irradiated twice with one month between the doses. Non-infected controls were similarly irradiated.

4.10 Flow cytometry

Kidneys were collected from euthanized zebrafish and placed into PBS supplemented with 1% fetal calf serum and kept on ice. In the first experiment (1 wk post irradiation) kidneys from four individual from each group were pooled. In the later time-point (5 weeks post 1.irradiation), fish were analyzed separately. Kidneys from untreated groups were used as controls. Kidneys were homogenized by pipetting the 1 ml volume 15 times. Prior to analysis, the samples were run through a 50 μm cell strainer. The sizes of different immune cell populations were determined with FACSCantoII (Beckton Dickinson) and the FACSDiva software. The results were further analyzed with FlowJo (Treestar Inc, Ashland, OR). The identification of lymphocytes, blood cell precursors, erythrocytes, granulocytes and monocytes was based on the cellular granularity and size according to (Traver, Paw et al. 2003). A total of 30,000 events per sample were collected for analysis.

4.11 Statistical analysis

The sample sizes for experimental fish groups were calculated with power and sample size program (version 3.1.2) using data from preliminary studies (Dupont, Plummer 1998). Statistical analysis was carried out using the GraphPad Prism software (5.02). Generally, as data was not normally distributed, a non-parametric two-tailed Mann-Whitney test was used. For estimation of the predictive value of *gata3/tbx21* and *foxp3* expression for activity of the disease a receiver operating characteristic analysis was carried out with a confidence interval of 95%. area under curve value of 0.5 indicates no connection and 1.0 indicates a perfect marker. In survival experiments, the log-rank Mantel-Cox test was used. In Rpf experiments, the plates with the same sample +/- Rpf were compared pair-wise, and a one-tailed paired t-test was used. P-values<0.05 were considered significant.

5 SUMMARY OF THE RESULTS

5.1 Adult zebrafish as a model for active, latent and reactivated TB (I)

Previously, only rabbits (Manabe, Dannenberg et al. 2003) and non-human primates (Flynn 2006) have been shown to exhibit the entire spectrum of TB disease similar to that seen in humans. To assess the applicability of the non-mammalian adult zebrafish-*M. marinum* model to study the mechanisms leading to the development of different disease outcomes, adult zebrafish were injected (*i.p.*) with either a low (34±15 cfu) or a high dose (2,029±709 cfu) of *M. marinum* (strain ATCC 927). A 32-week follow up study was carried out. Survival, histopathology, bacterial load, host gene expression, bacterial gene expression, bacterial cultures and effects of immunesuppression were studied to characterize the infection.

5.1.1 The outcome of *M. marinum* infection in adult zebrafish is dose dependent (I)

A high-dose infection (2,029±709 cfu, n=104) caused a progressive disease type with 64 % of the fish showing severe symptoms during the 32-week follow-up, whereas the low infection dose (34±15 cfu, n=180 fish) produced a more controlled infection with 25 % suffering from apparent symptoms (Figure 1A) The observed symptoms were typical of fish tuberculosis (Stamm, Brown 2004) and included uncoordinated swimming/immobility, abdominal swelling and occasional skin ulcers. The high-dose infection caused a high proportion of the fish (24 %) to develop an active primary infection with symptoms during the first 3 weeks, whereas in the low-dose group, none of the fish had developed visible symptoms by this time (Figure 1A).

To assess the development of mycobacterial load in the zebrafish, an *M. marinum* specific qPCR assay measuring the number of *M. marinum* 16S–23S ITS (internal transcribed spacer) DNA copies in fish tissues was developed. The 16S–23S ITS region is highly species specific (Gurtler, Stanisich 1996, Roth, Fischer et al. 1998), and it is present as one copy per *M. marinum* genome (Roth, Fischer et al. 1998). The

validation of the method was carried out by comparing the results with traditional plating methods as presented in the supplementary data of article I. The differences observed in clinical symptoms were in line with the differences in the mycobacterial loads measured from the internal organs of zebrafish samples at different time-points (Figure 1B): The high infection dose caused the bacterial loads to rapidly reach high levels (av.6.0×10^5 cfu/fish at 1 wpi). The bacterial burden stayed at this level for some weeks and then again increased between 6 and 8 wpi to a level of 3.0×10^6 cfu/fish by the end of the 32-week experiment. The growth pattern suggests that many of the fish that survived the first weeks of infection developed a slowly progressive, chronic disease. The bacterial loads in the low dose group grew steadily during the first weeks of infection reaching a stable level in the order of 10^5 cfu between 4 and 6 wpi generally staying at this level until the end of the experiment. The results were further supported by data gained through the quantification of the number of affected organs and granulomas in histology samples (Figures 1C and 1D in article I) showing that after 4 weeks, the disease remained stable in the low-dose group but progressed in the high-dose group. Thus, after the initial phase of bacterial growth, the low-dose infection group seemed to have developed a disease resembling latent, non-progressive TB.

It is still noteworthy that although the vast majority of the low-dose infection group remained asymptomatic, 25 % developed disease during the follow-up. Also the variation in the bacterial load was substantial starting from 4 wpi. Both these observations suggest that a disease spectrum developed in the zebrafish population infected with the low dose.

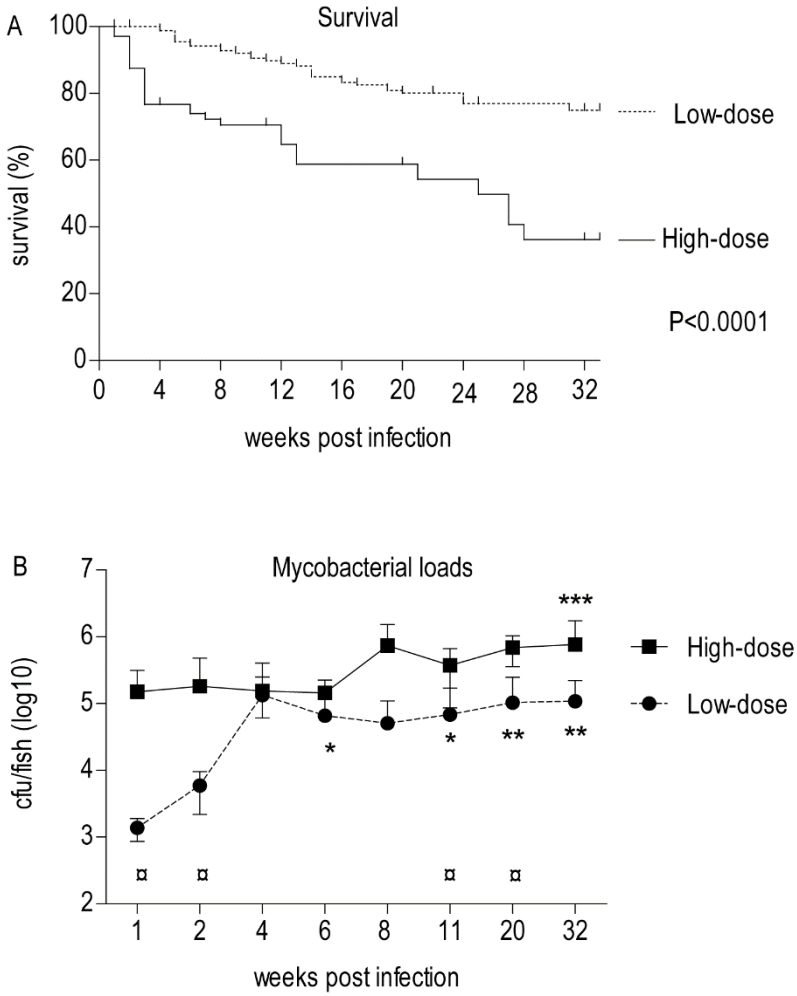

Figure 1. The outcome of *M. marinum* infection in the adult zebrafish is dose dependent. Adult zebrafish were *i.p.* infected with either a low (34±15 cfu) (n=180) or a high dose (2029±709 cfu) (n=104) of *M. marinum.* (A) Survival was followed for 32 weeks. (B) Fish were collected as samples (n=5 per group) at different time-points and the bacterial loads were determined with qPCR. ¤ Significant between the two dose-groups. Within low-dose group: * Significant compared to 1 wpi, ** Significant compared to 1 and 2 wpi. Within high-dose group: *** Significant compared to 1,2,4, 6 wpi.: Modified from article I.

5.1.2 Dormant mycobacteria exist in the zebrafish with a latent infection (I)

The rather stable bacterial numbers in the fish surviving the active initial phase of the infection prompted us to further characterize the bacterial populations present

in the surviving fish, as dormant mycobacteria are thought to be important for the establishment of latent mycobacterial infections in humans (Chao, Rubin 2010). The qPCR method used for bacterial quantification does not assess the metabolic state nor the viability of the bacteria, but reads the entire pool of mycobacterial genomes present in the sample. Furthermore, as dormant bacteria are viable but not culturable (Chao, Rubin 2010) the detection of these hiding population is a rather challenging task.

To assess the presence of dormant populations in adult zebrafish, two methods were developed. The first method was based on measuring the expression the gene encoding citrate synthase (*GltA1*), which has been found to be produced at high levels during *in vitro* induced dormancy in *Mtb* (Deb, Lee et al. 2009). As early as 4 wpi, a 17-fold higher expression of *GltA1* per bacterial genome was observed in the group infected with a low dose compared with the high-dose infected group supporting the idea that in a latent infection, the proportion of dormant mycobacteria is greater than in a more progressive infection (Figure 2A).

The second method relied on the utilization of a "bacterial cytokine" called Rpf, which has been documented to revert dormant *Mtb* into an actively diving state *in vitro* to allowing culture on solid media (Mukamolova, Kaprelyants et al. 1998). The hypothesis was that by comparing the number of culturable bacteria in the absence (to show non-dormant mycobacteria) or presence of Rpf (to show both dormant and non-dormant bacteria) the numbers of dormant bacteria could be assessed. After validating the method with *M. marinum in vitro*, it was successfully used to assess the presence of dormant mycobacteria in zebrafish organ homogenates. A dormant bacterial population could be detected in the low-dose group at a late time point (20 wpi) as shown by the 4-fold increase in the number of colonies grown in the presence of Rpf (Figure 2B). This effect was not seen in the group infected with a high initial dose supporting the idea that the low dose infection better replicates the characteristics of latent mycobacterial infection in terms of dormancy.

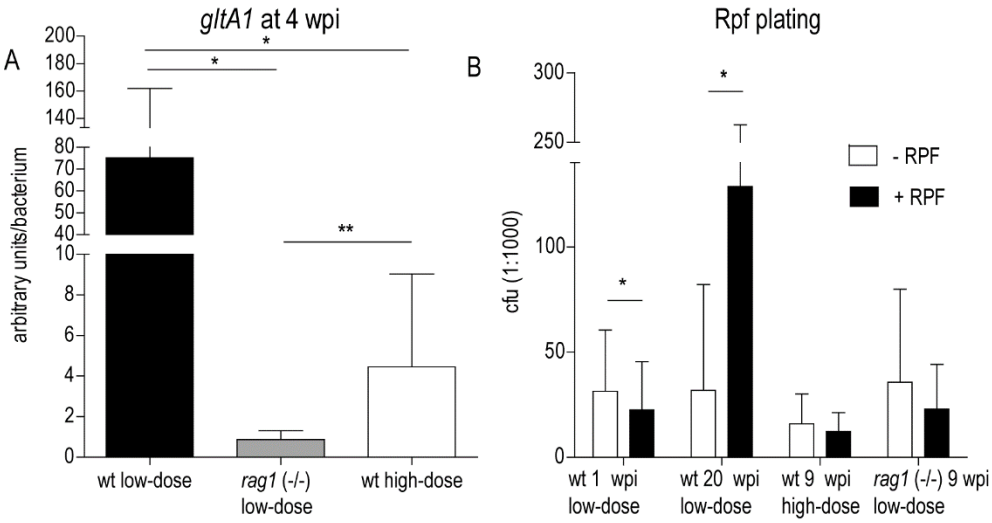

Figure 2. Dormant mycobacteria can be shown in adult zebrafish with a latent infection. (A) *GltA1* expression was measured from low-dose-infected *rag1* (−/−) and wild-type (wt) fish and high-dose infected wt fish and normalized to the total *M. marinum* load in each fish measured with qPCR (B) Parallel homogenate sample dilutions from low-dose-infected fish (wt or *rag1* (−/−)) or high-dose-infected wt fish were plated at different time points +/− Rpf to detect dormant mycobacteria. *P<0.05. Modified from article I.

5.1.3 Latency and dormancy in the *M. marinum* infection of adult zebrafish can be reversed by transient depletion of immune cells by gammairradiation (I)

As immunosuppressive treatments are known to cause reactivation of latent TB (Lin, Flynn 2010, Zack, Stottmeier et al. 1974), the next step was to test whether this situation could be modelled in the zebrafish. Two doses of gammairradiation (25 Gy with 1 month between the doses) was found to efficiently cause reactivation in the latently infected zebrafish population as proven by increased mortality (Figure 3A), a significant increase in mycobacterial load (an 106-fold increase compared to a latent infection) (Figure 3B) accompanied by the disappearance of the dormant bacterial population as determined with Rpf plating (Figure 3C&D).

As determined with flow-cytometry, the gammairradiation treatment reduced the number of granulocytes and monocytes by 47 % at 8 days post irradiation treatment, whereas the lymphocyte population was even more severely depleted (80 % reduction). By 5 weeks post irradiation, the immune cell numbers had returned to original levels (see Fig 5E&F in article I). The drastic effect of lymphocyte depletion

on maintaining control over latent infection clearly demonstrates the importance of adaptive immune cells in fish tuberculosis.

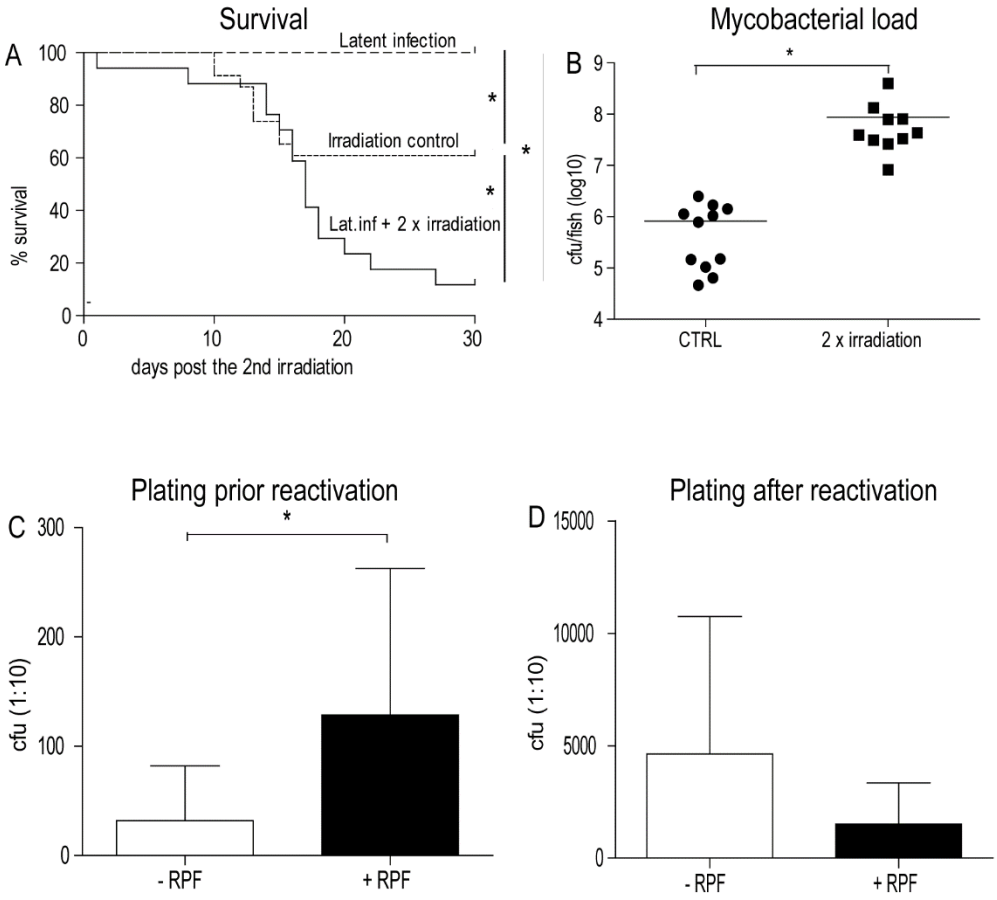

Figure 3. Gammairradiation causes reactivation of latent mycobacterial infection in the zebrafish. (A-B) Zebrafish (n=17) with a latent *M. marinum* infection were irradiated twice with 25 Gy with one month between the doses. Twice irradiated, non-infected zebrafish (n=23) as well as zebrafish with a latent infection (n=14) were included as controls. (A) Survival was followed for 30 days after the second dose. *P<0.05 between all groups. (B) During this period, moribund fish were collected 15–22 days after the second radiation dose. Bacterial loads were compared with those of similarly infected, non-irradiated control fish that were collected at the end-point of the experiment. *P<0.05 (C) Fish with a latent infection (n=7) were irradiated twice with 25 Gy with one month between the doses and plated +/− Rpf for 18 d after the second radiation dose. (C) Fish (n=6) with a latent infection were plated +/− Rpf. Modified from article I.

5.2 Adaptive immunity plays a central role in controlling mycobacterial infection in adult zebrafish (I&II)

Adaptive immunity and especially CD4+ T helper cells are regarded as highly important for controlling human TB (North, Jung 2004, Cosma, Sherman et al. 2003, O'Garra, Redford et al. 2013). A survival experiment presented in a previous study by Swaim et al. 2006 as well as the dramatic effects of induced depletion of lymphocytes (I) implied that this could also be the case in fish TB. To dissect the role of adaptive immunity in the fish TB model in more detail, two sets of experiments were carried out using the low infection dose. The first approach was to use *rag1* (-/-) fish lacking functional T and B cells (Wienholds, Schulte-Merker et al. 2002) and compare the progression of infection to wild type controls (I). The second approach utilized the natural disease spectrum developing within the zebrafish population to look for associations between the bacterial load and T cell responses (II).

5.2.1 Adaptive immunity restricts mycobacterial growth in zebrafish and supports bacterial dormancy (I)

Experimentation with *rag1* (-/-) fish demonstrated the role of adaptive immunity in restricting mycobacterial infection. In *rag1* (-/-) fish group, an *i.p.* infection with (34±15 cfu) caused severe symptoms in 43 % of the individuals during the 8 week follow-up, whereas the wild type group remained healthy (Figure 4A). Similarly, the bacterial loads in *rag1* (-/-) fish were significantly higher than in wild type fish (7.2-fold at 2 wpi and 13.4-fold at 7 wpi))(Figure 4B). The difference in bacterial load at 2 wpi suggest that antimycobacterial adaptive functions are operational to some extent prior to 2 wpi (I).

The measurement of mycobacterial dormancy-related gene expression (*GltA1*) together with Rpf platings indicated a role for adaptive immunity in inducing mycobacterial dormancy. *GltA1* expression at 4 wpi was 87.4-fold higher in the wild type fish than in *rag1* (-/-) mutants (Figure 2A). In line with the qPCR result, addition of Rpf on culture plates did not increase the number of culturable bacteria from *rag1* (-/-) (at 9 wpi), whereas in the wild type population, Rpf increased the number of colonies by 4-fold (Figure 2B). Together these results indicate that functional adaptive immune cells limit mycobacterial growth and are involved in induction of a latent infection with a dormant mycobacterial subpopulation.

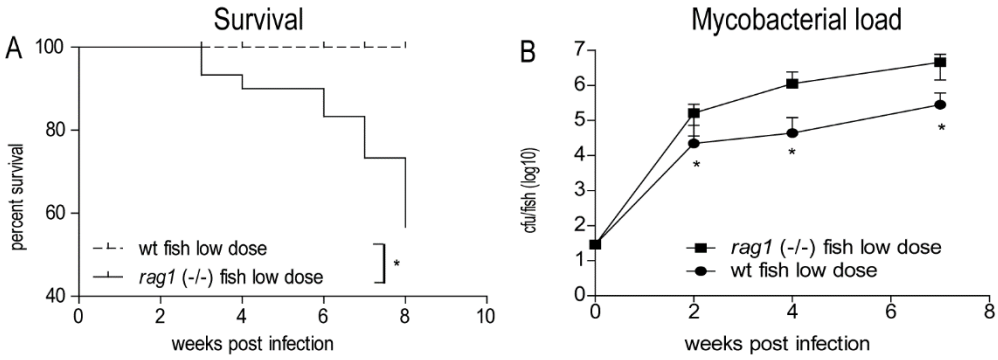

Figure 4. Adaptive immunity limits the progression of mycobacterial infection in zebrafish. (A) Adult wt and *rag1* (−/−) zebrafish were infected with a low dose of *M. marinum* (n=30) and followed for survival. *P<0.05 (B) Adult wt and *rag1* (−/−) fish were infected with a low dose of *M. marinum.* Average mycobacterial load was measured with qPCR at 2, 4, and 7 wpi (n=10 per time point). *P<0.05. Modified from article I.

5.2.2 High total T cell number is associated with limited *M. marinum* growth among fish with an asymptomatic infection (II)

To further elucidate the role of adaptive immune responses, the natural disease spectrum induced by a low-dose (21±7 cfu) *M. marinum* infection was exploited. A large group (~150) of wild type zebrafish were infected and the internal organs were collected at 2, 4 and 20 wpi. Mycobacterial loads were determined by qPCR, and at each time-point, subgroups were formed based on the load measurement. The 25 % with the lowest bacterial numbers were assigned the *Low* group and the 25 % with the highest bacterial loads the *High* group. The 50 % in between were named the *Medium* group. Between 8 and 20 weeks of infection, fish showing symptoms were collected to form a group of spontaneously reactivated fish (*Reactivated*). The work-flow of these experiments is presented in Figure 5.

Based on the expression of *cd3,* a general T cell marker encoding the T cell receptor, the fish organ samples revealed some level of association between the total T cell numbers and control of mycobacterial growth. A modest association was seen as early as 2 wpi, but it was more apparent at later time-points at 4 wpi and 20 wpi (Figure 6A-C). However, in the *Reactivated* group, which showed severe symptoms of infection and high bacterial loads, half of the individuals had high *cd3* expression (Figure 6D) suggesting that the assessment of the total number of T cells is not sufficient and more detailed analysis of the types of T cells associated with disease control is necessary.

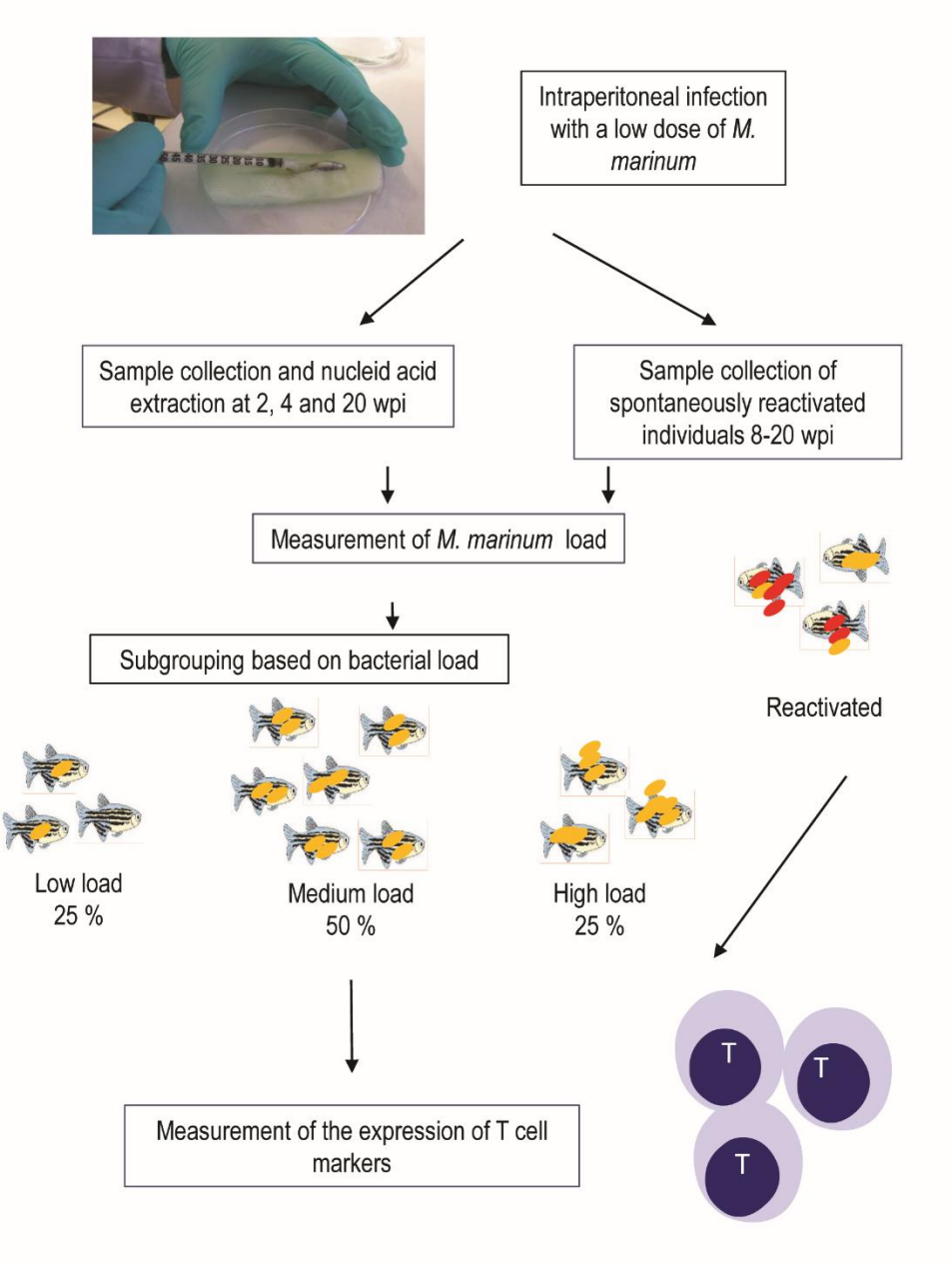

Figure 5. Work-flow chart on studying the association between the natural disease spectrum and T cell responses.

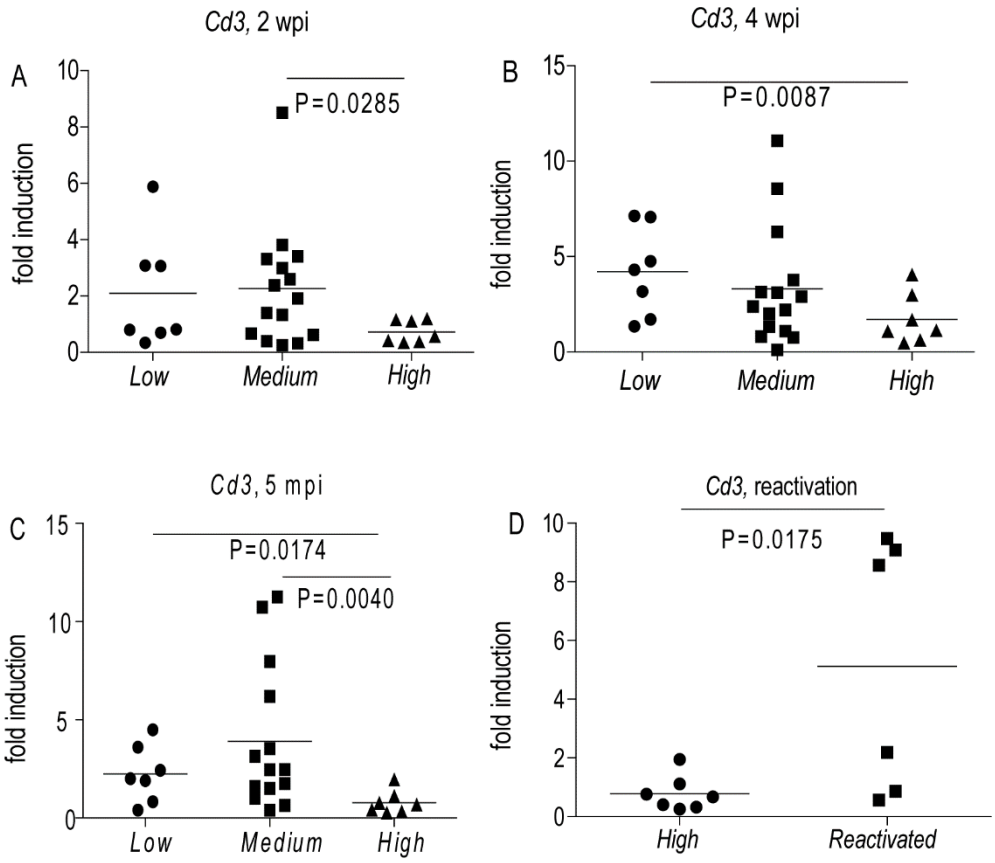

Figure 6. High total T cell number is associated with limited *M. marinum* growth. (A–C) Zebrafish infected with a low dose of *M. marinum* (21±7 cfu) were divided in subpopulations according to the bacterial load into upper quarter (*High*) (n=7), lower quarter (*Low*) (n=7) and a *Medium* group (n=15). The changes in the total T cell numbers were assessed in the different subpopulations by measuring *cd3* transcription with q-RT-PCR during a primary (2 and 4 weeks) or a late stage (5 months) mycobacterial infection. (D) The *cd3* levels of spontaneously reactivated fish were compared to those measured in latently infected fish in the *High* group at 5 months. Modified from article II.

5.2.3 T helper 2 type responses dominate in the groups with limited bacterial growth (II)

The expression of transcription factors commonly used as markers for Th differentiation in mice was measured of the zebrafish organs to look for associations between disease control and the type of T helper response in the zebrafish *M.*

marinum infection. T-box transcription factor 21 (tbx21) is a Th1 cell transcription factor important for Th1 lineage commitment, whereas gata3 is the major transcriptional switch of Th2 cell differentiation with an additional role in endothelial cell biology (Watral, Kent 2007). The central transcription factors responsible for Th lineage commitment are conserved from fish to humans (Willett, Cherry et al. 1997, Hoffmann 2003, Danilova, Hohman et al. 2004) and their expression could thus be measured to indirectly assess the proportions of the corresponding Th populations.

At 2 wpi there were no differences in these markers between the *Low, Medium* and *High* groups, but at 4 and 20 wpi, some differences became apparent (Figure 7). Th1 and Th2 cytokines are generally thought to functionally counteract each other (Hernandez-Pando, Orozcoe et al. 1996), and were therefore studied as a pair (Th2 induction divided by Th1 induction). The Th2 marker *gata3* dominated over the Th1 marker *tbx21* in the *Low* group and was essentially lacking in the *High* and *Reactivated* groups, showing an association between disease control and T helper 2 response. Th2 dominant response also correlated with mycobacterial dormancy as evidenced by dormancy gene *GltA1* measurements at 20 wpi (See Figure 4A in article II). At the level of cytokines IL4 and Ifnγ, the association between Th2 dominance and disease control was seen as early as at 2 wpi (Figure 8A-C). The expression of an array of additional markers related to Th2 and Th1 responses was measured to verify the result (Figure 3 in article II). Of note, the high expression of *igM* was associated with a better disease control providing one mechanistic explanation for better disease control (Figure 8D). IgM is the most abundant of the three immunoglobulins present in the fish (Lieschke, Currie 2007) and has been shown to be associated with IL4 signalling and Th2 response in the zebrafish (Zhu, Pan et al. 2012). In *rag1(-/-)* fish, which lack adaptive immunity and are generally unable to survive the challenge with *M. marinum* (Figure 4) there was no association between the bacterial load and the *gata3/tbx21* (see Figure 2 in article II).

Overall, a clear association between the bacterial loads and Th responses was found in this study. To assess whether the low Th2/Th1 ratio was the cause or the consequence of high bacterial loads, two experiments were carried out. The first one was designed to test the effect of the initial infection dose (2691±520 cfu vs. 21±7 cfu) on the average Th2/Th1 ratio at 4 weeks. The result showed that the initially larger bacterial dose can cause the lack of Th2 response compared to low-dose infection (Figure 7D) and suggests that lower Th2 response could also be a consequence of high bacterial loads. Thus, in the *High* subgroup among the low-dose infected fish (Figure 7A-C), the resulting Th1-balanced response can be a consequence of the inability to limit mycobacterial growth with innate mechanisms

during the early phase of infection. To test whether there is a natural spectrum in Th balance of the host that could lead to differences in the bacterial loads, a second control experiment was carried out (Figure 7E). Healthy zebrafish were injected with heat-killed *M. marinum* and the *gata3/tbx21* ratio was assessed at 10 days post injection. A vast natural variation in the Th2/Th1 ratio was seen in the responses to heat-killed bacteria showing that the zebrafish population is heterogeneous in terms of Th2/Th1 balance even in the absence of mycobacterial infection. Based on these results the causation could not be unambiguously assessed.

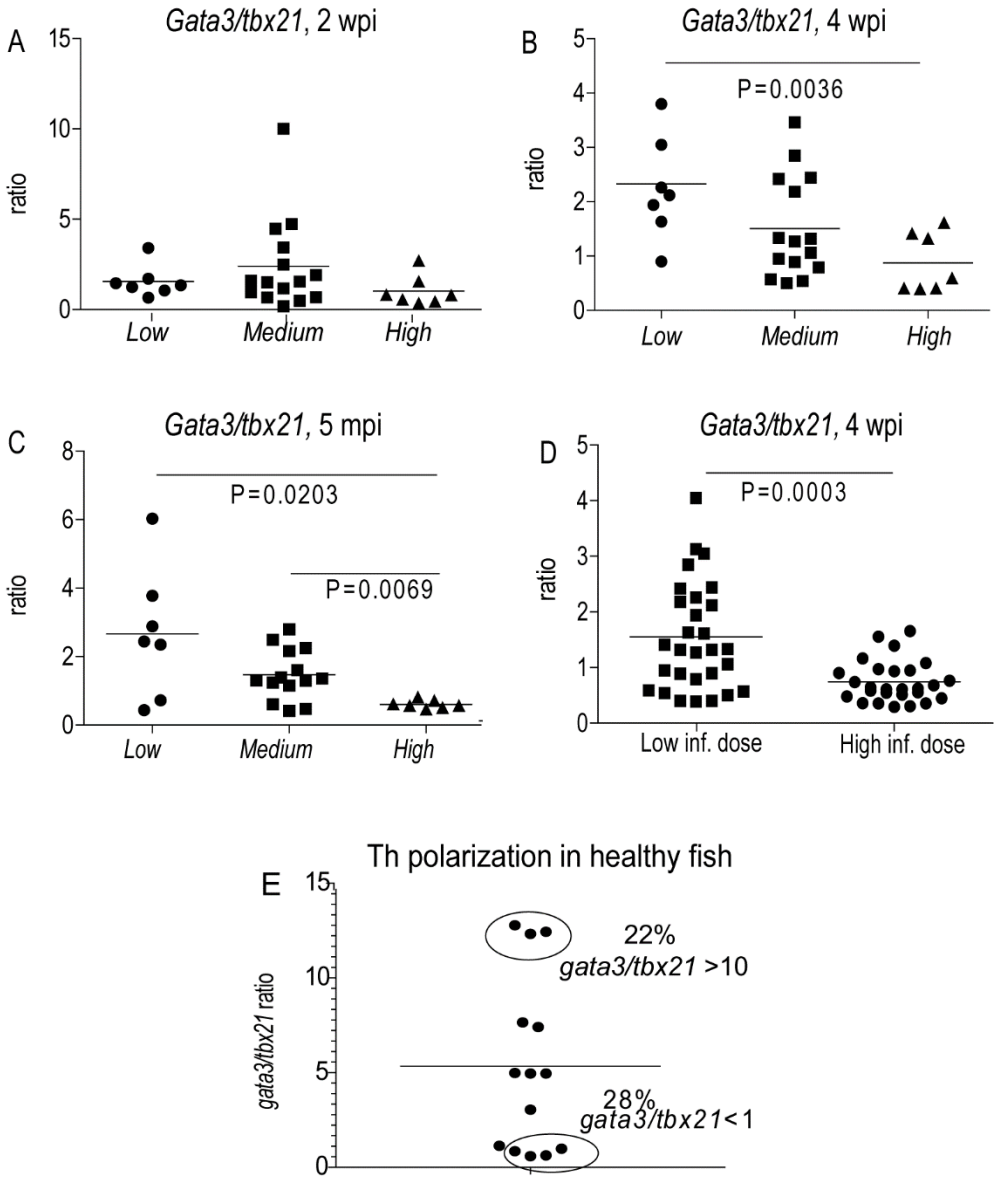

Figure 7. Th2 cells dominate the response in the zebrafish with limited mycobacterial growth. (A–C) *Tbx21* and *gata3* inductions wa measured in the different subpopulations at 2, 4 wpi and 5 mpi (months post infection). The *gata3/tbx21* ratio was calculated to determine the dominant Th type. (D) As a control experiment for assessing the effect of initially high bacterial load on *gata3/tbx21* ratio, WT zebrafish were infected with a high dose (2691±520 cfu) and the *gata3/tbx21* ratio of this group (n=25) was compared to those of the group (n=30) infected with a low dose (21±7 cfu) at 4 wpi. (E) To assess the natural polarization pattern of T cells with regard to *gata3/tbx21*, WT zebrafish (n=14) were stimulated by an intraperitoneal injection of heat-killed *M. marinum*. *Gata3/tbx21* ratio was determined 10 days post injection. Modified from article II.

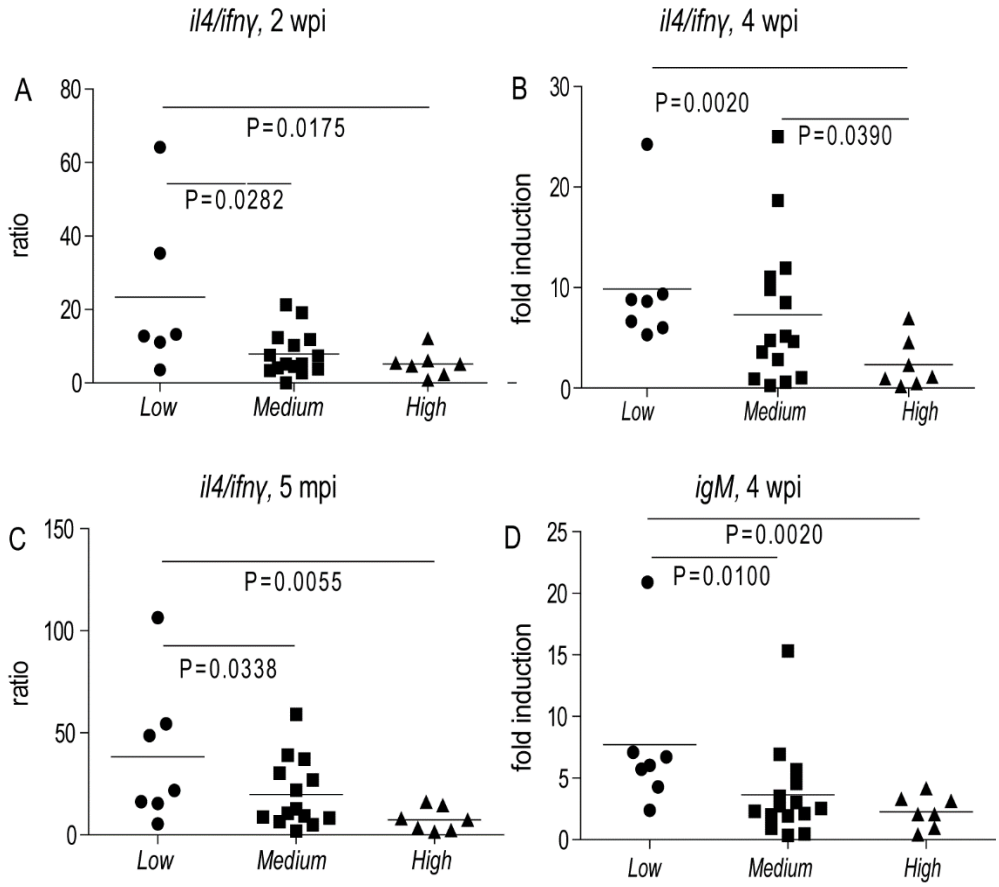

Figure 8. Th2 responses dominate in the zebrafish with limited mycobacterial growth. (A-C) The induction of selected type cytokines for Th1 (*Ifnγ1-2*) and Th2 response (*IL4b*) was measured in the different subpopulations at 4 wpi. The *IL4/Ifnγ* ratio of induction was calculated. (D) The expression of IgM was measured in the subpopulations at 4 wpi. Modified from article II.

5.2.4 Lacking Th2 response is prognostic for increased risk for reactivation (II)

Between 8 and 20 weeks after a low-dose infection, fish showing symptoms were collected to form a group of spontaneously reactivated fish (*Reactivated*). These fish had slightly higher bacterial loads compared to asymptomatic fish in the *High* group (Figure S1D in article II) and dormant populations were absent in both *Reactivated* and *High* groups. (Figure 4B in article II). It is thus likely that the disease in the fish in the *High* group was in the process of reactivation, although no sign of disease was

externally visible. The Th2/Th1 ratio in the *Reactivated* group was low and not significantly different from the *High* subgroup within the asymptomatic population. In ROC analysis, the high load individuals (*High*&*Reactivated*) could be reliably distinguished from the well-controlling population (*Low*&*Medium*) suggesting that measuring the Th2/Th1 balance could be one way of indirect assessment of bacterial loads and the activity of asymptomatic mycobacterial disease (Figure 4D in article II)

5.2.5 Induction of regulatory T cells associates with fulminant reactivation of latent mycobacterial infection (II)

As described in section 5.2.2, half of the fish in the *Reactivated* group had high T cell numbers (Figure 6D) and nevertheless succumbed to infection with high bacterial loads and severe symptoms. The T helper response distinguishing the symptomatic *Reactivated* fish from the asymptomatic *High* group was the induction of *foxp3* (*forkhead box P3*) used as a marker for regulatory T helper cells (Figure 9A). The difference remained visible even after normalization to the expression of *cd3* (Figure 9B).

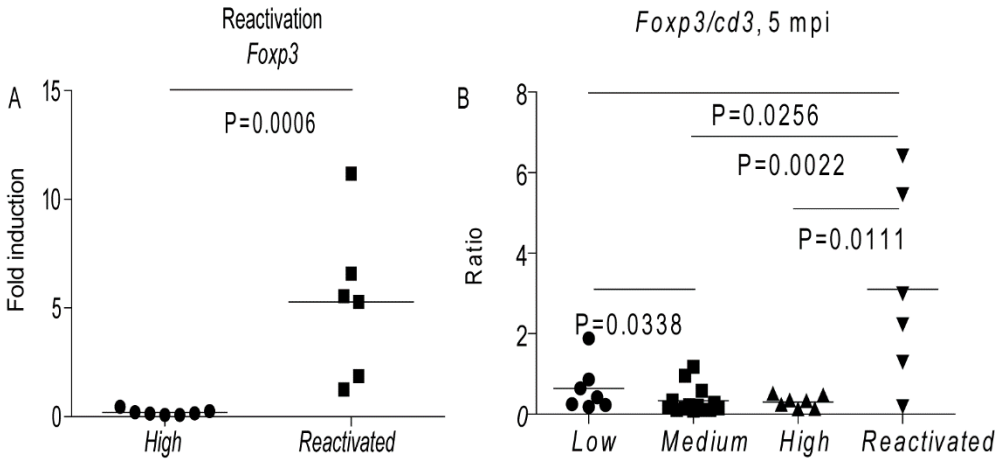

Figure 9. Induction of regulatory T cells associates with the spontaneous reactivation of mycobacterial infection in the zebrafish. (A) *Foxp3* induction of *Reactivated* group (fish showing symptoms between 8 and 20 weeks after an initial controlled phase) and *High* subpopulation at 5 mpi. (B) Induction of *foxp3* was normalized to *cd3* induction in the subgroups at 5 mpi and the *Reactivated* group. Modified from article II.

5.3 Immunomodulation prior to *M. marinum* infection enhances antimycobacterial innate responses leading to protection and sterilization of infection (III)

As seen in Figure 1B, during the first week of infection, bacterial growth was close to logarithmic in both dose groups suggesting that the immune system was unable to restrict mycobacterial proliferation during the early stage of infection. It seems that mycobacteria are efficiently hiding from the immune system until they reach a certain level. In the third part of this thesis the aim was to determine, whether the immune responses could be boosted prior to infection to more efficiently limit the bacterial growth in the early phase of the infection to potentially clear the infection. The scientific community is still struggling with developing treatments against TB that would result in the sterilization of the infection. Studying the nature of a protective innate responses creates important knowledge for the development of host-directed therapies. Such therapies could be used even in the absence of a functional adaptive immune system either alone or in combination with antibiotics with the aim of resolving the infection rather than limiting bacterial growth to establish latent infection.

5.3.1 Priming with heat-killed *Listeria monocytogenes* protects zebrafish against *M. marinum* infection (III)

To find ways to induce protective immune response in a preliminary experiment, groups of 10 fish were injected with a set of immunomodulatory agents including adjuvants, TLR-ligands and heat-killed bacteria 1 day prior to a low-dose *M. marinum* infection. The agents tested were LPS, paclitaxel, MDP, zymosan, heat-killed *M. marinum* (HKMm), *Listeria monocytogenes* (HKLm), *Streptococcus iniae* (HKSi), *Escherichia coli* (HKEc). PBS was used as an injection control. At 7 wpi, the bacterial loads were measured from the internal organs with qPCR: Apart from paclitaxel, which increased the median bacterial load and killed half of the fish, all modulators slightly decreased the median bacterial load (Figure 10A). However, HKLm had the most striking effect: 6/10 fish had entirely cleared the infection as opposed to 1/10 in the PBS control group. As the group sizes in this preliminary study were too small to gain conclusive results, the experiment with HKLm was repeated several. Repeating the experiment several times confirmed the protective effects of HKLm both in significantly reducing bacterial loads and increasing the number of individuals

without measurable mycobacteria (article III). The effect, although to a slightly lesser extent, could be seen even in the absence of adaptive immune responses in *rag1* (-/-) fish suggesting that modulation of innate immunity at an early time-point was sufficient to induce a sterilizing response.

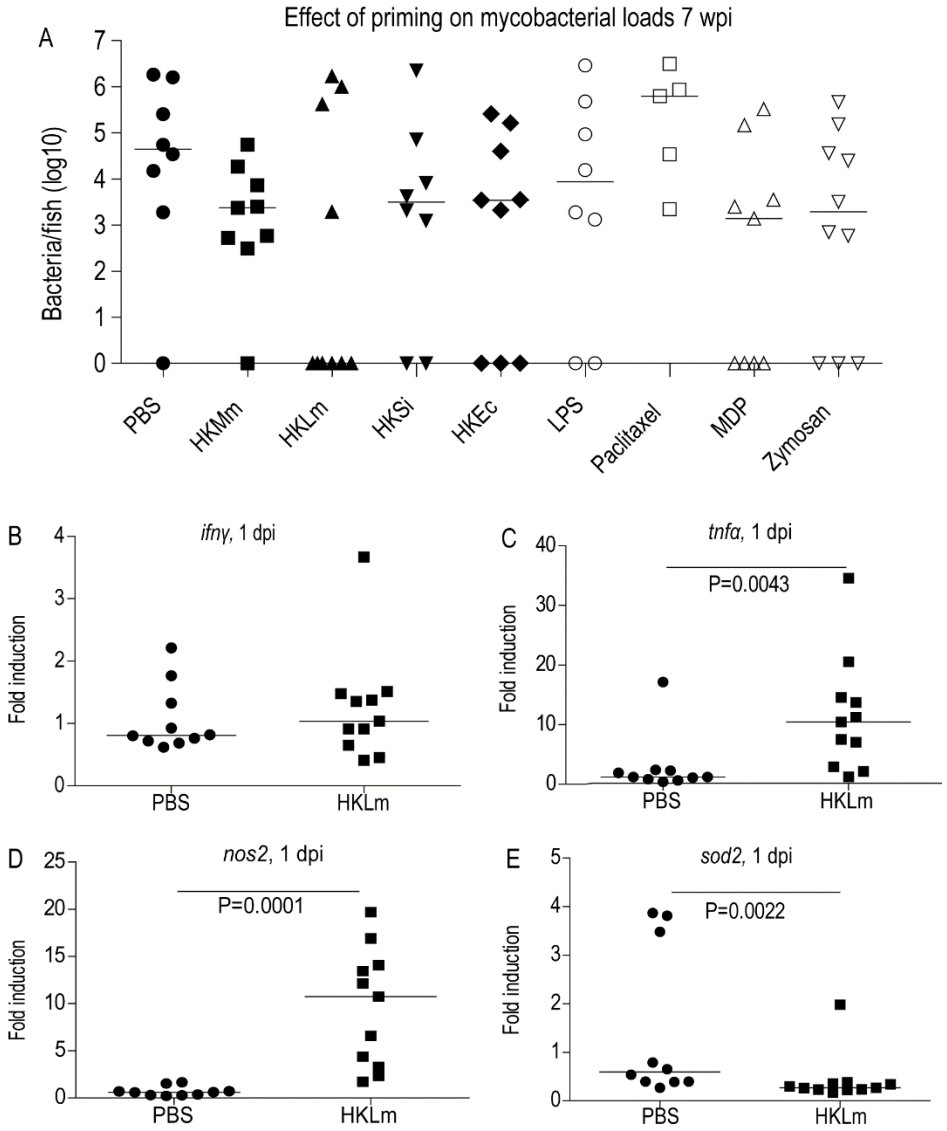

Figure 10. HKLm priming prior to *M. marinum* infection induces protective antimycobacterial responses. (A) In this preliminary experiment, zebrafish were primed with heat-killed *M. marinum* (HKMm), HKLm, heat-killed *S. iniae* (HKSi), heat-killed *E.coli* (KHEc), lipopolysaccharide (LPS), paclitaxel, muramyl dipeptide (MDP) or zymosan 1 day prior to *M. marinum* infection (16±4 cfu). Bacterial loads were measured from the internal

organs with qPCR at 7 wpi. (B-E) The effects of HKLm treatment on gene expression were studied 1 day post infection (2 days post priming) with qPCR. Modified from article III.

5.3.2 Priming with heat-killed *Listeria monocytogenes* causes increased *tnfa* and *nos2* and reduced *sod2* expression (III)

Looking for mechanistic explanations for the protective response induced by HKLm, changes in gene expression were measured at 1 dpi by RT-qPCR. The effect was likely not mediated through Ifnγ, as there were no differences in the expression of this cytokine at 1 dpi (Figure 10B). From a vast panel of genes analyzed, *tnfa, nos2b* and *sod2* were differentially expressed between PBS and HKLm-primed groups (Figure 10C-E). The median expression levels of *tnfa* and *nos2b* were substantially and significantly increased by HKLm (Figure 10D&E). The expression of *sod2* was slightly more downregulated in HKLm group compared to the PBS group (Figure 10F). These changes suggest that HKLm treatment boosted intracellular killing mechanisms leading to more efficient eradication of mycobacteria.

6 DISCUSSION

6.1 On the applicability of the zebrafish model for TB research

Although many details on mycobacterial pathogenesis have been elucidated using elegant *in vitro* models, animal models are indispensable in assessing the complex interplay between the host and the pathogen. Selecting an appropriate model is of utmost importance in the venture of extracting information that is relevant for medical applications. A huge proportion of studies on mycobacterial pathogenesis has been carried out in the mouse, although many aspects of the disease are not well replicated in this model (Dheda, Barry 3rd et al. 2016). *Mtb* is not a natural pathogen of mice (Comas, Coscolla et al. 2013) and experimental infections of mice with the human pathogen generally lead to a progressive disease instead of latency (McCune Jr, Tompsett et al. 1956). The mouse model differs from human TB also in terms of the structure of granulomas (Alexander, Liu 2006, Russell, Barry 3rd et al. 2010, Flynn 2006, Barry, Boshoff et al. 2009). When trying to understand the entity of tuberculosis, it is important to note the possible discrepancies between the human disease and the disease in an experimental model. Using the wrong model in the wrong context can lead to erroneous conclusions and hinder the development of new treatment strategies.

In this thesis, the adult zebrafish-*M. marinum* model was used to study the nature of immune responses related to the disease spectrum caused by this pathogenic mycobacterium. One of the most important benefits of this model is that *M. marinum* in a natural fish pathogen (van der Sar, Appelmelk et al. 2004, Decostere, Hermans et al. 2004, Tobin, Ramakrishnan 2008). Natural host-pathogen pairs have been molded by the forces of evolution and provide relevant knowledge on true virulence strategies and pathogenesis that essentially is a product of the complex interactions between the bacterium and the host.

One central difference between laboratory mice and laboratory zebrafish is that zebrafish are not inbred (Trede, Langenau et al. 2004), which on one hand can be seen as a down-side leading to large biological variation in experimental infections, but which in this study was seen as an advantage. Due to the variation in the genetic pool of the zebrafish, it was possible to see the entire spectrum of disease states

develop after a low-dose challenge with *M. marinum*. Importantly, also latent infection with a dormant mycobacterial population spontaneously developed in the zebrafish. Previously, a non-human primate model has been shown to develop a disease spectrum closely resembling that of human TB (Capuano III, Croix et al. 2003, Lin, Ford et al. 2014). However, ethical and economic considerations limit the use of the primate model, whereas in the zebrafish model, carrying out larger experiments is more feasible.

The small size of the zebrafish is a great advantage in that the entire animal can be collected to carry out a thorough analysis of the disease state and bacterial load at an organismal level. It is not practically feasible to study bacterial clearance in a larger animal. On the down-side, it is not possible to collect blood or tissue samples of a living fish to follow the course of infection within a single individual. Theoretically, using mutant zebrafish lines lacking pigmentation combined with fluorescent or bioluminescent bacteria should allow longitudinal studies on bacterial loads. However, the approach does not permit simultaneous measurement of immune responses or changes in bacterial gene expression, which was possible using nucleic acids extracted from euthanized fish in this study.

The immune system of the zebrafish is highly similar to humans as described in section **2.7.2 The immune system of the zebrafish.** Most importantly, adaptive immune cells are present in the zebrafish making it a highly convenient model for this study. The importance of adaptive immunity in the fish TB model could be reliably shown using the commercially available *rag1* (-/-) mutant. There are also some differences between the immune functions of fish and man that need to be recognized. The adaptive immune cells of zebrafish produces fewer antibody isotypes than humans (Willett, Cherry et al. 1997, van der Sar, Appelmelk et al. 2004, Danilova, Bussmann et al. 2005), and do not go through affinity maturation (Trede, Langenau et al. 2004, Meeker, Trede 2008, Lieschke, Trede 2009, Wakae, Magor et al. 2006). Regarding the innate arm of immunity, TLRs in the zebrafish show 50 % higher diversity, possibly allowing a wider repertoire of microbial patterns to be recognized by innate cells (Lieschke, Trede 2009). Interestingly, there is evidence that zebrafish have specific innate memory responses that give specific protection against re-infection (Hohn, Petrie-Hanson 2012) It has been suggested that the zebrafish might rely on the innate arm of immunity more heavily than mammals (Lewis, Del Cid et al. 2014), which should be noted also in the context of *M. marinum* infection: Although we were able to induce a sterilizing innate response by HKLm priming in the zebrafish, whether or not the human innate immune system has the capacity to respond in the same manner is not known. Also, the relative importance

of adaptive T helper responses and innate responses in determining the disease outcome might be different in fish TB compared to human TB.

An obvious topic to be discussed when using fish to model a pulmonary infection is the fact that fish do not have lungs. In humans, TB is transmitted through the lungs and it is also the most common tissue type to become infected (Dheda, Barry 3rd et al. 2016). In the fish, the natural route of *M. marinum* infection is through the gastrointestinal tract (Harriff, Bermudez et al. 2007). Experimental infections utilizing the natural route would be a more appropriate model for studying the early stages of infection, as natural entry through the mucosa likely induces a different type of immune activation than infection by injection, which unavoidably causes some level of tissue damage and pro-inflammatory activation. When collecting data for article I, I also infected fish by bathing to introduce the bacteria through the natural route. As only 50 % of the fish got colonized by bathing, the method was not chosen for further experiments. However, to study the natural early immune responses, colonization and transmission, the bathing infection model should be studied further, as it could provide highly relevant knowledge on these aspects of infection.

Although there are undoubtedly differences between human TB and *M. marinum* infection in fish, the pathology in general is strikingly similar (Parikka, Hammarén et al. 2012, Meijer 2016, Ramakrishnan 2013, Berg, Ramakrishnan 2012, Swaim, Connolly et al. 2006). The adult zebrafish model of TB nicely complements the assortment of *in vivo* models. Due to its small-size and cost efficiency, large scale experiments and iterative approaches are feasible in the zebrafish. Importantly, the adult zebrafish-*M. marinum* model is a potential platform for testing treatment strategies against dormant mycobacteria. Due to the small size and the sensitive qPCR-based method developed for detecting both actively dividing and dormant mycobacteria, bacterial clearance can be assessed in the zebrafish. Furthermore, testing novel vaccination strategies protecting against latent and reactivated infection could be tested in this model. The results extracted from preliminary fish experiments can then be further validated in mammalian models and ultimately in humans.

6.2 On the balanced innate immune response in mycobacterial infection

Innate immune cells are the first ones to encounter pathogenic mycobacteria, and it is reasonable to think that the early events of infection are central in determining the outcome of infection. In the case of TB, even a few inhaled bacilli are thought to be sufficient to cause infection (Rajaram, Ni et al. 2014). This is due to the efficient virulence strategies that allow the mycobacteria to survive inside phagocytic cells, inhibit transportation of antigens to lymph nodes, inhibit co-stimulation of T cells and essentially replicate within phagocytes for weeks prior to the activation of adaptive mechanisms (Chackerian, Alt et al. 2002, Geijtenbeek, Van Vliet et al. 2003, Simeone, Bobard et al. 2012, Houben, Demangel et al. 2012, Flynn, Chan 2003). As the virulence mechanisms are highly conserved between *M. marinum* and *M. tuberculosis* (Stinear, Seemann et al. 2008), the *M. marinum*-zebrafish model is a feasible system to test ways to circumvent mycobacterial virulence.

In the last article forming this thesis, HKLm was shown to lower bacterial loads and even increase bacterial clearance when injected into adult zebrafish one day prior to low-dose infection. To my knowledge, sterilization of mycobacterial infection has not previously been induded in any animal model. This protective effect was accompanied by the induction of *tnfa*, *nos2b* and reduction of *sod2*.

The role of Tnfα in controlling mycobacterial infections in general is rather well-established (Miller, Ernst 2008, Lin, Myers et al. 2010, Clay, Volkman et al. 2008). Importantly, in human genome-wide association studies, the locus encoding this cytokine has been linked with innate clearance (Cobat, Poirier et al. 2015). In the context of the early days of mycobacterial infection in zebrafish larvae, Tnfα is induced along with increased ROS production within macrophages leading to efficient intracellular killing (Roca, Ramakrishnan 2013). High mitochondrial ROS within macrophages has also been associated with enhanced intracellular killing of mycobacteria (reviewed by (Hall, Sanderson et al. 2014)). The down-regulation of Sod2, an enzyme that neutralizes mitochondrial ROS (Pias, Ekshyyan et al. 2003), seen in the context of HKLm priming is a likely protective mechanism in the early phase of *M. marinum* infection. Interestingly, in a recent human population study assessing the susceptibility to *M. leprae* infections, polymorphisms leading to reduced activity of Sod2 were linked to protection against infection (Ramos, Salomao et al. 2016). Thus, HKLm-induced down-regulation of the *sod2* transcription is a potential strategy to induce protection against mycobacterial infections in humans.

The production of NO is another well-known anti-mycobacterial strategy (Nicholson, Bonecini-Almeida Mda et al. 1996, Elks, Brizee et al. 2013). However, mycobacteria possess an array of evasion mechanisms counteracting the production of NO (Bhat, Srivastava et al. 2017, Elks, van der Vaart et al. 2014, Queval, Song et al. 2016), and the natural levels of NO in *Mtb* infection are not mycobacteriocidal (Jung, Madan-Lala et al. 2013). There are two orthologues of human *nos2* in the zebrafish, *nos2a* and *nos2b*, both of which contribute to the induction of NO production (Lepiller, Franche et al. 2009). Assessing the differences in gene expression caused by HKLm treatment, *nos2b* was found to be significantly up-regulated. The increased expression of *nos2b* and subsequent higher NO levels could provide an additional mechanism for enhanced intracellular killing of *M. marinum* during the early days of infection.

However, findings originated from the larval zebrafish model and confirmed in human populations have revealed that the levels of Tnfα dually modulate the pathogenesis of TB so that moderate production of this cytokine is associated with host-beneficial outcomes, whereas too low or too high levels lead to poor disease control (Tobin, Roca et al. 2012, Tobin, Vary Jr et al. 2010). In addition to the magnitude of the pro-inflammatory host response, the kinetics likely play a role in the determination of the disease outcome. In the larval zebrafish model of TB, it is known that in the beginning of infection, high Tnfα is useful and boosts intracellular killing, but at the later stages leads to necroptosis releasing mycobacteria to extracellular environment where they divide uncontrollably. (Roca, Ramakrishnan 2013) Similarly, generation of ROS initially leads to better intracellular killing, but when continued, causes necroptosis with detrimental effects (Roca, Ramakrishnan 2013). The effects of a prolonged HKLm treatment and treatment at a later time-point after the establishment of infection call for further investigation.

Keeping the results presented above in mind, the positive effects induced by a single injection of HKLm in the majority of zebrafish may depend on the host genotype: in a host with a naturally strong pro-inflammatory innate responses, further induction of these responses may lead to negative effects. The beneficial effects of HKLm injection may also be specific to the early stage of infection. Even in the population in which additional short boosting of pro-inflammatory responses during the early days of infection has positive effects, excessive or prolonged induction of Tnfα and ROS can potentially lead to heightened tissue pathology. Thus, development of host-directed therapies of TB will require detailed information on the type, magnitude and kinetics of the response as well as the genotype of the patient.

6.3 On the importance of adaptive immunity in controlling mycobacterial infection

Adaptive immunity, especially CD4+T helper cells, are deemed highly important for protection against primary active or reactivated mycobacterial disease (Boom, Canaday et al. 2003, Lin, Rutledge et al. 2012). Lowered levels of T and B cells in the whole blood samples are associated with active TB (Berry, Graham et al. 2010). HIV co-infection that abrogates the function of T helper cells increases the risk of reactivated tuberculosis (Pawlowski, Jansson et al. 2012). In mouse studies, the lack of helper T cells leads to hypersusceptibility to TB (Mogues, Goodrich et al. 2001). However, the detailed mechanisms of resistance mediated through T helper cells remain incompletely characterized, which is one of the obstacles hampering the development of an efficient TB vaccine (Mayer-Barber, Barber 2015).

In this thesis, one of the aims was to assess the importance of T cells in the zebrafish TB model. A lot of pioneering work on the mycobacterial pathogenesis has been carried out in zebrafish larvae. However, the adult zebrafish could provide an experimental platform in which also the role of adaptive immunity in mycobacterial infections could be assessed in a laboratory setting. A previous survival experiment carried out by (Swaim, Connolly et al. 2006) suggested a role for adaptive immunity in the zebrafish TB model. The first study of my thesis confirmed the survival result. Also, the mycobacterial loads were shown to reach higher levels in the absence if adaptive immunity (I). The importance of lymphocytes for the maintenance of latent state was further demonstrated in experiments, in which the depletion of lymphocytes by gammairradiation led to reactivation of mycobacterial infection (I). In the second part of the thesis (II), the total T cell numbers were found to be lower in the organs of individuals with higher mycobacterial loads during the early weeks of infection. These findings clearly demonstrated the importance of adaptive immunity in the *M. marinum* infection in the zebrafish and set the stage for using the adult model for further elucidating the adaptive mechanisms behind the vast disease spectrum.

The central transcription factors governing T helper polarization are conserved from fish to humans (Mitra, Alnabulsi et al. 2010). As antibodies were not available, the expression of these transcription factors was used as a read-out to measure the numbers of the corresponding T helper types during a mycobacterial infection. When divided into subgroups based on the mycobacterial load, the individuals with the poorest disease control and the highest bacterial loads were found to be deficient of Th2 type responses. In the individuals with limited bacterial growth, Th2 markers

were expressed at a high level. Looking at characteristic Th1 and Th2 gene expression signatures, the differences in Th2 response were more conspicuous between a poorly controlled and a well-controlled infection than in Th1 (II). An array of human studies assessing the cytokine profiles of T cells extracted from either patients with an active or a latent TB infection showed that active TB was associated with more Th1 type cytokines (Pollock, Whitworth et al. 2013, Caccamo, Guggino et al. 2009, Sester, Fousse et al. 2011, Sutherland, Adetifa et al. 2009). However, measuring Th2 responses were not mentioned in these studies. To my knowledge, the only study with an otherwise similar set up but with additional data on Th2 responses found that Th2 responses are higher in the latent population (Gourgouillon, de Lauzanne et al. 2012), in line with our findings in the fish.

Based on the data from the zebrafish model, determining the balance of Th2/Th1 of mycobacterium specific T cells might give more detailed information on the status of a subclinical mycobacterial infection and could help to allocate prophylactic treatment to those patients with the highest risk of reactivation. In the zebrafish, measuring *gata3/tbx21* from infected tissues was reliably associated with mycobacterial load and dormancy, which are difficult to directly assess in human patients. Of course, it is possible that the polarization of the cells in the blood is not the same as in the target tissue. The applicability of the diagnostic approach measuring the ratio of Th2/Th1 responses in human blood samples should be tested.

The result in the zebrafish model was surprising in the light of the traditional dogma on the importance of cell-mediated Th1 responses in controlling intracellular pathogens and Th2 responses, B cells and humoral antibody responses in controlling extracellular pathogens. As *Mtb* is known to be an intracellular pathogen, Th1 responses have been considered protective (Mayer-Barber, Barber 2015) whereas Th2 responses have been regarded irrelevant or even detrimental (Orme, Roberts et al. 1993, Hernandez-Pando, Orozcoe et al. 1996). However, it is now known that the ability of virulent mycobacteria to cause necrosis of phagocytic cells leads to the release of bacteria also to extracellular locations (Behar, Divangahi et al. 2010). Based on what has been discovered using the larval zebrafish model of TB (Roca, Ramakrishnan 2013) and also in human TB (Grosset 2003), it seems that a substantial proportion of mycobacteria actually reside in extracellular niches after the initial phases of infection rendering intracellular killing mechanisms useless at this stage of infection. Humoral responses from B cells reflected by the Th2 polarization should be able to limit the growth of such extracellular mycobacterial populations. Indeed, in the mouse model of TB, there is evidence that even though Th2 responses

are redundant during the early phase of infection, at a later stage, the mice lacking Th2 had higher mycobacterial loads compared to wild-type (Jung, LaCourse et al. 2002). In the zebrafish, the strong Th2 response was accompanied by an induction of IgM suggesting that increased production of antibodies is a likely protective mechanism during *M. marinum* infection. Another mechanism behind the beneficial effects of Th2 responses could be that they balance pro-inflammatory Th1 responses and limit the tissue pathology caused by excessive inflammation (Lugo-Villarino, Hudrisier et al. 2013).

After our publication describing the association of Th2 and humoral responses with limited mycobacterial growth in the zebrafish model, important findings on this subject have been published in human TB. First, Lu and colleagues published their *in vitro* results showing that patients with latent TB produce protective antibodies that improve the ability of macrophages to kill intracellular *Mtb* significantly better than antibodies from patients with active TB (Lu, Chung et al. 2016). Their result suggests that antibodies can also mediate protective effects by limiting the growth of intracellular mycobacteria. After that, another study by Li et al. showed that a portion of health care workers that did not develop active TB despite severe exposure produce antibodies which, when injected to mice prior to pulmonary TB challenge, protect the mice from infection (Li, Wang et al. 2017). Together, these results challenge the traditional view of antibodies being redundant in controlling TB infections. Based on what was seen in article II, I propose that both Th1 and Th2 responses are needed for controlling mycobacterial infections. Intracellular populations present especially during the early phases of infection require efficient Th1 as well as CD8+ responses. However, to be able to control the extracellular populations emerging at later stages and possibly to avoid excessively pro-inflammatory responses when mycobacterial loads are higher, Th2 responses are beneficial (Figure 11). These findings could have important implications in vaccine development, which traditionally has been aiming at induction of Th1 response and suppression of Th2. I propose that in order to succeed in the development of treatments against TB, the traditional dogma of Th1 as the protective immune response needs to be reassessed. An alternative interpretation of the correlation of high Th1 responses with active TB in humans is that these responses (at least in excess) are pathological rather than protective.

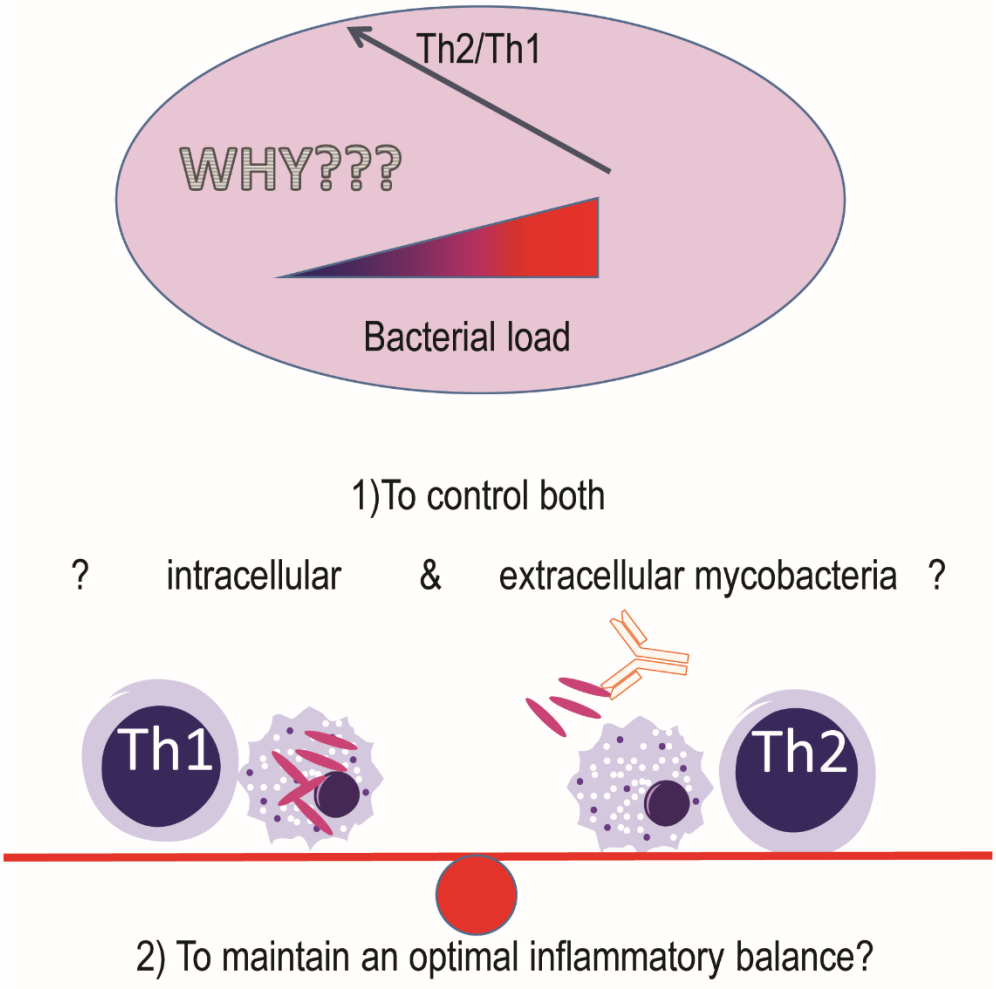

Figure 11. Potential explanations for the usefulness of Th2 in mycobacterial infection.

Although it is clear that adaptive immunity is generally needed to inhibit the development of active mycobacterial infection, in a minority of the cases, the host is able to mount an optimal adaptive response leading to clearance of the infection. In study II, 10 % of the individuals were able to clear the infection, whereas in 90 % of the cases mycobacteria managed to colonize the host. Also in human TB, more often than not, the mycobacteria manage to gain a foothold once they have entered the body (Verrall, G Netea et al. 2014). These numbers show that even in the presence of a functional adaptive immune system, mycobacterial infections are generally not cleared.

The finding that T cell epitopes in *M. tuberculosis* are hyperconserved (Comas, Chakravartti et al. 2010) as well as the large number of pattern recognition receptor agonists expressed by *Mtb* has prompted speculation that mycobacteria could actually orchestrate the activation of adaptive responses for their benefit (Russell 2013). The first step at which mycobacteria could harness adaptive responses is during the establishment of infection. There is evidence according to which mycobacteria induce T regulatory responses to allow replication to reach higher bacterial numbers during the early phases of *Mtb* infection in the mouse (Urdahl, Shafiani et al. 2011, Shafiani, Tucker-Heard et al. 2010, Scott-Browne, Shafiani et al. 2007). At 3 wpi in the mouse, the regulatory responses subside and Th1 responses gain foothold (Shafiani, Dinh et al. 2013). In study II, we showed that in *M. marinum* infection in the fish, regulatory T cells are again induced in spontaneously reactivated infection. In human studies, the high levels of mycobacterium-specific regulatory T cell responses have been associated with active TB (Wu, Huang et al. 2007, Ribeiro-Rodrigues, Resende Co et al. 2006). Interestingly, in HIV positive patients lacking T helper responses, the disease is not as efficiently transmitted (Corbett, Marston et al. 2006), suggesting that hi-jacking host adaptive responses could also provide a more efficient transmission of disease. These data imply that mycobacteria have highly developed virulence strategies that utilize the adaptive T helper immune response to allow the establishment of infection and later reactivation and transmission. The potential benefits of certain types of T helper responses should be kept in mind when designing vaccines against TB.

7 SUMMARY AND CONCLUSIONS

One third of the world's population has been estimated to be infected with tuberculosis substantiating the awesome success of *M. tuberculosis* as a human pathogen. The intimate co-evolution of *Mtb* and *Homo sapiens* has led to a situation in which more often than not, the host species is unable to eradicate the bacterium, and commonly, a latent, asymptomatic infection lurking within the host even for decades, ensues. As so far, the attempts to develop a sterilizing vaccine have failed and as antibiotics have limited and constantly decreasing efficacy against TB, beating this resilient bug calls for prompt solutions utilizing pioneering strategies.

In this thesis, I used an adult zebrafish-*M. marinum* model, which nicely recapitulates the central aspects of human TB. A small-dose intraperitoneal injection of *M. marinum* given to adult zebrafish caused a spectrum of different disease states including clearance, latency, primary active and reactivated infection. Mycobacteria were demonstrated to enter a dormant state during a latent infection in adult zebrafish. The adaptive immune response was found to be central in determining the disease outcome. The importance of adaptive immune response in controlling *M. marinum* infection in zebrafish was demonstrated by rapid bacterial growth and high mortality in mutant *rag1* (-/-) zebrafish lacking T and B cells as well as in irradiation experiments that caused depletion of adaptive immune cells and subsequent reactivation of latent infection. In addition, the higher number of T cells (CD3) was found to be associated with limited bacterial growth in the zebrafish.

Looking at the nature of T cell responses in more detail revealed a higher level of Th2 type responses in the individuals with limited bacterial growth than in individuals in which the bacterial numbers reached higher levels. Th1 cells were similarly induced irrespective of the bacterial load. The finding was surprising in that Th2 responses have been traditionally regarded dispensable in controlling mycobacterial infections. At least in the zebrafish, clearly, Th2 responses play a central role. It is likely that neither Th1 nor Th2 response alone is sufficient to control *M. tuberculosis* or *M. marinum*. A plausible scenario is that both are needed at different stages of infection to limit the growth of distinct mycobacterial

populations. Traditionally, Th1 is thought to be more efficient in controlling intracellular pathogens whereas Th2 is considered useful against extracellular pathogens. Although mycobacteria are infamous for their intracellular survival skills, they are nowadays also known to reside in extracellular niches where they could be efficiently targeted by Th2 responses.

A low Th2/Th1 ratio measured from infected tissue samples was reliably associated with a high bacterial load in the zebrafish model. The lack of Th2 responses was also evident in individuals with a spontaneously reactivated infection. Measuring the Th balance is therefore a potential way of indirectly determining the status of mycobacterial infection in the fish. Currently there are no means of directly assessing mycobacterial loads or the status of a latent mycobacterial infections in human patients. Such diagnostics would allow estimating the risk of reactivation and allocation of antibiotic treatment only to high-risk individuals. The biomarker potential of Th responses should be studied in human blood samples.

In a spontaneously reactivated infection, the average total T cell number was increased compared to fish with an asymptomatic infection. A more detailed assessment of the types of T cells revealed a specific induction of regulatory T helper cells in spontaneously reactivated infection. The finding is interesting and stirs up speculation upon the elegant pathogenic strategy of mycobacteria. Induction of regulatory T cells to suppress effector responses could be one mycobacterium-driven process to further mycobacterial survival and transmission. The role of regulatory T cell responses in the context of reactivation of human tuberculosis should be assessed in future investigations.

In the context of innate immunity to mycobacterial infections it was found that boosting pro-inflammatory responses by priming with heat-killed *L. monocytogenes* when bacterial loads are still rather low is protective and can even lead to clearance of *M. marinum* infection in the fish. These protective responses included induction of *tnfa, nos2* and down-regulation of *sod2*. However, it must be noted that prolonged induction of this type of immune response may not lead to a host-beneficial response. Using HKLm as a treatment at a later stage of infection needs to be further studied. Host-directed strategies in modulating innate immune responses in general is a promising field, but such strategies, due to the strong host component require customization to suit each patient individually. Also, it is unlikely that one type of immune response would be universally ideal in controlling tuberculosis, as there are so many variables that affect the course of infection such as co-infections, the mycobacterial strain, route of infection, infection dose and so on. In addition to the type of response, the magnitude as well as the kinetics of response likely play a central

role in the outcome of the disease. Some pathology is associated with insufficient response that allows bacterial replication, whereas in some cases, disproportionate inflammation causes severe symptoms. The optimal balance of the immune response will vary from one case to another.

Although there are undoubtedly differences between human the pathology of human and fish TB, the pathogenesis in general is astonishingly similar (Parikka, Hammarén et al. 2012, Meijer 2016, Ramakrishnan 2013, Berg, Ramakrishnan 2012, Swaim, Connolly et al. 2006). The adult zebrafish-*M. marinum* system nicely complements the assortment of *in vivo* models: Due to its small-size and cost efficiency, large scale experiments and iterative approaches are feasible in the zebrafish. As spontaneous latency with dormant mycobacteria is commonly seen in the adult zebrafish, it is potential platform for testing novel vaccination strategies against latent and reactivated infection as well as treatments targeted at dormant mycobacteria. With the sensitive qPCR-based method developed for detecting both actively dividing and dormant mycobacteria in all internal organs, bacterial clearance can be assessed in the zebrafish more reliably than any other animal. The zebrafish thus presents itself as a magnificent model system to look for sterilizing treatment strategies against tuberculosis.

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Milka Hammarén

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10 ORIGINAL COMMUNICATIONS

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Mycobacterium marinum Causes a Latent Infection that Can Be Reactivated by Gamma Irradiation in Adult Zebrafish

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Abstract

The mechanisms leading to latency and reactivation of human tuberculosis are still unclear, mainly due to the lack of standardized animal models for latent mycobacterial infection. In this longitudinal study of the progression of a mycobacterial disease in adult zebrafish, we show that an experimental intraperitoneal infection with a low dose (~35 bacteria) of *Mycobacterium marinum*, results in the development of a latent disease in most individuals. The infection is characterized by limited mortality (25%), stable bacterial loads 4 weeks following infection and constant numbers of highly organized granulomas in few target organs. The majority of bacteria are dormant during a latent mycobacterial infection in zebrafish, and can be activated by resuscitation promoting factor *ex vivo*. In 5–10% of tuberculosis cases in humans, the disease is reactivated usually as a consequence of immune suppression. In our model, we are able to show that reactivation can be efficiently induced in infected zebrafish by γ-irradiation that transiently depletes granulo/monocyte and lymphocyte pools, as determined by flow cytometry. This immunosuppression causes reactivation of the dormant mycobacterial population and a rapid outgrowth of bacteria, leading to 88% mortality in four weeks. In this study, the adult zebrafish presents itself as a unique non-mammalian vertebrate model for studying the development of latency, regulation of mycobacterial dormancy, as well as reactivation of latent or subclinical tuberculosis. The possibilities for screening for host and pathogen factors affecting the disease progression, and identifying novel therapeutic agents and vaccine targets make this established model especially attractive.

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Introduction

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis*, a highly specialized pathogen capable of evading the immune defense by various strategies. The success of the pathogen and the shortcomings of current medical interventions are reflected by the high prevalence of *M. tuberculosis* infection; one third of the world's population has been estimated to carry the pathogen and to have a latent, subclinical infection [1], which can be diagnosed using immunological sensitization to *M. tuberculosis* antigens [2]. Noteworthy, this asymptomatic infection is thought to consist of a variety of disease states that differ in bacterial phenotypes and burdens. [2,3].

According to the report of the World Health Organization (WHO), TB caused 1.7 million deaths and 9.4 million new cases in 2009, especially in developing countries. Approximately 5–10% of carriers develop an active disease during their lifetime [4], which reflects the spectrum of disease states within the population with latent TB [2,3]. This number is even higher in countries with a high prevalence of human immunodeficiency virus (HIV) [4]. The current preventive treatment against TB, the Bacille Calmette-Guérin (BCG) vaccine, protects children against the most severe forms of TB (TB meningitis or disseminated TB), but its efficacy in adults has been questioned and is thought to have limited or no protection against the disease [5,6]. A worrisome shortcoming is that BCG does not protect against the reactivation of latent, subclinical TB [7]. The prevalence of HIV seems to be one of the most important attributes to the increase in the number of active TB cases [5,8]. Tumor necrosis factor (TNF) neutralizing treatments often used in autoinflammatory diseases have also been found to increase susceptibility to TB [4,5], as do malnutrition, tobacco smoke, indoor air pollution, alcoholism, insulin dependent diabetes, renal failure, and immune suppressive treatments, such as glucocorticoids [4]. These factors may either

Author Summary

One third of the world's population has been estimated to be infected with *Mycobacterium tuberculosis*, which under the appropriate set of circumstances causes lethal lung disease. According to current understanding, mycobacteria can persist in their host without causing symptoms – a state referred to as latency or subclinical infection. However, if the immune system of the host becomes compromised, for example due to immunosuppressive medical treatments or HIV, the disease can become reactivated with detrimental consequences. The mechanisms leading to latency are not well understood. Latent tuberculosis responds poorly to antibiotics, and there is currently no effective vaccine against latent or reactivated tuberculosis. Using *Mycobacterium marinum*, a natural fish pathogen and a close relative of *M. tuberculosis*, we were able to induce a disease in adult zebrafish closely mimicking the human latent disease. We show that a dormant mycobacterial population is present in animals with a latent mycobacterial disease. Dormancy is also thought to occur in human tuberculosis. In addition, we present a method, with which the latent disease can be experimentally reactivated. Despite the evolutionary distance between man and fish, the zebrafish presents itself as a unique model for studying the mechanisms related to latency and reactivation.

cause the primary infection to progress, or an existing subclinical infection to reactivate. In general, the mechanisms for the reactivation of tuberculosis are not well established and warrant further investigation.

Various animal models have been used for studying mycobacterial infections with the ultimate aim of understanding human TB [8]. The zebrafish has lately been established as a new, genetically tractable model for studying host–mycobacterium interactions [9–11]. Zebrafish are naturally susceptible to *Mycobacterium marinum* [12–14], which is a close relative of *M. tuberculosis* [15]. *M. marinum*-induced disease in zebrafish shares the main pathological and histological features, including necrotic granulomas, with human TB [16] and is thus a highly attractive model for the human disease. Zebrafish larvae have been widely used for studying innate immune responses to *M. marinum* infection [9,11,17]. However, adaptive immune responses have also been reported to be essential for controlling human TB [18,19] and are also important for controlling *M. marinum* infection in adult zebrafish [10].

Studies on the latency, dormancy and reactivation of TB have been impeded by the lack of applicable animal models, as spontaneous latency without the help of chemotherapeutics has only been successful in the rabbit [20], and in macaque [21] models. Here, we show that a low-dose *M. marinum* infection spontaneously develops into a latent, non-progressive disease in adult zebrafish, with a static number of granulomas and a stable bacterial burden mainly consisting of dormant bacteria. The existence of a large dormant population of mycobacteria seems to be connected to the latent disease. In our model, the stable latent disease can be experimentally reactivated with γ-radiation, essentially mimicking the immune suppression-induced reactivation in human TB. This study thus presents a novel vertebrate platform suitable for large scale genetic screening, as a means of characterizing host and pathogen mechanisms underlying the transitions in TB from an acute infection to latency, and to a reactivated infection.

Results

A low-dose *M. marinum* infection leads to a latent disease with stable bacterial loads after 4 weeks

The lack of suitable and well-established animal models mimicking latent, subclinical TB in humans prompted us to investigate if such a model could be developed in zebrafish. First, we compared several methods for infecting adult zebrafish with their natural pathogen, *M. marinum*, to create a physiological infection model leading to a static phase after the primary active disease. We infected zebrafish either by injecting different bacterial doses into the abdominal cavity or by bathing, to find a suitable dose and an infection route inducing a latent infection with low mortality. The experimental groups were followed up to 32 weeks for survival. A high-dose intraperitonaeal (i.p.) infection (2,029±709 cfu) was characterized by high mortality (end-point mortality 64%), whereas most fish infected with a low dose (34±15 cfu) generally survived (end-point mortality 25%) (Figure 1A). A group of fish was also infected with 9,075 ± 2,681 cfu, but this dose lead to an extremely high mortality (80% mortality in 5 weeks)(data not shown) and the group was excluded from further characterizations. Bathing the fish in water containing 2.4×10^6 cfu/ml lead to an infection only in 50% of the individuals (determined by bacterial loads), which then developed a similar level of end-point mortality as the low-dose injected fish (data not shown). Because of the low incidence rate, bathing was not considered a suitable method for studying latent mycobacterial infection in adult zebrafish.

Latent human TB is diagnosed using tuberculin skin test (TST), interferon-γ release assays (IGRA) and characterized by a lack of clinical signs [2]. In our model, we are able to directly follow the progression of the disease by quantifying total mycobacterial burdens within the whole organism. For this purpose we developed a new, qPCR-based method specific for *M. marinum* (Supporting information, Text S1, Figure S1). In the high-dose group, an average bacterial load of 6.0×10^5 cfu/fish (SD = 6.5×10^5) was measured as early as 1 week post infection (wpi). Bacterial growth during the first week after injection was close to logarithmic, suggesting that the bacteria grew in an unrestricted manner. During the 32-week follow up, the average burdens rose to 3.0×10^6 cfu/fish (SD = 3.2×10^6), indicating that the high dose i.p. injection leads to a chronic progressive disease. Also in the low-dose group, the bacteria grew almost logarithmically during the first week of infection. The average bacterial load increased from the 1 weeks' 5×10^3 (SD = 3.1×10^3) to 4 weeks' 5.2×10^5 cfu/fish (SD = 1.1×10^6). After the four-week time point, however, the average bacterial burden ceased to grow, remaining at an unaltered level until the end of the experiment (at 32 weeks 4.4×10^5 cfu ± 4.4×10^5/fish) (Figure 1B). This result suggests that experimental infection of adult zebrafish by an i.p. injection of a small dose of *M. marinum* leads to an active primary infection, followed by a controlled state in most individuals.

Granuloma formation and spreading of the infection ceases at the onset of the stable state infection in the low-dose infection model

In order to get a more detailed and biologically relevant measure of the progression of the disease in our infection model, we carried out histological analyses at 2, 4, 8 and 20 wpi. Ziehl-Neelsen staining for mycobacteria was used for the quantification of granulomas and affected target organs. The gonads, pancreas, liver, muscle, mesentery, spleen, gut and kidney were specifically assessed for the presence of mycobacterial lesions. Early granulomatous structures characterized by cellular and bacterial

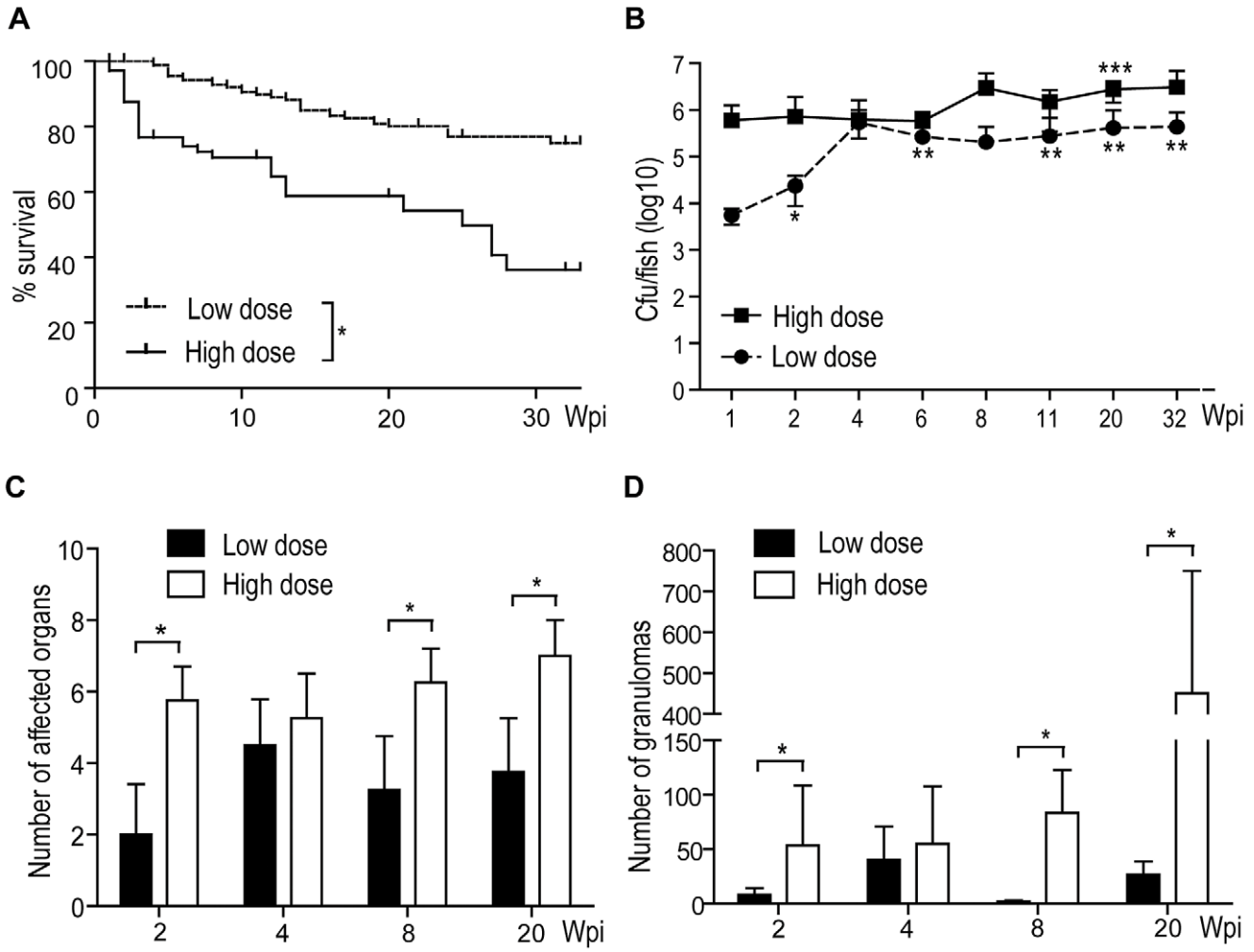

Figure 1. Zebrafish mortality, the development of bacterial load and the number of lesions have dose-dependent patterns. Adult zebrafish were i.p. infected with either a low (34±15 cfu) (n = 180) or a high dose (2029±709 cfu) (n = 104) of *M. marinum*. (A) Survival was followed for 32 weeks. * $P<0.05$ (B) The figure shows the average loads for 5 fish (except 32 wk high dose, n = 2). Low-dose statistics: * sig. diff. from 1 wk, ** sig. diff. from 1 and 2 wk. High-dose statistics: *** sig. diff. from 1, 2, 8, 11 and 20 wk. Low-dose vs. high-dose statistics: loads at time-points marked with † are sig. diff. (C) By default, 4 individuals per dose were analyzed by Ziehl-Neelsen staining (except 20 wk high dose, n = 3) per time-point The gonads, pancreas, liver, muscle, mesentery, spleen, gut and kidney were assessed and the number of organs with visible bacteria was determined. *$P<0.05$. (D) The total number of granulomas in a sample set for each individual was counted. * $P<0.05$.
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aggregation were formed by 2 wpi in both dose groups (Figure 2A–D). The general appearance of the structures developed in the course of the infection such that at 20 weeks, most granulomas were insulated from the surrounding tissue by a fibrotic and/or cellular cuff (Figure 2E–H).

Granulomas were counted in representative sample sets for each individual (Figure 1D). Unsurprisingly, the fish infected with a low dose had significantly less granulomas at 2, 8 and 20 weeks following infection than the high-dose infected fish. The number of granulomas thus seems to be determined by the initial dose. In the high-dose infection, the number of granulomas significantly increased between 4 and 20 weeks, whereas in the low-dose infection, the number did not increase after the first 4 weeks, further supporting the relevance of our model for latent TB.

The number of affected organs was found to be determined by the initial infection dose. At 2 wpi, the low-dose infected fish had lesions in ~2 organs (most often in the pancreas and gonads), whereas fish infected with the high-dose had bacteria in ~6 organs (pancreas, kidney, gonads, liver, muscle, spleen). The number remained relatively unaltered for the duration of the experiment (Figure 1C), with the exception of a slight increasing trend in the high-dose group between 2 and 20 weeks. In the low-dose group, an increase between 2 and 4 weeks was seen (not significant), but the number of affected organs then ceased to grow, suggesting that the infection was well-controlled.

In conclusion, the histological analysis supports the idea that the high-dose infection is progressive with an increasing number of granulomas in various target organs, whereas the low-dose infection resembles a latent infection with unaltered numbers of granulomas in few target tissues.

Cytokine responses to *M. marinum* differ between low-dose and high-dose infection

To build a more detailed understanding on the different outcomes between the high and low dose infection, the early immune responses were studied by measuring cytokine expression levels in the internal organs of infected fish by reverse transcription

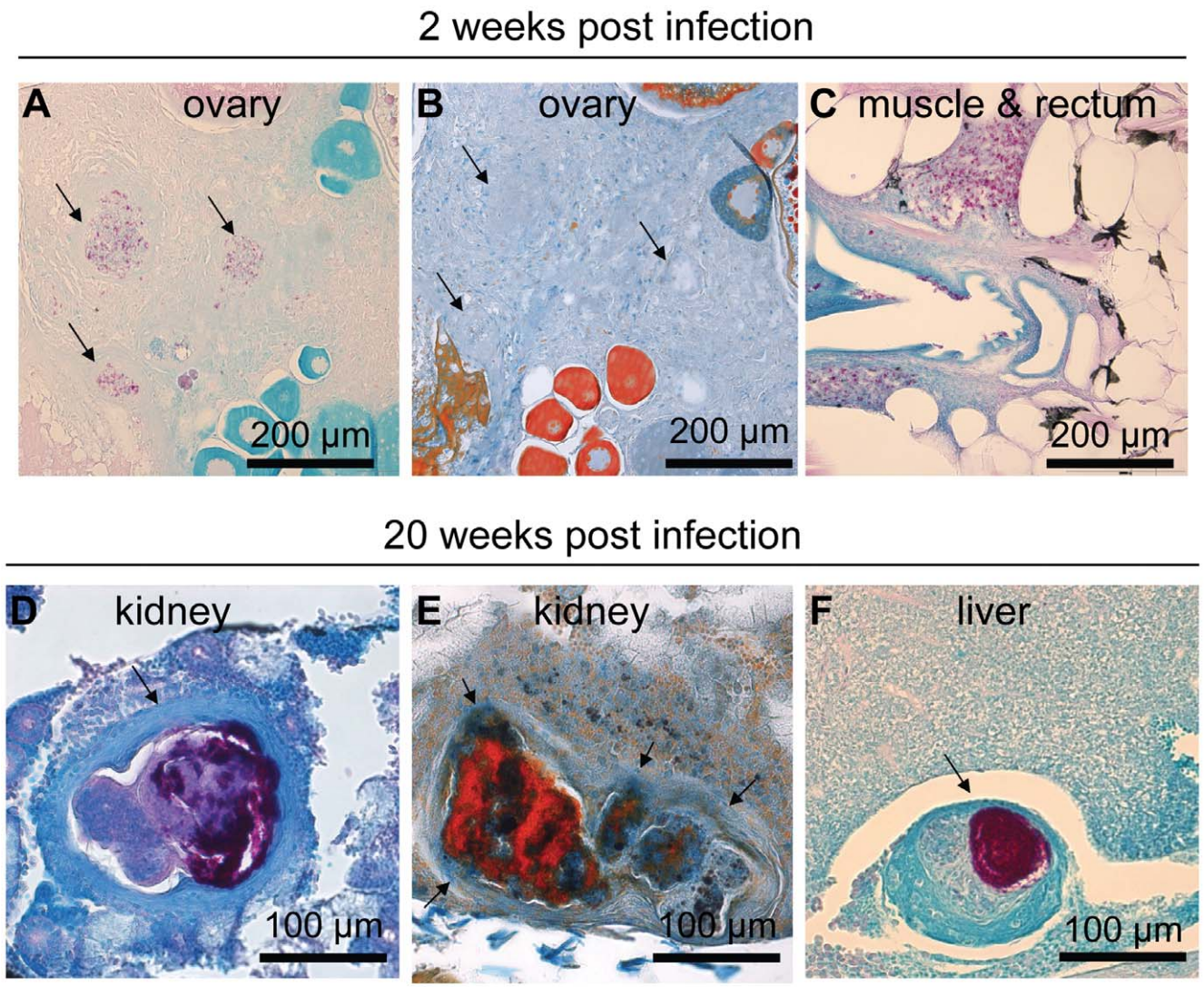

Figure 2. *M. marinum* induces the formation of granulomas that mature into well-defined structures during an infection. In fish infected with a low dose (34±15 cfu) of *M. marinum*, Ziehl-Neelsen staining at 2 wpi commonly reveals areas with free bacteria (C). Some slightly better formed and restricted areas containing bacteria, here referred to as early granulomas, are also seen (A), but as shown in (B) trichrome staining of the adjacent slide, encapsulation around the mycobacterial lesions is absent at the early stage of infection. At 20 weeks, fish that have survived have mature granulomas (D–F) many of which are multicentric surrounded by a fibrous capsule (D&E). (E) Trichrome staining shows the fibrous capsule in blue (F). The amount of bacteria inside granulomas has increased from the earliest time-points.
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quantitative PCR (q-RT-PCR). One day after infection, the high-dose infection caused an induction of *tumor necrosis factor alpha* (*TNFα, ZDB-GENE-050317-1*) by 6.5-fold (SD = 6.6), *interleukin 6* (*IL-6, ZDB-GENE-120509-1*) by 9.6-fold (SD = 10.4) and *interleukin 12* (*IL-12, ZDB-GENE-060724-1*) by 2.7-fold (SD = 1.8) (Figure 3C), but no induction was seen in *interleukin 1 beta* (*IL-1β, ZDB-GENE-040702-2*). Among the low-dose infected fish, only *IL-6* was induced but at a lower level, 3.9-fold induction, SD = 4.8, compared to high-dose infection at 1 dpi.

As the early innate responses are known to regulate the activation of adaptive responses, it was not surprising that differences in *interferon gamma 1–2* (*IFNγ1–2*, ZDB-GENE-040629-1) and *inducible nitric oxide synthase 2b* (*Nos2b*, ZDB-GENE-080916-1) levels were seen between the high and low dose groups at later time points (2–7 wpi). *Nos2b* was consistently more highly induced with the high dose than with the low dose at 2, 4 and 7 weeks (Figure 3D). The expression was at the highest level already at 2 wpi (high-dose group 1,508-fold, SD = 2,136, low-dose group 123-fold, SD = 167), after which the level declined in both dose groups, still remaining strongly induced.

In *IFNγ1–2* expression, the high dose caused a 13.7-fold induction (SD = 16) at 2 weeks. The low dose caused a more moderate 3.0-fold induction (SD = 2.8 (Figure 3F)), which was not different from the induction in the buffer-injected group. At 4 wpi, no difference was detected in *IFNγ1–2* levels. Noteworthy, at 7 wpi, the *IFNγ1–2* expression in the high-dose group had decreased to 1.8-fold induction (SD = 1.6), whereas in the low-dose group the level had increased to 8.8-fold (SD = 11.0), compared to uninfected controls. Thus, the kinetics of *IFNγ1–2* show a decreasing trend in the high-dose group and an increasing trend in the low-dose group, but the differences at late time-points are not significant. In conclusion, these results suggest that the strong early cytokine responses with the high infection dose are associated with *Nos2b* induction at an early phase of infection

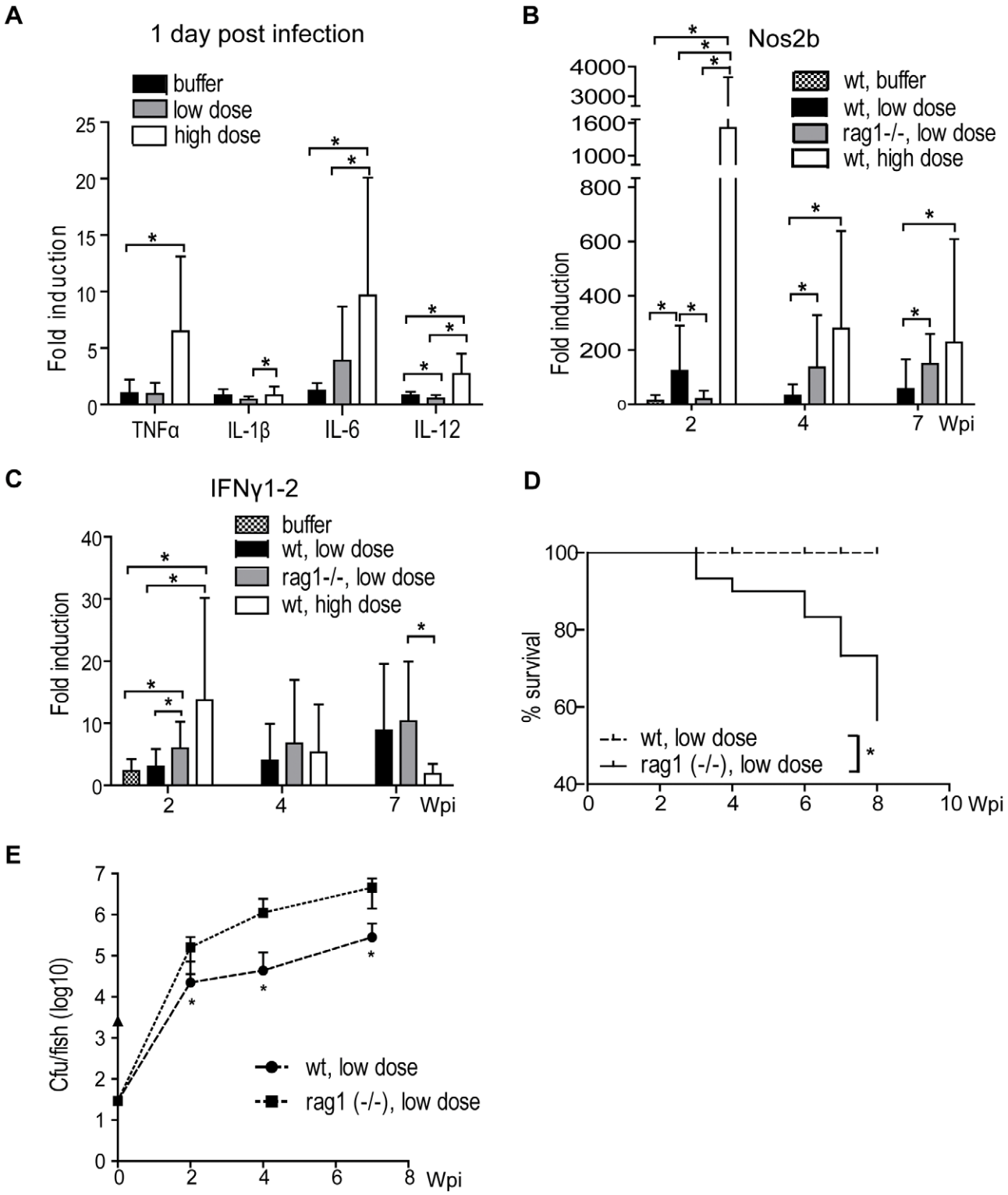

Figure 3. Bacterial dose and the presence of functional adaptive immunity define the outcome of mycobacterial infection. (A) The early cytokine response at 1 d post infection was measured from wt fish infected with a high (2029±709 cfu) or a low (34±15 cfu) dose or injected with sterile PBS buffer (n in each group 10–20). *P<0.05 (B) Wt fish were infected with a high or a low dose or sterile PBS buffer (for early time-points), and rag1 (−/−) fish were infected with a low dose Nos2b expression was measured with q-RT-PCR (n in each group was 9–20/time point). *P<0.05 (C) Fish were infected as in (B) and *IFNγ1–2* was measured with q-RT-PCR. *P<0.05. (D) Adult wt and rag1 (−/−) zebrafish were infected with a low dose (n = 30) and followed for survival. *P<0.05 (E) Adult wt and rag1 (−/−) fish were infected with a low dose. Average mycobacterial load was measured by qPCR at 2, 4, and 7 wpi (n = 10 per time point). *P<0.05.
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(2 wpi) and to the different kinetics of *IFNγ1–2* response between the two dose groups.

Adaptive immunity is required for the restriction of bacterial growth and the induction of latency

According to the current understanding on human TB, adaptive immunity is required for efficient control of the disease [18,19]. Survival results from a previous publication suggest a role for adaptive immunity in mycobacterial infection in the zebrafish [10]. We wanted to study whether adaptive immunity is required for the establishment of latency in the zebrafish. To this end, we used a recombination activating protein 1 (*rag1*) deficient zebrafish line, which lacks functional T and B cells [22].

First, we looked at the morbidity caused by a low dose of the type strain of *M. marinum* in rag1-mutant (−/−) zebrafish. Rag1 (−/−) fish, along with wild type (wt) controls, were infected with the low dose (34±15 cfu). The fish were euthanized at the end-stage of infection and survival curves were drawn (Figure 3D). None of the wt fish showed signs of disease during the 8-week follow up, whereas 43% of the rag1 (−/−) fish reached the end-stage of disease. DNA was extracted from the end-stage rag1 (−/−) fish and the mycobacterial load was measured by qPCR. The average load was 3.89×10^7 cfu/fish (SD = 3.68×10^7), which is similar to the levels measured from terminal stage *M. marinum* infected wt fish (data not shown), indicating that the rag1 (−/−) zebrafish had suffered from an end-stage *M. marinum* infection.

Dynamic disease progression among rag1 (−/−) fish was associated with elevated mycobacterial loads compared to wt controls during the first weeks of infection. Rag1 (−/−) and wt fish were infected with the low dose for determination of bacterial burdens by qPCR. Already at 2 wpi, the loads in the rag1 (−/−) fish were significantly higher (1.61×10^5 cfu/fish, SD = 1.25×10^5) than in the wt fish (2.22×10^4 cfu/fish SD = 4.99×10^4), indicating that the adaptive immune responses are used already by 2 wpi as a means of restricting the mycobacterial infection. During the following weeks, the bacterial burdens remained significantly higher in the rag1 (−/−) mutants (3.80×10^6 cfu, SD = 3.15×10^6) compared to wt fish (2.83×10^5 cfu, SD = 3.26×10^6 at 7 wpi).

Alongside with gene-expression measurements from wt fish, *Nos2b* (Figure 3B) and *IFNγ1–2* (Figure 3C) levels were measured from low-dose infected rag1 (−/−) fish. At 2 wpi, *Nos2b* expression was significantly lower in rag1 (−/−) fish (19.6-fold, SD = 30.3) compared to the wt fish (123-fold, SD = 16), suggesting that adaptive responses affect *Nos2b* induction during the early phase of infection preceding the latency. It is generally thought that in human TB, *Nos2* is induced as a result of IFNγ production by lymphocytes, leading to macrophage activation and control of mycobacterial growth. However, in the adult zebrafish model the *Nos2b* induction at 2 wpi is not likely to be mediated by an adaptive *IFNγ1–2* induction, as the measured *IFNγ1–2* levels were significantly higher in the rag1 (−/−) mutants (6.0-fold induction, SD = 4.5) than in the wt fish (3.0-fold induction, SD = 2.8). At 4 and 7 weeks, the situation was altered so that the rag1 (−/−) mutants had significantly higher *Nos2b* expression levels (induced 136-fold, SD = 193 and 149-fold, SD = 110, respectively) than those observed in the wt (induced 31.6-fold, SD = 42.0 and 56.6-fold induction, SD = 108, respectively). These results suggest that in the adult zebrafish model, the initial macrophage activation preceding the onset of latency is mediated by adaptive responses driving Nos2b induction, but unexpectedly, not via IFNγ.

Most mycobacteria enter a dormant state during a latent infection in adult zebrafish

In human TB, the majority of bacteria are thought to enter a dormant state in response to the stress caused by the immune response and hypoxia. Dormant bacteria are viable but not culturable (VBNC) [23]. This state has been shown to be reversible by the addition of a resuscitation promoting factor (Rpf) *in vitro* [24]. The role of dormancy and resuscitation in a latent mycobacterial infection is difficult to study in humans, as the putative dormant bacteria are not accessible for visualization and cannot be cultured [23]. To investigate, whether there is a dormant bacterial population in *M. marinum* infected adult zebrafish, we tested the effect of Rpf on the number of colonies cultured from fish with a latent infection.

First, we tested if hypoxic *M. marinum* cultures can be resuscitated by an addition of *Micrococcus luteus* Rpf on antibiotic plates. Of note, the standard method of assessing the effect of Rpf on mycobacterial growth in broth culture and most probable number assay could not be used due to the fast-growing contaminating normal flora from the gut. Dilutions of active logarithmic and old hypoxic *M. marinum* broth cultures were plated with and without Rpf. As expected, Rpf significantly increased the number of colonies plated from old, hypoxic, inactive cultures (2.4-fold increase) but did not increase the number of colonies of active bacteria (Figure 4A). Altogether, these results indicate that Rpf from *M. luteus* media is active on 7H10 plates and is able to cause resuscitation of a significant proportion of dormant *M. marinum* that do not otherwise grow on culture plates. This also confirms the role of Rpf as a resuscitating enzyme for *M. marinum*, resembling its well established function for *M. tuberculosis*.

Next, adult zebrafish were infected with the low dose, and the disease was allowed to develop for twenty weeks before the fish were collected for analysis. Parallel samples were analyzed in the presence and absence of Rpf on the plate. When the diluted samples from fish with a latent infection were plated in the presence of Rpf, the number of culturable *M. marinum* increased 4-fold (32 ± 50 cfu without Rpf compared to 129 ± 134 cfu with Rpf) (Figure 4A). For early infection stage samples (1 wpi), the addition of Rpf did not have a growth promoting effect (31 ± 29 cfu without Rpf, 21 ± 22 cfu with Rpf) (Figure 4). With the high infection dose, leading to a more progressive disease, the population of resuscitable dormant bacteria were not detected at 9 wpi using Rpf (Figure 4A). Similarly, in the low-dose infected rag1 (−/−) fish, Rpf did not increase the average number of culturable mycobacteria, suggesting that adaptive immunity has a role in the efficient induction of mycobacterial dormancy. These results indicate that a distinguishable dormant mycobacterial population exists in the zebrafish with a latent infection, whereas in the active infection bacteria are predominantly in a replicative form.

To further confirm the existence of dormant mycobacterial population in the zebrafish with a latent infection, we measured the expression levels of known dormancy-associated mycobacterial genes. Based on *M. tuberculosis in vitro* dormancy microarray data [25], *HspX* (MMAR_3484), *devr* (MMAR_1516), *tgs1* (MMAR_1519) and *GltA1* (MMAR_1381) were chosen for q-RT-PCR measurements. Of these, only *GltA1*, which encodes a metabolic enzyme called citrate synthase, had generally high enough expression levels for reliable quantification from fish with a latent infection. *GltA1* expression was measured at 4 wpi from high-dose infected wt fish and low-dose infected wt and rag1 (−/−) fish. The *GltA1* expression level normalized to the number of bacteria in the low-dose infected wt fish (75.2, SD = 86.8) was significantly higher than in the high-dose wt fish (4.46, SD = 3.55), supporting the idea that in latent infection the proportion of

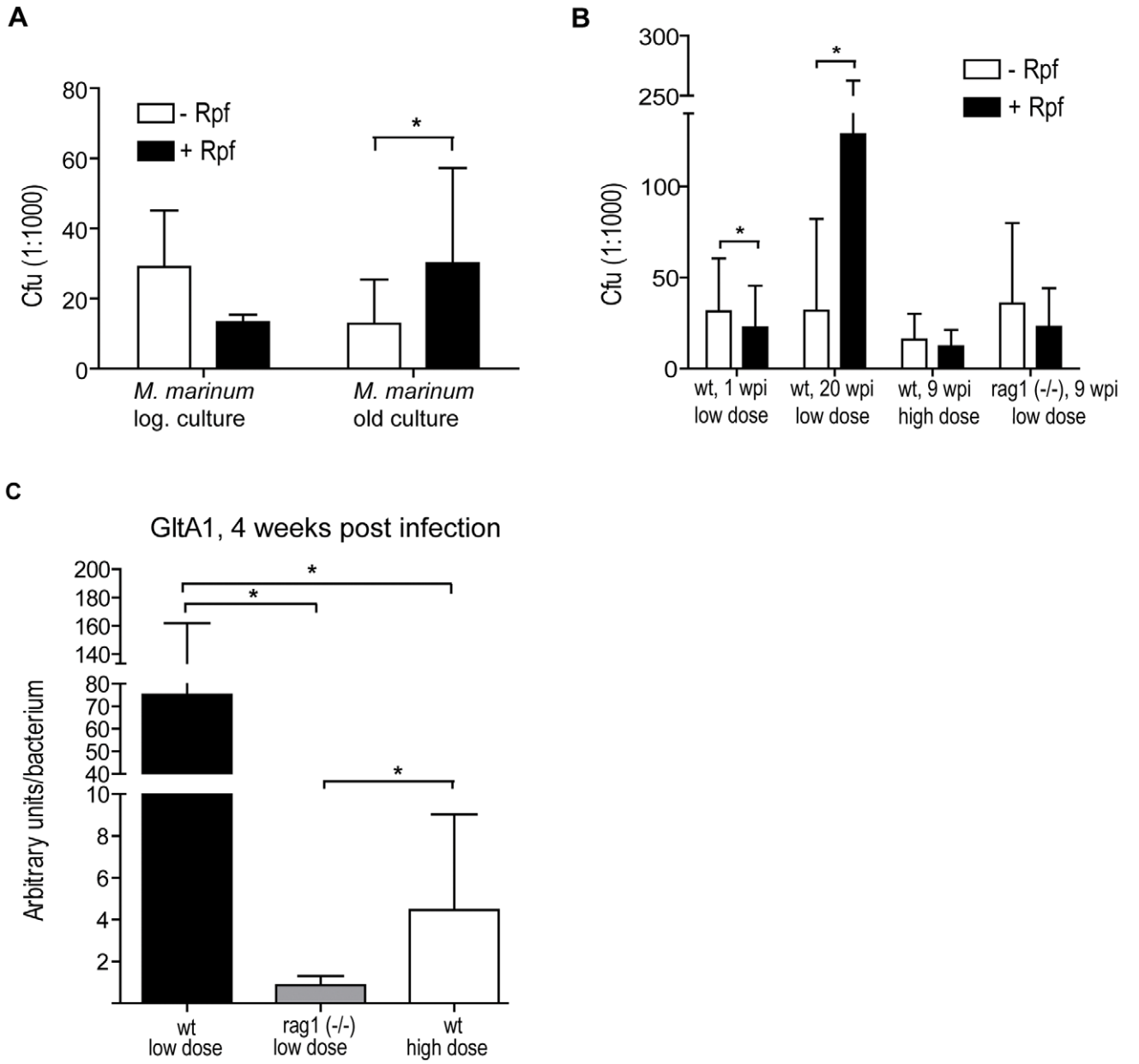

Figure 4. A major part of the mycobacteria are in a dormant state in latent infection. (A) Parallel dilutions of fresh logarithmic or old plateau phase *M. marinum* cultures were plated +/− Rpf to show the resuscitating effect of *Micrococcus luteus* Rpf on dormant *M. marinum*. (B) Parallel homogenate sample dilutions from low-dose (34±15 cfu) infected fish (wt or rag1 (−/−)) were plated at different time points +/− Rpf to detect dormant mycobacteria. (C) *GltA1* expression was measured from low-dose infected rag1 (−/−) and wt fish and high-dose infected wt fish and normalized to the total *M. marinum* load in each fish measured by qPCR. *P<0.05.
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dormant mycobacteria is greater than in a more progressive infection. The lowest *GltA1* expression/bacterium was seen in the low-dose infected rag1 (−/−) fish (0.86, SD = 0.44). The low *GltA1* expression in rag1 (−/−) fish, together with the plating result showing no resuscitating effect by Rpf in rag1−/− fish (Figure 4B), suggests that adaptive immunity plays a role in the induction of mycobacterial dormancy *in vivo*.

The reactivation of a latent mycobacterial infection in zebrafish can be induced by γ-irradiation

Various immunosuppressive medical treatments, such as glucocorticoids [4] and radiation treatment [26], are seen as factors that increase the risk of the reactivation of latent human TB. Having established a model for latent mycobacterial infection in adult zebrafish, we next moved on to test the effect of γ-irradiation as immunosuppressive treatment to reactivate latent mycobacterial infection. Fish were infected with the low dose (34 ± 15 cfu), and five months post infection, a group of fish was irradiated with 25 Gy. Survival was followed for 1 month post irradiation, and the bacterial load was determined at 2 weeks. As a single 25 Gy dose of γ-radiation did not seem to cause sufficient reactivation of the latent mycobacterial infection in our zebrafish model system (Figure S2), two 25 Gy doses were administered to a group of fish with a latent *M. marinum* infection with one month

between the doses. Survival was followed for one month after the second irradiation. To assess the changes in the mycobacterial numbers and lesions, moribund or recently dead fish were collected and analyzed either histologically or with *M. marinum*-quantification PCR. Two 25 Gy doses of γ-radiation caused some degree of early time-point mortality in both irradiated groups. However, in the non-infected group, no deaths occurred after 16 days from the second irradiation (total mortality 40%), whereas the infected, irradiated population continued to die, reaching an end-point mortality of 88% (Figure 5A). No deaths occurred in the non-irradiated latent infection group. The immunosuppressive treatment with two 25 Gy doses of γ-irradiation lead to a significant increase in mortality among zebrafish with a latent mycobacterial infection, suggesting reactivation of the disease.

To confirm that the increased mortality after the γ-irradiation was related to the progression of the mycobacterial infection, the bacterial burdens were determined. Fish collected for qPCR 15–22 days after the second γ-radiation dose had an average bacterial load of 8.7×10^7 cfu ($SD = 1.2\times10^8$), which was 106-fold higher compared to non-irradiated controls (average load 8.2×10^5 cfu, $SD = 8.1\times10^5$) (Figure 5B). A histological analysis of moribund individuals revealed vast areas of free bacteria not restricted to granulomas (Figure 5C,D). Based on these results, γ-irradiation-induced reactivation of latent mycobacterial infection in adult zebrafish is a highly promising model for investigating the cellular and molecular mechanisms involved in reactivated mycobacterial infections.

Gamma irradiation-induced depletion of lymphocyte populations is associated with the reactivation of latent mycobacterial infection

To characterize the effect of γ-irradiation on blood cells, the changes in different blood cell populations were analyzed using flow cytometry (FCM). The numbers of granulo/monocytes and lymphocytes were measured from kidney homogenates. First, the immediate effects of a 25 Gy dose of γ-irradiation were studied by analyzing changes one week after the treatment (Figure 5E). The average proportion of granulocytes and monocytes was reduced by 47%, however there was a striking 80% reduction in the lymphocyte population, compared to normal levels. The efficient depletion of lymphocytes was further verified using the fish lines Tg(lck:lck-EGFP) and Tg(rag2-GFP), which express GFP in T cells, or in T and B cells, respectively. With these fish, a 67% reduction in the T cell population (lck) and a 99% reduction in the B and T cell population (rag2) were seen one week after irradiation (Figure 5E). Despite the marked leukocyte depletion, one 25 Gy dose of γ-irradiation had not been sufficient for the reactivation of a latent mycobacterial infection in zebrafish, as no significant changes were seen in mortality rates (Figure S2A) or in bacterial burdens (Figure S2B). Therefore, we next studied the recovery of leukocytes after the first irradiation, as well as the short- term effect of the second 25 Gy dose (Figure 5F). Both lymphocyte and granulocyte/monocyte populations had recovered to normal levels by five weeks after the first 25 Gy dose. The second 25 Gy dose of γ-irradiation reduced the number of lymphocytes by 53% compared to the recovery levels (Figure 5F), whereas granulocytes were not significantly affected by the second treatment. These results suggest that the effective reactivation of a latent mycobacterial infection required two 25 Gy doses of γ-irradiation because of the rapid recovery of the lymphocyte and granulocyte/monocyte populations after the first treatment. In addition, the mechanism of reactivation in this model is most likely due to the specific depletion of lymphocytes rather than a decrease in granulocytes.

Immunosuppression by γ-irradiation leads to reactivation of the dormant mycobacterial population

To assess the changes in the dormant bacterial population after the reactivation, we plated samples in the presence and absence of Rpf at 2.5 weeks after the second 25 Gy irradiation dose. In the non-irradiated fish with a latent infection, the number of colonies were 4-fold higher in the presence of Rpf than in its absence (Figure 5H), whereas after double irradiation the resuscitating effect of Rpf could no longer be seen (Figure 5G). This result supports the idea of latency-associated mycobacterial dormancy, which is reversed in reactivated disease.

Discussion

During the last couple of decades, the prevalence of active TB has substantially increased. Many of these cases are likely to be due to the reactivation of latent TB as a consequence of various immune compromising factors, such as HIV [27], diabetes [28] and glucocorticoid treatment [29]. Currently, the reactivation of latent TB is one of the greatest challenges in the field of infectious diseases, as present vaccination strategies do not protect against this phase of infection [7]. The fact that multiresistant strains of *M. tuberculosis* are arising in many parts of the world [5,30] further complicates the control of this disease. Thus, more detailed information on the mechanisms of the host–pathogen interactions in a latent mycobacterial disease and its reactivation is indispensable.

In general, the *M. marinum* infection model in zebrafish is well established. As *M. marinum* is a common pathogen of zebrafish, it can be considered a more natural model for studying host–mycobacterium interaction, than is, for example the *M. tuberculosis* mouse model. The histopathology of mycobacterial lesions in zebrafish has been shown to be more similar to human TB than is the histopathology in the mouse model (reviewed in [14]). The genetic similarities between *M. marinum* and *M. tuberculosis* are well documented [15], including the currently known genes involved in virulence and in dormancy (Dos-regulon) [31]. Thus, it is likely that the characterization of phenomena involved in latent infections and dormancy in a *M. marinum* infection, is useful for understanding human latent TB.

The concept of latent TB is problematic, and a debate over the definition as well as the nature of latent TB is on-going [32]. "Latent TB" is a broad clinical definition diagnosed with indirect immunological reactions in the tuberculin skin test (TST) or the interferon-γ release assay (IGRA) in the absence of clinical symptoms [2]. These assays do not reveal whether there are viable bacilli present in the host, but rather, whether the host has been infected with the bacterium and developed an adaptive response against it. Thus, cases diagnosed with latent TB compose a heterogeneous group with different bacterial phenotypes and loads [2,3]. In studies on latent TB patients, DNA of *M. tuberculosis* has been shown to be generally present in the lung necropsy samples of individuals with a latent infection [33,34]. These findings are in harmony with the common latency paradigm stating that in most infected individuals mycobacteria become dormant and non-replicating in the hypoxic environment of the granuloma but can be resuscitated in non-restrictive circumstances [2]. Still, the presence of mycobacterial DNA, as such, does not reveal the metabolic status (dormancy) of the bacteria. The subject warrants further investigation in applicable animal models as well as in human cohorts.

In this study we set up a novel model for latent TB using experimental *M. marinum* infection of adult zebrafish. We showed that mycobacterial dormancy is a central feature of latent TB in the zebrafish. The importance of adaptive immunity in the

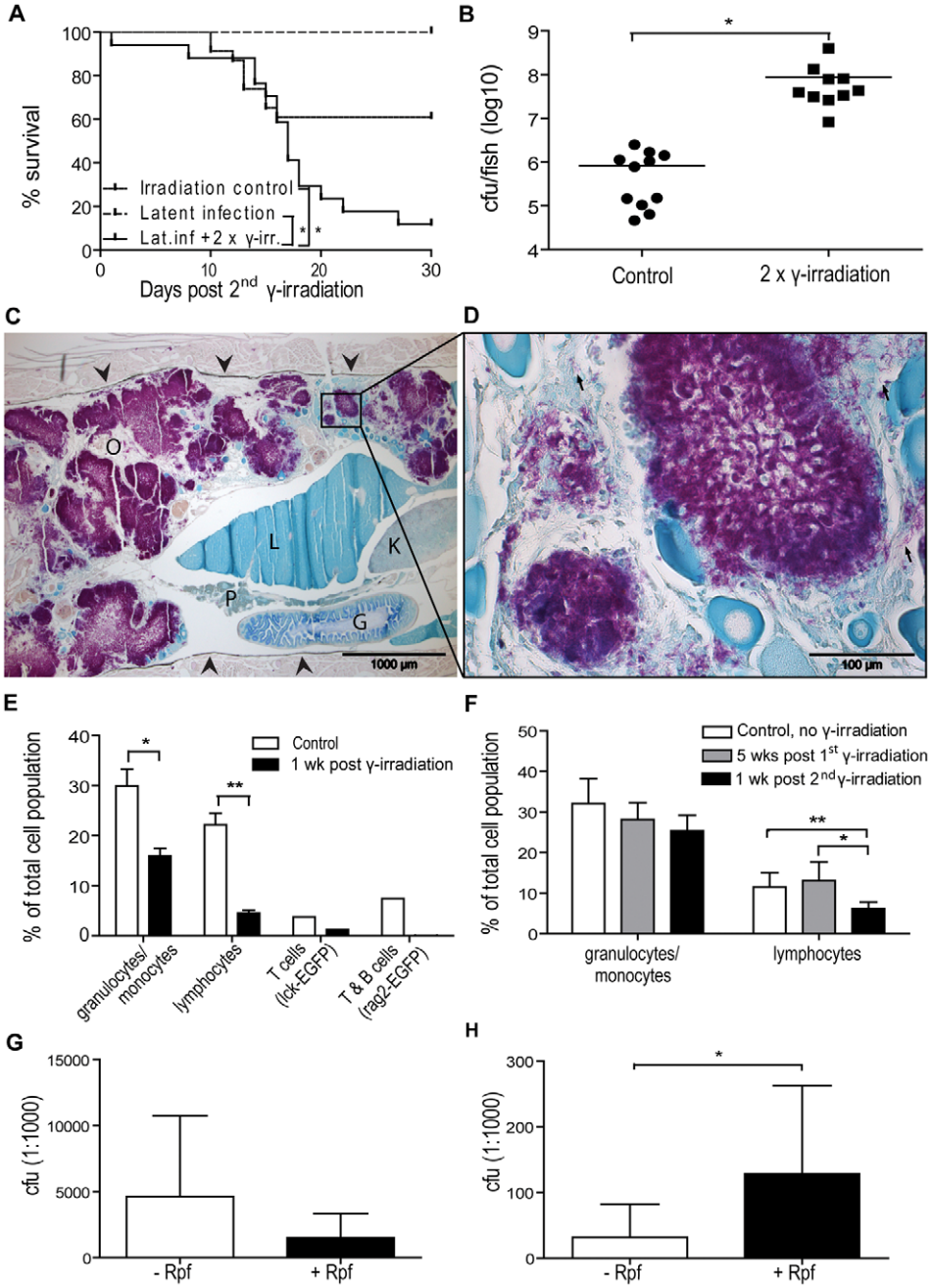

Figure 5. Gamma irradiation induces reactivation resulting in increased mortality due to uncontrolled growth of mycobacteria. (A–C) Zebrafish (n = 17) with a latent *M. marinum* infection were irradiated twice with 25 Gy with one month between the irradiations. Twice irradiated, non-infected zebrafish (n = 23) as well as zebrafish with a latent infection (n = 14) were included as controls. (A) Survival was followed for 30 days after the second dose. *P<0.05. (B) During this period, moribund or recently dead fish were collected 15–22 days after the second radiation dose. Bacterial loads were compared with those of similarly infected, non-irradiated control fish that were collected at the end-point of the experiment. *P<0.05 (C&D) A representative Ziehl-Neelsen stained sample from a reactivated fish showing large numbers of free mycobacteria (purple areas) in the zebrafish body cavity (C). The sides of the body cavity are marked with arrowheads O = ovary, P = pancreas, L = liver, G = gut, K = kidney. (D) A picture taken with a higher magnification showing individual rods (few examples pointed out with arrows). (E) Four groups of 4 adult zebrafish (1 rag2-gfp, 1 lck-gfp and 2 wild-type groups) were γ-irradiated with 25 Gy. Similar control groups were left untreated. Kidneys were collected 8 d post irradiation, pooled and analyzed by FCM. FSC-SSC -plots were gated based on cell size and granularity as described in [56] (gates shown in Figure S3) to assess the effect of irradiation on leukocyte populations. *P<0.05. For further verification of the effect of radiation on lymphocytes, a GFP gate was used for the rag2 and lck groups expressing GFP in B and T cells, or T cells, respectively. (F) Adult non-infected wt zebrafish were irradiated with 25 Gy once (grey bars) (n = 3) or twice (n = 7) (black bars) with one month between the doses. Leukocyte recovery and re-depletion were assessed by FCM. Non-irradiated fish (n = 4) were used as controls. *P<0.05 (G) Fish with a latent infection (n = 7) were irradiated twice with 25 Gy with one month between the doses and plated +/− Rpf for 18 d after the second radiation dose. (H) Fish (n = 6) with a latent infection were plated +/− Rpf.
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establishment of a latent disease in zebrafish was shown in a number of experiments carried out with rag1 (−/−) zebrafish that lack T and B cells. In addition, we developed a pioneering adult zebrafish model, in which an immunosuppressive radiation treatment was used for reactivation of the latent disease. With this model, various aspects of the currently poorly characterized process of latency, dormancy and reactivation can be studied in a simple vertebrate system.

As a first step, we had to be able to induce a non-progressive, but persistent, infection in adult zebrafish. Based on previous work in adult zebrafish, the severity of the disease is dependent on both the dose and the strain [10,35,36]. The type strain of *M. marinum* (ATCC 927) has previously been reported to produce a moderate infection in zebrafish, but previously only high doses have been used [35]. In our hands, a low dose of this strain delivered as an injection (i.p.) was found to be the most reliable means of inducing a latent infection. In addition to injecting, bathing in water infested with different concentrations of mycobacteria was also tested. Although bathing could provide a more natural route of infection through the gills or the gut, the low incidence rate achieved by this method made it unsuitable for this study. As our scope is to study latency and reactivation and not the natural course of initial colonization, i.p. injection was considered applicable for our purposes.

The non-progressive status of the experimental infection could be verified by quantifying bacterial loads in the fish using an in-house-developed qPCR assay, and by quantifying granulomas in full-length longitudinal sections. Most fish did not show any signs of disease, and the average bacterial burdens as well as the number of granulomas and affected organs cease to grow after 4 weeks of infection remaining at a static level in the majority of individuals. This essentially demonstrates the central features of the latent disease. The disease is present in the host and has the potential to reactivate under appropriate circumstances. A centrally important feature of our model is that the non-progressive state developed naturally between the host and the mycobacteria without further intervention, and lasted for the entire duration of the 8-month study in 75% of the individuals.

The results gained with the quantitative PCR method in our model showed that the total number of mycobacteria ceased to increase after the first weeks of infection and remained stable for the entire duration of the study. Bacteria entering a non-replicating, dormant state would be a reasonable explanation for the non-progressive bacterial burdens; which is also thought to happen in human TB. To examine whether the bacteria entered a dormant state in our model system, we carried out *ex vivo* plating experiments. Comparing the efficacy of *ex vivo* growth in liquid broth and solid plate has been previously used for showing dormant *M. tuberculosis* populations in chronically infected mice [37]. We used an alternative, specific method using resuscitation promoting factor (Rpf) from *Micrococcus luteus*. Rpf has been shown to resuscitate dormant *M. luteus* but also various mycobacterial species [24]. Homologous proteins with the same function have thereafter also been found to be present in actively dividing mycobacterial cultures [38], and the functions of these muralytic enzymes has been extensively studied in mycobacterial species [39]. Mutant *M. tuberculosis* strains without functional Rpfs have been shown to be less virulent and unable to reactivate *in vivo* [40,41].

Using *M. luteus* Rpf on solid plates, we found that the majority of the bacteria in most fish with a latent infection were actually in a dormant, viable but not culturable state, and could be resuscitated by the addition of Rpf. The resuscitable population of dormant mycobacteria seen in latent wt fish was absent in rag1 (−/−) fish lacking functional adaptive immunity. Also, the expression level of the known dormancy-associated enzyme, citrate synthase (*GltA1*), in wt fish was 87-fold higher than in the rag1 (−/−) fish, indicating that effective induction of mycobacterial dormancy is mediated by adaptive immune responses. The presence of Rpf on plates did not increase the number of culturable mycobacteria in samples representing the active phases of infection; namely the primary active disease with a low dose, a progressive disease with a high dose and the reactivated infection. These results suggest that dormancy of a high proportion of the total mycobacterial population is associated with the latent disease. In this study, there was variation in all the measured parameters within the experimental grou25ps with latent infection. This variation is most likely explained by differences in disease progression between individuals within the latent groups. Similar wide disease spectrum is thought to be present also in the human latent TB [2,3]. To characterize the underlying factors leading to this typical variation in disease outcomes, it would be beneficial to follow the disease progression in individuals instead of heterogeneous groups in studies using *in vivo* models of TB.

The early cytokine responses (*TNFα, IL-6, IL-1β, IL-12*) were measured on the first day of low-dose or high-dose infection. The high dose generally evoked a stronger pro-inflammatory response, which may have contributed to the high mortality in the beginning of the infection. Conversely, the low-dose infection seemed to avoid evoking strong responses. Of the measured cytokines, only *IL-6* was induced. IL-6 has been reported to be important in restricting mycobacterial growth [42] and in efficient protection by vaccination against TB in mice [43], and as such, may have had a role in the initiation of a latent disease in the zebrafish.

The differences in the disease progression were further studied at later time-points, where *Nos2b* and *IFNγ1-2* expression levels were measured. According to current hypothesis, IFNγ induces Nos2 in macrophages activating them to more efficiently destroy intracellular mycobacteria [44–46]. In our zebrafish model, *Nos2(b)* was clearly induced with both the low and the high dose at 2–7 wpi. In the high-dose infection group, the induction at 2 weeks was as high as ~1500-fold compared to baseline levels. Despite this strong induction, most of the fish succumbed to infection, perhaps due to insufficient phagocytic capacity. At the same time, *Nos2b* was not induced in rag1−/− fish at 2 wpi, and the bacterial burdens were already significantly higher than in the wt low dose animals. Based on this, adaptive responses mediate the *Nos2b* induction and are required for the restriction of mycobacterial growth already at this stage. However, the adaptive mechanism behind this induction in the mycobacterial disease in the zebrafish remains obscure, as *IFNγ1-2* was not induced at 2 weeks in the low-dose infected wt fish. Later on, at 4 and 7 wpi, an induction of Nos2b was also seen in rag1 (−/−) fish, indicating that the innate arm of immunity alone, to some extent, can induce the production of nitric oxide as a response to the high bacterial numbers.

According to the latest hypotheses on latent TB, the grand scheme is complex with various co-existing populations of mycobacteria in different niches and metabolic states. Some of these populations have been suggested to constantly probe the environment in search of prospects for reactivation (e.g. immunodeficiency), whereas others are in a less active state, waiting for resuscitation signals from the probing population. The proportion of bacteria in each population determines the disease status. Likely, a fully functional immune system is able to keep this small active population in line. In case of immunosuppression, the active population replicates and excretes resuscitation factors, leading to

reactivation of the dormant population [23]. Our findings in the zebrafish model support this elegant hypothesis.

Latent mycobacterial infection models have previously been set up in the rabbit [20], in the mouse [47,48], in the guinea pig [49] and in the macaque [21]. Some evidence on bacterial dormancy exists in the chronic disease of vaccinated mice [37]. The rabbit and macaque models were induced with a low bacterial inoculate similarly to our zebrafish model, whereas the mouse and guinea pig models utilize chemotherapeutics. The historical Cornell's model of latency in the mouse is artificially induced by antibiotic treatment. After the antibiotics are removed, a spontaneous reactivation occurs [47]. Without antibiotic treatment, the bacterial loads continue to increase, eventually leading to the death of the mouse. Others have utilized streptomycin auxotrophic strains of *M. tuberculosis* whose growth can be arrested in the absence of streptomycin, resulting in a paucibacillary state [48,49]. However, in our zebrafish model, the infection is induced with a naturally replicating strain and generally results in a latent disease without any antibiotic treatment. Thus, it is likely that the mycobacterial infection in the zebrafish more accurately models the natural course of infection that is determined by the interplay between the pathogen and the immune response of the host. In our model, as many as 75% of the fish survived the 8-month period and managed to restrict further bacterial growth, suggesting that a latent-type disease developed. The percentage of latent-type cases was much higher in zebrafish than in the macaque model (40%) [21]. In humans, 90–95% of TB cases are subclinical [4].

Having established a potential model for latent TB, we next set up a method for reactivation *in vivo*. So far, reactivation has only been established in non-human primate models in the context of simian immunodeficiency virus (SIV) [50], in a rabbit model with dexamethasone [20] and in a murine model with aminoguanidine [51]. Even though these models are likely to replicate the human TB-HIV co-infection and glucocorticoid-induced reactivation, respectively, the zebrafish could provide a useful and ethical model for large-scale experiments on reactivation. To our knowledge, irradiation has not been previously used for inducing reactivation of latent mycobacterial infections.

First, we tested whether irradiation could be used for the reactivation of the latent disease. Surprisingly, despite irradiation killing almost all lymphocytes and half of the granulo/monocytes, a single dose (25 Gy) was found to be insufficient for a general reactivation of the mycobacterial disease during the one-month follow-up period. This could be due to the combined effect of the rapid recovery of the leukocyte population after irradiation [52] and the low growth rate of mycobacteria. In adult zebrafish, leukocyte numbers have been reported to recover to pre-irradiation levels in 2 weeks after a 20 Gy dose [52]. However, when the 25 Gy was administered to latently infected fish twice, with one month between the doses, the desired effect on the mycobacterial disease was achieved. Mortality increased significantly compared to latently infected controls and similarly irradiated healthy fish. A descriptive histological analysis of moribund individuals also revealed vast areas of free bacteria outside granulomas. The mycobacterial loads in twice irradiated fish with end-stage infection had increased by ~100-fold compared to stable state levels. The kinetics of the bacterial outgrowth in these fish is in harmony with theoretical calculations of unrestricted bacterial growth. Leukocyte numbers have been reported to reach the lowest level 6–7 days after 20 Gy of radiation [52]. At this point, the bacteria should be able to grow without limitation. In liquid culture at 29°C, the *M. marinum* used in our laboratory doubles its numbers in 24 hours and thus a 100-fold increase would require ~7 days. Indeed, in the reactivation group, a steep drop in survival after 16 days concomitant with high bacterial loads was seen. Based on these results, the zebrafish model for the reactivation of a mycobacterial disease appears highly promising.

Radiation treatments, as is well known, have various biological effects. When considering irradiation as a method for reactivating a mycobacterial infection, some of these effects need to be discussed. Firstly, the dose used in this study (25 Gy) is high and would be lethal for mammals. Zebrafish seem to be relatively resistant to the acute adverse effects caused by irradiation, as the treatment *per se* did not cause mortality. The lethal dose for adult zebrafish has been reported to be as high as 40 Gy, possibly due to the smaller genome and the lower body temperature compared to mammals [52]. For reactivation purposes, the 25 Gy dose was administered twice, which led to efficient reactivation of the mycobacterial disease, but also caused a 40% mortality *per se*, which is slightly less than the mortality caused by a single dose of 30 Gy [52]. Secondly, γ-radiation is likely to have direct effects on *M. marinum*. An aspect to be considered in the context of the reactivation model is the possibility of causing mutations in the bacterial genome, in addition to affecting the immune cell numbers of the host. *In vitro* studies have previously shown that *M. tuberculosis* is twice as radioresistant as *E. coli* [26]. Still, with doses above 1 Gy the viability of *M. tuberculosis* is adversely affected in a dose-dependent manner [26]. This has probably been the basis for the historical X-ray treatment *against* TB. Of note, also mutations advantageous to the bacteria can occur, and can be enriched in the population if a selection pressure, such as antibiotics, is applied. In our experiments, however, there was no selection pressure, but rather the pressure from the host's side was transiently relieved. Based on our FCM data, the dampened immune suppression of the host, rather than an advantageous mutation in mycobacteria, is likely to be the trigger for reactivation. Still, as a precaution, special measures should be taken to prevent the release of irradiated mycobacteria into the environment.

In conclusion, we have set up a system in which a latent mycobacterial disease can be established and assessed in adult zebrafish. The majority of the bacteria present in zebrafish enter a dormant state, with a smaller bacterial population remaining active. We were also able to induce a transition from this stable state to a progressive mode using repeated γ-irradiation mimicking immune suppressive states that cause human TB to reactivate. The reactivated infection is characterized by the absence of dormant mycobacterial population, similarly to the active primary disease preceding latency. Currently, there is no vaccine that would give proper protection against the reactivation of the latent disease. Gamma radiation induced reactivation should be an applicable model for testing new vaccine candidates, as the T cells required for protective immunity against TB are resistant to γ-radiation [53]. Thus, this proof-of-concept model for the reactivation of latent TB in a non-mammalian vertebrate shows high promise as a tool for large-scale studies on the related mechanisms.

Materials and Methods

Zebrafish lines and maintenance

For most experiments, adult (5–8 month-old) wild-type AB zebrafish were used. In addition, adult, rag1 (−/−) hu1999 mutant fish (from ZIRC) were used. For the FACS analysis, transgenic lines Tg(lck:lck-EGFP)^cz2 and Tg (rag2:GFP)^zdf8 (from ZIRC) were also used. Fish were kept in a flow-through system with a light/dark cycle of 14 h/10 h and were fed with SDS 400 food twice daily.

Ethics statement

All experiments have been accepted by the Animal Experiment Board in Finland (under the Regional State Administrative Agency for Southern Finland) and were carried out in accordance with the EU-directive 2010/63/EU on the protection of animals used for scientific purposes and with the Finnish Act on Animal Experimentation (62/2006).

Licence for the zebrafish facility: LSLH-2007-7254/Ym-23, Licence for experiments: ESLH-2008-07610/Ym-23 and 20.10.2010 ESAVI-2010-08379/Ym-23.

Experimental infection

M. marinum (ATCC 927) was cultured similarly as described in [10] with the following modifications: culture at 29°C, concentration of Tween 80 0.2%. Bacteria were first cultured on plates for 1 wk, transferred into liquid medium for 4 d, diluted once ~1:10, cultured to an OD600 of 0.495–0.680, collected by centrifugation and diluted appropriately with sterile 0.2 M KCl +0.3 mg/ml phenol red (Sigma-Aldrich). For qPCR experiments PBS without phenol red was used. The fish were briefly anesthetized in 0.02% 3-aminobenzoic acid ethyl ester (pH 7.0) (Sigma-Aldrich) and intraperitoneally (i.p.) injected with 5 µl using an Omnican 100 30 G insulin needle (Braun, Melsungen, Germany). To verify the bacterial dose, samples of bacterial dilutions were taken while infecting, diluted when needed and plated onto 7H10 plates. The low dose was 34±15 cfu and the high dose 2029±709 cfu. In survival experiments, humane end point criteria approved by a national ethical board were followed. If any of the following criteria were fulfilled, the animals were euthanized: lack of response to touch, abnormal swimming, gasping, observable swelling, observable waisting or loss of scales.

Histology

Fish were euthanized by incubation in 0.04% 3-aminobenzoic acid ethyl ester (Sigma-Aldrich) pH 7.0. Heads and tails were removed and the fish were fixed in 10% phosphate buffered formalin pH 7.0 for 5–11 days at RT. After 1 week of decalcification with 20% EDTA-citrate pH 7.2 samples were rinsed with tap water, transferred through an ethanol series with increasing concentrations, put into xylene and longitudinally embedded in paraffin. 5 µm sections were cut; every 40th section was placed on a slide. The fish were sectioned thoroughly so that the entire kidney tissue lining the spine was included. The slides were stained with Ziehl-Neelsen staining and analyzed using the 200× magnification of an Olympus BX51 microscope. For Mallory's trichrome staining standard methods were used.

qPCR

DNA extraction from mycobacteria: For determination of the bacterial load, the peritoneal cavity of the euthanized fish was emptied. The organs were put into weighed metal bead containing homogenization tubes (Mobio, California, USA) and frozen at −80°C. The mass of the organ sample was determined. The self-prepared modified enzymatic lysis buffer MELB (20 mM Tris-HCl pH 8.0, 20 mM sodium EDTA pH 8.0, 1.2% Triton X 100) was added to the samples, which were homogenized in a volume of 575 µl using the PowerLyzer24 (Mobio) at speed 3,200 for 3×20 second cycles with 30 second pauses. An appropriate proportion (sample mass <25 mg) of the homogenate was taken for DNA extraction. The samples were sonicated in an m08 water bath sonicator (Finnsonic, Lahti, Finland) for 9 min. Lysozyme (Sigma-Aldrich) was added to a final concentration of 20 mg/ml and incubated at 37°C for 2 h. After incubation, MELB was added to the samples to equalize the volume of all samples to 180 µl. From this point on the QIAGEN DNeasy Blood & Tissue Kit manufactures protocol for DNA extraction from gram-positive bacteria was used. The DNA was eluted twice with a volume of 200 µl.

RNA-DNA co-extraction from infected zebrafish: Organs were collected as above, and homogenized in tubes with ceramic beads, 3200 rpmi, 3×40 s in 1.5 ml of TRI reagent (MRC, OH, USA). RNA extraction was carried out according to the manufacturer's protocol. DNA was extracted from the same sample for determination of the mycobacterial load by adding back extraction buffer (1:1) (4 M guanidine thiocyanate (Sigma-Aldrich), 50 mM sodium citrate, 1 M Tris) on top of the lower phase after phenol-chloroform phase separation. DNA was thereafter precipitated with isopropanol, washed with ethanol twice and dissolved in sterile ddH_2O.

Primers were designed for the *M. marinum* 16S–23S ITS sequence: F: 5′-caccacgagaaacactccaa-3′ R: 5′-acatcccgaaaccaa-cagag-3′. For quantification of mycobacterial load SENSIFAST NO-ROX SYBR was used. The final reaction solution had the following composition: 1× SENSIFAST NO-ROX SYBR GreenPCR Master Mix (stock 2×), 0.4 µM MMITS1 forward primer, 0.4 µM MMITS1 reverse primer 3 µl of template. Duplicate or triplicate dilutions were made for each sample. A standard curve was made by extracting the total DNA from a known amount of bacteria (logarithmic culture) and 10-fold using serial dilutions. DNA extracted from three healthy fish were included in order to determine the background signal. The qPCR was carried out using the BIO-RAD CFX96 cycler with the following settings: 1. 3 min 95°C, 2. 5 s 95°C, 3. 10 s 65°C, 4. 5 s 72°C, 5. 39 cycles from 2. to 4. 6. Melting curve 55–95°C at 0.5 intervals.

For q-RT-PCR, primer sequences can be found in the Supporting Information (Text S2). For gene-expression measurements, Bio-Rad iScript One-Step RT-PCR Kit with SYBR Green was used according to the manufacturer's instructions. The optimal annealing temperature of each primer pair were determined using melting curve analysis and agarose gel electrophoresis. The expression of *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*, ZDB-GENE-030115-1) was used for normalization of the host genes. The results from mycobacterial dormancy genes were normalized to the bacterial load measured from the corresponding DNA sample.

Bio-Rad CFX Manager software and GraphPad Prism 5.02 were used in the analysis. Using the standard curve, a concentration in units of bacterial genome copies was obtained for every sample. The bacterial load per fish (the visceral organs) could be calculated: (qPCR result (bact./µl of template) x qPCR sample dilution factor Y x total DNA eluate volume (µl) x homogenate dilution factor X). The limit of detection with the qPCR method was estimated to be ~10^3 cfu/fish.

Plating on antibiotic plates +/− Rpf

For production of secreted Rpf for resuscitation of dormant mycobacteria, *Micrococcus luteus* was cultured. An inoculate from a glycerol stock of *M. luteus* was revived in 10 ml of LB liquid medium at 37°C o/n in rotation (to an OD605 = 0.100). A 100 ml volume of lactate minimal medium, LMM (composition described in [54] with the exception of a lower concentration of lactate (0.5% w/v) [24] was inoculated with 4 ml of the o/n culture and was cultured aerobically in rotation (150 rpm) at 30°C for 4 days to an OD605 = 0.705. After centrifugation (10,000 g, 3 min) the supernatant was sterile filtered and aliquots were stored at −80°C. For Rpf plating an aliquot was thawed and 500 µl was absorbed

on each 7H9 antibiotic plates. On –Rpf plates, 500 µl of fresh, sterile LMM was absorbed. The concentrations of antibiotics on plates were as described in [55] and 20 µg/ml azithromycin (Sigma-Aldrich). Fish were homogenized with the same settings as for DNA extractions from mycobacteria in sterile PBS supplemented with 0.5% Tween 80 (v/v). Dilutions were plated +/− Rpf and incubated in the dark at 25°C for 15–17 days. *M. marinum* colonies were counted and the average load of culturable bacteria was determined. As controls, active logarithmic *M. marinum* broth cultures and old stationary *M. marinum* broth cultures that had been kept in closed bottles at +29°C for 5–8 months were plated +/− Rpf and cultured as described above.

Immunosuppression

Irradiation with 25 Gy was carried out with Gammacell 1000 irradiator in glass flasks with 5 fish/80 ml of water. In the reactivation experiments, low-dose infected fish were irradiated twice with one month between the doses. Non-infected controls were similarly irradiated.

Flow cytometry (FCM)

Nine days after irradiation, fish were euthanized and the kidneys were collected and placed into PBS supplemented with 1% fetal calf serum (FCS) on ice. In the first experiment (1 wk post irradiation) kidneys from each group of fish (4/group) were pooled. Fish were analyzed individually in the second one (5 weeks post 1.irradiation). Also kidneys from untreated groups of the same fish lines were similarly collected in order to determine a baseline. Kidneys were homogenized by pipetting the entire volume (1 ml) up and down 15 times. The samples were also filtered before analysis. Immune cell populations were determined using a FACSCantoII (Beckton Dickinson) and the FACSDiva software. The results were analyzed using FlowJo (Treestar Inc, Ashland, OR). Lymphocytes, blood cell precursors, erythrocytes and granulo/monocytes were identified based on the cellular granularity (SSC-A) and size (FSC), (see Figure S2) according to [56]. In addition, reporter fish lines Lck-GFP (T lymphocytes) and RAG-GFP (B and T lymphocytes) we used in some experiments to further confirm the identity of the immune cell populations. Total 30,000 events per sample were collected for analysis.

Statistical analysis

Statistical analysis was carried out using the GraphPad Prism software (5.02). For determination of statistical significance of differences in bacterial loads, number of granulomas, affected organs and leukocyte counts and gene-expression data, a non-parametric one-tailed Mann-Whitney test was used. In survival experiments, the log-rank Mantel-Cox test was used. In Rpf experiments, the plates with the same sample +/− Rpf were compared pair-wise, and a one-tailed paired t-test was used. P-values$<$0.05 were considered significant.

List of genes mentioned in the article

Zebrafish (*Danio rerio*):
Nos2b: ZDB-GENE-080916-1
IFNγ1-2: ZDB-GENE-040629-1
TNFα: ZDB-GENE-050317-1
IL-1β: ZDB-GENE-040702-2
IL-6: ZDB-GENE-120509-1
IL-12: ZDB-GENE-060724-1
GAPDH: ZDB-GENE-030115-1
M. marinum:
GltA1: MMAR_1381
HspX: MMAR_3484
DevR: MMAR_1516
GltA1: MMAR_1381
Tgs1: MMAR_1519

Supporting Information

Figure S1 qPCR and plating give similar results. Dilutions (1, 1:10, 1:1000) of mycobacterial culture (logarithmic growth phase) were added onto healthy fish organ samples. The amount of bacteria added was determined by plating dilutions of the culture (result shown as white bars). The samples were homogenized and the DNA was extracted. The bacterial concentration was determined by qPCR (result shown as black bars).
(TIF)

Figure S2 A single 25 Gy dose of gamma radiation is not sufficient for reactivation of latent tuberculosis. Latently infected adult zebrafish (n = 39) were γ-irradiated (25 Gy). Latently infected, non-irradiated zebrafish (n = 25) were used as controls. The effects of the irradiation were controlled by irradiating non-infected fish (n = 30). (A) Survival was followed for 28 days. * P$<$0.05 (B) To determine the bacterial load, 5 fish were collected 2 weeks after irradiation. Similarly infected non-irradiated controls were also collected.
(TIF)

Figure S3 Gamma irradiation depletes the lymphocyte population in adult zebrafish. 4 groups (1 Tg(rag2-GFP). 1 Tg(lck:lck-egfp) and 2 wt groups) of 4 adult zebrafish were γ-irradiated with 25 Gy or left untreated. Kidneys were collected 9 d post irradiation, pooled and analyzed by FCM. FSC-SSC –plots were gated based on [56] as follows: E = erythrocytes, G/M = granulocytes & monocytes, L = lymphocytes, P = blood cell precursors. The numbers by the gates show the percentage of cells within the gate of the total live population. For GFP-expressing lines (rag2 and lck) a GFP gate was also used. The GFP positive populations were reanalyzed on a FSC-SSC -plot. The lymphocyte population was most severely affected by irradiation, whereas the number of granulo/monocytes decreased less. An increase in the proportion of blood cell precursors was detected. A reanalysis of the GFP results verified that the GFP-expressing cells were mostly present within the lymphocyte gate.
(TIF)

Text S1 A qPCR-assay for quantifying of *M. marinum* load in adult zebrafish tissues.
(DOC)

Text S2 Sequences of the Q-RT-PCR primers used in the study.
(DOC)

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Author Contributions

Conceived and designed the experiments: M. Parikka, M.M. Hammarén, M. Rämet, A. Iivanainen. Performed the experiments: M.M. Hammarén, M. Parikka, S.-K.E. Harjula, N.J.A. Halfpenny, K.E. Oksanen, M.J. Lahtinen, E.T. Pajula. Analyzed the data: M.M. Hammarén, M. Parikka, M. Pesu. Contributed reagents/materials/analysis tools: M. Rämet, M. Parikka, M. Pesu. Wrote the paper: M.M. Hammarén, M. Parikka.

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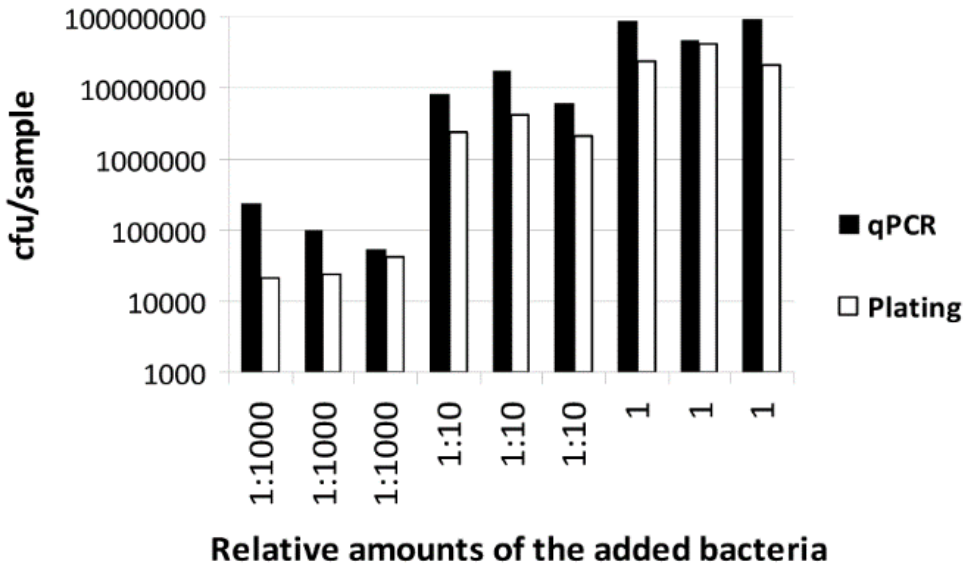
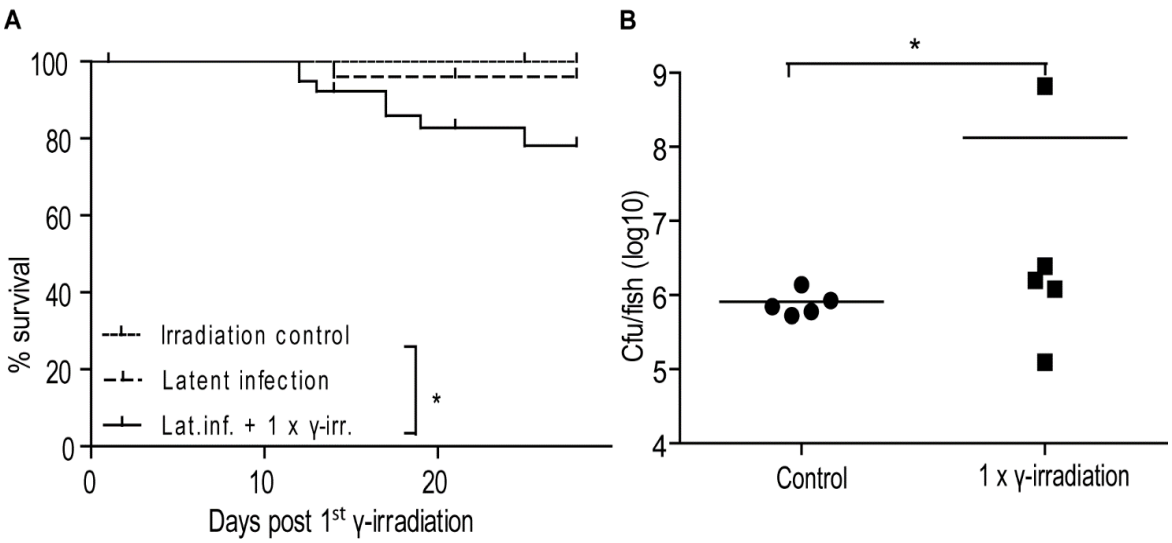


Figure S1, Parikka et al. 2012



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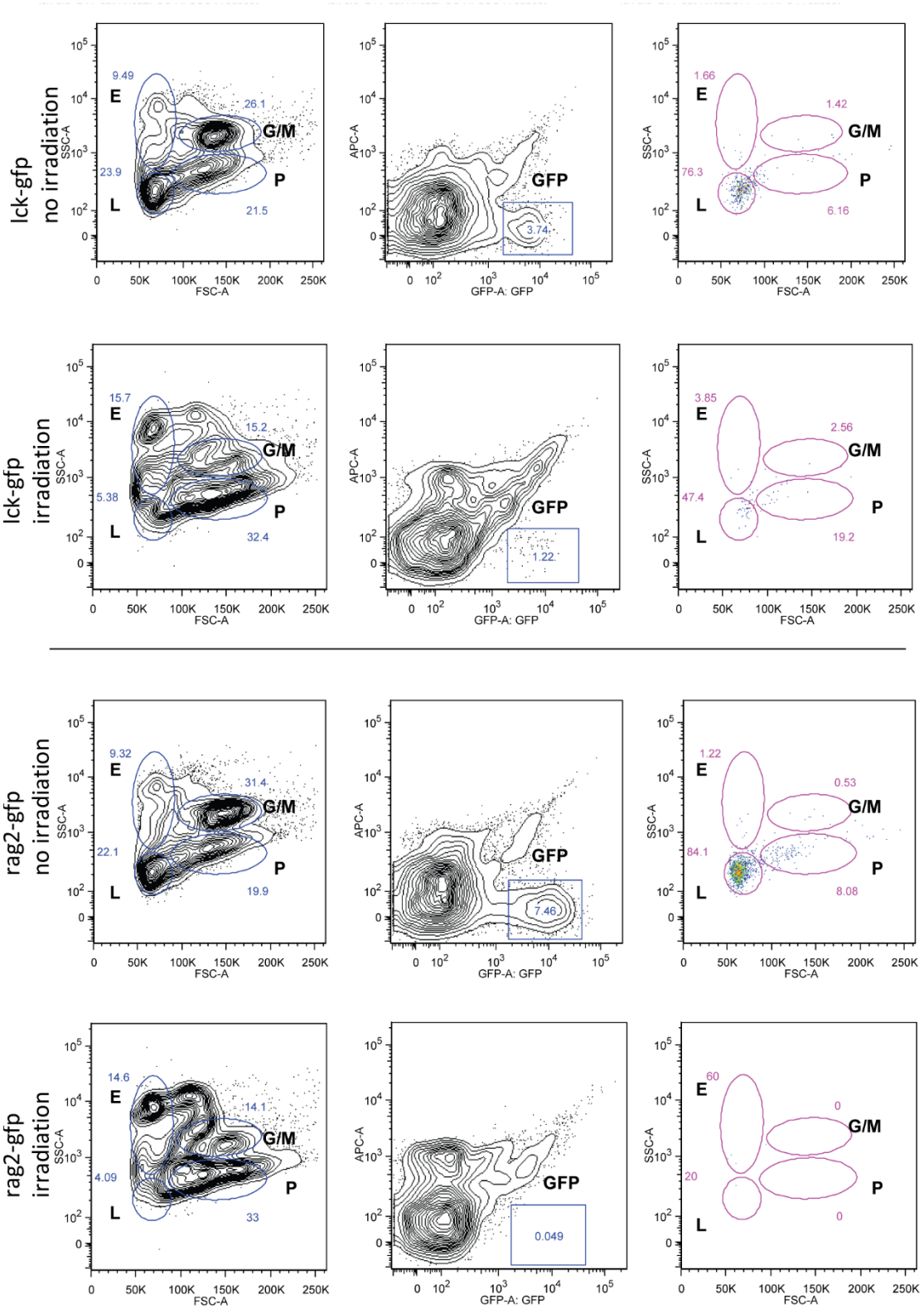

Figure S3, Parikka et al. 2012

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Adequate Th2-Type Response Associates with Restricted Bacterial Growth in Latent Mycobacterial Infection of Zebrafish

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Abstract

Tuberculosis is still a major health problem worldwide. Currently it is not known what kind of immune responses lead to successful control and clearance of *Mycobacterium tuberculosis*. This gap in knowledge is reflected by the inability to develop sufficient diagnostic and therapeutic tools to fight tuberculosis. We have used the *Mycobacterium marinum* infection model in the adult zebrafish and taken advantage of heterogeneity of zebrafish population to dissect the characteristics of adaptive immune responses, some of which are associated with well-controlled latency or bacterial clearance while others with progressive infection. Differences in T cell responses between subpopulations were measured at the transcriptional level. It was discovered that a high total T cell level was usually associated with lower bacterial loads alongside with a T helper 2 (Th2)-type gene expression signature. At late time points, spontaneous reactivation with apparent symptoms was characterized by a low Th2/Th1 marker ratio and a substantial induction of *foxp3* reflecting the level of regulatory T cells. Characteristic *gata3/tbx21* has potential as a biomarker for the status of mycobacterial disease.

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Introduction

Tuberculosis (TB) is a pulmonary disease spread worldwide. It is caused by an infection with *Mycobacterium tuberculosis*. Only 5–10% of infected individuals develop a primary active disease while the most common outcome of infection is a latent or subclinical disease with no evident symptoms. This latent disease has the inherent ability to reactivate after years or even decades of latency and is therefore a major global threat. The existing vaccine, the Bacille Calmette-Guérin (BCG), is not entirely safe and does not confer protection against latent or reactivated TB. Current antibiotic regimens have started losing their efficacy due to the spread of antibiotic resistance genes [1]. In total, the primary active infections and reactivated infections cause 1–2 million deaths yearly, which makes *M. tuberculosis* the deadliest bacterium for humans [2].

The dichotomy to a latent and active tuberculosis is an over-simplification, as the infection can actually lead to a wide spectrum of disease states ranging from a well-controlled (or even cleared) latent disease to fulminant, severe forms of TB. Within the latent population, a ''sub-spectrum'' exists leading to differences in the risk of reactivation [3]. The pathogenesis of tuberculosis has been widely studied for decades, but as it seems that TB is not a single disease but a spectrum of different outcomes, it remains poorly understood. Better understanding on the factors that contribute to the type of TB disease is crucial for the development future treatment strategies.

The TB spectrum is likely to arise from genetic variation both in the host and in different pathogen strains as well as from environmental factors. It is known that adaptive immunity and especially T helper (Th) cells are required for controlling the disease. HIV-infected individuals are more susceptible to active and reactivated tuberculosis due to the defective T lymphocyte response [4]. Mice lacking T helper responses are hypersusceptible to TB [5]. Based on the observations that IL-12 or IFN-γ deficient mice are unable to restrict mycobacterial infection, it was initially concluded that Th1 cells are the predominant mediators of protective immunity to *M. tuberculosis* [6–8]. In mice, observations of an early Th1 response (2–3 weeks post infection, wpi) followed by a Th2 response simultaneously with the onset of a chronic phase, have led to a presumption that Th2 response is detrimental to the host by leading to a failure of Th1 response to clear the

Author Summary

Tuberculosis is a common and potentially lethal lung disease spread worldwide. One third of the world's population is estimated to be infected with *Mycobacterium tuberculosis*, yet most individuals develop a latent disease which has the potential to reactivate. Some are thought to be able to clear the infection. The current vaccine does not give adequate protection against the disease, and due to incorrect use of antibiotics, resistance to treatment has substantially increased. There is an urgent need for novel treatment approaches, such as modulation of the host's immune response. However, the ideal immune response against tuberculosis is unknown. In addition, more accurate diagnostic tools are needed for distinguishing the high risk individuals among latent patients so that treatment could be given to those that are most likely to benefit from it. In this study, we used the *Mycobacterium marinum*-zebrafish model to study the T cell responses in mycobacterial infection. Utilizing the natural heterogeneity of the zebrafish population, we found associations between the disease severity (bacterial load) and the type and magnitude of T cell responses. Our results on typical T cell signatures are useful as diagnostic biomarkers as well as provide new understanding needed for therapeutic approaches based on immunomodulation.

infection [9,10]. Subsequently, it has also been shown that the lack of Th2 responses in IL-4, IL-13 or Stat-6 deficient mice does not lead to better resistance to *M. tuberculosis* infection but, instead, to increased bacterial burdens at later stages of infection [11]. Although the role of humoral immunity in response to mycobacterial infection is still unclear, there is evidence that Th2 responses are needed as well for optimal protective immunity [1,12]. Despite the emerging understanding of the roles of different Th subtypes in TB immunity, it is still not known what type of Th profiles are needed at different phases of infection to provide optimal protection. In part, this is due to the lack of suitable animal models for studying the full spectrum of disease outcomes, including latency and reactivation.

Several animal models have been developed with the aim of understanding the complex pathogenesis of tuberculosis. The murine model of pulmonary TB is well standardized and has made many valuable contributions to the knowledge of the disease pathomechanisms, especially on the role of T cells as mediators of protective immunity [13]. A major constraint of the model is that mice do not develop spontaneous latency although they can restrict the bacterial growth to chronic progressive infection. One of the rare animal models developing true latency is the Cynomolgus macaque. In the macaque, a low-dose *M. tuberculosis* infection leads to active primary disease in 50% and latent disease in 50% of individuals [14]. In the rabbit model of latent TB, the lung bacterial burdens start declining at 4 wpi following a primary phase with limited bacterial growth. In the rabbit TB model, different outcomes of infection can be induced by using mycobacterial strains with different virulence properties [15,16].

In addition to the mammalian models of TB, we have previously shown that infection of adult zebrafish with their natural pathogen, *Mycobacterium marinum*, can be used to model latent TB [17]. *M. marinum* is a close genetic relative of *M. tuberculosis*, and typically infects cold-blooded hosts, such as frogs and various freshwater and saltwater fish species [18]. *M. marinum* infection of zebrafish embryos has been established as an elegant model to dissect the innate mechanisms of protective host responses in active mycobacterial infection [19–21]. However, the full spectrum of mycobacterial disease outcomes can be observed only in the adult zebrafish, due to the full maturation of adaptive immune system after the first four weeks post fertilization [21,22]. In the adult zebrafish model, the injection of a low dose of *M. marinum* (ATCC 927 type strain) into the abdominal cavity leads to a systemic infection, characterized by an initial 3–4 week phase with rapid bacterial growth, followed in most individuals by a latent phase with stable bacterial burdens. In the latently infected fish, the majority of the mycobacterial population passes into a non-replicative state, dormancy, but can be experimentally reactivated by immunosuppression [17].

The wide disease spectrum typical of mycobacterial disease results from various host- and pathogen-associated factors. It is known that genetic determinants lead to an inherent, stable preference towards either T helper 1 or T helper 2 response that varies between human individuals [23,24]. As a starting point for our study, we hypothesized that the differences that control the T helper response might be associated with the establishment of the wide spectrum seen in TB patients and that differences in T cell polarity might be related to the progression of the disease. Taking advantage of the heterogeneity of the zebrafish population and the wide spectrum of mycobacterial disease outcomes in the zebrafish model, we set out to look for differences in T helper responses involved in regulating protective response. Finding such differences would 1) allow the use of T helper markers among latently infected individuals to distinguish between those at high or low risk of reactivation and 2) provide understanding on what type of T cell response gives the optimal protection against mycobacterial infection and allow development of novel kinds of therapeutic or preventive approaches.

Results

Expansion of T lymphocytes is associated with limited mycobacterial growth in the zebrafish

Our previous work [17] provided evidence that functional lymphocyte response is a prerequisite for latency and mycobacterial dormancy in the *M. marinum* infection of zebrafish. To further demonstrate the significance of lymphocyte responses in the immune defence against mycobacteria in zebrafish, we carried out adoptive transfer experiments on low-dose (21±7 cfu) *M. marinum*-infected *rag1* (−/−) fish. Spleen and kidney marrow cells were transferred from WT or *rag1* (−/−) zebrafish immunized with heat-killed *M. marinum* to *rag1* (−/−) recipients at 2 wpi. At 4 wpi, bacterial burdens were significantly lower in the fish that received transplants from immunized WT donors, compared to the fish that received transplants from *rag1* (−/−) donors (3.2×105 vs. 1.8×106). This indicates that heat-killed *M. marinum*-induced lymphocytes, rather than NK cells or other innate immune cells, transferred additional immune protection against *M. marinum* infection to *rag1*-deficient zebrafish. (Figure 1A).

In our current study of ~150 individuals, a total of 10% of zebrafish were able to clear the bacterial number below the detection limit of *M. marinum* q-PCR-analysis (~100 bacteria) (Figure S1B–D). These individuals capable of clearance were not detected in the groups that were collected at 2 wpi, suggesting that the clearance is likely to occur after the activation of adaptive responses. These results attest the significance of adaptive responses in the immune protection against zebrafish mycobacteriosis, and support the view of similarity of human and zebrafish anti-tuberculosis immunity.

In the current study, we aimed at further elucidating the details of adaptive immune response leading to a variety of infection outcomes. Unlike many other commonly used laboratory animals,

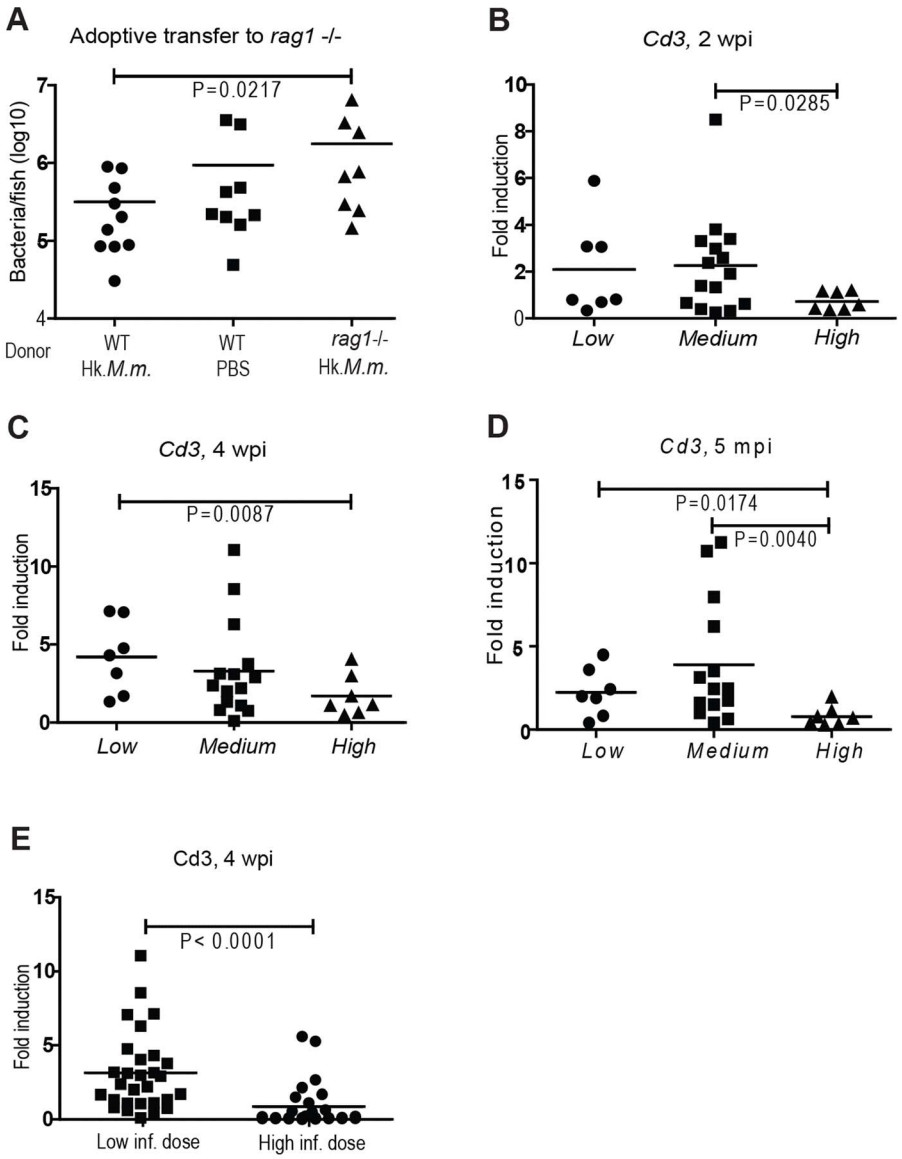

Figure 1. T cell numbers are higher in individuals with low bacterial loads. (A) In an adoptive transfer experiment, we transferred spleen and kidney cells to low-dose infected *rag1* (−/−) mutant fish 12 dpi. The donors were WT immunocompetent fish treated with heat-killed *M. marinum* (Hk.*M.m.*) or PBS and *rag1* (−/−) fish treated with Hk.*M.m.* 10 d prior to the adoptive transfer. The bacterial loads of the recipient fish were measured 4 wpi by q-PCR, n = 8–10/group. (B–D) At all time points of this study, zebrafish infected with a low dose of *M. marinum* (21±7 cfu) were divided in subpopulations according to the bacterial load into upper quarter (*High*) (n = 7), lower quarter (*Low*) (n = 7) and a *Medium* group (n = 15). The changes in the total T cell numbers were assessed in the different subpopulations of low-dose infected WT fish by measuring *cd3* transcription by q-RT-PCR during a primary (2 and 4 weeks) or a late stage (5 months) mycobacterial infection (E) As a control experiment for assessing the effect of initially high bacterial load on *cd3*, WT zebrafish were infected with a high dose (2691±520 cfu) and the *cd3* levels of this group (n = 25) were compared to those of the group (n = 30) infected with a low dose (21±7 cfu) at 4 wpi.
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zebrafish populations are genetically heterogeneous. This characteristic causes large variations and standard deviations in most studies utilizing this model, including our studies on mycobacterial infection. On the positive side, the zebrafish population provides starting material for studying the natural differences between individuals. To be able to elucidate the host factors affecting the outcome of infection, the variation in environmental and bacterial factors was first minimized: the bacterial strain, bacterial growth conditions, infection procedure, infection dose and housing of infected fish (water quality and temperature, feeding etc.) were carefully standardized.

WT adult zebrafish were infected with a low dose (21±7 cfu) of *M. marinum*, collected at various time points and divided into three subpopulations, based on their bacterial burdens. The subpopulations were named *Low* (25% of the population, individuals with the lowest bacterial burdens), *Medium* (50% of individuals) and *High* (25% of the population, individuals with the highest bacterial burdens, including the primary-progressive). The *Low*, *Medium* and *High* subpopulations were analyzed at various stages of mycobacterial disease: at 2 wpi (primary active disease), 4 wpi (the onset of latency in the majority of zebrafish) and 5 months post infection (mpi; late stage at which most individuals maintain latency). The

reactivation risk of latent mycobacterial infection is thought to increase with increasing bacterial load [25]. The bacterial burdens in the subgroups at different time points are shown in Figure S1.

To dissect the differences in the total T lymphocyte numbers between the *Low*, *Medium* and *High* subpopulations, we quantified the cluster of differentiation (*cd*) 3 levels from internal organs of the zebrafish by q-RT-PCR. Zebrafish *cd3* has been shown to be an ortholog of the mammalian T cell marker *cd3* [26]. Here, the *cd3* expression as a marker for T cell numbers in zebrafish was further validated as described in Figure S2. Induction of T cell expansion was similar in the *Low* and *Medium* groups, seen as a 2-fold induction in *cd3* expression level already at 2 wpi (Figure 1B) and peaking to 4-fold around 4 wpi (Figure 1C), compared to the *cd3* expression levels in non-infected zebrafish. The *High* group differed from the rest of the population by showing a modest T cell expansion (max. 1.7 ± 1.3), which was only seen at 4 wpi (Figure 1C). A similar pattern remained at a late stage of the infection (5 months, Figure 1D). To assess whether the limited T cell expansion is the cause or the consequence of enhanced bacterial growth in the *High* group, the low-dose-infected fish were compared with a group infected with a high dose of *M. marinum* (2691 ± 520 cfu). Based on our previous work [17] a high initial dose causes the bacterial load to be significantly higher than with a low initial dose during the first 2 weeks of infection and this difference will even out by 4 wpi. At 4 wpi, *cd3* expression levels were significantly lower in the high-dose group (Figure 1E), suggesting that the reduced T cell numbers in the *High* subpopulation may at least partly be affected by the rapid bacterial growth.

Taken together, these results indicate that an early T cell expansion associates with protective response against mycobacterial infection, as the fish with highest *cd3* expression levels were always found in the *Low* and *Medium* subgroups. However, individuals with modest lymphoproliferative response were found equally in all the three subgroups, suggesting that other factors besides efficient T cell expansion are required for mounting a protective response against mycobacterial infection.

Controlled mycobacterial infection is characterized by sufficient induction of Th2-type responses

As Th cells are potent orchestrators of immune responses during infection, it is reasonable to assume that in addition to total lymphocyte numbers, variation in Th response types may be an important factor underlying the wide spectrum of outcomes in mycobacterial infections. For zebrafish, antibody markers or reporter lines for FACS (fluorescence-activated cell sorting) analysis of different T lymphocyte populations are not available. To assess the Th1/Th2 balance of individuals with different infection outcomes, we measured the levels of master regulator transcription factors for Th1/Th2 lineage development, *T-box transcription factor 21 (tbx21)* and *gata3*, from the internal organs of infected zebrafish. Tbx21 is a Th1 cell transcription factor important for Th1 lineage commitment and gata3 is a well-known regulator of Th2 cell differentiation also playing a role in endothelial cell biology [27]. The central T cell transcription factors tbx21, gata3 and foxp3 have been identified in the fish [28–30]. The enrichment of *tbx21* and *gata3* in the zebrafish T cell population was validated as described in Figure S2. During infection, the alterations in the transcript levels of these transcription factors reflect the changes in the numbers of the corresponding T helper cells. The ratio of Th2/Th1 markers was used to assess the balance of T helper cell response. In addition, the induction of a Th2-type cytokine IL-4 (IL4b) [31] and a Th1-type cytokine IFN-γ (*ifnγ1-2*) was measured and the ratio was calculated.

At 2 wpi, the induction of both *gata3* and *tbx21* was significantly higher in *Low* and *Medium* than in the *High* group (Figure S3A&D). At this time point, there were no significant differences in the *gata3/tbx21* ratio between the three groups (Figure 2A). At 4 wpi, *gata3* was still significantly more induced in *Low* and *Medium* groups compared to *High* group (Figure S3B). However, the *tbx21* levels were similar in all groups (Figure S3E). As determined by the *gata3/tbx21* ratio, the *Low* group had developed a significantly more Th2-biased response than the *High* group by 4 wpi (Figure 2B), suggesting that insufficiency of Th2 cells is a differentiating factor between the *Low* and *High* individuals. The *il4/ifnγ* ratios generally followed a similar pattern (Figure 2D–E). Also at 2 wpi, the *il4/ifnγ* ratio was significantly higher in the *Low* group compared the other two, although there were no significant differences in the *gata3/tbx21* ratio at this time point. At 2 weeks, it is likely that the adaptive Th response is in the process of maturation conducted by the cytokines excreted by innate immune cells. Similar patterns were observed at the late time point 5 months post infection (Figure S3C&F, Figure 2C&F).

At 4 wpi, the Th2/Th1 balance was also assessed at the protein level by semi-quantitative Western blot analysis of gata3 and CXCR3 (a CXC chemokine receptor preferentially expressed on Th1 cells) from individuals in *Low*, *Medium* and *High* groups (Figure 2J, Figure S4). The results showed a similar trend as seen with q-RT-PCR analyses.

To assess the importance of functional, specific lymphocytes for the changes in the levels of the markers used in this study, we also carried out similar infection experiments in *rag1* (−/−) mutants. The fish were infected with a low dose (35 ± 18 cfu) and collected 4 wpi. We found that there was some induction of *gata3, tbx21, il4* and *ifnγ* in the infected *rag1* (−/−) mutants (Figures S2H&I and 2H&I) showing the proportion of lymphocyte-independent induction of these markers. However, the induction of *gata3*, *tbx21* and *il4* was significantly higher in the WT fish (Figures S2H&I and 2H) than in *rag1* (−/−) fish at 4 wpi. This clearly demonstrates the major contribution of functional lymphocytes in the changes seen in these markers during mycobacterial infection. The *rag1* (−/−) fish were grouped according to bacterial load (Figure S1E) as previously described for the WT fish, and association of the *gata3/tbx21* ratio and bacterial load was assessed. In the absence of functional lymphocytes no association was detected, implying that the differences in this ratio relevant to the course of mycobacterial infection seen in WT fish are indeed derived from lymphocytes. However, the expression levels of *ifnγ* were similar in *rag1* (−/−) and WT fish showing that the induction of this Th1-type cytokine in mycobacterial infection might not be as dependent on functional lymphocytes as the other markers used.

To assess whether the Th2/Th1 balance is directly influenced by the bacterial burden in the beginning of the infection, low-dose (21 ± 7 cfu) infected fish were compared to fish infected with a high mycobacterial dose (2691 ± 520 cfu) at 4 wpi. Average of *gata3/tbx21* ratio was found to be lower in the high-dose infected group (Figure 2K), suggesting that rapid bacterial growth can *lead to* changes in this ratio. To investigate whether the differences in the disease outcome could *result from* genetically defined Th1/Th2 preferences, we stimulated healthy WT zebrafish by an i.p. injection of heat-killed *M. marinum* and 10 days later, analyzed the *gata3* and *tbx21* transcript levels. The spectrum of individual Th2/Th1 responses was broad, similarly to that seen in humans [23]. The *gata3/tbx21* ratio varied from 0.6 to 12.8 within a group of 14 zebrafish. 22% of the individuals were substantially Th2-biased (*gata3/tbx21*>10), whereas 28% had a bias towards Th1 (*gata3/*

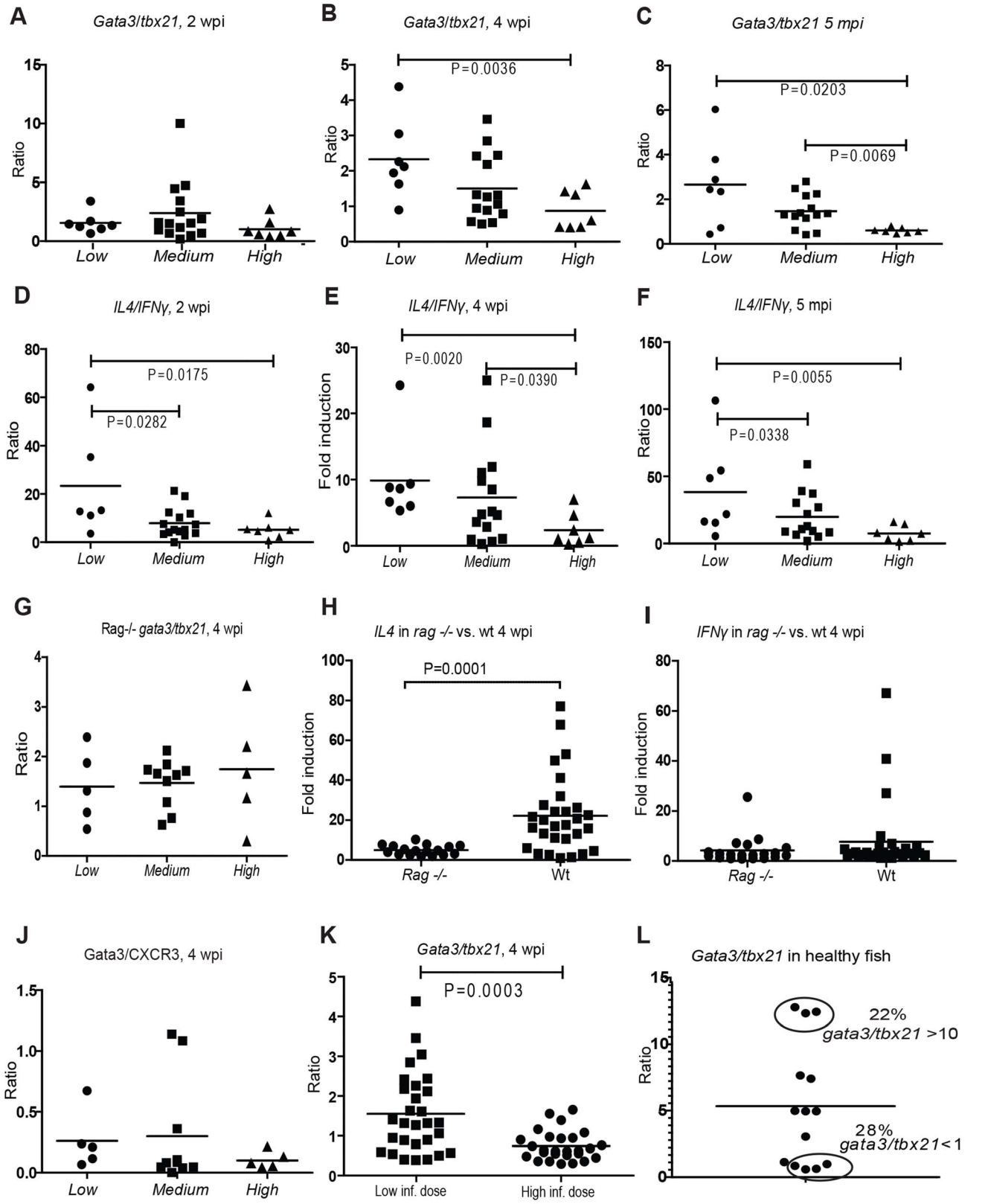

Figure 2. Controlled mycobacterial infection is characterized by Th2-type response from 4 weeks post infection. (A–C) *Tbx21* (*t-bet*) and *gata3* induction was measured in the different subpopulations at 2, 4 wpi and 5 mpi (months post infection). The *gata3/tbx21* ratio was calculated to determine the dominant Th type. (D–F) The induction of selected type cytokines for Th1 (*IFNγ1-2*) and Th2 response (*IL4b*) was measured in the different subpopulations. The *il4/IFNγ* ratio of induction was calculated. (G–I) *Rag1* (−/−) mutant zebrafish (n = 20) were infected with 35±18 cfu of *M. marinum* and analyzed at 4 wpi. (G) Grouping of mutant fish was carried out according to bacterial load similarly to wt fish (See Figure S1E). The association of *gata3/tbx21* with the bacterial load was assessed. (H) The induction of *il4* at 4 wpi was compared between wt and *rag1*

(−/−) fish. (I) The induction of *ifnγ* at 4 wpi was compared between wt and *rag1* (−/−) fish. (J) Semi-quantitative western blots were carried out at 4 wpi from a population of 20 fish. Gata3 antibody was used as the Th2 marker, and CXCR3 as the Th1 marker. The bacterial loads were measured from the corresponding DNA samples to allow grouping to subpopulations. (K) As a control experiment for assessing the effect of initially high bacterial load on *gata3/tbx21* ratio, WT zebrafish were infected with a high dose (2691±520 cfu) and the *gata3/tbx21* ratio of this group (n = 25) was compared to those of the group (n = 30) infected with a low dose (21±7 cfu) at 4 wpi. (L) To assess the natural polarization pattern of T cells with regard to *gata3/tbx21*, WT zebrafish (n = 14) were stimulated by an intraperitoneal injection of heat-killed *M. marinum*. *Gata3/tbx21* ratio was determined 10 days post injection.
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tbx21<1) (Figure 2L). This observation of inherent Th1/Th2 phenotypes in healthy fish suggests that genetic Th1/Th2 preferences may in part lead to the development of wide disease spectrum in mycobacterial infections. Based on these results, it seems plausible that both the bacterium and the host can affect the *gata3/tbx21* ratio during mycobacterial infection.

At any of the time points of the study, the average *gata3/tbx21* ratio was never >1.0 in the progressive *High* group, whereas Th2 dominant response (average *gata3/tbx21* 1.5–2.7) was seen in the *Low* group in the primary infection. Altogether, these results show that induction of Th2-type responses during the first four weeks of mycobacterial infection are associated with controlling the bacterial growth.

In order to further confirm the reliability of our markers and to characterize the response in the different subgroups, we measured a wider selection of Th2 and Th1 signature genes at 4 wpi: Th2: interleukins *il13* and *il4*, *immunoglobulin M (IgM) constant region*, *V-maf musculoaponeurotic fibrosarcoma oncogene (cmaf)*, *STAT6*, *St2* (Figure 3A–F) Th1: *il12*, *interferon gamma 1-2 (IFNγ1-2)*, *nitric oxide synthase 2b (Nos2b)*, *tumor necrosis factor alpha (TNFα)*;(Figure 3G–J). It has been previously shown that the expression levels of the selected Th2 marker genes are upregulated in zebrafish in response to recombinant IL-4 treatment, thus representing a Th2-type response in the zebrafish [31]. In line with *gata3* and *il4* markers, there was a significantly higher induction of Th2 signature cytokines and other Th2 related genes in the *Low* group than in the *High* group (Figure 3A–F). At the same time, Th1 markers showed a somewhat higher induction in the *High* group (Figure 3G–J). However, the differences in Th2 markers were more indicative of the bacterial burden and the disease state than those seen in Th1 markers. The *Low* group showed substantial *IgM* induction at 4 wpi differing significantly from the rest of the population (Figure 3C). Only three antibody classes exist in zebrafish, namely IgM, IgD, and fish-specific IgZ, whereas IgG, IgA and IgE are absent [32]. Secreted tetrameric IgM is the most abundant zebrafish serum immunoglobulin [32], and is induced by Th2-mediated IL-4 signaling [31]. Taken together, these data suggest that Th2-type cytokines participate in the control of bacterial growth during the first four weeks of primary mycobacterial infection.

Th2 dominance is beneficial for the maintenance of long-term latent mycobacterial infection

As zebrafish with higher T cell numbers and a Th2-biased response were able to control the infection most efficiently, we next wanted to study whether these protective features were relevant for the ability of maintaining long-term latency. Even though the individuals with a primary progressive disease had already been removed from the experiment, large variations in the bacterial numbers (Figure S1D) and dormancy (Figure 4A) were seen at a late time point, when the population was again divided into subgroups according to their bacterial loads at 5 mpi. The individuals with highest levels of dormancy-associated mycobacterial citrate synthase I (*GltA1*) expression were found in the *Low* group, whereas in the *High* group *GltA1* expression levels were significantly lower than in the other groups (Figure 4A). This shows that there is a spectrum even *within* the "latent" population that has survived the primary infection.

The total T cell number still seemed to play a role at this late time point so that the *High* group had significantly less T cells than the other groups (Figure 1D). However, there was no significant difference in the total T cell number between the *Low* and *Medium* group. The protective role of Th2-type responses associated with lower bacterial numbers remained even at this late time point (Figures 2C&F). These results indicate the importance of high T cell numbers and a tendency to Th2-biased responses in determining how well the infection is controlled. It is plausible that the ideal response for inducing stable latency is similar to that required for maintaining long-term latency.

As we followed the large population of fish infected with a low initial dose, we saw that from 8 wpi to 5 mpi, there was a 17% overall morbidity in the population (data not shown). Following an eight-week period of asymptomatic infection, until the end of the 5 month follow-up, these spontaneously reactivated individuals were collected as they started showing symptoms of mycobacterial disease. These individuals had a slightly higher bacterial load than the fish in the *High* group (Figure S1D) but did not differ in terms of dormancy (Figure 4A). Thus, it is likely that the disease is active in the fish in the *High* group as well, although the fish did not yet show external signs of disease. Based on the *gata3/tbx21* ratios, the Th2/Th1 balance was similar in the *High* and *Reactivated* groups (Figure 4B), differing significantly from the *Low* and *Medium* groups. As direct assessment of the bacterial load within a population of asymptomatic humans is difficult, measuring the Th2/Th1 balance from a blood sample could be used as a clinical biomarker for estimating the activity of the disease. We carried out a ROC-analysis of *gata3/tbx21* ratio and found that *gata3/tbx21* ratio had a high sensitivity and specificity as a marker in distinguishing the high load individuals (*High*&*Reactivated*) from the well-controlling population (*Low*&*Medium*)(Figure 4C).

Spontaneous reactivation is associated with pronounced *foxp3* induction

We next wanted to study whether there were some differences in the T cell responses between the progressive *High* and *Reactivated* groups. In the *Reactivated* group, half of the individuals showed high induction of *cd3*. This was surprising, as within all the other groups, high *cd3* was generally associated with better disease control. A possibly detrimental role of the induced T cell response in the *Reactivated* group could be explained by a *non-ideal polarization* of these cells. The most striking difference between the *High* and *Reactivated* group was the significantly higher induction of *foxp3* in the *Reactivated* group (Figure 4E), indicating a pronounced regulatory T helper (Treg) proliferation. When the expression levels of *gata3*, *tbx21* and *foxp3* were analyzed relative to *cd3* in the *Reactivated*, only *foxp3* was significantly different between the *Reactivated* and *High* group (P = 0.0006, 10-fold higher proportion than in the *High* group, Figure 4E). In fact, the induction of *foxp3* was higher in the *Reactivated* group compared to any of the asymptomatic groups (Figure 4F). This implies that the increase in

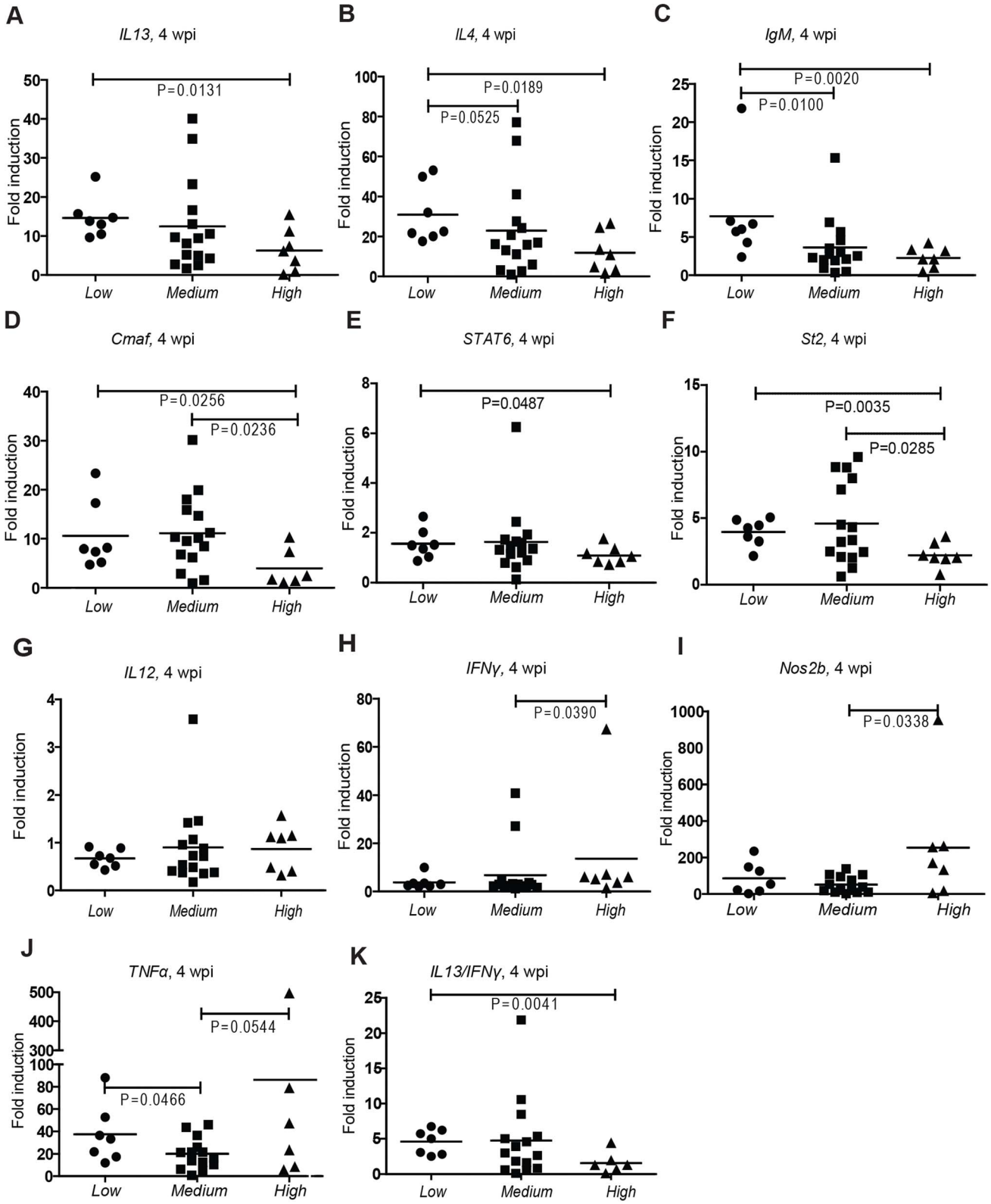

Figure 3. Controlled mycobacterial infection is distinguished from progressive infection by higher induction of Th2 markers. The groups with different bacterial loads were analyzed for typical Th2 (A–F) and Th1 (G–J) markers by q-RT-PCR at 4 wpi. (K) The ratio of the inductions of *il13* to *ifnγ* was also calculated in the different subgroups at 4 wpi.
doi:10.1371/journal.ppat.1004190.g003

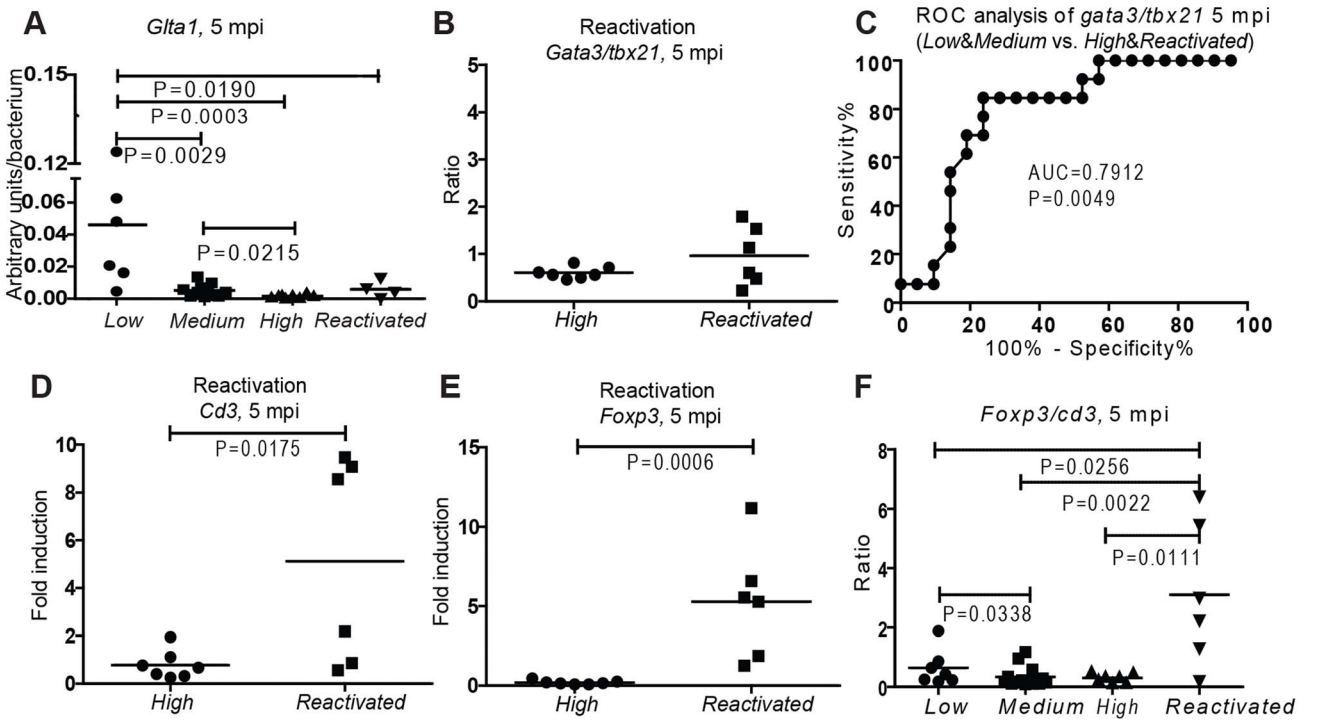

Figure 4. Low Th2/Th1 and high Treg are associated with activity of disease at late-stage mycobacterial infection. (A) The proportion of dormant bacteria in each non-stimulated subgroup was assessed by measuring the expression of a mycobacterial dormancy-associated gene *GltA1*. (B, D&E) The T cell inductions of *Reactivated* group (fish showing symptoms between 8 and 20 weeks after an initial controlled phase) and *High* group at 5 mpi. (C) ROC analysis of the *gata3/tbx21* ratio as a biomarker to distinguish individuals with a high bacterial burden from the individuals with a lower bacterial load (AUC = area under the curve). (F) Induction of *foxp3* normalized to *cd3* induction in the subgroups at 5 mpi and fish showing symptoms between 2 and 5 mpi.
doi:10.1371/journal.ppat.1004190.g004

foxp3 expression is higher than the increase in the total T cell number and thus, the increased proportion of *foxp3* cells could be used for distinguishing *Reactivated* individuals from the rest of the population at a late stage of infection. The role of foxp3 positive cells in reactivation of tuberculosis and its applicability in diagnostics warrants further investigation.

Discussion

It is known that T cell responses are essential in restricting mycobacterial growth in human tuberculosis as well as in various mammalian models for studying tuberculosis. The zebrafish is a newcomer in the field of immunology, and the components of its adaptive immune system have only recently been elucidated in more detail. It has been shown [33] that there are professional antigen presenting cells capable of inducing antigen-specific T cell responses in zebrafish. In our recent publication [34], we have demonstrated the presence of specific and protective immune responses against *M. marinum* infection in zebrafish. We have shown that vaccination of zebrafish with the Bacillus Calmette-Guérin (BCG) increases survival of adult zebrafish from infection with *M. marinum*. Furthermore, vaccination of zebrafish with plasmid DNA encoding mycobacterial antigens increases survival, reduces the spreading of bacteria as well as the number of granulomas in *M. marinum* infection, compared to vaccination with a control pDNA lacking the antigen-encoding sequence. *Rag1* (−/−) zebrafish lacking functional adaptive lymphocytes are not protected by the antigen-pDNA vaccine. In the antigen-pDNA - vaccinated zebrafish, interferon gamma expression levels are significantly higher during infection than in the control pDNA vaccinated fish, demonstrating the specificity of the anti-mycobacterial immune response induced in zebrafish.

For studies of zebrafish lymphocyte populations, antibody markers for FACS analysis are not available. To characterize the T cell responses of individuals with different infection outcomes, we measured expression levels of marker genes reflecting the total number of T cells and the Th profile from the internal organs of infected zebrafish. We also used the transgenic *lck:GFP* zebrafish as a tool to isolate T cells and to validate the T cell markers that were used in the study (Figure S2). We showed that the Th marker genes are enriched in sorted lck+ T cells and demonstrated a correlation between the *cd3* transcript level and the total number of lck+ cells from *lck:GFP* reporter fish. These assessments could only be done from uninfected zebrafish, because it is likely that *M. marinum* infection influences the expression of the GFP reporter gene driven by the *lck* promoter. It has been previously shown for other pathogenic mycobacteria that one of the mycobacterial virulence strategies is to inhibit host T cell receptor signaling by interfering with the expression and phosphorylation of lck [35–37]. Thus, the use of the *lck:GFP* reporter zebrafish line as a tool to study T cell responses in *M. marinum* infections would first require careful assessment of the effects of the bacterium on zebrafish *lck* expression. Instead, we have here relied on the use of several parallel markers reflecting the different Th profiles. In the future, development of new research tools is needed for a more detailed characterization of Th responses and their role in the pathogenesis of mycobacterial infection in the zebrafish model.

The results of the current study support the view that both Th1 and Th2 responses are induced in an optimal anti-TB-response. In

the well-controlling zebrafish individuals, both Th1 and Th2-type responses were efficiently induced and showed temporal and quantitative differences compared to the Th responses of progressive individuals. The individuals that are not capable of restricting bacterial growth and, subsequently, are likely to develop a progressive disease, maintain a more Th1-biased response at all stages of infection, not reflecting an excessive Th1-type response but, instead, a lack of Th2-type induction.

Our study shows that a low-dose *M. marinum* infection elicits different types of responses in different individuals. It is known that environmental factors, such as nutritional status or infections, influence the differentiation of T helper cells. In addition to environmental factors, it has recently been demonstrated in mice and humans that each mouse strain as well as each human individual has a genetically defined Th1/Th2 bias, and that the characteristic Th phenotype is sustained over the time [23]. Generally, genetic variation in the associated transcription factors, cytokines or cytokine receptors may define the inherent individual Th bias. It is known that there are various single nucleotide polymorphisms in the enhancer regions of human Th differentiation genes, and that these polymorphisms are related to the susceptibility to various disease states [24]. In the studied heterogeneous zebrafish population, the individual outcome of mycobacterial infection can be assumed to be affected by host genetic factors, including the inherent bias in T helper phenotype, as the variation in environmental and bacterial factors is minimal. Based on the results of this study, a Th1-type response is induced equally efficiently in both progressive and well-controlling individuals, but the lack of a Th2-type response causes the disease to progress in the (genetically) susceptible population. However, in a control experiment, in which zebrafish were infected with a high initial dose of *M. marinum*, we saw that the rapid growth of bacteria may also alter the Th2/Th1 balance tilting it towards Th1. Also, we saw that the high initial dose caused the total *cd3* expression levels to remain low suggesting bacterium-induced T cell inhibition. In humans, it is known that *M. tuberculosis* can cause apoptosis of specific T cells [38] and delayed activation of CD4-positive T cells [39]. It is likely that both the bacteria and the genetic determinants of the host are capable of affecting the T cell responses in mycobacterial infection, and it is challenging to distinguish the contribution of either alone. Also, additional host factors alongside with those related T cell responses are likely to affect the disease outcome.

As latent tuberculosis exists in a major part of the human population, its spontaneous reactivation is a serious global threat. Latent tuberculosis, when not initially caused by a resistant strain, can be treated with a 9-month isoniazid monotherapy that reduces the risk of reactivation by 60–90%. However, poor treatment compliance is a common problem in treating this asymptomatic disease, as only half of the patients complete therapy [40]. The poor compliance, in turn, affects the increased antibiotic resistance to isoniazid complicating the treatment of both latent and active tuberculosis. Therefore, it would be of paramount importance to be able to recognize the small population of latently infected individuals with a higher bacterial load and to allocate the treatment to only those who are most likely to benefit from it. At a late time point, at 5 months post infection, there was a zebrafish subpopulation present with a clearly more active disease, as determined by the total bacterial load and mycobacterial dormancy gene (*GltA1*) expression. The fish with a higher bacterial load had a lower *gata3/tbx21* ratio. Based on our results, analysis of the Th1/Th2 ratio from peripheral blood mononuclear cells could provide a correlate of activity of disease among the carriers of latent *M. tuberculosis* infection. The risk of reactivation is also thought to increase with increasing bacterial loads [25], and thus the Th1/Th2 ratio could have predictive value in evaluating the risk of reactivation of a latent infection. The potential of the Th1/Th2 ratio as a biomarker in the human population warrants further investigation.

The high induction of *foxp3* expression in spontaneously reactivated individuals is in line with a previous human study showing that quantification of Foxp3 from antigen-induced peripheral blood mononuclear cells can be used to discriminate between latent and active TB [41]. During infection, regulatory T cells (Treg) have an important role in controlling excessive inflammation to prevent tissue damage, but at the same time, their immunosuppressive function can prevent bacterial clearance [42]. The role of Treg cells has been investigated during the early response to TB infection, and there is evidence that *M. tuberculosis* induces the expansion of antigen-specific Treg cells thus delaying the priming of effector T cells in the lymph nodes and the subsequent arrival of T cells to the infection site [43]. As *M. tuberculosis* is capable of such exploitation of the Treg response as part of its virulence strategy during the early TB infection, it is plausible that similar pathogen-driven expansion of antigen-specific Treg cells could also play a role in the reactivation of latent TB and the subsequent transmission of the disease. On the whole, the role of Treg cells in reactivation of latent TB is highly interesting and calls for further characterization.

The existence of individuals that are able to clear mycobacterial infection illustrates that the optimal immune response to fight TB has already developed during the evolution. Adaptive mechanisms underlying mycobacterial clearance have so far remained enigmatic, and their better understanding will undoubtedly provide valuable knowledge for drug and vaccine development against tuberculosis. The zebrafish model is uniquely suitable for dissecting the natural spectrum of mycobacterial infection in large scale population studies. Analysis of the protective immunity leading to the eradication of bacteria in zebrafish can provide valuable knowledge for the development of new innovative approaches to prevention and treatment of tuberculosis.

The importance of Th1-type response in controlling mycobacterial infection is generally recognized because mycobacteria are (facultative) intracellular pathogens. The general – and simplified – paradigm of the reciprocal regulation between Th1 and Th2 responses has led to the idea that Th2 response in tuberculosis might inhibit the bacterial clearance by Th1 immunity. Therefore most tuberculosis vaccines currently under development aim at promoting an efficient Th1 response and inhibiting the induction of a Th2 response [44]. In the studied zebrafish population, 10% of the individuals were able to clear the infection after the activation of adaptive responses (>2 wpi). These clearers had a similar, Th2-biased response as the other individuals in the well-controlling *Low* and *Medium* subgroups. On the other hand, inability to induce Th2 responses seems to be a trait that is associated with progressive mycobacterial infection in the zebrafish. Our finding argues against the paradigm of Th2 response not being useful for controlling tuberculosis. If this holds true in human TB, the current therapeutic and preventive approaches promoting Th1 and inhibiting Th2-type response need to be thoroughly reconsidered.

Materials and Methods

Zebrafish lines and maintenance

For most experiments, adult (5–8 month-old) wild-type AB zebrafish were used. In addition, adult, *rag1* (−/−) hu1999 mutant fish and *lck:GFP* transgenic fish (both from ZIRC) were used. Fish

were kept in a flow-through system with a light/dark cycle of 14 h/10 h and were fed with SDS 400 food twice daily.

Ethics statement

All experiments have been accepted by the Animal Experiment Board in Finland (under the Regional State Administrative Agency for Southern Finland) and were carried out in accordance with the EU-directive 2010/63/EU on the protection of animals used for scientific purposes and with the Finnish Act on Animal Experimentation (62/2006). Permit for the zebrafish facility: LSLH-2007-7254/Ym-23, Permit for experiments: ESAVI/6407/04.10.03/2012, PH1267A and ESAVI/733/04.10.07/2013.

Experimental infection

M. marinum (ATCC 927) was cultured as described in [17]. In brief, bacteria were grown at 29°C in standard mycobacterium medium 7H9 (BD) with standard additives to an OD600 of 0.495–0.680 Anesthetized fish were intraperitoneally (i.p.) injected with 5 µl of bacteria suspended in sterile PBS using an Omnican 100 30 G insulin needle (Braun, Melsungen, Germany). The bacterial dose was verified by plating on 7H10 (BD) with the standard additives. The low infection dose was 21±7 cfu and the high dose 1783±364 cfu.

Injections with heat-killed *M. marinum*

M. marinum (ATCC 927) was transferred from 7H10 plate into 10 ml of liquid 7H9 medium with standard additives and cultured for 3–4 days at 29°C to an OD600 of 0.490. Pelleted bacteria were resuspended in PBS corresponding to half of the original culture volume. The bacteria were heat-killed at 100°C for 20 min and thereafter homogenized for 4 min with 4000 rpm using homogenization tubes from Mobio (California, USA) and Mobio PowerLyzer24 bead beater. Samples were plated on 7H10 and LB to verify proper killing. Heat-killed bacteria were injected in a volume of 5 µl *i.p* using Omnican 100 30 G insulin needles (Braun).

Transplantation of lymphatic cells from AB to *rag1* (−/−) fish

Kidney and spleen were collected from a euthanized AB fish in 20 µl of sterile PBS. The organs were gently homogenized by pipetting up and down ~20 times. 10 µl of this suspension was injected *i.p.* into an anesthetized recipient *rag1* (−/−) fish.

FACS sorting

For lymphocyte sorting experiments, *lck:GFP* fish were euthanized and their internal organs collected in ice-cold HBSS supplemented with 2% FBS (both from Life technologies, CA, USA). The tissue was mechanically disrupted by pipetting and passed through a 50 µm cell strainer to prepare single cell suspensions. Cells were washed twice with cold HBSS (+FBS), pelleted at 4°C, 300 g for 5 min and resuspended in 1 ml of the same buffer. 1 ml of Histopaque-1077 (Sigma-Aldrich, MO, USA) was then added under the cell suspension and lymphocytes and other mononuclear cells were enriched by centrifugation at room temperature for 20 min, 400 g. After centrifugation, the middle phase containing the target cells was transferred into a new tube, washed once and resuspended in HBSS (+FBS). Lck+ lymphocytes were sorted with FACSAria I (BD) (purity ≥95% based on GFP expression), collected by centrifugation and RNA was extracted using TRI reagent as described in [17].

q-PCR

The samples for gene expression analysis and mycobacterial quantitation were prepared using TRI reagent for DNA-RNA co-extraction (MRC, OH, USA) as previously described in [17]. RNA samples were treated with DNAse (Fermentas) according to the manufacturer's protocol. Bacterial loads were measured by q-PCR from DNA samples using SENSIFAST NO-ROX SYBR kit with *M. marinum*-specific primers as described in [17]. A dilution series of DNA extracted from mycobacterial culture was included in each run to allow absolute quantification. Gene expression was measured by q-RT-PCR using Bio-Rad iScript One-Step RT-PCR Kit with SYBR Green with various primers. Host genes were normalized to *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) or to *elongation factor 1 alpha (Ef1a)*, and the mycobacterial dormancy gene *GltA1* was normalized to the total bacterial load. q-RT-PCR results were analyzed using the ΔCt method. The induction of host genes was compared to a baseline RNA sample extracted from a pool of healthy, non-infected zebrafish and shown as fold induction compared to average normal levels. *GltA1* levels are shown in arbitrary units. Gene accession numbers and primer sequences can be found in Text S1.

Protein extraction and western blotting

For Western blotting, fish were collected 4 weeks after infection with 21±7 cfu. The peritoneal cavity of the euthanized fish was emptied and the collected organs were homogenized in 1.5 ml of TRI reagent (MRC, OH, USA) using the PowerLyzer24 bead beater. RNA-DNA co-extraction was carried out as described in [17]. After DNA extraction, the remaining interphase and organic phase were used for protein extraction according to the TRI reagent manufacturer's protocol. In brief, proteins were precipitated by adding 3 volumes of acetone and pelleted at 12,000 g for 10 min at 4°C. The protein pellet was washed three times with 0.8 ml of 0.3 M guanidine hydrochloride in 95% ethanol supplemented with 2.5% glycerol (v:v) and once with 1 ml of ethanol containing 2.5% glycerol (v:v). For solubilization of the protein pellet, 0.1 ml of 1% SDS per 10 mg of tissue sample was used. For Western blotting, 40 µg of total protein was resolved on a 10% SDS-PAGE gel and blotted onto Amersham Hybond ECL nitrocellulose membrane (GE Healthcare, Little Chalfont, UK). The following primary antibodies were used: anti-Gata-3 (IN) Z-Fish (AnaSpec, California, USA), anti-CXCR-3.2 (IN) Z-Fish (AnaSpec), anti-GFP antibody NB600-303 (Novus biological, Colorado, USA). In addition, actin was detected from all the membranes with anti-actin (MAB1501) antibody (Millipore, Temecula, USA) for sample normalization. IRDye infrared secondary antibodies (LI-COR Biosciences, Nebraska, USA) and Odyssey CLx (LI-COR) were used for target protein detection and Image Studio software (LI-COR) was used for protein quantitation. A representative image of the blots showing 10 individuals can be found in the Supplementary material (Figure S4).

Statistical analysis

Statistical analysis was carried out using the GraphPad Prism software (5.02). For determination of statistical significance of differences between the different groups, a non-parametric one-tailed Mann-Whitney test was used, if not stated otherwise. P-values$<$0.05 were considered significant. For estimating the predictive value of *gata3/tbx21* and *foxp3* expression for activity of the disease a ROC analysis was carried out with a confidence interval of 95%. AUC (area under curve) value of 0.5 indicates no connection and 1.0 indicates a perfect marker.

Supporting Information

Figure S1 Bacterial loads in the different subgroups at different stages of the infection. Organs from infected WT fish were collected at various time points (A) 2 wpi, (B) 4 wpi, (C) 7 wpi and (D) 5 mpi. The bacterial loads were measured by q-PCR. Based on the bacterial load, the fish were grouped in upper and lower quartile (*High* and *Low*, respectively) and the middle 50% (*Medium*). (D) Between 2 and 5 months post infection, fish showing external signs of disease were euthanized and labeled the *Reactivated* group. The bacterial loads of the *Reactivated* fish are shown with the bacterial loads from the fish collected at 5 mpi. (E) Organs were collected from low-dose *M. marinum*-infected *rag1* (−/−) fish at 4 wpi. The bacterial loads were measured by q-PCR and the fish were grouped as described for WT fish above.
(TIF)

Figure S2 Validation of markers by FACS-enrichment of T cells using *lck:GFP* reporter line. (A) The internal organs of non-infected *lck:GFP* reporter fish were collected and mononuclear cells (including lymphocytes) were enriched by Histopaque-1077 gradient centrifugation. The cells were then sorted based on size, granularity and GFP expression. (B–F) The marker gene expression was measured from sorted T cell samples by and compared to that measured from an unsorted tissue block. (G) The linear correlation between the T cell count of the sample and *cd3* expression measured by q-RT-PCR was assessed; $R2 = 0.81$. (H–I) *Rag1* (−/−) mutants and WT zebrafish were infected with a low dose of *M. marinum* and analyzed for *Tbx21* and *Gata3* expression by q-RT-PCR at 4 wpi.
(TIF)

Figure S3 The induction levels of *gata3* and *tbx21*. The induction levels of *gata3* (A–C) and *tbx21* (D–F) are shown separately in the different subgroups at 2 wpi (A&D), 4 wpi (B&D) and 5 mpi (C&F).
(TIF)

Figure S4 Semi-quantitative western blots on a Th1 and Th2 markers are in line with the results gained from q-PCR data. Western blots were carried out at 4 wpi from a population of 20 fish. Here shown as a representative the blots of 10 individuals (numbered 1–10). Th2/Th1 ratio was assessed with anti-Gata-3 (IN) and anti-CXCR-3.2 (IN) antibodies. Actin was detected for sample normalization.
(TIF)

Text S1 Primer sequences and the accession numbers of target genes.
(DOC)

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Author Contributions

Conceived and designed the experiments: MMH KEO MPe MR MPa. Performed the experiments: MMH KEO HMN BVL MPa. Analyzed the data: MMH KEO MPa. Contributed reagents/materials/analysis tools: MPe MR MPa. Wrote the paper: MMH KEO MPe MR MPa.

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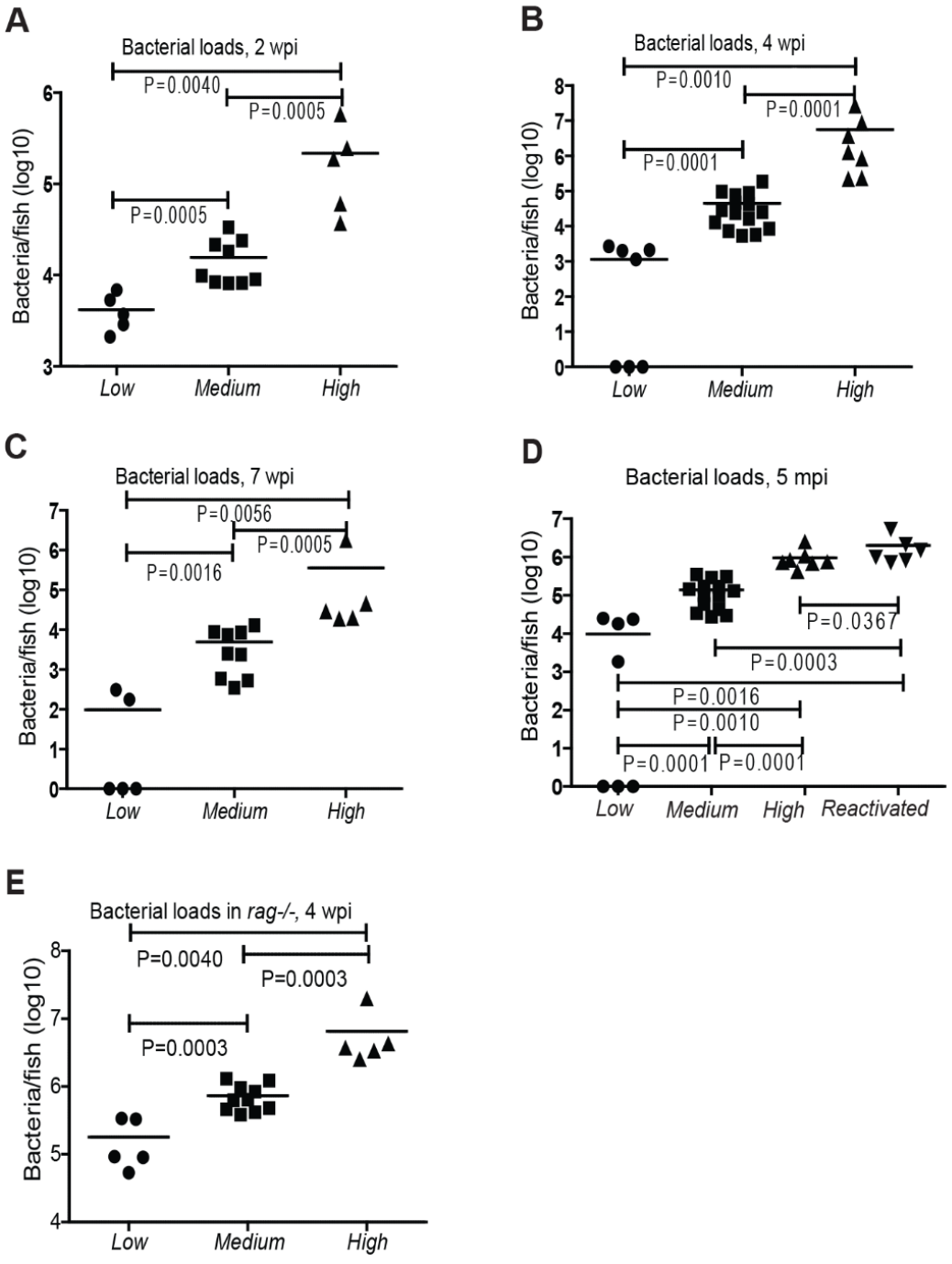

Figure S1, Hammarén et al. 2014

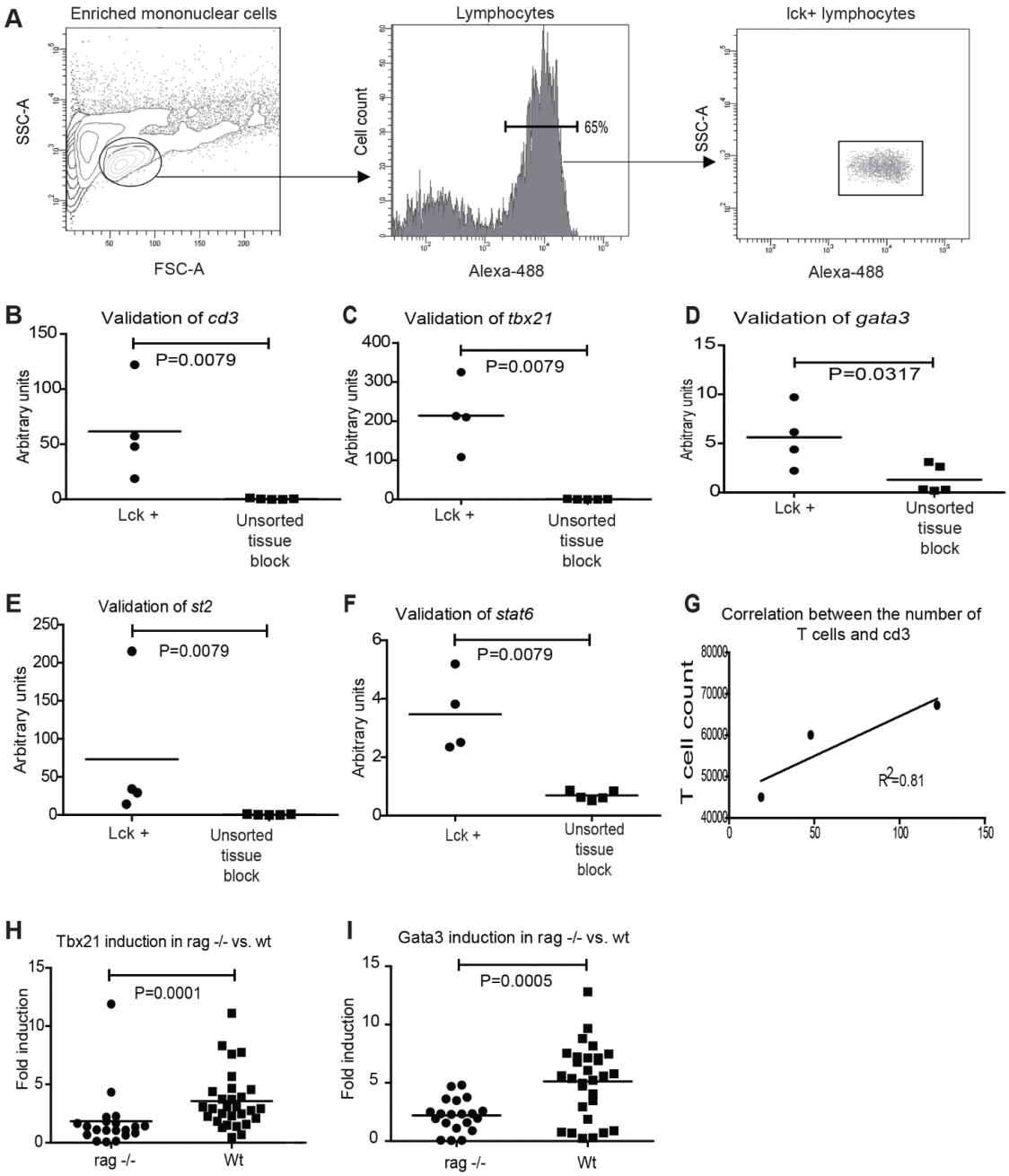

Figure S2, Hammarén et al. 2014

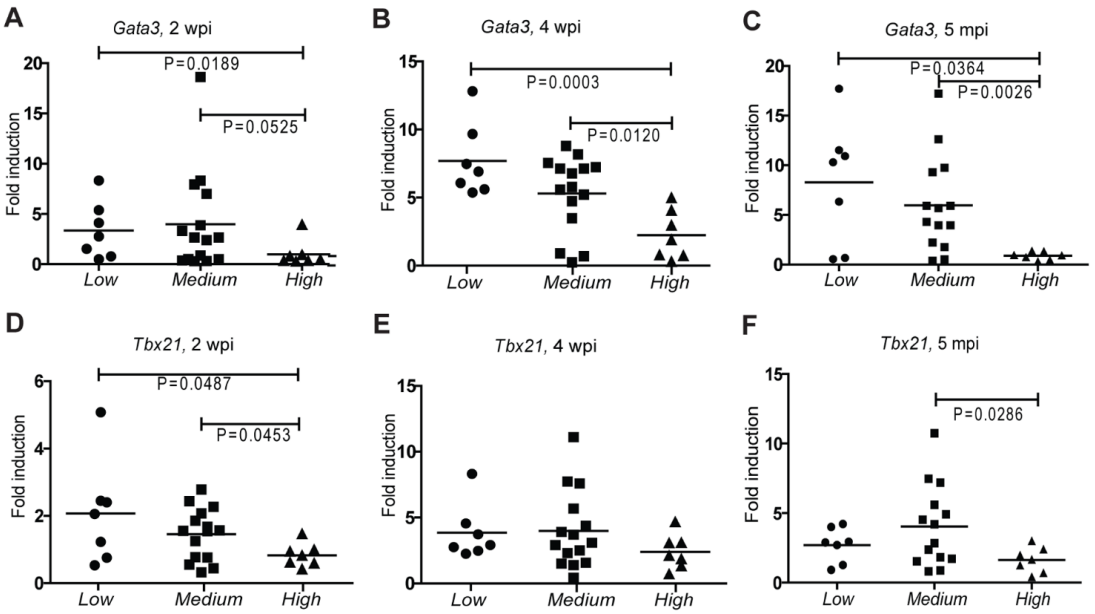

Figure S3, Hammarén et al. 2014

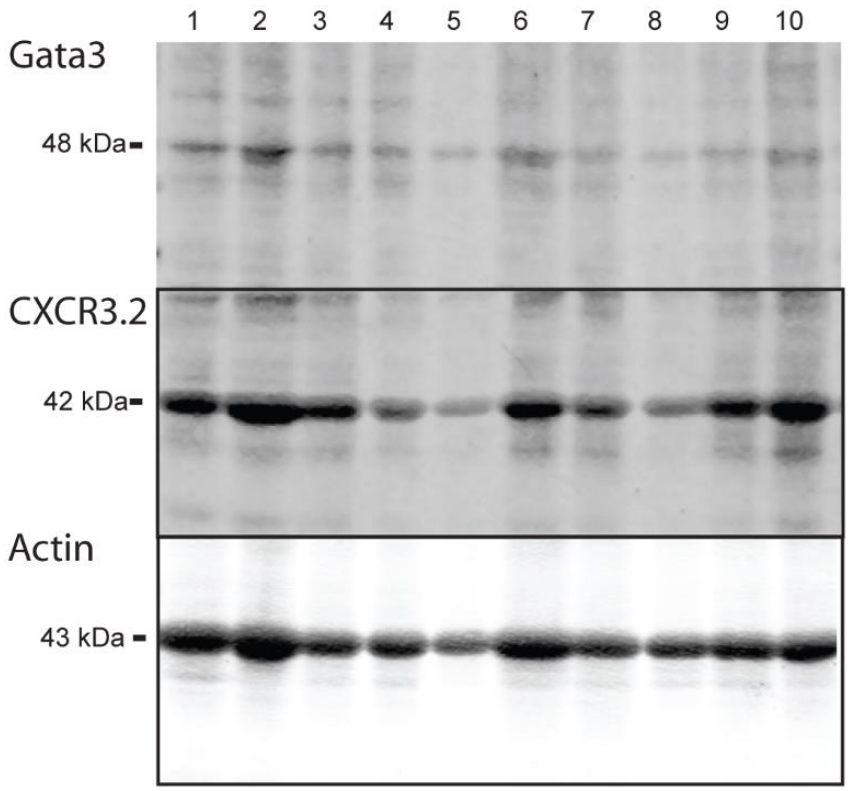

Figure S4, Hammarén et al. 2014