

## REVIEW

# Integrating fish models in tuberculosis vaccine development

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## ABSTRACT

Tuberculosis is a chronic infection by *Mycobacterium tuberculosis* that results in over 1.5 million deaths worldwide each year. Currently, there is only one vaccine against tuberculosis, the Bacillus Calmette–Guérin (BCG) vaccine. Despite widespread vaccination programmes, over 10 million new *M. tuberculosis* infections are diagnosed yearly, with almost half a million cases caused by antibiotic-resistant strains. Novel vaccination strategies concentrate mainly on replacing BCG or boosting its efficacy and depend on animal models that accurately recapitulate the human disease. However, efforts to produce new vaccines against an *M. tuberculosis* infection have encountered several challenges, including the complexity of *M. tuberculosis* pathogenesis and limited knowledge of the protective immune responses. The preclinical evaluation of novel tuberculosis vaccine candidates is also hampered by the lack of an appropriate animal model that could accurately predict the protective effect of vaccines in humans. Here, we review the role of zebrafish (*Danio rerio*) and other fish models in the development of novel vaccines against tuberculosis and discuss how these models complement the more traditional mammalian models of tuberculosis.

**KEY WORDS:** Tuberculosis vaccine, Zebrafish, Animal models, Mycobacteria

## Introduction

*Mycobacterium tuberculosis* (Mtb), the causative agent of tuberculosis (TB), has coexisted with the human population for thousands of years, causing high levels of mortality. Even today, despite extensive research and progress in the areas of prevention and treatment, TB remains the deadliest bacterial infection worldwide (Furin et al., 2019). According to the Global Tuberculosis Report 2019, 10 million new active disease cases are diagnosed each year, with 84% of the diagnoses confirmed in people living in 20 high-burden countries; for example, in regions of South-East Asia, Africa and the Western Pacific (WHO, 2019). Although TB affects all age groups in all parts of the world, the highest incidence is reported in low-income countries with a high human immunodeficiency virus (HIV) burden. Altogether, TB has been reported to be the cause of ~1.5 million deaths each year and the most common cause of death in HIV-positive individuals (Furin et al., 2019; WHO, 2019). Active TB (see Glossary, Box 1)

typically manifests as a pulmonary disease (Box 1) but more rarely, and especially among infants and immunocompromised people, a Mtb infection can progress into a disseminated disease, presenting, for example, as miliary or meningeal TB (Box 1) (Furin et al., 2019). In addition to active disease cases, 23% of the human population is estimated to carry Mtb in an asymptomatic and non-contagious form (Houben and Dodd, 2016). These ~1.7 billion people with a latent Mtb infection (Box 1) have a lifetime risk of developing active disease and thus represent a considerable reservoir of potential disease. However, estimating the true burden of latent TB is challenging due to the limitations of the available diagnostic tests for TB, which currently comprise the tuberculin skin test (TST; Box 1) and the interferon  $\gamma$  (IFN $\gamma$ ) release assay (IGRA; Box 1) (Furin et al., 2019; Behr et al., 2018).

Although the clinical presentation of TB has traditionally been divided into acute and latent infections, the overall disease spectrum of TB is highly variable (Fig. 1) (Drain et al., 2018; Cadena et al., 2017). In humans, the clinical symptoms of active TB include persistent cough, blood-stained sputum, weight loss and fever. Disease transmission occurs during the active phase through aerosol droplets containing Mtb (Sia and Rengarajan, 2019). TB is a granulomatous inflammatory disease, characterized by the presence of Mtb-containing immune cell clusters – granulomas – at the site of infection, mostly the lungs, but also in the lymph nodes and elsewhere in the body when the disease becomes disseminated (Sia and Rengarajan, 2019; Cadena et al., 2017). Granulomas form around macrophages infected with Mtb to prevent the dissemination of bacteria, but, at the same time, they are also the predominant site for Mtb replication and dissemination (Cadena et al., 2017). During the latent infection, bacteria inside the granulomas may become metabolically inactive, allowing them to persist in the human body for decades (Peddireddy et al., 2017). Even during this seemingly static and asymptomatic phase, the host-pathogen interactions in granulomas are highly dynamic, and the fate of infection (Fig. 1) is thought to be a result of continuous interplay between Mtb and its host (Cadena et al., 2017). Approximately 5–10% of the individuals with a latent infection develop active TB (WHO, 2019; Behr et al., 2018).

Owing to the complex pathophysiology and an incomplete understanding of the definitive steps leading to progression of the infection or reactivation of the latent disease, the outcome of an Mtb infection is poorly predictable. The conventional treatment of TB is a combination of four antibiotics – isoniazid, rifampin, pyrazinamide, and either ethambutol or streptomycin (Furin et al., 2019) – which requires long-term drug regimens lasting 6–9 months and is associated with poor patient compliance and frequent treatment failure (Furin et al., 2019; Munro et al., 2007). This also creates an excellent environment for the development of antibiotic-resistant Mtb, which has become a major problem in areas with a high TB incidence such as India, China and Russia (WHO, 2019). Although the global incidence and mortality are declining, the burden of TB in low-income countries remains high (WHO, 2019). In these countries, early and comprehensive diagnosis and treatment are

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**Box 1. Glossary**

**Active tuberculosis:** the contagious form of the disease. Symptoms include fever, cough, bloody cough, weight loss and night sweats. Granulomas and tuberculous lesions in infected tissues are detected with x-ray.

**Adaptive immune response:** confers specific protection against pathogens days or weeks after exposure and is responsible for the antigen-specific immunological memory protecting against a secondary infection.

**Bacillus Calmette–Guérin (BCG):** an attenuated *M. bovis* used as a vaccine for tuberculosis.

**Caseating granuloma:** granuloma with a necrotic core (see 'Necrotic granuloma core') with caseating insides with a cheese-like appearance.

**Cellular immune response:** adaptive immune response mediated by CD4<sup>+</sup> helper T cells that activate phagocytes and antigen-specific cytotoxic T cells (CD8<sup>+</sup>) to destroy intracellular pathogens or infected host cells.

**DNA vaccine:** a vaccine comprising a genetically engineered DNA construct encoding antigens that, after administration to the recipient, are expressed in host cells to elicit protective immune responses.

**Fibrotic capsule:** an epithelialized capsule built of fibroblasts that has formed around the infected cells to form a fibrous granuloma.

**Innate immune response:** non-specific and immediate defence mechanism comprising physiological barriers, receptors recognizing conserved patterns of foreign material, innate immune cells (such as macrophages, dendritic cells, granulocytes and natural killer cells), the complement system and inflammatory mechanisms.

**Interferon  $\gamma$  release assay (IGRA):** an assay to test the stimulation of leukocytes from a blood sample against tuberculosis antigens. Cannot distinguish between an active and latent infection.

**Latent tuberculosis:** non-contagious form of the disease. *M. tuberculosis* (*Mtb*) resides in granulomas without causing symptoms. Tuberculous granulomas can be visualized via x-ray. Latent disease can reactivate to an active infection in compromised immunity.

**Meningeal tuberculosis:** *Mtb* infection of the membranes enveloping the nervous system.

**Miliary tuberculosis:** *Mtb* infection that manifests across multiple tissues in 'grain-like' small lesions.

***Mtb* virulence factor:** a molecule produced by *Mtb* that is essential for colonization and survival of the bacterium inside the host organism.

**Mucosa-associated lymphoid tissue (MALT):** lymphoid tissue of mucosa in various sites of the body, including the gastrointestinal tract, respiratory tract and urogenital tract. Rich in lymphocytes, macrophages and antigen-presenting mucosal cells (microfold cells, M-cells).

**Necrotic granuloma core:** the core of the granuloma contains dead and dying cells as a result of non-apoptotic cell death.

**Pulmonary tuberculosis:** *Mtb* infection in the lung.

**Recombinant BCG (rBCG):** a genetically modified *M. bovis* BCG strain.

**Th1:** a subtype of CD4<sup>+</sup> T cells mediating a cellular immune response (see 'Cellular immune response').

**Th2:** a subtype of CD4<sup>+</sup> T helper cells mediating protection; for example, against extracellular parasites and suppressing the Th1 cell response.

**Th17:** a subtype of CD4<sup>+</sup> T helper cells mediating protection against intracellular pathogens through the recruitment of neutrophils and macrophages.

**Tuberculin skin test (TST):** test of reactivity to tuberculosis antigens. It is subject to false positives in BCG-vaccinated individuals, and cannot distinguish between an active and latent infection.

hampered by the poor availability of health services, and, as a consequence, from the estimated 10 million annual cases of TB, only 6.4 million are officially diagnosed and properly treated (WHO, 2019).

A more economical and practical approach for controlling TB is to prevent infections through vaccination. However, owing to variation in the protective efficiency of the current TB vaccine, Bacillus Calmette–Guérin (BCG; Box 1), the goal of eradicating TB

has not been achieved. While BCG protects young children from meningeal and miliary TB, it fails to prevent primary *Mtb* infections or latent disease reactivation (Dockrell and Smith, 2017; Mangtani et al., 2014). In addition, estimates of the effect of BCG on pulmonary TB vary significantly, ranging from 0% to 80% (Mangtani et al., 2014). Even though BCG protection reportedly lasts for up to 50 years (Aronson et al., 2004), in most cases, the protective effect wanes by adolescence, leaving the adult population poorly protected (Mangtani et al., 2017).

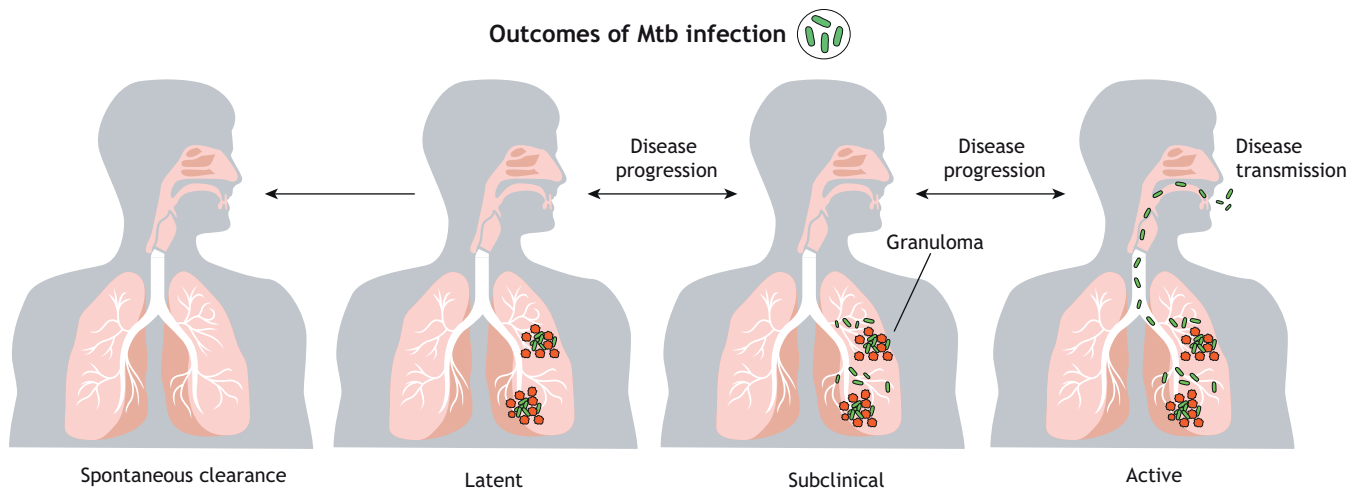
To address the shortcomings of the BCG vaccine and the problem of drug-resistant *Mtb*, a concerted research effort is underway to develop new anti-*Mtb* vaccines, and several vaccine candidates are currently in phases I-III of clinical trials (Table 1; also reviewed in detail in, for example, Hatherill et al., 2020; Martin et al., 2020). Although recent success with BCG revaccination, conferring 45.5% protection in previously BCG-vaccinated healthy adolescents (Nemes et al., 2018), paves the way for the success of other TB vaccines, many of the candidates that show protection in animal models fail in clinical trials. Therefore, reliable preclinical models with predictive value for human trials are needed to improve efficacy and save costs and time in vaccine development.

Mice are by far the most used model in the evaluation of novel TB vaccines, followed by other common mammalian models: guinea pig, rabbit, cattle and non-human primates (NHPs) (reviewed in Gong et al., 2020; Myllymäki et al., 2015). However, small mammalian models are limited by their lack of natural susceptibility to mycobacterial infection, whereas NHPs raise ethical concerns (Gong et al., 2020; Myllymäki et al., 2015). As an alternative to mammalian models, fish models, and especially zebrafish (*Danio rerio*), have gained popularity in TB vaccine development. Fish are naturally susceptible to *Mycobacterium marinum*, a close relative to *Mtb* that causes a disease resembling human TB (Hashish et al., 2018; Tobin and Ramakrishnan, 2008). The related pathogenesis of and host responses to *Mtb* and *M. marinum* infections, combined with the suitability of fish for large-scale antigen screens, hold promise for this model in supporting the preclinical evaluation of novel TB vaccine candidates.

In this Review, we discuss how fish models, especially the zebrafish, can complement the more traditionally used mammalian models for the development of novel TB vaccines. We start with a brief introduction to the common mammalian models of TB and discuss their advantages and limitations in light of recent data from preclinical and clinical studies. Finally, we highlight the potential of the zebrafish and other fish models in TB vaccine development by discussing the latest achievements made with these models.

**Mammalian models of TB**

Prior to clinical trials, the safety, immunogenicity and protective effect of each vaccine candidate is evaluated in animal models. The safety of the vaccine candidate is a prerequisite for progressing to clinical trials, while immunogenicity studies provide insights into proper vaccination schedules and doses for efficacy studies. The efficacy of candidate TB vaccines is tested in *Mtb* challenge studies, where protection in vaccinated animals is measured by, for example, survival, *Mtb* burden or the pathology score (McShane and Williams, 2014). In general, a vaccine candidate must demonstrate improved protection compared to BCG to progress to clinical trials. Owing to the complex pathophysiology of human TB, it is challenging to model all its aspects in one animal species and, therefore, TB vaccine candidates are preclinically validated in several species. In the following section and in Table 2, we briefly introduce the main mammalian models used in TB research.



**Fig. 1. Disease spectrum of pulmonary tuberculosis.** Pulmonary tuberculosis is the most common clinical manifestation of a *M. tuberculosis* (Mtb) infection. After primary infection, the disease can be spontaneously cleared by the immune system, remain latent, or progress into either subclinical or active infection. In latent, subclinical or active infection, Mtb (green) persist in the lung tissues in granulomas (red). In subclinical and active infection, free Mtb also reside in the lungs. In active disease, free Mtb are also secreted into the airways, which makes active disease contagious. Latent infection can progress into subclinical or active disease, and subclinical disease can progress into active disease, meaning that carriers of latent TB represent a significant disease reservoir.

### Mouse

The mouse is the most widely used model in TB vaccine development. Mice are relatively cost efficient, easy to maintain, and are supported by well-established genetic and immunological tools (Cooper, 2014). An Mtb infection in mice can be induced by aerosol, resulting in an active infection with a stable high Mtb burden and early death without a latent phase (Cooper, 2014). As Mtb is not a natural murine pathogen, many of the commonly used mouse strains (such as BALB/c and C57Bl/6) are resistant (Medina and North, 1998). Granulomas in mice also differ from those in humans as they lack necrotic cores (Box 1) and organized structure (Orme and Basaraba, 2014; Rhoades et al., 1998). To overcome these limitations and to better mimic the pathophysiology of human TB, researchers use immunodeficient or genetically modified strains, such as C3HeB/FeJ mice, which exhibit caseating granulomas (Box 1) (Lanoix et al., 2015; Driver et al., 2012). However, these lines show varying susceptibilities and lung pathologies, and thus different responses to drugs and vaccines, complicating the extrapolation of the results to humans. Despite their limitations, mice are frequently used in preclinical studies, where they offer a practical tool for evaluating the immunogenicity and the mechanisms of protection of novel TB vaccine candidates (Cardona and Williams, 2017). However, verification of the protective effect of the candidate in other models, which better recapitulate the complexity of human TB, is required.

### Guinea pigs

Guinea pigs are well suited for TB research. Although they are not natural hosts, guinea pigs are highly susceptible to Mtb via the airways and recapitulate the main features and symptoms of the acute TB in humans (Clark et al., 2014). Granulomas in Mtb-infected guinea pigs are similar to human granulomas, with corresponding cell types, necrotic cores and fibrotic capsules (Box 1) (Turner et al., 2003). However, an Mtb infection in guinea pigs is always progressive and fatal, and exhibits no latent phase (Clark et al., 2014; Grover et al., 2012; Williams et al., 2009). Compared to mice, higher costs and maintenance requirements, as well as scarcity of immunological reagents and genetic tools, limit the use of guinea pigs in TB research. In TB vaccine research,

guinea pigs are the preferred small mammals for testing the protective effects of novel TB vaccine candidates. Owing to the lack of latency, however, this model is not suited for studying vaccines against the reactivation of latent TB.

### Rabbit

Mtb and *Mycobacterium bovis* infections have been modelled in rabbits and, when introduced via the airways, both result in a spectrum of diseases similar to human TB (Peng et al., 2015). Although the ability to resist infection depends on the Mtb strain used (Manabe et al., 2003), rabbits are not particularly susceptible to Mtb and can contain the infection in a latent form that can then be experimentally activated by a corticosteroid treatment to model TB reactivation (Kesavan et al., 2009; Manabe et al., 2008). Similar to guinea pigs, the histology of granulomas in rabbits, with their necrotic, caseous cores, resembles that in humans. However, the use of rabbits in TB research is hampered by the lack of reagents and tools for research, as well as by high costs, poor availability of animals and ethical concerns (Peng et al., 2015). Although the presence of the entire disease spectrum of an Mtb infection in rabbits is a considerable advantage over other small mammalian models, rabbits are only selectively used in vaccine development and are more often utilized to study the rarer forms of human TB, cutaneous and meningeal TB (Peng et al., 2015).

### NHPs

As the genetically closest relatives to humans and natural hosts for Mtb, NHPs are the best models for human TB (Foreman et al., 2017). All aspects of human TB can be modelled in NHPs due to the similarities in immune response, disease pathology and clinical manifestation (Foreman et al., 2017; Capuano et al., 2003). Most importantly, NHPs recapitulate the whole spectrum of human TB and show similar heterogeneity in disease outcomes and granuloma pathology at the individual level (Lin et al., 2009; Capuano et al., 2003). One of the biggest advantages of NHPs is their ability to develop a long-lasting latent infection, although only about 40% of the infected individuals develop latency while the rest acquire an active disease (Lin et al., 2009; Capuano et al., 2003).

**Table 1. TB vaccines currently in clinical trials**

Vaccine	Composition	Mode of immunization	Phase	Results from efficacy studies in humans, if available	ClinicalTrials.com identifier/Reference
<b>BCG</b>					
BCG revaccination (IAVI)	Live <i>M. bovis</i> BCG	Pre-exposure	I/IIa	45% efficacy in BCG-vaccinated healthy adults	NCT02075203; Nemes et al., 2018
<b>Recombinant mycobacterial vaccines</b>					
VPM1002 (SII, Max Planck, VPM, TBVI)	Recombinant BCG (urease-deficient, BCG-expressing listeriolysin from <i>Listeria monocytogenes</i> )	Pre-exposure	II/III	–	NCT03152903; Nieuwenhuizen et al., 2017; Grode et al., 2013
MTBVAC (Biofabri, University of Zaragoza, TBVI)	Recombinant Mtb (deficient in PhoP and FadD26)	Pre-exposure	IIa	–	NCT03536117, NCT02933281; Arbués et al., 2013; Marinova et al., 2017
AEC/BC02 (Anhui Zhifei Longcom Biopharmaceutical)	A recombinant vaccine based on Mtb antigen Ag85B and ESAT-6–CFP-10 fusion protein	Pre-exposure, postexposure	I	–	NCT04239313; Lu et al., 2015
<b>Inactivated whole-cell or fragmented vaccines</b>					
RUTI® (Archivel Pharma)	Detoxified, fragmented Mtb in liposomes	Therapeutic	IIa	–	NCT02711735; Cardona, 2006; Nell et al., 2014; Vilaplana et al., 2011
Vaccine™ (Anhui Zhifei Longcom Biopharmaceutical)	Heat-killed <i>M. vaccae</i>	Postexposure, therapeutic	III	Faster sputum smear conversion and radiographic improvement in TB patients	NCT01979900; Yang et al., 2011; Bourinbaïar et al., 2020
DAR-901 (University of Dartmouth, Aeras)	Heat-inactivated <i>M. obuense</i>	Pre-exposure, therapeutic	IIb	39% efficacy against pulmonary TB in previously BCG-vaccinated, HIV-infected adults	NCT02712424; von reyn et al., 2017, 2010
Immuvac (MIP) (Cadila Pharmaceuticals)	Heat-killed <i>M. indicus pranii</i>	Therapeutic	III	No statistically significant difference in sputum smear conversion of vaccinated and unvaccinated TB patients	NCT00341328; Sharma et al., 2017
<b>Adjuvanted protein subunit vaccines</b>					
M72/AS01E (GSK, IAVI)	A fusion protein combining Mtb 32A and Mtb 39A+AS01 adjuvant	Postexposure	IIb	49.7% efficacy in adults infected with Mtb	NCT01755598; Van der Meeren et al., 2018; Tait et al., 2019
ID93+GLA-SE (IDRI)	A fusion protein combining four Mtb antigens (Rv2608, Rv3619, Rv3620, Rv1813)+GLA-SE adjuvant	Postexposure	I and IIa	–	NCT03722472, NCT03806686, NCT02465216; Penn-Nicholson et al., 2018
H56:IC31 (SSI, Valnevm Aeras)	ESAT-6, Ag85B, Rv2660c+IC31 adjuvant	Postexposure	IIb	–	NCT03512249; Suliman et al., 2019
GamTBvac (MoH Russia)	Ag85A, ESAT-6–CFP-10 with DBD+dextran-based adjuvant	Postexposure	II	–	NCT03878004; Tkachuk et al., 2017
<b>Viral vector vaccines</b>					
ChAdOx1.85A/MVA85A (aerosol/id/im) (University of Oxford, TBVI)	Modified vaccinia Ankara virus expressing Ag85A	Pre-exposure	I	–	NCT01954563; Manjaly Thomas et al., 2019
Ad5 Ag85A (McMaster University, CanSino)	Modified human adenovirus (Ad5) expressing Ag85A	Postexposure	I	–	NCT02337270; Jeyanathan et al., 2016
TB/FLU-04L (RIBSP)	Replicon-deficient influenza virus expressing Ag85A and ESAT-6	Postexposure	IIa	–	NCT02501421

Pre-exposure vaccine is given to people with no previous exposure to Mtb, whereas postexposure vaccine is given to people with latent Mtb infection. A therapeutic vaccine is used in conjunction with the drug therapy.  
 Ag85A/B, secreted antigen Ag85A/B; AS01, 3-O-desacyl-4'-monophosphoryl lipid A and the purified saponin, QS-21 in liposomes; CFP-10, 10 kDa culture filtrate antigen; DBD, dextran binding protein; ESAT-6, 6 kDa early secretory antigenic target; FadD26, long-chain fatty acid AMP ligase of Mtb; GLA-SE, toll-like receptor 4 agonist glucopyranosyl lipid adjuvant-stable emulsion; GSK, GlaxoSmithKline; IAVI, International AIDS Vaccine Initiative – a non-profit scientific research organization dedicated to addressing urgent, unmet global health challenges including HIV and tuberculosis; IC31, adjuvant combining an antibacterial peptide KLK5/5K1K and a TLR9 agonist, a synthetic ODNI1a, without a CpG motif; id, intradermal injection; IDRI, Infectious Disease Research Institute; im, intramuscular injection; MoH Russia, Ministry of Health of the Russian Federation; PhoP, possible two-component system response transcriptional positive regulator of Mtb; RIBSP, Research Institute for Biological Safety Problems; SSI, Serum Institute of India; TBVI, Tuberculosis Vaccine Initiative; VPM, Vakzine Projekt Management; –, not yet reported.

**Table 2. Overview of animal models in tuberculosis vaccine research**

	Non-human primates	Mouse	Rabbit	Guinea pig	Cattle	Zebrafish
Infecting mycobacterium species	Mtb	Mtb	Mtb, <i>M. bovis</i>	Mtb	<i>M. bovis</i>	<i>M. marinum</i>
Natural host to mycobacterial infection (yes/no)	Yes	No	No	No	Yes	Yes
Immune system	Innate and adaptive	Innate and adaptive	Innate and adaptive	Innate and adaptive	Innate and adaptive	Innate (<3 months), innate and adaptive (>3 months)
Infection route	Aerosol	Aerosol, intravenous	Aerosol	Aerosol	Aerosol	Larvae: caudal vein, duct of Cuvier, yolk sac; adults: intraperitoneal
Infection phases	Cleared, chronic, subclinical, latent, insipid, reactivation	Chronic, a latent infection model with antibiotic control	Chronic, latent, reactivation	Chronic	Chronic	High dose: chronic infection; low dose: latent infection with induced reactivation
Granulomas	Yes, most similar to human granulomas	Yes, but in most strains diffuse and different structure	Yes, necrotic and caseating	Yes, necrotic and caseating	Yes, necrotic and caseating	Yes, necrotic and caseating
Phase in vaccine development	Verification of the protective effect before clinical trials	Evaluation of immunogenicity and immune mechanisms	Not commonly used in vaccine studies, applied in study of cutaneous or meningeal TB	Evaluation of the protective effect	Human biomarker detection and postexposure vaccine development	Preclinical screening of novel vaccination and adjuvant targets
References	Foreman et al., 2017; Diedrich et al., 2010; Lin et al., 2009; Capuano et al., 2003	Cooper, 2014; Medina and North, 1998	Peng et al., 2015; Kesavan et al., 2009; Manabe et al., 2008; Manabe et al., 2003	Clark et al., 2014; Grover et al., 2012; Williams et al., 2009	Pesciaroli et al., 2014; Villarreal-Ramos et al., 2014; Cassidy, 2006; Buddle et al., 1999	Parikka et al., 2012; Swaim et al., 2006; Davis et al., 2002

Furthermore, reactivation of latent TB can be studied in a setting of simian immunodeficiency virus co-infection, thus mimicking the HIV-positive human population with a high TB risk (Diedrich et al., 2010). Although the advantages of NHPs as TB models are numerous, their use is limited due to notable economical and ethical constraints. In the preclinical evaluation of TB vaccine candidates, NHP models are invaluable for the verification of promising results obtained from smaller animals prior to progressing to clinical trials, but their use in early-phase examination is unjustified.

### Cattle

*M. bovis* is a close relative of Mtb and a natural pathogen of cattle and other domestic animals (Pesciaroli et al., 2014). Only a few bacilli can cause infection in susceptible hosts and the resulting disease is highly similar to human TB (Cassidy, 2006). As in humans, BCG confers partial protection against *M. bovis* in cattle and is used to prevent the disease and the associated economic losses in farmed animals and wildlife reservoirs (Villarreal-Ramos et al., 2014). For this reason, together with the similar immune responses observed in cattle and humans, cattle can be used in the development of TB vaccines. As other models, however, cattle have their drawbacks, including high costs and maintenance requirements, as well as the ethical and availability concerns of using these animals for research purposes.

### Mammalian models in preclinical evaluation of TB vaccines

Preclinical evaluation of several promising candidates is currently underway. Many of the novel strategies are based on recombinant BCGs (rBCG; Box 1) to improve the somewhat poor protection elicited by parenteral BCG. For example, an rBCG with a deletion in the Rv0198 gene locus encoding Zmp1 (see Box 2 for the full names of mycobacterial antigens discussed in this Review) has been evaluated in several animal models with encouraging results; compared to parenteral BCG, rBCGΔzmp1 conferred enhanced immunogenicity in mice and cattle and improved protection in guinea pigs (Sander et al., 2015; Khatri et al., 2014; Johansen et al., 2011). Another rBCG expressing the ESX-1 secretion system from *M. marinum* (BCG::ESX-1<sup>Mmar</sup>) showed improved protection compared to BCG in mice, as subcutaneous immunization decreased Mtb loads in the spleen and lungs after aerosol Mtb infection (Gröschel et al., 2017). Similarly, a recombinant BCG expressing the *Escherichia coli* heat-labile enterotoxin was more effective at reducing the bacterial burden in the murine lung compared to BCG (Nascimento et al., 2017).

Animal studies have also provided important insights into the relevant delivery routes of TB vaccines. The standard routes of immunization in mammals and humans include intradermal and intramuscular, which aim to elicit systemic immune responses (Stylianou et al., 2019; Dijkman et al., 2019; Brandt et al., 2004; Skeiky et al., 2004). However, in 1973, a study in NHPs showed that

### Box 2. Mycobacterial genes and proteins mentioned in the text

**Ag85:** secreted antigen Ag85  
**Cdh:** CDP-diacylglycerol pyrophosphatase  
**CFP-10:** 10 kDa culture filtrate antigen CFP-10  
**ESAT-6:** 6 kDa early secretory antigenic target  
**ESX-1:** 6 kDa early secretory antigenic target secretion system  
**MMAR\_4110:** an aldehyde dehydrogenase of *M. marinum*  
**MMAR\_4207:** conserved hypothetical membrane protein of *M. marinum*  
**PE\_31:** a PE protein family member possessing the characteristic N-terminal PE domain  
**PE5\_1:** a PE protein family member possessing the characteristic N-terminal PE domain  
**PPE15:** a PPE protein family member possessing the characteristic N-terminal PPE domain  
**RD4:** region of difference 4 locus  
**RpfB:** resuscitation-promoting factor B  
**RpfE:** resuscitation-promoting factor E  
**Rv1016c:** Mtb gene locus encoding a probable conserved lipoprotein LpqT  
**Zmp1:** probable zinc metalloproteinase

mucosal vaccination could enhance protection against airborne Mtb (Barclay et al., 1973). Since then, many other studies have supported the important role of mucosally activated lymphoid tissues (MALTs; Box 1) in protecting against a mycobacterial infection; for example, in mice (Aguilo et al., 2014) and in guinea pigs (Garcia-Contreras et al., 2008), although some also showed contradictory results, as in rhesus macaques (Sibley et al., 2016). The most recent evidence of the importance of a local immune response in clearing Mtb comes from an early-phase efficacy study in mice, which revealed that boosting BCG with ChadOx1.PPE15, a replication-deficient chimpanzee adenovirus expressing the Mtb antigen PPE15, elicited better protection against an aerosol Mtb infection in mice compared to BCG without a booster. However, the enhanced protection was only evident in mice immunized intranasally, but not in the intradermally vaccinated ones (Stylianou et al., 2018). Correspondingly, rhesus macaques immunized with BCG by endobronchial installation had reduced lung pathology and bacterial counts in the lungs compared to animals immunized with intradermal BCG (Dijkman et al., 2019). Interestingly, BCG vaccination of rhesus macaques through the intravenous route has been shown to elicit superior protection to intradermal or aerosol immunization (Darrah et al., 2020; Barclay et al., 1970). In a recent study by Darrah et al. (2020), intravenous immunization by BCG resulted in as much as a 100,000-fold reduction in thoracic Mtb count compared to intradermal administration, with six out of 10 animals having no detectable Mtb in any tissue analysed compared to 10/10 with signs of infection in the intradermal and aerosol immunization groups. Although perhaps not applicable as such in humans, these results – and the model used – provide important clues on the mechanisms of protection against TB.

The above-mentioned mammalian models are indispensable for the development of a novel TB vaccine, although, as noted, only NHPs can fully recapitulate the aspects of human TB. As we still have relatively limited data from clinical trials, the predictive value of each animal model has yet to be fully determined. Some TB vaccine candidates have shown good correlation between animal and human studies, as is the case for the candidate M72/AS01<sub>E</sub> (Tait et al., 2019; Van der Meeren et al., 2018), which was recently reported to confer 47.9% protection in otherwise healthy Mtb-infected adults in a phase IIb trial (Tait et al., 2019) (Table 1).

This success was preceded by preclinical safety and efficacy evaluation in mice, guinea pigs, rabbits and NHPs (Reed et al., 2009; Tsenova et al., 2006; Brandt et al., 2004; Skeiky et al., 2004). Particularly, the BCG prime-M72/AS01<sub>E</sub> boost strategy significantly reduced bacterial loads in the rabbit model of TB meningitis (Tsenova et al., 2006) and improved protection compared to BCG in cynomolgus macaques (Reed et al., 2009). However, in many cases, vaccines with an acceptable safety and efficacy profile in animals fail to show any effect in humans, as exemplified by the candidate MVA85A, a modified vaccinia Ankara virus expressing the Mtb antigen Ag85A (Tameris et al., 2013). At the preclinical stage, researchers observed improved protection elicited by MVA85A compared to BCG in mice and, as a booster for BCG, in guinea pigs and cattle (Vordermeier et al., 2009; Verreck et al., 2009; Williams et al., 2005; Goonetilleke et al., 2003). Conversely, although the BCG prime/MVA85A booster strategy in NHPs decreased the bacterial burden in the lungs, it did not improve protection compared to BCG (Verreck et al., 2009). The results from the MVA85A trial, although disappointing, evoked a conversation about the importance of not only the animal chosen, but also of optimizing the experimental setting – including Mtb strain, infection route, environmental factors – to better correspond to the situation in clinical trials (reviewed in detail in McShane and Williams, 2014).

As discussed in previous sections, no model is perfect, but they all serve specific purposes on the way to vaccine development from design to clinical translation (Table 2). As an alternative to mammalian models, several fish models, and particularly the zebrafish (*Danio rerio*), have recently been used in TB vaccine research. These vertebrate species provide practical, ethical and reliable models for conducting large-scale screens for TB vaccine antigens, as we discuss below.

### Zebrafish as a model for TB

The zebrafish model for TB was introduced in the early 21st century (Davis et al., 2002) and utilizes the natural relationship of the fish with *M. marinum*, a close relative of Mtb (Hashish et al., 2018; Tobin and Ramakrishnan, 2008). A *M. marinum* infection in zebrafish shares many similarities in its pathogenesis and host responses with human Mtb infection, including the whole spectrum of TB disease, as we discuss in the next section.

Zebrafish offer several advantages for modelling TB infections. They are easy to maintain and to modify genetically and can be used in large batches for experiments (Luukinen et al., 2018; Benard et al., 2012). Importantly, when using the fish pathogen *M. marinum* instead of Mtb, there is no need for biosafety considerations, which decreases the costs and space demands for the studies. Zebrafish embryos are optically transparent, and the availability of transgenic zebrafish lines with fluorescently labelled components of the immune system enable the observation of the responses of zebrafish embryos to a *M. marinum* infection in real time (Meijer and Spaink, 2011). However, zebrafish embryos fight a Mtb infection only with their innate immune system, and while this model provides an opportunity to study the function of the innate immune response (Box 1) without the influence of adaptive responses, it is not useful in vaccination studies. Adult zebrafish, on the other hand, possess a fully developed adaptive immune response (Box 1) and an inducible protective immunological memory against *M. marinum*, rendering them suitable for vaccine development (Swaim et al., 2006; Parikka et al., 2012; Oksanen et al., 2013).

The lack of lungs and lymph nodes in zebrafish is a considerable limitation and prevents the study of the disease pathology at these primary sites of human TB. Differences between the immune systems of zebrafish and humans, including different classes of

antibodies in fish (IgM, IgD and the fish-specific IgZ) and humans (IgG, IgM, IgD, IgE and IgA), the expansion of innate immune system-related genes due to the genome duplication in teleost fish, and the lack of basophilic neutrophils in zebrafish, might also affect disease pathology and host responses and, therefore, the immunogenicity and protective effect of a candidate vaccine (Stein et al., 2007; Danilova et al., 2005; Bennett et al., 2001). Additionally, although the natural relationship between fish and *M. marinum* is attractive for TB research, it must be noted that *M. marinum* and Mtb are not genetically identical and not all of the antigens are shared between the two species (Stinear et al., 2008; Tobin and Ramakrishnan et al., 2008). Finally, the limited availability of immunological reagents, such as zebrafish-specific antibodies, hampers the immunogenicity evaluation of novel vaccine candidates.

Next, we discuss the zebrafish as a model for developing a TB vaccine and summarize the latest advances achieved using this model. We begin by briefly discussing the related pathogenesis and host responses in *M. marinum* and Mtb infections.

### ***M. marinum* infection in zebrafish**

*M. marinum* represents the genetically closest relative of Mtb (Stinear et al., 2008; Tobin and Ramakrishnan, 2008) and is a common cause of TB disease in many ectotherms, such as frogs and fish (Hashish et al., 2018). The genes associated with *M. marinum* and Mtb pathogenesis are highly conserved and both pathogens exploit similar changes in genetic programmes to promote different disease stages in their hosts, again indicating related pathogenic mechanisms (Stinear et al., 2008; Tobin and Ramakrishnan, 2008). As a result, the chronic, progressive granulomatous infection caused by *M. marinum* is highly similar in its histopathology to human TB (Hashish et al., 2018). In adult zebrafish, depending on the bacterial dose, an experimental *M. marinum* infection can progress to an acute (high dose) or chronic (low dose) disease (Luukinen et al., 2018; Swaim et al., 2006; van der Sar et al., 2004; Prouty et al., 2003). *M. marinum* granulomas in zebrafish are well organized and histologically highly similar to Mtb granulomas, with their necrotic centres surrounded by infected macrophages, epithelial cells and infiltrating CD4<sup>+</sup> cells and neutrophils (Yoon et al., 2015; Yang et al., 2012; Swaim et al., 2006; van der Sar et al., 2004). Zebrafish granulomas also recapitulate other important aspects of human granulomas, including hypoxia and the influx and efflux of naïve and infected macrophages (Myllymäki et al., 2018a; Oehlers et al., 2015; Davis et al., 2002). Importantly, a low-dose *M. marinum* infection in zebrafish proceeds into the latent phase, characterized by necrotic and hypoxic granulomas with centrally organized bacteria and the ceasing of bacterial growth (Myllymäki et al., 2018a; Parikka et al., 2012). The reactivation of a latent *M. marinum* infection in zebrafish can be induced by  $\gamma$ -irradiation or by treatment with an immunosuppressive agent, leading to the disruption of granulomas and the loss of control of bacterial growth (Myllymäki et al., 2018a; Parikka et al., 2012).

The early events of the host-pathogen interaction, including bacterial recognition and phagocytosis by macrophages, bacterial evasion of phagolysosomal killing and granuloma formation are shared between Mtb and *M. marinum* (reviewed, for example, in Hodgkinson et al., 2019; Myllymäki et al., 2016), and have been extensively studied in zebrafish embryos. The adaptive immune response, however, is less explored in zebrafish. In humans, the adaptive immune response to Mtb is thought to be mainly mediated by the cellular immune response (Box 1). For their role in activating the antimicrobial mechanisms of macrophages, IFN $\gamma$ - and tumour

necrosis factor (TNF)-secreting CD4<sup>+</sup> cells of the Th1 (Box 1) subset have been shown to be the predominant cell type controlling Mtb in humans and animal models (Cooper et al., 1997, 1993; Flynn et al., 1993). Challenge studies in mutant zebrafish that lack active lymphocytes [*recombination-activating gene 1* (*rag1*)<sup>-/-</sup>] have confirmed that the control of an active and latent *M. marinum* infection depends on functional lymphocytes (Myllymäki et al., 2018a; Hammarén et al., 2014; Parikka et al., 2012; Swaim et al., 2006), and the presence of CD4<sup>+</sup> cells in granulomas suggests an important role for these cells in constraining a *M. marinum* infection (Yoon et al., 2015). In addition, an enhanced Th1 response was associated with reduced bacterial growth and improved survival in Interleukin 10 (Il10) mutant zebrafish with a progressive *M. marinum* infection (Harjula et al., 2018).

So far, most of the vaccine development has concentrated on evoking strong Th1 responses, although human and animal studies have provided conflicting data on the matter. When its efficacy as a booster for BCG was tested in phase IIb clinical trials, the vaccine candidate MVA85A induced long-lived CD4<sup>+</sup> cells expressing IFN $\gamma$ , TNF and IL2, but this response did not correlate with protection (Tameris et al., 2013). Correspondingly, no association between the elicited Th1 response and protection was seen in infants vaccinated with BCG (Kagina et al., 2010). In light of these and other studies, it is therefore evident that although a Th1/IFN $\gamma$  response is essential for protection against mycobacteria, other mechanisms, mediated by CD8<sup>+</sup>, unconventional and regulatory T cells, and by B cells, also play important roles (as reviewed in detail in Cardona and Cardona, 2019; Lu et al., 2016; Behar, 2013). It also means that correlates of protection, other than the currently used IFN $\gamma$ , would be valuable in the preclinical and clinical evaluation of TB vaccine candidates. A recent study in NHPs highlighted the role of the Th17 (Box 1) response, as the protection mediated by pulmonary immunization of rhesus macaques with BCG was associated with increased levels of Th17 cells but not with IFN $\gamma$  production (Dijkman et al., 2019). In addition, owing to the growing evidence from animal and human studies, there is now a consensus that the importance of the synergistic effect of Th1 and Th2 (Box 1), instead of Th1 alone, in fighting Mtb might be underappreciated and should not be neglected in the development of TB vaccines (Abebe, 2019; Loxton, 2019). Supporting this, an improved Th2 response was associated with better bacterial control during latency in the zebrafish-*M. marinum* model (Hammarén et al., 2014).

As noted above, a complete understanding of the mechanisms of protection in human Mtb infection is currently lacking, yet crucial for the development of novel vaccines. The zebrafish-*M. marinum* model, in which both the active and latent phase of the mycobacterial infection can be studied, provides a feasible platform. Along with the above-mentioned studies on the adaptive immune response to *M. marinum* infection, several transcriptome analyses of wild-type (Harjula et al., 2020; Ojanen et al., 2019; Hegedűs et al., 2009; van der Sar et al., 2009) and a hypersusceptible mutant (Harjula et al., 2020) zebrafish have also provided invaluable insights into host responses and the factors that affect host susceptibility to mycobacterial infection. In addition to providing insights into the protective mechanisms in human TB, adult zebrafish can also be used for the direct assessment of novel TB vaccine candidates and delivery methods, as we discuss next.

### **Modelling TB vaccine development in zebrafish**

As described above, a *M. marinum* infection in zebrafish recapitulates the disease spectrum of human TB, allowing the study of novel vaccines against both the primary infection and the

reactivation of a latent infection. In particular, large-scale screens to find the most immunogenic and protective Mtb antigen or antigen combination can be conducted in the ethical and cost-efficient zebrafish model. Zebrafish are usually vaccinated via intraperitoneal or intramuscular injections, although mucosal immunization by immersion can also be performed (Fig. 2) (Risalde et al., 2018; Oksanen et al., 2013). In the absence of biomarkers and reagents to assess the immunogenicity of candidate antigens in zebrafish, improved infection control reflected by survival rate, bacterial counts, number of granulomas and affected organs are used to evaluate the potential of vaccine candidates (Myllymäki et al., 2018a; Oksanen et al., 2013). Compared to inbred mice strains, zebrafish are genetically heterogeneous in laboratory conditions and the ability of individuals to resist *M. marinum* usually varies, but this can be overcome by using adequate group sizes based on power calculations (Balik-Meisner et al., 2018). Also, this recapitulates the natural diversity in the human population (Balik-Meisner et al., 2018; Churchill et al., 2004). The studies evaluating the efficacy of BCG and novel vaccine candidates in zebrafish are summarized in the following section and in Table 3.

### The protective effect of BCG in zebrafish

As the standard control for the preclinical and clinical evaluation of vaccine candidate efficacy, the effects of BCG against acute (high dose) or chronic (low dose) *M. marinum* infection have been tested in zebrafish. As with human Mtb, BCG does not prevent *M. marinum* infection but confers modest and variable protection in studied individuals (Su et al., 2019; Ru et al., 2017; Oksanen et al., 2016, 2013). This modest protection in zebrafish is likely to be associated with the poor replication of the BCG strain of *M. bovis* in zebrafish (Oksanen et al., 2016). Immunization with BCG improves the survival of zebrafish with acute *M. marinum* infection and lowers bacterial counts in chronic *M. marinum* infection (Oksanen et al., 2016, 2013). Importantly, this protective effect is enhanced by a booster DNA vaccination, which encodes the antigen combination Ag85B, ESAT-6 and RpfE (Oksanen et al., 2016). While we lack a complete understanding of the immunological mechanisms behind this protective effect in zebrafish, BCG is known to induce Th1-biased IL17-dependent responses in humans and in animal models (Gopal et al., 2012; Pitt et al., 2012; Umemura et al., 2007). Correspondingly, BCG injection in zebrafish induced the expression of several genes of the innate and adaptive immune responses, including those encoding IL17 and its receptor (Oksanen et al., 2016). The improved control of a *M. marinum* infection in vaccinated fish has also been associated with a restricted inflammatory response, mediated by TNF (Oksanen et al., 2016).

### Evaluating novel TB vaccine candidates in zebrafish

#### Recombinant BCG and other whole-cell vaccines

Vaccines based on live, recombinant or attenuated mycobacteria represent one of the most successful strategies for TB vaccine development, as demonstrated by their prevalence among the novel TB vaccine candidates at clinical trials (Table 1). These include the recombinant BCG VPM1002 (phase III) (Nieuwenhuizen et al., 2017) and recombinant Mtb MTBVAC (phase IIa) (Arbues et al., 2013), which improved the protective immune response compared to BCG in preclinical studies and are currently being evaluated in humans (Table 1). Complementing this success, zebrafish immunized with a BCG strain expressing genes of the virulence-associated region of difference (RD) 4 locus from Mtb had higher survival rates and lower bacterial counts in a chronic *M. marinum* infection compared to infected fish immunized with parental BCG

(Ru et al., 2017). Conversely, immunization of zebrafish with BCG expressing a mannosylated protein of Mtb encoded by the locus Rv1016c conferred decreased protection against *M. marinum* compared to immunization with parental BCG (Su et al., 2019). This poor protection compared to BCG was associated with impaired Th1 and Th17 responses and dendritic cell maturation in mice, suggesting that Rv1016c is an important Mtb virulence factor (Box 1) that allows escape from the host's immune system (Su et al., 2019).

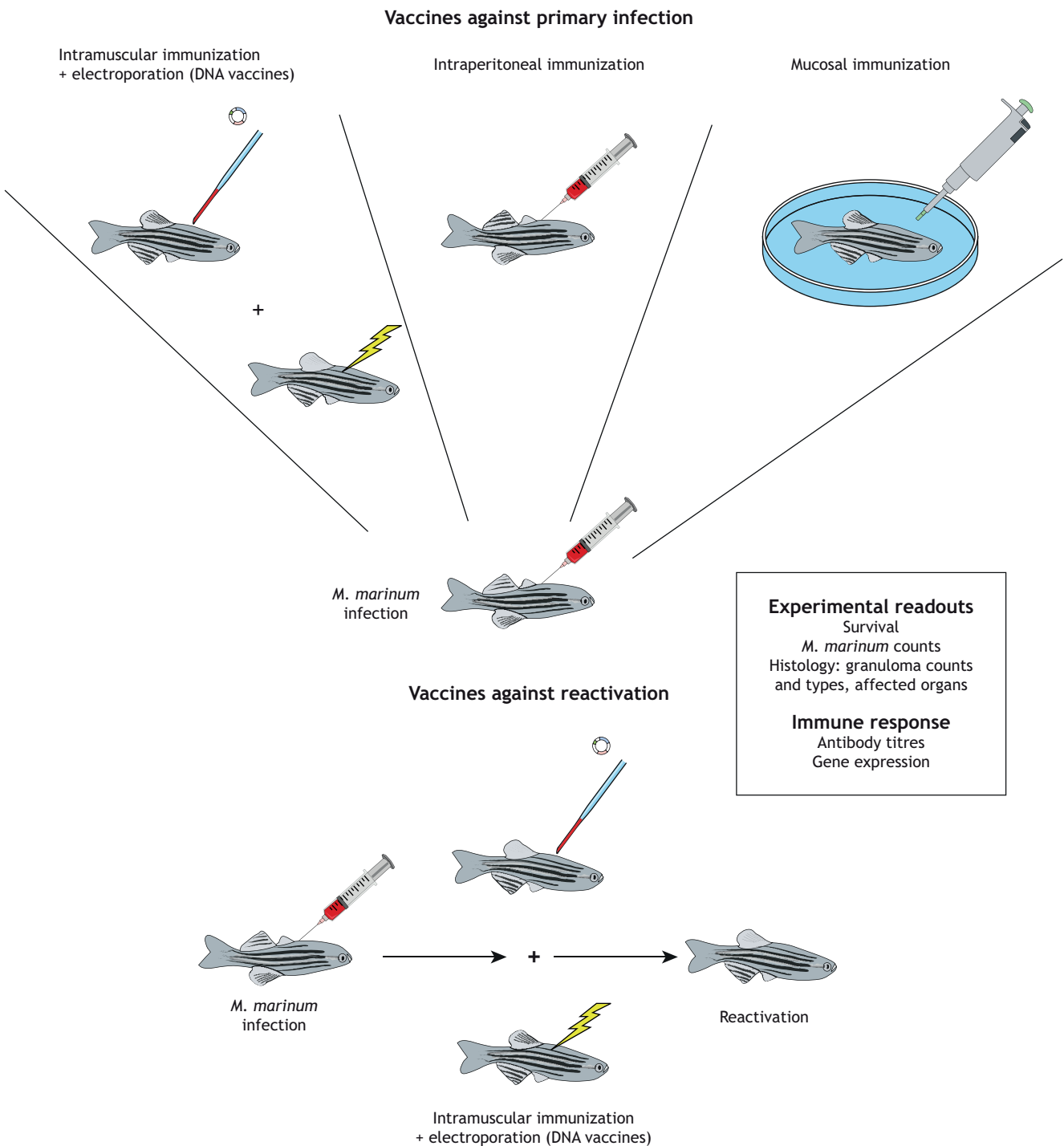
Along with recombinant BCG, other whole-cell vaccines, such as heat-killed *Mycobacterium vaccae* (Vaccae<sup>TM</sup>; Bourinbaier et al., 2020), *Mycobacterium obuense* (DAR-901; von reyn et al., 2017) or *Mycobacterium indicus pranii* (Immuvac; Sharma et al., 2017), or fragmented Mtb (RUTI; Nell et al., 2014), have advanced to clinical trials (Table 1). In zebrafish, heat-killed *M. bovis* and attenuated *M. marinum* elicit a protective immune response against chronic and acute *M. marinum* infection (López et al., 2018; Risalde et al., 2018; Cui et al., 2010). Interestingly, heat-killed *M. bovis* protected zebrafish against a *M. marinum* infection through mucosal vaccination by immersion, indicating that this clinically important vaccination route for a TB vaccine is also applicable to zebrafish (Risalde et al., 2018). In both systemic and mucosal immunization of zebrafish by heat-inactivated *M. bovis*, the protection was associated with the production of anti-*M. bovis* antibodies and with the increased expression of two genes related to the innate immune response, *interleukin 1b (il1b)* and *complement component 3a, duplicate 2 (c3a.2)*.

#### DNA vaccines

In addition to its poor efficiency, the use of BCG is also hampered by the risk of a disseminated infection in immunocompromised individuals, especially HIV-infected children (Hesseling et al., 2007; WHO, 2019), driving research into safer alternatives. Subunit vaccines, consisting, for example, of purified proteins or virus-like particles, or DNA vaccines (Box 1), are both safer than live vaccines and enable the introduction of multiple antigens to broaden the coverage and to direct the immune response to desired pathways (Hobernik and Bros, 2018; Suschak et al., 2017). Importantly, DNA vaccines also have less stringent storage requirements relative to other vaccine types, making them amenable for use in all parts of the world (Suschak et al., 2017). In the case of TB, DNA vaccines are also particularly useful in providing Mtb antigens that are expressed during different metabolic states and thus confer protection against different disease stages (Hobernik and Bros, 2018; Khademi et al., 2018). Owing to their potential, several DNA vaccines based on the most common Mtb virulence factors, including ESAT-6, Ag85 and PE/PPE family members (Box 2), combined with novel acute-phase or latency-associated antigens, have recently been tested in murine models (e.g. Liang et al., 2018, 2017; Tang et al., 2018). Although they have shown promise in their immunogenicity and protective effect in mice, no DNA vaccine against TB is currently in clinical trials. In fact, although DNA vaccines have been approved for veterinary use; for example, a horse vaccine against West Nile Virus and a salmon vaccine against the infectious hematopoietic necrosis virus (e.g. Collins et al., 2019), no DNA vaccine has yet been licensed for clinical use in humans (Collins et al., 2019; Hobernik and Bros, 2018).

Various combined DNA vaccines have been shown to protect zebrafish from acute and chronic *M. marinum* infections, including a DNA vaccine combining the Mtb antigens Ag85B, CFP-10 and ESAT-6 (Oksanen et al., 2013) and one that combined Ag85B, ESAT-6 and RpfE, which boosted the protective effect of BCG (Oksanen et al., 2016). To demonstrate the suitability of the





**Fig. 2. Vaccination routes and methods in zebrafish.** Zebrafish are usually infected by *M. marinum* via intraperitoneal injection or immersion. Three immunization routes (intramuscular, intraperitoneal and mucosal) are used in zebrafish to evaluate a vaccine's effect on the primary infection (upper panel). With DNA vaccines, the intramuscular injection of the expression construct is followed by electroporation of the target tissue. To assess a vaccine's effect on the reactivation of a latent *M. marinum* infection, zebrafish with a latent infection are vaccinated (using an intramuscular injection of a DNA vaccine followed by electroporation), after which the latent infection is activated by treatment with the immunosuppressant dexamethasone (lower panel). The figure also depicts the methods commonly used to evaluate the protective effect and the immunogenicity of the candidate vaccines in zebrafish.

zebrafish model for preclinical screening of novel TB vaccine antigens, Myllymäki et al. (2017) screened 15 *M. marinum* antigens in zebrafish for their protective efficacy against a primary *M. marinum* infection. These antigens have Mtb counterparts with immunogenic properties in mice and/or humans, and were used in a single dose to prime the immune response against a chronic or acute

*M. marinum* infection. The screen consisted of intramuscular injection of a plasmid encoding an antigen–GFP fusion protein with adequate antigen expression in the dorsal muscle. Expression of the PE5\_1, PE\_31, RpfE or Cdh antigens improved infection control in a chronic *M. marinum* infection. One antigen, RpfE, also improved the survival of zebrafish with acute infection.

Table 3. Vaccines against acute, chronic or reactivated latent *M. marinum* infections in zebrafish

Vaccines against an acute <i>M. marinum</i> infection						
First immunization Vaccine	Route	Second immunization Timepoint, vaccine	Control	High-dose infection Timepoint, Route	Effects of vaccination compared to control	Reference
BCG (Danish)	ip		PBS	strain 4 weeks, ATCC 927	71% higher survival	Oksanen et al., 2013
BCG (Danish)	im		PBS	4 weeks, ATCC 927	63% higher survival	Oksanen et al., 2013
pCMV-eGFP-Ag85B/CFP-10/ESAT-6	im+ep		pCMV-eGFP	3 weeks, ATCC 927	30% higher survival, 65% lower <i>M. marinum</i> counts	Oksanen et al., 2013
BCG (Danish)	ip		PBS	6 weeks, ATCC 927	39% higher survival	Oksanen et al., 2016
BCG (Danish)	im		PBS	6 weeks, ATCC 927	52% higher survival	Oksanen et al., 2016
BCG (Danish)	ip	4 weeks, pCMV-eGFP-Ag85B/ESAT-6/RpIF	pCMV-eGFP boosted	4 weeks, ATCC 927	50% higher survival	Oksanen et al., 2016
Attenuated <i>M. marinum</i> mutant L1D	ip	10 days, attenuated <i>M. marinum</i> mutant L1D	PBS	16 days, OSU-214	Increased survival	Cui et al., 2010
pCMV-eGFP-RpIF	im+ep		pCMV-eGFP	5 weeks, ATCC 927	60% higher survival	Myllymäki et al., 2017
Heat-killed <i>M. bovis</i> IV	ip	3 weeks, heat-killed <i>M. bovis</i> IV	PBS	2 weeks, ATCC 927	57% higher survival, decreased <i>M. marinum</i> counts, decreased number of <i>M. marinum</i> per granuloma, lower mean number of granulomas per fish, higher IgM titres against <i>M. bovis</i> P22, increased expression of <i>aktin2</i> , <i>c3a.2</i> and <i>il1b</i>	López et al., 2018
Heat-killed <i>M. bovis</i> IV	immersion	3 weeks, heat-killed <i>M. bovis</i> IV	PBS	2 weeks, ATCC 927	Decreased <i>M. marinum</i> counts, decreased number of <i>M. marinum</i> per granuloma, decreased number of granulomas per animal, higher IgM titres against <i>M. bovis</i> P22, increased expression of <i>c3a.2</i> and <i>il1b</i>	Risalde et al., 2018
Vaccines against a chronic <i>M. marinum</i> infection						
First immunization Vaccine	Route	Second immunization Timepoint, vaccine	Control	Low-dose infection Timepoint, Route	Effects of vaccination compared to control	Reference
pCMV-eGFP-Ag85B/CFP-10/ESAT-6	im+ep		pCMV-eGFP	strain 3 weeks, ATCC 927	66% fewer granulomas per animal, 38% lower number of affected organs per animal	Oksanen et al., 2013
BCG (Danish)	ip		PBS	6 weeks, ATCC 927	83% lower <i>M. marinum</i> counts	Oksanen et al., 2016
pCMV-eGFP-RpIF	im+ep		pCMV-eGFP	4 weeks, ATCC 927	88% lower <i>M. marinum</i> counts	Myllymäki et al., 2017
pCMV-eGFP-PE5_1	im+ep		pCMV-eGFP	4 weeks, ATCC 927	56% lower <i>M. marinum</i> counts	Myllymäki et al., 2017
pCMV-eGFP-PE_31	im+ep		pCMV-eGFP	4 weeks, ATCC 927	50% lower <i>M. marinum</i> counts	Myllymäki et al., 2017
pCMV-eGFP-Cdh	im+ep		pCMV-eGFP	4 weeks, ATCC 927	62% lower <i>M. marinum</i> counts	Myllymäki et al., 2017
rBCG(China): Rv1501-1508c	ip		BCG (China)	30 days, 535	1.73 log10 lower <i>M. marinum</i> counts, longer survival	Ru et al., 2017
rBCG(China): Rv1501-1516c	ip		BCG (China)	30 days, 535	2.25 log10 lower <i>M. marinum</i> counts, longer survival	Ru et al., 2017
Heat-killed <i>M. bovis</i> IV	ip	3 weeks, heat-killed <i>M. bovis</i> IV	PBS	2 weeks, ATCC 927	Decreased <i>M. marinum</i> loads, decreased number of <i>M. marinum</i> per granuloma, higher IgM titres against <i>M. bovis</i> P22, increased expression of <i>il1b</i>	López et al., 2018

Vaccines against the reactivation of a latent *M. marinum* infection

Low-dose infection Strain	Route	Vaccination Timepoint, vaccine	Route	Control	Reactivation (timepoint)	Effects of vaccination compared to control	Reference
ATCC 927	ip	5 weeks, pCMV-eGFP-MMAR_4110	im+ep	pCMV-eGFP	5 weeks	65% lower <i>M. marinum</i> counts	Niskanen et al., 2020
ATCC 927	ip	5 weeks, pCMV-eGFP-Ag85-ESAT6	im+ep	pCMV-eGFP	5 weeks	56% lower <i>M. marinum</i> counts	Myllymäki et al., 2018a,b
ATCC 927	ip	5 weeks, pCMV-eGFP-RpFB	im+ep	pCMV-eGFP	5 weeks	63% lower <i>M. marinum</i> counts	Myllymäki et al., 2018a,b
ATCC 927	ip	5 weeks, pCMV-eGFP-MMAR_4207	im+ep	pCMV-eGFP	5 weeks	85% lower <i>M. marinum</i> counts	Myllymäki et al., 2018a,b

Zebrafish studies of BCG or candidate TB vaccines, with information on administration routes, immunization schedules, the *M. marinum* strain used for infection and the effects of the vaccination on disease outcome are summarized. Only vaccines that produced a protective effect in zebrafish are included. The timepoints indicate the time interval between the designated and the previous treatment. Ag85B, secreted antigen Ag85B; Cdh, CDP-diacylglycerol pyrophosphatase; CFP-10, 10 kDa culture filtrate antigen CFP-10; ESAT-6, 6 kDa early secretory antigenic target; ep, intraperitoneal injection; im, intramuscular injection; ip, intraperitoneal injection; PBS, phosphate buffered saline; PE5\_1, a PE protein family member possessing the characteristic N-terminal PE domain; PE\_31, a PE protein family member possessing the characteristic N-terminal PE domain; RpFB, resuscitation-promoting factor B; RpFE, resuscitation-promoting factor E.

Owing to the high burden of latent TB, a vaccine that offers protection against reactivation would have a major impact. However, in the absence of a proper model for latent and reactivating Mtb infection, the studies that focus on these disease stages, and on the development of a vaccine that targets reactivation, have been limited. The zebrafish offers hope in this regard, with its naturally developing disease latency and spontaneous reactivation of a mycobacterial infection. To identify protective antigens against reactivation, 15 *M. marinum* antigens were screened in zebrafish for their protective effect against reactivation of a latent *M. marinum* infection (Myllymäki et al., 2018a). Fish with a latent infection were injected with a DNA vaccine expressing selected antigens prior to *M. marinum* reactivation by dexamethasone treatment. This screen revealed that two antigen candidates, RpFB and MMAR\_4207, and the antigen combination of Ag85 and ESAT-6, confer partial protection against reactivation (Myllymäki et al., 2018a). Thus, this study identified two novel antigen candidates and provided support for the use of zebrafish to investigate TB vaccines that prevent reactivation of a latent infection. In a recent study by Niskanen et al. (2020), an *in vitro* model for *M. marinum* latency and reactivation was used to identify mycobacterial genes specifically expressed during reactivation. When seven of these were tested as vaccine antigens, MMAR\_4110 was shown to be protective against the reactivation of *M. marinum* infection in zebrafish (Niskanen et al., 2020). Although MMAR\_4110, an aldehyde dehydrogenase, does not have a clear homologue in Mtb, the results suggest that the alcohol hydrogenases of Mtb could be potential target antigens for vaccine development.

The greatest disadvantage of DNA vaccines is their relatively poor immunogenicity in humans, and although their safety has been proven in several human trials (Hobernik and Bros, 2018), they harbour a risk for stable integration of exogenous DNA or inflammatory and autoimmune reactions due to long-term expression of antigens (Hobernik and Bros, 2018). To overcome the poor immunogenicity of DNA vaccines in humans, several strategies – including enhanced delivery, inclusion of molecular adjuvants and improved vectors – are constantly being explored (Suschak et al., 2017). Next, we discuss how zebrafish can help to advance one of these strategies, the development of delivery methods for DNA vaccines.

### Zebrafish as a model for vaccine delivery

The limited success of DNA vaccines in clinical trials has been linked to the poor expression of vaccine-encoded antigens and insufficient antigen presentation, which, in turn, are at least partly caused by the inefficient delivery of the vaccine to the recipient cells. Nanomedicine is one possible solution for improving DNA delivery. In addition to vaccines, nanobiotics have been suggested to improve delivery of therapeutics against TB (Batalha et al., 2019).

Delivery vehicles investigated in zebrafish include single-walled carbon nanotubes (SWCNTs), nanoliposomes and biocompatible non-toxic approved materials, such as polymer-based nanoparticles [e.g. poly(lactic acid) (PLA) or poly(lactic-co-glycolic acid) (PLGA)] and other polymers (Zhang et al., 2020; Crecente-Campo et al., 2019; Ji et al., 2019; Guo et al., 2018; Lovmo et al., 2017; Resseguier et al., 2017; Ruyra et al., 2014). Studies in both adult and larval zebrafish have demonstrated that nanoparticles can cross the tissue epithelium and be taken up by antigen-presenting cells (Zhang et al., 2020; Resseguier et al., 2017) and other leukocytes (Lovmo et al., 2017). Crecente-Campo et al. (2019), in turn, have investigated the effect of particle size on phagocytosis and antigen presentation by macrophages. They found

**Table 4. Vaccines against mycobacteriosis in fish**

Species	Immunization			Infection			Protective effect	Reference
	Vaccine	Route	Bacterial species, strain	Route	Route			
Sea bass ( <i>Dicentrarchus labrax</i> )	Heat-killed <i>M. marinum</i> <i>ipA::kan</i> mutant (deficient in <i>ipA</i> ) with (and without) 70% (W/W) ISA 760 VG SEPPIC, Montanide™ adjuvant	ip	<i>M. marinum</i> , Eliat WT	ip	Increased survival of vaccinated and boosted fish (with an adjuvant, 85%; without an adjuvant, 91%) compared to the control group injected with saline (with an adjuvant, 70%; without an adjuvant, 71%)	Ravid-Peretz et al., 2019		
Sea bass ( <i>Dicentrarchus labrax</i> )	Heat-killed <i>M. marinum</i> <i>ipA::kan</i> mutant	ip	<i>M. marinum</i> , Eliat WT	ip	Increased survival of vaccinated fish (75%) compared to the control group injected with saline (<20%)	Ziklo et al., 2018		
Amberjack ( <i>Seriola dumerili</i> )	BCG	im	<i>Mycobacterium</i> sp., TUMSAT-Msp001	im	Increased survival of vaccinated fish (68%) compared to the control group injected with PBS (2%)	Kato et al., 2011		
Amberjack ( <i>Seriola dumerili</i> )	BCG	im	<i>Mycobacterium</i> sp., TUMSAT-Msp001	immersion	Increased survival of vaccinated fish (59%) compared to the control group injected with PBS (18%)	Kato et al., 2011		
Amberjack ( <i>Seriola dumerili</i> )	BCG	ip	<i>Mycobacterium</i> sp., TUMSAT-Msp001	im	Increased survival of vaccinated fish (40%) compared to the control group injected with PBS (2%)	Kato et al., 2011		
Japanese flounder ( <i>Paralichthys olivaceus</i> )	BCG	im	<i>Mycobacterium</i> sp., TUMSAT-Msp001	im	Increased survival in vaccinated group (31%) compared to the control group injected with PBS (0%)	Kato et al., 2010		
Japanese flounder ( <i>Paralichthys olivaceus</i> )	Formalin-killed <i>Mycobacterium</i> sp.	im	<i>Mycobacterium</i> sp., TUMSAT-Msp001	im	No protective effect	Kato et al., 2010		
Striped bass ( <i>Morone saxatilis</i> )	Recombinant <i>Brucella abortus</i> encoding Ag85A from <i>M. bovis</i> BCG	ip	<i>M. marinum</i> , ATCC 972	im	No protective effect compared	Pasnik et al., 2003		
Juvenile hybrid striped bass ( <i>Morone saxatilis</i> × <i>M. chrysops</i> )	pCMV-85A, a pcDNA3.1 plasmid encoding Ag85A from <i>M. marinum</i>	im	<i>M. marinum</i> , ATCC 972	im	Increased survival of vaccinated fish (25 µg of DNA, 80%; 50 µg of DNA, 90%) compared to the control group injected with an empty vector (0%)	Pasnik and Smith, 2005		
Juvenile hybrid striped bass ( <i>Morone saxatilis</i> × <i>M. chrysops</i> )	pCMV-85A, a pcDNA3.1 plasmid encoding Ag85A from <i>M. marinum</i>	ip	<i>M. marinum</i> , ATCC 972	im	Increased survival of vaccinated fish (25 µg of DNA, 20%) compared to the control group injected with an empty vector (0%)	Pasnik and Smith, 2005		
Juvenile hybrid striped bass ( <i>Morone saxatilis</i> × <i>M. chrysops</i> )	pCMV-85A, a pcDNA3.1 plasmid encoding Ag85A from <i>M. marinum</i>	im	<i>M. marinum</i> , ATCC 972	im	Increased mean survival time of vaccinated fish compared to control group injected with an empty vector. Survival at day 28 postchallenge was 91%, 88% and 0% for fish injected with 25 µg or 50 µg of DNA or empty vector, respectively. 0% survival in all groups at the end of the experiment.	Pasnik and Smith, 2006		

Ag85A, secreted antigen Ag85A; im, intramuscular; ipA, mycobacterial invasion and intracellular persistence protein; ip, intraperitoneal; PBS, phosphate buffered saline; WT, wild type.

that smaller particles are more efficiently phagocytosed by macrophages (Crecente-Campo et al., 2019).

In larval zebrafish, lipopolysaccharide (LPS)- and polyinosinic: polycytidylic acid [poly(I:C)]-loaded nanoliposomes protected them from a sublethal challenge with *Aeromonas hydrophila* (Ji et al., 2019). Similarly, SWCNT-loaded recombinant proteins protected zebrafish against an *A. hydrophila* challenge, delivered both by bath immunization and by intraperitoneal injection (Guo et al., 2018). A similar study has been performed with nanoliposome-coated LPS and poly(I:C) against a spring viremia carp virus infection and *Pseudomonas aeruginosa* in adult zebrafish (Ruyra et al., 2014). The authors also discovered that lyophilised nanoliposomes provided comparable protection to the freshly prepared liposomes, further improving their usefulness (Ruyra, et al., 2014).

### Fish vaccines against mycobacterial infection

Besides zebrafish, several fish species have been used for modelling mycobacterial infections, as they are natural hosts to an aquatic mycobacterium. Armed with both innate and adaptive immunities, they also share key features of immune responses with humans (Rauta et al., 2012). Many of the studies have been designed with aquaculture in mind, but some information is also valid for human TB. However, vaccination and exposure strategies, such as balneation, that are commonly applied in aquacultural research are hardly applicable to mammals or humans. The most common models of a mycobacterial infection are the above-discussed zebrafish (Davis et al., 2002), medaka (Broussard and Ennis, 2007) and goldfish (Talaat et al., 1998), as these are aquarium sized and some unique genetic tools are available for each species. However, to our knowledge, medaka and goldfish have not been used for vaccination studies against mycobacterial species. Still, vaccination against mycobacteriosis has been studied in many other species important for commercial aquaculture, such as sea bass (*Dicentrarchus labrax*) (Ravid-Peretz et al., 2019; Ziklo et al., 2018), Japanese flounder (*Paralichthys olivaceus*) (Kato et al., 2010) and amberjack (*Seriola dumerili*) (Kato et al., 2011), and key results from vaccination experiments are displayed in Table 4. Notably, attenuated and killed mycobacteria (Ravid-Peretz et al., 2019; Ziklo et al., 2018), as well as BCG (Kato et al., 2011, 2010) and a DNA vaccine encoding Ag85A (Pasnik and Smith, 2005), have been shown to protect fish from mycobacteriosis. Studying the protective immune responses in these fish species could provide clues about the mammalian response to Mtb and help improve vaccine development efforts.

### Conclusions

As the role of the zebrafish in TB vaccine development begins to unfold, the relevance of the discoveries made in fish for humans remains to be determined. Despite the anatomical, genetic and physiological differences between zebrafish and humans, they appear to respond to the BCG vaccine and to antimicrobials that target mycobacteria in a similar way. These similarities, together with the novel insights into TB pathology gained from zebrafish, reflect the potential of this model for vaccine research. Compared to mammals, zebrafish are superior for large-scale screens, which allow the efficient discovery of protective antigens, antigen combinations and adjuvants to aid TB vaccine development. Furthermore, although not widely studied, fish also provide an attractive model for mucosal vaccine delivery. Mucosal administration through inhalation or oral delivery in humans does not require medical expertise or sterility, unlike invasive immunization methods, making it attractive for large-

scale vaccination campaigns (Stylianou et al., 2018). Although vaccination routes in fish are typically intramuscular or intraperitoneal, immersion- or oral-based vaccination strategies could provide important information on the permeability of compounds to MALTs. Although there is evidence of the existence of the counterparts for the mammalian antigen-presenting mucosal cells (microfold cells) in Atlantic salmon (Fuglem et al., 2010), carp (Rombout et al., 1985) and zebrafish (Brugman, 2016), the function of these cells needs further characterization to establish the similarities and differences between mammals and fish and to validate these fish species as appropriate models of mammalian MALTs.

Although the availability of zebrafish-specific antibodies is continuously increasing, the persistent scarcity of immunological reagents still forces most of the early-stage immunogenicity studies to be performed in mice, a model that only partially recapitulates human TB and requires significantly more resources. For the same reason, the potential of zebrafish as a model to reveal the yet unknown protective mechanisms against TB cannot be fully harvested. While it is unlikely that zebrafish or other fish models will replace mammalian models in preclinical evaluation of novel vaccine candidates, they do provide a useful model for efficient and cost-effective early-phase screening of novel vaccine candidates and, in this way, minimize the time, costs and number of higher vertebrates needed on the long path to a vaccine.

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### Competing interests

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### References

- Abebe, F. (2019). Synergy between Th1 and Th2 responses during *Mycobacterium tuberculosis* infection: A review of current understanding. *Int. Rev. Immunol.* **38**, 172-179. doi:10.1080/08830185.2019.1632842
- Aguilo, N., Toledo, A. M., Lopez-Roman, E. M., Perez-Herran, E., Gormley, E., Rullas-Trincado, J., Angulo-Barturen, I. and Martin, C. (2014). Pulmonary *Mycobacterium bovis* BCG vaccination confers dose-dependent superior protection compared to that of subcutaneous vaccination. *Clin. Vaccine Immunol.* **21**, 594-597. doi:10.1128/0014-0139.112.00700-13
- Arbues, A., Aguilo, J. I., Gonzalo-Asensio, J., Marinova, D., Uranga, S., Puentes, E., Fernandez, C., Parra, A., Cardona, P. J., Vilaplana, C. et al. (2013). Construction, characterization and preclinical evaluation of MTBVAC, the first live-attenuated *M. tuberculosis*-based vaccine to enter clinical trials. *Vaccine* **31**, 4867-4873. doi:10.1016/j.vaccine.2013.07.051
- Aronson, N. E., Santosham, M., Comstock, G. W., Howard, R. S., Moulton, L. H., Rhoades, E. R. and Harrison, L. H. (2004). Long-term efficacy of BCG vaccine in American Indians and Alaska Natives: A 60-year follow-up study. *JAMA* **291**, 2086-2091. doi:10.1001/jama.291.17.2086
- Balik-Meisner, M., Truong, L., Scholl, E. H., Tanguay, R. L. and Reif, D. M. (2018). Population genetic diversity in zebrafish lines. *Mamm. Genome* **29**, 90-100. doi:10.1007/s00335-018-9735-x
- Barclay, W. R., Anacker, R. L., Brehmer, W., Leif, W. and Ribi, E. (1970). Aerosol-induced tuberculosis in subhuman primates and the course of the disease after intravenous BCG vaccination. *Infect. Immun.* **2**, 574-582. doi:10.1128/IAI.2.5.574-582.1970
- Barclay, W. R., Busey, W. M., Dalgard, D. W., Good, R. C., Janicki, B. W., Kasik, J. E., Ribi, E., Ulric, C. E. and Wolinsky, E. (1973). Protection of monkeys against airborne tuberculosis by aerosol vaccination with bacillus calmette-guerin. *Am. Rev. Respir. Dis.* **107**, 351-358.
- Batalha, I. L., Bernut, A., Schiebler, M., Ouberai, M. M., Passemar, C., Klapholz, C., Kinna, S., Michel, S., Sader, K., Castro-Hartmann, P. et al. (2019). Polymeric nanobiotics as a novel treatment for mycobacterial infections. *J. Control. Release* **314**, 116-124. doi:10.1016/j.jconrel.2019.10.009

- Behar, S. M. (2013). Antigen-specific CD8(+) T cells and protective immunity to tuberculosis. *Adv. Exp. Med. Biol.* **783**, 141-163. doi:10.1007/978-1-4614-6111-1\_8
- Behr, M. A., Edelstein, P. H. and Ramakrishnan, L. (2018). Revisiting the timetable of tuberculosis. *BMJ* **362**, k2738. doi:10.1136/bmj.k2738
- Benard, E. L., van der Sar, A. M., Ellett, F., Lieschke, G. J., Spaink, H. P. and Meijer, A. H. (2012). Infection of zebrafish embryos with intracellular bacterial pathogens. *J. Vis. Exp.* **61**, 3781. doi:10.3791/3781
- Bennett, C. M., Kanki, J. P., Rhodes, J., Liu, T. X., Paw, B. H., Kieran, M. W., Langenau, D. M., Delahaye-Brown, A., Zon, L. I., Fleming, M. D. et al. (2001). Myelopoiesis in the zebrafish. *Blood* **98**, 643-651. doi:10.1182/blood.V98.3.643
- Bourinbaïar, A. S., Batbold, U., Efrementko, Y., Sanjagdorj, M., Butov, D., Damdinpurev, N., Grinshina, E., Mijidodorj, O., Kovolev, M., Baasanjav, K. et al. (2020). Phase III, placebo-controlled, randomized, double-blind trial of tableted, therapeutic TB vaccine (V7) containing heat-killed *M. vaccae* administered daily for one month. *J. Clin. Tuberc.* **18**, 100141. doi:10.1016/j.jctube.2019.100141
- Brandt, L., Skeiky, Y. A., Alderson, M. R., Lobet, Y., Dalemans, W., Turner, O. C., Basaraba, R. J., Izzo, A. A., Lasco, T. M., Chapman, P. L. et al. (2004). The protective effect of the *Mycobacterium bovis* BCG vaccine is increased by coadministration with the *Mycobacterium tuberculosis* 72-kilodalton fusion polyprotein Mtb72F in *M. tuberculosis*-infected guinea pigs. *Infect. Immun.* **72**, 6622-6632. doi:10.1128/IAI.72.11.6622-6632.2004
- Broussard, G. W. and Ennis, D. G. (2007). *Mycobacterium marinum* produces long-term chronic infections in medaka: a new animal model for studying human tuberculosis. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **145**, 45-54. doi:10.1016/j.cbpc.2006.07.012
- Brugman, S. (2016). The zebrafish as a model to study intestinal inflammation. *Dev. Comp. Immunol.* **64**, 82-92. doi:10.1016/j.dci.2016.02.020
- Buddle, B. M., Parlane, N. A., Keen, D. L., Aldwell, F. E., Pollock, J. M., Lightbody, K. and Andersen, P. (1999). Differentiation between *Mycobacterium bovis* BCG-vaccinated and *M. bovis*-infected cattle by using recombinant mycobacterial antigens. *Clin. Diagn. Lab. Immunol.* **6**, 1-5. doi:10.1128/CDLI.6.1.1-5.1999
- Cadena, A. M., Fortune, S. M. and Flynn, J. L. (2017). Heterogeneity in tuberculosis. *Nat. Rev. Immunol.* **2017**, 691-702. doi:10.1038/nri.2017.69
- Capuano, S. V., Croix, D. A., Pawar, S., Zinovik, A., Myers, A., Lin, P. L., Bissel, S., Fuhrman, C., Klein, E. and Flynn, J. L. (2003). Experimental *Mycobacterium tuberculosis* infection of cynomolgus macaques closely resembles the various manifestations of human *M. tuberculosis* infection. *Infect. Immun.* **71**, 5831-5844. doi:10.1128/IAI.71.10.5831-5844.2003
- Cardona, P.-J. and Cardona, P. J. (2019). Regulatory T Cells in *Mycobacterium tuberculosis* Infection. *Front. Immunol.* **10**, 2139. doi:10.3389/fimmu.2019.02139
- Cardona, P.-J. (2006). RUTI: a new chance to shorten the treatment of latent tuberculosis infection. *Tuberculosis* **86**, 273-289. doi:10.1016/j.tube.2006.01.024
- Cardona, P.-J. and Williams, A. (2017). Experimental animal modelling for TB vaccine development. *Int. J. Infect. Dis.* **56**, 268-273. doi:10.1016/j.ijid.2017.01.030
- Cassidy, J.-P. (2006). The pathogenesis and pathology of bovine tuberculosis with insights from studies of tuberculosis in humans and laboratory animal models. *Vet. Microbiol.* **112**, 151-161. doi:10.1016/j.vetmic.2005.11.031
- Churchill, G. A., Airey, D. C., Allayee, H., Angel, J. M., Attie, A. D., Beatty, J., Beavis, W. D., Belknap, J. K., Bennett, B., Berrettini, W. et al. (2004). The Collaborative Cross, a community resource for the genetic analysis of complex traits. *Nat. Genet.* **36**, 1133-1137. doi:10.1038/ng1104-1133
- Clark, S., Hall, Y. and Williams, A. (2014). Animal models of tuberculosis: guinea pigs. *CSH Perspect Med.* **5**, a018572. doi:10.1101/cshperspect.a018572
- Collins, C., Lorenzen, N. and Collet, B. (2019). DNA vaccination for finfish aquaculture. *Fish Shellfish Immunol.* **85**, 106-125. doi:10.1016/j.fsi.2018.07.012
- Cooper, A. M. (2014). Mouse model of tuberculosis. *Cold Spring Harb. Perspect Med.* **5**, a018556. doi:10.1101/cshperspect.a018556
- Cooper, A. M., Magram, J., Ferrante, J. and Orme, I. M. (1997). Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with *Mycobacterium tuberculosis*. *J. Exp. Med.* **186**, 39-45. doi:10.1084/jem.186.1.39
- Cooper, A. M., Dalton, D. K., Stewart, T. A., Griffin, J. P., Russell, D. G. and Orme, I. M. (1993). Disseminated tuberculosis in interferon gamma gene-disrupted mice. *J. Exp. Med.* **178**, 2243-2247. doi:10.1084/jem.178.6.2243
- Crecente-Campo, J., Guerra-Varela, J., Peleteiro, M., Gutiérrez-Lovera, C., Fernández-Mariño, I., Diéguez-Docampo, A., González-Fernández, Á., Sánchez, L. and Jos, é Alonso, M. (2019). The size and composition of polymeric nanocapsules dictate their interaction with macrophages and biodistribution in zebrafish. *J. Control. Release* **308**, 98-108. doi:10.1016/j.jconrel.2019.07.011
- Cui, Z., Samuel-Shaker, D., Watral, V. and Kent, M. L. (2010). Attenuated *Mycobacterium marinum* protects zebrafish against mycobacteriosis. *J. Fish Dis.* **33**, 371-375. doi:10.1111/j.1365-2761.2009.01115.x
- Daniilova, N., Bussmann, J., Jekosch, K. and Steiner, L. A. (2005). The immunoglobulin heavy-chain locus in zebrafish: identification and expression of a previously unknown isotype, immunoglobulin Z. *Nat. Immunol.* **6**, 295-302. doi:10.1038/nri1166
- Darrah, P. A., Zeppa, J. J., Maiello, P., Hackney, J. A., Wadsworth, M. H., Hughes, T. K., Pokkali, S., Swanson, P. A., Il, Grant, N. L., Rodgers, M. A. et al. (2020). Prevention of tuberculosis in macaques after intravenous BCG immunization. *Nature* **577**, 95-102. doi:10.1038/s41586-019-1817-8
- Davis, J. M., Clay, H., Lewis, J. L., Ghori, N., Herbolme, P. and Ramakrishnan, L. (2002). Real-time visualization of mycobacterium-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. *Immunity* **17**, 693-702. doi:10.1016/S1074-7613(02)00475-2
- Diedrich, C. R., Mattila, J. T., Klein, E., Janssen, C., Phuah, J., Sturgeon, T. J., Montelaro, R. C., Lin, P. L. and Flynn, J. L. (2010). Reactivation of latent tuberculosis in cynomolgus macaques infected with SIV is associated with early peripheral T cell depletion and not virus load. *PLoS ONE* **5**, e9611. doi:10.1371/journal.pone.0009611
- Dijkman, K., Sombroek, C. C., Vervenne, R., Hofman, S. O., Boot, C., Remarque, E. J., Kocken, C., Ottenhoff, T., Kondova, I., Khayum, M. A. et al. (2019). Prevention of tuberculosis infection and disease by local BCG in repeatedly exposed rhesus macaques. *Nat. Med.* **25**, 255-262. doi:10.1038/s41591-018-0319-9
- Dockrell, H. M. and Smith, S. G. (2017). What have we learnt about BCG vaccination in the Last 20 years? *Front. Immunol.* **8**, 1134. doi:10.3389/fimmu.2017.011134
- Drain, P. K., Bajema, K. L., Dowdy, D., Dheda, K., Naidoo, K., Schumacher, S. G., Ma, S., Meermeier, E., Lewinsohn, D. M. and Sherman, D. R. (2018). Incipient and Subclinical Tuberculosis: a Clinical Review of Early Stages and Progression of Infection. *Clin. Microbiol. Rev.* **31**, e00021-e00018. doi:10.1128/CMR.00021-18
- Driver, E. R., Ryan, G. J., Hoff, D. R., Irwin, S. M., Basaraba, R. J., Kramnik, I. and Lenaerts, A. J. (2012). Evaluation of a mouse model of necrotic granuloma formation using C3HeB/FeJ mice for testing of drugs against *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* **56**, 3181-3195. doi:10.1128/AAC.00217-12
- Flynn, J. L., Chan, J., Triebold, K. J., Dalton, D. K., Stewart, T. A. and Bloom, B. R. (1993). An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. *J. Exp. Med.* **178**, 2249-2254. doi:10.1084/jem.178.6.2249
- Foreman, T. W., Mehra, S., Lackner, A. A. and Kaushal, D. (2017). Translational research in the nonhuman primate model of tuberculosis. *ILAR J.* **58**, 151-159. doi:10.1093/ilar/ilx015
- Fuglem, B., Jirillo, E., Bjerkås, I., Kiyono, H., Nochi, T., Yuki, Y., Raida, M., Fischer, U. and Koppang, E. O. (2010). Antigen-sampling cells in the salmonid intestinal epithelium. *Dev. Comp. Immunol.* **34**, 768-774. doi:10.1016/j.dci.2010.02.007
- Furin, J., Cox, H. and Pai, M. (2019). Tuberculosis. *Lancet* **393**, 1642-1656. doi:10.1016/S0140-6736(19)30308-3
- Garcia-Contreras, L., Wong, Y.-L., Muttill, P., Padilla, D., Sadoff, J., DeRousse, J., Germishuizen, W. A., Goonesekera, S., Elbert, K., Bloom, B. R. et al. (2008). Immunization by bacterial aerosol. *Proc. Natl. Acad. Sci. USA* **105**, 4656-4660. doi:10.1073/pnas.0800043105
- Gong, W., Liang, Y. and Wu, X. (2020). Animal models of tuberculosis vaccine research: an important component in the fight against tuberculosis. *Biomed. Res. Int.* **2020**, 4263079. doi:10.1155/2020/4263079
- Goonetilleke, N. P., McShane, H., Hannan, C. M., Anderson, R. J., Brookes, R. H. and Hill, A. V. (2003). Enhanced immunogenicity and protective efficacy against *Mycobacterium tuberculosis* of bacille Calmette-Guérin vaccine using mucosal administration and boosting with a recombinant modified vaccinia virus Ankara. *J. Immunol.* **171**, 1602-1609. doi:10.4049/jimmunol.171.3.1602
- Gopal, R., Lin, Y., Obermajer, N., Slight, S., Nuthalapati, N., Ahmed, M., Kalinski, P. and Khader, S. A. (2012). IL-23-dependent IL-17 drives Th1-cell responses following *Mycobacterium bovis* BCG vaccination. *Eur. J. Immunol.* **42**, 364-373. doi:10.1002/eji.201141569
- Grode, L., Ganoza, C. A., Brohm, C., Weiner, J., III, Eisele, B. and Kaufmann, S. H. (2013). Safety and immunogenicity of the recombinant BCG vaccine VPM1002 in a phase 1 open-label randomized clinical trial. *Vaccine* **31**, 1340-1348. doi:10.1016/j.vaccine.2012.12.053
- Gröschel, M. I., Sayes, F., Shin, S. J., Frigui, W., Pawlik, A., Orgeur, M., Canetti, R., Honoré, N., Simeone, R., van der Werf, T. S. et al. (2017). Recombinant BCG Expressing ESX-1 of *Mycobacterium marinum* Combines Low Virulence with Cytosolic Immune Signaling and Improved TB Protection. *Cell Rep.* **18**, 2752-2765. doi:10.1016/j.celrep.2017.02.057
- Grover, A., Troudt, J., Arnett, K., Izzo, L., Lucas, M., Strain, K., McFarland, C., Hall, Y., McMurray, D., Williams, A. et al. (2012). Assessment of vaccine testing at three laboratories using the guinea pig model of tuberculosis. *Tuberculosis* **92**, 105-111. doi:10.1016/j.tube.2011.09.003
- Guo, Z., Lin, Y., Wang, X., Fu, Y., Lin, W. and Lin, X. (2018). The protective efficacy of four iron-related recombinant proteins and their single-walled carbon nanotube encapsulated counterparts against *Aeromonas hydrophila* infection in zebrafish. *Fish Shellfish Immunol.* **82**, 50-59. doi:10.1016/j.fsi.2018.08.009
- Hammarén, M. M., Oksanen, K. E., Nisula, H. M., Luukinen, B. V., Pesu, M., Rämét, M. and Parikka, M. (2014). Adequate Th2-type response associates with restricted bacterial growth in latent mycobacterial infection of zebrafish. *PLoS Pathog.* **10**, e1004190. doi:10.1371/journal.ppat.1004190

- Harjula, S. E., Ojanen, M. J., Taavitsainen, S., Nykter, M. and Rämetsä, M. (2018). Interleukin 10 mutant zebrafish have an enhanced interferon gamma response and improved survival against a *Mycobacterium marinum* infection. *Sci. Rep.* **8**, 10360. doi:10.1038/s41598-018-28511-w
- Harjula, S. E., Saralahti, A. K., Ojanen, M. J., Rantaperä, T., Uusi-Mäkelä, M. I., Nykter, M., Lohi, O., Parikka, M. and Rämetsä, M. (2020). Characterization of immune response against *Mycobacterium marinum* infection in the main hematopoietic organ of adult zebrafish (*Danio rerio*). *Dev. Comp. Immunol.* **103**, 103523. doi:10.1016/j.dci.2019.103523
- Hashish, E., Merwad, A., Elgami, S., Amer, A., Kamal, H., Elsadek, A., Marei, A. and Sitohy, M. (2018). *Mycobacterium marinum* infection in fish and man: epidemiology, pathophysiology and management; a review. *Vet. Q.* **38**, 35-46. doi:10.1080/01652176.2018.1447171
- Hatherill, M., White, R. G. and Hawn, T. R. (2020). Clinical development of new TB vaccines: recent advances and next steps. *Front. Microbiol.* **10**, 3154. doi:10.3389/fmicb.2019.03154
- Hegedűs, Z., Zakrzewska, A., Ágoston, V. C., Ordas, A., Rácz, P., Mink, M., Spaink, H. P. and Meijer, A. H. (2009). Deep sequencing of the zebrafish transcriptome response to mycobacterial infection. *Mol. Immunol.* **46**, 2918-2930. doi:10.1016/j.molimm.2009.07.002
- Hesseling, A. C., Marais, B. J., Gie, R. P., Schaaf, H. S., Fine, P. E., Godfrey-Faussett, P. and Beyers, N. (2007). The risk of disseminated Bacille Calmette-Guérin (BCG) disease in HIV-infected children. *Vaccine* **25**, 14-18. doi:10.1016/j.vaccine.2006.07.020
- Hobernik, D. and Bros, M. (2018). DNA vaccines-how far from clinical use? *Int. J. Mol. Sci.* **19**, 3605. doi:10.3390/ijms19113605
- Hodgkinson, J. W., Belosevic, M., Elks, P. M. and Barreda, D. R. (2019). Teleost contributions to the understanding of mycobacterial diseases. *Dev. Comp. Immunol.* **96**, 111-125. doi:10.1016/j.dci.2019.02.011
- Houben, R. M. G. J. and Dodd, P. J. (2016). The global burden of latent tuberculosis infection: a re-estimation using mathematical modelling. *PLoS Med.* **13**, e1002152. doi:10.1371/journal.pmed.1002152
- Jeyanathan, M., Damjanovic, D., Yao, Y., Bramson, J., Smail, F. and Xing, Z. (2016). Induction of an immune-protective T-Cell repertoire with diverse genetic coverage by a novel Viral-vectored Tuberculosis vaccine in humans. *J. Infect. Dis.* **214**, 1996-2005. doi:10.1093/infdis/jiw467
- Ji, J., Merino, S., Tomas, J. M. and Roher, N. (2019). Nanoliposomes encapsulating immunostimulants modulate the innate immune system and elicit protection in zebrafish larvae. *Fish Shellfish Immunol.* **92**, 421-429. doi:10.1016/j.fsi.2019.06.016
- Johansen, P., Fetteschoss, A., Amstutz, B., Selchow, P., Waeckerle-Men, Y., Keller, P., Deretic, V., Held, L., Kündig, T. M., Böttger, E. C. et al. (2011). Relief from Zmp1-mediated arrest of phagosome maturation is associated with facilitated presentation and enhanced immunogenicity of mycobacterial antigens. *Clin. Vaccine Immunol.* **18**, 907-913. doi:10.1128/CI.00015-11
- Kagina, B. M., Abel, B., Scriba, T. J., Hughes, E. J., Keyser, A., Soares, A., Gamielidien, H., Sidibana, M., Hatherill, M., Gelderbloem, S. et al. (2010). Specific T cell frequency and cytokine expression profile do not correlate with protection against tuberculosis after bacillus Calmette-Guérin vaccination of newborns. *Am. J. Respir. Crit. Care. Med.* **182**, 1073-1079. doi:10.1164/rccm.201003-0334OC
- Kato, G., Kondo, H., Aoki, T. and Hirono, I. (2010). BCG vaccine confers adaptive immunity against *Mycobacterium* sp. infection in fish. *Dev. Comp. Immunol.* **34**, 133-140. doi:10.1016/j.dci.2009.08.013
- Kato, G., Kato, K., Saito, K., Pe, Y., Kondo, H., Aoki, T. and Hirono, I. (2011). Vaccine efficacy of *Mycobacterium bovis* BCG against *Mycobacterium* sp. infection in amberjack *Seriola dumerilii*. *Fish Shellfish Immunol.* **30**, 467-472. doi:10.1016/j.fsi.2010.11.002
- Kesavan, A. K., Brooks, M., Tufariello, J., Chan, J. and Manabe, Y. C. (2009). Tuberculosis genes expressed during persistence and reactivation in the resistant rabbit model. *Tuberculosis* **89**, 17-21. doi:10.1016/j.tube.2008.08.004
- Khademi, F., Derakhshan, M., Yousefi-Avarvand, A., Tafaghodi, M. and Soleimanpour, S. (2018). Multi-stage subunit vaccines against *Mycobacterium tuberculosis*: an alternative to the BCG vaccine or a BCG-prime boost? *Expert Rev. Vaccines* **17**, 31-44. doi:10.1080/14760584.2018.1406309
- Khatiri, B., Whelan, A., Clifford, D., Petreira, A., Sander, P. and Vordermeier, H. M. (2014). BCG  $\Delta$ zmp1 vaccine induces enhanced antigen specific immune responses in cattle. *Vaccine* **32**, 779-784. doi:10.1016/j.vaccine.2013.12.055
- Lanoix, J. P., Lenaerts, A. J. and Nuermberger, E. L. (2015). Heterogeneous disease progression and treatment response in a C3HeB/FeJ mouse model of tuberculosis. *Dis Model Mech* **8**, 603-610. doi:10.1242/dmm.019513
- Liang, Y., Zhang, X., Bai, X., Xiao, L., Wang, X., Zhang, J., Yang, Y., Song, J., Wang, L. and Wu, X. (2017). Immunogenicity and therapeutic effects of a *Mycobacterium tuberculosis* rv2190c DNA vaccine in mice. *BMC Immunol.* **18**, 11. doi:10.1186/s12865-017-0196-x
- Liang, Y., Zhao, Y., Bai, X., Xiao, L., Yang, Y., Zhang, J., Wang, L., Cui, L., Wang, T., Shi, Y. et al. (2018). Immunotherapeutic effects of *Mycobacterium tuberculosis* rv3407 DNA vaccine in mice. *Autoimmunity* **51**, 417-422. doi:10.1080/08916934.2018.1546291
- Lin, P. L., Rodgers, M., Smith, L., Bigbee, M., Myers, A., Bigbee, C., Chiose, I., Capuano, S. V., Fuhrman, C., Klein, E. et al. (2009). Quantitative comparison of active and latent tuberculosis in the cynomolgus macaque model. *Infect. Immun.* **77**, 4631-4642. doi:10.1128/IAI.00592-09
- López, V., Rivalde, M. A., Contreras, M., Mateos-Hernández, L., Vicente, J., Gortázar, C. and de la Fuente, J. (2018). Heat-inactivated *Mycobacterium bovis* protects zebrafish against mycobacteriosis. *J. Fish Dis.* **41**, 1515-1528. doi:10.1111/jfd.12847
- Lovmo, S. D., Speth, M. T., Repnik, U., Koppang, E. O., Griffiths, G. W. and Hildahl, J. P. (2017). Translocation of nanoparticles and *Mycobacterium marinum* across the intestinal epithelium in zebrafish and the role of the mucosal immune system. *Dev. Comp. Immunol.* **67**, 508-518. doi:10.1016/j.dci.2016.06.016
- Loxton, A. G. (2019). B cells and their regulatory functions during Tuberculosis: Latency and active disease. *Mol. Immunol.* **111**, 145-151. doi:10.1016/j.molimm.2019.04.012
- Lu, J.-b., Chen, B.-w., Wang, G.-z., Fu, L.-l., Shen, X.-b., Su, C., Du, W.-x., Yang, L. and Xu, M. (2015). Recombinant tuberculosis vaccine AEC/BC02 induces antigen-specific cellular responses in mice and protects guinea pigs in a model of latent infection. *J. Microbiol. Immunol. Infect.* **48**, 597-603. doi:10.1016/j.jmii.2014.03.005
- Lu, L. L., Chung, A. W., Rosebrock, T. R., Ghebremichael, M., Yu, W. H., Grace, P. S., Schoen, M. K., Tafesse, F., Martin, C., Leung, V. et al. (2016). A functional role for antibodies in tuberculosis. *Cell* **167**, 433-443.e14. doi:10.1016/j.cell.2016.08.072
- Luukinen, H., Hammarén, M. M., Vanha-Aho, L. M. and Parikka, M. (2018). Modeling tuberculosis in *Mycobacterium marinum* infected adult zebrafish. *J. Vis. Exp.* **8**, 140. doi:10.3791/58299
- Manabe, Y. C., Dannenberg, A. M., Jr., Tyagi, S. K., Hatem, C. L., Yoder, M., Woolwine, S. C., Zook, B. C., Pitt, M. L. and Bishai, W. R. (2003). Different strains of *Mycobacterium tuberculosis* cause various spectrums of disease in the rabbit model of tuberculosis. *Infect. Immun.* **71**, 6004-6011. doi:10.1128/IAI.71.10.6004-6011.2003
- Manabe, Y. C., Kesavan, A. K., Lopez-Molina, J., Hatem, C. L., Brooks, M., Fujiwara, R., Hochstein, K., Pitt, M. L., Tufariello, J., Chan, J. et al. (2008). The aerosol rabbit model of TB latency, reactivation and immune reconstitution inflammatory syndrome. *Tuberculosis* **88**, 187-196. doi:10.1016/j.tube.2007.10.006
- Mangtani, P., Abubakar, I., Ariti, C., Beynon, R., Pimpin, L., Fine, P. E., Rodrigues, L. C., Smith, P. G., Lipman, M., Whiting, P. F. et al. (2014). Protection by BCG vaccine against tuberculosis: a systematic review of randomized controlled trials. *Clin. Infect. Dis.* **58**, 470-480. doi:10.1093/cid/cit790
- Mangtani, P., Nguindop-Djomo, P., Keogh, R. H., Sterne, J. A., Abubakar, I., Smith, P. G., Fine, P. E., Vynnycky, E., Watson, J. M., Elliman, D. et al. (2017). The duration of protection of school-aged BCG vaccination in England: a population-based case-control study. *Int. J. Epidemiol.* **47**, 193-201. doi:10.1093/ije/dyx141
- Manjaly Thomas, Z.-R., Satti, Z. R., Marshall, I., Harris, J. L., Lopez Ramon, S. A., Hamidi, R., Minhinnick, A., Riste, A., Stockdale, M., Lawrie, L. (2019). Alternate aerosol and systemic immunisation with a recombinant viral vector for tuberculosis, MVA85A: A phase I randomised controlled trial. *PLoS Med.* **16**, e1002790. doi:10.1371/journal.pmed.1002790
- Marinova, D., Gonzalo-Asensio, J., Aguilo, N. and Martin, C. (2017). MTBVAC from discovery to clinical trials in tuberculosis-endemic countries. *Expert Rev. Vaccines* **16**, 565-576. doi:10.1080/14760584.2017.1324303
- Martin, C., Aguilo, N., Marinova, D. and Gonzalo-Asensio, J. (2020). Update on TB vaccine pipeline. *Appl. Sci.* **10**, 2632. doi:10.3390/app10072632
- McShane, H. and Williams, A. (2014). A review of preclinical animal models utilised for TB vaccine evaluation in the context of recent human efficacy data. *Tuberculosis* **94**, 105-110. doi:10.1016/j.tube.2013.11.003
- Medina, E. and North, R. J. (1998). Resistance ranking of some common inbred mouse strains to *Mycobacterium tuberculosis* and relationship to major histocompatibility complex haplotype and Nramp1 genotype. *Immunology* **93**, 270-274. doi:10.1046/j.1365-2567.1998.00419.x
- Meijer, A. H. and Spaink, H. P. (2011). Host-pathogen interactions made transparent with the zebrafish model. *Curr. Drug Targets* **12**, 1000-1017. doi:10.2174/138945011795677809
- Munro, S. A., Lewin, S. A., Smith, H. J., Engel, M. E., Fretheim, A. and Volmink, J. (2007). Patient adherence to tuberculosis treatment: a systematic review of qualitative research. *PLoS Med.* **4**, e238. doi:10.1371/journal.pmed.0040238
- Myllymäki, H., Bäuerlein, C. A. and Rämetsä, M. (2016). The zebrafish breathes new life into the study of tuberculosis. *Front. Immunol.* **7**, 196. doi:10.3389/fimmu.2016.00196
- Myllymäki, H., Niskanen, M., Oksanen, K. E., Sherwood, E., Ahava, M., Parikka, M. and Rämetsä, M. (2017). Identification of novel antigen candidates for a tuberculosis vaccine in the adult zebrafish (*Danio rerio*). *PLoS ONE* **12**, e0181942. doi:10.1371/journal.pone.0181942
- Myllymäki, H., Niskanen, M., Luukinen, H., Parikka, M. and Rämetsä, M. (2018a). Identification of protective postexposure mycobacterial vaccine antigens using an immunosuppression-based reactivation model in the zebrafish. *Dis. Model. Mech.* **11**, dmm033175. doi:10.1242/dmm.033175

- Myllymäki, H., Niskanen, M., Oksanen, K. E. and Rämetsä, M. (2018b). Immunization of adult zebrafish for the preclinical screening of DNA-based vaccines. *J. Vis. Exp.* **140**, e58453. doi:10.3791/58453
- Myllymäki, H., Niskanen, M., Oksanen, K. E. and Rämetsä, M. (2015). Animal models in tuberculosis research - where is the beef? *Expert Opin. Drug Discov.* **10**, 871-883. doi:10.1517/17460441.2015.1049529
- Nascimento, I. P., Rodriguez, D., Santos, C. C., Amaral, E. P., Rofatto, H. K., Junqueira-Kipnis, A. P., Gonçalves, E., D'Império-Lima, M. R., Hirata, M. H., Silva, C. L. et al. (2017). Recombinant BCG Expressing LTAK63 Adjuvant induces Superior Protection against *Mycobacterium tuberculosis*. *Sci. Rep.* **7**, 2109. doi:10.1038/s41598-017-02003-9
- Nell, A. S., D'Iom, E., Bouic, P., Sabaté, M., Bossier, R., Picas, J., Amat, M., Churchyard, G. and Cardona, P.-J. (2014). Safety, tolerability, and immunogenicity of the novel antituberculous vaccine RUTI: randomized, placebo-controlled phase II clinical trial in patients with latent tuberculosis infection. *PLoS ONE* **9**, e89612. doi:10.1371/journal.pone.0089612
- Nemes, E., Geldenhuys, H., Rozot, V., Rutkowski, K. T., Ratangee, F., Bilek, N., Mabwe, S., Makhetha, L., Erasmus, M., Toefy, A. et al. (2018). Prevention of *M. tuberculosis* Infection with H4:IC31 Vaccine or BCG Revaccination. *New E. J. Med.* **379**, 138-149. doi:10.1056/NEJMoa1714021
- Nieuwenhuizen, N. E., Kulkarni, P. S., Shaligram, U., Cotton, M. F., Rentsch, C. A., Eisele, B., Grode, L. and Kaufmann, S. (2017). The recombinant bacille calmette-guérin vaccine VPM1002: ready for clinical efficacy testing. *Front. Immunol.* **8**, 1147. doi:10.3389/fimmu.2017.01147
- Niskanen, M., Myllymäki, H. and Rämetsä, M. (2020). DNA vaccination with the *Mycobacterium marinum* MMAR\_4110 antigen inhibits reactivation of a latent mycobacterial infection in the adult zebrafish. *Vaccine* **38**, 5685-5694. doi:10.1016/j.vaccine.2020.06.053
- Oehlens, S. H., Cronan, M., Scott, N. R., Thomas, M. I., Okuda, K. S., Walton, E. M., Beerman, R. W., Crosier, P. S. and Tobin, D. M. (2015). Interception of host angiogenic signaling limits mycobacterial growth. *Nature* **517**, 612-615. doi:10.1038/nature13967
- Ojanen, M. J., Uusi-Mäkelä, M. I., Harjula, S. E., Saralahti, A. K., Oksanen, K. E., Kähkönen, N., Määttä, J. A., Hytönen, V. P., Pesu, M. and Rämetsä, M. (2019). Intelectin 3 is dispensable for resistance against a mycobacterial infection in zebrafish (*Danio rerio*). *Sci. Rep.* **9**, 995. doi:10.1038/s41598-018-37678-1
- Oksanen, K. E., Halfpenny, N. J., Sherwood, E., Harjula, S. K., Hammarén, M. M., Ahava, M. J., Pajula, E. T., Lahtinen, M. J., Parikka, M. and Rämetsä, M. (2013). An adult zebrafish model for preclinical tuberculosis vaccine development. *Vaccine* **31**, 5202-5209. doi:10.1016/j.vaccine.2013.08.093
- Oksanen, K. E., Myllymäki, H., Ahava, M. J., Mäkinen, L., Parikka, M. and Rämetsä, M. (2016). DNA vaccination boosts Bacillus Calmette-Guérin protection against mycobacterial infection in zebrafish. *Dev. Comp. Immunol.* **54**, 89-96. doi:10.1016/j.dci.2015.09.001
- Orme, I. M. and Basaraba, R. J. (2014). The formation of the granuloma in tuberculosis infection. *Semin. Immunol.* **26**, 601-609. doi:10.1016/j.smim.2014.09.009
- Parikka, M., Hammarén, M. M., Harjula, S. K., Halfpenny, N. J., Oksanen, K. E., Lahtinen, M. J., Pajula, E. T., Iivanainen, A., Pesu, M. and Rämetsä, M. (2012). *Mycobacterium marinum* causes a latent infection that can be reactivated by gamma irradiation in adult zebrafish. *PLoS Pathog.* **8**, e1002944. doi:10.1371/journal.ppat.1002944
- Pasnik, D. J. and Smith, S. A. (2005). Immunogenic and protective effects of a DNA vaccine for *Mycobacterium marinum* in fish. *Vet. Immunol. Immunopat.* **103**, 195-206. doi:10.1016/j.vetimm.2004.08.017
- Pasnik, D. J. and Smith, S. A. (2006). Immune and histopathologic responses of DNA-vaccinated hybrid striped bass *Morone saxatilis* x *M. chrysops* after acute *Mycobacterium marinum* infection. *Dis. Aquat. Organ.* **73**, 33-41. doi:10.3354/dao073033
- Pasnik, D. J., Vemulapalli, R., Smith, S. A. and Schurig, G. G. (2003). A recombinant vaccine expressing a mammalian *Mycobacterium* sp. antigen is immunostimulatory but not protective in striped bass. *Vet. Immunol. Immunopat.* **95**, 43-52. doi:10.1016/S0165-2427(03)00099-0
- Peddireddy, V., Doddam, S. N. and Ahmed, N. (2017). Mycobacterial dormancy systems and host responses in tuberculosis. *Front. Immunol.* **8**, 84. doi:10.3389/fimmu.2017.00084
- Peng, X., Knouse, J. A. and Heron, K. M. (2015). Rabbit Models for Studying Human Infectious Diseases. *Comp. Med.* **65**, 499-507.
- Penn-Nicholson, A., Tameris, M., Smit, E., Day, T. A., Musvosvi, M., Jayashankar, L., Vergara, J., Mabwe, S., Bilek, N., Geldenhuys, H. et al. (2018). Safety and immunogenicity of the novel tuberculosis vaccine ID93+GLA-SE in BCG-vaccinated healthy adults in South Africa: a randomised, double-blind, placebo-controlled phase 1 trial. *Lancet. Resp. Med.* **6**, 287-298. doi:10.1016/S2213-2600(18)30077-8
- Pesciaroli, M., Alvarez, J., Boniotti, M. B., Cagiola, M., Di Marco, V., Marianelli, C., Pacciarini, M. and Pasquali, P. (2014). Tuberculosis in domestic animal species. *Res. Vet. Sci.* **97** Suppl, S78-S85. doi:10.1016/j.rvsc.2014.05.015
- Pitt, J. M., Stavropoulos, E., Redford, P. S., Beebe, A. M., Bancroft, G. J., Young, D. B. and O'Garra, A. (2012). Blockade of IL-10 signaling during bacillus Calmette-Guérin vaccination enhances and sustains Th1, Th17, and innate lymphoid IFN- $\gamma$  and IL-17 responses and increases protection to *Mycobacterium tuberculosis* infection. *J. Immunol.* **189**, 4079-4087. doi:10.4049/jimmunol.1201061
- Prouty, M. G., Correa, N. E., Barker, L. P., Jagadeeswaran, P. and Klose, K. E. (2003). Zebrafish-*Mycobacterium marinum* model for mycobacterial pathogenesis. *FEMS Microbiol. Lett.* **225**, 177-182. doi:10.1016/S0378-1097(03)00446-4
- Rauta, P. R., Nayak, B. and Das, S. (2012). Immune system and immune responses in fish and their role in comparative immunity study: a model for higher organisms. *Immunol. Lett.* **148**, 23-33. doi:10.1016/j.imlet.2012.08.003
- Ravid-Peretz, S., Colorni, A., Sharon, G. and Ucko, M. (2019). Vaccination of European sea bass *Dicentrarchus labrax* with avirulent *Mycobacterium marinum* (*ijpA::kan* mutant). *Fish Shellfish Immunol.* **90**, 317-327. doi:10.1016/j.fsi.2019.04.057
- Reed, S. G., Coler, R. N., Dalemans, W., Tan, E. V., DeLa Cruz, E. C., Basaraba, R. J., Orme, I. M., Skeiky, Y. A., Alderson, M. R., Cowgill, K. D. et al. (2009). Defined tuberculosis vaccine, Mtb72F/AS02A, emergence of protection in cynomolgus monkeys. *Proc. Natl. Acad. Sci. USA* **106**, 2301-2306. doi:10.1073/pnas.0712077106
- Resseguier, J., Delaune, E., Coolen, A., Levraud, J., Boudinot, P., Le Guellec, D. and Verrier, B. (2017). Specific and efficient uptake of surfactant-free poly(lactic acid) nanovaccine vehicles by mucosal dendritic cells in adult zebrafish after bath immersion. *Front. Immunol.* **8**, 190. doi:10.3389/fimmu.2017.00190
- Rhoades, E. R., Frank, A. A. and Orme, I. M. (1998). Progression of chronic pulmonary tuberculosis in mice aerogenically infected with virulent *Mycobacterium tuberculosis*. *Tuber Lung Dis.* **78**, 57-66. doi:10.1016/S0962-8479(97)90016-2
- Risalde, M. A., López, V., Contreras, M., Mateos-Hernández, L., Gortázar, C. and de la Fuente, J. (2018). Control of mycobacteriosis in zebrafish (*Danio rerio*) mucosally vaccinated with heat-inactivated *Mycobacterium bovis*. *Vaccine* **36**, 4447-4453. doi:10.1016/j.vaccine.2018.06.042
- Rombout, J. H., Lamers, C. H., Helfrich, M. H., Dekker, A. and Taverne-Thiele, J. J. (1985). Uptake and transport of intact macromolecules in the intestinal epithelium of carp (*Cyprinus carpio* L.) and the possible immunological implications. *Cell Tissue Res.* **239**, 519-530. doi:10.1007/BF00219230
- Ru, H., Liu, X., Lin, C., Yang, J., Chen, F., Sun, R., Zhang, L. and Liu, J. (2017). The impact of genome region of difference 4 (RD4) on mycobacterial virulence and BCG efficacy. *Front. Cell Infect. Microbiol.* **7**, 239. doi:10.3389/fcimb.2017.00239
- Ruyra, A., Cano-Sarabia, M., Garcia-Valtanen, P., Yero, D., Gibert, I., Mackenzie, S. A., Estepa, A., Maspoch, D. and Roher, N. (2014). Targeting and stimulation of the zebrafish (*Danio rerio*) innate immune system with LPS/dsRNA-loaded nanoliposomes. *Vaccine* **32**, 3955-3962. doi:10.1016/j.vaccine.2014.05.010
- Sander, P., Clark, S., Petretera, A., Vilaplana, C., Meuli, M., Selchow, P., Zelmer, A., Mohanan, D., Andreu, N., Rayner, E. et al. (2015). Deletion of *zmp1* improves *Mycobacterium bovis* BCG-mediated protection in a guinea pig model of tuberculosis. *Vaccine* **33**, 1353-1359. doi:10.1016/j.vaccine.2015.01.058
- Sharma, S. K., Katoch, K., Sarin, R., Balambal, R., Kumar Jain, N., Patel, N., Murthy, K., Singla, N., Saha, P. K., Khanna, A. et al. (2017). Efficacy and Safety of *Mycobacterium indicus pranii* as an adjunct therapy in Category II pulmonary tuberculosis in a randomized trial. *Sci. Rep.* **7**, 3354. doi:10.1038/s41598-017-03514-1
- Sia, J. K. and Rengarajan, J. (2019). Immunology of *Mycobacterium tuberculosis* Infections. *Microbiol. Spectr.* **7**. doi:10.1128/microbiolspec.GPP3-0022-2018
- Sibley, L., Dennis, M., Sarfas, C., White, A., Clark, S., Gleeson, F., McIntyre, A., Rayner, E., Pearson, G., Williams, A. et al. (2016). Route of delivery to the airway influences the distribution of pulmonary disease but not the outcome of *Mycobacterium tuberculosis* infection in rhesus macaques. *Tuberculosis* **96**, 141-149. doi:10.1016/j.tube.2015.11.004
- Skeiky, Y. A., Alderson, M. R., Ovendale, P. J., Guderian, J. A., Brandt, L., Dillon, D. C., Campos-Neto, A., Lobet, Y., Dalemans, W., Orme, I. M. et al. (2004). Differential immune responses and protective efficacy induced by components of a tuberculosis polyprotein vaccine, Mtb72F, delivered as naked DNA or recombinant protein. *J. Immunol.* **172**, 7618-7628. doi:10.4049/jimmunol.172.12.7618
- Stein, C., Caccamo, M., Laird, G. and Leptin, M. (2007). Conservation and divergence of gene families encoding components of innate immune response systems in zebrafish. *Genome Biol.* **8**, R251. doi:10.1186/gb-2007-8-11-r251
- Stinear, T. P., Seemann, T., Harrison, P. F., Jenkin, G. A., Davies, J. K., Johnson, P. D., Abdallah, Z., Arrowsmith, C., Chillingworth, T., Churcher, C. et al. (2008). Insights from the complete genome sequence of *Mycobacterium marinum* on the evolution of *Mycobacterium tuberculosis*. *Genome Res.* **18**, 729-741. doi:10.1101/gr.075069.107
- Stylianou, E., Harrington-Kandt, R., Beglov, J., Bull, N., Pinpathomrat, N., Swarbrick, G. M., Lewinsohn, D. A., Lewinsohn, D. M. and McShane, H. (2018). Identification and evaluation of novel protective antigens for the development of a candidate tuberculosis subunit vaccine. *Infect. Immun.* **86**, e00014-e00018. doi:10.1128/IAI.00014-18



- Stylianou, E., Paul, M. J., Reljic, R. and McShane, H. (2019). Mucosal delivery of tuberculosis vaccines: a review of current approaches and challenges. *Expert Rev. Vaccines* **18**, 1271-1284. doi:10.1080/14760584.2019.1692657
- Su, H., Peng, B., Zhang, Z., Liu, Z. and Zhang, Z. (2019). The *Mycobacterium tuberculosis* glycoprotein Rv1016c protein inhibits dendritic cell maturation and impairs Th1/Th17 responses during mycobacteria infection. *Mol. Immunol.* **109**, 58-70. doi:10.1016/j.molimm.2019.02.021
- Suschak, J. J., Williams, J. A. and Schmaljohn, C. S. (2017). Advancements in DNA vaccine vectors, non-mechanical delivery methods, and molecular adjuvants to increase immunogenicity. *Hum. Vaccin Immunother* **13**, 2837-2848. doi:10.1080/21645515.2017.1330236
- Suliman, S., Luabeya, A., Geldenhuys, H., Tameris, M., Hoff, S. T., Shi, Z., Tait, D., Kromann, I., Ruhwald, M., Rutkowski, K. T. et al. (2019). Dose optimization of H56:IC31 vaccine for tuberculosis-endemic populations. A double-blind, placebo-controlled, dose-selection trial. *Am. J. Respir. Crit. Care. Med.* **199**, 220-231. doi:10.1164/rccm.201802-0366OC
- Swaim, L. E., Connolly, L. E., Volkman, H. E., Humbert, O., Born, D. E. and Ramakrishnan, L. (2006). *Mycobacterium marinum* infection of adult zebrafish causes caseating granulomatous tuberculosis and is moderated by adaptive immunity. *Infect. Immun.* **74**, 6108-6117. doi:10.1128/IAI.00887-06
- Tait, D. R., Hatherill, M., Van Der Meeren, O., Ginsberg, A. M., Van Brakel, E., Salaun, B., Scriba, T. J., Akite, E. J., Ayles, H. M., Bollaerts, A. et al. (2019). Final Analysis of a Trial of M72/AS01E Vaccine to Prevent Tuberculosis. *N. Engl. J. Med.* **381**, 2429-2439. doi:10.1056/NEJMoa1909953
- Talaat, A. M., Reimschuessel, R., Wasserman, S. S. and Trucksis, M. (1998). Goldfish, *Carassius auratus*, a novel animal model for the study of *Mycobacterium marinum* pathogenesis. *Infect. Immun.* **66**, 2938-2942. doi:10.1128/IAI.66.6.2938-2942.1998
- Tameris, M. D., Hatherill, M., Landry, B. S., Scriba, T. J., Snowden, M. A., Lockhart, S., Shea, J. E., McClain, J. B., Hussey, G. D., Hanekom, W. A. et al. (2013). Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial. *Lancet* **381**, 1021-1028. doi:10.1016/S0140-6736(13)60177-4
- Tang, J., Cai, Y., Liang, J., Tan, Z., Tang, X., Zhang, C., Cheng, L., Zhou, J., Wang, H., Yam, W. C. et al. (2018). In vivo electroporation of a codon-optimized BER<sup>opt</sup> DNA vaccine protects mice from pathogenic *Mycobacterium tuberculosis* aerosol challenge. *Tuberculosis* **113**, 65-75. doi:10.1016/j.tube.2018.07.003
- Tkachuk, A. P., Guschin, V. A., Potapov, V. D., Demidenko, A. V., Lunin, V. G. and Gintsburg, A. L. (2017). Multi-subunit BCG booster vaccine GamTBvac: assessment of immunogenicity and protective efficacy in murine and guinea pig TB models. *PLoS ONE* **12**, e0176784. doi:10.1371/journal.pone.0176784
- Tobin, D. M. and Ramakrishnan, L. (2008). Comparative pathogenesis of *Mycobacterium marinum* and *Mycobacterium tuberculosis*. *Cell. Microbiol.* **10**, 1027-1039. doi:10.1111/j.1462-5822.2008.01133.x
- Tsenova, L., Harbacheuski, R., Moreira, A. L., Ellison, E., Dalemans, W., Alderson, M. R., Mathema, B., Reed, S. G., Skeiky, Y. A. and Kaplan, G. (2006). Evaluation of the Mtb72F polyprotein vaccine in a rabbit model of tuberculous meningitis. *Infect. Immun.* **74**, 2392-2401. doi:10.1128/IAI.74.4.2392-2401.2006
- Turner, O. C., Basaraba, R. J. and Orme, I. M. (2003). Immunopathogenesis of pulmonary granulomas in the guinea pig after infection with *Mycobacterium tuberculosis*. *Infect. Immun.* **71**, 864-871. doi:10.1128/IAI.71.2.864-871.2003
- Umehura, M., Yahagi, A., Hamada, S., Begum, M. D., Watanabe, H., Kawakami, K., Suda, T., Sudo, K., Nakae, S., Iwakura, Y. et al. (2007). IL-17-mediated regulation of innate and acquired immune response against pulmonary *Mycobacterium bovis* bacille Calmette-Guérin infection. *J. Immunol.* **178**, 3786-3796. doi:10.4049/jimmunol.178.6.3786
- Van Der Meeren, O., Hatherill, M., Nduba, V., Wilkinson, R. J., Muyoyeta, M., Van Brakel, E., Ayles, H. M., Henostroza, G., Thienemann, F., Scriba, T. J. et al. (2018). Phase 2b controlled trial of M72/AS01E vaccine to prevent tuberculosis. *N. Engl. J. Med.* **379**, 1621-1634. doi:10.1056/NEJMoa1803484
- van der Sar, A. M., Abdallah, A. M., Sparrius, M., Reinders, E., Vandenbroucke-Grauls, C. M. and Bitter, W. (2004). *Mycobacterium marinum* strains can be divided into two distinct types based on genetic diversity and virulence. *Infect. Immun.* **72**, 6306-6312. doi:10.1128/IAI.72.11.6306-6312.2004
- van der Sar, A. M., Spaik, H. P., Zakrzewska, A., Bitter, W. and Meijer, A. H. (2009). Specificity of the zebrafish host transcriptome response to acute and chronic mycobacterial infection and the role of innate and adaptive immune components. *Mol. Immunol.* **46**, 2317-2332. doi:10.1016/j.molimm.2009.03.024
- Verreck, F. A., Vervenne, R. A., Kondova, I., van Kralingen, K. W., Remarque, E. J., Braskamp, G., van der Werff, N. M., Kersbergen, A., Ottenhoff, T. H., Heidt, P. J. et al. (2009). MVA.85A boosting of BCG and an attenuated, phoP deficient *M. tuberculosis* vaccine both show protective efficacy against tuberculosis in rhesus macaques. *PLoS One* **4**, e5264. doi:10.1371/journal.pone.0005264
- Vilaplana, C., Gil, O., Cáceres, N., Pinto, S., Díaz, J. and Cardona, P. J. (2011). Prophylactic effect of a therapeutic vaccine against TB based on fragments of *Mycobacterium tuberculosis*. *PLoS ONE* **6**, e20404. doi:10.1371/journal.pone.0020404
- Villarreal-Ramos, B., Berg, S., Chamberlain, L., McShane, H., Hewinson, R. G., Clifford, D. and Vordermeier, M. (2014). Development of a BCG challenge model for the testing of vaccine candidates against tuberculosis in cattle. *Vaccine* **32**, 5645-5649. doi:10.1016/j.vaccine.2014.08.009
- von Reyn, C. F., Mtei, L., Arbeit, R. D., Waddell, R., Cole, B., Mackenzie, T., Matee, M., Bakari, M., Tvaroha, S., Adams, L. V. et al. (2010). Prevention of tuberculosis in Bacille Calmette-Guérin-primed, HIV-infected adults boosted with an inactivated whole-cell mycobacterial vaccine. *AIDS* **24**, 675-685. doi:10.1097/QAD.0b013e3283350f1b
- von Reyn, C. F., Lahey, T., Arbeit, R. D., Landry, B., Kailani, L., Adams, L. V., Haynes, B. C., Mackenzie, T., Wieland-Alter, W., Connor, R. I. et al. (2017). Safety and immunogenicity of an inactivated whole cell tuberculosis vaccine booster in adults primed with BCG: A randomized, controlled trial of DAR-901. *PLoS one* **12**, e0175215. doi:10.1371/journal.pone.0175215
- Vordermeier, H. M., Villarreal-Ramos, B., Cockle, P. J., McAulay, M., Rhodes, S. G., Thacker, T., Gilbert, S. C., McShane, H., Hill, A. V., King, Z. et al. (2009). Viral booster vaccines improve *Mycobacterium bovis* BCG-induced protection against bovine tuberculosis. *Infect. Immun.* **77**, 3364-3373. doi:10.1128/IAI.00287-09
- Williams, A., Goonetilleke, N. P., McShane, H., Clark, S. O., Hatch, G., Gilbert, S. C. and Hill, A. V. (2005). Boosting with poxviruses enhances *Mycobacterium bovis* BCG efficacy against tuberculosis in guinea pigs. *Infect. Immun.* **73**, 3814-3816. doi:10.1128/IAI.73.6.3814-3816.2005
- Williams, A., Hall, Y. and Orme, I. M. (2009). Evaluation of new vaccines for tuberculosis in the guinea pig model. *Tuberculosis (Edinb)* **89**, 389-397. doi:10.1016/j.tube.2009.08.004
- Yang, X. Y., Chen, Q. F., Li, Y. P. and Wu, S. M. (2011). *Mycobacterium vaccae* as adjuvant therapy to anti-tuberculosis chemotherapy in never-treated tuberculosis patients: a meta-analysis. *PLoS One* **6**, e23826. doi:10.1371/journal.pone.0023826
- Yang, C. T., Cambier, C. J., Davis, J. M., Hall, C. J., Crosier, P. S. and Ramakrishnan, L. (2012). Neutrophils exert protection in the early tuberculous granuloma by oxidative killing of mycobacteria phagocytosed from infected macrophages. *Cell Host Microbe* **12**, 301-312. doi:10.1016/j.chom.2012.07.009
- Yoon, S., Mitra, S., Wyse, C., Alnabulsi, A., Zou, J., Weerdenburg, E. M., van der Sar, A. M., Wang, D., Secombes, C. J. and Bird, S. (2015). First demonstration of antigen induced cytokine expression by CD4+ lymphocytes in a poikilotherm: studies in zebrafish (*Danio rerio*). *PLoS one* **10**, e0126378. doi:10.1371/journal.pone.0126378
- Zhang, C., Wang, G. and Zhu, B. (2020). Application of antigen presenting cell-targeted nanovaccine delivery system in rhabdovirus disease prophylactics using fish as a model organism. *J Nanobiotechnology* **18**, 24. doi:10.1186/s12951-020-0584-x
- Ziklo, N., Colorni, A., Gao, L. Y., Du, S. J. and Ucko, M. (2018). Humoral and cellular immune response of european seabass *Dicentrarchus labrax* vaccinated with heat-killed *Mycobacterium marinum* (*ijpA::kan* mutant). *J. Aquat. Anim. Health* **30**, 312-324. doi:10.1002/laah.10042