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# CHAPTER 1

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GENERAL INTRODUCTION



## TUBERCULOSIS

Tuberculosis (TB) is an infectious disease caused by the bacillus *Mycobacterium tuberculosis* or, less common, by closely related species of the *M. tuberculosis* complex [1, 2]. Typically, TB affects the lungs (pulmonary TB), but the disease can affect other sites of the body as well. Classical TB symptoms include a chronic cough, fever, night sweats, weight loss and fatigue [3]. Transmission of *M. tuberculosis* normally occurs when bacteria-containing droplets, which are expelled from patients with pulmonary TB by coughing or sneezing, are inhaled by new hosts.

TB is one of the oldest infectious diseases of man and continues to threaten human health. The oldest evidence of TB infection in humans is the discovery of characteristic TB lesions combined with *M. tuberculosis* ancient DNA and cell-wall specific lipid markers in 9,000-year-old human remains in an eastern Mediterranean settlement [4]. Today, TB remains an urgent health problem. In 1993, the World Health Organization (WHO) declared TB a global public health emergency and started specific programs to control the disease. Since 2006, the absolute number of TB cases has been falling moderately and TB mortality rates are considerably reduced [5]. However, TB is still the second leading cause of death from an infectious disease worldwide, after HIV infection. According to the most recent report of the WHO, globally there were an estimated 8.7 million new cases of TB in 2011 [5]. Most of these cases occurred in Asia (59%) and Africa (26%). In the same year, an estimated 1.4 million TB patients died of the disease [5].

One of the problems in the control of TB is the lack of an efficient vaccine. The current live attenuated vaccine strain, Bacillus Calmette-Guérin (BCG), was derived from *Mycobacterium bovis* and was first used in humans in 1921. Although it protects efficiently against meningitis and disseminated TB in young children, its efficacy for preventing pulmonary TB in adults varies extensively [6, 7]. Another problem in TB control is related to drug resistance. *M. tuberculosis* is naturally quite resistant to different antibiotics, but can be cured with a six to nine month antibiotic treatment regimen consisting of a combination of four different antibiotics. However, drug resistant *M. tuberculosis* strains have been emerging, mainly due to drug mismanagement, *i.e.* when the full course of treatment is not completed, or when patients with undiagnosed resistant TB receive inappropriate therapies. In 2011, there were an estimated 630,000 cases of multi-drug resistant TB among the total 12 million cases [5]. Even more alarming is the appearance of extensively-drug resistant *M. tuberculosis* strains and totally-drug resistant strains, which have been identified recently in India, Iran and Italy [8-10]. Probably, the fact that no new anti-TB drugs have been developed for half a century has contributed to the emergence of these untreatable *M. tuberculosis* strains.

## TUBERCULOSIS PATHOLOGY

While an estimated one-third of the world population is infected with *M. tuberculosis*, only 5-10% of the infected individuals develop active disease [11]. The vast majority of infections remains latent, a state in which bacilli are not cleared by

the immune system but reside in structures called granulomas or tubercles, after which the disease is named. Granuloma formation is initiated when *M. tuberculosis* is phagocytosed by alveolar macrophages in the lungs. Although macrophages have potent anti-microbial activity, *M. tuberculosis* is able to withstand the wide array of defense mechanisms of this host cell. Among others, *M. tuberculosis* has the ability to disrupt the maturation of the bacilli-containing phagosomes into phagolysosomes, to escape from the phagosome into the cytosol and to modulate the host immune responses [12-14], which collectively allows for its intracellular replication. Subsequently, the infected macrophages induce a proinflammatory immune response, which triggers the recruitment of additional macrophages and other immune cells to the infection site. Together, these cells give rise to an aggregate, or granuloma, which is highly organized and consist of a core of infected and uninfected macrophages, surrounded by a cuff of lymphocytes. Typically, macrophages in TB granulomas can fuse into multinucleated giant cells [15] or differentiate into epithelioid cells, which makes them resemble epithelial cells [16]. As the granuloma matures, fibroblasts encapsulate the core by forming a fibrous layer surrounding the granuloma. Occasionally, the center of the granuloma becomes necrotic. The necrotic center, called caseum, allows the outgrowth of large numbers of extracellular bacilli (Fig. 1). Granulomas can be maintained for decades, they can resolve, or they can progress to active disease when the immune status of the person declines. In the latter case, the necrotic center liquefies and cavitates and bacteria can enter the airways.

The granuloma response is usually described as a host-driven process required to contain infection and prevent dissemination of disease. Yet, in most cases, granulomas fail to eradicate the pathogen. Recently, there is a growing appreciation of the active role of the bacterium in promoting granuloma formation. Bacterial factors that stimulate the granuloma response have been identified, most prominently the type VII secretion system ESX-1 and mycobacterium-specific cell wall lipids such as trehalose dimycolate [17, 18]. However, a clear picture of the mechanisms operative in granuloma formation is currently lacking. As latency is

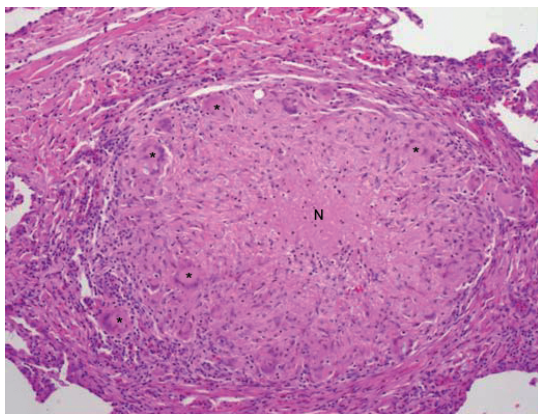


Figure 1. Granuloma in the lung of a TB patient. Hematoxylin-eosin stained section of a granuloma displaying abundant necrosis in the central region (N). Multinucleated giant cells, characterized by the typical horseshoe-like arrangement of the nuclei at the edge of the cell, are present at the periphery of the granuloma and are marked by asterisks. This figure was reprinted from [45] with permission from *Archives of Pathology & Laboratory Medicine*. Copyright 2010. College of American Pathologists.

a major obstacle for the eradication of *M. tuberculosis* infection, understanding the mechanisms underlying the process of granuloma formation will enhance the development of more effective therapies to control or eradicate this pathogen.

## STUDYING GRANULOMA FORMATION

Our current knowledge of the *M. tuberculosis* granuloma response is elusive for several reasons. First, working with *M. tuberculosis* has some important disadvantages: the extremely slow growth rate of the bacterium, *i.e.* 12 to 18 hours per cell division, and its pathogenic nature complicate experimental work. Second, because *M. tuberculosis* is exclusively a human pathogen, examination of its pathology *in vivo* in a natural host is virtually impossible. Mouse models are commonly used to study *M. tuberculosis* virulence, yet, they do not appear to be relevant models for granuloma formation. In contrast to human granulomas, murine granulomas are poorly organized, do not develop necrosis or hypoxia and contain relatively high numbers of bacteria [19-21]. Guinea pigs have also been used in tuberculosis research. These animals develop granulomas that are quite similar to their human counterparts in terms of their organization and formation of necrotic centers. However, guinea pigs are extremely susceptible to infection with *M. tuberculosis*, and granulomas are not able to contain the bacterial burden [19-21]. Another laboratory animal that can be used to study granuloma formation is the rabbit. Although rabbits are relatively resistant to *M. tuberculosis*, infection with *Mycobacterium bovis*, a member of the *M. tuberculosis* complex, results in pathology that is highly similar to that of human *M. tuberculosis* infection [19-21]. However, for both guinea pigs and rabbits, the paucity of knockout mutants in immune genes and immunological reagents severely limits analysis. Infection of non-human primates with *M. tuberculosis* most closely resembles human infection [19-21]. However, due to high costs, housing difficulties and ethical considerations, monkeys are not an ideal laboratory animal. Obviously, host-pathogen interactions can also be analyzed *in vitro*, but such models are limited in their ability to account for the complex host-pathogen interactions present *in vivo*. A third reason for the limited knowledge regarding the *M. tuberculosis* granuloma response is the laborious procedures required for analysis of granuloma formation, *i.e.* histological examination of tissues.

Despite these difficulties, in the past decade substantial progress has been made with respect to the study of the mycobacterial granuloma response. This is in part attributed to the use of the alternative model organism *Mycobacterium marinum*. *M. marinum* is one of the closest genetic relatives of *M. tuberculosis* outside the *M. tuberculosis* complex [22]. The genome of *M. marinum* is about 1.5 times the size of that of *M. tuberculosis*, which likely reflects its expanded host and environmental range, but orthologous coding sequences share an average amino acid identity of 85% [23]. The two species share many of the known virulence factors, and *M. tuberculosis* genes can functionally complement mutations in *M. marinum* orthologues and vice versa [23-26]. *M. marinum* has several advantages,

including its faster growth rate, *i.e.* a generation time of 4 to 6 hours, and its limited pathogenicity for humans. Because it grows optimally at 28-33°C, infection rarely leads to systemic disease in humans. *M. marinum* is primarily associated with human skin lesions called fish tank granulomas. Importantly, these local granulomas are often indistinguishable from *M. tuberculosis* dermal granulomas [27, 28]. *M. marinum* naturally inhabits aquatic environments and is a natural pathogen for poikilothermic species, such as fish and frogs.

The systemic granulomatous disease that is established by *M. marinum* in its natural hosts parallels the pathology seen in human tuberculosis, *i.e.* well-organized granulomas with differentiated macrophages and a central necrotic region are observed in different organs [29-35]. Conveniently, fish and frogs are ideal laboratory animals. Especially the zebrafish, *Danio rerio*, has become a popular model organism for the study of diverse human diseases because of its genetic tractability and short generation time.

The beauty of the zebrafish model is shown in studies using embryonic zebrafish. The embryos have the unique characteristic that they are transparent and therefore allow for real-time observation of host-pathogen interactions during infection. Using the zebrafish embryo *M. marinum* model, important advances have been made with respect to unraveling the mycobacterial granuloma response. First of all, infection in zebrafish embryos, which have not yet developed an adaptive immune system, clearly demonstrated that interactions of *M. marinum* with the innate immune system result in the formation of tight aggregates of macrophages. The presence of epithelioid and multinucleated giant cells and the activation of previously identified granuloma-specific *M. marinum* genes in these aggregates emphasize that they represent granulomas at their initial stage [36]. Furthermore, infection of zebrafish embryos with *M. marinum* revealed that although granulomas are necessary to contain the infection, the bacterium exploits the macrophage aggregates for its dissemination and expansion, and upon reinfection with *M. marinum*, infected cells traffic rapidly into preexisting granulomas [37-39]. Finally, with this model bacterial factors that promote granuloma formation have been identified [37, 40, 41].

## **AIM AND OUTLINE OF THIS THESIS**

The aim of the work described in this thesis was to exploit the possibilities offered by the zebrafish embryo *M. marinum* infection model to identify new mycobacterial virulence factors, more specifically, mycobacterial factors involved in the initiation of granuloma formation. By studying *M. marinum* mutants in a selection of virulence factors, the mechanism of their attenuation would be analyzed in detail in order to characterize the distinct steps in the early granuloma response.

The specialized type VII secretion system ESX-1 is known to be a major mycobacterial virulence factor required for granuloma formation [17]. Tubercle bacilli possess five different type VII secretion systems, named ESX-1 to 5. In **chapter 2**, an overview of the current knowledge of the specialized type VII secretion systems is given, and in particular their role in granuloma formation is discussed.

**Chapter 3** lays the foundation of the studies described in this work. In this chapter, the set-up of a screening platform for the identification of mycobacterial genes involved in initiation of the granuloma response is described. *M. marinum* mutants of a random transposon library were screened for their ability to initiate granuloma formation in zebrafish embryos. This is the first reported screen for mycobacterial granuloma determinants in an *in vivo* setting.

**Chapter 4** elaborates on **chapter 3**. The results of the complete zebrafish embryo screen are presented. Quite a few *M. marinum* mutants were identified for which initiation of granuloma formation was diminished. Both known and unknown mycobacterial granuloma determinants were identified, which validates our screen. To further address the relevance of the embryo screen, virulence of one of the early granuloma mutants was analyzed in adult zebrafish, which possess both an innate and an adaptive immune system.

**Chapter 5** focuses on an early granuloma mutant disrupted in the specialized secretion system SecA2. This secretion system has been implicated in virulence previously, yet, the responsible SecA2 substrates remained unknown [42]. Prior studies aiming at identifying SecA2 substrates concentrated on secreted material [43]. However, because the SecA2 system facilitates protein translocation across the inner membrane, we decided to use another approach to identify SecA2 substrates and compared cell wall fractions of the *secA2* mutant to its parent strain. Next, by overexpression of one of the identified SecA2 substrates in the mutant, we attempted to determine the role of this substrate in virulence.

**Chapter 6** describes the in-depth analysis of an early granuloma mutant with a mutation in a mannosyltransferase. This mannosyltransferase is required for the mannosylation of multiple domains of the cell wall glycolipids lipoarabinomannan (LAM) and lipomannan (LM) [44]. By complementation of the mutant with the *Mycobacterium smegmatis* mannosyltransferase orthologue, the LAM and LM mannosylation pattern was partially restored, and the contribution of the distinct mannosylated glycolipid domains to virulence could be investigated.

Finally, the overall results of this thesis are discussed in **chapter 7**.

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