CHAPTER 7

SUMMARIZING DISCUSSION

The pathological manifestation of tuberculosis is very typical in terms of the presence of granulomas, or cellular aggregates, that form around infection foci. Besides the host-protective role of containment of infection, granulomas have a host-detrimental role, as they provide a niche for bacterial persistence, contribute to the sequestration of bacteria from drugs and promote bacilli to adopt a persister state [1-3]. In approximately 90% of the individuals infected with *Mycobacterium tuberculosis*, a dynamic balance between host and pathogen maintains the disease in a subclinical stage, where bacilli reside in a latent state within granulomas. However, the bacilli can remain in the host for a lifetime and in roughly 10% of the cases reactivation and initiation of clinical disease occurs at some point in life [4]. Although this proportion of reactivation might seem low, 10% of the estimated 2 billion infected individuals represents a gigantic reservoir of renascent active bacilli that sustains disease and transmission [5]. Eradication of *M. tuberculosis* will only become realistic when a strategy is found to prevent granuloma formation or to kill bacilli within granulomas. To reach this goal, it is crucial to understand the mechanisms involved in the granuloma response. In this thesis, the *Mycobacterium marinum* zebrafish embryo model was used to investigate the effect of mycobacterial genes on the initial stages of granuloma formation. This led to the identification of mycobacterial factors that are required for the initiation of the granuloma response. This knowledge might be instrumental to the success of new therapeutic strategies directed against tuberculosis.

GRANULOMA MODELS

Recent studies indicating that granuloma formation is part of a pathogen-directed virulence program have fueled interest in the mycobacterial factors that influence the granuloma response. Different models of tuberculous granuloma formation have been developed that might be considered for the execution of medium- or high-throughput screens to delineate the mycobacterial mechanisms of granuloma formation.

One of these models is an *in vitro* human mycobacterial granuloma model [6-8]. In *in vitro* models, incubation of human peripheral blood mononuclear cells (PBMC) with either artificial beads coated with mycobacterial compounds or live mycobacteria induces cellular aggregates. It has been shown that these *in vitro* aggregates display similar characteristics to their human counterparts in terms of the cell types involved, morphological features, cellular differentiation levels and immunological responses of host cells [6-11]. These *in vitro* assays enable analysis of the molecular and cellular interactions occurring during the very first steps of the human granulomatous response. They allow for quick and relatively easy analysis of granuloma formation, *i.e.* aggregates develop within one week, the assay can be performed in tissue culture plates and formation of aggregates can be monitored by light microscopy. However, while much emphasis has been put on host cell dynamics within the *in vitro* model, the status of the bacteria has not been analyzed in detail. It would be relevant to evaluate how bacterial

persistence and gene expression patterns in *in vitro* models correlate to those in other granuloma models. Furthermore, it is not clear whether these models permit a comparative study of mycobacterial mutants for the identification of bacterial factors promoting the granuloma response. To date, mycobacterial mutants defective for granuloma formation in *in vitro* models have not been described. In fact, host cell aggregates have also been observed after incubation of PBMCs with the live attenuated vaccine strain *Mycobacterium bovis* BCG [6], while it is known that this strain is a poor inducer of granulomas *in vivo* [12]. Therefore, the currently described *in vitro* granuloma models are probably less suitable for the identification of mycobacterial mutants with a defect in their granuloma promoting activity. Another disadvantage of these *in vitro* models is that later events in granuloma formation cannot be investigated. Yet, *in vitro* models can serve as a valuable tool for studying the effects of mycobacterial factors on host cell morphology and differentiation that are typical for tuberculous granuloma formation, *e.g.* formation of epithelioid and multinucleated giant cells.

The mouse is a popular host for *M. tuberculosis* infections, and a large number of mouse tuberculosis models have been generated. Although mice are very useful experimental tools that can be used for virulence and drug studies, they are unreliable for the study of granuloma formation, because mice generally do not progress to latent infection and lack organized, necrotic granulomas. However, a few alternative mouse granuloma models more closely resembling human tuberculous granulomas have been described, that might offer possibilities for screening bacterial mutants. One such model is the so-called hollow fiber granuloma model. Subcutaneous implantation of *M. tuberculosis* encapsulated in semi-diffusible polyvinylidene fluoride (PVDF) hollow fibers in mice induces granuloma-like lesions within several weeks of infection [13]. In this extracellular microenvironment, soluble bacterial factors promote the recruitment of macrophages, lymphocytes and fibroblasts. The replicative and metabolic activity, antibiotic susceptibility and gene expression profiles of the bacilli in these artificial granulomas are comparable to those of *M. tuberculosis* in human latent tuberculosis infection. In contrast to the *in vitro* model, it has been demonstrated that the hollow fiber granuloma model allows for the identification of bacterial mutants that are specifically attenuated for persistence at the late stage of tuberculous infection [13, 14]. However, host-pathogen interactions in the hollow fiber model probably differ substantially from those that underlie human tuberculosis, because bacilli cannot be phagocytozed by host cells. The lesions that develop in this model are poorly characterized with respect to cell types, morphology and differentiation levels, so it remains to be established whether the diffuse and unstructured cellular infiltrates truly represent granulomas. Therefore, for identification of bacterial mutants involved in the initiation of granuloma formation, the hollow fiber granuloma model would not be preferred. Nevertheless, because the model seems to mimic latent granulomatous conditions, it might provide a unique opportunity to study mycobacterial factors involved in *M. tuberculosis* latency in the host.

A mouse model based on tail vein injection of *M. marinum* has also been developed for the study of mycobacterial pathogenesis [15]. A couple of weeks after infection, *M. marinum* induces macroscopic lesions in the tail that display many of the features found in human tuberculous granulomas, such as cellular organization, differentiation levels, and the presence of central necrosis. Although the choice of mice as a non-natural host for *M. marinum* is counterintuitive, this model provides the unique opportunity that necrotic granulomas develop locally in the context of an experimentally amenable mammalian immune system. Additionally, mutants attenuated for the induction of a granuloma response can be identified in this model [15, 16]. However, while a *M. marinum secA2* mutant rarely induces granulomas in zebrafish, it does initiate granulomas in mouse tails [16]. Therefore, it seems that early events of granuloma formation in the mouse tail model do not mimic that in a natural host, which questions the significance of the mouse tail model. Moreover, this model would not be particularly convenient for a medium- or high throughput screen to analyze single mutants of a mutant library for initiation of granuloma formation.

Another host organism that has become popular for the study of tuberculous granuloma formation is the zebrafish (*Danio rerio*). In response to infection with *M. marinum*, cellular aggregates arise that recapitulate bacterial and host cell properties of human tuberculosis granulomas [17-19]. Owing to their transparency, zebrafish embryos provide a tractable model to study the very first steps in tuberculous granuloma formation. Furthermore, zebrafish embryos facilitate dissection of innate immune mechanisms in granuloma formation because the adaptive immune system is not yet developed. The adult system allows for examination of the later steps of granuloma formation in the context of a fully developed immune system. Mutants defective for initiation of granuloma formation can be identified in both the embryo and the adult system [20, 21]. The major drawbacks of this model are the use of a model pathogen instead of *M. tuberculosis,* the use of a non-mammalian host, and a limited diversity of immunological tools. However, the low costs and large clutch size of zebrafish, the natural host-pathogen relation and the simplicity of monitoring initiation of granuloma formation in embryos offer ideal conditions for a medium- or high-throughput screen.

UTILITY OF THE *M. MARINUM* ZEBRAFISH EMBRYO SCREEN TO IDENTIFY MYCOBACTERIAL GRANULOMA DETERMINANTS

There has been much speculation about putative mycobacterial virulence factors, but only few have been confirmed using isogenic mutants in a relevant *in vivo* system. Using the natural *M. marinum* zebrafish embryo model, we have executed the first *in vivo* screen for mycobacterial granuloma determinants (**chapter 3**). Our results demonstrate the utility of this approach as we have identified 23 granuloma promoting genes (**chapter 4**). All but three have an orthologue in *M. tuberculosis*, and a substantial number of genes, or the pathways in which they are

involved, have been previously associated with *in vitro* or *in vivo* virulence of *M. tuberculosis*, validating the use of *M. marinum* as a surrogate for *M. tuberculosis*.

Notably, the only gene for which the described *M. tuberculosis* orthologue does not affect virulence is the glucosyltransferase *mmar_1681* [22]. One reason for this difference could be redundancy. For *M. tuberculosis*, it has been demonstrated that the orthologous glucosyltransferase encoded by *Rv3032* and GlgA have partially redundant glucosyltransferase activities [22]. In other mycobacterial species, such as *Mycobacterium leprae* and *Mycobacterium smegmatis*, only one of these two genes is functional. In that case, disruption of the only functional glucosyltransferase cannot be compensated for. The *M. marinum* genomic sequence reveals that the *glgA* orthologue is present. Also the observation that the *M. marinum* glucosyltransferase mutant does not substantially differ from the parental strain in terms of glucan/glycogen content and lipid profiles (R. van de Weerd and J. Geurtsen, unpublished results) supports the notion that, similar to *M. tuberculosis*, compensatory glucosyltransferase activity exists in *M. marinum*. Still, we cannot rule out that this situation is different upon infection. Another reason why our *M. marinum* glucosyltransferase results were not consistent with those obtained with *M. tuberculosis* might be the use of different model systems and read-outs. For analysis of virulence of the *M. tuberculosis* glucosyltransferase mutant, mice were infected intravenously and bacterial loads in lungs and spleens were monitored over the course of infection. Either determination of granulomatous structures in mice or a more relevant laboratory animal infected with the *M. tuberculosis* mutant, or examination of bacterial loads in organs of zebrafish infected with the *M. marinum* glucosyltransferase mutant could give a better insight into the differences between these mutant strains.

The use of the *M. marinum* zebrafish embryo model offers opportunities to answer important research questions that cannot easily be addressed using *M. tuberculosis* model systems. For example, whereas it has been well established that the accessory Sec secretion system, SecA2, is important for mycobacterial virulence, studies in *M. tuberculosis* failed to uncover the responsible SecA2 secreted substrates [23, 24]. Because the SecA2 pathway facilitates protein transport across the inner membrane, the most relevant location to search for SecA2 substrates is in the cell envelope. While optimal cell fractionation of *M. tuberculosis* is hampered by biosafety level III regulations, the biosafety level II regulations that apply to *M. marinum* allow for efficient isolation of cell envelope fractions. This enabled us to identify SecA2-dependent substrates in the *M. marinum* cell envelope that explain the virulence effect of SecA2 (chapter 5).

Another longstanding scientific enigma on which the *M. marinum* zebrafish embryo model has shed new light is the impact of mannosylation patterns of lipomannan (LM) and lipoarabinomannan (LAM) on mycobacterial virulence. LM and LAM are glycolipid constituents of the mycobacterial cell wall that are synthesized in a sequential order. As purified molecules, both structures modulate different aspects of host immunity, depending on their degree of mannosylation [25]. In contrast to these *in vitro* findings, studies using isogenic mycobacterial mutants producing LM

and/or LAM with an altered mannosylation profile showed no specific phenotype in mice or adult zebrafish [26-28]. Our analysis of a *M. marinum* mannosyltransferase mutant producing LM and LAM with a comparable modified mannosylation pattern in the context of innate immunity did reveal an effect on virulence (chapter 6). In accordance with the *in vivo* studies mentioned above, the presence of the adaptive immune system reduced the attenuated phenotype of the mannosyltransferase mutant. Hence, the possibility to study the impact of mycobacterial factors solely in the context of innate immunity in the zebrafish system contributes toward detailed understanding of the role of these factors in virulence.

Our screen was not comprehensive, as the *M. marinum* genome comprises over 5,400 predicted coding sequences [29] and a saturating screen would require more than 25,000 random transposon mutants. This implies that numerous granuloma determinants remain to be defined. The finding that well-described virulence determinants such as the major ESX-1 substrates ESAT-6 and CFP-10 (chapter 2) were not identified in our screen supports this conclusion. Obviously, continuation of the screen until virtually all genes are analyzed would complete our knowledge of the *M. marinum* granuloma determinants. Screening of such enormous numbers of mutants is only feasible with a less labor-intensive method. Recently, a high-throughput injection robot coupled to an automated read-out system has been developed for the study of *M. marinum* infection in zebrafish embryos [30]. In this set-up, bacteria are injected in the yolk sac of embryos at the very early developmental stage, which results in the formation of similar aggregates as observed with intravenous *M. marinum* infection. This system offers interesting possibilities for further studies.

Finally, it would also be highly interesting to determine which *M. marinum* genes are not involved in granuloma formation, for instance to establish which ESX-1 genes are dispensable for granuloma formation. The mutants with an unaffected granuloma phenotype from our screen have been stored, and could be investigated. To perform such an analysis, a high-throughput sequencing technology capable of sequencing transposon-chromosome conjunctions in very large pools of bacterial mutant DNA is desirable. Such a technology, called TraDIS, was developed recently [31] and its utility for the identification of *M. marinum* genes involved in growth *in vitro* has been demonstrated (E.M. Weerdenburg, manuscript in preparation).

POSSIBLE IMPLICATIONS OF THE *M. MARINUM* ZEBRAFISH EMBRYO SCREEN

Our screen has proven highly instrumental in discerning factors involved in the relatively poorly defined granuloma response from the microbe's perspective. Furthermore, the genes that we have identified might also represent new targets for anti-tuberculosis drug design or vaccine development.

The increasing prevalence of drug-resistant *M. tuberculosis* strains emphasizes the urgent need for new drugs to treat tuberculosis, which necessitates the

identification of new drug targets. To highlight the utility of our screen for the discovery of drug targets, DprE1 was recently shown to be the target of two new, possibly potent, classes of anti-mycobacterial agents that rapidly kill *M. tuberculosis in vitro* and *in vivo* [32]. DprE1 is part of a heteromeric membrane-associated enzyme complex required for the synthesis of the fundamental cell wall constituent arabinogalactan. Its partner is DprE2, which we identified in our screen. Hence, compounds targeting DprE2 could represent active *M. tuberculosis* inhibitors as well. Among the factors that we identified as granuloma determining factors (**chapter 4**), LGT, required for lipoprotein synthesis, might be another interesting drug target. The phenotype of the *lgt* mutant mimics that of the *dprE2* mutant in terms of *in vivo* infection levels and growth in culture, which suggests that both molecules are similarly important for pathogenicity. In contrast to what is found in *M. marinum*, *M. tuberculosis lgt* is essential [33], and thus even more crucial for this species. It is not clear how *M. marinum* compensates for *lgt* disruption, a second *lgt* homologue does not seem to be present in the *M. marinum* genome. Possibly, expression of the mutated *lgt* gene results in a truncated protein with residual activity. Because of its membrane localization, LGT is easily accessible for drugs and its absence from humans makes it an ideal drug target, as was also proposed previously [33].

In addition to drug targets, the identified genes might also represent mutation targets for construction of live attenuated *M. tuberculosis* vaccine strains. Development of new vaccines against tuberculosis is imperative because the current *M. bovis* BCG vaccine shows variable protection against pulmonary infection in adults. While BCG vaccination is effective against tuberculosis in infants, its protective effect is thought to decline during adolescence, particularly in endemic tuberculosis areas [34]. Currently, it is not known why BCG efficacy decreases. Previous exposure to environmental mycobacteria, helminths or viruses that compromise host immunity may contribute to the limited efficacy of BCG [34]. It has also been proposed that the absence of more than 100 *M. tuberculosis* genes in BCG resulted in over-attenuation of the vaccine strain, making it unable to persist long enough within the host to generate long-lasting protective immunity [35]. In line with this theory, the partial deletion of the *esx-1* locus incapacitates *M. bovis* BCG to translocate from the phago(lyso)some into the cytosol of its host cell. This translocation is required for strong induction of $CD8^+$ T cell activation [36]. Because $CD8^+$ T cells contribute to optimal immunity and protection against tuberculosis, this might also be a contributing factor to the insufficient level of immunity induced by BCG. Indeed, a recombinant *M. bovis* BCG strain producing the membrane-perforating listeriolysin from *Listeria monocytogenes* that enables bacterial entry into the cytosol showed superior protection against *M. tuberculosis* as compared to parental BCG [37]. This recombinant strain is currently being evaluated as a vaccine and has reached phase II clinical trials.

For the mutations identified in this work (**chapter 4**), those that a) do not inhibit ESX-1 functionality to permit bacterial translocation into the cytosol, b) allow the bacilli to persist long enough in host cells to activate immune responses, and

c) lead to severe attenuation in embryonic and adult zebrafish, might represent potential targets for construction of vaccine strains. Two of the 23 mutations meet these criteria. The first mutation is that of *secA2*. Strikingly, it has been shown that immunization of mice with a *M. tuberculosis secA2* deletion mutant with additional auxotrophic mutations confers markedly enhanced protection against challenge with *M. tuberculosis* when compared to BCG [38] and this mutant is currently being analyzed in a phase I clinical trial. The second mutation is that of *ppk1*. Besides analysis of ESX-1 secretion as circumstantial evidence for translocation, electron microscopy of *in vitro* infected macrophages indeed confirmed cytosolic localization of the *ppk1* mutant (M. van Zon and N.N. van der Wel, unpublished results). While the *ppk1* mutant is not attenuated for initial persistence in macrophages (unpublished results), its attenuation in adult zebrafish was as severe as that of an ESX-1 deficient mutant. This indicates that a *M. tuberculosis ppk1* mutant could be a safe and immunogenic vaccine strain. However, although direct evidence is lacking, it has been suggested that *M. tuberculosis ppk1* is essential [39]. Alternatively, *M. tuberculosis* mutants in other genes required for maintaining the inorganic polyphosphate balance could be explored as vaccine candidates. The *M. tuberculosis ppx* mutant might be such a mutant, as polyphosphate levels, *in vitro* and *in vivo* virulence of this mutant were affected [40]. Obviously, further studies are required to evaluate the vaccine potential of these types of mutant strains.

Finally, the zebrafish system can also be used for the evaluation of *M. marinum* mutant strains as candidates vaccine against fish tuberculosis [41]. Our attempts to assess the vaccine potential of the *ppk1* mutant in adult zebrafish were unsuccessful (unpublished results). However, for predicting the protective efficacy of vaccine candidates intended to eradicate the human pathogen, it would be more significant to use the corresponding *M. tuberculosis* mutants in relevant animal models such as mice or guinea pigs.

CONCLUDING REMARKS

The work described in this thesis has provided insight in the complex process of granuloma formation, which characterizes tuberculosis and challenges tuberculosis treatment. The role of several mycobacterial factors as important determinants of granuloma formation was elucidated using the *M. marinum* zebrafish embryo infection model. Results obtained with *M. marinum* appear highly relevant for *M. tuberculosis*, which emphasizes the potential of this model system. Our approach can lead to the further elucidation of the relatively poorly-defined granuloma response and might provide stepping stones for the development of effective tuberculosis therapies.

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