Cluster 2: The pathogen perspective: Rational design of novel vaccine and drug candidates

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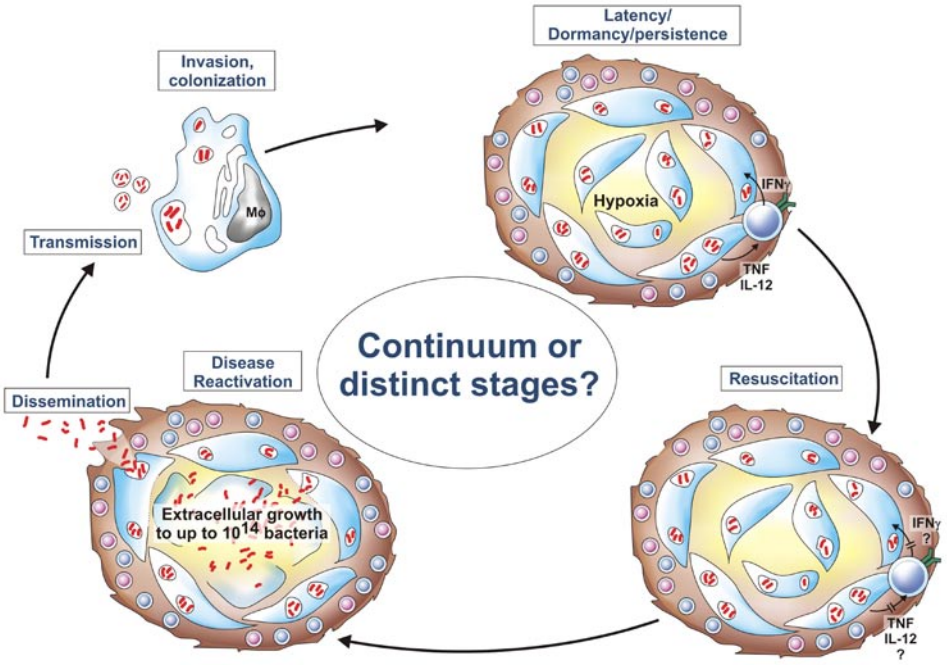

Executive summary

This cluster aims at a deeper understanding of the biology of the tubercle bacillus, *M. tuberculosis.* At the same time, we exploit our basic research to develop novel drugs and vaccines for the prevention and treatment of TB. The cluster is highly interdisciplinary. It ranges from global analysis of *M. tuberculosis* on the transcriptome and proteome level to structural and functional characterisation of distinct gene products as candidates for drug and/or vaccine development. It is furthermore accompanied by the development of new technologies for the elucidation of gene functions and interactions. Most of the projects are accomplished in close collaboration with in-house core facilities and external partners specialising in sequencing, transcriptomics, proteomics, bioinformatics and structural biology.

Global analysis of mycobacteria

[Global Analyses / Lee, Mattow, Rachman, Schreiber]

The overall objective of our transcriptomics and proteomics efforts is a better understanding of the biology of *M. tuberculosis* and the development of novel intervention measures for TB. Accordingly, our studies primarily aim at the identification of genes and proteins essential for pathogenicity, persistence and intracellular survival of mycobacteria, which we consider potential targets for vaccination, as well as diagnosis and chemotherapy

of TB. In the past, our analyses mainly focused on comparative profiling of virulent *M. tuberculosis* and attenuated *M. bovis* BCG strains (Mattow et al. 2003, Schmidt et al. 2004) as well as of *M. tuberculosis* strains with varying virulence such as H37Rv and its spontaneously derived avirulent mutant H37Ra. Since previous approaches failed to explain the avirulence of H37Ra we sequenced its genome using recently introduced massive parallel sequencing technology (454 sequencing). We identified four candidate genes that are mutated in the H37Ra strain and are essential for the virulent phenotype of H37Rv. One of these candidates acts as a transcriptional regulator. Therefore, it was also interesting to observe that target genes of this regulator are downregulated in H37Ra when compared to the H37Rv transcriptome (Rachman et al. 2006a). In addition, we have been using transcriptomics and proteomics to characterize targeted deletion mutants of *M. tuberculosis* (to identify downstream regulatory effects), and to monitor global changes in mycobacterial gene expression and protein abundance caused by altered physiological and/or environmental conditions.

[Transcriptomics of live tissue / Rachman]

DNA microarray experiments along with computational methods have been applied to carry out genome-wide expression profiling of *M. tuberculosis* isolated from infected human lung tissue and bone marrow-derived macrophages. These studies identified several genes differentially regulated during host infection (Rachman et al. 2006b, 2006c). Some of these genes have been selected for further analysis of their potential as drug targets. The results of these studies have also given us clues about characteristics that exist at the sites of pulmonary TB, which form the basis for further analyses. For example, results in global gene expression profiling of *M. tuberculosis* residing in caseous lesions of TB patients indicated an abundance of methylglyoxal (MG) at these sites. Moreover, a significant number of macrophages in these lesions undergo apoptosis. Therefore, we asked whether MG in macrophages determines apoptotic events during TB. We demonstrated elevated levels of MG and advanced glycation endproducts (AGE) during infection of macrophages and found abundant AGE in pulmonary lesions of TB patients (Rachman et al., in press). Global gene expression profiling of MG treated macrophages revealed a diverse spectrum of functions induced by MG including apoptosis and immune stimulation. Finally, we found that TNF-α and AGE interact in the induction of macrophage apoptosis. These results provide first evidence for an involvement of MG and AGE in TB.

[Role of MG and AGE in TB / Rachman]

[In vitro proteomics / Mattow]

2-DE-based comparative proteomic profiling of *in vitro* grown *M. tuberculosis* and bacilli isolated from phagosomes of infected host macrophages revealed several mycobacterial proteins unique to the intraphagosomal stage (Mattow et al. 2006) such as the conserved hypothetical protein Rv1130 which is currently being assessed for its role in intracellular survival of mycobacteria. We recently extended our transcriptomics and proteomics efforts to the functional characterisation of hypervirulent *M. tuberculosis* Beijing/W and *M.*

africanum isolates to identify signatures indicative for their hypervirulence. Preliminary results of the genome comparison of *M. tuberculosis* H37Rv and clinical isolates of the Beijing/W genotype family revealed DNA sequence differences in distinct genes which were induced *in vivo* . These differences may contribute to the differential virulence between *M. tuberculosis* H37Rv and clinical isolates of the Beijing/W genotype family. Therefore, characterisation of these genes in terms of function and structure may provide basic information for drug development against these hypervirulent strains.

Cooperations

- Stefan Niemann, National Reference Center for Mycobacteria, Borstel
- Michael Strong, David Eisenberg, Howard Hughes Medical Institute, University of California, Los Angeles (USA)
- George A. Kosmiadi, Central Tuberculosis Research Institute, Moscow (Russian Federation)
- Johannes Schuchhardt, MicroDiscovery GmbH, Berlin

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[Vaccines / Baumann, Behnck-Knoblau, Desel, Mir, Nasser Eddine, Reece, Steinhoff, Mittrücker, Schreiber]

Vaccine Research

The work of the vaccine research group is directed towards establishing novel vaccination strategies against TB in mouse models (Nasser Eddine and Kaufmann 2005; Kaufmann et al. 2006; Nasser Eddine et al., 2006; Baumann et al., 2006). Our main areas of interest are:

1. Construction of novel recombinant rBCG strains for live pre-exposure prime vaccination;
2. Construction of novel subunit vaccines mostly for post-exposure booster vaccination;
3. Development of mouse models for latent TB Infection (LTBI);
4. Impact of environmental mycobacteria on vaccine efficacy.

M. tuberculosis has a striking capacity to evade the host immune system and to prevent its elimination. As a response to the vigorous immune response encountered by *M. tuberculosis* after infection of humans, bacteria induce a dormancy program enabling survival within host macrophages. But once immune surveillance fails, dormant bacteria will be resuscitated, frequently resulting in active TB.

The only available vaccine against tuberculosis is *M. bovis* BCG, one of the safest vaccines known. However, its protective effect against pulmonary TB in adults is debatable and protection wanes with time. *M. tuberculosis* expresses different antigens during the dormant state as compared to active replication and immune mechanisms operative in active TB and in LTBI differ significantly. As a consequence, successful vaccination strategies should combine pre-exposure and post-exposure approaches to prevent active disease and protect LTBI harbouring dormant bacteria.

[Improvement of BCG / Nasser Eddine]

Our attempts to improve the efficacy of BCG against TB are aimed at increasing its immunogenicity. To this end we have constructed rBCGΔureC:hly (Grode et al. 2005) which has improved efficacy over BCG against pulmonary TB in the mouse. Even though the precise mechanisms of improved protection of the rBCGΔureC:hly over BCG remain to be fully elucidated, we assume that this vaccine strain (i) enhances cytosolic antigen loading of MHC I molecules in infected antigen presenting cells to prime CD8 T cells and (ii) promotes cross-priming by induction of apoptotic cell death of infected macrophages.

In an effort to compare the immune responses induced by rBCGΔureC:hly and BCG in bone marrow macrophages, we did not detect major differences in the host mRNA responses that were interrogated when using macrophages from adolescent (6-8 week) old mice. In contrast there were significant differences

between rBCGΔureC:hly and BCG induced responses if the macrophages were prepared from older mice (> 18 months). Currently, we are investigating the general implications of this phenomenon. rBCGΔureC:hly is expected to enter clinical trials in 2007 by the Vakzine Projekt Management GmbH who holds a world wide license for the further development towards application of the vaccine candidate.

Immunology

We aim at identifying antigens important for protection against both active TB and LTBI. The antigens so far known to induce protection comprise early-secreted antigens (CFP-10, ESAT-6, Ag85), the dormancy related antigens (HspX), and Rpf regulated genes (Rv3407). We have designed a DNA vaccine construct of antigen Rv3407 (Mollenkopf et al. 2004), Ag85A and HspX in a single ORF under a single promoter. Our construct incorporates 2A sequence technology affording segmentation of a polypeptide comprising multiple antigens into individual proteins. We are exploring this strategy to effectively deliver multiple antigens for multi-subunit vaccination approaches which could cover different stages of infection, and hence be suitable for vaccination both pre and post-exposure.

[Post exposure and boost vaccination / Baumann, Desel, Mir, Mollenkopf, Nasser Eddine]

To further address the need for new vaccines against dormant mycobacteria we use rBCGΔureC:hly as a carrier to express *M. tuberculosis* antigens associated with LTBI. We have prioritized antigens whose expression is upregulated during the dormant state for this purpose. Dormancy is linked to hypoxic conditions within the granuloma and surrounding tissue. DosR (Rv3133c) has been identified as the primary factor mediating the genetic response to reduced oxygen levels as well as exposure to low levels of nitric oxide. We will analyse this construct in prime-boost as well as in pre- and post-exposure conditions.

To facilitate harmonized efficacy testing of recently identified latency antigens in post-exposure vaccination experiments, dormancy models for *M. tuberculosis* infection are being established in cooperation with the Statens Serum Institute, Copenhagen and University of Oxford. The latency-associated Hspx was selected as a model antigen for dormancy. Each partner uses different vaccine formulations i.e. naked DNA, recombinant protein or MVA and also different drug regimes and mouse strains. In order to compare the different dormancy models, all vaccine constructs were exchanged. One model introduced by the Statens Serum Institute appears most promising and we are currently testing naked DNA vaccination with three DosR-regulated dormancy antigens in this model.

We participate in the Grand Challenge Consortium GC12-82 funded by the Bill and Melinda Gates Foundation aimed at developing a post-exposure vaccination schedule for LTBI. The final goal of this consortium is to combine a rBCG construct prime with a subunit vaccine boost to enhance protection against new infection with *M. tuberculosis* or reactivation of dormant *M. tuberculosis*. Our contribution involves construction of rBCGΔureC:hly expressing *M. tuberculosis* antigens associated with dormancy, starvation

[Construction of BCG-based latency vaccines / Reece]

and reactivation of *M. tuberculosis*. We have recently completed work on a single construct expressing Rv1733c, Rv2659c, associated with latent infection and Rv3407, associated with reactivation of TB. This construct will be further evaluated for protection in pre and post exposure murine models for TB.

BCG protects children from disseminated disease and generates a significant degree of protection in rodent models. However, vaccine trials in adults have given inconsistent results. Here, the efficiency of BCG-vaccination varies in different populations and regions from 80 to 0%, with a higher failure of protection in developing countries. Environmental mycobacteria (also called mycobacteria other than TB (MOTT)) are normal inhabitants of a wide variety of environmental reservoirs and occupy nearly every niche.

The range of efficiency in BCG-mediated protection, for which a previous exposure to environmental mycobacteria in developing countries has been postulated, and the need to develop a mucosal vaccine against TB initiated the following investigations.

i) To test the influence of pre-existing immunity induced by MOTT on oral BCG vaccination

ii) To investigate the impact of the endogenous intestinal flora on the efficacy of an oral vaccine against *M. tuberculosis*.

[Oral BCG vaccination / Steinhoff, Behnck-Knoblau, Mittrücker]

First, safety and efficiency of the oral route were analyzed and compared to the intravenous application of different BCG vaccine-strains. Next, the influence of MOTT exposure on BCG vaccination was analyzed in conventional (SPF) and germfree (GF) mice. We found that oral vaccination with BCG was safe and efficient in protecting against *M. tuberculosis* infection in SPF and in GF mice. Thus, the intestinal microflora does not seem to impact on oral BCG vaccination. Further, oral presensitisation with MOTT did not hamper growth and spread of the BCG Pasteur vaccine irrespective of the intestinal flora and had no influence on BCG mediated protection in SPF mice. First results in conventional animals indicate that MOTT given by drinking water were cleared on day 126. However, BCG vaccinated GF mice previously exposed to MOTT were highly sensitive to *M. tuberculosis* challenge (animals either died after challenge, or showed elevated *M. tuberculosis* titers in spleen and lung), despite unaltered BCG persistence. Vaccination studies with the BCG-Moreau strain gave similar results and demonstrated that MOTT-presensitized, GF animals had increased *M. tuberculosis* titers compared to controls (naïve mice). This indicates that previous MOTT-exposure interfered with protection against *M. tuberculosis* only in GF mice although it had no direct influence on the persistence of the BCG vaccine strain. To further study the influence of MOTT in GF mice, we currently compare the impact of MOTT presensitization on the course of *M. tuberculosis* infection in GF and SPF mice. Additionally, persistence of MOTT in GF mice is being analyzed.

In a complementary approach, mice were i.v. or orally vaccinated with BCG and challenged with *M. tuberculosis* via aerosol infection. Both after vaccination

and challenge infection, titers of mycobacteria and T cell responses against conserved mycobacterial antigens were determined in different tissues and at various time points post vaccination and post challenge. Intravenous vaccination resulted in dissemination of BCG to virtually all tissues analysed and mycobacteria-specific T cells were detected in spleen, lung and liver. After oral vaccination, mycobacteria were restricted to gut-associated tissues, and there was only sporadic dissemination into other organs. We did not detect specific T cells in any tissue analysed including mesenteric lymph nodes and lamina propria of the small intestine. However, both routes of vaccination resulted in similar protection against challenge infection with *M. tuberculosis*. Protection correlated with prompt accumulation of mycobacteria-specific $CD4^+$ and $CD8^+$ T cells in lungs of vaccinated and challenged mice. At later time points, frequencies and numbers of specific $CD8^+$ and particularly $CD4^+$ T cells in spleen and lung correlated with bacterial titers rather than the level of vaccination induced protection. In future studies we aim at characterizing vaccination induced T cells in detail. The long-term goal is to define phenotypic and functional characteristics that allow correlation of vaccination-induced T cell responses with protection against challenge with *M. tuberculosis*.

Cooperations

- Vakzine Projekt Management GmbH, Hannover
- Peter Andersen, Statens Serum Institute, Copenhagen (Denmark)
- Adrian Hill, University of Oxford (UK)
- JoAnne Flynn, University of Pittsburgh (USA)
- Tom Ottenhoff, Leiden University Medical Center, Leiden (NL)
- Jerry Sadoff, AERAS Global TB Vaccine Foundation, Rockville (USA)
- Gary Schoolnik, Stanford University, (USA)

Third Party Funding

- EU FP6 IP MUVAPRED
- EU FP6 IP TBVAC
- Bill & Melinda Gates Foundation Grand Challenges in Global Health GC12-82

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[Mycobacterial adjuvants / Niemeyer, Mattow, Schaible]

Dual capacity of mycobacterial Phosphatidylinnositol-mannosides (PIMs): Ability to stimulate immune responses via TCR and TLR mediated induction

Mycobacterial Phospatidyl-innositol mannoside (PIM) is the first mycobacterial lipid identified as antigenic and is recognized by murine and human NKT cells in the context of CD1d. However, mycobacterial lipids are also recognized by toll-like receptors (TLR) and thereby induce immune responses. We examined the expression of TLR2 and TLR4 on NKT cells. In contrast to a previous report showing that NKT cells express mRNA for TLR2, we could not detect intracellular or cell surface TLR2 or TLR4 expression. However, we found that PIM containing lipid fractions from *M. bovis* BCG possess the dual capacity to stimulate NKT cells directly by inducing a TLR2 mediated immune response. We fractionated and purified glcolipids from mycobacteria and found that not only crude extracts were able to activate via TLR2 but also semi-purified PIM. Lipid fractions with higher mannosylated PIM were recognized by TLR2 but failed to directly stimulate NKT cells via CD1d - TCR interactions. In contrast, lipid fractions containing lower mannosylated PIM were able to stimulate immune responses by both mechanisms. Similar results were observed when PIM containing fractions were treated with phospolipase. Since PIM induce an immune response by diverse routes we assessed the ability of PIM as a vaccine or adjuvant to protect against *M. tuberculosis* infection. We vaccinated mice with *M. bovis* BCG or PIM fractions that induced both TCR and TLR mediated immune responses. However, none of the tested lipid fractions, including a semipurified mix of PIM2 and Phospatidyl-innositol (PI), were effective either as vaccine or as adjuvant against *M. tuberculosis*. Moreover, we did not identify the precise antigen responsible for the activation of NKT cells and the induction of a TLR2 mediated responses.

Cooperations

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- Germain Puzo, Martine Gillerone, Université Toulouse (France)

Functional and structural characterisation of *M. tuberculosis* gene products

[Nasser Eddine, Warrier]

This endeavour benefits remarkably from a productive collaboration with structural biologists at the European Molecular Biology laboratory headed by Matthias Wilmanns. Thus far, this consortium has crystallized more than 30 gene products of *M. tuberculosis* based on our target selection. The availability of the genome sequence of *M. tuberculosis* provides a wide range of novel targets for the design of novel drugs and vaccines. We have applied functional genomic tools, such as transcriptomics and proteomics for the identification of genes and gene products involved in controlling vital aspects of the biology of *M. tuberculosis*. We apply molecular genetics, cell biology, structural biology and combinatorial chemistry to elucidate drug targets and identify new approaches for TB prevention and therapy.

[Rational drug development / Nasser Eddine, Warrier, Mattow]

Targeted genes were selected on different bases. Beside genes encoding key metabolic enzymes (e.g. fumarate reductase), enzymes involved in cell envelop processes (e.g. Rv2136c and *rml*), or information pathway components (e.g., *mrr*), several targets were selected based on genome-wide expression profiles of *M. tuberculosis* isolated from infected human lung tissue (e.g. Rv2642) assessed by microarrays.

[Crystal structures / Wilmanns, Nasser Eddine, Rachman]

We have isolated 12 different mycobacterial deletion mutants in order to understand the biological functions of crystallized gene products. Deletions were confirmed by molecular analysis and functional assays (where available) and compared to wild-type strains with respect to colony morphology, growth in culture and in macrophages and in animal experiments for attenuation and potential usefulness as vaccines. Additionally, downstream effects of inactivated enzymes have been studied by transcriptome (DNA-Arrays) and proteome analyses (2-DE and MALDI-MS). The combination of functional and structural analyses will provide a basis for drug discovery efforts (Janowski et al. 2006; Qingiun et al. 2006; Holton et al. in press).

In an attempt to better understand the biological role of distinct proteins in survival of *M. tuberculosis* in the host, small molecule inhibitors of these proteins are analysed for inhibition of survival of *M. tuberculosis* in liquid culture, in host macrophages and further during *in vivo* infection in a mouse model.
The first set of target *M. tuberculosis* proteins include Cyp51, a sterol demethylase, MptpA and MptpB, the pair of mycobacterial tyrosine phosphatases, and Antigen 85C, a mycolyl transferase. Further on, we are

also interested in unravelling the mode of action of these compounds on *M. tuberculosis* survival.

Cooperations

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- Combinature Biopharm AG, Berlin
- Forschungsinstitut für Molekulare Pharmakologie (FMP), Berlin
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Applying novel technologies to improve our understanding of the mycobacterial biology

[Beisiegel, Patzel, Schreiber, Köberle]

In TB research, the search for novel drug targets and antigens is severely hampered by insufficient knowledge of the biology of the pathogen. Currently only half of the mycobacterial genes in available databases have assigned functions where there are around 2000 genes whose biological role still remains largely enigmatic. Similarly, our knowledge of the regulatory circuits underlying the bacterial response to environmental stress and the host immune system is limited. Recently developed technologies to address these issues have not yet been applied to mycobacteria to investigate the role, function and interaction of mycobacterial genes and their respective protein products.

[Protein-protein interactions / Beisiegel]

The Tandem Affinity Purification (TAP) technique investigates protein-protein interactions by successive rigid purification of a tagged protein with its interacting complex partners. In contrast to other approaches like the Yeast Two Hybrid technique, interactions can be measured directly thus greatly facilitating our understanding of protein functions and underlying mechanisms. TAP has been established in yeast and is currently also being applied in *E.coli* and eukaryotic cell culture systems. We have successfully used TAP

to identify mycobacterial interaction partners and are currently investigating interactions of mycobacterial molecules with host proteins.

[ChIP-chip / Schreiber]

Chromatin immunoprecipitation (ChIP) combined with microarray based analysis (ChIP-chip) has been used widely in yeast, higher eukaryotes and *E. coli*. It determines the genomic DNA binding patterns of transcriptional regulators. We have established this technology to investigate the primary target genes of mycobacterial transcription factors essential for virulence and dormancy. The results will not only deliver novel insights into regulatory mechanisms but will also be combined with other binding and transcriptome data in order to describe and predict the behaviour of mycobacteria in the context of systems biology .

[RNAi / Patzel, Köberle]

Promising microbial and host-related targets identified with the TAP and ChIP technologies can be functionally validated using RNA interference (RNAi). In addition to conventional siRNA-mediated knock down of host genes we will make use of an advanced siRNA design and novel siRNA-based technologies for knocking down mycobacterial genes. The effects of functional gene knock down will be monitored *in vitro*, *ex vivo*, as well as *in vivo*, e.g. in experimental animal infection models (Patzel et al. 2005, 2006; Köberle et al 2006).

Cooperations

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- Tom Ottenhoff, Leiden University Medical Center, Leiden (NL)
- Jan Neefjes, The Netherlands Cancer Institute, Amsterdam (NL)

Third Party Funding

- RiNA Network (jointly funded by EFRE, BMBF, and Chiron Corp)
- BMBF Network PathoGenomikPlus

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