REPORT

New issues in tuberculosis

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Tuberculosis remains a major health problem worldwide. The disease is caused by *Mycobacterium tuberculosis* whose preferred habitat is the host macrophage. The immune response against tuberculosis is mediated by different subsets of T cells including both conventional CD4 and CD8 T cells as well as unconventional CD1 restricted and $\gamma\delta$ T cells. The CD1 restricted T cells are particularly remarkable because they recognise the glycolipids abundant in the mycobacterial cell wall. Although a vaccine, *M. bovis* BCG, is available which protects toddlers against miliary tuberculosis, it is ineffective in preventing pulmonary tuberculosis in adults. Therefore, a novel vaccine is urgently required. Knowledge about the functioning of different T cell populations during infection and disease provides the basis for rational vaccine design. We have constructed a recombinant BCG vaccine which, compared with wild-type BCG, induces superior protection not only against laboratory strains but also against clinical isolates of *M. tuberculosis*.

Tuberculosis remains a major global health threat having killed every fourth person of the eight to nine million people who developed this disease in 2003. Two billion people are infected with the aetiological agent, *Mycobacterium tuberculosis*. In the World Health Organization (WHO) European Region (which also includes the states of the former Soviet Union), 200 million people are currently infected with *M. tuberculosis*, resulting in 50 000 new cases of disease every year.¹ A rough indicator of the prevalence of individuals infected with *M. tuberculosis* can be estimated by multiplying the incidence rate of diseased individuals by a factor of 50–200. Although the overwhelming majority of infected individuals will harbour the pathogen lifelong, only 10% will develop active disease. These figures, however, are compounded by the global epidemic of acquired immunodeficiency syndrome (AIDS). In 2003 more than 15 million people were coinfecting with the human immunodeficiency virus (HIV) and *M. tuberculosis*, and of the approximately three million deaths of individuals with HIV infection, at least 600 000 died of tuberculosis. In fact, *M. tuberculosis* infection represents the major risk factor for mortality amongst individuals with HIV infection.

Tuberculosis can be treated with chemotherapy. However, the treatment is lengthy, requiring a combination of at least four drugs for six months. This long drawn out and complicated treatment schedule often affects compliance, thus resulting in development of resistant strains. In several countries, single resistant strains are responsible for more than 25% of all new cases of tuberculosis. In the Baltic states single resistant tuberculosis accounts for ~30% of all new cases. Although single resistance does not complicate drug treatment significantly, once an *M. tuberculosis* strain has developed resistance to one drug, development of multidrug resistance (MDR) is greatly increased. Worldwide, about 50 million people are now infected with MDR

M. tuberculosis strains, which accounted for 300 000 new cases in 2003.

MDR is defined as resistance against at least two first line drugs, generally isoniazid and rifampicin. However, almost 80% of all new cases of MDR tuberculosis are now due to resistance to three or more drugs. This not only hampers treatment for the affected individual but also exacerbates the cost of treatment by about 100-fold from about €10 to €1000 in developing countries and from €2000 to €200 000 in the industrialised nations. In other words, treatment of one case of MDR tuberculosis costs as much as treatment of 100 “normal” cases of tuberculosis. Obviously, this is a major issue in countries with low income rates and poorly developed public health systems. Even worse, cure of MDR tuberculosis can become impossible. In most infectious disease scenarios, the consequence of multiple risk factors is not additive but synergistic, and the combination of HIV and MDR tuberculosis is a fatal avalanche. Therefore, the coexistence of high prevalence of MDR tuberculosis and escalating HIV infection rates will not only increase the risk of tuberculosis outbreaks in individuals with HIV infection but also result in a dramatic spread of disease and development of additional mechanisms of drug resistance. This can be illustrated by comparing two polar scenarios. Given the optimal control measures for tuberculosis, infection of about 100 individuals will lead to five active cases of tuberculosis that then will newly infect one individual. In the worst case scenario—for example, coinfection with HIV and MDR tuberculosis, 50/100 infected individuals will develop active disease who will then spread the infection to 400 additional individuals. This is an amplification factor of about 400.

TUBERCULOSIS IN THE NEW EUROPEAN UNION MEMBER STATES

On 1 May 2004 the European Union (EU) admitted 10 new member states. Among them are several Eastern European states, including members of the former Soviet Union. This is a major challenge for the new EU not only from an economic standpoint but also from the public health point of view. Open borders between the EU member states, as much as they are desirable for many reasons, not least the political, will facilitate spread of infections. Of particular note is the situation with regard to tuberculosis. While we have witnessed decreasing incidence in the vast majority of the “old” EU member states, tuberculosis remains a significant health threat in most Eastern European entry countries (fig 1). With incidence rates ranging between 50/100 000 and 80/100 000 inhabitants, the situation is worst in the Baltic states of Estonia, Lithuania, and Latvia¹ followed by Hungary and Poland with incidence rates above 30/100 000 (which are in the same order of magnitude as the “old” EU member states of Portugal and Spain), and Slovakia and Slovenia with incidence rates above 20/100 000. In contrast, the reported

Abbreviations: BCG, bacille Calmette Guérin; hly, listeriolysin; MDR, multidrug resistance; MHC, major histocompatibility complex

Figure 1 Reported rates of incidence of tuberculosis in the 10 new member states of the European Union and Germany (2002).

incidence rates of 14/100 000 in the Czech Republic are comparable with those in most “old” EU member states, and those in Malta and Cyprus are amongst the lowest.

The situation is further worsened by two confounding issues that are not mutually exclusive: first, the burgeoning incidence of HIV and secondly, the escalating proportion of MDR tuberculosis cases (fig 2). Between 1999 and 2002 the rate of incidence of individuals with HIV infection increased about 80-fold in Estonia and about fivefold in Lithuania and Latvia.¹ It has been estimated that almost 1% of all adult Estonians are HIV positive. Most cases of HIV are found among young adults who share needles for injecting drugs. Of all new cases in Estonia 80% are younger than 25 years of age.

As expected, more recently, HIV is spreading further through sexual intercourse among homosexual and heterosexual individuals. Coinfection with HIV and *M. tuberculosis*

Figure 2 The high prevalence of multidrug resistant tuberculosis in Latvia, Lithuania, and Estonia.

increases the risk of developing tuberculosis almost 30-fold, and HIV infection is the major risk factor for developing active tuberculosis.

THE DISEASE AND PROTECTIVE ROLE OF THE UNDERLYING IMMUNE RESPONSE

Tuberculosis is a disease of the lung, which serves as both the port of entry for the pathogen as well as the major site of disease manifestation.² Pulmonary tuberculosis accounts for the vast majority of cases in adults and is transmitted by the aerogenic route. An individual with active pulmonary tuberculosis expels small droplets containing tubercle bacilli, which can be inhaled by another individual in the vicinity (fig 3). If these small droplets enter the alveolar space, the pulmonary dendritic cells and macrophages engulf the microorganisms. Some infected macrophages will remain in the lung tissue while some infected dendritic cells will migrate to the draining lymph nodes. T cells in the draining lymph nodes will be activated and on migration recognise the mycobacterial foci in the lung. Granulomatous lesions form and contain the bacteria, preventing development of active disease.³ In immunocompetent individuals infection is arrested at this stage. This is illustrated by the fact that of the two billion people infected with *M. tuberculosis* only eight to nine million will develop disease annually. Hence, the immune response stimulated during infection is highly efficacious in the vast majority of infected individuals. However, control of infection is incomplete and the pathogens are not eradicated, so that the risk of reactivation, even decades after infection, remains. Although it rarely happens, reinfection can also occur.³ If the balance between the host's defences and the persisting mycobacteria is tipped in favour of the pathogen, active disease occurs.^{2,4}

T lymphocytes are central to the control of *M. tuberculosis* infection.^{2,4} Several different T cell populations contribute to protection, and it appears that some of these T cells develop as a specific counter-measure against the mycobacteria (see fig 3). Without doubt, CD4 T cells which recognise the antigenic ligands presented by gene products of the major histocompatibility complex (MHC) II molecules are the main players in the field of tuberculosis control. In addition, CD8 T cells which recognise antigenic ligands presented by MHC I molecules participate in protective immunity. The contribution of the so called unconventional T cells—that is, $\gamma\delta$ T cells and CD1 restricted T cells, is less clear.² However, recent findings in non-human primates have confirmed those of experimental studies of tuberculosis in mice that $\gamma\delta$ T cells contribute to protection against tuberculosis.⁵ These $\gamma\delta$ T cells, as well as the CD1 restricted T cells, mitigate the dogma that T cells recognise MHC/peptide complexes only. The $\gamma\delta$ T cells are specific for small phospholipids—that is, low molecular weight metabolites comprising phosphate; the CD1 restricted T cells recognise the glycolipids abundant in the mycobacterial cell wall.

The survival strategy of *M. tuberculosis* in the infected host focuses on the macrophages that serve as the main habitat.⁶ Because macrophages have potent antimicrobial capacities, *M. tuberculosis* has developed mechanisms to overcome these host defence mechanisms, based mainly on the arrest of phagosome maturation (fig 4). A phagosome containing a microbe other than *M. tuberculosis*—for example *Escherichia coli*, will mature from early through to late stages accompanied by acidification of the phagosomal milieu. The acidic milieu of the late phagosome promotes fusion with lysosomes. Moreover, in the late phagosome, reactive oxygen and nitrogen metabolites form, which are critical for microbial killing. After the lysosomal enzymes find their pH optimum in the acidified phagosome, they degrade the microbes. However, by arresting phagosome maturation at an early

Figure 3 Infection, course of the disease, and the immune mechanisms activated in tuberculosis. IFN, interferon; IL, interleukin; LT, lymphotoxin; RN/OI, reactive nitrogen/oxygen intermediates; TNF, tumour necrosis factor; TLR, toll-like receptor.

stage, *M. tuberculosis* prevents its killing and degradation. Neutralisation of the phagosomal pH is critical for arrest of phagosomal maturation.

The antimicrobial capacities of macrophages need to be induced—that is, resting macrophages have only weak

antimicrobial capacities. Stimulation by appropriate cytokines activates the full spectrum. The T cell product interferon gamma ($\text{IFN}\gamma$) is the most potent mediator of this process² further supported by members of the tumour necrosis factor (TNF) family; $\text{TNF}\alpha$ is produced mostly by the macrophages themselves and $\text{TNF}\beta$ (or lymphotoxin) is a product of T lymphocytes. In addition, T cells can also lyse and directly destroy the mycobacteria in infected macrophages.

After engulfment by macrophages or dendritic cells, *M. tuberculosis* remains in the phagosome. Hence, its antigens have ready access to the MHC II molecules that pick up peptides in phagosomal compartments, which they then transport to the cell surface to present to CD4 T cells. In contrast, MHC I molecules are loaded in the cytosolic compartment. Hence, their main purpose is the presentation of viral antigens. The question therefore arises: How are mycobacterial antigens introduced into the MHC I processing pathway? The two most likely options are given below.⁷

Figure 4 Intracellular events following engulfment of *M. tuberculosis* by macrophages. MR, mannose receptor; LAMP-1, lysosome associated membrane protein-1; LBP, lysobisphosphatidic acid; SPR, surfactant protein receptor; TACO, tryptophan aspartate containing coat protein; TLR, toll like receptor (TLR).

- Recent experiments have shown that the phagosomal membrane is equipped with the MHC I processing machinery. This so called cross-presentation pathway can allow loading of MHC I molecules with peptides derived from phagosomal antigens.⁸⁻⁹
- M. tuberculosis* induces apoptosis in infected macrophages. This results in the formation of vesicles containing antigenic cargo that can be shuttled to bystander dendritic cells. Uptake of these vesicles directs the antigenic cargo to the MHC I presentation machinery.¹⁰ This mechanism has been termed as “cross-priming” (fig 5). On the basis of our recent findings, we consider cross-priming the major

pathway for presentation of antigens from *M. tuberculosis* to MHC I. While *M. tuberculosis* induces profound apoptosis in infected host cells, the vaccine strain *M. bovis* BCG (bacille Calmette Guérin) has poor apoptosis inducing activity. This difference could explain the fact that CD8 T cells are readily induced during *M. tuberculosis* infection but not in response to *M. bovis* BCG vaccination in the mouse.

We isolated vesicles from apoptotic macrophages infected with *M. tuberculosis* and determined the nature of their cargo as mycobacterial glycolipids and polypeptides.¹⁰ Addition of these vesicles to co-cultures of dendritic cells and specific T cells induced profound T cell stimulation. Both MHC I restricted and CD1b restricted T cells were stimulated as suggested by blocking experiments with appropriate anti-MHC I and anti-CD1b monoclonal antibodies.

While cross-priming appears essential for stimulation of MHC I and CD1 restricted T cells, it could also improve antigen presentation by MHC II for CD4 T cells. Macrophages, the preferred habitat of *M. tuberculosis*, have weaker antigen presenting capacity than dendritic cells. Moreover, it is well established that *M. tuberculosis* impairs antigen presentation via MHC II to CD4 T cells. Hence, antigen translocation through cross-priming from infected macrophages to bystander dendritic cells would also improve antigen presentation by MHC II molecules.²

CD1 and MHC I molecules have many similarities, although CD1 molecules are encoded outside the MHC.² The CD1 family has two groups: 1 and 2. Although the group 1 CD1 molecules are absent in rodents (mice and rats), they are present in humans, and their presentation spectrum makes them particularly interesting for tuberculosis. The most abundant glycolipids in the mycobacterial cell wall, such as mycolic acids, lipoarabinomannan, or glucose monomycolate, are ligands for group 1 CD1 molecules. It is

therefore likely that T cells with specificity for these glycolipids participate in the control of tuberculosis. The mycobacteria shed glycolipids, which in this way can be loaded onto CD1 molecules. Most frequently, however, the glycolipids are integrated into the phagosomal membrane and thus prevented from interacting directly with CD1 molecules. We recently showed that saposins promote loading of glycolipids onto CD1 molecules.¹¹ Saposins are derived from a precursor molecule, prosaposin, and four different saposin family members can be distinguished: saposin A, saposin B, saposin C, and saposin D. Saposins have been described as cofactors for enzymatic sphingolipid hydrolysis. Although enzymatically inactive, they facilitate interactions between the respective enzyme and its substrate. For example, saposin C acts as a cofactor for β -glucosidase in glucosyl ceramide degradation. We found that saposin deficient cell lines failed to stimulate T cells with specificity for CD1b/glycolipid antigen.¹¹ Reconstitution with saposin C, but not with saposin A, B, or D, fully restored glycolipid specific T cell stimulation. Moreover, we demonstrated co-localisation of lipoarabinomannan, CD1b, and saposin C in phagolysosomes. We assume that saposin C extirpates glycolipids from phagosomal membranes, guides the glycolipid to the CD1 molecule, and in this way facilitates interactions between mycobacterial glycolipids and CD1 molecules in the phagosomal compartment. Our studies dealt with glycolipid loading of the group 1 molecule, CD1b. Two more studies by other researchers published around the same time identified saposin involvement in glycolipid presentation by group 2 CD1 molecules.^{12, 13}

A RATIONAL VACCINE DESIGN

There is general agreement that satisfactory control of tuberculosis ultimately needs a novel vaccine as an adjunct

Figure 5 Cross-priming as a major mechanism for stimulation of major histocompatibility complex (MHC) I and CD1 restricted T cells. DC, dendritic cell; ER, endoplasmic reticulum; TAP, transporter associated with antigen presentation.

Figure 6 The different vaccination strategies against tuberculosis (TB). (A) Bacille Calmette Guérin (BCG) protects against childhood tuberculosis but not against adult tuberculosis. (C) An improved BCG could provide protection against both childhood and adult tuberculosis. (C) Subunit vaccines given as a boost after the BCG prime could improve the protective immune response.

to improved chemotherapy.² The French microbiologists Calmette and Guérin developed the current vaccine, BCG, in the early decades of the twentieth century. They attenuated *M. bovis* by serial passage in a bile enriched culture medium. The resulting vaccine proved safe and when given to a newborn child of a tuberculous mother protected the toddler from developing the disease. Since then, BCG has been given to more than three billion vaccinees with an excellent safety record.¹⁴ Without doubt, BCG protects against childhood tuberculosis, but unfortunately it fails to protect against the most common form of the disease, pulmonary tuberculosis in adults. While this deficit has been known for quite some time, proactive vaccine research programmes have only recently been initiated. Our increasing knowledge and understanding of the mechanisms underlying the immune response in the natural course of tuberculosis—the mechanisms that contain *M. tuberculosis* within the foci in 90% of infected individuals and the mechanisms absent in the 10% who develop disease—will be beneficial in designing a novel, rational vaccine against tuberculosis.

In general, two strategies can be distinguished (fig 6). The first is based on the assumption that the few selected antigenic peptides identified by CD4 T cells in the context of MHC II and perhaps some CD8 T cells with specificity for antigenic peptides bound to MHC I are sufficient for a protective immune response. This strategy aims at the construction of a subunit vaccine composed of one or a few antigens. The second strategy assumes that as many antigens as possible should be presented to activate a larger repertoire of T cell populations comprising conventional CD4 and CD8 T cells as well as unconventional T cells. This strategy forms the basis of viable attenuated vaccines. In fact, the strategies are not mutually exclusive and it is becoming increasingly clear that a combination of the best candidates derived from both approaches in a heterologous prime/boost regimen may provide the best possible option. In this scenario, pre-exposure priming with a highly efficacious attenuated vaccine strain should be followed by postexposure boosting with a potent subunit vaccine (see fig 6). Obviously,

the antigen composition of the subunit vaccine candidate is strongly influenced by the time of administration. Pre-exposure subunit vaccines should comprise antigens that are expressed by *M. tuberculosis* immediately after infection. In contrast, a postexposure subunit vaccine should depend on antigens expressed by dormant *M. tuberculosis* in the late stages of infection. An example of a promising pre-exposure vaccine antigen is Antigen 85 whereas HspX has been predicted as a good candidate for postexposure vaccination.^{3 15}

On the basis of comparative proteome analyses of *M. tuberculosis* H37Rv and *M. bovis* BCG, we have identified more than 30 gene products which are differentially expressed in *M. tuberculosis* and *M. bovis* BCG.^{16 17} The vaccine efficacy of these genes was determined in a high-throughput vaccination schedule using naked DNA constructs in the mouse model (Mollenkopf *et al*, submitted for publication). An antigen, Rv3407, was identified that induced a level of protection comparable with that induced by *M. bovis* BCG in the mouse system. This vaccine candidate was also used as a booster vaccine following prime vaccination with BCG (Mollenkopf *et al*, submitted for publication). Boosting the naked DNA encoding Rv3407 further increased the protective activity induced by BCG prime. This antigen, therefore, is an interesting candidate for further prime/boost vaccination trials.

We have decided to further improve the available vaccine BCG. Our strategy is based on the following assumption (fig 7): in addition to CD4 T cells, CD8 T cells significantly contribute to the protective immune response induced during natural infection with *M. tuberculosis*.³ Hence, *M. tuberculosis* is capable of stimulating CD8 T cells. Although *M. tuberculosis* remains entrapped in the early phagosome, it induces profound apoptosis in infected macrophages, thus initiating cross-priming, which ultimately leads to CD8 T cell stimulation. As discussed above, we consider cross-priming an essential mechanism of stimulation of CD8 T cells by *M. tuberculosis*. In contrast, BCG is an ineffectual inducer of CD8 T cells although it is capable of stimulating a profound CD4 T cell response. This is most probably due to the fact that BCG,

Figure 7 Rationale for the design of an improved bacille Calmette Guérin (BCG) vaccine. (A) The two different survival strategies of BCG and *L. monocytogenes*. (B) Conferring bacterial egression into the cytosol by integration of the encoding listeriolysin into the BCG genome. (C) Improved listeriolysin (hly) activity expressed by urease deficient (Δ Ure) BCG.

first, remains in the early phagosome and, secondly, fails to induce sufficiently strong apoptosis in infected macrophages.^{3–10} Hence, its antigens are more or less prevented from contact with MHC I molecules. To improve the capacity of BCG to stimulate CD8 T cells, we took advantage of our knowledge of the survival strategies of another intracellular bacterium, *Listeria monocytogenes*.¹⁸ After engulfment by the

macrophages, this microorganism egresses from the phagosome into the cytosol; listeriolysin (hly) is essential and sufficient for this translocation. Once in the cytosol, proteins secreted by *L. monocytogenes* can be introduced to MHC I molecules, thus leading to CD8 T cell stimulation. Because *L. monocytogenes* does not interfere with acidification of the phagosome, hly readily finds its pH optimum of 5.5. In contrast, BCG arrests phagosome maturation by neutralising the phagosomal pH to about 6.5–7. The mechanisms employed by *M. bovis* BCG/*L. monocytogenes* are depicted in fig 7.

By introducing the gene encoding hly into the BCG genome, we equipped this vaccine with the capacity to perforate the phagosomal membrane.¹⁸ Although current evidence suggests that rBCG-hly does not egress into the cytosol, we assume that it allows perforation of the phagosomal membrane, thus facilitating translocation of mycobacterial antigens into the cytosol where they can be loaded onto MHC I molecules (see fig 7). This rBCG-hly has been used as a vaccine against aerosol challenge with *M. tuberculosis*. Indeed, at later time points of tuberculosis (>20 weeks), protection induced by the rBCG-hly was found to be significantly better than that induced by wild-type BCG (Grode *et al*, submitted for publication).

To further improve the protective activity, we used a urease deficient BCG strain kindly provided by the group of B Gicquel, Paris. This BCG mutant lacks the urease gene (Δ UreBCG), and thus it impairs neutralisation of the phagosomal pH. Δ Ure rBCG was equipped with the hly gene, resulting in the Δ Ure rBCG-hly mutant. Indeed, preliminary evidence suggests that phagosomes harbouring Δ Ure rBCG-hly are acidified and, accordingly, provide an optimal pH for hly activity (Grode *et al*, submitted for publication). Most importantly, the protective capacity was further improved and Δ Ure rBCG-hly reduced the load of *M. tuberculosis* in aerosol infected mice by more than 2 log between week 4 and 13 after an aerosol challenge with *M. tuberculosis*. In contrast, wild-type BCG reduced the bacterial load by about 1 log over this period.

The laboratory strain *M. tuberculosis* H37Rv used in the experiments differs from clinical isolates in several aspects. Notably, the highly worrying members of the “Beijing/W” *M. tuberculosis* family which have started to conquer the world have several unique features.¹⁹ The family has a highly conserved genotype often encoding MDR. The Beijing/W family members, for example, were responsible for the MDR tuberculosis outbreak in New York in the early 1990s, and for numerous outbreaks of tuberculosis in the Beijing region of China.¹⁹ This family of highly related *M. tuberculosis* strains has become a major cause of MDR tuberculosis outbreaks, and it is suspected that it will become the most ubiquitous cause of tuberculosis worldwide. We, therefore, used a clinical Beijing/W isolate for testing the efficacy of Δ Ure rBCG-hly. Mice were vaccinated with wild-type BCG or Δ Ure rBCG-hly and later challenged with *M. tuberculosis* Beijing/W. Protective efficacy was assessed by determining colony forming units in lung homogenates between week 2 and 13. The BCG wild-type vaccine resulted in some protection at very early time points and then failed to protect against aerogenic infection with *M. tuberculosis* Beijing/W. In contrast, Δ Ure rBCG-hly reduced the bacterial load more than 100-fold over the whole duration of the experiment. Hence, this vaccine construct proved highly protective against a representative member of the most successful family of tubercle bacilli, Beijing/W (Grode *et al*, submitted for publication).

It could be argued that the high protective efficacy of Δ Ure rBCG-hly compared with wild-type BCG was made possible by increased virulence of the BCG strain. It is,

therefore, gratifying to see that severe combined immuno-deficiency (SCID) mice infected with rBCG-hly and, even more so, with ΔUre^rBCG-hly showed markedly longer survival times than SCID mice infected with wild-type BCG (Grode *et al.*, submitted for publication). We were thus encouraged to introduce this ΔUre^rBCG-hly strain into clinical trials. The vaccine construct has recently been licensed to the “Vakzine Projekt-Management GmbH” (Braunschweig, Germany), and we hope that it will be included into the vaccination efforts promoted by the Bill and Melinda Gates Foundation through Aeras (Washington, DC). We hope that this ΔUre^rBCG-hly strain will qualify as a candidate for pre-exposure vaccination with a superior BCG vaccine construct which can then be further improved by booster vaccination with the most successful subunit vaccine candidate (see fig 6).

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