The spectrum of latent tuberculosis: rethinking the biology and intervention strategies

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Abstract | Immunological tests provide evidence of latent tuberculosis in one third of the global population, which corresponds to more than two billion individuals. Latent tuberculosis is defined by the absence of clinical symptoms but carries a risk of subsequent progression to clinical disease, particularly in the context of co-infection with HIV. In this Review we discuss the biology of latent tuberculosis as part of a broad range of responses that occur following infection with *Mycobacterium tuberculosis*, which result in the formation of physiologically distinct granulomatous lesions that provide microenvironments with differential ability to support or suppress the persistence of viable bacteria. We then show how this model can be used to develop a rational programme to discover effective drugs for the eradication of *M. tuberculosis* infection.

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doi:10.1038/nrmicro2236
Published online
26 October 2009

Mycobacterium tuberculosis has evolved a highly efficient means of aerosol transmission that exploits immune-mediated damage to the lungs of individuals with active disease. Successful transmission can be detected by an antigen-specific T cell response in exposed contacts, and its extent is reflected in the estimate that one third of the global population has developed such a response. Around one in ten individuals in this infected population develop active tuberculosis (TB), most commonly within a few years after exposure, although they retain a lifetime risk of disease. This risk is significantly increased by immunosuppressive triggers, including HIV infection, tumour necrosis factor (TNF) neutralization therapy for other diseases, and diabetes, and can be decreased by prolonged preventive therapy with isoniazid[1]. Although current strategies for TB control focus on attempts to reduce transmission by prompt identification and treatment of infectious cases, it will be necessary to combine this with interventions to reduce the development of disease in the infected population if we are to approach the aim of TB elimination by 2050 (REF. 2) (BOX 1). In this Review, we discuss strategies for the development and application of drugs to achieve this goal.

The tuberculosis spectrum

Latent TB is defined solely by evidence of immunological sensitization by mycobacterial proteins in the absence of clinical signs and symptoms of active disease. Until recently sensitization has been defined by reactivity in the tuberculin skin test (TST). A drawback of using the TST is that the skin test reagent, purified protein derivative (PPD), contains crossreactive antigens that are also present in non-pathogenic mycobacteria. As a result, false positives can occur, for example in those previously immunized with the *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) vaccine. However, identification of recent conversion to a positive response by the TST has a well-recognized prognostic value[3]. Nevertheless, a major disadvantage is that such a conversion is often absent in those at greatest risk of disseminated TB; that is, children and adults infected with HIV. Recently, more specific tests of sensitization that rely on the *in vitro* release of interferon-γ (IFNγ) in response to specific *M. tuberculosis* antigens have been developed. There is consensus that these tests are less affected by prior exposure to BCG, but their effectiveness in detecting latent TB in children and HIV-infected adults remains debated[4]. Drawbacks that are common to both the

Box 1 | **The Global Plan to Stop TB**

Approximately nine million people develop tuberculosis (TB) every year, resulting in almost two million deaths. Infection is spread by individuals with active disease, particularly smear-positive disease that is characterized by high numbers of bacteria in the sputum. Infectiousness drops rapidly after the initiation of drug treatment, and prompt diagnosis and effective therapy are the main goals of current TB control programmes. Epidemiological modelling suggests that the detection of 70% of smear-positive cases, together with an 85% cure rate, will result in a self-limiting decline in TB incidence. The Global Plan to Stop TB (http://www.stoptb.org/globalplan) aims to halve the prevalence and mortality caused by TB by 2015, in line with the United Nations Millennium Development Goals. A longer term aim of the Global Plan to Stop TB is to eliminate TB (defined as an incidence of less than 1 per million of the global population) by 2050. To achieve this goal, reduced transmission by detection and treatment will have to be combined with interventions that prevent progression to disease following exposure. Treatment of latent TB with isoniazid for 9 months has been shown to significantly reduce the risk of disease; drugs that could achieve this goal by more feasible short-term therapy could represent a potent new weapon for TB control.

Tuberculin skin test
A method of diagnosing *Mycobacterium tuberculosis* infection by injecting tuberculosis antigens intradermally. A delayed type hypersensitivity response, dependent on the presence of sensitized T cells, is seen in those infected with *M. tuberculosis*. This does not distinguish latent infection from active tuberculosis.

Purified protein derivative
A precipitate of non-specific molecules from sterilized and filtered cultures of *Mycobacterium tuberculosis*.

Computed tomography
An imaging technique in which X-ray scans of a subject are compiled to generate a three-dimensional picture of various organs and structures (including brain and lungs).

Positron emission tomography
A nuclear medicine imaging technique using a positron-emitting probe that produces a three-dimensional image of biological processes within the scanned subject.

Granuloma
An organized structure comprising lymphocytes, macrophages, neutrophils and sometimes fibroblasts that often has a necrotic centre, which arises in response to continued antigenic stimulation in the presence of macrophages, for example in response to *Mycobacterium tuberculosis* infection.

TST and the IFNγ release test are that they do not distinguish latent infection from active disease, nor do they provide any direct evidence of the presence of viable bacilli. They determine simply that infection has at some point led to an acquired immune response that is detectable following re-challenge with antigen.

As a consequence of this purely clinical definition, TB is commonly thought of as having a simple binary distribution — active disease and latent infection. Although latent TB is generally loosely equated with bacterial containment in some inactive form, the current definition of latent TB includes a diverse range of individuals, from those who have completely cleared the infection to those who are incubating actively replicating bacteria in the absence of clinical symptoms[5]. Similarly, active TB in humans and non-human primates is characterized by diverse pathological presentations, ranging from sterile tissue, to caseous hypoxic lesions containing variable numbers of bacteria, to liquefied cavities with a massive load of replicating organisms. It is attractive to propose that this clinical diversity reflects the relative numbers, type and anatomical distribution of lesions. *M. tuberculosis* infection may therefore be better viewed as a continuous spectrum extending from sterilizing immunity, to subclinical active disease, to fulminant active disease, with conventional designations of latent infection and active disease corresponding to partially overlapping regions of biological heterogeneity[5] (FIG. 1). HIV infection skews this distribution in favour of the bacillus. Indeed, the greatly increased risk of disseminated disease in HIV-infected adults is well known, but there is also an increasing recognition of frequent subclinical active infection when this has been investigated by prevalence surveys[6].

Two important consequences arise from this model. First, it can be proposed that treatment of latent infection will be most effective when directed towards the part of the spectrum that corresponds to those at the highest risk of progression to disease, rather than to the entire two billion immunologically positive individuals. Second, drug development strategies that target bacterial populations on the basis of physiological requirements for persistence in different lesion types may be equally applicable to preventive therapy of latent infection and to improved treatment of active disease.

Insights into TB lesions from imaging

Pathologists analysing post-mortem tissues from humans have studied latent TB lesions extensively. The latent lesions they have characterized represent a subset of the heterogeneous lesions seen in active disease, with wide variations reported in the recovery of viable bacteria[7]. Exciting new opportunities are provided by recent advances in modelling latency in cynomolgus macaque monkeys[8,9], particularly when combined with high-resolution computed tomography (CT) and positron emission tomography (PET). CT provides structural data on the lung and lymph nodes, including a spatial map of granulomas (FIG. 2), and PET probes mark areas based on the function or phenotype of cells and processes in the tissue of interest. For example, the most commonly used PET probe in human cancer diagnosis is ^{18}F-fluorodeoxyglucose (FDG), which labels metabolically active cells. Integrating CT and PET images provides a detailed structural and functional map of the tuberculous disease process in the lungs.

The power of PET and CT imaging has rarely been applied to the study of infectious diseases in humans or animal models. However, we can learn from studies in cancer and neurobiology. The few reports of PET and CT imaging obtained from human patients with latent TB are invariably concerned with the diagnostic dilemma of differentiating latent tuberculosis from malignancies[10–12]. A comparison of the CT findings from patients with latent TB with those of patients with active TB confirms the pathological observations: CT scans of individuals with latent TB show a range of findings, some of which correspond to what is seen in patients with active disease[10]. The lesions that are most often reported are solitary pulmonary nodules ranging from a few mm to 20mm in size, although linear branching and consolidations have also been found[10]. This is probably because of a selection bias, as these lesions are the biggest challenge to the diagnostic radiologist, who must differentiate between TB or cancer. FDG–PET has been explored for its utility in improving the diagnostic accuracy of such exams, and as a result some limited information is available regarding latent TB lesions. Specifically, TB lesions are difficult to distinguish from malignancy on the basis of uptake of FDG, which varies tremendously among lesions. In at least two patients with latent TB FDG uptake was shown to be reduced following anti-TB chemotherapy[13]. Even this limited sample confirms the suggestion that latent infection can be depicted as a broad spectrum of conditions that overlap with those seen in active disease. In some patients this may be a slowly progressing form of the disease, in others a chronic, non-progressing percolating infection and in others the remnant of a waning infection. It remains to be seen whether any of these lesions harbour the truly metastable dormant bacilli that have been postulated to represent latency.

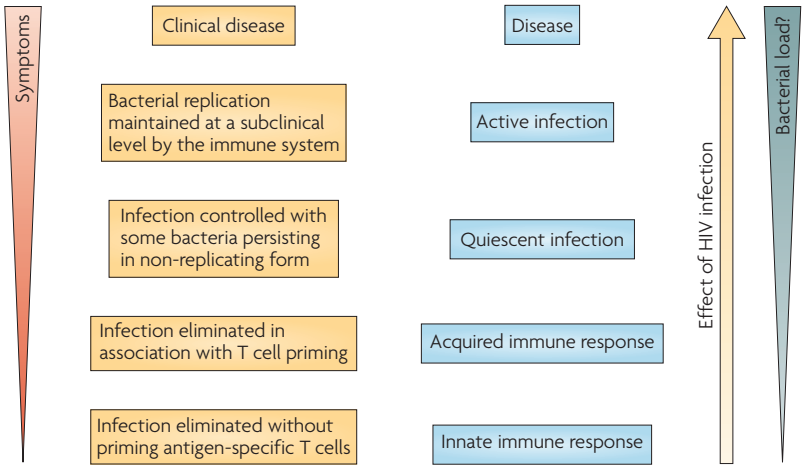

Figure 1 | **Tuberculosis infection as a spectrum.** The outcome of infection by *Mycobacterium tuberculosis* is generally represented as a bimodal distribution between active tuberculosis (TB) and latent TB on the basis of the presence or absence of clinical symptoms. We propose that latent TB is more usefully represented as part of a spectrum of responses to infection. One consequence of this model is that there may be a subpopulation within the group that is currently defined as having latent TB that should be preferentially targeted for preventive therapy. A second consequence is that efforts to develop drugs for effective treatment of latent TB would overlap the search for drugs that shorten treatment times for active TB.

These studies represent only the beginning of what is possible using functional imaging. There are numerous other PET probes that target different types of cells and processes, including hypoxia, cell proliferation and angiogenesis. These probes can provide additional information about the lesions and the process of disease establishment, progression and treatment. PET and CT technology allow detailed analysis of changes in individual tuberculous lesions over time and monitoring of the response of these lesions to treatment. Combining live imaging with the analysis of resected and post-mortem tissues provides the opportunity to generate lesion-based profiles of humans and animals infected with TB and to map lesion categories defined by imaging to cellular and molecular parameters.

Important key factors that facilitate the distinction between lesions include the response to treatment with existing and new drugs, the presence of immune cells and immune responses, bacterial gene expression, the presence of hypoxic regions, and drug permeability and retention. Early studies between the 1950s and the 1980s reported the levels of rifampicin and isoniazid, the two most effective anti-TB drugs, in different lesion types and lesion compartments, relative to blood plasma[14–16]. Although the read-outs used at the time have intrinsic limitations in terms of sensitivity and accuracy, these studies clearly indicated that only a small fraction of the drug present in plasma can reach some of the lesion compartments. Moreover, the rate of penetration into diseased tissue and sequestered infection sites is both drug specific and lesion specific, in agreement with what has been reported for non-TB drugs in abscess fluid[17,18]. There is a clear opportunity to refine and expand drug penetration studies in human and human-like TB lesions in animals by taking advantage of modern, innovative and state-of-the-art technologies for the quantification of small molecules. Modelling of the pharmacokinetics of first- and second-line drugs in plasma, lung tissue and various lesion types will allow the calculation of coefficients of penetration for each drug in each lesion type, providing insights on the physicochemical and other properties driving lesion penetration and on the location and type of lesion that is most resistant to drug dispersal.

A full analysis of tuberculous lesions will enhance the interpretation of PET and CT images from infected individuals to determine the efficacy of treatment or the state of disease. The opportunity to visualize latent lesions in humans and in non-human primates, as well as possibly watch reactivation occur, will provide data that will greatly enhance our understanding of latent TB infection, including the spectrum of latency and reactivation.

Pre-clinical animal models

To determine what animal model (or animal models) is best suited for evaluating drug efficacy against latent TB, a basic understanding of latent disease in humans is required. As discussed above, the pathology of TB in humans suggests that this form of the disease is characterized by a continuum of more or less quiescent and healed lesions[5] (FIG. 3). The few necropsies that were carried out on individuals with latent TB revealed consolidated fibrotic and necrotic lesions that had often become calcified[19,20]. One commonly accepted paradigm is that these arrested granulomas contain the bacilli that are responsible for disease reactivation, and that these bacilli have become dormant in response to hypoxic conditions, although this remains to be formally shown. Importantly, such structures could act as effective barriers to prevent the penetration of anti-TB drugs.

Although the mouse is by far the most practical and widely used animal model for drug discovery, TB in commonly used mouse strains does not recapitulate human pathology. Specifically, the granulomas are poorly organized and exclusively cellular (FIG. 3), they lack necrosis, fibrosis or hypoxia[21], the bacterial counts remain at high levels throughout the entire course of disease and the mice ultimately die of progressive infection[22]. These traits do not reflect what is seen (or assumed) in human latent disease.

In the Cornell mouse model, mice are treated with anti-TB drugs to reduce the bacterial load to undetectable levels, which has been suggested to mimic latent infection, as the mice have no obvious signs of disease. Subsequent spontaneous or steroid-induced regrowth of bacilli in a subset of these mice indicates that the bacteria persist and can undergo a form of reactivation[23]. It is possible that some bacterial subpopulations in the Cornell mouse model mimic those present in human latent TB, but in the absence of organized granulomas with caseous necrosis or mineralization, quantitative evaluation of drug efficacy against latent disease in this model is not reliable. Because mice are a practical animal model for the study of disease, efforts should focus on engineering the host and/or pathogen to provide more human-like immunopathology and environment-induced bacterial physiology[24].

Hypoxia
Low levels of oxygen in tissues.

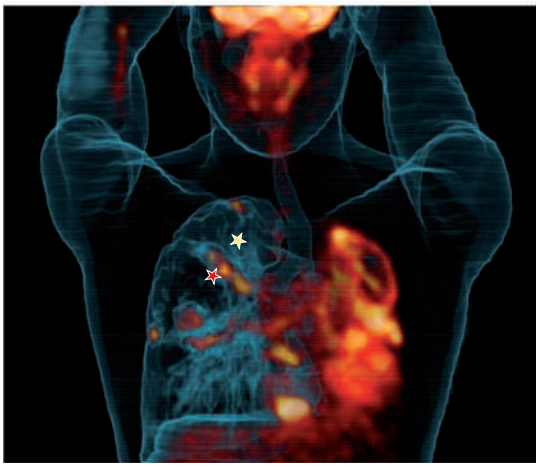

Figure 2 | **Positron emission tomography and computed tomography imaging.** An ^{18}F- fluorodeoxyglucose (FDG) positron emission tomography (PET) and computed tomography (CT) scan of a patient with tuberculosis with extensive bilateral disease and a complete collapse of the left lung. The right lung also shows extensive disease throughout and illustrates the variability of FDG–PET uptake among lesions within even a single infected patient. The yellow star illustrates one lesion that fails to take up FDG that lies immediately adjacent to a string of three lesions that take up label avidly (red star). These different types of lesion respond to chemotherapy with different kinetics, indicating that they represent distinct bacterial subpopulations in different microenvironments.

A rabbit model of latent TB has been proposed[25,26], which is characterized by persistent, host-contained paucibacillary infection that can be reactivated on immunosuppression. Although validation with standard anti-TB drugs is required to assess its experimental utility, the model seems to be attractive for the investigation of drug efficacy against persistent mycobacteria. In addition, strain variability (and prior vaccination) markedly alters the outcome of infection in rabbits, suggesting that bacterial factors could, in principle, be altered to create a suitable model[27].

Using non-human primates is undoubtedly the most costly and resource-intensive model, but they are the only species that reproduce the clinical characteristics of human latent TB (to the extent that we understand them)[8]. When infected with low doses of *M. tuberculosis*, approximately half of cynomolgus macaques survive for up to 3 years, and anecdotal evidence is that this period is indefinite, without clinical or radiographic signs of disease. Similarly to latently infected humans, these infected cynomolgus macaques respond positively to the TST in the absence of clinical symptoms. We have validated the clinically based classification of cynomolgus macaques as having active disease or latent infection using gross pathology, bacterial numbers and histology[92]. Histologically, the latent lesions show thick fibrosis, mineralization and/or central caseation (FIG. 3), which is also presumably the case in humans[8]. Hypoxia has been shown in chronically infected cynomolgus macaques[21]. The model is currently being validated with standard anti-TB drugs, and gene expression studies to characterize the metabolic state of the bacilli are underway. To test the ability of drugs to cure latent TB, it has to be shown that reactivation in response to immune suppression does not occur after drug therapy. Our preliminary data support the view that the non-human primate model can be used for this purpose. Combination of the non-human primate model with conditional gene silencing[28] techniques provides the first realistic experimental strategy for validation of drug targets for the treatment of latent TB.

It thus seems that non-human primates offer the most reliable model for testing the ability of candidate drugs to eradicate latent TB. But given the time, cost and compound requirements, this can only be accomplished when a candidate drug is about to enter full clinical development. For evaluation during early drug discovery stages, or even validating targets for latent disease, there is a clear gap in predictive and validated high-throughput *in vitro* assays. Such assays would attempt to recreate the environmental and immunological conditions present within discrete types of lesion, as seen in latent disease in humans and animal models, but require a much more detailed understanding of exactly what those conditions are. Combined with attractive pharmacokinetic and toxicological attributes, such assays must have the predictive value that is required to warrant efficacy studies in the non-human primate model.

Immunological characterization of lesion types

Both humans and non-human primates infected with *M. tuberculosis* show heterogeneity in lesion types. When infected with a low dose of *M. tuberculosis*, cynomolgus macaques develop active and latent disease in equal proportions. Both $CD4^+$ and $CD8^+$ T cells are found within the granuloma, and these can be tested for effector function, specificity and exhaustion. Differences between lesion types, as well as between the symptoms of monkeys with latent and active disease, may point to key features that are involved in the relative ability of granulomas to contain the infection. In addition, changes in immune function during drug treatment may point to differences in the mechanism by which the drug works. For example, we have observed increased lymphocyte infiltration in lesions during drug-induced healing, which supports the hypothesis that killing of bacteria or release of antigens during therapy causes increased lymphocyte activation, proliferation or migration to the lesions in the lungs.

Because of the wealth of immunological reagents for non-human primates, it is possible to test the necessity for various immune factors at different stages of disease and to study the mechanism by which these factors affect control of infection. For example, TB can be detected in individuals infected with HIV before a substantial loss of $CD4^+$ T cells[29], raising the possibility that depletion of $CD4^+$ T cells alone is not responsible for the increased risk of TB in patients with HIV. Studies comparing $CD4^+$ T cell depletion with simian immunodeficiency virus (SIV) infection (as a

Paucibacillary
Containing just a few bacilli, as is the case with granulomas that are caused by various mycobacterial diseases.

Caseation
Necrotic degeneration of bodily tissue into a soft crumbly cheese-like mass, where cellular outline is lost. In tuberculosis, this refers to the necrotic centre of a granuloma and is mainly driven by the immune response and is typical of tuberculosis pathogenesis.

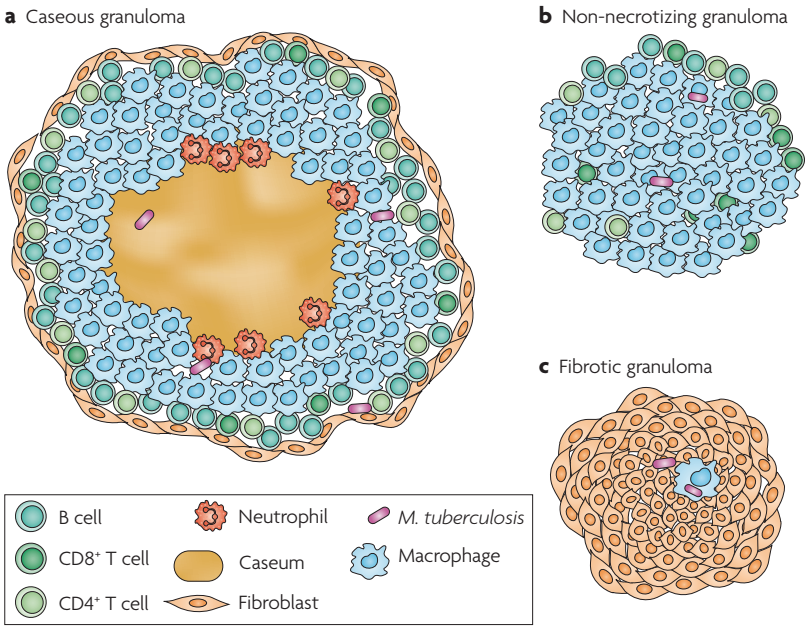

Figure 3 | **Tuberculous granulomas.** There are several granuloma types that can be found among humans and non-human primates, even within the same individual. **a** | The classic tuberculous granuloma, found in active disease and latent infection, is the caseous granuloma, which is composed of epithelial macrophages, neutrophils, a cuff of lymphocytes ($CD4^+$ and $CD8^+$ T cells and B cells) and sometimes surrounded by peripheral fibrosis. The centre of this type of granuloma is caseous, a necrotic state that probably consists of dead macrophages and other cells. This area is hypoxic. Mycobacteria in this granuloma can be found in macrophages (either in contact with T cells or not) in the hypoxic centre or possibly even in the fibrotic rim; this provides the mycobacteria with different microenvironments. **b** | The non-necrotizing granuloma is usually seen in active disease and consists primarily of macrophages and some lymphocytes; this lesion can be seen in guinea pigs and mice, albeit with more lymphocytes. *M. tuberculosis* bacilli are within macrophages in this lesion. **c** | Fibrotic lesions are seen mostly in latent tuberculosis but also in active disease and are composed almost completely of fibroblasts, with a minimal number of macrophages. Although it is possible to culture bacilli from some fibrotic lesions, it is not clear where the bacilli reside (possibly in macrophages or in the fibrotic area) or what the microenvironment is like.

surrogate for HIV) in cynomolgus macaques could potentially address these questions. Similarly, the role of $CD8^+$ T cells can be studied using CD8-depleting antibodies in cynomolgus macaques. Although $CD8^+$ T cells have been implicated in the control of TB in humans, there are no direct data showing that these cells are necessary for the containment of initial or latent infection. Finally, TNF neutralization, which is used to treat certain inflammatory diseases in humans, substantially increases the risk of TB reactivation[30]. Similar studies in cynomolgus macaques recapitulate the human data (L. Lin and J.F., unpublished observations) and therefore facilitate the study of the mechanisms by which TNF is important in the control of *M. tuberculosis* infection.

These studies, along with the investigation of the phenotypes and functions of immune cells in human granulomas from lung resection studies in combination with PET and CT imaging, provide a new look at the important host factors that work in concert with bacterial factors to determine the outcome of infection and maintenance of latent infection. It is likely that numerous host factors contribute to the initial containment of infection and prevention of reactivation. Although the balance of factors that contribute to these states may differ between hosts, they may lead to the same outcome. Mathematical modelling can provide insights into the balance of host responses as well as the relative importance of each response in acute and latent infection[31–33].

Characterization of subpopulations in latent TB

Much of the work on the transcriptional response of *M. tuberculosis* to various environmental conditions has focused on the hypoxic response, largely based on the pathologists' intuitive deduction that some tuberculous lesions appeared hypoxic. Analyses of mRNA transcripts from human lesions have indicated that the microenvironment supports differential responses by the bacteria, and this depends on the location of the bacilli relative to the outer lesion wall[34,35]. Specific stresses or conditions can lead to major metabolic realignment that also translates into important changes in drug susceptibility[36–39]. These observations, coupled with the introduction of a simple *in vitro* system for hypoxic adaptation of *M. tuberculosis*, led to a hypothesis that latent TB is a reservoir of organisms that are encased in caseous lesions under hypoxic conditions, whereas active TB is typified by bacilli replicating aerobically at the margin of liquefied cavities.

Although whole genome gene expression data are available from infected mice and from macrophages infected *in vitro*[40–43], evidence relating to the hypoxia hypothesis often relies on an interpretation of environmental conditions inferred from transcriptome analysis of just a handful of genes. This inference is complex for three reasons. First, transcriptional responses to different environmental cues often involve overlapping gene sets. For example, the two-component response regulator DosR, which is controlled by the sensor kinases–DosS and DosT and is central to hypoxic adaptation, is also upregulated by exposure to nitric oxide[44]; the siderophore-encoding mycobactin biosynthetic genes are upregulated by hypoxia and by iron limitation[45]; and genes of the methyl citrate cycle are regulated by hypoxia, low pH and iron restriction[45,46]. As a result the DosR hypoxia regulon is strongly induced even within apparently aerobic macrophage infections[40]. Second, it is now recognized that the hypoxia regulon comprises at least two subsets of genes that are differentially upregulated during initial adaptation and extended exposure to hypoxic environments[40,44,47]. The relevance of these adaptive responses has been validated by gene knockout studies in mice[48–52], although it should be noted that this animal model reproduces neither the pathology nor the complexity of human lesions[21]. In addition, expression data from human samples only give bulk, averaged signals and don't represent accurately the behaviour of bacterial subpopulations that are present in a single lesion[35].

Hypoxic response
Changes in gene expression induced by the incubation of *Mycobacterium tuberculosis* under anaerobic conditions. This comprises an initial transient response (by the DosR hypoxia regulon) followed by the enduring hypoxic response involving expression of a set of 230 genes.

Hypoxia regulon
A cluster of 48 genes that are controlled by the transcriptional regulator DosR, which is upregulated in response to hypoxia but also during exposure to nitric oxide, carbon monoxide, sodium dodecyl sulfate or low pH.

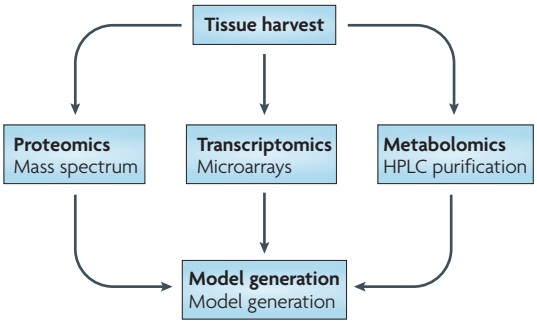

Figure 4 | **A systems biology approach to the pathology of tuberculosis.** Progress in understanding the complex biology of human tuberculosis — and applying this knowledge to drug development — will depend on being able to integrate multiple sources of data into *in silico* models that allow us to prioritise experiments and predict effective interventions. HPLC, high-performance liquid chromatography.

An important recent accomplishment was the demonstration that a subset of lesions in higher vertebrates (rabbits, guinea pigs and cynomolgus macaques) are, in fact, hypoxic and sensitive to metronidazole — a drug that is selective for TB adapted to hypoxia[21]. In the definitive experiment the oxygen tension was measured directly in rabbit lesions by surgically inserting a highly sensitive oxygen probe into lesions of live animals. Human clinical trials of metronidazole in patients with multidrug resistant strains are in progress (see ClinicalTrials.gov website), and studies in cynomolgus macaques indicate that metronidazole may be effective against both active and latent TB. This type of analysis helps to define the microenvironment in a certain lesion. These studies confirm the hypoxia hypothesis for a subset of lesions across animal models (except mice) and are consistent with the view that hypoxia makes at least some contribution to human TB.

Recent transcriptome data from active disease in humans adds further layers of complexity. Gene expression signatures from *M. tuberculosis* isolated directly from surgically removed lesions from patients with TB suggest that the bacilli probably experience nutrient deprivation and hypoxia in addition to various antimicrobial defence systems, conditions that damage DNA and conditions that encourage extensive remodelling of the cell wall[53]. A more recent study focused on *M. tuberculosis* isolated from patient sputum samples and revealed an enrichment for lipid bodies and a surprising upregulation of the DosR hypoxia regulon[54]. This highlights the difficulty in extrapolating from gene expression data to a set of environmental conditions. Although DosR is activated in response to hypoxia, it should be clear that bacteria in sputum are unlikely to be hypoxic and that in this case expression of DosR-regulated genes is not triggered by hypoxia.

These studies have given rise to a competing hypothesis that latent bacilli reside in a range of lesions that have different microenvironmental conditions, which drives them into a state in which they do not replicate or they replicate slowly. A corollary of this hypothesis is that a subset of the lesions in active disease is identical to those found in latent infection. The next challenge is to translate information from lesion analysis and transcriptional profiling into a metabolic framework as a platform for drug discovery.

Detection of the mRNA transcripts for genes within a metabolic pathway does not necessarily imply that these genes could be drug targets; the essential nature of the pathway during the relevant stage of host pathogenesis needs to be verified. Thus, although genes of the methylcitrate cycle seem to be upregulated *in vivo*, deletion of the corresponding genes does not affect virulence in the mouse model of TB infection[52]. Similarly, the recycling pathway for nicotinamide adenine dinucleotide (NAD) biosynthesis has been shown to be highly upregulated at both the gene expression and the enzymatic activity level during growth in host tissues, but inactivation of this pathway did not affect virulence in mice[55]. Genome-wide mutagenesis studies have been instrumental in elucidating the essential nature of genes that are required for initial growth in the mouse model[56]. However, results of such studies can only be interpreted in the light of the environment in which the transposon library was analysed, and differences in the availability of nutrients between different host models and in different lesions must be taken into account when extrapolating these data to human infection.

Most attempts at drug target validation have been based on studies that examined the role of individual enzymes. Understanding the full metabolic activity of an organism in a particular physiological state, instead of gradually identifying components that are activated during these conditions, is the ultimate means of drug target discovery (FIG. 4). A genome-scale network model of *M. tuberculosis* metabolism was designed based on the principles of flux balance analysis and was calibrated with experimental data generated from chemostat cultures of *M. bovis* BCG to predict growth rate, substrate consumption and biomass production in a pseudo-steady state[57]. Furthermore, the model predicted whether the genes are essential; these findings correlated with genome wide mutagenesis data. Indeed several anti-TB drug targets, including those of isoniazid (and thus ethionamide), pyrazinamide, ethambutol and cycloserine, were correctly predicted to be essential.

Flux balance analysis has also been used to study potential drug targets in sub-systems of the entire metabolic network. One group[58] developed a metabolic network based on the mycolic acid biosynthetic pathway to predict bottlenecks in this sub-system. Such metabolic models are of course only useful when nutrient availability under a particular physiologic state is known. Metabolomic studies of lung lesions will provide the groundwork for developing genome-scale metabolic networks that are relevant for drug discovery. Such measurements *in vivo* would be exceptionally technically challenging. *Ex vivo* studies of *M. tuberculosis* recovered from lesions were pioneered by Segal and Bloch in the 1950s[59,60]. More recent studies have indicated that metabolism immediately after recovery from the host to

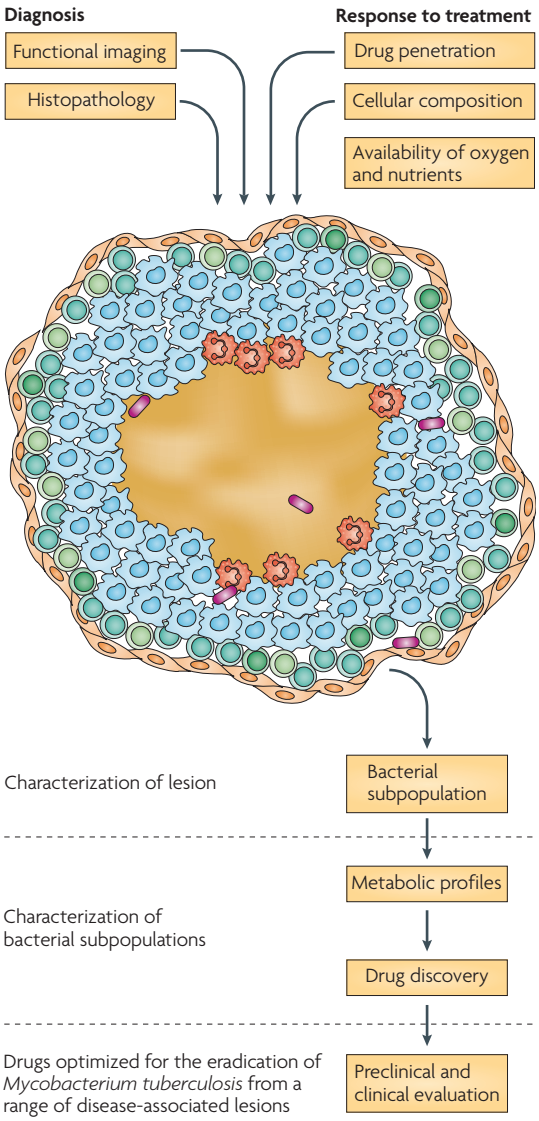

Figure 5 | **A lesion-based framework for the study of latent tuberculosis and drug development.** Latent and active tuberculosis (TB) in humans and non-human primates comprises a heterogeneous mixture of lesions that generate a range of physiological microenvironments associated with bacterial replication, persistence and killing. Characterization of the different types of lesion using state-of-the-art imaging, cellular and molecular techniques will provide a framework for understanding the biology of TB and for the development of drugs targeted against relevant bacterial subpopulations.

some extent recapitulates aspects of its *in vivo* metabolic activity[55], suggesting that *ex vivo* metabolomic studies of *M. tuberculosis* may provide clues to the metabolic capacity of this pathogen.

Discovery of drugs against latent TB

The most ambitious objectives for anti-TB drug discovery are to identify new drugs that effectively eradicate latent TB infection and reduce chemotherapy of active disease to 2 weeks. To achieve these goals we need to identify and kill those subpopulations of *M. tuberculosis* that are not eliminated efficiently with current antibiotics and thus are responsible for the prolonged treatments required for the various forms of TB (FIG. 5). A key issue complicating the discovery of cidal drugs is that we only have a limited understanding of the death-causing events that are induced by the existing drugs[61,62]. The physiological complexity underlying cidal activity is elegantly illustrated in a recent analysis of the mechanism of action of aminoglycosides[63]. At the heart of this difficulty is the fact that we lack a sensitive test to determine when a bacterium is dead, other than showing that it does not form a colony when placed on agar. One study[64] described a possible unifying death pathway in bacteria that involves the formation of hydroxyl radicals by oxidation of iron, with DNA and protein damage as the direct mechanism of death. Whether this holds true in a general sense remains to be seen. However, it seems likely that cidal compounds need to 'corrupt' or 'derail' cellular processes in a way that the bacterium cannot control or counteract. Instead of simply inhibiting an enzymatic activity to block a metabolic process, effective drugs might, for instance, have to trigger build-up of toxic intermediates, cause the release of bactericidal metabolites (for example, nitric oxide[65]) or collapse central homeostatic systems[66]. Such activities are currently difficult to predict owing to our limited understanding of bacterial metabolism. New cellular read-outs that accurately show when a bacterium is dead are urgently required to biologically annotate and evaluate hits.

To add an additional level of complexity, it has recently become clear that bacteria also show intrapopulation diversity; heterogeneity is observed at the single cell level within a single microenvironment. Phenotypic heterogeneity, defined as non-heritable and reversible variation in cellular parameters, is crucial for the persistence of bacterial subpopulations under selective pressures, including antibiotic stress[67]. Stochastically determined differences in growth rate or expression of a specific enzyme, for example, may result in a subpopulation of clonal organisms (referred to as persisters) even within a single microenvironment that might show differential antibiotic susceptibility. Differential behaviour of single bacilli is masked in standard batch culture read-outs that derive mean population values by pooling large numbers of cells. Importantly, new technologies that allow the analysis of bacterial behaviour at single cell resolution have made this persister problem easier to analyze[68]. Single cell analyses to determine the underlying subpopulation that is responsible for average cellular behaviour are an emerging field in TB research[67] and need to be implemented as an integral part in the drug discovery process for the evaluation and prioritization of potential targets.

Whole cell screens. Translating this individual lesion-based concept into something useful for drug discovery remains a crucial challenge. Although the target-led, genomics- and genetics-driven approach has provided the central strategy for drug discovery programmes in the pharmaceutical industry in recent decades, it has

Box 2 | **The Grand Challenges in Global Health project and drugs for latent TB**

A project to study fundamental biology and drug development strategies for latent tuberculosis (TB) was initiated in 2005 as part of a programme to tackle Grand Challenges in Global Health. The project, funded jointly by the Bill and Melinda Gates Foundation and the Wellcome Trust, brought together eight academic groups from four continents, with industrial expertise provided by the Novartis Institute for Tropical Diseases (NITD). The project focused in particular on exploring the role of hypoxia in latent TB and was organized in the form of three interlinked components.

The aim of the first component was to characterize TB lesions in freshly resected lung tissue from humans and non-human primates with active and latent TB. The presence of hypoxic regions was shown in lesions from non-human primates that matched the histopathology of a subset of lesions seen in humans with both active TB and latent infection. Efforts are continuing to determine whether particular patterns of bacterial transcription are associated with different lesion types.

The second component is directed towards drug discovery using several approaches. One is based on the use of conditional expression systems to identify genes that are required for the survival of *Mycobacterium tuberculosis* under hypoxic conditions, and targeting associated enzymes in high-throughput screens. Another is based on high-throughput screens using whole cell cultures to identify compounds that inhibit the ATP homeostasis, which is essential for viability during non-replicating persistence. In addition, there is a programme to optimize nitroimidazoles to release nitric oxide, which has been shown to be bactericidal for non-replicating, hypoxic organisms.

The final component addresses strategies for the evaluation of drugs with activity against hypoxic *M. tuberculosis*. This is achieved using positron emission tomography (PET) and computed tomography (CT) imaging to monitor the effect of drugs on individual lesions in a non-human primate model and in a clinical trial of the effect of adding metronidazole during salvage therapy for patients with multidrug-resistant TB. The potential use of immune biomarkers in field trials of preventive therapy is also being explored.

Many of the ideas and approaches outlined in this Review are based on results and unique collaborative environment facilitated by the Grand Challenges project.

been markedly ineffective in the area of antibacterials, in which potent enzyme inhibitors frequently fail to translate into agents that will kill, or even inhibit, growth[69]. Furthermore, many successful antibacterials have multiple targets, and several key anti-TB drugs, such as isoniazid, require metabolic activation after uptake by the bacillus to exert their cidal effects. The concept of an isolated, single-target approach to anti-TB drug discovery might therefore be questioned[70]. Whole cell screens allow the simultaneous screening of all targets that are essential for a particular physiological state of the organism. Together, these considerations have encouraged a move towards high-throughput screens based on monitoring of activity in whole bacteria. Subsequent identification of the mechanism of action of active compounds is achieved by sequencing the genome of spontaneous drug-resistant mutants[71,72], affinity purification[73] and comparative transcriptional fingerprinting with drugs that have a known mechanism of action[74]. As phenotypic, whole cell screening identifies compounds that kill the organism, it eliminates the first hurdle encountered in target-based approaches: translating activity against a target into its effect on the whole cell. The follow-up target recognition can result in the identification of new, compound-induced death mechanisms that cannot necessarily be predicted by genetics[75]. An example of such a phenotypic screening project is a work in progress within the Grand Challenges consortium (BOX 2): a high-throughput screen was carried out to identify hits that trigger the collapse of ATP homeostasis, the Achilles heel of dormant bacilli in hypoxic conditions[66]. Now corresponding targets are being identified using genetics and affinity purification. In addition, a biochemical respiratory vesicle-based assay and a series of *M. tuberculosis* strains containing externally regulatable respiratory chain functions are employed to identify the subset of hits that target energy metabolism.

Target-based screens. Even though whole cell screens have been successful, they also have limitations. Bacterial growth can be inhibited by different mechanisms, and secondary screens are required to identify the few useful compounds among the many that exhibit broad toxicity[69]. For those compounds that are not generally toxic, target identification often requires several of the experimental strategies mentioned above, all of which can, and frequently do, fail. In addition, lead optimization is greatly facilitated if the target of a compound is known and therefore more straightforward for hits from biochemical high-throughput screens than for hits from whole cell screens. In effect, target selection becomes simpler but hit-to lead drug development becomes more complicated. Target-based screens are also much more sensitive and compatible with new strategies for hit generation, such as fragment-based screens[76], which cannot be used in whole cell screens.

Why then have target-led approaches been so inefficient? To start with, the hit rate of high-throughput screening against bacterial targets is low: for instance, only 16 out of 67 high-throughput screening campaigns carried out by GlaxoSmithKline gave rise to hits[69]. This is possibly due to the limited chemical space covered by the compounds used in these assays. It could also be, at least in part, because cell permeability is not a factor in biochemical screens. This is difficult to engineer into a drug without knowing the physicochemical rules that determine uptake by the bacteria or by carrying out simple uptake assays. Mycobacteria have perhaps the least permeable outer cell wall of any known organism, far more difficult to penetrate than *Pseudomonas* spp., for example[77,78]. This layer is spanned by a single water-filled channel of known structure, the transport characteristics of which are poorly understood[79]. In addition, even if compounds can enter the bacterial cell, good enzyme inhibitors do not always

make good antibacterial agents. For example, the Grand Challenges consortium and NITD recently identified a large number of scaffolds that efficiently inhibited pantothenate kinase (PanK) and peptide deformylase (Def) *in vitro*. It has not been possible to isolate *M. tuberculosis* mutants with transposon insertions in the genes encoding PanK or Def[80], which suggested that PanK and Def are essential and that chemical inactivation of either enzyme should prevent the growth of *M. tuberculosis*. However, in contrast to a transposon insertion, which nearly always completely eliminates protein function, chemical inhibition of an enzyme within a cell is almost always incomplete, especially with inhibitors that have not yet been optimized for activity against their targets. In the case of PanK, none of the inhibitors reduced the growth of *M. tuberculosis,* despite their chemical diversity. Inefficient uptake might have contributed to the lack of activity of some PanK inhibitors, but this was not the case for the Def inhibitors[81].These compounds were taken up by the bacilli and stopped their growth[81], but the kinetics of growth inhibition were slow and the compounds were not cidal, resulting only in moderate killing efficacy in the TB mouse model despite adequate pharmacokinetic and pharmacodynamic parameters (V. Dartois and T.D., unpublished observations).

Therefore, we don't know which genes encode functions that are not only essential for growth but are also vulnerable to chemical inhibition and are thus good targets for biochemical screens. Gene silencing tools can help to solve this problem as they allow partial inhibition of a target in cellular assays and within animal models[28,82].

Our ability to transform compounds identified in target-based screens into antibacterial agents will increase once we better understand how small molecules penetrate the bacterial cell envelope and which of the processes that are essential for growth are susceptible to chemical inhibition. Information from transcriptional and metabolic profiling will help to predict vulnerable targets. Those targets should first be analysed by genetic or, preferably, chemical knockdown to verify vulnerability to partial inhibition. It is important that these validation experiments are carried out under several conditions that are representative of the proposed *in vivo* environment. Targets that are required for survival in diverse conditions, and are thus essential independently of the specific physiological state of the pathogen, obviously are particularly attractive. For example, nicotinamide cofactors are essential for the growth of *M. tuberculosis* and sudden NAD starvation causes cell death. Furthermore, inhibitors of NAD synthase are bactericidal in growing and non-replicating cells[55]. NAD synthase should allow us to test whether biochemical screens against targets that are vulnerable to chemical inhibition and essential under different *in vitro* conditions more frequently identify enzyme inhibitors that also kill *M. tuberculosis*.

Assessment of drug activity against latent TB

The advent of a new compound that is potentially active against latent TB in humans would present considerable logistic challenges when being evaluated. Historically, clinical trials to assess the value of a candidate for prophylactic intervention in individuals with latent TB have involved large numbers of patients and extensive follow-up studies. The two largest trials of isoniazid preventive therapy involved more than 50,000 participants and a 10-year follow-up period[83,84]. The past few years have witnessed a considerable expansion in the field of TB diagnostics and biomarkers, the main thrust being to better diagnose TB and to find a biological indicator of successful treatment response in active disease[85]. There is less research directed towards the discovery of markers that may change during the treatment of latent infection (which may therefore indicate successful treatment). Nevertheless there are interesting data showing that the IFNγ response to *M. tuberculosis* antigens approximately halves during drug treatment of latent TB[86–89]. This decrease may mark successful treatment, with the chemotherapeutic reduction in the number of bacteria corresponding to a reduced necessity to mount an immune response. In our own study we also documented a transient initial doubling of the IFNγ response at 1 month that was possibly due to an increase in antigen release following bacillary death[88]. These findings complement ongoing omics-based and larger clinical studies to determine whether a combination of markers monitored intensely can provide useful information.

The historical clinical trials of isoniazid[83] collected little information on subjects and evaluated efficacy based solely on relapse rates, assuming that all participants were more or less equally latent at the start and equally adherent with medication. In some small studies therapy with just isoniazid markedly affected TB treatment (with a reduction as high as 100% in the incidence of active disease in 261 participants at a Dutch marine training camp[90]), whereas in others the same regimen had only a modest effect[91]. The reasons for these discrepant outcomes are not widely discussed other than to say that adherence or duration were likely confounders.

But what if conventional wisdom on this point is incorrect and instead of poor adherence or duration the confounder was the different point in the spectrum of latency within which the individual cohorts started? The implication would be that specific groups of patients are at different risk of developing active disease. We propose that it is not one-third of the global population that is at imminent risk for the development of active disease and that the number is much smaller; the key issue is diagnosis of those patients who would benefit from intervention. The spectrum of latency concept allows us to envision a combination of imaging-enabled and perhaps biomarker tools to identify patients that have sub-clinical, but active, disease and targeting chemoprophylaxis to that specific population. Rather than requiring mass chemotherapy of one-third of the planet's population to achieve eradication, we could target the much smaller one in ten that will otherwise perpetuate the epidemic. In no small way, imaging has allowed us to envisage a solution to the problem of developing new chemotherapy for latent tuberculosis.

In summary, we propose to move away from the model of a binary distribution between active disease and latent infection towards a view of the outcome of infection with *M. tuberculosis* as a continuous spectrum generated by a range of lesions providing multiple microenvironments that support bacterial replication, persistence or killing. We anticipate that this model will help us to identify subpopulations of individuals who will benefit the most from intervention by preventive therapy, and to characterize subpopulations of bacteria that can be targeted by a new generation of anti-TB drugs customized for efficient sterilization of persistent infection.

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Acknowledgements.
We are grateful to colleagues in the Grand Challenges in Global Health project "Drugs for Treatment of Latent Tuberculosis" for stimulating discussion and to the Bill and Melinda Gates Foundation and the Wellcome Trust for financial support. This work was also funded (in part) by the Intramural Research Program of the National Institutes of Health (NIH), National Institute of Allergy and Infectious Diseases (NIAID) and by NIH RO1 HL075845 (J.L.F.). We also thank our Novartis colleagues in Singapore, Basel, Cambridge and La Jolla for discussions and collaboration.

DATABASES

Genome Project: http://www.ncbi.nlm.nih.gov/sites/entrez?db=genomeprj
Mycobacterium bovis bacillus Calmette–Guérin | *Mycobacterium tuberculosis*

FURTHER INFORMATION

Douglas Young's homepage: http://www3.imperial.ac.uk/cmmi/research/young
Global Plan to Stop TB website: http://www.stoptb.org/globalplan
Grand Challenges in Global Health website: http://www.grandchallenges.org/CureInfection/Challenges/Therapies/Pages/Tuberculosis.aspx

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