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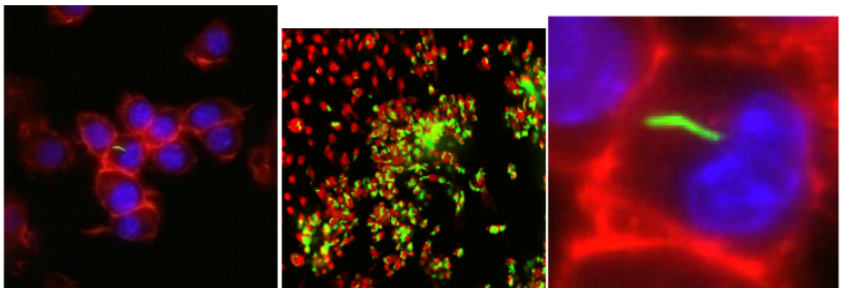
Français

Chémogénomique des Mycobactéries



Aims of the Project

Current tuberculosis treatment demands chemotherapy to be taken for more than six months, and inappropriate compliance often leads to the selection of multidrug resistant strains (MDR-TB) (Young et al., 2008). Recently, the emergence of extremely resistant strains (XDR-TB) has become a major world threat (Van Rie and Enarson, 2006). The scientific community agrees that the key to improving TB therapy relies on shortening its duration and making it more effective against XDR strains (Ginsberg and Spigelman, 2007). To this end, improved understanding of the fundamental biology of this complex disease will prove to be the key to radical advances in TB control (Young et al., 2008).

Pathology is intimately linked to the interplay between the host immune response and the persistence of the mycobacterium. In the lungs – the major affected organ of the disease – *M. tuberculosis* has a preferential tropism for alveolar macrophages, dendritic cells and type II alveolar pneumocytes (Armstrong and Hart, 1971; Bermudez and Goodman, 1996; Warner and Mizrahi, 2007). For the last four years, we have been undertaking a chemical genomics approach on intracellular *M. tuberculosis* to investigate the particular features of mycobacterial trafficking that enable the bacillus to avoid degradation within macrophages. This has been performed by directly monitoring the pathogen behaviour inside the host living cells using dynamic imaging confocal fluorescent microscopy, together with comprehensive image analysis (Christophe et al., 2009). Building on the considerable data generated by our three large scale screening approaches, we will further explore the molecular mechanisms contributing to *M. tuberculosis* intracellular survival.

First, we will extend our confocal image-based models to verify the current model of mycobacterial persistence within phagosomes.

Second, we will elucidate the function of *M. tuberculosis* virulence factors at the cellular level. More specifically, the second part of our project will focus on investigating the possibility of active transport of *M. tuberculosis* proteins into the host cell.

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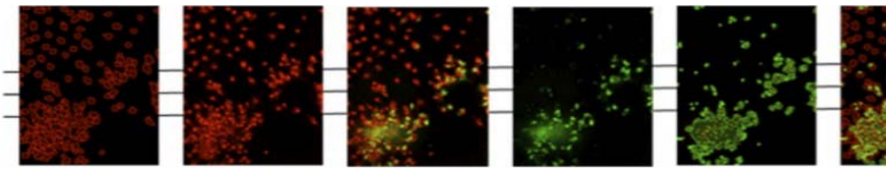
Third, we will undertake the characterisation of the signalling by the host cell effectors identified in our RNA interference screen.

Lastly, we will develop chemical modulators of host signalling pathways to determine whether boosting the host responses can contribute to more effective antitubercular chemotherapy.

The overarching aim of this project is to understand how *M. tuberculosis* controls host cell signalling pathways and how the macrophage becomes tolerant to the persistence of the tubercle bacillus to develop new therapeutic strategies for the full eradication of the bacillus from the mammalian host.



1. Quantitative Imaging of the *Mycobacterium tuberculosis* fate within host cells: Reassessment of the current dogmas of mycobacterium trafficking and replication

Work that led up to the Project: In the 1970s, J.A. Armstrong and P.D. Hart, using electron microscopy, suggested that the tubercle bacilli could replicate inside cultured macrophages by inhibiting the fusion of phagosomes with lysosomal degradative compartments (Armstrong and Hart, 1971). This discovery was further confirmed by extensive research from the groups of D. Russell, V. Deretic and J. Pieters (Pieters, 2008; Russell, 2001; Vergne et al., 2005), until being recently questioned by the work of P. Peters (van der Wel et al., 2007), which showed *M. tuberculosis* in the cytosol of phagocytic cells by electron microscopy. This dogma therefore needs to be readdressed to establish new correlations between intracellular localisation of the bacillus and its ability to remain in a dormant or metabolically active state. For instance, it is important to explicitly determine whether the bacterium being observed in a specific sub-cellular compartment is really one that will persist and to perform such analysis on a significantly large number of *M. tuberculosis* loaded cells in various settings. With the development of automated confocal fluorescence microscopy technologies, we have been able to establish a robust unbiased sub-cellular image analysis method enabling the quantitative analysis of intracellular trafficking and replication on several thousands of micrographs, and we will apply our technology to readdress this issue (Christophe et al., 2009; Fenistein et al., 2008; Lenseigne et al., 2007).

Methodology

1.1 Systematic multi-parameter quantitative image analysis of the sub-cellular trafficking of the tubercle bacillus

We have developed a confocal fluorescence-based imaging method for high throughput automated quantification of bacterial trafficking into lysosomes. To this end, mouse bone marrow-derived macrophages were incubated with mycobacteria that had previously been covalently labelled with the red fluorescent dye CypHer5. Macrophages were then pulsed with the

acidotropic green fluorescent dye LysoTracker DND-26 to label the lysosomal compartments. After nuclei labelling, sample images were acquired using an automated confocal microscope. An image analysis script was developed for automated quantification of infected cells, bacterial load and lysosomal compartments. A minimum of 1500 cells was automatically analyzed for each well and an average of four fields recorded per well. The fraction of bacteria co-localizing with LysoTracker-positive staining was found to be less than 10% in cells loaded with *M. tuberculosis* H37Rv, and in contrast, more than 50% with heat-killed H37Rv. Of particular interest, quantification of the percentage of cells containing detectable lysosomes led to similar results between strains and conditions. These data indicated that the amount of lysosomal compartments was a sufficient parameter to be used as a correlate of the localisation of mycobacteria in acidified vacuoles, independently of mycobacteria staining and detection.

Application of this assay to labelled-dormant and metabolically active mycobacteria, as well as extension to the use of specific cytosolic- and other organelle- fluorescence markers, will definitely contribute to determining the unbiased quantitative repartitioning of the bacterium within sub-cellular compartments within the host cells.

1.2 Image based analysis of the impact of *M. tuberculosis* replication on host cell death

Colonisation of macrophages by *M. tuberculosis* leads to severe host cell death after several days, which is not observed with the live attenuated BCG vaccine strain or even the *M. smegmatis* model strain at the same multiplicity of infection. Furthermore, *M. tuberculosis* induces much less cytotoxicity in type II pneumocytes compared to professional phagocytic cells. In the course of our screening studies, it has become apparent that mycobacterial-induced host cell death is still poorly understood and often ignored due to the absence of technologies enabling its accurate measurement during infection with fully virulent *M. tuberculosis,* which requires to be manipulated in containment category 3 facilities. Our aim here is to investigate when host cell death is triggered in the course of bacillus replication by quantitative imaging. In particular, we will investigate the different possible apoptosis pathways as well as autophagy process.

1.3 Dynamic 4D-Imaging of *M. tuberculosis* invasion in macrophages

Our objective is to perform concomitant dynamic analyses of both the mycobacteria and host cell features by live fluorescence microscopy in three dimensions and over time (4D). In depth analysis of the live mycobacterial phagosomes' physical characteristics – with regard to vesicle size, number, shape, speed, localisation, turnover, and capacity to fuse – will be performed. Subsequent kinetics studies will examine the same macrophages that have been labelled with multiple markers as mentioned above. This will allow us to further correlate phagosome maturation with mycobacterium replication and induction of apoptosis required for antigen presentation.

2 Bacterial protein effectors as modulators of *M. tuberculosis* intracellular localisation

Work that led up to the Project: Phagosome maturation arrest is one major strategy used by *M. tuberculosis* to survive inside host macrophages. To identify the pathogen determinants involved in this process, we have developed a confocal fluorescence-based imaging method for high throughput automated quantification of bacterial trafficking into lysosomes (Brodin et al., 2010). This method was applied to the screening of a library containing thousands of *M. tuberculosis* mutants, and we identified ten mutants that aberrantly trafficked into acidified vacuoles rapidly after phagocytosis. Molecular analysis of these mutants identified lesions in genes encoding lipid components previously reported to play a part in phagosome remodelling, such as the ESX-1 secretion system. In addition, we identified novel mycobacterial genes, such as those encoding PPE54 and the lipoprotein LppM, whose disruption led to rapid delivery of the bacilli into phago-lysosomes. Furthermore, complementation of Δ*ppe54*, Δ*lppM* and Δ*esx-1* mutants with large cosmids encoding the corresponding protein led to reversion to the wild type phenotype, confirming that the ESX-1 secretion system, PPE54 and LppM are directly involved in the blockage of phagosomal maturation. We now plan to explore the detailed mechanisms used by these bacterial protein effectors to trigger bacillus survival inside the host.

3 Host cellular factors contributing to *M. tuberculosis* intracellular replication

Work that led up to the Project: Global transcriptomics analyses have begun to provide new insights into the host-pathogen cross-talk of *M. tuberculosis* infection (Ehrt et al., 2001; Tailleux et al., 2008). However, this approach does not allow one to ascertain whether the modified expression of a particular gene has a direct impact on the bacterial intracellular fate. More recently, genomic RNAi screening in *Drosophila* S2 cells, using *M. fortuitum* as a model, allowed the first identification of cellular factors required for phagocytosis (Philips et al., 2005). Here, we performed the screening of an siRNA (small interfering RNA) library and uncovered key genes involved in the entry and replication of *M. tuberculosis* in mammalian macrophages. In particular, 7 and 14 siRNA duplexes were confirmed to induce a decrease and an increase in *M. tuberculosis* intracellular bacterial load, respectively. Among them, we found calcineurin (Jayachandran et al., 2007) and suppressor of cytokine signalling (SOCS) family member cytokine-inducible SH2-containing protein (CISH) as cellular factors promoting intracellular mycobacterial survival.

4 Chemical modulators of *M. tuberculosis* intracellular persistence

Work that led up to the Project: Until now, researches have been focused on the discovery of new antibacterial agents, using screening of whole microorganisms or target-based approaches. The latter often led to the development of inhibitors that turned out to be inactive in real *in vivo* settings (*e.g.*, *M. tuberculosis* infected macrophages). Therefore, we decided to undertake an unbiased non-target based approach. Our phenotypic cell-based model, whereby macrophages are infected with green fluorescent protein (GFP)–labelled *M. tuberculosis* and incubated with small molecules,

has been applied to the high throughput and high content screen (HT/HCS) of more than 200,000 compounds. This has enabled the identification of two new series of compounds that selectively interfere with the multiplication of *M. tuberculosis* within macrophages but are not active on the tubercle bacillus *per se* (Christophe et al., 2009). Two series of inhibitors showed strong activity at the micromolar range against intracellular *M. tuberculosis* replication but displayed no activity against extracellular, *in vitro* grown mycobacteria up to 100 M, clearly suggesting a putative cellular target. In addition, no obvious host cell cytotoxicity was observed at concentrations up to 100 M. The intracellular antibacterial activities of selected leads were further confirmed in more relevant primary human macrophages, which pave the way for successful development of these particular compounds.

Conversely, G. Kaplan recently reported that induction of *M. tuberculosis* intracellular growth *in vivo* in a latent TB animal model by the mild immunosuppressive phosphodiesterase PDE-4 inhibitor, CC-3052, could lead to full bacillus eradication when given in combination with the antitubercular drug isoniazid (INH) (G. Kaplan, Gordon Research Conference, Oxford, 18th August 2009). This preliminary result thus suggests that triggering bacterial replication could be a more appropriate therapeutic strategy than that of suppressing *M. tuberculosis* intracellular replication. To further explore this concept, and based on our RNAi screen results (whereby we showed that p38 MAP kinase (p38 MAPK) inhibition led to enhanced *M. tuberculosis* growth and, conversely, calcineurin inhibition led to decreased intracellular mycobacterial survival (Jayachandran et al., 2007)), we will investigate whether specific inhibitors of p38 MAPK and calcineurin could be suitable for drug development.

Outcomes of the Project:

- A comprehensive picture of the critical features of *M. tuberculosis* within live target cells provided by combined high content imaging and mycobacterial functional genomics;

- A better understanding on how *M. tuberculosis* controls host cell signalling pathways;

- The proof of concept about whether targeting the intracellular microorganism can lead to novel approaches for the fight against antibiotic resistance.

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