WP5 - Manipulation of phagosome maturation by pathogens & model testing

Deliverable 5.1 (Data array/pathogen genes)

The original plan was to use siRNA to knock-down target mycobacterial genes to establish their role in intracellular survival. However due to technical problems it has not been possible to use this approach. We have therefore switched to the more conventional approach of using mycobacterial mutants, which are attenuated in their intracellular survival as tools with which to probe the intracellular response of the host macrophage.

Many of the attenuated mutants reported in the literature were selected using murine host cells, so the initial work was to confirm reported phenotypes of a selected panel of mycobacterial using human cells. Therefore we screened mycobacterial mutants in human type 1 and type 2 macrophages (Verreck et al, 2004; Verreck et al, 2006) for their potential reduced or increased intracellular fitness. By determining bacterial counts our goal is to identify mutants that show modified intracellular fitness in macrophages for further transcriptome analysis of the infected macrophages. We expect that this will enable us to pinpoint host genes that are differentially regulated upon infection with these mutants and may therefore play a key role in phagosome maturation (Ehrt et al, 2001). This knowledge may help to gain deeper insight into the process of phagosome maturation and may reveal new host targets for therapeutic intervention.

We investigated a set of BCG mutants (see table below) generated by Stewart et al. (Rv3707c, Rv442c (PPE), Rv2301 (cutinase) and Rv1093 (*glyA*) (Stewart et al, 2005) that are unable to control early acidification of the phagosome in the murine macrophage cell line J774. Although these BCG mutants had previously been found to be attenuated for growth in J774 cells, no growth attenuation was observed in human type 1 or type 2 macrophages. In addition we studied a ureaseC deficient recombinant BCG vaccine strain created at the MPIIB that has been genetically modified to express listeriolysin (hly+) (Grode et al, 2005). In this strain absence of *ureC* results in increased acidification of the phagosome and thus inhibits maturation of the phago-lysosome, whereas listeriolysin is a protein that enables *Listeria monocytogenes* to escape from the phagosome of infected cells. The rationale behind this is to improve access of mycobacterial antigens to the MHC I pathway which would result in better CD8 T-cell stimulation and therefore better vaccine efficacy. Investigating the intracellular fitness of ΔureC hly+ rBCG in type 1 and type 2 macrophages we observed a transient reduction in intracellular fitness in both macrophage types at 72-120h post-infection. However, after 120h of incubation outgrowth of *ΔureC* hly+ rBCG was observed. This outgrowth at late time-points is due to increased apoptosis of macrophages, as a loss of cells was observed in the wells infected with *ΔureC* hly+ rBCG. We also tested BCG mutants with *in vitro* growth defects (*glpK* KO, *hspR* KO) (Beste et al, 2009) for their intracellular fitness in these cells. The *glpK* KO mutant grew significantly slower than the parental strain in

batch culture. The *hspR* KO mutant showed comparable growth to wild type in batch culture, however, it has been reported to be unable to switch to slow growth in chemostat experiments (Beste et al, 2009). Infection of type 1 and type 2 macrophages with the *glpK* KO mutant resulted in higher intracellular survival compared to the parental strain. The BCG mutant *ΔhspR* showed reduced intracellular fitness in human macrophages. Intracellular killing of *ΔhspR* was more pronounced in type 2 than in type 1 macrophages.

BCG mutants that were reported to show a reduced intracellular survival in the murine macrophage cell line J774 were not attenuated for growth in the human macrophages tested, which points to an important potential difference in the phagosome maturation between murine and human macrophages (Paul et al, 1996) or cell lines and primary macrophages and requires further investigation. The recombinant BCG vaccine strain *ΔureC* hly+ rBCG showed a transient decrease in intracellular fitness in human macrophages but induced apoptosis of host cells at later stages of infection. Therefore it will be difficult to isolate sufficient amounts of RNA from host macrophages infected with *ΔureC* hly+ rBCG to perform transcriptome analysis. Growth defects in BCG mutants appear to have a significant effect on their intracellular fitness depending on the nature of the growth defect. Slower growth in batch culture appears to correlate with higher intracellular fitness whereas the inability to switch to slow growth leads to a loss of intracellular fitness. We will therefore study the human macrophage host response of these “growth defect” mutants by analysing the macrophage transcriptome.

In order to further substantiate our hypothesis that mutants with growth defects *in vitro* have a modified susceptibility to phagosomal killing by macrophages we will investigate another two mutants (*M. tuberculosis ΔhspR* and *Δmce1*) with well-characterized *in vitro* growth defects (Beste et al, 2009). In addition to these mutants we plan to investigate other deletion mutants (targeting Rv3253, Rv0842, Rv2528c *mrr*, Rv2017, Rv2642), generated in house, in type 1 and type 2 human macrophages. Some of these mutants are already known to exhibit attenuated intracellular growth in murine macrophages. One mutant (Rv2528c *mrr*) has shown an enhanced fitness in murine macrophages.

List of mycobacterial candidate genes investigated for modulation of phagocytosis in host cells

Gene ID of drug target-candidate pathogen genes	knocked out protein/ information on the mutant	Reference	Intracellular fitness in human type 1 and type 2 macrophages
Rv3707c	CHP; unknown protein • predicted extracellular	Stewart et al, 2005	Reduced intracellular fitness was observed for these mutants upon infection of the murine macrophage cell line J774. However, in contrast to these experiments no reduced intracellular fitness was observed in human type 1 and type 2 macrophages.
Rv442c	PPE10; PPE proteins derive their name from a proline–proline–glutamic acid (PPE) motif • the PPE protein family is unique to Mycobacteria and is highly expanded in the pathogenic members of this genus		
Rv2301	cfp25; secreted cutinase		
Rv1093	glyA1; serine hydroxymethyltransferase		
Rv3696c	glpK; glycerol kinase • glpK is essential for glycerol metabolism • mutant exhibits dysgenic growth on plate and in batch culture	Beste et al, 2009	Infection of type 1 and type 2 macrophages with the glpK KO mutant resulted in higher intracellular survival compared to the parental strain.
Rv0353	hspR; heat shock protein repressor gene • negatively regulates several heat shock proteins that are consequently overexpressed in the KO mutant. • Exhibits identical growth during batch phase but significantly reduced fitness during slow growth rate (chemostat)	Beste et al, 2009	The BCG mutant ΔhspR showed reduced intracellular fitness in human macrophages. Intracellular killing of ΔhspR was more pronounced in type 2 than in type 1 macrophages
Recombinant BCG strain	ΔureC hly+ rBCG • absence of ureC results in increased acidification of the phagosome • genetically modified to express listeriolysin (hly), a protein that enables Listeria monocytogenes to escape from the phagosome of infected cells • vaccine candidate in phase I clinical trials	Grode et al, 2005	• reduced intracellular fitness in in human type 1 and type 2 macrophages at 72-120h postinfection • after 120h postinfection outgrowth of ΔureC hly+ rBCG was observed due to increased macrophage apoptosis followed by liberation of bacteria into the medium