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 genes in mycobacteria in order to establish their role in intracellular survival. However due to technical problems it proved impossible to use this approach. We therefore switched to the more conventional approach of using mycobacterial mutants 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 we first set out to confirm the reported phenotypes of a panel of mycobacterial-mutants using human cells. The intracellular fitness of mycobacterial mutants was tested in human type 1 and type 2 macrophages(Verreck et al., 2004; 2006), by determining bacterial counts with the aim to identify mutants with modified intracellular fitness in macrophages. The transcriptome of the macrophages would then be analysed and compared to wild-type infected and uninfected cells. This would 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 would provide deeper insight into the process of phagosome maturation possibly revealing new host targets for therapeutic intervention.

We investigated a set of BCG mutants (Stewart et al., 2005) (see table WP5). Rv3707c, Rv442c (PPE), Rv2301 (cutinase) and Rv1093 (glyA) are unable to control early acidification of the phagosome in the murine macrophage cell line J774 and showed attenuated growth (Stewart et al., 2005). However, no growth attenuation was observed in human type 1 or type 2 macrophages. A urease deficient recombinant BCG vaccine strain genetically modified to express listeriolysin (hly+) (Grode et al., 2005) was also tested. 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 aim of 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 ureC- 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 ureC- 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 ureC- hly+ rBCG. We also tested BCG mutants with in vitro growth defects (Beste et al., 2009) (glpK KO, hspR KO) for their intracellular fitness in human macrophages. The BCG glpK KO mutant grew significantly slower than the parental strain in batch culture. The BCG 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, although the BCG hspR- showed reduced intracellular fitness in human macrophages. Intracellular killing of BCG hspR- 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 between cell lines and primary macrophages, and requires further investigation. The recombinant BCG vaccine strain ureC- 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 ureC- 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 aimed at studying the human macrophage host response of these “growth defect” mutants by analysing the macrophage transcriptome.

To further test our hypothesis that mutants with growth defects in vitro have a modified susceptibility to phagosomal killing by macrophages we investigated another two mutants (M. tuberculosis hspR- and mce1-) with well-characterized in vitro growth defects (Beste et al., 2009). H37Rv mce1- grows like the wild type in batch culture; however, in chemostat experiments H37Rv mce1- exhibits a strong competitive advantage compared to the wild type at slow growth rate. Furthermore, H37Rv mce1- has been reported to be hypervirulent in mice in vivo (Weber et al., 2000; 2000; Lima et al., 2007). In contrast to these findings we did not observe any increased outgrowth of M. tuberculosis mce1- mutants in human macrophages in

vitro. We infected human macrophages with M. tuberculosis hspR- and observed a reduced intracellular fitness similar to its BCG counterpart

We tested in addition another two M. tuberculosis mutants generated at MPIIB: Rv2017- and Rv2528 mrr-. Rv2017 is possibly a transcriptional regulatory protein, and reduced intracellular fitness was observed in murine macrophages, however no reduced intracellular fitness was found in human type 1 and type 2 macrophages. Rv2528 mrr encodes a potential restriction protein, and previous analysis revealed the mutant was slightly hypervirulent relative to wild type in mice in vivo and murine macrophages in vitro. However, upon infection of human macrophages, Rv2528 mrr- showed only a very low, statistically insignificant, increase in intracellular fitness compared to the wild type.

We further tested the BCG metabolic mutant narG-, which lacks the ability to reduce nitrite under anaerobic conditions (Weber et al., 2000). It has been reported that BCG narG- fails to persist in immune competent (BALB/c) mice, but this attenuation was not reproduced in in human type 1 or type 2 macrophages. We also included the recombinant BCG strain complemented with RD1. RD-1 is the chromosomal locus that encodes for the ESAT-6 secretion system 1, that was deleted during attenuation of M. bovis to produce BCG. Enhanced virulence of BCG::RD1has been reported in immunodeficient mice in vivo (Brodin et al., 2006), however our investigations did not reveal any hypervirulence in human macrophages. Recent studies suggest that the RD-1 locus is critical in promoting IL-1beta secretion by infected mouse and human macrophages (Mishra et al., 2010; Dorhoi et al., 2012). Together these observations suggest that bacteria within the phagosome secrete virulence products encoded within the RD1 locus to control macrophage inflammatory responses, without any intracellular fitness penalty.

In summary we found four mycobacterial mutants that modulated phagocytosis and replication in human macrophages: M. bovis BCG mutants Rv3696c, Rv0353, recombinant BCG ureC- hly+ rBCG, and M. tuberculosis H37Rv Rv0353. Only minor differences were observed between type 1 and type 2 human macrophages. The recombinant BCG strain ureC- hly+ showed a transient decrease in intracellular fitness in human macrophages, but induced apoptosis of host cells at later stages of infection, making it impossible to isolate sufficient RNA amounts to perform host macrophages transcriptome analysis. Interestingly, three of the four mutants that modulate phagosome maturation have confirmed growth deficits. In vitro growth

defects in BCG and Mtb mutants appear to have a significant effect on their intracellular fitness. 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. BCG ureC- hly+ shows no growth deficit in batch culture, but remains untested in a chemostat.

The original goal of this workpackage was to analyse the transcriptome of infected macrophages in order to pinpoint host genes differentially regulated upon infection with mycobacterial mutant; this information would lead to a better understand of phagosome maturation and it manipulation by pathogens. M. tuberculosis and BCG mutants that influence phagosome maturation in human host cells were identified and characterised, but so far we have been unable to obtain usable data due to the low yield of RNA: one of the selected strains (ureC- hly+ rBCG) induced death of the infected cells, which further complicated the use of the strain in this study. Two of the other strains investigated, BCG hspR- and H37Rv hspR-, are carrying the same mutation, which further reduced the number of actual candidate genes to two, even though significant growth differences were observed depending on their background. However, due to the considerable discrepancies observed between the human and mouse macrophages used to test all the mutants, the validation of genes in the mouse model (D5.3) became obsolete.