WP4 - RNAi screens – maturation & model testing

Deliverable 4.1 (**Focused data array / pathogen survival**)

The two partners contributing activities to this work-package have established protocols and methods for the proposed screens using *M. tuberculosis* (LUMC) and *Salmonella* (NKI), as part of deliverable 4.1, and screening will commence in the second period of the project. The high throughput data acquisition nature of this project requires an extremely solid and robust system from which we can obtain reliable and reproducible data. **LUMC** have now developed and perfected the system, however we have experienced unanticipated delays on having to optimise separate systems for *Salmonella* and for *M. tuberculosis* and completion of this deliverable has been delayed to month 24.

Setting up the assay has taken longer than originally planned primarily due to fact we had multiple parameters to establish, namely selection of suitable cell lines that are easily transfectable with siRNA as well as being capable of phagocytosing mycobacteria. This aspect of our assay first led us to use the common monocytic cell line, THP-1, with which proved difficult to transfect with siRNA while maintaining an acceptable percentage of viable cells. Our search for suitable cells also lead us to generating clonal cell lines expressing CR3 and/or CD43 to enhance mycobacterial uptake in HeLa for instance, however the cell lines were not stable enough for the large screens we wished to perform, thus the reproducibility would have been compromised. We also lost a considerable amount of time using mycobacteria fluorescently labelled with Dylight compounds, which (a) caused extensive cording of the mycobacteria and (b) the fluorescence signal did not depreciate rapidly enough after phagosome lysosome fusion for the time frame of our experiments.

After circumventing the issue described above, the **LUMC** has now developed and perfected the system. Notably, we have:

- **Established a database** of 1300 target proteins to be knocked down using siRNA including Ras GTPases, GAPs and GEFs as well as kinases, phosphatases and genes identified by microarray to be induced only by live virulent mycobacteria in macrophages.
- **Explored suitable cell lines** for high throughput siRNA transfection and infection. HeLa are easily transfectable but non-phagocytic: suitable for optimising siRNA transfection conditions and *Salmonella* infection. MelJuSo are able to phagocytose *Mycobacterium tuberculosis,* are also easily transfectable and suitable for the screening stage of the project due to the high reproducibility of results obtained with these cells. THP-1 cells, although monocytic, are hard to transfect and thus only suitable for the verification stage of the project (smaller sample sizes).
- **Set up siRNA transfections** by optimising conditions to achieve up to 95% knockdown of AKT1 relative to scrambled siRNA control oligos in HeLa and MelJuSo cells and verified this

knockdown by immunoblotting. We verified that siRNA knockdown of AKT1 mimics the effects of a chemical inhibitor of AKT1 (H-89) on bacterial load after *Salmonella typhimurium* infection of HeLa cells. Verified that we are able to reproduce the results of our collaborators at Netherlands Cancer Institute (Kuijl, Neefjes *et al*.) by knocking down MARK2 which resulted in a decrease in *Salmonella typhimurium* bacterial load in HeLa cells. We studied the progression of siRNA knockdown of AKT1 in HeLa and MelJuSo cell lines by immunoblotting to determine the optimal time-point for infection with *Salmonella* or *M. tuberculosis*.

- **Infection protocols.** Optimised and streamlined protocols for infection of HeLa cells with *Salmonella* and MelJuSo cells with *Salmonella* and *M. tuberculosis* to increase our readout window. Verified that H-89 (chemical inhibitor of AKT1) treatment of HeLa and MelJuSo cells infected with *Salmonella* or *M. tuberculosis* mimics our own previous results in primary macrophages. Performed time-course experiments on MelJuSo cells infected with *M. tuberculosis* to determine the optimal time-point post infection for readout.
- **Set up two types of 96 well screening assays.** *HTS flow cytometry assay* (primary screen); a fast, robust and cost-effective semi-automated screening assay employing a flow cytometer coupled to a 96-well plate loader for assaying bacterial load and estimating host cell survival/proliferation using bacteria (*Salmonella*, *M. tuberculosis*) expressing green fluorescent protein. We verified flow cytometry results are in agreement with colony forming unit (CFU) assays and by fluorescent microscopy. *Dual-luciferase assay (*secondary screen*)* is an accurate luminescence screening assay employing bacteria expressing a firefly luciferase enzyme and human cell line expressing a *Renilla* luciferase assay enabling measurement of both bacterial and host cell viability within a single well.
- **Data analysis** Set up semi-automated data analysis for the screening stages of the project, providing a standardised computer readable data format for systems biologists.
- **Drug screen phosphatase assay.** We have also initiated pilot phosphatase inhibitor screens in mycobacterial infection which will help determine phosphatases involved in pathways in phagosome maturation
- **Our current activities are** performing the siRNA screens and plate validation assays.

In our previous screen at the **NKI** (Kuijl et al., 2007), we have shown that kinases involved in pathways related to cytoskeletal re-arrangements affect the maturation of *Salmonella* containing phagosomes.

We recently focused our study on ABL1 tyrosine kinases that have been shown to regulate Rac-dependent cytoskeletal dynamics in mammalian cells (Van Etten et al, 1994; Wang et al, 2001) suggesting its role in bacterial uptake (Ly and Casanova, 2009). So far we have demonstrated, in agreement with previous studies (Ly and Casanova, 2009), how impairment of ABL1 tyrosine kinase activity by using a specific ABL-inhibitor Gleevec (also called Imatinib; Khorashad et al.

2009) affects *Salmonella* uptake. Moreover, by using siRNA to knock down some tyrosine phosphatases that we previously identified as proteins impairing *Salmonella* infection (C. Kuijl, personal communication), and treating the same cells with Gleevec before their infection with the pathogen, we identified MTMR7 as a phosphatase having an important role in *Salmonella* internalization in MCF7 cells. More specifically, the silencing of tyrosine phosphatase MTMR7 (Mochizuki and Majerus, 2003), together with inactivation of tyrosine kinase ABL1 by Gleevec treatment, resulted in a strong synergistic decrease of *Salmonella* internalization, suggesting that these two proteins act together in regulating *Salmonella* infection. Our current goal is to understand how MTMR7 affects the *Salmonella* uptake and how this phosphatase acts together with the ABL1 kinase in regulating this process.

In addition, we have performed an siRNA screen for both kinases and phosphatases involved in the control of *Salmonella* uptake and intracellular growth. We identified some 10 kinases, and 14 phosphatases involved in the various aspects of *Salmonella* infection. At the same time, we generated a phosphatase chemical library (in collaboration with H. Ovaa, NKI) and expanded our kinase inhibitor library (in collaboration with H. Overkleeft, LIC, Leiden). We now aim at integrating the two data sets experimentally, since the substrates of most phosphatases are unknown. At the same time, we generated and purified a number of the phosphatases identified, to test their activity in the presence of the identified biologically active phosphatase inhibitors. We have now identified a number of target-lead combinations for these and are improving the compounds to generate selectivity. Of note, these compounds will be used in combination with silencing kinases or over-expressing their constitutive active form to generate biologically active phosphatase-kinase pairs; this is an important step to generate the data required for understanding kinase/phosphatase networks.

Finally, we have shown how to modify our bacteriostatic compound, the PKA inhibitor H-89 into an PKB/Akt1 inhibitor. We have now further modified this compound into a biologically active PKB/Akt1 inhibitors with low activity on PKA using a secondary modified compound library. In addition, we have generated potential PKD1 inhibitors on the basis of the H-89 core structure, which will also inhibit PKB/Akt1 activity and have low inhibitory effects of PKA as well as PKB/Akt1 using *in vitro* assays. We are building the third generation library of these structures to further improve selectivity and activity.

A paper showing the conversion of H-89 into PKB/Akt1 and PKD1 inhibitors is in preparation.

A paper describing the results of an siRNA library screen for factors controlling MHC class II expression and peptide loading is under revision (Paul et al.).

The use of resources is according to plan, see section 4 for more details.