WP4 - RNAi screens – maturation & model testing

The two partners contributing to this work-package have established protocols and methods for screening M. tuberculosis (LUMC) and Salmonella (NKI/LUMC) infected phagocytic cells. The project required high throughput data acquisition so it was essential to establish a solid and robust system from which we can obtain reliable and reproducible data. LUMC developed and perfected the basic system, and optimised separate procedures for the Salmonella and M. tuberculosis screens.
Developing the assay took longer than originally planned, primarily due to fact that we had to establish multiple parameters, namely selection of suitable cell lines that were both easily transfectable with siRNA, as well as capable of phagocytosing mycobacteria. Significant results include:

- Identification of cell lines suitable for high throughput siRNA transfection and bacterial infection. HeLa are easily transfectable but non-phagocytic: they are suitable for optimising siRNA transfection conditions and Salmonella infection. MelJuSo phagocytose M. tuberculosis, and are easily transfectable. The results obtained were highly reproducible, making them a suitable system for the project screens. THP-1 cells, although monocytic and able to phagocytose bacteria, are harder to transfect and thus only suitable for the verification and validation on smaller sample sizes of the primary hits obtained in the screens.

- Optimise siRNA transfections in HeLa and MelJuSo cells to achieve up to 95% knockdown of AKT1, relative to scrambled siRNA control oligos; this was verified by immunoblotting. We also confirmed that siRNA knockdown of AKT1 in Salmonella Typhimurium infected HeLa cells decreases the bacterial load, and so mimics the effects of H-89, a chemical inhibitor of AKT1. The optimal time point for bacterial infection after siRNA knockdown in HeLa and MelJuSo cells was determined by following the decrease in AKT1 signal using immunoblotting. During the screening stages of the project RNAi protocols were further optimised to enable efficient gene knock-down in the human monocytic cell line THP-1 and in primary human macrophages generated from monocytes isolated from buffy coats of healthy donors. Successful knockdown was verified by qPCR and immunoblotting.

- Infection protocols. We optimised and streamlined protocols for infection of HeLa cells with Salmonella and MelJuSo cells with M. tuberculosis to increase our readout window. We verified that H-89 (chemical inhibitor of AKT1) treatment of HeLa and MelJuSo cells infected with Salmonella or M. tuberculosis reproduced our previous results in primary macrophages. Time-course experiments on MelJuSo cells infected

with M. tuberculosis were performed to determine the optimal time-point post infection for readout.

- Set up a 96 well screening assay. Our HTS flow cytometry-based assay is a fast, robust and cost-effective semi-automated screen employing a flow cytometer coupled to a 96-well plate loader for measuring bacterial load using Salmonella or M. tuberculosis expressing DsRed, and estimating host cell survival/proliferation. Flow cytometry results are in agreement with colony forming unit (CFU) assays and fluorescence microscopy.

- Developed novel tools for studying intracellular trafficking of Salmonella and mycobacteria. These include bacteria (conditionally or constitutively) expressing stable or destabilized DsRed fluorescent protein and bacteria expressing multiple conditionally expressed reporter genes.

- Data analysis. We established semi-automated data analysis for the screening stages of the project, providing a standardised computer readable data format.

A paper outlining the RNAi and chemical compound screening assays is in preparation with intention to publish before 2013.

Deliverables: Utilizing the methods outlined above we performed siRNA and chemical compound screens on human cell lines infected with either Salmonella or M. tuberculosis. Promising preliminary screening results showing considerable bacterium-specific effects of host-targeted chemical compounds prompted us to perform all screens using both Salmonella and M. tuberculosis infected human cell lines. The overall focus was towards the study of regulatory proteins, relatively unbiased chemical compound libraries and integration of different screens to establish a comprehensive view of the regulatory host protein network for both Salmonella and M. tuberculosis. The focused RNAi array for Rab modifying proteins (Deliverable 4.1: Focused data array/pathogen survival) was abandoned in favour of an RNAi library for human deubiquitinases, which has a higher chance of identifying novel leads due to its unbiased nature (posttranslational protein modifications by deubiquitinases are not restricted to specific cellular mechanisms or protein families) and is as of yet left unexplored in the context of M. tuberculosis infection, increasing chances of generating high-impact results. RNAi screens for the human kinome were performed as part of Deliverable 4.3 (Focused data array/phagosome maturation). Both RNAi screens identified hits that markedly decreased or increased bacterial load of Salmonella or M. tuberculosis, or both in our cell-based assay. Results of these screens were fed into

WP1 for gene ontology (GO) analysis and constructing predictive clustering trees (PCTs).

Due to some delays in final assay development for the siRNA screens we decided to include chemical compound libraries as part of the drugome array (Deliverable 4.4: Drugome array/Salmonella). To identify druggable targets involved in regulation of phagosome maturation in human cell lines infected with Salmonella or M. tuberculosis, we performed four separate chemical compound screens:

- A Library of Pharmacologically Active Compounds (LOPAC; commercial, Sigma-Aldrich) was used to probe host regulatory networks for druggable targets regulating intracellular survival of Salmonella or M. tuberculosis. Results of this screen were fed into WP1 to develop an automated method for aggregation of known protein targets of chemical compounds in public repositories (PubChem, ChEMBL), outlined in the section on WP1 in this report. This provided a valuable protein target framework for identifying novel regulatory networks, due to its unbiased nature (chemical compounds may target any type of host protein). Targets were analyzed for enrichment, GO analysis was performed and PCTs were constructed to predict targets or combinations of targets for follow-up (WP1).

- A screen of the third generation PKB/Akt1 inhibitors derived from H-89 (H. Overkleeft, Leiden Institute of Chemistry) on human cells infected with Salmonella or M. tuberculosis led to the identification of several molecules with strong inhibitory effects on intracellular M. tuberculosis, Salmonella or both. Kinase profiling of a selection of compounds with distinct effects on bacterial load and PKB/Akt1 inhibition, identified candidate kinases that may regulate intracellular survival of Salmonella or M. tuberculosis. The role of these kinases in regulation of phagosome maturation is currently under investigation using RNAi. Of note, several kinases identified by this approach were also identified in the kinome RNAi screen. A paper on the PKB/Akt1 inhibitor screens is in preparation with intention to publish before 2013.

- A library of potential phosphatase inhibitors (H. Ovaa, Netherlands Cancer Institute) was screened on human cells infected with Salmonella or M. tuberculosis and resulted in the identification of a novel compound that reduced M. tuberculosis bacterial load.

- A library of autophagy inhibitors/activators (commercial, ENZO Life Science) was screened on human cells infected with Salmonella or M. tuberculosis, identifying

multiple chemical compounds affecting bacterial load of either Salmonella or M. tuberculosis or both. Of note, the top hit in this screen was structurally similar to two compounds from the LOPAC library screen, all three resulting in a decrease in M. tuberculosis bacterial load. These compounds all shared a deubiquitinase as a common target, which was also identified in the siDub screen.

Generally, all screens identified compounds or siRNA oligos that affected bacterial load of either Salmonella or M. tuberculosis or both. Hits from all the screens were integrated in a single network analysis to identify their possible interactions and/or participation in common processes. Here, targets identified in the LOPAC library screen were used to construct a general network consisting of targets with a regulatory role as well as targets with effector function, whereas the RNAi screens provided valuable data to identify crucial regulatory nodes within these networks. In addition to the unbiased, data-driven predictions performed in WP1, targets that were either identified in multiple independent screens or that complemented each other in the different regulatory networks identified using STRING or Ingenuity Pathway Analysis were selected for follow-up. Although the final analyses are still on going, the combined efforts have already led to the identification of several lead targets and testable hypotheses that are currently being tested experimentally. The combined efforts will be pulled together as Deliverable 4.5, and result in several papers with intention to publish in the course of 2013.

Interfere with the human kinome and validation of positive hits: 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. During this work-package we focused our studies on the ABL1 tyrosine kinases that have been shown to regulate Rac-dependent cytoskeletal dynamics in mammalian(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 before Salmonella infection (i.e two hours before infection) by using a specific ABL-inhibitor Gleevec (also called Imatinib;(Khorashad et al., 2009)) affects Salmonella uptake. The same result was obtained by using Gleevec-like inhibitors NTK-9 and NTK-12 synthesized by H. Owa's group (NKI). In an effort to investigate the pathways involved in phagosome maturation, we searched for proteins that can affect Salmonella internalization and/or replication together with the kinase ABL1.

By performing an siRNA screen for phosphatases, we identified 14 targets involved in the control of Salmonella uptake and intracellular growth (C. Kuijl, personal communication). The silencing of some tyrosine phosphatases that we previously

identified as proteins impairing Salmonella infection, and the treatment of the same cells with Gleevec before their infection with the pathogen, suggested that MTMR7 phosphatase, among others, might have a role in Salmonella infection 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 in Salmonella internalization/replication, suggesting that these two proteins might act together in regulating Salmonella infection. However, we were not able to reproduce these data in other cell lines, such HeLa cells, nor in other batches of MCF7 cells, suggesting that MTMR7 phosphatases possibly represent a false-positive hit of the phosphatases siRNA screen mentioned above.

Besides MTMR7, the silencing of some DUSP phosphatases revealed a synergistic decrease in Salmonella infection in MCF7 and HeLa cells, in combination with ABL1 kinase inhibition by Gleevec. In particular, the silencing of DUSP 27 and DUSP 11 in Gleevec-treated cells produced an additional effect in decreasing the Salmonella infection compared to Gleevec alone. At the same time, we generated a phosphatase chemical library (in collaboration with H. Ovaa, NKI) and purified a number of the phosphatases identified in order to test their activity in the presence of the biologically active phosphatase inhibitors. We have now identified one phosphatase inhibitor that inhibits DUSP 27, DUSP 11 and DUSP 3, and produces an additional effect in decreasing Salmonella infection when combined with Gleevec-inhibition of ABL1. This suggests that DUSP phosphatases work together with ABL1 kinase in controlling Salmonella infection. We are currently improving the phosphatase inhibitors to generate selectivity for one single phosphatase. These compounds will be used in combination with silencing of 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. A paper outlining the role of kinases and phosphatases in controlling Salmonella infection is in preparation with intention to publish before 2013, as part of Deliverable 4.5.

Finally, we have shown how to modify our bacteriostatic compound, the PKA inhibitor H-89 into a PKB/Akt1 inhibitor with low activity on PKA, by using a secondary modified compound library. In addition, we have generated potential PKD1 inhibitors based on the H-89 core structure, which will also inhibit PKB/Akt1 and have low inhibitory effects on PKA and 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.