WP2 - Assay Development and Phagosome characterization

During the project we successfully developed high-throughput, high-content screening assays using both murine and the more physiologically relevant primary human macrophages, derived from peripheral blood monocytes. We optimized the mycobacterial infection assay, combining the model organism Mycobacterium bovis bacillus Calmette-Guerin (BCG), genetically engineered to express GFP, with LysoTracker® Red DND-99 staining of lysosomes in the host cells. This provided a direct readout of the release of the phagosomal maturation block and degradative delivery of the bacteria to lysosomes when the system was perturbed.

Significant results • Establishment of the high-throughput transfection protocol for efficient RNA knock-down in murine cell lines and primary human macrophages in 384 well format, using the high throughput electroporation platform CELLAXES

- Upgrading of the QMPIA image analysis system for RNAi screens in both murine cell lines and primary human macrophages
- An RNAi library that targets ~300 genes (4 oligos per gene) was screened twice in M. bovis BCG-infected primary human macrophages
- The screen was analysed based on identification of rare phenotypes by binning of multi-parametric profiles
- A library of 2000 chemical compounds was also screened in M. bovis BCG-infected primary human macrophages
- Hits are being validated using laboratory and clinical isolates of M. tuberculosis in macrophage infection assays, measuring bacterial growth as the output
- The mode of action of hits identified in both chemical and RNAi screens is being inferred by comparing the multi-parametric phenotypic profiles obtained in both screens

Effective transfection protocols were established for infected human macrophages, but we encountered problems in identifying phenotypes associated with successful phagosome-lysosome fusion and mycobacterial degradation. Initial analysis using average values obtained from whole sample wells proved inadequate, so we implemented a binning analysis to improve the degree of separation between experimental and control samples. Finding the link between profiles obtained from the RNAi screen with those obtained from the chemical compounds screen proved more complex than initially expected: nevertheless by including additional functional genomics data we were able to identify host macrophage genetic nodes, such as

autophagy and rates of endocytosis trafficking, that when perturbed by chemical treatment, led to clearance of the intracellular pathogen.

Deliverables 2.1 (High-throughput Imaging assay) and 2.2 (Predictive models of phagosome maturation): We developed a phenotypic cell-based assay to use in an RNAi or chemical compound screen, aimed at systematically identifying host regulators of mycobacterial phagocytosis and, in the longer term, novel therapeutic targets. The optimised model uses primary human macrophages as host cell, infected with Mycobacterium bovis bacillus Calmette-Guerin (BCG) genetically engineered to express GFP.

The processes established to implement the screen involve: • transfection by electroporation to transfect primary human macrophages in optical 384-well plates with siRNAs, using the CELLAXES technology

- automated protocols to wash away the transfection medium, administer the bacteria to the host cells and wash away the non-internalized bacteria after the pulse period
- imaging the fixed plates at high resolution (40x) on an automated confocal microscopy platform (Opera, Perkin Elmer) equipped with an automated plate stacker
- the adaptation of image analysis software (Motion Tracking, developed in house by Prof. Yannis Kalaidzidis) to extract quantitative descriptors of the images and generate multi-parametric fingerprints of the possible phenotypes.

Deliverables 2.3 and 4.6 (data array/ phagosome maturation and endocytosis): The human macrophage model was used to perform a screen of a targeted library of 300 genes. Due to the large amounts of oligonucleotides needed for the electroporation technology, we decided to use a smaller library than the human kinome and phosphatome libraries originally proposed for these deliverables.

The screening campaign was preceded by an assay development phase in which we wanted to find positive controls: genes that, when down-regulated, would produce a phenotype resulting in degradation of intracellular bacteria (described by different parameters such as reduced intensity and number the bacteria, lysotracker signal associated with bacteria, all in the context of different host cells metrics).
We selected genes identified in the literature as having an effect on the host response to mycobacteria, but were unable to find a single gene that gave a

measurable phenotype in our assay. A double knockdown approach however, provided appreciable phenotypes. In particular the combinations of oligos targeting Coronin1 (Cor) and Pi3KCB gave a reproducible bacterial degradation phenotype.

The decision was taken then to perform the screen using a double knockdown geometry, in which a set of 266 genes, chosen following the rationale schematized in the figure below, were silenced in the presence of an oligo against Cor1A OR against Pi3KCB.

Microarray expression data from BCG-infected human macrophages (analysed by WP1: Saso Džeroski) was combined with the hits from an endocytosis genome wide screen performed in the Zerial lab (Collinet et al., 2010): the rationale being to enrich for genes whose knock-down has a functional impact on the regulation of endocytosis (deliverable 4.6), a process which shares components with mycobacterial phagocytosis. We had originally planned to directly apply our assay to a panel of genes comprising the kinome and a set of regulators of endocytosis, however this proved impracticable due to difficulties associated with scaling up the technology.

We have however increased the value of the screen by using a more relevant model system; primary human macrophages, instead of the originally proposed murine cell lines. In addition, we have also complemented the limited Functional Genomic activity, with a Chemical Genomic approach. The use of Chemical array data, and its integration with Functional Genomic data in the context of a different assay, has allowed us to produce interesting results regarding the mode of action of the compounds and the identification of host cellular pathways that could be exploited to re-establish the delivery of the pathogen to the phago-lysosome.

The analysis of a multi-parametric screen is a complex task, that was rendered even more complicated in this case by the fact that averaging the parameters measured in each well was insufficient to find phenotypes, due to the fact that only a subpopulation of cells in any given well shows a particular phenotypic response. In these circumstances we have found that dividing the negative control population into percentiles (binning) and comparing the population distribution in the corresponding parameter was a useful analysis method for scoring hits.
We have developed an R template implemented in KNIME for this subpopulation analysis. The script partitions the sorted population of a negative control into n equal sized bins (in this case n=5). Each bin covers a percentile of a parameter range. The

script then determines the count of objects in each parameter bin for each experimental well. Objects whose values lie either below or above the range of the negative control are attributed to either the lowest or highest bin respectively. The distribution of population counts is then used to compute z-score values relative to the negative control. This process is applied automatically for each parameter and each experimental condition in KNIME partitioning the population into a chosen number of bins. The final output of the template is a profile of n z-scores that we call 'z-score profiles'.

In this manner is it possible to select parameters that score changes in population distributions. To reduce the noise we also operate a parameter selection, based on the evaluation of the correlation of the object based measurements, taking into account only parameters that have <0.4 of Pearson correlation between each other. The result of this series of steps is the identification of bins of a discrete series of parameters (15), which are then used to calculate distance metrics, like the Mahalinobis distance, to define hits based on strength of the phenotype and to cluster genes in phenotype specific classes.

The result of the two different runs of the screen identified specific group of genes for each background, and we hypothesize that this is due to different epistatic relationships between the knockdown of the gene in the COR background and the PIK3CB background. A difficulty in providing a stable and definitive hit-list is the variability of the results from run to run. We will proceed with more runs to increase the statistical power of the analysis.

A complementary approach to find host regulators of bacterial survival was to use a Chemical Genomics screen in the same assay, to search for compounds able to stimulate the host cells to overcome the mycobacteria-induced phagosome-lysosome fusion block. This was achieved by screening a library of 2000 well-defined compounds, most of which are FDA approved drugs, in mycobacteria-infected primary human macrophages. In order to find compounds that were able to relieve the block without having a direct influence on bacterial uptake, we added the compounds to already infected cells.

The analysis in this case was more straightforward as the phenotypes identified were much more homogeneous in the cell population, compared to the functional genomics approach, meaning that averages derived from all the cells in a well had sufficient discriminative power. Phenotypic strength and clustering approach were

used to discriminate compounds with no effect from various phenotypic classes.

In particular we focused our attention to a cluster of 131 compounds having a profound effect on the bacterial fitness and, at the same time, having little or no influence on the host cell parameters. These are highlighted in the following scheme where the clusters are colour coded, with each box representing a Z value of a single parameter. We were able to show that the majority of the compounds in this cluster were not directly bacteriostatic, but only exerted their function through the host cells.
Three of these compounds were validated using one lab strain (H37Rv) and two clinical isolates of M. tuberculosis to infect primary human macrophages that were then treated with the compounds. Lysing infected macrophages and counting colony-forming units assessed bacterial viability.

The analysis of the chemical genomic campaign was certainly more straightforward than for the functional genomics screen, with chemicals give better phenotypes in the assay, but as expected the identity of the mode of action of the compounds remains problematic. In the absence of a direct functional genomic/chemical genomic link, we decided to approach the problem by integrating data from different screening campaigns to try to describe important host features in the reaction to the mycobacterial infection. In particular we have access to a highly granular set of data, the endocytosis genomic screen, in which every gene is quantitatively annotated in an assay describing endocytosis (Collinet et al., 2010): this assay was performed on HeLa cells, so direct phenotypic comparison was difficult. Consequently we decided to perform a bridging screen, in which we used the HeLa system and the endocytosis assay, but in presence of the same library as the mycobacteria assay. This resulted in a description of endocytic profiles of the compounds which were active in the mycobacteria assay.

By comparing the multi-parametric profiles of these phenotypes with those of the genome wide assay of endocytosis, we were able to perform an enrichment analysis of the cellular processes that genes with similar profile in the endocytosis assay shared. Using this technique we were able to predict the modes of action of the compounds in the active cluster of the mycobacteria-macrophage infection assay; this was then successfully validated experimentally for some of the hits.
The results of this activity are currently being summarized in a manuscript to be submitted for publication to “Cell: Host and Microbe”.