WP3 – RNAi screens: uptake and model testing

Deliverable 3.1 (Focused data array / uptake): The work in WP3 was delayed by the untimely and tragic death of Dr. E. Caron in July 2009. A cell biologist, Dr. Vania Braga, was recruited in September 2009 to manage WP3. Taken together with the high turnover of appointed post-doctoral staff (2 replacements in 18 months), this has had adverse effects on the project and led to delays in the progress of the experimental work.

Our initial screening work focused on setting up an appropriate experimental model to study M. bovis BCG uptake by the human macrophage-like cell line THP-1, suitable to use in an RNAi screen with a library targeting human mRNAs. We aim to (a) optimise a quantitative detection method that is suitable to test hypothesis-driven experiments, as well as medium-throughput screens; (b) validate the chosen model system and experimental conditions to identify and quantify signalling pathways relevant for bacterial uptake.

Identifying the receptor relevant for BCG internalization will facilitate mechanistic understanding of the signalling processes involved and how to interfere with them therapeutically. During the work to optimise the model system, we demonstrated that macrophages differentially uptake BCG found as single particles or as clumps. This is an interesting observation as mycobacteria characteristically grow as clumps, which show some similarities to biofilms formed by other bacteria, which provide environmental and survival advantages (Lasa, 2006). However, the functional consequences of clump uptake for phagosome maturation, intracellular mycobacteria survival or pathogenesis have not been investigated. In fact most research has focused on mycobacteria as a single particle because of the technical challenges of dealing with clumps.

An immunofluorescence-based assay was optimized to quantify levels of associated and internalised phagocytic targets, and differentiate between single or larger particles (Maryke Carsten, Imperial College). We demonstrated that in THP-1 macrophages, CR3 receptors are required for internalisation of single BCG, but not clumps. RhoA, involved in signalling pathways downstream of CR3, is also necessary for BCG uptake. Indeed, RNAi depletion of RhoA reduced the uptake of single BCG bacilli. Our data suggest that CR3 can act as a direct phagocytic receptor for BCG, and that BCG internalization utilises signalling from Rho small GTPases as shown for opsonized red blood cells.

The implications of our results are that, depending on the size of BCG particles presented to macrophages, different receptors are engaged and responsible for uptake. The corollary is that distinct intracellular signalling can be triggered and may influence the outcome of infection and immune response. To address the functional significance of our findings, we used two approaches. First, in collaboration with partners Dorhoi and Kaufmann (WP5), we determined that mycobacteria is found both as single cells and as clumps in the lungs of infected mice. Second we demonstrated that infection with single or clumped BCG leads to different patterns of cytokine production by human macrophages. The IL-1beta and IL-6 expression is significantly increased by clumped BCG at 48 hrs compared to single BCG. However, IL-4 and TNF-alpha expression was not detected at all when THP-1 monocytes were infected with either BCG size. These data suggest that single BCG and clumped BCG induce cytokine expression differentially, consistent with their internalisation through different signalling receptors.

Deliverable 3.3 (Finalized data array / uptake) We next proceeded to identify which signalling molecules could participate in the differential internalization of single or clumped BCG using RNAi to deplete Rho small GTPases GEFs, GAPs and effectors, including a number of kinases and phosphatases. The differential labelling detection method (associated or internalized particles) was successfully adapted for use with BCG uptake inTHP-1 macrophages seeded in 96-well plates. Custom-made software was developed by Dr. Chris Tomlinson (Centre for Integrative Systems Biology and Bioinformatics, Imperial College) to allow automated quantification from immunofluorescence images to generate four parameters: attached or internalized particle detection, single or clumped BCG.

The software is able to (i) identify specific BCG shapes by setting a threshold to eliminate debris and differentiate single (20-80 pixels) and clumps (81-500 pixels) at the amplification of acquired images; (ii) determine the localization of mycobacterium on or inside the THP-1 cells. Single and clump BCG were coloured in different colour inside the cells for quality control and visual analysis. The final output gives the details of each well: the number of cells, the total BCG count, the BCG attached to the cells, BCG internalised, the number of single BCG and clump BCG attached and internalised. Furthermore, software was thoroughly validated with a subset of images that had been visually analysed or semi-automatically by using ImageJ software.
RNAi screens were performed in triplicate. In collaboration with partners Barsacchi and Zerial (WP 2), the fluorescent images from the screen plates were obtained with an automated confocal microscope (Opera, Perkin Elmer) equipped with an

automated plate-stacker (10 images per well were collected with four channel DAPI, Cy3, Cy5 and FITC). A large-scale dataset of images was generated and analysed by the custom-made software, including statistics to identify the target proteins required for BCG uptake. We selected 30 proteins for further validation with four individual oligos to confirm the phenotype.

In summary, we have set up a robust assay to investigate the mechanisms of uptake of BCG by human macrophages. CR3 appears to be important for uptake of single bacteria but it is not required for attachment of bacteria to macrophages. Consistent with different receptors being required for single and clumped BCG uptake, the expression profile of cytokines was distinct if THP-1 macrophages were infected with single compared to clumped BCG. Finally, we have also completed the RNAi screen and developed software to identify the network of regulators mediating BCG uptake. In work continuing beyond the end of this project, we will (a) cross-reference our results with data provided by WP2, WP4 and WP5 and (b) test predictions and molecules identified in other work-packages (bacterial or host proteins) in the uptake of BCG. A manuscript on the characterisation of CR3 as a direct phagocytic receptor for BCG is being finalised for re-submission.