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. The above has had adverse effects and delays in the progress of the experimental work due to the impact on employed staff. A cell biologist, Dr. Vania Braga, has been recruited since Sep 2009 to manage WP3.

Initially, work concentrated on setting up an appropriate experimental model to study bacterial uptake by macrophages. We aimed to (a) optimise cell culture, infection and a quantitative detection method that are suitable to test hypothesis-driven experiments as well as medium-throughput screens; (b) validate the chosen model system and experimental conditions to identify signalling pathways relevant for bacteria uptake in a quantitative manner.

Several cell types were explored, including mouse primary macrophages. The macrophage-like cell line THP-1 was selected, as it is a human cell line suitable for use with an RNAi library targeting human mRNAs. The organism *M. bovis* BCG was chosen as a model of pathogenic mycobacteria and a series of experiments established optimal infection conditions (time course, MOI, etc). An immunofluorescence-based assay to quantify levels of associated and internalised phagocytic targets has been used, since it allows accurate single-cell based measure of phagocytosis (Caron & Hall, 1998). This differential labelling detection method (associated or internalized particles) was successfully adapted for use with BCG and in RNAi screens.

The receptor(s) that specifically mediate BCG phagocytosis are not well characterised, and identifying which receptor is relevant for BCG internalization will facilitate mechanistic understanding of the signalling processes involved. As macrophages have many different phagocytic receptors that may contribute to BCG internalization, we used a minimal model system, receptor-transfected non-phagocytic COS-7 cells to address this questins. This is a well established model that has been successfully used in the lab (Wiedemann et al, 2006; Groves et al, 2010). COS-7 cells expressing CR3 showed significantly increased association and uptake of BCG as opposed to expression of other receptors such as FcγR and CD43. To support the involvement of CR3 in BCG uptake, we used three approaches. First, the involvement of CR3 in THP-1 macrophages was tested by blocking CR3 function using specific antibodies. While BCG association was unaffected under these conditions, uptake of single bacteria was significantly reduced in macrophages. Second, a signalling-deficient mutant CR3 was no longer able to mediate BCG uptake by transfected COS-7 cells. Third, we tested whether RhoA, a known signalling pathway downstream of CR3, is also necessary for BCG uptake. Indeed, when RhoA was depleted by RNAi, uptake of single BCG was reduced. Our data suggest that CR3 can act as a direct phagocytic receptor for BCG and utilises known Rho small GTPases.

In parallel to these hypothesis-led experiments, we have also sett up a system for a large-scale RNAi screen of actin cytoskeleton regulators. Optimised conditions will be used in a 96-well format to identify the signalling network required for BCG uptake among Rho small GTPases, effectors, GEFs and GAPs. Software is being developed in collaboration with Chris Tomlinson (Centre for Integrative Systems Biology Imperial College) to allow automated large-scale quantification from immunofluorescence images obtained by the differential method described above (associated or internalized particle detection).

In summary, we have set up a robust assay to investigate the mechanisms of uptake of BCG by human macrophages. Direct uptake of BCG requires actin polymerisation, both for single and large clumps of bacteria. CR3 appears to be important for uptake of single bacteria but is not required for attachment to macrophages. When expressed in non-phagocytic cells only CR3 (of the receptors tested) was able to mediate uptake, requiring the signalling motif within the CR3 cytoplasmic tail and Rho activity. However we have experiences some delays in the screening but are now planning to complete this in the next period (i) finalise the RNAi screen, (ii) identify the network of regulators mediating BCG uptake (iii) cross-reference our results with data provided by WP2, WP4 and WP5 and (iv) test predictions and identified molecules identified in other work-packages (bacterial or host proteins) in the uptake of BCG. A no-cost extension is requested for 3 months to complete this work, details on the revised deliverable times are given in the management section.

A manuscript on the characterisation of CR3 as a direct phagocytic receptor for BCG is in preparation.