INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, remains one of the most significant infectious diseases mainly due to the spread of antibiotic resistance and the global threat of the HIV pandemic that often leads to secondary mycobacterial infections.
One hallmark of TB is the ability of the causing pathogen, *M. tuberculosis*, to survive and multiply inside macrophages. In more detail, these parasites inhibit phagosome-lysosome fusion to avoid their destruction and actively interfere with the immune responses of macrophages (Russell, 2001; Vergne *et al.*, 2004). Mycobacteria therefore have efficient means to modulate a series of signaling pathways upon engaging macrophage cell receptors and during their subsequent internalization. These processes are frequently studied using *M. avium* as a model of pathogenic mycobacteria (behaving like *M. tuberculosis*) and *M. smegmatis* as a model of non-pathogenic mycobacteria incapable of inhibiting phagosome-lysosome fusion (Pieters & Gatfield, 2002).

The aim for the course participants was to identify novel, key host factors involved in phagosome-maturation of macrophages during their encounter with pathogenic mycobacteria, such as the model organism *M. avium*. To achieve this, the participants acquired practical skills in automated, advanced light microscopy for screening RNAi libraries. On the theoretical side, recent advances in these technologies and the analysis of complex datasets via bioinformatics methods were discussed in lectures presented by leading experts in their respective fields. Additional lectures also gave in-depth overviews on the biology of *Mycobacterium* and its effects on the host immune system, as well as fundamental cell biological processes of vesicular transport. On the whole, this workshop presented a unique setting; it brought together students and post-docs from various fields to learn and apply novel technologies to advance our current knowledge of *Mycobacterium*-macrophage interaction on the molecular level.

Experimentally, the phagosome/endosome maturation and endosome-lysosome fusion has been studied using various approaches (Vergne *et al.*, 2004). Generally, changes in the physical and biochemical properties of maturing endosomes can be exploited to track this process. Advances in microscopy, and the design of novel fluorescent biochemical sensors have been highly useful for such studies. The course participants monitored the
Interaction between mycobacteria and macrophages with four different approaches. First, co-localization measurements were performed with fluorescent-labeled *M. avium* or *M. smegmatis* (alive or heat-killed) and macrophages-containing lysosomal markers. Secondly, pH sensors were used to track pH changes of macrophage-phagosomes containing mycobacterial strains or beads coated with *Mycobacterium* lipidic components. Thirdly, signaling events depending on tyrosine phosphorylation during *Mycobacterium*-macrophage interaction were followed using an elegant assay based on Fluorescence Resonance Energy Transfer (FRET). Finally, phagosome maturation was studied in a more general way using proteolysis as a read-out to identify phagosome-lysosome fusion. All four assays were streamlined for screening, either using RNA interference (RNAi) to knockdown specific host proteins (assay 1, 2 and 4), or to identify tyrosine-phosphorylated proteins on the whole genome scale upon ingestion of *Mycobacterium* by macrophages. The authors emphasize that the results presented are preliminary and are provided in order to illustrate the principle of the assays used. Nevertheless, we were able to establish the robustness of the four assays employed that may be useful in microscopy-based screening in the future.

RESULTS AND DISCUSSION

**Imaging-based RNAi-screens to identify modulators of phagosome-lysosome fusion in mycobacteria-infected macrophages**

First, we used a “straight-forward” approach to identify lysosomes that contained *M. smegmatis* or *M. avium* using fluorescent-labeled bacteria and specific compartmental dyes. The phagosome-lysosome fusion was scored by confocal microscopy, and the involvement of a selection of different macrophage proteins in this process was analyzed by RNAi knockdowns. For these assays murine macrophage cultured cells (cell line RAW 264.7) were grown on microscope slides that were pre-spotted in duplicate or quadruplicate with RNAi against a selection of macrophage proteins (Erfle *et al*, 2004). The cells were then pulsed with a lysosomal tracking dye, rhodamine-
gold, and subsequently infected with live or heat-killed fluorescent-labeled mycobacteria (GFP-\textit{M. smegmatis} / Oregon green-\textit{M. avium}) (Anes \textit{et al}, 2003).

Confocal images were analysed for a specific form of co-localization using Manders coefficients combined with the ImageJ software version 1.35h (available at http://rsb.info.nih.gov/ij/index.html) (M2; green bacteria co-localizing with the red marker compared to the total green signal). The Manders coefficients are independent of different signal intensities in the channels analyzed for co-localization (Manders \textit{et al}, 1996). In the current case, thresholding of the lysosomal channel allowed the deduction of the percentage of mycobacteria that were targeted to the lysosomes. This detection threshold for the lysosome channel overcomes the limitation of the exclusion volume inside the lysosomes as there will be enough co-localizing signal background to detect with the bacteria.

Results of a sub-genomic RNAi screen on live \textit{M. avium}-infected macrophages are shown in Figure 1. By inspection of these samples via confocal microscopy, we found that the bacteria were efficiently phagocytosed and were partially targeted to the lysosomes (data not shown). Quantitative analysis indicated that the lysosomal targeting could be most strongly inhibited by RNAi against the following genes – interleukin 1α (IL-1α), mitogen-activated protein kinase 13 (MAPK13), nucleoside diphosphate kinase-m1d (Ndpk-m1d) and polo-like kinase (PLK-1). As a rationale, the corresponding gene products are potential inhibitory targets for \textit{M. avium} to modulate the endosomal trafficking in macrophages. In contrast, RNAi against phospholipase D (PLD-1) and sphingosine-kinase (Sph-kinase) enhanced most significantly the lysosomal targeting of \textit{M. avium}. Hypothetically, boosting the activity of the corresponding gene products by \textit{M. avium} could inhibit the phagosome-lysosome fusion. We emphasize that further analyses are required to evaluate the importance of these primary findings.
The described assay appears as a powerful method to study phagosome-lysosome fusion, and should be applicable to whole genome wide screens after optimization of the following key factors. Currently, the efficiency of the RNAi treatments cannot be controlled easily, but could be improved by growing cells on dots of plasmid DNA that carry a selection marker and encode the specific RNAi molecules under inducible promoters. Furthermore, cell confluence is a critical parameter and can have a dramatic effect on the final results simply due to field selection in automatic confocal imaging. Increasing the number of RNAi spots per gene on the slide would strengthen the reliability of the assay.

**Imaging-based RNAi-screens for the identification of modulators of phagosome acidification**

Another way to track phagosome/endosome maturation is to monitor directly the pH of this compartment upon uptake of *Mycobacterium*, or upon ingestion of latex beads that were coated with the *Mycobacterium* surface lipid molecule TDM (Trehalose Dimycolate). Ulrich Schaible’s research group has recently shown that TDM inhibits phagolysosome fusion when coupled onto beads, resembling the mycobacterial phagosomes (Axelrod *et al*., in preparation). Such assays could be scaled up for screening purposes similar to the ones in the previous paragraph.

TDM-coated beads and *M. avium* were labeled with Fluorescein (FITC), a fluorescent pH-dependent dye, and Rhodamine was used as a reference dye whose fluorescence intensity is pH-independent. Macrophages were transfected with a variety of siRNAs. The TDM-coated beads and *M. avium* were loaded onto these transfected macrophages and phagocytosis was allowed to occur for 20 minutes. Data acquisition involved capturing images using an Olympus ScanR widefield screening microscope (excitation filter settings: Fluorescein with 475/30 nm, Rhodamine with 572/23 nm bandpass; Acquisition filter settings: DAPI/FITC/Texas Red Tripleband dichroic mirror and a triple band emission filter: 460/25 nm, 530/40 nm, 625/50 nm). As explained in more detail later, fluorescence from beads that were not phagocytosed was quenched by addition of Trypan Blue. The data was
analyzed using Image J version 1.35h, and a mask was created to remove overexposed data. A median filter of 2.0 was applied to all images.

A measure of the degree of acidification of the phagocytosed latex beads/bacteria was obtained using the ratio of the Fluorescein and Rhodamine fluorescence intensity for each image. The expected ratiometric value derived from normalized fluorescence intensities for a non-acidifying phagosome would be approximately 1 (without acidification the pH would not change and consequently FITC fluorescence intensity would be unaltered). However, a low pH-phagosome would have values less than 1 since FITC fluorescence intensity decreases, while Rhodamine fluorescence intensity is stable.

The histograms of the ratio values relative to the number of pixels of each image revealed that populations of TDM-coated beads/M. avium could be assigned to different pH compartments (data not shown). To screen for general changes in pH-values, the existence of different populations the obtained ratio values were plotted in histograms relative to the number of pixels of each image. Statistical analysis of the data was performed calculating the median (the middle value) and the mode (value(s) with the highest frequency) of the plotted data.

Experiments where the median approximated the mode indicated that the entire population of phagocytosed TDM-coated beads/M. avium were in compartments of similar pH. However, a difference between the median and the mode suggested that there were two distinct phagosomal populations with different pH values. Data for the uncoated bead controls and heat-killed M. avium revealed a comparable value for the median as well as for the mode of approximately 0.6, which indicates a phagosome population of low pH. However, TDM-coated beads and live M. avium had an average median of approximately 1, but a mode of 0.6, which suggests that the majority of the phagosomes did not acidify.
From the pH screen it is clear that inducible nitric oxide synthase (iNOS) and mitogen-activated protein kinase 14 (MAPK14) are important factors in the acidification of dead *M. avium*. Blocking these proteins prevents acidification since the median is approximately 1 and mode is approximately 0.6, thereby suggesting two distinct population in acidification. However, what is more interesting is that Iκβα, interleukin 10 (IL10) and MAPK13 inhibition results in a single pH phagosome population with a median and mode of approximately 0.6. This suggests that these three factors possibly prevent phagosome maturation.

Our results from the pH screen do not provide conclusive data on factors that influence phagosomal maturation. However, the screen indicates that the median and mode values are exceptional indicators of phagosomal maturation, based on pH.

**Quantitative imaging of host cell signalling networks upon host-pathogen interaction by FRET/FLIM**

Phagocytosed mycobacteria induce distinct signaling networks that influence the host immune response in favour of the pathogen. Therefore, we analyzed the host signaling networks involved in the early encounter between macrophages and mycobacteria via FRET by FLIM (Fig 2). We measured the tyrosine phosphorylation pattern of 32 YFP-tagged human proteins expressed in RAW 264.7 macrophages that could be hypothetically important during the early signaling events of *Mycobacterium*-macrophage interaction (Simpson *et al*, 2000). FRET was observed by monitoring the decrease in lifetime of the donor fluorophore (YFP-tagged proteins) as it transfers energy to an acceptor (Cy3.5-labelled generic antibody against phosphotyrosine, PY72) using FLIM (Wouters & Bastiaens, 1999). FLIM measurements were performed in the frequency domain allowing the determination of the lifetimes via the phase shifts and modulations of the exciting light (Squire & Bastiaens, 1999) (experimental details are outlined in Fig 2).
Experimentally, the tyrosine phosphorylation states of the 32 hypothetically responsive proteins to *Mycobacterium*-macrophage interaction were monitored after incubating macrophages with the pathogenic *M. avium* strain (for 5 and 60 minutes). In parallel, macrophages were incubated and assayed with the non-pathogenic *M. smegmatis* strain (for 5 and 60 minutes) or with immunoglobulin G (IgG) coupled latex beads (20 minutes). Differential analysis of the datasets for the two *Mycobacterium* strains allows the identification of tyrosine phosphorylated proteins involved in virulence/pathogenicity at the recognition and internalization of the mycobacteria (time-point at 5 minutes) or in conveying the block of phagosome-lysosome fusion (time-point at 60 minutes). Control experiments (phagocytosis of IgG coupled beads by macrophages) were performed to follow normal macrophage phagocytic functions.

Using the control IgG-coupled beads, we investigated the phosphorylation state of a set of 8 recombinant proteins and their role in Fcγ receptor (FcγR)-mediated phagocytosis. This revealed consistent phosphorylation patterns in cells expressing YFP-tagged epidermal growth factor receptor (ErbB1) and protein kinase C alpha (PKCα). Furthermore, punctuated phosphorylated structures with little FRET were detected in cells expressing YFP-Src wild type (WT) (Fig 3A). FRET was not observed in cells transfected with cDNA encoding MEK-YFP, Extracellular signal-regulated kinase (ERK)-YFP and Src-mutant-YFP. Signaling during phagocytosis is highlighted by the phosphorylation of two conserved tyrosine residues of FcγR located within the immunoreceptor tyrosine-based activation motif (ITAM), which are substrates for Src kinases (Sobota et al, 2005). This reinforces our results implying that YFP-Src WT tyrosine phosphorylation is necessary for binding and ingestion of IgG beads. The high phosphorylation levels of ErbB1 and PKCα detected by FLIM indicate their key role in the regulation of FcγR-mediated phagocytosis (Fig 3A,B). Furthermore, recent evidence demonstrated that PKCα...
associates with the nascent and fully formed phagosome (Breton & Descoteaux, 2000), and that it mediates respiratory burst in RAW264.7 cells (Larsen et al, 2000).

In the second part of our experiments, for both M. avium and M. smegmatis, results showed significant tyrosine phosphorylation, for PKCα and ErbB1 receptor (Fig 3B). These two proteins are probably involved in universal signaling events during phagocytosis rather than being associated with mycobacterial virulence. Accordingly, several other studies have shown the involvement of PKCα phosphorylation in phagocytosis of other mycobacteria (Lima et al, 2001), and other bacterial species (Coxon et al, 1998). Interestingly, a protein encoded by the non-characterized ORF556 was phosphorylated only 5 minutes after M. avium challenge (Fig 3B). In this case BLAST analysis showed putative homology with hypothetical proteins in Arabidopsis thaliana and Mus musculus, as well as some homology with a microtubule-associated protein from Drosophila melanogaster. Its early phosphorylation after encountering M. avium suggests a possible role in recognition and/or internalization of pathogenic mycobacteria.

A possible mechanism to explain the increase in phosphorylation is that activation of IgG-R triggers the assembly of NADPH oxidase, which leads to an increase in the steady state (SS) of superoxide, which spontaneously may dismutate to highly diffusible hydrogen peroxide. Protein tyrosine phosphatases (PTP) can be inactivated by hydrogen peroxide and shift the phosphorylation state of the cell. It has been demonstrated that this type of regulation has an impact in the SS of tyrosine phosphorylation in vivo (Reynolds et al, 2003).

**Monitoring phagosome maturation via proteolytic hydrolysis by fluorescent microscopy**

The previous assays gathered information on the interaction between Mycobacterium and macrophages. We complemented these studies during the workshop with investigations that followed phagosome maturation and
phagosome-lysosome fusion without using *Mycobacterium*, but IgG-coupled beads that are readily ingested by macrophages. Performing these experiments, we used the hydrolytic activity present in lysosomes as experimental readout. Recently, an assay based on spectro-fluorometry has been developed to monitor phagosome-maturation processes using the hydrolytic activities of the phagosomal compartment as the physiological indicator (Yates et al, 2005). For those studies, IgG-labeled polystyrene or silica beads were coated with hydrolysable probes, which can be cleaved either by proteases, lipases or galactosidases. Professional phagocytes readily take up the IgG-labeled and coated beads, and bead-containing endosomes are processed to fuse with the hydrolase-containing lysosomes. The bead-coating probes have been designed to display dramatic changes in their fluorescent properties upon hydrolytic cleavage after lysosome fusion (Yates et al, 2005).

One of the goals of this workshop was to establish such a hydrolytic endosome-maturation-assay using fluorescent microscopy. Rapid four-dimensional microscopy also allows the capture of individual phagosomal-endosomal fusion events, and such discrete events were recorded at the workshop (see below). Furthermore, an RNAi screen was used to identify proteins that may be involved in endosome maturation and/or endosome-lysosome fusion.

Comparing various beads coated with hydrolytic-sensitive markers (Yates et al, 2005), we found that carboxylated polystyrene beads (diameter ~2µm) coated with DQ Green BSA (Molecular Probes) were particularly useful to track proteolysis during phagosome maturation in real time by confocal microscopy. For this assay, we isolated peritoneal mouse macrophages, and incubated them with fluorescently coated, IgG-labeled beads. Bead uptake was surveyed by phase-contrast microscopy and changes in fluorescence in the green emission channel. Scaling up this assay for automated fluorescent image analysis requires a clear distinction of particle-coated beads that have been internalized and coated beads that are not ingested by macrophages. Such analyses are needed to process large datasets typically obtained in screens. In our analysis it appeared that coated beads in cell culture media were
prone to unspecific hydrolysis resulting in an increase in their green fluorescence after extended incubation (up to 1 hour). We were able to optimize this assay by preventing the occurrence of unspecific extracellular fluorescent beads employing the following method. Cells were incubated with DQ green BSA beads for 35 minutes at 37°C, then 0.1% Trypan Blue was added to the culture medium. Subsequently, this was washed off with PBS at different time points (1 minute, 7 minutes and 3 hours). Images were collected and fluorescence changes quantified (Fig 4A). It was found that after 7 minutes incubation with Trypan Blue, beads remaining outside were successfully quenched, i.e. initially their fluorescence decreased up to 20-fold followed by a mild recovery, however the fluorescence was always at least 3-fold lower than the original one, hence easily distinguishable from bright beads inside cells. We were therefore able to demonstrate the principle that Trypan Blue is not taken up by living cells containing the phagocytosed coated beads and that it is able to quench the fluorescence of beads that are not taken up. As indicated in Figure 4A, this strategy allows a clear inside/outside distinction of the fluorescent-coated beads, and allows a quantification of the hydrolytic enzymatic activity of the bead containing vesicles.

We used this optimized assay to perform high-resolution four-dimensional imaging to track the distribution of hydrolytic activity on the surface of the beads (Fig 4B). This indicated that green fluorescence appeared first at specific spots on the beads, and subsequently spread over the entire surface of the beads. These results were interpreted as distinct, individual fusion events between the bead-containing phagosome with small vesicles containing the hydrolytic enzymes. Possibly, this assay will allow detailed studies on phagosome maturation to establish a hierarchy of the appearance of various hydrolytic enzymes during the formation of lysosomes.

Finally, this assay allows screening of RNAi libraries or chemical compounds that interfere with phagosome maturation via hydrolytic activities. We performed an RNAi screen during the course of the workshop using target genes involved in signal transduction and vesicle trafficking. Our preliminary results (data not shown) suggest that
Rab7, PKA-RII, and sphingosine kinase might be three important targets for the referred pathway. Cells that were knocked down for these three target factors appeared to display a stronger green fluorescence. This indicates their possible involvement in controlling the maturation of the phagosomes, however further experiments are needed to clearly define their roles in this process.

CONCLUSION
We studied *Mycobacterium*-macrophage interaction with a focus on (1) phagosome maturation and (2) triggering host cell-signaling responses. This led to the establishment of four robust assays as described in detail above by monitoring either physical or biochemical parameters of the *Mycobacterium*-containing endosome or lysosome. Importantly, we were able to carry out preliminary light microscopy based screens based on our assays to identify key host factors involved in the interplay between the pathogen and the host.

Apart from the large amounts of data obtained during this workshop in such a short amount of time, the most impressive result of this event was the collective success of teachers and participants in developing state-of-the-art technological demanding approach essentially from scratch. This becomes even more impressive when one realizes that most course participants were hitherto not familiar with the techniques taught and applied during the workshop.

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FIGURE LEGENDS

Figure 1 | RNAi-screen identifies modulators of phagosome-lysosome fusion in *M. avium*-infected macrophages. Macrophage-like RAW267.4 cells were grown on microscope slides pre-spotted in duplicate (marked with an asterisk) or quadruplicate with RNAi against a selection of macrophage proteins. The cells were pulsed for 1 hour with the lysosomal-tracking dye rhodamine-gold and subsequently infected for 1 hour with live Oregon green-*M. avium*. Confocal images were analysed for a specific form of co-localization using Manders coefficients (M2; co-localized green with red as compared to the total green after thresholding, also see text). M2-values provide a direct measure of the percentage of bacteria inside lysosomes.

Figure 2 | Strategy for the quantitative detection of tyrosine phosphorylation signalling involved in host-pathogen interaction using FRET-FLIM microscopy (Simpson *et al*, 2000; Squire *et al*, 2000).

Figure 3 | (A) Fluorescence intensity and FRET–FLIM lifetime images of fixed RAW 264.7 cells that stably express YFP-recombinant proteins, after 20 min pulse with IgG-coupled beads. The lifetime values are presented for each pixel using the colour scale presented on the right side of the figure. Panels from left to right: YFP-recombinant proteins fluorescence intensity (first column), modulation lifetimes images before (τm, second column) and after acceptor photobleaching (τm-CY3.5/PY72, third column), phase lifetime images before (τϕ, fourth column) and after acceptor photobleaching (τϕ-CY3.5/PY72, fifth column). (B) Lifetime differences of positive hits identified by measurement of the lifetime difference of the donor fluorescence, before and after acceptor photobleaching (anti-phosphotyrosine antibody PY72 labelled with Cy3.5 fluorophore) after incubation with IgG-latex beads (20 minutes) or infected with *M. smegmatis* and *M. avium* (5 and 60 minutes).
Figure 4 | DQ green BSA coated beads can be used to track phagosome maturation in bone marrow derived macrophages. (A) Trypan Blue was used to quench fluorescence of non-phagocytosed beads that turn fluorescent due to non-specific hydrolysis. This treatment is crucial to allow image analysis of large datasets such as that obtained by screens. (B) Beads, coated with DQ green BSA, and Alexa Fluor 594 (control fluorophor) were phagocytosed by bone marrow derived macrophages isolated from mice. Upon ingestion, the fluorescence of the beads was tracked by confocal microscopy (Zeiss LSM510-META) in 3 dimensions over time (z-section thickness 200 nm; analysis with Zeiss LSM software). The green fluorescence indicates the hydrolysis of DQ green BSA by lysosomal proteases. The arrows indicate potentially discreet endosome-lysosome fusion events.