NEMO 2005

NON-MAMMALIAN **E**XPERIMENTAL **MO**DELS
FOR THE STUDY OF BACTERIAL INFECTIONS

Coordinator: Pr. P. Cosson (Pierre.Cosson@medecine.unige.ch)

Activity Report September 2005

NON-MAMMALIAN **E**XPERIMENTAL **MO**DELS FOR THE STUDY OF BACTERIAL INFECTIONS

Coordinator: Pr. P. Cosson (Pierre.Cosson@medecine.unige.ch)

To test the ability of a bacteria to cause a disease it is usually necessary to infect a mammalian host and allow the disease to progress. These experiments inflict significant suffering to the animals. Our general aim is to stimulate the emergence of a community of scientists using alternative non-mammalian hosts for the study of bacterial infections. Our common belief is that many experiments currently carried out using mammalian hosts could be advantageously replaced by the use of alternative non-mammalian hosts.

Our network of laboratories was created in Feb 2005, initially as an informal gathering of research groups involved in similar subjects. Our specific goals are:
1-To organize an annual meeting on the theme of Non-mammalian hosts for the study of bacterial infections, in order to stimulate exchanges among research groups.
2-To strengthen our research in this field through a series of collaborative works, for which we hope to find financial support.
3-To publicize the use of alternative non-mammalian hosts in the scientific community.

The five research groups currently implicated in this network are: P. Cosson (Geneva, CH), M.O. Fauvarque (Grenoble, FR), G. Greub (Lausanne, CH), H. Hilbi (Zurich, CH) and T. Soldati (Geneva, CH). We might invite a few other research groups to join us in the future.

In this Activity Report we describe briefly the results obtained in 2005, emphasizing planned or ongoing collaborative work within the network. The CVs of the five groups leaders implicated are also joined.

CONTENTS

EXTENSIVE ANALYSIS OF BACTERIAL VIRULENCE IN DICTYOSTELIUM

Pr.Dr. P. Cosson, Geneva Faculty of Medicine, Geneva, Pierre.Cosson@medecine.unige.ch

1. Background: the confrontation of Dictyostelium amoebae and pathogenic bacteria

Dictyostelium is a free-living amoebae which feeds upon bacteria. In its natural habitat (forest soil), it encounters numerous pathogenic bacteria. This confrontation of a predatory amoeba with pathogenic bacteria closely resembles the situation of a white blood cell attacking an invading bacteria in the human body. Our laboratory is using the amoeba *Dictyostelium* as a host to study the virulence of many bacterial pathogens. Our initial results (Cosson, 2002) indicate that a large number of pathogenic bacteria, both gram-positive and gram-negative can be analyzed in this system. We have also analyzed in detail bacterial virulence genes on Pseudomonas aeruginosa and Klebsiella pneumoniae (Benghezal, 2005, in press).

Dictyostelium appears as a remarkably predictive model of bacterial virulence, and a good alternative to mammalian hosts in many instances. In addition the Dictyostelium model will allow the identification of new host resistance genes using genetic tools not available in mammals.

2. Host genes that determine resistance to pathogenic bacteria

Since phagocytic cells (neutrophils and macrophages) form the first line of defense of the organism against invading microorganisms, their role in antibacterial defense is essential. Indeed, a few host genes specifically involved in the function of phagocytic cells have been linked to resistance to bacterial infections in mammals. This is notably the case of the Nramp1 protein, a cation transporter present in the membrane of the phagosomes, and which presumably influences the fate of intraphagosomal bacteria by influencing the ionic content of the phagosome (Forbes and Gros, 2001). In humans, mutations in NRAMP1 are associated with an increased susceptibility to bacterial infections, particularly mycobacteria. Similarly, mutations affecting genes encoding components of the NADPH-oxidase complex cause

susceptibility to bacterial diseases, in particular to *S. aureus* or *K. pneumoniae* infections (Fang, 2004; Reeves *et al*., 2002; Nathan and Shiloh, 2000).

An extensive analysis of host defense mechanisms is limited by ethical and practical restrictions to animal experiments, and it is clear that our current knowledge of the genetic basis of host resistance to bacterial infections is far from complete. In order to identify systematically host genes involved in resistance to pathogenic bacteria, we have isolated *Dictyostelium* mutants that show an increased susceptibility to at least one of three bacteria tested: *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* or *Bacillus subtilis*. We have thus isolated a new host resistance gene, temporarily named Resistin 1. Loss of Resistin 1 makes the cells susceptible to *Klebsiella pneumoniae* (Fig. 1) and to a lesser extent to *Pseudomonas aeruginosa*, but not to *Bacillus subtilis*. We are currently analyzing which cellular functions are deficient in these mutant cells, in particular whether mutant cells can phagocytose and kill Klebsiella bacteria as efficiently as wild-type cells. This will allow us to understand the role of Resistin 1 in the interaction with pathogenic bacteria. Interestingly the Resistin protein, like Nramp1 belongs to the family of cation transporters, suggesting that these proteins might also play a role in the confrontation of phagocytic cells to Klebsiella bacteria. This work will provide a better understanding of the function of the immune system and of its complex interactions with bacterial pathogens. It also allows the development of new tools that represent powerful alternatives to the use of animal models to study various aspects of the infectious process.

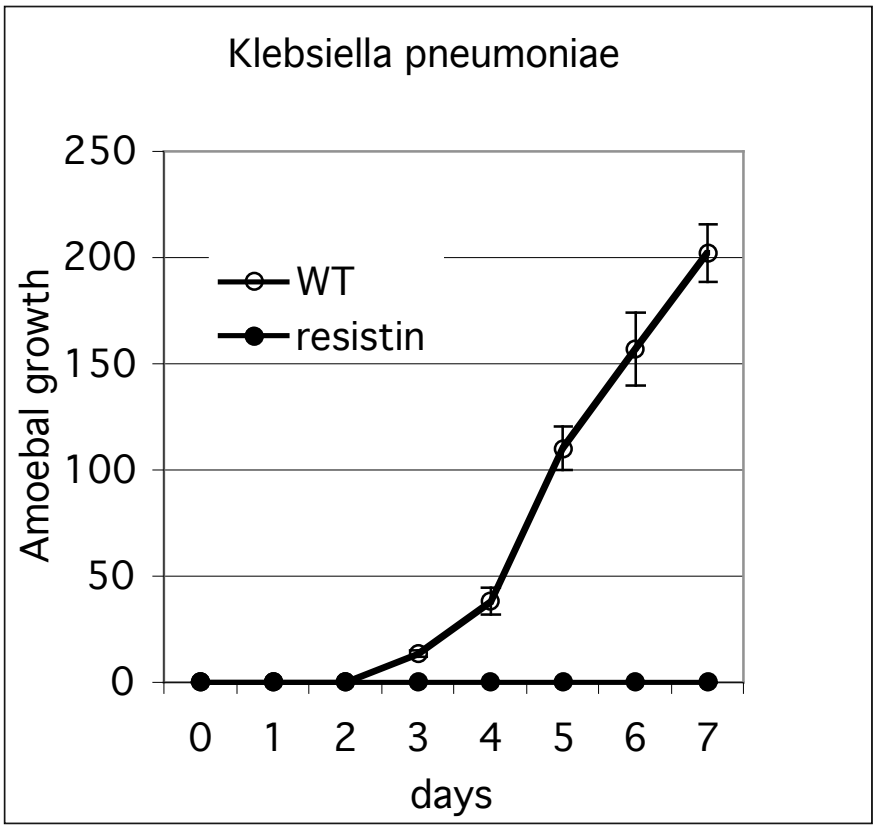


Fig. 1. Growth of *resistin* mutants on Klebsiella pneumoniae is inhibited. Interestingly the same mutant cells grew normally on non-pathogenic *B. subtilis* bacteria.

3. References.

Cosson, P., Zulianello, L., Join-Lambert, O., Faurisson, F., Gebbie, L., Benghezal, M., *et al* (2002) Pseudomonas aeruginosa virulence analyzed in a Dictyostelium discoideum host system. *J Bacteriol*. 184: 3027-3033.

Fang, F.C. (2004) Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nat Rev Microbiol*. 2: 820-832.

Forbes, J.R. and Gros, P. (2001) Divalent-metal transport by NRAMP proteins at the interface of host-pathogen interactions. *Trends Microbiol*. 9: 397-403.

Nathan, C. and Shiloh, M.U. (2000) Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc Natl Acad Sci U S A*. 97: 8841-8848.

Reeves, E.P., Lu, H., Jacobs, H.L., Messina, C.G., Bolsover, S., Gabella, G., *et al* (2002) Killing activity of neutrophils is mediated through activation of proteases by K+ flux. *Nature*. 416: 291-297.

BACTERIAL VIRULENCE AND INNATE IMMUNE RESPONSE: DROSOPHILA AS A MODEL SYSTEM

Dr Marie-Odile Fauvarque, CEA-Grenoble, France, marie-odile.fauvarque@cea.fr

1. Background: innate immune response in man and *Drosophila melanogaster*

Innate immune response is essential for the elimination of pathogens and for the activation of the adaptative immune system allowing the production of specific antibodies in Vertebrates. Innate immune response relies mainly on phagocytic cells ensuring both the phagocytosis of pathogens and the concomitant activation of conserved signalling pathways that triggers the production of inflammatory molecules and of antimicrobial peptides. These include NF-kB, Jun kinases- and p38-MAP Kinases-dependent pathways that are conserved from Drosophila flies to humans. In humans, phagocytic cells also produces oxygen reactive species through the activation of NADPH oxydase and NO synthases. These oxygen reactive species contribute efficiently to intracellular signalling, but may cause cell death by apoptosis, or tumor formation due to the oxidation of DNA molecule or the deregulation of cellular oncogenes. The understanding of mechanisms underlying innate immunity and pathologies associated with inflammation in response to bacterial infection is thus of outstanding interest for the future.

Genetic and molecular studies have revealed a striking conservation between the mechanisms that regulate insect host defence and the mammalian innate immune response. In *D. melanogaster*, activation of Pattern Recognition Receptors induces a humoral response that mainly consists in NF-kB-dependent antimicrobial peptide synthesis by the fat body (the functional equivalent of mammalian liver). Peptide synthesis is stimulated by two distinct signalling pathways, the Toll and the Immune deficiency (Imd) pathways, which are similar to the Toll-like receptor and the Tumour Necrosis Factor receptor signalling pathways in mammals, respectively. In addition, *Drosophila* blood cells of the hemocyte lineage can differentiate into phagocytes ensuring pathogens engulfment.

During infection, pathogens synthesize a vast number of virulence factors targeting host defence mechanisms. In particular, the type III secretion system is a cell-to-cell contact-dependent apparatus that uses a needle-like structure to deliver toxic bacterial proteins either to the cell membrane or directly into the host cell cytoplasm. The type III secretion system is found in many Gram-negative pathogens including *Pseudomonas aeruginosa*, an opportunist pathogen that causes nosocomial infections and is the major cause of death in cystic fibrosis patient. Others and we have shown recently that virulence factors characterized previously in mammalian systems, including quorum-sensing and type III secretion system, also play a role in *P. aeruginosa* virulence against *D. melanogaster* and *D. discoideum*.

2. New proteins involved in innate immunity in *Drosophila*

Original genetic approaches in drosophila led us to identify new proteins involved in innate immunity :
1) We developed a new transgenic approach that allowed us to analyze the contribution of the *P. aeruginosa* exotoxin S to bacterial virulence *in vivo*, and to identify new ExoS targets involved in inflammation and innate immune response. Among these, a cyclic GMP-dependent protein kinase might be involved in nitric oxide signalling following bacterial infection.
2) A genetic screen in Drosophila allowed us to identify conserved proteins involved in bacterial resistance. Notably, we selected a deubiquitinating enzyme that may regulate NF-kB-dependent antimicrobial peptide synthesis in response to infection.

We plan to study the implication of these candidate proteins in the regulation of innate immune signalling following bacterial challenge.

In parallel, we engaged a collaboration with the laboratory of Pr. P. Cosson in order to analyse the interaction of pathogenic bacteria with non-mammlian hosts, respectively Dictyostelium amoebae and Drosophila flies. The joint project is described below.

3. Extensive analysis of Pseudomonas virulence genes

Using the Dictyostelium system, a Tn5 insertional mutagenesis has been performed in P. Cosson's laboratory to identify new virulence genes in *P. aeruginosa*. Screening of 3,000 mutants led to the identification of twenty genes involved in bacterial virulence.

We have recently tested whether mutants exhibiting decreased virulence in a Dictyostelium system also show decreased virulence in Drosophila, where studying the course of Pseudomonas infections is reasonably easy. Our preliminary results (Fig. 1) indicate that the mode of infection is crucial to determine the role of a given gene product: all mutants analyzed were still pathogenic following septic injury into the thorax or induced only slightly delayed mortality, while when fed to the flies, mutant bacteria lost their pathogenicity compared to wild type controls. This suggests that the virulence genes identified in this study play a crucial role in the early stages of a natural infection (such as the passage of the intestinal epithelium), but are dispensable at later stages.

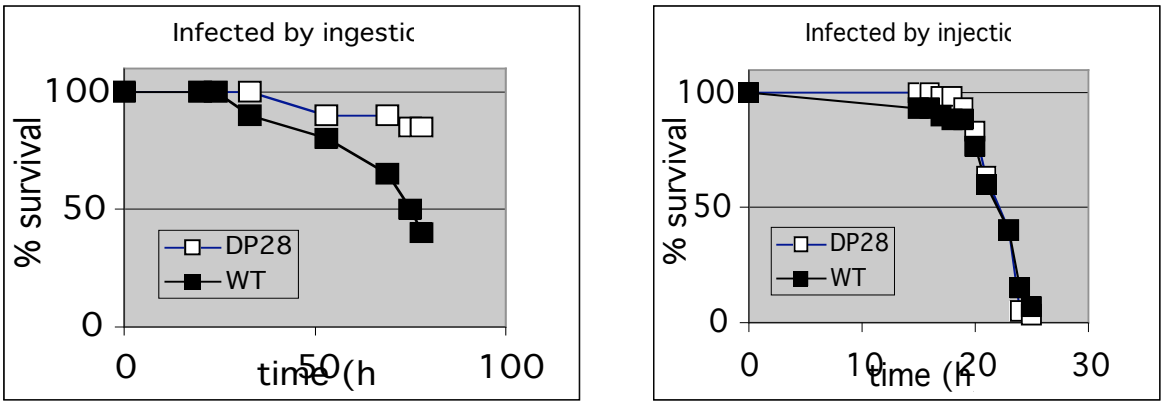


Figure 1. The specific role of pchH is apparent in the Drosophila infection model. Pseudomonas strains, either wild-type (WT) or mutated in pchH (DP28 mutant) were used to infect Drosophila flies either by ingestion (top panel) or by direct intrathoracic septic injury (lower panel). The survival of the infected flies is plotted as a function of time, revealing an important role for the pchH gene in the infectious process when initiated by ingestion of the bacteria.

Our results in the Drosophila model of infection confirm the role of these virulence genes in a living multicellular organism that possesses a complex innate immune system. We are now actively pursuing this study, and are further characterizing several of the new identified genes by elucidating their role in the induction of bacterial virulence genes, their role in the *Pseudomonas*/amoeba interaction and finally the mechanisms of virulence on various *Drosophila* mutants impaired in their innate immune system (i.e. NFkB-dependent pathways and cellular-dependent immunity). Once mechanisms of virulence would be partly elucidated in these organisms, they could be assessed in more complex mammalian systems in the long term.

This work is now reaching completion and should be submitted for publication in 2006.

4. Identification of host genes involved in interaction with pathogenic bacteria

Dictyostelium mutants with increased sensitivity to certain pathogenic bacteria (in particular *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) were recently obtained and characterized in the laboratory of Pierre Cosson including a gene called PHG1. A mutant Dictyostelium strain defective for PHG1 exhibited a specific susceptibility to *Klebsiella pneumoniae* bacteria due to its essential role in the intracellular killing of *K. pneumoniae* bacteria. On the contrary Dictyostelium *phg1* mutants did not show an increased sensitivity to *P. aeruginosa* bacteria.

PHG1 encodes a polytopic membrane protein with a N-terminal lumenal domain and nine potential transmembrane segments potentially involved in pathogen recognition. The existence of *phg1* orthologs in mammals and *Drosophila* (*Dphg1*) suggests that it may play similar function in these organisms. A Drosophila strain where *Dphg1* is deleted was recently obtained and its function in Dosophila immunity is being characterized (Bergeret and Fauvarque, unpublished results). Remarkably we recently observed that Drosophila *phg1*

mutants exhibited increased sensitivity to infection with *K. pneumoniae*, but not with *P. aeruginosa* (Fig. 2). This result illustrates the advantage of our combined approach of bacterial pathogenesis. Our current work is focused on several other genes recently identified in our laboratory and which, in the Dictyostelium system, were essential for resistance to *P. aeruginosa* bacteria. We are characterizing in more detail what is the precise role of the corresponding gene products during confrontation with *P. aeruginosa*, and we intend to test the role of the corresponding genes in the resistance of Drosophila flies to *P. aeruginosa* infections. The function of *phg1* and other essential phagocytosis genes identified in the amoebae could also be studied in *Drosophila* phagocytes by hemocyte-directed RNA interference (RNAi).

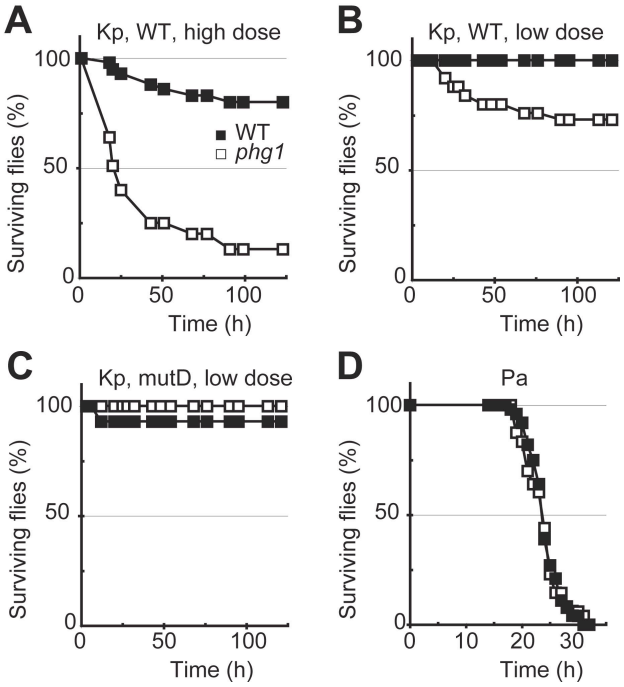


Figure 2. Drosophila *phg1* mutants exhibit an increased susceptibility to *K. pneumoniae* infections. Male Drosophila flies, either wild-type (WT) or mutant (*phg1*) were infected with a high dose of wild-type *K. pneumoniae* (A), a low dose of wild-type *K. pneumoniae* (B), a low dose of an avirulent *K. pneumoniae* mutant (C), or with wild-type *P. aeruginosa* (D).

A part of this work is now in press (Cellular Microbiology).

Conclusion:

The general aim of the drosophila team is to identify new mechanisms of innate immunity by original approaches in Drosophila and to elucidate the contribution of bacterial virulence factors in inhibiting host defense mechanisms. We will also analyze molecular and cellular dysfunction caused by a deregulation of these candidate genes *in vivo* during drosophila development and adult life. In parallel, we will perform *ex vivo* studies in cultured cells in order to determine the **contribution of human orthologs in both the immune response and pathologies associated with inflammation phenotypes**.

5. Recent publications

Avet-Rochex A, Bergeret, E., Attrée, I., Meister, M. and Fauvarque M-O. (2005) Pseudomonas aeruginosa ExoS toxin GAP domain affects Drosophila RhoGTPases and cellular immunity. *Cell. Microbiology*, in press

Raymond K., Bergeret E., Avet-Rochex A., Griffin-Shea R. and Fauvarque M.-O. (2004). A screen for modifiers of RacGAP(84C) gain-of-function in the Drosophila eye revealed the LIM kinase Cdi/TESK1 as a downstream effector of Rac1 acting during spermatogenesis. *J. Cell Science 117* : 2777-2789

Fauvarque, M.-O., Bergeret , E., Chabert, J. , Dacheux, D., Satre, M., Attrée, I. (2002) Role and activation of type III secretion system genes in *Pseudomonas aeruginosa*-induced *Drosophila* killing. *Microbial path. 32* : 287-295.

Raymond, K., Bergeret, E., Dagher, M. C., Breton, R., Griffin-Shea, R., and Fauvarque, M.-O. (2001) The Rac GTPase-activating Protein RotundRacGAP Interferes with Drac1 and Dcdc42 Signalling in *Drosophila melanogaster. J Biol Chem. 276* : 35909-35916

Bergeret E., Pignot-Paintrand, I., Guichard, A., Raymond, K., Fauvarque, M.O., Cazemajor, M., and Griffin-Shea, R. (2001). RotundRacGAP functions with Ras during spermatogenesis and retinal differentiation in *Drosophila melanogaster. Mol Cell Biol. 21* : 6280-6291

Fauvarque, M.O., Laurenti, P., Boivin, A., Bloyer, S., Griffin-Shea, R., Bourbon H.-M., and Dura, J.-M. (2001). Dominant modifiers of the *polyhomeotic* extra-sex-combs phenotype induced by marked *P* element insertional mutagenesis in *Drosophila*. *Genet Research. 78* : 137-148.

FREE-LIVING AMOEBAE AS A TOOL TO STUDY INTRACELLULAR PATHOGENS

Dr. G. Greub, Faculty of Biology and Medicine, Lausanne, Gilbert.Greub@chuv.hospvd.ch

1. Background: free-living amoebae and pathogenic bacteria

Free-living amoebae feed on bacteria, fungi and algae. However, some microorganisms evolved to become resistant to these protists. These amoebae-resistant microorganisms include established pathogens, such as *Legionella*, *Mycobacterium avium*, and *Listeria monocytogenes*, and emerging pathogens, such as *Parachlamydia acanthamoebae* (reviewed in (1;2)). Thus, free-living amoebae may be used as a tool for the culture of some intracellular bacteria and new bacterial species that might be potential emerging pathogens (2;3). Thus, we are using amoebae as cells in a cell culture system to study the biodiversity of intracellular hosts of free-living amoebae in water and since human are commonly exposed to water, the potential role played by these intracellular bacteria as agents of pneumonia. Their research focus on Chlamydia-like organisms, *Mycobacteria* and *Legionella pneumophila*.

Of note, free-living amoebae represent an important reservoir of amoebae-resisting bacteria that may, while encysted, protect the internalized bacteria from chlorine and other biocides. Greub *et al*. showed that biocides used for bronchoscope decontamination are poorly effective against amoebae (4), suggesting that amoebae may play the role of a protection for the internalized bacteria and might be at the origin of nosocomial transmission of amoebae-resisting bacteria, such as *Legionella* and *Mycobacteria* by inadequately disinfected bronchoscopes.

Free-living amoebae may also act as a Trojan horse and play a role in the selection of virulence traits and in adaptation to survival in macrophages (2). Indeed, intra-amoebal growth may modify morphologically the development of *Legionella pneumophila* (5), and this may be associated with increased virulence, as shown in HeLa cells (6).

2. Pathogenicity of *Mycobacterium kansasii* determined in Acanthamoeba

As described above, free-living amoeabe might also be used as a tool to determine the virulence of a given bacteria. Currently, we are studying virulent and avirulent *Mycobacteria* [*M. kansasii*] (7) for their ability to grow within *Acanthamoeba*. More precisely, we are using *Acanthamoeba castellanii* ATCC 30010 to test *Mycobacterium kansasii* virulence.

Mycobacterium kansasii is (with *Mycobacterium avium*), one of the more common mycobacterial species responsible for disease due to nontuberculous mycobacteria (7). However, little is known about its pathogenicity, mode of transmission, and natural reservoir. *M. kansasii* causes pulmonary disease similar to tuberculosis in immunocompetent patients and pulmonary, extrapulmonary, or disseminated disease in patients with various immunodeficiencies, in particular HIV infection. We showed that subtypes 1 and 2 are more pathogenic than subtype 3 (7). Consequently, in this project, we tested whether the growth of *Mycobacterium kansasii* in *Acanthamoeba* correlates with the virulence of *M. kansasii*.

Amoebae were infected at a multiplicity of infection of 1 bacteria per amoeba, and bacterial growth was monitored by Ziehl-Neelsen staining and seeding the homogeneized co-culture on 7H10 plates. As shown in Fig. 1, we could easily detect internalized *M. kansasii* by Ziehl-Neelsen staining.

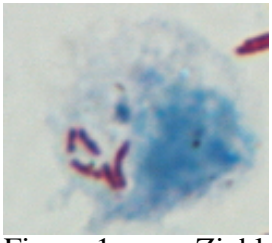


Figure 1 : Ziehl-Neelsen staining of an amoebal co-culture 7 days post-infection. The mycobacteria (dark) were clearly seen within the vacuoles of *Acanthamoeba castellanii* (grey). Magnification 1,000 x.

Moreover, the number of viable *M. kansasii* increased over time (colony forming unit on 7H10 plates; data not shown) and pathogenic subtypes 1 grew better in amoebae than non-pathogenic subtypes 3 (Figure 2).

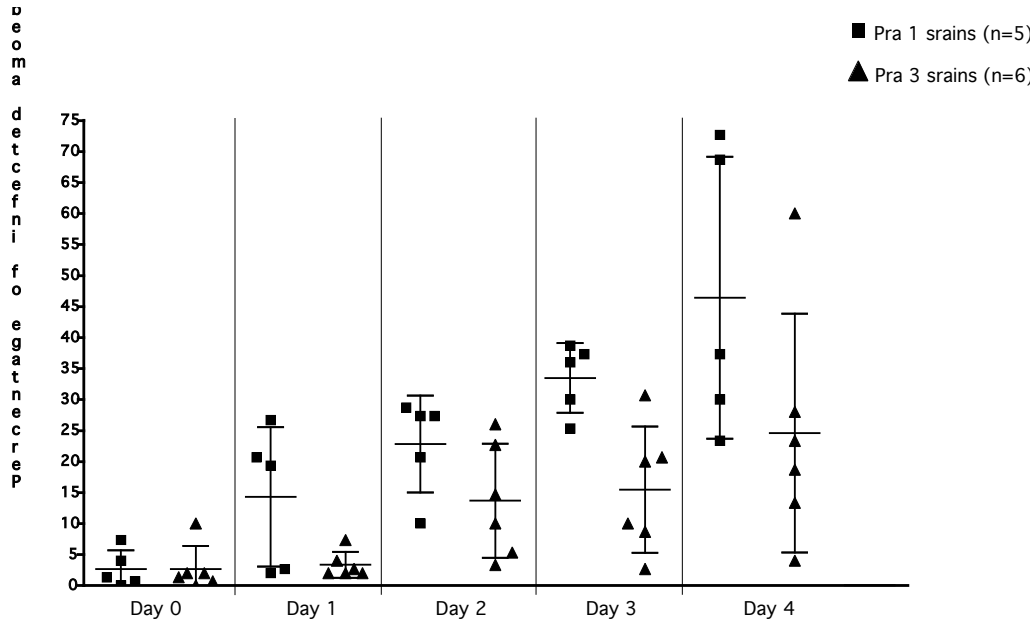


Figure 2 : Percentage of amoebae infected by pra 1 and pra 3 strains. Bacteria were detected after specific Ziehl-Neelsen staining, at least 100 amoebae were observed for each point.

These preliminary results demonstrate the usefulness of *Acanthamoeba castellanii* as a cell system to test mycobacterial virulence. In the next months, we intend to test the growth of various mycobacterial species (mainly isolated from patients) to test whether their potential pathogenic role correlates with their growth in *Acanthamoeba*.

Animal models are generally used to test bacterial virulence. Our work suggests that *Acanthamoeba* might represent an interesting easy alternative model to test bacterial virulence of amoebae-resisting bacteria such as *Legionella* and *Mycobacteria*.

3. References.

1. Greub G, Raoult D. Parachlamydiaceae: potential emerging pathogens. Emerg.Infect.Dis. 2002;8(6):625-30.
2. Greub G, Raoult D. Microorganisms resistant to free-living amoebae. Clin.Microbiol.Rev. 2004;17(2):413-33.
3. Greub G, La Scola B, Raoult D. Amoebae-resisting bacteria from human nasal swabs by amoebal co-culture. Emerg Infect Dis 2004;10(3):470-7.
4. Greub G, Raoult D. Biocides currently used for bronchoscope decontamination are poorly effective against free-living amoebae. Infect.Control Hosp.Epidemiol. 2003;24(10):784-6.
5. Greub G, Raoult D. Morphology of Legionella pneumophila according to their location within Hartmanella vermiformis. Res.Microbiol. 2003;154(9):619-21.
6. Garduno RA, Garduno E, Hiltz M, Hoffman PS. Intracellular growth of Legionella pneumophila gives rise to a differentiated form dissimilar to stationary-phase forms. Infect Immun JID - 0246127 2002;70(11):6273-83.
7. Taillard C, Greub G, Weber R, Pfyffer GE, Bodmer T, Zimmerli S et al. Clinical implications of Mycobacterium kansasii species heterogeneity: Swiss National Survey. J.Clin.Microbiol. 2003;41(3):1240-4.

AMOEBAE: A CELLULAR PATHOGENESIS MODEL FOR THE LEGIONNAIRES' DISEASE AGENT LEGIONELLA PNEUMOPHILA

Prof. Dr. Hubert Hilbi, Institute of Microbiology, ETH Zürich; hilbi@micro.biol.ethz.ch

1. Background: interaction of *Legionella pneumophila* with amoebae

Legionella pneumophila is an environmental bacterium and ubiquitously found together with predatory amoebae. However, rather than being digested by amoebae, *L. pneumophila* replicates within and ultimately kills these host cells (Fields, 1996; Steinert *et al.*, 2002; Hilbi, 2003). If inhaled via contaminated aerosols, *L. pneumophila* can replicate in macrophages of the human lung and cause the potentially fatal pneumonia Legionnaires' disease. Intracellular replication of *L. pneumophila* is a prerequisite for virulence – *Legionella* strains that do not show this trait are non-pathogenic.

The mechanism by which *L. pneumophila* is taken up by and replicates within amoebae and macrophages is very similar and depends on the bacterial Icm/Dot type IV secretion system (Hilbi *et al.*, 2001; Segal and Shuman, 1999; Solomon *et al.*, 2000). Within these phagocytes, *L. pneumophila* establishes a specific, replication-permissive phagosome by preventing phagosome/lysosome fusion (Horwitz, 1983), and by intercepting with secretory vesicles at endoplasmic reticulum (ER) exit sites (Kagan and Roy, 2002). Since many virulence traits of *L. pneumophila* have been evolutionarily selected for in their natural amoebae hosts, amoebae are good models to analyze cellular interactions between the opportunistic pathogen *L. pneumophila* and phagocytes.

Primary bone marrow-derived macrophages isolated from mice of the A/J strain are frequently used to study cellular interactions with *L. pneumophila* (Berger and Isberg, 1993). As alternatives, we employ macrophage cell lines and amoebae, including *Acanthamoeba castellanii* and the social amoeba *Dictyostelium discoideum*. Using *A. castellanii* as a selective host cell, we recently identified a cytotoxic *L. pneumophila* protein by screening a *Legionella* chromosomal library for multicopy suppressor plasmids (Albers *et al.*, 2005).

Dictyostelium is an excellent amoeba model to study interactions with *L. pneumophila*, since many genetic tools and mutants of this haploid phagocyte are available. Moreover, the genome sequence of *Dictyostelium* has just been published (Eichinger *et al.*, 2005). Lately, we demonstrated that the autophagy pathway is not required for intracellular replication of *L. pneumophila* within *D. discoideum* (Otto *et al.*, 2004). We are currently analyzing phagocyte and *Legionella* factors involved in cytotoxicity, phagocytosis, intracellular replication and survival in the environment.

2. Role of phosphoinositides and other host cell factors in phagocytosis and intracellular replication of *L. pneumophila*

Vesicle trafficking during phagocytosis, endocytosis and exocytosis is regulated by the metabolism of phosphoinositide (PI) lipids (De Matteis and Godi, 2004; Gillooly *et al.*, 2001). PI second messengers can be phosphorylated at different positions of the inositol ring, and as a result, recruit specific effector proteins to distinct membranes in a time- and organelle-dependent manner. Thus, intracellular membrane trafficking, actin remodeling, and receptor-mediated signal transduction is coordinated by specific kinases and phosphatases that phosphorylate or dephosphorylate PI lipids. PI metabolism is well characterized in *Dictyostelium*, and several mutants lacking defined PI kinases or phosphatases have been described, including strains deleted for class I PI(3) kinase-1 and -2 (D*PI3K1/2*) (Zhou *et al.*, 1995) or individual PI(5) phosphatases (D*PI5P1-4*) (Loovers *et al.*, 2003).

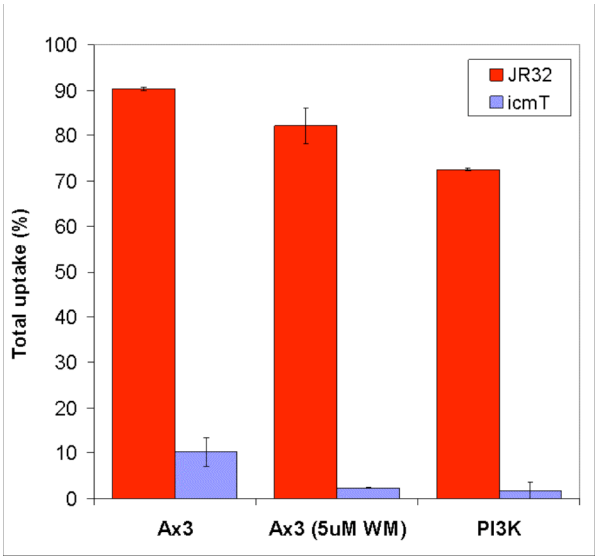
To analyze whether phosphoinositide metabolism plays a role in phagocytosis and intracellular replication of *L. pneumophila*, we compared wild-type *Dictyostelium* with either D*PI3K1/2* or cells treated with pharmacological inhibitors of PI3Ks (wortmannin, LY294002). Phagocytosis was quantified by flow cytometry using green fluorescent protein (GFP)-expressing *L. pneumophila* wild-type and *icmT* mutant (D*icmT*) strains.

Wild-type *L. pneumophila* is phagocytosed by amoebae and macrophages about 10 times more efficiently than *icm*/*dot* mutants (Fig. 1; Hilbi *et al.*, 2001), suggesting that *Legionella* employs a specific phagocytic pathway to enter phagocytes. Genetic

and pharmacologic data indicate that wild-type *L. pneumophila* is phagocytosed independently of PI(3) kinases, while uptake of D*icmT* is inhibited if functional PI(3) kinases are lacking. Contrarily, wild-type *L. pneumophila* replicates two orders of magnitude more efficiently in absence of PI(3) kinases (Fig. 1). Currently, we use markers of the *Legionella*-containing phagosome/ER (calnexin-GFP) or late endosomes/lysosomes (common antigen-1, CA-1) to compare intracellular trafficking of *L. pneumophila* in wild-type and D*PI3K1/2* mutant *Dictyostelium* strains.

As many as 6 class I PI3Ks and 13 other lipid kinases are found in the genome of *Dictyostelium* (Eichinger *et al.*, 2005). To study the role of these genes in intracellular replication of *L. pneumophila*, we intend to chromosomally delete some of them and test the interactions of the mutants with *L. pneumophila* as described above.

A



B

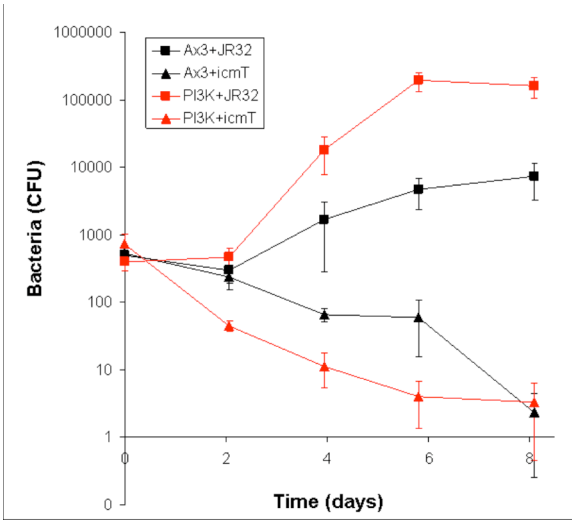


Fig. 1. Phagocytosis by *D. discoideum* and intracellular replication of *L. pneumophila* within *D. discoideum*. Phagocytosis of *L. pneumophila* was analyzed by flow cytometry using GFP-expressing wild-type *L. pneumophila* (JR32) and an *icmT* mutant (D*icmT*). (**A**) Deletion (PI3K) or inhibition (Wortmanin, WM) of PI3Ks inhibits phagocytosis of D*icmT* but not wild-type *L. pneumophila*, and (**B**) enhances intracellular growth of wild-type *L. pneumophila*.

In an unbiased approach to identify host cell factors involved in intracellular replication of *L. pneumophila*, we screened *Dictyostelium* mutant libraries generated by restriction enzyme mediated insertion (REMI) for mutants no longer sustaining growth of *Legionella*. The REMI mutants were infected with GFP-expressing *L. pneumophila*, and *Dictyostelium* amoebae harboring fluorescent bacteria were sorted by fluorescence activated cell sorting (FACS). This strategy warranted that for further analysis every single *Dictyostelium* cell contained at least one bacterium. The *Legionella*-infected *Dictyostelium* amoebae were then incubated for several days. Surviving cells were subjected to a second round of selection, seeded at < 1 cell per well in a 96 well plate and incubated again. Thus, 25 apparently *Legionella*-resistant *Dictyostelium* clones were obtained. The chromosomal insertion sites are currently being identified in collaboration with Prof. Hans Faix (University of Hannover, Germany). Interesting genes thus discovered will be characterized further.

3. References

Albers, U., Reus, K., Shuman, H.A., and Hilbi, H. (2005) The amoebae plate test implicates a paralogue of *lpxB* in the interaction of *Legionella pneumophila* with *Acanthamoeba castellanii*. *Microbiology* **151**: 167-182.

Berger, K.H., and Isberg, R.R. (1993) Two distinct defects in intracellular growth complemented by a single genetic locus in *Legionella pneumophila*. *Mol Microbiol* **7**: 7-19.

De Matteis, M.A., and Godi, A. (2004) PI-loting membrane traffic. *Nat Cell Biol* **6**: 487-492.

Eichinger, L., Pachebat, J.A., Glockner, G., *et al.* (2005) The genome of the social amoeba *Dictyostelium discoideum*. *Nature* **435**: 43-57.

Fields, B.S. (1996) The molecular ecology of *Legionellae*. *Trends Microbiol* **4**: 286-290.

Gillooly, D.J., Simonsen, A., and Stenmark, H. (2001) Phosphoinositides and phagocytosis. *J Cell Biol* **155**: 15-18.

Hilbi, H., Segal, G., and Shuman, H.A. (2001) Icm/dot-dependent upregulation of phagocytosis by *Legionella pneumophila*. *Mol Microbiol* **42**: 603-617.

Hilbi, H. (2003) Fressen und gefressen werden - Vom Umgang pathogener Bakterien mit Phagozyten. *Quartalhefte der Naturforschenden Gesellschaft in Zürich* **148/4**: 113-121.

Horwitz, M.A. (1983) The Legionnaires' disease bacterium (*Legionella pneumophilia*) inhibits lysosome-phagosome fusion in human monocytes. *J Exp Med* **158**: 2108-2126.

Kagan, J.C., and Roy, C.R. (2002) *Legionella* phagosomes intercept vesicular traffic from endoplasmic reticulum exit sites. *Nat Cell Biol* **4**: 945-954.

Loovers, H.M., Veenstra, K., Snippe, H., Pesesse, X., Erneux, C., and van Haastert, P.J. (2003) A diverse family of inositol 5-phosphatases playing a role in growth and development in *Dictyostelium discoideum*. *J Biol Chem* **278**: 5652-5658.

Otto, G.P., Wu, M.Y., Clarke, M., Lu, H., Anderson, O.R., Hilbi, H., Shuman, H.A., and Kessin, R.H. (2004) Macroautophagy is dispensable for intracellular replication of *Legionella pneumophila* in *Dictyostelium discoideum*. *Mol Microbiol* **51**: 63-72.

Segal, G., and Shuman, H.A. (1999) *Legionella pneumophila* utilizes the same genes to multiply within *Acanthamoeba castellanii* and human macrophages. *Infect Immun* **67**: 2117-2124.

Solomon, J.M., Rupper, A., Cardelli, J.A., and Isberg, R.R. (2000) Intracellular growth of *Legionella pneumophila* in *Dictyostelium discoideum*, a system for genetic analysis of host-pathogen interactions. *Infect Immun* **68**: 2939-2947.

Steinert, M., Hentschel, U., and Hacker, J. (2002) *Legionella pneumophila*: an aquatic microbe goes astray. *FEMS Microbiol Rev* **26**: 149-162.

Zhou, K., Takegawa, K., Emr, S.D., and Firtel, R.A. (1995) A phosphatidylinositol (PI) kinase gene family in *Dictyostelium discoideum*: biological roles of putative mammalian p110 and yeast Vps34p PI3-kinase homologs during growth and development. *Mol Cell Biol* **15**: 5645-5656.

THE AMOEBA DICTYOSTELIUM AS A MODEL HOST FOR MYCOBACTERIUM MARINUM

Dr. Thierry Soldati, Department of Biochemistry, University of Geneva, thierry.soldati@biochem.unige.ch

1. Background: amoebae and the mycobacterial plague

Tuberculosis is the most prevalent infectious disease in the world with over one-third of the global population estimated to be infected with the causative agent, *Mycobacterium tuberculosis*. Pathogenic *Mycobacteria* are taken up into lung macrophages by phagocytosis but the bacterium-containing phagosome is arrested at an early stage and does not mature into a degradative, lysosomal compartment. Therefore, rather than being destroyed by the macrophages, pathogenic *Mycobacteria* survive and replicate within these cells. *M. marinum* and *M. avium* are capable of infecting humans with *M. avium* becoming notable as an opportunistic pathogen in immuno-suppressed patients The genome of *M. marinum* is sequenced and bears significant homology to bacteria within the tuberculosis cluster, and thus *M. marinum* has been established as a potent model organism for the study of *M. tuberculosis* interactions with the host cell.

Dictyostelium discoideum as a model organism for the study of phagocytosis

We make use of a social amoeba, *Dictyostelium discoideum*, a professional phagocyte very similar to macrophages, but which is genetically and biochemically tractable and a very versatile experimental model organism. We have applied a phagosome purification method to characterise the molecular mechanisms of phagosome maturation within *Dictyostelium* (Lefkir et al, 2004; Gotthardt et al, 2002). We have also developed rapid freezing techniques that improve the preservation of organelle structure and antigenicity for light and electron microscopies (Neuhaus et al, 2002 and 1998; Neuhaus and Soldati 2000). Significantly, an infection protocol with *M. marinum* in *Dictyostelium* has recently been established (Solomon, et al, 2003), and we have optimised this protocol to establish infections for the study of *Mycobacteria*-containing phagosomes.

Dictyostelium discoideum as a multifacetted alternative to animal use

As mentioned above, *M. marinum* is an excellent model for the *Mycobacteria* of the tuberculosis cluster. Studies of systemic infections and granuloma formation are routinely performed using fish (such as *D. rerio*) and frogs. But, because the hallmarks of a persistent infection of macrophages can be recapitulated within

amoeba such as *Dictyostelium*, many aspects of the cellular infection can now be studied in a non-animal system. It is also worth noting that fresh-water amoeba, and among them *Acanthamoeba* might represent the natural reservoir for some *Mycobacteria* (see work from Dr. G. Greub, this application). This has an important impact on the understanding of the ecology of such pathogens, but also offers a new opportunity to test amoeba as a permissive host to grow difficult *Mycobacteria*, such as *M. leprae* that usually can be cultivated only in Armadillos. In parallel with tentatives to use metazoan hosts, such as *Drosophila* and *C. elegans*, the establishment of an infection system of *Mycobacteria* in *Dictyostelium* also widens the spectrum of genetically tractable and manipulatable hosts for such prevalent pathogens.

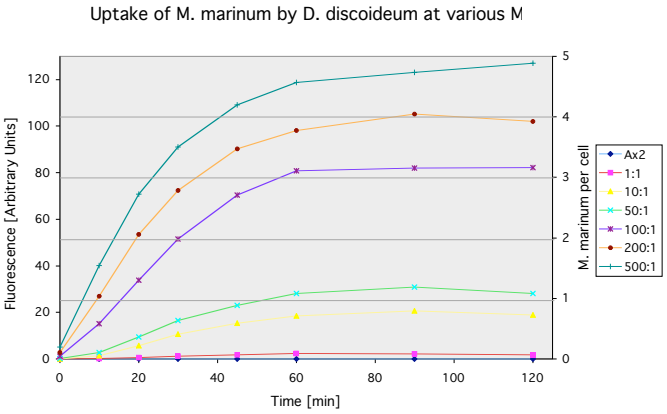
2. Virulence of *Mycobacterium marinum* in *Dictyostelium*

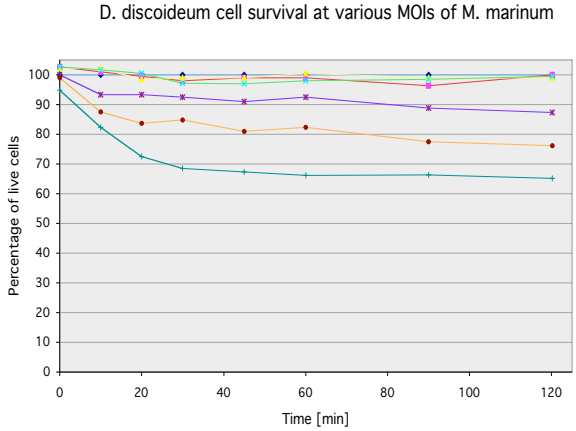
Overall the aim of our laboratory is to study phagosome biogenesis and to characterize the impact of *Mycobacteria* on this process. For example, we are investigating the interaction of phagosomes with F-actin *in vitro*, focusing on actin-nucleation and F-actin binding. We have recently used a FACS-based phagocytosis assay to identify and quantify the role of sterols during uptake and to monitor transit and exocytosis. In order to monitor the steps of maturation, we make use of cells that stably express GFP or mRFP-tagged GTPases of the Rab family such as the endosomal Rab5, 7, 4, 14 and 11. We also have strains expressing subunits of the vacuolar H-ATPase and vacuolin, as well as many other markers. The major aim of this present project is to understand the mechanisms involved in arresting phagolysosome maturation. Specifically, we want to characterize phagosomes containing *M. marinum* (*in vitro* and *in vivo*), to analyse their composition and to monitor the recruitment and trafficking of host proteins to and from that organelle.

The outcome of an infection is determined by complex interactions between the pathogen and the host, but our understanding of these processes is still rudimentary. The advantage of the *Dictyostelium* model-system is that both interaction-partners, the host and the pathogen, are easily experimentally manipulated. Recently, the conditions for a persistent *M. marinum* colonization of *Dictyostelium* have been established in the lab.

A major hurdle was to improve the efficiency of the existing infection protocol. Indeed, the preliminary observations can be made with an infection rate of less than 10-20%, but biochemical analysis and genetic screenings are rendered feasible only if the infection rate reaches almost 100%, e.g. one M. marinum per Dictyostelium cell. Increasing the MOI readily increased the number of cells infected, but concomitent cytotoxicity obliged us to look for an optimum. At an MOI of 100:1,

it takes less than 20 minutes to infect all the cells, whereas cytotoxicity is kept under 10% under these conditions.





The "persistence" arises from a complex and fragile equilibrium of interplays in which the intracellular pathogen arrests phagosome-maturation but does not kill the host cell. By manipulating this fragile equilibrium either in favor of *Dictyostelium* (meaning the host can clear/kill the pathogen) or the pathogen (meaning the pathogen kills the host) new potential candidates for regulating either host-defense or pathogen-infectivity can be identified. *Dictyostelium* cells can be easily manipulated using either random (e.g. REMI, restricted enzyme mediated insertion mutagenesis) or directed mutagenesis and sophisticated high-throughput methods (e.g. FACS-based cell sorting) can be used to identify mutants and subsequently the mutation(s) that lead(s) to clearing/killing of the pathogen. Similar approaches can be used in the pathogen in order to manipulate its pathogenicity. Random mutations can be introduced using transposon mutagenesis and screening can be carried out to identify mutants that kill/harm the host, *Dictyostelium*. Genetic analyses of these mutated organisms, the resistant host cells and the potent pathogen, will identify new factors involved in host defense and bacterium pathogenicity. Possible adaptations and answers of either organism according to changes within the host-pathogen equilibirium will allow further analysis of these dynamic and complex interactions.

3. References

Blancheteau, V., Gotthardt, D., Bosserhoff, A., Ruppert, T., Delorenzi, M., and Soldati, T. (2005) Time-resolved proteomic and functional clustering analyses of phagosome maturation, in preparation.

Bruegger, B., Gotthardt, D., Dieckmann, R., Blancheteau, V., Wieland, F., and Soldati, T. (2005) Time-resolved lipidomic analyses reveal massive membrane remodeling accompanying phagosome maturation, in preparation.

Gotthardt, D., Warnatz, H.J., Henschel, O., Brückert, F., Schleicher, M., and Soldati, T. High resolution dissection of phagosome maturation reveals distinct membrane trafficking phases Mol. Biol. Cell, (2002) 13, 3508-3520.

Lefkir, Y., Malbouyres, M., Gotthardt, Ozinsky, A., D., Bruckert, F., Aderem, A.A., Soldati, T., Cosson, P., and Letourneur, F. Involvement of the AP-1 adaptor complex in early steps of phagocytosis and macropinocytosis. Mol. Biol. Cell, (2004) 15, 861-869.

Neuhaus, E.M., Almers, W., and Soldati, T. Morphology and dynamics of the endocytic pathway in Dictyostelium discoideum Mol. Biol. Cell, (2002) 13, 1390-1407.

Neuhaus, E.M., and Soldati, T. A myosin I is involved in membrane recycling from early endosomes. J. Cell Biol., (2000) 150, 1013-1026.

Neuhaus, E., Horstmann, H., Almers, W., Maniak, M., and Soldati, T. Ethane-freezing/methanol-fixation of cell monolayers. A procedure for improved preservation of structure and antigenicity for light and electron microscopies. J. Struct. Biol., (1998) 121, 326-342

Solomon, J.M., Leung, G.S. and Isberg, R.R. (2003). Intracellular replication of *Mycobacterium marinum* within *Dictyostelium discoideum*: efficient replication in the absence of host coronin. *Infection and Immunity*. **71:** 3578-3586