Biology of Mycobacteria-containing phagosomes: acidification, fusion and actin nucleation

with an emphasis on *Mycobacterium avium* subspecies *paratuberculosis*

von dem Fachbereich Biologie
der Universität Hannover

zur Erlangung des Grades eines
DOKTORS DER NATURWISSENSCHAFTEN

Dr. rer. nat.

Genehmigte Dissertation
von

Dipl. -Biol. Mark Philipp Kühnel
Geboren am 14.12.1971, in Hannover
2002

Referent: Prof. Dr. P. Valentin-Weigand
Referent: Univ.-Prof. Dr. A. Brakhage
Tag der Promotion: 28.01.2002

To my Grandmother
and my Parents

Hiermit versichere ich, an Eides statt die folgende Studie selbstständig und ohne fremde Hilfe angefertigt zu haben.

_____

Mark Ph. Kühnel

Abstract - English

Pathogenic mycobacteria, such as *Mycobacterium tuberculosis* (*M.tb*) and *M.avium* ssp. *avium* (*M.avium*) facilitate disease by surviving within a potentially hostile environment, the macrophage phagosome. These bacteria inhibit phagosome maturation, especially acidification and fusion with lysosomes. In this study the pH and fusiogenicity of phagosomes containing *M. avium* ssp. *paratuberculosis* (*M.ptb*), the causative agent of paratuberculosis in ruminats was analysed, as well as the ability of these phagosomes to facilitate actin assembly, a process strongly linked to membrane fusion. Using the J774 macrophages cell line, we compared viable and killed *M. ptb* and *M. avium*, as well as two non pathogenic mycobacteria, Mycobacterium smegmatis and Mycobacterium gordonae. Analysis of the fusiogenicity revealed that phagosomes containing live *M.ptb* or *M. avium* were significantly inhibited in their ability to acquire some markers for the endocytic network, such as internalised calcein, BSA-gold or the membrane protein Lamp 2. However they were almost completely accessible to 70 kDa dextran and Lamp 1. Overall, the phagosomes containing dead pathogenic mycobacteria behaved similarly to the ones containing live non-pathogenic mycobacteria in all experiments. Using FITC-dextran in a new fluorescence-activated cell sorting (FACS)-based method, it was possible to determine the overall pH of the bulk of endocytic compartments, including phagosomes. For cells infected with live *M.ptb* or *M.avium* only a mild acidification to pH 6.3 was observed, whereas cells treated with killed *M.ptb* or *M.avium* showed substantial acidification of phagosome/endocytic compartments to a value of 5.2. After infection with *M.smegmatis* or *M.gordonae*, acidification was initially (1-5h after infection) inhibited, but increased after longer infection to levels similar to those with dead mycobacteria. Using a method developed for measuring actin assembly on Latexbead-phagosomes (LBP), isolated phagosomes containing live and killed *M.ptb* and *M.avium* were tested for their ability to nucleate actin. Phagosomes containing live *M.avium* or *M.ptb* were found to be inhibited in this process, whereas phagosomes containing killed mycobacteria nucleated actin similar to LBP controls. However, selected lipids could activate this process, leading to increased intracellular killing of *M.ptb* and *M.avium*.

Keywords: Mycobacterium, fusion, actin-nucleation

Abstract – Deutsch

Pathogene Mykobakterien wie *Mycobacterium tuberculosis* (*M.tb*) und *M. avium* ssp. *avium* (*M.avium*) verursachen Krankheiten durch ihr überleben in einer potentiell lebensfeindlichen Umgebung, dem Phagosom in Makrophagen. Diese Bakterien verhindern die Reifung des Phagosoms, insbesondere die Ansäuerung und Fusion mit Lysosomen. In dieser Arbeit wurde daher die Ansäuerung, Fusionseigenschaften, sowie die Fähigkeit zur Bildung von Aktinfilamenten an der Phagosomenmembran, ein Prozess der eng mit Fusion in Verbindung steht, von Phagosomen, die *M. avium* ssp. *paratuberculsosis*, den Erreger der Paratuberkulose der Widerkäuer, enthalten, untersucht. Unter Benutzung von J774 Mausmakrophagen wurden lebende und abgetötete *M.ptb* und *M.avium*, sowie zwei nicht-pathogene Mykobakterienspezies, *M.smegmatis* und *M.gordonae*, vergleichend untersucht. Die Untersuchungen der Fusionseigenschaften ergaben, dass Phagosomen, welche lebende *M.ptb* oder *M.avium* enthielten hinsichtlich ihrer Fähigkeit einige endosomale Marker, wie internalisiertes Calcein, BSA-Gold oder das Membranprotein Lamp 2, zu erhalten, inhibiert waren. Jedoch waren sie fähig, 70 kDa Dextran, und Lamp 1 zu erhalten. Abgetötete Mykobakterien zeigten verhielten sich in allen Experimenten vergleichbar zu lebenden nicht-pathogenen Mykobakterien. Durch Verwendung einer neu etablierten Fluoreszenz-aktivierten Zell Sortierungs (FACS)-Methode und FITC-Dextranpartikel, gelang es den durchschnittlichen pH-Wert des endosomalen Netzwerks in infizierten Zellen zu bestimmen. Zellen, die mit *M.avium* oder *M.ptb* infiziert worden waren zeigten nur eine milde Ansäuerung von pH 6,3, während Zellen, die abgetötete Mykobakterien internalisiert hatten eine deutliche Ansäuerung der Endosomen/Phagosomen auf pH 5,2 zeigten. Zellen die mit *M.smegmatis* oder *M.gordonae* infiziert worden waren wiesen zunächst eine Verhinderung der endosomalen Ansäuerung auf, diese Verstärkte sich jedoch mit der Zeit auf Werte, die mit denen von Zellen, die mit toten Mykobakterien behandelt worden waren, vergleichbar waren. Durch Verwendung einer Methode die zur Messung der Nukleierung von Aktin an Latexpartikel-haltigen Phagosomen (LBP) etabliert worden war, gelang es Mykobakterien-haltige Phagosomen auf ihre Fähigkeit Aktin zu nukleieren zu testen. Phagosomen die lebende *M.ptb* oder *M.avium* enthielten waren hinsichtlich dieses Prozesses signifikant inhibiert, wogegen Phagosomen, welche tote Mykobakterien enthielten Aktin vergleichbar zu den LBP nukeierten. Einige ausgewählte Lipide waren in der Lage diesen Prozess in Mykobakterien-haltigen Phagosomen zu aktivieren, was zu einer effizienteren Abtötung der Mykobakterien durch den Makrophagen führte.

Schlüsselworte: Mycobacterium, Fusion, Aktin

Index of chapters

Index of Tables

Index of Models

Index of Figures

A Introduction

Mycobacterial diseases like tuberculosis, paratuberculosis and leprosy cause a lot of problems in humans and animals all over the world. The focus of my thesis therefore deals with pathogenic mycobacteria and their effects on the cell biology of infected host cells with a special emphasis on M. *avium* subspecies *paratuberculosis*.

A.1 Mycobacteria

The genus Mycobacterium contains a number of strict and opportunistic pathogens that affect humans and animals alike. The best known mycobacterial pathogens include *Mycobacterium tuberculosis* and *M. bovis*, the infectious agents of tuberculosis, *M. leprae*, which causes leprosy in humans, *M. bovis* and *M. avium*, which cause tuberculosis in cattle and birds respectively, as well as *M. paratuberculosis*, which results in one of the most important diseases in ruminants, namely paratuberculosis or Johne´s disease.

The genus *Mycobacterium* is one of the oldest defined. The generic name *Mycobacterium* initially described a group of organisms that grew as mould-like pellicles on liquid media (Lehmann and Neumann, 1896). At the beginning of the last century, the characteristics used to define mycobacteria were the absence of motility, the morphology of the bacilli (rod shaped and slightly curved), and resistance to acid-alcohol following coloration with phenol fuchsin (Ziehl-Neelsen stain). This characteristic is now known to be due to the unique lipid composition of the bacterial cell wall (Barry, 2001). In the order *Actinomycetales* mycobacteria belong to the genus *Mycobacterium*, which is the single genus in the family of *Mycobacteriaceae* (Stackebrandt et al., 1997).

Mycobacteria are defined as aerobic, acid-alcohol fast, rod-shaped actinomycetes with occasional branching; aerial hyphae are normally absent, and the bacteria are non-motile, non-sporulating organisms that contain arabinose, galactose, and *meso*-diaminopimelic acid in the cell wall. The guanine/cytosine (GC)-deoxyribonucleic acid (DNA) base ratios are in a range of 62-70% (except for *M. leprae*, which has a GC base ratio of 58%); they have mycolic acids of high molecular weight (sixty to ninety carbons), which lack components with more than two points of unsaturation in the molecule (Goodfellow and Wayne, 1982). Although historically defined as non-encapsulated organisms, mycobacteria are now known to possess a capsule like

structure (Rastogi, 1993). Similarly, although initially considered as obligate aerobes, some species and strains are microaerophilic (Goodfellow and Wayne, 1982). Actinomycetes include diverse microorganisms, in both ecological and morphological terms (Goodfellow and Wayne, 1982).

Differentiation of mycobacteria from allied taxa (e.g. members of the Corynebacterium, Mycobacterium and Nocardia [CMN] group) can be easily performed on the basis of the ability to synthesise mycolic acids. Mycobacterial mycolic acids usually occur as complex mixtures of components that have oxygen containing moieties such as carboxy, keto or methoxy groups, in addition to the 3-hydroxy acid system. The members of the CMN group are the only micro-organisms that are able to synthesise mycolic acids (Goodfellow and Wayne, 1982); however on the basis of the number of carbon atoms and pyrolysis esters of the mycolic acids (as well as the GC content of the DNA), discrimination among various CMN group members is possible (Barry, 2001). For this reason the genus *Mycobacterium* is defined by the lengths of the carbon backbone, the number of unsaturated links, the presence of supplementary oxygenated functions, and the esters produced by pyrolysis.

Historically, the nomenclature of mycobacterial infections was limited to tuberculous or non tuberculous mycobacteria (which were also referred to as atypical mycobacteria). The former include *M. tuberculosis*, which is responsible for human tuberculosis*, M. bovis* and *M. avium*, which are responsible for bovine and avian tuberculosis*, M. africanum*, which also causes human tuberculosis and is essentially restricted to Africa. Others include *M. microtii*, a pathogen of small rodents, and the vaccine strain *M. bovis* BCG. Although this classification is sufficient for practical purposes, the exact taxonomic status of a number of newly described species, subspecies or subtypes is difficult to specify. This is best illustrated by the example of the pig pathogen belonging to the *M. avium*-complex (MAC) which, although known for years, suddenly became a focus of attention when infection of AIDS patients harbouring these bacteria were first detected in the 1980s. However, as the genomes of species become sequenced more precise classification by molecular techniques can soon be expected.

A.1.1 Mycobacterium avium complex (MAC)

Mycobacterium avium complex organisms (*M. avium, M. paratuberculosis* and *M. silvaticum*) are especially important in animal health care. *M. avium*, originally detected in birds, is also a pathogen in mammals (Tell et al., 2001; Thorel et al., 2001). *M. paratuberculosis* (*M. ptb*) is the causative agent of paratuberculosis or Johne´s disease in ruminants, and *M. silvaticum* causes disease in birds (Legrand et al., 2000). Although these three organisms were initially considered as separate species within the *M. avium* complex, more recent studies and molecular typing approaches using DNA-DNA hybridisation, restriction fragment length polymorphism (RFLP) and pulsed-field gel electrophoresis (PFGE), revealed that these organisms could be distinguished as three different subspecies within the single *M. avium* species, namely: *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *silvaticum* (Saxegaard and Beass, 1988; Thorel et al., 1990). Phenotypic and genetic differences in the existence of several insertion sequences have been found to exist between the three subspecies, e.g. mycobactin dependent growth and its stimulation by pyruvate on solid medium, in addition to the presence of the *IS900* in *M. ptb* and, on the other hand, the absence of *IS901* and *IS902* in *M. ptb*, in contrast to the other two subspecies and the specific presence of *IS1613* in *M. avium* (Legrand et al., 2000). Furthermore the three subspecies show different epidemology, animal hosts and reservoirs (Legrand et al.,2000). The combination of these parameters not only permits discrimination between the three subspecies, but also justifies the operational distinction between the three.

A.1.2 Clinical significance of Mycobacterium avium subspecies

Mycobacterium avium subspecies are involved in a variety of different diseases in different hosts. Of special interest is that bacteria of the MAC and especially *M. avium* subsp. *avium* is often found in human patients suffering from immunodeficiency like in HIV-infections (Horgen et al., 2000). Recently, a significant increase in the incidence of caseous lesions in the lymph nodes of slaughter pigs caused an extensive investigation in the Netherlands (Komijn et al., 1999). Of 2,899 animal groups examined, 158,763 animals in total, 5% showed caseous lesions in the submaxillary and/or mesenteric lymph nodes. Acid fast bacteria were detected in 41% of cases by microscopal examination of Ziehl-Neelsen stained smears. Isolation

and investigations of strains by *IS1245*-RFLP revealed that 90 out of 91 isolates were *M. avium* subsp. *avium.* When RFLP patterns were compared to patterns obtained from MAC isolates from 191 human patients, 75% similarity was detected, suggesting that pigs may be a reservoir for *M. avium* for humans. Alternatively, pigs and humans share common environmental sources of infection (Komijn et al., 1999). However, determination of MAC organisms is difficult because of the ubiquitous nature of these organisms.

Mycobacterium avium subsp. *paratuberculosis* (*M. ptb*) is the causative agent of paratuberculosis (Johne's disease), a chronic granulomatous enteritis in ruminants. Paratuberculosis is now one of the most widespread bacterial diseases of domestic animals and its impact on the world economies is enormous (Cocito et al., 1994). For the United States the yearly losses due to this disease had already been estimated in 1989 to exceed 1.5 billion U.S. dollars (Jones, 1989). Typically, animals are infected with *M. ptb* at an age of less than 6 months via oral faecal transmission; however, clinical signs such as chronic diarrhoea, emaciation, and dehydration usually develop after a 3-5 years incubation period (Chiodini et al., 1984). *M. ptb* has also been detected in the intestinal tissue of human patients with Crohn's disease, a chronic enteritis of unknown etiology showing pathological and clinical similarities to paratuberculosis (Chiodini, 1989), suggesting a possible zoonotic relevance.
Despite the importance of the disease, the molecular machinery that is involved in the entry and survival of *M. ptb* in the host is only poorly characterised. Current hypotheses on the mechanisms of pathogenicity are mainly based on the concepts developed for *M. tuberculosis* and *M. avium* subsp. *avium* (*M. avium*), although *M. ptb* differs significantly in its phenotypic features. *M. ptb* is characterised by prolonged growth rates *in vitro* (it takes up to 8 to 12 weeks before growth is visible on artificial media), growth dependence on mycobactin, its route of infection (oral), tissue tropism (intestine), and host specificity (obligatory pathogenic only for ruminants) (for review see Clarke, 1997; Valentin-Weigand and Goethe, 1999). Once inside the intestine, the pathogen seems to enter the mucosa via ileal dome M-cells and is then taken up by subepithelial macrophages in which it persists and multiplies (Momotani et al., 1988).

It is now generally accepted that the persistence of *M. ptb* in host macrophages is the crucial process in the establishment and progression of disease (Clarke, 1997; Valentin-Weigand and Goethe, 1999).

A.1.2.1 Immune response

The immune response to all pathogens is at least in part dependent on cytokines, which regulate all cells of the immune system. Mycobacteria are no exception to this rule. The inflammatory response to these bacteria is crucial to the control of the infection, but may also contribute to the chronic nature and associated pathology. Effective elimination of mycobacteria by the host, in general, requires the co-ordinate action of Th1 lymphocyte-derived IFN-γ and macrophage derived interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α, and granulocyte-macrophage colony stimulation factor (GM-CSF). Therefore several studies have addressed the role that these factors play in mycobacterial diseases, such as bovine and ovine paratuberculosis (Zurbrick et al., 1988; Zhao et al., 1997; Stabel, 1995; Adams et al., 1994, 1995, 1996; Begara-McGorum et al., 1998). Using a variety of different experimental approaches, including experimentally infected animals, as well as whole blood, blood derived monocytes/macrophages and cell culture systems, the cytokine production of infected cells was studied. Results of these experiments suggest that *M. paratuberculosis* can induce up-regulation of IL-1β, IL-6, TNF-α, and GM-CSF. These effects were reported to be similar in infections with both live and dead mycobacteria as well, as purified lipoarabinomannan (LAM). However it is not clear whether these cytokines facilitate inactivation of *M. paratuberculosis* by a mechanism that is the same or similar to their roles in infections with other mycobacteria. In fact there is evidence for *M. paratuberculosis*-specific mechanisms, since it has been reported that mRNA of INF-γ was significantly decreased in tissue of experimentally-infected lambs (Begara-McGorum et al., 1998), as well as by recent results, showing that INF-γ had only moderate effects on the viability of *M. paratuberculosis* in infected bovine macrophages (Zhao et al., 1997). These findings are in contrast to results obtained from other mycobacteria which have been described to be much more sensitive to INF-γ, which induces the production of nitric oxide (NO) in response to an infection. The limited antimycobacterial effect that was observed *in vitro* could be explained by

insufficient production of NO, which seems to kill bacteria when applied in a cell free system (Zhao et al., 1997).

It has been argued that progression of paratuberculosis is related to a shift in the immune response from a T-helper-1 (Th-1) like to a T-helper-2 (Th-2) like type of immune reaction. Recent studies based on lymphocyte stimulation assays and ELISAs using different antigens and animals in different stages of the disease not only supported this hypothesis, but indicated that the direction and extent of the shift seems to depend on the type of antigen and its predominant localisation during infection (Koets et al., 1999). These findings suggest that the host defence mechanisms are misdirected upon infection with *M. paratuberculosis* to favour survival of the bacteria. This hypothesis gains further support by recent results showing that persistence of *M. paratuberculosis* in mouse macrophages results in suppressed antigen presentation of exogenously added antigen (Goethe et al., 1999; Valentin-Weigand et al., 1997). Levels of MHC class II and co-stimulating molecules B7.1 and B7.2 remained unaltered, indicating that the level of defective antigen presentation occurred at the level of antigen processing. This is in contrast to findings obtained from infections with *M. tuberculosis*, which was found to suppress antigen presentation by downregulation of MHC class II and B7 molecules (Chan and Kaufmann, 1994; Gercken et al., 1994; Saha et al., 1994; Vanheyningen et al., 1997). These findings indicate that *M. paratuberculosis* is able to deactivate the immune response in a manner similar, but not identical to, the immunomodulating effects of other mycobacteria.

A.2 Macrophage functions

Mycobacteria are facultative intracellular pathogens that are able to survive and to multiply in phagosomes of infected macrophages. These organelles possess innate killing functions, most prominently fusion with lysosomes, production of reactive oxygen intermediates (ROI) and nitric oxide (NO), and acidification (see below). It is of great importance to understand how macrophage killing functions are regulated, since all of these functions may be inhibited by these pathogens. Therefore, an understanding of the mechanisms that regulate phagosome maturation provides a

basis to understand mycobacterial pathogenicity and will hopefully lead to new therapies against intracellular pathogens.

The macrophage is an early reacting 'sentry' cell that acts as one of the first line of defence against invading microbes in higher eucaryotic organisms. Macrophages represent a power that lies mostly dormant in the tissue throughout the body until aroused. Being 'the big eater', macrophages are capable of unleashing intense local destructive forces as well as generalised alarm reactions. In response to bacterial invasions, macrophages ingest and kill bacteria using a powerful weaponry synthesised by the oxidative burst, this leads to production of highly microbicidal ROI and NO intermediates (Ding et al., 1988; Akaki et al., 2000), and also initiates a series of reactions that greatly increase the defence capability of the host. These include a variety of different functions: 1.) Clotting and trapping of microorganisms on the surface of the macrophage by secretion of tissue factors. 2.) Secretion of leucotriene, which cause endothelial cells to generate surface platelet activating factor to promote leukocyte adherence. 3.) Secretion of chemoattractants that recruit leukocytes. 4.) Antigen processing and presentation to induce a specific humoral immune response. 5.) Secretion of cytokines such as IL-1, TNF-α, IL-6, and interferons, which have a wide range of actions, starting with fever induction and leading to a modulation of the whole immune response, including enhanced stimulation of the macrophages themselves. This somewhat mysterious process is known as macrophage activation.

Ingestion and killing of invaded bacteria are the key functions of these professional antigen presenting cells. The process called phagocytosis, results in formation of phagosomes and activation of secondary functions, and is therefore of special importance.

A.2.1 Phagocytosis

Phagocytosis is the mechanism of mainly specialised cells for the uptake of relatively large particles (>0.5 µm). The main cell types specialised for this mechanism are found in the first line of defence against invading pathogens, as they are able to dispose and kill them with great efficiency. These cells are called professional phagocytes. However, some microorganisms including mycobacteria have evolved methods to use phagocytosis as a way to invade these cells and set up residence, thereby avoiding direct destruction by serum antibodies and complement factors and

furthermore from detection by other immunological cells. In vertebrates, most of the professional phagocytes are found among two classes of leukocytes: polymorph nuclear leukocytes (neutrophils, eosinophils and basophils) and mononuclear phagocytes (monocytes and macrophages). These all originate from stem cells in the bone marrow (Greenberg et al., 1993; Allen and Aderem, 1995; 1996; Haas, 1998).

While professional phagocytes are much more efficient than other cells at internalising particles, most cells have some phagocytic activity, at least under some conditions. For example, thyroid and bladder epithelial cells phagocytose erythrocytes *in vivo*, and many different cell types have been shown to phagocytose particles in culture. These cells are called non-professional phagocytes (Rabinovitch, 1995). The main difference in phagocytic efficiency can probably be explained by different arrays of specialised phagocytic receptors expressed on the surface of the phagocytic cells, including Fc receptors, complement receptors, scavenger receptors, as well as mannose and galactose receptors. It is the transmembrane signalling from these receptors, that are usually cross-linked upon binding ligand, that is the direct cause of all ensuing cellular changes. In agreement with this, transfections of fibroblasts and epithelial cells with Fc receptors dramatically enhance their phagocytic abilities (Indik et al., 1995). However, it is clear that this is not the only difference between professional and non-professional phagocytes, and at present no single model can describe the complex mechanism by which phagocytosis is mediated.

The phagocytic process can be divided into three distinguishable steps: particle binding to the cell surface, formation of endocytic vesicles termed phagosomes and maturation of phagosomes to become phagolysosomes, in which internalised material is degraded. Importantly, specialised pathogens such as many mycobacteria have acquired the capacity to effectively block the latter function.

A.2.2 Binding to cell surface and formation of phagosomes -general

The problem for the innate immune system to discriminate between self and potential pathogens has been addressed by the evolution by a variety of different phagocytic receptors that recognise conserved motives on pathogens that are not found in higher eucaryotes. Most pathogens are recognised by more than one receptor, and these receptors are capable of cross-talking and synergising with each other. Additionally, many of these receptors have two functions, working as molecules for

cell-cell or cell substrate adhesion, as well as particle uptake. For example, adhesion mediated by the fibronectin receptor ($\alpha_5\beta_1$ integrin) activates the otherwise inactive complement receptor CR3 ($\alpha_M\beta_2$ integrin) to mediate phagocytosis (Pommier et al., 1983; Wright and Griffin, 1985). However, since adherent cells like J774 mouse macrophages often round up during phagocytosis, there may be a competition for cytoskeletal and membrane components during this process. This idea gains additional evidence from the fact that many cytoskeletal components that are known to participate in cell adhesion are also enriched in the phagocytic cup (Allen and Aderem, 1995 and 1996). By far the most prominent is actin (Boxer et al., 1974; Griffin et al., 1976; Wang et al., 1984) which is undoubtedly essential for the overall process. However, the precise mechanistic role of actin and its multiple interacting partners is only poorly understood, and very complex.

There are two general mechanisms for the interaction between receptors and particles. One is a direct interaction between innate structural determinants on the particle surface and the receptor. This is referred to as non-opsonic phagocytosis. The other is an indirect interaction of the receptor with host supplied opsonins, especially complement and immunoglobulins that have previously bound to the surface of the particle. This is referred to as opsonin-dependent phagocytosis. The best studied opsonin receptors are the Fc receptors, which bind to the Fc domain of IgG (FcγR) (Ravetch, 1997; Ravetch and Clynes, 1998; Unkeless et al., 1995), and the complement receptor 3 (CR3) that binds complement protein 3bi (Carroll, 1998; Sengelov, 1995). The specificity of Fc receptor-mediated phagocytosis relies both on the 'lock and key' interactions between Fab domains of antibodies, binding to their ligands on the infectious agents, and on the interactions of their opposite Fc domains with the Fc receptor on the cell surface. The complement receptors operate differently: here the CR3 binds the protein 3bi, which binds non-specifically to the carbohydrate surfaces of pathogens via the so-called 'alternative‘ pathway. After binding, the CR3 is not able to induce phagocytosis without additional stimuli, such as activators of PKC, cytokine stimulation or by attachment to the extracellular matrix (Wright and Griffin, 1985). So, in contrast to the FcγR, the complement receptor is not constitutively active, but more highly regulated.

Upon binding of a particle to specific phagocytic receptors on the surface of the phagocyte, actin is polymerised at the site of ingestion, and the particle is internalised

via an actin-based mechanism (Allison et al., 1971; Sheterline et al., 1984). Driven by actin polymerisation, and likely myosin V (Swanson et al., 1999; Titus, 1999), the plasma membrane encloses the particle before it buds off to form a vacuole enclosing the particle, the phagosome. In one prominent model for this process, internalisation of particles via tight interactions to cell surface receptors result in a 'zipper mechanism' in which pseudopods spread around the particle leading to a phagosome having a membrane tightly apposed around the particle (Swanson and Baer, 1995). On the other hand phagocytosis via CR3, is generally considered not to occur by pseudopod extension. Phagosomes derived by this pathway have been seen to form by particles sinking directly into the body of the cell. These phagosomes normally have a much less tightly apposed membrane relative to the non-complement-opsonised particles (Allen and Aderem, 1996; Kaplan, 1977).

A.2.3 Biogenesis of phagolysosomes

After phagosome formation, actin is usually shed from the organelle. The newly formed phagosomes, which have a composition more similar to the plasma membrane than the later stages of phagosomes (Lang et al., 1988), are not fully active in the sense that they have not yet acquired the whole machinery needed to kill and degrade internalised microorganisms. However, it should be mentioned that the oxidative burst, which is also important to kill pathogens, probably also occurs simultaneously with the uptake (Babior, 2000; Akaki et al., 2000). In order to be able to function at maximum efficiency in killing and degrading, the new phagosomes have to undergo a series of maturation steps, involving an extensive interaction with organelles of the endocytic pathway (Berón et al., 1995; Desjardins, 1995). Early in this process the young phagosomes recycle several plasma membrane molecules including Fc- and mannose receptors (Muller et al., 1983, Stahl and Ezekowitz, 1998), while they acquire markers of the early endocytic pathway such as Rab5 and EEA1 (Desjardins et al., 1994, Pitt et al., 1992, Scianimanico et al., 1999; Steele-Mortimer et al., 1999). 'Young' or immature phagosomes have been shown to fuse sequentially with endocytic organelles at different stages (early endosomes, late endosomes and lysosomes) as they mature, both *in vivo* and *in vitro* (Desjardins et al., 1997; Jahraus et al., 1998). Accordingly, the phagosomes acquire markers from late endosomes and lysosomes during the later maturation steps. These markers include LAMP molecules, Rab7 and 11, the H^+-ATPase, the cation-independent

mannose-6-phosphate receptor (CI-MPR), as well as many hydrolases including several cathepsins (Desjardins et al., 1994; Jahraus et al., 1994; Oh and Swanson, 1996; Pitt et al., 1992; Ramachandra et al., 1999). The sequential acquisition of hydrolases from the endocytic pathway suggests that they are transferred by sequential fusion with subsets of endosomes and lysosomes containing different levels of hydrolases (Claus et al., 1998).

The phospholipid composition of the phagosomes is also changed during phagosome maturation. Young phagosomes are enriched in phosphatidylcholine, whereas older phagosomes are enriched in sphingomyelin (Desjardins et al., 1994). All the changes in the phagosomes during their maturation results in the making of an organelle capable of performing its main task: to kill and degrade internalised pathogens, and then to further process their antigens for presentation at the cell surface in order to recruit and activate T-cells, the next key step in the immunological response (Harding, 1995; Ramachandra et al., 1999).

A.2.4 Phagosome membrane fusion and pH

Over the last decade the molecular machinery governing membrane fusion events has been extensively studied, including that of the endocytic pathway (Gonzalez and Scheller, 1999; Haas, 1998; Novick and Zerial, 1997; Pfeffer, 1999). The main regulators of membrane interactions appear to be Rab proteins, with their effectors, and the SNARE proteins (McBride et al., 1999). Many of these Rab and SNARE molecules have been identified on phagosomes, including Rab4, Rab5, Rab7 and Rab11 (Cox et al., 2000; Desjardins et al., 1994; Mosleh et al., 1998), synaptobrevin I and II, and NSF (Desjardins et al., 1997), as well as syntaxin 2,3 and 4 (Hackam et al., 1996). In addition, the Rab5 effectors EEA1 (Scianimanico et al., 1999; Stelle-Mortimer et al., 1999) and Rabaptin5 (Duclos et al., 2000) have also been found on phagosomes. These data strongly suggest that the mechanisms for fusion are the same for endocytic organelles and phagosomes.

Small GTPases and SNARE molecules have also been shown to be involved in endosome-phagosome fusion (Berón et al., 1995; Funato et al., 1997; Jahraus et al., 1998). Directionality for the phagosome maturation could be guided by these small GTPases. In many cell types Rab5 has been found to localise preferentially with early endosomes, clathrin coated vesicles and the plasma membrane (Bucci et al., 1992;

Chavrier et al., 1990), whereas Rab7 has been found on later endocytic and phagocytic structures (Meresse et al., 1995; Desjardins, 1995). As the phagosomes lose Rab5 and acquire Rab7 during their maturation, this would favour fusion with late endocytic compartments and thereby drive transformation of phagosomes towards phagolysosomes (Desjardins et al., 1994 and 1997). Retrieval of molecules from phagosomes (Pitt et al., 1992) may in part be occurring through recycling processes regulated by Rab4 and Rab11 (Cox et al., 2000), which have been shown to localise to endocytic recycling compartments (Ullrich et al., 1996).

The interaction between phagosomes and endocytic structures was previously believed to be a single complete fusion event between a newly formed phagosome and different endosomes and lysosomes to generate a phagolysosome (Rabinowitz et al., 1992). However, more recent observations indicate that reality seems to be far more complex. Desjardins et al (1994) proposed that rather than complete fusion of phagosomes with endosomes, many transient and dynamic interactions may occur between a phagosome and endocytic organelles that finally generate a phagolysosome. This model is referred to as the 'kiss and run' hypothesis (Desjardins, 1995; Storrie and Desjardins, 1996). According to this model phagosomes and endosomes move on cytoskeletal elements, fuse transiently at point sites, forming a fusion pore and then separate again in a process that would be both spatially and temporally regulated. Indeed, phagosomes have been shown to bind (Blocker et al., 1996), and to move on microtubules both *in vivo* (Blocker et al., 1998) and *in vitro* (Blocker et al., 1997) and video observations have shown transient contacts followed by a microtubule-dependent separation between phagosomes and endocytic organelles (Desjardins et al., 1994). In addition, phagosomes bind filamentous actin (F-actin) in a myosin Va dependent manner (Al-Haddad et al., 2001). In a kiss and run model, the exchange of material between the fusing organelles could be more selective than in a complete fusion event. Experiments in macrophages showed that this is indeed the case with phagosomes (Desjardins et al., 1997). Endosomes containing gold particles of different sizes were able to selectively transfer these to the phagosomes, with the smaller gold being more easily transferred than the bigger ones. This clearly indicated that complete mixing of both organelles was not occurring.

It is well known that phagosomes in general can fuse with all stages of the endocytic pathway, including lysosomes. Lysosomes are complex vacuolar organelles that

represent the late stages of the endocytic pathway, full of hydrolytic enzymes capable of degrading a wide range of macromolecules, including those present on microbes. The pH of this compartment is the lowest found in animal cells with a pH of 4.5-5.0. This highly acidic milieu resembles the conditions needed for optimal activity of the hydrolytic enzymes. The low pH is maintained by membrane ATP-dependent proton pumps, the vacuolar ATPases (Mellman et al., 1986; Ohkuma and Poole, 1978). Although phagosomes can be seen to fuse with single lysosomes, the formation of phagolysosomes is usually a rather continuous process of fusion events which lead to a maturation of the phagosome by transient fusion and fission events with endocytic organelles (Desjardins et al., 1994; Desjardins, 1995).

Within these phagolysosomes microbes are normally degraded by a variety of lytic enzymes, delivered by the lysosomes. This highly regulated function represents a significant antimicrobial mechanism of phagocytes. It seems that the antimicrobial activity of the phagolysosome is mediated by a combination of the degrading function of the lysosomal hydrolases and a direct effect of the acidification. However, the mechanisms by which hydrolases and acidification confer antimicrobial properties, as well as the mechanisms that regulates acidification of various endocytic compartments and the fusion and fission events of endosomes with phagosomes, are not fully understood. Elucidation of these mechanisms will greatly facilitate our understanding of how mycobacteria manage to escape the hostile environment of phagolysosomes.

A.3 Interaction of mycobacteria with macrophages

Intracellular microorganisms such as mycobacteria have evolved the ability to manipulate host those cell killing functions, in order to survive intracellularly. Mycobacterial species have been especially successful in this respect and molecular insights into how they attain this goal are know emerging.

A.3.1 Mycobacterial virulence factors and the cell envelope

Given the major differences observed among pathogenic and non-pathogenic mycobacteria regarding the ability to trigger cytokine secretion by macrophages

(Beltan et al., 2000) the components of the mycobacterial cell envelope play a important role, as specific cell wall components of pathogenic and non pathogenic mycobacteria have been found to modulate immune responses in the infected host. A recent review discussed aspects related to the interaction of *M. tuberculosis* with macrophages, and subsequent activation of mycobactericidal acitivity of macrophages (O`Brien et al., 1996). A model has been proposed involving *M. avium* and a group of amphipathic substances referred to as the GPL antigens, which are one of the key elements in the mycobacterial cell envelope. These serovar specific *M. avium* antigens (Brennan and Goren, 1979) localised in the exterior of the cell envelope (Barrow et al., 1980; Tereletsky and Barrow, 1983) tend to accumulate within the phagosomes in long term infections with mycobacteria (Rulong et al., 1991). GPLs, being relatively inert to macrophage degradation, not only accumulate in host macrophages, but also persist as the infection continues (Woodbury and Barrow, 1989; Hellio et al., 1988; Hooper et al., 1986), leading to changes in the macrophages (Lopez-Martin et al., 1994). The immunosuppressive role of *M. avium* lipids as a result of interaction with human monocytes (Tsuyuguchi et al., 1990), and of a GPL in *M. smegmatis* was demonstrated to modify membrane permeability (Sut et al., 1990). These effects on biological membranes may be extremely important, since the ability of immunocompetent cells to function properly in immune reactions such as cell-to-cell recognition, phagolysosomal fusion and organisation of the cytoskeleton by membrane dependent processes might be disturbed. *M. avium* GPLs result in a non specific inflammatory response when injected intraperitoneally into mice (Hooper and Barrow, 1988). Phenylalanine-containing lipids of *M. avium* have also been identified as immunosuppressive agents (Barrow et al., 1993). Although the lipopeptide core has not yet been identified to be a product of GPL degradation, some of the immunosuppressive effects may be due to other lipids structurally related to the GPL, or to metabolic products of GPL. Consequently, GPLs and related lipids may act as important immunomodulators of host responsiveness.

A.3.2 Phagosome-endosome Fusion

One of the most important functions of professional phagocytes is the uptake and killing of pathogens by phagocytosis. In order to avoid killing intracellular pathogens have evolved different strategies. In general, three strategies are possible: first,

pathogens like *Trypanosoma cruzi* and *Listeria monocytogenes* escape from the phagosome into the cytoplasma; second, pathogens like *Leishmania donovani* and *Coxiella burnetii* reside in a phagolysosome; and the third possibility is to reside in a phagosome that does not fuse with lysosomes.

In 1971 D'Arcy Hart and colleagues proposed that mycobacteria manage to survive in macrophages by inhibition of the phagosome-lysosome fusion (Armstrong and Hart, 1971; D'Arcy Hart et al., 1972). This hypothesis was based on studies of *M. tuberculosis* infected mouse-macrophages. EM analysis of infected macrophages with ferritin particles chased in lysosomes revealed that only viable mycobacterial phagosomes did not fuse with lysosomes (Armstrong and Hart, 1971). Since then, the apparent inaccessibility of mycobacterial phagosomes to the endocytic network has been intensively studied and characterised by the use of different endocytic markers which were found on the phagosomal membrane (Russell et al., 1996; Clemens, 1996; Deretic and Fratti, 1999).

Pathogenic mycobacteria were found to acquire markers from early endosomes like the transferrin receptor, Lamps, procathepsin D or Rab5 (Clemens, 1996), but not markers of lysosomes like cathepsinD (Clemens, 1996), subunits of the proton ATPase (Russell et al., 1996), Rab7 (Clemens et al., 2000), as well as gold particles previously chased in lysosomes (Clemens, 1996).

Emerging evidence, provided by recent studies including this one, have revealed that the situation is far more complex. The inaccessibility of phagosomes containing mycobacteria to the endocytic network seems to be dependent on the viability and pathogenicity of the bacteria, the kind of endocytic organelle whose fusion with phagosomes is to be monitored as well as the time of infection. In fact the mycobacterial phagosome is a highly dynamic, fusion-competent structure that fuse in a very selective way with specific endosomal compartments (Russell et al., 1996, and below). The factors that mediate the inhibition of fusion are therefore of special interest. In the case of the tubercle bacillus, this property has been attributed to bacterial surface sulpholipids derivatives of multiacylated trehalose 2-sulfate, a lysosomotropic polyanionic glycopeptide (Middlebrook, 1982; Goren, 1959, 1970; Goren et al., 1976; Hellio et al., 1988). Other important mycobacterial virulence factors include the lipidoglycans (such as lipoarabinomannan, LAM) which are able to modulate the cytokine secretion and macrophage effector functions (Vercellone et al., 1998). The ability of mycobacteria to survive and modulate immune responses in the

host is clearly related to the architecture of the bacterial envelope and the constituents within its boundary (Rastogi and Barrow, 1994; Rastogi, 1990). Another mechanism of fusion prevention might be mediated by ammonia. It has been demonstrated that some mycobacteria generate ammonia in cultures in concentrations up to 20 mM. These anions not only raise the vacuolar pH, but also affect the saltatory movement of lysosomes. However it is unlikely that the ability of ammonia to raise the intralysosomal pH is responsible for inhibition of fusion because it has been shown that other bases capable of raising the lysosomal pH stimulate fusion with phagosomes (Gordon et al., 1980). Apart from this, the general role of acidification for fusion of phagosomes with lysosomes is unclear (Mellman et al., 1986; Hackam et al., 1997; Downey et al., 1999). The precise mechanisms by which ammonia is involved in inhibition of fusion remains to be determined. In addition to inhibiting fusion, ammonia might work by inactivating lysosomal enzymes which are known to work best a low pH.

In mycobacteria, two enzymatic systems of producing ammonia have been intensively studied. The first is the mycobacterial urease, which catalyses the production of ammonia and carbon dioxide from urea (Clemens et al., 1995, Reyrat et al., 1995). Transfection experiments of an urease negative BCG strain with the urease gene from *M. tuberculosis* revealed that the transfected bacteria were capable of surviving and multiplying in alveolar macrophages of infected mice (Reyrat et al., 1996), although it remains to be determined if the loss of the urease affects the survival of *M. tuberculosis*. The second enzyme is the glutamine synthetase. This enzyme was found to be secreted by pathogenic mycobacteria, like *M. tuberculosis* and *M. bovis*, but not by non pathogenic *M. smegmatis* and *M. phlei,* and might therefore be involved in the production of ammonia via its participation in the nitrogen metabolism. Immuno EM revealed that the enzyme is secreted by mycobacteria into the lumen of the phagosome (Clemens and Horwitz, 1995). One of its putative functions of the released glutamine synthetase is to mediate the synthesis of poly [L-glutamic acid/glutamine], a cell wall structure found in pathogenic strains of mycobacteria (Harth et al., 1994; Harth and Horwitz, 1999). Interestingly, the glutamine synthetase inhibitor L-methionine-S-sulfoximine selectively blocks the growth of pathogenic mycobacteria, both in culture and in macrophages (Harth and Horwitz, 1999). Although it is likely that the mechanism by which glutamine synthetase confers a growth advantage through its role in cell wall synthesis, its

potential role in influencing the vacuolar pH and fusion by ammonia production remains a possibility.

A.4 Phagocytosis and the cytoskeleton – general

It is important to note that in 2000 de Chastelier et al. reported that the actin cytoskeleton of infected J774 macrophages infected with *M. avium* was significantly modified. This was the first time that an effect of mycobacteria on the actin cytoskeleton was reported. Both the actin and microtubular cytoskeleton is intimately involved in phagocytosis and in the intracellular biology of the phagosome in general. Actin is unquestionably involved in the uptake process but, until recently, the firm dogma in the field was that subsequent stages of phagosomes are devoid of actin. As discussed below, recent data show that actin is probably intimately involved at all stages of phagosome maturation. Soon after their formation, phagosomes also interact with microtubules. Since the biology of the phagosome is highly regulated and dependent on many of fusion events that have to be regulated, the question arises whether or not actin is involved in the trafficking of phagosomes and in killing of mycobacteria in macrophages. To answer this question a detailed analysis of the involvement on actin an mycobacteria is part of this thesis.

A.4.1 Phagocytosis and actin

Nearly nothing was known about mycobacterial phagosomes and actin at the start of my thesis. Most of our knowledge about phagosomes derived from the latex bead phagosome (LBP), a model system used extensively over the past decade in the group of Gareth Griffiths. They have shown that within 10 sec latex beads contacting J774 cells, transmembrane signalling leads to a significant, though transient rise in actin nucleation (Defaque et al., 2000). As discussed below this process is thought to drive the process of membrane outgrowth leading to phagosome formation. However, the main focus has been on the role of actin in the later stages of phagosomes and especially in relation to their fusion with endocytic organelles.

Actin is the most abundant protein in many cell types, including macrophages, where it can occupy up to 12 % of the total cell protein (Hartwig and Stossel, 1982). Macrophage actin, and all other non-muscle actins studied so far, is a 42 kDa globular protein (G-actin) indistinguishable from muscle actins in all important

aspects. Monomeric G-actin can assemble into double helical filaments, and the equilibrium distribution between monomers and polymers in cells is highly regulated. Actin is generally considered to be a cytoplasmatic protein, although recent evidence argues for a function in the nucleus (Rando et al., 2000; and Wada et al., 1998).

There are four mechanisms to assemble F-actin, namely, 1) a *de novo* actin nucleation, 2) uncapping of pre-existing filaments by release of end capping-proteins, 3) severing of intact filaments to generate free ends and 4) nucleation from the sides of pre-existing filaments. Up to now, it is still unknown how these four mechanisms contribute to the amount of total F-actin in cells (Chan et al., 1998; Eddy et al., 1997; Li et al., 1995; Shariff and Luna, 1990; Tilney et al., 1992; Welch et al., 1998). This conventional view of actin dynamics does not generally consider the role of membranes.

A.4.2 Membrane dependent actin nucleation

In cells, much of the actin assembly takes place on the cytoplasmic surface of membranes. Compared to the pure *in vitro* situation, the complexity of the mechanisms underlying the regulation of actin nucleation increases tremendously when cellular membranes are taken into consideration. These membranes contain hundreds of proteins and lipids that are able to interact in several signal transduction systems which regulate actin nucleation in a very specific and dynamic manner.

The general concept of actin filaments originating from, or close to the cytoplasmic surface of membranes, especially the plasma membrane, has been established by observations in a wide array of eukaryotic systems (Tilney et al., 1995; Tilney et al., 1992; Mooseker and Tilney, 1975; Tilney, 1976; Tilney et al., 1979; Tilney and Kallenbach, 1979; Tilney and Tilney, 1996). In all cases the actin filaments can be seen by EM to originate from ill-defined electron dense plaques on the cytoplasmic side of the plasma membrane (see below), and they always seem to be orientated with their barbed end facing towards these dense plaques. Such an orientation of actin filaments has also been seen in other systems (Carraway and Carraway, 1989; Cramer et al., 1997). Many studies argue that there is a link between membrane fusion and actin dynamics, especially during exo- and endocytosis (Burgoyne and Cheek, 1987; Lamaze et al., 1977; Riezman et al., 1996). However, little is known about the detailed mechanisms connecting these processes. Much of our knowledge

comes from studies of induced secretion of adrenal medullar cells (Aunis, 1998; Burgoyne and Cheek, 1987; Van Obberghen et al., 1975). Upon triggering of exocytosis in these cells, the actin cytoskeleton rapidly depolymerises until 30 seconds before a switch to a second assembly state, which lasts until the starting level is reached (Cheek and Burgoyne, 1986).

A.4.3 Actin and pathogens

Actin nucleation is a highly regulated process that is not only found in normal cell functions, but also reported to be important in some bacterial systems and contributes to pathogenicity of intracellular pathogens. Since these bacterial systems can be easily purified they proved to be a good system of analysing principles of actin nucleation on membranes.

De novo actin nucleation on membranes: the Listeria monocytogenes and Shigella flexneri systems

In cells, severing, uncapping and true nucleation may all contribute to actin assembly. While the relative importance of these different mechanisms is difficult to estimate there seems to be no doubt that the *de novo* process on membranes does indeed occur in cells (Hall and Nobes, 2000; Shariff et al.,1990; Tilney et al., 1992b; Norman et al., 1994; Li et al., 1995; Eddy et al., 1997; Chan et al., 1998) and in bacterial systems. Two bacterial systems have been extensively characterised, namely *Listeria monocytogenes* and *Shigella flexneri* (Cudmore et al., 1995; Tilney and Tilney, 1993; Theriot, 1995; Smith and Portnoy, 1997; Welch et al., 1997a; Lasa et al., 1998; Cossart and Lecuit, 1998; Carlier, 1998). It is believed that aspects of bacterial membrane actin assembly are relevant to the problem of actin nucleation as it occurs on a eukaryotic membrane.

Listeria is a facultative intracellular bacterium which escapes (via a lysogenic toxin) from its vacuole (the phagolysosome) to grow and divide within the cytoplasm. At this stage, the bacteria can recruit components of the cellular actin cytoskeleton in order to assemble actin around itself, initially as a cloud. After a short lag phase the system switches to a mode whereby actin is nucleated at one pole of the bacterium allowing the actin structure to dramatically grow as a vectorial comet from this pole (with the

barbed ends of filaments orientated towards the bacterial membrane) (Theriot, 1999). This comet propels the bacteria at speeds of 5-10μm/min through cells with cytosolic extracts (Theriot et al., 1992). When an actin tail is formed at one bacterial pole, a driving force is somehow provided, allowing the bacteria to be propelled and spread into neighbouring cells (Tilney and Tilney, 1993; Theriot, 1995). *Shigella* and some *Rickettsia* species are capable of undergoing the same series of events while vaccinia virus shows a variation on this theme (Cudmore et al., 1995).

The molecular mechanisms of actin-based motility of *Listeria* and *Shigella* have been most extensively characterised. The key to this characterisation was the development of *in vitro* motility assays using fluorescence labeled G-actin mixed with purified cell extracts such as platelets or Xenopus eggs, in which *Listeria* is able to move in the presence of ATP (Theriot et al., 1994; Welch et al., 1998). *Listeria*, being Gram-positive, is enveloped by one membrane (Anthony and Hill, 1988). A 610 amino acid integral outer membrane protein, Act A, is the key to this actin nucleation process (Smith et al., 1995) and when put on the surface of beads it can nucleate actin comet assembly from cytosol extracts (Cameron et al., 1999). Different heterologous expression experiments have clearly shown that when artificially targeted to membranes (mitochondria, the plasma membrane or the surface of *E. coli* bacteria that are normally unable to mediate actin assembly) the presence of Act A could induce membrane-associated actin polymerisation at the sites of expression (Kocks et al., 1995; Pistor et al., 1994). Thus at the membrane Act A is a true nucleator of actin polymerisation. The protein has its C-terminal end embedded in the bacterial membrane while its N-terminus extends outwards through the peptidoglycan domain, most likely as a dimer (Lasa et al., 1998; Cossart and Lecuit, 1998).The domain organisation of the analogous protein from *Shigella,* IcsA, has also been well-characterised (Cudmore et al., 1995). Act A binds to VASP which recruits the ARP2/3 complex, a seven-protein machine that can facilitate and extent the assembly of actin (Welch, 1999; Higgs and Pollard, 2001). The precise mechanisms of this complex reaction is currently unclear.

A.4.4 Regulators of membrane-dependent actin nucleation

A.4.4.1 Phosphoinositides and actin nucleation

During the assembly of actin an impressive body of evidence argues that specific lipids are recruited laterally along the membrane or synthesised at (or close to) the sites of actin nucleation (Shariff and Luna, 1992; Ha and Exton, 1993). Foremost among these are the now highly prominent phosphoinositides, which can co-localise with actin binding proteins on membranes subdomains (Fukami et al., 1994; Bubb et al., 1998). The beauty of these molecules lies in their simplicity. A relatively minor lipid that is essentially exclusively on the cytoplasmic leaflet of membranes, phosphatidyl inositol (PI, 3% of total lipids) exposes its five hydroxyl groups on its inositol ring to different lipid kinases and phosphatases. The inositol ring can be phosphorylated at position 4 by a phosphatidylinositol-4 kinase (PI4K). The phosphatidylinositol-3 kinases (type III) can also synthesise PI_3P from PI, as well as $PI_{3,4}P_2$ and $PI_{3,4,5}P_3$ but these pathways will not be described here. Two major products can be synthesised from PI_4P, $PI_{4,5}P_2$ (PIP_2) and $PI_{3,4}P_2$, by phosphorylation of PI_4P by a phosphatidyl inositol 4-P 5-kinase or by a PI3K (type I or II), respectively. One PI3K kinases can also synthesise $PI_{3,4,5}P_3$ (PIP_3). A large family of kinases has now been identified that carry out these functions. Many of these enzymes, which operate as peripheral membrane proteins, are highly specific for one reaction (e.g. PI_3 to PI_3P to $PI_{3,4}P_2$) but will not make $PI_{3,5}P_2$)

Other kinases and their products have been recently discovered, providing an alternative pathway for the synthesis of PIP_2 and PIP_3. PI_5P is a minor lipid in mammalian cells (Rameh et al., 1997), and its synthesis could allow the subsequent formation of $PI_{3,5}P_2$ (by a phosphatidyl inositol 5-P 4-kinase) as well as PIP_3 (by PI3K type I or II). Moreover, this phosphatidyl inositol 5-P 4-kinase could also produce a new lipid, $PI_{3,5}P_2$ (Rameh et al., 1997). These lipid phosphorylation events and their regulation have been described in detail in recent reviews (Carpenter and Cantley, 1996; Vanhaesebroeck et al., 1997; Martin, 1998).

The synthesis of these phosphoinositides can be transiently activated upon signaling in many, but not all systems (Martin, 1998). It now looks increasingly likely that these seven different phosphoinositides species are playing the roles of different lipid messengers that can recruit different effector molecules. In recent years there have

also been many molecular links identified between some of these lipids and an increasing number of membrane traffic events have been found (De Camilli et al., 1996; Martin, 1998).

Most of the observations on actin nucleation have focused on the prominent role of PIP_2, which in most cell models is a relatively minor lipid at rest: PIP_2 generally represents less than 0.4% of the total cellular phospholipids, and $PI_{3,4}P_2$, as well as PIP_3 (less than 0.01%) (Ireton et al., 1996). These lipids are essentially all in the cytoplasmic leaflet of membranes where they have the ability to bind *in vitro* to many actin-binding proteins.

A number of lines of evidence argue that the synthesis, and breakdown of different lipids is closely coupled to phosphoinositides actin assembly and disassembly. In many cells a rapid burst of PIP_2 coincides with actin assembly (15-30 sec, averaged over a population of cells) after ligand-receptor interactions (Apgar, 1995). In some cells PIP2 levels drop as actin is assembled 0-30sec for instance there is a 30% decrease in cellular PIP_2 levels in platelets in response to Fc receptor cross-linking (Gratacap et al 1998) and a 80% decrease in RBL cells in response to platelet activating factor (Stauffer et al., 1998). However, in some of these cell types the levels of PIP_3 and PA increase.

A number of ABP's bind phosphoinositides and especially PIP_2. Interestingly, these proteins fall into two different functional classes. First, there is the great majority that release their bound actin when they bind PIP_2. These proteins are logical candidates for the role of 'provider' of actin monomers which can be co-ordinately released from a patch of membrane as the ABPs bind newly synthesised PIP_2 (or any other PIP). Perhaps this process could be dynamically coupled with the binding site of the ABP to PIP_2 being so positioned (close to the nucleating machinery) so that the monomers would be funnelled into the growth sites (Pantaloni et al., 2001). We suspect that this 'funnelling' process requires some sophisticated protein machinery for inserting the monomers in their correct position. If so, it would go against the current belief that it is all a consequence of actin massaction and solution chemistry.

The second class of ABPS are those that can simultaneously bind (G and / or) F actin and PIP_2. These proteins, which include ezrin, radixin, moesin (ERM), and talin are strong candidates for being part of the 'core' actin assembly machinery in some membrane subdomain, where they can bind F (and G) actin, while remaining bound

to PIP_2 as well as the membrane. Ezrin and moesin have been shown to be essential for LBP actin nucleation (Defaque et al., 2000b).

A.4.4.2 Other signaling lipids involved in actin nucleation

It now seems clear that other lipids such as sphingosine-1-phosphate (S-1-P) (Bornfeldt et al., 1995; Yamamura et al., 1996; Spiegel and Merrill, 1996), phosphatidic acid (PA), lyso-PA (LPA) (Meerschaert et al., 1998) and diacylglycerol (Shariff et al., 1992) are also potential second messengers in the processes of actin assembly or disassembly. How these molecules could function in any signal cascade leading to actin assembly is completely open, but recent *in vitro* data have pointed out for a role of LPA in actin-gelsolin dissociation. LPA has been shown to release actin from actin-gelsolin complexes as efficiently as PIP_2 and PI_4P (Meerschaert et al., 1998) (note that PA and S-1-P had no effect in this system). LPA also inhibited the F-actin severing activities of gelsolin as well as similar actin severing proteins. Although the physiological relevance of these data remains to be determined, other results suggest that LPA could control actin reorganisation *in vivo*. For instance, LPA levels increase in platelets in response to thrombin activation and could then mediate actin-gelsolin dissociation; moreover, the dissociation of such complexes has been shown to be independent of PIP_2 or PIP_3 in some cell models, suggesting the involvement of other mechanisms in this case (Meerschaert et al., 1998 and refs therein).

Gelsolin is also linked to the signaling lipids in another interesting manner. It binds and activates phospholipase D (PLD) which synthesises PA from phosphatidyl choline (Steed et al., 1996). Increases in PA have been associated with actin assembly in some systems (Moritz et al., 1992; Ha et al., 1993). The propensity of gelsolin to activate PLD does not depend on Ca^{2+} but is greatly stimulated *in vitro* by the presence of PIP_2 (Steed et al., 1996), which can bind with high affinity to gelsolin (Yu et al., 1992) (table 3). This gelsolin/PLD/PIP_2 complex could be also linked to the tyrosine kinase c-src, since tyrosine phosphorylation of gelsolin by PP60 (c-src) was enhanced by PIP_2 vesicles (De Corte et al., 1997) and increased gelsolin-associated phosphatidyl inositol-3 kinase activity (Chellaiah 1998). The ability of gelsolin to affect phosphoinositide-signaling pathways is also reinforced by the fact that this molecule

can be isolated in complexes with other lipases and kinases, such as phospholipases (Kwiatkowski, 1999).

A.4.4.3 GTP-binding proteins and actin

The number of molecules implicated in the regulation of actin assembly is almost as large as the known signal cascade maps (Burridge and Chrzanowska-Wodnicka, 1996; Keenan and Kelleher, 1998; Martin, 1998). The small GTP-binding proteins of the Rho family stand out however as being key regulators of actin assembly. These 20-25 Kd GTPases must be prenylated at their COOH termini for efficient functioning and it is widely assumed that they are mostly bound to membranes in their active, GTP state. In this state they operate as molecular adaptors or switches on the surfaces of membranes, a process allowed by the isoprenyl lipid moity that is covalently attached to the C-terminus of these proteins (Zerial and Huber, 1995).
There are three major classes of these molecules known as Rho, Rac and cdc42. A fascinating aspect of these rho family proteins is that the type of receptors activated determines which member of the rho family is recruited to the sites where the receptors accumulate and where actin assembly occurs. The precise one recruited then seemingly regulates the size and morphology of the actin assembly patches on the membrane (Hall, 1998). When cdc42 is activated, for example, microvilli-like 'filopodia' are rapidly extended (Nobes and Hall, 1995; Ramesh et al., 1999). This GTPase has also been shown to directly regulate actin assembly by the use of cell-free systems, where rhodamine actin polymerisation could be followed on membrane vesicles (Ma et al., 1998) or in neutrophils or *Dictyostelium* lysates (Zigmond et al., 1997) upon cdc42 activation. In the case of rac (and in some cases coupled to the cdc42 signaling pathway), actin structures needed for more lamellar membrane processes of ruffling, phagocytosis and macropinocytosis are activated in many cell types (Nobes and Hall, 1995; Seastone et al., 1998). Rac is also responsible for actin assembly in activated platelets (Hartwig et al., 1995). Finally, the 'founder' member rho (comprising several members) (Ridley, 1997) regulates stress fiber bundle growth as well as focal adhesion points at the plasma membrane (Nobes and Hall, 1995; Guillemot et al., 1997). In macrophages, this pathway is also responsible for complement receptor-dependent phagocytosis, whereas the immunoglobulin receptor-dependent uptake is regulated by both cdc42 and rac (Caron and Hall, 1998; Zigmond, 1997; Schmidt and Hall, 1998).

By now, a multitude of cytoplasmic factors that interact with the rho family have been identified, as have a number of membrane components. For instance, these GTPases can interact with many different kinases and activate them (Schmidt and Hall, 1998; Hall, 1998). Links between these signaling pathways and the actin nucleation machinery on the membrane are not yet understood.

While the rho proteins are regulators of actin, and can be membrane bound in their active, GTP-bound state, it is not clear to which extent they are directly involved in the processes of actin nucleation on membrane surfaces .The fact that binding of Cdc-42 to N-wasp that is bound to the surface of PIP_2 vesicles leads to a robust nucleation of actin (Ma et al., 1998) implies that this complex can nucleate actin from eukaryotic membrane surfaces under some conditions in the presence of the ARP2/3 complex. Nevertheless, the precise role of the Arp2/3 complex, if any, in *de novo* nucleation on membrane surfaces is far from clear despite the prominence this complex has been given in recent reviews. It should also be noted that the assembly of actin in microvilli is known to be completely independent from Arp2/3, since these cells lack this complex.

A.4.5 Actin and membrane fusion

Many studies argue that F-actin can prevent fusion by creating a physical barrier in the space between the secretory granules and the plasma membrane and that depolymerisation of this actin is necessary for fusion to occur (Orci et al.,1972). This agrees with many studies on exocytosis making the same conclusion that the function of actin in the fusion process is to prevent it, by forming a barrier (Burgoyne et al., 1993; Muallem et al., 1995; Orci et al., 1972; Trifaro and Vitale, 1993; Trifaro et al., 1992; Vitale et al., 1995). However, this simple model does not fit for all membrane fusion events since actin can also act as a positive effector of exocytosis (Muallem et al., 1995; Orci et al., 1972), as well as endocytosis (Lamaze et al., 1997). Moreover, the cytochalasins, which are actin-specific drugs, and both stimulate or inhibit exocytosis in different cell systems (Aunis, 1998; Burgoyne and Cheek, 1987; Orci et al., 1972). Interestingly, Bernstein et al. (1998) recently showed that cycles of neuronal exocytosis correlated with cycles of actin polymerisation and depolymerisation. This is also consistent with the notion that actin is somehow involved in membrane fusion. A high resolution fluorescence microscopy study of

exocytosis by Lang et al. (2000), showing that movement of secretory granules is both mediated and limited by the actin cortex close to the membrane in PC-12 cells, further supported this idea.

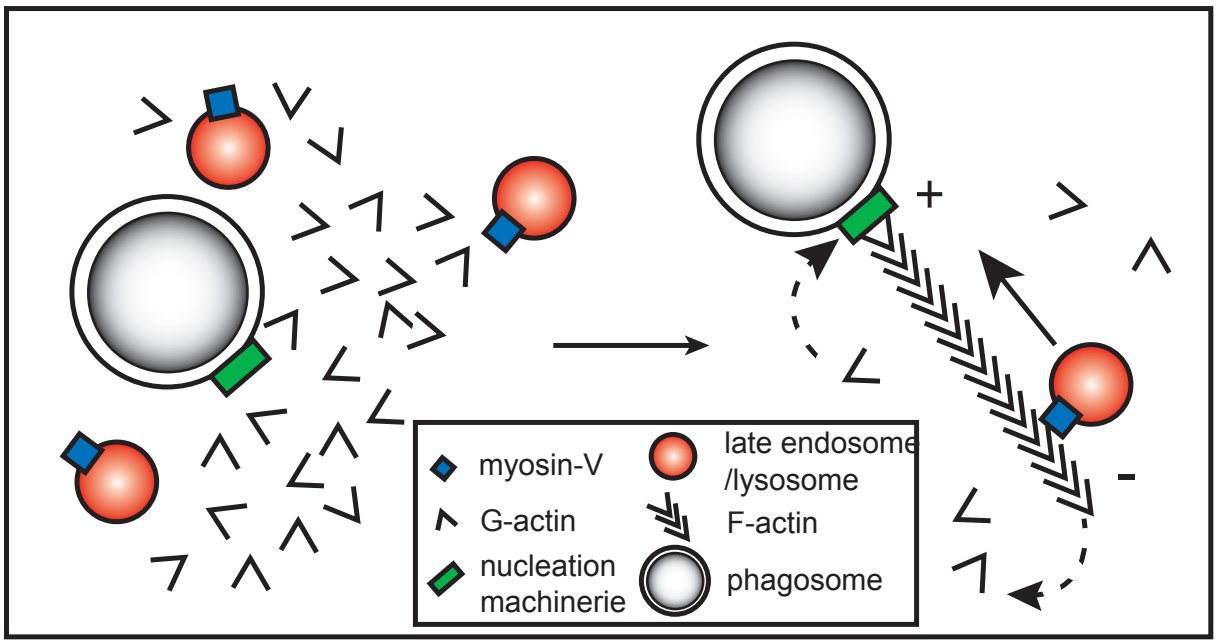
A.4.6 Actin and phagosome fusion

At the beginning of my thesis a link between actin and phagosome was just a speculation derived from various observations obtained from work on actin nucleation on latex bead phagosomes in the Griffiths group at the EMBL Heidelberg.

A.4.7 The latex bead phagosome (LBP) system

The LBP system developed by the Griffiths group has proven to be a powerful model system for studying many phagosome functions. The essence of this approach is to internalise (usually 1 µm) non-degradable latex beads (which can be conjugated to many ligands). After gentle homogenisation the LBP can be purified by taking advantage of the low density of the beads in order to float the LBP away from contaminants on a discontinuous sucrose step gradient. Of particular relevance to my thesis are the studies showing that the LBP can nucleate actin *in vitro* (even at 36 h after incubation start) and those describing how this actin can facilitate the docking of phagosomes to endocytic organelles, a process that facilitates their fusion (Egeberg et al., in prep). Using the LBP actin nucleation assay it has been shown that actin can be nucleated *de novo* on the membrane of the phagosome. In this immunofluorescence-based assay the LBP are mixed with rhodamine G-actin, that is buffered with the G-actin-sequestering protein, thymosin β4, plus a simple buffer, and a low (0.2 mM) or high (5 mM) level of ATP. After a 10-20 min incubation the percentage of labelled LBP is counted directly. If necessary the signal per phagosome can be evaluated by FACS (Defaque et al.,2000a, 2000b), or by computer assisted quantification of fluorescence images. The standard LBP assay works optimally at low (0.2 mM) ATP high level ATP (1-5 mM) was found to inhibit the nucleation of actin and provides a negative control. Importantly, and in contrast to all other *in vitro* assays developed for LBP (F-actin binding, a distinct process from nucleation; fusion; microtubule binding; microtubule motility), the actin nucleation assay is devoid of cytosol (as well as GTP). The use of this assay has revealed a role for Ezrin and/or moesin (Defaque et al., 2000a), gelsolin (Defaque et al., 2000b) as

well as the phosphoinositides PI_4P and $PI_{4,5}P_2$ (Defaque et al., submitted). As discussed in more detail in the Results and Discussion, recent data has greatly expanded this list into at least 23 different enzyme reactions that operate *in vitro* in this system (Bos, Anes, Kuehnel; joint first authors et al., manuscript in preparation).
In this process, as in all membrane systems examined, actin is nucleated on the membrane and then further grows by insertion of monomers on to the 'plus', or 'barbed' end of filaments at the membrane surface; the 'minus', or 'pointed' end is then distal to the membrane. One consequence of such an orientation, and of vectorial growth of the actin, is that, in the presence of cytosolic extracts and ATP, it can allow other (or the same) organelles that have bound myosin (such as endosomes) to move along the actin towards the phagosome (this model is refferred to as the **actin-track model**; Jahraus et al., 2001). Myosin-V on LBP has recently been shown to be one of the factors responsible for binding LBP to F-actin (Al Haddad et al., 2001).



Model 1: Actin-track model-general. F- actin is nucleated by a nucleation machinerie on the phagosomal membrane and other (or the same) organelles can bind to this F-actin via myosin Va. Since barbed-ends (+) of the actin filaments are facing towards the surface of the phagosomes, a myosin Va based transport would result in the organelles being brought in close proximity of each other and thereby increase fusion efficiency.

The use of the actin nucleation assay has been complemented in the latter two references by the use of TLC, HPLC and, in preliminary studies by mass-spectrometry, in order to analyse the lipids that become phosphorylated during *in*

vitro nucleation, as well as the total lipid composition. From these studies, which were initiated by Desjardins et al. (1994), and are being actively pursued in the Griffiths group, the *in vitro* synthesis of a number of lipids (such as PI_4P, $PI_{4,5}P_2$, $PI_{3,4,5}P_3$, phosphatidic acid and sphingosine-1-phosphate) have been detected; the levels of the different lipids vary greatly depending on the effector. This analysis of the lipids is being complemented by an exhaustive analysis of all the LBP proteins by 2D-gels and mass-spectrometry, by Michel Desjardins (Montreal) and Jerome Garin (Grenoble). Until now approximately 150 of the estimated 500 proteins have been identified (and many confirmed by immunoblotting) (Garin et al., 2001; Desjardins et al., 1994).

A.5 Goals of my thesis

With the background I provided in this introduction the experimental goals for my thesis can be summarised as follows:

PART A Characterisation of *M. avium* subspecies, and for comparison other non-pathogenic mycobacteria, in an *in vivo* infection system consisting of J774 mouse-macrophages with a focus on: a) phagosomal acidification, b) phagosomal fusion, and c) actin co-localisation. Effectors that are found in PART B to increase *in vitro* actin nucleation were then tested in the various *in vivo* assays to find out whether the actin track model (see above and Discussion) plays a role in mycobacteria infected cells.

PART B *In vitro* functions of phagosomes containing mycobacteria, focusing specifically on actin nucleation of *M. avium* subspecies- and *M. smegmatis*-containing phagosomes and, especially on the role of lipid effectors in regulating this process.

B. Experimental procedures

B.1 Material and liquids

B.1.1 Chemicals

β-mercaptoethanol	Merck
Acetone	Merck
Aprotinin	Sigma
Arachidonic acid	Sigma
MgATP	Merck
$AuCl_2$	Merck
BacLight viability kit	Molecular Probes
Cacaodylate	Sigma
$CaCl_2$	Sigma
Catalase	Sigma
CDP-choline	Calbiochem
Ceramide	Calbiochem
Chloroform	Merck
DABCO®	Fluka
DMEM	Gibco BRL
DTT	Sigma
EDTA	Sigma
EGTA	Sigma
Ethanol	Merck
FCS	PAA
Fish skin gelatin	Sigma
FITC-dextran 70 kDa	Sigma
Formvar	Merck
Glacial acetic acid	Merck
Glucose	Merck
Glucose oxidase	Sigma
Glutamine	Gibco BRL
Glutaraldehyde	Sigma
Glycerol	Merck

Glycine	Merck
HCL	Merck
Hepes	Sigma
K_2CO_3	Sigma
KCl	Merck
Kieselgel plates	Merck
Latex beads	Sigma
Leupeptin	Sigma
L-Glutamine	Gibco BRL
Methanol	Merck
Methylcellulose	Merck
$MgCl_2$	Merck
Middlebrook 7H10	Difco
Na_2HPO_4	Merck
NaCl	Merck
NaH_2PO_4	Merck
NaOH	Merck
NH_4Cl	Merck
NHS-fluorescein	Merck
NHS-Biotin	Molecular Probes
Nigericin	Sigma
NP40	Merck
Osmium tetraoxide (OsO_4)	Merck
Paraformaldehyde	Sigma
Penicillin	Gibco BRL
Pipes	Sigma
potassium oxalate	Sigma
Rhodamine	Sigma
Rhodamine-phalloidin	Sigma
Sodium tricitrate	Merck
Sphingosine	Calbiochem
Sphingosine-1-phosphate	Calbiochem
Streptavidin-TexasRed	Boehringer/Mannheim
Streptomycin	Gibco BRL

Sucrose	Merck
Tannic acid	Merck
Tris	Merck
Triton X100	Merck
Uranylacetate	Merck

Thymosin-β4 was a kind gift of Dr. W. Voelter. Acetone muscle powder was provided by Dr H. Faulstrich from the Max Planck Institute for Cell Biology (Heidelberg).

Preparation of rhodamine-actin

Rhodamine G-actin was self prepared by extraction of actin from acetone powder of rabbit muscle. The acetone powder was a kind gift of Dr. H. Faulstrich from the Max Planck institute for Cell Biology (Heidelberg). For this, 3 g of acetone powder were incubated in 60 ml of G-buffer on a stirrer for 30 min on ice. Following not dissolved material was removed by gentle filtration through a G4 scintered glass filter. The residue was washed again with 40 ml of G-buffer and filtered as described above. Combined filtrates were centrifuged for 30 min at 40k rpm in a Ti60 rotor at 4°C using a Beckmann ultra centrifuge to completely remove non-dissolved material. Following, MgATP was added to a final concentration of 1.5 mM, then $MgCl_2$ was added to a concentration of 3 mM and finally KCl was added to a final concentration of 50 mM. The suspension was then incubated at 25°C for 2 hours and following incubated at 4°C over night. After getting viscous KCl was added to the suspension to a final concentration of 0.8 M and stirred at 25°C for 15 min to polymerise actin. The suspension was then splitted into high speed tubes for a Ti70 rotor and centrifuged at 50k rpm at 4°C for 90 min. The F-actin is visible as a white spot on the bottom of the tubes. Pellets were combined and washed 3 times with ice cold G-buffer until homogenised using a dounce homogeniser. The suspension was transferred to dialyse tubes and dialysed against 500 ml of G-buffer at 4°C on a stirrer. After 3 hours G-buffer is changed and further incubated for 2 hours. F-actin was pelleted by centrifugation at 45k rpm using a 50Ti rotor at 15°C for 45 min. Following $MgCl_2$ was added to a final concentration of 2 mM and KCl to 50 mM to the supernatant. After 25 min incubation at RT the suspension became viscous and the concentration of actin was determined by Bradford analysis. The concentration was between 3-4 mg/ml. In

order to label actin with rhodamine, F-actin was layered on top of F-buffer (+glycerol) and sedimented by centrifugation through this cushion using a TL 100.4 rotor at 80k rpm for 60 min at 4°C. Pellets were resuspended in F-buffer (-glycerol) and sedimented again through F-buffer (+glycerol). After the second centrifugation the suspension was diluted to 1 mg/ml (24 μM) actin in F-buffer (-glycerol) and gently sonicated to disperse filaments. Following, 4 moles NHS-rhodamine were added to 1 mol of actin and incubated for 2 h at 4°C. Unbound NHS-groups were saturated by adding 10 mM Tris pH 8.0 and the F-actin was pelleted by centrifugation through F-buffer (+glycerol) as described above. Pellets were dissolved in G-buffer and sonicated gently to depolymerise F-actin. Following the suspension was dialysed against G-buffer for 24h at 4°C before actin was stored in liquid nitrogen in 20μl aliquots.

B.1.2 Media

DMEM:	DMEM	1	l
	Glutamine	4	mg/l
	FCS	10	%
	Penicillin	100	U/ml
	Streptomycin	100	μg/ml

Watson Reid:	L-asparagine	5	g/l
	D-glucose	10	g/l
	diammoniumcitrate	2	g/l
	Fe-ammoniumcitrate	75	mg/l
	NaCl	2	g/l
	KH_2PO_4	2	g/l
	$MgSO_4$ x 7 H_2O	2	g/l
	$ZnSO_4$ x 7 H_2O	10	mg/l
	$CaCl_2$ x 2 H_2O	2	mg/l
	Glycerol	63	ml/l

after sterilisation the pH is adjusted to 5.6 at room temperature and the following chemicals are added after sterile filtration:

	Sodium pyruvate	4	g/l

and for *M. paratuberculosis*

	mycobactin J	2	mg/l

Middlebrook 7H10:	Middlebrock 7H10 powder	19	g/l
	Middlebrock OADC enritchment	100	ml/l

and for *M. paratuberculosis*

	mycobactin J	2	mg/l

B.1.3 Buffers

Anti fading buffer:	catalase	5	μl (10 mG/ml)
	glucose oxidase	5	μl (10 mG/ml)
	Glucose	5	μl (1M)
	β-mercaptoethanol	0.42	μl
Blocking buffer LM:	fish skin gelatine	2	%
	in PBS (pH 7.4)		
Blocking buffer EM:	NH_4CL	50	mM
	in PBS (pH 7.4)		
BRB80-buffer:	Pipes pH 6.8	80	mM
	$MgCl_2$	1	mM
	EGTA	1	mM
Coating buffer:	$NaHCO_3$	100	mM
	Na_2CO_3	40	mM
Contrast solution for immunoEM:			
	methylcellulose	2	%
	uranyl acetate	0.3	%
F-buffer (+glycerol):	Hepes	10	mM
	MgATP	1	mM
	MgCl2	1	mM
	KCl	100	mM
	Glycerol	25	%

F-buffer (-glycerol):	Hepes	10	mM	
	MgATP	1	mM	
	$MgCl_2$	1	mM	
	KCl	100	mM	
F-buffer-rhodamine-tris:				
	MgATP	1	mM	
	$MgCl_2$	1	mM	
	KCl	100	mM	
	Tris pH 8.0	10	mM	
	DTT	1.5	mM	
	Glycerol	25	%	
Fixation buffer for TEM:	paraformaldehyde	5	%	
	glutaraldehyde	3	%	
	cacodylate	0.1	M	pH 6.9
	sucrose	0.09	M	
	$MgCl_2$	0.01	M	
	CaCl2	0.01	M	
Fixation buffer for ImmunoEM:				
	paraformaldehyde in PBS pH 7.4	0.4	%	
G-buffer:	Tris pH 8.0	2.0	mM	
	MgATP	0.1	mM	
	DTT	0.1	mM	
	$CaCl_2$	0.1	mM	

HB-buffer:	sucrose	0.25	M
	imidazole	3	mM
	leupeptin	0.5	ng/ml
	aprotinin	4	ng/ml
	pepstatin A	1	ng/ml
	DTT	1	mM

Lipid extraction solution I:

methanol	1	part
chloroform	1	part

Lipid extraction solution II:

methanol	1	part
HCL 1M	1	part

Lipid extraction solution III:

chloroform	75	parts
methanol	25	parts
a. dest	2	parts

RB-buffer:	Hepes pH 7.0	20	mM
	KCl	50	mM
	MgCl2	4	mM
	CaCl	0.2	mM
	MgATP	0.2	mM
	FSG	0.3	%

Running solution TLC:

chloroform	80	parts
acetone	30	parts
methanol	26	parts
glacial acetic acid	24	parts
a. dest	14	parts

pH calibration buffer:	nigericin	10	mM
	$MgCl_2$	1	mM
	KCl	105	mM
	NaH_2PO_4/Na_2HPO_4		
PBS (pH 7.4):	$Na_2HPO_4 \times H_2O$	100	mM
	KH_2PO_4	3	mM
	NaH_2PO_4	20	mM
	K_2HPO_4	3	mM
RB-buffer:	Hepes	200	mM
	KCl	500	mM
	$MgCl_2$	40	mM
	$CaCl_2$	2	mM

B.1.4 Solutions

Aprotinin:	4 mg/ml in a.dest; stored in 10 µl aliquots at-20°C
Arachidonic acid:	100 mM in ethanol; stored in 20 µl aliquots at -20°C
MgATP:	100 mM in a.dest; stored in 10 µl aliquots at -80°C
BacLight viability kit:	stored as 10 µl aliquots at -20°C
Catalase:	10 mg/ml in a. dest; stored in 10 µl aliquots at -20°C
CDP-choline:	250 mg/ml in a. dest; stored in 50 µl aliquots at -20°C
Ceramide:	1mg/ml in a.dest; stored in 20 aliquots at -20°C
DTT:	1M in a. dest; stored in 100 µl aliquots at -20°C
Fish skin gelatin:	0.3% in a. dest; stored in 100 µl aliquots at -20°C
FITC-dextran 70 kDa:	250mg/ml in a.dest; stored in 100 µl aliquots at -20°C
Glucose oxidase:	10 mg/ml in a. dest; stored in 10 µl aliquots at -20°C
Leupeptin:	0.5 mg/ml in a. dest; stored in 20 µl aliquots at -20°C
Methylcellulose:	2% in a.dest; stored in 10 ml aliquots at -20°C
NHS-fluorescein:	5 mg/ml in DMSO; stored in 20 µl aliquots at -20°C
NHS-Biotin:	1 mg/ml in DMSO; stored in 20 µl aliquots at -20°C
Nigericin:	100 mg/ml in ethanol; stored in 20 µl aliquots at -20°C
Paraformaldehyde:	16% in PBS; stored in 10 ml aliquots at -20°C

NHS-Rhodamine:	5 mg/ml in DMSO; stored in 50 µl aliquots at -20°C
Rhodamine-phalloidin:	0.1 mg/ml in a. dest; stored in 20 µl aliquots at -20°C
Sphingosine:	5 mM in ethanol; stored in 10 µl aliquots at -20°C
Sphingosine-1-phosphate:	5 mM in methanol; stored in 10 µl aliquots at -20°C
Streptavidin-TexasRed:	1 mg/ml in DMSO; stored in 20 µl aliquots at -20°C
Sucrose:	2.3 M in a.dest; stored in 10 ml aliquots at -20°C
Uranylacetate:	3% in a.dest; stored in 10 ml aliquots at -20°C

B.1.5 Antibodies

anti Lamp1:	1D4B	Hybridoma bank, University of Iowa
anti Lamp2:	ABL93	Hybridoma bank, University of Iowa
goat anti rat-FITC		Dianova [1)]
goat anti rat-TRITC		Dianova [1)]
goat anti rabbit-CY3		Dianova [1)]
rabbit anti *M. bovis*		Sigma

[1)] working solution as recommended by the manufactorer

B.2 Cells and cell culture

To investigate the intracellular survival of different mycobacteria, a cell culture infection model was established, consisting of the very well described mouse macrophage cell line J774A.1 and different strains of pathogenic or non-pathogenic (i. e. saprophytic) mycobacteria.

B.2.1 Macrophage cell culture

The mouse macrophage cell line J774A.1 (Ralph et al., 1975) was maintained in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin (referred to as complete medium) at 37°C and 8% CO_2. Passages were done every three days. For this cells were washed twice in 5 ml PBS and harvested by scraping in 10 ml complete medium per plate. Cell suspensions were diluted 1:4 with complete medium and seeded in new petri dishes for further maintenance. To avoid changes in the characteristics of the cell line, J774 cells were only used for up to 20 passages.

For infection experiments, the cells were maintained in antibiotic-free complete medium for 48 h and grown to confluency in petri dishes or seeded on glass coverslips and grown to semiconfluency.

B.2.2 Bacterial strains and growth conditions

The field isolate *M. paratuberculosis* 6783 has been described previously (Jark *et al.*, 1997). *M. smegmatis* mc^2155 (ATCC 19420) was kindly provided by G. Auling (Institut fuer Microbiologie, University of Hannover). *M. gordonae* (ATCC 14470) and *M. avium* (ATCC 25291) were obtained from the DSMZ (German Collection of Microorganisms and Cell Cultures; Braunschweig). All mycobacteria were grown on Watson Reid liquid medium (Jark *et al.*, 1997) at 37°C. For cultivation of *M. ptb,* Watson Reid medium was supplemented with Mycobactin J (1 mg/l). Growth of mycobacteria occurs mainly on the surface of the liquid, but not on the bottom of the flask. For protecting mycobacteria from sinking to the bottom of the flask during inoculation they were first incubated in a few drops of sterile pentane and then carefully layered on top of the medium and incubated for 2-3 months. For infection of macrophages and fluorescence labelling, mycobacteria were harvested by centrifugation for 10 min at 1000 x g and were resuspended in 5 ml of antibiotic-free complete medium. Homogenisation of the mycobacteria was done as follows. Bacterial suspensions were vortexed in the presence of glass beads (3 mm of diameter) for 5 min and incubated for another 2 min in a sonic waterbath (GFL) at high intensity, following they were centrifuged for 5 min at 50 x g. Microscopy confirmed that the resulting supernatant contained mainly single bacteria. Viability of bacteria was examined using the *Bac*Light system (see below). Bacterial suspensions from *M. ptb* contained between 70% to 80% live bacteria, those from *M. avium, M. gordonae,* and *M. smegmatis* approximately 95%. Heat-inactivation of bacteria was achieved by incubating bacterial suspensions with an optical density at 660 nm (OD_{660}) of 1.0 for 15 min at 85°C (Zurbrick and Czubrynski, 1987). Loss of viability was confirmed by serial platings (see below).

B.2.3 Determination of bacterial viability by fluorescence

Mycobacterial cultures were checked for viability before use. For this 10 µl of bacterial cultures were mixed with 10 µl of BacLight suspension and incubated for 5 min at RT. Bacteria were washed two times with PBS and resuspended in 100 µl of

PBS. Suspensions were examined microscopically using a Leica DML microscope equipped with an N2.1 filtersystem. Red bacteria were regarded as dead and green as live. At least 50 bacteria were counted.

B.2.4 Fluorescence labelling of mycobacteria

Mycobacteria were harvested as described above and diluted with ice cold PBS to an OD_{660} of 1.0. To 1 ml of the cell suspension 100 µl sulpho-NHS-biotin (5 mg/ml DMSO) were added and incubated on a shaker for 20 min at 4°C. Then, the bacteria were washed three times with 1 ml of cold antibiotic-free complete medium and centrifuged for 5 min at 13,000 rpm using a microcentrifuge. To determine labelling efficiency labelled and non-labelled bacteria were treated with 2% paraformaldehyde for 15 min at room temperature, washed three times with PBS, and incubated in PBS containing 5% FCS for 20 min to block non-specific binding sites. After three washing steps with PBS bacteria were incubated with 100 µl of PBS containing 1 µg/ml TexasRed-streptavidin for 20 min. After additional three washes with PBS the bacterial pellet was resuspended in 1 ml PBS and 10 µl of the mycobacterial suspension was mounted on a glass slide by heat fixation. Samples were examined microscopically using a Leica DML microscope equipped with an N2.1 filtersystem (Leica, Bensheim, Germany). Approximately 200 bacteria were counted. The labelling efficiency was between 90 to 95%.

B.2.5 BSA-coating of latex beads

Latex beads (1 µm in diameter, SIGMA) were diluted to an OD_{660} of 1.0 with PBS. One ml of the latex bead suspension was pelleted by centrifugation and washed 3 times in coating buffer (pH 9.6). Latex beads were then coated with BSA by shaking incubation for 18 hours at 4°C in coating buffer supplemented with 1 mg/ml BSA. Coated latex beads were washed twice with coating buffer and PBS containing 0.5 % FCS, and stored in PBS at 4°C until further use. The binding efficiency was determined by immunofluorescence microscopy using a rabbit anti-BSA antiserum and a FITC-labelled goat anti-rabbit antiserum. The labelling efficiency was between 94% and 98%.

B.2.6 Determination of phagosomal pH-values by FACS-analysis

J774 cells were seeded on petri dishes as described above. After 48 h of culture, antibiotic-free complete medium was replaced by antibiotic-free complete medium containing 1 mg/ml FITC-dextran. Cells were allowed to pinocytose FITC-dextran for 30 min at 37°C and 8% CO_2 after which monolayers were washed twice with warm PBS to remove non pinocytosed dextran. Then, antibiotic-free complete medium containing *M. ptb*, *M. avium*, *M. gordonae*, *M. smegmatis* or latex beads was added. After incubation for 1 hour at 37°C and 8% CO_2 cells were washed three times with ice cold PBS to remove extracellular particles and harvested by scrapings and suspended in 1 ml PBS on ice. The suspensions were dispensed in aliquots and centrifuged for 5 min at 500 x g and 4°C. Five aliquots were used to generate a pH-calibration curve as following. Cell pellets were resuspended in 500 µl of pH-calibration buffers containing 10 µM nigericin, 1 mM $MgCl_2$, 105 mM KCl and different ratios of KH_2PO_4 and $NaHPO_4$ to obtain pH-values of 5.0, 5.5, 6.0, 6.5, and 7.0 (Liang and Hughes, 1998). The tester aliquot, which did not have an experimentally altered pH, was resuspended in 500 µl of ice cold PBS. The probes were kept on ice and measured immediately by FACS-analysis using a FACScan equipment (Becton Dickinson, Heidelberg, Germany). A total of 10,000 events were determined. The average fluorescence intensities obtained from macrophages treated with calibration buffers were used to generate a calibration curve. The fluorescence emission obtained from the cells treated with calibration buffer of pH-values of 5.0, 5.5, 6.0, 6.5 were calculated as percentages of the fluorescence emission from cells treated with the calibration buffer of pH 7.0 which was set to 100%. The pH-value of the tester cell population was calculated by referring their fluorescence emission to the calibration curve.

For FACS-analysis of J774 cells infected for long-term periods (5 h, 24 h, 48 h, and 72 h) J774 cells were allowed to internalise mycobacteria or BSA-coated latex beads for 1 h. Following, monolayers were washed three times with warm PBS to remove extracellular particles, and complete medium containing 50 µg/ml gentamicin was added to kill remaining extracellular bacteria. After incubation for 4 h at 37°C and 8% CO2, cells were washed with antibiotic-free complete medium and fresh antibiotic-free complete medium was added. Medium was removed at 20 h, 44 h, and 68 h after infection and replaced by antibiotic-free complete medium containing 1 mg/ml FITC-dextran. After additional 4 hours cells were washed three times with ice cold

PBS, scraped in 1 ml of PBS, and the fluorescence emission was monitored as described above.

B.2.7 Viability assessment of intracellular mycobacteria

To determine mycobacterial viability during infections, J774 macrophages were infected as described above. Monolayers were washed two times with PBS and scraped off the plates in 1 ml of 1% Non-idet P40 in PBS. J774 cells were disrupted by 10 passages through a 24 gauge needle. Tenfold serial dilutions of the homogenates were prepared with PBS and 100 µl each plated on Middlebrook 7H10 agar plates with 10% Middlebrook OADC enrichment (Difco), for *M. ptb* the medium was supplemented with 2 mg/l of mycobactine J. After incubation for to 2-3 months (*M. ptb*) at 37°C colony forming units (CFU) were counted.

B.3 Microscopy

B.3.1 Confocal laser scanning microscopy

For confocal laser scanning microscopy J774 cells grown on glass coverslips were washed two times with PBS and then incubated with antibiotic-free complete medium containing 1 mg/ml FITC-dextran for 30 min at 37°C and 8% CO_2. The cells were washed three times with warm PBS and incubated with antibiotic-free complete medium containing sulpho-NHS-biotin labelled mycobacteria of an OD_{660} of 0.1 at 37°C and 8% CO_2. After one hour, the coverslips were washed three times with ice cold PBS and fixed with 2% paraformaldehyde for 15 min at 4°C. Following, cells were treated with 0.1% Triton X100 for 5 min. Cells were washed two times with DMEM and incubated in PBS containing 5% FCS for 20 min at room temperature. Then, PBS was replaced by 1 µg/ml TexasRed-streptavidin in PBS. After additional 20 min coverslips were washed with PBS and then mounted ”up side down” on slides using 10 µl of Mowiol® containing 10 mg/ml freshly prepared DABCO®. The slides were then sealed with nail polish and stored at 4°C until further examination. Fluorescence images were collected using a Leica DM-IRBE inverted confocal laser scanning microscope equipped with an immersion oil Plan-Apo 100x/1.4 n.a. lens and an argon/krypton laser (Leica). Settings allowed simultaneous co-localisation of FITC-dextran-labelled endosomes and TexasRed labelled mycobacteria. Computer acquisition and analysis of pictures was done using the Leica confocal laser scanning

standard software and Adobe Photoshop (Adobe Systems Incorporated, Amsterdam, Holland). The TexasRed fluorescence of the mycobacteria was analysed for co-localisation with the FITC-dextran fluorescence by overlaying the green and red pictures resulting in a yellow signal. Red fluorescent signals and yellow fluorescent signals which could be identified as single bacteria in terms of morphology were counted to a total of 200 events.

Analysis of phagosome lysosome fusion were performed as described by Oh and Straubinger (1996). Cells were washed two times with PBS and incubated in the presence of 200 µM calcein in antibiotic-free complete medium for 24 hours. Following, cells were washed two times in PBS and incubated in antibiotic-free complete medium containing sulpho-NHS-labelled mycobacteria as described above. After 1 h and 5 h coverslips were treated and analysed as described above. For the assessment of Lamp1 and Lamp2 in MCP J774 cells were infected as described above and analysed for co-localisation of mycobacteria phagosomes with late endosomal and lysosomal markers using monoclonal antibodies from rat against murine Lamp1 (clone 1D4B) and Lamp2 (clone ABL-93) at a dilution of 1:50 in PBS following fixation with 2% paraformaldehyde and permeabilisation with 0.1% Triton X100. Secondary FITC-conjugated goat anti-rat IgG antibody was used at a dilution of 1:300 in PBS. Samples were analysed with a Leica confocal scanning microscope (model SP2) with a x100 oil immersion objective and the appropriate filter set. Cells and phagosomes were clearly identifiable under DIC optics. Images were digitally acquired and processed using Adobe Photoshop™. Phagosomes from at least 20 cells per time point were counted.

B.3.2 Intracellular detection of mycobacteria by electron microscopy

For transmission electron microscopy (in cooperation with Manfred Rhode, GBF Braunschweig), infected cells were fixed for 1 hour on ice with a solution containing 3% (w/v) glutaraldehyde and 5 % (w/v) formaldehyde in cacodylate buffer. After several washings with cacodylate buffer, cells were fixed with 1% (w/v) osmium tetroxide in cacodylate buffer for 1 h at room temperature, washed again with cacodylate buffer, and scraped off the culture flask. Cells were centrifuged for 3 min at 2000 x g and embedded in 1.5% agar. Small cubes of the agar were dehydrated with a graded series of acetone and embedded in Spurr resin, and ultrathin sections

were cut with glass knives. After counterstaining with 0.5 % uranylacetat (pH 4.5) for 30 min at 40°C and with 1% lead citrate for 30 min at 20°C (Ultrostainer; Leica, Bensheim, Germany), sections were examined at calibrated magnifications with a Zeiss transmission electron microscope (EM910) at an acceleration voltage of 80 kV.

B.3.3 Quantification of Lamp 1 and 2 by immunoelectron microscopy

Cells were grown in 10 cm Petri dishes and infected as described above. After 5 h of incubation, to each dish containing 10 ml of DMEM, an equal volume of fixative consisting of 0.4% paraformaldehyde in 0.4 M potassium phosphate, pH 7.4, was added. After 10 min, the mixture was replaced by fresh fixative, and the cells were incubated for an additional 3 h at room temperature. Then, cells were washed with PBS, and the excess aldehyde was blocked by incubation for 10 min with 50 mM NH_4Cl in PBS. After additional washing with PBS, cells were scraped, pelleted, and the pellet was embedded in 2% low melting point agarose in PBS. After the agarose was hardened by chilling, small blocks were cut at -100°C with a Leica FCS cryomicrotome using a Drukker diamond knife. Sections were collected with 2.3 M sucrose–PBS and transferred to 100-mesh hexagonal grids coated with 1% Formvar and carbon, freshly glow discharged. Sections were thawed and blocked for 15 min with 0.5% fish skin gelatin, 20 mM glycine in PBS. Thawed sections were then labelled using the undiluted monoclonal antibodies to Lamp 1 and Lamp 2 and protein A–colloidal gold. Labelled grids were embedded in aqueous 2% methylcellulose, 0.3% uranyl acetate (Serva) to add contrast and prevent shrinkage upon drying. Grids were observed with a Zeiss EM-10 microscope at 80 kV. Pictures of at least 20 cells were analysed for numbers of gold particles bound per length of the phagosomal membrane as described by Griffiths (1993).

B.3.4 Labelling of macrophages with BSA-gold

To load lysosomes of J774 macrophages with either BSA-gold particles, cells were grown to confluence and incubated with 5 or 15 nm BSA-gold particles of an OD_{520}:5 for 1h, followed by a chase for 5h in DMEM without gold. BSA-gold particles of different sizes were prepared using tannic acid in sodium citrate to reduce gold chloride as described (Slot and Geuze, 1985)

B.4 Subcellular fractionation methods

B.4.1 Isolation of Mycobacteria containing phagosomes (MCP)

For isolation of MCP, 20x20cm petri dishes with a confluent layer of J774 cells were infected with 50 ml DMEM containing either *M. avium*, *M. paratuberculosis*, or *M. smegmatis* (live or heat killed) either non-labelled or labelled with NHS-fluorescein at a concentration of OD_{660} of 0.1 for 1 hour at 5 % CO_2 at 37°C. Non-internalised bacteria were removed by intensive washings with PBS and further incubated for another 1 or 24 hours. Following medium was removed and cells were washed again with PBS. Cells were harvested by scraping in 20 ml PBS and collected in 50 ml Falcon tubes. Cells were centrifuged at 1000 x g for 10 min, resuspended in 5 ml of HB-buffer and pelleted again. Pellets were resuspended in a final volume of 1.5 ml of HB buffer and lysed using a 1ml syringe fitted to a 22-gauge needle. Nuclei and intact cells were removed by gentle centrifugation at 800 x g at 4°C and supernatants were layered on top of a discontinuous sucrose gradient consisting of 2 ml 50% sucrose, 4 ml 37% sucrose and 4 ml 25 % sucrose and centrifuged at 24k rpm using a SW40 rotor for 60 min at 4°C. The band forming between the 50% and 37% sucrose contains the mycobacteria-containing phagosomes and can be collected in approximately 200 µl with a syringe by penetrating the tube wall with a 22 gauge needle. The phagosome suspension is diluted with HB to a final volume of 2 ml and layered on top of 1 ml 50% sucrose and centrifuged again in a SW55 rotor at 36k rpm for 30 min at 4°C. Mycobacteria-containing phagosomes are seen as a band above the 50 % sucrose and are collected as described above. Phagosomes are aliquoted in 20 µl portions and stored at -20 until further use.

In order to check the proportion of intact phagosomes, phagosomes containing dead *M. avium* were incubated with rabbit anti *M. avium* antiserum and detected with a goat anti rabbit-CY3 antibody. The ratio between phagosomes stained in red (not intact) and non-labelled phagosomes (intact) was determined and only preparations with above 65% intact phagosomes were used for further experiments.

B.4.2 Actin nucleation assay by fluorescence microscopy

This assay was described in detail by Defacque et al (2000b). Briefly, glass slides were coated with 0.5 % fish skin gelatine in water and air-dried prior to the experiment. A constant number of phagosomes (Blocker et al., 1997) was incubated

between a slide and a coverslip in RB buffer with 2 µM rhodamine G-actin, 6 µM thymosin β4 and an antifade reagent (Blocker et al., 1997) for 15 min at room temperature. The percentage of positive phagosomes was determined using a Zeiss Axioscope microscope. In all experiments described the errors reported are the standard deviations from at least three separate counts. For lipid treatments the stock lipids were dissolved as follows: Arachidonic acid (AA) and ceramide (Cer) in water (1 mg/ml), sphingosine (Sph) in ethanol (5 mM), spingosine-1-phosphate (S1P) in warm methanol (5 mM) and phosphatidyl-inositol-di-phosphate in ethanol (5 mM). For performing either the actin nucleation assay or survival experiments the phagosomes were mixed with the respective lipid at the following final concentrations: 250 µM AA, 100 µM Sph and S1P, 1 mM Cer and 150 µM $Pi(4,5)P_2$.

C. Results

C.1 Cellular level

C.1.1 The Infection Model

To study intracellular survival of mycobacteria the first step was to establish an infection model. For this the mouse macrophage cell line J774, which is widely used to study intracellular pathogens, including mycobacteria (Via et al., 1998; Schwab et al., 1994; Gahan et al., 2001), was chosen. Basically, the infection procedure described previously (Kuehnel, 1998) was used with some modifications (see below). To find an optimal MOI the concentration of mycobacteria was determined by optical density at 660 nm and the number of internalised bacteria in J774 macrophages was determined by microscopy of either Ziehl Neelsen- or fluorescence-stained infected macrophages. Using 5 ml of an OD_{660} of 0.1 per 10 cm petridish approximately 20-30 *M. ptb* were taken up per cell in 1 hour of time and over 90 % of macrophages were infected (Fig. 1). This concentration was used for further experiments.

Since there are no detailed information about *M. ptb*-macrophage interactions, the intracellular fate of *M. ptb* in J774 macrophages was first followed. To monitor survival of *M. ptb* within J774 cells serial platings with lysates from J774 cells that had been infected for 1 h, 24 h, 48 h, 72 h, and 96 h with *M. ptb,* strain 6783 were performed, and compared to J774 cells infected with *M. avium*, *M. smegmatis* and *M. gordonae*. The latter two are considered non pathogenic species and are not yet recognised as intracellular pathogens. In these experiments, non ingested mycobacteria were removed by several washing steps after infection of the cells for 1 h, and then the cells were cultured in medium containing 50 µg/ml gentamicin at 37ºC for 4 h. This was necessary to inhibit the growth of the remaining extracellular, fast growing *M. smegmatis*.

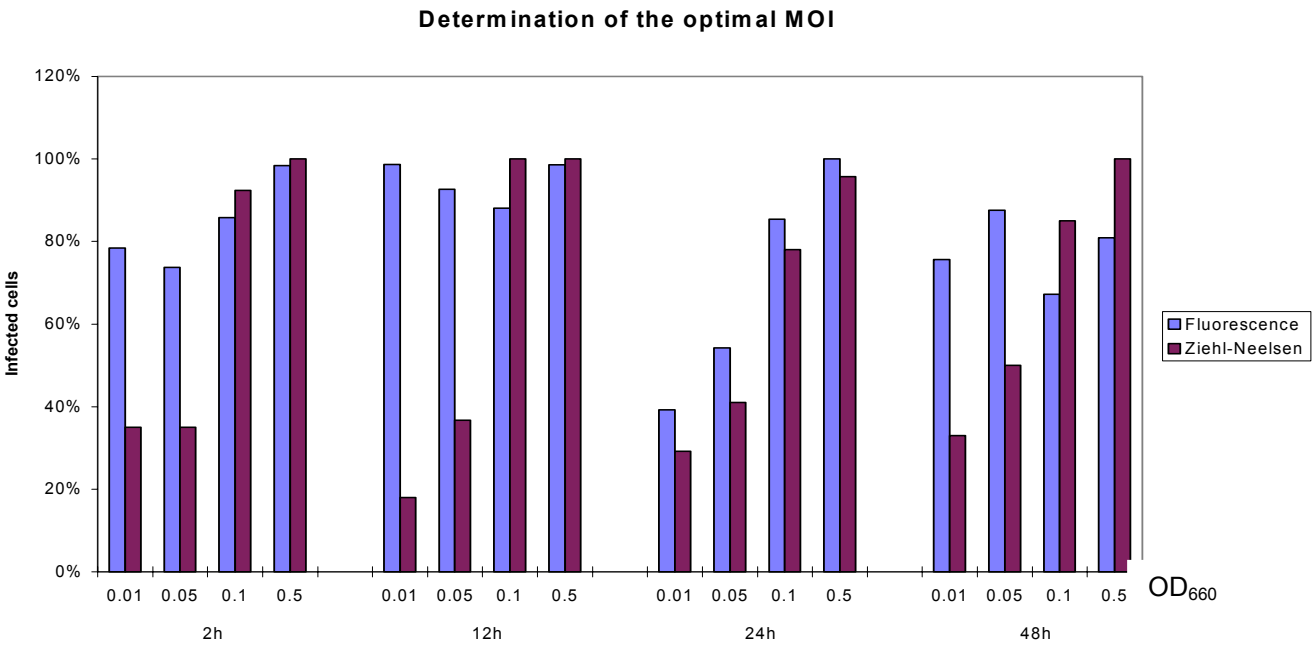


Figure 1: Determination of the optimal MOI.

Cells were infected with different concentrations of *Mycobacterium paratuberculosis* for two hours and after indicated time points, visualised by either using an anti *M. bovis* antibody or Ziehl-Neelsen staining. Using an OD_{660} of 0.1 for 2 hours as a standard infection resulted in an average infection of 80-90% of cells containing a well countable number of internalised bacteria of approximately 30 per cell, as viewed by Ziehl-Neelsen staining.

In preliminary experiments it was confirmed that at this concentration gentamicin did not influence intracellular viability of all tested mycobacteria (Fig. 2 and 3). As expected, *M. ptb* and *M. avium* were able to survive in J774 cells over the whole infection period (Fig. 2), whereas the non-pathogenic mycobacteria resisted to intracellular killing only for 24 h (Fig. 3).

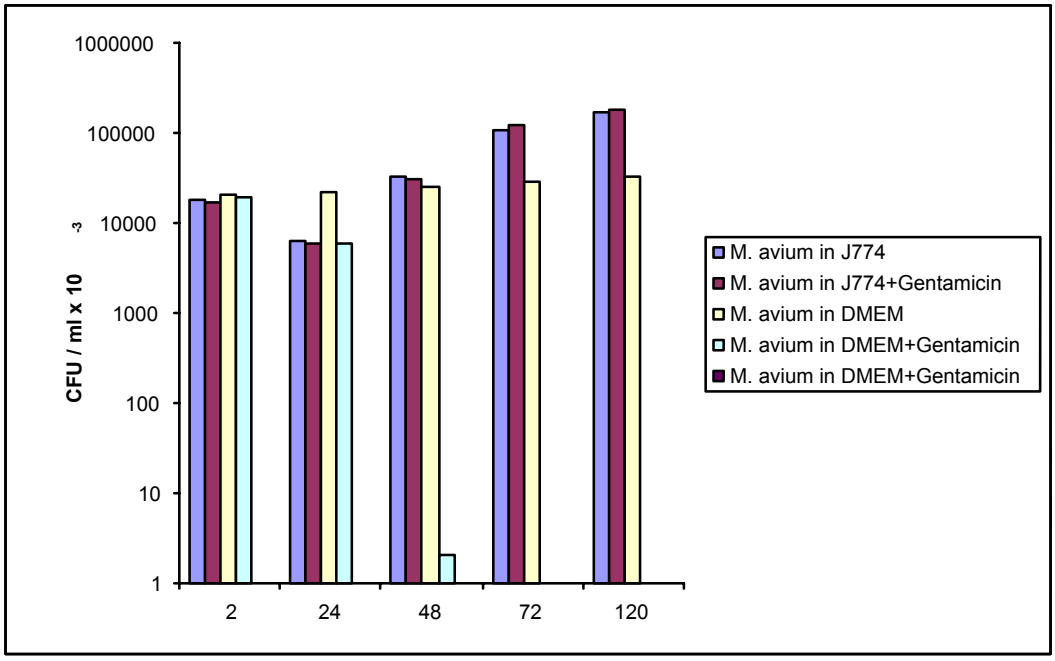


Figure 2: Effect of gentamicin on viability of *M. avium*.

J774 cells were either incubated with *M. avium* in the presence or absence of 50 μg/ml gentamicin or incubated in medium with or without gentamicin. The table shows one representative result from three individual experiments. *M. avium* incubated in medium containing the antibiotic at this concentration is killed within 48 hours, whereas bacteria in J774 cells are not affected by gentamicin.

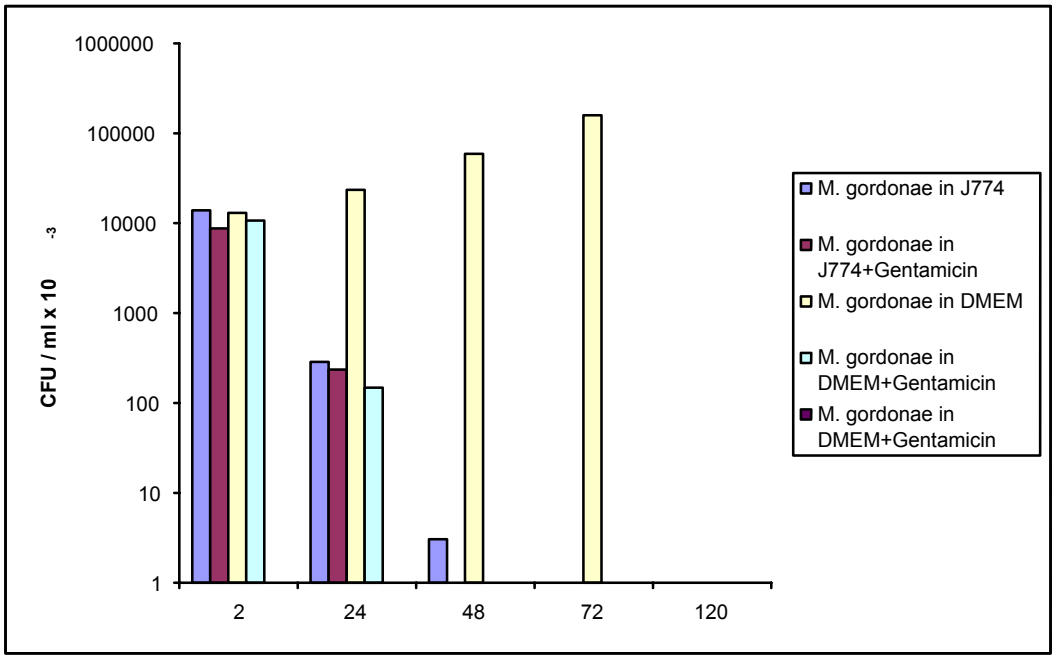


Figure 3: Effect of gentamicin on viability of *M. gordonae*.

J774 cells were either incubated with *M. gordonae* in the presence or absence of 50 μg/ml gentamicin or incubated in medium with or without gentamicin. The table shows one representative result from three individual experiments. Internalised *M. gordonae* are not affected by 50 μg/ml gentamicin, as they are killed as fast as the bacteria without antibiotic.

Then transmission electron microscopy (TEM) was performed using J774 cells that were infected with *M. ptb* and then harvested and examined 2 h, 24 h, 72 h, and 10 days after infection (representatively shown in Fig. 4).

At all examined times post infection, bacteria were mostly found as single organisms surrounded by an electron transparent zone in vacuoles with clearly defined membranes. Occasionally, the bacteria were found in large vacuoles with multiple organisms. At each time point, individual bacteria frequently were localised also in vacuoles where the vacuolar membrane was tightly apposed to the bacterial cell surface. It is mostly accepted that mycobacteria reside in large vacuoles containing several bacteria early in the infection and small vacuoles with single microorganisms after 2 to 3 days of infection. In contrast, sometimes also small vacuoles with single microorganisms were found early after infection (Fig. 4, A) and large vacuoles with several bacteria even after 10 days of infection (Fig. 4, E). Surprisingly, no significant apparent morphological differences were seen in macrophages infected with live versus dead bacteria. In both, intact and, to a much lesser extent, degraded bacteria could be seen inside the macrophages at early and late time points. The picture remained unchanged for up to 10 days (Fig. 4).

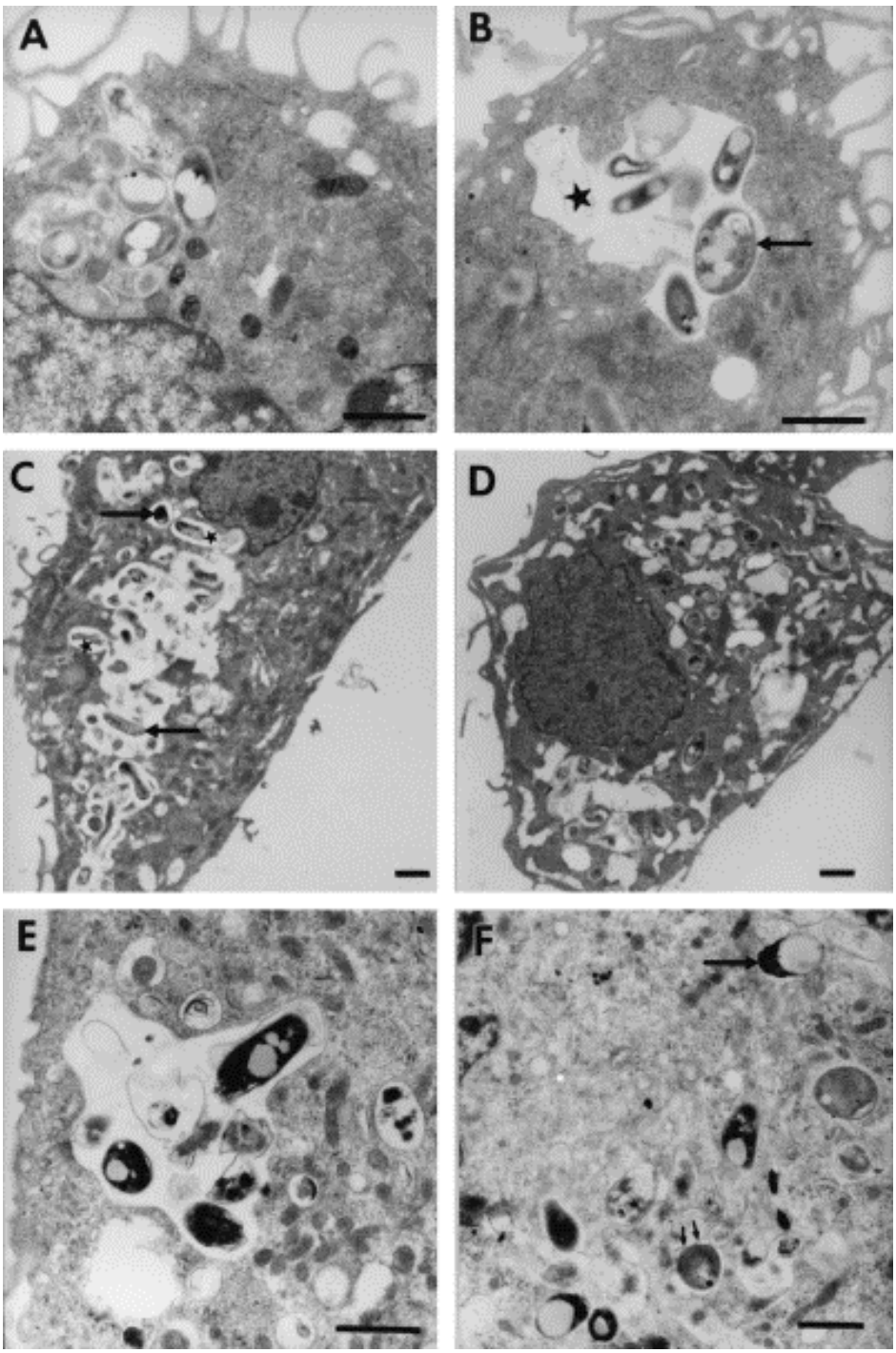


Figure 4: Intracellular localization of *M. ptb* in J774 macrophages.

J774 cells were infected with *M. ptb* strain 6783, and TEM pictures were taken after 2 h (A and B), 24 h (C), 72 h (D) and 10 days (E and F). Degraded material is indicated by big stars, bacteria are indicated by long arrows. The small arrows indicate bacteria tightly apposed to the vacuolar membrane, and small stars indicate electron-transparent zones. Bar = 2 µm.

C.1.2 Analysis of fusion of Mycobacteria containing phagosomes (MCP) with endocytic organelles

Fusion events of phagosomes containing mycobacteria have been extensively characterised by electron and light microscopy, as well as by biochemical approaches. The general conclusion from these studies was that the phagosome of live pathogenic mycobacteria fuse well with early endosomes, but fuse either poorly or not at all with late endosomes and lysosomes. One argument available in favor of this notion was that the pH of phagosomes of *M. avium* and *M. tuberculosis* was precisely that of the early endosomes (~6.3) (Clemens, 1996). As pointed out in the discussion, a survey of the literature reveals that, for the mycobacteria, the question of whether a specific phagosome acquires markers of the endocytic pathway depends greatly on the mycobacterial species, on the type of marker analysed, as well as on the conditions of the cells (see Clemens, 1996). For this reason, a number of different approaches was used to evaluate the fusion of phagosomes containing the pathogenic *M. ptb*, as well as *M. avium* and the non-pathogenic *M. smegmatis* and *M. gordonae*.

C.1.3 Accessibility of MCP to FITC-dextran

In the first assay the fluid phase marker 70 kDa FITC-dextran was used with two different labeling protocols. First, heat-killed, or viable *M. ptb*, *M. avium* or *M. gordonae* were allowed to bind and enter J774 cells over a 1 h period. At the end of this incubation an average of ~20 bacteria had been internalised per cell and in all experiments at least 98% of the cells were infected. Following, extensive washing steps to remove surface bound bacteria and further incubation of 15 min, FITC-dextran (1 mg/ml) was added to the medium and incubated for a further period of 4 h. The latter was done in the presence of gentamicin (50 µg/ml) in order to kill any remaining extracellular mycobacteria. After this time extensive colocalisation of FITC-dextran with internalised mycobacteria was observed by confocal microscopy for both live and killed *M. ptb*, *M. avium,* and *M. gordonae*. It was observed that cells that had phagocytosed only very few mycobacteria showed only poor colocalisation of FITC-dextran with mycobacteria, in contrast to cells that had internalised approximately 30-40 bacteria and more. Using an OD_{660} of 0.1 of mycobacteria turned out to have 90% of the bacterial phagosomes positive for FITC-dextran (Fig. 5A).

In a second experiment the FITC-dextran was added first to the cells for 30 min followed by a 15 min chase before adding the bacteria. After this protocol the bulk of the dextran would be expected to label later endocytic structures at steady state (at ~1h). After an additional incubation of 1 h, again, extensive fluorescence labeling of the phagosomes of all mycobacteria was seen by confocal microscopy (Fig. 5 B). Again the concentration of mycobacteria per macrophage was crucial for the colocalisation efficiency.

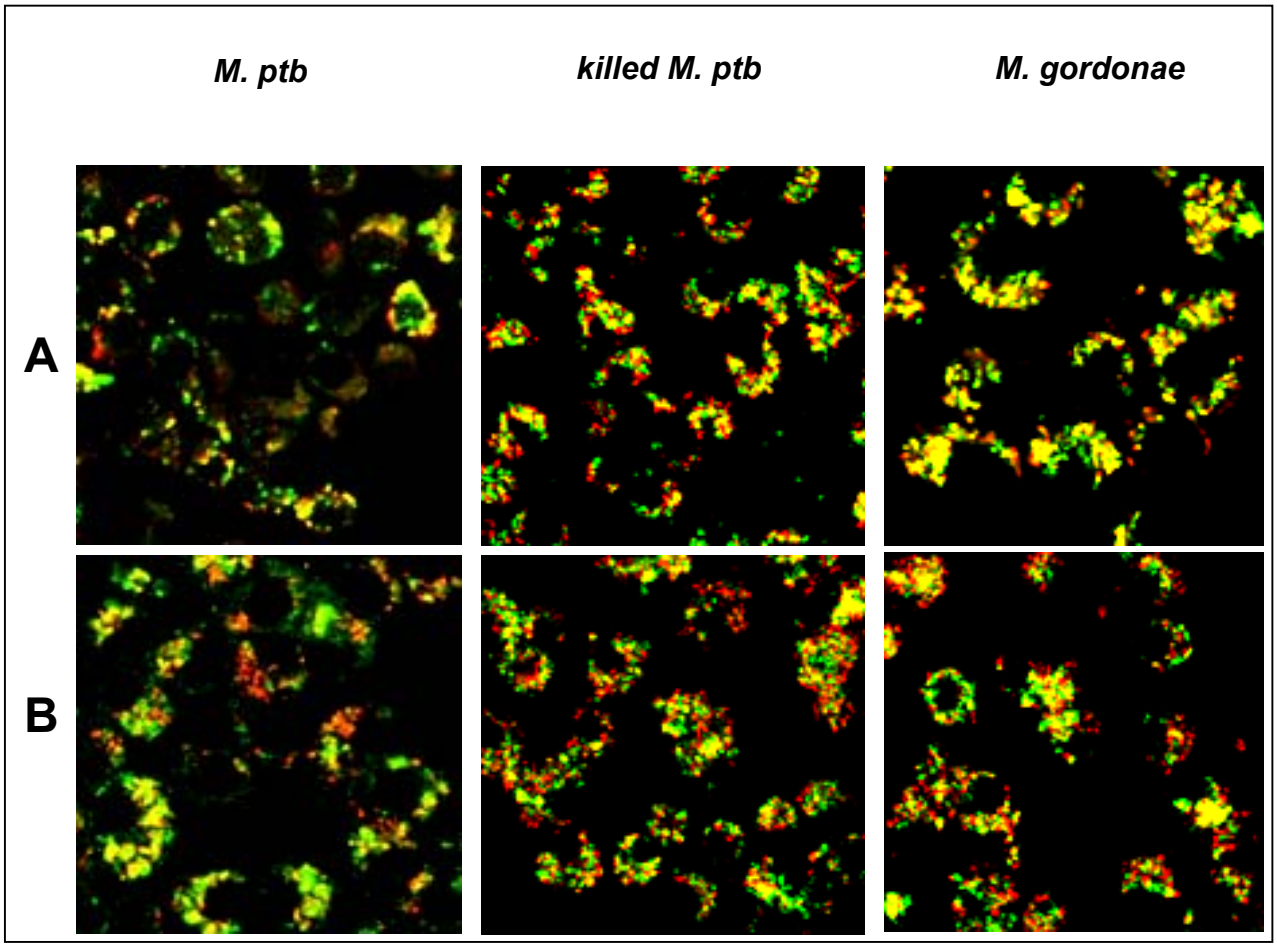


Figure 5: Mycobacteria-containing phagosomes are accessible to FITC–dextran.

A. J774 cells seeded on coverslips were infected with sulpho-NHS-biotin-labelled live or dead *M. ptb* or *M. gordonae* for 1 h. Then, monolayers were washed, and medium containing 50 µg ml^{-1} gentamicin and 1 mg ml^{-1} FITC–dextran was added. After incubation for 4 h, cells were washed, fixed, permeabilized, treated with Texas red–streptavidin and analysed by confocal scanning microscopy. Co-localization of *M. ptb* (red fluorescence) with FITC–dextran (green fluorescence) is indicated by yellow to orange fluorescence. B. J774 cells seeded on coverslips were incubated with 1 mg ml^{-1} FITC–dextran for 30 min. Then, cells were washed and incubated for 1 h with medium containing sulpho-NHS-biotin-labelled *M. ptb*, *M. avium* or *M. gordonae*. Coverslips were then treated as described in (A).

C.1.4 Acquisition of Lamp1 and 2 by MCP

For this approach we used immunofluorescence and confocal microscopy applying monoclonal anti-Lamp1 and anti-Lamp2 antibodies; both have been used widely as phagosomal markers (see Clemens, 1996). These two well-characterised marker proteins are mostly found in late endosomes and lysosomes in uninfected cells, although both follow a complex recycling route via early endosomes, trans-Golgi-network and the plasma membrane (see Griffiths, 1996). In most cells, including J774, the concentration of these proteins on the plasma membrane is relatively low. Accordingly, after phagosome formation the phagosomal membrane is low in Lamp1 and Lamp2 (Desjardins et al., 1994). In the case of latex bead phagosomes, as they mature they concentrate these membrane proteins until they are apparently fully equilibrated with late endocytic organelles (Desjardins et al., 1994).

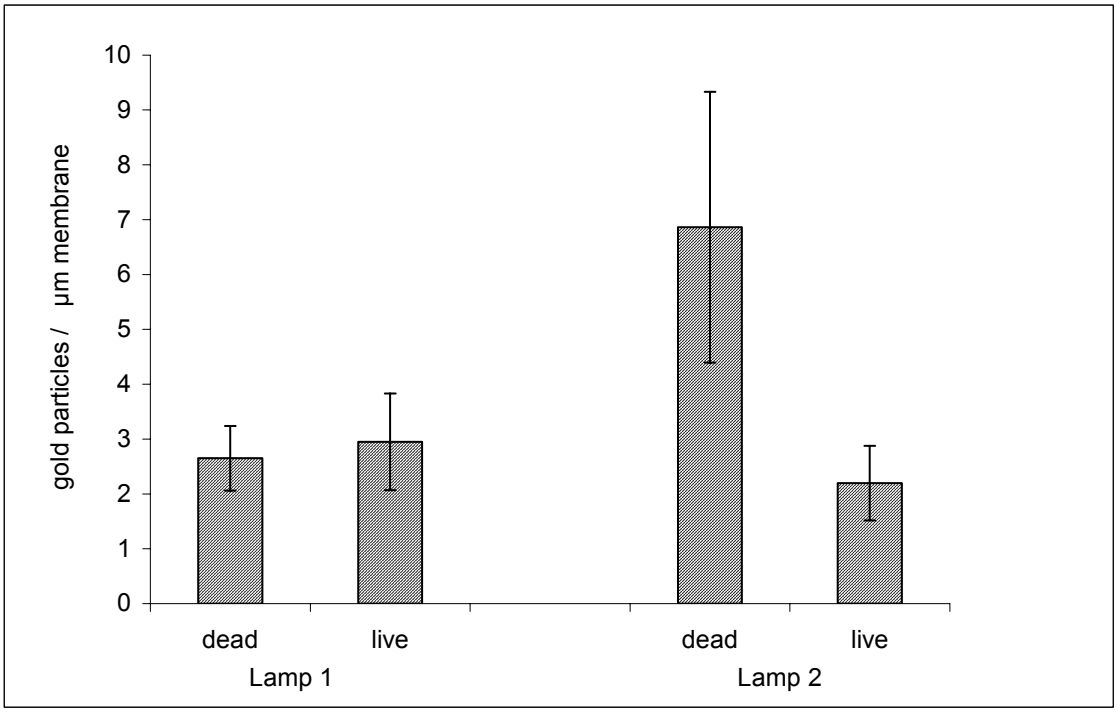


Figure 6: Density of LAMP 1 and 2 on phagosomal membranes in *M. avium*-infected macrophages.

J774 cells were infected and fixed 5 h after infection. Lamps were detected by immunoelectron microscopy as described in Experimental procedures and visualized using protein A–gold particles. Gold particles on phagosomal membranes in cells containing dead and live *M. avium* were counted, and the density per µm membrane was determined.

	1h		5h		24h	
	Lamp1 pos. (%)	Lamp2 pos. (%)	Lamp1 pos. (%)	Lamp2 pos. (%)	Lamp1 pos. (%)	Lamp2 pos. (%)
M. ptb	88 +/- 4	65 +/- 6	90 +/- 3	34 +/- 6	92 +/- 4	18 +/- 4
M. ptb dead	95 +/- 3	78 +/- 5	89 +/- 3	89 +/- 5	89 +/- 7	87 +/- 7
M. avium	93 +/- 6	52 +/- 6	99 +/- 1	22 +/- 7	95 +/- 4	24 +/- 5
M. avium dead	90 +/- 3	68 +/- 3	95 +/- 7	84 +/- 5	94 +/- 5	79 +/- 8
M. gordonae	96 +/- 4	64 +/- 7	92 +/- 4	75 +/- 8	90 +/- 5	67 +/-10

Table 1: Acquisition of Lamp1 and Lamp2 by MCP.

In this study a systematic investigation of the labeling of the phagosomes of different mycobacteria for both Lamp1 and 2 at different times after infection was carried out. As shown in Tab. 2, after 1 h of infection Lamp1 was evenly distributed in over 90% of both live and dead mycobacterial phagosomes at all time points. In contrast, in the case of live *M. ptb* and *M. avium* Lamp2 labeling co-localised with 50-65% of mycobacterial phagosomes at 1h of infection, but this parameter was considerably lower at 5 h and 24 h of infection, labelling only ~20 % of phagosomes after 24 h (Tab. 1). Although Lamp1 and 2 are generally indistinguishable in their trafficking routes our data argue that in mycobacteria-infected cells they appear to behave differently. The significance of the loss of Lamp2, but not Lamp1 at later times of infection requires further study.

In order to insure that the differences in Lamp1 and 2 labeling were correct the density of these markeres on membranes of phagosomes in infected cells was analysed by immunogold electron microscopy. For this cells infected with live and dead *M. avium* at 5 h post infection were selected and analysed with a cryo EM approach. The results are summarised in Fig. 6. Lamp1 is distributed at the same density on membranes of live and dead *M. avium* containing phagosomes, whereas Lamp2 is 2-5 fold more concentrated on membranes enclosing killed *M. avium* compared to membranes surrounding live *M. avium.*

C.1.4 Acquisition of gold particles by MCP

As a third approach to assess fusion we used colloidal gold particles visualised by EM. For this we internalised either 5 nm or a 15 nm BSA-gold probes, either together, or into two separate sets of cells. The rationale for these experiments are the findings

of Desjardins et al. (1997) that the size of the particle greatly influences its abilities to be transferred from endocytic organelles to phagosomes, with the small particles being more readily transferred. The BSA-gold was pulsed for 1 h and chased for 3 h before internalising the bacteria for 1 h followed by a 1 h or 5 h chase period. The cells were then fixed and processed for electron microscopy thin sections. A representative set of pictures is shown in Fig. 7. The percentage of bacterial phagosomal profiles containing at least one gold particle, indicative of fusion, was scored (Fig. 8). The results show that the assessment of fusion depended strongly on the type of mycobacteria used, on the size of the gold probe, and on the time in which the phagosomes have been allowed to mature. In agreement with Desjardins et al. (1997) the smaller gold probe was always more accessible to all the different types of phagosomes than was the larger gold, and, in general, the longer the intracellular life of the phagosome the more likely it was to get access to both sizes of gold.

The most accessible phagosomes to the 5 nm gold were those containing heat- killed *M. ptb*, with 85% of phagosomal profiles labelling for gold at both the 1 h and 5 h stages. It should be noted that in the absence of serial section reconstruction, we cannot rule out that all the phagosomes are accessible to gold in these experiments. In contrast, the 15 nm gold co-localised with only 12% of the phagosomes containing heat-killed *M. ptb* after 1 h but after 5 h over 50% of this phagosomes were accessible. The most striking observation in Fig. 8 is that viable *M. ptb* were quite effective in preventing access of both 5 nm and 15 nm gold particles, relative to dead pathogenic mycobacteria or *M. smegmatis*. In both cases the pool of accessible phagosomes rose only slightly with long incubation (15 nm gold) or actually fell, in the case of the 5 nm gold. This argues that these viable MCP can partially inhibit the transfer of these particles and, after a 1 h maturation, are quite effective in preventing further access of both gold markers.

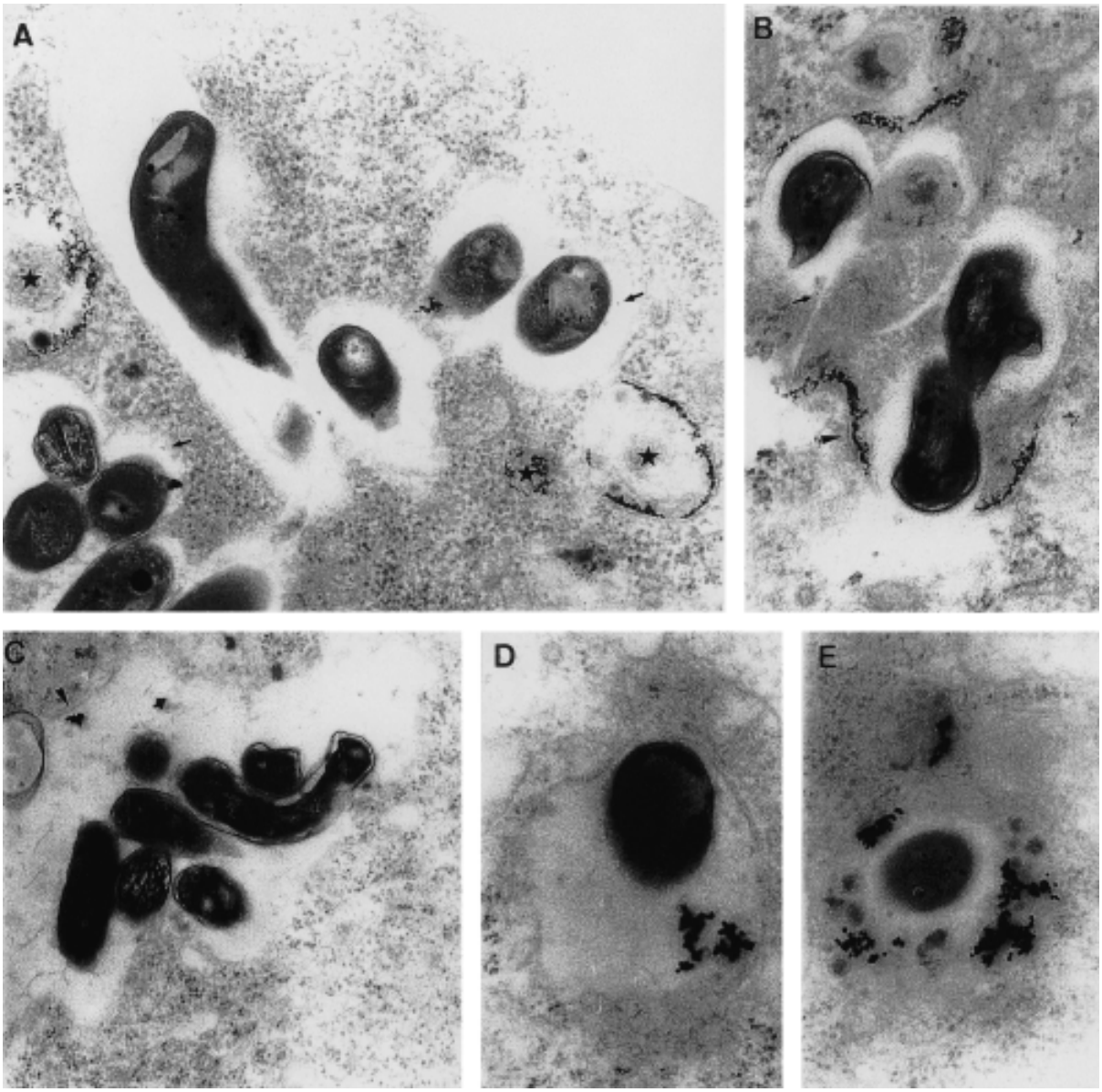


Figure 7: Co-localization of mycobacteria with BSA–gold.

Late endsomes/lysosomes (stars) of J774 cells were labelled in a 4 h chase with 5 nm and 15 nm BSA–gold and then infected with live (A) or dead (B–E) mycobacteria. Samples were fixed and prepared for electron microscopy at different time points (A and B, 5 h p.i.; C, 1 h p.i.; D, 5 h p.i.; E, 24 h p.i.). Intraphagosomal localization of gold particles is demonstrated by representative sections in which the phagosomal membranes were clearly visible (arrowheads). A. Phagosomes of live *M. ptb* are accessible to small numbers of 5 nm gold particles (small arrows, single gold; stars, gold-labelled late endosomes/lysosomes). B. Dead *M. ptb* acquire high numbers of 5 nm gold particles. C–E. Dead *M. ptb*-containing phagosomes were accessible to 15 nm gold particles.

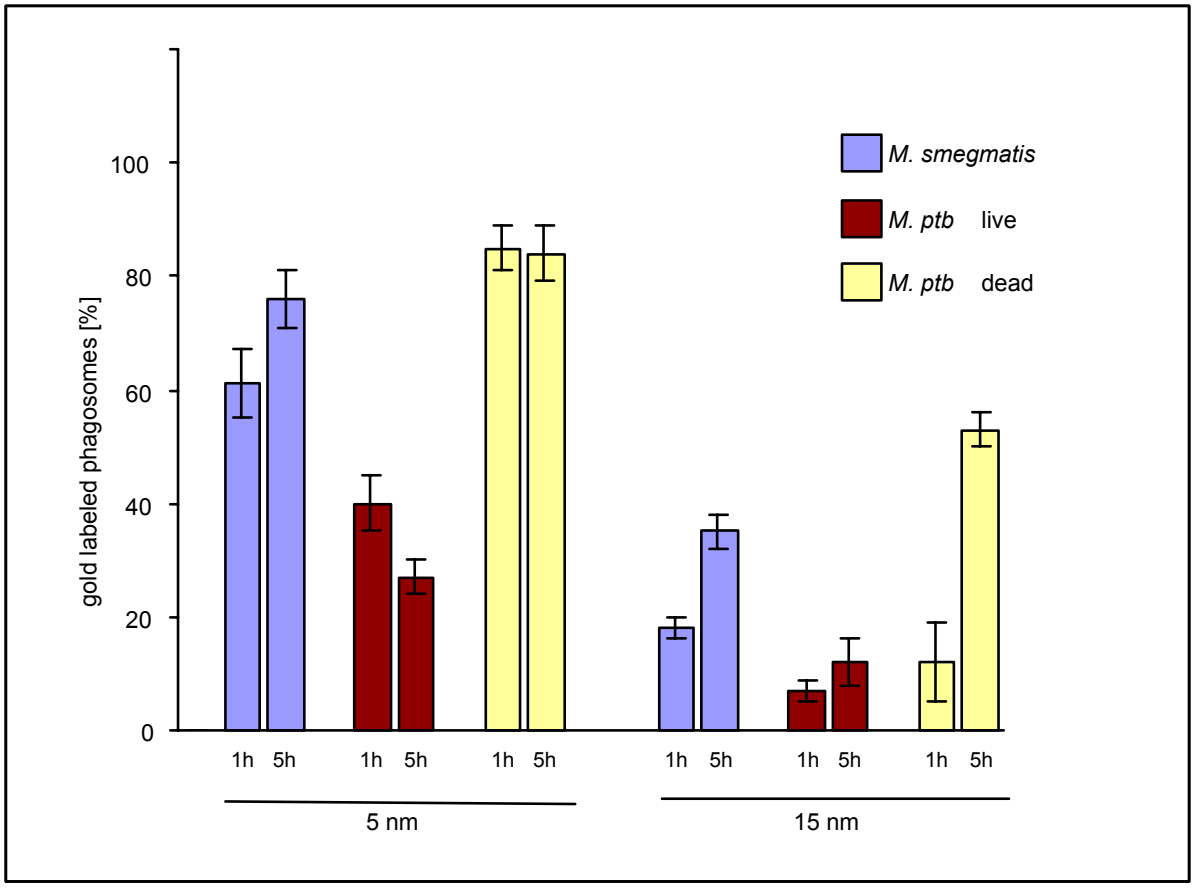


Figure 8: Quantitation of co-localization of mycobacteria with BSA–gold particles.

Late endsomes/lysosomes of J774 cells were labelled with 5 nm and 15 nm BSA–gold as described in the legend to Fig. 7. Samples were fixed and prepared for electron microscopy at the indicated time points after infection. Phagosomes containing at least one gold particle were counted as positive. At least 50 phagosomes were counted per sample. The numbers of phagosomes containing mycobacteria co-localized with gold particles are given as a percentage of all phagosomes counted.

C.1.5 Use of calcein to monitor phagosome lysosome fusion

The fourth approach took advantage of a method established by Oh and Staubinger (1996) for estimating fusion of *M. avium* with endocytic organelles in J774 cells. The basis of this method is to internalize the fluorochrome calcein into cells for 24 h and then to allow the cells to internalise for 1 h and 5 h mycobacteria that had been surface labeled with sulpho-NHS-biotin. After fixation calcein can be directly visualised by confocal microscopy, whereas the bacteria were detected by TexasRed-streptavidin. Colocalisation of the red bacteria with the green calcein resulted in a yellow fluorescence and was taken as an indication of fusion. Viable *M. avium,* for which a reduction in phagosome-lysosome fusion has been described (Frehel et al., 1986; Oh and Straubinger, 1996), served as positive control.

Quantification of multiple images revealed that at 1 h after infection 80%, and at 5 h after infection 75% of all heat-killed *M. ptb* colocalised with calcein-labeled endosomes, whereas only 30% and 27%, respectively, of live *M. ptb* were colocalised with calcein (Fig. 9). As expected, live *M. avium* colocalised only to 5% at 1 h and 3% at 5 h after infection. In contrast, non-pathogenic *M. gordonae* colocalised to 77% at 1 h after infection of J774 cells and to 68% at 5 h after infection (Fig. 9).

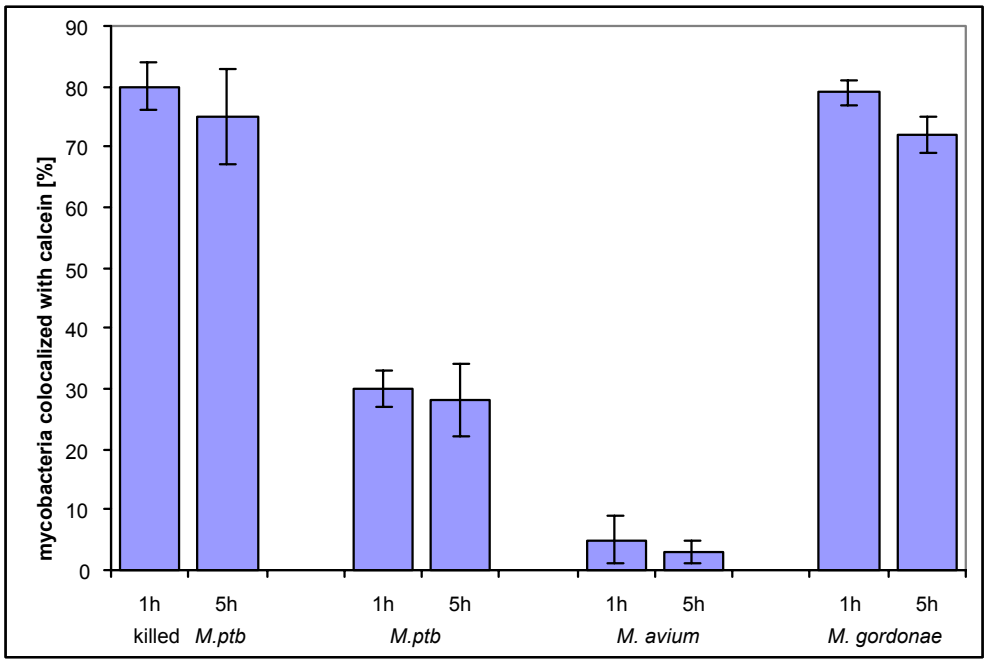


Figure 9*:* Phagosome–lysosome fusion in mycobacteria-infected J774 macrophages*.*

J774 cells seeded on coverslips were incubated for 24 h in medium containing 200 µM calcein. Then, medium was removed, and the cells were incubated with medium containing the indicated sulpho-NHS-biotin-labelled mycobacteria. After 1 h or 5 h, coverslips were removed, treated with Texas red–streptavidin, and co-localization of the mycobacteria with calcein was counted after visualization using a confocal microscope. Co-localization of calcein (green fluorescence) with *M. ptb* (red fluorescence) was indicated by yellow to orange fluorescence and counted. Each value is the mean percentage (± SD) of calcein-co-localized bacteria obtained from at least three independent experiments.

C.1.6 Effect of lipids on fusion in mycobacteria infected macrophages

In order to check if lipids that are able to increase actin nucleation (C.2.4) *in vitro* are able to have effects on fusion with acidic vesicles in mycobacteria infected macrophages, cells were infected as described and the chase was carried out either in the presence of 250 µM AA, 100 mM Cer or normal medium. Cells were fixed after 5 or 24 hours and fluorescence microscopy was performed using a Leica SP2

confocal microscope. At least 50 cells were analysed for colocalisation of calcein (green) with mycobacteria (red). The results are summarised in Table 2.

	5h			24h		
	control	+AA	+Cer	control	+AA	+Cer
M. avium	6 +/-2	42 +/-5	26 +/-5	8 +/-4	52 +/-3	36 +/-6
M. avium dead	44 +/-5	94 +/-4	75 +/-5	53 +/-4	96 +/-4	79 +/-4
M. smegmatis	67 +/-7	93 +/-2	90 +/-4	86 +/-6	95 +/-2	90 +/-5

Table 2: Effects AA and cer on fusion of MCP with lysosomes.

Results are given in [%] +/- standard deviation.

Dead *M. avium* and *M. smegmatis* infected cells were as expected colocalised with calcein even in non stimulated cells to a high extend (44-86%), whereas only 6% (+/-2) of live *M. avium* colocalised with the lysosomal marker. Incubation of infected cells with either AA or cer was found to increase colocalisation of calcein with MCP significantly at both time points. *M. avium* phagosome colocalisation with the lysosome marker was increased 4-fold upon treatment of cells with cer (from 6 +/-2% to 26 +/-5%) and upon treatemt with AA a 7-fold increase was observed (from 6 +/-2% to 42 +/-5%) at 5 hour infection. The same trend was also seen after 24 hour infection. A strong increase in colocalisation was also seen in cells infected with dead *M. avium* and *M. smegmatis.* Approximately 75-90% of dead *M. avium* and over 90% of *M. smegmatis* colocalised with calcein upon treatment of cells with AA or cer at both time points.

C.1.7 Analysis of the pH of endocytic and phagocytic compartments

It has been well established that the early endosomes have a pH of ~6-6.5 whereas the more heterogeneous late endocytic structures, including lysosomes, generally have a lower pH of ~4.5-5.5 (Mellman et al., 1986; Clague, 1998). It should be noted that the distinction between these various classes of late endocytic organelles has not yet been clearly defined (Storrie, 1988; Griffiths, 1996). Usually bacteria and non-degradable particles such as latex beads after being phagocytosed by the macrophage follow a standard route, in that their enclosing compartment fuse sequentially with all stages of the endocytic pathway, first with early endosomes and later with late endosomes and lysosomes (Duclos and Desjardins, 2000; Jahraus et

al., 1998). Such phagosomes tend to have a relatively acidic pH ~5 (Clemens, 1996). In contrast, phagosomes containing viable pathogenic mycobacteria, such as *M. avium* or *M. tuberculosis* appear to fuse continuously with early endosomes but their fusion with late endocytic organelles is generally considered to be reduced. In agreement with this dogma, the pH of these phagosomes is in the same range as early endosomes (pH 6-6.5) (Clemens, 1996).

The above data on phagosomal pH were obtained by the use of pH-sensitive fluorescent dyes that were bound to the bacteria. In these approaches only the pH of the phagosome is estimated. In this study an alternative approach was developed using FACS that allowed to estimate the global average of the pH of all compartments in which internalised FITC-dextran resides at steady state. In these experiments these include the bacteria enclosing phagosomes, which under our standard conditions appear to represent the bulk of the volume of the dextran positive compartments as confirmed by confocal laser scanning microscopy (Fig. 5 and 11B). Nevertheless, the average pH per cell of all the compartments containing FITC-dextran was estimated.

In order to be able to use a FACS-based pH analysis of the endocytic organelles in mycobacterial infected J774 cells, first a pH time course in non-infected cells was determined. J774 cells were labeled for 30 min with FITC-dextran and, either immediately (t=0), or after a further chase period of 30 min, 1 h, 2 h or 4 h, FACS-analysis was performed to determine pH. Each individual pH calculation that is provided was standardised relative to a standard curve obtained by measuring aliquots of the preparations in the presence of nigericin buffers with pH values from pH 5-7. To do this cells were scraped in 1.2 ml of PBS and aliquotted in 200 µl aliquots in 1.5 ml tubes. Each aliquot was washed with a phosphate buffer with a pH value ranging from pH 5-7, or PBS (tester). Following, cells were pelleted and resuspended in 200 µl of nigericin buffers containing the same pH value as the washing buffers, or PBS for the tester sample. FACS analysis was carried out and the average fluorescence intensity of the pH 7 sample was set as 100 %. All other fluorescence intensities were calculated as percentage intensities of the fluorescence intensity of pH 7. The intensity of the tester was then compared with the standard curve to yield the average pH of the tester sample. A typical standard curve with the tester from time point 1 h is given in Fig. 10. This procedure was done for every individual pH value that is provided in this study.

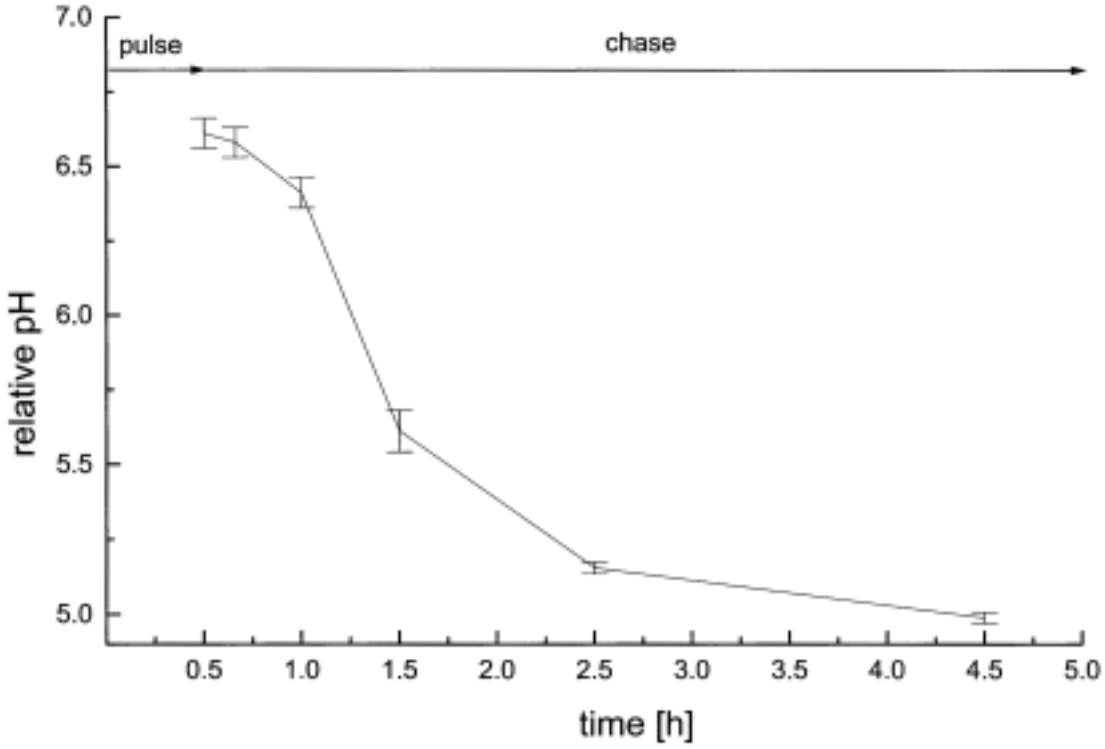


Figure 10: Time course of endosomal acidification in J774 macrophages.

J774 cells were incubated with 1 mg ml^{-1} FITC–dextran for 30 min. After the indicated chase times, cells were analysed by FACS as described in Experimental procedures. The pH-values were calculated by referring the fluorescence emission from the cells to the fluorescence emission of cells from a calibration curve. Each value represents the mean (± SD) of calculated pH-values from three independent experiments.

At t=0 of chase dextran-positive compartments had an average pH of ~6.6, which went down to ~6.4 after a chase period of 1 h (Fig. 10). Since dextran is expected to reach late endocytic compartments in J774 cells, as well as in primary macrophages, after ~15 min (Jahraus et al., 1998; Rabinowitz et al., 1992) it was surprising to see that the average pH after 1 h of FITC-dextran internalization showed a value of ~pH 6.4 consistent with the pH of the early endosomes. It can be suggested that the total volume of the early endosomes may be a significant fraction of the endocytic pathway in J774 cells, such that the dextran within its volume contributes more to the total pH than does that found in the late endocytic structures at the 1 h time point. Whatever the reason, after 4-5 h the FITC-dextran positive compartments reached a pH value of ~5, a value similar to that expected for the average of late endocytic compartments.

Presumably, by this time the marker had full access to the luminal spaces of the entire set of late endocytic organelles, in addition to filling the early endosomes. In any case, our data allowed us to operationally define two separate endocytic compartments in uninfected J774 cells, a set of early endosome having a pH value of ~6.3 and a set of late endosomes/lysosome compartments having a pH value of ~5. The value for the latter is presumably an over-estimate due to an unknown contribution of the FITC-dextran present in the pH of 6.3 early compartments in these experiments.

C.1.8 Analysis of endosomal pH in mycobacteria infected J774 cells

The next thing to analyse was the average pH of the phagocytic and endocytic compartments in cells infected with different mycobacteria or treated with BSA-coated latex beads (1 µm in diameter). For this, J774 cells were incubated with FITC-dextran for 30 min and, following a 15 min rinse and chase period at 37ºC, cells were subsequently treated with mycobacteria or latex-beads in the absence of FITC-dextran. The special interest was in comparing *M. ptb* with the well-characterised *M. avium*, and the non-pathogenic *M. gordonae* and *M. smegmatis*. *M. avium* and *M. tuberculosis* have been shown to reside in vacuoles that have a pH of 6.3 (Sturgill-Koszycki et al., 1994; Oh and Straubinger, 1996; Crowle et al., 1991; Clemens, 1996). Heat-killed mycobacteria, as well as inert latex beads are known to follow the normal phagosomal maturation process (Desjardins, 1995; Clemens, 1996) and provided a reference system in which the phagosomes would acidify maximally.

FACS-analysis revealed that fluorescence emission obtained from *M. ptb* and *M. avium*-infected cells was significantly higher than from cells, which had internalised heat-killed mycobacteria or BSA-coated latex beads. According to the individual calibration curves it was calculated that the fluorescence emission of phagosomes and labeled endocytic organelles of J774 cells infected with *M. ptb* or *M. avium* corresponded to pH-values of approximately 6.3 (SD+/-0.1) and 6.2 (SD+/-0.08), respectively. For cells that had phagocytosed killed *M. ptb* and latex beads pH values of approximately 5.3 (SD+/-0.05) and 5.1 (SD+/-0.1), were calculated respectively, indicating a normal progression of acidification (Fig. 11 A). These data show that *M. ptb* behaves similar to *M. avium* and, more intriguingly, they suggest that both bacteria lead to a partial inhibition of acidification of the bulk of the endocytic compartments in infected cells relative to uninfected cells.

FACS-analysis of J774 cells infected with *M. smegmatis* and *M. gordonae* revealed pH values in the FITC-containing compartments, after 1 h infection, of approximately 5.8 (SD+/-0.9) (Fig. 11 A), which was intermediate between the pathogenic *M. ptb* and *M. avium* on the one hand and the killed pathogenic bacteria or latex beads on the other.

Since a partial inhibition of acidification of the phagosomal and late endocytic compartments in macrophages infected with both pathogenic and non-pathogenic mycobacteria was observed, it was very interesting studying this effect over longer infection periods. For this, the approach was modified. Briefly, J774 cells were allowed to ingest live or heat killed mycobacteria for 1 h. After several washing steps to remove non ingested mycobacteria the cells were cultured in medium containing 50 µg/ml gentamicin at 37ºC for 4 h. Before FACS-analysis, the cells were allowed to endocytose FITC-dextran for 4 h. This was necessary to obtain effective labeling of phagosomes in macrophages infected for longer periods. Using these conditions a reasonable colocalisation of FITC-dextran with TexasRed-labeled mycobacteria was observed, as shown by confocal laser scanning microscopy (Fig. 11 B).

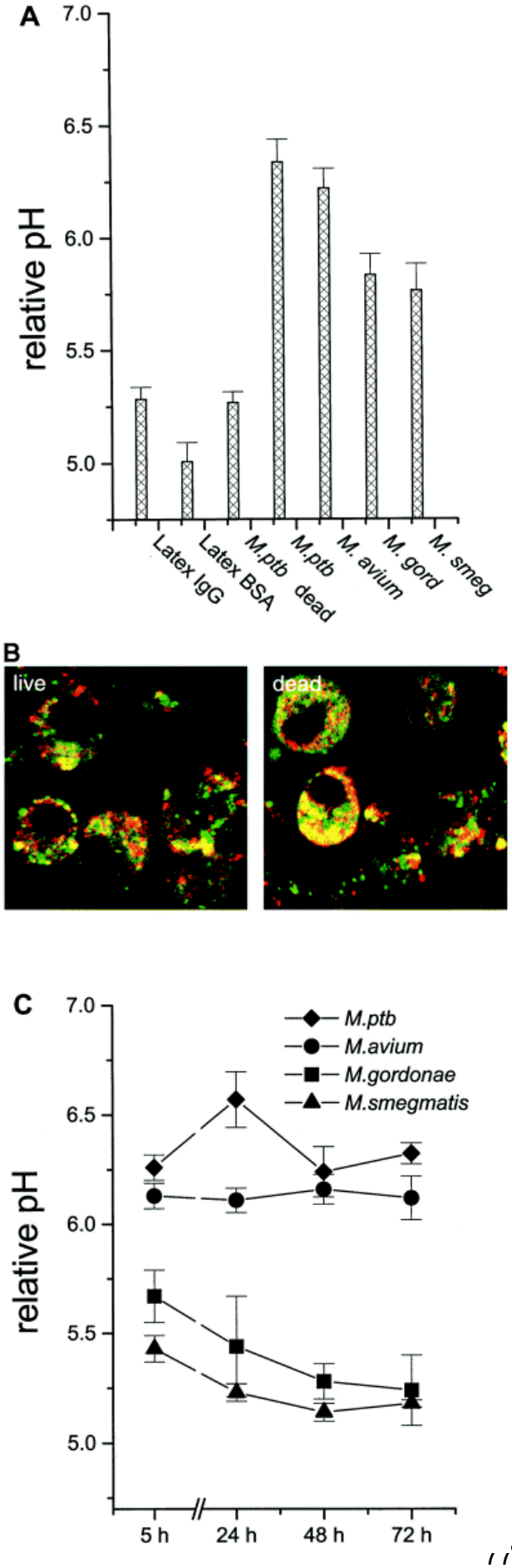


Figure 11: Analysis of pH in mycobacteria-infected J774 cells.

A. J774 cells were incubated with 1 mg ml-1 FITC–dextran for 30 min; then, FITC–dextran containing medium was removed, and the cells were incubated for 1 h with medium containing live or heat-inactivated *M. ptb* (dead *M. ptb*), *M. avium*, BSA-coated latex beads (Latex BSA), *M. smegmatis* (M. smeg) or *M. gordonae* (M. gord). Cells were analysed by FACS as described in Experimental procedures, and pH-values were calculated by referring the fluorescence emission from the cells to the fluorescence emission of cells from a calibration curve. Each value represents the mean (± SD) of calculated pH-values from three independent experiments.

B. Co-localization (yellow fluorescence) of *M. ptb* (red fluorescence) with FITC–dextran (green fluorescence) in J774 cells that had been infected with live or dead *M. ptb* for 24 h as visualized by confocal scanning microscopy described in Fig. 2.

C. J774 cells were incubated for 1 h with medium containing live *M. ptb*, *M. avium*, *M. smegmatis* or *M. gordonae*. For pH analyses, medium was removed, and the cells were incubated in fresh medium containing 1 mg ml-1 FITC–dextran for 4 h. After washing with PBS, cells were analysed by FACS as described in Experimental procedures, and pH-values were calculated by referring the fluorescence emission from the cells to the fluorescence emission of cells from a calibration curve. Each value represents the mean (± SD) of calculated pH-values from three independent experiments.

With this modified approach it was possible to analyze acidification within J774 cells up to 72 h after infection. As before, non-infected cells gave an average pH for the endocytic pathway of 5 also with this protocol (data not shown). At 5 h post infection the fluorescence emission obtained from *M. ptb*- and *M. avium*-infected cells corresponded to pH values only slightly lower than those observed at 1 h of infection, being pH 6.3 (+/- 0.06) for *M. ptb* and 6.1 (+/- 0.06) for *M. avium* (Fig.7 C). Surprisingly, this did not change up to 72 h of infection, indicating that the pH in the FITC-dextran containing organelles, including phagosomes that had reached apparent equilibrium at this pH. In contrast, cells infected with non pathogenic mycobacteria had a pH value of pH 5.7 (+/- 0.12) for *M. gordonae* and 5.4 (+/- 0.06) for *M. smegmatis* at 5 h post infection this value continuously decreased, reaching an overall pH of approximately 5.2 at 48 h post infection (Fig.7 C).

C.1.9 Localisation of actin in infected cells

It has been shown by Chastellier et al. (2000) that *M. avium* infected cells have a modified actin filament network and it has been proposed by the group of Griffiths (unpublished data), that actin is involved in fusion of different endosomes with phagosomes. Therefore, a light microscopical investigation of J774 cells infected with different mycobacteria was carried out to find out if changes in the actin network by mycobacteria are species specific or linked to the viability of the bacteria. For this J774 cells were infected with live and dead FITC-labeled *M. avium* or *M. smegmatis.* Cells were fixed after 2 and 24 h and actin was detected by rhodamine-phalloidin. LSM of infected cells revealed, that dead *M. avium* infected cells (Fig. 13), as well as cells infected with *M. smegmatis* (Fig. 12) showed a co-localisation of bacteria with actin. In all cases over 55% of the bacteria were colocalised with the red fluorescence of rhodamine-phalloidin at 2h post infection, increasing to over 78% after 24 h. Surprisingly the co-localisation of *M. avium* with the phalloidin staining was very low (14%)(Table 3) at both time points. Similar results were seen for live *M. ptb*, but these infections were not quantified. The results indicate, that pathogenic mycobacteria are able to prevent their phagosome from being attached to the actin filament network, or from starting nucleation of actin filaments on the phagosomal membrane.

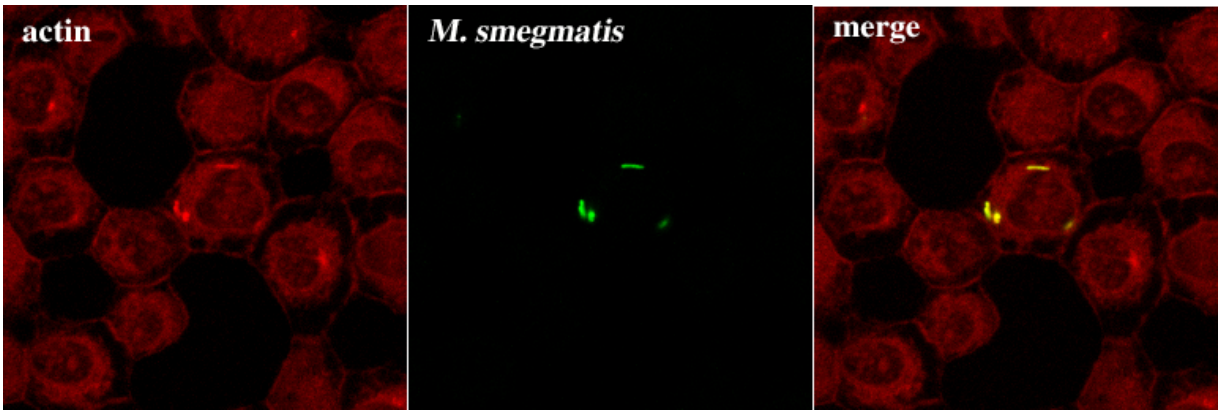


Figure 12: Co-localisation of *M. smegmatis* with actin as viewed by fluorescence microscopy.
Cells were infected with *M. smegmatis* for 1 hour and chased for 24 hours. Cells were fixed and actin was detected using rhodamine-phalloidine. At 24 h p.i. most of the bacteria were colocalised with actin.

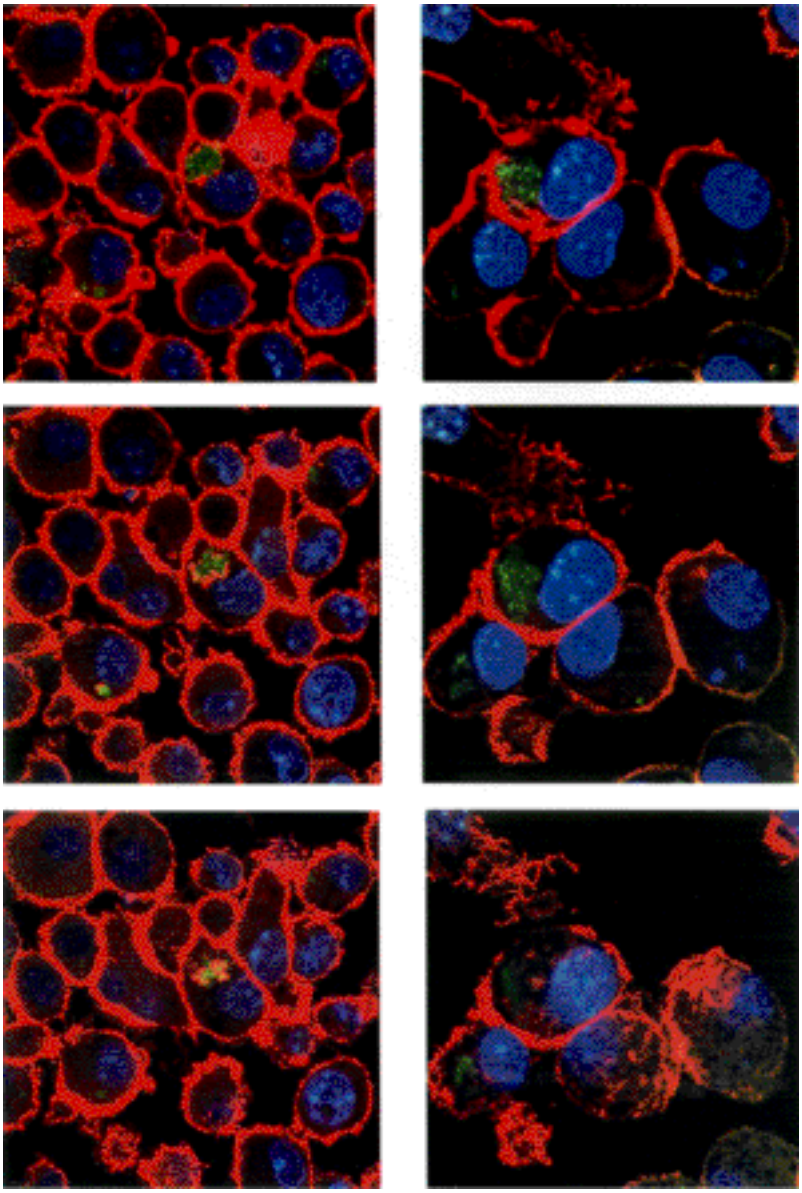


Figure 13: Co-localisation of *M. avium* with actin as viewed by fluorescence microscopy.

Representative pictures of cells infected with either dead (left) or live (right) *M. avium* for 1 hour and chased for 24 hours. Cells were fixed and actin was detected using rhodamine-phalloidine. Three subsequent layers of at least 20 infected J774 macrophages were analysed for colocalisation of actin with the bacteria. At 24 h p.i. only 14 % of live *M. avium* were colocalised. with actin (right), whereas over 50% of dead *M. avium* showed colocalisation with actin (left)

C.1.10 Effect of Arachidonic Acid (AA) on actin in infected macrophages

In order to check whether lipids that are able to increase actin nucleation *in vitro* (C.2.4) are able to have effects on actin localisation in mycobacteria infected macrophages, cells were infected as described and the chase was carried out in the presence of 250 μM AA. Cells were fixed after 24 hours and actin was detected by rhodamine-phalloidine (Fig. 14).

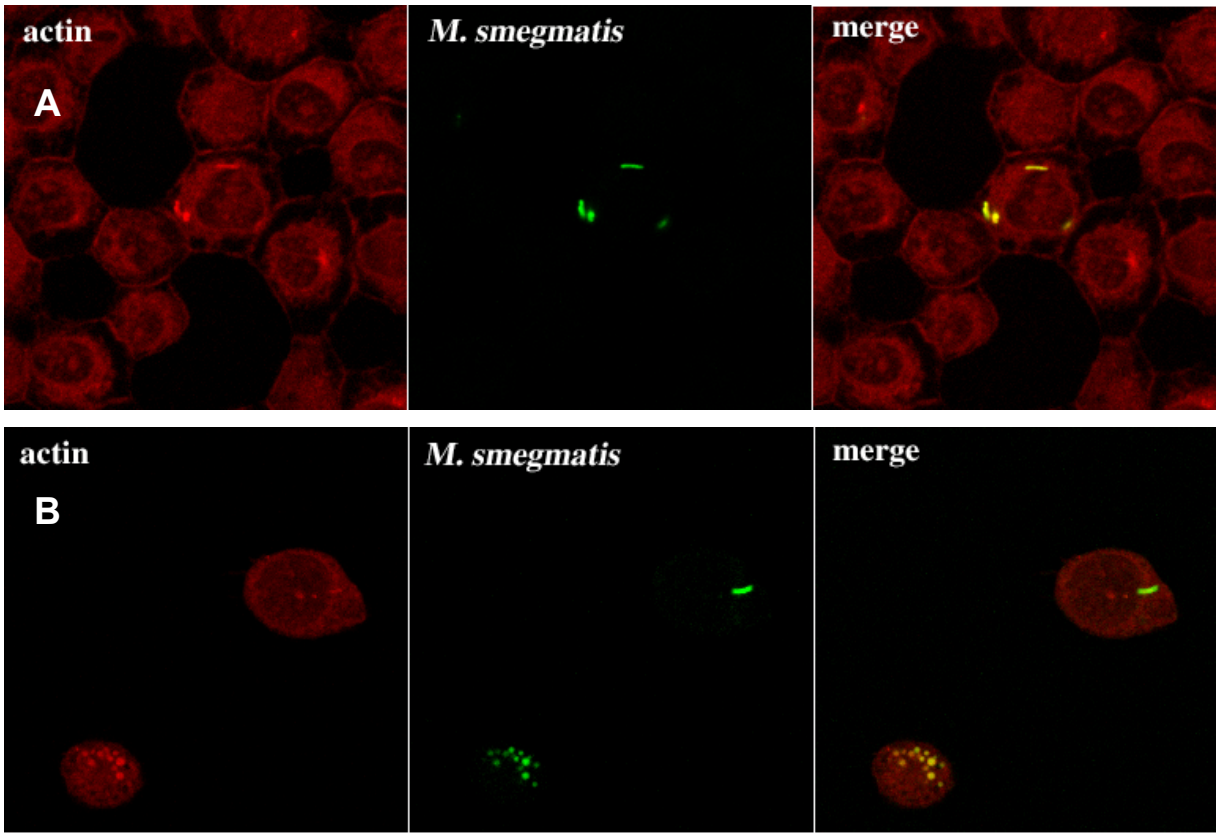


Figure 14: Effect of AA on co-localisation of *M. smegmatis* with actin.

Cells were infected with *M. smegmatis* for 1 hour and chased for 24 hours either in normal medium (row A) or in the presence of 250 μM AA (row B). Cells were fixed and actin was detected using rhodamine-phalloidine. At 24 h p.i. most of the bacteria were not only colocalised with actin as in A, but their phagosomes had changed their rod-shaped morphology towards round compartments (B), indicating degradation of the bacteria.

	2h		24h	
	Control [%]	+AA [%]	Control [%]	+AA [%]
M. avium	14 +/- 4	47 +/-5	22 +/-2	44 +/-3
M. avium dead	69 +/-4	86 +/-4	59 +/-3	78 +/-5
M. smegmatis	60 +/-5	90 +/-5	66 +/-6	94 +/-4

Table 3: Co-localisation of mycobacteria with actin in J774 macrophages.

Cells were infected with live or killed *M. avium* or *M. smegmatis* and incubated for 2 or 24 h with or without medium containing 250 µM AA.

After 2 and 24h the amount of *M. avium* colocalised with actin was significantly increased (Table 3) in AA stimulated cells and in addition a large number of the phagosomes of *M. smegmatis* and only few of *M. avium* had changed their morphology from a rod-shaped towards a round compartment, indicating intense degradation of bacteria.

C.2 *In vitro* studies of isolated phagosomes

C.2.1 Isolation of MCP

Since cells infected with live and dead as well as pathogenic and non pathogenic mycobacteria behaved so different in endosomal acidification, fusion and actin colocalisation the molecular mechanisms responsible for these differences were studied in more detail. For this a method to isolate intact phagosomes containing mycobacteria was established. Briefly, cells were infected with NHS-biotin or NHS-FITC labeled mycobacteria for 1 h and chased for another 2 h until they were harvested by scraping in ice cold PBS. Following cells were washed in HB buffer and disrupted by repeated passing through a neddle. Intact cells and nuclei were pelleted by gentle centrifugation and the broken cell material was layered onto a sucrose gradient consisting of 2 ml 50%, 4 ml 37% and 4 ml 25% sucrose and centrifuged for 45 min at 140,000 x g. Under these conditions the phagosomes were enriched between 50% and 37% sucrose. Phagosomes were harvested with a syringe and layered onto 1 ml of 50% sucrose and centrifuged again at 140,000 x g. Phagosomes formed a band above the 50% sucrose. After removing the supernatant with a pipette the phagosomes were collected using a

pasteur pipette and divided into 20 µl aliquots and following stored at -20°C until further use.

One aliquot was used for checking the intactness of phagosomes. For this NHS-biotin labeled MCP were incubated with streptavidin-TexasRed and counterstained with Syto9. Fluorescence microscopical analysis revealed that intact phagosomes were stained green from Syto9, whereas broken phagosomes were stained yellow to orange due to colocalisation the red fluorescence from TexasRed with green fluorescence from Syto9. Routinely 70% pf phagosomes in a preparation had intact membranes.

C.2.2 Actin nucleation of mycobacterial phagosomes

Defaque et al. (2000) were able to show that LBP nucleate actin *in vitro* and that fusion of LBP with endosomes was, at least in part, dependent on actin. Since we found that phagosomes containing pathogenic mycobacteria, in contrast to those containing dead bacteria, were inhibited in the aquisition of a variety of late endosomal markers, it was of special interest to find out whether these phagosomes were able to nucleate actin *in vitro* or not.

For this the basic assay described by Defacque et al (2000) was used and adopted for the use of isolated phagosomes containing either live FITC-labeled *M. avium* or *M. smegmatis* or dead *M. avium* or Latex beads. Actin is prevented from spontaneous polymerisation in eukaryotic cells by Tβ4 (Gondo et al., 1987; Cassimeris et al., 1992; Weber et al., 1992). Therefore, as a general strategy monomeric G-actin was buffered with a 3-fold excess of chemically synthesised Tβ4. Under these *in vitro* conditions, but in the absence of cytosol, Tβ4 inhibited the initial rate of spontaneous rhodamine-actin polymerization. Using fluorescence microscopy, rhodamine-actin dots and bundles of actin attached to phagosomes were clearly visible after 15 min of incubation (Fig. 15, 16).

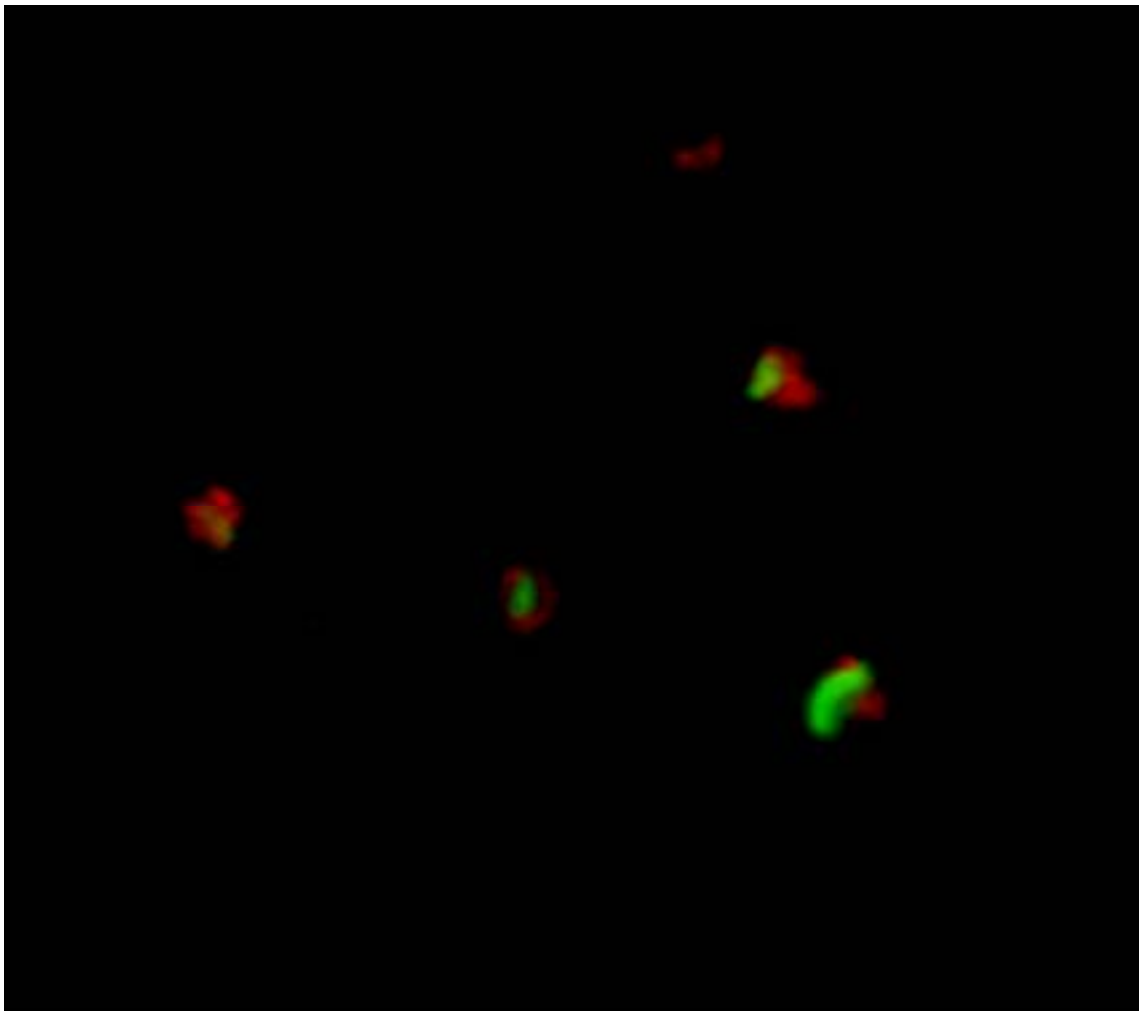


Figure 15: Actin nucleation on mycobacteria phagosomes.

Phagosomes containing killed FITC-labeled *M.avium* (green) were isolated from macrophages and the actin nucleation assay was performed under low ATP (0.2 mM) conditions. Actin is seen as red dots on the surface of the phagosomal membrane.

In a few cases a partial or even complete halo of actin could be seen around phagosomes (Fig. 15). The percentage of individual phagosomes with rhodamine-actin structures was determined (Fig. 16) by analysing at least 50 phagosomes per sample, and in an average of three experiments using 1 h chase and 2 h pulse phagosomes 4% (+/- 2%) of live *M. avium* and 6% (+/- 4%) of live

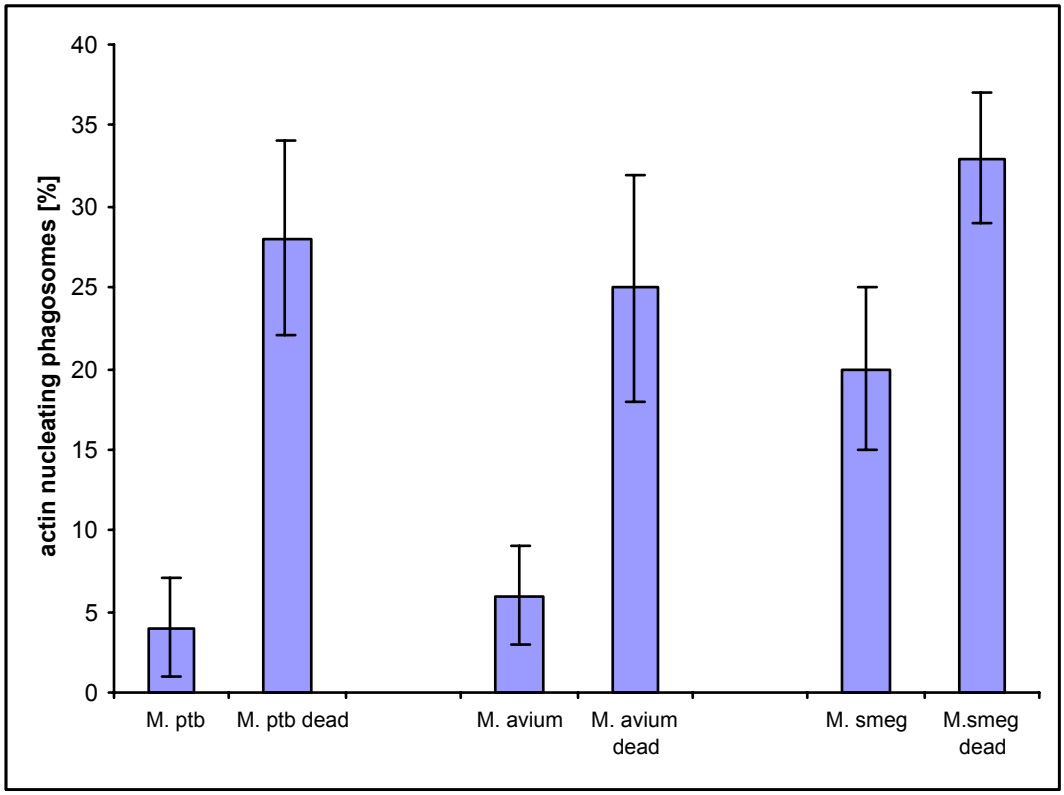


Figure 16: Quantitation of actin nucleation on 2 h mycobacterial phagosomes.

Results from at least three individual experiments are given as means +/- standard deviation. Live *M. avium* and *M. paratuberculosis* containing phagosomes are clearly inhibited in actin nucleation. Live *M. smegmatis* and killed mycobacteria phagosomes were found to have significantly higher amounts of actin nucleation (at the p=0.01 level using the student's t-test).

M. ptb phagosomes were found to be positive. As a positive controle for the system LBP were used which were 17% (+/-6%) positive under these conditions. In order to make sure that the observed effects are linked to phagosomes, pure bacteria were checked for nucleation. *M. avium* or *M. ptb* were only 1.5% (+/-1%) positive and pure latex beads were also only labeled with actin to a very low extent (0.9 ± 0.3% of positive beads). The percentage of positive phagosomes was significantly higher among phagosomes containing heat-killed mycobacteria. Phagosomes containing dead *M. avium* were positive at a level of 25% (+/- 8%) while phagosomes containing dead *M. ptb* were 28% (+/-6) positive. Even the 2 h phagosomes of live and dead *M. smegmatis* were found to have a significantly

higher amont of actin nucleation. Since the amount of phagosomes containing live *M. smegmatis* nucleating actin was as high or even higher than the number of phagosomes containing killed *M. avium* or *M. ptb*., the *M. smegmatis* phagosomes were considered as not generally inhibited in actin nucleation.

Analysis of actin nucleation of phagosomes at either high (5 mM) or low (0.2mM) ATP conditions revealed that, just like in the LBP system (Introduction A.4.7), the high concentrations of ATP inhibited the nucleation of actin (Fig. 17).

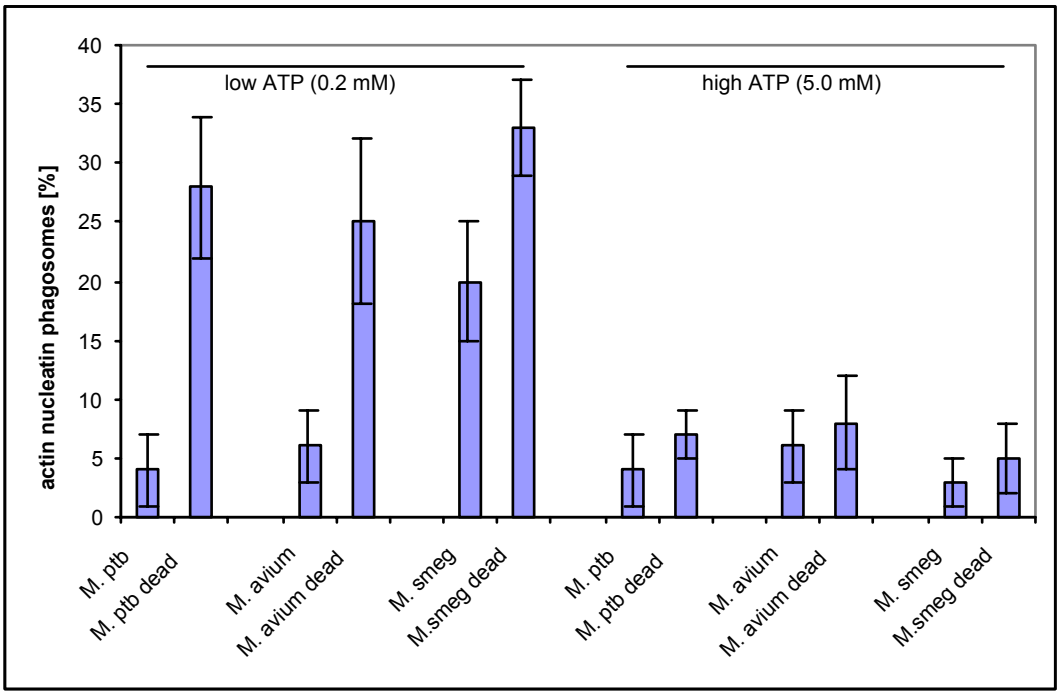


Figure 17: Effect of ATP on actin nucleation of mycobacterial phagosomes

Actin nucleation was performend using 2 hour *M. ptb*, *M. avium* and *M. smegmatis* containing phagosomes. An inhibitory effect was seen at all tested phagosomes at a significance level of p=0.01 using the student's t-test, except for live *M. avium* and live *M. ptb* containing phagosomes.

C.2.4 Influence of lipids on actin nucleation

Live *M. avium* and *M. paratuberculosis* containing phagosomes have shown to inhibit actin nucleation *in vitro*. Since lipids are known to affect actin nucleation in the LBP system (Kuehnel, Anes and Bos et al., submitted) and regulation of cellular actin assembly in general (Baer et al., 2001) the effect of endogenous

added lipids that are involved in the lipid signalling network found in the LBP system was tested. For this phagosomes used in the actin nucleation assay were incubated in $PI(4,5)P_2$, arachidonic acid (AA), sphingosine (Sph), sphingosine-1-phosphate (S1P) or ceramide (Cer) and the actin nucleation assay was performed. The percentage of actin nucleation positive phagosomes were counted using fluorescence microscopy. The results are summarised in Fig. 18, 19. These data show that four lipids, when added to phagosomes, are capable of activating actin nucleation on phagosomal membranes, at the p=0.01 level using the student's t-test, that are normally 'silent' in this process.

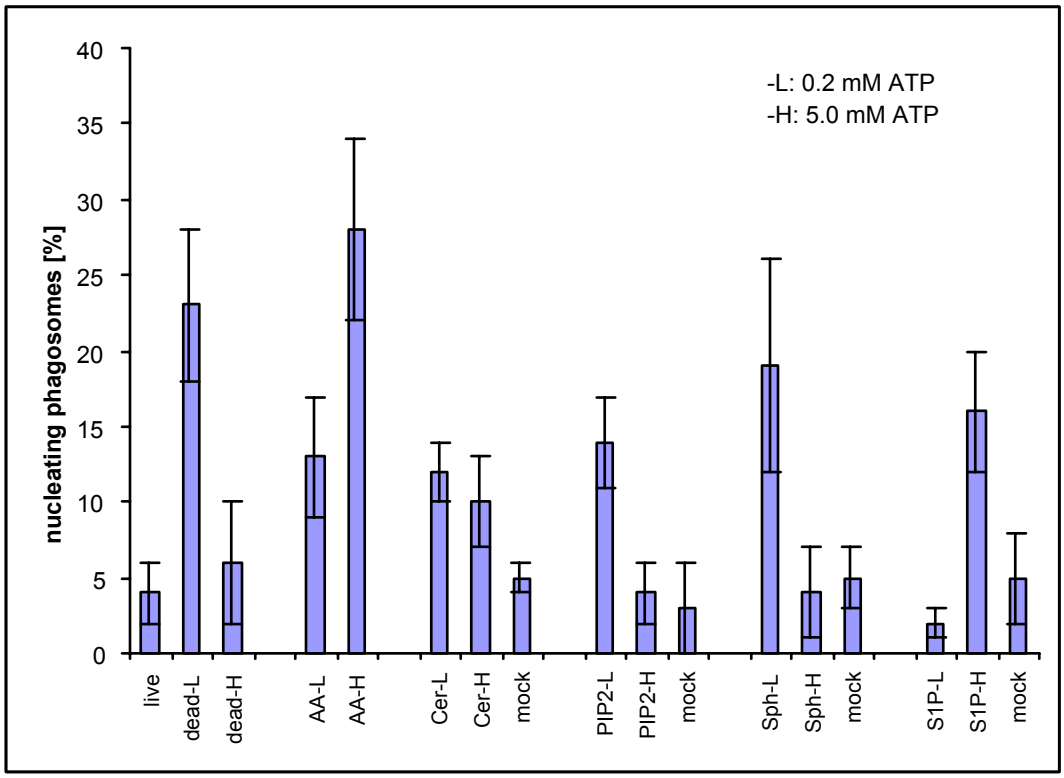


Figure 18: Effects of lipids on actin nucleation in *M. avium* containing phagosomes.

Actin nucleation was performed using 2h *M. avium* containing phagosomes in the presence of ether AA, Cer, $Pi(4,5)P_2$, Sph or S1P at concentrations discribed (B.4.2). Phagosomes that were incubated in the lipid solvents (mock) revealed no significant effect of solvents under these conditions.

In *M. avium* containing phagosomes AA was found to increase actin nucleation significantly at low (0.2 mM) ATP, but even significantly higher at high (5.0 mM)

ATP conditions. Cer increased actin nucleation at both high and low ATP conditions. Pi(4,5)P_2 and Sph stimulated at low ATP but not at high ATP conditions and S1P stimulated at high, but inhibited at low ATP concentrations (Fig. 17). Using 24 h *M. smegmatis* phagosomes the results regarding effects of lipids on actin nucleation were similar to results obtained from 2 h *M. avium* phagosomes.

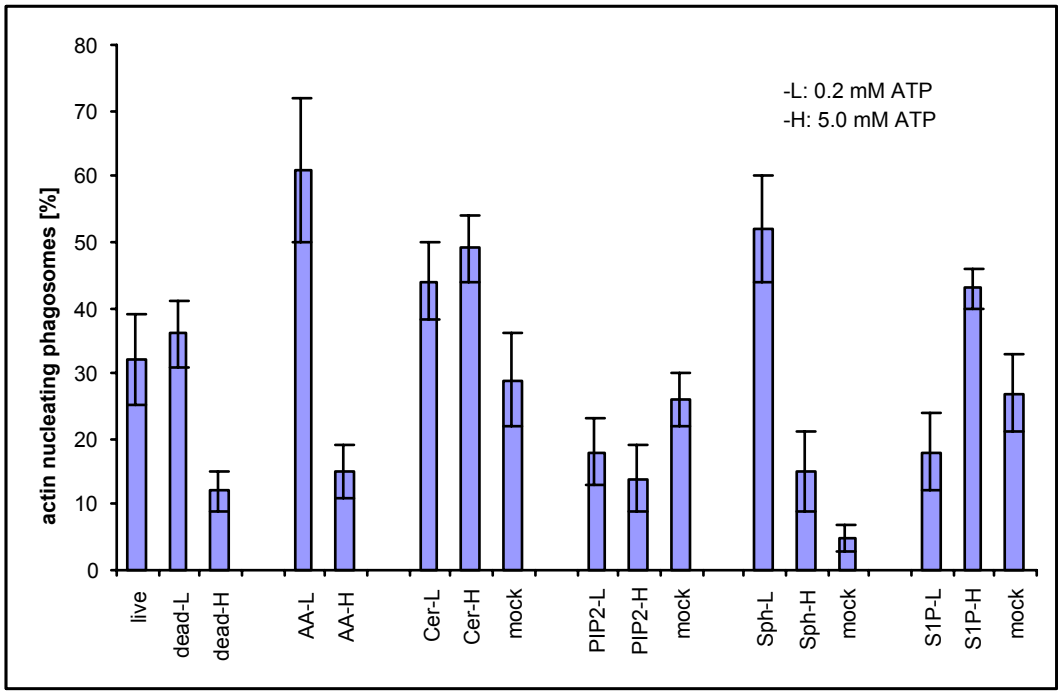


Figure 19: Effects of lipids on actin nucleation in *M. smegmatis* containing phagosomes.

Actin nucleation was performed using 24 h *M. smegmatis* containing phagosomes in the presence of either AA, Cer, Pi(4,5)P_2, Sph or S1P at concentrations indicated in (B.4.2). Solvent controles (mock) revealed no significant effect of solvents under these conditions.

C.2.5 Influence of lipids on bacterial killing

Live *M. avium* and *M. paratuberculosis* containing phagosomes have been shown to be inhibited in actin nucleation *in vitro*. Since I have shown that at least five lipids (AA, Cer, Sph, S1P and Pi(4,5)P_2) are able to switch on the nucleation of actin of mycobacterial phagosomes *in vitro*, the effects of lipids on the killing function of infected macrophages was addressed. For this macrophages were infected with *M. avium* and stimulated for 24 h with either AA, Cer or S1P or a combination of these three. After 24 h macrophages were disrupted and lysates were plated on middlebrock 7H10 agar plates. The results are summarised in Fig. 20.

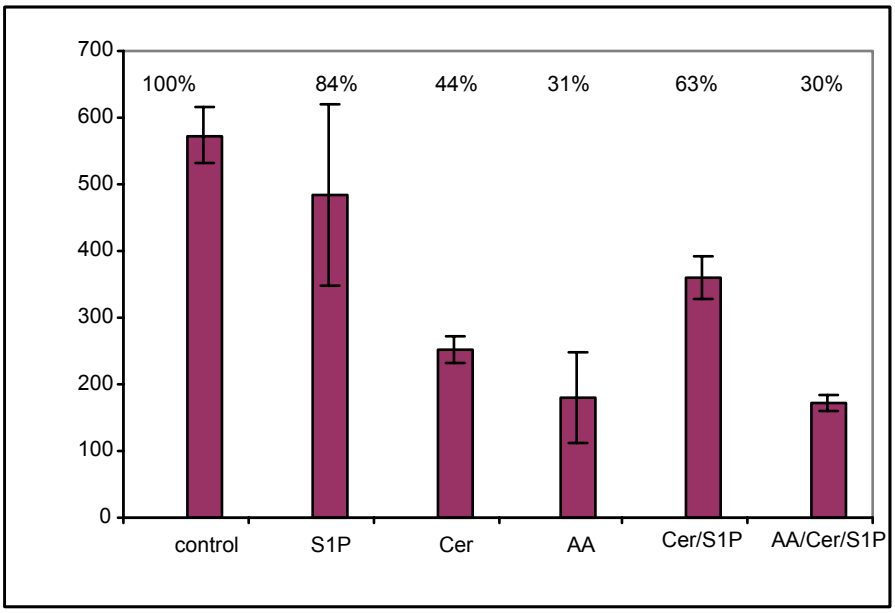


Figure 20: Effects of lipids on the killing of *M. avium* in infected macrophages.

J774 macrophages were infected with *M. avium* for 1h and then stimulated with lipids for 24 hours. Cells were lysed and lysates plated to determine CFUs (B.2.7). Two lipids (Cer and AA) were found to have a significant effect on intracellular survival of *M. avium*. Results of three individual experiments are given as mean +/- standard deviation. The numbers represent the percentage of the mean in comparison to the control.

After 24 h the number of bacterial CFUs found in infected macrophages that were stimulated with Cer was only 44% compared to controls and cells that were treated with AA had only 31% of CFUs. A combination of one or both of these

lipids with S1P had also a significant effect on CFUs. A combination of AA, Cer and S1P deceased the CFUs to 30 % and a combination of Cer and S1P decreased to 63% in comparison to controls.

C.3 Summary of results regarding the actin track model

The actin track model (see Introduction) proposes that an increase in actin nucleation can lead to an increase in fusion. If this applies to macrophages infected with pathogenic mycobacteria, some of these lipids might 1) stimulate actin nucleation (which, as shown above is inhibited) leading to 2) an increase in fusion which could result in 3) increased killing of the pathogens. These postulates were fulfilled in all cases:

1. Actin nucleation *in vitro*

I found that addition of AA, Cer, S1P or PI(4,5)P_2 led to significant increase of actin nucleation on live M. Avium-containing phagosomes. As shown in Fig. 18 a addition of these lipids at physiological concentrations to the assay significantly increased the number of phagosomes that could nucleate actin.

2. Actin *in vivo*

Addition of AA resulted in a co-localisation of actin (visualised by phalloidin) with intracellular M. avium and M. smegmatis.

AA was tested for its effect on *M. avium* infected macrophages. The percentage of phagosomes that were positively labelled with rhodamine-phalloidin was estimated for killed and live *M. avium*, as shown in Tab. 3.

3. Fusion

Treatment of cells with AA or Cer increased fusion of late endosomes/lysosomes with live M. avium-containing phagosomes as seen by fluorescence microscopy. Macrophages were internalised together with calcein for 2 hours to label acidic late endosomes lysosomes and following infected with *M. avium* for one hour.

After a five and 24 h hour chase period samples were fixed and co-localisation of calcein with mycobacteria was estimated. The results are summarised in Table 2.

4. Killing

Treatment of infected cells with AA or Cer (or combinations of Cer, S1P and/or AA) led to significant increase of killing of live M. avium.

Macrophages were infected with *M. avium* for 1 hour and then chased for another 24 hours in the presence of either AA, Cer, S1P, or a combination of the three. Then, cells were disrupted and plated onto Middlebrock 7H10 plates. After 3-4 weeks colony forming units were counted. Fig. 20 shows that treatment of macrophages with AA or Cer (or combinations of Cer, S1P and/or AA) led to a significant increase in the killing of *M. avium*.

D. Discussion

Since *M. ptb* causes a chronic disease in ruminants that is non-treatable and always accompanied with a lethal fate for cattle it is necessary to understand the pathogenicity of the bacterium and to identify the virulence mechanisms by which the disease is established. When I started this thesis only a few groups had published papers on the pathogenicity of *M. ptb* and its interaction with the host on a cellular basis.

D.1 *In vivo* analysis

For pathogenic mycobacteria in general a consensus had already been developed that these bacteria have the ability to block key phagosomal functions, acidification (Mellman et al, 1986), fusion (Desjardins et al., 1994 and 1995) as well as oxidative burst and antigen presentation (Ding et al., 1988; Akaki et al., 2001). It is widely thought that these inhibitory mechanisms help the pathogen to survive and multiply in what becomes a protected environment in the phagosomal lumen. Answering the question of how *M. ptb* affects phagosome acidification and fusion was a key goal I addressed (see below).

One significant problem in working with mycobacteria is their slow growth. Whereas non-pathogenic mycobacteria like *M. smegmatis* require only a few hours for doubling, pathogenic *M. tuberculosis* needs 12-24 hours while *M. paratuberculosis* requires 1-4 days. Therefore successful culture of *M. ptb* is measured in months. Within this time, *M. paratuberculosis* growing on the surface of the liquid medium is very sensitve to disturbances, as they die when they sink to the bottom of the medium.

It was then necessary to establish a reproducible cell culture infection system. For this J774 cells were used and the optimal conditions for infection were determined.

With this basis I was now able to start the experiments, focussing initially on acidification and later on fusion.

D.1.1 Phagosome acidification

Before I started a number of methods had been used to estimate the pH of phagosomes containing pathogenic mycobacteria such as *M. tb*. This was done by conjugation of a suitable fluorochrome to the bacterial surface. After internalisation, the phagosomal pH can be estimated since the emission spectrum of FITC varies with the pH, and standard fluorescence-based methods can be used to estimate this parameter. Using such methods, a number of groups had established that the pH of phagosomes enclosing pre-killed mycobacteria is in the range of 4.5-5.0, the same value as the lysosomes (Sturgill-Koszycki et al., 1994; Clemens, 1996; Storrie, 1986; Mellman, 1989). However, the phagosomes with the live pathogens maintain a pH at steady state that is identical to the early endosomes (pH about 6.3). Since these phagosomes can fuse continuously with the transferrin-rich early endosomes (but poorly with late endocytic organelles; see below) this pH result was taken to indicate that the lumen of phagosomes had reached an equilibrium with the early endosomes but they could not access the later, lower pH compartments. Moreover, Russell and colleagues had shown that these phagosomes are depleted (relative to 'normal' phagosomes) in sub-units of the vacuolar proton ATPase (Sturgill-Koszycki et al., 1994). The latter is normally enriched in late endocytic organelles (Storrie et al., 1996). Many experiments showed that this simple and attractive model fell short in a more detailed analysis.

I developed a FACS-based method that could analyse the average pH of late endocytic organelles and phagosomes. The key to this method is to internalise FITC-dextran either before, or after infection with mycobacteria. This marker is then 'chased' so that it reaches equilibrium in late endocytic organelles, and, if fusion is allowed in the system, also to phagosomes. As discussed in more detail below, the ability of various markers to move from late endocytic organelles to the phagosome of pathogenic mycobacteria depends greatly on many parameters that are poorly understood. In my hands, both protocols (adding FITC-dextran before or after infection) resulted in full access of the marker to the phagosomes of live *M. ptb*, as well as of *M. avium, M. smegmatis, M gordonae*

and of LBP. After 1 hour of internalisation the FITC-dextran uniformly labelled the phagosomes and, as expected, late endocytic organelles.

The basis of the FACS method is that the average pH of the FITC-positive compartments can be estimated by selecting a wavelength of 490nm and measuring fluorescence intensities of cells having internalised FITC-dextran. In order to calibrate the system, standard pH curves were done using a range of nigericin containing buffers from pH 4.5-8 as described in material and methods. Nigericin was used because as an ionophore it is able to make membranes permeable, so that the buffer system reaches all parts of the cell.

Early after infection with both pathogenic and non-pathogenic bacteria, an average pH of the phagosomal/lysosomal compartments of 6–6.3 was detected. My data argue that the presence of the pathogenic *M. ptb* and *M. avium* in J774 cells leads to a partial inhibition, not only of the lumen of the phagosome but also of the bulk volume of the endocytic pathway. In agreement with this notion, when labelled *M. ptb* were used for infection of J774 cells with the pH indicator dye acridine orange, a significant reduction in the number of highly acidic vesicles was seen relative to the number seen in uninfected cells, or in cells incubated with heat-killed bacteria (data not shown). A striking difference was seen after both 1 h and 5 h of infection, such that three ‘classes’ of phagolysosomes could be identified depending on the species. Cells infected with *M. ptb* had a calculated pH of 6.3; the same value was obtained for *M. avium*, in excellent agreement with Sturgill-Koszycki et al. (1994) and Oh and Straubinger (1996). In contrast, I found that phagolysosomes of dead *M. ptb* and *M. avium* had an average pH of 5–5.5, again in exellent agreement with Sturgill-Koszycki et al. (1994) and Oh and Straubinger (1996). Similar pH-values were seen with phagosomes of the non-pathogenic mycobacteria *M. gordonae* (pH 5.8) and *M. smegmatis* (pH 5.2) after 5 h infection times.

Earlier analysis of phagosomal acidification by using spectrofluorimetry of mycobacteria-infected macrophages (Sturgill-Koszycki et al., 1994; Oh and Straubinger, 1996) spanned only a relatively narrow time period of ~6 h. Using a modified approach, it was possible for me to analyse acidification of endosomal

compartments for up to 72 h. This analysis showed that *M. ptb* and *M. avium* remained in non-acidified compartments for up to 72 h, whereas J774 cells infected with non-pathogenic mycobacteria showed increasing acidification, reaching pH-values of 5.2 after 48h. This indicates that pathogenic mycobacteria possess mechanisms by which they can continuously inhibit not only phagosomal acidification but also late endocytic organelles. The lack of acidification seemed to protect *M. ptb* and *M. avium* from intracellular killing, as we found nearly the same number of viable bacteria (CFU) by serial dilution platings of 2 h infections and 72 h for *M. ptb* and even an increase in CFU between these two time points for *M. avium*. In contrast, the viability of the non-pathogenic mycobacteria was already greatly reduced after 24 h. This indicates that non-pathogenic mycobacteria, probably as a result of the initial inhibition of phagosomal acidification, can resist intracellular killing to some extent but are unable to survive for longer time periods. This argues that the inhibition of acidification is not restricted to the phagosome, but is more a general property of the endolysosomal system. This result opens up many questions for the future, such as

1) Is the degradation ability of the lysosomal system generally inhibited in pathogenic mycobacteria-infected cells?

2) Are membrane, or soluble factors, released from the bacteria able to be incorporated, via fusion events, in the membranes of endolysosomes?

3) Is the inhibition of endolysosomal acidification responsible for the (partial) inhibition of fusion with phagosomes?

4) Is homotypic fusion of late endosomes and lysosomes with themselves affected in any way?

D.1.2 Phagosome fusion

This ability of phagosomes to fuse with late endocytic organelles is a crucial function since it allows the cell to fill the phagosomes with hydrolytic enzymes. When this happens, the ability of pathogenic mycobacteria to survive decreases dramatically (Clemens, 1996). It is widely reported that the phagosomes of

pathogenic mycobacteria are inhibited in the fusion with late endocytic organelles. However, closer inspection of the actual published data reveals a number of complexities, as well as deviation from this dogma. The first important point is that, even in experiments that report a 'block' in fusion (i.e. inaccessibility of a particular marker) the percentage of phagosomes that is classed as fusion-negative is never 100%. For example, Ullrich et al. (1999) analysed the colocalisation of *M. avium* with procathepsin D and found that only 23% of phagosomes were positive for this marker an Clemens and Horwitz (1995), who analysed differences in phagosomes containing live vs. killed mycobacteria, observed fusion with BSA-gold (25nm) in 27% of phagosomes containing live and 70% of phagosomes containing killed mycobacteria.

Collectively these data argue that, in all these experiments, one is dealing with (at least) two functionally-distinct pools of phagosomes, those inaccessible to the marker, and those that acquire it. This phenomenon is generally ignored but deserves more study. Moreover, 'fusion' varies according to the state of the cell ('activated' versus quiescent macrophages) (Schaible et al., 1998), and other factors such as the time and load of infection (Clemens, 1996).

In order to increase the chances of making a more comprehensive conclusion about fusion I decided to use 4 (FITC-dextran, gold, Lamp and Calcein) different approaches and applied these not only to *M. ptb* but also to other species of mycobacteria, namely the non pathogenic *M. smegmatis* and *M. gordonae,* and the pathogenic *M. avium.*

Unfortunately, the collective data still do not allow a straightforward conclusion about the ability of the phagosome of pathogenic *M. ptb* and *M. avium* to fuse with late endocytic organelles. Some markers, such as FITC-dextran or Lamp 1 gain access to the majority of phagosomes, whereas others, such as Lamp 2, or pre-internalised colloidal gold were largely inaccessible. The significant difference between Lamp 1 and 2 was especially surprising, given that these markers invariably localise identically in many earlier studies (Storrie et al., 1996). The fact that the inhibition of transfer of Lamp 2 was seen with two

pathogenic mycobacteria tested argues that this is a selective process related to these bacteria. However, the mechanisms responsible for these observation await more detailed studies in the future.

D.2 *in vitro* analysis

The work on the role of actin in the latex bead phagosome (LBP) system established in the Griffith group at the EMBL (Heidelberg) provided the basis for the work on MCP. Of particular importance for my work was the fact that they had established a relatively simple light microscopy-based assay that could monitor the nucleation of actin on the LBP membrane. This assay had already revealed roles for ezrin/moesin (Defaque, 2000b), gelsolin (Defaque, 2000b), profilin (Griffiths, 2002, personal communication), as well as a large number of lipids (Defaque et al., submitted). During my visit to the EMBL I contributed to these studies by showing that arachidonic acid and CDP-choline were novel and powerful effectors of LBP actin nucleation (as well as mycobacterial containing phagosomes – see below). The high number of effectors and conditions that affect this system have been analysed by bioinformatic specialists, using methods described by Schuster et al. (2000). This system uses published information on known pathways and asks, which combinations of different reactions and metabolites (so called 'elementary modes') can potentially operate in any given multi-component system in a fashion that obeys the known thermodynamics and stoichiometry of the individual reactions.

The above approach on 23 components led to 128 predicted 'elementary modes', of which 14 were predicted to lead to an increase in ATP that could provide energy to stimulate actin polymerisation. I have recently tested the first of these (CDP-choline + PA + G-actin --› F-actin + PC + CMP) and found that CDP-choline indeed leads to a significant increase in actin nucleation on both LBP and live and killed *M. avium*-containing phagosomes. Moreover, in recent preliminary studies I also observed a dramatic effect of this reagent on the actin (labelled with rhodamine phalloidine) network of *M. avium* infected cells (unpublished results).

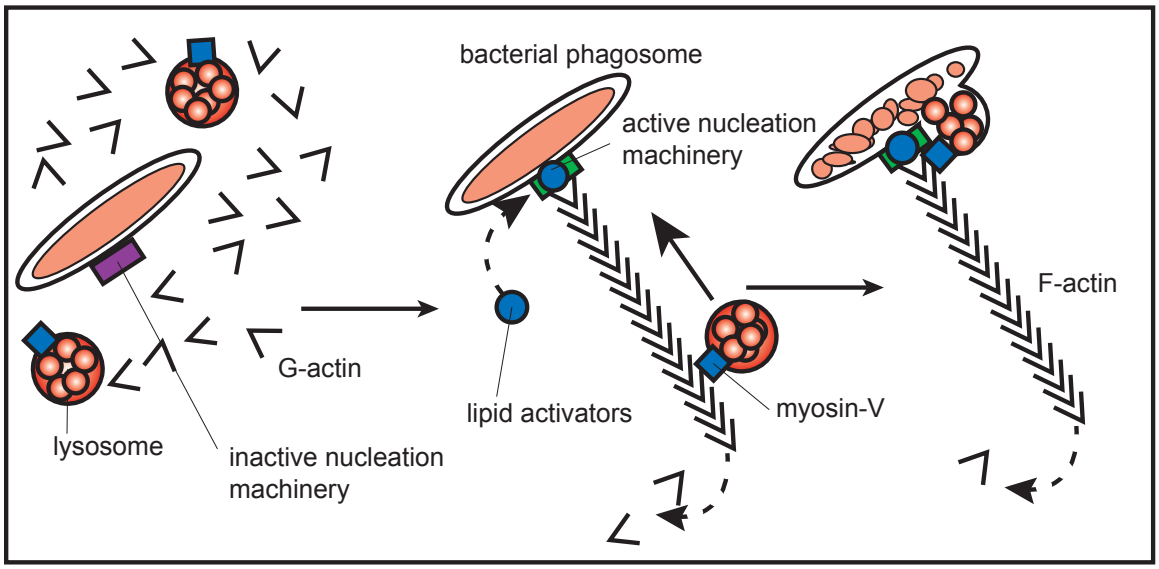
The goal of my EMBO short term fellowship visit to the EMBL was to isolate *M. ptb*- and *M. avium*-containing phagosomes in order to see whether the actin nucleation assay could be applied to these more complex systems. Remarkably, the project was successful in less than two weeks. By modifying existing protocols (Desjardins et al., 1994; Sturgill-Koszycki et al., 1996) I was able to isolate killed *M. avium* phagosomes in a condition pure enough to test the actin assay. Moreover, I was able to show that the magnitude of these phagosomes was intact. These phagosomes were then used for lipid analysis and for addressing actin nucleation.

D.2.1 Actin nucleation

The dead *M. avium* phagosomes turned out to be even more efficient in the actin nucleation assay than the LBP. Whereas only 10-20% of the latter routinely give a positive signal for actin, the killed *M. avium* phagosomes reproducibly give values in the range of 30-40%. Next live *M. avium* phagosomes were tested. These were evidently inhibited because the percentage of actin-positive phagosomes never exceeded 5% and the signal per phagosome was clearly lower than that seen with the dead phagosomes. This finding urged to test whether pathogen *M. avium* could also inhibit actin polymerisation *in vivo*. For this, mycobacteria infected cells were labeled with rhodamine-phalloidin after fixation and the distribution of actin was analysed by fluorescence microscopy (FM) and confocal laser scanning microscopy (LSM).

A strategy now emerged to combine the above findings with the actin track model proposed by Egeberg et al (see introduction). The latter proposes that cytosolic actin nucleated by membrane organelles such as phagosomes can assemble into bundles and networks along which the same, or different organelles can associate, via a membrane-bound myosin. Via such a system actin could facilitate the fusion of phagosomes and endocytic organelles (Egeberg et. al. – manuscript in preparation).

It is possible that LBP-bound myosin V is one myosin that can move LBP along (membrane-induced) F-actin tracks, since a collaborator of Griffiths, Sergei Kusnetzov has shown that phagosomes also bind to F-actin in the presence of cytosol (a distinct process from actin nucleation and that mysoin V is one of the key components responsible (Al-Haddad et al., 2001)). The stage was set for the following strategy.



Model 2: Actin-track model - killing of intraphagosomal bacteria. Phagosomes containing pathogenic mycobacteria are inhibited in actin nucleation. Upon activating the nucleation machinery with lipid activators, actin is nucleated at the phagosomal membrane These filaments provide tracks for lysosomes to bind, move towards the phagosome, fuse and thereby kill the phagocytosed bacterium.

In the LBP system a number of lipids had been identified that activated actin nucleation. If these effectors could also switch on this process in an infected cell the actin track model would argue that this could lead to an increase in fusion and, conceivably, also in pathogen killing. Remarkably, the strategy was very successful since:

1) Five lipids, Cer, Sp, S1P arachidonic acid (AA) and PI(4,5)P_2 (PIP_2) were found to stimulate actin nucleation by live *M. avium* phagosomes *in vitro*.

2) Cer and AA treatment of J774 cells infected with *M. avium* (my work) or *M. tb* (Kuehnel, Anes and Bos, in preparation) showed a significant increase in labelling for actin on phagosomes.

3) Live *M. avium* infected cells showed a significant increase in the level of fusion after treatment with AA.

4) Treatments of infected cells with AA or Cer (or combinations of Cer, S1P and/or AA) led to a highly significant increase in the rate of killing of *M. avium* and *M. tb* (Kuehnel, Anes and Bos, in preparation).

These data open the door to a new rationale for finding therapeutic compounds that can increase pathogen killing by targeting signaling cascades in the phagosomal membrane such that phagosomal killing functions are activated. In the experiments presented here this strategy was initiated by the finding that AA, Cer, S1P, Sph and Pi(4,5)P_2 could stimulate actin nucleation of pathogenic *M. avium* phagosomes *in vitro*. Next, these lipids were tested in infected macrophages and showed stimulation also of actin nucleation/localisation around intracellular phagosomes. In agreement with the actin track model this increase in actin was associated with an increase in fusion. A consequence of the latter was an increase in killing of *M. avium* and *M.tb* (Kuehnel, Anes and Bos, in preparation) in infected macrophages. It should be noted that AA (but not other lipids) has been already shown to increase in *M.tb* killing, and that this lipid activates the oxidative burst phenomenon and has synergistic effects with RNI (Akaki et al., 2001). Now it seems likely that the combination of activation of the oxidative burst in combination with an increased actin nucleation on MCP might be the key to killing of intracellular pathogenic mycobacteria.

E. Summary

A number of novel observations were made that are of importance for our understanding of the intracellular behaviour of the phagosome of pathogenic mycobacteria such as *M. paratuberculosis* and *M. avium*, as well as of non-pathogenic *M. smegmatis*:

1) I developed a novel method for determination of pH, not only of phagosomes but for the whole endocytic pathway. Using this method I was able to show that the pH, not only of the phagosomes, but also other parts of the endocytic network in *M. paratuberculosis* and *M. avium*, of infected macrophages were inhibited in acidification. This effect was found to be linked to phagosomes containing pathogenic mycobacteria like *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis,* and was absolutely dependent on the viability of the organisms.

2) A detailed description of the ability of these phagosomes to fuse with late endocytic organelles was carried out. The phagosomes of *M. avium* and *M. paratuberculosis* were largely inhibited in fusion with late endocytic organelles but were seen to acquire, at least to some extent, markers found in late endocytic organelles, such as Lamp1 and 5 nm gold particles.

3) An *in vitro* system for analysing actin nucleation on MCP was developed. Using this approach it was shown for the first time that pathogenic mycobacteria, but not non-pathogenic bacteria are inhibited in nucleating actin *in vitro*. Again, this effect is linked to pathogenic mycobacteria and viability of the organisms. The results in infected macrophages were essentially identical.

4) The latter method was applied to identify effectors that affect the membrane signaling molecules regulating actin nucleation. Five lipids were found to be able to stimulate actin nucleation *in vitro*.

5) In macrophages infected with pathogenic *M. avium* three lipids (AA, Cer and S1P) were found not only to stimulate actin nucleation, but also fusion as well as to increase killing of the mycobacteria. The latter set of experiments provides proof of principle of a new approach that has the potential to increase killing of

pathogens by targeting not the bacterium itself, but the signaling cascades in the phagosomal membrane that regulate key phagosomal killing functions.

F. References

Adams, J. L., Collins, M. T., and Czuprynski, C. J. (1996) Polymerase chain reaction analysis of TNF-alpha and IL-6 mRNA levels in whole blood from cattle naturally or experimentally infected with *Mycobacterium paratuberculosis*. Can J Vet Res **60**:257-62

Adams, J. L., and Czuprynski, C. J. (1994) Mycobacterial cell wall components induce the production of TNF-alpha, IL-1, and IL-6 by bovine monocytes and the murine macrophage cell line RAW 264.7. Microb Pathog **16**:401-11

Adams, J. L., and Czuprynski, C. J. (1995) Ex vivo induction of TNF-alpha and IL-6 mRNA in bovine whole blood by *Mycobacterium paratuberculosis* and mycobacterial cell wall components. Microb Pathog **19**:19-29

Akaki, T., Tomioka, H., Shimizu, T., Dekio, S., Sato, K. (2000) Comparative roles of free fatty acids with reactive nitrogen intermediates and reactive oxygen intermediates in expression of the anti-microbial activity of macrophages against *Mycobacterium tuberculosis.* Clin Exp Immunol **121**:302-10

Al-Haddad, A., Shonn, M. A., Redlich, B., Blocker, A., Burkhardt, J. K., Yu, H., Hammer, J. A., Weiss, D. G., Steffen, W., Griffiths, G., and Kuznetsov, S. A. (2001) Interaction between phagosomes and actin filaments in vitro: myosin-V mediates ATP-dependent phagosome binding. Mol Biol Cell, in press

Allen, L. A., and Aderem, A. (1996) Molecular definition of distinct cytoskeletal structures involved in complement- and Fc receptor-mediated phagocytosis in macrophages. J Exp Med **184**: 627-37

Allen, L. H., and Aderem, A. (1995) A role for MARCKS, the alpha isozyme of protein kinase C and myosin I in zymosan phagocytosis by macrophages. J Exp Med **182**: 829-40

Allison, A. C., Davies, P., and De Petris, S. (1971) Role of contractile microfilaments in macrophage movement and endocytosis. Nat New Biol **232**: 153-5

Anastasiadis, P. Z., and Reynolds, A. B. (2001) Regulation of Rho GTPases by p120-catenin. Curr Opin Cell Biol **13**:604-10

Anthony, B. F., and Hill, H. R. (1988) Gram-positive bacteria: an overview and summary of session. Rev Infect Dis **2**:345-50

Apgar, J. R. (1995) Activation of protein kinase C in rat basophilic leukemia cells stimulates increased production of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate: correlation with actin polymerization. Mol Biol Cell **6**:97-108

Armstrong, J. A., and d'Arcy Hart, P. (1975) Phagosome-lysosome fusion interaction in cultured macrophages infected with virulent tubercle bacteria. Reversal of the usual nonfusion pattern and observations on bacterial survival. J Experim Med **142**: 1-16

Armstrong, J. A., and d'Arcy Hart, P. (1971) Response of cultured macrophages to *Mycobacterium tuberculosis*, with observations of fusion of lysosomes to phagosomes. J Exp Med **134**: 713-740

Aunis, D. (1998) Exocytosis in chromaffin cells of the adrenal medulla. Int Rev Cytol **181**: 213-320

Babior, B. M. (2000) Phagocytes and oxidative stress. Am J Med **109**: 33-44

Barker, L.P., George, K.M., Falkow, S., and Small, P.L.C. (1997) Differential trafficking of live and dead *Mycobacterium marinum* organisms in macrophages. Infect Immun **65**: 1497-1504

Barrow, W. W., Carvalho de Sousa, J. P., Davis, T. L., Wright, E. L., Bachelet, M., and Rastogi, N. (1993) Immunomodulation of human peripheral blood mononuclear cell functions by defined lipid fractions of *Mycobacterium avium*. Antimicrob Agents Chemother **37**: 652-661

Barrow, W. W., Ullom, B. P., and Brennan, P. J. (1980) Peptidoglycolipid nature of the superficial cell wall sheath of smooth-colony-forming mycobacteria. J Bacteriol **144**: 814-822

Barry CE 3rd. (2001) Interpreting cell wall 'virulence factors' of *Mycobacterium tuberculosis*.Trends Microbiol **9**:237-41

Begara-McGorum I., Wildblood L. A., Clarke C. J., Connor K. M., Stevenson K., McInnes C. J., Sharp J. M., Jones D. G. (1998) Early immunopathological events in experimental ovine paratuberculosis. Vet Immunol Immunopathol **63**:265-87

Beltan, E., Horgen, L., and Rastogi, N. (2000) Secretion of cytokines by human macrophages upon infection by pathogenic and nonpathogenic mycobacteria. Microb Pathogen **28**: 313-318

Belvin M. P., and Anderson K. V. (1996) A conserved signaling pathway: the Drosophila toll-dorsal pathway. Annu Rev Cell Dev Biol **12**:393-416

Bendixen, P. H., Bloch, B., and Jorgensen, B. (1981) Lack of intracellular degradation of *Mycobacterium paratuberculosis* by bovine macrophages infected *in vitro* and *in vivo*: Light microscopic and electron microscopic observations. Am J Vet Res **42**: 109-113

Bernstein, B. W., DeWit, M., and Bamburg, J. R. (1998) Actin disassembles reversibly during electrically induced recycling of synaptic vesicles in cultured neurons. Brain Res Mol Brain Res **53**: 236-51

Berón, W., Alvarez-Dominguez, C., Mayorga, L., and Stahl, P. D. (1995) Membrane trafficking along the phagocytic pathway. Trends Cell Biol **5**: 100-104

Blocker, A., Griffiths, G., Olivo, J. C., Hyman, A., and Severin, F. (1998) A role for microtubule dynamics in phagosome movement. J Cell Sci **111**: 303-312

Blocker, A., Severin, F. F., Burkhardt, J. K., Bingham, J. B., Yu, H., Olivo, J. C., Schroer, T. A., Hyman, A. A., and Griffiths, G. (1997) Molecular requirements for bi-directional movement of phagosomes along microtubules. J Cell Biol **137**: 113-129

Blocker, A., Severin, F. F., Habermann, A., Hyman, A. A., Griffiths, G., and Burkhardt, J. K. (1996) Microtubule-associated protein-dependent binding of phagosomes to microtubules. J Biol Chem **271**: 3803-11

Bornfeldt, K. E., Graves, L. M., Raines, E. W., Igarashi, Y., Wayman, G., Yamamura, S., Yatomi, Y., Sidhu, J. S., Krebs, E. G., and Hakomori, S., (1995) Sphingosine-1-phosphate inhibits PDGF-induced chemotaxis of human arterial smooth muscle cells: spatial and temporal modulation of PDGF chemotactic signal transduction. J Cell Biol **130**:193-206

Bonecini-Almeida M., Chitale, S., Boutsikakis, I., Geng, J., Doo, H., He, S., and Ho, J. L. (1998) Induction of *in vitro* human macrophage anti-*Mycobacterium tuberculosis* activity: requirement for IFN-gamma and primed lymphocytes. J. Immunol **160**:4490-99

Boxer, L. A., Hedley-Whyte, E. T., and Stossel, T. P. (1974) Neutrophil action dysfunction and abnormal neutrophil behavior. N Engl J Med **291**: 1093-9

Brennan, P. J., and Goren, M. B. (1979) Structural studies on the type-specific antigens and lipids of the *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium scrofulaceum* serocomplex, *Mycobacterium intracellulare* serotype 9. J Biol Chem **254**: 4205-4211

Bubb, M. R., Baines, I. C., Korn, E. D. (1998) Localization of actobindin, profilin I, profilin II, and phosphatidylinositol-4,5-bisphosphate (PIP2) in Acanthamoeba castellanii. Cell Motil Cytoskeleton **39**:134-46

Bucci, C., Parton, R. G., Mather, I. H., Stunnenberg, H., Simons, K., Hoflack, B., and Zerial, M. (1992) The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway. Cell **70**: 715-28

Burgoyne, R. D., and Cheek, T. R. (1987) Reorganisation of peripheral actin filaments as a prelude to exocytosis. Biosci Rep **7**: 281-8

Burgoyne, R. D., Morgan, A., Robinson, I., Pender, N., and Cheek, T. R. (1993) Exocytosis in adrenal chromaffin cells. J Anat **183**: 309-14

Cain, C. C., Sipe, D. M., and Murphy, R. F. (1989) Regulation of endocytic pH by the Na+,K+/-ATPase in living cells. Proc Natl Acad Sci U S A. **86**: 544-548

Burridge, K., Chrzanowska-Wodnicka, M. (1996) Focal adhesions, contractility, and signaling. Annu Rev Cell Dev Biol **12**:463-518

Carlier, M. F. (1998) Control of actin dynamics. Curr Opin Cell Biol **10**:45-51

Caron, E., Hall, A. (1998) Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. Science **282**:1717-21

Carpenter, C. L., and Cantley, L. C. (1996) Phosphoinositide kinases. Curr Opin Cell Biol **8**:153-8

Carraway, K. L., and Carraway, C. A. (1989) Membrane-cytoskeleton interactions in animal cells. Biochim Biophys Acta **988**: 147-71

Carroll, M. C. (1998) The role of complement and complement receptors in induction and regulation of immunity. Annu Rev Immunol **16**: 545-68

Cassimeris, L., Safer, D., Nachmias, V. T., and Zigmond, S. H. (1992) Thymosin beta 4 sequesters the majority of G-actin in resting human polymorphonuclear leukocytes. J Cell Biol **119**:1261-70

Chan, A. Y., Raft, S., Bailly, M., Wyckoff, J. B., Segall, J. E., and Condeelis, J. S.(1998) EGF stimulates an increase in actin nucleation and filament number at the leading edge of the lamellipod in mammary adenocarcinoma cells. J Cell Sci **111**:199-211

Chan, A., Raft, S., Bailly, M., Wyckoff, J., Segall, J., and Condeelis, J. (1998) EGF stimulates an increase in actin nucleation and filament number at the leading edge of the lamellipod in mammary adenocarcinoma cells. J Cell Sci **111**: 199-211

Chan, J., and Flynn, J. L. (2000). Latent and reactivation tuberculosis. Einstein Q. In press

Chan, J., and Kaufmann, S. H. E. (1994) Immune mechanisms of protection. In Tuberculosis: Pathogenesis, Protection and Control, ed. BR BloomWashington, DC: Am Soc Microbio, pp. 389-415

Chavrier, P., Parton, R. G., Hauri, H. P., Simons, K., and Zerial, M. (1990) Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. Cell **62**: 317-29

Cheek, T. R., and Burgoyne, R. D. (1986) Nicotine-evoked disassembly of cortical actin filaments in adrenal chromaffin cells. FEBS Lett **207**: 110-4

Chiodini, R. J., van Kruiningen, H. J., and Merkal, R. S. (1984) Ruminant paratuberculosis (Johne's disease): The current status and future prospects. Cornell Vet **74**:218-262

Chiodini, R.J. (1989) Crohn's disease and the mycobacteriosis: a review and comparison of two disease entities. Clin Microbiol Rev **2**: 90-117

Clague, M. J. (1998) Molecular aspects of the endocytic pathway. Biochem J **336**: 271-282

Clarke, C. J. (1997) The pathology and pathogenesis of paratuberculosis in ruminants and other species. J Comp Path **116**: 217-261

Claus, V., Jahraus, A., Tjelle, T., Berg, T., Kirschke, H., Faulstich, H., and Griffiths, G. (1998) Lysosomal enzyme trafficking between phagosomes, endosomes, and lysosomes in J774 macrophages. Enrichment Of cathepsin h in early endosomes. J Biol Chem **273**: 9842-9851

Clemens, D. L. (1996) Characterization of the *Mycobacterium tuberculosis* phagosome. Trends Microbiol **4:** 113-118

Clemens, D. L., and Horwitz, M.A. (1995) Characterization of the *Mycobacterium tuberculosis* phagosome and evidence that phagosomal maturation is inhibited. J Exp Med **181:** 257-270

Cocito, C., Gilot, P., Coene, M., De Kesel, M., Poupart, P., and Vannuffel, P. (1994) Paratuberculosis. Clin Microbiol Rev **7:** 328-345

Cossart P., and Lecuit, M. (1998) Interactions of *Listeria monocytogenes* with mammalian cells during entry and actin-based movement: bacterial factors, cellular ligands and signaling. EMBO J **17**:3797-806

Cox, D., Lee, D. J., Dale, B. M., Calafat, J., and Greenberg, S. (2000) A Rab11-containing rapidly recycling compartment in macrophages that promotes phagocytosis. Proc Natl Acad Sci U S A **97**: 680-5

Cramer, L. P., Siebert, M., and Mitchison, T. J. (1997) Identification of novel graded polarity actin filament bundles in locomoting heart fibroblasts: implications for the generation of motile force. J Cell Biol **136**: 1287-1305

Crowle A, Ross, E. J., May, M. H. (1987) Inhibition by 1,25(OH)2-vitamin D3 of the multiplication of virulent tubercle bacilli in cultured human macrophages. Infect. Immun **55**:2945-50

Crowle, A. J., Dahl, R., Ross, E., and May, M. H. (1991) Evidence that vesicles containing living, virulent *Mycobacterium tuberculosis* or *Mycobacterium avium* in cultured human macrophages are not acidic. Infect Immun **59:** 1823-1831

Cudmore, S., Cossart, P., Griffiths, G., Way, M. (1995) Actin-based motility of vaccinia virus.Nature. **378**:636-8

D'Arcy Hart, P., Armstrong, J. A., Brown, C. A., and Draper, P. (1972) Ultrastructural study of the behavior of macrophages toward parasitic mycobacteria. Infect Immun **5**:803-7

D'Arcy Hart, P. (1975) Response of macrophages to bacterial infection with special reference to their lysosomes. Pathol Biol (Paris) **23**:451-2

De Corte, V., Gettemans, J., and Vandekerckhove, J. (1997) Phosphatidylinositol 4,5-bisphosphate specifically stimulates PP60(c-src) catalyzed phosphorylation of gelsolin and related actin-binding proteins. FEBS Lett **401**:191-6

Defacque, H., Bos, E., Garvalov, B., Barret, C., Roy, C., Mangeat, P., Shin, H. W., Rybin, V., and Griffiths, G. (2002) Phosphoinositides Regulate Membrane-dependent Actin Assembly by Latex Bead Phagosomes. Mol Biol Cell **13**:1190-202

Defacque, H., Egeberg, M., Antzberger, A., Ansorge, W., Way, M., and Griffiths, G. (2000) Actin assembly induced by polylysine beads or purified phagosomes: quantitation by a new flow cytometry assay. Cytometry **41**:46-54

Defacque, H., Egeberg, M., Habermann, A., Diakonova, M., Roy, C., Mangeat, P., Voelter, W., Marriott, G., Pfannstiel, J., Faulstich, H., Griffiths, and G. (2000) Involvement of ezrin/moesin in *de novo* actin assembly on phagosomal membranes. EMBO J **19**:199-212

Denis, M. (1991) Killing of *Mycobacterium tuberculosis* within human monocytes: activation by cytokines and calcitriol. Clin Exp Immunol **84**:200-6

Deretic, V., and Fratti, R.A. (1999) Mycobacterium tuberculosis phagosome. Mol Microbiol **31**: 1603-1609

Deretic, V., Via, L., Fratti, R. A., and Deretic, D. (1997) Mycobacterial phagosome maturation, rab proteins, and intracellular trafficking. Electrophoresis **18:** 2542-2547

Desjardins, M. (1995) Biogenesis of phagolysosomes: the 'kiss and run' hypothesis. Trends Cell Biol **5:** 183-186

Desjardins, M., Celis, J. E., van Meer, G., Dieplinger, H., Jahraus, A., Griffiths, G., and Huber, L. A. (1994) Molecular characterization of phagosomes. J Biol Chem **269**: 32194-32200

Desjardins, M., Huber, L. A., Parton, R. G., and Griffiths, G. (1994) Biogenesis of phagolysosomes proceed through a sequential series of interactions with the endocytic apparatus. J Cell Biol **124:** 677-688

Desjardins, M., Nzala, N. N., Corsini, R., and Rondeau, C. (1997) Maturation of phagosomes is accompanied by changes in their fusion properties and size-selective acquisition of solute materials from endosomes. J Cell Sci **110**: 2303-2314

Ding, A. H., Nathan, C., Stuehr, D. (1988) Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. J Immunol **141**:2407-12

Douvas, G., Looker, D. L., Vatter, A. E., and Crowle, A. J. (1985) Gamma interferon activates human macrophages to become tumoricidal and

leishmanicidal but enhances replication of macrophage-associated mycobacteria. Infect Immun **50**:1-8

Duclos, S., Diez, R., Garin, J., Papadopoulou, B., Descoteaux, A., Stenmark, H., and Desjardins, M. (2000) Rab5 regulates the kiss and run fusion between phagosomes and endosomes and the acquisition of phagosomeleishmanicidal properties in RAW 264.7 macrophages J Cell Sci. **113**:3531-41

Duclos, S., and Desjardins, M. (2000) Subversion of a young phagosome: the survival strategies of intracellular pathogens. Cell Microbiol **2**: 365-377

Egeberg, M. Kjeken, R., and Griffiths, G. – manuscript in preparation

Eddy, R. J., Han, J., and Condeelis, J. S. (1997) Capping protein terminates but does not initiate chemoattractant- induced actin assembly in Dictyostelium. J Cell Biol **139**: 1243-1253

Flesch, I., and Kaufmann, S. H. E. (1990) Activation of tuberculostatic macrophage functions by gamma interferon, interleukin-4, and tumor necrosis factor. Infect Immun **58**:2675-77

Flesch, I., and Kaufmann, S. (1987) Mycobacterial growth inhibition by interferon-g activated bone marrow macrophages and differential susceptibility among strains of Mycobacterium tuberculosis. J Immunol **138**:4408-13

Frehel, C., de Chastellier, C., Lang, T., and Rastogi, N. (1986) Evidence for inhibition of fusion of lysosomal and prelysosomal compartments in macrophages infected with pathogenic *Mycobacterium avium*. Infect Immun **52**: 252-262

Fuchs, R., Schmid, S., and Mellman, I. (1989) A possible role for Na+,K+-ATPase in regulating ATP-dependent endosome acidification. Proc Natl Acad Sci U S A **2**: 539-543

Fukami, K., Endo, T., Imamura, M., and Takenawa, T. (1994) alpha-Actinin and vinculin are PIP2-binding proteins involved in signaling by tyrosine kinase. J Biol Chem **269**:1518-22

Funato, K., Beron, W., Yang, C. Z., Mukhopadhya,y A., and Stahl, P. D. (1997) Reconstitution of phagosome-lysosome fusion in streptolysin O- permeabilised cells. J Biol Chem **272**: 16147-16151

Garin, J., Diez, R., Kieffer, S., Dermine, J. F., Duclos, S., Gagnon, E., Sadoul, R., Rondeau, C., and Desjardins, M. (2001) The phagosome proteome: insight into phagosome functions. J Cell Biol 2001 Jan **152**:165-80

Gercken, J., Pryjma, J., Ernst, M., and Flad, H. D. (1994) Defective antigen presentation by Mycobacterium tuberculosis-infected monocytes. Infect Immun **62**:3472-8

Goethe, R., Kuehnel, M. P. Darjii, A., Weiss, S., Rohde, M., Gerlach, G.F., and Valentin-Weigand, P. (1999) Interactions of *Mycobacterium avium* subsp. *paratuberculosis* with murine macrophage: Intracellular survival and modulation of macrophage functions. Colloquium on Paratuberculosis, Melburne, Australia

Gondo, H., Kudo, J., White, J. W., Barr, C., Selvanayagam, P., and Saunders, G. F. (1987) Nucleotide, OMIM, Protein Differential expression of the human thymosin-beta 4 gene in lymphocytes, macrophages, and granulocytes. J Immunol **139**:3840-8

Gonzalez, L. Jr., and Scheller, R. H. (1999) Regulation of membrane trafficking: structural insights from a Rab/effector complex. Cell **96**: 755-8

Goodfellow, M., and Wayne, L. G. (1982) Taxonomy and momenclatur. In: The biology of the mycobacteria, Vol. 1. Physiology, indentification and classification. Academic Press, London 471-521

Gordon, A. H, Hart, P. D., and Young, M. R. (1980) Ammonia inhibits phagosome-lysosome fusion in macrophages. Nature **286**:79-80

Goren, M. B., D'Arcy Hart, P., Young, M. R., Armstrong, J. A.(1976) Prevention of phagosome-lysosome fusion in cultured macrophages by sulfatides of Mycobacterium tuberculosis. Proc Natl Acad Sci U S A **73**:2510-4

Goren, M. B. (1970) Sulfolipid I of Mycobacterium tuberculosis, strain H37Rv. I. Purification and properties.Biochim Biophys Acta **210**:116-26

Gratacap, M. P., Payrastre, B., Viala, C., Mauco, G., Plantavid, M., Chap, H. (1998) Phosphatidylinositol 3,4,5-trisphosphate-dependent stimulation of phospholipase C-gamma2 is an early key event in FcgammaRIIA-mediated activation of human platelets. J Biol Chem **273**:24314-21

Greenberg, S., Chang, P., and Silverstein, S. C. (1993) Tyrosine phosphorylation is required for Fc receptor-mediated phagocytosis in mouse macrophages. J Exp Med **177**:529-34

Griffin, F. M. Jr., Griffin, J. A., and Silverstein, S. C. (1976) Studies on the mechanism of phagocytosis. II. The interaction of macrophages with anti-immunoglobulin IgG-coated bone marrow-derived lymphocytes. J Exp Med **144**: 788-809

Griffiths, G. (1996) On vesicles and membrane compartments. Protoplasma **195**:37-58

Griffiths, G., Back, R., and Marsh, M. (1989) A quantitative analysis of the endocytic pathway in baby hamster kidney cells. J Cell Biol **109**:2703-20

Griffiths, G., Matteoni, R., Back, R., and Hoflack, B. (1990) Characterization of the cation-independent mannose 6-phosphate receptor-enriched prelysosomal compartment in NRK cells. J Cell Sci **95**:441-61

Guerin, I., and de Chastellier, C. (2000) Pathogenic mycobacteria disrupt the macrophage actin filament network. Infect Immun **68**: 2655-62

Guillemot, J. C., Montcourrier, P., Vivier, E., Davoust, J., and Chavrier, P. (1997) Selective control of membrane ruffling and actin plaque assembly by the Rho GTPases Rac1 and CDC42 in FcepsilonRI-activated rat basophilic leukemia (RBL-2H3) cells. J Cell Sci **110**:2215-25

Ha, K. S., and Exton, J. H. (1993) Activation of actin polymerization by phosphatidic acid derived from phosphatidylcholine in IIC9 fibroblasts. J Cell Biol **123**:1789-96

Haas, A. (1998) Reprogramming the phagocytic pathway--intracellular pathogens and their vacuoles Mol Membr Biol **15**:103-21

Haas, A. (1998) NSF-fusion and beyond. Trends Cell Biol **8**: 471-3

Hackam, D. J., Rotstein, O. D., Bennett, M. K., Klip, A., Grinstein, S., and Manolson, M. F. (1996) Characterization and subcellular localization of target membrane soluble NSF attachment protein receptors (t-SNAREs) in

macrophages. Syntaxins 2, 3, and 4 are present on phagosomal membranes. J Immunol **156**: 4377-83

Hackam, D. J., Rotstein, O.D., Zhang, W.J., Demaurex, N., Woodside, M., Tsai, O., and Grinstein, S. (1997) Regulation of phagosomal acidification. Differential targetting of Na+/H+ exchangers, Na+/K+-ATPases, and vacuolar-type H+-ATPases. J Biol Chem **272:**29810-20

Hall, A. (1998) G proteins and small GTPases: distant relatives keep in touch. Science **280**:2074-5

Hall, A., and Nobes, C. D. (2000) Rho GTPases: molecular switches that control the organization and dynamics of the actin cytoskeleton. Philos Trans R Soc Lond B Biol Sci **355**:965-70

Harding, C. V. (1995) Intracellular organelles involved in antigen processing and the binding of peptides to class II MHC molecules. Semin Immunol **7**: 355-60

Harth, G., Zamecnik, P. C., Tang, J. Y., Tabatadze, D., and Horwitz, M. A. (2000) Treatment of *Mycobacterium tuberculosis* with antisense oligonucleotides to glutamine synthetase mRNA inhibits glutamine synthetase activity, formation of the poly-L-glutamate/glutamine cell wall structure, and bacterial replication. Proc Natl Acad Sci U S A **97**:418-23

Harth, G., Clemens, D. L., and Horwitz, M. A. (1994) Glutamine synthetase of Mycobacterium tuberculosis: extracellular release and characterization of its enzymatic activity. Proc Natl Acad Sci U S A **91**:9342-6

Harth, G., and Horwitz, M. A. (1999) An inhibitor of exported Mycobacterium tuberculosis glutamine synthetase selectively blocks the growth of pathogenic

mycobacteria in axenic culture and in human monocytes: extracellular proteins as potential novel drug targets. Exp Med **189**:1425-36

Harth, G., and Horwitz, M. A. (1999) Export of recombinant Mycobacterium tuberculosis superoxide dismutase is dependent upon both information in the protein and mycobacterial export machinery. A model for studying export of leaderless proteins by pathogenic mycobacteria. J Biol Chem **274**:4281-92

Hartwig, J. H., Bokoch, G. M., Carpenter, C. L, Janmey, P. A., Taylor, L. A., Toker, A., and Stossel, P. T. (1995) Thrombin receptor ligation and activated rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. Cell **82**:643-653

Hartwig, J. H., and Stossel, T. P. (1982) Macrophages: their use in elucidation of the cytoskeletal roles of actin. Methods Cell Biol **25**: 201-225

Hasan, Z., Schlax, C., Kuhn, L., Lefkowits, I., Young, D., Thole, J., and Pieters, J. (1997) Isolation and characterization of the mycobacterial phagosome: segregation from the endosomal/lysosomal pathway. Mol Microbiol **24:** 545-553

Hellio, R., Fréhel, C., Rauzier, J. Y., and Rastogi, N. (1988) Electron cytochamistry of mycobacteria: evidence that strongly acidic sulfate groups are present on the surface of H37Rv (virulent) strain of *Mycobacterium tuberculosis*. Curr Microbiol **17**: 235-242

Higgs, H. N., and Pollard, T. D. (2001) REGULATION OF ACTIN FILAMENT NETWORK FORMATION THROUGH ARP2/3 COMPLEX: Activation by a Diverse Array of Proteins. Annu Rev Biochem **70**:649-676

Hooper, L. C., and Barrow, W. W. (1988) Decreased mitogenic response of murine spleen cells following intraperitoneal injection of serovar-specific

glycopeptidolipid antigens from the *Mycobacterium avium* complex. Adv Exper Med Biol **239**: 309-325

Hooper, L. C., Johnson, M. M., Khera, V. R., and Barrow, W. W. (1986) Macrophage uptake and retention of radiolabeled glycopeptidolipid antigens associated with the superficial L1 layer of *Mycobacterium intracellulare* aerovar 20. Infect Immun **54**: 133-141

Horgen, L., Barrow, E. L., Barrow, W. W., and Rastogi, N. (2000) Exposure of human peripheral blood mononuclear cells to total lipids and serovar-specific glycopeptidolipids from *Mycoabcerium avium* serovars 4 and 8 results in inhibition of TH1-type- responses. Microb Pathogen **29**: 9-16

Indik, Z. K., Park, J. G., Hunter, S., and Schreiber, A. D. (1995) The molecular dissection of Fc gamma receptor mediated phagocytosis. Blood **86**: 4389-99

Ireton, K., Payrastre, B., Chap, H., Ogawa, W., Sakaue, H., Kasuga, M., and Cossart, P. (1996) A role for phosphoinositide 3-kinase in bacterial invasion. Science **274**:780-2

Jahraus, A., Storrie, B., Griffiths, G., and Desjardins, M. (1994) Evidence for retrograde traffic between terminal lysosomes and the prelysosomal/late endosome compartment. J Cell Sci **107**: 145-57

Jahraus, A., Tjelle, T. E., Berg, T., Habermann, A., Storrie, B., Ullrich, O., and Griffiths, G. (1998) *in vitro* fusion of phagosomes with different endocytic organelles from J774 macrophages. J Biol Chem **273**: 30379-30390

Jahraus, A., Egeberg, M., Hinner, B., Habermann, A., Sackman, E., Pralle, A., Faulstich, H., Rybin, V., Defacque, H., and Griffiths, G. (2001) ATP-dependent

membrane assembly of F-actin facilitates membrane fusion. Mol Biol Cell **12**:155-70

Jark, U., Ringena, I., Franz, B., Gerlach, G. F., Eyerbach, M., and Franz, B. (1997) Development of an ELISA technique for serodiagnosis of bovine paratuberculosis. Vet Microbiol **57**: 189-198

Joiner, K. A., Fuhrmann, S. A., Miettinen, H. M., Jasper, L. H., and Mellmann, L. (1990) *Toxoplasma gondii*: fusion competence of parasitophorus vacuole in Fc receptor transfected fibroblasts. Science **249:** 641-646

Jones, R. L. (1989) Review of the economic impact of Johne's disease in the United States,. In Johne's disease. Current trends in research, diagnosis and management. Miller, A.R. and Wood P.R. (ed.), Common-Wealth Scientific and Industrial Research Organisation, Melbourne, Australia, pp. 46-50

Kaplan, G. (1977) Differences in the mode of phagocytosis with Fc and C3 receptors in macrophages. Scand J Immunol **6**: 797-807

Keenan, C., and Kelleher, D. (1998) Protein kinase C and the cytoskeleton. Cell Signal **10**:225-32

Kocks, C., Marchand, J. B., Gouin, E., d'Hauteville, H., Sansonetti, P. J., Carlier, M. F., Cossart, P. (1995) The unrelated surface proteins ActA of Listeria monocytogenes and IcsA of Shigella flexneri are sufficient to confer actin-based motility on Listeria innocua and Escherichia coli respectively. Mol Microbiol **18**:413-23

Komjin, R. E., de Hass, P. E. W., Schneider, M. M. E., Eger, T., Nieuwenhuijs, J. H. M., van den Hoek, R. K., Bakker, D., van Zijderveld, F. G., and van Soolingen D. (1999) Prevalence of *Mycobacterium avium* in slaughter pigs in the

Netherlands and comparison of IS1245 restriction fragment length polymorphism patterns of porcine and human isolates. J Clin Microbiol **37**: 1254-1259

Koets, A. P., Rutten, V. P. M. G., Bakker, D., Muller, K.E., and VanEden, W. (1999) The T1 to T2 shift in bovine paratuberculosis and the role of activation of induced cell death, in: Proceedings of the Sixth international Colloquium on Paratuberculosis, Melburne, Australia

Kuehnel, M. P., Goethe, R., Habermann, A., Mueller, E., Rhode, M., Griffiths, G., and Valentin-Weigand, P. (2001) Characterization of the intracellular survival of *Mycobacterium avium* ssp. *paratuberculosis*: phagosomal pH and fusogenicity in J774 macrophages compared with other mycobacteria. Cell Microbiol **3**:551-566

Kuehnel, M. P. (1998) Charakterisierung der Internalisierung von *Mycobacterium avium* subspec. *paratuberculosis* in murine und bovine Makrophagen. Diplomarbeit

Kwiatkowski, D. J. (1999) Functions of gelsolin: motility, signaling, apoptosis, cancer. Curr Opin Cell Biol **11**:103-8

Lamaze, C., Fujimoto, L. M., Yin, H. L., and Schmid, S. L. (1977) The actin cytoskeleton is required for receptor-mediated endocytosis in mammalian cells. J Biol Chem **272**: 20332-20335

Lang, T., de Chastellier, C., Ryter, A., and Thilo, L. (1988) Endocytic membrane traffic with respect to phagosomes in macrophages infected with non-pathogenic bacteria: phagosomal membrane acquires the same composition as lysosomal membrane. Eur J Cell Biol **46**: 39-50

Lang, T., Wacker, I., Wunderlich, I., Rohrbach, A., Giese, G., Soldati, T., and Almers, W. (2000) Role of actin cortex in the subplasmalemmal transport of secretory granules in PC-12 cells. Biophys J **78**: 2863-77

Lasa, I., Dehoux, P., Cossart, P. (1998) Actin polymerization and bacterial movement. Biochim Biophys Acta **1402**:217-28

Legrand, E., Goh, K. S., Sola, Ca., and Rastogi, N. (2000) Description of a novel *Mycobacterium simiae* allelic variant isolated from Caribbean AIDS patients by PCR-restriction enzyme analysis and sequencing of *hsp* 65 gene. Molec Cell Probes **14**: 355-363

Lehmann, K. B., and Neumann, R. (1896) Atlas und Grundriss der Bakteriologie und Lehrbuch der speciellen bakteriologischen Diagnostik, 1st Ed., Munich

Li, R., Zheng, Y., and Drubin, D. G. (1995) Regulation of cortical actin cytoskeleton assembly during polarised cell growth in budding yeast. J Cell Biol **128**: 599-615

Liang, E., and Hughes, H. (1998) Characterisation of a pH-sensitive surfactant, dodecyl 1-2-(1'-imidazolyl)propionate (DIP), and preliminary studies in liposome mediated gene transfer. Biochim Biophys Acta **1369**: 39-50

Lopez-Martin, L. M., Quesada, D., Lakhadar-Ghazal, F., Tocanne, J. F., and Lanéelle, G. (1994) Interactions of mycobacterial glycopeptidolipids with membranes: influence of carbohydrate on induced alterations. Biochemistry **33**: 7056-7061

Lurie, M. (1942) Studies on the mechanism of immunity in tuberculosis. The fate of tubercle bacilli ingested by mononuclear phagocytes derived from intracellular bacteria. Immunol Today **19**: 491-94

Mackaness, G. (1969) The influence of immunologically committed lynphoid cells on macrophage activation *in vivo*. J Exp Med **129**: 973

Martin, T. F. (1998) Phosphoinositide lipids as signaling molecules: common themes for signal transduction, cytoskeletal regulation, and membrane trafficking. Annu Rev Cell Dev Biol **14**:231-64

McBride, H. M., Rybin, V., Murphy, C., Giner, A., Teasdale, R., and Zerial, M. (1999) Oligomeric complexes link Rab5 effectors with NSF and drive membrane fusion via interactions between EEA1 and syntaxin 13. Cell **98**: 377-86

McDonough, K. A., Kress, Y., and Bloom, B. R. (1993) Pathogenesis of tuberculosis: interaction of *Mycobacterium tuberculosis* with macrophages. Infect Immun **61:** 2763-2773

Meerschaert, K., De Corte, V., De Ville, Y., Vandekerckhove, J., and Gettemans, J. (1998) Gelsolin and functionally similar actin-binding proteins are regulated by lysophosphatidic acid. EMBO J **17**:5923-32

Mellman, I., Fuchs, R., and Helenius, A. (1986) Acidification of the endocytic and exocytic pathways. Anua. Rev Biochem **55**: 663-670

Meresse, S., Gorvel, J. P., and Chavrier, P. (1995) The rab7 GTPase resides on a vesicular compartment connected to lysosomes. J Cell Sci **108**: 3349-58

Middlebrook, G. (1982) Tuberculosis and medical science. Am Rev Respir Dis **125**:4-7

Momotani, E., Whipple, D. L., Thiermann, A. B. and Cheville, N.F. (1988) Role of M cells and macrophages in the entrance of *Mycobacterium paratuberculosis* into domes of ileal Peyer's patches in calves. Vet Pathol **25**: 131-137

Mooseker, M. S., and Tilney, L. G. (1975) Organization of an actin filament-membrane complex. Filament polarity and membrane attachment in the microvilli of intestinal epithelial cells. J Cell Biol **67**: 725-43

Mosleh, I. M., Huber, L. A., Steinlein, P., Pasquali, C., Gunther, D., and Meyer, T. F. (1998) Neisseria gonorrhoeae porin modulates phagosome maturation. J Biol Chem **273**: 35332-8

Muallem, S., Kwiatkowska, K., Xu, X., and Yin, H. L. (1995) Actin filament disassembly is a sufficient final trigger for exocytosis in nonexcitable cells. J Cell Biol **128**: 589-598

Muller, W. A., Steinman, R. M., and Cohn, Z. A. (1983) Membrane proteins of the vacuolar system. III. Further studies on the composition and recycling of endocytic vacuole membrane in cultured macrophages. J Cell Biol **96**: 29-36

Nobes, C. D., and Hall, A. (1995) Rho, rac and cdc42 GTPases: regulators of actin structures, cell adhesion and motility.Biochem Soc Trans **23**:456-9

Norman, J. C., Price, L. S., Ridley, A. J., Hall, A., Koffer, A. (1994) Actin filament organization in activated mast cells is regulated by heterotrimeric and small GTP-binding proteins. J Cell Biol **126**:1005-15

Novick, P., and Zerial, M. (1997) The diversity of Rab proteins in vesicle transport. Curr Opin Cell Biol **9**: 496-504

O'Brien, L., Roberts, B., and Andrew, P. W. (1996) *In vitro* interaction of *Mycobacterium tuberculosis* and macrophages: activation of anti-mycobacterial activity of macrophages and mechanisms of anti-mcobacterial activity. Curr Top Microbiol Immunol **215**: 97-130

Oh, Y. K., and Swanson, J. A. (1996) Different fates of phagocytosed particles after delivery into macrophage lysosomes. J Cell Biol **132**: 585-93

Oh, Y.K., and Straubinger, R.M. (1996) Intracellular fate of *Mycobacterium avium*: Use of dual-label spectrofluorometry to invastigate the influence of bacterial viability and opsonization on phagosomal pH and phagosome-lysosome interaction. Infect Immun **64**: 319-325

Ohkuma, S., and Poole, B. (1978) Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc Natl Acad Sci USA **75**: 3327-31

Orci, L., Gabbay, K. H., and Malaisse, W. J. (1972) Pancreatic beta-cell web: its possible role in insulin secretion. Science **175**: 1128-30

Ottenhof, T. H., Kumararatne, D., and Casanova, J. L. (1998) Novel human immunodeficiencies reveal the essential role of type-1 cytokines in immunity to normal and immunized animals. J Exp Med **75**: 247

Pantaloni, D., Le Clainche, C., and Carlier, M. F. (2001) Mechanism of actin-based motility. Science **292**:1502-6

Pfeffer, S. R. (1999) Transport-vesicle targeting: tethers before SNAREs. Nat Cell Biol **1**: 17-22

Pistor, S., Chakraborty, T., Niebuhr, K., Domann, E., and Wehland, J. (1994) The ActA protein of Listeria monocytogenes acts as a nucleator inducing reorganization of the actin cytoskeleton. EMBO J **13**:758-63

Pitt, A., Mayorga, L. S., Schwartz, A. L., and Stahl, P. D. (1992) Transport of phagosomal components to an endosomal compartment. J Biol Chem **267**: 126-32

Pitt, A., Mayorga, L. S., Stahl, P. D., and Schwartz, A. L. (1992) Alterations in the protein composition of maturing phagosomes. J Clin Invest **90**: 1978-83

Pommier, C. G., Inada, S., Fries, L. F., Takahashi, T., Frank, M. M., and Brown, E. J. (1983) Plasma fibronectin enhances phagocytosis of opsonised particles by human peripheral blood monocytes. J Exp Med **157**: 1844-54

Rabinovitch, M. (1995) Professional and non-professional phagocytes: an introduction. Trends cell biol **5**: 85-87

Rabinowitz, S., Horstmann, H., Gordon, S., and Griffiths, G. (1992) Immunocytochemical characterization of the endocytic and phagolysosomal compartments in peritoneal macrophages. J Cell Biol **116**: 95-112

Ralph, P., Prichard, J., and Cohn, M. (1975) Reticulum cell sarcoma: an effector cell in antibody-dependent cell-mediated immunity. J Immunol **114**: 898-905

Ramachandra, L., Noss, E., Boom, W. H., and Harding, C. V. (1999) Phagocytic processing of antigens for presentation by class II major histocompatibility molecules. Cell Microbiol **1**: 205-214

Ramachandra, L., Song, R., and Harding, C. V. (1999) Phagosomes are fully competent antigen-processing organelles that mediate the formation of peptide:class II MHC complexes. J Immunol **162**: 3263-72

Rameh, L. E., Tolias, K. F., Duckworth, B. C., and Cantley, L. C. (1997) A new pathway for synthesis of phosphatidylinositol-4,5-bisphosphate. Nature **390**:192-96

Ramesh, N., Anton, I. M., Martinez-Quiles, N., and Geha, R. S. (1999) Waltzing with WASP. Trends Cell Biol **9**:15-9

Rando, O. J., Zhao, K., and Crabtree, G. R. (2000) Searching for a function for nuclear actin. Trends Cell Biol **10**: 92-7

Rastogi, N. (1990) 5th forum in microbiology – killing intracellular mycobacteria: dogmas and realities. Res Microbiol **141**: 191-270

Rastogi, N. (1993) Mycobacteria as intracellular pathogens: current notions of pathogenicity, virulence, and drug resistance and their theri relationship to effective therapy. In: Antimicrobial agents and intracellular pathogens. CRC Press, Boca Raton, Florida 245-300

Rastogi, N., and Barrow, W. W. (1994) 11th forum in microbiology – laboratory and clinical aspects of the *Mycobacterium avium* epidemic: contributing factors associated with variability of drug susceptibility and immune responsiveness, and the multifaceted nature of pathogenicity. Res Microbiol **145**: 167-261

Rastogi, N., and Blom-Potar, M. C. (1990) A comparative study on the activation of J-774 macrophage-like cells by gamma-interferon, 1,25 dihydroxyvitamin D_3 and lipopetide RP-56142: Ability to kill intracellularly multiplying Mycobacterium tuberculosis and *Mycobacterium avium*. Zbl Bakt **273**: 344-361

Rastogi, N., and David, H. L. (1988) Mechanisms of pathogenicity in mycobacteria. Biochimie **70**: 1101-1120

Rastogi, N., Blom-Potar, M. C., and David, H. L. (1989) Comparative intracellular growth of difficult-to-grow and other mycobacteria in a macrophage cell line. Acta Leprol **7**: 156-159

Ravetch, J. V. (1997) Fc receptors. Curr Opin Immunol **9**: 121-5

Ravetch, J. V., and Clynes, R. A. (1998) Divergent roles for Fc receptors and complement *in vivo*. Annu Rev Immunol **16**: 421-32

Reyrat, J. M., Berthet, F. X., and Gicquel, B. (1995) The urease locus of *Mycobacterium tuberculosis* and its utilization for the demonstration of allelic exchange in Mycobacterium bovis bacillus Calmette-Guerin. Proc Natl Acad Sci U S A **92**:8768-72

Reyrat, J. M., Lopez-Ramirez, G., Ofredo, C., Gicquel, B., and Winter, N. (1996) Urease activity does not contribute dramatically to persistence of Mycobacterium bovis bacillus Calmette-Guerin. Infect Immun **64**:3934-6

Ridley, A. J. (1997) The GTP-binding protein Rho. Int J Biochem Cell Biol **29**:1225-9

Riezman, H., Munn, A., Geli, M. I., and Hicke, L. (1996) Actin-, myosin- and ubiquitin-dependent endocytosis. Experientia **52**: 1033-41

Rigby, W. (1988). The immunobiology of vitamin D. Immunol Today **9**: 54-58

Rockett, K., Brookes, R., Udalova, I., Vidal, V., Hill, A. V., and Kwiatkowski, D. (1998) 1,25-Dihydroxyvitamin D3 induces nitric oxide synthase and suppresses growth of *Mycobacterium tuberculosis* in a human macrophage-like cell line. Infect Immun **66**: 5314-21

Rook, G. A. W. (1988) The role of vitamin D in tuberculosis. Am Rev Respir Dis **138**: 768-70

Rook, G. A. W., Steele, J., Ainsworth, M., and Champion, B. R. (1986). Activation of macrophages to inhibit proliferationof *Mycobacterium tuberculosis*: comparison of the effects of recombinant gamma interferon on human monocytes and murine peritoneal macrophages. Immunol **59**: 333-38

Rulong, S., Aguas, A. P., Da Silva, P. P., and Silva M. T. (1991) Intramacrophagic *Mycobacterium avium* bacilli are coated by a multiple lamellar structure: freeze fracture analysis of infected mouse liver. Infect Immun **59**: 3895-3902

Russell, D. G., Dant, J., and Sturgill-Koszycki, S. (1996) *Mycobacterium avium*- and *Mycobacterium tuberculosis*-containing vacuoles are dynamic, fusion-competent vesicles that are accessible to glycosphingolipids from the host cell plasmalemma. J Immunol **156**:4764-73

Saha, B., Das, G., Vohra, H., Ganguly, N. K., and Mishra, G. C. (1994) Macrophage-T cell interaction in experimental mycobacterial infection. Selective regulation of co-stimulatory molecules on Mycobacterium-infected macrophages and its implication in the suppression of cell-mediated immune response. Eur J Immunol **24**:2618-24

Saxegaard, F., and Beass, I. (1988) Relationship between *Mycobacterium avium*, *Mycobacterium paratuberculosis* and 'wood pegeon mycobacteria'.

Determinations by DNA-DNA hybridization. Acta Pathol Microbiol Immunol Scand **96**: 37-42

Schmidt, A., Hall, M. N. (1998) Signaling to the actin cytoskeleton. Annu Rev Cell Dev Biol **14**:305-38

Smith, G. A., Portnoy, D. A., Theriot, J. A. (1995) Asymetric distribution of the Listeria monocytogenes ActA protein is required and sufficient to direct actin-based motility. Mol Microbiol **17**:945-951

Schorey, J., Carroll, M., and Brown, E. J. (1997) A macrophage invasion mechanism of pathogenic mycobacteria. Science **277:** 1091-1093

Schuster, S., Fell, D. A., and Dandekar, T. (2000) A general definition of metabolic pathways useful for systematic organization and analysis of complex metabolic networks. Nat Biotechnol **18**:326-32

Scianimanico, S., Desrosiers, M., Dermine, J. F., Méresse, S., Descoteaux, A., and Desjardins, M. (1999) Impaired recruitment of the small GTPase rab7 correlates with the inhibition of phagosome maturation by Leshmania donovani promastigotes. Cell Microbiol **1**: 19-32

Seastone, D. J., Lee, E., Bush, J., Knecht, D., and Cardelli, J. (1998) Overexpression of a novel rho family GTPase, RacC, induces unusual actin-based structures and positively affects phagocytosis in Dictyostelium discoideum. Mol Biol Cell **9**:2891-904

Sengelov, H. (1995) Complement receptors in neutrophils. Crit Rev Immunol **15**: 107-31

Shariff, A., and Luna, E. J. (1990) Dictyostelium discoideum plasma membranes contain an actin-nucleating activity that requires ponticulin, an integral membrane glycoprotein. J Cell Biol **110**: 681-92

Shariff, A., and Luna, E. J. (1992) Diacylglycerol-stimulated formation of actin nucleation sites at plasma membranes. Science **256**: 245-7

Sheterline, P., Rickard, J. E., and Richards, R. C. (1984) Fc receptor-directed phagocytic stimuli induce transient actin assembly at an early stage of phagocytosis in neutrophil eukocytes. Eur J Cell Biol **34**: 80-7

Sibley, L. D., Franzblau, S. G., and Krahenbuhl, J. L. (1987) Intracellular fate of *Mycobacterium leprae* in normal and activated mouse macrophages. Infect Immun **55**: 680-685

Silver, R. F., Li, Q., and Ellner, J. J. (1998) Expression of virulence of *Mycobacterium tuberculosis* within human monocytes: virulence correlates with intracellular growth and induction of tumor necrosis factor alpha but not with evasion of lymphocyte-dependent monocyte effector functions. Infect Immun **66**: 1190-1199

Slot, J. W., and Geuze, H. J. (1985) A new method of preparing gold probes for multiple-labeling cytochemistry. Eur J Cell Biol **38**: 87-93

Smith, G. A., Portnoy, D. A. and J. A. Theriot. (1995) Asymetric distribution of the *Listeria monocytogenes* ActA protein is required and sufficient to direct actin-based motility. Mol Microbiol **17**:945-951

Spiegel, S., and Merrill, A. H. Jr. (1996) Sphingolipid metabolism and cell growth regulation. FASEB J **10**:1388-97

Stabel, J. R. (1995) Temporal effects of tumor necrosis factor-alpha on intracellular survival of *Mycobacterium paratuberculosis*. Vet Immunol Immunopathol **45**:321-32

Stackebrandt, E., Rainey, F. A., and Ward-Rainey, N. L. (1997) Proposal for a new hierarchic classification system, *Actinobacteria*, classis nov. Int J Syst Bacteriol **47**: 479-491

Stahl, P. D., and Ezekowitz, R. A. (1998) The mannose receptor is a pattern recognition receptor involved in host defense. Curr Opin Immunol **10**:50-5

Stauffer, T. P., Ahn, S., and Meyer, T. (1998) Receptor-induced transient reduction in plasma membrane PtdIns(4,5)P2 concentration monitored in living cells. Curr Biol **8**:343-6

Steed, P. M., Nagar, S., and Wennogle, L. P. (1996) Phospholipase D regulation by a physical interaction with the actin-binding protein gelsolin. Biochemistry **35**:5229-37

Steele-Mortimer, O., Méresse, S., Gorvel, J. P., Toh, B. H., and Finlay, B. B. (1999) Biogenesis of Salmonella typhimurium-containing vacuoles in epithelial cells involves interactions with the early endocytic pathway. Cell Microbiol **1**: 33-49

Stockem, W., Hoffmann, H. U., and Gruber, B. (1983) Dynamics of the cytoskeleton in Amoeba proteus. I. Redistribution of microinjected fluorescein-labeled actin during locomotion, immobilization and phagocytosis. Cell Tissue Res **232**:79-96

Stockem, W., Naib-Majani, W., Wohlfarth-Bottermann, K. E., Osborn, M., and Weber, K. (1983) Pinocytosis and locomotion of amoebae. XIX.

Immunocytochemical demonstration of actin and myosin in Amoeba proteus. Eur J Cell Biol **29**:171-8

Storrie, B. (1988) Assembly of lysosomes: perspectives from comparative molecular cell biology. Int Rev Cytol **111**: 53-105

Storrie, B., and Desjardins, M. (1996) The biogenesis of lysosomes: is it a kiss and run, continuous fusion and fission process? Bioessays **18**: 895-903

Sturgill-Koszycki, S., Schaible, U. E., and Russell, D. G. (1996) *Mycobacterium*-containing phagosomes are accessible to early endosomes and reflect a transitional state in normal phagosomes biogenesis. EMBO J **15**: 6960-6968

Sturgill-Koszycki, S., Schlesinger, P., Chakraborty, P., Haddix, P. L., Collins, H. L., Fok, A. K., Allen, R. D., Gluck, S. L., Heuser, J., and Russell, D. G. (1994) Lack of acidification in *Mycobacterium* phagosomes produced by exclusion of the vesicular proton-ATPase. Science **263**: 678-681

Sut, A., Siruge, S., Sixou, S., Lakhadar-Ghazal, F., Tocanne, J. F., and Laneelle, G. (1990) Mycobacteria glycolipids as potential pathogenicity effectors: alteration of model and natural membranes. Biochemistry **29**: 8498-8502

Suter, E. (1952) The multiplication of tubercle bacilli within normal phagocytes in tissue cultures. J Exp Med **96**: 137

Swanson, J. A., and Baer, S. C. (1995) Phagocytosis by zippers and triggers Trends Cell Biol **5**: 89-93

Swanson, J. A., Johnson, M. T., Beningo, K., Post, P., Mooseker, M., and Araki, N. (1999) A contractile activity that closes phagosomes in macrophages. J Cell Sci **112**: 307-16

Tell, L. A., Woods, L., and Cromie, R. L. (2001) Mycobacteriosis in birds. In: Mycobacterial infections in domestic and wild animals. Rev Sci Tech Off Int Epiz **20**: 180-203

Tereletsky, M. J., and Barrow, W. W. (1983) Postpathogenic detection of glycopeptidolipids associated with the superficial L1 layer of *Mycobacterium intracellulare*. Infect Immun **41**: 1312-1321

Theriot, J. A. (2000) The polymerization motor. Traffic **1**:19-28

Theriot, J. A. (1995) The cell biology of infection by intracellular bacterial pathogens. Annu Rev Cell Dev Biol **11**:213-39

Theriot, J. A, Rosenblatt, J., Portnoy, D. A., Goldschmidt-Clermont, P. J., and Mitchison, T. J. (1994) Involvement of profilin in the actin-based motility of *L. monocytogenes* in cells and in cell-free extracts. Cell **76**:505-17

Theriot, J. A., Mitchison, T. J., Tilney, L. G., and Portnoy, D. A. (1992) The rate of actin-based motility of intracellular Listeria monocytogenes equals the rate of actin polymerization. Nature **357**:257-60

Thorel, M. F., Krichevsky, M., and Levy-Frebault, V. V. (1990) Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of *Mycobacterium avium*, and description of *M. avium* subsp. *avium* subsp. nov., *M. avium* subsp. *paratuberculosis* subsp. nov., and *M. avium* subsp. *silvaticum* subsp. nov. Int J Syst Bacteriol **40**: 254-260

Thorel, M. F., Krichevsky, M., and Lévy-Frébault, V. V. (1990) Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of *Mycobacterium avium*, and description of *Mycobacterium avium* subsp. *avium*

subsp. nov., *Mycobacterium avium* subsp. *paratuberculosis* subsp. nov., and *Mycobacterium avium* subsp. *silvaticum* subsp. nov. Int J Syst Bacteriol **40**: 254-260

Thorel, M. F., Huchzermeyer, H. F., and Michel, A. L. (2001) *Mycobacterium avium* and *Mycobacterium intracellulare* infection in mammals. In: Mycobacterial infections in domestic and wild animals. Rev Sci Tech Off Int Epiz **20**: 204-218

Tilney, N. L., Garovoy, M. R., Busch, G. J., Strom, T. B., Graves, M. J., and Carpenter, C. B. (1979) Rejected human renal allografts: recovery and characteristics of infiltrating cells and antibody.Transplantation **28**:421-6

Tilney, L. G. (1975) The role of actin in nonmuscle cell motility. Soc Gen Physiol Ser **30**: 339-88

Tilney, L. G. (1976) The polymerization of actin. II. How nonfilamentous actin becomes nonrandomly distributed in sperm: evidence for the association of this actin with membranes. J Cell Biol **69**: 51-72

Tilney, L. G., and Kallenbach, N. (1979) Polymerization of actin. VI. The polarity of the actin filaments in the acrosomal process and how it might be determined. J Cell Biol **81**: 608-23

Tilney, L. G., and Tilney, M. S. (1996) The cytoskeleton of protozoan parasites. Curr Opin Cell Biol **8**: 43-8

Tilney, L. G., Clain, J. G., and Tilney, M. S. (1997) Membrane events in the acrosomal reaction of Limulus sperm. Membrane fusion, filament-membrane particle attachment, and the source and formation of new membrane surface. J Cell Biol **81**: 229-53

Tilney, L. G., DeRosier, D. J., Weber, A., and Tilney, M. S. (1992) How Listeria exploits host cell actin to form its own cytoskeleton. II. Nucleation, actin filament polarity, filament assembly, and evidence for a pointed end capper. J Cell Biol **118**: 83-93

Tilney, L. G., Tilney, M. S., and DeRosier, D. J. (1992) Actin filaments, stereocilia, and hair cells: how cells count and measure. Annu Rev Cell Biol **8**: 257-74

Tilney, L. G., Tilney, M. S., and Guild, G. M. (1995) F actin bundles in Drosophila bristles. I. Two filament cross-links are involved in bundling. J Cell Biol **130**: 629-38

Titus, M. A. (1999) A class VII unconventional myosin is required for phagocytosis. Curr Biol **9**: 1297-303

Tjelle, T. E., Lovdal, T., and Berg, T. (2000) Phagosome dynamics and function. Bioessays **22**: 255-63

Toyohara, A., and Inaba, K. (1979) Transport of phagosomes in mouse peritoneal macrophages. J Cell Sci **94**:143-53

Trifaro, J. M., and Vitale, M. L. (1993) Cytoskeleton dynamics during neurotransmitter release. Trends Neurosci **16**: 466-72

Trifaro, J. M., Vitale, M. L., and Rodriguez Del Castillo, A. (1992) Cytoskeleton and molecular mechanisms in neurotransmitter release by neurosecretory cells. Eur J Pharmacol **225**: 83-104

Tsuyuguchi, I., Kawasumi, H., Takashima, T., Tsuyuguchi. T., and Kishimoto, S. (1990) *Mycobacterium avium-Mycobacterium intracellulare* complex-induced

suppression of T-cell proliferation *in vitro* by regulation of monocyte accessory cell activity. Infect Immun **58**: 1369-1378

Ullrich, O., Reinsch, S., Urbe, S., Zeria, I. M., and Parton, R. G. (1996) Rab11 regulates recycling through the pericentriolar recycling endosome. J Cell Biol **135**: 913-24

Unkeless, J. C., Shen, Z., Lin, C. W., and DeBeus, E. (1995) Function of human Fc gamma RIIA and Fc gamma RIIIB. Semin Immunol **7**: 37-44

Valentin-Weigand, P., Darji, A., ZurLage, S., Rohde, M., Gerlach, G.F., and Weiss, S. (1997) Defective antigen presentation by *Mycobacterium paratuberculosis*-infected macrophages. Immunobiology **2-4**: 334-335

Valentin-Weigand, P., and Goethe, R. (1999) Pathogenesis of *Mycobacterium avium* subspecies *paratuberculosis* infections in ruminants: still more questions than answers. Microb Infect **1**: 1121-1127

Vanhaesebroeck, B., Leevers, S. J., Panayotou, G., and Waterfield, M. D. (1997) Phosphoinositide 3-kinases: a conserved family of signal transducers. Trends Biochem Sci **22**:267-72

VanHeyningen, T. K., Collins, H. L., and Russell, D. G. (1997) IL-6 produced by macrophages infected with Mycobacterium species suppresses T cell responses. J Immunol **158**:330-7

Van Obberghen, E., Somers, G., Devis, G., Ravazzola, M., Malaisse-Lagae, F., Orci, L., and Malaisse, W. J. (1975) Dynamics of insulin release and microtubular-microfilamentous system. VII. Do microfilaments provide the motive force for the translocation and extrusion of beta granules? Diabetes **24**: 892-901

Vercellone, A., Nigou, J., and Puzo, G. (1998) Relationships between the structure and the roles of lipoarabinomannans and related glycoconjugates in tuberculosis pathogenesis. Frontiers Biosci **3**: 149-163

Via, L. E., Fratti, R. A., McFalone, M., Pagán-Ramos, E., Deretic, D., and Deretic, V. (1998) Effects of cytokines on mycobacterial phagosomes maturation. J Cell Sci **111**: 897-905

Vitale, M. L., Seward, E. P., and Trifaro, J. M. (1995) Chromaffin cell cortical actin network dynamics control the size of the release-ready vesicle pool and the initial rate of exocytosis. Neuron **14**: 353-63

Wada, A., Fukuda, M., Mishima, M., and Nishida, E. (1998) Nuclear export of actin: a novel mechanism regulating the subcellular localization of a major cytoskeletal protein. Embo J **17**: 1635-41

Walker, L., Lowrie, D.B. (1981) Killing of *Mycobacterium microti* by immunologically activated macrophages. Nature **293**: 69-70

Wang, E., Michl, J., Pfeffer, L. M., Silverstein, S. C., and Tamm, I. (1984) Interferon suppresses pinocytosis but stimulates phagocytosis in mouse peritoneal macrophages: related changes in cytoskeletal organization. J Cell Biol **98**: 1328-41

Weber, A., Nachmias, V.T., Pennise, C.R., Pring, M., and Safer, D. (1993) Interaction of thymosin beta 4 with muscle and platelet actin: implications for actin sequestration in resting platelets. Biochemistry **31**:6179-85

Welch, M. D., Rosenblatt, J., Skoble, J., Portnoy, D. A., and Mitchison, T. J. (1998) Interaction of human Arp2/3 complex and the Listeria monocytogenes ActA protein in actin filament nucleation. Science **281**: 105-8

Welch, M. D. (1999) The world according to Arp: regulation of actin nucleation by the Arp2/3 complex. Trends Cell Biol **9**:423-7

Welch, M. D, Mallavarapu, A., Rosenblatt, J. and Mitchison, T.J. (1997) Actin dynamics in vivo. Curr Opin Cell Biol **9**:54-61

Woodbury, J. L., and Barrow, W. W. (1989) Radiolabelling of *Mycobacterium avium* oligosaccharide determinants and use in macrophage studies. J Gen Microbiol **135**: 1875-1884

Wright, S. D., and Griffin, F. M. Jr. (1985) Activa
tion of phagocytic cells' C3 receptors for phagocytosis. J Leukoc Biol **38**: 327-39

Wright, S. D., Craigmyle, L. S., and Silverstein, S. C. (1983) Fibronectin and serum amyloid P component stimulate C3b- and C3bi-mediated phagocytosis in cultured human monocytes. J Exp Med **158**: 1338-43

Xu, S., Cooper, A., Sturgill-Koszycki, S., van Heyningen, T., Chatterjee, D., Orme, I., Allen, P., and Russell, D. G. (1994) Intracellular trafficking in *Mycobacterium tuberculosis* and *Mycobacterium avium*-infected macrophages. J Immunol **153**: 2568-2578

Yamamura, S., Sadahira, Y., Ruan, F., Hakomori, S., and Igarashi, Y. (1996) Sphingosine-1-phosphate inhibits actin nucleation and pseudopodium formation to control cell motility of mouse melanoma cells. FEBS Lett **382**:193-7

Yu, F. X., Sun, H. Q., Janmey, P. A., and Yin, H. L. (1992) Identification of a polyphosphoinositide-binding sequence in an actin monomer-binding domain of gelsolin. J Biol Chem **267**:14616-21

Zerial, M., and Huber, L. A. (1995) Guidebook to the small GTPases. ISBN 0 19 8599447

Zerial, M., and Stenmark, H. (1993) Rab GTPases in vesicular transport. Curr Opin Cell Biol **5**:613-20

Zhao, B., Collins, M.T., and Czuprynski, C.J. (1997) Effects of gamma interferon and nitric oxide on the interaction of Mycobacterium avium subsp. Paratuberculosis with bovine monocytes. Infect Immun **65**:1761-6

Zigmond, S. H., Joyce, M., Borleis, J., Bokoch, G. M., and Devreotes, P. N. (1997) Regulation of actin polymerization in cell-free systems by GTP-γ S and cdc42. J. Cell Biol **138**:363-374

Zurbrick, B. G., Follett, D. M., and Czuprynski, C. J. (1988) Cytokine regulation of the intracellular growth of *Mycobacterium paratuberculosis* in bovine monocytes. Infect Immun **56**:1692-7

Zurbrick, B. G., and Czuprynski, C. J. (1987) Ingestion and intracellular growth of *Mycobacterium paratuberculosis* within bovine blood monocytes and monocyte-derived macrophages. Infect Immun **55**: 1588-1593

F. Appendix

Abbreviations

AA	arachidonic acid
Cer	ceramide
CFU	colony forming units
DMEM	Dulbecco's Modified Eagle's Medium
FACS	fluorescent activated cell sorter
FCS	fetal calf serum
F-actin	filamentous actin
G-actin	globular actin
Lamp1/2	Lysosomal associated membrane protein 1/2
LBP	latex bead phagosome
MAC	*Mycobacterium avium* complex
MCP	mycobacteria containing phagosome
OADC	oleic-acid-dextrose-catalase
$Pi(3,4,5)P_3$	phospahtidylinositol-3,4,5-trisphosphate
$Pi(4,5)P_2$	phosphatidylinositol-4,5-bisphosphate
PiP	phosphatidylinositol-4-phosphate
PNS	post nuclear supernatant
S1P	sphingosine-1-phosphate
SM	sphingomyelin
Sph	sphingosine
Tβ4	thymosine-β-4
TNF-α	tumor necrosis factor- α
INF-γ	Interferon- γ

Acknowledgements

I wish to express my gratitude to my supervisor at the microbiology department in Hannover Prof. Peter Valentin-Weigand, who guided my way in the world of mycobacteria and who always had an open door to discuss anything and to my supervisor at the EMBL, Gareth Griffiths who, with an astonishing enthusiasm, has opened new doors to my way of seeing science and life.

Prof. Gerald F. Gerlach I would like to thank for being always helpful when needed.

Dr. Ralph Goethe. I would like to thank you for your nerves of iron. After all I learned quite a lot from you. Good luck.

Manfred Rhode, Beate Sodeik, Hans Joachim Schubert provided me with EM pictures, knowledge about confocal microscopy, FACS-analysis and always a good idea. Thanks for everything.

I am greatful to the two groups that I have been working in. Thanks to everybody from Hannover. Sabine, Achim, Petra, Anja, Ralph, Birgit, Matthias, Kathrin, Nora, Yenner, and Nadine, I had a lot of fun with you and there are things I will never forget: Sauna, dancing, conversations, tears, the 'MiBi-Fete' and breakfasts to name just a few. It was a nice time in Hannover.

Anja, Morten, Rune, Sybille, Sylvia, Laura, Birgit, Andrea, Evelyne and Jacomina, thanks for the great atmosphere in the lab. Working successfully is great, but working under these conditions is even greater.

Very special thanks to Elsa Anes. I never met such a positive person like you. I hope we will continue to work for a long time.

'Frau' Kirpal and 'Herr' Richter stay the way you are.

The support I get from my family was always of the greatest importance to me. Thanks to my wonderful sister Kathrin, to my parents and my grandmother. You were the soil in which I was able to grow.

Many thanks to all my friends, that were with me at all my ups and downs, especially to Adrian, Matthias, Jens, Andreas, Eiko, Achim, Susanne and last but not least Klaus.

Last but not least I would like to thank the woman that was with me in the years in Hannover, Beatrice. Good luck on your way.

Heidelberg, June 2002
Mark Philipp Kühnel